THE EFFECT OF SURGICAL MUCOSECTOMY ON THE INTESTINE AND ITS POSSIBLE CLINICAL CONSEQUENCES

Ph. D. Thesis

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LIST OF PUBLICATIONS

Full papers related to the subject of the thesis

1. **Urbán D,** Varga G, Érces D, Marei M, Cervellione RM, Keene D, Goyal A, Cserni T. Prolonged ischemia of the ileum and colon after surgical mucosectomy explains contraction and failure of "Mucus free" bladder augmentation. J Pediatr Urol (in press article, 10.1016/j.jpurol.2022.04.015) **IF 1.83**

2. **Urbán D**, Cserni T, Boros M, Juhász Á, Érces D, Varga G. Bladder augmentation from an insider's perspective: a review of the literature on microcirculatory studies. Int Urol Nephrol. 2021;53:2221-2230. **IF 2.37**

3. **Urbán D**, Marei MM, Hajnal D, Varga G, Érces D, Poles M, Imre D, Szabó A, Cervellione RM, Cserni T. Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol. 2020;16:20-26. **IF 1.622**

4. **Urbán D**, Kőnig R, Cserni T. A rövidbél-szindróma korszerű sebészi kezelése: autológ rekonstrukció és intestinalis rehabilitáció [Autologous reconstructive surgery and intestinal rehabilitation in the management of short bowel syndrome]. Orv Hetil. 2020;161:243-251. Hungarian. **IF 0.54**

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Response to a commentary related to the subject of the thesis

Marei MM, **Urbán D**, Cserni T. Response to commentary to 'Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplastsy'. J Pediatr Urol. 2020;16:29-30.

Full papers not related to the subject of the thesis

1. Cserni T, **Urban D**, Hajnal D, Érces D, Varga G, Nagy A, Cserni M, Marei M, Hennayake S, Kubiak R. Pyeloureteric magnetic anastomosis device to simplify laparoscopic pyeloplasty: a proof-of-concept study. BJU Int. 2021;127:409-411. **IF 5.588**
2. Szántó Z, Kovács O, Parham Choroumzadeh D, Lippai N, Csőszi T, **Urbán D**. A VATS lobectomia standardizálódása osztályunkon 2011–2017 között az onkológiai utánkövetés eredményeinek tükrében [Standardization of VATS lobectomies in our department between 2011-2017 in regard to results of their oncological follow-ups]. Magy Seb. 2019;72(3):98-102.

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LIST OF ABBREVIATIONS

AChE acetylcholinesterase

BE Barrett oesophagus

CLE confocal laser endomicroscopy

EDTA ethylenediaminetetraacetic acid

ENS enteric nervous system

ESD endoscopic submucosal dissection

$EtCO_2$ end-tidal carbon dioxide pressure

FI fluorescence imaging

GI gastrointestinal

HD Hirschsprung's disease

HE haematoxylin and eosin

ICG intraoperative indocyanine green

IDF incident-dark field illumination

LDF laser Doppler flowmetry

LSCI laser speckle contrast imaging

MFI microvascular flow index

NADPH-d dihydronicotinamide adenine dinucleotide phosphate diaphorase

NF neurofilament

nNOS nitric oxide synthase

OPS orthogonal polarisation spectral

PAI photoacoustic imaging

pCO_2 pressure of carbon dioxide

RBCV red blood cell velocity

SBS short bowel syndrome

SDF side-stream dark field imaging

SUMMARY

Mucosectomy may be part of many surgical procedures for several indications. Ex-vivo mucosectomy is also used by researchers and pathologist to explore and study 3D structure of the enteric nervous system. Besides, in-vivo mucosectomy with endoscopic submucosal dissection (ESD) became a standard minimally invasive treatment option for early non-invasive gastrointestinal (GI) cancers without regional lymph node metastases. ESD usually is well tolerated however scar formation, stricture at the surgical site with large areas resected is a known complication.

The ileum and colon are the most commonly used donor organs for bladder augmentation, however the presence of intestinal mucosa within the augmented bladder is associated with significant complications, such as urinary tract infection, stone formation and adenocarcinoma development. Not surprisingly, extensive research has been carried out to reduce the risk associated with the presence of intestinal mucosa in the augmented bladder. Composite flaps with cultured urothelium coverage after mucosectomy in experimental settings seemed to be a viable and promising approach but the experimental results were not translated into clinical practice and contraction and stricture of the intestinal flaps is still major concern. Similar experimental attempts have been made to create composite intestine transplanting small bowel mucosa in the colon after colonic mucosectomy to increase absorptive surface in severe short bowel syndrome assuming the colon will remain functional after mucosectomy.

The link between ischemia and postoperative fibrosis is relatively well known but the exact mechanism of stricture formation after mucosectomy is still less understood. Our main goal, therefore, was to study and characterize the effects of mucosectomy on the intestinal microperfusion and the enteric nervous system (ENS), to investigate and define the potential contribution of these intramural factors to the negative postoperative consequences.

We have carried out our investigation in two separate but inter-related studies using anesthetized minipigs. We have demonstrated that mucosectomy results in an abrupt cessation of the microcirculation of the intestinal wall without significant recovery within the warm ischemia time. Significant disruption of the ENS with broken reflex circuits of the intestinal segments were demonstrated histologically with prompt intestinal contractions in vivo. These findings may influence the direction of research in reconstructive surgery and urology: the stricture seen after extensive mucosectomy remains invertible, composite intestinal segments with transplanted mucosa may not be viable longer term if immediate microvascular and neurological damage of the intestinal segment are not addressed.

TABLE OF CONTENT

1. INTRODUCTION

Mucosectomy, the dissection and removal of the intestinal mucosa is technically feasible by conventional and minimally invasive surgical techniques. Ex-vivo mucosectomy is performed by researchers and pathologists as well to explore and study the function and structure of the enteric nervous system (ENS) [1–4]. Endoscopic submucosal dissection (ESD), a minimal invasive technique to remove early diagnosed gastrointestinal (GI) malignancies not invading the muscle layers and lymph nodes has become increasingly popular in recent times. Using natural body orifices, this approach can eliminate the peri-operative burden of open surgeries without compromising the oncological outcome [5,6]. Although ESD is well tolerated, several studies have reported strictures or stenosis requiring steroid treatment and multiple balloon dilatations [7–9].

Mucosectomy plays significant roles in reconstructive urology as well. Parts of the digestive system, especially ileum and colon are routinely used for bladder augmentation as donor organs. The presence of the intestinal mucosa in the augmented bladder is however, associated with complications such as urinary tract infection and stone formation due to mucus production, absorption of electrolytes from urine and adenocarcinoma development. Therefore, intensive research attention has been focused on “mucus free” bladder augmentation. Composite flaps with cultured urothelium coverage after mucosectomy seemed to be viable and promising approach [10]. Similar experimental attempts were made to create composite small bowel mucosa after colonic mucosectomy to increase the absorptive surface in severe short bowel syndrome assuming the colon will remain functional after mucosectomy [11–13]. However, despite the promising experimental results the findings cannot be translated into clinical practice because contraction of the intestinal flaps remained a major concern.

Limited knowledge is available on the background of stricture formation after mucosectomy, and in particular, on the role of microcirculation of the remaining intestinal wall. It is generally assumed that sero-muscular or sero-muculo-submucosal intestine still preserves its blood supply if the mesenteric vessels remained intact. The link between ischemia and fibrosis and stricture is relatively well known [14–17]. In this context it is essential to understand the impact of mucosectomy on the intramural microcirculation, we hypothesized that removal of the mucosa/submucosa may disrupt the intramural vascular plexuses compromising the perfusion of the remaining sero-muscular wall.

Similarly, the consequence of the disruption of the ENS in this scenario is less understood, and it is not known how the neural circuits responsible for the contraction and relaxation of the intestinal muscle layers react to surgical mucosectomy. We hypothesized that mucosectomy disrupts the ENS, which may cause contraction in the involved segments. This possibility has not been explored before in real-life or clinically relevant experimental conditions.

1.1 Stricture after mucosectomy in clinical practice

1.1.1 ESD and strictures in the GI tract

Endoscopic excision of large mucosal-submucosal lesions in esophagus is possible, however rate of complications i.e. stricture is increasing with the size of excision from 2% to 88% [14–16]. Approx. 66%-88% of patients with higher than 75% circumferential resection developed esophageal stricture [7,8]. At present multiple endoscopic balloon dilatation and systematic or local steroid therapy is available for treatment [7]. In an experimental study extracellular matrix covered stents were tested to prevent stricture and push the boundaries of mucosal resection. The effect was temporary, stricture formation was significantly more severe after stent removal than in the control group [17].

There are only a few reports about stricture after ESD in stomach [9,18]. However, its incidence strongly depends on the localisation and circumferential expansion of the lumen. All cases were reported where the resected ratio was at least 75% or higher of the whole circumference of the lumen [9]. Significant stenosis was localised on pre-pylorus, antrum and cardia [8,9].

In terms of complications the rectum seems to be the “most forgiving” space, because strictures were not reported after circumferential resection of under 90% [8,19]. However, in case of resection over 90% of the circumference, stricture was registered in 43.8% of patients. In case of total circumferential resection, the complication rate rises further (71.4%) [8,19]. Again, the management involves multiple balloon dilatations, and steroid treatment.

The difference in ESD limitation and complications may be explained with the different functional features of esophagus, stomach and rectum [8]. For example, rectum has larger lumen than the esophagus, in addition it holds the stool before defecation, which generates a constant pressure on the wall and it keeps the rectum dilated [8].

1.1.2 Short bowel syndrome (SBS)

The modern management of severe SBS involves medical bowel management and autologous reconstructive surgical interventions. The interventions are focusing on increasing the absorptive surface of the small bowel by controlled bowel expansion technique, which is mainly hydrostatic expansion of the residual small bowel and the reconstruction of the motility of the bowel segments i.e. reducing its extremely dilated diameter without losing further absorptive surface (bowel lengthening techniques) [20].

In SBS cases intact colon is usually available, but the change of the colonic mucosa is does not help in absorption of nutrients. Therefore creation of composite intestine from the colon with transplanted cultured small bowel mucosa or stem cells is the focus of present GI research [12,13]. The colonic mucosa is removed with surgical or chemical mucosectomy and epithelial organoid containing ileal stem cells is implanted. The neo-ileal mucosa demonstrates villus structures and crypts, and contains enterocytes, goblet cells, enteroendocrine cells, and Paneth cells [12,13]. In an experiment with human organoids, not just the villus-like structures with sodium-dependent bile acid uptake activity, but the lymphovascular system of the mucosa was restored too [13]. During mucosectomy it is crucial to remove the residual colonic mucosa and preserve the viability of submucosal layer. In an animal study the ambiguous consequences of surgical mucosectomy were emphasized, a heterogeneous surface with residual native mucosa and deep debridement-induced scar formation was demonstrated [11]. Chemical mucosectomy remains another option. Dithiothreitol solution and EDTA were found to be effective experimental means to remove the mucosal layer, however in this case the mesentery was temporary clamped to prevent systemic EDTA toxicity [11]. Thus, this approach is still far from being safe alternative in clinical practice [11].

To achieve the final goal “to turn the colon to small bowel” it is not enough to transplant the small bowel mucosa into it. The colonic segment should remain functional after mucosectomy. Contraction and/or motility problems in the composite bowel would lead to ultimate failure to treat SBS.

1.1.3 Reconstructive urology - bladder augmentation

At present, enterocystoplasty is the most favoured method for augmentation, while other procedures, such as auto-augmentation of the detrusor muscle, are less common. Stomach and colon can be used for bladder enlargement, but the preferred organ is the ileum [21]. The pros and cons of different GI segments used for bladder augmentation are summarised in Figure 1.

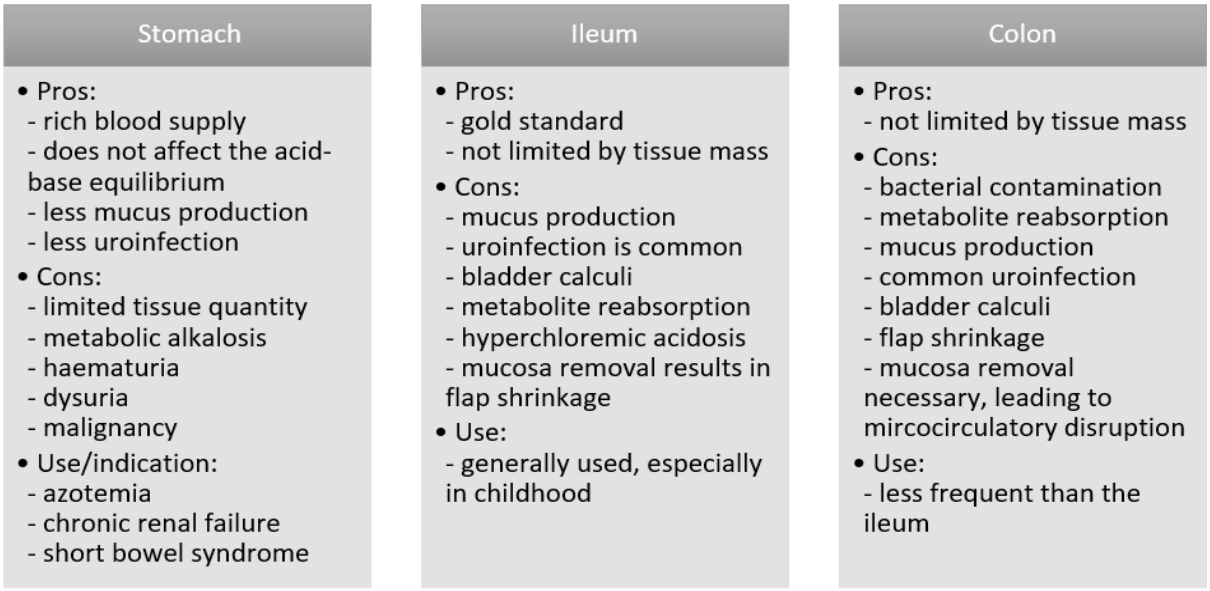


Figure 1 *Comparison of GI segments for bladder augmentation*
Source: Urbán D, Cserni T, Boros M, Juhász Á, Érces D, Varga G. Bladder augmentation from an insider's perspective: a review of the literature on microcirculatory studies. Int Urol Nephrol. 2021;53:2221-2230.

The presence of intestinal mucosa within the augmented bladder is associated with significant complications as increased infection rate due to mucus production, stone formation, absorption of electrolytes from urine and the long-term risk of adenocarcinoma [22]. Bladder augmentation with de-mucosalised (mucosectomised) intestine or stomach has been under research since the early 1950s. However, after the first promising experiments, the clinicians could not obtain acceptable results [23,24]. Thirty years later researchers tried to revive the procedure, achieving more success in rodents like rats and rabbits [25,26], but they were not able to reproduce good results in larger animals like dogs [27,28].

It had been suggested that the shrinkage of the flaps is possibly multifactorial, due to (a) chemically-induced fibrosis caused by the exposure of the raw surface to urine, (b) chronic infection, (c) prolonged postoperative decompression of the bladder and/or (d) ischemia occurring during the mucosectomy [26]. Researchers are now attempting to eliminate the

chemical irritation caused by urine on the mucosectomised raw surface of the flaps using urothelium, but despite the use of postoperative splints, the shrinkage of flaps remains a significant problem [10,29,30].

1.2 Mucosectomy and intramural circulation of the intestine

1.2.1 Anatomy of the intramural blood supply of small intestine and colon

To understand the consequences and boundaries of the mucosectomy / ESD the knowledge about the intramural circulation of the intestine is essential. The intramural vessels of the intestine are organised in subserosal, submucosal intermuscular and mucosal interconnected plexuses (Figure 2) [31].

