An Investigation of Biomarkers in Laryngopharyngeal Reflux

Dr John M Wood

MBBS BSocSci(Hons) BA(Hons)

A Thesis Submitted for the Degree of

Master Of Surgery

Flinders University of South Australia

January 2013

Flinders ENT, Department of Surgery
Flinders Medical Centre, Bedford Park
South Australia 5042

TABLE OF CONTENTS

ACKNOWLEDGMENTS

The work described in this thesis was performed at Flinders ENT, Department of Surgery, Flinders Medical Centre, Flinders University of South Australia, Bedford Park, South Australia.

This research was supported by the following funding:

- Garnett Passe and Rodney Williams Memorial Foundation Project Grant
- Flinders University Faculty of Health Sciences Research Seeding Grant

I would like to express my sincere thanks to Prof Simon Carney as my thesis supervisor, teacher and mentor. I greatly appreciate the support he has provided in clinical and research fields, and in the successful application for surgical training in Otolaryngology.

Many thanks to my co-supervisor Dr Damian Hussey, for his invaluable assistance, steerage, his astute and critical analysis at all the landmark moments, and for welcoming me into his laboratory.

I greatly appreciate the support and friendship of Dr Charmaine Woods, from Flinders ENT, Dept. of Surgery, whose guidance and patience in teaching me laboratory techniques allowed this project to progress, and for keeping each other sane in “The Dungeon”.

Many thanks for the work of Dr David Astill, Dept of Anatomical Pathology, Flinders University in providing expert histological analysis, and to Michelle Norman for preparing the histology.

To the staff of the Upper GI laboratory, many thanks for all your assistance.

And finally, to my wife Kate, and my children, Eleanor and Isaac, many thanks for your lasting love, support and enduring my absences.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

John Melville Wood

January 2013

PREFACE

A portion of this work has been published or presented as follows:

Publications

Wood JM, Hussey DJ, Woods CM, Watson DI, Carney AS. Biomarkers and laryngopharyngeal reflux. J Laryngol Otol 2011; **125**: 1218-24

Presentations

Wood, JM, Hussey DJ, Woods CM, Astill D, Watson DI, Carney, AS. The molecular biology of laryngopharyngeal reflux: Anatomical variations within the larynx. Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting, 3rd April 2012, Adelaide, South Australia.

Wood JM, Hussey DJ, Woods CM, Watson DI, Carney AS. Review of biomarkers in laryngopharyngeal reflux. Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting, 7th April 2011, Melbourne, Victoria.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AOR	Adjusted Odds Ratio
CA	Carbonic anhydrase
CD1d	Cluster of Differentiation 1d
CDH1	Epithelial cadherin (E-cadherin)
cDNA	Complementary DNA
CRNN	Cornulin (Squamous epithelial-induced stress protein 53kDa (SEP 53)
DIS	Dilation of intercellular spaces
DNA	Deoxyribonucleic acid
DGER	Duodenogastroesophageal reflux
GORD	Gastroesophageal reflux disease
H and E	Haematoxylin and eosin
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IL	Interleukin
KRT	Cytokeratin
LMA	Laryngeal mask airway
LOS	Lower oesophageal sphincter
LPR	Laryngopharyngeal reflux
MGMT	O-6-methylguanine-DNA methyltransferase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MUC	Mucin
NERD	Non-erosive reflux disease
NKT	Natural killer T cell
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
PTGS2	Prostaglandin-endoperoxide synthase-2
qtRT PCR	Quantitative real time reverse transcriptase polymerase chain reaction
RFS	Reflux finding scale

RIN	RNA integrity number
RSI	Reflux symptom index
SSRI	Selective serotonin re-uptake inhibitor
TNF-α	Tumour necrosis factor - α
UOS	Upper oesophageal sphincter
VEGF	Vascular endothelial growth factor
VHI	Voice Handicap Index

ABSTRACT

Laryngopharyngeal reflux (LPR) is an increasingly diagnosed disease in Otolaryngology, however it is a highly controversial topic. There is no gold standard diagnostic test and despite a wealth of articles, there is little understanding of the pathophysiological mechanisms underlying laryngeal damage. In addition, the response to anti-reflux medical treatment is highly variable, with a notable proportion failing to have any response. The lack of comprehension of the pathophysiology and definitive diagnosis limits the ability to conduct adequate investigation of treatment options. This study aimed to identify known and novel biomarkers in patients with LPR. Given evidence suggesting that LPR biomarker expression may vary across different areas of the larynx, biopsies were collected and analysed from sub-regions of the larynx.

Recruited patients completed the Reflux Symptom Index and the Reflux Finding Score was assessed at the time of biopsy collection under general anaesthetic. Biopsies were collected from 4 anatomically distinct locations in the larynx in both LPR and non-refluxing control patients. Sections were sent for histological examination and qRT-PCR analysis was conducted on 20 genes identified as being related to reflux and inflammation, including interleukins 6 (*IL-6*) and 8 (*IL-8*), prostaglandin-endoperoxide synthase-2, cytokeratins 8 and 14, mucin genes *MUC1, 2, 3B, 4, 5B, 6, 7*, and carbonic anhydrase III.

In patients with LPR, site-specific differences in gene expression were noted. The medial arytenoid area of the larynx was more susceptible to alterations in gene expression. Statistically significant differences were noted in genes related to intrinsic defences and inflammation, including *CD1d*, *TGFβ-1* and mucins.

Mucins play an important role in protecting the epithelium from fluctuations of pH, ionic concentration and hydration. They are also implicated in renewal and differentiation of the epithelium and modulation of cell-cycle progression. In patients with LPR, this study demonstrated significantly lower expression of the secreted gel-forming mucin genes in the medial arytenoid region (*MUC2* and *MUC5B*) and the posterior commissure (*MUC5B*).

Carbonic anhydrase (CA) is an integral component of laryngeal defence, increasing the pH of the mucosal surface. Expression of CA I, II and III are present in the normal larynx. Expression of CA-III is known to vary in the larynx between different locations in response to refluxate. *CA3* gene expression was lower in the false cord region in LPR patients, however this was not significantly different.

There is also evidence of an inflammatory process, with changes in *CD1d* expression, which is known to be decreased in epithelial inflammation and increase in *CRNN* and *TGFβ-1* noted in the medial arytenoid sub-site.

Consequently, there is significant evidence of molecular changes in laryngeal epithelium between patients with LPR compared to normal controls. This study identifies that these changes vary according to the sub-site of the larynx. Whilst the posterior commissure is most commonly identified as the area demonstrating macroscopic change consistent with LPR, this study has identified that the medial arytenoid is the area most likely to demonstrate a molecular change. With identified molecular changes in mucin expression (*MUC2* and *5B*), cytokeratin 14 and molecular markers of inflammation, this study provides increasing evidence for the diagnosis of LPR and potential markers for therapeutic monitoring.

1 CHAPTER 1: INTRODUCTION

1.1 Definition

Laryngopharyngeal reflux (LPR) is typically defined as the backflow of gastric contents in the larynx, pharynx, trachea and bronchus[1]. The consequences of such refluxate contribute to a broad spectrum of upper aerodigestive tract inflammatory symptoms and have been associated with numerous disorders, including reflux laryngitis, obstructive sleep apnoea, laryngeal cancer, otitis media with effusion, laryngeal granuloma and subglottis stenosis[2-4].

The idea that gastro-oesophageal reflux could lead to otorhinolaryngological manifestations was first considered in 1903, when Coffin considered that "reflux of gas from the stomach" and "hyperacidity" caused laryngeal and nasal symptoms in patients with voice hoarseness and post-nasal rhinorhoea[5]. It has been increasingly recognized over the last 30 years that extra-oesophageal manifestations of gastro-oesophageal reflux disease (GORD) lead to a distinct clinical spectrum of symptoms. The term "reflux" has been synonymous with GORD however it is increasingly clear that the oesophagus is not the only area damaged by refluxate. There is a significantly high burden of reflux related disease on the Australian community and a large health budget expenditure for both health services and pharmaceuticals[6]. It is associated with a considerable impairment in quality of life unless treated effectively. According to a recent global definition, GORD can cause oesophageal and extra-oesophageal

symptoms[7] and over the last few decades, extra-oesophageal reflux has also been recognized as an individual identity.

The World Congress of Gastroenterology defined gastro-oesophageal reflux disease as a "condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications[7]." This definition has the benefits of including patients without the classical symptoms such as heartburn and those who may be suffering from the complications of reflux. Extra-oesophageal complications were included in the spectrum of the disease, with the laryngeal symptoms forming one of the clinical syndromes identified (Figure 1).

Given the common mechanisms of development, GORD and LPR have been intimately linked, however it is becoming recognized that LPR is significantly different from GORD, with a pathophysiology that leads to disparate clinical presentations and response to treatment. Previously patients presenting with pyrosis and regurgitation have been classified as "*typical*" GORD and those with other symptoms, such as laryngeal manifestations, or chronic cough described as "*atypical".* Whilst this is a useful clinical distinction to make, it implies that the pathophysiology is similar. Increasingly the term "*silent*" reflux is being utilized, given the lack of classic reflux symptoms associated with this disease[8].

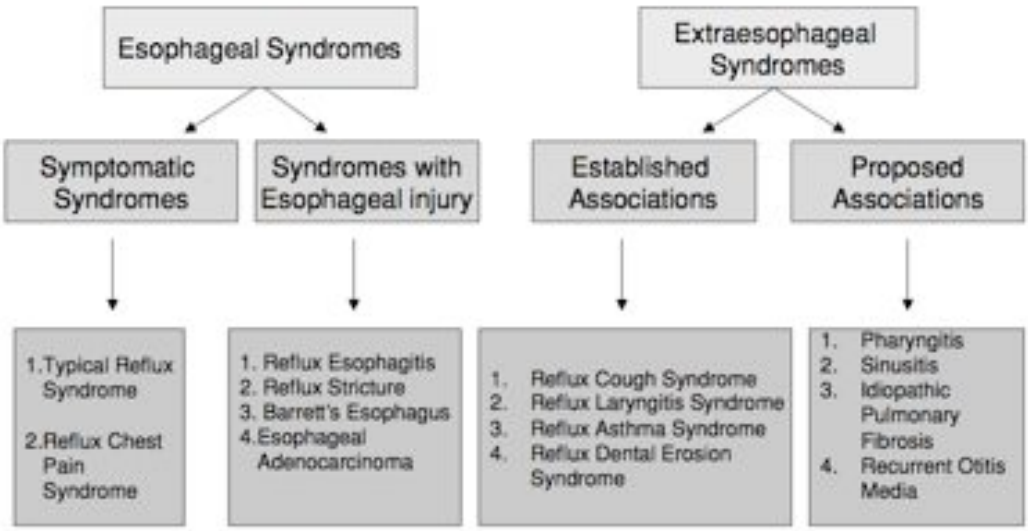


Figure 1 Montreal Classification of Gastro-oesophageal Reflux Disease

From Vakil et al. 2006[7].

1.2 Epidemiology

The prevalence of reflux, both LPR and GORD is difficult to determine. GORD is one of the most common diseases in the Western world[9,10], with the prevalence estimated at between 26% to 44%[11]. Such a variety in estimates identifies the difficulty of determining the true prevalence of GORD even though it is quite common, so it follows that estimating the true prevalence of (relatively uncommon) LPR is more of a problem. Numerous studies have attempted to quantify the incidence of GORD, however this is often hampered due to a lack of consensus over even the basic definition of the disease, given no internationally applied definition[9].

GORD has been described as a spectrum disease, with many patient subgroups ranging from symptomatic disease without mucosal lesions (Non-erosive reflux disease – NERD) to disease with significant complications including erosive oesophagitis, ulceration, strictures or Barrett's esophagus[11].

In a recent paper by the Australian Institute of Health and Welfare reviewed GORD prevalence estimated a rate of between 12.5% and 29.5%. This latter figure included patients that had GORD symptoms at any time in the past that may have resolved and consequently included a greater number. However difficulty remains in finding a true prevalence, given that the phenomenon of gastroesophageal reflux is common even in an

asymptomatic population and is identified in 65 – 75% of normal individuals[12].

Similarly it has been difficult to truly define the prevalence of LPR, with part of the problem being the difficulty of definitive diagnosis. Consequently much of the literature has considered the rates of LPR in already established disorders. Kuhn et al.[13] noted that, compared to a control group, a greater number of patients with vocal cord nodules also had LPR. Koufman et al.[14] prospectively analysed consecutive, newly presenting patients referred to their voice centre. Patients with both symptoms and findings consistent with LPR underwent an ambulatory 24-hour double probe pH monitoring. Nearly three quarters of those undergoing pH monitoring had abnormal studies, demonstrating reflux events of less than a pH of 4.0, into the oesophagus. It is of note that 50% of all these patients presenting with voice symptoms overall had pH probe demonstrated reflux into the larynx, at a pH less than 4.0, indicating that it is likely to be either a considerable cause, or confounder in the identification and management of voice and laryngeal symptoms. Furthermore, signs and symptoms related to reflux have been identified in 4 to 10% of all patients seen by Otolaryngologists[2]. Another study, using questionnaires to identify the prevalence of GORD and LPR, found 66% of respondents noting either GORD or LPR symptoms and 26% reporting both GORD and laryngeal symptoms[15]. This may be an overestimate given the majority of the community population they surveyed were recruited in hospital outpatient settings. However despite numerous

other publications addressing the pathogenesis of LPR, definitive epidemiological research is lacking.

The true prevalence still remains in doubt, with much controversy surrounding it. Some physicians suspect an over-diagnosis, and even mis-diagnosis, in many patients, whilst others believe it is considerably under-diagnosed[16]. The latter is more likely, given the lack of "classical" reflux symptoms associated with LPR, the difficulty in traditional methods for providing a definitive diagnosis and the lack of accord on the examination findings. Until a definitive diagnostic tool and substantive epidemiological data is collected, the actual prevalence and burden of disease remains unknown.

1.3 Anatomy of the Larynx

From the pharynx the upper aerodigestive tract must serve the competing functions of respiration and swallowing. The larynx is crucial to the maintenance and protection of the upper airway, toileting of the lower respiratory tract by coughing and for conducting the Valsalva maneuver. Sensation of the larynx is also important, providing important information regarding airway function and purity of the inhaled air, with resultant reflexes[17]. The production of voice, whilst important in our society, is not the primary function of the larynx, however voice disorders have been associated with significant levels of psychological distress[18]. Furthermore, voice disorder symptoms associated with LPR, such as hoarseness, chronic

cough and throat clearing were found to be associated with a poorer quality of life, with decreased self-esteem, increased relationship strain, fatigue and frustration[19].

The larynx is composed of 3 single cartilages, 3 paired cartilages, and intrinsic and extrinsic muscles with a mucosal coverage. It is divided anatomically, embryological and clinically into three major compartments: the supraglottis, glottis and subglottis[20] (Figure 2). The supraglottic region includes the epiglottis, arytenoid cartilages, aryepiglottic folds, vestibular folds and the laryngeal ventricles. The glottis includes the vocal cords with the anterior and posterior commissure and the subglottic region extends from 5 to 10mm below the true vocal fold to the inferior rim of the cricoid cartilage[20].

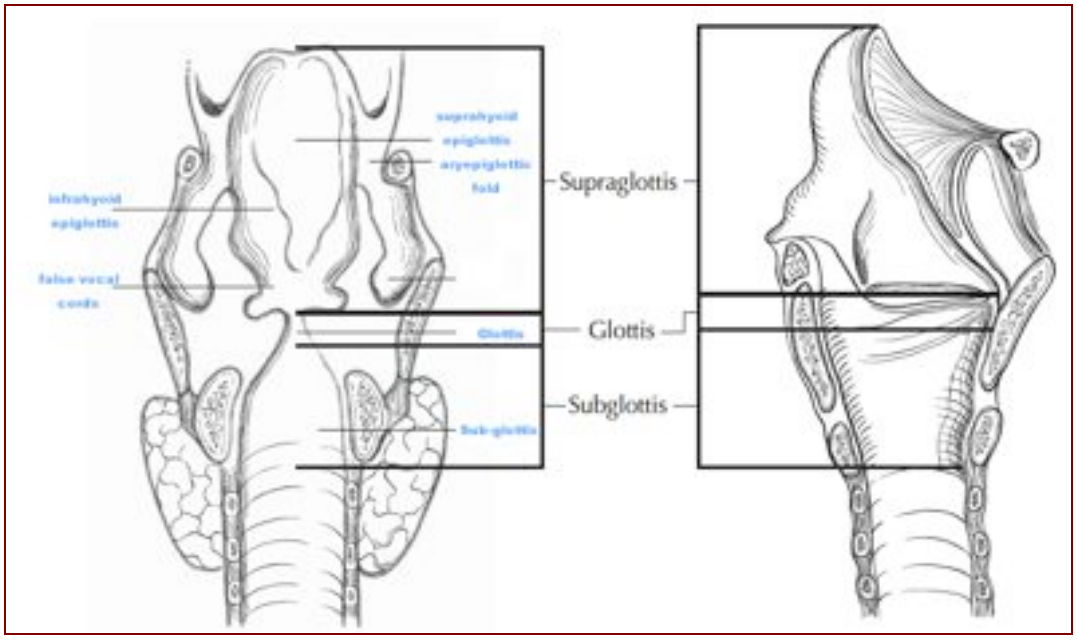


Figure 2 Laryngeal Compartments

Adapted from American Joint Committee on Cancer Staging Atlas, 2006[21].

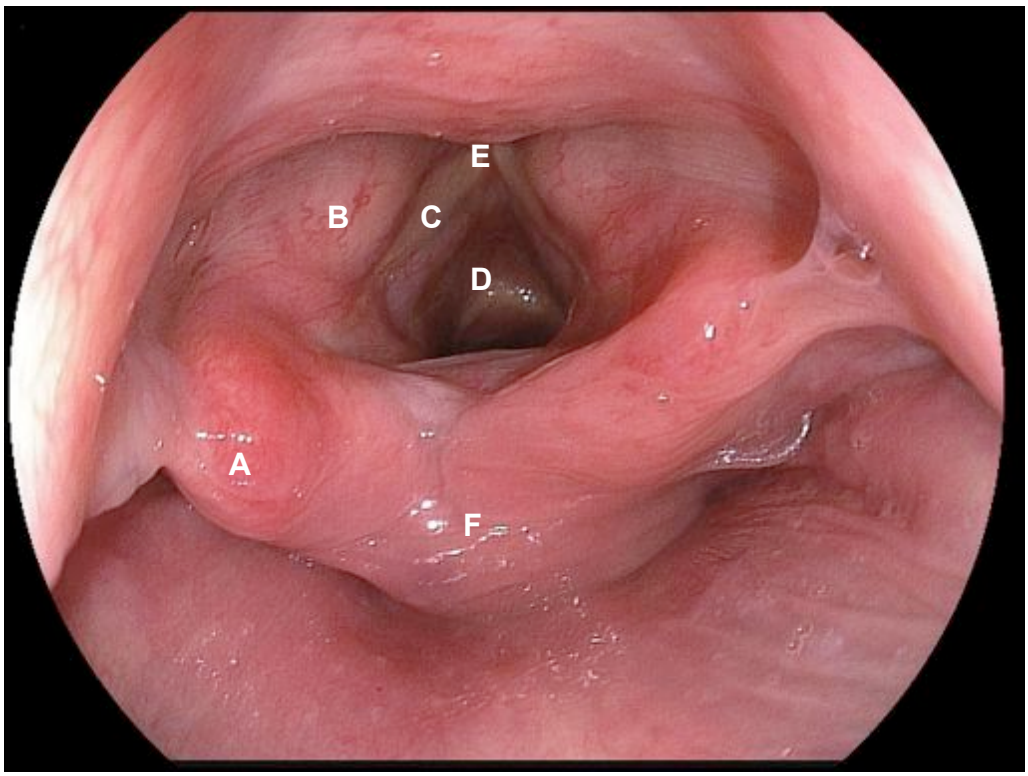


Figure 3 Anatomy of the larynx.

A: Arytenoid cartilages, B: false vocal cord (laryngeal ventricle), C: true vocal cord, D: subglottis, E: anterior commissure, F: posterior commissure. Photograph from Gastrolab [22]

There has, however, been some debate and discrepancy in describing both the anatomy and the histology of the human larynx. Whilst the sub-sites such as the epiglottis have defined boundaries, the concept of the posterior commissure has been questioned[23] and even proposed as being part of the subglottis[24]. The anterior commissure is easily identified, however the posterior commissure has been described as being based on a "clinical impression based on indirect and direct visual examination[25]." To identify it as part of the glottis has been argued as being incorrect, given the posterior end of the glottis is actually a wall. In practice, however, this is likely to be more semantics than of clinical importance, given researchers in LPR have described "posterior commissure" changes throughout the literature. Delahunty, in describing an association between LPR and posterior laryngitis identified the characteristic "interarytenoid heaping of mucosa[26]." This was further described in the literature by Kambic and Radsel[27], with Koufman[2] describing it as the most common laryngeal finding in patients with reflux laryngitis. Belafsky[28] described the posterior commissure as being in close proximity to the oesophageal inlet and hypertrophy of this region typically being graded as mild, moderate and severe. Mild hypertrophy coincides with a "mustache-like appearance" of posterior commissure mucosa, with swollen mucosa creating a straight line across the back of the larynx identified as being moderate. Severe hypertrophy was described when there is mucosa bulging into the airway. Consequently for this study the posterior commissure was identified as the area described by Stell et al.[29] in their morphological study larynx and comprises of the "band" of epithelium extending from the arytenoid eminences.

The mucosal lining of the larynx consists of both squamous and respiratory type columnar epithelium. There has, however, been a degree of discrepancy regarding descriptions of the mucosa lining. This may, in part, be due to the difficulty in accurately defining the sub-sites of the larynx, which, unlike the oesophagus, do not always possess a definitive "transition zone" or anatomically distinct landmark to separate distinct areas. The epiglottis is lined anteriorly with squamous epithelium. In the posterior aspect, the upper portion of the epiglottis is lined with squamous epithelium. Some studies have found the entire posterior aspect of the epiglottis to be lined with squamous mucosa[30], however recent consensus is that it becomes columnar epithelium inferiorly[31]. The vibratory margin of the vocal cord is stratified squamous epithelium[32]. Typically the remainder of the larynx is described as consisting of ciliated columnar epithelium, with the transition from squamous epithelium of the vocal cord to the respiratory epithelium being the landmark for the lower border of the glottic region[20]. (Figure 4) Despite this, there is evidence that the vestibular folds (false vocal cords) possess a variable mucosal lining. Stell et al.[30] studied 49 post-mortem larynges of non-smokers and found the vestibular folds were covered entirely in respiratory type epithelium in only 50%. Nearly 10% were noted to be entirely squamous, with the remaining 40% being a mixed respiratory and squamous epithelium. Unfortunately there is no further classification of whether these patients suffered from reflux, or had any laryngeal symptoms to account for any changes in mucosal lining. The posterior commissure mucosa has been inconsistently described, with some

studies identifying it as respiratory columnar epithelium[20] and others noting a solely squamous lining[25,29,33]. This may be due to the difficulty in identifying the true boundaries of the posterior commissure, with those defining the "posterior commissure" of the glottis, along the plane of the anterior commissure and encompassing the medial wall of the posterior larynx likely to find respiratory epithelium in continuity with the sub-glottis. However the current study considers the posterior commissure to be the area of the larynx between the arytenoid eminences, and, as described by Stell et al.[29] is comprised of squamous epithelium.

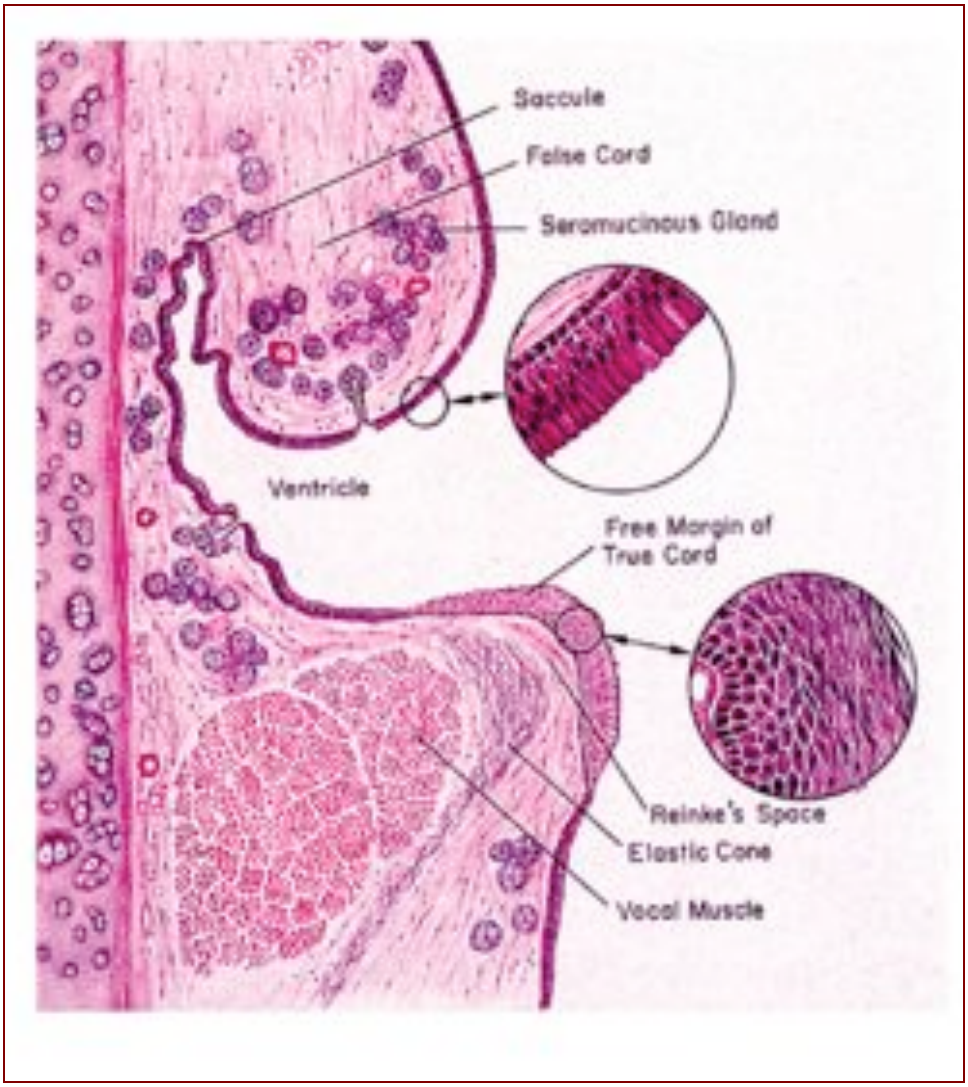


Figure 4 Histology of the Larynx

from Mills 2007 Histology for Pathologists[31].

1.4 Extra-oesophageal Reflux: LPR

LPR is clinically identified by a cluster of signs and symptoms suggestive of irritation or damage caused by extra-oesophageal reflux. The most common symptoms associated with LPR have been recognized as excessive phlegm, globus pharyngeus, throat clearing and sore throat[34]. Symptoms are likely to be caused by gastric refluxate, with evidence that not only acid refluxate, at a pH of less than 4.0, leads to inflammation, but there is evidence non-acid reflux (pH between 5 and 7) may also lead to injury in the larynx[35].

1.4.1 Reflux Laryngitis

Posterior laryngitis was first described in 1972[26] and is the most recognized of the LPR-associated stigmata and includes pachyderma, erythema and oedema of the arytenoid mucosa and hyperplastic interarytenoid tissue[26,36]. As a finding it has been noted to be highly suggestive of LPR, with one study finding 15 out of 20 patients with posterior laryngitis having reflux events during dual-channel pH monitoring[37]. However it is recognized that such findings are particularly subjective in nature and the degree of erythema, oedema and inflammation can be difficult to standardize on examination.

Pseudosulcus is another laryngoscopy finding first described by Koufman,[38] it is thought to represent infraglottic oedema, giving the appearance of a furrow or sulcus subglottically and extends back to the posterior commissure. It is distinguished from a true sulcus vocalis in that the sulcus

is at the free edge of the vocal fold and terminates at the vocal process in the latter. In a recent study, all patients with pseudosulcus had episodes of LPR during a 24-hour pH study, with 19 of the 20 patients studied reporting symptoms commonly associated with LPR. Consequently this study suggested pseudosulcus had a positive predictive value of 90% for LPR and as a relatively objective finding has been suggested a useful finding in diagnosis of LPR[36]. However in other studies there has been no statistical difference in the occurrence of pseudosulcus between patients with or without extra-oesophageal reflux and with or without GORD[34].

1.4.2 Globus Pharyngeus

Globus pharyngeus was first described by John Purcell in 1704, although Hippocrates noted globus nearly 2500 years ago. The word "globus," stems from the Latin word for "ball," essentially as the sensation is like something in the throat. It has a prevalence higher in women with Purcell describing and naming the symptom *globus hystericus*, having been linked with uterine dysfunction from which it was believed all hysteria arose[39]. Nearly always this symptom is described as a foreign body sensation in the throat. It is a common condition and has been associated with LPR in 40 – 80% of patients[34,40]. Multiple aetiologies of globus have been postulated, including lingual tonsil hypertrophy, cricopharyngeal spasm and even cervical osteophytes. Gastroesophageal reflux has been linked with globus since the late 1960s, however subsequent studies have widely divergent results

linking acid reflux to this symptom, with rates varying between 7 to 90% of patients with globus having acid reflux[41]. There is significant evidence now to suggest the role of non-acid reflux and that small amounts of reflux can lead to the development of LPR symptoms[35]. Consequently the assessment of globus by barium swallow and pH monitoring may miss a significant population suffering extra-oesophageal reflux.

1.4.3 Dysphagia

The term dysphagia is derived from the Greek *dys* meaning bad or disordered and *phago* meaning "eat." Swallowing itself is a complex physiological motion with a bolus passing from oral cavity to cervical oesophagus in around 2 seconds[2]. Throat pain and dysphagia are non-specific symptoms which can be attributed to a wide range of causes, including infectious, neoplastic, myopathic, neurologic, traumatic, inflammatory or idiopathic. Koufman[2] suggested there were three possible mechanisms of symptom production associated with GORD. Firstly, refluxate may lead to irritation of the laryngopharyngeal structures. Secondly, referred discomfort to this region from oesophageal dysfunction, or finally, from upper oesophageal sphincter dysfunction. Whilst dysphagia is commonly considered one of the symptoms of LPR, the pathophysiology is yet to be determined.

1.4.4 Chronic Cough

Chronic cough has been associated with LPR, however is a non-specific symptom. In the majority of cases this symptom is attributed to asthma, sinonasal disease or LPR. However it is important to consider less common causes including chronic pulmonary disease, chemical irritants, congestive heart failure, medications such as angiotensin-converting enzyme inhibitors[42] and rare conditions such as chronic eosinophilic pneumonia[43] .

A number of mechanisms have been proposed by which GORD and LPR induce cough, with neither mutually exclusive. Firstly aspiration of refluxate can occur at both a macroscopic and microscopic level. Large amounts of refluxate may be aspirated into the broncho-pulmonary tract and in these patients grade 3 or 4 oesophagitis is typically common. Microaspiration is consistent with small amounts of refluxate passing across the upper oesophageal sphincter. It is thought that these demonstrate laryngeal mucosal inflammation and associated cough and hoarseness[5].

Secondly the vagus-mediated oesophago-bronchial reflux mechanism has more recently been proposed and originates from the oesophageal receptors for cough rather than the laryngeal and bronchial receptors[5]. In addition to this stimulation, a “vicious cycle” is instigated, with cough increasing trans-diaphragm pressure, which induces relaxation of the lower oesophageal sphincter, increasing the likelihood of further reflux[5].

1.5 Consequences

Little is known of the long-term consequences and the natural history of LPR, however there are multiple documented associations in the literature, in all areas of the upper aerodigestive tract. The idea of reflux into the laryngopharynx was considered in the otolaryngological literature as far back as 1968[44], in the development of vocal cord granulomas. Other associations include laryngomalacia, as well as subglottic stenosis. The latter of these was reported in 1985 with the case of a recalcitrant subglottic stenosis which, despite other management, resolved once treated for reflux[45]. Such case reports are supported by recent research on the exposure of the subglottic columnar epithelium to acid and pepsin. Bulmer et al.[33] found this subglottic tissue was the most susceptible to damage of all the sub-sites of the larynx.

LPR has been considered a risk factor in the development of otitis media, particularly in children. Gastric reflux is particularly common in neonates and infants, with evidence that it may occur in nearly two thirds of infants at 4 months of age[46]. In a study of 509 patients undergoing myringotomy the presence of pepsin was detectable in 20% of middle ear fluid samples and those with purulent effusions were more likely to be pepsin 'positive.'[47]

The role of LPR in the development of laryngeal cancer is still widely debated. The most common risk factors for the development of this remain smoking and alcohol and the human papilloma virus. Vaezi et al.[48] conducted a matched case-control study of 96 patients with newly

diagnosed laryngeal cancer, finding GORD was significantly associated with laryngeal cancer. In their study, symptomatic GORD was significantly higher in the cancer patients, than controls (13.5% vs 5.7%) and for any given level of smoking, GORD increased the probability of developing laryngeal cancer. There was no interaction between smoking and GORD noted however. It is difficult to determine these causal relationships, as such studies are retrospective. Additionally the patient numbers required to determine such a causative association are likely to be much higher than have been currently studied, given that without following a patient for many years prior to diagnosis, the only method to determine any correlation would be to diagnose both the laryngeal cancer and GORD at the same time. By doing it in retrospect the question of the direction of causality remains open, given even the psychological impact of a cancer diagnosis may cause GORD[49,50]. Despite this, the concept of LPR induced chronic inflammation causing cancer still remains logical, given a similar aetiology in other cancers. Recently, one group of researchers, using the Human Cancer Pathway Finder Super Array found exposure of pepsin altered the expression of 27 genes implicated in carcinogenesis[51]. Furthermore, in animal models, exposing hamster cheeks to a known carcinogen, 7,12-dimethyobenzanthracene, found the application of pepsin lead to a statistically significant increase in tumour volume[52]. Overall the evidence supporting the role of LPR in the causation of cancer remains tenuous, with the literature remaining divided. In part, adequate studies are limited by numerous other factors, including the consensus of the definition and diagnosis of LPR.

1.6 Diagnosis

Asymptomatic gastroesophageal reflux has been reported in 65-75% of normal individuals[12]. Consequently the difficulty lies in using a test sensitive and specific enough to distinguish between non-symptomatic normal population and definitive refluxers. There is an abundance of literature considering diagnostic testing in GORD, with parameters becoming increasingly defined. Various methods have been considered in the literature for the diagnostic testing of LPR, however there is no current reliable "gold standard" test available.

1.6.1 pH Monitoring

Definitive diagnosis of LPR currently does not exist. The use of dual-probe 24 hour pH monitoring has previously been considered the "gold standard" test, yet it has significant problems. This may relate to the ubiquitous use of pH monitoring in the diagnosis of GORD.

Oesophageal pH monitoring has been widely used for the diagnosis of GORD, with a sensor typically placed proximal to the upper margin of the lower oesophageal sphincter (LES) at a point far enough away to avoid displacement into the stomach, particularly during swallowing when the oesophagus shortens. It would seem reasonable then to assume that to measure LPR it would be feasible to place a similar probe in the

hypopharynx or proximal oesophagus[53]. In practice there has been significant controversy regarding the placement, with some studies suggesting between 15 to 20cm above the LES, or either just below or up to 2cm above the upper oesophageal sphincter (UES).

Difference of opinion also exists as to the level of "normal" acid reflux. Some otolaryngologists believe that in some patients, any laryngeal acid exposure can cause signs and symptoms, even if occurring only once every day (or even less), particularly given there is no evidence that laryngeal mucosa has strong protective measure against acid[16]. Some researchers feel that any reflux into the larynx is abnormal. One study comparing signs and symptoms of LPR to pH monitoring found a lack of correlation between laryngeal symptoms and pH monitored laryngeal reflux[54] and this was consistent with other researchers[55].

Additionally, interpretation of pH monitoring results can be difficult and can depend on who is interpreting it. For example a gastroenterologist call a 24-hour impedance study normal with four or five episodes of acid reflux (pH less than 4.0) at a proximal sensor[16]. Smit et al[56] suggested that more than four episodes of laryngeal reflux is pathological, however, as previously mentioned, there is no standardized number of "normal" reflux events.

Such studies of LPR and pH monitoring have demonstrated evidence that LPR is different to GORD. The periods of LPR are shorter in duration

(seconds) and less frequent. Additionally they mostly occur in the upright position.

Other techniques, such as the questionnaire based Reflux Symptom Index (RSI), have previously been demonstrated to show a strong correlation with pH-documented reflux[57]. In addition, such monitoring is a significantly invasive procedure and is limited by patient compliance. Furthermore, as already stated, pH monitoring, although considered "gold standard" has a significant variability in results ranging from 20 to 50% and the guidelines by the American College of Gastroenterology stated that "available evidence does not support the routine use of proximal pH monitoring in clinical practice"[58].

