EVALUATION OF THE CELLULAR, HUMORAL AND MOLECULAR EFFECTS OF THE TEDUGLUTIDE ADMINISTRATION ON AN ANIMAL MODEL OF INTESTINAL ANASTOMOSIS

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Doctoral Programme in Health Sciences, Medicine, supervised by Professor Doctor Francisco José Franquera Castro e Sousa, Professor Doctor Maria Filomena Rabaça Roque Botelho, and Professor Doctor Ana Bela Sarmento Antunes da Cruz Ribeiro, and presented to the Faculty of Medicine of the University of Coimbra

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ADMINISTRAÇÃO DO TEDUGLUTIDE NUM MODELO ANIMAL DE
ANASTOMOSE INTESTINAL

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**Original articles**


Abstracts


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Bem hajam!
“Surgery is an Art and a Science in which the Hands are at the service of the Intelligence”

Jean d'Ormesson
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[Glp2]: Concentration of glucagon-like peptide 2

[GSH]: Cellular reduced glutathione content

α-Sma: α-smooth muscle actin

σ: Spearman’s correlation coefficient

ΔC₇: Threshold cycle

Δψmt: Mitochondrial membrane potential

ACS-NSQIP: American College of Surgeons National Surgical Quality Improvement Program

Adam: A desintegrin and metalloprotease (adamalysin)

Adams: A desintegrin and metalloprotease with thrombospondin motifs

AKT: Protein-kinase B

Alcam: Activated leukocyte cell adhesion molecule

APC: Allophycocyanin

Arg: Arginine

ARRIVE: Animal Research: Reporting In Vivo Experiments

ASA: American Society of Anesthesiology

ATP: Adenosine triphosphate

AUC: Area under the curve

auROC: Area under the Receiver Operating Characteristic Curve

AV: Annexin-V

Bcl2: B-cell chronic lymphocytic leukemia/lymphoma 2
bFGF: Basic fibroblast growth factor

*Bmi1*: Bmi1 protooncogene, Polycomb ring finger (B-cell-specific Moloney murine leukemia virus insertion site 1)

*Bmi1*: B-cell-specific Maloney murine leukemia virus insertion site 1

*Bmp*: Bone morphogenic protein

*Bmp4*: Bone morphogenic protein 4 ligand

cAMP-PKA-CREB: Cyclic adenosine monophosphate-Protein kinase A-cyclic adenosine monophosphate response element-binding protein

*CD166*: Cluster of differentiation 166

*CD24*: Cluster of differentiation 24

*CD31*: Cluster of differentiation 31

*CD44*: Cluster of differentiation 44

*CD45*: Cluster of differentiation 45

*CD90/Thy-1*: Cluster of differentiation 90/Thymocyte differentiation antigen 1

cDNA: Complementary deoxyribonucleic acid

*Cl*: Confidence interval

*CK18*: Cytokeratin 18

*Col1a1*: Collagen, type I, alpha 1, mRNA

*Col3a1*: Collagen, type III, alpha 1, mRNA

*Col4a1*: Collagen, type IV, alpha 1, mRNA

*Col5a1*: Collagen, type V, alpha 1, mRNA

*COPD*: Chronic obstructive pulmonary disease

*Cox2*: Cyclooxygenase 2

*CTGF*: Connective tissue growth factor
Ctgf: Connective tissue growth factor, mRNA

cWnt: Canonical Wingless

CXCL5: C-X-C motif chemokine ligand 5

DCF: 2',7'-dichlorofluorescein

DCFH₂: 2',7'-dichlorodihydrofluorescein

DCFH₂-DA: 2',7'-dichlorodihydrofluorescein diacetate

DHR 123: dihydrorhodamine 123

DDR: Discoidin domain receptors

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Early ap: Early apoptosis index

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

Egf: Epidermal growth factor, mRNA

EIA: Competitive enzyme immunoassay

ELISA: Enzyme-linked immunosorbent assay

EMA: European Medicines Agency

Epcam: Epithelial cell adhesion molecule

EPO: Erythropoietin

ErbB: Erythroblastic leukemia viral oncogene
ERK: Extracellular signal-regulated kinase

ESPEN: European Society for Clinical Nutrition and Metabolism

F

Fab: Antigen-binding fragment

FACS: Fluorescence-activated cell sorting

Fc: Crystallizable fragment

FDA: United States Food and Drug Administration

FGF2: Fibroblast growth factor 2

Fgf2: Fibroblast growth factor 2, mRNA

FGF7: Fibroblast growth factor 7 (keratinocyte growth factor 1, KGF1)

Fgf7: Fibroblast growth factor 7, mRNA

FGF-R1: Fibroblast growth factor receptor 1

FITC: Fluorescein-isothiocyanate

FL: Fluorescence detector

G

G&S: Gordon-Sweet's protocol

GALT: Gut-associated lymphoid tissue

GFRP: Growth factor-rich plasma

GH: Growth hormone

GLP-1: Glucagon-like peptide 1

GLP-2 MMB: GLP-2 mimetibody construct

GLP-2: Glucagon-like peptide 2

GLP-2R: Glucagon-like peptide 2 receptor

Glp2r: Glucagon-like peptide 2 receptor, mRNA
GM-CSF: Granulocyte-macrophage colony-stimulating factor
Gpr49: G Protein-coupled receptor 49
Grp78: 78 KDa glucose-regulated protein
GSH: Reduced glutathione
GSK3β: Glycogen synthase kinase 3 β

Hb: Hemoglobin
H&E: Hematoxylin-eosin
H₂O: Water
H₂O₂: Hydrogen peroxide
HB-EGF: Heparin-binding epidermal-like growth factor
Hbegf: Heparin-binding EGF-like growth factor, mRNA
HBOT: Hyperbaric oxygen therapy
HBSS: Hank’s balanced salt solution
Hepes: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution
Hif: Hypoxia-inducible factor 1
Hippo-YAP: Hippo-Yes-associated protein 1
HO: Hydroxyl radical
Hopx: Homeodomain-only protein
Hprt1: Hypoxanthine phosphoribosyltransferase 1, mRNA
HR: Hazard ratio

ic[GSH]: Cellular reduced glutathione content
IESC: Intestinal epithelial stem cells
IFN-γ: Interferon-γ
Ig: Immunoglobulin
Igf1: Insulin-like growth factor 1, transcript variant 1, mRNA
IGF-1: Insulin-like growth factor 1
IGF-BP: Insulin-like growth factor binding protein
IL: Interleukin
IL-1α: Interleukin-1 alpha
IL-2R: Interleukin-2 receptor
IL-4: Interleukin-4
IL-6: Interleukin-6
IM: Intramuscular
IMAGinE: International Multispecialty Anastomotic Leak Global Improvement Exchange
iNOS: Inducible nitric oxide synthase
IO: Intraoperative
IP: Intraperitoneal
IQR: Interquartile range

JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide
JC1 A/M: JC-1 aggregates/monomers ratio

Kgf: Keratinocyte growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Lap</td>
<td>Laparotomy</td>
</tr>
<tr>
<td>Late ap</td>
<td>Late apoptosis/necrosis index</td>
</tr>
<tr>
<td>LCA</td>
<td>Leucocyte common antigen</td>
</tr>
<tr>
<td>Lgr5/Gpr49</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>Lrig1</td>
<td>Leucine-rich repeats and immunoglobulin-like domains 1</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemo-attractant protein-1</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean intensity of fluorescence</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>Mmp 1</td>
<td>Matrix metallopeptidase 1 (interstitial collagenase), mRNA</td>
</tr>
<tr>
<td>Mmp 12</td>
<td>Matrix metallopeptidase 12, mRNA</td>
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<td>Mmp 2</td>
<td>Matrix metallopeptidase 2, mRNA</td>
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<tr>
<td>Mmp 3</td>
<td>Matrix metallopeptidase 3, mRNA</td>
</tr>
<tr>
<td>Mmp 9</td>
<td>Matrix metallopeptidase 9, mRNA</td>
</tr>
<tr>
<td>MO</td>
<td>Mercury orange dye</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney strain of murine leukemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
</tbody>
</table>
mTOR: Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
Mψ: mitochondrial membrane potential

NCBI: National Center for Biotechnology Information
N.t.: Not tested
NF-κB: Nuclear factor-kappa-light-chain-enhancer of activated B cells
NO: Nitric oxide
NOS: Nitric oxide synthase
NSAID: Non-steroid anti-inflammatory drugs

ONOO: Peroxynitrite
OR: Odds ratio

p: value of significance
PAS-AB: Periodic acid schiff-alcian blue
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
Pdgfb: Platelet-derived growth factor beta polypeptide, mRNA
PE: Phycoerythrin
Pecam-1: Platelet and endothelial cell adhesion molecule-1
Peroxides: Cytosolic peroxides level
PerCP-Cy5.5: Peridinin chlorophyll protein complex with cyanin-5.5
PGE2: Prostaglandin E2
PI: Propidium iodide
PI3K: Phosphatidylinositol 3-kinase
PPAR-γ: Peroxisome proliferator activator receptor-γ
Pro-MMP: Pro-metalloproteinase
PRP: Platelet-rich plasma

qRT-PCR: Quantitative real-time reverse-transcription polymerase chain reaction

Reac Sp: Reactive species generation in the mitochondria
Ref: Reference
Res: Resection
RNA: Ribonucleic acid
ROC: Receiver Operating Characteristic
ROS: Reactive oxygen species
RR: Relative risk
RS: Reactive species
R-spondins: Roof plate-specific spondins
RT: Room temperature
Sc: Subcutaneous
SDF-1: Stromal cell-derived factor-1
SMC: Smooth muscle cells
SPSS: Statistical Package for Social Sciences
STAT3: Signal transducer and activator of transcription-3
STEPS: Study of Teduglutide Effectiveness in Parenteral Nutrition-Dependent Short-bowel Syndrome Subjects
Tcf4: Transcription factor 4
Ted: Teduglutide
Tert: Telomerase reverse transcriptase
TGF-β: Transforming growth factor-β
Tgfβ1: Transforming growth factor, beta 1, mRNA
Th1: T-helper 1 lymphocytes
Th2: T-helper 2 lymphocytes
Timp1: TIMP metallopeptidase inhibitor 1, mRNA
Timp2: TIMP metallopeptidase inhibitor 2, mRNA
Timp: Tissue inhibitor of matrix metalloproteinases
tKGF: Truncated keratinocyte growth factor
TNF-α: Tumour necrosis factor-alpha
Tpl2: Tumor progression locus 2
Trem2: Triggering receptor expressed on myeloid cells 2
Tris: Tris(hydroxylmethyl)aminomethane
UK: United Kingdom

USA: United States of America

USP: United States Pharmacopeia

VEGF: Vascular endothelial growth factor

Vegfa: Vascular endothelial growth factor A, transcript variant 2, mRNA

Vs.: Versus

VSM: Vascular smooth muscle

Wnt: Wingless

Wnt3: Wingless ligand 3
Abstract & Resumo
Abstract

Despite recent progresses in surgical technique and perioperative care, failure of intestinal anastomotic healing remains one of the most feared complications in digestive surgery, exerting a profound adverse impact on the operative morbidity and mortality rates, oncologic and functional outcomes, and socioeconomic costs.

Teduglutide is an enterotrophic analogue of glucagon-like peptide 2 (GLP-2) approved for the pharmacological rehabilitation of short-bowel syndrome.

Present study aimed to clarify the potential of teduglutide as a promoting strategy for the improvement of intestinal anastomotic healing, on an animal model, through the influence on the cellular, humoral and molecular mediators of repair.

An experimental rat model of standard small-bowel anastomosis was used with evaluation at the third and seventh postoperative days. Structural assessment of the anastomosis included the macroscopic integrity and the histological and immunohistochemical examination of healing parameters, comprising reepithelialization, neoangiogenesis and fibroplasia. Cellular and molecular mediators of anastomotic healing were analyzed, including: putative epithelial stem cells response (using Lgr5, Bmi1 and the panel CD24/CD44/CD166/Grp78 surface markers by flow cytometry); cellular viability and death (with double staining with annexin-V/propidium iodide by flow cytometry); oxidative stress [quantification of cytosolic peroxides with 2',7'-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) probe, mitochondrial reactive species with dihydrorhodamine 123 (DHR 123) probe, total intracellular reduced glutathione with mercury orange staining and mitochondrial membrane potential with 5,5',6,6'-tetrachloro-1,1',3,3'-tethraethylbenzimidazolcarbocyanine iodide (JC-1) probe, by flow cytometry]; local and systemic inflammatory response (tissue and plasma concentrations of interleukine-1$\alpha$, macrophage chemo-attractant protein-1, tumor necrosis factor-$\alpha$, interferon-$\gamma$ and interleukine-4 by flow cytometric multiplexed bead assay); gene expression of main extracellular matrix components ($\text{Collagen, type I, alpha 1: Col1a1; Collagen, type III, alpha 1: Col3a1; Collagen, type IV, alpha 1: Col4a1; Collagen, type V, alpha 1: Col5a1}$) and remodeling factors, matrix metalloproteinases ($\text{Mmp; Mmp1 and Mmp13, Mmp2 and Mmp9, Mmp3, Mmp12 and}$
Teduglutide had no apparent relevant impact on the rate or severity of intestinal anastomotic leakage. A favorable influence of teduglutide on the reepithelialization and neoangiogenesis events of the proliferative phase of anastomotic repair was documented. Teduglutide was associated with an increase of subepithelial myofibroblasts density score, but no significant effect on the goblet, Paneth and glial cellular indexes was observed.

This growth factor was associated with an enhancement of type III collagen deposition on the submucosa at the seventh postoperative day, although with simultaneous reduction of type I collagen level in that layer, and a non-significant reduction of global anastomotic collagen content.

Teduglutide inhibited the gene modulation of fibrolysis in the predominantly inflammatory phase of anastomotic repair, while repressed the fibrogenesis in the proliferative stage. Teduglutide induced the upregulation of gene expression of *Timp1*, *Timp2* and *Col4a1*, and the downregulation of *Mmp3* and *Mmp12*, at the third postoperative day; and the repression of gene expression of *Timp1*, *Col3a1*, *Col4a1* and *Col5a1*, at the seventh day.

Teduglutide contributed to the expansion of the putative crypt base columnar stem cells pool at the seventh day and to the concomitant depletion of the putative “position +4” stem cells fraction. An increase (non-significant) of the overall putative intestinal epithelial stem cells was also observed in teduglutide-treated animals.
Teduglutide was associated with a non-significant prooxidative effect, with an increase of the cytosolic peroxides and mitochondrial reactive species levels and a reduction of the cellular reduced glutathione content. Those effects were coincident with an increase of cellular viability indexes and a non-significant decrease of early apoptotic events. No relevant influence on mitochondrial membrane potential was verified. A non-significant increase of tissue levels of the anti-inflammatory interleukin-4 at the seventh day, and a significant reduction of plasma levels of interferon-\(\gamma\) at the third day were observed in teduglutide-treated animals.

Teduglutide induced the upregulation of the gene expression of \(Igf1\), \(Vegfa\) and \(Ctgf\) and the downmodulation of \(Fgf2\), \(Fgf7\), \(Tgfb1\) and \(Glp2r\).

To conclude, the present study reflects the complexity of the intestinal anastomotic repair and points to a favorable influence of teduglutide on this process that deserves additional investigation.

**Keywords:** Teduglutide; Surgical anastomosis; Anastomotic leak; Reepithelialization; Physiologic angiogenesis; Extracellular matrix; Growth factor; Adult stem cells; Inflammation; Oxidative stress
Resumo

Apesar dos recentes progressos da técnica cirúrgica e suporte peri-operatório, a falência da cicatrização anastomótica intestinal constitui, ainda, uma das mais temíveis complicações da cirurgia digestiva, com um importante impacto adverso na mortalidade e morbidade operatórias, resultados oncológicos e funcionais e custos econômico-sociais.

O teduglutide é um análogo enterotrófico do glucagon-like peptide 2 (GLP-2) aprovado para a reabilitação farmacológica na síndroma do intestino curto.

Este estudo procurou analisar as potencialidades do teduglutide como estratégia adjuvante da cicatrização anastomótica intestinal, num modelo animal, através da sua influência nos mediadores celulares, humorais e moleculares do processo reparativo.

Foi utilizado um modelo experimental de anastomose intestinal estandardizada, em rato, com avaliação ao terceiro e ao sétimo dias pós-operatórios. A avaliação estrutural da anastomose incluiu a integridade macroscópica e os exames histológico e imunohistoquímico dos parâmetros de cicatrização, tais como reepitelização, neoangiogênese e fibroplasia. Foram analisados os seguintes mediadores celulares e moleculares da cicatrização anastomótica: resposta das putativas células estaminais epiteliais (usando os marcadores de superfície Lgr5, Bmi1 e o painel CD24/CD44/CD166/Grp78 por citometria de fluxo); viabilidade e morte celular (com marcação dupla com anexina V/iodeto de propídeo, por citometria de fluxo); stresse oxidativo [quantificação de peróxidos citoplasmáticos com sonda de diacetato de 2ʹ,7ʹ-diclorodihidrofluoresceína (DCFH-DA), espécies reactivas mitocondriais com sonda de dihidrorodamina 123 (DHR 123), glutatión reduzido intracelular com marcação com alaranjado de mercúrio e potencial de membrana mitocondrial com sonda de iodeto de 5,5ʹ,6,6ʹ-tetraclorotetraclorofluorofenilmetilhidrazina e (JC-1), por citometria de fluxo]; resposta inflamatória local e sistêmica (concentrações teciduais e plasmáticas de interleucina-1α, macrophage chemo-attractant protein-1, factor de necrose tumoral-α, interferon-γ e interleucina-4 por citometria de fluxo; expressão gênica de componentes da matriz extracelular (Collagen, type I, alpha 1: Col1a1; Collagen, type III, alpha 1: Col3a1; Collagen, type IV, alpha 1: Col4a1; Collagen, type V, alpha
I: Col5a1) e respectivos factores de remodelação, metaloproteinases (Mmp) da matriz 1, 13, 2, 9, 3, 12 e 14 (Mmp1 e Mmp13, Mmp2 e Mmp9, Mmp3, Mmp12 e Mmp14) e inibidores tecidulares das Mmp 1 e 2 (Timp1 e Timp2); assim como dos factores de crescimento potencialmente implicados na reparação anastomótica (Insulin-like growth factor 1, transcript variant: Igf1; Vascular endothelial growth factor A, transcript variant 2: Vegfa; Transforming growth factor, beta 1: Tgfb1; Connective tissue growth factor: Ctgf; Fibroblast growth factor 2: Fgf2; Fibroblast growth factor 7: Fgf7; Epidermal growth factor: Egf; Heparin-binding epidermal-like growth factor: Hbegf; Platelet-derived growth factor beta polypeptide: Pdgfb; Glucagon-like peptide 2 receptor: Glp2r) por transcrição reversa quantitativa da reacção em cadeia da polimerase em tempo real; e da concentração plasmática do Glp-2 (por imunoensaio enzimático competitivo).

O teduglutide não teve impacto relevante aparente na incidência e gravidade da deiscência anastomótica mas exerceu uma influência favorável nos processos de reepitelização e de neoangiogénese da fase proliferativa da cicatrização. Associou-se, ainda, a um aumento da densidade de miofibroblastos subepiteliais, sem modificação significativa dos índices de células caliciformes, de Paneth e gliais.

Este factor de crescimento associou-se ao aumento da deposição de colagéneo III na submucosa ao sétimo dia pós-operatório, embora com redução concomitante do colagéneo I na mesma camada, e à redução não significativa do teor global de colagéneo na anastomose.

O teduglutide inibiu a modulação génica da fibrólise na fase predominantemente inflamatória da reparação enquanto, pelo contrário, reprimiu a fibrogénese na fase proliferativa. O teduglutide aumentou a expressão génica de Timp1, Timp2 e Col4a1 e, reduziu a de Mmp3 e Mmp12, ao terceiro dia pós-operatório; e diminuiu a expressão génica do Timp1, Col3a1, Col4a1 e Col5a1, ao sétimo dia.

O teduglutide induziu a expansão das putativas células estaminais colunares basais das criptas e, concomitantemente, a depleção das células da “posição +4”. Nos animais tratados com teduglutide, observou-se, ainda, um aumento global não significativo das putativas células epiteliais intestinais.
O teduglutide associou-se a um efeito pro-oxidativo não significativo, com aumento dos níveis de peróxidos citoplasmáticos e de espécies reactivas mitocondriais e redução do glutatião reduzido celular. Estes efeitos foram acompanhados por um aumento do índice de viabilidade celular e uma redução não significativa dos eventos apoptóticos precoces. Não se verificou influência significativa no potencial de membrana mitocondrial. Nos animais tratados com teduglutide, observou-se um aumento não significativo dos níveis tecidulares da citocina anti-inflamatória interleucina-4 ao sétimo dia, assim como uma redução significativa da concentração plasmática de interferon-γ ao terceiro dia.

O teduglutide promoveu o aumento da expressão génica do \( lgf1 \), \( Vegfa \) e \( Ctgf \) e a repressão do \( Fgf2 \), \( Fgf7 \), \( Tgfb1 \) e \( Glp2r \).

Em conclusão, o presente estudo reflecte a complexidade da cicatrização anastomótica intestinal e sugere uma influência favorável do teduglutide neste processo que justifica uma investigação adicional.

**Palavras-chave:** Teduglutide; Anastomose cirúrgica; Fístula anastomótica; Reepitelização; Angiogénese fisiológica; Matriz extracelular; Factor de crescimento; Células estaminais adultas; Inflamação; Stresse oxidativo
Chapter 1

Introduction
1.1. Intestine structure and function

The intestine is characterized by a highly differentiated structure composed of four distinct layers: mucosa, submucosa, *muscularis propria* and serosa (Fig. 1.1). Mucosa consists of a single layer of columnar epithelium resting on the *lamina propria*, which contains loose connective tissue with blood and lymphatic vessels and some lymphoid tissue, and is separated from submucosa by the *muscularis mucosae*. In the small intestine, mucosa is organized in millions of villi and crypts of Lieberkühn that markedly increase its surface area. Submucosa contains connective tissue with blood vessels, lymphatics and the Meissner’s plexus of the enteric nervous system. *Muscularis propria* includes two concentric layers of smooth muscle (an inner circular and an outer longitudinal) and, between both, the Auerbach’s plexus. Serosa consists in a delicate sheet of loose connective tissue covered outside by the mesothelium (Piscaglia, 2014; Lloyd and Gabe, 2008).

![Image of intestinal cross-section](image-url)

Figure 1.1. Cross-section of human small bowel (hematoxylin-eosin staining; original magnification 2x). Intestinal wall is subdivided into four layers: mucosa, submucosa, *muscularis propria* and serosa (image kindly provided by M.A. Cipriano)
The gut is an organ with complex and intricate absorptive, digestive, defense, neuromotor, endocrine and metabolic functions. These functions include digestion and absorption of water, macro and micronutrients; defense (maintenance of the barrier function and participation on local and systemic immune processes) and modulation of the inflammatory response; mixture and propulsion of chyme (with stationary motility and propulsive peristalsis); clearance of microorganisms and particles; production of peptides with endocrine, paracrine and autocrine properties; metabolism of amino acids and synthesis of apoproteins. Small intestine is the largest endocrine organ and produces peptides that regulate the metabolism of glucose; appetite and food ingestion; gastric, biliary and pancreatic secretions; gastrointestinal motility and immune function (Lindberg et al., 2008; O'Mahony L, 2008).

The intestinal epithelium covering constitute the largest surface of contact of the human body with the external environment, with a total area over 200 m² (Lopetuso et al., 2013). The Gut-associated Lymphoid Tissue (GALT) is one of the greatest lymphoid organs, containing up to 70% of the body’s total number of immune cells, and acting as an inductor for the Mucosal-associated Lymphoid Tissue (MALT) (Fay et al., 2017; O’Mahony, 2008; Garcia Lorenzo Mateos et al., 2007; Wiest et al., 2003). The GALT comprises intraepithelial lymphocytes, lamina propria lymphocytes, Peyer’s Patches, and mesenteric lymph nodes. Peyer’s Patches interact with epithelial cells to induce the local immune responses to luminal antigens, mediating the interplay between antigen presenting cells and T cells and the release of cytokines from activated T cells (Fay et al., 2017).

Intestinal microbiota is constituted by a population of approximately 40 trillion of bacteria, of over 1,000 different species, predominantly (80%) of two dominant phyla (Firmicutes and Bacteroidetes) (Fay et al., 2017; Cabrera-Perez et al., 2017). Bacteriophagic particles, viruses, fungi and archaea are also constituents of gut microbiota. Firmicutes are Gram-positive bacterial taxa constituted by many commonly recognized genera (such as Clostridia, Streptococcaceae, Staphylococcaceae, Enterococcaceae, and Lactobacillae). Bacteroidetes, on the other hand, are Gram-negative bacteria composed mainly of Bacteroides species, which are obligate anaerobes. Human intestinal microbiota seems to be analogous to that of
mice, with concordances until the genus (for Firmicutes) and species levels (for Bacteroidetes) (Cabrera-Perez et al., 2017). Gut microbiota-host multidirectional interactions play a relevant role in the homeostasis preservation. Intestinal dysbiosis, defined as a disturbance of microbial composition and/or activity, seems to contribute for the development of several diseases, due to its adverse impact on gut barrier, immunity and endocrine function (Bachmann et al., 2017).

Regardless of the constant exposure to physical, biochemical, microbiological, and other forms of injury, the intestine maintains a relative state of homeostasis with preservation of the structure and function.

1.2. Intestinal anastomotic leakage

1.2.1. Definition and incidence

Despite recent progresses in surgical technique and perioperative care, failure of intestinal anastomotic healing, with subsequent leakage, dehiscence, stenosis or bleeding, remains a major source of morbidity and mortality in digestive surgery (Guyton et al., 2016; Bosmans et al., 2015; Shogan et al., 2013; Thompson et al., 2006). However, definition and reporting of anastomotic complications have been characterized by significant inconsistence. Furthermore, pathophysiology of intestinal anastomotic repair is still poorly understood (Guyton et al., 2016; Chadi et al., 2016; Vallance et al., 2016; Bosmans et al., 2015; Shogan et al., 2013).

Intestinal anastomotic leakage is one of the most ominous postoperative complications (Guyton et al., 2016). According to the International Multispecialty Anastomotic Leak Global Improvement Exchange (IMAGinE), anastomotic leakage may be defined as a defect in the integrity of a surgical junction between two hollow viscera leading to a communication between the intra and extraluminal compartments (Chadi et al., 2016; Shogan et al., 2013). This event may lead to a variety of anatomic and clinical consequences, ranging from a small contained abscess with spontaneous resolution to
a generalized peritonitis requiring urgent reoperation, and from a purulent discharge from a wound or drain to a life-threatening septic shock (Shogan et al., 2013). Anastomotic leakage may be classified, in accordance with the impact on the clinical management, as grade A (that does not imply an active therapeutic intervention), B (that demands nonoperative radiological or endoscopic treatment) or C (that requires reoperation) (Shogan et al., 2013).

Intestinal anastomotic fistula develops in 1 to 9% of cases, although this is probably an underestimated and underreported incidence. Rates of anastomotic leakage vary from 0.02 to 4% in enterocenteric and ileocolonic anastomosis, 0 to 2% in colocolonic anastomosis and 1 to 20% in colorectal, coloanal and ileoanal anastomosis (Chadi et al., 2016). The risk of disruption varies according to the anastomotic site. It is usually lower in small bowel and higher in colon and rectum, particularly in most distal locations (Hyman, 2009; Turrentine et al., 2015) (Fig. 1.2).

![Image](image_url)

**Figure 1.2. Frequency and mortality of gastrointestinal anastomotic leaks by anatomic site.** Retrospective review of 2,237 adult surgical gastrointestinal tract procedures with anastomosis prospectively collected from the American College of Surgeons National Surgical Quality Improvement Program database and published by Turrentine FE et al in 2015 (Turrentine et al., 2015)
1.2.2. Diagnosis and management

Early diagnosis and intervention are critical for the successful management of the anastomotic leakage and for the minimization of its deleterious consequences (Chadi et al., 2016). Nevertheless, timely diagnosis may constitute a major challenge since the initial clinical and radiological signs are often nonspecific (Chadi et al., 2016).

Most (79.4%) of the gastrointestinal fistula were identified within 30 days after surgery (median time of 11 days and mean of 28 days) and 94.3% within 90 days (Turrentine et al., 2015). Anastomotic leakage with mechanic and structure-related causes tend to be diagnosed earlier, whereas those with ischemia-related origin tend to present later (Chadi et al., 2016).

Surgeon’s intraoperative evaluation has a low accuracy for detection of anastomotic leakage in gastrointestinal surgery (Guyton et al., 2016; Shogan et al., 2013). After colorectal surgery, surgeons’ clinical risk assessment has a sensitivity of 38 to 62% and a specificity of 46 to 52%, not significantly influenced by the respective training level (Karliczeck et al., 2009). After colon cancer resection, the predictive capacity was also low (area under the receiver operating characteristic curve [auROC]=0.40, p=0.243) (Sammour et al., 2017).

The well-established intraoperative anastomotic air leak test, when feasible, seems to be effective in predicting satisfactory healing if it demonstrates an intact anastomosis (Vallance et al., 2016; Chadi et al., 2016). Intra-operative endoscopy may allow the assessment of the anastomosis integrity and has been shown to be safe and reliable, but the potential benefit of its routine use remains to be determined (Vallance et al., 2016). Intraoperative imaging may also be useful (Hirst et al., 2014).

Computerized tomography with luminal contrast and water-soluble contrast studies are the current preferred techniques for the diagnosis of intestinal anastomotic leakage, but are characterized by variable sensitivity and specificity and logistical constraints (Chadi et al., 2016; Hirst et al., 2014).

Several biomarkers of ischemia, inflammation and microbiological aggression, in the systemic circulation and in the peritoneal drainage fluid, were evaluated for the diagnosis of anastomotic disruption. Most commonly evaluated parameters were C-
reactive protein, procalcitonin and total leucocytes count, in the systemic circulation, and interleukin-6, interleukin-10 and tumor necrosis factor-α, in the peritoneal drainage fluid. These biomarkers are considered poor predictors of anastomotic leakage when isolated, but its combination showed improvement in the diagnostic accuracy (Su'a et al., 2017). Notwithstanding, serum C-reactive protein at the third postoperative day seemed to be a useful negative predictive test for the development of anastomotic leakage following colorectal surgery, with a pooled area under the curve of 0.81 (95% confidence interval [CI] 0.75 to 0.86); derived cut-off value was 172 mg/l, which corresponded to a high negative predictive value (97%) and a negative likelihood ratio of 0.26 to 0.33 (Singh et al., 2014). In a recent study, procalcitonin revealed high specificity and negative predictive values for anastomotic leakage in colorectal surgery at the third and fifth postoperative days (auROC of 0.775 and 0.862, respectively) and its combination with C-reactive protein significantly improved the diagnosis at the fifth day (auROC=0.901) (Giaccaglia et al., 2016). Combination of plasma C-reactive protein and calprotectin at the third postoperative day after colorectal surgery yielded sensitivity of 100%, specificity of 89%, positive likelihood ratio of 9.09 (95% CI 4.34-16), negative likelihood ratio of 0.00 (95% CI 0.00-0.89) ($p<0.001$) and a high negative predictive value (100%) (Reisinger et al., 2014). Furthermore, preoperative intestinal fatty acid-binding protein levels can be used for anastomotic leakage risk assessment as they predicted colorectal anastomotic disruption at a cut-off level of 882 pg/ml with sensitivity of 50%, specificity of 100%, positive likelihood ratio of “infinite” (95% CI 4.01-“infinite”), and negative likelihood ratio of 0.50 (95% CI 0.26-0.98) ($p<0.0001$) (Reisinger et al., 2014). Those biomarkers might be advantageous in providing real-time monitoring of leakage development and be useful as discharge criteria in enhanced recovery after surgery programs. Nevertheless, implementation of those parameters in the daily practice deserves additional investigation.

New strategies, including several diagnosis scores and decision algorithms, have been developed for detection of anastomotic leakage in the postoperative period of colorectal cancer surgery, such as the Diacole score based in 13 clinical signs and symptoms and proposed by Rojas-Machado SA et al in 2016 (Rojas-Machado et al., 2016). The ongoing Condor (Early Complication Detection After Colorectal
Surgery) prospective study is also expected to contribute for the development of a new diagnostic score (Kornmann et al., 2016). Those indexes require validation and further evaluation to determine whether they translate into improved patient outcomes. Further research is needed to address this important area.

After an early diagnosis, an optimized multidisciplinary approach is necessary, based on medical, radiologic, endoscopic and/or surgical interventions. Management often implies reoperation in a hostile abdomen, creation of an intestinal stoma, and the need for subsequent operations to restore intestinal continuity (Hyman, 2009).

Recent advances in the management of anastomotic leakage comprise the more widespread use of non-operative strategies, including guided percutaneous drainage, endoluminal stenting, endoscopic vacuum-assisted drainage and over-the-scope-clip system application, and also of minimally invasive approaches (laparoscopic or endoscopic) in operative interventions (Chadi et al., 2016; Vallance et al., 2016; Sparreboom et al., 2016a).

1.2.3. Consequences

Intestinal anastomotic leakage has an important negative impact on the morbidity and mortality rates, reoperation and readmission indexes, lengths of hospital stay, long-term oncological outcomes, functional results, patients’ quality of life, economic costs and surgeon-patient relationships (Gessler et al., 2017; Scarborough et al., 2017; Chadi et al., 2016; Midura et al., 2015; Shogan et al., 2013; Luján et al., 2011; Hyman, 2009).

In a retrospective review of 2,237 adult surgical gastrointestinal tract procedures with anastomosis prospectively collected from the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database, anastomotic leaks were associated with higher overall morbidity (98.0 vs. 28.4%; \( p < 0.0001 \)), return to the operating room (45.8 vs. 4%; \( p < 0.0001 \)), length of stay (13 vs. 5 days; \( p \leq 0.0001 \)), 30-day mortality (8.4 vs. 2.5%; \( p < 0.0001 \)), long-term mortality (36.4 vs. 20.0%; \( p \leq 0.0001 \)) and risk of death (adjusted hazard ratio [HR]=1.77; \( p = 0.008 \)); and decreased survival (log-rank test, chi-square=23.1; \( p < 0.0001 \)); as well as, increased hospitalization costs (chi-square [\( \chi^2 \)]=359.8; \( p < 0.0001 \); mean $16,085.39 hospital cost
for patients who had an intact anastomosis and did not experience complications, and mean $56,349.12 for those who experienced an anastomotic leak) (Turrentine et al., 2015).

In a retrospective study of 99,879 patients submitted to colorectal surgery, anastomotic leaks had a 30-days overall incidence of 6.18% and incurred additional length of stay and hospital costs of 7.3 days and $24,129, respectively (only within the first hospitalization); higher 30-day readmission rates (1.3 times) and postoperative infection rates (0.8–1.9 times) were also observed (p<0.001 for both) (Hammond et al., 2014).

In a meta-analysis of 34 nonrandomized studies analyzing the oncologic impact of anastomotic leakage following restorative colorectal cancer surgery, involving 78,434 patients, increased local recurrence (relative risk [RR] 1.90, 95% CI 1.48-2.44, I²=78%) and reduced overall survival (RR 1.36, 95% CI 1.24-1.50, I²=74%), cancer-specific survival (RR 1.41, 95% CI 1.19-1.68, I²=56%), and disease-free survival (RR 1.40, 95% CI 1.20-1.63, I²=86%) were documented (Ha et al., 2017). Similar results were observed by Wang S et al (Wang et al., 2017) after curative anterior rectum resection for rectal cancer, with greater local recurrence and decreased overall and cancer-specific survival. Additionally, Nachiappan S et al, prospectively verified a significantly reduced overall survival after anastomotic leakage treated with reoperation following elective colorectal cancer resection (HR 2.74, 95% CI 1.67-4.52, p<0.001) (Nachiappan et al., 2015). In a multicentric prospective study, anastomotic leakage after surgery for colorectal cancer constituted an independent predictor of the quality of life (β=-0.42, p<0.001) (Di Cristofaro et al., 2014). Other authors reported also deterioration of the quality of life with anastomotic leakage after colorectal cancer surgery (Brown et al., 2014; Marinatou et al., 2014; Ashburn et al., 2013). Anastomotic leakage constitutes an important indicator of the surgical quality of care and has aroused an increasing interest in performance metrics (Nikolian et al., 2017).

### 1.2.4. Etiology

Pathogenesis of the failure of intestinal anastomotic repair is multifactorial and insufficiently known. Interference of non-recognized determinant factors may explain
failures of anastomoses performed in a technically correct manner and in the absence of other apparent risk conditions (Guyton et al., 2016).

Differences in the cytokines response in early and late anastomotic leakages, with increased plasma concentrations of interleukin-1β, interleukin-6, interleukin-8 and interleukin-10 within the first five postoperative days in the first case and no significant modifications in late-onset dehiscence, may support the theory of different pathological mechanisms of disruption (Ellebæk et al., 2014).

A successful intestinal anastomosis requests a meticulous surgical technique and compliance with fundamental surgical principles, including the absence of ischemia, excessive tension and distal obstruction, adequate serosal incorporation and apposition; and an uncompromised waterproof-sealed lumen (Tabola et al., 2017; Guyton et al., 2016).

Nevertheless, at present moment, the blood perfusion adequate to the anastomotic healing is not accurately defined and there are no direct unequivocal evidences that the increase of tension prevents healing. In a recently published experimental study, tissue hypoxia was not a distinctive feature of the disrupted anastomotic tissues, even after segmental devascularization and the assertion that hypoxia plays a major role in the pathogenesis of anastomotic leak was considered still unconfirmed (Shakhsheer et al., 2017). At the present, intraoperative assessment of tissue perfusion with fluorescence real-time angiography is feasible and alters surgical strategy in a non-negligible proportion of patients, but its impact on the incidence of anastomotic dehiscence is still unknown (Mizrahi et al., 2017; Chadi et al., 2016; Vallance et al., 2016). In a recent study, fluorescence angiography during laparoscopic left-sided or anterior rectum resection implied revision of the surgical plan in 8% of cases, without postoperative development of leakage (Jafari et al., 2015).

Multiple technical aspects of anastomotic surgery have been extensively described and analyzed, including handsewn versus stapled and compression anastomosis, everting versus inverting, single versus double-layer anastomosis, anastomotic configuration, and suture types and materials, but none was considered highly determinant in the
outcome and no individual method of anastomosis construction was found to be superior (Kar et al., 2017; Guyton et al., 2016; Herrle et al., 2016; Gustafsson et al., 2015; Slieker et al., 2013; Neutzling et al., 2012; Choy et al., 2011). Thus, surgical technique is left to the surgeon’s preference (Guyton et al., 2016). However, although handsewn anastomosis is nonstandardized, a single-layer continuous technique using inverting sutures with slowly absorbable monofilament material seems preferable (Slieker et al., 2013).

In spite of extensive experimental research in the field of intestinal surgery (Nordentoft et al., 2015; Vakalopoulos et al., 2013), anastomotic sealing with tissue adhesives has not yet shown convincing results and has not been implemented into the regular clinical practice (Vakalopoulos et al., 2017; Vakalopoulos et al., 2013).

Nowadays, there is no high-level clinical evidence demonstrating benefits of sutureless dynamic compression anastomoses (including biofragmentable anastomotic rings, compression anastomotic rings, compression anastomotic clips and magnetic anastomoses) (Tabola et al., 2017; Bobkiewicz et al., 2017; Graves et al., 2017; Slessor et al., 2016; Li et al., 2016; D’Hoore et al., 2015; Massomi et al., 2013), intraluminal devices (Bakker et al., 2017; Lee et al., 2015; Morks et al., 2011) or external coating (with fibrin sealants, hyaluronic acid/carboxymethylcellulose, omental pedicle grafts and others) (Nasiri et al., 2017; Pomergaard et al., 2012; Hao et al., 2008) to reduce leakage rates. Nevertheless, the prophylactic transanal decompression tube is considered a likely effective and safe method of preventing anastomotic leakage after rectal cancer surgery (Zhao et al., 2017a).

Anastomotic fistula may occur after technically well-constructed anastomoses. Highly experienced and specialized surgeons working in high-volume centers continue to experience anastomotic leaks (Guyton et al., 2016; Shogan et al., 2013).

Emerging evidence suggests that gut microbiota has a strong influence on the intestinal anastomotic healing (Bachmann et al., 2017). After injury, gut microbiota supports epithelial cells migration, adhesion, restitution and proliferation; mucosal barrier integrity; immune cells activation; and vascularization. Akkermansia muciniphila and Lactobacillus species seem to reinforce wound healing and gut barrier function, stimulating prorestitutive signalling and increasing cellular migration and proliferation.
Reactive oxygen species and formyl peptide receptors are considered key molecular mediators of microbiota regulation of wound healing process. Microbiota-host cell interactions also modulate β-catenin signaling and, subsequently, the epithelial cell proliferation (Bachmann et al., 2017).

Gut microbiota seems to be influenced by the intestinal resection and anastomosis process and, also, by the perioperative management (including antibiotics prescription and mechanical bowel preparation), with emergence of species with a more aggressive tissue-destroying phenotype, such as Enterococcus. Lower levels of the usually cytoprotective Lactobacillaceae bacteria have also been evident in perianastomotic tissues (Bachmann et al., 2017). Shogan BD et al (Shogan et al., 2014) demonstrated that the colon anastomotic injury induced significant changes in the anastomotic tissue-associated microbiota, including a 500-fold and 200-fold increase in the relative abundance of Enterococcus and Escherichia/Shigela, respectively, with minimal repercussion in luminal microbiota. Functional profiling predicted the prevalence of bacterial virulence-associated pathways in postanastomotic tissues (Shogan et al., 2014). A correlation between the bacterial family Lachnospiraceae, low microbial diversity and intestinal colon anastomotic leakage, possibly in association with the body mass index, was found by van Praagh JB et al (van Praagh et al., 2016). The relative abundance of the Lachnospiraceae family (phylum Firmicutes) may be explained by the higher abundance of mucin-degrading Ruminococci in anastomotic failure cases (van Praagh et al., 2016). Gelatinase GelE of Enterococcus faecalis, a highly prevalent bacterium in leaking anastomotic tissues (Shogan et al., 2014), disrupts the intestinal integrity, induces inflammation and degrades collagen (Bachmann et al., 2017). The distinct core microbiota of the elderly, with greater proportion of Bacteroides species and distinct abundance patterns of Clostridium groups may contribute to the increased risk of leakage in those individuals (Bachmann et al., 2017).
1.2.5. Risk factors and prevention

Numerous patient, disease, and operation-related risk factors of anastomotic leakage have been recognized in clinical studies (Nikolian et al., 2017; Rencuzogullari et al., 2017; van Rooijen et al., 2016; Midura et al., 2015) (Table 1.1).

Table 1.1. Clinical relevant risk factors for gastrointestinal anastomotic leakage described in the literature

<table>
<thead>
<tr>
<th>Patient-related factors</th>
<th>Disease-related factors</th>
<th>Laboratorial factors</th>
<th>Operation-related factors</th>
<th>Other factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender 1-11</td>
<td>Neoadjuvant treatment</td>
<td>Hyperglycemia / high hemoglobin A1c 2</td>
<td>Emergent/urgent operation 4,9</td>
<td>Lack of mechanical bowel preparation 9</td>
</tr>
<tr>
<td>Young age 3</td>
<td>Chemotherapy 3,6,9</td>
<td>Anemia 15</td>
<td>Blood transfusions 1,5,9,11</td>
<td>Lack of selective gut decontamination 5</td>
</tr>
<tr>
<td>Smoking history 3,4,6,7,11</td>
<td>Radiotherapy 8,11</td>
<td>Leukocyte count &gt; 12,000/mm³ 1</td>
<td>Blood loss 5,9,11</td>
<td>Antibiotics omission 2</td>
</tr>
<tr>
<td>Alcohol excess 1-11,12</td>
<td>Bevacizumab 11</td>
<td>Thrombocytosis 4</td>
<td>Prolonged operating time 5,9,11</td>
<td>Number of hospital beds 92</td>
</tr>
<tr>
<td>Obesity 1,2,6,9,11</td>
<td>Neoplasia</td>
<td>Hypoproteinemia 2</td>
<td>Intraoperative complications 5,9,10</td>
<td>Contaminated/infected wounds 7</td>
</tr>
<tr>
<td>Malnutrition 7,11,12</td>
<td>Distal location 11</td>
<td>Hypoalbuminemia 1,3</td>
<td>Intraoperative hypotension 5</td>
<td>Perioperative fluid overload (&gt;8 l infusion in the first 3 days) 4</td>
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<td>ASA classification ≥3,7,9,11</td>
<td>Advanced stage 11</td>
<td>Abnormal sodium 12</td>
<td>Low central venous oxygen saturation 15</td>
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<tr>
<td>Comorbidities</td>
<td>Metastatic disease 11</td>
<td></td>
<td>Other concomitant surgical procedures 1</td>
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<td>Diabetes mellitus 1,3,5,7</td>
<td>Great size 9,11</td>
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<td>Rectum anastomosis 12</td>
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<td>COPD 1,7</td>
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<td>Low rectal anastomosis 1,8,9,10</td>
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<td>Cardiovascular disease 1,12</td>
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<td>Elective rectal cancer surgery 82</td>
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<td>Renal disease 1</td>
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<td>High ligation of inferior mesenteric artery 810</td>
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<td>Liver disease 1</td>
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<td>Splenic flexure mobilization 81</td>
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<td>Overall comorbidities 1,7,11</td>
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<td>Mechanical anastomosis 81</td>
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<td>Medications</td>
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<td>Stapler firing ≥ 3mm 9</td>
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<td>Steroids 1,8,12</td>
<td></td>
<td></td>
<td>Open approach colectomy 8</td>
<td></td>
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<tr>
<td>Immunosuppressors 6,11</td>
<td></td>
<td></td>
<td>Emergency colectomy for bleeding 81</td>
<td></td>
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<tr>
<td>Anticoagulants 2</td>
<td></td>
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<tr>
<td>Non-selective NSAID 1,7</td>
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There are no evidences that minimally invasive approaches (including laparoscopic and robotic interventions) (Juo et al., 2014) and standardized enhanced recovery after surgery protocols (Wang et al., 2014; Li et al., 2013) increase the risk of intestinal anastomotic leakage. Through the analysis of 8,442 cases of the National Surgical Quality Improvement Program database, Kiran RP et al (Kiran et al., 2015) demonstrated that combined preoperative mechanical bowel preparation with oral antibiotics (but not without) significantly reduces anastomotic leaks after colorectal surgery (odds ratio=0.57, 95% CI 0.35-0.94). Combined preoperative mechanical bowel preparation with oral antibiotics (and isolated oral antibiotics) was also associated with a significant reduction of anastomotic leak risk in a more recent analysis of 32,359 cases of the same database (Koller et al., 2017). Recently, Garfinkle R et al (Garfinkle et al., 2017) showed, on a conditional logistic regression of matched patients extracted from an analysis of 40,446 cases, that oral antibiotic preparation alone was protective of anastomotic leak (odds ratio=0.60, 95% CI 0.34-0.97). Similar results were obtained in other studies (Scarborough et al., 2015). A systematic review and meta-analysis suggested that, in elective gastrointestinal surgery, a combination of perioperative selective decontamination of the digestive tract and perioperative intravenous antibiotics may reduce the rate of anastomotic leakage compared with use of intravenous antibiotics alone (Roos et al., 2013).

Influence of new advances in perioperative care (such as surgical prehabilitation protocols, perioperative nutrition support and rational goal-directed perioperative fluid therapy) in the incidence of anastomotic complications need to be further evaluated.

Recently, Rojas-Machado et al (Rojas-Machado et al., 2016) proposed the Procole prognostic score (Prognostic Colorectal Leakage) for colorectal cancer surgery as a good predictive indication. The Reveal (Predictive Factors of Anastomotic Leakage after Colorectal Surgery) study of predictive factors after colorectal surgery is also ongoing (Jongen et al., 2016).

Development of prognostic indexes and predictive nomograms may be very important to estimate the risk of anastomotic leakage, but they require previous prospective external validation (Sammour et al., 2017; Rojas-Machado et al., 2016; Frasson et al.,
The identification of high-risk cases may allow the secondary prevention of anastomotic disruption (including the use of adjuvants of repair, anastomotic reinforcement and other strategies) and the establishment of adequate criteria for selective proximal virtual or classical diverting ostomies. These data may allow a more accurate risk assessment in the clinical practice and also risk adjustment for quality of care control (Nikolian et al., 2017). Nevertheless, although those numerous potential risk factors may contribute to anastomotic failure, the underlaying mechanisms remain poorly understood and speculative (Guyton et al., 2016).

Anastomotic leakage remains a critical, expensive and potentially life-threatening complication and an ongoing challenge (Guyton et al., 2016). An evidence-based and integrated approach based on the understanding of the pathophysiology and on clinical trials is necessary to prevent anastomotic complications. Surgical research for improvement of the understanding of the cellular, molecular and biochemical pathways of anastomotic healing and for the development of new preventive and early diagnostic strategies is needed. Great interest emerged on innovative perioperative interventions to improve anastomotic repair, especially in high-risk conditions.

