EXPRESSION OF TRANSIENT RECEPTOR POTENTIAL VANILLOID 4 (TRPV4) IN NERD VS NORMAL CONTROL AND ASSOCIATION WITH PH STUDY AND MANOMETRY PARAMETER

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TABLE OF CONTENTS

LIST OF TABLES, FIGURE AND PICTURES		PAGE
Figures		**v**
Pictures		**v**
Tables		**vi**
LIST OF ABBREVIATIONS		**vii**
ABSTRACT		**viii**
ABSTRAK		**ix**
1. INTRODUCTION		
1.1	**Background**	**1**
1.2	**Definitions**	**2**
1.3	**Epidemiology**	**2**
1.4	**Prevalence**	**3**
1.5	**Risk Factor**	**6**
1.6	**Clinical manifestation**	**7**
	1.6.1Definition and Recorded Variables	**8**
1.7	**Pathophysiology**	**9**
1.8	**Upper Esophageal Endoscopy**	**10**
1.9	**24 hour Ambulatory PH Impedance Monitoring**	**16**
1.10	**Manometry**	**19**
1.11	**Transient Receptor Potential Vanilloid 4 (TRPV4)**	**20**

2. OBJECTIVES AND HYPOTHESIS

2.1	**Study Question**	**27**
2.2	**Study Hypothesis**	**27**
2.3	**Study Ojectives**	
	2.3.1 General	**27**
	2.3.2 Specific	**27**

3. METHODOLOGY

3.1	**Study design**	**28**
3.2	**Study Populations and Sampling**	**28**
3.3	**Characteristic of Subjects**	**28**
	3.3.1 Inclusion criteria	**28**
	3.3.2 Exclusion Criteria	**29**
3.4	**Sample Size Calculation**	**29**
3.5	**Research Tool**	
	3.5.1 Interview/ Variables recorded in Data Entry form	**31**
	3.5.2 Endoscopy	**32**
	3.5.3 Immunohistochemistry	**33**
	3.5.4 Manometry	**35**
	3.5.5 24 Hour Ambulatory pH Impedance Monitoring	**35**
3.6	**Ethical Issue and Clearance**	**36**
3.8	**Statistical Analysis**	**37**
3.9	**Flow Chart of the Study Design**	**38**

4.0 RESULTS

4.1 Descriptive Analysis for baseline characteristic 39

4.2 Endosopic Finding 43

4.3 pH Study Result 45

4.4 Manometry result 46

4.5 Statistical Analysis 47

5.0 DISCUSSION 54

6.0 CONCLUSION 62

7.0 LIMITATION 63

8.0 RECOMMENDATION 64

9.0 REFERENCES 65

LIST OF FIGURES, PICTURES AND TABLES **PAGE**

Figure 1 GERD subgroup **4**

Figure 2 NERD prevalence by Ethnic Group **5**

Figure 3 Subcategories of NERD by using 24 H oesophageal PH monitoring **18**

Figure 4 Expression of TRPV4 transcripts in the mouse esophagus by in situ hybridization **24**

Figure 5 Immunohistochemical analysis of TRPV4 in Mouse Esophagus **25**

Figure 6 Gender Distribution among NERD **40**

Figure 7 Gender Distribution among control group **40**

Figure 8 Mean Age among NERD group **41**

Figure 9 Racial distribution among NERD and control group

Figure 10 Distribution of endoscopic finding among NERD group **44**

Figure 11 Distribution of endoscopic finding among control group **44**

PICTURES

Picture 1 Linear furrowing **12**

Picture 2 Circular ring **13**

Picture 3 White nodules/exudates **14**

Picture 4 Linear shearing/crepe paper mucosa **14**

Picture 5 Esophageal stricture **15**

Picture 6 Hiatal hernia **16**

Picture 7 Negative TRPV4 staining **48**

Picture 8 Positive TRPV4 staining **48**

TABLES

Table 1 Demographic data for baseline characteristics **43**

Table 2 24 hour pH Impedance Result **45**

Table 3 Manometry Result **46**

Table 4 Expression TRPV4 in NERD and non NERD **49**

Table 5 TRPV4 at 5cm, comparison mean difference using Independent T test **46**

Table 6 Comparison pH study parameter with TRPV4 positive and negative in NERD **50**

Table 7 Comparison different manometry parameter with TRPV4 positive and negative In NERD **50**

Table 8 Comparison demographic detail with TRPV4 **51**

Table 9 Gender association with TRPV4 in NERD group **51**

Table 10 Comparison demographic detail inTRPV4 positive and negative in non NERD **51**

Table 11Endoscopic with TRPV positive (NERD and non NERD) **52**

Table 11 Endoscopic with TRPV4 negative (NERD and non NERD) **52**

LIST OF ABRREVIATIONS

AB	**Avidin-Biotin**
AET	**Acid Exposure Time**
BMI	**Body Mass Index**
CGRP	**Calcitonin Gene Related Peptide**
DIS	**Dilated Intracellular Space**
EE	**Erosive Esophagitis**
EGD	**Esophagogastroduodenoscopy**
ERD	**Erosive Reflux Disease**
GEJ	**Gastroesophageal Junction**
GERD	**Gastroesophageal reflux disease**
HRM	**High Resolution Manometry**
IEM	**Ineffective Esophageal Motility**
IHC	**Immunohistochemistry**
LES	**Lower Esophageal Sphincter**
NERD	**Non erosive reflux disease**
PAR 2	**Proteinase Activated Receptor 2**
PBS	**Phosphate Buffer Solution**
RT-PCR	**Reverse Transcriptase Polymerase Chain Reaction**
TLESR	**Transient Lower Esophageal Sphincter Relaxation**
TRP	**Transient Receptor Potential**
TRPV4	**Transient Receptor Potential 4**

ABSTRACT

Background

Non erosive reflux Disease (NERD) has emerged as a real entity in the spectrum of gastroesophageal reflux disease (GERD).It may potentially represent the most common manifestation of reflux disease. Although numerous studies had been performed on NERD,none of the the studies had explored the expression of TRPV4 as a causative factor and the link between pH study and manometry. This knowledge is beneficial for new targeted treatment.

Methodology

This was a prospective study that was done on NERD and control patients from March 2017 until November 2017. A total of 55 patients - 39 NERD and 16 control patients were investigated. All patients had undergone EGD and multiple biopsies were taken. Notably, erosive esophagitis patients were excluded. Apart from that, the patients also went through pH study and manometry.

Results

The result from this research we showed that TRPV4 was expressed in both NERD and control patients. No association was discovered between TRPV4 and NERD, as well as among pH study, manometry and endoscopic features. Futhermore, TRPV4 was found to be higher in cells from the normal group as compared to the NERD group. Notably, p-value was insignificant but this may be caused by small sample size

Conclusion

This study was performed to determine the association between TRPV4 and NERD. No significant association was revealed between the expression of of TRPV 4 and the NERD group. Similarly, other parameters from pH study, manometry and endoscopic finding also did not show any significant association with TRPV4.

ABSTRAK

Latar belakang

Penyakit refluks bukan menyebabkan hakisan (*nonerosive reflux disease*, NERD) telah muncul sebagai suatu entiti utama dalam kalangan penyakit refluks gastroesofagus (*gastroesophageal reflux disease*, GERD). NERD berpotensi tinggi untuk mewakili manifestasi penyakit refluks yang paling kerap berlaku. Walaupun pelbagai kajian telah dijalankan terhadap NERD, tiada lagi kajian yang meneliti ekspresi TRPV4 sebagai faktor penyebab dan hubungannya dengan kajian pH 24 jam (24 hour pH study) dan manometri. Kajian ini adalah bermanfaat untuk sasaran rawatan yang baru.

Metodologi

Kajian prospektif ini dijalankan terhadap pesakit NERD dan sekumpulan pesakit normal (sebagai kawalan) dari Mac 2017 sehingga November 2017. Sejumlah 55 orang pesakit terlibat, iaitu 39 orang pesakit NERD dan 16 orang pesakit normal. Semua pesakit telah menjalani EGD dan beberapa biopsi telah diambil. Walau bagaimanapun, pesakit esofagitis erosif (*erosive esophagitis*) telah dikecualikan. Selain itu, pesakit juga menjalani kajian pH dan manometri.

Keputusan

Keputusan kajian membuktikan bahawa ekspresi TRPV4 terkandung dalam kedua-dua kumpulan NERD dan pesakit normal. Tiada sebarang hubungan dapat dikesan antara TRPV4 dan NERD, serta antara kajian pH, manometri, dan endoskopik. Tambahan pula, bagi jumlah sel yg mengekpesikan TRPV4 dikesan lebih tinggi dalam kumpulan normal berbanding

dengan kumpulan NERD bagi. Nilai p adalah tidak signifikan tetapi ini mungkin disebabkan oleh sampel kajian yang kecil.

Rumusan:

Kajian ini dijalankan untuk menentukan hubungan antara TRPV4 dan NERD. Daripada kajian tersebut, tiada hubungan yang signifikan didapati antara ekspresi TRPV4 dan kumpulan NERD. Tambahan pula, tiada hubungan yang signifikan didapati antara TRPV4 dengan parameter yang lain iaitu kajian pH, manometri, dan endoskopik.

1. INTRODUCTION

1.1 Background of GERD, NERD

Gastroesophageal reflux disease (GERD) represents an important medical problem in Western countries: about 20% of the population in Western countries complain of experiencing typical symptoms of this disease (heartburn and acid regurgitation)(Grande et al., 2012).

Gastroesophageal reflux disease (GERD) has been defined in the Montreal Consensus Report as a chronic condition that develops when the reflux of gastric contents into the esophagus in significant quantities causes troublesome symptoms with or without mucosal erosions and/or relevant complications. GERD is associated with a variety of symptoms and also with a variety of lesions including esophageal erosions (or 'mucosal breaks'), ulceration, stricture, Barrett's epithelium and esophageal adenocarcinoma. The cardinal symptoms of GERD are considered to be heartburn and regurgitation, but many patients report other symptoms referable both to the esophagus and to other, extra-esophageal locations(Armstrong, 2008).

GERD is classified into two types based on the endoscopic detection of mucosal lesions (such as erosions), which are endoscopically positive GERD and endoscopically negative GERD. The former type of GERD is known as reflux esophagitis and the latter is almost synonymous with non erosive reflux disease (NERD)(Yoshida, 2007). NERD should be defined as the presence of typical symptoms of gastroesophageal reflux disease caused by intraesophageal reflux (acidic or weakly acidic), in the absence of visible esophageal mucosal injury at endoscopy (Fass, Fennerty, & Vakil, 2001).

1.2 Definition of NERD

NERD is a subcategory of GERD characterized by troublesome reflux-related symptoms in the absence of esophageal mucosal erosions/breaks at conventional endoscopy and without recent acid-suppressive therapy (Modlin et al., 2009). According to the Montreal definition, NERD is a condition in which typical reflux symptoms, heartburn and regurgitation, are defined as troublesome in patients with negative endoscopy (Vakil, van Zanten, Kahrilas, Dent, & Jones, 2006). The absence of visible lesions on endoscopy and the presence of troublesome reflux-associated (to acid, weakly acidic or non-acid reflux) symptoms are the two key factors for the definition of NERD. This clinical entity requires instrumental diagnostic testing (endoscopy and esophageal impedance-pH testing) for its correct diagnosis. Using this technique, we now know that stimuli other than acid can evoke typical reflux symptoms. Fass and colleagues were the first to demonstrate that only 45% of NERD patients have an increased esophageal acid exposure, while the remaining 55% do not have an excess of acid in their esophagus. In the latter group, they identified a subgroup of patients with an esophagus hypersensitive to acid reflux and an additional one with an unclear association between heartburn and some kind of non-acid reflux.

1.3 Epidemiology

GERD is prevalent worldwide, and disease burden may be increasing. The range of GERD prevalence estimation was 18.1%-27.8% in North America, 8.8%-25.9% in Europe, 2.5%-7.8% in East Asia, 8.7%-33.1% in the Middle East, 11.6% in Australia and 23.0% in South America. Incidence per 1000 person-years was approximately 5 in the overall UK and US populations, and 0.84 in paediatric patients aged 1-17 years in the UK. Evidence suggests an increase in GERD prevalence since 1995 ($p<0.0001$), particularly in North America and East Asia(El-Serag, Sweet, Winchester, & Dent, 2014).

Zagari et al. performed a large epidemiologic study in the general population of two villages

in northern Italy and demonstrated a 23.7% (out of 1,033 subjects) prevalence rate of patients with reflux symptoms at least twice a week. Of those patients with reflux symptoms, 75.9% were found to have a negative endoscopy (Zagari et al., 2008).A US study on subjects who had their reflux symptoms controlled by antacids alone has shown that 53% of those subjects had no erosive esophagitis on gastrointestinal endoscopy (NERD) (Robinson et al 1998).

1.4 Prevalence

Over the years several studies have been conducted to determine the prevalence of NERD around the world. In Malaysia, cross-sectional study on consecutive patients with dyspepsia undergoing upper gastrointestinal endoscopy, 134 patients (13.4%) had endoscopic evidence of reflux oesophagitis while 254 patients (65.5%) were diagnosed as having NERD(Rosaida & Goh, 2004). A multicentre prospective study in Korea involving 25 536 subjects, was conducted to determine the prevalence rates and risk factors for erosive oesophagitis and non-erosive reflux disease (NERD) in Korean population. The study used well designed questionnaire and evaluated endoscopic findings. The result showed that 2019 (8%) and 996 subjects (4%) had erosive oesophagitis and non-erosive reflux disease respectively.(Kim et al., 2008). Similarly in Japan, a cross sectional study involving 10 837 subject had been conducted. Of that subjects, 733 (6.8%) presented with endoscopic reflux esophagitis (RE) and 1,722 (15.9%) were diagnosed as non-erosive reflux disease (NERD) (Minatsuki et al., 2013). The prevalence of NERD in medical check-up studies was reported from 3.1% to 4.0%, comprising about 70%-80% of GERD(Jung, 2011).

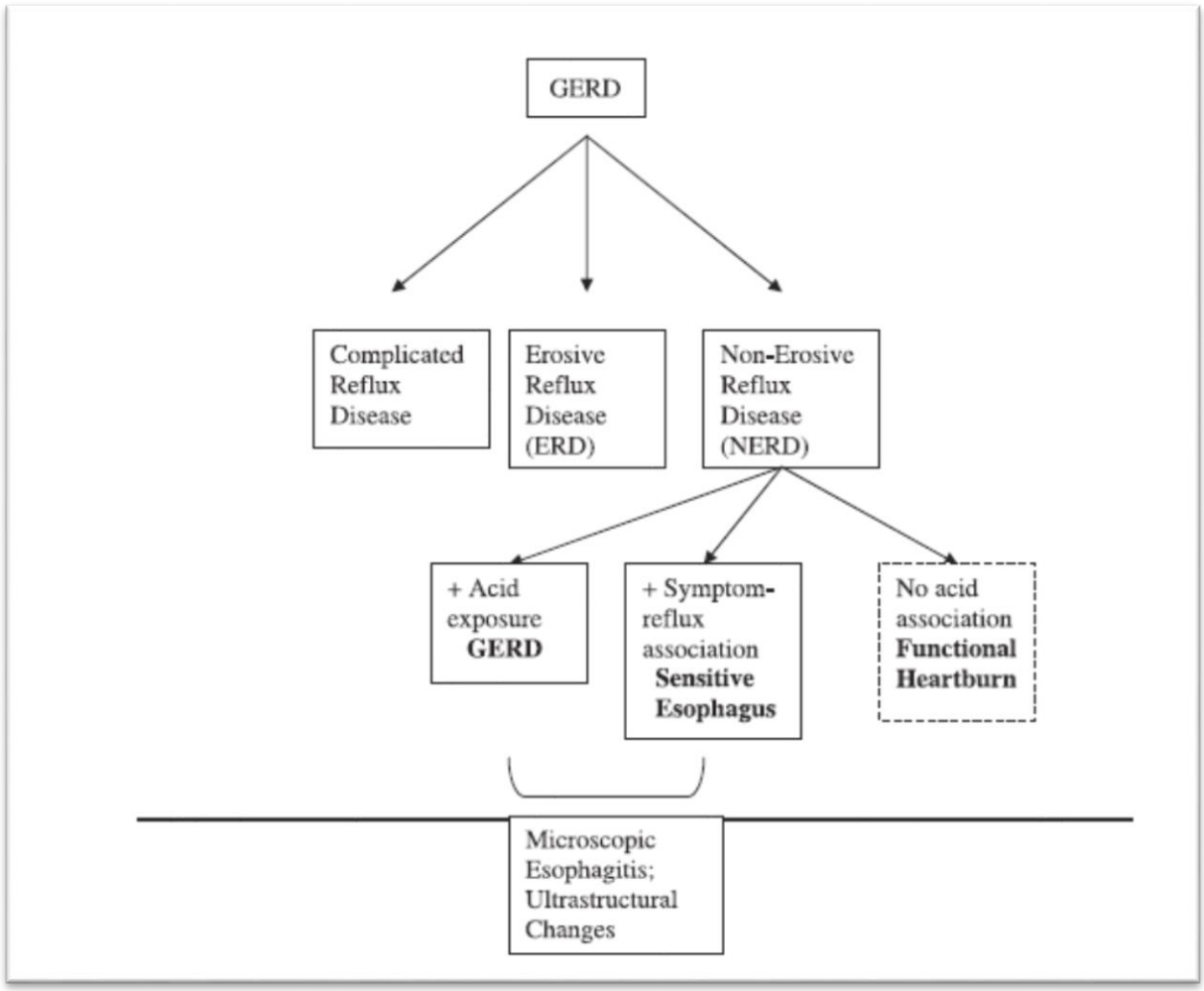


Figure 1: GERD subgroup

Source: Quickley et al , 2006

	Race (% within racial group)			
	Malay	Chinese	Indian	Total
All				
Control	125	352	135	612
RE	23 (11.2)	50 (10.4)	61(19.6)	134
NERD	58 (28.2)	81 (16.8)	115 (32.1)	254
GERD	81 (39.3)	131 (27.1)	176 (56.6)	388
Total	206	483	311	1000

Figure 2Distribution of GERD, RE, NERD by racial distribution in Malaysia

Source from Rosaida and Goh et al 2004

1.5 Risk factor

Multivariate analysis showed that the risk factors for erosive oesophagitis and NERD differed, i.e. those of erosive oesophagitis were male, a *Helicobacter pylori* eradication history, alcohol, body mass index ≥25 and hiatal hernia. In contrast, the risk factors for NERD were female, age <40 and ≥60 vs. 40–59 years, body mass index <23 and a monthly income <$1000, glucose ≥6.9mmol/L, smoking, a stooping posture at work and antibiotic usage (Kim et al., 2008).