Consequently there is a shift towards diagnosis and furthermore management, of LPR by clinical diagnosis based on a cluster of symptoms and signs in the larynx. The RSI and a rating scale for physical findings, the Reflux Finding Score (RFS) consequently provide a quantitative measurement of such signs and symptoms and are consistently utilized in literature. Despite well-documented limitations, these still remain the best standardised measurements of LPR in the light of no single pathognomonic change. It is well recognized that LPR is difficult to accurately diagnose with a single investigational modality, and recent studies have suggested that combining two modalities would increase the likelihood of a correct diagnosis. Park et al[59] studied 57 patients who complained of globus pharyngeus symptoms for longer than one month, comparing RFS, RSI, 24-

hour double-probe pH monitoring and the symptom checklist-90-revision(SCL-90-R) for each patient. They found individually the RSI had a sensitivity of 75% and specificity of 18.8%. RFS was found to have a sensitivity of 87% and specificity of 37%[59], suggesting the RFS was more sensitive than the RSI, but had a higher rate of false positives. 24-hour double probe pH monitoring was used as the "gold standard" against which these were compared, which further complicates these figures, given it has a well-documented significant variability in positive results according to examiners interpretation and false-positive or false–negative results[59]. Furthermore, combination of the RSI and RFS scores demonstrated an increased specificity, however sensitivity was decreased (sensitivity of 68%, specificity of 50%). Again this may well be complicated by the physical process of reflux, with non-acid refluxers still demonstrating signs and symptoms which are not picked up by the 24-hour dual probes.

1.6.2 Reflux Symptom Index

LPR is well recognized as having a cluster of symptoms quite different to gastroesophageal reflux. As previously mentioned, vocal fatigue, hoarseness, globus pharyngeus, chronic cough, post-nasal drip are all included as manifestations. Individually they may have many causes, however collectively they can provide an indication of LPR. Belafsky et al[60] developed a 9 item questionnaire utilising 25 patients with a clinical diagnosis of LPR and further confirmed with 24-hour double-probe pH

monitoring (Appendix 1). Patients were given the RSI and the 30-item Voice Handicap Index (VHI) at their first visit, then repeated before commencing a course of anti-reflux medication. Belafsky[60] found a high correlation between pre-treatment scores, concluding the measure possessed a high level of reproducibility[60]. At the time of development there was no validated instrument for the use of the otolaryngologist to assess outcomes in LPR patients. The RSI was developed as an outcome measure, particularly to measure the improvement in defined LPR symptoms following a trial of 6 months of anti-reflux medication. Belafsky [60] found that there was a similarity between the RSI in asymptomatic patients to those treated with 6 months of anti-reflux medication twice daily. An addition there was a significant improvement in the 'functional' subscale of the VHI and there was a correlation with improvement in the RSI. Patients who experienced a 5 point or greater improvement in RSI were 11 times more likely to experience a five point improvement in VHI[60]. It is of note that the mean RSI of LPR patients in this study was 21.2 and improved to 12.8 following treatment. Belafsky considered a score of greater than 13 as being abnormal, so although the symptom severity improves significantly, the mean score still lies very close to the abnormal range. This may suggest that a longer course is required, or additional management is needed.

Belafsky[60] developed the RSI, however the absolute cut off for abnormal result is arbitrary. His study considered 25 patients diagnosed with LPR on clinical and 24 hour pH double probe ambulatory monitoring. These patients were age and gender matched control from an asymptomatic group

with no evidence of LPR. In consideration of the control group, the 95% upper confidence limit for RSI was 13.6. From this figure he suggested that an RSI of greater than 13 would be abnormal. Despite this, other studies have utilized other values to determine "abnormal" results. It is particularly difficult to determine this cut off considering there is no universally accepted gold standard for the diagnosis of LPR.

In an epidemiological study sampling 2000 general practice patients in the UK, 30% of patients had an RSI greater than 10, with 75% of these patients also suffering GORD symptoms[61]. In addition, they considered BMI, recognizing 40% of those sampled with a normal BMI. Of the normal BMI population, 24% had an RSI over 10. This rate increased to 40% with patients having a BMI between 25-29 and 50% with a BMI greater than 30[61].

Oyer[62] suggested that although the RSI was a validated outcomes tool, its predictive value for LPR remained controversial. This was most noted when considering the effects of anxiety and depression in comparing pH monitoring measurements and RSI scores. They found that patients classified with a psychiatric disorder (eg depression or anxiety) had a significantly higher RSI score than those classified into the non-psychiatric group, however they had a significantly lower incidence of abnormal probe studies. This indicates that those with psychiatric disorders may have a lower threshold for reporting such symptoms. They suggested that an elevated RSI was a poor predictor for an abnormal pH probe study in the

psychiatric group, but a strong predictor for an abnormal probe study in non-psychiatric patients[62].

In addition, some research does not use the RSI at all, citing that most academic and non-academic otolaryngologists do not frequently use the RSI in clinical practice, as LPR is a clinical diagnosis of a cluster of symptoms, with clearly no single pathognomonic finding[63].

1.6.3 Reflux Finding Score

Laryngeal findings have been identified as being crucial to the diagnosis of LPR, partly due to the requirement to rule out any other pathology leading to the laryngeal symptoms. Belafsky et al.[57] developed the Reflux Finding Score (RFS) based on the 8 most common laryngeal findings identified in their voice centre that were representative of LPR (Appendix 2). The instrument scores range from a minimum of zero indicating no inflammation, to a maximum of 26.

Such scales are useful both clinically and from a research perspective to assist in diagnosis and to monitor improvement following the commencement of treatment. The RFS has the benefit of grading each of the laryngeal findings on a scale of severity. This is important to be able to quantify laryngeal inflammation, but also important given there is no single laryngeal finding pathognomonic of LPR. Multiple individual laryngeal findings have been considered as identifying the presence of LPR, with

posterior commissure hypertrophy previously being regarded as the sine qua non of reflux laryngitis, being diagnosed in 74% of patients with LPR[28]. (Figure 5) In addition, 15 of 20 patients identified with a posterior laryngitis were noted to have pharyngeal reflux events on dual-channel pH monitoring[37].

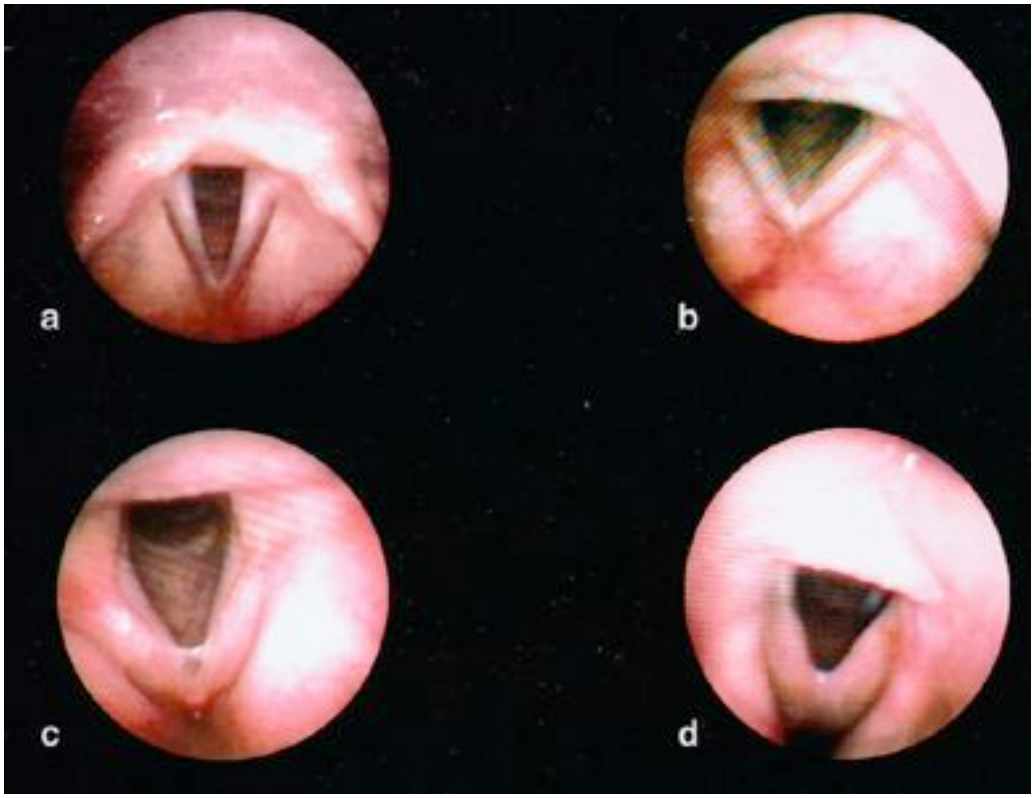


Figure 5 Posterior Commissure Hypertrophy

a) normal posterior commissure; b) mild hypertrophy, moderate vocal fold oedema, pseudosulcus vocalis; c) moderate hypertrophy (straight line across posterior larynx), pseudosulcus vocalis, moderate vocal fold oedema, partial ventricular obliteration, diffuse laryngeal oedema; d) severe posterior commissure hypertrophy, with severe vocal fold oedema, total ventricular obliteration and diffuse laryngeal oedema.

From Belafsky et al. 2001[57].

Whilst this is recognized as being the most frequent finding associated with LPR, other findings have also been considered equally important. Other research has focused on the laryngeal pseudosulcus as a predictor of LPR. The pseudosulcus refers to oedema of the undersurface of the vocal fold (Figure 6), and typically extends from the anterior commissure to the posterior larynx, and is located inferior to the striking zone of the vocal fold[64]. Comparatively, a sulcus vocalis stops at the vocal process and is found in the striking zone. A number of studies have considered the presence of a pseudosulcus to be predictive of LPR, with one study identifying it as having a positive predictive value for LPR of 90%, however this lacked a comparison control group[36]. Furthermore, other studies have reported a sensitivity of 70% and specificity of 77% and considered the presence of a pseudosulcus alone suggestive of LPR. It must be noted, however, that the ageing larynx can also demonstrate a pseudosulcus, with an inelastic vocal fold epithelium and atrophic vocal fold musculature[64], yet these changes occur rarely as a single finding in LPR.

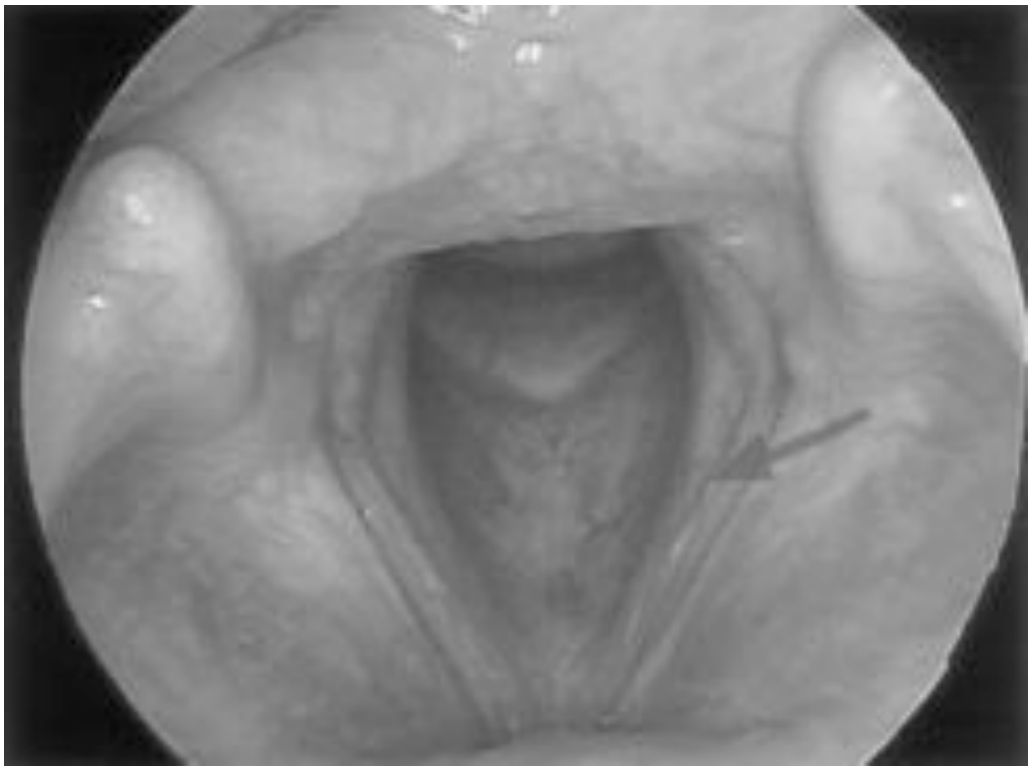


Figure 6 Laryngeal Pseudosulcus

Image of laryngeal pseudosulcus (arrow)

From Hickson et al. 2001[36]

It should be noted that the RFS scale considers signs of laryngeal inflammation and Belafsky was careful to note that these are not necessarily specific for LPR, but other laryngeal pathology, such as infection, neoplasia, autoimmune disorders and environmental toxins can result in an abnormal RFS[28]. However there are some subtleties within the scoring, such that localized erythema, such as that involving the mucosa only over the arytenoids, contributes 2 points, whilst diffuse laryngeal erythema contributes 4 points. Overall, such a scale has a number of benefits, including its ease of administration, has the ability to document severity and has sound inter- and intra-observer reliability[57], with increasing evidence that the identification of multiple mucosal signs improves the clinical diagnosis of LPR[65].

1.7 Pathogenesis of Reflux

The study of LPR has been historically linked with GORD, from initial diagnostic techniques such as pH monitoring, to sharing common management strategies, such as the commencement of proton pump inhibitors (PPIs) for treatment. However there are clear differences between the disease processes, with classically different symptom clusters, such that the majority of patients with LPR do not suffer heartburn[2]. For example, for injury to occur in the larynx from reflux there must be firstly agents in the refluxate that causes injury and secondly a failure, or loss, of the any intrinsic, or extrinsic defences. A number of injurious agents have been considered, including acid and pepsin. In addition Lipan et al.[66] described four anti-reflux barriers that exist to protect the larynx from injury: the gastroesophageal junction, oesophageal motor function and acid clearance, the upper oesophageal sphincter and laryngopharyngeal mucosal resistance.

1.7.1 Damaging Agents

Gastric refluxate has long been recognized as causing oesophagitis. As early as 1934, Asher Winkelstein considered that a number of his patients may be suffering symptoms resulting from the "irritant action on the mucosa of hydrochloric acid and pepsin[67]."

1.7.1.1 Acid

Hydrochloric acid (HCl) is an aggressive component of refluxed gastric contents and is recognized as causing oesophagitis, with increasing severity noted with increasing acid exposure. There seems to be strong evidence of the role for acid, with a positive response to a trial of PPI being diagnostic of LPR. The current parameters for pH probe studies identifies an acid reflux episode when a reflux event is below a pH of 4.0[68]. It is well known that laryngeal epithelium is more sensitive to injury by gastric refluxate than oesophageal mucosa[68]. Whilst up to 50 episodes of reflux in the distal oesophagus may be considered physiologically normal, Koufman[2] described as few as 3 episodes per week in the proximal oesophagus may lead to laryngeal injury.

Multiple studies have identified damage to laryngeal mucosa, in a number of laryngeal sub-sites, such as the posterior commissure, the vocal folds and supragolttis, on exposure to acid at pH levels of 2.0. Bulmer et al[33] found these areas were resistant to damage following incubation in a test solution of pH 4.0, however there was injury to the subglottic region, an area of columnar epithelium. Despite this, it has been postulated that the impact of gastric HCl on the laryngopharynx is diminished due to secretions from the salivary glands and oesophagus[35]. There is evidence of weak acidic reflux episodes (pH <5.0) and even non-acid reflux episodes lead to laryngeal

injury[68]. Furthermore multiple studies have identified a synergistic effect of both acid and pepsin in effecting inflammatory changes in the larynx[69,70].

1.7.1.2 Pepsin

Pepsin is a proteolytic enzyme, with its precursor, pepsinogen, released in the stomach from chief cells. It is increasingly implicated in LPR due to its proposed role in both acid and non-acid reflux. Multi-channel intra-luminal pH monitoring impedance studies have identified episodes of gastric reflux that are either non-acidic or weakly acidic in symptomatic patients[71], suggesting mucosal injury might be caused by non-acid refluxate components such as bile salts and pepsin. The damaging effects of pepsin in an acidic environment have been well described previously[2], with an optimum activity at a pH of 2.0[72]. Recent research has proposed that pepsin is a causative agent of laryngeal damage in non-acidic reflux[71,73-75].

Whilst pepsin is inactive at a pH of 6.5[72], it is irreversibly inactivated at a pH of 8.0[76]. Recently it has been shown that at 37^{o}C pepsin remains stable at a pH of 7.0 for more than 24 hours, retaining nearly 80% of its original activity on re-acidification. With a mean pH of 6.8[75], the larynx may contain stable 'active' pepsin, potentially causing more damage with subsequent reflux episodes. Additionally there is evidence that such pepsin is actively transported into and remains in, laryngeal epithelial cells[75,76]. The pH of intracellular structures such as Golgi bodies and lysosomes lie between 4.0

to 5.0. Whilst the laryngeal mucosa is exposed to inactive pepsin, intracellular uptake into this micro-environment allows for the acidification of pepsin. This may lead to intracellular injury. [76]

Furthermore, a significant association has been found between the presence of pepsin in laryngeal epithelia in patients with reflux-attributed laryngeal disease and depletion of two laryngeal protective proteins; carbonic anhydrase isoenzyme III (*CA3*) and Sep70, a squamous epithelial stress protein[76,77]. It is of note that both of these proteins are depleted after exposure to pepsin and not in response to low pH alone, suggesting a specific role for pepsin in laryngeal damage.

1.7.1.3 Bile

Whilst recent research has suggested pepsin and acid play a role in the pathogenesis of LPR, few studies have considered the role of non-acidic duodenogastroesophageal reflux (DGER). Clinical studies have demonstrated that duodenal secretions are capable of refluxing into the stomach and oesophagus[78,79]. There is also evidence that a bilious reflux causes injury to the oesophageal mucosa, with a graded increase in oesophagitis with increasing exposure to biliary pigment in symptomatic patients. Such duodenal secretions have been demonstrated to be capable of causing damage, with evidence that bile salts lead to oesophageal mucosal injury, however there is a variability in injury according

to the type of bilious reflux and the acidity of the refluxate. Interestingly conjugated bile salts had been found to cause mucosal injury at a pH of 1.2 to 1.5, whereas un-conjugated salts were found to increase mucosal permeability and injury at a pH of 7.0 or higher, but not at a lower pH[80]. In an experimental setting, conjugated bile acids are more injurious to mucosa at an acidic pH, whilst chenodeoxycholic acid is more active at a pH of 5.0 to 8.0[80].

Furthermore, recent research[80] exposed the laryngeal mucosa of rats to taurocholic acid and chenodeoxycholic acid at a range of pH 1.5 to 7.4. Using a negative control, this study found taurocholic acid was injurious to laryngeal mucosa at a pH of 1.5, where as chenodeoxycholic acid caused the maximum inflammation at a pH of 7.4.

Previous research has demonstrated a role of bile acids in oesophagitis, Barrett's metaplasia, dysplasia and oesophageal adenocarcinoma[81]. In addition, induction of cyclo-oxygenase-2 (COX-2) expression has been implicated as a mechanism in carcinogenesis[82]. Sung et al[83] studied the effect of bile salts on cultured hypopharyngeal cells, finding chenodeoxycholate induced the up-regulation of mRNA as well as COX-2 protein in a dose-dependent manner.

The above indicates that bile has a mechanism for generating laryngeal injury, in both acidic and non-acidic environments. However it remains to be determined whether the same mechanism occurs in the human larynx. In

addition, whilst hydrochloric acid and pepsin appear to have a synergistic effect, the relationship between bile salts and pepsin is more complex. A number of studies have demonstrated that bile salt, particularly taurine-conjugated salts, actually reduce pepsin proteolytic activity at a pH of 2. Furthermore, a recent study noted that whilst pepsin activity was pH dependent, bile acids did not attenuate the activity of pepsin [84]. Consequently the role and interactions of all these components of refluxate are yet to be determined.

1.8 Laryngeal Defences

1.8.1 Gastroesophageal junction

Ultimately the reflux of gastric contents up the esophagus, and potentially into the larynx and pharynx, is a failure of the lower oesophageal sphincter (LOS) to control this retrograde flow. On occasion such retrograde flow is desirable, for example to allow an urgent expulsion of gas, or emesis of noxious agents. Consequently the LOS cannot be a simple, unchangeable one-way valve.

Lying in the chest and abdomen, the LOS is sensitive to intra-thoracic and intra-abdominal pressure. The LOS pressure itself varies with inspiration, rising with diaphragmatic contraction secondary to the external force of the crural fibres[85]. The transition from intra-thoracic to intra-abdominal sphincter is noted when measuring with manometric tracing and is recognized as the

respiratory inversion point. This is the point at which the pressure of the oesophagus changes from negative to positive with inspiration, then from positive to negative with expiration[86]. Consequently the primary line of defence against reflux is this integrity, affected by the intrinsic lower esophageal sphincter, extrinsic compression of the LOS by the crural diaphragm, the intra-abdominal location of the LOS and integrity of the phrenoesophgeal ligament.

There are multiple theories emerging on how reflux breaches the gastroesophageal junction. Firstly, transient depressions in lower oesophageal sphincter pressure, a recognized physiological phenomenon, may be increased in frequency in patients with GORD[66]. GORD symptoms have been attributed to both increased frequency of LOS sphincter relaxations and increased frequency of acid reflux during these relaxations. No study has yet demonstrated a relationship between these relaxations of sphincter and LPR.

Secondly, hypotension of the lower oesophageal sphincter has been proposed as playing a role in GORD and LPR. Grossi et al.[87] demonstrated transient relaxations in the lower oesophageal sphincter were the main cause of distal reflux, also noting that hypotension of the LOS was more likely to cause proximal reflux. Logically this proximal reflux is more likely to reach the laryngopharynx[88], which may suggest that hypotension of the LOS is likely to play a causative factor in LPR. Despite this, studies measuring basal LOS pressures in LPR patients have failed to find a significant

difference to those in the control groups[88,89]. It is possible that due to the infrequent episodes of reflux required to cause laryngeal inflammation, monitoring over relatively short periods fails to find a significant difference between normal and patients refluxing to the laryngopharynx.

1.8.2 Oesophageal motor function and acid clearance

Normal motor function of the oesophagus allows boluses to be pushed by a strong peristaltic motion from the cricopharynx down into the stomach. These waves are either primary, triggered by the pharyngeal swallow, or secondary. These secondary waves are triggered by stimulation of the oesophageal mucosa. Of these it would appear that the primary peristalsis is the most important for returning refluxate back to the stomach[90]. Dysfunctional oesophageal motor function has consequently been considered of significance in LPR. A number of studies have found impaired oesophageal motility, measured by oesophageal acid clearance, or manometric measurement, in patients with LPR[91,92]. Of further interest was a conclusion that the primary oesophageal dysfunction associated with LPR was not as severe as that found in patients with GORD[91], this would lead to less refluxate exposure in patients with LPR. Whilst GORD is manifest by significant exposure of the oesophagus to acid reflux, a reduced exposure time experienced by the patients with LPR may provide enough refluxate to damage the larynx, without causing the typical oesophageal symptoms associated with GORD[66].

1.8.3 Upper oesophageal sphincter

The upper oesophageal sphincter (UOS) is the high-pressure zone located between the pharynx and cervical oesophagus, protecting the reflux of food into the airway and air into the digestive tract. The UOS tonically constricts, relaxing to allow boluses of food or fluids with swallowing. Studies measuring UOS pressures found similar average pressure levels in patients with LPR to controls[88,93]. However whilst the average pressure itself was not significantly different, the duration of tonic pressure was nearly double in the control group compared to a group with GORD[66]. Torrico et al[94] found nearly all reflux events were associated with such an increase in UOS pressure. Consequently a shortened period of UOS pressure may allow greater opportunity for refluxate to enter the laryngopharynx. To date, however, no studies have measured this.

1.8.4 Mucosal Resistance

Once past the upper oesophageal sphincter, intrinsic mucosal defences remain the sole barrier to refluxate. Significant amounts of research have concentrated on the role of both damaging agents, and the mucosal response to refluxate, both *in vitro* and *in vivo* studies. Despite this, the role of inflammatory mediators has yet to be determined, however may play a role in both the defences and the propagation of signs and symptoms.

1.8.4.1 Inflammatory Markers:

Damage to mucosal linings of the oesophagus is well recognized on the macroscopic level, with mucosal ulceration, or columnar-lined distal oesophagus. At a more subtle level, injuries to this lining can occur at the histological and microscopic level and is recognized in the presence of inflammation. Additionally ultra-structural changes in the intercellular gaps of the mucosa have been recognized on electron microscopy which correlate with mucosal injury[95]. Such damage has significant consequences, particularly given chronic inflammation is associated with carcinogenesis. In the oesophagus, chronic inflammation of the squamous epithelium from GORD is recognized as leading to intestinal metaplasia, dysplasia and eventually oesophageal adenocarcinoma[96]. Given the aetiology of LPR is similarly due to the refluxate of gastric contents, it is possible that a comparable pathological process may ensue, including the potential for tumourgenesis in the larynx.

Prior to these macroscopic and microscopic changes more subtle molecular changes alter the expression of genes involved in aspects of the cell cycle such as cellular repair, proliferation and migration. Consequently research has suggested that a more sensitive assessment of mucosal injury may lie in the measurement of the genes involved in these processes[96] and progress our identification of changes beyond subjective viewing with endoscope or microscope.

1.8.4.2 Interleukin 8

At the molecular level, some pathways associated with inflammation are similar to those involved in carcinogenesis. The nuclear factor-κB is one such pathway that activates interleukin-8 (IL-8) as a major downstream product. Nuclear factor-κB is a transcription factor that regulates many genes involved in the inflammatory process and is known to increase the expression of genes for many cytokines, enzymes and adhesion molecules in chronic inflammatory diseases[97]. It is known to reside in cytoplasm of most cells as an inactive heterodimer consisting of p50 and RelA subunits complexed to the inhibitory IκB, which prevents the migration of the heterodimer into the nucleus. When stimulated NF-κB translocates to the nucleus and binds to its specific site and up-regulates the transcription of a variety of genes that are involved in the inflammatory and immune response[98]. Further research has suggested NF-κB has a role in regulating cell proliferation, tumour development and cell transformation, with altered levels of NF-κB expression seen in a number of tumours[98]. Several studies have found NF-κB to have an anti-apoptotic function in breast cancer and hepatocellular carcinoma[99,100]. Conversely, inhibition of NF-κB may lead to cellular apoptosis[101]. It is now well recognized as having an important role in progression of cancer of the oesophagus[96,98]. In fact many studies have also demonstrated an over-expression by tumour cells of IL-8, a major product of the NF-κB pathway.

IL-8 is recognized as a unique protein that possesses dual roles in inflammation and carcinogenesis and is recognized to be directly up-

regulated by NF-κB activation[102], although additional hormone response elements and NF-IL-6 consensus site have also been characterized on the IL-8 gene promoter[103]. The biological effects of IL-8 are mediated through binding of IL-8 to two cell-surface G protein-coupled receptors, called CXCR1 and CXCR2. These two receptors have markedly distinct ligand-binding pharmacology, with CXCR1 being activated in response to binding of IL-8 and granulocyte chemotactic protein-2. CXCR2 is activated by multiple CXC-chemokines, including growth-related oncogenes (GROα, β and γ), neutrophil-activating peptide and granulocyte chemotactic protein-2[103].

Oesophageal damage from reflux demonstrates a well-defined progression from intestinal metaplasia, dysplasia through to oesophageal adenocarcinoma. In one study considering a wide range of oesophageal biopsies, a progressive increase in *IL-8* mRNA expression was found, corresponding to worsening mucosal injury, with the highest expression found in adenocarinoma[102]. Such findings would suggest that *IL-8* is associated with the progression of mucosal injury in GORD and the significant increase in IL-8 expression with the development of dysplasia would suggest a role for IL-8 mRNA levels as a biomarker for disease progression in patients with intestinal metaplasia[102]. Consequently as a marker for inflammation, IL-8 may provide a potential biomarker for similar refluxate changes in the larynx.

1.8.4.3 Interleukin 6

Similarly interleukin-6 (*IL-6*) is a multifunctional cytokine that plays a pivotal role in the acute inflammatory pathway. There is evidence that reflux induced damage in the oesophagus leads to increased gene expression of *IL-6*, consistent with an inflammatory state. A recent study considered changes in gene expression in oesophageal mucosal biopsies from patients, with non-erosive reflux and erosive reflux groups compared to controls[104]. Overall they found expression of *IL-6* was increased in both the non-erosive and erosive reflux groups. In addition, levels of *IL-6* increased according to the degree of reflux pathology, consistent with increasing inflammation. Similarly, following treatment, such levels decreased.

It would be reasonable to suggest similar patterns of gene expression would be responsible for mediating inflammation in LPR, which is proposed as having a similar aetiology, just at a more proximal anatomic site. However studies into inflammatory gene expression in LPR are scarce in the literature. One of the few studies investigating inflammatory cytokines in LPR did so comparing any change between pre- and post-treatment with a PPI following diagnosis by oesophageal manometry, 24-hour pH monitoring and videolaryngostroboscopy[105]. Taking tissue biopsies from the posterior commissure, changes in gene expression were measured in a number of common mediators of inflammation, including *IL-6*, *IL-8*, transforming growth factor-β 1 (*TGFβ-1*) and vascular endothelial growth factor (*VEGF*). They found no significant change in gene expression of inflammatory cytokines following a 10-week course of 20mg twice-daily dose of rabeprazole. Given

the lack of control group, it was difficult in this study to identify if there was a true increase in gene expression in the LPR patients prior to treatment. In addition, a 10-week course at such a dose may not be of sufficient length, or strength to provided a therapeutic benefit. Furthermore it highlights the difficulty in diagnosis of LPR for research purposes. Consequently the role of such acute inflammatory markers remains uncertain in LPR.

1.8.4.4 Transforming Growth Factor β - 1

Transforming growth factor β - 1 (TGFβ-1) belongs to a group of cytokines which have a diverse range of actions, including regulating epithelial cell growth, differentiation, motility, organization, apoptosis and tumourgenesis[106]. Its role in inflammation is complex, with some studies noting increased levels in fibroproliferative disorders of many organs, including airways and lung parenchyma[107]. In addition, a study investigating subglottic stenosis found TGFβ-1 was stimulated in tracheal injury, promoting the transformation of tracheal fibroblasts into myofibroblasts[107]. Furthermore, exposure to gastric juice promoted a similar transformation, suggesting that such refluxate may play a role in such stimulation. A study investigating the time of exposure to acid and pepsin in posterior commissure biopsies and false vocal fold found a significant relationship between time, level of pH and exposure to pepsin, with statistically significant changes in expression of *TGFβ-1* and *VEGFA* in the posterior commissure region. It is of interest to note that the false vocal fold was more sensitive to a pH effect in this study[108].

1.8.4.5 Carbonic anhydrase III

Carbonic anhydrase (CA) is an integral component of the intrinsic defence system and is effective by catalyzing the reversible hydration of carbon dioxide. This produces bicarbonate ions that are then actively pumped into the extracellular space where acidic refluxate can be neutralized. In the oesophagus this plays a significant role with carbonic anhydrase capable of increasing the pH of gastroesophageal refluxed residual acid from 2.5 to close to neutral[109].

There are eleven identified carbonic anhydrase isoenzymes[110], with demonstrated differences in activity, susceptibility to inhibitors and tissue distribution. CAI, II, III and IV have been demonstrated to be expressed by oesophageal epithelium and changes in distribution occur in inflamed oesophageal biopsy specimens.[110] CAIII has been noted to be both increased in expression and to undergo a redistribution from the basal to the suprabasal cell layers in inflamed oesophageal mucosa from patients with GORD[110,111]. It is thought that these changes are due to refluxate and represent attempts to counteract damage[112]. It has been proposed that an increase in CAIII expression may be due to basal cell hyperplasia, which is a histological sign of oesophagitis[110].

Recent research has demonstrated that CAI, II and III are present in normal laryngeal epithelial cells to a variable extent[110,113]. CAIII has been demonstrated in the squamous epithelial cells of the oesophagus and in the

posterior commissure area of the larynx[112]. CAI and CAII have been demonstrated in both the vocal cord and inter-arytenoid areas and CAIII throughout the laryngeal epithelium. Expression of CAIII in patients with laryngopharyngeal reflux is noted to differ between laryngeal biopsy locations[110]. In the presence of laryngopharyngeal reflux and pepsin in particular, CAIII expression in the vocal fold is potentially decreased allowing further damage to occur from acidic refluxate. Conversely CAIII may increase in the posterior commissure as a response to laryngopharyngeal reflux[74], with symptom severity correlating with CAIII levels[113]. Given the larynx possesses areas of respiratory type epithelium in addition to squamous epithelium, there remains the possibility that certain areas of the larynx may vary in response to laryngopharyngeal reflux, although a large scale study looking at epithelial type in all areas of the larynx in LPR is currently lacking.

1.8.4.6 E-cadherin

The cadherin family of molecules are calcium dependent cell-cell adhesion molecules and mediate homophilic adhesion. E-cadherin is recognized as having a crucial role in the maintenance of epithelial integrity and barrier function[112]. As such, damage to this barrier from refluxate may also lead to a breach of the mucosal barrier. Pepsin has been proposed to damage structures by digesting intracellular structures that maintain cohesion between cells[112]. Reduced levels of E-cadherin in response to laryngopharyngeal reflux have been demonstrated[114] and it is not clear

whether this down-regulation is due to the refluxate components, such as acid and pepsin, or as a result of an inflammatory response associated with laryngopharyngeal reflux.

Decreased expression of E-cadherin has been associated with poor prognostic factors in head and neck squamous cell carcinomas, including vascular invasion and decreased survival[115]. There is strong evidence that E-cadherin is a tumour suppressor, with the loss of E-cadherin being a key first step in tumour invasion[115]. Consequently decreased E-cadherin in the presence of laryngopharyngeal reflux may play a component in the development of laryngeal symptoms and may contribute to the development of laryngeal carcinoma in the setting of reflux.

1.8.4.7 Mucins

Mucus is a selective physical barrier covering the luminal surfaces of epithelial organs in the body. It forms a gel which protects the extracellular milieu on one side and the plasma membrane and cell interior on the other side. In addition to protection, mucus assists in lubrication and transport. Throughout the body mucus has specific functions. In the gastrointestinal tract it protects epithelial cells from autodigestion, where as in the cervix it protects the uterine cavity and controls the survival and penetration of spermatozoa[116]. Clearly, mucins provide multiple functions in a variety of environments and with interfaces varying from air, food, fluids and colonization with microorganisms. Such environments are susceptible to

diverse ranges of hydration, oxygenation, ionic concentration and pH[117]. It is hardly surprising then that there are currently 20 known mucin genes, which encode the backbone of mucins and of these, 16 have been identified in the airway[116]. They have a crucial role in maintaining homeostasis, promoting cell survival and regulate the local molecular microenvironment near the cell surface.

Mucins are high molecular weight glycoproteins and possess a protein backbone and oligosaccharide side chains. These side chains are attached by O-glycosidic linkages to either a serine or threonine reside. They are categorized into either secreted, or transmembrane (Table 1). Secreted mucins can further be classified into gel-forming mucins (such as *MUC2*, *MUC5A*, *MUC5B*) and non-gel-forming (MUC 7, *MUC8*).

There is evidence that inflammatory mediators and bacterial products may affect specific mucin gene expression. Interleukin-9 (IL-9) and 13 have been demonstrated to up-regulate mucus expression in airway inflammation and *MUC2* expression is up-regulated by tumour necrosis factor - α (TNF-α)[116]. Furthermore *MUC5B* expression has demonstrated to be up regulated by IL-6, *17* in addition to TNF-α.

Samuels et al.[118] studied laryngeal biopsies from a small group of patients with laryngopharyngeal reflux (n =3) and controls (n =2). Mucin 1-5, 7, 9, 13, 15, 16, 18-20 were detected in normal laryngeal epithelium, with 6, 8 and 17 absent. Of these, *MUC1* and *MUC4* were the predominant transmembrane

mucins and *MUC5AC*, *MUC5B* and *MUC2* the major constituents of airway mucus in the laryngeal epithelium. In the patients with laryngopharyngeal reflux, there was a decreased expression of *MUC5AC*, *-5B* and *-2*. This would lead to an overall decrease in mucin secretion from the laryngeal epithelium resulting in decreased protection from further reflux episodes. This is consistent with a gastroesophageal reflux disease model in which oesophageal mucin secretion was also reduced in patients with reflux oesophagitis[119].

Alterations in mucin expression have been identified in a variety of inflammatory conditions including gastritis, peptic ulcer disease and intestinal neoplasia and inflammatory bowel diseases. A lower level of *MUC3* expression has also been noted in laryngopharyngeal reflux patient samples[118]. *MUC3* has been noted to play an active role in epithelial cell restitution[120]. Specifically *MUC3A* mucins are thought to play a role in the maintenance of intestinal epithelium during hypoxic conditions[121] and modulating cell migration and apoptosis to promote wound healing[120].

Recently, mucins were suggested to be involved in the pathogenesis of cancer. Recent studies have indicated that *MUC1* and *MUC4* may modulate various pathways contributing to cell growth[122]. *MUC1* is known to be over-expressed in pancreatic and colon cancers and over 90% of breast cancers[123]. Multiple effects of *MUC1* on tumourgenesis are recognised. Firstly it is known to act as a natural ligand of galectin-3 in human cancer cells and the interaction between galectin-3 and cancer-associated *MUC1*

enhances cancer cell-endothelial adhesion, which may promote metastasis[124]. Secondly, as a transmembrane protein, its cytoplasmic tail binds with the ErbB family of growth factor receptor tyrosine kinases and potentiates ErbB-dependent signal transduction in the *MUC1* transgenic breast cancer mouse model. *MUC1* activation is thought to increase cell proliferation by activating extracellular signal-regulated kinases[122] and it plays a role in protection against oxidative stress-induced cell death[122].

