



**Studies on the pathophysiology of Bile Acid-
Induced inflammatory airways disease**

Adil A Aldhahrani

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Medical School
Institute for Cell and Molecular Biosciences
Newcastle University

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ABSTRACT

Background:

Gastro-oesophageal reflux and aspiration may be associated with airway disease. Bile acid aspiration has been shown to be prevalent among patients with advanced airway disease, which raises the concern that recurrent microaspiration of bile acids may cause airway epithelial injury which may lead to inflammatory airway disease.

Objectives:

To investigate the possible links between bile acids and lung injury which may cause pro-inflammatory cytokine release using BEAS- 2B, NCI-H292, 16HBE14o- and Calu-3 cell lines. Moreover, this study aimed at exploring whether primary bronchial epithelial cell cultures taken from lung transplant patients (PHBECs), tracheal epithelial cells (upper airway cells) from the subglottic area cells, BEAS-2B and 16HBE14o- cell lines undergo EMT like processes after stimulation with primary and secondary bile acid. Finally, the role of duodeno-gastro-oesophageal refluxate in mucus production from NCI-H292 and Calu-3 cell lines was investigated.

Methods:

BEAS-2, 16HBE14o-, NCI-H292 and Calu-3 cell lines were cultured and stimulated with Bile acid (BAs). Pro-inflammatory markers including IL-8, IL-6 and GMCSF were measured. Primary bronchial epithelial cell cultures taken from lung transplant patients and tracheal epithelial cells from the subglottic area cells were cultured. The effect of individual primary and secondary bile acids were evaluated by 48 hour challenge. Post-challenge TGF β 1, MMP9 and pro-collagen concentrations were measured using commercial ELISAs. RNA was isolated to determine the changes in expression of an epithelial marker E-cadherin and the mesenchymal marker fibronectin at a molecular level using a quantitative real-time PCR. The NCI-H292 and Calu-3 cell lines were also used as an *in vitro* model system to study MUC gene regulation. Cells were stimulated with bile acids and mucin content of the culture medium measured using an indirect ELISA and quantitative real-time PCR was used to evaluate MUC5AC and MUC5B expression in NCI-H292 and Calu-3 cell lines.

Results:

The cells used in this study elevated levels of IL-8, IL-6 and GM-CSF released after treatment by primary and secondary bile acids. IL-8, IL-6 and GM-CSF are common findings in airways and lung disease where reflux and aspiration have been implicated as a possible injury. Significantly greater concentration of TGF β 1, MMP9 and pro-collagen were measured in cultures of PHBEs, tracheal epithelial cells, BEAS-2B and 16HBE14o- cell lines treated with BAs. E-cadherin expression significantly decreased while fibronectin was significantly increased. MUC5AC and MUC5B levels were induced in response to stimulation of Calu-3 and NCI H292 cells with BAs.

Conclusion:

The NCI-H292, Calu-3, BEAS-2B and 16HBE14-o cell lines are sensitive and useful models to study human respiratory processes and diseases related to BAs aspiration-induced lung injury. Aspiration of bile acids may cause cell injury, inflammation and cell death relevant to the pathophysiology of chronic airways disease. Furthermore, Bile acids stimulated EMT related processes in PHBEC, tracheal epithelial cells, BEAS-2B and 16HBE14O-cell lines. The Calu-3 and NCI H292 cells can be used as an *in vitro* model system to study MUC5AC and MUC5B gene regulation.

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LIST OF ABBREVIATIONS

AEC	: Airway Epithelial Cells
ASMCs	: Airway Smooth Muscle Cells
ATCC	: American Type Culture Collection
BALF	: Bronchoalveolar lavage fluid
BAs	: Bile Acids
BARs	: Bile Acid receptors
BEBM	: Bronchial Epithelial Basal Medium
BEGM	: Bronchial Epithelial Growth Medium
BOS	: Bronchiolitis Obliterans Syndrome
CA	: Cholic acid
cDNA	: Complementary DNA
CDCA	: Chenodeoxycholic acid
CF	: Cystic fibrosis
CLO	: Columnar lined oesophagus
COPD	: Chronic obstructive pulmonary disease
DAMPs	: Damage-associated molecular patterns
DCA	: Deoxycholic acid
DGOR	: Duodenogastro-oesophageal reflux
DMEM	: Dulbecco's modified eagles medium
GM-CSF	: Granulocyte-macrophage colony-stimulating factor
GORD	: Gastro-oesophageal reflux disease
DMSO	: dimethylsulphoxide
ECM	: Extracellular-matrix
EERD	: Extra-oesophageal reflux disease
EGF	: Epidermal growth factor
ELISA	: Enzyme linked immunosorbent assay

EMEM	: Eagle's Minimum Essential Medium
EMT	: Epithelial to Mesenchymal Transition
ENT	: Ear, nose and throat
EORD	: Extra-oesophageal reflux disease
ERD	: Erosive Reflux Disease
FCS	:Fetal Calf serum
FEV1	: Forced expiratory volume in 1 second
FVC	: Forced vital capacity
FXR	: Farnesoid X receptor
H2RAs	: Histamine-2 receptor antagonists
ICP	: Intrahepatic cholestasis of late pregnancy
IL-1RA	: Interleukin-1 receptor antagonist
IL-8	: Interleukin-8
IL-6	: Interleukin-6
ILD	: Interstitial lung disease
IFN	: Interferon
IPF	: Idiopathic pulmonary fibrosis
ISS	: Idiopathic Subglottic Stenosis
LPR	: Laryngopharyngeal reflux
LCA	: Lithocholic acid
LES	: lower oesophageal sphincter
M	: Molar
MAPK	: Mitogen-activated Kinase
MET	: Mesenchymal Epithelial Transition
MMP	: Matrix metalloproteinase
NO	: Nitric oxide
μmol/l	: Micro moles per litre
SAGES	: Society of American Gastroenterological Surgeons

PAMPs	: Pathogen-associated molecular patterns
PGP	: pan-neuronal marker protein gene product
PAP	: pulmonary alveolar proteinosis
PCL	: Periciliary layer
PGDF	: Platelet-derived growth factor
pO ₂	: Pressures of oxygen
qRT-PCR	: Real-time reverse transcription-PCR
RDS	: Respiratory distress syndrome
RNA	: Ribonucleic acid
RV	: Respiratory Volume
TGF β	:Transforming Growth Factor- β
TNF- α	:Tumour necrosis factor alpha
α -SMA	: α -Smooth muscle actin

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DECLARATION

This thesis is based on research performed in the Institute for Cell and Molecular Biosciences, Newcastle University. I performed all the work and analysis of the results

PUBLICATIONS

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Aldhahrani A, Pearson J, Ward C. Bile acid aspiration and human Airway Epithelial Cell injury. In *Proceedings of the Physiological Society 2015*. The Physiological Society [Abstract].

CHAPTER 1: GENERAL INTRODUCTION

1.1 The respiratory system and airway epithelium

The human respiratory tract Figure 1-1 (Tu *et al.*, 2013) comprises the upper airways, which include the nose, sinuses, mouth and throat; and the lower airways: the trachea, bronchi and lungs. Within the lungs, the bronchi divide into increasingly smaller tubes called bronchioles and ultimately into alveoli, which are the air sacs where gas exchange occurs. This study will focus on the epithelial cells – of which there are various types – which line the system.

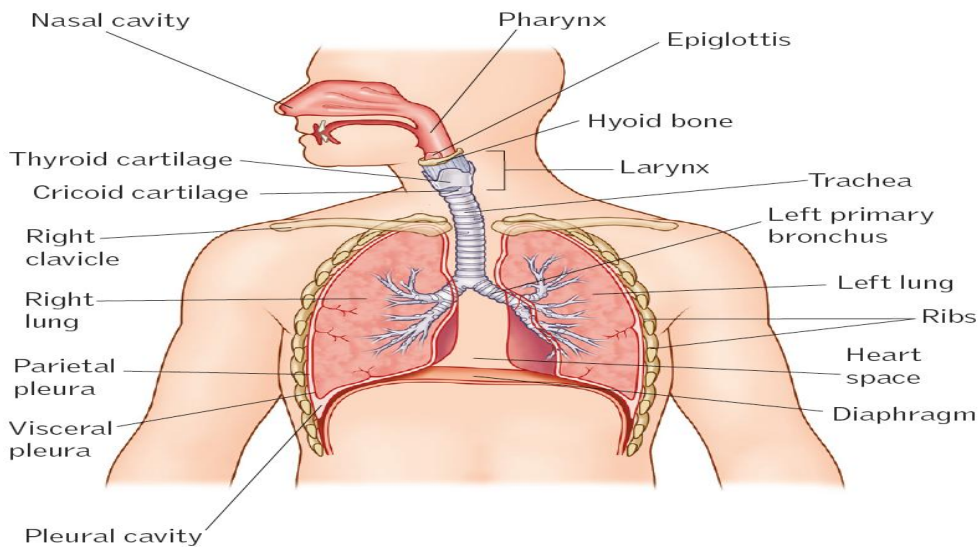


Figure 1-1: The anatomy of the respiratory tract (Tu *et al.*, 2013).

1.2 Anatomy of the airway epithelium

The airway epithelium includes a range of specialised cell types, each with a specific function that contributes to lung homeostasis. At least eight different types of epithelial cell are found in the system and, according to their location these fall into one of three categories: basal, ciliated columnar and non-ciliated secretory columnar cells (Knight and Holgate, 2003). Epithelial function is also assisted by immune and inflammatory cells, which migrate to the airway epithelium via the basement membrane. An example of these different cell types is seen in Figure 1-2 below, which shows a cross-section through the airway wall including the underlying mesenchyme.

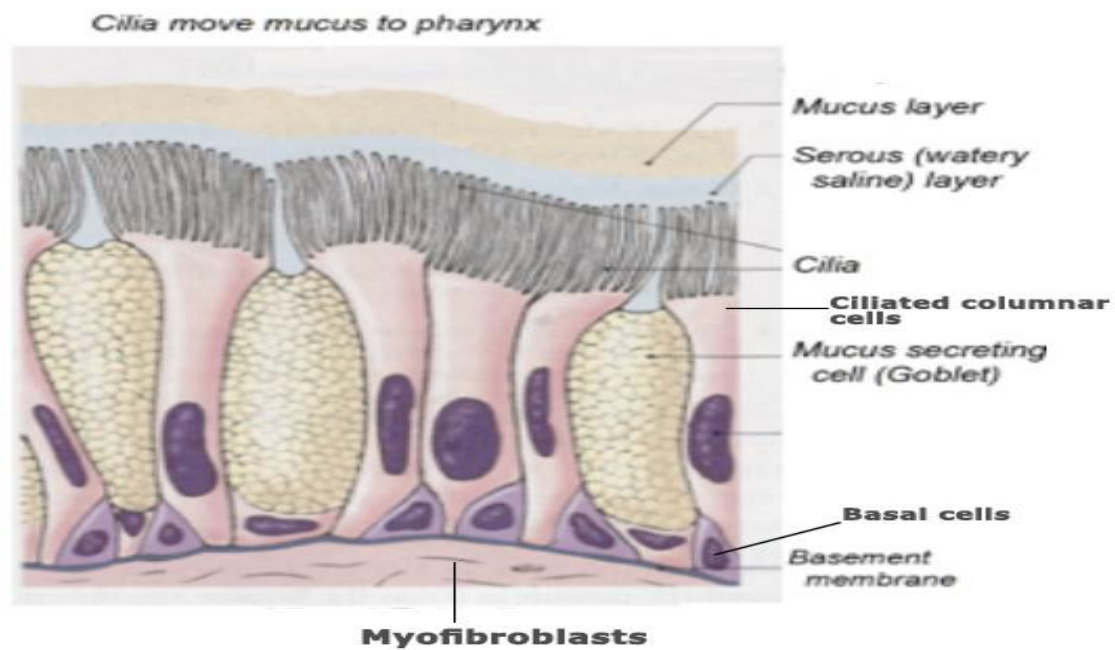


Figure 1-2: Schematic cross-section of the airway wall. The airway is lined with an epithelium, anchored together with several different junctions. The underlying mesenchyme is also shown consisting of myofibroblasts, smooth muscle cell bundles, blood vessels, and afferent nerve endings positioned deep into the airway. Drawing is not to scale (West, 2012).

The airway epithelium is particularly susceptible to damage by external factors, such as chemical toxins and other pollutants, bacteria, viruses, dust and smoke particles. However, the cells in this layer are arranged in an adhesive mechanism to ensure three dimensional structure and normal function is maintained. Hemidesmosomes are extra-cellular matrix (ECM) junctions, which connect basal cells to the basement membrane, conferring the tissue with increased mechanical integrity (Green and Jones, 1996). Interactions between basal and columnar cells are assisted by desmosomes, which are intercellular adhesion molecules of the cadherin family. These also function to create trans-cellular networks within the tissue, further increasing its mechanical integrity (Green and Jones, 1996). Interactions between columnar cells are aided by belt-like tight junctions (zona occludens), which regulate cellular transport. E-cadherin adherens junctions also help maintain cell adherence (Roche *et al.*, 1993). It is thought that the airway epithelium is pseudostratified in large airways, becoming columnar and cuboidal as it progresses through the airways to the lower airways (Crystal *et al.*, 2008).

1.2.1 Basal cells

Despite relative abundance, the density of basal cells varies according to the other cell types in different regions of the airway (Wang *et al.*, 1992). Basal cells are pyramidal in shape, with a large nucleus (Evans *et al.*, 2001) and use hemidesmosomes to attach other epithelial cells to the basement membrane. It is believed that epithelial stem cells originate from the basal cells, before differentiating into ciliated and secretory columnar cells in the upper part of the tract (Boers *et al.*, 1998). Basal cells have been discovered to secrete various active molecules including cytokines, chemokines, and growth factors.

1.2.2 Columnar secretory cells

Found within the epithelial layer's apical surface, the predominant secretory cells in the upper airways are mucus-secreting goblet cells, which are the system's primary mucus source. The primary role of mucus is to protect the airway epithelium by trapping toxins or small particles. Moreover, mucus stops airway epithelium drying out. These cells are characterized by membrane-bound electron-lucent acidic mucin granules (Jeffery and Li, 1997) and are involved in mucociliary clearance. Diseases such as asthma and COPD cause the number of these cells to increase, resulting in the characteristic productive cough (Lumsden *et al.*, 1984). In smaller airways (terminal bronchioles), Clara cells are predominant and also contain electron dense granules (Jeffery and Li, 1997). Xenobiotic compounds are metabolised via p450 monooxygenases in Clara cells, which also produce bronchiolar surfactant (Knight and Holgate, 2003) and can absorb and secrete ions (Van Scott *et al.*, 1987). Stem cells for the lower airway epithelium are found in areas of high Clara cell population and low mucous and basal cell population (Hong *et al.*, 2001). The serous cell is a different type of secretory cell similar to the mucus cell, but with a more electron dense granule content. Normally only found in rodent airways, these rare cells have occasionally been observed in the smaller airways of the human lung (Rogers *et al.*, 1993).

1.2.3 Columnar ciliated cells

More than half of all airway epithelial cells are ciliated (Knight and Holgate, 2003). These cells are involved in the transport of mucus and foreign objects in a directional capacity from the lower to the higher airway. Until recently, it was believed that these cells could not differentiate any further and were derived from secretory or basal cells. However, studies into the effects of naphthalene injury on mouse epithelia showed that these cells can trans-

differentiate, signalling a potential role in epithelial repair (Lawson *et al.*, 2002; Park *et al.*, 2006).

1.2.4 Alveolar type I cells

Of the two types of epithelial cells found in the alveoli, type I cells are squamous thin cells, covering 90-95% of the alveolar surface (Crapo *et al.*, 1982). They are particularly vulnerable to injury and do not divide. They are vital in maintaining alveolar cellular composition and the thin blood-gas barrier they form is vital to gas exchange (Williams, 2003). They also play an important role in alveolar epithelial homeostasis (Berthiaume *et al.*, 2006). Type I cells do not exhibit specific molecular markers and are difficult to isolate *in vitro*. Consequently, type II cells have received more scientific attention.

1.2.5 Alveolar type II cells

The remaining 5% of the alveolar epithelium is made up of the cuboidal type II cells. These are progenitors for type I cells, which cannot reproduce on their own. Type II cells are therefore vital in maintaining alveolar health following injury (Sugahara *et al.*, 2006).

1.2.6 Other cell types surrounding the airway epithelium

There are a range of other cell types and tissues proximal to the airway epithelium. These include: fibroblasts, blood vessels, smooth muscle, afferent nerve ending and immune cells. The latter include mast cells, lymphocytes, dendritic cells, and macrophages. When an external threat is present, these immune cells migrate to the epithelium to play a defensive role (Rennard *et al.*, 1994). Intra-epithelial nerves are populous in the bronchial epithelium. They have been found to penetrate the basement membrane to form a network between the epithelial cells and the basal lamina, as revealed by immunoreactivity for the pan-neuronal marker protein gene product (PGP) 9.5 (Adriaensen and Scheuermann, 1993). Below the basement membrane, fibroblasts secrete growth and attachment factors, which are involved in tissue repair and airway remodelling (Pollak and Lefebvre, 1992). They also stimulate bronchial epithelial cell migration via the production of chemotactic cytokines, which further contributes to the airway repair (Shoji *et al.*, 1989). Airway Smooth Muscle Cells (ASMCs), present between the trachea and the alveoli, control airway wall contraction and relaxation. These are governed by intracellular signalling (Hall, 2000), through which phospholipase C, adenylyl cyclase, and ion channel pathways control a series of homeostatic mechanisms. ASMCs play a critical role in asthma pathology, which involves increased bronchial SMC

mass and airway inflammation. It is therefore suggested that the airway hyper-responsiveness exhibited by asthma patients may be caused by ASMC abnormality (Borger *et al.*, 2006). ASMC responses are affected by cytokines in both directions (constrictor or relaxant) and therefore demonstrate a degree of immune function (Hakonarson *et al.*, 2001).

1.2.7 Basement membrane

The basement membrane is structurally composed of the extracellular matrix (ECM) proteins, anchoring filaments of laminin 5, collagen IV, and proteoglycans, and is found under the airway epithelium. In healthy bronchi, this layer is about 10nm (Jones, 2012). Further down (away from the epithelial cells), the composition is mainly made up of collagen III.

1.3 Functions of the airway epithelium

1.3.1 Epithelial barrier function and adhesion mechanisms

As was described in the previous section, the airway epithelium serves primarily to protect the airways from external threats, including toxic gases, pathogens, viruses, bacteria and allergens, including pollen and dust mite antigens (Knight and Holgate, 2003).

1.3.2 Mucociliary clearance and protective factors secretions

Further to the airway epithelium's protective role, secretory epithelial cells produce mucus, which traps foreign objects. Coordinated ciliary beating then assists in transporting these out of the tract (Bals *et al.*, 2004). Anti-oxidants and anti-bacterial agents, such as lactoferrin, lysozyme, and opsonins as well as anti-proteases and $\alpha 1$ -Anti-trypsin such as tissue inhibitors of metalloproteinases are produced by epithelial cells (Velden and Versnel, 1998).

1.3.3 Epithelial inflammatory potential

Injury to the airway epithelium triggers a pro-inflammatory response. This causes the production of such mediators as TGF- β , cytokines, arachidonic acid products and including IL-8 and IL-6.

1.4 Gastro-oesophageal reflux

1.4.1 Definition

Gastro-oesophageal reflux (GOR) is, most of the time, a normal response caused when the lower oesophageal sphincter becomes relaxed and gastric contents travel up into the oesophagus. Generally, this is harmless and there are no symptoms (Herbella and Patti, 2010). Under a specific set of conditions however, GOR may become pathological, leading to gastro-oesophageal reflux disease (GORD) (Morehead, 2009; Herbella and Patti, 2010). Vakil et al. define GORD as “a condition which develops when the reflux of gastric contents causes troublesome symptoms and/or complications” (Vakil et al., 2006). However, it is discussed that this definition does not have any quantitative that can be used to measure the incidence and prevalence of the GORD (McCarthy, 2013). Heartburn and regurgitation are the primary symptoms of GORD, although since these are also associated with over eating and indigestion, this makes distinguishing chronic GORD more difficult. The following definition has therefore been proposed: “GORD is the incidence of heartburn or regurgitation once or more daily” (McCarthy, 2013). Classifications of GORD exist which are tighter still, including: heartburn or regurgitation without erosions on endoscopic examination (Non-erosive Reflux Disease, NERD), or with erosions present (Erosive Reflux Disease, ERD). A range of risk factors have been identified in the development of GORD, including alcohol, tobacco, obesity, diabetes asthma, hiatus hernia, old age and pregnancy. Furthermore, even in non-obese patients, obstructive sleep apnoea is also identified as a risk factor (Morehead, 2009).

1.4.2 Diagnosis of GOR

1.4.2.1 Endoscopy

In addition to mucosal biopsies, visual investigation can indicate the severity of GORD (McCarthy, 2013). This method is also important in ongoing assessment of the disease, including checking for oesophagitis (by tracking morphologic changes in the squamous epithelium) and columnar lined oesophagus (CLO), caused by columnar metaplasia. CLO in particular can indicate the onset of oesophageal adenocarcinoma (aka Barrett’s Carcinoma). Endoscopy is therefore a vital investigative tool in identifying potential GORD-related oncologic complications (Lenglinger *et al.*, 2006).

1.4.2.2 Ambulatory pH testing

Ambulatory pH testing is an established standard method for monitoring GORD (Hirano, 2006). This involves placing a probe 5cm above the lower oesophageal sphincter and measuring the length of time the pH is less than 4 (Lenglinger *et al.*, 2006). For accuracy, some monitors use two probes, one proximal and one distal. Of course, non-acidic reflux is not detected using this method (Hirano, 2006).

1.4.2.3 Impedance-pH monitoring

When the reflux is non-acidic (or of low acidity), more sensitive methods are used. A technique involving combined multichannel intraluminal impedance and pH monitoring is able to measure all types of reflux as well as bolus transport (Figure 1-3) (Hong and Vaezi, 2009). This is considered more accurate than the previously dominant method of 24h pH monitoring.

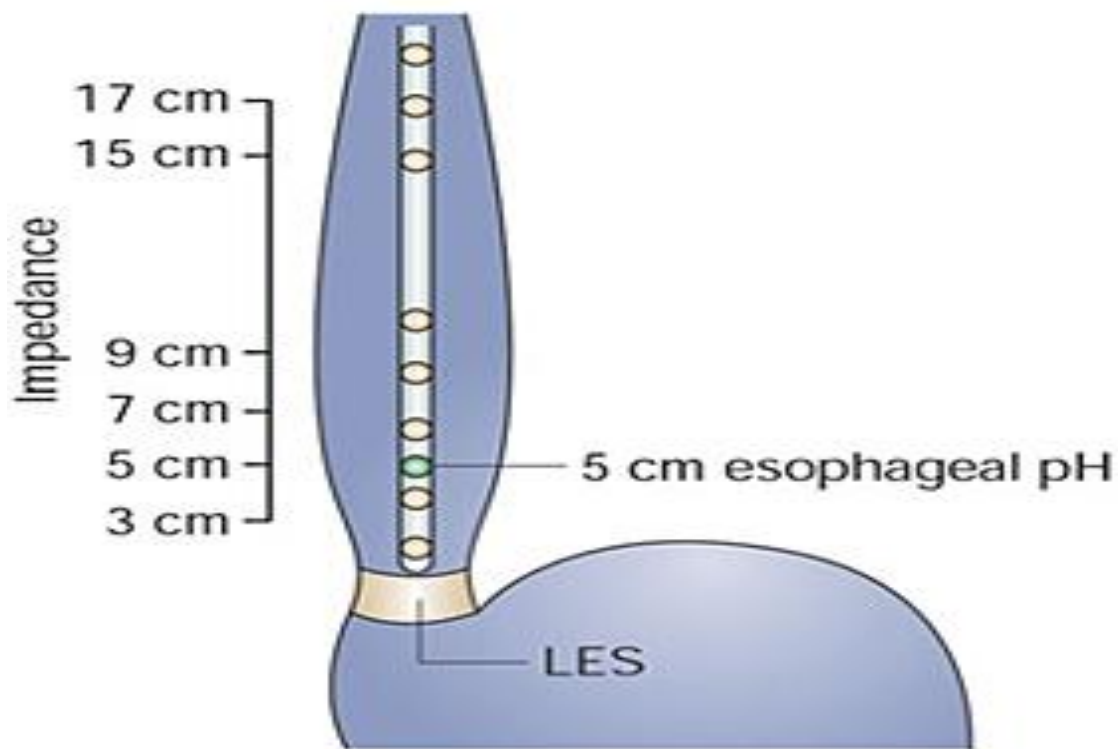


Figure 1-3: Impedance-pH catheter. The pH electrode is located 5 cm above the lower oesophageal sphincter (LES) and the distal electrode in the stomach, allowing impedance measurements at 3, 5, 7, 9, 15 and 17 cm overhead the LES (Tutuian and Castell, 2006).

Ambulatory impedance monitoring has been in use since 1991 and is agreed as a reliable method for measuring GOR. An impedance probe is used, with electrode rings placed at various intervals to measure the refluxate electrical conductivity. This data, in addition to pH data yields information on exposure of the oesophagus to gastroduodenal contents and its acidity levels (Hong and Vaezi, 2009).

Patients who presented with symptoms of reflux (heartburn and regurgitation), but for whom endoscopy revealed nothing, previously underwent oesophageal pH testing. However, since proton pump inhibitors became the dominant empirical method in diagnostic testing, more objective methods are now used for patients who are unresponsive to acid suppressants, prior to considering fundoplication (Hirano and Richter, 2007; Kahrilas *et al.*, 2008).

Where oesophagitis is observed visually, ambulatory 24-hour pH monitoring is considered acceptably sensitive (77%-100%) and specific (85%-100%). In cases where GORD cannot be verified by endoscope, the results are less easy to interpret (0-71% sensitive and 85-100% specific) (Kahrilas and Quigley, 1996). Impedance monitoring is more than 90% sensitive and specific, compared to both manometry and pH monitoring. This has led to consensus that

impedance monitoring is the best available method for testing the implications of reflux symptoms. Ambulatory pH testing may be considered an accurate test of oesophageal reflux, but is far less sensitive in cases of endoscopy negative GORD, which may indicate an acid-sensitive oesophagus. Both devices are considered far less efficacious in patients who do not respond to proton pump inhibitor therapy (Sifrim *et al.*, 2004).

1.4.2.4 Bilitec monitoring

When pH levels seem normal, Bilitec monitoring can be used in place of ambulatory pH testing (Lenglinger *et al.*, 2006), to identify bile refluxate in the oesophagus and measure its concentration. This method is not without its disadvantages; refluxate can become trapped in the sensor and a special diet is required. Biomarkers in bronchioalveolar lavage fluid may be used to get around these factors (Hirano, 2006), but the method is generally unpopular among patients and is rarely taken up.

1.4.2.5 Bravo capsule

A more tolerable option is the Bravo Capsule (Medtronic, MN, USA) (Hirano, 2006). The capsule is applied to the lower oesophageal mucosa via either a catheter or endoscope and measures pH levels over about 24h, transmitting data to a wireless receiver.

1.4.3 Composition of gastric juice

Gastric juice is secreted by cells which have a structural arrangement referred to as 'gastric pits'. These comprise several gastric glands and can be found all along the gastric mucosa. The juice itself is usually transparent and is made up of a mixture of: hydrochloric acid (HCl), mucin, lipase, pepsin, intrinsic factor, peptides, nucleic acids and electrolytes. Parietal cells secrete HCl, which maintains gastric conditions at between pH2-3. This is necessary for pepsin activation, which plays a defensive role against pathogens in the stomach and intestine. Gastric pH (hypochlorhydria) can be elevated by disease, bile reflux, or pharmaceuticals, which may cause bacterial overgrowth in the stomach. These bacteria can produce nitrite and nitroso-compounds, one of the causes in the atrophy-metaplasia-dysplasia carcinoma pathway (Lu *et al.*, 2010).

1.4.4 Treatment

Treatment options are intended to: provide symptom relief, oesophageal healing, prevent and manage other complications associated with GORD, and maintain oesophageal and mucosal remission (Fock and Poh, 2010)

1.4.4.1 Lifestyle modifications

All medications have side effects to a varying degree. Less severe cases of GORD can and should therefore be managed through lifestyle changes, including diet and weight-loss measures, bed-head elevation, smoking cessation and avoiding postprandial recumbency for at least 3h. It has been suggested, however, that in spite of the fact that smoking, citric acid, or fatty foods can raise oesophageal pH, GORD outcomes show no significant improvement when these factors are removed (Fock and Poh, 2010).

1.4.4.2 Antacids

Instantaneous, albeit temporary relief can be provided by antacids, which are widely accessible, but require frequent ingestion. However, they only treat the discomfort and not the causes of GORD (Herbella and Patti, 2010). Furthermore, some antacids, such as metoclopramide, can cause side effects, such as depression, drowsiness and involuntary spasms (Ganzini *et al.*, 1993), as well as diarrhoea, constipation and interference with the absorption of other drugs (McCarthy, 2013).

1.4.4.3 Acid suppression therapy

Histamine-2 receptor antagonists (H2RAs) and proton pump inhibitors are two classes of medications which can result in acid suppression.

1.4.4.3.1 Histamine-2 receptor antagonists

H2RAs (e.g. cimetidine, ranitidine, famotidine and nizatidine) can heal mucosal damage and relieve symptoms, and so have significantly improved the control of GORD-related symptoms. Furthermore, the same efficacy as antacid treatment can be achieved with only a twice-daily treatment. However, they are less suitable for long-term treatments, due to the potential for tolerance (Hatlebakk and Berstad, 1996) and are also less effective in controlling acid secretion than PPIs (Katz and Tutuian, 2001).

1.4.4.3.2 Proton pump inhibitors (PPIs)

PPIs have become established as the dominant therapy for gastric acid-related diseases (McCarthy, 2013). Patients who were on proton pump inhibitor (PPI) therapy had significantly reduced oesophageal acid exposure and a reduced number of reflux events; and, as antisecretory compounds, are more effective – and last longer - than Histamine-2 receptor antagonists (H2RAs) (Fock and Poh, 2010). Five PPIs are currently on the market: omeprazole, lansoprazole, rebeprazole, pantoprazole and esomeprazole. The risks associated with PPI are very low. However, levels of pepsin and bile were comparable in both patients who were on PPIs and those that were not. This suggests that while PPI treatment can reduce gastric acid secretion, it cannot prevent gastric aspiration (Blondeau *et al.*, 2008b) so, acid suppression alone may not be totally effective (Patel and Hurst, 2013). Acid suppression also has side effects, including: increased potential for: fractures; risk of infection; greater susceptibility to atrophic gastritis; hypomagnesaemia; *Clostridium difficile*-associated diarrhoea; as well as vitamin B12 and iron deficiencies (Bredenoord *et al.*, 2013).

1.4.4.4 Anti-reflux surgery

Some GORD patients are appropriate candidates for anti-reflux surgery. This has been shown by longitudinal studies to enable better symptom control than medical therapeutic interventions (Fock and Poh, 2010). The Nissen fundoplication procedure is commonly performed on children with GOR (Georgeson, 2009), in which a 1.5-2cm fundus is wrapped around the oesophagus, using a bougie to control the tightness of the wrap. As a result, pressure at the lower oesophagus is increased, limiting the change of reflux occurring (Dallemane *et al.*, 1991). Patients may experience complications after surgery, such as dysphagia, post-vagotomy symptoms and gas-bloat syndrome. The Society of American Gastro-Enterological Surgeons (SAGES) has recommended, based on the high reoccurrence of GORD symptoms, that surgery should only be performed as a last resort; after other medical management options have been exhausted, in complicated circumstances (e.g. Barrett's Syndrome), with atypical symptoms (e.g. asthma), or when age or medication expenses make management difficult (Dumonceanu *et al.*, 2013).

1.4.5 Extra-oesophageal reflux disease

At a conference in 2006, the Montreal Consensus Group distinguished two types of GORD syndrome: oesophageal and extra-oesophageal. The latter is subdivided into either ‘established’ or ‘proposed’ (Figure 1-4) (Vakil *et al.*, 2006). When describing GOR that has breached the upper sphincter of the oesophagus to interfere with the laryngeal or pharyngeal mucosa (Figure 1-5), ENT specialists use the terms: extra-oesophageal reflux disease (EORD) or laryngopharyngeal reflux (LPR). LPR can result in a wide range of symptoms, which may cause concomitant symptoms to be overlooked (e.g. heartburn), which increases the complexity of diagnosis. In cases such as this, 24h pH monitoring and laryngoscopy are usually used (Kavitt and Vaezi, 2013).

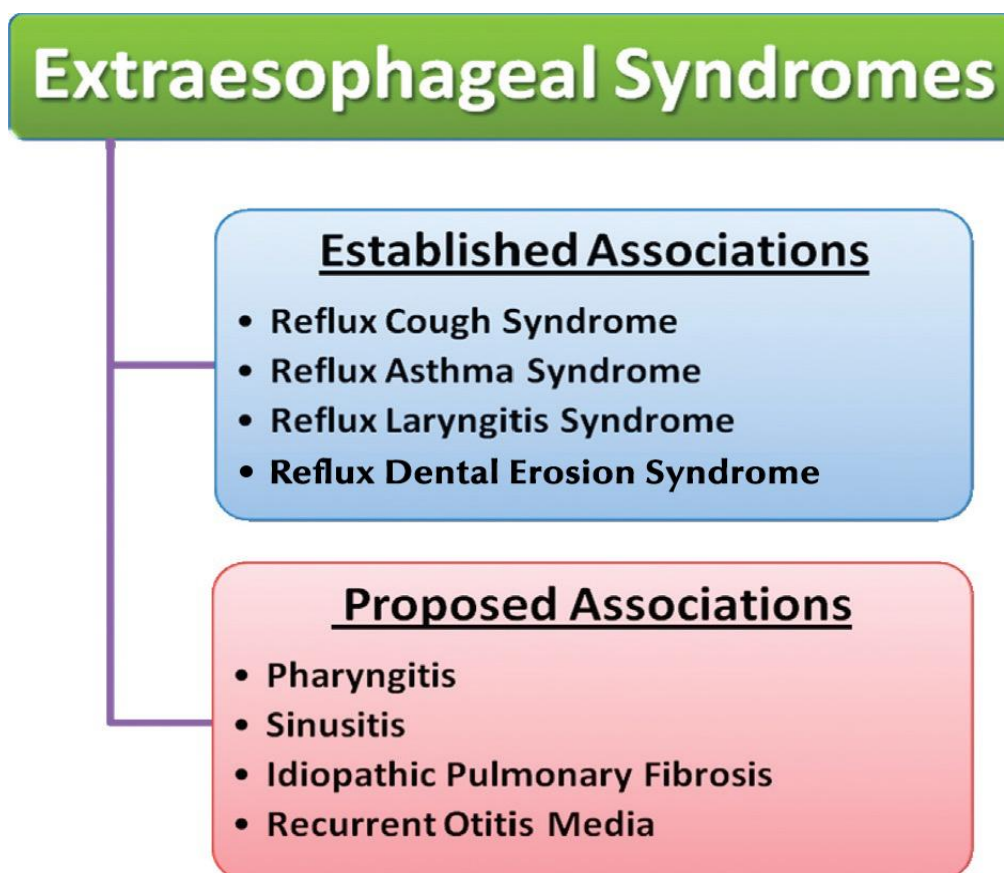


Figure 1-4: Classifications of extra-oesophageal syndromes linked to GORD in agreement with Montreal Consensus Conference (Vakil *et al.*, 2006).

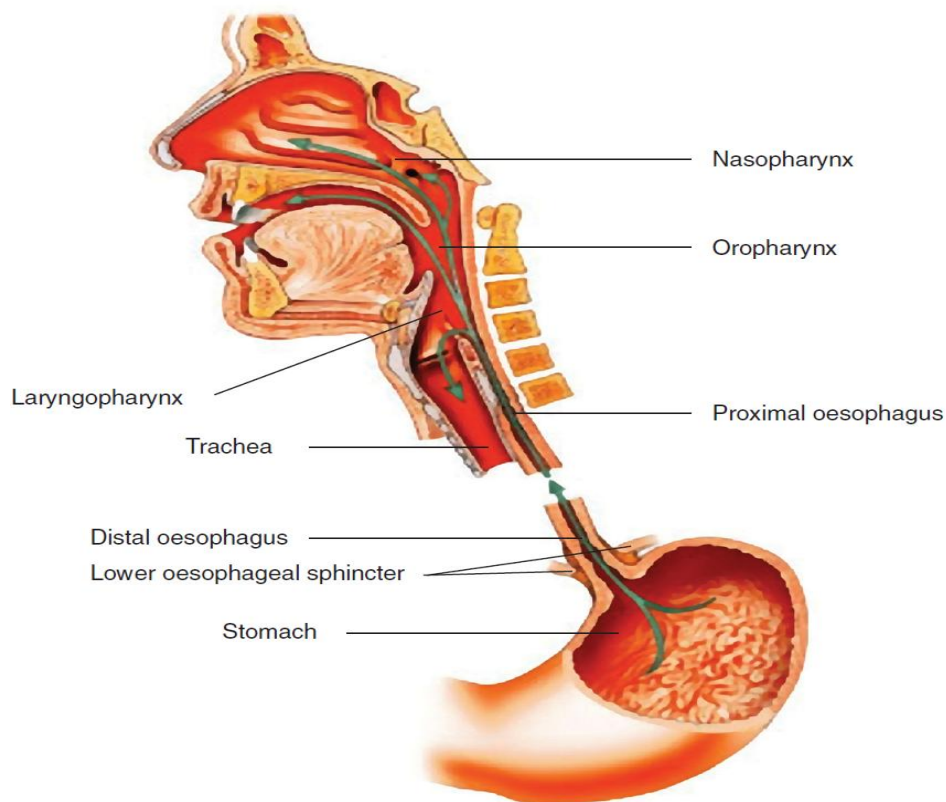


Figure 1-5: Extra-oesophageal pass from stomach to oesophageal. Laryngopharyngeal reflux results when refluxate reaches the vulnerable mucosa of the pharynx, larynx, oral cavity and nasal cavity (Patel and Hurst, 2013).

1.4.6 Aspiration

When material, such as food, liquids, particles, blood, secretions of bacteria enter the airways, this is called aspiration (Raghavendran *et al.*, 2011). Sometimes, small volumes of refluxate are inhaled into the lungs (microaspiration), which can lead to pulmonary parenchymal lesions. These cases are rarely associated with the more traditional respiratory symptoms and so are harder to diagnose. Consequently, microaspiration has been deemed a ‘silent’ phenomenon (Lee *et al.*, 2010).

1.4.7 Duodenogastro-oesophageal reflux (DGOR)

Refluxate may contain bile salts, pancreatic enzymes, stomach enzymes and duodenal contents, as well as HCl. The severity of oesophageal mucosal damage is heavily affected by these compounds, which are regurgitated through the pylorus into the stomach, then refluxed into the oesophagus. Bile acids have toxic effects on the colonic mucosa (Turjman and Nair, 1981; Owen *et al.*, 1984), hepatocytes (SchÖLmerich *et al.*, 1984) and gastric mucosa (Gadacz and Zuidema, 1978; Gillen *et al.*, 1988). However, their toxicity to the oesophageal mucosa is less well-researched (Nasr *et al.*, 2013). A strong evidence base exists linking oesophageal acid exposure (OAE) to both mucosal damage and Barrett's oesophagus and its associated complications (Legrand *et al.*, 1990; Iftikhar *et al.*, 1993; Takahashi *et al.*, 2011). However, It has been suggested that excessive DGOR may also have a damaging effect on the gastric mucosa (Stern *et al.*, 1984). Indeed, patients with a range of airway diseases were found to have increased bile acid concentrations in their BAL fluid. In comparison to aspiration of gastric contents, aspiration of bile acid may lead to non-cardiac pulmonary oedema, derived from the increased severity of pneumonitis. These findings are critical to the understanding of GORD, as they challenge the established idea that oesophagitis and other GORD symptoms were caused only by reflux of HCl. As a result of this misconception, the majority of therapies were aimed at gastric parietal cells, which produce gastric acid. A study of acid reflux (via pH monitoring) and reflux of duodenal juice (via bilirubin monitoring) among 53 GORD patients, found that two-thirds of the patients had a mixture of both types of reflux (Kauer *et al.*, 1995). Furthermore, higher bilirubin levels were found in patients with Barrett's metaplasia. These results provide a potential explanation for why PPI therapy is only partially successful in symptom control in some patients and why, despite PPI therapy, some patients have increased risk of developing Barrett's oesophagus.