1.3. Intestinal anastomotic healing

Intestinal anastomotic healing is a continuous multicellular multimolecular dynamic process involving complex interactions between multiple signaling networks and rigorous spacial and temporal control. Repair progression requires the coordinated interplay of specific cell types, cell surface receptors, extracellular matrix proteins and bioactive factors (Rijcken et al., 2014; Greaves et al., 2013; Thompson et al., 2006). Inadequate anastomotic healing may conduct to fistula and dehiscence, when insufficient, or to fibrosis and stenosis, when uncontrolled and excessive (Rijcken et al., 2014).

Traditionally, the wound healing model considers three overlapping phases: inflammatory, proliferative and reparative (Rijcken et al., 2014; Greaves et al., 2013) (Fig. 1.3).
Figure 1.3. Graphic representation of the classical model of the intestinal anastomotic healing. Anastomosis repair develops in three overlapping phases. Inflammatory stage (left) is characterized by inflammatory cells infiltration, extracellular matrix degradation, wound debridement and provisional closure. Proliferative phase (middle) comprises the reepithelialization, angiogenesis and fibroplasia processes. Reparative stage (right) develops with remodeling of extracellular matrix, wound maturation and contraction. Adapted from Rijcken E et al., 2014

1.3.1. Inflammatory, proliferative and remodeling phases

**Inflammatory phase**

In the inflammatory stage, during the first one to four days, the coagulation process origins thrombin, an inducer of platelet degranulation, and triggers the release of bioactive mediators that stimulate the recruitment and activation of inflammatory cells. Influx of inflammatory cells to the wound site is facilitated by vasodilation and increase of vascular permeability. These cells participate in the wound debridement, extracellular matrix degradation, antigen presentation, phagocytosis and release of reactive oxygen species, inflammatory cytokines, chemokines and growth factors that amplify the repair process. Neutrophils are the most abundant inflammatory cells in the early stages, followed by monocytes and classically activated macrophages (M1), which are considered the most important regulatory cell type in the exudative phase (Rijcken et al., 2014; Greaves et al., 2013; Delavary et al., 2011). Provisional wound closure and debridement were therefore ensured (Rijcken E et al., 2014). Molecular mediators of inflammatory phase of repair include vasoactive amines (histamine and serotonin), plasma proteases (bradykinin and complement factors), coagulation factors
(such as fibrinopeptides and platelet-activating factor), arachidonic acid metabolites (thromboxanes, prostaglandins and leukotrienes), cytokines (interleukins and tumor necrosis factor-α), growth factors (platelet-derived growth factor, transforming growth factor β, vascular endothelial growth factor and insulin-like growth factor) and reactive oxygen and nitrogen species (Rijcken et al., 2014; Greaves et al., 2013).

**Proliferative phase**

Proliferative phase is characterized by reepithelialization, angiogenesis and fibroplasia and occurs, generally, between the second and the fourteenth postoperative days (Rijcken et al., 2014; Greaves et al., 2013). Activated macrophages (M2) contribute to the transition of inflammatory to proliferative phase of healing, suppressing the inflammatory response and the adaptive T-helper 1 lymphocytes (Th1)-immunity and favoring a T-helper 2 lymphocytes (Th2) response, which has been related to fibrogenesis. M2 macrophages constitute a relevant source of transforming growth factor β and promote angiogenesis and extracellular matrix deposition (Delavary et al., 2011).

Reepithelialization process begins within minutes to hours of injury, to obtain the reestablishment of the epithelial continuity, and is based on restitution, crypt stem cells proliferation and differentiation, and crypt fission (Bloemendaal et al., 2016; Rijcken et al., 2014; Greaves et al., 2013; Iizuka M and Konno S, 2011). Restitution occurs along the exposed and new basement membrane and implies cellular adhesion and cytoskeletal remodeling (Rijcken et al., 2014; Iizuka and Konno, 2011). Neighboring epithelial cells suffer reorganization of the actin cytoskeleton, lose their columnar polarity, acquire a flattened morphology and migrate into the wound to restore barrier integrity (Iizuka and Konno, 2011).

Molecular mediators of intestinal epithelial wound repair include growth factors (transforming growth factor α, transforming growth factor β, epidermal growth factor, heparin-binding epidermal growth factor, hepatocyte growth factor, fibroblast growth factor 2, keratinocyte growth factor, insulin-like growth factors 1 and 2, cytokines, chemokines and their receptors, prostaglandin E2, cyclooxygenases 1 and 2, toll-like
receptors 2, 5 and 9, hypoxia-inducible factor and dietary factors such as glutamine (Iizuka and Konno, 2011). Most important involved signaling pathways were Rho family, for modulation of cytoskeletal actin reorganization; extracellular signal-regulated kinase (ERK)1/ERK2, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt, for regulation of epithelial proliferation and survival; nuclear factor-κB (NF-κB), for epithelial cells protective function; signal transducer and activator of transcription-3 (STAT3) for regulation of immune homeostasis and triggering receptor expressed on myeloid cells 2 (Trem2) for promotion of M2 macrophages activation (Iizuka and Konno, 2011).

Angiogenesis is the complex process of ingrowth of new microvessels in the wound that restores microcirculation and allows reestablishment of adequate perfusion, normoxia and nutrient delivery to the regenerating tissue. Endothelial cells from the capillaries in the margin migrate into the wound, suffer pseudopodia, proliferate and form new capillaries (Greaves et al., 2013). Fibroblast growth factor 2 is the most important pro-angiogenic factor in the early phase after injury, decreasing then towards the seventh day (Greaves et al., 2013). Vascular endothelial growth factor is determinant to the angiogenesis and is upregulated in the first three to seven days after the injury, promoting endothelial cell migration and proliferation, and vascular permeability (Greaves et al., 2013). Other important angioproliferative factors include tissue hypoxia, nitric oxide, tumor necrosis factor-α and angiopoietin 1. Proangiogenic effects of hypoxia are mediated through hypoxia-inducible factor 1, which upregulates the vascular endothelial growth factor, endothelial nitric oxide synthase and heme oxygenase 1 expressions (Greaves et al., 2013).

In the proliferative phase, fibroplasia, the process of extracellular matrix deposition and basement membrane restoration, provides a scaffold for further cellular influx, adhesion and differentiation during the healing process (Greaves et al., 2013). Fibrogenesis results from the activation and synergy of mesenchymal cells, mainly fibroblasts and myofibroblasts, that are the key effectors of tissue repair (Greaves et al., 2013; Speca et al., 2012). Number of fibrogenic cells in the wound increase by proliferation, transdifferentiation and desdifferentiation between fibroblast, myofibroblast and smooth muscle phenotypes, epithelial-to-mesenchymal and
endothelial-to-mesenchymal transitions and migration from non-affected tissues to the
wound (chemotaxis and chemokinesis) (Greaves et al., 2013; Speca et al., 2012).

Fibroblasts, the most important cell type in the wound at the fourth day, produce a
collagen-rich extracellular matrix that gradually replace temporary fibrin matrix and
increase tissue biomechanical strength (Speca et al., 2012; Thompson et al., 2006).
Myofibroblasts (subepithelial myofibroblasts and intersticial cells of Cajal) are highly
contractile cells with a phenotype between fibroblasts and smooth muscle cells that
participate in tissue growth and repair (Speca et al., 2012). Myofibroblasts produce
high levels of extracellular matrix and basement membrane components, particularly
collagen, glycosaminoglycans, tenasin and fibronectin, and participate in the
modulation of the inflammatory reaction by the secretion of chemokines and
cytokines (Speca et al., 2012). Myofibroblasts generate traction forces and initiate
wound closure and contraction (Greaves et al., 2013). Other cells types involved in
the fibrogenesis are stellate cells, pericytes, local and bone marrow-derived stem cells
(Greaves et al., 2013; Speca et al., 2012).

Extracellular matrix is an active and dynamic structure that participates in the
regulation of the inflammatory response and the healing process by focal adhesions
with immune and non-immune cells (Speca et al., 2012). It has anchorage-related
functions and constitutes a reservoir for mediators such as cytokines, chemokines and
growth factors (including vascular endothelial growth factor, transforming growth
factor β and fibroblast growth factor 2) (Gattazzo et al., 2014). Extracellular matrix
assists endothelial cell proliferation and migration and determines the outcome of
vessel organization (Greaves et al., 2013). Extracellular matrix degradation is mediated
by the fine balance between matrix-degrading enzymes (including matrix
metalloproteinases) and their inhibitors (such as tissue inhibitors of matrix
metalloproteinases) (Speca et al., 2012). Adequate wound tension and cytokines
profile constitute determinant factors of the mechanical properties of extracellular
matrix, with an important role in the restoration of mechanotransduction and tissue
homeostasis (Gattazzo et al., 2014; Greaves et al., 2013).

Most important molecular mediators of fibrogenesis process include growth factors
(transforming growth factor β1, connective tissue growth factor, platelet-derived
growth factor, insulin-like growth factors 1 and 2, epidermal growth factor and vascular endothelial growth factor), cytokines, chemokines, reactive oxygen species, endothelins 1, 2 and 3, components of the renin-angiotensin system, peroxisome proliferator activator receptor-γ (PPAR-γ), mammalian target of rapamycin (mTOR), matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Speca S et al., 2012).

Remodeling phase

In the reparative phase, the last and longest phase of wound healing process, which may elapse up to six months (Rijcken et al., 2014), extracellular matrix undergoes continuous synthesis, breakdown and remodeling by proteolytic enzymes (including matrix metalloproteinases), leading to wound maturation and contraction (Greaves et al., 2013). Extracellular matrix acquires progressively a predominantly collagenous structure, with reduced cellularity (Greaves et al., 2013). Deposited collagen suffers rearrangement and cross-linking and type III collagen is replaced by type I collagen (Delavary et al., 2011). Remodeling is mainly promoted by transforming growth factor β and platelet-derived growth factor (Rijcken et al., 2014) and is influenced by the predominance of a Th2 or a Th1 response (Delavary et al., 2011).

1.3.2. Particularities of intestinal healing

Despite fundamental similarities, the healing process of the intestinal anastomosis differs from that of the cutaneous tissue. Intestinal anastomotic wound environment is characterized by the particular microbiota, the biomechanical stress resultant from motility and mass propulsion, and the higher susceptibility to vascular perfusion disturbances (Thompson et al., 2006).

Gut microbiota composition, including aerobic and anaerobic microorganisms, is different from the skin flora (Rijcken et al., 2014) and seems to exert a relevant influence on the anastomotic healing process (Bachmann et al., 2017).
The importance of mechanical forces in the intestinal healing process was demonstrated by Kovalenko PL et al (Kovalenco et al., 2012) who verified that the repetitive deformation may favor mucosal repair via ERK signaling, whereas the increase of luminal pressure may inhibit it by an ERK-independent mechanism. Loss of the repetitive distension may decrease mucosal healing in the defunctionalized bowel. Moreover, the increased luminal pressure above anastomoses (or in a spastic bowel disease) could further inhibit mucosal restoration, despite peristaltic reccuring strain (Kovalenco et al., 2012).

Collagen in the gastrointestinal tract includes subtypes I, III and V and is produced by fibroblasts and smooth muscle cells, while, in the skin, only subtypes I and III are produced and solely by fibroblasts (Bosmans et al., 2015).

In comparison with cutaneous repair, intestinal anastomotic healing is characterized by a proportionally faster increase of the physical strength (especially in small intestine), possibly as a result of differences in the degree of expression and time course of proinflammatory and antiinflammatory cytokines and growth factors between these two wound sites (Zubaidi et al., 2015; Bosmans et al., 2015; Alzoghaibi and Zubaidi, 2014; Alzoghaibi, 2013; Zubaidi et al., 2010). The intestinal serosal layer, nonexistent in the skin, provides a matrix for fibroblasts and has an important role in the wound strength (Bosmans et al., 2015).

Collagenase activity exerts a relevant role the intestinal anastomotic repair and it is responsible for the low wound biomechanical strength in the early postoperative period (Bosmans et al., 2015). In fact, in the first two to three days of the postoperative period, a transient reduction of the anastomotic strength occurs, due to the degradation of collagen by the proteinase activity in the wound (Thompson et al., 2006). Thereafter, strength increases by synthesis and deposition of new collagen in the predominantly proliferative phase of the healing process. Tensile strength, a rarely used measure for anastomosis assessment, reflects the suture-holding capacity of the preexisting collagen of perianastomotic tissue, in all the surface area of the wound edge, and correlates with collagen synthesis after the fourth postoperative day. Tensile strength is regained at a slower rate than the bursting pressure. Bursting pressure reflects the weakest site of the anastomosis and is usually used as an evaluation
parameter only in first few days after the operation. Bursting pressure of a small intestine anastomosis is approximately 50% of normal at the second and third postoperative days and approaches 100% at the seventh day, whereas a tensile strength similar to the unwounded tissue is reached only four weeks after surgery (Thompson et al., 2006).

A better understanding of the pathophysiology of intestinal anastomotic disturbances, insufficiently known at the present (Guyton et al., 2016), may lead to more targeted and efficacious preventing interventions, with a potential relevant clinical and socioeconomic impact.

1.4. Adjuvant interventions on intestinal anastomotic healing

Numerous experimental studies have been undertaken on the role of adjuvants of intestinal anastomotic healing, either in standard or high-risk contexts (Yauw et al., 2015) (Table 1.2).

The perioperative strategies to improve anastomosis repair described in those studies included pharmacological interventions, nutrients and probiotics administration, stem cell-based therapies, anastomotic reinforcement, among many others (Yauw et al., 2015).

The most studied pharmacologic interventions included the administration (topic or systemic) of growth factors and hormones (granulocyte-macrophage colony-stimulating factor, insulin-like growth factor, platelet-rich plasma, growth hormone and erythropoietin), matrix metalloproteinases (mmp) inhibitors, prostacyclin analogues, antibiotics, allopurinol, superoxide dismutase, β-D-glucan and aprotinin (Yauw et al., 2015). Nutrients manipulation comprised short-chain fatty acids, glutamine, arginine, vitamin A/retinoid acid, vitamin C and zinc supplementation. Other types of adjuvant interventions included the use of tissue adhesives (cyanoacrylates, fibrin glues, polyethylene glycol adhesives and albumin-based adhesives) and alternative forms of
anastomotic reinforcement, hyperbaric oxygen therapy, ischemic preconditioning and electromagnetic field stimulation (Vakalopoulos et al., 2017; Yauw et al., 2015).

### Table 1.2. Potential adjuvant strategies for intestinal anastomotic healing analyzed in experimental studies

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
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<tbody>
<tr>
<td><strong>Pharmacological adjuvants</strong></td>
<td></td>
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<tr>
<td>Growth factors</td>
<td>Insulin-like growth factor, keratinocyte growth factor, granulocyte-macrophage colony-stimulating factor, epidermal growth factor, basic fibroblast growth factor, vascular-endothelial growth factor, heparin-binding epidermal growth factor, glucagon-like peptide 2, platelet-derived growth factor, transforming growth factor β, platelet-rich plasma</td>
</tr>
<tr>
<td>Hormones</td>
<td>Growth hormone, erythropoietin, melatonin, anabolic steroids, estrogen, progesterone, ghrelin, leptin, triiodothyronine, adrenomedullin, somatostatin analogues (octreotide, lanreotide), placental lactogenic hormone, somatomammogenic chorionic hormone, thyroid stimulating hormone</td>
</tr>
<tr>
<td>Active peptides</td>
<td>Matrix metalloproteinases inhibitors (aprotinin, nanomostat mesylate, soybean trypsin inhibitor, others), prostataglandins (E1, E2); β-D-glucan, activated protein C, antithrombin III, caffeic acid phenethyl ester, pentadecapeptide BPC 157, calcitonin gene-related peptide, s-methylisothiouria hemisulphate, poly-l-lysine and poly-l-glutamate, enterosan; coagulation factor XIII, flavonoids, neurotensin, pancreatic polypeptides, substance P, carnitine, catalase, adenosine triphosphate, amelogenin, aminoguanidine, β-aminopropionitrile, neurokinin 1 receptor antagonist, parthenolide, regenerating agent 11</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Allopurinol, superoxide dismutase, dioxime+hesperidine, N-(3-aminomethyl)benzyl)acetamidine</td>
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<tr>
<td>Vasodilators</td>
<td>Prostacyclin analogues (iloprost, others), sildenafil, bosentan, nitroglycerine, papaverine, talafadil, trapidil, perfluorane</td>
</tr>
<tr>
<td>Other drugs</td>
<td>Pentoxifylline, acetylcysteine, simvastatin, ranitidine, famotidine, ketocon, piroxicam, flurbiprofen, nifedipine, dopamine, bromopride, neostigmine, phenytoin, low molecular weight heparin, lisinopril, mescaline, montelukast, tacrolimus, tranexamic acid, amifostine, bupivacaine</td>
</tr>
<tr>
<td>Antibiotics/anti sepsis</td>
<td>Neomycin, metronidazole, gentamicin, levamizol, cephalosporins, oxynhydril, levamisole, amikacin, doxycycline, imipenem, penicillin, taurocholic acid, kanamycin+chloramphenicol, kanamycin+cephalotin, penicillin/streptomycin/chloroformurep (chloramphenicol and dihydrostreptomycin); chlorhexidine, iodine, silver-nanoparticle-coated sutures; and others</td>
</tr>
<tr>
<td>Organic compounds</td>
<td>Polyphenols (reservatrol, proanthocyanidins), ethyl pyruvate, guar gum, honey, tualang honey, gingko biloba extract, aloe vera, algae, oxidized cellulose, castor oil, copaiba oil, shark cartilage, thymoquinone, extracts of aroeira do sertão, Schinus terebinthifolius, Jatropha gossypiifolia, Passiflora edulis</td>
</tr>
<tr>
<td>Other compounds</td>
<td>Chitosan, propolis, vinpocetine, cholerat toxin, interleukin 2, methylene blue, pyrrolidine dithiocarbamate, taurocholate, cartilage bone marrow extract, radioprotective treatments (WR-2721 and ribose-cysteine) and others</td>
</tr>
<tr>
<td>Nutrients</td>
<td>Glutamine, short-chain fatty acids (mostly butyrate), arginine, branched-chain amino acid mixture, glycin, methionin; vitamin A/retnioic acid, vitamin B5, vitamin C, zinc; whey, lactulose, ketone bodies, kefir, polyphosphate; and others</td>
</tr>
<tr>
<td>Probiotics</td>
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<tr>
<td>Stem cell therapy</td>
<td>Adipose stem cells, bone marrow-derived mesenchymal stem cells</td>
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<tr>
<td><strong>Anastomotic reinforcement</strong></td>
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<tr>
<td>Tissue adhesives</td>
<td>Tissue adhesives (cyanoacrylates, fibrin glues, polyethylene glycol adhesives and albumin-based adhesives; biological coating (omentum, peritoneum, collagen fleece, small intestine submucosa, amniotic membrane and others); synthetic matrices and patches (polyglactin, polyglycolic acid, polypropylene, alginate and others)</td>
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<tr>
<td>Other therapies</td>
<td>Hyperbaric oxygen, ischemic preconditioning, electromagnetic field stimulation, positively charged dieethylaminoethyl cross-linked dextran bead particles/electrical stimulation, laser stimulation, radiofrequency, extracorporeal shockwave therapy, hyperoxygenated solution lavage, other types of oxygen supplementation, ozone gas, circadian rhythm alteration</td>
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</table>

*From references Vakalopoulos et al., 2017; Hyoju et al., 2017; Zhao et al., 2017b; Kizitan et al., 2016; Aznan et al., 2016; De la Portilla et al., 2016; Yauw et al., 2015; Sipahi et al., 2014; Silva et al., 2014; Cueto et al., 2014; Vakalopoulos et al., 2013; Pommergaard et al., 2012*

**Pharmacologic adjuvants**

In a systematic review of 75 experimental studies on colorectal anastomosis repair published in 2014 (Oines et al., 2014), 56 different adjuvant substances were tested.
Subsequent meta-analysis identified seven compounds with the potential to reproducibly improve anastomotic healing under non-complicated conditions, including the growth factors and hormones insulin-like growth factor 1, growth hormone and erythropoietin; broad-spectrum inhibitors of matrix metalloproteinases [associated with an increase of the weighted mean of bursting pressure of 48 mmHg (95% CI 31-66)]; immunomodulators, such as iloprost and tacrolimus [that increased the mean bursting pressure by 60 mmHg (95% CI 30-89) and 29 mmHg (95% CI 4-53), respectively]; and hyperbaric oxygen therapy [that enhanced the mean bursting pressure by 24 mmHg (95% CI 13-34) on the third and fourth postoperative days]. In those studies, anastomotic healing could be improved by various mechanisms, including the stimulation of epithelialization, angiogenesis and fibrogenesis, and the inhibition of the degradation of extracellular matrix, among others (Oines et al., 2014).

Another systematic review of 65 preclinical studies and 48 compounds was recently undertaken and the meta-analysis suggested that postoperative hyperbaric oxygen therapy significantly improved colonic anastomotic healing in rat models complicated by bowel ischemia, with a significant increase of the bursting pressure by a mean of 28 mmHg (95% CI 17-39 mmHg, \( p < 0.00001 \)) (Nerstrom et al., 2016). Favorable effects of granulocyte-macrophage colony-stimulating factor were demonstrated in the context of segmental ischemia and obstructive jaundice (Nerstrom et al., 2016). Iloprost was found to be beneficial for the early healing of colonic anastomosis in rats with intestinal obstruction and in those exposed to chemotherapy. Positive actions of N-acetylcysteine after ischemia/reperfusion injury and radiotherapy were also verified (Nerstrom et al., 2016).

Matrix metalloproteinases inhibitors have been shown to improve the biomechanical properties in animal models of colonic anastomotic healing (Krarup et al., 2017; Krarup et al., 2013; Ågren et al., 2011), preventing the collagen degradation and preserving the integrity of extracellular matrix (Krarup et al., 2013). Although selective metalloproteinases inhibition seems to increase the breaking strength and to reduce the anastomotic leakage in experimentally obstructed colon (Krarup et al., 2017), on the contrary, non-selective inhibition was found to increase anastomotic disruption, possibly by impeding reepithelialization (Rehn et al., 2015). In humans, broad
spectrum matrix metalloproteinases inhibitors were associated with detrimental side effects, mainly the musculoskeletal syndrome, due to the high structural homology and overlapped substrate specificity of metalloproteinases (Amar et al., 2017; Cui et al., 2017). Future research should focus on the development of new generation matrix metalloproteinases inhibitors (with high specificity and selectivity for the targeted metalloproteinases), determination of the ideal timing and duration of the administration, and appropriate delivery system (probably by topical administration).

Iloprost is a stable analogue of prostaglandin I₂, which is a potent endogenous vasodilator and also a cryoprotective compound on the intestinal epithelium. Iloprost may improve intestinal anastomotic healing (Oines et al., 2014) through the stimulation of intestinal perfusion and angiogenesis (Galanopoulos et al., 2011).

Hyperbaric oxygen therapy (HBOT) seems to improve colorectal anastomotic healing, particularly in the context of ischemia (Nerstrom et al., 2016; Boersema et al., 2016; Yildiz et al., 2013). Oxygen may stimulate collagen synthesis, matrix deposition, angiogenesis, epithelialization, and eradication of some bacteria. Besides, hyperbaric oxygen therapy may act through the inhibition of inducible nitric oxide synthase protein (iNOS) expression and the suppression of proinflammatory agents (Boersema et al., 2016). Logistical issues and potential side effects (including middle ear and pulmonary barotraumas, central nervous system and pulmonary oxygen toxicities, ocular adverse events, increase of blood pressure, pulmonary edema and hypoglycemia in diabetics) may limit hyperbaric oxygen therapy clinical applications (Heyboer et al., 2017).

**Anastomotic reinforcement**

The external reinforcement of colonic anastomoses failed to show convincing results in a systematic review of 40 studies and 20 different coating materials (Pommergaard et al., 2012). In humans, fibrin glue has shown positive, however not significant, results; omental pedicle grafts demonstrated no benefit and hyaluronic acid/carboxymethylcellulose use was considered deleterious (Pommergaard et al., 2012). Other coating materials were evaluated only in preclinical context and
demonstrated overall contradictory results, despite some positive effects of the coating with amniotic membrane, polypropylene mesh and collagen matrix-bound coagulation factors (Pommergaard et al., 2012).

Tissue adhesives are among the most studied strategies as gastrointestinal anastomotic sealants, mostly on animal but, also, in clinical studies (Vakalopoulos et al., 2013). According to the systematic review of 48 clinical and experimental studies published by Vakalopoulos KA et al in 2013 (Vakalopoulos et al., 2013), results for the sealing of small intestine and colorectal anastomoses remain inconclusive and predominantly based on animal research. Promising results of fibrin glue sealing on small intestine anastomosis were documented and, for colorectal anastomoses, fibrin glue appeared more advantageous than cyanoacrylates (Vakalopoulos et al, 2013). Nevertheless, in some experimental studies, cyanoacrylate glues showed encouraging effects, particularly in high-risk conditions (Boersema et al., 2017; Wu et al., 2015; Wu et al., 2014).

Nordentoft T et al (Nordentoft et al., 2015) demonstrated in a systematic review of 28 experimental studies that fibrin glue did not have a consistently positive influence on the healing of gastrointestinal anastomoses and considered plausible that the potential positive effects of fibrin glue sealing were related mainly to its mechanical effect. Likewise, Stergios K et al (Stergios et al., 2017) found no evidence to support the use of fibrin sealants as adjunct of colorectal anastomotic healing in the context of severe diabetes.

Furthermore, the ideal tissue adhesive has not yet been found (Vakalopoulos et al., 2017). In a recent experimental research, cyanoacrylates exhibited mild clinical and immunohistopathological effects while maintaining high anastomotic strength and were considered promising as colonic anastomotic sealants. On the contrary, albumin based adhesives (gelatin-resorcinol-formaldehyde and glutaraldehyde-albumin glues) were considered the least suitable. Polyethylene glycol adhesives (polyethylene glycol, trilisine amine, blue dye and N-hydroxy succinimide) and fibrin glue showed low mechanical strength (Vakalopoulos et al., 2017).
Regardless numerous experimental studies in colorectal surgery, anastomotic sealing with tissue adhesives has not yet been applied into the everyday clinical practice (Vakalopoulos et al., 2017).

Despite extensive research, no substantial evidence was documented to justify the implementation of any of the referred promoting strategies of intestinal anastomotic repair for routine clinical use. Growth factors and hormones (insulin-like growth factor, growth hormone, granulocyte-macrophage colony-stimulating factor and erythropoietin), reinforcement with tissue adhesives, hyperbaric oxygen therapy (HBOT), inhibitors of matrix metalloproteinases and iloprost were the most promising interventions (Vakalopoulos et al., 2017; Nerstrom et al., 2016; Oines et al., 2014; Vakalopoulos et al., 2013; Pommergaard et al., 2012). Logistical constraints, absence of approval for human application and the risk of undesired side-effects, such as the potential development of anastomotic strictures, peritoneal adhesions and carcinogenesis, were some of the limitations of the described adjuvants. Unfortunately, at the moment, no convincing conclusions were drawn about its clinical applicability, efficacy and safety. More experimental and clinical studies are needed before those strategies can be recommended for clinical practice. Research on anastomotic healing may benefit also from a more systematic approach.

### 1.5. Growth factors as promoters of intestinal anastomotic healing

Several experimental studies analyzing the effects of growth factors, hormones and analogues on the gastrointestinal anastomotic healing suggested potential interferences in every phases of the repair process (Rijcken et al., 2014). Insulin-like growth factor 1, vascular endothelial growth factor, epidermal growth factor, heparin-binding epidermal growth factor, basic fibroblast growth factor, transforming growth factor β and platelet-derived growth factor participate in the cellular migration and proliferation, angiogenesis and extracellular matrix synthesis; and its exogenous administration appears to increase the mechanical strength of intestinal anastomosis (Rijcken et al., 2014; Oines et al., 2014).

In present investigation, a literature search was conducted including all relevant articles published between January 1, 2000 and July 22, 2017. Prospective controlled
studies that investigated a growth factor or hormone with the purpose of promoting intestinal anastomotic healing under uncomplicated or complicated conditions were included. The search was performed using the PubMed version of Medline database and two different syntaxes (search #1 and search #2, described in the Supplementary Table S1). Articles selected through title and abstract screening were then subjected to full-text analysis. Review articles and studies on other types of anastomosis (for example, gastrojejunal, esophagojejunal, pancreatojejunal and bilioenteric anastomosis) were excluded.

A total of 395 and 111 records were identified in search #1 and in search #2, respectively. Article titles and abstracts were screened and 39 eligible articles were identified. Cross-references from the included studies were manually reviewed and 10 other articles were found. Evaluation of the reporting quality and of the internal or external validity was not formally performed.

A comprehensive review of 49 experimental studies was undertaken (Table 1.3). In fourteen articles (28.6%), full-text was unavailable and only the abstract was analysed.

Nineteen growth factors and hormones were assessed for potential improvement of intestinal anastomotic healing. They included insulin-like growth factor 1, granulocyte-macrophage colony-stimulating factor, basic fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor, platelet-derived growth factor, heparin-binding epidermal growth factor, glucagon-like peptide 2, transforming growth factor β, erythropoietin, growth hormone, melatonin, leptin, ghrelin, octreotide, lanreotide, thyroid hormone, adrenomedullin and nandrolone. Effects of platelet-rich plasma and growth factor-rich plasma were also analyzed. Majority of studies were performed in a rodent model (n=43; 87.8%), were conducted in high risk context (n=32; 65.3%), involved colonic anastomosis (n=38; 77.6%), recurred to systemic administration of the drug (n=30; 61.2%) and reported clearly beneficial effects of the investigated compound (n=42; 85.7%). Complicated anastomotic conditions included ischemia, hypoxia, intra-abdominal sepsis, chemotherapy (systemic or intraperitoneal), radiotherapy, pharmacological immunosuppression (corticosteroid treatment or other), intestinal obstruction, obstructive jaundice, open abdomen and chemically-induced colitis.
Most investigated compounds were insulin-like growth factor 1, granulocyte-macrophage colony-stimulating factor, platelet-rich plasma, growth hormone and erythropoietin. All seem to potentially increase the bursting pressure of the anastomosis (Table 1.3).

Insulin-like growth factor 1, growth hormone and granulocyte-macrophage colony stimulating factor were shown to be beneficial to intestinal anastomotic healing in most of the experimental studies. In humans, insulin-like growth factor 1 is used in cases of growth hormone insensitivity and severe insulin-resistance and is associated with relevant side effects, such as hypoglycemia, hyperplasia of lymphoid tissues, retinal edema and severe myalgias (Frysak et al., 2015; Rosenbloom, 2009). Growth hormone is approved for the treatment of adult patients with short-bowel syndrome, but is associated with relevant adverse effects, including peripheral edema, arthralgias, sleep disturbances and others (Pironi et al., 2016).

Granulocyte-macrophage colony-stimulating factor is a pleiotropic cytokine that activates granulocyte and macrophage cell lineages. It is also known to have an important function in wound repair (Rho et al., 2015), with influence on inflammation, reepithelialization, neovascularization (Mann et al., 2006) and immune response (Yan et al., 2017). In a previously mentioned systematic review and meta-analysis of pre-clinical studies, Nerstrom M et al (Nerstrom et al., 2016) assessed the efficacy of therapeutic agents against colonic anastomotic leakage on high-risk conditions; positive effects of granulocyte-macrophage colony-stimulating factor were found in the context of segmental ischemia and obstructive jaundice, although it failed to show a significant favorable influence in chemotherapy (Nerstrom et al., 2016). Nowadays, granulocyte-macrophage colony-stimulating factor is approved for the treatment of adults with acute myeloid leukemia and submitted to bone marrow/peripheral blood stem cell transplantation and its potential side effects included fluid retention, respiratory and cardiovascular symptoms, renal and hepatic dysfunctions (Francisco-Cruz et al., 2014).

Platelet-rich plasma (PRP) is an autologous blood-derived product enriched in platelets, growth factors, adhesive proteins, angiogenic factors, chemokines, cytokines, clotting factors and their inhibitors, membrane proteins and immune mediators, and used in a variety of clinical applications (Pavlovic et al., 2016). Most important growth
factors comprised in the platelet-rich plasma are vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, insulin-like growth factor, transforming growth factor β and hepatocyte growth factor. These bioactive molecules are involved in clotting, inflammation, chemotaxis, mitogenesis, cellular differentiation and host defense. Growth factor-rich plasma is characterized, in comparison with platelet-rich plasma, by a moderate platelet concentration and absence of leucocytes and fibrin scaffold (Giusto et al., 2017). In humans, platelet-rich plasma is being increasingly used as a biological enhancer for tissue healing in the treatment of musculoskeletal soft tissue injuries, with a low incidence of side effects, although with insufficient clear evidences of benefit (Moraes et al., 2014). Heterogeneity of platelet-rich plasma preparations may influence their biological properties and impair the evaluation of efficacy (Kaux et al., 2017). Overall, results of platelet-rich plasma and growth factor-rich plasma as adjuvants of anastomotic healing were inconsistent (Giusto et al., 2017) (Table 1.3).

Platelet-derived growth factor, transforming growth factor β, epidermal growth factor, basic fibroblast growth factor, vascular endothelial growth factor and platelet-derived formulas were clinically studied for the topical treatment of diabetic lower-extremity ulcers (Martí-Carvajal et al., 2015; Barrientos et al., 2014) and its safety profiles were considered unclear (Martí-Carvajal et al., 2015).

Erythropoietin (EPO) is the main hormonal regulator of erythropoiesis and recombinant erythropoietin emerged as the leading drug for the treatment of anemia from chronic kidney disease and other causes. Side effects of erythropoietin include arterial hypertension, vascular access thrombosis and major adverse cardiovascular events, including myocardial infarction and stroke (Palmer et al., 2014).

A previously referred meta-analysis (Oines et al., 2014) identified pharmacologic compounds with the potential to improve colonic anastomotic healing under non-complicated conditions, including insulin-like growth factor 1, growth hormone and erythropoietin, which were associated with an enhancement of the weighted mean anastomotic bursting pressure of 61 mmHg (95% CI 43-79), 21 mmHg (95% CI 7-35) and 45 mmHg (95% CI 14-76), respectively (Oines et al., 2014).
Table 1.3. Animal studies on the potential promoting effects of the exogenous administration of growth factors, hormones and analogues on the intestinal anastomotic healing published from 2000 (n=49)

<table>
<thead>
<tr>
<th>Study</th>
<th>Drug</th>
<th>Location</th>
<th>Context</th>
<th>Species</th>
<th>Sample size</th>
<th>Dosage and schedule</th>
<th>Route</th>
<th>Time of evaluation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuchs TF et al, 2012</td>
<td>IGF-1</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>48</td>
<td>IO</td>
<td>Local (coated suture)</td>
<td>Day 3</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Rijcken E et al, 2010</td>
<td>IGF-1</td>
<td>Colon</td>
<td>Dextran sodium sulphate colitis</td>
<td>Rats</td>
<td>120</td>
<td>IO</td>
<td>Local (coated suture)</td>
<td>Days 1, 3 and 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Ingle RA et al, 2008</td>
<td>IGF-1</td>
<td>Colon</td>
<td>Mycophenolate mofetil</td>
<td>Rats</td>
<td>63</td>
<td>1 mg/kg/day</td>
<td>IP</td>
<td>Days 2, 4 and 6</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Zacharakis E et al, 2007</td>
<td>IGF-1</td>
<td>Colon</td>
<td>S-fluorouracil</td>
<td>Rats</td>
<td>80</td>
<td>2 mg/kg/day</td>
<td>IP</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Zacharakis E et al, 2003b</td>
<td>PDGF-BB</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rabbits</td>
<td>40</td>
<td>Local</td>
<td>Day 4 Beneficial (only in IP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rijcken E et al, 2017</td>
<td>EGF</td>
<td>Small intestine</td>
<td>Ischemia</td>
<td>Rabbits</td>
<td>16</td>
<td>2 μg/kg/day</td>
<td>IO</td>
<td>Local</td>
<td>Days 3 and 7</td>
</tr>
<tr>
<td>Mantzoros I et al, 2004a</td>
<td>GM-CSF</td>
<td>Small intestine</td>
<td>Ischemia</td>
<td>Rats</td>
<td>144</td>
<td>50 μg</td>
<td>IO</td>
<td>Local</td>
<td>Days 3 and 7</td>
</tr>
<tr>
<td>Demirer S et al, 2004a</td>
<td>GM-CSF</td>
<td>Small intestine</td>
<td>Ischemia</td>
<td>Rats</td>
<td>144</td>
<td>50 μg</td>
<td>IO</td>
<td>Local</td>
<td>Days 3 and 7</td>
</tr>
<tr>
<td>Erden E et al, 2002</td>
<td>GM-CSF</td>
<td>Colon</td>
<td>S-fluorouracil</td>
<td>Rats</td>
<td>45</td>
<td>50 μg</td>
<td>IO</td>
<td>Local</td>
<td>Day 3 Beneficial (only after S-FU)</td>
</tr>
<tr>
<td>Demirer S et al, 2002</td>
<td>GM-CSF</td>
<td>Colon</td>
<td>Long-term methylprednisolone</td>
<td>Rats</td>
<td>80</td>
<td>50 μg/kg/day or 50 μg SC</td>
<td>Local or SC</td>
<td>Day 3</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Akiner R et al, 2017</td>
<td>VEGF-A and FGF-2 (gene therapy)</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>40</td>
<td>1 μg VEGF-A and 1 μg bFGF, IO</td>
<td>Local (plasmid delivery)</td>
<td>Day 4</td>
<td>Beneficial (VEGF-A, FGF-2 and combination)</td>
</tr>
<tr>
<td>Ishii M et al, 2009</td>
<td>VEGF-A</td>
<td>Colon</td>
<td>Normal</td>
<td>Rabbits</td>
<td>10 μg</td>
<td>IO</td>
<td>Local</td>
<td>Days 3 and 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Radulacu A et al, 2011</td>
<td>HB-EGF</td>
<td>Small intestine</td>
<td>Normal</td>
<td>Mice</td>
<td>149</td>
<td>800 μg/kg/day</td>
<td>Enteral</td>
<td>Days 3 and 6</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Radstone HA et al, 2010</td>
<td>GLP-2 and GLP-2</td>
<td>Colon</td>
<td>Hypoxia</td>
<td>Rats</td>
<td>48</td>
<td>GLP-2: 100 μg/kg or 2 μg/kg, 2 id. on days 0 and 3</td>
<td>SC</td>
<td>Day 5</td>
<td>Not clearly beneficial</td>
</tr>
<tr>
<td>Saydenoglu K et al, 2003b</td>
<td>PFGT-BB</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>40</td>
<td>Local (PEG loaded sponge)</td>
<td>Day 7</td>
<td>Beneficial (only in ischemia)</td>
<td></td>
</tr>
<tr>
<td>Migal J et al, 2008</td>
<td>TGF-β1 (gene therapy)</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>24</td>
<td>At day 0 or 3</td>
<td>Local (plasmid delivery)</td>
<td>Day 6</td>
<td>Beneficial (when administered at the 3rd day)</td>
</tr>
<tr>
<td>Gupta G et al, 2017</td>
<td>PRP and GPRP</td>
<td>Small intestine</td>
<td>Normal</td>
<td>Pigs</td>
<td>8</td>
<td>100 μg</td>
<td>IO</td>
<td>Local</td>
<td>Day 8</td>
</tr>
<tr>
<td>Szczurek A et al, 2016</td>
<td>PRP</td>
<td>Colon</td>
<td>Intra-abdominal sepsis</td>
<td>Rats</td>
<td>50</td>
<td>50 μg</td>
<td>IO</td>
<td>Local</td>
<td>Day 7</td>
</tr>
<tr>
<td>Zhou B et al, 2014</td>
<td>PRP</td>
<td>Colon</td>
<td>Open abdomen</td>
<td>Rats</td>
<td>30</td>
<td>10 μg</td>
<td>IO</td>
<td>Local</td>
<td>Day 7</td>
</tr>
<tr>
<td>Yamaguchi R et al, 2012</td>
<td>PRP</td>
<td>Small intestine</td>
<td>Normal</td>
<td>Rats</td>
<td>48</td>
<td>100 μg</td>
<td>IO</td>
<td>Local</td>
<td>Day 5</td>
</tr>
</tbody>
</table>
Table 1.3. Animal studies on the potential promoting effects of the exogenous administration of growth factors, hormones and analogues on the intestinal anastomotic healing published from 2000 (n=49) - Continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Drug</th>
<th>Location</th>
<th>Context</th>
<th>Species</th>
<th>Sample size</th>
<th>Dosage and schedule</th>
<th>Route</th>
<th>Time of evaluation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresno L et al, 2010</td>
<td>PRP</td>
<td>Small intestine</td>
<td>Normal</td>
<td>Pigs</td>
<td>35</td>
<td>IO</td>
<td>Local</td>
<td>Days 1, 2, 3, 4 and 7</td>
<td>Not clearly beneficial</td>
</tr>
<tr>
<td>Yal S et al, 2008</td>
<td>PRP</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>30</td>
<td>IO</td>
<td>Local</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Ozal Turku, U et al, 2012</td>
<td>EPO</td>
<td>Colon</td>
<td>Radiotherapy</td>
<td>Rats</td>
<td>32</td>
<td>500 IU/Kg/day 7 days</td>
<td>IM</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Kzemmer DA et al, 2010</td>
<td>EPO</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>20</td>
<td>5 IU/kg, 24 h before and at day 0</td>
<td>SC</td>
<td>Days 3 and 5</td>
<td>Beneficial at day 5</td>
</tr>
<tr>
<td>Farouquzzaman SK, 2009</td>
<td>EPO</td>
<td>Colon</td>
<td>Intestinal obstruction</td>
<td>Pigs</td>
<td>20</td>
<td>500 IU/kg</td>
<td>SC</td>
<td>Beneficial only in obstruction</td>
<td></td>
</tr>
<tr>
<td>Moran M et al, 2007</td>
<td>EPO</td>
<td>Colon</td>
<td>Intestinal obstruction</td>
<td>Rats</td>
<td>40</td>
<td>500 IU/Kg/day, 7 days</td>
<td>SC</td>
<td>Day 7</td>
<td>Beneficial only in obstruction</td>
</tr>
<tr>
<td>Kuper MA et al, 2016</td>
<td>GH</td>
<td>Colon</td>
<td>Everolimus</td>
<td>Rats</td>
<td>48</td>
<td>2.5 mg/Kg/day 7 days before and 7 days after</td>
<td>SC</td>
<td>Day 7</td>
<td>Beneficial in normal and everolimus contexts</td>
</tr>
<tr>
<td>Adas M et al, 2013</td>
<td>GH and hyperbaric oxygen</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>80</td>
<td>GH 2 mg/Kg/day from day 0 to 6, hyperbaric O$_2$ 3 hours after surgery and continued for 4 days</td>
<td>SC</td>
<td>Day 4</td>
<td>Beneficial in combination</td>
</tr>
<tr>
<td>Wang P et al, 2009</td>
<td>GH and fibrin glue</td>
<td>Small intestine</td>
<td>Traumatic shock with peritonitis</td>
<td>Pigs</td>
<td>163</td>
<td>2 IU/Kg/day, 7 days</td>
<td>SC</td>
<td>Day 10</td>
<td>Fibrin glue and combination beneficial (GH not beneficial)</td>
</tr>
<tr>
<td>Tao TM et al, 2006</td>
<td>GH</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>22</td>
<td>2 mg/kg/day, 7 days before and 4 days after</td>
<td>SC</td>
<td>Day 4</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Yarimkaya A et al, 2003</td>
<td>GH or nandrolone</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>70</td>
<td></td>
<td></td>
<td>Day 3 and 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Çağlıküleç M et al, 2002</td>
<td>GH</td>
<td>Small intestine</td>
<td>Obstructive jaundice</td>
<td>Rats</td>
<td>40</td>
<td>2 mg/kg/day</td>
<td>SC</td>
<td>Day 7</td>
<td>Beneficial in jaundice and normal contexts</td>
</tr>
<tr>
<td>Ozen IO et al, 2007</td>
<td>Melatonin</td>
<td>Small intestine and colon</td>
<td>Bacterial peritonitis</td>
<td>Rats</td>
<td>32</td>
<td>5 and 10 mg/kg/day, 5 days starting in the day before</td>
<td></td>
<td></td>
<td>Beneficial (dose-dependent effect)</td>
</tr>
<tr>
<td>Özdenen O et al, 2007</td>
<td>Melatonin</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>1 mg/kg or 10 mg/kg, at day 0 and 2</td>
<td>SC</td>
<td>Days 3 and 7</td>
<td>Not beneficial</td>
<td></td>
</tr>
<tr>
<td>Ceran C et al, 2013</td>
<td>Ghrelin</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>20</td>
<td>10 mg/kg/day, 7 days</td>
<td>IP</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Karaman K et al, 2012</td>
<td>Thyroid hormone (T3)</td>
<td>Colon</td>
<td>Normal</td>
<td>Rats</td>
<td>30</td>
<td>400 μg/100 g at day 1</td>
<td>SC</td>
<td>Days 3 and 7</td>
<td>Beneficial at day 7</td>
</tr>
<tr>
<td>Karatepe O et al, 2011</td>
<td>Adrenomedullin</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>40</td>
<td>2 μg/day from day 0 to 3</td>
<td>SC</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Colak T et al, 2007</td>
<td>Octreotide</td>
<td>Colon</td>
<td>5-fluorouracil</td>
<td>Rats</td>
<td>40</td>
<td>20 μg/kg/day</td>
<td>SC</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Demireriklis H et al, 2002</td>
<td>Lanreotide</td>
<td>Colon</td>
<td>Intestinal obstruction</td>
<td>Rats</td>
<td>16</td>
<td>5.4 mg/kg, 2 days before</td>
<td>IM</td>
<td>Day 7</td>
<td>Beneficial</td>
</tr>
<tr>
<td>Tazelen A et al, 2004</td>
<td>Leptin</td>
<td>Colon</td>
<td>Ischemia</td>
<td>Rats</td>
<td>48</td>
<td>1 mg/kg, 2 id</td>
<td>IP</td>
<td>Day 7</td>
<td>Beneficial in normal and ischemic contexts</td>
</tr>
</tbody>
</table>

bFGF, Basic fibroblast growth factor; EGF, Epidermal growth factor; EPO, Erythropoietin; GH, Growth hormone; GFRP, Growth factor-rich plasma; GLP-2, Glucagon-like peptide 2; GLP-2 MMB, GLP-2 mimetibody construct; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HB-EGF, Heparin-binding epidermal-like growth factor; IGF-I, Insulin-like growth factor I; PDGF, Platelet-derived growth factor; PRP, Platelet-rich plasma; tKGF, Truncated keratinocyte growth factor; TGF-β, Transforming growth factor β; VEGF, Vascular endothelial growth factor. IM, intramuscular; IO, intraoperative; IP, intraperitoneal; SC, subcutaneous. * Six anastomosis per animal  † Article full-text not retrievable
In spite of some promising results, numerous experimental studies on growth factors as promoters of intestinal anastomotic healing are characterized by poor quality, including low internal validity (high risk of selection, performance and detection bias) and reporting quality (Bosmans et al., 2016; Yauw et al., 2015). Its interpretation is hampered by the heterogeneity of animal models, anatomic localizations of the anastomosis, surgical techniques, context (normal or high-risk), evaluated endpoints and outcome measures, and studied growth factors, including type and administration schedule (dosage, route of delivery, timing and duration). Safety profiles and high cost may constitute additional disadvantages of these drugs. Therefore, further research is necessary to support the use of growth factors in this context.

1.6. Glucagon-like peptide 2 and teduglutide

Glucagon-like peptide 2

Glucagon-like peptide 2 (GLP-2) is a potent and relatively specific gastrointestinal growth factor with intestinotrophic, antisecretory and transit-modulating properties (Drucker and Yusta, 2014).

GLP-2 is a 33-amino acid peptide produced in the enteroendocrine L cells of the intestine, predominantly located in the distal ileum and colon, with glucagon-like peptide 1 (GLP-1), intervening peptide 2 and glicentin, after cleavage of proglucagon by prohormone convertase 1 and 3 (Drucker and Yusta, 2014; Janssen et al., 2013) (Fig. 1.4).

GLP-2 is produced in response to nutritional, hormonal and neural stimulation (Drucker and Yusta, 2014). Secretion occurs in a biphasic pattern in humans (Janssen et al., 2013; Marathe et al., 2013). Early postprandial secretion elapses between 30 to 60 minutes and is probably due to the stimulation by neural and endocrine pathways; second peak develops between 90 to 120 minutes by direct stimulation of intestinal L cells by digested nutrients (including glucose, fatty acids and dietary fibers) (Janssen et al., 2013; Marathe et al., 2013). After ingestion of nutrients, plasma levels of GLP-2 and GLP-1 increase two to five-fold, depending of the size and composition of the meal.
Plasma concentrations of GLP-2 increase, also, in response to intestinal injury or major resection (Drucker and Yusta, 2014).

Figure 1.4. Peptide products of the posttranslational processing of proglucagon in enteroendocrine cells of the intestine. Proglucagon is cleaved by proconvertases 1 and 3 leading to the formation of GLP-2, GLP-1, IP-2 and glicentin. GLP-2, Glucagon-like peptide 2; GLP-1, Glucagon-like peptide 1; GRPP, Glicentin-related pancreatic polypeptide; IP-2, Intervening peptide 2; PC1/3, Prohormone convertase 1/3. Adapted from Janssen et al., 2013

After release, biologically active GLP-2(1-33) is rapidly degraded, through cleavage at the alanine residue in position “2” from the N-terminus, by the ubiquitously expressed exopeptidase dipeptidylpeptidase IV, resulting in the generation of its metabolite GLP-2(3-33) (Drucker and Yusta, 2014; Janssen et al., 2013). GLP-2(3-33) is a pharmacological antagonist of glucagon-like peptide 2 receptor (GLP-2R), although it exerts also a weak agonist activity (Drucker and Yusta, 2014). The enzymatic inactivation and the renal clearance control the elimination of bioactive GLP-2 (Drucker and Yusta, 2014).

