Splenic nerve bundle stimulation in acute and chronic inflammation

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Publication date
2024
Document Version
Final published version

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COLOFON

The printing of this thesis was financially supported by Galvani Bioelectronics and Catharina Ziekenhuis.

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Cover design: Juul ten Hove
Layout and design: Anna Bleeker, persoonlijkproefschrift.nl

Splenic nerve bundle stimulation in acute and chronic inflammation

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 12 januari 2024, te 13.00 uur

door David Jan Brinkman
geboren te Amersfoort

Promotiecommissie

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GENERAL INTRODUCTION AND OUTLINE

The human immune system protects the body against external threats such as bacteria, viruses, and fungi, and clears internal defects like malignant cells. It comprises the innate and adaptive immune system. The innate immune system functions as an early detection and elimination system by recognizing foreign bodies such as bacteria leading to phagocytosis and initiating an inflammatory response using numerous mediators called cytokines. These cytokines regulate antigen-presentation by dendritic cells and activate other immune cells such as T- and B-cells. These are part of the adaptive immune system and develop a more specialized response to specific pathogens, followed by immunological memory.

The whole immune system works in a delicate balance and is in the absence of infection under constant influence of microbial, dietary, and metabolic products.[1] However, dysregulation is not uncommon and subsequent inflammatory processes can lead to acute and chronic inflammatory based diseases.

One example of an acute inflammatory response is related to the surgical inflammatory response. Surgery inevitably causes an inflammatory response throughout the body, which is vital for sustaining adequate plasma volume, protection against infections and the initiation of wound healing processes. The immune response is initiated by the migration of monocytes to the surgical site, which release cytokines such as TNF-α and interleukin (IL-)6.[2] Plasma levels of these cytokines peak between 4 and 12 hours after surgical trauma and attract other immune cells to the surgical site, cause leukocytosis and induce the production of C-reactive protein (CRP) and other acute phase proteins in the liver.[3-6] This initial pro-inflammatory response is counteracted by anti-inflammatory cytokines such as IL-10. In an ideal situation, there is balance between all the different elements of the surgical inflammatory response.[7] However, in abdominal surgery, opening the peritoneal cavity and subsequent handling of the intestine during abdominal surgery triggers a neurogenic response, leading to local inflammation of the intestinal muscular layer and influx of leucocytes. This, together with a systemic inflammatory response to surgery, is pivotal in the pathophysiology of postoperative ileus following abdominal surgery, a major cause of postoperative morbidity.[8] An increased systemic inflammatory response as early as 24 hours after surgery, as measured by inflammatory markers such as IL-6, CRP and TNF has also been associated with other postoperative complications (Rettig TC Ann Surg, 2016; Watt DG, Surgery, 2015).[9,10] Postoperative CRP levels have shown a strong correlation with complications in patients undergoing open versus laparoscopic colorectal surgery regardless of surgical approach.[11] Plasma cytokine concentrations, including IL-6, on the first postoperative day have a predictive value on gastroesophageal anastomotic leakage at an early stage in patients undergoing esophagectomy.[12] These changes in IL-6 often preceded the increase in CRP levels.

Based on these findings, some investigators have explored the effects of modulating the inflammatory response during the perioperative period for patients undergoing high-risk surgical procedures, including esophagectomy and pancreatectomy.[13] A recent randomized controlled (unblinded) trial showed that applying transcutaneous stimulation of auricular branch of the vagus nerve reduced CRP and IL-6 levels on postoperative day 1 in patients undergoing lung lobectomy. This was accompanied by a significant reduced incidence of postoperative pneumonia and a shorter hospitalization time.[14] In addition, a study in patients undergoing colorectal surgery evaluated the effect of gum chewing just before and after the surgery, as the

autonomic nervous system can be activated by gum chewing, on postoperative complications, length of hospital stay, and systemic inflammatory parameters.[15] Although these treatments showed some evidence that they may be beneficial in patients undergoing abdominal surgery, more high quality randomized controlled trials are needed to validate these effects.

The immune system maintains a physiological form of self-reactivity, primarily for the selection of lymphocytes and immune homeostasis. However, in up to 9% of the general population this autoreactivity results in pathogenesis known as autoimmune diseases.[16] Inflammatory bowel diseases (IBD) are chronic autoimmune diseases that are primarily located in the intestine. ulcerative colitis (UC) and Crohn's disease (CD) are recognized as two distinctive subtypes.[17,18]

Extensive research has identified immune cells, cytokines and other components that are responsible for the pathogenesis. This resulted in new therapies as biological disease mediated anti-inflammatory drugs (bDMARDS, also called biologicals) and the more recent evaluated and approved targeted synthetic (ts) DMARDs, the Janus Kinase (JAK) inhibitors. However, non-responders remain and these treatments tend to show reduced disease modulating effect over time.[19] Besides the lack of response to therapy, patients discontinue medication because of side effects. As a result, the need for the development of new therapeutic strategies remains.

The autonomic nervous system (ANS) has long been known as a critical regulator of intestinal function and much evidence now exists to suggest that it also plays an important role in the development of IBD. Dramatic changes in the ANS in IBD are apparent from the cellular to the molecular level ultimately leading to altered communication between the ANS and effector cells of the intestine. An extensive review of this interaction is the subject of chapter 1 of this thesis. The role of ANS dysfunction in IBD needs to be further explored, but current data points in the direction that influencing or restoring ANS function may have an effect on inflammation in IBD.

SPLENIC NEUROVASCULAR BUNDLE STIMULATION

Discoveries in the field of neuroscience have shown how the autonomic nervous system (ANS) is involved in controlling the immune functions. Borovikova et al. demonstrated that vagus nerve stimulation (VNS) was able to decrease systemic TNF-α levels in a rodent model of endotoxemia.[20] This phenomenon is now commonly known as the "inflammatory reflex".[21,22] From an evolutionary perspective, it seemed plausible that the autonomic nervous system exerts control over the immune system since it can act in a rapid and integrative manner and controls other physiological processes such as heart rate and blood pressure. Also in a rodent model of postoperative ileus in which gastrointestinal motility is impaired, electrical VNS was able to diminish inflammation and restore motility function of the gastrointestinal tract.[23] This was confirmed via the administration of enteral nutrition, which activates that afferent vagus nerve endings, hereby setting the anti-inflammatory reflex in motion.[24] From clinical studies with patients suffering from depression, it was demonstrated that VNS could be applied in humans.[25] The first studies have been published that investigated the effect of VNS in patients with IBD and rheumatoid arthritis (RA), with promising results.[26-28]. VNS exerts its potential immunomodulatory effects indirectly, relying on evoking neural signals in vagal fibers to the celiac ganglion where in turn signaling may be triggered in adjacent adrenergic neurons projecting to the spleen.[29] However, the vagus nerve also controls most, if not all, organs of the body, and therefore many off target effects such as bradycardia can

occur. Such effects are expected to strictly limit the stimulation parameter range of VNS in which immunomodulatory effects can be achieved without side effects.

Extensive evidence exists demonstrating that the efferent arm of the "inflammatory reflex" controls systemic immune responses via nerves going to the spleen.[30,31] The spleen is innervated by the splenic nerves.[32] This neuronal plexus runs along the splenic artery (SpA), together forming a neurovascular bundle, until it enters the splenic parenchyma where it releases catecholamines, subsequently modulate immune cells.[33] It has been shown that splenic neurovascular bundle stimulation (SpNS) is as effective as VNS in models of endotoxemia and arthritis without the significant cardiovascular off-target effects seen there for VNS. [34,35] Evidence from immune cells and tissue collected in porcine and human studies have provided extensive evidence of immunomodulatory effect from SpNS and suggests it differs from biological or targeted synthetic DMARDs.[36-38]

The importance of the splenic neurovascular bundle in the cholinergic anti-inflammatory pathway led to the believe that these nerves could be targeted for therapeutic interventions. Therefore, it is attracting to explore the effect of SpNS in the context of IBD and the modulation of the surgical inflammatory response. Although this novel, innovative treatment seems promising, it should be investigated whether there is evidence that stimulation of the splenic neurovascular bundle could have the same immunomodulating effects in humans.

OUTLINE OF THIS THESIS

This thesis consists of two parts. In the first part, the concept of SpNS is further explored in the context of IBD. The second part focuses on the justification and feasibility of SpNS during major, upper gastrointestinal surgery.

PART I: Splenic neurovascular bundle stimulation for experimental colitis

Although many studies indicate that neuromodulation might serve as a therapy for IBD, the experimental basis is narrow and needs to be further explored. In **Chapter 1**, the current literature was reviewed on the interactions between the nervous system and immune system in the gut summarizing the findings of experimental studies that have investigated neuromodulation in experimental colitis studies. VNS is the predominant form of neuromodulation that has been investigated in the context of IBD. The working mechanism, however, remains mostly unclear. $ChAT^{+}$-T cells are thought to play an important role in the spleen following VNS and are also present in the gut. In **Chapter 2**, the role of these T-cells was investigated in three different models of colitis. Since the spleen and the splenic neurovascular bundle are essential for the immunomodulatory effect of VNS, the effect of direct SpNS during experimental colitis was investigated. This is the subject of **Chapter 3**.

PART II: Surgical relevance and feasibility of splenic neurovascular bundle stimulation in humans

There should be an anatomical and functional basis for the application of SpNS in humans. The theory underlying SpNS has been addressed in rodents, but there is only scarce information about the sympathetic innervation of the human spleen and its relation with immune cells. To this end, the sympathetic innervation of the human spleen is further investigated in **Chapter 4**. It remains topic of debate whether an increased inflammatory response following pancreatic surgery can predict postoperative complications and whether laparoscopy can reduce the

inflammatory response. In **Chapter 5** it is explored whether inflammation markers in blood early in the postoperative period are associated with complications in open and laparoscopic pancreaticoduodenectomy. The final two chapters focus on the safety and feasibility of SpNS in humans. The anatomical variability of the splenic artery was explored in **Chapter 6**, which also revealed information in relation to the design of the neuromodulation device used for the clinical trial in **chapter 7**. This final chapter describes a first-in-human early feasibility trial in patients undergoing easophagectomy determining the safety and feasibility of temporarily cuff implantation around the splenic neurovascular bundle and subsequent stimulation. In addition, preliminary evidence for the effect of SpNS on the postoperative inflammatory response was obtained.

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PART I

SPLENIC NEUROVASCULAR BUNDLE STIMULATION FOR EXPERIMENTAL COLITIS

01

NEUROIMMUNE INTERACTIONS IN THE GUT AND THEIR SIGNIFICANCE FOR INTESTINAL IMMUNITY

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Cells 2019

ABSTRACT

Inflammatory bowel diseases (IBD) have a complex, multifactorial pathophysiology with an unmet need for effective treatment. This calls for novel strategies to improve disease outcome and quality of life for patients. Increasing evidence suggests that autonomic nerves and neurotransmitters, as well as neuropeptides, modulate the intestinal immune system, and thereby regulate the intestinal inflammatory processes. Although the autonomic nervous system is classically divided in a sympathetic and parasympathetic branch, both play a pivotal role in the crosstalk with the immune system, with the enteric nervous system acting as a potential interface. Pilot clinical trials that employ vagus nerve stimulation to reduce inflammation are met with promising results. In this paper, we review current knowledge on the innervation of the gut, the potential of cholinergic and adrenergic systems to modulate intestinal immunity, and comment on ongoing developments in clinical trials.

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic, debilitating conditions that have a major impact on quality of life of an increasing amount of patients worldwide.[1] Extensive experimental and clinical work has been conducted to understand the etiology of IBD and to develop corresponding therapies. Current treatment strategy for IBD is mainly based on a step-up approach, starting with relatively mild immunomodulatory agents such as 5-aminosalicylic acid and steroids, followed by immunosuppressants like thiopurines and methotrexate, before considering more aggressive drugs (i.e. biologicals) and eventually surgery as a last resort. However, this strategy is not equally effective for all patients and is accompanied by substantial costs and side-effects. The necessity for development of novel therapies has ignited innovative views on how IBD and other immune-mediated diseases should be treated. In this respect, it is acknowledged that the nervous system is a regulator of immune function which can potentially be harnessed to achieve immunosuppression in IBD. Especially the vagus nerve and its main neurotransmitter acetylcholine (ACh) have been put forward, since stimulation of the vagus nerve (VNS) was shown to reduce local and systemic inflammation in animal models of endotoxemia, arthritis, and colitis.[2-5] Clinical trials are therefore currently being conducted to determine the beneficial effects of VNS in patients.[6,7]

The mechanism via which VNS can reduce inflammation is yet to be fully elucidated. Although the intestine is densely innervated, it is not clear whether vagal efferent nerves actually innervate mucosal cells. This might indicate a role for other types of nerves.[8] Furthermore, it was demonstrated that all sorts of immune cells in the gut could be the target of a wide variety of neurotransmitters such as ACh, but also epinephrine, norepinephrine (NE), nitric oxide, and a large number of neuropeptides that act as immune modulators. This review aims to delineate the role of neuronal innervation in reducing the progression and relapse of IBD and to indicate potential directions for new developments on neuromodulatory treatments.

INNERVATION OF THE GUT

The autonomic nervous system regulates key functions of the gastrointestinal tract such as motility, secretion and vasoregulation, and acts autonomous in that its activities are not under direct conscious control. It represents the extrinsic control of the intestine and is classed as in sympathetic and parasympathetic branches based on anatomy and neurotransmitter function. The sympathetic and parasympathetic systems originate in the central nervous system (with cell bodies in the brainstem and spinal cord), while the intrinsic neurons of the enteric nervous system (ENS) reside within the wall of the gastrointestinal tract. The ENS is a distinct part of the central nervous system that can act either independently or in response to external triggers originating from sympathetic and parasympathetic nerves. The ENS is composed of small aggregations of nerve cells, called enteric ganglia, that form nerve fibers innervating effector tissues such as the intestinal muscular layer, blood vessels and gastroenteropancreatic endocrine cells. It is divided into the myenteric plexus (MP) and the submucosal plexus (SMP). The MP, also known as Auerbach's plexus, is the outer of the two major ENS plexuses and is comprised of the neurons that are located between the muscle layers of the gastrointestinal tract. It reaches from the esophagus to the internal sphincter and is primarily involved in the regulation of smooth muscle motor patterns like peristalsis. The SMP (Meissner's plexus) is the network of neurons located between the muscle layers and the

mucosa. Its function is to regulate reflexes like secretion and absorption as well as the smooth muscle motor function. The SMP is present in the small and large intestines, but is lacking in the esophagus and the greater part of the stomach. The ENS contains neurotransmitters such as ACh and is believed to regulate intestinal immunity.[9] The crosstalk between the gut and the nervous system also comprises neuropeptides that are important mediators between the nervous system and neurons or other cell types in effector tissues. These small proteins such as substance P (SP), calcitonin-gene related peptide (CGRP), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), serotonin, somatostatin, and corticotropin-releasing factor are important in multimodal neuronal communication. Of note, the distribution of these peptides has been widely studied. The abovementioned neuropeptides originate from the dorsal root ganglia.[10] Although a direct link has not been established yet, it is very likely that these molecules act as messengers in the gut-brain axis.

Most of the parasympathetic innervation of the intestine is through the vagus nerve, especially given the recent observation of the sacral preganglionic innervation to the lower gut is sympathetic of nature, not parasympathetic.[11] Preganglionic neurons of vagal efferents originate from the motor neurons of the dorsal motor nucleus and synapse with postganglionic neurons within the MP. The parasympathetic nervous system (PNS) is cholinergic and ACh is the neurotransmitter that is released at both ends which binds to muscarinic and nicotinic receptors. The distal part of the colon is innervated by pelvic nerves that arise from S2, S3 and S4 nerve roots of the sacral plexus. The stomach and upper gastrointestinal tract are most densely innervated by parasympathetic nerves.

Sympathetic innervation arises from preganglionic fibers from the thoracolumbar intermediolateral nucleus of the spinal cord and synapses with postganglionic noradrenergic neurons in prevertebral and paravertebral ganglia at the site of the gastrointestinal tract. The celiac-mesenteric ganglia provide innervation to the stomach, the small intestine and the proximal part of the large intestine. The remaining part of the large intestine is innervated through the inferior mesenteric ganglia. The rectum is innervated by fibers originating from the pelvic ganglia. In contrast to the vagus nerve, sympathetic nerves spread throughout the entire depth of the gastrointestinal wall where they influence physiological functions such as motility, secretion and intestinal vasculature.[12-14] Like the vagal fibers, sympathetic nerves synapse with the MP and SMP. The main neurotransmitter of the sympathetic nervous system (SNS), NE, binds to adrenergic GPCR receptors, which contain α and β subunits, with each several subtypes.

In the early 90s it was already discovered that patients with IBD suffer from autonomic dysfunction.[15] This is reflected by a reduced pan-enteric innervation pattern leading to functionally reduced neuronal activity. For instance, inflammation in the intestine is shown to reduce the innervation of the stomach.[16] Innervation of the colon is subject to change following inflammatory processes in the gut mucosa. In line with this, colitis is associated with loss of neurons, altered neurochemical content and reduced neurotransmitter release in both animals as patient populations. This concerns the PNS and SNS as well as the ENS.[17] Moreover, both noradrenergic and cholinergic neuronal pathways mediate stress-induced reactivation of colitis in the rat.[18] This suggests a pivotal role for the autonomic nervous system in regulating intestinal immunity.

THE ANTI-INFLAMMATORY PATHWAY AND CHOLINERGIC MODULATION OF INTESTINAL INFLAMMATION

The cholinergic anti-inflammatory pathway

The influence of neurons on inflammation in the gut first came to attention when the cholinergic anti-inflammatory pathway (CAIP) was described (figure 1). This pathway has been suggested to work as a reflex mechanism via which the nervous system can control excessive immune reactions.[19] The mechanism has been proposed after it was shown that VNS could attenuate the inflammatory response in an endotoxemia model.[5] Immunity and inflammation are crucial defense mechanisms to protect against potential threats. Obviously, regulation of these processes is essential, since uncontrolled functioning could lead to autoimmune disorders. The nervous system, using rapid nerve signals and promptly acting neurotransmitters, might have developed into a control mechanism to sustain excessive inflammatory reactions, leading to a homeostatic immune environment. The etiological reason for such a neuronal immune-regulatory system can be supported by the integrative, direct acting, and targeted reactivity that the neuronal system has in favor over humoral immunoregulation, with slow acting secreted and diffusing mediators. Initially, it was suggested that the CAIP was a clear-cut system in which immune cells are targeted by the vagus nerve via its neurotransmitter ACh. However, several anatomical and physiological controversies about the CAIP desired further elucidation of this theory.

The CAIP is commonly proposed as a reflex mechanism in which the vagus nerve functions as both the afferent and efferent part.[22] Various experimental studies have indeed shown that afferent vagal nerve fibers can be activated by different stimuli to control inflammation. Afferent fibers can react to pathogen-associated molecules such as endotoxin and viral nucleic acids, but also to ATP and cytokines which are released by host cells during the course of inflammation.[23] Vagal afferents further contain receptors for hormones such as cholecystokinin, which are released after duodenal intake of high-fat nutrition.[24] The contribution of the efferent part of the vagus nerve is mostly substantiated by the positive effect of VNS in models such as endotoxemia and ileus.[5,25] However, this interpretation overlooks the reality that VNS activates not solely efferent but also afferent fibers. Secondly, it was observed that the spleen was necessary for the anti-inflammatory effect of VNS.[26] This is conflicting, since the spleen is sympathetically innervated and no connections are found between the vagus nerves and splenic nerve bundles thus far, although superior parts of the spleen receive cholinergic innervation in the mouse.[20,27] The role of the spleen in the CAIP is further discussed below. On the efferent part of the anti-inflammatory pathway, Martelli and colleagues have pointed out that the greater splanchnic nerves are a likely candidate to exert this function rather than the vagus nerve.[21,28] Recently, they have shown with electrophysiological experiments that the vagus nerve could indeed activate the sympathetic splanchnic nerves via a route involving central systems. Strikingly, cutting the splanchnic nerves completely abolished the anti-inflammatory effects of VNS, suggesting that the splanchnic nerves represent the efferent arm of the anti-inflammatory pathway.[29] Instead of focusing on intervention of the vagus nerve through electrical stimulation, stimulating the bilateral splanchnic nerves might therefore be an interesting therapeutic option and should be investigated in future experimental disease models such as those where vagal nerve stimulation has been successful (e.g. RA, IBD, sepsis models).

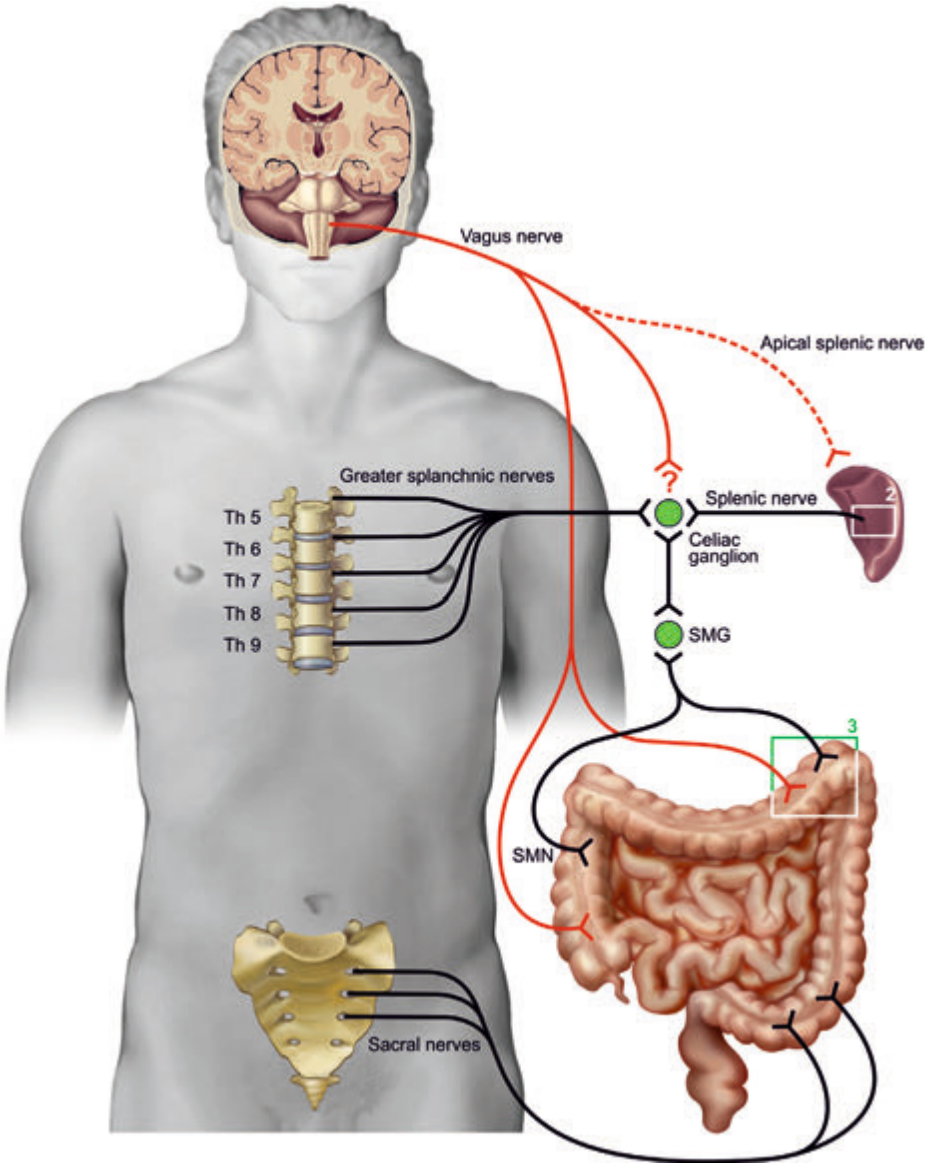

Figure 1. Schematic overview of the existing theories on the (cholinergic) anti-inflammatory pathway. The theory of the cholinergic anti-inflammatory pathway entails that efferent fibers suppress inflammation via the splenic nerve bundles. The nerve bundles that innervate the spleen are sympathetic of nature, although cholinergic innervation of the superior pole of the murine spleen was also described (via an apical nerve[20]). It is hypothesized that vagal fibers and the splenic nerve synapse in the celiac ganglion (CG), but no anatomical studies have established this thus far. An alternative theory assumes that the greater splanchnic nerves comprise the anti-inflammatory pathway.[21] Both sympathetic and cholinergic nerves innervate the large intestine, although the distal part only receives innervation from sympathetic nerves that originate from the sacrum. SMG = superior mesenteric ganglion; SMN = superior mesenteric nerves.

VNS in experimental colitis and underlying mechanisms

The potential anti-inflammatory effects of VNS gave rise to the idea that the vagus nerve is a prospective target for the treatment of IBD. This was substantiated by the fact that vagotomy exacerbated acute and relapsing dextran sulfate sodium (DSS)-induced colitis in rodents.[3,30,31] In these experiments, ventral and dorsal truncal branches of the vagus nerve were cut at the level of the diaphragm. In vagotomized animals, disease outcomes such as histology scores worsened while colonic inflammatory cytokine levels increased. Vagotomy did not change the course of colitis in macrophage-deficient mice, underlining previous findings that gut macrophages are the end-target of the vagus nerve.[25,32] Of particular importance is that vagotomized animals needed a pyloroplasty to sustain food passage, indicating a non-selective effect of the vagotomy.[3,30,31] A more selective approach was recently performed.[33] In this study, only the vagal branches that project to the intestines were cut. Interestingly, unlike ligation of the vagus branches at the more proximal cervical level, this approach did not affect the severity of colitis.[33] This observation suggests that vagotomy performed at the more distal level targeting vagal branches supplying the intestine does not suffice to modulate the disease. Probably, vagal immunomodulation of colitis (or IBD) requires other (non-vagal) neural branches that are critically dependent of the cervical vagus, being afferent of efferent in nature.

Hereafter, the potential of VNS to improve IBD was investigated in several colitis models. In general, a beneficial effect of VNS is shown on colitis outcomes such as disease activity index (DAI), macroscopic and histologic scores and colonic cytokine levels in 2,4,6-trinitrobenzene sulphonic acid (TNBS)- and oxazolone-induced colitis.[4,34-36] Stimulation parameters vary between experiments, which makes it difficult to compare experiments. The indiscriminate effect of VNS was also shown in these studies, where changes in heart rate following VNS were reported. However, in humans this might be dependent on the location of the stimulator (left or right vagus nerve)[37] and stimulating the vagus nerve below the diaphragm might prevent off-target effects.[38] A detailed overview of performed animal studies can be appreciated in Table 1.

Type of stimulation or denervation	Colitis model	Species	Study	Location	Stimulation details	Main outcomes
Vagotomy	Acute DSS	C57BL/6 mice	Ghia 2006[3]	Subdiaphramatic	-	DAI ↑ Macroscopic score ↑ Histology score ↑ MPO ↑ Colonic cytokines ↑
			Di Giovangiulio 2016 [31]	Subdiaphramatic	-	DAI ↑ Weight loss ↑ Survival rate ↓
			Willemze 2018[33]	Intestine specific (celiac branch vagus)	-	DAI = Weight loss = Colonic cytokines =
	Relapsing DSS	C57BL/6 mice	Ghia 2007[30]	Subdiaphramatic	-	DAI ↑ Macroscopic score = Histology score ↑ MPO ↑ Colonic cytokines ↑
VNS	TNBS	Sprague–Dawley rats	Meregnani 2011[34]	Left cervical vagus	3h per day 1 mA, 5 Hz, 500 µs, 10s ON, 90s OFF	Weight loss ↓ Histology score ↓ Colitis index ↓ MPO ↓ Colonic cytokines =
			Sun 2013[35]	Left cervical vagus	3h per day 0.25 mA, 20 Hz, 500 µs, 30s ON, 5 min OF	DAI ↓ Weight loss ↓ Macroscopic score ↓ Histology score ↓ MPO ↓ Colonic cytokines ↓
			Jin 2017[36]	Left cervical vagus	3h per day 1–3 mA, 5 Hz, 500 µs 10s ON, 90s OFF	DAI ↓ Weight loss ↓ Macroscopic score ↓ Histology score ↓ MPO ↓ Plasma cytokines ↓
	Oxazolone	Balb/c mice	Meroni 2018[4]	Right cervical vagus	5 min per day 1 mA, 5 Hz, 1000 µs	Survival rate ↑ Histology scores = Colonic and serum cytokines ↓

Type of stimulation or denervation	Colitis model	Species	Study	Location	Stimulation details	Main outcomes
Sympathectomy	TNBS	Sprague-Dawley rats	McCafferty 1997[39]	Systemic (6-OHDA)	-	Macroscopic score ↓ Histology score ↓ MPO ↑
	Acute DSS	BALB/c mice	Straub 2008[40]	Systemic (6-OHDA)	-	Colon length ↑ Histology score ↓
		C57BL/6 mice	Willemze 2018[33]	Superior mesenteric nerve	-	DAI ↑ Weight loss = Colonic cytokines =
	Relapsing DSS	BALB/c mice	Straub 2008[40]	Systemic (6-OHDA)	-	Colon length ↓ Histology score ↑
	Spontaneous	IL10 -/- mice	Straub 2008[40]	Systemic (6-OHDA)	-	Histology score ↑ Colonic cytokines ↑
		RAG1 -/- mice	Willemze 2019[41]	Systemic (6-OHDA)	-	Weight loss = Colon weight = Histology score ↑ Colonic cytokines =
				Superior mesenteric nerve	-	Weight loss = Colon weight ↑ Histology score ↑ Endoscopy score = Colonic cytokines ↑
SNS	Acute DSS	Sprague-Dawley rats	Willemze 2018[33]	Superior mesenteric nerve	5 min twice daily 0.2 mA, 10 Hz, 2000 µs	DAI ↓ Weight loss = Histology = Colonic cytokines = Endoscopy score =

Table 1. Overview of studies that investigated cutting of nerves or nerve stimulation in models of colitis. VNS = vagus nerve stimulation; SNS = sympathetic nerve stimulation; DSS = dextran sulfate sodium; TNBS = trinitrobenzenesulfonic acid; DAI = disease activity index; MPO = myeloperoxidase; 6-OHDA = 6-hydroxydopamine

Following the discovery of the anti-inflammatory potential of VNS, mechanistic studies were performed to delineate the underlying mechanism. The vagus nerve mainly exerts its function via the neurotransmitter ACh. Multiple receptors exist for ACh, which are traditionally classified in muscarinic and nicotinic cholinergic receptors, based on their working mechanism. Nicotinic receptors are directly linked to ion channels, whereas muscarinic receptors are G protein-linked receptors that affect cell signaling via e.g. cyclic adenosine monophosphate (cAMP). Following the initial study on VNS, it was demonstrated that VNS was dependent on the α7 nicotinic acetylcholine receptor (α7nAChR).[42] This receptor is located in the brain but is also present on immune cells such as macrophages, dendritic cells and T-cells.[43-45] When located on neurons, activation causes swift desensitization via the modulation of intracellular calcium. When located on non-neuronal cells such as immune cells however, different mechanisms are described, including classical ion flux, modulation of cAMP or inhibition of p38 mitogen-activated protein (MAP)-kinases, ultimately leading to an inhibition of the release of pro-inflammatory cytokines like tumor necrosis factor TNF-α.[46] Stimulation of the α7nAChR, both through pharmacological agonists and VNS, has been shown to be potentially beneficial for a wide variety of diseases ever since. These include fatty liver disease, kidney ischemia, arthritis and schizophrenia.[6,47-50] In colitis models, selective agonists for α7nAChR reduced immune cell infiltration and disease severity, but have also been shown to worsen colitis.[51-53] The relevance of α7nAChRs was also demonstrated in a model of postoperative ileus, which is a postsurgical state of intestinal hypomotility with an inflammatory origin.[54] Matteoli et al showed that surgical inflammation and intestinal transit in mice were improved by VNS. The end targets of VNS were found to be macrophages that express α7nAChR and lie in close proximity to cholinergic myenteric neurons that are believed to communicate with vagal efferents.[32]

Although the α7nAChR is a plausible target of VNS in sepsis models, vagotomy has been shown to worsen colitis independent of α7nAChR as well.[31] Therefore, the influence of other cholinergic receptors in the setting of colitis should not be overlooked. Nicotinic receptors such as α_5 are for instance protective in colitis, while VNS improved phagocytic capacities of intestinal macrophages via $\alpha_2\beta_4$ receptors.[55,56] Colitis could also be ameliorated by muscarinic receptor agonists, although these agonists are believed to primarily act on receptors that are located in the central nervous system.[44,57] In the colonic epithelium however, colitis affects muscarinic receptors that protect against cytokine-induced barrier dysfunction and maintain intestinal mucosal homeostasis, indicating a local role for muscarinic receptors in immunological homeostasis.[58-60]

Besides neuronal sources of ACh, immune cells such as T- and B-cells potentially participate actively in the cholinergic system and the anti-inflammatory pathway through their production of ACh. This is already extensively reviewed by Fuji and colleagues.[61,62] In brief, these cells are characterized by the presence of choline acetyltransferase (ChAT), which is the rate-limiting enzyme for the synthesis of ACh. Immunological activation of T-cells upregulates the expression of ChAT, indicating an increase in the production of ACh. This can subsequently regulate immune function, since immune cells express all types of muscarinic receptors (M1-M5) and a vast amount of nicotinic receptors. In the intestine, $ChAT^+$-T-cells have already been linked to the production of antimicrobial peptides and microbial diversity, though the participation of ACh immune cells in colitis is not clarified yet.[63]

IMPORTANCE OF THE SPLEEN IN MEDIATING THE CAIP

In further research on the CAIP, the spleen was recognized as an essential part as splenectomy and ablation of the splenic nerve in rodents abolished the effects of VNS.[26,64] Cutting the splenic nerve bundles also abrogated the positive effects of muscarinic receptor agonists on colitis.[57] Two decades ago, it was already demonstrated that splenic nerve activity increases in response to endotoxemia.[65] The sympathetic splenic nerve bundles surround the splenic artery and derive from the greater splanchnic nerves after synapsing in the celiac ganglia. Immunohistochemical studies in rodents revealed that the splenic nerve bundles enter the spleen via the splenic artery and its terminal branches. There, the majority of nerve branches are found in the white pulp, where they form a possible synaptic-like connection with leukocytes.[12,66] Various studies suggest that the spleen is also innervated by parasympathetic fibers, but strong histological evidence for this statement is lacking in current literature.[67] The participation of the spleen in the anti-inflammatory pathway has given rise to a great deal of debate. Although small branches of the vagus nerve reach the celiac ganglia, no anatomical evidence can be found for a connection between the vagus nerve and the splenic sympathetic neurons in tracing studies.[27]

ChAT$^+$-T-cells provided an explanation for this problem. Rosas-Ballina et al. demonstrated that following VNS, ChAT$^+$-T-cells release ACh in the spleen, thereby possibly relaying the parasympathetic signal.[68] Furthermore, it was found that VNS increased plasma NE via the α7nAChR located on splenic sympathetic neurons. It is therefore suggested that ACh released by ChAT$^+$-T-cells acts on the α7nAChRs that are located on splenic sympathetic nerves, resulting in the release of NE. This subsequently leads to reduction of pro-inflammatory cytokines and systemic inflammation by NE acting on the β-adrenergic receptors on myeloid cells such as macrophages (figure 2A).[69] Indeed, immunomodulation via the splenic nerve fibers appears to be dependent on β-adrenergic receptors and can inhibit the production of TNFα independently of α7nAChR.[70,71]

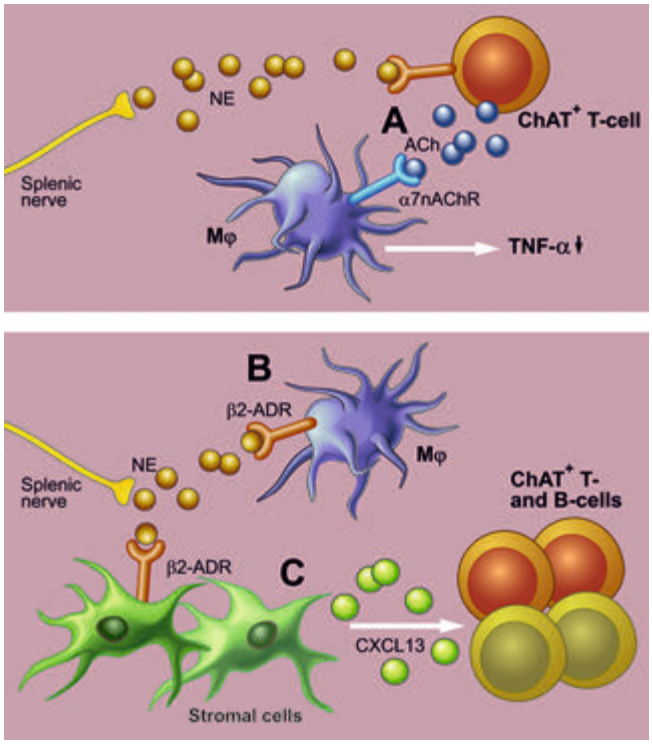

Figure 2. Mechanisms via which stimulation of the splenic nerve can control inflammation. (A) Stimulation of the splenic nerve causes the release of norepinephrine (NE), which binds to receptors on choline acetyltransferase (ChAT)$^+$ T-cells. These cells produce acetylcholine (ACh), which reduces the production of inflammatory cytokines such as tumor necrosis factor (TNF)-α by binding to the α7 nicotinic acetylcholine receptor (α7nAChR) of macrophages.[68] (B) Released NE could directly bind to β_2-adrenergic (β2-ADR) on macrophages (or other target cells). (C) Upon activation by NE, splenic stromal cells produce chemokines such as CXCL13, which control the distribution of ChAT$^+$ lymphocytes.[72] Mφ = macrophage

Although this is an elegant theory, there should be a clear anatomical connection in the spleen that lies at the basis of the interaction between immune cells and neurons. Rat studies have demonstrated such a relationship, however only few ChAT$^+$-T-cells and -B-cells are actually located near sympathetic neurons in the murine spleen.[72] Possibly, ChAT$^+$-cells are affected in an indirect manner via the adrenergic activation of stromal cells in the spleen expressing CXCL13, a chemokine that can recruit B-cells through their receptor CXCR5.[72] β-adrenergic activation has previously been shown to control lymphocyte egress in secondary lymphoid organs and might therefore be the mechanism via which splenic nerve stimulation could inhibit systemic inflammation.[73] Alternatively, NE that is released by the sympathetic neurons could act directly on β-adrenergic receptors of macrophages (figure 2B and C).[74] Nevertheless, stimulation of the splenic nerve bundles with either electrical stimulation[20] or ultrasound[75] was able to reduce symptoms in mouse models of rheumatoid arthritis.

Translational studies like recently performed by Verlinden et al, will further clarify whether neurons in the spleen have the potential to influence immune cells.[76] It was already demonstrated that septic patients show a loss of sympathetic splenic nerves, indicating a regulating role in disease.[77] Nevertheless, recent insights also show that the spleen might not be

as essential as previously believed, since the anti-inflammatory properties of splanchnic nerve stimulation are spread across other abdominal organs such as the liver and adrenal glands.[78]

SYMPATHETIC MODULATION OF GUT IMMUNITY

It has been recognized for a long time that the SNS is a strong modulator of inflammatory activation. Colonic macrophage TNF and IL-6 secretion has been shown to be regulated by sympathetic innervation.[79] Moreover, the anti-inflammatory prospect of sympathetic neurotransmitters such as epinephrine and NE has been described in different disease models like arthritis and sepsis.[74,80] The a- and b-adrenergic receptors are, like cholinergic receptors, present on nearly all types of immune cells. There are however differences in the expression of these receptors, since for instance monocytes show greater β-adrenergic receptor density than lymphocytes.[81] Especially the β_2-adrenergic receptor is involved in the suppression of pro-inflammatory cytokine release following stimuli such as lipopolysaccharide *in vitro* and lymphocyte expansion.[73,82,83] β_2-adrenergic receptor stimulation controls inflammation by driving rapid IL-10 secretion.[84] NE therefore has gained increasing recognition as a modulator of intestinal inflammation. Moreover, in a DSS-colitis model sympathetic denervation lead to worsening of the DSS-induced colitis, whereas sympathetic stimulation caused improvement.[85] This anti-inflammatory effect of NE acting on β_2-adrenergic receptors was highlighted in a human study by the finding that patients with IBD that were using b-blockers had an increased risk of developing a disease relapse compared to patients with IBD that were not using b-blockers.[57] Next to this direct anti-inflammatory effect, high concentrations of NE can also result in apoptosis in different cell types.[86-90] In lymphocytes, this is mediated by again, the β_2-adrenergic receptor.[91] This counts as an anti-inflammatory mechanism as well, in the case that pro-inflammatory cells are targeted.

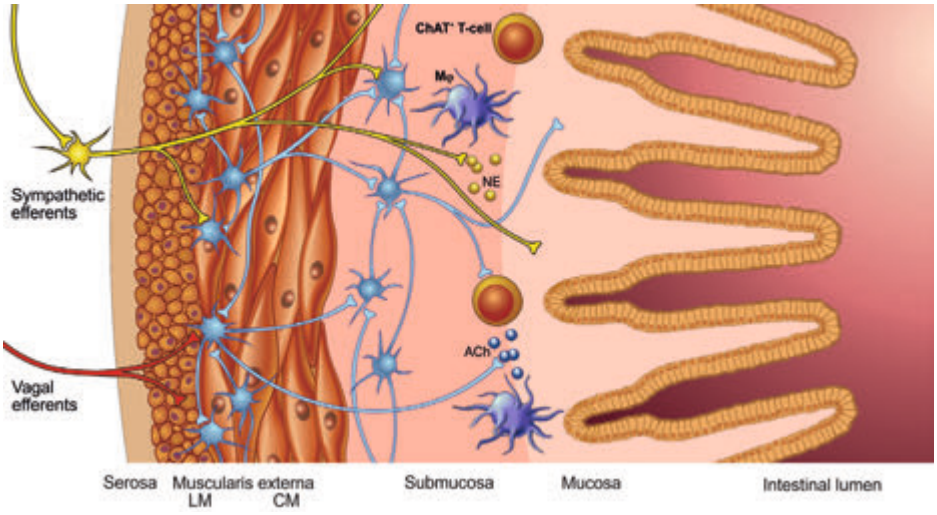

Figure 3. Innervation of the intestinal wall. Both sympathetic and cholinergic nerves innervate the intestinal wall, where they synapse with the enteric nervous system (ENS). Only the sympathetic nerve fibers reach the mucosal layer of the intestine, where they can interact with immune cells. ChAT+ T-cells reside in the intestinal wall, possibly contributing to immunological homeostasis. Mφ = macrophages; NE = norepinephrine; ACh = acetylcholine.

Of note, the antagonizing effects of sympathetic activity must be appreciated at the receptor level. That is, in low concentrations (10^{-9} to 10^{-7} M), NE binds to α-adrenergic receptors that have pro-inflammatory properties, whereas in higher concentrations (10^{-7} to 10^{-5} M) NE has more affinity for β-adrenergic receptors with its anti-inflammatory effects. The opposite applies for adenosine, another neurotransmitter of the SNS that binds to A1 or A2 adenosine receptors.[92] Together with the facts that sympathetic nerve fibers are lost in inflammatory conditions and NE levels are decreased, a normally present anti-inflammatory b-adrenergic zone becomes a pro-inflammatory a-adrenergic zone.