1.4.8 Relationship between lung disease and GOR

Patients with advanced stages of lung diseases often exhibit GOR, suggesting that recurrent microaspiration – and consequently further lung injury may be partially caused by reflux (Allaix *et al.*, 2013). No reliable diagnostic tool has yet been identified to measure the impact of aspiration on clinical outcomes (Meyer and Ganesh Raghu, 2013). Lung diseases can be caused by GOR either by a) neural reflex mechanism, in which reflux is limited to the lower oesophagus, or b) proximal GOR, in which refluxate breaches the upper sphincter of the oesophagus, causing injury to the upper airway. Furthermore, if aspiration occurs, this increases the risk of lung disease (Morehead, 2009).

GOR has been implicated in the lung damage in Idiopathic Pulmonary Fibrosis (IPF) in patients with GOR and also hiatus hernia. GOR has also been implicated in a range of other chronic lung diseases included COPD, CF and asthma. In these cases, aspiration may be a contributing factor, rather than viral or autoimmune causes, as initially believed (Mays *et al.*, 1976).

A number of key factors of GORD-related complications beyond the oesophagus have been suggested: In a study of 100 pathological reflux patients, a correlation was established between weak oesophageal peristalsis and slow clearance of the oesophagus, and symptoms in the respiratory system following reflux. The study concluded that inadequate oesophageal motility may indicate increased respiratory symptoms following reflux events. pH testing also indicates a sequential link between reflux and cough. Where the cough preceded a reflux event, this is assumed to cause an increase in abdominal pressures. When reflux precedes a cough, it is believed that this causes the cough. Reflux symptoms including heartburn, regurgitation and dysphagia have limited correlation with reflux measured via pH (Pellegrini *et al.*, 1979).

Decreased oesophageal motility was found in 16 out of 24 systemic sclerosis patients (67%) and this was associated with impaired lung compliance (Denis *et al.*, 1981). Another study of patients with systemic sclerosis found that 13 were affected by reflux, which was implicated in their associated pulmonary problems. Patients underwent endoscopy, mucosal biopsies at both ends of the oesophagus, pulmonary function tests, oesophageal manometry and pH monitoring probes. Peristalsis was not detected in 77% of patients. Of these, some patients exhibited the presence of acid in their upper oesophagus. Based on these findings, it was suggested that the pulmonary complications associated with systemic sclerosis may be due to

chronic aspiration of gastric juices. However, it is worth acknowledging that many of these patients exhibit end-stage oesophageal disease symptoms with zero peristalsis – so this study does not necessarily apply to the average GORD patient (Johnson *et al.*, 1989).

A study of 70 GORD patients aimed to find a correlation between coughing and oesophageal reflux, using a dual-sensor pH probe. The study's hypothesis proposed that when cough and distal reflux were concomitant, then a vagal reflux arc due to oesophageal irritation was responsible for the accompanying symptoms. However, when reflux went further, reaching the upper oesophagus, then the cough was more likely to be caused by microaspiration, and this was more prevalent in patients with low oesophageal motility (Patti *et al.*, 1993).

Previously, this data had been limited to individual case studies. However, the association between respiratory complications and reflux in population settings was confirmed by El-Serag and Sonnenberg. Using a case control study of 200,000 US veterans, it was found that where erosive oesophagitis was present, indicating severe GORD, there was a 1.36 odds ratio (OR) of pulmonary fibrosis, a 1.28 OR of chronic bronchitis and a 1.22 OR of chronic obstructive pulmonary disease (COPD). This data, despite not proving a certain causal effect, nevertheless makes a connection between GORD and advanced lung disease (ALD) (el-Serag and Sonnenberg, 1997; Sweet *et al.*, 2009). Another study on the association between reflux and O₂ desaturation used pulse oximetry monitoring and a 24h pH impedance. The study found a positive correlation between reflux episodes at both ends of the oesophagus and with O₂ desaturation. These findings could enable more accurate ways to predict reflux episodes. These events were found to decrease following anti-reflux surgery (Wilshire *et al.*, 2013).

1.5 Bile Acids

Hepatocytes in the liver synthesise bile acids, which serve digestive and secretory functions, are water-soluble and aid lipid absorption in the small intestine. Using cholesterol as a starting point, a number of pathways are responsible for the production of bile acids (Monte *et al.*, 2009). Primary bile acids are first synthesised by the catabolism of cholesterol into cholic acid (CA) and chenodeoxycholic acid (CDCA). Bacterial enzyme action in the colon aids primary BA deconjugation into free bile acids and further enzymatic action converts CA into deoxycholic acid (DCA) and CDCA into lithocholic acid (LCA). More specifically, the hydroxyl (OH) group is detached from the 7th carbon atom by the enzyme 7 alpha-dehydroxylase. DCA and LCA are moved to the canaliculi of the liver through the portal vein to join the new primary BAs and re-conjugate with glycine or taurine. This is a closed-system recycling process, involving the bile flow from the liver, via the biliary tract to the intestine then back to the liver. This process of 'enterohepatic circulation' enables recycled bile to be stored in the gall bladder for release after meals. This usually happens in two bursts, resulting in up to eight bursts per day (Jenkins and Hardie, 2008).

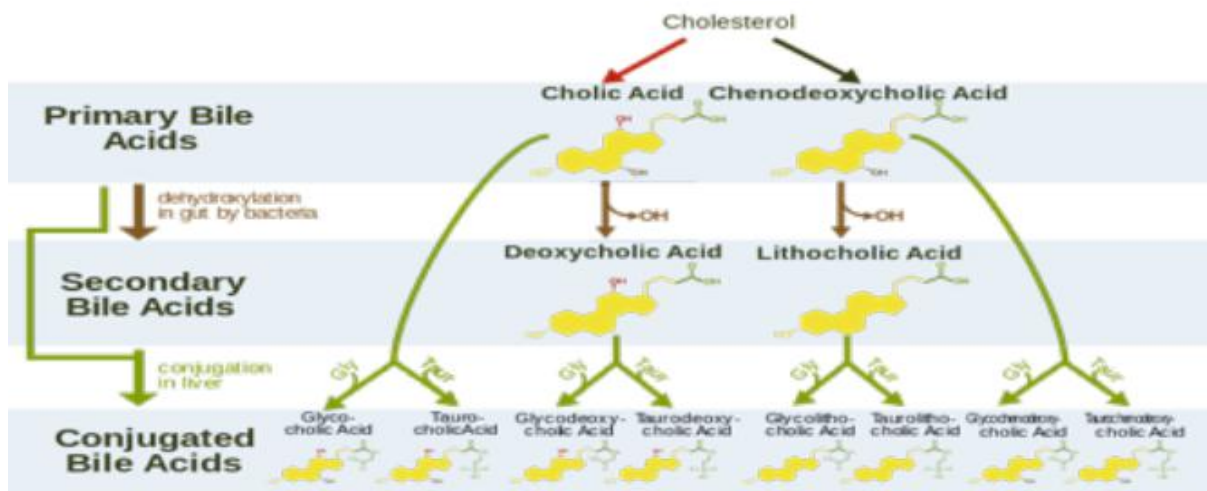


Figure 1-6: The classical pathway for bile acid (BA) formation. In human hepatocytes, the two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesised as a result of cholesterol metabolism. CA and CDCA differ in their hydroxylation at C-12~. These primary BAs are usually conjugated to glycine or taurine before they enter biliary flow. Once they reach the digestive tract, microbes in the gut can remove the conjugated glycine or taurine, transforming primary BAs into secondary BAs via dehydroxylation at C-7. CA is transformed into deoxycholic acid (DCA) and CDCA is transformed into lithocholic acid (LCA). Most BAs then re-enter enterohepatic circulation returning to the liver where they are re-conjugated to taurine or glycine. Image from: [https://upload.wikimedia.org/wikipedia/commons/thumb/c/c4/Bile_acid_differentiation.svg/655px-Bile_acid_differentiation.svg.png] [accessed on 11 September, 2016].

Most circulating bile acid is conjugated to glycine or taurine. Unconjugated BAs tend to remain in the colon, where the glycine or taurine subunits are removed via microbial modification (Monte *et al.*, 2009). Gastro-oesophageal reflux disease (GORD) patients undergoing therapy which targets acid suppression can exhibit overgrowth of bacteria in the intestine. In turn, this causes BAs to deconjugate, resulting in a greater number of unconjugated BAs in circulation (Figure 1-6) (Solaas *et al.*, 2000).

1.5.1 Evidence for the presence of Bile Acid in the airway

Reports of BA presence within the airway have increased over the last decade and this has similarly been associated with lung function decline. BAs may reach the lung in one of two ways; they may be aspirated during reflux episodes, or via increased circulatory BAs in blood. In a porcine lung model, BA aspiration caused severe chemical pneumonitis, and rabbit models, injected BAs caused pulmonary damage (Kaneko *et al.*, 1990; Porembka *et al.*, 1993; Perez and Briz, 2009).

Gastroesophageal reflux (GOR) and subsequent BA aspiration causes lung injury in adults. In transplant patients, immunosuppressive drugs and iatrogenic vagal nerve injury may induce GOR, or it may already be present prior to lung transplant (D'Ovidio and Keshavjee, 2006). Following lung transplantation, BA aspiration may be increased and has been implicated in the onset of bronchiolitis obliterans syndrome (BOS). BOS, defined by a loss of lung function can be associated with inflammatory cells in and around the airway and also fibrosis (D'Ovidio *et al.*, 2005). BOS patients who have also received a lung transplant have been reported to exhibit greatly increased BA levels in BAL fluid, up to 32 $\mu\text{mol/L}$ compared to normal level which is from 0 to 16 $\mu\text{mol/L}$ (D'Ovidio *et al.*, 2005).

BA aspiration is injurious to the pulmonary system (el-Serag and Sonnenberg, 1997; Wilshire *et al.*, 2013). Cell membranes are particularly susceptible to bile. *In vitro* studies on Type II pneumocytes have demonstrated this (D'Ovidio *et al.*, 2005; D'Ovidio and Keshavjee, 2006). BAs also have a detergent effect. In the lungs however, damage to the mucus barrier may result in disruption to the lipids in the surfactant. Consequently, the type II pneumocytes, whose primary role is the production of surfactant protein and phospholipids are particularly prone to damage.

A groundswell of research is emerging which implicates the role of BA reflux in Barrett's oesophagus and cancer (Attwood *et al.*, 1992; Stamp, 2002). Increased bile exposure was found in Barrett's metaplasia patients, as well as heightened levels of secondary BAs within their refluxate (Nehra *et al.*, 1998). By comparison, Barrett's patients with more advanced conditions, such as early adenocarcinoma exhibited increased BA exposure (Stein *et al.*, 1999).

Studies of rabbits have shown that macrophage function can be inhibited by bile acids which affect lipopolysaccharide (LPS) mediated cytokine production and phagocytosis. Bile acids may also cause down-regulation of Interferons (IFNs)-mediated signal transducers. By implication, it has been inferred that innate immunity receptors on both monocytes and macrophages may be generally down-regulated (D'Ovidio and Keshavjee, 2006). If this is the case, then the risk of local infection may be increased, triggering an up-regulation of a more aggressive adaptive immune response (D'Ovidio and Keshavjee, 2006). This may be further exacerbated by damage to the surface epithelial cells (D'Ovidio *et al.*, 2005). Bile acids have also been associated with elevated neutrophils, IL-8, bacterial and fungal presence, decreased pulmonary surfactant and higher inflammatory scores on transbronchial biopsy (D'Ovidio and Keshavjee, 2006; Blondeau *et al.*, 2008b).

Studies into the pathogenesis of cholestatic liver injury in rats, hamsters and rabbits have identified the monohydroxy- bile acid, LCA, as hepatotoxic (Palmer and Ruban, 1966; Miyai *et al.*, 1977; Hofmann, 1999b). The research into LCA-induced cholestasis has yielded important understandings of: bile canalicular membrane biochemistry (Kakis and Yousef, 1978); low solubility of LCA causing crystalline plug formation (Miyai *et al.*, 1977); and greater knowledge around the transport of canalicular export pumps to and from the canalicular membrane (Kubitz *et al.*, 2004).

Cystic Fibrosis (CF) causes acid GOR in both children and adults, which can damage and compromise pulmonary functioning (Robinson and DiMango, 2014). Studies of CF patients have detected bile acids in the lower airway via tandem mass spectrometry. Their presence poses a threat of injury even after lung transplantation (Brodie *et al.*, 2015). In chronic bronchitis, airway wall destruction and the build-up of intraluminal secretions also lead to reduced lung function in CF patients (Rowe and Clancy, 2006). As a consequence, the need for antibiotics and bronchodilators increases and this may further affect the gastric and duodenal flora and may impair lower oesophageal sphincter (LES) function, leading to the

worsening of GOR (Blondeau *et al.*, 2010). Bile acids have been found in the saliva of a third of child CF patients (Blondeau *et al.*, 2010), indicating a potentially heightened aspiration risk. Studies have never clarified whether GOR is a primary phenomenon of CF, or secondary to cough or physiotherapy. The link between this and GOR, aspiration and CF-related respiratory systems is still under debate (Vic *et al.*, 1995; Blondeau *et al.*, 2010).

Reflux-related aspiration has also been diagnosed through analysis of sputum BAs (Perng *et al.*, 2007). GOR patients were made to produce sputum, which was then analysed to compare the concentrations of BA and TGF-beta 1 levels. It was found that GOR patients had higher bile acid concentrations in comparison to controls ($p < 0.005$), which also correlated to TGF-beta 1. TGF-beta 1 can play a role in fibroblast proliferation. It has been found recently that over half of CF patients have BAs in their sputum (Pauwels *et al.*, 2012).

GOR in children has been linked with wheezing and chronic coughing (Bauman *et al.*, 1996). Neuro-disabled children often exhibit high incidences of GOR and are often admitted to hospital due to recurrent respiratory problems (Trinick *et al.*, 2012). In these cases, aspiration may occur either directly, or result from reflux. Nevertheless, there remains some uncertainty around the relationship between GOR, aspiration, and respiratory symptoms. Increased BA levels have been found in ventilator-assisted pneumonia (VAP) patients, indicating that neutrophilic inflammation in patient airways may be caused by CDCA-induced IL-8 production via the activation of mitogen-activated protein (MAP) kinases (Wu *et al.*, 2009).

Recently, a study conducted by McDonnell assessed the inflammatory and cytotoxic effects of chenodeoxycholic, cholic, deoxycholic and lithocholic acids at concentrations 0, 12.5, 25, 50 and 100 $\mu\text{mol/L}$ showing they present a challenge to human bronchial epithelial cells (HBEs). IL-8 was measured by ELISA within culture supernatants, wherein the viability of the cells was analysed using MTT. It was also found that exposure to BA reduced the viability of the cells in a concentration-dependent manner. The BA-induced IL-8 release was more significant at lower acidic levels up-to the point of full cell deterioration. Given these results, this model would suggest that BA cytotoxicity within the pulmonary epithelium are affected by acidity as well as being dependent on the concentration level (O'Toole *et al.*, 2015).

Song et al (2011) have reported that in human oesophageal epithelial cells conjugated bile acid are potent inducers of MUC5AC expression, a mucin that is usually only produced in the stomach and tracheobronchial epithelium. They demonstrated that the conjugated bile acid glycochenodeoxycholate, taurocholic acid, glycocholic acid and taurodeoxycholic acid, which are major components of bile refluxate in the oesophagus, are strong inducers of MUC5AC mucin expression in the oesophageal cells. Since MUC5AC is an important mucin in the airway, this finding may be relevant for understanding how bile acid could affect mucin secretion and mucociliary clearance in the lung (Song *et al.*, 2011).

There is considerable variability in the levels of detection of bile acids in reflux studies. This is not only due to the different methods of detection but variability between individuals and the time of day samples were collected. A common assay is the 3α hydroxylase method described by Fausa & Skålhegg (Fausa and Skålhegg, 1977). This assay is not affected by pH but the presence of food or colourants can interfere with results (Collins *et al.*, 1984). Mass spectrometry assays are useful method. However, kits developed to look at pathological levels in blood may not be suitable for detection of the lower levels found in aspiration.

There is considerable variability in the lower limit of detection of mass spectrophotometric assays; Collins et al suggested $62.5\mu\text{mol/L}$ (Collins *et al.*, 1985), Klokkenburg claims $5\mu\text{mol/l}$ (Klokkenburg *et al.*, 2009), Biostat, who produce the commercially available assay claim a lower limit of detection $1\mu\text{mol/L}$ and the Leuven group have claimed an accuracy of $0.2\mu\text{mol/L}$ (Blondeau *et al.*, 2008a). These levels are lower than serum bile acid levels ($<8\mu\text{mol/L}$) (D'Ovidio *et al.*, 2005). One group have found this type of assay to be unreliable (Gotley *et al.*, 1990). Certain operations can affect the concentration of intra-gastric bile acid concentrations; 90% of the normal population will have intra-gastric bile acids concentrations of less than $250\mu\text{mol/L}$ (Gotthard *et al.*, 1985). Intra-gastric levels up to $34,260\mu\text{mol/L}$ have been reported after the formation of a gastro-jejunostomy (Watt *et al.*, 1984).

Duodenogastric reflux can occur early in the morning or following a meal (Klokkenburg *et al.*, 2009). Oesophageal bile acid levels which are considered normal – even in Barrett's oesophagus – are between 0 and $200\mu\text{mol/L}$ and very rarely exceed $1000\mu\text{mol/L}$. No bile acids are detected at all in about 25% of patients with reflux (Gotley *et al.*, 1991). If bile acids are detected above stomach level, this indicates duodenogastric reflux, in which both bile and gastric reflux are combined, making for a weak-acid refluxate (pH 4-7).

Individuals who fast have exhibited serum bile levels of between 1-10 μ M, although these levels are known to spike under certain conditions, such as food ingestion, or intrahepatic cholestasis of late pregnancy (ICP). The latter is related to a hormone imbalance, which causes BA clearance from serum to be less effective. If ICP is left unchecked, this may cause foetal BAs to accumulate, which can be dangerous. In ICP, bile acid concentrations in the bloodstream can reach 40-60 μ M in the mother and 60-120 μ M in the new born (Zecca *et al.*, 2004; Zecca *et al.*, 2008; Keely, 2010). ICP new born exhibiting respiratory distress syndrome (RDS) have been found to have BAs in their BAL, which was not found in non-RDS controls (Zecca *et al.*, 2008). BAs are taken up for circulation, indicated by increased serum BA levels, then reach the lung.

Due to the lack of reliable assay techniques, there is a paucity of in-depth studies on specific types and levels of BA in lungs and airways. Indeed, BAL analysis has been the most popular form of study so far. This method has inherent problems as it involves perfusion of the airways with 100-200 ml of fluid (180ml common), resulting in BAL appearing more dilute in terms of BAs. Furthermore, out of the many different conjugations of BA, it is not yet known which type is most common in the lungs. Age, diet or medication can also have an effect on the type of BA in circulation and can alter taurine-conjugated secondary BA ratios. Pathogenic colonisation of the airways may also cause conjugated primary BAs to degrade, thereby increasing the levels of unconjugated secondary BAs in the lung. Most research therefore has concentrated on the presence in the airways of the major BA forms in circulation, which are CA, CDCA or DCA (Perng *et al.*, 2008; Zecca *et al.*, 2008; Aseeri *et al.*, 2012).

1.5.2 Bile Acid receptors (BARs)

BARs are responsible for regulating the homeostasis of BAs. They are found throughout the enterohepatic system and include the G-protein coupled receptor (TGR5) and the Farnesoid X receptor (FXR). Macrophages including liver Kupffer cells and T-lymphocytes also have BARs and indeed, FXR is believed to have a counter-regulatory effect on such cells (Renga *et al.*, 2009; Vavassori *et al.*, 2009). For example, the presence of FXR on macrophages suppresses IL-6 and IL1 β production (Vavassori *et al.*, 2009), suggesting a modulatory role for BARs in the immune system. If this is the case, then this may have a bearing on the role of inflammation in a number of GOR-related disorders, such as Barrett's oesophagus or

chronic lung allograft rejection, which presents clinically as obstructed lung function or BOS (Ward *et al.*, 2005; Hodge *et al.*, 2007).

1.6 Inflammation

Following mechanical or chemical trauma, or an infection, inflammation is the body's response and involves a broad range of physiological reactions, such as: enhanced release of a range of cytokines which cause the activation and migration of leukocytes, plasma protein seepage, proximal vasodilation and distal constriction of blood vessels. The role of inflammation is vital to pathogen clearance and wound healing. Visible symptoms include swelling and redness, as well as increased body temperature and pain. Monocytes (macrophages are monocyte that have entered tissue) and neutrophils travel towards the inflamed tissue (Malmström *et al.*, 2013).

1.6.1 Cytokines and chemokines

Structural cells and immunocytes, such as epithelial and endothelial cells, as well as fibroblasts, release proinflammatory cytokines and chemokines. These are released in response to a range of stimuli, such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), nitric oxide (NO) and other cytokines. Cells communicate on both the paracrine and autocrine levels via cytokines, which include peptides, proteins and glycoproteins. These are either pro- or anti-inflammatory, or they may also serve a chemotactic or growth function (Borthwick *et al.*, 2013).

1.6.2 Main cytokines implicated in chronic inflammatory lung diseases

Many studies have documented that IL-6, IL-8 and GM-CSF play a role in the regulation of airway inflammation and pathophysiology of lung disease (Ruiz *et al.*, 2002). *In vitro* studies have shown that these pro-inflammatory cytokines inhibit apoptosis of granulocytes (Brach *et al.*, 1992; Lee *et al.*, 1993; Leuenroth *et al.*, 1998; Castro-Alcaraz *et al.*, 2002). In addition IL-8 has been of specific interest in studies investigating the pathogenesis of airway diseases, due to its important effects on neutrophil cell biology (Keatings *et al.*, 1996).

1.6.2.1 Interleukin-6 (IL-6)

IL-6 is a 22-27 kDa protein with a variable molecular mass according to its glycosylation state and whose cytokine function may be either pro- or anti-inflammatory (Shain *et al.*, 2009). As a proinflammatory, IL-6 plays a role in the activation of monocytes, T-cell differentiation and B-cell maturation. It also serves as a pyrogen, inducing fever in response to infection. Non-leucocytes, including fibroblasts, astrocytes, endothelial, epithelial and malignant cells also secrete IL-6 (Rincon and Irvin, 2012). As an anti-inflammatory cytokine, IL-6 can upregulate the production of interleukin-1 receptor antagonist (IL-1RA) and IL-10, as well as inhibit the activity of IL-1 and tumour necrosis factor alpha (TNF- α). Macrophages, fibroblasts, T-cells and muscle cells are all able to secrete IL-6 (Hirano, 1998).

Research into lung diseases is beginning to identify IL-6 as a key player. Previous research has focussed on other cytokines such as IL-4, IL-5, IL-13 and IL-17, while IL-6 was overlooked, simply being considered a by-product of airway inflammation. Recently however, IL-6 has been implicated in the development of both asthma and possibly COPD. It has therefore taken centre stage in the research and treatment of these and similar chronic lung diseases (Rincon and Irvin, 2012).

1.6.2.2 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is synthesised by fibroblasts, macrophages, endothelial, epithelial and mast cells. It is a 14 kDa glycosylated protein which forms homodimers that play a role in the transformation of stem cells into granulocytes (such as eosinophils, basophils and neutrophils), as well as monocytes. Viral, bacterial, fungal or protozoal infections cause blood serum concentrations of GM-CSF to increase. Increased levels have also been observed in tumour-bearing animals and also following irradiation of bone marrow. GM-CSF production – observed *in vitro* – is stimulated by IL-1 and TNF α . In bone marrow, GM-CSF is involved in haematopoiesis (Lim *et al.*, 2013).

The granulocyte macrophage colony-stimulating factor receptor, which is also called cluster of differentiation 116 (CD116), is used for GM-CSF signalling. CD116 is a dimer produced by myeloblasts and neutrophils. It comprises an α chain, specific to CD116, whose role is to recognise and bind to ligands, and a β chain, responsible for signal transduction – the β chain is also part of the IL-3 and IL-5 receptors. A chain ligand binding causes the α and β subunits to dimerise, as well as phosphorylation of the β chain on tyrosine residues via JAK kinases.

An Shc adaptor protein associated with the phosphorylated β subunit, leads to activation of other downstream signalling molecules (Broughton *et al.*, 2012).

As a strong chemoattractant for neutrophils and macrophage activator, GM-CSF plays a key role in oxidative metabolism as well as stimulating both phagocytic and microbicidal responses, improving the cytotoxicity of macrophages (Sorgi *et al.*, 2012) and neutrophils (Katano *et al.*, 2010).

GM-CSF is also heavily involved in host defence and surfactant homeostasis regulation in the lung. Deficiency or signal pathway disruption of GM-CSF in mice potentially leads to impaired macrophage capacity, phagocytosis, cell adhesion and pathogen killing, which can lead to pulmonary alveolar proteinosis (PAP) (Ohashi *et al.*, 2012).

1.6.2.3 Interleukin 8 (IL-8)

IL-8 is produced by mesenchymal, airway and parenchymal cells in the lungs. It stimulates angiogenesis, regulates leukocyte trafficking and causes mucin secretion (Strieter, 2002). IL-8 production indicates the occurrence of damage. BO positive patients were found to have increased IL-8 levels, although the stage of BO development was not found to make a difference to the IL-8 levels (D'Ovidio *et al.*, 2005). The lowest IL-8 levels in BAL were found in control subjects; higher levels were found in stable lung transplant patients; and the highest levels were detected in BOS patients (Zheng *et al.*, 2000). IL-8 is produced as a damage response by fibroblasts, alveolar macrophages, smooth muscle, epithelial and endothelial cells. In this context, IL-8 is a neutrophilic chemoattractant and an angiogenic factor.

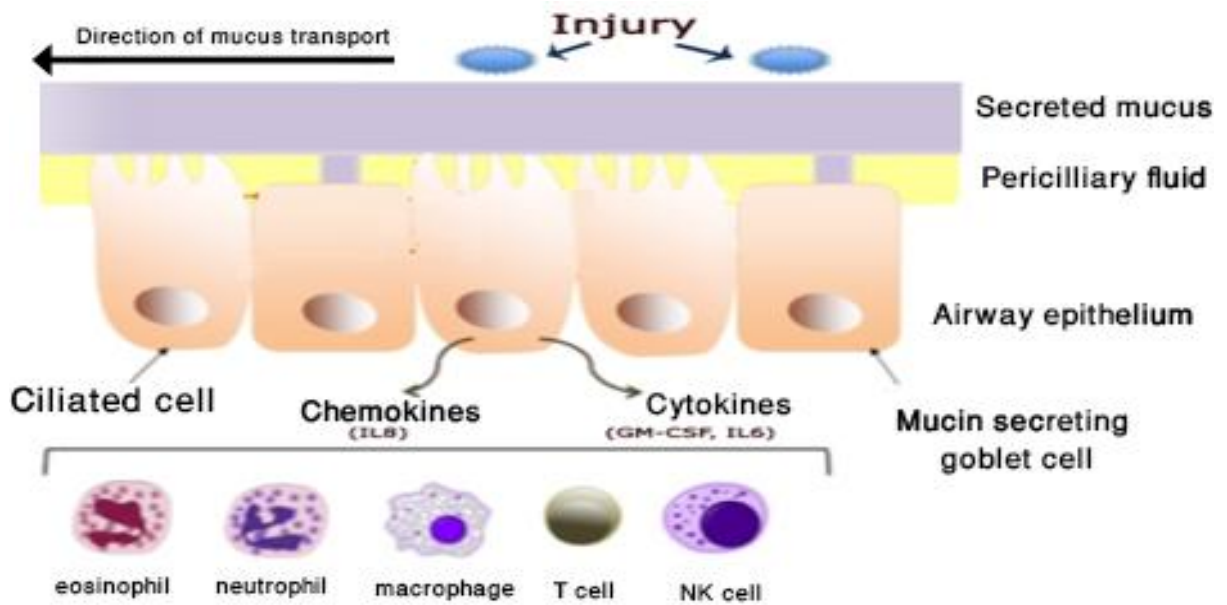


Figure 1-7: The airway epithelium. In response to injury, epithelial cells produce inflammatory chemokines and cytokines that recruit and activate immune cells. These cells include: neutrophils, eosinophils, macrophages, T-lymphocytes and natural killer (NK) cells which are activated to amplify host responses and regulate innate immunity (Adapted from Varielle., 2011 (Vareille et al., 2011)).

1.7 Airway mucus

Airway mucus forms a physical protective barrier between the epithelial surface and the external world. Mucins – either secretory or tethered to a membrane – are glycoproteins which form part of the constituents of respiratory mucus, along with lipids, proteins, ions and water. Submucosal tracheobronchial glands and epithelial goblet cells secrete these components to form mucus. Mucus is a significant part of the innate immune system as well as mucociliary defence. It is antibacterial and has a humidifying effect in inhaled air. It also physically traps and ejects particles, pathogens and other foreign bodies from the respiratory tract (Rose and Voynow, 2006). When inhaled particles or microbes reach the lung they become attached to the mucus gel layer and are cleared from the lungs by the action of the cilia moving the mucus layer up and out of the lungs (Pearson *et al.*, 2016). When mucus production is inhibited or disordered, this is implicated in a number of diseases, including CF, COPD and asthma. Overproduction of mucus leads to chronic airflow limitation, neutrophilia and suppuration. Lung transplant patients have sometimes exhibited disordered mucus homeostasis (Veale *et al.*, 1993), although further research is required into this subject.

Coughing and ciliary activity are the primary governors of mucus homeostasis, and lungs should contain very little or no mucus (Kim, 1997). Various complications may arise from hypersecretion, such as obstruction, infection, discomfort and higher mortality rates. Excess mucus may also transport foreign particles into the bronchial tree, rather than ejecting them from the airway (Kim, 1997). Diseases of the respiratory tract lead to increased mucus secretion, which can cause obstruction and in fact, the inflammatory/immune response can lead to airway remodelling (including goblet cell hyperplasia) and mucin gene regulation (Rose and Voynow, 2006).

1.7.1 Effect of aspiration

There is a dearth of research on the effects of aspiration on the mucus layer. Aspirated pepsin and bile salts can lead to increased vulnerability of the epithelium by disrupting the mucus layer which protects it. One consequence of this is increased mucus production, which may lead to airway obstruction.

Mucus is made up of mucins, which are a family of macromolecules (usually $> 2 \times 10^6$ Da), high in carbohydrate (50-90%). These complex glycoproteins exhibit heavy glycosylation, of which O-glycosylation causes numerous tandem repeats of proline, serine and/or threonine. The MUC backbone is made up of between 1.1 and 15 kilobases and can contain up to 11,000

amino acids, which make up 10-50% of the weight of the MUC (Rose and Voynow, 2006). At least 21 different types of mucin are produced by humans. In human airways, predominant mucins include MUC5AC, produced by goblet cells and MUC5B, produced by glandular mucosal cells. MUC7 is generated by both mucosal and serosal cells in the salivary glands, although saliva itself contains predominantly MUC5. Localised serous cells in the submucosal glands produce MUC5 in the larger airways of 15-20% of healthy individuals. As a result, mucociliary clearance and coughing are ineffective in terminal and respiratory bronchioles.

Two layers combine to form the airway mucus barrier: the periciliary layer (PCL) overlies the cells and the cilia, and is around 10 µm thick. Sitting on top of this is the mucus gel layer, which is around 60µm thick. It was previously believed that the PCL was a low viscosity fluid that allowed free movement of the cilia. However, it has been shown by Kesimer et al. (Kesimer *et al.*, 2013) and Button et al. (Button *et al.*, 2012) that mucins (MUC5AC and MUC5B) from the gel layer do not enter the PCL, although keratan sulphate, a glycosaminoglycan associated with membrane-bound mucins, MUCs 1, 4 and 16, is found in the PCL. Furthermore, MUCs 4 and 20 were expressed on the cilia, whilst the goblet cell surface exhibited MUC16, with MUC1 in the microvilli. The goblet cells appeared to have broad pillars of MUC16 extending through the PCL and into the gel layer and because MUC16 is co-released with MUC5B, it has been speculated that these pillars may be a mechanism for channelling MUC5B into the gel layer.

1.7.2 Mucus secretion

Mucus secretion can be caused by cytokine and leukocyte secretion. Mucins are produced under a range of circumstances, including stimulation by smoke, bacteria, matrix metalloproteinases, lipopolysaccharide, reactive oxygen species, neutrophil elastase, growth factors, triphosphates (cell injury markers) and bacterial by-products. These may have a direct effect or they may cause leukocyte stimulation (Kim, 1997; Jackson, 2001). Lipopolysaccharide has been shown *in vitro*, to increase MUC5AC, MUC5B and IL-8. These findings implicate goblet cells in playing a key role in mucosal immunity (Smirnova *et al.*, 2003). Disruption of these regulating factors can cause hypersecretion via the secretory cascade within minutes. This can be injurious, leaving the lungs vulnerable to damage or infection (Rose and Voynow, 2006).

1.8 Epithelial to Mesenchymal Transition (EMT)

1.8.1 What is Epithelial to Mesenchymal Transition (EMT)?

EMT allows epithelial cells to acquire a mesenchymal phenotype via multiple biochemical changes. This elevates its capacity for migration, resistance to apoptosis, invasiveness and boosts extracellular matrix (ECM) component production (Figure 1-8) (Kalluri and Neilson, 2003).

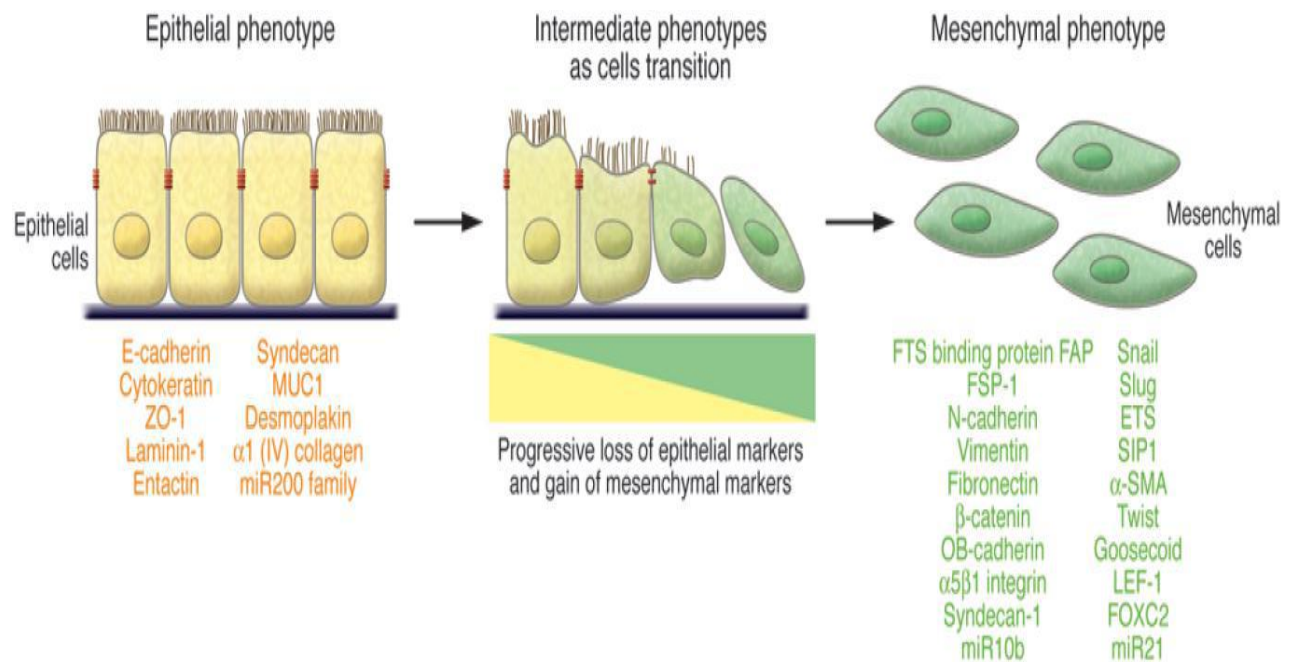


Figure 1-8: The changes in epithelial cells during EMT. EMT includes a change of a polarized epithelial cell into a movable and ECM component secreting mesenchymal cell (Kalluri and Weinberg, 2009).

The EMT comprises three different subtypes:

Type 1: Occurring during implantation, embryogenesis and organ development;

Type 2: Linked with tissue regeneration and organ fibrosis;

Type 3: Linked with cancer development.

In summary, type 1 EMT can lead to the generation of secondary epithelial cells by forming mesenchymal cells, which may undergo Mesenchymal Epithelial Transition (MET). However, there is no link between this and any circulatory proliferation of an invasive phenotype. Type 2 EMT however, generates fibroblasts and similar cells related to the post-inflammatory repair system and tissue reconstruction. It stops when the inflammation is subdued. If inflammation continues unabated, then so does the type 2 EMT; fibroblasts

continue to be generated and organ fibrosis and destruction may ensue. Type 3 EMT is found in cancer cells, enabling them to metastasize and invade (Kalluri and Weinberg, 2009).

1.8.2 Molecular Markers

Mesenchymal properties are gained only after the epithelial cell has passed through a number of stages. Further to this, markers on the epithelial cell, including E-cadherin and cytokeratins are expressed at a lower rate. These play a role in maintaining structural integrity as well as in intercellular contact. When adhesion proteins are no longer expressed, cellular organisation breaks down, resulting in enhanced fibroblastic protein expression, including fibronectin and vimentin (Ward *et al.*, 2005).

1.8.2.1 E-cadherin

Of the epithelial cell markers, E-Cadherin is a major structural protein. It plays a significant role in intercellular adhesion and in adherens junction maintenance. One of the primary EMT events is the inhibition of E-cadherin expression, which is caused by a number of EMT-inducing transcription factors (Daniele *et al.*, 2016). The adherens junctions define apico-basal polarity and are located at the basolateral membrane. Homotypic interactions, which are calcium-dependent, occur in the extracellular tail of adherens junctions, serving to connect adjacent epithelial cells. At the same time, E-cadherin has a cytoplasmic component, which mediates mechanical stability through interaction with the actin and microtubule cytoskeletons through linked β -, α -, γ - or p120-catenin proteins (Schmalhofer *et al.*, 2009).

E-cadherin expression also plays an indirect role in β -catenin or NF- κ B transcription (Solanas *et al.*, 2008). It has been established that these proteins are involved in EMTs during tumour invasion and that E-cadherin prevents these from reaching the nucleus, thereby suppressing their transcriptional activation role (Brabletz *et al.*, 2005; Maier *et al.*, 2010).

Due to its significant role in both cell adhesion and also EMT-related down-regulation, it is suggested that loss of E-cadherin may be used as a prognostic marker for the progress of cancers (Fendrich *et al.*, 2009; Tryndyak *et al.*, 2010; Hong *et al.*, 2011), although it does not necessarily indicate the risk of mortality (Ucvet *et al.*, 2011).

1.8.2.2 Cytokeratins

Epithelial markers, otherwise known as Cytokeratins, are intermediate filaments that are predominantly expressed by epithelial cells: within these are a wide array of proteins that vary in affinity, isoelectric pH values and molecular weight (Kirfel *et al.*, 2003). The expression of cytokeratins varies among different epithelia types in their different developmental stages, and may also be used as an adjunctive tool for histological diagnosis and epithelial classification (Chu and Weiss, 2002; Azevedo *et al.*, 2008). EMT is a process whereby epithelial cells acquire a mesenchymal phenotype and lose their epithelial characteristics. The invasive and metastatic potential of these cells is subsequently increased by EMT (Guarino, 2007). The EMT process is characterised by the upregulation of mesenchymal markers such as fibronectin and vimentin, and the downregulation of epithelial markers such as E-cadherin and cytokeratin (Koliijn *et al.*, 2015).

1.8.2.3 α -smooth muscle actin (α -SMA)

α -SMA (Mesenchymal markers) has been described as a smooth muscle cell differentiation marker (Skalli *et al.*, 1989). EMT-related differentiation and myofibroblasts have been linked with increased α -SMA expression, allowing for cell population identification and for EMT process assessment in fibrosis (Chai *et al.*, 2003; Wu *et al.*, 2007). α -SMA expression involves a small GTPase Rho and its effector, Rho kinase, which are also associated with fibrosis-associated EMT, particularly in cytoskeleton reorganisation (Masszi *et al.*, 2003). This manifests through stress fibre formation, which are contractile and tension exerting actin filaments that play a key role in cell motility (Alberts *et al.*, 2002).

