

From nociception to perception

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FROM NOCICEPTION TO PERCEPTION: BREAKING DOWN THE PROCESS OF GUT-BRAIN SIGNALLING

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FROM NOCICEPTION TO PERCEPTION: BREAKING DOWN THE PROCESS OF GUT-BRAIN SIGNALLING

PROEFSCHRIFT

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GENERAL INTRODUCTION

Visceral pain & functional gastrointestinal disorders

Pain originating from the internal organs, or visceral pain, is a common sensation in both health and disease. As with somatic pain, visceral pain can vary in intensity, ranging from mild abdominal discomfort to debilitating pain. However, whereas somatic pain tends to be well defined and localized, visceral pain is often described as a diffuse and vague sensation. Moreover, visceral pain often occurs without the presence of tissue damage or inflammation. Increased sensitivity to physiological internal stimuli such as intestinal distension can be the cause of abdominal pain in these cases. The perception of internal bodily processes is called interoception.[10] In most individuals, interoception does not result in conscious sensations. Others have increased interoceptive awareness, and thus become consciously aware of their internal bodily states.[7] When non-noxious (internal) stimuli are perceived as painful, this is referred to as visceral hypersensitivity. The latter is considered a key phenomenon in functional visceral pain.[20]

Chronic visceral pain in the absence of an identifiable organic cause is a hallmark symptom of many disorders traditionally referred to as functional gastrointestinal disorders (FGIDs).[11] Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are prototypical examples, with reported prevalence rates in Western populations of around 10% for both disorders.[28] Irritable bowel syndrome is characterized by lower gastrointestinal symptoms, with altered bowel habits and associated abdominal pain. Functional dyspepsia entails upper gastrointestinal symptoms, with meal-related symptoms (early satiation or postprandial fullness) and/or epigastric pain. The prevalence of these disorders is highest in females, typically presenting at younger age. Symptoms often vary significantly over time, with a natural course of frequent remission and relapse. Although relatively common, the management of chronic visceral pain in functional gastrointestinal disorders remains challenging.[14; 27] The complexity of mechanisms involved in generating the subjective sensation of visceral pain from a stimulus highlights the difficulty of the development of effective treatment strategies.

Innervation of the gastrointestinal tract

The gastrointestinal tract receives dual innervation from vagal and spinal afferents. [21] The vagus nerve innervates the entire gastrointestinal tract with the exception of the distal two thirds of the colon. All respective afferent neurons have their cell bodies located in the nodose ganglia. Sensory information from the gut is subsequently transduced toward the nuclei of the solitary tract (NTS) located in the medulla oblongata. Spinal afferents, which include lumbar splanchnic and sacral pelvic nerves, innervate the distal parts of the colon. The cell bodies are located in dorsal root ganglia and sensory information is transduced to the spinal dorsal horn. Importantly, visceral afferentation is far less dense than somatic, with a high level of divergence within the central nervous system. This is expected to cause the difficulty in localizing visceral pain.[31]

Peripheral input

The generation of visceral nociceptive input primarily depends on molecular transducers located on afferent neurons.[5] Transient receptor potential (TRP) channels constitute a large family of cation channels that serve an important sensory role in many parts of the body, including the gastrointestinal tract. TRP channels respond to a multitude of stimuli, including mechanical, chemical and thermal. Various endogenous and exogenous compounds are known stimuli, with different compounds activating different channels. For example, vanilloid compounds such as capsaicin (the pungent principle of chili peppers), activate transient receptor potential vanilloid 1 (TRPV1). Mustard oil is a known exogenous stimulus of transient receptor potential ankyrin 1 (TRPA1), whereas peppermint oil activates transient receptor potential melastatin 8 (TRPM8).[18] Endogenous agonists include protons (TRPV1), prostaglandins and various cytokines (TRPA1). Moreover, TRP channels can become sensitized upon stimulation, resulting in a stronger activation upon subsequent exposure to stimuli. Increased sensitivity and/or expression of TRP channels on visceral afferents is postulated to result in peripheral hypersensitivity. Studies using rectal balloon distension have provided evidence for such increased pain sensitivity in IBS.[1; 36] On the other hand, experimental studies using rectal mucosal biopsy material have not consistently shown increased expression of TRP channels (including TRPV1), even in cases of proven visceral hypersensitivity.[35]

Inflammation has been identified as the primary cause of sensitization of TRP channels.[30] Much can therefore be learned from intestinal inflammatory conditions such as inflammatory bowel disease (IBD). In contrast to IBS, IBD is characterized by profound inflammation and tissue injury. However, even after the inflammation subsides, patients often maintain abdominal pain symptoms. This is the case in up to one third of patients, who report IBS-like symptoms during remission.[13] Exploring the effect of inflammation on TRP channel functioning is crucial to understanding pain symptoms in these patients. It should be noted that low-grade inflammation has been considered a pathophysiological factor in IBS, but the importance remains a subject of debate.[29]

Nociceptive functioning is also determined by the neuronal microenvironment. This includes the extracellular matrix, one of the essential components of connective tissue.[4] Indeed, previous studies have indicated a link between common hereditary connective tissue disorders known as hypermobility spectrum disorders (HSD), and functional gastrointestinal disorders (including IBS and FD).[15; 16] HSD is characterized by symptomatic joint hypermobility (*i.e.* the capability to move joints beyond normal limits),

and encompasses disorders that can be placed on a continuum between asymptomatic joint hypermobility at one end and hypermobile Ehlers Danlos Syndrome (hEDS) on the other end. All subjects presenting with symptomatic joint hypermobility but not satisfying the diagnostic criteria for hEDS can be defined as HSD. This patient group was previously often referred to as (benign) joint hypermobility syndrome (JHS) or EDS type 3, for which separate but similar diagnostic criteria were used.[8] IBS and FD have been found to be significantly more common in subjects with HSD, as compared to subjects without HSD.[15] Vice versa, HSD was significantly more common in patients with an FGID as compared to patients with an organic GI disorder in one study (39% vs. 27.5%, respectively).[16] The putative effect of HSD on nociception is threefold. First, given the regulatory role of the extracellular matrix in neuronal functioning, it seems likely that connective tissue alterations affect nociception both in the periphery and in the central nervous sytem. Moreover, similar to the increased mobility of joints, the biomechanical properties of the gut could be different as well. As such, changes in the degree of intestinal stretch could have an effect on visceral mechanosensitivity. Lastly, psychopathology including anxiety and depressive disorders is more common among subjects with HSD,[2] further increasing pain susceptibility and the risk of developing chronic pain.

Central processing

After the generation of peripheral input, the nociceptive signal is transmitted to various relay stations before reaching the brain. After reaching the dorsal horn, sensory information travelling through spinal afferents can follow several routes, each integrating the sensory information in a specific manner. Sensory input travelling through the spinothalamic tract reaches the somatosensory cortices, providing the sensory discriminatory aspect. On the other hand, input travelling from the dorsal horn through the spinoreticular and spinomesenphalic tracti reaches the insular and cingulate cortices, giving rise to the affective aspect of visceral pain (see Figure 1.1). In a quantitative meta-analysis of functional brain imaging studies, it was reported that regions implicated in emotional arousal (i.e. the affective component of visceral pain) are more strongly activated in FGID patients.[33] In contrast, healthy controls demonstrate stronger cognitive modulation, thereby inhibiting nociceptive input at the central level.

For the vagus nerve, the primary relay station is the NTS. Few studies have been able to investigate the transmission of nociceptive input from the NTS in humans due to its small size and location within the brainstem. Given that the majority of visceral sensory information travels through the NTS, it is expected that much can be learned from studying this brainstem nucleus and its connections within the brain.



Figure 1.1. Schematic drawing of possible routes of a nociceptive stimulus in the distal colon. (reproduced with permission from Matthews and Aziz)[23]. Abbreviations: pACC, perigenual anterior cingulated cortex; MCC, mid-cingulate cortex.

Gut-brain axis

Communication between gut and brain is considered bidirectional; this is referred to as the gut-brain axis.[25] Many studies have explored the extensive implications of the gut-brain axis in health and disease. Common anecdotal examples entail the feeling of "stomach butterflies" when falling in love, or abdominal discomfort when experiencing psychological distress. Indeed, anxiety has been shown to increase colonic transit, whilst depressive symptoms are associated with constipation.[17] These observations are extremely relevant to understanding pathophysiological mechanisms in FGIDs, as comorbid affective disorders are highly prevalent. Not only can long-lasting abdominal complaints cause affective symptoms, affective symptoms on their turn can result in the perpetuation of abdominal pain. This vicious circle often proves difficult to disrupt in clinical practice. Given the central role of the gut-brain axis in FGID symptomatology, FGIDs are now commonly referred to as disorders of gut-brain interaction (DGBIs), as per the Rome IV consensus in 2016.[24] With the recognition of the gut-brain axis as biological basis for FGIDs, treatments are targeted accordingly.[26] Neuromodulators, for example antidepressants and antipsychotics, are now increasingly being explored as therapeutic options for disorders of gut-brain interaction. Postulated mechanisms

of action include modulation at the level of the affective component of pain (i.e. emotional circuits) or increasing descending modulation of nociceptive signals. [12] Moreover, neurogenesis could potentially reverse structural abnormalities, bringing brain circuits back to their premorbid state if treatment is continued for a sufficient amount of time (e.g. 6-12 months).[3; 6] In a non-pharmacological approach, hypnotherapy, meditation and cognitive behavioral therapy appear to be promising therapeutic options for disorders of gut-brain interaction.[19; 22; 32] Reconceptualizing FGIDs as DGBIs can facilitate the implementation and acceptance of these therapeutic entities both among patients and practitioners and also helps in decreasing the negative connotation associated with the term 'functional' which is too often used as a synonym for psychological comorbidity.

Due to the mixture of sensory, emotional and cognitive input that determines perception, pain is a highly subjective sensation and therefore difficult to measure. Furthermore, no biomarkers for either visceral pain or disorders of gut-brain interaction exist. End-of-day symptom diaries are currently recommended by drug regulatory authorities to assess treatment response in IBS.[9; 34] Daily assessment provides a good overview of symptoms. A diary can be easily incorporated in a smartphone application, making it more user-friendly and data collection more reliable. A disadvantage of endof-day diaries is that they are completed retrospectively, resulting in minor recollection bias. Moreover, once daily assessment does not capture the high temporal variability of symptoms during daytime. These limitations are overcome by the experience sampling method (ESM), which employs random and repeated assessments at multiple time points across momentary states in daily life and thereby provides a detailed overview of symptoms experienced during the day. ESM can also capture symptom triggers, as queries regarding nutrition, environment (current location and company) and positive/negative affect are included. More in-depth understanding of the mechanisms involved in visceral pain can not only increase efficacy of therapies, but also support the development of biomarkers that enable selection of patients for certain therapies. Developing high-quality symptom measurement tools is therefore an important aspect of visceral pain research.

AIMS & OUTLINE OF THIS THESIS

Chronic visceral pain is a common symptom in functional gastrointestinal disorders, now referred to as disorders of gut-brain interaction, yet still poorly understood. Given the important differences with somatic pain, extrapolating earlier findings from somatic fields is insufficient and more focused research regarding visceral pain is necessary. In this thesis, we approach visceral pain mechanisms and abnormalities leading to increased pain sensitivity from both a peripheral and central perspective. To this end, the current thesis is divided into three major parts. The first part focusses on molecular transducers of visceral pain, in particular transient receptor potential channels. The second part explores the association between hypermobility spectrum disorders (i.e. hereditary non-inflammatory connective tissue abnormalities) and visceral pain. The third part describes central pain processing, pain perception and measurement of visceral pain symptoms.

Nociceptors such as transient receptor potential channels are at the starting point of visceral pain. Given the divergent (homeostatic) processes that these channels are involved in, one cannot simply use potent antagonists to prevent visceral pain. In order to fully harness the potential of TRP channels as therapeutic targets, it is necessary to increase our understanding of these intricate receptors. In the first part of this thesis, we explore the functioning and involvement of several important TRP channels in visceral pain. In **chapter 2**, we review current literature regarding the role of TRPV1, TRPV4, TRPA1 and TRPM8 in irritable bowel syndrome. In order to find potential analogies and/or differences in states of chronic intestinal inflammation, we reviewed current literature regarding TRPV1 and TRPA1 in inflammatory bowel disease in **chapter 3**. Finally, several studies have indicated that visceral sensitivity decreases with age. This natural analgesic effect could serve as an example in the development of new treatment strategies. In **chapter 4**, we explored age-related changes in visceral nociception and related alterations in TRP channels.

Abdominal symptoms and functional gastrointestinal disorders are highly prevalent among individuals with hypermobility spectrum disorders. The association between connective tissue disorders and FGID/DGBI should be explored as it offers pathophysiological insights and as such, possible therapeutic targets. Importantly, the mechanisms involved are expected to be at play in both the peripheral and central nervous system given the common facilitating role of connective tissue in neuronal functioning. In chapter 5, we provide a review of current literature on joint hypermobility syndrome/ Ehlers-Danlos hypermobility type (now commonly referred to as hypermobility spectrum disorders). Unfortunately, few studies have been performed to increase our understanding of GI physiology alterations in HSD. Anecdotal evidence has suggested that colonoscopy is more difficult in individuals with HSD, possibly due to laxity of intestinal tissue and increased pain sensitivity. In **chapter 6**, we describe a prospective exploratory study performed at the endoscopy unit, reporting on pain scores during colonoscopy and cecal intubation time in HSD and non-HSD individuals. In chapter 7, we assess characteristics of IBS patients with and without HSD, with a focus on GI symptomatology, affective symptoms and perceived quality of life.

In the third part of this thesis, we report on several studies at the level of central pain processing and perception, i.e. measurement of symptoms in clinical trials. **Chapter 8** describes a neuroimaging study in healthy volunteers using a novel visceral pain model consisting of the duodenal infusion of capsaicin. During infusion, functional magnetic

resonance imaging (fMRI) was used to investigate brain responses, with a particular focus on the nucleus of the solitary tract. Given the inherent methodological difficulties of fMRI, it can be challenging to develop a valid data preprocessing pipeline. In **chapter 9**, we describe the technical aspects of quality of several data cleaning approaches in order to increase signal-to-noise ratio.

The final step after central pain processing entails the experience of pain. Given its subjective nature, high quality measurement instruments are necessary to provide the best representation of patient symptoms. The latter is true for both research as well as clinical practice, as detailed information on symptoms is needed to be able to provide appropriate care. **Chapter 10** describes a comparative study of digital instruments to measure gastrointestinal symptoms.

Finally, we summarize the main findings of this thesis in **chapter 11**, highlighting future implications and possibilities for further research. A schematic overview of various key aspects of visceral pain – forming the outline of this thesis – is provided in Figure 1.2.



Figure 1.2. Schematic overview of the various aspects of visceral pain discussed in this thesis.

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PART I

MOLECULAR TRANSDUCERS OF VISCERAL PAIN

CHAPTER 2

Review article: Transient receptor potential channels as possible therapeutic targets in irritable bowel syndrome

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Aliment Pharmacol Ther. 2017 Nov; 46(10):938-952.

ABSTRACT

Introduction. Abdominal pain in irritable bowel syndrome (IBS) remains challenging to treat effectively. Researchers have attempted to elucidate visceral nociceptive processes in order to guide treatment development. Transient Receptor Potential (TRP) channels have been implied in the generation (TRPV1, TRPV4, TRPA1) and inhibition (TRPM8) of visceral pain signals. Pathological changes in their functioning have been demonstrated in inflammatory conditions, and appear to be present in IBS as well. We here aim to provide a comprehensive review of the current literature on TRP channels involved in visceral nociception. In particular, we emphasise the clinical implications of these nociceptors in the treatment of IBS.

Methods. Evidence to support this review was obtained from an electronic database search via PubMed using the search terms "visceral nociception", "visceral hypersensitivity", "*irritable bowel syndrome*" and "transient receptor potential channels". After screening the abstracts the articles deemed relevant were cross-referenced for additional manuscripts.

Results. Recent studies have resulted in significant advances in our understanding of TRP channel mediated visceral nociception. The diversity of TRP channel sensitization pathways is increasingly recognised. Endogenous TRP agonists, including poly-unsaturated fatty acid metabolites and hydrogen sulphide, have been implied in augmented visceral pain generation in IBS. New potential targets for treatment development have been identified (TRPA1 and TRPV4), and alternative means of affecting TRP channel signalling (partial antagonists, downstream targeting and RNA-based therapy) are currently being explored.

Conclusions. The improved understanding of mechanisms involved in visceral nociception provides a solid basis for the development of new treatment strategies for abdominal pain in IBS.

INTRODUCTION

Irritable bowel syndrome (IBS) is a gastrointestinal disorder characterized by chronic recurrent abdominal pain and alterations in bowel habit. The pathophysiology of IBS is incompletely understood, which poses obstacles in the search for effective therapeutic approaches. While the defecation pattern can generally be managed adequately with pharmacotherapy, abdominal pain tends to be difficult to treat effectively in IBS patients. In the search for new therapeutic strategies, accumulating interest has been given to peripheral mechanisms of nociception as a key target to develop novel analgesics for IBS-related pain. It is now widely accepted that an altered visceral sensitivity through abnormal endogenous pain processing plays an important role in the pathogenesis. This can result both from peripheral and central sensitization processes.[62] By virtue of peripheral sensitization of nociceptive afferents, increased nociceptive discharge can result in the generation of pain symptoms.[61] The responsiveness of these nociceptive afferents or nociceptors, is determined by the expression of specific channels sensing noxious stimuli.[49] The discovery of sensory transducer molecules, including the transient receptor potential (TRP) channel family has opened a new horizon in understanding peripheral nociceptive processes. TRP channels constitute a family of nonselective cation channels. Several members of this family, of which the Vanilloid 1 capsaicin receptor (TRPV1) has been studied most extensively, have been identified to function as integrators and transducers of nociceptive signals in both somatic and visceral pain. However, as nearly all sensory neurons and several non-neuronal cell types express TRPV1, its role is not limited to nociception. TRP channels indeed appear to have a broad spectrum of functions in the human body, a topic that has been reviewed in detail, elsewhere.[50] This review will focus on current knowledge with regards to the potential role of TRP channels in the pathogenesis of pain symptoms in IBS, with particular emphasis on visceral nociception. Specifically, we will summarise their clinical implications and discuss the future of TRP channel targeted therapy.

METHODS

Evidence to support this review was obtained from an electronic database search via PubMed by two of the authors (AB & ZW) using the search terms "visceral nociception", "visceral hypersensitivity", "irritable bowel syndrome" and "transient receptor potential channels". The last date of the search was 21st of July 2017. After screening the abstracts the articles deemed relevant were cross-referenced for additional manuscripts.

IRRITABLE BOWEL SYNDROME PATHOPHYSIOLOGY

Several mechanisms have been hypothesized to play a role in the pathogenesis of IBS, including disturbances in microbiota, low-grade inflammation, immune activation, intestinal barrier dysfunction and altered bile salt absorption. Discussing these mechanisms separately is beyond the scope of this article. A thorough overview is provided in a recent review article.[47] We would like to emphasise that IBS is a heterogeneous disease. Even identical symptoms are likely caused by different processes. [47] Grouping IBS patients on the basis of stool pattern thus promotes heterogeneity, resulting in varying results with different cohorts. This aspect is also relevant when studying the role of TRP channels in visceral pain generation in IBS. Indeed, low-grade mucosal inflammation has been proposed as an important pathophysiological factor in IBS.[88] Researchers have since demonstrated a sensitizing effect of inflammatory mediators on various TRP channels, as will be discussed below. It is important to note that inflammation does not seem to be required to maintain visceral sensitization, as two recent clinical studies investigating the effects of mesalazine in IBS failed to demonstrate any benefits. [9; 71] On the other hand, post-inflammatory sensitization can provide a theoretical explanation for IBS-like symptoms after gastro-enteritis, known as post-infectious IBS, and after achieving endoscopic and biochemical remission in inflammatory bowel disease (IBD). However, it would be inadequate to assume inflammation as the sole driving factor of visceral hypersensitivity. Shortcomings of our current knowledge on TRP channel sensitization should be recognized.

SENSORY INNERVATION OF THE INTESTINE

Nociceptive signalling from the oesophagus to the proximal colon is conducted through the vagal nerve. Information from mid to distal colon and rectosigmoid is carried by the lumbar splanchnic and sacral pelvic nerves. The vagal nerve contains the peripheral terminals of pseudo-unipolar neurons with their cell bodies located in the nodose ganglia. Visceral sensory information from the vagal nerve supply is transduced into the solitary nuclei located in the medulla oblongata. The splanchnic and pelvic nerves contain axons of pseudo-unipolar neurons arising from a dorsal root ganglion (DRG). Peripheral sensory information from this supply is transduced into the dorsal horn of the spinal cord and ascends via the spinothalamic tract.[18] Peripheral nociceptive signalling is extensively modulated by the central nervous system, resulting in suppression or augmentation of the nociceptive input. These central processes determine whether nociceptive signalling (sensing and transmitting noxious stimuli) is perceived as pain (unpleasant experience).[38]

Sensory afferents of the vagal and spinal nerves have previously been divided in different subclasses.[21] Based on their sensitivity to mechanical stimuli, afferents

were divided in mucosal, muscular, serosal and mesenteric fibre classes.[61] Mucosal afferents were defined as responsive to fine tactile and chemical stimuli, whereas serosal and mesenteric afferents respond to noxious mechanical stimuli.[62] Sensing of intermediate (physiological) distension was attributed to muscular afferents. An additional class specific to the pelvic pathway, the muscular-mucosal class, responds to tactile and distension stimuli. It should be noted that evidence supporting the suspected anatomical distribution described above is currently lacking. Song et al. have attempted to morphologically identify specialised afferent axonal structures in the guinea pig intestine.[92] They were able to demonstrate mechanosensitive fibres in the mesenteries and preparations of isolated mucosa/submucosa of the ileum and colon. However, no mechanosensitive fibres were observed in preparations of isolated muscle layers (with intact myenteric ganglia and serosa). Afferent functioning therefore appears to depend on molecular characteristics rather than the location within the gut wall.[12; 62] Functional

Channel	Implications in IBS	Study type	Reference
TRPV1	Sensitized and/or upregulated in colonic tissue samples of IBS patients, resulting in enhanced capsaicin sensitivity.	Human studies	[2; 60; 101; 106; 110]
		Animal study	[72]
	Sensitized by inflammatory mediators.	Combined study	[105]
	Expression profiles regulated epigenetically,	Human study	[110]
	likely influenced by psychological stress through glucocorticoids and/or catecholamines.	Animal studies	[51; 52]
	Indirectly involved in mechanosensation.	Animal study	[13; 20]
TRPV4	Elevated levels of endogenous agonist 5,6-EET in the supernatant in of colonic biopsies from IBS-D patients.	Human study	[29]
	Sensitized by inflammatory mediators.	Animal studies	[26; 27; 90]
	Putative direct mechanosensitive nociceptor in humans.	Human study	[76]
TRPA1	Functional coupling with TRPV1.	Animal study	[3]
	Activated by hydrogen sulphide, present in IBS-D patients with small bacterial overgrowth.	Human study	[8]
	Sensitized by inflammatory mediators.	Animal studies	[66; 102]
	Likely to act as a directly mechanosensitive nociceptor	Animal studies	[19; 20]
	in hyperalgesia.	Human study	[109]
TRPM8	Inhibits chemo- and mechanosensory responses of TRPA1 and TRPV1.	Animal study	[42]
	Potentially protective against nociceptor sensitization through anti-inflammatory effects.	Animal study	[87]
	TRPM8 polymorphisms are associated with slower colonic transit and an increased risk of IBS-C and IBS-M in humans.	Human study	[45]

Table 2.1. Data on TRP channels in IBS

differences in nociceptor transducer molecules and their divergent expression along sensory afferents determine the physiological role of these afferents.[12] Understanding the functioning of individual TRP channels may provide further insights into nociceptive processes and sensitization mechanisms. Below we will discuss in detail the TRP channels that have been identified as key players in visceral nociception. These include TRPV1, TRPV4, TRPA1 and TRPM8. An overview of current data on these channels and their implications in IBS is provided in Table 2.1.

TRP channel regulation

In order to understand TRP channel functioning, one must be aware of the complex molecular modulation that these channels are subjected to. Modulatory processes can either result in sensitization or desensitization of the respective afferent.[83] While desensitization prevents nociceptive signaling, sensitization of nociceptors enhances their discharge (i.e. potentiates the nociceptor response to a second stimulus).

Several mechanisms are involved in TRP channel regulation.[83] First, gene expression can be altered through DNA methylation, resulting in gene silencing. Second, posttranslational modifications (e.g. phosphorylation and dephosphorylation) affect channel functioning. Phosphorylation cascades can be initiated by various sensitizing agents (discussed below). Depending on the agent, different phosphorylation pathways are involved (e.g. protein kinase A, protein kinase C, calmodulin-dependent kinase).[54] In contrast, dephosphorylation reduces TRP channel sensitivity to stimuli. Finally, TRP channels can be degraded by movement to intracellular lysosomes,[57] or mobilized from intracellular pools to the cell membrane (translocation).[54] All of these processes are kept in balance in physiological conditions, but can be disrupted in disease. Currently, we are only beginning to understand the role of these modulatory processes in IBS. Considering our growing knowledge on TRP channels in visceral pain, future studies may focus on this unexplored field to guide treatment development.

TRPV1

Of all TRP channels, TRPV1 has been studied most extensively. Studies investigating the expression patterns of TRPV1 in mice have demonstrated the channel's presence along the entire gastrointestinal tract.[108] Although human studies are more scarce, the expression of TRPV1 in the oesophagus and colon is now well documented, and the channel is suspected to be present in the human small intestine as well.[100] Immunostaining of human colon biopsies has demonstrated TRPV1-positive fibres throughout the mucosa, with a particular abundance in the submucosal plexus.[2; 101] Activation of these fibres by noxious stimuli results in action potential generation and pain sensation. TRPV1 is activated by noxious heat (> 42 °C), protons (pH < 6) and the vanilloid capsaicin, the pungent principle in hot peppers.[101] In addition, several

Channel	Exogenous agonists	Physical stimuli	Endogenous agonists
TRPV1	Capsaicin (red pepper), polygodial (mountain pepper), piperine (black pepper), gingerol (ginger), olvanil, resiniferatoxin, camphor, diphenylboronic anhydride, double-knot toxin (DkTx), vanillotoxin (tarantula toxin), phenylacetylrinvanil, 2-aminoethoxydiphenyl borate (2-APB), evodiamine, cannabidiol, cannabigerol	(Thermal >42°C)	Acid (pH < 6), lipoxygenase products (e.g. 12-(S)- hydroperoxyeicosatetraenoic acid (12S-HPETE), 15-(S)- hydroperoxyeicosatetraenoic acid (15S-HPETE), leukotriene B4 (LTB4), 5-(S)-hydroxyeicosatetraenoic acid (5S-HETE)), reactive oxygen species (ROS), adenosine, ATP, lysophosphatidic acid, polyamines (e.g. spermine, spermidine, and putrescine) and conjugates of biogenic amines (e.g. N-arachidonylethanolamine (anandamide), N-arachidonoyldopamine, N-oleoylethanolamine (OLEA), N-arachidonolylserine, N-hexadecanamide, and various N-acyltaurines and N-acylsalsolinols)
TRPV4	Bisandrographolide A (BAA), alpha-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (4α-PDH), apigenin, GSK1016790A and RN1747	Mechanical and thermal (>24°C)	Citric acid, dimethylallyl pyrophosphate, anandamide, arachidonic acids and epoxyeicosatrienoic acid metabolites (e.g. 5,6-epoxyeicosatrienoic acid (5,6- EET) and 8,9-EET, which also mediate TRPV4 activation by cell swelling)
TRPA1	Allyl isothiocyanate (AITC), cinnamaldehyde (cinnamon), allicin (garlic), carvacrol and thymol (oregano), curcumin (turmeric), capsiate (capsinoid), acrolein, menthol, icilin, nicotine, URB597, chlorobenzylidene malononitrile (tear gas), formalin, α,β -unsaturated aldehydes, auranofin, PF-4840154, cannabichromene, cannabidiol (CBD), tetrahydrocannabinol (THC) and apomorphine (agonist in low micromolar range and antagonist in higher concentration)	Mechanical and thermal (<15°C and >25°C)	Prostaglandins (e.g. prostaglandin A1, 8-iso-prostaglandin A2, and 15-deoxy- Δ-prostaglandin J2), 4-Hydroxnonenal (4-HNE, a lipid peroxidation product), 4-oxononenal (4-ONE), methylglyoxal, reactive oxygen species (ROS), cytokines (e.g. TNF-α and IL-6), bradykinin and hydrogen sulphide
TRPM8	Menthol, icilin, linalool, geraniol, WS-3, WS-12, WS-23, PMD38, hydroxycitronellal, FrescolatMGA, FrescolatML, CoolactP, Cooling Agent 10, cis- and trans-p- menthane3 and CPS-36	Thermal (<28°C)	Unknown

Table 2.2. Identified agonists and physical stimuli of TRP channels discussed in this paper.[4; 11; 23; 56; 57; 103]



Figure 2.1. Schematic depiction of nociceptive afferent innervation of the intestine. Proximal (blue) neurons travel through the vagal nerve. These neurons transduce sensory information through the nodose ganglia into the solitary tract nuclei. Distal (red) neurons travel through the splanchnic and pelvic nerves (two distinct systems). Both the splanchnic and pelvic nerves' somata reside in the dorsal root ganglia. In addition, splanchnic nerves travel through prevertebral ganglia (not shown). The nociceptive afferents within these neurons presumably have their nerve endings (top inset) in the mucosa/submucosa and mesenteries. [92] Various stimuli (shown at the top) can activate the nociceptors, depending on the expressed TRP channels (see Table 2). Stimuli include exogenous agonists (e.g. capsaicin or menthol), physical stimuli (e.g. mechanical or thermal) and endogenous agonists (e.g. prostaglandins or lipoxygenase products). Activation of nociceptors by these stimuli results in action potential generation and pain sensation. In addition, inflammatory mediators are released (neurogenic inflammation), which can result in TRP channel sensitization and local tissue inflammation.

NG = nodose ganglion, STN = solitary tract nucleus, DRG = dorsal root ganglion, S = splanchnic nerves, P = pelvic nerves

compounds have been identified as endogenous agonists. These include inflammatory mediators such as lipoxygenase products and prostaglandins, and endocannabinoids such as anandamide (see Table 2.2). Furthermore, TRPV1 seems to be involved in afferent signalling of mechanical stimuli,[49] but its exact mechanism is still poorly understood. Whether the mechanosensory properties of TRPV1 are related to indirect effects on neuronal excitability or interactions with other TRP channels, remains to be established.[13]

The use of potent chemical activators such as capsaicin has provided valuable information on the functioning of TRPV1-expressing afferents. Upon activation, in addition to the generation of an action potential, these sensory afferents release proinflammatory sensory neuropeptides; calcitonin-gene related peptide (CGRP) produces local vasodilation and substance P (SP) increases venular and capillary permeability leading to plasma protein extravasation and oedema formation, collectively referred to as neurogenic inflammation.[96] TRPV1-expressing sensory neurons can therefore influence GI vascular, immune and smooth muscle function, as well as sensitize surrounding nociceptors.[49] Under physiological conditions, these effects are counteracted by the anti-inflammatory effects of somatostatin, which has also been shown to be released by capsaicin-sensitive afferents.[44] Sustained disruptions in the balance of these proand anti-inflammatory neuropeptides may result in the pathological sensitization of nociceptive afferents, as well as local tissue inflammation (see Figure 2.1). Importantly, these processes do not seem to be limited to TRPV1, but also apply to the other TRP channels discussed in this review.

Sensitization of TRPV1-expressing afferents has been demonstrated in IBS patients by increased perceptive responses to capsaicin in multiple studies.[40; 89; 101] Gonlachanvit et al. demonstrated that diarrhoea predominant IBS (IBS-D) patients experience greater abdominal burning after a single ingestion of a spicy meal or standard meal in combination with a capsaicin capsule, compared to healthy controls.[40] As symptoms developed within one hour after ingestion, proximal gut hypersensitivity to capsaicin was suggested to exist in these patients. Schmulson et al. showed a significantly decreased rectal pain threshold in IBS patients after a 7-day chilli rich diet compared to a diet without chilli, suggesting TRPV1-induced visceral hyperalgesia.[89] More recently, van Wanrooij et al. studied the effects of rectal capsaicin application in IBS patients.[101] Patients reported increased pain intensity, a similar effect lacking in healthy volunteers. Furthermore, the pain response appeared to be independent of anticipatory anxiety, suggesting a direct capsaicin effect on nociceptive mucosal afferents.

Mechanisms underlying the increased capsaicin sensitivity in IBS patients have been studied extensively. Akbar et al. demonstrated upregulation in sigmoid mucosal samples of IBS patients.[2] This increase correlated with symptom severity, suggesting that an increase in afferent discharge through TRPV1 activation might be directly related to pain symptom generation. Our earlier study corroborated these findings, demonstrating increased transcription of TRPV1 in IBS patients, which also strongly correlated with symptom severity.[60] More recently, two studies confirmed the augmented expression of TRPV1 in colonic biopsies of IBS-D patients.[106; 110] It should be noted that the overall density of innervation has been shown to be increased in IBS patients. Increased TRPV1 sensitivity may therefore be due to axonal sprouting rather than isolated TRPV1 upregulation, possibly as a result of increased nerve growth factor expression. [55; 106] On the other hand, Van Wanrooij et al. were unable to objectify increased numbers of mucosal TRPV1 in colonic biopsies of IBS patients, as compared to healthy controls.[101] Even when the IBS patient group with visceral hypersensitivity (defined by decreased discomfort threshold during rectal distension) was analysed separately, no significant upregulation of TRPV1 was found. The question whether increased capsaicin responsiveness in IBS relates to individual TRPV1 sensitivity or TRPV1 expression thus remains without a decisive answer.

TRPV1 functioning in (post-)inflammatory conditions

As discussed above, the mediators of neurogenic inflammation are known to sensitize TRPV1. In addition, systemic inflammatory mediators have been shown to be involved in both sensitization and activation of TRPV1 (see Table 2.2).[48] Their potential relevance to IBS pathophysiology is evident, as many have postulated a role for subclinical inflammation in IBS.[47] However, inflammation has been argued to be within the physiological range in IBS.[88] Moreover, a study measuring poly-unsaturated fatty acids in colon biopsy material from IBS-D patients and healthy controls, failed to demonstrate differences in concentrations of lipoxygenase products (TRPV1 agonists). [29] It is possible that the role of inflammatory mediators in TRP channel sensitization is limited to post-inflammatory hyperalgesia, as encountered in post-infectious IBS and IBD in remission. Animal studies have provided evidence for TRPV1 mediated post-inflammatory hyperalgesia, using experimental colitis models induced by dextran sodium sulphate. After recovery from colitis, TRPV1 deficient mice showed no pain-related behavioural responses or increased visceromotor responses to colorectal distension, whereas these responses were readily observed in wildtype mice.[72]

Another explanation for inflammation mediated hyperalgesia in IBS could be related to histamine. Barbara et al. observed increased numbers of mucosal mast cells in close proximity to sensory nerves in colon biopsies of IBS patients,[10] and these findings have been confirmed in a more recent study.[106] Moreover, Wouters et al. demonstrated an increased Ca²⁺ response and increased number of responding neurons to capsaicin in histamine pre-treated biopsy specimens of healthy volunteers.[105] Immunostaining showed co-expression of histamine receptor H1 (HRH1) and TRPV1 on submucosal neurons in both IBS patients and healthy controls. A functional coupling of these receptors therefore appears likely. In a proof-of-concept trial, the same study group demonstrated a significant decrease in abdominal pain scores in IBS patients after 12 weeks of treatment with the HRH1 antagonist ebastine, as compared to placebo. Unfortunately, not all patients reported pain relief, emphasising the heterogeneity of the IBS patient population.

Chronic stress and epigenetics

IBS is often described to be a disorder of the gut-brain axis. In this model, psychological stress is generally accepted as a key factor influencing GI symptoms and vice versa. Importantly, animal models have suggested both glucocorticoid- and catecholamine-mediated TRPV1 upregulation.[52; 112] In addition, epigenetic mechanisms may regulate the effects of chronic stress on TRPV1 expression. Increased histone acetylation of the TRPV1 promoter has been demonstrated in chronic stress models in rats, resulting in TRPV1 upregulation in DRG derived neurons.[51] Furthermore, the epigenetics of visceral pain perception have been investigated in diarrhoea predominant IBS patients.[110] Two miRNAs known to decrease TRPV1 expression, miR-199a and miR-199b, were found to be significantly downregulated and shown to correlate with pain scores. Taken together, these results indicate epigenetic alterations, possibly under the influence of psychological stress, modulate TRPV1 functioning in IBS.

Activation of sensitized nociceptors

Currently identified endogenous agonists of TRPV1, as well as the other TRP channels discussed in this review, are primarily related to inflammation (see Table 2.2) . As already noted, (subclinical) inflammation is not the sole underlying mechanism in IBS. Furthermore, it is unknown whether the concentrations of endocannabinoids known to activate TRPV1 in vitro are high enough in vivo in order to achieve activation.[64] This constitutes a significant gap in our knowledge of peripheral nociception in IBS, as it remains unclear what stimuli ultimately activate sensitized nociceptors in vivo. Although capsaicin is a common dietary constituent, it is unlikely to be a major factor in abdominal pain generation in IBS. Current understanding of intestinal signalling suggests that nociceptive signals are generated by exciting sensitized nociceptors as a result of mechanical stimulation or distension. These mechanical stimuli could be related to physiological motor responses of the intestine.[88] High amplitude colonic contractions have been shown to be of a magnitude above nociceptive thresholds in visceral hypersensitivity. Therefore, mechanical stimuli generated by the gut itself may be responsible for the generation of pain symptoms through sensitized nociceptors.

TRPV4

Studies investigating expression patterns of Transient Receptor Potential Vanilloid 4 (TRPV4) in the human colon have demonstrated immunoreactivity in the submucosa and serosa.[36] Initially termed vanilloid receptor-related osmotically activated channel (VR-OAC), this channel has been implicated in the detection of osmolarity changes.[74] In addition, TRPV4 is now known to sense strong acidosis, temperatures >24°C and, among others, the synthetic phorbol ester alpha-phorbol 12,13-didecanoate (4 α PDD) (see Table 2.2). Currently identified endogenous agonists include anandamide and the poly-unsaturated fatty acid metabolites 5,6-epoxyeicosatrienoic acid (5,6-EET) and 8,9-EET.

Accumulating evidence points toward a role of TRPV4 in mechanosensation. [49; 76] Under basal conditions, TRPV4 is thought to primarily sense high threshold mechanical stimuli.[26] Comparing TRPV4 knockout and wildtype mice, responses to noxious distension pressures were diminished in TRPV4 knockouts. In contrast, responses did not differ at innocuous pressures. TRPV4 is however considered to play a major role in visceral hypersensitivity.[26; 90] Intracolonic administration of 4α PDD has been shown to induce hyperalgesia in mice. [26] In IBS, several pathways have been proposed to result in visceral hypersensitivity through TRPV4 sensitization. The effects on TRPV4 of known mediators of visceral hypersensitivity, serotonin and histamine, were investigated in one study.[27] Serotonin and histamine administration was demonstrated to result in potentiated TRPV4 responses to 4α PDD. The same research group postulated a role for proteases in mediating visceral hypersensitivity. [28] Subsequent studies have supported this theory.[26; 90] Activation of Protease-Activated Receptor (PAR₂), a channel that is also coexpressed with TRPV4 in afferents innervating the colon, resulted in visceral hyperalgesia in wildtype mice. Because hyperalgesia was lacking in TRPV4 knockout mice, this channel was suspected to be the downstream effector of PAR, mediated visceral hypersensitivity.[26]

Human studies on visceral TRPV4 functioning are currently limited. In one study researchers acquired supernatant of colonic biopsies from IBS-D patients.[29] Intracolonic administration of the supernatant resulted in visceral hypersensitivity in mice. This was concluded to be TRPV4 mediated, as injection of TRPV4 targeted small interfering RNA prevented the effect. Subsequently, potential TRPV4 agonists in the supernatant were quantified. The concentration of 5,6-EET was found to be significantly elevated and correlated with patients' abdominal pain severity and frequency. Interestingly, 5,6-EET production was linked to PAR₂ activation, as PAR₂ agonist peptide induced 5,6-EET synthesis in sensory neurons. The authors therefore suggested 5,6-EET to be an endogenous TRPV4 agonist with a major role in visceral hypersensitivity in IBS-D patients. No significant differences in poly-unsaturated fatty acid metabolite concentrations in supernatants have been observed in constipation predominant IBS (IBS-C) patients, mixed bowel habit IBS (IBS-M) patients and healthy volunteers.

TRPA1

To date, our knowledge on Transient Receptor Potential Ankyrin 1 (TRPA1) functions in visceral nociception is mostly limited to animal models. TRPA1 is thought to primarily act as a chemosensor, responding to various irritants and spices, among others cinnamaldehyde (cinnamon) and allyl isothiocyanate (AITC), the pungent compound in mustard oil, horseradish and wasabi.[49] Currently identified endogenous agonists include prostaglandins and products of oxidative stress (see Table 2.2). Furthermore, TRPA1 has been implicated in temperature sensation, although its responsiveness has long been debated. Recently, a U-shaped temperature-activation curve was demonstrated using human TRPA1 in lipid bilayer and whole-cell patchclamp recordings.[79] This study group observed TRPA1 activation in temperatures below 15 degrees Celsius, as well as temperatures above 25 degrees, but little activation to temperatures in between.

In addition to the above, TRPA1 has been studied intensively for its suspected mechanosensitive properties. It appears to have no role in sensing high pressure distension under basal conditions.[25] However, TRPA1 has been demonstrated to be an important mediator of visceral hyperalgesia.[19; 20] Several animal studies have indicated that the mechanical stimulus threshold can be decreased upon chemical activation with mustard oil. These findings have recently been confirmed in an ex vivo study using human colonic tissue.[109] Moreover, TRPA1 can be sensitized via PAR₂ activation. Similar to TRPV4, TRPA1 may therefore be one of the effectors of protease mediated visceral hypersensitivity.

Several characteristics of TRPA1 add to the complexity of its functioning. TRPA1 is almost exclusively expressed in TRPV1-positive neurons. Both channels have been shown to interact with eachother.[69] Similar to TRPV1, TRPA1 can be desensitized upon repeated stimulation.[3] In addition, TRPA1 activation can be reduced upon repeated capsaicin application, a process referred to as cross-desensitization.[3] Indeed, Brierley et al. showed that TRPA1 knockout mice were responsive to capsaicin, but lacked mechanical desensitization that normally follows afterwards.[20] Thus, whereas TRPV1 is responsible for the direct response to capsaicin, the subsequently reduced mechanosensory function appears to be TRPA1-mediated. These results are in line with the general belief that TRPV1 itself is not directly mechanically gated,[13] as the reduced response to mechanical stimuli upon chemical desensitization of TRPV1 relies on TRPA1.