The association between the SNS and the inflamed intestine is reciprocal as the intestinal inflammation can also affect the nerves and its adrenergic activity. Sympathetic innervation is markedly decreased in inflamed colonic tissue of IBD patients, as well as in various colitic mouse models. In IBD patients, a loss of tyrosine hydroxylase $(TH)^+$ nerve fibers was demonstrated, with TH being the rate-limiting enzyme for the production of epinephrine and NE. Furthermore, a marked preponderance of pro-inflammatory SP^+ fibers was found.[40] The increased presence of nerve repellent factors such as semaphorin 3C, a protein that exerts repulsive actions against sympathetic fibers specifically, possibly contributes to this differential loss.[93] Fontgalland and colleagues suggest that somatostatin and NOS, both anti-inflammatory neuropeptides, also interfere with the nerve loss as both somatostatin- and NOS-labeled nerves are reduced in inflamed tissue.[94]

The loss of sympathetic nerves in inflamed tissue would logically result in a reduction in sympathetic neurotransmitter levels. Significantly lower NE levels were indeed found in Crohn's disease patients compared to healthy controls.[95] This is supported by the fact that the release of NE from sympathetic nerve terminals is restricted in inflamed tissue.[96-98] The inflammation induced inhibition could augment to the chronicity of the inflammation as NE negative immune regulation is dampened. Moreover, next to the anti-inflammatory role of the SNS that is the focus of this review, the main role of sympathetic nerves lies in vasoregulation. Loss of these nerves results in an impaired blood flow, which could add up to sustaining the inflamed environment. The SNS can exert opposing pro- and anti-inflammatory functions, depending on the concentration of neurotransmitters and neuropeptides (that is reliant on their release and the presence of sympathetic nerves), the amount and availability of receptors, the receptor affinity and the timing of sympathetic activity. No consensus exists on the role of the SNS as well as the inflammatory milieu in continuance of the inflammatory processes. Interestingly, a similar discussion on the direction of neuroanatomical change endures in the field of rheumatology.[99-101] It has still to be elucidated whether the changes in sympathetic activity are the result of chronic inflammation or vice versa. It could be a combination as the SNS seems to act conflicting. Exerting pro-inflammatory effects have been observed at the early inflammatory phase and anti-inflammatory in the chronic phase of inflammation.[40] Different experimental models of colitis (acute and chronic) might provide more clarity on the role of the SNS in different stages of the disease. However, both loss of sympathetic activity and local inflammation may lead to an unfavorable situation that supports the ongoing disease processes.

NEUROPEPTIDES

Various subtypes of previously mentioned neuropeptide receptors are also present on immune cells suggesting a possible impact of these molecules on immunity. Due to the anti-inflammatory properties, the therapeutic potential of these have been studied extensively, both in experimental and clinical setting. In TNBS-induced colitis administration of VIP induced a remarkable amelioration with lower levels of pro-inflammatory chemokines and cytokines, inhibition of Th1 responses and induction of a Th2 immune response. Administration of CGRP had a similar effect.[102,103] In rat CGRP protected the colonic mucosa against TNBS in both the early and late phase of inflammation, while the antagonist of CGRP, hCGRP, exacerbated TNBS-induced inflammation.[104] Results on substance P are conflicting with studies showing beneficial effects in DSS- and TNBS-induced colitis models[105,106] and studies showing the opposite.[107,108] NPY, one of the most abundant peptides in the autonomic nervous system, and serotonin have a pro-inflammatory effect.[109-114] However, these results have not been supported by clinical evidence and hence, clinical significance of these results remains unclear.

NEURONAL INNERVATION AND MICROBIOTA

Not only does the SNS impact the intestinal immunity directly, it has also been suggested that there is an association with the gut microbiota. Firmicutes (F) and Bacteroidetes (B) are two major phyla of the domain Bacteria and dominate the human intestinal microbiota. An increased F/B ratio has been associated with obesity [115] and IBD[116]. Therefore, these days it is being used as marker for pathological conditions.[117] Yang and colleagues made use of a new bone marrow chimera mouse model lacking b-adrenergic receptor 1 or 2 and showed a significant shift in the Bacilli class of Firmicutes in the colon, more specifically in the family of Lactobacillaceae. This was in line with the findings that were published by Bartley et al[117] who showed that reduced b-adrenergic signaling leads to beneficial shifts in the gut microbiota. However, no significant change was found in F/B ratio. Intriguingly, in both studies the sympathetic depletion lead to a beneficial shift in the gut microbiota composition (i.e., comparable diversity and richness, and decreased Proteobacteria phylum) associated with immune suppression.[118] This is in sharp contrast with the anti-inflammatory effects of NE on cytokine level.

The role of the PNS in modulation of the gut microbiome is still equivocal. Late research in Parkinson's Disease, a common neurogenerative disorder, has demonstrated that atrophy of the vagus nerve represents an important route of disease progression.[119] This pathogenesis has been associated with changes in the gut microbiota composition and inflammation.[120-122] However, this could also be the cause of Parkinson Disease rather than the result, since VNS did not alter gut microbiota compositions in mice in another study focusing on Amyotrophic Lateral Sclerosis.[123] In this study, during surgery the animals of the experimental group received one hour of VNS. Chronic use of VNS might affect the microbiota and these studies are therefore warranted.

The intestinal microbiome greatly interacts with the mucus barrier. The intestinal epithelium is covered by a dense layer of mucus that functions as the primary defense barrier hosting antimicrobial peptides and preventing bacterial translocation into underlying tissues. Goblet cells (GC) are simple columnar epithelial cells that act as the primary source of mucins that form the mucus layer. Interestingly, ulcerative colitis but not Crohn's Disease has been associated with a defective colonic mucus layer and a reduced number of GC.[124] Various types

of GC exist and their differentiation is based on their location and function. The surface GC secrete mucins continuously to maintain the mucus layer, whereas GC located in the intestinal crypts secrete mucins upon stimulation. The secretion is party controlled by the PNS, since it has been shown that acetylcholine induces rapid transient increase in mucus secretion in mouse, rat, rabbit and human colon.[125-128] The critical role for the autonomic nervous system is stressed by the finding of altered GC differentiation in Hirschprung disease. The congenital aganglionosis leads to increased GC differentiation and proliferation resulting in changed mucus properties, increasing the susceptibility for inflammation.[129] Taken together, the autonomic nervous system is able to modulate the intestinal microbiota, possibly through the PNS and assured through the SNS.

IMPACT OF THE AUTONOMIC NERVOUS SYSTEM ON INTESTINAL EPITHELIAL PROLIFERATION

Mucosal healing is considered to be a major prognostic factor in the management of IBD. The SNS as well as the PNS have been linked to enhanced cell proliferation and tissue regeneration in multiple organs.[130] Recent studies show that the nervous system can also alter intestinal epithelial cell proliferation.[131] However, the mechanism of this effect has not been identified yet. It can be either indirectly (via food intake,[132] or inflammation as previously described), or directly. The latter could be due to the fact that sympathetic nerves come in close contact to the intestinal epithelium and hence autonomic neurotransmitters can bind to receptors on proliferating cells in the intestinal epithelium.

Surgical and chemical ablation of autonomic nerves with subsequent loss of neurotransmitters has been associated with an alteration in intestinal epithelial cell proliferation. Various studies have aimed to identify the underlying pathway. However, results are indecisive on whether autonomic neuronal activity would be anti- or pro-proliferative. After SNS or PNS denervation, epithelial cell proliferation has shown to either decrease[130,133-135] or increase.[136] Results are time dependent and effects seem to recover due to compensatory mechanisms, such as upregulation of adrenergic or cholinergic receptor expression, compensation by non-denervated branch, and modulation of the ENS.[137] No data exist on change in epithelial cell proliferation after sympathetic or parasympathetic stimulation, rather than denervation. Potentially, this has a more long-lasting and substantial effect.

Both sympathetic and parasympathetic neurotransmitter receptors are expressed in the crypt, villus and epithelial stem cells (figure 4 and 5).[138,139] NE and ACh released from these terminals could bind to receptors on stem cells, transit amplifying cells,[131] a combination of these or interact with other cells involved in epithelial cell proliferation pathways. $Lgr5^+$ crypt base columnar cells, which lie deep in the crypts of Lieberkühn are known for their important role in cell proliferation. However, cell types like Paneth cells, interspersed among the stem cells, or stroma cells such as (myo)fibroblasts and glia cells could also take part in these processes, since they are in close proximity. It is likely that they impact epithelial cell proliferation and differentiation partly through the release of cytokines. Cytokines influence the expression of tight junctions and stem cell proliferation and have therefore a pivotal role in modulating the intestinal epithelial barrier and its underlying cells. This also accounts for other factors, such as neuropeptides. For instance, SP has a pro-proliferative role in epithelial cell growth.[140,141]. Its receptors are present in colonic mucosa.

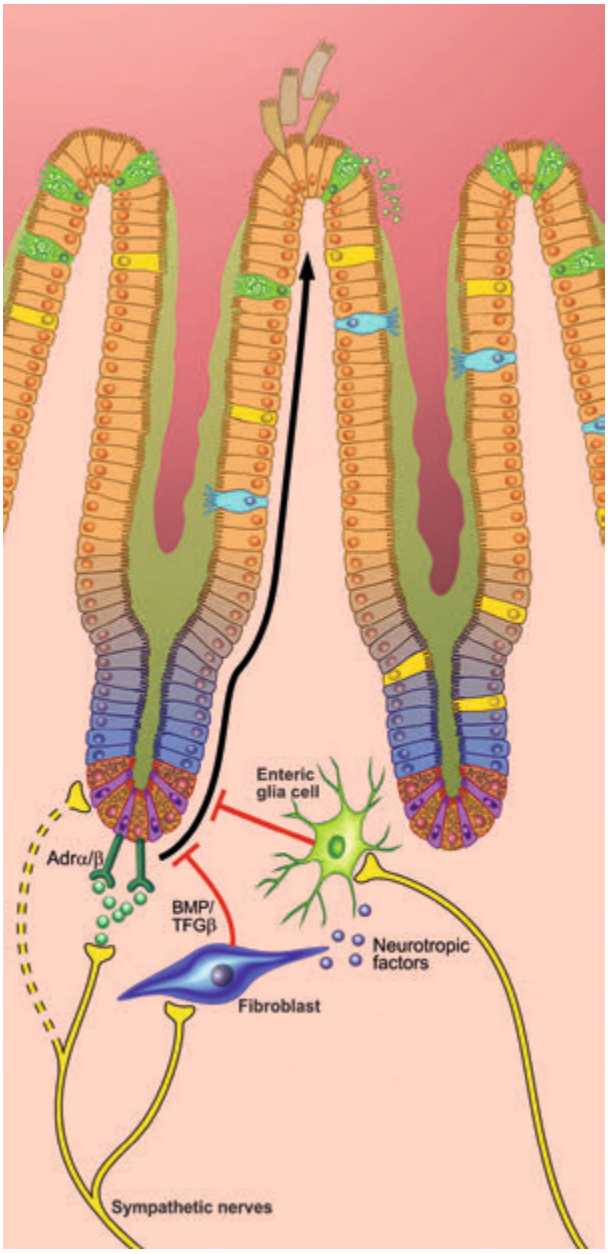

Figure 4. Proposed model of innervation of the intestinal crypt. Sympathetic nerves can affect the proliferation in the crypt in multiple ways. Sympathetic neural activity inhibits proliferation through fibroblasts (that produce bone morphogenetic protein (BMP) and transforming growth factor (TGF-β)) and enteric glia cells that express adrenergic receptors. Enteric glia cells can also produce neurotrophic factors that are critical in the growth, survival and differentiation of nerves. In addition, adrenergic receptors are present on cells within the crypt, suggesting that sympathetic neural activity affects proliferative processes directly.

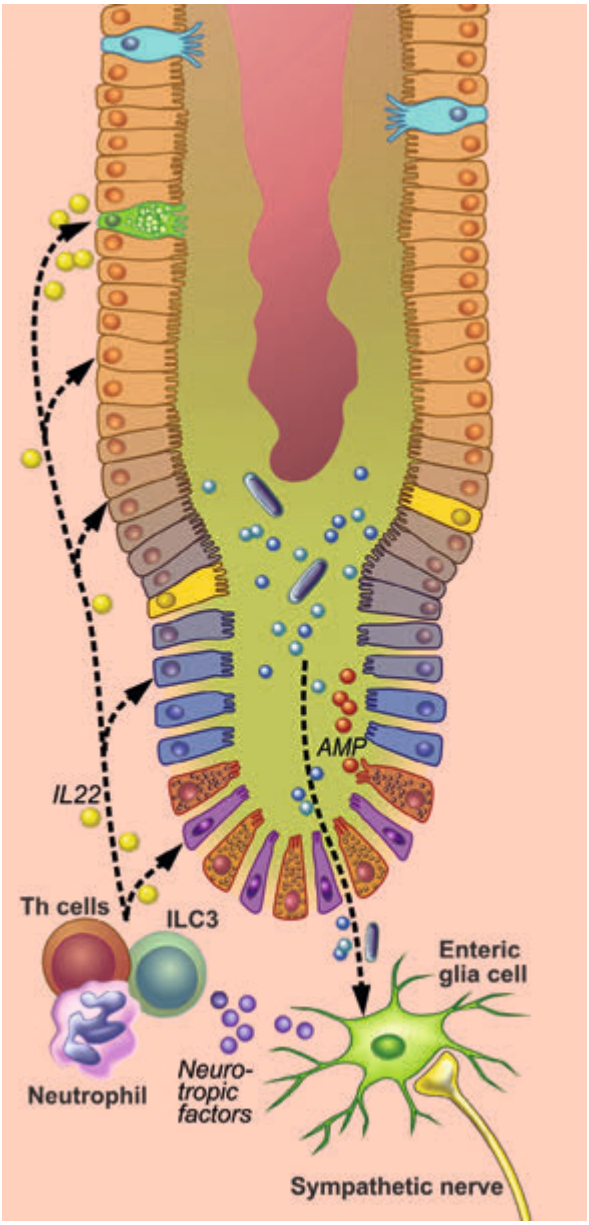

Figure 5. Inflammatory processes in the intestinal crypt. In inflammatory state, enteric glia cells are activated by pro-inflammatory cytokines and factors like antimicrobial peptides. Under influence of sympathetic neural activity neurotrophic factors are produced and extruded. Inflammatory cells like neutrophils, T-helper (Th-)cells and type 3 innate lymphoid cell (ILC3), interleukin (IL-)22 is produced, that plays a pivotal role in modulating inflammation and stimulating host defense/antimicrobial peptide secretion.

Enteric glial cells (EGC) reside beneath the epithelial layer. Their astrocyte-like shape suggests a communicating role with the central nervous system. Neunlist and colleagues showed that EGC inhibit intestinal cell proliferation through a transforming growth factor (TGF)-β_1-dependent pathway[142]. This anti-proliferative effect was underlined by another study that demonstrated an additive upregulation of differentiation-related genes via activation of peroxisome proliferator-activated receptor γ, PPARγ. [143]. Conversely, in a more recent study genetic ablation of EGC did not alter intestinal epithelial proliferation. Despite this, it is thought that EGC might influence the epithelium when "activated" by certain infectious or immunological conditions. Glia can adopt pro- or anti-inflammatory phenotypes depending on the context[144] and EGC regulate the neurotoxic effects of intestinal inflammation.[145] Given the fact that these cells express β_2-adrenergic receptors [146], they could play a role in the proposed interaction between the SNS and epithelial proliferation.

The role of the PNS in epithelial cell proliferation remains debatable, since no direct innervation of the epithelium has been demonstrated yet. It is likely that the ENS plays an important role in this pathway connecting the PNS to the intestinal epithelial barrier through ACh signaling. Also, denervation of the PNS alters the proliferative rate of the intestinal epithelium while the ENS remains intact.[133-135,147] Despite the fact that the ENS as well as the PNS exert their functions through ACh, hindering the demonstration of the parasympathetic attribution to epithelial proliferation, this suggests that the PNS can modulate the proliferation directly.

Thus, modulation of (para)sympathetic activity could have a beneficial effect in proliferative processes in the intestinal epithelium either directly or indirectly via various cell types and could hence act as new therapeutic target in processes such as wound healing in IBD or postoperatively. Further research is needed to enlighten the involved pathways.

CLINICAL STUDIES IN THE FIELD OF BIOELECTRONICS

The extensive connections between the autonomic nervous system and the intestine together with the prevalence of the intestinal disturbances or diseases that are associated with neuronal activity makes the innervation of the gut an appealing target for new treatment methods. So far, various clinical trials have investigated the use and efficacy of (para)sympathetic neuromodulatory techniques in the treatment of inflammation. Bioelectronic Medicine, mainly represented by VNS, opens new therapeutic avenues for treatment modalities for IBD. It has already proven its efficacy in rheumatoid arthritis,[6] sepsis,[148] kidney ischemia-reperfusion injury[48] and Crohn's Disease.[7,34,35] Trials on application of VNS are still ongoing in postoperative ileus, juvenile idiopathic arthritis and systemic lupus erythematosus. A small clinical trial performed by Bonaz et al demonstrated that cervical VNS was able to reduce clinical and biological symptoms in 5 out of 7 subjects with active Crohn's Disease.[7] Well-designed, randomized-controlled studies have to be conducted to confirm these promising results. Since cervical VNS also influences physiological functions such as heart rate, stimulation of the abdominal branches of the vagus nerve might be a more targeted approach.[149]

In recent years, developments on non-invasive neuromodulatory techniques has been of interest. Transcutaneous VNS (tVNS) appeals promising as no surgical implantation is required. Instead, the auricular concha that is innervated by the vagus nerve can be stimulated transcutaneously.[150,151] Several devices have been investigated, such as The Cerbomed NEMOS stimulator (Erlangen, Germany) and the electroCore LLC gammaCore device (Basking Ridge,

NJ, USA), which was originally designed to treat primary headache delivering electrical signals to the cervical part of the vagus nerve. Lerman et al have shown that tVNS through use of the gammaCore device decreased cytokine and chemokine levels in healthy individuals.[152] Although the advancement in these noninvasive techniques is encouraging, risk for noncompliance should be taken into account.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last decades, many studies have revealed an important role of the autonomic nervous system in modulating intestinal immunity. Through research into the role of both the SNS as well as the PNS, the role of the SNS has been cued as fundamental in the intervention of immune processes. Many open questions however remain. Further research is needed to elucidate the specific cells under influence of neurotransmitters in both healthy and diseased conditions since data are conflicting. For instance, specialized epithelial cells including those present in the crypt stem cell niche, express neurotransmitter receptors but whether such cells are truly innervated and functionally affected remains to be established. Local neurotransmitter concentrations critically direct the types of receptors activated especially in the case of adrenergic receptor classes, further complicating conclusive studies in this field. Nonetheless, it is evident that the autonomic nervous system has great potential to serve as therapeutic target for inflammatory diseases and to that end the advancement of new neuromodulatory techniques has to be pursued. The importance of inflammatory reflexes in regulating acute and chronic intestinal inflammatory disorders is emerging and the ENS appears as a pivotal element linking sympathetic and more particularly vagal inputs to the immune system.

Clinically, an added immune regulatory function by neural interfacing may provide us with a powerful tool to enhance remission in IBD patients. Such applications may be more feasible and less invasive than originally thought, making use of implantable devices. Neural interfacing technology provides the basis for mapping neural signals and for Bioelectronic Medicines. Electrode-based interfaces must be adapted to interrogate visceral nerve activity effectively, but more pre-clinical work seems necessary to determine this as a true treatment paradigm.

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02

ACETYLCHOLINE-PRODUCING T-CELLS AUGMENT INNATE IMMUNE DRIVEN COLITIS BUT ARE REDUNDANT IN T-CELL DRIVEN COLITIS

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American Journal of Physiology – Gastrointestinal and Liver Physiology 2019

ABSTRACT

Clinical trials suggest that vagus nerve stimulation presents an alternative approach to classical immune suppression in Crohn's disease. T-cells capable of producing acetylcholine ($ChAT^+$ T-cells) in the spleen are essential mediators of the anti-inflammatory effect of vagus nerve stimulation. Besides the spleen, $ChAT^+$ T-cells are found abundantly in Peyer's patches of the small intestine. However, the role of $ChAT^+$ T-cells in colitis pathogenesis is unknown. Here, we made use of $CD4^{cre}ChAT^{fl/fl}$ mice ($CD4ChAT^{-/-}$ mice), lacking ChAT expression specifically in $CD4^+$ T-cells. Littermates ($ChAT^{fl/fl}$ mice) served as controls. In acute dextran sulfate sodium (DSS)-induced colitis (7 days of 2% DSS in drinking water), $CD4ChAT^{-/-}$ mice showed attenuated colitis and lower intestinal inflammatory cytokine levels compared to $ChAT^{fl/fl}$ mice. In contrast, in a resolution model of DSS-induced colitis (5 days of 2% DSS followed by 7 days without DSS),

$CD4ChAT^{-/-}$ mice demonstrated a worsened colitis recovery and augmented colonic histological inflammation scores and inflammatory cytokine levels as compared to $ChAT^{fl/fl}$ mice. In a transfer colitis model using $CD4^+CD45RB^{high}$ T-cells, T-cells from $CD4ChAT^{-/-}$ mice induced a similar level of colitis compared with $ChAT^{fl/fl}$ T-cells. Together, our results indicate that $ChAT^+$ T-cells aggravate the acute innate immune response upon mucosal barrier disruption in an acute DSS-induced colitis model, while they are supporting the later resolution process of this innate immune driven colitis. Surprisingly, ChAT expression in T-cells seems redundant in the context of T-cell driven colitis.

INTRODUCTION

Vagus nerve stimulation (VNS) is well recognized for its anti-inflammatory potential. It can lower inflammatory cytokine levels and dampen inflammation in animal models for sepsis, ileus, colitis and arthritis.[1-6] Currently, VNS is investigated in complex immune disorders like Crohn's disease (CD) and rheumatoid arthritis. In clinical trials, five out of seven patients with CD responded to VNS, while fifty percent of patients with rheumatoid arthritis were in remission or had a good response to VNS.[7,8] The mechanism by which VNS improves CD is likely to be indirect since vagal cholinergic fibers do not reach the inflamed colonic mucosa.[9] Furthermore, vagal denervation did not affect the course of colitis,[10] in line with the observation that vagal innervation of the intestine, as identified with retrograde tracing, is sparse in the mucosal and submucosal layer of the intestine.[11] In general, it is recognized that the anti-inflammatory effect of VNS in endotoxemia involves activation of the sympathetic, adrenergic splenic nerve and the presence of acetylcholine-producing T-cells in the spleen.[12,13] Choline acetyltransferase (ChAT), the rate-limiting enzyme required for the synthesis of acetylcholine, can be used as marker for acetylcholine-producing T-cells.[13-15] ChAT$^+$ T-cells in the spleen have a regulatory, memory phenotype (CD44^{high}CD62L^{low}CD45RB^{low} cells).[13] Upon activation, splenic ChAT$^+$ T-cells produce interleukin (IL)-10, interferon (IFN)-γ and IL-17, indicating that ChAT expression in the spleen is not restricted to a specific T-cell subset.[13] A recent study showed that mice surviving sepsis after cecal ligation and puncture have higher vagus nerve activity and an increased number of ChAT$^+$ T-cells in the spleen.[16] Although most studies have focused on splenic ChAT$^+$ T-cells, it was demonstrated that blood-born ChAT$^+$ T-cells are important in regulating blood pressure by secreting acetylcholine and that ChAT$^+$ T-cells play a vital role in controlling viral infection.[17,18]

We and others demonstrated that ChAT$^+$ T-cells also reside in the intestinal mucosa and Peyer's patches (PPs).[13,14,19] Because many non-neuronal cells in the intestine express receptors for acetylcholine, ChAT$^+$ T-cells likely interact with their direct surrounding through secretion of acetylcholine.[20-24] Interestingly, ChAT expression in T-cells increased upon activation via adrenergic β_2 receptors.[19] We showed that these ChAT$^+$ T-cells stimulate antimicrobial peptide expression and that mice lacking ChAT expression in CD4$^+$ T-cells display a higher microbiota diversity in the small intestine.[19] However, the relevance of ChAT$^+$ T-cells in an inflamed setting, such as colitis, is unclear. To this end, we aimed to investigate the role of ChAT$^+$ T-cells in diverse models of experimental colitis to study their role in innate, wound healing and effector T-cell derived functions.

In this study, we established that ChAT$^+$ T-cells are abundant in colon and PPs as compared to spleen and peripheral blood. Surprisingly, ChAT$^+$ T-cells aggravate DSS-induced colitis whilst ameliorating colitis in a DSS-induced resolution model. ChAT$^+$ T-cells do not play a role in the pathogenic effector function of T-cells a T-cell driven colitis model.

MATERIALS AND METHODS

Mice

Adult female C57BL/6 mice (Charles River Laboratories, Maastricht, The Netherlands), adult male and female ChAT-enhanced green fluorescent protein (eGFP)[25] mice and adult male and female Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Adult male and female CD4^{cre}ChAT$^{fl/fl}$ mice (described earlier, from this point referred to as CD4ChAT$^{-/-}$

mice[19]) were bred in our own facility. Mice were housed under specific pathogen free conditions in individually ventilated cages in the animal research institute of the Academic Medical Center in Amsterdam. Animals were maintained on a 12/12 hours light/dark cycle under constant temperature (20°C ± 2°C) and humidity (55%) and *ad libitum* food and drinking water. Mice were handled in accordance with guidelines of the Dutch Central Committee for Animal Experiments and the local Animal Research Ethics Committee of the University of Amsterdam. The same ethics committee approved the experimental protocols.

Flow cytometry

In order to determine the frequency of $ChAT^{+}$ T-cells, we collected the PPs, mesenteric lymph nodes (MLNs), spleen and colon in RPMI-1640 (ThermoFisher Scientific, Landsmeer, The Netherlands) on ice and blood in a heparin tube from four adult ChAT-eGFP mice (2 male and 2 female) and four adult female C57BL/6 mice. Colon tissue was cut open longitudinally, cut in 0.5 cm pieces and washed with phosphate buffered saline (PBS). Subsequently, it was incubated in calcium/magnesium-free Hanks' balanced salt solution (HBSS; Lonza, Basel, Switzerland) supplemented with 2% (v/v) fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands) containing 5 mM EDTA (ThermoFisher Scientific) for 20 minutes, shaking at 230 rpm at 37 °C. After incubation, we washed the tissue pieces with PBS and cut them into 0.1 cm pieces. Tissue pieces were incubated for 40 minutes under vigorous shaking at 37 °C with calcium/magnesium-free HBSS supplemented with 2% FCS, 62.5 µg/ml Liberase TL and 200 µg/ml DNase I (both enzymes from Roche Applied Bioscience, Almere, The Netherlands). The colon cell suspension was passed through a 40 µm cell strainer. PPs, MLNs and spleens were mashed and flushed through a 40 µm cell strainer. Erythrocytes were lysed in the spleen and blood samples by incubation with erythrocyte lysis buffer (containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 5 minutes on ice. All cell suspensions were washed with RPMI-1640, resuspended in FACS buffer (PBS with 0.5% Bovine Serum Albumin (BSA), 0.01% NaN_3 and 0.3 mM EDTA) and stained with PeCy7-conjugated CD4 (RM4-5; Affymetrix, Vienna, Austria). Cell staining was analyzed using a FACS Fortessa (BD Bioscience, San Jose, CA, USA).

To determine the frequency of ChAT expression in the cell subset that is used for T-cell transfer, we collected spleens from four adult female ChAT-eGFP mice and four adult female C57BL/6 mice, and mashed and flushed them through a 40 µm cell strainer. Erythrocytes were lysed by incubation with erythrocyte lysis buffer for 5 minutes on ice. Cell suspensions were washed with RPMI-1640, resuspended in FACS buffer and stained with PeCy7-conjugated CD4 and APC-conjugated CD45RB (C363-16A; Biolegend, San Diego, CA, USA). Cell staining was analyzed using a FACS Fortessa.

Dextran sulfate sodium (DSS)-induced colitis

For the acute DSS-induced colitis model in mice, 2% (w/v) DSS (TdB Consultancy, Uppsala, Sweden) was added to the drinking water for seven consecutive days. For the resolution model of DSS-induced colitis, 2% (w/v) DSS was added to the drinking water for five consecutive days, followed by normal drinking water for a subsequent seven days, making the total experiment length 12 days. Daily replacement of drinking water with fresh DSS solutions was performed. For the acute model, ten adult male or female mice (from both genotypes: $CD4ChAT^{-/-}$ and $ChAT^{fl/fl}$) received DSS, five extra mice of each genotype were kept on normal drinking water as control groups. For the resolution model, nine adult male or female mice (from both genotypes: $CD4ChAT^{-/-}$ and $ChAT^{fl/fl}$) received DSS, five extra mice of each genotype were kept

on normal drinking water as control groups. Gender and age were equally distributed over the groups. During the study, bodyweight and behavior were monitored daily. After seven or twelve days, mice were sacrificed and disease activity index was determined, measuring inflammation (0-3), diarrhea (0-3) and occult blood in the stool (0-3) as described before.[26] We measured colon wet weight and colon length. Colon weight per cm, indicating edema, was used as disease parameter. Colon tissue was cut in half longitudinally for histopathology. The other half of the colon was cut in half and used for measuring cytokine mRNA levels and protein concentrations.

Two percent DSS in the drinking water for seven consecutive days was given to ten ChAT-eGFP mice to address frequency changes due to acute DSS-induced colitis. Five extra ChAT-eGFP mice receiving normal drinking water served as control group. Both male and female adult mice were used and gender was distributed equally over the groups. During the study, bodyweight and behavior were monitored daily. After seven days, the animals were sacrificed and PPs and MLNs were collected, mashed, and flushed through a 40 µm cell strainer. Cell suspensions were washed with RPMI-1640, resuspended in FACS buffer and stained with PeCy7-conjugated CD45 (30-F11; Biolegend, San Diego, CA, USA), DAPI (Brunschwig, Amsterdam, The Netherlands), PE-conjugated CD3 (145-2C11; BD Bioscience), and PerCP-Cy5.5-conjugated CD4 (RM4-5; Affymetrix). Cell staining was analyzed using a SH800S Cell Sorter (Sony Biotechnology, Weybridge, United Kingdom).

T-cell transfer colitis

We applied the T-cell transfer model as described.[27,28] In short, $CD4^{+}CD45RB^{high}$ cells were isolated from spleens with magnetic bead depletion and fluorescence-activated cell sorting. Spleens from $CD4ChAT^{-/-}$ mice and littermate controls ($ChAT^{fl/fl}$ mice) were used. Eleven $Rag1^{-/-}$ mice per group were transferred with $3x10^{5}$ $CD4^{+}CD45RB^{high}$ cells intraperitoneally (i.p.). Five $Rag1^{-/-}$ mice did not receive the cell transfer and served as control group. To check if $ChAT^{+}$ T-cells could be traced back at the end of the transfer experiment, four $Rag1^{-/-}$ mice were injected with $CD4^{+}CD45RB^{high}$ cells from spleens of ChAT-eGFP mice. Adult male and female $Rag1^{-/-}$ mice were randomly divided over the groups, creating identical groups concerning gender and age. Bodyweight and behavior were assessed three times a week. Animals reaching bodyweight loss of 20% from start and showing sickness behavior were sacrificed before seven weeks. In that case, all parameters were taken along, tissues were still assessed and last bodyweight carried forward was used in the analysis of the bodyweight over time. After three, five and seven weeks, endoscopy was performed under anesthesia with 3% isoflurane/O_2 to assess colonic inflammation. The Olympus URF type V endoscope (Zoeterwoude, The Netherlands) was rectally inserted for a maximum of 5 cm and videos of the endoscopy were recorded using a Medicap USB200 Medical Digital Video Recorder (Roermond, The Netherlands), when retracting the endoscope. A blinded and trained technician determined the murine endoscopic index of colitis severity (MEICS), consisting of wall thickening, vascularity, visible fibrin, granularity and stool consistency, scored between 0 and 3.[29] This determined a total endoscopy score ranging from 0 to 15. After seven weeks, the animals were sacrificed. Disease activity index was determined, measuring inflammation (0-3), diarrhea (0-3) and occult blood in the stool (0-3) as described before.[26] Spleen weight was measured, a measurement for inflammation and successful T-cell transfer. We measured colon wet weight and colon length. Colon weight per cm, indicating edema, was used as a disease parameter. Colon tissue was cut

in half longitudinally for histopathology. The other half of the colon was cut in half and used for measuring cytokine mRNA levels and protein concentrations.

Blood was collected from four $Rag1^{-/-}$ mice that received cell transfer from ChAT-eGFP mice to determine the frequency of ChAT expressing cells seven weeks after transfer. Blood from five $Rag1^{-/-}$ mice that received cell transfer from $ChAT^{fl/fl}$ mice was used as control. Erythrocytes were lysed by incubation with erythrocyte lysis buffer for 5 minutes on ice. Cell suspension was washed with RPMI-1640, resuspended in FACS buffer and stained with PeCy7-conjugated CD4. Cell staining was analyzed using a FACS Fortessa.

Histopathology

Colon tissue was fixed in 4% formalin according to protocol and embedded in paraffin for routine histology. A blinded and experienced researcher evaluated formalin-fixed hematoxylin and eosin (HE) stained tissue sections microscopically and scored the sections based on four (T-cell transfer colitis) or five (DSS-induced colitis) characteristics of inflammation explained in Table 1 and Table 2. This resulted in the mouse colitis histology index (MCHI), based on Koelink *et al*.[30]

	0	1	2	3
Goblet cell loss	None	< 10%	10-50%	> 50%
Crypt density	Normal	Decrease of < 10%	Decrease of ≥ 10%	
Hyperplasia	None	Slightly increased crypt length	2-3 times increased crypt length	> 3 times increased crypt length
Submucosal infiltrate	None	Individual cells	Infiltrate(s)	Large infiltrate(s)

Table 1. MCHI for T-cell transfer colitis model.[30] Goblet cell loss is weight 1x, crypt density 2x, hyperplasia 2x and submucosal infiltrate 3x. Total score ranges from 0 to 22.

	0	1	2	3
Goblet cell loss	None	< 10%	10-50%	> 50%
Crypt density	Normal	Decrease of < 10%	Decrease of ≥ 10%	
Hyperplasia	None	Slightly increased crypt length	2-3 times increased crypt length	> 3 times increased crypt length
Submucosal infiltrate	None	Individual cells	Infiltrate(s)	Large infiltrate(s)
Area involved	None	< 10%	10-50%	> 50%

Table 2. MCHI for DSS-induced colitis model.[30] All subscores are weight equal. Total score ranges from 0 to 14.

RNA isolation, cDNA synthesis and quantitative PCR analysis

We extracted RNA from frozen colon tissue after homogenization of the samples in TriPure isolation reagent according to manufacturer's instructions (Roche Applied Science). RNA from the DSS-induced colitis experiments was cleaned with the Bioline ISOLATE II mini kit (GC biotech B.V., Alphen a/d Rijn, The Netherlands) according to manufacturer's protocol. cDNA was synthesized using dNTPs (ThermoFisher Scientific), Random primers (Promega, Leiden, The Netherlands), Oligo dT primers (Sigma, Zwijndrecht, The Netherlands), Revertaid and Ribolock (both ThermoFisher Scientific). Quantitative polymerase chain reaction (PCR) was performed using SensiFAST SYBR No-ROX (GC biotech B.V.) on a LightCycler 480 II (Roche Applied Science) to analyze expression levels of murine IL-1β, IL-6, IL-10, IL-17A, tumor necrosis

factor (TNF)-α, IFN-γ, mucin 2 (Muc2), inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1) using LinRegPCR software.[31] For normalization, murine reference genes hypoxanthine phosphoribosyltransferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (RPLP0), cyclophilin and eukaryotic translation elongation factor 2 (EEF2) were selected, after analysis for stability in geNorm.[32] Primers (synthesized by Sigma) are listed in Table 3.

Gene	Forward sequence	Reverse sequence
IL-1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
IL-6	GAGTTGTGCAATGGCAATTCTG	TGGTAGCATCCATCATTTCTTTGT
IL-10	TGTCAAATTCATTCATGGCCT	ATCGATTTCTCCCCTGTGAA
IL-17A	AAAGCTCAGCGTGTCCAAACA	CTTCATTGCGGTGGAGAGTC
TNF-α	TGGAACTGGCAGAAGAGGCACT	CCATAGAACTGATGAGAGGGAGGC
IFN-γ	TACTACCTTCTTCAGCAACAGC	AATCAGCAGCGACTCCTTTTC
Muc2	TGAAGACCGAGATTGTGCCC	AGATGACGTTGAGCTGGGTG
iNOS	CACCAAGCTGAACTTGAGCGA	GCCCCATAGGAAAAGACTGCA
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
HPRT	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
GAPDH	GACAACTCATCAAGATTGTCAGCA	TTCATGAGCCCTTCCACAATG
RPLP0	CCAGCGAGGCCACACTGCTG	ACACTGGCCACGTTGCGGAC
Cyclophilin	ATGGTCAACCCCACCGTGT	TTCTGCTGTCTTTGGAACTTTGTC
EEF2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA

Table 3. Primer sequences for qPCR

Protein concentrations of cytokines in intestinal tissue

Frozen colon tissue was homogenized on ice in Lysis Buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl{\cdot}6H_2O$, 1 mM $CaCl_2$, 1% Triton) with protease inhibitor cocktail (Roche Applied Science), pH 7.4, diluted 1:1 with PBS. In these tissue lysates, protein concentrations of IL-6, IL-10, TNF-α, IFN-γ and monocyte chemoattractant protein (MCP)-1 were measured with a mouse inflammation kit by BD cytometric bead assay (CBA; BD Bioscience) according to manufacturer's protocol, with the exception that reagents were 10 times diluted.

Statistical analysis

Graphs were made with Prism 7.0 (GraphPad Software, La Jolla, CA). For all experiments, we used a Kolmogorov-Smirnov test to determine normality of distribution. In IBM SPSS Statistics Version 25 (IBM Corporation, Armonk, NY), an independent T-test or Mann-Whitney U test (2 groups) or an ANOVA or Kruskal-Wallis test (> 2 groups) was used to check for statistical significance. If the ANOVA analysis gave a significant difference, then a Bonferroni correction for multiple comparisons was done. If the Kruskal-Wallis analysis gave a significant difference, a pairwise comparison with post-hoc Dunn's test was performed. All data are expressed as mean (if distributed normally) or median (if not distributed normally) plus standard deviation (SD), interquartile range (IQR) or individual data points. P-value (P) < 0.05 was considered significant.

RESULTS

ChAT+ T-cells are enriched in the intestine compared to peripheral blood and spleen.
We first investigated the frequency of ChAT$^+$ T-cells in the spleen, colon, MLNs, PPs, and peripheral blood. Examples of the flow cytometry plots are shown in Figure 1A. The percentage of ChAT$^+$ T-cells was highest in the colon and PPs, followed by the MLNs (Figure 1B). Surprisingly, although its pivotal role in the anti-inflammatory reflex of VNS is described extensively,[13,33-35] the frequency of ChAT$^+$ T-cells in the spleen was much lower than in the structures involved in intestinal immunity. The frequency of ChAT$^+$ T-cells in the PPs is in line with earlier data.[19] Because ChAT$^+$ T-cells are highly enriched in the intestine, we hypothesized that these cells are crucial for maintaining gut immune homeostasis.

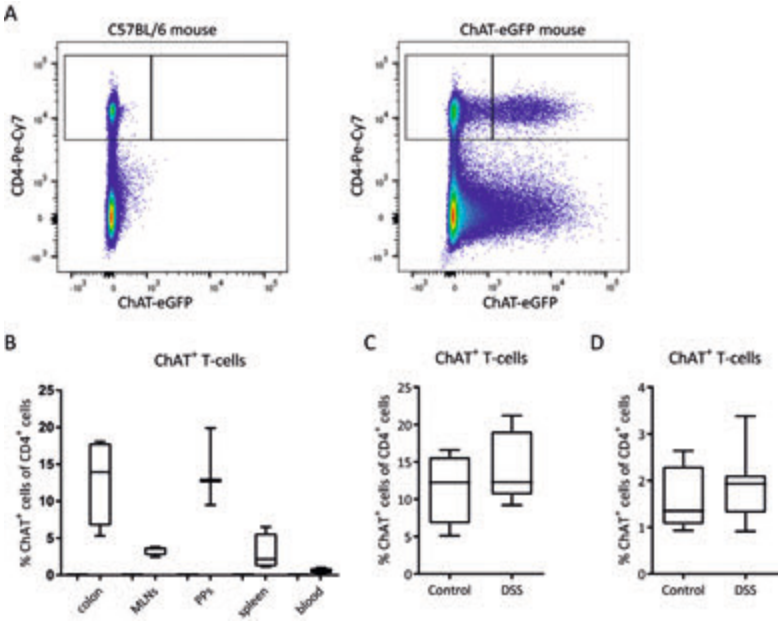

Figure 1. Frequency of ChAT$^+$ T-cells in different organs. (A) Example of the gating of ChAT$^+$CD4$^+$ T-cells. Shown is spleen tissue. (B) Quantification of the ChAT$^+$ T-cell frequency of the total CD4$^+$ population measured by flow cytometry in colon, mesenteric lymph nodes (MLNs), Peyer's patches (PPs), spleen and peripheral blood of four C57BL/6 mice (all female, left boxes) and ChAT-eGFP mice (2 male and 2 female, right transparent boxes). (C-D) ChAT$^+$ T-cell frequency from the total CD4$^+$ cell population measured by flow cytometry in PPs (C) and MLNs (D). Shown is the difference between ChAT-eGFP mice on normal drinking water (Control) and ChAT-eGFP mice that are treated with 2% dextran sulfate sodium (DSS) for seven consecutive days (DSS). N = 4-9, both male and female. Representative of two independent experiments is shown. Data are expressed as Box-Whisker-Plots indicating the median ± Min-Max. We tested for statistical significant differences with a Mann-Whitney U test. P-value < 0.05 was considered significant.

ChAT+ T-cells worsen inflammation in acute DSS-induced colitis.
We measured the frequency of ChAT$^+$ T-cells during acute DSS-induced colitis using ChAT-eGFP mice. The frequency of ChAT$^+$ T-cells in the PPs as well as the MLNs slightly increased after DSS exposure, however not significant (P=0.71 and P=0.70 respectively; Figure 1C-D). Next, we tested the effect of acute DSS-induced colitis in CD4ChAT$^{-/-}$ mice and ChAT$^{fl/fl}$ mice. ChAT deficiency did not affect bodyweight loss over time (Figure 2A). Clinical parameters such as

disease activity index and histology showed a trend towards a worsened colitis in $ChAT^{fl/fl}$ mice as compared to $CD4ChAT^{-/-}$ mice, although these parameters were not increased with statistical significance (disease activity index P=0.22; histology P=0.62; Figure 2B-D). This was also evident with respect to cytokine levels in the colon (Figure 2E-F). IL-6 expression was significantly higher in the $ChAT^{fl/fl}$ mice compared with the $CD4ChAT^{-/-}$ mice (resp. mean: 1 vs. 0.35; P=0.04; Figure 2F) while Muc2 expression was reduced in $CD4ChAT^{-/-}$ mice as compared to $ChAT^{fl/fl}$ mice (resp. mean: 0.76 vs. 1; P=0.02; Figure 2F) reflecting goblet cell depletion.