In a large Japanese population study, results from univariate analyses demonstrated that gender, *Helicobcter pylori* infection, BMI, pepsinogen (PG) I/II ratio, alcohol intake, and smoking are statistically significant factors for EE. It also showed that H. pylori infection, female gender, higher pepsinogen (PG) I/II ratio, younger age, smoking, higher BMI, and alcohol drinking are positively associated factors for NERD. For age, gender, and H. pylori infection, the directions of correlation for NERD were opposite to those for EE indicating that NERD is an utterly different disorder from EE. (Minatsuki et al., 2013).

Wu et al. evaluated the clinical characteristics of patients with NERD in comparison to those with erosive esophagitis. Each patient underwent endoscopy, esophageal manometry, acid perfusion test, and ambulatory 24-hour esophageal pH monitoring. The authors found that NERD patients had a significantly higher prevalence of functional bowel disorders such as functional dyspepsia and irritable bowel syndrome, psychological disorders, and positive acid perfusion test. Patients with erosive esophagitis were characterized by higher prevalence of hiatal hernia, greater esophageal acid exposure, and more esophageal dysmotility. Carlsson et al., 1998 compared the clinical characteristics of patients with NERD and those with erosive esophagitis. In the NERD group, 60% were female; the mean age was 49 yr; mean weight was 80.5 kg for males and 69.5 kg for females; 23% were smokers; 59% were alcohol consumers; 80% had symptom duration longer than 12 months; 29% had hiatal hernia; and 34% were positive for *Helicobacter pylori*. The erosive esophagitis group was similar to

the NERD group in term of their mean age, smoking and alcohol consumption, prevalence and duration of heartburn, and status of *Helicobacter pylori* infection. However, there were more males (59%) in the erosive esophagitis group, increased prevalence of hiatal hernia (56%), and increased weight of both males and females (86 kg and 76 kg, respectively).

1.6 Clinical manifestation

Typical manifestations of GERD are heartburn or acid regurgitation, however atypical or extra-esophageal symptoms might also be present including respiratory symptoms, such as chronic cough, asthma or laryngitis, dental erosions, non-cardiac chest pain (NCCP) or sleep disturbance.
A large study involving 25 centers in Denmark and Sweden reported on 424 patients with troublesome heartburn associated with NERD (Kim et al, 2008). Heartburn are commonly described a burning sensation behind sternum, rising up to throat and neck. Regurgitation presents as bitter or sour taste in the mouth. Regurgitation is less common than heartburn, more difficult to control with anti reflux treatment. It is exacerbated when one bend over or assume a supine position, or assuming supine position. (Simmonds, 2011).

On the basis of symptoms, the differential diagnosis between GERD and NERD is really challenging. Grande et al, demonstrates heartburn is significantly higher in NERD than in patients with erosive esophagitis. Patients with NERD also complained of extraesophageal symptoms and retrosternal pain more often than EE patients and had a lower incidence of dysphagia. Nonetheless, these differences were not statistically significant.

1.6.1Definition and recorded variables

1. Gastroesophageal reflux disease (GERD)

Gastroesophageal reflux disease (GERD) is a condition in which the stomach contents (food or liquid) leak backwards from the stomach into esophagus (Simmond 2011) . GERD is further divided into erosive relux disease(ERD) and non erosive reflux disease (NERD) based on upper endoscopy finding (Locke et al 1997).

2. Non erosive reflux disease (NERD)

NERD is a distinct pattern of GERD. Defined as troublesome reflux related symptoms in the absence of esophageal mucosal erosions / break at conventional endoscopy (Simmonds, 2011)

3. Heartburn

Heartburn is commonly used to describe burning sensation behind sternum rising up towards the throat and neck (Simmond 2011)

4.Regurgitation

Regurgitation presents as a bitter or sour taste in the mouth. Regurgitation is less common than heartburn, more difficult to control with anti reflux treatment, it is exacerbated when bending over or assuming the supine position (Simmonds 2011)

5.Odynophagia

Odynophagia is painful swallowing , in the mouth (oropharynx) or esophagus. It can occur with or without dysphagia.

6.Dysphagia

Dysphagia is difficulty in swallowing , it may be a sensation that suggests difficulty in passage of solids or liquids from the mouth to the stomach, a lack of pharyngeal sensation or various other inadequacies of the swallowing mechanism .

1.7 Pathophysiology

Recent studies have provided greater insight into the pathophysiology and symptom generation in NERD. The major concepts in the pathophysiology include the pattern of mucosal response to gastric contents during reflux and on mucosal factors that may affect symptom perception. The pathophysiology as reduced ability to clear acid from the esophagus following reflux events in patients with erosive disease is uncommon in NERD patients.However, the latter group is characterized by greater esophageal sensitivity in the proximal esophagus.

The potential explanations for the symptom generation in NERD include microscopic inflammation, visceral hypersensitivity (stress and sleep), and sustained esophageal contractions.It has been observed that acid exposure disrupts intercellular connections in the esophageal mucosa, producing dilated intercellular spaces (DIS) and increasing esophageal permeability, allowing refluxed acid to penetrate the submucosa and reach chemosensitive nociceptors. DIS has been observed in both NERD and erosive disease without significant specificity as is also found in 30% of asymptomatic individuals. DIS has been found to regress with acid suppression.

Peripheral receptors are shown to be mediating esophageal hypersensitivity due to acid reflux including upregulation of acid sensing ion channels, increased expression of TRP receptor and prostaglandin E-2 receptor (EP-1). It is suggested that visceral hypersensitivity plays a more important role in NERD while esophageal acid exposure more on erosive esophagitis (Justin CY Wu, 2008). Three broad mechanisms are believed to underlie visceral hypersensitivity: peripheral sensitisation, central sensitisation and psychoneuroimmune interactions. Detection of TRP channel in alimentary tract is postulated to have lead to visceral hypersensitivity. Activation of TRP channels generates signals that are transmitted to the central nervous system via either vagal or spinal nerves that lead to pain stimulus (Knowles & Aziz, 2008).

1.8 Upper gastroesophageal endoscopy

As stated in the definition, the diagnosis of NERD depends on exclusion of erosive disease by esophagogastrduodenoscopy (EGD). The diverse characteristics of NERD are apperent on endoscopy. Erosions are absent in these patients, but changes such as reddish or whitish discoloration are sometimes seen in areas of esophageal mucosa. Others patients may display normal esophageal mucosa.

The Los Angeles (LA) classification describes four grades of esophagitis severity A to D(Lundell et al., 1999)based on extent of esophageal lesions known as " mucosal breaks " are used for ERD classification.

Grade A: one or more mucosal break < 5 mm in length

Grade B: at least one mucosal break >5mm long, but not continuous between tops of adjacent mucosal folds

Grade C: At least one mucosal break that is continuous between tops of adjacent mucosal folds, but which iinvolved less than 75% of circumference

Grade D: mucosal break that involves 75% of the luminal circumferences

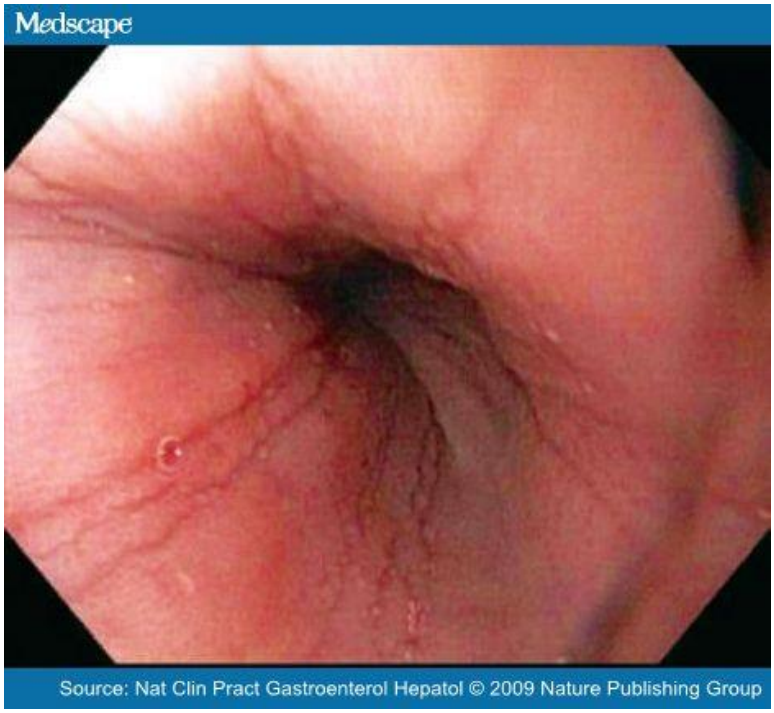
Although (NERD) is called endoscopy negative reflux disease it is suggested that mucosal changes in NERD patients may be too subtle to be detected by conventional endoscopy. Narrow-band imaging (NBI) was introduced for better visualization of mucosal and microvascular patterns at the esophagogastric junction of NERD patients with normal endoscopy. This technique utilizes spectral narrow band filters and enables imaging of superficial tissue structures such as capillary and mucosal patterns without the use of dye. It was demonstrated that the presence of microerosions and increased vascularity at the squamocolumnar junction were the best predictors for GERD diagnosis.(Sharma et al., 2007) NERD patients appear to have intrapapillary capillary loops and microerosions identified on NBI than controls. As a result of this, , the term "minimal change esophagitis" was introduced, and further studies claimed that these mucosal changes can be detected in many patients with NERD. among this histological changes were basal cell hyperplasia, dilated intercellular spaces and papillary elongation(Savarino et al., 2013).

Distinguishing NERD patients from those with eosinophilic esophagitis has become a major area of interest in the past few years. Dellon et al. compared clinical, endoscopic, and histologic findings between eosinophilic esophagitis and GERD. Features that independently predict eosinophilic esophagitis included younger age, dysphagia, food allergy, esophageal rings, linear furrows, white plaques or exudate, absence of hiatal hernia, a higher maximum eosinophil count, and the presence of eosinophil degranulation in the biopsy specimen. (Dellon et al.)

1.8.1Linear Furrowing

Linear or longitudinal furrows are vertical esophageal lines or ridges in the esophageal wall

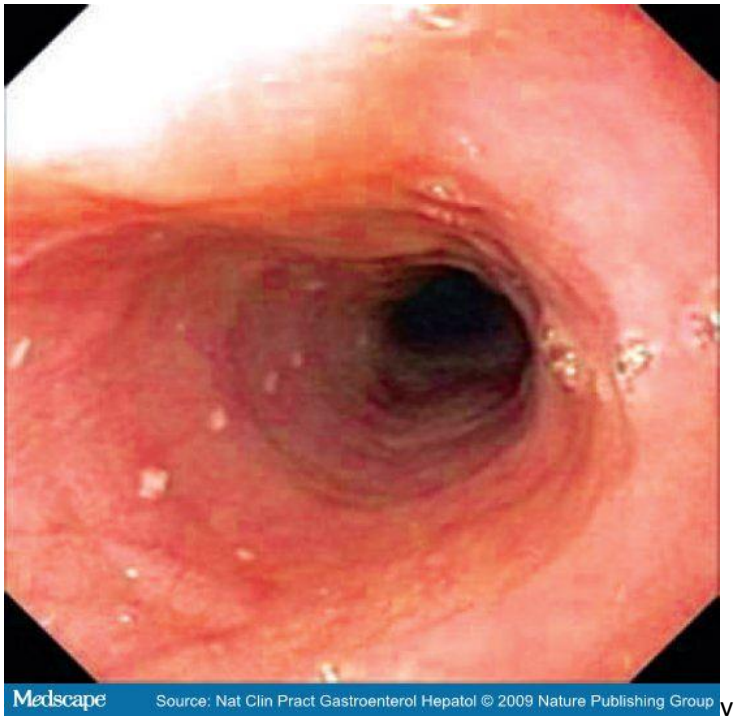
(Carr and Watson 2011)



Picture 1 Linear Furrowing (www.medscape.com)

1.8.2 Circular rings

Circular ring or esophageal rings are defined as multiple rings that may be fine, web like or thickened. It also termed “corrugated” or “ringed” esophagus (Carr and Watson 2011)



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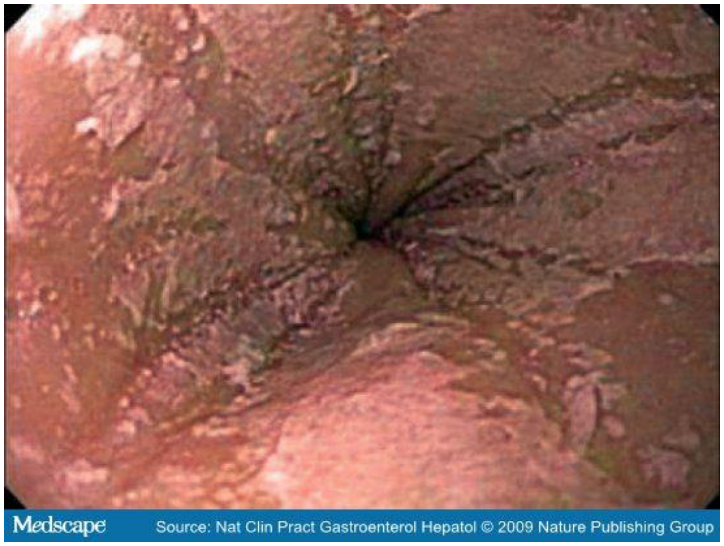
Picture 2 Circular ring (www.medscape.com)

1.8.3 White nodules/exudates

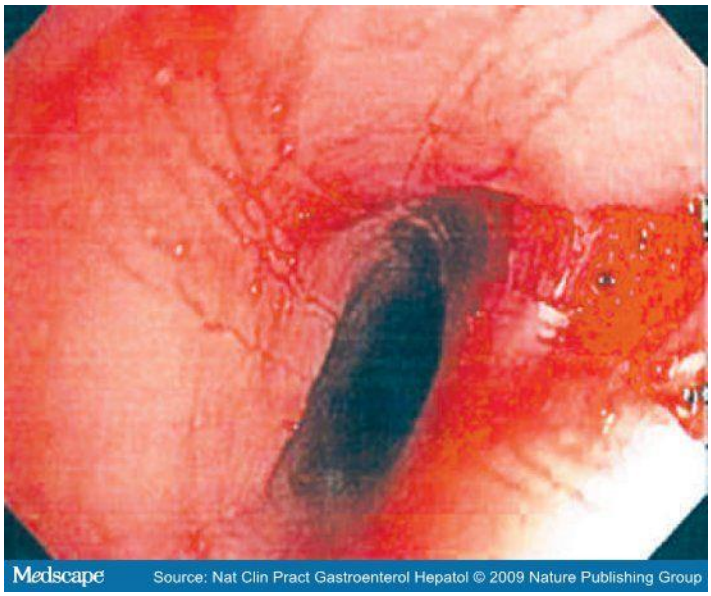
Whitish nodules or exudates is described as patches of whitish papules that can be seen as 1-2mm in diameter and can be scattered along the length of mucosal surface of the esophagus (Carr and Watson 2011). They resemble small patches of candida albicans but actually represent eosinophilic microabscesses (Carr and Watson 2011)



Picture 3 White nodules/exudates (www.medscape.com)

1.8.4 Crepe paper mucosa/ linear shearing

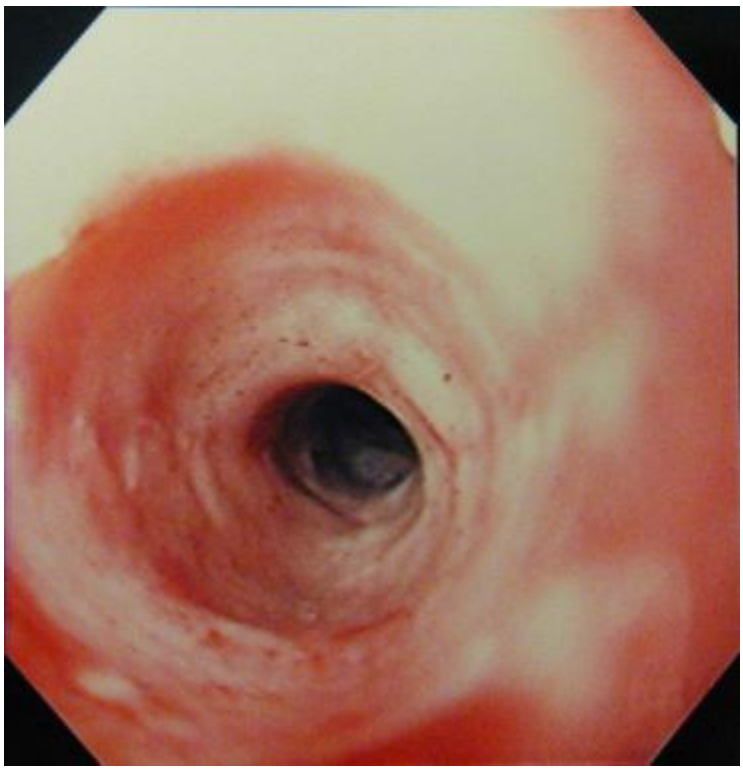
Crepe paper mucosa is described as mucosal abrasions or shearing that occur upon minimal contact. It is phenomenon when the fragile mucosa can fracture with passage of the endoscope if the esophagus in narrow in calibre (Carr and Watson 2011)



Picture 4 Linear shearing/crepe paper mucosa (Carr and Watson 2011)

1.8.5 Esophageal stricture

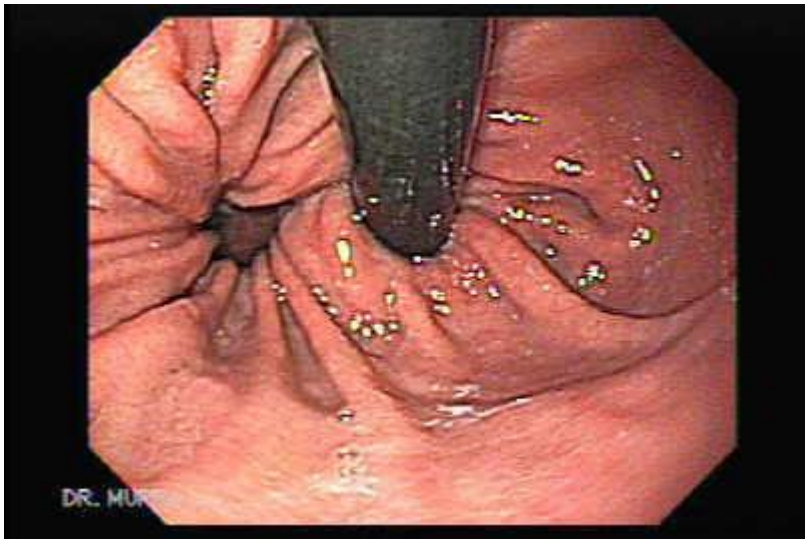
The esophageal stricture is described as narrowed esophagus with fixed internal diameter, and has poor expansion on air insufflation (Carr and Watson 2011). The narrowing of esophagus can be benign or malignant.