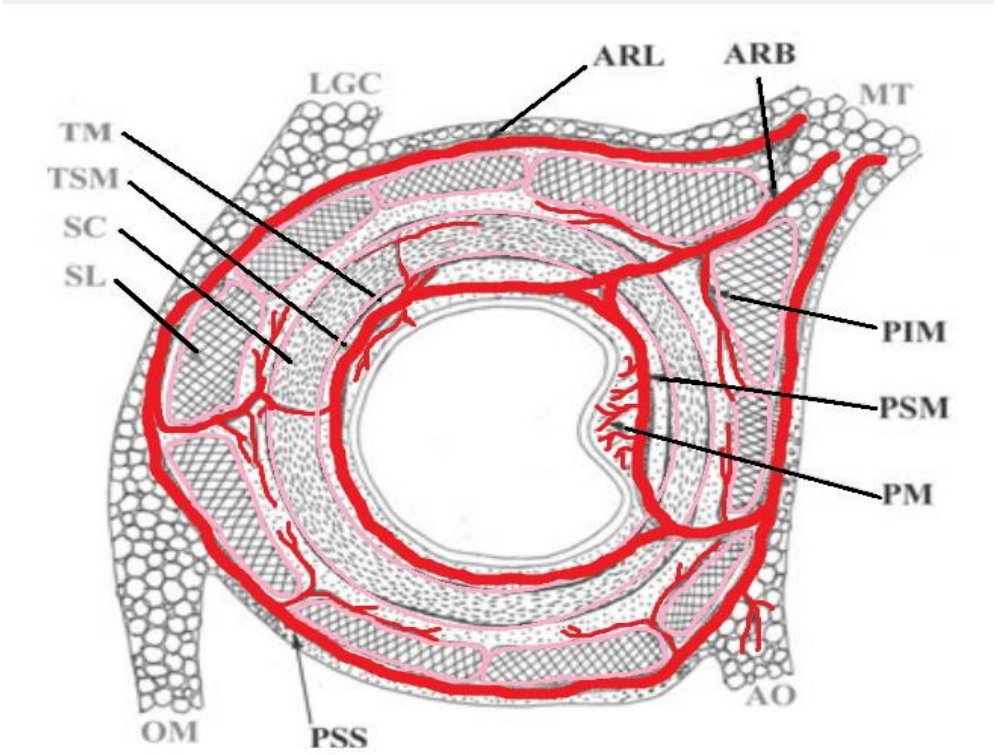


Figure 2 '*The scheme of the intramural plexuses in cross-section of the large intestine wall.* ***MT:*** *mesocolon transversum;* ***LGC:*** *ligamentum gastrocolicum;* ***TM:*** *tunica mucosa;* ***TSM:*** *tunica submucosa;* ***SC:*** *stratum circulare;* ***SL:*** *stratum longitudinale;* ***OM:*** *omentum majus;* ***AO:*** *appendix omentalis,* ***ARL:*** *arteria recta longa;* ***ARB:*** *arteria recta brevis;* ***PIM:*** *plexus intermuscularis;* ***PSM:*** *plexus submucosus;* ***PM:*** *plexus mucosus;* ***PSS:*** *plexus subserosus.'*
Source: Kachlik D, Baca V, Stingl J. The spatial arrangement of the human large intestinal wall blood circulation. J Anat 2010;216:335–43. https://doi.org/10.1111/j.1469-7580.2009.01199.x.

The subserosal vascular plexus: Structure of intramural circulation commences with vasa recta, which are branched from *arteria marginalis* (Drummond's marginal artery) or arcades and they run toward to the serosa at straight [31]. When vasa recta reach the serosa they richly give collaterals and form the subserosal plexus. The short vasa recta feed mesocolic/mesenterial region, adjacent intestinal wall and external muscle layer [31]. At the antimesenteric border the long vasa recta turn back and make numerous anastomosis with each other and short vasa recta too (Figure 3).

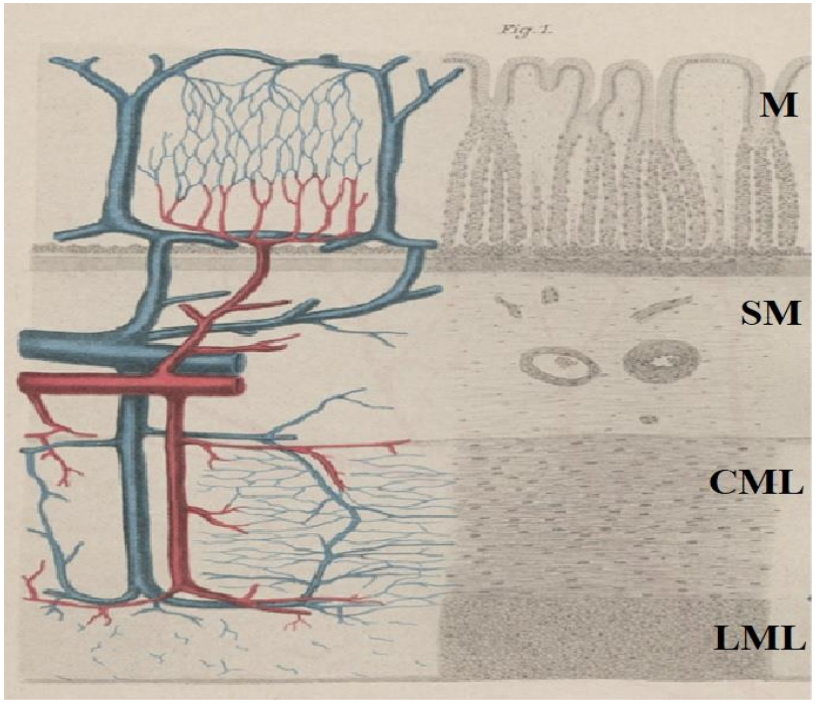


Figure 3 *Intramural vasculature of gastric wall. This schematic diagram demonstrates the central role of submucosal plexus, because these arteries supplying the mucosa and also a significant part of the muscle layer.* **M** *Mucosa;* **SM** *Submucosal layer;* **CML** *Circumferential muscle layer;* **LML** *Longitudinal muscle layer.*
Source: Rau W, Hohaus C, Jessen E. A Differential Approach to Form and Site of Peptic Ulcer. Sci Rep 2019;9:8683. https://doi.org/10.1038/s41598-019-44893-x.

The intermuscular plexus: Centrifugal branches of the vasa recta, -also known as recurrent muscular branches- turn back from the submucosal plexus to the muscle layers (circumferential and longitudinal) and they compose the intermuscular plexus. Microdissection and corrosion casting showed that these recurrent branches serve as the main arterial source, however subserosal plexus also send off branches, but their role is insignificant in human specimen [31].

Since the submucosal plexus has such a crucial role in arterial supplementation of muscle wall it is likely that destruction of this layer (removal of submucosa or ESD) may result in significant ischemia of the muscularis propria. Whether the direct branches from the subserosal plexus already labelled "insignificant" could replace them remains a question. No study measured the effect of the mucosectomy (ESD) on the microcirculation of the remaining intestine (Figure 4).

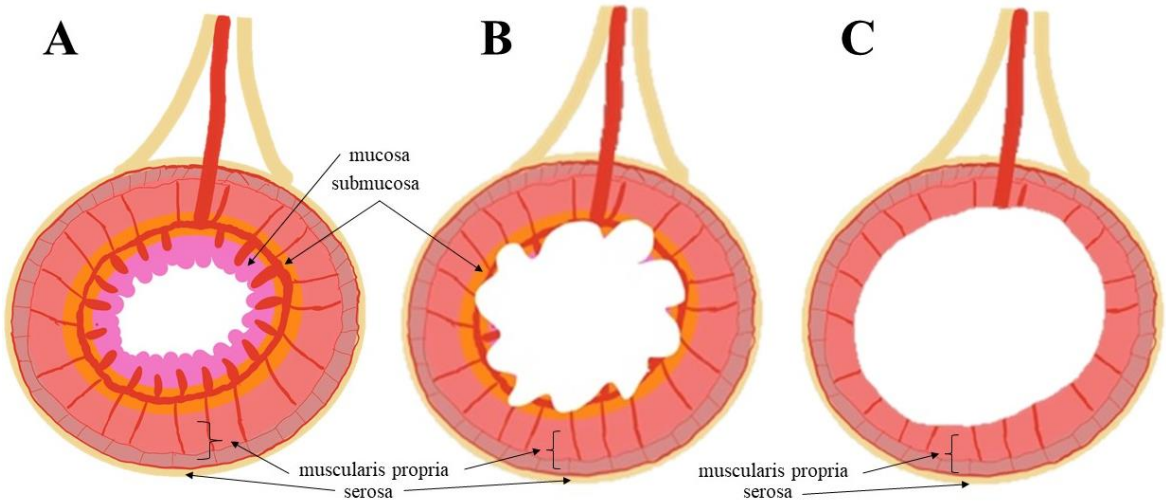


Figure 4 *Schematic diagram of intramural circulation after mucosectomy.* **A***: arterial blood supply of the intact intestine;* **B:** *blood supply after ESD;* **C:** *blood supply after total removal of submucosa. Note after mucosectomy the remaining sero-muscular layers receive blood supply only directly from the thin branches of the vasa recta*

The submucosal plexus: The long vasa recta penetrate the muscle layer and create the submucous plexus in the loose connective tissue (Figure 3, Figure 4). Two direction is optional for outgoing vessels, the centrifugal and the centripetal. Mucosal plexus consists of centripetal branches from submucosal plexus [31]. Gallavan et al. observed the main role of submucosal plexus *in vivo* dog models, where the arterial pO_2, pCO_2, and pH were measured during prandial phase [32]. The results indicated that the mucosal layer via this plexus obtains approx. 75% of the whole intramural blood flow, while muscle layer receives only 25% [32,33].

The mucosal plexus: The formation of mucosal arterioles shows difference between the colon and small intestine, this diversity originated from their physiological function. In small intestine can be found the 'Fountain-like' arrangement, where the arterioles without branches reached the top of villi, additionally 'step ladder' and 'tuft type' presented with deep interconnections [31,33]. However, in the colon this classification cannot be applied, lower capillary density was detected in this area, which shows relationship with excessive water reabsorption [31].

1.2.3 The correlation between ischemia and fibrosis, stricture in the GI system

There is colloquial evidence for close relationship between ischemia, fibrosis and contraction [34–37]. An example for systemic hypoxia induced intestinal stricture is necrotising enterocolitis (NEC) in premature neonates, where the link between NEC and intestinal stricture is well established [38]. Almost 30 % of NEC patients develop intestinal stricture in the long run [39]. Posttraumatic ischemic stenosis of the small bowel is rare condition, but a good example for local ischemia. After blunt trauma mesenteric defect causes ischemia of the corresponding bowel and result in stenotic, fibrotised and strictured bowel segment [40–42]. Another proof for local ischemia is when the superior rectal artery is divided below Sudeck's point during sigmoidectomy, this has been reported to cause ischemic stricture [43].

1.3 In-vivo imaging of tissue microcirculation

There are several techniques available for study tissue microcirculation in-vivo in real time (Figure 5). Intact microvascular flow is recognized as a primary factor in the healing of GI anastomoses; there is therefore a high demand for reliable, easy-to-use intra- or postoperative diagnosis. Not surprisingly, a wide range of clinically available methods have been developed and tested with variable success. In Figure 5 the currently available microcirculation measurement techniques is demonstrated.

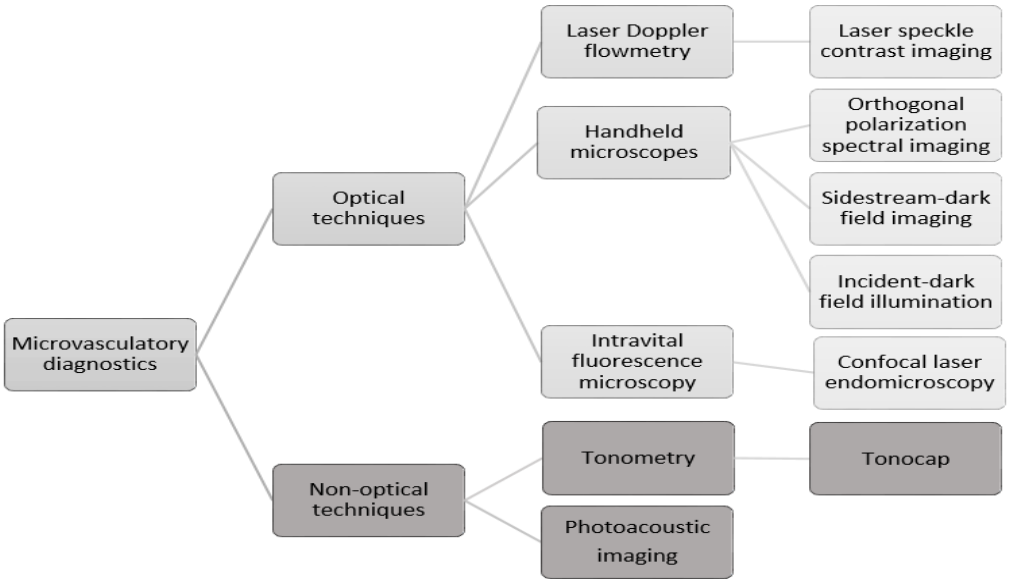


Figure 5 *Groups of diagnostic tools by microcirculation measurement technique*
Source: Urbán D, Cserni T, Boros M, Juhász Á, Érces D, Varga G. Bladder augmentation from an insider's perspective: a review of the literature on microcirculatory studies. Int Urol Nephrol. 2021;53:2221-2230.

Devices with laser Doppler flowmetry (LDF) can measure red blood cell velocities endoscopically in the mucosal microcirculation [44]. Simple and non-invasive, LDF is capable of real-time detection; however, it uses arbitrary units like the majority of other optical techniques, and zero-point calibration is missing. The evaluation requires experience because of the heterogeneity and overlay of vessels [44,45]. It is therefore not recommended as a first choice in intraoperative measurements. Laser speckle contrast imaging (LSCI) also provides arbitrary units and colour-coded images [44]. Although it is more sensitive than LDF and has fewer artefacts, the clinical feasibility of LSCI has not been confirmed, because clinical interpretation of its arbitrary units is difficult and it could be misleading.

Fluorescence imaging (FI) has gained renewed popularity in recent years. Here a laser beam illuminates the tissue, where a fluorescent molecule is activated [44]. The quality of perfusion is usually assessed with indocyanine green (ICG), and this technique may predict the potential of anastomotic necrosis intraoperatively. However, quantitative parameters have not yet been described [44]. Moreover, images taken with this technique could be overestimated because ICG molecules can infiltrate the congested tissue to some extent simply by diffusion from the healthy zone [46].

Confocal laser endomicroscopy (CLE) is based on the principles of intravital fluorescence microscopy combined with optical fibres. With the added value of confocality, fluorescence imaging can evaluate functional capillary density, blood cell velocity, changes in permeability, vasoconstriction and dilation [47]. Confocal images can provide information not just on the microcirculation, but also on the morphology of intestinal villi and crypts [48,49]. Fluorescein isothiocyanate–dextran enables us to see the capillary system, while acridine orange dye shows epithelial loss, which may be crucial to determining viability at the resection line. Moreover, CLE is successfully used for an "optical biopsy" to conduct a morphological analysis for ulcerative colitis and removal of tumour tissue [50–53]. In fact, CLE might be a reliable asset for diagnostics, with limited invasiveness, ease of use and preservation of the physiology of the organ being great advantages.

Tonometry has changed over time. Modern devices with infrared sensors, calibrations and automatically sampled gases can detect the partial pressure of carbon dioxide (pCO_2) and calculate intramucosal pH. Studies have confirmed the predictive potential of mucosal pCO_2 in septic and critically ill patients and intra- and postoperative monitoring [54,55]. Indeed, the levels of mucosal oxygen and CO_2 saturation are clinically relevant parameters of microcirculation in humans [56,57]. Mucosal pCO_2 detection is appropriate for intraoperative control of microcirculation and capable of continuous postoperative monitoring after esophagectomy or other surgery [55]. Easy clinical application and the possibility for long-term monitoring during the postoperative period in intensive care units also represent advantages.