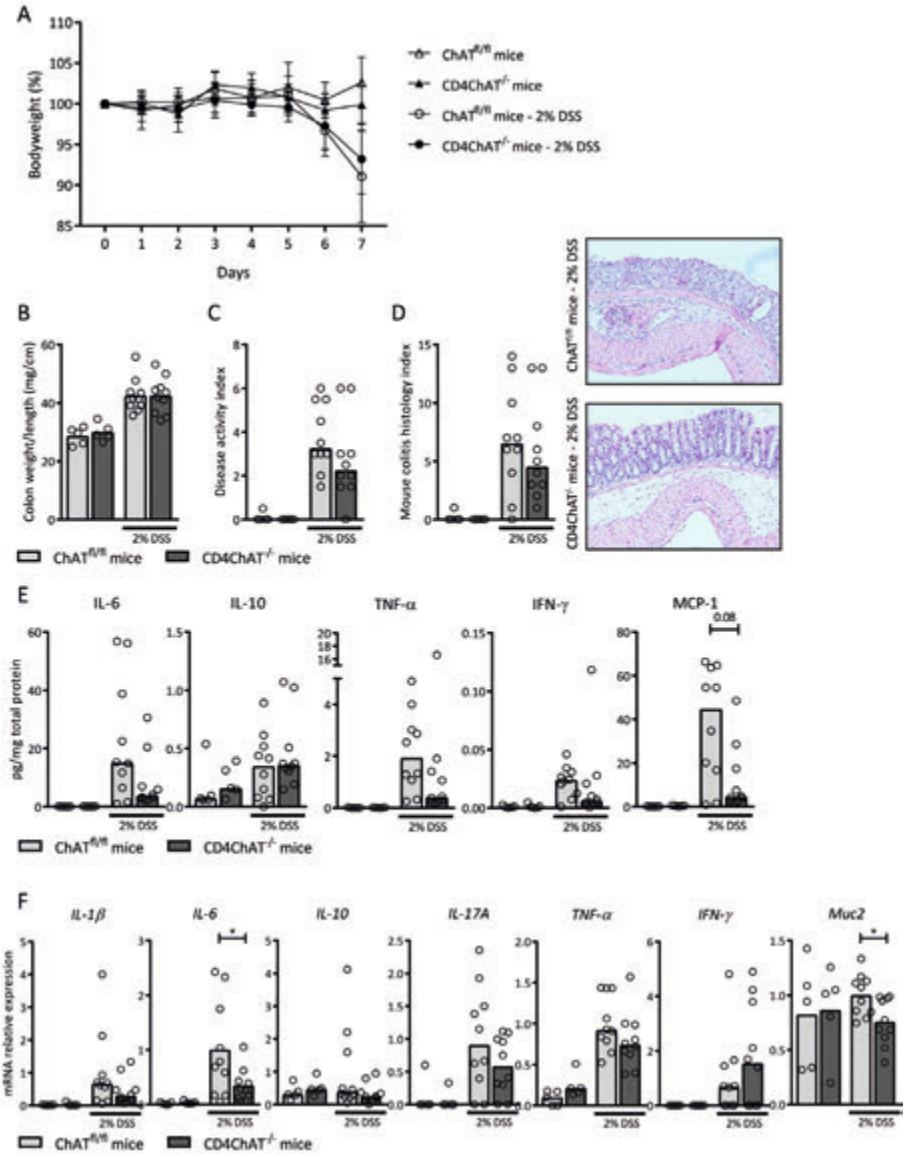

Figure 2. ChAT^{+} T-cells worsen inflammation in acute dextran sulfate sodium (DSS)-induced colitis. (A) Bodyweight loss of mice over time, compared with day 0. Data are expressed as mean and standard deviation (SD). (B) Colon weight as a marker of edema and inflammation, normalized for colon length. (C) Disease activity index for colitis based on Melgar *et al*.[26] (D) Mouse colitis histology index, based on Koelink *et al*.[30] Two representative pictures are shown of a hematoxylin and eosin staining, magnification 100x. (E) Protein levels of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 in colon homogenates, normalized for total protein levels. (F) mRNA levels of IL-1β, IL-6, IL-10, IL-17A, TNF-α, IFN-γ and mucin 2 (Muc2) in colon homogenates. mRNA levels are normalized for reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0) and relative compared to ChAT$^{fl/fl}$ mice receiving 2% DSS. N = 5-10, both male and female mice. Data are expressed as mean (B) or median (C, D, E and F (except IL-6 and Muc2)) and individual data points. We tested for statistical differences between ChAT$^{fl/fl}$ mice and CD4ChAT$^{-/-}$ mice receiving DSS with an independent T-test or a Mann-Whitney U test. $P < 0.05$ was considered significant. * $P < 0.05$

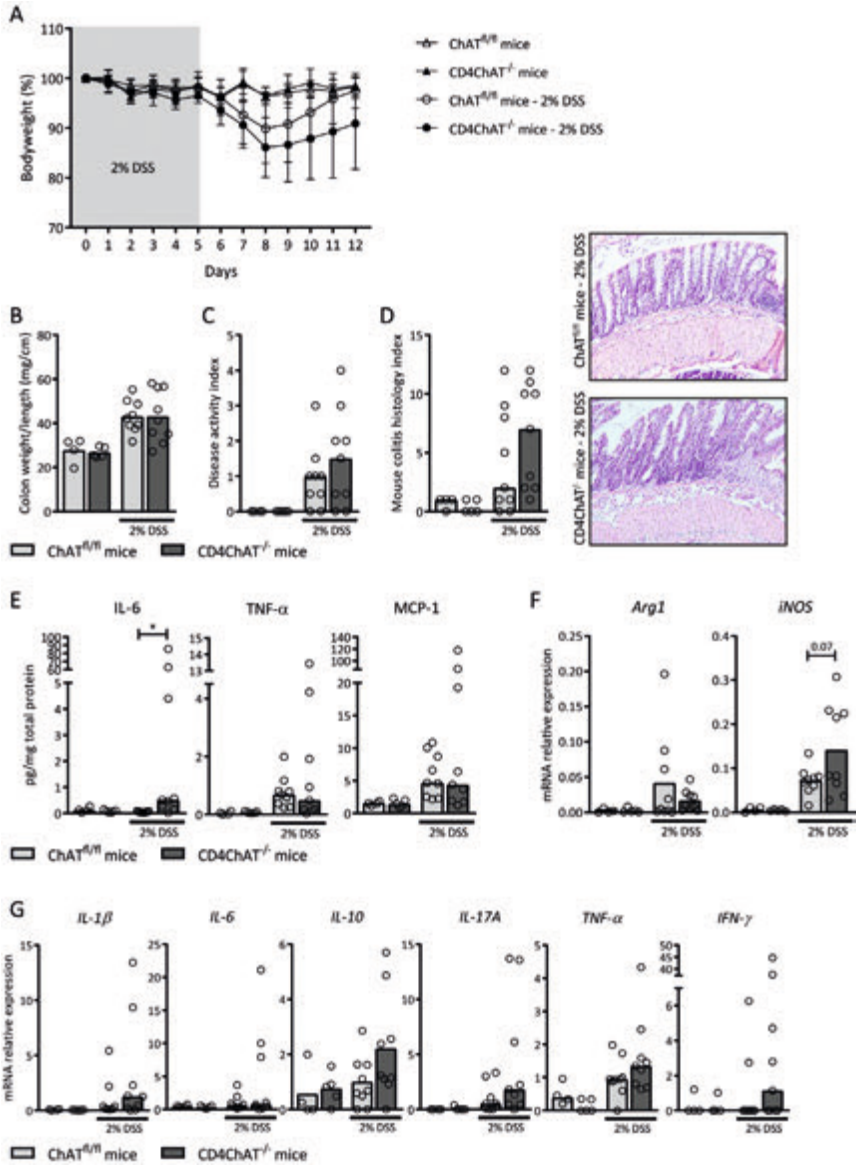

Figure 3. ChAT⁺ T-cells support resolution of intestinal inflammation induced by dextran sulfate sodium (DSS). (A) Bodyweight loss of mice over time, compared with day 0. Data are expressed as mean and standard deviation (SD). (B) Colon weight as a marker of edema and inflammation, normalized for colon length. (C) Disease activity index for colitis based on Melgar *et al*.[26] (D) Mouse colitis histology index, based on Koelink *et al*.[30] Two representative pictures are shown of a hematoxylin and eosin staining, magnification 100x. (E) Protein levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 in colon homogenates, normalized for total protein levels. IL-10 and interferon (IFN)-γ were below detection limit. (F & G) mRNA levels of inducible nitric oxide synthase (iNOS) and Arginase 1 (Arg1) IL-1β, IL-6, IL-10, IL-17A, TNF-α and IFN-γ in colon homogenates. mRNA levels are normalized for reference genes cyclophilin and eukaryotic translation elongation factor 2 (EEF2) and relative compared to ChAT$^{fl/fl}$ mice receiving 2% DSS. N = 4-9, both male and female. Data are expressed as mean (B) or median (C, D, E and F (except IL-10)) and individual data points. We tested for statistical differences between ChAT$^{fl/fl}$ mice and CD4ChAT$^{-/-}$ mice receiving DSS with an independent T-test or a Mann-Whitney U test. $P < 0.05$ was considered significant. * $P < 0.05$

ChAT+ T-cells support resolution of intestinal inflammation induced by DSS.

Next, we investigated the role of ChAT^{+} T-cells during resolution of inflammation, in a 5-day DSS colitis model followed by a recovery period.[26] CD4ChAT$^{-/-}$ mice displayed reduced recovery after DSS-induced colitis compared to ChAT$^{fl/fl}$ mice represented by a reduced bodyweight (Figure 3A), a higher, not statistically significant, colitis histology index (P=0.19; Figure 3D) and higher IL-6 protein levels in the colon as compared to ChAT$^{fl/fl}$ mice (resp. median 0.51 vs. 0.05; P<0.01; Figure 3E), possibly reflecting a lack of cholinergic inhibition of IL-6 protein secretion as observed earlier.[36] Arg1, a marker for anti-inflammatory M2 macrophages, was not clearly different between the ChAT$^{fl/fl}$ and CD4ChAT$^{/-}$ mice. However, iNOS, a marker for inflammatory M1 macrophages, was increased in the CD4ChAT$^{-/-}$ mice, although not significantly (P=0.07; Figure 3F). This trend was also seen at mRNA level of several inflammatory cytokines (Figure 3G). Of note, not all cytokines were expressed at detectable levels in the resolution phase of inflammation.

ChAT does not participate in the pathophysiology of colitogenic T-cells in a transfer model of colitis.

Since DSS-induced colitis is mainly characterized by innate driven inflammation, we also investigated the role of ChAT^{+} T-cells in a model of T-cell driven colitis. ChAT expression was found in the CD4^{+}CD45RB^{high} T-cells that are transferred to induce colitis (Figure 4A). Seven weeks after transfer we found an increased frequency, compared to the frequency in the transferred population, of ChAT^{+} T-cells in the blood of Rag1$^{-/-}$ mice injected with T-cells from ChAT-eGFP mice (Figure 4B), indicating that the transferred population has expanded and retained ChAT expression.

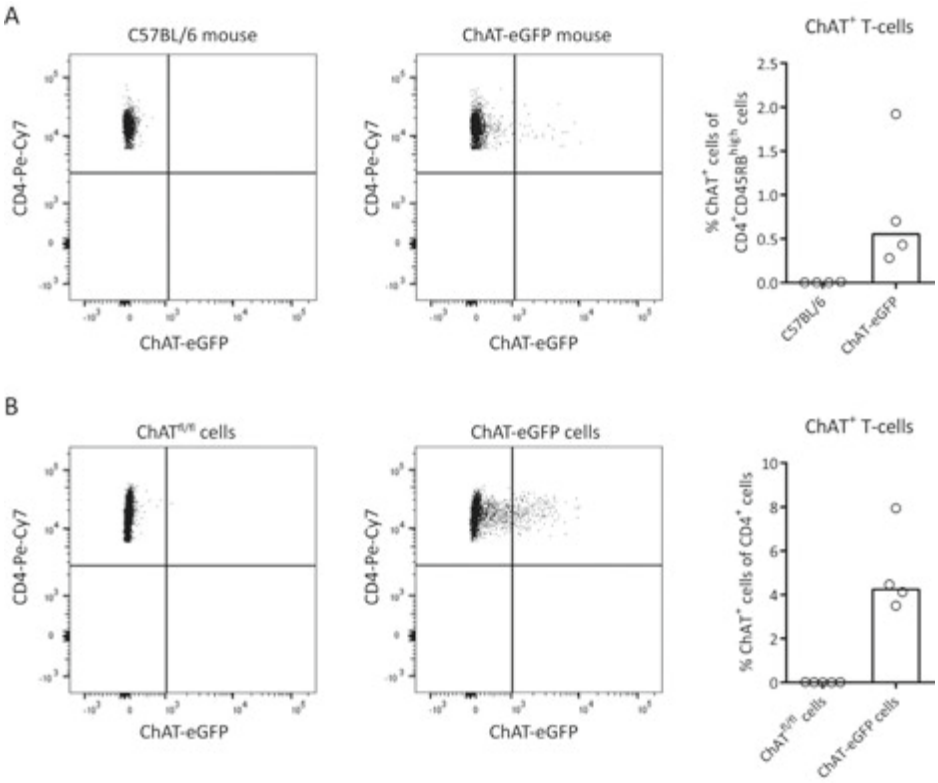

Figure 4. ChAT^{+} T-cell frequency before and after T-cell transfer. (A) Gating strategy and quantification of ChAT^{+} T-cells in the T-cell population of the spleen that is used for T-cell transfer. Spleens from four ChAT-eGFP and four C57BL/6 mice are used (both male and female). (B) Gating strategy and quantification of ChAT^{+} T-cells in peripheral blood of Rag1$^{-/-}$ mice seven weeks after T-cell transfer of ChAT$^{fl/fl}$ mice or ChAT-eGFP mice. Five Rag1$^{-/-}$ mice receiving cells from ChAT$^{fl/fl}$ mice and four Rag1$^{-/-}$ mice receiving cells from ChAT-eGFP mice were used. Male and female mice were equally distributed among groups. Median and individual data points are shown.

Rag1$^{-/-}$ mice transferred with CD4^{+}CD45RB^{high} T-cells from spleens from CD4ChAT$^{-/-}$ mice or ChAT$^{fl/fl}$ mice lose weight starting after four weeks until the end of the experiment at a similar rate (Figure 5A). Endoscopic assessment of colonic inflammation showed that after three weeks inflammation was not apparent, but endoscopy score was increased after five and seven weeks compared to non-transferred mice (Figure 5B). However, there was no difference between CD4ChAT$^{-/-}$ or ChAT$^{fl/fl}$ T-cell transferred mice. Spleen weight, colon weight/length ratio, disease activity index and the colitis histology index were all increased in both groups receiving T-cell transfer compared to non-transferred mice (Figure 5C-F), indicating successful induction of colitis. In the colon, an upregulation of inflammatory cytokines, like IL-1β, IL-6, TNF-α and IFN-γ, both at mRNA expression and protein level was evident (Figure 5G-H). No significant differences were seen between CD4ChAT$^{-/-}$ or ChAT$^{fl/fl}$ T-cell transferred mice in colon cytokine levels.

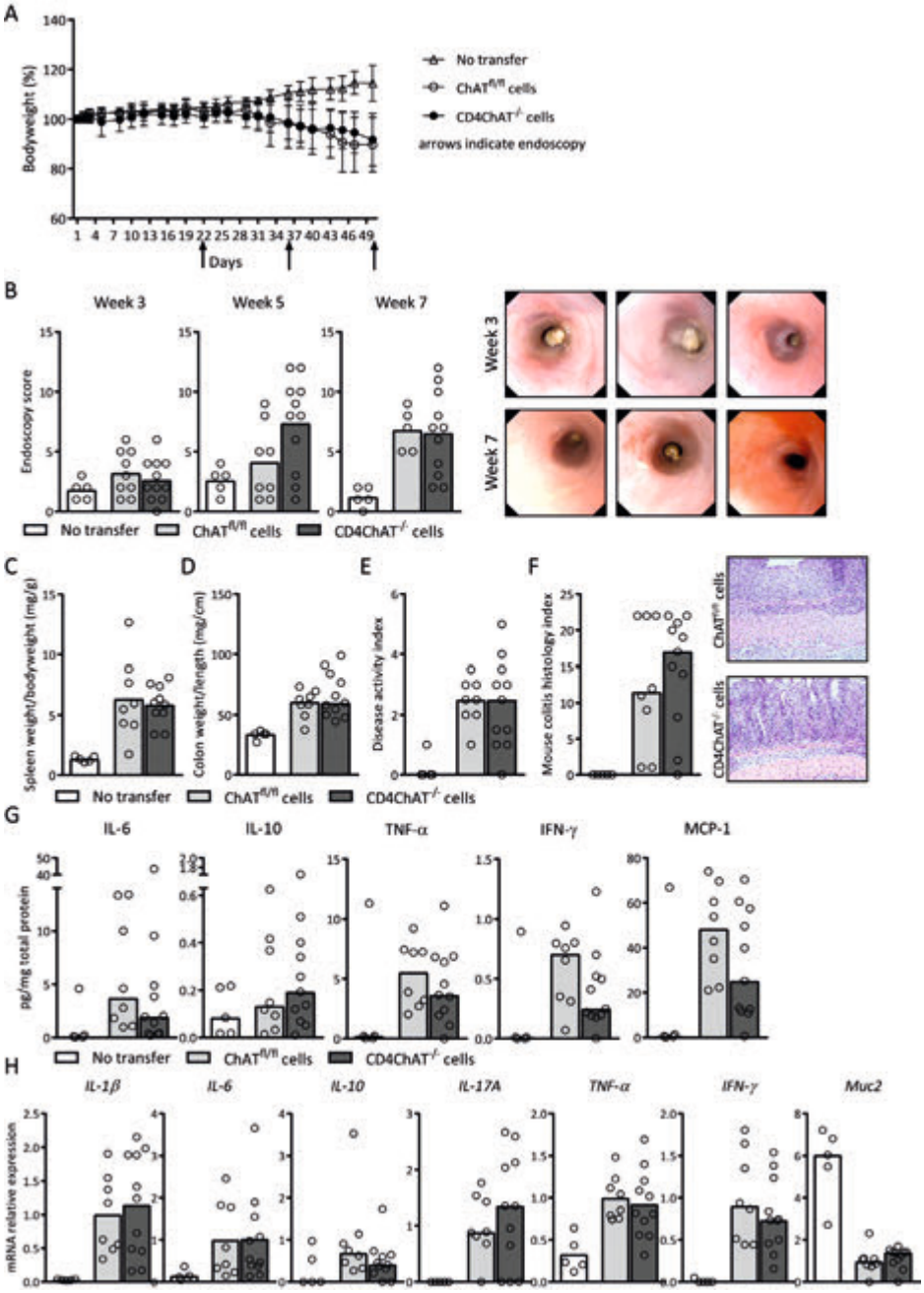
A
Bodyweight (%)
No transfer
ChAT fl/fl cells
CD4ChAT -/- cells
arrows indicate endoscopy
Days
B
Week 3
Week 5
Week 7
Endoscopy score
No transfer
ChAT fl/fl cells
CD4ChAT -/- cells
C
Spleen weight/bodyweight (mg/g)
D
Colon weight/length (mg/cm)
E
Disease activity index
F
Mouse colitis histology index
G
IL-6
IL-10
TNF-α
IFN-γ
MCP-1
pg/mg total protein
H
IL-1β
IL-6
IL-10
IL-17A
TNF-α
IFN-γ
Muc2
mRNA relative expression

Figure 5 (previous page). ChAT does not participate in the pathophysiology of colitogenic T-cells in a transfer model of colitis. (A) Bodyweight loss of mice over time, compared with day 0. Data are expressed as mean and standard deviation and last weight carried forward is used for animals that were sacrificed before the endpoint at seven weeks. (B) Endoscopy score as marker for intestinal inflammation based on Becker *et al*.[29] Measured after three, five and seven weeks. Three animals are used as example to the right, snapshots from the movies from week three and week seven are taken. (C) Spleen weight relative to total bodyweight as measurement of successful T-cell transfer. (D) Colon weight as a marker of edema and inflammation, normalized for colon length. (E) Disease activity index for colitis, based on Melgar *et al*.[26] (F) Mouse colitis histology index, based on Koelink *et al*.[30] Two representative pictures are shown of a hematoxylin and eosin staining, magnification 100x. (G) Protein levels of (IL)-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 in colon homogenates, normalized for total protein levels. (H) mRNA levels of IL-1β, IL-6, IL-10, IL-17A, TNF-α, IFN-γ and mucin 2 (Muc2) in colon homogenates. mRNA levels are normalized for reference genes hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relative compared to $Rag1^{-/-}$ mice receiving T-cell transfer from $ChAT^{fl/fl}$ mice. N = 5-11, both male and female. Data are expressed as mean (B and C) or median (D, E, F, G and H (except IL-1β, IL-6 and TNF)) and individual data points. We tested for statistical differences between $Rag1^{-/-}$ mice receiving T-cell transfer from $ChAT^{fl/fl}$ mice or $CD4ChAT^{-/-}$ mice with an independent T-test or a Mann-Whitney U test. $P < 0.05$ was considered significant.

DISCUSSION

We show that a significant proportion of T-cells in the spleen and PPs expresses ChAT, and that the frequency of $ChAT^+$ T-cells is higher in the intestine compared to the spleen and peripheral blood. In the spleen, these cells may relay the adrenergic signal from splenic nerve fibers and produce acetylcholine to act on cholinergic receptors of other immune cells such as monocytes.[37] In earlier work, we characterized the phenotype and the function of these $ChAT^+$ T-cells in the healthy intestine.[19] Because of the anti-inflammatory properties of acetylcholine and the fact that $ChAT^+$ T-cells also reside in the intestine, these cells are potential candidates to fulfil an autocrine or paracrine role in intestinal inflammation, while acknowledging that B-cells, macrophages and dendritic cells are also reported to express ChAT and the latter also express CD4.[14] However, to what extent this is relevant in myeloid cell function in our model is unclear[38] especially as we demonstrated earlier that the $ChAT^+$ subset in PPs lacked markers specific for myeloid cells such as CD11b and CD11c, and were not identified as ILCs.[19]

In the present study, we investigated $ChAT^+$ T-cells in different colitis models. Our results suggest a dual role of $ChAT^+$ T-cells in colitis since the outcome depends on the stage of the colitic process. $ChAT^+$ T-cells aggravate acute DSS-induced colitis, but are seemingly required to secure a proper resolution of innate immune driven inflammation, whereas the expression of ChAT is redundant in T-cell driven colitis. It is important to emphasize that the differential role of $ChAT^+$ T-cells in these models may be due to the fact that in each of these models different cells play an effector role.

We did not measure acetylcholine levels in the intestine in these experiments. Acetylcholine is a neurotransmitter with a very short half-life, predominantly exerting an autocrine and paracrine function, which is rapidly broken down and notoriously hard to measure. Noteworthy, cholinergic fibers are the main source of acetylcholine in the intestine.[39]

In DSS-induced colitis, DSS causes damage to the intestinal epithelial layer and triggers a strong innate immune response.[40] Leukocytes are recruited upon inflammation, producing inflammatory cytokines like TNF-α and these leukocytes are potential target cells of acetylcholine produced by the $ChAT^+$ T-cell. In addition to this, $ChAT^+$ T-cells present in lymphoid structures in the intestine are activated by the innate immune system and produce pro-inflammatory mediators like IFN-γ and IL-17A, contributing to the inflammatory process.

This is likely since we showed before that ChAT^{+} T-cells in the intestine in a homeostatic condition produce these factors.[19] Additionally, we showed in the current study that TNF-α and IFN-γ protein concentration appear to be lower in CD4ChAT$^{-/-}$ mice receiving DSS as compared to ChAT$^{fl/fl}$ mice (Figure 2). The decrease in TNF-α and IFN-γ protein concentration was not seen on gene expression levels, which could be explained by the fact that inflamed tissue also contains recruited circulating cells that are not necessarily influenced at the site of inflammation. The trend towards lower TNF-α protein concentration in the CD4ChAT$^{-/-}$ mice is surprising in the context of papers that show that ChAT^{+} T-cells are capable of reducing the TNF-α secretion of inflammatory monocytes and macrophages, and are important in the anti-inflammatory effect of VNS.[13,33,41] However, these studies did not focus on the particular phenotype of ChAT^{+} T-cells in the intestine and what they produce besides acetylcholine. It is unlikely that acetylcholine itself has an inflammatory effect in acute colitis, since there is widespread evidence showing that acetylcholine has potent anti-inflammatory effects on innate immune cells and intestinal epithelial cells through muscarinic and nicotinic AChRs.[42-45]

The role of ChAT^{+} T-cells in the resolution of inflammation has not been previously addressed. The vagus nerve is described to stimulate resolution in peritoneal inflammation[46] and after *Escherichia coli* infection.[47] One may hypothesize that the production of acetylcholine by ChAT^{+} T-cells in the intestine creates an autocrine trigger affecting their differentiation and effector function depending on the tissue microenvironment and colitis stage. Alternatively, T-cell derived acetylcholine may affect pro-inflammatory mediator levels such as IL-6 and iNOS.[48] The greater importance ChAT^{+} T-cells in the resolution phase of inflammation compared to its role during active inflammation fits with a purported activity of acetylcholine on epithelial cells to boost antimicrobial peptide levels and secure homeostasis.[19] The data from the DSS-induced colitis experiments suggest a bivalent role for CD4^{+}ChAT^{+} T-cells. This could indicate that other CD4^{+} cells such as dendritic cells may be involved. However, only a small portion of CD3^{-}CD4^{-} cells express ChAT and these cell populations were not different between DSS and control groups (data not shown).

The T-cell transfer colitis model is a model for T-cell driven inflammation in contrast to DSS-induced colitis which is a model for innate immune mediated inflammation. We did not find a role for ChAT^{+} T-cells as CD4ChAT$^{-/-}$ T-cells produced similar colitis as compared to ChAT$^{fl/fl}$ T-cells. Although we were able to trace back the ChAT^{+} T-cells in peripheral blood seven weeks after transfer, the frequency of ChAT^{+} T-cells in the transferred T-cell population is limited and may have not been sufficient to alter the disease course. Another study showed that co-transfer of ChAT^{+} T-cells reduced hallmarks of the disease.[14] However, these cells have a regulatory phenotype, which could be the primary cause of the inhibition of T-cell transfer colitis. Our results suggest that the relative low amount of ChAT^{+} T-cells in the CD4^{+}CD45^{high} T-cell population that is transferred does not reduce the colitic potential of pathogenic T-effector cells.

Critical next steps would be to phenotype ChAT^{+} T-cells in the different phases of intestinal inflammation and to determine the frequency and phenotype of ChAT^{+} T-cells in the intestine of patients with IBD. A first study with patients with ulcerative colitis shows less immunoreactivity for non-neuronal ChAT in the mucosa.[49] Since adrenergic activation controls the generation of ChAT^{+} T-cells in our previous work and VNS is supposed to work via adrenergic pathways, it would be intriguing to investigate if nerve stimulation therapies such as VNS can influence the phenotype of ChAT^{+} T-cells.

In conclusion, our study reveals a dual role of ChAT^{+} T-cells in intestinal inflammation, aggravating the acute innate immune response while supporting the resolution process. This difference might be explained by a change in phenotype or activity of the cell depending on the phase of inflammation. This knowledge is important in optimizing the current field of nerve stimulation in complex immune disorders and emphasizes the need for a disease-, location- and time-specific approach.

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03

ELECTRICAL STIMULATION OF THE SPLENIC NERVE BUNDLE AMELIORATES DEXTRAN SULFATE SODIUM-INDUCED COLITIS IN MICE

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Journal of Neuroinflammation 2022

ABSTRACT

Background: Vagus nerve stimulation has been suggested to affect immune responses, partly through a neuronal circuit requiring sympathetic innervation of the splenic nerve bundle and norepinephrine (NE) release. Molecular and cellular mechanisms of action remain elusive. Here, we investigated the therapeutic value of this neuromodulation in inflammatory bowel disease (IBD) by applying electrical splenic nerve bundle stimulation (SpNS) in mice with dextran sulfate sodium (DSS)-induced colitis.

Methods: Cuff electrodes were implanted around the splenic nerve bundle in mice, whereupon mice received SpNS or sham stimulation. Stimulation was applied 6 times daily for 12 days during DSS-induced colitis. Colonic and splenic tissues were collected for transcriptional analyses by qPCR and RNA-sequencing (RNA-seq). In addition, murine and human splenocytes were stimulated with lipopolysaccharide (LPS) in the absence or presence of NE. Single-cell RNA-seq data from publicly available data sets were analyzed for expression of β-adrenergic receptors (β-ARs).

Results: Colitic mice undergoing SpNS displayed reduced colon weight/length ratios and showed improved Disease Activity Index scores with reduced Tumor Necrosis Factor α mRNA expression in the colon compared with sham stimulated mice. Analyses of splenocytes from SpNS mice using RNA-seq demonstrated specific immune metabolism transcriptome profile changes in myeloid cells. Splenocytes showed expression of β-ARs in myeloid and T cells. Cytokine production was reduced by NE in mouse and human LPS-stimulated splenocytes.

Conclusions: Together, our results demonstrate that SpNS reduces clinical features of colonic inflammation in mice with DSS-induced colitis possibly by inhibiting splenic myeloid cell activation. Our data further support exploration of the clinical use of SpNS for patients with IBD.

BACKGROUND

Inflammatory bowel diseases (IBD) are debilitating conditions that greatly affect the daily life of patients. At present, treatment modalities for IBD mainly consist of anti-inflammatory agents with increasing intensity if disease remains uncontrolled. However, pharmaceutical treatment is accompanied with substantial side effects and has a high financial burden. In this light, novel therapies that can improve disease outcome of patients with IBD are warranted.

It is increasingly acknowledged that the autonomic nervous system possesses a regulatory activity towards the immune response. Vagus nerve stimulation (VNS) was found to improve clinical outcomes in small scale clinical trials in patients with rheumatoid arthritis and Crohn's disease.[1-3] Similarly, in experimental colitis rat models, VNS decreased disease parameters and colonic cytokines.[4-7] Despite a positive outlook for the use VNS as immunosuppressive treatment, it has become evident that VNS affects many organs, acts on immune cells indirectly,[8] and therefore has off-target effects such as cardiovascular changes restricting its clinical application.[9]

It has been shown that the anti-inflammatory effect of VNS in experimental endotoxemia rests on innervation of the spleen through the splenic nerve bundle.[10,11] Accordingly, anti-inflammatory activity in experimental colitis of cholinergic agonists relies on splenic innervation.[12-14] Since the anatomical and functional connection between the vagus nerve and the sympathetic splenic nerve bundle remain topic of debate, therapeutic efficacy of nerve stimulation might be improved through directly targeting the splenic nerve bundle instead of the vagus nerve.[15] Interestingly, it has been reported that stimulation of the splenic nerve bundle could be as effective as VNS in murine models of endotoxemia and arthritis.[9,16] Moreover, ultrasound stimulation of the splenic nerve bundle improves disease outcome in a setting of dextran sulfate sodium (DSS)-induced colitis.[17] Yet, thus far the effect of electrical splenic nerve bundle stimulation (SpNS) in experimental colitis is not well understood.

In this study, we demonstrate that electrical SpNS using implanted cuff electrodes in mice with DSS-induced colitis ameliorated colitis. Further, we investigated the potential role of adrenergic receptor (AR) activation in splenic myeloid cells that could be mediating this effect.

METHODS

Animals

Female C57BL/6N mice (8-12 weeks old) were purchased from Charles River Laboratories (Maastricht, the Netherlands). The animals were housed under specific pathogen free conditions in individually ventilated cages in the animal facility at the Amsterdam UMC, location Academic Medical Center (AMC), in Amsterdam. Animals were maintained on a 12/12 hours light/dark cycle under constant condition of temperature (20°C ± 2°C) and humidity (55%) with *ad libitum* food and water. Mice handling and experimental protocols were in accordance with the local guidelines and approved by the local Animal Research Ethics Committee.

Surgical implantation and stimulation of cuff electrodes around the splenic neurovascular bundles and the cervical vagus nerve

First, an incision was made in the skin of the head and the skull was cleaned to attach the head mount, Preci-Dip (RS components, Haarlem, the Netherlands) with dental cement (Super-

Bond C&B, Sun medical, Hofmeester Dental, Rotterdam, the Netherlands) according to the manufacturer's protocol. Second, an incision was made in the left flank to reach the spleen and to place the cuff electrode (100 µm micro cuff sling, CorTec GmbH, Freiburg, Germany) around the splenic artery, or an incision was made in the neck to expose the left cervical vagus nerve to place the cuff. The wires of the cuff were led subcutaneously to the skull and attached to the head mount. The whole procedure was performed in mice under anesthesia with 2-2,5% isoflurane/O_2. Pre-operatively and 24 hours post-operatively, meloxicam (Metacam) 1 mg/kg (Boehringer, Ingelheim am Rein, Germany) and enrofloxacin (Baytril) 10 mg/kg (Bayer Healthcare, Whippany, NJ, USA) were administered subcutaneously. Before the start of DSS treatment, mice had a recovery period of 10 days and were tethered and single housed 5 days before start of stimulation. Before start of stimulation, mice were paired based on weight and then randomly allocated (1:1) to the sham or stimulation group (Table 1). Stimulation started at the same day as the treatment with DSS and was performed 6 times per 24 hours (every 4 hours) for 2 minutes with a biphasic pulse (650 µAmp, 10 Hz, 100 µs per phase). Mice were observed during the first stimulations for behavioral changes and altered breathing patterns. Before, during and after the experiment, cuff functioning was ensured by measuring impedance using a Minirator MR Pro (NTI Audio, Essen, Germany). If the impedance was >25 kΩ before start of stimulation, the mouse was allocated to the sham group. Animals that received sham stimulation underwent the same surgical procedure with cuff placement, were tethered to a wire but no actual stimulation was performed.

Surgical splenic denervation

Via a midline incision the spleen and splenic artery were located. Selective denervation was achieved by cutting the catecholaminergic nerve fibres running along the splenic artery. Sham-operated animals underwent a laparotomy without denervation. Surgery was performed on anesthetized mice by injecting intraperitoneally (i.p.) a mixture of fentanylcitrate/fluanisone (Janssen, Beerse, Belgium) and midazolam (Roche, Woerden, the Netherlands). Finadyne (Intervet, De Bilt, the Netherlands) was injected subcutaneously pre- and postoperatively. Before the start of the DSS-induced colitis experiment, mice had a recovery period of two weeks.

Acute SpNS and endotoxemic shock

Seven days following implantation, animals were injected i.p. with a lethal dose of lipopolysaccharide (LPS; 400 µg). Electrostimulation was applied using a PlexStim V2.3 (Plexon) starting at -10, 0 and +20 minutes relative to LPS injection. Mice were electrically stimulated with the same parameters as above at 16, 20, 24, 30, 34 and 38 hours after LPS injection. Survival was followed over 4 days. Serum was collected at 90minutes after LPS injection and assessed for Tumor Necrosis Factor (TNF)-α levels. Again, controls were fully Cortec implanted mice, which did not receive electrical stimulation (sham). Electrostimulation were rectangular charged-balanced biphasic pulses (650 µAmp, 10 Hz, 100 µs per phase) for 2 minutes. For TNF-α, retro-orbital blood sampling was performed under isoflurane anesthesia. TNF-α levels were measured by ELISA (Mouse TNF-alpha DuoSet, R&D Systems) following manufacturer instructions.

Mouse ID	Impedance according to manufacturer	Impedance before DSS	Impedance during DSS	Impedance after DSS	Group
#26	3.7	10	11	11	Stim
#27	4.6	13			Sham
#28	4.8	13			Sham
#29	3.6	18	14	15	Stim
#30	4.5	18			Sham
#31	6.8	17	14	13	Stim
#32	4.8	>50			Sham
#33	5.1	16			Sham
#34	3.6	9.7	11	11	Stim
#35	4.4	14			Sham
#36	3.9	18	14	14	Stim
#37	4.6	21			Sham
#38	3.6	15	14	13	Stim
#39	5.1	26			Sham
#40	3.9	19	13	12	Stim
#41	5.1	18	14	14	Stim
#42	5.9	20	21	1	Stim
#43	3.9	16	16	15	Stim
#44	4.6	20			Sham
#45	4.3	2.4			Sham
#46	6.9	17	15	14	Stim
#47	4.1	20			Sham
#48	4.6	17			Sham
#49	4.2	18	14	13	Stim
#50	4.6	17	16	16	Stim
#51	4.8	27			Sham
#52	4.8	18	15	16	Stim
#53	6.2	14	11	12	Stim
#54	5.1	35			Sham
#55	5.1	>50			Sham
#56	4.8	23			Sham

Table 1. Impedance measurements. DSS = dextran sulfate sodium

Dextran sulfate sodium (DSS)-induced colitis

Two percent (w/v) DSS (TdB Consultancy, Uppsala, Sweden) was added to the drinking water for 5 consecutive days. Daily replacement of drinking water with fresh DSS solutions was performed. After 5 days, DSS drinking water was replaced by normal drinking water and animals were followed for weight and behavior for the subsequent 7 days, making the total experiment length 12 days. During the study, bodyweight was monitored daily. At the end of the

study, mice were sacrificed, and the colon weight and length were measured, as parameter for colitis. Then, the tissue was snap-frozen in liquid N_2 and stored at -80°C or put in 10% formalin for further processing. The disease activity index (DAI), ranging from 0-9, was used to assess the clinical outcome of the DSS-induced colitis. DAI was determined by combining scores of stool consistency (0-3), occult blood in the stool (0-3), and macroscopic inflammation (0-3).[18]

Endoscopy was performed at day 8 after start of DSS. Endoscopy was performed under anesthesia with 3% isoflurane/O_2 to assess colonic inflammation. The Olympus URF type V endoscope (Zoeterwoude, the Netherlands) was rectally inserted for a maximum of 5 cm and videos of the endoscopy were recorded using a Medicap USB200 Medical Digital Video Recorder (Roermond, the Netherlands), while retracting the endoscope. A blinded and trained technician determined the murine endoscopic index of colitis severity (MEICS), consisting of wall thickening, vascularity, visible fibrin, granularity, and stool consistency, with each component scoring between 0 and 3.[19]

Histology

Swiss rolls of the distal colon were fixated in 10% formalin. Afterwards the tissue was embedded at the Pathology department of the Amsterdam UMC, location AMC, in paraffin for routine histology. A blinded and experienced pathologist evaluated formalin-fixed hematoxylin and eosin (HE) stained tissue sections microscopically. The pathologist scored the distal colon based on eight characteristics of inflammation.[20] This resulted in a total histology score ranging from 0 to 24.

RNA isolation and RNA sequencing analysis

RNA was extracted from snap-frozen colon tissue after homogenization of the samples in TriPure isolation reagent (Roche Applied Science, Almere, the Netherlands) according to the manufacturer's instructions. For RNA sequencing (RNA-seq), RNA quality was assured using the Bioanalyzer (Agilent, Santa Clara, USA) where samples with a RIN score of >8 were used for further analyses. mRNA was converted into cDNA with the KAPA mRNA HyperPrep Kit (Roche), whereupon the cDNA was prepared for sequencing on the HiSeq4000 at the Core Facility Genomics, Amsterdam UMC, in a 50 bp single-ended fashion to a depth of 40 million reads per sample. The raw reads were checked for quality using FastQC (v0.11.8) and MultiQC (v1.7)[21,22] and were subsequently mapped using STAR (v2.7.3) against mouse genome GRCm38.[23] Post-alignment processing was done using SAMtools (v1.9),[24] whereupon reads were counted using featureCounts as found in the Subread package (v1.6.4).[25,26] Gene features were obtained from Ensembl (v98).[27] Resulting counts were imported and analyzed in the R statistical environment (v3.6.2) using packages obtained from Bioconductor (v3.10).[28] DESeq2 was used to perform the pairwise differential expression analyses.[29] Plots were made using pheatmap (v1.0.12) and ggplot2 (v3.3.1).[30,31]

cDNA synthesis and quantitative PCR analysis

For quantitative polymerase chain reaction (qPCR), RNA was further cleaned from DSS with the Bioline ISOLATE II RNA mini kit (GC biotech B.V., Alphen a/d Rijn, the Netherlands). cDNA was synthesized using dNTPs (ThermoFisher Scientific, Landsmeer, the Netherlands), Random primers (Promega, Leiden, the Netherlands), Oligo dT primers (Sigma, Zwijndrecht, the Netherlands), Revertaid and Ribolock (ThermoFisher Scientific) according to the manufacturer's instructions. PCR was performed using SensiFAST SYBR No-ROX (GC biotech B.V.) on a LightCycler 480 II (Roche Applied Science) to analyze expression levels of genes of

interest using LinRegPCR software.[32] For normalization the reference genes hypoxanthine phosphoribosyltransferase (*H*prt), cyclophilin, Non-POU Domain Containing Octamer Binding (*Nono*) and ribosomal protein lateral stalk subunit P0 (*Rplp0*) were selected, after analysis for stability in geNorm.[33] Primers (synthesized by Sigma) are listed in Table 2.

Gene	Forward sequence	Reverse sequence
IL-1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
Il-6	GAGTTGTGCAATGGCAATTCTG	TGGTAGCATCCATCATTTCTTTGT
Il-10	TGTCAAATTCATTCATGGCCT	ATCGATTTCTCCCCTGTGAA
Il-12	AGACCCTGCCCATTGAACTG	CGGGTCTGGTTTGATGATGTC
TNF-α	TGGAACTGGCAGAAGAGGCACT	CCATAGAACTGATGAGAGGGAGGC
Mmp-7	TCTGCATTTCCTTGAGGTTG	AGGAAGCTGGAGATGTGAGC
Acod-1	ACTCCTGAGCCAGTTACCCT	GGTGGTTCACTTTCAAGCCG
Cxcl1	CCACACTCAAGAATGGTCGC	TCTCCGTTACTTGGGGACAC
Cxcl2	CCCAGACAGAAGTCATAGCCAC	TGGTTCTTCCGTTGAGGGAC
S100a8	ACTTCGAGGAGTTCCTTGCG	TGCTACTCCTTGTGGCTGTC
S100a9	TGGGCTTACACTGCTCTTACC	GGTTATGCTGCGCTCCATCT
Hprt	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Cyclophilin	ATGGTCAACCCCACCGTGT	TTCTGCTGTCTTTGGAACTTTGTC
Nono	AAAGCAGGCGAAGTTTTCATTC	ATTTCCGCTAGGGTTCGTGTT
Rplp0	CCAGCGAGGCCACACTGCTG	ACACTGGCCACGTTGCGGAC

Table 1. Primer sequences for qPCR

Protein concentrations of cytokines in intestinal tissue

Snap-frozen colon tissue was homogenized on ice in Greenberger Lysis Buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl{\cdot}6H_2O$, 1 mM $CaCl_2$, 1% Triton) with protease inhibitor cocktail (Roche Applied Science), pH 7.4, diluted 1:1 with phosphate buffered saline (PBS). In these tissue lysates, protein concentrations of IL-6, IL-10, IL-12, TNF-α, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 were measured with a mouse inflammation kit by BD cytometric bead assay (CBA; BD Bioscience, San Jose, CA, USA) according to manufacturer's protocol, with the exception that reagents were 10 times diluted.

Adrenergic cell culture assays

Murine spleens were obtained from female C57BL/6 mice. Human splenic tissue was obtained from patients that underwent distal pancreatectomy for pancreatic cancer during which the spleen was taken out as part of the procedure. Splenic tissue was obtained in consultation with a pathologist to assure that tissue was not affected by any tumorous tissue. The Medical Ethics research Committees United (MEC-U, Nieuwegein, the Netherlands) approved the protocol (registration number W16.182) and patients provided informed consent for the use of their material. Spleens or splenic tissue were immediately homogenized on a 70 µm cell strainer and suspended in RPMI-1640 medium (supplemented with 10% fetal calf serum (FCS; Bodinco, Alkmaar, the Netherlands), 100 U/ml pen/strep (Lonza, Basel, Switzerland), 2 mM L-Glutamine (ThermoFisher Scientific) to obtain a single cell suspension. Cells were counted using the Coulter Counter (Beckman Coulter, Indianapolis, USA) and were plated using $1*10^6$ cells per well. All conditions were performed in triplicate. Cells were pre-treated with norepinephrine

(NE) and/or propranolol (both 1 mM, Sigma-Aldrich, Saint Louis, USA) 30 minutes before exposure to LPS 100 ng/mL (Bio-Connect, Huissen, the Netherlands). Murine TNF-α was assessed after 4 hours in supernatant with ELISA (R&D systems, Minneapolis, USA) according to the manufacturer's instructions. Human cytokines (TNF-α, IL-6, and IL-8) were assessed by means of BD cytometric bead assay (CBA; BD Bioscience, San Jose, CA, USA) according to manufacturer's protocol, with the exception that reagents were 10 times diluted.

