PERIPHERAL AND CENTRAL MECHANISMS IN ACID INDUCED VISCERAL PAIN HYPERSENSITIVITY IN THE HUMAN OESOPHAGUS

by

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ABSTRACT

Introduction: Gastro-oesophageal reflux disease (GORD) affects 40% of Western populations. Many symptoms persist despite the resolution of inflammation or acid exposure. In these patients, visceral pain hypersensitivity (VPH), modulated peripherally and centrally, is believed to be important.

Study 1: Full thickness human oesophageal samples were collected from 10 patients undergoing oesophagectomy. Using immunohistochemistry, the samples were stained with PGP 9.5, synapthophysin, TRPV1, TRPV4 and ASIC3. Within healthy areas of the human oesophagus, immunoreactive nerve fibres and ganglia were identified in the sub-epithelium and myenteric plexus. Within the epithelium, nerve endings were absent; however, ASIC3 was reliably identified in epitheliocytes.

Study 2: Oesophageal biopsy samples from 36 individuals were collected during upper GI endoscopy; erosive oesophagitis, non-erosive reflux disease and controls. Symptoms were profiled using symptom severity scores, adapted from a validated questionnaire. Oesophageal mucosal biopsies were taken 3-5 cm above the lower oesophageal sphincter. Specific features of oesophageal mucosal epithelial ASIC3 were identified by immunohistochemistry, which were associated positively with an increase in symptom severity.

Study 3: Oesophageal VPH can be induced in healthy volunteers using an established model of acid infusion. In total, 85 retrospective infusions from 57 volunteers were studied, and VPH was present in 70%. The model was shown to be safe and reproducible, but reliability of the model for drug studies was only valid provided control measures were observed.

Study 4: Pregabalin is a centrally acting calcium channel blocker currently used for somatic neuropathic pain and partial seizures. Using the model described in study 3, a prospective, double-blinded, cross-over, placebo-controlled study in 15 healthy volunteers was performed. After an initial screening visit, the volunteers were randomised to either pregabalin or placebo. Pregabalin prevented or attenuated VPH at 30 and 90 minutes after acid sensitisation.

Conclusion: The evidence for central sensitisation was further consolidated with the pregabalin study and peripheral sensitisation by the ASIC3 study. The evidence is important in the detection of predominant mechanisms underlying the symptoms of patients with GORD for both cohort development and targeted treatment.

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To my parents…
I am here because you were there

STATEMENT OF ORIGINALITY

The author wishes to certify that all the work presented in this thesis is original and that all experiments, the acquisition and analysis of resulting data and the subsequent production of this manuscript were performed by him unless clearly stated otherwise.

In particular, the following data included were not acquired by me.

Chapter 2: Additional TRPV1 optimisation using numerous alternative antibodies, immunofluorescence techniques and Western blotting.

Chapter 4: The clinical studies used for retrospective analysis. However, I was involved in assisting in some of these clinical studies.

PUBLICATIONS AND PRESENTATION

Some of the results presented in this thesis have already been published, in part, in the following journals:

PUBLICATIONS

PAPERS

1. **Chua YC**. Improving Immunohistochemistry. *Immunology* 2007; 14.3: 35-38.

2. Matthews PJ, Knowles CH, **Chua YC**, Delaney C, Hobson AR, Aziz Q. The effects of concentration and frequency of acid infusion on the development and maintenance of esophageal hyperalgesia in a human volunteer model. *American Journal of Physiology* 2008; 294: 914-917.

3. Sharma A, Paine P, Rhodes S, Warburton F, **Chua YC**, Aziz Q. Autonomic response to human esophageal acidification predicts the development of visceral hyperalgesia. Submitted to *Neurogastroenterology and Motility (NGM)*

4. **Chua YC**, Q Aziz. Perception of gastro-esophageal reflux. Accepted for publication in *Best practice and research: Clinical Gastroenterology*.

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1. Sharma A, Paine P, Unsworth B, Parvez K, Spibey K, **Chua YC**, Aziz Q. Autonomic responses to distal esophageal acidification and their relationship to sensitisation in a human model of visceral pain hypersensitivity. *Gastroenterology* 2007; 132 issue 4, supplement 1: A155.

2. **Chua YC**, Willert RP, Knowles CH, Delaney C, Hobson AR, Aziz. Development of a physiological model of gastro-oesophageal reflux disease (GORD) in healthy volunteers. *British Journal of Surgery* 2008; 95 issue 4: 29.

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6. Ng K, **Chua Y**, Gresty M, Marreddy U, Williams S, Baker G, Andrews P, Sanger GJ, Ban V, Chey S, Aziz Q. Identification of psychophysiological biomarkers of nausea using a novel visual induction method. *Gut* 2010; 59, Issue 4: 72.

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PRESENTATIONS TO LEARNED SOCIETIES

INTERNATIONAL

1. Sharma A, Paine P, Unsworth B, Parvez K, Spibey K, **Chua YC**, Aziz Q. Autonomic responses to distal esophageal acidification and their relationship to sensitisation in a human model of visceral pain hypersensitivity (Oral). *American Gastroenterological Association (AGA) during Digestive Diseases Week,* Washington, May 2007

2. **Chua YC**, Aziz Q, Knowles CH. Molecular studies of nociception in human esophageal hypersensitivity (Oral). *World Congress of Esophageal Diseases (OESO),* Monaco, April 2008

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4. **Chua YC**, Sharma A, Ng KS, Jafari J, Knowles CH, Aziz Q. Pregabalin prevents development of visceral pain hypersensitivity (VPH) in a model of esophageal acid sensitisation in healthy volunteers [Mid-point results] (Poster). *American Gastroenterological Association (AGA) during Digestive Diseases Week,* Chicago, May-June 2009

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7. **Chua YC,** Hughes SF, Chinaleong J, Peiris M, Hubball A, Ng KS, Levey P, Preston S, Patel B, Chaudry A, Martin JE, Aziz Q, Knowles CH. Esophageal epithelial ASIC3 is associated with increase in severity of symptoms in patients with gastro-oesophageal reflux disease (Oral). *World Congress of Oesophageal Diseases (OESO),* Boston August 2010

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reproducibility of the human model of oesophageal hypersensitivity (Poster). *Society for Academic and Research Surgery (SARS),* Birmingham January, 2008

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List of abbreviations

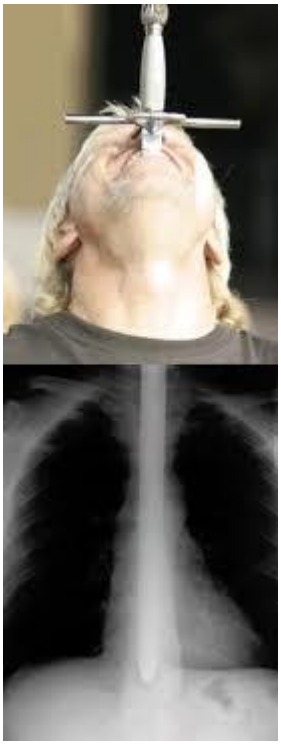
AA	Arachidonic acid
AMPA	Amino-m-ethylene-phosphonic acid
ANS	Autonomic nervous system
ASIC	Acid sensing ion channel
ASIC3	Acid sensing ion channel 3
ATP	Adenosine triphosphate
BDNF	Brain derived natriuretic peptide
BMI	Body mass index
CaMKII	Calmodulin-dependent protein kinase
CEP	Cortical evoked potential
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CO2	Carbon dioxide
COX	Cyclo-oxygenase
CBT	Cognitive behavioural therapy
CRF	Corticotrophin-releasing factor
CS	Central sensitisation
CSI	Cardiac sympathetic index
CVT	Cardiac vagal tone
DAB	Diaminobenzamine
DIS	Dilated intercellular spaces
DRG	Dorsal root ganglion
ECF	Epirubicin, Cisplastin and 5-Flurouracil
ECG	Electrocardiogram
EO	Erosive oesophagitis
FGID	Functional gastro-intestinal disorders
FH	Functional heartburn
GI	Gastro-intestinal
GORD	Gastro-oesophageal reflux disease
GSK	Glaxo-Smith-Kline
HCL	Hydrochloric acid
H&E	Heamatoxylin and eosin
HIV	Human immunodeficiency virus
HPA	Hypothalamo-pituitary axis
IBS	Irritable bowel syndrome
IF	Immunofluorescense
IHC	Immunohistochemistry
IMS	Industrial methylated spirit
IRTX	Iodoresiniferatoxin
KPa	Kilo-pascal
LOS	Lower oesophageal sphincter
mA	Mili-amperes
MAP	Mean arterial pressure
mBP	Mean blood pressure

MHRA	Medicine and Health Regulatory Agency
mRNA	messenger ribonucleic acid
NERD	Non-erosive reflux disease
NGF	Nerve growth factor
NIHR	National Institute for Health Research
NK1	Neurokinin 1
NMDA	N-methyl-D-Aspartate
NO	Nitic oxide
NPFF	Neuropeptide FF
NPSF	Neuropeptide SF
NST	Nucleus solitary tract
NTS	Nucleus tractus solitarii
OGD	Oesophago-gastro-duodenoscopy
PAF	Platelet activating factor
PGP 9.5	Protein gene product 9.5
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Parasympathetic nervous system
PGE_2	Prostaglandin E_2
PS	Peripheral sensitisation
PT	Pain threshold
P2X3	P2X purinoreceptor 3
RT-qPCR	Real time quantitative polymerase chain reaction
REC	Reserarch ethics committee
SEC	Sustained (o)esophageal contraction
SNS	Sympathetic nervous system
SSRI	Selective serotonin receptor inhibitor
STAI-S	Spileberger trait anxiety index (State)
STAI-T	Spileberger trait anxiety index (Trait)
TLOSR	Transient lower oesophageal sphincter relaxation
Trk B	Tyrosine kinase receptor B
TRPV1	Transient receptor potential vanilloid 1
TRPV4	Transient receptor potential vanilloid 4
UKCRN	United Kingdom Clinical Research Network
UOS	Upper oesophageal sphincter
VAS	Visual analogue scale
VPH	Visceral pain hypersensitivity
VR	Vanilloid receptor

1 INTRODUCTION: ANATOMY AND PHYSIOLOGY OF THE OESOPHAGUS, THE PERCEPTION OF GASTRO-OESOPHAGEAL REFLUX AND THE ROLE OF VISCERAL PAIN HYPERSENSITIVITY

1.1 THE HUMAN OESOPHAGUS

The oesophagus is part of the gastro-intestinal tract, connecting the oral cavity to the stomach. Food, once chewed, tasted, lubricated in the mouth and confirmed as safe by the brain for ingestion, is transported into the stomach via the oesophagus. Within this passage, food is propelled into the stomach for digestion and disinfection by highly acidic gastric secretion. The length and tone of this muscular tube also allow gastric content to remain in place and not be easily regurgitated, even during vigorous physical activities. This allows nutrition to remain within the body and prevents corrosive digestive juices from contaminating the oesophagus itself as well as the mouth, teeth and vocal cords. Swallowing solids can even take place when one is upside down or in outer space, with peristalsis alone without the help of gravitational force. Gastric content can also be expelled retrogradely against gravity in vomiting and reflux. Although physically it appears to be just a simple muscular tube connecting the mouth and stomach, muscular composition and innervation are such that this tube can sense a multitude of stimuli, propel a food bolus inwards and outwards and form areas of high tone (sphincters) which contract and relax appropriately. All of these functions are only possible due to the extensive nerve supply, receptors and musculature arrangements within this complex organ. Many of the physiological roles of the oesophagus can be altered in pathological states, and to understand how these are altered in disease states.



1.2 ANATOMICAL CONSIDERATION

The oesophagus, although a single tube, can be divided into upper, middle and lower parts. This distinction is not just physical, but has different embryological bases which determine the anatomy of the organ including muscular composition, nerve innervation, vascular supply and drainage. The embryological origin of the upper oesophagus is from branchial arches 4 and 5[1], which form mainly the striated muscular components of the upper and middle oesophagus, while the lower oesophagus originates from mesenchyme of the somites[1], which forms the smooth muscle layers of the middle and lower oesophagus.

1.2.1 Muscle layers

The oesophagus measures 18-25 cm in length and is mainly a muscular organ[1]. The muscular composition of the oesophagus is mainly striated in the upper oesophagus, and as it progresses to the lower oesophagus, it becomes more mixed with smooth muscle. By the lower third of the oesophagus, it is mainly smooth muscle, composed of the outer longitudinal muscle and inner circular layer[2]. The longitudinal muscle is arranged in fascicular columns, which are more distinct in the upper oesophagus and they merge into a single sheet of muscle towards the distal oesophagus[1]. The circular muscle is arranged as a set of concentric circles and provides peristaltic contractions. Accessory bands of muscles connect the oesophagus to adjacent structures.

The two oesophageal sphincters – the upper oesophageal sphincter (UOS) and lower oesophageal sphincter (LOS) are muscular sphincters. The oesophagus maintains the separation of between different organs; proximally, the oropharynx and distally, the stomach. The UOS is mainly a functional sphincter, an area packed with highly sensitive nerves and reflexes that initiate swallowing and regurgitation. The LOS, on the other hand, is both a functional as well as an anatomical sphincter, and acts as a high pressure zone before the oesophagus forms the stomach. The high tone is maintained by constant smooth muscle contraction controlled by nervous plexi and neuro-hormonal factors. In addition, the anatomy of the LOS represents an area of thickened musculature, corresponding to the diaphragmatic ring, which enters the abdomen at an angle. These mechanisms form the LOS and prevent the reflux of gastric contents as well as allowing the entry of food bolus into the stomach when necessary.

1.2.2 Layers of the oesophagus

Although essentially a muscular tube, the oesophagus is organised into distinct layers, which allow for movements between the layers that are optimum for a dynamic tube. Within these layers, blood vessels, nerves complexes, receptors and connective tissues exist. The luminal surface of the oesophagus is lined by non-stratified stratified squamous epithelium[2], which is adapted to withstand abrasions. The epithelium is rich in receptors and sensitive nerves endings[3] that respond to a range of stimuli; therefore, an intact epithelium is essential in normal oesophageal sensation. The range of sensory biomarkers and proteins important in oesophageal sensation is discussed further in section 1.1.2.1: Peripheral sensitisation. Below the epithelium are the lamina propria and muscularis mucosae[4]. All three layers constitute the mucosal layer[4]. The submucose is mainly connective tissue and contains the sub-mucosal nerve plexus. The third layer is the muscular coat, consisting of inner circular and outer longitudinal muscles[1]. The anterior aspect of the oesophagus is protected by the serosa.

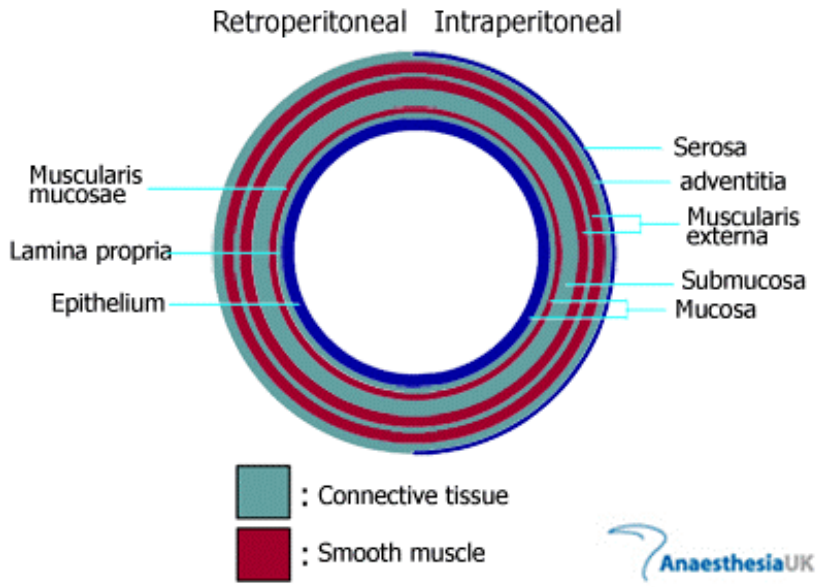


Figure 1.1: Different layers of the oesophagus including retroperitoneal and intraperitoneal aspects of the oesophagus. (Anaesthesia UK: training site of Royal College of Anaesthetics, UK)

1.2.3. Arterial supply

The arterial supply to the oesophagus is based on the division of its upper, middle and lower parts. Arterial supply to the upper oesophagus derives from the branches of the inferior thyroid artery, which originates from the subclavian artery[2]. The middle oesophagus is supplied by terminal branches of the bronchial arteries, while the lower oesophagus derives its blood supply from the left gastric artery[2]. However, the oesophagus is highly vascularised, with an extensive anastamotic network and collaterals. There is considerable overlap between territories.

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1.2.4 Venous drainage

The venous drainage of the oesophagus is less well demarcated by the three areas. The upper and middle parts of the oesophagus drain into the systemic venous circulation via the azygous system[2]. The lower third empties into the left gastric vein, which is part of the hepatic portal system[2]. This area of anastamoses between the systemic and portal system is where oesophageal varices arise from portal hypertension, in turn obstructing venous drainage from the distal oesophagus that drains into the portal vein. This causes pressure build up in the venous drainage of the distal oesophagus.

1.2.5 Innervation and control

1.2.5.1 Intrinsic nerve supply

Based on histological layers, as suggested by the existence of double muscular complexes (one more superficially at the muscularis mucosae, and the other deeper muscle complex with the two layers of muscle – inner circular and outer longitudinal), it is hardly surprising that there are also two separate plexi within the wall of the oesophagus. Associated within the submucosal layer is Meissner's plexus, which innervates the muscularis mucosae and secretory glands. Within the deeper muscular layer, between the longitudinal and circular muscle layers, is the myenteric Auerbach's plexus, which controls contractions of the circular and longitudinal layers. The myenteric plexus also exists in the striated muscle of the upper oesophagus, although its function there is less clear. Together, the submucosal (Meissner's) and myenteric (Auerbach's) plexi form the intrinsic innervation of the oesophagus. A network of fibres is believed to connect the two plexi[1]. The coordination of spontaneous peristalsis by the smooth muscle is controlled by the intrinsic system,

which is independent from extrinsic control.

1.2.5.2 Extrinsic nerve supply

A) Motor innervation

The main motor supply of the oesophagus is the vagus nerve, which supplies the upper and lower oesophagus. Branches supplying the upper oesophageal muscles and upper oesophageal sphincter come from the nucleus ambiguus, whereas the vagal efferents for the distal oesophagus and lower oesophageal sphincter originate from the dorsal motor neuron of the vagus nerve[1]. Although the vagus controls most of the motor function of the oesophagus, it is mainly a *sensory* nerve, with up to 85% of its nerve fibres being sensory[5]. This is discussed further below.

B) Sensory innervation

The sensory nerves of the oesophagus are less well studied to date compared to motor nerves. The sharpness of sensation in the viscera is also much less discrete compared to somatic sensation. However, sensory modalities that exist within the oesophagus are quite wide ranging including thermo-, chemo- and mechano-sensations. Sensory innervation can be divided into two systems, namely vagal afferents, which are mainly parasympathetic, and spinal afferents, which are predominantly sympathetic[6]. These systems share some similar activation pathways[7] and interact accordingly[6]. For example, the vagus system, had also been shown to reduce hyperalgesia by reducing the release of epinephrine from adrenals[8], as well as reducing sympathetically controlled neurogenic inflammation[9].

Vagus afferents

Vagal fibres are mainly unmylelinated C-fibres that have their cell bodies in the nodose ganglia before projecting into the nucleus solitary tract (NST). From the NST, these fibres form synapses with second-order neurons and project to the brainstem, hypothalamus, amygdala and cerebral cortex[5 10]. Vagal afferents are traditionally thought to mediate physiological sensations such as satiety and nausea[11]. However, related to its physiological motor function, the vagus nerve is also believed to be sensitive to mechanosensation[11]. Vagal afferents have receptors in the mucosa, which

respond to mucosal fine stroking as well as tension receptors in the oesophageal wall that are responsive to distension[12]. Smooth peristalsis requires both effective sensation of distension as well as contractions. Vagal afferents are also enriched with receptors responsive to polymodal intra-luminal stimuli[8] including osmo[13], chemo[14] and thermo[15] sensations.

Spinal afferents

The current understanding is that mucosal nerve endings, located in the lamina propria of the mucosa, sense intra-luminal stimuli[3]. These nerve endings are mainly spinal afferents whose cell bodies are found in dorsal root ganglia. From the spinal cord, they travel into the thalamus and primary sensory cortical areas. The distribution of nerve endings is thought to vary according to stimuli. Acid sensing nerve endings are believed to be superficial in the epithelium, whereas nerve endings deeper in the muscle and serosa are believed to be important for mechano sensation. Currently, many of the roles of these nerve endings can merely be speculated upon based on their anatomical locations. Systematic studies of oesophageal sensation have been difficult because the reporting of sensation is subjective and requires effective communication to the researcher. Animal studies have limited contribution and human studies are limited by difficulty in obtaining full thickness samples. As a result, many of the studies that have researched oesophageal sensation are controversial and inconclusive.

1.2.5.3 Autonomic Nervous System

As discussed above in motor supply, the predominantly parasympathetic[13] vagus provides motor innervation to the muscular layers as well as secretory function to the mucosal glands. The origins of PNS for the vagus are the nucleus ambiguous and dorsal motor nucleus. Sympathetic innervation of the oesophagus originates mainly from the thoracic sympathetic chain (T1-10), with the first thoracic ganglion frequently joined to the cervical ganglion to form the stellate ganglion[2]. The sympathetic system regulates vascular smooth muscle tone, and to a lesser extent the parasympathetic system, oesophageal contractile and secretory functions. Sympathetic activation had been traditionally believed to increase LOS tone and cause contraction[16] via the adrenergic system[17]; however, a cat study did not show the benefit of sympathetic modulation in influencing LOS function in combination with baclofen [18].

1.2.5.4 Central nervous system

The two main areas for oesophageal sensation within the brain, including pain processing, are the thalamus and cerebral cortex. The thalamus, located in the diencephalon at the dorsal end of the brainstem, forms the central core of the brain. It is an important centre for relaying and integrating important sensory and motor messages from the periphery to the cortical areas. It also integrates factors such as consciousness, attention, memory and emotions. The cortical areas of oesophageal sensation are the cingulate, insular, sensory, parietal occipital, and prefrontal regions based on human studies [19-21]. The significance of each area and cortical activity in relation to pain/acid sensation is discussed further in section 1.5.3.3: Cortical mechanisms.

Since the motor function of the oesophagus is mainly involuntary, much of the known information of the role that the central nervous system plays in its function pertains to sensation. However, there is limited evidence of motor representation of oesophagus in the cortex in swallowing studies. A sophisticated human study using transcranial magnetic stimulation and magnetic resonance imaging by Hamdy, *et al*. showed that swallowing musculature is discreetly and somatotopically represented in the motor and pre-motor cortex of both hemispheres asymmetrically – and not influenced by the dominant handedness. The loci for myolohyoid, pharynx and oesophagus were discreet, with the oesophageal locus predominantly in the pre-motor cortex[22]. Cortical swallowing motor pathways from each hemisphere interact and their excitability is modulated by sensory input[23]. For example, stimulation of afferent branches of the cranial trigeminal and vagus nerves have been shown to facilitate cortical swallowing pathways in the brainstem[24], which will be discussed further below.

1.3 PHYSIOLOGY AND FUNCTION

1.3.1 Swallowing

Swallowing is functionally divided into three stages, namely oral, pharyngeal and oesophageal reflecting the anatomical structures involved[23]. Functional activation of

these centres is divided further into two – cortical centres to initiate and modulate volitional swallowing[25 26], whilst the brainstem swallowing centre coordinates the sequence of reflex events in swallowing via cranial nerves (trigeminal, glossopharyngeal, vagus and hypoglossal)[26] to the protect airway and simultaneously generate coordinated muscle contraction and relaxation, leading to oesophageal peristalsis[23].

Peristalsis in swallowing is created by coordinated contraction and relaxation of the smooth muscle complex, all of which is coordinated by the cortex and swallowing centre. These contractions are also coordinated by local reflexes via the intrinsic plexi. The external innervation of the oesophagus, sensory, motor and ANS modulates peristalsis. The primary function of the oesophagus is to allow the ingestion of food and nutrients necessary for metabolism and growth in humans.

1.3.2 Prevention of Reflux

Related to the main function of swallowing and entry of food into the stomach, the lower oesophageal sphincter (LOS) also functions to prevent the reflux of gastric contents into the oesophagus. The LOS, as described above, is a muscular as well as a functional sphincter dependent on the maintenance of muscular tone and its angle. Any inability of the LOS to relax and contract appropriately would result in diseased states. Dysphagia and achalasia occur when the oesophagus and LOS fail to relax in a coordinated way to allow the entry of food. Gastro-oesophageal reflux disease (GORD) is associated with the spillage of gastric contents into the oesophagus. Many factors normally contribute to the prevention of this backflow from happening, and in GORD one or more of these mechanisms may fail (discussed below in: Pathogenesis of GORD).

1.4 GASTRO-OESOPHAGEAL REFLUX DISEASE

Gastro-oesophageal reflux disease (GORD) is a common condition. Estimates suggest that between 20 and 44% of Western populations having symptoms of GORD at least once a month and 20% weekly[5 6]. GORD can be divided broadly into erosive oesophagitis (EO) and non-erosive reflux disease (NERD). EO sufferers demonstrate signs of inflammation or superficial ulcers on endoscopy, but NERD patients do not

have any abnormalities. Of all the patients with GORD, it is estimated that between 50 and 70% have NERD[7 8].

1.4.1 Pathogenesis of acid reflux

1.4.2.1 Anatomical defects

Increased oesophageal acid exposure in GORD has several potential causes, some related primarily to physiological dysfunction and others to anatomic defects of the gastro-oesophageal junction, such as in a hiatus hernia. The importance of hiatus hernias in the pathogenesis of GORD is obscured by imprecise definitions that have led to wide variations in estimates of their prevalence across populations. Diagnosis could be made based on radiological or endoscopic methods. A hiatus hernia occurs when the stomach protrudes through the diaphragm into the chest cavity. In so doing, the LOS is compromised, and the reflux of gastric contents (including acid) into the oesophagus can occur easily. From an epidemiological standpoint, hiatus hernias and GORD share similarities in that both become more common with age and obesity. However, since the LOS is also functional sphincter, GORD and acid reflux could occur without the presence of anatomical distortion like a hiatus hernia.

1.4.2.2 Transient lower oesophageal sphincter relaxations (TLOSRs)

The LOS contracts and relaxes appropriately to allow the inward passage of food into the stomach for digestion, and prevents the reflux of gastric content. Even in the presence of normal anatomy, inappropriate and uncoordinated relaxation of the LOS can cause reflux. This phenomenon is referred to as 'transient lower oesophageal sphincter relaxations' (TLOSRs). In a study in human volunteers, gastro-oesophageal reflux was shown to be unrelated to low steady-state basal LOS pressure, but rather occurred during inappropriate transient relaxations[27]. TLOSRs mostly tend to occur after eating due to the distension of the proximal stomach[28 29], although they can occur spontaneously, too. It is thought to be a vagally mediated reflex from gastric mechano-distension[28 30]. A study in humans after fundoplication showed that TLOSRs were reduced, although gastric accommodation was not altered – indicating that the receptive field for TLOSRs was located within the region affected by fundoplication; i.e. proximal stomach[29] close to the oesophagus.

1.4.2.3 Motility and gastric emptying

Poor oesophageal clearance and high intra-gastric pressure have been implicated in increasing acid reflux, whilst ineffective oesophageal motility has been implicated in poor oesophageal acid clearance, especially in the supine position[31], together with reduced salivation[32]. These factors reduce the 'push' or forward factors for oesophageal clearance and the neutralisation of acid. Increased intra-gastric pressure or motility, on the other hand, prevent clearance and can therefore contribute backward pressure and increased reflux[27]. Cisapride, a prokinetic agent, has been shown in combination with Ranitidine to reduce the number and duration of reflux episodes in patients with oesophagitis[33]. In the same study, cisapride was also shown to enhance oesophageal clearance[33]. Although many of these factors may be relevant to nocturnal GORD symptoms while in the supine position, ineffective persistaltic clearance and TLOSRs were found to be most important and relevant[27] .

1.4.2.4 Sustained oesophageal contractions

Contractions of the longitudinal smooth muscle could occur spontaneously. These contractions are termed 'sustained oesophageal/esophageal contractions', more commonly known as SECs, which have been demonstrated to increase the wall thickness of the oesophagus[34], which corresponds to heartburn symptoms[35 36]. In most patients (70%), SECs precede heartburn symptoms and are reproduced in 75% of Bernstein positive subjects[35]. While this provides an explanation for symptoms associated with acid exposure, the mechanisms for symptoms remain speculative. Contractions may cause acid reflux or SECs may trigger pain by causing transient ischaemia or neuronal activation.

1.4.2 GORD symptoms

GORD symptoms can be very wide ranging, from the classic feeling of heartburn[37], indigestion and bloating to some very atypical presentations such as recurrent aspiration pneumonia[38] and tooth decay[39]. Heartburn is typically described as burning chest discomfort extending from the upper abdomen to the chest and sometimes to the jaw. As the name suggests, this symptom is often confused with cardiac chest pain. Regurgitation is another common symptom of GORD, often associated with an acidic taste in the mouth. When heartburn or acid regurgitation dominate the patient's symptoms, they have very high specificity but low sensitivity for GORD[37]. Other

common symptoms include feelings of sickness, bloating and an acidic taste in the mouth.

1.5 VISCERAL PAIN HYPERSENSITIVITY IN OESOPHAGUS

1.5.1 Role of visceral pain hypersensitivity in GORD

In patients with EO, inflammation and/or erosions are assumed to cause the symptoms. However, NERD patients exhibit evidence of abnormal acid exposure times but no evidence of erosions. In these patients, increased sensitivity to acid is suggested by a significantly shorter lag time and higher pain score to acid infusion in comparison to healthy volunteers[40], Barrett's patients and even EO. This also demonstrates that sensitivity to acid in fact is not dependent on erosive changes in the oesophageal mucosa. Furthermore, abnormal responses to the Bernstein test[41] have been demonstrated in the majority of patients who use antacids and report chronic heartburn but have normal acid contact time[41]. This suggests that these patients are hypersensitive to the physiological reflux of acid. In addition, some patients have all the hallmarks and symptoms of GORD, but do not have objective evidence of abnormal acid exposure. These patients are classified as having functional heartburn (FH). Symptoms in FH are considered to be due to excessive sensitivity to physiological amounts of acid.

In both the presence and absence of inflammation, acid is the main triggering factor in symptom generation in GORD. The corrosive effects of acid can be sensed by sensory nerves and receptors in the oesophageal epithelium (Chapter 2). Damage and inflammation will also facilitate dilation of the intercellular spaces and exposure to acid of the sub-epithelial nerves, leading to further symptom generation. Repeated exposure of these nerves could lead to their sensitisation so that symptoms could occur with even minor or physiological reflux episodes; a phenomenon described as *visceral pain hypersensitivity (VPH)*. The following sections will deal with peripheral and central VPH mechanisms.