Orthogonal polarisation spectral (OPS) imaging was used by the first generation of handheld microscopes, which were able to directly visualise human microcirculation without contrast materials [58]. It is still appropriate for intraoperative measurement, but image stabilisation is difficult. Sidestream dark field imaging (SDF) illuminates the tissue with green LED light with higher resolution, but it still requires direct contact with the tissue [59]. In addition, movements, such as heartbeat, compression and breathing, may distort results [44]. Incident-dark field illumination (IDF) was the next generation of handheld devices; it was completely digital and showed a significantly higher rate of total vessel density [60]. Moreover, it has a shortened pulse time, a digital stabiliser and reduced weight. In brief, OPS, SDF and IDF can all measure the important parameters of microvascular flow, including total vessel density, the proportion of perfused vessels and the flow heterogeneity index, as recommended by a roundtable consensus meeting on this subject [61].

In our Institute there is a significant experience with hand held microscopes and they routinely used. Previously OPS was utilised, which was reliable to analyse microcirculation. In Study II Cytocam Braedius was applied (Figure 6), which is the successor of the OPS and based on IDF technology (Figure 6). This device is able to describe tissue microcirculation with quantitative parameters, however the original software of Cytocam could not calculate red blood cell velocity (RBCV), therefore we had to develop a new software for this purpose. The CapScan is an image analysing software, which is capable of exportation of analysing metrics of capillary. The main source of analysing is a video file, which is presented in a video viewer for the user (Figure 7). In the viewer, it is able to stop, or analyse the video per frame. In the software image stabilizer algorithm is integrated. At first the user assigns the vessel which will be analysed, then the user marks the width of the capillary. In vectorial top view of vessels there are several cross sections, thus the software removes these cross section from the marked capillary and it presents the plan of the examined vessels only.

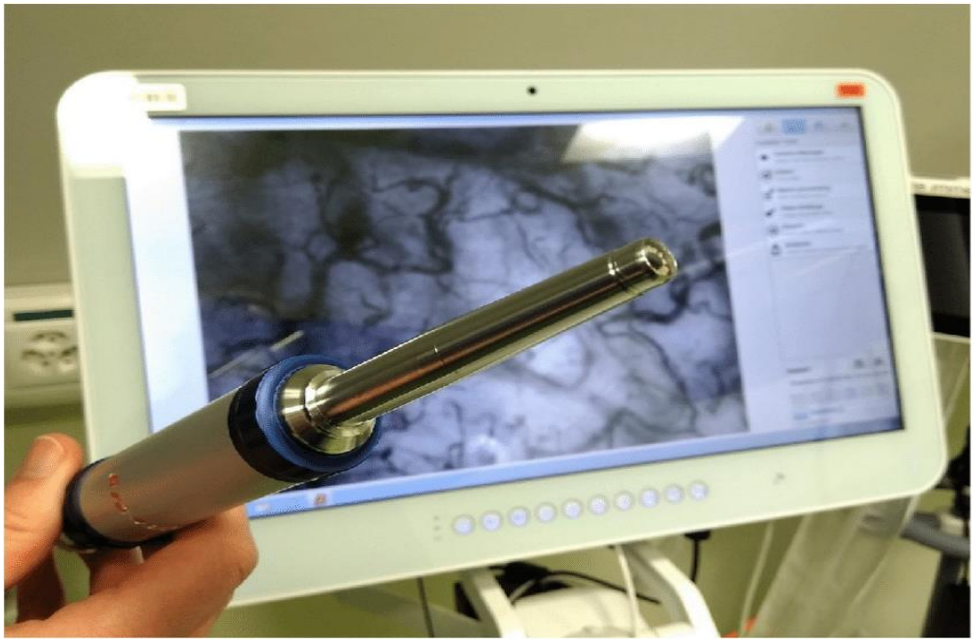


Figure 6 *Cytocam Braedius, the handheld microscope unit and the attached monitor showing the capillaries*

To define the red blood cell velocity in another window the identified vessel is presented, where the movement of red blood cells could be marked frame by frame by the user. If on the following frame the location of the red blood cell does not marked, the program ignores it. The program registrates the pixel location of the user given points, then it projects this data to the original video. After all it calculates the red blood cell velocity from the collected data.

Usage of Cytocam is relatively straightforward, but direct connection with tissue sample is required, moreover the hand oscillation and compression generated artefacts decrease the efficiency of analysing. To solve this issue special device holder was used. Another disadvantage of this instrument that there is no laparoscopic version, thus clinical application during minimally invasive surgery is not resolved. A more recent technology in the preclinical phase is photoacoustic imaging (PAI). Pulses of non-ionising laser light are absorbed by tissues, and the heat, which is caused by energy absorption, results in a thermoelastic expansion [62]. The accompanying sound effect can be detected by high-sensitivity ultrasonic transducers. Haemoglobin- and melanin-containing tissues can be effectively examined with this method. The main limitations of this technique are that a handheld version is currently unavailable and greater penetration depths are necessary. [63].

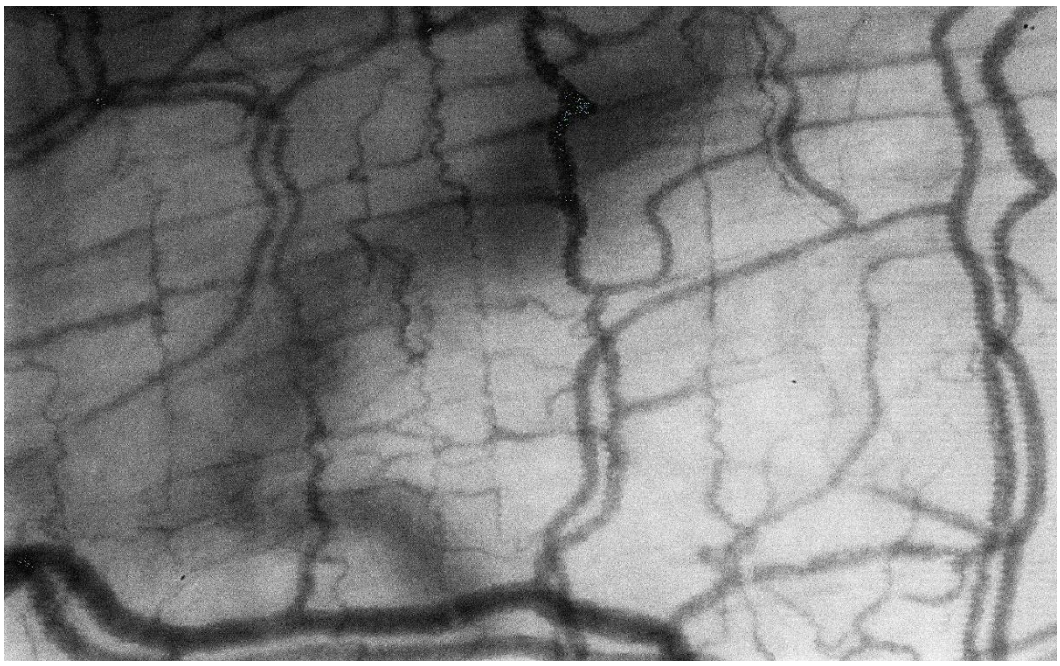


Figure 7 *Snapshot of serosal capillaries and microcirculation with Cytocam Braedius handheld microscope*

1.4 Mucosectomy and the ENS

1.4.1 The ENS

An intact ENS is essential to maintain the intestinal integrity and function. ENS is an organised mesh of ganglionated plexuses (Figure 8). The two main parts of ENS are the myenteric and submucosal plexuses [64]. The myenteric plexus is built up by nerve strands and small ganglia which are localised between the circumferential and longitudinal muscle layers (Figure 9) as a ring along the whole GI tract. It communicates with central nervous and

autonomic nervous system, moreover it interacts with deep submucosal plexus too [64]. The motoneurons primarily located in the myenteric plexus innervates muscularis propria thereby it is responsible for peristaltic movement. The fine balance of the excitatory and inhibitory neural circuits responsible for normal muscle tone and motility does not exists if the ENS is not fully developed and intact. Increased baseline muscle tone is observed in HD, where the myenteric plexus is under-developed and inhibitory motoneurons are missing. Therefore, the muscle layer is unable to relax, which is the diagnostic feature of HD in anorectal manometry. Increased AchE-positivity is a diagnostic histological feature of HD, which comes from the hypertrophied AchE-positive uninhibited excitatory nerve endings [65]. Similarly, decreased peptidergic and nitrergic innervation (inhibitory motoneurons) has been hypothesized as an etiology for intrinsic pelvico-ureteric junction obstruction because of failure to relax to stimuli (urine bolus), and it is at least suspected as an etiology of hypertrophic pyloric stenosis [66,67].

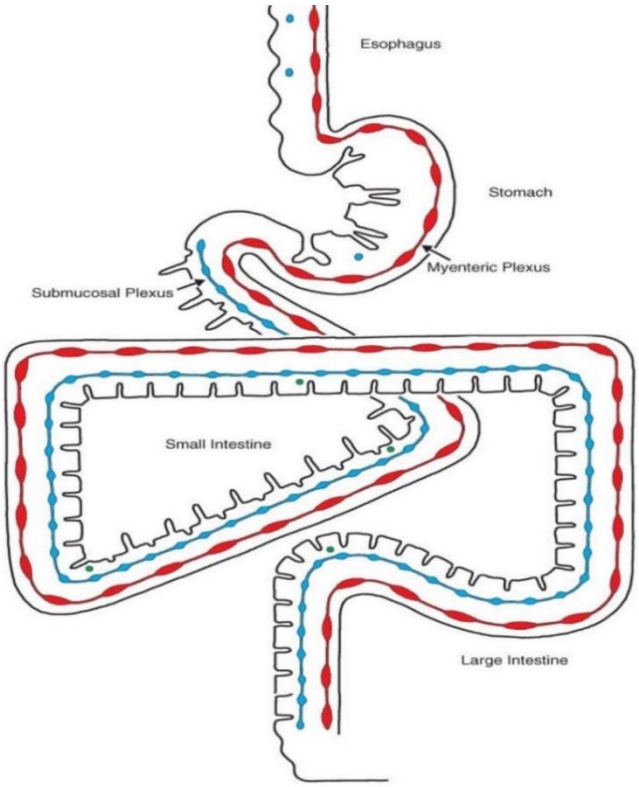


Figure 8 *Distribution of enteric ganglia in whole GI tract. The myenteric ganglia form a continuous plexus from the upper esophagus to the internal anal sphincter (red), and the submucosal plexus (blue), which is organised in the small and large intestines. Isolated ganglia occur in the gastric and esophageal submucosa and in the mucosa throughout the digestive tract.'*

Source: Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012;9:286–94. https://doi.org/10.1038/nrgastro.2012.32.

Submucosal plexus can be found in small and large intestine (Figure 8). In the stomach and the esophagus scattered ganglia can be detected instead of organised plexus (Figure 8). The Submucosal plexus can be divided for inner and outer plexuses. Submucosal plexus mostly has sensory function, however there is a significant structural difference in the ENS of small rodents and large mammals. While in motor neurons of small rodent are located only in the myenteric plexus, in larger mammals the submucosal plexus also contains nitrergic inhibitory motor neurons in inner submucosal plexus, which are responsible for muscle relaxation [64,68]. This may explain why researchers did not see flap contraction after mucosectomy in rodents with sero-muscular bladder augmentation while others observed this in large animals like dogs [25–28].

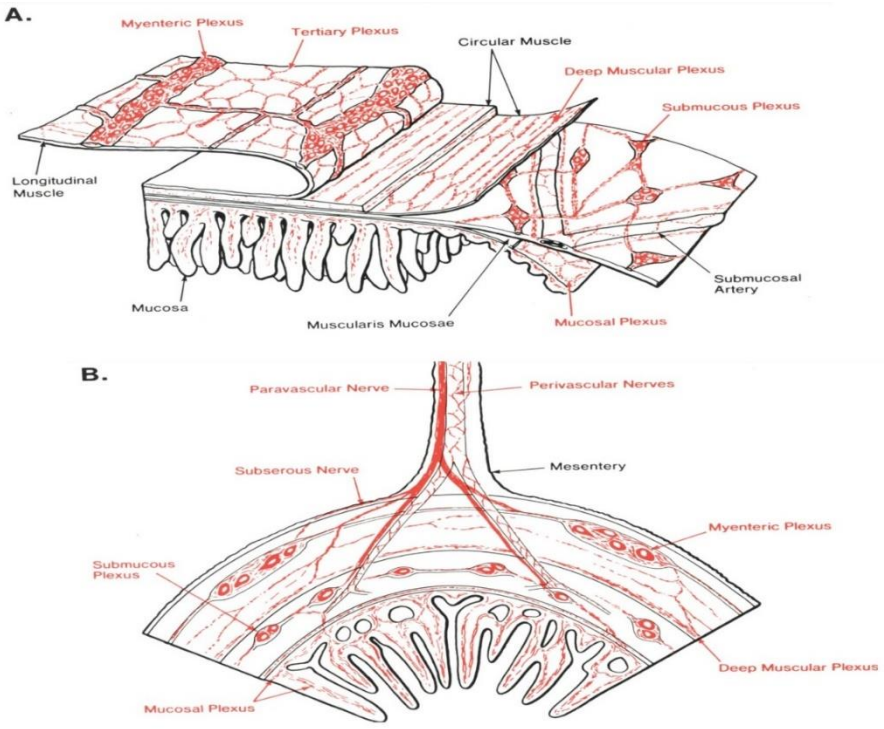


Figure 9 *Localisation of myenteric and submucosal plexuses in the intestinal wall.* ***A:*** *Myenteric and submucosal plexuses between the layer of intestinal wall. B: Cross section of the intestinal wall with ENS plexuses* [69].

Source: Furness JB, Costa M. Types of nerves in the entereic nervous system. Commentaries in the Neurosciences, Pergamon; 1980, p. 235–52. https://doi.org/10.1016/B978-0-08-025501-9.50016-8.

Since the ENS is an interconnected structure and its integrity is important to maintain muscle tone and in large mammals submucosal plexus clearly contains motoneurons it is highly likely that mucosectomy, ESD or removal of submucosal layer will destroy these structures, however this has not been demonstrated (Figure 10).

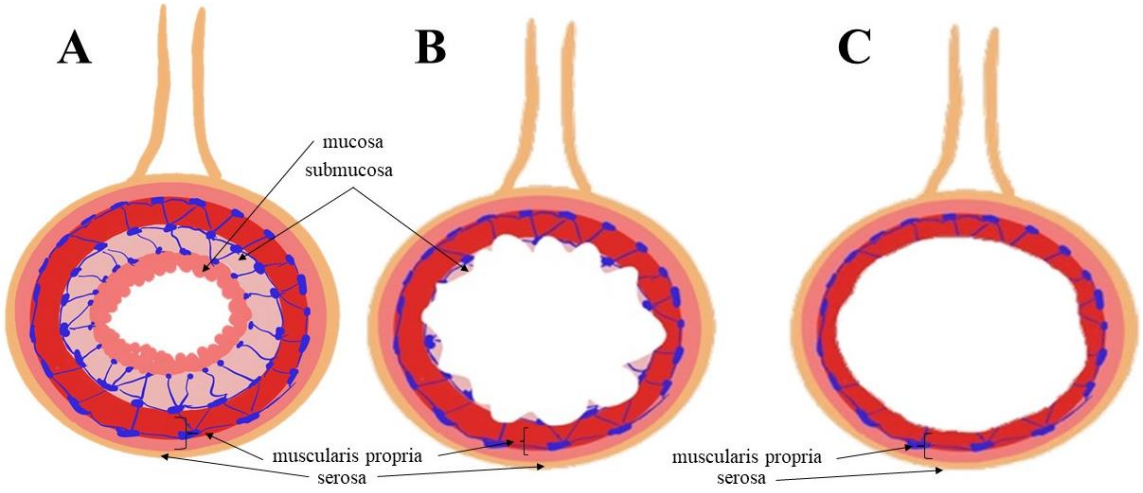


Figure 10 *Schematic diagram of ENS.* **A***: plexuses of the intact intestine;* **B:** *ENS after ESD;* ***C:*** *ENS after total removal of submucosa. Note: after mucosectomy the interconnected ganglia are disconnected*

1.4.2 Techniques to study the ENS

The traditional pathological techniques, -paraffin embedded tissue block and frozen section, - able to analyse plexuses only in two dimensions. The whole-mount technique opened a new way to examine the spatial location of plexuses in three dimension (Figure 11) [70].