TRPA1 functioning in (post-)inflammatory conditions

TRPA1 expression has previously been investigated in inflamed human colonic tissue, showing upregulation in IBD patients with active inflammation.[69] Evidence explaining the role of TRPA1 in inflammation however remains contradictory, with reports of both pro- and anti-inflammatory effects. Although its effect on inflammation is enigmatic, TRPA1 itself is undoubtedly affected by inflammatory mediators. Indeed,

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the endogenous agonists of TRPA1 are related to inflammation (see Table 2.2). Furthermore, studies investigating the effects of chemically induced colitis in mice demonstrated sensitization of visceral afferents to mechanical stimuli. These effects were observed in wildtype mice, but not in TRPA1 deficient mice.[25; 66; 102]

Since TRPV1, TRPV4 and TRPA1 have all been shown to be involved in inflammation induced visceral hyperalgesia, one could assume that combined sensitization of these channels provides a particularly potent mechanism to induce hypersensitivity. Synergistic effects of TRPV1 and TRPA1 inhibition have indeed been demonstrated in the attenuation of colorectal distension-associated pain behaviour at high pressures in rats.[102] Unfortunately, this concept has not yet been proven in IBS. Cenac et al. have measured endogenous agonists of TRPA1, TRPV1 and TRPV4 (primarily inflammatory mediators) in the supernatant of colon biopsy material of IBS patients.[29] They demonstrated elevated levels of endogenous agonists of TRPV4, but not TRPA1 or TRPV1. Another study involved peripheral blood mononuclear cell supernatants from IBS-D patients.[56] The supernatants were shown to induce mechanical hypersensitivity in vitro in colonic afferent neurons. Cytokine concentrations in the supernatants were subsequently measured, showing elevated levels of TNF- α , soluble IL-2, IL-6, IL-10, IL-1 β and the chemokines CCL3 and CCL4. Combined with the expression profiles of the receptors of these signalling molecules in colonic nerves, the authors proposed TNF- α , IL-6, IL-10 and IL-1 β as possible mediators of mechanical hypersensitivity in IBD-D. The mechanism of action of TNF- α was demonstrated to be TRPA1 dependent, as its sensitizing effect was abolished in the presence of a TRPA1 antagonist. In contrast, the selective inhibition of TRPV1 using low doses of capsazepine had no effects on mechanical hypersensitivity induced by TNF-a. In addition, a more recent study by the same group demonstrated IL-6 mediated mechanical hypersensitivity to be TRPA1 dependent as well.[23] Whether an interaction of sensitized TRP channels plays an important role in IBS thus remains to be elucidated.

Future studies should include different approaches covering the diversity of potential pathophysiological mechanisms in IBS. For example, elevated levels of hydrogen sulphide, an endogenous TRPA1 agonist, were recently demonstrated in IBS-D patients with small bacterial overgrowth.[8] These findings demonstrate that mechanisms of TRP channel sensitization may vary among patients.

TRPM8

Transient Receptor Potential Melastatin 8 (TRPM8) appears to be one of the least studied TRP channels in humans. Only very recently TRPM8 polymorphisms have been demonstrated to be associated with slower colonic transit and an increased risk of IBS-C and IBS-M in humans.[45] However, data on TRPM8 functioning is mainly based on animal studies, limiting our understanding of its role in visceral pain generation. Our current knowledge of TRPM8 is that it has a role in thermosensation (primarily low temperatures).[77] Several chemical compounds are able to activate TRPM8, among others menthol and icilin. Many will acknowledge that the sensation of mentholated liniments is difficult to describe. Cold and burning perceptions alternate upon application. Because of these opposing sensory inputs, menthol is thought to also activate channels other than TRPM8. Indeed, some authors have pointed to menthol-induced TRPA1 activation in order to explain the diverse psychophysical sensations after topical application of menthol.[58] Moreover, coupling of TRPM8 to both TRPV1 and TRPA1 has been demonstrated previously.[42] As mentioned above, AITC is able to cause mechanical hypersensitivity through TRPA1 activation. This effect, however, does not occur after pretreatment with icilin. In contrast, icilin-induced mechanical desensitization is absent in TRPA1 deficient mice, indicating that the effect was TRPA1 mediated. Likewise, capsaicin is able to cause mechanical desensitization, but not after icilin pre-treatment.[42] TRPM8 is therefore thought to inhibit chemo- and mechanosensory responses of TRPA1 and TRPV1, and thus provide antinociceptive effects through cross-desensitization.

In addition, visceral TRPM8 is thought to have a role in inflammation. Human studies have revealed increased TRPM8 expression in colonic biopsy material from IBD patients as compared to healthy controls.[87] Several experimental colitis models in mice have suggested protective effects of TRPM8 activation. In these studies, icilin treatment significantly attenuated induced colitis in wildtype mice, but not in TRPM8 deficient ones.[33; 53; 87] The protective effects of TRPM8 activation have been linked to CGRP, which co-localizes with TRPM8 in the human colon.[33] Although the pro-inflammatory effects of CGRP in neurogenic inflammation are evident, primarily consisting of vasodilation, anti-inflammatory effects of the neuropeptide have been observed as well.[41; 99] De Jong et al. demonstrated expression of the components of the CGRP receptor, calcitonin receptor-like receptor (CLR) and receptor activity modifying protein-1 (RAMP1), on CD11c+ dendritic cells in the murine spleen.[33] CGRP knockout mice were shown to have higher levels of pro-inflammatory cytokines (including TNF- α and IL-6) in chemically induced acute colitis, as compared to wildtype mice. Moreover, CD11c+ dendritic cells were found to be co-localized with CGRP positive fibres in the murine colon. CGRP was therefore thought to exert a protective role in colitis via inhibition of the release of pro-inflammatory cytokines, through interaction with local dendritic cells. Indeed, TRPM8 deficient mice showed significant improvement in disease activity after treatment with recombinant CGRP, whereas no effect of treatment was observed in wildtype mice with induced colitis. Paradoxically, enhanced CGRP expression levels have been observed in mucosal fibres of TRPM8 deficient mice.[87] This discrepancy has yet to be clarified, although it is likely that a disrupted colonic CGRP release prevents the neuropeptide from reaching its effector. Taken together, these results suggest that TRPM8 upregulation is a protective mechanism aimed at mitigating tissue inflammation. Therefore, TRPM8 could theoretically protect against nociceptor sensitization by inflammatory mediators.
NON-NEURONAL TRP CHANNEL EXPRESSION

As already mentioned, TRP channels are expressed by a multitude of cell types including those of non-neuronal origin (both intestinal and extra-intestinal).[35] Examples of non-neuronal cells expressing TRP channels include vascular smooth muscle cells, endothelial cells, keratinocytes and intestinal epithelial cells. The possible involvement of the latter in IBS pathophysiology should not be overlooked. Increased intestinal permeability has been demonstrated in IBS-D patients, [80] and has been associated with visceral hypersensitivity.[111] In two studies, TRPV4 activation with 5,6-EET and 4αPDD resulted in increased intestinal permeability.[32; 107] One of the proposed mechanisms was via downregulation of tight junction proteins. However, conflicting results have been obtained for the role of TRPV1 in regulating intestinal permeability. Capsaicin has previously been shown to increase permeability. In contrast, the endocannabinoid-like compound oleoylethanolamine has been shown to be able to both increase and decrease intestinal permeability via TRPV1.[59] Similarly, the role of TRPA1 in the regulation of intestinal permeability remains controversial. Fothergil et al. demonstrated decreased trans-mucosal resistance in colon tissue from mice after AITC and cinnamaldehyde administration.[37] We demonstrated no effects on small intestinal permeability with the administration of cinnamaldehyde in twelve healthy controls.[63] It therefore remains unclear to what extent TRP channels contribute to IBS pathophysiology via altering intestinal permeability.

MOTILITY EFFECTS OF TRP CHANNEL ACTIVATION

Although we here focus on the role in visceral nociception, it should be noted that TRP channels are known to affect gut motor function as well.[13] The effects of TRP channel activation on motility are not only channel dependent, but also location dependent. For example, TRPV1-positive fibres located in the lower oesophageal sphincter that are exposed to gastric acid cause a local inhibitory reflex, lowering the intraluminal pressure.[14] On the other hand, application of capsaicin in the distal colon and rectum in mice has been shown to cause fast transient colonic contractions followed by a delayed sustained contraction.[75] These results indicate that different effector pathways are involved depending on the location. Suggested effectors are the tachykinin receptors (mainly NK1 and NK2), which respond to neuropeptides released upon TRP channel activation, as discussed above.[30] Indeed, the contractility lowering effect in the oesophagus was demonstrated to be NK1 dependent via local substance P release, whereas NK2 activation was shown to be responsible for long lasting contractility in the distal colon.[14; 75] Additional pathways are likely to be involved however, as fast transient colonic contractions were shown to be inhibited by NK1 antagonists. Other TRP channels have also been implicated in motility. Whereas TRPV4 has

previously been demonstrated to inhibit colonic motility in mice via reduced NOdependent calcium release, TRPA1 has been implicated in both reduced and increased motor activity.[36; 85; 91] Taken together, the effects on gut motility emphasise the involvement of TRP channels in IBS pathophysiology, as they may account for both the altered defecation pattern as well as pain symptoms encountered in the syndrome.

CLINICAL IMPLICATIONS

Although all of the TRP channels discussed above are suggested to have a role in visceral pain generation, only two (TRPV1 and TRPM8) have been implied in the treatment of IBS. Generally, two strategies are exploited in blocking TRP channels. One is the direct inhibition by administration of antagonists, the other one by repeated

	TRPV1	TRPV4	TRPA1
Antagonists	Potent analgesics, but thermoregulation interference with first generation compounds[39; 93; 95]	Reduction of human nociceptor mechanosensitivity ex vivo[76]	Tested in neuropathies with good safety profiles[86]
	Modality-selective antagonists currently being developed[73]		Several antagonists currently evaluated in clinical trials[86]
Desensitization	Six weeks of chilli treatment effective in IBS-D patients, but short-term adverse effects may limit adherence[6]	Evidence currently lacking	Repeated TRPA1 stimulation results in desensitization[3]
Cross- desensitization	Inhibition of chemo- and mechanosensory responses with peppermint oil*	Inhibition of chemo- and mechanosensory responses with peppermint oil*	Reduced mechanosensitivity through repeated capsaicin administration[3]
Downstream targets	NK1 antagonists may provide anti-hyperalgesic effects[70]	Evidence currently lacking	Evidence currently lacking
	Improved abdominal pain and stool pattern in female IBS-D patients with NK2 antagonist ibodutant[98]		
	Somatostatin analogues may provide anti-hyperalgesic effects[17; 43; 81; 84]		
	Improved abdominal pain scores in IBS patients with HRH1 antagonist ebastine[105]		
RNA-based therapy	Plausible[94]	Plausible[94]	Plausible[94]

Table 2.3. Summary of therapeutic strategies in relation to the TRI	P channels discussed in this paper.
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*Effects mediated by TRPM8 activation[42]

stimulation in order to desensitize the channel and its respective nerve terminal. In addition, several alternative techniques for TRP channel targeted therapy have been developed, that will be discussed hereafter. A summary of the optional therapeutic strategies related to each TRP channel is provided in Table 2.3.

TRP antagonists

The discovery of TRPV1 as a key player in nociception led to the development of TRPV1 antagonists as novel therapeutics in pain control. However, investigators soon encountered a major hurdle, as the first compounds interfered with thermoregulation. Several first-generation compounds were stranded in pre-clinical trials as they elicited marked hyperthermia. [93; 95] Moreover, interspecies differences in TRPV1 functioning further complicated research. While one TRPV1 antagonist appeared to be safe in animals, its first human clinical trial was prematurely halted as hyperthermia was observed in three out of four patients.[39] Interestingly, while TRPV1 has been identified as a thermosensor, its temperature threshold is well above physiological body temperature. It is possible that adverse effects are not TRPV1 related and represent an off-target effect. Additionally, classical TRPV1 antagonists impair the noxious heat sensation and may therefore increase burn risk. Together, these results disfavoured the development of non-selective TRPV1 antagonists. Therefore, new strategies are being explored in order to tackle thermoregulation related adverse events in TRPV1 targeted therapy. Several second generation modality specific antagonists have been developed, as it has been observed that hyperthermia is less severe with compounds that are full antagonists for capsaicin, but not for protons.[15] Two recent phase-I trials reported no side effects of two modality-selective TRPV1 antagonists.[1; 7] Their potency as analgesics however remains to be established in future studies.

Desensitization

The desensitizing properties of TRPV1 agonists such as capsaicin have been exploited for many years by topical preparations in the treatment of neuropathic pain.[34]

At this moment, only one small randomized crossover study investigated the effects of six weeks of chilli treatment in IBS-D patients.[6] At the end of treatment, patients reported significantly decreased post-prandial abdominal burning sensations. Similar positive effects have been demonstrated in epigastric pain in patients with functional dyspepsia.[16] An important drawback of capsaicin therapy however, is its short-term aggravating effects, possibly limiting adherence in the clinical setting. Therefore, more studies are needed to evaluate efficacy and feasibility in a larger IBS population. In addition, it remains to be established at which dose, frequency and length of administration repeated capsaicin is able to, if all, induce desensitization of TRPV1-positive nerve endings in the gut.

Ultra-rapid desensitization using potent agonists offer a theoretical background for achieving a fast analgesic response. One of these ultra-potent TRPV1 agonists is resiniferatoxin, which causes sustained calcium influx, resulting in a cytotoxic intracellular free calcium concentration and consequent axonal damage of TRPV1positive neurons.[22] Unfortunately, its potency also poses challenges. Similar to first generation TRPV1 antagonists, resiniferatoxin increases the heat pain threshold. Moreover, high or repeated systemic doses of resiniferatoxin induce long-lasting damage to TRPV1-positive neurons,[68] rendering it not suitable for therapeutic applications in IBS.

Cross-desensitization

Another mechanism aimed at analgesia is related to TRPV1 channel crossdesensitization. As mentioned above, TRPM8 activation is thought to provide antinociceptive effects through subsequent inhibition of TRPV1 and TRPA1. Peppermint oil, containing the TRPM8 agonist menthol, exploits these beneficial effects and is registered for the use in IBS in several countries.[65] It should be noted that the exact mechanism of action remains to be elucidated. Moreover, its effects appear to reach beyond TRP channel signalling as calcium channel mediated smooth muscle relaxation has been observed in vitro in human colon tissue without the involvement of TRPM8.[5]

Multiple small trials have evaluated the efficacy of peppermint oil in IBS. Side effects of this herbal therapy are rather mild, with heartburn being the most common.[65] One meta-analysis reported that 75% of IBS patients experience improvement in abdominal pain compared with only 27% in patients receiving placebo.[65] In a more recent trial, patients received either a sustained release peppermint oil preparation ensuring drug release in the small intestine, or a placebo. Patients receiving peppermint oil reported a significantly greater reduction in abdominal pain or discomfort compared with patients receiving placebo after a treatment duration of 28 days.[24] New preparations have been developed recently ensuring peppermint oil release in the colon, trials are ongoing and data on the efficacy of these new formulations will be reported in the near future.

Downstream targets of therapy

Drugs that target molecules downstream of TRPV1 provide an attractive pharmacological alternative to direct TRPV1 inhibition. As TRPV1 activation is accompanied by the release of sensory neuropeptides inducing neurogenic inflammation and sensitization of surrounding nociceptors, it makes sense to target mediators (or their respective receptors) of this process. Indeed, antagonists of the tachykinin NK1 and NK2 receptors of substance P and neurokinin A respectively, are being developed for various purposes, among others for symptom relief in IBS. For example, ibodutant, a neurokinin-2 receptor antagonist, has been shown to improve abdominal pain and stool pattern in female IBS-D patients.[98] Moreover, neurokinin-1 receptor antagonists such as aprepitant are currently being used as anti-emetics and have been suggested as analgesics as well.[31; 70] Unfortunately, several pre-clinical studies demonstrated that NK1 antagonists lacked analgesic effects.[46] It should be noted that animal models studying the effects of NK1 antagonists simulated *somatic* pain, whereas the NK1 receptors are more relevant in visceral pain.[70] Moreover, NK1 antagonists are thought to function as anti-hyperalgesic agents rather than analgesics as they do not affect baseline nociception, but attenuate nociceptive responses sensitized through inflammation.[46] Although at first sight this does not appear favourable, inhibiting the sensitized state rather than the normal resting state may actually make NK1 antagonists excellent candidates for treating visceral pain. The most important advantage would be that physiological functions may remain unaltered. In fact, similar mechanisms likely explain the beneficial effects of the HRH1 antagonist ebastine in IBS patients, which has been discussed above.[105] As TRPV1 sensitization after histamine pre-treatment has been shown to be HRH1 mediated, the effects of ebastine on abdominal pain are thought to be related to the inhibition of the sensitized state of TRPV1.

One additional mediator of neurogenic inflammation has been implicated as a potential target in the development of analgesic therapies. Somatostatin, originating from capsaicin-sensitive sensory afferents, counteracts pro-inflammatory neuropeptides CGRP and substance P. Indeed, the potent analgesic effects of somatostatin have long been recognized.[84] Early studies using the somatostatin analogue octreotide have demonstrated increased thresholds of visceral sensory perception in IBS patients.[17; 43] Moreover, anti-hyperalgesic effects of selective agonists of somatostatin receptor 1 and 2 have been reported in induced visceral hypersensitivity in mice.[81] Therefore, somatostatin analogues or receptor agonists may provide additional therapeutic strategies in visceral pain. Clinical studies will need to substantiate their applicability in IBS patients.

RNA-based therapy

The observation of decreased expression of several TRPV1 targeting miRNAs in IBS patients suggests an opportunity for RNA-based therapy. The therapeutic potential of post-transcriptional gene silencing by RNA interference is increasingly being recognized.[94] The major benefit of synthetic siRNAs is their high target specificity, preventing the suppression of unrelated genes. Unfortunately, several challenges have yet to be overcome before RNA-based therapy can become a therapeutic option in IBS. The oral bioavailability of oligonucleotides is limited, confining the possible modes of administration to more invasive approaches. Furthermore, the effects are only

transient, demanding repeated treatment. Therefore, there is a need for stable TRPV1 targeting siRNAs that are readily absorbed in the GI tract.

Exploiting new targets

Our current knowledge on the role of visceral TRPV4 and TRPA1 in humans is limited. Nonetheless, data from animal studies, discussed above, suggest that these channels are viable targets for IBS therapy. The previous discovery of elevated levels of the endogenous TRPV4 agonist 5,6-EET in IBS-D patients and the putative implication of TRPV4 in mechanical hypersensitivity justify the need for further research. TRPA1-targeted therapy has been explored in animal models of visceral pain. However, contrasting evidence was found demonstrating attenuated visceral nociception in rodents with both TRPA1 antagonists as agonists. [67; 82; 102] It is unclear whether the mechanism of action of the latter was based on desensitization. Arguing against such a mechanism is the observation that reduced abdominal contractions were observed in mice after a single oral dose of a TRPA1 agonist. Furthermore, one TRPA1 antagonist has recently shown efficacy in patients with painful diabetic neuropathy, and several other TRPA1 antagonists have entered the phase of testing in clinical trials.[86] Although the true potential of these drugs will need to be explored, their safety profile appears to be more favourable than that of early TRPV1 antagonists. Given this advantage, we expect a boost in TRPA1 targeted therapy development in the near future.

New targets may continue to be identified. TRP channel regulatory processes in IBS constitute relatively unexplored terrain (see "TRP channel regulation"). Improved understanding of the underlying molecular processes may help identify targets that modulate TRP channel functioning.

CHALLENGES OF TRP CHANNEL TARGETED THERAPY

Although the possibilities of TRP channel targeted therapy appear endless, treatment development has been plagued by many challenges over the past years.[57] As mentioned above, the first TRPV1 antagonists were associated with thermo-regulatory side effects. To date, the mechanisms by which these side effects arise are unknown. New selective agents may provide a solution to this issue. However, other challenges are to be expected. [57] This is mainly provoked by two factors. First, TRP channels are expressed in a wide range of tissues (see section "Non-neuronal TRP channel expression"). Targeting TRP channels consequently affects systems other than nociceptors. For example, a systemically administered TRPV4 agonist resulted in endothelial dysfunction and cardiovascular collapse in one study.[104] TRPV1 expressed in the central nervous system is thought to have a role in mood disorders. Although conflicting data exists, TRPV1 antagonism may exacerbate depressive symptoms.[64] Regulatory relations

between organ systems further complicate the matter. TRPV1-expressing neurons are thought regulate immunological functions, and interference with this function could prove detrimental in systemic inflammatory conditions.[97] The other major issue of TRP targeted treatment development is related to the large diversity of possible stimuli of each channel. Even selective agents will not immediately overcome this hurdle. For example, a new TRPV1 antagonist that does not cause hyperthermia, was shown to potentiate proton-induced calcium influx in one study.[73] This unforeseen effect could prove problematic in the upper gastrointestinal tract, as it may result in excessive TRPV1 activation by gastric acid (e.g. physiological gastro-oesophageal reflux).

In addition, several limitations currently exist in the study of TRP channels and their role in nociception. Basic research is complicated by the lack of quality reagents. Antibodies used to investigate expression patterns possess poor specificity. A similar issue is encountered with agonists/antagonists used to investigate TRP channel functioning, which presents a major problem in understanding TRP biology.[78] Furthermore, studies regarding TRP channel involvement in visceral pain generation mostly focus on inflammation. Although animal colitis models have provided a wealth of information, alternative mechanisms of TRP channel sensitization should be explored. As discussed above, IBS is highly heterogeneous. Further insights in the mechanisms underlying IBS are needed in order to expand our knowledge of peripheral nociception and ultimately guide IBS treatment development.

CONCLUSIONS

In summary, TRPV1, TRPV4, TRPA1 and TRPM8 have been shown to play important roles in visceral pain generation and inhibition, making them potential targets in the treatment of IBS. TRPV1 antagonists have proven to be potent analgesics, but it remains difficult to produce compounds with an acceptable safety profile. Different strategies targeting TRPV1 (i.e. modality-selective antagonism) or downstream molecules (NK1 or NK2 antagonists or somatostatin agonists) may solve this issue. TRPV1 desensitization strategies may provide suitable alternatives, yet short-term adverse effects may limit treatment adherence. In contrast, TRPV1 cross-desensitization with peppermint oil is attractive because of the low prevalence of adverse effects. The mechanism of action of peppermint oil however appears to reach beyond TRP channel signalling. In addition, TRPV4 and TRPA1 provide promising new targets. In our opinion, TRPA1 represents an important candidate for the development of new treatments of visceral pain. Its putative implication in mechanosensation in hyperalgesia but apparent lack of such function under basal conditions suggests a major role in IBS. Moreover, early TRPA1 antagonists have proven to be safe in clinical trials, rendering TRPA1 targeted therapy less of a pharmaceutical challenge than TRPV1 inhibition.

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CHAPTER 3

Role of TRPV1 and TRPA1 Ion Channels in Inflammatory Bowel Diseases: Potential Therapeutic Targets?

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Chapter 3

ABSTRACT

Inflammatory bowel diseases (IBD) have long been recognized to be accompanied by pain resulting in high morbidity. Transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) ion channels located predominantly on the capsaicin-sensitive sensory neurons play a complex role in hyperalgesia and neurogenic inflammation. This review provides an overview of their expression and role in intestinal inflammation, in particular colitis, that appears to be virtually inconsistent based on the thorough investigations of the last twenty years. However, preclinical results with pharmacological interventions, as well as scarcely available human studies, more convincingly point out the potential therapeutic value of TRPV1 and TRPA1 antagonists in colitis and visceral hypersensitivity providing future therapeutical perspectives through a complex, unique mechanism of action for drug development in IBD.

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic relapsing and remitting inflammatory disorders of the bowel. [74; 75] The two major groups of IBD are Crohn's disease (CD) and ulcerative colitis (UC), each having its own disease characteristics. The cardinal symptom in both disorders however, is abdominal pain, which often causes significant morbidity. Transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) ion channels located predominantly on the capsaicin-sensitive sensory neurons play a complex role in hyperalgesia and neurogenic inflammation, and although their role in colitis is seemingly contradictory, there is a growing evidence on their involvement in IBD. The aim of this review is therefore to provide an overview of their expression and role in intestinal inflammation, in particular colitis.

CD is characterized by lesions affecting the entire gastrointestinal tract, UC per definition is limited to the colon. In addition, whereas inflammation is usually confined to the mucosa in UC, it often extends beyond the muscular layers in CD (i.e., transmural inflammation). Microscopically, one can sometimes identify characteristic differences, with non-caseating granulomas present in CD and crypt abscesses in UC. Even clinical differences exist, as bloody stools are commonly seen in UC but far less often in CD. On the other hand, perianal disease (including fistulas and perianal abscesses) is more suggestive for CD than UC. Other symptoms are encountered in both UC and CD, including malaise, fatigue, diarrhoea and loss of appetite.

As mentioned above, disease activity varies during the course of the disorder, meaning that both the extent of inflammation and patient symptoms change over time. In order to monitor disease activity, both clinical and biochemical assessments are used, as well as endoscopic follow-up. Currently commonly used biomarker is fecal calprotectin (a non-invasive inflammatory marker that correlates well with disease activity in both UC and CD).[11] No other biomarkers have yet been established as an instrument to monitor disease activity in clinical practice.

Although biochemical/endoscopic signs of disease activity are important to monitor, it should be noted that IBD patients often report symptoms during clinical and biochemical remission. These symptoms may include abdominal pain, bloating, or a feeling of incomplete rectal evacuation, accompanied by a disturbed bowel pattern. Since these symptoms are compatible with irritable bowel syndrome, they are often referred to as "IBS-like". In clinical practice, treatment of these IBS-like symptoms in IBD patients remains particularly challenging. Increasing understanding of the biological and molecular background of these phenomena can therefore potentially contribute to the development of novel treatment paradigms.

INFLAMMATORY BOWEL DISEASES (IBD) AND RELATED PAIN

Inflammatory states have long been recognized to be accompanied by pain, as captured by the early Latin definition of inflammation (calor, rubor, tumor and dolor).[34] In line with this definition, abdominal pain is a cardinal symptom of IBD.[10] Inflammation is likely the primary cause of abdominal pain in active IBD and one of the main suspects in the pathophysiology of ongoing complaints during remission, although the latter is still a subject of discussion.[6] Over 30% of UC patients in remission and almost double this percentage in quiescent CD patients have IBS-like symptoms.[68] On the basis of pain symptoms, one could expect visceral hypersensitivity to be present in active IBD and in symptomatic quiescent IBD patients. Already in the late seventies, Farthing and colleagues studied sensory responses to rectal balloon distensions in patients with ulcerative colitis, as compared to healthy controls. [20] Not surprisingly, it was demonstrated that patients with active ulcerative colitis tolerated a far smaller balloon volume than healthy controls. Similar findings were later reported by two other research groups.[44; 61] Moreover, Rao and co-workers showed that hyperalgesia largely subsided upon remission. Finally, Hoboken and colleagues observed rectal hypersensitivity in UC patients in remission that reported IBS-like symptoms. However, these IBS-like symptoms do not appear to be associated with initial extent of the disease.[68] Moreover, no differences have been found in fecal calprotectin between UC patients in remission with or without IBS-like symptoms.[32; 78] It is therefore more likely that the acute inflammatory phase induced lasting changes in visceral nociception in these patients, rather than low-grade mucosal inflammation causing ongoing symptoms during remission.

Paradoxically, two more recent studies reported similar or even higher rectal discomfort thresholds in IBD patients (either active disease or in remission) as compared to healthy controls.[7; 13] However, important differences with the above mentioned studies should be kept in mind when interpreting these results. First, Bernstein and co-workers studied CD patients with isolated ileal involvement only, with balloon distensions consequently being applied to non-inflamed tissue. The observed higher rectal discomfort thresholds in CD patients during a ramp distension protocol would therefore more likely represent a form of central compensation due to a chronic inflammatory state and subsequent nociceptive signaling, rather than an effect at the peripheral level. The same research group conducted a similar study in UC patients. Using a threshold tracking paradigm, which is considered a non-biased distension protocol as the direction of each step depends on the patient's response during the previous distension, [81] it was demonstrated that UC patients had similar rectal discomfort thresholds as healthy volunteers. It should be noted, however, that UC patients in this study were either in remission or were reported to have only mild disease activity, with most patients being asymptomatic. Results therefore do not necessarily contradict the earlier balloon distension studies, in which patients often had significant disease activity.

TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 AND ANKYRIN 1 PAIN SENSING ION CHANNELS

Transient receptor potential (TRP) ion channels comprise more than 30 structurally related ion channels, divided into the TRPC (Canonical), the TRPV (Vanilloid), the TRPM (Melastatin), the TRPP (Polycystin), the TRPML (Mucolipin), the TRPA (Ankyrin) and the TRPN (NOMPC) subfamilies based on their sequence homology. [56] Most of them are non-selective cation channels, however, they exhibit differences in permeability and selectivity.[1] These ion channels are tetramers composed of six transmembrane domains, with a pore formed by the hydrophobic region between the fifth and sixth segments. They can assemble as homo- or heterotetramers to form functional units.[41] The physiological role of TRP channels ranges from store-operated calcium channels to thermo-, mechano- and chemosensors.

The most investigated members of the family in relation to gastrointestinal inflammation include vanilloid 1 (TRPV1), ankyrin 1 (TRPA1). They are located predominantly on the capsaicin-sensitive sensory neurons, but several non-neural expressions have recently been described that drew great attention to this research area. [21] In general, they are activated by a variety of exogenous chemicals and endogenous mediators making them important regulatory structures in inflammatory and pain processes. Here we focus on TRPV1 and TRPA1, since there are many experimental and clinical results describing their expression and importance in the gastrointestinal tract most importantly in the colon.

TRPV1 and TRPA1 are polymodal nociceptors playing an important role in thermo- mechanical- and chemo-sensation, and play a complex role in hyperalgesia and neurogenic inflammation. Their endogenous activators are often produced during inflammation, e.g., lipoxygenase products, the acidified pH of the inflamed tissue, and the gastrointestinal mucosa is frequently exposed to their exogenous agonists, such as capsaicin, allyl isothiocyanate, allicin etc. ingested by food. Furthermore, TRPV1 and TRPA1 are capable of functional interaction, such as heterologous desensitization,[65] since the majority of TRPA1 expressing nerve fibers co-express TRPV1.[70] Both ion channels can be sensitized by a variety of other mechanisms, such as prostaglandins, bradykinin and proteases, e.g. cathepsin expressed by immune cells, via the protease-activated receptor 2 (PAR2) present on both capsaicin-sensitive nerve endings and the immune cells themselves.[3; 12]

TRPV1 and TRPA1 in IBD Patients

Putative evidence points toward sensitization and even activation of TRPV1 by various inflammatory mediators, as indicated by the multiplicity of animal studies reported below. In vitro studies using human embryonic kidney cells (HEK293 cells) transfected with rat TRPV1 cDNA have reported TRPV1 sensitization by various mediators of

inflammation as well, including prostaglandin E2 and prostaglandin I2, bradykinin, nerve growth factor and the chemokine CCL3.[51; 67; 84; 85] Moreover, in a study with rectal biopsy material from healthy volunteers, pre-incubation with histamine was shown to potentiate TRPV1 responses. Inflammation associated tissue acidification would furthermore appear to be an obvious route for TRPV1 activation.[54] Indeed, intradermal and intramuscular low pH injections in healthy volunteers were shown to elicit moderate pain responses, which were potentiated by the injection of prostaglandin E2.[64] Similarly, Jones and colleagues demonstrated that topical application of capsaicin potentiated pain responses to iontophoresis of protons.[28] It should be noted, however, that desensitization after repeated topical capsaicin application did not reduce acid-induced pain responses, suggesting that other receptors are involved as well. Nonetheless sufficient data indicates TRPV1 is sensitized in inflammatory conditions, thus potentially resulting in hyperalgesia and abdominal pain in IBD.

Although TRPV1 appears to be sensitized by inflammatory mediators, studies on the expression of TRPV1 in inflamed human intestine, and in particular colon tissue have yielded contradictory results (Tables 3.1 and 3.2). Yiangou and co-workers previously investigated TRPV1 immunoreactivity in colonic tissue samples from IBD patients who underwent a colectomy due to refractory disease, using tissue samples obtained from resections due to non-obstructing carcinoma as controls.[83] It was demonstrated that TRPV1 immunoreactivity was greatly increased in the colonic nerve fibers of patients with IBD as compared to controls. These findings were recently corroborated by a study with samples from 60 IBD patients (30 patients with UC and 30 patients with CD). [46] Whereas Yiangou observed increased expression in the submucosa only, Luo and colleagues reported increased TRPV1 expression in both the mucosa and infiltrating inflammatory cells. On the other hand, we observed decreased levels of TRPV1 mRNA in biopsy material from patients with active and inactive CD and UC as compared to healthy controls.[37] In line with our findings, Rizopoulos and colleagues very recently reported decreased TRPV1 expression in mucosal biopsy material from UC patients, as compared to colonic resections from non-IBD patients.[62] Importantly, TRPV1 expression does not appear to be correlated with disease activity, arguing against a role for the extent of inflammation. Regardless of the direction of regulation, inflammation induced changes in TRPV1 expression are likely reversible. Akbar and co-workers found no differences in TRPV1-immunoreactivity in rectosigmoid biopsies when comparing samples from asymptomatic quiescent IBD patients and healthy volunteers, but did find increased TRPV1 expression in quiescent IBD patients with abdominal pain.[2] Similarly, we did not observe significant differences in TRPV1 transcription in sigmoid colonic mucosal samples from (primarily asymptomatic) quiescent UC patients, as compared to healthy controls.[33]

		Sampling Method/			Relation to Abdominal Complaints and/or Disease	
Disease Activity	Ion Channel	Location	Methods	Results; Number of Patients	severity	Ref
Active CD	TRPV1	Resection (colectomy)	IHC (computerized image analysis)	upregulated in submucosa; n = 6	Not reported	[83]
		Colon biopsy – affected and non-affected regions	IHC (computerized image analysis)	upregulated in mucosa and infiltrating inflammatory cells; <i>n</i> = 30	No significant correlation between disease severity and TRPV1 expression	[46]
		Distal colon biopsy	IHC, qPCR	downregulated mRNA n = not reported	Not reported	[37]
		Colon biopsy	IF	downregulated mRNA n = 6	Not reported	[6]
	TRPA1	Distal colon biopsy	IHC, qPCR	upregulated mRNA n = not reported	Not reported	[37]
		Colon biopsy	IF	upregulated mRNA $n = 7$	Not reported	[6]
CD in remission	TRPV1	Distal colon biopsy	IHC, qPCR	downregulated mRNA n = not reported	Not reported	[37]
		Rectosigmoid biopsy	IHC	upregulated in symptomatic quiescent patients; 3.9-fold increase in median number of TRPV1-immunoreactive fibers (CD and UC combined) n = 9	Significant correlation between TRPV1 expression and abdominal pain score	[2]
CD – disease activity unknown	TRPA1	Surgical samples of fibrotic regions (colon)	IHC	Denser immunoreactivity in mucosal and submucosal layers n = 3	Not reported	[27]
		Biopsy from fibrotic regions (colon)	IHC, RT-PCR	upregulated mRNA and protein levels $n = 8$	Not reported	[38]

Table 3.1. Alterations in TRPV1 and TRPA1 transcription/expression in Crohn's disease (CD) patients with fold-changes where available. (IHC: immunohistochemistry, IF: immunofluorescence).

3

TRP channels in IBD

Disease Activity	Ion Channel	Sampling Method/ Location	Methods	Results: Number of Patients	Relation to Abdominal Complaints and/or Disease Severity	Ref
Active UC	TRPV1	Resection (colectomy)	IHC (computerized image analysis)	upregulated in submucosa $n = 3$	Not reported	[83]
		Colon biopsy – affected and non-affected regions	IHC (computerized image analysis)	upregulated in mucosa and infiltrating inflammatory_cells; n = 30	No significant correlation between disease severity and TRPV1 expression	[46]
		Distal colon biopsy	IHC, qPCR	downregulated mRNA	Not reported	[37]
		Colon biopsy	IHC (manual counting by two observers)	downregulated protein $n = 26$	No significant correlation between clinical features and TRPV1 expression	[62]
UC in remission	TRPV1	Distal colon biopsy	IHC, qPCR	downregulated mRNA	Not reported	[37]
		Colon biopsy	IHC (manual counting by two observers)	downregulated protein $n = 24$	No significant correlation between clinical features and TRPV1 expression	[62]
		Rectosigmoid biopsy	IHC	upregulated in patients with IBS-like symptoms; 3.9-fold increase in median number of TRPV1-immunoreactive fibers (CD and UC combined) n = 11	Significant correlation between TRPV1 expression and abdominal pain score	[2]
		Rectosigmoid biopsy	qPCR	No significant difference in mRNA levels between asymptomatic patients and healthy controls n = 34	Not reported	[33]

Table 3.2. Alterations in TRPV1 and TRPA1 transcription/expression in ulcerative colitis (UC) patients with fold-changes where available.

Chapter 3

There is little data regarding the expression and function of TRPA1 in IBD patients. However, results appear to be less contradictory than with TRPV1 (Tables 3.1 and 3.2). In our study with biopsy material from patients with active and inactive CD and UC, we found a significant TRPA1 mRNA upregulation. Similarly, Bertin and co-workers found TRPA1 to be upregulated in patients with active UC and CD, although the difference was non-significant because of small sample size (n = 13). Triple immunofluorescence staining for TRPV1, TRPA1 and CD4 demonstrated that infiltrating CD4+ T cells were also positive for TRPV1 and TRPA1.[9] Moreover, a significantly higher number of these cells was found in the colonic tissue samples of both UC and CD patients. Two other studies also reported increased TRPA1 expression in stenotic regions in the colon of CD patients, in samples obtained surgically and endoscopically. These studies suggested TRPA1 to be anti-fibrotic. Using a culture medium containing normal human intestinal myofibroblasts (InMyoFibs), it was demonstrated that adding type I collagen to the medium enhanced TRPA1 expression.[27] When fibrosis was elicited by transforming growth factor β 1, knockdown of TRPA1 with siRNA resulted in enhanced fibrogenic effects.[27]

ANIMAL MODELS OF IBD

Unfortunately ideal IBD models with real translational value do not exist in animals, because they cannot completely mimic the complexity of the multifactorial psychosomatic disease. Moreover, as colitis models usually involve short-term administration of an irritating substance, these rather represent acute inflammation. This is a significant limitation of these models in the context of their representation of IBD, which is a chronic disease. In addition to the duration of administration, the type of irritating substance being used determines the characteristics of the model. The fact that model specifics can influence the results should be taken into consideration in their interpretation. We have to rely on well-established and characterized mechanism models exhibiting most autoimmune and inflammatory components of the human disease.[77] Since human studies revealed a potential role of TRPA1 and TRPV1 receptors in the pathogenesis of IBD, but only expression changes could be detected in the human samples, preclinical tests are essential to have a better insight into the pathogenesis of IBD, investigate functional alterations including the role of these ion channels, as well as perform pharmacological interventions.

Besides chemical induction and bacterial infection (with *Salmonella typhimurium* and *Salmonella dublin* or invasive-adherent *Escherichia coli*) several transgenic and knockout strains have been developed in order to investigate the specific pathophysiologic alterations in IBD.

Administration of 1%–5% dextran sulfate sodium (DSS) in the drinking water of animals is a widely used method of chemically-induced colitis by disrupting the tight junctions between

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the intestinal epithelial cells and inducing inflammation through exposing the lamina propria to bacterial and other toxins, infective agents and antigens.[45] The consequent inflammatory cascade with a characteristic symptomatology (bloody diarrhea, weight loss and histopathology with inflammation limited to the mucosa, as well as cytokine profile) is considered to model UC with several limitations such as great variability between the experimental paradigms including concentration, molecular weight and sulphate content of DSS, intestinal flora, strain differences, administration protocol and timing, as well as the endpoints.[57]

Further UC-related rodent models include the intrarectal administration of oxazolone and acetic acid. Oxazolone induces a characteristic T helper 2 (Th2) predominant immune response associated with epithelial cell loss in the colon, acetic acid administration evokes direct chemical damage (erosions, ulcerations accompanied by crypt abnormalities) in the distal colon.

In contrast to these UC-like models, trinitrobenzene or dinitrobenzene sulfonic acid (TNBS/DNBS) colitis is associated with profound transmural infiltration of inflammatory cells and Th1-mediated immune response, thus resembling more to CD.[31]

For the investigation of T-cell-mediated pathogenesis of colitis, IL-7 overexpressing and T-cell receptor α chain (*TCRa*) deficient (knockout: KO) mice are used in acute and chronic models, respectively, associated with neutrophilic and lymphocytic infiltration.[50; 80] Other mouse strains developing spontaneous colitis include Wiskott-Aldrich syndrome protein (*WASP*) KO mice with characteristically elevated levels of Th2 cytokines.[53] Meanwhile, 25% of mice lacking the multidrug resistance 1a gene (*Mdr1* α KO) also show similar symptoms due to a decreased production of IL-10 and functional Treg cells.[55] Furthermore, *IL-2* KO, as well as guanine nucleotide-binding protein subunit α -2 (*Gai2*) KO mice exhibit UC-like phenotype with crypt abscess formations and ulcerations.[63; 66]

Expression of TRPV1 and TRPA1 in Animal Colon

In the gastrointestinal tract TRPV1 is often co-expressed with TRPA1 in capsaicinsensitive extrinsic sensory nerves, especially in the primary sensory neurons of the dorsal root ganglia. The density of these TRPV1 positive fibers increase from proximal to distal regions of the colon in mice.[49] Furthermore, during DSS colitis the proportion of DRG neurons expressing TRPV1, and their relative TRPV1 mRNA levels increase with a subsequently elevated release of sensory neuropeptides, such as calcitonin generelated peptide (CGRP) and substance P (SP).[18] Although the role of TRP-expressing afferents in inflammation is undisputable, there is growing evidence on the expression of TRPV1 and TRPA1 in intrinsic sensory neurons of the myenteric and submucosal plexuses [4; 37; 49; 60] as well as on the surface epithelial cells of colonic mucosa.[29; 37; 60] The importance of sensory-immune interactions in colonic inflammation is also supported by the expression of TRPV1 and TRPA1 on inflammatory cells like mucosal macrophages, as well as CD4+ T cells (Table 3.3 and 3.4).[8; 9; 37]

mRNA	Location	Method	Model, Animal Species/Strain	Ref
TRPV1	isolated crypts, submucosal and muscle layers of distal, middle and proximal colon	qPCR	intact male Wistar rats	[29]
	upregulated in colonic DRG to the distal colon in DSS-colitis		2.5% DSS-treated C57BL/6 mice	[18]
	unaltered in distal colon, cell type not specified	_	DSS colitis - male C57BL/6 mice	[37]
	CD4+ T cells	_	primary cell culture from C57BL/6 spleen	[8]
TRPA1	muscularis externa and mucosa of duodenum, ileum and colon; cell type not specified	_	intact C57BL/6 mice	[60]
	surface epithelium of middle colon	ISH	intact male Wistar rats	[29]
	isolated crypts, submucosal and muscle layers of distal, middle and proximal colon	qPCR	intact male Wistar rats	[29]
	upregulated in distal colon, cell type not specified	_	DSS colitis - male C57BL/6 mice	[37]

Table 3.3. mRNA expression of TRPV1 and TRPA1 in the animal colon (ISH: in situ hybridization).

Table 3.4. Protein expression of TRPV1 and TRPA1 in the animal colon (IHC: immunohistochemistry).