One study found high expression of *MUC1* in patients with laryngeal dysplasia and cancer[125]. High expression was also reported in the "control" patients' larynx, however these samples were not from healthy controls. The role of *MUC1* in the context of laryngeal pathology remains unclear and further research is required to characterize *MUC1* expression in patients with laryngopharyngeal reflux through to laryngeal cancer.

MUC4 is expressed in epithelial surfaces of the oral cavity, eye, salivary glands and many other epithelial surfaces to protect and lubricate. In a retrospective analysis of laryngeal cancer specimens *MUC4* was identified in nearly half of the specimens[126]. In this study, the presence of *MUC4* was associated with a trend to better survival in patients with advance stage non-metastatic laryngeal cancer. In contrast other research has shown pancreatic, bile duct and lung cancers over-express *MUC4* and *MUC4* is associated with a poorer prognosis in these tumours[122]. Consequently whilst there are proposed mechanisms for tumour progression in other cancers, the role of *MUC4* in laryngeal cancer is still unclear.

Table 1: Mucin Genes in the Aerodigestive Tract

Gene	Localization	Primary Tissue Expression
MUC1	Transmembrane	Breast, pancreas
MUC2	Secreted	Jejunum, ileum, colon
MUC3	Transmembrane	Colon, small intestine, gallbladder
MUC4	Transmembrane	Airways, colon
MUC5AC	Secreted	Airways, stomach
MUC5B	Secreted	Airways, submandibular glands
MUC6	Secreted	Gastric stomach, ileum, gallbladder
MUC7	Secreted	Sublingual and submandibular glands
MUC8	Secreted	Airways
MUC9	Both	Fallopian tubes
MUC12	Transmembrane	Colon, airways, reproductive tract
MUC13	Transmembrane	Colon, trachea, kidney, small intestine
MUC15	Transmembrane	Colon, airways, small intestine, prostate
MUC16	Transmembrane	Ovarian epithelial cells
MUC17	Transmembrane	Duodenum, colon, stomach
MUC18	Transmembrane	Airways, lungs, breast
MUC19	Secreted	Salivary glands, trachea
MUC20	Transmembrane	Placenta, colon, lung, prostate liver

Modified from Samuels et al. 2008[118]

1.9 Treatment options for LPR

1.9.1 Lifestyle Factors

It has long been recognized that food and beverages which contain caffeine, such as coffee, teas and carbonated beverages, alcohol, chocolate and peppermints can weaken the oesophageal sphincter[127]. Even decaffeinated teas and coffees still contain enough caffeine to affect the sphincters. Acidic foods may also further complicate symptoms. The majority of foods have a pH range between 2.5 and 6.0. Foods with a pH above 4.6 are classed as low-acid foods, examples of which include meats, poultry, seafood, milk and many fresh vegetables. Franco[127] suggested that acid and spicy foods may directly irritate the throat lining, leading to inflammation and potentially providing re-activation of the pepsin deposited in the laryngopharynx from LPR.

Koufman[128] conducted a study on patients with LPR recalcitrant to treatment by PPI twice daily and an H2-receptor antagonist at night. The patients were placed on a strict low acid diet (defined as no food with a pH below 5), for 2 weeks, to 4 weeks. There was a statistically significant drop in both the RSI and RFS at follow up. The mean RSI dropped from 14.9 to 8.6, with the mean RFS dropping from 12.0 to 8.3. Koufman felt one of the main issues was the acidification of the American diet, related in part to the prevention of bacterial growth which prolongs shelf life of foods.

It seems reasonable to assume that reduction in food and lifestyle factors which promote reflux are likely to improve LPR symptoms. However there are few studies confirming this. Koufman's study highlights that there are likely to be other, possibly confounding factors which impact on symptoms in patients with LPR and few studies have considered dietary factors in the management of LPR.

1.9.2 Proton Pump Inhibitors

Given the proposed aetiology of LPR, and the close association with GORD, the use of acid suppression treatment has had a significant role in the management of the disease. However such associations are difficult to confirm, as even pH probe demonstrated reflux, whilst confirming the presence of refluxate, does not truly prove causality and in addition such studies have not predicted response to acid-suppression treatment[129].

Despite this, a proton pump inhibitor is typically commenced in the clinical situation, with a satisfactory response being therapeutic for the patient and also considered diagnostic of LPR. Research investigating the efficacy of PPI use in LPR is still widely variable, and probably reflects the heterogeneous clinical response to treatment and the likely wide spectrum of disease. Furthermore the presence of abnormal proximal reflux on pH monitoring does not predict response to therapy[129].

Altman et al.[130] reviewed published clinical practice guidelines on the management of GORD and LPR, identifying 13 key articles, with the majority of experts agreeing that empirical treatment with PPI is the current recommendation, however the dose and the length of treatment is widely debated. Despite this, a meta-analysis reviewing eight randomised controlled trials found a non-significant symptom reduction compared to placebo.

Furthermore, the length of time required to treat successfully and the rate of response is still unknown. Generalised laryngeal oedema and erythema have been described as responding to appropriate medical therapy, however there has been no significant change in the degree of posterior commissure pachyderma in some studies PPI treatment for LPR, even up to over 3 years of treatment[131]. It has been proposed that this may represent an irreversible histopathological transformation. It has been described that exposure to gastric secretions in this area can promote epithelial hyperplasia of the prickle and basal cell layers and some keratinisation[27]. Consequently it may represent that the patient has been exposed to LPR, however may not currently be suffering. Furthermore the question remains on whether the symptoms are due to the reflux event itself, or due to these histopathological changes.

In addition there are increasingly recognized side effects of PPI use. PPIs are not without their problems with a number of adverse consequences reported in the literature. A large (n = 13556) case control study suggested

that long term use of PPI significantly increased the risk of hip fracture (AOR: 2.65), with the strength of association increasing with duration of therapy[132]. The proposed mechanism for this was still uncertain, but may be related to the change in acidity of the stomach environment inhibiting the release of ionised calcium from insoluble calcium salts.

Furthermore, there is evidence that ongoing use of PPIs may change the distribution of gastrointestinal tract defences, with use for greater than one month relaying a ten-fold increase in the risk of developing Campylobacter related diarrhoea, an increased risk of Salmonella infection and of the development of Clostridium difficile in hospitalised patients[133].

In summary, PPI use is considered the mainstay of treatment for LPR, however there are a number of issues related to its use. Firstly, there is a treatment group which remains unresponsive to PPI use. Research into the characteristics of this group is still pending, and likely to be awaiting a satisfactory diagnostic test and the ability to monitor treatment outcomes. Secondly the use of PPI, whilst providing an improvement in symptoms in a significant population group, still possess risk in prescribing and as such should be utilised judiciously. Lastly, the time required to provide relief remains yet to be determined.

1.9.3 Alginates

Other management strategies studied includes the use of liquid alginate suspension. Liquid alginates have been utilized for the treatment of gastric reflux for many years and are thought to work by providing a mechanical anti-reflux barrier within the fundus of the stomach[134]. One study compared the use of 10mL of Gaviscon Advance with a non-treatment group. Treatment outcomes were measured using the RSI and RFS at 2, 4 and 6 months[134]. Whilst there was a significant improvement in RSI and RFS scores from the commencement of the study to month 2, and 6 months in the treatment group, there was also a statistically significant improvement in RSI from commencement to both month 2 and 6 for RSI in the non-treatment group. It was notable that there was no statistically significant improvement in RFS for the non-treatment group. This would indicate that there may be a symptomatic improvement, without any significant change in examination findings. This may be due, in part, to a placebo type effect, or represent symptomatic improvement predating a significant change in RFS. Despite this, there was evidence that treatment with Gaviscon Advance demonstrated statistically significantly greater improvement in RSI than no treatment after 2 months and 6 months. Further research is required to determine its role either as a sole agent, or together with a PPI in LPR.

1.9.4 Antidepressants

Selective serotonin reuptake inhibitors (SSRIs) such as citalopram 20mg, have been demonstrated to prolong the acid perfusion time to induce a

perception of heartburn. Additionally they have been described as being highly efficacious in patients with non-cardiac chest pain of presumed esophageal origin[135]. Tricyclic antidepressants have also been considered similarly[135]. Whilst commencing such medication may provide symptomatic relief, surely the damage afforded to the area of reflux would still be occurring. Additionally given GORD typically responds to short courses of anti-reflux medication, it may well be the case that LPR, which we know often requires much longer term treatment, may not respond to this at all. This may be the case given the acid bolus is the sensitising agent in oesophageal reflux and a delayed time to perception would allow the esophagus with its typical protection systems, time to clear the bolus. The pathophysiology of LPR, with its more insidious mechanism, may not prove responsive to such management and the utilization of such medications has been hypothesized, but is not routine.

1.9.5 Surgical

Fundoplication has been recognized as providing a significant improvement in patients with typical esophageal reflux symptoms. However patients with LPR, by definition fall into the atypical symptomatic population. Consequently there remains uncertainty to the level of improvement following fundoplication for this population. Undoubtedly there are subgroups of patients with symptoms who would demonstrate greater improvements than others. There is a well described and understood mechanism for typical symptoms of esophageal reflux. Equally much less is

known about the pathophysiology for these atypical symptoms and consequently the results of both medical and surgical managements have been difficult to qualify, however some studies have proposed that patients with LPR were a favourable group for successful surgery for reflux[136]. There seem to be some factors which make improvement following surgery more likely.

Similar to patients with GORD, those with LPR symptoms, who demonstrated a response to pre-operative trial blockers of a PPI, also noted improvement following fundoplication[137]. Such improvement was measured by a greater than 3 point improvement on a 10 point symptom questionnaire[136]. In addition, patients who demonstrated acid above the cricopharyngeal level, demonstrated by dual probe pH testing, also demonstrated success following fundoplication[136]. Additionally, other studies have suggested that pharyngeal pH monitoring may predict patients most likely to benefit from surgical therapy[136,137].

Conceptually fundoplication as the management of LPR should provide greater improvement than acid suppression alone, given the significance of pepsin in refluxate. Fundoplication should significantly reduce the volumetric bolus to the larynx, providing greater defence than PPI alone. However, a recent review of 893 consecutive patients following fundoplication found there was variability in improvement. Patients presenting with both throat and classical GORD symptoms had a similar improvement overall to those with just classic GORD symptoms. However, patients presenting with only

throat symptoms and with objective evidence of reflux on 24-hour pH probe monitoring had a significantly poorer outcome, with less than 50% of patients improving following surgery[138].

Such heterogeny in treatment outcomes, whether it be PPI use, or fundoplication, implies a wide spectrum of disease and the likelihood of LPR sub-groups. At present there is not sufficient understanding of the pathophysiology to categorise patients, however this may become important in selecting a successful treatment modality in the future.

1.10 Conclusions

Despite the abundance of research literature, there are many significant controversies regarding LPR. It is still not known whether LPR is a laryngeal symptom extending from GORD, or whether it is a separate clinical entity. Some experts are suggesting the latter, given a different pattern of symptoms, with the presence of an upright reflux pattern and typically normal oesophageal motility testing in patients with LPR[130]. Additionally, there is significant discord on what actually constitutes abnormal pharyngeal exposure to refluxate, with the sensitivity of pH testing reported to be as low as 40%[139,140].

Previous literature supports a variability in mucosal response within the sites of the larynx, with the most commonly biopsied site typically being the posterior commissure and the vocal fold. A recently published study highlights the difficulty in the clinical diagnosis of LPR, particularly on identifying mucosal signs alone and calls for further research into mucosal changes in LPR[65]. In addition, given the histology of the larynx varies according to the sub-site, no single study has considered mucosal changes in all sub-sites of the human larynx.

1.11 Hypotheses

- The mRNA expression of inflammatory and mucosal defence genes will differ between patients clinically diagnosed with laryngopharyngeal reflux and a control population.

- The mucosal expression patterns will vary according to the laryngeal sub-site between patients clinically diagnosed with laryngopharyngeal reflux and a control population.

1.12 Aims

Consequently the aims of this project were to determine if there is a group of molecular markers which are altered in patients with LPR compared with a non-refluxing population. Secondly, this study aims to further identify molecular differences in the sub-sites of the larynx. Previous literature has noted differing responses throughout the larynx[33,74,112] often focusing on the posterior commissure and vocal fold. Bulmer et al.[33] noted a variability throughout the sub-sites of porcine larynges using histological analysis, measures of optical density and a DNA assay to quantify tissue damage. However no study has previously considered all aspects of the human larynx.

Such markers, or set of markers, together with a specific laryngeal location, may provide a mechanism for monitoring the response to treatment and potentially identifying those patients that may fail empirical management.

2 CHAPTER 2: METHODS

2.1 Patient Recruitment

Ethical approval was sought and provided by the Southern Adelaide Clinical Human Research Ethics Committee (SAC HREC). Patients were recruited from those referred to the ENT Department at a tertiary teaching hospital and associated private consulting rooms with a clinical history (including hoarseness, chronic cough, globus pharyngeus) and examination suggestive of LPR, and undergoing a panendoscopy or microlaryngoscopy under general anaesthetic. Given the possibility that chronic irritation from refluxate may lead to laryngeal cancer[48], patients with suspected malignancies were also identified and included in the initial patient recruitment group.

Control patients were recruited from patients on elective surgery waiting lists from Otolaryngology, Orthopaedic and General Surgery units. Patients were included if they had no clinical history or examination findings consistent with reflux. Additionally if they had a history of upper aerodigestive tract inflammatory conditions they were excluded from the control population. For ethical reasons the examination and collection of research biopsies in the control group could not alter the type of anaesthetic they would receive for their primary procedure, including type of endotracheal tube. Consequently patients undergoing procedures in which the anaesthetist would typically utilise a supraglottic airway, such as a laryngeal mask airway (LMA) were excluded from the study due to being unable to assess and biopsy the

larynx. Other exclusion criteria included being under the age of 18, or over 80 years of age or a patient decision to withdraw from the study.

Patients were contacted prior to their day of surgery for their primary procedure, and consented for laryngeal examination and collection of biopsies from the 4 sub-sites of the larynx. Patients were given the Reflux Symptom Index (Appendix 1) to complete prior to their procedure.

Laryngoscopy and collection of tissue biopsies were typically conducted by either myself, or the overseeing consultant ENT surgeon. Laryngeal examination was conducted under general anaesthetic utilizing appropriate scopes, typically Storz Lindholm (Figure 7), or Kleinsasser laryngoscopes, with suspension where available. Assessment of the larynx was conducted utilizing the previously validated Reflux Finding Score[57] (Appendix 2).

Tissue biopsies were taken from the 4 sub-sites with 2mm cupped microlaryngoscopy biopsy forceps (OP304R: B Braun/Aesculap, Melsungen, Germany) (Figure 8). Tissue biopsies were collected in a standardized order, commencing with the true vocal cord (away from the vibrating edge to avoid any impact on voice), then the false vocal cord, the medial arytenoid and finally the posterior commissure (Figure 9). Separate biopsy forceps were utilized to collect each sample to avoid contamination from each site. If there was evidence of a likely tumour, then biopsies were taken of the lesion for diagnostic histopathology, and samples taken of the lesion and adjacent the lesion for research purposes. Haemostasis was achieved with

temporary placement of neuropatties soaked in 1:10 000 adrenaline solution on the mucosa.

2.2 Tissue Storage

Biopsy samples, averaging 0.0015g, were placed in RNA*later*® (Ambion USA), ensuring there was greater than 5 volumes of RNA*later* per tissue sample. The tubes were labeled and catalogued according to site. These were stored at 4°C overnight, as per the RNA*later* manufacturer's protocol, before being stored at -20°C for later analysis. A representative piece, approx 1/3 the size of the specimen was cut to send for histology. If the specimen size did not allow sufficient material for histological examination, the entire specimen was kept for RNA processing only.

2.3 Patient Groups

Patients were separated into 3 groups. Patients with a clinical history and examination consistent with LPR, and an RSI greater than 12, and RFS greater then 6, were classed as the LPR group[57,60]. Patients with no history or examination findings consistent with reflux, were classed in the non-LPR group. The third group of patients were an intermediate group, however given the diagnostic difficulty with LPR, this middle group, with an uncertain diagnosis, were not included in the analysis.

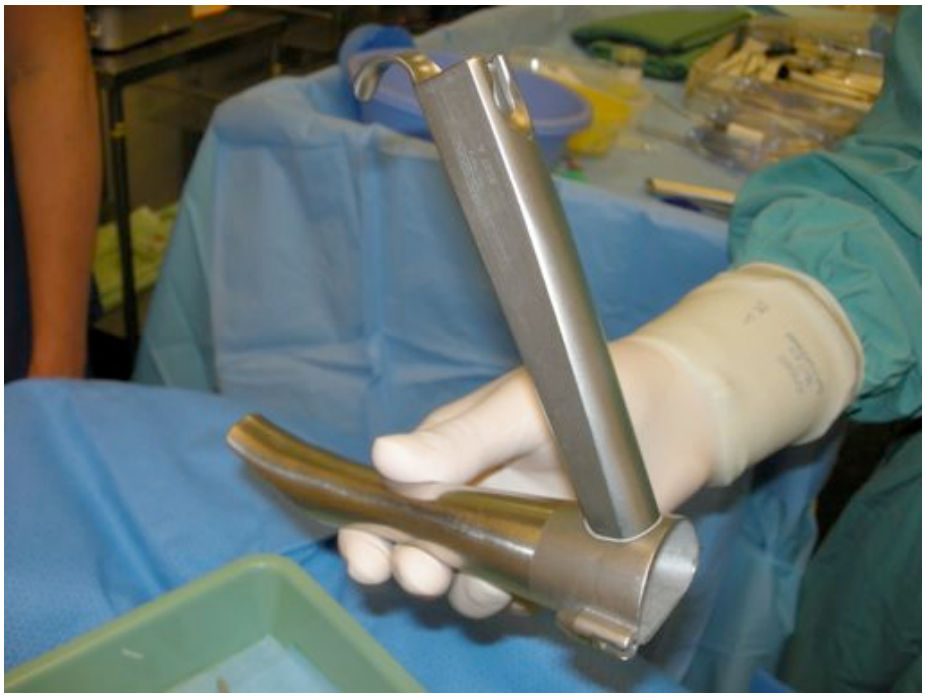


Figure 7: Lindholm Laryngoscope

(author's own photograph)

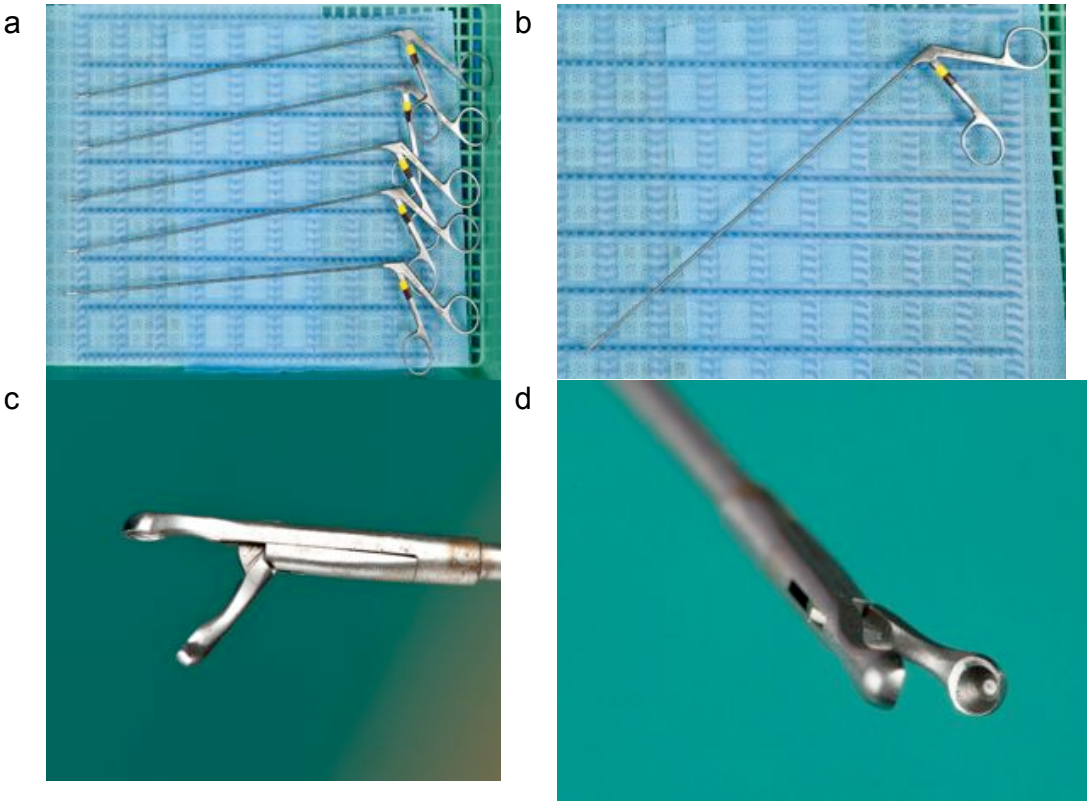


Figure 8: Microlaryngeal 2mm Cupped Forceps

a) research biopsy set, b) individual 2mm cupped forcep, c) & d) close up of cupped forcep tip.

(Photography courtesy of Flinders Medical Centre Dept of Medical Illustration)

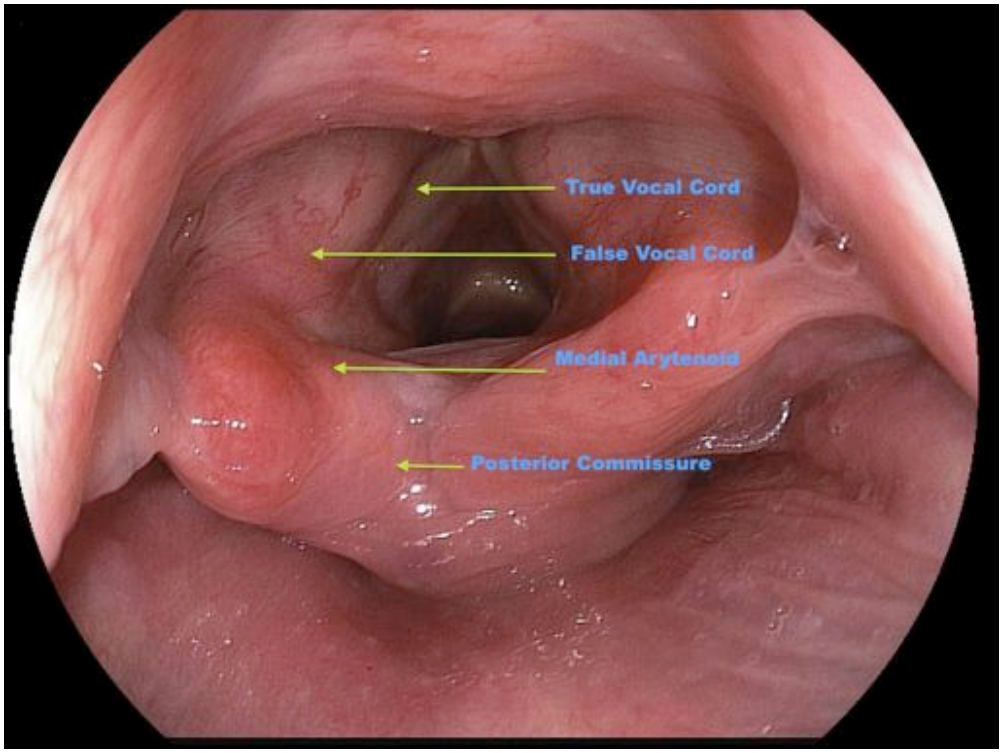


Figure 9: Position of Biopsy Collection

Adapted from Gastrolab, 2012[22].

2.4 Histological Analysis

A portion of each specimen, where possible, was sent for histological analysis. The samples were embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H and E). These were analysed by an expert histopathologist, who was blinded to the site and group of the sample. Samples were assessed for tissue type (squamous or columnar), evidence of metaplasia or dysplasia, and inflammation

2.5 QIAzol RNA Extraction Protocol

The QIAzol RNA extraction protocol was utilized based on the recommendation by a QIAGEN technical development representative, to ensure the downstream integrity for the real-time RT-PCR. All materials were supplied in QIAGEN miRNeasy Mini Kit (Catalogue No. 217004, QIAGEN, Valcencia, CA, USA), RNA extraction was conducted according to the manufacturer's protocol (QIAGEN, Valencia, CA, USA).

Samples were thawed, and weighed prior to extraction. RNA isolation commenced with homogenization of tissue in 700μL of QIAzol (QIAGEN, Valencia, CA, USA), using a plastic pestle attached to a vertical Dremel rotary drill (Dremel, Illinois, USA). Chloroform was added, enabling separation of aqueous and organic phases when centrifuged, with minimal protein and DNA contamination of the RNA.

The upper aqueous phase, containing the RNA, was collected, whilst the remaining interphase and organic phase containing the genomic DNA and cellular debris and proteins, were stored at -80°C for later use, if required. The aqueous phase was mixed with 100% ethanol to provide appropriate binding conditions with the silica-gel membrane when added to the RNAeasy spin column (QIAGEN, Valencia, CA, USA). Buffer RWT (QIAGEN, Valencia, CA, USA), a buffer containing guanidine and ethanol was washed through the column via centrifuge, assisting in protein removal, and enhance binding of the RNA. As the downstream analysis would be sensitive to very small amounts of DNA, QIAGEN recommend the utilization of the DNase digestion protocol. DNase incubation mix was added to the RNA bound to the membrane on the column, and incubated at 25 - 30°C for 15 minutes. The DNase incubation mix was then again washed through with Buffer RWT via centrifuge.

Buffer RPE (500μL), a buffer high in ethanol content, was added to the column and washed through via centrifuge to remove further salts from the column. The RNA was then eluted by adding 30μL of RNase free water, into which it readily dissolves, and centrifuged. From this solution of 30μL, 1μL was removed and placed in an Eppendorf tube for the spectrophotometric assessment of the resulting RNA concentration. The remaining RNA sample was then stored at -20°C for later processing.

2.5.1 Spectrophotometric Assessment of RNA Concentration

The RNA concentration of each sample was measured using a Biophotometer (Eppendorf, North Ryde, NSW). The Biophotometer was set for RNA, with a dilution factor of 1/60, measuring wavelengths at 230, 260, 280 and 320 nm.

2.6 RNA Bioanalysis

It is well recognised that the accuracy of gene expression quantification is influenced by quality of the RNA, with potentially low quality RNA compromising the results of downstream processes such as RT-PCR[141]. Consequently RNA quality was assessed using the Agilent RNA 6000 Pico Assay Protocol (Agilent Technologies, Waldbronn, Germany). For analysis on the Pico-chip samples were first prepared to achieve a concentration of 2.5ng/μL using ultra pure water, and then processed according to the manufacturer's protocol. Results were quantified as a RNA Integrity Number (RIN). In keeping with previous research[141,142], samples with a RIN higher than 5 were considered to be of suitable quality for downstream analysis. Samples with a RIN lower than 5 were not utilized for further analysis.

2.7 Quantitative Real Time Reverse Transcription Polymerase Chain Reaction Analysis

2.7.1 cDNA Synthesis

Complementary DNA (cDNA) derived from messenger RNA (mRNA) is utilized for quantitative PCR analysis. cDNA was prepared utilizing the RT^2 First Strand Kit (Catalogue No. 330401, QIAGEN, Valcencia, CA, USA), following the manufacturer's protocol. Where possible as close to 320ng of RNA (total volume 10uL) was incorporated in each reaction and the Thermocycler (Eppendorf, North Ryde, NSW, Australia) was used for incubations. The cDNA was diluted to a final volume of 111uL and stored at 4°C until required.

2.7.2 RT^2 SYBR Green Mastermix

All RT^2 SYBR Green Mastermix vials (QIAGEN, Valencia, CA) were pooled to ensure uniformity of reagents throughout the entire protocol. To prepare individual sample mastermix solutions, 52μL of sample cDNA was added to 260μL of RT^2 SYBR Green Mastermix and 208μL of ultra pure water. This 'sample master mix' provided enough prepared template for all reactions on each array. All Mastermix samples were frozen to ensure consistency with only one freeze/thaw cycle per sample.

2.7.3 Polymerase Chain Reaction

A commercially prepared Custom RT^2 Profiler PCR Array (SABiosciences, Corporation, Frederick, MD, USA) for the genes of interest was utilized (Table 2). Where possible all four samples from each patient were analysed on the one array. A number of duplicate reactions were also conducted where space permitted on an array ring to ensure inter-array reliability. PCR Mastermix samples were thawed for each PCR run. As per the manufacturer's protocol, 20μL of PCR Mastermix was pipetted into each well. Real-time PCR was performed using the Rotor-Gene 6000 (Corbett Life Science, Sydney, NSW, Australia), commencing at 95°C for 10 minutes, then 40 cycles of 15 seconds at 95°C then 30 seconds at 60°C. Melt analysis occurred from 65°C–95°C rising by 1°C/4 sec.

2.8 Quantitative RT PCR Analysis

The cycle take off data and melt curve data were reviewed using Rotor-Gene Software (Version 1.7; Corbett Life Sciences, Sydney, NSW, Australia). Take-off values for the positive and negative controls were analysed to ensure the assays performed satisfactorily and genomic DNA contamination was not a concern. Melt curves for every gene were analysed to ensure amplification of a single product for each assay. Quantitative real-time RT-PCR analysis was then performed using Q-Gene software[143]. The expression of each gene was normalized to the commonly used housekeeping gene HPRT[144]. GraphPad Prism Version 5.0b for Mac

(GraphPad Software, San Diego California, USA, www.graphpad.com) was used for statistical analysis, using the Mann Whitney U test, with a *p* value less than 0.05 considered significant.

Table 2: PCR Array Genes

Background	Gene marker	Gene Abbreviation
Squamous/Columnar changes	Cytokeratin-8	*KRT8*
	Cytokeratin-14	*KRT14*
Inflammation	Interleukin-6	*Il-6*
	Interleukin-8	*IL-8*
	Cyclooxygenase-2	*PTGS2*
	Cornulin/Squamous epithelial heat shock protein 53	*CRNN*
	Antigen-presenting glycoprotein CD1d	*CD1d*
	Vascular endothelial growth factor A	*VEGFA*
	Transforming Growth Factor β-1	*TGFβ-1*
	O-6-methylguanine-DNA methyltransferase	*MGMT*
Mucosal Defences	Mucin 1	*MUC1*
	Mucin 2	*MUC2*
	Mucin 3B	*MUC3B*
	Mucin 4	*MUC4*
	Mucin 5B	*MUC5B*
	Mucin 6	*MUC6*
	Mucin 7	*MUC7*
	Carbonic anhydrase-III	*CA3*
	Epithelial-cadherin	*CDH1*

3 CHAPTER 3: RESULTS

3.1 Demographics

56 patients were consented for microlaryngoscopy and research biopsy collection, with the age ranging between 20 and 84 years of age, with a median age of 50 years. Of these there were 31 males and 25 females. Of these 10 patients had no tissue collected at the time, due to either a laryngeal mask airway being utilised, or in one case, an inability to successfully intubate the patient.

Patients (n = 10) with a history and examination findings consistent with LPR, and RSI greater than 12, and RFS greater than 6 were included in the LPR group. The ranges for these patients for the RSI lay between 12 and 45, and for the RFS between 6 and 17. Five patients with other pathology, such as a diagnosis of laryngeal carcinoma, were excluded from the group given another pathology was likely contributing to at least the RSI. Of these, 4 were smokers or ex-smokers. The control group (n = 9) consisted of patients with no history or examination findings consistent with LPR, and RSI and RFS scores below the above cut off. Non of these patients were smokers. In practice the RSI for these patients ranged from 0 to 6, well below the cut off for inclusion in the LPR group, and the highest RFS being 5 (although in a patient with an RSI of 0). The median RFS score for this group was 1.5, and was 4 for the RSI. The remaining patients remained in an intermediate group, in which patients had either elevated RSI or RFS.

The average age in the LPR group was higher than the non-LPR group, which was significantly different (p = 0.007) (Table 3). There was higher ratio of males to females in the LPR group also. There was a significant difference for the RSI scores (p < 0.0001) and the RFS scores (p < 0.0001) between the LPR and the non-LPR group.

3.2 Histological Analysis

Not all of the samples were of adequate size to be sectioned for histological analysis. Of all the 78 samples processed, 52 samples were of adequate size to be sectioned, and categorized in the LPR or non-LPR group. Histological assessment by an expert pathologist identified sub-site specific tissue types in the larynx (Table 4). The medial arytenoid and false vocal cord sub-sites in the non-LPR group were most often found to contain columnar epithelium (Figure 10). In the LPR group, these two areas were more likely to demonstrate squamous epithelium. The posterior commissure region was the only area to histologically demonstrate hyperplasia in 3 of the specimens. In addition, there was histological evidence of metaplasia in the medial arytenoid region in the LPR group (Figure 11). Both LPR and non-LPR group demonstrated evidence of inflammation histologically (Table 5). No samples demonstrated any evidence of dysplasia.

Table 3: Demographics

	Non-LPR group (n = 9)	LPR Group (n = 10)	*p* value
Average Age	38	55	*p* = 0.007*
Male/Female	3/6	12/6	*p*= 0.1**
Average RSI	3.6	19.5	*p < 0.0001**
Average RFS	2	10.3	*p* < 0.0001*

Demographic data with statistically significant differences highlighted with * in red box, "*p*" values for Mann Whitney U test.