Picture 5 Esophageal stricture (www.medscape.com)

1.8.6 Hiatal hernia

Hiatus hernia refers to herniation of elemens of the abdominal cavity most commonly stomach, into the mediastinum, through esophageal hiatus of the diaphragm. The main types of hiatal hernia are sliding type and para esophageal type (Nabh 2013)



Picture 6 Hiatal hernia (www.medscape.com)

1.9 24 Hour Ambulatory pH Impedance Monitoring Study

The important improvements in the definition of NERD were established with the advent of esophageal impedance-pH testing; tool use for the diagnosis and subclassification of GERD. 24-hour impedance pH monitoring enables detection of acidic, weakly acidic and nonacidic reflux and correlation with symptoms. This technique is able to identify three subsets of NERD (i.e.,patients with an excess of acid, with a hypersensitive esophagus [to weakly acidic reflux], or with nonacid-reflux related symptom) and patients with functional heartburn.

A reflux episode was defined as a pH decrease below 4 pH units at the distal esophageal sensor lasting $\geq$ 4 s. If the pH decreased to below 4 pH units in the middle esophagus or both the middle and proximal esophagus, simultaneously with a similar pH decrease in the distal oesophagus, the reflux episode was defined as propagated (proximal reflux). The duration of each reflux episode was assessed at the three esophageal sensors. The acid exposure time was defined as pathological if the percentage of time during which pH < 4 exceeded the upper limits of normal values in the total

recording time (5%) at the level of the distal esophagus.(Smout, Breedijk, van der Zouw, & Akkermans, 1989) .

Fass and colleagues were the first to demonstrate that only 45% of NERD patients have an increased esophageal acid exposure, while the remaining 55% do not have an excess of acid in their esophagus. Using this technique, we now know that stimuli other than acid can evoke typical reflux symptoms. In a study comparing between NERD and ERD, there is distinct differences in clinical and physiologic characteristics between NERD and ERD patients. Patients with high-grade reflux esophagitis had the highest esophageal acid exposure, whereas NERD patients had lower acid exposure (J. C. Wu, Cheung, Wong, & Sung, 2007).

Recently, one study involving 150 NERD patients off PPI therapy found that an increased esophageal acid exposure was present only in 42% of cases. The remaining 58% of patients had normal esophageal acid exposure and among them, 32% and 26% respectively, had a positive and negative symptom association probability(Savarino et al., 2008).

Ambulatory 24-hour esophageal pH monitoring is essential for diagnosing NERD, especially after the recent introduction of the new definitions for functional heartburn by the Rome III Committee for Functional Esophageal Disorders.(Drossman, 2006). Functional heartburn is defined as “episodic retrosternal burning in the absence of pathological gastroesophageal reflux, pathology-based motility disorders, or structural explanations. The Rome III Committee for Functional Esophageal Disorders redefined the functional heartburn group, and consequently NERD, by primarily incorporating the hypersensitive esophagus group and those patients with negative symptom association who are responsive to PPI treatment back into the NERD group.

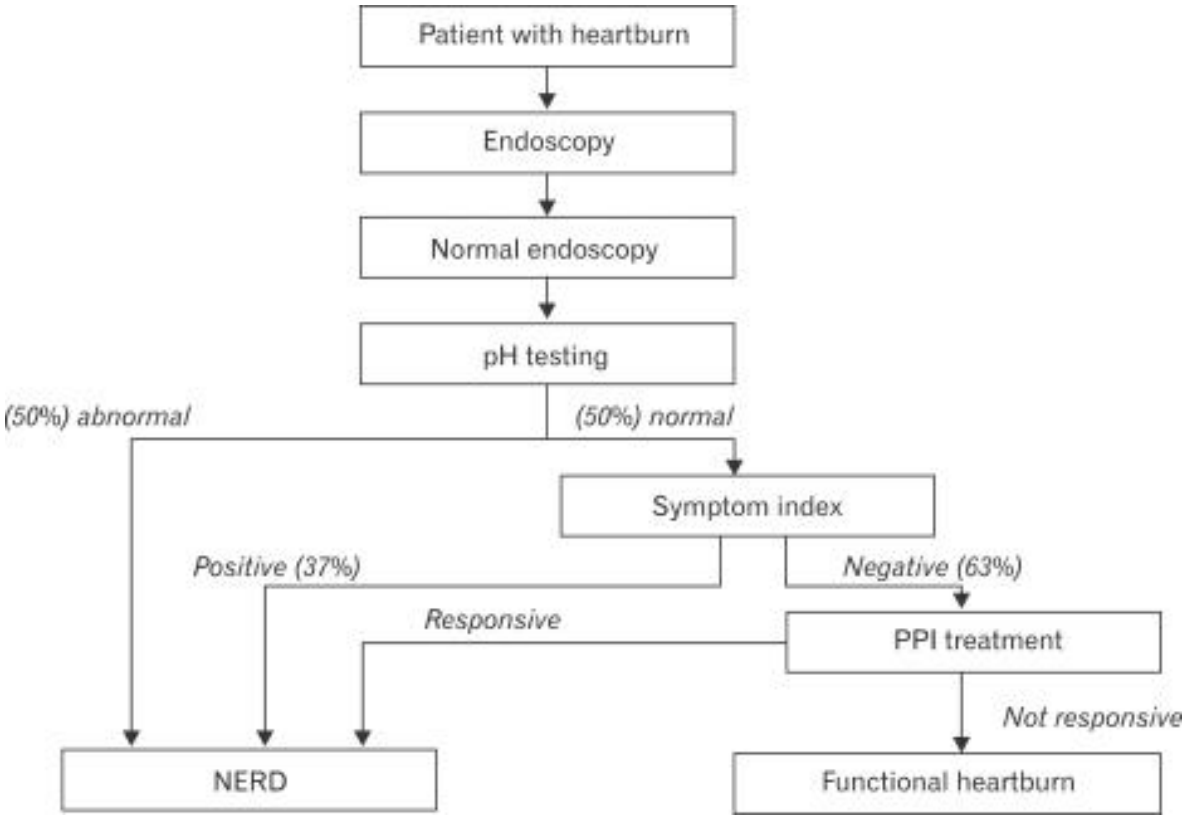


Figure 3 Algorithm for NERD and functional heartburn based on Rome III criteria

1.10 Manometry

Esophageal motility abnormalities are among the main factors implicated in the pathogenesis of gastroesophageal reflux disease. The pathogenesis of GERD is multifactorial, involving transient lower esophageal sphincter (LES) relaxations (TLESRs) as well as other LES pressure abnormalities (i.e., hypotensive LES). Moreover, other factors contributing to the pathophysiology of GERD include impairment of the esophagogastric junction (EGJ) (i.e., hiatal hernia), ineffective esophageal acid and bolus clearance, delayed gastric emptying and impaired mucosal defensive factors.(Castell, Murray, Tutuian, Orlando, & Arnold, 2004) The anti-reflux barrier, consisting of LES, crural diaphragm (CD), angle of His and normal thorax-abdomen pressure gradient, prevents reflux of gastric contents into the esophagus, whereas esophageal peristalsis helps to clear the refluxate and reduce exposure to noxious components of gastric juice. The main motility abnormalities contributing to the occurrence of refluxes in GERD are impairment of the GEJ (i.e., TLESRs, hypotensive LES, anatomic distortion of the GEJ) and ineffective esophageal motility (IEM).(Martinucci et al., 2014).

Manometric studies were performed to evaluate the LES for amplitude, length and capacity of relaxation upon swallowing. The features and morphology of the swallowing complexes were analyzed together with the propagation of peristaltic waves in the body of the esophagus.
HRM combined with multichannel impedance monitoring (HRM-MI) allows a simultaneous and more accurate analysis of the reflux episodes and esophageal motility. Transient lower esophageal sphincter (LES) relaxations (TLESRs) are the most important mechanism leading to gastroesophageal reflux in patients with gastroesophageal reflux disease (GERD) as well as in healthy subjects.
There were several studies comparing the esophageal motility function and acid exposure in NERD and EE patients. In general, erosive reflux disease patients had lower LES pressure, amplitude of distal esophageal peristalsis, and higher rate of ineffective peristalsis. These manometric abnormalities were associated with high esophageal acid exposure (J. C. Wu et al., 2007) .

Frazzoni et al showed that the mean LES pressure was significantly lower in ERD and NERD patients than in controls and functional heartburn patients. The mean distal esophageal wave amplitude was lower in patients with EE than in patients with NERD, functional heartburn and controls. In addition, the prevalence of hiatal hernia was significantly higher in EE and NERD than in functional heartburn subjects and controls. In line with these results, GERD patients have a greater prevalence of abnormally low LES pressure, IEM and hiatal hernia compared with patients with functional heartburn and healthy controls. From the study, IEM gradually increased from controls and functional heartburn to NERD and from erosive reflux disease to Barret's Esophagus patients.

A study performed with HRM coupled with simultaneous fluoroscopy that investigated the esophageal motor events leading to esophagogastric junction opening during TLESRs in healthy subjects showed that esophageal shortening and inhibition of the crural diaphragm always occur before esophagogastric junction opening and the occurrence of a common cavity (Pandolfino et al.)

Another study comparing TLESR between NERD patient and healthy subjects showed TLESRs in NERD patients are associated more often with reflux episodes than in healthy subjects (Ribolsi, Holloway, Emerenziani, Balestrieri, & Cicala, 2014)

1.11 Transient Receptor Potential Vanilloid 4 (TRPV4)

The etiology of esophageal mucosal injury is complex, since it may involve the reflux of gastric acid, bile acid, and pancreatic juice, external factors such as drugs and alcohol, or functional factors such as esophagogastric motility. The mechanism of esophageal mucosal injury has gradually been understood at the molecular biological level. It is particularly important that pro-inflammatory factors, such as inflammatory cytokines (interleukin-6 and-8), leukocytes and oxidative stress, have been demonstrated to be involved in the development of gastroesophageal reflux disease (GERD) including non erosive reflux disease (NERD).

In addition, nociceptors such as acid-sensitive vanilloid receptors, protease-activated receptors and substance P have also been implicated in the pathogenesis of neurogenic inflammation in NERD patients with esophageal hypersensitivity (Yoshida et al., 2013).

TRPs are intrinsic membrane proteins that allow the passage of cations. Except for TRPM4 and TRPM5, all TRP channels are Calcium-permeable cation channels but their selectivity towards cations varies greatly among different TRPs –(Boesmans, Owsianik, Tack, Voets, & Vanden Berghe, 2011). The activation mechanism of TRP channels is unclear in many cases, but known activators include specific agonists such as capsaicin (TRPV1) and mustard oil (TRPA1), an increase in intracellular Ca2+ (TRPM4, 5), temperature (heat: TRPV1, 2, 3, 4, TRPM4, 5; cold: TRPM8, TRPA1), mechanical or osmotic stress (TRPV4, TRPC) and phospholipase C (PLC) activation. Cell swelling activates TRPV4 via the PLA2-pathway (Boesmans et al., 2011).

In the periphery, activation of sensory nerve endings, which feed into nociceptive pathways of the central nervous system (CNS), give rise to the sensation of pain. The threshold for pain has to be high enough not to interfere with normal physiology, but low enough that it can be evoked before marked tissue damage occurs . In order to achieve this function "nociceptive" nerve endings express a variety of ion channels and receptors which transduce mechanical and chemical stimuli or regulate neuronal excitability (Brierley, Hughes, Harrington, Rychkov, & Blackshaw, 2010).

The mammalian transient receptor potential (TRP) superfamily comprises of 28 TRP cation channels that can be subdivided into six main subfamilies: the TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPML (Mucolipin) and the TRPP (Polycystin) channels (Ramsey et al., 2006). Six mammalian genes TRPV1–TRPV6 code for the members of the TRPV subfamily.

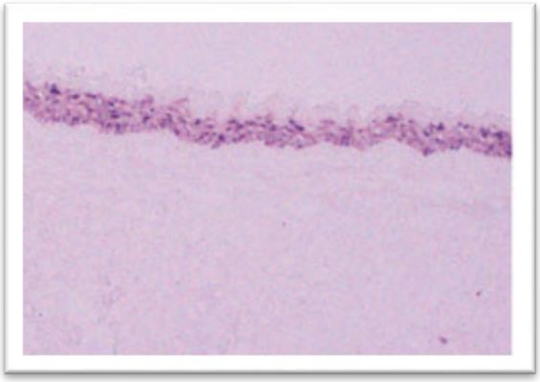
TRPV,a subgroup of TRPV channels,has been most commonly studied in the pathogenesis of GERD and NERD. It is predominantly expressed on unmyelinated and some thinly myelinated sensory neurons that can be activated by capsaicin, noxious heat, acidosis (pH < 5.9), depolarization and endovanilloids (Voets et al., 2002)

TRPV1 mRNA and protein expression were examined in the esophageal mucosa of non-erosive reflux disease (NERD) and erosive esophagitis (EE) patients which correlated to esophageal acid exposure. The result showed NERD and EE patients presented increased TRPV1 receptors mRNA and protein, although no correlation with acid exposure was demonstrated. Increased TRPV1 in the esophageal mucosa may contribute to symptoms both in NERD and EE patients and possibly account for peripheral mechanisms responsible for esophageal hypersensitivity in NERD patient. (Guarino et al., 2010).

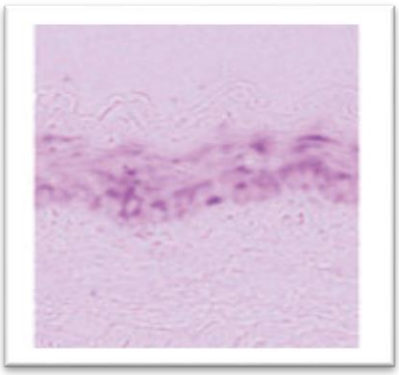
The transient receptor potential vanilloid 4 (TRPV4) is also subtype TRP channel family. TRPV4 is a calcium-permeable channel that is activated by mechanical or osmotic (hypotonicity) stress. The first study reporting the expression of TRPV4 in the gut showed that retrogradely labeled neurons from the gut expressed TRPV4 transcript (Zhang, Jones, Brody, Costa, & Brookes, 2004). Later on, TRPV4, proteinase-activated receptor-2 (PAR2) and calcitonin gene-related peptide (CGRP) were expressed in the same neurons at intestinal neuron, suggesting that TRPV4 is present on sensory neurons. (Sipe et al., 2008)

Cenec e al, 2008 performed TRPV4 immunostaining in whole colonic tissues in mice, and demonstrated like others, that TRPV4 was expressed on neurons, but also that TRPV4 was strongly expressed in intestinal epithelial cells, and in unidentified cells present in the submucosa and in the muscular layer . Another study investigated the expression of TRPV4 in the mouse esophageal , using TRPV4 expression at the mRNA and protein levels using reverse transcription-polymerase chain reaction (RT-PCR), in situ hybridization, and immunohistochemistry. It was found that TRPV4 mRNA was expressed in the mouse esophageal epithelium, in situ hybridization analysis was carried out to ascertain the localization which showed this was mainly located in the intermediate and basal cells of the epithelium. Immunohistochemically analyses performed showed that TRPV4 immunoreactivity was only detected within the esophageal epithelia (Figure 1). At higher magnification, the immunoreactivity was most prominent in the basal layer of the epithelium, and a moderate immunoreaction for the protein was observed in the intermediate layer TRPV4 expression human esophageal tissue.(Shikano et al., 2011)

Another study was conducted to determine TRPV4 expression in the human esophagus and its precise location.. To determine the precise location of the TRPV4 protein within the esophageal mucosa fluorescent immunohistochemistry was performed on human esophagus tissue. There was strong positive TRPV4 immunoreactivity in the basal cells of the esophageal epithelium, and this immunoreactivity grew weaker as cells emerged closer to the luminal surface.(Ueda, Shikano, Kamiya, Joh, & Ugawa, 2011).