Protein	Location	Method	Model, Animal Species/Strain	Ref
TRPV1	intrinsic sensory neurons of the myenteric plexus-longitudinal muscle of ileum and colon	IHC	intact Sprague-Dawley rats and Dunkin-Hartley guinea pigs of both sexes	[4]
	mucosa, submucosal layers, myenteric plexus and mucosal layer of rectum, distal, transverse and proximal colon	_	male ddY mice	[49]
	immunopositive neuron fiber density is higher in the distal than the proximal colon	_	intact and 2.5% DSS-treated C57BL/6 mice colon	[18]
	enteric ganglia, epithelial cells of the distal colon, myenteric and submucosal plexuses, mucosal macrophages, leukocytes	_	male C57BL/6 mice	[37]
	membrane of resting CD4+ T cells immunoblotting, flow cytometry, confocal microscopy		primary cell culture from C57BL/6 spleen	[8]
TRPA1	distal colonic epithelial cells, myenteric and submucosal plexuses, interstitial macrophages	IHC	male C57BL/6 mice	[37]
	myenteric and submucosal ganglia; surface epithelial cells of small and large intestines	_	intact C57BL/6 mice	[60]
	surface epithelium of middle colon	_	intact male Wistar rats	[29]
	membrane of resting CD4+ T cells	IHC, confocal microscopy	primary cell culture from C57BL/6 spleen	[9]

Role of TRP Channels in Animal Models of Colitis

The role of TRP channels, in particular TRPV1 and TRPA1 is virtually contradictory in the pathogenesis of IBD. Several studies have been focused on elucidating the mechanism by which these channels might mediate pro-inflammatory and/or antiinflammatory effects (Table 3.5).

Goso and co-workers provided the first evidence for a protective role of TRPV1expressing peptidergic sensory nerves via the release of the protective neurotransmitter CGRP upon acute co-administration of capsaicin in a TNBS-induced colitis model [60]. Administration of TRPV1 agonists, resiniferatoxin (RTX) or high dose capsaicin, induces a sustained functional denervation of TRPV1-expressing extrinsic neurons, thus it provides a method in animal models for the investigation of these sensory afferents and the released neurotransmitters. The results of this chemical desensitization are not coherent, since pro-inflammatory and protective roles have also been described. Neonatal capsaicin desensitization, as well as the administration of the TRPV1 antagonist capsazepine have been reported to significantly attenuate macroscopic damage score, myeloperoxidase (MPO) activity increase (peroxidase enzyme released from neutrophil granulocytes in the inflamed tissues) and inflammatory histopathological alterations compared to normal DSS-treated rats attributing the colitogenic effect to SP released from the nerve terminals of TRPV1-expressing sensory fibers.[35] Meanwhile, Utsumi and coworkers found opposing results in the same model after adult treatment by high doses of capsaicin, which exacerbated colitis and reduced the inflammation-induced upregulation of both SP- and CGRP-positive fibers.[76] However, they described that TRPV1 and TRPA1 gene deletion decreased colitis severity and the upregulation of SP-positive nerve fibers without influencing protective CGRP-positive nerves. Similarly, neonatal capsaicin denervation resulted in more severe colitis in the oxazolon-induced model, but exacerbation was not accompanied by changes in the expression and distribution of CGRP- and SP-immunoreactive nerves in the colon.[43] These virtually contradictory pro- and anti-inflammatory effects of neuropeptides released from the TRPV1/A1expressing fibers during chemically-induced colitis were further investigated in the TNBS model, where abrogated CGRP release in the isolated colon preparations and dorsal root ganglia were observed in *Trpa1*, but not in *Trpv1* gene-deficient mice. They showed that this mechanism is mediated via the sustained sensitization of TRPA1 by TNBS covalently binding to the cysteine and lysine residues in the cytoplasmic N-terminus of the receptor protein. TNBS induces similar severe acute colitis in wildtype and *Trpv1-*^{-/-}, but reduced inflammation in *Trpa1-^{-/-}* mice or wildtype animals treated with the TRPA1 antagonist HC-030031. Sensory denervation, as well as SP gene-deletion abolished both TNBS and DSS-induced colitis, while in CGRP-deficient mice TNBS induced a more severe colitis further supporting the opposing actions of the sensory neuropeptides released from the same nerve terminals.[18; 19] Trpv1-deficiency did not affect disease

Approaches	Results	Animal Strain/Species	Model	Ref
TRPV1 antagonist	reduces colitis severity	Sprague-Dawley rats	5% DSS + capsazepine	[35]
		female BALB/c mice	5% DSS + capsazepine/ JNJ 10185734	[36]
		Sprague-Dawley rats	TNBS + capsazepine	[22]
		female Wistar rats	TNBS + BCTC	[79]
		<i>IL10^{-/-}Trpv1^{-/-}</i> mice	<i>IL10^{-/-}</i> -induced spontaneous colitis + SB366791	[8]
TRPV1 agonist	<i>attenuates</i> colitis/visceral hyperalgesia	male Sprague-Dawley rats	TNBS + capsaicin	[24]
		male BALB/c mice	DNBS + curcumin	[47]
		male Sprague-Dawley rats	5% DSS + curcumin	[82]
TRPV1 gene	decreases colitis	<i>female Trpv1^{-/-}</i> mice	2% DSS	[73]
deletion		IL10 ^{-/-} Trpv1 ^{-/-} mice	IL10 ^{-/-} -induced spontaneous colitis	[8]
		male <i>Trpv1</i> -/-mice	2% DSS	[76]
	aggravates colitis	female Trpv1-/-mice	DNBS	[48]
	does not affect colitis severity	female Trpv1 ^{-/-} mice	5% DSS	[73]
		Trpv1 ^{-/-} mice	TNBS	[19]
		Trpv1 ^{-/-} mice	2.5% DSS	[39]
	protects against chronic pain during recovery	<i>Trpv1</i> ^{-/-} mice	2.5% DSS	[39]
	decreases CD4+ T cell activation and cytokine production	<i>IL10^{-/-}Trpv1^{-/-}</i> mice	IL10 ^{-/-} -induced spontaneous colitis	[8]
TRPA1 antagonist	reduces colitis severity	C57BL/6 mice	TNBS + HC-030031; DSS + HC- 030031	[19]
	reverses visceromotor response	female Wistar rats	TNBS/ethanol + TCS-5861528	[79]
TRPA1 gene	decreases colitis	Trpa1-/- mice	TNBS, 2% DSS	[19]
deletion		male Trpa1 ^{-/-} mice	2% DSS	[76]
	aggravates colitis	male Trpa1 ^{-/-} mice	2% DSS	[37]
		IL10 ^{-/-} Trpa1 ^{-/-} mice	IL10 ^{-/-} -induced spontaneous colitis	[9]
	<i>increases</i> TRPV1 channel activity in CD4+ T cells, <i>increases</i> CD4+ T cell activation and proinflammatory cytokine production	IL10 ^{-/-} Trpa1 ^{-/-} mice	<i>IL10</i> ^{+,-} induced spontaneous colitis	[9]
Capsaicin-	aggravates colitis	female BALB/c mice	oxazolone	[43]
induced sensory desensitization		male <i>Trpv1^{-/-}</i> , <i>Trpa1^{-/-}</i> mice	2% DSS	[76]
	alleviates colitis	Sprague-Dawley rats	5% DSS	[35]
RTX-denervation	alleviates colitis	C57BL/6 mice	TNBS, 2% DSS	[19]

Table 3.5. Role of TRPV1 and TRPA1 in animal models of colitis (*Trpv1*^{-/-}, *Trpa1*^{-/-} gene deleted mice were bred on C57BL/6 background).

severity, only prevented chronic pain development during the recovery phase of DSSinduced colitis.[39] However, this result was challenged by other studies demonstrating the pathogenic role of TRPV1 by gene-deleted mice exhibiting less severe DSS-induced colitis, concluding that inflammatory mediators activate the TRPV1 receptor and induce neurogenic inflammatory components by releasing SP, neurotensin, vasoactive intestinal polypeptide and galanin.[72; 76] Meanwhile, Massa and co-workers found more severe DNBS-induced colitis in *Trpv1*^{-/-} mice, suggesting a protective role of TRPV1.[48] Bertin and co-workers proposed non-neuronal TRPV1 and TRPA1-mediated proinflammatory mechanisms in colitis. They showed that both channels are present on mouse and human CD4+ T cells and play an important regulatory role in their activation and the production of proinflammatory cytokines, such as interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-10 and tumor necrosis factor α (TNF α). In a spontaneous *IL10^{-/-}* colitis model both genetic deletion and pharmacologic inhibition of TRPV1 resulted in attenuated inflammation. They provided clear experimental evidence in a T cell adoptive transfer model that TRPV1-expressing CD4+ T cells are involved in colitis pathogenesis.[8] In the same experimental paradigm TRPA1 was described to exert protective actions by restraining TRPV1 activity on these immune cells, thus controlling their activation and inflammatory functions.[9] The protective role of TRPA1 was also supported by TRPA1-mediated downregulation of proinflammatory neuropeptides SP, neurokinins A, B (NKA, NKB) and NK1 receptor, as well as cytokines and chemokines like TNF α , IL-1 β , monokine induced by gamma interferon (MIG) and monocyte chemotactic protein-1 (MCP-1).[37]

Pharmacological interventions with curcumin had anti-inflammatory and antihyperalgesic effects in colitis models.[47; 82] Although in these studies curcumin was interpreted and discussed as a TRPV1 agonist, it is important to note that curcumin is a non-selective compound having a typical pleiotropic effect including direct antioxidant activity, anticancer and antimicrobial properties mediated by a wide range of targets, even the TRPA1 receptor.[40; 42; 52] Considering that TRPA1 is almost exclusively expressed in TRPV1-positive neurons and both channels are known to interact,[65; 70] crossdesensitization could have a role in the actions of curcumin. Furthermore, curcumin was also described as a TRPV1 antagonist, because it inhibited capsaicin-evoked potentials. [86] In a clinical study, curcumin was reported to significantly reduce relapse rate in UC patients not via the activation, but the inhibition of TRPV1 either directly or by ways of cross-desensitization of TRPA1.[25] However, we should be cautious when drawing conclusions regarding TRPV1 involvement based on the results of curcumin administration.

Cannabinoids have also shown beneficial effects in animal colitis models.[17; 48] Changes in the endocannabinoid system during intestinal inflammation have been described and TRPV1-associated effects could be involved in the anti-inflammatory effects of cannabinoids.[16; 17; 26] More than two-fold increase of anandamide also acting as a TRPV1 agonist was described in the human UC biopsy samples.[17] A single oral dose of the endocannabinoid palmitoylethanolamide (PEA) was shown to increase 2-arachidonoylglycerol (2-AG) blood levels in human volunteers.[59] In HEK-293 cells transfected with human recombinant TRPV1, PEA significantly enhanced 2-AG induced activation and desensitization of TRPV1. It was therefore speculated that 2-AG is responsible for the protective effect of PEA during an induced inflammatory response. [58] In a study using colonic explants of six quiescent IBD patients, it was demonstrated that treatment with PEA and cannabidiol (CBD) suppressed secretion of inflammatory mediators in explants exposed to inflammatory cytokines that was counteracted by the TRPV1 antagonist.[16]

Other TRPV1 and TRPA1 antagonists also showed mainly protective actions. TRPV1 blockade by the non-selective antagonist capsazepine, JNJ 10185734, BCTC and SB366791 in various models of colitis exerted anti-inflammatory actions supporting the pathogenic role of TRPV1 in experimental IBD.[8; 22; 35; 36; 79] Moreover, both intraperitoneal and intrathecal administrations of TRPV1 and TRPA1 antagonists exerted analgesic actions in rat colitis models highlighting central nervous system mechanisms.[79]

CONCLUSION, DRUG DEVELOPMENTAL PERSPECTIVES

TRPV1 and TRPA1 expression, and experimental data regarding its role in colitis appears to be virtually inconsistent. Activation of these receptors on sensory nerve terminals mediates neurogenic inflammation via the release of SP and CGRP, resulting in increased vascular permeability, plasma protein extravasation and inflammatory cell activation. Meanwhile, anti-inflammatory sensory neuropeptides, such as somatostatin and opioid peptides released simultaneously from the same nerve ending exert antiinflammatory and analgesic actions both locally and systemically through getting into the circulation. Furthermore, these ion channels on vascular smooth muscle and inflammatory cells such as macrophages and T helper cells mediate both proand anti-inflammatory functions. Therefore, the overall role of TRPV1 and TRPA1 in experimental colitis is dependent on 1) the diversity of the expression of these ion channels on sensory nerves, immune cells, epithelial cells and vascular smooth muscle cells, [21] 2) the consequent activation-induced release of broad range of pro- and antiinflammatory mediators including sensory neuropeptides and cytokines exerting divergent mechanisms, 3) the complex interactions of the co-expressed TRPV1 and TRPA1 receptors (Figure 3.1), 4) differences of the experimental models, protocols and paradigms (species, strain, concentration and composition of the chemicals, duration, intensity, complex mechanisms of the injury), as well as several limitations of the models.[57]



Figure 3.1. The complex interactions of TRPV1 and TRPA1 and their virtually contradictory role in colitis. Panel A demonstrates the afferent and efferent (sensocrine) functions (yellow arrows) of the capsaicin-sensitive sensory nerve fibers. Panel C (without inflammation) and D (during inflammation) depict an enlarged schematic section of the colon cross section (panel B) focusing on the expression and interaction of TRPV1 (green double lines) and TRPA1 (orange double lines) in the colon mucosa. Neurogenic inflammation is mediated via the release of SP and CGRP (blue dots represent neurotransmitters, such as SP, CGRP and somatostatin), resulting in increased vascular permeability, plasma protein extravasation and inflammatory cell activation. Meanwhile, anti-inflammatory sensory neuropeptides, such as somatostatin and opioid peptides released simultaneously from the same nerve ending exert anti-inflammatory and analgesic actions both locally and systemically through getting into the circulation. Furthermore, these ion channels on vascular smooth muscle and inflammatory cell such as macrophages (MP) and T helper cells (Th1) mediate both pro- (+) and anti-inflammatory (-) effects by regulating the release of cytokines (IFN- γ , IL-2, IL-10, TNF α are represented as red dots).

However, preclinical results with pharmacological interventions, as well as scarcely available human studies, more convincingly point out the potential therapeutic value of TRPV1 and TRPA1 antagonists in colitis and visceral hypersensitivity providing future therapeutical perspectives for small molecule candidates. The first generation TRPV1 compounds thoroughly investigated in a broad range of clinical trials as novel analgesic and anti-inflammmatory drugs interfered with thermoregulation, elicited severe hyperthermia [69; 71] and raised heat pain thresholds with consequently increased burn risk.[5; 23] Therefore, they could not be registered for the clinical practice. Second generation new drugs with different inhibition sites on the TRPV1 and/or TRPA1 antagonists without the hyperthermic side effect could provide solutions to these problems.[14] Their clinical efficacies are currently intensively investigated, but they could open new perspectives through a complex, unique mechanism of action for drug development in IBD.[15; 30]

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CHAPTER 4

Age-related decrease in abdominal pain and associated structural- and functional mechanisms: an exploratory study in healthy individuals and irritable bowel syndrome patients

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Chapter 4

ABSTRACT

Introduction. The world population is ageing, resulting in increased prevalence of age-related comorbidities and healthcare costs. Limited data are available on intestinal health in elderly populations. Structural and functional changes, including altered visceroperception, may lead to altered bowel habits and abdominal symptoms in healthy individuals and irritable bowel syndrome (IBS) patients. Our aim was to explore age-related changes in gastrointestinal symptoms and underlying mechanisms.

Methods. In total, 780 subjects (IBS patients n = 463, healthy subjects n = 317) from two separate studies were included. Subjects were divided into different age groups ranging from young adult to elderly. Demographics and gastrointestinal symptom scores were collected from all participants using validated questionnaires. A subset of 233 IBS patients and 103 controls underwent a rectal barostat procedure to assess visceral hypersensitivity. Sigmoid biopsies were obtained from 10 healthy young adults and 10 healthy elderly. Expression of the visceral pain-associated receptors transient receptor potential (TRP) Ankyrin 1 (TRPA1) and Vanilloid 1 (TRPV1) genes were investigated by quantitative RT-PCR and immunofluorescence.

Results. Both elderly IBS and healthy individuals showed significantly lower scores for abdominal pain (p<0.001) and indigestion (p<0.05) as compared to respective young adults. Visceral hypersensitivity was less common in elderly than young IBS patients (p<0.001). Relative TRPA1 gene transcription, as well as TRPA1 and TRPV1 immunoreactivity were significantly lower in healthy elderly versus healthy young adults (p<0.05). Surprisingly, no significant association was found between the abdominal pain symptoms and the expression of the nociceptive ion channel expression.

Conclusions. Our findings show an age-related decrease in abdominal pain perception. This may in part be related to decreased TRPA1 and/or TRPV1 receptor expression. Further studies are needed to reveal precise underlying mechanisms and the associations with intestinal health.

INTRODUCTION

Ageing affects gut functioning on several levels; for example motility, gut microbiota composition, local immune and inflammatory responses and sensory functions (e.g. gustatory and nociceptive) [5; 24] are reported to change with age. Perturbations in these functions can be linked to common gastrointestinal (GI) disorders in the elderly: for example constipation and diverticulitis occur more frequently with ageing [8; 9]. In addition, acute inflammatory gastrointestinal diseases, such as appendicitis and cholecystitis, can be more difficult to diagnose as their presentation in the elderly is more often atypical or silent, in part related to less severe abdominal pain [18]. On the other hand, decreased nociceptive signaling could be of benefit in disorders of gut-brain interactions that are characterized by chronic recurrent pain, such as irritable bowel syndrome (IBS). Studies using balloon distentions in the esophagus and rectum demonstrated an increased visceral pain threshold in the elderly [12; 13], and abdominal pain was shown to be inversely correlated with age during a nutrient challenge in the form of enteral feeding solution [7]. The decreased sensitivity to visceral pain appears to translate to lower prevalence rates of IBS, as a meta-analysis showed that the odds of IBS in those aged 50 years and older are significantly lower than in those younger than 50 years [15]. Moreover, two population-based studies demonstrated the disappearance of abdominal pain with ageing [3; 22].

Currently, little is known about the mechanisms behind the decline in sensory signaling in the gut with increasing age. Elucidating these mechanisms could potentially provide new insights for the development of novel (visceral) pain management strategies, in particular with respect to different therapeutic responses related to age. From a molecular perspective, the responsiveness of visceral afferents to noxious stimuli is determined by the expression and activity of sensory transducer molecules including transient receptor potential (TRP) channels [2]. TRP channels constitute a family of nonselective cation channels. Several members of this family, including transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1), have been identified to function as transducers of nociceptive signals in both somatic and visceral pain [2]. It has therefore been hypothesized that a reduction in functioning or expression of TRPV1 and TRPA1 in visceral afferents is responsible for reduced abdominal pain with age. Significantly decreased responses to the TRPV1 agonist capsaicin in colonic high threshold mechanosensitive afferents was observed in 24-month-old mice when compared to 3-month-old ones, which were unrelated to the expression of TRPV1 in DRG neurons [10]. Similarly, in human ileal and sigmoid biopsy samples, a decreased baseline firing rate and blunted response to bradykinin were shown in elderly compared to young individuals (with a cut-off of 65 years of age) [31].

Given the limited data with regards to alterations in TRP channel expression, signaling and function with ageing and abdominal pain, our aim was to explore age-

related changes in gastrointestinal symptoms including nociception, and particularly their relationship with TRPV1 and TRPA1 immunoreactivity and mRNA expression profiles in sigmoid biopsies. In addition to subjective abdominal symptom ratings we studied visceral sensitivity across age groups using rectal balloon distension in IBS patients and healthy controls.

METHODS

The present manuscript is based on data from two separate studies. One study was part of a larger project on the effect of pectin on GI function, hereafter referred to as the 'biopsy study' [1; 30]. This involved a randomized, double-blind, placebo-controlled trial in healthy young adults (18-40 years) and healthy elderly (65-75 years), which had been registered in the US National Library of Medicine (NCT02376270). The second study is part of a larger cohort study on the phenotypic and genotypic characterization of IBS patients, hereafter referred to as the 'Maastricht IBS (MIBS) cohort' [17; 20], which is registered in the US National Library of Medicine (NCT00775060). Subjects aged between 18 and 75 years were included in this primary-tertiary care cohort study. IBS was diagnosed using the Rome III criteria in all patients. Fulfilment of criteria was checked by means of interview by an experienced clinical researcher (medical doctor or last year medical doctor in training). In addition, healthy controls (*i.e.* no current or past gastrointestinal disorders) were included in this cohort study. Patient characteristics of the Maastricht IBS cohort, including symptom scores, intestinal permeability, serotonin metabolism and visceral hypersensitivity has been previously

Healthy subjects (biopsy study)	IBS patients and healthy controls (Maastricht IBS cohort)	
18-40 years: Young adults (n = 52)	IBS patients	Healthy controls
65-75 years: Elderly (n = 48)	18-39 years: Young adults (n = 191) 40-64 years: Middle-aged adults (n = 209) 65-75 years: Elderly (n = 63)	18-39 years: Young adults (n = 90) 40-64 years: Middle-aged adults (n = 84) 65-75 years: Elderly (n = 43)
Gastrointestinal Symptom Rating Scale (n = 100)	Gastrointestinal Symptom Rating Scale ($n = 463$ IBS patients, n = 217 healthy controls)	
Visceral pain-associated parameters (sigmoid biopsies) - TRPA1 and TRPV1 gene mRNA expression (n = 20) - TRPA1 and TRPV1 nerve fibre stainings (n = 16)	Visceral hypersensitivity (rectal barostat procedure; n = 233 IBS pat	ients, n = 103 healthy controls)

Table 4.1. Overview of available da	ata
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reported elsewhere [17; 20; 21; 26; 27; 29]. The current exploratory study was specifically aimed at investigating age-related changes. Both studies had been approved by the Maastricht University Medical Center+ (MUMC+) Ethics Committee. All study procedures were performed in compliance with Good Clinical Practice Guidelines and according to the revised Declaration of Helsinki. All subjects gave written informed consent prior to participation. An overview of the obtained data per study and sample sizes is provided in Table 4.1. Details on the methods are provided below.

Gastrointestinal Symptom Rating Scale (GSRS) questionnaire

The GSRS was completed by all subjects in both studies. This 15-item questionnaire encompasses a range of gastrointestinal symptoms, including abdominal pain, bloating, flatulence, frequency of bowel movements, stool consistency, urgency, and the feeling of incomplete evacuation [25]. Symptoms are combined into five clusters, which include abdominal pain, reflux, diarrhoea, indigestion and constipation. The intensity of symptoms is scored on a 7-grade Likert scale (1, no symptoms at all; 2, minimal symptoms; 3, mild symptoms; 4, moderate symptoms; 5, rather serious symptoms; 6, serious symptoms; and 7, very severe symptoms).

Sigmoid biopsies – TRPA1 and TRPV1 analyses

In the biopsy study, tissue samples were obtained from the sigmoid colon by means of standard flexible sigmoidoscopy and snap frozen in liquid nitrogen upon storage at -80C until further analysis. No bowel preparation was used. Biopsies were obtained after the supplementation period, and only subjects receiving placebo were included for TRP analyses. Note that placebo consisted of the polysaccharide maltodextrin. Maltodextrin is completely digested and absorbed in the small intestine and therefore has no significant effect on the colon [14].

TRPV1 and TRPA1 mRNA expression

Sample homogenisation of biopsy specimen was performed in 1 mL TRI Reagent (Molecular ResearchCentre Inc., Cincinatti, OH, USA) and total RNA was isolated with Direct-Zol RNA MiniPrep isolation kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. Samples were then measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) to assess RNA quantity and purity. After treatment with deoxyribonuclease I enzyme (Zymo Research, Irvine,CA) total RNA (100 ng) was reverse transcribed with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. To amplify trancripts, real-time qPCR was performed on a Stratagene Mx3000P qPCRSystem (Agilent Technologies, Santa Clara, CA) using Luminaris HiGreen LowROX qPCR Master Mix (Thermo Fisher Scientific) (Bohonyi et al, 2017). The following primer pairs were used to amplify the genes of interest: Trpv1 (NM_080706.3) (sense): 5'-CAGCTCAATTGCTGTGCAGGTTA-3' and (antisense): 5'-TGCCAGTATGGATGGAAGTGGAA-3'; Trpa1 (NM_007332.2) (sense): 5'-ATGGACAGCTTGGTTACCTCCAC-3' and (antisense): 5'-CAGCACTCTGCTGGTTTGTATGAA -3'. All reactions were measured in triplicates and the geometric mean of their Ct values were calculated which was normalized to transcripts of the glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (NM_001289746.1) (sense): 5'- CCTGCACCACCAACTGCTTA -3' and (antisense): 5'-TAGAGGCAGGGATGATGTTCTG-3'; used as reference gene. Primers with similar efficiencies were used and melt curve analyses were performed to verify primer specificity. The determination of relative messenger RNA (mRNA) expression levels was performed according to the comparative Ct method.

TRPV1 and TRPA1 immunofluorescent staining

Five µm frozen sections of sigmoid biopsies were fixed on Superfrost Plus Microscope Slides (Thermo Fischer Scientific, Waltham, USA) and stained with a 1:100 dilution of guinea pig polyclonal anti-TRPV1 (GP14100, Neuromics, Edina, MN, USA) and 1:200 dilution of rabbit polyclonal anti-TRPA1 (orb86362, Biorbyt, Cambridge, United Kingdom) primary antibodies. Slides were incubated with VECTASTAIN[®] ABC-Peroxidase Kit- Guinea Pig IgG (PK-4007, BioMarker Ltd., Budapest, Hungary) and HISTOLS peroxidase kit - rabbit IgG (30011.R500 Histopathology Ltd, Pécs, Hungary). The reaction was then visualized by 1:2000 dilution of Tyramide Signal amplification kit with HRP-goat anti-rabbit IgG and Alexa Fluor 594 and 488 tyramide (T20925 and T20922, respectively; Life Technologies, Paisley, UK). Nuclear counterstaining for fluorescent microscopy was performed by Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) in 1.5 ug/mL concentration (Vector Laboratories, Burlingame, CA, USA). TRPV1 and TRPA1 immunopositivity was quantified by counting TRPA1 and TRPV1 positive nerve fiber-like structures per area of lamina propria performed by an experienced pathologist who was blind to the treatment order (BK). Incubating sigmoid mucosa with Tris-buffered saline instead of the primary antibodies served as negative control, while sections of previously sampled human dorsal root ganglia expressing TRPV1 and TRPA1 abundantly were used as positive control. The antibody specificity has been validated by preabsorption of the respective blocking peptides (P14100 Neuromics, Edina, MN, USA for TRPV1 and AAP35205 Aviva Systems Biology, San Diego, CA, USA) as described previously [11].

Rectal barostat (balloon distension)

Participants arrived in the hospital after an overnight fast. Rectal perception was measured using an electronic barostat (Distender II; G&J Electronics, Toronto, ON,

Canada, part: C7-CB-R) and a balloon of non-compliant material (Mui Scientific, Missisauga, ON, Canada, part: C7-2CB-R), which was lubricated with KY-gel (Johnsson & Joshnsson, Longhorne, PA, USA). The procedure was executed according to our standardised protocol as described previously [16]. Abdominal pain was scored using a visual analogue scale (VAS) ranging from 0 to 100 mm. For subjects who did not complete the full protocol, VAS-scores for the remaining pressure steps were padded using the highest VAS-score achieved over the protocol thus far. Visceral hypersensitivity was defined as a VAS-score of ≥ 20 mm at a pressure of 26 mmHg or lower.

Data visualization & statistical analysis

All data plots were created using GraphPad Prism 9. Barcharts with error bars include superimposed individual data points. Tabular data are presented as mean \pm standard deviation (SD) for continuous variables and proportions for categorical outcomes. Continuous variables were found to be normally distributed. Statistical analyses were performed in R Statistical Software version 3.6.3 (2020-02-29). Univariate comparisons were performed using an independent samples t-test for continuous variables and χ^2 test for dichotomous variables. A two-sided alpha value of 0.05 was used. Given the hypothesis-generating/exploratory nature of the current study, significance level was not corrected for multiple comparisons.

RESULTS

Subject characteristics

The baseline characteristics of subjects from both studies are displayed in Table 4.2. In the healthy subject subgroup of the MIBS cohort, young and middle-aged subjects were more often female than elderly subjects. Gender was equally distributed in IBS patients (Maastricht IBS cohort) and healthy subjects from the biopsy study. A significantly higher proportion of healthy young adults and middle-aged subjects in the Maastricht IBS cohort was female, as compared to healthy elderly. The BMI of elderly subjects was significantly higher across all groups, as compared to their younger counterparts. Proton pump inhibitors were more frequently used by elderly subjects in the IBS patient subgroup, as well as in the biopsy study, as compared to the respective young subjects. Selective serotonin reuptake inhibitors were more frequently used by middle-aged IBS patients, as compared to elderly IBS patients.

Abdominal symptoms & visceral hypersensitivity

Median and mean GSRS scores are shown in Figure 4.1 and Supplementary Table 4.1, respectively. Scores for abdominal pain were significantly lower in elderly as compared to young subjects in all groups (in both IBS patients and healthy subjects). In IBS patients, scores for diarrhoea were also lower in elderly as compared to young subjects. In healthy subjects from both the Maastricht IBS cohort and the biopsy study, scores for indigestion were lower among elderly subjects.

Visceral hypersensitivity was found to be less common among elderly IBS patients as compared to young adults and middle-aged patients (Figure 4.2). In the youngest age category ranging from 18-39 years, visceral hypersensitivity was found in 63.0% of IBS patients. In middle-aged adult (40-64 years) and elderly (65-75 years) IBS patients, visceral hypersensitivity was found in 40.0% and 25.0%, respectively. A χ^2 test on all three age categories indicated a significant difference between at least two age groups (χ^2 (1, N = 233) = 17.65, p <0.001). Post-hoc testing revealed that visceral hypersensitivity was significantly more common in young-adults versus elderly (χ^2 (1, N = 128) = 12.75, p <0.001) and in young-adults versus middle-aged patients (χ^2 (1, N = 205) = 10.85, p = 0.001). Overall low percentages of visceral hypersensitivity were found in all age groups of healthy controls (7.1%, 4.4% and 10.0% respectively).

TRPA1 and TRPV1 immunoreactivity & mRNA expression profiles in healthy subjects

TRPA1, TRPV1 and TRPA1/TRPV1 composite immunoreactivity was significantly lower in elderly subjects (Figure 4.3A). In addition, TRPA1 and TRPV1 immunoreactivity demonstrated a strong correlation (r = 0.865, p < 0.01, Figure 4.3B). Relative mRNA expression of TRPA1, but not TRPV1, was significantly lower in elderly subjects (Figure 4.3C). Representative immunofluorescence slides of TRPA1 and TRPV1 stainings in young adults and elderly are shown in Figure 4.3D. A moderate correlation was found between TRPA1 and TRPV1 relative mRNA expression levels, though this correlation failed to reached statistical significance (r = 0.442, p = 0.051, Supplementary Figure 4.1A). Immunoreactivity and relative mRNA expression levels correlated moderately for both TRPA1 (r = 0.530, p = 0.038, Supplementary Figure 4.1B) and TRPV1 (r = 0.566, p = 0.022, Supplementary Figure 4.1C. Abdominal pain scores correlated moderately with TRPV1 relative expression (r = 0.502, p =0.024, Supplementary Figure 4.2B). No significant correlations were found between abdominal pain scores and TRPA1 relative expression, nor with TRPA1 and TRPV1 immunoreactivities (Supplementary Figure 4.2).

	IBS patients (M	(IBS)		Healthy subjec	ts (MIBS)		Healthy subjec	ts (biopsy study)
	Young adults	Middle-aged	Elderly	Young adults	Middle-aged	Elderly	Young adults	Elderly
Ν	191	209	63	06	84	43	52	48
Age	26.9 ± 6.1	52.5 ± 7.2	70.3 ± 4.1	23.9 ± 4.1	55.1 ± 7.4	68.4 ± 2.5	23.1 ± 4.3	69.7 ± 2.8
(yrs, mean±SD)								
Female (%)	77.0	72.2	65.1	72.2**	60.7*	41.9	57.7	43.7
BMI	$23.7 \pm 4.9^{***}$	25.7 ± 4.3	26.4 ± 4.1	$22.5 \pm 2.9^{**}$	25.2 ± 4.2	25.0 ± 3.4	$22.9 \pm 2.7^{***}$	25.8 ± 2.7
$(kg/m^2, mean \pm SD)$								
Symptom duration (yrs, mean \pm SD)	7.2 ± 6.9***	17.3 ± 15.7	21.6 ± 18.7	N.A.	N.A.	N.A.	N.A.	N.A.
IBS subtype (%)				N.A.	N.A.	N.A.	N.A.	N.A.
IBS-C	17.2	21.3	21.3					
IBS-D	33.9	36.6	27.9					
IBS-M	45.2	34.7	45.9					
IBS-U	3.8	7.4	4.9					
Medication (%)								
Idd	11.5***	33.0	30.2	1.1	3.6	7.0	**0	12.5
NSAID	24.1*	28.7	38.1	13.3	21.4	25.6		
SSRI	9.4	17.2*	6.3	2.2	2.4	4.7		
Motility + drugs	17.8	18.2	23.8	0	0	0		
Motility - drugs	14.1	16.3	12.7	0	0	0		ı

Table 4.2. Baseline characteristics of IBS patients (MIBS cohort) and healthy subjects (MIBS cohort and biopsy study).

Abbreviations: IBS-C = IBS constipation predominant subtype, IBS-D = IBS diarrhoea predominant subtype, IBS-M = IBS mixed subtype, IBS-U = IBS unspecified subtype, PI = proton pump inhibitor, NSAID = non-steroidal anti-inflammatory drug, SSRI = selective serotonin reuptake inhibitor. ropord 5

Motility + drugs: e.g. polyethylene glycol, motility – drugs: e.g. loperamide.

MIBS age groups: 18-39 years (young adults), 40-64 years (middle-aged adults), 65-75 years (elderly)

Biopsy study age groups: 40-64 years (middle-aged adults), 65-75 years (elderly)

4



Figure 4.1. Boxplot showing GSRS scores in IBS patients (panel A), healthy subjects from the Maastricht IBS cohort study (panel B) and healthy subjects from the biopsy study (panel C). Plots include median, interquartile range and minimum and maximum values.

Independent t-tests were used for comparison of mean GSRS scores per age group. * P < 0.05 ** P < 0.01 *** P < 0.001



Figure 4.2. Occurrence of visceral hypersensitivity in healthy (control) subjects and IBS patients, based on rectal barostat data (semi-random staircase protocol) from the MIBS cohort. χ^2 tests were used for comparing prevalence of visceral hypersensitivity per age group.



Figure 4.3 (panel A-D). Panel A: Immunoreactivity values (mean number of stained fibers per mm² of lamina propria) of TRPA1, TRPV1 and combined (TRPA1/TRPV1 composite) of sigmoid biopsies from healthy young adults and healthy elderly. Panel B: Correlation of immunoreactivity of TRPA1 and TRPV1 (mean number of stained fibers per mm² of lamina propria), demonstrating co-expression. Panel C: TRPA1 and TRPV1 expression (relative to GADPH as reference gene) in young adults and elderly. Panel D: immunofluorescence images demonstrating abundant staining of TRPV1 and TRPA1 in sigmoid biopsies from young adults, but not in the elderly.

Abbreviations: DAPI = 4',6-diamidino-2-phenylindole (cellular immunofluorescence stain).

DISCUSSION

To the best of our knowledge this is the first study to explore associations between agerelated changes in abdominal symptoms and TRPA1 and TRPV1 expression profiles in humans. First, our study corroborates the previously reported finding that abdominal pain decreases with age. Importantly, this decrease was not limited to healthy subjects, as it was also observed in a large group of IBS patients. We have previously reported that a significant proportion of IBS patients demonstrates lower gastrointestinal symptom scores over a follow-up period of five years as compared to their baseline values [29]. In the current study, we found that other gastrointestinal symptoms, such as diarrhoea and indigestion also decreased with ageing in IBS patients and healthy subjects, respectively. It should be noted that, based on these symptom reports alone, one cannot differentiate between an effect of ageing on visceral nociception and IBS natural disease progression. Hence, in the current study we have attempted to explore potential underlying mechanisms of decreasing abdominal pain scores with ageing in both IBS patients and healthy individuals.

In line with the decreased abdominal pain scores, we observed that visceral hypersensitivity measured by rectal barostat procedure, was significantly less common among elderly IBS patients than younger patients. This reduction was also apparent when comparing young adults and middle-aged IBS patients, pointing to a gradual decline in visceral sensitivity with age. Visceral hypersensitivity was an infrequent finding in healthy volunteers in all age groups, hence no decline with age was observed in this subgroup of healthy controls.

In a subgroup of healthy subjects, we used immunofluorescence to measure TRPA1 and TRPV1 protein expression in sigmoid biopsies of both healthy young adults and elderly subjects. These TRP channels have been implicated to play important roles in the transduction of nociceptive information from the gut and several studies have shown upregulation of TRPV1 in the colonic mucosa of IBS patients [2]. TRP channels have therefore been implicated as potential therapeutic targets for the treatment of chronic abdominal pain. Here we found, for the first time in human colonic tissue, that TRPA1 and TRPV1 immunoreactivity was significantly lower in biopsies from healthy elderly subjects compared to their younger counterparts. Given the role of these molecular transducers in visceral nociception, we subsequently explored associations between TRPA1 and TRPV1 immunoreactivity and abdominal pain scores. Only TRPV1 mRNA expression correlated moderately with abdominal pain. No significant correlations were found between abdominal pain scores and TRPA1 mRNA expression, nor with immunoreactivity of TRPA1 or TRPV1. It should be pointed out, however, that abdominal pain scores were rather low in this specific subgroup of healthy subjects, with a maximum pain score of only 3 (on a 7-point Likert scale.

Another important observation is the strong correlation between TRPA1 and TRPV1 immunoreactivity, which is in line with previous findings indicating that TRPA1 is almost exclusively expressed on TRPV1-positive neurons [2].

Using quantitative RT-PCR, we found that TRPA1, but not TRPV1, mRNA expression levels were lower in elderly subjects. The discrepancy between the protein and mRNA expression levels can in part be explained by the fact that PCR encompasses TRP mRNA from all cellular origins, including non-neuronal (e.g. epithelial), whereas for immunofluorescence staining was predominantly quantified for (morphologically defined) neural fibres. Moreover, not all mRNA is converted into protein and this may also be affected by ageing (*i.e.* mRNA decay). The latter was also found in a mouse study, where TRPV1 protein expression decreased by ageing in dorsal root ganglion (DRG) neurons, but TRPV1 mRNA expression levels were not altered [28]. In line with these findings, we observed only modest (but statistically significant) correlations between immunoreactivity and expression levels from the same TRP channel (Supplementary Figure 4.1B and C). Another question is to which extent neural elements are affected by potential mRNA decay. Findings in literature are somewhat incongruent in this respect. Cibert-Goton et al. found decreased afferent responses to capsaicin, but only in multiunit and not in single-unit recordings [4]. In addition, McGuire et al. found no effect of age on single unit responses to von Frey hair probing, bradykinin or ATP [19]. On the other hand, Keating et al. showed that the decreased response to capsaicin was observed in single-unit recordings, which would rather suggest age-related loss in TRPV1 channel function.

It has been postulated that neurodegeneration, in particular of the enteric nervous system, plays a key role in the deterioration of gut functioning with increasing age [23; 24]. Indeed, the ENS regulates most gastrointestinal functions, ranging from absorption and secretion to motility, afferent signaling, and also immune and inflammatory responses. Thus, decreased functioning of the ENS would reflect on all these functions. A limitation of the current study is that we did not obtain specific neural stainings in the biopsy materials. It is therefore unclear whether the decreased TRPV1 and TRPA1 immunoreactivity was due to decreased expression on afferent fibers, or due to neurodegeneration (*i.e.* lower density of neural fibers). The distinction is particularly difficult to make for gastrointestinal biopsy specimens, as TRPV1 and TRPA1 themselves are not solely involved in nociception, but have been implicated in visceral motility and permeability as well [2]. In order to obtain a complete view of the enteric nervous system one would require intestinal resection material instead of mucosal biopsy specimens.

When exploring associations between TRPA1 and TRPV1 expression levels and abdominal pain scores, a moderate correlation was found for TRPV1 expression only. One should realise that pain is a highly complex mechanism, as not only biological factors (*e.g.* neural density and afferent functioning), but also psychological factors (*e.g.* cognitions

Chapter 4

and stress) play a substantial role in the ultimately perceived sensation after an applied stimulus. As a result, there is a non-linear relationship between peripheral nociceptive input and pain sensation. Cognitions about the meaning of pain (and other abdominal symptoms) as well as coping strategies may be particularly important in the context of decreased abdominal symptoms with ageing. Unfortunately, these factors are rather subjective and therefore, difficult to measure, and as such, have not been included in the present study. Although one can only speculate about the importance of altered coping strategies with ageing, our study provides evidence that decreased sensory function of the gut involves biological changes in visceral afferents, as indicated by the decreased TRPA1 and TRPV1 expressions. Changes in abdominal symptoms over long periods of time may however also be related to habituation [6], especially since our elderly IBS patients reported a significant longer duration of IBS symptoms compared to the younger patient groups. Nevertheless, such an effect is unlikely to play a role when using the rectal barostat procedure, which was novel to all subjects. We therefore think that further research is warranted for investigating the association between reduced abdominal symptoms and visceral hypersensitivity with age, and changes in neural TRP channel expression profiles. The latter should also account for neural density, as current evidence cannot rule out that the decreased nociceptive sensitivity could be driven by neurodegeneration rather than specific alterations in afferent functions. In this sense, age-related changes in nociceptive signaling can potentially have beneficial effects in patients experiencing chronic pain as a result of nociceptive overdrive or other forms of visceral hypersensitivity.

Another limitation of our paper is that we did not obtain biopsies from IBS patients. Had we done so, we could have assessed whether there are any age-related changes in expression profiles that are specific to IBS. Therefore, we consider our study exploratory in nature and further studies will be warranted to examine exact underlying mechanisms in different (patient) populations. Results presented in the current study need to be interpreted in light of the exploratory nature. This is particularly important with our biopsy results, where sample sizes (and as such statistical power) were limited. Finally, one should keep in mind that data from two separate studies was used, which poses methodological limitations (e.g. variability in subject populations).

Understanding the processes of the age-related decrease of the intensity of abdominal sensations can have important therapeutic repercussions. First, the decreased perception of adverse stimuli is beneficial for patients with chronic abdominal pain, such as in IBS. Given this observation, patients can be reassured that their symptoms are likely to diminish over time. Moreover, insights regarding this natural analgesic effect of ageing could prove valuable for the development of novel (visceral) pain management strategies.

CONCLUSION

An age-related decrease in the reporting of abdominal pain is found in both IBS patients and healthy individuals. Furthermore, the prevalence of visceral hypersensitivity in IBS patients decreases with age. This may be attributed to biological changes, such as decreased TRPA1 and/or TRPV1 receptor expression in the intestinal epithelium. Further studies are needed to specify the underlying mechanisms and the association with intestinal health.

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SUPPLEMENTARY MATERIAL



Supplementary Figure 1 (panel A-C). Panel A: Correlation between TRPA1 relative expression and TRPV1 relative expression. Panel B: Correlation between TRPA1 immunoreactivity (mean number of stained fibers per mm² of lamina propria) and TRPV1 relative expression. Panel C: Correlation between TRPV1 immunoreactivity and TRPV1 relative expression.