Single-cell RNA-sequencing database studies

Publicly available single-cell RNA-sequencing (scRNA-seq) datasets from two C57BL/6JN mouse spleens were downloaded from the Gene Expression Omnibus (GEO; GSE109774).[34] Alignment was done against GRCh38 using Cell Ranger Software (v3.1.0; 10x Genomics, Inc., Pleasanton, CA, USA) and subsequently datasets were imported in Rstudio (v1.3.1093).[35] Seurat was used to import, integrate, and cluster data, and plots were made with ggplot2 (v3.3.3).[31,36] Cells were filtered for dead cells that were identified by a low (500-5000) gene count. Clusters were identified by Louvain clustering method, and annotation was performed with use of known markers.[34] Thereafter, expression of *Adrb2* (encoding for β_2-AR) was assessed. The top 13 principal components were used to calculate the t-distributed stochastic neighbor embedding (t-SNE).

Data presentation and statistical analysis

Graphs and statistical analyses were made with Prism 8.3 (GraphPad Software, La Jolla, CA, USA). For all data, a Kolmogorov-Smirnov test was used to determine normality of distribution. Data are shown as mean (if distributed normally) plus standard deviation or median (if not distributed normally), and individual data points. A *P*-value < 0.05 was considered significant. Data were compared with the independent t-test or Mann-Whitney U test as appropriate. Survival was plotted using Kaplan-Meier's curves and differences between groups were estimated using the log-rank test. In case of multiple groups, a one-way ANOVA and Dunnett's multiple comparison test were used.

RESULTS

Recently, it was shown that SpNS could decrease disease severity in a mouse model of arthritis and that SpNS causes the release of splenic NE, which in turn can reduce monocyte and macrophage LPS-induced cytokine secretion via β_2-AR.[9] A much debated issue is whether NE targets β_2-AR on macrophages or T cells that produce acetylcholine.[8] Therefore, the expression of *Adrb2* in murine splenocytes was examined using a publicly available scRNA-seq datasets. A total of 13,365 features were retrieved in 9,568 individual cells. After quality control, the remaining 9,407 cells were annotated to main cell populations (i.e., T, B, natural killer cells (NK), dendritic cells, and macrophages) using previously reported markers (Fig. 1A).[34] Subsequent feature analysis showed a marked expression of *Adrb2* in a cluster of cells belonging to the B cell population, when compared with other cell populations (log2FoldChange = 0.51, padj = 8.98E-18). In addition, *Adrb2* expression by a cluster of cells belonging to the macrophage population was found to be significantly increased when compared to other cell populations (log2FoldChange = 0.31, padj = 0.009) (Fig. 1B).

Hereafter, LPS-induced inflammatory activation on both murine and human splenocyte cultures was investigated. In line with earlier investigations, NE decreased the release of TNF-α following LPS challenge in murine and human splenocytes. This effect was abrogated by pre-treatment with propranolol, demonstrating a mechanism involving β-ARs (Fig. 1C, D).

As earlier studies demonstrated that vagotomy aggravates colitis outcome in a DSS-induced animal model through splenic innervation, and VNS ameliorates Crohn's disease in patient trials, we hypothesized that disrupting the splenic nerve bundle (SplX) would worsen colitis.[37] As expected, mice receiving DSS in drinking water lost weight, showed an increased DAI and had a shortened colon due to edema compared to control mice (Additional file 1). The expression of inflammatory colonic cytokines was also increased.

However, splenic nerve bundle denervation did not alter these outcomes significantly when compared to sham-operated animals. Hence, we could not demonstrate a tonic and intrinsic role for splenic innervation for regulation of immune responses in this model. Notably, VNS using chronic nerve cuffs around the left cervical vagal branch did not ameliorate disease in the setting of DSS-induced colitis (Additional file 2). To address the role of splenic nerve activity in the immune response in DSS-induced colitis, we next assessed

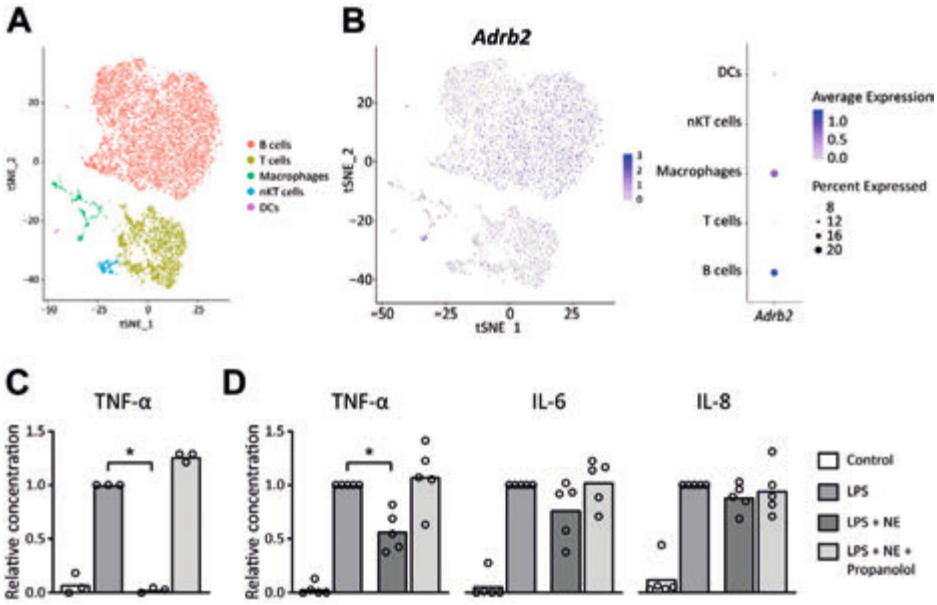

Fig. 1. Distribution of *Adrb2* in murine splenocytes and decrease of pro-inflammatory cytokines by NE in murine and human splenocyte cultures. **A** Cell type clusters visualized by t-SNE clustering (color coding) based on the expression of known marker genes. **B** t-SNE and dotplot showing *Adrb2* expression per cell type. N = 2 mice. **C** Protein levels of TNF-α in supernatant from murine splenocytes after treatment with LPS and pretreatment with norepinephrine and/or propranolol. N = 3 mice. **D** Protein levels of TNF-α, IL-6 and IL-8 in supernatant from human splenocytes after treatment with LPS and pretreatment with norepinephrine and/or propranolol. N = 5 subjects. For both murine and human data, protein levels were relative compared with LPS condition. * indicates a significant difference compared to LPS treated splenocytes. Statistical differences were assessed with a one-way ANOVA and Dunnett's multiple comparison test. *t-SNE* t-distributed stochastic neighbor embedding, nKT natural killer T cell, DC dendritic cell, *Adrb2* adrenergic receptor β2, *TNF* tumor necrosis factor, *IL* interleukin, *LPS* lipopolysaccharide, NE norepinephrine

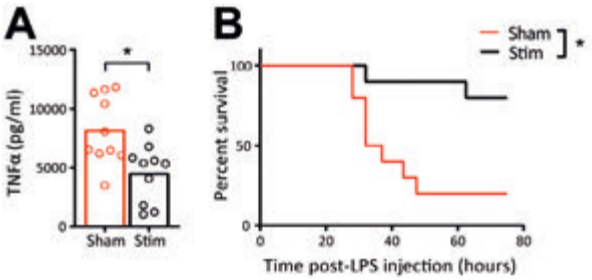

Fig. 2 SpNS reduces systemic TNF-α and survival after LPS injection. A Systemic TNF-α levels 90 min after LPS injection. B Survival after LPS injection. N = 4–10 per group. Data are expressed as mean and individual data points. *indicates a significant difference compared to control. Statistical differences between Sham and Stim mice were assessed using an independent t-test. Statistical difference in survival was assessed using the log-rank test. $P < 0.05$ was considered significant. *SpNS* splenic nerve plexus stimulation, *TNF* tumor necrosis factor; *LPS* lipopolysaccharide

the effect of SpNS by implanting a cuff around the splenic artery and surrounding nerve bundle (Additional file 3). Electrical stimulation of the nerve bundle can be varied in amplitude, current, and frequency. Using the stimulation parameters depicted in Additional file 3, SpNS led to a reduced induction of systemic TNF-α and increased survival in LPS-injected mice (Fig. 2A, B). Using the stimulation parameters established, we next implanted cuff electrodes around the splenic nerve bundle and artery and tested the stimulation regiment in conscious, freely moving mice, in the setting of DSS-induced colitis. SpNS applied 6 times daily for 12 days reduced the colon weight/length ratio (40.4 vs. 51.8 mg/cm; $P = 0.01$) and DAI (0 vs. 1.5; $P = 0.06$) when compared with sham stimulated mice (Fig. 3A). No clear difference in the loss of bodyweight over time was observed (Fig. 3B). At day 8, during the peak of DSS-induced colitis, comparable endoscopic scores (4.3 vs. 6.1; $P = 0.14$) were found in the animals treated with SpNS versus animals that underwent sham-stimulation (Fig. 3C). At the end of the experiment, spleen weight (98.5 vs. 133.0; $P = 0.07$), histology scores (4 vs. 11.5; $P = 0.18$), and protein expression of cytokines in the colon were not significantly different between mice that received SpNS and sham stimulated mice, although trends towards amelioration of colitis in SpNS-treated mice were evident (Fig. 3D-F).

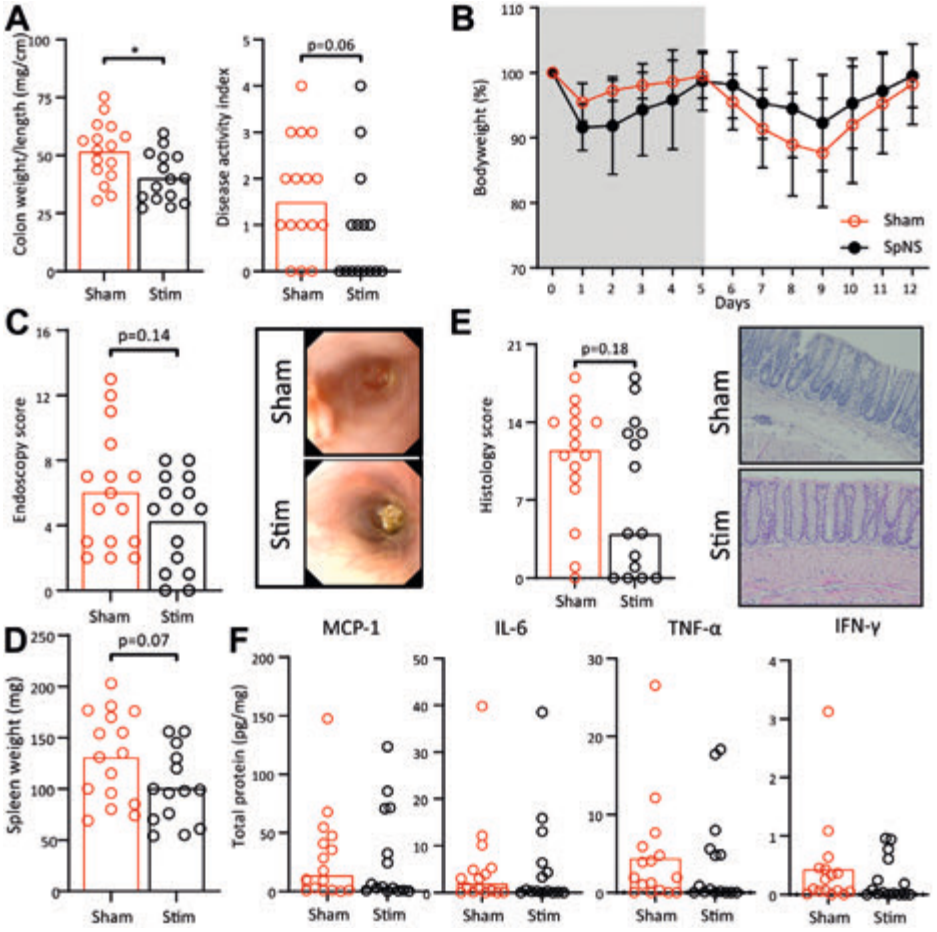

Fig 3. SpNS improves DSS-induced colitis. **A** Colon weight/length and Disease Activity Index at day 12. **B** Bodyweight loss of mice over time, compared with day 0. Data are expressed as mean and SD. **C** Endoscopy score at day 8 and two representative images. **D** Spleen weight. **E** Histology score and two representative images are shown of a hematoxylin and eosin staining, magnification 10×. **F** Protein levels of MCP-1 IL-6, TNF-α and IFN-γ in colon homogenates, normalized for total protein levels. N = 15–16 per group. All mice (both sham and stim) were implanted with a cuff electrode, were allowed to recover for 10 days and then received DSS in drinking water for 5 days followed by a 7 day recovery period. Data are expressed as mean or median and individual data points. Statistical differences between sham and stim mice were assessed using an independent t-test or a Mann–Whitney U test. $P < 0.05$ was considered significant. DSS dextran sulfate sodium, SpNS splenic nerve plexus stimulation, MCP monocyte chemoattractant protein, IL interleukin, TNF tumor necrosis factor, IFN interferon

Because SpNS ameliorated clinical features of colitis, we aimed to identify molecular events following SpNS. Transcriptional profiling by means of RNA-seq was performed on spleens from mice with DSS-induced colitis that received sham stimulation and mice with DSS-induced colitis that received SpNS. The top 50 genes that were differentially expressed between sham stimulated mice and SpNS mice are listed in Fig. 4A. The downregulation of nitric oxide synthase 2 (*Nos2*) and gene signatures associated with immune metabolism prompted us to further focus on genes and pathways that are involved in cellular immune metabolism, which is known to be influenced by β_2-AR activation.[38] For instance, Hypoxia-Inducible Factor

(HIF)-1α marks the cellular response to systemic oxygen levels in various cells, including activated macrophages.[39] Expression of genes in the HIF-1α signaling pathway was significantly reduced in SpNS spleens ($P = 0.007$), while expression of genes encoding proteins relevant to oxidative phosphorylation was relatively increased in SpNS spleen cells ($P < 0.001$, Fig. 4B). This suggests that SNS favors the metabolic state of oxidative phosphorylation over glycolytic states in stimulated splenocytes, corresponding to our earlier observation of NE-simulated macrophages[38] and characteristics of macrophages found in tissue mucosa of colitis in remission.[40]

Next, to explore the underlying mechanisms through which SpNS affected intestinal inflammation, we performed bulk RNA-seq. Differentially expressed genes in colon were assessed between mice that did not receive stimulation or DSS, sham stimulated mice with DSS-induced colitis, and SpNS-treated mice with DSS-induced colitis (Additional file 4). Comparing the transcriptomes of mice with DSS-induced colitis (without SpNS) and control mice indicated an expected induction of genes encoding pro-inflammatory cytokines, such as TNF-α, and genes involved in wound healing and remodeling, such as Matrix Metalloproteinase (MMP)-7 (Additional file 5).

When comparing sham stimulated and SpNS mice with DSS colitis, 87 genes were differentially expressed in the colonic tissue of which the top 40 are shown in Fig. 5A. Genes that were significantly different were predominantly found in the domains of leukocyte chemotaxis and migration. Noteworthy is the SpNS-induced downregulation of S100A8 and S100A9, which encode subunits of calprotectin, the clinical biomarker for IBD.

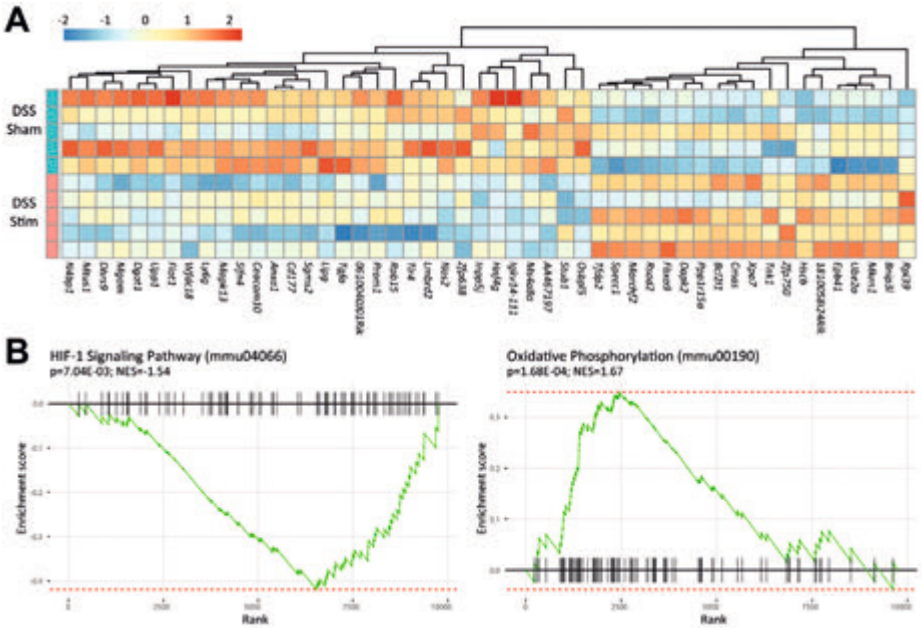

Fig. 4 SpNS induces transcriptomic changes in the spleen. **A** Heatmap of the top 50 differentially expressed genes when comparing sham and SpNS mice. **B** GSEA plots showing depletion and enrichment of HIF-1 signaling pathway and Oxidative phosphorylation pathway associated genes among the SpNS mice. *SpNS* splenic nerve plexus stimulation, *GSEA* gene set enrichment analysis, *NES* normalized enrichment score, *HIF* hypoxia-inducible factor

Genes of interest for colonic inflammation were validated in a second independent experiment using qPCR. Indeed, TNF-α was significantly reduced in mice that received SpNS compared with sham stimulated animals (0,44 vs. 1.00 respectively, $P = 0.009$; Fig. 5B). As we showed in the spleen, genes in the HIF-1α signaling pathway were reduced, although this effect was less pronounced in the colon compared with the spleen (Fig. 5C).

DISCUSSION

Here, we demonstrate that SpNS induced transcriptional changes in the spleen, and reduced clinical signs of colitis such as colon oedema and histological parameters scored in the assessment of colitis, while having a limited effect on colon inflammation. The results of this study support both *ex* and *in vivo* studies in models of endotoxemia demonstrating SpNS can be used to reduce inflammatory activation of splenocytes.[16,41,42] Recently, the effect of both electrical and ultrasound SpNS was found to improve inflammation in two different animal models of arthritis.[9,43] Similarly, ultrasound stimulation of the spleen and splenic nerve bundle improved DSS-induced colitis.[17] Although it could not be excluded that other organs were affected by this stimulation, the immune modulatory effects were not observed in splenectomized animals and therefore ultrasound was thought to target the spleen and splenic nerve bundle directly. Our results largely corroborate the observations made by Nunes *et al.* regarding the weight loss and decrease in colon density and histology scoring after stimulation. In line, in our studies a negative regulation of expression of various cytokines by SpNS was noted, depending on the starting time of stimulation after the induction of DSS-induced colitis. This highlights the complexity of the DSS model, in which duration of DSS and moment of outcome measurement vary throughout literature.[44,45] The fact that both electrical (this study) and ultrasound (Nunes *et al.*) SpNS reduce disease severity following DSS-induced colitis strengthens the idea that SpNS might hold potential as a therapy for patients with IBD, although further mechanistic studies are warranted.

The parameters used in this study were similar to the stimulation settings that were used in an earlier reported model of experimental arthritis.[9] Interestingly, the anti-inflammatory effects of SpNS in the arthritis experiments were more profound than what we have found in our study. It may well be that the pathology in the collagen-induced arthritis model is primarily dependent on systemic cells and cytokines, and is therefore better controlled through SpNS compared to DSS-induced colitis, which is a more mucosal inflammatory process. Noteworthy, Guyot *et al*. focused on stimulation of a nerve branch that innervated the cranial pole of the spleen, which consists of cholinergic and adrenergic fibers in contrast to the adrenergic, arterial branches. Conversely, in our present study we focused on stimulation of the nerves that run along the artery, because this bundle resembles human anatomy regarding splenic innervation more accurately and is surgically accessible in human.[46,47]

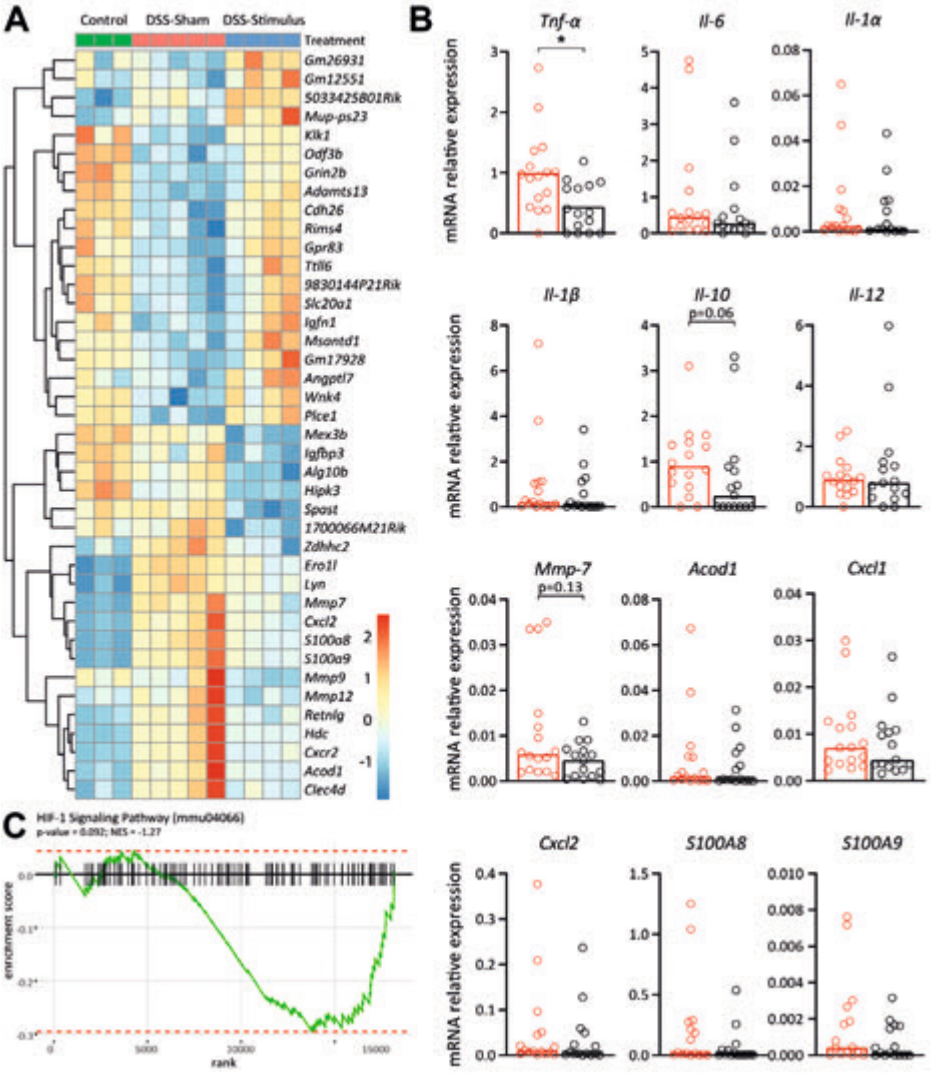

Fig.5 SpNS induces transcriptomic changes in the colon. A Heat map of top 40 differently expressed genes when comparing between sham and SpNS mice. B mRNA levels of TNF-α, IL-6, IL-1α, IL-1β, IL-10, IL-12, MMP-7, ACOD1, CXCL1, CXCL2, S100A8 and S100A9. mRNA levels are normalized against reference genes Nono and RPLP0. N = 15–16 per group. C GSEA plots showing depletion of HIF-1 signaling pathway associated genes among the SpNS mice. Data are expressed as mean (TNF-α) or median (other) and individual data points. Statistical differences between sham and stim mice were assessed using an independent t-test or a Mann–Whitney U test. $P<0.05$ was considered significant. *DSS* dextran sulfate sodium, *SpNS* splenic nerve plexus stimulation, *HIF* hypoxia-inducible factor, *Nono* non-POU domain-containing octamer-binding protein, *RPLP0* ribosomal protein lateral stalk subunit P0, *TNF* tumor necrosis factor, *IL* interleukin, *MMP* matrix metalloproteinase, *ACOD* cis-aconitate decarboxylase, *CXCL* chemokine (C-X-C motif) ligand, *S100* S100 calcium-binding protein, *GSEA* gene set enrichment analysis

While VNS has been found to result in anti-inflammatory effects in various experimental conditions such as sepsis, postoperative ileus, rheumatoid arthritis and kidney disease, we were unable to demonstrate any anti-inflammatory effects of VNS in our DSS-induced colitis model.[48-51] Histologic examination of the cuff showed intact neural tissue and stimulation parameters used were similar to other studies, making it unlikely that VNS was taking place insufficiently, or vagus nerve bundles were damaged.[15,52] There could be several other explanations for this contradiction. In earlier studies VNS was shown efficacious in reducing colitis in rats under 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and only improved survival from oxazolone-induced colitis in mice, both representing more detrimental models of colitis.[6,53] For the DSS-induced colitis model no beneficial effect of stimulation has been reported previously, although vagotomy did worsen colitis.[37] Furthermore, VNS did not improve the histology score in all studies, which is the conventional outcome parameter for experimental colitis.[4] While we could not replicate the anti-inflammatory effect of VNS, it still holds potential as it seems to improve outcome of patients with Crohn's disease in a limited, non-randomized controlled clinical trial.[2] The off-target effects such as heart rate depression remain undesirable, but this might be prevented by application of subdiaphragmatic VNS.[7]

Although we discuss indirect modulation of colitis through the splenic nerve plexus and vagus nerve, it should be noted that sympathetic nerve stimulation or stimulation of other nerves directly innervating the colon such as the superior mesenteric nerve and sacral nerves have all been shown to improve experimental colitis in earlier studies. [54-56] This underlines the potential of neuromodulation as a treatment for IBD patients.

Our analysis showed that inflammatory gene transcription during DSS-induced colitis is counteracted by SpNS in spleen and colon. Our approach is one of a preventive intervention into DSS-induced colitis, rather than a treatment of an established colitis, which is a limitation of this study. Another drawback of the current study is that the observed signal could well be the result of an interplay between the proportional representation of various cell types as well as changes in their transcriptional profiles. From earlier studies we know that SpNS causes transcriptional changes in the T and B cell populations present in the spleen.[43] However, we previously demonstrated that the AR activity affects the macrophage potential to adapt their metabolic profile *in vitro*, supporting our interpretations in the current study.[38] Inflammatory macrophage activation blunts oxidative phosphorylation, thereby preventing repolarization, strictly directing inflammatory cell expression.[39] Having identified SpNS-associated differences in the expression of HIF-1α and oxidative phosphorylation, it is enticing for us to suggest that SpNS affects macrophage activation quite specifically. This is also supported by the scRNA-seq results showing a large portion of macrophages expressing *Adrb2*. The latter could at least in part provide a mechanism for the beneficial action of splenic nerve bundle activation on immune driven pathology such as in colitis or collagen-induced arthritis.[9] Nonetheless, future studies are necessary to disentangle which SpNS-associated differences are the result of cellular heterogeneity and the transcriptome thereof.

CONCLUSIONS

In conclusion, we demonstrated the effects of SpNS in a murine model of colitis and showed that SpNS ameliorated clinical features of colitis and caused differential expression of genes in the spleen and colon involved in inflammation. Future experimental studies should focus on the connection between the spleen and colon to gain more insight in the therapeutic mechanisms of SpNS. Meanwhile, a clinical trial in patients undergoing esophagectomy will investigate safety and feasibility of SpNS for application in humans (www.clinicaltrials.gov; NCT04171011).

Additional figures can be accessed via the journal site: https://jneuroinflammation.biomedcentral.com/articles/10.1186/s12974-022-02504-z

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PART II

SURGICAL RELEVANCE AND FEASIBILITY OF SPLENIC NEUROVASCULAR BUNDLE STIMULATION IN HUMANS

04

AGE-RELATED VARIATION IN SYMPATHETIC NERVE DISTRIBUTION IN THE HUMAN SPLEEN

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Frontiers in Neuroscience 2021

ABSTRACT

Introduction: The cholinergic anti-inflammatory pathway (CAIP) has been proposed as an efferent neural pathway dampening the systemic inflammatory response via the spleen. The CAIP activates the splenic neural plexus and a subsequent series of intrasplenic events, which at least require a close association between sympathetic nerves and T cells. Knowledge on this pathway has mostly been derived from rodent studies and only scarce information is available on the innervation of the human spleen. This study aimed to investigate the sympathetic innervation of different structures of the human spleen, the topographical association of nerves with T cells and age-related variations in nerve distribution.

Materials and Methods: Spleen samples were retrieved from a diagnostic archive and were allocated to three age groups; neonates, 10–25 and 25–70 years of age. Sympathetic nerves and T cells were identified by immunohistochemistry for tyrosine hydroxylase (TH) and the membrane marker CD3, respectively. The overall presence of sympathetic nerves and T cells was semi-automatically quantified and expressed as total area percentage. A predefined scoring system was used to analyze the distribution of nerves within different splenic structures.

Results: Sympathetic nerves were observed in all spleens and their number appeared to slightly increase from birth to adulthood and to decrease afterward. Irrespective to age, more than halve of the periarteriolar lymphatic sheaths (PALSs) contained sympathetic nerves in close association with T cells. Furthermore, discrete sympathetic nerves were observed in the capsule, trabeculae and red pulp and comparable to the total amount of sympathetic nerves, showed a tendency to decrease with age. No correlation was found between the number of T cells and sympathetic nerves.

Conclusion: The presence of discrete sympathetic nerves in the splenic parenchyma, capsule and trabecular of human spleens could suggest a role in functions other than vasoregulation. In the PALS, sympathetic nerves were observed to be in proximity to T cells and is suggestive for the existence of the CAIP in humans. Since sympathetic nerve distribution shows interspecies and age-related variation, and our general understanding of the relative and spatial contribution of splenic innervation in immune regulation is incomplete, it remains difficult to estimate the anti-inflammatory potential of targeting splenic nerves in patients.

INTRODUCTION

The cholinergic anti-inflammatory pathway (CAIP) comprises an efferent neural pathway that dampens the systemic inflammatory response via the spleen and is suggested to involve sequential activation of the efferent vagus nerve and the splenic plexus.[1,2] Others have put forward that instead of the efferent vagus nerve, this pathway involves the greater splanchnic nerve.[3] Irrespective, activation of the splenic plexus results in a cascade of intrasplenic events, starting with the release of norepinephrine (NE).[4] Studies have demonstrated that NE then activates adrenergic receptors on $CD4^+$ $ChAT^+$ T lymphocytes, which in turn produce and secrete acetylcholine (ACh).[5-7] ACh then inhibits the release of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) from activated macrophages via nicotinergic receptor signaling.[7-10]

Morphological evidence for the presence of sympathetic nerves in proximity to splenic T lymphocytes was provided earlier by Bellinger et al.[11,12] In a study on rat spleens, they observed sympathetic nerves diverging into T lymphocyte specific white pulp areas, also known as periarteriolar lymphatic sheaths (PALSs). In the PALSs these nerves were in close proximity to T lymphocytes and formed synaptic connections.[13] The presence of sympathetic nerves which could release NE in the proximity of T lymphocytes in the human spleen might hold potential as a therapeutic target for immune related disease and knowledge on the anatomical configuration of splenic innervation in humans is therefore essential.

The presence of sympathetic nerves at the medio adventitial junction in human spleens has been described in various studies,[14-17] however, innervation of T cell specific lymphoid tissue has only been reported once.[18] In the latter study, sympathetic innervation patterns in spleens of end-stage sepsis patients were investigated and sympathetic nerves were observed to be in close association with lymphocytes in the PALS of the control group (trauma patients who died after hemorrhagic stroke). The results of this study were descriptive and it remains unclear whether this was a common feature and observed in all PALSs, or only occasionally. Since, PALS related sympathetic nerves were seldom observed in end-stage sepsis patients, the authors suggested this difference to be disease-related.[18] However, other factors, such as aging, are known to contribute to decline of sympathetic innervation as well, as shown in the rat spleen and human cerebral arteries.[11,12,19] If human splenic innervation is subject to age-related decline as well, this information is of relevance because it might determine the window of application of anti-inflammatory neuromodulation along age. Since the age profile of a substantial number of patients of the control group in the study of Hoover et al.[18] was lacking, as well as comprehensive data on the prevalence of PALS related sympathetic nerves, our understanding of human splenic innervation remains incomplete.

Therefore, in this study, quantitative and semi-quantitative analytical methods were used to investigate the distribution of sympathetic nerves in human spleens of various age groups. Although the PALS is considered to represent the primary structure of T cell neuromodulation, T cells migrate through the spleen and exposure to NE might occur at any location they pass while entering or exiting the spleen. Therefore, blood vessels, red pulp, trabeculae and the capsule were evaluated for the presence of sympathetic nerve tissue as well.

MATERIALS AND METHODS

Tissue Samples

A total of 26 paraffin embedded splenic samples were provided the Pathology Department of the University Medical Center Utrecht. Samples were divided into three age groups, being 40 weeks of gestation (from now on referred to as neonatal), 10–25 years and 25–70 years. This study was approved by the Medical Ethical Committee (#18-167) as a "non-Medical Research Act" study and the Biobank of the University Medical Center Utrecht approved to use the rest biomaterial for this research (biobank #18-284). None of the individuals was known with immunological or splenic clinical conditions. Table 1 contains data on age, sex, and cause of death.

Samples were obtained from the splenic hilar region and were cut in the transversal plane. Paraffin embedded splenic samples were cut on a microtome (Leica 2050 Super Cut, Nussloch, Germany) and 5 µm thick sections of splenic tissue were collected on glass slides, air dried and subsequently heat fixed for 2 h on a slide drying table of 60°C (Medax, 14801, Kiel, Germany). All slides were deparaffinized, rehydrated and further processed for histochemical or immunohistochemical staining. Hematoxylin/Eosin (HE) was used to evaluate technical tissue quality, to generate a tissue overview, and to screen for general pathological changes. A double T and B cell staining, using antibodies against specific membrane proteins, being CD3 and CD20 respectively, was used to screen the white pulp for distinct pathological abnormalities. To quantify and compare the overall presence of sympathetic nerves and T cells, and the distribution of sympathetic nerves in the PALS and other splenic structures (capsule, trabeculae, rep pulp and arteries), a double staining for sympathetic nerves and T cells was performed. In this procedure antibodies against CD3 and tyrosine hydroxylase (TH), were used, the latter being an enzyme involved in the synthesis of NE. The general nerve marker, protein gene product 9.5 (PGP9.5) was used on adjacent slides to confirm neural identity of TH-immune reactive (IR) structures.

Staining Procedures

Tissue sections were dewaxed in xylene and rehydrated through graded alcohols prior to histochemical or immunohistochemical staining. Prior to immunohistochemistry, sections were pre-treated with Heat Induced Epitope Retrieval (HIER) in citrate buffer (pH6.0) for 20 min at 95°C.

Hematoxylin/Eosin Staining

Tissue sections were stained with hematoxylin for 10 min at room temperature (RT). After rinsing in running tap water, sections were dipped in ethanol 50%, stained with eosin for 1 min and dehydrated in graded alcohols and xylene. Slides were coverslipped with Entellan (Merck, Darmstadt, Germany).

#	Sex	Age	Cause of death
25–70 years (*N* = 7)			
1	F	46	Pancreatic tail cyst
2	F	66	Myocardial infarct
3	F	52	Subarachnoid hemorrhage
4	F	48	Traumatic motor bike accident
5	M	66	Unknown
6	M	26	Arrhythmia
7	M	29	Long QT syndrome
10–25 years (*N* = 7)			
8	F	14	Acute unexpected death, probably due to cardiac arrest
9	M	16	Arrhythmia
10	M	11	Sudden unexpected death due to coronary artery anomaly
11	M	11	New diabetes mellitus with keto-acidosis
12	F	12	Unknown
13	M	11	Herniation of the sigmoid due to congenital mesenterial defect
14	F	24	Lung emboly
40 weeks (*N* = 12)			
15	M	40 weeks 2 days	Perinatal asphyxia
16	F	39 2/7 weeks 2 days	Perinatal asphyxia
17	M	40 weeks 1 day	Perinatal asphyxia
18	M	42 1/7 weeks 3 days	Perinatal asphyxia
19	F	41 weeks 10 days	Perinatal asphyxia
20	M	40 6/7 weeks 4 days	Perinatal asphyxia
21	M	40 2/7 weeks 1 days	Perinatal asphyxia
22	M	40 2/7 weeks 1 day	Perinatal asphyxia, congenital heart defect
23	M	40 2/7 weeks 4 days	Perinatal asphyxia
24	M	41 5/7 weeks 3 days	Perinatal asphyxia
25	F	35 weeks 1 day	Perinatal asphyxia, first born of dichorionic twin
26	M	41 6/7 weeks 7 days	Perinatal asphyxia

Table 1. Patient profiles. Age is represented in years for the 25–70 and 10–25 years age groups and in weeks of gestation and postnatal days for the 40 weeks group (e.g., 41 6/7 w 7 = 41 weeks and 6 days of gestation where after the newly born lived for 7 days).

Single Immunohistochemical Staining Procedures (PGP9.5 and Tyrosine Hydroxylase)

fter the HIER procedure, sections were incubated with 5% Normal Human Serum (NHS) in TBS prior to incubation with rabbit anti human PGP9.5 antibody (1:2000 in TBS-T + 3% BSA, 48 h, 4°C, Dako, Glostrup, Denmark) or rabbit anti-human TH (1:1500 in TBT-T + 1% BSA, overnight RT, Pel-Freez, Rogers AR). Visualization of bound antibodies was performed with undiluted Brightvision Poly-Alkaline Phosphatase (AP) Goat-anti-Rabbit (ImmunoLogic, Amsterdam, Netherlands) and Liquid Permanent Red (LPR, Dako). All sections were counterstained with hematoxylin (Klinipath), dried on a hotplate for 15 min at 60°C and coverslipped with Entellan

(Merck). Tris-buffered saline with 0.05% Tween20 (TBS-T) was used for all regular washing steps. Negative controls were obtained by incubation with TBS-3% BSA without primary antibodies. Human vagus nerve – and sympathetic trunk sections were included as a positive control for general – and sympathetic nerve tissue respectively. Both, staining procedures and positive controls, have been used in previous studies in which they proved to be valid to detect small nerves (including single nerve fibers) and to serve as proper controls, respectively.[20,21]

Sequential Double Immunohistochemical Staining Procedure (CD20/CD3 and CD3/TH)
After HIER, sections were incubated with 3% Normal Goat Serum (NGS) (CD20/CD3) or 5% NHS (CD3/TH). In the first staining sequence, sections were incubated with CD20 or CD3 antibodies (details of used antibodies, including dilution, incubation time are presented in Table 2) and visualized with Brightvision Poly-AP Goat-anti-Mouse or Goat-anti-rabbit Mouse (ImmunoLogic) respectively followed by PermaBlue plus/AP (Diagnostics Biosystems, Pleasanton, United States). Prior to the second staining sequence a HIER in citrate buffer (pH6.0, 15 min RT) was performed, removing unbound antibodies but leaving chromogens unhanged.[22] Sections were then incubated with 3% NGS (CD20/CD3) or 5% NHS (CD3/TH) followed by incubation with CD3 and TH antibodies, where after they were visualized with Brightvision Poly-AP Goat-anti-Rabbit (ImmunoLogic) and LPR (Dako, Glostrup, Denmark). Sections were dried on a hotplate for 15 min at 60°C and coverslipped with Entellan (Merck, Darmstadt, Germany). Tris-buffered saline with 0.05% Tween20 (TBS-T) was used for all regular washing steps. Negative controls were obtained by incubation with TBS-3% BSA without primary antibodies. Human spleen sections that were previously confirmed to show proper staining for B cells, T cells and sympathetic nerves were included as positive controls.

Double stain	Staining sequence	Primary antibody	Host	Vendor	Dilution, incubation time and temperature	Secondary antibody	Chromogen
CD3/TH	1	CD3	Rabbit	Dako A0452	1:50, 90 min, RT	Brightvision-anti-Rabbit/AP	PermaBlue
	2	TH	Rabbit	Pel-Freez P40101	1:1500, overnight, RT	Brightvision-anti-Rabbit/AP	LPR
CD20/CD3	1	CD20	Mouse	Dako M0755	1:400, 90 min, RT	Brightvision-anti-Mouse/AP	PermaBlue
	2	CD3	Rabbit	Dako A0452	1:100, 90 min, RT	Brightvision-anti-Rabbit/AP	LPR

Table 2. Detailed information on antibodies used in sequential double staining procedures.

Microscopic Evaluation

Hematoxylin/eosin and CD3 and CD20 stain was evaluated by bright field microscopy. The chromogen LPR was used to visualize sympathetic nerves. This marker has stable fluorescent characteristics and allows the user to alternately use bright field and fluorescent microscopy on the same slide. This can be beneficial as both modalities have their own advantages, e.g., fluorescent microscopy is more sensitive allowing small nerves to be more easily recognized, whereas bright field allowed better discrimination between lymphocytes and other cells. Instead of using a band pass filter suited for LPR, a long pass filter was used which allowed emission of a broader range of wave lengths. This resulted in a green/yellow autofluorescence of connective tissue, which was used to determine if the observed nerve extended beyond,

e.g., perivascular connective tissue. All samples were studied using a DM6 microscope (Leica, Nussloch, Germany) with an I3 fluorescent filter.

Image Acquisition

Single images were captured at various magnifications. These images were either brightfield or fluorescent images, depending on which modality appeared most suited to visualize the structures of interest. Both brightfield and fluorescent tile scans (stitched overlapping images) were captured for digital image analysis. Tile scans were either used to quantify the total amount of sympathetic nerves and T cells or to automatically select PALS regions which were then further studied in detail regarding their innervation (all tile scans were obtained using a 10x objective). Image acquisition was performed using a DM6 microscope with a motorized scanning stage, a I3 fluorescent filter, a DFC7000 T camera and LASX software (all from Leica, Nussloch, Germany).

Quantitative Analysis of General Sympathetic Nerve and T Cell Presence

Bright field and fluorescent tile scans of CD3 and TH stained slides, respectively, were optimized and analyzed in Fiji (ImageJ with additional plugins).[23] Optimization of the images included removal of irrelevant tissue (e.g., hilar connective tissue and vasculature), artifacts and large trabecular arteries with large surrounding nerves. Both the total splenic tissue area and the area of TH and CD3-IR tissue were selected using standardized thresholds and data was expressed in pixels. The overall area occupied by sympathetic nerves and T cells was expressed as area% with respect to the total tissue area. Table 3 contains an overview of the different parameters investigated in this study, including a short description of the quantification method and how the data are expressed.

Quantitative analysis of:	**Method of quantification**	**Expressed as**
General sympathetic nerve presence	Automated counting of TH-IR pixels	Area %
General T cell presence	Automated counting of CD3-IR pixels	Area %
Semi-quantitative analysis of:	**Method of quantification**	**Expressed as**
# PALSs with sympathetic nerves	Automated selection of PALSs and manually counting of + PALSs	%
Sympathetic nerve density in:		
PALS	Microscopic evaluation of automated selected PALS for the relation of sympathetic nerves with T cells	Score 1–3
Capsule	General microscopic evaluation	Score 1–3
Trabeculae	General microscopic evaluation	Score 1–3
Red pulp	General microscopic evaluation	Score 1–3
Arteries	General microscopic evaluation	Score 1–3

Table 3. Studied parameters, their method of quantification and data expression.