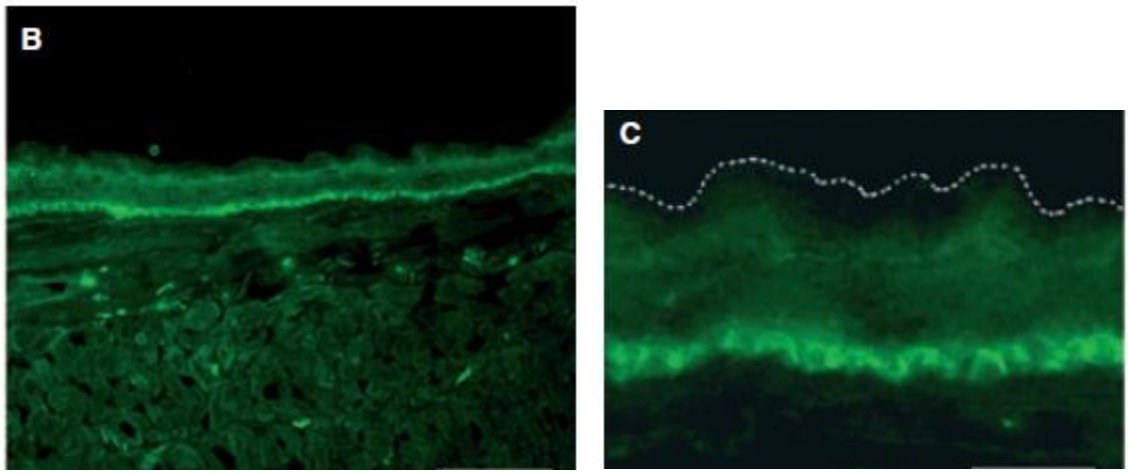
Strong expression of TRPV4 transcripts was observed in the basal and intermediate layers of the esophageal epithelium at low magnifications when TRPV4 antisense probe was used



Strong expression of TRPV4 transcripts was observed in the basal and intermediate layers of the esophageal epithelium at high magnifications when the TRPV4 antisense probe was used

Figure 4 Expression of TRPV4 transcripts in the mouse esophagus by in situ hybridization.

SOURCE: Shikano et al, 2011



(B) TRPV4 immunoreactivity was only found in the epithelium at low magnification

(C) At higher magnification, strong immunoreactivity for the protein was observed in the basal layer of the epithelium and moderate immunoreactivity was detected in the intermediate layer

Figure 5 Immunohistochemical analysis of TRPV4 in mouse esophagus

There are few proposed mechanism how TRPV4 lead to NERD:

1.It is suggested that visceral hypersensitivity plays a more important role in NERD while esophageal acid exposure is related to erosive esophagitis .(Justin CY Wu, 2008). Three broad mechanisms are believed to underlie visceral hypersensitivity: peripheral sensitisation, central sensitisation and psychoneuroimmune interactions. Detection of TRP channel in alimentary tract is postulated to have led to visceral hypersensitivity. Activation of TRP channels generates signals that are transmitted to the central nervous system via either vagal or spinal nerves that lead to pain stimulus (Knowles & Aziz, 2008)

2. TRPV4 may function as a multimodal receptor that regulates a variety of calcium-dependent cellular events, including proliferation, differentiation, and the formation of cell-to-cell junctions. Proliferation in the basal cell layer is important in the repair of reflux-induced injury; however, both eosinophilic esophagitis and gastrointestinal reflux disease (GERD) are characterized by basal cell hyperplasia. Recent studies suggested that the impairment of the esophagus begins in the basal cell layer of the esophageal epithelium. Dilated intercellular spaces caused by breaks in the epithelial junctional barrier are reported feature of reflux damage to the human esophageal epithelium

2.0 OBJECTIVES AND HYPOTHESIS

2.1Study Questions

Is there any association between TRPV4 expression and NERD?

Does TRPV4 play a role in NERD pathophysiology?

2.2 Study Hypothesis

NERD is associated with TRPV4 expression

2.3 Study Objectives

2.3.1 General objectives

This study is to determine the expression of TRPV4 in NERD subjects' vs normal subjects

2.3.2 Specific objectives

1.To compare TRPV4 expression in NERD and normal subjects

2. To compare different pH study parameter between TRPV4 positive and negative subjects

3. To compare different manometry parameter with TRPV4

4. To determine association between TRPV4 expression and demographic data

5. To compare association of TRPV4 expression with endoscopic finding

3.0 METHODOLOGY

3.1 Study Design

Prospective, cross sectional study

3.2 Study Population

The population is all patients from gastrointestinal clinic and wards from Hospital Universiti Sains Malaysia who undergo OGDS

3.3 Characteristic of subjects

3.3.1 Inclusion criteria

Patients:

1. Age 18 years and above
2. GERD symptoms (Using GERD Q questionnaire, score>8)
    - dysphagia
    - food impaction
    - heartburn
    - chest pain
    - nausea and /or vomiting
    - abdominal pain
    - refractory reflux
    - odynophagia
    - weight loss

- hoarseness of voice

Exclusion criteria

1. Coagulation disorders (iatrogenic or inherited)
2. Fungal or esophageal infection
3. Pregnancy
4. Endoscopic finding of erosive esophagitis (Los Angelas classification grade A to D)
5. Patient disagree for OGDS
6. Patient with psychological/neurological disease that do not allow them to have upper endoscopy examination
7. Evident of eosinophilic esophagitis on HPE

3.4 Sample Size Calculation

<u>For objective 1 - To compare TRPV4 expression in non erosive reflux disease (NERD) and non NERD at 5cm and 15cm</u>

Sample size calculation - cannot be calculated because no previous data available.

Sample size will based on Objective 2 and 3

Propose statistical analysis – using independent T test

For objective two – comparing the expression TRPV4 and pH study in NERD - Two proportion formula (independent observation)

From previous study, Joh T. et al. showed that 11.8% of patients with NERD (normal endoscopy finding) had abnormal ambulatory 24-hours esophageal pH study (Joh et al., 2007)

The Power and Sample Size Program version 3 (January 2009) was used to calculate the sample size, with P0 = 0.12 (probability among control) and P1 = 0.37 (probability among exposure). With the level of significant of $\alpha = 0.05$ and the power of the study being 80%, the sample size per group was calculated as follow:

n = required sample size, m (ratio between 2 group)=1:1

Anticipated drop out rate =10%

Power = 0.8

Po = 0.12

P1 = 0.37

n = 48

n = 48 x2

n = 96

For objective three – comparing the expression TRPV4 and manometry in NERD - Two means formula

(independent observation)

From previous study, Impedance High resolution Manometry Analysis of patients with Non Erosive Reflux Disease, Clinical Gastroenterology and Hepatology 2014, mean TLESR in NERD group is 11.7 with standard deviation 7.86. With the level of significant of $\alpha = 0.05$ and the power of the study being 80%, the sample size per group was calculated as follow

Standard deviation (SD) 7.86

Detectable difference 5

Anticipated drop out rate 20%

n = 49 for each arm

n= 49 x 2

n= 98

Objective 4 and 5 – cannot be calculated as this has not been done before. Will be based on objective 2 and 3

3.5 Research Tool

EQUIPTMENTS AND MATERIAL USED FOR RESEARCH

1. Patient's folder
2. GERD Q Questionaire
3. Endoscopy – Olympus model Evis Exera II
4. PH probe (Medical Measuring System or MMS, Amsterdam, Netherland)
5. Manometry (Medical Measurement System or MMS, Amsterdam, Netherland)
6. TRPV4Goat Antibody, Santa Cruz Biotechnology, Dallas, Texas , USA
7. Power and sample size program version 3 and licensed SPSS version 22.0

3.5.1 Interview and Variable recorded in Data Entry form

Patient will be interviewed for GERD symptoms and undergo EGD. Patients who fulfilled study criteria would be invited to participate in the study. After signing the informed consent form, baseline data from patients clinical notes will be reviewed and recorded. All subjects were interviewed by a single doctor before endoscopy.Symptoms during presentation – symptoms are identified and assessed based on frequency of symptoms, intensity and duration of symptoms (GERD Q Questionnaire)

3.5.2 Endoscopy- Olympus model Evis Exera II

Upper endoscopy (EGD) was performed by trained personnel (Gastroenterologist) using the Olympus Evis Exera II (Olympus, Japan). At the time of endoscopy, features of specific endoscopic findings were observed (rings, linear furrows, strictures either at proximal/middle or distal esophagus, white plaques, narrow esophageal calibre, decreased mucosal vascularity, congestive esophageal mucosa , erosive esophagitis, hiatal hernia or normal esophagus) will be reviewed and documented in the EGD data collection form (Appendix 2) and picture of endoscopic findings will be taken. All patients who have erosive esophagitis were excluded from the study.

Two biopsies using standard biopsy forceps (Boston scientifi) were obtained from lower and upper third of esophagus, which were located approximately 5cm and 15cm above gastroesophageal junction epithelium in all patients. The rationales for taking 2 biopsies were due to:

Patients with non-erosive reflux disease and, to a lesser extent, patients with erosive reflux disease, are sensitive to acid in the oesophagus, being more sensitive to proximal acid than distal. (Thoua, Khoo, Kalantzis, & Emmanuel, 2008). Thus biopsies were taken at 5cm (proximal) and 15cm (distal) above GEJ.

All biopsies samples taken would be placed inside formalin containing bottle and then dispatched to pathology laboratory in Pathology Department of Hospital University Sains Malaysia. All biopsies that have been processed by the technician in the Pathology Department of HUSM would be reviewed and interpreted by a single pathologist.

3.5.3 Immunohistochemistry

i. All steps are carried out at room temperature in humidified chamber

ii. Sufficient volumes of reagents is applied to completely cover the section: 100ul is usually adequate, or 1-3 drops of working solutions

iii. Suction is used to remove reagents after each step, drying specimen between steps is avoided

iv. After preparation of tissue, sections incubated for 5- 10 minutes in 0.1 -1% hydrogen peroxide diluted in PBS, deionized H2O or methanol to quench endogenous peroxidase activity. Then, washed in PBS twice for 5 minutes each

v. Section incubated for one hour in 1.5% blocking serum in PBS (mixing bottle)

vi. Section incubated with primary antibody TRPV4 (SANTA CRUZ BIOTECHNOLOGY, USA) for 30 minutes at room temperature or overnight at 4 degrees Celsius. Optimal antibody concentration was determined by titration, ranging from 0.5 – 5.0 ug/ml, diluted in 1.5% blocking serum in PBS (from mixing bottle). Wash with 3 changes of PBS for 5 minutes each

vii. Section incubated for 30 minutes with biotinylated secondary antibody ImmunoCruz™ goat ABC Staining System (SANTA CRUZ BIOTECHNOLOGY, USA) as prepared in mixing bottle 2 or approximately 1ug/ml. Wash with 3 changes of PBS for 5 minutes each

viii. Section incubated for 30 minutes with AB enzyme reagents (AB mixing bottle) . Wash with 3 changes of PBS for 5 minutes each

ix. Section incubated in 1-3 drop peroxidase substrate (substrate mixing bottle) for 30 seconds - 10 minutes or until desired stain intensity developed. The stain maybe checked for staining by rinsing with H2O and viewing under a microscope. Additional peroxidase substrate was added if necessary and continues to incubate. Wash section in deionized H2O for 5 minutes.

x. Section counterstained in Gill formulation hemotoxylin for 5-10 seconds . Immediately wash with several changes deionized H2O

xi. Destain with acid alcohol and bluing reagents. Wash with tap water

xii. For paraffin embedded tissue sections, dehydrate done with 2x 95% ethanol for 10 seconds each, 2x 100% ethanol for 10 seconds each, 3x xylenes for 10 seconds each. Wipe off excess xylenes

xiii. Immediately add 1-2 drops of permanent mounting medium and cover with a glass cover slip.

xiv. Observe by light microscopy.

- Cytoplasmic immunostaining activity of TRPV4 in esophageal epithelial is analyzed under light microscope under 400 magnifications.
- Scoring of immunohistochemical expression of TRPV4 is based on combined score of qualitative and quantitative analyses. The intensity (qualitative) of immunohistochemical staining is evaluated by dividing the cytoplasmic staining reactions into four score groups:

  0 = negative staining,

  1 = weak cytoplasmic staining intensity,

  2 = moderate cytoplasmic staining intensity,

  3 = strong/intense cytoplasmic staining intensity.

- The immunohistochemical staining was quantified from a total of 100 cells as follows:

  0 = no positive staining,

  1 = < 25% of cells show cytoplasmic staining positivity,

  2 = 25–50% of cells show cytoplasmic reactivity,

  3 = > 50% of cells showing cytoplasmic reactivity

- A combined score for immunohistochemical staining was obtained by adding the qualitative and quantitative scores; these sums were then divided into three main groups:

  score = 0: no immunoreactivity;

  score = 1–3: weak immunoreactivity; and

  score = 4–6: strong immunoreactivity.

3.5.4 Manometry

A solid state probe (Medical Measurement System or MMS, Amsterdam, Netherlands) that consists of 36 pressure channels and 18 impedance sensors will be placed across the oesophagus and stomach in all volunteers. Procedures were conducted by trained staff who had performed more than 200 cases over 2 years. Subject will be explained on the procedure, which usually lasts about half an hour. First, the catheter is inserted nasally after given lignocaine spray in the sitting position. The subject will then assume a standing position, followed by a period of rest of approximately 30-40 s to record the resting pressure of the lower esophageal sphincter (LES). Upon completion of all test swallows, the probe can be removed.

In this study, we measure

1. Mean LES
2. IRP4
3. DCI index
4. Distal latency
5. % weak peristalsis

3.5.5 24-Hour Ambulatory PH impedance Monitoring

The pH probe (Medical Measurement System MMS; Amsterdam, Nethelands) was calibrated with buffers at pH 4.0 and 7.0. The probe consisted of one pH sensor located at 5 cm from the tip of catheter, and 6 impedance sensor spaced regularly above the pH sensor. The procedures was conducted by staff who were properly trained and familiar with the devices.The upper border of the LES was determined first using the manometry, and the pH sensor was placed 5 cm above the upper

border. After lignocaine spray given to patient, the catheter was passed nasally, typically in the sitting position. Recordings were started when the probe was placed in its correct location.

Patients were instructed to record any events in a diary. Subject will be allowed home or for those who prefer to stay in the hospital, the admissions to ward were arranged. After completed 24-hours, the probe was then removed.

The analysis of the pH monitoring for our study included the following parameters:

1. Total percentage of total reflux time ph <4
2. De meester score
3. Total reflux
    - Acid (supine and recumbent)
    - Nonacid (supine and recumbent)

3.6 Ethical Issue and Clearance

To meet this requirement, ethical clearance was sought and obtained from the USM Human Research Ethics Committee. The Ethical Committee approval reference number is USM/JEPem/16100401. Furthermore, this study was also conducted in accordance to the principles of ethics on human research as laid down by the Declaration of Helsinki (18th World Medical Association General Assembly, 1964).(Appendix 1)

3.7 Statistical Analysis

Data was analysed using IBM Statistical Package for Social Sciences (SPSS) version 22.0 Armonk, NY: IBM Corp. Data were presented as mean and standard deviation if normally distributed or if not then median for continuous data. The prevalence data were expressed as percentage (%).