In preclinical models, inhibition of dipeptidylpeptidase IV activity slightly increases the effects of GLP-2, but treatment with this enzyme inhibitors does not significantly expand the mucosal epithelium in normal rats (Drucker and Yusta, 2014).
The activity of GLP-2 is transduced through a G protein-coupled receptor (glucagon-like peptide 2 receptor - GLP-2R) expressed almost exclusively in the intestinal tract, stomach and central nervous system (in rodents). GLP-2R expression is considered high in the proximal small intestine, particularly in jejunum, and decrease distally along the longitudinal axis (Drucker and Yusta, 2014; Baldassano et al., 2013; Janssen et al., 2013). Nevertheless, the study of El-Jamal N et al (El-Jamal et al., 2014) did not confirm the decreasing gradient of GLP-2R toward the distal gut. More limited expression of GLP-2R was also observed in the lung, cervix, vagal afferents and heart (Drucker and Yusta, 2014; Janssen et al., 2013), as well as in the liver, pancreas, spleen, bladder, mesenteric fat and lymph nodes (El-Jamal et al., 2014). GLP-2R is highly selective for GLP-2 and reacts only weakly to equimolar concentrations of structurally related peptides such as glucagon, GLP-1 and glucose-dependent insulinotropic polypeptide (Drucker and Yusta, 2014). In the intestine, at the cellular level, GLP-2R was identified in subepithelial myofibroblasts, enteric neurons and some enteroendocrine cells, but, surprisingly, was not detected in crypt epithelial cells and enterocytes, which seem to constitute the main targets of GLP-2 action in vivo (Drucker and Yusta, 2014; Janssen et al., 2013; Dubé et al., 2007).

Signaling pathways transducing the multiple actions of GLP-2 in the target cells of the intestine remain largely unknown. Most GLP-2 effects are indirect and secondary to endocrine, paracrine, autocrine and neural signals activated by the GLP-2R (Drucker and Yusta, 2014; Janssen et al., 2013; Rowland and Brubaker, 2011).

GLP-2 demonstrates a complex and indirect mechanism of action with intricate signaling pathways and multiple mediators’ participation (including insulin-like growth factors 1 and 2, ErbB superfamily of ligands, keratinocyte growth factor, vasoactive intestinal polypeptide, endothelial nitric oxide synthase, and vascular endothelial growth factor) (Drucker and Yusta, 2014; Janssen et al., 2013; Brinkman et al., 2012; Rowland and Brubaker, 2011; Bulut et al., 2008; Dubé et al., 2007). According to the proposed model for the mechanism of action on intestinal epithelial crypt cells, GLP-2 acts via GLP-2R binding on subepithelial myofibroblasts, leading to the release of insulin-like growth factor 1 and, likely, other growth factors, such as ErbB ligands (Rowland and Brubaker, 2011). Insulin-like growth factor 1 binds to the insulin-like
growth factor 1 receptor (IGF-1R) located on crypt epithelial cells and transactivates the ErbB receptor, leading to proliferative responses including phosphatidylinositol-3-kinase (PI3K/Akt) and canonical Wingless (cWnt)/β-catenin signaling (Rowland and Brubaker, 2011) (Fig. 1.5).

**Figure 1.5. Proposed mechanism for the action of GLP-2 on the intestinal crypt cells.** GLP-2 activates the GLP-2R on the subepithelial myofibroblasts leading to the release of IGF-1 (and likely other growth factors, such as ErbB ligands), which then binds to the IGF-1R expressed on the crypt epithelial cells. IGF-1R may transactivate the ErbB-R and origin proliferative responses. ErbB-R, ErbB receptor, GLP-2, Glucagon-like peptide 2; GLP-2R, Glucagon-like peptide 2 receptor; IGF-1, Insulin-like growth factor 1; IGF-1R, Insulin-like growth factor 1 receptor. Adapted from Rowland and Brubaker, 2011

PI3K/Akt signaling activates β-catenin in intestinal epithelial stem and progenitor cells via phosphorylation at Ser552, through a mechanism involving Ras activation and glycogen synthase kinase 3β (GSK3β) phosphorylation. Role of the IGF-1R and/or ErbB receptor expressed by the subepithelial myofibroblasts has not been established, but it may mediate the autocrine effects of myofibroblasts-derived factors, leading to
the release of other growth factors. Several studies suggest also a dose-dependent activation of the cAMP-protein kinase A–cAMP response elements (CRE) B (cAMP-PKA-CREB) pathway by the ligand binding to GLP-2R and inhibition of GSK3β and B-cell lymphoma 2 (Bcl-2) (Rowland and Brubaker, 2011).

Main biological effects of GLP-2 are related to the regulation of energy absorption and maintenance of intestinal mucosa morphology, function and integrity (Janssen et al., 2013). Physiological actions of endogenous GLP-2 receptor signaling include the adaptation of intestinal mucosal growth to refeeding (via the activity of ErbB ligands), the resistance to injury and the antimicrobial mucosal defense mechanisms (through the Paneth cell functions). Proglucagon-derived axis seems to play an important role in the normal human intestinal development and function (Drucker and Yusta, 2014; Janssen et al., 2013).

Several studies, particularly of animal but also clinical experimentation, have suggested that exogenous GLP-2 administration may induce expansion of intestinal absorption surface (through stimulation of proliferation in the epithelial crypt cells and inhibition of apoptosis in the crypt and villi); improvement of macronutrients digestion and absorption (increasing the expression and activity of digestive enzymes and transporters); reduction of intestinal permeability and reinforcement of barrier function (upregulating tight junction proteins zonula occludens-1 and occludin); stimulation of mesenteric perfusion (predominantly in the pancreas, duodenum and jejunum) and portal blood flow; inhibition of gastrointestinal motility, gastric acid hypersecretion and intestinal chloride secretion; repair after injury; anti-inflammatory and antioxidant effects; proangiogenic actions; and increase of distraction enterogenesis (Drucker and Yusta, 2014; Janssen et al., 2013; Sueyoshi et al., 2013; Dubé et al, 2007). Extra-gastrointestinal effects of GLP-2 administration seems to include the stimulation of glucagon secretion (without significant modification of glucose homeostasis), inhibition of bone reabsorption, control of the appetite, and reduction of food intake, gut motility and blood pressure (after direct administration in the central nervous system of rodents) (Drucker and Yusta, 2014).

Potential for GLP-2 to induce carcinogenesis remains controversial (Drucker and Yusta, 2014; Trivedi et al., 2012). Several studies have demonstrated that the
enterotrophic effects of GLP-2 and its analogues are exerted indirectly through multiple mediators (including insulin-like growth factor 1, ErbB ligands and receptors), with activation of signaling pathways that have been implicated in the development of intestinal cancer (Trivedi et al., 2012), namely phosphatidylinositol 3 kinase/protein kinase B and β-catenin (Kannen et al., 2013). Previous experimental studies suggested that sustained pharmacological GLP-2 (and its analogues) administration may promote, rather induce, tumor growth in rodent models of carcinogen-induced and inflammation-associated carcinogen-induced neoplasia (Drucker and Yusta, 2014; Trivedi et al., 2012). Ability of exogenous or endogenous GLP-2R signaling to modify the growth of preexisting intestinal tumors depends on the precise experimental model used (Drucker and Yusta, 2014; Trivedi et al., 2012).

Activation of GLP-2R signaling seems to protect the small and large bowels in several experimental models of intestinal injury, including short-bowel syndrome and major small-bowel resection, parenteral nutrition and fasting-associated gut hypoplasia, chemical enteritis and/or colitis, postchemotherapy and postradiotherapy enteritis, antigen-induced inflammatory bowel disease, infectious enteritis, ischemic insult, immune-mediated hypersensitivity lesion, sepsis, mucosal dysfunction and reduced barrier function (Drucker and Yusta, 2014; Janssen et al., 2013). Degree of mucosal protection was highly dependent on the specific models and timing of GLP-2 administration (Drucker and Yusta, 2014).

Teduglutide

Teduglutide [GLP-2(2-33)] is a long-acting dipeptidylpeptidase IV-resistant equivalent of GLP-2(1-33), obtained through the substitution of alanine by glycine at position “2” that confers higher biological potential and longer half-life (Drucker and Yusta, 2014; Yazbeck et al., 2009; Drucker et al., 2002) (Table 1.4).
In human, half-life of teduglutide is increased compared with native GLP-2 (thirty minutes versus seven minutes and, following subcutaneous injection, 180 to 330 minutes versus 60 to 90 minutes, respectively) (Berg et al., 2014; Naimi et al., 2013).

Teduglutide is currently accepted for pharmacological rehabilitation of patients with short-bowel syndrome associated intestinal failure (Burness and McCormak, 2013) and also considered a promising medication for moderate-to-severe Crohn’s disease (Blonski et al., 2013). In short-bowel context, teduglutide seems to increase energy, fluid, mineral and electrolyte absorption, weight gain, lean body mass, bone density, plasma levels of citrulline, urine output and creatinine clearance; and to decrease parenteral nutrition and/or fluid therapy requirements (Drucker and Yusta, 2014). Several studies have demonstrated that, in patients with short-bowel syndrome, teduglutide treatment is safe, well tolerated and efficacious, with improvement of intestinal absorption and reduction of parenteral support requirements (Naberhuis et al., 2016; Austin et al., 2016). In the study STEPS (Study of Teduglutide Effectiveness in Parenteral Nutrition-Dependent Short-bowel Syndrome Subjects) II, 93% of the patients who completed 30 months of treatment achieved clinical response (defined as a reduction of weekly parenteral support volume of at least 20% from baseline), with a mean parenteral support reduction of 66% (corresponding to 7.6±4.9 l/week), and 33% obtained full enteral autonomy (Schwartz et al., 2016). Teduglutide was recently approved by the European Medicines Agency for treatment of patients with short-bowel syndrome related parenteral support dependence despite optimized medical and dietetic treatment, aged more than one year and who are stable following a period of postsurgical intestinal adaptation (Kim and Keam, 2017; Billiauws et al., 2016).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (Homo sapiens) GLP-2</td>
<td>His - Ala - Asp - Gly - Ser - Phe - Ser - Asp - Glu - Met - Asn - Thr - Ile - Leu - Asp - Asn - Leu - Ala - Ala - Arg - Asp - Phe - Ile - Asn - Trp - Leu - Ile - Gin - Thr - Lys - Ile - Thr - Asp</td>
</tr>
<tr>
<td>Rat (Rattus norvegicus) GLP-2</td>
<td>His - Ala - Asp - Gly - Ser - Phe - Ser - Asp - Glu - Met - Asn - Thr - Ile - Leu - Asp - Asn - Leu - Ala - Thr - Arg - Asp - Phe - Ile - Asn - Trp - Leu - Ile - Gin - Thr - Lys - Ile - Thr - Asp</td>
</tr>
<tr>
<td>Teduglutide</td>
<td>His - Gly - Asp - Gly - Ser - Phe - Ser - Asp - Glu - Met - Asn - Thr - Ile - Leu - Asp - Asn - Leu - Ala - Ala - Arg - Asp - Phe - Ile - Asn - Trp - Leu - Ile - Gin - Thr - Lys - Ile - Thr - Asp</td>
</tr>
</tbody>
</table>

*From references Drucker and Yusta, 2014; Drucker et al., 2002. Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartate; Glu, Glutamate; Gin, Glutamine; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Ph e, Phenylalanine; Ser, Serine; Thr, Tryptophan
Furthermore, in the recently published guidelines of the European Society for Clinical Nutrition and Metabolism, teduglutide was considered the first choice for carefully selected patients with chronic intestinal failure who are candidates for growth factor treatment (Pironi et al., 2016).

Adverse events associated with treatment with GLP-2 analogues include headache, nausea, vomiting, abdominal pain, abdominal distention, ostomal site complications, injection site complaints, nasopharyngitis and development of non-neutralizing antibodies (Drucker and Yusta, 2014; Janssen et al., 2013). Proproliferative and antiapoptotic activities of GLP-2 rise concerns about its carcinogenic risk (Drucker and Yusta, 2014; Trivedi et al., 2012), particularly at long-term and emphasize the importance of careful screening and follow-up of human subjects treated with GLP-2 analogues (Drucker and Yusta, 2014). Nevertheless, as mentioned before, the potential for GLP-2 and its analogues to induce carcinogenesis remains controversial (Drucker and Yusta, 2014; Trivedi et al., 2012; Iakoubov et al., 2009; Koehler et al., 2008; Thulesen et al., 2004). Tumor promotion effects seem to be associated with the exposition to teduglutide plasma concentrations much higher than those obtained with the daily recommended dose (Kim and Keam, 2017). Furthermore, there have been no reports of intestinal dysplasia in patients with short-bowel syndrome-associated intestinal failure treated for up to two years with teduglutide (Schwartz et al., 2016).

Teduglutide has a considerable therapeutic potential, particularly in short-bowel syndrome (preventing or minimizing the parenteral nutrition dependence), but also in others types of acute and chronic intestinal dysfunction, such as inflammatory bowel diseases, radiation and chemotherapy enteropathies, necrotizing enterocolitis, and ischemia/reperfusion lesions (Drucker and Yusta, 2014; Yazbeck et al., 2009; Wallis et al., 2007). However, cellular and molecular mechanisms underlying biological action of GLP-2, precise therapeutic indications, optimal administration methods and adverse effects remain unclear and additional studies are required.
Chapter 2

Framework, hypothesis & objectives
2.1. Framework & hypothesis

Regardless of continuous improvements in surgical technique and perioperative care, intestinal anastomotic failure remains, as mentioned before, one of the most serious postoperative complications, due to its incidence and deleterious consequences (Guyton et al., 2016; Chadi et al., 2016; Vallance et al., 2016; Bosmans et al., 2015; Shogan et al., 2013).

Anastomotic failure has a multifactorial etiology and its pathogenesis continues to be insufficiently understood (Guyton et al., 2016; Chadi et al., 2016; Vallance et al., 2016; Bosmans et al., 2015; Shogan et al., 2013). Therefore, further research on the underlying pathophysiological mechanisms of intestinal wound healing and on innovative perioperative management to improve anastomotic repair, especially in high-risk context, persists necessary.

Glucagon-like peptide 2 (GLP-2) is a gastrointestinal growth factor with a relevant role on the control of energy absorption and on the preservation of intestinal mucosa morphology and function (Drucker and Yusta, 2014). GLP-2 demonstrates a complex, indirect and poorly understood mechanism of action with intricate signaling pathways and multiple mediators’ participation (Drucker and Yusta, 2014; Janssen et al., 2013; Rowland and Brubaker, 2011). GLP-2 exogenous administration has been associated with intestinotrophic, antisecretory, transit-modulating and antiinflammatory effects (Drucker and Yusta, 2014).

Teduglutide is a long-acting equivalent of GLP-2 (Drucker and Yusta, 2014) currently accepted for pharmacological rehabilitation of patients with short-bowel syndrome associated intestinal failure and considered safe, well tolerated and efficacious in this context (Kim and Keam, 2017; Billiauw et al., 2017; Naberhuis et al., 2016; Austin et al., 2016).

Intestinal anastomotic repair is a complex and well-orchestrated multicellular multimolecular process, regulated by cytokines, chemokines, growth factors, reactive oxygen species and other factors (Nerstrom et al., 2016; Rijcken et al., 2014; Oines et al., 2014), and thus, susceptible to the influence of GLP-2 and its analogues. Intestinotrophic and cytoprotective properties of GLP-2, particularly the promotion of
epithelial proliferation, neoangiogenesis and blood perfusion, suggest a potential favorable influence on the inflammatory and proliferative phases of intestinal anastomotic healing. In fact, previous experimental studies indicated potential beneficial effects on anastomotic repair from the exogenous administration of growth factors that are considered mediators of the GLP-2 intestinotrophic actions, including insulin-like growth factor 1, epidermal growth factor, heparin-binding epidermal growth factor, fibroblast growth factor 7/keratinocyte growth factor and vascular endothelial growth factor (Nerstrom et al., 2016; Rijcken et al., 2014; Oines et al., 2014; Drucker and Yusta, 2014; Janssen et al., 2013; Rowland and Brubaker, 2011).

Knowledge of the potential influence of teduglutide on the intestinal anastomotic repair is relevant, not only for patients with short-bowel syndrome undergoing intestinal anastomosis (including autologous intestinal reconstruction procedures) during treatment with this growth factor, but also to explore its potential role as a perioperative adjuvant strategy. Relative specificity of the GLP-2 effects, consequence of the predominant gastrointestinal expression of its receptor (GLP-2R) (El-Jamal et al., 2014; Drucker and Yusta, 2014; Janssen et al., 2013; Baldassano et al., 2013) may constitute an advantage in this context.

Any anastomotic healing-promoting intervention could have a potential enormous positive clinical and socioeconomic impact. As referred previously, albeit numerous experimental studies on this subject, results were insufficient to allow the translation into the daily practice. GLP-2 seems to be critically involved in the physiologic process of intestinal anastomotic repair and, therefore, teduglutide may represent a new therapeutic resource, particularly in high-risk conditions.

In regard to this framework, it was hypothesized that teduglutide may interfere with the cellular, humoral and molecular mediators of intestinal repair and exert a favorable influence on the anastomotic healing (Fig. 2.1).
2.2. Objectives

The present study aimed to analyze the effects of teduglutide administration on the early stages of the intestinal anastomotic healing on an animal model and to elucidate about the underlying cellular, humoral and molecular mechanisms.

An experimental rat model of standard intestinal anastomosis was proposed with evaluation at the third and the seventh postoperative days, corresponding presumably to the predominantly inflammatory and proliferative phases, respectively (Rijcken et al., 2014). Experiment outline includes the evaluation of the anastomosis outcome with an anastomotic complication score and the assessment of structural parameters of anastomotic healing, including inflammatory cells influx, reepithelialization, neoangiogenesis and fibroplasia. Analysis of the cellular, humoral and molecular mediators of anastomotic healing was programmed, including cellular viability and death, oxidative stress, local and systemic inflammatory response, growth factors, putative intestinal epithelial stem cells, extracellular matrix components and remodeling factors. Correlation of the results with Glp-2 plasma concentrations and Glp-2 receptor (Glp-2r) tissue gene expression was also planned.
The ultimate goal of this experiment was to better understand the intestinal repair and to anticipate the role of teduglutide as a promoting strategy of the anastomotic healing.

For this purpose, the following five specific objectives were addressed:

- To analyse the response of putative intestinal epithelial stem cells to teduglutide treatment in the context of intestinal anastomosis repair – Chapter 3;

- To evaluate the influence of teduglutide administration on the intestinal anastomosis outcome, at the macroscopic and microscopic levels – Chapter 4;

- To investigate the effects of teduglutide on the cellular viability and death processes, oxidative stress and mitochondrial function, and inflammatory response on the context of intestinal anastomotic healing – Chapter 5;

- To study the response of tissue growth factors involved in the intestinal anastomotic repair to the teduglutide postoperative administration – Chapter 6;

- To assess the effects of teduglutide short-term administration on the fibrogenesis process of the intestinal anastomotic healing – Chapter 7.
Chapter 3

Response of putative intestinal epithelial stem cells to teduglutide on an animal model of intestinal anastomosis
This chapter was partially published as:


3.1. Abstract

Previous studies suggest that intestinal epithelial stem cells (IESC), critical drivers of homeostasis and regeneration, include two subpopulations: crypt-based columnar and “position +4” stem cells, identified by Lgr5 and Bmi1 biomarkers, respectively. Teduglutide is an enterotrophic long-acting counterpart of glucagon-like peptide 2. This study investigated the response of putative IESC to surgical injury and teduglutide administration on an animal model of intestinal resection and anastomosis. Wistar rats \( (n=62) \) were distributed into four groups: “Ileal Resection and Anastomosis” versus “Laparotomy”, subsequently subdivided into “Postoperative Teduglutide Administration” versus “No Treatment”; and sacrificed at third or seventh postoperative days, with ileal sample harvesting. Flow cytometry was used to analyze IESC with monoclonal antibodies against Lgr5, Bmi1 and also CD44, CD24, CD166 and Grp78 surface markers. Surgical trauma induced an increase of putative epithelial stem cells population at third day \((9.2\pm2.8 \text{ vs. } 5.5\pm2.7\%,\ p=0.0001)\), which was more intense and involved all subpopulations after ileal resection. At seventh day, teduglutide was significantly associated with higher proportion of Lgr5+/Bmi1- cells \((5.9\pm0.4 \text{ vs. } 2.8\pm1.9\%,\ p=0.005)\) and, on the contrary, lower percentage of Lgr5+/Bmi1+ cells \((0.03\pm0.04 \text{ vs. } 1.87\pm0.22\%,\ p=0.049)\) after ileal resection; and higher proportion of Lgr5'/Bmi1+ cells \((1.8\pm0.6 \text{ vs. } 1.2\pm1.3 \%),\ p=0.028)\) after isolated laparotomy. After surgery, Lgr5+/Bmi1- and Lgr5-/Bmi1+ subpopulations demonstrated an inverse correlation and both correlated negatively with Grp78 labeling index. Lgr5'/Bmi1+ and CD44+/CD24low+/CD166+/Grp78+ cells proportions exhibited a high grade positive correlation. Those observations support the existence of two epithelial stem cells subpopulations with distinct behavior after surgical injury and teduglutide treatment.
3.2. Introduction

Intestinal epithelium is one of the most dynamic sites of cell turnover, demonstrating a relevant self-renewing capacity, achieved by crypt stem cell proliferation and crypt-to-villus multilineage differentiation, which constitutes the basis of tissue homeostasis and repair (Barker, 2014; Vanuytsel et al., 2013; Tesory et al., 2013; Barker et al., 2012). Resident stem cells located in the crypt base provide rapidly proliferating transit-amplifying cells that differentiate and give origin to all the seven cellular types of the epithelium (Barker, 2014) (Fig. 3.1).

**Figure 3.1. Crypt-to-villus architecture of the intestinal epithelium.** Stem cells located at the crypt base (crypt base columnar and “+4” cells) provide rapidly proliferating transit-amplifying cells that differentiate and migrate up to the villus tip, where they undergo anoikis. Differentiated epithelial cells comprise enterocytes, enteroendocrine cells, goblet cells, Paneth cells, tuft cells, microfold (M) cells and cup cells. Under homeostatic conditions, transit along the crypt-villus axis takes three to five days. Paneth cells constitute an exception because they are renewed only every three to six weeks and follow a downward migratory path from the transit-amplifying compartment to the crypt bottom. Adapted from Barker N, 2014 and Smith RJ et al., 2017
Recently, an unifying model of adult intestinal epithelial stem cells (IESC) emerged, considering two functionally different populations, coexisting and interconvertible, corresponding to crypt base columnar stem cells and “position +4” stem cells, respectively (Barker, 2014; Clevers, 2013; Barker et al., 2012; Rizk and Barker, 2012; Yan et al., 2012). Crypt base columnar cells, intercalated between Paneth cells at the base of the crypts, are considered actively cycling cells, very sensitive to canonical wingless (wnt) signaling and susceptible to radiation injury, which give origin to transit-amplifying cells on an everyday basis, ensuring the homeostatic self-renewal. “Position +4” stem cells, localized preferentially at the fourth position from the crypt base, immediately above the Paneth cells compartment, are considered slow-cycling reserve cells that guarantee injury-induced regeneration (Barker, 2014; Clevers, 2013; Barker et al., 2012; Rizk and Barker, 2012; Yan et al., 2012; Tian et al., 2011) (Fig. 3.2).

**Figure 3.2. Model of adult intestinal epithelial stem cells.** Two functionally different epithelial stem cells populations are considered to exist: crypt base columnar and “position +4” stem cells. Crypt base columnar stem cells, intercalated with Paneth cells at the base of the crypts, are considered actively cycling cells and continuously generate rapidly proliferating transit-amplifying cells, which subsequently differentiate into the mature lineages of the villi, ensuring the homeostatic self-renewal of the epithelium. “Position +4” stem cells, localized immediately above the Paneth cells, are considered slow-cycling reserve cells that can restore the crypt base columnar cells compartment following injury. Adapted from Barker N, 2014
Stem cells homeostasis is maintained by a neutral drift clone dynamics guaranteed by the stem cell niche and the complex interplay among multiple signaling pathways, including wnt, hedgehog, bone morphogenic protein (bmp), notch, hippo-yap and ephB/ephrin B (Table 3.1; Fig. 3.3) (Bloemendaal et al., 2016; Barker, 2014; Gracz and Magness, 2014; Vanuytsel et al., 2013).

### Table 3.1. Intestinal epithelial stem cell signaling pathways

<table>
<thead>
<tr>
<th>Signaling pathway</th>
<th>Upward gradient (from crypt base)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt</td>
<td>Decreasing</td>
<td>Most important factor in the maintenance of the self-renewing phenotype of IESC; Induction of Paneth cells differentiation; Regulation of crypt-to-villus migration (through cell-contact dependent EphB/Ephrin signaling).</td>
</tr>
<tr>
<td>Notch</td>
<td>Decreasing</td>
<td>Preservation of IESC self-renewal (in synergism with wnt); Promotion of absorptive lineage commitment through lateral inhibition (and inhibition of secretory lineage differentiation).</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Increasing</td>
<td>Sensing of the epithelial integrity; Confinement of the wnt responsive compartment to the base of crypts (through bmp4); Maturation and homing of underlying stromal cells; Modulation of the proinflammatory response.</td>
</tr>
<tr>
<td>Bmp</td>
<td>Increasing</td>
<td>Prevention of IESC overproliferation and maintenance of an undifferentiated state; Differentiation of enteroendocrine cells; Complete maturation of the secretory cell lineage.</td>
</tr>
<tr>
<td>Hippo/Yap</td>
<td>Increasing</td>
<td>Suppression of the proliferation of differentiated cells through wnt and notch inhibition (prevention of uncontrolled proliferation without differentiation)</td>
</tr>
<tr>
<td>EphB/Ephrin</td>
<td>Decreasing for EphB3 and EphB4; Increasing for EphB1 and EphB2</td>
<td>Coordination of cellular migration; Modulation of actin cytoskeleton.</td>
</tr>
</tbody>
</table>

Bmp, Bone morphogenic protein; Bmp4, Bone morphogenic protein 4 ligand; IESC, Intestinal epithelial stem cells; Hippo-Yap, Hippo-Yes-associated protein; Wnt, Wingles. * From references Bloemendaal et al., 2016; Gracz and Magness, 2014; Vanuytsel et al., 2012

The stem cell niche is the complex and dynamic microenvironment in which epithelial stem cells reside and that regulates its behavior during tissue homeostasis and regeneration (Smith et al., 2012). The stem cell niche is constituted by epithelial and mesenchymal components, including Paneth cells, subepithelial myofibroblasts, smooth muscle cells of the *muscularis mucosae*, lymphatic and vascular endothelial cells, bone-marrow derived stromal cells, neural cells, intraepithelial lymphocytes, extracellular matrix, mesenchymal stem cells, among others (Tesori et al., 2013; Roth et al., 2012). In the niche, the bidirectional dynamic regulatory network between epithelial and

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65
mesenchymal cells includes the hedgehog, bmp and wnt pathways. Hedgehog signaling from differentiated epithelial cells induces stromal synthesis of Bmp ligands. Active Bmp signaling within epithelial cells contributes to the maintenance of the differentiated state and the reduction of proliferative activity. Production of canonical wnt ligands by Paneth and pericryptal mesenchymal cells promotes epithelial stem cells self-renewal. Secretion of Bmp antagonists by the underlying mesenchymal cells protects epithelial stem cells from the differentiation induced by bmp ligands (Smith et al., 2017).

Figure 3.3. Proposed signaling pathways in the crypt-villus axis. Wingless (wnt) and notch signaling are highly active at the base of the crypts, contributing to the stem cell renewal. On the contrary, bone morphogenic protein (bmp), hedgehog (Hh) and hippo signaling are predominant at the villi, inducing differentiation, and their activity decrease towards the crypts. Hh signaling from the differentiated epithelial cells induces the synthesis of bmp ligands in the mesenchymal cells, in a paracrine way, which contribute to the maintenance of the differentiated state and to the inhibition of proliferation. Production of wnt ligands by Paneth cells and pericryptal mesenchymal cells promotes epithelial stem cells self-renewal and induces the Paneth cells differentiation. Secretion of bmp antagonists by the underlying pericryptal mesenchymal cells protects epithelial stem cells from the differentiation induced by bmp ligands. Adapted from Bloemendaal ALA et al., 2016 and Smith RJ et al., 2017

According to genetic marking and lineage tracing studies, leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5/Gpr49) and B-cell-specific Moloney
murine leukemia virus insertion site 1 (Bmi1) seem to be markers that identify the two functionally different intestinal epithelial stem cells (Yan et al., 2012).

Lgr5 is a wnt/β-catenin target gene that encodes an orphan G-protein coupled receptor for the wnt agonists roof plate-specific spondins (R-spondins) 1 to 4 (Barker, 2014; Clevers, 2013; Tesori et al., 2013; Kemper et al., 2012; Barker et al., 2012). Lgr5 has been considered a specific marker of crypt base columnar epithelial stem cells (Barker et al., 2012; Barker, 2010; Sato et al., 2009; Barker et al., 2007).

Bmi1 is a polycomb ring finger oncogene that encrypt a polycomb group protein, component of the transcriptional polycomb repressive complex 1, considered to be involved in the modulation of the stem cell self-renewal, pluripotency and lineage specification in several tissues (Barker et al., 2012; Rajasekhar et al., 2007). Bmi1 is the most widely recognized marker of “position +4” intestinal epithelial stem cells (Rizk and Barker, 2012; Sangiorgi et al., 2008). Nevertheless, specificity of Bmi1, as well as that of other proposed markers of “position +4” epithelial stem cells such as Tert, Hopx and Lrig1, has been challenged by the expression analysis with single-molecule in situ hybridization techniques (Koo et al., 2014; Clevers, 2013;).

Teduglutide is a long-acting counterpart of glucagon-like peptide 2 (GLP-2), which is a relatively specific gastrointestinal growth factor crucial for the maintenance of intestinal mucosa morphology, function and integrity (Drucker and Yusta B, 2014; Burness and McCormak, 2013).

Regeneration of the epithelial architecture after the intestinal disruption triggered by surgical resection and anastomosis is based on a wound repair process that includes restitution, crypt stem cell proliferation and differentiation, and crypt fission (Bloemendaal et al., 2016; Rijcken et al., 2014; Iszuka et al., 2011). Influence of teduglutide on crypt base columnar and “position +4” stem cells dynamics in the perioperative context of intestinal resection is not yet well understood.

This study aimed to analyze the response of putative intestinal epithelial stem cells to surgical injury and teduglutide short-term administration on an animal model of intestinal resection and anastomosis.
3.3. Methods

3.3.1. Ethical statement

Study protocol was approved by the institution’s Ethics Committee (Official Letter n° 32-06-09) and was implemented in consonance with the institutional and national guidelines respecting animals’ protection in experimental research. Experiment was conducted according to the “Animal Research: Reporting in Vivo Experiments” (ARRIVE) guidelines (Kilkenny et al., 2010a; Kilkenny et al., 2010b).

3.3.2. Animals

Adult male Wistar albinus rats (Rattus norvegicus albinus) weighting 250 to 300 g were acclimatized to the laboratory environment for five days before the experimental study, kept in temperature (22±1ºC) and humidity (50±10%) controlled ventilated cages, with light/dark cycles of 12 hours, and maintained on water and standard rodent diet (5L79 Purina rat and mouse 18% chow; Charles River Laboratories, Wilmington, Massachusetts, USA) ad libitum. Animals were supplied by the animal colony of the Laboratory for Experimental Research of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal.

3.3.3. Study Design

Rats were randomly assigned into four different experimental groups: (1) “Res Ted +”, (2) “Res Ted –”, (3) “Lap Ted +” and (4) “Lap Ted –”. Group “Res Ted +” rats were submitted to standard ileal resection and anastomosis (Res), teduglutide postoperative administration (Ted) and sacrifice at the third or seventh day, respectively (two subgroups) (Fig. 3.4). In group “Lap Ted +”, a laparotomy was performed (Lap; without resection), teduglutide was administered and sacrifice occurred at the third or seventh day, respectively (two subgroups). Group “Res Ted –” rats were subjected to intestinal resection and anastomosis, and were sacrificed at the third or seventh postoperative days, respectively (two subgroups). Finally, in group “Lap Ted –”, laparotomy was
performed and sacrifice occurred at the third or seventh days, respectively (two subgroups). “Lap” and “Ted -” groups were considered controls. Expected number of animals to be involved in the study corresponded to approximately eight for each subgroup.

**Figure 3.4. Study design.** Adult male Wistar albinus rats were randomly distributed into four groups: “Ileal Resection and Anastomosis” (“Res”) versus “Laparotomy” (“Lap”), each one split into “Postoperative Teduglutide Administration” (“Ted +”) versus “No Treatment” (“Ted -”). Evaluation was performed at the moments of the operation and sacrifice, at the third or seventh postoperative days (eight subgroups), with ileal segment harvesting and blood collection. Tissue baseline values of “Ileal Resection and Anastomosis” groups were considered for comparison with postoperative results of the “Laparotomy” groups; tissue samples recovered at the sacrifice in these animals corresponded to the perianastomotic segments.

**3.3.4. Operative Procedures**

All the operative procedures were accomplished by the same investigator after a period of two hours solid fasting (water was never restricted), with clean surgical technique, under anesthesia with intraperitoneal injection of a combination of ketamine hydrochloride (75 mg/kg; Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg; Laboratórios Vitória, Amadora, Portugal).

In “Res” groups, a 10-cm length ileal resection was performed, preserving distal 5 cm, after a 3-cm midline laparotomy. Continuity was reestablished by a standardized end-to-end anastomosis with eight equidistant full-thickness polydioxanone United States Pharmacopeia (USP) 6/0 stitches (PDS II; Ethicon, Johnson-Johnson Intl, Cincinnati,
USA). Abdominal wall was closed with two running sutures (muscle-aponeurotic and cutaneous) of braided coated polyglactin 910 USP 4/0 (Surgilactin, Sutures Limited, Wrexham, UK) and natural silk USP 4/0 (Surgisilk, Sutures Limited, Wrexham, UK), respectively. In “Lap” groups, a 3-cm midline laparotomy (without resection) was performed, with mild manipulation of the small-bowel. In the first postoperative day, ingestion was restricted to water with 5% glucose (at a 1:1 ratio) and, then, *ad libitum* oral hydration and chow were reassumed.

Rats were monitored on a daily basis throughout the entire length of the experiment. Quotidian evaluation included the activity grade, sleep patterns, food and water ingestion, elimination and other. Operative morbidity and mortality were recorded. A daily health score of the animals was calculated, as described previously by van Landeghem L *et al* (Van Landeghem *et al.*, 2012a), by the sum of points attributed by the four following parameters: activity (active = 1 and stationary = 0), posture (normal = 1 and hunched = 0), pelage (normal grooming, smooth and healthy-looking fur = 1 and lack of grooming and rough fur = 0) and dehydration (none = 1 and dehydrated = 0; dehydration was tested by gently raising a small piece of dorsal skin and was considered to be present if the skin stayed up in a tent or only slowly retracted back to normal shape). Overall health score, ranging from “0” to “4”, was determined every day during the three or seven day-time courses following the operation (according to the study groups). Any rat assigned an overall health score of “0” was euthanized in accordance with the protocol. Survival data corresponded to those animals that did not fulfilled the health score criteria for euthanasia.

At the third or the seventh postoperative days, animals were sacrificed by cervical displacement and subjected to relaparotomy with ileal resection (10-cm length, retaining distal 3 cm). During relaparotomy, the peritoneal cavity was carefully inspected.

### 3.3.5. Drug regimen

In “Ted +” groups, teduglutide (American Peptide Company, Sunnyvale, California, USA) was administered subcutaneously during the postoperative period (from the
operation day) at 200 μg/kg body weight per day, diluted in 0.25 ml phosphate buffered saline pH 7.4 (PBS pH 7.4, Gibco, Life Technologies, Carlsbad, California, USA) after reconstitution in agreement with the manufacturer’s recommendations.

Teduglutide, [Gly2]GLP-2 peptide, was purchased in a lyophilized form. Purity of the product was 98.8% (as confirmed by high performance liquid chromatography and mass spectral analysis), molecular weight was 3752.1 Da and the white lyophilized powder contained 91.8% of peptide. Lyophilized peptide was stored in a freezer at -20°C under dry conditions for maximum stability and was warmed to room temperature prior to opening and dissolving. Necessary amount of peptide for the day was aliquoted, the vial was resealed tightly and the remaining was stored at -20°C. Peptide was dissolved in 5% ammonium hydroxide in water to a concentration of 1 mg/ml and the product was then diluted with phosphate buffered saline pH 7.4.

3.3.6. Tissue sampling

In “Res” groups, a 10-cm length ileal segment was carefully removed, opened at the mesenteric side, gently rinsed with normal saline solution and divided in four samples: one, the most distal, with 4 cm and three, with 2 cm. First 4-cm length ileal fragment was split into three similar longitudinal strips (each one corresponding to one third of the circumference) that were immediately subjected to further processing: tissue dissociation and cell separation procedures (for cellular characterization by flow cytometry, in this study), homogenization (for determination of tissue levels of cytokines by flow cytometric bead assay and for quantitative real-time reverse-transcription polymerase chain reaction - qRT-PCR), and fixation with 10% formaldehyde (Sigma-Aldrich, Sintra, Portugal) followed by paraffinization (for histological examination), respectively. Other three contiguous ileal specimens were retrieved and prepared for subsequent studies: one fixed in 10% formaldehyde for histological examination; one placed in a cryotube, frozen in liquid nitrogen and stored at -80°C; and the remaining preserved in RNA-later (Sigma-Aldrich, Sintra, Portugal), respectively and successively (Fig. 3.5 A). Samples for molecular studies were weighted
on a high-precision digital scale (Kern 770 Electronic Analytical Balance; Kern & Sohn GmbH, Ziegrlei, Balinger, Germany).

Figure 3.5. Tissue harvesting. A. Initial resection. A 10-cm length ileal resection was performed with preservation of distal 5 cm. Four contiguous ileal segments were recovered: one with 4-cm and three 2-cm length. Most distal sample was divided into three longitudinal strips for histological examination (A) and molecular studies (flow cytometry and qRT-PCR) (B and C); the other three contiguous specimens were prepared, fixed in 10% formaldehyde for histological examination (D), frozen in liquid nitrogen and stored at -80˚C (E), and preserved in RNA-later (F), respectively. B. Relaparotomy. A 10-cm ileal resection retaining distal 3 cm was undertaken. Four contiguous ileal segments (one with 4-cm and three 2-cm length) were retrieved. Most distal ileal sample, with the anastomosis in the middle, was divided into three longitudinal sections for histological examination (A) and molecular studies (flow cytometry and qRT-PCR) (B and C); the other three contiguous specimens were prepared, fixed in 10% formaldehyde for histological examination (D), frozen in liquid nitrogen and stored at -80˚C (E), and preserved in RNA-later (F), respectively.

In all groups, during relaparotomy, the procedure was analogous to the previously described (Fig. 3.5 B). In “Res” groups, distal samples recovered at the sacrifice corresponded to the anastomotic segment and included the anastomosis in the
middle. Tissue baseline values of “Res” groups were considered for comparison with postoperative results of the “Lap” group.

3.3.7. Intestinal tissue dissociation and cell separation procedure

Cells were isolated from one ileal longitudinal strip, immediately after excision, by a standardized adaptation of the collagenase/dispase isolation technique outlined by Evans GS et al (Evans et al., 1992) and Dekaney CM et al (Dekaney et al., 2007; Dekaney et al., 2005), to obtain a cellular population predominantly constituted of epithelial and some stromal cells.

Summarily, the ileal sample was cleaned of mesentery, flushed with Hank’s balanced salt solution (HBSS, Gibco, Life Technologies, Carlsbad, California, USA), cut into pieces of approximately 3 mm length and washed six times in 10 ml of HBSS on an orbital shaker (80 cycles/minute). Fragments were cut into 1 mm pieces and shaken vigorously in HBSS containing 2 ml of enzyme solution, on a shaking platform, for 25 minutes at room temperature. Enzyme solution contained 0.1 mg/ml of dispase type II (Gibco, Life Technologies) and 5 mg/ml of collagenase type IV (Gibco, Life Technologies) in HBSS.

Digested tissue was further disaggregated by vigorous up and down hand pipetting for five minutes, transferred to a conical tube and allowed to sediment under gravity for one minute; then, the supernatant was removed into a new tube and sedimentation was repeated twice. Subsequently, 4 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) in fetal bovine serum 10% (DMEM 10%) (Gibco, Life Technologies) was added to supernatant, mixed and spin at 470x g for three minutes. Pellet was collected, resuspended in 4 ml of DMEN 10% and the procedure was repeated once. Cell pellets were combined in DMEN 10% and passed through a 70 μm filter to obtain a single cells suspension. Isolated cells in phosphate buffered saline (PBS, pH 7.4, Gibco, Life Technologies) were centrifuged at 1000x g, at room temperature, for five minutes, resuspended in 500 μl of PBS and distributed for ten different tubes for flow cytometry (50 μl/vial). Three vials were used for multiparameter flow cytometry.
cellular immunophenotyping and the remaining for additional studies (four for oxidative stress study and one for cell viability and death evaluation).

3.3.8. Multiparameter flow cytometry cellular phenotyping

Characterization of the isolated cells from rats’ ileum was performed by multicolor flow cytometry. Single cells suspensions were stained with monoclonal antibodies directed against cell surface markers using a direct immunofluorescence technique, following the manufacturer’s instructions.

Identification of the putative epithelial stem cell populations was accomplished with the cell surface markers Lgr5 and Bmi1 (Barker et al., 2014), markers of crypt base columnar and “position +4” stem cells, respectively. Epithelial stem cells population (Lgr5+ and/or Bmi1+ cells) was considered to include three fractions: Lgr5+/Bmi1− [corresponding presumably to crypt base columnar stem cells (Barker et al., 2012; Yan et al., 2012; Sato et al., 2009; Barker et al., 2007)], Lgr5−/Bmi1+ [representing possibly putative “position +4” stem cells (Barker et al., 2012; Rizk and Barker, 2012; Yan et al., 2012; Sangiorgi et al., 2008)] and Lgr5+/Bmi1+ (double positive) cells.

Additionally, putative intestinal epithelial stem cells were studied with a combination of CD44, CD24, CD166 and Grp78 surface markers according to Wang F et al (Wang et al., 2013) (Fig. 3.6). These authors proposed the use of CD44+/CD24low/CD166+/Grp78low and CD44+/CD24low/CD166+/Grp78+ combinations to identify putative epithelial stem cells and transit-amplifying cells, respectively (Wang et al., 2013). Cytokeratin 18 (CK18), CD31 and CD45 surface markers were used to recognize the epithelial, endothelial and hematopoietic cellular differentiations, respectively; vimentin, α-smooth muscle actin (α-Sma) and desmin markers were used to label mesenchymal cells (Rekhtman and Bishop, 2011). Main characteristics of the stem cell surface markers and references of the monoclonal antibodies used in present study were included in Supplementary Tables S2 and S3, respectively. The flow cytometry panel used is represented in Supplementary Table S4. Antibodies combinations included: Anti-Lgr5 / Anti-Bmi1 + Anti-goat IgG Phycoerythrin (PE) / Anti-CD45 / Anti-CD31; Anti-desmin / Anti-CK18 / Anti-α-Sma + Goat anti-rabbit
IgG-Peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5) / Anti-vimentin; Anti-78 KDa glucose-regulated protein (anti-Grp78) / Anti-CD166+Anti-goat IgG PE / Anti-CD24 / Anti-CD44.

**Figure 3.6. Fluorescence-activated cell sorting (FACS) strategy.** Method for sequentially gating putative intestinal epithelial stem cells using CD44, CD24, CD166 and Grp78 surface markers, after intestinal tissue dissociation and cell separation procedures, as previous description of Wang et al. (Wang et al., 2013). According to these authors, CD44 and CD24 markers may be used to select epithelial crypt cells and to exclude fractional differentiated cells in the lower crypt, respectively; combination of CD44, CD24 and CD166 may further exclude differentiated cells from the villi and crypts. The use of CD44+/CD24low/CD166+/Grp78low and CD44+/CD24low/CD166+/Grp78+ combinations was proposed to identify putative epithelial stem cells and transit-amplifying cells, respectively. CD44+/CD24hi/CD166+ fraction may correspond to secretory cells in the lower region of crypts as result of the high expression of CD24 and CD166 in that location (Wang et al., 2013).

Isolated cells were resuspended in the staining medium at a concentration of one million of cells per 100 μl per tube and incubated with 0.5 μg of monoclonal antibodies according to the supplier's concentrations, for 15 minutes, at room temperature, in the absence of light. Next, cells were incubated with 2 ml of lysis buffer (BD BioSciences, San Jose, California, USA) for 10 minutes, at room temperature, in the dark. Then, cells were washed with PBS pH 7.4 (Gibco, Life Technologies) by centrifugation at 300x g for five minutes and resuspended in 400 μl of that buffer solution. A similar process was followed for the secondary antibody staining.

Cells analysis and data acquisition were performed using a six-parameter, four-color, FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA) and the CellQuest software (version 0.3, BD BioSciences, San Jose, California, USA). For each
sample, 5 x 10^4 events per tube were acquired. Results were analyzed and quantified using the Paint-a-Gate 3.02 program (BD BioSciences). Experiments were carried out in duplicate. Results were expressed in terms of percentage (%) of positive cells expressing each protein.

3.3.9. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18 software package (SPSS, Chicago, Illinois, USA). Type of distribution of variables was determined using Shapiro-Wilk and Kolmogorov-Smirnov-Lillifors tests. Data were indicated as medians and interquartile ranges (median ± IQR) or numbers (%). Non-parametric continuous variables were compared by Mann-Whitney U test, Wilcoxon matched pairs test, and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons (for comparisons across multiple groups). Correlations were determined by the Spearman’s rank correlation coefficient (σ). Categorical variables were compared by Qui-square test. Differences were considered statistically significant at a level of 95% (p<0.05).

3.4. Results

3.4.1. Animals’ postoperative course

Sixty-two animals were studied. There were two cases of mortality associated with anastomotic leakage (in “Res Ted -” and “Res Ted +” groups, respectively) and one of indeterminate etiology (in “Res Ted -” group), that were excluded from further analysis.

Fifty-nine animals completed the study and were included into the following groups: “Res Ted +” (15, eight of them sacrificed on the third day), “Res Ted -” (13, five sacrificed at third day), “Lap Ted +” (16, eight sacrificed on the third day) and “Lap Ted -” (15, seven sacrificed at third day) (Fig. 3.7).
Morbidity included one case of small scurf at the teduglutide injection site. Postoperative daily health score used by van Landeghem L et al (van Landeghem et al., 2012a) amounted to four points in all animals except in the cases of mortality at day of death (two points).

![Diagram of animal distribution for study groups](image)

**Figure 3.7. Distribution of the animals for the study groups.** Adult male Wistar albinus rats were randomly allocated into four groups: “Ileal Resection and Anastomosis” (“Res”) versus “Laparotomy” (“Lap”), each one subdivided into “Postoperative Teduglutide Administration” (“Ted +”) versus “No Treatment” (“Ted -”). Evaluation was accomplished at the moments of the operation and sacrifice (Sac), at the third or seventh postoperative days (eight subgroups), with ileal segment harvesting and blood collection. Tissue baseline values of “Ileal Resection and Anastomosis” groups were considered for comparison with postoperative results of the “Laparotomy” groups; tissue samples recovered at the sacrifice in those animals corresponded to the peri-anastomotic segments.

### 3.4.2. Flow cytometry expression profile of cells isolated from rats’ ileum at the baseline

In the flow cytometry analysis of rats’ small intestine preparations derived from the initial ileal resection, surface markers of epithelial (CK18⁺), mesenchymal (vimentin⁺), smooth muscle (desmin⁺) and hematopoietic (CD45⁺) differentiation were demonstrated in 23.5±12.3 (18.7-49.7)%, 16.2±5.9 (11.7-21.6)%, 16.7±1.3 (9.3-19)% and 6.2±4.2 (1.9-16.9)% of isolated cells, respectively (Supplementary Figure S1).

Lgr5 and Bmi1 surface cell markers were expressed in 3.5±1.5 (2.1-5.5)% and 3.2±2.5 (1.2-5.9)% of all cells, respectively; nearly 26.7% of Lgr5⁺ cells coexpressed the Bmi1
marker. In basal conditions, putative epithelial stem cells (Lgr5⁺ and/or Bmi1⁺ cells) amounted to 5.5±2.7 (3.1-9.7)%; approximately 48.7% of them were Lgr5⁺/Bmi1⁻, 34.5% Lgr5⁻/Bmi1⁺ and the remaining exhibited overlapping expression of those biomarkers.