1.5.2 Peripheral mechanisms

Gastrointestinal pain is mediated by spinal visceral afferent fibres, with a probable important contribution from vagal afferent fibres.[42] When activated, mechanoreceptors and chemo-sensitive receptors (resident in mesentery, serosa and submucosa) depolarise Aδ- and C-fibres. The ability to transduce noxious mechanical, chemical or thermal stimuli into generator currents able to depolarise such fibres is a property of transducer channels such as transient receptor potential (TRP) channels. TRPV1, TRPV4 and TRPA1 channels have been shown to have a role in GI nociception, as have acid-sensing ion channels (ASICs) and $P2_x$ purinoceptors[43]. In the presence of tissue inflammation or injury, there is an up-regulation of pain transmission. The ability to enhance pain transmission to the brain in these situations is important, as heightened bodily awareness can alter behaviour, which in turn aids in the protection of injured sites and the promotion of healing. Research in somatic pain has suggested that both peripheral and central mechanisms can increase nociceptive transmission following inflammation or injury to tissues. Peripheral mechanisms include *peripheral sensitisation (PS)*, which is an inflammatory mediator-induced facilitation of nociceptor activity in peripheral tissues. *Peripheral sensitisation* causes pain hypersensitivity at the site of injury or inflammation, also known as primary hyperalgesia. Here, inflammatory products including bradykinin, histamine, 5HT, prostanoids, proteases and cytokines permit nociceptor firing at reduced thresholds.

Receptors that may be involved in PS are described below:

- TRPV channels – Transient receptor potential vanilloid receptors, or vanilloid receptors (VR). This is a group big of receptors. They are cation-selective ligand-gated ion channels and can be activated by different stimuli. The most studied is the TRPV1 subtype, also known as the capsaicin receptor. TRPV1 is expressed by most small sensory nerves and is sensitive to heat, hydrogen ions and capsaicin (and other endogenous capsaicin-like substances) [44]. Whilst immediate sensitisation is mediated by an increased probability of channel opening in response to these stimuli, increased levels of TRPV1 protein have also been observed in the mucosa of patients with some visceral hypersensitivity states, with or without evident inflammation[45] [46], including the oesophagi of patients with gastro-oesophageal reflux disease (GORD)[3]. Apart from acid, TRPV1 is also responsible for the sensation of heat from capsaicin. Most

patients with GORD also report sensitivity to hot drinks and spicy foods.

- Purine receptors – P2X Purinoceptors are ATP-dependent, ligand-gated membrane cation channels. There are currently seven subtypes known. In particular, P2X3 expression in the dorsal root ganglion is increased in rats with chronic oesophageal acid infusion[47].
- ASICs – These 'acid-sensing ion channels' are amiloride-sensitive, voltage-insensitive epithelial Na channels. Subtypes 1-3 are sensitive to acid and possibly mechanical stimuli. They have been shown to be upregulated in mucosal inflammation and stimulated by NGF and serotonin. One in particular, acid sensing ion channel 3 (ASIC3), as the name suggests, has been known to sense acid (fluctuations in pH) but is better known as an important mechanoreceptor[48] and nociceptor in end organs. It is known to be fairly ubiquitous in the central nervous system (CNS), and exists in sensory nerve endings[49]. However, the mechanism of action in its role as a sensory nociceptor has been linked to pH fluctuations[50 51 52] and inflammation in the oesophagus and stomach in animal studies. This will be discussed further in Chapters 2 & 3.

1.5.3 Central sensitisation

In normal circumstances, the presence of stimuli will activate the peripheral receptors as mentioned above. Action potentials would then be generated via activation of Na/K channels, and impulses will be sent to the spinal cord via peripheral afferent nerves. Repetitive stimulation or high intensity stimuli can cause a constant firing of action potential to the spinal cord[53]. Enhanced nociceptor input in turn activates intracellular signalling cascades within spinal dorsal horn neurones, leading to central sensitisation and amplified responses to noxious and innocuous inputs due to facilitated excitatory synaptic responses and depressed inhibition[54 55 56]. This facilitation is triggered by the pre-synaptic release of neurotransmitters and neuromodulators such as glutamate[57 58], substance P[59 60], brain-derived neurotrophic factors (BDNF)[61] and prostaglandins[62 63]. These neurotransmitters and neuromodulators activate ligand-gated ion channels (NMDA-receptor-glutamate)[60], metabotrophic receptors (metabotrophic glutamate receptor (mGluR)-glutamate and NK1-substance P[64]) and tyrosine kinase receptors (Tyrosine Kinase (Trk) B--BDNF[61]) and increase intracellular calcium via release from intracellular stores and calcium inflow[54] (Figure 1.2). Consequently, calcium-

dependent enzymes such as protein kinase A[65], protein kinase C [66] and tyrosine kinases are activated, leading to phosphorylation of the NMDA receptors[54]. This dramatically changes NMDA receptor kinetics and reduces its voltage-dependent magnesium block[66], thus augmenting its subsequent responsiveness to glutamate and increasing synaptic strength, enabling previously sub-threshold inputs to activate the cell[54 56 67]. This increase in gain alters receptive field properties and pain sensitivity, causing tissue hypersensitivity far beyond the site of injury.

In addition to producing central sensitisation, which occurs within seconds of appropriate spinal dorsal horn neurone activation, nociceptive input also generates an activity-dependent change in transcription in the dorsal root ganglion and dorsal horn neurones[54 56 67]. These changes occur in response to a complex mechanism involving both an increase and a modification of constitutively expressed genes, as well as the induction of novel genes [68 69] . This phenotypic shift results in allodynia (previously, non-nociceptive stimuli induced pain) as well as hyperalgesia (heightened sensitivity to a previously painful stimulus). These changes take hours to manifest but, when established, lead to long-lasting changes in stimulus response characteristics. Evidence that central sensitisation is a major component of somatic hypersensitivity has already been demonstrated in human somatic pain models[70]. In animal studies, direct electrophysiological recordings from spinal neurones also suggest that central sensitisation is important in visceral hypersensitivity [71 72 73]. However, until recently, similar studies in man were not possible due to a lack of suitable human experimental models and non-invasive techniques available to assess visceral afferent pathways.

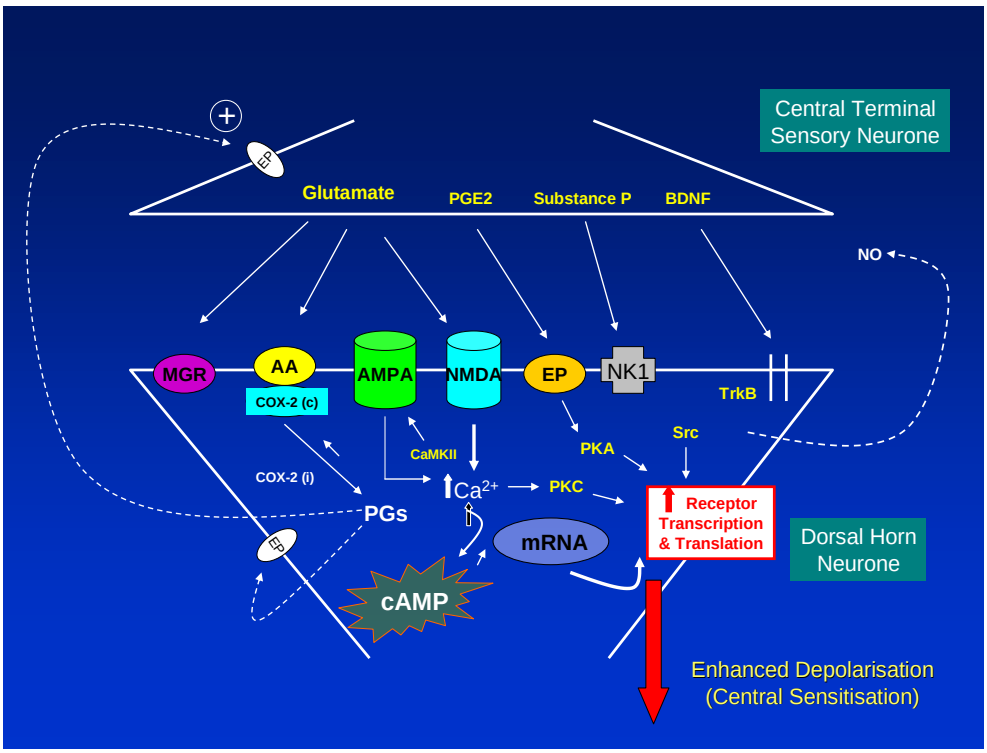


Figure 1.2: Shows receptor mechanisms underlying the induction of central sensitisation. The central terminals of primary nociceptors release glutamate, substance P, brain-derived neurotrophic factors (BDNF) and prostaglandin E_2 (PGE_2). Glutamate binds to ionotropic AMPA (Amino-Methylene-Phosphonic Acid), NMDA (N-Methyl-D-Aspartate) receptors and to metabotropic glutamate receptors (MGR). Substance P and BDNF bind to the G protein coupled neurokinin 1 (NK-1) receptor and the tyrosine kinase receptor B (TrkB), respectively, and PGE_2 binds to the EP1 receptor on the postsynaptic membrane. An increase in intracellular Ca^{2+} concentration triggers the activation of protein kinases A and C (PKA & C) and Ca^{2+}-calmodulin-dependent protein kinase (CaMKII). These kinases and tyrosine kinase Src phosphorylate the NMDA and AMPA receptors leading to a potentiation in activity. Nitric oxide (NO) is also produced, which has a positive feedback effect on presynaptic glutamate release. Central prostaglandin production is increased via arachadonic acid (AA) by cyclooxygenase-2 (COX-2) induction.

1.5.4 Human oesophageal models of peripheral and central sensitisation

Specific experimental models of VPH in the human oesophagus in reaction to acid infusion have been described. Sarkar, *et al.*, using either saline or acid infusion in a double blind randomised manner on the distal oesophagus, demonstrated a drop in pain threshold to electrical stimuli within the exposed distal and non-exposed proximal oesophagus after acid but not the saline infusion in healthy volunteers [74] (Figure 1.3). In addition, a decrease in patient threshold was also demonstrated on the anterior chest wall after oesophageal acidification. Although the primary hyperalgesia/allodynia at the site of infusion is believed to be due to PS, the secondary hyperalgesia/ allodynia in the proximal oesophagus and anterior chest wall, distant from the acid infusion, is believed to be due to the sensitisation of spinal dorsal horn neurones[74 75 76].

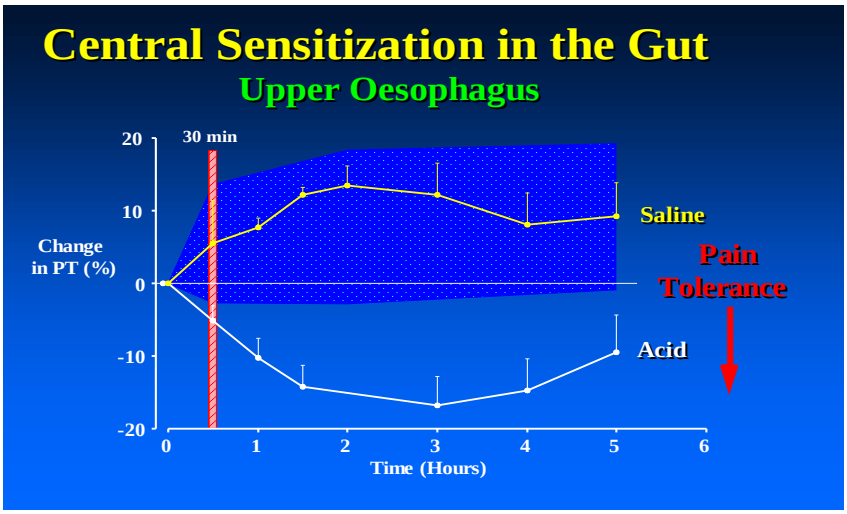


Figure 1.3: Shows the change in pain threshold on the upper oesophagus after acid/saline infusion in the lower oesophagus. (Reproduced from Sarkar et al., Lancet 2000)[74]

Sarkar, *et al*. demonstrated that in patients with gastro-oesophageal reflux disease, oesophageal pain hypersensitivity to experimental acid infusion can be reversed by acid suppression with proton pump inhibitors [77]. The mechanism of this reduced sensitivity to acid may be related to the fact that treatment of acid reflux removes peripheral afferent input to the spinal cord, which may in turn reduce the degree of resultant central sensitisation. Patients with functional heartburn have also been speculated to have heightened sensation to other peripheral inputs, such as bile reflux [78]. However, pain sensitivity to oesophageal chemical stimuli is far from understood, especially when one considers that even in patients with documented acid reflux in pH studies, the majority of events are not perceived [79].

Reproducibility studies with the acid-induced VPH model have demonstrated variations in the magnitude of sensitisation between subjects[80]. Recently, state anxiety was implicated as a factor that may modulate the degree of sensitisation to oesophageal acidification within this model[81] [82]. It can be speculated that psychological state modulates pain sensitivity and the degree of central sensitisation in response to injury by moderating descending spinal inhibitory and facilitatory influences.

Drewes, *et al*. demonstrated that the induction of pain hypersensitivity to acid infusion is also associated with enhanced oesophageal contractions[83]. The same group also demonstrated that acid sensitisation of the oesophagus made it more sensitive to heat rather than cold[84], which may be related to the peripheral up-regulation of receptors such as TRPV1. Other variations to the acid perfusion model were explored and gender differences observed. For instance, females were shown to report a wider anatomical area of pain referral over the anterior chest wall after acid infusion in comparison to males, whereas males reported increased sensitivity to chemical and mechanical stimuli[85]. This is important in the clinical context in explaining gender differences in reporting of symptoms. It has also been demonstrated that sensitisation can occur in different areas of the gut after acid-induced sensitisation of the distal oesophagus. The oesophagus, duodenum and rectum were all more sensitive to mechanical distension after acid sensitisation rather than saline[86]. As the innervation of these organs overlaps at the level of the spinal cord, it is likely therefore that this

cross-sensitisation is related to central sensitisation of spinal dorsal horn neurons caused by the acid infusion.

1.5.5 Cortical mechanisms

A major limitation of most visceral hypersensitivity studies is that they rely on subjective methods of reporting sensation intensity[87]. To overcome this issue, a commonly used neuro-physiological technique, cortical evoked potentials (CEPs), has been adopted for use as a more objective correlate of oesophageal sensation. CEPs allow the recording of cortical neuronal electrical fields generated in response to peripheral nerve stimulus. Signal averaging of cortical electrical activity up to 200 oesophageal stimuli is conducted to generate an optimal signal-to-noise ratio, and a temporal pattern of cortical activation is obtained. As a result of the excellent temporal resolution of this technique (1millisecond) it is possible to study the conduction velocity of afferent neuronal transmission from the oesophagus to the cortex. Using this technique before and after oesophageal acid infusion, a consistent reduction in CEP latency to oesophageal electrical stimulation has been described, which demonstrates that the facilitation of afferent pathway conduction accompanies the CS (Figure 1.4) [88]. In a recent study in NERD and functional heartburn patients[89], there was a correlation between pain threshold and acid exposure, with increased oesophageal sensitivity being associated with a lower DeMeester score. Thus, reflux-negative (functional heartburn) patients had lower pain thresholds compared to both reflux positive patients and controls. Cortical-evoked potentials were normal in reflux-negative patients, but significantly delayed in the reflux-positive group. This suggests that increased oesophageal pain sensitivity in functional heartburn patients is associated with heightened afferent sensitivity, as normal latency evoked potential responses could be elicited with reduced afferent input. Similar differentiation in the afferent response using cortical evoked potentials has also been shown in subgroups of patients with non-cardiac chest pain[90].

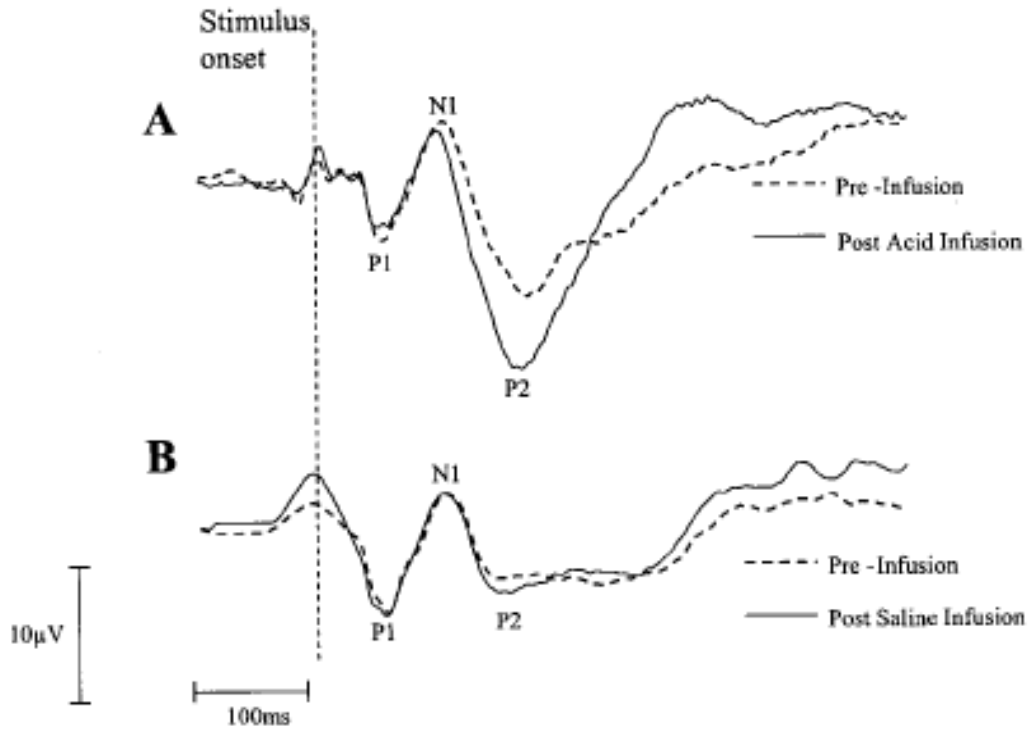


Figure 1.4: Shows increased proximal oesophageal afferent pathway sensitivity following distal oesophageal acidification. Distal oesophageal acid infusion is associated with a reduction in the latency of the evoked potential response from the non-acid exposed proximal oesophagus. No change in latency occurs following saline infusion. These results support the involvement of central sensitisation in the mediation of VPH. (Reproduced with permission from Sarkar S, Hobson AR, Furlong PL, Woolf CJ, Thompson DG, Aziz Q. Central neural mechanisms mediating human visceral hypersensitivity. Am J Physiol Gastrointest Liver Physiol 2001;281(5):G1196-202)[91].

Functional Magnetic Resonance imaging has also been used to study the brain processing of acid-induced oesophageal hypersensitivity. Lawal, *et al.* studied brain processing to mechanical stimulation of the proximal oesophagus following the infusion of acid or control buffer solution into the distal oesophagus[20]. Following distal oesophageal acid infusion, both subliminal and conscious levels of proximal oesophageal distensions caused a significant increase in brain activity in both the cingulate and the insular cortices in comparison to the control buffer solution[20]. This suggests the development of acid-induced sensitisation of the oesophagus to

mechanical distention and indicates that this sensitisation is accompanied by increased activity in brain areas processing both sensory (insular cortex) and cognitive (cingulated cortex) aspects of sensation.

In a recent fMRI study in GORD patients[92], acid-induced heartburn was associated with an increase in activity in the sensory/motor, parieto-occipital, cingulate and prefrontal regions, as well as the insula. Activation of similar areas was also observed in healthy subjects not experiencing heartburn to the administered acid infusion. However, activity in these regions occurred more rapidly and with greater intensity in GERD patients than in healthy controls in response to acid exposure, which suggests the presence of heightened afferent pathway sensitivity in the patients.

1.5.6 Autonomic nervous system

The role of the ANS in modulating oesophageal sensitivity has also been demonstrated. For instance, increased basal sympathetic activity and lower vagal activity, as measured by the power spectral analysis of heart rate variability, are associated with increased sensitivity to oesophageal acid perfusion in patients with NCCP compared with healthy controls[93]. In addition, the heightened sensitivity to oesophageal acid infusion in GORD patients, as compared to healthy controls, has been shown to be associated with reduced vagal tone during infusion[94]. These data support the concept of neuro-humoral influences on the susceptibility to symptoms such as heartburn.

1.5.7 Stress and hormones

Animal studies have shown that stress (early maternal separation) induces visceral hyperalgesia, colonic dysmotility and anxiety-like behaviour[95 96]. Stress could affect VPH by modulating ANS as described above. In addition, stress is also associated with activation of the hypothalamic pituitary axis, while the Corticotrophin-releasing factor (CRF) is believed to play an important role[96] in altering the physiology, permeability and secretory functions of the gut[97] during stress. Induction of stress is associated with increased paracellular permeability and dilated intracellular spaces (DIS) in the oesophagus[98]. A previous link between DIS with NERD[99] and the development of symptoms of acid reflux[100 101] has also been demonstrated.

Furthermore, stressful situations often alter cognitive interpretation of stimuli and alter pain reporting[102].

1.6 RESEARCH RELEVANCE

The perception of symptoms in GORD is a multi-factorial process. The origin of symptoms in GORD is obviously acid in the oesophagus. Acid is corrosive and the normal human oesophagus can sense acid but is also very resilient to it. While in most individuals intermittent acid exposures can cause the occasional heartburn that we all experience from time to time, in some individuals VPH can be triggered due to peripheral and central sensitisation of primary afferent and spinal dorsal horn neurons, causing more persistent and chronic symptoms. The future treatment of GORD patients' symptoms requires comprehensive understanding and profiling of all the factors influencing VPH including ANS dysfunction, emotions, stress and cognition, all of which are current areas of research interest.

1.6.1 Gaps in Knowledge

Within the VPH model, many individual pathways and interactions still need to be explored further, as outlined below.

Research agenda in VPH

1. *Molecular markers* of afferent receptor sensitisation in oesophageal mucosal biopsies need to be identified.
2. The use of neurophysiological techniques to *identify subgroups* of patients with true afferent nerve hypersensitivity or psychological causes needs to be explored.
3. The role of *stress and autonomic nervous system* in mediating oesophageal peripheral and central sensitisation needs elucidation.
4. Treatment with anti neuropathic pain *drugs* needs to be explored in patients with oesophageal pain hypersensitivity refractory to acid suppression.

Specifically, my studies aim to explore and fill in gaps on issues in VPH that are *clinically relevant*. For peripheral mechanisms, there are still many uncertainties surrounding biomarkers and nerves associated with acid induced symptoms in humans (Chapters 1 & 2). For central mechanisms, human models have been used to induce VPH, but the reproducibility and problems with this model have not been systematically studied (Chapter 4), while a practical oral agent and how it could prevent VPH has never been found (Chapters 5).

1.6.2 Cohort development

The identification of both PS and CS biomarkers is important to help identify and classify patients where these mechanisms are *predominant*. This is important in furthering our understanding of the development of symptoms in patients with acid reflux, which is different from inflammation or acid exposure per se.

1.6.3 Therapeutic potential

With the identification of these biomarkers or pathways, it might be possible in some cases to alter the role of these factors in the development of symptoms. They can also be exploited as investigative tools. As mentioned, many of the changes within VPH have evolutionary or protective roles; therefore, potential modulation needs to be done with care. This is only possible if we further our understanding in PS and CS in the context of VPH.

2 DISTRIBUTION OF PERIPHERAL MARKERS AND NERVE FIBRES IN FULL THICKNESS SAMPLES OF HUMAN OESOPHAGUS

2.1 INTRODUCTION

To further explore the role of specific neuronal afferent receptors in acid-mediated oesophageal visceral pain hypersensitivity (VPH), in-vitro studies of full-thickness human oesophageal samples permit the distribution of these receptors in different layers of the oesophagus to be determined. These experiments form the basis of the selection of suitable mucosal biomarkers for subsequent studies (Chapter 3).

2.1. 1 Aims and objectives

1. To determine the distribution of neuronal elements (ganglia and nerve fibres) in surgically excised full-thickness oesophageal samples;
2. To determine the distribution of protein expression for selected neuronal receptors (TRPV1, TRPV4, ASIC3) in surgically excised full-thickness oesophageal samples.

2.1. 2 Rationale

Little is known of oesophageal afferent innervations in comparison with other gut regions, even in rodent species, with particular difficulties obtaining full-thickness oesophageal tissue, which hinders studies in this area and the distribution of neuroreceptors in humans. In contrast, mucosal biopsies are readily available in humans at upper GI endoscopy. Such biopsies have value in the diagnosis of epithelial diseases such as cancer, dysplasia and metaplasia (Barrett's oesophagus)[103 104] and can also provide information on the presence of inflammatory cells in the context of GORD[105]. In other areas of the GI tract, notably the small and large intestine, mucosal biopsies have been used to provide information on the neurochemistry of terminal processes in relation to submucosal/myenteric intrinsic and extrinsic afferents [106 107 46 108]. Studies of visceral pain have frequently sought increases in the expression of key

neuronal proteins involved in damage-sensing, on the basis that these are upregulated to induce a phenotypic switch during sensitisation[109]. Central to this premise are observations from basic studies of the somatic nervous system of rodents, where regulation of cation channels by nerve growth factor (NGF) is conferred by axonal transport to the periphery from the DRG [110] [111] [112] [113] [114]. Such mature proteins might thus be found in increased quantities in biopsies that contain nerve endings from visceral primary (spinal) afferents. Although less studied than elsewhere, other scholars have demonstrated that small fibres in lamina propria papillae (including those obtained by endoscopic biopsy) can be immunostained for TRPV1 in the oesophagus [3]. These studies did not, however, use full-thickness tissue to study the broader distribution of such proteins, which we deem necessary to determine accurately the size, depth and number of biopsies as well as the exact methods of protein quantification that would subsequently be required for future biopsy studies as part of the overall thesis.

2.2 SUBJECTS AND METHODS

2.2.1 National research ethics approval process

Approval for the experiments in Chapters 2 and 3 was granted by the East London and the City Research Ethics Committee III (submitted 19th July 2007). Dr Chua and Mr CH Knowles attended the interview on 19th July 2007. Provisional approval was given subject to minor amendments. Final approval was awarded 27th November 2007: REC: 07/H0705/67. Barts and the London NHS Trust Joint Research Office acted as sponsors. Data were constantly updated on the National Institute for Health Research (NIHR) via the UKCRN (UK Clinical Research Network) website. Further details on the ethical approval process are attached in Appendix 2.01.

Research samples were coded and anonymised at source. A link to clinical details was kept by the investigator in a secure folder and as a hard copy only.

2.2.2 Subjects

Ten subjects undergoing oesophagectomy for oesophageal adenocarcinoma (n = 9) and benign stricture (n = 1) were recruited, all of whom gave written consent for their involvement in the study (Table 2.1). All were male, aged 48-80 (mean 65) years. Eight out of the nine cancer patients had received neo-adjuvant chemotherapy. For oesophageal adenocarcinoma, the main combination chemotherapy was Cisplastin and 5-Flurouracil, along with gastro-oesophageal adenocarcinoma with ECF (Epirubicin, Cisplastin, and 5-Flurouracil). Chemotherapy could have had a significant effect on epithelium and enteric neurones but it was necessary to use these samples; as it was only in this situation I could realistically obtain full-thickness human oesophageal samples (limitations of these samples discussed in section 2.4.2). Surgery was performed 4-6 weeks after the end of chemotherapy. The tumour stages and histological grades are listed in the table below. No subject withdrew consent during the study.

Sex	***Age***	***Chemo-therapy***	***Reflux***	***Barrett's***	***Diagnosis***	***Stage***	***Histology Grade***
M	48	No	No	No	Benign stricture	N/A	N/A
M	74	Yes	No	Yes	Adenocarcinoma	T3N1	Poorly differentiated
M	71	Yes	Yes	Yes	Adenocarcinoma	T1N1	Well differentiated
M	69	Yes	No	No	Adenocarcinoma	T2N3	Poorly differentiated
M	59	Yes	No	No	Adenocarcinoma	T2N0	Poorly differentiated
M	57	Yes	No	No	Adenocarcinoma	T1N0	Well differentiated
M	69	Yes	Yes	No	Adenocarcinoma	T3N1	Poorly differentiated
M	65	Yes	No	No	Adenocarcinoma	T2N0	Poorly differentiated

M	61	Yes	No	No	Adenocarcinoma	T3N0	Poorly differentiated
M	80	No	No	Yes	Adenocarcinoma	T2N0	Poorly differentiated

Table 2.1: Clinical and tumour details of the 10 oesophagectomy samples

2.2.3 Tissue acquisition and fixation

Surgical samples were collected immediately after resection from theatre and transferred directly to the Department of Morbid Anatomy, Barts and the London Trust. Whole specimens were examined by a Consultant Pathologist with sub-specialist expertise in GI pathology (Dr Joanne Chin-Aleong). Following gross inspection, an area at the margin of the specimen was identified for research and summarily sharp dissected from the main specimen (avoiding histological diagnostic compromise). The samples were invariably distal oesophago-gastric samples from laparoscopic assisted oesophagectomy. The full-thickness oesophageal samples were taken from the proximal margin of the resected samples and were therefore mainly distal oesophageal samples. The distal margin would constitute gastric border. Two blocks were created from each specimen by division with one half snap-frozen in liquid nitrogen and stored at -80 C and another paraffin fixed (methodology below).

2.2.4 Tissue preparation

Formalin fixation was performed in 10% neutral formalin for at least 24 hours, with the specimen orientated and pinned mucosa-up on a small piece of cork to avoid contraction. Fixed samples were then embedded in paraffin cassettes by the laboratory technicians and stored for future sectioning. (For the protocol regarding paraffin fixation, please refer to Appendix 2.03). Three to five micron sections were cut using a standard microtome on request. Slides were cut by laboratory technicians and incubated in 37 ºC before immunohistochemistry staining.

2.2.5 Control tissues

Formalin-fixed, paraffin wax-embedded human colon, kidney and liver samples, as well as rat cerebella and dorsal root ganglia (DRG), were obtained from the Pathology

group, Blizard Institute of Cell and Molecular Science (BICMS), Queen Mary University, London control tissue bank.

2.2.6 Immunohistochemistry methods

2.2.6.1 Background to method

Immunohistochemistry (IHC) is a scientific method for detecting antigenic proteins in cells and tissues, using labelled antibodies as reagents for the optical detection and localisation [115]. As such, it is an indirect method of antigen detection and the specificity of antibody binding to the chosen epitope is paramount. Immunohistochemistry involves a number of steps. Samples collected are often 'fixed' (e.g. paraffin fixation) or frozen. 'Unmasking' of antigens which may be hidden by fixation process (e.g. formalin cross-links) with antigen retrieval is the necessary first step. A number of techniques usually involving heating and alkaline treatment have been described. Although the actual molecular mechanisms underlying the processes for antigen retrieval are not known, antigen retrieval methods are thought to reverse protein modification inflicted by formalin during the fixation process [116].