There are eight steps that were followed for this statistical analysis:

a) Data exploration and cleaning

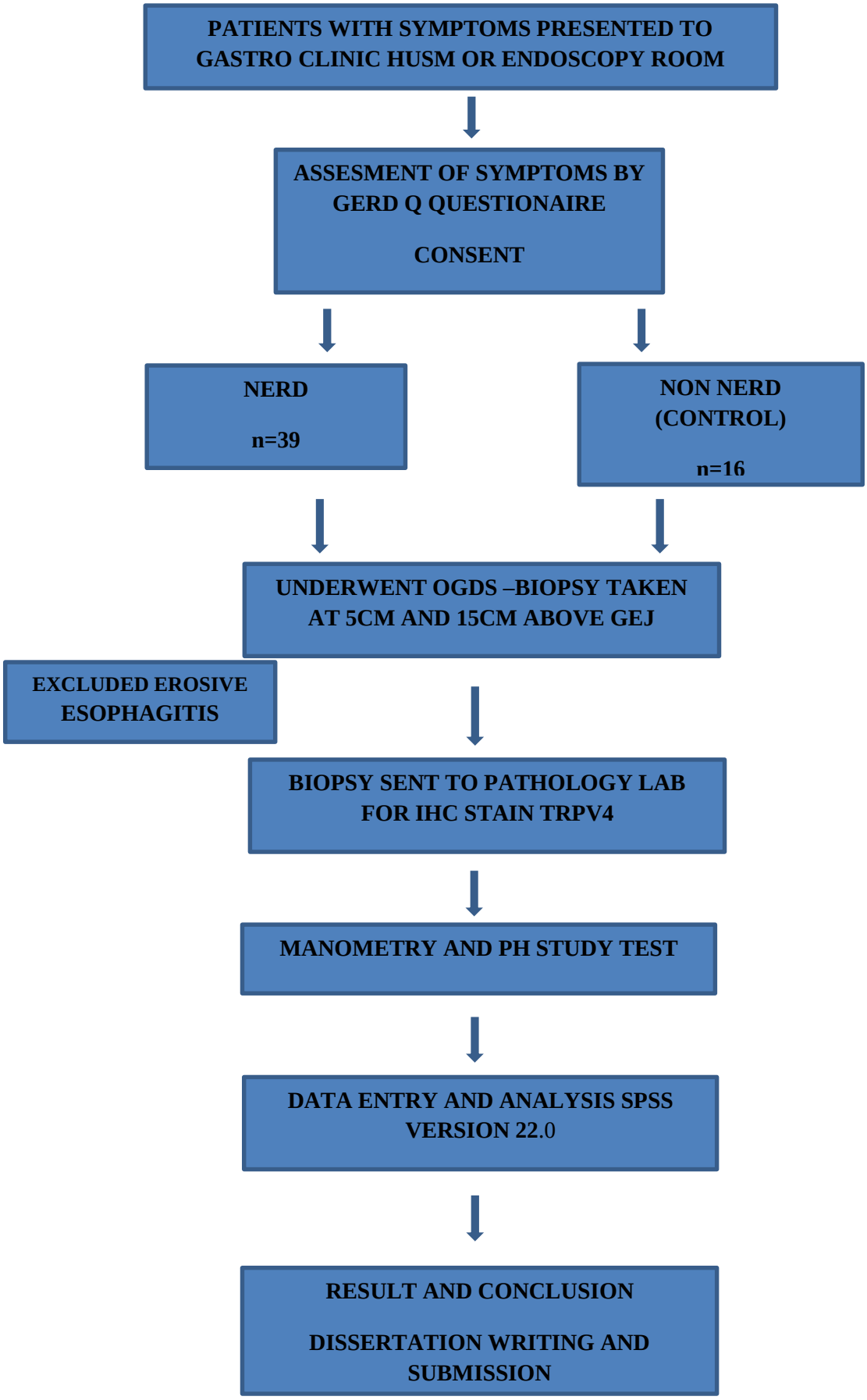
b) Descriptive analysis

c) Analysis based on each objectives:

	OBJECTIVE	ANALYSIS
1.	To compare TRPV4 expression in NERD and normal subjects	When data is normally distribute – Independent T test When data is not normally distribute- non parametric Mann Whitney will be used
2.	To compare different pH study parameter between TRPV4 positive and negative subjects	
3.	To compare different manometry parameter with TRPV4	
4.	To determine association between TRPV4 expression and demographic data	When data met assumption analysis Pearson Chi Square will be used When data did not met assumption analysis Fisher exact test will be used
5.	To compare association of TRPV4 expression with endoscopic finding	

d) Interpretation, presentation and write up

FLOW CHART OF STUDY DESIGN



4.0RESULTS

A total of 55 patients were screened and 39 were included in NERD group based on symptoms assessment and endoscopic findings.

Patient are considered as NERD when,

i. Typical reflux symptom – assessed by GERD Q (score > 8)
ii. No evidence of erosive esophagitis on endoscopy

For the control group, 16 were recruited. Among this were patients were those who volunteered and symptomatic patients but GERD Q score <8.

Total patient for both group were 55, NERD= 39 and non NERD= 16.In both group, patient were offered to continue with pH study and manometry for further evaluation. All patients with erosive esophagitis were excluded from the study.

4.1 Descriptive analysis for baseline characteristic

A total 55 patient were included in the study, 39 in NERD and 16 in control group.
In the NERD group, 59% (23) patients were male and 41% (16) were female. In control group 43.8% (7) were male and 56.3% (9) were female. The mean (SD) age in NERD were 46 (14.38). Majority of the patients involved in this study were Malay 77% (30) and non-Malay (23%). In comparison, the control group, 43.8% (7) were male while 56.3% (9) were female. 93 % of the patients were Malay and 7 % were non malay.

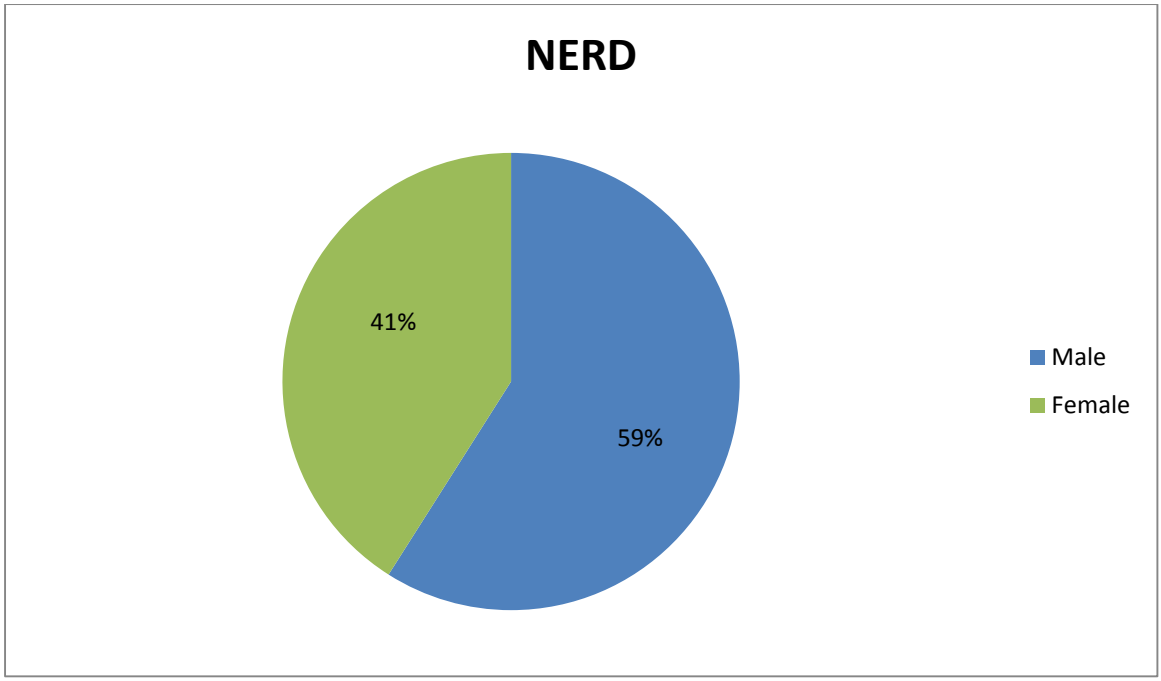


Figure 6 Gender distribution among NERD

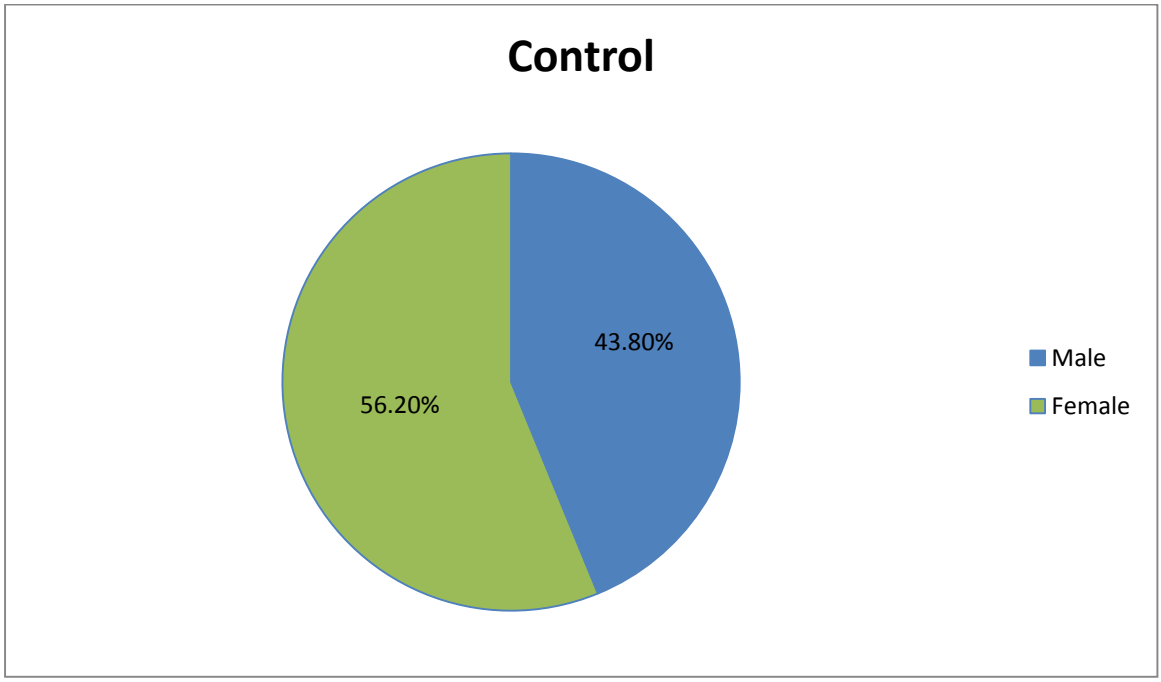


Figure 7 Gender Distribution among control group

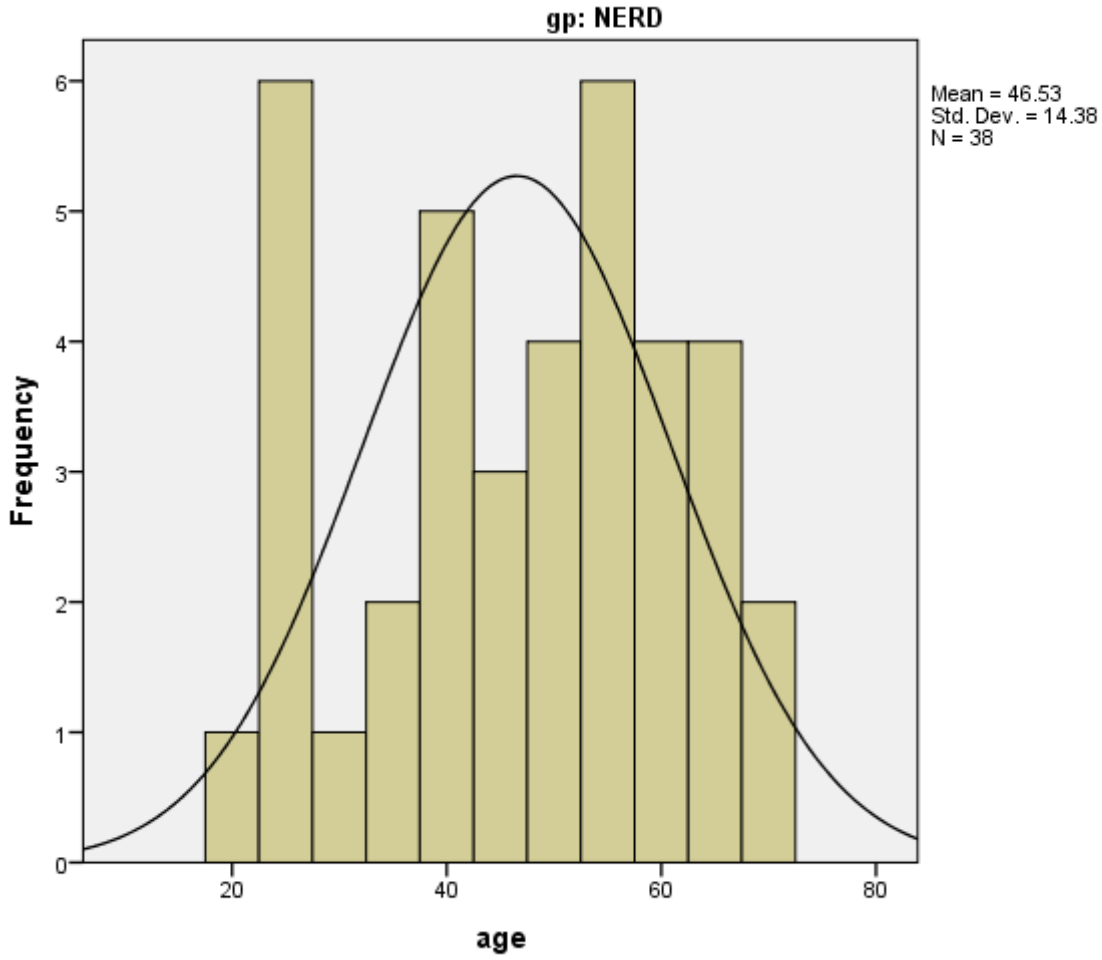


Figure 8 Mean Age among NERD group

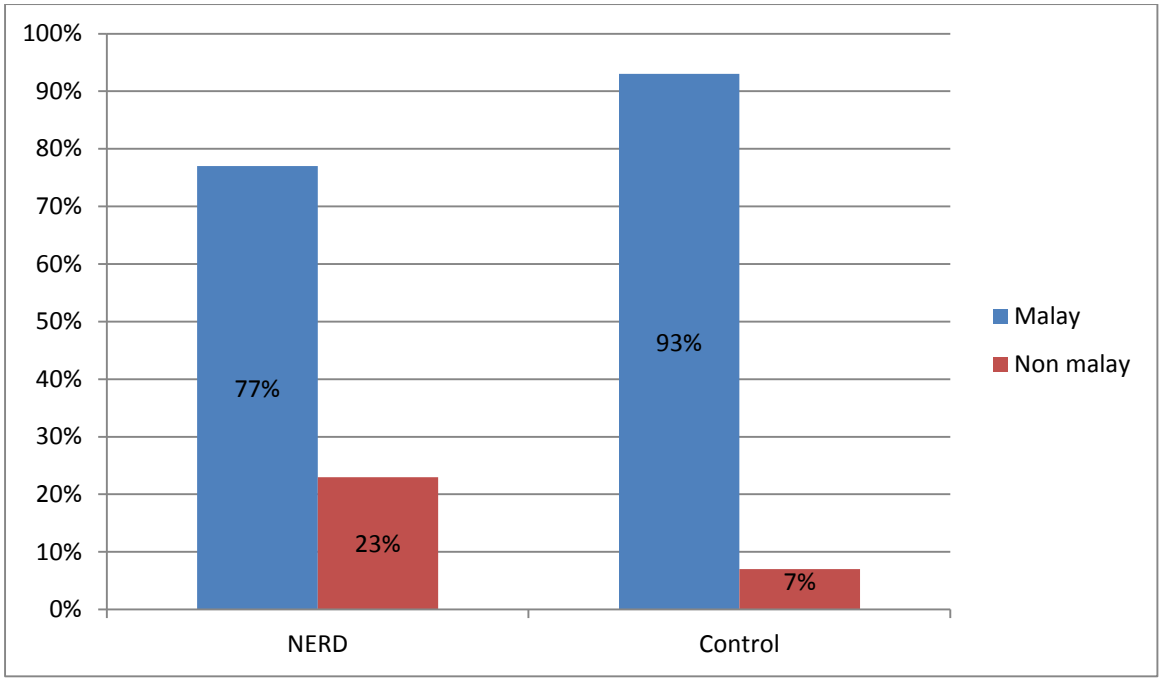


Figure 9 Racial Distribution among NERD and Control group

Table 1 Demographic data for baseline characteristic

Parameter	NERD		Control	
	No of patients (%)	Mean (SD)	No of patients (%)	Mean (SD)
Age		46 (14.38)		50 (28)
Gender				
Male	16 (41%)	9(56.3 %)	9 (56.3%)	
Female	23 (59%)	7 (43.8%)	7 (43.8%)	
Race				
Malay	30 (77%)	15 (93%)	15 (93%)	
Non Malay	9 (23%)	1 (7%)	1 (7%)	

4.2 Endoscopic finding

The most common endoscopic finding in NERD group was white exudates which were documented in 64% (25) of the patient , and this was followed by circular rings 44% (17), congested mucosa 41% (16) , linear furrowing 21 % (8), hiatal hernia (18 %) , decreased mucosa 15 % (6), linear shearing 8% (3) and stricture 3% (1). (Figure 4)

In control group, white exudates documented with 25 % (4) of total patients, followed by circular rings 12.5 % (2). The other features are negative in group.