3.4.3. Putative epithelial stem cells response to surgical injury

Surgical trauma induced a significant expansion of putative epithelial stem cells population (Lgr5⁺ and/or Bmi1⁺) at the third postoperative day (9.2±2.8 vs. 5.5±2.7%, \(p=0.0001\)), with normalization at the seventh. That response was significantly more intense after ileal resection, in comparison with isolated laparotomy (10.9±1.2 vs. 7.9±2.2%, \(p=0.036\)), and involved all the three subpopulations (predominantly Lgr5⁻/Bmi1⁺ cells), including Lgr5⁺/Bmi1⁻ cells that did not suffer any increase after laparotomy (Fig. 3.8-3.9).

**Figure 3.8.** Evolution of epithelial stem cell markers expression after ileal resection. Putative intestinal epithelial stem cell markers (Lgr5 and Bmi1) expression, in cells isolated from rats' ileum, at third and seventh days after ileal resection and anastomosis, in animals not submitted to teduglutide administration (n=13), as detected by flow cytometry. Samples recovered at sacrifice corresponded to the anastomotic segment. Data were expressed as percentage of cells expressing the marker (median±interquartile range). * \(p<0.05\)
3.4.4. Putative epithelial stem cells response to teduglutide administration

After ileal resection and anastomosis, teduglutide administration was significantly associated with higher proportion of Lgr5⁺/Bmi1⁻ cells at the seventh day (5.9±0.4 vs. 2.8±1.9%, p=0.005) and lower percentage of Lgr5⁻/Bmi1⁺ cells at both moments (0.05±0.03 vs. 4.91±0.28%, p=0.003 at the third day; 0.03±0.04 vs. 1.87±0.22%, p=0.049 at the seventh day; Fig. 3.10-3.11). After isolated laparotomy, higher percentage of Lgr5⁺/Bmi1⁺ cells was observed in teduglutide-treated animals at the seventh day (1.8±0.6 vs. 1.2±1.3 %, p=0.028; Fig. 3.11). After laparotomy alone, teduglutide treatment was not associated with relevant modifications of the Lgr5⁺/Bmi1⁻ subpopulation.
Figure 3.10. Dot plots. Representative dot plots of flow cytometry analysis of cells isolated from the ileum of a teduglutide-treated rat, at the baseline (left) and at the third day after intestinal resection and anastomosis (right), demonstrating the expression pattern of Lgr5 and Bmi1 surface markers. An increase of the proportion of Lgr5+ and/or Bmi1+ cells and of the Lgr5+/Bmi1-, Lgr5-/Bmi1+ and Lgr5+/Bmi1+ fractions was observed at the third day in comparison with baseline.

Figure 3.11. Expression of epithelial stem cells markers in the different groups of study. Expression of putative intestinal epithelial stem cell markers in cells isolated from rats’ ileum, at third and seventh postoperative days, in the different study groups (n=59), as detected by flow cytometry analysis. Results were presented in percentage (%) of cells expressing the specific marker and corresponded to the median (±interquartile range) of each represented group. Kruskall-Wallis test was used for comparisons. “Res”, Ileal Resection; “Lap”, Laparotomy; “Ted +”, With postoperative teduglutide administration; “Ted -”, Without teduglutide administration.
Considering all the studied animals \((n=59)\), teduglutide postoperative administration was significantly associated with an enrichment of putative intestinal epithelial stem cells population at the seventh day after the operation \((7.4\pm1.1 \text{ vs. } 5\pm3.4\%, \ p=0.0001)\); higher proportion of \(\text{Lgr}5^+\text{/Bmi}1^+\) cells \((3.8\pm3.1 \text{ vs. } 2.8\pm1.8\%, \ p=0.015)\) and \(\text{Lgr}5^+/\text{Bmi}1^+\) cells \((1.5\pm0.7 \text{ vs. } 0.8\pm1.1\%, \ p=0.001)\) was also observed at that time point (Fig. 3.12).

**Figure 3.12. Expression of putative epithelial stem cells markers after surgery (ileal resection or isolated laparotomy).** Expression profile of cells isolated from rats’ ileum, at third and seventh postoperative days, after ileal resection and isolated laparotomy, according to the teduglutide administration in all groups \((n=59)\), as detected by flow cytometry analysis. Results were presented in percentage \((\%\) of cells expressing the specific marker (median±interquartile range). Mann-Whitney U test was used for comparisons. “Teduglutide +”, With postoperative teduglutide administration; “Teduglutide -”, Without teduglutide administration

### 3.4.5. Correlations profile of putative epithelial stem cells at the moment of sacrifice

After the operation, at the moment of sacrifice, putative epithelial stem cells pool \((\text{Lgr}5^+ \text{ and/or Bmi}1^+ \text{ cells})\) correlated mainly with \(\text{Lgr}5^+/\text{Bmi}1^+\) fraction \((\sigma=74.6\%,\)
Moreover, at that moment, Lgr5⁺/Bmi1⁻ and Lgr5⁻/Bmi1⁺ subpopulations correlated inversely ($\sigma=-30.4\%$, $p=0.019$), contrary to the observed in basal condition ($\sigma=52.1\%$, $p=0.003$); particularly after ileal resection ($\sigma=-69\%$, $p=0.0001$) and teduglutide administration ($\sigma=-71.9\%$, $p=0.0001$). Lgr5⁺/Bmi1⁻ and Lgr5⁻/Bmi1⁺ fractions correlated negatively with Grp78 labeling index. Lgr5⁺/Bmi1⁺ and CD44⁺/CD24low⁺/CD166⁺/Grp78⁺ cells proportions demonstrated a significant and high-grade correlation ($\sigma=87.1\%$, $p=0.0001$) in the postoperative period. CD44⁺/CD24low⁺/CD166⁺/Grp78low⁺ cells did not correlated significantly with Lgr5⁺, Bmi1⁺ and Lgr5⁺/Bmi1⁻ fractions, demonstrating only a low grade correlation with Lgr5⁻/Bmi1⁺ and Lgr5⁺/Bmi1⁺ cells.

### Table 3.2. Correlations between the proportion of putative epithelial stem cells at the moment of sacrifice

<table>
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<th>(σ / p)</th>
<th>Lgr5⁺/Bmi1⁻ cells</th>
<th>Lgr5⁻/Bmi1⁺ cells</th>
<th>Lgr5⁺/Bmi1⁺ cells</th>
<th>Lgr5⁺ or Bmi1⁺ cells</th>
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<tr>
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<tr>
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<td>$p=0.016$</td>
<td>$p=0.0001$</td>
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<tr>
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<tr>
<td></td>
<td>$p=0.016$</td>
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<td>CD44⁺/CD24low⁺/CD166⁺/Grp78⁺ cells</td>
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<td>-37.5%</td>
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<td>$p=0.001$</td>
<td>$p=0.003$</td>
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</table>

*Relevant correlations between the proportion of putative epithelial stem cells isolated from rats' ileum, at the moment of sacrifice, in all the studied animals (n=59), as determined by flow cytometry. Samples recovered at sacrifice in rats submitted to ileal resection corresponded to the anastomotic segment. Spearman's correlation coefficient ($\sigma$) and value of significance ($p$) were presented.

### 3.5. Discussion

Intestinal epithelial stem cells have been characterized mainly by genetic lineage tracing strategies combined with transplantation and development of organotypic culture systems (Barker et al., 2012). Until recently, the identification of putative epithelial stem cells using cell surface markers was hampered by the unavailability of reliable
antibodies for fluorescence-activated cell sorting or immunohistochemical analysis (Barker et al., 2012; Rizk and Barker, 2012; Kemper et al., 2012). In this study, analysis of the dynamics of putative intestinal epithelial stem cell populations was accomplished by flow cytometry with a combination of cell surface markers (Lgr5 and Bmi1). Cell separation method used in this experiment included mechanical tissue dissociation, enzymatic digestion, rotation, filtration and density-based steps. Procedure yielded a heterogeneous population including cells with epithelial, mesenchymal, muscle and hematopoietic differentiation. Interpretation of the results of the cellular characterization by flow cytometry must be cautious due to the heterogeneity of the isolated population, potential modifications of the cell surface phenotype induced by the isolation method, and non-specific antibody binding (Tomlinson et al., 2013; Gracz and Magness, 2012).

Present study supports the existence of two functionally different putative epithelial stem cells subpopulations, identified by the Lgr5 and Bmi1 markers, with distinct behaviors after surgical injury and teduglutide treatment: Lgr5⁺/Bmi1⁻ and Lgr5⁻/Bmi1⁺ cells [corresponding presumably to crypt base columnar stem cells and to putative “position +4” stem cells, respectively (Barker et al., 2012; Yan et al., 2012)].

As outlined above, surgical injury, especially the ileal resection, induced a significant expansion of putative epithelial stem cells population (Lgr5⁺ and/or Bmi1⁺ cells) at the third postoperative day, which reverted at the seventh. The expansion of epithelial stem cells compartment observed after ileal resection, with increase of all three subpopulations, may have been related with activation of stem cells proliferation or cellular plasticity and lineage reversibility, as adaptive mechanisms after injury, contributing to the reestablishment of epithelial continuity. In reality, according to previous studies, crypt regeneration following injury seems to be based on the reactivation of reserve “position +4” stem cells (Tian et al., 2011; Takeda et al., 2011) or plasticity (dedifferentiation) of committed progenitors, the transit-amplifying cells (Tetteh et al., 2016; Barker, 2014; Buczacki et al., 2013; van Es et al., 2012). As suggested formerly by other authors (Tetteh et al., 2016; Buczacki et al., 2013), the replenishment of Lgr5-positive stem cells pool may derive from plasticity of both their absorptive and secretory-lineage progeny. In present study, less intense aggression
induced by isolated laparotomy did not seem to be sufficient to significantly modify the Lgr5+/Bmi1− fraction of putative crypt base columnar stem cells.

Postoperative teduglutide administration, during seven days, was significantly associated with expansion of intestinal epithelial stem cells population and higher proportions of Lgr5+/Bmi1− cells and Lgr5+/Bmi1+ cells. Previously, other experimental studies suggested that Glp2 may stimulate epithelial stem cells and transit-amplifying cells proliferation, namely of cells at the positions “+3” to “+10” (after acute administration), “+15” to “+20” (after chronic treatment), “+1” to “+4” and “+4” from the crypt base (Rowland and Brubaker, 2011). Although the signaling networks responsible for the transduction of Glp-2 effects are still insufficiently known, Wnt/β-catenin, one of the most important epithelial stem cells signaling pathways, seem to be critically involved (Bloemendaal et al., 2016; Drucker and Yusta, 2014; Janssen et al., 2013; Rowland and Brubaker, 2011).

In this experiment, teduglutide treatment during seven days after ileal resection and anastomosis was significantly associated with enrichment of putative crypt base columnar stem population (Lgr5+/Bmi1− cells) whereas, on the contrary, induced depletion of putative “position +4” stem cells (Lgr5+/Bmi1+ fraction).

Increase of Lgr5+/Bmi1− fraction might have derived from proliferation on the same compartment, recruitment of the Lgr5+/Bmi1+ cells or plasticity of transit-amplifying cells to revert to a stem cell phenotype, as previously demonstrated in the literature (Tetteh et al., 2016; Buczacki et al., 2013; van Es et al., 2012). Lgr5+/Bmi1+ cells might have constituted a reservoir that was activated after injury to replenish the Lrg5+/Bmi1− pool, as described by others authors (Tian et al., 2011; Takeda et al., 2011), or to contribute directly to the transit-amplifying compartment. Similarly, Van Landegherm L et al (van Landegherm et al., 2015; van Landegherm et al., 2012b) demonstrated that insulin-like growth factor 1 (Igf 1), a critical mediator of the enterotrophic effects of Glp2 (Drucker and Yusta, 2014; Rowland and Brubaker, 2011; Bortvedt et al., 2012), activates two intestinal epithelial stem cells populations, via distinct regulatory pathways, to promote growth of normal intestinal epithelium and crypt regeneration after irradiation. Indeed, according to those authors studies, Igf 1 increases the percentage of Sox9-low intestinal epithelial stem cells (actively cycling)
and percentage of those cells in M-phase, but does not expand Sox9-high cells (reserve intestinal epithelial stem cells) (van Landegherm et al., 2015; van Landegherm et al., 2012b). According to Buczacki SJ et al (Buczacki et al., 2013), approximately 20% of the intestinal Lgr5^+ cells are quiescent secretory precursors, coexpressing Lgr5 and all “position +4” markers, that can revert into actively cycling Lgr5^+ crypt base columnar stem cells upon injury, contributing to regeneration.

In this investigation, variations of putative epithelial stem cells pool (Lgr5^+ and/or Bmi1^+ cells) depended mainly of Lgr5^+/Bmi1^+ fraction, as demonstrated by a significant and high positive correlation, suggesting that “position +4” stem cells might constitute the main effectors of surgical injury stem-cells response.

Inverse correlation between Lgr5^+/Bmi1^- and Lgr5^-/Bmi1^+ subpopulations after surgery, opposed to that observed in basal condition, was in accordance with a potential interconversion between putative crypt base columnar and “position +4” stem cells, particularly after ileal resection and teduglutide administration.

Lgr5^+/Bmi1^- and Lgr5^-/Bmi1^+ subpopulations of putative epithelial stem cells correlated moderate and negatively with Grp78 labeling index, as expected. In reality, Grp78 is considered a marker of endoplasmic reticulum stress signaling that is induced at the transition from the stem cells to the transit-amplifying cells in the early phase of differentiation process (Heijmans et al., 2013).

In 2013, Wang F et al (Wang et al., 2013) described a method of isolation and characterization of putative intestinal epithelial stem cells based on a surface markers combination and a colony-formation assay, using immunohistochemistry, real-time reverse-transcription polymerase chain reaction and fluorescence-activated cell sorting. In 2016, Nefzger CM et al (Nefzger et al., 2016) proposed also a combinational cell surface marker-mediated strategy for the isolation of putative crypt base columnar stem cells, transcriptionally and functionally equivalent to the putative intestinal stem cells extracted from Lgr5-Green fluorescent protein (Grp) models (CD31^-/CD45^-/CD166^low/CD24^med/CD44^high/Grp78^neg/low/Epcam^high/EphB2^high). In present study, Lgr5^-/Bmi1^+ subpopulation, the putative “position +4” stem cells, demonstrated a significant high positive correlation with CD44^+/CD24^low/CD166^+/Grp78^+ cells that were considered by Wang F et al (Wang et al., 2013) to correspond to transit-amplifying
cells. Nevertheless, surprisingly, CD44+/CD24low/CD166+/Grp78low cells, perceived by those authors as epithelial stem cells, did not demonstrated a significant correlation with Lgr5+/Bmi1- cells and showed only a low-grade correlation with Lgr5-/Bmi1+ and Lgr5+/Bmi1+ cells.

In conclusion, the present study corroborates the existence of two functional putative epithelial stem cells subpopulations (Lgr5+/Bmi1- and Lgr5-/Bmi1+) with distinct behavior after surgical injury and teduglutide administration. Surgical injury seems to induce an increase of putative epithelial stem cells population at the third day. Teduglutide administration during seventh days after surgical intervention was associated with expansion of the intestinal epithelial stem cells pool (particularly of putative crypt base columnar cells); nevertheless, it was associated with a depletion of putative “position +4” stem cells in the peri-anastomotic segment after ileal resection.

Current results improve the understanding of the role of the intestinal epithelial stem cells dynamics in the intestinal repair. A better knowledge of intestinal epithelial stem cells modulation during the anastomotic healing might offer new insights into the development of perioperative adjuvant pharmacological interventions, including the use of growth factors such as teduglutide, and could be extrapolated also to other contexts. Additional studies are necessary to completely explain the observed putative epithelial stem cells behavior.
Chapter 4

Influence of teduglutide on the intestinal anastomosis outcome on an animal model
This chapter was partially published as:


4.1. Abstract

Intestinal anastomotic failure continues to be one of the most relevant surgical incidents. Teduglutide is an enterotrophic long-acting equivalent of glucagon-like peptide 2. This study evaluated the effects of teduglutide short-term administration on the intestinal anastomosis outcome on an animal model. Wistar rats (n=31) were submitted to ileal resection and standard anastomosis, with or without postoperative teduglutide treatment, and sacrificed at the third or at the seventh day, with tissue sampling. Macroscopic outcome of the anastomosis was characterized according to the Anastomotic Complication Score. Histological parameters of anastomotic healing were assessed with the modified Houdart-Hutschenreiter’s classification after hematoxylin and eosin staining. Collagen content and distribution were evaluated with the Gordon and Sweet’s technique. Neoangiogenesis and epithelial proliferation indexes were determined with anti-CD31 and anti-Ki67 immunostaining, respectively. Paneth and goblet cells were studied with periodic acid shiff-alcian blue pH 2.5; subepithelial myofibroblasts and glial cells with anti-actin, smooth muscle and anti-S-100 immunostaining. Teduglutide administration had no apparent impact on the rate or severity of anastomotic leakage. Teduglutide was associated with higher levels of reepithelialization (p=0.022) and of neoangiogenesis (neovessels density=16.0±10.8/mm² vs. 5.3±3.4/mm², p=0.0001) at the seventh postoperative day. Lower expression of type I collagen (p=0.015) and higher content of type III collagen (p=0.007) in the submucosa were also documented, at the seventh day, in teduglutide-treated animals. This growth factor was also associated with an increase of subepithelial myofibroblasts density score at the seventh day, whereas no relevant influence was observed on Paneth, goblet and glial cells indexes. Those findings indicate a positive impact of teduglutide on the reepithelialization and neoangiogenesis events of the proliferative phase of intestinal anastomotic repair. Additional studies are necessary to clarify its effects on the fibrogenesis process.
4.2. Introduction

Teduglutide is an enterotrophic long-acting equivalent of glucagon-like peptide 2 (GLP-2), which is a gastrointestinal growth factor with a relevant participation in the maintenance of intestinal mucosa integrity and function (Drucker and Yusta, 2014).

Intestinal anastomotic healing is a dynamic and highly regulated process of cellular, humoral and molecular mechanisms classically divided into three sequential overlapping stages (Thompson et al., 2006; Rijcken et al., 2014). In the exudative phase, during the first one to four days, coagulation process triggers the release of bioactive mediators that stimulate the recruitment of inflammatory cells. These cells participate in the wound debridement, extracellular matrix degradation, antigen presentation, and phagocytosis. The proliferative phase is characterized by reepithelialization, angiogenesis and fibroplasia and usually runs between the second and the fourteenth postoperative days. In the reparative phase, extracellular matrix remodeling lead to wound stabilization and contraction (Rijcken et al., 2014).

Regardless of continuous improvements in surgical technique and care, intestinal anastomotic failure persists as a relevant surgical problem (Guyton et al., 2016; Bosmans et al., 2015).

Present investigation evaluated the influence of teduglutide administration on the intestinal anastomosis structural outcome, at the macroscopic and microscopic levels, on an animal model.

4.3. Methods

4.3.1. Ethical statement

Experiment was authorized by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (License nº 32-06-2009) and conducted according to the institutional and national animals’ defense recommendations.
4.3.2. Animals and experimental design

Adult male Wistar albinus rats weighting between 250 and 300g were housed in stainless steel cages under controlled temperature (22±1°C) and relative humidity (50±10%) conditions, with dark/light cycles of 12 hours. Animals were maintained on unrestricted access to standard rodent diet and tap water, except for two hours fasting before surgery (only with free access to water).

Animals were randomly allocated into two groups: “Postoperative Teduglutide Administration” (“Ted +”) versus “No Treatment” (“Ted -”). Evaluation was performed at the time of the operation and at the time of sacrifice, on the third or seventh postoperative day (four subgroups), with ileal harvesting. Blinded assessment was guaranteed in the microscopic evaluation procedures.

4.3.3. Anesthesia and operative details

All surgical procedures were implemented by the same surgeon under clean conditions. Rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg; Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg; Laboratórios Vitória, Amadora, Portugal).

After a 3-cm midline laparotomy, a 10-cm length ileal resection was undertaken, 5 cm upstream of ileocecal valve. An end-to-end anastomosis using eight full-thickness equidistant polydioxanone USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl., Cincinnati, USA) restored the intestinal continuity. Abdomen was closed with two running sutures (muscle-aponeurotic and cutaneous) of braided coated polyglactin 910 USP 4/0 (Surgilactin, Sutures Limited, Wrexham, UK) and natural silk USP 4/0 (Surgisilk, Sutures Limited), respectively. On postoperative period, ingestion of 5% glucose in water (1:1) was consented in the first day, succeeded by ad libitum oral hydration and chow. Daily surveillance of the animals was undertaken and a health score was calculated as described previously by van Landeghem L et al (van Landeghem et al., 2012a).
Surgical mortality and morbidity were recorded. At the third or seventh postoperative day, animals were sacrificed by cervical displacement and subjected to a relaparotomy with ileal resection (10-cm length, retaining distal 3 cm).

4.3.4. **Teduglutide administration**

In “Ted +” groups, teduglutide (American Peptide Company, Sunnyvale, California, USA) was administered subcutaneously in the postoperative period (from the operation day), 200 μg/kg body weight per day, diluted in 0.25 ml phosphate buffered saline pH 7.4 (PBS; Gibco, LifeTechnologies, Carlsbad, California, USA), after reconstitution in agreement with the manufacturer’s instructions.

4.3.5. **Tissue harvesting**

One of three similar longitudinal strips of the most distal 4 cm of each ileal specimen (with the anastomosis in the middle), corresponding to one third of the circumference, was retrieved, after cautious washing with normal saline solution. One segment of ileum, immediately adjacent to the 4-cm ileal specimen previously described, 2-cm length, was also recovered. Samples were fixed in 10% formaldehyde, dehydrated and embedded in paraffin, and then 4 μm sections were prepared.

4.3.6. **Macroscopic structural evaluation of the anastomosis**

During relaparotomy, the peritoneal cavity was rigorously checked for anastomotic leakage, intra-abdominal abscess, peritonitis or intestinal obstruction. At the macroscopic examination, “in situ” normal anastomotic healing was considered as apparent anastomosis integrity, with no evidence of leakage or dehiscence, peri-anastomotic collections or obstruction. Anastomotic leakage was defined as a defect in the integrity of the surgical junction between two intestinal loops leading to a communication between the intra and extraluminal compartments (Chadi et al., 2016; Shogan et al., 2013). Macroscopic outcome of the anastomoses was characterized by
the Anastomotic Complication Score proposed by Bosmans JWAM et al (Bosmans et al., 2016) for animal research, as follows: 0: No adhesions or abnormalities; 1: Adhesion to fat pad, clean anastomosis underneath; 2: Adhesion to intestinal loop, abdominal wall or other organ; 3: Anastomotic defect found underneath adhesion, no other abnormalities; 4: Signs of possible contamination; 5: Clear anastomotic complication; spread of pus, obstruction at the anastomosis, signs of peritonitis; 6: Fecal peritonitis; death due to peritonitis. Intra-abdominal adhesions are assessed and semiquantitatively graded using the standard Hulka scale (0 point: No adhesions; 1 point: Single, easily dissectible adhesion; 2 points: Multiple, easily dissectible adhesions; 3 points: Single, dense adhesion; 4 points: Multiple, dense adhesions) (Cakmak et al., 2009).

4.3.7. Microscopic structural assessment of the anastomosis

Anastomotic samples were retrieved according to the former explanation and, for each one, at least two 4 μm sections were systematically analyzed, after having been staining with hematoxylin and eosin (H&E) or Gordon and Sweet’s protocol or subjected to immunohistochemistry, following standard protocols.

Analysis was performed by an expert pathologist blinded to the experimental groups using a conventional binocular light microscope (Nikon Labophot, Nikon, Tokio, Japan). Photomicrographs were obtained on a light microscope (Nikon Eclipse 50i, Nikon Corporation) equipped with a digital camera (Nikon Digital Sight DS-Fi1, Nikon Corporation) and recorded with the integrated software.

4.3.7.1. Healing parameters according to the modified Houdart-Hutschenreiter’s classification

Anastomotic healing was evaluated with an adaptation of the Houdart-Hutschenreiter’s histopathological assessment scale (Cakmak et al., 2009), which included the following criteria: mucosal reepithelialization, presence of inflammatory granulomas and formation of granulation tissue (infiltration of inflammatory cells,
neovascularization, presence of fibroblasts and development of fibrosis), muscle layer
destruction (ischemic necrosis occurrence, continuity interruptions and inflammatory
infiltration) and local inflammatory response (infiltration of neutrophils, lymphocytes,
histiocytes and giants cells) (Table 4.1). Granulation tissue was morphologically
identified as tissue containing numerous fibroblasts, inflammatory cells and
microvessels within a loose matrix of collagen, at the center line of the anastomotic
wound (Thompson et al., 2006).

Table 4.1. Histological parameters of intestinal anastomotic healing (modified
Houdart and Hutschenreiter’s classification)a

<table>
<thead>
<tr>
<th>1. Mucosal reepithelialization</th>
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<td>Grade 0</td>
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<td>Grade 1</td>
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<td>Grade 2</td>
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<td>Grade 3</td>
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<th>2. Inflammatory granuloma and formation of granulation tissue</th>
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<tr>
<td>Inflammatory cell presence</td>
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<tr>
<td>Grade 0</td>
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<td>Grade 1</td>
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<td>Grade 2</td>
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<th>3. Muscle layer destruction</th>
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<tr>
<td>Ischemic necrosis</td>
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<td>Grade 0</td>
</tr>
<tr>
<td>Grade 1</td>
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<td>Grade 2</td>
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<th>4. Wound inflammatory infiltration</th>
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<tr>
<td>Neutrophils</td>
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<td>Grade 0</td>
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<td>Grade 1</td>
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<td>Grade 2</td>
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<td>Grade 3</td>
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N.c., Not considered. a Adapted from reference Cakmak et al., 2009
Five high-power fields (x400 magnification; 0.95 mm² area/field) from at least two 4-μm sections, stained with hematoxylin and eosin (H&E) following standard procedures, were evaluated per anastomosis.

### 4.3.7.2. Evaluation of collagen content and distribution

For each animal, at least three 4-μm cut sections of the anastomotic segment, obtained from the formalin-fixed and paraffin-embedded block, were stained with Gordon and Sweet's silver impregnation technique, according to standard protocol (Gordon and Sweets, 1936; Saxena, 2010), and systematically studied. Gordon and Sweet's staining is based on the argyrophilic properties of reticulin fibers. Reticulin fibers are thin fibers composed of type III collagen that form a delicate stromal network and acquire a black color against a gray to light pink background, while fibrous tissue composed of type I collagen appears brown (Gordon and Sweets, 1936; Saxena, 2010).

Succinctly, 4 μm sections were deparaffinized, hydrated and submitted to the Gordon and Sweet’s protocol. Procedure included oxidation in 1% acidified potassium permanganate for five minutes (“oxidation”), discoloration with 2% oxalic acid for two minutes, treatment in 2.5% iron alum (ferric ammonium sulphate) for 15 to 30 minutes (“sensitization”), impregnation in ammoniacal silver solution for two minutes, reduction with 10% aqueous formalin for two minutes (“developing”), toning in 0.2% gold chloride (sodium tetrachloroaurate) for three minutes (“toning” ) and fixation in 5% aqueous sodium thiosulphate for three minutes; all those steps were always followed by rinsing with distilled water. After a new washing in distilled water, sections were counterstained with van Gieson’s stain (mixture of picric acid and acid fuchsine) for two minutes, dehydrated, cleared and mounted.

For each sample, two standard regions from the lamina propria, submucosa and muscularis propria layers, and from the granulation tissue, were evaluated at 200x magnification. Thickness, orientation and distribution of collagen fibers were observed. A semiquantitative analysis was performed, based on the intensity of staining in the different layers or regions, using a visual analogue score: a score of “0” was considered as no staining, “1” as mild staining, “2” as moderate staining, and “3” as
intense staining. The mean value obtained for each bowel layer or region was considered.

4.3.7.3. Determination of the neoangiogenesis score

Angiogenesis was evaluated by the capillary density, using immunohistochemical staining of endothelial cells with the monoclonal antibody anti-CD31, as stated by Mall JW et al (Mall et al., 2003).

Deparaffinized 4 μm sections of the anastomotic segment obtained according to previous description were treated with ultra-cell conditioning solution (Ultra CC1; Ventana Medical Systems, Inc., Tucson, Arizona, USA) and heated in a microwave oven (52 minutes at 96°C) for antigen retrieval. Thereafter, sections were incubated with anti-CD31 (Pecam-1) rat monoclonal antibody (Biocare Medical, Pacheco, California, USA), at 1:50 dilution, at 37°C for 44 minutes. Sections were treated with the amplification kit (Ventana Medical Systems, Inc.) and with the antibody diluent (Ventana Medical Systems, Inc.). Finally, they were counterstained with hematoxylin (Ventana Medical Systems, Inc), submitted to bluing reagent application (Ventana Medical Systems, Inc), dehydrated and mounted.

Two 4 μm sections of each sample were scanned systematically at 40x magnification to identify three areas with the highest microvessel density at the anastomosis site ("hot spots"). Microvessels were then counted in each vision field at 200x magnification (corresponding to an area of 3.8 mm²). The angiogenesis score corresponded to the mean number of capillary vessels identified in the three 200x magnification vision fields.

4.3.7.4. Analysis of the epithelial proliferation index

Epithelial proliferation index was assessed using immunohistochemical staining with the monoclonal antibody anti-Ki67, a proliferation marker present during all active phases of the cell cycle (while absent in quiescent G0 cells) (Inglin et al., 2008; Rekhtman and Bishop, 2011).
Deparaffinized 4 μm sections of the perianastomotic ileum were treated with ultra-cell conditioning solution (Ultra CC1; Ventana Medical Systems, Inc.) and heated in a microwave oven (36 minutes at 95°C). Sections were incubated with anti-Ki67 rabbit monoclonal primary antibody (Ventana Medical Systems, Inc.) at 37°C for 36 minutes. Sections were treated with antibody diluent (Ventana Medical Systems, Inc.), counterstained with hematoxylin (Ventana Medical Systems, Inc.), submitted to bluing reagent application (Ventana Medical Systems, Inc.), dehydrated and mounted.

A dark brown nuclear staining (of any intensity) was regarded as positive. The ratio between Ki67-labelled cells and the total number of cells per crypt was calculated after counting cells, at 400x magnification, in five contiguous and randomly selected longitudinal well-oriented sections of crypts (those in which it was possible to visually follow without interruption the continuation of a crypt into the villus).

4.3.8. Other histopathological analysis

Sections of 4 μm of the ileal perianastomotic specimens, recovered as previously stated, were stained with periodic acid schiff-alcian blue pH 2.5 (PAS-AB pH 2.5) or subjected to immunohistochemistry. Analysis was undertaken by a pathologist with the formerly reported equipment.

4.3.8.1. Estimation of the density of Paneth cells and goblet cells

For global assessment of the density of Paneth cells and goblet cells, a periodic acid schiff-alcian blue pH 2.5 (PAS-AB) staining protocol was used. PAS-AB pH 2.5 is a staining method used to detected polysaccharides (such as glycogen) and mucosubstances in tissues; acidic mucins appeared in blue, neutral mucins in magenta, mixtures of both in blue/purple and nuclei in deep blue (Rekhtman and Bishop, 2011; Ellis, 2014).

Briefly, slides were deparaffinized and hydrated, rinsed in distilled water, treated with 3% aqueous acetic acid for two minutes, stained with 1% alcian blue pH 2.5 (copper phthalocyanins solution in acetic acid and distilled water) for 30 minutes and washed well in running water for 10 minutes. Sections were then treated with 1% aqueous
periodic acid for 10 minutes, rinsed in distilled water, stained with Schiff's reagent (produced by treating basic fuchsin with sulfurous acid) for 15 minutes and washed well in running water for 10 minutes. Nuclei were stained with Mayer hematoxylin for 10 minutes and sections were washed in running water for 10 minutes. Sections were dehydrated, cleared and mounted.

For goblet cells evaluation and quantification, two contiguous well-oriented, full-length crypt-villus units per animal were randomly selected and scored, at 200x magnification. Mean number of identified goblet cells was considered.

Paneth cells are cells of the nonendocrine secretory lineage, with typical morphology (pyramidal-shaped) and secretory granules, localized at the bases of the crypts of Lieberkühn, interspersed with granule-free crypt base columnar cells (Roth et al., 2012; Parry et al., 2013). Paneth cells were counted in six contiguous and randomly selected longitudinal well-oriented sections of crypts, at 200x magnification, in ileal cross-sections. Mean value was considered.

4.3.8.2. Study of subepithelial myofibroblasts and enteric nervous system

Evaluation of subepithelial myofibroblasts in the lamina propria of the distal ileum was performed using immunohistochemical staining with the monoclonal antibody anti-actin, smooth muscle.

Subepithelial myofibroblasts were identified as α-smooth muscle actin-expressing stromal cells located in the lamina propria, under the epithelial layer and subjacent to the basement membrane, with characteristic morphology (spindle-shaped with slender cytoplasmic processes or with an enlarged cytoplasm), and forming a network around the crypts. Alfa(α)-smooth muscle actin remains the most typical intestinal myofibroblast marker, although not specific, also being expressed by pericytes, lymphatic lacteal smooth muscle cells, muscularis mucosae smooth muscle cells and bone marrow-derived mesenchymal stromal cells (Roulis et al., 2016; Mifflin et al., 2011).
Sections were deparaffinized with EZ Prep concentrate (10x) solution (Ventana Medical Systems, Inc., Tucson, Arizona, USA) and heating at 72°C. Deparaffinized sections were treated with ultra-cell conditioning solution (Ultra CC1; Ventana Medical Systems, Inc.) and heated in a microwave oven, 36 minutes at 95°C, for antigen retrieval. Then, sections were incubated with anti-actin, smooth muscle mouse monoclonal antibody (Cell Marque, Sigma-Aldrich, Rocklin, California, USA), at 1:100 dilution, at 37°C for 32 minutes. Sections were rinsed with reaction buffer concentrate (10x) (Ventana Medical Systems, Inc.), a Tris based buffer solution. Finally, they were counterstained with hematoxylin (Ventana Medical Systems, Inc), submitted to bluing reagent application (Ventana Medical Systems, Inc), dehydrated and mounted.

Assessment was performed in well-oriented crypt-villus axis of five randomly selected fields of vision, at 200x magnification (each field corresponding to 3.8 mm²). A semiquantitative analysis of subepithelial myofibroblasts was performed considering: “1” as normal morphology, distribution and density, “2” as moderate increase of distribution and density and “3” as marked increase of distribution and density.

Enteric nervous system study was performed with immunohistochemistry through the expression of glial marker S-100 in the submucosal and myoenteric plexus of rats’ ileum.

Sections were deparaffinized with high temperatures (72°C) and the EZ Prep concentrate (10x) solution (Ventana Medical Systems, Inc.). Deparaffinized sections were treated with ultra-cell conditioning solution (Ultra CC1; Ventana Medical Systems, Inc.) and heated in a microwave oven (eight minutes at 95°C) for antigen retrieval. Sections were submitted to enzymatic digestion with protease 3 (Ventana Medical Systems, Inc.) (one drop), an endopeptidase of the serine protease family, and were incubated with anti-S100 polyclonal rabbit antibody (Dako, Glostrup, Denmark), at 1:1000 dilution, at room temperature, for 24 minutes. Sections were treated with reaction buffer concentrate (10x) (Ventana Medical Systems, Inc.). Finally, they were counterstained with hematoxylin (Ventana Medical Systems, Inc.), submitted to bluing reagent application (Ventana Medical Systems, Inc.) for four minutes, dehydrated and mounted.
Expression of glial marker S-100 in the submucosal and myoenteric plexus was evaluated in four sections of rats' ileum, with light microscopy, at 100x magnification. Morphology, distribution and density of S-100 immunoreactive glial cells and nerve fibers were studied and a semiquantitative scoring was done as follows: “1” as few glial cells and fibers with a regular morphology and distribution; “2” as numerous glial cells and fibers; and “3” as dense networks of glial cells and fibers.

4.3.9. Statistical analysis

Statistical Package for Social Sciences (SPSS) for Windows, version 18 (SPSS, Chicago, Illinois, USA) was used for the statistical analysis. Testing for normality was performed with Shapiro Wilk and Kolmogorov-Smirnov-Lillifors tests. Non-parametric continuous variables were compared by Mann-Whitney U test and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons (for comparisons across multiple groups). Data were expressed as median and interquartile range (median ± IQR) or number (%). A $p<0.05$ was considered statistically significant.

4.4. Results

4.4.1. Macroscopic structural evaluation of the anastomosis

Thirty-one animals were studied: 16 were included into “Ted +” group (eight of them sacrificed on the third day) and 15 into the “Ted –” group (six killed at third day).

There were two cases of anastomotic leakage associated with mortality (in “Res Ted -” and “Res Ted +” groups, respectively), both with an Anastomotic Complication Score of “6” (Bosmans et al., 2016). In a third case of mortality, there were no signs of anastomotic complication (death of unknown etiology) and the Anastomotic Complication Score was “1” (adhesion to fat pad and clean anastomosis underneath). These cases were omitted from additional analysis.

In the surviving animals, there was no evidence of anastomotic fistulae, intra-abdominal abscess, peritonitis or intestinal obstruction. Anastomotic Complication Score (Bosmans et al., 2016) was lower or equal to “2” in all these animals. Semiquantitative
assessment of intra-abdominal adhesions did not exceed one point in the Hulka scale (single, easily dissectible adhesion) (Cakmak et al., 2009) in any case. Morbidity included one case of small scurf at the teduglutide injection site.

Teduglutide was not associated with significant impact on the macroscopic outcome of the anastomosis, namely on the rate or severity of anastomotic leakage evaluated with the Anastomotic Complication Score (Bosmans et al., 2016). Operative mortality and morbidity rates were not significantly different in animals with or without teduglutide treatment: 6.3 vs. 13.3%, n.s. and 6.3% vs. 0%, n.s., respectively; as well as, the anastomotic complication score (Bosmans et al., 2016) and the intra-abdominal adhesion index according to the Hulka scale (Cakmak et al., 2009): 0±0 (0-6) vs. 1±2 (0-6), p=0.06 and 0±0 (0-1) vs. 0±1 (0-1), p=0.08, respectively.

4.4.2. Microscopic structural assessment of the anastomosis

4.4.2.1. Parameters of anastomotic repair in animals not submitted to teduglutide administration

In the animals not submitted to teduglutide treatment, histopathological assessment of the anastomotic repair according to the modified Hourdard-Hutschenreiter’s classification revealed higher infiltration of inflammatory cells (p=0.012) and higher grade of fibrosis (p=0.003) in the granulation tissue at the seventh postoperative day, in comparison with the third day; higher number of giant cells in the wound was also observed at that time point (p=0.028) (Fig. 4.1; Table 4.2).

According to the Gordon and Sweet’s silver staining, the anastomotic segment of animals not submitted to teduglutide demonstrated significantly higher global content of collagen at the seventh day than on the third day (types I and III; p=0.009), especially in the granulation tissue (p=0.008); and also, higher expression of type I collagen (p=0.002), particularly in the granulation tissue (p=0.003) and in the muscularis propria (p=0.018) (Table 4.3; Fig. 4.2). Type I/type III collagens ratio was higher at the seventh day, although not significantly [1.9±0.9 (1.1-2.3) vs. 1.0±0.8 (0.6-1.5), n.s.]. At the third day, granulation tissue did not express type I collagen, which was observed only in the submucosa.
4.4.2.2. Parameters of anastomotic repair in teduglutide-treated animals

Higher level of reepithelialization was documented at the seventh postoperative day in teduglutide-treated animals \((p=0.022)\) according to the modified Hourdart-Hutschenreiter’s scale (Table 4.2; Fig. 4.3). Furthermore, higher neoangiogenesis score (neovessels density=16.0±10.8/mm² vs. 5.3±3.4/mm², \(p=0.0001\); Fig. 4.4 and 4.5) and enhanced epithelial proliferation index (81.0±11.0 vs. 74.8±19.0%, n.s.; Fig. 4.6 and 4.7) were verified, at the last moment of postoperative evaluation, in animals that underwent teduglutide treatment.

Teduglutide administration during seven postoperative days was associated with reduction of the global content of collagen (with a statistically not significant difference), as well as lower expression of type I collagen, without statistical significance in all the layers except in the submucosa [2.0±0.0 (1.0-3.0) vs. 3.0±0.0 (2.0-3.0) points, \(p=0.015\)]. Nevertheless, higher content of type III collagen in the submucosa [1.0±0.0 (1.0-1.0) vs. 0.0±0.0 (0.0-0.0) points, \(p=0.007\)] was observed in
teduglutide-treated animals at that moment (Table 4.3). At the seventh day, a not-significant reduction of the levels of type I collagen in the lamina propria, muscularis propria and granulation tissue, as well as of type III collagen in the muscularis propria and granulation tissue, were observed in animals treated with teduglutide (Fig. 4.8 and Fig. 4.9). No relevant effects of teduglutide on global type I/type III collagens ratio were evident.

Table 4.2. Intestinal anastomotic healing according to the modified Houdart-Hutschenreiter’s classification*

<table>
<thead>
<tr>
<th>Score</th>
<th>Third postoperative day</th>
<th>Seventh postoperative day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mucosal reepithelialization</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 1 (1-2)</td>
</tr>
<tr>
<td>2. Granulation tissue formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cells presence</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
</tr>
<tr>
<td>Neovascularization</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
</tr>
<tr>
<td>Fibroblasts presence</td>
<td>1 ± 1 (1-2)</td>
<td>1 ± 0 (1-1)²</td>
</tr>
<tr>
<td>Fibrosis development</td>
<td>1 ± 0 (1-1)</td>
<td>0 ± 1 (0-1)²</td>
</tr>
<tr>
<td>3. Muscle layer destruction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic necrosis</td>
<td>0 ± 1 (0-1)</td>
<td>1 ± 1 (0-1)</td>
</tr>
<tr>
<td>Muscle layer continuity</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
</tr>
<tr>
<td>Inflammatory infiltration</td>
<td>2 ± 1 (1-3)</td>
<td>1 ± 2 (1-3)</td>
</tr>
<tr>
<td>4. Wound inflammatory infiltration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 1 (1-2)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1 ± 1 (0-1)</td>
<td>1 ± 1 (0-2)</td>
</tr>
<tr>
<td>Histiocytes</td>
<td>0 ± 1 (0-1)</td>
<td>1 ± 1 (0-1)</td>
</tr>
<tr>
<td>Giant cells</td>
<td>0 ± 1 (0-1)</td>
<td>0 ± 1 (0-1)²⁺</td>
</tr>
</tbody>
</table>

Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted +” groups, teduglutide was administered after the operation. For each sample, five high-power fields from at least two 4 μm sections stained with hematoxylin-eosin were evaluated per anastomosis. Parameters were graded into four categories (0: absent, 1: mild, 2: moderate, 3: intense). Data were presented as median ± interquartile range (minimum-maximum). Kruskal-Wallis test was used. ² “Ted –” (seventh day) vs. “Ted –” (third day), p<0.05; ³ “Ted +” (seventh day) vs. “Ted –” (seventh day), p<0.05; ⁴ “Ted +” (third day) vs. “Ted –” (third day), p<0.05; ⁵ “Ted +” (third day) vs. “Ted –” (third day), p<0.05; ⁶ “Ted +” (third day) vs. “Ted –” (third day), p<0.05; ⁷ “Ted +” (seventh day) vs. “Ted –” (third day), p<0.05. * Adapted from reference Cakmak et al., 2009.
Table 4.3. Collagen content and distribution in the ileal anastomotic segment

<table>
<thead>
<tr>
<th>Score</th>
<th>Study groups</th>
<th>Third postoperative day</th>
<th>Seventh postoperative day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ted -</td>
<td>Ted +</td>
<td>Ted -</td>
</tr>
<tr>
<td>Total collagen</td>
<td>6 ± 3 (5-8)</td>
<td>5.5 ± 1 (5-7)</td>
<td>11.5 ± 2 (9-15)</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>3 ± 0 (3-3)</td>
<td>3 ± 1 (2-3)</td>
<td>7 ± 1 (6-8)</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>3 ± 3 (2-5)</td>
<td>3 ± 2 (2-4)</td>
<td>4 ± 3 (3-7)</td>
</tr>
<tr>
<td>Type I/type III collagens ratio</td>
<td>1 ± 0.8 (0.6-1.5)</td>
<td>1 ± 0.8 (0.5-1.5)</td>
<td>1.9 ± 0.9 (1.1-2.3)</td>
</tr>
<tr>
<td>Total collagen in granulation tissue</td>
<td>1 ± 1 (0-1)</td>
<td>1 ± 1 (0-1)</td>
<td>4.5 ± 2 (3-6)</td>
</tr>
<tr>
<td>Type I collagen in lamina propria</td>
<td>0 ± 0 (0-0)</td>
<td>0 ± 0 (0-0)</td>
<td>1 ± 1 (0-1)</td>
</tr>
<tr>
<td>Type I collagen in submucosa</td>
<td>3 ± 0 (3-3)</td>
<td>3 ± 1 (2-3)</td>
<td>3 ± 0 (2-3)</td>
</tr>
<tr>
<td>Type II collagen in muscularis propria</td>
<td>0 ± 0 (0-0)</td>
<td>0 ± 0 (0-0)</td>
<td>1 ± 1 (0-2)</td>
</tr>
<tr>
<td>Type II collagen in granulation tissue</td>
<td>0 ± 0 (0-0)</td>
<td>0 ± 0 (0-0)</td>
<td>3 ± 1 (2-3)</td>
</tr>
<tr>
<td>Type III collagen in lamina propria</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
</tr>
<tr>
<td>Type III collagen in submucosa</td>
<td>0 ± 2 (0-2)</td>
<td>0 ± 1 (0-1)</td>
<td>0 ± 0 (0-0)</td>
</tr>
<tr>
<td>Type III collagen in muscularis propria</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 0 (1-1)</td>
<td>1 ± 2 (1-3)</td>
</tr>
<tr>
<td>Type III collagen in granulation tissue</td>
<td>1 ± 1 (0-1)</td>
<td>1 ± 1 (0-1)</td>
<td>2 ± 1 (1-3)</td>
</tr>
</tbody>
</table>

Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Collagen content and distribution were evaluated with the Gordon and Sweet’s silver staining technique. Intensities of staining were graded in four categories (0: absent, 1: mild, 2: moderate, 3: intense) and total scores were the mean values obtained for each bowel layer or area. Data were presented as median±interquartile range (minimum-maximum). Kruskal-Wallis test was used. * “Ted -” (seventh day) vs. “Ted +” (third day), p<0.05; † “Ted +” (seventh day) vs. “Ted -” (seventh day), p<0.05; ‡ “Ted +” (third day) vs. “Ted -” (third day), p<0.05; § “Ted +” (third day) vs. “Ted -” (seventh day), p<0.05; ¶ “Ted +” (seventh day) vs. “Ted -” (third day), p<0.05; ‖ “Ted +” (seventh day) vs. “Ted +” (third day), p<0.05

4.4.3. Density scores of Paneth cells, goblet cells, subepithelial myofibroblasts and glial cells

In animals not submitted to teduglutide administration, this study demonstrated, in comparison with baseline, lower number of Paneth cells per crypt in the perianastomotic tissue at the third and the seventh days [3±0.7 (2.3-3.2) vs. 5.3±1 (4-7), p=0.0001 and 4.1±1.2 (3.2-5) vs. 5.3±1 (4-7), p=0.007], lower number of goblet cells per crypt-villus unit at the seventh day [40±10 (31.5-47) vs. 53.3±10.5 (38.5-72), p=0.002]; higher expression of anti-actin, smooth muscle in subepithelial myofibroblasts at the third and the seventh days [2±0 (2-2) vs. 1±0 (1-1), p=0.0001 and 2±0 (2-2) vs. 1±0 (1-1), p=0.0001] and higher expression of glial marker S-100 in the submucosal and myoenteric plexus at the seventh postoperative day [2±0 (1-3) vs.
$1 \pm 0$ (1-1), $p=0.0001$ and $2 \pm 1$ (1-3) vs. $1 \pm 0$ (1-1), $p=0.0001$, respectively] (Fig. 4.10 to 4.13).

Figure 4.2. A and B. Ileal anastomosis at the seventh postoperative day, in an animal not submitted to teduglutide administration (Gordon and Sweet’s staining; original magnification 100x). Anastomosis demonstrated high collagen content (especially type III). Type I collagen (light purplish brown) was observed mainly in submucosa and deep layers of granulation tissue, and also in lamina propria and muscularis propria. Dense deposition of type III collagen (bright black color) was observed in the granulation tissue and muscularis propria. * m, mucosa; sm, submucosa; mp, muscularis propria; gt, granulation tissue
Figure 4.3. Ileal anastomosis at the seventh postoperative day, in a teduglutide-treated animal (hematoxylin-eosin staining; original magnification 100x). Slight reepithelialization, moderate neovascularization, abundant fibroblasts presence, and regular infiltration of inflammatory cells, mainly of polymorphonuclear neutrophils, were documented.

Figure 4.4. Neoangiogenesis in the anastomotic segments determined using immunohistochemical staining with the monoclonal antibody anti-CD31. Neoangiogenesis score corresponded to the mean number of capillary vessels identified in three 200x magnification vision fields (median±interquartile range). Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Kruskal-Wallis test with pairwise comparisons was used.
Figure 4.5. Neoangiogenesis in the anastomotic segment evaluated with immunohistochemical staining of the endothelial cells with the monoclonal antibody anti-CD31. A. Neoangiogenesis at the seventh day after ileal resection and anastomosis in a rat not submitted to teduglutide administration (CD31; original magnification 200x). B. Neoangiogenesis at the seventh day after ileal resection and anastomosis in a teduglutide-treated rat (CD31; original magnification 200x)
Figure 4.6. Epithelial proliferation evaluated in the rats’ perianastomotic segment using immunohistochemical staining with the monoclonal antibody anti-Ki67.