** "*p*" values for Chi-square test.

(RSI = Reflux Symptom Index; RFS = Reflux Finding Score)

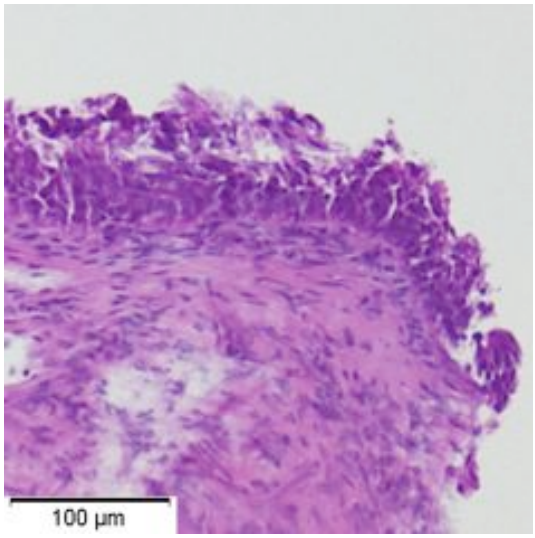
Table 4: Histological Analysis

	Non-LPR Group			LPR Group		
	Column	**Squam**	**Mix**	**Column**	**Squam**	**Mix**
Medial Arytenoid	5	1	0	2	4	2
False Vocal Cord	5	0	0	4	4	1
True Vocal Cord	1	2	2	2	4	1
Posterior Commissure	0	3	0	0	9	0

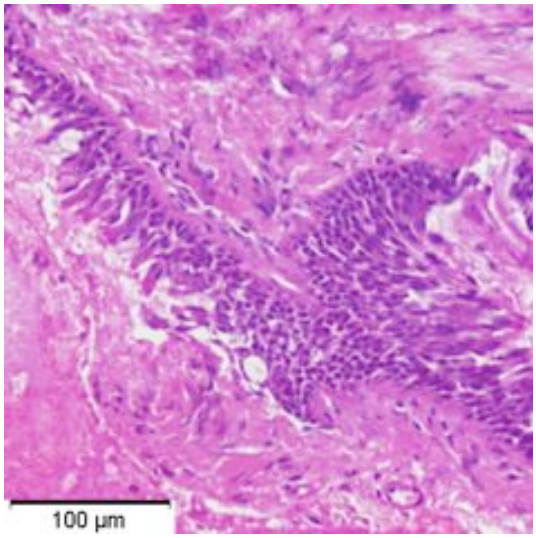
Column = columnar epithelium; Squam = squamous epithelium, Mix = mixed

Table 5: Histological Evidence of Inflammation

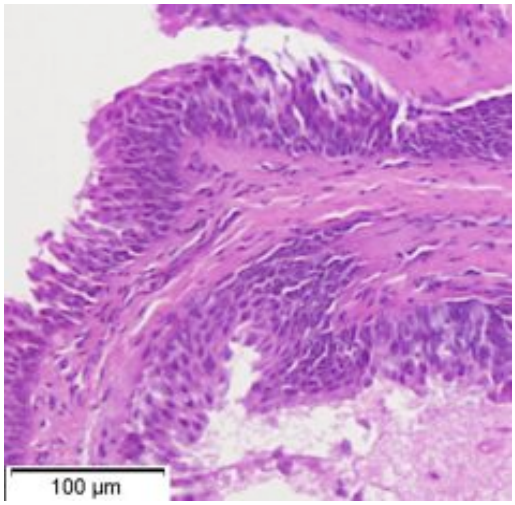
	Non-LPR Group			LPR Group		
	-	+/-	+	-	+/-	+
Medial Arytenoid	1	4	1	0	2	1
False Vocal Cord	0	1	1	0	3	0
True Vocal Cord	0	0	0	0	1	1
Posterior Commissure	0	0	0	0	1	1



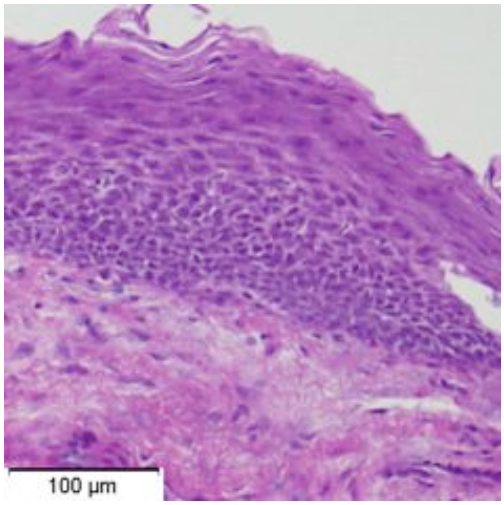
a) Medial arytenoid columnar epithelium



b) False vocal cord columnar epithelium



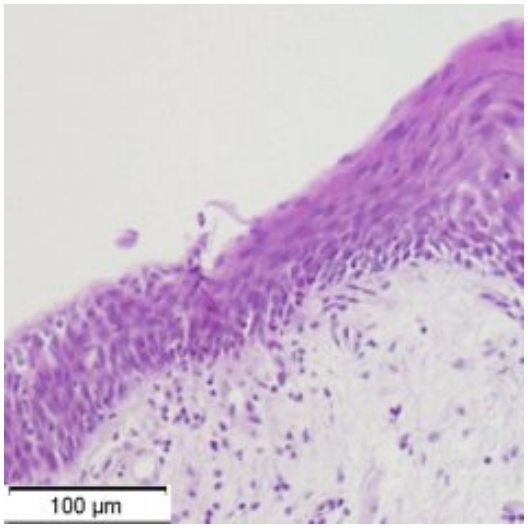
c) True Vocal cord squamous epithelium (and columnar)



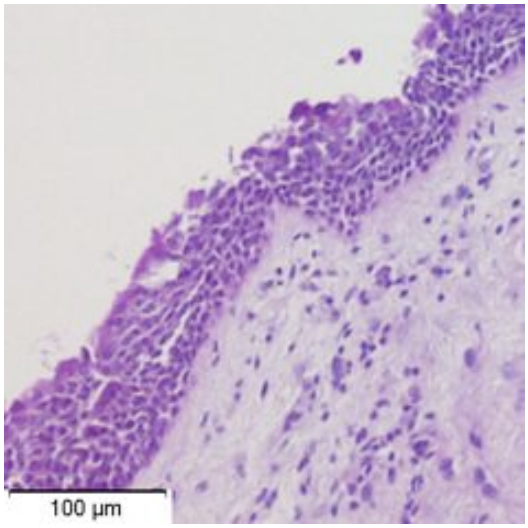
d) Posterior commissure squamous epithelium

Figure 10: Histology of all 4 regions in a Non-LPR patient

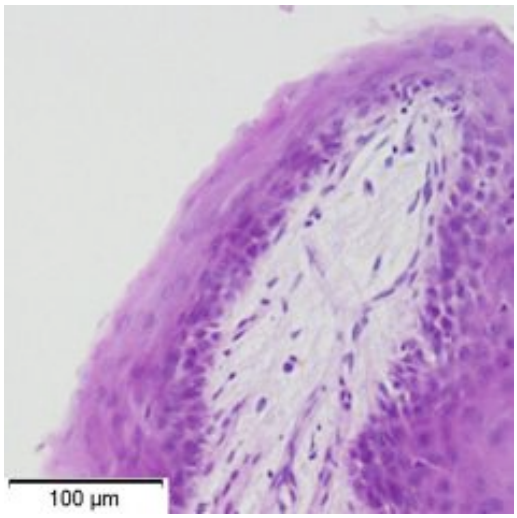
Histology of biopsies from Patient Number 006: RSI 5, RFS 2.
Microscopy at 100µm



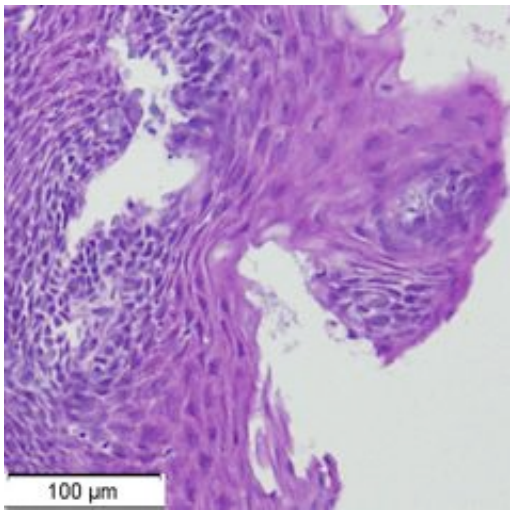
a) Medial arytenoid columnar to squamous metaplasia



b) False vocal cord columnar epithelium



c) True Vocal cord squamous epithelium



d) Posterior commissure squamous epithelium

Figure 11: Histology of all 4 regions in an LPR Patient

Histology of biopsies from Patient Number 15: RSI of 23, RFS of 11. Microscopy at 100µm

3.3 PCR Analysis

Biopsy samples were analysed for LPR (n = 10) and non-LPR (n = 9) groups. Following RNA isolation, quantitative real-time reverse transcription PCR was conducted. The melt curves and take-off values for each PCR plate were analysed to confirm amplification of a single PCR product with the same melt curve profile in reactions from the tissue samples, which was not present in the negative control reactions. The housekeeping genes from each plate were analysed. Human Genomic DNA Control (HGDC) determined non-transcribed genomic DNA contamination, Reverse Transcription Controls (RTC) tested the efficiency of the RT2 First Strand Synthesis, and Positive PCR Controls (PPC) tested the efficiency of the reaction itself. The latter of these two confirmed inter-well and intra-plate consistency. No significant inconsistency was identified.

HPRT was used as a housekeeping gene, as it has similar gene expression in all cells. There was no significant difference in gene expression of *HPRT* in each laryngeal sub-site between control and LPR groups (Table 6). Consequently *HPRT* was utilized to normalize all the genes of interest to calculate relative expression.

Table 6: Statistical Analysis of HPRT Gene Expression

	p -value
Medial Arytenoid	0.717
False Vocal Fold	0.949
True Vocal Fold	0.450
Posterior Commissure	0.328

"p-values" for two-tailed Mann Whitney U test for *HPRT* Gene expression between LPR and non-LPR groups.

Data graphs displaying the relative expression for LPR and non-LPR groups are presented for each gene in Figure 12 to Figure 31, with statistical comparisons presented in Table 7. There was no significant difference in gene expression for *IL-6*, *IL-8*, *PTGS2* and *MGMT* between the LPR and non-LPR group. Expression of *KRT14* demonstrated a significant difference between the LPR and non-LPR groups, with higher levels of expression in the LPR group, however this was confined to only the medial arytenoid (p = 0.015) and posterior commissure (p = 0.030) sub-sites of the larynx.

Differences in mucin gene expression varied between genes. *MUC1*, *4*, *6* and *7* demonstrated no significant difference between the LPR and non-LPR groups throughout all the sub-sites of the larynx. *MUC3B* demonstrated a trend towards lower expression in the medial arytenoid in the LPR group (p = 0.084), however this was not statistically significant. Statistically significant differences in expression were observed for the secretory mucins, *MUC2* and *MUC5B.* The medial arytenoid region demonstrated a significant difference in gene expression between LPR and non-LPR patients for both *MUC2* (p = 0.0020 and *MUC5B* (p = 0.0013) with lower expression noted in the LPR group. There was no significant difference in expression of *MUC2* in the other sub-sites of the larynx. In addition to the medial arytenoid, expression of *MUC5B* was significantly lower in the LPR group, compared to the non-LPR group in the posterior commissure (p = 0.041) but not the remaining sub-sites.

CA3 expression demonstrated a trend to be lower in the false vocal cord region of the larynx in the LPR group (p = 0.086), however this did not achieve statistical significance. The medial arytenoid, true vocal cord and posterior commissure did not demonstrate any significant difference in expression of *CA3*.

Expression of *CRNN* was significantly higher in the medial arytenoid region of the larynx in the LPR group (p = 0.007), however there was no significant difference in the other sub-sites. The medial arytenoid also demonstrated significant differences in *CD1d* (p = 0.024) and *TGFß1* (p = 0.042), both demonstrating lower in expression in LPR patients. The remaining sub-sites of the larynx did not demonstrate any significant differences. Expression of *CDH1* was significantly higher in the LPR group compared to the non-LPR group noted in the medial arytenoid (p = 0.049). This elevation was not noted in the remaining sub-sites of the larynx.

Overall, the medial arytenoid and posterior commissure both demonstrated statistically significant differences in gene expression between the LPR and non-LPR groups. It is of note that the medial arytenoid sub-site demonstrated the most variations in gene expression in the LPR group.

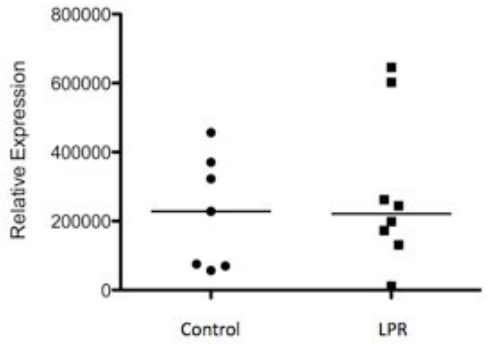
The outliers for each of the genes were assessed, patient records were reviewed to ensure that there was no confounding factors contributing to this. The outliers noted in the VEGFA were noted to be of a high expression, and occurred in both the LPR and non-LPR groups. Further analysis of

these outliers identified that within each group they were not related to individual patients and additionally such changes were not consistent throughout all genes.

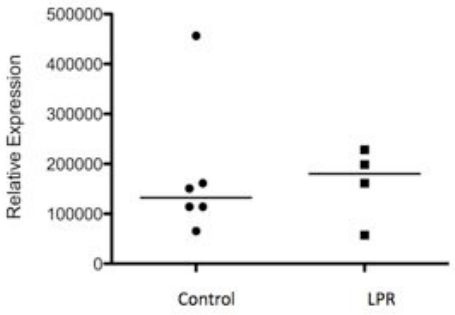
Table 7: Gene Expression Statistical Analysis

Background	Gene	Medial Arytenoid	False Vocal Cord	True Vocal Cord	Posterior Comm.
Squamous/ Columnar	***KRT14***	0.015*	0.165	0.714	0.030*
	KRT8	0.247	0.391	0.537	0.623
Inflammation	***IL-6***	0.536	0.476	0.662	0.539
	IL-8	0.613	0.285	0.360	0.902
	PTGS2	0.772	0.914	0.360	0.110
	CRNN	0.007*	0.114	0.931	0.303
	CD1d	0.024*	0.610	0.714	0.540
	MGMT	0.181	0.450	0.310	0.935
	TGFβ-1	0.042*	0.453	0.712	0.806
	VEGFA	0.450	0.109	0.314	0.566
Mucosal Defences	***MUC1***	0.450	0.762	0.926	0.805
	MUC2	0.002*	0.476	0.931	0.653
	MUC3B	0.084	0.200	0.464	0.221
	MUC4	1.00	0.521	0.781	0.326
	MUC5B	0.013*	0.352	0.792	0.041*
	MUC6	0.954	0.454	0.178	0.712
	MUC7	0.613	0.762	0.178	0.653
	CA3	0.600	0.086	0.082	0.288
	CDH1	0.049*	0.914	0.855	0.367

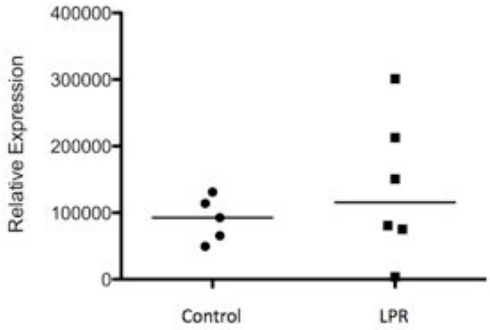
: *p* values for two-tailed Mann Whitney U test for difference in gene expression between LPR and non-LPR groups normalized to HPRT. "*" results highlighted in red statistically significant.



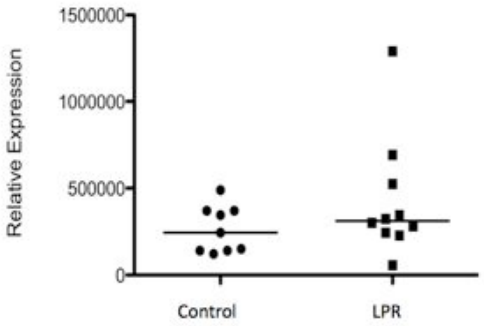
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

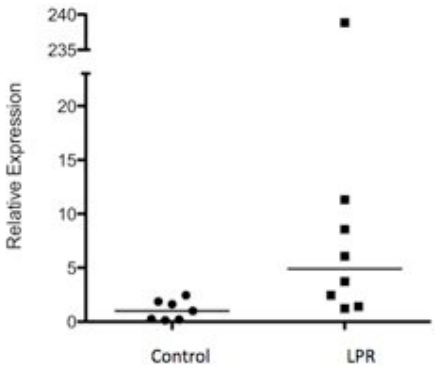


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

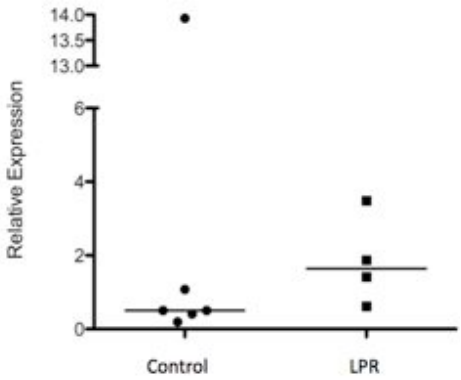


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

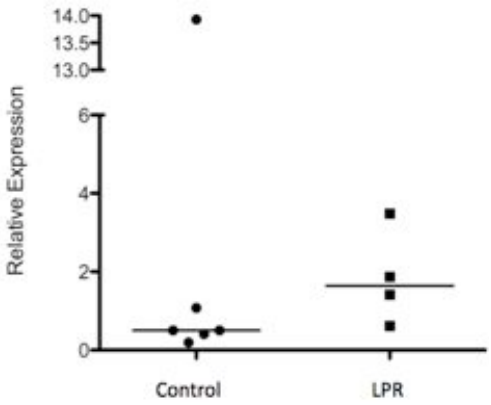
Figure 12: HPRT Relative Expression



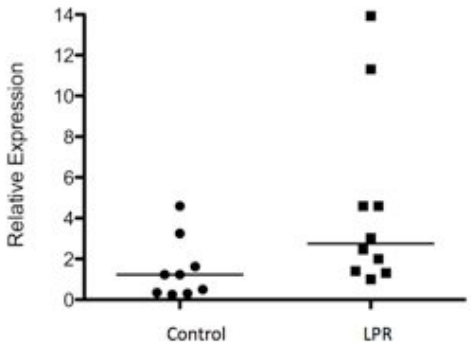
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

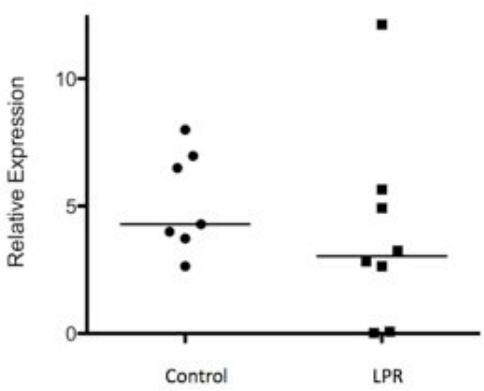


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

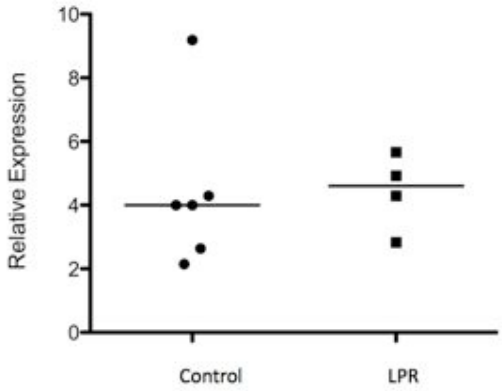


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

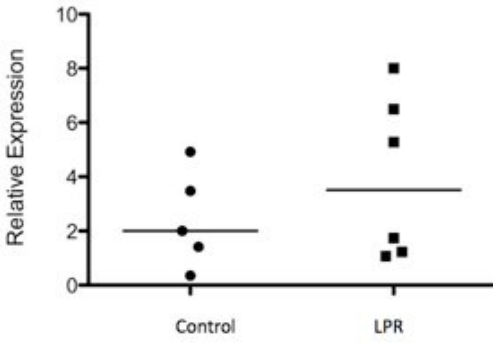
Figure 13: KRT14 Relative Expression Normalised to HPRT



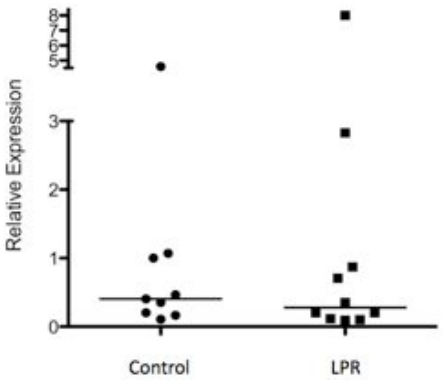
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

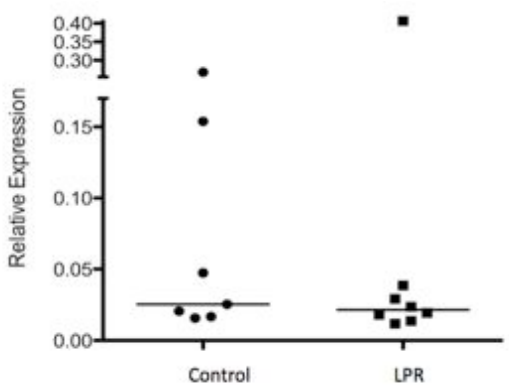


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

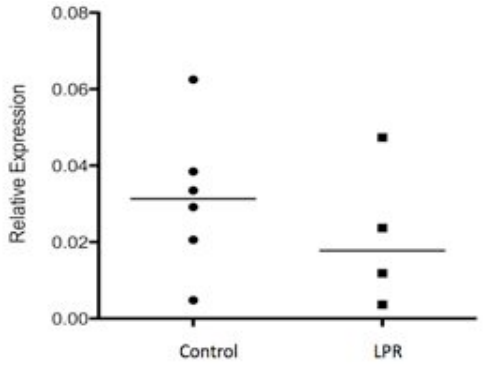


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

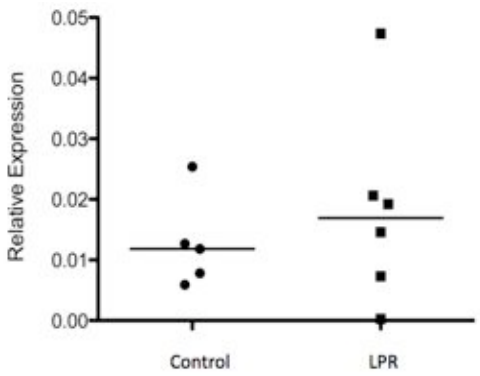
Figure 14: KRT8 Relative Expression Normalised to HPRT



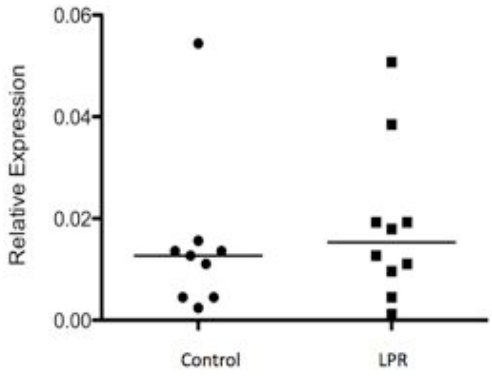
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

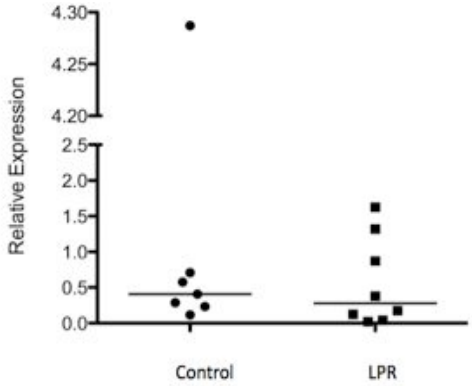


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

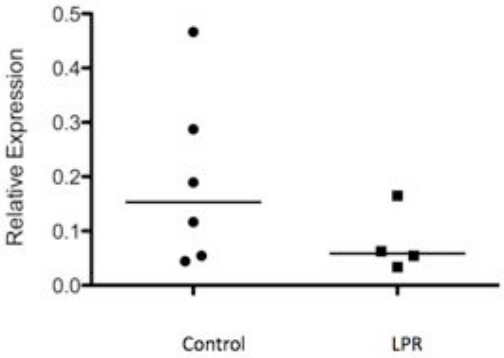


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

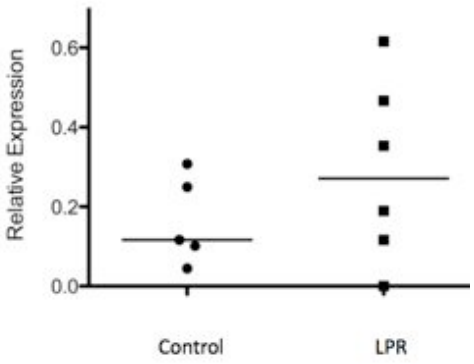
Figure 15: IL-6 Relative Expression Normalised to HPRT



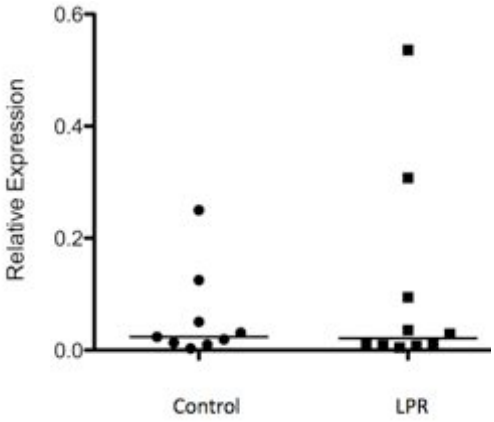
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

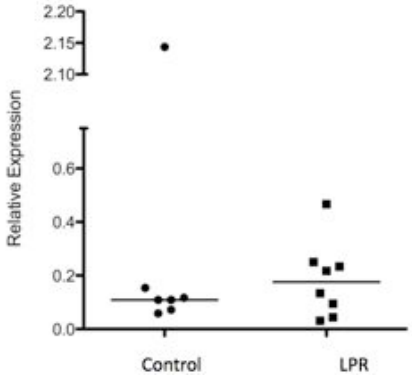


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

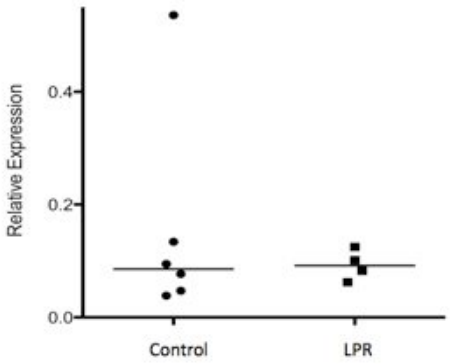


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

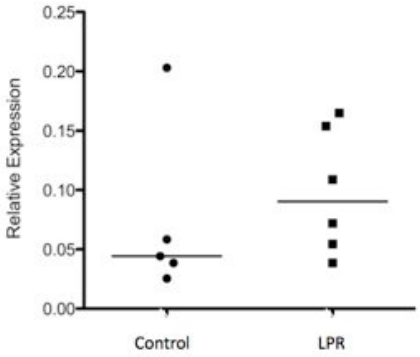
Figure 16: IL-8 Relative Expression Normalised to HPRT



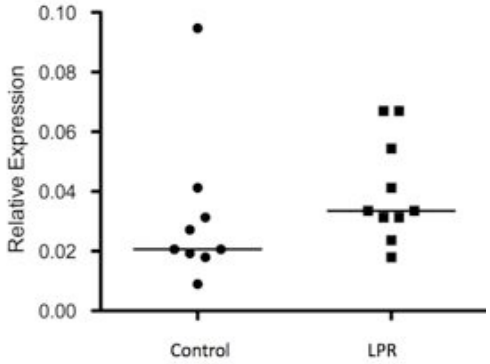
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

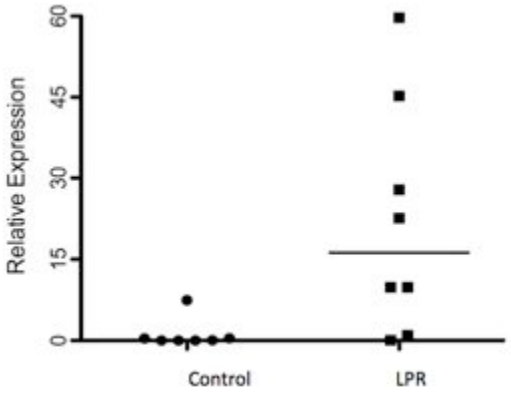


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

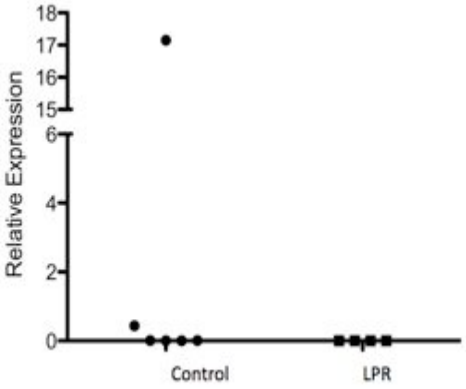


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

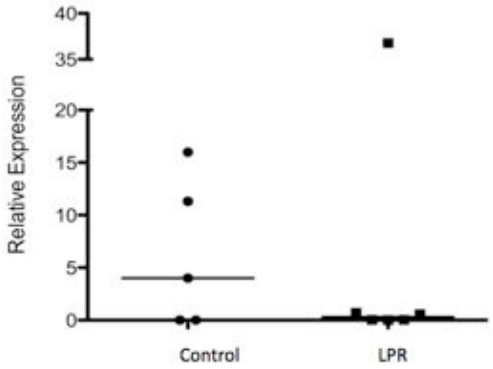
Figure 17: PTSG2 Relative Expression Normalised to HPRT



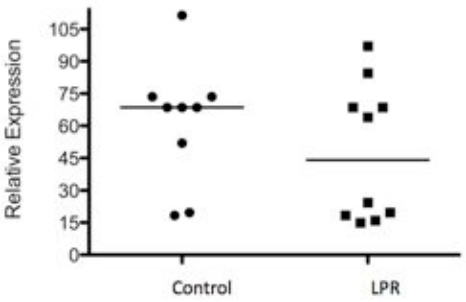
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

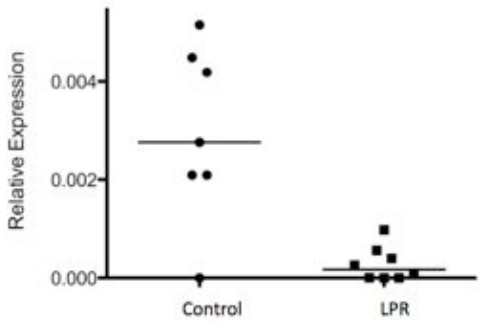


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

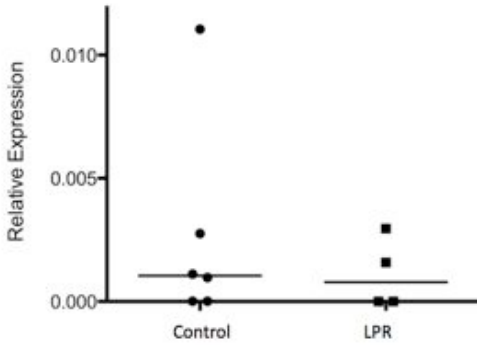


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

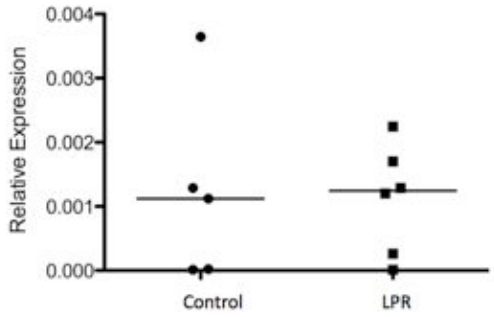
Figure 18: CRNN Relative Expression Normalised to HPRT



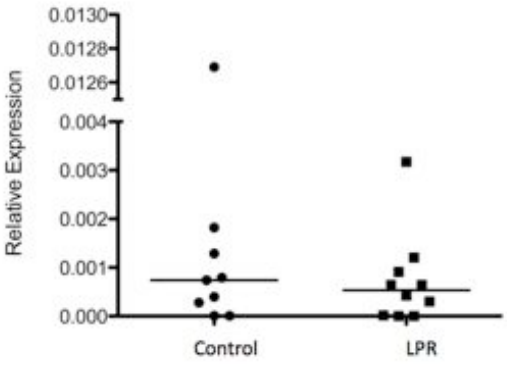
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

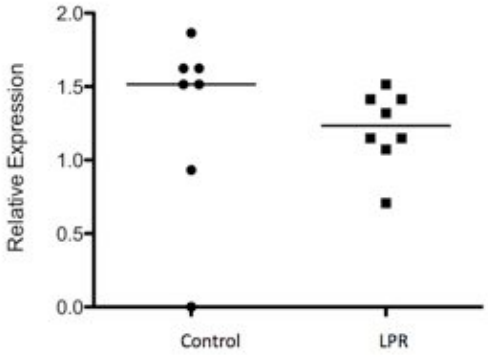


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

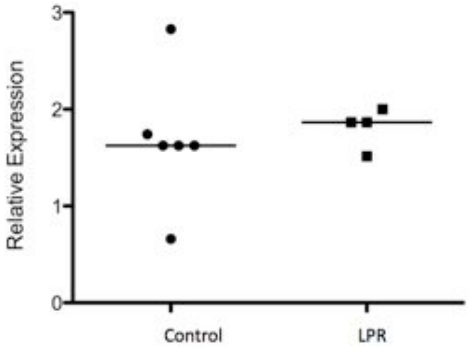


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

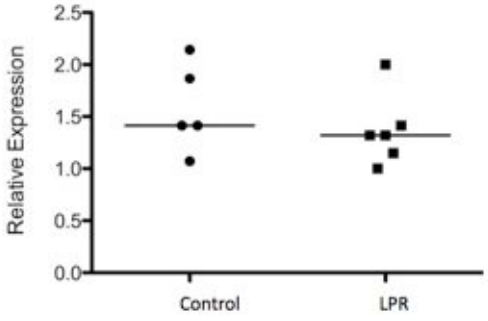
Figure 19: CD1d Relative Expression Normalised to HPRT



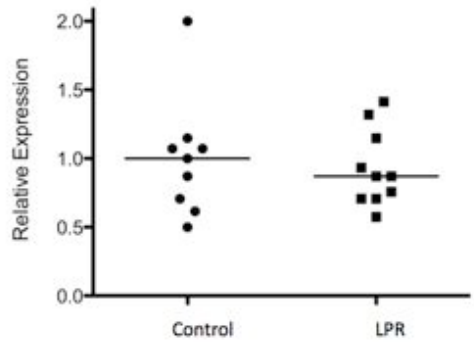
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

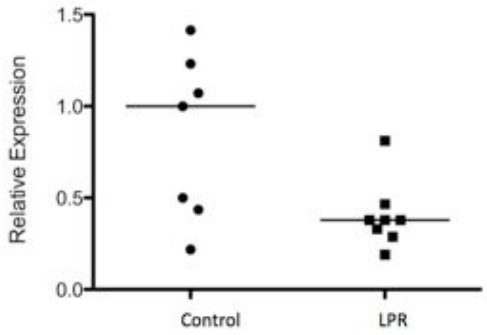


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

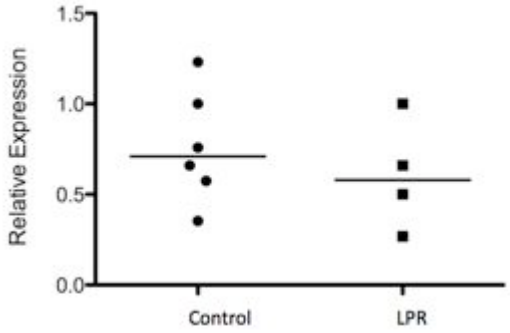


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

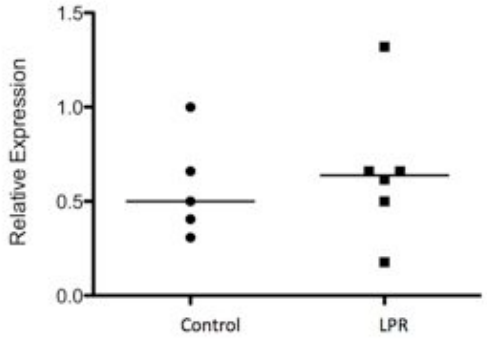
Figure 20: MGMT Relative Expression Normalised to HPRT



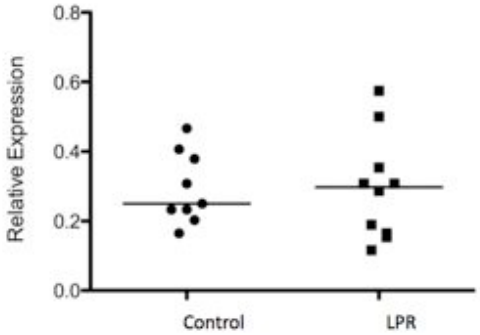
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

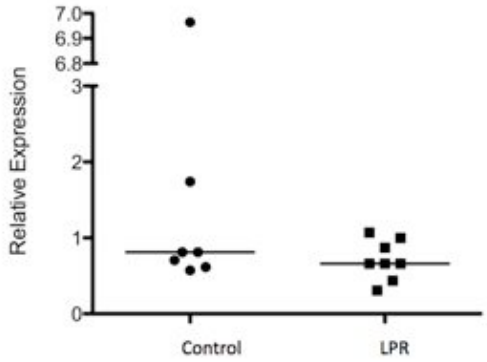


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

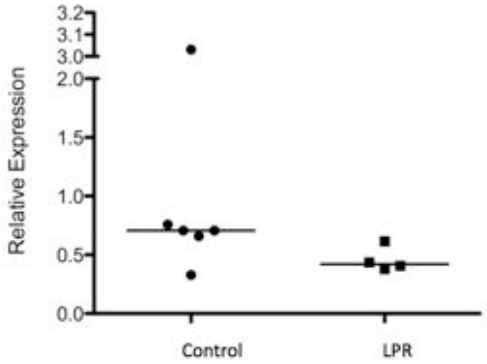


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

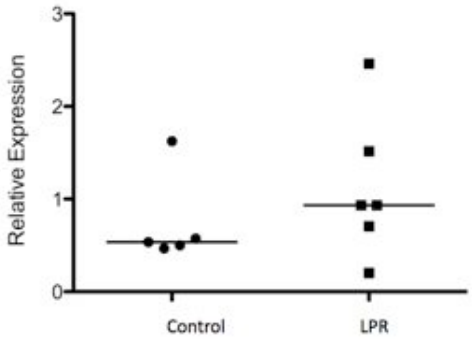
Figure 21: TGFβ-1 Relative Expression Normalised to HPRT



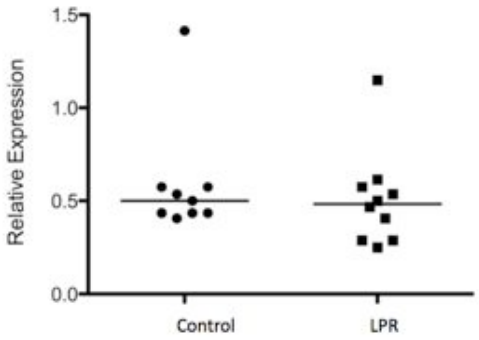
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

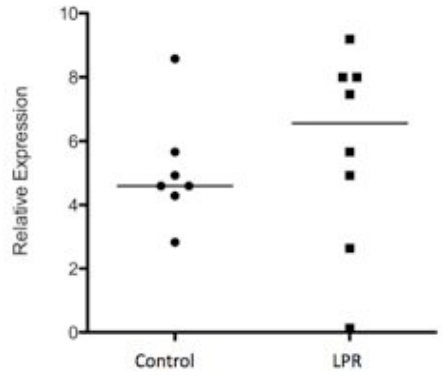


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

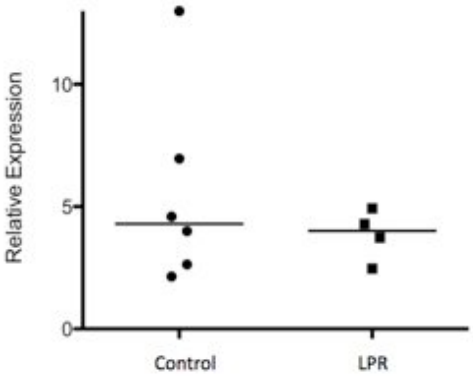


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

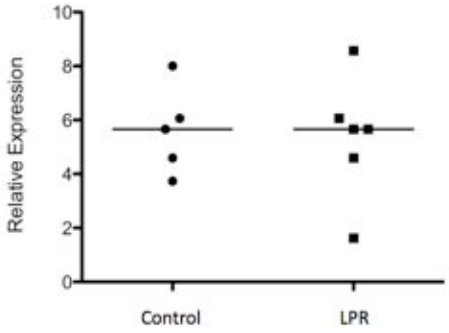
Figure 22: VEGFA Relative Expression Normalised to HPRT