1.8.2.4 Fibronectin

Fibronectin (Mesenchymal markers) is involved in cell adhesion (Alberts *et al.*, 2002). A glycoprotein, it forms part of the extracellular matrix and is recognized by integrins. It is protected, in either it's soluble or fibrillar form, by Fibroblast-Specific Protein 1 (FSP1) expressing cells and plays a critical role in cell attachment and migration, metastasis and cytoskeletal assembly. EMT-inducing conditions lead to enhanced fibronectin production and it can therefore be used as a marker of EMT (Strutz *et al.*, 2002).

1.8.3 Transforming Growth Factor- β (TGF β) induction of EMT

Within a range of cell types, including epithelial, endothelial and hematopoietic cells, the inhibition of cell proliferation can be caused by the multi-functional cytokine, TGF- β . As well as these effects, it is also responsible for inducing a variety of extracellular matrix proteins, and subsequently the stimulation of tissue fibrosis.

However, within the pulmonary microenvironment, the chronic overexpression of TGF- β often results in fibrosis (Samet, 2000; Willis and Borok, 2007). In areas where a lung tumour does become established, this often leads to the dysregulation of the TGF- β signalling cascade, such that an increase in the manufacture of TGF- β contributes to potent EMT induction, apoptosis resistance and immune evasion (Roberts and Wakefield, 2003; Massagué, 2008). TGF β in EMT *in vitro* studies have been demonstrated to down-regulate epithelial markers, including cytokeratins and E-cadherin. Conversely, the same studies have revealed the regulation of mesenchymal markers, such as α -smooth muscle actin, N-cadherin, fibronectin and vimentin (Moustakas and Heldin, 2012).

As TGF β mediates EMT via the activation of Smad2 and Smad3, this in-turn mediates EMT (Piek *et al.*, 1999; Valcourt *et al.*, 2005), and via the Smad pathway, HMGA2 is also regulated, which leads to the elicitation of EMT (Thuault *et al.*, 2006). In turn, as TFs such as Snail1 and Twist1 are up-regulated, the working of HMGA2 and Smads together also leads EMT to being induced (Thuault *et al.*, 2008; Tan *et al.*, 2012). Suppressing the expression of E-cadherin is possible through the interaction of Snail1 and activated Smads (Vincent *et al.*, 2009). In alveolar epithelial cells (AECs), TGF- β 1 has been reported in recent studies to induce EMT, as well as differentiating fibroblasts into myofibroblasts and was also demonstrated among IPF patients to be up-regulated (Trulock *et al.*, 2005; Willis *et al.*, 2005; Câmara and Jarai, 2010).

Moreover, following chronic exposure to TGF- β 1 *in vivo* and *in vitro*, alveolar epithelial cells have been demonstrated to undergo EMT: this is found to result in a loss of epithelial proteins such as E-cadherin and the cytokeratins; and also leads to the acquisition of mesenchymal proteins, including collagen type I and fibronectin (Willis and Borok, 2007). A role for differentiation and growth factors in the induction of EMT has been indicated by numerous previous studies: these include TGF β , Notch, Wnt, integrins and receptor tyrosine kinases (RTK). The cross-communication that exists between these factors enables the remodelling of the architecture and function of the cells via protein networks and gene programmes

(Moustakas and Heldin, 2007). The activation of effector proteins, including PI3K, MAPK and NF κ B, Src, Rho GTPases and integrin-linked kinase (ILK), occurs through signalling pathways such as inflammation or hypoxia. In turn, this facilitates the regulation of EMT (Moustakas and Heldin, 2007). Further, in the progression of cancer and embryogenesis, TGF β significantly induces EMT (Moustakas and Heldin, 2012). The platelet-derived (PDGF) growth factor is induced by TGF β , whose receptors and ligands are also significant in inducing EMT, metastasis within the hepatocellular carcinoma and invasion (Fischer *et al.*, 2007). Furthermore, as the process of EMT begins, the Jagged1, Notch ligand and Hey1 the target gene, are induced by TGF β in a manner that is dependent on Smad3 (Zavadil *et al.*, 2004). Over the course of the EMT process, TGF β also represses the inhibition of the differentiation protein (Id3 and Id2) (Thuault *et al.*, 2006). These proteins act as antagonists to the proteins of the basic helix-loop-helix (bHLH), which include the EMT-inducing transcription factor and Twist1. Further, Id2 and Id3 are also targeted by the BMP7 signalling pathway, which may to some extent explain the role BMP7 has in the TGF β -induced inhibition of EMT, in-turn resulting in MET (Kowanetz *et al.*, 2004).

1.8.4 Matrix metalloproteinase (MMP)-9 and EMT

The deterioration of various components in extracellular remodelling is caused by an enzyme-group known as MMPs. These are synthesised and released by neutrophils, airway epithelial cells and alveolar macrophages (Kim *et al.*, 2008b). In studying COPD patients, a correlation has been identified between the activity of MMP-12 and emphysema levels (Chaudhuri *et al.*, 2012), and it has also been demonstrated that FEV1 is reduced by polymorphisms in MMP12 and MMP1 (Tzortzaki *et al.*, 2006). Furthermore, an elevated level of gelatinase B MMP-9 and neutrophil collagenase MMP-8 has also been identified among COPD patients (4). While the former mostly originates from macrophages and neutrophils, these may also be manufactured by epithelial cells, mast cells, smooth myocytes and fibroblasts. Due to its collagen-lytic and elastolytic activity, extracellular matrix turnover is critically dependent on MMP-9. Furthermore, the activity of several biological factors, such as proteinases (e.g., MMP-13), cytokines (e.g., IL-1, VEGF) and/or their inhibitors (e.g., α 1-antitrypsin) are moderated under MMP-9.

This is because of its broad substrate specificity that also allows it to cleave extracellular matrix components. Further, the BAL fluid of COPD patients has been found to contain heightened levels MMP-9, which was found to come from BAL macrophages that express an increased level of MMP-9 when compared to control subjects (Barnes and Hansel, 2004).

A similar case can be observed in the serum of COPD patients, where the inflammatory response can be found to involve MMP-9, that indicates the COPD stage (Piesiak *et al.*, 2011). Further, MMP-9 is becoming the target of the ongoing research into COPD therapy (Wu *et al.*, 2012). Among certain lung diseases, a neutrophil-predominant response is exhibited, in which the MMP-9 produced by the neutrophil is able to obscure epithelial and other cell-produced MMP-9; a process that may be instrumental in the pathogenesis of the COPD. Levels of MMP-9 are elevated, and are found to be involved in asthma, inflammation, IPF and emphysema (Daniele *et al.*, 2016). Moreover, EMT has recently come to appear as a set of changes in sequence that may lead to migratory and invasive properties becoming expressed by epithelial cells.

It has been demonstrated *in vivo* on tumour biopsies and on developmental models, as well as in a range of tumoural and non tumoural *in vitro* systems, that the expression of MMPs (and MMP9 in-particular) is most evidently instrumental to processes of EMT. In accordance to this, the use of transcription factors (snail, ETS, β -catenin) to regulate MMPs, that are known to regulate pathways of EMT, have now been firmly established. As a consequence, MMPs are rather considered as target genes of EMT pathways and MMP expression as a late event of the EMT. In part, this may be because of direct up-regulation of the gene transcription of MMP by the factors driving EMT (Gilles *et al.*, 2005).

Nevertheless, changes in EMT have been shown to be initiated by MMP9. Also, the ability to cleave E-cadherin has been demonstrated among certain MMPs, thereby inducing the fragility of E-cadherin complex and changes in EMT (Coussens *et al.*, 2002; Overall and López-Otín, 2002). As MMP-9 is a Type IV collagenase, it is therefore able to distort basement membranes, which has been identified to cause EMT among patients of the lung transplant procedure (Ward *et al.*, 2005).

1.9 Overall aim

Gastroesophageal reflux (GOR) and subsequent BA aspiration have been implicated in several disorders of airways, including lung transplant rejection, COPD, CF and asthma. Taken together, the studies described in (1.5) provide evidence to support the existence of bile acid in the airways. Bile acids are present and can sometimes be associated with decreasing lung function, no direct correlation has yet emerged to indicate whether bile acids play a pathophysiological role in the airways.

My research focused on the hypothesis that bile acids may induce inflammatory lung disease. This thesis brings together a series of experiments to investigate the possible links between any primary bile acids (cholic acid and chenodeoxycholic acid) and secondary bile acids (lithocholic acid and deoxycholic acid) present in the lung and damage caused to the epithelium.

There are three overall aims of this study. The first is to investigate the effects of bile acids on the expression of inflammatory markers that are important in airway diseases. The second is to investigate the link between bile acids that may injure airway epithelial cells and epithelial–mesenchymal transition (EMT) that may leading to airway fibrosis. Finally, to explore the use of the NCI-H292, Calu-3 cell lines as potential *in vitro* models to test the hypothesis that the presence of bile acids causes mucus hypersecretion.

The thesis was divided into chapters as follow:

Chapter one: General introduction

Chapter two: Material and methods

Chapter three: Establishing an *in-vitro* model of human bronchial epithelial cell injury using cell lines

- In chapter three, human bronchial epithelial cell lines BEAS-2, 16HBE14o-, NCI-H292 and Calu-3 cell lines will be cultured to assess the effect of bile acids challenge. The effect of cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid will be measured by assessment of inflammatory cytokine production. Markers will include IL-8, IL-6 and GMCSF which are important in inflammatory lung disease.

Chapter four: Bile acid Induced Epithelial to mesenchymal transition (EMT)

- In chapter four, human bronchial epithelial (PHBE) cells, primary human epithelial cells from the subglottis area, BEAS-2B and 14HBE14-O cell lines will be cultured. The cells will be stimulated to investigate the role of BAs in instigating EMT by evaluate E-cadherin expression (epithelial marker) and fibronectin expression (a mesenchymal marker). The effect of primary bile acids (Cholic acid and chenodeoxycholic acid) and secondary bile acids, lithocholic acid and deoxycholic acid) will be measured by assessment using ELISA of TGF β 1, (TGF- β 1 is a potent EMT inducer), MMP9 and Pro-collagen production (can cause inflammation, translocation, further EMT and other damage).

Chapter five: The role of duodeno-gastro-oesophageal refluxate in mucus production from NCI-H292 and Calu-3 cell lines

- In chapter five the NCI-H292 and Calu-3 cell lines that have been used as an *in vitro* model system were used to study MUC5 gene regulation. Cells were stimulated with bile acids and mucin content of the culture medium measured using a periodic acid-Schiff's (PAS) assay. To confirm and extend the PAS findings MUC5AC and MUC5B will be measured using indirect ELISA. Finally, quantitative real-time PCR will be used to evaluate MUC5AC and MUC5B expression in NCI-H292, Calu-3 cell lines.

Chapter six: General discussion and conclusion

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

2.1.1 General practice

As per the University's policy on Health and Safety, the work as follows has been performed within a biosafety level II laboratory. Further, the form Control of Substances Hazardous to Health (COSHH) and the form Biological Control of Substances Hazardous to Health (BIOCOSH) were both reviewed before the work was conducted and were signed off by the University's Committee on Biological safety.

Cell culture procedures were performed in a containment level II microbiological safety cabinet. This was routinely cleaned with 70% ethanol prior to use. Additionally, all tissue culture equipment was sprayed with 70% ethanol before use. For routine culture, cell lines were grown in either 25cm² or 75cm² plastic tissue culture flasks (Corning, UK).

The cell types were cultured in specific medium according to American Type Culture Collection (ATCC) recommendations, which were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Adherent cell lines were placed horizontally in the incubator to allow for cell adherence, while suspension cells were grown in flasks.

2.1.2 Culture medium

2.1.2.1 RPMI 1640 Medium

To support the growth of suspension cell lines, RPMI 1640 medium (Sigma, 5886) was used. Cells were grown in this medium after the addition of 10% Fetal Calf serum (FCS), 2 mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (Sigma, UK), and 50µg/ml amphotericin B (Lonza, USA).

2.1.2.2 Eagle's Minimum Essential Medium (EMEM)

EMEM with Earle's acid medium was used to support the growth of the cell lines. Complete medium was obtained after the addition of 10% FCS, 50µg/ml amphotericin B (Lonza, USA), 100U/ml penicillin, 2 mM L-glutamine and 100 µg /ml streptomycin (Sigma, UK).

2.1.2.3 Bronchial Epithelial Cell Growth Medium (BEGM)

In order to prepare BEGM complete medium (Lonza) supplements supplied by Lonza were added into 500ml BEBM base medium: these were bovine pituitary extract 2ml (final concentration 0.004 ml/ml), insulin 0.5 ml (5 µg/ml), hydrocortisone 0.5 ml (0.5 µg/ml), retinoic acid 0.5 ml (0.1 ng/ml), transferrin 0.5 ml (10 µg /ml), triiodothyronine 0.5 ml (6.7 ng/ml), epinephrine 0.5 ml (0.5 µg/ml), Epidermal Growth Factor human recombinant 0.5 ml (10 ng/ml), L-glutamine (to 100 U/ml) and penicillin/streptomycin (Final concentration 100U/mL) (Sigma, UK).

2.2 Cell lines and Primary cells

2.2.1 Normal human bronchial epithelial cells

By using normal human bronchial epithelial (NHBE) cells as to primary cultures, it is possible to provide an *in vitro* representation of the native airway epithelium. Numerous key attributes of the normal airway epithelium are exhibited by such (NHBE) cell cultures. Furthermore, research into the biology of cells within the airway can be facilitated through the use of an expanded pool of *in vitro*-like epithelial cells (Gray *et al.*, 1996).

During bronchoscopies, airway brushings were taken from lung transplant patients and these bronchial epithelial cells were suspended in RPMI 1640 basal medium. Following centrifugation at 1000 rpm, for 5 minutes at room temperature cell pellets were re-suspended in 4 ml of complete BEGM (Lanza). Cell suspensions were transferred to a collagen pre-coated T25cm flask 30 µg/ml PureCol (Advanced BioMatrix, San Diego, CA, USA) and incubated in 5% CO₂ at 37°C. Growth medium replaced every subsequent 48 hours until (PHBECs) reached a confluence of 80-95%.

2.2.2 BEAS-2B

Derived from normal human epithelial cells immortalised through the hybridisation of simian virus 40 adenovirus 12 (Reddel *et al.*, 1988). BEAS-2B is considered a human bronchial epithelial cell line. BEAS-2B cells are maintained as sub-confluent, otherwise they induce squamous differentiation and BEAS-2B is available from the ATCC (CRL-9609). Furthermore, these are popular for research on the function and structure of airway epithelial cells as they were used to maintain the morphology of an epithelial cell *in vitro* as well as release IL-8 and IL-6 in addition to other cytokines (Swanson *et al.*, 2009). Within a humidified atmosphere containing 5 % CO₂ at a temperature of 37°C, the BEAS-2B cells

were grown in 75-cm² flasks in BEGM medium. The cells were passaged every 4-6 days to maintain sub-confluent. The cells were cultured and used for experiments at passage 6-23 and at density of 1 x 10⁵ cells/cm² on 24 well.

2.2.3 Calu-3

Calu-3 (ATCC HTB-55) is a human bronchial epithelial cell line originally sourced from a bronchial adenocarcinoma in a 25-year-old Caucasian male (Itani *et al.*, 2007; Zhu *et al.*, 2010). Calu-3 cells structurally can form organised cell layers with tight junctions. They show typical airway epithelial properties, for example, generation of high levels of secretion parts that are similar to those of the serous cells of the tracheobronchial organ. Further, the expression of mucus genes MUC1 and MUC5AC and the secretion of MUC5/5AC mucins and secretory granules are also attributes that these cells feature (Berger *et al.*, 1999). Within an EMEM medium containing 2mM L-glutamine, 10% FCS, streptomycin (100 µg/ml) and penicillin (100 U/ml); the Calu-3 cells were cultured in a humidified atmosphere containing 5% CO₂ and at a temperature of 37°C. The cells were cultured and used for experiments at passage 2-33 and at density of 1 x 10⁵ cells/cm² on 24 well plates.

2.2.4 NCI-H292

NCI-H292 cells (ATCC; CRL-1848), are a bronchial epithelial cell line derived from a mucoepidermoid carcinoma. NCI-H292 cells react to an assortment of airway injuries including tobacco smoke. This leads to up regulation of different endpoints connected with inflammation IL-6 and IL-8, airway remodelling MMP-1, GM-CSF, and mucin overproduction MUC5AC (Newland and Richter, 2008). In addition NCI-H292 cells have been widely utilized as a lung model for toxicological assessment (Newland *et al.*, 2011). The NCIH292 cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100U/ml), streptomycin (100 µg/ml) and 50µg/ml amphotericin B in a humidified 5% CO₂ climate at 37°C. Monolayer cells were routinely subcultured each 3-4 days. Cells were washed twice with sterile PBS and passaged using a 5 mL 0.05% trypsin: 0.02% EDTA 1x solution. The cells were cultured and used for experiments at passage 14-52 and at density of 1 x 10⁵ cells/cm² on 24 well plates.

2.2.5 16HBE14o- Cells

16HBE14o- cells were acquired from Dr. Dieter Gruenert, University of California, San Francisco USA. The cell line 16HBE14o- was derived from an infant male heart-lung patient (at 1 year of age) by transforming (large T-antigen SV40) cultured bronchial epithelial cells. While this cell line is neither licenced nor commercially available, epithelial polarised cell mono layers are formed and epithelial morphology is maintained. This includes tight junction formation and high trans-epithelial resistance (Cozens et al., 1994). Further, other differentiated features that characterise the native epithelium are also found to be expressed (Gruenert et al., 1995). Moreover, over the course of many passages, high viability is also demonstrated. What is controversial however is their ability to secrete mucus and form cilia (Ehrhardt et al., 2008; Pohl et al., 2009). Within an EMEM medium containing 2mM L-glutamine, 10% FCS, streptomycin (100 µg/ml) and penicillin (100 U/ml); the 16HBE14o-cells were cultured in a humidified atmosphere containing 5% CO₂ and at a temperature of 37°C. Furthermore, a fibronectin coating solution was used to pre-coat the T75 flasks. At a confluency of 80-90%, the cells were passaged by first washing with Hebes buffered saline (HBS), following by allowing the cells to detach using 5 mL 0.05% trypsin: 0.02% EDTA solution. The cells were cultured and used for experiments at passage 62-108 and at density of 1×10^5 cells/cm² on 24 well plates.

2.3 Cell count

Cells were counted using an improved Neubauer chamber haemocytometer (Figure 2-1). Ten microlitres of cells suspended in medium were placed onto the chamber secured with a glass cover slip. The number of cells at each corner was counted, as well as the middle square in the centre. The average was taken and multiplied by 5 to include all 25 squares and multiplied 104 to obtain the number of cells per ml.

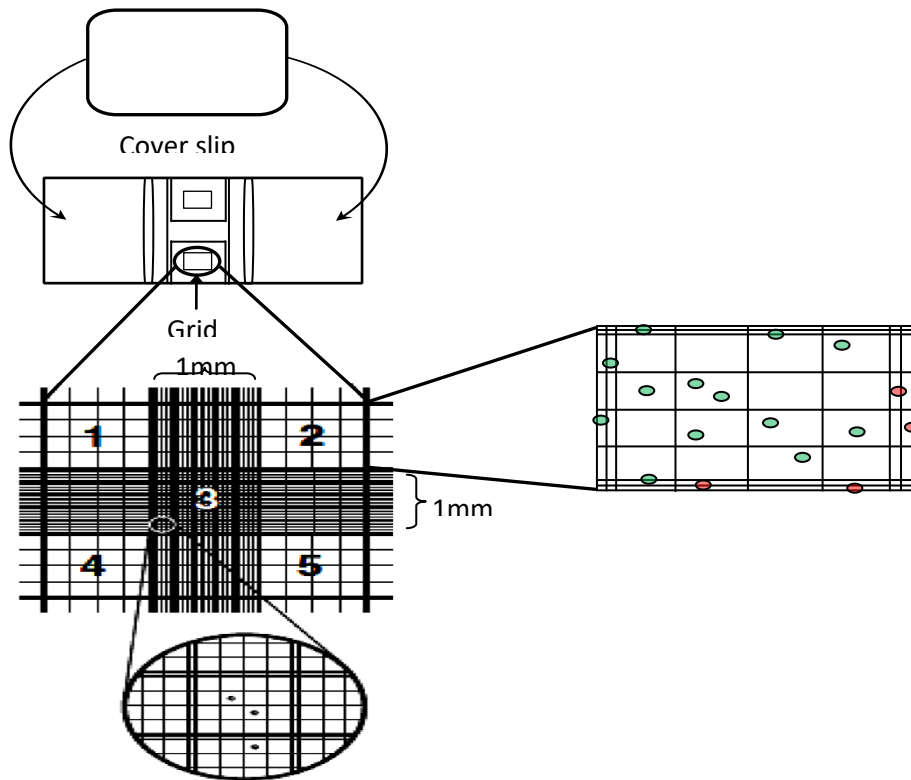


Figure 2-1: Illustration of a hemocytometer slide. After placing the coverslip on the slide, a cell suspension is loaded and cells are counted in each square. Cells touching the middle line on the top and left are included in the counting (green) while those touching right and bottom are excluded from the count (red) (Martin, 1994).

2.4 Cell cryopreservation

Keeping in mind the end goal to maintain a supply of the cell lines and to hold cells from an early passage, cryopreserved cells were frozen in liquid nitrogen. Cells in their log development stage (70-80% confluent in flask) were re-suspended in freezing medium, which comprises of 10% dimethylsulphoxide (DMSO-Sigma) in complete medium or FCS. Cells were frozen in batches of 5-10 million cells for each cryovial (Corning, UK). The vials were set in a Nalgene cell freezing container filled with isopropyl alcohol which provides a progressive reduction in temperature of 1 degree Celsius per minute when stored at - 80°C overnight. On the second day, the cells were moved into a liquid nitrogen tank for long term tissue banking. For cell recovery, cells were defrosted by quick warming in a 37°C water bath. Once defrosted, the cells were moved promptly into 10-15 ml of fresh pre-warmed medium and incubated at 37°C.

2.5 Epithelial Cell passage

When the cells in the culture flask approached 75-80% confluence, they were passaged, permitting further propagation. Five ml of 0.05% trypsin; 0.02% EDTA was added to the flask, then incubated at 37°C for 2-5 minutes until cells detached . Five ml of complete medium that contain FCS was then added to inhibit the trypsin and to help re-suspend the cells. Thereafter, in order to pellet the cells, the solution was centrifuged at 1100rpm for 7 minutes and at a temperature of 10°C; before seeding into 6 and 24 well plate, these were mixed carefully with 12ml of complete epithelial medium.

2.6 Primary bronchial epithelial Cell (PHBECs), tracheal epithelial cells and cell lines stimulation

At about 85-90% confluence, plates containing primary cells or cell lines were rested for 24 hours in serum free medium. Bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid) were dissolved in methanol to prepare stock solution that was (100 mmol/L) in this experiment. To achieve the experimental concentration different concentrations were prepared by diluting stock solution in resting medium. These dilutions were at least 1000 fold leaving vanishingly small amounts of methanol. After this process, they were added to PHBECs, tracheal epithelial cells and cells lines to achieve stimulation. A total of three repeated cultures and four repeated wells of PHBECs, tracheal epithelial cells and cell lines (n= 12) was carried out. The following bile acid concentrations were used: 1, 2, 5, 10, 15, 16, 17, 18, 20, 30, 50, 75, and 100µmol/L at incubation times of 24 and 48h. After

these times, samples of the cell culture supernatants, as well as control samples, were collected to measure cytokine concentrations using ELISA assays (R&D Systems). The control samples consisted of cells incubated with resting medium. The data presented in the thesis are all 48 hour results because stimulation for 24 hours did not produce any changes in the cell viability compared to control suggesting damage was time dependent. Moreover, in developing an ELISA, secretion was measured in cell supernatants that were treated for 24 hr with bile acids, but this did not significantly induce secretion of pro-inflammatory mediators suggesting damage and bile acid stimulation was time dependent. This fits in with other studies by our group. In these studies the effect of individual primary and secondary bile acids were evaluated on PBECs after stimulation for 24 and 48 hours. Cell death, Interleukin 8 (IL-8) and Interleukin 6 (IL-6) production were measured by ELISA. They found that 48 hours treatment was more effective compared to 24 hour (Aseeri, 2012). A time course for mRNA studies was not performed. It might be predicted that peak mRNA effects would occur before the 48 hour time point and further studies could evaluate this.

2.7 Cell viability assay

2.7.1 CellTiter-Blue™ Assay

2.7.1.1 Assay principle

The CellTiter-Blue™ Assay (Promega, USA) is based on cellular reduction of resazurin to the absorption product resorufin. It is based on the principal that viable cells are able to metabolize and reduce the dye; whereas the capacity to reduce the dye of dead cells rapidly diminishes once their membranes are compromised (Rich Moravec and Riss, 2003).

2.7.1.2 Titre-blue Assay

Cells were incubated, under experimental conditions, for 48h, before being assessed using the Titre-blue Assay (Promega, USA). The medium in the 48 well plate was discarded and cells in 4 wells were killed using 200µl ice-cold methanol for 5 minutes. 100µl of CellTiter-Blue was added to each well followed by 500µl of resting medium (serum- free medium). The plate was incubated for 2-4 hours, after which the 24 well plate was plated out in the 96-well microtiter plate. The ratio of live to dead cells was used to generate a standard curve (Figure 2-2), divided into five regions: 100% live cells (resting medium); 25:75% dead: live cells; 50:50%; 75:25% dead: live cells; and 100% dead cells (positive control). Absorbance measurements were used to monitor results. As the absorption maximum is 600nm for

resazurin and 573 nm for resorufin according to the manufacturer's data, absorbance was measured at 560nm and 600nm was used as the reference wavelength.

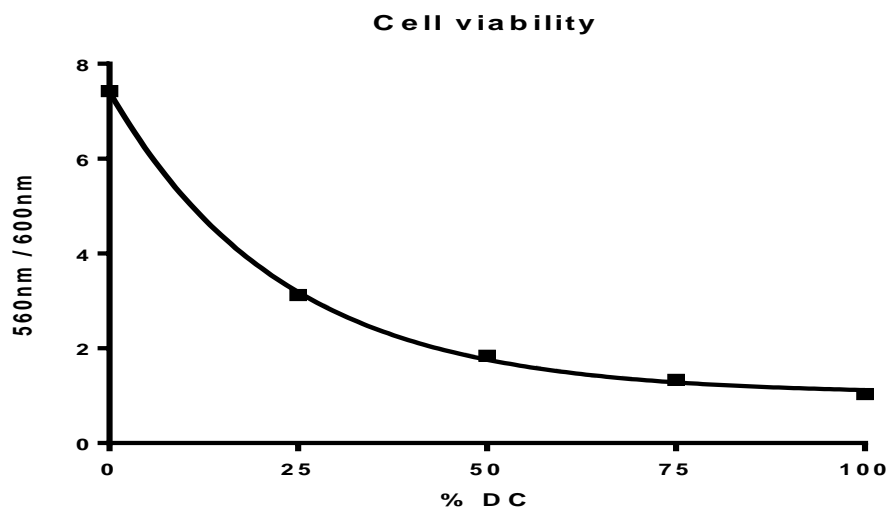


Figure 2-2: Standard curve of methanol killed cells measured via cell viability assay (Promega USA) (mean \pm SD; n=6).

2.7.2 MTT assay

2.7.2.1 Assay principle

Behind the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay is the principle that mitochondrial activity is steady for the majority of viable cells and that a reduction or increase in the viable cell count can therefore be correlated linearly to the mitochondrial activity. When formazan crystals are reduced from the tetrazolium salt MTT, this is seen to reflect the level of mitochondrial activity within the cells that can be solubilised for homogenous measurement. As a result, measuring the formazan concentration reflected in optical density (OD) using a plate reader at 570 and 690 nm can be used to identify the decrease or increase in the number of viable cells (van Meerloo *et al.*, 2011).

2.7.2.2 Assay procedure

The MTT assay was used for several experiments. This is mainly used to assess cell viability in the presence of a drug or toxin. MTT (TOXI Sigma, St. Louis, USA) was dissolved at 5mg/mL in Hank's salt solution and filtered to avoid MTT aggregates. The solution was added to each well in an amount equal to 10% of the culture medium volume and the plate was incubated for a further 2-4 hours. Formazan crystals developed which were dissolved in MTT Solubilisation Solution equal to the original culture medium volume. A gyratory shaker

was then employed to accelerate dissolution. Spectrophotometric measured absorbance of 570 and background at 690nm was used to measure well absorbance. It is noted, that although this procedure was employed, these metabolic assays do not distinguish between cytotoxic and cytostatic drugs or compounds, therefore the accuracy surrounding low cell numbers is questionable. The standard curve was made up of: 100% live cells (resting medium); 25:75%; 50:50%; 75:25% dead: live cells; and 100% dead cells. This was used to determine the percentages of viable cells.

2.8 Secretion analysis

2.8.1 Enzyme-Linked Immunosorbent Assay (ELISA)

To measure levels of cytokine and chemokines DuoSet ELISA kits were purchased from R&D systems. ELISA kits provide a sandwich ELISA. The assay was carried out as detailed in the kit protocol and is described below.

- Coating with capture antibody

A 96-well microplate was prepared with a 1/100 solution of capture antibody (100µl, R&D Systems, USA) and reagent buffer (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20mM Trizma base, 150mM NaCl), pH 7.2-7.4) at 100µl per well. The plate was incubated overnight at room temperature.

- Wash step

The contents of the plate were discarded and wells were then emptied and washed three times using 300µl wash buffer per well (0.05% Tween 20 in phosphate buffer solution, pH 7.2-7.4). The plate was dried by inversion and by patting the plate with force on paper towels.

- Blocking step

The plate blocked with 1% bovine serum albumin solution in phosphate buffer (PBS) (300µl). The plate was incubated at room temperature for 1 hour.

- Standards and samples

After the plate was aspirated and blotted dry as in wash step, 100µl of standards diluted in 100µl of PBS or 100µl of cell culture supernatant samples were added in duplicate. An adhesive plate sealer was applied to the plate, which was then incubated at room temperature for a further two hours and the washing stage was repeated.

- Detection antibody

100µl of detection antibody was added to each well, a new adhesive strip applied, and the plate was incubated for two hours at room temperature.

- Development

Following a further wash step, 100µl of streptavidin horseradish peroxidase (HRP) diluted in reagent diluent was added to each well and incubated for 20 minutes out of direct light at room temperature. A substrate solution (1:1 mixture of colour reagent A (H₂O₂) and colour reagent B (tetramethylbenzidine)) was added to each well (100µl) and the plate was incubated away from direct light for a further 20 minutes at room temperature. The addition of 50µl stop solution (2N H₂ SO₄) was required for each well and the plate was placed on the plate shaker for 1 minute at 100rpm.

- Analysis

The optical density (OD) of the contents of each well was immediately determined using a microplate reader set at 450nm and corrected at 570nm to correct for optical imperfections.

- Determination of cytokine and chemokines in sample from standard

The average OD value was derived for the standard and sample wells, the blank cytokine and chemokines standard OD value was subtracted from each mean value. GraphPad (version 6) was used to create a standard curve with a 4 parameter logistic curve fit. The sample values were interpolated from the standard curve.

Protein name	Capture antibody origin and concentration	Detection antibody origin and concentration	Reagent diluent	Substrate	Detection range	Supplier & catalogue number
IL-8	Mouse anti-human IL-8 4µg/ml	Biotinylated goat anti-human IL-8 20ng/ml	0.1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/ml	R&D Systems & DY208
IL-6	Mouse anti-human IL-6 2µg/ml	Biotinylated goat anti-human IL-6 50ng/ml	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	9.38-600 pg/ml	R&D Systems & DY206
GM-CSF	Mouse anti-human GM-CSF 2µg/ml	Biotinylated mouse anti-human IL-8 0.5µg/ml	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	15,62-1000 pg/ml	R&D System & DY215
MMP9	Mouse anti-human MMP9 1µg/ml	Biotinylated goat anti-human MMP9 12.5ng/ml	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/ml	R&D Systems & DY911
Pro-collagen	Mouse anti-human Pro-Collagen 4µg/ml	Biotinylated goat anti-human Pro-Collagen 100ng/ml	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/ml	R&D Systems & DY6220-05
TGFβ1	Mouse anti-TGFβ1 antibody 2µg/ml	Biotinylated chicken anti-human TGFβ1 antibody 100ng/ml	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/ml	R&D Systems & DY240

Table 2-1: Composition and concentrations of reagents and antibodies used in ELISA. TMB- 3,3',5,5'-Tetramethylbenzidine.

2.8.2 Determination of MUC5B mucin concentration by indirect ELISA

The concentration of MUC5B mucin within the medium that was collected from stimulated Calu-3 and NCI-H292 cells was measured in duplicate:

- Sample and Standard coating

100µl of the sample was coated onto a 96 well plate (Maxisorp, NUNC). At the same time, the standard of purified human saliva MUC5B mucin was diluted in PBS ranging from 0 - 8µg/ml, was coated onto the well by 100µl. Further, the plate was incubated at room temperature and covered overnight.

- The washing stage

On the following day, the plate was washed three times (0.05% Tween 20 in PBS), and was then blotted dry by using paper towels.

- The blocking stage

After incubating the plate overnight, the plate was carefully dried and the residue was discarded. Accompanying this, 300µl of 1% casein in PBS was added to block unoccupied binding sites. The plate was then incubated at room temperature for 2 hours.

- Primary antibody

100µl of the primary monoclonal antibody for MUC5B (EPR6920 raised in rabbit (Abcam); (1:200 dilution in 0.5% Tween20 in PBS and 0.1% casein) was added to each well. The plate was then incubated at room temperature for 2 hours.

- Secondary antibody

The washing stage was repeated, and then 100 µl of the secondary antibody was added to each well and incubated for 2 hours at room temperature (horseradish peroxidase-conjugated goat anti-rabbit (Dako); in this stage, 1 in 5000, diluted using an antibody diluent (0.1% casein and 0.05% Tween20 in PBS).

- The development stage

The solution was discarded following incubation and the washing stage was repeated to dry. Additionally, this stage saw the inclusion of 100µl of peroxidase substrate ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) was added and left to develop at room temperature for 20 minutes. As a final step, at 20 minutes the reaction was stopped with 1% SDS and a plate reader was used to measure an absorbance at 405 nm (Tecan M200).

2.8.3 The quantification of MUC5AC mucin concentration through indirect ELISA

MUC5AC mucin in cell culture supernatant collected from stimulated Calu-3 and NCI-H292 cells was detected using indirect ELISA.

- Standard and sample coating

The standard of purified pig gastric mucin was diluted in PBS ranging from 0 - 8µg/ml, 100µl was coated onto 96 well plate in duplicate (Maxisorp, NUNC). Similarly 100µl of each sample was coated onto the plate. The plate was covered and incubated overnight at room temperature.

- The washing stage

The following day the plate was emptied and washed three times with the wash buffer (0.05% Tween 20 in PBS) and blotted dry on clean paper towels.

- The blocking stage

Following its incubation overnight, the residue was discarded from the plate followed by a thorough dry. 300µl of 1% casein in PBS was added to block unoccupied binding sites. The plate was then incubated at room temperature for 2 hours.

- Primary antibody

100µl of the primary mouse monoclonal antibody for MUC5AC (45M1 epitope Life Technologies); diluted 1 in 100 in antibody diluent (0.1% casein and 0.05% Tween20 in PBS)) which was added. The plate was then left to incubate at room temperature for 2 hours.

- Secondary antibody

The washing stage was repeated, and then 100 µl of the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse (Dako); (1:5000 dilution in 0.5% Tween20 in PBS and 0.1% casein) was added for a total of 2 hours.

- The development stage

After washing as previously described, 100µl of peroxidase substrate ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) was added and incubated at room temperature for 20 minutes. The reaction was stopped after 20 minutes, with 1% SDS and a plate reader was used to measure absorbance at 405 nm (Tecan M200).

2.8.4 Periodic Acid Schiff (PAS) assay

2.8.4.1 Principle of PAS Staining

PAS is a staining method used to detect glycoproteins, such as mucins, through the oxidation of glucose or sugar with adjacent –OH groups by periodic acid. Periodic acid oxidises compounds having free hydroxyl group (-OH group), resulting in aldehydes, which react with the Schiff reagent to form a purple or red stain that can be detected by spectrophotometry. The glycoprotein content of the apical fluid was measured using a periodic acid-Schiff's (PAS) assay (Mantle and Allen, 1978).

2.8.4.2 Assay procedure

Aliquots of cell culture supernatant were thoroughly mixed diluted 1:10 in deionised water and 200µl sample in duplicate was located into 96 well plate for each PAS analysis. For the standard curve, purified papain digested porcine mucin was used (1mg/ml in deionised water; mucin obtained from Professor Jeff Pearson, Newcastle University) and diluted into 200, 100, 80, 60, 40, 20 and 0 µg/ml samples. The cell supernatant sample and mucin standard curve were combined with 20µl of periodic acid mixture (made from 2.5 ml of 7% acetic acid plus 5 µl of 50% periodic acid). The plate was incubated at 37°C for 45 min. Schiff's solution was made by combining 6 ml of Schiff's reagent with 0.1g sodium metabisulfite, which was also incubated in the 37°C water bath for 45min. 20µl of Schiff's solution was then added to each sample (including mucin standard curve and negative control samples) and left to develop for 30 minutes at room temperature. The absorbance in each sample was read using a Tecan plate reader at a wavelength of 555 nm.

2.9 Molecular biology techniques

2.9.1 RNA biology

Based on the function, RNA can be divided into three main types. Firstly, (i) Messenger RNA (mRNA) is transcribed from DNA by RNA polymerase and serves as a template for the synthesis of proteins. Second, (ii) Ribosomal RNA (rRNA) is one of 2 main components of ribosomes; the other is proteins (ribosomes = rRNA + proteins). These ribosomes become associated with mRNA in the cytoplasm. Lastly, (iii) Transfer RNA (tRNA) contains a specific amino acid at one end and anticodon region which can recognise and bind mRNA. The tRNA binds to the mRNA codon and transfers amino acids to the ribosomes, in order to synthesise protein. For this particular study, the focus was mRNA, since it represents potential gene expression in physiological and pathological conditions. Therefore, it allows measurement of changes in gene expression after the treatment of cells with a variety of cytokines.

2.9.2 Molecular biology

A level of precaution is required, specifically when RNA isolation and amplification is performed. All reagents used for this procedure and other molecular biology use, should have a high degree of purity. These reagents are also RNase free which provides protection during the RNA isolation process. All reagents used for RNA isolation in this project were available commercially and at a molecular biology grade. All areas used for RNA isolation were decontaminated by using RNase removal spray (Sigma). Pipettes were cleaned with 70% ethanol. Filter sterile tips and autoclaved sterile Eppendorfs were used throughout.

2.9.3 RNA extraction

Gene expression modulation, which may be caused by a particular stimulus or disease, can be analysed by isolating RNA. This study used the RNEasy Mini-kit (Qiagen) to extract RNA longer than 200 nucleotides from cultured cells. This kit takes advantage of the selective binding properties of a silica-gel membrane. Cells were pelleted and homogenised in 350µl Buffer RLT. To this, 350µl of 70% ethanol was added via pipette. The lysate was then added to an RNEasy mini-column and centrifuged for 15 seconds at 10,000rpm (MIKRO 200, Hettick Zentrifugen, DJB Labcare Ltd, Bucks, UK). A proportion of the mixture flowed through, and was discarded. To the remaining mixture, 350µl of RW1 buffer (Qiagen) was added and the column was centrifuged for 15 seconds at 1000 rpm. The Flow-through was again discarded and DNase digestion was carried out on the column. The wash buffer, centrifugation and flow-through discard process were repeated and 500µl RPE elution buffer (Qiagen) was then added. The centrifuge and flow-through discard process were repeated once again and another 500µl RPE elution buffer added. This time, the column was centrifuged for two minutes at 10,000rpm. Flow-through was once again discarded and the mixture was transferred to a fresh tube, which was centrifuged at 13,000rpm to dry. The mixture was transferred once again and 50µl RNase-free water was added by pipette to the membrane. This was centrifuged once more for one minute at 10,000rpm. The eluate was removed from the tube and passed through the column once more as described to obtain a higher RNA concentration. RNA was stored at -80°C.