Proliferation index corresponded to the mean percentage of Ki67-labelled cells per crypt identified in five longitudinal sections of the crypts at 400x magnification (median±interquartile range). Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Kruskal-Wallis test with pairwise comparisons was used.

Teduglutide administration was associated with an increase of the expression of anti-actin, smooth muscle in subepithelial myofibroblasts at the seventh day (Table 4.4; Fig. 4.14-4.15). No significant modifications of the goblet and Paneth cells densities nor of the expression of S-100 in the submucosal and myoenteric plexus were observed with teduglutide therapy (Table 4.4; Fig. 4.16-4.17).

4.5. Discussion

In this study, teduglutide had no apparent significant impact on the macroscopic outcome of the anastomosis evaluated by the Anastomotic Complication Score (Bosmans et al., 2016). This fact may have been related with a probably underpowered study (type II statistical error originated by a small sample size).

Rats have been considered suitable animal models to analyze the intestinal anastomotic healing (Bosmans et al., 2016) using histological, cellular, humoral or molecular parameters as primary outcomes, with clear advantages in the availability, costs, handling and housing requirements. However, the evaluation of clinical
outcomes may be influenced by rodents’ strong resistance to infection and/or high efficient intra-abdominal immune system (Pommergaard et al., 2011).

**Figure 4.7.** Epithelial proliferation evaluated in the perianastomotic segment using immunohistochemical staining with the monoclonal antibody anti-Ki67. **A.** Epithelial proliferative activity at the seventh day after ileal resection and anastomosis in a rat not submitted to teduglutide administration (Ki67; original magnification 400x). **B.** Epithelial proliferative activity at the seventh day after ileal resection and anastomosis in a teduglutide-treated rat (Ki67; original magnification 400x)
Figure 4.8. Ileal anastomosis at the third postoperative day in a teduglutide-treated animal, with Gordon and Sweet's staining (original magnification 100x).
Anastomosis demonstrated low collagen content, mainly restricted to type III (bright black color). Granulation tissue did not express type I collagen (light purplish brown), which was observed only in the submucosa.

Figure 4.9. Ileal anastomosis at the seventh postoperative day in a teduglutide-treated animal, with Gordon and Sweet's staining (original magnification 200x).
Granulation tissue demonstrates moderate content of type I and type III collagens (light purplish brown and bright black colors, respectively).
Figure 4.10. Paneth cells density at the seventh day after ileal resection and anastomosis in a rat not submitted to teduglutide treatment (periodic acid schiff-alcian blue pH 2.5 staining; original magnification 400x)

Figure 4.11. Goblet cells density at the seventh day after ileal resection and anastomosis in a rat not submitted to teduglutide treatment (periodic acid schiff-alcian blue pH 2.5 staining; original magnification 200x)
Figure 4.12. Expression of the glial marker S-100 in the submucosal and myoenteric plexus of the perianastomotic ileum, at the seventh day after ileal resection and anastomosis, in a rat not submitted to teduglutide administration (original magnification 100x)

Figure 4.13. Glial cells and fibers of the submucosal and myoenteric plexus of the perianastomotic ileum, at the seventh day after ileal resection and anastomosis, in a rat not submitted to teduglutide administration (anti-S100 immunostaining; original magnification 200x)
Table 4.4. Subepithelial myofibroblasts, glial cells and nerve fibers in the perianastomotic segment

<table>
<thead>
<tr>
<th>Score</th>
<th>Study groups</th>
<th>Third postoperative day</th>
<th>Seventh postoperative day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepithelial myofibroblasts (lamina propria)</td>
<td>2 ± 0 (2-2)</td>
<td>2.5 ± 1 (2-3)</td>
<td>2 ± 0 (2-2)</td>
</tr>
<tr>
<td>Glial cells and fibers (submucosal plexus)</td>
<td>2 ± 0 (2-2)</td>
<td>2 ± 0 (2-2)</td>
<td>2 ± 0 (1-3)</td>
</tr>
<tr>
<td>Glial cells and fibers (myoenteric plexus)</td>
<td>2 ± 0 (2-2)</td>
<td>2 ± 0 (2-2)</td>
<td>2 ± 1 (1-3)</td>
</tr>
</tbody>
</table>

Subepithelial myofibroblasts of rats’ ileum were evaluated with immunohistochemical staining with the monoclonal antibody anti-actin, smooth muscle. A semiquantitative assessment was performed in well-oriented crypt-villus axis of five randomly selected fields of vision, at 200x magnification, considering: “1” as normal morphology, distribution and density, “2” as moderate increase of distribution and density and “3” as marked increase of distribution and density. Glial cells and nerve fibers of rats’ ileum were analyzed with immunohistochemistry through the expression of the glial marker S-100 in the submucosal and myoenteric plexus. A semiquantitative evaluation was undertaken in four sections of rats’ ileum, at 100x magnification, considering: “1” as few glial cells and fibers with a regular morphology, distribution and density; “2” as numerous glial cells and fibers and “3” as dense networks of glial cells and fibers. Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Data were presented as median-interquartile range (minimum-maximum). Kruskal-Wallis test was used. *“Ted +” (seventh day) vs. “Ted –” (seventh day), p<0.05; **“Ted +” (seventh day) vs. “Ted +” (third day), p<0.05

Figure 4.14. Cross-section of the perianastomotic ileum, at the seventh day after ileal resection and anastomosis, in a teduglutide-treated rat (anti-actin, smooth muscle; original magnification 100x)
Figure 4.15. Subepithelial myofibroblasts (arrow) in the lamina propria of the perianastomotic ileum, at the seventh day after ileal resection and anastomosis, in a teduglutide-treated rat (anti-actin, smooth muscle; original magnification 400x)

Figure 4.16. Paneth cells density in the rats’ perianastomotic ileum using the periodic acid schiff-alcian blue pH 2.5 staining protocol. Paneth cells were counted in six contiguous and randomly selected longitudinal well-oriented sections of crypts, at 200x magnification (median±interquartile range). Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Kruskal-Wallis test with pairwise comparisons was used
Goblet cells density in the rats’ perianastomotic ileum using the periodic acid schiff-alcian blue pH 2.5 staining protocol. Goblet cells were counted in two randomly selected and contiguous, well-oriented, full-length crypt-villus units per animal, at 200x magnification (median±interquartile range). Animals (n=28) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. In “Ted+” groups, teduglutide was administered after the operation. Kruskal-Wallis test with pairwise comparisons was used.

Histological assessment has been considered an appropriate and very valuable outcome measure for the anastomotic healing study (Bosmans et al., 2016).

The higher grade of fibrosis, the enhanced global collagen content in the wound, and the higher type I/type III collagens ratio (although not statistically significant) observed in this experiment at the seventh postoperative day were in accordance with the expected evolution of anastomotic healing (Rijcken et al., 2014; Thompson et al., 2006). In fact, an intensification of the fibrogenesis process occurs in the proliferation phase, when fibroblasts and myofibroblasts produce a more collagenous and less cellular matrix, increasing the tissue biomechanical resistance (Thompson et al., 2006).

A significant increase of the neoangiogenesis index was observed in the perianastomotic segment of the teduglutide-treated animals, at the seventh day, confirming the proangiogenic effects of GLP-2 described in the literature (Dubé et al., 2007).

Teduglutide administration during seven postoperative days was associated with a significantly more intense wound reepithelialization and an increase of the epithelial
proliferative activity (although not statistically significant). In fact, several studies demonstrated that exogenous GLP-2 prescription induces expansion of the intestinal epithelial surface, through stimulation of the epithelial cells proliferation in the crypts and inhibition of the apoptosis in crypts and villi (Drucker and Yusta, 2014; Janssen et al., 2013; Dubé et al., 2007).

Gordon and Sweet’s impregnation technique suggested lower expression of type I collagen and higher content of type III collagen in the submucosa of teduglutide-treated animals at the seventh day. No statistically relevant effects on global type I/type III collagen ratio were evident in these animals. The interpretation of these results must consider the limitations of the Gordon and Sweet’s staining method, which constitutes a semiquantitative morphometric assessment, although advantageous in allowing the evaluation of the content and topographic distribution of collagen in the different layers of ileum.

Majority of collagen, key component of extracellular matrix, is located at the submucosal layer, which ensures most of the biomechanical resistance of the anastomosis (Thompson et al., 2006). In the intestine, types I and III collagens predomine and amount to 68% and 20%, respectively (Thompson et al., 2006; Gelse et al., 2003). Granulation tissue is characterized by an enhanced proportion of type III collagen (to approximately 30 to 40%) and a simultaneous decrease of type I collagen (Thompson, 2006). Anastomotic suture-holding capacity is transiently reduced in the first two or three days of the postoperative period, which is related to the increase of collagen degradation by matrix metalloproteinases; thereafter, in the proliferative phase of the healing process, strength increases due to enhanced de novo synthesis and deposition of collagen (Thompson, 2006).

Synthesis and deposition of collagens are complex processes (Gelse et al., 2003). Fibrogenesis is completed only when collagen forms a stable, cross-linked and remodeled aggregation (Chen et al., 2009; Gelse et al., 2003). No necessary correlation exists between collagen content and mechanical strength of the anastomosis (Rijcken et al., 2014; Hendriks et al., 1990), and other factors, such as collagen subtypes and cross-linking, also appeared to be relevant (Rijcken et al., 2014).
Paneth and goblet cells, subepithelial myofibroblasts and enteric nervous system have deserved less interest in the anastomotic healing research. Nevertheless, Paneth cells and goblet cells have an important influence in the microbiota-host interactions and in the intestinal repair (Bosmans et al., 2017; Hou et al., 2017). In fact, recent evidence points to a strong impact of gut microbiota on the intestinal anastomotic healing (Bachmann et al., 2017). Previous studies suggested that teduglutide preferentially increase the proportion of enterocytes rather goblet cells (although this effect appears to be model specific), whereas others reported an increase of mucin-positive cells after chronic treatment (Rowland and Brubaker, 2011). Furthermore, teduglutide seems to induce wingless/β-catenin signaling pathway (Drucker and Yusta, 2014) that is involved the promotion of Paneth cells differentiation (Bloemendaal et al., 2016). Additionally, GLP-2 receptors in subepithelial myofibroblasts and enteric neurons are thought to play a crucial role in the mediation of this growth factor effects (Drucker and Yusta, 2014; Janssen et al., 2013; Rowland and Brubaker, 2011). Therefore, investigation of the response of those cells to teduglutide treatment in the perioperative context seems to be justified.

In this study, a significant reduction of the density of goblet and Paneth cells in the postoperative period was observed. Teduglutide had no relevant influence of on goblet and Paneth cells density indexes.

Goblet cell secretion exerts an important role in the regeneration of the functional epithelial mucous layer, a dynamic structure determinant in the maintenance of the intestinal defense barrier. A functional mucous layer influences positively the healing of colonic anastomosis (Bosmans et al., 2017; Fay et al., 2017). Mice deficient in the mucin 2 gene (Muc2) lack a functional mucous layer and demonstrated higher rate of leakage after proximal colonic anastomosis; as well as, higher plasma levels of intestinal fatty acid-binding protein (a marker of enterocyte damage); tendency for more intense leucocytes infiltration and less collagen deposition and neoangiogenesis; and a tendency towards higher systemic bacterial load at the third postoperative day (Bosmans et al., 2017).

Paneth cells are highly specialized secretory cells, located at the base of the crypts and intercalated between crypt base epithelial stem cells. In addition to its role as key
mediators of host-microorganisms’ interactions, through the production of cryptidins, defensins and lysozyme, Paneth cells are critical elements of epithelial stem cell niche, through the secretion of growth signaling factors crucial for the stem cells maintenance, including canonical wingless and notch ligands, epidermal growth factor and transforming growth factor β (Hou et al., 2017; Tesori et al., 2013; Smith et al., 2012). Loss of Paneth cells in vivo origins the reduction of Lgr5-positive stem cells pool and the presence of Paneth cells in cultures in vitro increases the capacity of those cells to form multipotent and self-renewing organoids (Sato et al., 2011). Recently, Parry L et al (Parry et al., 2013) demonstrated the important participation of Paneth cells in the mediation of epithelial response to injury, in two mouse models of deletion of the transcription factor β-catenin within the crypt; Paneth cells were very sensitive to β-catenin loss and their absence compromised the recovery of the crypts from the damage (Parry et al., 2013). In 2012, Yilmax OH et al (Yilmaz et al., 2012) demonstrated that the stem cell niche regulates the size and activity of intestinal epithelial stem cells pool according to the nutrient availability, through repression of mammalian target of rapamycin complex 1 (mTORC1) signaling in Paneth cells. In their study, caloric restriction was associated with increase of proliferative Lgr5-positive cells and Paneth cells, mild mucosal atrophy and reduction of transit-amplifying proliferating cells (Yilmaz et al., 2012).

Importance of biomechanical forces and mechanotransduction in the intestinal healing process has been documented (Kovalenco et al., 2012). In present study, S-100 expression in glial cells and fibers increased at the seventh day (in relation with baseline), both on the submucosal and the myoenteric plexus. Recently, the regenerative potential of the enteric nervous system after ileal anastomosis was also suggested by Pfeifle VA et al (Pfeifle et al., 2017), in a rat model, although with a faster recovery of the myoenteric plexus in comparison with the submucosal plexus.

In this analysis, subepithelial myofibroblasts were identified by α-smooth muscle actin expression, morphologic and location criteria, without recourse to other markers (such as vimentin or CD90/Thy1) or to ultrastructural characteristics (Roulis et al., 2016; Mifflin et al., 2011). Teduglutide was associated with an increase of subepithelial myofibroblasts density score at the seventh day. Subepithelial myofibroblasts seem to constitute relevant elements in the regeneration process. Previous studies suggest that
Subepithelial myofibroblasts may regulate epithelial stem cell function, as constituents of the stem cell niche (Hou et al., 2017; Roulis et al., 2016); participate in extracellular matrix remodeling and mechanical regulation of the tissues; and be involved in antigen presentation and immunoregulation (Roulis et al., 2016). Lamina propria mesenchymal cells, including subepithelial myofibroblasts, seem to express bone morphogenic protein signaling (Bmp) antagonists (including noggin, gremlin1, gremlin2, and chordin-like1), non-canonical wingless (Wnt) molecules (such as Wnt 2b, Wnt 4, Wnt 5a, and Wnt 5b) and the Wnt/β-catenin agonists R-spondin 1 or 2. Therefore, they are considered to contribute for the maintenance of proliferating and non-differentiated epithelial stem cells and to the equilibrium between proliferation and differentiation. In fact, Wnt signaling, transduced by β-catenin/transcription factor 4 (Tcf4) is fundamental for the maintenance of proliferating, non-differentiated status of stem cells in the crypts. Bmp signaling antagonizes Wnt/β-catenin pathway, inhibiting self-renewal and promoting differentiation. Non-canonical Wnt expression has been considered to participate in the control of Wnt/β-catenin signaling in the stem cell niche, adjusting epithelial proliferation and differentiation. Subepithelial myofibroblasts are also considered to interact with immune cells types and to be able to sense inflammatory, bacterial and damage signals, activating tumor progression locus 2 (Tpl2)-cyclooxygenase 2 (Cox2)-prostaglandin E2 (PGE2) pathway and promoting epithelial cells proliferation (Roulis et al., 2016).

In current investigation, short extension of the ileal resections (10-cm length), lower than 20% of total small bowel length (Pénzes and Skála, 1977; Miller, 1971), allowed adequate tissue harvesting for baseline analysis without inducing a short-bowel syndrome. Extent of treatment (three or seven days) was chosen based on the expect course of the intestinal anastomotic healing, corresponding presumably to the predominantly inflammatory and proliferative phases (Rijcken et al., 2014). Besides, the intestinotrophic effects of GLP-2 and its analogues have been demonstrated, in rat models, after treatment periods of three to ten days (Kaji et al., 2008). Proliferative effects of GLP-2 or its long-acting analogues have been detected, indeed, as early as six hours after administration to mice (Austin et al., 2016).

Present study did not include the mechanical evaluation of anastomotic repair, namely the determination of the bursting pressure, measure of anastomotic integrity, and of
the tensile strength, reflex of suture-holding capacity of the perianastomotic tissue (Pommergaard et al., 2014; Thompson et al., 2006). Although widely used, often as major end points, these parameters demonstrated relevant limitations related with the heterogeneity, reproducibility and accuracy of the determination methods, particularly in small animals (Pommergaard et al., 2014; Vakalopoulos et al., 2013), and may interfere with subsequent histological and biomolecular analysis. Furthermore, anastomotic bursting pressure may not correlate with the integrity of the anastomosis and the clinical outcome (Vakalopoulos et al., 2013).

Despite several limitations (including the small number of cases and the semiquantitative analysis), results of present investigation denote a favoring impact of teduglutide short-term postoperative administration on the reepithelization and neoangiogenesis events of the proliferative phase of intestinal anastomotic repair. Effects of teduglutide on the fibrogenesis process deserve more investigation.

Additional studies are necessary to clarify the potential benefit of teduglutide in the high-risk context of intestinal anastomotic repair, including its efficacy, safety and applicability.
Chapter 5

Effects of the perioperative administration of teduglutide on the cellular viability and death, oxidative stress and inflammatory response
This chapter was partially published as:

5.1. Abstract

Teduglutide is an intestinotrophic long-acting modified form of glucagon-like peptide 2, authorized for pharmacological rehabilitation of short-bowel syndrome. Present study intended to investigate the inflammatory and redox responses to teduglutide on an animal model of intestinal anastomosis and laparotomy. Wistar rats (n=62) were allocated into four groups: “Ileal Resection and Anastomosis” versus “Laparotomy”, each one split into “Postoperative Teduglutide Administration” versus “No Treatment”; and euthanized at the third or the seventh day. Ileal and blood samples were recovered at the baseline and at the sacrifice. Flow cytometry was used to study the inflammatory response (IL-1α, MCP-1, TNF-α, IFN-γ and IL-4 concentrations), oxidative stress (cytosolic peroxides, mitochondrial reactive species, intracellular glutathione and mitochondrial membrane potential levels) and cellular viability and death (with annexin-V/propidium iodide double staining). Postoperative teduglutide treatment was associated with higher cellular viability index and lower early apoptosis ratio at the seventh day; higher cytosolic peroxides level at the third day and mitochondrial generation of reactive species at the seventh day; as well as higher tissue concentration of IL-4 and lower tissue pro-to-antiinflammatory cytokines ratio at the seventh day. Those findings suggested an intestinal prooxidative and antiinflammatory influence of teduglutide on the perioperative context with a potential interference in the intestinal anastomotic healing.
5.2. Introduction

Teduglutide is a long-acting recombinant analogue of human glucagon-like peptide 2 (GLP-2) authorized for the pharmacological rehabilitation of short-bowel syndrome (Drucker and Yusta, 2014). GLP-2 has been considered to have an important antiinflammatory activity, documented in animal models of chemically induced ileitis and colitis, promoting the reduction of local expression of interleukin (IL)-1β, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and the increase of IL-10 and IL-4 levels (Ivory et al., 2008; Sigalet et al., 2007). Similarly, other authors observed improvement of the damage score and decrease of tissue expression of IL-1, IL-7 and TNF-α, with polyethylene glycosylated porcine GLP-2, in a rodent model of ulcerative colitis (Qi et al., 2017). Furthermore, the antiinflammatory effects of GLP-2 were also recently demonstrated on an experimental rat model of necrotizing enterocolitis, with the reduction of ileal interstitial TNF-α and IL-6 levels, and the improvement of clinical sickness score and survival rate (Nakame et al., 2016).

Intestinal inflammatory and redox responses to the perioperative administration of teduglutide are not yet well understood. It may be hypothesized that teduglutide influences inflammatory and redox reactions with a potential interference in the intestinal anastomotic healing. Present study intended to investigate the inflammatory and redox responses to teduglutide short-term perioperative treatment on an animal model of intestinal anastomosis and laparotomy.

5.3. Methods

5.3.1. Study layout

Experiment was ratified by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (Official Letter nº 32-06-2009) and implemented in consonance with the national recommendations for animals' safety.

Adult male Wistar albinus rats, weighting 250 to 300 g, were randomly allocated into four groups: “Ileal Resection and Anastomosis” (“Res”) versus “Laparotomy” (“Lap”), each one split into “Postoperative Teduglutide Administration” (“Ted +”) versus “No Treatment”
Evaluation was accomplished at the operation and euthanasia, at the third or the seventh postoperative day [presumptively consonant with the inflammatory and proliferative stages of the intestinal anastomotic repair (Rijcken et al., 2014)], with ileal harvesting and blood collection. Blinded assessment was guaranteed in all the laboratorial analysis. All the operative interventions were executed by the same surgeon, after two hours solid fasting, with clean surgical technique and under anaesthesia with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg; Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg; Laboratórios Vitória, Amadora, Portugal).

In “Res” groups, a 10-cm length ileal resection was completed, retaining distal 5 cm, through a 3-cm midline laparotomy, and concluded with an end-to-end anastomosis with eight equidistant full-thickness polydioxanone USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl., Cincinnati, USA); abdominal wall was closed with muscle-aponeurotic and cutaneous continuous sutures of braided coated polyglactin 910 USP 4/0 (Surgilactin, Sutures Limited, Wrexham, UK) and natural silk USP 4/0 (Surgisilk, Sutures Limited), respectively. In “Lap” groups, a 3-cm midline laparotomy was carried out with mild handling of the small bowel.

In the first postoperative day, 5% glucose in water at a 1:1 ratio was provided and, then, ad libitum rodent diet and hydration were restored. At the third or seventh postoperative day, animals were euthanized by cervical displacement and a relaparotomy with ileal resection was undertaken (10 cm length, conserving distal 3 cm). In “Ted +” groups, teduglutide (American Peptide Company, Sunnyvale, California, USA) was applied subcutaneously in the postoperative period (including on the day of the operation), 200 μg/kg/day, after preparation in agreement with the manufacturer’s recommendations.

5.3.2. Tissue and blood sampling

Three similar longitudinal strips of the most distal 4-cm length of each ileal specimen, each one corresponding to one third of the circumference, were carefully retrieved for cell isolation procedure, homogenization and 10% formaldehyde fixation,
respectively. In “Res” groups, tissue samples obtained at the sacrifice corresponded to the anastomotic segment and included the anastomosis in the middle. Tissue baseline values of “Res” groups were considered for comparison with postoperative results of the “Lap” groups. Blood samples of 1 ml were collected before the operations, into ethylenediaminetetraacetic acid (EDTA)-containing tubes, stabilized with 0.1 mg/ml of aprotinin from bovine lung (Sigma-Aldrich, Sintra, Portugal) and 0.037 mg/ml of nicotinonitrile dihydrochloride hydrate (Sigma Aldrich), and centrifuged for 20 minutes at 1500x g and 4°C. Plasma aliquots were maintained at -80°C.

5.3.3. Intestinal tissue homogenization

Briefly, fragments from one ileal longitudinal strip, with approximately 1 mm, were rapidly introduced in a mixture of protease inhibitors (1 ml/100 mg) and submitted to mechanical homogenization using a little microstrainer. Inhibitors cocktail was previously prepared by adding aprotinin from bovine lung (Sigma-Aldrich), leupeptin hemisulfate salt (Sigma-Aldrich) and pepstatin A (Sigma-Aldrich) (1 μl of each, all diluted in a 10 mg/ml stock concentration) to 10 ml of phosphate buffered saline 7.4 (PBS 7.4, Gibco, Life Technologies, Carlsbad, California, USA) and stored on ice. Preparation was sonicated twice with one short pulse of ten seconds (50% amplitude modulation on vector rotation), cooled during ten seconds and distributed for two tubes of 1.5 ml. Sonication (one pulse of ten seconds) was repeated and centrifugation was undertaken, 14000x g, for ten minutes, at 4°C. Supernatant was removed to a new tube (pellet was preserved on ice for posterior RNA extraction in further studies). Centrifugation was repeated twice and supernatant was removed, aliquoted (100 μl) and stored at -80°C until further use.

5.3.4. Intestinal tissue dissociation and cell separation procedure

Cells were isolated from one ileal longitudinal strip, immediately after excision, by a standardized adaptation of the collagenase/dispase isolation technique proposed by
Evans GS et al (Evans et al., 1992) and Dekaney CM et al (Dekaney et al., 2007; Dekaney et al., 2005), formerly delineated in the Chapter 3, to produce a preparation mainly composed of epithelial and some mesenchymal cells.

5.3.5. Cellular viability and death study

Cell viability and death were analyzed by flow cytometry that is considered the most adequate method to study these processes on an individual cell basis, providing quantitative data with high efficiency and low operator-dependent interferences (Galluzzi et al., 2012; Kroemer et al., 2009).

Annexin-V (AV) is a 35 kDa calcium-dependent phospholipid-binding protein that adheres with high affinity to phosphatidylserine (Sgonc and Gruber, 1998). Phosphatidylserine, a phospholipid normally found at the inner surface of the plasma membrane of viable nucleated cells, is exposed at the surface of cells undergoing apoptosis, by translocation from the inner to the outer leaflet of the plasma membrane, and serves as a recognition signal for elimination of apoptotic cells by macrophages. Therefore, fluorescein-isothiocyanate-labeled annexin-V is a marker of phosphatidylserine extracellular exposure, an apoptotic signal on the cell surface, which occurs early in the apoptotic cycle (Galluzzi et al., 2012; Kroemer et al., 2009; Sgonc and Gruber, 1998).

Annexin-V assay is rapid, specific for an early event in the apoptotic process and does not require fixation. However, phosphatidylserine exposure is not exclusive of apoptotic cell death (it also constitutes an early characteristic of parthanatos and netosis); it may be reversible, independent from apoptosis (and even from cell death) and impaired in autophagy-deficient cells in apoptosis. Furthermore, Annexin-V may bind to intracellular phosphatidylserine when plasma membranes are ruptured (Kroemer et al., 2009).

Propidium iodide (PI), a plasma membrane-impermeant fluorochrome in viable cells, binds stoichiometrically to deoxyribonucleic acid (DNA), intercalating between the bases, of the late apoptotic and necrotic cells (which are characterized by the loss of plasma membrane integrity and the disruption of nuclear membrane), causing red
fluorescence in the nucleus (Aubry et al., 1999). Propidium iodide is used as exclusion vital chromatinophilic dye to quantify cells with sub-G1 DNA content in presence of plasma membrane permeabilization (Galluzzi et al., 2012; Kroemer et al., 2009).

In this experiment, annexin-V/propidium iodide double staining was used to evaluate viability and death in isolated cells, with the Annexin-V-FITC Apoptosis Detection Kit (Immunostep, Salamanca, Spain). Briefly, one million cells were washed once with 1 ml of phosphate buffered solution (PBS, pH 7.4, Gibco, Life Technologies, Carlsbad, California, USA) by centrifugation at 1000x g for five minutes and resuspended in 100 μl of cold binding buffer. Binding buffer was prepared with 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution (Hepes)/sodium hydroxide (pH 7.4), 140 mM sodium chloride, 2.5 mM calcium chloride. In this assay, one million cells were incubated in binding buffer with 5 μL of fluorescein-isothiocyanate-labeled annexin-V and 5 μl of propidium iodide, during 15 minutes, at room temperature, in the dark, following the manufacturer’s recommended protocol. After incubation time, cells were diluted in 400 μL of ice-cold binding buffer, and analyzed by flow cytometry within one hour. Subsequently, cells were excited at a wavelength of 525 nm for annexin-V and 640 nm for propidium iodide. Fluorescent green staining of the plasma membrane indicated apoptosis by release of annexin-V to the outer leaflet of the plasma membrane; red staining of deoxyribonucleated acid with propidium iodide indicated the loss of plasma membrane integrity typical of late apoptotic and necrotic cells.

Analysis was accomplished using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA). For each assay, 1x10^6 cells were used. At least 10.000 events were collected by acquisition using CellQuest software (version 0.3, BD BioSciences, San Jose, California, USA) and analyzed using Paint-a-Gate 3.02 software (BD BioSciences). Cells were defined according to the positivity for annexin-V (AV) and/or propidium iodide (PI) labelling. Results were presented as percentage of early apoptotic (AV+/PI-), late apoptotic/necrotic (AV+/PI+), necrotic (AV-/PI+) and viable cells (AV-/PI-).
5.3.6. Oxidative stress study

Subsequent parameters were analysed in the isolated cells from rats’ ileum by flow cytometry: peroxides levels in cytosol, with 2’,7’-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) probe; reactive species production in the mitochondria, with dihydrorhodamine 123 (DHR 123) probe; intracellular reduced glutathione (GSH) content, with mercury orange staining; and mitochondrial membrane potential, with 5,5’,6,6’-tethrachloro-1,1’,3,3’-tethraethylbenzimidazolcarbocyanine iodide probe (JC-1). Analysis was fulfilled using the flow cytometer and software outlined above, conforming to formerly detailed techniques (Gonçalves et al., 2013; Almeida et al., 2008). For each assay, 1x10$^6$ cells were used; at least 50,000 events were collected. Results were revealed as mean fluorescence intensity (MFI) values. Experiments were carried out in duplicate.

5.3.6.1. Monitoring reactive oxygen species generation in the cytosol

Intracellular reactive oxygen species production was measured with the fluorochrome 2’,7’-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) probe, which is the most widely used fluorochrome for detection of intracellular oxidative stress (Dikalov et al., 2014; Kalyanaraman et al., 2012; Gomes et al., 2005). DCFH$_2$-DA is a stable, non-fluorescent and cell permeable compound that is converted to 2’,7’-dichlorodihydrofluorescein (DCFH$_2$) by intracellular esterases; when oxidized by reactive oxygen species, the deesterified product is converted to 2’,7’-dichlorofluorescein (DCF), a fluorescent compound with excitation/emission wavelengths of 498/522 nm, respectively (Gomes et al., 2005). Despite some limitations, DCFH$_2$-DA was considered a simple, rapid and sensible probe for intracellular reactive oxygen species detection, including hydrogen peroxide (H$_2$O$_2$; in presence of cellular peroxidase), peroxynitrite (ONOO$^-$) and hydroxyl radical (HO$^-$) (Gomes et al., 2005). DCFH$_2$-DA assay is also used to analyze cell death (Kroemer et al., 2009). Overgeneration of intracellular radical oxygen species, whose levels DCFH$_2$-
DA was considered to reflect, precedes often the mitochondrial permeability transition occurring in intrinsic apoptosis and regulated necrosis.

The assay was performed according to a previously stated procedure (Almeida et al., 2008; Halliwell and Whiteman, 2004) with the DCFH$_2$-DA probe (Molecular Probes, Life Technologies Corporation, Carlsbad, USA). A cell suspension of $1 \times 10^6$ cells per milliliter was incubated with 5 μM of DCFH$_2$-DA dissolved in dimethylformamide 1 mM, to obtain a final concentration of 5 μM, for 45 minutes, at 37°C, in a humidified atmosphere of 5% carbon dioxide, in the dark. Cells were washed with phosphate buffered saline pH 7.4 (PBS 7.4; Gibco, Life Technologies Corporation) by centrifugation at 300x g for five minutes and resuspended in 400 μl of the same buffer solution. Analysis was performed with an excitation and emission wavelengths of 488 and 525 nm, respectively.

5.3.6.2. Monitoring reactive species production in the mitochondria

Mitochondria reactive species (RS) were detected with dihydrorhodamine 123 (DHR 123) probe (Cardoso et al., 1998). DHR 123 is a probe widely used to detect several reactive species production in the mitochondria (including hypochlorous acid and peroxynitrite) (Gomes et al., 2005). DHR 123 is a nonfluorescent, noncharged dye that easily penetrates cell membranes and is oxidized, by intracellular oxidants, to rhodamine 123, a fluorescent cationic and lipophilic compound, with excitation and emission wavelengths of 505 and 529 nm, respectively, which tends to accumulate in the mitochondria, held there by the membrane potential (Gomes et al., 2005).

DHR123 (DHR123, Life Technologies Corporation, Carlsbad, USA) was added to one million cells per milliliter in phosphate buffered saline, at a final concentration of 5 μg/ml and incubated for 10 minutes in a shaking water bath, at 37°C, in the dark. Then, cells were washed, resuspended in phosphate buffered saline, and kept on ice for immediate detection by flow cytometry using the previously described equipment.
5.3.6.3. Determination of total intracellular reduced glutathione content

Determination of the intracellular glutathione (GSH) expression, the most abundant nonprotein thiol in mammalian cells and an important antioxidant defense, was performed by flow cytometry using the fluorescent compound mercury orange [1-(4-chloromercuryphenoylazo)-2-napthol]. This compound binds stoichiometrically to mercurial sulfhydryl groups of intracellular nonprotein thiols with formation of fluorescent products. Restriction of staining time allows a selective marking, as this compound reacts faster with GSH than with the sulfhydryl groups of proteins (five minutes versus eight hours, approximately) and the reaction product emits an intense red fluorescence when excited with an argon laser at a wavelength of 488 nm (O’Connor et al., 1988).

One million of cells were washed with phosphate buffered saline by centrifugation, 300x g, five minutes and resuspended in 1 ml of phosphate buffered saline. Cells suspension was incubated with 4 μL of mercury orange (MO; Sigma-Aldrich, Sintra, Portugal) in acetone (Sigma-Aldrich, Sintra, Portugal) 10 mM, at a final concentration of 40 μM, for 15 minutes, at room temperature, in the dark. After washing the cells twice with cold phosphate buffered saline by centrifugation at 300x g for five minutes, cells were resuspended in 400 μl of the same buffer solution and kept on ice. Analysis was performed at the excitation wavelength of 488 nm, by flow cytometry, using the aforementioned equipment.

5.3.6.4. Mitochondrial membrane potential measurement

Integrity of the inner mitochondrial membrane was evaluated by the gradient potential across this membrane, the mitochondrial membrane potential (Δψmt), using the fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetracyanobenzimidocarbocyanine iodide (JC-1) and flow cytometry, according to a previously outlined method (Martinou and Youle, 2011; Almeida et al., 2008). This lipophilic cationic probe is a potentiometric dye able to selectively enter the mitochondria (Gomes et al., 2005). JC-1 mitochondrial membrane potential-sensitive fluorochrome allows the
visualization of the energized mitochondria, monitorization of the mitochondrial membrane potential through the change of emission spectra and detection of the mitochondrial membrane permeabilization (Galluzzi et al., 2012; Kroemer et al., 2009). Mitochondrial membrane potential determines the selective uptake of JC-1 by mitochondria, emitting fluorescence at different wavelengths. When the membrane potential is high, aggregate formation in the mitochondria is predominant and red/greenish-orange fluorescence (590 nm) is emitted after excitation at 490 nm; on the contrary, as the mitochondrial membrane potential decreases, or if the membrane is depolarized, JC-1 forms monomers in the cytosol that emit green fluorescence (529 nm). Thus, the ratio of red/greenish-orange and green fluorescence determined by flow cytometry, which corresponds to aggregates/monomers quotient, provides an estimate of the mitochondrial membrane potential. An increase of the aggregates/monomers ratio indicates an increase in the mitochondrial membrane potential (Gomes et al., 2005). Additionally, JC-1 constitutes a cytofluorometric method for cells viability and death analysis, contributing to the definition of cell death (through identification of irreversible mitochondrial transmembrane potential dissipation) and the characterization of death modality, as occurs in intrinsic apoptosis and parthanatos (Galluzzi et al., 2012; Kroemer et al., 2009).

Briefly, one million cells were washed with phosphate buffered saline through centrifugation at 300x g during five minutes, resuspended in 1 ml of phosphate buffered saline and incubated with 1 μl of JC-1 dye (Molecular Probes, Life Technologies Corporation, Carlsbad, USA), in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Sintra, Portugal) at 5 mg/ml concentration, for 15 minutes, at 37°C, in the dark. At the end of the incubation period, cells were washed twice in cold phosphate buffered saline by centrifugation at 300x g for five minutes, resuspended in 400 μl of same buffer solution and analyzed by flow cytometry. Results were presented as aggregates/monomers fluorescence intensities ratio, calculated through the quotient between mean fluorescence intensities (MFI) of aggregates and monomers.
5.3.7. Evaluation of tissue and systemic inflammatory response

A multiplex cytokine bead array approach was used to measure the expression of inflammatory cytokines, using the Rat Cytokine 5plex Kit FlowCytomix (eBioscience, Affymetrix, Vienna, Austria) produced for quantification of rats’ homogenized tissue and plasma levels of interleukine-1α (IL-1α), macrophage chemo-attractant protein (MCP-1), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukine-4 (IL-4).

Flow cytometric multiplexed bead assay is characterized by high sensitivity, specificity, reproducibility and cost-efficiency (Tighe et al., 2013). Succinctly, the biological test sample and a recombinant protein standard mixture are combined with a mixture of sets of micrometer scale beads, each impregnated with a fluorescent dye, stably coated with a capture antibody to the desired analyte (cytokine) to be detected. Beads can be differentiated by their sizes and by their distinct spectral addresses. After incubation and washing, a mixture of labeled detection antibodies (biotin-conjugated) specific for the desired cytokines and labeled with the same reporter dye (streptavidin-phycoerythrin) is added. After further washing, the detection antibody-cytokine-capture antibody-bead complex is analyzed through a flow cytometer. Each bead has the potential to have both the cytokine captured, and the specific detection antibody reporter-dye bound. Proportion of reporter bound depends upon cytokine concentration in the original biological solution allowing quantitative analysis of each bead. Calibration to bead sets incubated with dilution series of analytes allows quantitative interpretation (Tighe et al., 2013).

In the Rat Cytokine 5plex Kit FlowCytomix, analyte sensitivity [defined as the concentration resulting in a fluorescent intensity significantly higher than that of the dilution medium (mean+2standard deviations)] was determined to be the following: IL-1α=8.5 pg/ml, MCP-1=0.8 pg/ml, TNF-α=4.3 pg/ml, IFN-γ=0.8 pg/ml and IL-4=0.3 pg/ml. Biological samples standardization was attentive to increase stability and representativeness of the cytokine measurement. Reagents were prepared following the manufacturer’s recommendations (described in detail in Supplementary Data S1). Standard and test samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA) as stated in the producer’s instructions. Mean
fluorescence intensities (MFIs) of the serially diluted standard samples were calculated and used to generate the standard curves of each cytokine that model protein concentration as a function of the MFI. Cytokine concentrations present in the test sample were calculated, using the corresponding standard curves and dilution factors. All the samples were run in duplicate. Results were presented in pg/ml. Tissue concentrations were normalized to the total protein content of the sample, estimated by the bicinchoninic acid protein assay (Smith et al., 1985) recurring to bovine serum albumin as standard, and expressed as pg/μg protein. Pro-to-antiinflammatory cytokines ratio ([IL-1α]+[TNF-α]+[IFN-γ]/[IL-4]) and T-helper 1 lymphocytes (Th1)-to-T-helper 2 lymphocytes (Th2) cytokines quotient ([TNF-α]+[IFN-γ]/[IL-4]) were calculated (Ramirez et al., 2013; Menger and Vollmar, 2004).

### 5.3.8. Bicinchoninic acid protein assay

Total protein content of the homogenate of each tissue specimen was determined by the bicinchoninic acid protein assay (Smith et al., 1985) using bovine serum albumin as reference. Bicinchoninic acid is a stable water-soluble compound capable of origin an intense purple chromophore complex with cuprous ion in an alkaline milieu. Bicinchoninic acid allows monitoring cuprous ions (Cu⁺) produced in the reaction of proteins with alkaline cupric ions (Cu²⁺) (biuret reaction), creating chromophore complexes (that display a strong absorbance at 562 nm) proportional to a broad range of protein concentrations. Bicinchoninic acid protein assay is a simple, high sensitivity, one-step analysis, less susceptible to common interferences (like nonionic detergents and buffer salts) than the Lowry’s technique (Reichelt et al., 2016; Sapan et al., 1999; Smith et al., 1985).

In summary, protocol included the preparation of a working mixture of bicinchoninic acid solution (containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1N sodium hydroxide, pH 11.25) and 4% copper sulphate [4% (w/v) CuSO₄·5H₂O], in a proportion of 50:1, and of calibration protein standards from bovine serum albumin (2000, 1500, 1000, 750, 500, 250, 125 and 25 μg/ml). All protein standards (25 μl of each) and the biological sample (5 μl of the homogenate in 20 μl of
water) were pipetted into a microwell plate, and 200 μl of the working mixture was added mixing thoroughly. Plate was covered, incubated at 37°C, for 30 minutes, in a water bath, and then, cooled to room temperature. Absorbance at 562 nm of all the samples was measured within ten minutes in an enzyme-linked immunosorbent assay (ELISA) equipment (Synergy™ HT Multi-Mode Microplate Reader, Biotek Instruments, Winooski, Vermont, USA). Protein concentration of the biological sample was calculated from the calibration plot constructed from protein standards and results were expressed in μg/ml.

5.3.9. Statistical analysis

Statistical analysis was completed using the SPSS Software version 18.0 (SPSS, Chicago, Illinois, USA). Testing for normality was performed with Shapiro Wilk and Kolmogorov-Smirnov-Lillifors tests. Data were indicated as median and interquartile range (median±IQR). Comparison of non-parametric continuous variables was undertaken with Mann-Whitney U test and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons. Correlations were determined by the Spearman’s rank correlation coefficient (σ). A probability value of p<0.05 was considered to denote statistical significance.

5.4. Results

5.4.1. Postoperative outcome

Fifty-nine animals concluded the experiment and were comprised into the following groups: “Res Ted +” (15, eight of them sacrificed on the third day), “Res Ted –” (13, five sacrificed at third day), “Lap Ted +” (16, eight sacrificed on the third day) and “Lap Ted -” (15, seven sacrificed at third day).
5.4.2. Cellular viability and death

In animals not submitted to teduglutide treatment, ileal perianastomotic segments demonstrated a significant decrease of viable cells proportion between the third and seventh days ($p=0.01$) (Fig. 5.1).

Teduglutide administration was associated with an increase of viability index in the cells isolated from the perianastomotic segment at the seventh day ($p=0.005$) (Fig. 5.2).

Global evaluation of the effects of postoperative teduglutide treatment underscored the increase of cellular viability ($p=0.0001$) and decrease of early apoptosis occurrences at the seventh day ($p=0.001$) (Fig. 5.3).

Figure 5.1. Viability and death in cells isolated from rats’ ileum, at the third and seventh days after ileal resection and anastomosis, in animals not submitted to teduglutide treatment. Analysis was performed by flow cytometry using double staining with Annexin-V/Propidium iodide. Animals ($n=13$) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. Samples recovered at sacrifice corresponded to the anastomotic segment. Baseline values were considered for comparison. Data were explicit as percentage (%) of viable, early apoptotic, late apoptotic/necrotic (late apop/necrotic) and necrotic cells (median:interquartile range). Kruskal-Wallis test with pairwise comparison was used.
Viability and death in cells isolated from rats’ ileum, at the third and seventh days after the operation, in the different groups of study. Analysis was performed by flow cytometry using double staining with Annexin-V/Propidium iodide. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or seventh postoperative day. In groups “Res Ted +” and “Lap Ted +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Data were explicited as percentage (%) of viable, early apoptotic, late apoptotic/necrotic (late apop/necrotic) and necrotic cells (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.

5.4.3. Oxidative stress

Intestinal anastomotic healing induced a prooxidative influence, particularly evident at the third day, which was expressed by an increase of cytosolic peroxides level (p=0.0001) and reactive species generation in the mitochondria (p=0.005) and by a reduction of mitochondrial membrane potential (p=0.001) and cellular reduced glutathione content (p=0.001) until the seventh day (Fig. 5.4). Teduglutide treatment appeared to reinforce the prooxidative effects of anastomotic repair, although without reaching statistical significance (Fig. 5.5). When considering all the operated animals, postoperative teduglutide administration was significantly associated with an increase of the cytosolic peroxides level at the third day (p=0.042), as well as of the mitochondrial generation of reactive species at the seventh day (p=0.011) (Fig. 5.6). Teduglutide influence on the mitochondrial membrane potential appeared to be not relevant.
Figure 5.3. Viability and death in cells isolated from rats’ ileum, at the third and seventh days after the operation, according to the teduglutide administration.

Analysis was performed by flow cytometry using double staining with Annexin-V/Propidium iodide. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lop) and sacrificed on the third or seventh postoperative day. In groups “Teduglutide +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomatic segment. Data were explicitied as percentage (%) of viable, early apoptotic, late apoptotic/necrotic (late apop/necrotic) and necrotic cells (median±interquartile range). Mann-Whitney U test was used.

Figure 5.4. Oxidative stress evaluation in cells isolated from rats’ ileum, at the third and seventh days after ileal resection and anastomosis, in animals not submitted to teduglutide treatment. Assessment was performed by flow cytometry using DCFH2-DA, DHR123 and JC-1 fluorescent probes and mercury orange to determine cytosolic peroxides level, mitochondrial reactive species generation, mitochondrial membrane potential (mV) and cellular reduced glutathione content ([GSH]), respectively. Animals (n=13) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. Samples recovered at sacrifice corresponded to the anastomatic segment. Baseline values were considered for comparison. Data were presented as mean fluorescence intensity (MFI) values (median±interquartile range). Results of JC-1 probe were expressed as aggregates/monomers ratio. Kruskal-Wallis test with pairwise comparison was used.
Oxidative stress evaluation in cells isolated from rats' ileum, at the third and seventh days after the operation, in the different groups of study. Assessment was performed by flow cytometry using DCFH$_2$-DA, DHR123 and JC-1 fluorescent probes and mercury orange to determine cytosolic peroxides level, mitochondrial reactive species generation, mitochondrial membrane potential (m$\psi$) and cellular reduced glutathione content ([GSH]), respectively. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or seventh postoperative day. In groups “Res Ted +” and “Lap Ted +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Data were presented as mean fluorescence intensity (MFI) values (median±interquartile range). Results of JC-1 probe were expressed as aggregates/monomers ratio. Kruskal-Wallis test with pairwise comparison was used.

5.4.4. Local inflammatory response

Anastomotic repair induced a significant upregulation of tissue IL-1$\alpha$ (p=0.0001), MCP-1 (p=0.026) and TNF-$\alpha$ (p=0.0001) until the third day, with a drop of TNF-$\alpha$ thereafter; as well as of IFN-$\gamma$ between the third and seventh days (p=0.034) (Fig. 5.7). An increase of pro-to-antiinflammatory cytokines ratio until the seventh day (623.8±422.5 vs. 7098.5±7396.5, p=0.0001) and of Th1-to-Th2 cytokines ratio, especially until the third day (0.0±0.9 vs. 118.2±49.3, p=0.0001) were observed in the perianastomotic segment.

Teduglutide administration was associated with higher tissue levels of IL-4 at the seventh day after isolated laparotomy (p=0.036) (Fig. 5.8).
When considering all the studied animals, teduglutide treatment was associated with a higher expression of IL-4 at the seventh day after the operation \((p=0.0001)\) (Fig. 5.9), concomitant with a lower pro-to-antiinflammatory and Th1-to-Th2 cytokines ratios \((409.3\pm1768.1 \text{ vs. } 2864.5\pm6613.6, \ p=0.012 \text{ and } 0.0\pm2.2 \text{ vs. } 3.0\pm7.3, \ p=0.017, \ \text{respectively})\).

![Figure 5.6](image-url)

**Figure 5.6. Oxidative stress evaluation in cells isolated from rats’ ileum, at the third and seventh days after the operation, according to teduglutide administration.** Assessment was performed by flow cytometry using DCFH2-DA, DHR123 and JC-1 fluorescent probes and mercury orange to determine cytosolic peroxides level, mitochondrial reactive species generation, mitochondrial membrane potential \((\Delta m\psi)\) and cellular reduced glutathione content \((\text{[GSH]})\), respectively. Animals \((n=59)\) were submitted to ileal resection and anastomosis \((\text{Res})\) or laparotomy \((\text{Lap})\) and sacrificed on the third or seventh postoperative day. In groups “Teduglutide +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Data were presented as mean fluorescence intensity \((\text{MFI})\) values (median±interquartile range). Results of JC-1 probe were expressed as aggregates/monomers ratio. Mann-Whitney U test was used.
Figure 5.7. Analysis of tissue inflammatory response in rats’ ileum by flow cytometric multiplexed bead assay, at the third and seventh days after ileal resection and anastomosis, in animals not submitted to teduglutide treatment. Animals (n=13) were submitted to ileal resection and anastomosis and sacrificed on the third or the seventh postoperative days. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Baseline values were considered for comparison. Cytokines concentrations were determined as function of fluorescence intensities and normalized to the protein content of the sample. Data were expressed as pg/mg (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.

Figure 5.8. Analysis of tissue inflammatory response in rats’ ileum by flow cytometric multiplexed bead assay, at the third and seventh days after the operation, in the different groups of study. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or the seventh postoperative days. In groups “Res Ted +” and “Lap Ted +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Cytokines concentrations were determined as function of fluorescence intensities and normalized to the protein content of the sample. Data were expressed as pg/mg (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.
Figure 5.9. Analysis of tissue inflammatory response in rats’ ileum by flow cytometric multiplexed bead assay, at the third and seventh days after the operation, according to the teduglutide administration. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or the seventh postoperative days. In groups “Teduglutide +”, teduglutide was administered after the operation. Samples recovered at sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Cytokines concentrations were determined as function of fluorescence intensities and normalized to the protein content of the sample. Data were expressed as pg/mg (median±interquartile range). Mann-Whitney U test was used.