Following antigen retrieval, all samples are blocked with normal horse serum to provide a non-specific peptide block. In some cases, specific serum compatible to the choice of secondary antibody should be used. An additional avidin/biotin block is used if the positive control happens to be derived from the kidney or liver, each of which contains endogenous biotin[117]. After the blocking steps, the specific primary antibody is applied and incubated with the tissue. Excess antibody matter is washed prior to the application of a species-specific secondary antibody, which is conjugated to a tag such as biotin or a fluorophore. This secondary antibody complex is applied and binds to the primary antibody before the application of a detection reagent containing a chromagen such as diaminobenzidine (DAB) or fluorophore that binds to the antibody conjugate. Gill's haematoxylin provides a contrasting counter-stain of nuclei and cellular structures to give strong colours that aid microscopic visualisation. Primary antibody specificity should be checked using positive control tissue that is known to contain the antigen. Negative controls, where the primary antibody is omitted, should

also be included in each experiment[118]. The compositions of all solutions and reagents are detailed in Appendix 2.02, and kits are now available to simplify methods.

2.2.6.2 Indirect immunohistochemistry kit-based system

Indirect immunohistochemical staining was performed using a kit-based system. Initially, this was known as the ABC kit (Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, California, USA); however, the same company updated the kit to a newer version, known as the 'RTU kit' (R.T.U. Vectastain Universal Elite: Vector Laboratories, Burlingame, CA, USA), which is also an avidin-biotin-peroxidase complex method.

In brief, tissue sections were de-waxed in two changes of xylene for 2 minutes and dehydrated in two changes of industrial methylated spirit (IMS) for another 2 minutes. After washing in running tap water for 5 minutes, thermal antigen retrieval was carried out by microwaving or water bathing sections in a citrate buffer (Vector Laboratories, Burlingame, California, USA) for a duration specified in specific optimisation schedules. Sections were allowed to cool for 5 minutes and washed again in running tap water for 5 minutes. Endogenous peroxidase activity was blocked by placing sections in methanol containing 3% H_2O_2 for 15 minutes. After further washing in running tap water for 5 minutes and soaking in the wash buffer (Dako, Cambridgeshire, UK) for 2 minutes, the sections were then incubated in normal horse serum (supplied in the kit) for 20 minutes at room temperature. Excess blocking serum was tipped off and the sections were incubated with primary antibodies in a dilution specified in the optimisation schedule, usually for 40 minutes at room temperature, unless otherwise stated. Sections incubated with antibody diluent served only as negative controls. Primary antibodies were washed off with wash buffer and the sections incubated with secondary biotinylated antibodies from the kit (Universal secondary antibody-anti rabbit and mouse, Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature. The secondary antibody was washed off with wash buffer and sections incubated in avidin complex solution (in the kit) for 30 minutes at room temperature, washed again with wash buffer and then incubated in diaminobenzidine (DAB) solution (Bio-Genex-Laboratories, San Ramon, California, USA) for a further 5 minutes. After further washing in running tap water

for 5 minutes, the sections were counter-stained in Gill's haematoxylin for 2 minutes, washed in running tap water for 5 minutes, dehydrated twice in alcohol for 2 minutes, cleared twice in xylene for 2 minutes and mounted in Canada balsam (VWR International, Leicestershire, UK).

2.2.7 Visualisation of immunostaining (microscopy)

All 10 samples were examined microscopically on all antibodies. Sections were analysed at magnifications of X10, X25 and X40 using a light microscope (Leitz Dialux 20, Leica Microsystems UK Ltd., Milton Keynes, UK). The presence and distribution of immunostaining in epithelial cells, nerve fibres and ganglia were noted, and a representative area for each gut region was photographed with a digital camera using the Leica IM50 Image Manager software (Leica Microsystems, UK). All specimens were reviewed by me and independently by another doctoral student (Dr Andrew Hubball). They were randomly selected to be examined by a post-doctoral research fellow (Dr Madusha Peiris) to check the findings.

2.2.8 Antibodies

2.2.8.1 Generic neuronal markers

In order to map the distribution of neuronal endings and cell bodies in the full thickness oesophageal tissue, the generic nerve marker protein gene product 9.5 (PGP 9.5) was used. PGP 9.5 is a member of the ubiquitin hydrolase family of proteins known to be confined to neural and neuroendocrine cells[119]. PGP 9.5 can be used to gain a measure of the total neural count for subsequent determination of functional subsets of neurons using other expressed proteins[120].

Synaptophysin is a glycoprotein present in the membrane of neuronal pre-synaptic vesicles in the brain, spinal cord and retina, adrenal medulla vesicles, neuromuscular junctions and endocrine cells[121 122 123]. It is thus employed in IHC to label synapses[124 125]. The optimisation schedule for these two antibodies had already been largely determined by laboratory staff. Details of the antibodies and concentrations are shown in Table 2.2.

Antibody	*Kit & Dilution*	*Positive control*	*Clonality*	*Antigen retrieval*
PGP 9.5, Serotec, Kidlington UK	RTU kit 1:2000	Rat cerebellum	Polyclonal rabbit anti-human	- microwave 10 minutes
Synaptophysin, Abcam, Massachusetts, USA	ABC kit 1:200	Rat cerebellum	Polyclonal rabbit anti-rat	- microwave 25 minutes

Table 2.2: Generic neuronal antibodies and optimisation schedule

2.2.8.2 Specific neuronal receptors

Within the full thickness human oesophagus, the ubiquity, specificity and relationship between these markers and nerves needed to be explored. Apart from the relevance of determining the overall distribution, the feasibility and reproducibility of these receptors in small and superficial biopsy samples needed to be validated for subsequent studies. Immunostaining was performed for TRPV1, TRPV4 and ASIC3. A schedule of all antibodies is shown in Table 2.3.

TRPV1

Significant problems were encountered for TRPV1 in respect of unreliability and poor specificity of antibodies with high variability between manufacturers and production batches. In total, four different commercially available antibodies were tested. Despite assistance from more experienced scientists, it was impossible to stain reliably using TRPV1 (see discussion), with the exception of a single batch of antibody from ThermoRicher Leicestershire, UK, which specifically stained the positive controls – human DRG (selective staining of small neurons) and colonic neurons (as previously published[46]) – and produced a band at 95KPa (as predicted)[126 127] using Western blotting (Appendix 2.07). A subsequent antibody from the same commercial supplier failed to yield specific staining in the oesophagus, and on subsequent retesting as above failed also on controls and blotting. Immunostaining using the GSK, Abcam

and Neuromics TRPV1 antibody is included preliminarily, but the results are critically discussed below. A schedule of antibodies is shown in Table 2.3.

Antibody	***Kit & Dilution***	***Positive control***	***Clonality and Immunogen***	***Antigen retrieval***
TRPV1 GSK	ABC kit (IF) 1:1000, 24 hr	Human colon/ Rat DRG	Rabbit anti-human TRPV1	- microwave 25 minutes
TRPV1 Abcam Massachusetts USA	ABC kit (IF) 1:3000	Human colon/ Rat DRG	Polyclonal rabbit anti human (amino acid 602 076 TRPV1)	-microwave 25 minutes
TRPV1 Neuromics, Minneapolis, USA	ABC kit (with species specific secondary antibody) 1:8000, 24 hr	Human colon/ Rat DRG	Polyclonal rabbit anti-rat (amino terminus of TRPV1)	- microwave 25 minutes
TRPV1 ThermoFisher Leicestershire, UK	RTU kit 1:500	Human colon/ Rat DRG	Polyclonal rabbit anti-human (TRPV1)	- microwave 10 minutes
Only one batch of ThermoFisher antibody produced a band at the predicted range in Western blotting with good neuronal staining on positive control (DRG and colon). The Western blotting band is attached in Appendix 2.07 for reference.				

Table 2.3: TRPV1 antibody optimisation schedule for four separate antibodies

TRPV4

Recommended optimisation of TRPV4 was available in the laboratory, but my own run of optimisation of this antibody was conducted on a human colon prior to use in my research samples.

ASIC3

Recommended optimisation of ASIC3 was available in the laboratory using a dilution of 1:25, but my own run of optimisation of this antibody, conducted prior to use in the samples, confirmed a dilution of 1:50. The manufacturer recommended a human kidney as a positive control, so using this dilution we achieved a good comparable standard of positive control. As this dilution was considered high, the manufacturer was contacted. Staining of the positive control was comparable, but with different laboratory protocols the final dilution used varied.

Antibody	***Kit & Dilution***	***Positive control***	***Clonality and Immunogen***	***Antigen retrieval***
TRPV4 Millipore (Chemicon), Massachusetts, USA	ABC kit 1:20 RTU kit 1:50	Human liver	Polyclonal rabbit anti-rat (amino acids 853-871 of TRPV4)	ABC- microwave 25 minutes RTU- water bath 40 minutes
ASIC3 Abcam, Massachusetts USA	RTU kit 1:50	Human kidney	Polyclonal rabbit anti-human (N-terminal amino acids 73-122 of ASIC3)	- microwave 10 minutes

Table 2.4: Specific antibodies and optimisation schedule for TRPV4 and ASIC3

2.3 RESULTS

2.3.1 Histology of the oesophageal mucosa

H&E staining confirmed a thick squamous epithelial layer (approximately 35 cells thick), although there was variation between individuals and the thickness of the epithelium.

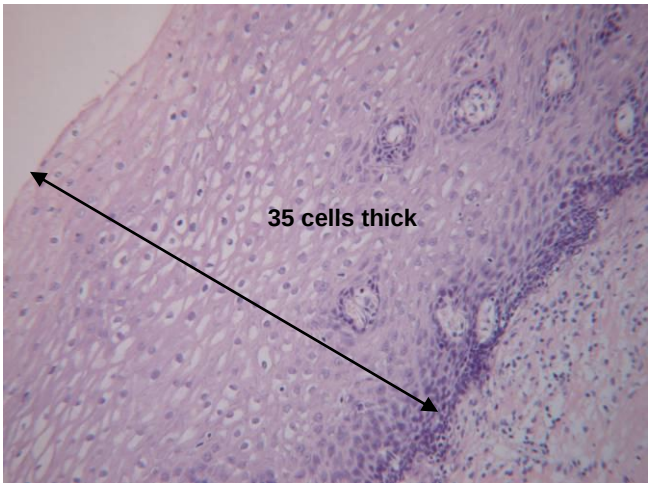


Figure 2.1: H&E staining of a representative oesophageal mucosa, demonstrating the epithelial layer and normal cellular morphology. Magnification X25.

2.3.2 Generic neuronal markers

2.3.2.1 PGP 9.5

Ganglia and neurons: Within the distal oesophageal myenteric layer, ganglia were clearly identified and well outlined by PGP 9.5 staining. Ganglionic density was measured at a median 6 per 5mm^2 (range 3-11 per 5mm^2), more than previous reports in 1992 of 2.8 ganglia per 10mm^2 in the distal oesophagus[128], perhaps due to technical improvements. Our examination area was limited to 5mm^2 due to the limited length of tissues vs. total sample area. Individual cell bodies were easily identified with a median of 4 neurons per ganglion (range 2-6 neurons per ganglion), which was more consistent with previous reports of 5 neurons per ganglion[128].

Nerve fibres: PGP 9.5 stained numerous small nerves in the sub-epithelium. These extended into the epithelial papillae, but were absent from the epithelium. This pattern was observed in all 10 samples.

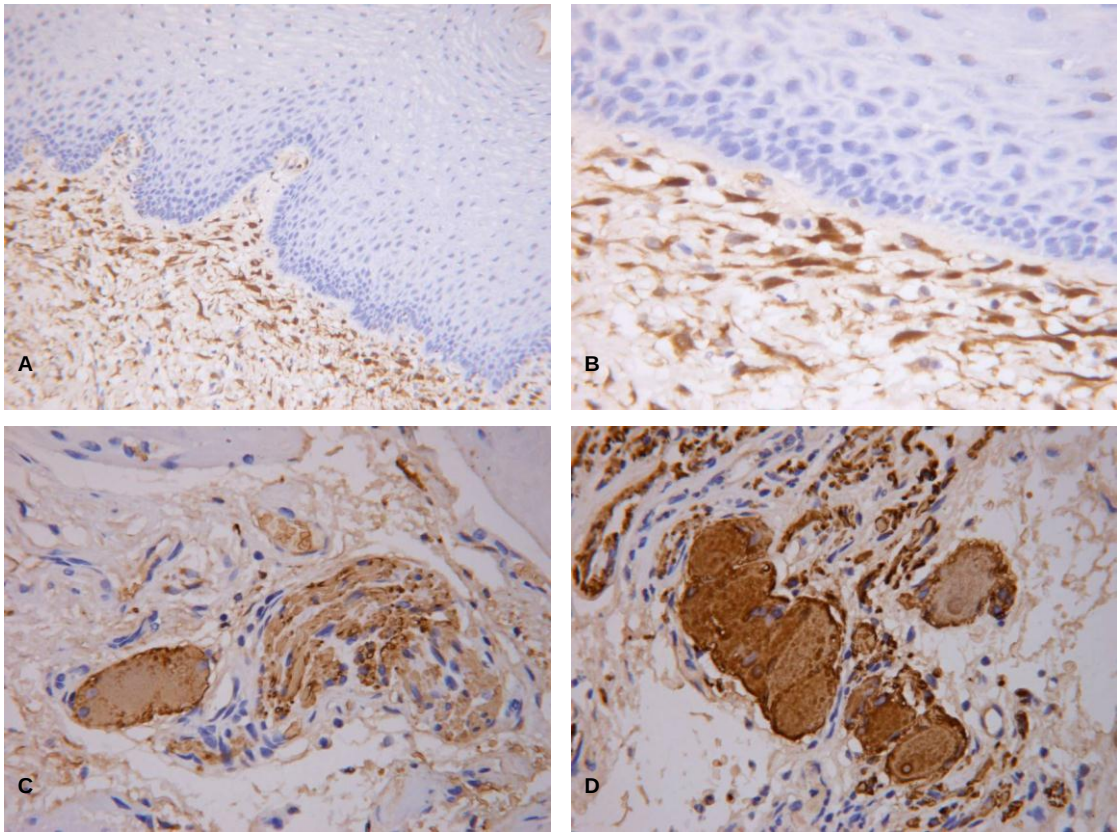


Figure 2.2: PGP 9.5 in a human oesophagus. (A) Nerve endings in lamina propria X10,(B) Neurons in lamina propria X25 (note the absence of nerve fibres in the epithelial layer). (C&D) Myenteric ganglia staining, both images X 25 magnification.

2.3.2.2 Synaptophysin

Synaptophysin stained abundant synapses around the neurons within the myenteric ganglia. Elsewhere they were not detected. Notably, as with PGP9.5, no staining was evident within the epithelium. These findings were again consistent for all 10 samples.

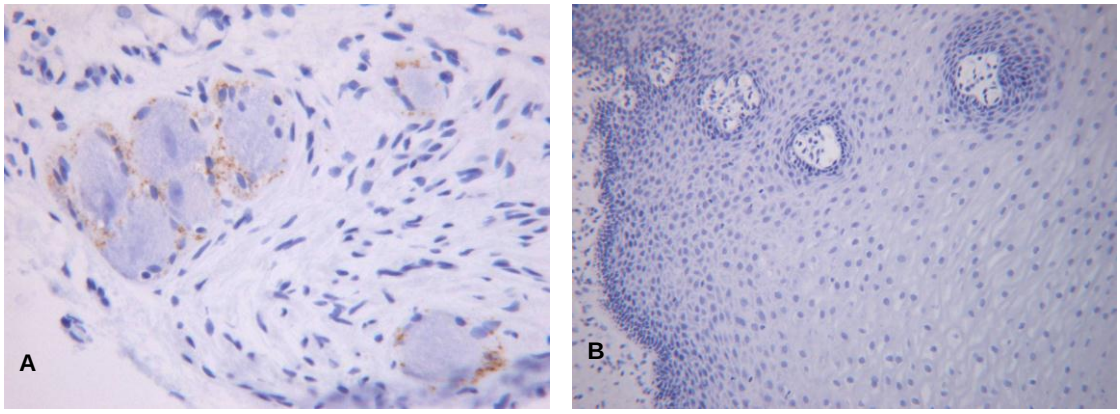


Figure 2.3: (A) Synaptophysin in human oesophagus, myenteric ganglia, magnification X 25. (B) Epithelium X10, with no synapses detected in the epithelial layer.

2.3.2 Specific neuronal receptors

2.3.2.1 TRPV1

Immunohistochemistry results for GSK, Abcam and Neuromics antibodies accepting unreliability are shown in Figure 2.4. In these results, neuronal TRPV1 was detected occasionally on the luminal surface of epithelium. Epithelial TRPV1, although more regular, exhibited a peculiar pattern in the superficial epithelium, which was not considered to represent specific immunoreactivity. Using the Neuromics antibody, we noted weak staining of neuronal cell bodies within the myenteric ganglia and also of the basal layers of the squamous epithelium, although this varied in intensity (Figure 2.5).

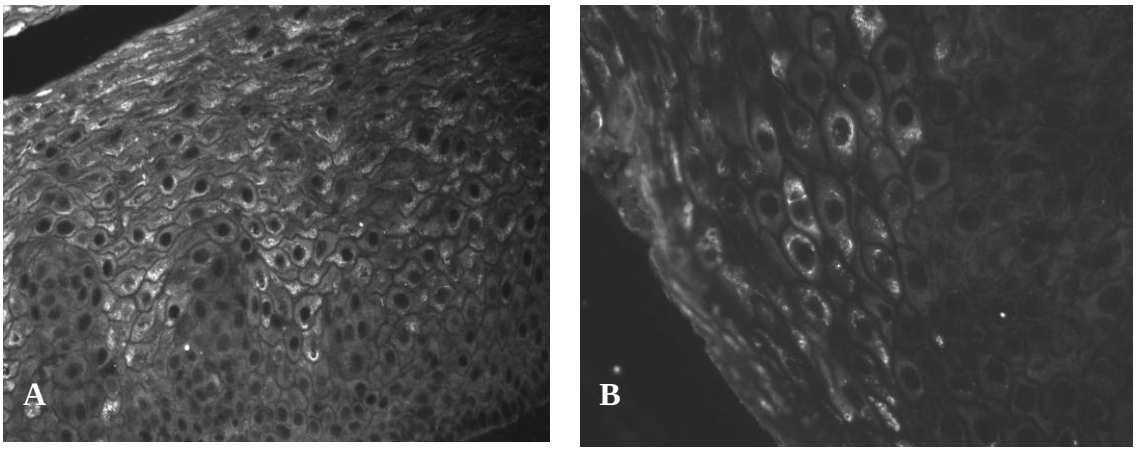


Figure 2.4: Human oesophageal TRPV1 staining with immunofluorescence with; (A) GSK antibody (B) Abcam antibody staining mainly epithelial cell cytoplasm. No ganglionic or neuronal staining detected. X 40 Magnification (Immuno-fluorescence with frozen sections courtesy of Dr Madusha Peiris)

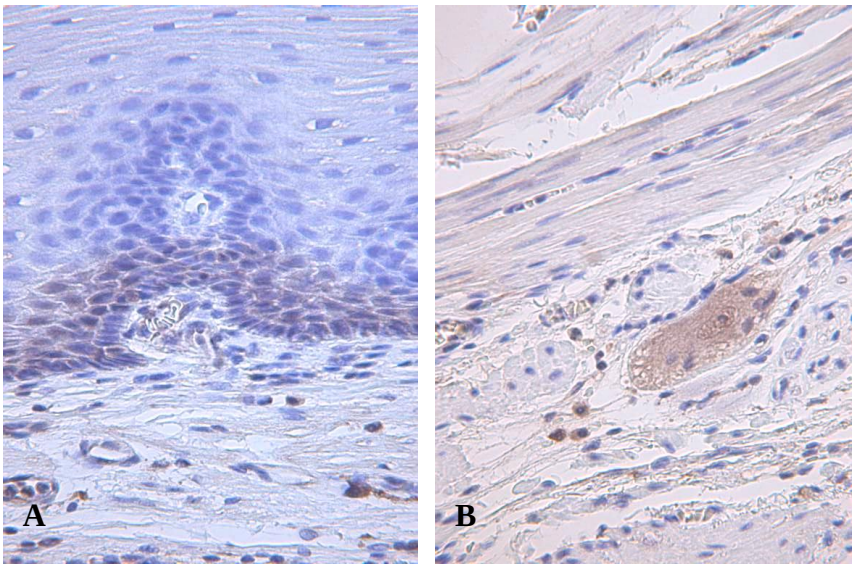


Figure 2.5: (A) Neuromics TRPV1 in human oesophagus; lack of staining in epithelium and sub-epithelium with no staining of TRPV1 immunoreactive nerve fibres detected, X 25 magnification. (B) TRPV1 staining ganglion in myenteric plexus X 25 magnification.

2.3.2.2 TRPV4 (Figure 2.6)

TRPV4 antibody stained the cytoplasm of myenteric neurons in all 10 samples. Weak nuclear staining was also detected in most cells throughout the squamous epithelium. In addition, in a sample with Barrett's metaplasia, TRPV4 staining was much stronger in the epithelium.

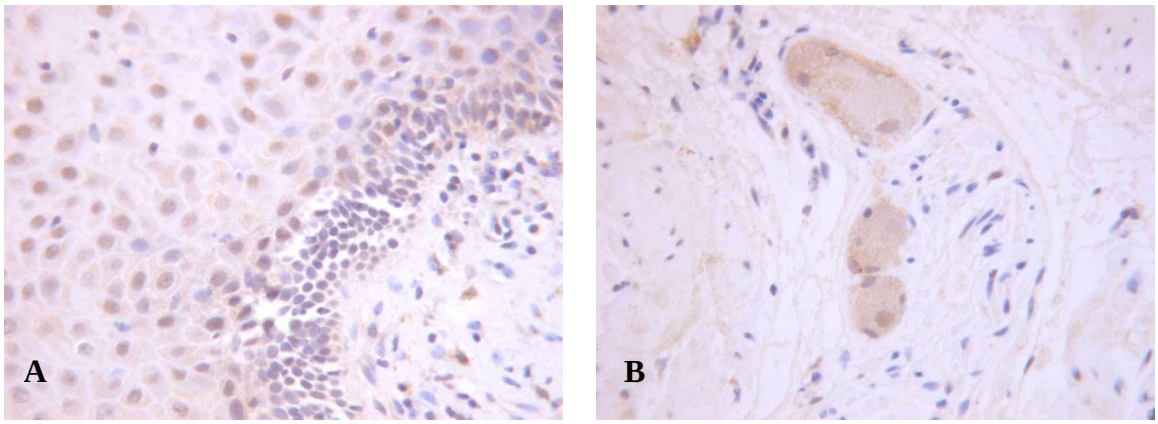


Figure 2.6: (A) TRPV4 in human oesophagus, epithelium and lamina propria, X25 magnification. (B) TRPV4 in human oesophagus, ganglia in myenteric plexus, X25 magnification.

2.3.2.3 ASIC 3 (Figures 2.7-2.8)

Strong cytoplasmic staining was detected in myenteric ganglia neurons in all 10 samples (Figure 2.8). Myenteric ganglia were positive for ASIC3 (Figure 2.8). No clearly distinct staining of nerve fibres was observed in the sub-epithelium in comparison with PGP9.5 (Figure 2.7). ASIC3, however, produced strong epithelial staining with noticeable variation in different samples (from intense to almost negligible). In addition, apart from the difference in intensity of staining, there were also differences in the distribution and morphology of ASIC3 staining in the epithelium, which will be discussed in Chapter 3. This difference may represent different states of receptor activation and could be exploited in the biopsy study with patients.

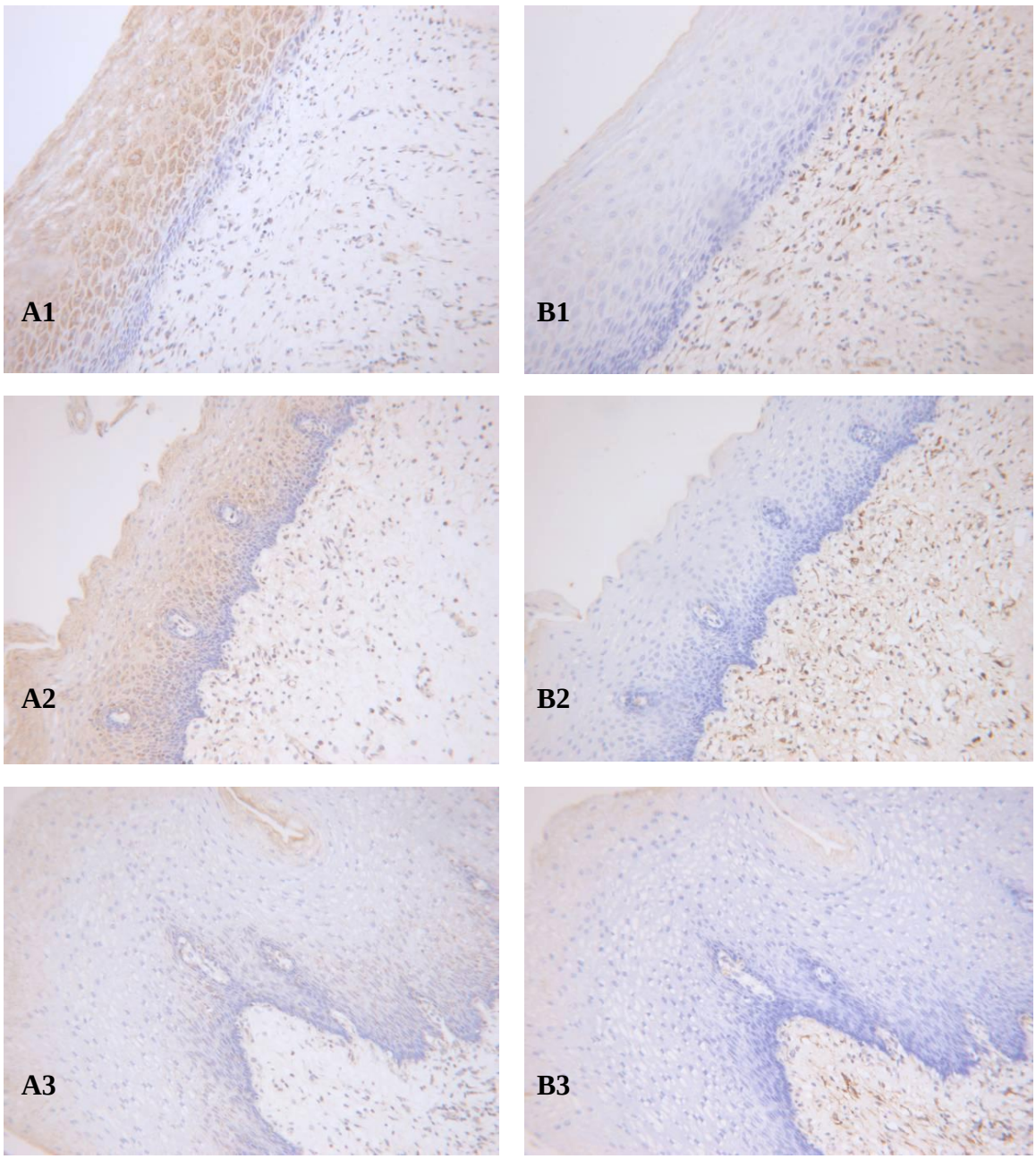


Figure 2.7: Paired images from consecutive cuts of human oesophagus stained with ASIC3 (A1, A2, A3) and PGP 9.5 (B1, B2, B3) in X10 magnification. Lack of overlap between areas stained with ASIC3 and PGP 9.5 suggested minimal ASIC3 immunoreactive neuronal endings. The 3 pairs represented the spectrum of ASIC3 staining intensity in the epithelium; A1- high intensity ASIC3 staining, A2-medium ASIC3 staining and A3- minimal ASIC3 staining.

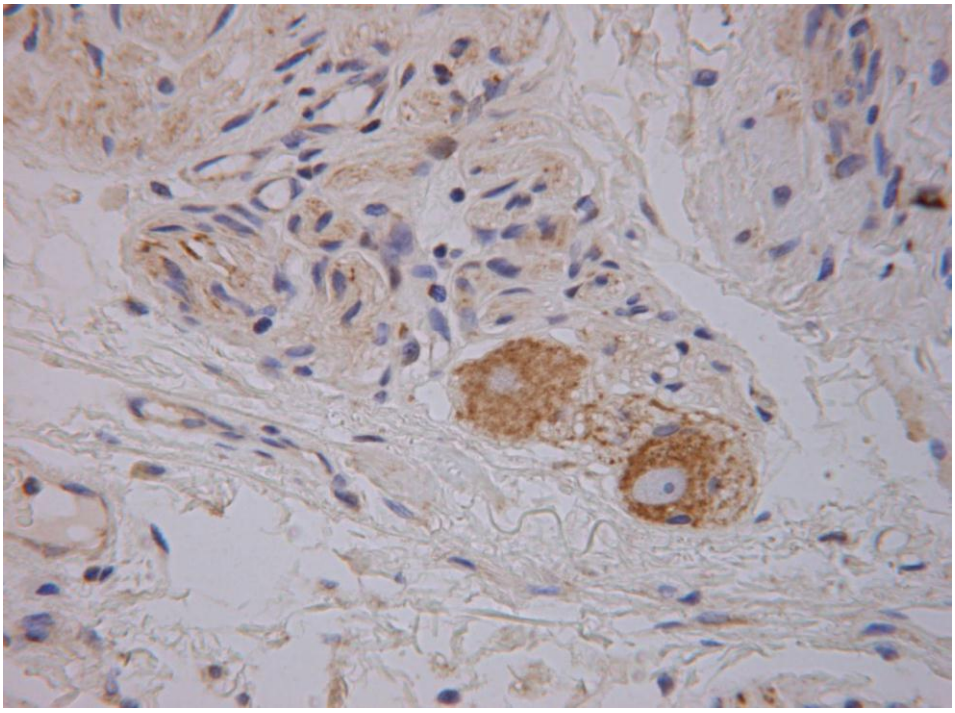


Figure 2.8: ASIC3 staining in deep myenteric layers of full-thickness oesophageal samples showing neuronal ganglia. Magnification X25.

2.4 DISCUSSION

2.4.1 Summary of immunohistochemical staining

The main findings of immunostaining are summarised in Table 2.5.

Antibody	Ganglia	Neurons	Epithelium
PGP 9.5	+	+	-
Synaptophysin	+(periganglionic synapses)	-	-
TRPV1	+/-	-	+/-
TRPV4	+	-	+/-
ASIC3	+	-	+

Table 2.5: Summary of antibody staining using IHC in human full-thickness oesophageal samples

2.4.2 Limitations

The study has several significant limitations that must be acknowledged and discussed.

Human samples: these were necessarily obtained for patients with disease. Nine patients had adenocarcinoma and one had a benign stricture. For each patient, it could be supposed that chronic acid exposure may have contributed to the disease process, although only two were documented to have symptomatic GORD. Indeed, three showed evidence of Barrett's metaplasia, which is a consequence of chronic GORD. Furthermore, eight had undergone neo-adjuvant chemotherapy with ECF combination chemotherapy. Chemotherapeutic agents suppress mitosis including in the epithelium, although in all patients usually 4-6 weeks lapsed between the completion of chemotherapy and operation. Adenocarcinoma of the oesophagus can also affect receptor expression in the human oesophagus and receptors have been used as predictors of treatment responses to neo-adjuvant chemotherapy in oesophageal cancer [129]. Chemotherapy is also well documented to induce neuronal damage including peripheral neuropathy [130] [131], which could affect quality of my neuronal staining. Chemotherapy was also shown to be associated with vagal changes[132] and in the human oesophagus, shown to induce dysphagia, further suggesting neuronal involvement[133]. In summary, these specimens from patients receiving neo-adjuvant chemotherapy cannot be considered 'normal'.