Supplementary Figure 2 (panel A-D). Correlation between abdominal pain and A) TRPA1 relative expression, B) TRPV1 relative expression, C) TRPA1 immunoreactivity (mean number of stained fibers per mm² of lamina propria) and D) TRPV1 immunoreactivity.

PART II

VISCERAL PAIN IN HYPERMOBILITY SPECTRUM DISORDERS

CHAPTER 5

Gastrointestinal disorders in joint hypermobility syndrome/Ehlers-Danlos syndrome hypermobility type: a review for the gastroenterologist

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ABSTRACT

Background. Joint hypermobility syndrome (JHS) / Ehlers-Danlos syndrome hypermobility type (EDS-HT) is the most common hereditary non-inflammatory disorder of connective tissue, characterized by a wide range of symptoms, mainly joint hyperextensibility and musculoskeletal symptoms. A majority of patients also experiences gastrointestinal (GI) symptoms. Furthermore, JHS/EDS-HT has specifically been shown to be highly prevalent in patients with functional GI disorders, such as functional dyspepsia and irritable bowel syndrome.

Purpose. The aim of this review is to examine the nature of gastrointestinal symptoms and their underlying pathophysiology in JHS/EDS-HT. In addition, we consider the clinical implications of the diagnosis and treatment of JHS/EDS-HT for practising clinicians in gastroenterology. Observations summarized in this review may furthermore represent the first step towards the identification of a new pathophysiological basis for a substantial subgroup of patients with functional GI disorders.

INTRODUCTION

Joint hypermobility syndrome (JHS) / Ehlers-Danlos syndrome hypermobility type (EDS-HT) is presumably the most common hereditary non-inflammatory disorder of connective tissue laxity, mainly characterized by joint hyperextensibility in association with musculoskeletal symptoms. JHS/EDS-HT is recognised as a multisystem disorder [9]. For instance, autonomic dysfunction, chronic pain syndromes, urinary dysfunction, anxiety and mood disorders, as well as structural disorders such pelvic organ prolapse have been described in JHS/EDS-HT patients [9], (see figure 5.1) and occur more frequently in JHS/EDS-HT compared to age and gender matched healthy controls [8]. Notably, there is a high prevalence of gastrointestinal (GI) symptoms in patients with JHS/EDS-HT, such as epigastric discomfort, gastro-oesophageal reflux and constipation [10; 48; 65]. In one study, GI symptoms were reported to be particularly prominent in the JHS/EDS-HT patients who also had high levels of fatigue, cutaneous changes, orthostatic, immune, urogynaecological, visual and respiratory problems and reduced quality of life metrics [19]. However, there are considerable inter-individual differences in the clinical presentation of JHS/EDS-HT, thereby complicating its diagnosis and management [19].



Figure 5.1. Systemic manifestations of joint hypermobility syndrome / Ehlers Danlos syndrome hypermobility type. Adapted from Castori et al. [9]

It should be noted that although EDS-HT shows a significant phenotypic overlap with the joint hypermobility syndrome (JHS), overall consensus regarding the exact relation between these syndromes is currently lacking, mainly because their genetic background is incompletely understood [11; 35]. To date, there are three different opinions concerning the overlap between EDS-HT and JHS: (a) EDS-HT and JHS are the same disorder in all circumstances; (b) EDS-HT and JHS are partially overlapping conditions and correspond to the same disorder in selected circumstances (e.g. familial cases); (c) EDS-HT and JHS are two always separated disorders. Available literature however is scarce of data in support to any of the above hypotheses [11]. Because of this lack of evidence, we use the term JHS/EDS-HT throughout this review article.

A central defining feature of JHS/EDS-HT is generalized joint hypermobility, which refers to the characteristic of being able to actively and/or passively move joints beyond normal limits [15]. Joint hypermobility can affect a few joints (localized or monoarticular joint hypermobility) or several joints in multiple body sites (generalized joint hypermobility, gJHM). gJHM, in the absence of other symptoms, is considered to be a harmless trait and may even confer advantages for certain areas of endeavour such as ballet dancers and gymnasts [56]. In Western populations the prevalence of gJHM is in the order of 10-20%, and even higher rates have been described in Asian and African population groups [24; 33]. The reported population prevalence of JHS/EDS-HT of 1:5000 suggests that the risk of developing the syndrome in an individual with gJHM is small [41].

Generalized joint hypermobility can be screened for by using a simple validated fivepoint questionnaire, but is formally evaluated using the Beighton score (see table 5.1). The definitive diagnosis of JHS/EDS-HT however requires additional criteria to be fulfilled. Whereas the Villefranche criteria (1997, table 5.2) are traditionally used for the diagnosis of EDS-HT, JHS is diagnosed using the Brighton criteria (1998, table 5.2) [4; 32]. The

Table 5.1. Assessment of generalized joint hypermobility

Five-point questionnaire for generalized joint hypermobility (screening tool). Answering yes to 2 or more of these questions suggests hypermobility with sensitivity of 85% and specificity 90%.

- 1. Can you now (or could you ever) place your hands flat on the floor without bending your knees?
- 2. Can you now (or could you ever) bend your thumb to touch your forearm?
- 3. As a child, did you amuse your friends by contorting your body into strange shapes or could you do the splits?
- 4. As a child or teenager, did your kneecap or shoulder dislocate on more than one occasion?
- 5. Do you consider yourself "double-jointed"?

Beighton Score (clinical assessment of joint hypermobility). Score 0-9.

- 1. Passive dorsiflexion of the little fingers beyond 90 degrees (one point for each hand). Two points
- 2. Passive apposition of the thumbs to the flexor aspects of the forearms (one point for each thumb). Two points
- 3. Hyperextension of the elbows beyond 10 degrees (one point for each elbow). Two points.

4. Hyperextension of the knees beyond 10 degrees (one point for each knee). Two points.

 Forward flexion of the trunk with knees fully extended so that the palms of the hands rest flat on the floor. One point.

Table 5.2. Diagnostic criteria	for JHS (left) and EDS-HT (right)
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1998 Brighton Classification for Joint Hypermobility Syndrome (JHS) [32]

Villefranche criteria for the hypermobility type of Ehlers-Danlos Syndrome [4]

Synarome ()113) [32]		Dunios Synurome [4]			
Ma	jor criteria	Ma	ijor criteria		
1.	Beighton score of 4/9 or greater (either currently or historically)	1.	Generalized joint hypermobility (defined as Beighton score ≥ 5/9)		
2.	Arthralgia for longer than 3 months in 4 or more joints	2.	Skin involvement: hyperextensibility or smooth velvety skin		
Minor criteria		Minor criteria			
1.	Beighton score of 1, 2, or 3/9 (0, 1, 2, or 3 if aged 50þ)	1. 2.	Recurrent joint dislocations Chronic limb/joint pain		
2.	Arthralgia (for 3 months or longer) in 1–3 joints, back pain (for 3 months or longer), or spondylosis, spondylolysis, spondylolisthesis	3.	Positive family history		
3.	Dislocation/subluxation in more than one joint or in one joint on more than one occasion				
4.	Soft tissue rheumatism: 3 or more lesions (eg, epicondylitis, tenosynovitis, bursitis)				
5.	Marfanoid habitus (tall, slim, span/height ratio >1.03 upper: lower segment ratio <0.89, arachnodactyly [positive Steinberg/wrist signs])				
6.	Abnormal skin: striae, hyperextensibility, thin skin, papyraceous scarring				
7.	Eye signs: drooping eyelids, myopia, or antimongoloid slant				
8.	Varicose veins, hernia, or uterine/rectal prolapse				
A a and un	liagnosis of JHS requires 2 major criteria, or 1 major d 2 minor, or 4 minor, or 2 minor in the presence of an equivocally affected first-degree relative.	The ind lea	e presence of at least 1 major and 1 minor criteria is licative, a definitive diagnosis of EDS-HT requires at st 2 major criteria		

reader can appreciate the considerable overlap between the two different diagnostic criteria. International consensus currently considers the Brighton criteria as an extension of the Villefranche criteria and is therefore more often applied in clinical practice [9]. In addition, the Brighton criteria are more suitable for assessing adults considering the natural loss of joint mobility by age, whereas the Villefranche criteria are more adequate for evaluating children and young adults [15]. However, more recently, the diagnostic criteria are being harmonized by a group of international experts and the new criteria are expected to be published towards the end of 2016.

Interestingly, JHS/EDS-HT has specifically been shown to be highly prevalent in patients with functional GI disorders (FGIDs) [10; 26; 29; 48; 65], which include irritable bowel syndrome (IBS) and functional dyspepsia (FD). FGIDs are common, affecting up to 20% of the general population and accounting for more than one third of gastroenterologists' workload in secondary care [1]. Though the severity of complaints varies, it has been demonstrated that FGIDs exert a marked socioeconomic burden through increased absenteeism and healthcare utilization [57]. An important aspect is the chronic character of complaints and significant comorbidities associated with FGID e.g. fibromyalgia, autonomic dysfunction and psychopathology, which can have profound effects on quality of life in these patients [51]. It is tempting to assume that patients presenting with signs and symptoms representative of JHS/EDS-HT constitute a separate clinical subgroup of patients with FGIDs in whom therapeutic considerations may differ from those without JHS/EDS-HT. The aim of this review is to examine the nature of GI symptoms and their underlying pathophysiology in JHS/EDS-HT with a particular focus on the functional GI disorders encountered in these patients and to consider the clinical implications of the diagnosis for practising clinicians.

METHODS

Evidence to support this review was obtained from an electronic database search via PubMed using the following search Medical Subject Headings (MESH) terms: Ehlers-Danlos hypermobility type OR Ehlers- Danlos type III OR joint hypermobility syndrome OR JHS AND gastrointestinal manifestations OR gastrointestinal symptoms OR functional gastrointestinal disorders). This search strategy yielded 83 articles. Articles discussing generalized joint hypermobility, thereby focusing on the trait rather than the syndrome were excluded from further analysis. Although these may also include JHS/EDS-HT patients, this population was considered too heterogeneous, which could complicate the generalizability of results. For the same reason, articles discussing different EDS subtypes than JHS/EDS-HT were excluded. Furthermore, case reports were excluded from analysis because we believe these might not give an appropriate representation of the JHS/EDS-HT population and only describe the most catastrophic phenotypes. We would however like to refer the reader to a recent review summarizing these papers [12]. All residual articles discussing GI manifestations in JHS/EDS-HT or JHS were further examined for useful references that did not emerge in the initial search. A total of 11 articles that focussed on GI manifestations in JHS/ EDS-HT were selected for inclusion. One study found a high prevalence of gJHM in a paediatric population of patients with FGIDs, however, as it did not meet the inclusion criteria for the current manuscript, we have not provided further details [39]. Unfortunately, no studies discussing GI manifestations in paediatric JHS/EDS-HT patients were identified, limiting evidence of the current review to the adult population. In addition to the systematic search on the gastrointestinal manifestations in JHS/ EDS-HT as described above, we investigated current literature to explore non-GI manifestations in JHS/EDS-HT in a narrative approach to merge parallel findings in order to offer a reasonable insight into pathogenesis.

RESULTS

JHS/EDS-HT and functional gastrointestinal disorders

Several reports have been published to describe the putative relationship between JHS/ EDS-HT and GI symptoms [10; 26; 29; 48; 65] (see supplementary table 5.1).

In 2010, Castori et al. published a pilot study on manifestations of JHS/EDS-HT in 21 patients, in which GI symptoms were present in 86% (18/21) of patients, including dyspepsia (66.7%), gastroesophageal reflux (57.1%), recurrent abdominal pain (61.9%) and constipation/diarrhoea (33.3%) [10].

Fikree et al. were the first to perform a prospective evaluation to investigate the relationship between JHS/EDS-HT and FGIDs. The prevalence of JHS/EDS-HT was found to be 33% in an unselected group of patients referred to their secondary care gastroenterology clinic [29]. In addition, these patients were compared to patients who were referred from the rheumatology clinic with a previously confirmed diagnosis of JHS/EDS-HT and were characterized by higher prevalence of various musculoskeletal features and higher Beighton scores. A high prevalence of GI symptoms was found in all groups, though symptoms were most common in rheumatology referrals (see table 5.3). Moreover, these patients were more likely to be diagnosed with a FGID (see figure 5.2).

	Non-JHS/EDS-HT (CI)	JHS/EDS-HT Unselected (CI)	OR adjusted (CI)	JHS/EDS-HT Rheumatology (CI)	OR adjusted for age and gender (CI)
Alternating bowel habit	30.4 (25.6 - 35.6)	38.6 (31.2 - 46.3)	1.38 (0.96 - 2.03)	65.8 (49.4 - 79.9)	1.70 (1.27 – 2.27)
Abdominal pain	83.2 (76.9 - 85.4)	90.3 (83.2 - 93.4)	1.62 (0.91 – 2.9)	100 (91.4 – 100)	2.04 (1.20 - 3.45)
Globus	19.1 (15.1 – 23.6)	27.2 (20.7 - 34.4)	1.46 (0.94 - 2.26)	47.7 (32.5 - 63.3)	1.64 (1.21 – 2.22)
Heartburn	23.5 (19.2 – 28.3)	33.0 (26.1 - 40.4)	1.66 (1.1 – 2.5)	25.0 (13.2 - 40.3)	1.29 (0.96 - 1.75)
Water brash	18.5 (14.5 – 22.9)	30.9 (24.1 -38.3)	2.02 (1.3 - 3.1)	29.5 (16.8 - 45.2)	1.59 (1.17 – 2.16)
Dysphagia	10.6 (7.5 – 14.3)	1647.1 (11.0 – 22.4)	1.59 (0.92 – 2.7)	31.8 (18.6 – 47.6)	1.79 (1.25 – 2.56)
Early satiety	42.8 (37.7 - 48.0)	53.4 (45.7 - 61.0)	1.4 (0.98 – 2.1)	79.1 (64.0 – 90.0)	1.68 (1.26 – 2.23)
Postprandial fullness	27.1 (22.6 - 32.1)	41.4 (34.0 – 49.1)	1.74 (1.2 – 2.6)	61.4 (45.5 -75.6)	1.72 (1.29 – 2.29)
Regurgitation	11.4 (8.3 – 15.2)	17.5 (12.2 – 24.1)	1.57 (0.93 – 2.6)	33.3 (19.6 - 49.5)	1.70 (1.20 – 2.42)
Bloating	47.9 (42.6 - 53.2)	54.3 (46.6 - 61.8)	1.15 (0.79 – 1.67)	88.6 (75.4 - 96.2)	1.60 (1.20 – 2.14)

 Table 5.3.
 Comparison of prevalence of GI symptoms in non-JHS/EDS-HT patients, JHS/EDS-HT patients

 from the unselected group and JHS/EDS-HT patients referred from rheumatology.

Reprinted from A prospective evaluation of undiagnosed joint hypermobility syndrome in patients with gastrointestinal symptoms. CIs for proportions and ORs are presented in brackets.

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More recently, Fikree et al. prospectively examined the relationship between JHS/ EDS-HT and FGIDs in a case-control design study comparing patients with a diagnosis of a FGID or an organic GI disorder [26]. There was a significantly higher prevalence of JHS/EDS-HT in the FGID group (39% versus 27.5%, p = 0.002). Interestingly, the prevalence of JHS/EDS-HT was 51% in patients with postprandial distress syndrome, a subtype of functional dyspepsia.

In 2013, Zeitoun and co-workers performed a cohort study of EDS patients and found that gastro-oesophageal reflux (heartburn and regurgitation) and dyspepsia (epigastric pain, nausea, postprandial fullness and belching) were present in around 70% of the subjects [65]. Furthermore, 48% of all participants met criteria for IBS (using the Rome III criteria) and 36% had functional constipation. In addition, these authors reported a greatly reduced GI-related quality of life index.

More recently Nelson et al. performed a retrospective review of patients diagnosed with EDS, of which 71% JHS/EDS-HT [48]. Of 687 patients, 378 (55%) had GI symptoms, most notably abdominal pain (56%), nausea (44%), constipation (42%), heartburn (38%), irritable bowel like-symptoms (30%), vomiting (25%), and diarrhoea (23%). In EDS patients who underwent physiology testing, abnormal gastric emptying was observed in 22.3%; 11.8%

delayed and 10.5% accelerated. Colonic transit was abnormal in 28.3%; 19.6% delayed and 8.7% accelerated. Rectal evacuation disorder was confirmed in 18/30 (60%) patients who underwent anorectal physiology measurements. The finding of GI dysmotility in these patients is consistent with a previously observed high prevalence of dysmotility in patients with gJHM attending tertiary care neurogastroenterology clinics [64].

Table 5.4 shows a summary of prevalence data on GI symptoms in JHS/EDS-HT from the currently published studies.

Symptom	Range	Median (IQR)	References
Nausea	32 - 71%	45 (35 - 65)	[29; 48; 65]
Vomiting	14 - 25%	14	[29; 48]
Globus	(1% *) 27 - 48%	27	[29; 48]
Heartburn	25 - 69%	38 (29 - 63%)	[10; 29; 48; 65]
Regurgitation	(4% *) 18 - 69%	33 (8 - 60%)	[10; 29; 48; 65]
Water brash	(1% *) 30 - 31%	30	[29; 48]
Belching	(3% *) 41- 71%	43 (13 - 65%)	[29; 48; 65]
Bloating	(17% *) 54 - 89%	54	[29; 48]
Dysphagia	11% - 63%	24 (13 - 55%)	[29; 48; 65]
Dyspepsia	(11% *) 34- 63%	43 (17 - 63%)	[10; 29; 48]
Epigastric pain	79%		[65]
Postprandial fullness	(7% *) 41 - 67%	51 (16 - 66%)	[29; 48; 65]
Abdominal pain	56 - 100%	76 (58 - 98%)	[10; 29; 48]
IBS-like symptoms	30 - 47%	39	[48; 65]
Constipation	19 - 42%	35 (23 - 41%)	[10; 29; 48; 65]
Diarrhoea	23 - 42%	32 (25 - 40%)	[10; 29; 48; 65]
Alternating bowel habit	39 - 66%	52	[29]

 Table 5.4. Gastrointestinal symptoms in JHS/EDS-HT patients (Collective data from all studies included for analysis)

*Globus, regurgitation, water brash, belching, bloating, dyspepsia and postprandial fullness were less prevalent in the study of Nelson et al. (values shown in brackets).

(IQR = interquartile range, given when available)

JHS/EDS-HT AND ORGANIC DISORDERS

Hiatus hernia

Zeitoun et al. reported that 19 of 72 EDS patients (26.4%) that had undergone upper endoscopy were shown to have a hiatal hernia [65]. This corresponds to the number reported by Al-Rawi et al. investigating the presence of hiatal hernias in patients with gJHM (22%) [2].

Pelvic organ prolapse and defecatory disorders

Pelvic organ prolapse has been extensively investigated in the past, though mainly in gJHM [47]. One small study found a high prevalence (75%) of pelvic organ prolapse in women with EDS [8]. Another study demonstrated the frequent occurrence of defecatory problems in JHS/EDS-HT patients, most notably straining (61.7%), incomplete evacuation (63%) and digitation (33.3%) [45]. Furthermore, the majority (73.3%) of the JHS/EDS-HT group had a clinically significant prolapse compared to 35% of the control group (P < 0.001).

In an earlier study, Manning et al. investigated the association between obstructive defecation and lower urinary tract dysfunction in JHS/EDS-HT [43]. It was demonstrated that women presenting with lower urinary tract dysfunction also frequently had symptoms of obstructed defecation. Furthermore, patients with obstructed defecation were more likely to show symptoms of JHS/EDS-HT (70.6% versus 50.0%, p < 0.001). JHS/EDS-HT was therefore considered an important factor in the above mentioned association.

Inflammatory bowel disease

Fikree et al. demonstrated a relatively high prevalence of JHS/EDS-HT in patients with Crohn's disease (8/25, 32%), but not in patients with ulcerative colitis (8/38, 21%) [26]. An earlier study found a significantly higher prevalence of gJHM in Crohn's disease (70.7%) compared to ulcerative colitis (35.7%) (P = 0.0063) and healthy controls (25.3%) (P < 0.0001). No differences were found in the proportion of gJHM between UC patients and healthy controls (P = 0.3). The prevalence of JHS/EDS-HT was relatively low in both groups although it was higher in Crohn's than in UC (12.2% Crohn's disease, 3.57% UC). [61].

Coeliac disease

One small study demonstrated that the prevalence of coeliac disease was 16% in the JHS/EDS-HT group, which is significantly higher than the estimated population prevalence (1%) [17]. These results were broadly corroborated in another prospective case controlled study where JHS/EDS-HT was found in 4 out of 13 (31%) patients attending GI clinics with a new diagnosis of coeliac disease [26]. More recently, Laszkowska et al. performed a nationwide population-based cohort study in Sweden, reporting a 49% increased risk of JHS/EDS-HT in coeliac disease patients (95%CI = 1.07-2.07; p = 0.018) [40].

Supplementary table 5.2 shows a summary of prevalence data on organic GI disorders in JHS/EDS-HT from the currently published studies. It is important to note that the majority of studies performed in JHS/EDS-HT patients to assess GI symptoms or abnormalities (either functional or organic) have a number of limitations. The patient

populations investigated are generally highly selected as these comprise patients attending secondary/tertiary care clinics. We already mentioned the impact of higher Beighton scores on GI symptoms. These studies may have therefore included more severe cases of JHS/EDS-HT. Furthermore, several of the studies are based on a small number of patients. Thus these findings are not necessarily generalizable to the majority of patients with JHS/EDS-HT, most of whom remain undiagnosed and in the community. Primary care studies investigating GI symptoms in JHS/EDS-HT are currently scarce. Fikree et al. demonstrated that healthy students who met criteria for JHS/EDS-HT were more likely to experience multiple GI symptoms in particular postprandial distress syndrome, as compared to healthy non-JHS/EDS-HT students [27]. Finally, comparison of different studies is troublesome due to large differences in methodology (i.e. diagnostic criteria used, healthcare setting and study design). We were therefore unable to perform a meta-analysis of data.

RELEVANT NON-GI MANIFESTATIONS IN JHS/EDS-HT

There is growing evidence that patients with JHS/EDS-HT have an increased prevalence of chronic pain syndromes, autonomic dysfunction and psychopathology Because, these factors are also co-morbid with functional GI disorders [23; 58], the evidence for their involvement with JHS/EDS-HT will be described below.

Chronic widespread pain and opiate use

A large survey conducted in the Netherlands among EDS patients (including 157 with hypermobility subtype) showed that 98% of patients experienced chronic, mostly musculoskeletal pain [60]. Pain severity changed over time but remained continuously present in 85% of patients. It has recently been postulated that chronic widespread pain in EDS is due to the persistent nociceptive input as a result of joint abnormalities that triggers central sensitization in the dorsal horn neurons [20]. Moreover, such central sensitization may also be the cause of generalized hyperalgesia, which has been demonstrated in both children and adults with JHS/EDS-HT [52]. It should be noted however that other factors than musculoskeletal pain may contribute to chronic pain in EDS patients. For example, a high prevalence of small fibre neuropathy has been reported in JHS/EDS-HT, resulting in neuropathic pain [13].

In the Dutch survey mentioned above, 89% of the EDS patients who had pain were on one or more analgesics with 23% being on tramadol. Fikree et al. found similar numbers of opioid use in JHS/EDS-HT patients referred by the rheumatologist (29.6%) [29]. Opioid use is particularly relevant in these patients because of their potential to exacerbate GI symptoms and result in opioid induced bowel dysfunction and potentially narcotic bowel syndrome, which makes management even more challenging [21; 25].

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Autonomic dysfunction

JHS/EDS-HT patients can experience symptoms suggestive of (pre)syncope (i.e. lightheadedness, dizziness and actual fainting), as well as palpitations and chest pain. Hakim et al. reported symptoms of pre-syncope in 41% of JHS/EDS-HT patients, compared to only 15% in the control group [34]. A subset of these symptoms may be explained by the association of JHS/EDS-HT with postural tachycardia syndrome (PoTS). PoTS is defined as an elevation of the heart rate of at least 30 bpm within 10 minutes after standing upright, or a heart rate > 120 bpm after standing. The prevalence of PoTS in JHS/EDS-HT patients reached 15% in one study [31]. Autonomic dysfunction, such as PoTS, orthostatic hypotension and/or uncategorized orthostatic intolerance are seen in three out of four patients with JHS/EDS-HT [46]. PoTS can be treated with a combination of non-pharmacological approaches, a structured exercise training program, and often some pharmacological support. Several hypotheses have been formulated to explain why orthostatic intolerance is so strongly associated with JHS/ EDS-HT. These include amongst others, altered vascular tissue elasticity, impaired peripheral vasoregulation, physical deconditioning and neuropathies [18; 34; 59].

PoTS, independent of EDS status, is associated with GI symptoms and with dysmotility of the stomach, small bowel and colon, as demonstrated by a number of previous studies [37; 42; 62]. Patients with GI symptoms who meet the criteria for JHS/EDS-HT have significantly higher autonomic scores for orthostatic domains (p<0.001) compared to patients without JHS/EDS-HT [29].

Psychiatric comorbidity

Since the late 80's, anxiety disorder have been described to appear more commonly in individuals with gJHM [5]. More recently, these associations have also been found in JHS/EDS-HT, as extensively reviewed by Sinibaldi et al. [54]. Furthermore, panic disorder, agoraphobia, simple phobia and dysthymic disorder or major depression were all found to have a strong association with JHS/EDS-HT [6]. A 15-year follow-up cohort study of JHS/EDS-HT patients showed a cumulative incidence of panic disorder of 41.4% in the JHS/EDS-HT group, compared to only 1.9% in the non-JHS/EDS-HT control group [7].

The observed prevalence of anxiety and other psychiatric comorbidities is higher in JHS/EDS-HT than other chronic pain conditions, but the basis for this is not fully understood [55]. Several studies have hypothesized this to be related to increased interoception, which refers to the concept of sensory bodily information that is continuously assessed and interpreted in relation to certain expectations, which results in a person becoming aware of his/her autonomic bodily state. This interoceptive sensitivity enhances the salience of previously innocuous stimuli, meaning that bodily prediction error signals (e.g. increased heart rate) are attributed to ambiguous environmental stimuli, which in turn become potential threats [16]. Interoceptive sensitivity can be associated with enhanced emotional responsiveness resulting in anxiety and hypervigilance [53]. It has been confirmed through questionnaires that people with gJHM score higher on a body awareness index [22], a factor which can contribute to the development of anxiety.

Other possible hypotheses put forward to explain the increased psychiatric manifestations in JHS/EDS-HT include the fact that a greater perception of joint instability and the frequency with which this impacts on activities of daily living can contribute to anxiety [55]. Furthermore, frequent falling due to autonomic dysfunction can sometimes result in kinesiophobia (fear of movement) - an excessive, irrational and debilitating fear of physical movement and activity resulting from a feeling of vulnerability to painful injury or re-injury [14]. In addition, a considerable phenomenological overlap exists between symptoms of both PoTS and anxiety (i.e. panic, dizziness, palpitations) and it is possible that these symptoms of orthostatic intolerance may exacerbate anxiety, particularly so when combined with enhanced interoception.

A recent study demonstrated a significant association of abdominal, joint and neuropathic pain with having a psychiatric disorder in EDS [36]. However, other GI manifestations, notably symptoms of dyspepsia and reflux, were not significantly affected by the presence of psychopathology [29]. This would suggest that the contribution of psychopathology to GI manifestations in JHS/EDS-HT may be symptom-dependent and primarily related to pain.

DISCUSSION

Scientific evidence suggests an association between JHS/EDS-HT and GI disorders, and these patients meet the criteria for FGIDs, specifically those with upper GI symptoms. [26; 29; 48; 65]. Interestingly, postprandial distress syndrome, a subtype of dyspepsia, showed the strongest association with JHS/EDS-HT in one study [26], and symptoms of this disorder including bloating and postprandial fullness were consistently associated with JHS/EDS-HT in multiple studies (see table 5.6) but the aetiology behind this remains to be elucidated. Another important observation is that JHS/EDS-HT patients with lower Beighton scores and modest musculoskeletal involvement seem to experience fewer GI symptoms [29]. It could be speculated that these patients would have a better prognosis concerning GI symptoms compared with their more hypermobile peers, though this hypothesis will need to be assessed in future studies.

The prerequisite step to the development of a more complete understanding of the relationship between JHS/EDS-HT and GI symptoms is the objective assessment as to whether such symptoms are attributable to any demonstrable physiological or anatomical abnormalities therein. It should be noted that, to date, only a preliminary study has examined this specifically. In this study, esophageal motility was evaluated in 17 JHS/

EDS-HT with dysphagia symptoms. This study, as of yet available in abstract form, has shown that 59 % (10/17) of patients had evidence of esophageal dysmotility [28]. No other studies have specifically examined the physiological and anatomical basis of GI symptoms in JHS/EDS-HT. Though several studies described the findings of physiological investigations in JHS/EDS-HT, including Nelson et al.[48], as discussed in detail above, it is difficult to interpret these in the absence of a clinical background of the patients. It therefore remains challenging to ascertain the pathophysiology of GI symptoms and FGIDs seen in JHS/EDS-HT. We here formulate a number of hypotheses on pathogenesis, including ones related to abnormal motility, altered biomechanical properties (e.g compliance), increased sensitivity and changes in the gut-brain axis, which will be discussed in more detail below.

It is tempting to assume that GI symptoms in JHS/EDS-HT would relate to altered motility and biomechanics of the GI tract as a result of the connective tissue anomaly. It is possible that variations in connective tissue and, in particular, in the extracellular matrix (ECM) composition in JHS/EDS-HT are responsible for laxity of joints, pelvic floor and other sites including the GI tract, most notable the intestinal wall. A previous study in gJHM found evidence for significant anorectal anatomical abnormalities, including rectoceles [47] – no such studies have yet been performed in JHS/EDS-HT. To date, no collagen defects and/or associated mutations have been found in JHS/EDS-HT. Only in 5-10% of patients a mutation in TNXB, the gene that codes for tenascin-X, a glycoprotein in the ECM that is responsible for organization and maintenance of the matrix itself, was linked to an autosomal recessive form of JHS/EDS-HT [49; 66]. Notably, we have previously reported that GI symptoms are encountered frequently in tenascin-X deficient patients [30]. For the remainder of patients, JHS/EDS-HT is a diagnosis of exclusion.

Interestingly, investigations aimed at assessing GI motor function, such as reported by Nelson et al., have demonstrated both increased *and* delayed gastric emptying and colonic transit, which would suggest involvement of other mechanisms than merely alterations in the mechano-elastic properties of the intestinal wall. Moreover, very recently, animal studies demonstrated that changes in mechano-elastic properties of the intestine result in altered mechano-sensory afferent responses [63]. Indeed, considerable scientific evidence exists to support a role for the extracellular matrix in nerve function. Neurons express certain receptors that enable them to interact with the surrounding connective tissue. Furthermore, the ECM can affect maturation and function of synapses in the peripheral and central nervous system (for review, see [3]). More specifically, in vitro studies have demonstrated that the ECM microenvironment has profound influence on the differentiation of neuronal subtypes innervating smooth muscle cells of the intestine [50]. In fact, the composition of the ECM was able to influence cholinergic and nitrergic neurotransmission in these neural cells and their responses to electrical field stimulation. This suggests significant effects of the ECM on the neurodevelopment of the enteric nervous system. It remains to be established, however, whether such neurodevelopmental changes are present in JHS/EDS-HT and whether this indeed has an effect on the development of GI abnormalities and symptoms through influencing afferent signaling.

We hypothesize that presumed changes in afferent signalling may result in a) increased peripheral discharge of nociceptive information (peripheral sensitization) or b) augmentation of afferent information at the level of spinal dorsal horn neurones (central sensitization). Sensitization refers to a reduction in the threshold for perception of sensory stimuli arising from visceral organs. A commonly accepted hypothesis in the development of functional GI disorders is indeed related to visceral hypersensitivity [38]. We therefore speculate that neurodevelopmental changes as a result of ECM abnormalities in JHS/EDS-HT may render intestinal afferents more prone to sensitization. However, no studies have specifically examined the presence of visceral hypersensitivity in JHS/EDS-HT, let alone to establish whether this is related to alterations in afferent signalling.

In summary, although an association between GI symptoms and JHS/EDS-HT appears to have been consistently reported, it remains to be established what the exact nature of the connective tissue anomaly is in relation to GI function and symptom generation. Although several hypotheses have been formulated (see figure 5.3 for schematic overview), the scarcity of data warrants further mechanistic studies to identify the pathophysiological basis of this association. We believe that systematic evaluation of GI sensorimotor function and genotypical characterisation of patients with overlap between JHS/EDS-HT and FGIDs is an important research area in the future.



Figure 5.3. Diagram illustrating possible pathways for the pathogenesis of functional gastrointestinal disorders in joint hypermobility syndrome / Ehlers Danlos syndrome hypermobility type.

CONCLUSIONS AND RECOMMENDATIONS FOR CLINICAL PRACTICE

We here provide recommendation for the clinical management of patients with JHS/ EDS-HT presenting with GI symptoms, which are based on expert opinion rather than scientific evidence due to the current paucity of data.

- **Recognition.** It is important for gastroenterologists to consider the diagnosis of JHS/EDS-HT in patients with FGIDs and multiple musculoskeletal symptoms. When JHS/EDS-HT is suspected, the patient should be referred to a specialist that is familiar with the disorder (i.e. clinical geneticist or rheumatologist) in order to establish a definitive diagnosis.
- **Multidisciplinary approach.** Recognition of comorbidities as part of a multisystem disorder warranting a multidisciplinary approach is an important clinical imperative. Pointing out to patients that the comorbidities of JHS/EDS-HT, including its gastrointestinal manifestations, are the result of a multi-system disorder, may aid patients' psychological health by providing reassurance and a potential explanation of their symptoms.
- **Opioid use.** An attempt should be made to decrease the use of opioids and to consider replacing them with pain modulators (such as tricyclic antidepressant, gabapentinoids or serotonin-norepinephrine reuptake inhibitors) instead. The efficacy of pain modulators in this group of patients of course requires further study.
- Nutritional aspects. Attention should be given to maintain an optimal nutritional status and prevent the development of nutritional deficiencies. A number of potentially beneficial dietary supplements have been reported for the classical subtypes of EDS, including carnitine, co-enzyme Q10, high doses of vitamin C (1500 mg per day), and various chondral protectors [44]. The exact role for these supplements in the therapy for JHS/EDS-HT remains to be further elucidated.

Studies have shown a remarkable association between FGIDs and JHS/EDS-HT, suggesting that about 50% of FD patients and 40% of IBS have JHS/EDS-HT. This observation may indeed represent the first step towards the identification of a new pathophysiological basis for a substantial subgroup of FGID patients. The discovery of an association of a disorder of connective tissue with hitherto unexplained GI symptoms has raised the prospect of identification of important therapeutic targets in the signalling and interaction between ECM and intestinal neural cells. It is of interest that despite extensive advances in our understanding of gut sensorimotor function, the aspect that is least studied is the role of connective tissue in gut function. Not very long ago – against all odds – Helicobacter pylori was found to be the cause for peptic ulcer disease. Time will tell whether the putative association between JHS/EDS-HT and FGIDs has a similar potential within neurogastroenterology.

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Study group	Study design	Patient population	Primary findings
Castori et al. 2010	Prospective pilot study	21 JHS/EDS-HT patients, recruited from genetics outpatient clinic. New diagnoses confirmed through Villefranche criteria.	Gastrointestinal symptoms in 86%. Dyspepsia (66.7%), gastroesophageal reflux (57.1%) and recurrent abdominal pain (61.9%)
Zeitoun et al. 2013	Cohort study	108 JHS/EDS-HT patients, recruited through French EDS patient support group. Established diagnoses.	Heartburn/regurgitation (68.7%), dysphagia (62.6%) and epigastric pain (78.8%)
Fikree et al. 2014	Prospective cross- sectional study	552 new consecutive patients recruited at gastroenterology clinics, with unknown JHS/ EDS-HT status. 150 diagnosed with JHS/EDS-HT through Brighton criteria. 54 JHS/EDS-HT patients with established diagnoses.	Heartburn (33%), water brash (30.9%) and postprandial fullness (41.4%) High prevalence of FGIDS in JHS/EDS-HT groups (57.5% and 91% respectively)
Fikree et al. 2015	Case-control study	641 consecutive patients recruited at gastroenterology clinics, divided in 336 FGIDs and 305 organic disorders. New diagnoses of JHS/EDS-HT in 131 and 84 patients respectively.	Higher JHS/EDS-HT prevalence in FGIDs (39% versus 27.5%) Highest JHS/EDS-HT prevalence in gastroduodenal disorders (44%)
Nelson et al. 2015	Retrospective study	271 JHS/EDS-HT patients at Mayo clinic (enteric neuroscience and medical genetics). Established diagnoses (Villefranche criteria).	Abdominal pain (56%), nausea (44%), constipation (42%) and heartburn (38%) Abnormal gastric emptying in 22.3% and abnormal colonic transit in 28.3%;

Supplementary table 1. Summary of studies investigating gastrointestinal manifestations in JHS/EDS-HT

Supplementary table 2. Association between organic disorders and JHS/EDS-HT

Study group	Study design	Patient population	Primary findings
Zeitoun et al. 2013	Cohort study	108 patients JHS/EDS-HT, recruited through French EDS patient support group. Established diagnoses.	72 patient underwent upper endoscopy, of which 19 were shown to have a hiatal hernia (26.4%)
Danese et al. 2011	Prospective pilot study	31 recruited from a genetics clinic with an established JHS/EDS-HT diagnosis	Coeliac disease (16%) confirmed through biopsy
Carley et al. 2000	Retrospective study	8 patients with established JHS/ EDS-HT diagnosis	Pelvic organ prolapse (75%)
Mastoroudes et al. 2013	Case-control study	60 patients with established JHS/ EDS-HT diagnosis recruited at tertiary hypermobility clinic and 60 matched healthy controls	Pelvic organ prolapse (73%)

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Prevalence of JHS/EDS-HT in organic disorders				
Study group	Study design	Patient population	Primary findings	
Manning et al. 2003	Case-control study	838 patients that had been referred for urodynamic analysis in the past and 148 matched healthy controls	404 patients with obstructed defecation (%JHS/EDS-HT 70.6), 397 patients without obstructed defecation (%JHS/EDS-HT 50.0)	
Fikree et al. 2015	Case-control study	641 consecutive patients recruited at gastroenterology clinics, divided in 336 FGIDs and 305 organic disorders. New diagnoses of JHS/EDS-HT in 131 and 84 patients respectively.	Crohn's disease 32% Ulcerative colitis 21%	
Fikree et al. 2015	Case-control study	641 consecutive patients recruited at gastroenterology clinics, divided in 336 FGIDs and 305 organic disorders. New diagnoses of JHS/EDS-HT in 131 and 84 patients respectively.	Coeliac disease (30%)	

CHAPTER 6

Colonoscopy is safe and not associated with higher pain scores in patients with Hypermobile Spectrum Disorder – results from an exploratory prospective study

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ABSTRACT

Introduction. Patient perception of colonoscopy varies greatly. Young slender women and irritable bowel syndrome (IBS) patients appear to be at risk for peri-procedural pain. Recent evidence suggests a high prevalence of joint hypermobility related connective tissue disorders in this population. Therefore, we aimed to investigate whether hypermobility spectrum disorder (HSD) is associated with increased pain during colonoscopy.

Methods. We prospectively included patients undergoing routine colonoscopy. Subjects were assessed for HSD using the 2017 criteria, and IBS and functional dyspepsia using the Rome III criteria. After colonoscopy and recovery from sedation, patients were asked to report pain scores on a 100mm visual analogue scale (VAS). In addition, cecal intubation time was measured, endoscopists scored the difficulty of the procedure (100mm VAS), and procedure related adverse events were registered.

Results. Of 200 included patients, 22 (11%) met criteria for HSD. A female predominance was observed in HSD patients (86.4% vs. 49.4%, p<0.001). A crude linear regression model demonstrated that pain scores were 13.30mm higher in HSD vs. non-HSD patients (95%-CI 0.07 – 26.53, p=0.049). When subsequently correcting for possible confounding factors, however, this difference in pain scores could be explained by a confounding effect of female gender. Cecal intubation time, perceived procedural difficulty and complication rate did not differ significantly between groups.

Conclusions. HSD seems not to be a predictor of painful colonoscopy, probably due to female gender roling as a confounding factor. In addition, performing colonoscopy is not more complicated in HSD vs. non-HSD patients, nor is it associated with more adverse events.

INTRODUCTION

Ehlers-Danlos syndrome (EDS) is a non-inflammatory heritable connective tissue disorder resulting from defects in collagen structure, synthesis, or processing. Its manifestations frequently involve the cutaneous and musculoskeletal systems such as skin laxity and joint hypermobility [11]. The combined prevalence of all types of EDS appears to be at least 1 in 5,000 individuals worldwide [23] but good quality epidemiological data are lacking. The hypermobility type (hEDS) is the most common (80-90%); type 4 (vascular EDS) accounts for 3–6% of all EDS and is considered the most serious because it is associated with vascular rupture. A key defining feature of EDS is joint hypermobility, which refers to the characteristic of being able to actively and/or passively move joints beyond normal limits. Joint hypermobility can vary in intensity as well as in location, ranging from localized hyperflexibility (*i.e.*, involving fewer than five joints) to generalized joint hypermobility (G-JHM), in which at least five different joints are affected.

As far as gastroenterology practice is concerned, evidence suggests that EDS may pose particular risk for spontaneous or instrumentation-related intestinal rupture [3; 5; 12; 16; 18; 20]. A recent retrospective study showed, however, that this increased risk was enterily accounted for by the vascular EDS patients but not other EDS subtypes, such as hEDS [13]. Another anecdotal but yet scientifically unproven concern is related to colonoscopy being more painful or difficult for the endoscopist in EDS patients. This is assumed to be the result of technical difficulty of the colonoscopy by increased tissue laxity, causing the colon to stretch relatively easy upon intubation thus hindering the negotiation of tortuous curves.

In the current study, we therefore aimed to explore in a pilot study whether hypermobile patients presenting for a colonoscopy had more painful experiences or had an increased risk for adverse events. Joint hypermobility was assessed according to the novel 2017 criteria for "Hypermobility Spectrum Disorder" (HSD). There is a fairly broad continuum of joint hypermobility and related symptoms, ranging between, at one end, asymptomatic generalized joint hypermobility-someone who has no symptoms apart from their joints' capacity to move beyond normal limits—through to hypermobile EDS (hEDS), at the other end. Within this spectrum, the category of HSD represents patients with symptoms related to their joint hypermobility but who do not meet the full (new and stricter) criteria for hEDS (see Figure 6.1). By definition, patients presenting with symptoms and generalized joint hypermobility (G-JHM) would be classified as HSD. HSD will therefore include most people who have been previously diagnosed with joint hypermobility syndrome (JHS) or benign joint hypermobility syndrome (BJHS) and some people who previously had the diagnosis of EDS type 3. Patients with JHS have previously been shown to have increased risk of chronic pain syndromes, functional gut disorders e.g. functional dyspepsia and IBS, and visceral hypersensitivity [4; 9]. These factors are known to be associated with increased pain/decreased tolerability during colonoscopy [9] [7].