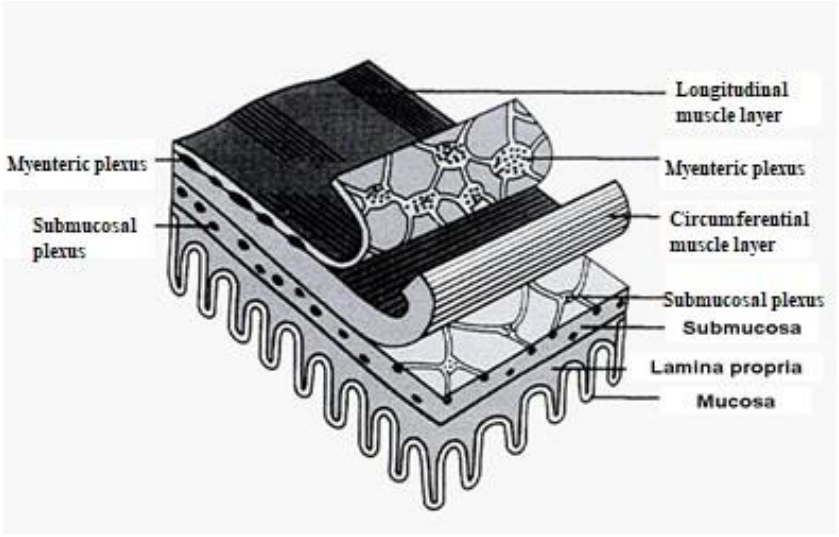


Figure 11 *Schematic diagram of whole-mount preparation. Separation of tissue layers reveals the spatial localisation of plexuses.*
Source: Cserni T. Experimental and Clinical Data to the Surgery of the Ileocaecal Junction in Childhood 2007.
http://webpac.lib.unideb.hu:8082/WebPac/CorvinaWeb?action=cclfind&resultview=long&cclltext=idno bibDEK00625982.

Briefly summarizing the procedure, the rest of mesentery/mesocolon and serosa could be removed from the specimen with dissection microscope [70]. With fine forceps the submucosal layer can be separated from muscle layer (Figure 12). Thereafter the circular muscle fibres can be peeled off one by one from the longitudinal muscle (Figure 13) [70].

Figure 12 *Whole-mount preparation. Removal of submucosa with dissection microscope. The submucosa is peeled off, and the circular muscular fibres can be visualized.*
Source: Cserni T. Experimental and Clinical Data to the Surgery of the Ileocaecal Junction in Childhood 2007.
http://webpac.lib.unideb.hu:8082/WebPac/CorvinaWeb?action=cclfind&resultview=long&ccltext=idno bibDEK00625982.

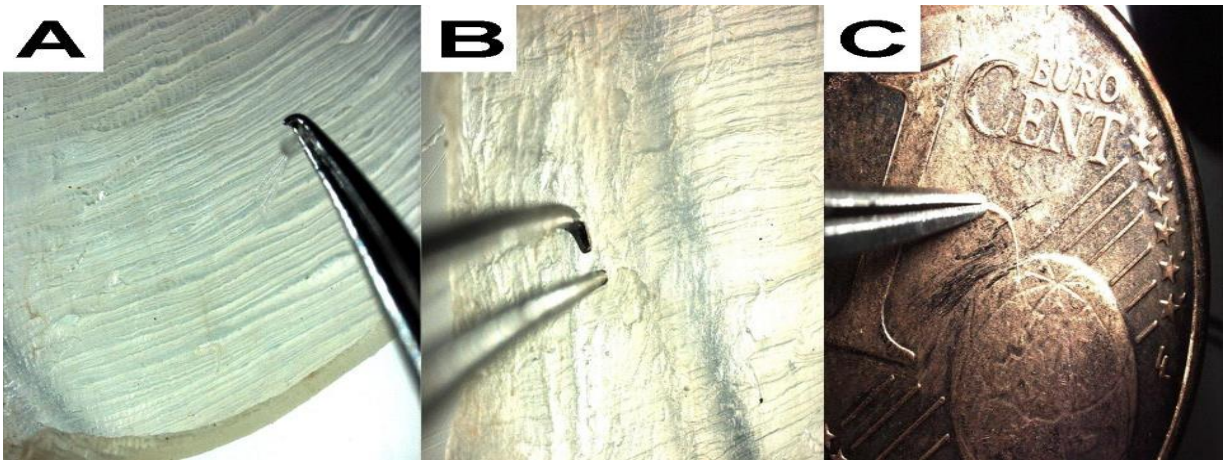


Figure 13 *Steps of whole-mount preparation, muscle fibres removal.* ***A:*** *Peel off the circular muscle fibre* ***B:*** *The longitudinal muscle layer can be visualized after removal of circular muscle fibres* ***C:*** *Size of A circular muscle fibre*
Source: Cserni T. Experimental and Clinical Data to the Surgery of the Ileocaecal Junction in Childhood 2007.
http://webpac.lib.unideb.hu:8082/WebPac/CorvinaWeb?action=cclfind&resultview=long&ccltext=idno bibDEK00625982.

Reduction of longitudinal muscle fibres is beneficial in voluminous specimen, to improve of visibility of plexuses, however extensive removal of longitudinal fibres may cause damage of plexuses [70]. To increase efficiency of whole-mount preparation and mitigate of potential damage, visualization of plexuses with NADPH-d dyeing is considerable, because the native plexuses are not visible with microscope [70].

2. MAIN GOALS

Our goal was to study and understand the effect of surgical mucosectomy on the intestine to explain the background and origin of the major complication, stricture and contraction seen with the mucosectomised flaps.

1. We aimed to assess the effect of surgical mucosectomy on the microcirculation of the colon and the ileum in the same experimental setting and monitor intramural microcirculation for possible recovery beyond the known warm ischemia time.
2. We aimed to evaluate the effect of mucosectomy on the dimension of the intestinal flaps and assess the changes in the anatomy of the ENS.

3. MATERIAL AND METHODS

3.1 Experimental animals and anaesthesia in Study I and II.

13 female Vietnamese minipigs (weighing 45±8 kg) were used (n=5 in Study I, n=8 in Study II). The animal experiments were performed according to EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and carried out in strict adherence to the NIH guidelines for the use of experimental animals. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation, with the license number V/1637/2013 and V/148/2013.

The animals were kept under conventional circumstances, in standard cages. They were fed with commercially available mixed food, fasted 24 hrs before surgery and always had free access to water. Anaesthesia was induced with an intramuscular injection of a mixture of ketamine (20 mg/kg) and xylazine (2 mg/kg) and maintained with a continuous infusion of propofol (2%; 50 μl/kg/min i.v.), via a cannulated ear vein. Endotracheal tube was inserted, and the animals were ventilated mechanically, with a volume-controlled ventilator. The tidal volume was set at 8-9 ml/kg, and the respiratory rate was adjusted to maintain the end-tidal carbon dioxide pressure (EtCO2) to 35-45 mmHg. Norocarp S (carprofen; 4 mg/kg) and normal saline infusion were administered via the ear vein catheter. Heart rate, O_2 saturation (pulse

oxymetry), $EtCO_2$ (capnometry) and body temperature were continuously monitored perioperatively.

3.2 Protocol in Study I

3.2.1 Surgical procedure

First, the ileal and colonic segments were isolated and detubularised along the anti-mesenteric line. The bowel was kept warm with 0.9% saline solution. The detubularised bowel strips were placed on wet gauze. The mesenteric pedicle was not clamped. The width and the microcirculation were recorded on the serosal and mucosal surfaces. Mucosectomy was then performed. In the sero-musculo-submucosal group (Group A), only the mucosa was scraped off with the back of a forceps from the bowel at the level of the mucosal lamina propria (Figure 14 A). Surgical loops with 2.5 X magnification were used to make sure that no mucosal islands are left behind. In the seromuscular group (Group B), the mucosa and the submucosa were peeled off the seromuscular layer, as one layer and in one piece (Figure 14 B). It was easy to separate the submucosa from the muscular layers at one corner of the flaps with a fine pair of forceps. Injection of saline into the submucosa was not necessary. The microcirculation was rerecorded with IDF side stream videomicroscopy (Cytoscan Braedius, The Netherlands), in each group, after the mucosectomy procedure, on the serosal and the raw surface, as well, at 0, 5, 15, 30, 60, 90, 120, 180 minutes.

3.2.2 Quantifying microcirculation

Microcirculatory videos were performed with IDF-imaging device (Cytocam, Braedius medical, Huizen, Netherlands), in accordance with international standard recommendations [61]. The camera is fully digital and contains a high-resolution sensor, with a pixel size of 1.4 micron. Video files were saved directly to the Braedius CytoCam HDD in AVI format. Optical magnification of 4x was used, to provide a 1.55 x 1.16 mm field of view.

The Microvascular flow index is a semi-quantitative and summarized score, which is the mean value of four quadrant measurements. Categorical values are given based on predominant flow (no flow = 0, intermittent = 1, sluggish = 2, continuous = 3). Red blood cell velocity (RBCV) being a quantitative marker, which was measured using the CapScan software, for this purpose, programmed by our team. RBCV is presented in meter/second (μm/s).

3.3 Protocol in Study II

3.3.1 Surgical procedure

In each animal, 3 five-cm-long ileum and colonic segments were isolated and detubularized along the mesenteric line, one of those three segments represented the control group, and one represented each of the experimental groups (Group I and II). Similarly, three 5x5 cm large segments were created in each animal from the stomach at the greater curvature preserving the right gastroepiploic artery. The small intestine was kept warm with 0.9% saline solution. The detubularized bowel and stomach flaps were placed on wet gauze and the width was measured with linear ruler under no tension.

The mucosectomy was performed in 2 groups (a segment from each animal was allocated to each group). In Group I (sero-musculo-submucosal flap group), only the mucosa was scraped off with the back of the forceps from all segments at the level of lamina propria mucosae (Figure 14 A). In Group II (sero-muscular flap group), the submucosa was separated from the sero-muscular layer at the edge of the flap with fine forceps and peeled away from the sero-muscular layer in one piece (Figure 14 B). The mucosectomised flaps of both groups than were placed on wet gauze and measured again with a linear ruler with no tension.

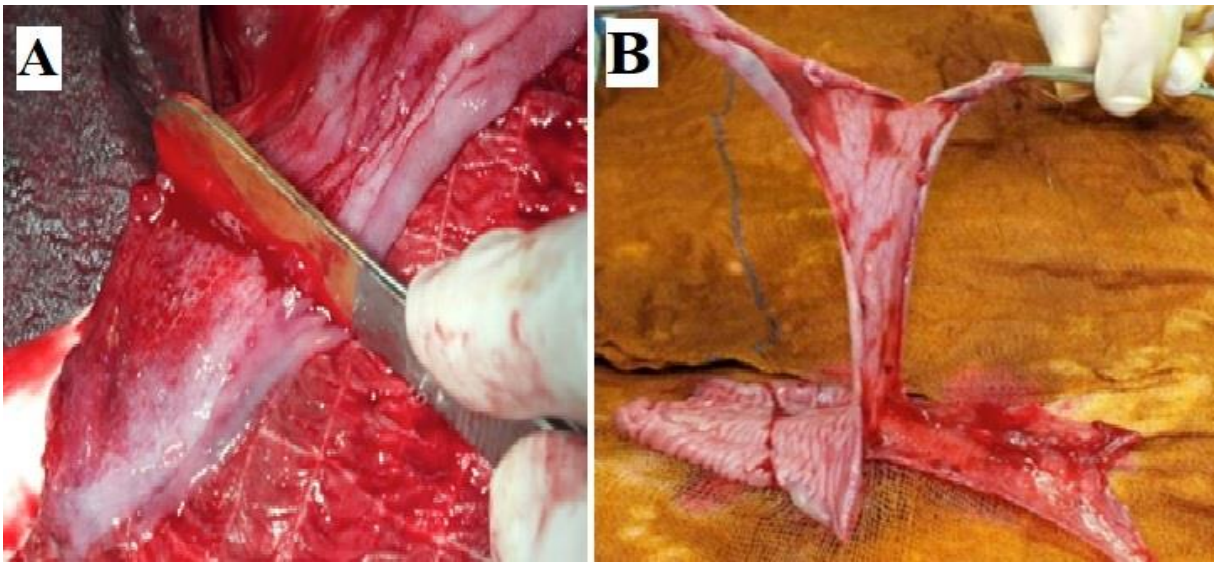


Figure 14 Surgical removal of mucosa. **A:** *Group I-Mucosectomy, mucosa is removed with the back of the forceps.* ***B:*** *Group II-Seromuscular Flap, the mucosa and submucosa is peeled off from the sero-muscular flap (with Tamás Cserni's permission)*

3.3.2 Histological procedures

3.3.2.1 Haematoxylin and eosin (HE) and immunohistochemistry staining

In Group I. histology samples were taken from the sero-musculo-submucosal flaps. In Group II. the specimens were taken from the sero-muscular layer and the mucosa-submucosa layer which was peeled away. After fixation in 4% formalin, the specimens were embedded in paraffin and staining was done with conventional HE and neurofilament (NF) immunohistochemistry with monoclonal mouse anti-human neurofilament protein clone 2F11, Code Nr. M0762 (DakoCytomation). For standard NADPH-diaphorase (NADPH-d) and acetylcholine esterase (AchE) enzyme histochemistry, 1x1 cm specimens were excised from the mucosa-submucosal layer, which was pealed way from the sero-muscular layer. The whole mount preparations were incubated in 1 mg/mL β-NADPH (Sigma), 0.25 mg/ml nitro blue tetrazolium and 0.3% Triton-X in 0.05 mol/l Tris-HCL buffer (pH 7.6) at 37 ºC for 2 hours and then left in staining solution at room temperature. After the desired staining intensity was achieved, the specimens were washed in PBS for 15 minutes and processed by the standard AchE histochemistry. The specimens were placed in 10 mL of an incubating solution (65 mM sodium acetate buffer, pH 6.0, 1.7 mM acetylthiocholine iodide, 5 mM sodium citrate, 3 mM cupric sulfate and 0.5 mM potassium ferricyanide) for 100 minutes at 37 ºC. Tissues were then rinsed twice in 0.1 M Tris buffer (pH 7.6) and mounted on Polysine slides (BDH) using Glycergel mounting medium (DakoCytomation). The slides were examined with an Olympus BX 63 light microscope and they were scanned with a 3DHistech Panoramic MIDI II device. The animals were sacrificed after the procedure. The specimens were compared and contrasted to each other.

3.4 Statistical analysis

3.4.1 Study I

Data analysis was performed with a statistical software package (Sigmaplot 13.0.0/2017 for Windows by Systat Software Inc., Jandel Scientific, Erkrath, Germany, 2017). Normality of data distribution was analysed with the Shapiro–Wilk test. The Friedman ANOVA on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed with Dunn’s method. Differences of extra- and intraluminal side within the groups (Group A-I, Group B-I, Group A-C and Group B-C) were analysed with the Mann-Whitney

test. Median values and 75th and 25th percentiles are provided in the figures; $p<0.05$ were considered significant.