The IHC biopsies were obtained from slightly different areas of the oesophagus, depending on the availability of a specimen that could be safely dissected away from the main structure without compromising routine diagnosis and oncological staging. Nevertheless, all biopsies came from the distal oesophagus, as evidenced by the diagnoses of adenocarcinoma and the types of surgery performed, namely laparoscopic assisted oesophagectomy (as mentioned in section 2.2.3). This is important because neuronal distribution may differ from proximal to distal oesophagus. From the lower oesophagus towards the lower oesophageal sphincter (LOS), there is evidence that atypical myenteric plexus localisation causes spuriously low neuronal counts in lower oesophagus in opossum study[134]. This is believed to

result from myenteric plexus lying in more variable planes at it approaches the LOS[135].

The method used, IHC, is an indirect measure of the presence and distribution of protein in the sample. IHC has limitations concomitant on the quality of the antibodies, which is especially relevant to the study of human samples where polyclonal antibodies are most commonly employed (as in this study). In this protocol, there were several limitations including:

a. The antibodies were not validated using other methods. While PGP9.5 and synaptophysin were already well-validated in the laboratory (and produced specific staining in the expected distribution), the antibodies to neuronal receptors were not. A known positive control and negative control were used for all antibodies; however, other methods such as the purchase of a blocking peptide were not. Thus, although the basic method can be deemed successful, it is not possible to conclude that binding is specific to the receptor studied.
b. Immunohistochemical protein expression was not confirmed by other methods such as Western blotting[136 137] or by relating findings to the expression of mRNA using RT-qPCR[136 137 126] or other techniques. This would not be of relevance to neuronal expression (without considerable enrichment, e.g. by laser capture micro-dissection), but could have been used to study epithelial expression – this is further discussed in Chapter 3.
c. Variable techniques of fresh frozen section versus paraffin fixed samples. This applied mainly to TRPV1 staining as discussed in 2.4.3.2.

The proteins studied were not comprehensive in respect of those that could be of relevance to acid sensing in the human oesophagus. The classes of relevant receptors were discussed in Chapter 1. Those selected were chosen on the basis of more extensive evidence from previous studies, as outlined below.

2.4.3 Previous studies and current evidence

Despite the limitations outlined above, the findings merit discussion in the context of previous studies, with particular focus on the anatomical location of these receptors.

2.4.3.1 Oesophageal neuronal distribution based on immunohistochemical markers

The main finding was that our prior assumption that nerves would be evident within the epithelial layer was clearly incorrect. This assumption was based mainly on TRPV1 studies[126 138]. On the basis of H&E staining, standard histological texts are not helpful in describing the localisation of small sensory nerve endings in the oesophageal epithelium. Classic histopathological descriptions and visualisations of oesophageal innervation have been limited to the intrinsic plexi including submucosa plexus (Meissner's) and myenteric plexus (Auerbach's), as described in Chapter 1. The distinction between these plexuses in isolated small clinical samples is recognised as being difficult – even by histopathologists – due to the often thick muscularis mucosae in the human oesophagus [4].

As standard H&E is not the best method for detecting small nerve endings, more specialised methods have been used including silver staining and, more recently, specific antibodies such as PGP 9.5 or peripherin as part of the immunohistochemistry process. Using silver staining of nerves, a detailed study looking at the human oesophagus, visualised nerves in the sub-epithelial lamina propria[139]. More conclusively, using the same techniques as in the present study (PGP 9.5 immunohistochemistry) for human volunteers and patients, Newton et al. found that nerve endings did not penetrate the epithelium, but innervation in the sub-epithelium (especially the papillae) did increase in the presence of inflammation[140]. The former observation is consistent with the PGP 9.5 results of the current study.

2.4.3.2 TRPV1

Based on a pilot study demonstrating the up-regulation of TRPV1 immunoreactive nerve fibres in patients with erosive oesophagitis compared to normal volunteers[3], we originally aimed to characterise the distribution of TRPV1 immunoreactive nerve fibres in human oesophageal biopsy samples, and then correlate the derived data with detailed symptoms as well as endoscopic disease classification. As a pilot to a study of biopsy samples, it was necessary to chart distribution in full-thickness tissues (for validation purposes). Since this time, two key publications on the subject have emerged. In 2006, Bhat & Bielefeldt demonstrated that increased immunoreactive TRPV1-free nerve endings in epithelium and papillary areas were associated with

increased acid exposure time in patients with GORD, but not correlated with symptom severity assessed by questionnaire[141].

In a more recently published study, oesophageal mucosal biopsies from patients with NERD and EO demonstrated increases in TRPV1 in both RT-qPCR and Western blotting [126] compared to healthy volunteers. Immunohistochemistry was completed to control for inflammatory cells, but not for the localisation of TRPV1 expression. Furthermore, no figure was given showing either TRPV1 immunoblotting or immunostaining. The antibody (Millipore, Massachusetts, USA) was tried within our department but failed to produce satisfactory immunoblotting or staining. Thus, although this increase correlated with acid exposure time, as well as symptom severity in patients with GORD[126], the quantification methods (without describing distribution) of TRPV1 may be due to epithelial TRPV1 as opposed to its neuronal counterpart. Assuming that the expression of TRPV1 shown in this study is not methodologically flawed, it still remains much more likely that the authors described *epithelial* expression. This very important point was discussed and accepted by Guarino, *et al*. as a likely possibility. They also accepted that neural tissues were unlikely to be present in biopsy tissues (consistent with our findings, i.e. without enrichment, there are few if any neuronal elements in mucosal biopsies).

In this study, we have mainly used paraffin-fixed samples. Frozen sections were used with the immunofluorescence technique using GSK and Abcam antibodies. These antibodies using fresh frozen sections indeed yielded better quality staining of TRPV1 compared to those using paraffin fixed sections (Neuromics and Thermo-Fisher). The GSK antibody produced the best quality staining but unfortunately this went out of production. It is noteworthy to mention this was the antibody of choice in other recently published studies in TRPV1 in human gastro-intestinal tract[109 3 45]. The highly variable technique between studies could explain for variable observations. All antibodies still produced epithelial TRPV1 staining rather than neuronal. Neuronal TRPV1 was noted in deep myenteric layer with only Neuromics antibody. The significance of epithelial TRPV1 staining is discussed in section 2.4.4.2.

2.4.3.3 TRPV4

In the mouse gastrointestinal tract, using in-situ hybridisation methods, mRNA-enriched TRPV4 nerve fibres were localised in the serosal aspect of the colon, but not the mucosal nerves believed to be important in mechano-sensation in the colon[137]. TRPV4 protein has also been described in rat peripheral sensory nerves, although requiring extensive preparation[142]. In this study, the role of TRPV4 in nociception induced by hypotonicity was explored. Neuronal TRPV4 was believed to be upregulated the same way as neuronal TRPV1 – in the DRG and transported peripherally to the relevant sensory nerves. In this study, TRPV4 was ligated for three days to dampen the distal transport of TRPV4, and only with this measure was TRPV4 visualised in the saphenous nerve proximal to ligature[142]. In a separate study, TRPV4 was found in mouse DRG, but only in 10% of the neurons found mainly in the lung, kidney and sensory organs such as the skin, tongue and cochlear[143]. Unsurprisingly, non-neuronal TRPV4 was detected in areas in association with fluid such as renal cortical cells surrounding the ventricles in brain[144] and the bladder[145]. Our results, employing simple immunohistochemistry, showed that oesophageal ganglionic staining was occasional.

2.4.3.4 ASIC3

Immunoreactive ASIC3 has been clearly demonstrated to be present in rat DRG as well as 20.6% of nodose (vagal) ganglia[146]. Further evidence of neuronal ASIC3's co-existence with Calcitonin gene-related peptide (CGRP) hints towards its role as a nociceptor, while its existence in metaboreceptive neurons suggests a role in acid sensation [51]. ASIC3 in the rat oesophagus was detected in the epithelium (prickle cell layer), nerve fibres and neurons, but only in the deep myenteric layer[136]. This is fairly consistent with our findings in humans.

2.4.4 Interpretation of results

2.4.4.1 Oesophageal mucosal nociception

The most striking and robust finding of this study was the absence of neuronal elements within the epithelial layer. This finding was unexpected based on previous publications[3] [138], and necessarily presented a major problem for anticipated future

investigations (Chapter 3), since the planned mucosal biopsies could not be used to study nerves. However, perhaps this finding is not so surprising. The oesophagus, with its deep squamous epithelium (from our samples about 35 cells thick on average), is presumably teleologically able to withstand swallowing potentially very hot or cold hazardous substances and also because of its adjacency to an organ whose pH varies from 1.4-2.9 [147] and whose intervening barrier is acknowledged not to be absolute (even in the absence of significant GORD, humans still have some acid exposure)[148] [149]. Thus, from an evolutionary perspective, it would be deleterious for nerves to lie in a position where they might be responsive to such environmental changes but unable (unlike the buccal cavity) to readily expel the offending content.

Nevertheless, it is a fact that the oesophagus is a site of acute algesia in response to noxious stimuli – anyone who has swallowed a hot potato or very cold drink knows this, and numerous studies of healthy volunteers and patients confirm responses to polymodal stimuli such as distension, electrical and thermal stimulations[15] [150] [151] . Furthermore, it is a site of chronic pain from infiltration, e.g. by a tumour[152] [153] and, of course, more commonly by GORD [154] [155].

The question of how stimuli reach the sensitive nerve endings in the sub-epithelium to activate them, and perhaps lead to peripheral sensitisation, is pertinent. This facilitation of stimuli across the mucosal epithelium may broadly occur due to three separate, additive or co-operating processes:

- Dilated intracellular spaces (DIS) facilitate the access of acid to nerve endings: This now well established mechanism is supported by three studies on rabbits by the Siffrim group in Leuven, which used in-vitro permeability studies and electron microscopy to evaluate DIS. In response to acid, DIS is potentiated by bile salts in short duration exposures[156]. Secondly, such exposures lead to DIS in the non-exposed proximal oesophagus[157]. Finally, this process is greatly augmented by concomitant stress conditions[98].
- Epithelial erosions permit the direct access of acid stimuli to nerves: Erosive oesophagitis and inflammation are often associated with pain. Direct access of stimuli to the nerve endings by breaks in epithelial integrity could lead to PS and

therefore increased immunoreacting TRPV1. This theory, although attractive, is not supported by evidence. Several studies attest to greater sensitivity in NERD rather than EO groups of patients[158]. In addition, one study demonstrated that patients with NERD often report more severe acid symptoms compared to EO patients [159]. Further, a study of patients with EO showed these to be *hyposensate* to mechanical stimuli compared to normal volunteers[150]. Thus, an intact epithelium may be associated with more PS than one that is demonstrably eroded.

- Epitheliogenic nociception: The concept that an intact superficial epithelium has intrinsic mechanisms of transducing damaging stimuli to underlying nerves is receiving increasing attention following studies that show that in-vitro mucosal preparations (e.g. cat oesophagi) can respond to acid on the luminal surface by the basal secretion of inflammatory mediators such as Substance P, CGRP[160] and the platelet activating factor (PAF) [161]. Such responses could be experimentally blocked by the TRPV1 antagonist IRTX. The same study went on to show the presence of TRPV1 in the epithelium by immunoblotting[161]. A similar study in relation to PAF release was very recently performed in a human oesophageal epithelial cell line[162]. This line of evidence, if true, has relevance to the findings below.

2.4.4.2 TRPV1

The problems of specificity with various TRPV1 antibodies were covered above. In short, we were unable to show any convincing immunostaining for TRPV1. On this basis, the immunoreactive TRPV1 nerve was dropped as a useful biomarker for studies in Chapter 3. The question of whether superficial epithelial TRPV1 expression (as noted with one antibody) has true functional relevance in nociceptive signalling is discussed below.

There is currently no reason to believe why epithelial TRPV1 should not respond to the same stimuli as neural TRPV1 such as heat, acid and capsaicin. For instance, one animal study showed that complete knockout of TRPV1 attenuated oesophagitis in mice[163]. While this may be the case, epithelial TRPV1 challenges the current understanding of PS involving the retrograde activation of messages from the oesophagus to DRG, and increased protein synthesis in the spinal cord and downstream to the nerves. TRPV1 activation, and therefore PS, could occur within the

epithelium itself, which is supported by emerging evidence as described above in epitheliogenic transduction. Even with a reliable antibody, this could only be explored using mRNA techniques to explore actual up-regulation. A previous study by Guarino, *et al.* addressed this relationship between protein and mRNA. The authors accepted that the protein measured in immunoblotting could well be epithelial TRPV1, as it was not visualised by immunohistochemistry. This again added to the evidence and strength of argument for epithelial TRPV1. Crucially for a mechanistic study, RT-qPCR should be ideally controlled with specific and serial *timing* after sensitisation. In patients this is not realistic, so I did not pursue this course of action in the biopsy study.

2.4.4.3 TRPV4

TRPV4 has been shown to be an osmoreceptor[144] and a mechanoreceptor[164]. In the mammalian viscera, evidence of the involvement of TRPV4 receptors in VPH comes from mechanical distension of the colon in rodents[165 166 137]. The role of TRPV4 in mechano-sensation could be relevant in the specific context of VPH and functional GI conditions in general.

Expression of TRPV4 has been shown to be low in mouse gastro-oesophageal vagal afferents, with knockouts not showing altered vagal function to mechano-sensation, [137 167] in contrast to reduced behavioural responses to somatic pain [143]. To the best of our knowledge, there are no morphological studies of TRPV4 immunostaining in human full-thickness oesophagi. Although our findings suggest that TRPV4 is localised to the myenteric ganglia and basal epithelial layers, the relevance of this observation in the context of acid-induced oesophageal hyperalgesia is unfortunately not substantiated by animal studies.

2.4.4.3 ASIC3

ASIC3, as its name suggests, is considered an important acid-sensing ion channel and seemed a logical target for study. The current results also show varying intensity of ASIC3 staining in the epithelium of a full-thickness oesophagus as well as different morphology in cellular expression. Currently, there is strong evidence for ASIC3 as a mechano-sensitive receptor in mouse vagal gastro-oesophageal afferents[48]. Of course,

there are potentially numerous other epithelial receptors apart from ASIC3; nevertheless, such findings are in keeping with ASIC3 having a role for mechanoreceptors in pathological acid reflux, since GORD has a continuum of motility disturbances associated with poor coordination and inappropriately high intra-gastric pressure and lower oesophageal relaxation. Increased ASIC3 in patients with more severe acid reflux should (in theory) have scientific justification and relevance in the context of VPH, as it is involved in both sensation and motility. More recently, the role and evidence of *epithelial* ASIC3 in the oesophagus and acid-related VPH have emerged, making it an even more suitable candidate. This will be discussed further in Chapter 3.

2.5 CONCLUSIONS

On the basis of the study of a full-thickness human oesophagus, and accepting limitations, it can be concluded that:

1. There are no (or at least very infrequent) nerve fibres in the oesophageal epithelium based on established neuronal markers;
2. Of the neuronal receptors stained, only ASIC3 had the potential for study in mucosal biopsies based on its strong and apparently differential staining in oesophageal epithelium.

3 MOLECULAR BASIS OF HUMAN VISCERAL PAIN HYPERSENSITIVITY IN THE OESOPHAGUS: THE RELATIONSHIP OF SYMPTOMS TO MUCOSAL ASIC3 EXPRESSION

3.1 INTRODUCTION

Acid-sensing ion channels (ASICs) were introduced in Chapter 1 (1.5.2: Peripheral sensitisation) and the rationale for studying epithelial ASIC3 in Chapter 2. This chapter covers the systematic study of ASIC3 expression in mucosal biopsies in relation to GORD.

3.1.1 Background

3.1.1.1 ASIC3

ASICs are members of the epithelial sodium channels/degenerin family, which is associated with neurotransmission[168]. ASIC3, previously known as DRASIC (dorsal root acid- sensing ion channel)[169], is present in rodent CNS and in peripheral somatic sensory neurons such as those innervating the muscle and skin[51 170]. An earlier functional study demonstrated that ASIC3 is more closely associated with mechano-transduction rather than acid sensing[48]. In this seminal study, it was shown that knockouts of ASIC3 mice exhibited reduced sensitivity to mechano-sensation (measured as the recorded number of spikes/per second) after von Frey/stretch stimulations, but crucially stimulation to mucosal receptors remained unchanged. These results highlighted the separation between epithelial and neuronal receptors in sensation.

Although ASICs in sensory neurons function primarily as mechanosensors[171 48], they have also been known to contribute to nociception accompanying tissue acidosis[169 172]. ASIC subunits can form H+ gated cation channels capable of acting as effective neuronal acid sensors within a wide pH range [173 174]. Furthermore, NGF and serotonin

(both of which are increased in mucosal inflammation) stimulate ASIC3 transcription in peripheral sensory neurones through direct interaction with the promoter region of the ASIC3 gene[175 176].

3.1.1.2 Distribution of ASIC3 in humans

Human ASIC3 has been cloned from a foetal cDNA library [177] and testes[178], and shows an 84% sequence identity with rat ASIC3 [169]. Human ASIC3 has been demonstrated in dorsal root ganglia[179], sensory ganglia[177] and in a variety of other tissues including bronchial cells, tracheal cells, human small airway epithelial cells[180] as well as the intestine[179].

3.1.1.3 The role of ASIC3 in acid-induced VPH

The distribution and role of ASIC3 have been studied in acid-exposed and non-acid-exposed regions of the GIT in experimental animals[48] and, to a lesser extent, in humans[179], the latter confirming up-regulation during inflammatory conditions. Specific to its role as an acid sensor are two experimental studies. Akiba, *et al*.[136] used luminal CO2 to produce changes mimicking acid infusion. The use of an established ASIC3 inhibitor (amiloride derivative[181 182]) was shown to attenuate the hyperaemic response of the oesophagus to this stimulus[136]. ASIC3 was also noted to be present in rat *epithelial* cells, in particular stratum spinosum (prickle cells)[136]. The second study, conducted by Wultsh et al., explored the sensory role of ASIC3 in the presence of gastritis[183]. Using a rat model, acid (HCL) or saline was infused for 2 hours in the normal and inflamed stomachs (gastritis) of conscious rats. Activation of neurons in the nucleus of the tractus solitarii (NTS) of the brain stem was visualised by immunohistochemistry using c-Fos. Exposure of normal gastric mucosa to acid caused a three-fold increase in C-Fos activity compared to saline in wild-type rats[183]. Knockout of ASIC3 did not affect this observation. In the same model, using rats pre-treated with iodoacetamide in drinking water for 7 days (to induce gastritis), the 41% increase in NTS c-Fos-positive neurons after acid infusion in the wild-type rats was absent in ASIC3 knockouts. Thus, ASIC3 appears to have a role in acid sensing in pathological conditions characterised by mucosal inflammation.

3.1.2 Rationale

In Chapter 2, it was established that nerve fibres were absent within the human oesophageal epithelium. ASIC3, however, could be considered a promising epithelial biomarker based on the demonstration of varying intensity and distribution of sub-cellular immunostaining. Given the acknowledged role of ASIC in acid and the damage sensing outlined above, it was reasonable to study ASIC3 in mucosal biopsies in this study.

3.1.3 Aims

The aims were to:

1. Assess epithelial ASIC3 expression in mucosal biopsies of patients within the spectrum of GORD;
2. Correlate the severity of common GORD symptoms with mucosal expression of ASIC3.

3.2 SUBJECTS AND METHODS

3.2.1 National research ethics approval

This body of work was submitted in the same ethics application as the full-thickness oesophagus study (Chapter 2). The updating of the patient enrolment for this part was done via the UKCRN (United Kingdom Clinical Research Network), study reference number 4823 (https://portal.ukcrn.org.uk).

3.2.2 Subjects

Patients within or without the spectrum of GORD aged 18-60 years old, without other significant past medical histories, were recruited from those attending the Hermann Taylor endoscopy department of the Royal London Hospital.

3.2.3 Inclusion and exclusion criteria

Patients with conditions that could affect normal physiology on pain and reporting were excluded from the study. Specific inclusion and exclusion criteria were:

Inclusion criteria

1. Male or female subjects aged 18−60 years (GI physiology and sensation could be different in those aged <18 or >60 years);
2. Main presenting complaint or symptomatic evidence of GORD;
3. Patients undergoing investigation for anaemia, cancer surveillance or unexplained weight loss without acid-related symptoms volunteered as controls.

Exclusion criteria

1. The participants were required to speak English (as the study was dependent on the subjective reporting of pain thresholds and adverse effects, which could not be undertaken through an interpreter);
2. Chronic serious systemic diseases (except acid-reflux related conditions) including cardiac, liver, renal, metabolic, malignancies, neurological or psychiatric as assessed by medical assessment/endoscopy admission clerking;
3. Currently using recreational drugs or pain killers;
4. Pregnancy due to higher incidence of physiological acid reflux in pregnant women and normal physiology not fully examined and appreciated;
5. BMI (Body Mass Index) > 35.

3.2.4 Patient recruitment process

Participant information sheets were sent to the patients before their endoscopy appointment dates. Some patients called to express interest in participation prior to arrival, while others expressed interest on arrival at the endoscopy department. Patients who agreed to participate all consented to research at the same time as the routine clinical consent. Subsequently, they underwent a quick medical assessment (structured medical assessment) and completed questionnaires on symptom severity. They were reminded of the second (multi-modal) visit and contact details of the researcher if they were interested.

3.2.5 Endoscopy and biopsy

The subjects underwent routine standard trans-oral oesophagogastroduodenoscopy (OGD) using Olympus gastroscopes (GIF XQ 260 2.8mm channel, Olympus, Olympus Europa, Hamburg, Germany). Additional biopsies were obtained using tooth biopsy forceps (Captura biopsy forceps with a spike, 2.4mm, Cook Medical, Indiana, USA). Oesophageal biopsies were taken at a fixed position 3-5 cm above the lower

oesophageal sphincter (LOS). In patients with GORD, biopsies were taken *between* oesophageal erosions or *away* from actual erosions to obtain epithelium. Each patient contributed four biopsies for the research. Two biopsies were snap-frozen in liquid nitrogen and the remaining two immersed in formalin fixation for 24 hours at room temperature. Frozen samples were stored at -80 °C and the formalin-fixed samples processed by lab staff into anonymous paraffin-fixed cassettes and stored as such. Full protocols for fixation and tissue preparation were as described in Chapter 2, Appendix 2.03. Protocols of the endoscopy unit in terms of safety and recovery were observed for all patients.

3.2.6 Questionnaires

A structured medical assessment was used as well as a validated questionnaire, developed and routinely used in the department, which was used to assess the type and severity of GORD symptoms. The six specific symptoms scored using VAS were adapted from ReQuest™ (validated GORD) questionnaires [184] to quantify subjective symptom severity. The actual full protocol of ReQuest™ questionnaires had to be filled in for four weeks, usually to monitor treatment progress, which was neither practical nor necessary for this study. Thus, we only used the ReQuest™ scores (1-10) as a single 'snapshot' to assess current symptom severity, filled in during the visit by the patients. The ReQuest™ questionnaire was used by kind permission of the author (Prof KD Bardhan). The six symptoms assessed were (copy of questionnaire available in Appendix 3.01):

1. General feeling
2. Acid complaints
3. Upper abdominal/stomach complaints
4. Lower abdominal/digestive complaints
5. Nausea
6. Sleep disturbances

3.2.7 Multi-modal sensitivity tests

A specifically commissioned catheter has been validated in previous studies in the human oesophagus (Figure 3.1)[15 185]. Training was provided at the University of Aalborg, Denmark with my collaborators, who were also responsible for inventing the instrument.

This training took place during my five-week attachment in Denmark from 6th February to 4th March 2007. All stimuli were repeated three times and stopped as soon as the subjects reached their pain threshold. This threshold was recorded.

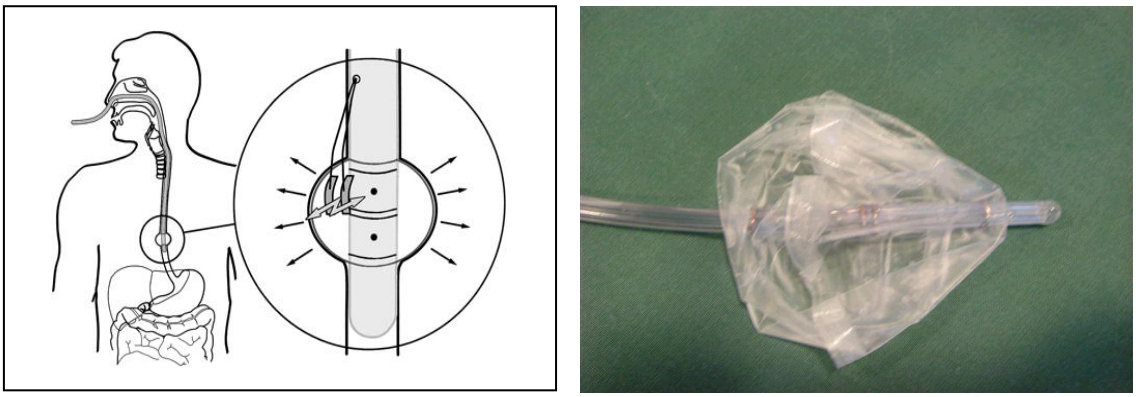


Figure 3.1: The oesophageal multi-modal catheter. (A) Schematic representation of the balloon of the multi-modal catheter. Thermal and mechanical stimulations were delivered via water perfusion and monitored for intra-balloon pressure monitor as well as with a temperature probe. The balloon is also equipped with impedance planimetry technology for estimating the cross-sectional area if desired[186]. (B) The balloon ex-vivo.

Thermosensitivity: Cold and heat stimuli were given with a recirculation of 5ml of water in a polyurethane bag, which was in a tubing system primed with cold water. The probe had two perfusion channels attached to a manually controlled pump system, where water was infused into one channel and simultaneously sucked out of the other. Water temperature was maintained by the thermostatically controlled water bath maintained at 65°C. A thermal electrode was placed into the probe to record the temperature of water in the exit channel. As warm water was infused, the water temperature varied from 20-60°C, recorded in 10°C increments. For each infusion, the subjects rated sensation and pain on a visual analogue scale of (VAS) 1-10 (Appendix 3.02).

Mechano-sensitivity: Using the same balloon, water was infused at a fixed temperature of 38°C at an infusion rate of 10ml/minute until sensation was first felt (first constant sensation) and then to the maximum tolerated (maximum tolerated volume: pain felt).

The maximum volume was set at 25ml. Subjects were required to complete VAS scores for sensation and pain.

Electrical Stimulation: The multi-modal catheter incorporates a metal bipolar ring electrode at its distal tip to deliver electrical stimuli. Thresholds to stimulation measured as intensity in mA, at which the subject first reported sensation and discomfort, were determined in a stepwise fashion, increasing the intensity of stimulation in increments of 2 mA on three separate occasions, from which mean threshold values were calculated. Patients were then asked to rate these sensations on a VAS. Discomfort was defined not as the maximal pain that could be tolerated, but rather as the level at which further stimulation would not be desirable. An electrical current was also applied via electrodes to the foot, to act as somatic control for the same protocol.

3.2.7 Cellular analysis

Detailed paraffin fixation, immunohistochemistry methods and sectioning were as described for the full-thickness oesophageal samples in Chapter 2 and Appendix 2.02-2.06. From the paraffin fixation cassettes, 3-5 micron sections were cut using a microtome (Shandon Finese E+, ThermoFisher Scientific, Leicestershire UK), mounted on glass slides (VWR International, Leicestershire, UK) and incubated at 37ºC in an oven before use. These sections were processed for H&E to characterise the presence or absence of oesophagitis, ulceration and other cellular changes such as metaplasia (Barrett's oesophagus), as well as confirming that the biopsies were indeed of oesophageal squamous epithelium. Immunohistochemistry (IHC) using the ASIC3 antibody (Abcam, Massachusetts USA) was performed based on the optimisation schedule from Chapter 2. Validation of this antibody in Western blotting was performed by the manufacturer (Appendix 3.03), while H&E was performed in a similar fashion to that outlined in Chapter 2 (Appendix 2.02-2.04.).

3.2.8 Outcome measures

ASIC3 immunostaining acted as the primary outcome measure. The results were expressed based on morphology and distribution of epithelial immuno reactivities carried out by two researchers (myself and Dr Andrew Hubball), with the slides

anonymised and blinded to their clinical details. H&E staining reporting was performed blind by a consultant pathologist with an interest in upper GI diseases (Dr Joanne Chin-Aleong).

3.2.9 Data analysis

The original sample size calculation was performed on the basis of projected results for TRPV1. The main analysis was intended to be a scatter graph of symptom scores vs. TRPV1, with a 95% confidence interval for correlation coefficient. This assumed a standard deviation of 10% estimated from data in a previous study [187]. A total sample size of 48 patients or more would have to have 80% power to detect a significant correlation ($r \geqq 0.8$). Since there were no preceding data on epithelial ASIC3 in the human oesophagus, it was deemed reasonable to continue to use the calculation based on TRPV1, since this sample size also lay within a feasible range for recruitment.

Softwares used for analysis were; Excel (Microsoft Corp, USA) and graph-pad prism (Graph-pad software Inc, California, USA). Tests for normality, median and inter-quartile ranges were initially performed. Further comparisons of data were then performed using; Fisher exact test for analysis of ASIC3 features in different disease classification, Wilcoxon paired test for comparison of proportion of patients with ASIC3 features across 2 symptom-based groups, and Kuskal-Wallis test for comparison of symptom scores across 3 patient groups based on clinical classification.

3.3 RESULTS

3.3.1 Subjects

Out of a total of 44 patients who consented to their involvement in the study, three sets of slides went missing in transition and five failed to satisfy the inclusion and exclusion criteria. The reasons were undisclosed or overlooked during consent, but were then found in medical records or discovered during the endoscopy itself (one with previous treated pancreatic cancer, severe diabetic neuropathy, discovery of upper GI mass, discovery gastric polyp and over the age limit with cardiac ischeamia). Thus, only 36 sets of samples from corresponding patients were analysed, 17 of which were female (mean age 42.5 years, range 19-59 years).

3.3.2 Disease classification

Patients referred *without* overt acid-related symptoms, and presenting complaints based on clinical details (referral letters and medical assessments), were classified as controls. Those with acid-related reflux were separated based on OGD findings to either the erosive oesophagitis (EO) or non-erosive reflux disease (NERD) group. As this last cohort did not have an objective measure from pH studies, they could represent both NERD as well as functional heartburn (FH). In total, of the 36 patients, 14 were healthy controls, 18 NERD/FH and four EO. With modern treatment and the wide use of effective acid suppressant treatment, patients with erosions were rare.

H&E analyses for the patients showed normal to mild inflammation in 34 slides. Moderate inflammation was found in two samples. No severe inflammation considered pathological was found. However, the aim was to assess mucosal sensation and epithelial receptors; therefore, biopsies were obtained *away* from areas of erosion/inflammation. As a result, the disease classification was based entirely on *clinical symptoms/endoscopic* findings and not histopathology.