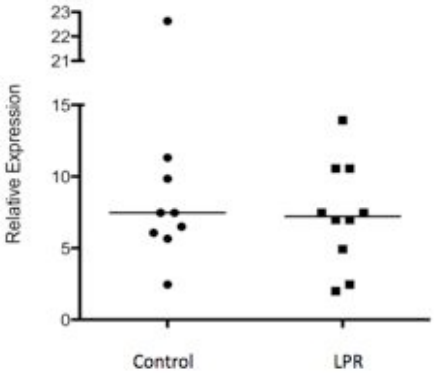
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

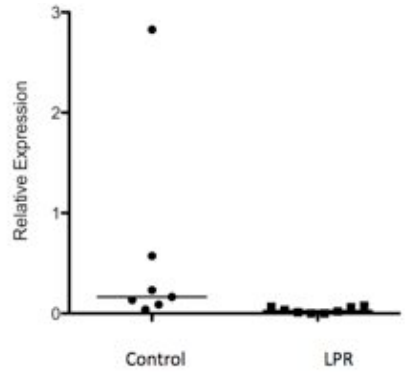


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

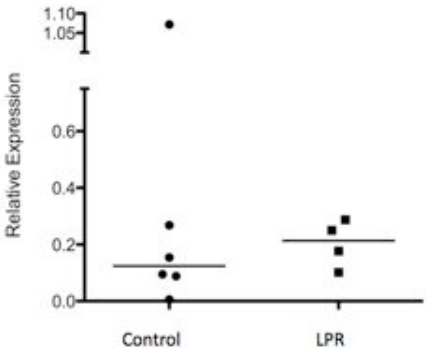


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

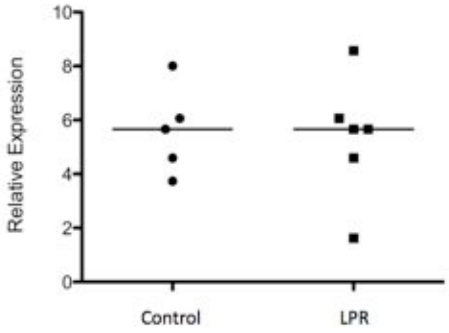
Figure 23: MUC1 Relative Expression Normalised to HPRT



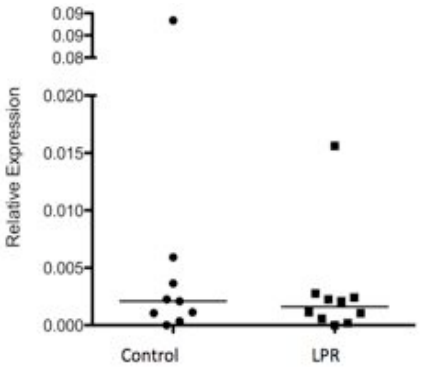
a) Medial arytenoid
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

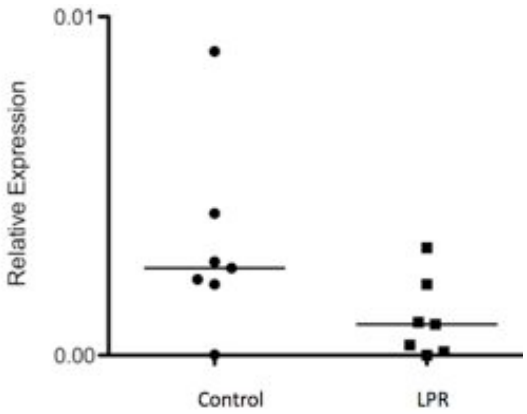


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

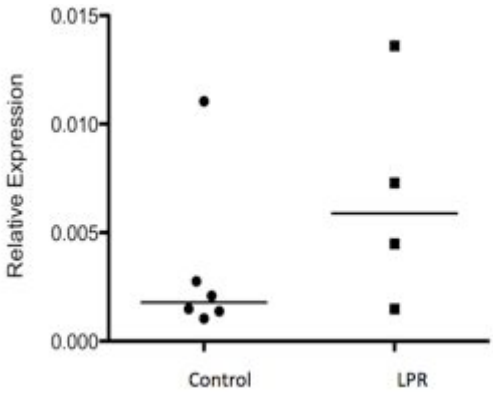


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

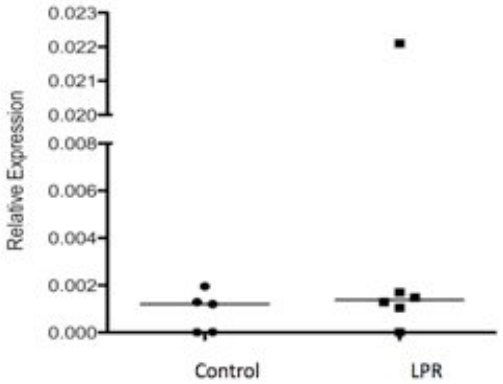
Figure 24: MUC2 Relative Expression Normalised to HPRT



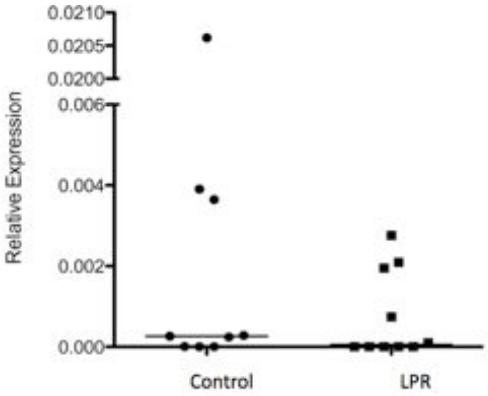
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

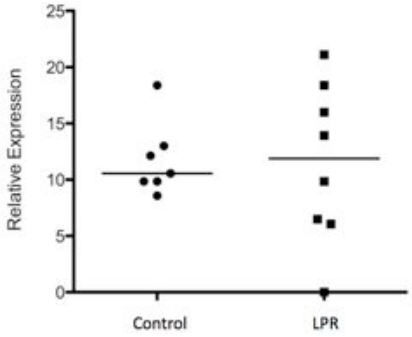


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

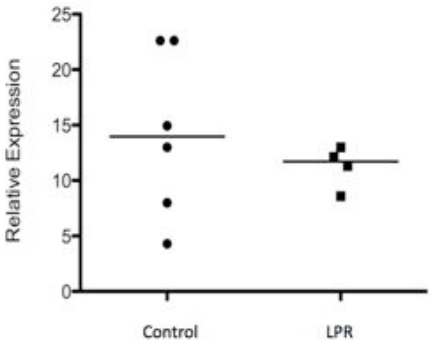


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

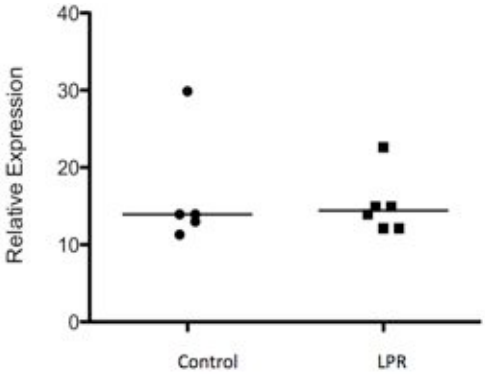
Figure 25: MUC3B Relative Expression Normalised to HPRT



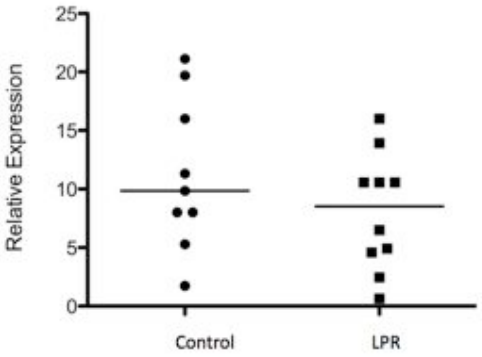
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

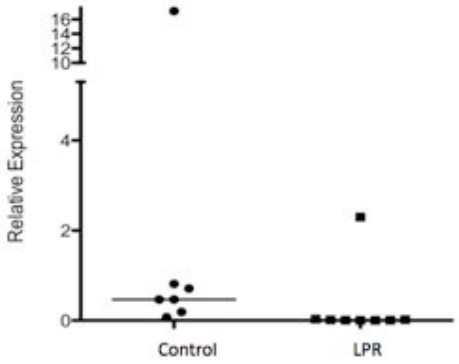


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

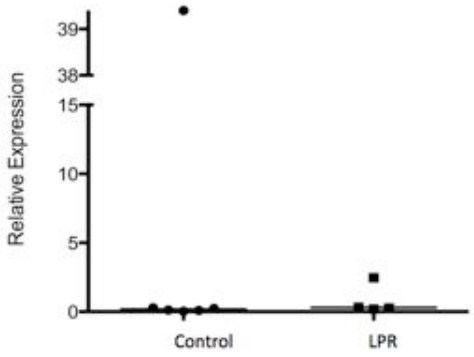


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

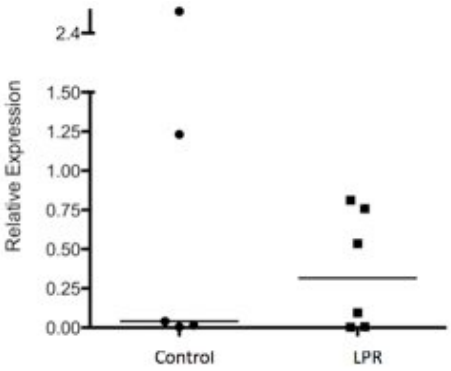
Figure 26: MUC4 Relative Expression Normalised to HPRT



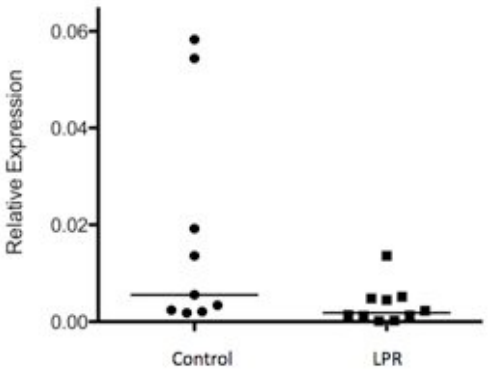
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

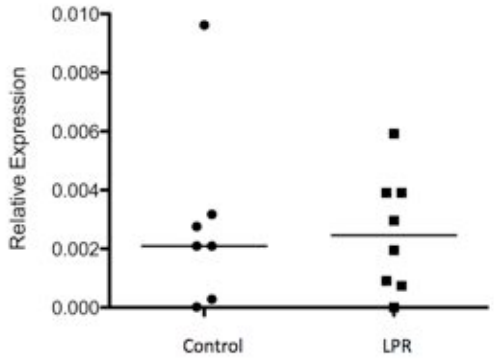


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

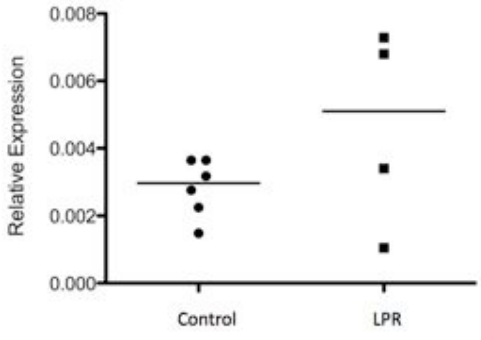


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

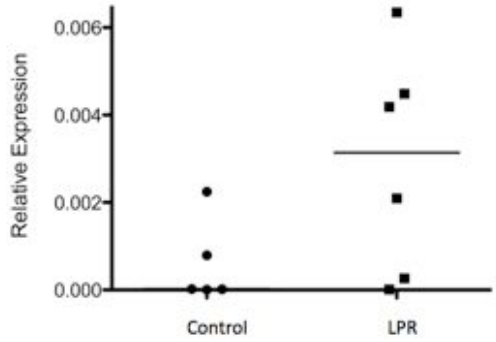
Figure 27: MUC5B Relative Expression Normalised to HPRT



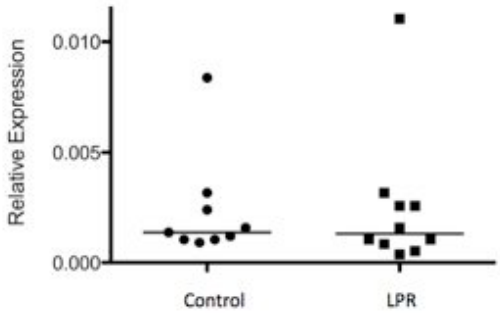
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

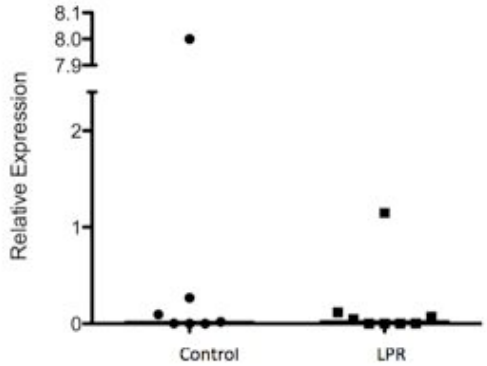


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

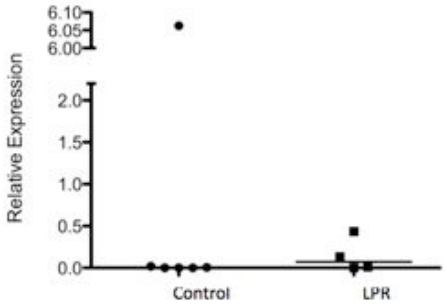


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

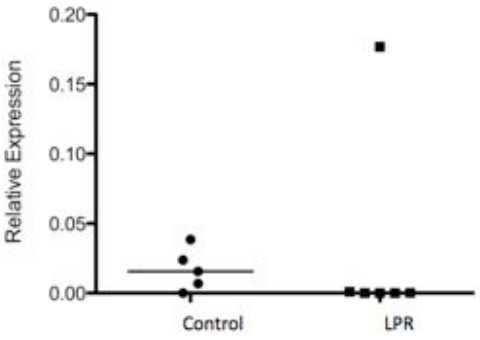
Figure 28: MUC6 Relative Expression Normalised to HPRT



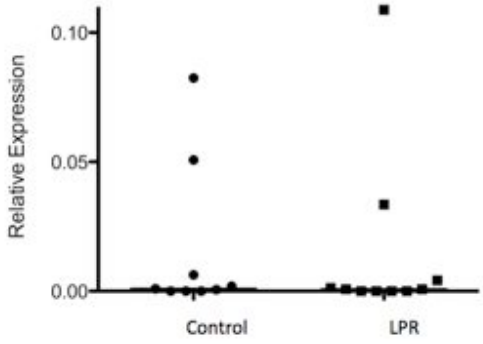
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

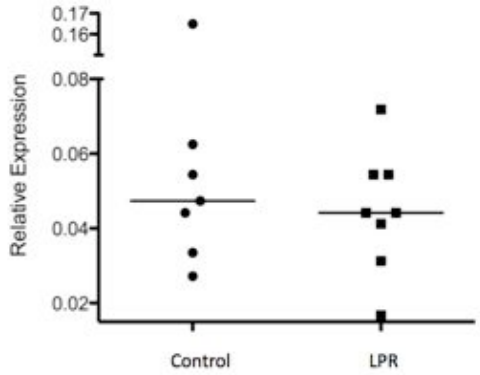


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

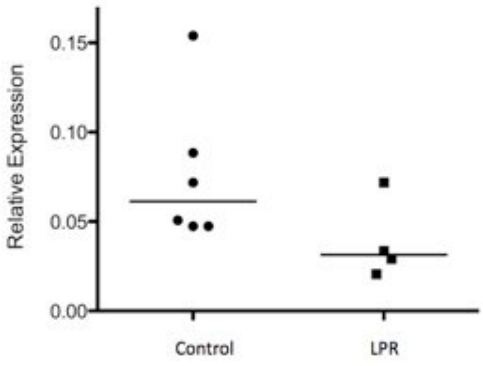


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

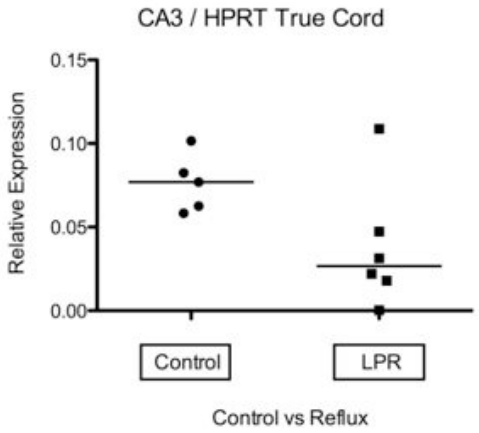
Figure 29: MUC7 Relative Expression Normalised to HPRT



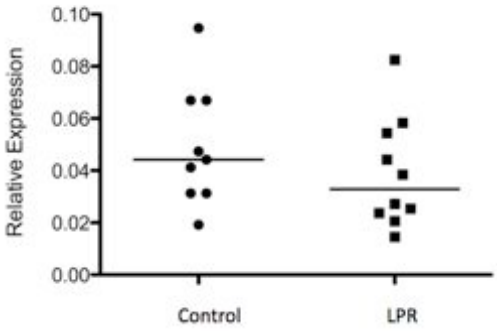
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)

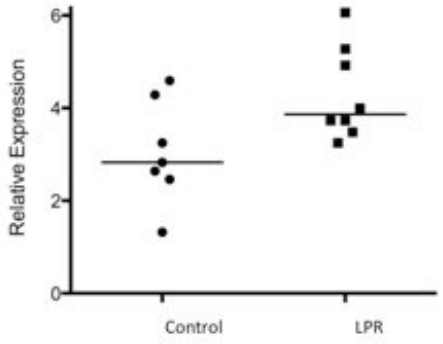


c) True vocal cord
Control (n = 5) vs LPR (n = 6)

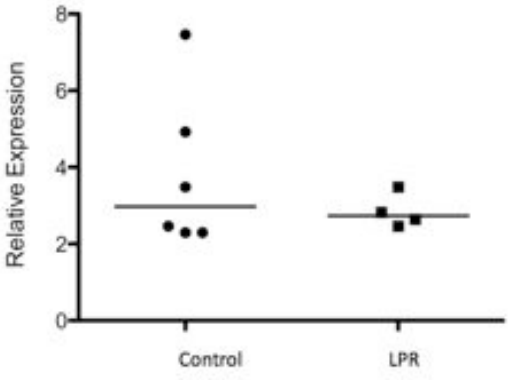


d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

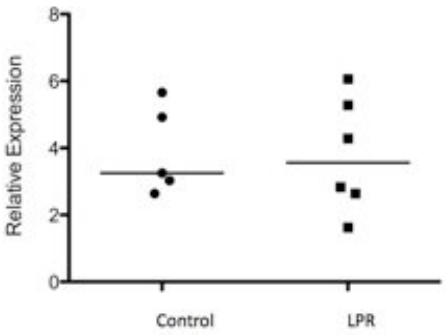
Figure 30: CA3 Relative Expression Normalised to HPRT



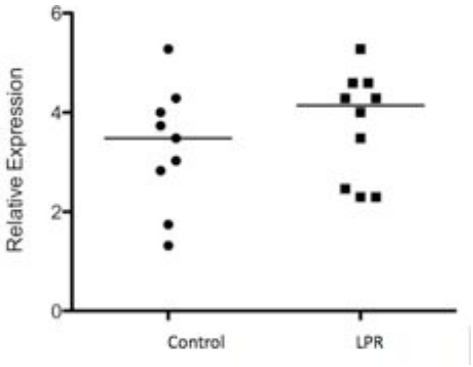
a) Medial arytenoid:
Control (n = 7) vs LPR (n = 8)



b) False vocal cord
Control (n = 6) vs LPR (n = 4)



c) True vocal cord
Control (n = 5) vs LPR (n = 6)



d) Posterior commissure
Control (n = 9) vs LPR (n = 10)

Figure 31: CDH1 Expression Relative Expression Normalised to HPRT

4 CHAPTER 4: DISCUSSION

This study investigated a clinical population diagnosed with LPR, and compared them to a group of asymptomatic patients. The results demonstrated that there are mucosal differences between the two patient groups and that the medial arytenoid region is the area demonstrating the most differences between the LPR and control groups.

4.1 Histopathology

Given the widely discrepant descriptions of the mucosal lining of the supraglottis[30] in the literature it was essential to classify the epithelium by histological examination. Previous literature has reported patches of squamous mucosa intermixed with ciliated epithelium in both the supraglottis and subglottic regions[31]. Stell et al. concluded that the vestibular folds (false vocal cord) were covered entirely by respiratory epithelium in 50% of cases and mixed respiratory and squamous epithelium in about 40%[30]. The patients in our normal group demonstrated mainly columnar epithelium in the medial arytenoid and false vocal cord regions, with none of the non-LPR group demonstrating any squamous epithelium in the false vocal cord area. The LPR group demonstrated areas of squamous epithelium in both the medial arytenoid and false vocal cord. It is possible that squamous metaplasia occurs in response to ongoing LPR induced irritation and inflammation in the larynx. Stell's findings may have been due to either active or passive smoking which was much more prevalent in northern England at the time of these earlier studies.

4.2 Cytokeratins 8 & 14 (*KRT8* and *14*)

Given the histological differences noted as a possible consequence of exposure to refluxate, tissue markers for squamous changes were measured by PCR, demonstrating a significant increase in cytokeratin 14 (*KRT14*) expression in the LPR group, in both the medial arytenoid and posterior commissure.

Keratins form part of the cytoskeleton of the epithelial cells, providing structural support in the cytoplasm and protect the cells against mechanical and non-mechanical stresses. *KRT14* is a marker of non-keratinising squamous epithelium[145]. As a molecular marker it identifies the earliest stages of squamous metaplasia, even when not identifiable on routine H & E staining[146]. Although squamous metaplasia was noted in some of the samples on histological examination in this study, it is possible earlier changes of metaplasia in other individuals might be detectable by *KRT14* in our study group.

There is increasing evidence that such a programmed change occurs by direct transdifferentiation, as identified in mouse models[147]. The epithelium of the developing mouse oesophagus undergoes a change from columnar to stratified squamous tissue, with an accompanying replacement of markers of columnar epithelium, cytokeratin 8 and 18 (*KRT8* and *18*), by *KRT14*. The proposed model by Yu et al.[147] demonstrated a conversion in the basal layer

from *KRT8* to the *KRT14* positive cell lineage. Further immunohistochemical analysis determined a temporal expression for *KRT8* and *14* markers during the course of development from columnar to stratified squamous tissue[147]. Initially, as columnar epithelium, the tissue was *KRT8* positive, and *KRT14* negative. The expression of *KRT8* decreased in the basal layers, and was replaced by *KRT14* expressing cells. For a period, however, the suprabasal layers still consisted of *KRT8* positive cells. This may explain the results in the current study, given there was no significant change in *KRT8*, yet the expression of *KRT14* in the LPR group was higher.

Our current study demonstrated the medial arytenoid histology as being mostly columnar epithelium in the non-LPR group, with an increased amount of squamous epithelium noted in the LPR group. This is also reflected by the *KRT14* expression, with a statistically significant increase in the LPR group. There was a slight decrease in *KRT8* expression in the medial arytenoid, however this was not significant. Consequently this may represent evidence of a squamous change in this laryngeal sub-site, with both the increased expression in the basal layer of *KRT14*, and the persisting KRT8 in the suprabasal layers of the medial arytenoid sub-site. This would be in keeping with a chronic irritation or persistent injury to this area[148]. It is of note that the histologist clearly identified an area of squamous metaplasia in one of the medial arytenoid biopsies from the LPR group, representing a likely response to chronic irritation.

Histopathology examination of the posterior commissure demonstrated entirely squamous epithelium in the posterior commissure in both the LPR and non-LPR patients. This is consistent with the requirement for a more durable epithelial layer in this area. As such no difference in expression of the columnar marker *KRT8* was expected given there would theoretically be no metaplastic process. However there was a significant difference in *KRT14* between the two groups. This keratin predominates in the basal cells in stratified epithelia[149]. The posterior commissure, by its anatomical location alone, is the area first in line to damage by refluxate. There is evidence in the oesophagus that hyperplasia of the basal layer occurs in the presence of reflux, and has been proposed to be due to epithelial repair mechanisms in response to this injury[104]. A recent study on the effect of refluxate on the oesophageal epithelium found hyperplasia of this basal layer was associated with increased expression of *KRT14*, and additionally this expression correlated with increasing severity of reflux[104]. Given the damaging effects of refluxate on the posterior commissure, and the well described "posterior commissure hypertrophy" it is possible that a similar response is occurring in this area. Our study found the *KRT14* expression increased in the posterior commissure biopsies in the LPR group, suggesting a response to injury. Van Roon et al.[104] identified that *KRT14* could consequently be a surrogate tissue marker of reflux. Examination of the posterior commissure data revealed 2 outliers, with high expression of *KRT14* in the LPR group. One of these outlying patients had a particularly high RSI and RFS, however this was not the case for the other patient. Consequently, whilst *KRT14* could be proposed as a marker of LPR, there is

no evidence that there is a definitive relationship between severity and expression, which would add further weight to this argument. Further research into *KRT14* expression may provide further information on its exact role in the development of hyperplastic and metaplastic change in response to reflux.

4.3 Inflammatory Markers

4.3.1 IL-6 and IL-8

In the context of repeated episodes of reflux it would be reasonable to assume that a mucosal inflammatory response would be present. It is well documented that cytokine mediators have a significant role in the pathogenesis of inflammatory conditions and levels of these are elevated in oesophageal mucosa in response to reflux. Recently, research considering inflammatory markers in GORD found an increase in IL-6, with a graded expression according to the severity of reflux[104]. In addition, IL-8 has been recognised as being elevated in the oesophageal mucosa of patients with oesophagitis, and has a significant chemotactic activity, attracting leukocytes, particularly neutrophils[150].

IL-6 is typically produced at the site of inflammation, and plays a crucial role in the acute phase response. Inflammation is a complex phenomenon, fundamentally a protection response. It is differentiated into acute and chronic, depending on the nature of the stimuli, and the response. Acute

inflammation is defined as being rapid in onset, of short duration and lasting for hours to days, with characteristic changes of exudation of fluid and plasma proteins and emigration of leucocytes (mainly neutrophils)[151]. However persisting stimuli, or a failure of the reaction to subside may lead to chronic inflammation. Other sources of chronic inflammation include immune-mediated inflammatory diseases and prolonged or repeated exposure to toxic agents[151]. IL-6 is well recognized in acute inflammation where it plays a significant role as an inducer of the production of most of acute phase proteins. However, IL-6 has a complex role in inflammation, and its role depends on the type of inflammation.

A recent study by Bathoorn et al[152] investigated the effect of pepsin on bronchial epithelial cells. Using an experimental *in vitro* model, human bronchial cells were exposed to pepsin at pH concentrations of 1.5, 2 and 2.5. They found pepsin induced cytotoxicity that was pH-dependent, with the most significant injury at the lowest pH. In addition, IL-6 and IL-8 release was greatest at the lower pH levels.

The current study demonstrated no significant difference in expression of IL-6 and IL-8 between patients with LPR and controls. Whilst the immediate response to acid and pepsin challenges may demonstrate acute inflammatory changes *in vitro*, patients with LPR may have been suffering symptoms for many months. Consequently, this may be representing a chronic inflammatory condition, rather than an acute response. There has been a number of studies finding changes in IL-6 gene expression in GORD,

however such changes have not been able to be replicated in studies considering LPR[105].

Unlike GORD, patients with LPR are likely to have significantly fewer episodes of refluxate, at a possibly higher pH. Such episodes may not be severe enough to instigate the acute inflammatory reaction identified *in vitro* studies but irritating enough to trigger a low grade, chronic inflammation. Whist frank ulceration is rare, laryngeal granulomas are not uncommon as a result of chronic exposure to refluxate[153].

Despite the wealth of knowledge of inflammatory mediators in GORD, there is little understanding of the translation to LPR, which is increasingly identified as having important differences in the inflammatory pathophysiology[105]. It is recognised that there is little objective evidence investigating inflammation in the posterior commissure[105]. Previous experimental work using an *in vitro* cell culture model found increased expression of pro-inflammatory markers in response to exposure to pepsin, even in non-acidic enviroments[154].

In vivo experiments have considered changes in *IL8* in response to treatment with the PPI rabeprazole, in LPR patients, but there was no comparison of gene expression between LPR patients, and a non-refluxing control group[105]. *IL8* expression was found to be significantly increased in biopsies from the posterior commissure in patients who had previously been using PPIs. With the lack of a control group it becomes difficult to identify a

true difference, or if those using PPIs prior to becoming enrolled in the trial were patients with more severe symptoms, or of longer chronicity of symptoms leading to greater mucosal changes.

Our study, comparing a non-refluxing population with an LPR population found no significant difference between any of the sub-sites of the larynx for gene expression of *IL6* and *IL8*. It is of note that other studies investigating inflammatory mediators in LPR also failed to find a significant change in *IL6*[105]. IL-6 is released during significant tissue trauma, with multiple studies identifying an increase in expression in the oesophageal mucosa, with greater severity of insult[104,155], with the levels if IL-6 corresponding to the degree of trauma to the cell, and predicting the magnitude of cell damage. Consequently, the severity of mucosal damage from LPR may not reach a threshold high enough to warrant a measurable change in *IL6* gene expression, however, given this study considered mRNA expression changes, it may be possible that there is a change in protein.

4.3.2 PTGS2

Prostaglandin-endoperoxide synthase 2 (also known as COX-2) (*PTGS2)* has a demonstrated role in the sequence of change from normal squamous epithelium, metaplasia, dysplasia to invasive neoplasia[156]. The cyclooxygenases of which COX-2 is one, are the rate-limiting enzymes converting arachidonic acid to prostaglandin. This isoform is rapidly induced by stimuli, and is increasingly recognized as having a role not only in

inflammation, but also promoting carcinogenesis, and in the growth of existing tumours[157].

A number of studies have recognized increased expression of COX-2 in oesophageal mucosa exposed to gastric refluxate in both animals and humans[158] and conversely, in some studies, a COX-2 inhibitor reduced the risk of developing oesophageal carcinoma[159]. Over expression of COX-2 has been proposed to induce tumourgenesis and in addition has been found in squamous cell carcinomas (SCC) of the head and neck[157]. One study found COX-2 expression gradually increasing from normal epithelium through dysplasia to poorly differentiated SCC[157]. In addition patients with intestinal metaplasia were also noted to have an increased expression[160].

There is a paucity of literature investigating the role of COX-2 in the laryngopharynx. One of the few studies investigating the expression of COX-2 in response to LPR collected biopsies from the anterior and posterior pillars around the palatine tonsils during tonsillectomy[83]. The tissue was exposed to bile salts and an increase the expression of COX-2 mRNA was detected. Such a study highlights the difficulties in collecting adequate biopsies in the clinical setting for LPR investigations. The indication for the patients to undergo tonsillectomy was not identified but if from an adult population, would likely be due to recurrent tonsillitis. Such repeated infection and resultant inflammation may have altered the true response to the injurious challenges studied. Additionally, the area from which these biopsies were collected may not be readily translatable to the human larynx,

particularly as this current study demonstrates a significant variation in epithelia structure within the larynx alone, let alone much higher up at the junction of the oral cavity and oropharynx.

Consequently, it is not surprising that this current study did not demonstrate a significant difference in gene expression between the LPR and non-LPR group with respect to COX-2. Examination of the outliers of this gene found that although there was a single patient with increased COX-2 expression in the control group for each sub-site, these were from separate patients. Given its role in tumourgenesis, the absence of such a difference between the two groups may suggest that refluxate into the larynx may not, in itself, provide a significant enough injury to progress to dysplasia or cancer. It must be recognized that this study considered mRNA expression, not COX-2 protein levels.

In addition, a number of studies have demonstrated that COX-2 can exert a regulatory effect on VEGF production[156]. Both *VEGFA* and COX-2 demonstrated no significant difference between the LPR and non-LPR group. This would suggest that the process driving metaplastic changes in the oesophagus in response to reflux may be different to the mucosal response in the larynx.

4.3.3 MGMT

O^6-Methylguanine-DNA methytransferase (*MGMT*) is a DNA repair protein, and whilst its role in LPR is uncertain, it has previously identified

associations with oesophageal squamous cell carcinoma, lung cancer, melanoma and cancers of the upper aerodigestive tract[161]. A recent study identified hypermethylation and expression of *MGMT* in Barrett's esophagus, with hypermethylation detected in 100% of Barrett's intraepithelial neoplasia, 88.9% of Barrett's metaplasia, but only 21.4% of normal oesophageal mucosa[161,162]. There was also significant down-regulation of *MGMT* transcripts and protein expression noted, which correlated with disease progression. The current study found no significant difference of gene expression in any of the laryngeal sub-sites between LPR and non-LPR groups however, as previously mentioned, this study did not measure protein expression. This would indicate that hypermethylation may not play a significant role in mucosal injury in LPR but further research is required to investigate this further.

4.3.4 TGFβ-1

There are many other important cytokines involved in inflammation. TGFβ-1 has a multitude of functions and as a growth factor is one of the few that have an inhibitory function[73]. It is a cytokine recognized as regulating cell replication and differentiation, bone formation, angiogenesis, haematopoiesis, cell cycle progression and cellular migration[106]. TGFβ-1 is known to have a wide range of effects that stimulate mesenchymal cells however it has a significant inhibitory effect on epithelial proliferation[106]. A number of studies have also found the administration of topical TGFβ-1 improved healing in ulcers, incisional and excisional wounds[163]. This cytokine has regulatory effects on a wide range of cell types, with a role in

regulating epithelial cell growth, differentiation, motility, organisation and apoptosis[106].

Furthermore *TGFβ-1* may have a role in tumourgenesis, acting as a tumour suppressor early on. Later however, it is thought to promote angiogenesis and immunosuppression, providing an environment suitable for rapid tumour growth by acting both on tumour cells and the local environment[106]. Consequently the role of *TGFβ-1* in any inflammatory condition, including LPR, appears to be complex with a variety of responses. The current study identified decreased expression of *TGFβ-1* in patients with LPR which may indicate a decreased ability to repair, with the potential for early loss of tumour suppression. On closer analysis, it is of note that more than half of the non-LPR group had a relatively elevated expression of *TGFβ-1* compared to the LPR group, with the resulting statistically significant result. However there appeared to be a bi-modal distribution in the non-LPR group, the significance of which is uncertain. Consequently further research is required to determine the relative expression of *TGFβ-1* in both a non-LPR population, and consequently if there is significantly less expression in LPR patients of *TGFβ-1* in the medial arytenoid.

There is unfortunately a paucity of studies looking at *TGFβ-1* considering the role this cytokine has in LPR[105,108]. Thibeault et al.[105] found a significant increase in *TGFβ-1* gene expression following PPI treatment in patients with LPR but only in the sub-group of patients who had already been taking PPIs. This may indicate either different phenotypes of disease or greater

inflammation and consequently symptoms in that group. The current study found a significant decrease in the non-treated LPR group, consistent with the study conducted by Thibeault et al[105]. More specifically, there was a lower gene expression of *TGFβ-1* in the medial arytenoid region. As an area typically of columnar epithelium and close to the posterior commissure, this region would likely receive the majority of the refluxate, and any impairment in wound healing would be significantly detrimental. Our results confirm that *TGFβ-1* remains an important cytokine for future research, especially regarding the role it may have in carcinogenesis, possibly exacerbated or initiated by LPR.

4.3.5 VEGF-A

Similar to *TGFβ-1*, Vascular Endothelial growth factor A (*VEGFA*) has multiple roles, being a mediator of vascular hyperpermeability, angiogenesis and inflammation[164]. There is increasing literature identifying the role of VEGF in other reflux related diseases, particularly in Barrett's oesophagus[156]. In addition, it is recognized that COX-2 exerts a regulatory effect on VEGF production. In both Barrett's oesophagus and colon cancer it has been reported that COX-2 expression stimulates angiogenesis by inducing VEGF[165]. Vallböhmer et al.[166] compared oesophageal biopsies from patients with normal squamous oesophageal mucosa, through to Barrett's and adenocarcinoma. They found that expression of both COX-2 and VEGF was significantly up-regulated in patients with metaplasia, dysplasia and cancer when compared with controls, with a sequential increase in expression noted between each of these groups. It is of interest

to note that there was no significant change in epidermal growth factor receptor (EGFR), which is over-expressed in squamous cell carcinoma of the oesophagus.

VEGF has a significant role in inflammation and repair, with cell migration, chemotaxis and induction of vascular permeability, epithelialization and collagen deposition all identified as being stimulated by it[167]. Its role in LPR is still unclear, with experimental *in vitro* studies identifying gene expression changes in both false vocal fold fibroblast cultures in response to acid bolus and in post-cricoid fibroblast cultures in response to both acid and pepsin[108]. The most significant changes were measured during the first 60 seconds after exposure. Additionally, there was a noticeable difference between the post-cricoid and false vocal cord mucosal responses, with the false vocal cord tissue being more resistant to pepsin than the post-cricoid tissue. Such a response conflicts with other studies finding significant injury caused by both acid and pepsin[33].