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We therefore hypothesized that patients with HSD have higher pain scores during colonoscopy versus non-HSD patients. In addition, we also assessed the rate of adverse events and other technical details related to colonoscopy to ascertain potential differences related to the patients HSD status.



Figure 6.1. Visualisation of the spectrum of hypermobility disorders. On the left asymptomatic generalised joint hypermobility, on the right hEDS, with HSD covering the range from right to left. The top of the figure displays extra-articular manifestations. EDS, Ehlers–Danlos syndrome; hEDS, hypermobility type Ehlers–Danlos syndrome; FGDs, functional gastrointestinal disorders; G-HSD, generalised hypermobility spectrum disorder; GJH, generalised joint hypermobility; H-HSD, historical hypermobility spectrum disorder; JH, joint hypermobility; L-HSD, localised hypermobility spectrum disorder; P-HSD, peripheral hypermobility spectrum disorder; POTS, postural orthostatic tachycardia syndrome. (Reprint from Castori *et al.*[6])

METHODS

Study design

This study was performed at the endoscopy department of the Maastricht University Medical Center (Maastricht UMC) in Maastricht, the Netherlands, a secondary/ tertiary referral hospital. The study protocol has been approved by the Maastricht UMC Committee of Ethics (IRB identifier 15-4-258) and was executed according to the revised Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013).

Participants

All patients aged between 18 and 75 years undergoing a colonoscopy with regular sedation were considered eligible for inclusion. Patients were excluded when undergoing a colonoscopy in the context of the national screening program for colorectal cancer (due to age bias – joint hypermobility is strongly influenced by age [22]), follow-up colonoscopy for inflammatory bowel disease or colorectal cancer, or when having a history of previous extended abdominal surgery - due to bias towards more painful colonoscopy). Patients with a history of uncomplicated cholecystectomy, appendectomy, and/or hysterectomy were considered eligible. Procedures under propofol-based sedation were excluded from the study due to the presumed better tolerability of this method. All participants gave their written informed consent prior to inclusion.

Study procedures

Clinical assessments

All subjects were assessed for HSD using the 2017 criteria by a trained clinical researcher [6]. HSD is defined by generalized joint hypermobility (as assessed using physical examination according to the Beighton score, in addition to the presence of certain clinical features which do not fit the diagnosis of hEDS on the 2017 classification for hEDS, i.e joint trauma, chronic pain, orthopaedic manifestations (pes planus, valgus deformity) etc., as a result of joint hypermobility (see https://www.ehlers-danlos.com/ heds-diagnostic-checklist/). None of the patients investigated had a diagnosis of HSD, hEDS or JHS prior to inclusion.

In addition, participants were assessed for irritable bowel syndrome (IBS) and functional dyspepsia (FD) using the Rome III criteria [8] as potential confounders.

Colonoscopy

Colonoscopies were performed either by a consultant gastroenterologist (n = 10) or trainee (n = 21). The endoscopists were blinded to the HSD status of the patient. Pentax HD+ colonoscopes (HOYA Corporation, PENTAX Lifecare Division, Showanomori Technology Center, Japan) with carbon dioxide (CO_2) insufflation were used in all procedures. Furthermore, all colonoscopies were performed under conscious sedation with the use of midazolam, with the addition of an opiate (either pethidine or fentanyl). Standard dosage applied was 2.5mg for midazolam, 25µg for fentanyl, and 25mg for pethidine. An additional dose was administered as needed, using a maximum total dosage of 5.0mg for midazolam, 50µg for fentanyl, and 50mg for pethidine (considered 'high dose'). The level of sedation was noted on an ordinal scale from 1 (awake) to 5 (unresponsive) after the endoscopy was completed. There were a minimum of one endoscopist and two assisting nurses present during all procedures. The nurses were

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present to monitor the patient during the procedure and in addition, all patients were monitored by pulse oximetry and blood pressure measurement every 5 min.

Patient reported outcomes

After the colonoscopy, when patients had recovered adequately from sedation, they were asked to report peri-procedural pain scores on a 100mm visual analogue scale (primary outcome measure). In addition, the patient was asked to score perceived discomfort using the same scale.

Endoscopist reported outcomes

Endoscopists were requested to report the degree of technical difficulty of the colonoscopy on a 100mm visual analogue scale directly after the colonoscopy. In addition, endoscopists were asked to provide an indication of their experience level (*i.e.*, the number of colonoscopies performed). Experience level was divided in five categories: having performed 0-200 colonoscopies, 200-500 colonoscopies, 500-1500 colonoscopies, 1500-5000 colonoscopies, or more than 5000 colonoscopies.

Assisting nurse reported outcomes

Nurses assisting the colonoscopy were asked to report the level of discomfort of the patient using the Modified Gloucester Discomfort Scale, ranging from 1 (meaning no discomfort) to 5 (meaning severe discomfort) [19].

Procedure related outcomes

Sedative and analgesic dose administered before and during endoscopy were registered immediately. During colonoscopy, cecal intubation time (from anal insertion to caecum) was recorded. Patients in whom the caecum was not reached, were excluded from the analyses of mean intubation time. Macroscopic diagnoses were registered in order to correct for possible confounding (e.g. inflammation, diverticulosis). The adequacy of bowel preparation was described using the Boston Bowel Preparation Scale (BBPS) [14]. Finally, the occurrence of adverse events was strictly monitored and registered.

Sample size calculation

Sample size calculation was based on the primary outcome measure, patient-reported pain scores during colonoscopy, using a visual analogue scale. Mean VAS scores during regular colonoscopy have been reported as 32 mm with a standard deviation of 21.6 mm [17]. A difference in VAS scores of 15 mm was considered clinically relevant, based on earlier studies investigating pain scores during colonoscopy [15; 17]. To be confident of finding differences in reported discomfort scores of at least 15 mm with a power of 80% and two-sided alpha of 0.05, a sample size of 33 subjects is required in the smallest

group. Since we initially expected to find a prevalence of joint hypermobility as a trait of about 30% [10], a total sample size of 110 subjects was required. As the prevalence of joint hypermobility was found to be substantially less after inclusion of 110 patients, we included additional patients up to a total of 200 patients. This did not, however, result in a change in the prevalence of hypermobility or the diagnosis of HSD. Considering this unaltered prevalence, a sample size of 330 patients would have been necessary to detect a difference of 15mm in VAS scores. Consequently, we did not have sufficient power to detect a 15mm difference in VAS scores.

Statistical analysis

Study participants fulfilling 2017 criteria for hypermobility spectrum disorder or not are referred to as 'HSD' and 'non- HSD,' respectively. Data are presented as mean \pm standard deviation (SD) for normally distributed continuous variables and proportions for categorical outcomes. Univariate comparison of patient characteristics between HSD and non-HSD patients was performed using an independent samples t-test for continuous variables and χ^2 test via cross-tabulation for dichotomous variables. Significance level was corrected for multiple comparisons according to Bonferroni (p < 0.0033 for 15 comparisons).

Subsequently, a crude (unadjusted) linear regression analysis was performed using the primary outcome measure (patient reported pain scores) as dependent variable while introducing HSD status as the sole covariate. In addition, multivariable linear regression analysis was performed, adjusting for sex, age, BMI, previous abdominal surgery, FD, IBS, active inflammation, diverticulosis, bowel cleanliness, sedative and analgesic dose, and endoscopist training level. Variance inflation factors revealed no multicollinearity issues. Because of a suspected non-normal distribution, a sensitivity analysis was performed after a two-step datatransformation of patient reported pain scores [24]. After transformation, data were visually confirmed to be normally distributed. As linear analysis with the transformed data yielded similar results, the results of linear regression reported below are based on the original data.

All analyses were performed using IBM SPSS Statistics, version 23 (IBM Statistics for Macintosh, Chicago, IL, USA).

RESULTS

Study participants

During the study period, 348 eligible patients were contacted for participation. A total of 200 patients gave informed consent and were included in the study (mean age 56.4 ± 13.4 ; 52.7% female). Twenty-two patients (11%) met criteria for HSD, who were predominantly female (see Table 6.1). One patient had G-JHM not meeting the criteria

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for HSD and was therefore included in the control group. The prevalence of G-JHM was therefore 11.5%.

FD was more common in HSD compared to non-HSD (50.0% vs. 14.0%, p<0.001). When corrected for age and gender in a logistic regression model, this failed to reach the predefined statistical significance level, although a positive trend remained (ORadj 3.62, 95%CI 1.35 – 9.72, p=0.01). IBS prevalence rates demonstrated a similar trend, with a higher rate in HSD patients. No differences were found in macroscopic diagnoses (i.e., diverticulosis, macroscopic inflammation, presence of polyps) (see Table 6.1).

	Non-HSD	HSD	
	(n=178)	(n=22)	P-value
Age (mean ± SD)	57.1 ± 13.3	50.3 ± 13.5	0.026
Female, n (%)	87 (49.4%)	19 (86.4%)	0.001
BMI (mean ± SD)	26.7 ± 4.3	27.0 ± 4.7	0.746
IBS, n (%)	46 (25.8%)	10 (45.5%)	0.056
FD, n (%)	25 (14.0%)	11 (50.0%)	<0.001
Macroscopic inflammation, n (%)	20 (11.2%)	1 (4.5%)	0.331
Diverticulosis, (%)	70 (39.3%)	8 (36.4%)	0.773
Presence of one or more polyps, n (%)	73 (41.0%)	6 (27.3%)	0.207
BBPS* (mean ± SD)	8.0 ± 1.2	7.9 ± 1.6	0.855
High dose midazolam, n (%)	43 (24.2%)	12 (54.5%)	0.003
High dose opiate**, n (%)	116 (65.1%)	18 (81.8%)	0.117
Level of sedation***, n (%)	Awake: 39 (45.3%) Drowsy: 36 (41.9%) Sleeping, responsive to speech: 11 (12.8%)	Awake: 5 (71.4%) Drowsy: 1 (14.3%) Sleeping, responsive to speech: 1 (14.3%)	n.a.
Adverse events			
Mild desaturation, n (%) Vasovagal episode, n (%)	18 (10.1%) 8 (4.5%)	3 (13.6%) 0 (0%)	0.618 0.309
Previous uncomplicated abdominal surgery n (%)	35 (19.7%)	7 (31.8%)	0.187
First-time colonoscopy n (%)	93 (52.2%)	11 (50%)	0.842
Indication for colonoscopy Adenoma follow-up Abdominal nain/discomfort	33 (18.5%) 55 (30.9%)	3 (13.6%)	0.494
Altered stool pattern Rectal blood loss	41 (23.0%) 42 (23.6%) 7 (2.0%)	7 (31.8%) 2 (9.1%)	
rammai colon cancer	/ (3.9%)	1 (4.3%)	

Table 6.1. Patient characteristics

*BBPS = Boston Bowel Preparation Scale.

**Either high dose of fentanyl (50 μg) or high dose of pethidine (50 mg).

*** incomplete data; level of sedation was not registered for all patients. P-values not calculated.

Significance level corrected for multiple comparisons (Bonferroni): p < 0.0033

Patient-reported pain scores

A crude linear regression model demonstrated that pain scores were 13.30mm higher (95%-CI 0.07 - 26.53, p=0.049) in HSD vs. non-HSD patients. When subsequently correcting for possible confounding factors, however, no significant effect on pain scores was found for HSD. The effect of HSD in the crude linear regression appeared to be primarily related to the confounding effect of female gender (see Supplementary data). Indeed, female gender was the strongest predictor of a higher pain score (B: 12.15mm, 95%-CI 2.71 – 21.59, p=0.012). In addition, endoscopist training level appeared to have a role in patient pain perception, with lower pain scores when the procedure was performed by a more experienced endoscopist (B: -4.36, 95%CI -7.59 to -.473, p = 0.027, calculated with the use of experience categories, see methods section). No other clinically relevant confounders were identified in the current study, including age, BMI, presence of IBS or FD, active inflammation, diverticulosis, bowel cleanliness, sedative and analgesic dose (see Supplementary data). Less than 20% of variability in pain scores was explained with the use of the complete regression model (R-squared=0.197). In addition to the above, no significant association was found between Beighton score (representing trait joint hypermobility) and patient reported pain scores.

Discomfort scores

Neither patient- nor nurse-reported discomfort scores differed significantly between HSD and non-HSD patients (patient reported: 35.77mm \pm 27.46 versus 42.59mm \pm 29.84 (on a 100mm VAS), p=0.278; nurse reported: 2.57 vs 3.00 (on a 5-point Likert scale), p=0.132. There was a fairly good correlation between patient and nurse-reported scores: 0.64, p < 0.001.

Procedure related outcomes

Overall cecal intubation rate was 93.0%; there was no significant difference between failure to reach the cecum (13.6% HSD vs. 6.1% non- HSD, p=0.193). Endoscopist-reported technical difficulty scores were comparable (39.98mm \pm 28.97 versus 40.73mm \pm 30.97, p=0.910). Similarly, cecal intubation time was comparable between both patient groups: mean cecal intubation time in HSD patients was 10 minutes and 34 seconds (SD 7 minutes and 36 seconds) versus 12 minutes and 14 seconds (SD 7 minutes and 27 seconds) in non-HSD patients, p=0.358.

A significantly higher proportion of patients with HSD were administered high doses of midazolam (54.5% vs. 24.2%, p=0.003). However, of all patients receiving high-dose midazolam, 83.6% was female, hence gender was likely a confounding factor. Indeed, when corrected for gender, binary logistic regression showed that the odds of high dose midazolam use were not significantly higher in HSD patients (ORadj 2.29, 95%CI 0,88 - 5.98, p=0.09).

Adverse events

No serious adverse events were reported. Reported adverse events included 21 cases of mild oxygen desaturation (10.5%), all involving only a minor and transient reduction in saturation with a minimum reported oxygen saturation of 80%. In addition, 8 vasovagal episodes (4.0%) were reported, which composed of transient hypotension and/or bradycardia or excessive perspiration. Adverse event rates were similar between groups receiving low and high dose midazolam. Similarly, rates did not differ between patient groups (see Table 6.1). VAS pain scores did not differ significantly between patients who experienced an adverse event and those who did not (data not shown).

Sensitivity analysis

Analyses were rerun when defining the groups as follows: patients with G-JHM (hence including all patients with HSD, n=23) and patients withtout G-JHM (n=177). No differences were detected in any of the outcome parameters when compared to the initial analyses (data not shown).

DISCUSSION

Our results from this exploratory study indicate that HSD, which is a disorder clinically closely related to hypermobile Ehlers-Danlos syndrome, and largely overlapping with the now defunct category of JHS, is not independently associated with higher patient reported pain scores during colonoscopy. Although an initial crude linear regression model indicated higher pain scores in HSD patients, subsequent analyses revealed a strong confounding effect of female gender and endoscopist experience. Similarly, there was no association between Beighton score and patient reported pain scores, indicating that also less severe phenotypes of joint hypermobility in the general population seem not to be related to periprocedural pain. Furthermore, we found no significant difference in patient- and nurse-reported discomfort scores or the occurance of adverse events between HSD and non-HSD patients. Lastly, endoscopist-reported technical difficulty scores and cecal intubation time did not differ significantly between HSD and non-HSD patients. It therefore appears that colonoscopy in patients with HSD is neither more painful nor less safe than in non-HSD patients. This conclusion might be relevant for both patients and pracitioners when making clinical decisions for colonscopy referral.

Our hypothesis of higher pain scores in HSD patients was driven by anecdotal evidence in addition to the reported higher prevalence of the female gender and functional GI disorders in patients previously defined as having JHS, according to pre-2017 nosology. Both the high prevalence of functional GI disorders (in particular functional dyspepsia) and female predominance in HSD patients were corroborated in the current study, albeit

the higher prevalence of IBS failed to reach statistical significance. While functional dyspepsia and IBS often co-exist as manifestations of visceral hypersensitivity [1], the explanations why HSD is more strongly linked with functional dyspepsia than IBS remain speculative. It is possible that the upper GI tract is more sensitive to the extracellular matrix alterations seen in HSD [25]. Certain extracellular matrix molecules that have been linked to hypermobility are also involved in upper GI sensory function [2]. However, more in-depth studies investigating connective tissue alterations in both the upper and lower GI tract, and their correlations with symptomatology, are required to answer this question.

It should be noted that patients with HSD more often needed higher doses of midazolam. One might speculate that the reason patients did not experience more pain was related to the higher level of sedation. However, the level of sedation did not appear to correlate with patient reported pain scores in the regression model and was not a confounder. In addition, we observed that of all patients receiving high-dose midazolam, 83.6% was female. Therefore, the higher midazolam dosages in the HSD group appeared to be mainly gender-related, as also demonstrated by a binary logistic regression analysis.

A strength of the current study is that prospective recruitment took place via the general colonoscopy programme in a secondary/tertiary clinic, without pre-selection for specific GI diseases. Participants included primary (through open-access for GPs), secondary, and tertiary care patients, resulting in a study population that reflects the general hospital population. Furthermore, the evaluation of HSD status was performed by a trained researcher after the colonoscopy in order to reduce bias in the patient-, endoscopist-, and nurse-reported outcome measures.

Several limitations of the current exploratory study have to be mentioned. First, the rather small sample size limits the generalisability of the findings. In order to detect a difference in VAS scores of 15mm, we would have needed to include 330 patients albeit this would probably not have impacted the prevalence and therefore the effect size would not have been influenced either. Therefore, we assume that no clinically relevant effect of HSD on perceived pain during colonoscopy is to be expected in a larger patient sample. Nevertheless, the lack of a statistical association between HSD and pain scores could be due to a type II error. A possible explanation for the lower than expected prevalence of HSD is the relatively high age of the study population, as compared to a previous study where the prevalence of joint hypermobility syndrome (JHS) was found to be 33% [10]. In addition, the study by Fikree et al. demonstrated that patients with joint hypermobility syndrome were significantly younger than patients not fulfilling criteria for JHS. A similar trend was observed in the current study, although this age difference was not statistically significant after Bonferroni correction. In fact, it is well-known that joint hypermobility decreases by age [21].

Second, we identified patients with HSD who need not necessarily fulfil the 2017 criteria for hEDS. Therefore, current results cannot be readily extrapolated to patients with hEDS or any other EDS subtype. On the other hand, we believe the population with HSD represents a clinical relevant patient group to endoscopists (11% of the current overall patient population).

In conclusion, we have not found patients with hypermobility spectrum disorder to perceive colonoscopy as more painful, when correcting for confounders, in particular gender, nor for level of sedation. Neither was colonoscopy associated with more adverse events in this patient group. Gastroenterologists therefore do not need to defer from colonoscopy, although findings of this current exploratory study should be corroborated in larger populations.

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SUPPLEMENTARY DATA

Model		Coefficients		Sig.	95,0% Confidence Interval for B	
	В	Std. Error	r	Lower Bound	Upper Bound	
1	(Constant)	33,657	2,225	,000	29,270	38,045
	HSD	13,297	6,708	,049	,069	26,526

Initial crude linear regression analysis

Model R-square 0.019

Mo	del	Coefficients		Sig.	95,0% Confidence Interval for B	
		В	Std. Error	_	Lower Bound	Upper Bound
2	(Constant)	26,691	2,964	,000	17,844	29,537
	HSD	5,276	6,672	,430	-7,885	18,438
	Gender	18,149	4,189	,000	9,885	26,414
3	(Constant)	26,711	21,178	,209	-15,085	68,508
	HSD	3,399	7,022	,629	-10,459	17,257
	Gender	12,417	4,813	,011	2,918	21,917
	Abdominal surgery	1,095	5,160	,832	-9,089	11,279
	Functional Dyspepsia	9,226	5,743	,110	-2,109	20,560
	Irritable Bowel Syndrome	5,769	4,631	,214	-3,370	14,909
	Age	,346	,182	,059	-,014	,705
	Body Mass Index	-,191	,467	,683	-1,112	,731
	BBPS	-1,834	1,672	,274	-5,135	1,467
	Macroscopic inflammation	-2,245	6,563	,733	-15,198	10,708
	Diverticulosis	-,510	4,581	,912	-9,552	8,532
	Endoscopist experience	-4,036	1,805	,027	-7,599	-,473
	Sedative dose (low/ high)	7,705	5,456	,160	-3,063	18,472
	Analgesic dose (low/ high)	5,601	4,683	,233	-3,641	14,844
	Number of earlier colonoscopies	,813	,584	,560	-1,935	3,561

Multivariable linear regression analysis

a. Dependent Variable**: Patient reported pain score** on 100 mm Visual Analogue Scale (VAS) R-square **model 2**: 0.106

R-square model 3: 0.197

Do Irritable Bower Synarome patients with and without Hypermobility Spectrum inisorders differ in gastrointestinal symptoms, psychological omorbidities, and Quality of Life?

> Lisa Vork, Abraham B. Beckers, Zsa Zsa R.M. Weerts, e, Adam D. Farmer, Qasim Aziz, Martine A.M. Hesselink, Daisy M.A.E. Jonkers, Ad A.M. Masclee, Daniel Keszthelyi

> > (Manuscript submitted)

APTER 7

PART III

VISCERAL PAIN PROCESSING AND PERCEPTION

CHAPTER 8

Evidence for engagement of the nucleus of the solitary tract in processing intestinal chemonociceptive input irrespective of conscious pain response in healthy humans

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Chapter 8

ABSTRACT

Neuroimaging studies have revealed important pathomechanisms related to disorders of gut-brain interactions, such as irritable bowel syndrome and functional dyspepsia. More detailed investigations aimed at neural processing in the brainstem, including the key relay station of the nucleus of the solitary tract (NTS), have hitherto been hampered by technical shortcomings. To ascertain these processes in more detail, we used multi-echo multiband 7T functional magnetic resonance imaging (fMRI) and a novel translational experimental model based on a nutrient-derived intestinal chemonociceptive stimulus. In a randomized cross-over fashion, subjects received duodenal infusion of capsaicin (the pungent principal in red peppers) and placebo (saline). During infusion, fMRI data and concomitant symptom ratings were acquired. Of 26 healthy female volunteers included, 18 were included in the final analysis. Significantly increased brain activation over time during capsaicin infusion, as compared to placebo, was observed in brain regions implicated in pain processing, in particular the NTS. Brain activation in the thalamus, cingulate cortex and insula were more pronounced in subjects who reported abdominal pain (visual analogue scale > 10mm), as compared to subjects who experienced no pain. On the contrary, activations at the level of the NTS were independent of subjective pain ratings. The current experimental paradigm therefore allowed us to demonstrate activation of the principal relay station for visceral afferents in the brainstem, the NTS, which was engaged irrespective of the conscious pain response. These findings contribute to understanding the fundamental mechanism necessary for developing novel therapies aimed at correcting disturbances in visceral afferent pain processing.

INTRODUCTION

Neuroimaging studies have proven to be invaluable for exploring the distinct properties of visceral sensory processing. Specifically, processing of afferent input from the gastrointestinal tract has received increasing attention as a key mechanism in disorders of gut-brain interaction (DGBI). These include highly prevalent conditions such as functional dyspepsia (FD) and irritable bowel syndrome (IBS), both characterized by visceral pain as a cardinal symptom [30]. The perception of visceral pain requires integration of interoceptive signals with emotional-cognitive inputs in the brain, which occurs in a non-linear fashion[30]. Previous studies focusing on gut-brain interactions in the context of visceral pain have primarily employed mechanical stimuli, such as balloon distension[51]. These studies have uncovered key brain regions including the perigenual anterior cingulate cortex, anterior midcingulate cortex, anterior insula, thalamus, secondary somatosensory cortex and amygdala[51]. More recently, the relevance of these regions across different types of visceral pain (esophageal, gastric and rectal distension) has been corroborated in analyses from fMRI studies investigating various types of somatic and visceral pain[55]. In addition to the ability to discriminate visceral from somatic pain, distinct neural features were shown across different types of visceral pain. The specificity of such features is intruiging and calls for more in-depth understanding from different visceral models.

We therefore consider possible knowledge gaps with regards to studying visceral nociception using neuroimaging. *First*, ecological validity could be improved by looking beyond mechanical stimulation and including chemical stimuli, which in daily life originate from dietary intake. *Additionally*, administering stimuli to areas of the gastrointestinal tract that are accessible through a minimally invasive manner, such as the esophagus and rectum, may be less relevant within the general context of DGBI pathophysiology. The duodenum, in particular, has been implicated as a key region in this respect[56], but until now has remained unexplored in visceral pain neuroimaging studies. *Finally*, previous experiments performed in the early 2000s have identified key primary afferent processing regions in the brainstem, which purportedly include the nucleus of the solitary tract (NTS)[4; 9]. These studies have formed the cornerstone of early brainstem research in visceral pain processing, but have been limited in spatial resolution and spatial specificity by the technology available at that time. Technological advancements from the last two decades have vastly improved the localization accuracy of the brainstem MRI signal.

We therefore utilized multi-echo multiband 7T fMRI, specifically designed to study brain regions, in particular the brainstem, that existing work has not been able to capture properly due to technical limitations of standard whole-brain 1.5 and 3T fMRI [33]. In addition, we used a novel translational experimental model based on a nutrient-derived intestinal chemonociceptive stimulus, reflective of the clinical phenomenon of mealinduced exacerbation of pain in DGBI[7]. We hypothesized that the NTS, a key relay station for visceroceptive input, would show increased activity during chemostimulation of the duodenum. In addition, we hypothesized that the aforementioned brain regions known to be responsive to visceral pain would be engaged, and that the BOLD response in these regions would correlate with subjective pain ratings obtained during scanning.

METHODS

Subjects

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center/University of Maastricht and registered on clinicaltrials. gov (NCT02551029). Subjects gave written informed consent and received a monetary compensation for participation. Only females were included, given that the majority of DGBI patients is female, as well as to select a homogeneous population. Subjects aged between 18 and 65 years and with a BMI between 18 and 30 kg/m² were considered eligible. Subjects did not have any significant medical history, did not meet Rome IV criteria for irritable bowel syndrome or functional dyspepsia and were all right-handed.

Sample size

The current study was the first to investigate NTS engagement during a duodenal chemonociceptive stimulus using multi-echo multiband 7T fMRI, yielding a high number of repeated measurements. Both the multi-echo acquisition (which enables multi-echo independent component analysis) and repeated measurements (resulting in reduced within-subject variance) are associated with increased statistical power[25]. Therefore, a sample size of 21 subjects was deemed sufficient for the current study during conceptualization. This sample size is in line with earlier brain imaging studies, which provided statistical evidence using small collectives, that is, between 12 and 21 participants.

Experimental design

The study was performed in randomized cross-over fashion with the administration of a solution of the transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin or saline in the duodenum in two separate scanning sessions. We have previously successfully implemented this model outside the scanner environment to induce pain and other upper gastrointestinal sensations[54]. The test days were separated by a washout period of at least one week. At the start of each scanning visit a nasoduodenal tube (Bengmark, Flocare[®], Zoetermeer, the Netherlands) was positioned under fluoroscopic guidance. In all subjects fMRI scanning was first commenced without
the administration of any substance. After a baseline scanning period of 6 minutes, the duodenal infusion of one of two substances (capsaicin or saline) was started automatically, as the infusion pump (EmpowerMR Injector System, Bracco Injeneering S.A., Switzerland) was programmed with a fixed delay. Web-based randomization software (http://www.randomization.com) determined the order of the substances administered. Randomization was counterbalanced in order to limit confounding sequence effects. Subjects were blinded to the substance being administered. Duodenal infusion lasted 20 minutes for both the capsaicin and saline. Post-infusion functional scanning continued for 10 minutes. The total duration of each functional run was 36 minutes, and was completed without interruption in every subject. During scanning, subjects completed Visual Analogue Scales (VAS) every three minutes for three symptoms: abdominal pain, abdominal discomfort and abdominal burning. This resulted in a total of 13 VAS measurement time points (each including 3 symptoms). An MRI-compatible joystick was used to gather VAS scores on a continuous scale, with at one end 0mm, meaning that the respective symptom was not present, and at the other end 100mm, which translated to the symptom being unbearable. VAS scales were back-projected onto a screen visible to the subject by a mirror while in the scanner. Each scale was visible for a fixed duration of 6 seconds, in order to allocate each VAS score to the corresponding fMRI images.

All subjects received a dose of 1.2 mg of capsaicin, which is slightly less than the 1.5 mg previously used[54], as we needed the necessary adjustments for flow velocity considering the setting of scanner experiments. This dose corresponds to the daily intake of capsaicin in Europe[11]. The capsaicin solution was administered at a rate of 6 ml/min (concentration 0.01mg/ml) yielding a total administered volume of 120 ml. Physiological NaCl 0.9% (B. Braun) was used for saline infusion. Given that the capsaicin was dissolved in alcohol, the same amount of alcohol (0.46ml 96%) was added to the final saline solution in order to rule out any (brain activity) effect related to alcohol administration. The same flow rate was used for saline as for capsaicin infusion.

MRI and physiological data collection

Blood oxygenation level-dependent (BOLD) fMRI data were collected on a Siemens Magnetom 7T scanner (Siemens Healthineers, Erlangen, Germany) using a 32-channel Nova Medical head coil. Functional MRI data were acquired with a multi-echo EPI (ME-fMRI) sequence[36; 37].

Each fMRI scan consisted of 1350 volumes per echo time, yielding 4050 images in total. Concurrent with MRI scanning, pulse rate and respiration signals were collected with the use of a pulse oximeter (left index finger) and respiratory belt, respectively.

MRI data analysis

Preprocessing was performed using a combination of Statistical Parametric Mapping (SPM12, Wellcome Centre for Human Neuroimaging, UCL, London, UK), CONN toolbox (19.b)[57], the Oxford Centre for Functional MRI of the Brain (FMRIB) Software Library (FSL; v.6.0), and TE Dependent ANAlysis (tedana v0.0.8)[23; 24]. For details, please see supplementary methods.

Data were analyzed with SPM12 using a previously developed pharmacological MRI analysis method[58]. As a part of the first-level analyses, the 225 volumes acquired during the pre-infusion period were used as baseline for each condition (capsaicin versus saline). No significant differences were found when comparing baseline scans between conditions (data not shown). All remaining volumes were then equally divided into 45 time bins of 25 volumes (40 seconds per time bin). Signal averages for each time bin were compared with the baseline average per condition using regression analysis. Signal changes were subsequently subtracted between conditions, resulting in one t-contrast per time bin comparing the brain response between capsaicin and saline relative to pre-infusion baseline. A high pass filter of 2160 s (number of volumes x TR) was used to minimize the influence of very low frequency noise in the BOLD signal.

Second-level voxel-wise analysis was performed on the t-contrasts from the first-level analysis. For this purpose, a repeated-measures ANOVA model was applied to compare the difference in signal change (relative to baseline) between capsaicin and saline over time bins at the group level, with the condition-by-time interaction effect being the effect of interest. The resulting statistical parametric map was thresholded at the voxel level at p < 0.05 with family-wise error correction. A pre-defined mask encompassing pain-responsive regions was used, as obtained from the Neurosynth meta-analytic registry (https://neurosynth.org/analyses/terms/pain/). The Neurosynth pain mask is based on z-scores from a two-way ANOVA testing of 516 studies for the presence of a non-zero association between term "pain" and voxel activation using a false discovery rate (FDR) criterion of 0.01.

In order to aid interpretability, a cluster extent threshold of 10 voxels was used for all analyses. Additional region-of-interest and laterality analysis was performed for the NTS (see supplementary methods).

Subjective responses and their relation to the BOLD signal

VAS scores were calculated as differences compared to baseline (Δ VAS). Given the high number of zero values in the subjective responses, the distribution of the Δ VAS scores was strongly right-skewed. A Wilcoxon signed-rank test (proc univariate, SAS 9.4 [SAS Institute, Cary, NC, USA]) was therefore used for comparing mean Δ VAS scores.

Correlational analyses between fMRI BOLD data and VAS scores were deemed unfeasible due to the non-normal distribution. A response criterion was therefore introduced. Groups were defined based on a response criterion for abdominal pain ratings during capsaicin infusion. This criterion was set at a maximum VAS score of 10mm, allowing to differentiate subjects who only perceived supraliminal non-painful sensation at most ('non-responders'), from subjects who perceived at least minimal to moderate abdominal pain ('responders').

Photoplethysmographic signal processing and analysis

Photoplethysmographic (PPG) data was used to compute pulse rate variability (PRV). PRV, analogous to heart rate variability, is considered a parameter for cardiac parasympathetic activity[5; 43]. Specifically, the root mean square of successive differences (RMSSD) is one of the widely used and well-validated measures of heart (and pulse) rate variability[34; 35]. Although the RMSSD is generally calculated from ECG data, it can also be calculated accurately from PPG data in healthy subjects at rest[43]. Marginal linear mixed models (SAS proc mixed) were used to compare RMSSD change from baseline between conditions using SAS 9.4 (SAS Institute, Cary, NC, USA). For more details, see the supplementary methods.

All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Study subjects

Twenty-six participants were included in the study. Subjects' mean age was 25 years (\pm SD 4.4), with a mean BMI of 22.7 kg/m² (\pm SD 1.9). Two subjects were excluded because of failure to position the nasoduodenal tube within the duodenum, two subjects were excluded based on excessive movement during scanning (confirmed with the ART-toolbox after visual quality control, >20% of volumes with framewise displacement >0.9mm), one for inadequate fMRI data quality (significant ghosting effects suspected to be related to a dreadlocked hairstyle), one subject requested termination of scanning due to urinary urgency and two subjects dropped out for logistic reasons (inability to plan second scanning visit). This resulted in a study population of 18 subjects that was used for data analysis.

Subjective syptom reporting

The intensity of symptoms elicited varied across individuals (Figure 8.1 and Supplementary Figure 8.1), in line with our previous observations[54]. The group mean abdominal pain score was 9.8 mm (on a 100mm visual analogue scale [VAS], ±SD 19.7 mm, range 0 - 89.5 mm). Three subjects did not report any symptoms during capsaicin infusion. VAS scores for abdominal pain during and after saline infusion were

significantly lower as compared to the ratings during and after capsaicin infusion: 0.6 mm (\pm SD 2.4 mm, range 0 – 25.2 mm, Wilcoxon signed-rank test, W = 248, p = 0.001). Based on the responder criterion, 8 participants were abdominal pain responders and 10 participants non-reponders.



Figure 8.1. Mean ΔVAS scores (along with standard error of the mean) for abdominal pain (panel A), abdominal burning (panel B) and abdominal discomfort (panel C). VAS scores obtained during test days with capsaicin infusion were subtracted by VAS scores obtained during test days with saline infusion. Individual ΔVAS scores are shown on the background in each panel (not included in the figure legend).

VAS scores for abdominal discomfort during and after capsaicin infusion were significantly higher as compared to VAS scores during and after saline infusion, with a mean of 11.5 mm for capsaicin (\pm SD 19.5 mm, range 0 – 80.9 mm) and 4.5 mm for saline (\pm SD 10.5 mm, range 0 - 48,0 mm, Wilcoxon signed-rank test, W = 244, p = 0.004). Mean VAS for abdominal burning was 6.6 mm for capsaicin (\pm SD 15.6 mm, range 0 – 73.8 mm) and 2.3 mm for saline (\pm SD 8.6 mm, range 0 – 54.0 mm, Wilcoxon signed-rank test, W = 221.5, p = 0.026). For all symptoms, VAS scores were significantly higher during the infusion period as compared to the post-infusion period (data not shown).

Abdominal pain and discomfort demonstrated a strong correlation (Spearmans ρ = .74, p < 0.001), whereas correlation between abdominal pain and burning was more modest (Spearmans ρ = .54, p < 0.001).

BOLD response

Capsaicin significantly increased the blood oxygenation level-dependent (BOLD) signal (condition-by-time interaction effect) in the majority of regions included in our mask consisting of pre-defined regions of interest, including the right subgenual anterior cingulate cortex, bilateral anterior midcingulate cortex, bilateral insula (across all subregions), left postcentral gyrus, right supramarginal gyrus, bilateral Rolandic operculum, left superior temporal gyrus, right putamen, right amygdala, bilateral ventral thalamus, left cerebellum (regions 4,5 and 8), nucleus cuneiformis, and the posterior region of the medulla oblongata (Figure 8.2 and Supplementary Table 8.1). The latter was of particular interest given the primary hypothesis of NTS activation.



Figure 8.2. Brain responses to capsaicin compared to saline at group level (change from baseline). Primary effect of interest: condition-by-time interaction effect. Parametric map thresholded at the voxel-level p<0.05 FWE-corrected using a pre-defined mask consisting of pain-responsive regions (https://neurosynth. org/analyses/terms/pain/<u>see Online Methods</u>). Color scale reflects F-values. Y and z represent MNI coordinates in respective directions. Images are in neurological convention. Abbreviations: ACC_pre = pregenual anterior cingulate cortex, MCC_ant = anterior midcingulate cortex, Ins_ant = anterior insula, Ins_mid = middle insula, Ins_post = posterior insula, Operc = rolandic operculum, VPL = ventroposterolateral nucleus (thalamus), VL = ventrolateral nucleus (thalamus), PostC = postcentral gyrus, Put = putamen, Hip = hippocampus.

Brainstem responses

To aid differentiation of active brainstem nuclei, analyses were repeated with unsmoothed data, as shown in Figure 8.2. We observed several distinct clusters in the medulla oblongata. A bilateral activation was seen in the upper medulla (Figure 8.3A and B), whereas the lower medulla comprised one posteriorly centered cluster (Figure 8.3C). A high resolution brainstem atlas was used for identification of active brainstem clusters[49]. A - primarily right-sided - portion of the typical V-shape of the NTS can be recognized in Figure 8.3, with the most caudal part displayed in Figure 8.3C. The latter represents the commissural nucleus[49], which corresponds to the NTS subregion that preferentially receives projections specifically from visceral TRPV1-positive vagal fibers[17]. In addition, the axial slice shown in Figure 8.3B (and E) encompasses a larger right-sided cluster, which includes purported nucleus ambiguus (Namb) and spinal trigeminal nucleus (SpV)[49]. Finally, one smaller cluster could be seen just posterior to the left NTS, which includes purported dorsal motor nucleus of the vagus nerve (DMNX), see Figure 8.3E. Given the presence of second-order projections from the NTS to the SpV, Namb and DMNX[53], we believe that the activations of these regions are downstream from the primary NTS activation. Namb and DMNX are both premotor nuclei implicated in the generation of autonomic response patterns evoked by physiological and various sensory stimuli[42].





Differences in BOLD response according to perceptive response

We subsequently compared BOLD responses between groups according to the perceptive responses, i.e. responders vs. non-responders. Results are shown in Figure 8.4 and Supplementary Table 8.3. BOLD responses particularly in the ventral thalamus, pregenual anterior cingulate cortex, and anterior insula were more pronounced in responders compared to non-responders.

Interestingly, we here observed an increased BOLD response at the location of the NTS (y coordinate -44, z coordinates between -52 and -58) both in responders and non-responders. The fact that no difference was seen between responders and non-responders was confirmed by an additional region-of-interest analysis (Supplementary Table 8.3).





We further examined differences in BOLD response between responders and nonresponders at the level of the thalamus, an important relay station to the cortex downstream from the NTS, using high-quality parcellations that included 16 functional zones (Supplementary Table 8.4). According to the perceived pain response, significant differences in BOLD response were found for the mediodorsal nucleus, ventral lateral nucleus and intralaminar nuclei of the thalamus. In addition, in order to ascertain the potential role for other subcortical sensory relay stations more rostral to the NTS, we also examined, *a posteriori*, activation in the parabrachial complex and locus coeruleus in responders and non-responders, but this provided no evidence for their involvement as ROI analysis did not demonstrate significant clusters (Supplementary Table 8.3). Similarly, we investigated further the activation of the periaqueductal gray matter (PAG), which was included in the pain mask. For the PAG, we did observe an active cluster just above the extent threshold (i.e. 10 voxels) but in responders only (Supplementary Table 8.4). When comparing responders with non-responders however, we did not observe a significant difference.



Figure 8.5. Mean RMSSD (along with standard error of the mean) plotted as change from baseline at the whole group level (panel A), and separately for responders (panel B) and non-responders (panel C) as identified by the abdominal pain response criterion. Post-hoc paired t-tests were used to identify specific time windows at which there were significant differences between conditions, significant windows were highlighted with asterisks (*: p<0.05, **:p<0.008 [adjusted for multiple comparisons]).

Autonomic outflow: heart rate variability

We finally ascertained the effects of capsaicin infusion on autonomic outflow, as a functional peripheral readout of the intervention, using pulse rate variability as calculated from photoplethysmographic signal of the pulse wave. We observed a significant increase in the root mean square of successive beat-to-beat difference (RMSSD), which is a marker that reflects on cardiac parasympathetic activity, during capsaicin infusion. Such changes were not observed during or after saline infusion, resulting in a significant difference between conditions (marginal linear mixed model, main effect of condition F(1,12)=5.49, p=0.037). Moreover, the increase in RMSSD after capsaicin was driven by the abdominal pain responder group, resulting in a significant difference between responders (marginal linear mixed model, condition-by-responder interacton effect F(1,11)=5.49, p=0.039) (Figure 8.5).

DISCUSSION

Brainstem nuclei are assumed to play a key role in the processing of visceral afferent input, although investigating their function in more detail has hitherto been hampered by technical challenges. Here, by combining dedicated multi-echo multiband 7T fMRI with a nutrient-derived intestinal chemonociceptive stimulus in healthy individuals, we demonstrate activation of the principal relay station for vagal afferents, the nucleus of the solitary tract (NTS), which was engaged irrespective of the conscious pain response.

Complex statistical analytic methods and computational algorithms aside, advances in the field of neuroimaging in relation to DGBIs have more recently been limited by the lack of relevant technical developments that allow more specific identification of brainstem nuclei. These challenges are related to the small size of many brainstem structures; MRI signal distortion due to physiological noise (generated by chest motion and the propagation of cardiac pulse pressure waves); and magnetic susceptibilityinduced distortions because of its proximity to air-filled cavities[44]. Given the length of the paradigm in the current study and the near whole brain coverage, an EPI image resolution of 2.2mm isotropic was deemed the maximum, despite the high-power field strength. This was considered sufficient for NTS imaging because of the added benefits of multi-echo acquisition and ultra-high field strength, providing increased signal-tonoise and contrast-to-noise ratio. The latter is corroborated by a recent 7T fMRI study mentioned previously in which NTS engagement was reported after applying a 2mm Gaussian smoothing kernel (using single echo EPI), resulting in a target resolution similar to our unsmoothed data[46].

In addition, we here applied a duodenal chemonociceptive stimulus, that has hither tho not been used within the context of neuroimaging. Given the observed NTS activation, the question arises which conduit was used to transmit the afferent input from the intestinal mucosa to the brainstem. The upper GI tract receives dual innervation, vagal and thoracolumbar spinal, where the former is believed to transmit chemical and the latter more nociceptive stimuli[13]. The NTS has been hypothesized to receive input from both systems[1]. Considering the – at least on average - relatively low intensity of the pain perceived following the stimulus administered, one would assume the principal involvement of the vagal pathway. This proposition is supported by the fact that cardiac parasympathetic activity, as reflected in part by the RMSDD, increased over the course of the intervention, whereas a decreased parasympathetic activity would be expected of a more salient, spinally-mediated acute painful stimulus[18]. However, it was indeed the pain-responders who demonstrated this increase in RMSDD in particular, which would be contrary to this commonly accepted view. On the other hand, previous studies have failed to show a relationship between perceived pain intensity and changes in heart rate variability[31], which we believe is reflective of the complexity of neural mechanisms, integrating perceptual, interoceptive and cognitive elements, that are involved in the control of autonomic outflow[18].

