An Investigation of Biomarkers in Laryngopharyngeal Reflux

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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

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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.

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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.