2.9.4 Analysis of RNA concentration and purity

The RNA sample should be free from contaminating substances, including proteins, DNA, phenol, ethanol and salts. It is known that any contamination can decrease the efficiency of reverse transcription, leading to reduced amplification. A Nanodrop spectrophotometer (No-1000 NanoDrop®) was used for determination of RNA concentration and purity. The machine measures the absorbance at 230, 260 and 280 nm and uses these values as a guide to nucleic acid purity and concentration. One microliter of extracted RNA was applied to the Nanodrop after blanking with 1 μ l of RNase-free water. The concentration was obtained in ng/ μ l. The purity of isolated RNA sample was evaluated by examining 260/280 and 260/230 ratios. The reading around 2 for both of the ratios is considered as a pure sample free from protein and phenol contamination and suitable for cDNA synthesis. Figure 2-3 shows an example of RNA concentration measurement and purity using the Nanodrop.

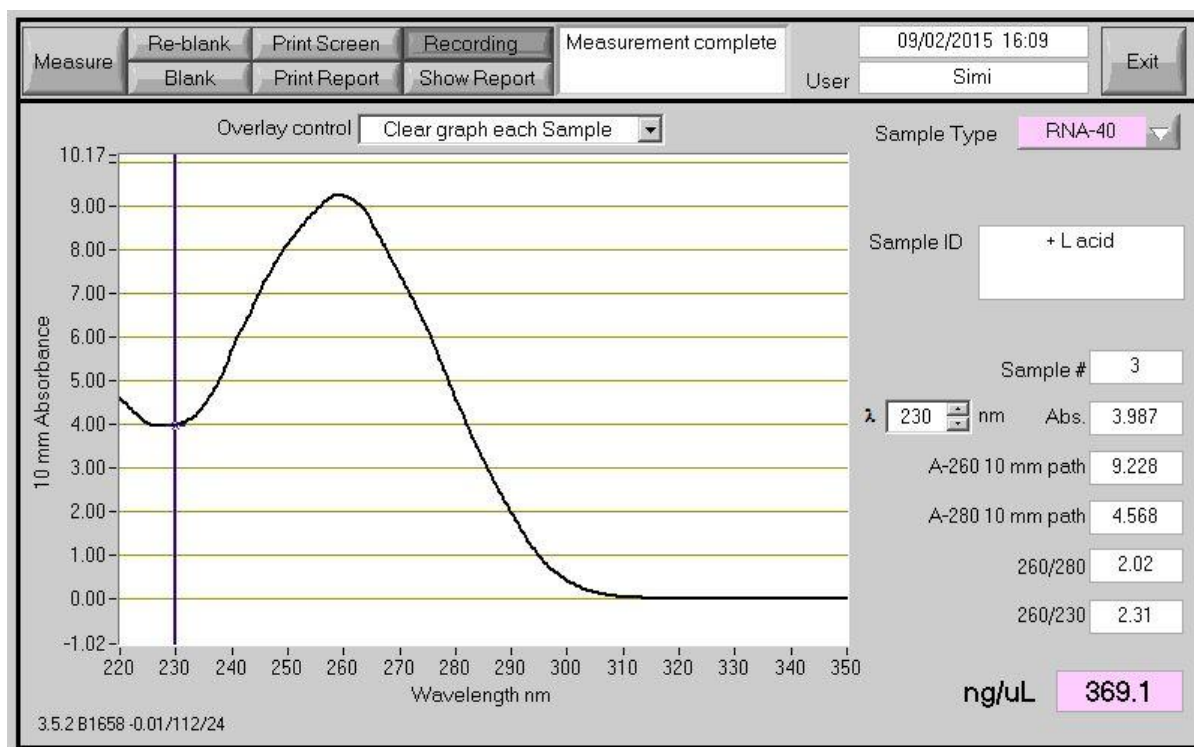


Figure 2-3: Examination of RNA purity and concentration using a Nanodrop instrument.

2.9.5 Reverse-Transcriptase PCR (cDNA)

A Tetro cDNA synthesis kit (Bioline) was used in accordance with the manufacturer's guidelines to reverse transcribe the RNA isolated in the above process to obtain complementary DNA (cDNA) within a 20 μ l reaction mixture. The reaction mixture was made up of 4 μ l 5X RT buffer, 1 μ l (0.5 μ g/ μ L) oligo-dT Random Primer, 1 μ l (200 U/ μ L) Tetro reverse transcriptase enzyme, 1 μ l (10U/ μ L) RNase Inhibitors, 1 μ l (10 mM) dNTP mix, 200ng RNA sample and the rest RNase-free H₂O (see table 2-2). These mixtures were vortexed, then centrifuged briefly to collect the contents at the bottom of the tube. A thermal cycler (G-Storm, Surrey, UK) was used to incubate the samples at 25°C for 10 min. These were then transcribed in the same device at 42°C for 15 minutes followed by heating at 95°C for 5 minutes. This produced cDNA, which was then stored at -20°C and used as a q-PCR template.

components	Tetro cDNA synthesis
RNA	2 μ l equivalent
5X RT buffer	4 μ l
10 mM dNTP mix	1 μ l
Oligo (dT) 18 Primer Mix (0.5 μ g/ μ L)	1 μ l
RNase Inhibitor (10 U/ μ L)	1 μ l
Tetro reverse Transcriptase (200 U/ μ L)	1 μ l
RNase free water	Variable
	20 μ l total volume

Table 2-2: Contents of first strand Tetro cDNA synthesis reaction.

2.9.6 Real time-polymerase chain reaction

2.9.6.1 General principle

Quantitative polymerase chain reaction (q-PCR) is a precise technique for the measurement of changes in gene expression. This procedure requires a specific sequence of DNA to be amplified in a geometric way, producing many copies of the original sequence. The amplified product can be measured either at the end point of the reaction in "traditional" PCR or during the amplification process in the real-time PCR method. Q-PCR has many advantages in comparison to the traditional in the context of the processing time and accuracy. Traditional PCR is regarded as a qualitative method in determining the presence or absence of a particular gene of interest at the end point of the reaction, q-PCR is able to detect small differences in the level of the expression between examined samples. In contrast to traditional PCR, in q-PCR the amplification and quantification occur simultaneously. An ideal amplification plot has an exponential, linear and plateau phases, in which the amplicon is quantified as the amplification is in progress and reaches plateau when the reaction components are depleted. The PCR product is determined in the exponential phase and crosses the threshold level in a cycle known as the threshold cycle. The detection process in this method requires the presence of fluorescent reagents that detect the amount of the amplified product. These reagents are either sequence specific or non-sequence specific. An example of sequence specific reagent is the 5' nuclease probe such as the Taqman assay.

2.9.6.2 TaqMan assay

Modulation in gene expression was determined using TaqMan® Gene Expression Assays, primer-probes (or 5' nuclease assay). This assay, in the presence of exonuclease activity of TaqMan DNA polymerase, uses two primers and a probe. This probe is a non-extendable DNA sequence, modified to include a quencher at the 3' end and a fluorescence reporter at the 5' end. It is capable of binding in either an exon boundary spanning or non-exon spanning manner to the desired gene's complementary sequence. The proximity of the reporter (fluorescence emitting) to the quencher (fluorescence absorbing) prevents excess light from escaping.

2.9.6.3 PCR efficiency

PCR efficiency is defined as the rate at which a PCR product is generated, it is represented as a percentage value with a maximum PCR amplification efficiency of almost 100%. If the PCR amplicon is doubled during each cycle, it is seen to reflect 100% efficiency. This value is estimated from a standard curve specific for each reaction, prepared by serial dilutions of cDNA sample. In this curve, a log of input nucleic acids on X-axis is plotted against CT values on the y-axis. The gradient of the semi-log regression is determined to evaluate the amplification efficiency by this equation: $\text{efficiency} = 10^{(-1/\text{gradient})-1}$. The gradient value equal to -3.32 represents 100% amplification efficiency. A value that is more negative than this reflects an amplification process less than 100%, while more positive value reflects sample quality or pipetting problems. In this project five human primer/probe combinations were used; E-Cadherin (Hs01023894-m1-CDH1), fibronectin (Hs00365052m-1FN1), MUC5AC (Hs00873651_mH), MUC5B (Hs00861595_m1) and HPRT1 (Hs02800695-m1) (Life Technologies). The expression of each test gene was normalised against expression of the housekeeping gene, HPRT1. Figure 2-4 shows the efficiency curves for these primer/probe combinations performed using 10 times serial dilution from cDNA sample.

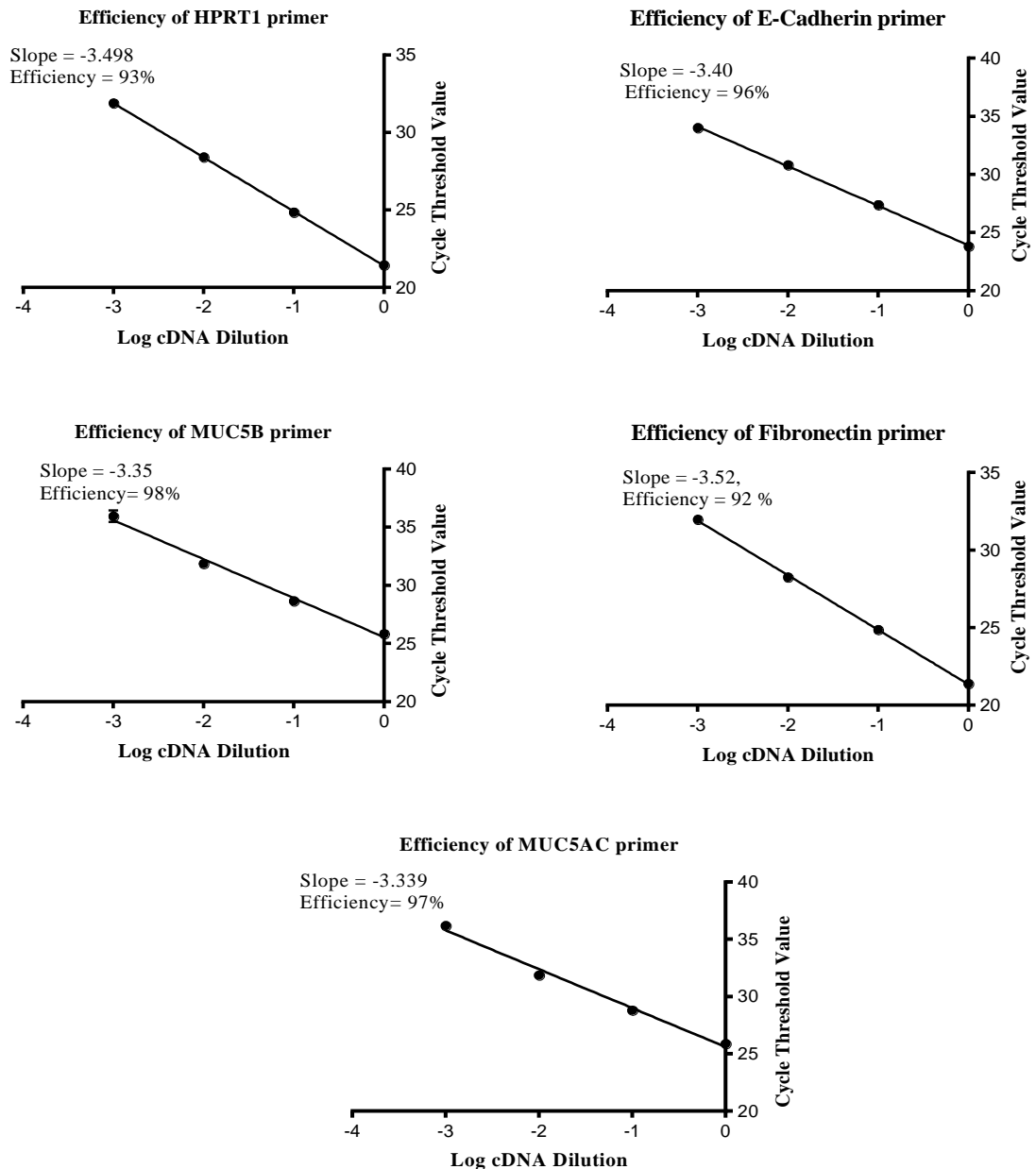


Figure 2-4: The standard curve for tenfold dilution series of cDNA for E-Cadherin, fibronectin, MUC5AC, MUC5B and HPRT transcripts. The reactions were run on an Applied Biosystems instrument (StepOnePlus) for 40 cycles. For gradient and efficiency values, the data were analysed by linear regression converting x values to a log scale.

2.9.6.4 Real-time PCR detection

To determine the change in expression of a particular gene at a molecular level a quantitative PCR reaction was performed. This was done by using a cDNA template transcribed from the isolated RNA. PCR reaction was prepared in 20 μ l total volume by mixing 10 μ l of 2X master mix (SensiFAST™ Probe Hi-ROX Kit (Bioline)), containing TaqMan DNA polymerase enzyme and buffers essential for its activity, 7 μ l distilled water molecular grade, 2 μ l of cDNA and 1 μ l of desired primer mix (purchased from Life Technologies, ready to use). The

mixture was amplified using an Applied Biosystems StepOnePlus. The amplification process was carried out for 40 cycles, in the following sequential steps: 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds followed by 20 seconds 60°C. Each PCR run contains a target gene in parallel with a housekeeping gene. In addition, each run had negative control wells where the RNA sample and master mix mixture were checked separately for any contamination.

2.9.6.5 Data analysis-comparative $\Delta\Delta\text{CT}$ value

The comparative or relative method of quantification requires the CT (Cycle threshold) value and arithmetic or application formulas in order for a result to be achieved. The CT is defined as the number of cycles that are needed for the background level to be exceeded by the fluorescence signal. Calculating the target gene's level of expression is made by comparing the CT values of both control and activated cells. The CT values of both the samples of interest and the control are first normalised to the reference gene or endogenous housekeeping gene. In order for a valid result to be obtained, the efficiency or amplifying both the reference and the target used in the experiment must be greater than 90% and approximately or close to equal. Here are the below steps in order to calculate the comparative CT ($\Delta\Delta\text{CT}$):

Step 1: To calculate the standard deviation and mean values of the replicate samples.

The mean standard deviations and CT values are calculated by using Microsoft® Excel software.

Step 2: In order to calculate the ΔCT value.

The ΔCT is calculated as the following:

$$\Delta\text{CT} = \text{CT (target)} - \text{CT (reference)}$$

Step 3: To calculate the ΔCT value's standard deviation.

The standard deviation (SD) of the ΔCT value is calculated from the standard deviations of the reference and the target values with the formula below:

$$\text{SD} = (\text{SD target}^2 + \text{SD reference}^2)^{1/2}$$

Step 4: Calculating the $\Delta\Delta\text{CT}$ values.

The $\Delta\Delta\text{CT}$ value is calculated as:

$$\Delta\Delta\text{CT} = \Delta\text{CT (treated sample)} - \Delta\text{CT (non-treated sample)}$$

Step 5: Calculating the standard deviation of $\Delta\Delta\text{CT}$ values.

The calculation of $\Delta\Delta\text{CT}$ requires the subtraction of the ΔCT calibrator (un-treated) sample. This is a subtraction of an arbitrary constant; thus, the SD of the $\Delta\Delta\text{CT}$ value is comparable to the SD of the ΔCT value.

2.10 Statistical analysis

All data are represented as mean \pm Standard Error of the Mean (S.E.M), and in this study, n = the number of repeat experiments. GraphPad Prism v6 (Windows) was used for statistical analysis, with comparison between two groups using unpaired Student's t-test. Experiments comparing three or more matched groups used a repeated one-way analysis of variance (ANOVA), next a Bonferroni post-hoc test, comparing all pairs. As is standard, P values of ≤ 0.05 were considered significant. In this study, * refers to $p < 0.05$, ** refers to $P < 0.01$ and *** $p < 0.001$.

CHAPTER 3: ESTABLISHING AN *IN-VITRO* MODEL OF HUMAN BRONCHIAL EPITHELIAL CELL INJURY USING CELL LINES

3.1 Introduction

The airway epithelium plays a critical role in healthy as well as diseased lungs, primarily by providing a physical barrier to foreign particles, such as particulates and smoke, in addition to viruses and bacteria. Moreover, it enables a range of pro-inflammatory, anti-microbial and regulatory functions, and has the capacity to regenerate. Studies of the airway epithelium have been carried out since the late 1970s (Mathias *et al.*, 1996). It has long been known that bronchial secretions are also involved in the initiation and augmentation of pulmonary host defence mechanisms. The latter involves generating and disseminating a range of mediators for various responses, including chemotaxis, inflammatory cell differentiation and activation in both diseased and non-diseased conditions (Brown *et al.*, 2008).

Many chronic degenerative diseases, such as lung allograft rejection, are characterised by epithelial damage related to pro-inflammatory cytokines. Symptoms include bronchial cell injury and necrosis, alongside leukocyte, neutrophil and macrophage infiltration, followed by accelerated inflammatory lung disease (Todd and Palmer, 2011).

IL-8 is a neutrophilic chemoattractant and an angiogenic factor. Immunostaining has been used to identify IL-8 localisation in peribronchial lesions (Zheng *et al.*, 2000). IL-6 plays a role in the activation of monocytes, T-cell differentiation and B-cell maturation. It also serves as a pyrogenic factor, inducing fever in response to infection (Rincon and Irvin, 2012). GM-CSF is involved in macrophage activation and is a strong chemoattractant for neutrophils. It stimulates microbicidal and phagocytic activity, oxidative metabolism and improves cytotoxicity of both macrophages (Sorgi *et al.*, 2012) and neutrophils (Katano *et al.*, 2010).

Evaluation of a broad array of epithelial cell lines has found that those mimicking adult human airway epithelial behaviours can be used to expand knowledge of the airway epithelium.

This study investigated four such cell lines to compare inflammatory mediator expression, both basal and inducible. The most physiologically relevant cell type to evaluate airways damage was arguably primary human bronchial epithelial cells (PHBECs). However, these

are costly and not easy to culture (Stewart *et al.*, 2012). Consequently, cell lines were investigated in terms of their ability to provide a useful model to complement the use of PHBECs. It was found that BEAS-2B cells were able to secrete IL-6, IL-8 and other cytokines while maintaining epithelial cell morphology *in-vitro*. These qualities make BEAS-2B a popular cell line for airway epithelial structure and functional studies (Swanson *et al.*, 2009).

The mucoepidermoid carcinoma-derived NCI-H292 cell line responds to cigarette smoke in the same way as PHBECs and provides a useful model for mucus production studies (Binker *et al.*, 2009). The virally transformed bronchial epithelial line, 16HBE14o-, is used frequently in studies of ion transportation and barrier function (Gruenert *et al.*, 1988; Cozens *et al.*, 1994). Finally, the human bronchial submucosal gland-derived Calu-3 cell line provides a model for the study of airway surface liquid, mucins and similar immunologically active substances (Shen *et al.*, 1994), alongside pro-inflammatory cytokines involved in airway inflammation and epithelial damage (Hamid *et al.*, 1999).

Asthma and (COPD) are common, chronic airway pathophysiologies that include airflow obstruction. This airflow obstruction is associated with endothelin(s) production, peptides that cause significant vaso and broncho-constriction (Ucvet *et al.*, 2011). Furthermore, the metabolism of arachidonic acid generates certain mediators, including cysteinyl leukotrienes, which cause smooth muscle contraction, which consequently changes cell chemo-attraction and vascular permeability, thereby resulting in excess mucus production.

Therefore, the findings described in this chapter aim to establish a model for airway epithelial cell injury that is relevant to chronic degenerative diseases. Primary and secondary bile acids (BAs) were introduced to the cell lines described above to shed light on this. Pro-inflammatory cytokine levels (IL-8, IL-6 and GM-SCF) were measured by Enzyme-Linked Immuno-Sorbent Assay (ELISA) with the aim of establishing a connection between BAs and epithelial damage. These candidate marker cytokines were chosen on the basis of prior studies which had found these factors to be released by damaged cells, and that showed they are upregulated during infection. For patients with inflammatory lung diseases, these are some of the primary exacerbating factors (damage and infection).

3.2 Aims of the study

On the basis of the evidence for the role of epithelial-derived cytokines and other mediators in the evolution of airway inflammation, this study aims to:

- Determine the sensitivity of BEAS-2B, Calu-3, 16HBE14-O and NCI-H292 human airway epithelial cell lines to primary and secondary BAs
- From this, to determine the optimum BAs concentration profile that may injure cells or stimulate them to an inflammatory phenotype
- To therefore investigate the effects of primary and secondary BAs on the basal expression of inflammatory mediators in the human airway epithelial cells, using the data established by preliminary experimentation in the cell lines to make the best use of the limited primary cells.

3.3 Methods

3.3.1 Cell line stimulation

After reaching 80-95% confluence in 24-well plates, cell lines were rested for 24 hours as described in chapter 2, section 2.2. They were then exposed to individual primary and secondary BAs, at the concentrations reported in section (see chapter 2, section 2.6).

3.3.2 Cell viability assay

The Titer-Blue Assay and MTT assays were used. A standard curve was produced using known ratios of live to dead cells, and cells were killed using a five-minute exposure to ice-cold methanol. The standard curve was used to determine the percentages of viable cells and comprised: 100% live cells (resting medium); 25:75% dead:live cells; 50:50%; 75:25% dead:live cells; and 100% dead cells (see chapter 2, sections 2.7.1 and 2.7.2).

3.3.3 ELISA

A commercially available ELISA method based on antibody pairs was used to determine human IL-8, IL-6 and GM-CSF concentrations in cell culture supernatants from differentiated cell lines (see chapter 2, section 2.8.1). This assay uses an analyte-specific monoclonal antibody pre-coated onto a microplate. After adding the sample, the relevant protein binds to the antibody. Following the addition of an enzyme-linked analyte-specific detection antibody, the substrate solution develops colour (via enzyme digestion) in proportion to the protein concentration in the sample. The signal intensity was then measured.

3.4 Results and discussion

3.4.1 Morphology of cell lines used in this study

One of the aims of this project was to culture and process bronchi-derived 16HBE14o-, BEAS-2B (virally transformed for immortality), Calu-3 and NCI-H292 (cancerous) human cell lines. These lines all have different transformation properties. Different conditions were used for different cell lines; their morphology are illustrated Figure 3-1. The ‘cobblestone’ morphology indicates healthy cells in submerged culture. The cells are clearly adherent with very few floating or fibroblast-like cells; nevertheless, they have a tendency to clump and expand towards other colonies, rather than migrating. Furthermore, I was able to re-establish these cells in culture, even after cryopreservation.

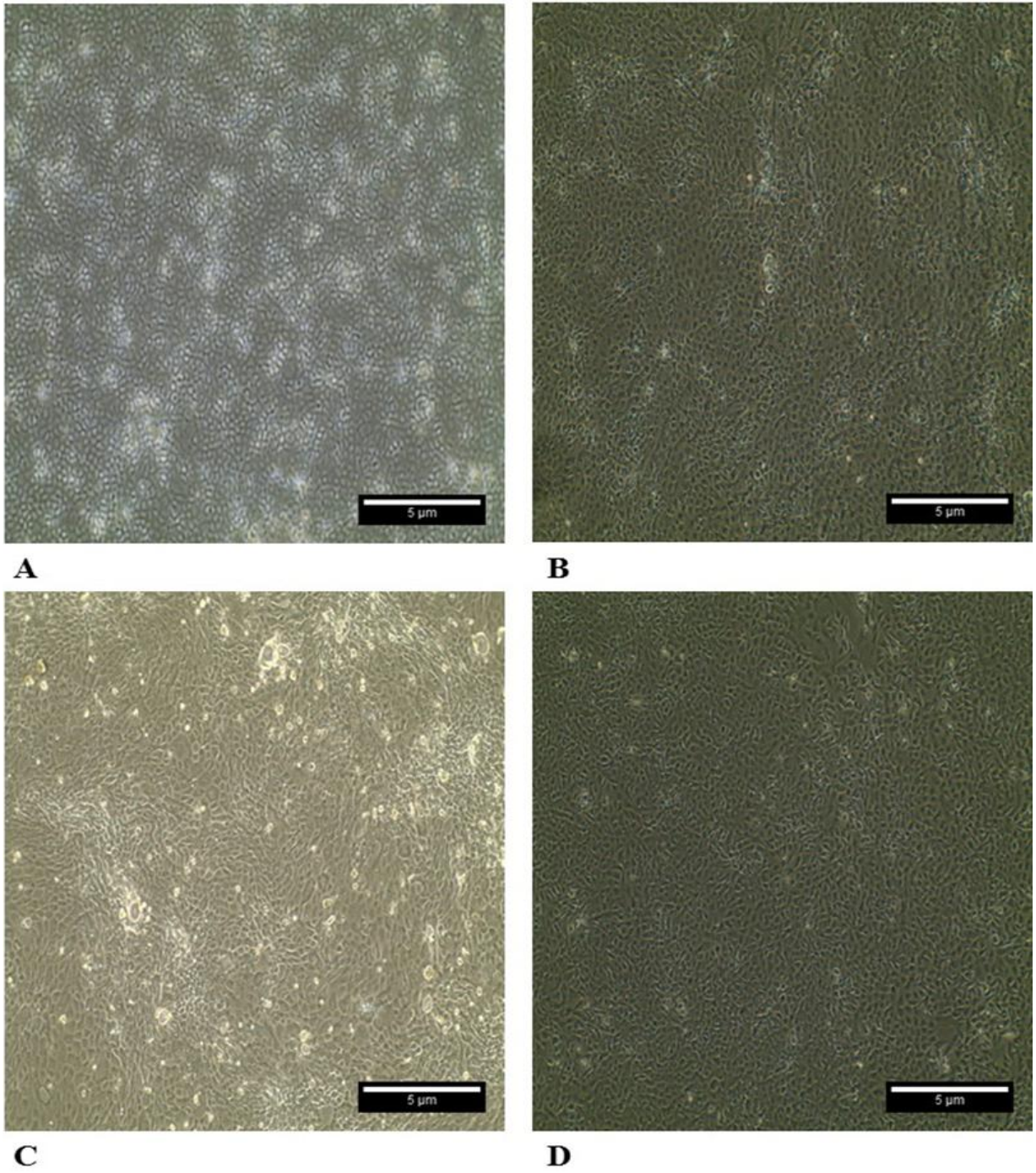


Figure 3-1: Morphology of cell lines used in the study. 10x magnification of (A) NCI-H292, (B) BEAS-2B, (C) Calu-3 and (D) 16HBE14o- cell lines cultured in complete medium in 24 or 6-well cell cultures at 37°C with 5% CO₂ for 24 hours. These cells have the typical appearance of healthy cell lines in submerged culture.

3.4.2 Influence of individual bile acid on 16HBE14o- cell viability and inflammatory cytokine (IL-8, IL-6 and GM-CSF) release

16HBE14o- cell monolayers at about 80-90% confluence were cultured in resting medium with 15-100 $\mu\text{mol/L}$ cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) for 48 hours. Lithocholic acid (LCA) was used in concentrations of 1 to 20 $\mu\text{mol/L}$, also for 48 hours.

Both CellTiter-Blue (repeated culture $n=3$ with repeated wells $n=12$; overall $n=12$) and MTT (repeated culture $n=3$ with repeated wells $n=12$; overall $n=12$) were used to assess cell viability. Both the CellTiter-Blue and MTT assays were used to calculate viability for two plates. The results revealed no significant difference between the two assessment methods.

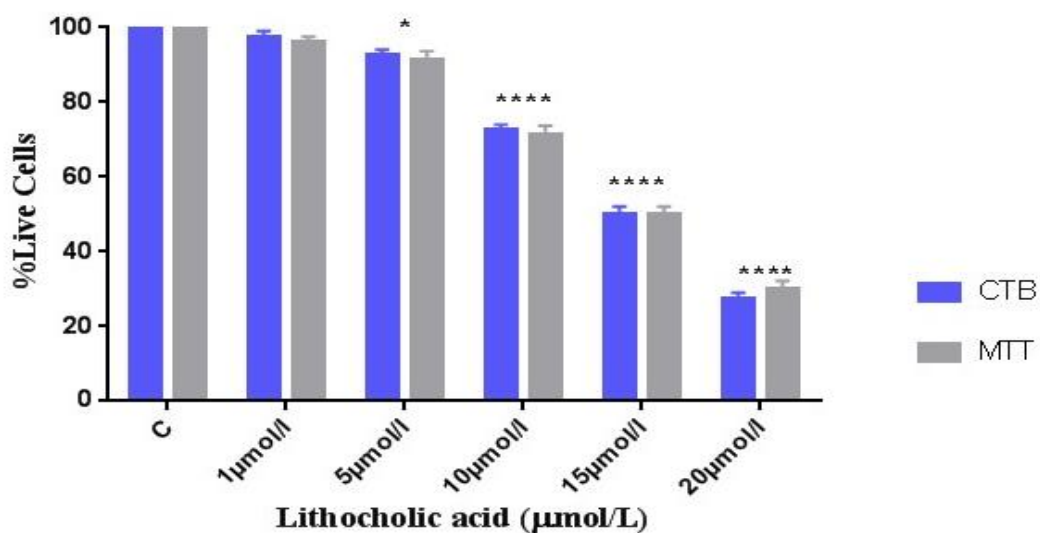
The cell viability was found to be affected depending on the BAs concentration. There was an inverse relationship between BAs concentration and measured cell viability, with increasing concentration and decreasing cell viability in a dose-dependent manner.

The results revealed that cell viability reduced significantly in response to exposure to 10 $\mu\text{mol/L}$ of LCA to $73\pm 3\%$ of control and then decreased with 15 $\mu\text{mol/L}$ LCA to $50\pm 3\%$ of control. This decreased further to the lowest cell viability level at 20 $\mu\text{mol/L}$ LCA with $28\pm 3\%$ viable cells (Figure 3-2A).

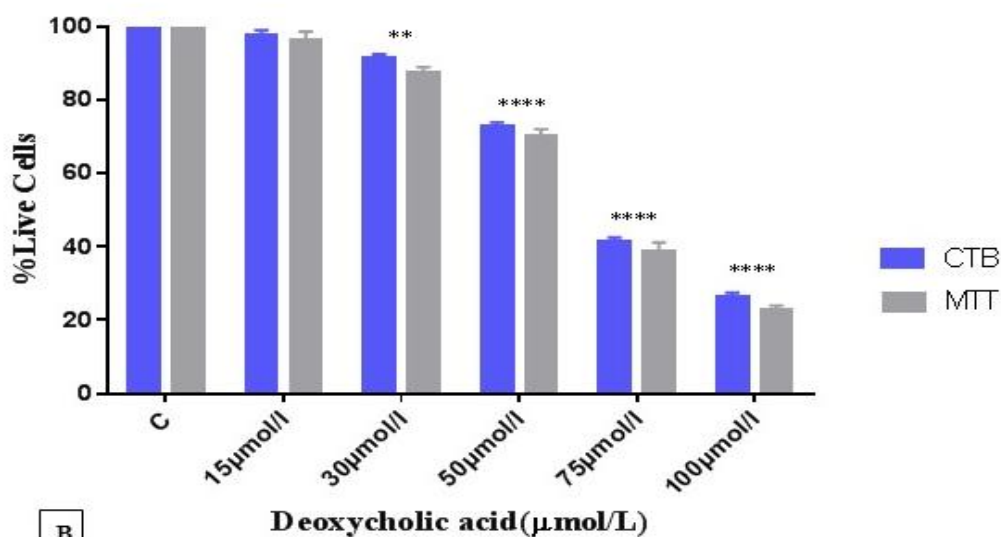
The cell viability was reduced significantly by DCA at 50 $\mu\text{mol/L}$ ($73\pm 3\%$) and the cell viability then decreased with 75 $\mu\text{mol/L}$ DA to $41\pm 3\%$. This decreased further to the lowest cell viability level at 100 $\mu\text{mol/L}$ DA with $26\pm 2\%$ viable cells (Figure 3-2B).

Furthermore, cell viability decreased significantly in response to exposure to 75 $\mu\text{mol/L}$ of CA with $85\pm 3\%$. This decreased further to the lowest cell viability level at 100 $\mu\text{mol/L}$ CA ($73\pm 3\%$). CA did not affect cell viability at concentrations 15, 30 and 50 $\mu\text{mol/L}$ (Figure 3-3A).

Furthermore, cell viability decreased significantly by CDCA at 50 $\mu\text{mol/L}$ with $82\pm 2\%$. The cell viability then reduced with 100 $\mu\text{mol/L}$ CDCA to $50\pm 3\%$. No difference was found in the cell viability at lowest CDCA concentrations, 15 and 30 $\mu\text{mol/L}$, compared with the control (Figure 3-3 B).

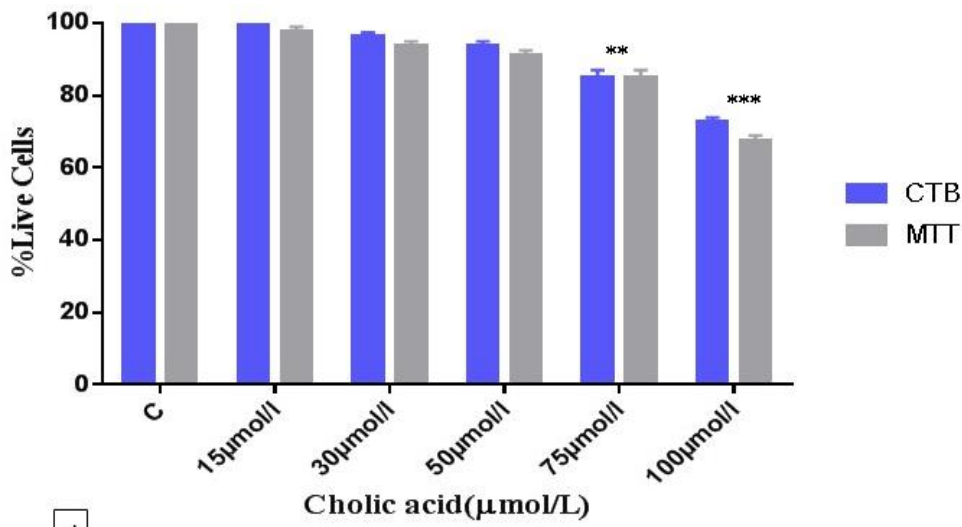


A

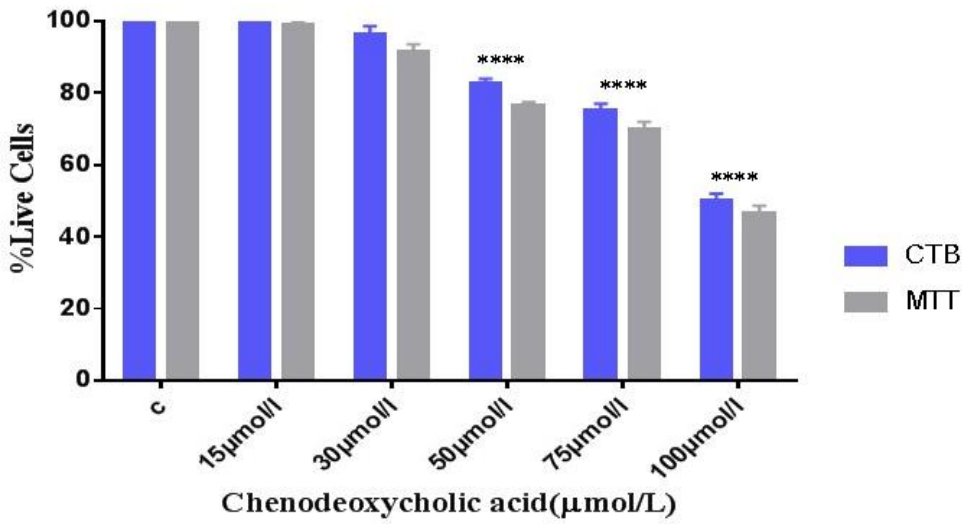


B

Figure 3-2: Effects of BAs on 16HBE14o- cell proliferation. 16HBE14o- cell viability following stimulation with secondary Bas, (A) Lithocholic and (B) Deoxycholic acids at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue or MTT reagent. The data are representative of three independent experiments (n=12). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA).



A



B

Figure 3-3: Effects of BAs on 16HBE14o- cell proliferation. 16HBE14o- cell viability following stimulation with primary BAs (A) Cholic (B) Chenodeoxycholic acids at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue or MTT reagent. The data are representative of three independent experiments (n=12). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.

IL-8, IL-6 and GM-CSF production by the 16HBE14o- cell line was analysed following stimulation by BAs. The 16HBE14o- cells were cultured and stimulated with primary BAs (CA and CDCA) and the secondary BA (LCA and DCA).

IL-8 levels increased by a significant amount when stimulated with 10 and 15 $\mu\text{mol/L}$ LCA but not at 1, 5 and 20 $\mu\text{mol/L}$. IL-8 levels increased significantly at 10 $\mu\text{mol/L}$ LCA to 136 ± 24 pg/mL compared to the control, 41 ± 12 pg/mL, and increased to the maximum level at 15 $\mu\text{mol/L}$ LCA of 340 ± 3 pg/mL

IL-6 levels also significantly increased following stimulation by 1 $\mu\text{mol/L}$ or above of LCA. The result revealed that the highest level of IL-6 production was at 10 $\mu\text{mol/L}$ LCA with 294 ± 30 pg/mL compared with the control (33 ± 3 pg/mL), and then declined as concentration of LCA increased to 20 $\mu\text{mol/L}$.

GM-CSF levels increased significantly at 5 $\mu\text{mol/L}$ LCA with 284 ± 15 pg/mL and increased further to the maximum level at 10 $\mu\text{mol/L}$ LCA with 707 ± 35 pg/mL. The level of GM-CSF production then fell off at 15 $\mu\text{mol/L}$ LCA with 258 ± 37 pg/mL but remained significantly higher than the control, 14 ± 3 pg/mL. LCA at concentration 20 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF level (Figure 3-4 and Table 3-1).

16HBE14o- cells

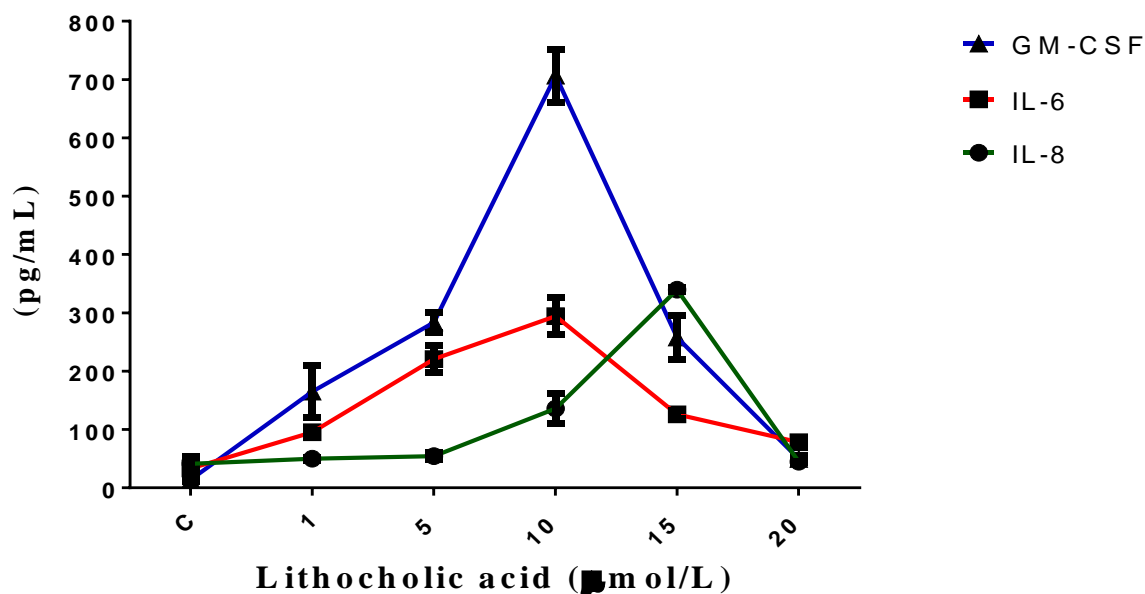


Figure 3-4: Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA. The data are representative of three independent experiments (n=6) (see table 3-1). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	1	5	10	15	20
IL-8 (pg/ml)	_____	_____	↑**	↑****	_____
IL-6 (pg/ml)	↑**	↑**	↑***	↑****	↑*
GM-CSF (pg/ml)	↑**	↑****	↑****	↑****	_____

Table 3-1: The effect of different levels of Lithocholic acid on the 16HBE14o- cell line following stimulation for 48 hours, \uparrow Increase demonstrated * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

IL-8, IL-6 and GM-CSF production by the 16HBE14o- cell line was analysed following stimulation with DCA. The IL-8 levels at 30, 50 and 75 $\mu\text{mol/L}$ DCA increased significantly, but 15 and 100 $\mu\text{mol/L}$ DCA displayed no increase. The highest level of IL-8 release occurred with 75 $\mu\text{mol/L}$ DCA to 864 ± 32 pg/mL, compared with the control (41 ± 8 pg/mL).