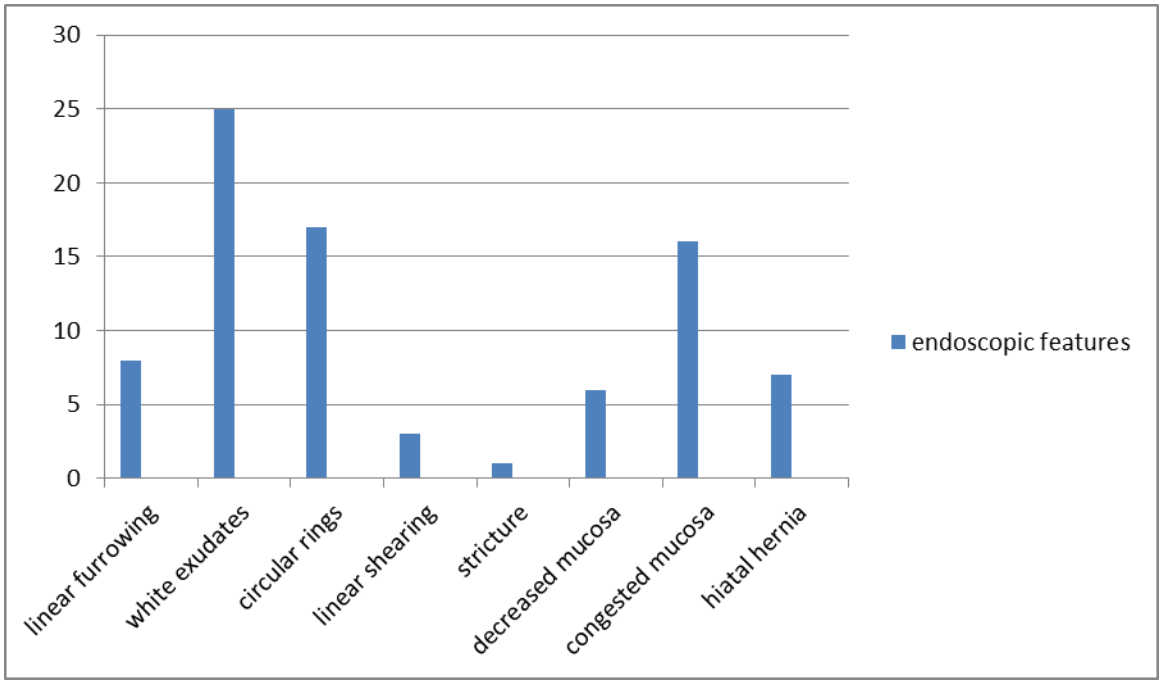


Figure 10 Distribution of endoscopic findings among NERD group

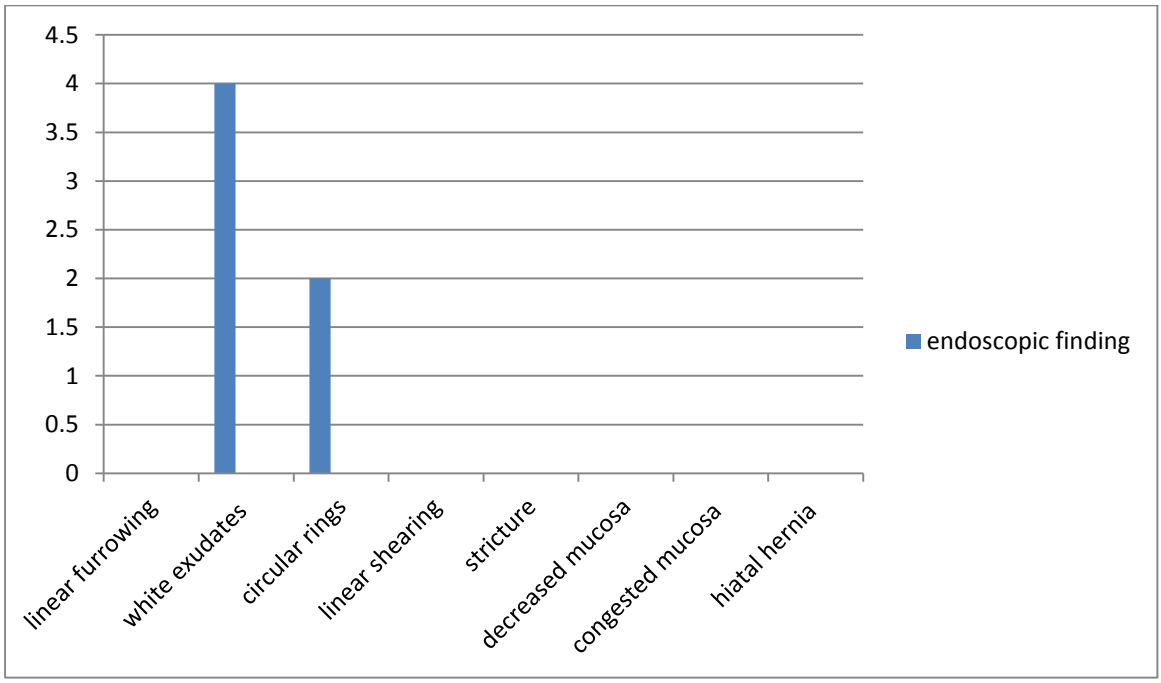


Figure 11 Distribution of endoscopic finding in non NERD group

4.3 pH Study Result

Table 2 showed the result for 24 hour pH impedance study. In NERD group, 39 patients completed 24 hours ambulatory pH impedance study. No patients from control group agreed to undergo the procedure.

Table 2 pH study result

Variables	**Median (IQR)**
De meester score	11.8 (12.15)
Total reflux	32 (27.8)
Acidic reflux	16.9 (23.5)
Non Acid reflux	13.5 (23)
Acidic reflux (upright)	10.5 (25.5)
Acidic reflux (supine)	2.0 (4.0)
Non acid reflux (upright)	2.0 (4.0)
Non acid reflux (supine)	1.0 (3.0)
% pH less than 4 (total)	1.3 (3.4)
% pH less than 4 (upright)	2.0 (3.6)
% pH less than 4 (supine)	0.05 (2.2)

4.4 Manometry Result

Table 3 depicted manometry result in this study. In NERD group, 39 subjects completed manometry test. No data for control group as patient unwilling to undergo the procedure.

Table 3 Descriptive analysis for Manometry parameter

Variables	**Median (IQR)**
Mean LES	21 (17)
IRP4	7.3 (11.4)
DCI	762 (1375)
Distal latency	6.8 (1.6)
% weak peristalsis	10 (60)

4.5 Statistical Analysis

Univariate analysis using Fisher exact test was used to compare TRPV4 positive in NERD and non NERD group. Scoring of immunohistochemical expression of TRPV4 is based on combined score of qualitative and quantitative analyses. The intensity (qualitative) of immunohistochemical staining is evaluated by dividing the cytoplasmic staining reactions into four score groups:

0 = negative staining,

1 = weak cytoplasmic staining intensity,

2 = moderate cytoplasmic staining intensity,

3 = strong/intense cytoplasmic staining intensity.

The quantitative immunohistochemical staining was quantified from a total of 100 cells as follows:

0 = no positive staining,

1 = < 25% of cells show cytoplasmic staining positivity,

2 = 25–50% of cells show cytoplasmic reactivity,

3 = > 50% of cells showing cytoplasmic reactivity

A combined score for immunohistochemical staining was obtained by adding the qualitative and quantitative scores; these sums were then divided into three main groups:

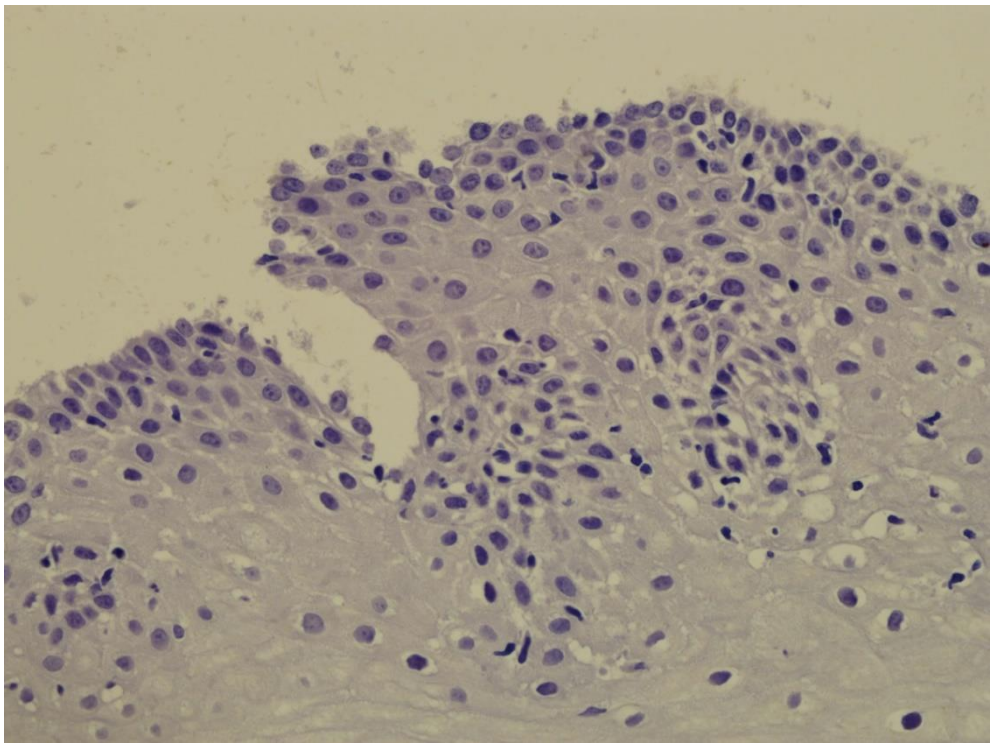
score = 0: no immunoreactivity;

score = 1–3: weak immunoreactivity; and

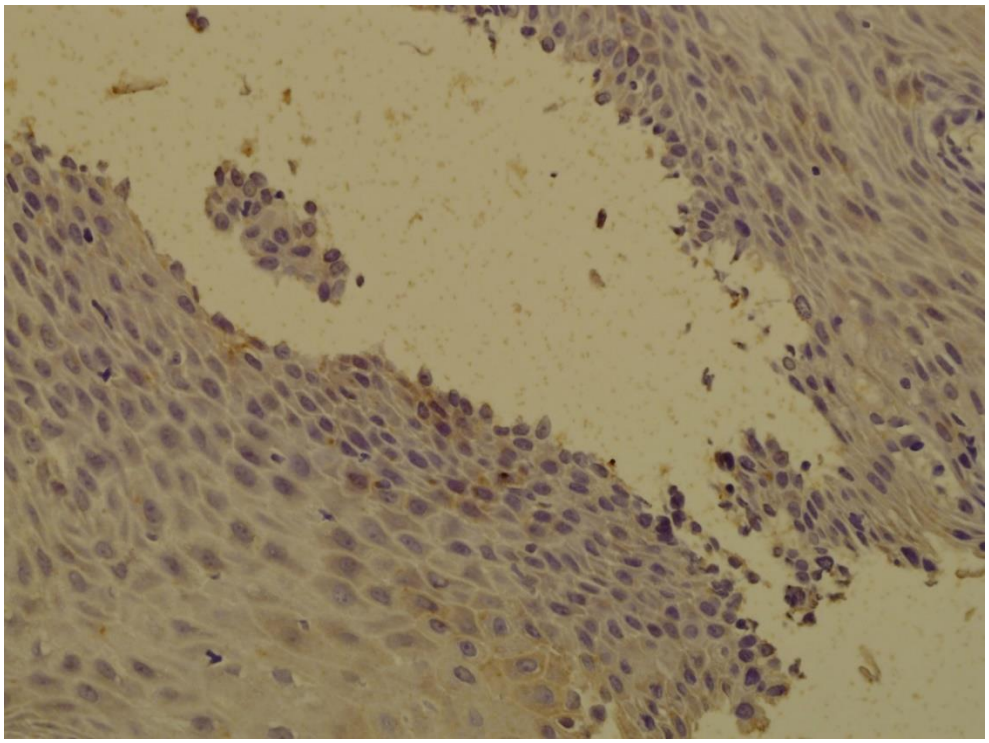
score = 4–6: strong immunoreactivity.

Staining is consider positive when score 1 and above

Immunohistochemistry using TRPV4 SANTA CRUZ, Biotechnology, Dallas, Texas, USA



Picture 7Negative stain for TRPV4



Picture 8 Positive stain for TRPV4

Table 4 Expression of TRPV4 in NERD and non NERD

	NERD, n(%)	Non Nerd, n(%)	p value
			0.71
TRPV 4 positive	7(17.9)	4(25.0)	
TRPV 4 negative	32(82.1)	12(75.0)	
Total	39	16	

Table 5 TRPV4 at 5cm, compare mean difference using Independent T test

	NERD , Mean (SD)	Normal , Mean (SD)	T stat (df)	p value
Intensity	0.21 (0.07)	0.25 (0.11)	-0.33(53)	0.74
Cell stained	0.21 (0.07)	0.31 (0.15)	-0.71(53)	0.48

The TRPV4 expression at 5cm is positive in NERD group, 17.9 % (7) whereas in non NERD group 25% (4) positive, p-value 0.71. We further evaluate the positive result, looking at intensity and number of cell stained with immunorectivity for the staining for both group. From this evaluation, for NERD, the mean for intensity staining in NERD group 0.21 (0.075) as compared to non NERD group 0.25 (0.112). p value is 0.745.

For cell stained in NERD group the mean (SD) was 0.21 (0.075) and in non NERD group the mean (SD) was 0.31 (0.151). p value is 0.482 thus indicating no statistically significant between two group with cell stained .

Biopsies at 15cm yielded only one positive result from NERD thus no statistical analysis carried out.

Objective 2 to compare pH study with 24 hour pH impedance study in NERD group.

Table 6 Comparison different pH study parameters with TRPV4 positive and negative in NERD

Ph study parameter	TRPV4 positive, Median (IQR)	TRPV4 negative, Median (IQR)	Z	P value
De meester score	5.00(23.11)	4.01(11.74)	-0.13	0.89
Total reflux	21.00(37.00)	34.00(27.000)	-1.13	0.25
Reflux (A)	14.00(24.00)	13.00(24.00)	-0.72	0.47
Reflux (NA)	7.00(47.00)	14.00(20.00)	-1.17	0.24
Upright (A)	11.00(22.00)	10.00(27.00)	-0.53	0.59
Upright (NA)	6.00(35.00)	12.00(18.00)	-0.87	0.38
Supine (A)	2.00(3.00)	2.00(5.00)	-0.81	0.41
Supine (NA)	1.00(1.00)	1.00(3.00)	-1.03	0.30
% ph<4 (upright)	2.40(8.70)	2.00(3.20)	-0.13	0.89
% ph <4 (supine)	0.40(3.90)	0.00(2.10)	-0.42	0.67
% ph <4 (total)	1.50(7.00)	1.20(3.40)	-0.16	0.86

For objective 3, comparing manometry parameter with TRPV4 positive and negative in NERD, no significant association is seen.

Table 7 Comparison manometry parameters with TRPV4 positive and negative in NERD

Manometry	TRPV4 positive, Median (IQR)	TRPV4 negative, Median (IQR)	Z	P value
Mean LES	29.00(16.00)	20.50(14.40)	-1.69	0.09
IRP4	8.60(8.30)	6.20(11.30)	-1.04	0.29
DCI	1080.00(2049.00)	759.00(1297.00)	-0.15	0.88
Distal latency	6.90(1.30)	6.70(2.00)	-0.51	0.60
% weak peristalsis	0.30(10.00)	20.00(57.75)	-1.67	0.09

Table 8 To compare demographic detail with TRPV4 positive and negative in NERD

Demographic	**TRPV4 positive, Median (IQR)**	**TRPV4 negative, Median (IQR)**	**Z**	**p-value**
Age	58.50(19.00)	44.00(24.00)	-1.62	0.10
	NERD, n(%)	Non Nerd, n (%)		
Female	16(41.0)	9(56.3)		0.37
Male	23(59.0)	7(43.8)		

In NERD group, insignificant result for demographic data compared with TRPV4 negative and positive

Table 9 To compare sssociation between gender and TRPV4 in NERD group

Gender	**TRPV4 positive, n(%)**	**TRPV4 negative, n(%)**	**p-value**
Female	5(71,4)	11(34.4)	0.10
Male	2(28.6)	21(65.6)	

Table 10 To compare demographic detail withTRPV4 positive and negative in non NERD

Gender	**TRPV4 positive (n%)**	**TRP4 negative (n%)**	**p-value**
Female	2 (50%)	7 (58.3%)	p>0.95
Male	2 (50%)	5 (41.7%)	

In non NERD group, insignificant result for demographic data compared with TRPV4 negative and positive

Table 11 To compare endoscopy finding with NERD and non NERD in TRPV4 positive

Endoscopy	**TRPV4 positive**		**p-value**
	NERD, n(%)	**NON NERD,n(%)**	
Linear furrowing	0(0.0)	0(0.0)	-
White exudates	4(57.1)	0(0.0)	0.19
Circular rings	2(28.6)	0(0.0)	0.49
Linear shearing	0(0.0)	0(0.0)	-
Stricture	0(0.0)	0(0.0)	-
Decreased mucosal	0(0.0)	0(0.0)	-
Congested mucosal	3(42.9)	0(0.0)	0.23
Hiatal hernia	2(28.6)	3(75.0)	0.24

Table 12 To compare endoscopy finding in NERD and non NERD in TRPV4 negative

There is no statistically insignificant between endoscopy finding and TRPV4 positive in NERD and non NERD

Endoscopy	**TRPV4 negative**		**p-value**
	NERD, n(%)	**NON NERD, n(%)**	
Linear furrowing	8(25.0)	0(0.0)	0.08
White exudates	21(65.6)	4(33.3)	0.08
Circular rings	15(46.9)	2(16.7)	0.09
Linear shearing	3(9.4)	0(0.0)	0.55
Stricture	1(3.1)	0(0.0)	>0.95
Decreased mucosal	6(18.7)	0(0.0)	0.16
Congested mucosal	13(40.6)	0(0.0)	0.009*
Hiatal hernia	5(15.6)	9(75.0)	<0.001*

Among TRPV positive group, there was no significant association between endoscopic findings and NERD or non-NERD.

Among TRPV negative group, there was a significant association between congested mucosa and NERD or non-NERD ($p=0.009$). There was also a significant association between hiatal hernia and NERd or non-NERD ($p<0.001$). Other endoscopic findings among TRPV negative group showed no significant association.

5.0 DISCUSSION

Non erosive reflux disease is a gastroesophageal reflux disease (GERD) with distinct pattern. Notably, many studies on NERD showed that it has different mechanism compared to GERD. This study has been conducted particularly to identify immunohistochemical markers that are associated with NERD.

The transient receptor potential molecule that has 6 subtypes has been postulated to play a role in pathophysiology of NERD. Earlier research had proven TRPV1 was expressed in NERD. Guarino et al stated that non-erosive reflux disease (NERD) and EE patients showed increased TRPV1 receptors mRNA and protein, although no correlation with acid exposure was demonstrated. Increased TRPV1 in the esophageal mucosa may contribute to symptoms both in NERD and EE patients. Plus, it may possibly account for the peripheral mechanisms responsible for esophageal hypersensitivity in NERD patients.