3.3.3 ASIC3 immunohistochemistry

3.3.3.1 Distribution

ASIC3 staining was present in the epithelium of most samples. Distribution, however, varied from the deep stratum basale to stratum spinosum, to the superficial luminal aspect. There were some samples with almost no staining. Interestingly, some samples exhibited a nucleated superficial layer of cells that looked almost like the stratum lucidum/corneum of the keratinised stratified squamous epithelium of the skin. However, since the oesophageal epithelium is non-keratinised and this layer was not previously described, this is simply referred to as the ‘luminal layer’.

3.3.3.2 Morphology

Most ASIC3 immunostaining was found surrounding the nuclei, termed ‘perinuclear’, in the stratum spinosum layer. However, within the stratum spinosum, which is the thickest layer of the epithelium, some staining was also noted to predominate on the cytoplasmic membrane of the cells (membrane-bound), giving the appearance of an

area free from staining between the basophilic nucleus and the brown (DAB) ASIC3 stain. Membrane-bound staining tended to be located in the more superficial aspect of the stratum spinosum compared to perinuclear staining.

3.3.3.3 Analysis

The above description can be considered as four separate characteristics of ASIC3 staining based on anatomical location and cellular morphology. These four features were assessed as present or absent, as detailed in table 3.1.

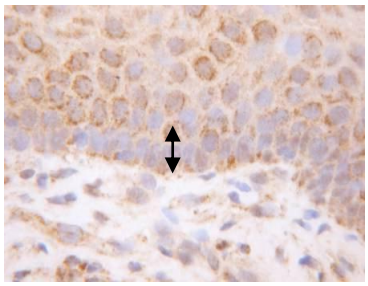
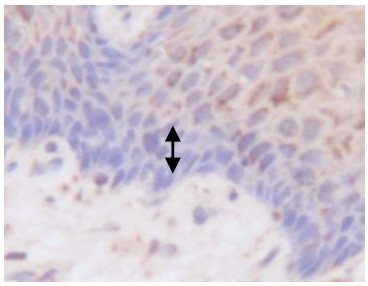
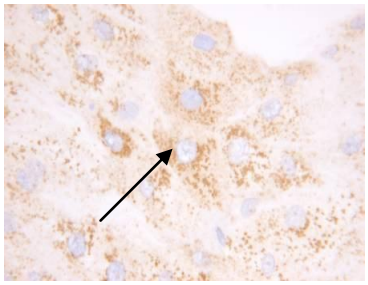
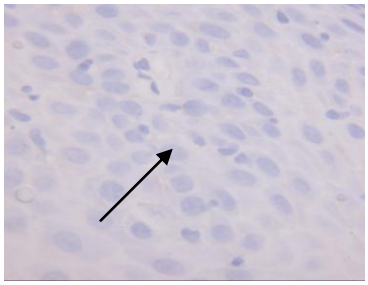
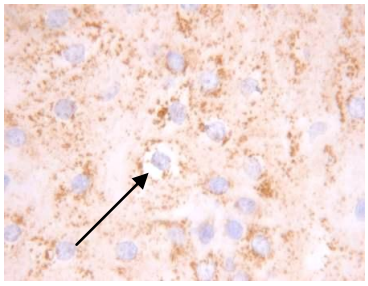
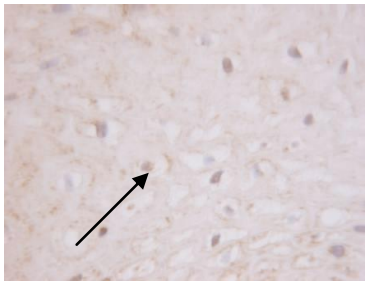
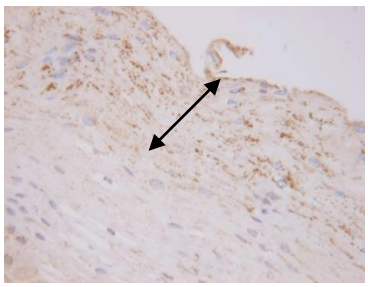
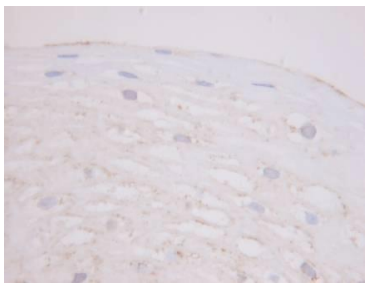
Characteristic	*Positive*	*Negative*
Stratum Basale Basal layer (BL)		
Stratum Spinosum I) Perinuclear (PN)		
II) Membrane bound (MB)		
Stratum lucidum Luminal layer (LL)		

Table 3.1: 4 separate ASIC3 features for human oesophageal biopsies. For each feature, points were given as either 1=positive, 0=negative. Analysis was performed on the percentages of patients expressing particular features.

3.3.4 Distribution of ASIC3 based on disease classification

Based on the presence or absence of these characteristics, analysis was undertaken in respect of disease classification based on endoscopic and clinical findings (as

described in 3.3.2: Disease Classification)

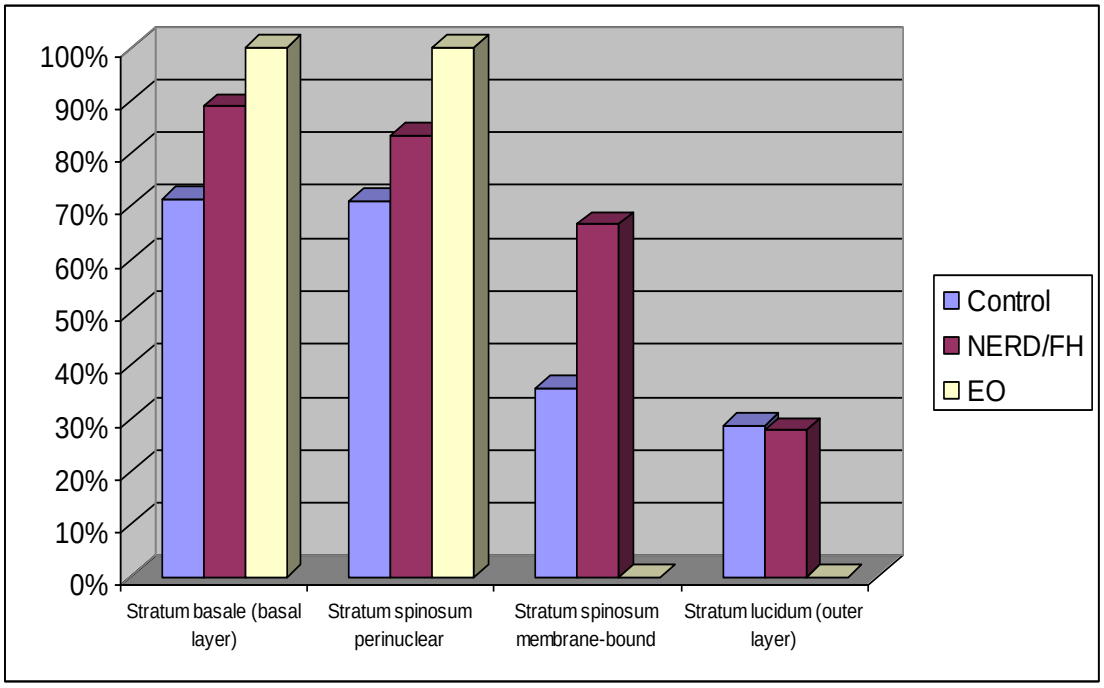


Figure 3.2: ASIC3 immunohistochemical characteristics in the controls, patients with NERD/FH and EO. The membrane-bound feature was statistically significant between patients with NERD/FH and EO, with p=0.028 for the Fisher exact test.

3.3.5 Expression of ASIC3 based on histological evidence of inflammation

As only two slides were considered to have moderate inflammation, compared to 34 with normal to mild inflammation, no analyses were undertaken.

3.3.6 Expression of ASIC3 based on symptom severity

On the basis of dividing patients into two groups in line with their scores (<5 or ≥ 5), proportions with certain staining characteristics could be compared (Table: 3.2). Controls were included in symptom assessment, as many of the symptoms assessed were generic, including general feeling, sleep and nausea. It was important to identify if these features were markers for symptoms independent of inflammation or erosions. In general, *all* characteristics were seen with a greater prevalence in patients in the higher scoring group, i.e. more severe symptomatic group (symptoms < 5, median 51.50% [34.05-79.47], symptoms ≥5, median 64.71% [35.39-86.61]. The three symptoms in particular, demonstrating the most consistent pattern of correlation, were acid symptoms, lower GI complaints and sleep complaints (Figure 3.3).

Symptoms	*Severity*	*BL*	*PN*	*MB*	*LL*
General Feeling	<5	13/16 (81%)	11/16 (69%)	8/16 (50%)	5/16 (31%)
	≥5	16/20 (80%)	18/20 (90%)	9/20 (45%)	4/20 (20%)
Acid complaints	<5	16/20 (80%)	13/20 (65%)	8/20 (40%)	5/20 (25%)
	≥5	14/16 (88%)	16/16 (100%)	8/16 (50%)	4/16 (25%)
Upper GI discomfort	<5	16/19 (84%)	15/19 (79%)	7/19 (37%)	5/19 (26%)
	≥5	13/17 (76%)	14/17 (82%)	9/17 (53%)	4/17 (24%)
Lower GI discomfort	<5	17/22 (77%)	18/22 (82%)	9/22 (41%)	5/22 (23%)
	≥5	13/14 (93%)	11/14(79%)	7/14(50%)	4/14(31%)
Nausea	<5	21/26 (81%)	20/26 (77%)	12/26(50%)	7/26 (27%)
	≥5	9/10 (90%)	9/10 (90%)	4/10 (40%)	2/10 (20%)
Sleep disturbances	<5	12/15 (80%)	11/15 (73%)	6/15 (40%)	3/15 (20%)
	≥5	18/21 (86%)	18/21 (86%)	10/21(48%)	6/21 (29%)

Table 3.2: Percentage of patients with symptom scores (<5 or ≥ 5), with each ASIC3 staining characteristic. Key: BL-basal layer, PN-perinuclear, MB-membrane bound, LL-luminal layer. Difference between the two symptom severity groups, for all ASIC3 characteristics.

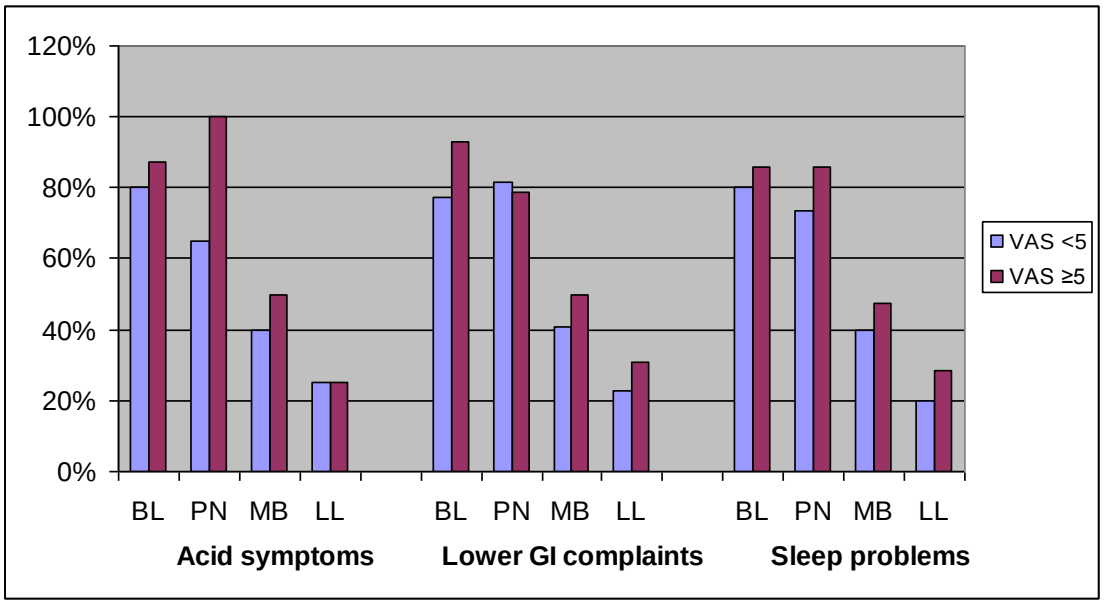
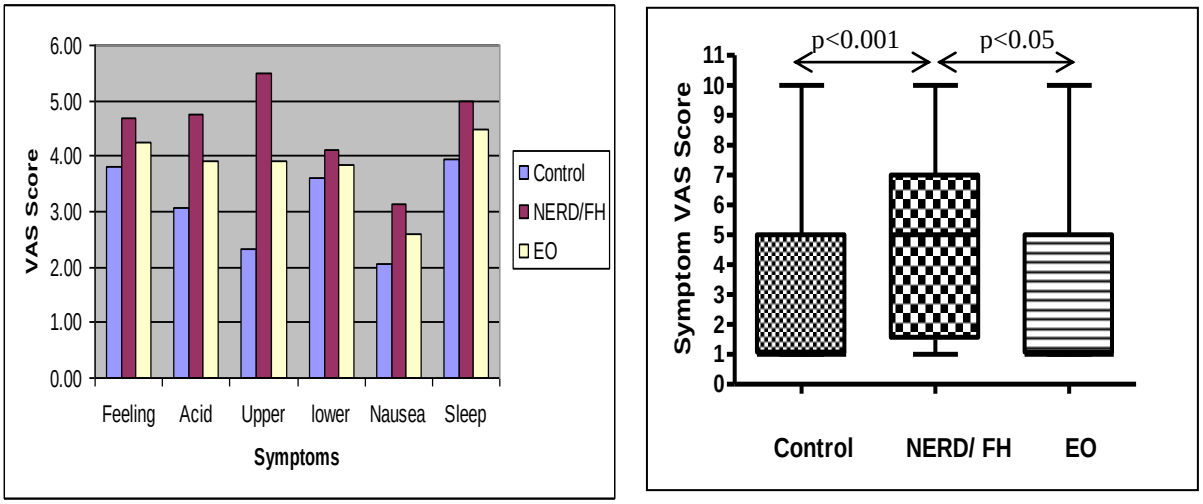


Figure 3.3: Comparison of ASIC3 staining characteristics for symptoms with the most differences between two symptom severity groups.

3.3.7 Symptom severity based on disease classification

A VAS assessment of symptom severity demonstrated that symptoms were, in general, worse in NERD/FH patients for all six symptoms assessed (Median VAS: controls-1, NERD/FH-5, EO-1. NERD/FH compared to EO, $p<0.05$, and control patients, Dunn's multiple comparison test, $p<0.001$) (Figure 3.4). Although the controls were referred for non-acid-related symptoms, it was interesting that their scores were not consistently 1 when questioned (out of 14 controls, 10 scored the minimum 1 for both 'acid' or 'upper GI complaints'. Also, a number of generic symptoms were assessed).



A *B*

Figure 3.4: (A) VAS scores of six individual symptoms based on ReQuest™ for controls, patients with NERD/FH and EO. B) Pooled symptom severity scores for the same three groups of patients, Kruskal-Wallis=0.0003, Dunn's multiple comparison test, control vs. NERD/FH p<0.001 and NERD/FH vs. EO p<0.05.

3.3.8 Multimodal sensitivity testing

Despite successful piloting in five healthy volunteers, out of the patients who had consented to oesophageal biopsies, only three actually underwent multi-modal oesophageal sensitivity testing and one failed intubation. Although oral intubation of the catheter was successful in healthy volunteers, it was not acceptable to most patients, especially without the use of local anaesthetic spray. Furthermore, intubation of the semi-rigid multimodal catheter was almost akin to oral intubation with a gastroscope, without the benefit of direct vision and a navigation system.

3.4 DISCUSSION

3.4.1 Summary of the findings

Four separate ASIC3 immunostaining characteristics were determined in the human oesophageal mucosal biopsies based on morphology and anatomical distribution, namely basal cell staining, stratum spinosum – perinuclear or membrane bound – and luminal layer staining. These four features were generally lowest in the controls and highest in NERD patients. EO patients expressed maximal basal cell staining as well as perinuclear appearance in the stratum spinosum. The more superficial features were not detected in the EO cohort, perhaps because of erosions. Patients with higher symptom severity were also more likely to exhibit evidence of all four ASIC3 characteristics.

3.4.2 Limitations

The patients were classified based on clinical assessments and clinical endoscopic findings. Patients considered as controls did *not* have symptoms of acid as their main presenting problems, yet their symptom scores were not consistently at the minimum of 1. Perhaps occasional acid reflux occurred even in healthy subjects and some of the symptoms assessed were very generic (e.g. general feeling, sleep problems, lower GI discomfort). It indicated the tool is sensitive to normal population complaints. I thought it was important to retain the study's classification based on the participants' presenting complaints (rather than to reassign them based on symptom scores) because in the general healthy population, individuals do experience acid reflux and upper GI pain. Therefore, this control group, although symptomatic when questioned in detail, did not feel their acid-related symptoms were particularly problematic, and were thus *not* representative of FH or NERD. Similarly, being referred for an OGD, they could not be representative of an entirely 'normal' population either. The observed effect of the results, therefore, would likely be greater if NERD/FH data were compared to completely healthy volunteers rather than patient controls.

Patients with acid-related presenting complaints as the reason for OGD were considered to be EO if they had evidence of erosions on the endoscopy. The remaining patients were classified as NERD/FH based on symptoms and the endoscopy (no

erosions). Ideally, subjects needed to undergo pH monitoring to differentiate NERD from FH. However, this would have involved two additional visits on top of the endoscopy and multi-modal oesophageal stimulation visit – this would have impinged on the GI physiology department's clinical service provision, which was already in high demand. Moreover, the aim of the study was mainly to correlate ASIC3 (or other markers) with the development and *severity of symptoms* rather than to another disease-based biomarker.

Smoking should also be added as an exclusion criterion. Smoking is well-known to cause and contribute to acid reflux[188]. Smokers therefore are likely to suffer from chronic acid reflux[189] and may have changes in receptor expression in the epithelium. These changes are likely to be non-representative of healthy sensation. In addition, smoking is also likely to affect the cellular development and morphology of the oesophageal epithelium itself. For example, smoking has been known to contribute to development of Barrett's oesophagus as well as oesophageal adeconocarcinoma[190 191].

Irrespective of whether patients were in or outside the GORD spectrum, we correlated *symptoms to the expression of ASIC3.* On the basis that symptoms might also be deemed more clinically relevant to real-life patients, the six symptoms chosen were adapted from a validated GORD questionnaire (ReQuest^{TM} questionnaire), which has previously proven to be a useful tool for monitoring the progress of GORD treatment including newly available acid suppressant treatment[192]. We originally planned to have an objective and comprehensive multi-modal oesophageal stimulation visit to profile the subjects' oesophageal sensitivities in detail, but this was not possible for the reasons mentioned above.

The next main limitation was the rudimentary and essentially qualitative (binary) methods used to describe ASIC3 distribution. Since ASIC3 is present in all groups to some degree, any future predictive model would require a more detailed scoring system based on *ASIC3 quantitation* using either automated microscopic quantification methods of analysis on the immunohistochemistry, or immunoblotting of mucosal biopsy samples. With adequate controls and appropriate equipment, quantification methods using digital computer assisted method had proved reliable[193], consistent and reduced observer bias[194] . Automated quantification is recommended

for more complicated analysis where traditional methods may have limitations[195]. The density of each morphology sub-type in the relevant layer should be better quantified. As such, the current findings of increased ASIC3 with increasing symptoms must be considered speculative at best. In future, perhaps final patient scores might be plotted against the severity of symptoms and extrapolated to provide a correlation line that has the potential to be developed into a predictive guide to symptom severity.

The final limitation is the lack of any clear understanding of the significance of different morphological characteristics of ASIC3 staining patterns. Broadly, the results show that there is more ASIC3 in general in patients with GORD than for the controls, and its distribution changes slightly. Both observations could have been greatly supported by first qualitative demonstration of an appropriate mRNA transcript (by PCR) and the former by quantitative data obtained from alternative methods, especially qRT-PCR. It has been noted that the current theory of retrograde neuronal transportation suggests that the up-regulation of protein synthesis for *neuronal* proteins occurs in the DRG and that this could thus not be captured in epithelial biopsies. However, this is not true of *epithelial* proteins, where it is likely that protein synthesis occurs within the epithelial granular layer, where mitotic activity is high and continues as these cells mature towards the luminal surface. RT-qPCR will certainly have a role in exploring the mechanisms of epithelial protein up-regulation in a future study.

3.4.3 Clinical relevance

3.4.3.1 ASIC3 as biomarker of GORD

Although the preliminary results are insufficient to recommend ASIC3 as a biomarker at the current time, the quest to assert whether ASIC3 could function as a biomarker of peripheral sensitisation remains. The only statistically significant results were the ‘membrane bound’ appearance of ASIC3 being significantly different between the groups. This was limited by the small numbers and difficult to comment. More importantly, was the pattern being established in the other morphology sub-groups seemed to indicate acid exposure or disease sub-types were important to trigger ASIC3 expression in the epithelium; increasing from controls to EO. As mentioned

above in 3.4.2, we hope to look for ASIC3 as a marker for symptom severity, not as another disease marker of EO, NERD or FH. I speculate that the stratum spinosum 'membrane-bound' appearance to be clinically more important as hinted by the statistical significance (despite small numbers), as well as it likely to represent the physiologically active morphology of ASIC3 important in luminal sensation (being found in the more superficial layer of epithelium and on the epithelial cell membrane itself).

The role of ASIC3 has been attributed mainly to sensation[50], but the experimental use of an ASIC3 inhibitor has also been shown to reduce hyperaemic[136] response to acid. ASIC3 is not unique in this role; TRPV1 is also associated with inflammation, with several studies demonstrating clearly that TRPV1 activation leads to neurogenic[196 161] and epithelial[197 198] inflammatory mediator release, e.g. Substance-P, CGRP and PAF, which form an important peripheral sensitisation (PS) mechanism[160 161]. Neither a cause nor an effect of this relationship has been established for ASICs, but they might function similarly. In keeping with this hypothesis is the finding that NERD patients had more ASIC3 characteristics. NERD patients will present with the highest symptomatology and most consistent VPH to a variety of stimuli including acid[158 159]. However, NERD patients by definition have minimal physical evidence of inflammation. Therefore, it is unlikely that inflammation either leads to, or is caused by, increased ASIC3 expression; rather, ASIC3, by some way mediating signals from acid exposure of the epithelium, may lead to neuronal sensitisation in the absence of any major effect on inflammation.

3.4.3.2 ASIC3 and symptoms

The percentage of patients with certain ASIC3 characteristics increased with the severity of symptom profiles. An increase in symptom severity was associated with an increase in all four ASIC3 features. This association was more pronounced for three symptoms: acid complaints, sleep disturbance and lower GI discomfort (also defined as 'indigestion' in the questionnaire).

The first association is in keeping with the general hypothesis of ASIC3 as an acid sensory and a nociceptive receptor in published studies[136 183]. The associations with sleep disturbance and indigestion are more difficult to explain, as these are more

general symptoms and we postulate that they could relate to the role of ASIC3 in mechano-transduction[48]. Page, *et al*.[48] established a clear relationship between the expression of oesophageal ASIC3 and increased action potentials leading to changes in contractility and motility. An increase in ASIC3 in GORD symptoms may be responsible for altered motility; this, aside from the sensation of altered pH per se, could contribute to further symptoms. An increase in the firing of action potential resulting from an increase in ASIC3[48] could also be interpreted as a negative feedback mechanism providing distal visceral relaxation and promoting better gastric emptying, in order to relieve intra-gastric pressure and reduce reflux.

3.4.3.3 Modulation of ASIC3 as a potential treatment for GORD symptoms

Although the use of an ASIC inhibitor has been efficacious in reducing animal hyperaemic[136] responses to acid infusion in the rat oesophagus, the action of ASIC3 in gut motility and mechano-sensation need to be explored further before translation into human therapy. Clinical utility of the ASIC3 receptor antagonist will have to presumably clear the same hurdles as TRPV1 in terms of ubiquitous expression and thus adverse events and generic side effects. Novel and experimental ASIC3 modulating agents will be discussed in Chapter 6.

3.6 CONCLUSIONS

This study showed that epithelial ASIC3 in biopsy samples is present, but epithelial ASIC3 characteristics have weak association with clinical classification. Similarly, there was a significant but modest association between ASIC3 characteristics with an increase in symptom severity. Although there were several limitations in this study, overall it highlighted some interesting ASIC3 cellular expression, important clinical associations, relevance of epithelial biomarker and feasibility. Future studies are required to determine whether ASIC3 is a practical/useful marker of GORD symptoms.

4 HUMAN MODEL OF VISCERAL PAIN HYPERSENSITIVITY: THE MODEL, REPRODUCIBILITY AND VARIABILITY

4.1 INTRODUCTION

4.1.1 The need for a VPH model

Visceral pain hypersensitivity (VPH) is a concept that has evolved rapidly over the last few decades and is considered to be an important patho-physiological mechanism for the development of visceral pain in functional gastro-intestinal disorders (FGIDs). However, studies in patients with established FGIDs make it difficult to tease out the individual mechanisms involved in the development of VPH, as quite often these patients present with multiple factors that may have an impact on our understanding of VPH. These include psychological and physiological factors, which are much easier to control for in healthy volunteer studies. Animal VPH models have leaned away from the oesophagus to observe pathophysiological changes in other organs in response to experimental induction of injury, inflammation or both[74 199]. In man, these regions, e.g. the colon and rectum, have been the subject of extensive studies in patients with inflammatory disease states, such as ulcerative colitis, and in functional disorders characterised by hypersensitivity, such as IBS[200], but have not been well-modelled in healthy volunteers[201 202] for reasons of accessibility/acceptability.

For the above reasons, work conducted in the department has focused on understanding both the causes of VPH and the factors that modulate it. This has been performed by developing and using a healthy volunteer model of acid-induced VPH to explore hypersensitivity in human oesophagi. However, for this model to be accepted as a standard, it is important to demonstrate that it is reproducible. This is particularly relevant when a particular model is considered for use in pharmacological studies. Prior to the start of my interventional study using this VPH model, I have analysed the results of previous studies to determine its reproducibility. For the purpose of the rest

of the chapter, and to make it easier to link to subsequent chapters, VPH in the oesophagus will be referred to as 'oesophageal' visceral pain hypersensitivity (VPH).

4.1.2 The human VPH model

In the human oesophagus, common clinical conditions where VPH is believed to play an important role include non-erosive reflux disease (NERD) and functional heartburn (FH) (refer to Chapter 1). A human model using acid perfusion in the oesophagus to induce VPH in healthy volunteers was developed in the department. This was a robust model of human oesophageal sensitisation using acid infusion in the distal oesophagus, demonstrating the induction of pain hypersensitivity both in the distal acid exposed region (primary hyperalgesia), the proximal non-acid exposed oesophagus and in the area of somatic referral on the anterior chest wall (secondary hyperalgesia) [74] (Chapter 1: 1.5.4: Human models of peripheral and central sensitisation). This secondary hyperalgesia is attenuated by Prostaglandin E2 receptor-1 (EP-1) receptor antagonism [203], and both can be prevented and reversed with Ketamine, a N-Methyl D-Aspartate (NMDA) receptor antagonist [75], suggesting that central sensitisation (CS) is an important mechanism for the development of secondary hyperalgesia in this model. Indeed, cerebral cortical responses to this sensitisation model have now also been studied[204], which demonstrate a reduction in the latency of these potentials after oesophageal acid infusion – suggesting sensitisation of afferent pathways. This model has been used extensively in many studies including drug trials[76 205 206].

4.1.3 Aims

The first aim was to explore the reproducibility and variability of sensitisation in the model of acid-induced oesophageal hypersensitivity, through a retrospective analysis of departmental studies. Secondary aims were to identify limiting factors within the model and ways to overcome these problems.

4.2 SUBJECTS AND METHODS

4.2.1 Subjects

As some subjects participated in more than one study, 57 volunteers contributed to 85 separate 'infusions'. These studies were carried out between 2002 and 2006. These 57 healthy volunteers (age range 20-58 years old, but due to a strict coding system, sex distribution could not be determined for pooled data) were from previous studies performed in GI Sciences at Manchester University.

4.2.2 Methodology

A total of six studies were included – five drug trials and one physiological study. The data collected were taken from the placebo and 'screening' arms of these drug and physiological studies, which were conducted by two main researchers. Screening studies were performed in two drug trials to identify sensitisers to acid infusion before randomisation. The importance of introducing a screening visit is discussed further in section 4.4: Discussion. The results of these studies regarding the effects of specific drugs on the model have been published previously[76 205 206] (Table 4.1). I analysed the data retrospectively from these studies.

4.2.3 Acid Infusion and Electrical Stimulation Model

The human oesophageal VPH model is a validated model involving the infusion of physiological HCl (0.15M) via a naso-oesophageal tube to the distal oesophagus. This acid infusion lasted for 30 minutes. Baseline pain thresholds (PTs) to electrical stimulation in the oesophagus were measured in the proximal as well as distal oesophagus before and after acid infusion. The timing of post-acid electrical stimulation varied from study to study (Table 2.1). The electrical stimuli were delivered via electrical simulation catheter (with proximal and distal bipolar rings) at a frequency of 0.3 Hz, 500microseconds duration; intensity 0-100mA. Further technical details and equipment used for this model are described in greater detail in Chapter 5 where they was used in a drug study (Section 5.2.5 and 5.2.6).

Intervention used in the studies	*Timing of PT measurements post-acid*	*Number of Subjects*	*Visit type*
1. Ketamine[76]	TI, T30, T60, T90, T120	13	Placebo
2. Paracoxib[205]	TI, T60, T120, T180	6	Placebo
3. Valdecoxib[205]	TI, T30, T60, T90	10	Placebo
4. Neurokinin-1 receptor antagonist[206]	T30, T60, T90, T120, T150	9	Placebo
5. Glycine antagonist	TI, T30, T60, T90, T120	17	Placebo Screening
6. ANS physiological characterisation	TI, T30, T60, T90, T120	13	Screening

Table 4.1: Recruitment of subjects from six different studies involving 85 separate distal oesophageal acid infusions (TI =immediately, T30=30 minutes, T60=60 minutes, T90=90 minutes, T120=120 minutes, T150=150 minutes T180=180 minutes, all calculated from after completion of acid infusion)

4.2.4 Statistics

Only data from electrical stimulation of the proximal oesophagus after distal oesophageal acidification were included in the analysis, as these were most relevant to my studies. Raw data from the different studies were compiled and analysed. These compilations and raw data analyses were performed using Excel (Microsoft Corp, USA), SPSS (IBM Corp, New York, USA) and Stata (Stata Corp LP Texas USA). The methods used included tests for normality, means (with 95% CI) and medians (with inter-quartile ranges). For the main comparisons of maintenance of sensitisation across experimental time points (4.3.2: Time Effects), the results were analysed with a linear mixed model with 'change in PT' as an outcome, along with adjustments made for repeated measurements per person per study (GLLAMM command in Stata). For the sub-analysis looking at the order of visits, p value and difference in means were calculated from longitudinal regression, which also controlled for the same individuals and randomisation allocation. Placebo effect analysis was performed similar to order effect, controlling for the same individuals only.