Semi Quantitative Analysis of Sympathetic Nerve Presence and Density in Various Splenic Areas

All samples contained large and small sympathetic nerves. Large nerves run with penetrating and trabecular arteries, whereas small nerves occur as discrete entities or are associated with smaller vascular structure from which they occasionally extent to the surrounding tissue. Only small nerves were evaluated in this study, since they are of relevance in regulation of local processes, such as immune cell function.

Periarteriolar Lymphatic Sheaths For each sample a series of PALS regions was automatically selected and further studied in detail. No difference was made in types of PALS (being follicle associated PALS or non-follicle associated PALS). Automated selection was performed in Fiji, using tile scans of CD3/TH-stained slides. A threshold was set to select all CD3-IR areas, which were turned into solid regions using a blur function. Solid regions of 40.000 pixels or more were then selected. This approach resulted in a selection of 23–60 PALSs of substantial size. A maximum of 40 selected PALSs were then evaluated for the presence of sympathetic nerves. The number of positive PALSs was counted and expressed as percentage of the total number of studied PALSs. The association of sympathetic nerves with T cells was then graded as follows; 1: only one or two sympathetic nerves were observed to extend beyond the connective tissue of the vessel wall and to be in close proximity to T cells immediately lining the vessel wall, 2: multiple sympathetic nerves extended beyond the connective tissue of the vessel wall and were in close proximity to T cells immediately lining the vessel wall and 3: comparable to 2, but nerves extended beyond T cells immediately lining the vessel wall.

Capsule, Trabeculae, Red Pulp and Arteries All samples were studied microscopically using a 20x objective and alternately switching between brightfield and fluorescent microscopy for reasons described in section “Microscopic Evaluation.” Nerve density in various splenic areas was quantified by means of scoring according to the following grading scale 0: complete absence, 1: low density, 2: moderate density, and 3: high density. These scores were assigned when the observation was representative for the whole sample. With respect to the arteries, a division was made into large and small arteries. Large arteries represented penetrating arteries, also referred to as trabecular arteries (these were surrounded by a substantial amount of connective tissue). Small arteries represented arteries that could be observed in the red and white pulp.

Prior to scoring, various samples of the different age groups were evaluated by the observers in order to obtain a general idea of the extent of PALS innervation, the relation of PALS related sympathetic nerves with T cells, and to low and high nerve densities in other areas. Each sample was examined independently by two observers (CC and DB) who were blinded for the age group. When there was disagreement between the observers the samples were re-examined and scored by consensus.

Statistical Analysis

Statistical analysis and graph conception were performed using Graphpad Prism 8. A Kruskall–Wallis test was used to compare the three age groups for their general sympathetic nerve and T cell presence, and, for sympathetic nerve density in various splenic areas. An uncorrected Dunn’s test was used to provide a p-value for each separate group comparison. All parameters were expressed as median followed by their inter quartile range. Association

between the general presence of sympathetic nerves and T cells was tested by means of Pearson's correlation coefficient.

RESULTS

All spleens showed well defined white pulp with distinct T and B cell regions (PALS and follicles, respectively) and red pulp with well-defined splenic cords red pulp sinusoids were more indistinctly present. All samples contained blood vessels of various sizes, trabeculae and most samples contained a significant bit of capsule. No pathological abnormalities were observed. TH-IR structures showed comparable patterns to PGP9.5-IR structures in adjacent slides, confirming their nerve identity. Figure 1 shows examples of normal splenic morphology.

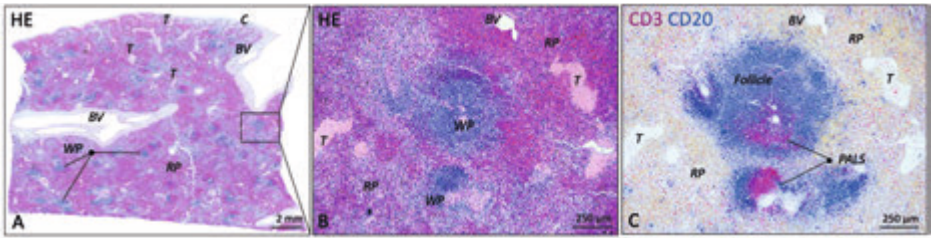

Figure 1. Microscopic images of a normal spleen. **(A)** Overview image of a splenic sample of a patient from the 10–20 years group (HE staining). White and red pulp can be clearly distinguished as well as vascular structures and connective tissue structures such as trabecula**e. (B)** Close up image of the boxed splenic region in **(A)**, showing normal splenic pulp morphology with clear white and red pulp areas (HE staining). **(C)** Similar region as in **(B)**, showing the presence of T and B cells (CD3 and CD20, respectively) in periarteriolar lymphatic sheaths (PALSs) and follicles, respectively. BV, blood vessel; C, capsule; WP, white pulp; RP, red pulp; T, trabecula.

General Sympathetic Nerves and T Cells Presence and Their Age-Related Variation

Sympathetic nerves were detected in 26/26 samples (100%). Nerves were mostly observed surrounding vascular structures and to a lesser extent as discrete structures in the PALS, capsule, trabeculae and red pulp. T cells were observed in all samples and were primarily present in the PALS and to a lesser extent in follicles and in the red pulp. General sympathetic nerve presence was higher in the 10–25 group (0.1 [0.09–0.18]) compared to the neonatal group (0.02 [0.01–0.07], $p = 0.0034$) as well as compared to the 25–70 group (0.04 [0.02–0.05], $p = 0.0192$) (Figure 2A). No significant difference in T cell presence was observed between the different age groups (Figure 2B). No correlation was found between sympathetic nerve and T cell presence ($r = 0.076$, $p = 0.71$). Table 4 contains an overview of quantified median data per age group and lists age-related significant differences. Data of all separate individuals can be found in the Supplementary Data.

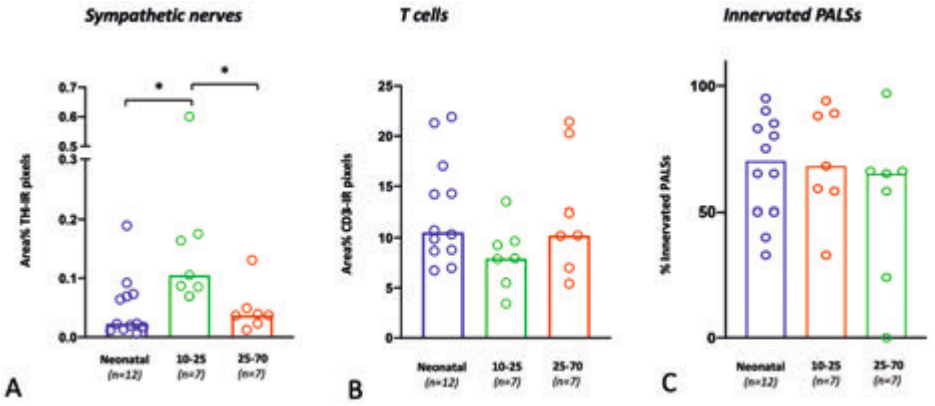

Figure 2. Age-related variations in general T cell and sympathetic nerve presence and the percentage of innervated PALS. **(A)** The general presence of sympathetic nerves per sample is calculated as the number of TH positive pixels and expressed as area % with respect to the total area of each sample. **(B)** The general presence of T cells per sample is calculated as the number of CD3 positive pixels and expressed as area % with respect to the total area of each sample. **(C)** The number of sympathetic innervated PALSs per sample expressed as percentage of the total selected number of PALSs in each sample. ∗P < 0.05.

Sympathetic Nerve Presence in the Periarteriolar Lymphatic Sheaths and Its Age-Related Variation

In 25/26 subjects (96%), sympathetic nerves were occasionally observed to extend beyond the adventitial lining of the central artery into the lymphatic tissue where they were in close proximity to T cells. To gain an objectified understanding of the number of innervated PALSs, a series of PALSs was automatically selected for each sample and further studied in detail (Figure 3A). PALSs with sympathetic nerves in close proximity to T cells were observed in 614 of the in total studied 1027 PALSs (60%). No significant difference in the percentage of innervated PALSs was observed between the different age groups (Figure 2C). All PALSs that contained sympathetic nerves were additionally evaluated with respect to the number of nerves that were in association with T cells and whether these nerves would travel further into the lymphatic tissue. Of the in total 1027 studied PALSs, 302 (29%) PALSs contained one paravascular nerve in proximity to T cells (score 1), 249 (24%) PALSs contained multiple nerves (score 2) and 63 (6%) PALSs contained nerves which traveled further into the lymphatic tissue (score 3) (Figures 3B–H contains examples of all scores). The neonatal group showed a higher percentage of PALSs with a score 1 (38 [28.5–60]) compared to the 25–70 group (21 [8–41], p = 0.0059). For the other scores no age-related differences were observed.

	Neonatal	10–25 years	25–70 years	Significant difference
General sympathetic nerve presence (Area %)	0.02	0.10	0.04	10–25 > neonatal $p = 0.0034$
	(0.01–0.07)	(0.09–0.18)	(0.02–0.05)	10–25 > 25–70 $p = 0.0192$
General T cell presence (Area %)	10.55	7.95	10.23	NS
	(8.70–16.34)	(5.50–9.66)	(7.02–20.29)	
# PALSs with sympathetic nerves (%)	70 (50–95)	68 (58–89)	65 (24–66)	NS
Score 1 (%)	38	34	21	Neonatal > 25–70 $p = 0.0059$
	(28.5–60)	(21–41)	(8–41)	
Score 2 (%)	10	9	8	NS
	(5–14.75)	(4–19)	(1–11)	
Score 3 (%)	4	6	9	NS
	(0–9.5)	(0–12)	(0–11)	
Sympathetic nerve density in				
Capsule	2	0	0	Neonatal > 10–25 $p = 0.0003$
	(1–2)	(0–0)	(0–1.25)	Neonatal > 25–70 $p = 0.008$
Trabeculae	1	1	1	NS
	(1–2)	(1–3)	(1–2)	
Large arteries	3	2	2	Neonatal >25–70 $p = 0.0098$
	(2–3)	(2–3)	(1–2)	
Small arteries	3	2	2	NS
	(2–3)	(2–3)	(1–2)	
Red pulp	2	1	0	Neonatal > 25–70 $p = 0.0016$
	(1-2)	(0-2)	(0-1)	

Table 4. Data is expressed as median values (interquartile range is placed between brackets). Observed significant differences between groups are listed including their p-value.

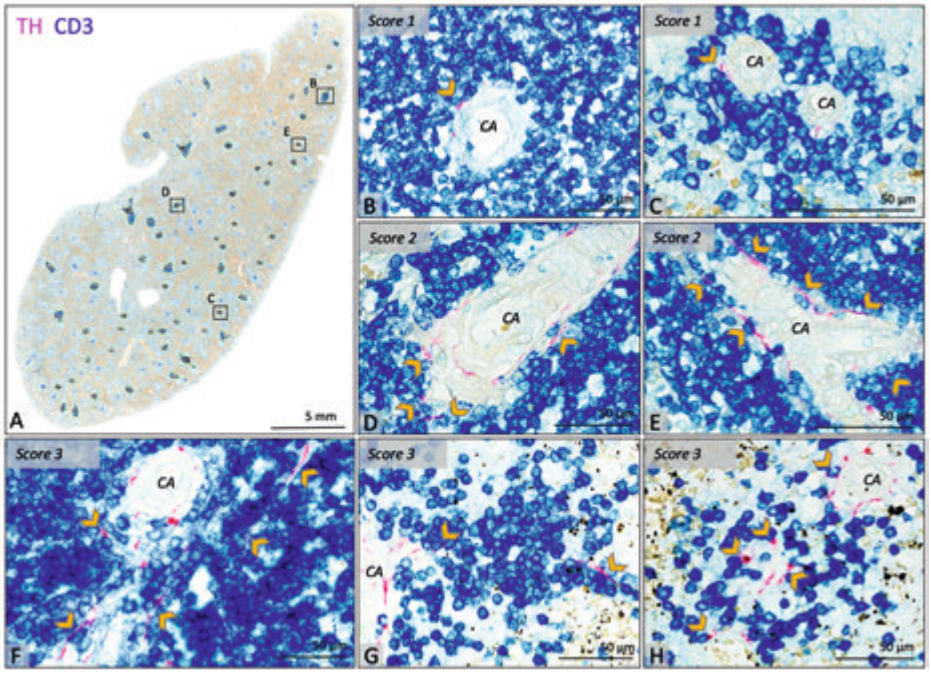

Figure 3. Data is expressed as median values (interquartile range is placed between brackets). Observed significant differences between groups are listed including their p-value. Bright field microscopic images of PALS related sympathetic nerves in CD3/TH double stained splenic tissue slides of various neonatal individuals. **(A)** Overview image of a splenic sample showing automatically selected T cell regions which were further investigated for the presence of sympathetic nerves and their relation with T cells. **(B–E)** Close up images of the marked regions in (A) representing PALSs with a score 1 or 2. **(F–H)** Close up images of PALSs with a score 3 (all from different neonatal individuals). Score 1: Only one or two sympathetic nerves were observed to extend beyond the connective tissue of the vessel wall of the central artery (CA) and to be in close proximity with T cells immediately lining the vessel wall. Score 2: multiple sympathetic nerves extended beyond the connective tissue of the CA and were in close proximity with T cells immediately lining the vessel wall. Score 3: comparable to 2, but nerves extended beyond T cells immediately lining the vessel wall. CA, central artery; Arrow heads: pointing out sympathetic nerves that are in close proximity with T cells, but might be obscured by the blue stain.

Sympathetic Nerve Density in Other Splenic Areas and Its Age-Related Variation

Capsule

In 25/26 subjects (96%) a substantial amount of capsule was present of which 15 (60%) contained sympathetic nerves. These nerves were scattered and were mainly observed in the part of the capsule which was in close proximity to the hilum, where vascular structures with surrounding nerves entered the spleen. In the hilar region, the capsule was less distinct and showed continuity with hilar specific structures such as the adventitia of incoming vascular structures or the connective tissue of suspending splenic ligaments (Figures 4A,B). Most observed capsular nerve tissue was present in the more superficial and middle part of the capsule (Figures 4D–F) and only sporadically in the deeper part, where it was in direct contact with the red pulp (Figure 4C).

Trabeculae

All subjects showed trabeculae which, as a result of the cutting plane of the samples, were observed either as immediate extensions of the capsule, or as discrete structures deeper in the parenchyma (Figure 5A). The deeper parts of the trabeculae frequently contained large vascular structures (Figures 5C,E). In 25/26 (96%) subjects, trabecular sympathetic nerves were present to some extent and were often observed in the deeper parts of trabeculae, whereas the trabeculae that extended immediately from the capsule were mostly devoid of nerves. Trabecular sympathetic nerves were observed as discrete structures (Figures 5B,D,F), or as a nerve plexus surrounding vascular structures (Figures 5C,E). In both cases nerves could extend up to the external border of the trabecular tissue where nerves were in proximity to the surrounding red pulp.

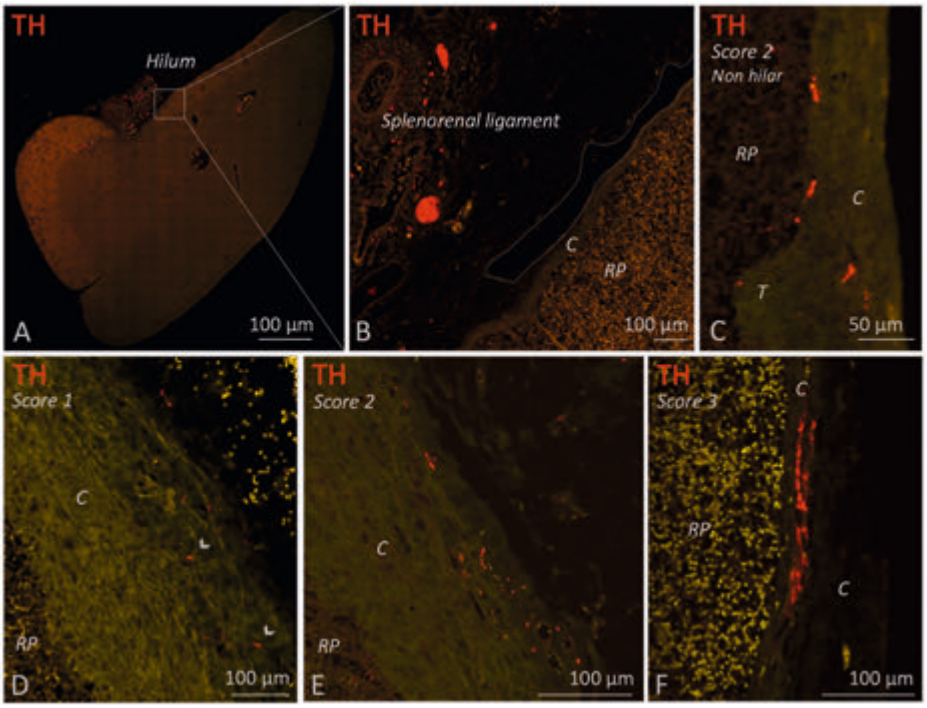

Figure 4. Fluorescence microscopic images of various capsule related sympathetic nerve densities (TH staining). **(A)** Overview image of a neonatal spleen. Large blood vessels with a perivascular nerve plexus reside in the splenorenal ligament and enter the spleen at the hilum. **(B)** Close up images of the boxed region in **(A)**. The splenorenal ligament contains connective tissue, vascular structures and sympathetic nerves. The lining of the splenorenal ligament reflects over the hilar capsule and on its distal continuation thins out (dotted line shows the lining of the ligament). **(C)** Close up image of a part of a capsule obtained from a non-hilar part of a neonatal spleen. A moderate density (score 2) of sympathetic nerves can be observed. **(D,E)** Close up images of hilar capsule samples of spleens of individuals from the 25–70 years age group containing a low (score 1) and moderate (score 2) density of sympathetic nerves, respectively. **(F)** Close up image of hilar capsule sample of a neonatal spleen with a high (score 3) density of sympathetic nerves. Capsular nerves were mostly observed in the more superficial and middle part of the capsule and only sporadically in the deeper part where they were in direct contact with the red pulp (as shown in C). C, capsule; RP, red pulp; T, trabecula. Arrow heads: small capsular nerves.

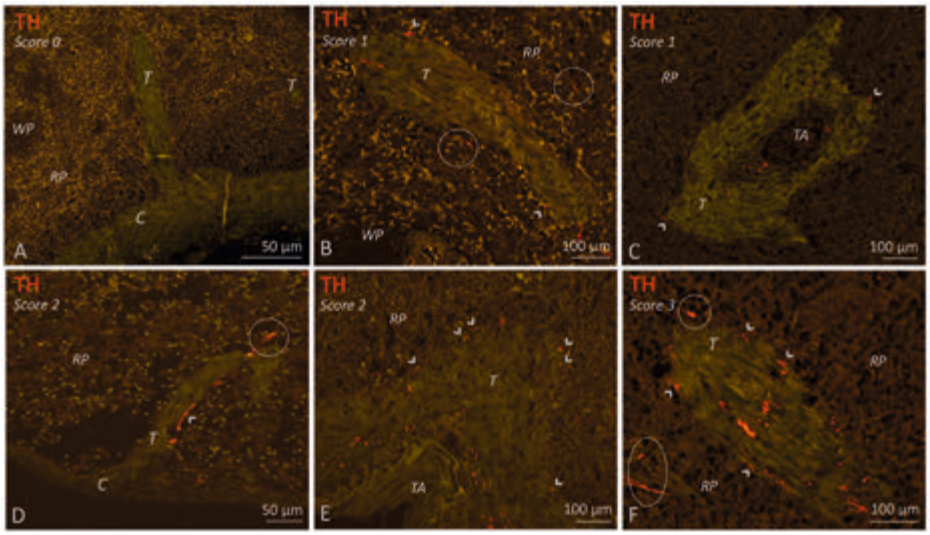

Figure 5. Fluorescence microscopic images of various trabecula related sympathetic nerve densities (TH staining) in different neonatal individuals. **(A)** Spleen without trabecular sympathetic nerves (score 0). **(B)** Trabecula with a low density (score 1) of sympathetic nerves. A few small nerves are present within the connective tissue of the trabecular and a few nerves can be observed on its outer margin where they are in proximity to the RP. **(C)** Trabecula with a low density (score 1) of sympathetic nerves. Perivascular nerves can be observed in the adventitia of a small blood vessel (trabecular artery) and in the connective tissue of the trabecula from where it diverges to its outer margins where a few nerves are bordering the RP. **(D)** Trabecula extending from the capsule with a moderate density (score 2) of sympathetic nerves. Most nerves are in proximity to the RP and on its cranial site a nerve extents into the RP. Sympathetic nerves in parts of trabeculae directly extending from the capsule were very sparse. **(E)** Comparable to **(C)** but this figure contains a larger trabecular artery and shows a moderate density (score 2) of sympathetic nerves. **(F)** Trabecula with a high density (score 3) of sympathetic nerves which diverge to the trabecula's outer border to be in proximity to the RP.

Arteries

All 26 subjects had clear recognizable vascular structures of various sizes which were to some extent surrounded with perivascular sympathetic nerves (Figure 6). In case of splenic artery branches in the hilum or large incoming trabecular arteries, nerve tissue was presented as large nerve bundles running in the adventitia or trabecular connective tissue, respectively, or as finer neural structures. In case of smaller arteries, nerves were organized in a more delicate network that were in close proximity to the vessel wall. Occasionally, perivascular nerves extended beyond the adventitial connective tissue. This was observed in central arteries, trabecular arteries and to a lesser extent in small arteries in the red pulp.

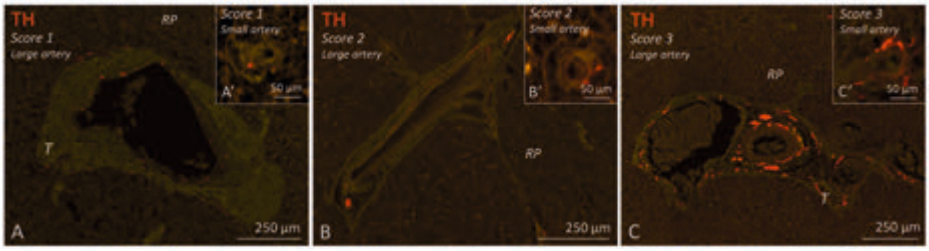

Figure 6. Fluorescence microscopic images of various of blood vessel related sympathetic nerve densities (TH staining) in different 25–70 years age group individuals. Each figure contains a representative example of a large vessel and a small vessel with a specific amount of sympathetic nerves (score 1–3). (A–C) Splenic samples with a low, moderate or high density of sympathetic nerves surrounding large arteries (score 1-3). (A'–C') Splenic samples with a low, moderate, or high density of sympathetic nerves surrounding small arteries (score 1–3). RP, red pulp; T, trabecula.

Red Pulp

The red pulp of all subjects contained clearly recognizable lymphoid tissue, also known as the splenic cords (Figure 7A). Sinusoids were easily recognized if they contained a substantial amount of blood, but otherwise were less distinct. Sympathetic nerves were observed in the red pulp of 20/26 (77%) subjects. These nerves comprised either small solitary nerves in between the red pulp (Figures 7B,D), or nerves bordering parenchymal trabeculae (Figures 5B,E,F; 7C) or small vascular structures (Figures 5F, 7A,D,E).

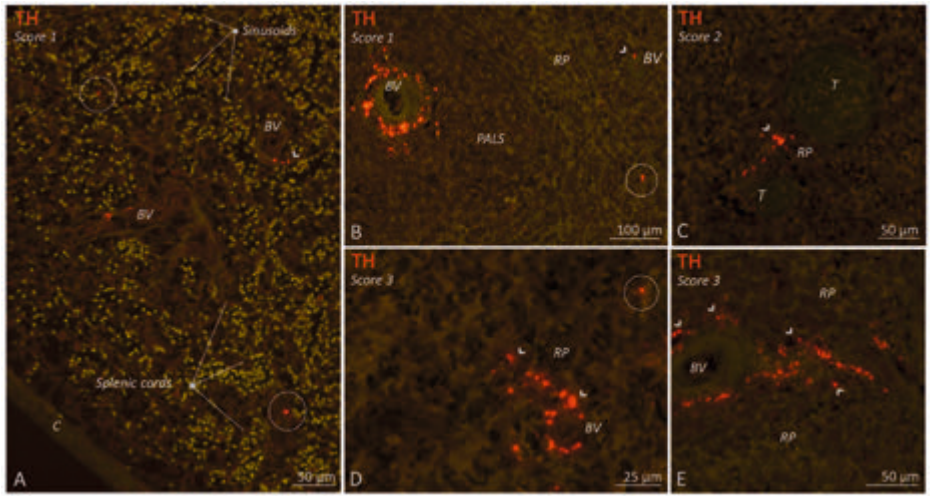

Figure 7. Fluorescence microscopic images of various red pulp related sympathetic nerve densities (TH staining) in different 10–25 year age group individuals. Sympathetic nerves are present as discrete structures running in the splenic cords (encircled structures) or as nerves that originate from a perivascular or trabecular plexus and from there diverge further into the red pulp (RP) (arrow heads). **(A)** Splenic sample with clear splenic cords and sinusoids and a low density (score 1) of sympathetic nerves. **(B–E)** Various examples of spleens with RP related nerves either as a low density (score 1), a moderate density (score 2) or a high (score 3) RP related sympathetic nerve density. C, capsule; BV, blood vessel; PALS, periarteriolar lymphatic sheath; RP, red pulp; T, trabecula.

Age-Related Variations

Age-related differences were observed with respect to nerve density in the capsule, large arteries and the red pulp. The neonatal group showed a significant higher nerve density in its capsule compared to both the 10–25 and 25–70 group, and, surrounding its large arteries and in its red pulp when compared to the 25–70 group. No age-related differences were observed in trabeculae and small arteries. Table 4 contains detailed information on median group data and p-values.

DISCUSSION

This study shows that the human spleen contains sympathetic nerves, not only associated with the splenic vasculature, but also as discrete structures in the PALS, capsule, trabeculae and red pulp. Furthermore, the presence of sympathetic nerves shows a mild tendency to decrease with age. These findings are of relevance for understanding the role of splenic sympathetic nerves in regulation of the systemic immune response in humans and for the development of neuromodulatory anti-inflammatory therapies.

Sympathetic nerves were observed in all spleens but their presence was most prominent in the 10–25 age group, suggesting that from birth their number increases whereafter it decreases from adulthood on. This observation fits in with the fact that organ systems, including the peripheral nervous system, mature after birth until the onset of adulthood whereafter they subsequently show signs of aging.[24] More specifically, animal studies have shown that an age-related decline applies for splenic sympathetic innervation as well.[11,12,25]

A decrease in T cell presence has been put forward as another explanation for a decrease in sympathetic nerve abundance.[18] The authors observed the presence of T cells to correlate to that of sympathetic nerves and suggested the lack of specific nerve growth factors produced by these T cells to be of relevance. In the current study, however, no correlation between the general presence of sympathetic nerves and T cells (both expressed as area%) was found, thereby further emphasizing aging to be the most plausible explanation for the observed decline of sympathetic innervation in spleens of healthy persons.

In 96% of the studied individuals, PALSs were observed to contain sympathetic nerves that extended beyond the adventitia of central arteries and to be in apposition with T cells. So far PALS innervation in humans have only been reported once.[18] The authors, however, did not supply information on the number of innervated PALSs per individual, deeming it impossible to estimate whether PALS innervation represented a structural entity of normal healthy spleens or a more coincidental heterogeneous finding. The current study shows that human PALSs innervation was observed in 60% of the in total 1027 studied PALSs and therefore represents a structural phenomenon. Furthermore, no significant age-related differences could be determined with respect to the innervation of the number of PALSs, or the extent to which sympathetic nerves were in proximity to T cells.

Sympathetic innervation patterns observed so far in human PALSs, however, seem to differ significantly from rodent species. In rats, mice and rabbits, nerves were more densely present and also traveled further into the parenchyma.[12,26,27] This questions whether splenic plexus stimulation in humans, with only a few T cells of the PALS in direct contact with sympathetic nerves, would target enough of these cells to establish a similar systemic anti-inflammatory effect as observed in rodents. It is, however, known that vagus nerve stimulation in humans

results in a systemic anti-inflammatory response.[28] Since vagus nerve stimulation activates the splenic plexus this effect must be elicited by splenic sympathetic nerves, potentially involving different components and/or locations than what is known for the prevailing CAIP NE-Ach-TNF mechanism.[1,2] In the following part various alternative options for explaining this effect will be discussed in the light of our findings.

Most T cells are migratory cells and reside in the PALS for only a certain amount of time whereafter they disseminate to the red pulp and return to the systemic circulation. If, during this migration, enough $CD4^+$T cells pass sympathetic nerves and short term synaptic connections are formed, a phenomenon referred to as short term plasticity,[29] a significant amount of adrenergic receptors on $CD4^+$T cells might indeed get activated. Another potential mechanism to overcome this issue might be volume transmission; a process wherein a neurotransmitter is not released in a synaptic cleft but is expelled into the extracellular matrix and reaches its effector cells by diffusion.[30] Volume transmission of NE could result in adrenergic activation in increased numbers and more distant T cells other than the ones in direct contact with sympathetic nerves. Furthermore, the splenic white pulp appears to contain low amounts of acetylcholinesterase, and volume transmission of Ach (secreted by activated T cells in the PALS) might occur as well, thereby further expanding the indirect effector scope of sympathetic nerves.[31]

Other studies have casted doubt on the prevailing mechanisms of the cholinergic anti-inflammatory pathway involving the sequence of NE release, ACh production, and subsequent TNFα reduction through cholinergic receptor activation on macrophages, both with respect to the mechanism itself and with respect to its location.[32,33] In a recent study wherein the relationship between sympathetic neurons and $ChAT^+$ lymphocytes were mapped in complete mouse spleens, it was shown that overall few $ChAT^+$ T cells were juxtaposed to sympathetic fibers and that their distance to these fibers exceeded that of traditional synapses (Murray et al., 2017). Moreover, the authors showed that sympathetic innervation was involved in homing of $ChAT^+$T cells to appropriate physical regions in the spleen by increasing the expression of the chemokine CXCL13 in stromal cells.[33] Such homing processes are vital as it conjoins the right cells for a properly aligned immune response (reviewed by Zhao et al[34]). In case of conjoining the key mediators of the intrasplenic NE-ACh-TNFα mechanism, this requires homing of $ChAT^+$ T cells towards macrophage rich areas such as the marginal zone and the red pulp, where adrenergic receptors on these T cells need to be activated prior to release of ACh in proximity to these macrophages. Interestingly, vagus nerve stimulation in mice specifically attenuated TNFα production by splenic macrophages in these two areas 30 minutes after endotoxin administration.[32] The authors observed nerve terminals adjacent to these TNFα producing macrophages, but did not provide information on the local presence of $ChAT^+$ T cells. With the above discussed topics in mind, it would be more plausible that the intrasplenic NE-Ach-TNFα mechanism occurs in the marginal zone and red pulp, instead of the PALS. Further support for favoring the red pulp and marginal zone over the PALS as designated immune regulation areas, is the difference in T cell transit time. T cell passage through the red pulp and marginal zone takes 5 and 50 min respectively whereas passage through the PALS (from the perivascular area to the macrophage rich marginal zone/red pulp) takes 2.5–6 hours[35-37] and the latter may take too long to provoke the fast systemic response which peaks at 90 min after electrical stimulation.[3,32,38]

According to recent literature, human spleens do not have a marginal zone but their red pulp is considered to be morphologically and functionally comparable to mice and rats (reviewed by Steiniger[39]). In contrast to previous studies[14-17] the current study showed that, although rare, red pulp innervation is present in all age groups albeit it more prominent in younger individuals. Sporadically, discrete nerves were observed within the red pulp, but most of the red pulp innervation was supplied either by trabecular nerves which extended to the outer margins of the trabecular connective tissue, or by nerves positioned outside the connective tissue surrounding small red pulp vascular structures. These nerves always remained in proximity to the trabeculae and vascular structures and never traveled deeper into the red pulp. In human fetuses, capsular nerves have also been observed to extend into the red pulp.[16] In the current study, however, capsular nerves have been observed in all age groups, but only rarely extended into the red pulp or reached the inner capsular margins contacting the red pulp and therefore were not considered to generally contribute to red pulp innervation.

Thus, comparable to the PALS, the red pulp in humans contains significant less sympathetic innervation when compared to other animals in which red pulp innervation was already considered sparse.[11] Therefore, again one could question whether splenic plexus stimulation in humans would target a sufficient amount of red pulp effector cells to provoke the effect observed in animals.[32] While searching the literature for the role of the stromal cells in immune regulation, a more elegant and subtle mechanism, which potentially requires little direct innervation of red pulp immune cells, was found. In the spleen stromal cells reside in both the white and red pulp where they represent the main cellular components of the reticular framework, a connective tissue scaffold which provides support for splenic immune cells and guidance for their migration.[40] As shown by a transmission electron microscopic study in guinea pigs, the reticular framework is composed enveloping reticular cells which enclose connective tissue components and occasionally a sympathetic axon or free nerve endings.[41] The connective tissue space of the framework was shown to be continuous and to contain meshwork like spaces.[41] The author referred to these meshwork like spaces as catecholamine canals since he hypothesized them to facilitate diffusion of released sympathetic neurotransmitters over larger distances throughout the reticular framework. With the exception of follicles, the reticular cells of the reticular framework are represented by contractile myofibroblasts.[42] Contraction of these myofibroblasts results in exposure of migrating immune cells to the content of catecholamine canals; sympathetic nerve endings or previously secreted and diffused NE.[41] Adrenergic signaling can than modulate the immune response, which in case of diffused NE does not require direct immune cell innervation. The reticular framework might equal the more recent discovered splenic conduit system in mice; an interconnected tubular network that functions as a transport system for fluid, small molecules and particles (including antigens) and is covered with fibroblast reticular cells which support migratory lymphocytes.[43,44] Whether the human spleen contains catecholamine cannals or a conduit system has not been established yet.

Overall, it can be concluded that, apparent age-related and interspecies differences in splenic sympathetic nerve distribution and density exists. It is, however, uncertain if and to what extent these differences are of significance for NE-ACh-TNFα mechanism based anti-inflammatory therapies in humans. Although experimental studies have shown an indisputable role for sympathetic nerves, ChAT+ T cells and macrophages, it is, however, not completely understood how and where the various elements of the prevailing intrasplenic

NE-ACh-TNFα mechanism interact and whether unknown intermediate elements are required. In order to be able to extrapolate experimental data to humans and to estimate whether targeting splenic sympathetic nerves in humans could be beneficial, additional experimental and morphological studies are required and alternative or additional mechanisms should be taken into consideration.

Study Limitations

The use of single tissue sections will result in a 2D representation of sympathetic nerves in relation with surrounding structures. This makes it difficult to truly estimate whether the evaluated sympathetic nerves represent small, local tissue innervating (discrete) nerves or that they might be part of larger en route nerves. This bias should be kept in mind when interpreting data on sympathetic nerve quantification. However, since the same bias applies to all samples, we consider its influence on group comparison data negligible.

CONCLUSION

Although less extensive when compared to other animals, human spleens contain sympathetic nerves, not only associated with vascular structures but also as discrete entities. In the PALS, these nerves were in proximity to T cells, suggesting the potential existence of a CAIP in humans. Alternative locations involved in neuroimmune regulation might be represented by the capsule, trabeculae and red pulp since these structures contain discrete sympathetic nerves as well. Since splenic sympathetic nerve distribution and density shows interspecies variation and our general understanding of the relative and spatial contribution of splenic innervation in immune regulation is incomplete, it remains difficult to estimate the anti-inflammatory potential of targeting splenic sympathetic nerves in humans. Future studies should focus on the anti-inflammatory efficacy of targeting these nerves in humans and further characterize the underlying mechanism. Splenic sympathetic innervation density slightly decreases from adulthood on and these age-related variations might be of relevance when developing sympathetic nerve based anti-inflammatory therapies.

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05

THE INFLAMMATORY RESPONSE AFTER LAPAROSCOPIC AND OPEN PANCREATODUODENECTOMY AND THE ASSOCIATION WITH COMPLICATIONS IN A MULTICENTER RANDOMIZED CONTROLLED TRIAL

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HPB 2019

ABSTRACT

Background

The systemic inflammatory response seen after surgery seems to be related to postoperative complications. A reduction of the inflammatory response through minimally invasive surgery might therefore be the mechanism via which postoperative outcome could be improved. The aim of this study was to investigate if postoperative inflammatory markers differed between laparoscopic (LPD) and open pancreatoduodenectomy (OPD) and if there was a relationship between inflammatory markers and the occurrence of postoperative complications.

Methods

A side study of the multicenter randomized controlled LEOPARD-2 trial comparing LPD to OPD was performed. Area under the curve (AUC) for plasma inflammatory markers, including interleukin (IL-) 6 , IL-8 and C reactive protein (CRP) levels, were determined during the first 96 postoperative hours and compared between LPD and OPD, Clavien-Dindo ≥III complications, and postoperative pancreatic fistula (POPF) grade B/C.

Results

Overall, 38 patients were included (18 LPD and 20 OPD). The median AUC of IL-6 was 627 (195-1378) after LPD vs. 338 (175–694)pg/mL after OPD, (p=0.114). The AUC of IL-8 and CRP were comparable. IL-6 levels were higher in patients with a Clavien-Dindo ≥III complication (634[309–1489] vs. 297[171–680], p=0.034) and POPF grade B/C (994[534–3265] vs. 334[173–704], p=0.003). In patients with a POPF grade B/C, IL-6 levels tended to be higher after LPD, as compared to OPD (3533[IQR 1133–3533] vs. 715[IQR 39 –1658], p=0.053).

Discussion

LPD, as compared to OPD, did not reduce the postoperative inflammatory response. IL-6 levels were associated with postoperative complications and pancreatic fistula.

INTRODUCTION

An increased systemic inflammatory response as early as 24 hours after surgery, reflected by inflammatory markers such as interleukin (IL) 6, C reactive protein (CRP) and tumor necrosis factor alpha (TNF-alpha), is associated with postoperative complications.[1-4] Minimally invasive surgery may reduce the surgical trauma and diminish this initial inflammatory response. A reduction in systemic inflammatory markers, particularly IL-6 plasma levels, after laparoscopic as compared to open surgery has indeed been confirmed for appendectomy[5], cholecystectomy[6], colectomy[7], and esophagectomy[8].

It is currently unclear whether this mechanism also holds true for laparoscopic pancreatoduodenectomy (LPD). Two recent single-center randomized controlled trials from highly experienced centers reported reduced intraoperative blood loss and decreased length of hospital stay after LPD compared to open pancreatoduodenectomy (OPD).[9,10] Despite these positive results, concerns regarding the safety of LPD have been raised. Several cohort studies have reported on increased rates of postoperative pancreatic fistula, postoperative bleeding, readmission, and 30-day mortality, especially in low-volume centers.[11-16]

In the Netherlands, LPD was introduced through a nationwide training program[17], after which the multicenter randomized controlled LEOPARD-2 trial was conducted comparing laparoscopic with open pancreatoduodenectomy.[18] This trial was prematurely terminated because of safety concerns due to a higher mortality after LPD.[19] The current study was performed as part of the LEOPARD-2 trial and set out to investigate if markers of inflammation differed postoperatively between LPD and OPD. Furthermore, the aim was to assess if inflammatory markers were related to postoperative complications.

METHODS

The present study was a side study during the first phase of the LEOPARD-2 trial; a multicenter, patient-blinded, parallel-group, randomized controlled superiority trial comparing LPD with OPD. The study protocol and primary results of this trial have been published.[18,20] The study was performed in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines. The protocol was approved by the institutional review board of Amsterdam UMC, location AMC, and verified by the institutional review boards of all other participating hospitals. The LEOPARD-2 trial was registered in the Dutch trial registry (NTR5689).

Study population

Participating patients were adults with an indication for elective pancreatoduodenectomy because of a malignant, pre-malignant, or symptomatic benign disease in the pancreatic or peri-ampullary region. Exclusion criteria were preoperative suspicion of vascular tumor involvement, a body mass index of >35 kg/m^2, and neoadjuvant radiotherapy for pancreatic cancer. The study flow chart is shown in Figure 1.

Study endpoints

Primary outcome was the inflammatory response as measured by plasma cytokine IL-6 levels during the first 96 hours after incision. The area under the curve (AUC) of IL-6, reflecting the total systemic levels of this cytokine during the study time frame, was compared between LPD and OPD.

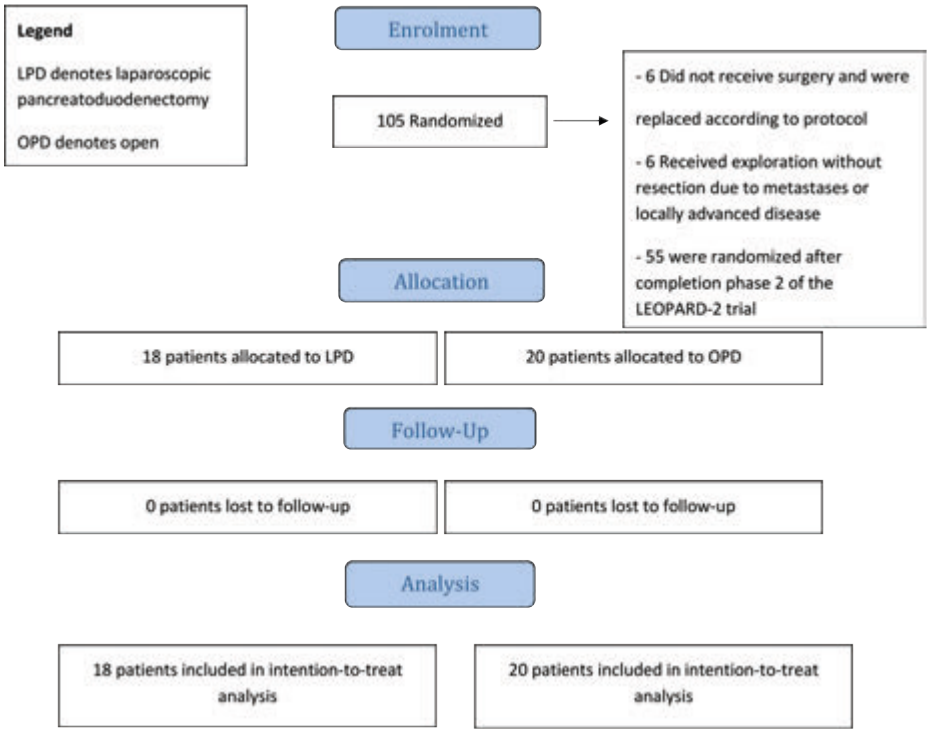

Figure 1. Flow-chart of patient inclusion.

The most important secondary outcomes were the association between inflammatory markers and the occurrence of postoperative complications, e.g., Clavien-Dindo grade ≥III complications and pancreatic fistula grade B/C. Based on the clinical experience that pancreatic fistulas seem to have a more severe clinical course in LPD compared to OPD, a separate analysis comparing IL-6 levels in patients with a pancreatic fistula grade B/C between LPD and OPD was performed. Other inflammation markers measured included serum levels of TNF-alpha, IL-1β, IL-8, IL-12p70 (pro-inflammatory cytokines), IL-10 (anti-inflammatory cytokine), and CRP after LPD and OPD.