This is the first study to establish the association between TRP channel membrane TRPV4 and NERD. The research is fundamental as it aims to establish the reasons behind the generations of symptoms and explore new targeted treatment for NERD. To that end, 39 NERD patients and 16 control patients were monitored during the research. All patients with EE were excluded from the study. This study was conducted in HUSM Kubang Kerian, Kelantan, where the majority ethnic group is Malay.

Research in Asia, reported that NERD affects different ethnicities at different rates: 60 -90% in Chinese, 65% in Indians and 72 % in Malay (Chen & Hsu, 2013). Rosaida et al. claimed that independent risk factor for NERD is highest for Indian race followed by Malays. In the current research , the majority of NERD patients (77%) and the control group (93%) were made up of Malays. This was aligned with the demographic background of the state of Kelantan, whereby 95% of the population are Malay (Vital Statistic Malaysia 2007, Jabatan Perangkaan Malaysia).

Male and female from present research were almost equally distributed at 51% and 49 respectively. As study in Sweden by Fass (2007) depicted that 60 % of the NERD group were female and the mean age in the research was 49 years old. In addition, Carlson et al also stated that 60% of NERD patient were female with the mean age of 49 years old. Another study on NERD by Minatsuki et al, (2013) showed that females were more affected by NERD than males. This was supported by another review article which demonstrated that the female population with age between 40-50 years old were more likely to have NERD compared to EE (Chen and Hsu, 2013). In this study, in theNERD group, 59% (23) patients were male and 41% (16) were female. In control group 43.8% (7) were male and 56.3% (9) were female.

The difference between EE (Erosive esophagitis) and NERD in endoscopic finding was only erosive lesions. In EE, the Lost Angeles classification was used to decribe the esophagitis.Conversely, no such classification was available for NERD. There was no such classification although nonspecific macroscopic esophageal changes are observed. The microscopcic changes in NERD such as (i) basal cell hyperplasia , (ii) focal or diffuse infiltration by polymorphonuclear and (iii) dense non-follicular infiltration of mononuclear inflammatory cell. Most of endoscopic changes ie linear furrow, linear shearing, circular ring were observed in eosinophilic esophagitis compared to NERD.(Dellon et al.) Minimal information is available in the literature regarding endoscopic changes in all normal endoscopic findings such as white exudates, circular ring. Plus they were mention recently in details in eosinophilic esophagitis population only.This may be due to the lack of recognition for these endoscopic changes previously.

The first objective was to examine the expression of TRPV4 in NERD and normal population. Samples were taken at 5 cm and 15cm above GEJ junction. The result depicted positivity towards TRPV4 at 5cm whereas at 15cm only one patient from NERD group showed positive result. Univariate analysis via Fisher exact test was applied to compare the expression both NERD and

control group. As a result, there was no significant differences between both groups with p- value of 0.71.

The next stage was the analysis to compare the intensity and quantification of cells stained in positive result from both groups. For intensity, mean (SD) of NERD and normal group were 0.21 and 0.25, p value of 0.74.The quantitative score of cells, for the quantified positive cells from a total of 100 cells, showed the mean (SD) were 0.21 (0.075) in the NERD group and 0.31 (0.151) in normal group. From the results, it was evident that control group had higher mean score for cells stained with TRPV4 compared to NERD group.Nevertheless, p- value was statically insignificant but this could be misleading due to small sample size. As this was first study in the area, the result cannot be compared to other study.

The second objective was, to compare pH study parameter with TRPV4 positive and negative in NERD group. In their study, Martinez et al (2002) reported that 45.1% of the NERD groups demonstrated an abnormal 24-h pH study. In this study, total reflux mean number of acid reflux event was 95 $\pm$ 9.4, whereas the mean time pH < 4 (%) was 6.0 $\pm$0.9 for total, 6.2 $\pm$ 0.9 for upright and 4.9 $\pm$1.2 for supine. The result for our study showed median De Meester score was 11.8 (12.15) with total reflux 32 (27.8). Furthermore, the median (IQR) for acid reflux was16.9 (23.5) and non acid reflux was 13.5 (23). For median (IQR) % pH less than 4 was 1.3 (3.4) for total, 2.0 (3.6)for upright and 0.05 (2.2) for supine. We compare different pH study parameter with TRPV4 positivity. For non acid reflux, the median (IQR) for TRPV positive was 7 (47) as compared to 14 (20) intoTRPV4 negative. Reflux can be characterized into acid and non-acid reflux; the latter can be subdivided in weakly acid and weakly alkaline reflux. Acid reflux has been defined as a reflux event associated with drop in esophageal pH <4, weakly acid when associated with a pH drop between 4 and 7 and weakly alkaline when reflux event is not associated with a pH drop <7. Data support a role for non-acid reflux as a cause of symptoms in some NERD patients, especially those who do not respond to treatment

with proton pump inhibitor (Karamanolis & Tutuian, 2013).The result why median IQR for non acid reflux was higher in TRPV4 negative could be due to false negative result as limitation in detection method or TRPV4 was inactive form as no TRPV4 agonist was applied.

Esophageal motility was hypothesized to influence the generation of symptom in GERD. (Frazzoni, De Micheli, Zentilin, & Savarino, 2004) claimed that NERD and functional heartburn differed in terms of prevalence of hiatal hernia, mean LES relaxation and number of upright acid exposure. Notably, mean LES in the study was 15.3 ± 8.9.Apart from that a research comparing esophageal function test among the Chinese population demonstrated mean (SD) for different manometry parameters were (15.3±8.9) for LES pressure, (7.5±4.8) for IRP4, (751.9±856.2) for DCI, 55(49.5)for ineffective esophageal motility , 10(9.0) for hiatal hernia (Gao, Gao, Chen, Qian, & Zhang, 2017). In our current research, the analysis for manometry patients produced median (IQR) mean LES of 21 (17), IRP4 of 7.3 (11.4), DCI of 762 (1375) and percentage of weak peristalsis of 10 (60). The mean LES was higher while the percentage of weak peristalsis was lower compared to earlier studies explained above. This indicates that NERD was less severe form of GERD. Plus , the comparison between manometry parameter and TRPV4 expession did not yield positive result. Nonetheless, it was worth to note that the readings median (IQR) for percentage weak peristalsis were marked differently, 0.3 and 20 respectively. Although p value > 0.05, the marked differences implied possible correlation although it was statistically insigfinicant. This could partly be attributed secondary to small sample size.

Objective 4 was to determine association of demographic data and TRPV4 in NERD and control group. In NERD group, there is no significant association between gender and age with TRPV4 positive and negative (p- value 0.101) . For nonNERD group, the result is similar, no significant association between geder and TRPV4 negative and postive. However, the result cannot be compared as no other study had been done on the topic.

The endoscopic findings, of TRPV positive group, depicted no significant association between endoscopic findings among NERD or non-NERD groups. In contrast, in TRPV4 negative group, there was a significant association between congested mucosa and NERD or non-NERD ($p=0.009$) as well as between hiatal hernia and NERD or non-NERD ($p<0.001$), as expected in NERD group.

Transient receptor potential (TRP) cation channels are involved in majority of cellular functions. During the last decade, there has been growing interest in the physiological and pathological roles of TRPV4 in the gastrointestinal tract. A vast amount of evidence was accumulated on the important role of these cation channels in different regulatory aspects of the alimentary tract. This provides pharmacological opportunity to target TRPs as a strategy to treat various gastrointestinal disorders.

Few studies that demonstrated the presence of TRPV4 in intestinal epithelial cells also identified in the presence of TRPV4 and infiltrated CD45-positive cells in IBD (Crohn's disease and ulcerative colitis).Vernoglle et al. stated there were strong role for TRPV4 for intestinal inflammation and potentially IBD.Although TRPV4 still has few issues to be addressed, it can be researched as new targeted drugs for IBD.

Shikano et al. (2010) showed that TRPV4 transcripts and its immunoreactivity were expressed in the basal and intermediate layers of the mouse' s esophageal epithelium. Its physiological role included regulation of cell volume coupled to various physiological processes, such as cell proliferation, differentiation, and migration. Furthermore, a Japanese study demonstrated that TRPV4 transcripts and protein were present in human esophagus .(Ueda et al., 2011). In both studies, the expression of TRPV4 at the mRNA and protein levels was examined using reverse transcription-polymerase chain reaction (RT-PCR), insitu hybridization, and immunohistochemistry.

Further investigations are needed to clarify the pathological contributions of TRPV4. Notably, this paper outlines the first study to further investigate roles and functions of TRPV4 in NERD. The expression of TRPV4 was assumed to influence pathophysiological aspect and generation of symptoms in NERD. Using IHC method, primary and secondary antibodies from Santa Cruz Biotechnology, Texas USA ,TRPV4 immunoreactivity was detected in esophageal tissue in NERD snd control group. This correlated with previous study finding that found expression TRPV4 in human esophagus.

However this research study did not find significant association between TRPV4 positivity and NERD. The result depicted that, the expression was similar in both groups but slightly higher in normal population than the NERD groups for cell stained. These outcomes may be influenced by several factors including experimental method. Previous research implemented RT-PCR, IHC and in-situ hybridization to confirm expression and localisation. For IHC, immunoreactivity was read using fluorescence microscopy. In contrast, the detection in this study only use IHC method and the immunoreactivity was examined using light microscopy only. Application of these methods to confirm presence of receptor is a better approach as it can exclude false positive and false negative.

Pathogenesis of NERD is theorised that visceral hypersensitivity was superior than acid exposure. Three broad mechanisms were believed to underlie visceral hypersensitivity: namely peripheral sensitisation, central sensitisation and psychoneuroimmune interactions. The TRPV4-receptor was expressed in human and mouse esophageal cells. Additionally, stimulation of the receptor caused the release of adenosine triphosphate (ATP), which is responsible for local inflammation and mediating TRPV1 activation. Studies have shown that the expression of the TRPV1- receptor is higher in inflamed esophageal mucosa as well as in the mucosa of patients with NERD. In the GI tract, TRPV4 occurs primarily in fibers of extrinsic primary afferent neurons. Nevertheless, some epithelial and other cells have also been reported to stain positively for this TRPV channel subunit.

TRPV4 is present in the nodose ganglion, DRG, stomach, small intestine and colon of rodents. Retrograde labeling illustrated vagal afferent neurons projecting to the murine forestomach contained TRPV4. In these neurons, TRPV4 is coexpressed with TRPV1, TRPV2 and TRPA1 to various degrees (Zhang et al., 2004) . This ignited that TRPV4 and TRPV1 may coexist together and ATP release from TRPV4 will lead to TRPV1 activation.Consequently resulted in esophageal inflammation and symptoms generation in NERD. This research we examined TRPV4 expression but excluded TRPV1. Nonetheless, the research by (Guarino et al., 2010) showed that TRPV1's expression was higher in NERD compared to control group.

The result of the current study confirmed TRPV4's expression but the factors that activate TRPV4 were not assessed. The literature depicted , TRPV4 channels can be activated by endogenous substances including (i) arachidonic acid (AA), (ii) endocannabinoids anandamide and(iii) 2-arachidonyl glycerol (2-AG), and (iv) cytochrome P-450 metabolites of AA (like epoxyeicosatrienoic acid) (v) 4a-PDD and (vi) hypotonic stimuli.(Shikano et al., 2011). In colon, The TRPV4 agonist-evoked sensitisation of colonic afferent nerve fibers to mechanical stimuli is associated with mechanical hyperalgesia, as the visceromotor response to colorectal distension is enhanced.

A research that aimed to prove the significance of TRPV4 in visceral hypersensitivity symptoms in colon used TRPV4 agonist (4alphaPDD) to activate TRPV4. This resulted in the activation of activation a cationic current and calcium influx in the colonic projections of DRG neurons and which caused dose-dependent visceral hypersensitivity.(Cenac et al., 2008). Possible explaination was that, TRPV4 expressed in normal control was in its inactivated form. Plus, TRPV4 in NERD needs to be stimulated with TRPV4 agonist prior to inducing visceral hypersensitivity.

Another notable issue was the sample size. From the results, we did see positivity in both group eventhough it was not statistically significant in both groups. For quantitative score of cells, for quantified positive cell from a total of 100 cells, showed that the mean (SD) was 0.21 (0.075)for

NERD and 0.31 (0.151) for the normal group. From the results, control group had higher mean score for cell stained with TRPV4 compared to the NERD group.The insignificant p value is likely attributed to the small sample size. A bigger sample size with improved method of detection (as mentioned earlier) will allow the researcher to analyse the trend of positivity in both group. This may generate new information regarding role and regulation of TRPV4 in NERD.

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6.0 CONCLUSION

The prospective, cross- sectional research between adult of NERD patients and control patients examined the expression of TRPV4 in NERD and the association link between pH study and manometry. It was carried out from March 2017 till November 2017. With the exclusion of EE, 39 NERD patients and 16 control patient were included in the research.

The research discovered that TRPV4 was expressed in both NERD and control patients. No difference in the expression of TRPV4 in NERD and control group.Further there was no association between TRPV4 expression and 24 H pH study and monometry .

7.0LIMITATIONS

1. Small sample size
2. No data for pH and manometry for control patients as the patients refused to undergo another invasive procedure after upper gastroesophageal endoscopy with prolonged procedure time
3. Detection method limited to IHC

8.0 RECOMMENDAIONS

1. To get larger sample size to improve the accuracy in future studies
2. To consider RT-PCR and in situ hybridization on top of IHC as the detection method for TRPV4
3. Usage of TRPV4 agonist to activate TRPV4 receptor in the demonstration of visceral hypersensitivity
4. To acquire data for pH study and manometry in control patients for proper comparison

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Appendix 1

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan, Malaysia.
T: 609 - 767 3000 samb. 2351/2362
F: 609 - 767 2351
E: jepem@usm.my
www.jepem.kk.usm.my

27th March 2017

Dr. Nazihah Azis
Department of Medicine
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/16100401
Protocol Title : Expression of TRPV4 (Transient Receptor Potential Vanilloid 4) in Non-Erosive Reflux Disease.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/16100401**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **27th March 2017** until **26th March 2018.**

Study Site: Hospital Universiti Sains Malaysia.

The following researchers also involve in this study:

1. Prof. Dr. Lee Yeong Yeh
2. Dr. Faezahtul Arbaeyah Hussain
3. Dr. Muhammad Ilham Mohd Hafiz

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (English version)
2. Patient Information Sheet and Consent Form (Malay version)
3. Questionnaire
4. OGDS Data Collection Form

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2015: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2015: Study Protocol Amendment Submission Form.**
3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2015: Study Protocol Amendment Submission Form.**

4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2014: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2015.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2014: Final Report Form.**

Please note that forms may be downloaded from the JEPeM-USM website: www.jepem.kk.usm.my

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs) and Local Regulations and Standards in Ethical Review.

Thank you.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

PROF. DR. MOHD SHUKRI OHMAN
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia) JEPeM
Universiti Sains Malaysia

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

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Date of meeting : 23rd January 2017
Venue : Meeting Room, Division of Research & Innovation, USM Kampus Kesihatan.
Time : 9.00 a.m – 2.00 p.m
Meeting No : 352

Members of Committee of the Jawatankuasa Etika Penyelidikan (Manusia), JEPeM Universiti Sains Malaysia who reviewed the protocol/documents are as follows:

	Member (Title and Name)	Occupation (Designation)	Male/ Female (M/F)	Tick (✓) if present when above items, were reviewed
Deputy Chairperson :	Professor Dr. Mohd Shukri Othman	Deputy Chairperson of Jawatankuasa Etika Penyelidikan (Manusia), JEPeM USM	M	✓ (Deputy Chairperson)
Secretariat:	Miss Siti Fatihah Ariffin	Research Officer	F	✓
Members :				
1.	Mr. Harry Mulder	Community Representative	M	✓
2.	Dr. Haslina Taib	Lecturer, School of Dental Sciences	F	✓
3.	Associate Professor Dr. Mohtar Ibrahim	Lecturer, School of Medical Sciences	M	✓
4.	Dr. Mujahid Bakar	Lecturer, School of Health Sciences	M	✓
5.	Professor Dr. Nik Hazlina Nik Hussain	Lecturer, School of Medical Sciences	F	✓
6.	Professor Dr. Nor Hayati Othman	Lecturer, School of Medical Sciences	F	✓
7.	Associate Professor Siti Hawa Ali	Lecturer, School of Health Sciences	F	✓
8.	Dr. Soon Lean Keng	Lecturer, School of Health Sciences	F	✓
9.	Mrs. Zawiah Abu Bakar	Community Representative	F	✓

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

PROFESSOR DR. MOHD SHUKRI OTHMAN
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

Appendix 2

MAKLUMAT KAJIAN

Tajuk Kajian: Kehadiran TRPV4 kepada penyakit "Non Erosive Refkux Disease"

Nama Penyelidik: ***Prof Lee Yeong Yeh*** ***(MMC 36810)***
Dr Faedzahtul Arbaieyah ***(MMC 44984)***
Dr Sharifah Emilia ***(MMC33693)***
Dr Nazihah Azis ***(MMC 48974)***

PENGENALAN

Anda dipelawa untuk menyertai satu kajian penyelidikan secara sukarela melibatkan endoscopi terhadap trek atas gastrousus dan pengambilan beberapa biopsi ketika menjalani pemeriksaan endoskopi (OGDS) . Melalui pengambilan biopsi ini maka pesakit yang menghidapi 'nonerosive reflux disease' dan faktor-faktor pesakit yang berkaitan dengan masalah ini boleh dikaji secara lebih lanjut. Kemudian ,anda akan ditemubual mengenai simptom-simptom anda dan akan diberikan markah berdasarkan keterukan dan kekerapan simptom-simptom tersebut oleh Dr penyelidik.