IL-6 levels were elevated significantly by 30, 50, 75 and 100 $\mu\text{mol/L}$ DCA concentrations. The highest level of IL-6 production was at 50 $\mu\text{mol/L}$ DCA to 330 ± 3 pg/mL compared with the control 36 ± 2 pg/mL and then declines as the concentration of DCA increases up to 100 $\mu\text{mol/L}$. DCA at concentration 15 $\mu\text{mol/L}$ did not cause a significant increase in IL-6 levels.

16HBE14o- cells also produce GM-CSF. These cells were exposed to DCA for 48 hours at concentrations ranging from 15 to 100 $\mu\text{mol/L}$. The GM-CSF levels increased significantly at 30 $\mu\text{mol/L}$ DCA with 153 ± 5 pg/mL and increased further to the maximum level at 50 $\mu\text{mol/L}$ DCA with 318 ± 15 pg/mL. The level of GM-CSF production then fell off at 100 $\mu\text{mol/L}$ DCA with 47 ± 2 pg/mL but was still significantly higher than the control at 12 ± 2 pg/mL. DCA at concentration 15 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF levels (Figure 3-5 and Table 3-2).

16HBE14o- cells

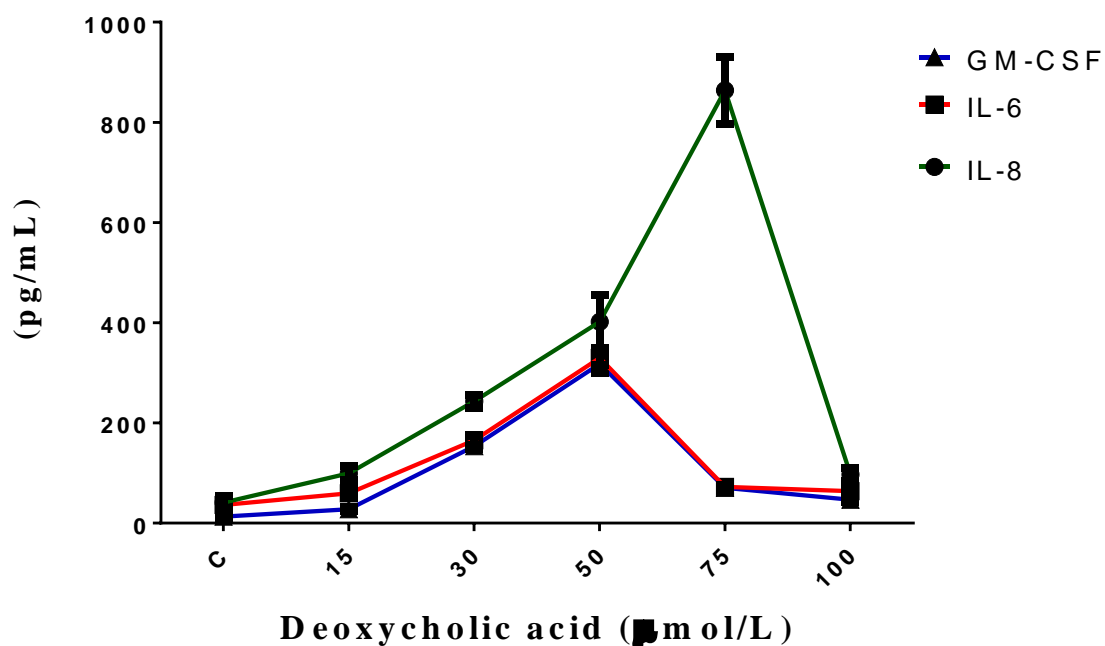


Figure 3-5: Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with DCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA. The data are representative of three independent experiments (n=6) (see table 3-2). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Deoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	_____	↑**	↑****	↑*****	_____
IL-6 (pg/ml)	_____	↑**	↑****	↑*	↑*
GM-CSF (pg/ml)	_____	↑**	↑****	↑*	↑*

Table 3-2: The effect of different levels of Deoxycholic acid on the 16HBE14o- cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

IL-8, IL-6 and GM-CSF production by 16HBE14o- cells increased at 48 hours after stimulation with CA concentrations ranging from 15 to 100 $\mu\text{mol/l}$. IL-8 levels at 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CA were significantly increased. The highest level of IL-8 release occurred with 100 $\mu\text{mol/L}$ CA to 825 ± 32 pg/mL compared with the control (50 ± 9 pg/mL).

The IL-6 levels increased significantly at 15 $\mu\text{mol/L}$ CA with 103 ± 5 pg/mL and increased further to the maximum level at 75 $\mu\text{mol/L}$ CA with 413 ± 16 pg/mL. The level of IL-6 production then fell off at 100 $\mu\text{mol/L}$ CA with 322 ± 22 pg/mL but remained significantly higher than the control at 32 ± 3 pg/mL.

The GM-CSF levels also significantly increased following stimulation by 30 $\mu\text{mol/L}$ or above of CA. The result revealed that the highest level of GM-CSF production was at 100 $\mu\text{mol/L}$ CA with 330 ± 20 pg/mL compared with control at 31 ± 5 pg/mL. CA at concentration 15 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF levels (Figure 3-6 and Table 3-3).

16HBE14o- cells

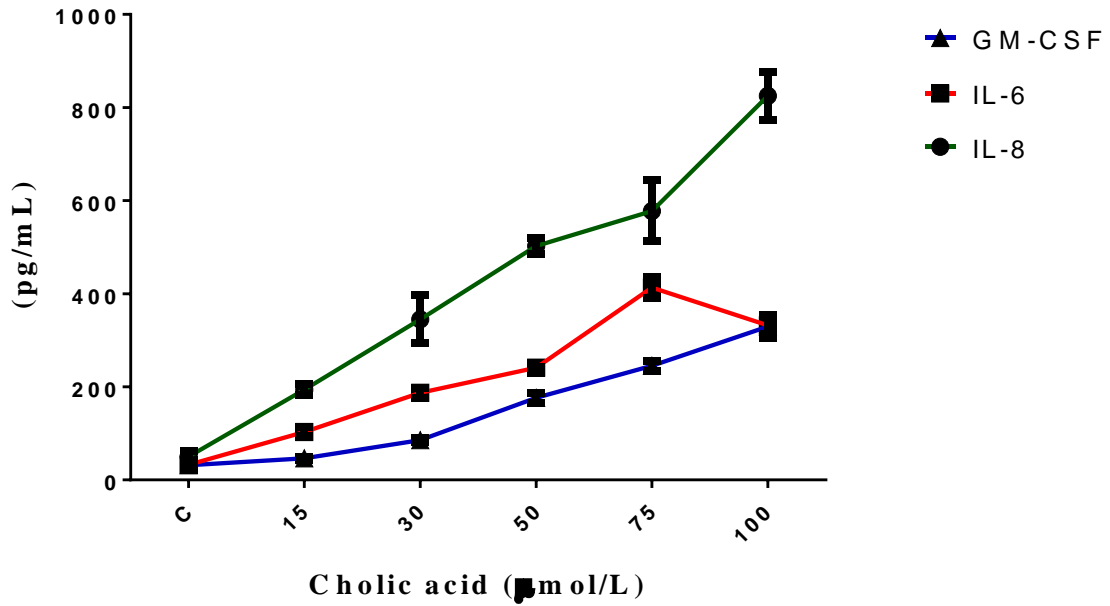


Figure 3-6: Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with CA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-3). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Cholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	\uparrow^{**}	\uparrow^{**}	\uparrow^{***}	\uparrow^{***}	\uparrow^{***}
IL-6 (pg/ml)	\uparrow^{**}	\uparrow^{***}	\uparrow^{***}	\uparrow^{***}	\uparrow^{***}
GM-CSF (pg/ml)	_____	\uparrow^{**}	\uparrow^{***}	\uparrow^{***}	\uparrow^{***}

Table 3-3: The effect of different levels of cholic on the 16HBE14o- cell line following stimulation for 48 hours, \uparrow Increase demonstrated * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

CDCA stimulation of cultured 16HBE14o- at concentrations from 15 to 100 μ mol/L for 48 hours was compared with controls without CDCA. The IL-8 levels increased significantly at 15 μ mol/L CDCA to 577 \pm 22 pg/mL and rose further to the highest level at 75 μ mol/L CDCA to 1075 \pm 32 pg/mL compared to the control (41 \pm 12 pg/mL). The level of IL-8 production then decreased at 100 μ mol/L CDCA but remained significantly higher than the control.

Moreover, the findings showed a significant increase in IL-6 at 15, 30, 50, 75 and 100 μ mol/L of CDCA. The highest level of IL-6 production was at 50 μ mol/L CDCA with 423 \pm 20 pg/mL compared with control (29 \pm 3 pg/mL) and then declined as the concentration of CDCA increased up to 100 μ mol/L.

ELISA detected the GM-CSF following CDCA stimulation of 16HBE14o- cells. The CDCA significantly upregulates GM-CSF production at 30, 50, 75 and 100 μ mol/L, but not at 15 μ mol/L. GM-CSF production increased significantly at 30 μ mol/L CDCA TO 65 \pm 9 pg/mL and rose further to a maximum level at 100 μ mol/L CDCA to 640 \pm 20 pg/mL compared with the control at 14 \pm 3 pg/mL (Figure 3-7 and Table 3-4).

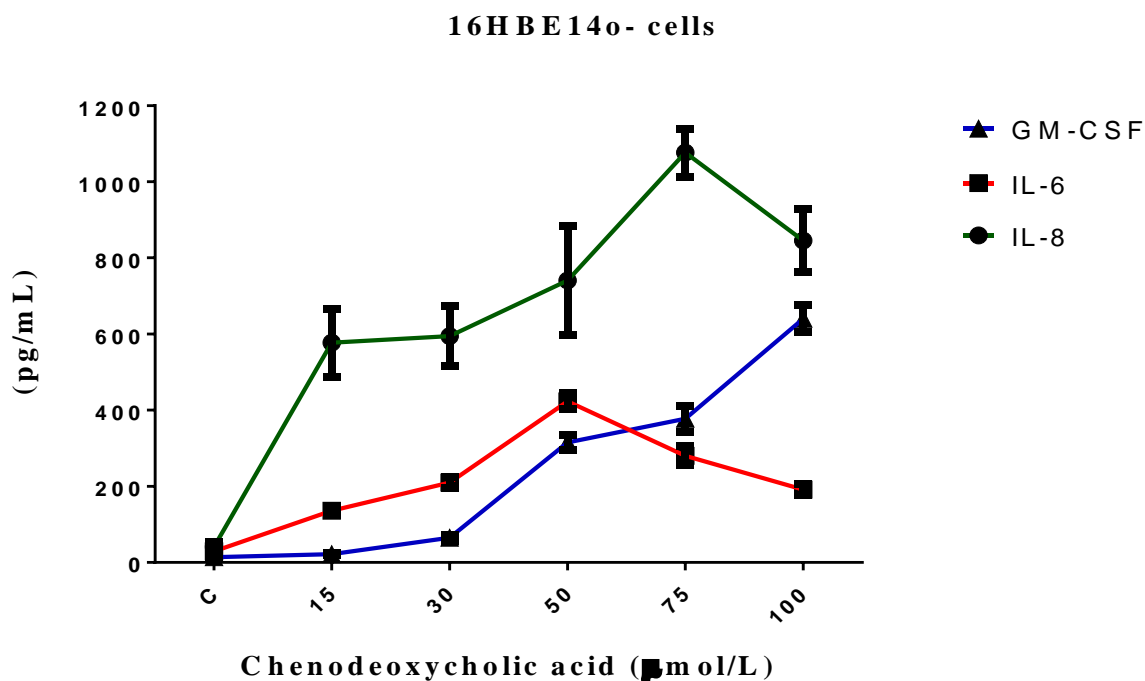


Figure 3-7: Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with CDCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-4). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Chenodeoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑***	↑***	↑***	↑***	↑**
IL-6 (pg/ml)	↑***	↑***	↑*****	↑***	↑***
GM-CSF (pg/ml)	_____	↑*	↑***	↑***	↑*****

Table 3-4: The effect of different levels of Chenodeoxycholic acid on the 16HBE14o- cell line following stimulation for 48 hours, ↑ Increase demonstrated * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ***** = $p < 0.0001$ compared with control, ANOVA).

3.4.3 Influence of individual BAs on BEAS-2B cell viability and inflammatory (IL-8, IL-6 and GM-CSF) release

Both the CellTiter-Blue and MTT assays were used to calculate viability. The results revealed no significant difference between the two methods of assessment. BEAS-2B cells were exposed to CA, DCA and CDCA in concentrations from 15 to 100 $\mu\text{mol/L}$ for 48 hrs, and LCA concentrations from 1 to 20 $\mu\text{mol/L}$.

The cell viability was found to be affected, depending on the BAs concentration. There was an inverse relationship between BAs concentration and measured cell viability, with increasing concentration and decreasing cell viability in a dose-dependent manner.

The results showed that cell viability reduced significantly in response to exposure to 5 $\mu\text{mol/L}$ LCA to $78\pm 3\%$ of control and then decreased further with 10 $\mu\text{mol/L}$ LCA to $46\pm 3\%$. This decreased further to the lowest cell viability level at 20 $\mu\text{mol/L}$ LCA with $7\pm 3\%$ (Figure 3-8 A).

Cell viability was reduced significantly by DCA at a concentration of 50 $\mu\text{mol/l}$ ($94\pm 3\%$). However, 15 and 30 $\mu\text{mol/l}$ DCA did not affect cell viability (Figure 3-8 B).

Cell viability reduced significantly in response to exposure to 50 $\mu\text{mol/L}$ CA to $88\pm 3\%$ of control. This decreased further to the lowest cell viability level at 100 $\mu\text{mol/L}$ CA ($53\pm 3\%$). The lowest concentrations (15 and 30 $\mu\text{mol/L}$) of CA did not affect cell viability (Figure 3-9 A).

Cell viability was reduced significantly by CDCA at concentration 50 $\mu\text{mol/l}$ to $88\pm 2\%$ of control. The cell viability was greatly reduced by 100 $\mu\text{mol/L}$ of CDCA to $53\pm 3\%$ of control. No difference was found in the cell viability at 15 and 30 $\mu\text{mol/L}$ CDCA compared with the control (Figure 3-9 B).

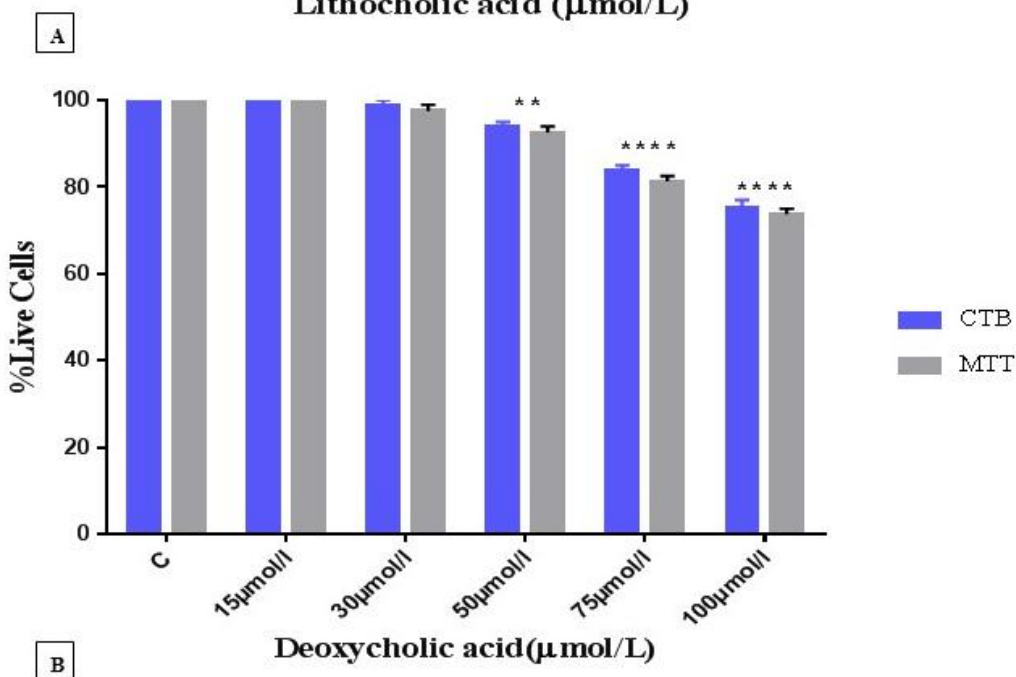
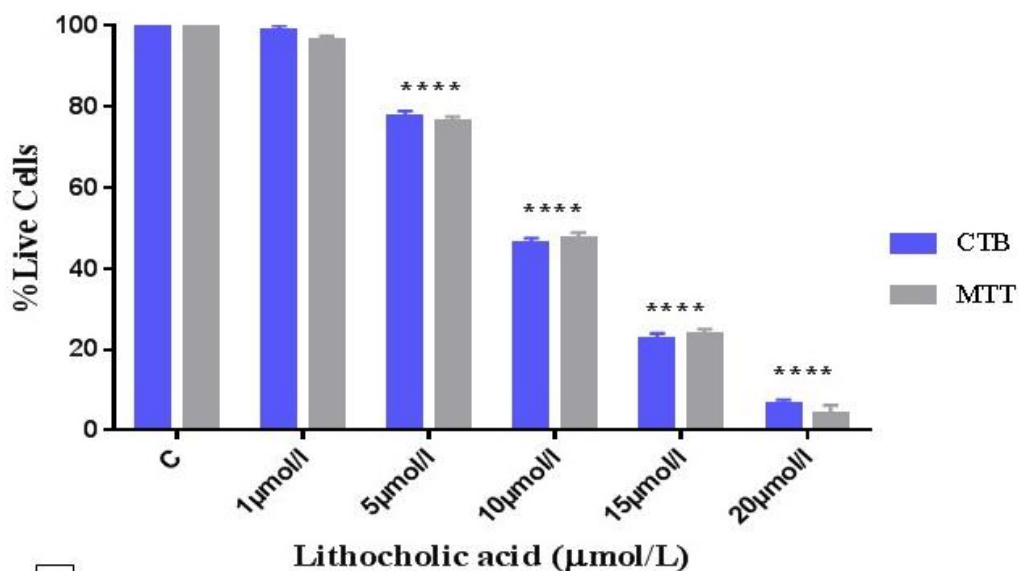
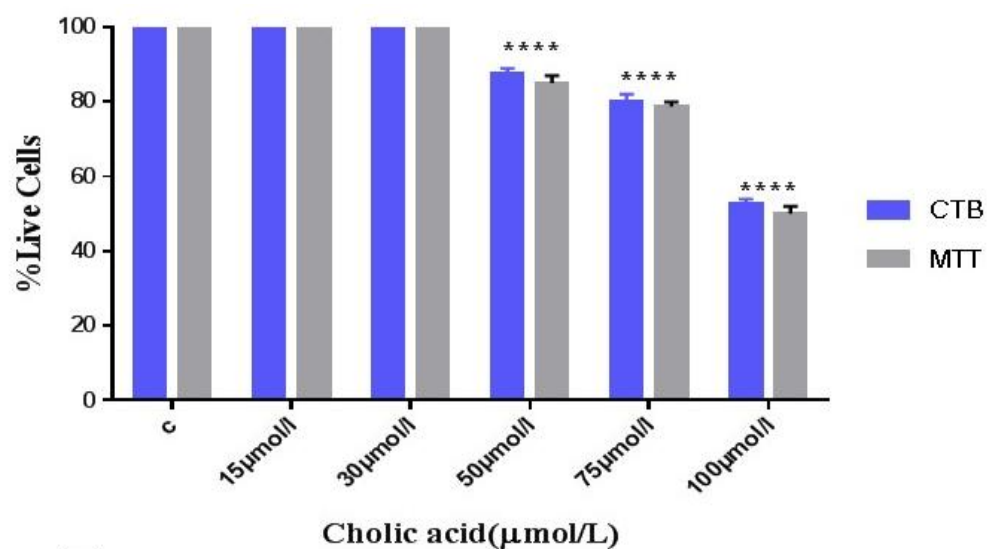
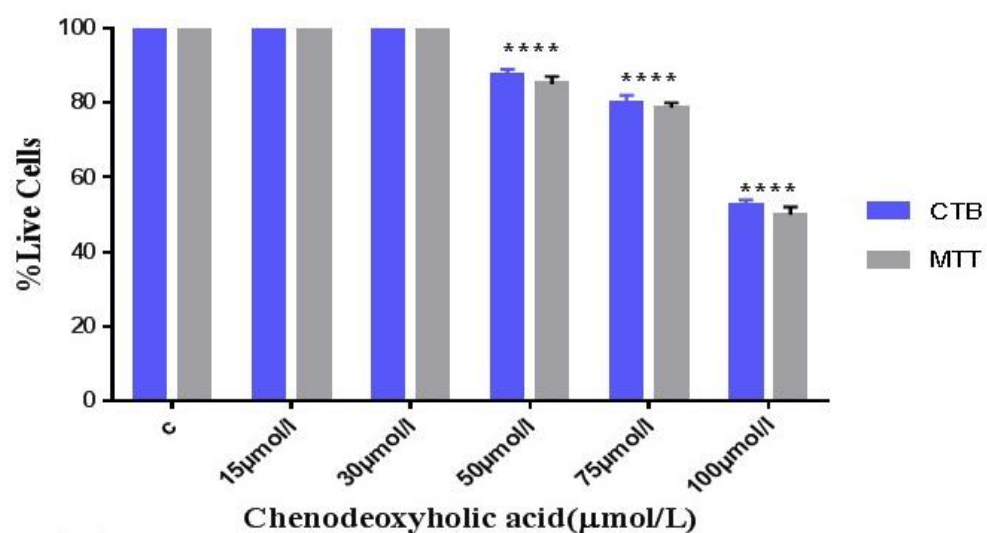


Figure 3-8: Effects of BAs on BEAS-2B cell proliferation. BEAS-2B cell viability following stimulation with secondary BAs (A) Lithocholic (B) Deoxycholic acid, at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue or MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.



A



B

Figure 3-9: Effects of BAs on BEAS-2B cell proliferation. BEAS-2B cell viability following stimulation with primary BAs (A) Cholic (B) Chenodeoxycholic acids at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue or MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.

BAs affect IL-8, IL-6 and GM-CSF secretion by BEAS-2B cells, which was investigated by treating cells with LCA in concentrations from 1 to 20 $\mu\text{mol/l}$. The experiment showed a significant increase in IL-8, IL-6 and GM-CSF released by bile acid challenged BEAS-2B cells compared to untreated controls. LCA caused IL-8 levels to increase significantly, but this was not observed at a concentration of 1 $\mu\text{mol/L}$.

IL-8 levels increased significantly at 5 $\mu\text{mol/L}$ LCA to 608 ± 3 pg/mL and increased further to the maximum level at 10 $\mu\text{mol/L}$ LCA (905 ± 12 pg/mL). The level of IL-8 production subsequently fell off at 20 $\mu\text{mol/L}$ LCA, but was still significantly higher than the control.

There was a significant increase in IL-6 levels with LCA at concentrations of 10, 15 and 20 $\mu\text{mol/L}$, but not at 1 and 5 $\mu\text{mol/L}$. The result revealed that the highest level of IL-6 production was at 10 $\mu\text{mol/L}$ LCA to 206 ± 2 pg/mL compared with the control at 15 ± 2 pg/mL and then declines as the concentration of LCA rose to 20 $\mu\text{mol/L}$.

The GM-CSF levels increased significantly at 5 $\mu\text{mol/L}$ LCA to 208 ± 4 pg/mL and rose further to the maximum level at 10 $\mu\text{mol/L}$ LCA to 332 ± 11 pg/mL compared with the control 23 ± 3 pg/mL. The level of GM-CSF production then fell off at 20 $\mu\text{mol/L}$ LCA, but was still significantly higher than the control. LCA did not cause GM-CSF levels to increase (Figure 3-10 and Table 3-5).

BEAS-2B cells

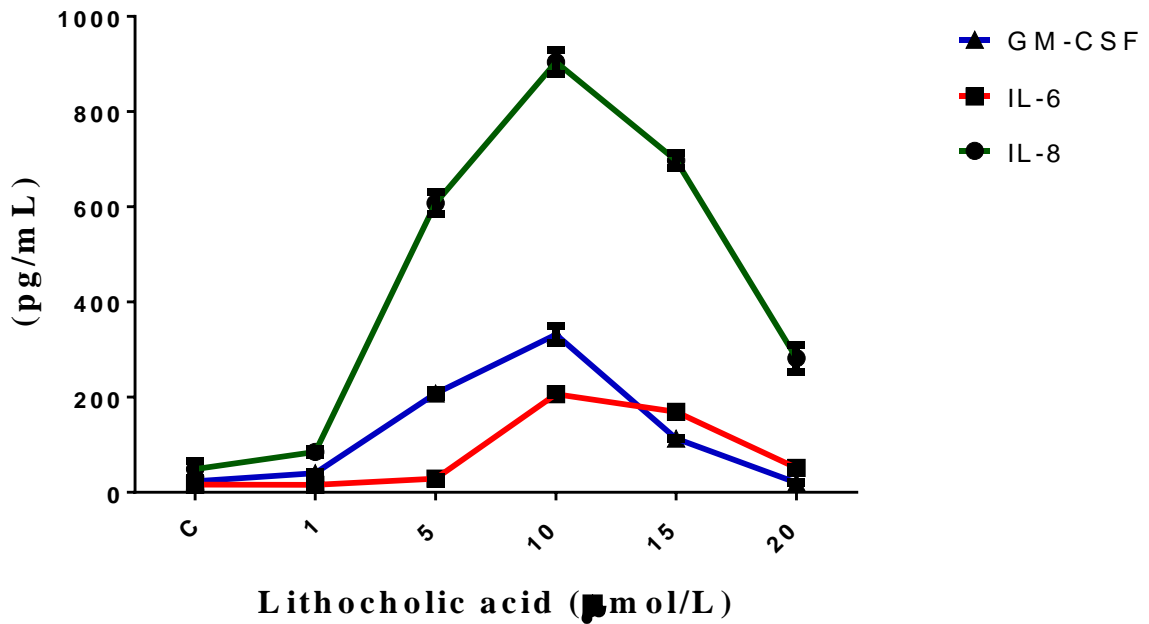


Figure 3-10: Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-5). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	1	5	10	15	20
IL-8 (pg/ml)	_____	↑*****	↑*****	↑*****	↑****
IL-6 (pg/ml)	_____	_____	↑*****	↑*****	↑**
GM-CSF (pg/ml)	_____	↑*****	↑*****	↑***	_____

Table 3-5: The effect of different levels of Lithocholic acid on the BEAS-2B cell line following stimulation for 48 hours, \uparrow Increase demonstrated * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Cells were treated with DCA in concentrations ranging from 15 to 100 $\mu\text{mol/l}$. IL-8, IL-6 and GM-CSF release from BEAS-2B cells also increased significantly. It can be observed that IL-8 levels increased significantly at 30 $\mu\text{mol/L}$ DCA to 243 ± 16 pg/mL and increased further to the maximum level at 75 $\mu\text{mol/L}$ DCA of 955 ± 15 pg/mL compared with the control: 56 ± 6 pg/mL. The level of IL-8 production then fell off at 100 $\mu\text{mol/L}$ DCA but were still significantly higher than the control. However, no significant increase was seen using a concentration of 15 $\mu\text{mol/L}$ DCA.

There was a significant increase in IL-6 levels with DCA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 10 $\mu\text{mol/L}$. The highest level of IL-6 secretion was with 100 $\mu\text{mol/L}$ DCA, 218 ± 8 pg/ML, compared with the control 17 ± 5 pg/mL.

Furthermore, the GM-CSF levels increased significantly at 30 $\mu\text{mol/L}$ DCA to 175 ± 3 pg/mL and increased further to the maximum level at 75 $\mu\text{mol/L}$ DCA, 343 ± 2 pg/ML, compared with the control, 31 ± 3 pg/mL. The level of GM-CSF production then decreased at 100 $\mu\text{mol/L}$ DCA but remained significantly higher than the control. However, no significant increase was observed using a concentration of 15 $\mu\text{mol/L}$ DCA (Figure 3-11 and Table 3-6).

BEAS-2B cells

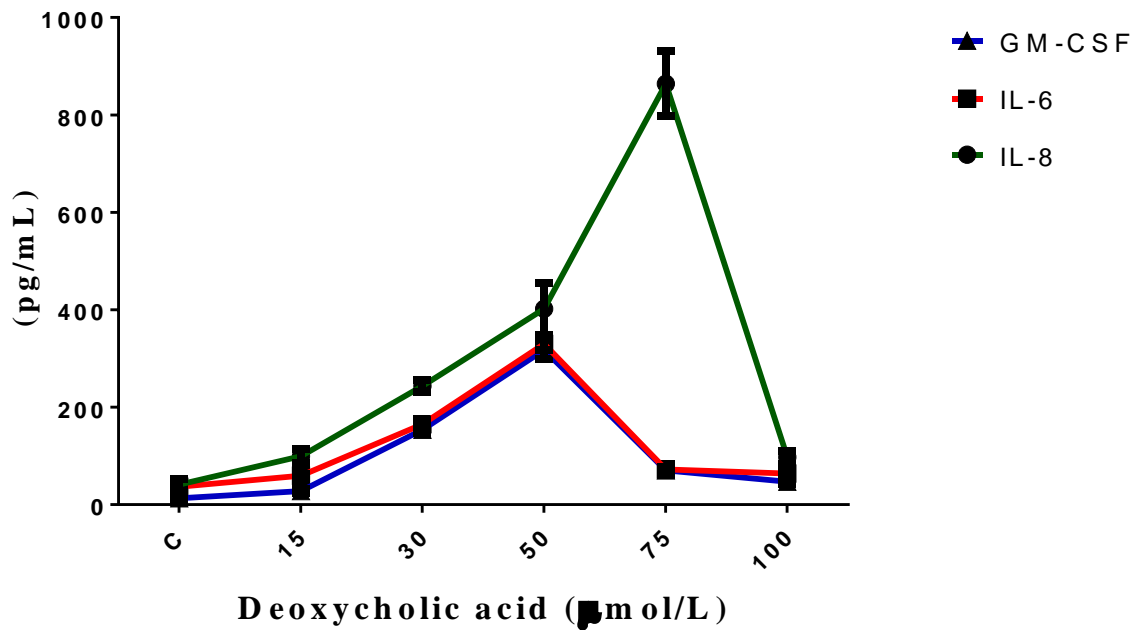


Figure 3-11: Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with DCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-6). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Deoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	_____	↑****	↑*****	↑*****	↑*****
IL-6 (pg/ml)	_____	_____	↑**	↑*****	↑*****
GM-CSF (pg/ml)	_____	↑*	↑**	↑***	↑****

Table 3-6: The effect of different levels of Deoxycholic acid on the BEAS-2B cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

ELISA analysis detected IL-8, IL-6 and GM-CSF in conditioned medium following CA stimulation of BEAS-2B. IL-8 levels increased significantly with CA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 30 $\mu\text{mol/L}$. The highest level of IL-8 secretion was with 100 $\mu\text{mol/L}$ CA, 243 ± 6 pg/ml, compared with the control, 51 ± 3 pg/ml.

IL-6 levels were significantly elevated by all CA concentrations except 15 $\mu\text{mol/L}$. The highest level of IL-6 secretion was with 100 $\mu\text{mol/L}$ CA, 277 ± 3 pg/ml, compared with the control, 18 ± 2 pg/ml.

Furthermore, the GM-CSF levels increased significantly at 30 $\mu\text{mol/L}$ CA and increased further to the maximum level at 75 $\mu\text{mol/L}$ CA of 326 ± 2 pg/ml compared to the control 29 ± 3 pg/ml. The level of GM-CSF production then decreased at 100 $\mu\text{mol/L}$ CA while remaining significantly higher than the control. However, no significant increase was observed using a concentration of 15 $\mu\text{mol/L}$ CA (Figure 3-12 and Table 3-7).

BEAS-2B cells

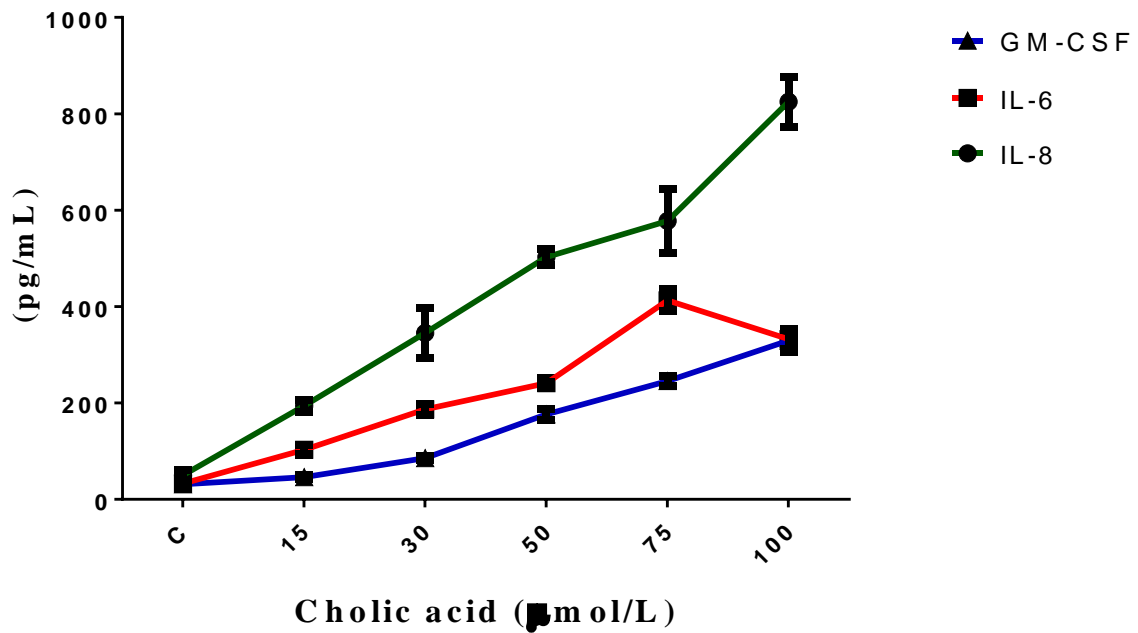


Figure 3-12: Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with CA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-7). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Cholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	_____	_____	↑**	↑**	↑**
IL-6 (pg/ml)	_____	↑**	↑***	↑*****	↑*****
GM-CSF (pg/ml)	_____	↑***	↑*****	↑*****	↑*****

Table 3-7: The effect of different levels of Cholic acid on the BEAS-2B cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Concentrations from 15 to 100 $\mu\text{mol/l}$ CDCA were tested. IL-8 levels increased significantly, but this was not observed at concentration of 15 $\mu\text{mol/L}$ CDCA. IL-8 levels increased significantly at 30 $\mu\text{mol/L}$ CDCA to 373 ± 6 pg/mL and increased to the maximum level at 50 $\mu\text{mol/L}$ CDCA of 759 ± 12 pg/mL compared to the control, 56 ± 9 pg/mL. The level of IL-8 production then fell off at 100 $\mu\text{mol/L}$ CDCA but was still significantly higher than the control.

IL-6 levels were increased with CDCA at concentrations of 30, 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 $\mu\text{mol/L}$. However, the maximum output of IL-6 occurred at 100 $\mu\text{mol/L}$ CDCA to 272 ± 3 pg/mL compared with the control 27 ± 3 pg/mL.

The GM-CSF was detected by ELISA following CDCA stimulation of BEAS-2B cells. The CDCA significantly upregulates GM-CSF production at 30, 50, 75 and 100 $\mu\text{mol/L}$. GM-CSF production increased significantly at 30 $\mu\text{mol/L}$ CDCA to 50 ± 4 pg/mL and increased further to the maximum level at 50 $\mu\text{mol/L}$ CDCA of 234 ± 7 pg/mL compared to the control, 27 ± 3 pg/mL. The level of GM-CSF production then decreased at 100 $\mu\text{mol/L}$ CDCA but was still significantly higher than the control (Figure 3-13 and Table 3-8).

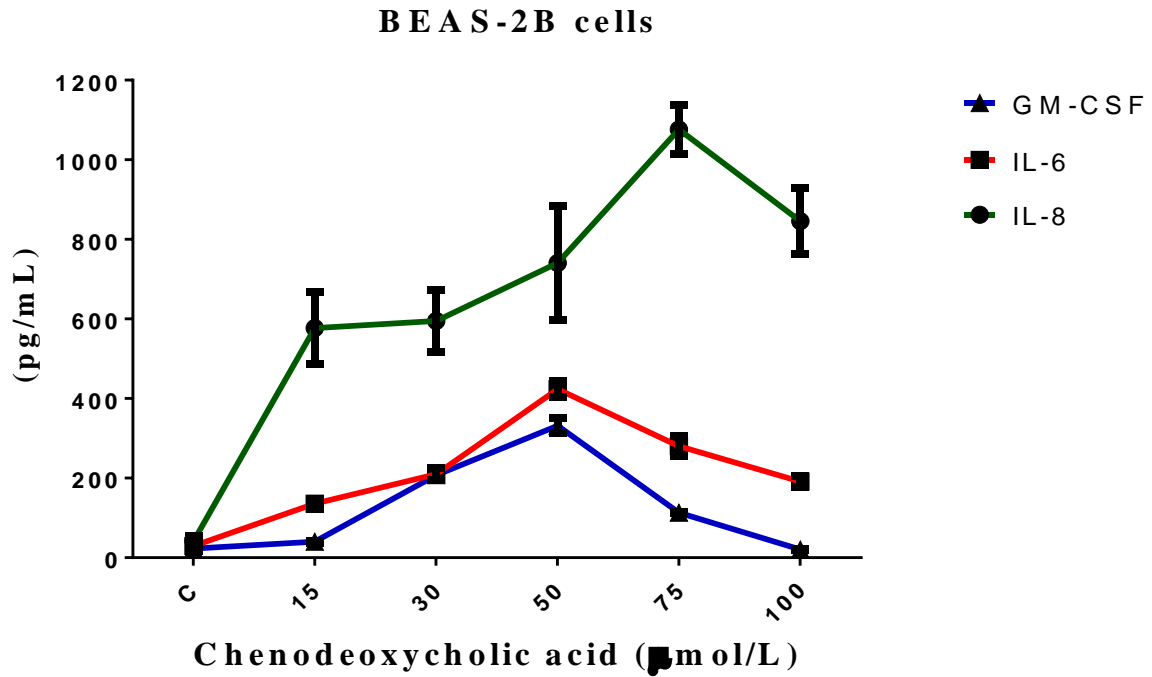


Figure 3-13: Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with CDCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-8). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Chenodeoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	_____	↑***	↑****	↑****	↑***
IL-6 (pg/ml)	_____	↑**	↑***	↑***	↑***
GM-CSF (pg/ml)	_____	↑*	↑***	↑**	↑*

Table 3-8: The effect of different levels of Chenodeoxycholic acid on the BEAS-2B cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

3.4.4 Influence of individual BAs on NCI-H292 cell viability and inflammatory (IL-8, IL-6 and GM-CSF) release

Cultured NCL-H292 in complete medium (RPMI) in 24-well cell plates, were stimulated with both primary and secondary BAs at a range of concentrations. After 48h, cell viability was calculated by counting, using the CellTiter-Blue Cell Viability and MTT Assays.

The results revealed that cell viability reduced significantly in response to exposure to 10 $\mu\text{mol/L}$ LCA with $93\pm 43\%$ viability and the cell viability then decreased at 15 $\mu\text{mol/L}$ LCA with $85\pm 4\%$. This decreased further to the lowest cell viability level at 20 $\mu\text{mol/L}$ LCA with $14\pm 6\%$. LCA at 1 to 5 $\mu\text{mol/L}$ did not cause any cell death (Figure 3-14A).

Furthermore, cell viability reduced significantly in response to exposure to 30 $\mu\text{mol/L}$ DCA with $90\pm 3\%$ and the cell viability then decreased with 75 $\mu\text{mol/L}$ DCA to $50\pm 5\%$. This decreased further to the lowest cell viability level at 100 $\mu\text{mol/L}$ DCA ($13\pm 3\%$) (Figure 3-14B).

CA and CDCA at 15 to 100 $\mu\text{mol/L}$ did not cause any cell death, which suggests that secondary BAs have greater cytotoxicity than primary BAs. Both the CellTiter-Blue and MTT assays were used to calculate viability for two plates. The results analysis found no significant difference between the two assessment methods (Figure 3-15 A & B).