4.3 RESULTS

4.3.1 Threshold of sensitisation

In the pooled data from the six studies, distal oesophageal acidification resulted in a fall in PTs at all time intervals in the proximal oesophagus post-acid infusion. The average change in PT after an infusion was a reduction of 7.4mA (-8.4 to –6.4 $p<0.001$). Individuals with both absent and exaggerated sensitisation to acid were noted. A 6mA decrease was used in most studies to define sensitisation to acid infusion, and at this threshold analyses at just 60 minutes after acid (T60 being the consistent time point used in all studies) sensitisation were noted in 74% of infusions (63 out of 85 separate acid infusions). The use of 6mA in the early studies was arbitrary. However, looking at the different time points, variations in the number of subjects who sensitised were noted when different thresholds were used to define sensitisation in this model (Table 4.2).

PT threshold	***Percentage sensitisers at all time points***
5mA	73.3%
6mA	69.8%
7mA	59.1%

Table 4.2: The magnitude of 'change in PT' in the proximal oesophagus to define sensitisation after distal oesophageal acid infusion and the corresponding percentages of participants who sensitised.

4.3.2 Time effects

There were no statistically significant differences in the change in PT from 30 minutes to 120 minutes after acid infusion (Figure 4.1 and Table 4.3).

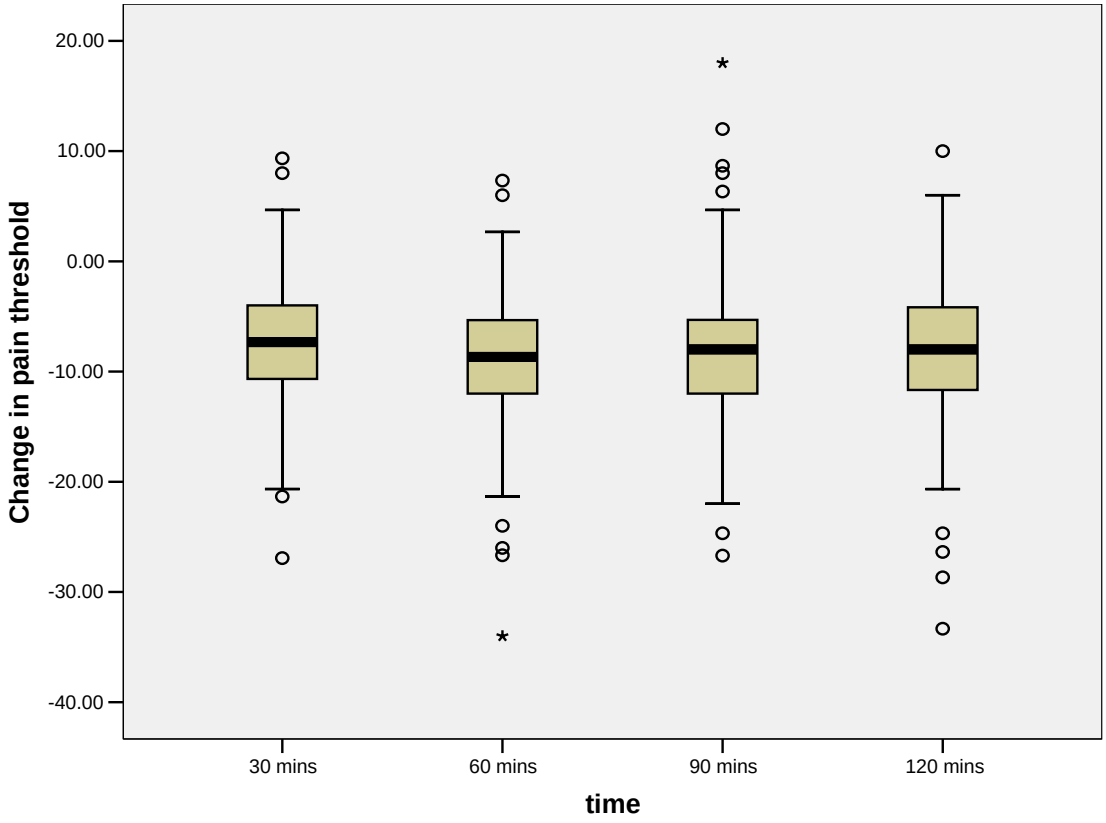


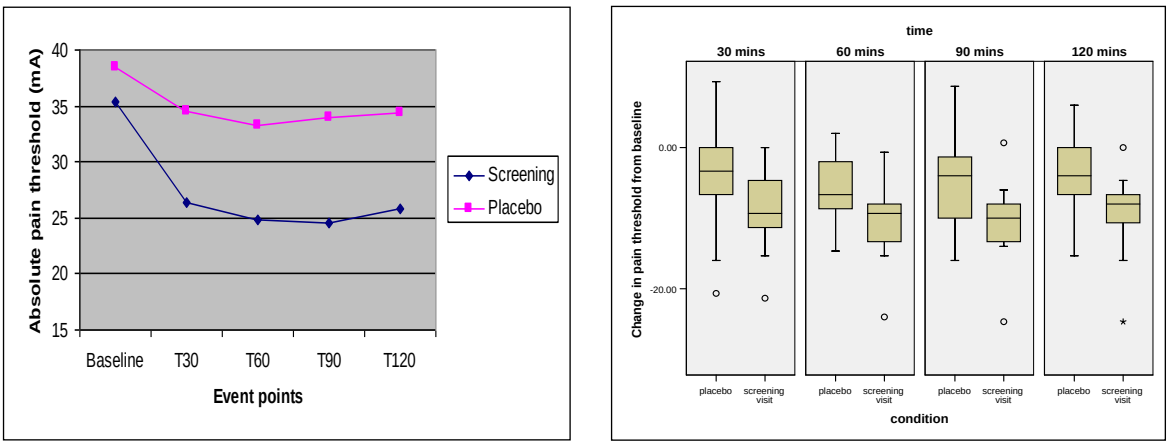
Figure 4.1: The drop in PT and its maintenance in the proximal oesophagus after distal oesophageal acid infusion. Compared to the baseline, the drop in PT across all time points was significant. Using linear mixed model with 'change in PT' as an outcome adjustments made for repeated measures per person per study, p<0.001. The main bar line represents mean, with 95% CI within the main box.

Reduction in PT	***Mean (95% CI)***	***p-value***
30 mins	-7.4(-8.4 to -6.3)mA	<0.001
Difference between 60 mins and 30 mins	-0.4(-1.5 to 0.7)mA	0.482
Difference between 90 mins and 30 mins	0.3(-0.8 to 1.5)mA	0.577
Difference between 120 mins and 30 mins	0.0(-1.1 to 1.2)mA	0.963

Table 4.3: The reduction in PTs at different time points post acid infusion (comparison of PT at 30 minutes was in relation to the baseline).

4.3.3 Placebo effect

Comparing the screening visit with the placebo visit, the placebo effect is present, as demonstrated by the attenuation in sensitisation (+5.4mA; CI 2.8mA to 7.9mA; p<0.001). Again, this comparison was possible in one study only, which had both a screening visit as well as a placebo visit (Study 5). However, since the 'screening' visit took place prior to randomisation, effectively it was always performed prior to the placebo visit; hence, it is important to determine whether the observed differences in PT during the placebo visit in comparison to the screening visit were truly reflective of the placebo effect or related to order effects (Figure:4.2)



A *B*

Figure 4.2: (A) Absolute PT during screening and placebo visits in the proximal oesophagus; note baseline PT 35- 38mA. (B) The 'change in PT' in the proximal oesophagus after distal oesophageal acidification in the placebo visit compared to the screening visit was p<0.001 for all time points, using longitudinal regression controlling for same individuals. Main bar was mean value with 95% CI in the box. Both these charts are from a single study with placebo and screening visits. N=17.

4.3.4. Order Effect

Study 5, with 17 subjects (age 20-49 years, 8 males), produced information on the order of visits, and a screening visit was performed. Comparable numbers of subjects were subsequently randomised to either drug or placebo for the trial. These visits in the trial *post randomisation* were classed as visit 1 and visit 2. The average absolute PT for all time points in visit 2 (including baseline) was 6.8mA (95%CI: 3.1mA to

10.4 mA; p<0.001) higher than visit 1 (Figure 4.3). However, using the linear mixed model with a 'reduction in PT' as an outcome, there was no statistically significant difference in the change in PT after acid compared to the baseline between visit 1 and visit 2. Therefore, although absolute PTs increased with the second visit, the magnitude of change in PT remained the same for all time points (Figure 4.4).

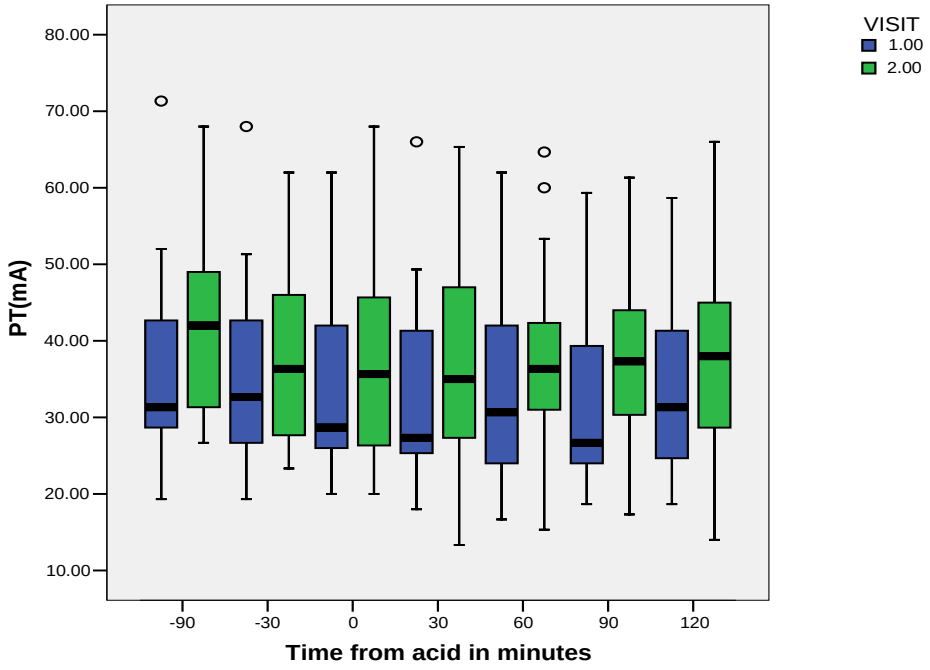


Figure 4.3: The absolute PTs in the proximal oesophagus in the first compared to the second post-randomisation visit, p<0.001 for all time points using longitudinal regression which controlled for the same individuals and randomisation allocation. Main bar was mean with 95% CI within the main box. N=17.

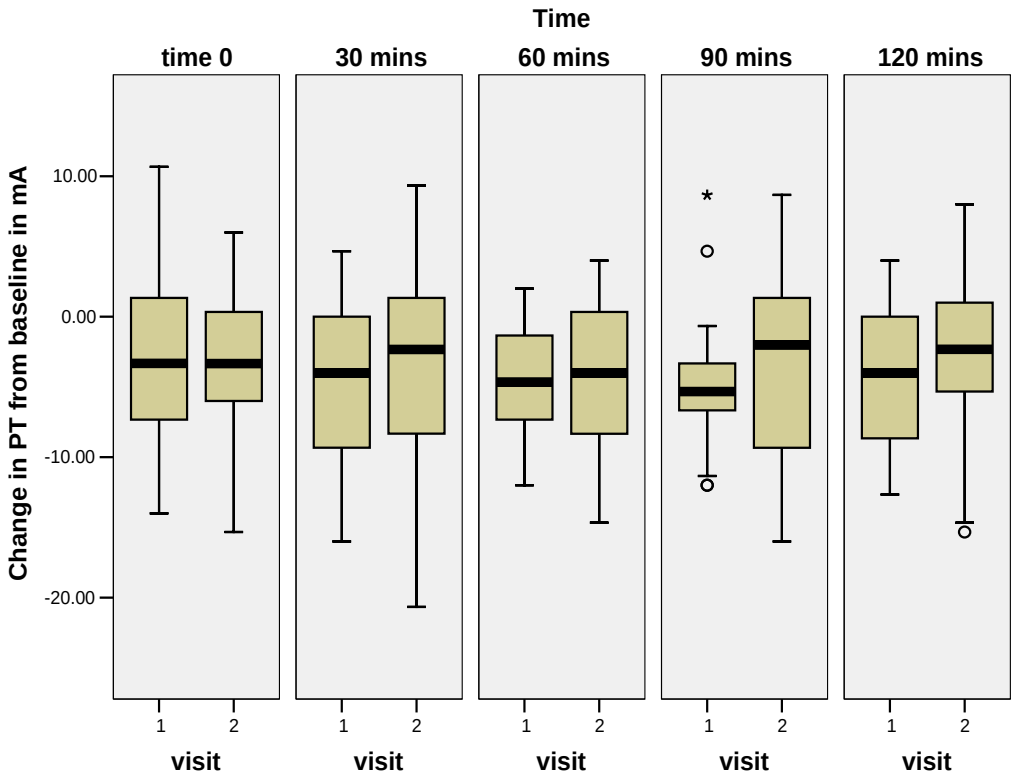


Figure 4.4: The 'change in PT' in the proximal oesophagus after distal oesophageal acidification in the first and second post-randomisation visits at all-time points, p=0.221. P was based on longitudinal regression which controlled for the same individuals and randomisation allocation. Main bar was mean with 95% CI within the main box.

4.4 DISCUSSION

The traditional definition or threshold of sensitisation was set between 5 to 7mA and varied between studies. This threshold range was originally set arbitrarily, but thought to be clinically significant. Now, with the benefit of more studies completed using this model, I was in a better position to review and identify a more precise threshold. From one of the studies with absolute values available for both the screening and placebo visits, the data confirmed that the mean baseline pain threshold (PT) in the proximal oesophagus during electrical simulation was 35mA for the screening visit and 38 mA for the placebo visit. A drop in PT of 5-7mA after acid sensitisation corresponded to a change of roughly 13-20% compared to mean baseline PT (prior to acid sensitisation). Ideally, selecting a higher threshold would ensure more significant change, but that had to be balanced with a realistic threshold to capture the majority of the population.

If the magnitude of sensitisation was defined as a reduction in pain threshold of 6mA, then sensitisation occurred in 70% of participants for all time points. Increasing this to 7mA dropped sensitisation to 59% of the study population. Taking these two factors into account, 6mA is an optimum threshold for representing a significant clinical change, and it still captures more than two-thirds of the population.

From the data, there seemed to be a wide variation in the degree of sensitisation, and up to one-quarter of the volunteers in the general population did not sensitise. However, in those who did, sensitisation was shown to be maintained for up to 120 minutes after acid infusion. Furthermore, there did not seem to be significant changes between time points within these 120 minutes. Future studies could shorten the post acid stimulation time points to two events. In most studies investigating the effect of pharmacological and non-pharmacological intervention to influence sensitisation, non-sensitisers would not be useful recruits. Therefore, we learnt that an initial screening visit is necessary to exclude these non-sensitisers. The ideal study model for studies intending to use this model would use a crossover method, allowing a suitable period of washout time depending on the half-life of the drug used. Analysis of results using crossover design would allow pairing of the same subject, avoiding the need for case control or matching in the case of parallel design. With a wide variation in the distribution of data, case control or parallel design would require a high number of subjects. However, one disadvantage of the crossover design which requires an additional screening visit is that it would require the participant to attend multiple visits. Therefore, we need to examine the effects of multiple visits and acid infusion on this model, especially after the mandatory screening visit.

In one of the studies above, a screening visit was introduced. In this study, I examined PT and sensitisation in the two visits post-randomisation, where they were either subjected to a placebo or drug to explore if the sensitisation and robustness of this model would last up to the third visit. Although there seemed to be a consistently higher absolute PT for the later visit compared to the earlier one across all time points, the degree of sensitisation (defined as *change* in PT) was maintained for up to 120 minutes (Figure: 4.4). This further strengthens the argument that a crossover study

design is robust, as multiple visits did not seem to diminish the sensitising effects of distal acid infusion.

It is good practice to have a placebo arm in any interventional studies, including drug studies. In most interventional studies, the placebo effect is present. In drug studies using this model, we noted a significant placebo effect compared to the screening visit. Therefore, for an intervention to be considered effective, it has to exert an effect above that of the placebo, as order effects can occur. As already discussed, there was no *change* in the degree of sensitisation between visits post-randomisation. However, although it was possible for me to study the order effect between the screening and all the subsequent visits, since either a drug or placebo had been introduced post-randomisation, therefore, comparison of the order effects between these visits would not be valid. Order effects should be best studied in a prospective study involving multiple visits (3 or more) controlling for all other factors without introduction of drug or placebo. Below is a table summarising all of the factors influencing sensitisation (Table 4.3).

Influencing factor(s)	***Observed effect(s)***	***Recommendation(s)***
Definition of sensitisation threshold	PT drop of 6mA represented a change of up to 20% in PT. It also captured almost 70% of the population	A sensitisation threshold of 6mA to be used consistently
Sensitisation does not occur in everyone	70% sensitised across all time points at 6mA	An initial screening visit to rule out non-sensitisers for drug studies
Variation in magnitude of sensitisation	Wide variation existed within population	Crossover model, where volunteers will act as their own control group; in parallel group, use matched population or larger sample size will be required
Time effect	Sensitisation lasted up to 120 minutes and maintained	Duration of study and protocol could be

	at 4 separate time points; 30, 60, 90 and 120 minutes post acid infusion	simplified to less than 120 minutes and less time points
Placebo effect	Placebo effect detected	Placebo control group must be used
Order effect	Increase in absolute PT after visit 1, but 'change' in PT not affected	To analyse results on 'change' in PT

Table 4.4: Summary of potential limiting factors on the model, how they influence the model and recommended solutions.

4.4 CONCLUSIONS

Distal oesophageal acidification within this VPH model induced a reduction in PT in the proximal oesophagus in the majority of subjects (up to three-quarters), and the magnitude of sensitisation remained comparable for studies using multiple visits. Limiting factors within this model were identified and provided appropriate controls were taken (the introduction of a mandatory screening visit, placebo controlled arm, cross-over study design), this is a robust model for human studies of pharmacological and non-pharmacological modulation of human oesophageal acid induced VPH. Using findings from this experience and the model, we designed and explored the effect of Pregabalin in influencing acid-induced oesophageal VPH.

5 PREGABALIN PREVENTS THE DEVELOPMENT OF VISCERAL PAIN HYPERSENSITIVITY IN A MODEL OF OESOPHAGEAL ACID-INDUCED SENSITISATION IN HEALTHY VOLUNTEERS

5.1 INTRODUCTION

5.1.1 Background

Gastro-oesophageal reflux disease (GORD) causes symptoms that can interfere with quality of life. It is a very common disorder, with 20–44% of Western people experiencing GORD symptoms at least once a month and 20% weekly[207 208]. GORD can be divided on the basis of endoscopic findings into erosive oesophagitis (EO) and non-erosive reflux disease (NERD)[209]. Other patients are defined as having functional heartburn (FH) when symptoms occur in the absence of abnormal acid exposure. Studies of acid exposure across the GORD spectrum have revealed an increasing prevalence of abnormal oesophageal acid exposure times, as defined by 24-hour pH monitoring while one progresses from FH and NERD through to EO[210 211]. This suggests that a factor other than acid reflux must be responsible for symptom generation in patients with NERD and FH [212]. It is proposed that visceral pain hypersensitivity (VPH) is more common in patients with NERD and FH in comparison to EO and may cause symptoms in response to either physiological amounts of acid reflux or other luminal stimuli[213 214]. The VPH mechanism is unclear but involves (amongst other potential mechanisms) the dilatation of epithelial intercellular spaces[98] and exposure of sub-epithelial nerves to acid[215 216]. This leads to the sensitisation of peripheral afferent nerves (peripheral sensitisation (PS)) and spinal dorsal horn neurons[77] (central sensitisation (CS)) . Once CS is established, it can continue to potentiate pain after initial peripheral stimulus is discontinued.

In addition to the above mentioned VPH mechanisms, psychological stress can also increase sensitivity to acid[217] as well as exacerbate symptoms of GORD[218]. Relaxation, on the other hand, reduces pain during experimental oesophageal acid exposure[219]. Furthermore, stress has a direct effect on the autonomic nervous system

(ANS), and there is recent preliminary evidence that acid exposure in the oesophagus alters the ANS profile differently in healthy subjects more susceptible to developing VPH compared to those who are more resistant[93 220]. However, the roles of stress and ANS in influencing the effectiveness of treatment for VPH are not known.

A model in which the infusion of acid in the distal oesophagus induces sensitisation at the site of acid infusion (primary hyperalgesia), as well as in the proximal oesophagus (secondary hyperalgesia) due to PS and CS, respectively, has been described previously [77 203 221]. Interestingly, anxiety induction exacerbates secondary hyperalgesia in this model. Furthermore, this model has been used to study the effects of various drugs on VPH[75 203 205 206]. For instance, Ketamine, an N-methyl D-Aspartate (NMDA) receptor antagonist, was effective in both preventing and reducing secondary hyperalgesia induced by experimental acid infusion[75]. However, Ketamine has considerable central side effects and requires intra-muscular or intravenous administration, making it impractical for routine use in practice.

Pregabalin is a specific ligand of alpha-2-delta type 1 and 2 subunits of voltage gated calcium channels[222], which can be given orally and act centrally. Pregabalin is structurally related to GABA but does not interact with GABA[223] or NMDA receptors[224]; however, it reduces pain modulators including substance P[225] and glutamate in the brain[224]. It is a gabapentinoid-like gabapentin, but has more predictable pharmacological effects and achieves therapeutic outcomes at lower doses, thereby reducing dose-related side effects. It is effective in non-inflammatory somatic pain including neuropathic pain[226] and pain associated with diabetic neuropathy[227 228] and fibromyalgia[229 230 231]. Recently, it has been demonstrated that Pregabalin increases pain thresholds to rectal distension in patients with irritable bowel syndrome (IBS) [232].

5.1.2 Aims

The aim of this study was to determine the effect of Pregabalin on reducing acid-induced oesophageal secondary hyperalgesia (compared to the placebo) in this model (primary outcome). Secondary outcomes were established to determine whether:

1. Pregabalin affects subjective acid pain and discomfort

2. Psychological states or traits influence the magnitude of acid-induced VPH pre and post Pregabalin
3. An individual's autonomic profile at baseline and in response to oesophageal acid infusion is associated with the effectiveness of Pregabalin.

5.2 PATIENTS AND METHODS

5.2.1 Study design

A prospective, double-blinded, placebo-controlled, cross-over study was conducted over three visits (CONSORT[233 234 235]diagram: Figure 5.2). Volunteers who sensitised to acid at the screening visit were included in subsequent studies. After visit 1, eligible volunteers were randomised (performed by the Barts and the London NHS Trust pharmacy using a computer-generated randomisation schedule) and given medications with written instructions. Visits 2 and 3 were undertaken after completing the course of medication.

5.2.2 Pregabalin dosing

Based on the results of previous studies[236 237], Pregabalin (Pfizer, Kent, UK) was self-administered 75 mg twice daily for three days, 150mg twice daily on the fourth day and 150mg in the morning of the return visit on the fifth day (12 x 75mg capsules total provided in a single bottle). Medications and instructions were given at the end of visits 1 and 2. The interval between visit 1 (screening visit) and visit 2 was at least seven days, as retrospective analysis of departmental data has shown sensitisation is reproducible within one week. However, the interval between visits 2 and 3 was at least two weeks to allow time for the complete washout of Pregabalin. Volunteers were asked to return the medication bottle on subsequent visits.

5.2.3 Subjects

Adult healthy volunteers, aged 18 to 60, were recruited by advertisement. All had normal medical assessments including a detailed medical interview, and none was taking any regular medication. Urine tests were performed at all visits to exclude pregnancy (First Step FS208 Euromed Limited, UK) and drugs of abuse (Triage 8 TM, Biosite San Diego USA). An oesophageal manometry was carried out on each

subject at the screening visit to exclude motility disorders and to locate the lower oesophageal sphincter (LOS). Written informed consent was obtained from the participants after the nature and the purpose of the trial had been explained. The protocol was approved by the local ethics committee (Research ethics committee reference: 07/MRE08/39 and MHRA (Medicine and Healthcare products Regulatory Agency). Details of the ethical application and MHRA are set out in Appendix 5.01-5.02.

5.2.4 Intubation and set-up

All experiments were conducted with subjects seated on a couch, having fasted for a minimum of six hours and usually from the night before. A short medical interview was performed during all visits, including general health, and at visits 2 and 3 a short interview on side effects and compliance. The catheter and pH probe assembly was passed trans-nasally into the oesophagus without local anesthetic. Only water-based gel (KY Jelly, Johnson & Johnson) was used to lubricate the catheters. Electrical stimulations of the oesophagus and a control area (foot) were performed at four time points: baseline prior to acid infusion and 30 minutes and 90 minutes after 30-minute continuous acid infusion.

5.2.5 Oesophageal acid infusion

Hydrochloric acid (HCl; 0.15 mol/L) was infused through a port in the electrical stimulation catheter (Gaeltec, Isle of Skye, UK), sited 3 cm above the lower oesophageal sphincter (LES) at a constant rate of 8 ml/min for 30 minutes via an infusion pump (Graseby Medical, Hertfordshire, UK). Previous studies have indicated that this acid concentration induces VPH in the majority of healthy subjects[74 88]. A twin-channel pH catheter (Synectics Medical, Enfield, UK) measured pH in both the proximal oesophagus (at the site of electrical stimulation) and in the distal oesophagus (at the site of acid infusion) for the duration of each study.

5.2.6 Electrical stimulation

Oesophageal electrical stimulation was delivered using a pair of 1cm spaced silver-silver chloride bipolar ring electrodes (Gaeltec, Isle of Skye, UK), as described in detail previously[76 205 206]. To act as a somatic control, pain thresholds to electrical

stimulation were determined on the dorsum of the right foot using silver-silver chloride electrodes with 2 cm spacing: frequency 0.3 Hz, square wave pulses of 500 μs duration; intensity 0 - 100 mA (Model DS7, Digitimer Ltd, Welwyn Garden City, UK).

5.2.7 Outcomes

Primary outcome: pain threshold to electrical stimulation was recorded in the proximal oesophagus (19 cm above the lower oesophageal sphincter) as the lowest intensity in mA at which the subject reported pain to 2 mA stepwise stimulus increments. Measurements were taken at baseline, 30 minutes and 90 minutes after the completion of acid infusion. An average of three readings was taken for each time point (Figure 5.1)

Secondary outcomes

(1) Visual analogue scale (VAS) ratings of pain and unpleasantness intensity were assessed subjectively for acid infusion. A score of 0 was completely without pain or unpleasantness, 3= mild, 5= moderate, 7= severe but tolerable, 10= maximum possible pain/ unpleasantness.

(2) Psychological assessments: State and trait anxiety were assessed using the Spielberger Trait Anxiety Index (STAI) questionnaire: STAI-S for anxiety state and STAI-T for anxiety trait. Both questionnaires were completed at the start of the screening visit, with the STAI-S assessed again at each separate visit following drug administration, as well as after volunteers were connected to the autonomic equipment and had shown stable autonomic recordings. This was prior to intubation and acid infusion.

(3) Autonomic recordings: In all experiments, non-invasive arterial blood pressure, pulse, ECG and respiratory rate were continuously monitored. Efferent parasympathetic activity was measured by deriving a continuous cardiac vagal tone (CVT) index (Neuroscope, MediFit Instruments Ltd, London UK)[238-240]. Sympathetic activity was measured using R-R interval data to produce the Cardiac Sympathetic Index (CSI) [241]. A modified Einthoven's lead II electrocardiogram was acquired by the Neuroscope using three pre-jelled ECG electrodes (ConMed Cleartrace ECG electrodes, ConMed, Swindon UK). Electrode sites were below the lateral aspects of the right and left clavicles and the left mid-clavicular line below the left nipple. Blood pressure was measured continuously with a Portapress non-invasive blood pressure

monitor (Finapres Medical Systems, Paasheuvelweg 34a, NL-1105 BJ, Amsterdam ZO, Netherlands). Respiration rate was monitored via a transducer placed around the lower chest (Smartbelt Braebon Medical Corp, New York, USA). All autonomic recordings were acquired continuously during the study period, but separated by marking of various time periods in the protocol including the five events: baseline, pre-acid electrical stimulation during the entire 30-minute acid infusion, as well as 30 minutes and 90 minutes post acid electrical stimulations. The mean of three periods, as demarcated by the markings for three electrical stimuli for each event point, were used for ANS measures during electrical stimulation. During acid infusion the mean of the whole 30-minute period was used.

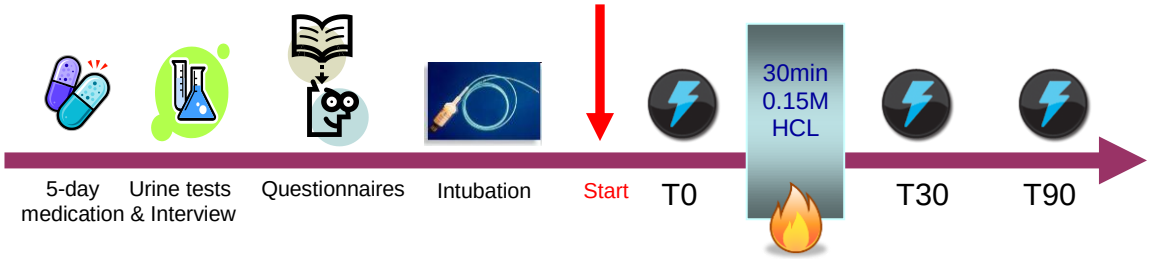


Figure 5.1: Protocol for the sequence of events and outcomes during each study visit

5. 2.8 Data Analysis

Retrospective analysis of data from 57 subjects (aged 20-58 years) confirmed that subjects who were successfully recruited into previous studies (sensitised) had average PT (proximal oesophagus) changes of -7.4mA (-8.4 to -6.3) at 30 minutes post acid infusion. This was maintained for up to 120 minutes [242]. On the basis of intent to treat, and using a more conservative estimate of -6mA, 16 subjects were required to detect this difference with 90% power at the 5% significance level. Data on heart rate, R-R interval, CVT and mean arterial pressure (MAP) were extracted from the Neuroscope. R-R interval data were reformatted for entry into the Cardiac Metric program (CMet) [243] for the calculation of the Cardiac Sympathetic Index [241]. All data were cleaned and analysed prior to un-blinding. Outcomes took the form of normal and non-normal data, and appropriate parametric and non-parametric data analyses employed using proprietary software (GraphPad Software, Inc., California, USA). 'Column statistics' command weas used to test for the normality of data, median and data spread. As

outcome data were non-parametric, the Wilcoxon paired test was used to compare outcomes for placebo and Pregabalin visits.