Study procedures

Baseline characteristics, intra-operative measurements (blood loss, operation duration, conversion and vascular resections) and postoperative outcomes and complications were prospectively collected with the use of standardized case report forms. Blood samples were drawn just before the first incision, and subsequently 6, 24, 48 and 96 hours after incision. Systemic IL-6 levels have been shown to peak between 4-12 hours after surgery.[21-24] Samples were collected up to 96 hours after surgery to account for inflammation markers with a more latent post-surgical onset (e.g., CRP). Samples were collected into ethylenediaminetetraacetic acid- (EDTA), heparin-, and citrate-coated tubes and processed within one hour. Platelet-poor plasma was obtained by centrifuging blood at 2000 ×g for 15 minutes at 4 °C. The supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until further processing. Samples were analyzed following completion of the inclusions by an operator who was blinded to the treatment allocation (D.B.). Plasma levels of IL-6, IL-8, IL-1β, IL-12p70, TNF-alpha, and IL-10 were determined using a cytometric bead array (BD CBA Human Inflammatory Cytokines

Kit, BD Biosciences, San Jose, CA) according to the manufacturer's instructions (theoretical limits of detection: IL-6: 2.5 pg/mL, IL-8: 3.6 pg/mL, IL-1β: 7.2 pg/mL, IL-12p70: 1.9 pg/mL, TNF-alpha: 3.7 pg/mL, IL-10: 3.3 pg/mL). CRP samples were assayed on a BM/Hitachi 705 instrument (Boehringer, Mannheim, Germany).

Definitions

The pancreatic and peri-ampullary region were defined as the pylorus, the duodenum, the pancreatic head, and distal bile duct. Conversion was defined as any LPD in which an incision was used for other reasons than trocar placement or specimen extraction, and was further classified into non-urgent and urgent conversions.[25] Complications were categorized according to the Clavien-Dindo scoring system.[26] Only major complications, i.e., Clavien-Dindo grade ≥III, were reported. Pancreatic surgery-specific complications, such as postoperative pancreatic fistula (POPF), hepaticojejunostomy (HJ) leakage, delayed gastric emptying (DGE), and post pancreatectomy hemorrhage (PPH) were classified using the International Study Group on Pancreatic Surgery (ISGPS) definitions. Only grade B and C complications were reported.[27-30]

Sample size calculation

The sample size calculation was based on previously published studies on laparoscopic versus open colectomy and esophagectomy and a study on minimally invasive versus open pancreatic necrosectomy.[7,8,31] A 35% reduction in the area under the curve, from the start of the procedure to 96 hours after the start of the procedure, in cytokine IL-6 plasma levels was expected with laparoscopy (from 250 to 162.5 pg/mL), with a standard deviation (SD) of 80 pg/mL. Significance level (α) was set to 0.05 and power (1-β) to 0.8. The sample size in each arm needed, calculated using the independent Student's t-test, was 14 patients. Including 10% of cross-over from the LPD to OPD, a 1% loss to follow-up (based on previous studies), and 10% of patients presenting with metastasized disease (included, but used as oversampling), a total of 20 patients were allotted to each group, so in total 40 patients were necessary.

Statistical analysis

Analysis was performed according to the intention-to-treat principle. Laparoscopic procedures converted to open were analyzed in the laparoscopic group. Sensitivity analysis excluding converted patients was performed. Primary and secondary outcomes were cross-checked with data from primary sources. Inflammatory marker values were analyzed using an AUC analysis. AUCs were compared depending on the data distribution. An independent Student's t-test was used for normally distributed ordinal variables, while the Mann-Whitney U test was used for data with a non-parametric distribution. Additionally, inflammation marker values were presented as a function of time after incision. For comparison of normally distributed continuous variables, the independent Student's t-test was used and values were expressed as mean (SD). Continuous non-normally distributed variables were compared using the Mann-Whitney *U* test and values are expressed as median and interquartile range (IQR). Numbers and frequencies are presented for dichotomous data and compared using the Chi-square or Fisher's exact test as appropriate. The association between IL-6 and operative time was studied with use of a scatter plot and corresponding correlation coefficients (*r*) were calculated. The association between IL-6, IL-8, and CRP (AUC) and Clavien-Dindo grade ≥III complications and POPFs grade B/C were assessed by comparing the inflammatory markers between patients with and without these complications. Differences with a two-tailed *p*-value < 0.05 were considered statistically significant.

RESULTS

Baseline characteristics

The first 38 patients of the 105 patients randomized in the LEOPARD-2 trial were included. The sample size calculation incorporated 10% (4 patients) oversampling because of metastatic disease, but, during the trial no blood samples were drawn from metastatic patients. Baseline characteristics were comparable between patients randomized for LPD and OPD (Table 1). All intra- and postoperative outcomes are presented in Table 2. In the LPD group, 3 patients died because of postoperative complications, which included 1 patient because of bowel ischemia after intraoperative vascular damage, 1 patient with post pancreatectomy hemorrhage, and 1 patient with a grade C pancreatic fistula.

	LPD (N = 18)	**OPD (N = 20)**	***p*-value**
Female, N	12	8	0.100
Age, y, mean (SD)	74 (62-79)	69 (61-73)	0.196
BMI, kg/m^2, mean (SD)	25 (23-29)	26 (24-29)	0.534
Diabetes mellitus, N	2	4	0.663[#]
Biliary drainage, N	8	11	0.516
Karnofsky score, median (IQR)	80 (78-90)	80 (80-90)	0.874
ASA physical status, N			0.452
1	3	2	
2	9	14	
3	6	4	
1	3	2	
2	9	14	
3	6	4	

Table 1. Baseline characteristics. Abbreviations: LPD, laparoscopic pancreatoduodenectomy; OPD, open pancreatoduodenectomy; SD:, standard deviation; BMI, body mass index; IQR, interquartile range; ASA: American Surgical Association. [#]Fisher's Exact test was used.

Inflammatory response

The median AUC of IL-6 was 627 (IQR 195 – 1378) after LPD vs. 338 (IQR 175 – 694) pg/mL after OPD ($p = 0.114$) (Figure 2A). IL-6 levels were comparable between LPD and OPD at different time points (Figure 2D). These results remained consistent when conversions were analyzed in the OPD group (per protocol analysis, data not shown). The median AUC of IL-8 and IL-8 plasma levels at different time points were non-significantly higher after LPD, as compared to OPD (Figure 2B and 2E, respectively). The same applied to the median AUC of CRP and CRP plasma levels at different time points (Figure 2C and 2F, respectively). Plasma levels of IL-1β, IL-12p70, and TNF-alpha were below the detection limit. Figure 5 (Appendix) shows that no statistically significant correlation between IL-6 levels and operative time for LPD ($r = 0.09$) and OPD ($r = -0.15$) and combined ($r = 0.19$) was present.

	LPD (N = 18)	OPD (N = 20)	*p*-value
Conversion from laparoscopy to open**, N**	3	-	
Vascular **resection, N**	2	2	>0.999*
Operative time, min, median (IQR)	445 (403-530)	295 (224-330)	0.001
Blood loss, mL, median (IQR)	300 (200-550)	500 (300-800)	0.057
Postoperative **pancreatic fistula grade B/C, N**	3	5	0.697*
Hepaticojejunostomy leak grade B/C, N	4	2	0.395*
Delayed gastric emptying grade B/C, N	8	4	0.164*
Post pancreatectomy hemorrhage **grade B/C, N**	2	3	>0.999*
Clavien-Dindo **grade ≥III N**	11	8	0.194
≥III	4	6	
Grade IV	4	2	
Grade V	3	0	
Readmission, N	2	4	0.663*
Length of hospital stay, median (IQR)	13 (9-25)	11 (7-25)	0.714

Table 2. Intra- and postoperative outcomes. Abbreviations: LPD, laparoscopic pancreatoduodenectomy; OPD, open pancreatoduodenectomy; Min, minutes; IQR, interquartile range. #Fisher's Exact test was used.

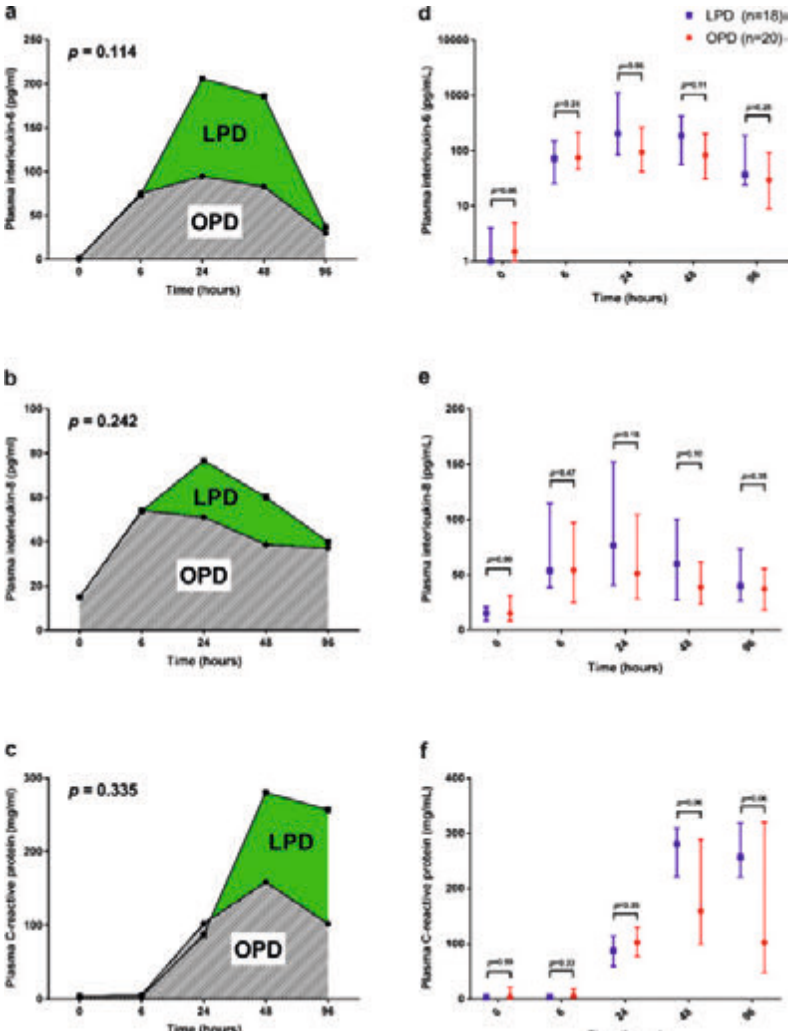

Figure 2. IL-6 **(A)**, IL-8 **(B)** and CRP **(C)** plasma levels after LPD and OPD. Figures **D, E** and **F** show the corresponding levels at each consecutive time point.

Association between inflammatory response and postoperative complications

Patients who developed Clavien-Dindo grade ≥III complication had higher IL-6 levels (634 [IQR 309 – 1489] vs. 297 [IQR 171 – 680] pg/mL, $p = 0.034$) and higher CRP levels (521 [IQR 397 – 633] vs. 374 [IQR 173 – 440] pg/mL, $p = 0.005$) compared to patients without these complications (Figure 3). Similar results were seen for CRP in patients with and without a Clavien-Dindo grade ≥III complication (Figure 3). AUC values of IL-6 (994 [IQR 534 – 3265] vs. 334 [IQR 173 – 704] pg/mL, $p = 0.003$), IL-8 (446 [IQR 215 – 773] vs. 182 [IQR 122 – 265] pg/mL, $p = 0.007$), and CRP (625 [IQR 554 – 718] vs. 380 [IQR 203 vs. 520] pg/mL, $p < 0.001$) were significantly higher for patients with a POPF grade B/C compared to patients without a POPF grade B/C (Figure 4). In patients with a POPF grade B/C, a trend was seen to higher IL-6 levels after LPD, as compared to OPD (3533 [IQR 1133 – 3533] vs. 715 [IQR 395 – 1658], $p = 0.053$) (Figure 4).

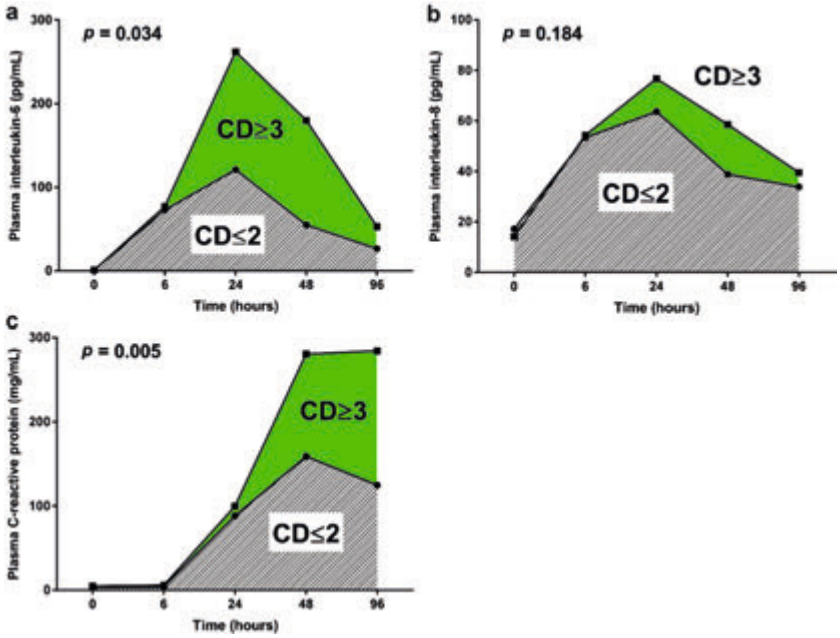

Figure 3. IL-6 **(A)**, IL-8 **(B)** and CRP **(C)** plasma levels in patients who did or did not develop a Clavien Dindo ≥ III complication.

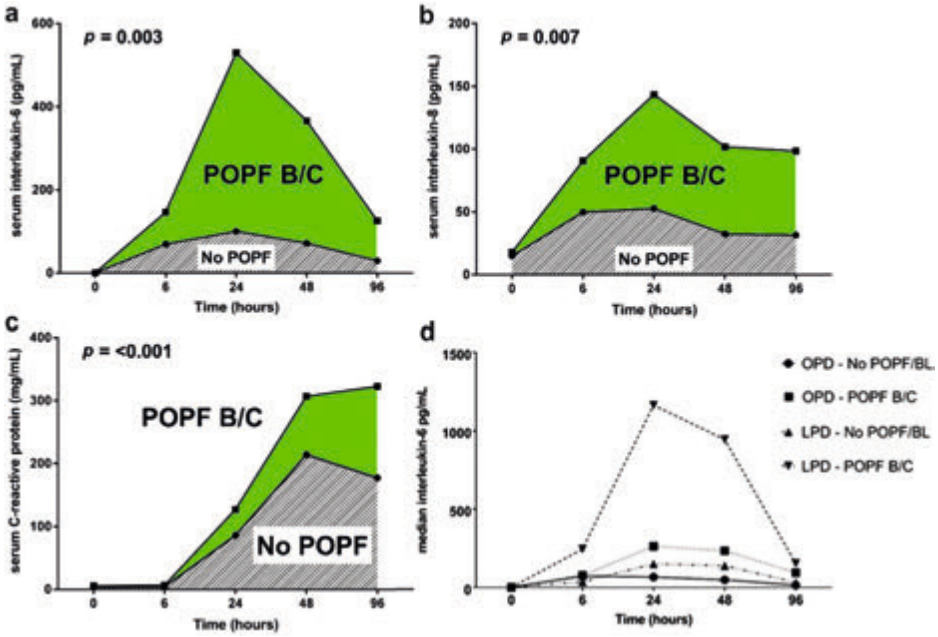

Figure 4. IL-6 **(A)**, IL-8 **(B)** and CRP **(C)** plasma levels in patients who did or did not develop a POPF grade B/C and **(D)** IL-6 levels for LPD and OPD separatelyv

DISCUSSION

In contrast to earlier findings from other laparoscopic gastrointestinal procedures, this side study of the multicenter LEOPARD-2 trial demonstrated that LPD did not reduce IL-6 levels compared to OPD. Furthermore, it was found that levels of IL-6 and CRP were higher in patients with a Clavien-Dindo grade ≥III complication and POPF grade B/C. The peak in IL-6 levels occurred at 24 hours after surgery whereas complications are mostly diagnosed later. IL-6 as a marker could therefore improve the early detection of major postoperative complications. Interestingly, in the subgroup of patients with a POPF grade B/C, IL-6 levels were (non-significantly) higher after LPD compared to OPD, possibly indicating a more severe inflammatory response to POPF after LPD.

Minimally invasive surgery aims to reduce the surgical trauma, lower the systemic inflammatory response, fasten postoperative recovery, and ultimately reduce the risk of postoperative complications.[32] The benefits of the laparoscopic approach regarding mitigating the inflammatory response have been confirmed for several types of gastrointestinal surgery (e.g., bowel surgery). However, the findings of the current study do not support these earlier outcomes.[5-8,31] There are several possible explanations for this result. Pancreatoduodenectomy is a highly complex and lengthy procedure. The benefit of laparoscopy could be insufficient to compensate for the extent of the procedure. This is however contradicted by findings in esophagectomy, another complex gastrointestinal procedure, where the laparoscopic approach did reduce systemic inflammatory markers.[8] Unfortunately, no operative times for laparoscopic and open esophagectomy were reported in that study. A more likely explanation for the relative high inflammatory response in the LPD group is the frequent occurrence of complications after pancreatoduodenectomy. The difference in rate of Clavien-Dindo grade ≥III complications after LPD and OPD was not statistically significant but it could still have been a relevant factor. Also, the potentially higher IL-6 response after LPD compared to OPD in patients with a POPF grade B/C could indicate that complications after LPD have a more fulminant clinical course. This finding does correspond to the authors clinical experience with LPD. A final explanation for these outcomes was that surgeons had not completed the learning curve for LPD, which could be as high as 80-120 procedures for major gastrointestinal procedures.[18,33,34] Since surgeons who participated in the trial had performed 20-40 LPDs, it may well be that pancreatic anastomoses were created inadequately, leading to local and systemic inflammation and very early POPF. However, similar rates of POPF grade B/C were reported between LPD and OPD in the LEOPARD-2, PLOT, and PADULAP trials.[9,10,18]

An increasing body of evidence suggests a role for systemic IL-6 levels in the prediction of complications after gastrointestinal surgery. In accordance with the tendency in our results, several studies found that high IL-6 plasma levels at 24 hours were associated with the occurrence of major complications after gastrointestinal surgery.[1,4] IL-6 is a cytokine that is released early in the inflammatory cascade by immune cells such as T-cells and macrophages, including Kupffer cells. Systemic IL-6 levels peak between 4-12 hours and induce the production of acute phase proteins such as CRP in the liver.[21,22,35] CRP plasma levels are commonly used as a clinical decision tool because of its predictive value, which was also found in our study.[36] The early peak of IL-6 makes it a potential early predictor of postoperative complications, especially since IL-6 values at 24 hours have been proven as accurate as CRP levels at 72 hours

in the prediction of postoperative complications.[1] Future studies should assess the value of IL-6 levels in clinical practice to aid decision making.

The results of this study should be interpreted in the context of some limitations. First, this study has a relatively small sample size. Although the study was adequately powered to show a reduction in IL-6, the results did not meet the hypothesized reduction of IL-6. A larger study would be required to draw definite conclusions on the relation between LPD and OPD and IL-6 but also to further examine the association between IL-6 and postoperative complications. Second, different outcomes may be found in centers with more experience in LPD. As mentioned, the surgeons in the current trial had performed 20-40 LPD procedures before the start of the trial. This was deliberately designed so that the trial results would be applicable for surgeons after the first phase of the learning curve, rather than for surgeons with extensive experience. Third, fluid administration during surgery was not measured but could have decreased levels of inflammatory markers due to dilution.

The major strength of this study is the randomized design, allowing for blood sampling in patients who were randomly allocated to LPD or OPD, which dismissed the influences of surgical case selection.[37] Therefore, to this date, this study provides the highest level of evidence on the inflammatory response after LPD vs. OPD. It would be very interesting to compare the present findings to a series with LPD and robot-assisted pancreatoduodenectomy in more experienced centers. It is possible that with more experience the inflammatory response does decrease to similar or even lower levels compared to OPD.

In conclusion, LPD did not reduce the inflammatory response (e.g., IL-6) compared to OPD. Also, an association was seen between IL-6 and CRP levels and major complications and POPF. Peak levels of IL-6 occurred as early as 24 hours after surgery and could therefore improve the early detection of major postoperative complications compared to CRP. In patients with POPF grade B/C, IL-6 levels were higher after LPD compared to OPD. Future research should focus on the relevance of IL-6 levels in clinical decision making.

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06

MORPHOMETRIC ANALYSIS OF THE SPLENIC ARTERY USING CONTRAST-ENHANCED COMPUTED TOMOGRAPHY (CT)

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Surgical and Radiologic Anatomy 2020

ABSTRACT

Purpose

To evaluate the morphology and course of the splenic artery, which might impact the surgical implantation of systems that stimulate the nerves surrounding the splenic artery. Experimental studies indicate that these nerves play an important part in immune modulation, and might be a potential target in the treatment of autoimmune diseases.

Methods

This retrospective cohort study made use of contrast-enhanced CT images from 40 male and 40 female patients (age 30-69) that underwent a CT examination of the aorta, kidneys or pancreas. Anatomic features were described including total splenic artery length, calibers, tortuosity, the presence of arterial loops and the branching pattern of the splenic artery.

Results

No age-gender related differences could be found related to tortuosity or branching pattern. The length of splenic artery in contact with pancreatic tissue decreased with increasing age but was not different between genders. Artery diameters were wider in male compared to female subjects. Loops of variable directions, that represent a part of the artery that curls out of the pancreatic tissue, were identified in each age-gender category and were present in nearly all subjects (86%).

Conclusion

This study suggests that although some anatomic features of the splenic artery are subject to factors as age and gender, the tortuosity of the splenic artery is not age-dependent. Most subjects had one or multiple loops, which can serve as a target for neuromodulatory devices. Future studies should investigate whether splenic nerve stimulation is safe and feasible.

INTRODUCTION

There is a growing body of experimental data to support the hypothesis that electrical stimulation of the nerves that project to the spleen can modulate immune responses, making it a potential target for neuromodulation in the treatment of chronic inflammatory conditions.[1-3] These nerves form a plexus around the splenic artery before innervating the spleen.[4,5] Therefore, the course of the splenic artery can impact the therapeutic potential of splenic nerve stimulation in humans.

The study of splenic artery anatomy has been complicated by the potential effect of aging on splenic artery tortuosity, as well as the effect of using different methodologies for characterizing the arterial anatomy.[6] An example of this is the assessment of proximity of the splenic artery to the pancreas where gross anatomic and radiographic studies have demonstrated different results.[7-9] In an autopsy series, Pandey et al. reported that the splenic artery was within the substance of the pancreas in 23.1% cases while in a cohort of patients studied by cross-sectional imaging Zhu and colleagues reported this relationship in 63.3% of cases. Determining the actual area where the splenic artery is in direct contact with the pancreas could provide a better characterization of their anatomic relationship and might help to indicate if placement of a neuromodulatory device is feasible.

It is thought that the splenic artery elongates and becomes more tortuous with age, which increases separation between the splenic artery and the pancreas.[6] This increased separation may allow a safer approach for device implantation on the splenic artery, lymphadenectomy or to select cases for vessel sparing distal pancreatectomy. Our understanding of this important anatomic detail is however largely derived from cadaveric studies, where degradation and formalin fixation may result in altered vascular and pancreatic anatomy.[6,7,10] Furthermore, it is not systematically described in literature how splenic artery diameters vary between splenic origin and branching the splenic hilum, which can impact design of neuromodulatory devices.

To address these limitations, and for the preparation of a clinical trial (www.clinicaltrials.gov; NCT04171011), we have undertaken a study to characterize arterial tortuosity, branching and anatomic proximity of the artery to the pancreas in a single cohort of patients using 3D reconstructed images from abdominal CT angiograms. Furthermore, we addressed the effects of age and gender difference on these anatomic features.

MATERIALS AND METHODS

Subjects

Data for this study were collected using randomly chosen contrast-enhanced CT images from subjects that underwent a CT examination of the aorta, kidneys or pancreas in the Catharina Hospital Eindhoven, the Netherlands between January 1, 2009 and January 1, 2018. All CT scans were acquired with an iCT scanner (Koninklijke Philips N.V., the Netherlands). Images were obtained using a standardized protocol. Scanning conditions were a voltage of 100 kV, gantry rotation time of 0.75s, pitch of 0.914 and automated detector collimation. Field of view (FOV) was 250-500 mm dependent on patient size with a 512 matrix size. Only scans with a slice thickness of 1 mm were used. Subjects received between 80 and 120 mL nonionic contrast medium (Iomeron 300, Bracco, Italy). The Medical research Ethics Committee United (MEC-U, Nieuwegein, the Netherlands) approved the study (reference number W18.127)

and waived the requirement of informed consent. In compliance with the EU General Data Protection Regulation (GDPR), subjects received a letter of notification that their data was used in this study. If there were any objections, subjects could refuse the use of their data. CT images were selected by an experienced radiologist (JN) and assessed for quality (adequate opacification of the splenic artery, and the absence of artifacts that would impact the reading and interpretation of the CT images). Exclusion criteria were previous pancreatic or splenic surgery, pancreatic cancer, splenomegaly, spleen cancer and Hodgkin lymphoma. Additionally, patients with conditions that affect normal vascular anatomy or interfered with the performing the necessary measurements, were excluded from the study at the discretion of the analysts or supervising radiologist. Subjects (sex ratio 1:1) were proportionately allocated in four age decades between 30 and 69 years (30-39, 40-49, 50-59 and 60-69). Because of the limited availability of CT imaging in younger patients, a lower limit of 30 years was chosen. An upper limit of 69 years was selected to create a study population representing the target group for potential neuromodulatory device implantation.

Data collection

Scans from selected subjects were analyzed using image analysis software (Intellispace Portal, Koninklijke Philips N.V., The Netherlands). Image analysis was performed by two qualified registrars (DB and ST), supervised by the radiologist (JN) for quality assurance. Data were systemically acquired using electronic case report forms (Research Manager, de research manager, Deventer, The Netherlands). Subject characteristics, if available, were extracted from the clinical record, including age, height, weight and body mass index.

Measurements

It was determined whether each splenic artery originated from the celiac trunk. Maximum calibers were measured at the origins of the celiac and splenic arteries and at predefined points related to the origin of the splenic artery (25, 50 and 75% of the total splenic artery length). The total length of the splenic artery was defined as the length through the center of the vessel from the origin of the splenic artery to the point in the splenic hilum where main branching occurred. The relation of the splenic artery to the pancreas was described as the part of the splenic artery that is in direct contact with the pancreas as a percentage of the total splenic artery length, i.e. no distinct layer of tissue present between the artery and pancreas (Fig. 1). The tortuosity index of the splenic artery was calculated by the analysis software, normalizing the uncoiled length of the artery between the origin of the splenic artery and its termination in the splenic hilum, defined as Y, to the linear distance between the origin of the splenic artery and its termination in the splenic hilum, defined as X. The tortuosity index is calculated as the ratio (Y/X).[6] The branching pattern in the splenic hilum was described. Furthermore, incidence of proximal polar branching was determined, i.e. arterial branches that branch off to the splenic poles before main branching in the splenic hilum. Splenic artery loops were defined as segments of the splenic artery, where there was a distinct layer of tissue, between the artery and the pancreas and because of its direction, easy to access during laparoscopy. To give a better idea on where each loop is located, a distinction was drawn between proximal and distal loops. Proximal loops are approximately located in the first 50% of the splenic artery and distal loops in the last 50%.

Statistical analysis

Prism 8.3 (GraphPad Software, La Jolla, CA) was used to create graphs and perform statistics. Data were tested for normality, but since the majority of data was non-normally distributed, all data were presented as median [interquartile range], and compared with the Mann-Whitney U test if needed. In case of multiple groups, a Kruskall-Wallis and Dunn's multiple comparison test were used. Association between tortuosity index and other variables was tested by means of Spearman's rank correlation coefficient. Female and male patients were combined in four age categories to assess the influence of age on the presence of loops (0 or ≥1) with the Fisher's exact test. A *P*-value < 0.05 was considered significant.

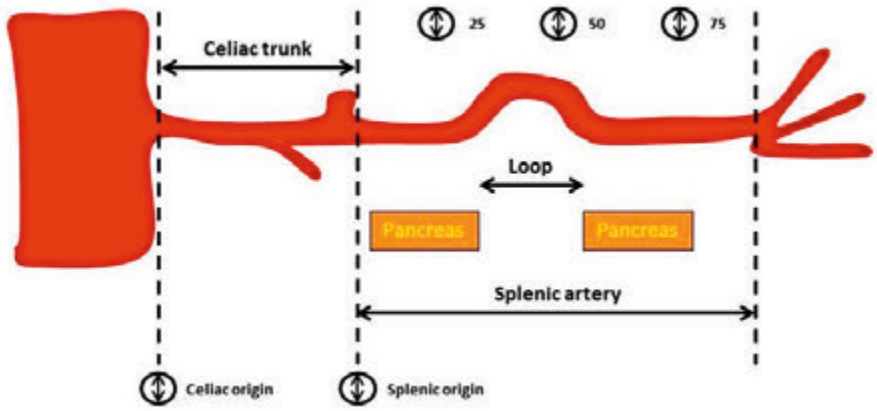

Fig. 1 Schematic overview of the measurements of the celiac trunk and splenic artery. = caliber

RESULTS

Study population and splenic artery characteristics

A total of 80 subjects were included. Subject characteristics such as height, weight and BMI are presented in table 1. The total splenic artery length and the calibers of the splenic artery are depicted in table 1 and figure 2. Each subject had a splenic artery that originated from the celiac trunk. The splenic artery tended to elongate with age in female subjects, with longer splenic artery lengths in the 60 group compared to the 40 group ($P = 0.02$), while this trend was not seen in male subjects. Splenic artery diameter was greater in male subjects compared to in female subjects at all predefined locations ($P < 0.05$). In both males and females, the caliber of the splenic artery is at its largest at the origin and becomes narrower during its course before branching in the splenic hilum.

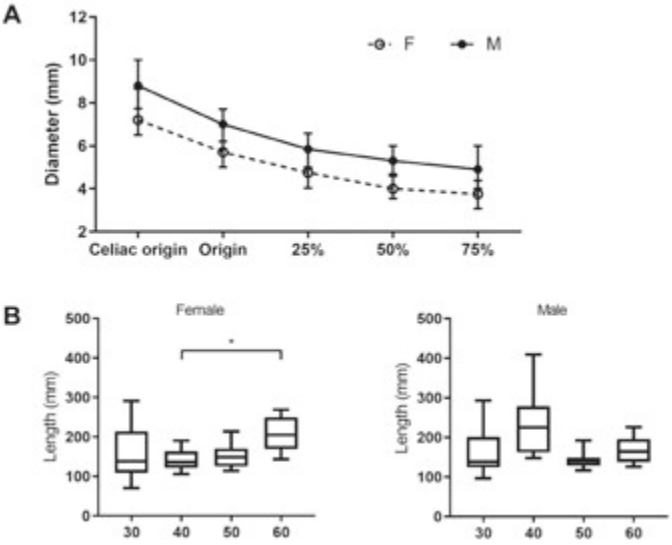

Fig. 2 Splenic artery caliber at standardized locations. (A) Data are presented as median with interquartile range for both female and male subjects. (B) Data are presented as median (line), interquartile range (boxes) and range (whiskers). Statistically significant differences ($P < 0.05$) between age groups as showed by Dunn's multiple comparison test are indicated by *.

Relation to the pancreas and other organs

The percentage of total splenic artery length that is in contact with pancreatic tissue decreased with age (Fig. 3). In the study population, the splenic artery was rarely in contact with organs other than the pancreas. In 20% of subjects, a short segment of the splenic artery was in contact with the stomach and in one subject (1.2%) there was contact with the left kidney.

Tortuosity

The tortuosity index of the splenic artery is presented in figure 4 for all age-gender categories. There was a high variability within each age-gender category and no correlation was observed between age and tortuosity index. Furthermore, the tortuosity index was not correlated with the percentage of the splenic artery that is in contact with the pancreas (r=-0.12; $P = 0.31$) and, although statistically significant, poorly correlated with the caliber of the splenic artery at 50% of the total length (r=0.19; $P = 0.04$).

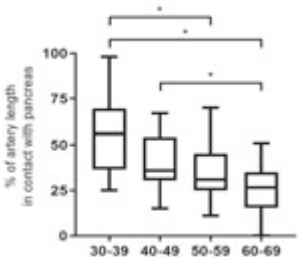

Fig. 3 Percentage of total splenic artery length that is in contact with the pancreas. Data are presented as median (line), interquartile range (boxes) and range (whiskers). Kruskall Wallis test showed $P < 0.0001$. Statistically significant differences ($P < 0.05$) between age groups as showed by Dunn's multiple comparison test are indicated by *.

		Female				Male			
		30-39 (30F)	40-49 (40F)	50-59 (50F)	60-69 (60F)	30-39 (30M)	40-49 (40M)	50-59 (50M)	60-69 (60M)
Length (cm)		169 [157-176]	170 [165-172]	171 [169-172]	165 [162-168]	183 [176-186]	182 [181-187]	178 [171-183]	178 [176-185]
Weight (kg)		61 [53-67]	72 [63-85]	65 [59-77]	79 [71-93]	81 [67-91]	93 [88-99]	94 [83-109]	91 [83-109]
BMI (kg/m^2)		21.1 [20.2-22.9]	24.3 [21.3-29.9]	23.0 [20.0-25.2]	29.2 [24.8-34.6]	25.1 [20.2-26.3]	28.0 [26.4-30.7]	28.7 [27.7-33.4]	27.6 [24.5-32.7]
Splenic artery length (cm)		138 [109-214]	137 [122-163]	149 [125-171]	205 [170-250]	139 [124-202]	225 [162-279]	141 [129-150]	165 [139-196]
Celiac origin (mm)		6.9 [6.1-8.1]	8.0 [7.1-8.9]	7.0 [6.0-8.0]	8.0 [6.7-9.4]	8.8 [7.7-9.4]	8.4 [7.4-10.1]	8.5 [7.0-9.3]	9.9 [8.0-10.3]
Splenic origin (mm)		5.9 [4.9-6.5]	5.5 [4.9-5.9]	5.5 [5.0-6.3]	5.3 [4.8-6.9]	6.7 [6.3-7.8]	7.2 [5.6-7.7]	7.0 [5.8-7.3]	6.4 [5.4-7.1]
Caliber at 25% of length (mm)		4.9 [3.9-5.0]	4.2 [3.4-4.6]	5.0 [4.0-5.0]	4.7 [3.9-5.9]	5.7 [5.1-6.4]	5.1 [4.0-7.1]	6.0 [5.8-7.3]	5.3 [4.5-6.3]
Caliber at 50% of length (mm)		4.1 [3.5-4.7]	4.0 [3.5-4.3]	4.5 [4.0-5.3]	4.0 [3.0-4.9]	5.3 [4.4-5.6]	5.3 [4.6-6.2]	6.0 [5.0-6.0]	4.6 [4.1-6.5]
Caliber at 75% of length (mm)		4.2 [3.6-4.7]	3.5 [2.9-3.7]	4.0 [3.0-5.0]	3.6 [3.6-4.6]	4.2 [3.8-6.0]	4.7 [4.1-5.7]	5.5 [4.0-6.3]	4.9 [4.0-6.0]
Loops	No loops	4	1	0	0	4	1	1	0
	1 loop	3	9	4	2	1	4	7	7
	2 or more loops	3	0	6	8	5	3	2	3

Table 1 Subject and splenic artery characteristics for each age-gender category. Data are presented as median [IQR] or as count.

Loops

The number of loops in each age-gender category is shown in table 1. The portion of subjects without any loops decreased with age ($P = 0.002$). Arterial loops were evenly distributed between the proximal and distal splenic artery and the median diameters were comparable between proximal and distal loops (5.1 [4.5-6.3] vs. 5.0 [4.2-6.0] mm, respectively, $P = 0.33$). The anatomic paths taken by the loops were highly variable, see representative cross-sectional images (Fig. 5), although there more loops oriented cranially as the arteries traveled away from the pancreas.

Branching pattern

For the total study population, the median number of branches of the main splenic artery, in the splenic hilum, was 2 [2-3]. In 59% of subjects (47/80), there were also branches from the proximal splenic artery that coursed to the hilum, projecting to the cranial or caudal pole of the spleen separate from the main splenic artery. The median length of these early branches to the splenic hilum was 37 mm (range 6-266 mm).

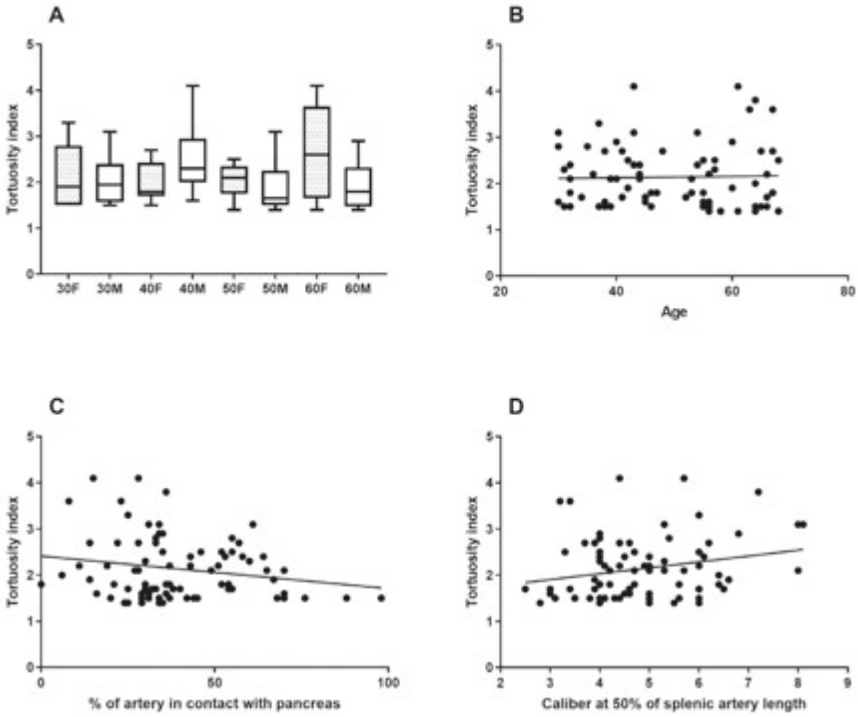

Fig. 4. Tortuosity index of the splenic artery. Age-gender categories **(A)** and correlation of tortuosity index with age **(B)**, contact with the pancreas **(C)** and calibers at 50% of total splenic artery length. Data are presented as median (line), interquartile range (boxes) and range (whiskers) **(A)** or plotted as one point for each subject **(B-D)**.

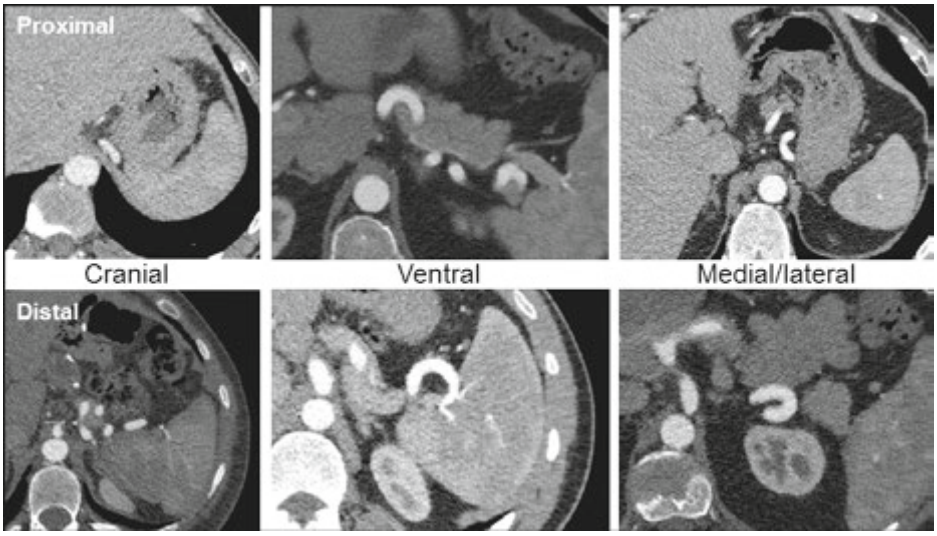

Fig. 5. Common anatomic directions of proximal and distal splenic artery loops.

DISCUSSION

In this study, we used cross-sectional imaging to evaluate splenic artery morphology and relationships of the splenic artery to adjacent structures in a cohort of patients stratified by age and gender. We found that splenic artery loops as potential sites for neuromodulatory device placement were present in a large number of patients. We also identified a high degree of variability of splenic artery length, caliber and tortuosity. We demonstrated a novel finding that the length of the splenic artery in direct contact with the pancreas decreases with advancing age. Remarkably, this observation was not the result of increasing arterial tortuosity which did not vary significantly between the different age-gender categories.

In this cohort of patients, the splenic artery was found to gradually decrease in diameter as it traveled from its origin until it terminated with branching in the splenic hilum. Additionally, male subjects were found to have vessels with larger diameters than females. While these observations have not been previously reported for the splenic artery, they are consistent with findings in the coronary bed, as well the upper extremity arteries.[11,12] Our findings of splenic artery length and the pattern of terminal branching were consistent with previous studies.[7,10,13,14] Another noteworthy observation in this study is the finding of proximal polar branches, in which an arterial branch arises directly from the main splenic artery, traveling to the cranial or caudal pole of the spleen without passing through the hilum. These were present in 59% of all subjects, which is remarkably higher than a different imaging study (5%), but in agreement with cadaver work (51%).[7,10] While these differences may reflect different protocols used for imaging, it is possible that these represent true differences between the patient populations rather than being accounted for by gender or an age related phenomenon.

The observations in this study not only add to our understanding of splenic artery morphology but also demonstrate the value of using CT angiography for defining splenic artery anatomy and its relationship to regional anatomic structures, which aids in treatment planning for a range of procedures such as distal pancreatectomy, lymphadenectomy for oncologic

resections and endovascular interventions.[9,10,15,16] Beyond these traditional indications, these observations may be important in the development of a new therapy, currently being investigated, which uses stimulation of the splenic plexus to modulate immune responses in subjects suffering from chronic inflammatory diseases.[1] In humans, the splenic neural plexus has been shown to run in close proximity with the splenic artery.[17] Furthermore, it has been shown that loops are surrounded by a substantial amount of nerve tissue.[4] The present study, using cross sectional imaging of living subjects, is the first to report on the anatomy of the splenic artery as it relates to contact with the pancreas and to explore this anatomy as it may impact accessibility of the splenic artery and associated nerve plexus. This is significant as this relationship impacts the ease of surgical dissection in the perivascular space while minimizing the potential for injury to local structures.

To accomplish this, the relationship between the splenic artery and pancreas was characterized by measuring the proportion of the total length of the splenic artery that is in contact with the pancreas as well as the presence and characteristics of splenic artery loops, segments where the artery is distinct from the pancreas. We demonstrated that the amount of the splenic artery that was in contact with the pancreas decreases with age. These observations did not correlate with increases in tortuosity or diameter, which is consistent with the hypothesis that this is not solely due to generalized arterial enlargement. Splenic artery loops, as described in the study, were present in most patients and equally distributed throughout the course of the splenic artery. This comparison may, however, have been limited by a large amount of variability within the age-gender categories. Comparison of these findings with those of other studies is difficult, since the relation to the pancreas was described in a different way. Zhu et al used the most inferior part of the splenic artery as a reference point, whereas Zheng and colleagues described whether the splenic artery was located supra- or intra-/retropancreatically.[9,10] It can however be stated that there is a high variation in these studies, since different rates of supra- and intrapancreatic courses are reported.[8-10] In our opinion, determining the actual area where the splenic artery is in direct contact with the pancreas provides a better characterization of the relationship between the splenic artery and the pancreas.