3.4.2 Study II

Data analysis was performed with a statistical software (Sigmaplot 13.0.0/2017 for Windows by Systat Software Inc.). To find the differences between the sero-musculo-submucosal, the sero-muscular and the control group, we used Saphiro-Wilk normality test, one and two-way ANOVA. P values < 0.05 was considered significant.

4. RESULTS

4.1 Study I

Both the MFI and RBCV showed an abrupt reduction of microcirculation on both surfaces of the remaining intestinal flap in both, the ileum and the colon. Slightly better values were seen on the colon (Group A-C), but even these values remain far below the preoperative (control) results. The values on the raw surface of the bowel flaps was slightly higher than that measured on the serosal surface. Some recovery of the microcirculation was noted after 60-90 minutes, but this remained significantly lower than the preoperative control values. The results are summarized in Figures 15 and 16.

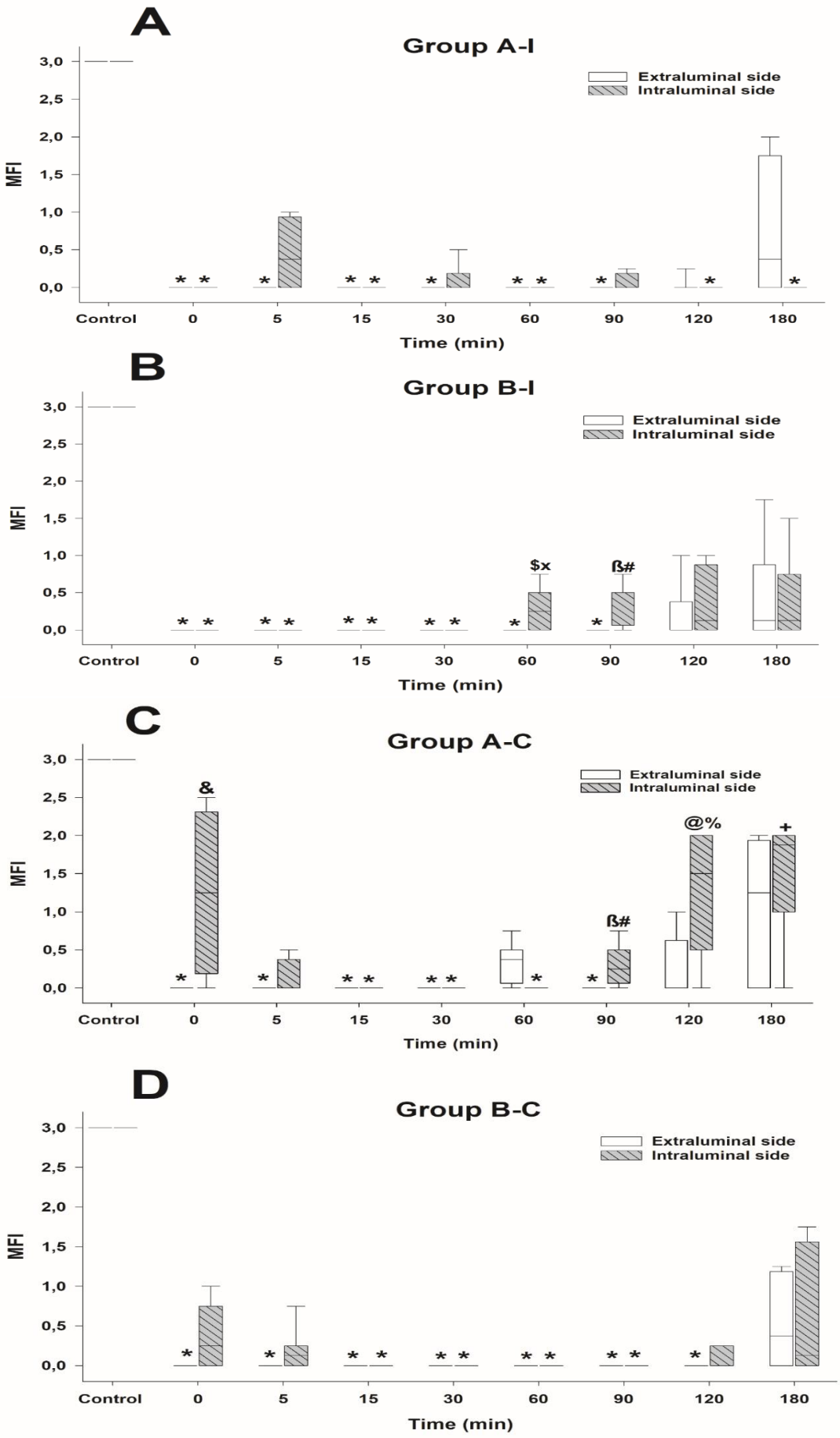
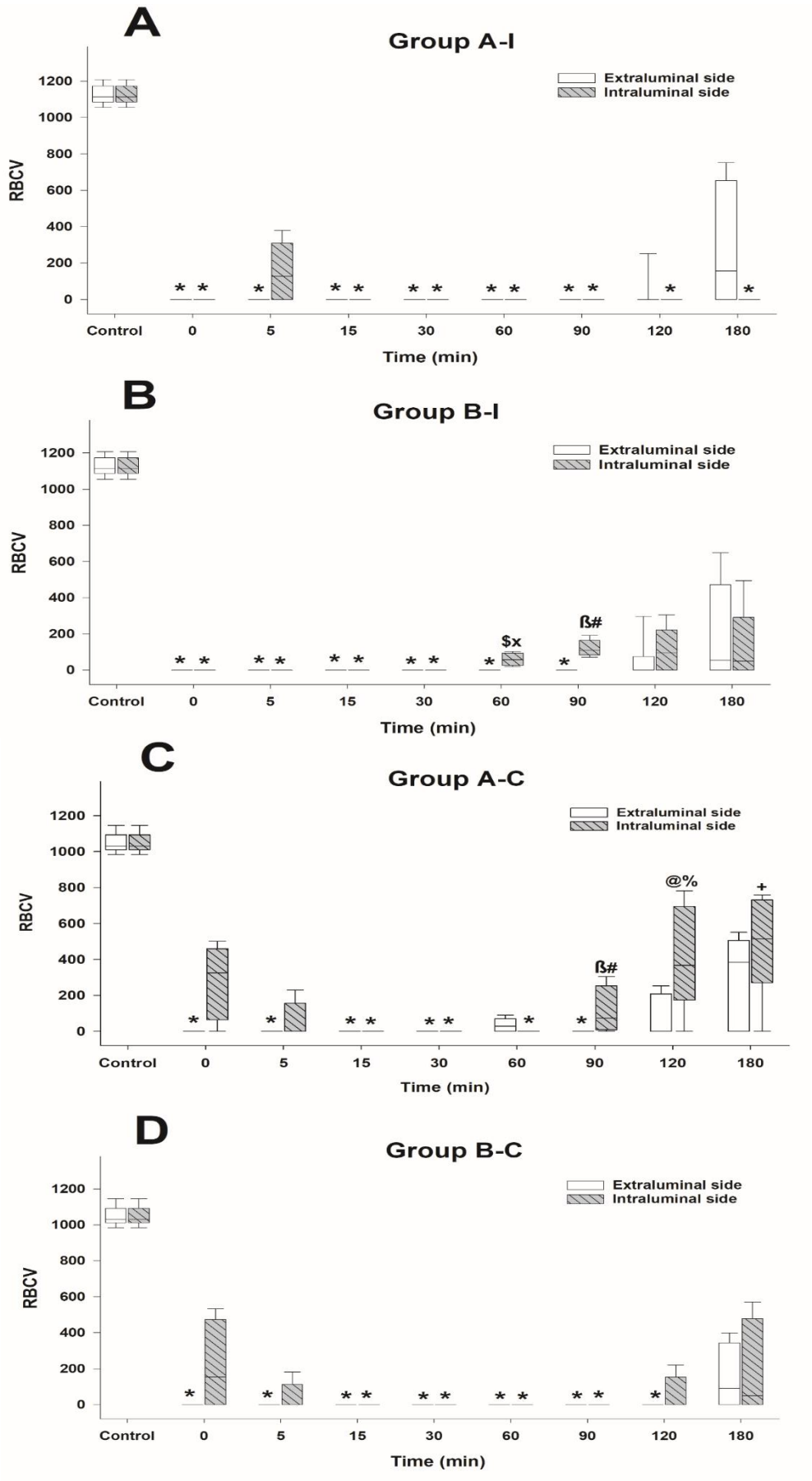

A
Group A-I
Extraluminal side
Intraluminal side
MFI
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Control
0
5
15
30
60
90
120
180
Time (min)
B
Group B-I
Extraluminal side
Intraluminal side
$x
ß#
C
Group A-C
Extraluminal side
Intraluminal side
&
@%
+
ß#
D
Group B-C
Extraluminal side
Intraluminal side

Figure 15 *Microvascular Flow Index **A:** Group A-I (ileal sero-musculo-submucosal group) box plot diagram demonstrates results of the extraluminal side with white and the intraluminal side with white and line pattern; **B:** Group B-I (ileal seromuscular group) box plot diagram demonstrates results of the extraluminal side with white and the intraluminal side with white and line pattern; **C:** Group A-C box plot diagram demonstrates results of the extraluminal side with grey and the intraluminal side with grey and line pattern; **D:** Group B-C box plot diagram demonstrates results of the extraluminal side with grey and the intraluminal side with grey and line pattern.*

All of the plots demonstrate the median values (horizontal line in box plots) and the 25th (lower whisker) and 75th (upper whisker) percentiles, ($p<0.05$).

** $p<0.05$ vs. baseline values. & $p<0.05$ Group A-C extra- vs. intraluminal side 0 min; $ $p<0.05$ Group A-C extra- vs. intraluminal side 60 min; # $p<0.05$ Group A-C extra- vs. intraluminal side 90 min; @ $p<0.05$ Group A-C extra- vs. intraluminal side 120 min; x $p<0.05$ Group A-C vs. Group B-C extraluminal side 60 min; ß $p<0.05$ Group A-C vs. Group B-C intraluminal side 90 min; % $p<0.05$ Group A-C vs. Group B-C intraluminal side 120 min; + $p<0.05$ Group A-C vs. Group B-C intraluminal side 180 min; $ $p<0.05$ Group B-I extra- vs. intraluminal side 60 min; # $p<0.05$ Group B-I extra- vs. intraluminal side 90 min; x $p<0.05$ Group A-I vs. Group B-I intraluminal side 60 min; ß $p<0.05$ Group A-I vs. Group B-I intraluminal side 90 min.*

Source: Urbán D, Varga G, Érces D, Marei M, Cervellione RM, Keene D, Goyal A, Cserni T. Prolonged ischemia of the ileum and colon after surgical mucosectomy explains contraction and failure of "Mucus free" bladder augmentation.


A
Group A-I
Extraluminal side
Intraluminal side
RBCV
1200
1000
800
600
400
200
0
Control
0
5
15
30
60
90
120
180
Time (min)
B
Group B-I
Extraluminal side
Intraluminal side
RBCV
$x
ß#
Control
0
5
15
30
60
90
120
180
Time (min)
C
Group A-C
Extraluminal side
Intraluminal side
RBCV
ß#
@%
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Control
0
5
15
30
60
90
120
180
Time (min)
D
Group B-C
Extraluminal side
Intraluminal side
RBCV
Control
0
5
15
30
60
90
120
180
Time (min)

Figure 16 *Red Blood Cell Velocity* ***A:*** *Group A-I (ileal sero-musculo-submucosal group) box plot diagram demonstrates results of the extraluminal side with white and the intraluminal side with white and line pattern;* ***B:*** *Group B-I (ileal seromuscular group) box plot diagram demonstrates results of the extraluminal side with white and the intraluminal side with white and line pattern;* ***C:*** *Group A-C box plot diagram demonstrates results of the extraluminal side with grey and the intraluminal side with grey and line pattern;* ***D:*** *Group B-C box plot diagram demonstrates results of the extraluminal side with grey and the intraluminal side with grey and line pattern.*

All of the plots demonstrate the median values (horizontal line in box plots) and the 25th (lower whisker) and 75th (upper whisker) percentiles, ($p<0.05$).

** $p<0.05$ vs. baseline values; # $p<0.05$ Group A-C extra- vs. intraluminal side 90 min; @ $p<0.05$ Group A-C extra- vs. intraluminal side 120 min; ß $p<0.05$ Group A-C vs. Group B-C intraluminal side 90 min; % $p<0.05$ Group A-C vs. Group B-C intraluminal side 120 min; + $p<0.05$ Group A-C vs. Group B-C intraluminal side 180 min. * $p<0.05$ vs. baseline values $ $p<0.05$ Group B-I extra- vs. intraluminal side 60 min; # $p<0.05$ Group B-I extra- vs. intraluminal side 90 min; x $p<0.05$ Group A-I vs. Group B-I intraluminal side 60 min; ß $p<0.05$ Group A-I vs. Group B-I intraluminal side 90 min.*

Source: Urbán D, Varga G, Érces D, Marei M, Cervellione RM, Keene D, Goyal A, Cserni T. Prolonged ischemia of the ileum and colon after surgical mucosectomy explains contraction and failure of "Mucus free" bladder augmentation.

4.2 Study II

There was no significant difference in the original size of the segments from the stomach, ileum and colon, measured across the different animals (n=5). The stomach contracted to a lesser extent of its original width, 92.82 ± 7.86% in Group (I) and 82.24 ± 6.96% in Group (II). The ileum contracted to 81.68 ± 4.25% in Group (I) and to 72.675 ± 5.36% in Group (II). The flap shrinkage was most significant in the colon, down to 83.89 ± 15.73% in Group (I) and to 57.13 ± 11.51% in Group (II). One-way ANOVA test showed a significant difference ($p < 0.05$) for each segment between Group (I) and (II), comparing the stomach to the ileum and the ileum to the colon (Figure 17). The shrinkage of ileal flap is demonstrated in Figure 16.