Penyakit 'Nonerosive reflux disease' bukanlah sesuatu yang baru dan telah banyak kajian telah dibuat di seluruh dunia termasuk di Malaysia. Pesakit-pesakit yang menghidapi '' mempunyai tanda-tanda yang tidak spesifik seperti 'pedih pada bahagian perut (heartburn)' , susah untuk menelan , terasa makanan tersekat ketika menelan , sakit dada dan pelbagai tanda lain . Tanda-tanda ini tidak spesifik untuk satu penyakit sahaja di mana ia boleh didapati juga dalam penyakit lain seperti 'erosive esophagitis', 'eosinophilic esophagitis ' dan untuk membezakan penyakit-penyakit ini hanyalah melalui pengambilan biopsi sahaja.

Pesakit-pesakit yang memenuhi kriteria akan dipelawa untuk meneruskan pemeriksaan lanjut iaitu pemonitoran pH-impedance secara 24 jam dan manometry, yang mana pemeriksaan ini dapat memberi penerangan yang lebih lanjut sebab-sebab yang lebih mendalam berkaitan dengan simptom-simptom pesakit.

Sebelum anda bersetuju untuk menyertai penyelidikan ini, adalah penting untuk anda membaca dan memahami borang ini. Pengambilan biopsi hanyalah pada satu masa endoskopi ini sahaja. Kami menjangkakan penyertaan sebanyak 100 pesakit dalam kajian ini.

TUJUAN KAJIAN

Tujuan utama kajian ini dijalankan adalah untuk mengkaji keterukan simptom, perubahan esophagus melalui pememerhatian endoscopi, dan kehadiran receptor TRPV4 di dalam tisu esophagus pesakit yg menghidapi NERD yang menjalani OGDS di HUSM. Kemungkinan juga maklumat yang diperolehi daripada kajian ini akan dapat digunakan oleh pihak penganjur di masa hadapan bertujuan untuk melihat perkara-perkara yang berkaitan dengan penyakit ini secara lebih lanjut.

KELAYAKAN PENYERTAAN

Anda mestilah:
Berumur 18 tahun dan ke atas
Mengalami symptom GERD seperti (GERD Q Questionaire)

- sukar menelan
- tersekat ketika manelan
- pedih ulu hati
- sakit dada
- loya dan muntah
- sakit perut
- sakit ketika menelan
- kurang berat badan
- serak suara (hoarseness of voice)

Anda tidak boleh mengikuti kajian ini sekiranya:

- Berumur kurang dari 18 tahun
- Mempunyai masalah pendarahan/koagulasi
- Mempunyai jangkitan fungus atau jangkitan saluran esophagus.
- Mengalami masalah perdarahan salur pemakanan (upper gastrointestinal bleeding) ketika kajian dibuat.
- Anda sedang menerima rawatan menggunakan inhalasi atau sistemik kortikosteroid dalam
- Tempoh 30 hari sebelum kajian dijalankan.
- Mempunyai masalah penyakit hati kronik.
- Anda sedang mengandung

- Endoscopy menunjukkan ciri-ciri ‘Erosive Esophagitis’
- Anda mengalami masalah psikologi/neurologi yang tidak membenarkan anda menjalani prosedur endoskopi.

PROSEDUR-PROSEDUR KAJIAN

Setelah anda bersetuju untuk menyertai kajian ini, 4 biopsi akan diambil daripada saluran esophagus anda semasa anda menjalani OGDS pada 5cm and 15cm.Kemudian, maklumat dan sejarah kesihatan akan diambil dripada rekod kesihatan anda sebelum ini. Tisu tersebut akan dihantar ke makmal patologi untuk ujian selanjutnya. Tisu tersebut akan disimpan dan mungkin akan digunakan pada masa akan datang dan anda berhak menolak penyimpanan tisu tersebut. Anda akan dipelawa untuk meneruskan pemeriksaan melalui kaedah pemonitoran manometry dan pH-impedance secara 24 jam ambulatory yang mana anda dibenarkan untuk pulang atu meneruskan pemonitaran di dalam wad bagi tujuan merekod simptom-simptom anda. Ujian endoscopi mungkin menagmbil masa 15 minit, manakal manometry selama 30 minit hingga ke satu jam. PH study pula akan dijalan selama 24 jam.

RISIKO

Sekiranya anda menyertai kajian ini, risiko yang anda mungkin alami adalah sama seperti yang telah diterangkan oleh doktor sebelum anda menjalani pemeriksaan endoskopi. Risiko ini berkaitan dengan prosedur endoskopi OGDS seperti ketidakselesaan semasa prosedur, serak suara selepas prosedur dan risiko yang berkaitan dengan ubat pelali sekiranya digunakan semasa prosedur. Anda tidak akan merasa sakit disebabkan oleh biopsi yang diambil. Risiko berkaitan dengan biopsi pada bahagian esofagus adalah sangat kecil seperti pendarahan. Manakala bagi pemonitoran pH-impedance secara 24 jam ambulatory, anda akan berasa sedikit tidak selesa kerana ia dijalankan selama 24 jam.
Jika apa-apa maklumat penting yang baru dijumpai semasa kajian ini yang mungkin mengubah persetujuan and untuk terus menyertai kajian ini, anda akan diberitahu secepat mungkin.

MELAPORKAN PENGALAMAN KESIHATAN

Jika anda mengalami apa-apa kecederaan, kesan buruk, atau apa-apa pengalaman kesihatan yang luarbiasa semasa kajian ini, pastikan anda memberitahu jururawat atau Dr. Nazihah binti Azis **[No.**

Pendaftaran Penuh Majlis Perubatan Malaysia: 48974] di talian 0129626481 secepat mungkin. Anda boleh membuat panggilan pada bila-bila masa, siang atau malam, untuk melaporkan pengalaman sedemikian.

PENYERTAAN DALAM KAJIAN

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda berhak menolak untuk menyertai kajian ini atau anda boleh menamatkan penyertaan anda pada bila-bila masa, tanpa sebarang hukuman atau kehilangan manfaat yang sepatutnya anda perolehi.

Penyertaan anda juga mungkin boleh diberhentikan oleh doktor yang terlibat dalam kajian ini tanpa persetujuan anda. Sekiranya anda berhenti menyertai kajin ini, doktor yang terlibat di dalam kajian ini atau salah seorang kakitangan akan berbincang dengan anda mengenai apa-apa isu perubatan berkenaan dengan pemberhentian penyertaan anda.

MANFAAT YANG MUNGKIN [Manfaat terhadap Individu, Masyarakat, Universiti]

Anda mungkin menerima maklumat tentang kesihatan anda dari apa-apa pemeriksaan fizikal dan ujian makmal yang bakal dilakukan dalam kajian ini.
Anda akan dibayar sedikit pampasan untuk perbelanjaan pengangkutan anda yang berkaitan dengan penyertaan anda anda dalam kajian ini.
Maklumat yang didapati dari kajian ini akan memanfaatkan pihak penyelidik, bidang perubatan amnya, dan memanfaatkan pesakit pada masa depan..

PERSOALAN

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini atau hak-hak anda, sila hubungi;

Dr Nazihah binti Azis
Jabatan Perubatan
Pusat Pengajian Sains Perubatan
USM Kampus Kesihatan
012-9626481

Sekiranya anda mempunyai sebarang soalan berkaitan kelulusan Etika atau sebarang pertanyaan dan masalah berkaitan kajian ini, sila hubungi;

En. Mohd Bazlan Hafidz Mukrim
Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM
Pusat Inisiatif Penyelidikan -Sains Klinikal & Kesihatan
USM Kampus Kesihatan.
No. Tel: 09-767 2354 / 09-767 2362
Email : bazlan@usm.my/jepem@usm.my

KERAHSIAAN

Maklumat perubatan anda akan dirahsiakan oleh doktor dan kakitangan kajian. Ianya tidak akan dedahkan secara umum melainkan jika ia dikehendaki oleh undang-undang.
Data yang diperolehi dari kajian yang tidak mengenalpasti anda secara perseorangan mungkin akan diterbitkan untuk tujuan memberi pengetahuan baru.

Rekod perubatan anda yang asal mungkin akan dilihat oleh pihak penyelidik, Lembaga Etika kajian ini dan pihak berkuasa regulatori untuk tujuan mengesahkan prosedur dan/atau data kajian klinikal. Maklumat perubatan anda mungkin akan disimpan dalam komputer dan diproses dengannya.

Dengan menandatangani borang persetujuan ini, anda membenarkan penelitian rekod, penyimpanan maklumat dan pemindahan data seperti yang dihuraikan di atas.

TANDATANGAN

Untuk dimasukkan ke dalam kajian ini, anda atau wakil sah anda mesti menandatangani serta mencatatkan tarikh halaman tandatangan (Lihat contoh Borang Keizinan Pesakit di **LAMPIRAN S** atau **LAMPIRAN G (untuk sampel genetik)** atau **LAMPIRAN P**).

Appendix 3

OGDS Data Collection Form

Research Tile : Expression TRPV4 in Non Erosive Reflux Disease

Researcher's Name: ***Prof Lee Yeong Yeh*** ***(MMC 36810)***
Dr Faedzahtul Arbaieyah ***(MMC 44984)***
Dr Sharifah Emilia ***(MMC 33693)***
Dr Nazihah Azis ***(MMC 48974)***

PATIENT ID : _____

SEX : _____ **AGE :** _____

ENDOSCOPIC FEATURE		SEVERITY		
		Mild	Mod	Sever
1. Linear furrowing , vertical lines of the esophageal mucosa	Y☐ N☐			
2. White exudates , white specks , nodules , granularity	Y☐ N☐			
3. Circular rings	Y☐ N☐			
4. Linear shearing / crepe paper mucosa with passage of endoscope or dilator	Y☐ N☐			
5. Stricture : proximal , middle or distal	Y☐ N☐			
6. Narrow calibre esophagus	Y☐ N☐			
7. Decreased mucosal vascularity	Y☐ N☐			
8. Congested esophageal mucosa	Y☐ N☐			
9. Erosive esophagitis	Y☐ N☐			
10. Hiatal hernia	Y☐ N☐			
11. Lamina propia fibrosis	Y☐ N☐			
12. Normal appearing esophagus	Y☐ N☐			
13. Others -	Y☐ N☐			

LAMPIRAN P

Borang Keizinan bagi Penerbitan Bahan yang berkaitan dengan Pesakit/ Subjek (Halaman Tandatangan)

Tajuk Kajian:	**Kehadiran TRPV4 kepada penyakit "Non Erosive Reflux Disease"**	
Nama Penyelidik:	***Prof Lee Yeong Yeh***	***(MMC 36810)***
	Dr Faedzahtul Arbaieyah	***(MMC 44984)***
	Dr Sharifah Emilia	***(MMC 33693)***
	Dr Nazihah Azis	***(MMC 48974)***

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini.

Dengan menandatangani mukasurat ini, saya memahami yang berikut:

- Bahan yang akan diterbitkan tanpa dilampirkan dengan nama saya dan setiap percubaan yang akan dibuat untuk memastikan ketanpanamaan saya. Saya memahami, walaubagaimanapun, ketanpanamaan yang sempurna tidak dapat dijamin. Kemungkinan sesiapa yang menjaga saya di hospital atau saudara dapat mengenali saya.
- Bahan yang akan diterbitkan dalam penerbitan mingguan/bulanan/dwibulanan/suku tahunan/dwi tahunan merupakan satu penyebaran yang luas dan tersebar ke seluruh dunia. Kebanyakan penerbitan ini akan tersebar kepada doktor-doktor dan juga bukan doktor termasuk ahli sains dan ahli jurnal.
- Bahan tersebut juga akan dilampirkan pada laman web jurnal di seluruh dunia. Sesetengah laman web ini bebas dikunjungi oleh semua orang.
- Bahan tersebut juga akan digunakan sebagai penerbitan tempatan dan disampaikan oleh ramai doktor dan ahli sains di seluruh dunia.
- Bahan tersebut juga akan digunakan sebagai penerbitan buku oleh penerbit jurnal.
- Bahan tersebut tidak akan digunakan untuk pengiklanan ataupun bahan untuk membungkus.

Saya juga memberi keizinan bahawa bahan tersebut boleh digunakan sebagai penerbitan lain yang diminta oleh penerbit dengan kriteria berikut:

- Bahan tersebut tidak akan digunakan untuk pengiklanan atau bahan untuk membungkus.
- Bahan tersebut tidak akan digunakan di luar konteks – contohnya: Gambar tidak akan digunakan untuk menggambarkan sesuatu artikel yang tidak berkaitan dengan subjek dalam foto tersebut.

Nama Pesakit (Dicetak atau Ditaip)

Nama Singkatan atau **No. Pesakit**

No. Kad Pengenalan Pesakit

T/tangan Pesakit

Tarikh (dd/MM/yy)

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan (Dicetak atau Ditaip)

Tarikh (dd/MM/yy)

Nota: i) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran

LAMPIRAN S

Borang Keizinan Pesakit/ Subjek
(Halaman Tandatangan)

Tajuk Kajian: **Kehadiran TRPV4 kepada penyakit "Non Erosive Refkux Disease"**

Nama Penyelidik:

Prof Lee Yeong Yeh	***(MMC 36810)***
Dr Faedzahtul Arbaieyah	***(MMC 44984)***
Dr Sharifah Emilia	***(MMC 33693)***
Dr Nazihah Azis	***(MMC 48974)***

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini **termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian** dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.
- Semua soalan-soalan saya telah dijawab dengan memuaskan.
- Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Pesakit untuk simpanan peribadi saya.

Nama Pesakit (Dicetak atau Ditaip)	**Nama Singkatan & No. Pesakit**
No. Kad Pengenalan Pesakit (Baru)	**No. K/P** (Lama)
Tandatangan Pesakit atau Wakil Sah	**Tarikh** (dd/MM/yy) (Masa jika perlu)

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan (Dicetak atau Ditaip)	**Tarikh** (dd/MM/yy)
Nama Saksi dan Tandatangan	**Tarikh** (dd/MM/yy)

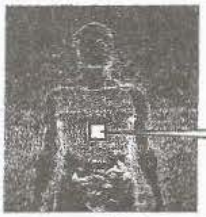
Nota: i) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran

Unit Fungsi GI dan Motiliti,
Hospital USM, Kubang Kerian
Soal Selidik GERD (*GastroEsophageal Reflux Disease*)

Nama : _____ Jantina : Lelaki / Perempuan Tinggi : _____ sm
No. Pendaftaran : _____ Umur : _____ Berat : _____ kg
Nombor K/P : _____ BMI : _____

(A) Sila jawab soalan yang tersenarai di bahagian ini. Jawapan anda dapat membantu doktor anda memberi rawatan yang sewajarnya bagi mengatasi masalah kesihatan anda supaya anda dapat menikmati kehidupan anda dengan sempurna.

Tanda-tanda gejala yang dialami oleh setiap individu berkemungkinan berlainan. Jawab soalan tersebut mengikut tanda-tanda gejala yang dialami oleh anda sendiri berdasarkan dalam tempoh 7 hari yang lepas.



No.	Soalan (berdasarkan tempoh 7 hari yang lepas)	Kekerapan (hari)			
A1	Berapa kerap anda berasa seperti panas dan / atau pedih di kawasan ulu hati?	0 hari	1 hari	2-3 hari	4-7 hari
A2	Berapa kerap anda berasa seperti cecair atau makanan bergerak ke arah atas menuju ke tekak atau mulut?	0 hari	1 hari	2-3 hari	4-7 hari
B1	Berapa kerap anda mengalami kesakitan di kawasan ulu hati?	0 hari	1 hari	2-3 hari	4-7 hari
B2	Berapa kerap anda berasa loya atau perasaan hendak muntah?	0 hari	1 hari	2-3 hari	4-7 hari
C1	Berapa kerap anda mengalami kesukaran tidur lena disebabkan pedih ulu hati dan/atau rasa hendak muntah?	0 hari	1 hari	2-3 hari	4-7 hari
C2	Berapa kerap anda mengambil ubat-ubatan tambahan (seperti Gaviscon, Zantac, Omesec) untuk mengatasi masalah pedih ulu hati atau rasa hendak muntah?	0 hari	1 hari	2-3 hari	4-7 hari

(B) Bahagian pengiraan skor ini akan diisi oleh staf kesihatan yang bertugas.
Merujuk kepada jawapan yang diberikan oleh pesakit, tandakan ' √ ' pada ruang yang berkaitan.

No.	Skor				Jumlah mata
A1	0 hari (0 mata)	1 hari (1 mata)	2-3 hari (2 mata)	4-7 hari (3 mata)	mata
A2	0 hari (0 mata)	1 hari (1 mata)	2-3 hari (2 mata)	4-7 hari (3 mata)	mata
B1	0 hari (3 mata)	1 hari (2 mata)	2-3 hari (1 mata)	4-7 hari (0 mata)	mata
B2	0 hari (3 mata)	1 hari (2 mata)	2-3 hari (1 mata)	4-7 hari (0 mata)	mata
C1	0 hari (0 mata)	1 hari (1 mata)	2-3 hari (2 mata)	4-7 hari (3 mata)	mata
C2	0 hari (0 mata)	1 hari (1 mata)	2-3 hari (2 mata)	4-7 hari (3 mata)	mata
				Jumlah Skor	mata

Keputusan :	
Jumlah skor 0-8 mata	Less likely GERD
Jumlah skor 9-18 mata	Highly suggestive of GERD

_____ _____
Tarikh Nama & Tandatangan Staf

SOAL SELIDIK GERD (Gastroesophageal Reflux Disease)