In contrast to what was found in cadaver studies, this study has been unable to demonstrate that the tortuosity of the splenic artery increases with age.[6,7] This difference could be because this study was limited to patients who were between 30- and 69-years of age, while the cadaver studies also included fetuses, neonates and young children. Our study did not include data from children or young adults because of limited availability of clinical CT imaging in these age categories. As such, it cannot account for any increases in arterial dimensions or tortuosity that have been described in these younger patient groups.[6] Given the high variation in each age-gender category in this study, it seems likely that factors other than age have a greater influence on the development of tortuosity of the splenic artery. Alternatively, this may reflect difference between measurements taken in decompressed arteries as compared to those taken in vivo where they are distended by arterial pressure.

It is important to bear in mind the possible limitations to this study. First, this is a retrospective study of CT images previously acquired as part of the patient's standard care. Underlying conditions of subjects could have had an influence on the course of the splenic artery, although an effort was made to identify factors with known relationships to altered anatomy and structure and to include these prospectively in the study's exclusion criteria. Another

limitation of this study design is that, by using angiography, only the caliber of the splenic artery lumen was measured, rather than the external diameter of the splenic artery including the arterial wall. This also limits the assessment of smaller arteries such as pancreatic branches. Histopathological studies and ultrasonography could provide additional information on the wall thickness of the splenic artery should total artery diameter be required.[18,19]

In summary, this study describes the characteristics of the splenic artery, with specific attention for age-gender differences and the relation to the pancreas. This study supports the idea that there are segments of the splenic artery that are discrete from the pancreas and are approachable at locations without causing significant damage to the pancreas or other structures. Clinical studies are ongoing to confirm that the splenic artery and nerve plexus are accessible and if a device can safely be implanted in a clinical surgical setting (www.clinicaltrials.gov; NCT04171011).

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07

SPLENIC ARTERIAL NEUROVASCULAR BUNDLE STIMULATION IN ESOPHAGECTOMY: A FEASIBILITY AND SAFETY PROSPECTIVE COHORT STUDY

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Frontiers in Neuroscience 2022

ABSTRACT

The autonomic nervous system is a key regulator of inflammation. Electrical stimulation of the vagus nerve has been shown to have some preclinical efficacy. However, only a few clinical studies have been reported to treat inflammatory diseases. The present study evaluates, for the first time, neuromodulation of the splenic arterial neurovascular bundle (SpA NVB) in patients undergoing minimally invasive esophagectomy (MIE), in which the SpA NVB is exposed as part of the procedure. This single-center, single-arm study enrolled 13 patients undergoing MIE. During the abdominal phase of the MIE, a novel cuff was placed around the SpA NVB, and stimulation was applied. The primary endpoint was feasibility and safety of cuff application and removal. A secondary endpoint included the impact of stimulation on SpA blood flow changes during the stimulation, and an exploratory point was C-reactive protein (CRP) levels on postoperative day (POD) 2 and 3. All patients successfully underwent placement, stimulation, and removal of the cuff on the SpA NVB with no adverse events related to the investigational procedure. Stimulation was associated with an overall reduction in splenic arterial blood flow but not with changes in blood pressure or heart rate. When compared to historic Propensity Score Matched (PSM) controls, CRP levels on POD2 (124 vs. 197 mg/ml, p=0.032) and POD3 (151 vs. 221 mg/ml, p= 0.033) were lower in patients receiving stimulation. In conclusion, this first-in-human study demonstrated for the first time that applying a cuff around the SpA NVB and subsequent stimulation are safe, feasible, and may have an effect on the postoperative inflammatory response following MIE. These findings suggest that SpA NVB stimulation may offer a new method for immunomodulatory therapy in acute or chronic inflammatory conditions.

INTRODUCTION

The autonomic nervous system has a critical role in regulating immune function, via the 'inflammatory reflex'.[1,2] The efferent arm of the inflammatory reflex controls the systemic immune responses via nerves, traveling with the splenic artery (SpA).[3,4] These nerves form a neurovascular bundle (NVB)[5] which contains fibers that enter the splenic parenchyma where neurotransmitters, in particular catecholamines, are released from nerve endings that subsequently modulate immune cells.[5] Exogenous electrical activation of neural pathways targeting the spleen has been shown to modulate systemic cytokine production in rodents, using either upstream activation of the cervical vagus nerve, or near end-organ activation of the SpA NVB.[4,6-9] This mechanism has also been investigated in large animal studies where acute activation of the SpA NVB, in terminally anesthetized pigs, has been shown to reduce the production of tumor necrosis factor α and interleukin 6 following endotoxemia.[10] In this model, SpN stimulation (SpNS) has also been shown to produce amplitude- and frequency-dependent changes in SpA blood flow, which are directly correlated to nerve activation.

Clinical evidence from feasibility studies of electrical cervical vagus nerve stimulation suggests that such interventions have beneficial effects on inflammatory and clinical parameters of patients with rheumatoid arthritis and Crohn's disease.[11-13] While these clinical findings are supportive of the role of the inflammatory cascade in these diseases, application of this therapy for inflammatory diseases is to date not approved as standard of care. Challenges with VNS at the cervical level range from off-target effects such as bradycardia and hoarseness or more serious side-effects including effects on respiration, heart rate variability or stimulation-induced obstructive sleep apnea.[14-17] Such off-target affects are due to the activation of low threshold cervical vagal fibers to the larynx, pharynx, heart, and lungs.[18] Activation of the sympathetic path directly at SpN may avoid such side effects and allow the full neuro-immunomodulatory effect to be accessed.

To further evaluate the potential of SpNS in humans, a cuff was developed that can accommodate the pulsating features of the SpA and can effectively stimulate the SpA NVB in humans. The laparoscopic placement of the cuff and assessment of stimulation parameters were first evaluated in farm pigs.[19]

In the current study, we evaluated the feasibility and safety of temporarily placing the cuff around the SpA NVB and delivering acute stimulation in patients undergoing minimally-invasive esophagectomy (MIE). This procedure was selected for this evaluation since it requires exposure of the splenic NVB as part of the procedure and cuff placement can occur with little or no additional dissection of the splenic artery. In addition, MIE is a surgery with a known postoperative inflammatory response. Studies suggest that the postsurgical inflammatory response is associated with increased postoperative morbidity and mortality as reviewed by Straatman et al.[20] and attenuation of an excessive inflammatory response through SpNS may therefore reduce morbidity and mortality following MIE.

MATERIALS AND METHODS

Study design and patients

This intra-operative study was a first-in-human, single-arm open-label, single-center trial that was performed at the Catharina Hospital Eindhoven, a district teaching hospital in the Netherlands. The principles of Good Clinical Practice and Declaration of Helsinki were followed. The study protocol was approved by the Medical Ethics Committees United (MEC-U, Nieuwegein, the Netherlands) under reference numbers NL70757.100.73 and R19.048. The institutional review board of the Catharina Hospital also approved the protocol and the informed consent. All patients provided written informed consent before enrolment in the study. The study protocol was registered with Clinicaltrials.gov under NCT04171011.

At the point in the standard MIE when the lymphadenectomy was performed, the splenic NVB was being isolated, and an investigational cuff was implanted laparoscopically around the NVB and connected to an external pulse generator. An ultrasound transducer was introduced to the abdomen and placed on the NVB to visualize splenic arterial BF during stimulation. The NVB was stimulated two to four times (with intervening pauses to observe post-stimulation recovery) using parameters selected to cause a change in splenic arterial blood flow (a marker of NVB activation). Stimulation parameters were adjusted over the course of the study based on experience with prior study participants. Blood samples were taken before, and at certain pre-defined time points after, stimulation and recovery. The cuff and ultrasound transducer were removed after completion of stimulation, and the surgery was continued. Surgeon experience applying and removing the cuff was documented, in addition to the responses to NVB stimulation. Postoperative safety was followed through 7-days post-surgery (or day of discharge if earlier).

Safety was reviewed by the principal investigator (MDL) and a sponsor-appointed Medical Monitor (EI) after three and six patients had completed the study. Additionally, all patients were monitored for adverse events (AE) and were followed until the AE events were resolved.

Participants were eligible for inclusion if they underwent MIE. Other inclusion criteria were age equal or above 21, male or female of non-reproductive potential, a confirmed splenic artery loop on preoperative computed tomography angiography (CTA), and normal blood pressure or hypertension managed with medication and who were otherwise deemed fit for surgery. Exclusion criteria were previous splenectomy, an existing implantable device, active pancreatitis, or history of severe pancreatitis with complications, hepatic or splenic disease, use of oral steroids 4 weeks prior to inclusion, current use immunosuppressive agents or biologicals, and use of anticoagulants within 1 week of surgery. Potential study participants were pre-screened by the treating physician or a member of the research team as they were referred for curative treatment of esophageal carcinoma. When all eligibility criteria were met, the patient was informed and written informed consent was obtained by the treating physician (GAPN or MDL). During a run-in period at the start of the study, three patients were included to optimize the techniques for cuff placement and ultrasound recording. Outcome analysis of these run-in patients was similar as for the other patients.

Neuromodulation system

The Galvani stimulation system (Galvani System) consists of an implantable cuff connected to a 95 cm lead (models 10303-95 and 10305-95, investigational product; supplied sterile),

an extension cable (model F7830DIN, Fiab SpA, Italy supplied sterile), and an external pulse generator (Inomed ISIS-HC Neurostimulator (Model 504185); Inomed Medizintechnik GmbH, Germany) (Figure 1A). The implantable cuff and lead are an investigational device manufactured by Galvani Bioelectronics. The cuff is composed of 2 outer arms (with electrodes) and a middle arm (no electrode) (Figure 1B), designed to interface with the nerves surrounding the splenic artery. Two versions of the cuff were available in the study, the '6 mm' (which accommodated NVBs up to 7.8 mm in diameter) and the '7 mm' (NVBs up to 9.1 mm). The stimulation cuff diameter ranges were based on a previous study, which provided insight on the diameter of the human splenic artery using CT angiography.[21] The proximal end of the cuff contains a connector for connection to the external pulse generator, the ISIS neurostimulator, via the extension cable. The ISIS Neurostimulator and accompanying software were used according to the manufacturer's instructions.

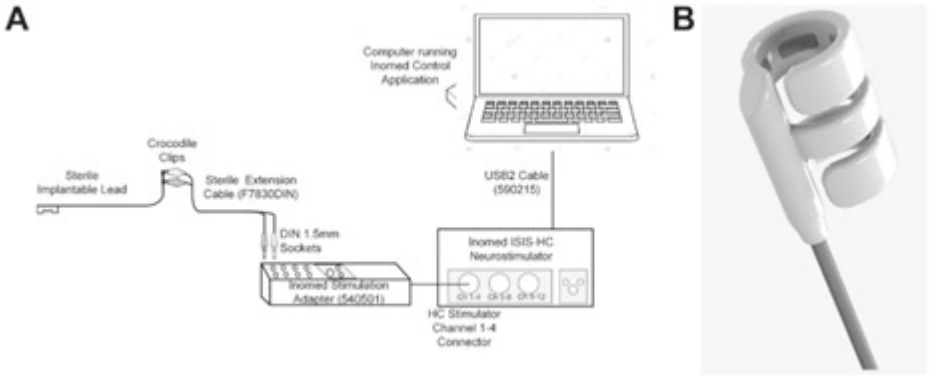

Figure 1. (A) Stimulation set-up. A laptop with the stimulation software (Inomed Control Application) was connected to the Inomed Neurostimulator (Model 504185). Via the extension cable, the neurostimulator was connected to the sterile implantable cuff. **(B)** The distal end of the cuff. This is a cuff which was composed of two arms with electrodes and one middle arm, designed to fit around the SpA NVB.

Procedures

All participants in this study were undergoing Minimal Invasive Esophagectomy with intrathoracic anastomosis (MIE) and creation of gastric conduit as previously described.[22,23] During the abdominal phase of this procedure, a lymphadenectomy is routinely performed and the splenic artery is partially exposed. This procedure is highly similar to the laparoscopic procedure that was performed in pigs.[19] The stomach is mobilized before the splenic arterial loop is visualized to place the cuff. Due to the anatomical differences between human and pigs the spleen does not have to be retracted. At this point in the procedure, the PI and attending anesthetist determined if it was appropriate to conduct the experimental protocol. If the investigational procedure progressed, the splenic NVB was dissected from the attached soft tissue if not already done as part of the standard procedure. Following mobilization, the cuff was introduced through a trocar into the abdomen and was manipulated in the abdomen using atraumatic graspers and positioned around the splenic NVB. The other end of the cuff was connected to the external pulse generator via the extension cable. A single impedance measurement was taken to ensure proper function of the cuff. If there was a high impedance (>2.5kΩ), the cuff was repositioned or replaced with a second cuff. Next the ultrasound transducer, (L51K, Hitachi Medical Systems B.V., Reeuwijk, the Netherlands), was introduced

into the abdominal cavity through one of the trocars, to collect velocity profiles during the protocol. The intraoperative stimulation was performed according to the predefined stimulation scheme, with increasing amplitudes over the course of the trial. Total experimental procedure time (including additional dissection, cuff placement, optimization of Doppler imaging, stimulation, and cuff removal) was not allowed to exceed 40 minutes. The surgeon performing the implantations (MDL) had completed a training program consisting of education on the use of the neuromodulation device and performing implantations in human cadavers.

All stimulations used biphasic symmetric pulses delivered at 10 Hz frequency, 400 µs pulse width per phase, and 1 minute duration. The current amplitude varied from 8 mA (3.2 µC) to 20 mA (8 µC), based on the results of previous studies and to investigate the effect of step-up stimulation on safety and target engagement.[10,24] Based on preclinical data, three stimulations were considered to be optimal.[8,10] For the run-in patients, 1 to 5 stimulations could be applied. The amplitudes were slowly ramped-up over the course of the trial with maximal 3 stimulations per patient in the main cohort. In figure 2B and table S1 the different amplitudes used per patient are shown. Based on the physiological changes upon stimulation, the PI could decide to increase, decrease or maintain the stimulation amplitude, or not proceed with the next stimulation. Impedance measurements were taken before each stimulation to confirm good contact and functionality of the cuff. After each stimulation, splenic arterial blood flow and cardiovascular metrics had to return to baseline before initiating a new stimulation. After the last stimulation, a final impedance measurement was taken before the cuff was removed from the SpA NVB and abdominal cavity.

Splenic artery blood velocity measurements (Peak Systolic Velocity (PSV), End Diastolic Velocity (EDV) and Velocity Time Integral (VTI)) and were obtained using pulse wave Doppler. A laparoscopic ultrasound transducer (L51K, Hitachi Medical Systems B.V., Reeuwijk, the Netherlands) was placed distally from the cuff on the splenic NVB. The probe was attached to an Ultrasound System from Hitachi (V70) and Doppler images were obtained according to the manufacturer's instructions. The pulse wave Doppler signal was optimized intraoperatively by a trained investigator (DJB). Briefly, the probe was placed in parallel to the splenic artery by the surgeon. A center point in the artery was chosen to measure arterial blood flow and angle correction (max. angle of 60 degrees) and beam steering were used for correction of the Doppler signal.. If a constant arterial flow curve was observed, PSV, EDV and VTI were recorded as still frame flow velocity profiles and values from a minimum of three adjacent beat flow velocities were averaged. These values were recorded twice prior to each stimulation as a baseline, and twice during each stimulation (at 15 and 40 seconds). If the ultrasound probe moved between baseline measurements and stimulation, new baseline measurements were performed. All images and measurements were subjected to a quality review at the end of the study, with independent reading by a single investigator (BC). This included a review of image quality, selection of images for analysis, and evaluation of the measurements that were performed.

Heart rate (HR) and mean arterial pressure (MAP) were continuously monitored using a patient monitoring system (GE Healthcare, Chicago, Illinois, United States) as is standard care for MIE. In parallel with the blood velocity measurements, two baseline recordings for HR and MAP were manually documented from the system as well as during each stimulation at 15 and 40 seconds.

CRP and leukocyte count were measured on postoperative day (POD)2, 3 and 4 as part of the standard care. CRP levels were determined in the morning (at 08.00 am) by immunoturbidimetric assay (Roche/Hitachi cobas C system, Roche).

End of follow-up was defined as POD7, day of hospital discharge, or conclusion of any ongoing SAEs/AEs.

Outcomes

The primary objectives of this study were to evaluate the safety and feasibility of the temporary placement, stimulation, and removal of an investigational cuff around the SpA NVB. Feasibility was demonstrated by the proportion of participants in whom the cuff was successfully applied to the splenic NVB and removed. Safety analysis evaluated all adverse events and serious adverse events (SAEs) that resulted from stimulation after cuff application, stimulation, and removal. SAEs were collected from informed consent until POD7 and AEs and device deficiencies from the day of the intraoperative procedure (day 0) until POD7. All AEs deemed unresolved or ongoing at the time of the POD7 or at the day of discharge assessment were monitored until the event was resolved, stabilized, or otherwise explained. Adverse events were to be graded as mild, moderate, or severe. A serious event caused hospitalization or prolongation of participant hospitalization, a life-threatening illness or injury, a medical or surgical intervention to prevent life threatening illness, injury or permanent impairment to a body structure or a body function or chronic disease. Furthermore, the principal investigator and the sponsor had to determine whether the event was related to the study device, study procedure or surgical procedure.

A Secondary outcome was SpA BF changes as measured by the PSV, EDV and the VTI. Exploratory outcomes included changes in heart rate (HR) and mean arterial blood pressure (MAP), white blood cell count at day 2-4 and C-reactive protein (CRP) plasma levels at POD2-4.

Statistical analysis

Due to the exploratory nature of this study, no power calculation for sample size was performed. After the run-in period (three patients), the number of ten participants was deemed sufficient to determine whether further research and development in this therapeutic area are warranted. To evaluate white blood cell count and CRP levels, patients in the trial were compared to patients that underwent MIE in 2019-2020 at the Catharina Hospital (n=65). Propensity score matching was used to select specific controls for the trial patients. Because of the small sample size of the trial patient group, only three covariates were chosen to include in the propensity score matching analysis. These covariates included the occurrence of complications which are known to influence the postoperative inflammatory after surgery. A logistic regression analysis was performed on postoperative occurrence of any Clavien Dindo complication ≥3, pneumonia and anastomotic leakage. The resulting propensity variables were used to select controls for the trial patients. A caliper width of 0.2 was chosen as recommended to minimize error rate. SPSS version 26.0 (IBM, Armonk, NY) was used to perform propensity score matching and this resulted in a matched control for each of the trial patients.[25] SPSS was also used to perform group comparisons on CRP and leucocyte levels using parametric or non-parametric tests as appropriate. Graphs were made with Prism 8.3 (GraphPad Software, La Jolla, CA, USA).

Role of the funding sources

The sponsor of this study was Galvani Bioelectronics. The report presents the results of a sponsored study. The funding sources had a role in study design, data collection, analysis, and interpretation, and writing of this report. The corresponding author had full access to all the study data and the final responsibility to submit for publication.

RESULTS

Between November 2019 and September 2020, 37 patients were pre-screened for eligibility for this study. Of these patients, three patients were not eligible (history of pancreatitis, use of anti-inflammatory agents or creation of colon interposition instead of gastric conduit), six patients could not participate (COVID-19 pandemic or logistic reasons that hampered cuff availability) and 14 patients did not provide informed consent. Fourteen subjects were consented to participate in the study; in one patient, the study procedures could not take place due to COVID-19 pandemic restrictions. This resulted in 13 patients participating in this feasibility study. Patients included in the study had an age range from 51 to 77 years and had a BMI range 19.1 to 33.8. The patient demographics are depicted in Table 1.

Patient	Age (yrs)	Sex	Preoperative status (ASA grade)	Surgery duration (min)	Blood loss (mL)
001	51	M	2	274	150
002	60	F	2	278	150
003	54	M	2	232	150
004	77	M	3	241	150
006	69	F	2	232	100
007	63	M	3	239	100
008	62	F	2	253	100
009	69	M	2	263	100
010	57	M	2	256	150
011	65	F	2	245	100
012	66	M	2	280	100
013	64	M	3	233	150
014	68	M	2	364	470

Table 1. Patient demographics and surgical data

Patients' lumen diameter measured by CT angiography scan ranged from 4.0 mm to 7.2 mm, and the 6 mm cuff size was selected for all participants prior to the surgery. In ten out of thirteen patients the preoperative CT measurements correlated with the cuff fit (Table 2). In three patients a loose cuff fit was observed.

Patient	Artery diameter (mm)	Proximal arm fit	Middle arm fit	Distal arm fit	Comments by surgeon
001	5.1	Loose fit	Good fit	Good fit	Cuff seemed slightly too large
002	4.1	Good fit	Good fit	Good fit	No comments
003	5.5	Loose fit	Loose fit	Loose fit	Cuff is too large
004	5.0	Good fit	Good fit	Good fit	Cuff appeared to be somewhat loose
006	5.3	Good fit	Good fit	Good fit	No comments
007	5.7	Good fit	Good fit	Good fit	No comments
		Good fit	Good fit	Good fit	No comments
008	5.2	Loose fit	Loose fit	Loose fit	Better fit of distal arm
009	7.2	Good fit	Good fit	Good fit	Excellent fit
010	5.5	Loose fit	Loose fit	Loose fit	Very loose fit
011	5.2	Good fit	Good fit	Good fit	No comments
012	5.4	Loose fit	Loose fit	Loose fit	Loose fit of all three arms
013	4.0	Good fit	Good fit	Good fit	Good fit with additional fat tissue
014	6.5	Good fit	Good fit	Good fit	No comments

Table 2. Artery diameter and cuff fit

Placement of the cuff on the SpA NVB, applying stimulation and subsequently removing the cuff was successful in all patients. All cuffs were positioned in a single attempt except for the first patient where two attempts were made, and cuff placement in all patients was completed within 3 minutes, with 9 out of 13 placed in 1 minute or less. In one patient, the implanted cuff needed to be replaced because high impedance values (>2.5Ω) were measured on the cuff after placement on the NVB.

All impedance measurements after implantation were within acceptable levels. All stimulations applied were of 1 minute duration with 100% current delivery. Amplitude levels ranged from 8 mA to 20 mA (3.2-8 µC at 400µs pulse width) with three stimulations applied to each individual participant, except for the first run-in participant who received two stimulations and the third run-in participant who received four stimulations. The starting amplitude in the first patient (001) was 10 mA. Because this caused a considerable decrease in blood flow (figure 2B), a lower starting amplitude (8 mA) was chosen for patient 002. Thereafter, it was deemed safe to increase the starting amplitude in every three patients. Stimulation levels by participant are listed in Figure 2B with the accompanying blood flow changes (VTI) per stimulation at 15 seconds after start of stimulation and in Supplementary Table S1. During the study a total of 39 stimulations were delivered to the thirteen participants.

Eleven patients experienced a total of 24 adverse events, of these six were determined SAEs in six patients (Table 3). None of these were attributed to placement or removal of the cuff nor were they attributed to the stimulation, but they were all related to the MIE procedure.

Placement of the ultrasound transducer could be performed in all patients. Duplex ultrasound data were monitored from 36 stimulations in twelve participants. In one patient (002) the data was not recorded due to a technical issue with the ultrasound system. A consistent and reproducible decrease in splenic arterial blood flow was observed as represented by VTI except in one patient (011, Figure 2A-B). The blood flow decrease peaked at 15 seconds after start of stimulation and at 40 seconds (Supplementary Figure S1), the blood flow change decreased

and optically returned to baseline levels typically within 60 to 180 seconds after cessation of stimulation. The reduction appeared to be stimulation amplitude dependent, and a correlation was observed between the stimulation amplitude and all blood flow metrics (VTI, PSV and EDV at 15 seconds after start stimulation, Figure 2C), although statistical correlation is only found for the participants in the main cohort (excluding run-in patients, R-square value – 0.8247, $p<0.05$). Cardiovascular endpoints were continuously monitored during the study procedure. Small changes in HR and MAP were observed, however, no clinically significant observations were made (Supplementary figure S2). All observed changes returned to baseline within one minute after the end of stimulation. Impedance measurements taken after stimulation and prior to cuff removal confirmed that the cuff was still electrically functional in all participants. Day of discharge and post-operative complications were captured but based on the small study population no conclusion could be drawn from this data.

Patient	Day	Event	Serious	Severity	Relationship to NVB stimulation
001	2	Mucus plugging	Yes	Severe	No
002	1	Tachycardia	No	Mild	No
	2	Dyspnea	No	Mild	No
	5	Pneumonia	No	Severe	No
	6	Anastomotic leakage	Yes	Severe	No
	7	Hypokalemia	No	Moderate	No
003	1	Reduced control over left arm	No	Mild	No
004	0	Hypotension	No	Mild	No
	2	Colon herniation	Yes	Moderate	No
006	6	Trush	No	Mild	No
007	4	Atrial fibrillation	No	Severe	No
	5	Pneumonia	Yes	Severe	No
	18	Anxiety	No	Mild	No
	18	Excessive postoperative pain	No	Moderate	No
008	6	Pneumonia	Yes	Mild	No
009	5	Wound infection	No	Mild	No
	0	Excessive postoperative pain	No	Moderate	No
010	11	Pleural effusion	Yes	Moderate	No
	11	Hypokalemia	No	Moderate	No
	24	Delirium	No	Mild	No
011	1	Increased liver enzymes	No	Mild	No
012	-	-	-	-	-
013	-	-	-	-	-
014	0	Hyperglycemia	No	Mild	No
	1	Tachycardia	No	Mild	No
	3	Subcutaneous emphysema	No	Mild	No

Table 3. Adverse events.

In a post-hoc analysis, CRP plasma levels in the intervention group were significantly lower on POD3 when compared to a cohort of patients that underwent MIE (n=65) in 2019 and 2020 in the same institution (150.5±19.0 vs. 206.7±11.6 mg/L, respectively; p= 0.043, Figure 3A). After propensity score matching (PSM), CRP plasma levels in the intervention group (n=13) were significantly reduced compared to the PSM cohort (n=13) on POD2 (124.2±20.3 vs. 196.7±24.7 mg/ml; p=0.032) and POD3 (150.5±19.0 vs. 220.5±24.4; mg/L p=0.033). On POD4, no significant differences in CRP levels between the cohorts were observed (SpNS vs PSM p=0.413; SpNS vs 2019-2020 p=0.323). The number of leucocytes on any postoperative day (Figure 3B) did not differ between groups. Baseline characteristics (Table 4) were not statistically different between matched groups.

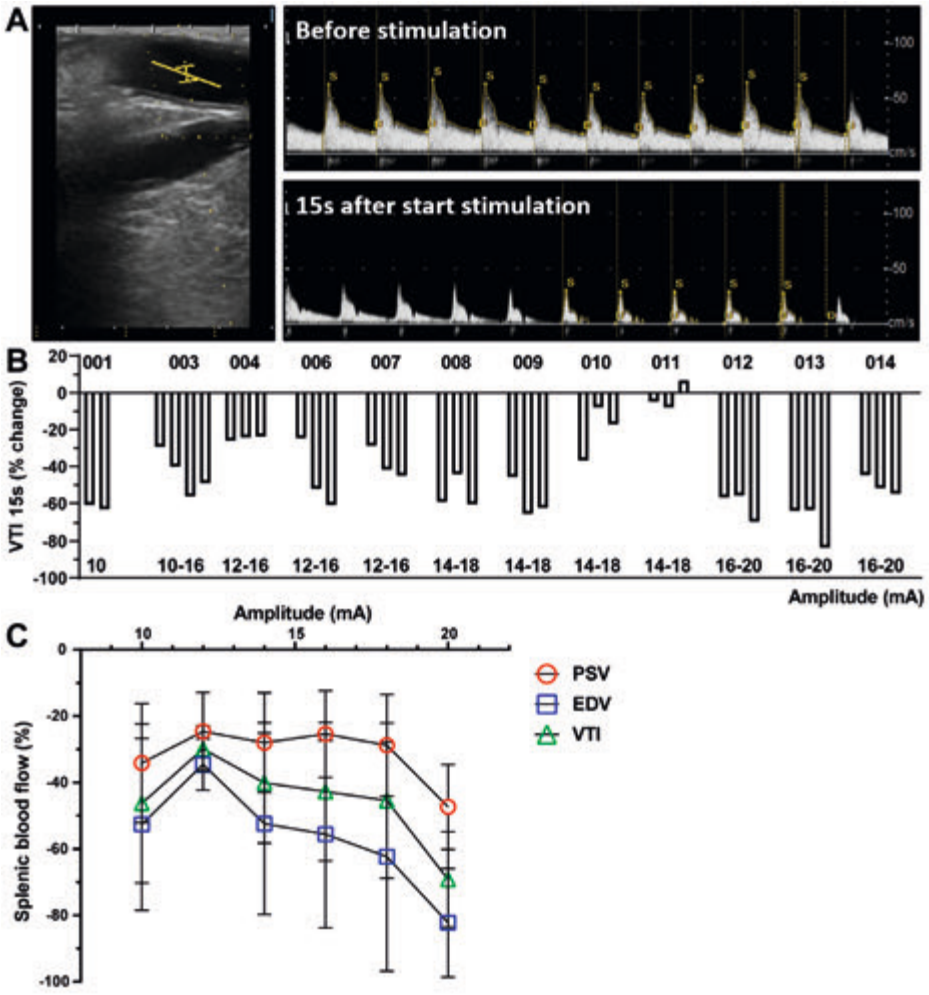

Figure 2. SpNS caused a reproducible splenic arterial blood flow change. (**A)** Representative ultrasound image of the SpA (left). The ultrasound probe was placed parallel to the splenic artery. A center point (yellow lines) in the artery was chosen to measure Doppler flow velocity profiles before (upper) and after start of stimulation (lower). (**B)** Change in blood flow (VTI) presented as percentage change compared to baseline 15 seconds after start of SpNS as presented per patient for each stimulation. The amplitudes of the stimulation are listed below (**C)** Effect of increasing stimulation amplitudes on decrease in PSV, EDV and VTI, averaged over all patients, compared to baseline 15 seconds after start of SpNS. SpA, splenic artery; SpNS, splenic nerve bundle stimulation; VTI, Velocity Time Integral; PSV, Peak Systolic Velocity; EDV, End Diastolic Velocity.

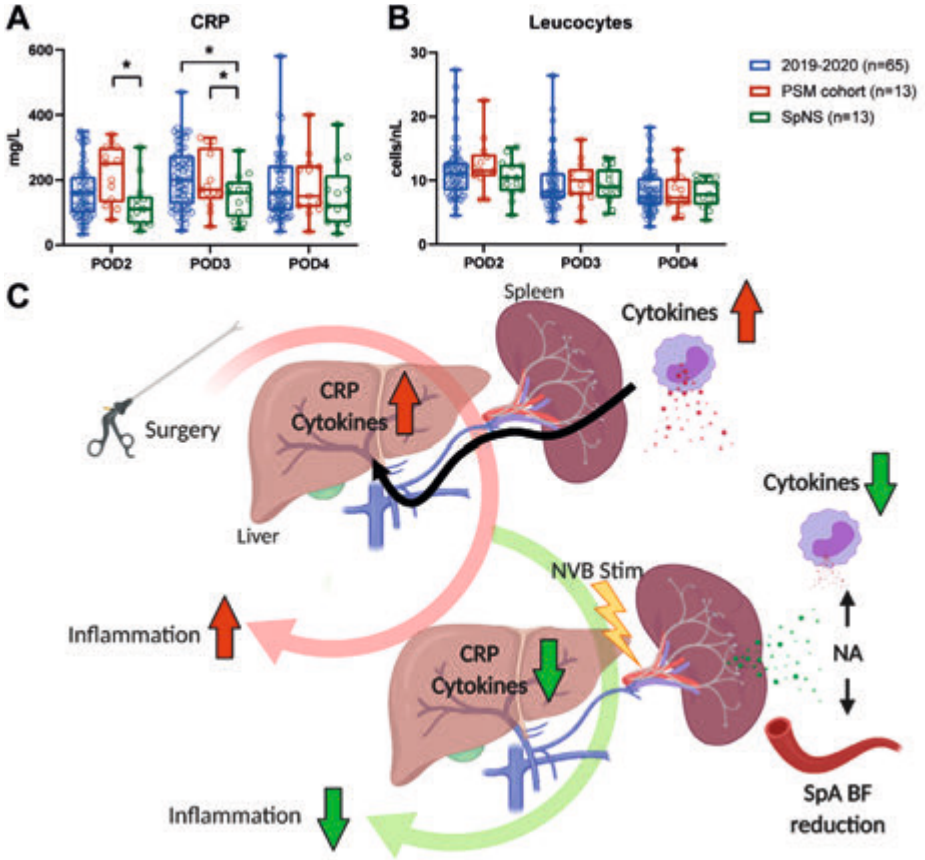

Figure 3. Postoperative inflammation was lower in patients that underwent SpNS compared to matched controls.(**A)** Plasma CRP levels and (**B)** Blood leucocytes on POD2-4 in all patients that underwent MIE in 2019, patients that underwent SpNS and patients that were matched to SpNS patients based on the occurrence of major complications, pneumonia and anastomotic leakage. Bars indicate mean and standard deviation. (**C)** Schematic representation of the putative mechanism of action. Surgical procedures trigger immunological responses. The accumulation of cytokines and the consequent production of CRP by the liver are biomarkers of postoperative inflammation and correlate with postoperative recovery. When stimulation of the splenic nerve is applied via a stimulation lead placed around the SpA NVB, norepinephrine (NA) is released within the spleen. The neurotransmitter causes contraction of the vascular bed with consequent transient decrease in splenic arterial blood flow that can be used as a biomarker of nerve engagement. At the same time NA binds to spleen resident immune cells and suppresses their ability to produce cytokines. * is P<0.05.

DISCUSSION

This first-in-human study demonstrated for the first time that applying a cuff around the splenic neurovascular bundle and subsequent stimulation are safe, feasible and may alter the postoperative inflammatory response following MIE.

None of the adverse events were attributed to placement or removal of the cuff nor were they attributed to the stimulation, but they were all considered to be part of the normal postoperative outcomes for patients that undergo MIE.

	2019-2020 (n=65)	Matched cohort (n=13)	SpA NVB stimulation (n=13)
Age, years (SD)	63.9 (9.6)	65.2 (13.0)	63.0 (7.0)
Sex, male (%)	54 (83)	9 (70)	9 (70)
BMI, kg/m^2 (SD)	27.2 (4.7)	25.6 (4.1)	26.0 (4.8)
Preoperative status, ASA ≥3 (%)	17 (27)	2 (15)	3 (23)
Comorbidity (%)	44 (68)	7 (54)	6 (54)
Cardiovascular comorbidity (%)	33 (51)	7 (54)	3 (23)
Pulmonary comorbidity (%)	9 (14)	1 (8)	3 (23)
Diabetes (%)	9 (14)	0 (0)	2 (15)
Pathological tumor stage, ≥3 (%)	28 (44)	4 (31)	7 (54)
Surgery duration, min (SD)	248 (36)	243 (34)	262 (34)
Blood loss, mL [IQR]	100 [50-150]	150 [50-250]	150 [100-200]
Any 30 day complication (%)	35 (54)	7 (54)	6 (46)
≥CD3 (%)	17 (26)	4 (30)	4 (30)
Cardiopulmonary complication (%)	21 (32)	6 (46)	6 (46)
Pneumonia (%)	12 (18)	3 (23)	3 (23)
Anastomotic leakage (%)	6 (9)	1 (8)	1 (8)
Infection (incl. wound infection, %)	1 (2)	1 (8)	1 (8)

Table 4. Baseline characteristics and postoperative complications of all patients that underwent esophagectomy in 2019-2020 (excluding trial participants), the participants in the pilot trial and a complication matched cohort from the 2019-2020 group. Values are shown as continuous (SD or IQR) or number (%). Comparisons were made using the t test, Mann Whitney U test or Chi square test as appropriate. BMI-body mass index, ASA-American Society of Anesthesiologists Classification, CD-Clavien Dindo

Although no adverse events (e.g. additional bleeding) related to the dissection of the SpA NVB were observed, it must be considered that the MIE procedure includes exposure or partial exposure of the SpA NVB as part of the obligatory lymph node dissection. In this pilot trial, the potential safety risks were mitigated by including a small number of patients, using one single experienced surgeon and providing extensive training on the device and surgical placement.

The cuffs used in this study were specifically designed to be delivered laparoscopically to the abdomen and fit around the human SpA NVB. In general, intraoperative cuff fit around the SpA was satisfactory, although a loose fit was observed in some patients, this is to be expected as the cuff was designed for chronic implantation where the NVB would be less dissected than in the MIE and NVB diameter could be affected by vasoconstriction or other factors. Nonetheless, SpNS led to an overall consistent and reproducible decrease in splenic arterial blood flow, which correlated with the stimulation amplitude in the patients participating in the main cohort and indicated nerve target engagement. Based on these observations and previous studies in pigs showing that a blood flow change in the SpA during SpNS directly correlated with the evoked compound action potential, it can be concluded that SpNS was successful in target nerve engagement in the current study.[10] Reduction in SpA BF could therefore be used as a real-time biomarker for nerve engagement during intra-operative stimulation.[26]

Stimulation of the SpA NVB showed a minimal, variable effect on HR and MABP during MIE in patients under anesthesia. The changes were not statistically correlated to the stimulation amplitude. This is in contrast with the effects seen in terminally anesthetized pigs.[11] The differences can be because of the anesthetic agents used for a major surgery like MIE, or due to the anatomical and physiological differences between species.

Increasing experimental evidence has indicated that neural stimulation can reduce inflammation in acute and chronic animal models.[7,9] Although the current study was not designed and powered to show an effect on systemic inflammation, it was demonstrated that CRP plasma levels in the intervention group were reduced on POD2 and 3 when compared to a post-hoc analysis in a PSM control group. A few studies have provided insight in the interpretation of CRP levels after major abdominal surgery.[27-29]. Postoperative CRP levels have shown a strong correlation with complications in patients undergoing open versus laparoscopic colorectal surgery regardless of surgical approach[20.] Elevated CRP levels on the third POD after major abdominal surgery have been found to identify patients at an increased risk for developing major complications.[26] In this publication, a prediction model with a two-cut-off system is suggested. This system consists of a safe discharge criterion with CRP levels below 75 mg/L at POD 3, and at 215 mg/L (probability 20%) to serve as a predictor of severe complications. Other studies mention a single cut-off value of CRP levels between 140-190 mg/L for an increased risk of severe complications[20,27,28]. Although this data was based on several major abdominal surgeries, and not specifically MIE, CRP levels in the GAL1018 patients were 150.5mg/L on POD3, indicating that neuromodulation could induce a clinical relevant reduction in CRP levels and reduce severe postoperative complications following major abdominal surgery.

Because feasibility and safety were the primary aim of this study, no power analysis was performed on the reduction of postoperative CRP levels. The true value of these exploratory results needs to be further confirmed in a statistically powered randomized controlled trial. Interestingly, the number of leucocytes, the cells mainly responsible for production of inflammatory mediators, did not show a difference between groups, suggesting that stimulation had a direct effect on the inflammatory process.

These findings are in line with what is expected based on the experimentally found underlying mechanism which has been previously shown[11,19] and which have been summarized in Figure 3C. Recently, it has been shown in chronically implanted pigs that SpA NVB stimulation using the same cuff as used in this clinical study, is well tolerated and reduces inflammation and promotes resolution of inflammation.[19]

In conclusion, this first-in-human study represents a significant advancement towards the feasibility and safety of splenic neuromodulation in humans and supports the progress towards the development of bioelectronic medicine for patients with inflammatory conditions targeting the near-organ autonomic nervous system of the spleen. Future randomized larger studies are needed to substantiate the current results. Therefore, an acute randomized control trial in patients undergoing MIE is in preparation.

Supplementary figures can be accessed via the journal site:
https://www.frontiersin.org/articles/10.3389/fnins.2022.1088628/full

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

In this thesis, we performed translational studies to examine the anti-inflammatory potential and feasibility of splenic neurovascular bundle stimulation (SpNS) in both acute and chronic inflammation. We performed experimental studies to investigate the role of acetylcholine-producing T-cells and the effect of electrical SpNS on the course of colitis in mice. Additionally, pragmatic studies were carried out on the innervation of the human spleen, the inflammatory response after major abdominal surgery and the feasibility of SpNS during surgery as a foundation for SpNS as a clinical therapy for both acute and chronic inflammatory conditions.

Acetylcholine-producing T-cells (ChAT+ T-cells) and SpNS in experimental models of colitis
The discovery of a link between the nervous system and immune system was substantiated by exploring the underlying mechanisms. $ChAT^+$ T-cells were proposed to be the missing link between sympathetic and parasympathetic nerve systems in the spleen.[1] $ChAT^+$ T-cells are believed to play an important role in the anti-inflammatory effect of vagus nerve stimulation (VNS) and is involved in the regulation of blood pressure, viral clearance and gut homeostasis.[2,3] $ChAT^+$ T-cells were more abundant in the intestine compared to the spleen, indicating involvement in gut immune homeostasis. Previously, the phenotype and function of $ChAT^+$ T-cells was evaluated in healthy intestine.[3] Comparing $CD4ChAT^{-/-}$ mice and control littermates ($ChAT^{fl/fl}$ mice) in the acute inflammation model of dextran sulfate sodium (DSS) colitis, a dual role for $ChAT^+$ T-cells was found. During treatment with DSS, $CD4ChAT^{-/-}$ mice showed less intestinal inflammation than $ChAT^{fl/fl}$ mice, while in the healing phase $CD4ChAT^{-/-}$ mice appeared to underperform compared to the controle mice. It is likely that $ChAT^+$ T-cells contribute to the inflammatory process, since it was previously shown that $ChAT^+$ T-cells produce IFN-γ and IL-17, while in the healing phase acetylcholine plays an important anti-inflammatory role.[3] A limitation of this study is that ChAT was deficient in all T cells and not only the intestine. Furthermore, the influence of other cells that are reported to express ChAT cannot be ruled out, such as B cells, macrophages, and dendritic cells (with dendritic cells also expressing CD4).[4] The therapeutic possibilities of these results, via nerve stimulation or pharmacological modulation of $ChAT^+$ T-cells, are yet to be determined.

The use of nerve stimulation as a treatment for inflammatory bowel diseases has been explored in recent years. Although some promising results were shown with vagus nerve stimulation (VNS) studies, more selective approaches are needed to reduce unwanted side-effects such as bradycardia and respiratory depression, caused by the wide innervation pattern of the vagus nerve.[5-7] We have demonstrated an ameliorating effect of electrical SpNS on the course of DSS colitis in mice, and investigated mechanistic pathways as described in **Chapter 3**. Furthermore, we showed predominant expression of β_2-adrenergic receptors on myeloid cells and reduction of LPS-induced cytokine expression in human splenocytes in vitro. Our studies complement other experimental studies in which electrical SpNS was shown to reduce inflammation in murine and porcine models of sepsis and rheumatoid arthritis.[8-10] In a recent study performed in rats, electrical SpNS protected against myocardial ischemia-reperfusion injury,[11] escorted by ultrasound SpNS that improved myocarditis, pneumonia, arthritis and DSS colitis in murine models.[12-15] Ultrasound stimulation is a non-invasive technique, that can reduce risk of surgical complications and could be well-tolerated by patients. On the other hand, electrical stimulation techniques have been more extensively studied and allow for a more predictable and reproducible modulation of nerve activity. One other advantage is that for implantation of an electrical stimulatory device less hospital visits are needed than

repeated ultrasound stimulation treatments. Studies that compare ultrasound and electrical SpNS should be carried out to investigate which technique is superior.