This current study however, did not demonstrate a significant difference between any of the sub-sites of the larynx in mRNA expression, however this study did not measure tissue protein levels. It is possible that *VEGFA* mediated inflammation may not play a significant role in LPR, however in the absence of measuring this protein expression it is not possible to be certain and further work is required to confirm or refute this.

4.4 Laryngeal Defences

4.4.1 Mucins

Mucins consist of high molecular weight glycoproteins. The mucins are categorised into two primary classes: secreted gel-forming mucins, and transmembrane mucins. *MUC2*, *5A*, *5B*, *6*, *7*, *8* and *19* are gel-forming mucins and have been demonstrated to be present in the aerodigestive tract [Table 4]. Typically expressed in epithelial cell types, they provide protection in relatively harsh environments such as exposure to fluctuations in pH, ionic concentration, hydration and oxygenation[70]. Accordingly whilst their primary function is of protection, lubrication and transport, there are further implications that they have a role in the renewal and differentiation of epithelium, modulation of the cell cycle progression, adhesion and signal cell transduction[118] with a role in cell homeostasis and promotion of cell survival.

It is well described in GORD that the oesophageal mucosa secretes soluble mucus in response to excessive exposure to acid[104]. At the primary protective level this may lead to increased mucosal protection in response to the refluxate. In addition, alteration in the expression of the transmembrane mucins may lead to alterations of the cell cycle. This is of note given the increased expression of *MUC1* and *MUC3* in the pre-cancerous Barrett's oesophagus mucosa[104]. In addition *MUC6* has been identified in normal oesophageal mucosa, metaplastic columnar oesophageal mucosa and oesophageal adenocarcinoma[104].

Samuels et al.[118] identified the mucin expression profile in both the normal larynx (n =2) and in larynges of patients with LPR (n=3), identified by RSI and RFS. The normal larynx demonstrated predominant expression of the transmembrane mucins *MUC1* and *MUC4*, with expression of *MUC2*, *5A* and *5B* as the major airway gel-forming mucins. Other mucins important in oesophageal reflux, such as *MUC6*, were not identified in either the control or LPR groups. However, in addition to the low numbers sampled, only the posterior commissure area was biopsied. The current study utilised biopsies from multiple sub-sites of the larynx, and found a significant difference in the gel-forming mucins *MUC2* and *5B*, with the gene expression of both significantly lower in the LPR group, in the medial arytenoid region. In addition, *MUC5B* expression was significantly lower in the LPR group in the posterior commissure.

MUC2 was only significantly different in the medial arytenoid region. Such a difference may indicate a failing of mucosal defence in the presence of refluxate. In addition loss of this may reduce the resistance of this area of the larynx to further reflux boluses.

4.4.2 Carbonic Anhydrase III

It is well recognised that the larynx lacks many of the significant defence mechanisms that are present in the oesophagus. In addition to the mucins, the carbonic anhydrases play a role in mucosal defences by catalysing the reversible hydration of carbon dioxide, allowing bicarbonate ions to be actively pumped into the extracellular space. Johnston et al. [74] proposed

that this would not only neutralise refluxed gastric acid but, by increasing luminal pH, indirectly reduce the activity of pepsin present in the refluxate. Of the eleven isoenzymes the oesophagus has been identified as expressing carbonic anhydrase I, II, III and IV in the epithelium. There is a significant buffering effect of carbonic anhydrase system being able to increase the pH of acid boluses in the oesophagus from 2.5 to nearly neutral[109]. In response to refluxate, immunofluorescence studies have demonstrated a re-localisation of CA-III from the basal layers in normal oesophageal epithelium to the supra-basal layers in the inflamed oesophageal tissue. This may represent an increased buffering capacity of the inflamed tissue, providing greater protection in acid-challenged tissue.

CA-III has been identified in the normal larynx[110] and in LPR[75]. Furthermore, there is evidence that expression of *CA3* may vary throughout sub-sites of the larynx, particularly in response to refluxate. Expression of *CA3* in the control group demonstrated a slightly higher expression in the true cord than in the other regions. This may represent one of the few intrinsic defences for the true cord region, which relies on other areas of the larynx to produce protective mucin. The variability of expression of *CA3* in the normal larynx is lacking in current literature, however early studies into *CA3* expression noted its depletion in patients with LPR[110]. Comparing biopsies from the laryngeal ventricle, vocal fold and posterior commissure, found a depletion of *CA3* in both the ventricle and vocal fold regions, however no significant change in the posterior commissure[74].

This current study found no significant change in the posterior commissure between LPR and non-LPR groups. Gene expression of *CA3* appeared to decrease in both the true, and false vocal cord biopsies in the LPR group however this was not statistically significant.

A decrease in *CA3* expression in the true and false vocal cords would concur with previous research suggesting that there is a loss of intrinsic defences within sub-sites of the larynx in the presence of refluxate. This loss is not specific to histological tissue type with both the squamous vocal cord and the columnar ventricle demonstrating this decrease.

The posterior commissure maintained its expression of *CA3* and notably, in an earlier study was actually increased[113]. Consequently the posterior commissure appears to display a persistence of the intrinsic defences in the setting of LPR. This may explain part of the mechanism identifying why laryngeal pathology, such as carcinoma arising from this region, is such a rarity[113]. However the loss of *CA3* is not likely to be the sole event, given there was no significant change in the medial arytenoid region, however there were multiple other molecular changes in this area.

4.4.3 CRNN

Cellular defence pathways are also thought to play a role in the mucosal response to LPR[77]. These have been described as being molecular "chaperones" which regulate the folding and unfolding of cellular proteins[168]. *CRNN*, also known as squamous epithelial heat shock protein 53 (*SEP53*) is

one such that has a role in repairing protein damage after cell injury[169]. Animal models of oesophageal injury, using porcine epithelium, noted a significant up-regulation of *SEP53* in both hyperplastic and hyperkeratotic lesions[169]. Interestingly there was much lower, and dysregulated expression in oesophageal adenocarcinoma cells.

Whilst it is recognised that these changes were noted in oesophageal mucosa, an increase in expression in *SEP53* is in contrast to Johnston et al.[77] who found significantly decreased levels of *SEP70* and slightly less *SEP53* in the posterior commissure area of the larynx when measured by Western blot analysis. This current study considered further the sub-sites of the larynx. It is of note that there was higher relative expression of *SEP53* in the posterior commissure region compared to the other regions of the larynx in both groups. It is possible that this may be due to this area being the most susceptible to injury through refluxate, in addition to being squamous epithelium. This study identified a significant difference in *SEP53* in the medial arytenoid region in the LPR group, with higher expression noted in this group. This may reflect its role as a "molecular chaperone" for cellular repair processes, or potentially its increasingly recognised role in the immune system[170]. Additionally a significant component of such a difference is likely to be related to the increased level of squamous epithelium, rather than columnar, found on histological analysis in this study.

4.4.4 CD1d

The mucosal immune response is varied in the presence of LPR. Recent research has focused on the mucosal immune response to refluxate, particularly given its position at the junction of the IgA - dominated upper, and IgG - dominated lower airways[171]. The mucosal immune response has increasingly being studied. One study found a significant increase in CD8+ lymphocytes in the laryngeal epithelium biopsied from the true vocal cord, suggesting that in the this sub-site there may be an accumulation of CD8+ T cells in the luminal epithelial layer[171].

Further investigation into *CD1d* found significantly more expression in the superficial (luminal) layers of the vocal cord in patients with LPR. They described a change in expression from MHC Class $I^{hi}CD1d^{lo}$ in the basal layers to a gradual transition to MHC Class $I^{lo}CD1d^{hi}$ epithelial cells in the luminal layers. However the role of changes in expression of *CD1d* in inflammatory conditions is not entirely clear. *CD1d* has been associated with abnormal host immune responses in primary biliary cirrhosis[172], rheumatoid arthritis[173] and inflammatory bowel disease. What is known is that *CD1d* is a crucial member of the immune system in the presentation of glycolipid antigens to natural killer T cells (NKT cells)[173].

CD1d is ubiquitous in the intestinal epithelium and its down-regulation has been noted in microscopic colitis[174]. It was thought that this may be demonstrating an immunoregulatory dysfunction in the colonic mucosa. Additionally, *CD1d* is recognised as inducing production of

immunoregulatory cytokines such as interleukin-10. Consequently reduction of *CD1d* may contribute to the pathogenesis of this colitis by reducing the activation of NKT cells or the production of IL-10.

Our results did not backup the findings of increased expression of *CD1d* in the squamous vocal cord as found by Rees et al.[171]. However their study utilised a quantitative immunofluoresence technique and their biopsy samples were of the squamous vocal cord. Our population demonstrated a significant difference in the medial arytenoid region of the larynx, using a gene expression method, with a lower expression noted in the LPR group. However, we did not measure post-transcriptional expression. In addition other factors can influence the expression of *CD1d*. A recent study of cervical epithelium found *CD1d* down regulated in human papillomavirus – positive cells, *in vivo* and *in vitro*, on flow cytometry, however it is of note that the *CD1d* mRNA levels were not affected.

4.4.5 CDH1 (E-cadherin)

Epithelial cadherin (E-cadherin) is encoded by the *CDH1* gene and has a significant role in the intercellular interaction and adhesion. There is also significant evidence that it is crucial for epithelial integrity and barrier functions[112]. It is widely recognised that in both the oesophagus and larynx abnormal exposure to refluxate can cause increased paracellular permeability[113]. As the primary barrier to the passage of solutes through the paracellular space, permeability can be affected by the integrity of the intercellular junction. These are composed of an E-cadherin-catenin

complex, known as adherens junctions, with E-cadherins being recognised as important in cell-cell adhesion and tight junction complex composed of zonula occludens 1, and occludin. Potentially any damage to these could contribute to the loss of normal defences, allowing the mucosa to be more susceptible to further injury.

Dilatation of intercellular spaces (DIS) between squamous epithelial cells has been identified in studies of reflux exposed oesophageal mucosa. This is considered a morphological marker of acid damage to this tissue[175]. Furthermore, there is evidence it improves following treatment with a PPI[176]. One study analysed biopsies from the inter-arytenoid region of the posterior larynx for evidence of DIS[175]. Using a computer assisted morphometric system a statistically significant difference between the patients with LPR and the control group was identified. Interestingly there was no correlation between severity of reflux symptoms and the intercellular space distance.

Acid and pepsin have previously been identified as being able to break down the barrier and affect the epithelial permeability through injury to the junctional complex in oesophageal mucosa[177]. In addition, previous studies have found a decreased expression of E-cadherin in the biopsied areas of the larynx using immunohistochemical technique[112,113]. Gill et al.[112] found 50% of their samples from the vocal fold, ventricle and posterior commissure demonstrated either a partial or complete loss of E-cadherin expression. The pathophysiology of this change is poorly understood, with proposed mechanisms including exposure to pepsin or secondary to the inflammatory

response associated with LPR[112]. What is recognised is that E-cadherin is similarly expressed in both normal squamous epithelium and metaplastic columnar epithelium of the oesophagus[178,179].

This study demonstrated higher expression of *CDH1* in the LPR group, limited to the medial arytenoid region. This is contrary to the findings in previous literature, where dysplasia of the epithelium demonstrated a decrease in e-cadherin, most likely a reflection of the deterioration of squamous defences. Such a change in our study may be related to the histological differences noted between the two groups in the medial arytenoid, with the control group demonstrating normal columnar epithelium, compared to the largely squamous LPR group.

4.5 Laryngeal Sub-sites

The molecular studies regarding LPR can be classified as either animal studies[33,75], *in vitro*, using human biopsies[175] or a combination of these[113,180]. Of the work researching human tissue, the majority of these have considered the posterior commissure and one other sub-site, such as the vocal cord, or ventricle. This is hardly surprising given this is the area most noted demonstrating changes indicative of LPR. Fewer studies have compared more than 2 sub-sites at a time[74,112]. Subject numbers in each study are typically small, highlighting the difficulty in recruiting suitable

patients for such studies. Each study has almost unanimously found differences between laryngeal sub-sites in response to LPR.[74]

This study concurred with previous research in finding a variability of mucosal response within the larynx in the presence of LPR. Of most importance, however, is that the area demonstrating the greatest number of differences in gene expression was the medial arytenoid. The medial arytenoid was typically columnar epithelium in the non-LPR group, whereas the LPR group was noted to have squamous epithelium, or a combination of both. Such a histological change may well represent an attempt to provide an epithelium of greater resilience to the repeated, intermittent insult of LPR.

This medial arytenoid region also demonstrated the greatest number of molecular marker differences between the LPR and non-LPR group, with significant differences noted in expression of inflammatory genes such as *TGFβ-1*, *CD1d* and *CRNN*. The position of the medial arytenoid region is closer to the posterior commissure than the false vocal cord biopsies, which may indicate that this columnar epithelium is receiving more damaging refluxate than the false vocal cord, leading to greater changes in this region.

Additionally there was a decrease in gene expression of the secretory mucins *MUC2* and *MUC5B* in the medial arytenoid. Such a difference in one of the intrinsic mucosal defences between the LPR and non-LPR groups may represent an impairment, or loss of these defences in this region. Loss of mucin in this region may allow this area to be more susceptible to further

damage from refluxate. Alternatively, rather than a pathological change with decrease in laryngeal defences, such a difference may represent an adaptive change from the less resilient columnar epithelium to the more durable squamous epithelium. Such differences in *MUC2* expression were specific only to the medial arytenoid, however *MUC5B* demonstrated this also in the posterior commissure - already known to be squamous tissue on histological analysis in both patient groups. It is of note, however, that there were 2 outliers in the non-refluxing group which, were the groups more homogenous, may not have provided such a significantly different result.

This change in mucin expression may be complicated by the difference noted in histology from columnar to squamous tissue which in itself may lead to such a difference. In one study of Barrett's oesophagus, *MUC2* expression was significantly higher in columnar epithelium with goblet cells, than in columnar epithelium without[181]. In another study only mild superficial staining of *MUC1* was noted in the normal squamous epithelium, however *MUC2* was expressed in the Barrett epithelium and in dysplasia[182]. If the change in gene expression is purely due to the change in histology, then this could imply that an adaptive change is occurring, with such tissue being more protective to the larynx than the columnar epithelium even with the mucin.

However, within this study, there was a statistically significant difference of *MUC5B* of LPR and non-LPR patients in both the medial arytenoid (columnar epithelium) and also in the posterior commissure, an area entirely

identified as being squamous epithelium. Consequently this may indicate a failure of laryngeal defences in the presence of LPR however it is likely, on balance, that there is a combination of both factors finally affecting mucin expression.

4.6 Limitations

It must be qualified that there were notable differences between the two groups. Firstly, the average age for the LPR group was 17 years older than the non-LPR group. Such an age difference may contribute to the difference between groups. However, in their meta-analysis, Dent et al.[9] found the effect of age on the prevalence of GORD was uncertain. Two studies identified a slight but significant association. A study from a General Practitioner database in the United Kingdom found the incidence of GORD increased in both sexes until the age of 69, then decreased. Such a trend was also identified in other populations, although at different ages. It is not entirely clear what percentage of these people would also suffer LPR, however it is possible a similar distribution exists. Additionally, there is a suggestion in histological analyses of the larynx that the frequency of squamous epithelium in the larynx may be related to age[30], however the extent and the temporal parameters at which this occurs is not known. This

may be related to the “older” larynx being exposed to more airway irritants over time than a younger population.

Other studies have also demonstrated gene expression varies with age. A recent meta-analysis of 27 data sets, using profiles from mice, rats and humans, found 55 genes to be consistently over-expressed with age and 17 under-expressed[183]. It is of interest to note that the majority of over-expressed genes were related to inflammation and immune responses and those under-expressed related to energy metabolism, alterations in genes related to apoptosis and the cell cycle. Consequently it is clear that the age difference may provide a confounding variable, however it is difficult to determine the extent it may have influenced results in this study.

In addition, there was a noticeable difference between the ratio male to female in each group. The impact this has on the data is again difficult to determine, with a meta-analysis including 4 cross sectional studies and one longitudinal study, finding no significant association between sex and GORD[9]. Whilst it is recognised that LPR is likely a separate entity to GORD, there is currently a lack of any strong evidence that sex is a contributing factor for the development of the condition. However it is well recognised that the larynx is a hormone sensitive organ, with identifiable changes attributable to testosterone, progesterone and oestrogen[184]. Furthermore there is evidence that the greatest voice change after puberty occurs in females after menopause related to both hormone changes and subsequent muscular atrophy[184] Such changes may be represented by changes in gene

expression throughout the larynx, or even a change in susceptibility to damaging agents. Consequently as further research identifies specific sub-groups of LPR, both gender and age may become increasingly important. With a larger numbers future studies would be able to assess whether such changes contribute to any significant differences in gene expression in patients with LPR.

Despite the lack of evidence on prevalence, there is evidence that the human larynx may demonstrate sex related histological differences. Stell et al[30] in their study of 328 human larynges found the mucosa of the vestibular folds (false vocal cords) were significantly more likely to be entirely columnar epithelium and the laryngeal surface of the epiglottis more likely to possess more extensive, but not greater incidence of, islets of squamous epithelium in males. This may again provide a confounding variable, with greater proportion of males in the LPR group and histologically a greater proportion of squamous epithelium identified in the false vocal cord and medial arytenoid regions. However Stell's study was conducted more than 30 years ago, and as such, practices such as smoking may have had a greater influence on the male population undergoing autopsy than female. It is of note that our LPR group did possess a number of smokers. The effect of chronic irritation on the larynx has been well documented, and may lead to both squamous changes of respiratory epithelium through to laryngeal carcinoma[30,185]. Further examination of the data did not demonstrate that there was an over representation of smokers in the outliers. Whilst the role

of smoking in tumourgenesis is well documented, further research is now considering an additive effect of smoking and reflux to laryngeal injury[186]

It must also be made note of the number of significant outliers identified throughout many groups. In large population studies these may not contribute significantly to sway a statistical result, however with the low subject numbers in this study, including those discounted in the study due to poor RNA integrity, such outliers may have a significant effect on the analysis. In addition, such small population numbers may lead to missing a true statistical difference for some genes, with the false and true vocal cord biopsies being the most likely affected given their lower numbers of individual biopsies.

Patients were selected according to clinical history and examination, and on the RSI and RFS score. The diagnosis of LPR is typically a clinical decision, based on history and examination, with often a trial of PPI used to confirm. However to conduct research into LPR does not allow for the latter. The use of 24-hour pH probe has been utilised in earlier research for diagnosis and still nominally remains the "gold-standard" for diagnosis. It is becoming increasingly clear that the use of pH probes is not so much gold-standard but extremely limited, given that although the probe can detect acidic reflux, there is now a large body of evidence that this acid is not the sole aetiological factor with bile, pepsin and even air being considered as other causative agents[176]. Newer diagnostic techniques continue to be

developed, particularly multichannel intraluminal impedence monitoring[187] however their use is not yet widespread[176].

Regardless of these limitations, this study has demonstrated significant mucosal differences in a clinically diagnosed and symptomatic population when compared to control tissues from multiple sites in the larynx. To our knowledge, this is the first study of this type and has been possible because of the altruistic consent of those control patients who risked some extra discomfort and possible complication to facilitate this research.

A number of biopsies of the true vocal cord were found to have columnar in addition to squamous epithelium. This is likely to be due to the fact that biopsies were collected from the superior (and possibly too lateral) surface of the vocal fold, to avoid the very real risk of significant voice change if the free edge of the vocal cord was biopsied. This may well be the contributing factor why this study did not demonstrate the previously identified differences in gene expression in this region. In addition, there were a number of patient biopsies excluded from analysis due to the poor quality of extracted RNA. This reduction in the final number of tissue samples available for analysis has no doubt impacted upon the power of the study and may have affected the results drawn from the true vocal cord samples.

4.7 Summary

Overall, this study demonstrated a number of important differences in gene expression between a clinical population suffering from LPR and controls. Previous research has demonstrated that there are differences between the individual subsites of the larynx, however no single study has considered all areas together, A population of patients identified with LPR were compared to an asymptomatic group, with a mixed group. Samples from the 4 subsites of the larynx were biopsied under general anaesthetic following completion of the RSI questionnaire. The RFS was scored for each patient at the time of surgery. Sections of the samples were sent for histological analysis and prepared for quantitative real time reverse transcription PCR analysis on 20 previously identified genes.

The results from this project identified the mucosa of the medial arytenoid region of larynx as the sub-site with the most genes demonstrating a significant difference in gene expression. This is a novel finding, particularly given a large proportion of the literature on LPR has focussed on the posterior commissure, due to the well described hypertrophy which occurs in this area in the presence of LPR. The histopathology demonstrated this area to be overwhelmingly columnar epithelium in the normal group, however there was a notable increase in the presence of squamous epithelium in the patients with LPR, suggesting there may be metaplastic events occurring. How often, or over what time period is required to develop

this change is still to be determined, as is the point at which this injury becomes symptomatic. With increasing technology, the ability to both examine and biopsy the larynx is becoming easier. The advent of transnasal oesophagoscopy, including the ability to pass biopsy forceps through the scope would allow for biopsies without general anaesthesia. Such techniques are now possible, and would allow for possibly not only a definitive diagnosis, but the opportunity to monitor treatment effect. Our current findings would suggest that further research should include biopsies of the medial arytenoid area to identify further molecular changes. This area would be a safe and entirely suitable site for biopsying in order to monitor or research treatment effects. In addition, the ability to conduct such biopsies in as minimally invasive manner as possible would combat the small numbers limits this study.

4.8 Conclusions

This study has considered the first hypothesis, confirming that there are significant differences, both histologically and in gene expression, between the LPR group and the control group. This suggests that in a clinically diagnosed population, mucosal changes are identifiable in LPR.

In addition to the histological analysis, differences in expression of a number of inflammatory markers in the LPR group compared with controls was noted. *TGFβ-1*, *CD1d*, *CRNN* and *CDH-1* all demonstrated a significant

difference. However, a number of the cytokines related to the NF-κB inflammatory process demonstrated no significant difference between the two groups.

Secondly, this study found site-specific changes within the larynx, which are likely to be related to LPR. Hill et al.[131] suggested that the posterior commissure may not be specific enough to demonstrate changes in response to treatment. This study would suggest that the medial arytenoid is a region more sensitive to LPR changes than the posterior commissure for a number of reasons. Firstly as a mainly columnar epithelium histologically it is more susceptible to injury and secondly, it demonstrated the greatest molecular changes. A number of biomarkers have been identified, including *MUC2*, *5B*, *KRT14*, and inflammatory markers such as *CD1d*, *SEP53* and *TGFβ-1*.

5 APPENDICES

5.1 Appendix 1: Reflux Symptom Index

Within the last month, how did the following problems affect you? *Circle the appropriate response*	0 = No Problem 5 = Severe Problem					
1. Hoarseness or a problem with your voice	0	1	2	3	4	5
2. Clearing your throat	0	1	2	3	4	5
3. Excess throat mucus or post nasal drip	0	1	2	3	4	5
4. Difficulty swallowing food, liquids, or pills	0	1	2	3	4	5
5. Coughing after you ate, or after lying down	0	1	2	3	4	5
6. Breathing difficulties, or choking episodes	0	1	2	3	4	5
7. Troublesome or annoying cough	0	1	2	3	4	5
8. Sensations of something sticking in your throat, or a lump in your throat	0	1	2	3	4	5
9. Heartburn, chest pain, indigestion, or stomach acid coming up	0	1	2	3	4	5
TOTAL =						

Belafsky et al. 2002[60].

5.2 Appendix 2: Reflux Finding Score

The Reflux Finding Score (RFS)	
Subglottic edema	0 = absent 2 = present
Ventricular obliteration	0 = none 2 = partial 4 = complete
Erythema/hyperemia	0 = none 2 = arytenoids only 4 = diffuse
Vocal fold edema	0 = none 1 = mild 2 = moderate 3 = severe 4 = polypoid
Diffuse laryngeal edema	0 = none 1 = mild 2 = moderate 3 = severe 4 = obstructing
Posterior commissure hypertrophy	0 = none 1 = mild 2 = moderate 3 = severe 4 = obstruction
Granuloma/granulation tissue	0 = absent 2 = present
Thick endolaryngeal mucus	0 = absent 2 = present
TOTAL =	

5.3 Appendix 3: Professor J Wilson's Examiner's Report

FLINDERS UNIVERSITY

EXAMINER'S REPORT FORM

Examiner's Name: Professor Janet Wilson

Candidate's Name: John Wood

Degree for which thesis submitted: Master of Surgery

Thesis title: Biomarker expression in laryngopharyngeal reflux desease

1. Examiners are invited to make specific comments and suggestions on the report in the space provided hereunder. Additional comments may be attached on separate sheets but an explicit recommendation should be made in the place provided on the reverse of this form.

2. Would you please **submit the report by** **2 March 2013**

To: Ms Ashleigh Mernar
Faculty of Health Sciences
Flinders University
GPO Box 2100
ADELAIDE SA 5001 Australia

REPORT:

attached as separate Word doc

Report (cont)

After examination of the thesis I recommend that :
(Please tick as appropriate)

☐ (a) the degree for which the candidate has submitted this thesis for examination should be awarded, or

☑ (b) the degree for which the candidate has submitted this thesis for examination should be awarded, subject to the completion of amendments (specified by the examiner) to be carried out to the satisfaction of the supervisor and the Head of School (or nominee, where this is the same person);

***Note:** Amendments may range from the correction of spelling or typographical errors and small changes to the text, to changes to the structure and substance of some chapters of the thesis which could be completed to the satisfaction of the supervisor and the Head of School (or nominee, where this is the same person) without being returned to the examiner.*

or

☐ (c) the degree should not be awarded, but the candidate should be permitted to revise and re-submit the thesis or take a further examination, or both;

***Note:** This applies to changes which require a reshaping of the basic structure and substance of the thesis and extensive rewriting to bring it to an acceptable standard. In such cases the University expects that the re-submitted thesis would be examined by the original examiners, unless circumstances make this impossible or undesirable.*

or

☐ (d) in the case of a candidate who has submitted a thesis for the degree of Doctor of Philosophy, an appropriate Masters degree should be awarded, or

☐ (e) a degree should not be awarded and the candidate should not be allowed to present for the degree again.

(Delete as appropriate)

I ~~object~~/ do not object to my name being released to the candidate.

EXAMINER'S SIGNATURE: Jane A Wilson. DATE: 21/4/2013

Please note: the candidate's thesis should be returned with this form.

An investigation of Biomarkers in laryngopharyngeal reflux

Examiner report Prof Janet A Wilson Newcastle University 20th of April, 2013

Page xv "Reflux Finding Index" ? Reflux Finding Score

page 2, second sentence should read "the consequences of such refluxate **contribute** to the spectrum of upper aerodigestive tract inflammatory symptoms and **have** been associated"

page 3, "disease **as** a"

page 6, Koufman 2000. It is not strictly true to say that 50% of the series had "pH probe demonstrated reflux into the larynx". The proximal pH probe was placed in the hypopharynx behind the laryngeal inlet, 1 cm above the upper oesophageal sphincter. A single episode was regarded as abnormal.[Based on the analysis of 20 patients from the authors had reported more than 10 years previously.] In other words a further interpretation other than the two offered on this page is that there was some on-going lack of clarity about the precision of the normal range.

Page 8, sentence beginning the final paragraph requires to be rephrased.

Page 12, line nine should end "epiglottis to be lined"

page 18, idiopathic and eosinophilic cough should probably be included[1, 2].

Page 20, sentence referenced with 49 a noun is missing.

The first sentence is somewhat misleading. There is a distinction to be drawn between physiological post-prandial transient oesophageal sphincter relaxations and pathological levels of gastro-oesophageal reflux. If pathological reflux were present in 75% of the population then we could assume that a similar proportion of patients with voice disorders at the very least would, like their non-voice patient counterparts, have reflux but this is not really the thrust of the argument.

Page 22, as per the comment on page 6 it is not correct to say that Noordzij(3) measured laryngeal reflux. Reflux was measured 1 cm above the upper oesophageal sphincter. Eubanks (4) measured reflux just under 2 cm above and approximately 3 cm below the upper sphincter. There are in fact very few papers where reflux has been measured within the endo larynx i.e. beyond the aryepiglottic folds.

Page 23, line 2, have not has.

Page 24, line 2, verb missing after RFS. Line 6 ' missing at examiners.

Page 25, line 1, the correlation of the two measures provides some evidence of validity but not reproducibility.

Page 26, discussion of the general practice RSI scores. The correlation with BMI is not commented on. Presumably the association indicates some further validity of the RSI. However how much of this association was due to a change in the GORD item of the RSI and how much was due to the extra oesophageal

symptoms it contains? Oyer found that "The mean RSI of the +PSY group was higher than that of the -PSY group ($p < 0.05$), but the +PSY patients actually had a lower incidence of abnormal probe studies ($p < 0.02$).". You do not comment on this although I suggest that it was due to a greater tendency towards symptom reporting in those with an affective disorder.

Page 29, sentence "this is in contrast" does not make sense.

Page 32 try to avoid consecutive sentences beginning with however. The second one could begin "for example".

In general there is probably an excessive use of 'however' throughout.

Page 33, second sentence is slightly ambiguous. Was there a context to the sentence which was deleted? Or should it read there seems rather than there seemed?

I think sentence has been missed. Second last line of the first paragraph again I suggest replacing the word normal with physiological.

In the second paragraph the use of the word buffer here I think is inappropriate. Bulmer's group use the word buffer to describe the control condition in a pH of 7.4.

Page 34, the first use of reference 73 should be reference 72 on the activity and stability of pepsin.

Last sentence on that page needs to be rephrased. First of all the use of the term pH twice is redundant and secondly I think the meaning needs to be clearer. The external exposure of the larynx is to pepsin in a pH range at which it is inactive, whereas the intracellular Micro environment allows the acidification of pepsin.

Page 35, the key reference to the epithelial stress proteins is I think a different reference(5).

Page 36, reference is made to ref 76 – the correct spelling of senior author =Sasaki. This is on p. 153. On the same page there is a typo in the journal title of reference 78.

Page 37, the penultimate sentence of the top paragraph is incomplete.

Page 38, five lines from the foot spelling laryngopharynx.

Page 39, top line fails, not fail. The subject is monitoring.

Page 40, 1.8.3, Duration of pressure – which pressure? 1.8.4 word missing in last sentence.

Page 40-1 clarify the distinction between columnar-lining and intestinal metaplasia.

Page 41, line 3 do you mean intercellular gaps?

Page 42, third last line, effects not effect.

Page 43, sentence beginning "oesophageal damage" more or less replicates the sentence on page 41 referenced to number 92. Same paragraph after the duplicate period sign, the sentence beginning "It was noted" needs to be rephrased. So does the last sentence in that paragraph there the double use of marker could be improved upon.

Page 44, top line- from patients (no, after patients). The first line of the next paragraph needs to be rephrased. There are few places where the conditional tense is used twice in this way. It would be cleaner to

say "it is reasonable to propose that similar patterns of gene expression may be responsible". "Which is proposed as a similar aetiology" does not make sense. Do you mean " which has been considered to have"?
Page 46, middle of page "increased in expression and to undergo".
Page 47, line 9, larynx possesses. This study which is lacking is not strictly an epidemiological study but rather a large scale characterisation study.
Page 49, The third paragraph last sentence in addition is repeated.
Page 54, third paragraph first sentence- Commencement and commenced are not both needed in this sentence, nor is the final "is". The final sentence of this paragraph effectively repeats the final sentence of the paragraph above.
Page 56, the word to should be of.
Page 57, the reference to a significant placebo effect is incorrect here. The control group had no treatment and therefore cannot have had a placebo effect unless it is postulated that they were getting some the key is support from the follow-up process. I agree there is a placebo effect in the treatment of throat symptoms which is quite sizeable and has been estimated in other studies where a placebo was in fact used.
Page 58, line 3, omit "the benefits of". I think reference missing that has been designated 131. There is the literature describing the effect of anxiolytic medication on oesophageal muscle contractions (going back many years). We cannot be sure that because of medication has a primary psychotropic use it does not also have autonomic effects which could influence the biology of reflux.

Page 59, line 9, either? In the following sentence demonstrated is used twice, the second instance could be deleted.

Page 63, line 1 "if there is a group".

Methods

page 65 what information was given to the patients in respect of the impact of true vocal cord biopsy on post endoscopy voice quality? (Accepting that you have tried to avoid the free margin). Is there any exclusion for example of performing / professional voice users? I think it would be helpful to include the inclusion and exclusion criteria as presented to the ethics committee either here or as an appendix. How did you take account of the fact that the reflux finding scoring was not an upfront assessment by an independent observer but was being undertaken by the researchers in a group of patients whom you already strongly suspected might be suffering from LPR? It is also not clear how you dealt with patients who may have been identified from the non-otolaryngology waiting lists who turned out to have symptoms or signs of reflux? Did some of these end up in the intermediate group? Or even in the index group? Was the protocol to assess the larynx in these patients with flexible endoscopy prior to their GA or was the laryngeal

assessment done under anaesthetic? This needs to be clarified. I assume that the otolaryngology control recruits had had the endoscopy but what about the orthopaedic and general surgery patients? How many were in fact recruited from each of these three sources?

I think this is very important because you are going on to ascribe considerable significance to the differences between what at the end of the day were small groups of patients, whose age and sexes were not matched. So we need to have a high level of clarity about the derivation of the comparison groups.

The Devil's Advocate position or the sceptical position if you like is that the RSI is a measure of throat symptoms, albeit with a single item adding in gastro-oesophageal reflux. Second at the RFS is a measure of laryngitis and that neither of these instruments links directly to reflux. So the most parsimonious interpretation of your design is that you have identified a group who definitely have symptoms and signs of throat abnormality and a group who do not and that it is on their differences that you will proceed to draw conclusions.

Page 66, line 7 "were typically conducted".

Page 74, penultimate line, was also conducted.

Page 75, 2.8 line 1, were reviewed. Final line – less than.

Page 78, demographics. As indicated above, I think the reader would welcome greater clarity about the selection process. I think it is appropriate that we should see in more detail how the 46 left after exclusion of the non-biopsy patients were distributed. How many were recruited as controls and how many patients? Following analysis of clinical features, there were 27 patients in the intermediate group. How many of these came from what was originally described as the control group and how many from the ENT patients suspected of having LPR? I think a flow diagram would be very helpful to summarise these points. Also it would give some additional information on the RSI and RFS scores of patients having completely unrelated interventions.

Page 80, sex ratio. The appropriate test is chi-square test which like your Mann-Whitney is not significant at present sample size. Obviously however you do have twice as many males as females in the LPR group while the converse is true in the non-LPR group. With a bigger sample size this will be statistically different. Given that the larynx is a hormonally sensitive organ this difference should feature in the discussion.

Page 81, Histological analysis I do not think you say at any point how many of these patients were smokers If you are going to ascribe significance to squamous epithelium this is an important consideration and requires to be reported.

Page 82, it is difficult to orientate the false cord biopsy. The true cord specimen likewise would benefit from having the two different sorts of epithelium labelled. If this is a biopsy from the upper surface how are the various layers shown orientated?

Page 83, these specimens are somewhat different because they all appear to have a tissue air interface. Was any of these specimens regarded by your pathologist as being dysplastic?

I am not a laboratory scientist but with reference to table 5 on page 85 it is customary to show data as well as the probability of there being a statistical difference. The data in figure 12 should therefore be shown alongside the relevant table or at least on a succeeding page. As far as I can see you do not make any explanatory comment as to why some of the tests were performed in only a proportion. This should be included here.

Taking table 6 on page 88 as a whole, it is clear that the majority of the differences are situated in the medial arytenoid, as you comment yourself at the foot of page 87.

Was there any difference in the presence of an endotracheal tube which might have been in contact with the medial arytenoid between those who were designated as LPR sufferers and those designated as controls?

In respect of the Posterior Commissure, as you have 19 comparisons one would expect by chance one would be statistically significant.

Considering the graphs, for quite a number of assays there is a single outlying control with a very high value. Is this always the same individual? It would be customary to comment on this in the results particularly as the values seem in some cases several times higher than any observed even in the index group. For example the MUC5B relative expression on page 99, this would be much less significant were that outlier to be excluded. The CD1d relative expression on the other hand looks to be quite distinctly different between the two groups in the arytenoid area. The graphs would be easier to relate back to the table if the two forms of data presentation were shown in the same order!!

In the table it would be helpful for the inflammatory markers at least to indicate that one of the three markers is higher in the patient group, 2 lower.

Discussion

page 110, line 2, we do not know that the control population were not refluxers. They could have been silent refluxers. It is only acceptable to say they were a group of patients lacking conventionally accepted symptoms and signs of LPR. As stated above I'm concerned that the differences are confined to the medial

arytenoid area and that it has not been made entirely explicit that the proportion of patients with endotracheal tubes in contact with this region was the same in both groups.

You consider the potential role of cigarette smoking here but as stated above I'm not sure that we are ever told how many of each group were current or past cigarette smokers. I think there is also literature indicating that the use of asthma inhalers may generate epithelial changes. I recall an abstract by Prof CE Pope but I cannot find the reference at the present time.

Page 112, final lines. The finding of metaplasia should be included in the results. I'm not sure what the direction of travel of this discussion of the markers for squamous change is. It would be helpful at the outset of this section 4.2, to have a general statement on how you interpret the findings with respect to both of the cytokeratins and the fact that they are differently different between the two groups. In particular this refers to the second paragraph on page 112.