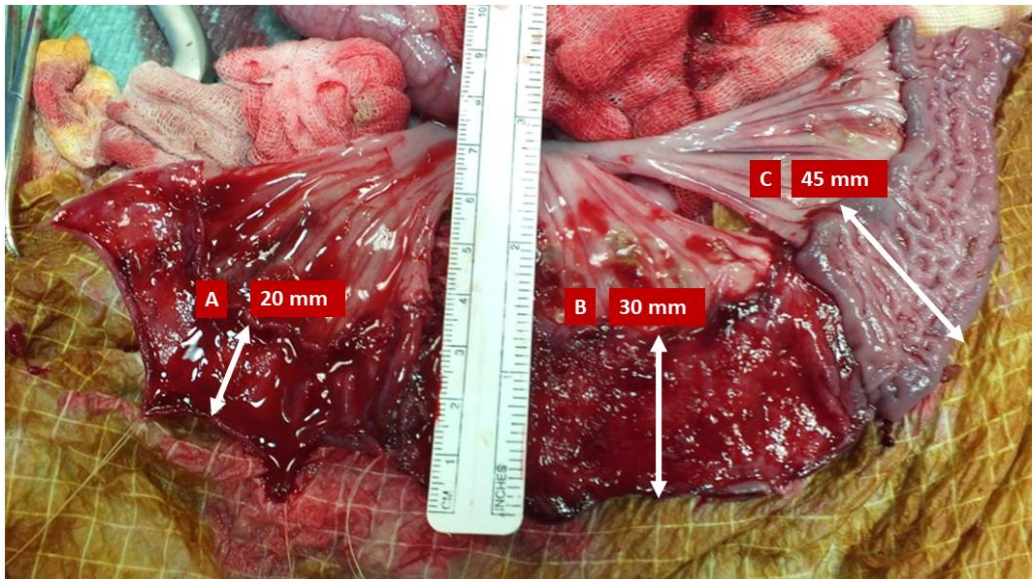

Figure 16 *Shrinkage of ileal segments* **A:** *Group II-Seromuscular Flap* **B:** *Group I-Mucosectomy* **C:** *Control group- Intact ileal flap (with Tamás Cserni's permission)*

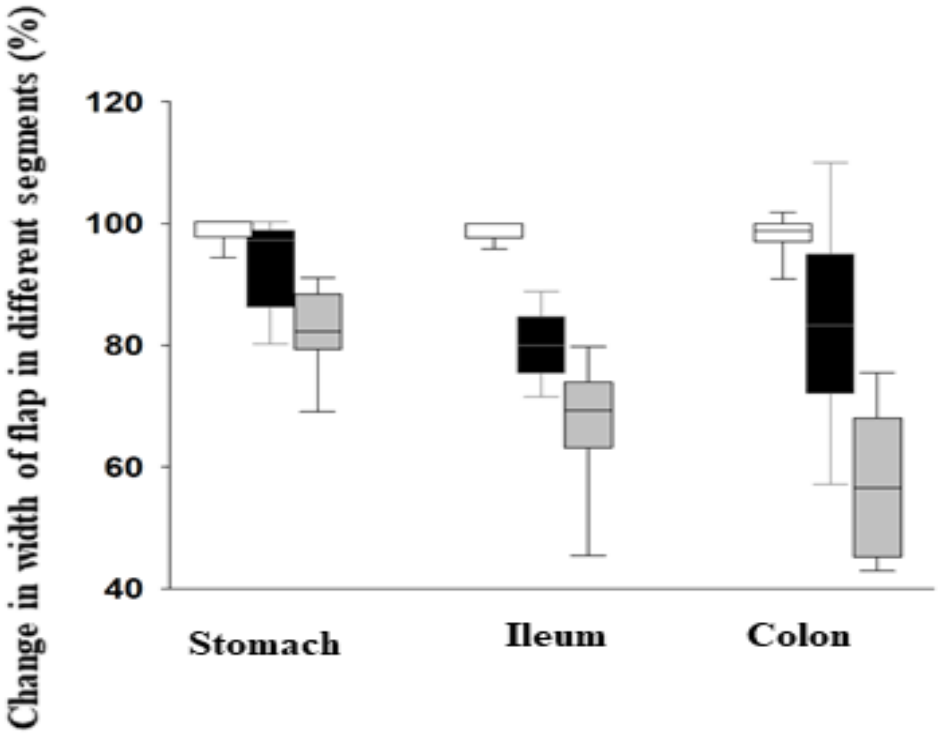

Figure 17 *The width of the flaps in the control (white), the mucosectomy (Group I.) (black) and the sero-muscular groups (Group II.) (grey). Two-way ANOVA test showed significant difference (p< 0,05) in each segment between Group I. and II, between stomach and ileum and between ileum and colon.*

Source: Urbán D, Marei MM, Hajnal D, Varga G, Érces D, Poles M, Imre D, Szabó A, Cervellione RM, Cserni T. Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol. 2020;16:20-26.

4.2.1 HE staining

The same features were observed consistently in all flaps. In Group (I), small mucosal remnants were observed on the top of the muscularis mucosae. In Group (II), the submucosal layer was completely missing.

4.2.2 Neurofilament immunohistochemistry

In Group (I), all plexuses, the superficial and deep submucosal and the myenteric plexuses were intact in all flaps (Figure 18 A, 19 A, 19 C). In Group (II), irrespective of whether it was ileum, colon or stomach, only the myenteric plexus could be observed intact (Figure 18 B, 19 B, 19 D). In any individual slide, only 1-2 submucosal ganglia were occasionally found loosely attached to the sero-muscular flap (Figure 18 B, 17 D) and the vast majority of the deep and superficial ganglia were missing. These ganglia could be observed in the removed submucosa-mucosal layer (Figure 18 C, 19 B & 19 D).

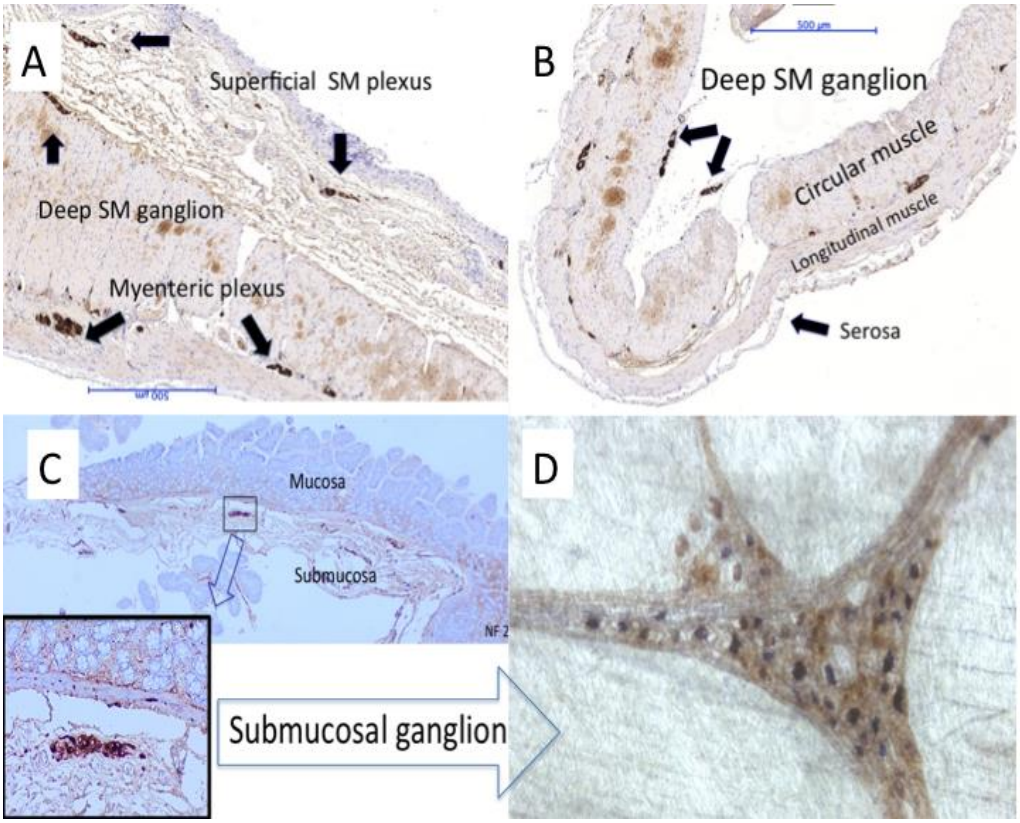

Figure 18 *Ileal flap.* ***A:*** *In Group I. all plexuses, the superficial and deep submucosal and the myenteric plexuses were intact (Neurofilament immunostaining)* ***B:*** *In Group II. Only 1-2 submucosal ganglia were attached loosely to the sero-muscular flap, vast majority of the deep and superficial ganglia are missing. (Neurofilament immunostaining)* ***C:*** *Submucosal ganglia can be observed in the removed submucosa-mucosal layer. (Neurofilament immunostaining)* ***D:*** *The full plexus of submucosal ganglia was found on the surface of the submucosal layer used to face to the muscle layers. Cholinergic neurons stained brown while nitrergic neurons stained blue in the submucosal ganglia. (Whole-mount preparation, NADPHd and AchE enzyme histochemistry).*

Source: Urbán D, Marei MM, Hajnal D, Varga G, Érces D, Poles M, Imre D, Szabó A, Cervellione RM, Cserni T. Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol. 2020;16:20-26.

4.2.3 Whole-mount preparation and AchE and NADPH-d histochemistry

The full plexus of submucosal ganglia was found on the surface of the submucosal layer that was previously facing the muscle layers. Cholinergic neurons stained brown while nitrergic neurons stained blue in the submucosal ganglia (Figure 18 D).

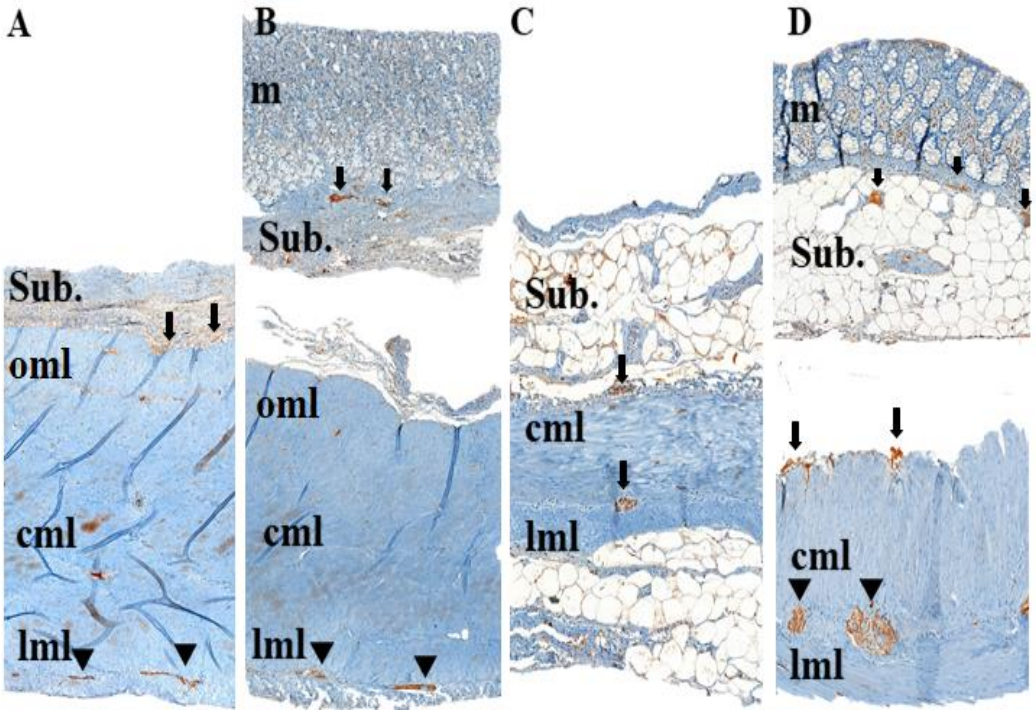

Figure 19 ***A****: stomach flap after scraping off the mucosa (Group I.)* ***B:*** *Stomach flap after total removal of the mucosa-submucous layers (Group II.) the removed mucosa on the top, the sero-muscular layers on the bottom.* ***C:*** *Colon flap after scraping off the mucosa (Group I.)*
D: *Colon flap after total removal of the mucosa-submucous layers (Group II.) the removed mucosa on the top, the sero-muscular layers on the bottom.* ***Arrowheads*** *– submucous plexus;* ***arrows*** *– myenteric plexus;* ***m*** *– mucosa;* ***sub*** *– submucosa;* ***oml*** *- oblique muscle layer;* ***cml*** *– circular muscle layer;* ***lml*** *– longitudinal muscle layer;* ***s*** *– serosa.*

Source: Urbán D, Marei MM, Hajnal D, Varga G, Érces D, Poles M, Imre D, Szabó A, Cervellione RM, Cserni T. Mucosectomy disrupting the enteric nervous system causes

contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol. 2020;16:20-26.

5. DISCUSSION

5.1 Experimental Study I

In a previous study we had demonstrated that mucosectomy results in an abrupt gross reduction of the capillary blood flow in the ileum [71]. The colon was not studied, and the length of the ischemic period was not recorded. However, every organ tolerates warm ischemia for a certain time period. The warm ischemia time of ileum was found to be up to 45 minutes in rats [72], and 2 h in dogs [73]. Strand-Amundsen et al. investigated viability of the porcine intestine after mesenteric obstruction [74]. Light microscopy showed loss of crypt epithel, congestion and bleeding in lamina propria, neutrophil infiltration, wavy myocytes and focal necrosis in both muscular layers after 3 hours of ischemia and 8 h of reperfusion. Electron microscopy demonstrated inflammation, cell death, fine-vacuolization of the sarcoplasm, slightly swollen mitochondria, focal single cell necrosis, swollen cell nuclei after 3 h of ischemia and 3 h of reperfusion. Three hours of total ischemia of the small bowel followed by reperfusion was considered to be the upper limit for viability in porcine mesenteric ischemia model [73]. In humans the warm ischemia cut off time of the ileum for microvascular transplantation is considered between 1-2 hrs [75].

In the present study we demonstrated that the microcirculation does not recover within the warm ischemia time of the ileum and colon after mucosectomy. Only minimal residual circulation was detected after mucosectomy. There was a degree of recovery in the RBCV, however both remained far below the control (preoperative) values. The partial re-establishment of circulation was seen after 60-90 minutes, sufficient to prevent acute necrosis. This is in accordance with our earlier observation where the flaps used for augmentation remained viable, even though they contracted [71]. When the muscularis propria and the muscularis mucosae are damaged, peristalsis and the movement of the villi will be lost. Regenerated scar tissue might not uphold sufficient peristalsis, and may lead to later stricture [76].

With the advancement of ESD, it has become clear that extensive removal of mucosa-submucosa (above 75% of the circumference) results in stricture in esophagus, stomach or colorectum) [7–9,15,16,19]. In our experiment the removal of mucosa/submucosa corresponds to this situation, the stricture seen after excessive ESD could be in accordance with our finding.

In this sense, our study first provides a possible link between mucosectomy and ischemia and explains stricture after excessive ESD-induced mucosectomy.

Our results provide the first clear evidence that surgical mucosectomy in both ileum and colon induces ischemia beyond the warm ischemia time. This will inevitably result in flap fibrosis and contraction. Vascularisation is key to the long-term success of all bladder augmentations and must be the focus if we are to achieve mucus free composite flaps. We did not observe any significant difference between the 2 mucosectomy processes (Group A and B). One would expect better results in Group A, with sero-musculo-submucosal flaps, where the submucosus vascular plexus is not destroyed. This is however not the case according to our findings. The microcirculation of the sero-mucular-submucosal flaps also dropped significantly and only partially recovered showing no significantly better values than the seromuscular flaps. This is in accordance with our experience in the earlier study where we found sero-mucular-submucosal flaps contracted after augmentation [71,77]. Furthermore, it is known that due to mucosal regrowth sero-muscular-submucosal flaps may not be ideal to eliminate mucus from augmented bladder.

In our previous study we tried to re-vascularise the flaps after mucosectomy with omentum, but it failed to prevent contraction of ileum [71]. It is likely that by the time the blood vessels from the omentum had established neo-vascularisation, the bowel flaps have already suffered irreversible ischemic damage. Pre-vascularisation of the donor organ perhaps with omentum before mucosectomy in the future might remain an option. But again, it will be difficult to establish neovascularisation if previous vascular structure is intact. In addition, mucosectomy causes damage to the enteric nervous system which may compromise results [77]. Mucus free bladder augmentation using intestinal segment is an ideal goal but remains difficult to achieve. Surgical removal of the mucosa result in severe prolonged ischemia as well as damage to the enteric nervous system both in the ileum and the colon with resultant fibrosis and contraction of these flaps.

5.2 Experimental Study II

Augmentation with de-mucosalised intestinal segment is still not fully reliable and did not gain widespread popularity. The main concern is the mechanism of flap shrinkage, which is still poorly understood. This study provided evidence that mucosectomy has a significant immediate effect on the dimensions of the GI segments, irrespective of whether it is a stomach, ileum or colon. Our results also offer an explanation to why shrinkage of the mucosectomised flaps is unavoidable in large animals, while in previous studies on smaller mammals in which motor neurons are only located in the myenteric plexus, this phenomenon has not been observed.

During ENS development, the Neural Crest cells first form the myenteric plexus immediately outside the circular muscle layer. The mesenchyma-derived longitudinal muscle layer then forms outside this, sandwiching the myenteric plexus after it has been formed, the submucous plexus is then formed by the neuroblasts, which migrate from the myenteric plexus across the circular muscle layer and into the submucosa, and from that point neurons extend into the mucosa [78]. The neurons that project to the mucosa include intrinsic sensory [79] and secretomotor neurons [80,81]. The submucosal plexus acts as a bridge of signals between the musculosa and the mucosa of the gut [82]. Submucosal neurons connect to each other, to the mucosa and to the myenteric plexus, so that disruption of this fine network at any level detrimentally affects the physiology of motility and vascularity [83–85].