An important observation we made here was that the degree of BOLD response to capsaicin was related to the subjects' perceptive response, i.e. more pronounced in responders versus non-responders. This observation is in line with the postulate that there is a direct relationship between visceral stimulus salience (subliminal, liminal or supraliminal) and the extent of brain activation, although this phenomenon has hitherto only been described for cortical responses [16; 47], but not for the brainstem. Interestingly, no differences in BOLD activation was observed between responders and non-responders on the level of the NTS and the differences only became apparent when looking at the thalamus (and more rostral areas). It remains unknown which levels of the neuraxis support the differences in thalamic activation that are related to perceptive responses. The PAG showed activation in a small cluster in responders only, with no significant difference between responders and non-responders, rendering the exact involvement of PAG uncertain. In addition, we did not identify activation in any other sensory relay nuclei, such as the locus coeruleus or the parabranchial nucleus, albeit these analyses were performed *a posteriori* and might have been limited in extent. Nevertheless, we speculate that monosynaptic projections from the NTS directly to the thalamus[8] may have substantial influence in determining whether the nociceptive signal will result in painful perception. As for the origin of the thalamic input, we investigated the activation of the different thalamic subnuclei. Those showing the most pronounced activation, i.e. the mediodorsal nucleus, ventral lateral nucleus and intralaminar nuclei, have all been implicated to receive input from the vagus nerve[14], with the mediodorsal nucleus projecting interoceptive information to the insula and orbital frontal cortex[48]. Therefore, we believe that this observation supports the postulate that differences in perceptive responses primarily originate from vagal pathways as opposed to spinal pathways, as the latter would be expected to yield a more pronounced BOLD response in the ventral posterolateral nucleus[48].

More detailed understanding of the processing of afferent input via the vagus nerve derives its relevance from the fact that the vagus is the major bidirectional highway of gut-brain connection. The afferent branch of the vagus nerve has therefore been the focus of multiple studies examining its effects on gut-brain communication, and more broadly the microbiota-gut-brain axis, in both health and disease[27]. Therefore, although this study was performed in healthy volunteers, findings can potentially carry important clinical implications. First, they allow fundemantal insight necessary for the development of novel therapeutic strategies aimed at the correction of disturbed visceral afferent pain processing. One of these recent developments include the use of transcutaneous stimulation of the auricular branch of the vagus nerve. Initial studies have provided evidence for its efficacy when using subliminal electrical stimuli to the auricular vagus for the treatment of functional abdominal pain and IBS in adolescents[19; 22]. The authors assumed that this therapy acts by virtue of NTS stimulation, and heart rate variability was able to predict clinical response[20], although the exact mechanisms of action admittedly remain to be elucidated. Second, the experimental capsaicin-pain model and ultra-high field imaging technology applied here also allows their future application for investigating underlying pathomechanisms in conditions related to altered visceroperception beyond FD and IBS, such as other DGBIs, i.e. reflux hypersensitivity, functional heartburn or chronic nausea and vomiting syndrome, but more broadly also eating disorders, including avoidant-restrictive food intake disorder (ARFID).

Although this study was limited by its sample size, which could imply that findings are idiosyncratic to this particular experimental context, rather than truly reflecting the mental operations under study[21], the cortical activations correspond to those described in previous studies examining visceral pain.[51] In addition, the magnitude of alterations in both brain activation and autonomic outflow were clearly delineated according to the individuals' pain perception, suggesting that these differences indeed reflect a distinct biological response. While we eventually included 18 subject in the final analysis instead of the 21 originally planned, a recent study using 7T brainstem imaging to show activation of the NTS in 16 subjects that became available at the time we had completed data collection, corroborated the adequacy of our sample size[46]. In addition, we acknowledge that localization of the NTS as based on an atlas that is not provided in a normalized (common) brain space such as MNI is difficult. We therefore complemented our analysis of the NTS by using a hand-drawn NTS ROI based on highresolution brainstem scans from a subset of subjects, which confirmed the medullary clusters to be part of the NTS. In addition, a recent study using vagus nerve stimulation at 7T fMRI reports similar coordinates of the NTS in the y and z direction, further corroborating our findings.[45] Further, the use of a pre-defined mask as opposed to

whole-brain analysis means that the results primarily provide high sensitivity but lower specificity by excluding regions that are not of interest.

Another limitation is the overall low pain ratings, making distribution not normal and cut-offs therefore somewhat arbitrary by necessity. This might emphasize the importance of a wider range of other visceral symptoms and aversive sensations, such as nausea and early satiation, which were not assessed in detail here, but make this type of investigation more relevant also to adjacent fields and clinical conditions, as mentioned above.

CONCLUSION

In conclusion, we here exploited the benefits of multi-echo multiband 7T fMRI to identify central processing of visceral afferent input to the brainstem arising from the duodenal region. Using advanced imaging and a novel stimulation paradigm, we were able to identify activation of the brainstem, corresponding to the key anatomical area of the nucleus of the solitary tract, assumed to result from vagal afferent excitation. At this level of the gut-brain axis, nociceptive processing appears to operate regardless of the perceptive responses. The differentiation according to the perceived pain seems to occur more downstream along the afferent gut-brain axis. The results presented here can further prompt future work on the exploration of fundamental mechanisms related to how pain emerges from nociception as well as new therapeutic approaches to treating visceral pain conditions, for instance by targeting specific areas within the gut-brain axis to downregulate disturbed visceroception and hyperalgesia.

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SUPPLEMENTARY METHODS

Study population

In addition to the abovementioned criteria, subjects were all non-smokers and did not excessively consume alcohol (defined as >15 units per week). All subjects were required to not regularly consume spicy food, as capsaicin-sensitive afferents can become desensitized after repeated exposure[3]. The presence of affective disorders, early life trauma or significant gastrointestinal symptoms were considered exclusion criteria. All subjects completed questionnaires one day prior to the first scanning visit in order to screen for these factors: Patient Health Questionnaire-9 (PHQ-9), Generalized Anxiety Disorder assessment (GAD-7), Early Trauma Inventory Self Report Short Form (ETISR-SF) and Gastrointestinal Symptom Rating Scale –IBS (GSRS-IBS). In addition, shortly before each scanning visit, subjects completed the Brief Pain Inventory (BPI-SF). All these questionnaires were indicative of the lack of any significant affective or pain disorders (such as baseline pain experience) that may affect fMRI results (data not shown).

Subjects were asked to refrain from spicy food intake for one week prior to scanning, and to not use caffeine or alcohol 24 hours prior to scanning. All subjects were studied after a 12-hour overnight fast.

MRI data acquisition

MRI data acquisition was performed using an interleaved ascending simultaneous multi-slice acquisition with a multi-band factor of 2. This sequence used the following parameters: 2.2 mm isotropic voxel size, 50 slices, repetition time (TR) = 1.6 s, echo time ET1 = 10.6 ms, ET2 = 26.08 ms, ET3 = 41.56 ms, flip angle = 64°, bandwidth = 2362 Hz/Px, echo spacing = 0.53 ms, generalized autocalibrating partially parallel acquisitions (GRAPPA) acceleration factor R = 3. Slices were tilted to fully incorporate the brainstem while minimizing the inclusion of air-filled cavities. Because of tilting, image acquisition of part of the parietal cortex was limited up to the postcentral gyrus. Given the length of the task and the near whole brain coverage, the EPI image resolution of 2.2mm isotropic was deemed the maximum. This was considered sufficient for NTS imaging because of the added benefits of multi-echo acquisition and ultra-high field strength. The latter is corroborated by a recent 7T fMRI study mentioned previously in which NTS engagement was reported after applying a 2mm Gaussian smoothing kernel (using single echo EPI), resulting in a resolution similar to our unsmoothed data[46].

Prior to functional scanning, two anatomical scans were acquired, one whole-brain MP2RAGE sequence (Magnetization Prepared - RApid Gradient Echo[28]), as to be used for registration purposes, and one high-resolution MT-TFL sequence with medulla

oblongata and lower pons coverage only (resolution 0.4x0.4x0.5mm), specifically designed to aid visualization of small brainstem nuclei (e.g. the NTS), as previously described[40].

After completion of each functional run, five additional volumes were acquired in the opposite phase-encoding direction, later to be used for estimation and correction of susceptibility-induced distortion. These recordings were considered a backup for physiological denoising if needed. Given the good quality of multi-echo denoising, physiological recordings were not used for denoising. However, photoplethismographic signal data was used for the calculation of pulse rate variability (see section 'Photoplethysmographic signal processing and analysis').

MRI preprocessing (details)

fMRI images were first corrected for slice timing differences using manually defined slice timings (extracted from the DICOM header) as applicable for multiband acquisitions, and subsequently motion corrected using an SPM-based script that estimated motion on ET1 images, after which the same transformations were applied to all three echoes. TE-dependence analysis was performed on slice time and motion corrected multi-echo data. An adaptive mask was generated, in which each voxel's value reflects the number of echoes with usable data. A monoexponential model was fit to the data at each voxel using log-linear regression in order to estimate T2* and S0 maps. For each voxel, the value from the adaptive mask was used to determine which echoes would be used to estimate T2* and S0. Multi-echo data were then optimally combined using the t2s combination method[38]. Principal component analysis followed by the stabilized Kundu component selection decision tree was applied to the optimally combined data for dimensionality reduction[23]. Independent component analysis was then used to decompose the dimensionally reduced dataset. Component selection was performed to identify BOLD (TE-dependent), non-BOLD (TE-independent), and uncertain (low-variance) components using the Kundu decision tree[23].

After applying tedana, the optimally combined and denoised data was corrected for susceptibility-induced distortion (estimated using topup, FSL[2]). Finally, ART-based (Artifact Detection Tools) motion outlier detection, segmentation, normalization to 2mm MNI space and minimal smoothing were applied with use of the CONN toolbox. A 4mm FWHM Gaussian smoothing kernel was chosen in order to maintain sufficient spatial resolution for brainstem analyses, as well as to limit extension of cardiorespiratory physiological noise near the brainstem surface and thereby prevent false-positive results[33]. Additionally, brainstem analyses were repeated with unsmoothed data in order to provide best possible spatial resolution and aid differentiation of active brainstem nuclei.

Post-processing, scrubbing was applied for censoring residual artefactual volumes as identified by the ART-toolbox (global BOLD signal changes above 5 standard deviations), realignment parameters were regressed out and spatially diffuse noise was removed by

means of the anatomical component-based noise correction method (aCompCor), as implemented in the CONN toolbox[39; 57]. Confound-corrected timeseries were used for the analyses described below.

Region-of-interest analysis

A region-of-interest (ROI) analysis within the NTS was performed when using the response criterion for abdominal pain as described above. The ROI of the NTS was hand drawn from high-resolution anatomical brainstem scans (see MRI and physiological data collection) using Duvorney's atlas as a reference. These estimates were generated from scans that were not affected in quality in any way whatsoever by artefacts, or other sources of distortion (nine out of eighteen scans). Drawings were made per subject and subsequently summed using SPM Imcalc to create the NTS ROI (see supplementary Figure 8.2). The resulting NTS ROI was used as a group level template, and all subjects (n = 18) were included in the ROI analysis.

In addition, a ROI analysis was performed within the thalamus using the CANlab combined atlas (based on data from Morel et al.[32]). This atlas contains 16 parcellations of the thalamus (plus one of the hypothalamus): anteromedial nuclei, anteroventral nuclei, habenular nuclei, pulvinar region, intralaminar nuclei, lateral dorsal nuclei, lateral geniculate nuclei, lateral posterior nuclei, medial geniculate nuclei, mediodorsal nuclei, wentral nuclei, ventral anterior nuclei, ventral lateral nuclei, ventral medial nuclei, ventral nuclei and ventral posterior medial nuclei. Similar to the NTS ROI analysis, we used the response criterion for abdominal pain for the thalamus ROI analysis, comparing responders and non-responders.

Finally, we performed ROI analyses, *a posteriori*, on the parabrachial nucleus and locus coeruleus. As these nuclei did not constitute the primary focus of this study, our high-resolution MT-TFL brainstem scans did not provide adequate coverage of these nuclei. We therefore used the Harvard Ascending Arousal Network Atlas for ROI analysis of the parabrachial nucleus and locus coeruleus[10]. It should be noted that this atlas is based on the post-mortem scan of a 53-year-old single subject. Given that the locus coeruleus is known to lose volume over age[26], it is possible that the location in the template does not perfectly translate to our younger study population, and this additional exploratory analysis should therefore be treated with the necessary caution.

A voxel-level threshold of p<0.05 (FWE-corrected) and extent threshold of 10 voxels was used for all ROI analyses (i.e. thresholds similar to the primary analyses).

Post-hoc laterality analysis

A post-hoc laterality analysis was carried out given the greater extent of activation in the right medulla oblongata at the location of the NTS. A box-shaped region-ofinterest was drawn at MNI coordinates -4 -42 -53 and 4 -42 -53 for left and right brainstem respectively, corresponding to the peak coordinates of the active clusters in the medulla, with x-y-z dimensions 3, 3 and 4mm (covering the entirety of the largest cluster). The location of the resulting binary files was checked visually and was confirmed to overlap with the observed active brainstem clusters. The SPM-based extension AveLI was used to calculate the laterality index from the left and rightsided region-of-interest[29]. In short, this tool calculates a laterality index for a range of thresholds and averages them to one index value. The resulting average laterality index can be considered "threshold-free" and thus circumvents the issue of threshold dependence of laterality. First level contrast images were used for calculations. This yielded 45 (averaged) laterality indices for each subject, corresponding to one index per timebin. The mean laterality index (with 95% confidence interval) was subsequently calculated per subject (see Supplementary Table 8.3). A one-sample t-test was used to test whether the resulting group mean was significantly different from zero.

Photoplethysmographic signal processing and analysis (details)

One separate physiological data file was stored per functional scan. All PPG data files were processed with the use of a custom written MATLAB script (MATLAB R2020a). Systolic peaks were detected using the peak detection method and were considered as the pulse times for the calculation of pulse intervals and PRV. Diagnostic figures were created to inspect data quality, which included pulse rate plotted against time, as well as pulse interval. Automated iterative peak detection highlighted potential ectopic and skipped beats. All signals, specifically highlighted regions, were checked manually for potential influential outliers in pulse rate, potential ectopic and skipped beats or noise. These beats were marked as errors for later correction of PRV. The deletion method was used for correction to calculate PRV (RMSSD). Samples (i.e. time windows) with more than 5% of the beats marked as errors were excluded from the analysis. If the baseline sample included >5% beats marked as errors, the entire visit was excluded from the analysis, as change from baseline could not be calculated in that case. This approach resulted in the exclusion of 55 out of 216 samples, leaving 161 samples for PRV analysis.

In addition to the PPG data, we processed log files containing information on respiration during each functional scan. A comparison of respiratory rate between conditions demonstrated no significant differences (data not shown), indicating that the PRV data was not confounded by respiratory rate.

After physiological data processing, a custom MATLAB script was used to compute the RMSSD. RMSSD was calculated first for the 6-minute baseline period, and subsequently over fixed time intervals of 5 minutes, as is currently considered the standard. Mean RMSSD was computed per 5-minute interval and plotted for both scanning conditions along with the standard error of the mean. For statistical analysis, the RMSSD change from baseline was calculated per subject for each condition (i.e. capsaicin and saline).

This change from baseline approach was used given the fact that heart rate variability measures are known to show significant day-to-day variability[15; 52], even though no differences in baseline RMSSD were found in this particular case (marginal linear mixed model, main effect of condition F(1,15)=1.45, p=0.25).

RMSSD (change from baseline) constituted the dependent variable, condition (capsaicin versus saline) and timebin constituted the within-subject independent variables, and their main effects as well as two-way interaction were included in the model. In order to account for the potential covariance between baseline RMSSD and its subsequent change from baseline, the addition of baseline RMSSD as continuous covariate of no interest to the model was tested, but did not reach significance (p=0.89) nor significantly impacted on the other effects in the model. The Kronecker product of an unstructured and a first order autoregressive variance-covariance matrix for the within-subject variables condition and time fitted the data best based on the lowest value of Akaike's Information Criterion. The main effect of condition constituted the effect of interest testing the hypothesis of a significant difference in RMSSD between conditions over the entire timecourse. To additionally test for the effect of responder status, an additional between-subject independent variable (responder versus non-responder) was added to the model, including its two-way interactions with the other variables, with the condition-by-responder interaction constituting the effect of interest testing the hypothesis of a difference in RMSSD response to capsaicine versus saline in responders versus non-responders.

SUPPLEMENTARY RESULTS

Supplementary Table 1. Differential brain response to duodenal capsaicin infusion	versus	saline
(condition*time interaction effect): regions demonstrating increased BOLD activity.		

Anatomical region	Subregion	Hemisphere	x	у	z	Number of voxels	F-value
Anterior cingulate cortex	Subgenual	Right	4	32	0	37	12.20
Insula	Anterior	Left	-42	12	-12	37	11.20
	agranular complex	Right	42	18	-6	27	8.38
	Anterior ventral insula	Right	34	20	2	19	5.21
	Granular	Left	-36	-20	12	37	15.16
	complex	Right	36	-14	10	18	5.13
	Middle insula	Left	-44	14	-8	45	10.17
		Right	42	16	-4	20	5.54
	Posterior area 1	Left	-38	-18	2	11	7.43
		Right	36	-16	8	42	13.94
	Posterior area 2	Left	-36	-6	8	13	4.26
		Right	38	-14	8	27	9.05
Postcentral gyrus		Left	-48	-18	28	32	7.93
Rolandic operculum		Left	-58	-6	10	146	7.57
		Right	40	-18	20	272	10.81
Superior temporal gyrus		Left	-52	-28	16	34	8.20
Amygdala		Right	26	4	-22	21	4.91
Thalamus	Ventroanterior	Left	-4	-10	2	47	7.25
	Ventroposterior	-	-20	-24	4	40	8.02
	Ventroanterior	Right	6	-20	4	84	10.39
	Ventroposterior		10	-24	2	88	16.15
Cerebellum	Region 4,5	Left	-6	-60	-14	20	7.36
Medulla oblongata	Posterior	Mid/Right	2	-42	-56	48	16.10

*A voxel-level threshold of p<0.05 (FWE-corrected) was used within a

Anatomical regions identified with the use of Automated Anatomical Labeling (AAL3), Melbourne Subcortex atlas (for thalamus subdivisions) and Human Connectome Project (for insula subdivisions)[12; 41; 50]

Supplementary Table 2. Differential brain response to duodenal capsaicin infusion versus saline (condition*time interaction effect): regions demonstrating decreased BOLD activity.

						Number of	
Anatomical region	Subregion	Hemisphere	х	у	z	voxels	F-value
Midcingulate cortex	Anterior	Left	-4	-6	36	52	10.83
	Anterior	Right	8	10	36	20	5.87
Supramarginal gyrus		Right	56	-26	38	75	10.10
Prefrontal cortex	Mid orbitofrontal	Right	30	52	-12	14	4.55
Putamen		Right	30	12	-2	74	7.93
Cerebellum	Region 8	Left	-22	-72	-52	27	8.23
Nucleus cuneiformis		Right	10	-28	-22	56	11.02

*A voxel-level threshold of p<0.05 (FWE-corrected) was used within a

Anatomical regions identified with the use of Automated Anatomical Labeling (AAL3), Melbourne Subcortex atlas (for thalamus subdivisions) and Human Connectome Project (for insula subdivisions)[12; 41; 50]

Supplementary Table 3. Mean laterality index per subject (averaged over 45 timebins). Laterality index values range from -1 to 1, where negative values indicate right-hemisphere dominance, and positive values indicate left-hemisphere dominance. Laterality index values are depicted in ascending order.

Subject number	Mean laterality index	95% Confidence interval	
7	-0.67	-0.700.64	
4	-0.66	-0.690.64	
8	-0.53	-0.600.46	
2	-0.51	-0.540.49	
12	-0.42	-0.450.38	
10	-0.40	-0.430.37	
14	-0.35	-0.380.31	
6	-0.31	-0.380.24	
5	-0.22	-0.270.17	
11	-0.13	-0.200.06	
18	-0.07	-0.11 - 0.04	
3	-0.01	-0.05 - 0.03	
13	0.02	-0.04 - 0.08	
15	0.07	0.01 - 0.12	
17	0.07	-0.03 - 0.18	
1	0.23	0.18 - 0.28	
9	0.27	0.21 - 0.33	
16	0.33	0.24 - 0.43	

A laterality index was calculated post-hoc (see Methods, section *Post-hoc laterality analysis*) for the medullar clusters of activation, as the cluster was substantially larger on the right side (see Figure 2). The laterality index indicated a weak right dominance, with a group average of -0.18 (95%CI -0.34 - -0.03, one-sample t-test indicating significant difference from zero: t(17)=-2.45, p = 0.026). Although the NTS also receives input from splanchnic afferents that can be excited by capsaicin, which is assumed to be equilateral, we postulate that the modest laterality observed here is related to the vagal anatomy of the infusion site. Importantly, the distal duodenum is primarily innervated by the right vagus nerve (via the celiac branch), while also receiving some innervation from the left vagus nerve via the gastroduodenal branch[6].

Supplementary Ta	ble 4. Uifferential brain	response to duodenal	capsaicin infusioi	n versus saline	e tor res	ponde	rs, non	-responders and respor	nders > non-responders.
Anatomical region	Subregion	Group	Hemisphere	Activity	x	y	z	Number of voxels	F-value
Anterior cingulate	Subgenual	Responders	Right	~	9	36	-9	31	5.74
cortex		Non-responders	1	ns					1
		Responders > non- responders		su	ı	ı	ī	1	1
Anterior cingulate	Pregenual	Responders	Left	+	-4	38	24	33	5.57
cortex		Non-responders	Left	→	-4	18	30	53	5.27
		Responders > non- responders	Right/left	←	-4	16	30	101	10.75
Midcingulate cortex	Anterior	Responders	Right	~	4	14	32	31	5.96
			Left	→	-4	-4	36	14	9.31
		Non-responders	Right	→	4	-4	36	36	9.50
			Left	→	-10	10	40	41	6.20
		Responders > non-	Right/left	÷	0	4	44	37	9.13
		responders	Right/left	÷	0	14	32	47	7.98
Insula	Anterior agranular	Responders	Right	←	42	18	-9	20	19.08
	complex		Left	÷	-44	10	×,	46	11.90
	Anterior ventral insula	I	Right	~	36	22	-2	38	13.76
	Granular complex	I	Left	¢	-36	-20	12	31	9.35
	Middle insula	I	Right	÷	40	18	-4	21	11.71
			Left	~	-40	16	0	48	15.01
	Posterior area 1	I	Right	Ļ	36	-16	8	13	7.57
			Left	Ļ	-40	-8	-10	13	7.37
	Posterior area 2		Left	Ļ	-44	8	-10	18	11.27

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	1-3		TT		1	;	1	N	
Anatomical region	Autoregion	Vion sociality	r off.	Activity	x	~ :	7	Number of voxels	F-Value
	Amerior agrammar complex	non-responders	reit	_	00-	71	-14	10	0.00
	Anterior ventral insula	I	Right	÷	38	22	-4	11	5.84
	Granular complex	I	Left	←	-38	-20	12	19	6.78
	Middle insula	I	Left	←	-32	14	8	33	8.15
	Posterior area 1	l	Right	÷	40	-18	2	28	7.34
	Posterior area 2	I	Right	¢	44	-10	4	11	7.08
			Left	←	-40	-2	2	22	5.42
	Anterior agranular complex	Responders > non- responders	1	ns	I	1		1	
	Anterior ventral insula	I	Right	~	38	22	-2	35	15.79
	Granular complex	I	1	ns				. 1	1
	Middle insula	I	Left	÷	-40	16	0	26	10.43
	Posterior area 1	I	1	ns					1
	Posterior area 2	I	Left		-44	æ	ş	14	5.88
Postcentral gyrus		Responders	Left	→	-60	-18	16	21	6.43
		Non-responders	Right	←	28	-34	56	54	8.40
			Left	÷	-30	-30	52	29	11.56
		Responders > non-	Right	→	28	-34	56	71	7.67
		responders	Right	÷	28	-34	56	50	7.67
			Left	→	-54	-10	18	45	6.57

Supplementary Table (continued)	4. Differential brain	response to duodenal o	capsaicin infusior	n versus salin	e for re:	sponde	rs, non	-responders and respo	nders > non-responders.
Anatomical region Su	bregion	Group	Hemisphere	Activity	x	y	z	Number of voxels	F-value
Supramarginal		Responders	Right	→	54	-24	38	33	8.39
gyrus			Left	→	-58	-24	44	31	7.61
		Non-responders	Right	→	56	-30	38	48	7.98
		Responders > non- responders	Left	→	-58	-24	44	29	10.89
Temporal superior		Responders	Right	←	56	0	2	18	5.99
gyrus			Left	~	-46	2	8-	76	13.31
		Non-responders	Right	←	56	-30	14	63	9.36
			Left	←	-54	-14	12	21	4.49
		Responders > non-	Right	←	48	-30	16	37	6.36
		responders	Left	←	-48	8	8-	24	5.28
Prefrontal cortex Mi	d orbitofrontal	Responders	Mid	→	0	38	-14	15	6.13
		Non-responders	1	ns				1	
		Responders > non- responders	Left	←	-2	36	-12	13	7.53
Rolandic operculum		Responders	Right	←	58	-2	12	146	8.95
			Left	→	-48	4	2	74	5.76
		Non-responders	Right	←	46	-12	16	193	9.04
			Left	←	-42	-20	18	123	8.51
		Responders > non-	Right	+	56	8	6	95	7.04
		responders	Left	→	-48	4	2	41	5.29
Putamen		Responders	1	ns				1	1
		Non-responders	Right	→	30	10	-4	23	6.50
			Left	→	-30	-2	-9	12	3.92
		Responders > non- responders	1	ns	I	1	1		

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Supplementary Ta (continued)	ible 4. Differential brain	response to duodenal	capsaicin infusior	ı versus saline	e for res	ponder	s, non-	responders and respo	nders > non-responders.
Anatomical region	Subregion	Group	Hemisphere	Activity	x	y	z	Number of voxels	F-value
Thalamus	Dorsoposterior	Responders	Left	÷	-16	-30	6	16	4.94
	Dorsoanterior	I	Right	÷	6	-18	12	16	6.12
	Ventroposterior	I	Right	÷	12	-24	2	74	12.70
			Left	÷	8-	-20	-2	64	13.40
	Ventroanterior	I	Right	←	6	-16	-2	157	17.06
			Left	÷	-9	8-	0	195	19.73
	Ventroanterior	Non-responders	Right	←	-12	-14	10	12	3.82
	Ventroposterior	I	Right	÷	8	-22	2	36	7.72
	Dorsoanterior	Responders > non-	Left	←	- 14	-14	10	27	6.14
	Dorsoposterior	_responders		ns					
	Ventroanterior	I	Right	÷	2	-16	12	66	13.99
			Left	←	-9	-9	0	188	19.01
	Ventroposterior	1	Left	←	-8	-20	-2	36	9.79
Periaqueductal grey		Responders	mid	←	0	-28	-10	10	9.61
		Non-responders	1	ns				1	
		Responders > non- responders	1	su	1	1			,
Medulla oblongata	Posterior	Responders	Right/left	←	4	-42	-56	39	9.25
		Non-responders	Right/left	←	2	-42	-54	80	11.65
		Responders > non- responders	Left	←	-6	-42	-48	19	7.99

Functional neuroimaging of visceral nociception

(continued)								
Anatomical region Subregion	Group	Hemisphere	Activity	x	y	z	Number of voxels	F-value
Nucleus of the solitary tract:	Responders	Right/left	¢	-4	-44	-52	18	7.85
Region-of-interest analysis	Non-responders	Right	¢	2	-42	-54	10	10.66
(a b) (o) ()	Responders > non-		su					1
	responders							
Parabrachial complex:	Responders	1	ns					1
Region-of-interest analysis	Non-responders	1	ns				. 1	1
(a posteriori)	Responders > non-		ns		,	,		
	responders							
Locus coeruleus:	Responders	1	ns				. 1	
Region-of-interest analysis	Non-responders	1	ns				. 1	1
(a posteriori)	Responders > non-	. 1	ns					
	responders							

*A voxel-level threshold of p<0.05 (FWE-corrected) was used within a pre-defined mask containing pain-responsive regions (see Online Methods). Corrected p-value for all clusters reported above was <0.001. Anatomical regions identified with the use of Automated Anatomical Labeling (AAL3), Melbourne Subcortex atlas (for thalamus subdivisions), Human Connectome Project (for insula subdivisions) and Harvard Ascending Arousal Network Atlas (parabrachial complex and locus coeruleus)[10, 12, 41, 50]

**ns: no significant results

Supplementary Table 5. Differential brain response to duodenal capsaicin infusion versus saline. Regionof-interest analysis using thalamus parcellations from CANIab combined atlas (based on data from Morel et al[32]) for responders, non-responders and responders > non-responders.

Subregion	Group	Hemisphere	Activity	x	у	z	Number of voxels	F-value
Mediodorsal	Responders	Right	1	4	-14	0	100	12.46
nucleus		Left	↑	-4	-18	2	61	14.29
	Non-responders	-	ns	-	-	-	-	-
	Responders > non-responders	Right	↑	4	-18	2	63	7.08
		Left	1	-2	-18	2	60	13.63
Intralaminar	Responders	Right	†	6	-16	-2	72	17.06
nuclei		Left	1	-2	-18	0	53	13.42
	Non-responders	Right	1	12	-24	0	23	6.06
	Responders > non-responders	Right	1	6	-16	-2	13	10.34
		Left	1	-2	-18	0	53	16.01
Ventral lateral	Responders	Right	†	20	-18	4	30	10.02
nucleus		Left	1	-10	-12	2	96	11.44
	Non-responders	Left	Ļ	-12	-14	10	30	3.82
	Responders > non-responders	Right	†	12	-8	6	10	5.24
		Left	†	-12	-12	0	110	9.74
Ventral	Responders	Left	†	-6	-8	0	19	19.73
anterior	Non-responders	Left	Ļ	-8	-8	0	10	4.40
nucleus	Responders > non-responders	Right	1	12	-6	6	14	5.01
		Left	1	-6	-6	0	21	19.01
Ventral medial	Responders	Right	1	12	-14	-2	14	10.22
nucleus	Non-responders	-	ns	-	-	-	-	-
	Responders > non-responders	Right	1	10	-10	-2	11	7.39
Ventral	Responders	Right	1	18	-22	4	12	7.27
posterolateral	Non-responders	-	ns	-	-	-	-	-
nucleus	Responders > non-responders	Left	1	-10	-16	12	11	7.82
Ventral	Responders	Left	1	-8	-18	-2	14	11.08
posteromedial	Non-responders	-	ns	-	-	-	-	-
nucleus	Responders > non-responders	-	ns	-	-	-	-	-
Pulvinar	Responders	Right	1	10	-26	2	28	12.67
nuclei		Left	†	-10	-26	0	39	10.09
	Non-responders	Right	†	16	-24	2	19	4.04
	Responders > non-responders	Left	1	-10	-26	0	18	7.11

No significant clusters were found in the following nuclei: anteromedial nucleus, anteroventral nucleus, midline nucleus, habenular nucleus, lateral geniculate nucleus, medial geniculate nucleus, lateral dorsal nucleus and lateral posterior nucleus.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Boxplots demonstrating delta VAS scores (subtracted between conditions) for abdominal pain, abdominal burning and abdominal discomfort. Red lines indicate median, red diamonds indicate te mean. Red plus signs represent outliers (> 1.5 * interquartile range).



Supplementary Figure 2. Region-of-interest (ROI) drawing of the NTS. Hand drawn from high-resolution anatomical brainstem scans.



Supplementary Figure 3. Time course of the delta brain response (change from baseline) following infusion of capsaicin versus placebo. Cluster locations correspond to coordinates in Supplementary Table 1.

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Supplementary Figure 4 Time course of the delta brain response (change from baseline) following infusion of capsaicin versus placebo. Cluster locations correspond to coordinates in Supplementary Table 1.



Supplementary Figure 5. Time course of the delta brain response (change from baseline) following infusion of capsaicin versus placebo. Cluster locations correspond to coordinates in Supplementary Table 1.

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Supplementary Figure 6. Time course of the delta brain response (change from baseline) following infusion of capsaicin versus placebo. Cluster locations correspond to coordinates in Supplementary Table 2.



Right NTS: responders (left) vs non-responders (right)



Supplementary Figure 7. Time course of the delta brain response (change from baseline) following infusion of capsaicin versus placebo in the medulla oblongata. Top panel demonstrates results at group level, lower panel compares time course at the location of the right NTS in responders versus non-responders.

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CHAPTER 9

Comparing the efficacy of data-driven denoising methods for a multi-echo fMRI acquisition at 7T

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ABSTRACT

In functional magnetic resonance imaging (fMRI) of the brain the signal is dominated by (physiological) noise. Imaging at ultrahigh field strength is becoming increasingly popular as it offers increased spatial accuracy. The latter is of particular benefit in brainstem neuroimaging given the small cross-sectional area of most nuclei. Physiological noise scales with field strength in fMRI acquisitions, however. Although this problem is in part solved by decreasing voxel size, it is clear that adequate physiological denoising is of utmost importance in brainstem-focused fMRI experiments. Multi-echo sequences have been reported to facilitate highly effective denoising through TE-dependence of Blood Oxygen Level Dependent (BOLD) signals, in a denoising method referred to as multi-echo independent component analysis (ME-ICA). It has not been explored previously how ME-ICA compares to other data-driven denoising approaches at ultrahigh field strength. In the current study, we compared the efficacy of several denoising methods, including anatomical component based correction (aCompCor), Automatic Removal of Motion Artifacts (ICA-AROMA), ME-ICA, and a combination of ME-ICA and aCompCor. We assessed several data quality metrics, including temporal signal-to-noise ratio (tSNR), delta variation signal (DVARS) and spectral density of the global signal. Moreover, we looked at the ability of each method to uncouple the global signal and respiration. In line with previous reports at lower field strengths, we demonstrate that after applying ME-ICA, the data is best post-processed in order to remove spatially diffuse noise with a method such as aCompCor. Our findings indicate that ME-ICA combined with aCompCor and ICA-AROMA are highly effective denoising approaches for multi-echo data acquired at 7T. ME-ICA combined with aCompCor potentially preserves more signal-of-interest as compared to ICA-AROMA.

INTRODUCTION

Functional magnetic resonance imaging (fMRI) exploits the small magnetic field distortions induced by deoxyhemoglobin to capture changes in cerebral oxygen supply and, indirectly, neuronal activity [13]. To facilitate measurement of these blood oxygenation level-dependent (BOLD) signal changes, fMRI relies on imaging techniques sensitive to susceptibility effects, such as gradient-echo echo-planar imaging (EPI). Given that the fractional change in the MRI signal as a result of most functional tasks does not exceed a few percent, the majority of the temporal signal variation can be classified as noise. The validity of an fMRI experiment that relies on the detection of small task-induced BOLD changes is therefore largely dependent on the efficiency of the applied data cleaning method(s). Various sources of noise can been identified, each warranting its own approach for mitigating confounding effects [14]. Common sources of noise include: 1) background noise, for example thermal noise (originating from the subject, and to a lesser extent scanner hardware) and spurious radiofrequency noise from the environment, 2) physiological noise, originating from cardiac and respiratory activity, and low frequency drifts due to intrinsic fluctuations in blood flow and metabolism, and 3) motion-related noise. Many approaches to denoising have been proposed over the past two decades, including methods that simply regress out respiratory and cardiac cycles or locally fit to those fluctuations [8], sample at multiple TE [24], are based on ICA or PCA and subsequent filtering or a combination of these. Although all fMRI studies are affected by these types of noise, studies focusing of the brainstem can be particularly challenging [15]. As a consequence of the close proximity of the basilar artery, pulse pressure waves cause relatively large displacements in the brainstem as compared to cortical regions. Similarly, the adjacent cerebrospinal fluid filled spaces, which flow correlates with the cardiac cycle [10], further increases brainstem motion. Being closer to air-filled cavities, such as the sinuses and the chest, also renders the brainstem prone to susceptibility artefacts. The complexity of these artefacts increases due to respiration, as it results in cyclic variability in air content in the chest [5].

Given the small size of most brainstem nuclei – often only a few millimeters wide – high spatial resolutions and tissue specificity are a prerequisite for (functional) brainstem imaging. Scanning at ultra-high field strength (7T and above) can facilitate such high resolutions. One should realize though that – although mitigated by higher spatial resolutions – physiological noise can become an even more dominant source of noise at higher field strengths [5; 25]. It will be clear that, for a brainstem fMRI experiment to succeed, highly efficient physiological denoising methods are warranted. The performance of such methods at 7T, in particular for brainstem imaging, has not been compared previously.

Previous resting state fMRI studies at 1.5T and 3T have demonstrated that multiecho EPI (ME-EPI) acquisitions enable the application of more effective data cleaning methods than single echo acquisitions [7; 11]. In addition, acquiring images at multiple echo times reduces signal dropout [17], which can be particularly problematic in the brainstem. Finally, if an experiment requires scanning both brainstem and cortical areas, the high T2* variability between these areas can be accounted for by the use of T2* maps calculated from ME-EPI data [22].

The aim of the current analysis was to investigate the performance of various data cleaning methods for multiband multi-echo fMRI at 7T in order to increase signal-tonoise ratio, using a dataset recently obtained to investigate brain(stem) responses to an intestinal chemonociceptive stimulus [3]. The performance of several data-driven denoising approaches was compared. These methods included anatomical component based correction (aCompCor), Automatic Removal of Motion Artifacts (ICA-AROMA) and multi-echo independent component analysis (ME-ICA). We hypothesized that ME-ICA combined with aCompCor, which fully exploits the benefits of multi-echo imaging, would yield the highest data quality based on the assessed quality metrics.

METHODS

Participants and data acquisition

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center/University of Maastricht and registered on clinicaltrials.gov with identifier NCT02551029. Subjects gave written informed consent. Eighteen female healthy volunteers participated in the study (mean age 25 years [±SD 4.4], mean BMI 22.7 kg/m² [±SD 1.9]). Only females were included due to the primary study aims, as described elsewhere in detail [3].

BOLD fMRI data were collected on a Siemens Magnetom 7T scanner (Siemens Healthineers, Erlangen, Germany) using a 32-channel Nova Medical Head Coil. Functional MRI data were acquired with a ME-EPI sequence using an interleaved ascending simultaneous multi-slice acquisition with a multi-band factor of 2. This sequence used the following parameters: 2.2 mm isotropic voxel size, 50 slices, repetition time (TR) = 1.6 s, echo times TE1 = 10.6 ms, TE2 = 26.1 ms, TE3 = 41.6 ms, flip angle = 64°, bandwidth = 2362 Hz/Px, echo spacing = 0.53 ms, GRAPPA acceleration factor R = 3, 1350 volumes with three echoes each. Slices were tilted to fully incorporate the brainstem, the primary focus of the study, while at the same time minimizing the inclusion of air-filled cavities. Because of tilting, image acquisition of part of the parietal cortex was limited up to the postcentral gyrus. Five additional volumes using the same parameters but with opposite phase encoding were acquired for the purpose of susceptibility

distortion correction. Concurrent with image acquisition, pulse rate and respiration signals were collected with the use of a pulse oximeter (left index finger) and respiratory belt, respectively.

Data pre-processing

Data preprocessing was performed using a combination of Statistical Parametric Mapping of the Wellcome Department of Cognitive Neurology of London (SPM12), CONN toolbox (19.b)[26], the Oxford Centre for Functional MRI of the Brain (FMRIB) Software Library (FSL; v.6.0), and TE Dependent ANAlysis (*tedana* v0.0.8)[11; 12].

fMRI images were first corrected for slice timing using manually defined slice timings (extracted from the DICOM header) as applicable for multiband acquisitions, and subsequently motion corrected using an SPM-based script that estimated motion on TE1 images, where after the same transformations were applied to all three echoes. TE-dependence analysis was performed on slice time and motion corrected multi-echo data.

An adaptive mask was generated, in which each voxel's value reflects the number of echoes with usable data. A monoexponential model was fit to the data at each voxel using log-linear regression in order to estimate T2* and S0 maps. For each voxel, the value from the adaptive mask was used to determine which echoes would be used to estimate T2* and S0. Multi-echo data were then optimally combined using the t2s combination method [18].

After *tedana*, the preprocessing pipeline branches, providing five different datasets (see Figure 9.1). The optimally combined non-denoised dataset was corrected for susceptibility-induced distortion (estimated using topup, FSL [2]), segmented and normalized to MNI space. Minimal smoothing using a 4 mm Gaussian kernel was performed as per the preprocessing pipeline described elsewhere [3]. The resulting dataset was considered the "uncleaned" dataset, hereafter referred to as ME-uncleaned. The ME-uncleaned dataset was denoised using the anatomical component based correction method (aCompCor) [4]. Alternatively, the ME-uncleaned dataset was denoised using Automatic Removal of Motion Artifacts (ICA-AROMA, aggressive option) [21].

In addition to the non-denoised dataset, *tedana* provided an optimally combined dataset that was denoised using ME-ICA. To this end, principal component analysis followed by the stabilized Kundu component selection decision tree was applied to the optimally combined data for dimensionality reduction (Kundu et al., 2013). Independent component analysis was then used to decompose the dimensionally reduced dataset. Component selection was performed to identify BOLD (TE-dependent), non-BOLD (TE-independent), and uncertain (low-variance) components using the Kundu decision tree (Kundu et al., 2013). The resulting denoised dataset was corrected for susceptibility-induced distortion, segmented, normalized to MNI space and finally smoothed using a 4mm Gaussian kernel (similarly to the ME-uncleaned data). This dataset was considered

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rid of spatially focal noise [20], and hereafter referred to as ME-ICA-focal. Spatially diffuse noise was subsequently removed from the ME-ICA-focal dataset using aCompCor, and hereafter referred to as ME-ICA-global. Finally, a high pass filter of 0.008 Hz (125 seconds) was applied to all datasets.



Figure 9.1. Flowchart demonstrating preprocessing pipeline, which branches after TE-dependent analysis (*tedana*). Intensity normalization and nuisance regressors were applied after *tedana* in order to prevent distortion of T2* estimations. Dataset names are shown at the bottom of the flowchart: these names are referred to throughout the manuscript.

Data quality assessment

In order to assess data quality, various quality metrics were calculated and compared between denoising methods.

1. Temporal Signal-to-Noise Ratio (tSNR) was calculated for all datasets using the formula \bar{S}/σ_N where \bar{S} represents the mean fMRI signal and σ_N the standard deviation (SD) of the noise. The mean tSNR (and SD) was calculated across subjects for each cleaning method. This was performed first using a subject specific grey matter mask. Additionally, tSNR was calculated using a brainstem mask. Finally, tSNR ratio maps were created, where the group level tSNR map of each denoising method was divided by the tSNR map of the ME-uncleaned dataset. These tSNR ratio maps were used to identify which regions benefited the most of each respective data cleaning method.