Page 113, to what extent did you observe a corresponding hyperplasia in your own participants?

Page 114, as above it would be helpful to know whether there was any visible inflammatory infiltrate or any difference in the degree of the presence of inflammatory cells between the two groups or indeed given the very small numbers possibly a correlation between the numbers and the levels in each specimen.

Page 115, the scatter grams for the two cytokines seem to indicate 2 individuals, 1 in each group, who are high outliers. Is the identity of the top scorer in each group at each site the same person or was the highest level at the four sites observed in a number of different individuals?

Page 116, as per my comment above, was there any histological evidence of chronic inflammation?

Page 117, third last line enzymes

page 118, second line should read in both animals and humans rather than in both animal and human studies to make sense. 5th line, second paragraph, should say an increase in the expression.

Page 121, is it true to say that there was a lower expression in the LPR group in the medial arytenoid region? The difference is I agree greatest at this site, but that surely is mostly due to the fact that about half of the medial arytenoid control samples had elevated expression (which might be associated with lower susceptibility to mucosal damage).

Page 122, last line, it's has no' unless it is a contraction of it is.

Page 123, the last few lines rightly belong in the results.

Page 124, first paragraph, Should be - there are further implications

page 126, penultimate paragraph, omit in at "in one study". Although as you say in the true and false vocal cords the LPR cohort had lower C A 3 gene expression than the controls, it is also worth commenting on the relative expression in the controls among the four sites it looks as if the levels were highest in the true cord followed by the false cord and then the posterior areas. How might you explain this?

Page 127, 4.4.3. It would be helpful to summarise the overall patterns observed before embarking on this discussion. Essentially the diagrams on page 104 indicate that there was very little expression in the true and false cords, that both groups showed relatively high levels with the wide distribution in the posterior commissure. The majority of medial arytenoid expression was in the LPR group and to a lesser extent than in the posterior commissure.

Page 130, final sentence of 4.4.4 needs to be rephrased. 4.4.5 middle of the first paragraph junctions should be plural. Final paragraph first sentence also needs rephrased

page 131, sentence beginning "using a computer" does not have a principal verb. I do not understand the final paragraph of this page. Gill et al demonstrated reduced e cadherin in LPR. Your graph on page 108 appears in the medial arytenoid region to show a marked reduction in the LPR group such that they are all clustered **below** the range of the controls (less one exceptional control with zero expression). Yet you say in the text that you demonstrated **higher** expression in the LPR group.

Page 132, 4.5 line 3, suggest "most have considered"

fourth line from the bottom, the greatest number of differences.

Page 133, line 1, alternatively it could reflect the age difference between LPR and controls given that the arytenoids can be an area of high pressure contact particularly in cough, and also particularly in men who were the predominant gender in the "LPR" group. You refer to this under limitations but in some ways it reflects a more systematic issue around the interpretation of your results which centres on attempts to map the changes observed to those that others have observed in exploring the LPR case.

You say that the medial arytenoid is closest to the posterior commissure which may explain its changes however one might have expected these to be maximal in the posterior commissure **itself** rather than the area "closest to the posterior commissure"? - I realise to some extent you account for this by saying that the posterior commissure is more squamous and therefore the markers are different but if postulating that the epithelium change is as a longer term result of the same insult, it becomes a rather tenuous argument in the absence of any longitudinal observation.

7

I agree with your comments at the foot of the page about the influence of outliers. We are told however very little about the characterisation of the patients you studied from your original cohort of recruits, and therefore no further comment is possible.

4.6 **limitations**

the fact that there are clearly documented potential age differences make it all the more surprising that you did not make more strenuous efforts to age match your controls. Over what period of time was the cohort recruited?

Page 136, second paragraph, the issue here is not so much whether there are gender differences in LPR but, given the uncertain nature of the LPR condition, although there are gender differences in other causes of change which might be open to misinterpretation as LPR. Your comments about on smoking allude to this but again I'm not certain as to why there is no indication of the smoking history in **your** sample. I believe it is only on page 138 we learn that patients excluded were omitted because of the poor quality of extracted RNA. This should be expanded upon in the methods section.

Page 139, the summary or what in effect should be a general synthesis is too brief. You have studied a great many factors in a small number of subjects with potential confounding variables.

Some form of unifying statement as to how this particular work has corroborated contradicted or added to existing literature is required. Also some form of either diagrammatic or verbal description of a working hypothesis which would unite the major components of your research is required. You refer to papers where trans nasal fibre-optic biopsies were taken. Do you feel that this is a way forward which would get round some of the problems of small numbers in the present series? Does Bioinformatic array have a place here? I think it is also worth some further reflection on the principle of gender here.

You have given quite some consideration to the cancer progression aspects of this although as you rightly state there is a very weak link presently in the evidence base between neoplasia and any exposure to chemical irritant in gastric secretion. Indeed it is quite possible that some of the reflux link may be a reflex phenomenon whereby excess coughing may be caused by vehicle stimulation than them being been no direct chemical affliction in the larynx.

We all know that adenocarcinoma of the lower third of the oesophagus is much more prevalent in men and occurs about a decade earlier in men than in women. Within the larynx glottic tumours have a massive male preponderance but the relative proportion of women in the supraglottis is higher. This changes which you observe in the false cord you might wish to relate back to gender. I appreciate it is difficult because of the

small numbers but as far as I can see you have not explored other potential influences on your markers than your original categorisation as control versus LPR.

Given that we as readers do not exactly see how the two cohorts were derived, and given the rather soft criteria of RSI and RFS, I think it would be valuable to look at age and sex effects or at least do a preliminary comparison along these lines to see if either is worth pursuing.

Reference 119,? The journal title

reference 144 is now in print(6)

it is preferable to have a standardised different format with all journal titles either spelt in full or abbreviated

References

1. Jaimes-Hernandez J, Mendoza-Fuentes A, Melendez-Mercado CI, Aranda-Pereira P. Chronic eosinophilic pneumonia: autoimmune phenomenon or immunoallergic disease? Case report and literature review. Reumatologia clinica. 2012 May-Jun;8(3):145-8. PubMed PMID: 22196999. Epub 2011/12/27. eng.
2. Morice AH, McGarvey LP, Dicpinigaitis PV. Cough hypersensitivity syndrome is an important clinical concept: a pro/con debate. Lung. 2012 Feb;190(1):3-9. PubMed PMID: 22186805. Epub 2011/12/22. eng.
3. Noordzij JP, Khidr A, Desper E, Meek RB, Reibel JF, Levine PA. Correlation of pH probe-measured laryngopharyngeal reflux with symptoms and signs of reflux laryngitis. Laryngoscope. 2002 2002;112(12):2192-5. PubMed PMID: MEDLINE:12461340.
4. Eubanks TR, Omelanczuk PE, Maronian N, Hillel A, Pope CE, 2nd, Pellegrini CA. Pharyngeal pH monitoring in 222 patients with suspected laryngeal reflux. J Gastrointest Surg. 2001 2001;5(2):183-90; discussion 90-1. PubMed PMID: MEDLINE:11331482.
5. Johnston N, Dettmar PW, Lively MO, Postma GN, Bellafsky PC, Birchall M, et al. Effect of pepsin on laryngeal stress protein (Sep70, Sep53, and Hsp70) response: role in laryngopharyngeal reflux disease. Annals of Otology, Rhinology & Laryngology. 2006;115(1):47-58. PubMed PMID: 16466100.
6. Ferlito A, Devaney KO, Woolgar JA, Slootweg PJ, Paleri V, Takes RP, et al. Squamous epithelial changes of the larynx: diagnosis and therapy. Head Neck. 2012 Dec;34(12):1810-6. PubMed PMID: 21971762. Epub 2011/10/06. eng.

5.4 Appendix 4: Response to Professor Wilson's Report

Prof Wilsons's Comments	**Corrections/Response**
Page xv "Reflux Finding Index" ?Reflux finding score	Changed to "Reflux finding score"
Page 2, "the consequences of such refluxate **contribute** to the spectrum of upper aerodigestive tract inflammatory symptoms and **have** been associated	Changed to "the consequences of such refluxate **contribute** to the spectrum of upper aerodigestive tract inflammatory symptoms and **have** been associated"
Page 3, "disease **as** a"	Changed to "disease **as** a"
Page 6 Koufman 2000. It is not strictly true to say 50% of the series had "pH probe demonstrated reflux into the larynx…"	The issues related to pH probe and lack of clarity regarding precision of normal range are addressed later in the thesis in section 1.6.1 pH Monitoring.
Page 8, sentence beginning the final paragraph to be rephrased	Changed to "The larynx is composed of 3 single cartilages, 3 paired cartilages, and intrinsic and extrinsic muscles with a mucosal coverage"
Page 12, line nine "epiglottis to **be** lined"	Changed to "epiglottis to **be** lined"
Page 18 "idiopathic and eosinophilic cough should probably be included"	Change to "such as angiotensin-converting enzyme inhibitors **and rare conditions such as chronic eosinophilc pneumonia**"
Page 20, sentenced referenced with 49, a noun is missing	Changed to "to a known carcinogen 7,12-dimethyobenzthracene, found the subsequent application of pepsin lead to…"
Page 22. The first sentence is somewhat misleading	Changed to "Asymptomatic gastroesophageal reflux has been reported in 65-75% of normal individuals…"
Page 22. As per comment page 6	Acknowledges the difficulty, and lack of research into measuring endolaryngeal reflux.
Page 23 "have" not "has"	Changed to "have"
Page 24 verb missing after RFS	Changed to "the RFS **was** more sensitive…"
Page 25 the correlation of the two measures provides some evidence of validity but not reproducibility	Belafsky (2002) found good construct validity in comparison between the RSI and Voice Handicap Index. Additionally Belafsky et al found the measure was highly reproducible, comparing pre-treatment groups. Changed to "…between pre-treatment scores, **concluding the measure possessed** a high level of reproducibility."

Page 26 Oyer et al. “The mean RSI of the +PSY group was higher than that of the –PSY group…?	Added “. This indicates that those with psychiatric disorders may have a lower threshold for reporting such symptoms.”
Page 29 sentence “This is in contrast” does not make sense	Changed to “Comparatively, a sulcus vocalis stops at the vocal process and is found in the striking zone.”
Page 32 consecutive sentences beginning with “however”	Changed to “For example,…”
Page 33, “seems” rather than “seemed”	Changed to “there seems to be…”
Page 33 suggest replacing word normal with physiological	Changed to “…may be considered physiologically normal…”
Page 33 inappropriate use of “buffer”	Changed to “following incubation in a **test solution** of pH 4.0…”
Page 34 First use of ref 73 should be reference 72	Changed to add ref 72.
Page 34: last sentence should be rephrased	Changed to “The pH of intracellular structures such as Golgi bodies and lysosomes lie between 4.0 to 5.0. Whilst the laryngeal mucosa is exposed to inactive pepsin, intracellular uptake into this micro-environment allows for the acidification of pepsin. This may lead to intracellular injury.”
Page 35. Key reference to epithelial stress protein is 5	Key reference is “Effect of pepsin in laryngeal stress protein (Sep 70, Sep53, Hsp 70).
Page 36 ref 76 – author incorrectly spelt, typo Ref 78	Endnote updated, Changed on references
Page 37. Sentence incomplete	Changed to “Furthermore, a recent study noted that whilst pepsin activity was pH dependent, bile acids did not attenuate the activity of pepsin.”
Page 38 typo	Changed to “laryngopharynx”
Page 39 “fails” not “fail”	Changed to “monitoring over short periods fails to find a significant difference”
Page 40 Duration of pressure – which pressure?	Changed to “The UOS tonically constricts, relaxing to allow boluses of food or fluids with swallowing. Studies measuring UOS pressures found similar average pressure levels in patients with LPR to controls[88,93]. However whilst the average pressure itself was not significantly different, the duration of tonic pressure was nearly double in the control group compared to a group with GORD”
Page 40-1 clarify intestinal metaplasia	Changed to “ injuries to this lining can occur at the histological and microscopic level and is recognized in the presence of inflammation.”
Page 41 “changes in intra-	Change to “changes in intercellular gaps”

cellular gaps”	
Page 42 third last line “effects” not effect	Changed to “effects”
Page 43 “it was noted..” to be rephrased	Changed to “..a progressive increase in *IL-8* mRNA expression was found, corresponding to worsening mucosal injury, with the highest expression found in adenocarinoma.”
Page 44 “from patients”	Changed to from patients
Page 44 rephrase 2nd paragraph first sentence	Changed to: “It would be reasonable to suggest similar patterns of gene expression would be responsible for mediating inflammation in LPR.”
Page 46. “increased in expression and to undergoes…”	Changed to “increased in expression and to undergo…”
Page 47 “larynx possesses”	Changed to “possesses”
Page 47 the “lacking” study is not strictly epidemiological	Changed to “although a large scale study looking at epithelial type in all areas of the larynx in LPR is currently lacking”
Page 49 “in addition” is repeated	Changed to “**Furthermore** *MUC5B* expression has …
Page 54 commenced used twice in same sentence	Changed to “Despite this, a proton pump inhibitor is typically commenced in the clinical situation…”
Page 56 the word to should be of	Changed to “an increased risk of Salmonella infection and **of** the development of C diff…”
Page 57 “reference to a significant placebo effect here is incorrect”	Changed to: “This would indicate that there may be a symptomatic improvement, without any significant change in examination findings. This may be due, in part, to a placebo type effect, or represent”
Page 58 “omit the benefits of”	Changed to “Whilst commencing such medication…”
Page 59, line 9 “either.”	Deleted “either”
Page 63 “if there is”	Changed to “if there is a group”
Page 67 “were typically conducted”	Changed to “were typically conducted”
Page 74 penultimate line “was also conducted”	Remains as “A number of duplicate reactions were also conducted…”
Page 75 2.8 Line 1 “were reviewed,” & final line “less than”	Changed to “were reviewed…” and “less than 0.05”
Page 78. Information regarding control group patients and normal ENT patients	Population is as described. Addition of smoking numbers.
Page 80 “discussion should include female:male ratio”	Discussed in 4.6 Limitations. Chi-squared calculated for table 3 Demographics
Page 81 – how many were smokers	Smoking numbers added. And discussed further in limitations

Page 82 – difficult to orientate false cord biopsy, label true cord specimen epithelium	Arrows identifying epithelium placed.
Page 83	Added "No samples demonstrated any evidence of dysplasia
Page 85 – table 5	Table utilized to demonstrate relative expression of HPRT is similar between each subsite. P-values demonstrate this.
Page 88, medial arytenoid – was there any difference between endotracheal tube	Response: as previously mentioned all patients had an endotracheal tube. Type and size of endotracheal tube was not recorded in the study
Discussion of graphs	Outlier discussed in the Discussion (ie no single patient), and graph order re-arranged to match table.
Page 110, line 2	Changed "non-refluxers" to "asymptomatic"
Page 112. Finding of metaplasia should be included in the results.	Evidence of metaplasia already discussed in results (2nd last sentence of Histopathology results).
Page 113. To what extent did you observe hyperplasia	Hyperplasia was noted in 3 specimens histologically – all from the posterior commissure region.
Page 114. ?any inflammatory infiltrate or presence of inflammatory cells	Both LPR and non-LPR demonstrated signs of inflammation to a similar amount. (See table at end of response)
Page 115. Scatter grams for 2 cytokines indicate 2 individuals one in each group who are high outliers – are they the same person?	Different individuals as mentioned in discussion.
Page 116. ?chronic inflammation	As above
Page 117. Third last line: enzyme	Changed to "enzymes"
Page 118.	Changed to "…in both animals and humans and conversely, in some studies…"
Page 121. –is it true to say there was lower expression in LPR group (or higher expression in non-LPR) of TGFB-1. Half of patients in non-LPR are elevated cf LPR	Added: "On closer analysis, it is of note that more than half of the non-LPR group had a relatively elevated expression of *TGFβ-1* compared to the LPR group, with the resulting statistically significant result. However there appeared to be a bi-modal distribution in the non-LPR group, the significance of which is uncertain. Consequently further research is required to determine the relative expression of *TGFβ-1* in both a non-LPR population, and consequently if there is significantly less expression

	in LPR patients of *TGFβ-1* in the medial arytenoid."
Page 122	Changed to "Its"
Page 123 the last few lines belong in results	Moved to end of results section and reworded.
Page 124: first paragraph – change "is" to "are"	Changed to "there are further implications"
Page 126 "omit "in one study"	Changed to "Comparing biopsies from the laryngeal ventricle, vocal fold…"
Page 126 Comment on expression of CAIII in each site	There was a slightly higher expression of *CA3* noted in the controls group for the true cord compared with the remainder of the larynx. This may occur as the true cord lacks significant other methods of defences. There are no goblet cells present, such as in the columnar epithelium of the medial arytenoid and false cord. Changed to "Expression of *CA3* in the control group demonstrated a slightly higher expression in the true cord than in the other regions. This may represent one of the few intrinsic defences for the true cord region, which relies on other areas of the larynx to produce protective mucin. The variability of expression of *CA3* in the normal larynx is lacking in current literature, however early studies into *CA3* expression noted its depletion in patients with LPR[110]."
Page 126 (Section 4.3.3 CRNN):	Added: "It is of note that there was higher relative expression of *SEP53* in the posterior commissure region compared to the other regions of the larynx in both groups. It is possible that this may be due to this area being the most susceptible to injury through refluxate, in addition to being squamous epithelium. This study identified a significant difference in *SEP53* in the medial arytenoid region in the LPR group, with higher expression noted in this group"
Page 130, final sentence 4.4.4 to rephrase. 4.4.5 middle 1st paragraph should be plural. Final paragraph first sentence should be rephrased.	Changed to: ", however it is of note that…" Changed to "Dilatation of intercellular spaces (DIS) between squamous epithelial cells has been identified in studies of reflux exposed oesophageal mucosa
Page 131 Sentence starting "using a computer" lacks a verb	Changed to "Using a computer assisted morphometric system a statistically significant difference between the patients with LPR and the control group was identified"
Page 131 – Final paragraph not consistent -	CDH1 graph incorrect results – replaced with correct graph. Text otherwise as intended.
Page 132 4.5 Line 3 Suggest "Most have	Prefer to remain as "…the majority of these have considered…"

considered"	
Page 132 the "greatest number…"	Changed to "the greatest number of differences…."
Page 133: Could changes be related to age.	The difference between median age in the groups was addressed in the limitation section. It is acknowledged that such a difference may bias the results, particularly given the small sample. In addition, it is acknowledged that the difference in distribution of sex between the two groups may also play a role.
Page 133: Medial arytenoid being closest to post. Commissure: Why don't you expect the PC to have changes. (second paragraph)	This paragraph compares the results of gene expression between the 2 areas of columnar epithelium – the false cord and the medial arytenoid, and provides a suggestion as to why the MA region demonstrated greater change than the FC, lying closer to the oesophagous. That the posterior commissure is squamous in epithelium means it has a better protection, but still demonstrated evidence of differences in gene expression, consistent with hypertrophy. This study considered each patient only once, and as such the opportunity for identifying longitudinal change was not possible. However this study does now provide the framework for future studies to monitor molecular changes, potentially in response to treatment.
Page 135. Age differences. Why were the patients not matched?	There is a difference between the median ages for each group, which may bias the results. However a matched-pairs design was not intended for the study at this stage. However, the benefits of such a study are recognised, and may provide more robust results.
Page 136: Pts excluded because of poor quality RNA	This is discussed in 2.6 RNA Bioanalysis. Samples, (rather than patients) with RIN less than 5 were not utilised for further analysis. Sentence added to this section to clarify this.
Page 139 Summary is too brief.	Summary expanded. Future possibilities considered. Also consideration of transnasal oesophagoscopy + biopsies considered.
Page 139 Consider age and sex effects	Added: "However it is well recognised that the larynx is a hormone sensitive organ, with identifiable changes attributable to testosterone, progesterone and oestrogen[184]. Such changes may be represented by changes in gene expression throughout the larynx, or even a change in susceptibility to damaging agents. Furthermore there is evidence that the greatest voice change after puberty occurs in females after menopause related to both hormone changes and subsequent muscular atrophy[184] Consequently as further

	research identifies specific sub-groups of LPR, both gender and age may become increasingly important. With a larger numbers future studies would be able to assess whether such changes contribute to any significant differences in gene expression in patients with LPR."
Ref 119	Journal title corrected
Ref 144	Now in print – updated
Journal titles	Correction to abbreviate all titles.

Histological Evidence of Inflammation

	Non-LPR Group			LPR Group		
	-	+/-	+	-	+/-	+
Medial Arytenoid	1	4	1	0	2	1
False Vocal Cord	0	1	1	0	3	0
True Vocal Cord	0	0	0	0	1	1
Posterior Commissure	0	0	0	0	1	1

5.5 Appendix 5: Mr G Rees' Examiner's Report

FLINDERS UNIVERSITY

EXAMINER'S REPORT FORM

RECEIVED
03 JUL 2013

Examiner's Name: Dr Guy Rees

Candidate's Name: Dr John Wood

Degree for which thesis submitted: Master of Surgery

Thesis title: Biomarker expression in laryngopharyngeal reflux disease

---

1. Examiners are invited to make specific comments and suggestions on the report in the space provided hereunder. Additional comments may be attached on separate sheets but an explicit recommendation should be made in the place provided on the reverse of this form.

2. Would you please **submit the report by** **8 June 2013**

To: Ms Ashleigh Merriel
Faculty of Health Sciences
Flinders University
GPO Box 2100
ADELAIDE SA 5001 Australia

REPORT: [illegible]

Report (cont)

An Investigation of Biomarkers in Laryngopharyngeal Reflux

Master of Surgery thesis
John M Wood

Review by Guy Rees July 2013

Spelling and grammatical errors

Abstract

Page xv, para 1, line 2:
Laryngopharyngeal reflux (LPR) is an increasingly diagnosed disease in Otolaryngology, however **(it)** is a highly controversial one

Page xvi, para 3, line 1:
Carbonic anhydrase (CA) is an integral component of laryngeal **defense**. This spelling is not consistent with the predominantly English rather that American format throughout the thesis. I would recommend the word **defence** be used.

Main body of thesis

Page 16, 1.4.2 Globus pharyngeus, line 5
'..and naming the symptom globus hystericus, having been **lin(k)ed** with uterine dysfunction...'

Page 17, 1.4.3 Dysphagia, line 11
'...or finally **(, from)** upper oesophageal sphincter dysfunction.

Page 19, 1.5 Consequences line 4-5
'The idea of reflux into the laryngopharynx was considered in the otolaryngological literature as far back as 1968,..' **Is there a quote missing from this point?**

Page 19, line 7
'The latter of these was reported in 1985 with a case of recalcitrant subglottic stenosis...' **again, missing quote?**

Page 19, Para 2 line 6
'..those with purulent effusions were more likely to be pepsin **"positive [44]"**

Should be 'positive'[44]

Page 21, 1.6.1 pH Monitoring, line 3-4

'...test, yet has demonstrated significant problems, however its use stems from its ubiquitous use in GORD.'
Suggest re-write as: '...test, yet it has significant problems. This may relate to the ubiquitous use of pH monitoring in the diagnosis if GORD.'

Page 23, para 2, line 6
'Despite well documented limitations (,) these still remain the best (,) standardi(z)ed of LPR...)
Again, lack of conformity in test to English vs American text and suggest utilise comma one and drop comma two.

Page 23, para 2, line 8-9
'It is well known that LPR is difficult to accurately diagnose on a single modality and recent studies have suggested combing two modalities..'

Re write as: 'It is well known that LPR is difficult to accurately diagnose **with** a single **investigational** modality, and recent studies have suggested **that combining** two modalities..'

Page 29 line 8
'...found in the striking.'
Re write as '...found in the striking **zone**.'

Page 29 line 14
'...aging larynx...'
Rewrite as '...**ageing** larynx...'

Page 33. 1.7.1.1 Acid. Para 1 , line 4
'...of the role for acid, with a response to a trial of PPI...'
Rewrite as '...of the role for acid, with a **positive** response to a trial of PPI...'

Page 33, para 1, line 8-9
'Whilst up to 50 episodes of reflux in the oesophagus may be normal, Koufman described as few as 3 episodes per week may lead to laryngeal injury'.
Rewrite as :
Whilst up to 50 episodes of reflux in the **distal** oesophagus may be normal, Koufman described as few as 3 episodes per week in the **proximal oesophagus** may lead to laryngeal injury'.

Page 39. 1.8.2 Oesophageal motor function and acid clearance. Line 13-17
'Whilst GORD is manifest by significant exposure of the oesophagus to acid reflux, a reduced exposure time experienced by patients with LPR may rovide enough refluxate to damage the larynx, without causing typical oesophageal symptoms associated with GORD'
I think there should be a quote to back this statement up, central as it is to the argument made by the author.

Page 43, para 2, line 5
'...corresponding to worsening mucosal injury [98]..'

Second fullstop to be removed.

Page 44, para2, line 3
'...as a similar aetiology, just higher anatomically'
Reads better as: '...as a similar aetiology, just at a more proximal anatomic site.'

Page 72, para 2, line 5
'...was removed an placed in an eppendorf tube...'
Probably should be re-written as : '...was removed an placed in an **Eppendorf** tube...'

Scientific construct of thesis

In the study described by Dr Wood, patients presenting to an ENT clinic with laryngopharyngeal symptoms were assessed by a Reflux Symptom Index (RSI). Following this, they had a fibroptic nasolaryngoscopy in the clinic and had scoring of the mucosal appearance using a Reflux Finding Score (RFS). Following this, patients were admitted for microlaryngoscopy and biopsies were taken of planned areas within the larynx. This tissue was then examined histologically and for inflammatory changes using gene expression markers.

In the results of this investigation, Dr Wood proposes that patients with a combination of elevated RSI and RFS scores suggestive of laryngopharyngeal reflux (LPR) be termed the LPR group, and those with low RSI and RFS scores be the control group.

In comparison of the two groups, differences in histopathology and gene expression of inflammatory and mucosal defenses (sic) are noted for some sub sites within the larynx.

In the discussion of these differences, Dr Wood, proposes that LPR causes laryngeal changes, which can be identified through the histologic and gene expression variation.

As the author mentions, repeatedly through the text, the diagnosis of LPR is difficult to make, despite the use of the RSI and RFS scoring systems, pH monitoring and response to therapeutic challenge. Dr Wood has gone to some length to support his theory that the RFS and RSI scores can be used as surrogates for the presence of LPR. This is the first major challenge to the construct of the paper. Ideally, all patients would have had a pre biopsy 24 hr pH study with dual / multichannel monitoring to establish the presence and severity of reflux. In addition, a repeat of the RFS and RSI scores following a period of treatment of reflux as a therapeutic trial may have added to the validity of the diagnosis of LPR.

On reading Peter Belafsky's paper looking at RSI (Journal of Voice 2002), it is interesting to note that his patient group (n=25) had all had a dual channel pH study where a diagnosis of reflux was made. It does not describe what threshold

was used to make this diagnosis, nor any measure of manometric changes. He then uses a therapeutic trial of this group of patients and compares their voice changes (Voice Handicap Index - VHI) with his Reflux Symptom Index after a period of therapy. A correlation of changes of VHI and RSI is used to give validation to the RSI scoring system. In this study, the use of a control group (normal VHI / RSI) and a placebo arm to the study (PPI vs placebo) may have improved the validation of this scoring system.

Peter Belafsky's other paper, developing a Reflux Finding Score was published in Laryngoscope in 2001, and documents 40 patients who had LPR diagnosed by dual channel pH probe (again, no actual results of the study and no manometry) who were examined sequentially during therapy for LPR with PPI. The construct of this research is stronger, as it uses photodocumentation and recording of the RFS by two ORL specialists, who were blinded to the patient identity and timing of the examination. Although the study used four time points (pre treatment and 2,4 and 6 months post onset of treatment), there was no placebo arm to the study. Also, it is interesting that Dr Belafsky attributes the response seen to the use of PPI therapy, and does not mention any Speech Pathology therapy during the study. It is highly likely that patients would have been given information and probably treated by a speech pathologist during the study, and so the impact of PPI as the sole agent causing improvement in RFS cannot be accepted.

Despite the criticism of the RSI and RFS tools of investigation, I accept that Dr Wood has identified a group of patients with laryngo-pharyngeal symptoms and signs which may relate to LPR. Certainly they appear to have inflammatory changes in the larynx, as seen on photodocumentation and on histologic / gene profile studies. There is a possibility, howeverm that these patients may have have laryngeal inflammation due to other causes. The tendency to throat clear is common in patients with laryngeal irritation, and characteristically, the medial surfaces of the arytenoid cartilages and vocal processes of the vocal cord are brought forcibly into contact. As a consequence, mechanical deberidement of the mucosal layer and exposure of the underlying epithelium may lead to inflammatory changes, the appearance, histology and gene profiles of which may be identical to those seen on patients with LPR.

There is another paper quoted by Dr Wood : Susan Thibeault's study in The Laryngoscope 2007 of Gene Expression Changes of Inflammatory Mediators due to LPR. In this study, biopsies were taken of laryngeal mucosa pre and post a 10 week period of PPI therapy. The first point of interest in this study was that all patients had a laryngoscopic exam with findings consistent with a diagnosis of LPR (Vaezi Score), and then went on to have a pH study. Of the initial 42 patients, 10 had a normal pH study. This tends to suggest that the RFS may not be as specific an identifier of LPR as might be thought. The next issue with this study was that all biopsies were taken from the interarytenoid area (posterior commissure) only, and showed no changes of inflammatory markers associated with PPI therapy over a short time period.

Discussion of results

Dr Wood identified 56 patients to enter his study, which, following losses to the study, resulted in 9 patients who acted as controls and 10 who were diagnosed as having LPR. As Dr Wood has shown, there were significant demographic differences between these groups in terms of age and sex ratio. Dr Wood explains in the text the reasons for accepting these differences, and the lack of impact on the study results, which I accept. I would have liked to see the actual ranges of RSI and RFS for the two groups. This was only partially explained in the text. I am given to understand that the two groups were sufficiently different to spate them without risk of overlap of diagnosis / pathology. The addition of the ranges could aid with this.
The overall numbers of the study are small, and this leads to difficulty with statistical analysis, particularly as each subject is assessed multiple times with different statistical measures. The risk of identifying an anomaly in error is high in these cases, and the use of Dunn or Bonferoni corrections for multiple analyses could be used. I appreciate that the likelihood of delivering a statistically significant result would be blunted by this statistical change. I would rely on the opinion of a qualified statistician to clear this issue.

The results section is well constructed and presents data in an easy to understand manner. The discussion of these results attempts to tie the hypothesis of LPR to epithelial changes.

The histologic findings on squamous metaplasia on the medial surface of the arytenoid and false vocal cord area in comparison the normal population shows that there are secondary effects of inflammation on cell maturation.

The measures of cytologic inflammation and changes in mucosal defen(s)e (sic) show similar changes, consistent with a mild chronic inflammatory insult impacting on the medial arytenoid and posterior commissure, again in contradistinction to the normal group.

On page 133, Dr Wood comments that '...difference may represent an adaptive change from the less resilient columnar epithelium to the more durable squamous epithelium.' This is a critical statement in the thesis. Are the findings histologically and in gene studies actually a protective response to a source of chronic injury? It may be that changes in the epithelial structure and function of the larynx in the presence of an irritative agent may protect against that agent. The changes in voice which occur with laryngeal inflammation may relate to epithelial changes with impact on vocal cord function, mucous consistency and muscle tensioning patterns and are secondary events to the protective changes. These changes are adaptations to a long history of inflammation which would be expected to take a long period after treatment for resolution to take place.

Summary of analysis of paper

There are a number of typographical / grammatical errors which should be addressed as indicated above.

I draw attention to the issues in the diagnosis of LPR using the RSI and RFS. I believe that Dr Wood has created a strong argument to use these investigational tools as a surrogate for the diagnosis of LPR, but there may be an issue regarding the specificity of theses tools.
I also note that there are other causes of laryngeal inflammation that may give the same symptoms, laryngeal findings and possibly identical histologic and gene profile measures.
The result of these comments is that we may be looking at a heterogeneous group of patients, some of whom may have LPR and others not, leading to difficulty interpreting results.

Overall, I support this thesis, subject to modifications as above.

Guy Rees
July 2013

5.6 Appendix 6: Response to Mr Rees' Report

Mr Rees' comments	Corrections/Response
Page XV "Laryngopharyngeal reflux (LPR) is an increasingly diagnosed disease in Otolaryngology, however **(it)** is a highly controversial one.	"it" added to sentence.
Page xvi, para 3, line 1 Carbonic anhydrase (CA) is an integral component of laryngeal **defence**.	Corrected to: English "defence" utilised throughout thesis for consistency.
Page 16, 1.4.2 Globus Pharyngeal, Line 5 "…and naming symptoms globus hystericus, having been **lin(k)ed** with uterine dysfunction	Corrected to: "…and naming the symptom globus hystericus, having been **linked** with uterine dysfunction.
Page 17, 1.4.3 Dysphagia, line 11 "…or finally **(, from)** upper oesophageal sphincter dysfunction."	Corrected to: "... or finally, **from** upper oesophageal sphincter dysfunction
Page 19 1.5 Consequences line 4-5 "The idea of reflux into the laryngopharynx was considered in the otolaryngological literature as far back as 1968" quotation missing?	Quotation added: Delahunty JE, Cherry JC. Experimentally produced vocal cord granulomas. Laryngoscope 1968;78:1941-7.
Page 19, line 7 "The latter of these was reported in 1985 with a case of recalcitrant subglottic stenosis…" ?missing quote	Quotation added: Little FB, Koufman JA, Kohut RI et al Effect of gastric acid on the pathogenesis of subglottic stenosis. Ann Otol Rhinol Laryngol 1985;94:516-519
Page 19, Para 2, Line 6. "..those with purulent effusions were more likely to be pepsin '**positive**'	Apostrophe's altered
Page 21, 1.6.1 pH Monitoring, line 3-4 '…test, yet has demonstrated significant problems, however its use stems from its ubiquitous use in GORD.' Suggest re-write	Changed to: "…, yet it has significant problems. This may relate to the ubiquitous use of pH monitoring in the diagnosis of GORD.'
Page 23, para 2, line 6 "Despite well documented limitations (,) these still remain the best (,) standardi(z)ed of LPR…" Inconsistent with English vs American text, utilise 1st, not second comma.	Changed to "standardised" First comma utilised, second comma removed.

Page 23, Para 2, line 8-9 "It is well know that LPR is difficult to accurately diagnose on a single modality…"	Changed as per recommendation: "It is well know that LPR is difficult to accurately diagnose with a single investigational modality, and recent studies have suggested that combining these two modalities.."
Page 29, line 8 '…found in the striking."	Changed to: '…found in the striking **zone**.' (as per Prof Wilson).
Page 29 line 14. 'aging larynx"	Changed to '…ageing larynx…'
Page 33 1.7.1.1 Acid, Para 1, line 4 '..of the role for acid, with a response to a trial of PPI…"	Changed to '..of the role for acid, with a **positive** response to a trial of PPI…'
Page 33, para1, line 8-9 "Whilst up to 50 episodes of reflux in the oesophagus may be normal, Koufman described as few as 3 episodes per week may lead to laryngeal injury.'	Changed to: "Whilst up to 50 episodes of reflux in the **distal** oesophagus may be considered physiologically normal, Koufman described as few as 3 episodes per week **in the proximal oesophagus** may lead to laryngeal injury.' (also changed with Prof Wilson)
Page 39. 1.8.2, Line 13-17 'Whilst GORD is manifest by significant exposure…a reduced exposure time experienced by patients with LPR may provide enough refluxate to damage the larynx…" Quotation needed	Quotation: Lipan MJ, Reidenberg, JS, Laitman JT. Anatomy of reflux: A growing health problem affecting the structures of the head and neck. The Anatomical Record (Part B: New Anat) 2006;289B:261-270.
Page 43, para 2, line 5 '…corresponding to worsening mucosal injury[98]..'	Second fullstop removed. Sentenced altered following Prof Wilson's comments.
Page 44, para 2, line 3 '…as a similar aetiology, just higher anatomically'	Changed to: '…as a similar aetiology, just at a more proximal anatomic site."
Page 72, para 2, line 5 'was removed and placed in an eppendorf tube…"	Changed to '…was removed and placed in an **Eppendorf** tube.."
Comment regarding ranges of RSI and RFS in "scientific construct of thesis" response	Ranges added to demographics.

Response to Scientific construct of Thesis:

Diagnosis of LPR:

This thesis proposes that there will be molecular differences between patients with signs and symptoms consistent with laryngopharyngeal reflux (LPR), and those who are asymptomatic. In his Examiner's comments, Mr Rees suggests that a pre-biopsy 24-hour pH dual probe/multichannel monitoring to establish the presence and severity of reflux. Furthermore, treatment of reflux as a therapeutic trial, with repeating RFS and RSI may have added validity of the diagnosis of LPR.

LPR is undeniably a difficult diagnosis, and lacks a "gold standard" test. Consequently any research considering LPR will attract commentary regarding definitive diagnosis. According to Friedman et al[188], diagnosis of LPR was made in a number of ways: (1) symptomatic response to a proton pump inhibitor (PPI); (2) endoscopic assessment of the larynx; or (3) observation of acidic reflux events using a pH probe. Of these, the first and second were utilised in this study using the Reflux Symptom Index[60] and Reflux Finding Score[57]. 24-Hour pH probe monitoring has been considered the gold standard[189], however is not without significant issues, in addition to being a further invasive test. Vaezi et al.[190] noted whilst proximal oesophagus pH sensors had a greater than 90% specificity, they had poor sensitivity and reproducibility. This has been repeated in other studies[191-193]. Furthermore Vaezi et al noted that the presence of an abnormal proximal

oesophagus did not predict response to a PPI either[194]. It is also recognised that there is a lack of consensus regarding the optimal pH criteria for LPR[195], and even the number of times per week required to cause injury significant enough to lead to symptoms.