5.4.5. Systemic inflammatory response

After ileal resection and anastomosis, an increase of plasma IFN-γ levels until the third day was documented; on the other hand, a decrease of IL-1α and TNF-α concentrations until the seventh day (p=0.0001 and p=0.0001, respectively) was also observed (Fig. 5.10).

Plasma pro-to-antiinflammatory and Th1-to-Th2 cytokines ratios increased until the third day after ileal resection and anastomosis (32.7±11.7 vs. 47.3±12.3, p=0.017 and 16.2±6.1 vs. 23.1±8.2, n.s., respectively) and decreased thereafter (47.3±12.3 vs. 8.2±2.3, p=0.0001 and 23.1±8.2 vs. 8.2±2.3, p=0.0001, respectively).
Teduglutide administration was associated with lower plasma levels of IL-1α, IFN-γ and TNF-α at the seventh day after laparotomy ($p=0.0001$, respectively); lower levels of IL-4 at both moments of evaluation after laparotomy ($p=0.013$ and $p=0.0001$, respectively); as well as lower plasma concentrations of IFN-γ at the third day after ileal resection and anastomosis ($p=0.004$) (Fig. 5.11).

When analyzing all the animals, teduglutide administration was associated with lower plasma levels of IFN-γ at the third day ($p=0.026$) and of IL-1α ($p=0.004$), TNF-α ($p=0.002$), IFN-γ ($p=0.0001$) and IL-4 ($p=0.0001$) at the seventh day (Fig. 5.12). That growth factor was not associated with significant modifications of plasma pro-to-antiinflammatory or Th1-to-Th2 cytokine ratios.

**Figure 5.10.** Analysis of systemic inflammatory response by flow cytometric multiplexed bead assay, at the third and seventh days after ileal resection and anastomosis, in animals not submitted to teduglutide treatment. Animals (n=13) were submitted to ileal resection and anastomosis and sacrificed on the third or seventh postoperative day. Baseline values were considered for comparison. Data were transmitted as plasma concentration (pg/ml) (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.
Figure 5.11. Analysis of systemic inflammatory response by flow cytometric multiplexed bead assay, at the third and seventh days after the operation, in the different groups of study. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or seventh postoperative day. In groups “Res Ted+” and “Lap Ted+”, teduglutide was administered after the operation. Data were transmitted as plasma concentration (pg/ml) (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.

Figure 5.12. Analysis of systemic inflammatory response by flow cytometric multiplexed bead assay, at the third and seventh days after the operation, according to the teduglutide administration. Animals (n=59) were submitted to ileal resection and anastomosis (Res) or laparotomy (Lap) and sacrificed on the third or seventh postoperative day. In groups “Teduglutide +”, teduglutide was administered after the operation. Data were transmitted as plasma concentration (pg/ml) (median±interquartile range). Mann-Whitney U test was used.
5.4.6. Relevant correlations between cellular viability and death indexes, oxidative stress parameters and cytokines levels at the sacrifice

In the postoperative evaluation of cells isolated from the rats’ ileum, cytosolic peroxides level, mitochondrial reactive species generation and mitochondrial membrane potential level correlated moderately and significantly with cellular viability (σ=50.4%, p=0.0001; σ=61.9%, p=0.0001 and σ=-49.8%, p=0.0001, respectively) (Table 5.1).

### Table 5.1. Relevant correlations between cellular viability and death indexes, oxidative stress parameters and tissue levels of cytokines at the sacrifice (n=59)^[a]

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<th>Late ap</th>
<th>Necrosis</th>
<th>Peroxides</th>
<th>Reac Sp</th>
<th>iGSH</th>
<th>mΨ</th>
<th>[IL-1α]</th>
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<th>[IL-4]</th>
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^[a] Analysis was performed by flow cytometry. Cellular viability and death indexes were determined using annexin-V/propidium iodide; cytosolic peroxides level (Peroxides), reactive species generation in the mitochondria (Reac Sp), mitochondrial membrane potential (mΨ) and cellular reduced glutathione content (iGSH) with DCFH-DA, DHR123 and JC-1 fluorescent probes and mercury orange staining, respectively; and tissue levels of cytokines by flow cytometric multiplex bead assay. Spearman’s correlation coefficient (σ) and value of significance (p) were presented. Early ap, Early apoptosis index; Late ap, Late apoptosis/necrosis index
Correlations between the levels of cytosolic peroxides and mitochondrial reactive species and tissue concentrations of IL-1α, MCP-1 and TNF-α were positive, while those of reduced glutathione and mitochondrial membrane potential levels were inverse.

5.5. Discussion

Intestinal anastomotic healing is a complex process that progresses in three imbricated steps: inflammatory, proliferative and remodeling (Rijcken et al., 2014; Thompson et al., 2006). In this study, the repair process induced a significant decrease of the percentage of viable cells isolated from the perianastomotic segment between both postoperative moments of evaluation. Intestinal anastomotic healing was characterized by a relevant and sustained prooxidative influence, particularly evident at the third day, including an increase of the cellular oxidative burden and a decrease of the mechanisms of protection against the oxidative injury, as expected to occur during the infiltration of the wounded tissues by inflammatory cells (Rijcken et al., 2014; Speca et al., 2012).

Inflammatory response and balance between pro and antiinflammatory cytokines are important in determining the outcome of the wound healing process (Zubaidi et al., 2015). Moreover, in a recently published meta-analysis, increased postoperative peritoneal levels of interleukine-6 (IL-6) (at the first, second and third days) and TNF-α (at the third, fourth and fifth days) were significantly associated with clinical relevant anastomotic leakage after colorectal surgery and were considered to contribute to its early detection (Sparreboon et al., 2016b). Higher plasma levels of IL-6 at the first day were also significantly associated with anastomotic leakage after colorectal surgery, but with a poor predictive capacity (Sammour et al., 2016). Nevertheless, in current context, local peritoneal cytokine levels appear to be of greater clinical relevance than systemic levels. Previous animal studies reported that systemic IL-6 administration exerts a negative effect on the healing of colonic anastomoses (Sammour et al., 2016).
However, description of the cytokines profiles and their time course during the intestinal anastomotic healing has not always been consistent in the literature (Zubaidi et al., 2015; Alzoghaibi and Zubaidi, 2014; Zubaidi et al., 2010).

In this experiment, tissue and plasma levels of inflammatory cytokines were determined by flow cytometry using a multiplex bead array approach. This was a rapid and simple method that allowed simultaneous quantification of multiple cytokines in a small sample volume (Tighe et al., 2013). The obtained results demonstrated high variability and, in some cases, did not reach the sensibility threshold of the assay (including some IL-1α and TNF-α determinations). Accurate measurement of cytokines expression is often hampered by stability and representativeness problems. In fact, cytokines have a short half-life and are susceptible to absorption, release from cells and degradation during sample collection and handling (Keutsterns et al., 2013).

In the present study, anastomotic healing activated a predominant tissue proinflammatory and Th1 response. Repair process promoted a significant upregulation of IL-1α, MCP-1 and TNF-α until the third day; and of IFN-γ between the third and the seventh days. Similarly, Seifert GJ et al (Seifert et al., 2014) demonstrated an upregulation of tissue IL-1α and IL-1β gene expressions on the second day and a downregulation in the later course until the eighth day, although without a consistent regulation of IFN-γ gene expression. Other authors described upregulation of IL-1β until the seventh day after an ileal anastomosis (Zubaidi et al., 2015; Zubaidi et al., 2010) and increase of IFN-γ expression between the third and seventh days (Zubaidi et al., 2015); however, contrary to the present findings, they observed a decrease of TNF-α levels at the third postoperative day (Zubaidi et al., 2015; Zubaidi et al., 2010).

In this investigation, downregulation of IL-4 at the seventh postoperative day was concordant with the literature (Zubaidi et al., 2010) and the MCP-1 kinetic profile in the anastomotic segment was analogous to that previously demonstrated by Alzoghaibi MA and Zubaidi AM (Alzoghaibi and Zubaidi, 2014).

Current results revealed a predominant proinflammatory and Th1 systemic response at the third day after intestinal resection and anastomosis and a prevailing antiinflammatory and Th2 systemic reaction at the seventh day, in concordance with
the literature (Murakami et al., 2007). As reported by previous experimental and clinical studies, the initial proinflammatory phase of the host response during the early postoperative period is followed by the antiinflammatory cytokines production by Th2 lymphocytes (Choileain and Redmond, 2006). Surgical stress induces a shift in the Th1/Th2 cell balance towards Th2 (Ramirez et al., 2013; Murakami et al., 2007; Decker et al., 1996), suggesting downregulation of cell-mediated and upregulation of antibody-mediated immunity, proportional to the magnitude of injury (Ramirez et al., 2013; Decker et al., 1996).

This study demonstrated a favorable influence of teduglutide on intestinal cellular viability in the perioperative context, particularly after ileal resection and anastomosis, and confirms its anti-apoptotic effects. Previous studies demonstrated that GLP-2 increases the intestinal epithelial proliferation in the crypts and inhibits the apoptosis in the crypts and villi (Drucker and Yusta, 2014).

Furthermore, present findings suggested a prooxidative influence of teduglutide and did not corroborate other authors’ conclusions pointing to an antioxidant effect (Arana et al., 2017; Lei et al., 2016; Drucker and Yusta, 2014; Janssen et al., 2013; Arda-Pirincci et al., 2011). In fact, Arda-Pirincci et al (Arda-Pirincci et al., 2011) demonstrated that teduglutide pretreatment prevented tumor necrosis factor-alpha/actinomycin D-induced intestinal oxidative injury, in a mouse model, with reduction of lipid peroxidation (malondialdehyde levels) and glutathione levels, glutathione peroxidase and superoxide dismutase activities, and a marked increase in catalase activity. In 2016, Lei Q et al (Lei et al., 2016) observed that exogeneous GLP-2 improves the intestinal antioxidant capacity, namely the tissue glutathione level, in a mouse model of total parenteral nutrition therapy. Dissimilar results of present study may be explained by the use of different models of intestinal injury (chemical/inflammatory versus surgical agression), materials used for oxidative stress analysis (homogenates of intestinal tissue instead of isolated cells’ preparations) and teduglutide administration schedules (timing and posology).

In this investigation, postoperative prooxidative effect of teduglutide was expressed by an increase of peroxides level in the cytosol at the third day and of reactive species generation in the mitochondria at the seventh day. Those parameters of oxidative
stress correlated positively with cellular viability index suggesting absence of deleterious effects on cellular death promotion and, on the contrary, a favorable influence on viability; indeed, in present analysis, teduglutide was associated with proviability and antiapoptotic effects. Although excessive intestinal oxidative stress may negatively influence anastomotic repair through disruption of cell signaling (including of the redox modulation of effector cells proliferation and differentiation), irreversible oxidation of macromolecules and induction of cell death (Circu and Aw, 2012; Aw, 2012), as demonstrated by other authors (Teke et al., 2013; Poyrazoglu et al., 2011), a certain level of redox stimulus is necessary to the normal course of the wound healing process (Rijcken et al., 2014; Greaves et al., 2013; Speca et al., 2012).

In current experiment, teduglutide postoperative treatment was related with a significant upregulation of tissue IL-4 expression at the seventh postoperative day and a shift of the postoperative local balance towards an antiinflammatory and Th2 response at that time point, although those effects did not reach statistical significance when analyzing exclusively the anastomotic segments. Upregulation of IL-4 at the seventh day may promote the transition from a primarily inflammatory state to a more proliferative phase of healing. IL-4 has been associated with activation and differentiation of fibroblasts, production of collagens, upregulation of matrix metalloproteinase 2, matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1, inhibition of the Th1 response and stimulation of Th2 cells (Speca et al., 2012). Present results suggest that teduglutide may influence anastomotic healing through the interference in the pro and antiinflammatory pathways, with a prevailing antiinflammatory effect at the seventh postoperative day.

In conclusion, in present study, intestinal anastomotic healing was characterized by a local prooxidative and proinflammatory response. Surgical injury associated with intestinal resection and anastomosis induced a systemic predominantly proinflammatory and Th1 cytokines reaction at the third day and an antiinflammatory and Th2 response at the seventh day.

Postoperative teduglutide treatment was significantly associated with a tissue prooxidative and antiinflammatory influence, with potential interference in the inflammatory and proliferative phases of the intestinal anastomotic healing.
Chapter 6

*Tissue growth factors profile after teduglutide administration on an animal model of intestinal anastomosis*
This chapter was partially published as:


6.1. Abstract

Teduglutide is an enterotrophic analogue of glucagon-like peptide 2 (GLP-2) with an indirect and poorly known mechanism of action, approved for the rehabilitation of short-bowel syndrome. This study aimed to analyze the response of tissue growth factors to surgical injury and teduglutide administration on an animal model of intestinal anastomosis. Wistar rats (n=59) were distributed into four groups: “Ileal Resection and Anastomosis” or “Laparotomy”, each one subdivided into “Postoperative Teduglutide Administration” or “No Treatment”; and sacrificed at the third or the seventh day, with ileal and blood harvesting. Gene expression of insulin-like growth factor 1 (Igf1), vascular endothelial growth factor a (Vegfa), transforming growth factor β1 (Tgfβ1), connective tissue growth factor (Ctgf), fibroblast growth factor 2 (Fgf2), fibroblast growth factor 7 (Fgf7), epidermal growth factor (Egf), heparin-binding epidermal-like growth factor (Hbegf), platelet-derived growth factor b (Pdgfb) and glucagon-like peptide 2 receptor (Glp2r) was studied by quantitative real-time reverse-transcription polymerase chain reaction. Plasma levels of Glp-2 were determined by competitive enzyme immunoassay. Upregulation of Fgf7, Fgf2, Egf, Vegfa and Glp2r at the third day, and of Pdgfb at the seventh day, was verified in the perianastomotic segments. Teduglutide administration was associated with higher fold-change of relative gene expression of Vegfa (3.6±1.3 vs. 1.9±2.0, p=0.0001), Hbegf (2.2±2.3 vs. 1.1±0.9, p=0.001), Igf1 (1.6±7.6 vs. 0.9±0.7, p=0.002) and Ctgf (1.1±2.1 vs. 0.6±2.0, p=0.013); and lower fold-change of Tgfβ1, Fgf7 and Glp2r. These results underscore the recognized role of Igf1 and Hbegf as molecular mediators of the effects of teduglutide and suggest that other humoral factors, like Vegfa and Ctgf, may also be relevant in the perioperative context. Induction of Vegfa, Igf1 and Ctgf gene expressions might indicate a favorable influence of teduglutide on the intestinal anastomotic healing.
6.2. Introduction

Failure of intestinal anastomotic repair persists as a major source of morbidity and mortality in digestive surgery and one of the most feared postoperative complications (Guyton et al., 2016; Bosmans et al., 2015; Shogan et al., 2013). Intestinal anastomotic healing is a multicellular multimolecular compelling process driven by the interplay of multiple signaling pathways and precisely controlled in terms of space and time (Rijcken et al., 2014) (Table 6.1). Teduglutide is a long-acting modified form of glucagon-like peptide 2 (GLP-2), which is an intestinal growth-promoting factor with intestinotrophic, antisecretory, transit-modulating and antiinflammatory effects. GLP-2 demonstrates a complex, indirect and poorly understood mechanism of action that appears to be mediated, at least partially, by insulin-like growth factor 1, ErbB superfamily of ligands, fibroblast growth factor 7, and vascular endothelial growth factor (Drucker and Yusta B, 2014). Response of tissue growth factors, key mediators of the anastomotic repair, to teduglutide administration in the perioperative context of intestinal anastomosis is still to be defined.

This study intended to analyze the response of tissue growth factors to surgical injury and teduglutide short-term administration on an animal model of intestinal anastomosis.

### Table 6.1. Simplified overview of the intestinal anastomotic healing

<table>
<thead>
<tr>
<th>Phase</th>
<th>Timing</th>
<th>Predominant cell types</th>
<th>Relevant mediators</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>1 to 4 days</td>
<td>Platelets, neutrophils, M1 macrophages, fibroblasts</td>
<td>PDGF, TGF-β, VEGF, IGF-1, cytokines, chemokines, coagulation factors, plasma proteases, arachidonic acid metabolites, vasoactive amines, ROS</td>
<td>Coagulation, inflammation, debridement, ECM degradation, protection, provisional wound closure</td>
</tr>
<tr>
<td>Proliferation</td>
<td>2 to 14 days</td>
<td>Fibroblasts, myofibroblasts, smooth muscle cells, M2 macrophages, lymphocytes, epithelial cells, endothelial cells</td>
<td>PDGF, TGF-β, FGF-2, IGF-1, VEGF, EGF, HB-EGF, cytokines, chemokines</td>
<td>Reepithelialization, neoangiogenesis, fibroplasia, definitive wound closure</td>
</tr>
<tr>
<td>Remodeling</td>
<td>14 to 180 days</td>
<td>Fibroblasts, lymphocytes</td>
<td>PDGF, TGF-β</td>
<td>Collagen cross-link, maturation of the wound</td>
</tr>
</tbody>
</table>

ECM, extracellular matrix; EGF, Epidermal growth factor; FGF-2, Fibroblast growth factor 2; HB-EGF, heparin-binding epidermal growth factor; IGF-1, Insulin-like growth factor 1; PDGF, Platelet-derived growth factor; ROS, Reactive oxygen species; TGF-β, Transforming growth factor β; VEGF, Vascular endothelial growth factor. Adapted from Rijcken E et al., 2014
6.3. Methods

6.3.1. Study protocol and surgical procedures

Experiment was approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (Official Letter nº 32-06-2009) and performed according to the institutional and national animals’ protection guidelines.

Adult male Wistar *albinus* rats were randomly allocated into four groups: “Ileal Resection and Anastomosis” (“Res”) or “Laparotomy” (“Lap”), each one subdivided into “Postoperative Teduglutide Administration” (“Ted +”) or “No Treatment” (“Ted -”). Assessment was performed at the operation and sacrifice moments, at the third or the seventh postoperative day (eight subgroups), with ileal segment harvesting and blood collection. Blinded evaluation was accomplished in the laboratorial analysis. Animals weighting 250 to 300 g were harboured in ventilated cages with a controlled environment of temperature (22±1°C), relative humidity (50±10%), and light-dark cycles of 12 hours; and with free access to water and standard rodent diet.

All the surgical interventions were performed by the same surgeon after two hours solid fasting, with clean surgical technique and under anaesthesia with intraperitoneal ketamine hydrochloride (75 mg/kg; Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg; Laboratórios Vitória, Amadora, Portugal). In “Res” groups, a 10-cm length ileal resection was undertaken, 5 cm upstream of ileocecal valve, after a 3-cm abdominal wall midline incision. A standard end-to-end anastomosis was constructed with eight equidistant full-thickness polydioxanone USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl., Cincinnati, USA). Abdominal wall was closed with muscle-aponeurotic and cutaneous running sutures of braided coated polyglactin 910 USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl., Cincinnati, USA). Abdominal wall was closed with muscle-aponeurotic and cutaneous running sutures of braided coated polyglactin 910 USP 4/0 (Surgilactin, Sutures Limited, Wrexham, UK) and natural silk USP 4/0 (Surgisilk, Sutures Limited), respectively. In “Lap” groups, animals were subjected to a 3-cm midline laparotomy (without resection) with gentle manipulation of the small bowel.

In “Ted +” groups, teduglutide (American Peptide Company, Sunnyvale, California, USA) was administered in the postoperative period (from the operation day), 200 μg/kg/day, subcutaneously, dissolved in 0.25 ml phosphate buffered saline pH 7.4 (PBS, pH 7.4, Gibco, Life Technologies, Carlsbad, California, USA), after preparation.
according to the manufacturer’s recommendations. In the first postoperative day, ingestion of water with 5% glucose at a 1:1 ratio was allowed and then unrestricted oral hydration and chow were reassumed. Daily surveillance was performed and operative mortality and morbidity were registered. At the third or seventh postoperative day, animals were sacrificed by cervical displacement and a relaparotomy with ileal resection was performed (10-cm length, preserving distal 3 cm).

6.3.2. Tissue and blood collection

Three similar longitudinal strips of the most distal 4-cm length of each ileal operative specimen, each one corresponding to one third of the circumference, were carefully recovered, after gentle washing with normal saline solution, for homogenization and additional procedures, respectively. Tissue baseline values of “Ileal Resection and Anastomosis” groups were considered for comparison with postoperative results of the “Laparotomy” groups; tissue samples recovered at the sacrifice in those animals corresponded to the perianastomotic segments.

Blood samples of 1 ml were drawn in the morning, before the operations, from the tail vein, into polyethylene therephthalate K3 ethylenediaminetetraacetic acid (K3EDTA) vacutainers. Samples were stabilized immediately with 0.1 mg/ml of aprotinin from bovine lung (Sigma-Aldrich, Sintra, Portugal) and 0.037 mg/ml of dipeptidylpeptidase IV competitive inhibitor nicotinonitrile dihydrochloride hydrate (Sigma-Aldrich) and centrifuged for 20 minutes at 1500x g and 4°C. Plasma aliquots were stored at -80°C.

6.3.3. Intestinal tissue homogenization

As mentioned in the Chapter 5, pieces from one ileal longitudinal strip recovered in consonance with precedent report, with approximately 1 mm, were promptly added to a mixture of protease inhibitors in a proportion of 1 ml/100 mg and underwent mechanical homogenization. Inhibitors preparation was formerly prepared by
misting aprotinin from bovine lung (Sigma-Aldrich), leupeptin hemisulfate salt (Sigma-Aldrich) and pepstatin A (Sigma-Aldrich) (1 μl of each, all diluted in a 10 mg/ml stock concentration) with 10 ml of phosphate buffered saline (PBS, pH 7.4, Gibco, Life Technologies) and stored on ice. Misture was sonicated twice with one short pulse of ten seconds, cooled during ten seconds and distributed into two tubes of 1.5 ml. Sonication (one pulse of ten seconds) was repeated and centrifugation was undertaken, 14000x g, for 10 minutes, at 4°C. Supernatant was removed to a new tube and pellet was preserved on ice for posterior ribonucleic acid (RNA) extraction.

6.3.4. Analysis of gene expression levels of growth factors and glucagon-like peptide 2 receptor

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) was used to characterize the messenger ribonucleic acid (mRNA) expression profile of genes of growth factors potentially involved in the anastomotic healing: Insulin-like growth factor 1, transcript variant: Igf1; Vascular endothelial growth factor A, transcript variant 2: Vegfa; Transforming growth factor, beta 1: Tgfb1; Connective tissue growth factor: Ctgf; Fibroblast growth factor 2: Fgf2; Fibroblast growth factor 7: Fgf7; Epidermal growth factor: Egf; Heparin-binding EGF-like growth factor: Hbegf; Platelet-derived growth factor beta polypeptide: Pdgfb; Glucagon-like peptide 2 receptor: Glp2r. Quantitative RT-PCR multistep protocol required high-quality RNA purification, optimal conversion of RNA to complementary deoxyribonucleic acid (cDNA) (reverse-transcription), amplification of the cDNA using polymerase chain reaction (PCR); and sensitive and accurate real-time detection of amplification products (Fraga et al., 2008; Nolan et al., 2006).

Total RNA was extracted from the homogenates of the longitudinal strips of ileum using the Isolate II RNA Mini Kit (Bioline, London, UK). One microgram of isolated total RNA was used for reverse-transcription, which was accomplished with the Tetro cDNA Synthesis Kit (Bioline) and using random hexamers. Real-time PCR primers were designed with the Beacon Designer (Premier Biosoft, Palo Alto, USA) and obtained from Sigma-Aldrich (Sintra, Portugal). All the genes included in this study were described in the National Center for Biotechnology Information (NCBI) Gene
database (http://www.ncbi.nlm.nih.gov/) as indicated in Supplementary Table S5. Quantitative RT-PCR was performed on a Bio-Rad iQ5 real-time PCR instrument (BioRad, Hercules, California, USA) using the SensiFAST SYBR & Fluorescein Kit (Bioline). Detailed description of the qRT-PCR protocol was included in Supplementary Data S2.

Data were analyzed by relative mRNA quantification (Pfaffl, 2004) using Hypoxanthine phosphoribosyltransferase 1 (Hprt1) as housekeeping gene internal control. All normalized values of samples corresponding to the moment of sacrifice were divided by the normalized value of the baseline (that is arbitrarily set to one), and expressed as fold variations. In rats that underwent laparotomy without resection, relative quantification was performed using mean ΔCT (threshold cycle) at baseline value of rats submitted to ileal resection.

**RNA extraction**

Succinctly, biological samples were first lysed and homogenized in the presence of guanidinium thiocyanate, a chaotropic salt that immediately deactivated endogenous RNases to ensure purification of intact RNA. After homogenization, ethanol was added to the sample. Lysate was filtered and the RNA adjusted to RNA binding conditions. Sample was then processed through a spin column containing a silica membrane to which the RNA binds. Genomic DNA contamination was removed by a DNase I digestion during the preparation. Any impurities such salts, metabolites and cellular components were removed by simple washing steps of the silica membrane with two different buffers. High-quality purified total RNA was then eluted in RNase-free water and stored at -80°C.

**Reverse-transcription (conversion of RNA to cDNA)**

The Tetro cDNA Synthesis Kit (Bioline) was used, which contains moloney strain of murine leukemia virus (MMLV) reverse transcriptase and is suitable for the production of templates for RT-PCR amplification.
Real-time polymerase chain reaction

In real-time PCR, the amount of product formed was monitored by the proportional fluorescence of the dye (fluorescein) introduced into the reaction, and the number of amplification cycles required to obtain a particular amount of DNA molecules was registered. Assuming a certain amplification efficiency between 90-105%, which usually is close to a doubling of the number of molecules per amplification cycle, it was possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample (Fraga et al., 2008; Nolan et al., 2006; Pfaffl, 2004).

Each sample was monitored for fluorescein and signals were regarded as significant if the fluorescence intensity exceeded 10-fold of the standard deviation of the baseline fluorescence, defined as threshold cycles (CT). CT were selected in the line in which all samples were in the logarithmic phase. For each sample, PCR was performed in duplicate.

6.3.5. Determination of glucagon-like peptide 2 plasma concentrations

Plasma levels of glucagon-like peptide 2 (Glp-2) were determined by competitive enzyme immunoassay (EIA) using the Glp-2 EIA Kit 96-Well Plate (Phoenix Europe GmbH, Karlsruhe, Germany), designed for quantification of rat total [Arg34]Glp2 in plasma, in accordance to the manufacturer’s recommended protocol (described in detail in Supplementary Data S3). Threshold of sensitivity of the assay was 0.16 ng/ml and typical range was 0-100 ng/ml. Cross-reactivity with rat and human GLP-2 was 100% and with rat and human GLP-1/[92-128]proglucagon was 5%. All determinations were done in duplicate.

Enzyme-linked immunosorbent assay (ELISA) protocol is characterized by high specificity and sensitivity, as well as broad analytical range and reproducibility. However, disadvantages of this procedure include the inability to distinguish between bioactive and inactive compounds, different binding affinity of the antibodies, narrow dynamic range, auto-antibody interference, high costs, requirement of large sample
volumes, and feasibility of the measurement of only one analyte at a time on a given sample volume (Tighe et al., 2013).

According to the basic principles, key event of the competitive immunoassay was the process of competitive reaction between the sample targeted peptide (or a standard peptide) and the biotinylated peptide with the primary antibody bound to the secondary antibody that coats the wells of a microtiter plate (Gan and Patel, 2013). Briefly, a secondary antibody, with nonspecific binding sites blocked, was immobilized on a solid phase, a 96 wells microtiter plate. The crystallizable fragment (Fc) of a capture primary antibody, with specificity for the peptide molecules, binded to the secondary antibody that coated the immunoplate. Biological sample peptide and standard peptide (containing a known concentration of recombinant Glp-2) competed with a biotinylated peptide for the antigen-binding fragment (Fab) of the primary antibody. Unbound protein was removed through extensive washing. Then, a streptavidin conjugated with the enzyme horseradish peroxidase (streptavidin-horseradish peroxidase complex) was added, which interacted with the biotinylated peptide. Excess of free enzyme conjugates was removed by washing. A chromogenic substrate (3,3',5,5'-tetramethylbenzidine) was added, which was chemically converted by the activity of the enzyme horseradish peroxidase coupled to the primary antibody, resulting in a color change. The intensity of the yellow color (that corresponded to the enzyme activity) was inversely proportional to the amount of peptide bound to the primary antibody in biological sample and standard solutions and directly proportional to the amount of biotinylated peptide-streptavidin-horseradish peroxidase complex. Finally, the optical density of the reaction was measured with a spectrophotometer (by the increased absorbance at 450 nm) and compared with the optical density of the known standard samples to determine peptide concentrations. Since the increase in absorbance was inversely proportional to the amount of captured peptide in the biological sample, the latter could be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of peptide (Phoenix Europe GmbH, 2017). Glp-2 plasma concentrations were calculated using the corresponding standard curve and the microplate reader with Gen5 software (Synergy HT, Biotek, Winooski, Vermont, USA), and expressed in ng/ml.
6.3.6. Statistical analysis

Results were analysed with Statistical Package for Social Sciences (SPSS) version 18 software package (SPSS, Chicago, Illinois, USA). Shapiro-Wilk and Kolmogorov-Smirnov-Lillifors normality tests were used. Data were indicated as medians and interquartile ranges (median ± IQR) or numbers (%). Non-parametric continuous variables were compared by Mann-Whitney U test and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons. Correlations were calculated by the Spearman’s rank correlation coefficient (\( \sigma \)). Categorical variables were compared by Qui-square test. The established threshold for statistical significance was 95% (\( p<0.05 \)).

6.4. Results

6.4.1. Postoperative outcome

Fifty-nine animals finished the protocol and were comprised into the groups: “Res Ted +” (15, eight of them sacrificed on the third day), “Res Ted −” (13, five sacrificed at third day), “Lap Ted +” (16, eight sacrificed on the third day) and “Lap Ted −” (15, seven sacrificed at third day).

6.4.2. Response of tissue growth factors gene expression to ileal resection and anastomosis

In the perianastomotic segments, upregulation of gene expression of Fgf7 (fold-change: 12.6±2.8), Fgf2 (fold-change: 6.1±2.2), Egf (fold-change: 2.7±2.2), Vegfa (fold-change: 2.7±1.0) and Glp2r (fold-change: 2.3±2.1) at the third postoperative day (Fig. 6.1 A); as well as of Pdgfb (fold-change: 2.0±1.5), Igf1, Egf, Hbegf, Vegfa and Glp2r at the seventh day was verified (Fig. 6.1 B). Moreover, downregulation of Igf1 (fold-change: 0.2±0.3), Hbegf (fold-change: 0.4±0.5), Tgf\( \beta \)1 (fold-change: 0.4±0.3) and Ctgf at the third day (Fig. 6.1 A); as well as of Tgf\( \beta \)1 (fold-change: 0.5±0.3), Fgf7 (fold-change: 0.5±0.4), Ctgf (fold-change: 0.5±0.3) and Fgf2 at the seventh day was also observed (Fig. 6.1 B).
Figure 6.1. Fold-changes of relative gene expression of growth factors and Glp2 receptor in the rats' ileum, at the third (A) and the seventh (B) days after operation, determined by qRT-PCR. Animals (n=59) were submitted to ileal resection and anastomosis (“Res”) or laparotomy (“Lap”) and sacrificed on the third or seventh postoperative day. In groups “Res Ted +” and “Lap Ted +”, teduglutide was administered after the operation. Samples recovered at the sacrifice from rats that underwent ileal resection corresponded to the anastomotic segment. Values were normalized to Hprt gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection (n=28). Results were expressed as median±interquartile range. Kruskal-Wallis test with pairwise comparisons was used. * p<0.05; ** p<0.01; *** p<0.001
Glp2r expression was upregulated after ileal resection and anastomosis, particularly at the third postoperative day, while it was downregulated after isolated laparotomy.

In the perianastomotic segments, lower fold-change of relative gene expression of Vegfa and Ctgf at the third day, and of Fgf7 at the seventh day, was observed in comparison with the ileal samples recovered after isolated laparotomy (Fig. 6.1).

6.4.3. Response of tissue growth factors gene expression to isolated laparotomy

In the ileal samples recovered after isolated laparotomy, upregulation of gene expression of Vegfa (fold-change: 4.1±1.1), Ctgf (fold-change: 3.4±1.6) and Egf at the third postoperative day; and of Egf (fold-change: 3.4±1.3), Fgf7 (fold-change: 3.1±1.0), Vegfa and Hbegf at the seventh day was documented (Fig. 6.1). Furthermore, downregulation of Tgfb1 (fold-change: 0.1±0.1), Pdgfb (fold-change: 0.3±0.1), Fgf2 (fold-change: 0.5±0.3), Igf1, Fgf7, Hbegf and Glp2, at the third day after the operation; as well as of Ctgf (fold-change: 0.2±0.1), Glp2r (fold-change: 0.5±0.4), Tgfb1 (fold-change: 0.5±0.1), Fgf2 and Pdgfb at the seventh day, was also evident.

6.4.4. Response of tissue growth factors gene expression to teduglutide postoperative administration

In the anastomotic segments, teduglutide was significantly associated with upmodulation of gene expression of Igf1 (2.0±0.9 vs. 0.3±0.3, p=0.028), and downmodulation of Fgf2 (0.5±0.3 vs. 6.1±2.2, p=0.028) and Fgf7 (0.1±0.0 vs. 12.6±2.8, p=0.0001) at the third day; as well as upregulation of Vegfa (2.2±1.7 vs. 1.6±0.6, p=0.045) and Ctgf (3.0±1.4 vs. 0.5±0.3, p=0.014), and downregulation of Tgfb1 (0.0±0.0 vs. 0.5±0.3, p=0.0001), Pdgfb (0.0±0.1 vs. 2.0±0.5, p=0.001) and Glp2r (0.4±0.2 vs. 1.1±0.6, p=0.002) at the seventh day (Fig. 6.1).

When considering all the animals (those submitted to ileal resection or isolated laparotomy), at the third postoperative day, teduglutide was significantly associated
with a higher fold-change of relative gene expression of \textit{Igf1} (5.6±8.0 vs. 0.7±0.7, \(p=0.0001\)) and \textit{Hbegf} (1.5±2.8 vs. 0.6±0.4, \(p=0.001\)); and lower fold-change of \textit{Fgf2} (0.5±0.2 vs. 0.8±5.5, \(p=0.002\)) and \textit{Fgf7} (0.2±0.7 vs. 1.3±11.4, \(p=0.0001\)) (Fig. 6.2). At the seventh postoperative day, teduglutide was significantly associated with higher fold-change of relative gene expression of \textit{Vegfa} (3.6±2.0 vs. 1.5±0.6, \(p=0.0001\)), \textit{Ctgf} (1.1±2.4 vs. 0.3±0.4, \(p=0.0001\)) and \textit{Hbegf} (3.0±1.6 vs. 1.3±0.7, \(p=0.004\)); and lower fold-change of \textit{Tgfβ1} (0.02±0.04 vs. 0.5±0.5, \(p=0.0001\)), \textit{Fgf7} (0.4±0.4 vs. 0.9±2.6, \(p=0.007\)) and \textit{Glp2r} (0.4±0.2 vs. 1.0±0.7, \(p=0.0001\)) (Fig. 6.2).

**Figure 6.2.** Fold-change of relative gene expression of growth factors and \textit{Glp2} receptor, in the ileum of rats from all groups (n=59), according to the teduglutide administration. Relative gene expression was determined at the moment of sacrifice (third or seventh day, after ileal resection and anastomosis or after laparotomy) by qRT-PCR. Samples recovered at the sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to \textit{Hprt} gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection (n=28). Results were expressed as median±interquartile range. Mann-Whitney U test was used. \textit{Teduglutide +}, Postoperative teduglutide administration; \textit{Teduglutide -}, Without teduglutide administration.
In all the animals ($n=59$), at the sacrifice, teduglutide was significantly associated with higher fold-change of relative gene expression of Vegfa ($3.6\pm1.3$ vs. $1.9\pm2.0$, $p=0.0001$), Hbegf ($2.2\pm2.3$ vs. $1.1\pm0.9$, $p=0.001$), Igf1 ($1.6\pm7.6$ vs. $0.9\pm0.7$, $p=0.002$) and Ctgf ($1.1\pm2.1$ vs. $0.6\pm2$, $p=0.013$); and lower fold-change of Tgfβ1 ($0.1\pm0.4$ vs. $0.4\pm0.4$, $p=0.002$), Fgf7 ($0.4\pm0.6$ vs. $1.2\pm3.0$, $p=0.0001$) and Glp2r ($0.5\pm6.1$ vs. $1.0\pm0.9$, $p=0.042$) (Fig. 6.3).

Figure 6.3. Fold-change of relative gene expression of growth factors and Glp2 receptor, in the ileum of rats from all groups ($n=59$) at the moment of sacrifice, according to the teduglutide administration. Relative gene expression was determined at the moment of sacrifice (third or seventh day, after ileal resection and anastomosis or after laparotomy) by qRT-PCR. Samples recovered at the sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to Hprt gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection ($n=28$). Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration.

6.4.5. Glucagon-like peptide 2 plasma concentrations

Median baseline levels of plasma Glp-2 were $0.906\pm0.578$ (0.385-4.140) ng/ml. In animals not submitted to teduglutide treatment, plasma levels of Glp-2 suffered a relevant increase between the third and the seventh days after ileal resection ($0.798\pm0.312$ vs. $2.549\pm4.007$ ng/ml, $p=0.0001$) and after laparotomy ($0.836\pm0.766$ vs.
1.239±0.549 ng/ml, p=0.03) (Fig. 6.4 A). At the seventh postoperative day, Glp-2 plasma levels were higher after ileal resection than after laparotomy (2.549±4.007 vs. 1.239±0.549 ng/ml, n.s.) (Fig. 4B).

**Figure 6.4.** Glp-2 plasma levels as determined by competitive enzyme immunoassay at the third and seventh days after the operation in animals not submitted to teduglutide treatment (A) and in the different groups of study (B). Animals (n=59) underwent ileal resection and anastomosis (Res) or laparotomy (Lap) and were sacrificed at the third or at the seventh postoperative days. In groups “Res Ted+” and “Lap Ted+”, teduglutide was administered after the operation. Baseline values were considered for comparison. Data were presented ng/ml (median±interquartile range). Kruskal-Wallis test with pairwise comparison was used.
Teduglutide-treated animals demonstrated higher Glp-2 plasma levels at the third day after ileal resection and anastomosis (2.407±1.507 vs. 0.798±0.312 ng/ml, n.s.) and lower concentrations at the seventh day (1.187±0.845 vs. 2.549±4.007 ng/ml, n.s.), although without achievement of the statistical significance threshold (Fig. 6.4 B).

When analyzing all operated animals (submitted to ileal resection or laparotomy), teduglutide administration was associated with higher levels of Glp-2 at the third day (1.354±1.703 vs. 0.836±0.364 ng/ml, p=0.011) and at the seventh day (1.5±1.039 vs. 1.392±1.192 ng/ml, n.s.); no significant differences were observed when considering simultaneously both moments of sacrifice (1.5±1.039 vs. 1.234±0.786 ng/ml, n.s.).

6.4.6. Correlations between gene expressions of growth factors and Glp2 receptor at the sacrifice

At the sacrifice, Glp2r relative gene expression correlated directly with Egf, Hbegf, Ctgf, Fgf7, Tgfβ1 and Igf1 gene expressions and inversely with Glp2 plasma levels (Table 6.2).

Plasma levels of Glp-2 at the moment of sacrifice correlated significantly and negatively with relative gene expression of Glp2r, Ctgf, Fgf7 and Egf; no significant correlations with Igf1, Vegfa and Hbegf mRNA expressions were observed.

5.5. Discussion

Present findings, namely the upregulation of Fgf7, Fgf2, Egf and Vegfa gene expression levels at the third postoperative day, and of Pdgfb, Vegfa, Egf and Igf1 at the seventh day, in the perianastomotic segments, reenforce the recognized participation of these growth factors in the wound healing process (Rijcken et al., 2014; Greaves et al., 2013). Upregulation of Pdgfb and Igf1 gene expression occurred in the predominantly proliferative phase, whereas that of Vegfa was verified in both inflammatory and proliferative stages, as predicted (Rijcken et al., 2014; Greaves et al., 2013). However, downregulation of Tgfβ1 and Ctgf gene expressions observed in the anastomotic segment at the third and at the seventh postoperative days was unexpected, giving the
relevant participation of those growth factors in the anastomotic repair (Rijcken et al., 2014; Seifert et al., 2014).

Table 6.2. Correlations between relative gene expressions of growth factors and Glp2 receptor in rats' small intestine and plasma levels of Glp2 at the moment of sacrifice (n=59)a

<table>
<thead>
<tr>
<th>σ / p</th>
<th>Igf1</th>
<th>Vegfa</th>
<th>Tgfb1</th>
<th>Ctgf</th>
<th>Fgf2</th>
<th>Fgf7</th>
<th>Egf</th>
<th>Hbegf</th>
<th>Pdgfb</th>
<th>Glp2r</th>
<th>Plasma [Glp2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igf1</td>
<td>-27.7%</td>
<td>-31.4%</td>
<td>34.2%</td>
<td>p=0.009</td>
<td>p=0.016</td>
<td>30.4%</td>
<td>p=0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegfa</td>
<td>-28.6%</td>
<td>-41.3%</td>
<td>-28.6%</td>
<td>-28.2%</td>
<td>34%</td>
<td>36.7%</td>
<td>29.6%</td>
<td>48.2%</td>
<td>p=0.024</td>
<td>p=0.001</td>
<td></td>
</tr>
<tr>
<td>Tgfb1</td>
<td>34.2%</td>
<td>-28.6%</td>
<td>-28.2%</td>
<td>34%</td>
<td>p=0.009</td>
<td>p=0.005</td>
<td>p=0.024</td>
<td>p=0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctgf</td>
<td>-28.2%</td>
<td>27.3%</td>
<td>34%</td>
<td>p=0.032</td>
<td>p=0.038</td>
<td>-32.6%</td>
<td>37%</td>
<td>-40.7%</td>
<td>p=0.001</td>
<td>p=0.002</td>
<td></td>
</tr>
<tr>
<td>Fgf2</td>
<td>-27.7%</td>
<td>30.3%</td>
<td>34%</td>
<td>p=0.036</td>
<td>p=0.021</td>
<td>31.8%</td>
<td>p=0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fgf7</td>
<td>-31.4%</td>
<td>-41.3%</td>
<td>34%</td>
<td>p=0.016</td>
<td>p=0.009</td>
<td>44.4%</td>
<td>37.6%</td>
<td>-40.5%</td>
<td>p=0.004</td>
<td>p=0.002</td>
<td></td>
</tr>
<tr>
<td>Egf</td>
<td>36.7%</td>
<td>27.3%</td>
<td>53.2%</td>
<td>p=0.005</td>
<td>p=0.038</td>
<td>44.4%</td>
<td>p=0.001</td>
<td>52.7%</td>
<td>-31.9%</td>
<td>p=0.016</td>
<td></td>
</tr>
<tr>
<td>Hbegf</td>
<td>31.8%</td>
<td>44.3%</td>
<td>p=0.015</td>
<td>p=0.001</td>
<td>39.9%</td>
<td>p=0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pdgfb</td>
<td>29.6%</td>
<td>-52.6%</td>
<td>p=0.024</td>
<td>p=0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glp2r</td>
<td>30.4%</td>
<td>48.2%</td>
<td>37%</td>
<td>37.6%</td>
<td>52.7%</td>
<td>39.9%</td>
<td>-30.6%</td>
<td>p=0.022</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma [Glp2]</td>
<td>-40.7%</td>
<td>-40.5%</td>
<td>-31.9%</td>
<td>-30.6%</td>
<td>p=0.022</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Relative gene expressions of growth factors and Glp2 receptor and postoperative plasma levels of Glp2 ([Glp2]) were determined by qRT-PCR and competitive enzyme immunoassay, respectively. Spearman’s rank correlation coefficient (σ) and level of significance (p) were presented. Igf1: insulin-like growth factor 1, transcript variant 1, mRNA; Vegfa: vascular endothelial growth factor A, transcript variant 2, mRNA; Tgfb1: transforming growth factor, beta 1, mRNA; Ctgf: connective tissue growth factor, mRNA; Fgf2: fibroblast growth factor 2, mRNA; Fgf7: fibroblast growth factor 7, mRNA; Egf: epidermal growth factor, mRNA; Hbegf: heparin-binding epidermal-like growth factor, mRNA; Pdgfb: platelet-derived growth factor beta polypeptide, mRNA; Glp2r: glucagon-like peptide 2 receptor, mRNA.

In fact, Seigert GJ et al. (Seifert et al., 2014) demonstrated, recently, a consistent upregulation of tissue Tgfb1, Ctgf and Igf1 gene expressions after ileoileal anastomosis. Epigenetic modifications triggered by environmental factors, including DNA methylation, histone modifications of DNA and RNA interference by regulatory noncoding RNA (such as microRNAs) (Mann and Mann, 2013) may have contributed to these results.
In this experiment, teduglutide administration was significantly associated with higher fold-change of relative gene expression of \textit{Igf1}, \textit{Hbegf}, \textit{Vegfa} and \textit{Ctgf}, as well as with lower fold-change of \textit{Tgfβ1} and \textit{Fgf7}, in the postoperative period.

Induction of \textit{Igf1} messenger RNA expression in teduglutide-treated animals documented in this study was according to the literature (Drucker and Yusta, 2014) and was observed at the third day, both in the anastomotic segments and in the ileal samples recovered after isolated laparotomy. \textit{Igf1} is considered a critical mediator of the enterotrophic effects of Glp-2 (Bortvedt \textit{et al.}, 2012), although not systematically required (Drucker and Yusta, 2014). \textit{Igf1} promotes growth of small intestinal epithelium and participates in the fibroplasia, modulating the proliferation of fibroblasts and myofibroblasts and the synthesis of collagen (Latella \textit{et al.}, 2013; Bortvedt \textit{et al.}, 2012). Several studies demonstrated that this growth factor administration improves the healing parameters, on animal models of colonic anastomosis, in standard and high-risk contexts (Rijcken \textit{et al.}, 2014; Oines \textit{et al.}, 2014).

An increase of \textit{Hbegf} messenger RNA expression levels in teduglutide-treated animals was verified in current study, at both postoperative time points (although statistically significant only at the seventh day after isolated laparotomy). Involvement of the ErbB ligand-ErbB signaling pathway in the proliferative actions of Glp-2 was suggested in previous experiments (Drucker and Yusta, 2014). Studies about the refeeding-induced mucosal proliferation revealed also the importance of ErbB signaling for the actions of endogenous Glp-2 (Drucker and Yusta, 2014).

\textit{Hbegf} has mitogenic and chemotactic effects on epithelial cells, fibroblasts and smooth muscle cells; promotes extracellular matrix synthesis and angiogenesis; modulates vasodilatation and preserves microcirculatory blood flow; improves intestinal motility; and demonstrates antiinflammatory effects (Rijcken \textit{et al.}, 2014; Yang \textit{et al.}, 2014; Radulescu \textit{et al.}, 2011). Moreover, this growth factor seems to preserve the intestinal mucosa and to restore gut barrier function after intestinal injury (Yang \textit{et al.}, 2014). Indeed, a potent intestinal cytoprotective effect of \textit{Hbegf} on intestinal epithelial cells (including stem cells), endothelial cells, pericytes, immunocytes and neuronal cells has been demonstrated in animal models of necrotizing enterocolitis, ischemia/reperfusion injury, and hemorrhagic shock and resuscitation (Yang \textit{et al.}, 2014). Furthermore, in
2011, Radulescu A et al (Radulescu et al., 2011) demonstrated, on an animal model, that exogenous Hbegf promoted intestinal anastomotic repair, and that the Hbegf (-/-) knockout mice had worse healing scores and higher morbidity and mortality rates after intestinal anastomosis.

Induction of Vegfa and Ctgf gene expression levels observed in this study in teduglutide-treated animals at the seventh day suggest that these growth factors may be also relevant as downstream mediators of Glp-2 effects in the perioperative context.

Vegfa has an important participation in wound healing, as it promotes the early events of angiogenesis (namely endothelial cell migration, proliferation and differentiation) and lymphangiogenesis (Rijcken et al., 2014; Barrientos et al., 2008). In 2011, Adas G et al (Adas et al., 2011) demonstrated a favorable impact of Vegfa plasmid delivery on the healing of ischemic colonic anastomosis on an animal model, including enhanced fibroblast activity, collagen deposition, angiogenesis, hydroxyproline levels and bursting pressure. After intraoperative local Vegfa administration in a rabbit model of colonic anastomoses, Ishii M et al (Ishii et al., 2009) found improved bursting pressure, increased hydroxyproline levels and, also, significantly enhanced submucosal capillary vascular counts.

Ctgf is considered a key determinant in the formation and maintenance of connective tissues and in the wound repair process (Speca et al., 2012; Jacobson and Cunningham, 2012; Barrientos et al., 2008). In fact, this growth factor promotes the proliferation, differentiation and chemotaxis of fibroblasts, the epithelial-mesenchymal transition, the extracellular matrix formation and remodeling, the reepithelialization (by stimulation of cell migration) and the angiogenesis (Speca et al., 2012; Jacobson and Cunningham, 2012; Barrientos et al., 2008). Discrepant responses of Ctgf and Tgfβ1 gene expressions to teduglutide administration, observed in this study, were surprising because Ctgf is controlled by Tgfβ1 in a Smad-dependent way and acts as a downstream mediator of Tgfβ action on connective tissue cells (Speca et al., 2012; Barrientos et al., 2008).