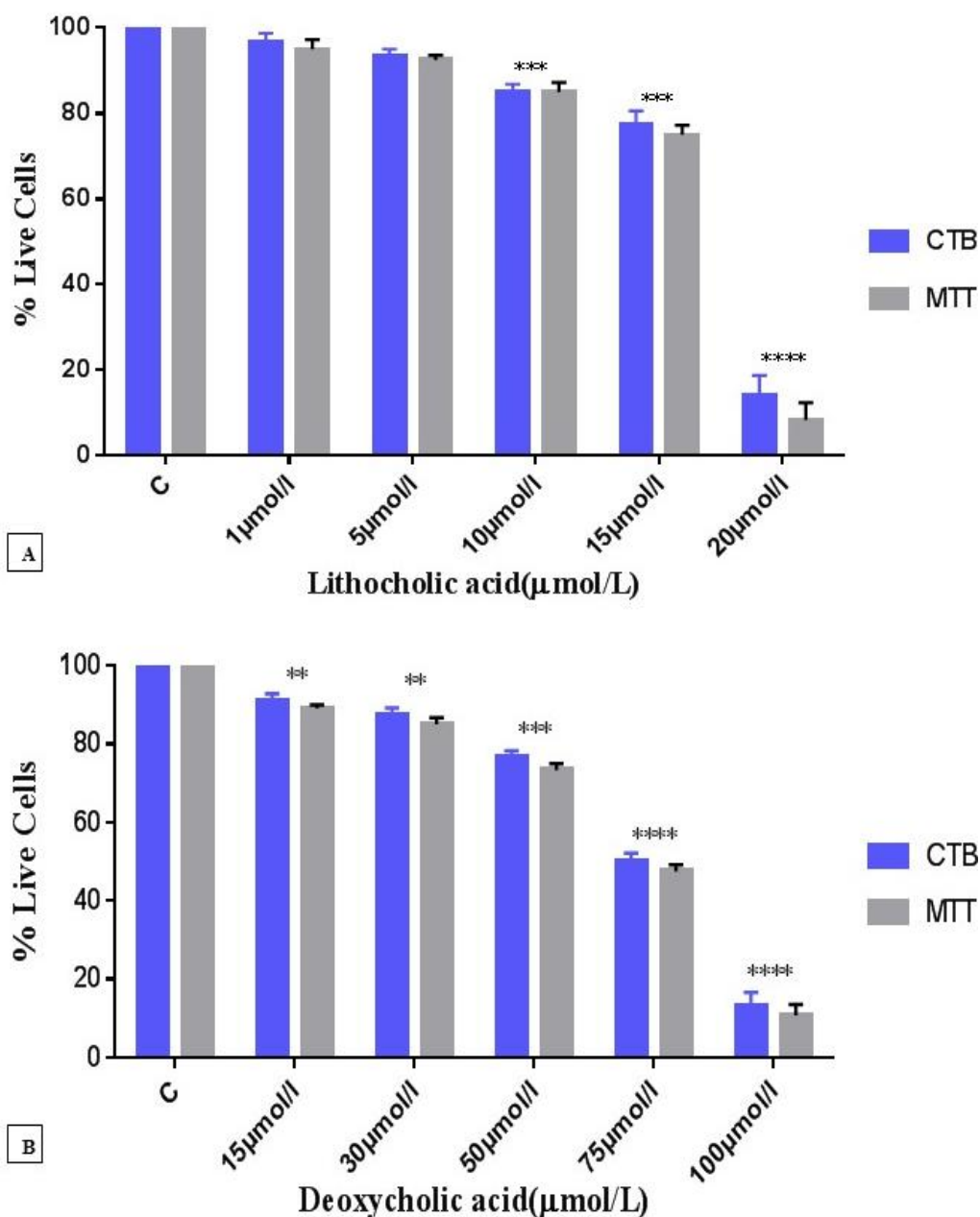
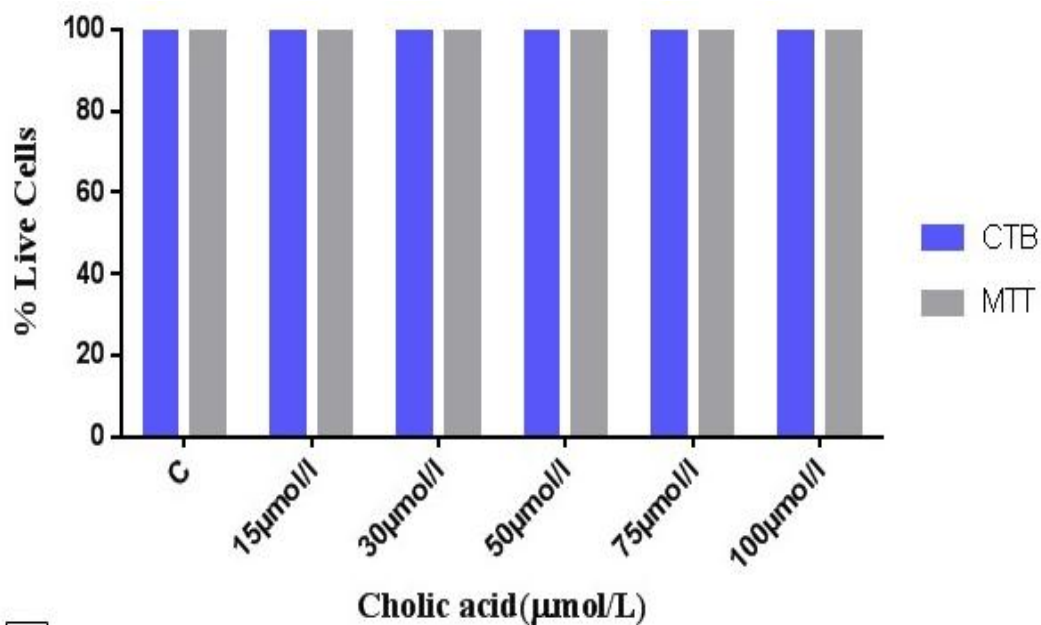
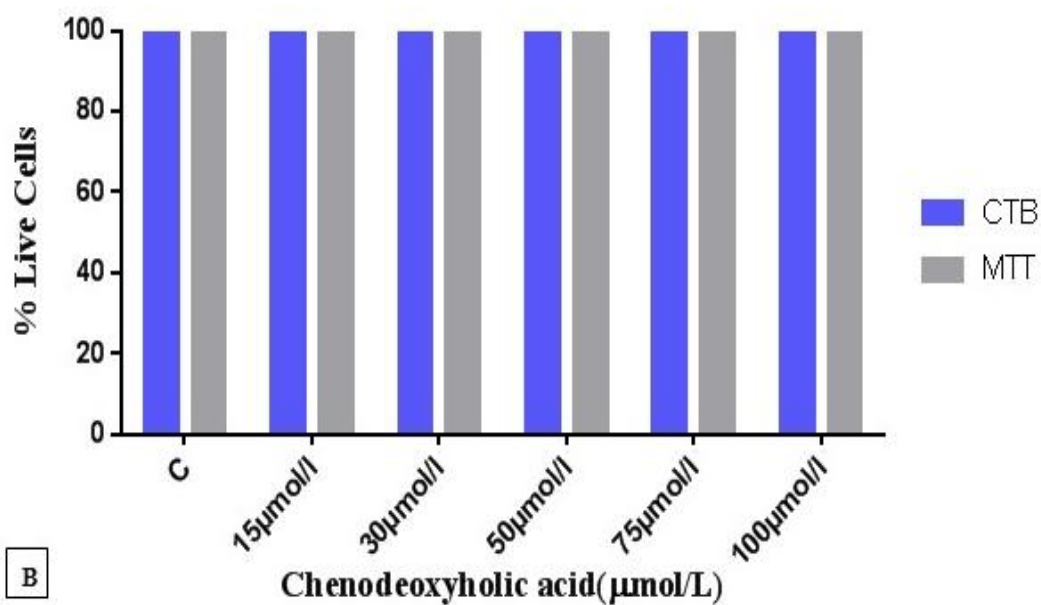


Figure 3-14: Effects of BAs on NCI-H292 cell proliferation. NCI-H292 cell viability following stimulation with secondary BAs (A) Lithocholic and (B) Deoxycholic acids, at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol prior to adding the CellTiter-Blue and MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.



A



B

Figure 3-15: Effects of BAs on NCI-H292 cell proliferation. NCI-H292 cell viability following stimulation with primary BAs (A) Cholic and (B) Chenodeoxycholic acids at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue and MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.

IL-8, IL-6 and GM-CSF production by the NCL-H292 cell line lung carcinoma was also analysed following stimulation by BAs. NCI-H292 cells were cultured and stimulated with primary BAs (CA and CDCA) and the secondary BAs (DCA) at a range of concentrations from 15 to 100 μ mol/L for 48 hours. LCA at 1 to 20 μ mol/L was also used and the results compared with the control.

At concentrations of 1, 5, 10, 15 and 20 μ mol/L LCA significantly increased IL-8 levels. However, the highest level was 690 \pm 24 pg/mL with 1 μ mol/L LCA compared with the control, 61 \pm 4 pg/mL. The level subsequently fell to 107 \pm 3 pg/mL with 20 μ mol/L LCA.

A significant increase was observed in IL-6 levels with LCA at concentrations of 5, 10 and 15 μ mol/L, 20 μ mol/L LCA did not significantly increase IL-6 output due to cell death. The result revealed that the highest level of IL-6 production was at 15 μ mol/L LCA to 304 \pm 2 pg/mL compared with the control, 17 \pm 2 pg/mL.

GM-CSF production by NCL-H292 cells increased significantly following stimulation with LCA at 1, 5, 10 and 15 μ mol/L, but this was not observed at 20 μ mol/L LCA due to cell death. The highest level of GM-CSF secretion was at 5 μ mol/L LCA with 327 \pm 2 pg/mL compared with the control, 18 \pm 2 pg/mL (Figure 3-16 and Table 3-9).

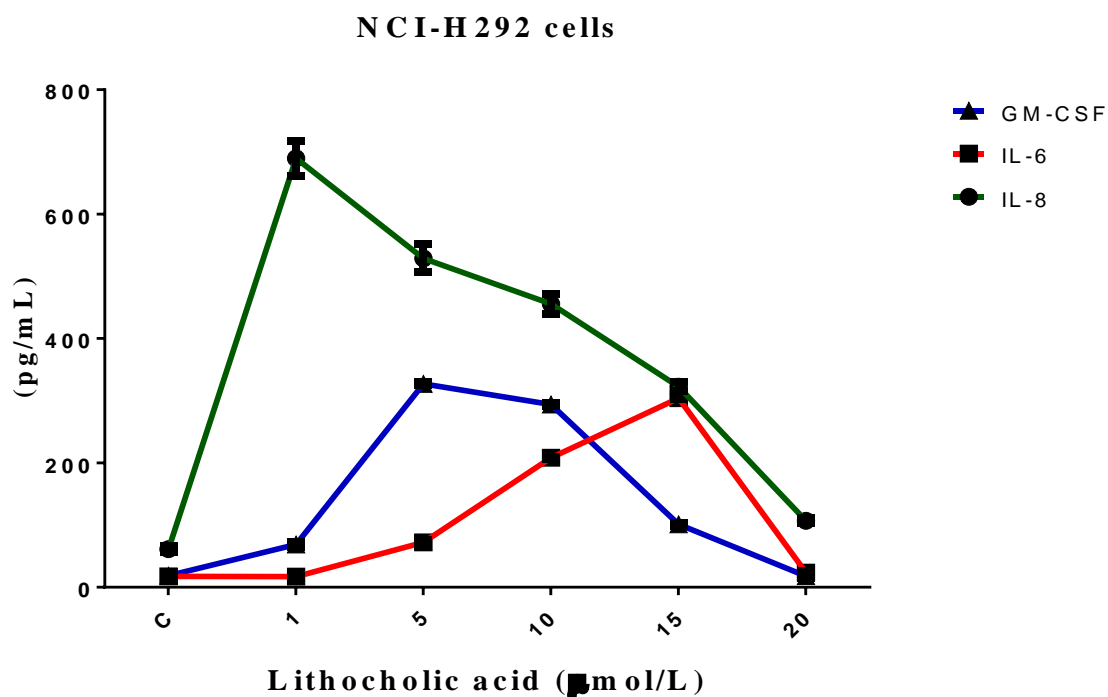


Figure 3-16: Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-9). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	1	5	10	15	20
IL-8 (pg/ml)	↑****	↑****	↑****	↑****	↑**
IL-6 (pg/ml)	_____	↑**	↑****	↑****	_____
GM-CSF (pg/ml)	↑*	↑****	↑****	↑*	_____

Table 3-9: The effect of different levels of Lithocholic acid on the NCL-H292 cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

IL-8, IL-6 and GM-CSF production by the NCL-H292 cell line lung carcinoma was also analysed following stimulation by DCA.

DCA at concentrations of 15, 30, 50, and 75 $\mu\text{mol/L}$ significantly increased IL-8 levels. The highest level of IL-8 secretion was at 1 $\mu\text{mol/L}$ DCA with $670\pm 23\%$ compared with the control, $32\pm 4\%$. The IL-8 levels then decreased to a level not significantly different from control at 100 μmol DCA.

The IL-6 levels at 30, 50 and 75 $\mu\text{mol/L}$ DCA increased significantly, but 15 and 100 $\mu\text{mol/L}$ DCA did not. The highest level of IL-6 production was at 218 ± 3 pg/mL with 75 $\mu\text{mol/L}$ DCA compared with the control (19 ± 2 pg/mL).

GM-CSF levels increased significantly at 30 $\mu\text{mol/L}$ DCA with 251 ± 3 pg/mL and increased to the maximum level at 50 $\mu\text{mol/L}$ DCA with 439 ± 8 pg/mL . The level of GM-CSF production then fell off at 75 $\mu\text{mol/L}$ DCA with 132 ± 2 pg/mL but remained significantly higher than the control 24 ± 3 pg/mL . DCA at concentration 15 and 100 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF levels (Figure 3-17 and Table 3-10).

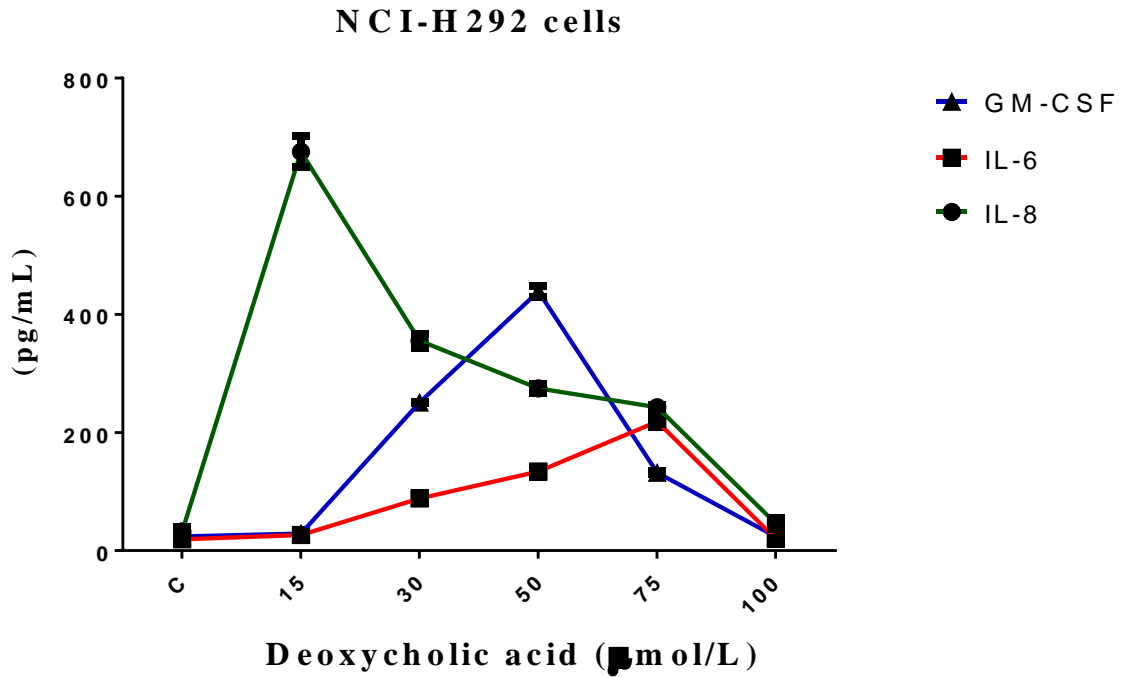


Figure 3-17: Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with DCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA. The data are representative of three independent experiments (n=6) (see table 3-10). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Deoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑****	↑****	b↑****	↑***	_____
IL-6 (pg/ml)	_____	↑***	↑***	↑****	_____
GM-CSF (pg/ml)	_____	↑***	↑****	↑****	_____

Table 3-10: The effect of different levels of Deoxycholic acid on the NCI-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

IL-8, IL-6 and GM-CSF production by the NCL-H292 cell line lung carcinoma was also analysed following stimulation by CA.

CA at concentrations of 15, 30 and 50 $\mu\text{mol/L}$ significantly increased IL-8 levels. The highest level of IL-8 secretion was at 15 $\mu\text{mol/L}$ CA at with $516 \pm 2 \text{ pg/mL}$ compared to the control, $37 \pm 3 \text{ pg/mL}$.

IL-6 levels at 50, 75 and 100 $\mu\text{mol/L}$ CA increased significantly, but 15 and 30 $\mu\text{mol/L}$ CA did not increase it. The highest level of IL-6 release occurred with 100 $\mu\text{mol/L}$ CA, to $124 \pm 2 \text{ pg/mL}$ compared with the control ($21 \pm 2 \text{ pg/mL}$).

All CA concentrations significantly elevated GM-CSF levels except 15 $\mu\text{mol/L}$. The highest level of GM-CSF secretion was with 100 $\mu\text{mol/L}$ CA to $429 \pm 3 \text{ pg/mL}$ compared with the control, $21 \pm 2 \text{ pg/mL}$ (Figure 3-18 and Table 3-11).

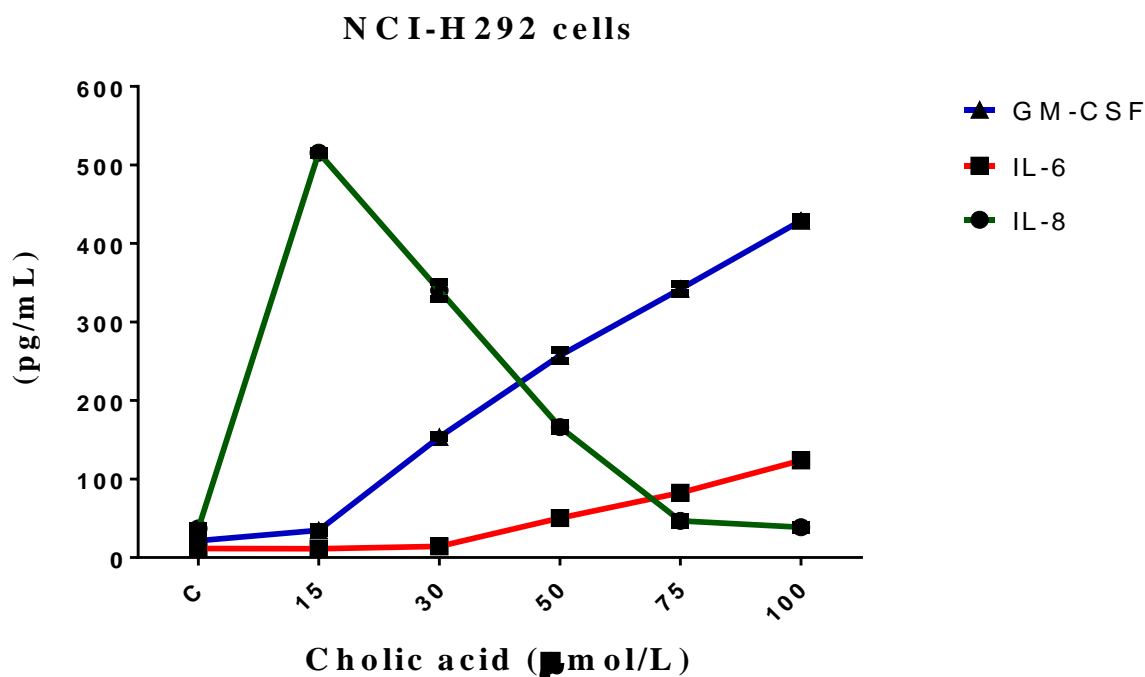


Figure 3-18: Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with CA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-11). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Cholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑***	↑***	↑**	_____	_____
IL-6 (pg/ml)	_____	_____	↑***	↑***	↑****
GM-CSF (pg/ml)	_____	↑***	↑***	↑****	↑****

Table 3-11: The effect of different levels of Cholic acid on the NCL-H292 cell line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

IL-8, IL-6 and GM-CSF production by the NCI-H292 cell line was analysed following stimulation by CDCA. NCI-H292 cells were cultured and stimulated with CDCA.

IL-8 levels increased by a significant amount when stimulated with 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CDCA. IL-8 levels increased significantly at 15 $\mu\text{mol/L}$ CDCA to 433 ± 21 pg/mL compared with the control, 41 ± 5 pg/mL and increased to the maximum level at 30 $\mu\text{mol/L}$ CDCA to 508 ± 17 pg/mL.

Moreover, IL-6 levels significantly increased following stimulation by 30 $\mu\text{mol/L}$ or above of CDCA. The result revealed that the highest level of IL-6 production was at 100 $\mu\text{mol/L}$ CDCA with 265 ± 3 pg/mL compared with the control (33 ± 3 pg/mL).

The GM-CSF levels increased significantly with CDCA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 30 $\mu\text{mol/L}$. However, the highest level of GM-CSF secretion with 100 $\mu\text{mol/L}$ CDCA to 319 ± 9 pg/mL compared with the control 13 ± 2 pg/mL (Figure 3-19 and Table 3-12).

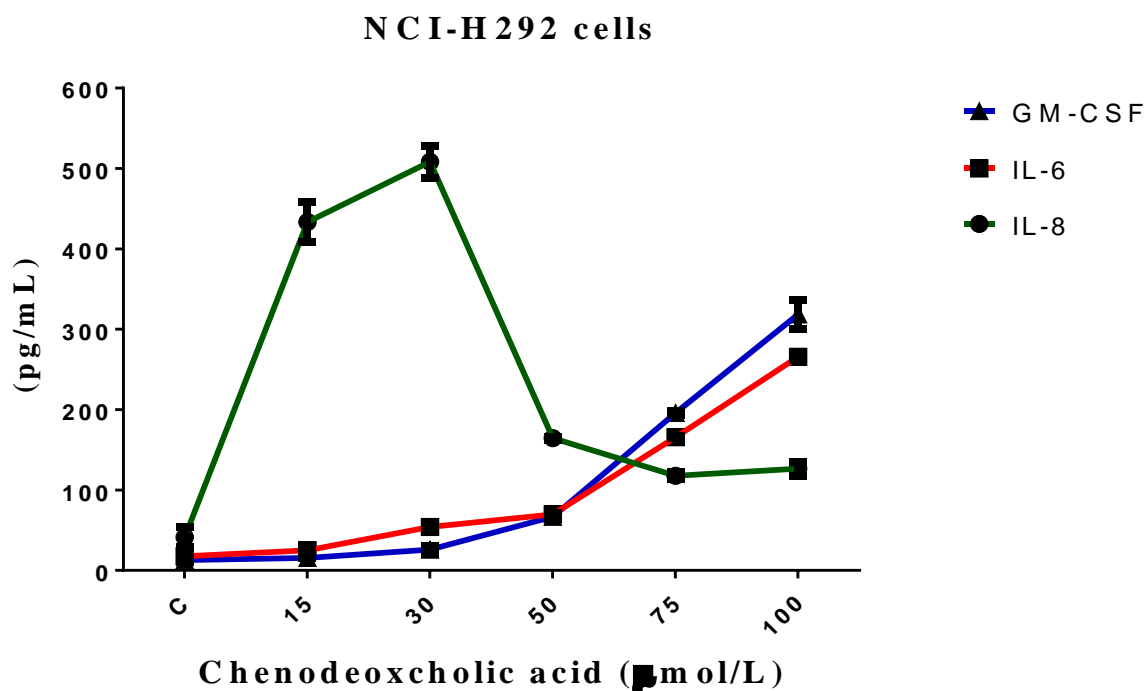


Figure 3-19: Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with CDCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-12). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Chenodeoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑****	↑****	↑***	↑**	↑**
IL-6 (pg/ml)	_____	↑**	↑**	↑****	↑****
GM-CSF (pg/ml)	_____	_____	↑***	↑****	↑****

Table 3-12: The effect of different levels of Chenodeoxycholic acid on the NCI-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

3.4.5 Influence of individual bile acids on Calu-3 cell viability and inflammatory (IL-8, IL-6 and GM-CSF) release

Calu-3 cells were exposed to LCA, DCA, CA and CDCA in concentrations ranging from 15 to 100 $\mu\text{mol/l}$ for 48 hrs. Their viability was then assessed using CellTiter-Blue and MTT assays. The results revealed no significant difference between the two methods of assessment.

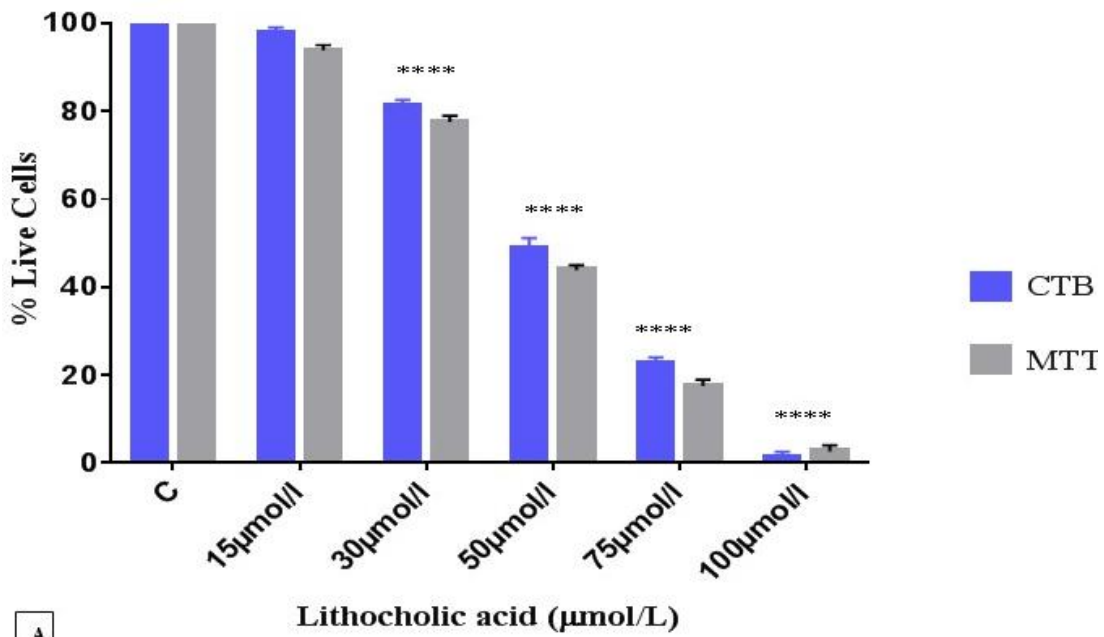
The cell viability was found to be affected depending on the BAs concentration. There was an inverse relationship between BAs concentration and measured cell viability, with increasing concentration and decreasing cell viability in a dose-dependent manner.

The results revealed a significant reduction in cell viability in response to 30 $\mu\text{mol/L}$ of LCA with $81 \pm 3\%$. Subsequently, the cell viability decreased with 75 $\mu\text{mol/L}$ LCA to $23 \pm 3\%$. This fell further to the lowest cell viability level at 100 $\mu\text{mol/L}$ LCA with $2 \pm 2\%$ (Figure 3-20A).

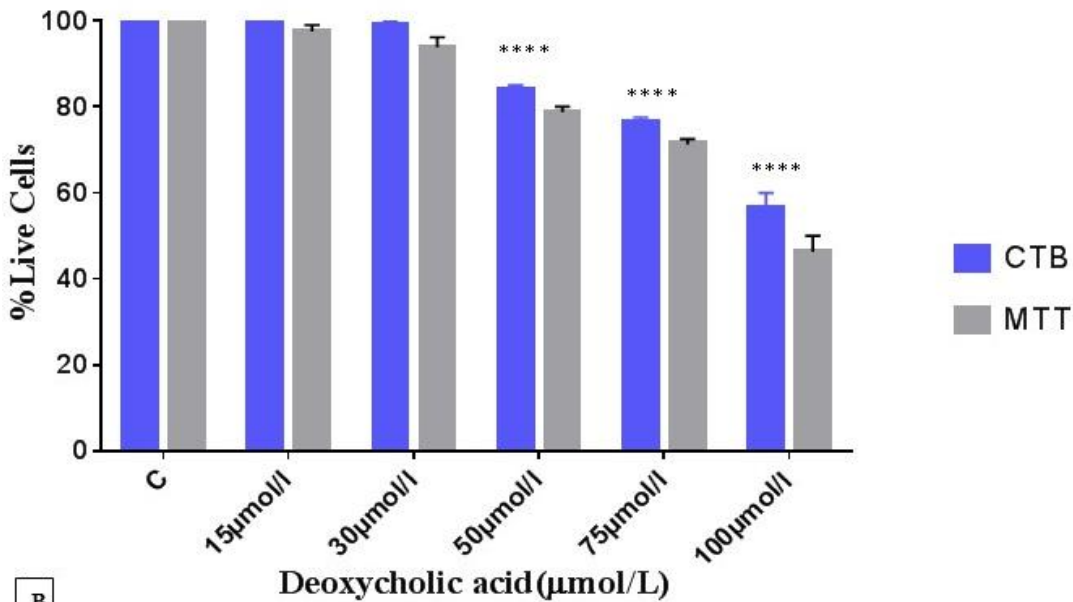
Cell viability was reduced significantly by DCA at concentration 50 $\mu\text{mol/l}$ ($84 \pm 2\%$). However, cell viability was not affected by 15 and 30 $\mu\text{mol/l}$ DCA (Figure 3-20 B).

Furthermore, cell viability reduced significantly in response to exposure to 50 $\mu\text{mol/L}$ CA with $93 \pm 3\%$. This decreased further to the lowest cell viability level at 100 $\mu\text{mol/L}$ CA ($73 \pm 3\%$). The lowest concentration (15 and 30 $\mu\text{mol/L}$) of CA did not affect cell viability (Figure 3-21A).

Cell viability was also decreased significantly by CDCA at concentration 50 $\mu\text{mol/l}$ with $87 \pm 3\%$. The cell viability then reduced by 100 $\mu\text{mol/L}$ CDCA with $65 \pm 6\%$. No difference was found in the cell viability at lowest CDCA concentrations, 15 and 30 $\mu\text{mol/L}$, compared with the control (Figure 3-21 B).



A



B

Figure 3-20: Effects of BAs on Calu-3 cell proliferation. Calu-3 cell viability following stimulation with secondary BAs (A) Lithocholic (B) Deoxycholic acids, at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M.. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the Titer-Blue or MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.

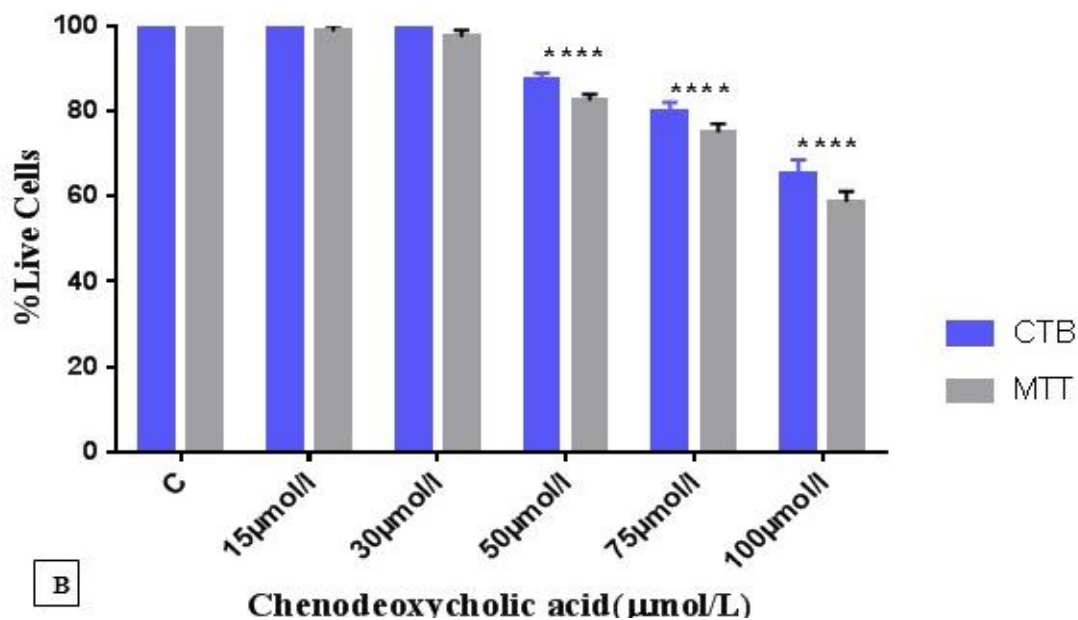
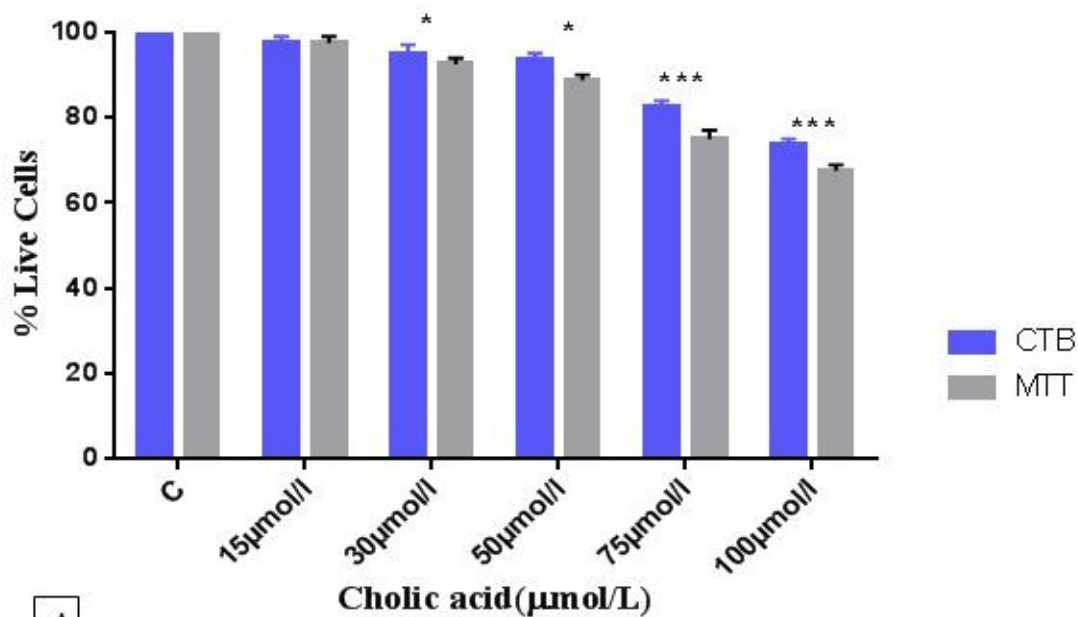


Figure 3-21: Effects of BAs on Calu-3 cell proliferation. Calu-3 cell viability following stimulation with primary BAs (A) Cholic (B) Chenodeoxycholic acids, at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M.. Statistical analysis was performed to test the effect of BAs compared with control. P values of 0.05 or less were regarded as significant. Negative controls were measured by fixing the cells in methanol before adding the Titer-Blue or MTT reagent. The data are representative of three independent experiments (n=6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate levels of significant change in viability comparing cells after the treatment compared to control, ANOVA.

Calu-3 cells produced significantly higher levels of IL-8 following LCA stimulation at concentrations of 15, 30, 50 and 75 μmol . The IL-8 levels increased significantly at 15 $\mu\text{mol/L}$ LCA with 571 ± 13 pg/mL and increased further to the maximum level at 50 $\mu\text{mol/L}$ LCA with 1289 ± 2 pg/mL. The level of IL-8 production then fell off at 75 $\mu\text{mol/L}$ LCA with 318 ± 9 pg/mL but remained significantly higher than the control 41 ± 5 pg/mL. LCA at concentration 100 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF levels.

There was significant increase in IL-6 levels with LCA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 30 $\mu\text{mol/L}$. The result revealed that the highest level of IL-6 production was at 50 $\mu\text{mol/L}$ LCA to 206 ± 2 pg/mL compared with control 12 ± 2 pg/mL.

GM-CSF production by Calu-3 cells increased significantly after stimulation with LCA at 15, 30, 50 and 75 $\mu\text{mol/L}$, but this was not observed at 100 $\mu\text{mol/L}$ LCA due to cell death. The highest level of GM-CSF secretion was at 50 $\mu\text{mol/L}$ LCA with 632 ± 12 pg/mL compared with the control 14 ± 3 pg/mL (Figure 3-22 and Table 3-13).

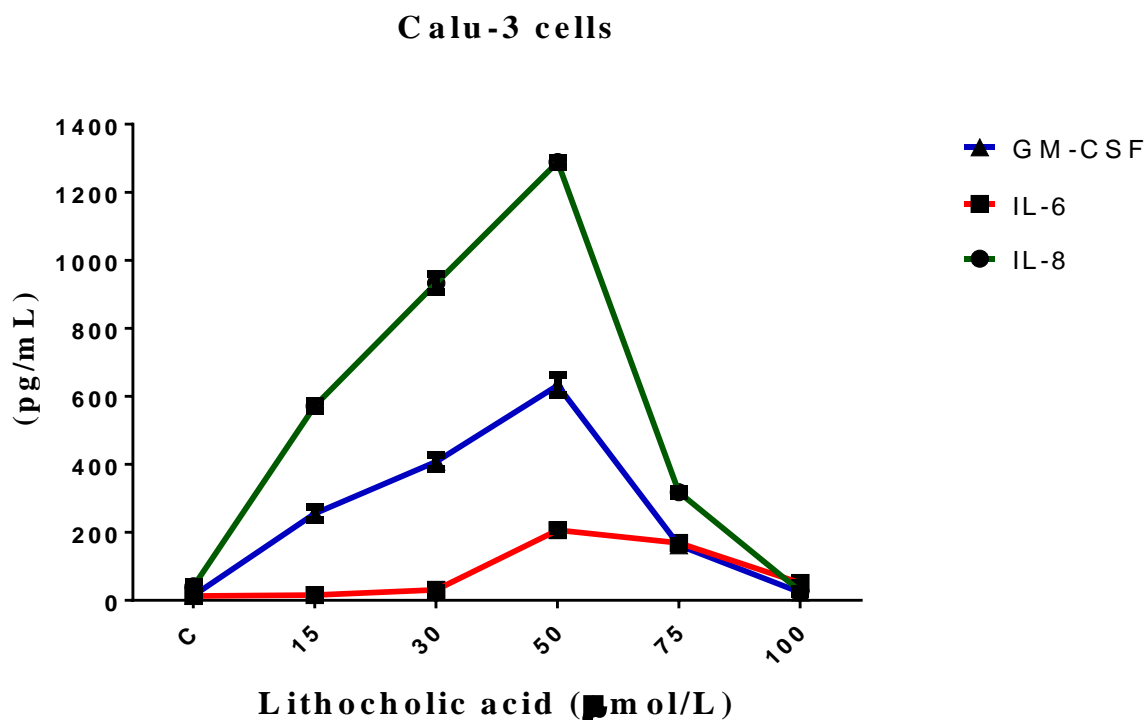


Figure 3-22: Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-13). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑***	↑***	↑*****	↑**	_____
IL-6 (pg/ml)	_____	_____	↑*****	↑*****	↑***
GM-CSF (pg/ml)	↑***	↑***	↑*****	↑**	_____

Table 3-13: The effect of different levels of Lithocholic acid on the Calu-3 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

DCA stimulation of cultured Calu-3 at concentrations from 15 to 100 $\mu\text{mol/L}$ for 48 hours was compared with controls without DCA. The IL-8 levels increased significantly at 15 $\mu\text{mol/L}$ to 388 ± 24 pg/mL and rose further to the highest level of 75 $\mu\text{mol/L}$ to 1199 ± 22 pg/mL compared to the control (41 ± 12 pg/mL). The level of IL-8 production then decreased to 100 $\mu\text{mol/L}$ but remained significantly higher than the control.