- 2. Delta variation signal (DVARS), i.e. the frame-to-frame root mean square change in fMRI signal was calculated using the formula $\sqrt{\frac{1}{v}\Sigma_i[x_{i,t} - x_{i,t-1}]^2}$ where $\sqrt{}$ is the number of non-null voxels and X the fMRI signal in voxel *i* at time *t*, as per [1]. DVARS was used to identify and compare artefactual signal changes in the fMRI time series – likely originating from subject motion – that remained after each data cleaning method. A qualitative comparison between DVARS for each data cleaning method and subject motion was performed at the subject level using a framewise displacement calculation [19].
- 3. Power spectral density was estimated using the multitaper method as available in MATLAB 2018a. Power spectra were calculated from fMRI time series at the subject level for each data cleaning method. Power spectra were subsequently normalized to the amplitude of the respective uncleaned series. Finally, power spectra were averaged for each data cleaning method. An adequate denoising method was expected to suppress non-neuronal frequencies (> 0.1 Hz [9]) while simultaneously preserving power spectral density amplitude in the low frequency range (< 0.1 Hz).</p>
- 4. It has been demonstrated previously that the respiratory cycle correlates significantly with the global signal [20]. This has been reported to be due to a) changes in blood gasses and b) motion related to respiration. Adequate denoising methods should uncouple the global BOLD signal from respiration. Correlation between the variability in the global signal and the variability in the respiratory cycle was calculated as described by [20]. Signals from the respiratory belt recordings were z-scored, and the envelope of each signal was calculated using the built-in MATLAB function ('peak' method). Finally, the standard deviation of the respiratory envelope was calculated to capture variability in respiratory patterns.

RESULTS

Temporal Signal-to-Noise Ratio (tSNR)

The tSNR values per data cleaning method are plotted in Figure 9.2 for whole-brain and brainstem separately (panel A and B, respectively). Each data cleaning method significantly improved tSNR, as shown in Table 9.1. The highest tSNR was achieved with the use of AROMA. Slightly lower tSNR values (as compared to whole-brain) were found when applying a brainstem mask (Figure 9.2B), although overall results were comparable, including the highest value for AROMA. Subsequently, we assessed the improvement in tSNR as compared to the uncleaned data using tSNR ratio maps (Figure 9.3). A relative uniform increase throughout the brain in tSNR was found with the use of aCompCor. For ME-ICA-focal, ME-ICA-global and ME-AROMA, the increase in tSNR was most apparent around the edges of the brain and across tissue boundaries (e.g. around the ventricles). When looking into the relative improvement in tSNR for whole-brain and brainstem separately, we found that for ME-ICA-focal and ME-ICA-global gain in tSNR was higher in the brainstem as compared to whole-brain (Table 9.2). ME-AROMA showed a higher gain in tSNR at the whole-brain level as compared to the brainstem. ME-aCompCor showed comparable gains in tSNR at the whole-brain and brainstem level.



Figure 9.2. Violin plots of tSNR values per data cleaning method, panel A: tSNR at whole-brain level, panel B: tSNR at brainstem level.

Table 9.1. Comparison of tSNR subject means per data cleaning method (versus uncleaned data) using	а
paired-samples T-test.	

ME-aCompCor > ME-uncleaned	t(35) = 19,90	P < 0.001
ME-ICA-focal > ME-uncleaned	t(35) = 23,93	P < 0.001
ME-ICA-global > ME-uncleaned	t(35) = 40,39	P < 0.001
ME-AROMA > ME-uncleaned	t(35) = 34,60	P < 0.001
ME-ICA-focal > ME-aCompCor	t(35) = 0,03	P = 0.97
ME-ICA-global > ME-ICA-focal	t(35) = 24,50	P < 0.001
ME-AROMA > ME-ICA-global	t(35) = 15,53	P < 0.001

Table 9.2. Relative improvement in tSNR at the whole-brain and brainstem level, as compared to MEuncleaned (uncleaned data)

	Whole-brain ratio	Brainstem ratio	
ME-aCompCor	1.43	1.46	
ME-ICA-focal	1.57	1.74	
ME-ICA-global	2.13	2.23	
ME-AROMA	2.90	2.53	



Figure 9.3. tSNR ratio maps: tSNR maps for each data cleaning method are divided by the tSNR map of the ME-uncleaned dataset. Color scale ranges from blue to green to red (low – high increase in tSNR, respectively).

Delta variation signal (DVARS)

The variability (standard deviation) of the DVARS per data cleaning method is displayed in Figure 9.4. Each data cleaning method significantly improved DVARS, as shown in Table 9.3. The lowest variability in DVARS was found when using AROMA.

In order to make a qualitative comparison of artefactual signal changes remaining after each data cleaning method, we plotted DVARS with framewise displacement (displacement between neighboring voxels) for two individual subjects: one subject demonstrating low movement during scanning (mean framewise displacement of 0.06 mm) and one subject demonstrating high movement (mean framewise displacement of 0.19 mm). DVARS was plotted as timeseries for each data cleaning method; peaks in signal change can be compared to peaks in framewise displacement (Figure 9.5).

ME-aCompCor > ME-uncleaned	t(35) = 7,62	P < 0.001
ME-ICA-focal > ME-uncleaned	t(35) = 23,32	P < 0.001
ME-ICA-global > ME-uncleaned	t(35) = 26,26	P < 0.001
ME-AROMA > ME-uncleaned	t(35) = 26,75	P < 0.001
ME-ICA-focal > ME-aCompCor	t(35) = 15,18	P < 0.001
ME-ICA-global > ME-ICA-focal	t(35) = 10,31	P < 0.001
ME-AROMA > ME-ICA-global	t(35) = 8,57	P < 0.001

Table 9.3. Comparison of DVARS subject means per data cleaning method using a paired-samples T-test.



Figure 9.4. Violin plot of DVARS standard deviation values per data cleaning method.

Power Spectral Density

Power spectra averaged per data cleaning method are displayed in Figure 9.6. Data cleaning with ME-ICA only (ME-ICA-focal) had little impact on the power spectrum both in the low and high frequency range. Data cleaning with aCompCor had a marked impact on the power amplitude at frequencies > 0.1 Hz, whilst maintaining the peak in the power spectrum at frequencies around 0.02 Hz (within the BOLD frequency range). Frequencies above > 0.1 Hz were further suppressed when combining ME-ICA with aCompCor (ME-ICA-global), though at the cost of spectral density in the BOLD frequency range. With the use of AROMA, the power spectrum in both the low and high frequency range was heavily suppressed.



Figure 9.5. Upper two panels display framewise displacement (FD): panels on the left correspond to a subject demonstrating low movement (FD = 0.06 mm), panels on the right correspond to a subject demonstrating high movement (FD = 0.19 mm). DVARS is plotted for each data cleaning method (red) against DVARS for the uncleaned data (blue).



Figure 9.6. Power spectra averaged per data cleaning method.

Correlation between breathing and global signal

The correlation between the variability of the global signal and variability of the respiratory envelope was calculated for each dataset. A strong correlation was found for the uncleaned dataset (r = 0.70, p = 0.002). No significant correlation was found between global signal and respiratory envelope for any of the cleaned datasets: ME-aCompCor (r = 0.40, p = 0.111), ME-ICA-focal (r = 0.39, p = 0.127), ME-ICA-global (r = 0.28, p = 0.271), ME-AROMA (r = 0.42, p = 0.095).



Figure 9.7. Correlation between respiration and global signal. Figure shows scatterplots and least-square lines with on the y-axis the variability (standard deviation) of the global signal of each dataset and on the x-axis the variability of the respiratory cycle, calculated as the standard deviation of the envelope of the z-scored respiratory belt signal.

Discussion

The current study is the first to investigate the efficacy of data cleaning methods of multi-echo fMRI data acquired at 7T. Given the absence of a so-called ground truth when using BOLD data one can only examine quality measures related to the data itself. In the current study we have used several measures that are largely unrelated to each other, with each measure highlighting different aspects of the data. We here demonstrate significant tSNR improvements with each of the used data cleaning methods. tSNR improvements showed a similar pattern for whole-brain and brainstem data only. AROMA resulted in the highest tSNR, followed by ME-ICA combined with aCompCor (ME-ICA-global). Notably, the tSNR ratio maps showed that for both the ME-AROMA and ME-ICA-global dataset, tSNR improvements were strongest around the edges of the brain and brainstem. This is in line with the previous finding that motion artefacts are most profound across the edges of the brain [16; 23]. Moreover, differences in proton density across different tissues, for example around the ventricles, similarly result in increased noise [6]. Denoising is therefore likely to have a stronger effect on these regions.

When comparing variability in DVARS, we found the lowest values for AROMA. In a qualitative analysis, it appears that denoising has a stronger effect on DVARS for subjects with higher degrees of motion, likely due to higher baseline signal variation. It should be noted that strong peaks related to motion remain visible in the DVARS timecourse even after applying ME-ICA or AROMA. One might therefore consider using additional steps

such as scrubbing in order to censor remaining artefactual scanning volumes, which is how we preprocessed the data for our primary analysis, as discussed elsewhere [3].

We subsequently compared power spectral density of each dataset. Whilst tSNR and DVARS were superior for ME-AROMA and ME-ICA-global, the power spectrum of these datasets indicated a strong reduction of both high (non-neuronal) and low (neuronal) frequencies, with the spectrum being most suppressed after applying AROMA. It is therefore possible that, although AROMA is highly effective in removing noise, the approach is too aggressive in the sense that it also removes signal of interest. It should be noted that AROMA can be performed with more lenient settings, as incorporated in the software itself ("non-aggressive" option). Due to the data size and number of comparisons, we have not used both options of AROMA here; hence, we cannot draw conclusions on data quality after running AROMA with the non-aggressive option. It is possible that the non-aggressive option would retain more signal of interest, but this would likely also result in less effective removal of noise. This was also demonstrated in a study comparing data cleaning methods applied to single-echo data acquired at 1.5T, including both options of AROMA [7]. This study furthermore applied the aggressive option of AROMA to multi-echo data, comparing it to ME-ICA. Given that resting state fMRI data was used, the authors compared connectivity maps after each data cleaning method. The authors stated that ME-ICA more effectively preserved functional connectivity (i.e. signal of interest) as compared to AROMA.

We finally looked at the ability of each data cleaning method to uncouple the global signal and respiratory cycle, as it has previously been demonstrated that respiration strongly affects the BOLD signal [20]. We indeed found a strong correlation when analyzing the uncleaned dataset. After each data cleaning method, the correlation between the global signal and respiratory cycle was no longer significant. This contrasts the previous finding of Power et al, where the correlation between global signal regression or aCompCor. This discrepancy could be both data and software related. First, the study by Power and colleagues was performed using 3T data. The proportion of physiological noise increases substantially with higher field strengths [25], which could affect the relationship between the global signal and respiratory cycle. Moreover, the software for ME-ICA has evolved over time. The first versions were developed to function using AFNI modules [11], whereas the version used here (*tedana*) is a standalone python-based program.

CONCLUSIONS

AROMA and ME-ICA combined with aCompCor encompass highly effective denoising options for multi-echo 7T fMRI data, as compared to the commonly used aCompCor. Combining ME-ICA and aCompCor appears to have an additive effect based on the

quality metrics used here, making it superior to ME-ICA alone. When using AROMA with the aggressive denoising option, it is possible that a portion of signal of interest is falsely classified as noise. When using AROMA, empirical testing of both the aggressive and non-aggressive option per dataset might therefore be the best choice.

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CHAPTER 10

Digital instruments for reporting of gastrointestinal symptoms in clinical trials: comparison of end-of-day diaries versus experience sampling method

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ABSTRACT

Introduction. Questionnaires are necessary tools for assessing symptoms of disorders of gut-brain interaction in clinical trials. We previously reported on the excellent adherence to a smartphone app used as symptom diary in a randomized clinical trial in irritable bowel syndrome (IBS). Other sampling methods, such as the experience sampling method (ESM), are better equipped to measure symptom variability over time, provide useful information regarding possible symptom triggers and are free of ecological and recall bias. The high frequency of measurements, however, could limit the feasibility of ESM in clinical trials.

This study aimed to compare adherence rates of a smartphone-based end-of-day diary and ESM for symptom assessment in irritable bowel syndrome and functional dyspepsia (FD).

Methods. Data from four separate studies were included. Patients with IBS participated in a randomized controlled trial, which involved a smartphone end-of-day diary of 2+8 weeks (pre-treatment + treatment period), and an observational study, during which patients completed ESM assessments using a smartphone application for one week. Patients with FD participated in a randomized controlled trial, which involved a smartphone end-of-day diary of 2+12 weeks (pre-treatment + treatment period), and an observational study, during which patients completed ESM assessments using a smartphone application for one week. Adherence rates were compared between these two symptom sampling methods.

Results. Twenty-five patients with IBS and fifteen patients with FD were included. Overall adherence rates for the end-of-day diaries were significantly higher than for ESM (IBS: 92.7% versus 69.8%, FD: 90.1% versus 61.4%, respectively).

Conclusions. We here demonstrate excellent adherence rates for smartphone applicationbased end-of-day diaries as used in two separate clinical trials. Overall adherence rates for ESM were significantly lower, rendering it more suitable for intermittent sampling periods rather than continuously during longer clinical trials.

INTRODUCTION

Disorders of gut-brain interaction (DGBI) are highly prevalent disorders, with a recent multinational survey study indicating that over 40 percent of the worldwide population suffers from at least one DGBI [12]. Among the most common DGBI disorders are irritable bowel syndrome (IBS) and functional dyspepsia (FD), which are characterized by lower and upper gastrointestinal symptoms, respectively, including abdominal pain, fullness, bloating, constipation, and diarrhea. Per definition, the diagnosis of these disorders is symptom based, according to the Rome IV criteria [5]. By extension, the evaluation of treatment response in clinical trials in DGBI relies completely on patient reported outcome measures (PROMs). It is therefore of utmost importance that clinical trials use symptom sampling methods that are able to produce an accurate representation of the symptomatology as experienced by the patient. As such, paper symptom diaries have been scrutinized as they are prone to fake adherence, as subjects can fake or backfill written answers outside of the proposed time-window to forge good adherence [7]. Thereby, the use of paper retrospective diaries introduces ecological and recall bias.

End-of-day symptom diaries are currently recommended by drug regulatory authorities to assess treatment response in IBS [3, 14]. The widespread dissemination of the smartphone during the previous two decades creates possibilities for more advanced symptom sampling methods. Recently we reported on a digital end-of-day symptom diary using a smartphone application in a randomized controlled trial in IBS [16]. We observed a very high adherence for the diary smartphone application of almost 88% during a treatment period of eight weeks. End-of-day diaries in any form, however, are not free of abovementioned ecological and recall bias, as a result of their retrospective nature. These limitations are currently best overcome by the experience sampling method (ESM), which employs random and repeated assessments at multiple time points across momentary states in daily life and thereby provides a detailed overview of symptoms experienced during the day. Previously developed ESM instruments for FD and IBS use a measurement frequency of ten times a day [11; 15]. It should be noted that the high frequency of this sampling method might raise concerns regarding adherence during clinical trials with a duration of several weeks or longer. In our previous IBS trial using the end-of-day diary, adherence declined over time. Logging fatigue is considered the underlying cause of this decline in adherence. It could be hypothesized that this mechanism impairs adherence even more in methods with a higher sampling frequency such as ESM. Hence, although ESM proved to be the method with more accurate real-life representation of daily symptoms, its feasibility in longer duration clinical trials is still unknown. In order to draw conclusions regarding this question, a better understanding of ESM adherence and decline thereof is required.

In the present exploratory study, we sought to compare adherence for end-of-day diaries as used in two randomized controlled trials with adherence for ESM in two separate observational studies. We hypothesized that overall adherence would be superior with the use of the end-of-day diary, as compared to ESM. Moreover, we hypothesized that adherence would remain more stable over time for the end-of-day diary as compared to ESM.

METHODS

The present study is based on data from two randomized controlled trials (RCTs) and two observational studies. For each study type, one study focused on IBS and one on FD. The RCTs employed end-of-day diaries, whereas the observational studies used momentary assessments (ESM). The Rome IV criteria were used as inclusion criteria in each study as per the disorder being investigated. All four studies had been approved by the Maastricht University Medical Center+ (MUMC+) Ethics Committee. All study procedures were performed in compliance with Good Clinical Practice Guidelines and according to the revised Declaration of Helsinki. All subjects gave written informed consent prior to participation.

Although including data from multiple studies, all patients included in the present paper were required to have participated in both an RCT and observational study (on the same disorder), in order to reduce variability across individuals. All patients thereby completed both end-of-day diaries during a longer period of time and ESM for a period of one week (not simultaneously). Details on each study are provided below and an overview of sampling characteristics is presented in Table 10.1. The exact queries in each sampling method are provided in Supplementary Tables 10.1-10.4.

Randomized controlled trials - end-of-day diaries

The RCT in IBS has been discussed in detail elsewhere [17]. In brief, the primary aim was to investigate the efficacy of peppermint oil, a conventional small-intestinal and a novel ileocolonic release formulation, in patients with IBS (NCT02716285). In this randomized, placebo-controlled trial IBS patients between 18 and 75 years were included. Patients completed an end-of-day diary using a smartphone application during a pre-treatment period (two weeks) and treatment period (eight weeks), as described previously [16]. At the core, this diary consisted of one question regarding abdominal pain experienced each day (to be scored on an 11-point numerical scale). After completing the abdominal pain question, subjects were asked about adverse events and sporadic medication use. During the day, patients had the option to report on defecation according to the Bristol stool chart [8]. Patients were instructed to register abdominal pain daily between 6PM and 12PM. Finally, psychological comorbidities were assessed at baseline using the GAD-7 and PHQ-9 questionnaire. The second RCT is an ongoing trial that investigates the efficacy of nortriptyline, in patients with FD (NCT03652571). In this randomized, placebo-controlled trial, FD patients between 18 and 65 years are included. Patients complete an end-of-day diary using a smartphone application during a pre-treatment period (two weeks) and treatment period (twelve weeks), the application is similar to the one used in the RCT in IBS. In the RCT in FD however, the diary consists of five symptom questions, corresponding to the five core symptoms of FD: epigastric pain, epigastric burning, early satiety, postprandial fullness, and upper abdominal bloating [13]. In addition to these five questions, subjects are asked about adverse events and sporadic medication use. Patients are instructed to register symptoms daily between 7.00PM and 12.00PM. There is no registration of bowel movements in this trial, as an altered bowel habit is not a core symptom in FD. Finally, psychological comorbidities were assessed at baseline using the GAD-7 and PHQ-9 questionnaire.

Observational studies – ESM

ESM data from IBS patients were obtained from a validation study of a newly developed patient-reported outcome measure (based on the ESM) for the use in IBS (NCT02880722) [15]. IBS patients and healthy volunteers between 18 and 70 years were included in this study. Both groups completed an end-of-day paper diary and ESM for a period of one week. The ESM was incorporated in a custom-made smartphone application. The ESM consisted of ten assessments randomly timed between 7.30AM and 10.30PM. Each assessment was preluded by an auditory signal and the application was programmed to enable completion of the assessment within ten minutes after the auditory signal. Subjects were instructed to complete as many assessments as possible, but to pass over questionnaires when completing was impossible (e.g. when driving). Assessments covered five different domains, which included physical status (e.g. abdominal pain), defecation (since the previous auditory signal), psychological factors (e.g. positive and negative affect), environment (e.g. current location and company), and nutrition and drug use, as described previously [15]. In total, the ESM for use in IBS consisted of 32 items (per assessment).

ESM data from FD patients were obtained from a separate validation study of a newly developed patient-reported outcome measure (based on the ESM) for the use in FD (NCT04204421) [6]. FD patients and healthy volunteers between 18 and 75 years were included in this study. Both groups completed an end-of-day diary and ESM for a period of one week. The diary and ESM were incorporated in the same custommade smartphone application. ESM was used similar as in the IBS ESM study, with ten assessments randomly timed between 7.00AM and 10.00PM. Assessments in the FD ESM study covered four domains, which included the same domains as in the IBS ESM study [11]. In total, the ESM for use in FD consisted of 33 items (per assessment).

	RCT in IBS	Observational study in IBS	RCT in FD	Observational study in FD
Sampling method	Digital end-of-day diary (smartphone application)	End-of-day paper diary and digital ESM (smartphone application) ^a	Digital end-of-day diary (smartphone application)	Combined digital end-of-day diary and ESM (smartphone application)
Sampling duration	Two weeks pre- treatment plus eight weeks treatment	One week	Two weeks pre- treatment plus twelve weeks treatment	One week
Sampling frequency	Once a day	Ten times per day	Once a day	Ten times per day
Sampling timeframe	Between 6PM and 12PM	Between 7AM and 10PM	Between 7PM and 12PM	Between 7AM and 10PM
Push notification(s)	Once at 10pm	Randomly timed	Once at 9pm	Randomly timed
Number of items	3 (plus Bristol stool chart) ^b	32 (five domains)	7 ^b	33 (four domains)
Estimated time investment	15-30 seconds	2-3 minutes (per assessment)	15-30 seconds	2-3 minutes (per assessment)

Table 10.1. Overview of sampling specifics per study.

^a Note that the end-of-day diaries are similar to the ones used in the corresponding RCT. In the observational study however, end-of-day diaries were completed on paper.

^b Including adverse event and sporadic medication use queries.

Statistical analyses and data plots

Adherence was the primary outcome measure. For both the end-of-day diaries and ESM, overall adherence was calculated as the percentage of completed assessments throughout the study. For visualizing adherence over time, weekly adherence rates were plotted for the clinical trials (end-of-day diaries) and daily adherence rates for the observational studies (ESM). In addition, for ESM we also calculated overall adherence as the number of days that ≥ 6 of the 10 assessments were completed, as described previously [10; 15]. The latter can be considered more appropriate when evaluating adherence of sampling methods such as ESM, where an excess of measurements is provided in order to obtain sufficient data during the day [4; 10].

All data was plotted using MATLAB R2018a. Linear mixed models were performed using the lme4 function in R Statistical Software version 3.6.3 (2020-02-29) [1]. In each model (per study), adherence to the application constituted the dependent variable and time constituted the within-subject independent variable. A restricted maximum likelihood estimation method and first order autoregressive variance-covariance matrix for the within-subject variable time fitted the data best based on the lowest value of Akaike's Information Criterion.

Given the exploratory nature of the study we did not perform sample size calculation.

RESULTS

In total 25 patients with IBS and 15 patients with FD were included in our analysis for adherence comparison. An overview of subject characteristics is provided in Table 10.2. Both IBS and FD patients were more frequently female (72 and 73.3%, respectively). For FD patients, the time between participation in the two studies was significantly longer than for IBS patients (12.5 vs 7.6 months, respectively [t(26.12)=4.30, p<0.001]). All subjects in the IBS and FD studies participated in the ESM observational study after participating in the RCT.

Adherence rates for the end-of-day diary in the IBS RCT during the pre-treatment period, treatment period, and total study duration (both periods combined) were 93.4%, 92.6%, and 92.7%, respectively. Overall adherence, i.e. the percentage of total completed assessments, for ESM during the observational IBS study was 69.8%.

Adherence rates for end-of-day diary in the FD RCT during the pre-treatment period, treatment period, and total study duration were 92.9%, 89.7%, and 90.1%, respectively. Overall adherence for ESM during the observational FD study was 61.4%.

It ought to be noted that, for trials using the ESM method, completion of ≥ 6 of the 10 questionnaires per day is considered as being adherent, as described previously [10; 15]. This type of adherence calculation can be considered more representative for sampling methods such as ESM. When using this approach, overall adherence was 79.4% and 64.8% for the ESM IBS and ESM FD study, respectively. Adherence in the latter was noticeably lower due to the effect of 4 outliers (adherence < 15%) in this relatively small group. 3 out of 4 subjects reported a specific reason for low adherence, which included 1) a technical error (subject did not receive push notifications on smartphone), 2) attending funeral of a close relative and 3) not able to complete most assessments during day job.

Weekly adherence to the end-of-day diaries and daily adherence to the ESM application are shown in Figure 10.1. Linear mixed models did not demonstrate a significant decline in adherence over time in either RCT (main effect of time, IBS RCT F(1,224)=2.24, p=0.136, FD RCT F(1,194)=0.87, p=0.769). A minor decline in adherence over time can be observed in the lower panels corresponding to the ESM studies, which was not statistically significant (linear mixed models, main effect of time, ESM IBS study F(1,149)=3.41, p=0.067, ESM FD study F(1,89)=1.23, p=0.270).

Cumulative completed assessments are plotted against the total number of assessments for each study and each subject in Figures 10.2-10.5. Single subject plots can be compared as subplot positions in the figures correspond to the same subject.



Figure 10.1. Adherence to each symptom assessment application. For end-of-day diaries, the weekly adherence is shown (i.e. percentage of completed assessments for each week). For ESM, the daily adherence is shown (i.e. percentage of completed assessments for each day [out of 10 measurements]).



Figure 10.2. Cumulative completed diary assessments in the IBS RCT. Red line indicates maximum number of assessments, blue line indicates actual number of completed assessments. Note that subplot positions in this figure correspond to the same subject in Figure 3. (X-axis: assessment number, Y-axis: completed assessments)

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Figure 10.3. Cumulative completed ESM assessments in the observational IBS study. Red line indicates maximum number of assessments, blue line indicates actual number of completed assessments. Note that subplot positions in this figure correspond to the same subject in Figure 2. (X-axis: assessment number, Y-axis: completed assessments)



Figure 10.4. Cumulative completed diary assessments in the FD RCT. Red line indicates maximum number of assessments, blue line indicates actual number of completed assessments. Note that subplot positions in this figure correspond to the same subject in Figure 5. (X-axis: assessment number, Y-axis: completed assessments)

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Figure 10.5. Cumulative completed ESM assessments in the observational FD study. Red line indicates maximum number of assessments, blue line indicates actual number of completed assessments. Note that subplot positions in this figure correspond to the same subject in Figure 4. (X-axis: assessment number, Y-axis: completed assessments)

		Observational		Observational	
	RCT in IBS	study in IBS	RCT in FD	study in FD	
Number of subjects in the present study	N = 25		N = 15		
Time between study participations, months					
Mean (SD)	76(+21)		$125(\pm 26)$		
Range	1 - 17		7 - 18		
Age ^a , years					
Mean (SD)	35.9 (± 12.8)		41.4 (± 15.2)		
Range	22 - 59		18 - 64		
Gender, n (%)					
Female	18 (72.0%)		11 (73.3%)		
Educational level, n (%)					
No education	0		0		
Low	2 (8.0%)		1 (6.7%)		
Moderate	12 (48.0%)		5 (33.3%)		
High	11 (44.0%)		9 (60.0%)		
IBS or FD subtype, n (%)	14 (56.0%) - Diarrhea 5 (33.3%) - Postprandial 3 (12.0%) - Constipation 4 (26.7%) - Epigastric pa 4 (16.0%) - Mixed 6 (40%) - Overlap 4 (16.0%) - Undefined 6 (40%) - Overlap		stprandial distress igastric pain lap		
IBS or FD severity ^b					
Mean score (SD)	228.8 (± 24.5)		83.2 (± 22.8)		
Mild, n (%)	7 (28.0%)		-		
Moderate, n (%)	13 (52.0%)		-		
Severe, n (%)	5 (20.0%)		-		
Psychological comorbidities ^c Anxiety					
Mean score (SD)	4.2 (± 2.9)		3.3 (± 2.8)		
Minimal, n (%)	14 (56.0%)		12 (80.0%)		
Mild, n (%)	9 (36.0%)		2 (13.3%)		
Moderate, n (%)	2 (8.0%)		1 (6.7%)		
Depression					
Mean score (SD)	5.0 (± 2.7)		4.9 (± 4.2)		
Minimal, n (%)	14 (% 56.0%)		8 (53.3%)		
Mild, n (%)	10 (40.0%)		6 (40%)		
Moderate, n (%)	1 (4.0%)		1 (6.7%)		

Table 10.2. Summary of patient demographic and baseline characteristics.

^a Age upon entering first study.

^b For IBS symptom severity, the Irritable Bowel Syndrome Severity Scoring System (IBS-SSS) was used. Scores were defined as: <175 mild, 175-300 moderate, and >300 severe case of IBS.

For FD symptom severity, the Nepean Dyspepsia Index (NDI - symptom scale) was used (continuous scale only, no validated severity categories).

^c For anxiety, the GAD-7 was used, and for depression the PHQ-9. Scores were defined as: ≥ 5 mild, ≥ 10 moderate, and ≥ 15 severe level of anxiety or depression.

DISCUSSION

In the current study we explored adherence rates for smartphone applications used for symptom assessment in IBS and FD. In line with our previous findings in a large RCT in IBS [16], we found an excellent overall adherence of 90% for a digital end-of-day diary in an ongoing trial in FD patients. Given the fact that these diaries only enable the logging of symptoms experienced during the day that the diary is being filled in, fake adherence (i.e., backfilling) is completely prevented. Indeed, the FDA recommends daily symptom assessment in IBS trials, which is best facilitated by the digital (smartphone) framework presented here [14]. The high overall adherence rates as observed in the FD and in the IBS RCT's confirm the feasibility of these digital diaries in clinical trials. Importantly, the RCT in FD involves five diary questions as opposed to a single question in the diary used in the IBS RCT. In addition, the FD RCT is 4 weeks longer in duration than the IBS RCT. It is encouraging that regardless of the added burden, adherence for the end-of-day diary in the FD RCT is still excellent.

In our previous IBS RCT study where we included 189 patients, we reported a small but significant decrease in adherence for the completion of daily diaries over the study duration. Such a decrease in adherence can be referred to as logging fatigue. In the current study, we found no evidence of logging fatigue in the subset of the IBS RCT, nor in the FD RCT. We hypothesized that with more frequent assessments, such as with ESM, logging fatigue could become more of an issue. Interestingly, in the current study, we found no significant decline in adherence rates over time in both ESM studies. However, since both studies were of relatively short duration (7 days), we cannot draw any conclusions on possible declining adherence rates over time when ESM is used for longer periods.

Overall adherence rates for the ESM studies were evidently lower than the end-of-day diary adherence rates, though in line with rates found in previous studies [9; 10]. Even when taking into account that it is generally not feasible to complete all ESM assessments and calculating ESM adherence as the number of days that ≥ 6 of the 10 assessments are completed, ESM adherence rates were still noticeably lower. To a large extent, this will likely be related to the nature of measurements. For the end-of-day diaries, subjects can choose a suitable moment between 6 (or 7)PM and 12PM, and can do so each day as per their own schedule. For ESM on the other hand, measurements are by their very definition timed at random moments and should be completed within 10 minutes after the assessment was announced. ESM is therefore likely to involve measurements at times when the subject is not able to complete the symptom assessment, especially as there will always be measurements within working hours. Furthermore, it is easy to miss a haptic or auditory signal on your cellphone. Moreover, the ESM assessments were far more extensive than the end-of-day diaries as they involved multiple domains, e.g. physical, psychological, environmental and nutritional. Indeed, it was demonstrated in a systematic review of studies including electronic diaries of various lengths that the extent of the
diary used was negatively associated with adherence [9]. The large difference in overall adherence rates between ESM and end-of-day diaries may reflect on the higher burden of ESM. We therefore think that it is not feasible to use ESM continuously during a trial of several weeks, especially because adherence already tends to be lower in studies of longer duration [2]. A solution could be to use ESM intermittently (e.g. one week in every four weeks), complementing the end-of-day diary. In this way, the end-of-day diary provides a strong continuous measurement framework, where ESM can be used at fixed periods to examine changes in symptom variability and symptom triggers over time (i.e. during treatment), in addition to analyze the complexity of factors contributing to symptom perception. However, the responsiveness (i.e., the sensitivity to detect change over time) of ESM has not yet been evaluated.

Finally, one should appreciate the differences in acquired data when using ESM or the end-of-day diary. As already mentioned above, ESM provides more detailed information on symptoms and their possible triggers. Our preference for the end-of-day diary as a continuous measurement framework primarily relates to clinical trials, as this is also in accordance to current recommendations from regulatory authorities. It is possible that in some situations the more detailed data outweighs the drawback of the higher number of missing values. This could especially be the case in clinical practice of functional disorders, where additional information on symptom triggers is extremely valuable.

A limitation of the current study is the small size of the study population. This is mitigated by the within-subject nature of the study, as the obligatory participation in both an end-of-day diary and ESM study limits subject specific effects on adherence, aiding a better comparison of assessment methods. As mentioned above, the possibility of selection bias cannot be excluded, as subjects that are willing to participate in more than one study could have a very strong motivation, which may translate to unrepresentatively high adherence rates. On the other hand, the overall adherence rate of our subset of subjects from the IBS RCT was only a few percent higher than the whole group adherence rate, arguing against such selection bias. Finally, since all subjects participated in the ESM studies after participation in an RCT, a carry-over treatment effect could have affected logging adherence. However, this would likely have influenced adherence during the RCT itself as well, and we observed stable adherence during both RCTs. Moreover, we previously observed no effect of GI symptoms on adherence rates. The latter also suggests that variation in duration between ESM and RCT participation is less relevant.

CONCLUSION

In conclusion, we here demonstrate excellent adherence rates for smartphone application-based end-of-day diaries as well as good adherence to two ESM-based applications. Overall adherence rates for ESM were evidently lower, as would be

expected given the nature of the methodology, but possibly also reflecting on the larger burden of this sampling method given the higher number of cues and questions to be answered. Even though we could not demonstrate a decline in response rate with ESM over a period of 7 days, it seems unfeasible to use ESM continuously in clinical trials over several weeks. Given the added value of ESM however, researchers should consider complementing end-of-day diaries with intermittent periods of ESM.

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SUPPLEMENTARY INFORMATION

Supplementary table 1. Diary questions in IBS RCT

Diary question		Symptom scale
1.	How would you rate your abdominal pain today? Think about the worst abdominal pain today.	(0 = no pain, 10 = worst possible pain)
2.	Did you experience any side effects of the treatment?	Yes/no
2a.	Please specify the side effect	
2b.	Please rate the severity of the side effect	(0 = not severe, 10 = very severe)
2c.	Please specify the duration of the side effect	minutes,hours
3.	Did you use any medication today (besides your usual medication)?	Yes/no
3a.	Please specify the medication	
3b.	What was the dosage of the medication?	µg,mg,gram,ml,other
3c.	How many times did you take the medication?	times
3d.	What was the reason for taking this medication?	

Supplementary table 2. Diary questions in FD RCT

Diary question		Symptom scale
1.	Today, I had pain in my upper abdomen. If yes, specify the worst abdominal pain today.	(0 = not at all, 10 = very severe)
2.	Today, I had a burning feeling in my upper abdomen. If yes, specify the worst feeling today.	(0 = not at all, 10 = very severe)
3.	Today, I was feeling bloated. If yes, specify the worst feeling today.	(0 = not at all, 10 = very severe)
4.	Today, I had a heavy feeling in my abdomen. If yes, specify the worst feeling today.	(0 = not at all, 10 = very severe)
5.	Today, I was not able to finish a regular sized meal.	(0 = able, 10 = not able)
6.	Did you experience any side effects of the treatment?	Yes/no
6a.	Please specify the side effect	
6b.	Please rate the severity of the side effect	(0 = not severe 10 = very severe)
6c.	Please specify the duration of the side effect	minutes,hours
7.	Did you use any medication today (besides your usual medication)?	Yes/no
7a.	Please specify the medication	
7b.	What was the dosage of the medication?	µg,mg,gram,ml,other
7c.	How many times did you take the medication?	times
7d.	What was the reason for taking this medication?	

	Physical status	Answer scale
1	I am having abdominal pain	0 (none) - 10 (very much)
1a	This pain is located in the following part(s) of my abdomen:	Figure abdominal regions
2	I am having intestinal gas	0 (none) - 10 (very much)
2a	The intestinal gas is causing discomfort	0 (none) - 10 (very much)
3	I am having rumbling sounds coming from my abdomen	0 (none) - 10 (very much)
4	My abdomen feels bloated	0 (none) - 10 (very much)
4a	The bloating makes me feel uncomfortable	0 (none) - 10 (very much)
4b	The bloating is accompanied by a swollen abdomen	0 (none) - 10 (very much)
5	I have the urge to open my bowels	0 (none) - 10 (very much)
6	I am feeling sick	0 (none) - 10 (very much)
7	I am suffering from burping	0 (none) - 10 (very much)
8	I am feeling heartburn	0 (none) - 10 (very much)
9	I am feeling full	0 (none) - 10 (very much)
10	I am having palpitations	0 (none) - 10 (very much)
11	I am sweating	0 (none) - 10 (very much)
12	I am short of breath	0 (none) - 10 (very much)
13	I feel dizzy	0 (none) - 10 (very much)
14	My muscles are hurting	0 (none) - 10 (very much)
15	My joints are hurting	0 (none) - 10 (very much)
16	I feel an urge to pass urine	0 (none) - 10 (very much)

Supplementary table 10.3. ESM questions in observational IBS study

Defecation

17	Since the last beep, I have had the feeling that I had to open my bowels time(s)	0, 1, 2, 3, 4, more than 4 times
17a	Since the last beep, I have actually opened my bowels time(s)	0, 1, 2, 3, 4, more than 4 times
17b	It looked like this:	Bristol Stool Score 1 - 7
17c	I had to strain	0 (none) - 10 (very much)
17d	It feels like my bowels are not completely empty	0 (none) - 10 (very much)

Mood and psychological factors

18	I am feeling good	0 (none) - 10 (very much)
19	I am feeling low	0 (none) - 10 (very much)
20	I am feeling anxious	0 (none) - 10 (very much)
21	I am feeling irritated	0 (none) - 10 (very much)
22	I am feeling stressed	0 (none) - 10 (very much)
23	I am feeling relaxed	0 (none) - 10 (very much)
24	I am worried	0 (none) - 10 (very much)

Supplementary table 10.3. ESM questions in observational IBS study (continued)

	Context and environment	
25	Where am I?	At home, at someone else's home, work/school, public place, on my way, somewhere else
26	What am I doing (just before the beep)?	Resting, work/school work, household work/ shopping, hygiene/self-care, eating/drinking, relaxing/recreation, sports, travelling, something else
27	I feel (un)comfortable doing this	-5 (extremely uncomfortable) – 5 (extremely comfortable)
28	My symptoms are limiting my current activities	0 (none) - 10 (very much)
29	Who is with me?	Partner/children, friends, housemates, colleagues, family (other than those who live in your house), acquaintances, strangers/others, no one
29a	I find this company (un)pleasant	-5 (extremely unpleasant) - 5 (extremely pleasant)
	Nutrition and drug use	
30	I have eaten since the last beep	Breakfast, lunch, dinner, a snack, none of these
31	I ate ago	More than 15 min., 15–30 min., 30 min1 hour, more than 1 hour
32	Since the last beep I have used	Caffeine (coffee) / theine (tea), nicotine (smoking), alcohol, drugs, medication, none of these
32a	This was following medication:	Medication for abdominal pain, other pain relief, medication for stomach acidity, medication for nausea, medication to stop diarrhea, medication for constipation, something else

Context and environment

Supplementary table 4. ESM questions in observational FD study

Physical status—Upper abdomen	Answer scale
I am having a full feeling in my upper abdomen	0 (none) - 10 (very much)
I am having a heavy feeling in my upper abdomen	0 (none) - 10 (very much)
My abdomen feels bloated	0 (none) - 10 (very much)
I am having pain in my upper abdomen	0 (none) - 10 (very much)
I am having a burning feeling in my upper abdomen	0 (none) - 10 (very much)
I am having pain in my lower abdomen	0 (none) - 10 (very much)
I feel nauseous	0 (none) - 10 (very much)
Since the last beep, I have actually vomited time(s)	0, 1, 2, 3, 4, 5, more than 5 times
I am suffering from bothersome burping	0 (none) - 10 (very much)
I am having a burning feeling behind the breastbone	0 (none) - 10 (very much)
10 I am bringing up stomach contents into the mouth and/or nose	0 (none) - 10 (very much)
Nutrition, medication, and substance use	
I have eaten since the last beep	Breakfast, lunch, dinner, a snack, none of these
	Physical status—Upper abdomenI am having a full feeling in my upper abdomenI am having a heavy feeling in my upper abdomenMy abdomen feels bloatedI am having pain in my upper abdomenI am having a burning feeling in my upper abdomenI am having pain in my upper abdomenI am having pain in my upper abdomenI feel nauseousSince the last beep, I have actually vomited time(s)I am suffering from bothersome burpingI am having a burning feeling behind the breastbone10 I am bringing up stomach contents into the mouth and/or noseNutrition, medication, and substance useI have eaten since the last beep

	1	
11a	I ate ago	Less than 15 min., 15-30 min., 30 min1 hour, more than 1 hour
11b	I am able to finish a normal sized meal	0 (none) - 10 (very much)
12	Since the last beep I have used	Caffeine (coffee)/theine (tea), nicotine (smoking), alcohol, drugs, medication, carbonated beverages, milk products, fruit juices, none of these
12a	This was medication for:	Abdominal pain, other pain relief, stomach acidity, nausea, diarrhea, constipation, something else

	Physical status—General complaints	
13	I am having palpitations	0 (none) - 10 (very much)
14	I am sweating	0 (none) - 10 (very much)
15	I am short of breath	0 (none) - 10 (very much)
16	I feel dizzy	0 (none) - 10 (very much)
17	I have pain on my chest	0 (none) - 10 (very much)
18	I feel tired	0 (none) - 10 (very much)
19	Since the last beep, I have had the feeling that I had to open my bowels time(s)	0, 1, 2, 3, 4, 5, more than 5 times
20	Since the last beep, I have actually opened my bowels time(s)	0, 1, 2, 3, 4, 5, more than 5 times
20a	It looked like this:	Bristol Stool Form Scale
20b	I had to strain	0 (none) - 10 (very much)
20c	It feels like my bowels are not completely empty	0 (none) - 10 (very much)

Supplementary table 4. ESM questions in observational FD study (continued)

	Psychological aspects	
21	I am feeling good	0 (none) - 10 (very much)
22	I am feeling relaxed	0 (none) - 10 (very much)
23	I am feeling low	0 (none) - 10 (very much)
24	I am feeling anxious	0 (none) - 10 (very much)
25	I am feeling irritated	0 (none) - 10 (very much)
26	I am feeling stressed	0 (none) - 10 (very much)
27	I am worried	0 (none) - 10 (very much)
	Context and environment	
28	Where am I?	At home, at someone else's home, work/school, public place, on my way, somewhere else
29	What am I doing (just before the beep)?	Resting, work/school, household work/shopping, hygiene/self-care, eating/ drinking, relaxing/ recreation, sports, travelling, something else
30	I feel (un)comfortable doing this	-5 (extremely uncomfortable) – +5 (extremely comfortable)
31	My symptoms are limiting my current activities	0 (none) - 10 (very much)
32	Who is with me?	Partner/children, friends, housemates, colleagues, family (other than those who live in your house), acquaintances, strangers/ others, no one
33	I find this company (un)pleasant	-5 (extremely unpleasant) – +5 (extremely pleasant)



General discussion

General discussion

GENERAL DISCUSSION

In this thesis we describe various key aspects of visceral pain, from peripheral sensory input to the subjective experience of pain. Part 1 focuses on the role of TRP channels in visceral nociception. Previous studies have implicated TRPV1, TRPV4 and TRPA1 in visceral hypersensitivity in IBS patients, as discussed in detail in **chapter 2**. The discovery of TRP channel involvement in (visceral) pain led to the development of various antagonistic compounds as novel analgesics. However, due to the broad functional profile of these channels, the developed compounds resulted in various serious adverse effects including thermoregulatory dysfunction and disturbed thermosensation. Moreover, TRP channel expression is not confined to neural tissue; non-neuronal expression is widespread and we are only beginning to understand TRP function across different tissue types (e.g. pulmonary, vascular and skin).[13; 20] A change in approach to TRP targeted treatment is necessary to fully harness the therapeutic potential of these receptors. New treatments need to address the alterations in TRP channel functioning in visceral hypersensitivity, without interfering with their physiological functions.