Present data indicated that the teduglutide administration was associated with downregulation of Tgfβ1 gene expression in the anastomotic segments at both
moments of evaluation. This fact raises concern about a potential negative impact on the anastomotic healing, since Tgfβ participates in all phases of this process (Rijcken et al., 2014; Barrientos et al., 2008).

Tgfβ is a pleiotropic polypeptide hormone that modulates the mucosal immune response and the tissue remodeling in the gut. In fact, Tgfβ downregulates the production of proinflammatory cytokines, promotes the differentiation of regulatory T-cells and induces the production of secretory immunoglobulin A (Biancheri et al., 2014; Speca et al., 2012). This growth factor also regulates extracellular matrix turnover and exerts an important role in tissue physiologic remodeling and wound repair in the intestine. Indeed, Tgfβ promotes the recruitment, proliferation, differentiation and activation of extracellular matrix-producing cells (Biancheri et al., 2014; Latella et al., 2013; Speca et al., 2012; Barrientos et al., 2008) and the epithelial and endothelial-mesenchymal transition (Speca et al., 2012). It also stimulates extracellular matrix production and deposition (enclosing types I and III collagens, fibronectin and proteoglycans) and inhibits its degradation (including through the inhibition of matrix metalloproteinases 1, 3 and 9) (Latella et al., 2013; Barrientos et al., 2008; Speca et al., 2012). Tgfβ promotes epithelial restitution inducing the migration of epithelial cells across the wound margin (Biancheri et al., 2014; Speca et al., 2012), stimulates the recruitment of inflammatory cells and macrophage-mediated tissue debridement (Barrientos et al., 2008), promotes angiogenesis (thought upregulation of Vegf) (Speca et al., 2012; Barrientos et al., 2008) and participates in wound contraction (Barrientos et al., 2008). Several cell types can produce and respond to TGF-β, including epithelial cells, macrophages, regulatory T-cells, myofibroblasts, and dendritic cells (Biancheri et al, 2014). Adenoviral-mediated transfer of Tgfβ1 on an animal model of colonic anastomoses, through intraluminal local administration, was associated with a significant increase of the anastomotic bursting pressure (Migaly et al., 2004).

The downregulation of Fgf7 gene in animals submitted to teduglutide treatment documented in the present study (statistically significant only in the anastomotic segments at the third day and in the ileal samples recovered after isolated laparotomy at the seventh day) was also unexpected, because this growth factor has been proposed as one of the mediators of Glp-2 action, particularly on the colonic mucosa
Drucker and Yusta B, 2014). Fgf7 (also known as keratinocyte growth factor 1, Kgf1) is a mitogenic growth factor with an important role in the intestinal epithelial growth, maintenance and repair and in the preservation of the barrier function (Rijcken et al., 2014; Cai et al., 2012; Barrientos et al., 2008). A favorable effect of this growth factor on the intestinal mucosal protection has been demonstrated, on experimental studies, in the context of chemically-induced inflammatory bowel disease, chemotherapy and radiation mucositis, ischemia/reperfusion syndrome, short-bowel syndrome and total parenteral nutrition (Cai et al., 2012). Intraperitoneal administration of truncated Kgf on an animal model of colonic anastomosis was associated with enhanced anastomotic bursting pressure, lower inflammatory activity on histological examination and higher crypt cell proliferation rates (Egger et al., 2001).

In present study, an increase of Glp-2 plasma levels after ileal resection was documented in the postoperative period and was congruent with the literature. In fact, several animal experiments demonstrated an increase of plasma Glp-2 concentrations following intestinal resections, with a positive correlation with its magnitude (Tappenden, 2014; Muto et al., 2013; Garrison et al., 2009; Koopmann et al., 2009; Martin et al., 2005).

Posology of teduglutide in our experiment was based on previous published experimental and clinical studies on the intestinotrophic properties and pharmacodynamic characteristics of GLP-2 and its analogues (Qi et al., 2017; Burness and McCormak, 2013; Arda-Pirincci et al., 2012; Alters et al., 2012; Kaji et al., 2009; Kaji et al., 2008; Martin et al., 2004).

As outlined above, most of the Glp-2 effects are indirect and secondary to endocrine, paracrine, autocrine and neural signaling activated by the Glp2r (Drucker and Yusta, 2014). In present study, higher correlation coefficients were presupposed between relative mRNA expressions of Glp2r and Igf1, Hbegf and Fgf7 gene expression levels, because these growth factors have been recognized as molecular downstream mediators of the Glp2r signaling in gastrointestinal tract (Drucker and Yusta, 2014). Gene expressions of Fgf2 and Vegfa, two of the most important mediators of neoangiogenesis (Greaves et al., 2013), did not correlated significantly as awaited. In
relation to the Glp-2/Glp2r axis, current investigation indicated that tissue Glp2r expression may be negatively affected by increased Glp-2 plasma concentrations.

In conclusion, results of present study underscore the recognized role of Igf1 and Hbegf as molecular mediators of the effects of teduglutide and suggest that other humoral factors, like Vegfa and Ctgf may be also relevant in the perioperative context of intestinal anastomosis. A negative influence of teduglutide on postoperative Tgfβ1 relative gene expression was also indicated.

Albeit the negative impact on postoperative tissue Tgfβ1, induction of Vegfa, Igf1 and Ctgf gene expressions might indicate a favorable influence of teduglutide on the intestinal anastomotic healing.
Chapter 7

Teduglutide effects on gene modulation of fibrogenesis on an animal model of intestinal anastomosis
This chapter was partially published as:

7.1. Abstract

Teduglutide is an enterotrophic analogue of glucagon-like peptide 2 approved for the rehabilitation of short-bowel syndrome. This study purposed to analyze the effects of teduglutide administration on the gene regulation of fibrogenesis during the intestinal anastomotic healing on an animal model. Wistar rats (n=62) were assigned into four groups: “Ileal Resection and Anastomosis” or “Laparotomy”, each one subdivided into “Postoperative Teduglutide Administration” or “No Treatment”; and sacrificed at the third or at the seventh days, with ileal sample harvesting. Gene expression of matrix components and remodeling factors [matrix metalloproteinases (Mmp) and tissue inhibitors of metalloproteinases (Timp)], and growth factors was studied by quantitative real-time reverse-transcription polymerase chain reaction. Net collagen deposition was assessed through the Collagen-to-Mmp-to-Timp ratio of fold-change of relative gene expression. Gene expression profiles revealed a balance towards net degradation of collagen at the third day of the intestinal anastomotic healing. Teduglutide appeared to be associated with an overall accumulation of collagen at the third day of the anastomotic repair, attributable to the upregulation of Timp1 and Timp2 and Collagen type IV alpha 1 and downregulation of Mmp3 and Mmp12; and to a net degradation of collagen at the seventh day, derived from repression of Collagen type III alpha 1, Collagen type V alpha 1, Collagen type IV alpha 1 and Timp1 expression. Teduglutide seemed to be associated with a favorable influence on fibrogenesis at the third day of the intestinal anastomotic repair and to a trend to fibrolysis at the seventh day.
7.2. Introduction

Extracellular matrix is a dynamic non-cellular three-dimensional network of macromolecules with particular physical, biochemical and biomechanical properties that confers structural stability to the tissues and supports organ function and repair (Gattazzo et al., 2014). In the intestine, extracellular matrix constitutes a key element of the epithelial stem cell niche (Gattazzo et al., 2014; Speca et al., 2012). Extracellular matrix is constituted by collagen (the most important structural component), proteoglycans, hyaluronan, elastin and elastin-associated proteins, fibronectin, laminins, and matricellular proteins (Theocharis et al., 2016). Remodeling was accomplished by extracellular matrix modifying-proteins that include proteases, such as the matrix metalloproteases [matrix metalloproteinases (mmps), a disintegrins and metalloproteases (adams), adams with thrombospondin motifs (adamts)], the plasminogen/plasmin system and the cathepsin proteases, and tissue inhibitors of metalloproteinases (timps) (Cui et al., 2017; Amar et al., 2017; Theocharis et al., 2016; Speca et al., 2012). Matrix macromolecules are synthesized and secreted by several cell types, including fibroblasts and epithelial, endothelial and immune cells. Extracellular matrix modulates cellular growth, migration, differentiation and survival, preserves homeostasis, and drives morphogenesis, through the interaction with surface receptors, such as integrins, discoidin domain receptors (DDR), CD44, syndecans and glypicans (Theocharis et al., 2016).

In the gut, majority of collagen is contained within the submucosal layer, which is the main responsible for the biomechanical resistance of the anastomosis, particularly during the inflammatory phase of wound healing (Thompson et al., 2006). Fibrogenesis derives from the activation of mesenchymal cells, mainly fibroblasts and myofibroblasts, which produce a collagen-rich extracellular matrix that gradually replace temporary fibrin matrix (Speca et al., 2012; Thompson et al., 2006). Collagen content in the perianastomotic tissues results from a precisely controlled equilibrium between synthesis and catabolism (Ågren et al., 2011).

Collagens are trimeric molecules formed by three polypeptide α chains, which contain the sequence repeat (glycine–X–Y)n (X being usually proline and Y 4-hydroxyproline). These repeats allow the formation of a triple helical conformation, which is the
structural feature characteristic of the collagen superfamily (Theocharis et al., 2016; Gelse et al., 2003). Most abundant collagen types in the intestine (I, III and V) belong to the fibrillar group (Gelse et al., 2003). Type I collagen predominates (approximately 68%), followed by type III (20%) and type V (12%) (Thompson et al., 2006). Those collagens are characterized by the aggregation into supramolecular structures with a typical quarter-staggered fibril-array (Gelse et al., 2003) (Table 7.1; Fig. 7.1A).

Table 7.1. Classification and chain composition of the most abundant collagens in the intestine\(^a\)

<table>
<thead>
<tr>
<th>Collagen group</th>
<th>Collagen type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>α1[I] , α2[I]</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>α1[III]</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>α1[V] , α2[V]</td>
<td>α1[V] , α2[V] α3[V]</td>
</tr>
<tr>
<td>Network-forming collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>α2[IV]</td>
<td>α3[IV] α4[IV] α5[IV]</td>
</tr>
<tr>
<td></td>
<td>α5[IV] , α6[IV]</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Adapated from Theocharis et al., 2016

All fibril-forming collagens are composed of a large continuous triple helix bordered by the amino and carboxyl-propeptides, respectively, and are synthesized as precursors, the procollagens, which are secreted in the extracellular space (Ricard-Blum and Ruggiero, 2005). Tropocollagen triple helices suffer self-assembly and regular staggering to form fibrils that are stabilized by cross-links and packed together into large fibers (Theocharis et al., 2016). Type I collagen triple helixes are generally constituted by a heterotrimer with two identical α1(I)-chains and one α2(I)-chain and are usually incorporated into aggregates containing either type III or type V collagens. Type III collagen is a homotrimer of three α1(III) chains frequently integrated in mixed fibrils (with type I collagen). Type V collagen is heterotrimer of three different α-chains (α1, α2 and α3) and generally forms heterofibrils with types I and III collagens in interstitial matrixes (Gelse K et al., 2003).
Figure 7.1. Structural features of the most abundant collagens in the intestine. Types I, III and V collagens are fibrillar collagens (A), which are characterized by the aggregation into supramolecular structures with a quarter-staggered fibril-array. Type IV collagen (B), a network-forming collagen, is the most important component of basement membranes and forms a more flexible triple helix conjunct organized into a meshwork (Theocharis et al., 2016; Gelse et al., 2003). Adapted from Gordon MK and Hahn RA, 2010
Collagen V is the most heterogeneous among the fibrillar collagens; in addition to the heterotrimer $[\alpha 1(V)]_2\alpha 2(V)$ found in most tissues, several other chain associations exist, including hybrid molecules (Ricard-Blum and Ruggiero, 2005). Type IV collagen, a network-forming collagen, is the most proeminent structural component of basement membranes, where it forms a stable two-dimensional supramolecular aggregate (Gelse et al., 2003). There are six $\alpha$-chains that can constitute type IV collagen trimmers but the most frequent chain composition is $[\alpha 1(IV)]_2\alpha 2(IV)$ (Gordon and Hahn, 2010) (Table 7.1; Fig. 7.1B).

Extracellular matrix-degrading enzymes play critical roles in the tissue remodeling and repair (Theocharis et al., 2016). Mmps, also designed matrixins, are a group of at least 28 calcium-activated zinc-dependent endopeptidases (23 in humans), collectively capable of degrading all extracellular matrix components. Moreover, mmps participate in the inflammatory and immune responses, angiogenesis, reepithelialization and proteolytic activation or degradation of chemokines, cytokines, cell-surface molecules and growth factors (such as insulin-like growth factor, transforming growth factor $\beta$ and fibroblast growth factors) (Levin et al., 2017; Cui et al., 2017; Biancheri et al., 2014; Speca et al., 2012; Ravi et al., 2007). Main characteristics of some relevant mmps potentially involved in the intestinal anastomotic repair are listed in Table 7.2.

Control of the extracellular matrix turnover during the anastomotic wound healing process depends on a rigorous balance between synthesis and degradation, and involves interactions between extracellular matrix components, proteases (including mmps) and proteases inhibitors (comprising timps) (Ågren et al., 2011; Baker EA, 2003). Failure to maintain the homeostasis of the extracellular matrix, with disturbances in the balance between synthesis and degradation of its components, seems to constitute an important factor in the pathogenesis of intestinal anastomotic failure (dehiscence or stenosis) (Ågren et al., 2011; Pasternak et al., 2010; Ågren et al., 2006; Stumpf et al., 2005) (Fig. 7.2).
<table>
<thead>
<tr>
<th>MMP</th>
<th>Collagen substrates</th>
<th>Non-collagen ECM substrates</th>
<th>Other targets</th>
<th>Physiological processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>I, II, III, VII, VIII, X, gelatin</td>
<td>Aggrecan, nidogen, perlecan, proteoglycan link protein, serpins, tenasin-C, versican</td>
<td>Casein, α1-antichymotrypsin, α1-antitrypsin, α1-proteinase inhibitor, IGF-BP-3 and -5, IL-1β, L-selectin, ovostatin, pro-TNF-α, SDF-1</td>
<td>Wound healing, Immune response</td>
</tr>
<tr>
<td>MMP13</td>
<td>I, II, III, IV, gelatin</td>
<td>Aggrecan, fibronectin, laminin, perlecan, tenasin</td>
<td>Casein, plasminogen activator 2, pro-MMP9 and 13, SDF-1</td>
<td>Tissue remodeling, Immune response</td>
</tr>
<tr>
<td>MMP2</td>
<td>I, II, III, IV, V, VII, X, XI, gelatin</td>
<td>Aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein, versican</td>
<td>Active MMP9 and 13, FGF-R1, IGF-BP-3 and 5, IL-1β, pro-TNF-α, TGF-β</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>MMP9</td>
<td>IV, V, VII, X, XIV, gelatin</td>
<td>Aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein, versican</td>
<td>CXCL5, IL-1β, IL-2-R, plasminogen, pro-TNF-α, SDF-1, TGF-β</td>
<td>Cellular apoptosis</td>
</tr>
<tr>
<td>MMP3</td>
<td>II, III, IV, IX, X, XI, gelatin</td>
<td>Aggrecan, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan, proteoglycan link protein, versican</td>
<td>Casein, α1-antichymotrypsin, α1-proteinase inhibitor, antithrombin III, E-cadherin, fibrinogen, IGF-BP-3, L-selectin, ovostatin, pro-HB-EGF, pro-IL-1β, pro-MMP-1, -8, and -9, pro-TNF-α, SDF-1</td>
<td>Tissue remodeling</td>
</tr>
<tr>
<td>MMP12</td>
<td>IV, gelatin</td>
<td>Elastin, fibronectin, laminin</td>
<td>Casein, plasminogen</td>
<td>Immune response</td>
</tr>
<tr>
<td>MMP14</td>
<td>I, II, III, gelatin</td>
<td>Aggrecan, elastin, fibrin, fibronectin, laminin, nidogen, perlecan, proteoglycan, tenasin, vitronectin</td>
<td>αβ3 integrin, CD44, pro-MMP2 and 13, pro-TNF-α, SDF-1, α1-proteinase inhibitor, tissue transglutaminase</td>
<td>Tissue remodeling, Immune response, Morphogenesis, Cellular apoptosis</td>
</tr>
</tbody>
</table>

CD44, Cluster of differentiation 44; CXCL5, C-X-C motif chemokine ligand 5; ECM, Extracellular matrix; IGF-BP, Insulin-like growth factor binding protein; IL, Interleukin; IL-2R, Interleukin 2 receptor; FGF-R1, Fibroblast growth factor receptor 1; MMP, Metalloproteinase; Pro-HB-EGF, Pro-heparin-binding epidermal-like growth factor; Pro-MMP, Pro-metalloproteinase; Pro-TNF-α, Pro-tumor necrosis factor alpha; SDF-1, Stromal cell-derived factor-1; TGF-β, Transforming growth factor beta. Adapted from Cui N et al., 2017

Overexpression of MMP1, MMP2 and MMP9 in biopsies obtained perioperatively from patients’ colon was associated with an increased risk of subsequent anastomotic leakage (Stumpf et al., 2005). In previous clinical studies, MMP2, MMP8 and MMP9 were found to be significantly elevated in the abdominal fluid drainage of patients with anastomotic leakage after colorectal surgery. Indeed, the peritoneal fluid level of MMP9 was considered a potential early marker to diagnose colorectal anastomotic leakage at a preclinical stage (Cini et al., 2013). Additionally, the treatment with Mmp inhibitors has been shown to improve the biomechanical properties in animal models.
of colonic anastomotic healing (Krarup et al., 2017; Krarup et al., 2013; Ågren et al., 2011).

Figure 7.2. Simplified schematic representation of the extracellular matrix turnover. Extracellular matrix (ECM) homeostasis is maintained by a delicate equilibrium between continuous synthesis and degradation. Synthesis of ECM components, mainly collagens, by fibroblasts and myofibroblasts, is balanced with the activity of the ECM-degrading enzymes and its inhibitors, such as tissue inhibitors of metalloproteinases. During the intestinal anastomotic healing, abnormal ECM degradation may contribute to the development of leakage, while excessive deposition may conduct to fibrosis and stenosis (Theocharis et al., 2016; Agren et al., 2011). Adams, A disintegrins and metalloproteases; Adamts, Adams with thrombospondin motifs; Mmps, matrix metalloproteinases

Teduglutide is an enterotrophic analogue of glucagon-like peptide 2 approved for the rehabilitation of short-bowel syndrome (Drucker and Yusta B, 2014).

At present, response of the intestinal fibrogenesis to teduglutide administration in the perioperative context of intestinal anastomosis is not well understood. This study proposed to analyze the effects of teduglutide short-term treatment on the gene
modulation of fibrogenesis during the intestinal anastomotic healing, on an animal model, including the gene expression levels of the main extracellular matrix components and remodeling factors, mmps and timps.

7.3. Methods

7.3.1. Study protocol

Adult male Wistar albinus rats were randomly assigned into four groups: “Ileal Resection and Anastomosis” (“Res”) or “Laparotomy” (“Lap”), each one subdivided into “Postoperative Teduglutide Administration” (“Ted +”) or “No Treatment” (“Ted -”). Evaluation was conducted at the moments of the operation and sacrifice, at the third or the seventh postoperative day (eight subgroups), with recovering of ileal segments (except during isolated laparotomy). All the laboratorial analyses were performed in a blinded manner for the experimental groups.

Study was approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal and undertaken in consonance with institutional and national animals’ protection guidelines.

7.3.2. Animals

Animals weighting 250 to 300 g were acclimatized to the laboratory environment for five days before experimental study; kept in temperature (22±1°C) and humidity (50±10%) controlled ventilated cages, with light/dark cycles of 12 hours; and maintained on water and standard rodent diet ad libitum.

7.3.3. Surgical interventions

All the operative procedures were executed by the same surgeon after a period of two hours fasting (water was never restricted) and determination of the preoperative
weight, with clean surgical technique and under anesthesia with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg, Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg, Laboratórios Vitória, Amadora, Portugal).

In “Ileal Resection and Anastomosis” (“Res”) groups, a 10-cm length ileal resection was performed, preserving distal 5 cm, after a 3-cm midline laparotomy. Continuity was restored by a standard end-to-end anastomosis with an interrupted single-layer full-thickness suture of eight equidistant polydioxanone USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl., Cincinnati, USA). Abdominal wall was closed in two layers (muscle-aponeurotic and cutaneous) of continuous sutures, with braided coated poliglactin 910 USP 4/0 (Surgilactin, Sutures Limited, Wrexham, UK) and natural silk USP 4/0 (Surgisilk, Sutures Limited), respectively. In “Laparotomy” (“Lap”) groups, a 3-cm midline laparotomy (without resection) was performed with gentle manipulation of the small bowel.

In the first postoperative day, ingestion of water with 5% glucose at a 1:1 ratio was allowed and, then, ad libitum oral hydration and chow were reassumed. Rats were monitored on a daily basis during the entire extent of the experiment. Operative morbidity and mortality were recorded. At the end of study, at the third or seventh postoperative day, animals were sacrificed by cervical displacement and a relaparotomy with ileal resection was performed (10-cm length, preserving distal 3 cm). During relaparotomy, peritoneal cavity was carefully inspected for anastomotic leakage, intra-abdominal abscess, peritonitis or intestinal obstruction; intra-abdominal adhesions were semiquantitatively graded using the Hulka scale (Cakmak et al., 2009).

7.3.4. Teduglutide administration

In “Ted +” groups, teduglutide (American Peptide Company, Sunnyvale, California, USA) was prepared following the manufacturer’s recommendations and administered subcutaneously in the postoperative period (starting on the day of the operation), 200 μg/kg per day, dissolved in 0.25 ml phosphate buffered saline pH 7.4 (PBS 7.4, Gibco, LifeTechnologies, Carlsbad, California, USA).
7.3.5. Tissue harvesting

Most distal segment, with 4-cm length, was carefully removed from each ileal operative specimen. Sample was opened at the mesenteric side, washed in normal saline solution and divided into three similar longitudinal strips, each one corresponding to one third of the circumference. These three longitudinal samples were prepared for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) after tissue homogenization, and additional studies, respectively, as mentioned in previous chapters.

In “Ileal Resection and Anastomosis” (“Res”) groups, distal samples recovered at sacrifice corresponded to the anastomotic segment and included the anastomosis in the middle; tissue baseline values of these animals were considered for comparison with postoperative results of the “Laparotomy” (“Lap”) groups.

7.3.6. Intestinal tissue homogenization

Intestinal tissue homogenization procedure was previously detailed in Chapter 5. Succinctly, fragments from one ileal longitudinal strip recovered as previous depiction were immediately introduced in a mixture of protease inhibitors in a proportion of 1 ml/100 mg and submitted to mechanical homogenization. Inhibitors cocktail was formerly prepared by adding aprotinin from bovine lung (Sigma Aldrich, Sintra, Portugal), leupeptin hemisulfate salt (Sigma Aldrich) and pepstatin A (Sigma Aldrich) (1 μl of each, all diluted in a 10 mg/ml stock concentration) to 10 ml of phosphate buffered saline pH 7.4 (PBS pH 7.4, Gibco, LifeTechnologies) and stored on ice. Preparation was sonicated twice with one short pulse of ten seconds, cooled during ten seconds and distributed into two tubes of 1.5 ml. Sonication (one pulse of ten seconds) was repeated and centrifugation was undertaken, 14,000x g, for ten minutes, at 4°C. Supernatant was removed to a new tube and pellet was preserved on ice for posterior ribonucleic acid extraction.
7.3.7. Analysis of gene expression levels of extracellular matrix components and remodeling factors

Quantitative real-time RT-PCR (qRT-PCR) was used to characterize the messenger ribonucleic acid (mRNA) expression profile of extracellular matrix-related genes in rats’ ileum. Gene expression of main extracellular matrix components (Collagen, type I, alpha 1: Col1a1; Collagen, type III, alpha 1: Col3a1; Collagen, type IV, alpha 1: Col4a1; Collagen, type V, alpha 1: Col5a1) was studied simultaneously with that of genes involved in extracellular matrix remodeling, matrix metalloproteinases (collagenases Mmp1 and Mmp13, gelatinases Mmp2 and Mmp9, stromelysin Mmp3, elastase Mmp12 and membrane-type Mmp14) and tissue inhibitors of metalloproteinases 1 and 2 (Timp1 and Timp2).

Total RNA was obtained from the homogenate of the longitudinal strip of ileum with the Isolate II RNA Mini Kit (Bioline, London, UK). One microgram of isolated total RNA was used for reverse-transcription, which was executed with the Tetro cDNA Synthesis Kit (Bioline) and using random hexamers. Real-time PCR primers were designed with the Beacon Designer (Premier Biosoft, PA, USA) and were obtained from Sigma-Aldrich (Sintra, Portugal). All the genes included in this study were described in the National Center for Biotechnology Information (NCBI) Gene database (http://www.ncbi.nlm.nih.gov/) as shown in Supplementary Table S6. Quantitative RT-PCR was accomplished on a Bio-Rad iQ5 real-time PCR instrument (BioRad, Hercules, California, USA) using the SensiFAST™ SYBR & Fluorescein Kit (Bioline). For each sample, PCR was performed in duplicate. Data were interpreted by relative mRNA quantification (Pfaffl, 2004) using Hypoxanthine phosphoribosyltransferase 1 (Hprt1) as housekeeping gene internal control. All normalized values of samples corresponding to the moment of sacrifice were divided by the normalized value of the baseline (that was arbitrarily set to one) and expressed as fold variations. In rats that underwent laparotomy without resection, relative quantification was performed using mean ΔC_T (threshold cycle) at baseline value of rats submitted to ileal resection. Genes were considered upregulated when the fold-change of relative expression was higher than “1.5” and downregulated when it was lower than “0.5”.
Net collagen deposition in rats’ small bowel at the moment of sacrifice was assessed using the method proposed by Sandler et al (Strup-Perrot et al., 2004; Sandler et al., 2003). It was assumed that no net collagen deposition or degradation occurred in control baseline samples. Thus, the Collagen-to-Mmp-to-Timp value of fold-change of relative expression was set to “1” for baseline controls and used as a reference. Briefly, relative change of gene expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to the Timp; fold-change for collagen mRNA was then divided by the fold-change value for the Mmp/Timp quotient to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Values obtained reflected a tendency toward “collagen deposition” relatively to the steady state when above one and toward matrix “degradation” when inferior to one. “Net collagen deposition” or “degradation” were considered to exist when at least 70% of the studied ratios, an arbitrary cut-off, were higher or lower than one, respectively.

7.3.8. Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 18 software (SPSS, Chicago, Illinois, USA). Shapiro-Wilk and Kolmogorov-Smirnov-Lillifors tests were used to assess the type of distribution of variables. Data were indicated as medians and interquartile ranges (median ± IQR). Comparison of non-parametric continuous variables was performed with Mann-Whitney U test and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons. Correlations were determined by the Spearman’s rank correlation coefficient (\( \rho \)). A \( p<0.05 \) was considered statistically significant.

7.4. Results

7.4.1. Postoperative course

Fifty-nine animals completed the study and were included into the groups: “Res Ted +” (15, eight of them sacrificed on the third day), “Res Ted −” (13, five sacrificed at third
day), “Lap Ted +” (16, eight sacrificed on the third day) and “Lap Ted -” (15, seven sacrificed at third day).

7.4.2. Relative gene expression levels of extracellular matrix components in the postoperative period

Analysis of relative gene expression in the perianastomotic ileal segments, on animals not submitted to teduglutide treatment, at the third day, revealed upregulation of Col1a1, reflected on the respective fold-change of expression between baseline and that time point (2.08±1.27), as well as, of Mmp2 (2.32±1.15), Mmp3 (7.90±1.00), Mmp13 (2.29±0.82) and Mmp14 (8.13±5.03); downregulation of Col4a1 (0.47±0.25), Timp1 (0.33±0.41), Timp2 (0.04±0.07) and Mmp1 (0.46±1.40) also occurred (Fig. 7.3 A). At the seventh day, upregulation of Col1a1 (3.44±1.99), Col3a1 (2.76±1.16), Col5a1 (3.68±2.43), Timp1 (2.26±1.23), Mmp3 (6.28±6.87), Mmp12 (2.30±2.63), Mmp13 (12.46±7.18) and Mmp14 (1.86±1.69) and downregulation of Col4a1 (0.80±0.38), Mmp1 (0.93±0.75), Mmp2 (0.88±0.27) and Mmp9 (0.56±0.36) were also verified (Fig. 7.3 B).

In animals not submitted to teduglutide administration, “net degradation” of collagen was observed at the third postoperative day, in the perianastomotic segments and in the ileal samples recovered after isolated laparotomy, as demonstrated in 95% and 70% of the 56 analyzed Collagen-to-Mmp-to-Timp ratios of fold-change of relative gene expression, respectively; “net deposition” was documented in the perianastomotic ileum at the seventh day (in 70% of the ratios) and “net degradation” was evident after isolated laparotomy at the same moment (in 73% of the ratios).
Figure 7.3. Fold-changes of relative gene expression of collagens, matrix metalloproteinases (Mmps) and tissue inhibitors of metalloproteinases (Timps) in cells isolated from rats’ ileum, at third (A) and at seventh days (B) after operation, determined by qRT-PCR. Wistar rats (n=59) were assigned into four groups: “Ileal resection and anastomosis” (“Res”) or “Laparotomy” (“Lap”), each one subdivided into “Postoperative Teduglutide Administration” (“Ted+”) or “No Treatment” (“Ted−”), and sacrificed at third or at seventh days, with ileal sample harvesting. Samples recovered at the sacrifice in rats that underwent ileal resection corresponded to the anastomotic segment. Values were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection (n=28). Results were expressed as median±interquartile range. Kruskal-Wallis test with pairwise comparisons was used. * p<0.05; ** p<0.01; *** p<0.001
7.4.3. Relative gene expression of extracellular matrix components after teduglutide administration

At the third day of the anastomotic healing, teduglutide treatment was associated with higher fold-change of expression of *Col4a1* (1.73±0.52 vs. 0.47±0.25, *p*=0.023), *Timp1* (4.51±4.41 vs. 0.33±0.41, *p*=0.004), *Mmp1* (2.35±1.32 vs. 0.46±1.40, *p*=0.021), *Timp2* (0.65±0.31 vs. 0.04±0.07, *p*=0.0001), and lower fold-change of *Mmp3* (0.62±0.67 vs. 7.90±1.00, *p*=0.002) and *Mmp12* (0.22±0.20 vs. 1.44±0.95, *p*=0.01), in comparison with controls (“Res Ted -“ group rats) (Fig. 7.3 A).

At the seventh day of the anastomotic repair, teduglutide administration was associated with lower fold-change of expression of *Col3a1* (0.28±0.49 vs. 2.76±1.16, *p*=0.0001), *Col4a1* (0.16±0.19 vs. 0.80±0.38, *p*=0.01), *Col5a1* (0.74±0.57 vs. 3.68±2.43, *p*=0.025), *Timp1* (0.04±0.07 vs. 2.26±1.23, *p*=0.0001), *Mmp2* (0.15±0.44 vs. 0.88±0.27, *p*=0.013), *Mmp12* (0.24±0.25 vs. 2.30±2.63, *p*=0.0001), *Mmp13* (0.76±0.67 vs. 12.46±7.18, *p*=0.002) and *Mmp14* (0.45±0.40 vs. 1.86±1.69, *p*=0.002) (Fig. 7.3 B).

In animals submitted to teduglutide treatment, “net deposition” of collagen was observed in the perianastomotic segments at the third day, as demonstrated in 80% of the 56 studied Collagen-to-Mmp-to-Timp ratios of fold-change of relative gene expression, while “net degradation” was evident at the seventh day in 75% of the studied quotients (Fig. 7.4-7.7).

In animals submitted to teduglutide administration after isolated laparotomy, “net deposition” of collagen was documented in the ileal segments at the third day (as demonstrated in 91% of the studied Collagen-to-Mmp-to-Timp ratios) and “net degradation” at the seventh day (evident in 71% of the studied parameters).

Globally, at the third postoperative day (after ileal resection or isolated laparotomy), teduglutide administration was associated with higher fold-change of *Coll1a1* (5.15±7.27 vs. 0.23±1.87, *p*=0.0001), *Coll3a1* (1.92±1.46 vs. 0.14±1.04, *p*=0.001), *Coll4a1* (1.36±1.22 vs. 0.47±0.18, *p*=0.005), *Timp1* (3.77±2.68 vs. 0.83±1.23, *p*=0.0001), *Timp2* (2.14±1.98 vs. 0.41±0.41, *p*=0.0001) and *Mmp2* (2.65±1.96 vs. 0.54±2.11, *p*=0.01) (Fig. 7.8).
Figure 7.4. Collagen (1α1/3α1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats’ perianastomotic segments (n=28), at the third postoperative day, according to the teduglutide administration*. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values. Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration. *All the differences were statistically significant except: Col3α1/mmp1:timp1 and Col3α1/mmp1:timp2

At the seventh postoperative day, teduglutide administration was associated with lower fold-change of Col3α1 (0.59±0.58 vs. 1.96±1.46, p=0.0001), Col5α1 (0.69±0.39 vs. 1.53±3.05, p=0.008), Timp1 (0.07±0.40 vs. 1.73±1.90, p=0.0001), Mmp1 (0.64±0.58 vs. 1.30±1.22, p=0.002), Mmp2 (0.33±0.40 vs. 0.95±0.54, p=0.0001), Mmp12 (0.49±1.10
vs. 1.19±2.34, p=0.02), Mmp13 (0.33±0.65 vs. 5.67±11.73, p=0.0001) and Mmp14 (0.45±0.39 vs. 1.30±1.17, p=0.0001) (Fig. 7.8).

Figure 7.5. Collagen (1a1/3a1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats' perianastomotic segments (n=28), at the seventh postoperative day, according to the teduglutide administration*. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values. Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration. * All the differences were statistically significant except: Col1a1/mmp2:timp2, Col1a1/mmp3:timp2, Col1a1/mmp12:timp2, Col1a1/mmp14:timp2, Col3a1/mmp2:timp2, Col3a1/mmp9:timp2 and Col3a1/mmp14:timp2.
Collagen (4α1/5α1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats’ perianastomotic segments (n=28), at the third postoperative day, according to the teduglutide administration*. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values. Results were expressed as median±interquartile range. Mann-Whitney U test was used. *Teduglutide +, Postoperative teduglutide administration; *Teduglutide -, Without teduglutide administration. *All the differences were statistically significant except: Col5a1/mmp1:timp1, Col5a1/mmp2:timp1, Col5a1/mmp3:timp1, Col5a1/mmp3:timp1, Col5a1/mmp13:timp1, Col5a1/mmp13:timp2, Col5a1/mmp2:timp2, Col5a1/mmp3:timp2, Col5a1/mmp9:timp2, Col5a1/mmp12:timp2, Col5a1/mmp13:timp2, Col5a1/mmp14:timp2

When considered all the animals (submitted to ileal resection or isolated laparotomy), at the moment of sacrifice (at the third or the seventh day), teduglutide treatment was associated with higher fold-change of Collα1 (3.79±5.04 vs. 0.33±2.64, p=0.0001) and Timp2 (0.90±1.71 vs. 0.44±0.64, p=0.003) (similar to the verified at the third day); and
lower fold-change of Col5a1 (0.70±0.47 vs. 0.95±1.42, p=0.005), Mmp12 (0.43±0.40 vs. 0.98±1.17, p=0.004), Mmp13 (0.17±0.41 vs. 1.57±8.34, p=0.0001) and Mmp14 (0.58±0.58 vs. 1.30±2.38, p=0.001) (Fig. 7.9).

Figure 7.7. Collagen (4a1/5a1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats’ perianastomotic segments (n=28), at the seventh postoperative day, according to the teduglutide administration*. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values. Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration. *All the differences were statistically significant except: Col4a1/mmp1:timp2, Col4a1/mmp2:timp2, Col4a1/mmp12:timp2, Col4a1/mmp14:timp2, Col5a1/mmp1:timp2, Col5a1/mmp2:timp2, Col5a1/mmp12:timp2 and Col5a1/mmp14:timp2
Figure 7.8. Fold-changes of relative gene expression of collagens, matrix metalloproteinases (Mmps) and tissue inhibitors of metalloproteinases (Timps), determined by qRT-PCR, in rats’ ileum at the moment of sacrifice (after ileal resection and anastomosis or isolated laparotomy), according to the teduglutide administration (n=59). Samples recovered at the sacrifice in rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection (n=28). Results were expressed as median±interquartile range of fold-change. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration.

Globally, teduglutide administration favored “net collagen deposition” (evident in 75% of the 56 analyzed Collagen-to-Mmp-to-Timp ratios), while “net collagen degradation” was observed in not treated animals (evident in 77% of the ratios). In teduglutide-treated animals, significantly higher values were observed in 43% of the Collagen-to-Mmp-to-Timp ratios, while lower values were presented in only 5% (Fig. 7.10 and 7.11).
Figure 7.9. Fold-changes of relative gene expression of collagens, matrix metalloproteinases (Mmps) and tissue inhibitors of metalloproteinases (Timps), determined by qRT-PCR, in rats’ ileum of all groups (n=59) at the moment of sacrifice (third or seventh day; after ileal resection and anastomosis or isolated laparotomy), according to teduglutide administration. Samples recovered at the sacrifice in rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to Hypoxanthine phosphoribosyltransferase 1 gene and fold-changes were generated by comparison with baseline values of rats submitted to ileal resection (n=28). Results were expressed as median ± interquartile range of fold-change. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide −, Without teduglutide administration

7.5. Discussion

In present study, global gene expression profiles revealed, a balance towards fibrolysis (net degradation of collagen) at third day of intestinal anastomotic healing, associated with upregulation of Mmp2, Mmp3, Mmp13 and Mmp14 expression and downregulation of Timp1 and Timp2; and towards fibrogenesis (deposition of collagen) at seventh day, associated with upregulation of Col1a1, Col3a1, Col5a1 and Timp1 expression and downregulation of Mmp1, Mmp2 and Mmp9. Upmodulation of stromelysin Mmp3, collagenase Mmp13, membrane-type Mmp14 and Col1a1 was
observed consistently throughout the repair, as well as, downregulation of Col4a1 and collagenase Mmp1.

Figure 7.10. Collagen (1a1/3a1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats’ ileum of all groups (n=59), at the moment of sacrifice (third or seventh day; after ileal resection and anastomosis or isolated laparotomy), according to the teduglutide administration. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold-change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine Phosphoribosyltransferase 1 gene and fold-changes were generated by comparing with baseline values of rats submitted to ileal resection (n=28). Samples from rats that underwent ileal resection corresponded to the anastomotic segment. Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration. * p<0.05; ** p<0.01, *** p<0.001
Figure 7.11. Collagen (4a1/5a1)-to-Mmp-to-Timp ratio of fold-change of relative gene expression in rats' ileum of all groups (n=59), at the moment of sacrifice (third or seventh day; after ileal resection and anastomosis or isolated laparotomy), according to the teduglutide administration. Relative expression of Mmp was calculated by determining the fold-change of the Mmp mRNA expression relative to its relevant inhibitor Timp; fold-change for collagen mRNA was then divided by the fold-change value for the relevant Mmp/Timp to yield the fold-change in the ratio of collagen deposition to collagen degradation compared with the baseline. Relative expressions were normalized to Hypoxanthine Phosphoribosyltransferase 1 gene and fold-changes were generated by comparing with baseline values of rats submitted to ileal resection (n=28). Samples from rats that underwent ileal resection corresponded to the anastomotic segment. Results were expressed as median±interquartile range. Mann-Whitney U test was used. Teduglutide +, Postoperative teduglutide administration; Teduglutide -, Without teduglutide administration. * p<0.05; ** p<0.01, *** p<0.001

Recently, Seifert GT et al demonstrated upregulation of Mmp2, Mmp3, Mmp9, Mmp12, Mmp13 and Mmp14 during ileal anastomotic healing (Seifert et al., 2014). Similarly, Krarup PM et al (Krarup et al., 2013) found a profound increase of Mmp8, Mmp9 and Mmp12 gene and protein expressions, a modest enhancement of Mmp13
gene expression and of Mmp2 gene and protein expressions, in colon anastomosis, at the third day of the healing process, comparing with non-injured adjacent colon; Mmp3 was not significantly upmodulated. In 2006, Ågren MS et al showed, in a rat model, that hydroxyproline concentration of a colonic anastomosis decreased to 43% at third postoperative day, more intensely in the suture-holding zone, and returned to the baseline levels at the seventh day (restoring a similar content in suture and suture-free areas) (Ågren et al., 2006).

According to the gene expression analysis, teduglutide appeared to be associated with a trend to increase the overall deposition of collagen at the predominantly inflammatory phase of the anastomotic healing (third postoperative day), as suggested by the Collagen-to-Mmp-to-Timp ratios of relative mRNA expression. This effect was attributable to the reduction of fibrolysis, related with the induction of gene expression of Timp1 and Timp2 and downmodulation of Mmp3 and Mmp12 (as well as, of Mmp9, Mmp13 and Mmp14, although not significant); furthermore, an upregulation of Col4a1 was also observed.

On the contrary, at the proliferative phase of anastomotic repair (seventh day), teduglutide was associated with a trend to decrease collagen deposition, possibly due to the reduction of fibrogenesis explained by the downmodulation of Timp1, Col3a1, Col4a1 and Col5a1, and Col1a1 (not significant).

Nevertheless, globally, teduglutide seemed to be associated with a predominantly profibrogenic effect, mainly derived from the downregulation of elastase Mmp12, collagenase Mmp13 and membrane-type Mmp14.

Downregulation of Mmp13 and Mmp14 was the most consistent effect of teduglutide, observed simultaneously in the anastomotic segments and in the ileal samples recovered after isolated laparotomy, both at third and at seventh day. Mmp14 is a membrane-type Mmp that potentiates other Mmps, degrades types I and III collagens and other extracellular matrix molecules, participates on the myofibroblasts migration and promotes wound healing (Ravi et al., 2007). Mmp13 is a collagenase with a large substrate specificity, able to cleave fibrillar collagens (including types I and III) and gelatin and to potentiate the inflammatory response (Cui et al., 2017; Ravi et al., 2007).
Interferences with the number, activation and secretion of extracellular matrix-producing cells, as well as with the inflammatory response, oxidative stress and growth factors profile may be involved on the teduglutide effects on fibrogenesis. In fact, fibrogenesis involves complex interactions between cells, extracellular matrix, cytokines, chemokines and growth factors (Latella et al., 2013; Speca et al., 2012). Activated myofibroblasts, the extracellular matrix-producing cells, are derived from resident mesenchymal cells (fibroblasts, subepithelial myofibroblasts and smooth muscle cells), epithelial and endothelial cells, stellate cells, pericytes and bone marrow stem cells (Latella et al., 2013; Speca et al., 2012). Relevant molecular mediators of extracellular matrix production include transforming growth factor β, connective tissue growth factor, platelet-derived growth factor, insulin-like growth factors 1 and 2, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factors, cytokines, chemokines and products of oxidative stress, among others (Latella et al., 2015; Latella et al., 2013; Speca et al., 2012).

In previous chapter of this thesis, it was demonstrated that teduglutide postoperative administration (after ileal resection or isolated laparotomy) was associated, at third day, with higher fold-change of relative gene expression of insulin-like growth factor 1 and heparin-binding epidermal-like growth factor and lower fold-change of fibroblast growth factor 2 and fibroblast growth factor 7; while, at seventh day, it was related with higher fold-change of relative gene expression of vascular endothelial growth factor a, connective tissue growth factor and heparin-binding epidermal-like growth factor and lower fold-change of transforming growth factor β1, fibroblast growth factor 7 and glucagon-like peptide 2 receptor.

In present study, promotion of fibrogenesis at third postoperative day might have result from teduglutide upregulation of insulin-like growth factor 1 expression, while tendency to collagenolysis at seventh day might have derived from the inhibition of transforming growth factor β1 signaling. Insulin-like growth factor 1 is a recognized critical mediator of the enterotrophic effects of glucagon-like peptide 2 (Drucker and Yusta, 2014; Bortvedt et al., 2012) that plays an important role on the modulation of the proliferation of fibroblasts/myofibroblasts and on the deposition of collagen (Latella et al., 2013; Bortvedt et al., 2012). Transforming growth factor β is a
pleiotropic cytokine that regulates extracellular matrix turnover (Biancheri et al., 2014), induces mesenchymal cells activation and differentiation into myofibroblasts, and stimulates extracellular matrix synthesis (Latella et al., 2013). Both transforming growth factor β (and its receptors) and insulin-like growth factor 1 are overexpressed in fibrostenotic Crohn’s disease and in animal models of intestinal fibrosis (Latella et al., 2015; Latella et al., 2013). Furthermore, a recent systematic review and meta-analysis on pharmaceutical interventions for the improvement of colonic anastomotic healing, demonstrated that insulin-like growth factor 1 improved anastomosis stability on experimental models (Oines et al., 2014).

Main limitations of present investigation must be considered in the interpretation of the results, namely the small number of studied animals in each group and the lack of protein expression analysis. The first shortcoming may have contributed to increase the dispersion of the results and, in some cases, to the difficulty to reach statistical significance. Regarding to the methodology, quantitative RT-PCR allowed a relative quantification of mRNA transcript content, but the presence of RNA does not always reflect the protein levels. Potential interferences in collagen synthesis and deposition at posttranscriptional, posttranslational and postsecretional levels may not have been detected (Chen et al., 2009; Gelse et al., 2003), such as disruption of the formation and stabilization of collagen triple helices, assembly into fibrils, or cross-linking (Chen et al., 2009). Accordingly, any therapeutic implications of this study require further investigation.

Favorable influence of teduglutide treatment on the overall deposition of collagen observed at the third day of the intestinal anastomotic healing, demonstrated in gene expression analysis, might reduce the probability of anastomotic failure, in the period of higher risk. In fact, in the first two to three days of postoperative period, a transient reduction of the anastomotic strength occurs, associated to the degradation of extracellular matrix proteins (especially preexisting mature collagen) by the proteinase activity in the wound. Thereafter, strength increases by de novo synthesis and deposition of collagen, in the predominantly proliferative phase of the healing process (Thompson et al., 2006). Burst pressure of a small intestine anastomosis is approximately 50% of normal at the second and third postoperative days, whilst a
tensile strength similar to the unwounded tissue is only reached four weeks after surgery (Thompson et al., 2006). Seifert GT et al (Seifert et al., 2014) observed that the number of genes consistently up or downregulated peaked at the second postoperative day and decreases until the eighth day, reflecting, according to those authors, the clinically most important phase of intestinal anastomotic healing.

Although the decrease of collagen deposition at the seventh day of the anastomotic repair might raise apprehension about the susceptibility to dehiscence, the limited temporal extent of the potential profibrogenic effect of teduglutide might reduce the risk of anastomotic stenosis and peritoneal adhesions, while protecting against fistulization during the higher risk period.

Results of present study might also be relevant to indicate potential effects of teduglutide on the progression of intestinal fibrotic diseases, increasingly recognized as major causes of morbidity and mortality (Latella et al., 2015), such as inflammatory bowel diseases, radiation enteropathy, postsurgical intestinal adhesions, and others. Teduglutide was indeed considered a promising therapeutic strategy in Crohn’s disease, targeting the intestinal barrier function (Blonski et al., 2013; Buchman et al., 2010).

In conclusion, in present study, gene expression profiles revealed a balance towards net degradation of collagen at third day of the intestinal anastomotic healing and towards net deposition at seventh day. Teduglutide appeared to be associated with a favorable influence on fibrogenesis at the third day of the intestinal anastomotic repair, considered the period of higher risk of failure, and to a trend to fibrolysis at the seventh day.

Further research is necessary to confirm those results and to determine the cellular and molecular mechanisms underlying to the teduglutide effects on fibrogenesis.
Chapter 8

General discussion and concluding remarks
Regardless of progresses in surgical technique and perioperative care, failure of intestinal anastomotic healing remains one of the most feared complications in digestive surgery (Guyton et al., 2016; Bosmans et al., 2015). Indeed, intestinal anastomotic leakage has a profound adverse impact on the operative morbidity and mortality rates, oncologic and functional outcomes, patients’ quality of life and socioeconomic costs (Gessler et al., 2017; Chadi et al., 2016; Midura et al., 2015; Shogan et al., 2013; Luján et al., 2011). An early detection and a timely integrated therapeutic intervention are critical for the successful management of the anastomosis complications (Chadi et al., 2016). Nevertheless, accurate risk stratification and control of relevant susceptibility factors are of utmost clinical and socioeconomic relevance.

Anastomotic healing is a complex multicellular and multimolecular process susceptible to innumerable interferences and its pathophysiology remains to be completely elucidated (Guyton et al., 2016).

Numerous experimental studies have been undertaken on the role of adjuvants of intestinal anastomosis healing. However, many of those studies were characterized by low internal validity and reporting quality and, also, by significant heterogeneity. Furthermore, efficacy and safety issues, as well as clinical applicability and economic constraints were frequent limitations. Until now, no clear evidences were documented to support the implementation of any of those strategies for the routine clinical use (Nerstrom et al., 2016; Yauw et al., 2015; Rijcken et al., 2014; Oines et al., 2014).