This understanding is in keeping with our observations that flap shrinkage occurred in both of our studied groups (I) and (II), albeit to a lesser degree when only the mucosa was scrapped in group (I), whilst it occurred more significantly when the submucosal plexus was fully disrupted in group (II). The flap shrinkage that we recorded was an instant phenomenon after mucosectomy. This can be explained with the disrupted nerve fibres connecting submucous and myenteric ganglia. In that situation, the inhibitory motoneurons located in the submucosa cannot suppress the myenteric cholinergic motoneurons, which being un-inhibited would induce an increased muscle tone and contraction of the muscle fibres.

As sensory nerve fibres are present in the mucosa, we can speculate that disrupting the nerve fibres between the mucosa and the ENS (as in our group (I) specimens) may influence the fine reflex circuits of the intestine responsible for motor integrity, including both muscle tone and contractility. Disruption of the submucosa (as in our group (II) specimens), would obviously have a more accentuated effect [83–85].

Increased baseline muscle tone is observed in Hirschsprung's disease (HD) where the myenteric plexus is under-developed and inhibitory motoneurons are missing. The muscle layer is therefore unable to relax, which is the diagnostic feature of HD in anorectal manometry. Increased AchE-positivity is a diagnostic histological feature of HD, which comes from the hypertrophied AchE-positive un-inhibited excitatory nerve endings [65]. The fine balance of the excitatory and inhibitory neural circuits responsible for normal muscle tone and motility does not exists if the ENS is not fully developed and intact. Similarly, decreased peptidergic and nitrergic innervation (inhibitory motoneurons) has been hypothesised as an aetiology for intrinsic pelviureteric junction obstruction due to failure to relax to stimuli (urine bolus), and it is at least suspected as an aetiology of hypertrophic pyloric stenosis [66,67,86].

Zhang et al (2011) found that botulinum toxin infiltration of the mucosectomised gastric flaps is able to prevent flap contraction (shrinkage) [29]. A clear explanation as to how and why did this happen was not given by the authors, but this clearly supports our hypothesis. It is plausible that non-specific paralysis of the imbalanced nerve circuits of the stomach after mucosectomy resulted in muscle relaxation i.e. prevented the shrinkage of the flaps. Other drugs, like local anaesthetics, nitric oxide (NO) gas, NO synthase (nNOS) and botulinum toxin can prevent contractions, however we did not pursue testing this because the accurate administration and dosage of these drugs would have complicated the study as confounding factors and most likely would have had a temporary effect of variable and unknown duration.

There is a significant difference between the different organs in the extent of flap shrinkage. It affected the stomach lesser and the colon greater. We can only speculate that this is affected by the anatomy and spatial pattern of the muscle layers of these organs. The more developed the individual layers (circular, oblique and longitudinal), the more likely they can oppose sliding and hold-on to one-another thus oppose contraction and shrinkage. The flaps were de-tubularised along the longitudinal axis, thus the width of the flaps we measured was coming from the length of the detubularised circular muscle fibres. Consequently, the circular muscle fibres had the most significant effect. The presence of a complete sheet of longitudinal and oblique muscle layers in the stomach would keep the circular fibres more fixed than in the ileum, which lacks an oblique layer, and even more than in the colon, which lacks an oblique layer and has a poorly developed longitudinal layer, arranged in three equally spaced lines (*taeniae coli*). Additionally, there may be subtle differences between the ENS of the stomach, ileum and colon which may contribute to these differences.

One can argue that surgical manipulation alone may have induced the contraction of the bowel flaps. However, the control group, where bowel segments were isolated and detubularised was exposed to a degree of surgical manipulation as well, although admittedly, scraping-off the mucosa or pealing the submucosa is a stronger stimulus than de-tubularisation. The only difference between the control group (on one hand) and Groups I. and II. (put together, on the other hand) is the mucosectomy, therefore the phenomenon of contraction (shrinkage) that was reliably observed must be the result of the mucosectomy. Furthermore, the flap shrinkage was significantly more expressed in Group (II) versus Group (I) due to the proven more disruption of the ENS in Group (II). Obviously, the surgeon can compensate for the shrinkage of the flaps by harvesting longer bowel loops for augmentation, but this has to be done before performing the mucosectomy. However, it is highly likely the bowel segment will still behave like one with HD i.e. will not be able to relax, therefore we can expect less compliance once augmentation is done with mucosectomy. Our main message is that; this should be considered when planning an augmentation using a de-mucosalised flap. It is noteworthy that, augmentation with sero-musculo-submucosal intestinal flaps does not result in a totally mucus-free bladder, as the mucosa will regrow [87–89]. Despite that we were very meticulous and used surgical loops with 2.5x magnification during the mucosectomy, we have seen mucosal island on the flaps after HE staining.

In our previous study, we found that significant disruption of the intrinsic blood supply and microcirculation of the flaps may result in long term ischemia and fibrosis [71]. By highlighting this, together with our current observation of damaged ENS and acute shrinkage, we aim to contribute to the discussion pertinent to microvascular and neuronal aspects of experimental efforts to remove or replace the mucosal coverage of the gastro-intestinal flaps used for augmentation.

The limitations of our study include the fact that we did not monitor the shrinkage in the long term, however we did not observe an improvement during the 2-hour duration of the experiment in each pig. In our previous animal study, where microcirculation of the mucosectomised ileal flaps was assessed before and after mucosectomy and the flaps were used for augmentation in a reverse fashion, we were able to demonstrate the long-term shrinkage of both types of these flaps [27].

6. SUMMARY OF THE NEW RESULTS AND CONCLUSIONS

1. Surgical mucosectomy results in an immediate and severe cessation of the microcirculation within the intestinal wall and the microvascular blood flow is not recovering within the warm ischemia time. No significant difference was found between the ileum and colon in this respect. This finding may offer explanation for the stricture seen after mucosectomy.
2. Surgical mucosectomy disrupts the ENS of the intestine resulting in immediate shrinkage. No significant difference was found between the stomach, the ileum and the colon, the damaged reflex circuits of the intestine may provide another explanation for shrinkage seen after mucosectomy.
3. The changes were present in the intestinal segments after sero-musculo-submucosal and sero-muscular technical approaches as well.

POSSIBLE FUTURE DIRECTIONS

Understanding the direct effect and consequences of mucosectomy on the intestine may help future surgeons to prevent or treat postoperative strictures. Today, the stricture seen after extensive mucosectomy remains invertible, if the damage of the microvascular supply and the ENS is not addressed, stricture remains a standard consequence of mucosectomy. In clinical practice, composite intestinal segments with transplanted mucosa, urothelium for bladder augmentation or small bowel mucosa in SBS may not be long-term options until the problems of microvascular and neurological damages of the intestinal segments are not solved.

Our findings may influence the direction of research in reconstructive surgery and urology as well. It may be worth considering prevascularisation of the affected segments. The omentum could be connected to the serosal surface of the intestine which is later subjected to mucosectomy. Nevertheless, in our previous experiments we have tried to vascularise the intestine with omentum after mucosectomy without significant success. Likewise, restoration of the ENS may not be possible today, however botulinum toxin might be used to paralyze AchE neurons and relax intestinal muscle temporary. Non-toxic chemical mucosectomy remains another option.

7. REFERENCES

[1] Cserni T, Paran S, Puri P. The effect of age on colocalization of acetylcholinesterase and nicotinamide adenine dinucleotide phosphate diaphorase staining in enteric neurons in an experimental model. J Pediatr Surg 2007;42:300–4. https://doi.org/10.1016/j.jpedsurg.2006.10.031.

[2] Cserni T, O' Donnel A, Paran S, Puri P. Correlation of enteric NADPH-d positive cell counts with the duration of incubation period in NADPH-d histochemistry. Pathol Oncol Res 2009;15:103–7. https://doi.org/10.1007/s12253-008-9081-5.

[3] Cserni T, Paran S, Puri P. New hypothesis on the pathogenesis of ileocecal intussusception. J Pediatr Surg 2007;42:1515–9. https://doi.org/10.1016/j.jpedsurg.2007.04.025.

[4] Cserni T, Paran S, Kanyari Z, O'Donnell A-M, Kutasy B, Nemeth N, et al. New insights into the neuromuscular anatomy of the ileocecal valve. Anat Rec (Hoboken) 2009;292:254–61. https://doi.org/10.1002/ar.20839.

[5] Ahmad NZ, Abbas MH, Abunada MH, Parvaiz A. A Meta-analysis of Transanal Endoscopic Microsurgery versus Total Mesorectal Excision in the Treatment of Rectal Cancer. Surg J (N Y) 2021;7:e241–50. https://doi.org/10.1055/s-0041-1735587.

[6] Carmichael H, Sylla P. Evolution of Transanal Total Mesorectal Excision. Clin Colon Rectal Surg 2020;33:113–27. https://doi.org/10.1055/s-0039-3402773.

[7] Park CH, Yang D-H, Kim JW, Kim J-H, Kim JH, Min YW, et al. Clinical Practice Guideline for Endoscopic Resection of Early Gastrointestinal Cancer. Clin Endosc 2020;53:142–66. https://doi.org/10.5946/ce.2020.032.

[8] Ohara Y, Toyonaga T, Tanaka S, Ishida T, Hoshi N, Yoshizaki T, et al. Risk of stricture after endoscopic submucosal dissection for large rectal neoplasms. Endoscopy 2016;48:62–70. https://doi.org/10.1055/s-0034-1392514.

[9] Iizuka H, Kakizaki S, Sohara N, Onozato Y, Ishihara H, Okamura S, et al. Stricture after endoscopic submucosal dissection for early gastric cancers and adenomas. Dig Endosc 2010;22:282–8. https://doi.org/10.1111/j.1443-1661.2010.01008.x.

[10] Turner A, Subramanian R, Thomas DFM, Hinley J, Abbas SK, Stahlschmidt J, et al. Transplantation of autologous differentiated urothelium in an experimental model of composite cystoplasty. Eur Urol 2011;59:447–54. https://doi.org/10.1016/j.eururo.2010.12.004.

[11] Avansino JR, Chen DC, Hoagland VD, Woolman JD, Stelzner M. Orthotopic transplantation of intestinal mucosal organoids in rodents. Surgery 2006;140:423–34. https://doi.org/10.1016/j.surg.2006.03.012.

[12] Tait IS, Evans GS, Flint N, Campbell FC. Colonic mucosal replacement by syngeneic small intestinal stem cell transplantation. Am J Surg 1994;167:67–72. https://doi.org/10.1016/0002-9610(94)90055-8.

[13] Sugimoto S, Kobayashi E, Fujii M, Ohta Y, Arai K, Matano M, et al. An organoid-based organ-repurposing approach to treat short bowel syndrome. Nature 2021;592:99–104. https://doi.org/10.1038/s41586-021-03247-2.

[14] Espinel J, Pinedo E, Ojeda V, Del Rio MG. Multiband mucosectomy for advanced dysplastic lesions in the upper digestive tract. World J Gastrointest Endosc 2015;7:370–80. https://doi.org/10.4253/wjge.v7.i4.370.

[15] Spadaccini M, Belletrutti PJ, Attardo S, Maselli R, Chandrasekar VT, Galtieri PA, et al. Safety and efficacy of multiband mucosectomy for Barrett's esophagus: a systematic review with pooled analysis. Ann Gastroenterol 2021;34:487–92. https://doi.org/10.20524/aog.2021.0620.

[16] Alzoubaidi D, Graham D, Bassett P, Magee C, Everson M, Banks M, et al. Comparison of two multiband mucosectomy devices for endoscopic resection of Barrett's esophagus-related neoplasia. Surg Endosc 2019;33:3665–72. https://doi.org/10.1007/s00464-018-06655-0.

[17] Schomisch SJ, Yu L, Wu Y, Pauli EM, Cipriano C, Chak A, et al. Commercially available biological mesh does not prevent stricture after esophageal mucosectomy. Endoscopy 2014;46:144–8. https://doi.org/10.1055/s-0033-1344997.

[18] Tsunada S, Ogata S, Mannen K, Arima S, Sakata Y, Shiraishi R, et al. Case series of endoscopic balloon dilation to treat a stricture caused by circumferential resection of the

gastric antrum by endoscopic submucosal dissection. Gastrointest Endosc 2008;67:979–83. https://doi.org/10.1016/j.gie.2007.12.023.

[19] Kantsevoy SV. Stenosis after colorectal endoscopic submucosal dissection: When to expect, how to manage? Gastrointest Endosc 2017;86:370–1. https://doi.org/10.1016/j.gie.2017.04.031.

[20] Urbán D, Kőnig R, Cserni T. [Autologous reconstructive surgery and intestinal rehabilitation in the management of short bowel syndrome]. Orv Hetil 2020;161:243–51. https://doi.org/10.1556/650.2020.31655.

[21] Jednak R. The evolution of bladder augmentation: from creating a reservoir to reconstituting an organ. Front Pediatr 2014;2:10. https://doi.org/10.3389/fped.2014.00010.

[22] Kropp B, Cheng E. Bladder Augmentation: Current and Future Techniques. In: Docimo S, Canning D, Khoury A, editors. Clinical Pediatric Urology, 5th Edition, London: Informa Healthcare; 2007, p. 871–910.

[23] Shoemaker WC, Bower R, Long DM. A new technique for bladder reconstruction. Surg Gynecol Obstet 1957;105:645–50.

[24] Shoemaker WC. Reversed seromuscular grafts in urinary tract reconstruction. J Urol 1955;74:453–75. https://doi.org/10.1016/s0022-5347(17)67305-1.

[25] Oesch I. Neourothelium in bladder augmentation. An experimental study in rats. Eur Urol 1988;14:328–9. https://doi.org/10.1159/000472971.

[26] Salle: Fibrosis of the seromuscular segment - Google Tudós n.d. https://scholar.google.com/scholar_lookup?title=Fibrosis%20of%20the%20seromuscular%20segment&author=J.L.%20Salle&publication_year=2000&pages=28-29 (accessed February 5, 2022).

[27] Salle: Determining factor in the contraction of de-epithe... - Google Tudós n.d. https://scholar.google.com/scholar_lookup?title=Determining%20factor%20in%20the%20contraction%20of%20de-epithelialised%20gastric%20flap%20for%20bladder%20augmentation&author=J.L.%20Salle&publication_year=1997 (accessed February 5, 2022).

[28] Cheng E, Rento R, Grayhack JT, Oyasu R, McVary KT. Reversed seromuscular flaps in the urinary tract in dogs. J Urol 1994;152:2252–7. https://doi.org/10.1016/s0022-5347(17)31652-x.

[29] Zhang Y, Liu G, Kropp BP. Re-epithelialization of demucosalized stomach patch with tissue-engineered urothelial mucosa combined with Botox A in bladder augmentation. BJU Int 2012;110:E106-112. https://doi.org/10.1111/j.1464-410X.2011.10845.x.

[30] Hidas G, Lee HJ, Bahoric A, Kelly MS, Watts B, Liu Z, et al. Aerosol transfer of bladder urothelial and smooth muscle cells onto demucosalized colonic segments for bladder augmentation: in vivo, long term, and functional pilot study. J Pediatr Urol 2015;11:260.e1-6. https://doi.org/10.1016/j.jpurol.2015.02.020.

[31] Kachlik D, Baca V, Stingl J. The spatial arrangement of the human large intestinal wall blood circulation. J Anat 2010;216:335–43. https://doi.org/10.1111/j.1469-7580.2009.01199.x.

[32] Gallavan RH, Chou CC, Kvietys PR, Sit SP. Regional blood flow during digestion in the conscious dog. Am J Physiol 1980;238:H220-225. https://doi.org/10.1152/ajpheart.1980.238.2.H220.