Our results also add to the understanding of the cholinergic anti-inflammatory pathway, which is a major topic of debate among the fields of neuroimmunology and bioelectronics. As described in the general introduction and more extensively in **Chapter 1,** various mechanisms have been proposed to explain the connections between the parasympathetic vagus nerve, $ChAT^+$ T-cells, the α7 nicotinic acetylcholine receptor, the sympathetic splenic nerve bundle, norepinephrine and different immune cells. Our findings suggest that norepinephrine that is released upon SpNS acts directly on macrophages rather than on $ChAT^+$ T-cells that act as an intermediate. This is supported by a recent study, in which norepinephrine (in vitro) and SpNS (in vivo) reduced TNF-α release following LPS in absence of $CD4^+$ T-cells.[16] The study also elaborated on the connection between the vagus nerve and splenic nerve bundle, rejecting the hypothesis that vagus nerve stimulation could not induce splenic nerve activity.[17,18] Even though the exact mechanism of the anti-inflammatory pathway remains disputed, experimental evidence indicates that direct stimulation of the splenic nerve bundle and the spleen provides an unique therapeutic opportunity for acute and chronic inflammatory disorders.

Sympathetic innervation of the human spleen and the association with immune cells

Next, the relevance and feasibility of SpNS in humans was explored. Studies are mostly performed in rodents, which are notoriously different compared to humans.[19] It is therefore critical to make a timely translation to the clinical setting. In rodents, the existence of sympathetic nerve tissue is abundant and associated with lymphocytes and macrophages.[20,21] We have collected human splenic tissue and performed histological analyses to evaluate the presence of sympathetic nerve fibers and there relation to immune cells. Although some sympathetic nerve fibers were found in close relation to T-cells, this was not apparent in all samples. Also, sympathetic nerve tissue in the spleen seemed dependent on the age of the individuals that were included. It has also been suggested that a decrease in sympathetic nerve tissue can occur as a consequence of sepsis, although the influence of age could not be excluded by the authors.[22] A recent study further underlined interspecies differences. Spleens from pigs were found to be richly innervated with sympathetic nerve tissue, while in human spleens nerves barely exited the adventitia of arterial vessels.[23] Additional mechanisms via which SpNS can interact with immune cells should therefore be considered.

The inflammatory response in major abdominal surgery and the feasibility of SpNS

Surgery inevitably causes an inflammatory response that is important in recovery, such as in wound healing. However, studies suggest a relationship between an increased inflammatory response and the incidence of postsurgical complications. For instance, high levels of interleukin(IL)-6 and C reactive protein (CRP) are associated with complications after major abdominal surgery.[24-26] We evaluated inflammatory markers after open and laparoscopic pancreatoduodenectomy and also analyzed the relationship with to complications. We confirmed the association between early high IL-6 levels (at 24 hours after start of surgery) and complications that occurred later in the postoperative period, such as pancreatic leakage. There results substantiate that a decrease of the inflammatory response after major abdominal surgery may be an important target to lower the incidence of postoperative complications.

In order to perform electrical SpNS in humans, a neuromodulatory device needed to be developed that can be placed around the splenic artery an surrounding nerve fibers. The relation

of the artery with the pancreas could give accessibility issues during placement of a device. An anatomical study was performed by collaborators, whilst we performed a study that included abdominal contrast-enhanced CT scans of 80 subjects.[27] This study helped us to provide splenic artery diameters that were essential in the development of the neuromodulatory device that was used in the feasibility study. We also observed that nearly all subjects had a splenic artery that was at least for some part distinct of the pancreas, although this seemed age-dependent.

After development of the neuromodulatory device (cuff electrode), other studies were then performed by our collaborators to evaluate splenic arterial blood flow as a parameter for nerve engagement, to validate stimulation parameters and to test the possibility of the device to be laparoscopically implanted in a large animal.[10,28,29] Eventually, we could perform a pragmatic feasibility study with the newly developed cuff electrode in patients undergoing esophagectomy, which was the result of close collaboration between engineers, neuroscientists, technicians and clinicians. In this feasibility study, we found that placement and removal of a cuff electrode around the splenic artery and surrounding nerve was safe and feasible. Furthermore, we could measure a significant and reproducible decrease in arterial blood flow, confirming engagement of the splenic nerve bundle. This was the first time electrical SpNS could be successfully performed in humans, without any apparent complications related to placement of the device or the stimulation itself. This study can precede future studies that examine the anti-inflammatory effect of patients with chronic inflammatory conditions such as rheumatoid arthritis or inflammatory bowel diseases. An exploratory outcome of our study was the reduction of postoperative C-reactive protein levels, a recognized inflammatory marker, in the study subjects compared to patients from a historic cohort. Although these results were exploratory, further studies investigating the use of SpNS as a preventive measure for postsurgical complications should be pursued.

FUTURE PERSPECTIVES

In recent decades, the connection between the nervous and immune systems has led to an emerging area of research that offers a novel perspective on immune regulation, opening doors to innovative therapeutic approaches. As technology continues to advance and our understanding of the neuro-immune interactions deepens, nerve stimulation therapies offer hope for more effective and sustainable approaches to treat inflammatory disorders. There were high hopes for VNS, but clinical trials never passed the pilot phase. This is probably due to unwanted side-effects, inadequate stimulation parameters or because anti-inflammatory effects in follow-up studies were disappointing and suffered from publication bias. Research should therefore not solely focus on VNS. Fortunately, two clinical trials are under way to investigate the safety, feasibility and early efficacy of electrical splenic neurovascular bundle stimulation in patients with rheumatoid arthritis (clinicaltrials.gov; NCT04955899 and NCT05003310). A clinical trial is also being conducted on ultrasound SpNS (NCT05417854) as a treatment for rheumatoid arthritis and together these studies will provide more clarity on SpNS as a viable therapy for chronic inflammatory conditions. Other neural pathways, such as the sacral nerves and pancreatic nerve have been explored in rodents, but their clinical relevance has to be established.[30,31] Experimental work remains essential in the field of neuroimmunology, but it should have translational implications. Therefore, well-designed, randomized controlled studies should be in the center of attention in the coming years.

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APPENDICES

SUMMARY

The immune system is essential in host-defense against internal and external pathogenic threats. It acts in a subtle homeostasis, which can tilt to an environment that is harmful for the host when disrupted. This can lead to an acute excessive inflammatory response in the recovery period of major abdominal surgery or to chronic inflammation in auto-immune diseases such as inflammatory bowel diseases (IBD). The immune system is under control of the autonomic nervous system, which provides potential therapeutic options to treat excessive immune reactions. In this thesis, splenic nerve stimulation (SpNS) was investigated in rodent models of colitis as a potential treatment for IBD. Moreover, in experimental colitis we examined the role of specific cells, $ChAT^{+}$ T-cells, that play a role in the connection between the nervous system and immune system. Thereafter, safety and feasibility of neuromodulation of the splenic arterial neurovascular bundle (SpA NVB) were evaluated in patients undergoing minimally invasive esophagectomy (MIE).

Chapter 1 discusses the available literature on the interaction between the immune system and nervous system in the intestine. The chapter provides an overview on the cholinergic anti-inflammatory pathway, the influence of both parasympathetic and sympathetic factors in immunomodulation of the gut, and other neural components that can affect the immune system. Studies that investigate the role of stimulation or elimination of intestinal innervation on experimental colitis are summarized. Vagus nerve stimulation (VNS) is most frequently evaluated in animal models of colitis and is showed to ameliorate colitis outcome. While the precise mechanism is widely debated, it is postulated that afferent fibers from different peripheral organs that run up through the vagus nerve carry signals to the brain stem where the brain then modulates the inflammatory homeostasis through efferent fibers that run down the vagus nerve and synapse with the splenic nerve at the ciliac ganglion that in turn modulate the immune cells passing through the spleen.. Following all the experimental work, clinical trials have been initiated that investigated the therapeutic of potential of vagus stimulation in IBD. However, VNS is associated with off targets effects such bradycardia, which might be prevented by a near organ neuromodulation approach as described in **Chapters 3 and 7**.

One of the disparities of the cholinergic anti-inflammatory theory is the connection between the parasympathetic vagus nerve and the sympathetic splenic nerve plexus. Mechanistic studies have demonstrated that a specific type of T-cell might relay the parasympathetic signal. The acetylcholine producing T-cells (defined by their expression of choline acetyltransferase (ChAT), an enzyme involved in the synthesis of acetylcholine) reside in the spleen and are activated upon norepinephrine release by the splenic nerves. Recent studies have shown that these $ChAT^{+}$ T-cells are also present in the gut, where they act as an important non-neuronal source of acetylcholine in the gut. In **Chapter 2** it was shown that $ChAT^{+}$ T-cells reside in the gut and the role of $ChAT^{+}$ T-cells in experimental colitis was further investigated. This was performed by making use of $CD4ChAT^{-/-}$ mice, that specifically lacked ChAT expression in all T cells. These mice were examined in three types of rodent colitis models. In a model of acute dextran sulfate sodium (DSS) colitis, $CD4ChAT^{-/-}$ mice suffered from less severe colitis compared with $ChAT^{fl/fl}$ (control) mice based on mRNA and protein expression of inflammatory cytokines in the colon. In contrast, $CD4ChAT^{-/-}$ mice showed a reduced potential to recover in a resolution model of DSS colitis compared to the wildtype as indicated by disease activity and histology scores, indicating that the $ChAT^{+}$ T-cell might undergo changes in its phenotype and function

during the different phases of inflammation. No differences between CD4ChAT$^{-/-}$ mice and ChAT$^{fl/fl}$ mice were found in the T cell transfer colitis model. These experiments revealed that the role of ChAT^{+} T-cells is more complex and additional experiments are warranted.

In **chapter 3** the therapeutic effect of electrical SpNS was investigated in a mice model of experimental colitis. SpNS was able to decrease various outcomes of colitis, such as histology scores and colon weight/length ratio and can reverse transcriptomic changes that were induced by DSS. In contrast to what other studies have demonstrated before, VNS did not improve outcome of DSS colitis in our hands, which might indicate that SpNS is more effective in reducing colitis due to the more selective approach.

Following the animal work, it was investigated whether SpNS has therapeutic potential in humans. **Chapter 4** investigates the innervation of the human spleen and the association between sympathetic nerves and T-cells. By performing a histologic study on postmortem splenic tissue, it was discovered that T-cells are in close proximity of neurons. Aging seems to be associated with a decrease in overall sympathetic innervation and with a reduced presence of neurons in T-cell regions of the spleen. This suggests that direct contact of neurons with T-cells might not be the only mechanism via which SpNS can modulate inflammation.

In **chapter 5** we investigated the association between the acute systemic inflammatory response and complications in pancreatic surgery. Increased inflammatory markers such as serum interleukin (IL)-6 and C-reactive protein (CRP) preceded major complications, underlining previous studies and suggesting that mitigation of the inflammatory response following abdominal surgery can prevent the development of complications.

The splenic nerve bundle runs alongside the splenic artery, which has a variable course and morphology. This impacts surgical accessibility and electrical cuff placement for SpNS. In **Chapter 6** the course of the splenic artery was evaluated by assessing the relation to the pancreas and the presence and characteristics of loops that are potentially surgically accessible. Contrast-enhanced computed tomography (CT) images were used from patients in different age and gender categories. It was demonstrated that the contact of the splenic artery with the pancreas decreases and the diameter of the splenic artery increases with age, however, much variation existed within gender and age categories. The vast majority of subjects (86%) demonstrated one or more splenic arterial loops. Loops outside of the pancreatic tissue were found in most subjects and were present along the entire course of the splenic artery. These loops can act as a target for the placement of neuromodulatory devices.

Finally, **Chapter 7** describes a first-in-human pilot trial which investigates the safety and feasibility of SpNS during esophagectomy. We demonstrated that cuff electrode placement in 13 participants was successful and could be performed safely. Stimulation was associated with an overall decrease in splenic artery blood flow, which served as a biomarker for successful SpNS, was shown in all patients using intra-abdominal Doppler ultrasound imaging. No changes in blood pressure or heart rate were observed following stimulation. When compared to historic Propensity Score Matched (PSM) controls, a decrease in serum CRP was observed on postoperative day 2 and 3 in stimulated participants. This study paves the path to future trials, investigating the therapeutic potential of SpNS in both surgical patients as well as patients that suffer from chronic inflammatory conditions such as IBD.

NEDERLANDSE SAMENVATTING

Het immuunsysteem is essentieel voor het bestrijden van interne en externe factoren die ziekte kunnen veroorzaken. Als het immuunsysteem echter doorslaat kan er sprake zijn van een situatie waarin het systeem schadelijk is voor het lichaam zelf. Voorbeelden hiervan zijn een buitensporige acute ontstekingsreactie na buikchirurgie of auto-immuunziekten zoals inflammatoire darmziekten. Het immuunsysteem wordt gecontroleerd door het autonome zenuwstelsel. Dit biedt de mogelijkheid voor nieuwe therapieën om bovenmatige ontstekingsreacties te behandelen. In dit proefschrift hebben we miltzenuwstimulatie onderzocht in verschillende muismodellen voor darmontsteking. Deze muismodellen hebben we ook gebruikt om de rol van een specifieke cel te onderzoeken die de neurotransmitter acetylcholine produceert, de $ChAT^+$ T-cel. Vervolgens hebben we de veiligheid en haalbaarheid van neurostimulatie van de miltslagader en de omliggende zenuwbundels onderzocht in patiënten die een slokdarmoperatie ondergingen.

In **hoofdstuk 1** vatten we de beschikbare literatuur samen en bespreken we de interactie tussen het immuunsysteem en het zenuwstelsel in de darmen. Het hoofdstuk geeft een overzicht van wat bekend is over de zogenoemde 'cholinergic anti-inflammatory pathway' en bespreekt de invloed van de twee onderdelen van het autonome zenuwstelsel (het parasympatische en het sympathische systeem) en andere onderdelen van het zenuwstelsel die een rol kunnen spelen in de regulering van het immuunsysteem in de darm. Daarbij hebben we de studies samengevat die het effect van zenuwstimulatie op darmontsteking onderzoeken en hebben we gekeken naar studies die juist gepoogd hebben te verhinderen dat zenuwen de darm kunnen beïnvloeden. Vagusstimulatie is de vorm van zenuwstimulatie die het meest onderzocht wordt en lijkt darmontsteking in muizen en ratten te verbeteren. Er lijkt sprake van een reflexmechanisme, waarbij het sensibele (afferente) deel van de vaguszenuw de ontsteking detecteert en dit doorgeeft aan de hersenstam. Via efferente zenuwen wordt daarna de ontsteking in de darm gedempt, waarbij studies laten zien dat dit via de miltzenuw en milt plaats vindt. Om het effect van vagusstimulatie verder te onderzoeken zijn er ook kleine klinische studies uitgevoerd waaraan mensen hebben deelgenomen met inflammatoire darmziekten zoals de ziekte van Crohn. Vagusstimulatie gaat echter gepaard met bijwerkingen zoals een trage hartslag. Dit kan mogelijk voorkomen worden door zenuwstimulatie specifieker toe te passen (zoals besproken in **hoofdstuk 3 en 7**), waardoor het doelorgaan (de darm) beter wordt bereikt.

Binnen de theorie van de 'cholinergic ant-inflammatory pathway' bestaat nog onduidelijkheid over hoe het parasympatische en sympathische deel van het autonome zenuwstelsel met elkaar verbonden zijn. Onderzoeken laten zien dat een bepaald type T-cel hierbij een rol speelt. Deze T cellen bezitten het enzym choline acetyltransferase (ChAT), waardoor de cellen in staat zijn om de neurotransmitter acetylcholine te synthetiseren. Dit is de belangrijkste neurotransmitter van het parasympatische systeem. Hoewel de miltzenuw sympathisch is, zorgen deze $ChAT^+$ T-cellen er mogelijk voor dat na miltzenuwstimulatie een parasympatische neurotransmitter vrijkomt, waardoor ontsteking gedempt kan worden. In **hoofdstuk 2** wordt aangetoond dat $ChAT^+$ T-cellen ook aanwezig zijn in de darmen en de rol van deze cellen is verder onderzocht in verschillende muismodellen voor darmontsteking. Dit werd uitgevoerd door gebruik te maken van $CD4ChAT^{-/-}$ muizen, die specifiek in T cellen het gen missen om ChAT aan te maken. In een acuut ontstekingsmodel (het DSS-colitis model), bleken $CD4ChAT^{-/-}$ muizen minder darmontsteking te ontwikkelen dan de wildtype muizen (de 'normale' muizen),

gebaseerd op klinische factoren zoals ziekteactiviteit en weefselonderzoek. Opmerkelijk genoeg bleek dit bij een herstelmodel voor darmontsteking precies andersom en werden er meer ontstekingseiwitten gemaakt in wildtype muizen vergeleken met CD4ChAT$^{-/-}$. Mogelijk hebben ChAT^{+} T-cellen een alternatieve rol en functie tijdens de verschillende fases van ontsteking. In een laatste model, het T cel transfer model, werden geen verschillen gevonden tussen de twee muissoorten. Onze experimenten laten zien dat de rol van ChAT^{+} T-cellen complex is en dat meer studies nodig zijn om dit te doorgronden.

In **hoofdstuk 3** is het effect onderzocht van elektrische miltzenuwstimulatie in een muismodel voor darmontsteking. Miltzenuwstimulatie verbeterde verschillende uitkomsten voor darmontsteking in vergelijking met placebobehandeling en was ook in staat om veranderingen die worden veroorzaakt door darmontsteking op het niveau van genexpressie tegen te gaan. In tegenstelling tot eerdere studies lieten onze experimenten geen voordeel zien van vagusstimulatie, wat mogelijk betekent dat miltzenuwstimulatie een effectievere manier is om darmontsteking te behandelen.

Hierna is verder onderzocht of miltzenuwstimulatie toegepast kan worden in mensen. In **hoofdstuk 4** is de innervatie van de milt onderzocht en of er een relatie bestaat tussen sympathische zenuwen en T cellen. Hiervoor werd post mortem miltweefsel gebruikt van personen uit verschillende leeftijdsgroepen, waarbij bevestigd werd dat T cellen inderdaad zich in de buurt van zenuwen bevinden. Als de leeftijd toeneemt lijkt de hoeveelheid sympathisch zenuwstelsel af te nemen er zijn er minder zenuwen in de buurt van T cellen. Dit geeft mogelijk aan dat miltzenuwstimulatie mogelijk niet afhankelijk is van T cellen en dat er andere mechanismen zijn die een rol spelen.

In **hoofdstuk 5** is onderzocht wat de relatie is tussen de acute systemische inflammatoire respons en complicaties na alvleesklierchirurgie. Verhoogde ontstekingseiwitten zoals interleukine 6 en C-reactief proteïne waren geassocieerd met het optreden van grote complicaties. Deze bevindingen bevestigden de resultaten van eerdere studies en toont aan dat het verminderen van de inflammatoire response na buikchirurgie het ontwikkelen van complicaties mogelijk kan tegengaan.

The miltzenuwen bevinden zich als een bundel langs de miltslagader. Deze slagader kent een grillig beloop, waardoor het plaatsen van een stimulator rondom de arterie mogelijk bemoeilijkt wordt. In **hoofdstuk 6** is het verloop van de miltslagader onderzocht, met name ten opzichte van de alvleesklier en de aanwezigheid van plaatsen die benaderbaar zijn voor het plaatsen van een stimulator. Hiervoor is gebruik gemaakt van CT beelden van mannen en vrouwen van verschillende leeftijden. Bij het overgrote deel van de patiënten (86%) bevond een deel van de miltslagader zich buiten de alvleesklier. Zowel bij de oorsprong van de miltslagader als dichter bij de milt was dit het geval. Dit betekent dat de miltslagader benaderbaar is en dat het plaatsen van een stimulator mogelijk moet zijn.

Ten slotte wordt in **hoofdstuk 7** een pilot studie beschreven waarin voor het eerst miltzenuwstimulatie is toegepast in mensen. De studie was gericht op veiligheid en haalbaarheid van miltzenuwstimulatie tijdens slokdarmchirurgie. De resultaten laten zien dat het plaatsen van een stimulator mogelijk is en dat dit veilig kon gebeuren bij 13 deelnemers. Tevens werd aangetoond dat stimulatie zorgde voor een daling in de bloedstroomsnelheid in de miltslagader (gemeten door middel van Doppleronderzoek tijdens de stimulatie), wat een bevestiging is dat

de miltzenuwen daadwerkelijk gestimuleerd werden. Er werden geen veranderingen gezien in systemische bloeddruk of hartslag door de stimulatie. Het ontstekingseiwit C-reactief proteïne op dag 2 en 3 na de operatie was lager in de deelnemers aan de studie dan in statistisch vergelijkbare patiënten die eerder een slokdarmoperatie hadden ondergaan. Dit onderzoek dient als basis voor toekomstige studies om de effectiviteit van miltzenuwstimulatie verder te onderzoeken, als behandeling voor zowel buitensporige acute ontstekingsreacties na buikoperaties als voor chronische aandoeningen zoals inflammatoire darmziekten.

PHD PORTFOLIO

Name PhD student:	Daan Brinkman
PhD period:	January 2016 – October 2020
Name PhD supervisor:	Prof. Wouter de Jonge

1. PhD training

		Year	Workload ECTS)
General courses			
-	Basic Laboratory Safety	2016	0.3
-	Good Clinical Practice (Catharina Hospital)	2016	1.0
-	Medical writing in English (Catharina Hospital)	2016	1.5
-	Practical Biostatistics	2016	1.1
-	Oral Presentation	2017	0.6
-	Project Management	2017	0.6
-	Didactical Skills	2020	0.6
Specific courses			
-	Laboratory Animals	2016	2.9
-	Laparoscopic Doppler	2019	1.2
-	Systematic Review	2019	0.7
Seminars, workshops and master classes			
-	Weekly department seminars Tytgat Institute	2016-2020	8.0
-	Seminars in gastroenterology and hepatology, AMC, Amsterdam, NL	2016-2019	0.5
-	DDW Highlights, Amsterdam, the Netherlands	2017	0.1
-	Science Night Catharina Hospital, the Netherlands	2018	0.1
-	DDW Highlights, Amsterdam, the Netherlands	2018	0.1
-	Science Night Catharina Hospital, the Netherlands	2019	0.1
Presentations			
Poster presentation			
-	The effect of intestinal manipulation on healing of the intestinal anastomosis. Digestive Disease Week, Washington D.C., USA	2018	0.5
-	Eye-tracking to differentiate viewing behavior of surgeons and trainees during laparoscopic pancreatoduodenectomy. International Hepato-Pancreato-Biliary Association World Congress, Geneva, Switzerland	2018	0.5
-	Acetylcholine secreting T-cells contribute to innate immune driven colitis. United European Gastroenterology Week, Barcelona, Spain	2019	0.5
Oral presentation			
-	The effect of sarcopenia and visceral obesity on the inflammatory response in colorectal surgery. Voorjaarsvergadering Nederlandse Vereniging voor Gastro-enterologie en Hepatologie, Veldhoven, the Netherlands	2016	0.5
-	Eye-tracking as a tool to differentiate physicians with different grades of experience in performing laparoscopic pancreaticoduodenectomy. Digestive Disease Days, Veldhoven, the Netherlands	2018	0.5
-	Eye tracking oogbeweging en blikveld analyse bij chirurgen en arts-assistenten tijdens het bekijken van laparoscopische pancreaschirurgie. Science Night Catharina Hospital, Eindhoven, the Netherlands	2018	0.5
-	The preoperative fecal lipidome but not fecal microbial diversity predicts postoperative ileus in elective colorectal surgery. Digestive Disease Days, Veldhoven, the Netherlands	2019	0.5
-	De inflammatoire respons na laparoscopische en open pancreatoduodenectomie en de relatie met postoperatieve complicaties in een multicenter, gerandomiseerde studie. Chirurgendagen, Veldhoven, the Netherlands	2019	0.5
-	Non-invasive electrical splenic nerve stimulation ameliorates DSS-induced colitis. Digestive Disease Days, Veldhoven, the Netherlands *	2020	0.3
-	Miltzenuwstimulatie in de muis reduceert lokale inflammatie in de darm. Chirurgendagen, Veldhoven, the Netherlands *	2020	0.3
-	Non-invasive electrical splenic nerve stimulation ameliorates DSS-induced colitis. Digestive Disease Week, Chicago, USA *	2020	0.3

* = accepted, but cancelled due to COVID-19 pandemic

(Inter)national conferences

-	Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie en Hepatologie (NVGE), Veldhoven, the Netherlands	2016	0.5
-	Chirurgendagen, Veldhoven, the Netherlands	2016	0.5
-	Chirurgendagen, Veldhoven, the Netherlands	2017	0.5
-	Digestive Disease Days, Veldhoven, the Netherlands	2018	0.5
-	Digestive Disease Week, Washington D.C., USA	2018	1.0
-	International Hepato-Pancreato-Biliary Association World Congress, Geneva, Switzerland	2018	1.0
-	Digestive Disease Days, Veldhoven, the Netherlands	2019	0.5
-	Chirurgendagen, Veldhoven, the Netherlands	2019	0.5
-	United European Gastroenterology Week, Barcelona, Spain	2019	1.0

Other

-	Journal Club Research group (2/month)	2016-2019	8.0
-	Annual PhD Retreat AG&M	2016-2019	4.0

2. Teaching

		Year	Workload (Hours/ ECTS)
Supervising			
-	Biomedical Student Robin Haring, research internship, 6 months	2018-2019	3.0
-	Several medical students at Catharina Hospital	2016-2019	1.5

3. Parameters of Esteem

		Year
Grants		
-	DPCG-IPSEN Science Travel Award	2019
-	Travel grant NVGE	2020
Awards and Prizes		
-	Most contributing participant, AG&M PhD Students Retreat	2017

LIST OF PUBLICATIONS

This thesis

1. **Brinkman DJ**, Gupta I, Matteucci P, Ouchouche S, De Jonge WJ, Coatney RW, Salam T, Chew DJ, Irwin E, Yazicioglu RF, Nieuwenhuijzen GAP, Vervoordeldonk MJ, Luyer MDP. *Splenic arterial neurovascular bundle stimulation in esophagectomy: a feasibility and safety prospective cohort study*. Frontiers in Neuroscience 2022

2. **Brinkman DJ**, Simon T, ten Hove AS, Zafeiropoulou K, Welting O, Hamersveld PHP, Willemze RA, Li Yim AYF, Verseijden C, Hakvoort TBM, Luyer MD, Vervoordeldonk MJ, Blancou P, de Jonge WJ. *Electrical stimulation of the splenic nerve bundle ameliorates dextran sulfate sodium-induced colitis in mice*. Journal of Neuroinflammation 2022

3. Cleypool CGJ, **Brinkman DJ**, Mackaaij C, Nikkels PGJ, Nolte MA, Luyer MD, de Jonge WJ, Bleys RLAW. *Age-Related Variation in Sympathetic Nerve Distribution in the Human Spleen.* Frontiers in Neuroscience 2021

4. **Brinkman DJ**, Troquay S, de Jonge WJ, Irwin ED, Vervoordeldonk MJ, Luyer MDP, Nederend J. *Morphometric analysis of the splenic artery using contrast-enhanced computed tomography (CT).* Surgical and Radiologic Anatomy 2020

5. Willemze RA, **Brinkman DJ**, Welting O, Van Hamersveld HP, Verseijden C, Luyer MD, Wildenberg ME, Seppen J, De Jonge WJ. *Acetylcholine-producing T-cells augment innate immune driven colitis but are redundant in T-cell driven colitis.* American Journal of Physiology – Gastrointestinal and Liver Physiology 2019

6. van Hilst J*, **Brinkman DJ***, de Rooij T, van Dieren S, Gerhards MF, de Hingh IH, Luyer MD, Marsman HA, Karsten TM, Busch OR, Festen S, Heger M, Besselink MG; Dutch Pancreatic Cancer Group. *The inflammatory response after laparoscopic and open pancreatoduodenectomy and the association with complications in a multicenter randomized controlled trial.* HPB 2019

7. **Brinkman DJ***, Ten Hove AS*, Vervoordeldonk MJ, Luyer MD, de Jonge WJ. *Neuroimmune Interactions in the Gut and Their Significance for Intestinal Immunity.* Cells 2019

*contributed equally

Other publications

8. Ten Hove AS, **Brinkman DJ**, Li Yim AYF, Verseijden C, Hakvoort TBM, Admiraal I, Welting O, van Hamersveld PHP, Sinniger V, Bonaz B, Luyer MD, de Jonge WJ. *The role of nicotinic receptors in SARS-CoV-2 receptor ACE2 expression in intestinal epithelia*. Bioelectronic Medicine 2020

9. Peters EG, Pattamatta M, Smeets BJJ, **Brinkman DJ**, Evers SMAA, de Jonge WJ, Hiligsmann M, Luyer MDP. *The clinical and economic impact of postoperative ileus in patients undergoing colorectal surgery.* Neurogastroenterology & Motility 2020

10. **Brinkman DJ,** van Hilst J, Luyer MD. *Internal herniation following laparoscopic pancreatoduodenectomy.* BMJ Case Reports 2020

11. van Hilst J, de Rooij T, Bosscha K, **Brinkman DJ,** van Diederen S, Dijkgraaf MG, Gerhards MF, de Hingh IH, Karsten TM, Lips DJ, Luyer MD, Busch OR, Festen S, Besselink MG; Dutch Pancreatic Cancer Group. *Laparoscopic versus open pancreatoduodenectomy for pancreatic or periampullary tumours (LEOPARD-2): a multicentre, patient-blinded, randomised controlled phase 2/3 trial.* The Lancet Gastroenterology and Hepatology 2019

12. de Rooij T, van Hilst J, Topal B, Bosscha K, **Brinkman DJ**, Gerhards MF, de Hingh IH, Karsten TM, Lips DJ, Luyer MD, Marsman HA, van Rijssen LB, Steen MW, Busch OR, Festen S, Besselink MG; Dutch Pancreatic Cancer Group. *Outcomes of a Multicenter Training Program in Laparoscopic Pancreatoduodenectomy (LAELAPS-2).* Annals of Surgery 2019

13. Smeets BJJ, **Brinkman DJ**, Horsten ECJ, Langius JAE, Rutten HJT, de Jonge WJ, Luyer MDP. *The Effect of Myopenia on the Inflammatory Response Early after Colorectal Surgery.* Nutrition and Cancer 2018

14. Dutch Snapshot Research Group. *Benchmarking recent national practice in rectal cancer treatment with landmark randomized controlled trials.* Colorectal Disease 2017

DANKWOORD

Het is tijd voor het dankwoord. Een zeer belangrijk onderdeel van een proefschrift en waarschijnlijk ook het meest gelezen. En dat is terecht. Dit proefschrift was niet tot stand gekomen zonder de hulp van een heel aantal mensen.

Allereerst wil ik alle **patiënten** bedanken die mee hebben gedaan aan de verschillende studies. Bovenal de patiënten die mee hebben gedaan aan de zenuwstimulatiestudie in hoofdstuk 7 ben ik enorm dankbaar. Meedoen aan een studie die een experimentele behandeling onderzoekt vergt moed en dat kan niet genoeg benadrukt worden.

Wouter, eind 2015 ontmoetten wij elkaar toen Misha het een goed idee vond dat ik de uitvoerder ging worden van een PhD project dat draaide om miltzenuwstimulatie. Ik kwam langs op het Tytgat Instituut en ik herinner me je energie en enthousiasme voor dit nieuwe project. Ik kreeg een rondleiding en werd meteen voorgesteld als nieuwe collega. Het voelde daardoor meteen vertrouwd en je hebt ervoor gezorgd dat het lab voor mij, als simpele en onwetende dokter, geen moment een plek was waar ik me niet op mijn gemak voelde. Je hebt een onbegrensd talent om van elk experiment, mislukt of geslaagd, het positieve te zien en dat zorgde ervoor dat ik altijd optimistisch ben geweest over een succesvol einde van mijn PhD. Je netwerk en vermogen tot samenwerken met verschillende onderzoekers maken jaloers en zijn je grootste kracht. Dit inspireert me enorm. Aan het eind van het project was je ook in staat om samen met mij prioriteiten te stellen om het geheel tot een goed einde te brengen. Het resultaat, een echte samenwerking tussen het lab en de kliniek, mag er denk ik zijn. Verder moet ik benadrukken wat een feest het was om onderdeel van jouw onderzoeksgroep te zijn geweest. Vele diners, zeiluitjes, congressen en borrels worden vooral op jouw initiatief georganiseerd en bezocht en zijn o zo belangrijk voor de sfeer in de groep. Dank daarvoor. Je onderzoeksgroep wordt steeds groter en er lijkt geen einde te komen aan de stroom projecten waar je bij betrokken bent. Ik kan niet wachten om te zien wat je verder gaat bereiken.

Beste **Misha**, ik kan me niet eens voorstellen hoe mijn carrière was verlopen zonder jou. Mijn semi-artsstage bij jou in het Catharina zorgden ervoor dat ik mijn opleiding tot arts kon afmaken en hebben me laten kennis maken met het doen van onderzoek. Ik weet nog goed dat ik daarna in de supermarkt stond en je me opbelde omdat ‘er een fantastisch promotietraject klaar ligt. Het is iets met zenuwstimulatie, het duurt 4 jaar en je zult af en toe in het lab moeten werken, in Amsterdam. Graag hoor ik het wel meteen van je.’ Dit belletje is kenmerkend voor je. Recht op je doel af, enthousiast over onderzoek vanuit je motivatie om het voor je patiënten beter te doen en altijd denkend in mogelijkheden. Ik heb hier veel ontzag voor en ik hoop in mijn verdere loopbaan jouw energie voor patiëntenzorg te benaderen. Ondertussen ben je ook nog prof geworden, een terechte beloning voor al het werk dat je hebt verzet. Dat je buiten je werk in het ziekenhuis nog tijd hebt voor andere dingen is bijna niet voor te stellen, maar je hebt ook nog een prachtig gezin en bent een beest op de fiets. Het fietsvirus heb je ook bij mij aangewakkerd. In 2019 hebben we met een grote groep van het Catharina meegedaan aan Alpe d’Huzes. Deze bijzondere week zal ik nooit vergeten en ik vond het erg bijzonder dat we de zesde en laatste keer samen naar boven reden. Dat wil zeggen de eerste kilometer van die laatste beklimming, want daarna was je er alweer vandoor. Met jouw doorzettingsvermogen ben ik benieuwd wat je hoogleraarschap ons verder gaat brengen.

Dan mijn andere copromotor, **Margriet**. Ik ben dankbaar dat je onderdeel bent geworden van mijn begeleidingsteam. Dit proefschrift was er niet gekomen zonder jouw goede ideeën en kritische blik. Je werd gedurende het proces zo veel meer dan 'iemand van de industrie' die controleerde of er wel voldoende progressie was. Je wilde de projecten tot een goed einde brengen en hebt hier alles voor gedaan. Je hielp me bij het opzetten van experimentele studies en bij het vragen van METC goedkeuring en je was altijd bereid tot het kritisch lezen van manuscripten en het doen van voorstellen om het eindresultaat beter te maken. Je was een onmisbare schakel tussen het werk in Eindhoven, Amsterdam en Stevenage en ik kan me niemand voorstellen die er beter in zou zijn geweest dan jij. Daarnaast ben ik je dankbaar voor de vele sociale activiteiten. Onder andere de borrels in Stevenage en Nice waren geweldig. Ook het diner met Paul en Misha bij Vane was memorabel. We hebben nog steeds een afspraak staan voor een diner om de klinische studie te vieren, laten we die vooral niet vergeten.

Beste leden van de **promotiecommissie,** prof. dr. R.E. Mebius, prof. dr. M.G. Netea, prof. dr. P.J. Tanis, prof. dr. S.E. la Fleur, dr. J.P.M. Derikx en dr. G. Matteoli, dank voor uw tijd en beoordeling van mijn proefschrift. Ik zie er naar uit om met u van gedachten te wisselen tijdens de verdediging.

Er is nog een heel aantal mensen dat ik wil bedanken. Firstly, the lovely people from the **Tytgat Institute.** I highly appreciate the time I have spent at the Tytgat Institute. I learned a lot about basic science and performing experiments, and I was able to train my presentation skills with the numerous progress reports and journal clubs. The diverse group of people at the Tytgat is really out of the ordinary. I have to thank the members of **group Wouter** for all the assistance with experiments and for the fun social activities we did together throughout the years. Special thanks to **Tanit**, who held my hand during my first steps in the lab. Ook wil ik **Caro** en **Theo** bedanken, zonder jullie hulp was ik nergens in het lab. **Andrew**, dank voor alle hulp met de RNA sequence experimenten en met het begrijpen ervan. **Rose**, ik keek enorm naar je op tijdens mijn eerste jaren op het Tytgat. Je efficiëntie, arbeidsethos en strakke planning waren inspirerend. Dank voor de samenwerking. **Olaf** en **Patricia**, zonder jullie was geen enkel dierexperiment van de grond gekomen, laat staan voltooid, en daarvoor ben ik jullie enorm dankbaar.

Cindy, toevallig zijn we onderdeel geworden van elkaars promotietraject. We hebben min of meer samen besloten dat we de humane innervatie van de milt beter in beeld moesten brengen en ik ben trots op de studie die we uiteindelijk tot stand hebben gebracht. Ik heb veel geleerd van je anatomische kennis en ben onder de indruk van je enthousiasme en verwondering. Dank voor alle discussies en de samenwerking.

Joost, dank voor je begeleiding tijdens de CT-studie. Alles wat ik vroeg kon meteen en je hebt me goed op weg geholpen op de afdeling radiologie. De resultaten van de studie waren essentieel voor de ontwikkeling van de cuff elektrode en daarom ben ik je erg dankbaar voor je hulp.

This whole project would not have been possible without the financial support of **Galvani Bioelectronics.** I want to thank all the people that were involved in the preclinical work that preceded the clinical trial. Many thanks to **Bob**, for all your animal work but especially for inviting me to Minneapolis and teaching me about Laparoscopic Doppler. **Paul**, thank you for supervising the cuff design but also for the laughs we had in Eindhoven and other places. **Isha,** thanks for coming to Eindhoven to carry out the trial and for performing all the calculations on blood flow and stimulation parameters. **Eric**, thank you for overviewing the training program

and for our collaboration during the CT project. **David** and **Annabel**, you were my regulatory approval buddies and you performed a lot of work behind the scenes that made my work on the floor a lot easier. I want to thank you all for an experience that will benefit me forever.

Dear **Thomas** and **Philippe**, our sister researchers in Nice. We were working simultaneously to unravel the mysteries around splenic nerve stimulation. I highly appreciate you sharing your methods and results. I have great memories of the Galvani conference in Nice that you hosted and cherish our scientific discussions. I wish you all the best for the future.

Dan wil ik nog **Jony**, **Thijs** en **Mark** bedanken. We kwamen erachter dat we allemaal geïnteresseerd waren in de inflammatoire respons na pancreaschirurgie en bundelden onze krachten. Ik ben dankbaar dat ik een klein onderdeel mocht zijn van jullie pancreasonderzoeksmachine. Wat jullie hebben neergezet verdient enorm veel respect en mag als voorbeeld dienen voor hoe onderzoek nationaal georganiseerd moet worden.

Een promotietraject is wat mij betreft niet mogelijk zonder de nodige afleiding. Mijn tijd in Eindhoven was prachtig en ik heb me altijd enorm thuis gevoeld, mede dankzij **Ricardo**, **Emiel** en **Robert-Jan.** Borrels op Stratumseind, carnaval en concerten van Axwell en Ingrosso leken ongeveer elke week plaats te vinden. Bedankt aan iedereen van de zolder, in het bijzonder **Koen**, **Marijn**, **Pio**, **Emmeline**, **Robin**, **Anne-Rave**, **Checca**, **Eef**, **Poodt**, **Van Basten Batenburg**, **Laura**, **Niels** en **Hageman**. Het was fijn om elkaar af en toe te ondersteunen, maar met name de gezelligheid en de onzin die we samen beleefden zullen me lang bijblijven.

Mijn vrienden uit Maastricht spelen zo'n grote rol in mijn leven dat ik dit moment zeker wil gebruiken om ze te bedanken. Natuurlijk moet ik de echte vrienden bedanken, met name **Kik**, **Djimmie**, **Boj**, **Pjèr**, **Tjester**, **Dex**, **Petrik**, **Stenlie**, **Hans**, **Tjuk**, **Storm**, **Zjeraar**, **Duko**, **Frits** en **Bil.** Het is niet normaal hoeveel plezier er was in Maastricht. Ik kan niet genoeg benadrukken hoe bijzonder ik het vind dat dit nu nog steeds zo is in Amsterdam, Den Bosch, Eindhoven, Den Haag, Les Eyzies, of waar we ook maar mogen zijn. Jullie hebben me gevormd tot wie ik ben en houden me met beide benen op de grond. Wat mij betreft zou het elke week zomerweekend of geldweekend mogen zijn en wordt er vanaf nu alleen nog maar naaibal gespeeld. Jongens van de jaarclub, we kennen elkaar nu vijftien jaar en we zijn al zoveel hoogtepunten verder. Dank voor de vele fietsvakanties, borrels in Breda, Maastricht of Schin op Geul en de weekenden weg. In het bijzonder dank aan **Erik**, **Tibor**, **Bas** en **Rik.** Heel speciaal dat we elkaar nog zo vaak zien en dat we zo'n groot onderdeel zijn van elkaars leven. Dank aan **Marit**, **Elisabeth**, **Olivier** en **Reinoud** voor het intensieve maar geweldige jaar als bestuur van Circumflex. Ik gebruik wat ik tijdens dat jaar heb geleerd nog dagelijks en zonder jullie was het niet hetzelfde geweest. Het zomerhuisje in Zweden en de clubs op Ibiza moeten binnenkort maar ingewijd worden als nieuwe borrellocatie.

Gijs en **Boudewijn**. Samen in de jaarclub, samen semi-arts in Eindhoven, samen arts-onderzoeker bij Misha. Eindelijk mag ik in jullie voetsporen treden met dit proefschrift. Ik kan me geen betere paranimfen bedenken dan jullie. De presentatie die ik over jullie heb gegeven tijdens jullie promotiefeest doet al geen recht meer aan wat jullie in de tussentijd nog meer bereikt hebben en daar kijk ik met veel bewondering naar. Dank dat jullie onderdeel zijn van mijn leven.

Lieve **schoonfamilie**, wat hebben we al mooie jaren beleefd samen. Ik heb me vanaf het eerste moment bij jullie thuis gevoeld en voel me echt onderdeel van de familie. De interesse in het

promotieavontuur van Anne en mij heb ik altijd op prijs gesteld en de afleiding tijdens de vele borrels en diners heeft ons genoeg energie gegeven om te komen tot waar we nu zijn.

Lieve familie, jullie mogen zeker niet ontbreken. **Carrien** en **Pieter**, we zijn helemaal onze eigen weg gegaan en lijken elkaar de laatste jaren steeds meer te vinden. Mijn jeugdjaren hebben me gemaakt tot wie ik ben en daar zijn jullie een groot onderdeel van geweest, iets waar ik enorm dankbaar voor ben. Ik ben trots op jullie. **Papa** en **mama**, dank voor jullie onvoorwaardelijke steun. Ik heb alle mogelijkheden gekregen die ik nodig heb gehad en ik besef me steeds meer hoe waardevol dat is. Dit proefschrift was echt niet mogelijk geweest zonder jullie.

Lieve **Anne**, ik ben zo trots op je. Promoveren tijdens je coschappen, verschillende carrièremoves binnen een paar jaar tijd en zonder na te denken verhuizen naar Eindhoven in coronatijd. Je moet het maar kunnen. We hebben het maar mooi voor elkaar gekregen om op dezelfde dag te promoveren. Onze beide moeders zeiden dat we net zo goed konden gaan trouwen, maar die houden we nog even tegoed. Ik ben dankbaar dat je in mijn leven bent. En dat is echt niet alleen omdat je zulke mooie figuren voor me kon maken met Illustrator of omdat ik je kon gebruiken als pre-reviewer voor alle manuscripten. Nee, elke dag met jou is een feestje. De afgelopen vijf jaar waren een geweldig avontuur en ik ben blij dat we onze plek in Den Haag hebben gevonden. Je bent de leukste die er is.

12.01
2024