Teduglutide is a long-acting analogue of glucagon-like peptide 2 (GLP-2) with enterotrophic properties, approved for the pharmacological rehabilitation of short-bowel syndrome (Drucker and Yusta, 2014), and considered safe and well tolerated (Kim and Keam et al., 2017; Billiauws et al., 2017; Naberhuis et al., 2016; Austin et al., 2016).

Present study evaluated the influence of teduglutide short-term administration on the cellular, humoral and molecular mediators of intestinal anastomotic healing on an animal model. The ultimate goal of this investigation was to clarify the potential of teduglutide as a promoting strategy for improvement of intestinal anastomotic healing. In order to achieve that goal, several aspects were addressed, including the influence
of teduglutide on the intestinal anastomosis clinical and structural outcome; the response of putative intestinal epithelial stem cells to teduglutide in the context of anastomosis repair; the effects of teduglutide on the cellular viability and death processes, oxidative stress, and tissue and systemic inflammatory response; perioperative tissue growth factors profiles after teduglutide administration; and the effects of teduglutide on the gene regulation of fibrogenesis during anastomosis repair.

**Intestinal anastomosis healing process**

Relevant aspects of the intestinal anastomosis healing were analyzed in this investigation. The role of the intestinal epithelial stem cells modulation in intestinal repair was analysed by multiparameter flow cytometry cellular phenotyping. Present findings corroborated the existence of two functional, interconvertible, putative epithelial stem cells subpopulations with distinct behavior after surgical injury and teduglutide administration and also with different correlation profiles with redox, inflammatory and growth factors signaling parameters (additional data on Supplementary Tables S7 and S8). A significant and high positive correlation was documented between putative crypt base columnar subpopulation proportion and parameters suggesting a local prooxidative influence, albeit accompanied by a positive correlation with cellular viability index; the opposite behavior was observed with the putative “position +4” stem cells fraction. Redox biology seems to play an important role in the intestinal epithelium homeostasis and repairing efficiency (Circu and Aw, 2012). The referred subpopulations of stem cells correlated in opposite direction with tissue proinflammatory interleukin 1α (IL-1α) and plasma macrophage chemo-attractant protein 1 (MCP-1) levels, and fibroblast growth factor 7 gene expression levels. IL-1 cytokine family is involved in the preservation of gut mucosal homeostasis, including the integrity of epithelial barrier and the promotion of epithelial repair (Lopetuso et al., 2013). Fibroblast growth factor 7 is a potent mitogenic growth factor with a relevant role in intestinal growth, maintenance and repair (Cai et al., 2012). This study allowed a better insight on the role of the intestinal epithelial stem cells modulation in intestinal repair.
Infiltration of the anastomotic wound by inflammatory cells and fibroblasts, and development of reepithelialization, neoangiogenesis and fibroplasia were observed by histological and immunohistochemical analysis. Anastomosis repair induced a relevant and sustained prooxidative influence (including an increase of the levels of cytosolic peroxides and mitochondrial reactive species and a reduction of the mitochondrial membrane potential and intracellular reduced glutathione content); a proinflammatory/T-helper 1 lymphocytes tissue response; an initial proinflammatory/T-helper 1 lymphocytes and a late antiinflammatory/T-helper 2 lymphocytes systemic reaction. During anastomotic repair, upregulation of gene expression of fibroblast growth factor 2, fibroblast growth factor 7, epidermal growth factor, vascular endothelial growth factor a, at the third day, as well as of platelet-derived growth factor b, at the seventh day, was observed. Gene expression profiles of extracellular matrix components and remodeling factors revealed a balance towards fibrolysis at the third day of the intestinal anastomotic healing, associated with upregulation gene expression of matrix metalloproteinase (Mmp) 2, Mmp3, Mmp13 and Mmp14; and downregulation of collagen type IV alpha 1 and tissue inhibitors of metalloproteinase (Timp) 1 and Timp2; and towards fibrogenesis at the seventh day, attributable to the upregulation of Collagens type I alpha 1, Collagen type III alpha 1 and Collagen type V alpha 1 and Timp 1.

**Teduglutide effects on intestinal anastomosis healing**

In this study, teduglutide was not associated with apparent relevant impact on the rate or the severity of anastomotic leakage. This fact may have been related with the probably low statistical power of the study and, also, with the high efficiency of intra-abdominal immune system of rodents (Pommergaard et al., 2011).

Nevertheless, present findings suggest potential effects of teduglutide on the reepithelialization, neoangiogenesis and fibroplasia events of the intestinal anastomotic healing (Fig. 8.1). Impact of this growth factor exogenous administration on the cellular, humoral and molecular mediators of the anastomosis repair was summarized in Figures 8.2 and 8.3.
Figure 8.1. Illustration depicting the main potential effects of teduglutide on the intestinal anastomotic healing, in a rodent model, suggested by the present investigation. The inhibition of extracellular matrix degradation (at gene expression level) was observed at the third day. The promotion of reepithelialization and neoangiogenesis, and the trend towards the reduction of extracellular matrix deposition (at gene expression level) were documented at the seventh day.

In this study, teduglutide appeared to be characterized by a context and time-dependent response.

A favorable effect of teduglutide on the reepithelialization and neoangiogenesis events of the proliferative phase of anastomotic repair was documented in histological and immunohistochemical analysis.

Teduglutide appeared to promote the reepithelialization in the predominantly proliferative phase of anastomotic healing, as demonstrated by histological examination. This effect may have be related with the expansion of the putative crypt base columnar stem cells pool and the concomitant reduction of the putative “+4 position” stem cells fraction, observed in teduglutide-treated animals. Therefore, the mechanism underlying the promotion of epithelialization may have involved the proliferation of putative crypt base columnar stem cells, and/or the conversion of
putative “+4 position” stem cells to crypt base columnar cells and/or to transit-amplifying progenitors that differentiated into mature epithelial cells.

**Figure 8.2.** Schematic overview of the influence of teduglutide on the cellular, humoral and molecular mediators of the intestinal anastomotic repair at the third day, suggested by the present study. “4” stem cells, Putative “+4 position” epithelial stem cells; CB stem cells, Putative crypt base columnar epithelial stem cells; Cellular GSH, Cellular reduced glutathione; Collα1, Collagen, type I, alpha 1, mRNA; Col3α1, Collagen, type III, alpha 1, mRNA; Col4α1, Collagen, type IV, alpha 1, mRNA; Col5α1, Collagen, type V, alpha 1, mRNA; Ctgf, Connective tissue growth factor; IFN-γ, Interferon-γ; Igf1, Insulin-like growth factor 1, transcript variant 1, mRNA; Il-4, Interleukin 4; Fgf2, Fibroblast growth factor 2, mRNA; Fgf7, Fibroblast growth factor 7, mRNA; Hbegf: Heparin-binding epidermal-like growth factor, mRNA; Mmp3, Matrix metalloproteinase 3, mRNA; Mmp 9, Matrix metalloproteinase 9, mRNA; Mmp12, Matrix metalloproteinase 12, mRNA; Mmp13, Matrix metalloproteinase 13, mRNA; Mmp14, Matrix metalloproteinase 14, mRNA; Mt reactive species, Mitochondrial reactive species; Pdgfb: Platelet-derived growth factor beta polypeptide, mRNA; Timp1, TIMP metalloproteinase inhibitor 1, mRNA (coding for metalloproteinase inhibitor 1); Timp2, TIMP metalloproteinase inhibitor 2, mRNA; Tgfb1, Transforming growth factor, beta 1, mRNA; Vegfa, Vascular endothelial growth factor A, transcript variant 2, mRNA. ↑ Trend to increase or upmodulation (not statistically significant); ↓ Trend to decrease or downmodulation (not statistically significant)

The promotion of the epithelialization was associated with an increase of cellular viability index and a trend (not statistically significant) to higher epithelial proliferation index, higher tissue concentrations of interleukin 4 (IL-4), lower tissue concentrations of interferon γ (IFN-γ), and higher levels of oxidative stress parameters, such as
cytosolic peroxides and mitochondrial reactive species. This was congruent with the important role of the redox biology in the regenerative potential of the intestinal epithelium, namely in cell signaling, modulation of proliferation and differentiation and regulation of death (Circu et al., 2012). The trend to a prooxidative influence may favor the intestinal epithelial stem cells progression from a proliferative to a differentiated state (Circu et al., 2012).

Figure 8.3. Schematic overview of the influence of teduglutide on the cellular, humoral and molecular mediators of the intestinal anastomotic repair, at the seventh day, suggested by the present study. “4” stem cells, Putative “+4 position” epithelial stem cells; CB stem cells, Putative crypt base columnar epithelial stem cells; Cellular GSH, Cellular reduced glutathione; Col1a1, Collagen, type I, alpha 1, mRNA; Col3a1, Collagen, type III, alpha 1, mRNA; Col4a1, Collagen, type IV, alpha 1, mRNA; Col5a1, Collagen, type V, alpha 1, mRNA; Ctgf, Connective tissue growth factor; IFN-γ, Interferon-γ; Igf1, Insulin-like growth factor 1, transcript variant I, mRNA; IL-4, Interleukin 4; Fgf2, Fibroblast growth factor 2, mRNA; Fgf7, Fibroblast growth factor 7, mRNA; Hbegf, Heparin-binding epidermal-like growth factor, mRNA; Mmp3, Matrix metalloproteinase 3, mRNA; Mmp9, Matrix metalloproteinase 9, mRNA; Mmp12, Matrix metalloproteinase 12, mRNA; Mmp13, Matrix metalloproteinase 13, mRNA; Timp1, TIMP metalloproteinase inhibitor 1, mRNA (coding for metalloproteinase inhibitor 1); Timp2, TIMP metalloproteinase inhibitor 2, mRNA; Tgfβ1, Transforming growth factor, beta 1, mRNA; Vegfa, Vascular endothelial growth factor A, transcript variant 2, mRNA. ↑ Trend to increase or upmodulation (not statistically significant); ↓ Trend to decrease or downmodulation (not statistically significant)
The favorable effect of teduglutide on reepithelialization may have been related with the upregulation of gene expression of insulin-like growth factor 1 at the third day and of connective tissue growth factor at the seventh day (Latella et al., 2013; Speca et al., 2012; Bortvedt et al., 2012). Increase of subepithelial myofibroblasts density evidenced in teduglutide-treated animals may have been relevant because they are important constituents of the stem cells niche, participating in the regulation of epithelial stem cells behavior in the regeneration process (Smith et al., 2012), and are considered cellular mediators of the GLP-2 and teduglutide effects, through the release of insulin-like growth factor 1 and likely other growth factors (Drucker and Yusta, 2014; Rowland and Brubaker, 2011). Teduglutide treatment was not associated with relevant modifications of the number of Paneth cells per crypt, which also represent a critical element of the epithelial stem cells niche, nor of the number of goblet cells per crypt-villus unit.

Teduglutide administration was associated with an induction of the neovessels development in the perianastomotic tissue at the seventh day, as evaluated by anti-CD31 immunostaining of endothelial cells. This effect may have resulted from the upmodulation of the gene expression of the vascular endothelial growth factor a, promoter of endothelial cells migration and proliferation (Greaves et al., 2013), as well of the connective tissue growth factor, at the seventh day, and of insulin-like growth factor 1, at the third day. Nevertheless, teduglutide was associated with downregulation of the gene expression of fibroblast growth factor 2 at the third day, considered the most important proangiogenic factor in the early phase after injury (Greaves et al., 2013).

In relation to the fibrogenesis process, teduglutide was not associated with significant differences on the global collagen content of the perianastomotic tissue nor on the type I to type III collagens ratio, as indicated by the semiquantitative assessment with the morphometric method of Gordon-Sweet’s histological staining.

Nevertheless, a trend to the repression of fibrolysis was observed, at gene expression level, at the predominantly inflammatory phase of anastomotic repair (third day), counteracting the trend to extracellular matrix degradation induced by the surgical injury. In fact, upmodulation of gene expression of Timp1, Timp2 and Collagen type IV alpha 1, and downregulation of gene expression of Mmp3 and Mmp12 (and of Mmp9,
Mmp13 and Mmp14, albeit not statistically significant) was verified at that time point. Expression of the genes coding alpha I chains of types I and III collagens was also induced, although without reaching the threshold of statistical significance. Inhibition of fibrolysis at the third day may have been related with the upregulation of gene expression of insulin-like growth factor 1, and of heparin-binding epidermal-like growth factor (not significant).

On the contrary, at the predominantly proliferative phase of the reparative process (seventh day), gene expression of extracellular matrix components and remodeling factors suggested an inhibition of fibrogenesis. This effect may have been related with the downregulation of gene expression of Timp1 and genes coding types III, IV and V collagens alpha chains. Repression of Collagen type I alpha 1 expression (not significant) was also observed. These events were concordant with a trend (albeit not significant) to the reduction of global collagen content of the perianastomotic tissues (at the expense of type I collagen) on the semiquantitative evaluation with the Gordon-Sweet's method; nevertheless, a significant increase of type III collagen content in the submucosa was documented with this technique. A plausible simultaneous reduction (not significant) of fibroblasts infiltration of the anastomosis was revealed by the histological analysis. Gene downmodulation of fibrogenesis at the seventh day may be explained by the inhibition of gene expression of transforming growth factor β1, a powerful profibrogenic growth factor (Biancheri P et al., 2014; Latella et al., 2013).

The trend to the inhibition of fibrolysis demonstrated at the third day by gene expression analysis suggests that teduglutide may protect against anastomotic failure in the period of lower biomechanical resistance and, therefore, of higher risk (Bosmans et al., 2015; Thompson et al., 2006). Nevertheless, the decrease of collagen deposition and the downmodulation of the relative gene expression of transforming growth factor β1 at the seventh day, suggested by the gene expression analysis, causes uncertainty about the increase of dehiscence susceptibility.

Transforming growth factor β has a relevant participation in the tissue remodeling and in the modulation of the mucosal immune response (Biancheri et al., 2014), and interfere in the all the phases of anastomosis healing (Rijcken et al., 2014). Transforming growth factor β signaling is characterized by tissue and context-
specificity of cell responses (Zhang, 2018; Budi et al., 2017). The composition and compartmentalization of the receptor complexes define the activation of signaling pathways and the interactions with Smad-interacting transcription factors and co-regulators determine the cell-type specificity of the transcription responses to the ligands (Budi et al., 2017). Regulation of the production of this pleiotropic cytokine is particularly complex and occurred at several levels, including transcription, translation, secretion and activation (Cebinelli et al., 2016). Several cell types can both produce and respond to transforming growth factor β, including epithelial cells, monocytes/macrophages, regulatory T-cell and myofibroblasts (Biancheri et al., 2014). Genetic polymorphisms (Cebinelli et al., 2016) and epigenetic modifications triggered by environmental factors, including deoxyribonucleic acid (DNA) methylation, histone modifications of DNA and ribonucleic acid (RNA) interference by regulatory noncoding RNA may have interfered with present results (Mann and Mann, 2013).

Despite of the intestinal transit-modulating properties attributed to the exogenous administration of GLP-2 (Drucker and Yusta, 2014), no significant impact of teduglutide on glial cells and fibers density was testified in present analysis.

Most of the teduglutide effects were observed after seven days of treatment, with exception of the promising increase of the fibrogenesis (documented at the third day). Although the short-term extent of perioperative treatment may minimize the risk for undesired tumor promoting effects, the potential use of teduglutide as adjuvant of the intestinal anastomotic healing should exclude, until further information, individuals with active or suspected malignancy.

At present, the study of the pathophysiology of the intestinal anastomosis healing is hampered by the complexity (that hinders a holistic approach) and the absence of objective methodological resources for in vivo dynamic evaluation and monitoring of the repair progression. The currently used animal models, mainly rodent, are not completely representative of the pathophysiology of the anastomotic failure in the human patients.

Main limitations of this investigation must be considered in the interpretation of the results and may reduce the translational impact of present findings.
Unfortunately, the small sample size may have introduced a statistical type II error.

Present study did not include the functional assessment of anastomosis, namely the determination of the bursting pressure and the tensile strength (Pommergaard et al., 2014; Thompson et al., 2006). Nevertheless, regardless the widespread use, these parameters have demonstrated relevant limitations related with the heterogeneity, reproducibility and accuracy of the methodology (especially in small animals), and also with the lack of reliable correlations with the structural integrity of the anastomosis and the clinical outcome (Pommergaard et al., 2014; Vakalopoulou et al., 2013).

Regarding to the specific methodology, study of growth factors and extracellular matrix remodeling factors was implemented at gene expression level, which does not always reflect tissue protein expression and activity levels, as posttranscriptional, posttranslational and postsecretional interferences may occur. Moreover, overall collagen content on the perianastomotic tissue may not correlate with the functional outcome. A complex biosynthetic and depositional pathway leads to the formation of the insoluble collagen matrix but the fibrogenesis is completed only when collagens formed a cross-linked and remodeled matrix (Chen et al., 2009).

An analysis of the teduglutide influence on the early small-bowel anastomotic healing in standard context was performed. Notwithstanding, extend the study to high-risk conditions (such as ischemia), to other segments of intestinal tract (including colon and colorectal anastomosis) and to the predominant remodeling phase could be also relevant.

A systemic therapy, through subcutaneous administration, was undertaken, based on previous experimental studies (Qi et al., 2017; Burness and McCormak, 2013; Arda-Pirincci et al., 2012; Alters et al., 2012; Kaji et al., 2009; Kaji et al., 2008; Martin et al., 2004) and no direct dose-effect assessment was accomplished. Previous studies suggest different effects according to the variations of GLP-2 dose and delivery method (Kaji et al., 2008). Other administration schedules could be studied in future studies. Local therapy, through direct perianastomotic injection, use of coated sutures, concomitant sealing with adhesives or impregnated matrixes, may be more advantageous and to allow a sustained but low-dosage release of the growth factor at the anastomotic site, reducing the potential undesired side-effects.
New methodological resources for the evaluation of intestinal anastomotic healing might be used in further investigations, such as the high-resolution probe-based confocal laser endomicroscopy and the spectral domain polarization-sensitive optical coherence tomography (De Palma et al., 2014; Son et al., 2014).

Notwithstanding the aforementioned limitations, present study contributed to improve the knowledge of the potential impact of teduglutide on the intestinal anastomotic repair (Fig. 8.4). This insight is relevant to explore its potential role as a perioperative healing-adjuvant strategy, but also for the patients with short-bowel syndrome undergoing intestinal anastomotic procedures in the course of this growth factor therapy.

**Figure 8.4. Schematic representation of the influence of teduglutide administration on the intestinal anastomotic repair process.** Col3a1, Collagen, type III, alpha 1, mRNA; Col4a1, Collagen, type IV, alpha 1, mRNA; Col5a1, Collagen, type V, alpha 1, mRNA; Ctgf, Connective tissue growth factor; IFN-γ, Interferon-γ; Igf1, Insulin-like growth factor 1, transcript variant 1, mRNA; IL-4, Interleukin 4; Fgf2, Fibroblast growth factor 2, mRNA; Fgf7, Fibroblast growth factor 7, mRNA; Mmp3, Matrix metalloproteinase 3, mRNA; Mmp12, Matrix metalloproteinase 12, mRNA; Pdgfb, Platelet-derived growth factor beta polypeptide, mRNA; Timp1, TIMP metalloproteinase inhibitor 1, mRNA (coding for metalloproteinase inhibitor 1); Timp2, TIMP metalloproteinase inhibitor 2, mRNA; Tgfβ1, Transforming growth factor, beta 1, mRNA; Vegfa, Vascular endothelial growth factor A, transcript variant 2, mRNA.

† Trend to increase or upmodulation (not statistically significant); v Trend to decrease or downmodulation (not statistically significant)
Further research should address the impact of the teduglutide on gut microbiome, which emerged as an important determinant of the anastomosis outcome, and to explore the influence of genetic polymorphisms in the susceptibility of anastomotic dehiscence.

Teduglutide therapeutic potential, demonstrated in this study, could be explored in other contexts, including in the reestablishment of the gut homeostasis in the intestinal dysfunction of the critically ill patients. Previous studies, including of Costa BP (Costa et al., 2017), suggested that biomarkers of intestinal dysfunction, such as citrullinemia, were associated with disease severity and mortality in intensive care units, namely in severe trauma patients. Intestinal dysfunction, with autodigestion and release of bacterial and toxic intestine-derived mediators, has been hypothesized to play a central role in the pathophysiology of systemic inflammatory response syndrome, multiple organ failure, sepsis and death in the critical illness.

*To conclude, the present study reflects the complexity of the anastomotic repair and points to a favorable influence of teduglutide on the intestinal anastomotic healing that deserves additional investigation.*


Bosmans JWAM, Jongen ACHM, Bouvy ND, Derikx JPM (2015) Colorectal anastomotic healing: Why the biological processes that lead to anastomotic leakage should be revealed prior to conduction intervention studies. BMC Gastroenterology. 15:180-186


Pfaffl MW (2004) Quantification strategies in real-time PCR. In A-Z of quantitative PCR (Editor: S.A. Bustin); International University Line (IUL); La Jolla, CA, USA; 87-112


Supplementary data
Referent to Chapter 1: Introduction

Table S1. Search strategy for the literature review on growth factors and hormones as adjuvants of the intestinal anastomotic healing

<table>
<thead>
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Referent to **Chapter 3: Response of putative intestinal epithelial stem cells to teduglutide on animal model of intestinal anastomosis**

Table S2. Characteristics of the putative intestinal epithelial stem cell surface markers used in the study

<table>
<thead>
<tr>
<th>Cell marker</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>Lgr5/Gpr49</td>
<td>Wnt/β-catenin-target gene that encodes a receptor for the wnt-agonists R-spondins. Specific marker of crypt base columnar intestinal stem cells.</td>
</tr>
<tr>
<td>Bmi1</td>
<td>Polycomb ring finger oncogene that encrypts a component of the polycomb repressive complex 1. Modulation of stem cells self-renewal, pluripotency and lineage specification in several tissues. Involvement in cancer initiation and progression and in embryonic development. Most widely recognized marker of &quot;position +4&quot; intestinal epithelial stem cells.</td>
</tr>
<tr>
<td>CD44</td>
<td>Class I transmembrane glycoprotein with several isoforms. Cell surface molecule whose role is primarily governed by various posttranslational modifications. Receptor for hyaluronic acid in the extracellular matrices. Induction of adhesion and migration. Ubiquitous expression in many normal cell types, including in most epithelial and lymphatic tissues; and, also, in human hematopoietic, mesenchymal and adipose-derived stem cells. No expression in undifferentiated human embryonic stem cells. Variant 9 emerged as marker of cancer stemness in a variety of solid tumors; other variants were suggested as cancer stem cell surface markers in several tumors (head and neck squamous cell, breast, colon, liver, ovarian, pancreas and gastric cancers).</td>
</tr>
<tr>
<td>CD24</td>
<td>35-60 kDa glycosyl phosphatidylinositol-linked sialoprotein. Role in cell-cell and cell-matrix interactions. Rare expression in normal tissues except B-cell precursors, neutrophils, neuronal cells, and certain epithelial cells. High expression in undifferentiated human embryonic stem cells. Cancer stem cell surface marker expressed in breast, gastric and pancreatic cell cancers.</td>
</tr>
<tr>
<td>CD166/Alcam</td>
<td>Type I membrane glycoprotein, which is a member of the immunoglobulin superfamily. Participates in cell-cell and cell-matrix interactions. Expression detected in many epithelial cells. Weak expression in undifferentiated human embryonic stem cells. Marker for human adipose-derived stromal stem cells and intestinal stem cells. Marker of colorectal cancer stem cells.</td>
</tr>
<tr>
<td>Grp78</td>
<td>Endoplasmic reticulum chaperone that assists proper protein conformation. Repressor of the unfolded protein response that plays an important role in the early regulation of intestinal stem cells differentiation. Marker of endoplasmic reticulum stress and activity of unfolded protein response that seems to be induced at the transition from stem cell to transit-amplifying cells in the beginning of the differentiation process.</td>
</tr>
</tbody>
</table>

*Bmi1, Polycomb ring finger oncogene (B cell-specific Maloney murine leukemia virus integration region 1); CD24, Cluster of differentiation 24; CD44, Cluster of differentiation 44; CD166/Alcam, Cluster of differentiation 166/Activated leukocyte cell adhesion molecule; Grp78, 78 KDa glucose-regulated protein; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor/G protein-coupled receptor 49; Wnt, wingless. * Data from references Kim and Ryu, 2017; Barker, 2014; Clevers et al., 2013; Wang et al., 2013; Barker et al., 2012; Levin et al., 2010; Rajasekhar et al., 2007*
Table S3. Monoclonal antibodies used in the characterization of rats’ small intestine cells by flow cytometry

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Antibody name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lgr5</strong> (Gpr49)</td>
<td>Anti-Gpr49/Lgr5 (Alexa Fluor 488)</td>
<td>Antibodies-online</td>
</tr>
<tr>
<td><strong>Bmi1</strong></td>
<td>Bmi1 Polycomb Ring Finger Oncogene antibody</td>
<td>Antibodies-online</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td>Rabbit F(ab’)'2 anti-goat IgG H&amp;L (Phycoerythrin)</td>
<td>Abcam</td>
</tr>
<tr>
<td><strong>CD45</strong> (LCA)</td>
<td>PerCP/Cy5.5 anti-rat CD45</td>
<td>Biolegend</td>
</tr>
<tr>
<td><strong>CD31</strong> (Pecam-1)</td>
<td>Mouse anti-rat CD31 (Alexa Fluor 647)</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td><strong>Desmin</strong></td>
<td>Anti-desmin (Alexa Fluor 488)</td>
<td>Antibodies-online</td>
</tr>
<tr>
<td><strong>CK18</strong></td>
<td>Anti-cytokeratin 18 (RGES3PE)</td>
<td>Santa Cruz Antibodies</td>
</tr>
<tr>
<td><strong>α-Sma</strong></td>
<td>Anti-alfa-smooth muscle actin (EP5368)</td>
<td>Abcam</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td>Goat anti-rabbit IgG-PerCP-Cy5.5</td>
<td>Santa Cruz Antibodies</td>
</tr>
<tr>
<td><strong>Vimentin</strong></td>
<td>Vimentin (D21H3) XP Rabbit mAb (Alexa Fluor 647 Conjugate)</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td><strong>Grp78</strong></td>
<td>Anti-Grp78 (10C3) antibody (DyLight 488)</td>
<td>Abcam</td>
</tr>
<tr>
<td><strong>CD166</strong> (Alcam)</td>
<td>Mouse/rat Alcam/CD166 antibody</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td>Rabbit F(ab’)'2 anti-goat IgG H&amp;L (Phycoerythrin)</td>
<td>Abcam</td>
</tr>
<tr>
<td><strong>CD24</strong></td>
<td>PerCP/Cy5.5 anti-mouse CD24</td>
<td>BioLegend</td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td>APC anti-mouse/human CD44</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

α-Sma, α-smooth muscle actin; APC, Allophycocyanin; Bmi1, Polycomb ring finger oncogene (B-cell-specific Moloney murine leukemia virus insertion site 1); CK18, Cytokeratin 18; CD24, Cluster of differentiation 24; CD31, Cluster of differentiation 31; CD44, Cluster of differentiation 44; CD45, Cluster of differentiation 45; CD166, Cluster of differentiation 166; Grp78, 78 KDa glucose-regulated protein; LCA, Leukocyte common antigen; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; PE, Phycoerythrin; PerCP-Cy5.5, Peridinin chlorophyll protein complex with cyanin-5.5
Table S4. Distribution of the antibodies used in the characterization of rats’ small intestine cells by flow cytometry

<table>
<thead>
<tr>
<th>Detection channel</th>
<th>Vial 1</th>
<th>Vial 2</th>
<th>Vial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL 1</td>
<td>Anti-Lgr5</td>
<td>Anti-desmin</td>
<td>Anti-Grp78</td>
</tr>
<tr>
<td>FL 2</td>
<td>Anti-Bmi1+Rabbit anti-goat IgG PE</td>
<td>Anti-CK18</td>
<td>Anti-CD166+Rabbit anti-goat IgG PE</td>
</tr>
<tr>
<td>FL 3</td>
<td>Anti-CD45</td>
<td>Anti-α-Sma+Goat anti-rabbit IgG-PerCP-Cy5.5</td>
<td>Anti-CD24</td>
</tr>
<tr>
<td>FL 4</td>
<td>Anti-CD31</td>
<td>Anti-vimentin</td>
<td>Anti-CD44</td>
</tr>
</tbody>
</table>

α-Sma, α-smooth muscle actin; Bmi1, Polycomb ring finger oncogene (B-cell-specific Moloney murine leukemia virus insertion site 1); CK18, Cytokeratin 18; CD24, Cluster of differentiation 24; CD31, Cluster of differentiation 31; CD44, Cluster of differentiation 44; CD45, Cluster of differentiation 45; CD166, Cluster of differentiation 166; FL, Fluorescence detector; Grp78, 78 KDa glucose-regulated protein; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; PE, Phycoerythrin; PerCP-Cy5.5, Peridinin chlorophyll protein complex with cyanin-5.5
Figure S1. Flow cytometry expression profile of cells isolated from rats’ ileum at the baseline (n=28). Results were presented in percentage (%) of cells expressing the specific marker and corresponded to the median (±interquartile range). α-Sma, α-smooth muscle actin; Bmi1, Polycomb ring finger oncogene (B-cell-specific Moloney murine leukemia virus insertion site 1); CK18, Cytokeratin 18; CD24, Cluster of differentiation 24; CD31, Cluster of differentiation 31; CD44, Cluster of differentiation 44; CD45, Cluster of differentiation 45; CD166, Cluster of differentiation 166; Desm, Desmin; Grp78, 78 KDa glucose-regulated protein; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; Vim, Vimentin
Referent to Chapter 5: Effects of the perioperative administration of teduglutide on the cellular viability and death, oxidative stress and inflammatory response

S1. Determination of cytokines concentrations in the rats’ homogenized intestinal tissue and in the plasma

Expression of inflammatory cytokines was performed by multiplex cytokine bead array approach, using the Rat Cytokine 5plex Kit FlowCytomix (eBioscience, Affymetrix, Vienna, Austria) produced for quantification of rats’ homogenized tissue and plasma levels of interleukine-1α (IL-1α), macrophage chemo-attractant protein (MCP-1), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukine-4 (IL-4).

Biological samples standardization was attentive to increase stability and representativeness of the cytokine measurement. Reagents were prepared following the manufacturer’s recommendations. Assay buffer, the biotin-conjugate mixture and the bead mixture were prepared by adding the assay buffer concentrate (phosphate buffered saline with 10% bovine serum albumin; 10x) to distilled water (50:450 ml), diluting each biotin-conjugate (1:20) and diluting each bead set (1:20) and washing it once with rat reagent dilution buffer (RRDB), respectively. Standard mixture was prepared by reconstituting the lyophilized standard with distilled water, making a 1:20 dilution of the reconstituted standards all diluted in the same vial to get Standard 1 and proceeding to further serial dilutions. Streptavidin-phycoerythrin solution was prepared by adding 176 μl of the streptavidin-phycoerithrin concentrate to 5324 μl of the assay buffer (1x). Diluted standard mixture (25 μl) dilution “1” to “7” was pipetted into designated tubes. Procedure included addition of 25 μl of the assay buffer (1x) to the blank tubes, of 25 μl of the standard mixture dilution “1” to the tube which is designated for cytometer setup, of 25 μl of the sample (supernatant from the intestinal tissue homogenization or plasma) to designated tubes, of 25 μl of bead mixture to all tubes and of 50 μl of biotin-conjugate mixture to all tubes. Tubes were then incubated two hours at room temperature (18° to 25°C) protected from light. One milliliter of the assay buffer (1x) was added to all tubes, which were then washed twice by centrifugation (five minutes at 200 x g) discarding the supernatant carefully and leaving 100 μl of liquid in each tube; streptavidin-phycoerithrin solution (50 μl) was added to
all tubes followed by incubation for one hour at room temperature (18 to 25°C) protected from light. This procedure was repeated once except for adding 500 μl of the assay buffer (1x) to the 100 μl of the liquid remaining in each tube (instead of the streptavidin-phycoerythrin solution) and the samples were ready for analysis on the flow cytometer. Standard and test samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA) as stated in the producer’s recommendations. Mean fluorescence intensities (MFIs) of the serially diluted standard samples were calculated and used to generate the standard curves of each cytokine that model protein concentration as a function of the MFI. Cytokine concentrations present in the test sample were calculated, using the corresponding standard curves and dilution factors.
Referent to **Chapter 6: Tissue growth factors profile after teduglutide administration on an animal model of intestinal anastomosis**

### Table S5. Primers used in gene expression analysis of growth factors and Glp2 receptor by qRT-PCR

<table>
<thead>
<tr>
<th>Gene*</th>
<th>GenBank Accession nº</th>
<th>Orientation</th>
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<th>Size (bp)</th>
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<tr>
<td>Rat Igf1</td>
<td>NM_001082477</td>
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<td>Rat Vegfa</td>
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</tr>
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<td>86</td>
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<tr>
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<td>Reverse</td>
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<td>Reverse</td>
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</tr>
<tr>
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<td>Reverse</td>
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<tr>
<td>Rat Pdgfb</td>
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<td>Reverse</td>
<td>CTGTTCCAGGGCTTTTCC</td>
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</tr>
</tbody>
</table>

* Igf1: Insulin-like growth factor 1, transcript variant 1, mRNA; Vegfa: Vascular endothelial growth factor A, transcript variant 2, mRNA; Tgfb1: Transforming growth factor, beta 1, mRNA; Ctgf: Connective tissue growth factor, mRNA; Fgf2: Fibroblast growth factor 2, mRNA; Fgf7: Fibroblast growth factor 7, mRNA; Egf: Epidermal growth factor, mRNA; Hbegf: Heparin-binding EGF-like growth factor, mRNA; Pdgfb: Platelet-derived growth factor beta polypeptide, mRNA; Glp2r: Glucagon-like peptide 2 receptor, mRNA; Hprt1: Hypoxanthine phosphoribosyltransferase 1, mRNA (housekeeping gene) (Rattus norvegicus)
S2. Protocol of analysis of gene expression levels of growth factors and glucagon-like peptide 2 receptor by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

RNA extraction

Total RNA was extracted from the homogenates of the longitudinal strips of ileum using the Isolate II RNA Mini Kit (Bioline, London, UK). Succinctly, biological samples were first lysed and homogenized in the presence of guanidinium thiocyanate, a chaotropic salt that immediately deactivated endogenous RNases to ensure purification of intact RNA. After homogenization, ethanol was added to the sample. Lysate was filtered and the RNA adjusted to RNA binding conditions. Sample was then processed through a spin column containing a silica membrane to which the RNA binds. Genomic DNA contamination was removed by a DNase I digestion during the preparation. Any impurities such salts, metabolites and cellular components were removed by simple washing steps of the silica membrane with two different buffers. High-quality purified total RNA was then eluted in RNase-free water and stored at -80°C.

In particular, pellet obtained in the previously outlined protocol of intestinal tissue homogenization was sonicated twice with one short pulse of 10 seconds followed by cooling intervals of 30 seconds. According to the protocol, up to 5x10⁶ eukaryotic cells were collected by centrifugation and all supernatant was removed. Cells were directly lysed by adding 600 μl of lysis buffer RLY (with a guanidine salt) and 6 μl of β-mercaptoethanol, a denaturing agent, to the cell pellet and vortexed vigorously. Lysate was loaded on an Isolate II filter placed on a collection tube (2 ml) and a centrifugation for one minute at 11,000x g was performed to obtain a clear lysate with reduced viscosity to facilitate efficient binding of RNA to the column membrane. Isolate II filter was discarded, the flow-through was transferred into a new 1.5 ml microcentrifuge tube; 350 μl of 70% ethanol was added and mixed by vortexing (2 × 5 seconds). One Isolate II RNA mini column was placed in a collection tube (2 ml) for each preparation. Lysate was pipetted up and down two to three times and all loaded onto the column. A centrifugation for 30 seconds at 11,000x g was performed and the column was
placed in a new collection tube (2 ml). A 350 μl of membrane desalting buffer (MEM) was added and a centrifugation at 11,000x g for one minute was done to dry the membrane. DNase I (RNase-free) stock solution was prepared by reconstitution of lyophilized DNase I in RNase-free water (adding 540 μl of water for the 250 preparations kit), incubation for one minute at room temperature and mixing by gently swirling. A DNase I reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube. For each isolation, 10 μl of reconstituted DNase I was added to 90 μl of reaction buffer for DNase I (RDN) and mixed by gently flicking the tube. DNase I reaction mixture (95 μl) was applied directly onto the center of silica membrane. Incubation at room temperature for 15 minutes was done. Then, the silica membrane was washed and drying (three steps): wash buffer RW1 (200 μl) was added to the Isolate II RNA mini column (to inactivate the DNase I), a centrifugation for 30 seconds at 11,000x g was done and the column was placed into a new collection tube (2 ml); wash buffer RW2 (600 μl), prepared by adding 96-100% ethanol to wash buffer RW2 concentrate (100 ml x 3 for the 250 preparations kit), was added to the Isolate II RNA mini column, a centrifugation for 30 seconds at 11,000x g was done, the flow-through was discarded and the column was placed back into the collection tube; wash buffer RW2 (250 μl) was added to the Isolate II RNA mini column, a centrifugation for two minutes at 11,000x g was done (to dry the membrane completely) and the column was placed into a nuclease-free (1.5 ml) collection tube. RNA was eluted with 60 μl of RNase-free water and centrifuged at 11,000x g for one minute. Eluted RNA was placed on ice immediately to prevent degradation by RNases and freeze at -80°C for long-term storage.

Reverse-transcription (conversion of RNA to cDNA)

The Tetro cDNA Synthesis Kit (Bioline) was used for reverse-transcription. According to the protocol, priming premix was prepared on ice, in a RNase-free reaction tube, with total RNA (1 μg), random hexamer primer mix as a primer (1 μl), free nucleotides (dNTP) mix 10 mM (1 μl), Reverse-transcription buffer (5x) (4 μl), ribosafe RNase inhibitor (1 μl), tetro reverse transcriptase (moloney strain of murine leukemia virus reverse transcriptase; 200 U/μl; 1 μl) and diethylpyrocarbonate-treated
water (up to 20 μl) (Bioline). Solutions were vortex and centrifuged briefly before use. Mixture was mixed gently by pipetting and samples were incubated for 10 minutes at 25°C followed by 30 minutes at 45°C. Reaction was terminated by incubation of the mixture five minutes at 85°C and chilling on ice.

**Real-time polymerase chain reaction**

Quantitative RT-PCR was performed on a Bio-Rad iQ5 real-time PCR instrument (BioRad, Hercules, California, USA) using the SensiFAST SYBR & Fluorescein Kit (Bioline).

During the procedure, distinct areas were maintained for reaction set-up and PCR amplification to prevent any DNA contamination. One microliter of the reverse-transcription product of each sample was used for real-time PCR. A PCR mastermix was prepared by mixing 2x SensiFAST SYBR & fluorescein mix (10 μl; 1x), 10 μM forward primer (0.8 μl; 400 nM), 10 μM reverse primer (0.8 μl; 400 nM), template (100 ng cDNA per reaction) and water up to 20 μl. Magnesium chloride concentration in the (1x) reaction mix is 3 mM. A two-step cycling was performed and included one cycle of polymerase activation (two minutes at 95°C) and 40 cycles of denaturation (five seconds at 95°C), annealing and extension (20 seconds at 60°C). After the reaction had reached completion, a melting point analysis was performed. Melt peak chart was established following the instruments instructions (checking every 0.5°C between 50 and 95°C with intervals of one second). Quality control of the procedure was done, including the detection of the presence of contaminating DNA that may affect the reliability of the data; accordingly, no-reverse-transcriptase and no-template controls were set up. Purity of each PCR product was checked by analyzing the amplification plot and dissociation curves. Each sample was monitored for fluorescein and signals were regarded as significant if the fluorescence intensity exceeded 10-fold of the standard deviation of the baseline fluorescence, defined as threshold cycles (C_T). C_T were selected in the line in which all samples were in the logarithmic phase. For each sample, PCR was performed in duplicate. Data were analyzed by relative quantification.
S3. Protocol for determination of glucagon-like peptide 2 plasma concentrations by competitive enzyme immunoassay

Plasma levels of glucagon-like peptide 2 (glp-2) were determined by competitive enzyme immunoassay (EIA) using the Glp-2 EIA Kit 96-Well Plate (Phoenix Europe GmbH, Karlsruhe, Germany).

Following the manufacturer’s protocol, procedure began with extraction of peptides from plasma. Rats’ plasma collected, as stated before, was acidified with 1% trifluoroacetic acid and centrifuged at 3000x g for 20 minutes at 4°C; supernatant acidified plasma solution was loaded onto a pre-equilibrated SEP-column containing 200 mg of C-18; eluent collected into a polystyrene tube was evaporated to dryness using a centrifugal concentrator and then lyophilized.

Enzyme immunoassay protocol began with standard peptide and quality controls preparation. Assay buffer solution (1x), used to dilute or reconstitute all other reagents and samples, was prepared by dilution of the assay buffer concentrate (20x; 50 ml) with 950 ml of distilled water and mixing. Standard peptide was centrifuged, diluted with 1 ml of assay buffer solution (1000 ng/ml), vortexed gently and allowed to sit for at least 10 minutes at room temperature; standard peptide was centrifuged and vortexed immediately before use. Assay buffer (0.9 ml) was added to each of five tubes. Serial dilutions were prepared by adding 0.1 ml of the prepared standard to Tube 1 (100 ng/ml) and mixing well; transferring 0.1 ml of Tube 1 to Tube 2 (10 ng/ml), mixing well; transferring 0.1 ml of Tube 2 to Tube 3 (1 ng/ml), mixing well; transferring 0.1 ml of Tube 3 to Tube 4 (0.1 ng/ml), mixing well and transferring 0.1 ml of Tube 4 to Tube 5 (0.01 ng/ml) and mixing well. Positive control was centrifuged and rehydrated with 200 μl of the assay buffer solution, allowed to sit for at least five minutes and mixed thoroughly.

Primary antibody (rabbit anti-peptide IgG) and biotinylated peptide were reconstituted with 5 ml of the assay buffer solution, allowed to sit for at least five minutes and mixed thoroughly. Enzyme solution was prepared by pipetting 12 μl of the streptavidin-horseradish peroxidase into 12 ml of the assay buffer solution, after centrifugation (3000-5000 rpm) during five seconds, and vortexing thoroughly.
Fifty microliters of prepared peptide standards (#1 to 5), rehydrated positive control and plasma sample were added to the designated wells (in duplicate) leaving two wells as “blank” and two wells as “total binding” (exclusively with the assay buffer solution). Reconstituted primary antibody and prepared biotinylated peptide (25 μl of each) were added to each well except the “blank”. Immunoplate was sealed with the acetate plate sealer and incubated for two hours, at room temperature (20 to 23°C) with orbital shaking at 300 to 400 rpm. Next, plate sealer was removed and solutions were decanted from the plate. Four careful washes were performed, with 350 μl of the assay buffer solution per well, decanting and tapping firmly after each wash to remove residual buffer. Streptavidin-horseradish peroxidase solution (100 μl) was added to each well. Plate was covered with sealer and incubated for one hour, at room temperature, with moderate shaking (300 to 400 rpm) on the microtiter plate shaker. Plate sealer was removed and solutions decanted from the plate. Four careful washes were performed, with 350 μl of the assay buffer solution per well, decanting and tapping firmly after each wash to remove residual buffer. The 3,3′,5,5′-tetramethylbenzidine substrate solution (100 μl) was added to each well, avoiding unnecessary exposure to light; plate was covered with sealer and incubated for one hour, at room temperature, with moderate shaking (300 to 400 rpm) on the plate shaker. Blue color was formed in wells of the peptide standards with intensity inversely proportional to the concentration of the peptide. Sealer was removed and 100 μl of stop solution (2N hydrochloric acid) was added to each well; plate was shaken by hand to ensure complete mixing of solution in all wells. Blue color turned to yellow after acidification. Absorbance (optical density) was read at 450 nm in a microtiter plate reader within 20 minutes. Difference of absorbance units was recorded.

A standard curve was constructed using the serial dilutions of peptide standards (known concentrations of standard peptide on a logarithmic scale versus its corresponding optical density reading on a linear scale). This analysis modeled the peptide concentration as a function of the optical density. Standard curve showed reverse sigmoidal shape and an inverse relationship between peptide concentrations and the corresponding absorbance. As the standard concentration increases, the yellow color decreases, thereby reducing the optical density absorbance. Then, the
Glp-2 plasma concentrations were calculated using the corresponding standard curve and the microplate reader with Gen5 software (Synergy HT, Biotek, Winooski, Vermont, USA), and expressed in ng/ml.
Referent to **Chapter 7**: Teduglutide effects on gene regulation of fibrogenesis on an animal model of intestinal anastomosis

Table S6. Primers used in gene expression analysis of extracellular matrix components by qRT-PCR

<table>
<thead>
<tr>
<th>Gene*</th>
<th>GenBank Accession nº</th>
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*Col1a1: Collagen, type I, alpha 1, mRNA (coding for collagen alpha-1(I) chain); Col3a1: Collagen, type III, alpha 1, mRNA; Col4a1: Collagen, type IV, alpha 1, mRNA; Col5a1: Collagen, type V, alpha 1, mRNA; Timp1: TIMP metallopeptidase inhibitor 1, mRNA (coding for metalloproteinase inhibitor 1); Timp2: TIMP metallopeptidase inhibitor 2, mRNA; Mmp1: Matrix metallopeptidase 1 (interstitial collagenase), mRNA; Mmp2: Matrix metallopeptidase 2, mRNA; Mmp3: Matrix metallopeptidase 3, mRNA; Mmp9: Matrix metallopeptidase 9, mRNA; Mmp12: Matrix metallopeptidase 12, mRNA; Mmp13: Matrix metallopeptidase 13, mRNA; Mmp14: Matrix metallopeptidase 14 (membrane inserted), mRNA; Hprt1: Hypoxanthine phosphoribosyltransferase 1, mRNA (housekeeping gene) (Rattus norvegicus)
Refer to Chapter 8: General discussion and concluding remarks

<table>
<thead>
<tr>
<th>Table S7. Relevant correlations between putative intestinal epithelial stem cells and other parameters*</th>
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<tr>
<td>(σ / p)</td>
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<tr>
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<td><strong>Viability and death</strong></td>
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<tr>
<td>p</td>
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<tr>
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<tr>
<td>Mitochondrial reactive species</td>
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<td>Cellular reduced glutathione</td>
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<td>p</td>
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<td>Mitochondrial membrane potential</td>
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<td><strong>Tissue cytokines level</strong></td>
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<tr>
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<tr>
<td><strong>Plasma cytokines concentration</strong></td>
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<td>p</td>
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* Relevant correlations of the proportion of putative intestinal epithelial stem cells isolated from rats’ ileum, at the moment of sacrifice, in all the studied animals (n=59), as determined by flow cytometry, versus cellular viability and death indexes, oxidative stress parameters, tissue and plasma levels of cytokines. Samples recovered at the sacrifice in rats submitted to ileal resection corresponded to the anastomotic segment. Viability and death analysis was performed by flow cytometry with Annexin-V/Propidium iodide; cytokines levels were determined by flow cytometric bead assay; peroxides production in the cytosol, reactive species generation in the mitochondria, mitochondrial membrane potential and cellular reduced glutathione content were evaluated by the DCF expression, DHR123 production, aggregates/monomers ratio of JC-1 probe and mercury orange staining determined by flow cytometry, respectively. Spearman’s correlation coefficient (σ) and value of significance (p) were presented.
Table S8. Relevant correlations between putative intestinal epithelial stem cells and tissue gene expressions of growth factors and plasma levels of glucagon-like peptide 2^a

<table>
<thead>
<tr>
<th>a / p</th>
<th>Lgr5^+ / Bmi1^- cells</th>
<th>Lgr5^- / Bmi1^- cells</th>
<th>Lgr5^- / Bmi1^- cells</th>
<th>Lgr5 or Bmi1^- cells</th>
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<tbody>
<tr>
<td>Gene expression of growth factors/receptor</td>
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<tr>
<td>Igf1</td>
<td></td>
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<tr>
<td>Vegfa</td>
<td>62.5%</td>
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<td>Tgfβ1</td>
<td>-39.7%</td>
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<tr>
<td>Ctgf</td>
<td>30.7%</td>
<td>43.5%</td>
<td>54.7%</td>
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<td>Fgf2</td>
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<td>Fgf7</td>
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<td>51.9%</td>
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<tr>
<td>Egf</td>
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<tr>
<td>Pdgrb</td>
<td>-37.8%</td>
<td>-26.1%</td>
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<tr>
<td>Glp2r</td>
<td>49.7%</td>
<td></td>
<td>34.5%</td>
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<td>Plasma Glp2 levels</td>
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<td>[Glp2]</td>
<td>-57%</td>
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</table>

^a Relevant correlations of the proportion of putative epithelial stem cells isolated from rats’ ileum, at the moment of sacrifice, in all the studied animals (n=59), as determined by flow cytometry, versus relative gene expressions of growth factors and glucagon-like peptide 2 receptor (Glp2r), and postoperative plasma levels of glucagon-like peptide 2 (Glp-2). Relative gene expressions of growth factors and Glp2r and postoperative plasma levels of Glp-2 were determined by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and by competitive enzyme immunoassay, respectively. Samples recovered at sacrifice in rats submitted to ileal resection corresponded to the anastomotic segment. Spearman’s correlation coefficient (σ) and value of significance (p) were presented.