IL-6 levels significantly increased following stimulation by 30 $\mu\text{mol/L}$ or above of DCA. The result revealed that the highest level of IL-6 production was at 100 $\mu\text{mol/L}$ with 218 ± 8 pg/mL compared with control 6 ± 2 pg/mL. DCA at concentration 15 $\mu\text{mol/L}$ did not cause a significant increase in GM-CSF levels.

Furthermore, the GM-CSF levels increased significantly at 30 $\mu\text{mol/L}$ of DCA and increased further to the maximum level at 75 $\mu\text{mol/L}$ of 680 ± 22 pg/mL compared with the control 12 ± 2 pg/mL. The level of GM-CSF production then decreased at 100 $\mu\text{mol/L}$ DCA but remained significantly higher than the control. However, no significant increase was observed using concentrations of 15 $\mu\text{mol/L}$ DCA (Figure 3-23 and Table 3-14).

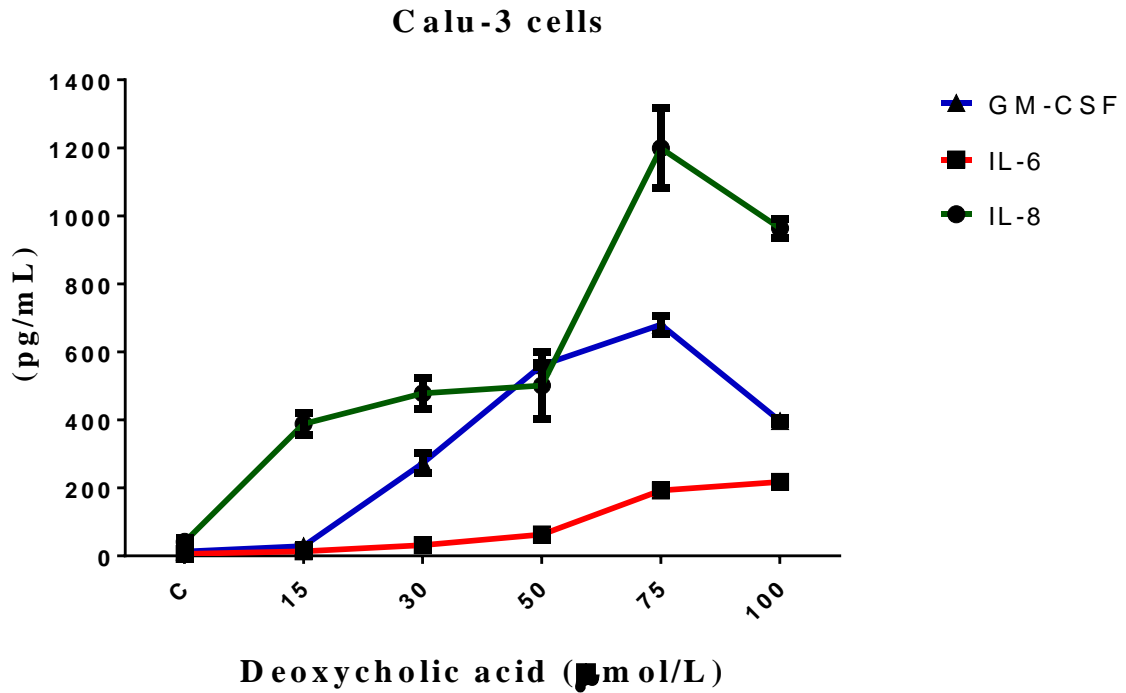


Figure 3-23: Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with DCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA. The data are representative of three independent experiments (n=6) (see table 3-14). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Deoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	\uparrow^{***}	\uparrow^{***}	\uparrow^{***}	\uparrow^{*****}	\uparrow^{*****}
IL-6 (pg/ml)	=====	\uparrow^{**}	\uparrow^{***}	\uparrow^{*****}	\uparrow^{*****}
GM-CSF (pg/ml)	=====	\uparrow^{***}	\uparrow^{***}	\uparrow^{*****}	\uparrow^{***}

Table 3-14: The effect of different levels of Deoxycholic acid on the Calu-3 cell line following stimulation for 48 hours. \uparrow Increase demonstrated. * Significant stimulation of cytokine release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Stimulation of Calu-3 cells with CA at concentrations of 15, 30, 50, 75, and 100 $\mu\text{mol/L}$ for 48 hours caused a significant increase in IL-8. The IL-8 levels increased significantly at 15 $\mu\text{mol/L}$ to 380 ± 21 pg/mL and increased further to the highest level of 75 $\mu\text{mol/L}$ to 1146 ± 11 pg/mL compared with the control (41 ± 12 pg/mL). The level of IL-8 production then decreased to 100 $\mu\text{mol/L}$ but remained significantly higher than the control.

The IL-6 levels increased significantly at 15 $\mu\text{mol/L}$ CA with 275 ± 4 pg/mL and increased further to the maximum level at 30 $\mu\text{mol/L}$ CA with 422 ± 11 pg/mL. The level of IL-6 production subsequently decreased at 75 $\mu\text{mol/L}$ CA with 91 ± 2 pg/mL but remained significantly higher than the control 17 ± 3 pg/mL. CA at concentration 100 $\mu\text{mol/L}$ did not cause a significant increase in IL-6 levels.

GM-CSF levels increased significantly with CA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 30 $\mu\text{mol/L}$. The highest level of GM-CSF secretion with 100 $\mu\text{mol/L}$ CA to 165 ± 9 pg/mL compared with the control, 31 ± 2 pg/mL (Figure 3-24 and Table 3-15).

Calu-3 cells

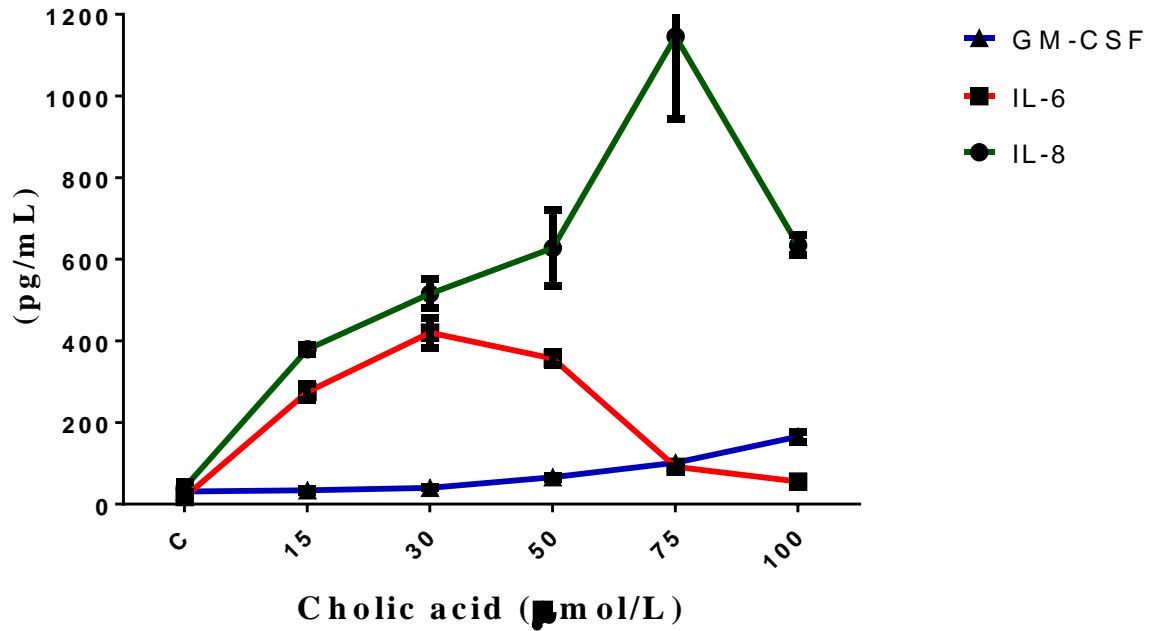


Figure 3-24: Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with CA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-15). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Cholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑**	↑***	↑***	↑*****	↑***
IL-6 (pg/ml)	↑***	↑*****	↑*****	↑**	_____
GM-CSF (pg/ml)	_____	_____	↑***	↑*****	↑*****

Table 3-15: The effect of different levels of Cholic acid on the Calu-3 cell line following stimulation for 48 hours, ↑ Increase demonstrated * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Stimulation of Calu-3 cells with CDCA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused a significant increase in IL-8. The IL-8 levels increased significantly at 15 $\mu\text{mol/L}$ CDCA with 544 ± 7 pg/mL and increased to the maximum level at 75 $\mu\text{mol/L}$ CDCA with 1363 ± 20 pg/mL. The level of IL-8 production subsequently decreased at 100 $\mu\text{mol/L}$ CDCA with 852 ± 23 pg/mL but remained significantly higher than the control at 39 ± 5 pg/mL.

Figure 25 illustrates the levels of IL-6 released from Calu-3 cells after stimulation by CDCA for 48 hours. The findings showed a significant increase in IL-6 at 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CDCA. The highest level of IL-6 production was at 50 $\mu\text{mol/L}$ CDCA with 508 ± 7 pg/mL compared with control (5 ± 2 pg/mL) and then declined as the concentration of CDCA increased up to 100 $\mu\text{mol/L}$.

The GM-CSF production by Calu-3 cells increased significantly following stimulation with CDCA at 15, 30, 50, 75 and 100 $\mu\text{mol/L}$. The highest level of GM-CSF secretion was at 75 $\mu\text{mol/L}$ CDCA with 750 ± 3 pg/mL compared with the control, 13 ± 3 pg/mL (Figure 3-25 and Table 3-16).

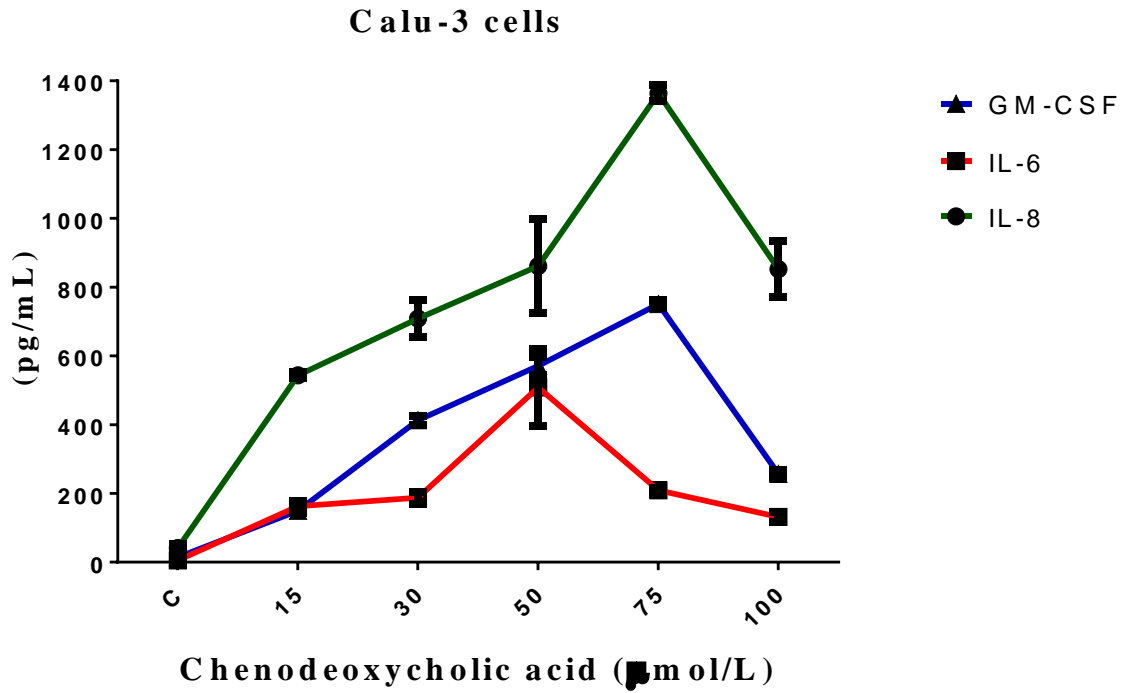


Figure 3-25: Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with CDCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA the data are representative of three independent experiments (n=6) (see table 3-16). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Chenodeoxycholic acid				
	15	30	50	75	100
IL-8 (pg/ml)	↑***	↑***	↑****	↑****	↑***
IL-6 (pg/ml)	↑**	↑**	↑****	↑***	↑**
GM-CSF (pg/ml)	↑***	↑***	↑****	↑****	↑***

Table 3-16: The effect of different levels of Chenodeoxycholic acid on the Calu-3 cell line following stimulation for 48 hours, ↑ Increase demonstrated * Significant stimulation of cytokines release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

3.5 Discussion

In this present age, lung disease is still a major cause of the world's mortality and morbidity, and it is on the rise. For example, COPD is the fifth largest cause of death worldwide, while interstitial lung disease (ILD) and cystic fibrosis (CF) are common and lead to significant consequences for the individuals who have them: this leads to a low quality of life and even death. All the above mentioned diseases are contributed by either chronic or acute inflammation, which results in using anti-inflammatory medication, such as steroids (Rincon and Irvin, 2012).

The association between lung disease and GORD has been suspected for a long time (Pearson and Wilson, 1971), and it is known that high incidences of GORD impact those suffering from asthma, idiopathic pulmonary fibrosis, COPD and CF (Feigelson *et al.*, 1987; Tobin *et al.*, 1998). Moreover, reflux is commonly discovered among recipients of a lung transplant, and the pathogenesis of bronchiolitis obliterans syndrome (BOS) (Stovold *et al.*, 2007) has also been associated to microaspiration of refluxate.

Furthermore, it has been identified that refluxate from GORD patients contains levels of BA at a millimolar level (Lefebvre *et al.*, 2009). Moreover, BAs may be contained in duodeno-gastro-oesophageal refluxate, which has a wide range of biological consequences (Lefebvre *et al.*, 2009).

Of the bronchoalveolar lavage (BAL) samples from individuals with CF who had undergone the lung transplantation procedure, 60% were found by Blondeau *et al.* to contain bile acids. Moreover, a series of other studies from the Leuven group points towards a potential role for the aspiration of BAs in the pathophysiology of BOS (Blondeau *et al.*, 2008a).

In advanced CF lung disease, Brodlie *et al.* demonstrated recently that BAs measured using tandem mass spectrometry can be traced in the lower airway. Moreover, following successful lung transplantation (Brodlie *et al.*, 2015), this potential source of injury continues.

In respiratory infection and inflammation, the role of BAs is undergoing a broader investigation, although researchers still poorly understand the underlying mechanisms. Within the body, the majority of BAs exist in their conjugated forms (Hofmann, 1999b).

A study was conducted by McDonnell et al that assessed the inflammatory and cytotoxic effects of primary (chenodeoxycholic and cholic) as well as secondary (deoxycholic and lithocholic) BAs at 0, 12.5, 25, 50 and 100 $\mu\text{mol/L}$ on human bronchial epithelial cells (HBEs), in addition to and independent of the injury induced by gastric acid, within an experimental model of duodeno-gastro-oesophageal aspiration. Furthermore, IL-8 was measured by ELISA in culture supernatants, wherein the viability of the cells was analysed using an MTT assay. It was also found that the exposure to BA reduced the viability of the cells in a concentration-dependent manner. BA-prompted IL-8 release was greater with higher combined concentrations and at lower acidic levels up to the point of cell death. Given these results, this model suggests that the BA cytotoxicity and inflammation within the pulmonary epithelium are both affected by acidity, in addition to being dependent on the concentration level (O'Toole et al., 2015).

The purpose of this chapter was to establish how the presence of BAs impacted the production of pro-inflammatory cytokines by airway epithelial cells. This was achieved by analysing the changes in the levels of IL-8, IL-6 and GM-CSF produced by epithelial cells.

Four cell lines were selected in the present study as lung tissue models: BEAS-2B, Calu-3, NCI-H292 and 16HBE14o- cells. In this case, the main finding was that cells confronted with the presence of BAs at 1, 5, 10, 15, 20, 30, 50, 75 and 100 $\mu\text{mol/L}$ released IL-8, IL-6 and GM-CSF throughout a 48-hour period, but at varying levels.

It has been reported that BAs lose their toxicity as a result of conjugation (Debruyne *et al.*, 2002; Blau *et al.*, 2007), although most of the BAs in humans already exist in their conjugated form (Hofmann, 1999b). Proton pump inhibitors and acid suppression therapies, used in the treatment of GORD, can cause these conjugated BAs to deconjugate (Theisen *et al.*, 2000), resulting in higher levels of deconjugated BAs in GORD patients undergoing such interventions (Theisen *et al.*, 2000). It has been suggested that therapies which suppress stomach acids result in increased bacterial growth in the stomach, causing BAs to deconjugate (Dettmar *et al.*, 2011).

Furthermore, BAs such as DCA, CDCA and LCA detected in airway secretions have all been found to be unconjugated (Wu *et al.*, 2009), which is significant to GORD patients. This study, therefore, sought to investigate the effects of gastric refluxate on the airway by using BA concentrations similar to the physiological bile acid content found in the gastric refluxate that reaches the patient's airway (BA concentrations ranging from 1 $\mu\text{mol/l}$ to 100 $\mu\text{mol/l}$).

Existing knowledge around the effects of BAs on airways is limited. Up to now, research has tended to focus on BAL studies, with less attention being given to investigating and quantifying specific BA concentrations in the lungs. This may be partly to do with the low reliability of acid assays and also difficulties associated with sampling procedures.

Previously, the highest concentration of BA reported in BAL was 240 μ mol/L (D'Ovidio *et al.*, 2005). BAL samples are diluted by 100-200-fold, meaning that the BA levels detected are significantly less than those found in patients' airways. In this study a range of between 1 - 100 μ mol/L for each individual bile acid was used, so are well within the physiological range expected in gastric refluxate.

According to the results: in NCI-H292, both LCA and DCA have an impact on cell viability. It was found that a 50 μ mol/L concentration of DCA acid caused cell death of 23 \pm 4%, and that 100 μ mol/L results in cell death of 88 \pm 4%. Further, it was also found that LCA at \geq 5 μ mol/L presents an impact on cell viability. Conversely, CDCA and CA at 15 to 100 μ mol/ml were not sufficient for cell death to occur; this would indicate that primary BAs have lower cytotoxicity than secondary BAs.

On the other hand, the observed difference in the effect of LCA between other cells and Calu-3 cells was made clear by this study. In BEAS-2B, NCI-H, and 16HBE14o- cells, the viability of the cells saw a reduction when exposed to concentrations from 5 to 20 μ mol/L, as lithocholate is normally a minor part of bile acid circulation (Wu *et al.*, 2012). Calu-3 cells were only impacted by LCA when the concentration was raised to 30 μ mol/L or higher.

It was further demonstrated by this study the NCI-H292 production of IL-8, GM-CSF and IL-6 became significantly affected when provoked by BAs. When confronted with BAs, significant levels of all three cytokines were released by the Calu-3 cells. Both Calu-3 and NCI-H292 cells derive from the lung tissue of humans and were initially considered as a useful model for inflammation within the airway. Carcinoma cells express an intercellular adhesion molecule-1 (Carney *et al.*, 1985), and secrete IL-8 and IL-6; the manufacture of these substances can be increased by TNF- α and IL-13 within the NCI-H292 cells (Bloemen *et al.*, 1993). As this is a tumour cell line, however, this study has acknowledged that certain attributes will differ from those found in the normal bronchial epithelium.

The resulting impacts of both secondary and primary BAs on the viability of BEAS-2B were studied. The viability of the cells was to some extent affected by all the bile acids that were

used. Most interesting was the finding that the impact of LCA could be observed at 5-10 times lower concentration than with other bile acids.

In addition to potentially impacting upon the viability of cells, my data demonstrates that the stimulation of BEAS-2B cells by BAs is associated with the release of GM-CSF, IL-6, and IL-8. BEAS-2B cells retain the morphology of an epithelial cell *in-vitro* (Reddel *et al.*, 1988) and release IL-8 and IL-6 in addition to other cytokines (Ke *et al.*, 1988). Under submerged conditions, BEAS-2B cells are commonly used as an *in-vitro* model of human airways epithelium; especially within studies on inhalation toxicity. BEAS-2B expressed both the differentiated epithelial cell marker cytokeratin 8 and the basal epithelial cell marker cytokeratin 5 when placed under submerged conditions. Further, the expression of cytokeratin 8 in BEAS-2B cells when placed under these submerged conditions indicates that, between the cells, there does exist some differentiation (Cha *et al.*, 2007; Veranth *et al.*, 2007; Petecchia *et al.*, 2009).

In conjunction with tumour necrosis factor alpha (TNF- α), the impact of IL-17 on the expression and manufacture of IL-8, and GM-CSF in BEAS-2B cells has been a focus of recent studies. Through these studies, it was revealed that IL-17A and TNF- α demonstrated the potential to induce the manufacture of IL-8 and GM-CSF synergistically, possibly via the MAPK pathways in human bronchial epithelial cells (Honda *et al.*, 2016). Within the paper by Mullol *et al.*, the impact of endothelins (ETs) on the secretion of IL-8, IL-6 and GM-CSF in a bronchial epithelial cell line (BEAS-2B) was investigated. The resulting data from this paper confirmed that IL-6, IL-8 and GM-CSF (Mullol *et al.*, 1996) are indeed generated by BEAS-2B cells.

Within my study, derived from bronchial surface epithelial cells (Cozens *et al.*, 1994), the immortalised cell line 16HBE14o- was introduced to the presence of BAs to assess the extent to which the cells would retain viability. One finding was that DCA and LCA (secondary bile acids) were much more cytotoxic when compared to CDCA and CA (primary bile acids).

In fact, Wu *et al.* echoes these same results, where the human epithelial cells in their study were exposed to DCA and LCA, and found that those cells had detached and were floating to the surface. At concentrations $\geq 1\mu\text{mol/L}$ on 16HBE14o-, the impact of LCA on the viability of the cells did vary. One example of this variability was where a different curve is observed with LCA, whereby $10\mu\text{mol/L}$ caused a cell death of $26\pm 3\%$, and $20\mu\text{mol/L}$ led to a cell death of $70\pm 3\%$. Furthermore, the study on secondary and primary BAs by Wu *et al.*, revealed

levels of these BAs that were similar to or greater than those observed in ventilator-associated aspiration pneumonia (Wu *et al.*, 2009).

In addition to potentially impacting upon the viability of the cells, my data demonstrated that when 16HBE14o- cells came into contact with BAs, this led to release of IL-6, IL-8 and GM-CSF; confirming that cytokines are released by 16HBE14o- cells.

The findings also corroborate the results from other studies, For example, according to a study conducted by Chow *et al.*, the 16HBE14o- cells and human bronchial epithelial cells became “chemically injured” as a result of being exposed to poly-L-arginine as a surrogate of the eosinophil cationic protein. Moreover, measured by a real-time PCR, the expression levels of IL-8 and IL-6 mRNA were increased. Furthermore, cell culture medium gathered from basolateral and apical compartments, and ELISA was used to quantify the levels of IL-8 and IL-6. Subsequently, this study demonstrated clearly the bronchial epithelia produces a polarised secretion of IL-8 and IL-6 when damaged by poly-L-arginine. The orchestration of how epithelial cells respond to inflammation may, therefore, be significantly impacted by this apically directed secretion of cytokines (Chow *et al.*, 2010).

Bathoorn *et al.* examined in a recent study the impact of pepsin in inducing cytotoxicity and inflammation to cells within the patient’s airways. To examine this impact, 16HBE14o- cells were introduced to pepsin. This study hypothesised that when at a lower pH, concentration of pepsin (0.125 mg/mL) would induce a release of pro-inflammatory cytokines and increase overall cytotoxicity. This *in-vitro* model showed that epithelial injury and the release of inflammatory mediators, involving IL- 6 and IL-8 can be caused by pepsin (Bathoorn *et al.*, 2011).

In my study, one further goal was to compare MTT and CTM assay results for cells that came into contact with BAs in similar conditions. However, for all cell lines following exposure to BAs, similarities in the viability data from both assays were found. Given the identical conditions of the controlled experiment, similar results were expected as mitochondrial enzymes reduce the reagents from both MTT and CTB.

Although the precise mechanisms for this are yet to be established, my study did find that the exposure to BAs does have an effect on the BEAS-2B, NCI-H292, 16HBE14o- and Calu-3 cells. However, what we do know is that BAs do disrupt cationic permeability and cell

membranes, as demonstrated in type II pneumocytes *in-vitro* (D'Ovidio *et al.*, 2005; D'Ovidio and Keshavjee, 2006), which could explain the findings of this study to some extent.

Necrosis and apoptosis are the two broad mechanisms that cause cell death in acute lung injury, which is itself a complex pathophysiological process (Jernigan *et al.*, 2004). Necrosis, which is rarer in lungs than elsewhere in the body due to the dual pulmonary blood flow and ventilation in the alveoli, is thought to be caused by an abrupt decrease in oxygen tension leading to ischemia (Walmrath *et al.*, 1994; Sato *et al.*, 2003). Apoptosis, which is a process of programmed cell death, can occur in the epithelium in response to environmental toxins, allergens, viral infection or drugs (White, 2011). Lung injury can induce apoptosis in neutrophils, macrophages and alveolar cells (Jernigan *et al.*, 2004). Bile acids are known to induce apoptosis (Lapre *et al.*, 1992), which in turn leads to compensatory cell proliferation, thereby implicating bile acids in the initiation and promotion of cancers. Studies of apoptosis and necrosis were beyond the scope of my thesis. This would be a very interesting area to look into.

In future work apoptosis could be identified with Annexin V staining. Annexin V has a high affinity for the phosphatidylserine that becomes exposed on the outside of the plasma membrane of apoptotic cells. If the Annexin V is labelled with green fluorescent fluorescein (FITC) apoptotic cells are stained brightly green. These can be differentiated from necrotic cells which stain positively with a nuclear probe. For example Propidium Iodide or Ethidium homodimer III (EtD-III) are nucleic acid stains which are not permeant to live or apoptotic cells but which stain necrotic cells leading to red fluorescence. Apoptotic and necrotic cells can therefore be counted by counting green and red cells (Van Engeland *et al.*, 1998). In further work it would be seem very reasonable to speculate that a shorter time course of BA stimulation might lead to apoptosis and that prolonged treatment might cause necrosis; this would be a good thing to investigate in further studies.

BAs, in their non-ionised, lipophilic form, are able to traverse the cell membrane at pH levels between 3 and 6. Intracellular pH levels are much higher, ionising the BAs and thereby trapping them within the cell. BA levels within the cell can reach up to eight times that of luminal levels, causing damage to both cells and their tight junctions (Gotley *et al.*, 1991), potentially leading to other injuries (Jenkins and Hardie, 2008; Klokkenburg *et al.*, 2009). Further to this, BAs can introduce pores or act as mobile carriers in membranes, modify their cation permeability and lead to cytotoxicity through excessive influx or release of cations

such as Ca^{++} . This may result in irreversible damage to the cellular metabolic processes (Morgan et al., 1986). Elevated Ca^{++} has been observed to cause a number of different outcomes, including activation of Ca^{++} -dependent proteases (Shields et al., 1987), ATP depletion, complement attack on the membrane becoming activated (Morgan et al., 1986), cytoplasmic or membrane-associated protein cross-linking, and triggering of increased phospholipase activity (Tyler et al., 1978). The build-up of intracellular Ca^{++} is widely recognised as an early event in certain types of cell injury, although whether this is true for all cells remains unanswered (Lauterburg, 1987).

Ca^{2+} signals can be studied in a range of contexts through the use of Ca^{2+} sensitive fluorescent dyes. Such indicators are non-invasive and provide a useful way of measuring acute changes in Ca^{2+} concentrations. A wide variety of indicators are available, with different spectral characteristics, brightness levels, affinities and targeting properties for different cellular domains. Such variation enables the exploration of both temporally and spatially diverse Ca^{2+} signals. Furthermore, Ca^{2+} readouts can be multiplexed with other cellular functions (Bootman *et al.*, 2013).

Within this study, the concentrations of LCA covered the range 0 – 20 $\mu\text{mol/L}$ and therefore included the concentration of $2.8 \pm 0.6 \mu\text{mol/L}$ identified in CF lung disease; the viability of PHBECs (Brodie *et al.*, 2015) was affected by low, $5 \mu\text{mol/L}$, concentrations. Additionally, when compared to other bile acids, the effect of LCA occurred at a 5-10 times lower concentration. Our understanding of LCA-induced cholestasis as a hepatotoxic factor in liver injury (Palmer and Ruban, 1966; Hofmann, 1999a) has been enhanced by studies conducted on mammals, such as hamsters, rats and rabbits. This could open up future research on factors such as: the variation in retrieval or transfer of canalicular export pumps to and from the canalicular membrane (Kubitz *et al.*, 2004); the biochemical changes that occur with the bile canalicular membrane (Kakis and Yousef, 1978); and also the formation of crystalline plug in bile canaliculi due to the poor solubility of LCA (Miyai *et al.*, 1977). Alongside other studies, this may contribute to an integrated ‘aerodigestive paradigm’ about reflux-related chronic lung diseases.

In terms of where aspiration and reflux are implicated in lung disease, all cases appear to be linked to the presence of either an acute or chronic inflammation, which has led frequently to the use of steroids as a form of anti-inflammatory medication (Morehead, 2009). Overall, the data gathered from my study supports the hypothesis that a cell’s exposure to BAs can

substantially alter the production of the cytokines known to contribute to airway injury. Upon reflection, this explains why chronic inflammation and a decrease in lung function are associated with aspiration by respiratory disease patients, and may also be a call for new approaches to patient therapy.

Overall, the data presented in this chapter has succeeded in demonstrating that levels of IL-6, IL-8 and GM-CSF that are commonly discovered in airways and in cases of lung disease where aspiration and reflux have been shown to be a possible cause of injury. Arguably, the most physiologically relevant cell type to assess the extent of the damage within the airways was the primary human bronchial epithelial cells (PHBECs). Although expensive and challenging to culture (Stewart *et al.*, 2012). Therefore four related cell lines were used to compare inflammatory mediator expression, both basal and inducible. Following assessment, the results confirm that BEAS-2B, NCI-H292, 16HBE14o- and Calu-3-cell lines constitute models that are both sensitive and effective for studying the human respiratory process, in addition to the diseases associated with lung injury induced by aspirating BAs.

CHAPTER 4: EFFECT OF BILE ACIDS ON EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) IN RESPIRATORY DISEASES

4.1 Introduction

Fibrotic responses can be stimulated by damage to an injured airway epithelium (Adamson *et al.*, 1988; Dai *et al.*, 1998). A growing number of experimental lung fibrosis studies have shown that cytokines and fibroblast promoting growth factors (which are also involved in migration, proliferation, and myofibroblast differentiation) are involved in the process of epithelial-mesenchymal transition (EMT), of airway epithelial cells (Kim *et al.*, 2006; Kim *et al.*, 2009).

EMT refers to a phenomenon in which epithelial cells have a plastic quality, which enables them to take on mesenchymal form and function. This is critical to embryonic development and also in recovery from acute injury in adults (Walser *et al.*, 2015). EMT can become pathogenic or unregulated in cases of chronic injury or inflammation and in epithelial derived cancers, referred to as Types I, II, or III EMT (Tian *et al.*, 2015). This may lead to pulmonary fibrosis, a chronic lung disease which involves extracellular matrix (ECM) accumulation, lung architecture remodelling and an increased risk of cancer (Todd *et al.*, 2012). Moreover, ECM is involved in the early dissemination of tumour cells through weakening cell-cell contacts, increasing migratory tendencies and increasing anoikis resistance.

The airway epithelial sheet is formed through cell adhesion mechanisms, out of a tightly connected mesh of polygonal epithelial cells (Sohal *et al.*, 2014). An initial change when epithelial cells transform is the inhibition of E-cadherin synthesis, a transmembrane glycoprotein found mainly in adherens junctions (Yang and Liu, 2001). Various studies have implicated E-cadherin as a marker of differentiated epithelial phenotype (Arias, 2001; Zeisberg *et al.*, 2003). It is also critical in epithelial polarity maintenance and therefore the development of tight junctions (Tunggal *et al.*, 2005). EMT consists of a number of steps, of which E-cadherin downregulation is the first, in both cancerous and non-cancerous cells. Acquisition of the mesenchymal phenotype is characterised by an increase in mesenchymal markers, including fibronectin, which plays a role in tissue repair, cell migration and adhesion, and a number of other processes (Miettinen *et al.*, 1994). EMT and epithelial damage are also characterised by the matrix metalloproteinase (MMP) 9 production from

epithelial cells. MMP-9 is a type IV collagenase. Collagen type IV, forms the reticular basement membrane, upon which epithelial cells lie. By disrupting the airway epithelium's basement membrane, MMPs can cause inflammation, translocation, further EMT and other damage (Kalluri and Neilson, 2003).

Within the tumour microenvironment, *in vivo*, various growth factors and cytokines can induce EMT. The transforming Growth Factor (TGF)- β 1 family of growth factors, which consists of over 30 different types, all with significant biological roles, is a particularly effective and well-known EMT inducer in either normal or pathological conditions. TGF- β 1 regulates both cell plasticity and cell proliferation pleiotropically, inhibiting normal epithelial cell proliferation on one hand (thereby opposing cancer promotion), but stimulating fibroblast proliferation on the other. It also interacts with immune system elements, promotes angiogenesis and induces EMT, all of which impact negatively on patients with a fibrotic disease and/or with cancer.

Patients with a range of airway diseases are prone to gastroesophageal reflux (GOR) and extra-oesophageal reflux disease (EORD). Given that airway epithelial cells can generate fibroblast-promoting growth factors, and that exposure to bile acids (BAs) is known to cause a fibrotic response, the hypothesised, causative link between respiratory disorders and GOR is biologically plausible. Asthma sufferers are prone to GOR (Hsu *et al.*, 2005), which is especially common in severe cases (Leggett *et al.*, 2005). Severe COPD cases have exhibited pathologic reflux (Casanova *et al.*, 2004) and a positive correlation is found between GOR symptoms and COPD exacerbation (Rascon-Aguilar *et al.*, 2006). Patients with idiopathic pulmonary fibrosis are also particularly vulnerable to GOR (Tobin *et al.*, 1998; Raghu *et al.*, 2006). Even after lung transplantation, aspiration of duodenogastroesophageal refluxate is prevalent. This is correlated with bronchiolitis obliterans, which has a fibrotic pathophysiology (D'Ovidio *et al.*, 2005).

It has been suggested that EORD is involved in the pathophysiology of a range of otolaryngology illnesses, including laryngitis, cough, hoarseness, postnasal drip disease, sinusitis, otitis media, dysphagia, laryngeal cancer and idiopathic subglottic stenosis (ISS) (Toohill and Jindal, 1997). Laryngeal cancer and idiopathic subglottic stenosis (ISS) is usually confined to the first two tracheal rings and the subglottic region. This rare, non-specific inflammatory process, which mainly affects women aged between 30 and 50, is

slowly progressive and has no known cause. It is identified in the upper trachea/lower larynx by a short segment of stenosis (Grillo *et al.*, 1993; Benjamin *et al.*, 1997).

Lung injury and airway fibrosis are associated with bile acid (BA) aspiration (19-23). A correlation has been found between BA concentration in bronchoalveolar lavage fluid (BALF) and the degree of lung fibrosis in Idiopathic Pulmonary Fibrosis (IPF) patients. It is therefore suspected that microaspiration of BAs may play a key role in IPF development and/or progression (Savarino *et al.*, 2013).

It is hypothesised in this chapter that injured airway epithelial cells may undergo EMT, a process that may be the mechanism leading to various respiratory diseases, including chronic obstructive pulmonary fibrosis (COPD), pulmonary fibrosis (PF), asthma and lung rejection. The second hypothesis is that BA aspiration may be a potential link to subglottic airway inflammation and ISS, with the potential that this important pathology is reflux-mediated. The studies which investigated these hypotheses involved challenge experiments in the epithelial cell lines BEAS-2B and 16HBE14o-. These studies were complimented by experiments with primary human epithelial cells including human bronchial epithelial cells from lung transplant recipients. The experiments involved stimulating the cells with BAs to establish if they underwent EMT.

4.2 Aims

The primary experimental aims, described in this chapter are as follows:

- Investigate the role of BAs in instigating EMT, using primary human bronchial epithelial cell cultures (PHBECs) from lung allografts, tracheal epithelial cells (upper airway cells) from the subglottic area and immortalized cell lines (BEAS-2B and 16HBE14o-)
- Evaluate E-cadherin expression in primary cells (primary bronchial human epithelial cell cultures from lung allografts (PHBECs), Tracheal epithelial cells (upper airway cells) from the subglottic area cells and cell lines (BEAS-2B and 16HBE14o-) stimulated with BAs, via quantitative real-time PCR.
- Examine the expression profile of fibronectin (a mesenchymal marker protein) in primary cells (primary bronchial human epithelial cell cultures from lung allografts (PHBECs), Tracheal epithelial cells (upper airway cells) from the subglottic area cells and cell lines (BEAS-2B and 16HBE14o-) stimulated with BAs, via quantitative real-time PCR.
- Assess TGF- β 1, MMP9 and pro-collagen production from challenged epithelial cells using an enzyme-linked immunosorbent assay (ELISA).

4.3 Materials and methods

4.3.1 Cell Culture

PHBECs were isolated (described in chapter 2, section 2.2) from stable lung transplant recipients. The BEAS-2B and 16HBE14o- cell lines were cultured in six-well plates. Dr Jason Powell (Newcastle University) supplied Tracheal epithelial cells (upper airway cells) from the subglottic area. Bronchial epithelial cell growth medium (BEGM) (see chapter 2, section 2.1.2.3) was used for PHBECs, tracheal epithelial cells culture and BEAS-2B cells. EMEM (see chapter 2, section 2.2.1.2) was used for 16HBE14o- cells culture.

4.3.2 Stimulation of PHBECs, Tracheal epithelial cells (upper airway cells), BEAS-2B and 16HBE14o- cells with bile acids

The cells were exposed to the following BAs: LCA, DCA, CA and CDCA. A range of BA concentrations (1, 5, 10, 15, 20, 30, 50, 75 and 100 $\mu\text{mol/L}$) were used to stimulate EMT in BEAS-2B and 16HBE14o- cell cultures (see chapter 2, section 2.6). The challenge was carried out for 48 hours, following which, the cells underwent the MTT and Titre-blue Assays to determine cell viability (described in chapter 2, sections 2.7.1 and 2.7.2)

4.3.3 ELISA

ELISA was used to determine the presence of TGF β 1, MMP-9 and Pro-collagen peptides before and after BS stimulation of the cells (See chapter 2, section 2.8.1).

4.3.4 Molecular biology

RNA was extracted using the RNEasy Mini-kit (Qiagen). Isolated RNA was reversibly transcribed using a Tetro cDNA synthesis kit (Bioline), and a quantitative PCR reaction was performed in order to determine specific gene expression changes at a molecular level (see chapter 2, section 2.9).

4.4 Results

4.4.1 Expression of EMT markers and epithelial activation in airway epithelium

4.4.1.1 Effect of BAs on the regulation of an epithelial marker E-cadherin and a Mesenchymal marker fibronectin in primary human bronchial epithelial cell cultures from lung allografts (PHBEC)

EMT can be identified by gene expression changes, notably by a decrease in E-cadherin (an epithelial marker) and an increase in Fibronectin (a mesenchymal marker). PHBECs were exposed for 48 hours to LCA, DCA, CA and CDCA at 1 and 10 $\mu\text{mol/L}$, using TGF β 1 at 5ng/ml as a positive control. These concentrations of the bile acids were the highest concentration that did not cause high cell death based on cell viability. Cell viability was confirmed at >90% (Figure 4-1) RT-PCR analysis of E-cadherin expression revealed a significant ($P < 0.001$) decrease with CA and CDCA. CA at concentration 10 $\mu\text{mol/L}$ induced three-fold decreases and 1 $\mu\text{mol/L}$ induced two-fold decreases, showing a negative concentration dependency effect. CDCA at 1 and 10 $\mu\text{mol/L}$ caused a three-fold decrease in E-cadherin mRNA, showing no concentration dependency effect. PHBECs treated with LCA and DCA at 1 and 10 $\mu\text{mol/L}$ do not decrease E-cadherin expression (Figure 4-2).