[33] Neil Granger D, Holm L, Kvietys P. The Gastrointestinal Circulation: Physiology and Pathophysiology. Comprehensive Physiology, John Wiley & Sons, Ltd; 2015, p. 1541–83. https://doi.org/10.1002/cphy.c150007.

[34] Cheng W, Heitmiller RF, Jones B. Subacute ischemia of the colon esophageal interposition. Ann Thorac Surg 1994;57:899–903. https://doi.org/10.1016/0003-4975(94)90199-6.

[35] Shavell VI, Saed GM, Diamond MP. Review: cellular metabolism: contribution to postoperative adhesion development. Reprod Sci 2009;16:627–34. https://doi.org/10.1177/1933719109332826.

[36] Strowitzki MJ, Ritter AS, Kimmer G, Schneider M. Hypoxia-adaptive pathways: A pharmacological target in fibrotic disease? Pharmacol Res 2019;147:104364. https://doi.org/10.1016/j.phrs.2019.104364.

[37] Lim DR, Hur H, Min BS, Baik SH, Kim NK. Colon Stricture After Ischemia Following a Robot-Assisted Ultra-Low Anterior Resection With Coloanal Anastomosis. Ann Coloproctol 2015;31:157–62. https://doi.org/10.3393/ac.2015.31.4.157.

[38] Phad N, Trivedi A, Todd D, Lakkundi A. Intestinal Strictures Post-Necrotising Enterocolitis: Clinical Profile and Risk Factors. J Neonatal Surg 2014;3:44.

[39] Necrotizing Enterocolitis - Pediatrics. MSD Manual Professional Edition n.d. https://www.msdmanuals.com/en-gb/professional/pediatrics/gastrointestinal-disorders-in-neonates-and-infants/necrotizing-enterocolitis (accessed March 2, 2022).

[40] Lee-Elliott C, Landells W, Keane A. Using CT to reveal traumatic ischemic stricture of the terminal ileum. AJR Am J Roentgenol 2002;178:403–4. https://doi.org/10.2214/ajr.178.2.1780403.

[41] Lien GS, Mori M, Enjoji M. Delayed posttraumatic ischemic stricture of the small intestine. A clinicopathologic study of four cases. Acta Pathol Jpn 1987;37:1367–74. https://doi.org/10.1111/j.1440-1827.1987.tb00469.x.

[42] Bryner UM, Longerbeam JK, Reeves CD. Posttraumatic Ischemic Stenosis of the Small Bowel. Archives of Surgery 1980;115:1039–41. https://doi.org/10.1001/archsurg.1980.01380090019005.

[43] Yamazaki T, Shirai Y, Sakai Y, Hatakeyama K. Ischemic stricture of the rectosigmoid colon caused by division of the superior rectal artery below Sudeck's point during sigmoidectomy: report of a case. Surg Today 1997;27:254–6. https://doi.org/10.1007/BF00941656.

[44] Jansen SM, de Bruin DM, van Berge Henegouwen MI, Strackee SD, Veelo DP, van Leeuwen TG, et al. Optical techniques for perfusion monitoring of the gastric tube after esophagectomy: a review of technologies and thresholds. Diseases of the Esophagus 2018;31. https://doi.org/10.1093/dote/dox161.

[45] de Bruin AFJ, Tavy ALM, van der Sloot K, Smits A, Ince C, Boerma EC, et al. Can sidestream dark field (SDF) imaging identify subtle microvascular changes of the bowel during colorectal surgery? Tech Coloproctol 2018;22:793–800. https://doi.org/10.1007/s10151-018-1872-4.

[46] de Bruin AFJ, Kornmann VNN, van der Sloot K, van Vugt JL, Gosselink MP, Smits A, et al. Sidestream dark field imaging of the serosal microcirculation during gastrointestinal surgery. Colorectal Dis 2016;18:O103-110. https://doi.org/10.1111/codi.13250.

[47] Laemmel E, Genet M, Le Goualher G, Perchant A, Le Gargasson J-F, Vicaut E. Fibered confocal fluorescence microscopy (Cell-viZio) facilitates extended imaging in the field of microcirculation. A comparison with intravital microscopy. J Vasc Res 2004;41:400–11. https://doi.org/10.1159/000081209.

[48] Diana M, Dallemagne B, Chung H, Nagao Y, Halvax P, Agnus V, et al. Probe-based confocal laser endomicroscopy and fluorescence-based enhanced reality for real-time assessment of intestinal microcirculation in a porcine model of sigmoid ischemia. Surg Endosc 2014;28:3224–33. https://doi.org/10.1007/s00464-014-3595-6.

[49] Kovács T, Varga G, Erces D, Tőkés T, Tiszlavicz L, Ghyczy M, et al. Dietary phosphatidylcholine supplementation attenuates inflammatory mucosal damage in a rat model of experimental colitis. Shock 2012;38:177–85. https://doi.org/10.1097/SHK.0b013e31825d1ed0.

[50] Liu Z, Luo X, Jiang W, Chen D, Chen W, Li K, et al. Real-time in vivo optical biopsy using confocal laser endomicroscopy to evaluate distal margin in situ and determine surgical procedure in low rectal cancer. Surg Endosc 2019;33:2332–8. https://doi.org/10.1007/s00464-018-6519-z.

[51] Tian Y, Zheng Y, Teng G, Li J, Wang H. Imbalanced mucosal microcirculation in the remission stage of ulcerative colitis using probe-based confocal laser endomicroscopy. BMC Gastroenterol 2019;19:114. https://doi.org/10.1186/s12876-019-1037-6.

[52] Lee J, Jeh SU, Koh DH, Chung DY, Kim MS, Goh HJ, et al. Probe-Based Confocal Laser Endomicroscopy During Transurethral Resection of Bladder Tumors Improves the Diagnostic Accuracy and Therapeutic Efficacy. Ann Surg Oncol 2019;26:1158–65. https://doi.org/10.1245/s10434-019-07200-6.

[53] Liem EIML, Freund JE, Savci-Heijink CD, de la Rosette JJMCH, Kamphuis GM, Baard J, et al. Validation of Confocal Laser Endomicroscopy Features of Bladder Cancer:

The Next Step Towards Real-time Histologic Grading. Eur Urol Focus 2020;6:81–7. https://doi.org/10.1016/j.euf.2018.07.012.

[54] Schröder W, Beckurts KTE, Stähler D, Stützer H, Fischer JH, Hölscher AH. Microcirculatory changes associated with gastric tube formation in the pig. Eur Surg Res 2002;34:411–7. https://doi.org/10.1159/000065709.

[55] Schröder W, Stippel D, Gutschow C, Leers J, Hölscher AH. Postoperative recovery of microcirculation after gastric tube formation. Langenbecks Arch Surg 2004;389:267–71. https://doi.org/10.1007/s00423-004-0493-8.

[56] Bludau M, Vallböhmer D, Gutschow C, Hölscher AH, Schröder W. Quantitative measurement of gastric mucosal microcirculation using a combined laser Doppler flowmeter and spectrophotometer. Dis Esophagus 2008;21:668–72. https://doi.org/10.1111/j.1442-2050.2008.00856.x.

[57] Hölscher AH, Schneider PM, Gutschow C, Schröder W. Laparoscopic ischemic conditioning of the stomach for esophageal replacement. Ann Surg 2007;245:241–6. https://doi.org/10.1097/01.sla.0000245847.40779.10.

[58] Groner W, Winkelman JW, Harris AG, Ince C, Bouma GJ, Messmer K, et al. Orthogonal polarization spectral imaging: a new method for study of the microcirculation. Nat Med 1999;5:1209–12. https://doi.org/10.1038/13529.

[59] Bertschy C, Bawab F, Liard A, Valioulis I, Mitrofanoff P. Enterocystoplasty complications in children. A study of 30 cases. Eur J Pediatr Surg 2000;10:30–4. https://doi.org/10.1055/s-2008-1072319.

[60] van Elteren HA, Ince C, Tibboel D, Reiss IKM, de Jonge RCJ. Cutaneous microcirculation in preterm neonates: comparison between sidestream dark field (SDF) and incident dark field (IDF) imaging. J Clin Monit Comput 2015;29:543–8. https://doi.org/10.1007/s10877-015-9708-5.

[61] De Backer D, Hollenberg S, Boerma C, Goedhart P, Büchele G, Ospina-Tascon G, et al. How to evaluate the microcirculation: report of a round table conference. Crit Care 2007;11:R101. https://doi.org/10.1186/cc6118.

[62] Xu M, Wang LV. Photoacoustic imaging in biomedicine. Review of Scientific Instruments 2006;77:041101. https://doi.org/10.1063/1.2195024.

[63] Steinberg I, Huland DM, Vermesh O, Frostig HE, Tummers WS, Gambhir SS. Photoacoustic clinical imaging. Photoacoustics 2019;14:77–98. https://doi.org/10.1016/j.pacs.2019.05.001.

[64] Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012;9:286–94. https://doi.org/10.1038/nrgastro.2012.32.

[65] Teitelbaum DH, Coran AG. Chapter 99 - Hirschsprung's Disease and Related Neuromuscular Disorders of the Intestine. In: Grosfeld JL, O'Neill JA, Coran AG, Fonkalsrud EW, Caldamone AA, editors. Pediatric Surgery (Sixth Edition), Philadelphia: Mosby; 2006, p. 1514–59. https://doi.org/10.1016/B978-0-323-02842-4.50102-9.

[66] Vanderwinden JM, Mailleux P, Schiffmann SN, Vanderhaeghen JJ, De Laet MH. Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. N Engl J Med 1992;327:511–5. https://doi.org/10.1056/NEJM199208203270802.

[67] Knerr I, Dittrich K, Miller J, Kummer W, Rösch W, Weidner W, et al. Alteration of neuronal and endothelial nitric oxide synthase and neuropeptide Y in congenital ureteropelvic junction obstruction. Urol Res 2001;29:134–40. https://doi.org/10.1007/s002400000165.

[68] Structure of the Enteric Nervous System. The Enteric Nervous System, John Wiley & Sons, Ltd; 2007, p. 1–28. https://doi.org/10.1002/9780470988756.ch1.

[69] Furness JB, Costa M. TYPES OF NERVES IN THE ENTERIC NERVOUS SYSTEM Abbreviations: CCK, cholecystokinin; EPSP, IPSP, excitatory and inhibitory, respectively, postsynaptic potential; 5-HT, 5-hydroxytryptamine; VIP, vasoactive intestinal polypeptide. In: Smith AD, Llinás R, Kostyuk PG, editors. Commentaries in the Neurosciences, Pergamon; 1980, p. 235–52. https://doi.org/10.1016/B978-0-08-025501-9.50016-8.

[70] Cserni T. Experimental and Clinical Data to the Surgery of the Ileocaecal Junction in Childhood 2007. http://webpac.lib.unideb.hu:8082/WebPac/CorvinaWeb?action=cclfind&resultview=long&ccltext=idno bibDEK00625982.

[71] Cervellione RM, Hajnal D, Varga G, Rakoczy G, Kaszaki J, Keene D, et al. Mucosectomy impairs ileal microcirculation and results in flap contraction after

experimental ileocystoplasty. J Pediatr Urol 2017;13:81.e1-81.e5. https://doi.org/10.1016/j.jpurol.2016.11.007.

[72] Illyés G, Hamar J. Sequence of morphological alterations in a small intestinal ischaemia/reperfusion model of the anesthetized rat. A light microscopy study. Int J Exp Pathol 1992;73:161–72.

[73] Robinson JW, Haroud M, Winistörfer B, Mirkovitch V. Recovery of function and structure of dog ileum and colon following two hours' acute ischaemia. Eur J Clin Invest 1974;4:443–52. https://doi.org/10.1111/j.1365-2362.1974.tb00418.x.

[74] Strand-Amundsen RJ, Reims HM, Reinholt FP, Ruud TE, Yang R, Høgetveit JO, et al. Ischemia/reperfusion injury in porcine intestine - Viability assessment. World J Gastroenterol 2018;24:2009–23. https://doi.org/10.3748/wjg.v24.i18.2009.

[75] Chen S-H, Tang Y-B, Chen H-C. Survival of transferred ileum after ischemia time longer than 1 hour: a clinical result different from animal studies. J Am Coll Surg 2013;217:300–5. https://doi.org/10.1016/j.jamcollsurg.2013.03.018.

[76] Horgan PG, Gorey TF. Operative assessment of intestinal viability. Surg Clin North Am 1992;72:143–55. https://doi.org/10.1016/s0039-6109(16)45632-x.

[77] Urbán D, Marei MM, Hajnal D, Varga G, Érces D, Poles M, et al. Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol 2020;16:20–6. https://doi.org/10.1016/j.jpurol.2019.08.019.

[78] Martucciello G, Pini Prato A, Puri P, Holschneider AM, Meier-Ruge W, Jasonni V, et al. Controversies concerning diagnostic guidelines for anomalies of the enteric nervous system: a report from the fourth International Symposium on Hirschsprung's disease and related neurocristopathies. J Pediatr Surg 2005;40:1527–31. https://doi.org/10.1016/j.jpedsurg.2005.07.053.

[79] Bornstein JC, Furness JB. Correlated electrophysiological and histochemical studies of submucous neurons and their contribution to understanding enteric neural circuits. J Auton Nerv Syst 1988;25:1–13. https://doi.org/10.1016/0165-1838(88)90002-1.

[80] Frieling T, Cooke HJ, Wood JD. Electrophysiological properties of neurons in submucosal ganglia of guinea pig distal colon. Am J Physiol 1991;260:G835-841. https://doi.org/10.1152/ajpgi.1991.260.6.G835.

[81] Rolle U, Nemeth L, Puri P. Nitrergic innervation of the normal gut and in motility disorders of childhood. J Pediatr Surg 2002;37:551–67. https://doi.org/10.1053/jpsu.2002.31610.

[82] Puri P, Rolle U. Development of the Enteric Nervous System. In: Holschneider AM, Puri P, editors. Hirschsprung's Disease and Allied Disorders, Berlin, Heidelberg: Springer; 2008, p. 13–20. https://doi.org/10.1007/978-3-540-33935-9_2.

[83] Román V, Bagyánszki M, Krecsmarik M, Horváth A, Resch BA, Fekete E. Spatial pattern analysis of nitrergic neurons in the developing myenteric plexus of the human fetal intestine. Cytometry A 2004;57:108–12. https://doi.org/10.1002/cyto.a.10112.

[84] Brandt CT, Tam PK, Gould SJ. Nitrergic innervation of the human gut during early fetal development. J Pediatr Surg 1996;31:661–4. https://doi.org/10.1016/s0022-3468(96)90669-7.

[85] Foxx-Orenstein AE, Kuemmerle JF, Grider JR. Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. Gastroenterology 1996;111:1281–90. https://doi.org/10.1053/gast.1996.v111.pm8898642.

[86] Wang Y, Puri P, Hassan J, Miyakita H, Reen DJ. Abnormal innervation and altered nerve growth factor messenger ribonucleic acid expression in ureteropelvic junction obstruction. J Urol 1995;154:679–83. https://doi.org/10.1097/00005392-199508000-00091.

[87] Dewan PA, Close CE, Byard RW, Ashwood PJ, Mitchell ME. Enteric mucosal regrowth after bladder augmentation using demucosalized gut segments. J Urol 1997;158:1141–6. https://doi.org/10.1097/00005392-199709000-00114.

[88] Jednak R, Schimke CM, Barroso U JR, Barthold JS, González R. Further experience with seromuscular colocystoplasty lined with urothelium. J Urol 2000;164:2045–9.

[89] Fraser M, Thomas DF, Pitt E, Harnden P, Trejdosiewicz LK, Southgate J. A surgical model of composite cystoplasty with cultured urothelial cells: a controlled study of gross

outcome and urothelial phenotype. BJU Int 2004;93:609–16. https://doi.org/10.1111/j.1464-410x.2003.04675.x.

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9. ANNEX