5.3 RESULTS

5.3.1 Subjects and recruitment

A total of 21 subjects were recruited (Figure 5.2). Two subjects could not tolerate nasal intubation (even though intubations were successful), while a further two showed no evidence of oesophageal sensitisation following acid infusion during the screening visit and were excluded as per protocol. One subject decided not to continue with the study post-randomisation, leaving 16 subjects who completed all three visits. All were naïve subjects never previously subjected to this model of acid perfusion. Of the 16 subjects who completed the study, one subject was excluded from analysis. This volunteer reached maximum pain threshold (100mA) for all stimuli for the second and third visits, and thus any difference between the placebo and drug would not be detected. Results from a total of 15 subjects were analysed: age 21-56 (median 31) years, six female.

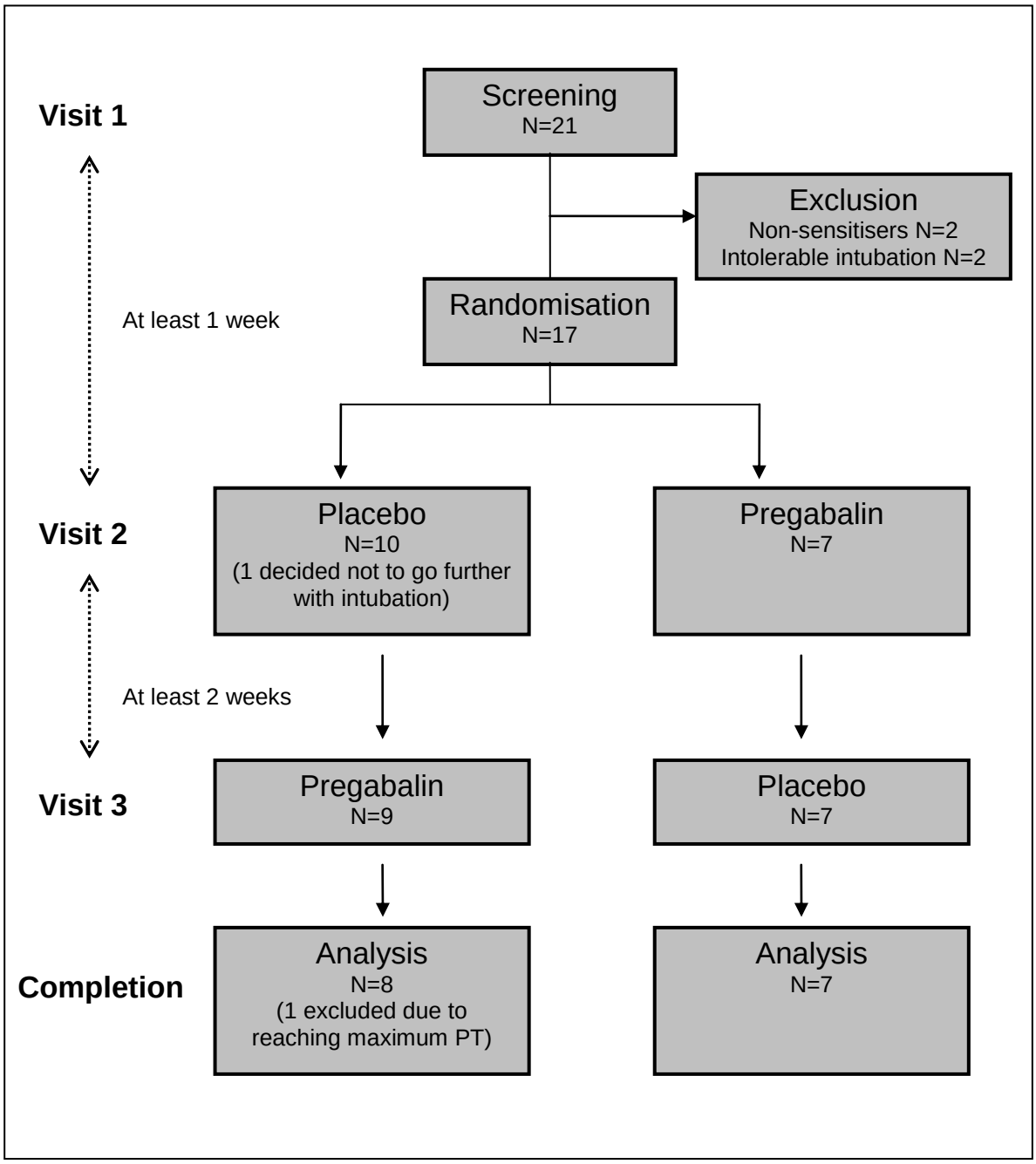


Figure 5.2: CONSORT flow diagram[233-235] illustrating the progress of volunteers through this trial including recruitment, enrollment, randomisation, withdrawal and completion. Final analysis: N=15.

5.3.2 Oesophageal acid infusion

During acid infusion, the pH level fell to < 2 in the distal oesophagus but remained > 6 in the upper (unexposed) oesophagus. The most common symptom reported with acid

infusion was nausea. Other sensations included a cold sensation in the chest region and the feelings of hunger and/or heartburn.

5.3.3 Drug administration and adverse events

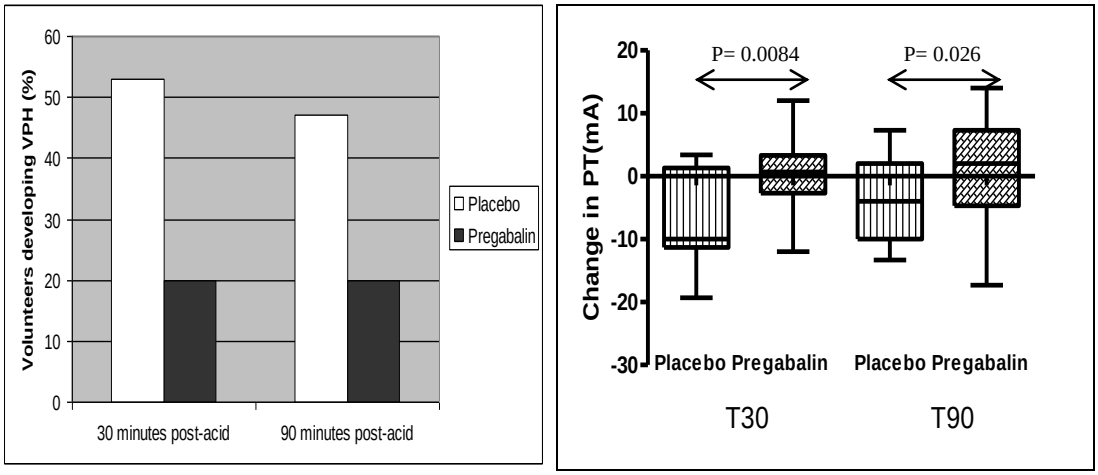
All 15 subjects complied fully with medication instructions. Compliance was acceptable based on the number of capsules found in the returned bottles and self-reporting, while the mean number of capsules remaining per visit (12 in total) were placebo (0.47, CI 0.005-0.9) and drug (0.33, CI -0.07 to 0.7). As expected, mild drowsiness was the most common side effect reported[244-246]. Euphoria was reported in one subject. Dizziness was also reported in two subjects, once after the placebo visit and once after the Pregabalin visit. Seven subjects were completely symptom free from any symptoms at all visits.

5.3.4 Primary endpoints

At the screening visit, all subjects demonstrated proximal oesophageal sensitisation following distal acidification (as per inclusion criteria). The proportion of sensitisers was significantly reduced from 53% to 20% at 30 minutes and 47% to 20% at 90 minutes after treatment with Pregabalin in comparison to the placebo (Figure 5.3). There was no difference in the absolute values of pain thresholds (PT) at baseline in subjects after receiving the placebo or Pregabalin (median 36mA (15.3-41.3) vs. 30 (17.3-48.7), p=0.42). However, Pregabalin prevented the development of acid-induced VPH in the proximal oesophagus (Figure 5.3 B). At 30 minutes, the median change in PT for all subjects was -10.0mA (-11.3 to +1.3) after treatment with the placebo, compared to an increase of +0.70mA (-2.7 to +3.3) after Pregabalin (P = 0.0084). This pattern was also observed at 90 minutes: the change in PT was -4.0mA (-10 to +2.0) after the placebo and +2.0mA (-4.7 to 7.3) after Pregabalin (P = 0.026).

Control area: Pregabalin had no significant effect on baseline somatic (foot) pain compared to the placebo (placebo 38mA (24 to 46) vs. Pregabalin 36.7mA (31 to 73), p=0.77). At 30 minutes post acid infusion, there was no significant change in PT between the placebo (median 0mA (CI 0 to 3.3)) and Pregabalin (median = 0mA (CI -2 to 2.7), p=0.36). Likewise, there was no difference at 90 minutes: median change in

PT = 0mA (CI -7 to 4) after the placebo vs. -1.3mA (CI -4 to 6) after Pregabalin, p=0.90.

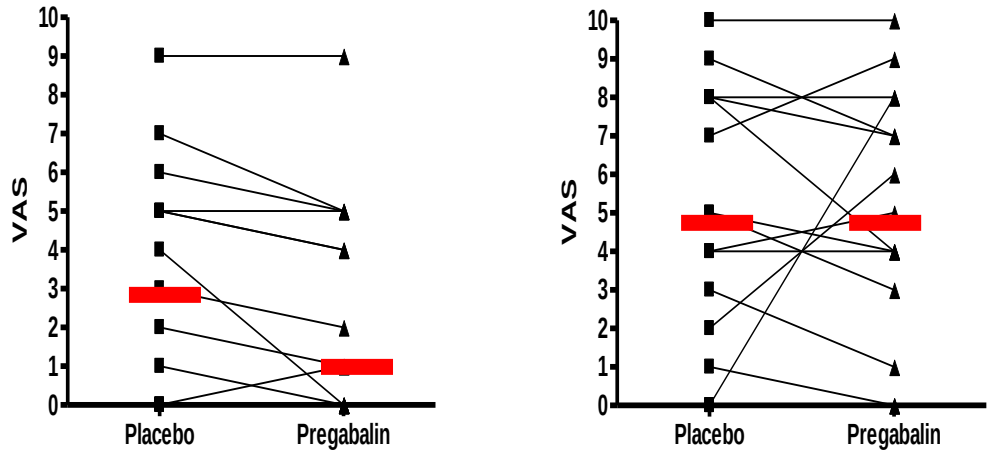


A B

Figure 5.3: (A) Percentage of volunteers who developed sensitisation at 30 minutes and 90 minutes after the placebo and Pregabalin. (B) Change in PT following acid infusion with the placebo and Pregabalin at 30 and 90 minutes. The reduction in PT following Pregabalin administration was significantly reduced at 30 and 90 minutes in comparison to the placebo.

5.3.5 Secondary endpoints

(1) *Pregabalin reduced subjective VAS scores for pain and unpleasantness following acid infusion*: Median VAS was 1 out of 10 for pain for oesophageal acid infusion after treatment with Pregabalin compared to 3 out of 10 after treatment with the placebo (p=0.027). The median VAS score for unpleasantness during acid infusion was unchanged at 5 out of 10 after both Pregabalin and the placebo (p=0.76) (Figure 5.4).



A B

Figure 5.4: VAS scores during acid infusion with placebo and Pregabalin treatment: (A) VAS score for pain was lower with Pregabalin in comparison to the placebo, P = 0.027; (B) VAS score for unpleasantness was similar for the placebo and Pregabalin visits, P = 0.76.

(2) Effect of anxiety state and trait: The anxiety state STAI-S scores were almost identical for the placebo and Pregabalin visits, with very narrow 25-75 percentile ranges (median of 52 (48-53) vs. 51 (50-52) respectively p=0.76). STAI-T did not predict the degree of sensitisation with the placebo or Pregabalin at 30 or 90 minutes (Linear regression of STAI-T vs. sensitisation: Placebo: 30 mins: r2 = 0.12 (P = 0.19), 90 mins: r^2 = 0.001 (P = 0.96); Pregabalin: 30 mins: r2 = 0.22 (P = 0.07), 90 mins r^2 = 0.03 (P = 0.54)).

(3) Effect of Pregabalin on autonomic functions: Taking the combined event point during each visit, Pregabalin significantly reduced parasympathetic variables (CVT) (p=0.0001) but increased sympathetic tone (CSI) in comparison to the placebo (p=0.0007). Looking at table 5.1, this reduction in CVT was evident at the baseline for Pregabalin in comparison to the placebo (p=0.01). In contrast to the other time points of the study, during acid infusion CVT was higher for the Pregabalin visit in comparison to the placebo visit (p=0.03); a higher sympathetic tone (CSI) during acid infusion was also observed for Pregabalin than for the placebo (p=0.04)

	Baseline	T0	Acid	T30	T90
CVT					
Placebo	6.00 (4.54-8.53)	7.18 (5.25-10.15)	6.16 (4.71-10.33)	8.36 (5.53-11.37)	9.02 (4.86-9.02)
Pregabalin	4.86 (3.81-8.13)	5.90 (4.51-7.92)	6.59 (4.78-7.80)	7.91 (4.83-10.34)	7.80 (5.15-11.90)
p-value	***0.01***	***0.05***	***0.03***	***0.04***	***0.15***
CSI					
Placebo	3.08 (2.31-3.64)	2.68 (2.24-3.48)	3.09 (2.26-3.51)	2.83 (2.07-3.58)	2.65 (1.94-3.60)
Pregabalin	3.17 (2.66-3.77)	3.50 (2.63-4.04)	3.43 2.92-4.33)	3.11 (2.25-4.23)	3.02 (2.59-4.18)
p-value	*0.1*	*0.38*	***0.04***	*0.24*	*0.17*
mBP					
Placebo	98.90 (83.40-104.10)	99.90 (88.90-106.90)	114.00 (102.10-116.30)	102.30 (99.00-106.10)	100.20 (93.50-108.60)
Pregabalin	98.10 (83.35-108.40)	91.50 (83.15-108.30)	105.00 (89.00-113.60)	102.30 (98.20-106.50)	100.60 (89.15-108.70)
p-value	*0.9*	*0.76*	*0.58*	*0.86*	*0.95*
HR					
Placebo	74.10 (68.00-77.70)	72.40 (66.30-80.70)	76.50 (67.7-81.30)	71.30 (67.30-79.30)	70.80 (64.0-78.90)
Pregabalin	71.30 (69.90-77.40)	72.50 (70.40-76.10)	73.00 (64.70-76.80)	69.40 (64.00-76.40)	67.70 (62.25-75.15)
p-value	*0.9*	*0.5*	*0.15*	*0.86*	*0.22*

Table 5.1: Median values (with 25th to 75th percentile range) of ANS parameters across all time points during the placebo and Pregabalin visits. P values were based on the Wilcoxon test.

5.4 DISCUSSION

This randomised crossover study of healthy volunteers who had previously developed VPH on acid exposure has shown that, in comparison to placebo, Pregabalin prevented the development of VPH to electrical stimulation in the non-exposed proximal oesophagus after acid exposure in the distal oesophagus, reduced the subjective sensation to acid infusion and lowered the parasympathetic tone, except during acid infusion, when the parasympathetic tone was higher. State and trait anxiety levels did not influence baseline acid sensitivity or the effects of Pregabalin.

In the current study, the demonstration of the anti-hyperalgesic effect of Pregabalin in our acid-induced oesophageal hypersensitivity model suggests a central mechanism of action, i.e. a reduction in central sensitisation. This is in keeping with data from rodents that suggest that the primary site of action of Pregabalin (and the related molecule gabapentin) is central[247 248]. Both drugs are considered to modulate the

processing of nociceptive signals at the dorsal horn[249 250], with evidence that both can also modulate descending inhibition of pain processing by higher brain centres [247 248].

The efficacy of Pregabalin in reducing hyperalgesia in somatic neuropathic pain has been extensively demonstrated in chronic pain conditions[226 251 252 253]. Similarly, evidence is emerging of the antihyperalgesic effects of Pregabalin in animal[254] and human models[232] of visceral pain. For example, in rodents, Pregabalin has been shown to reduce hyperalgesia to colonic rectal distension in the rat following injection with bacterial lipopolysaccharide[255]. Furthermore, in a recent preliminary study in patients with IBS, Pregabalin reduced rectal hyperalgesia to distension[232]. The distinction between the analgesic and anti-hyperalgesic effects of Pregabalin may be dose-related. In rodent studies, using injections of either trinitrobenzene sulfonic acid or lipopolysaccharide in the colon, the anti-hyperalgesic effects of intra-peritoneal Pregabalin were obvious at lower doses[254 255], but at higher doses analgesic effects were also observed[255]. In addition, in studies of patients with chronic neuropathic pain, e.g. post-herpetic neuralgia and diabetic peripheral neuropathy, doses up to 600mg per day, either in fixed doses or an escalating regime of up to 12 weeks[253 256], were associated with significant improvements in pain. It is thus possible that had higher doses of Pregabalin been administered in the current study, or been continued for longer, an analgesic effect may have been observed in addition to the anti-hyperalgesic effects.

While PS and CS are considered to be important mechanisms in VPH, there is also increasing evidence that anxiety and stress are important in the development and perception of symptoms in GORD patients, particularly in NERD and FH [257] sufferers. Such effects have been observed in clinical GORD studies as well as human experimental studies using functional imaging methods and oesophageal stimulation[258 259]. The effect of acute stress is thought to be mediated by specific brain areas modulating descending spinal inhibitory and excitatory pathways[259] (as well as HPA and ANS changes)[260-262]. Thus, we aimed to also explore whether the effects of Pregabalin were influenced by state or trait anxiety or by changes in autonomic functions. The anxiety state and trait questionnaires did not yield any positive correlations with the degree of sensitisation and response to Pregabalin. This may be because the sample size calculation for this study was based on the number required to

achieve a difference in pain thresholds in the two groups. Furthermore, only healthy volunteers were recruited, whose scores for STAI-S were uniformly low and therefore unlikely to influence sensitisation. It is noteworthy that a recent study of the model used has demonstrated that experimentally induced anxiety increases secondary hyperalgesia to distal oesophageal acidification[82 220].

Interpretation of the autonomic data is more complex. The classic fight/flight response to stressful events such as acute pain is one of sympathetic activation and parasympathetic withdrawal, which was mimicked by acid infusion in the placebo arm of our study. Pregabalin modulated this classic ANS response to pain by preventing PNS withdrawal, although sympathetic co-activation was also observed. Pregabalin has previously been shown in rats to be more effective in sympathetically-maintained rather than sympathetically-independent pain[263]. Our findings are therefore consistent with Pregabalin being more efficacious in sympathetically dominant states such as during acid infusion, perhaps because in such situations the prevention of PNS withdrawal is important in reducing pain processing given the established antinociceptive role of the PNS. A previous study has confirmed that increased SNS and lowered vagal parasympathetic activity were associated with increased sensitivity to acid in patients with FH compared to volunteers[264]. We might expect, therefore, that Pregabalin would also be effective in sympathetically dominant states including highly anxious VPH NERD and FH patients. The co-activation of SNS and PNS during acid infusion was also observed in our previous study[265] of oesophageal pain, indicating that SNS activation is not always associated with a reciprocal drop in PNS[266 267], but instead a co-activation of PNS is believed to have a compensatory role in response to tachycardia[265] or other physiological changes resulting from SNS activation in an attempt to optimise or normalise physiology[266].

There may be a question as to whether some of the observed ANS changes were directly caused by Pregabalin or were the indirect effect of a less painful state after treatment. The baseline data (Table 5.1) prior to electrical stimulation demonstrated that even at this point, CVT was lower for the Pregabalin in comparison to the placebo. Furthermore, baseline electrical thresholds were similar for treatment with either the placebo or Pregabalin. Together, this suggests that Pregabalin may lead to

primary ANS changes and thence modulate VPH rather than the other way round. On the other hand, the cause and effect relationship of Pregabalin on sensation caused by the acid infusion was less clear. However, assuming a blunted PNS withdrawal response to acid after Pregabalin treatment was responsible for this reduced pain, the prevention of PNS withdrawal or promotion of PNS elevation by other pharmacological and non-pharmacological means should have the same effects by reducing acid-induced pain. These questions are currently being explored by further studies.

While we acknowledge that Pregabalin should not be used as a substitute for acid suppressant treatment, it may have a role in symptom reduction in patients that have previously been sensitised by acid exposure. It has the potential to be used in conjunction with an acid suppression treatment. In these patients, if VPH has developed from previous acid exposures, even a small amount of acid could trigger symptoms in the absence of physical changes or damage detected by endoscopy.

Reliance on the self-administration of capsules inevitably leads to questions of compliance; however, this was equal and good for both drug and placebo. Assuming the placebo effect is unaltered by the actual dose, the significant treatment effect observed would be likely to be even greater had compliance been 100%. Inevitably, in most drug studies, placebo effects exist, which is why a placebo controlled design is usually necessary. In this study, all subjects included in the study sensitised in the screening visit after distal acid infusion, as per our inclusion criteria. However, post randomisation, for the placebo visit and Pregabalin visits, the rates of sensitisation were 53% and 20%, respectively. Pregabalin seemed to have an effect above placebo in reducing sensitisation. Furthermore, the screening visit was always the first visit, while the post randomisation placebo and Pregabalin happened on either visits 2 or 3. The first visit is often the most anxious visit for naïve volunteers. The observed placebo effect in comparison to the screening visit could have been influenced by this order effect.

5.5 CONCLUSION

In this study, we have shown that in healthy volunteers Pregabalin prevents and reduces oesophageal pain hypersensitivity in the oesophagus after distal acidification. It has been shown to be effective in relatively small doses and for a short duration of treatment. The use of smaller doses is likely to minimise potential side effects; however, using larger doses and different regimes could be explored further and benefit more refractory patients. We believe Pregabalin may have a role in the reduction of symptoms in patients with NERD and FH where VPH is a feature, and a 'proof of concept' study in these patients would be warranted to explore this further.

6 CONCLUSION: TOWARDS COHORT DEVELOPMENT, TARGETTED THERAPY AND FUTURE DIRECTIONS

6.1 SUMMARY OF EXPERIMENTS

The studies in the previous chapters explored the roles of peripheral sensitisation (PS) and central sensitisation (CS) in the context of acid-induced visceral pain hypersensitivity (VPH). In this final chapter, the clinical relevance and integration of both PS and CS concepts in clinical practice will be discussed, together with new developments and limitations of this model.

1. Chapter 2 (Full-thickness oesophageal samples) – Nerve fibres were absent within the human oesophageal epithelium. The epithelial acid-sensing ion channel (ASIC3) was identified in full-thickness human oesophagus and considered a suitable candidate in oesophageal mucosal biopsy samples.
2. Chapter 3 (Oesophageal biopsies from GORD patients) – Epithelial ASIC3 morphology and distribution were correlated with symptoms of GORD. The three main findings were: i) ASIC3 in the basal layer of the epithelium and its perinuclear expression increases from control to NERD and EO; ii) The expression of ASIC3 in epithelium is associated with an increase in acid complaints, indigestion and sleep problems; iii) NERD/FH patients had the most severe symptoms (even more than EO) suggesting superficial epithelial biomarkers, including epithelial ASIC3 have important roles in symptoms and PS.
3. Chapter 4 (Reproducibility of human model of VPH) – Acid infusion in the distal oesophagus reproducibly reduces the pain threshold (PT) to electrical stimulation in the non-exposed proximal oesophagus. This observation is maintained from 30 minutes to 120 minutes after acid infusion. Sensitisation is noted in up to 70% of the population
4. Chapter 5 (Pregabalin) – In a prospective, randomised, placebo-controlled, double-blinded, cross-over study in healthy volunteers, a 5-day treatment

programme with oral Pregabalin reduced and attenuated the drop in PT after acid infusion in the proximal oesophagus (CS). Treatment with Pregabalin was also associated with the prevention of parasympathetic withdrawal during acid infusion.

6.2 FUTURE RESEARCH DIRECTIONS

The studies and analyses performed answered some questions, but open further issues and research ideas. Future direction and work from these studies need to explore further issues

1. Chapter 2 (Full-thickness oesophageal samples) – Some of the difficulties in replicating previous work in TRPV1 immuno-staining could result from technical variability. Fresh frozen sections could be explored further as nerves are less likely to be damaged by formalin fixation. A substitute for the GSK antibody, which had proven to be effective needed to be sought. However, by not finding a neuronal marker, epithelial receptors and the important role of epithelium in the context of PS in the human oesophagus were explored and highlighted (Section 2.4.4.1). This area merits further research and is very relevant and consistent with emerging evidence. Density and expression of epithelial ASIC3 could be an area for future research.
2. Chapter 3 (Oesophageal biopsies from GORD patients)- Quantitative methods are essential, if epithelial ASIC3 density was to be used as an objective measure for symptoms in GORD patients (explored in section 3.4.2). Future studies recruiting larger number of patients are required to explore this further and to achieve greater statistical significance.
3. Chapter 4 (Reproducibility of human model of VPH) – Although from retrospective analyses the acid infusion model seemed robust, the question of reproducibility of the model after multiple acid infusions needed to be explored prospectively and systematically. This can be best achieved by a study replicating multiple visits (and acid infusion) without a drug or placebo.
4. Chapter 5 (Pregabalin) –Pregabalin has proven to be efficacious in preventing CS in proximal oesophagus after distal oesophageal acidification via prevention of cardiac vagal tone (parasympathetic) withdrawal during acid. It is worth exploring if a pure autonomic modulating drug such as atropine/

autonomic modulating behaviour (deep breathing, relaxation, cognitive-behavioural therapy) could influence VPH (Refer section 6.4.3)? These studies are currently underway, where I am also a co-researcher.

6.3 CLINICAL IMPLICATIONS

6.3.1 Treatment pathway

Based on the hypotheses and findings from my studies, I propose a potential treatment algorithm (Figure 6.1) for GORD patients with symptoms resistant to medical or surgical acid suppression.

These patients are a challenge in primary and secondary care. I believe they represent a heterogeneous group of patients and therefore the algorithm outlined below will help to identify important mechanisms which will allow targeted treatment in homogenous groups of patients, where oesophageal VPH is an important mechanism.

6.3.2 Cohort development

As mentioned in previous chapters, PS and CS are important mechanisms of oesophageal VPH; however, many different factors including ANS profiles, stress and personality types modulate PS and CS. Nevertheless, in many patients there is likely to be one dominant factor which needs to be identified. A useful starting point would be identifying the existence of VPH (Figure 6.1), which could be done by pH monitoring or the Bernstein acid perfusion test. Patients demonstrating good symptom correlation with acid exposure, although acid exposure time might be within normal limits, would be classified as FH. In this group, VPH is expected to be high. Patients with poor symptom correlation with excessive symptoms and normal acid exposure time may still have VPH, so a simple Bernstein test could be performed to confirm this hypothesis.

A practical starting point would be to trial Pregabalin treatment. In responders, CS has to be important and they may continue on this treatment in the long term if the benefit persists. Pregabalin could be important not just as treatment tool, but also as an investigative tool. With non-responders, oesophageal mucosal biopsy to detect

receptor abnormality could help to identify patients where PS is important. Again, although effective PS modulating drugs are currently limited in the market, the existence of receptor abnormalities is important in identifying these patients as a separate cohort. Those who do not respond to Pregabalin or show receptor abnormalities could be referred for tertiary consultation to a neurogastroenterologist. In these patients, specific ANS and psychological profiling could be performed and specific pharmacological and non-pharmacological treatment used. This would also involve a personalised treatment plan.

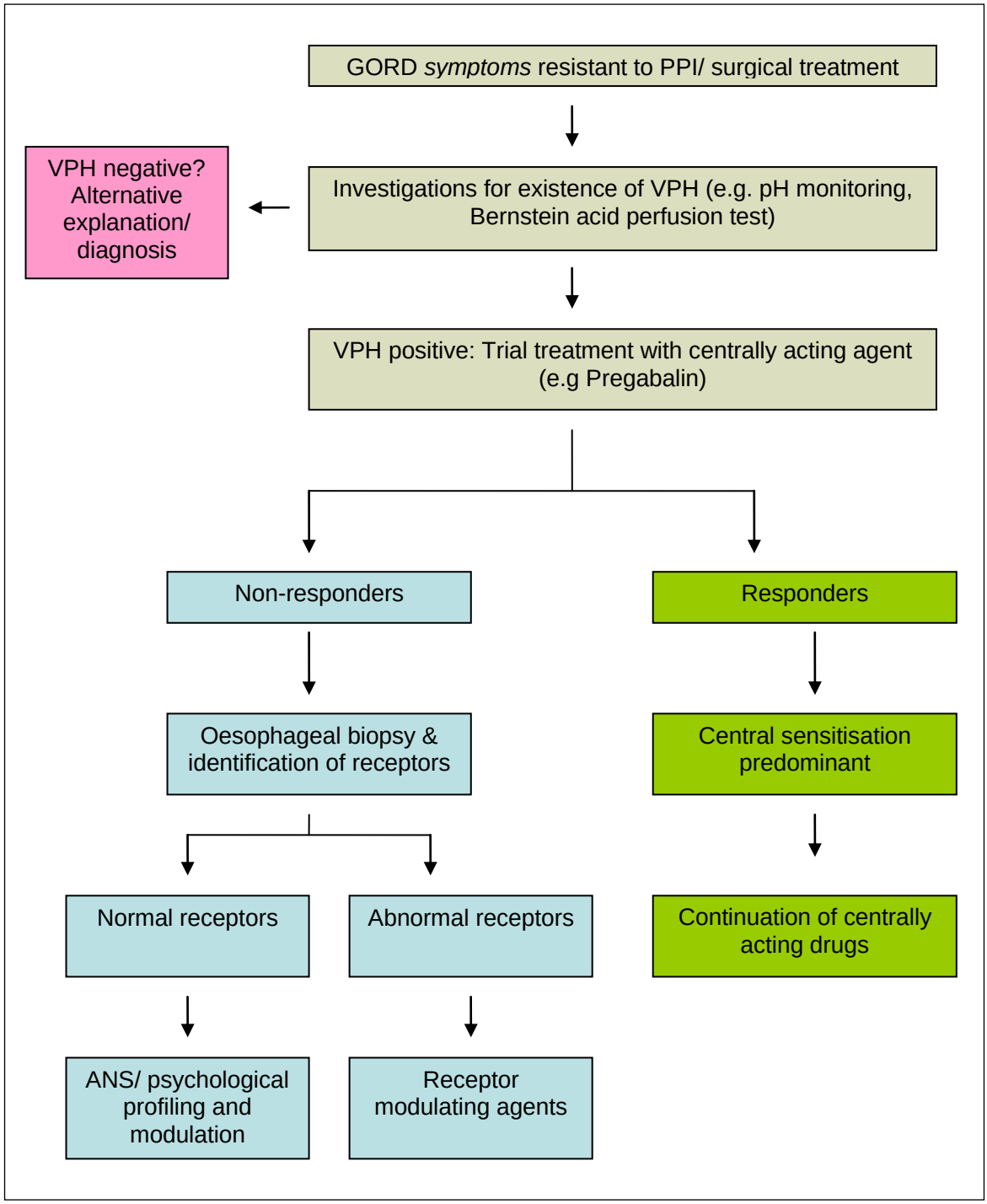


Figure 6.1: A summary to consolidate the main concepts of the proposed treatment pathway for patients where VPH is important, i.e. cohort identification, specific investigations and targeted treatment.

6.3.3 Targeted therapy

With cohort development, it may be possible to identify predominant and underlying patho-physiological abnormalities in VPH GORD patients who are resistant to acid

suppression therapy. These patients can then be managed with targeted treatment aiming at both PS and CS. These agents are discussed further in 6.3: Future developments.