Because of this it is difficult to say without some uncertainty whether symptoms solely related to LPR. Mr Rees correctly discusses that these patients are particularly prone to chronic throat clearing, which would tend to bring the medial arytenoid region and vocal processes together forcibly. Such an impact may lead to ongoing inflammation, particularly in this region in which we identified mucosal changes. It could be argued that the mechanical injury in this population could be the main cause of injury. Future research, including subgroup analysis of the RSI, identifying patients who rated highly on the second item “Clearing your throat,” and correlating this to medial arytenoid changes would be useful in a study with a sufficiently large enough population. The current study does not have sufficient population to warrant such a subgroup analysis. Furthermore the symptom of throat clearing, and even chronic coughing, are typical symptoms of LPR, and so the symptomatic response to the irritation may also be perpetuating the symptoms. Such a situation complicates the management. Treating successfully the underlying cause may still leave a patient with habitual throat clearing, and continuing physical irritation. Again identifying this population would be difficult, as mucosal biopsies may well show inflammatory changes which could potentially be attributed to either

pathophysiology. Ideally a way of demonstrating the continuing presence of reflux to this area would allow a method of differentiating between the two groups. Consequently further research would also need to focus on a method for definitive diagnosis, whether this be through pH monitoring or newer technologies such as the detection of aerosolised acid in the pharynx, detecting pepsin in saliva, or high resolution endoscopy with narrow band imaging[196]. Such technologies are in the development and research stage but may allow a differentiation for a more specific diagnosis to be made. In summary this study progresses the literature of LPR by identifying biomarkers, and a specific site which demonstrates significant mucosal change in the larynx.

6 REFERENCES

1. Wang L, Xiung L, Liu Y-L, et al. Correlation of pepsin-measured laryngopharyngeal reflux disease with symptoms and signs. Otolaryngol Head Neck Surg 2010;143:765-71.
2. Koufman JA. The otolaryngologic manifestations of gastroesophageal reflux disease (GERD): A clinical investigation of 225 patients using ambulatory 24-hour pH monitoring and an experimental investigation of the role of acid and pepsin in the development of laryngeal injury. Laryngoscope 1991;101:1-78.
3. Abd El-Fattah AM, Abdul Maksoud GA, Ramadan AS, et al. Pepsin assay: A marker for reflux in pediatric glue ear. Otolaryngol Head Neck Surg 2007;136:464-70.
4. Wise SK, Wise JC, DelGaudio JM. Gastroesophageal reflux and laryngopharyngeal reflux in patients with sleep-disordered breathing. Otolaryngol Head Neck Surg 2006;135:253-57.
5. De Benedetto M, Monaco G, Marra F. Extra-laryngeal manifestations of gastro-oesophageal reflux. Acta Otorhinolaryngol Ital 2006;26:256-59e.
6. Miller G, Pan Y. Gastro-oesophageal reflux disease. In: General practice in Australia, health priorities and policies 1998 to 2008. Britt H, Miller G, editors. Canberra: Australian Institute of Health and Welfare; 2009. p. 269-78.
7. Vakil N, van Zanten SV, Kharilas P, et al. The Montreal definition and classification of gastroesophageal reflux disease: A global evidence-based consensus. Am J Gastroenterol 2006;101:1900-20.

8. Wiener GJ, Tsukashima R, Kelly C, et al. Oropharyngeal pH monitoring for the detection of liquid and aerosolized supraesophageal gastric reflux. J Voice 2009;23:498-504.
9. Dent J, El-Serag HB, Wallander M-A, et al. Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut 2005;54:710-17.
10. Heading RC. Prevalence of upper gastrointestinal symptoms in the general population: a systematic review. Scand J Gastroenterol Suppl 1999;231:3-8.
11. Pace F, Bianchi Porro G. Clinical spectrum, natural history and epidemiology of GERD. In: Gastroesophageal reflux disease. Granderath FA, Kamolz T, Pointer R, editors. Vienna: Springer; 2006. p. 1-11.
12. Kotby MN, Hassan OE, El-Makhzangy AMN, et al. Gastroesophageal reflux/laryngopharyngeal reflux disease: a critical analysis of the literature. Eur Arch Otorhinolaryngol 2010;267:171 - 79.
13. Kuhn J, Toohill RJ, Ulualp SO, et al. Pharyngeal acid reflux events in patients with vocal cord nodules. Laryngoscope 1998;108:1146-49.
14. Koufman J, Amin M, Panetti M. Prevalence of reflux in 113 consecutive patients with laryngeal voice disorders. Otolaryngol Head Neck Surg 2000;123:385-58.
15. Connor NP, Palazzi-Churas KLP, Cohen SB, et al. Symptoms of extraesophageal reflux in a community-dwelling sample. J Voice 2007;21:189-202.

16. Sataloff RT. Laryngopharyngeal reflux: Concepts and controveries. Ear Nose Throat J 2008;87:549-54.
17. Woodson GE. Laryngeal and pharyngeal function. In: Cummings Otolaryngology: Head and Neck Surgery. Flint PW, Haughey BH, Lund VJ, Niparko JK, Richardson MA, Robbins KT, Thomas JR, editors 5 ed. Philadelphia: Mosby; 2010.
18. Merrill RM, Anderson AE, Sloan A. Quality of life indicators according to voice disorders and voice-related conditions. Laryngoscope 2011;121:2004-10.
19. Lenderking WR, Hillson E, Crawley JA, et al. The clinical characteristics and impact of laryngopharyngeal reflux disease on health-related quality of life. Value in Health 2003;6:560-65.
20. Thurnher D, Moukarbel RV, Novak CB, et al. The glottis and subglottis: An otolaryngologist's perspective. Thorac Surg Clin 2007;17:549-60.
21. AJCC. Larynx. In: AJCC Cancer staging atlas. Greene FL, Compton CC, Fritz AG, Shah J, Winchester DP, editors. New York: Springer; 2006.
22. Gastrolab. Larnyx. In; 2012. p. Larynx
23. Hirano M, Kurita S, Kiyokawa K, et al. Posterior glottis. Morphological study in excised human larynges. Ann Otol Rhinol Laryngol 1986;95:576-81.
24. McIlwain JC. The posterior glottis. J Otolaryngol 1991;20:1-24.
25. Tucker JA, Tucker ST. Posterior commissure of the human larynx revisited. J Voice 2010;24:252-59.

26. Delahunty JE. Acid laryngitis. J Laryngol Otol 1972;86:335-42.

27. Kambic V, Radsel Z. Acid posterior laryngitis. Aetiology, histology, diagnosis, and treatment. J Laryngol Otol 1984;98:1237-40.

28. Belafsky PC. Abnormal endoscopic pharyngeal and laryngeal findings attributable to reflux. Am J Med 2003;115:90S-96S.

29. Stell PM, Gregory I, Watt J. Morphometry of the epithelial lining of the human larynx I. The glottis. Clin Otolaryngol 1978;3:13-20.

30. Stell PM, Gudrun R, Watt J. Morphology of the human larnyx III. The supraglottis. Clin Otolaryngol 1981;6:388-93.

31. Mills SE. Larnyx and Pharynx. In: Histology for Pathologists. Mills SE, editor 3rd ed. Philadelphia: Lippincott Williams and Wilkins; 2007.

32. Sataloff RT, Heman-Ackah YD, Hawkshaw MJ. Clinical anatomy and physiology of the voice. Otolaryngol Clin N Am 2007;40:909-29.

33. Bulmer DM, Ali MS, Brownlee IA, et al. Laryngeal mucosa: Its susceptibility to damage by acid and pepsin. Laryngoscope 2010;120:777-82.

34. Ylitalo R, Lindestad P, Hertegard S. Pharyngeal and laryngeal symptoms and signs related to extraesophageal reflux in patients with heartburn in gastroenterology practice: a prospective study. Clinical Otolaryngology 2005;30:347-52.

35. Pearson JP, Parikh S. Review article: nature and properties of gastro-oesophageal and extra-oesophageal refluxate. Aliment Pharmacol Ther 2011;33 (Suppl. 1):2-7.

36. Hickson C, Simpson CB, Falcon R. Laryngeal pseudosulcus as a predictor of laryngopharyngeal reflux. Laryngoscope 2001;111:1742-45.

37. Ulualp SO, Toohill R, Hoffmann R, et al. Pharyngeal pH monitoring in patients with posterior laryngitis. Otolaryngol Head Neck Surg 1999;120:672-77.

38. Koufman JA. Gastroesophageal reflux and voice disorders. In: Diagnosis and Treatment of Voice Disorders. Rubin, Sataloff, Korovin, Gould, editors. New York - Tokyo: Igaku-Shoin Publishers; 1995. p. 161-75.

39. Harar R, Kumar S, Saeed M, et al. Management of globus pharyngeus: review of 699 cases. Journal of Laryngology and Otology 2004;118:522-27.

40. Curran AJ, Barry MK, Callanan V, et al. A prospective study of acid reflux and globus pharyngeus using a modified symptom index. Clin Otolaryngol 1995;20:552-54.

41. Harar R, Kumar S, Saeed M, et al. Management of globus pharyngeus: review of 699 cases. J Laryngol Otol 2004;118:522-27.

42. Murry T, Tabaee A, Aviv JE. Respiratory retraining of refractory cough and laryngopharyngeal reflux in patients with paradoxical vocal fold movement disorder. Laryngoscope 2004;114:1341-45.

43. Jaimes-Hernandez J, Mendoza-Fuentes A, Melendez-Mercado CI, et al. Chronic eosinphilic pneumonia: autoimmune phenomenon or immunoallergic disease? Case report and literature review. Reumatologia Clinica 2012;8:145-48.

44. Delahunty JE, Cherry JC. Experimentally produced vocal cord granulomas. Laryngoscope 1968;78:1941-47.

45. Little FB, Koufman JA, Kohut RI, et al. Effect of gastric acid on the pathogenesis of subglottic stenosis. Ann Otol Rhinol Laryngol 1985;94:516-19.

46. Nelson SP, Chen EH, Syniar GM, et al. Prevalence of symptoms of gastroesopahgeal reflux during infancy: a pediatric practice based survery from pediatric practice research group. Arch Pediatr Adolesc Med 1997;151:569 - 72.

47. O'Reilly RC, He Z, Bloedon E, et al. The role of extraesophageal reflux in otitis media in infants and children. Laryngoscope 2008;118:1-9.

48. Vaezi MF, Qadeer MA, Lopez R, et al. Laryngeal cancer and gastroesophageal reflux disease: a case-control study. Am J Med 2006;119:768-76.

49. Ozer E. Which one is the etiologic factor: Gastroesophageal reflux disease or larynx cancer? Laryngoscope 2005;115:1323-24.

50. Kamolz T, Pointner R. Gastroesophageal reflux disease. Heart-burn from a psychological view. Minerva Gastroenterol Dietol 2004;50:261-68.

51. Johnston N. Review article: uptake of pepsin at pH 7 - in non-acid reflux - causes inflammatory, and perhaps even neoplastic, changes in the laryngopharynx. . Aliment Pharmacol Ther 2011;33:13 - 20.

52. Allen J, Tinling SP, Johnston N, et al. Review article: effects of pepsin and alginate in an animal model of squamous cell carcinoma. Aliment Pharmacol Ther 2011;33:21 - 28.

53. Muderris T, Gokcan MK, Yorulmaz I. The Clinical value of pharyngeal pH monitoring using a double-probe, triple-sensor catheter in patients with laryngopharyngeal reflux. Arch Otolaryngol Head Neck Surg 2009;135:163-67.

54. Noordzij JP, Khidr A, Desper E, et al. Correlation of pH Probe-Measured Laryngopharyngeal Reflux With Symptoms and Signs of Reflux Laryngitis. Laryngoscope 2002;112:2192-95.

55. Eubanks TR, Omelanczuk PE, Maronian N, et al. Pharyngeal pH monitoring in 222 patients with suspected laryngeal reflux. J Gastrointest Surg 2001;5:183-90.

56. Smit CF, Mathus-Vliegen EMH, Devriese PP, et al. Laryngopharyngeal reflux: monitoring technique and normal values. Clinical Otolaryngology & Allied Sciences 1998;23:187.

57. Belafsky PC, Postma GN, Koufman J. The validity and reliability of the reflux finding score. Laryngoscope 2001;111:1313-17.

58. Hirano I, Richter JE. ACG practice guidelines: esophageal reflux testing. Am J Gastroenterol 2007;102:668-85.

59. Park KH, Choi SM, Kwon S, et al. Diagnosis of Laryngopharyngeal Reflux Among Globus Patients. Otolaryngol Head Neck Surg 2006;134:81-85.

60. Belafsky PC, Postma GN, Koufman J. Validity and reliability of the reflux symtom index (RSI). J Voice 2002;16:274-77.

61. Tawakir K, Penney S, Pothula V, et al. The prevalence of laryngopharyngeal reflux in the population. Clinical Otolaryngology 2009;34 Supplement:97.

62. Oyer SL, Anderson LC, Halum SL. Influence of anxiety and depression on the predictive value of the Reflux Symptom Index. Ann Otol Rhinol Laryngol 2009;118:687-92.

63. Vaezi MF. We Should Learn From Important Negative Results. Laryngoscope 2006;116:1718.

64. Belafsky PC, Postma GN, Amin MR, et al. Symptoms and findings of laryngopharyngeal reflux. Ear Nose Throat J 2002;81:10 - 13.

65. Powell J, Cocks HC. Mucosal changes in laryngopharyngeal reflux - prevalence, sensitivity, specificity and assessment. Laryngoscope 2012.

66. Lipan MJ, Reidenberg JS, Laitman JT. Anatomy of reflux: A growing health problem affecting structures of the head and neck. The Anatomical Record (Part B: New Anat) 2006;289B:261-70.

67. Bardhan KD, Strugala V, Dettmar PW. Reflux revisited: Advancing the role of pepsin. Int J Otolaryngol 2012;Vol 2012:13 pages.

68. Zeleník K, Matousek P, Urban O, et al. Globus pharyngeus and extraesophageal reflux: Simultaneous pH <4.0 and pH <5.0 analysis. Laryngoscope 2010;120:2160-64.

69. Adhami T, Goldblum JR, Richter JE, et al. The role of gastric and duodenal agents in laryngeal injury: An experimental canine model. Am J Gastroenterol 2004;99:2098-106.

70. Wood JM, Hussey DJ, Woods CM, et al. Biomarkers and laryngopharyngeal reflux. J Laryngol Otol 2011;125:1218-24.

71. Samuels TL, Johnston N. Pepsin as a marker of extraesophageal reflux. Ann Otol Rhinol Laryngol 2010;119:203-08.

72. Piper DW, Fenton BH. pH stability and activity curves of pepsin with special reference to their clinical importance. Gut 1965;6:506-08.

73. Ylitalo R, Baugh A, Li W, et al. Effect of acid and pepsin on gene expression in laryngeal fibroblasts. Ann Otol Rhinol Laryngol 2004;113:866-71.

74. Johnston N, Knight J, Dettmar PW, et al. Pepsin and carbonic anhydrase isoenzyme III as diagnostic markers for laryngopharyngeal reflux disease. Laryngoscope 2004;114:2129-34.

75. Johnston N, Dettmar PW, Bishwokarma B, et al. Activity/stability of human pepsin: Implications for reflux attributed laryngeal disease. Laryngoscope 2007;117:1036-39.

76. Johnston N, Wells CW, Blumin JH, et al. Receptor-mediated uptake of pepsin by laryngeal epithelial cells. Ann Otol Rhinol Laryngol 2007;116:934-38.

77. Johnston N, Dettmar PW, Lively MO, et al. Effect of pepsin on laryngeal stress protein (Sep70, Sep53, and Hsp70) response: Role in laryngopharyngeal reflux disease. Ann Otol Rhinol Laryngol 2006;115:47-58.

78. Bollschweiler E, Wolfgarten E, Pütz B, et al. Bile reflux into the stomach and esophagus for volunteers older than 40 years. Digestion 2005;71:63-64.

79. Tack J, Koek G, Demedts I, et al. Gastroesophageal reflux disease poorly responsive to single-dose proton pump inhibitors in patients without Barrett's esophagus: acid reflux, bile reflux or both? Am J Gastroenterol 2004;99:989-90.

80. Saskai CT, Marotta J, Hundal J, et al. Bile-induced laryngitis: is there a basis in evidence. Ann Otol Rhinol Laryngol 2005;114:192-97.

81. Sfhirvanni VN, Ouatu-Lascar R, Kaur BS, et al. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: ex vivo induction by bile salts and acid exposure. Gastroenterology 2000;118:487-96.

82. Zhang F, Subbaramaiah K, Altorki N, et al. Dihydroxy bile acids activate the transcription of cyclooxygenase-2. J Biol Chem 1998;273:2424-28.

83. Sung M, Roh H, Park S, et al. Bile acid inducts cyclo-oxygenase-2 expression in cultured human pharyngeal cells: a possible mechanism of carcinogenesis in the upper aerodigestive tract by laryngopharyngeal reflux. Laryngoscope 2003;113:1059-63.

84. Ali MS, Parikh S, Chater P, et al. Bile acids in laryngopharyngeal refluxate: Will they enhance or attenuate the action of pepsin? Laryngoscope 2012;Oct 15.

85. Merati AL. Reflux and cough. Otolaryngol Clin N Am 2010;43:97-110.

86. Maish M. Esophagus. In: Sabiston Textbook of Surgery: The Biological Basis of Modern Surgical Practice. Townsend CM, Beauchamp RD, Evers BM, Mattox KL, editors 18th ed. Philadelphia: Saunders; 2008.

87. Grossi L, Ciccaglione AF, Marzio L. Transient lower oesophageal sphincter relaxations play an insignificant role in gastro-oesophageal reflux to the proximal oesophagus. Neurogastroenterol Motil 2001;13:503-09.

88. Shaker R, Milbrath M, Ren J, et al. Esophagopharyngeal distribution of refluxed gastric acid in patients wiht reflux laryngitis. Gastroenterology 1995;109:1575-82.

89. Ylitalo R, Lindestad PA, Ramel S. Symptoms, laryngeal findings, and 24-hour pH monitoring in patients with suspected gastroesophago-pharyngeal reflux. Laryngoscope 2001;111:1735-41.

90. Bremner RM, Hoeft SF, Costantini M, et al. Pharyngeal swallowing: the major factor in clearance of esophageal reflux episodes. Ann Surg 1993;218:364-69.

91. Postma GN, Tomek MS, Belafsky PC, et al. Esophageal motor function in laryngopharyngeal reflux is superior to that in classic gastroesophageal reflux disease. Ann Otol Rhinol Laryngol 2001;110:1114-16.

92. Knight RE, Wells JR, Parrish RS. Esophageal dysmotility as an important co-factor in extraesophageal manifestations of gastroesophageal reflux. Laryngoscope 2000;110:1462-66.

93. Ulualp SO, Toohill RJ, Kern M, et al. Pharyngo-UES contractile reflux in patients with posterior laryngitis. Laryngoscope 1998;108:1354-57.

94. Torrico S, Kern M, Aslam M, et al. Upper esophageal sphincer function during gastroesophageal reflux events revisited. Am J Physiol Gastrointest Liver Physiol 2000;279:G262-G67.

95. Tobey NA, Hosseini SS, Argote CM, et al. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium Am J Gastroenterol 2004;99:13-22.

96. Oh DS, DeMeester SR, Vallbohmer D, et al. Reduction of interleukin 8 gene expression in reflux esophagitis and Barretts' esophagus with antireflux surgery. Arch Surg 2007;142:554-60.

97. Barnes PJ, Karin M. Nuclear factor-κB -- a pivotal transcription factor in inflammatory disease. New England Journal of Medicine 1997;336:1066-71.

98. Abdel-Latif MMM, O'Riordan J, Windle HJ, et al. NF-κB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. Ann Surg 2004;239:491-500.

99. Sovak MA, Bellas RE, Kim DW, et al. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 1997;100:2952-60.

100. Tai D-I, Tsai S-L, Chang Y-H, et al. Constitutive activation of nuclear factor B in hepatocellular carcinoma. Cancer 2000;89:2274-81.

101. Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α induced cell death. Science 1996;274:782-84.

102. Oh DS, DeMeester SR, Vallböhmer D, et al. Reduction of interleukin 8 gene expression in reflux esophagitis and Barretts' esophagus with antireflux surgery. Arch Surg 2007;142:554-60.

103. Waugh DJJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res 2008;14:6735-41.

104. Van Roon AH, Mayne GC, Wijnhoven BP, et al. Impact of gastro-esophageal reflux on mucin mRNA expression in the esophageal mucosa. J Gastrointest Surg 2008;12:1331-40.
105. Thibeault SL, Smith ME, Peterson K, et al. Gene expression changes of inflammatory mediators in posterior laryngitis due to laryngopharyngeal reflux and evolution with PPI treatment: A preliminary study. Laryngoscope 2007;117:2050-56.
106. Chin D, Boyle G, Parsons P, et al. What is transforming growth factor-beta (TGF-B). Br J Plast Surg 2004;57:215-21.
107. Jarmuz T, Roser S, Gal A, et al. Transforming growth factor-B1, myofibroblasts, and tissue remodeling in the pathogenesis of tracheal injury: Potential role of gastroesophageal reflux. Ann Otol Rhinol Laryngol 2004;113:488-97.
108. Ylitalo R, Thibeault S. Relationship between time of exposure of laryngopharyngeal reflux and gene expression in laryngeal fibroblasts. Ann Otol Rhinol Laryngol 2006;115:775-83.
109. Tobey NA, Powell DW, Schreiner VJ, et al. Serosal bicarbonate protects against acid injury to rabbit esophagus. Gastroenterology 1989;96:1466-77.
110. Axford SE, Sharp N, Ross PE, et al. Cell biology of laryngeal epithelial defenses in health and disease: preliminary studies. Ann Otol Rhinol Laryngol 2001;110:1099 - 108.
111. Christie KN, Thomson C, Xue L, et al. Carbonic anhydrase isoenzymes I, II, II, and IV are present in human esophageal epithelium. J Histochem Cytochem 1997;45:35-40.

112. Gill GA, Johnston N, Buda A, et al. Laryngeal epithelial defenses against laryngopharyngeal reflux: Investigations of E-cadherin, carbonic anhydrase isoenzyme III, and pepsin. Ann Otol Rhinol Laryngol 2005;114:913-21.

113. Johnston N, Bulmer D, Gill GA, et al. Cell biology of laryngeal epithelial defenses in health and disease: Further studies. Ann Otol Rhinol Laryngol 2003;112:481- 91.

114. Reichel O, Mayr D, Durst F, et al. E-cadherin but not β-catenin expression is decreased in laryngeal biopsies from patients with laryngopharyngeal reflux. Eur Arch Otorhinolaryngol 2008;265:937-42.

115. Kurtz KA, Hoffman HT, ZImmermann MB, et al. Decreased E-cadherin but not B-catenin expression is associated with vascular invasion and decreased survival in head and neck squamous carcinomas. Otolaryngol Head Neck Surg 2006;134:142-46.

116. Ali M, Pearson JP. Upper airway mucin gene expression: A review. Laryngoscope 2007;117:932-38.

117. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 2004;4:45-60.

118. Samuels TL, Handler E, Syring ML, et al. Mucin gene expression in human laryngeal epithelia: effect of laryngopharyngeal reflux. Ann Otol Rhinol Laryngol 2008;117:688-95.

119. Namiot Z, Sarosiek J, Marcinkiewicz M, et al. Declined human esopahgeal mucin secretion in patients with severe reflux esophagitis. Dig Dis Sci 1994;39:2523-29.

120. Ho SB, Dvorak LA, Moor RE, et al. Cysteine-rich domains of muc3 intestinal mucin promote cell migration, inhibit apoptosis, and accelerate wound healing. Gastroenterology 2006;131:1501-17.

121. Louis NA, Hamilton KE, Canny G, et al. Selective induction of mucin-3 by hypoxia in intestinal epithelia. J Cell Biochem 2006;99:1616-27.

122. Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alteration in the growth and survival of cancer cells. Oncogene 2010;29:2893-904.

123. Gendler SJ. MUC1, the renaissance molecule. J Mammary Gland Biol Neoplasia 2001;6:330-53.

124. Zhao Q, Guo X, Nash GB, et al. Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface. Cancer Res 2009;69:6799-806.

125. Jeannon J-P, Stafford FW, Soames JV, et al. Altered MUC1 and MUC2 glycoprotein expression in laryngeal cancer. Otolaryngol Head Neck Surg 2001;124:199-202.

126. Paleri V, Pearson JP, Bulmer D, et al. Expression of mucin gene products in laryngeal squamous cancer. Otolaryngol Head Neck Surg 2004;131:84-88.

127. Franco RA. Laryngopharyngeal reflux. Allergy and Asthma Proceedings 2006;27:21-25.

128. Koufman JA. Low-acid diet for recalcitrant laryngopharyngeal reflux: Therapeutic benefits and their implications. Ann Otol Rhinol Laryngol 2011;120:281-87.

129. Zerbib F, Stoll D. Management of laryngopharyngeal reflux: an unmet medical need. Neurogastroenterol Motil 2010;22:109-12.

130. Altman KW, Prufer N, Vaezi MF. A review of clinical practice guidelines for reflux disease: Toward creating a clinical protocol for the otolaryngologist. Laryngoscope 2011;121:717-23.

131. Hill RKMD, Simpson CBMD, Velazquez RMDP, et al. Pachydermia is Not Diagnostic of Active Laryngopharyngeal Reflux Disease. Laryngoscope 2004;114:1557-61.

132. Yang Y, Lewis J, Epstein S, et al. Long-term proton pump inhibitor therapy and risk of hip fracture. JAMA 2006;296.

133. Cheung TK, Lam PKY, Wei WI, et al. Quality of life in patients with laryngopharyngeal reflux. Digestion 2009;79:52-57.

134. McGlashan JA, Johnstone LM, Sykes J, et al. The value of a liquid alginate suspension (Gaviscon Advance) in the management of laryngopharyngeal reflux. Eur Arch Otorhinolaryngol 2009;266:243-51.

135. Lindstrom DRMD, Wallace JMD, Loehrl TAMD, et al. Nissen Fundoplication Surgery for Extraesophageal Manifestations of Gastroesophageal Reflux (EER). Laryngoscope 2002;112:1762-65.

136. So JBY, Zeitels SM, Rattner DW. Outcomes of atypical symptoms attributed to gastroesophageal reflux treated by laparoscopic fundoplication. Surgery 1998;124:28 - 32.

137. Kaufman JA, Houghland JE, Quiroga E, et al. Long-term outcomes of laparoscopic antireflux surgery for gastroesophageal reflux disease (GERD) - related airway disorder. Surg Endosc 2006;20:1824 - 30.

138. Ratnasingam D, Irvine T, Thompson SK, et al. Laparoscopic antireflux surgery in patients with throat symptoms: A word of caution. World J Surg 2011;35:342-48.

139. Shaker R, Bardan E, Gu C, et al. Intrapharyngeal distribution of gastric refluxate. Laryngoscope 2003;113:1182-91.

140. Vaezi MF. Treatment with PPIs Should NOT Be Preceded by pH Monitoring in Patients Suspected of Laryngeal Reflux. Am J Gastroenterol 2006;101:8-10.

141. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine 2006;27:126-39.

142. Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7:3.

143. Simon P. Q-Gene: Processing quantitative real-time RT-PCR data. Bioinformatics 2003;19:1439-40.

144. de Jonge HJM, Fehrmann RSN, de Bont ESJM, et al. Evidence based selection of housekeeping genes. PLoS One 2007;2:e898.

145. Vaidyanathan S, McDicken IW, Mansour P, et al. Detection of early squamous metaplasia in bladder biopsies of spinal cord injury patients by immunostaining for cytokeratin 14. Spinal Cord 2003;41:432-34.

146. Ferlito A, Devaney KO, Woolgar JA, et al. Squamous epithelial changes of the larynx: Diagnosis and therapy. Head Neck 2012;34:1810-16.

147. Yu W-Y, Slack JMW, Tosh D. Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus. Developmental Biology 2005;284:157-70.

148. Ferlito A, Devaney KO, Woolgar JA, et al. Squamous epithelial changes of the larynx: Diagnosis and therapy. Head Neck 2011;[E-Pub] Accepted 20 May 2011.

149. Porter RM, Lane EB. Phenotypes, genotypes and their contribution to understanding keratin function. Trends Genet 2003;19:278-85.

150. Harnett KM, Fieder F, Behar J, et al. Viewpoints on acid-induced inflammatory mediators in esophageal mucosa. J Neurogastroenterol Motil 2010;16:374-88.

151. Robbins SL, Kumar V. Robbins and Cotran Pathologic Basis of Disease, 8th ed. Philadelphia: Saunders Elesevier; 2010.

152. Bathoorn E, Daly P, Gaiser B, et al. Cytotoxicity and induction of inflammation by pepsin in acid in bronchial epithelial cells. International Journal of Inflammation 2011;(2011):5 pages.

153. Martins DH, Dias NH, Santos DC, et al. Clinical, histological and electron microscopic aspects of vocal fold granulomas. Braz J Otorhinolaryngol 2009;75:116-22.

154. Samuels TL, Johnston N. Pepsin as a causal agent of inflammation during nonacidic reflux. Otolaryngol Head Neck Surg 2009;141:559-63.

155. Waage A, Brandtzaeg P, Halstensen A, et al. The complex pattern of cytokines in serum from patients with meningococcal septic shock.

Association between interleukin-6, interleukin-1, and fatal outcome. J Exp Med 1989;169:333-38.

156. Zampeli E, Karamanolis G, Morfopoulos G, et al. Increased expression of VEGF, COX-2, and Ki-67 in Barrett's esophagus: Does the length matter? Dig Dis Sci 2012;57:1190-96.

157. Goto R, Hoshikawa H, Fujii T, et al. Clinicopathological significance of cyclooxygenase-2 expression in hypopharyngeal squamous cell carcinoma. Oncology Reports 2008;19:645-50.

158. Singh P, Singh N, Palit G. Analysing the role of COX-2 in acute oesophagitis and in melatonin-exerted protection against experimental reflux oesophagitis in rats. J Pharm Pharmacol 2011;63:1572-80.

159. Mayne GC, Watson DI, Hussey DJ. COX-2 mRNA is increased in oesophageal mucosal cells by a proton pump inhibitor. ANZ J Surg 2012;82:691-96.

160. Erkan G, Gönül II, Kandılcı U, et al. Assessment of COX-2 expression presence and severity by immunohistochemical method in patients with chronic active gastritis and intestinal metaplasia. Turk J Gastroenterol 2012;23:478-84.

161. Doecke J, Zhao ZZ, Panedeya N, et al. Polymorphisms in MGMT and DNA repair genes and the risk of esophageal adenocarcinoma. Int J Cancer 2008;123:174-80.

162. Baumann S, Keller G, Puhringer F, et al. The prognostic impact of O6-methylguanine-DNA methyltransferase (MGMT) promotor

hypermethylation in esophageal adenocarcinoma. Int J Cancer 2006;119:264-68.

163. Beck LS, Deguzman L, Lee WP, et al. TGF-beta 1 accelerates wound healing: reversal of steroid-impaired healing in rats and rabbits. Growth Factors 1991;5:295-304.

164. Eming SA, Kreig T. Molecular mechanisms of VEGF-A action during tissue repair. J Investig Dermatol Symp Proc 2006;11:79-86.

165. Möbius C, Stein HJ, Spieß C, et al. COX2 expression, angiogenesis, proliferation and survival in Barrett's cancer. European Journal of Surgical Oncology 2005;31:755-59.

166. Vallböhmer D, Peters JH, Kuramochi H, et al. Molecular determinants in targeted therapy for esophageal adenocarcinoma. Arch Surg 2006;141:476-82.

167. Bao P, Kodra A, Tomic-Canic M, et al. The role of vascular endothelial growth factor in wound healing. J Surg Res 2009;153:347-58.

168. Welch WJ, Brown CR. Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones 1996;1:109-15.

169. Nelson L, Anderson S, Archibald AL, et al. An animal model to evaluate the function and regulation of the adaptively evolving stress protein SEP53 in oesophageal bile damage responses. Cell Stress Chaperones 2008;13:375-85.

170. Little TJ, Nelson L, Hupp T. Adaptive Evolution of a stress response protein. PLoS One 2007;2:e1003.

171. Rees LE, Pazmany L, Gutowska-Owsiak D, et al. The mucosal immune response to laryngopharyngeal reflux. Am J Respir Crit Care Med 2008;177:1187-93.

172. Tsuneyama K, Yasoshima M, Harad K, et al. Increased CD1d expression on small bile duct epithelium and epitheliod granuloma in livers in primary biliary cirrhosis. Hepatology 1998;18:620-23.

173. Kojo S, Tsutsumi A, Goto D, et al. Low expression levels of soluble CD1d gene in patients with rheumatoid arthritis. J Rheumatol 2003;12:2524-28.

174. Ge Y, Rampy BA, Wang HL, et al. Reduced CD1d expression in colonic epithelium in microscopic colitis. Appl Immunohistochem Mol Morphol 2006;14:309-13.

175. Franchi A, Brogelli B, Massi D, et al. Dilation of intercellular spaces is associated with laryngopharyngeal reflux: an ultrastructural morphometric analysis of laryngeal epithelium. Eur Arch Otorhinolaryngol 2007;264:907-11.

176. Park S, Chun HJ, Keum B, et al. An electron microscopic study - correlation of gastroesophageal reflux disease and laryngopharyngeal reflux. Laryngoscope 2010;120:1303-08.

177. Tobey NA, Hosseini SS, Caymaz-Bor C, et al. The role of pepsin in acid injury to esophageal epithelium. Am J Gastroenterol 2001;96:3062-70.

178. Jankowski J, Newham P, Kandemir O, et al. Differential expression of e-cadherin in normal, metaplastic and dysplastic esophageal mucosa - a putative biomarker. Int J Oncol 1994;4:441-48.

179. Feith M, Stein HJ, Mueller J, et al. Malignant degeneration of Barrett's esophagus: the role of the Ki-67 proliferation fraction, expression of E-cadherin and p53. Dis Esophagus 2004;17:322-27.

180. Gill GA, Buda A, Moorghen M, et al. Characterisation of adherens and tight junctional molecules in normal animal larynx; determining a suitable model for studying molecular abnormalities in human laryngopharyngeal reflux. Journal of Clinical Pathology 2005;58:1265-70.

181. Hahn JP, Blount PL, Ayub K, et al. Intestinal differentiation in metaplastic, non-goblet columnar epithelium in the esophagus. Am J Surg Pathol 2009;33:1006-15.

182. Niv Y, Fass R. The role of mucin in GERD and its complications. Nat Rev Gastroenterol Hepatol 2012;9:55-59.

183. de Magalhaes JP, Curado J, Church GM. Meta-anaylsis of age-related gene expression profiles identifies common signatures of aging. Bioinformatics 2009;25:875-81.

184. Kadakia S, Carlson D, Sataloff RT. The effect of hormones on the voice. J of Singing 2013;69:571-74.

185. Koskinen W, Brondbo K, Dahlstrand HM, et al. Alcohol, smoking and human papillomavirus in laryngeal carcinoma: a Nordic prospective multicenter study. J Cancer Res Clin Oncol 2007;133:673-78.

186. Zen JH, Negro AD, C NJA, et al. Experimental model of smoking and simulation of reflux with acid and pepsin in rats. Acta Cirurgica Brazileira 2012;27.

187. Lee BE, Kim GH, Ryu DY, et al. Combined dual channel impedance/pH-metry in patients with suspected laryngopharyngeal reflux. J Neurogastroenterol Motil 2010;16:157-65.
188. Friedman M, Hamilton C, Samuelson CG, et al. The value of routine pH monitoring in the diagnosis and treatment of laryngopharyngeal reflux. Otolaryngol Head Neck Surg 2012;146:952-58.
189. Friedman M, Hamilton C, Samuelson CG, et al. The value of routine pH monitoring in the diagnosis and treatment of laryngopharyngeal reflux. Otolaryngol Head Neck Surg 2012;146:952 - 58.
190. Vaezi MF, Schroeder PL, Richter JE. Reproducibility of proximal probe pH parameters in 24-hor ambulatory esophageal pH monitoring. Am J Gastroenterol 1997;92:825-29.
191. Jacob P, Kahrilas P, Herzon G. Proximal esophageal pH-metry in patients with 'reflux laryngitis'. Gastroentrology 1991;100:305-210.
192. Cool M, Poelmans J, Feenstra L, et al. Characteristics and clinical relevance of proximal esophageal pH monitoring. Am J Gastroenterol 2004;99:2317-23.
193. Ikuo J, Richter JE. ACG practice guidelines: esophageal reflux testing. Am J Gastroenterol 2007;102:668-85.
194. Vaezi MF, Richter JE, Stasney CR, et al. Treatment of chronic posterior laryngitis with esomeprazole. Laryngoscope 2006;116:254-60.
195. Vailati C, Mazzoleni G, Bondi S, et al. Oropharyngeal pH monitoring for laryngopharyngeal reflux: Is it a reliable test before therapy? J Voice 2013;27:84-89.

196. Fox M. Review article: identifying the causes of reflux events and symptoms - new approaches. Aliment Pharmacol Ther 2011;33:36 - 42.