TRP channel alterations in visceral hypersensitivity

Evidence regarding the expression of TRP channels in visceral hypersensitivity remains contradictory. Several studies have found increased expression of TRPV1 in colon biopsy material from IBS patients.[3; 16; 19] Others failed to demonstrate such an increase for either TRPV1, TRPV4 or TRPA1.[35] An important factor explaining these discrepancies is likely the heterogeneity of IBS. The question then arises what drives the upregulation of TRP channels in some IBS patients, whilst others maintain normal expression patterns. It could be postulated that increased expression of TRP channels is related to inflammation. Increased levels of inflammatory mediators have been reported in IBS patients in several studies, though not consistently across subtypes. [10; 37] Others have argued that inflammation is within physiological levels in most IBS patients.[28] IBD could therefore serve as a pathophysiological example, demonstrating the impact of inflammation on TRP channel expression and functioning. Interestingly, studies on intestinal expression profiles of TRP channels in IBD patients are similarly contradictory, as demonstrated in chapter 3. These results underpin the complexity of processes involved in TRP channel regulation. Compensatory mechanisms might be at play in both IBS and IBD due to the chronic character of these disorders, rebalancing TRP channel expression over time. Furthermore, it is unclear how inflammation, or any other trigger for that matter, would result in the upregulation of neuronal TRP channels. The latter would need to entail an additional signaling pathway between the axon and its cell body, as gene transcription cannot take place in the axon itself. Understanding these regulatory processes is necessary to develop targeted treatment.[20]

Chapter 11

Although increased expression of TRP channels has not been consistently demonstrated in IBS, potentiation of these channels is recognized as a key mechanism in peripheral sensitization. Indeed, several studies have found decreased pain thresholds to rectal distension after capsaicin administration,[35] or increased abdominal burning after spicy meal ingestion in IBS patients as compared to healthy controls.[30] A study by Balemans et al. demonstrated sensitization of TRPA1 and TRPV4 in IBS patients, with increased Ca2+ responses in rectal submucosal neurons to TRPA1/TRPV4 agonists.[5] A possible treatment approach is therefore to return TRP channels to their pre-sensitized states. This allows for normal receptor function to continue, resulting in fewer side effects. For example, histamine has been demonstrated to be a potent sensitizer of TRPV1, TRPV4 and TRPA1; histamine receptors are therefore being explored as therapeutic targets for reversal of sensitization. [2; 5; 37] The histamine receptor antagonist ebastine was shown to effectively reduce visceral hypersensitivity, as compared to placebo.[37] More recently, endogenous anti-inflammatory lipid mediators known as resolvins were shown to reverse histamine-induced visceral hypersensitivity in a preclinical study.[24] In another approach, cross-desensitization of TRPV1 and TRPA1 via administration of menthol (peppermint oil) was found to provide abdominal pain relief in IBS patients, as compared to placebo, although not meeting the stringent criteria form symptom relief of the FDA/EMA.[36] Future studies will need to address alternative mechanisms of TRP channel sensitization, in order to find more routes to reverse the process.

Ageing and TRP channel functioning

Previous studies have indicated that abdominal pain(sensitivity) decreases with increasing age. In **chapter 4** we describe a study exploring gastrointestinal symptom scores among IBS patients and healthy volunteers in different age categories. In a small subgroup of healthy volunteers sigmoid mucosal biopsies were taken in order to investigate associated changes in TRP channel expression. Our study corroborates previous findings indicating an age-associated decrease in abdominal pain. We furthermore found that TRPV1 and TRPA1 expression was significantly lower in elderly as compared to young adults. It should be noted that we did not provide neural stainings in our study, hence we were not able to quantify neural density in our biopsy samples. This is important to address in future studies as ageing is associated with neurodegeneration. The decreased TRP expression patterns could therefore be related to decreased afferentation rather than downregulation of the TRP channels per se. If the decreased pain sensitivity in elderly is related to TRP channel downregulation however, the mechanisms involved should be explored further as these would hold significant therapeutic potential in visceral pain.

Stimulus origin in visceral hypersensitivity

Although a large body of evidence exists regarding the implication of TRP channels in visceral hypersensitivity, relatively little is known about the origin of stimuli that cause abdominal pain in disorders of gut-brain interaction. Dietary constituents are likely to have a role as nociceptive input, with pungent compounds among the obvious culprits. However, although exclusion diets, such as the popular low FODMAP diet, can provide significant symptom relief, the results are often not satisfactory.[23] In addition, exclusion diets are difficult to adhere to, particularly for longer periods of time. Other types of stimuli are likely to have a role in pain generation. Although normal colonic contractions are well below the nociceptive thresholds in healthy individuals, this is not the case for high amplitude contractions in the context of visceral hypersensitivity. [28] Moreover, it has previously been shown in IBS-D patients that the amplitude and frequency of high amplitude contractions of the colon is increased.[11] Indeed, over 90% of high amplitude contractions coincided with abdominal pain symptoms in these patients. Thus, mechanical stimuli generated by the gut itself may be responsible for the generation of pain symptoms through sensitized nociceptors. Similarly, a recent study using the gastric infusion of fructans (a typical FODMAP) has shown that small bowel motility and colon gas and volume was similar in IBS patients and healthy controls, yet IBS patients perceived significantly more symptoms and this was related to altered brain responses in pain-related regions. These findings suggest the presence of a dysfunctional signaling pathway along the gut-brain axis resulting in painful sensations of otherwise normal intestinal reactions.[38]

Another possibility is that endogenous compounds activate sensitized TRP channels. For example, poly-unsaturated fatty acids levels were increased in mucosal samples from IBS-D patients,[10] and hydrogen sulphide levels were increased in IBS-D patients with bacterial overgrowth.[6] These compounds are known agonists of TRPV4 and TRPA1 respectively, and could result in increased nociceptive input.

Understanding visceral sensory mechanisms allow us to gain further insight and develop novel and more effective therapies for visceral pain. In this sense, the capsaicinpain model described in **chapter 8** (see further below) allows for examining mechanisms of action of different therapeutic entities aimed at decreasing pain.

Pain sensitivity in hypermobility spectrum disorders

In part 2 of this thesis, we describe the association between HSD and disorders of gutbrain interaction. **Chapter 5** provides a comprehensive review regarding this association and serves a hypothesis-generating role. Abdominal symptoms in HSD might be related to aberrant mechanical properties of the gut and associated motility alterations, altered sensory function/perception due to changes in neuronal functioning, or a combination of both. For example, it can be postulated that, similar to increased tissue laxity of skin and joints, intestinal tissue has altered compliance in HSD patients, subsequently affecting gut motor function. At the level of sensory function, a preclinical study in tenascin-X deficient mice indicated increased density and sensitivity of nociceptive neurons in colonic mucosa. [4] Furthermore, previous studies have indicated that individuals with joint hypermobility have a high level of interoceptive awareness. [12; 22] The enhanced interoceptive awareness was also linked increased levels of anxiety, possibly through maladaptive appraisal tendencies toward body sensations resulting in catastrophic thinking.[22] Functional studies in HSD patients focusing on motility (e.g. high-resolution manometry or gastric emptying) and pain sensitivity (e.g. balloon distension studies) are required to provide further insights regarding the involved pathophysiological mechanisms. Unfortunately, still relatively few of such studies have been performed. A small study (n = 30) in HSD patients with dysphagia and reflux symptoms used high-resolution manometry and 24 hour pH-impedance.[14] Minor motility disorders were reported in 40% of patients and 53% of patients was found to have pathological reflux. A control group with similar gastrointestinal symptoms without HSD was not available in the study, making it difficult to interpret results of the functional tests. In two recent studies among FD patients (54-55% of which fulfilled criteria for HSD), gastric emptying, gastric accommodation and gastric hypersensitivity were evaluated.[8; 9] No significant differences were found between FD patients with or without HSD. We looked at difficulty of colonoscopy in HSD versus non-HSD (Chapter 6), as we expected the putative increased colonic tissue laxity to result in a more difficult procedure. However, we found no significant differences in endoscopist-reported difficulty scores or cecal intubation time for HSD patients as compared to non-HSD. We furthermore found no evidence for HSD related visceral hypersensitivity. Although patient-reported pain scores were significantly higher in the HSD group, this difference was found to be related to a confounding effect of gender as the majority of HSD patients was female, which are known to perceive colonoscopy as more painful.

The heterogeneity of disorders of gut-brain interaction such as IBS and FD is often seen as a major hurdle in therapy development. Treatment that proves effective in one subgroup often lacks efficacy in another. Thus, phenotyping of patients is a significant aspect of interest. In this light, HSD patients with a disorder of gut-brain interaction have been considered to represent a separate clinical entity. We have therefore looked at gastrointestinal symptomatology and affective comorbidities in IBS patients with and without HSD (**chapter 7**). We found no significant differences between groups. In combination with the abovementioned findings in available physiological studies, it seems unlikely that HSD associated disorders of gut-brain interaction form a separate clinical entity. Rather, we postulate a shared pathophysiological mechanism. This shared mechanism also applies to the overlap of somatic pain syndromes such as fibromyalgia with IBS.[33] Moreover, both IBS and fibromyalgia are highly prevalent among HSD patients. Increased pain sensitivity due to peripheral and/or central sensitization likely plays a significant role in this context.[7] Previous studies indicating generalized hyperalgesia in HSD underline this pathophysiological basis.[26; 29] Considering this mechanistic overlap, gastrointestinal symptoms in HSD would not require an alternative therapeutic approach, at least not on the basis of current findings. Nevertheless, similar to the treatment of disorders of gut-brain interaction, attention needs to be paid to associated comorbidities (e.g. affective disorders or somatic pain disorders) in order to provide relevant referrals.

Processing of nociceptive input

In the third part of this thesis we focus on the central processing of visceral nociceptive input and subsequent subjective response. Using a novel visceral pain model, consisting of the duodenal infusion of capsaicin, we investigated how nociceptive input from the gut enters the brainstem, and to which regions it is subsequently transduced (chapter 8). Few neuroimaging studies on visceral pain have included the brainstem as region of interest due to the difficulties to accurately visualize it.[31] Our study was the first to employ multi-echo echo-planar imaging at ultrahigh field strength (7 Tesla) during a visceral pain stimulus, providing an unprecedented signal-to-noise ratio for the brainstem in this research field. We furthermore specifically chose a nutrient-related chemical stimulus (i.e. capsaicin). We believe that this entails a more valid representation of gastrointestinal symptoms as compared to supraphysiological mechanical or thermal stimuli. Upon administration of capsaicin, we found a significant activation of the nucleus of the solitary tract (NTS). Multiple regions implied in pain processing were also significantly activated, including the thalamus, insula and anterior cingulate cortex. Interestingly, whilst (sub)cortical activations were more pronounced in subjects who reported abdominal pain (responders) as compared to subjects who were unresponsive (non-responders), activations at the level of the NTS were comparable between groups. This observation highlights the difference between nociception and pain, as nociceptive input does not necessarily result in the subjective experience of pain. Processes involved in the latter are of great interest in pain research given their therapeutic potential. As the NTS is the final location where activations upon stimulation do not defer between abdominal pain responders and non-responders, we believe that it serves an important role in determining whether nociceptive input is perceived as painful. These findings might also explain the efficacy of treatments aimed at modulating vagal activity in abdominal pain, including breathing exercises, mindfulness, and transcutaneous vagal nerve stimulation.[15; 21; 32] It is possible that - similar to the classical gate-control theory of pain - increased vagal tone induced by these activities results in competition with nociceptive input at the level of the NTS.

Chapter 11

It is important to point out that our neuroimaging study was performed in healthy volunteers. It is therefore unclear which brain regions would be differentially activated during capsaicin infusion in patients with disorders of gut-brain interaction as compared to controls. The use of our stimulus paradigm in patients would be unfeasible due to high patient burden. Previous studies using short-duration mechanical or thermal stimuli have included IBS patients, however. In 2011, Tillisch et al. performed a meta-analysis on rectal balloon distension studies, identifying differences in brain responses during a visceral pain stimulus in IBS patients and healthy controls.[34] The researchers reported that regions directly linked to afferent processing were similarly activated in patients and controls. On the other hand, while patients showed greater activations of regions implicated in emotional arousal, healthy controls demonstrated stronger activations of cognitive modulatory regions. It was therefore postulated that dysregulation of central modulatory processes causes stronger subjective pain responses upon visceral stimulation in IBS patients. Whereas healthy controls effectively modulate afferent input, minimizing the subjective pain experience to solely reflect the stimulus itself, IBS patients couple the stimulus to previous experiences resulting in increased negative affect and pain. Furthermore, enhanced interoceptive awareness further increases emotional arousal, resulting in high levels of anxiety.[25] Anxiety is known to affect memory formation, which forms the basis of fear conditioning. Increased anxiety in IBS patients is thought to result in hypervigilance, amplifying the pain response upon repeated exposure.[17] The latter also highlights the importance of treating affective symptoms in disorders of gutbrain interaction; untreated affective symptoms reinforce pain and stimulus avoidance resulting in significant morbidity. The mediating role for interoceptive awareness in anxiety should be considered in its treatment, possibly through cognitive behavioral therapy aimed at restoring beliefs regarding visceral perceptions.

In contrast to the implication of cortical regulatory processes, the role of the brainstem in transduction and modulation of nociceptive input in disorders of gutbrain interaction is relatively unexplored. It seems likely that increased nociceptive input due to peripheral sensitization would result in increased activation of the NTS. On the other hand, previous studies have shown imbalances in the autonomic nervous system in IBS patients, including dominance of sympathetic activity.[1; 18; 27] It is possible that the latter affects the modulation of nociceptive input in the vagal relay station. Studies combining brainstem neuroimaging with the assessment of autonomic activity in disorders of gut-brain interaction are needed to answer these questions.

Considering the methodological difficulties inherent to neuroimaging, in particular the processing of obtained data, it is of utmost importance to continuously evaluate the quality of the data throughout a project. Data cleaning, or so-called denoising, is necessary as only a fraction of the fMRI signal entails signal of interest. As a result, the choice of a denoising method can have significant impact on the results of an fMRI experiment. Various denoising approaches have been described in the past, however none have compared their efficacy in the cleaning of multi-echo 7T data. We have therefore applied several data cleaning methods to our data and compared their efficacy (**chapter 9**). In terms of several data quality metrics, our data cleaning approach as used in **chapter 8** proved highly effective as compared to alternative methods.

Pain perception and symptom registration

Pain perception is derived from the complex integration of peripheral and central factors. How these factors are integrated is likely individual dependent. Detailed patient phenotyping, in part by high quality symptom registration, is necessary to explore exactly how we subconsciously modulate pain perception. The experience sampling method (ESM) has shown to be a valuable tool for assessing symptom triggers. Queries incorporated in this measurement instrument include aspects beyond the perceived symptom itself; contextual information such as current location, someone's company, previous meal and current affective state is also gathered. Moreover, momentary assessments prevent recollection bias, which has been shown to be significant even with the use of end-of-day diaries. On the other hand, the high frequency of measurements with ESM results in lower adherence as compared to end-of-day diaries (**chapter 10**). For prolonged registration periods (e.g. clinical trials), a combination of both with continuous measurement using end-of-day diaries and intermittent use of ESM would offer the benefits of both measurement tools.

Conclusion and future implications

Understanding the mechanisms underlying pain requires the breakdown of involved processes into peripheral and central factors. The current thesis provides a comprehensive outline of such factors, highlighting some of the alterations in the processing and perception of pain found in disorders of gut-brain interaction. Significant inter-individual differences between patients exist, however, with separate factors being of varying importance in different patients. In order to identify the origin of increased visceral pain sensitivity in individual patients, we need to improve monitoring of symptoms and associated physiological responses. ESM is of significant value in this context as it not only provides detailed mapping of perceived symptoms, but also explores symptom triggers. Moreover, ambulant autonomic measurements are becoming more readily available with the advent of smartwatches. Common options now include heart rate monitoring combined with heart rate variability and skin conductance measurements. As surrogate markers these physiological measurements can provide an indication of (para)sympathetic activity (i.e. autonomic function). Combining ESM and physiological monitoring could further aid patient stratification and thereby guide patient-tailored treatments.

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ADDENDUM

Summary Nederlandse samenvatting Impact paragraph Dankwoord List of publications Curriculum Vitae

SUMMARY

Chronic visceral pain is a hallmark symptom of many disorders of gut-brain interaction (DGBI). These disorders can be characterized by upper abdominal pain, such as in functional dyspepsia (FD), or lower abdominal pain, such as in irritable bowel syndrome (IBS). Although both FD and IBS are highly common with reported prevalence rates in Western populations of around 10%, their treatment remains challenging. The complexity of mechanisms involved in generating the subjective sensation of visceral pain from a stimulus highlights the difficulty of the development of effective treatment strategies.

In this thesis, we approach visceral pain mechanisms and abnormalities leading to increased pain sensitivity from both a peripheral and central perspective. To this end, the current thesis is divided into three major parts. The first part focusses on molecular transducers of visceral pain, in particular transient receptor potential channels. The second part explores the association between hypermobility spectrum disorders (i.e. hereditary non-inflammatory connective tissue abnormalities characterized by increased mobility of joints) and visceral pain. The third part describes central pain processing, pain perception and measurement of visceral pain symptoms.

Part I - molecular transducers of visceral pain

In chapter 2, we review current literature regarding the role of the different transient receptor potential (TRP) channels, i.e. TRPV1, TRPV4, TRPA1 and TRPM8 in IBS. Per TRP channel, we provide an overview of gastrointestinal expression patterns, mechanisms of activation and sensitization, and their putative implication in chronic visceral pain in IBS. Possible therapeutic targets were highlighted. We finally described various mechanisms of TRP channel targeted therapy, including (selective) antagonism, (cross-)desensitization, downstream targeted therapy and, RNA-based therapy (experimental). Given the important role of TRP channels in visceral pain generation and inhibition, and the various options for regulating their function, it seems likely that TRP channel targeted therapy will become an effective option for visceral pain management in IBS and other DGBIs. The primary challenge in treatment development is related to the large amount of physiological functions of TRP channels. Expanding our knowledge on TRP channel functioning in (patho)physiological conditions will aid future treatment development.

In order to find potential analogies and/or differences in states of chronic intestinal inflammation, we reviewed current literature regarding TRPV1 and TRPA1 in inflammatory bowel disease (IBD) in **chapter 3**. TRPV1 and TRPA1 not only play a complex role in hyperalgesia, but also in (neurogenic) inflammation. Regarding the latter, the role of these ion channels in IBD is seemingly contradictory. Activation of

Addendum

these receptors on sensory nerve terminals mediates neurogenic inflammation via the release of sensory neuropeptides (such as substance P and calcitonin gene-related peptide, CGRP), resulting in increased vascular permeability and inflammatory cell activation. Meanwhile, anti-inflammatory sensory neuropeptides, such as somatostatin and opioid peptides released simultaneously from the same nerve ending exert antiinflammatory and analgesic actions both locally and systemically. It remains to be elucidated which processes result in tipping the scale to a net pro-inflammatory or antiinflammatory effect. Till date however, preclinical studies more convincingly point out the potential therapeutic value of TRPV1 and TRPA1 antagonists in colitis and visceral hypersensitivity.

In **chapter 4**, we explored age-related changes in visceral nociception and related alterations in TRP channels, as this natural analgesic effect could serve as an example in the development of new treatment strategies. Previous studies pointed to a decrease in abdominal pain symptoms with ageing. Our study corroborated these findings, showing lower abdominal pain scores in elderly versus young adults in both healthy volunteers and IBS patients. We furthermore found that visceral hypersensitivity, as assessed using a rectal barostat procedure, was significantly less common in elderly IBS patients as compared to young adults. Investigating possible underlying mechanisms, we found that relative TRPA1 gene transcription, as well as TRPA1 and TRPV1 immunoreactivity were significantly lower in healthy elderly versus healthy young adults. We therefore concluded that decreased visceral sensitivity in elderly may be attributed to decreased TRPA1 and/or TRPV1 receptor expression in the intestinal epithelium. Future studies will need to assess these biological changes on a larger scale and include assessments for neural density, as neurodegeneration could also have role in the decreased visceral sensitivity in elderly.

Part II - visceral pain in hypermobility spectrum disorders

Abdominal symptoms and DGBIs are highly prevalent among individuals with hypermobility spectrum disorders (HSD). In **chapter 5**, we provide a review of current literature on abdominal symptoms in joint hypermobility syndrome/Ehlers-Danlos hypermobility type (now commonly referred to as HSD). Although a wide range of abdominal symptoms was reported in HSD patients, upper gastrointestinal symptoms, including bloating and postprandial fullness appeared to be the most common. Underlying mechanisms remain to be elucidated. Unfortunately, few studies have been performed to increase our understanding of GI physiology alterations in HSD. Two recent studies in FD patients by Carbone et al. did not identify gastric motility or sensitivity differences when dividing groups in HSD and non-HSD patients. We can therefore only speculate regarding the underlying mechanisms of abdominal symptoms in HSD. We hypothesized that changes in afferent signaling (e.g. due to extracellular matrix alterations) result in increased peripheral discharge of nociceptive information (peripheral sensitization) and/or augmentation of afferent information at the level of spinal dorsal horn neurons (central sensitization). More in-depth mechanistic studies are required to investigate these hypotheses.

Anecdotal evidence suggests that colonoscopy is more difficult in individuals with HSD, possibly due to laxity of intestinal tissue and increased pain sensitivity. In **chapter 6**, we describe a prospective exploratory study performed at the endoscopy unit, reporting on pain scores during colonoscopy and cecal intubation time in HSD and non-HSD individuals. A total of 200 patients was included, of whom 22 (11%) met criteria for HSD. Although initial linear regression demonstrated higher pain scores in HSD patients, this was found to be related to a confounding effect of female gender. After correction for confounding factors, we found no differences in pain scores, cecal intubation time or endoscopist-reported procedural difficulty. It should be noted that the number of HSD patients was relatively low, however. Moreover, the sedative of choice at the endoscopy unit, midazolam, is known to cause anterograde amnesia in a subset of patients, hence patient may not fully recall their experience after colonoscopy. For assessing visceral pain sensitivity in HSD, different approaches will be necessary. Nonetheless we conclude that HSD status appears irrelevant in colonoscopy practice.

In **chapter** 7, we assess characteristics of IBS patients (n = 258) with and without HSD, with a focus on GI symptomatology, affective symptoms and perceived quality of life. When comparing IBS patients with HSD to their non-HSD counterparts, no apparent differences in gastrointestinal symptoms, depression, anxiety, and quality of life were found. Future studies should focus on differences in additional somatic symptoms (including overlapping DGBIs), autonomic symptoms, and somatization scores, in order to unravel the question of whether IBS patients with a comorbid diagnosis of joint hypermobility or HSD represent a specific subgroup within the IBS population.

Part III - visceral pain processing and perception

In the third part of this thesis, we report on several studies at the level of central pain processing and perception, i.e. measurement of symptoms in clinical trials. **Chapter 8** describes a neuroimaging study in 18 healthy volunteers using a novel visceral pain model consisting of the duodenal infusion of capsaicin. During infusion, functional magnetic resonance imaging (fMRI) at ultrahigh field strength (7 Tesla) was used to investigate brain responses, with a particular focus on the nucleus of the solitary tract (NTS), the primary relay station of visceral afferents in the brainstem. Significantly increased brain activation over time during capsaicin infusion, as compared to placebo, was observed in brain regions implicated in pain processing, in particular the NTS. Brain activation in the thalamus, cingulate cortex and insula were more pronounced in subjects who reported abdominal pain (visual analogue scale > 10mm), as compared to subjects who experienced no pain. On the contrary, activations at the level of the NTS

were independent of subjective pain ratings. These findings prompt the exploration of fundamental mechanisms related to how pain emerges from nociception as well as new therapeutic approaches to treating visceral pain conditions, for instance, by targeting specific areas within the gut-brain axis to downregulate disturbed visceroception and hyperalgesia.

From a methodological point of view, fMRI experiments are inherently difficult. Most notably, it can be challenging to develop a valid data preprocessing pipeline, which is necessary to enable robust analyses. In **chapter 9**, we describe the technical aspects of quality of several data cleaning approaches in order to increase temporal signal-to-noise ratio (tSNR; among other data quality metrics). Optimized tSNR is of particular interest in brainstem neuroimaging, as large vessel and air-filled cavities proximity results in a generally lower tSNR as compared to cortical brain regions. Using our imaging paradigm described in **chapter 8**, which included a multi-echo sequence optimized for brainstem imaging, we compared several data cleaning approaches. Optimal results were achieved with multi-echo independent component analysis (ME-ICA) combined with anatomical component based correction (aCompCor), which was also our approach of choice in **chapter 8**.

The final step after central pain processing entails the experience of pain. Given its subjective nature, high quality measurement instruments are necessary to provide the best representation of patient symptoms. **Chapter 10** describes a comparative study of digital instruments to measure gastrointestinal symptoms. In this study, we included 25 IBS patients and 15 FD patients. All subjects were required to have participated in a randomized controlled trial (which incorporated a digital end-of-day diary) and observational study (which used momentary assessments, i.e. the experience sampling method [ESM]). We found excellent adherence rates for the end-of-day diaries as well as good adherence to the ESM-based applications. Overall adherence rates for ESM were evidently lower, as would be expected given the nature of the methodology (and consequently, higher patient burden). Nonetheless, ESM does have added value over end-of-day diaries due to the frequent and more extensive measurements. Researchers should choose between these sampling methods based on their research questions, or try to combine them optimally (e.g. use ESM at intervals).

Finally, we summarize the main findings of this thesis in **chapter 11**, highlighting future implications and possibilities for further research.

NEDERLANDSE SAMENVATTING

Storingen in de hersen-darm communicatie worden gekenmerkt door chronische buikpijn (ook wel viscerale pijn) en komen zeer frequent in de algemene populatie voor. Voorbeelden hiervan zijn het prikkelbare darmsyndroom (PDS), waarbij klachten vooral onderin de buik worden gerapporteerd, en functionele dyspepsie (FD), waarbij de klachten meer in de bovenbuik worden waargenomen. Zowel PDS als FD komen bij circa 10% van de Westerse bevolking voor. Toch blijft de behandeling van deze aandoeningen in veel gevallen uitdagend. Voor een groot deel hangt dit samen met de complexiteit van het proces van pijnprikkel in maag en darm tot (subjectieve) pijnbeleving. Om tot een effectieve behandeling te komen dient dit proces van begin tot einde begrepen te worden.

In dit proefschrift onderzoeken we de mechanismen van viscerale pijn, met specifiek aandacht voor afwijkingen die kunnen leiden tot verhoogde pijn gevoeligheid. Hierin wordt onderscheid gemaakt tussen het "perifeer" en "centraal" niveau. Het perifeer niveau duidt op de zenuwuiteinden in de darm die een pijnprikkel waarnemen. Het centraal niveau verwijst naar de verwerking van een prikkel in de hersenen en de uiteindelijke subjectieve ervaring. Deze onderverdeling komt ook terug in de drie delen van dit proefschrift. In **deel I** wordt de focus gelegd op moleculaire pijnsensoren op de zenuwuiteinden, de zogeheten transient receptor potential (TRP) channels. **Deel II** van dit proefschrift gaat over de associatie tussen hypermobiele gewrichten en viscerale pijn. **Deel III** gaat over de centrale verwerking van pijnsignalen, pijnperceptie en het meten van pijn.

Deel I – moleculaire pijnsensoren

In **hoofdstuk 2** voorzien we een overzicht van de huidige literatuur betreffende de rol van een viertal verschillende TRP channels bij buikpijn, specifiek bij PDS. Iedere TRP channel is gevoelig voor andere prikkels, in het overzicht beschrijven we dan ook de diverse prikkels die leiden activatie van deze pijnsensoren. Daarnaast kunnen deze pijnsensoren op verschillende manieren overgevoelig (gesensitizeerd) worden. Dit laatste is van groot belang bij PDS en andere aandoeningen van de hersendarm-as. In deze context beschrijven we dan ook potentiele behandelmogelijkheden. Zo kunnen TRP channels bijvoorbeeld direct geremd/geblokkeerd worden, of minder gevoelig gemaakt worden door herhaaldelijke blootstelling aan een prikkel (desensitizatie). Gezien de centrale rol van TRP channels bij het ontstaan van pijnprikkels achten we de kans groot dat toekomstige behandelingen zich hierop zullen richten. Een van de nadelen is momenteel nog dat TRP channels, naast de vorming van pijnprikkels, allerlei belangrijke functies hebben, zoals het regelen van de temperatuur in het lichaam. Ontregeling van hiervan is zeer onwenselijk. Wanneer we de functie van TRP channels beter begrijpen kunnen we mogelijk gericht de verhoogde pijngevoeligheid zoals bij PDS aanpakken, zonder andere functies negatief te beïnvloeden.

Een van de mogelijkheden waarop TRP channels gevoeliger worden voor prikkels is door ontsteking (inflammatie). Andersom is het ook mogelijk dat TRP channels na prikkel blootstelling leiden tot ontsteking via een proces genaamd neurogene inflammatie. In **hoofdstuk 3** geven we een overzicht van de rol van TRPV1 en TRPA1 bij belangrijke ontstekingsziekten van de darm (inflammatory bowel disease, IBD). De huidige literatuur laat wisselende resultaten zien. Niet alleen toegenomen ontsteking, maar ook remming van het ontstekingsproces na activatie van TRPV1 en TRPV1 werd beschreven in diverse studies. Welke processen uiteindelijk de doorslag geven tot toegenomen ontsteking is momenteel onduidelijk. Wel is duidelijk dat remming van TRP channels leidt tot het terugdringen van ontsteking en het verminderen van de pijngevoeligheid.

Uit eerdere onderzoeken is gebleken dat buikpijn minder voorkomt op hogere leeftijd. In hoofdstuk 4 beschrijven we een eigen onderzoek waarin we keken naar buikpijnscores in verschillende leeftijdscategorieën. Dit deden we bij zowel gezonde vrijwilligers als bij PDS-patiënten. Bij beide groepen waren de buikpijnscores lager in ouderen (65+) versus jongvolwassenen (18-40 jaar). In de praktijk kunnen we ook uitspraken doen over de pijngevoeligheid middels een specifiek onderzoek, de zogeheten rectale barostat. Dit onderzoek maakt gebruik van een ballon in de endeldarm. Deze wordt geleidelijk computergestuurd met lucht gevuld. Bij hogere pijngevoeligheid zal bij beperkte vulling pijn aangegeven worden. In ons onderzoek bleek dat oudere PDS-patiënten significant minder vaak een hoge pijngevoeligheid hadden ten opzichte van jongvolwassen patiënten. Tot slot hebben we gekeken naar verschillen in de dichtheid (expressie) van TRP channels in darmbiopten van een groep gezonde vrijwilligers. Hieruit bleek dat de biopten van ouderen een beduidend lagere dichtheid TRP channels vertoonden ten opzichte van jongvolwassenen. De verlaagde pijngevoeligheid bij ouderen is daarom mogelijk gelinkt aan het verminderde voorkomen van deze moleculaire pijnsensoren in de darm. Belangrijk om op te merken is wel dat wij niet hebben kunnen kijken naar de zenuwvoorziening van de darm in het geheel. Dit is relevant omdat we weten dat de zenuwvoorziening bij toegenomen leeftijd achteruit gaat. Hiervoor dient in toekomstig onderzoek gecorrigeerd te worden.

Deel II – viscerale pijn en hypermobiliteit

Hypermobiliteit, d.w.z. overmatig flexibele gewrichten, komt veel voor in de algehele populatie. Vaak heeft men hier weinig last van, en kan dit ook leiden tot bepaalde voordelen (zoals bij sporters of muzikanten), maar soms gaat dit gepaard met uitgebreide gewrichtsklachten. Gezien de uiteenlopende presentatie wordt tegenwoordig gesproken van hypermobiliteits spectrumstoornissen (hypermobility spectrum disorders, HSD). Studies hebben aangetoond dat mensen met HSD vaker een aandoening van de hersendarm-as hebben dan mensen zonder HSD. In **hoofdstuk** 5 zetten we deze studies op een rij. Met name klachten van de bovenbuik, zoals een opgeblazen gevoel en een vol gevoel na de maaltijd, lijken vaak voor te komen bij HSD. Helaas is nog onduidelijk waarom buikklachten vaker voorkomen bij HSD. Bij HSD heeft het bindweefsel een afwijkende samenstelling, waardoor het bijvoorbeeld slapper is dan normaal bindweefsel. Bindweefsel komt in het hele lichaam voor en vervult een onderhoudende functie voor verschillende andere weefsels, waaronder zenuwweefsel. Zo zou dit mogelijk ook kunnen zorgen voor een gevoeligere zenuwvoorziening in de darm, of een afwijkende prikkelverwerking in de hersenen.

Uit praktijkervaringen werd eerder verondersteld dat mensen met HSD meer pijn ervaren bij een onderzoek van de darm (coloscopie). In **hoofdstuk 6** beschrijven we studie waarin we keken naar de pijnervaring van mensen met en zonder HSD die een coloscopie ondergingen. Ook keken we naar de totale duur van het darmonderzoek, en vroegen we de uitvoerende arts de moeilijkheid van de procedure te scoren. In totaal includeerden we 200 patiënten, waarvan 22 (11%) HSD bleken te hebben. Hoewel de HSD-groep gemiddeld hogere pijnscores rapporteerde, bleek dit bij nader onderzoek gerelateerd aan het groter aantal vrouwen in deze groep (uit eerder onderzoek is gebleken dat vrouwen meer pijn ervaren bij een darmonderzoek). Het darmonderzoek leek daarnaast niet langer te duren bij patiënten met HSD, noch werd het onderzoek als moeilijker ervaren door de arts. Wij concludeerden daarom dat de HSD-status van een patiënt irrelevant is bij een coloscopie.

In **hoofdstuk** 7 beschrijven we een onderzoek binnen 258 patiënten met PDS. Hierbij maakten we onderscheid tussen patiënten met en zonder HSD, en keken we naar potentiele verschillen in buikklachten, stemmingsklachten en kwaliteit van leven. Op genoemde vlakken bleken PDS-patiënten met en zonder HSD niet van elkaar te verschillen. Op basis van huidig onderzoek hebben we daarom geen reden om aan te nemen dat HSD-patiënten met een aandoening van de hersendarm-as een separate entiteit vormen. Bij buikklachten heeft HSD-status daarom geen directe behandelconsequenties.

Deel III - Viscerale pijn verwerking en perceptie

In het derde deel van dit proefschrift beschrijven we een aantal onderzoeken gericht op de centrale component van pijn, in het bijzonder pijnprikkel verwerking en perceptie van pijn. **Hoofdstuk 8** gaat over een neurowetenschappelijk onderzoek bij 18 gezonde vrijwilligers. In dit onderzoek maakten we gebruik van een nieuw experimenteel model om viscerale pijn te bestuderen. Hiertoe werd een dunnedarmsonde ingebracht via de neus, en werd over twee verschillende testdagen een zoutoplossing en een oplossing met rode peper extract (capsaïcine) toegediend. De toediening werd hierbij geblindeerd (proefpersonen wisten niet welke oplossing op een dag gegeven werd). Tijdens toediening werden met behulp van een speciale MRI-scanner beelden verkregen waaruit de hersenactiviteit over de tijd berekend kon worden. Gebieden in de hersenstam die eerder niet in detail bekeken konden worden, konden nu goed in beeld gebracht worden. Zo kon de plek waar het pijnsignaal als eerste in de hersenen binnenkomt bestudeerd worden. Met het gebruik van ons pijnmodel was er een groot verschil in de mate van pijn die werd ervaren door proefpersonen. Hiervan gebruikmakend konden ook verschillen in hersenactiviteit bestudeerd worden. Opvallend was dat de activaties in de hersenstam op groepsniveau niet verschilden wanneer mensen met lage pijnscores werden vergeleken met mensen met hogere pijnscores. In hoger gelegen hersengebieden daarentegen kon dit onderscheid wel gemaakt worden. Een potentieel verantwoordelijk gebied voor dit onderscheid in pijnervaring werd aangewezen. Gericht vervolgonderzoek is nodig om al dan niet te kunnen bevestigen dat dit hersengebied inderdaad een rol speelt bij het (selectief) doorlaten van pijnsignalen.

De zogeheten functionele MRI (fMRI) experimenten zoals hierboven beschreven zijn zowel in de uitvoering als analyse technisch uitdagend. De verkregen data dient namelijk uitgebreid bewerkt te worden voordat deze gebruikt kan worden. Daarbij kan de data op veel verschillende manieren bewerkt worden teneinde het reële signaal van ruis te scheiden; hierin dienen goed onderbouwde keuzes gemaakt te worden. In **hoofdstuk 9** beschrijven we een aantal mogelijke databewerking strategieën, waarbij een vergelijking gemaakt wordt op basis van verkregen datakwaliteit. De meest optimale strategie is ook de aanpak zoals toegepast in **hoofdstuk 8**.

Na de centrale verwerking van een pijnprikkel volgt de uiteindelijke perceptie van pijn. Gezien het subjectieve karakter van pijn is het van groot belang om gedegen meetinstrumenten te gebruiken om patiënt symptomen in kaart te brengen. In **hoofdstuk 10** beschrijven we twee methoden om symptomen in beeld te brengen. Een methode maakt gebruik van een dagboek dat wordt ingevuld op een smartphone. Vragen over buikklachten worden hier eenmaal daags, aan het einde van de dag, ingevuld. De andere methode is de experience sampling method (ESM), waarbij de gebruiker tienmaal per dag middels een geluidssignaal gevraagd wordt enkele vragen over buikklachten en contextuele vragen (gezelschap, activiteit, genuttigde voeding) te beantwoorden. Dit laatste is door zowel frequentie als het aantal vragen een grotere tijdsbelasting. Dit zagen we ook terug in de mate waarin de vragen beantwoord werden. Toch lijkt ESM goed uitvoerbaar en kan het van grote waarde zijn om beter inzicht te krijgen in het klachtenpatroon. Wanneer behoefte is aan informatie op detailniveau dient ESM overwogen te worden.

In **hoofdstuk 11** sluiten we af met een overzicht van de uitgevoerde studies, diens implicaties en mogelijkheden voor toekomstig onderzoek.

Impact paragraph

IMPACT PARAGRAPH

Disorders of the gut brain interaction (DGBIs) are highly prevalent disorders, with an overall prevalence of 40% worldwide. Many DGBI, including irritable bowel syndrome (IBS) and functional dyspepsia (FD), are characterized by chronic visceral pain. Chronic visceral pain constitutes a substantial clinical challenge with largely unmet medical needs, as underlying mechanisms are incompletely understood. Factors of influence are related to cognition, affect and behaviour, including learning and expectations around pain, and comorbid mood and anxiety disorders. Chronic visceral pain is associated with high patient burden and decreased quality of life, and results in increased healthcare seeking behaviour. Similar to somatic chronic pain syndromes, the misuse of opioids is major problem. In the USA for example, this misuse has reached epidemic proportions. Importantly, opioids can result in a paradoxical effect in visceral pain, further escalating symptoms upon opioid continuation, which is referred to as narcotic bowel syndrome. New treatment strategies in chronic visceral pain are necessary to meet the needs of this large patient group, and to reduce healthcare resource utilization and associated costs.

In the first part of this thesis, we focus on molecular aspects of pain, in particular transient receptor potential (TRP) channels as promising targets for visceral pain management. Studies indicate sensitization and/or increased expression of TRP channels in IBS. Unfortunately, direct antagonism of these channels has shown to be associated with various side effects, mainly due to the involvement of TRP channels in a wide range of physiological functions (e.g. thermosensation and thermoregulation). The reversal of sensitization and overexpression could provide a better approach, as this does not alter physiological functions. More detailed insights in these processes is necessary to develop such therapies. We have therefore here reviewed TRP channel involvement in visceral pain in inflammatory and non-inflammatory conditions. It is unclear to what extent inflammation has a role in TRP channel regulation in chronic visceral pain in DGBIs. Other mechanisms will need to be explored in future studies. For example, we have shown that ageing results in decreased visceral sensitivity and decreased TRPV1 and TRPA1 expression. This natural analgesic process could provide new clues for TRP channel regulation, and therefore holds significant therapeutic potential in chronic visceral pain.

One of the major challenges in the development of new pain treatment strategies relates to the large heterogeneity of DGBI patients. Phenotyping, even in specific syndromes such as IBS and FD, is difficult. It is likely that underlying mechanisms in visceral hypersensitivity differs among patients, affecting treatment outcome. In the second part of this thesis, we investigated the overlap between DGBI and hypermobility spectrum disorder (HSD), a condition characterized by increased mobility of joints. IBS and FD are highly prevalent among HSD patients, and vice versa, we showed a high prevalence of HSD in a cohort of IBS patients. However, we did not find differences in gastrointestinal symptomatology or comorbid affective disorders in HSD and non-HSD IBS patients, arguing against a separate clinical entity. We furthermore found no evidence for increased pain sensitivity during colonoscopy in HSD patients versus non-HSD. HSD patients with DGBI therefore do not seem to require an alternative management approach. Alternate identifiers will need to be explored to optimise DGBI patient phenotyping.

In the third part of this thesis, we have investigated the propagation and integration of visceral pain signals using a novel visceral pain model. The brainstem has a central role in the relay of visceral signals, which in particular includes the nucleus of the solitary tract (NTS), the principal relay station for visceral afferents. We demonstrated NTS activation following capsaicin infusion in the duodenum in healthy individuals. Moreover, the NTS was activated irrespective of the subjective pain response. The NTS, or projections from the NTS, appear to serve an important role in determining which signals are ultimately perceived as painful. It can be speculated that stimulation of the NTS, such as via transcutaneous vagus nerve stimulation (tVNS), might interfere with nociceptive signals, disrupting their propagation. As such, we have recently acquired ethical approval for a study combining our visceral pain model with tVNS (active versus sham stimulation during capsaicin infusion). This study will help to identify whether the NTS is indeed an attractive treatment target for chronic visceral pain, and thereby expand basic scientific evidence for existing treatment options, such as tVNS, deep breathing exercises and mindfulness.

Finally, the incorporation of detailed symptom registration methods in research could aid treatment development. In this thesis, we report on experience sampling method (ESM), also known as ecological momentary assessment, as an easy to use and highly informative symptom sampling method. Adding ESM aids patient stratification, as for example symptom patterns and triggers differ between patients. It will be particularly interesting to add ESM to neuroimaging studies, to investigate whether phenotype-based patient stratification reflects on brain activity patterns. Such combinations could then help identify patient subgroups, possibly aiding the discovery of specific underlying mechanisms of pain sensitivity, development of new treatments and finally the prediction of treatment outcome.
Dankwoord

DANKWOORD

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CURRICULUM VITAE

Abraham Bèr (Bram) Beckers was born on December 30, 1991 in Arnhem, the Netherlands. He attended primary school in Stein and finished secondary education at Groenewald in Stein in 2010. In the same year, he started studying biomedical sciences at Maastricht University. In 2011, he started medical school at the same university. After graduating with distinction in 2017, Bram started his PhD at the division of Gastroenterology-Hepatology of Maastricht University Medical Center, under the supervision of prof. dr. A.A.M. Masclee and prof. dr. D. Keszthelyi. The



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