6.4 FUTURE DEVELOPMENTS

Drug studies are currently being conducted to alter different mechanisms for the development of VPH. These drugs can be divided into those that work peripherally and those that act more centrally.

6.4.1 Peripheral sensitisation modulating drugs

In the modulation of peripheral sensitisation, the antagonist or agonist of a particular candidate receptor is an attractive proposition. The modulation of receptor TRPV1 is the most studied to date in the context of VPH and pain in general (somatic and visceral) (Table 6.1), so it therefore acts as a good example to illustrate PS modulating drugs, although it is by no means the only receptor involved in VPH.

TRPV1 antagonists are believed to act either directly by blocking the channel pore or indirectly by blocking the Ca2+ channel[268]. For many years, the only known TRPV1 antagonist was ruthenium red, which is a general TRPV inhibitor[269]. Capsazepine is the newest TRPV1 antagonist, which is more specific than ruthenium red, but using it produces its own set of problems. Capsazepine is a relatively weak antagonist[270 271] and therefore for it to achieve therapeutic effects, high concentrations are required, which can cause it to bind to Ca2+[272] and acetylcholine receptors[271]. This binding causes non-specific anti-cholinergic effects because Capsazepine binds to nicotinic acetylcholine receptors[271]. In addition, its effects vary for heat-induced pain versus acid-induced pain as well as between species[273]. The latter problem has made the translation of efficacy from rodents to humans unpredictable and unreliable. Despite these problems, though, currently numerous products and trials using TRPV1 modulation are in place. These include SB-366791 from GlaxoSmithKline (GSK), which is a competitive inhibitor of TRPV1[274], Abbott laboratories have come out with A-425619, a promising TRPV1 antagonist[275], and Johnson and Johnson have created JNJ17203212, another TRPV1 antagonist effective in bone cancer pain[275].

To date, the repertoire of active ligands on the ASIC3 channel has been limited to amiloride derivatives, non-steroidal anti-inflammatory drugs and gadolinium[276]. For amiloride, trials are mainly at the pre-clinical/animal study stages including an amiloride derivative, which is an effective ASIC3 inhibitor[181] and is efficacious in acid-related pain in rodent gastritis models[136] (Chapter 2). There are now newer amiloride[182] derivatives including amidine[181]. In addition, gadolinium has also been shown to bind to ASIC3 and effectively reduce electrophysiological response to low pH in rat oocytes[277]. More recently, toxin from a sea anemone was discovered and found to be an effective ASIC3 inhibitor, at least in cultured sensory neurons[276]. The ASIC3 blocker is important not just in the context of acid-induced VPH, but also low pH states such as chronic ischemic pain[278].

The use of agonists, on the other hand, is expected to hyper-stimulate specific receptors, and as a result desensitise them. The mechanism is believed to be related to excessive Ca2+ influx, which in the long term can cause terminal nerve degeneration[279]. Nerve terminals are able to regenerate, and, in theory, the use of TRPV1 agonists should not produce permanent change. There is evidence for the ablation of TRPV1 containing neurons by capsaicin injection[54 280 281], and capsaicin has been used clinically to alleviate neurogenic pain in herpes zoster, diabetic and HIV peripheral neuropathy[44 282 283]. Intrathecal administration of TRPV1 agonists has shown efficacy in reducing pain from osteosarcoma in dogs and reducing sensation to thermal stimuli[284]. Intrathecal administration is believed to work more effectively by acting on TRPV1 in dorsal root ganglia (DRG) in the spinal cord, but in the context of VPH this would constitute modulating CS. There is limited evidence on the use of ASIC3 agonists. Mammalian neuropeptides FF (NPFF)[285] and SF (NPFF)[286] have been known to activate (although they did not bind to) ASIC3, but unfortunately these two peptides are still very new and non-specific.

Receptor	Mechanism(s)	Condition	Evidence
Immunoreactive TRPV1	Increase TRPV1 immunoreactivity in sensory nerves is associated	Faecal urgency	Chan, *et al*[287]

	with increased rectal hypersensitivity and faecal urgency		
Immunoreactive TRPV1	Increased immunoreactivity in sensory neurons in colon associated with increased pain	IBS, colonic motility	Akbar *et al.*[109]
Immunoreactive TRPV1	TRPV1 is sensitive to low pH, heat and capsaicin. These factors tend to aggravate symptoms in GORD patients	Erosive oesophagitis[3]	Matthews *et al.*[3]
Immunoreactive TRPV1	Increase free nerve endings in epithelium and papillary areas is associated with increased acid exposure time	GORD (with evidence of increased acid exposure)	Bhat *et al.*[141]
TRPV1 gene and protein	Increased TRPV1 protein and gene in patients with NERD and EO, but no correlation with acid exposure time. Likely to cause symptoms	NERD and EO	Guarino *et al.*[288]

Table 6.1: Peripheral receptors studied in the context of 'human' VPH

TRPV1 is not the only receptor associated with VPH; rather, it is the most established. However, the principles are the same as we find with other receptors, as there are many with known associations with VPH and their modulation has proven to be effective (Table 6.2). Nevertheless, these receptors often also have physiological functions. In one animal study, using the TRPV1 antagonist caused significant hyperthermia[289]. ASIC3, as mentioned in Chapter 2, is also involved in mechano-sensation and motility[48]. These good examples illustrate that while many of these

receptors may be associated with pain, they also play an important part in homeostatic function modulations, and hence care should be exercised in their modulation (discussed further in 6.4: Limitations).

6.4.2 Central sensitisation-modulating drugs

Central modulating agents include a very wide-ranging group of drugs, namely modulators of spinal CS, ANS and stress responses as well as prokinetic agents. In particular, the success of the CRF receptor antagonist and alpha-2 receptor agonist clonidine has highlighted that symptoms in IBS can be treated by HPA axis and ANS modulation. Gabapentin and Pregabalin have also been shown to be effective in reducing pain caused by rectal distension, relevant in irritable bowel syndrome (IBS) patients[232 290]. The table below is a summary of modulators that have been proven effective in different functional gastro-intestinal disorders (FGIDs), their mechanisms of action and clinical application.

Drug	Mechanism(s)	Conditions	Evidence
Corticotrophin-releasing factor (CRF) Receptor antagonist	Blunting of stress-related, anxiety-like behaviour in the gut especially colonic motility. It improves GI motility, visceral perception and negative mood response to GI stimulation	Irritable bowel syndrome (IBS)	Sagami, *et al.*[291 292]
Alpha2 Adrenergic Agonist (Clonidine)	It reduces pain perception, autonomic function reactivity and anxiety. Colonic transit not affected	Diarrhoea predominant IBS	Camilleri, *et al.*[293]
CCK Antagonist	Improves oesophageal contraction, inhibit gallbladder contractions[294 295], improves gastric emptying and colonic transit[296 297]	IBS, Functional dyspepsia	Varga, *et al.*[295]

NMDA Antagonist (Ketamine)	Inhibition of acid induced central sensitisation in healthy human volunteers [76]	Oesophageal hypersensitivity to acid	Willert, *et al.*[75]
Gabapentin	Reduces rectal mechano-sensation and increases rectal compliance	Diarrhoea predominant-IBS	Lee, *et al.*[290]
Pregabalin	It increases sensory distension threshold to normal in IBS patients with rectal hypersensitivity	IBS	Houghton, *et al.*[232]

Table 6.2: Some examples of drugs relevant to human clinical conditions related to VPH.

6.4.3 Non-pharmacological modulation of ANS

There is general consensus that activation of the sympathetic nervous system is pro-nociceptive, while activation of the parasympathetic nervous system is anti-nociceptive. Support for this concept is also available in our study of oesophageal VPH, where the efficacy of Pregabalin was associated with a reduction in withdrawal of the parasympathetic tone during acid infusion in comparison to the placebo. This raises the possibility that the modulation of the ANS may be efficacious in hypersensitivity states. While ANS can be effectively modulated by pharmacological interventions, the logical question to ask would be whether non-pharmacological interventions that modulate the ANS can also be effective in modulating VPH. The obvious benefit of non-pharmacological interventions is the avoidance of side effects inherent with many drugs. Non-pharmacological measures may include cognitive behavioural therapy (CBT), breathing and relaxation exercises, etc. Current and future work is continuing within the research group to explore whether CS to oesophageal acid infusion can be affected by the non-pharmacological modulation of ANS, including by exercise and breathing methods, in which I am involved.

6.4.4 Novel VPH modulating agents specific to GORD

Pharmacological agents used to modulate the PS and CS arms of VPH have been better studied in IBS than in functional heartburn and dyspepsia. Although the modulators of PS and CS have been discussed above, there are, however, other non-specific neuromodulators that have some efficacy in VPH seen in GORD. These are discussed briefly below.

The selective serotonin re-uptake inhibitor (SSRI) Citalopram has been shown to reduce symptoms (heartburn, chest pain and regurgitation) in GORD patients with evidence of positive symptom correlation to acid exposure on pH impedance, but only with normal acid exposure time, suggesting that they are hypersensitive to physiological amounts of acid reflux[298]. Symptoms as defined above were reported in 23% vs. 67% (p=0.047) of patients taking Citalopram or a placebo for six months, respectively. A major criticism of this study was that assessments of anxiety and depression levels were not performed. The question remains as to whether Citalopram reduces symptom reporting by direct analgesic effect or indirectly by its anxiolytic and antidepressant effects.

My own Pregabalin study is now ready to move to proof of concept in GORD patients. This study demonstrated that a reduction of CS is associated with changes in autonomic nervous system (ANS). A clear relationship between ANS and VPH was discussed in Chapters 1 and 2.

6.5 CONCLUDING REMARKS

The VPH concept is very much in line with the theory of evolution. Within VPH exists great variability between individuals and past experience, so it is not hard to imagine survival benefits from VPH in our distant past. Ingesting highly acidic or noxious chemicals can cause death, so the ability of the body (the viscera) to recognise this at a subconscious level is necessary to avoid future contact with the same substance. Interaction with the ANS further consolidates that VPH is linked intricately to survival instincts. In times of high stress and danger, the human body is hyper vigilant to potential dangers and subsequently lowers perception thresholds. However, human lifestyles and experience are constantly changing. Eating in modern life, while

still necessary for human existence, has evolved into a high art; it covers social, cultural and pleasure roles in equal measure. Foods are highly processed, cooked and generally safe and free from contamination from soil and dirt. The role of VPH in survival has somehow eroded. The nature of stress has also evolved from running away from predators and hunting for food to one of meeting a deadline in an office or passing an important viva examination. However, the human evolutionary process is a dynamic process – as the environment changes, the human adapts.

One final point of importance in human evolution is the existence of great variability between individuals, which promotes a rich diversity of properties within the human gene pool. VPH is within the spectrum of human variability, as pain is, after all, a subjective experience. At what point is sensation considered to be unpleasant? Is the elimination of bodily sensation necessarily always good? Should we remove the offending stimuli or sensation? Pain and interpretation are ultimately a result of the interaction of past experience, cognitive processes, emotion and the essential ingredients of being alive. As we age, most of our sensations numb and we yearn for the youthful days when all sensations, including pain, were once so acute, precise and exciting!

APPENDICES

Chapter 2

2.01 NATIONAL RESEARCH ETHICS APPROVAL PROCESS

The ethical application and approval for both parts of the experiments were made in a single application.

Application

The application was made via the electronic National Research Ethics Committee (NREC) application form, together with all the attachments. This was submitted to the committee below.

Committee

The ethics application was submitted to the East London and the City Research Ethics Committee 3, competent to consider studies involving human tissues. The committee was chaired by Dr David Ingram and a panel of other clinicians, lay members and administrators.

The Interview

The committee met on 19th July 2007 to consider our application. The outcome was one of provisional approval subject to some recommended changes. The scientific justification and overall structure of all the studies remained intact. The main recommended point was to make the risks of serious injuries from gastroscopy more explicit to patients and volunteers taking part in the biopsy study (Chapter 5). The current accepted risk is quoted as 1/10,000 per gastroscopy. In addition, the patients recruited would have the gastroscopy done anyway, thus the scope itself was not for research, but rather for the procurement of the biopsy samples.

Approval

The recommended changes were made and submitted for consideration by the chair, Dr Ingram. Several draft changes were made, but everything was finally approved on 27th November 2007. The approval reference number is 07/H0705/67

R&D

The study was sponsored by the Bart's and the London NHS Trust, with the R&D office as the official sponsor body. As a result, this study also obtained Department of

Health funding.

Registration of trial website

To remain compliant, accrual data for the study were constantly updated on the National Institute for Health Research (NIHR) via the UKCRN (UK Clinical Research Network) website, which also enables UKCRN to fund the Trust appropriately for costs incurred by the NHS. The accruals of this study were regularly updated with UKCRN. The other benefit included allowing the participants to verify independently the legitimacy of the research in which they were involved.

2.02 SOLUTIONS AND REAGENTS

All solutions and reagents were supplied by VWR International, Lutterworth, Leicestershire, UK, Dako, Ely, Cambridgeshire, UK (Dako), Bio-Genex Laboratories, San Ramon, CA, USA (Bio-Genex). The Vectastain Universal Elite ABC kit and R.T.U. Vectastain Universal Elite were supplied by Vector Laboratories Inc., Burlingame, CA, USA (Vector), IMS = 100% industrial methylated spirits were prepared in-house.

Generic solutions

Endogenous peroxidase blocking solution	6ml H_2O_2 194ml dH_2O
Acid Alcohol	300ml dH_2O 700ml IMS 10ml conc. HCL (added to 990ml of the above)

Heamatoxylin and Eosin

Gill's Haematoxylin	1L dH_2O 625ml ethylene glycol, 10g haematoxylin 1g sodium iodate

	176g aluminium sulphate
	50ml glacial acetic acid
	825ml dH_2O
1% Eosin	1g eosin Y
	100ml dH_2O

Immunohistochemistry

Endogenous peroxidase	3ml 30% H_2O_2
block (3%)	97ml 100% methanol

REAL™ Antibody diluent (DAKO)

Wash buffer (DAKO)	0.05mol/L Tris HCl
	0.15mol/L NaCl
	0.05% Tween 20

Vectastain Universal Elite ABC reagents or
RTU Vectastain Universal Elite reagents (Vector labs)

Diaminobenzidine	1ml substrate buffer
tetrachloride (DAB) solution	1 drop DAB chromagen
(Bio-Genex)	

Phosphate buffered saline (PBS)
Mounting medium for immunohistochemistry: Canada balsam

Antigen retrieval

Citrate buffer solution (Vector)	Citrate buffer stock 100ml
	dH_2O 900ml

2.03 FIXATION AND PREPARATION OF TISSUES

1. Fix tissues with 10% formalin or other fixatives for 24-48 hours at room temperature.
2. Fixative volume should be 5-10 times tissue volume.
3. Trim fixed tissues into appropriate size, shape and place in embedding cassettes.
4. The process for the paraffin embedding schedule is as follows – in most modern laboratories, including in our department, this is automated and machine operated (Shandon Excelsier ES, ThermoFisher Scientific, Leicestershire, UK):
    - Formalin 1 hour and 30 minutes
    - Alcohol (ethanol), six changes, 1 hour each
    - Toluene (Xylene substitute,) three changes, 1 hour each
    - Paraffin wax, three changes, 1 hour and 20 minutes each
    - Embedding tissues into paraffin blocks
5. Trim paraffin blocks as necessary and cut at 3-10 um (5 um is commonly used).
6. Place paraffin ribbon in water bath at about 40-45 ºC.
7. Pick up sections on negatively charged microscope slides (VWR International, Leicestershire, UK) and allow water to drain off.
8. Dry sections overnight at 37 ºC.

2.04 HAEMATOXYLIN AND EOSIN STAINING

1. Dewax sections in xylene (2x 2 min)
2. Remove xylene with IMS (2x 2 min)
3. Wash in running tap water for 5 min
4. Stain in Gill's haematoxylin for 20 min
5. Wash in running tap water for 5 min
6. Differentiate by dipping in 1 % acid alcohol

7. Wash in running tap water for 5 min
8. Stain in eosin for 3 min
9. Dip in tap water and blot
10. Dehydrate rapidly in IMS (2x 2 min)
11. Clear in xylene (2x 2 min)
12. Mount in Canada balsam

2.05 INDIRECT IMMUNOHISTOCHEMISTRY

ABC kit (Vectastain Universal Elite ABC: PK-6200)

1. De-wax sections in **Xylene** (2 X 2 minutes)
2. Dehydrate sections in **alcohol** (2 X 2 minutes)
3. Place sections in **endogenous peroxidase** block for 15 minutes (97ml methanol, 3ml hydrogen peroxide)
4. Wash sections in tap water for 2 minutes
5. If **antigen retrieval** is required, go to the procedure below (25 minutes microwave)
6. Transfer to tap water and wash for 2 minutes
7. Soak sections in a trough of wash buffer for 3 minutes
8. Wipe around the sections and apply the PAP pen (ImmEdge pen, Vector labs)
9. Apply **normal horse serum** for 5 minutes
10. Tip off the horse serum
11. Apply **primary antibody** at the appropriate dilution in antibody diluent (Dakocytomation) for 40 minutes
12. Wash off the antibody with wash buffer (twice) and flick the slide to remove excess
13. Apply the **Universal Biotinylated secondary antibody (secondary antibody from the kit)** for 30 minutes. (Add 2 drops of normal horse serum to 5ml of wash buffer, and then add 2 drops of biotinylated secondary antibody. Vortex this solution)
14. Make up the Avidin complex solution. This must stand for 30 minutes before use. (Add 2 drops of reagent A to 5ml of wash buffer, and then add 2 drops of reagent B. Vortex solution. Leave to stand 30 minutes, and drop for 30 minutes)

15. Wash off the antibody with wash buffer (twice) and flick the slide to remove excess
16. Apply the **Avidin complex** solution for 30 minutes
17. Wash off with wash buffer X2 and flick the slide to remove excess
18. Apply the appropriate **DAB solution** (DAB capsule in distilled water)
19. Wash in running tap water for 5 minutes
20. Counter-stain in Gill's haematoxylin for 2 minutes
21. Blue in running tap water for 5 minutes
22. Differentiate in 1% acid alcohol for 2 seconds (appropriately 2 dips) if necessary
23. Wash in running tap water for 5 minutes
24. Dehydrate, clear and mount

RTU kit (R.T.U. Vectastain Universal Elite)

1. Place the sections into a plastic staining rack
2. Dewax sections in **Xylene** for two changes of 2 minutes each
3. Remove the Xylene in **Alcohol** for two changes of 2 minutes each
4. Place the sections into the sink and wash in tap water for 5 minutes
   If **antigen retrieval** is required, refer to the appropriate optimisation schedule specific to each antibody
5. If required, carry out **Avidin/biotin block** (Avidin (Bottle A in Kit) for 15 minutes, wash with buffer and Biotin (bottle B in kit) for 15 minutes)
6. Prepare the **3% endogenous peroxidase** (10ml distilled water, 300microlitres of hydrogen peroxide)
7. 3% endogenous peroxidase for 15 minutes
8. Remove the rack, place in sink and wash in tap water for 5 minutes
9. Wash out the black plastic staining with distilled water and wash buffer
10. Soak wash buffer for 5 minutes
11. Prepare an immuno-staining tray by adding a few drops of wash buffer to create a moist chamber
12. Taking each slide in turn, wipe around the section to remove excess wash buffer, and then using a PAP pen draw a line across the slide above and below the section

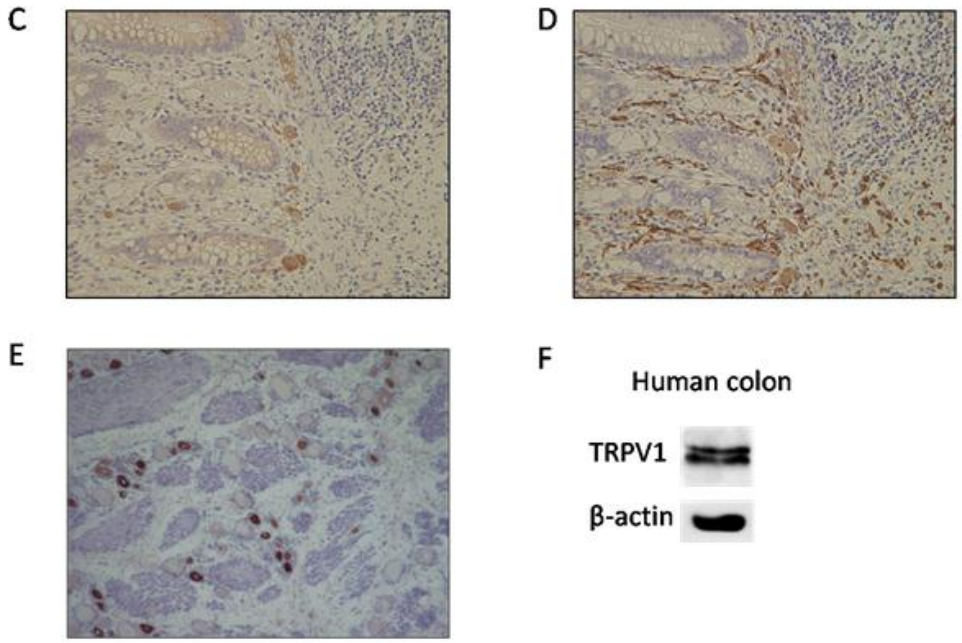
13. Place the section onto the rack in the immuno staining tray and cover with wash buffer. Do not allow the sections to dry out
14. When all of the sections are on the rack, tip off the wash buffer
15. Apply sufficient drops of normal blocking serum, **Normal horse serum**, (yellow bottle from RTU kit) to cover each section
16. Put on the lid of the immuno staining tray and leave for 20 minutes
17. Prepare the primary antibody at the appropriate dilution using antibody diluent
18. Tip off the normal horse serum
19. Apply the **primary antibody** to each test section and the positive control section
20. Apply antibody diluent alone to the negative control section
21. Replace the immuno staining lid and leave for 40 minutes
22. Rinse off the primary antibody with wash buffer
23. Cover each section with wash buffer, replace the immuno staining tray lid and leave for 2 minutes. Rinse with wash buffer, cover each section again and leave for a further 2 minutes
24. Tip off the wash buffer
25. Apply sufficient drops of Universal Biotinylated **secondary antibody** (Blue bottle from RTU kit) to cover each section
26. Replace the immuno staining tray lid and leave for 30 minutes
27. Rinse off the secondary antibody with wash buffer
28. Cover each section with wash buffer, replace the immuno staining tray lid and leave for 2 minutes. Rinse with wash buffer, cover each section again and leave for a further 2 minutes
29. Tip off the wash buffer
30. Apply sufficient drops of the Vectastain Elite **Avidin Biotin Complex** Reagent (Grey bottle from RTU kit) to cover each section
31. Replace the immuno staining tray lid and leave for 30 minutes
32. Rinse off the ABC reagent with wash buffer
33. Cover each section with wash buffer, replace the immuno staining tray lid and leave for 2 minutes. Rinse with the wash buffer, cover each section again and leave for a further 2 minutes
34. Into a yellow-topped tube place 1ml of substrate buffer from the Biogenex Two Component DAB kit

35. To this add 1 drop of the DAB chromogen from the kit and mix well
36. Tip off the wash buffer from the slides and apply the DAB solution to each of the slides for 5 minutes
37. Wash the slides in tap water for 5 minutes
38. Load the slides into a machine staining rack ensuring that all of the sections are facing in the direction of the arrow on the rack
39. The remainder of the technique is carried out using machines and involves staining the sections with the dye haematoxylin. This stains the nuclei a light blue colour, which contrasts against the brown immunocytochemical staining. The sections are then dehydrated in alcohol to remove water and washed in Xylene before having a glass cover-slip placed over the section to protect it and to produce a permanent preparation.

2.06 ANTIGEN RETRIEVAL

1. Place sections into a plastic staining rack, leaving a gap between the slides
2. Place the rack into a large glass staining trough containing exactly 1000ml of the antigen retrieval buffer
3. Cover the trough with cling film and make several holes in the cling film
4. Place the trough in the microwave and run at full power for 25 minutes (for the Step kit, this step is always the same and fixed for 25 minutes. This varies with the Dako kit, timing and microwave setting specific to the individual optimisation schedule)
5. With care, remove the trough from the microwave
6. Carefully remove the cling film
7. Leave sections to stand in the hot buffer for 5 minutes
8. Return to the original technique

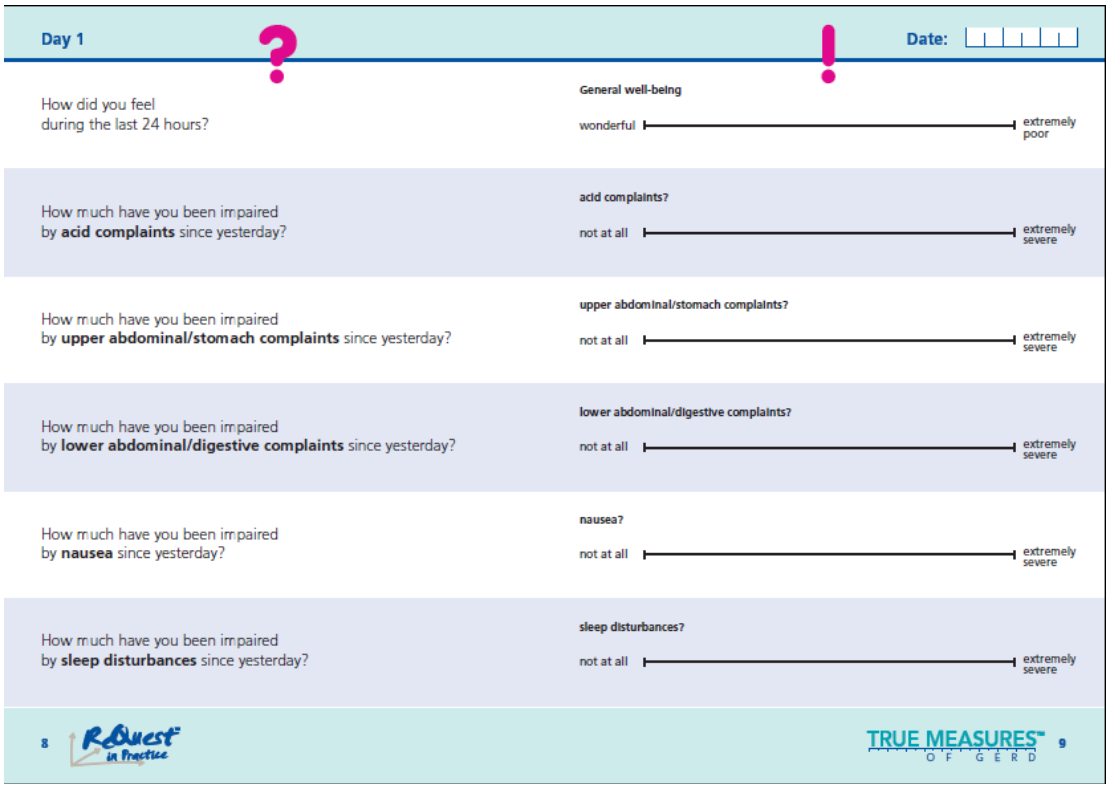
2.07 ADDITIONAL TRPV1 VALIDATION



Human colon TRPV1 immunohistochemistry (IHC) staining and immunoblotting with the TRPV1 antibody (ThermoFisher). C&D) human colonic TRPV1, E) Human DRG with small neurons staining F) Bands in Western blotting. This band on the immunoblot could not be repeated with a subsequent batch of antibody intended for the oesophagus (Courtesy of Dr George Boundouki)

Chapter 3

3.01 ReQuest™ QUESTIONNAIRE



Day 1 Date:

How did you feel during the last 24 hours?
General well-being
wonderful — extremely poor

How much have you been impaired by **acid complaints** since yesterday?
acid complaints?
not at all — extremely severe

How much have you been impaired by **upper abdominal/stomach complaints** since yesterday?
upper abdominal/stomach complaints?
not at all — extremely severe

How much have you been impaired by **lower abdominal/digestive complaints** since yesterday?
lower abdominal/digestive complaints?
not at all — extremely severe

How much have you been impaired by **nausea** since yesterday?
nausea?
not at all — extremely severe

How much have you been impaired by **sleep disturbances** since yesterday?
sleep disturbances?
not at all — extremely severe

8 ReQuest in Practice TRUE MEASURES OF GERD 9

3.02 VISUAL ANALOGUE SCALE FOR MULTI-MODAL STIMULI

Milestones of VAS (scale of 0-10)

0- No perception

1- Vague perception* (first sensation)

2- Definite perception of mild sensation

3- Vague perception of moderate sensation

4- Definite perception of moderate sensation

5- Pain threshold

6- Mild pain

7- Moderate pain* (need to stop)*-*

8- Pain of medium intensity

9- Intense pain

10- Unbearable pain

3.03 WESTERN BLOTTING OF ABCAM ASIC3 ANTIBODY

Western blotting performed on ASIC3 antibody (Ab 49333) to validate the specificity of this antibody by the manufacturer. It is recommended for use in immunohistochemistry in human paraffin fixed samples (c/o: Abcam)

ASIC3 antibody (ab49333) at 2.5 µg/ml + Jurkat cell lysate at 10 µg
Secondary Antibody: HRP conjugated anti-Rabbit IgG at 1/50000 dilution
Predicted band size: 59 kDa
Observed band size: 59 kDa
Gel concentration 12%

Chapter 5

5.01 NATIONAL RESEARCH ETHICAL APPROVAL PROCESS

Application

The application was made via electronic the National Research Ethics Committee (NREC) application form, together with all the attachments. This was submitted to the committee below.

Committee

The ethics application was submitted to the North West 5 Research Ethics Committee, competent to consider studies involving drug trial. The committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004 and is authorised to carry out the ethical review of clinical trials of medicinal products. The committee was chaired by Dr Donal Manning, Vice-chair Dr J Pearson, statistician Mr R Swindell, a panel of other clinicians, lay members and administrators. The site specific assessment (SSA) was assessed by East London & The City Research Ethics Committee 3 for Barts and the London site.

The Interview

The ethics application was submitted for consideration on 15th May 2007. The outcome was one of provisional approval subject to some recommended changes. The scientific justification and overall structure of all the studies remained intact. Statistical clarification, consolidation of the volunteer information sheet and a mention of Pfizer Ltd in the information sheet were required.

Approval

With the changes made, on 4th July 2007 final approval was given. The approval reference number is 07/MRE08/39. This study also required a separate approval by Medicine and Health Regulatory Agency (MHRA).

R&D

The study is sponsored by the Joint Research & Development Office, Barts and the London NHS Trust as the official sponsor body.

Registration of the trial website

The R&D office and Department of Health recommended registering the trial with Current Controlled Trials (CCT) Ltd on their website called 'ISRCTN'. This study

registration can be checked at www.controlled-trials.com/ISRCTN40924266.

5.02 MEDICINE AND HEALTH REGULATORY AGENCY (MHRA)

Details for the MHRA are EudraCT number: 2006-000499-33, MHRA number: 21313/0026/001, Protocol number: 2006neuro06. Final MHRA approval was obtained on 4th May 2007. Documents for audits and inspections were updated during the study period. Annual safety reports were made and this study was closed with MHRA on 16th April 2010.

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