Because E-cadherin mRNA is downregulated by BA treatment, I also used RT-PCR to analyse treated PHBECs for fibronectin expression (a mesenchymal protein). RT-PCR analysis of Fibronectin expression revealed that CA at 1 and 10 $\mu\text{mol/L}$ caused a six-fold increase in fibronectin mRNA, showing no concentration dependency. CDCA at 10 $\mu\text{mol/L}$ caused seven-fold increase in fibronectin mRNA and 1 $\mu\text{mol/L}$ caused five-fold increase in fibronectin mRNA, showing a positive concentration dependency effect. PHBECs treated with LCA and DCA at 1 and 10 $\mu\text{mol/L}$ showed no significant increase in Fibronectin expression (Figure 4-3).

In general, looking at Figure 4-2 and Figure 4-3 it can be observed that primary BAs affected E-cadherin and fibronectin expression more than secondary BAs. Moreover, this was not concentration dependent.

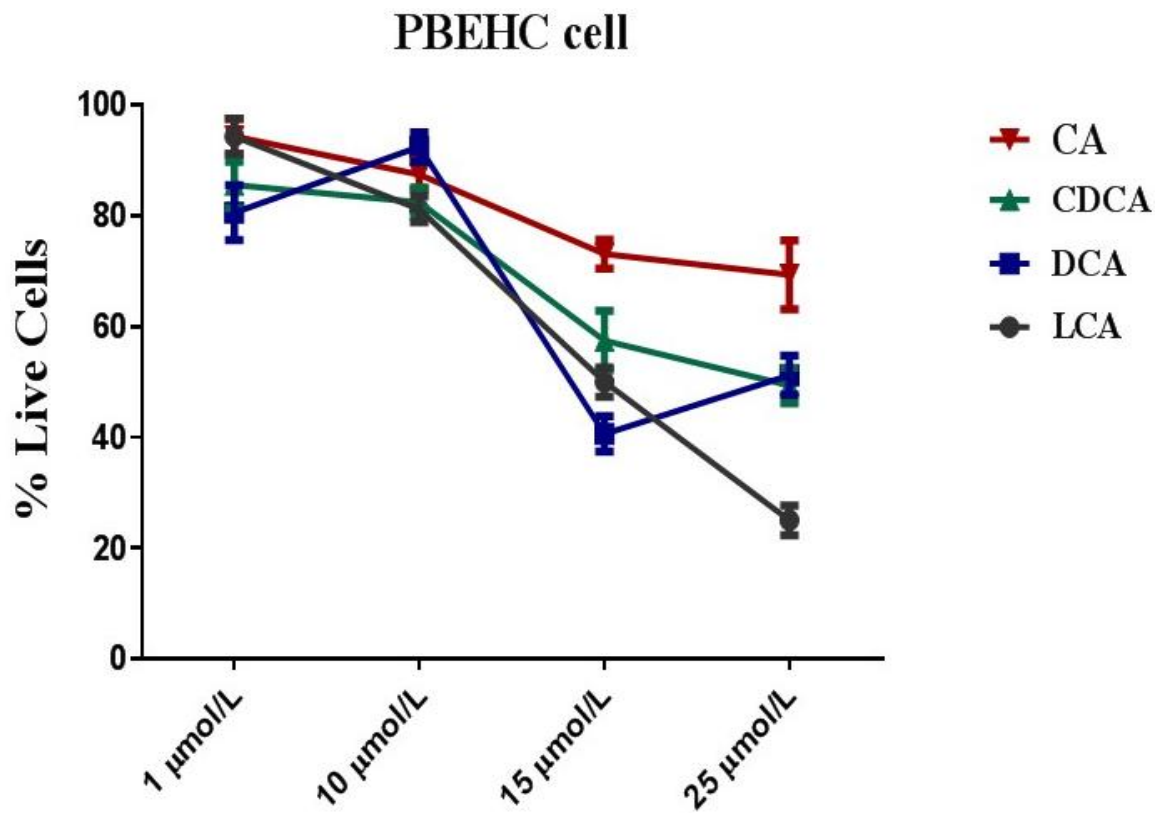


Figure 4-1: Effects of BAs on PBEHC cell proliferation. PBEHC cell viability following stimulation with BAs Lithocholic, Deoxycholic, Chenodexychoic and Cholic acids, at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test effect of BAs compare with control. Negative controls were measured by fixing the cells in methanol before adding the Titre-Blue reagent. The data are representative of three independent experiments (n=6).

E-Cadherin expression in PHBECs

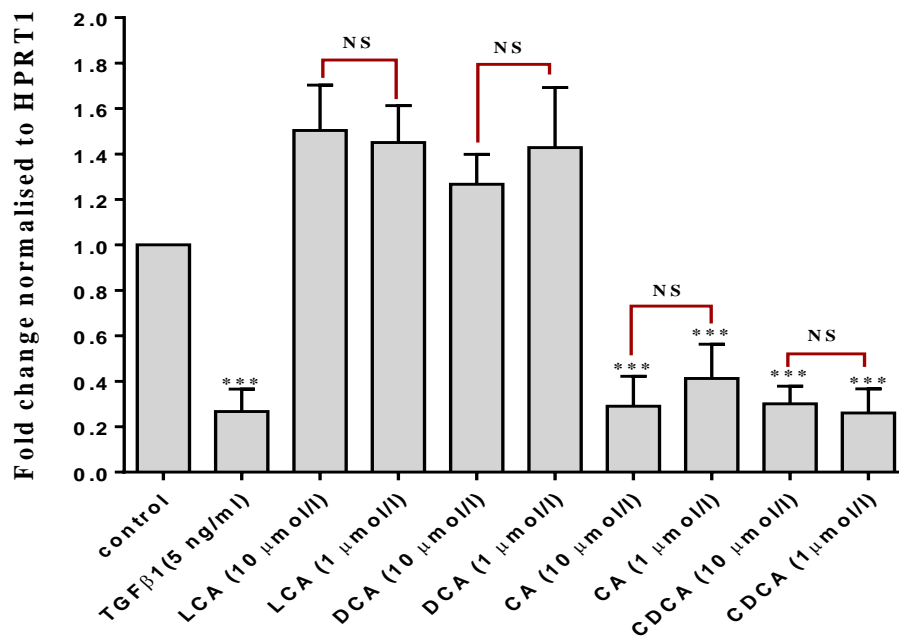


Figure 4-2: E-cadherin mRNA expression following BA and TGF- β 1 treatment. Confluent PHBECs were cultured for 48 hours under various conditions as follows: medium containing 5ng/ml of TGF- β 1 or 1 and 10 μ mol/L of LCA, DCA, CA or CDCA. HPRT1 mRNA was used to normalize E-cadherin RNA levels, which were presented as mean \pm SEM (n=3). The E-cadherin mRNA was significantly downregulated in TGF- β 1, CA and CDCA treatment groups, whereas E-cadherin gene expression levels were similar to the control in the LCA and DCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to control, ANOVA). For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

Fibronectin expression in PHBECs

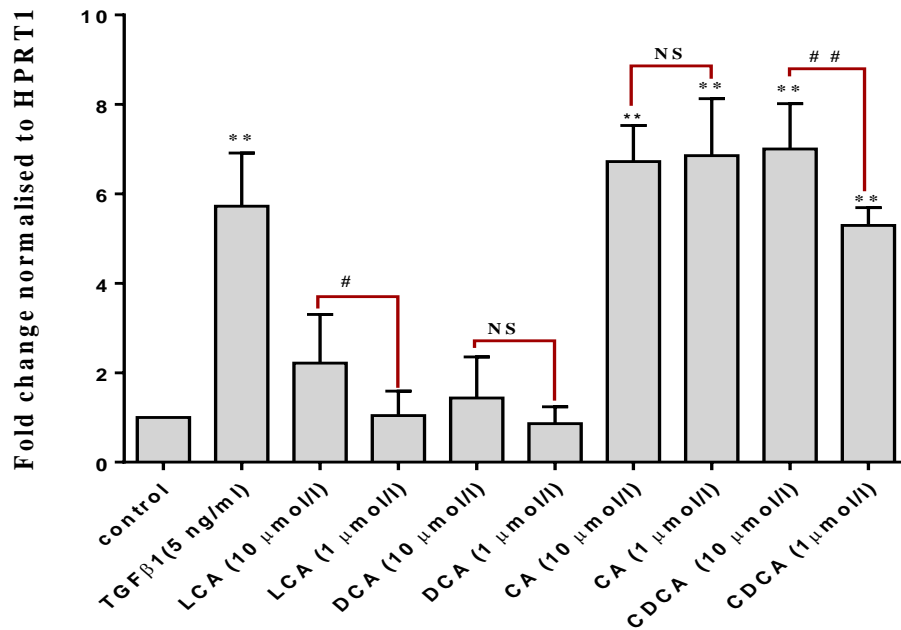


Figure 4-3: Fibronectin mRNA expression following BA and TGF-β1 treatment. Confluent PHBECs were cultured for 48 hours under various conditions as follows: medium containing 5ng/ml of TGF-β1 or 1 and 10μmol/L of LCA, DCA, CA or CDCA. HPRT1 mRNA was used to normalize Fibronectin RNA levels, which were presented as mean ±SEM (n=3). The Fibronectin mRNA was significantly upregulated in TGF-β1, CA and CDCA treatment groups, whereas Fibronectin gene expression levels were similar to the control in the LCA and DCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to control, ANOVA). For pairwise analysis Student's t-test #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ and ####: $p < 0.0001$. (NS- not significant).

4.4.1.2 Effect of BAs on epithelial marker E-cadherin and mesenchymal marker fibronectin regulation in tracheal epithelial cells (upper airway cells) from the subglottic area

EMT can be identified by gene expression changes, notably by a decrease in E-cadherin (an epithelial marker) and an increase in Fibronectin (a mesenchymal marker).

Epithelial cells were exposed for 48 hours to LCA, DCA, CA and CDCA at concentrations of 1 and 10 $\mu\text{mol/L}$, using TGF β 1 at 5 ng/ml as a positive control. These concentrations of the bile acids were the highest concentration that did not cause high cell death based on cell viability. Cell viability was confirmed at >90% (Figure 4-4). RT-PCR analysis of E-cadherin expression revealed a significant ($P < 0.001$) decrease at both concentrations of CA and CDCA and 10 $\mu\text{mol/L}$ of LCA. CA at concentration 1 and 10 $\mu\text{mol/L}$ induced three-fold decreases, showing no concentration dependency effect. CDCA at 1 and 10 $\mu\text{mol/L}$ caused two-fold decreases, showing no concentration dependency effect. LCA at 10 $\mu\text{mol/L}$ caused a two-fold decrease in E-cadherin mRNA. No significant decrease was observed in cells stimulated with 1 $\mu\text{mol/L}$ LCA (Figure 4-5).

Because E-cadherin mRNA is downregulated by BA treatment, I also used RT-PCR to analyse treated primary human subglottic cells for fibronectin expression (a mesenchymal protein). It was found that CA at 1 and 10 $\mu\text{mol/L}$ caused a three-fold increase and 10 $\mu\text{mol/L}$ LCA caused a three-fold increase in fibronectin, showing no concentration dependency effect. CDCA at 1 $\mu\text{mol/L}$ caused a three-fold increase and CDCA at 10 $\mu\text{mol/L}$ caused a four-fold increase in fibronectin mRNA, showing no concentration dependency effect. No significant decrease was observed in cells stimulated with DCA at 10 $\mu\text{mol/L}$ and LCA at 1 $\mu\text{mol/L}$ (Figure 4-6).

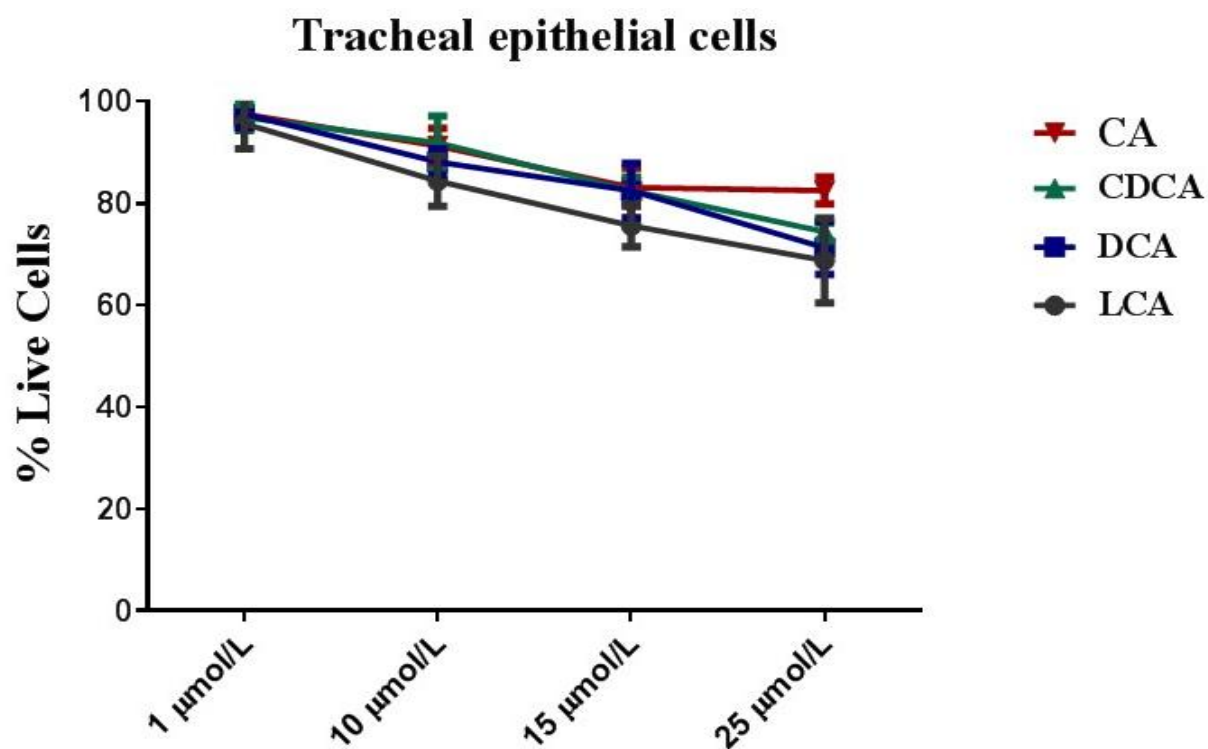


Figure 4-4: Effects of BAs on tracheal epithelial cell proliferation. Tracheal epithelial cell viability following stimulation with Lithocholic, Deoxycholic, Chenodexycholic and Cholic acids at varying concentrations. Viability is measured as a percentage of the control after 48h. Bars represent mean \pm S.E.M. Statistical analysis was performed to test effect of BAs compare with control. Negative controls were measured by fixing the cells in methanol before adding the CellTiter-Blue reagent. The data are representative of three independent experiments (each experiment n=6 wells).

E-Cadherin expression in tracheal epithelial cells

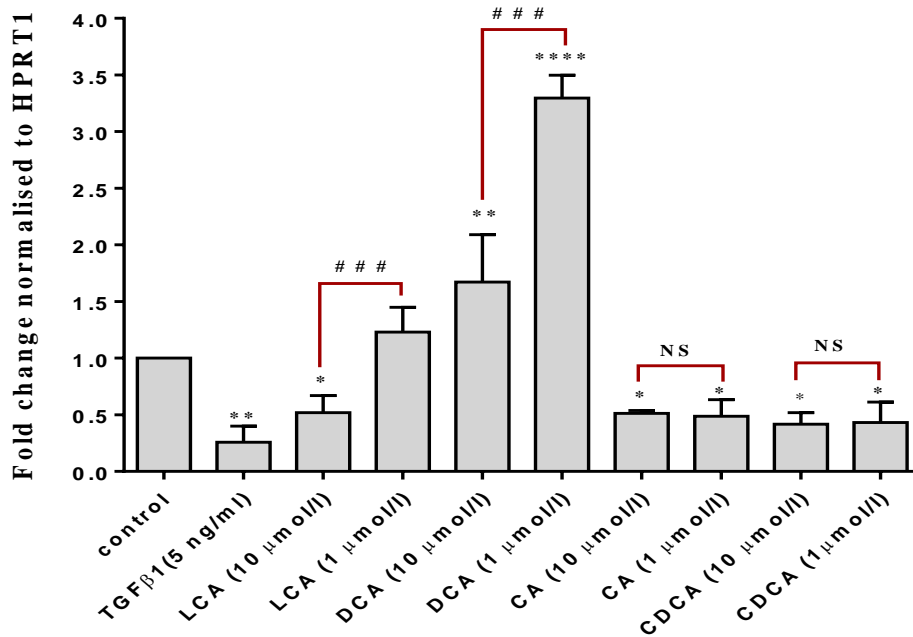


Figure 4-5: E-Cadherin mRNA expression following BA and TGF-β1 treatment. Confluent tracheal epithelial cells from the subglottic area were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5ng/ml of TGF-β1 or 1 and 10 μmol/L of LCA, DCA, CA and CDCA. HPRT1 mRNA was used to normalize E-cadherin mRNA levels, which were presented as mean ± SEM (n=3). The E-cadherin mRNA was significantly downregulated in TGF-β1, 10 μmol/L LCA, 1 and 10 μmol/L CA and CDCA treatment groups, whereas E-cadherin gene expression levels were similar to the control in 1 μmol/L LCA and DCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p < 0.05, ##: p < 0.01, ###: p < 0.001 and ####: p < 0.0001. (NS- not significant).

Fibronectin expression in tracheal epithelial cells

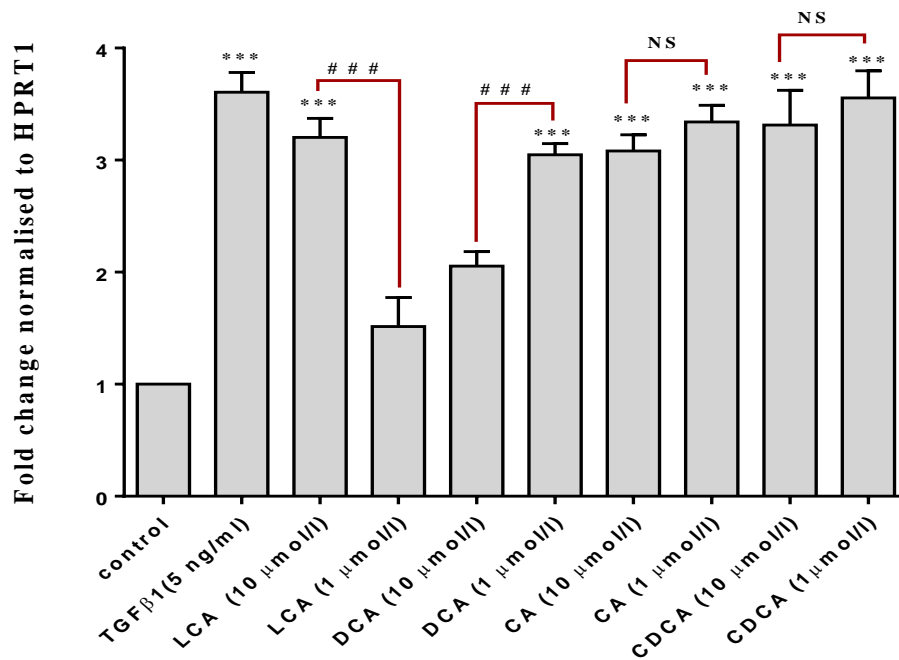


Figure 4-6: Fibronectin mRNA expression following BA and TGF-β1 treatment. Confluent tracheal epithelial cells from the subglottic area were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5ng/ml of TGF-β1 or 1 and 10 μmol/L of LCA, DCA, CA and CDCA. HPRT1 mRNA was used to normalize Fibronectin mRNA levels, which were presented as mean ± SEM (n=3). The Fibronectin mRNA was significantly upregulated in TGF-β1, 10 μmol/L LCA, 1 μmol/L DCA, 1 and 10 μmol/L CA and CDCA treatment groups, whereas Fibronectin gene expression levels were similar to the control in 1 μmol/L LCA and 10 μmol/L DCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p < 0.05, ##: p < 0.01, ###: p < 0.001 and ####: p < 0.0001. (NS- not significant).

4.4.1.3 Effect of BAs on regulation of epithelial marker E-cadherin and Mesenchymal marker fibronectin in the human bronchial epithelial cell line (BEAS-2B) cells

EMT can be identified by gene expression changes, notably by a decrease in E-cadherin (an epithelial marker) and an increase in fibronectin (a mesenchymal marker). In chapter three, I demonstrated that the optimum BAs concentration profile that may injure BEAS-2B cells by using CellTiter-Blue to assess cell viability. In this chapter I evaluate E-cadherin and fibronectin expression in BEAS-2B cells after stimulated with BAs at concentration which did not have significant effect on the cell viability. Cell viability was confirmed at >90%. (Figure 3-8 and Figure 3-9). BEAS-2B cells were exposed for 48 hours to the following BA concentrations: LCA at 1 and 5 $\mu\text{mol/L}$; DCA at 15 and 30 $\mu\text{mol/L}$; CA at 30 and 50 $\mu\text{mol/L}$; and CDCA at 30 and 50 $\mu\text{mol/L}$. The positive control was TGF β 1 at a concentration of 5 ng/ml.

A real-time (RT) PCR was used to quantify E-cadherin expression, which significantly ($P < 0.001$) decreased in response 5 $\mu\text{mol/L}$ LCA with two-fold decrease. DCA at concentration 30 $\mu\text{mol/L}$ caused a three-fold decrease in E-cadherin mRNA and 15 $\mu\text{mol/L}$ caused five-fold decrease in E-cadherin mRNA, showing a positive concentration dependency effect. CA at concentration 50 $\mu\text{mol/L}$ induced a two-fold decrease. CDCA at 50 $\mu\text{mol/L}$ caused a two-fold decrease and 30 $\mu\text{mol/L}$ caused a three-fold decrease in E-cadherin mRNA, showing no concentration dependency effect. However, 1 $\mu\text{mol/L}$ LCA and 30 $\mu\text{mol/L}$ CA had no significant effect (Figure 4-7).

Having established that exposure to BAs causes a downregulation of E-cadherin mRNA, I then used RT-PCR to test for fibronectin expression. Treatment with 5 $\mu\text{mol/L}$ LCA caused a three-fold increase and 1 $\mu\text{mol/L}$ LCA caused a two-fold increase in fibronectin mRNA, showing a positive concentration dependency effect. DCA at 15 $\mu\text{mol/L}$ caused a three-fold increase and CA at 50 $\mu\text{mol/L}$ caused a two-fold increase in fibronectin mRNA. CDCA at 30 $\mu\text{mol/L}$ caused a three-fold increase in fibronectin mRNA. However, 30 $\mu\text{mol/L}$ DCA, 30 $\mu\text{mol/L}$ CA and 50 $\mu\text{mol/L}$ CDCA had no significant effect (Figure 4-8).

E-Cadherin expression BEAS-2B cells

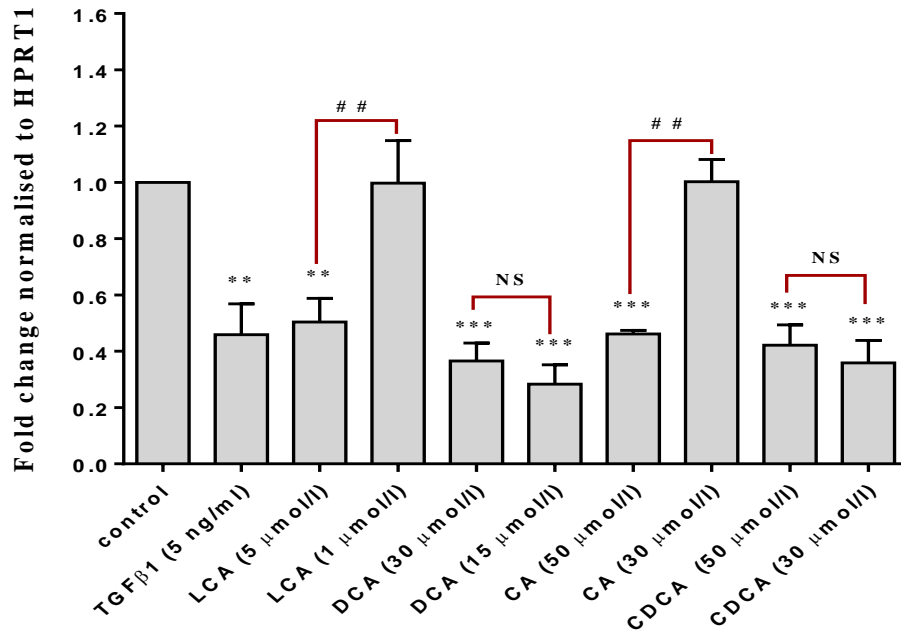


Figure 4-7: E-Cadherin mRNA expression following BA and TGF-β1 treatment. Confluent BEAS-2BS cells were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5ng/ml of TGF-β1 (positive control) or 1 and 5 μmol/L of LCA, 15 and 30 μmol/L of DCA, 30 and 50 μmol/L of CA and CDCA. HPRT1 mRNA was used to normalize E-cadherin RNA levels, which were presented as mean ±SEM (n=3). The E-cadherin mRNA was significantly downregulated in TGF-β1, DCA, CDCA, 5 μmol/L LCA and 50 μmol/L CA treatment groups, whereas E-cadherin gene expression levels were similar to the control in 1 μmol/L LCA and 30 μmol/L CA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

Fibronectin expression BEAS-2B cells

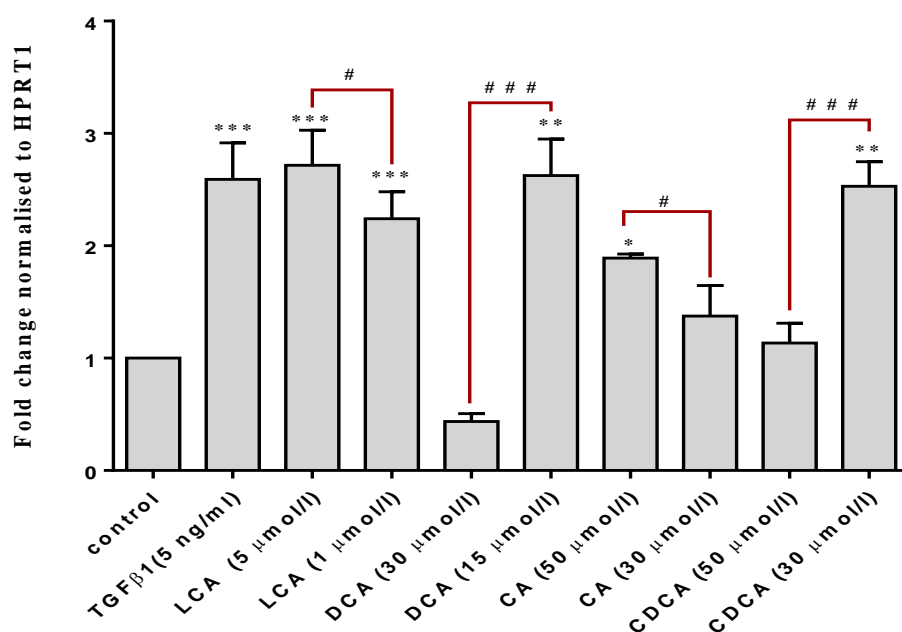


Figure 4-8: Fibronectin mRNA expression following BA and TGF-β1 treatment. Confluent BEAS-2BS cells were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5 ng/ml of TGF-β1 or 1 and 5 μmol/L of LCA, 15 and 30 μmol/L of DCA, 30 and 50 μmol/L of CA and CDCA. HPRT1 mRNA was used to normalize Fibronectin mRNA levels, which were presented as mean ±SEM (n=3). The Fibronectin mRNA was significantly unregulated in TGF-β1, LCA, 15 μmol/L DCA, 50 μmol/L CA and 30 μmol/L CDCA treatment groups, whereas Fibronectin gene expression levels were similar to the control in 30 μmol/L DCA, 30 μmol/L CA and 50 μmol/L CDCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p < 0.05, ##: p < 0.01, ###: p < 0.001 and ####: p < 0.0001. (NS- not significant).

4.4.1.4 Effect of BAs on epithelial marker E-cadherin and mesenchymal marker fibronectin regulation in 16HBE14o- cells

EMT can be identified by gene expression changes, notably by a decrease in E-cadherin (an epithelial marker) and an increase in Fibronectin (a mesenchymal marker). In chapter three, I demonstrated that the optimum BAs concentration profile that may injure 16HBE14o- cells by using CellTiter-Blue to assess cell viability. In this chapter I evaluate E-cadherin and fibronectin expression in 16HBE14o- cells after stimulating with BAs at concentrations which did not have significant effect on the cell viability. Cell viability was confirmed at >90% (Figure 3-2 and Figure 3-3).

16HBE14o- cells were exposed for 48 hours to LCA at concentrations of 1 and 10 $\mu\text{mol/L}$, DCA at 15 and 30 $\mu\text{mol/L}$, CA at 50 and 75 $\mu\text{mol/L}$ and CDCA at 30 and 50 $\mu\text{mol/L}$, using TGF β 1 at 5 ng/ml as a positive control. RT-PCR analysis of E-cadherin expression revealed a significant ($P < 0.001$) decrease at all concentrations. LCA at concentration 10 $\mu\text{mol/L}$ caused five-fold decreases and at 1 $\mu\text{mol/L}$ caused a three-fold decreases in E-cadherin mRNA, showing no concentration dependency effect. DCA at concentration 30 $\mu\text{mol/L}$ caused a five - fold decrease and 15 $\mu\text{mol/L}$ caused two-fold decreases in E-cadherin, showing no concentration dependency effect. CA at concentration 75 $\mu\text{mol/L}$ caused a five-fold decrease and 50 $\mu\text{mol/L}$ caused four-fold decrease in E-cadherin mRNA. CDCA at 50 $\mu\text{mol/L}$ caused a five-fold decrease and 30 $\mu\text{mol/L}$ caused a two-fold decrease in E-cadherin mRNA, showing no concentration dependency effect (Figure 4-9).

Because E-cadherin is downregulated by BA treatment, I also used RT-PCR to analyse treated 16HBE14o- cells for fibronectin expression. It was found that fibronectin expression increased at all concentrations of LCA, DCA, CA and CDCA. Treatment with 10 $\mu\text{mol/L}$ LCA caused a two-fold increase and 1 $\mu\text{mol/L}$ LCA caused a three-fold increase in fibronectin mRNA, showing a negative concentration dependency effect. DCA at 30 $\mu\text{mol/L}$ caused a four-fold increase and 15 $\mu\text{mol/L}$ caused a three-fold increase in fibronectin mRNA, showing a positive concentration dependency effect. CA at concentration 75 $\mu\text{mol/L}$ caused a four-fold increases and 50 $\mu\text{mol/L}$ caused three- fold increases in fibronectin mRNA, showing a positive concentration dependency effect. CDCA at 50 $\mu\text{mol/L}$ caused a five-fold increase and 30 $\mu\text{mol/L}$ caused a four-fold increase in fibronectin mRNA, showing a positive concentration dependency effect (Figure 4-10).

E-cadherin expression in 16HBE14o- cells

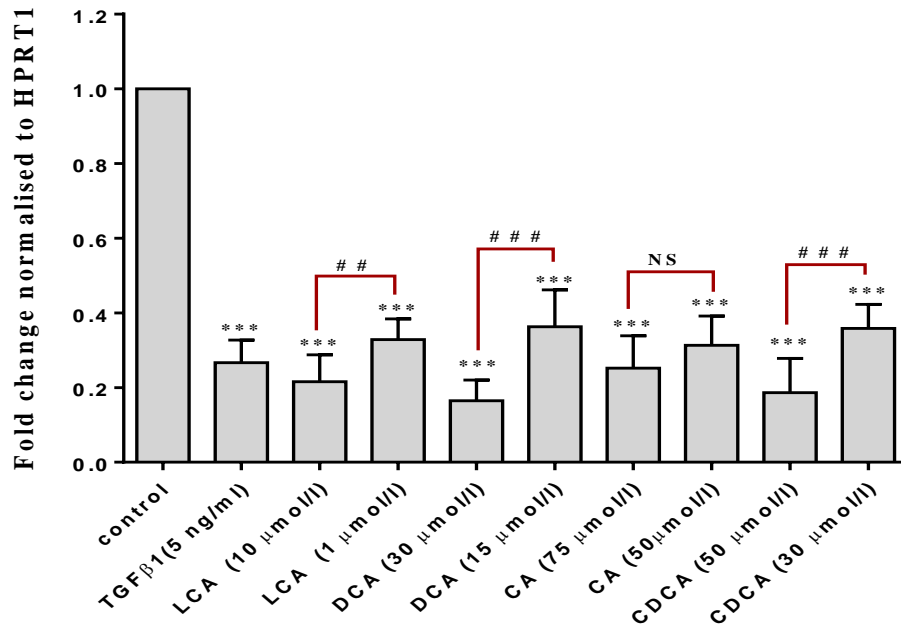


Figure 4-9: E-cadherin mRNA expression following BA and TGF-β1 treatment. Confluent 16HBE14o- cells were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5ng/ml of TGF-β1 or 1-10 μmol/L LCA, 15-30 μmol/L DCA, 50-75μmol/L CA and 30-50 μmol/L CDCA. HPRT1 mRNA was used to normalize E-cadherin mRNA levels, which were presented as mean ±SEM (n=3). The E-cadherin mRNA was significantly downregulated in TGF-β1, LCA, DCA, CA and CDCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

Fibronectin expression in 16HBE14o- cells

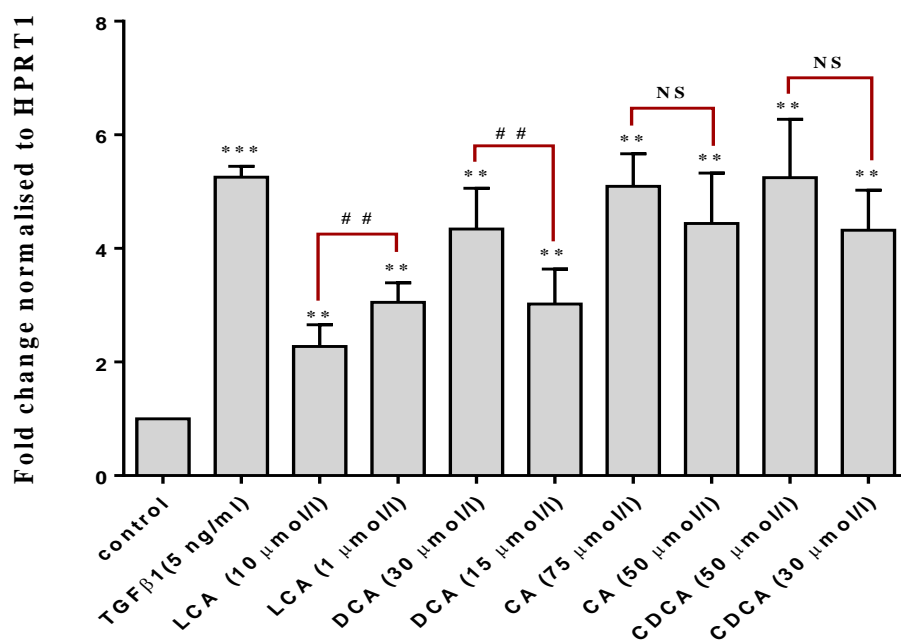


Figure 4-10: Fibronectin mRNA expression following BA and TGF-β1 treatment. Confluent 16HBE14o- cells were cultured for 48 hours under various conditions as follows: medium (unstimulated control), medium containing 5ng/ml of TGF-β1 or 1-10 μmol/L LCA, 15-30μmol/L DCA, 50-75 μmol/L CA and 30-50 μmol/L CDCA. HPRT1 mRNA was used to normalize Fibronectin mRNA levels, which were presented as mean ±SEM (n=3). The Fibronectin mRNA was significantly upregulated in TGF-β1, LCA, DCA, CA and CDCA treatment groups. The data are representative of three independent experiments (each experiment n=6 wells). (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared to control, ANOVA). For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.2 Measurement of TGFβ1 expression

4.4.2.1 Measurement of TGFβ1 protein expression in primary human bronchial epithelial cell cultures from lung allografts (PHBEC)

TGF-β1 is accepted as a master switch driver of EMT (Sime and O'Reilly, 2001; Borthwick *et al.*, 2012). TGF-β1 production by PHBECs was assessed using ELISA after BA exposure at concentrations of 1 and 10 μmol/L (Figure 4-11). These concentrations of the bile acids were the maximal concentration that did not cause high cell death based on cell viability. Cell viability was confirmed at >90% (Figure 4-1). Little or no TGF-β1 was produced by control cells, whereas exposure to LCA, DCA, CA and CDCA (at 1 and 10 μmol/L for 48h) resulted in a significant increase in TGF-β1 secretion compared to control as follows: LCA at 1 μmol/L caused 1106±107 pg/ml TGF-β1 to be produced ($p < 0.001$) and LCA at 10 μmol/L generated 639±77 pg/ml TGF-β1 ($p < 0.001$), showing a negative concentration dependency effect. DCA at 1 μmol/L caused 269±34 pg/ml TGF-β1 to be produced ($p < 0.001$) and DCA at 10 μmol/L generated 527±54 pg/ml TGF-β1 ($p < 0.01$), showing a positive concentration dependency effect. CA at 1 μmol/L caused 773±51 pg/ml TGF-β1 to be produced and CA at 10 μmol/L generated 856±62 pg/ml TGF-β1 ($p < 0.001$), showing no concentration dependency effect. CDCA at 1 μmol/l caused 797±53 pg/ml TGF-β1 to be produced and CDCA at 10 μmol/L generated 585±44 pg/ml TGF-β1 ($p < 0.001$, showing a negative concentration dependency effect. The mean level of TGF-β1 production in control cells was 90±18 pg/ml.

TGFβ1 levels produced by PHBEC

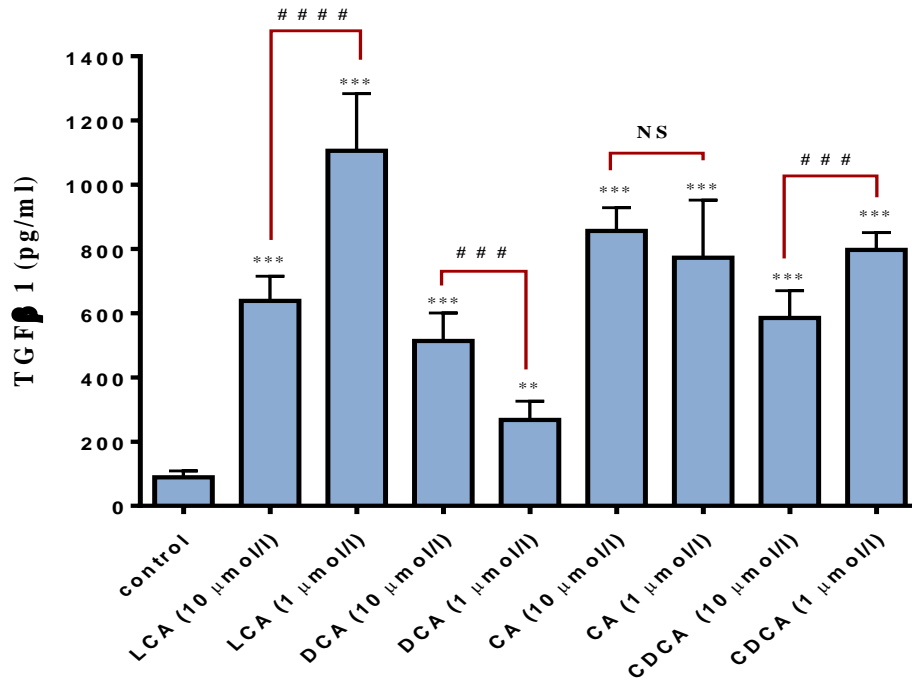


Figure 4-11: ELISA of TGFβ1 levels in culture medium produced by primary human bronchial epithelial cell cultures from lung allografts (PBEC) treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean ± S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.2.2 Measurement of TGFβ1 protein expression in tracheal epithelial cells (upper airway cells) from the subglottic area

In order to ascertain if damaged tracheal epithelial cells released TGF-β1, I carried out an experiment in which primary human epithelial cells from human subglottic cells were exposed to 1 and 10 μmol/L concentrations of LCA, DCA, CA and CDCA for 48 hours. Maximal non-lethal conditions for bile acid stimulation were determined via cell viability assay. Cell viability was confirmed at >90% (Figure 4-4). TGF-β1 levels were measured using ELISA. TGF-β1 production by tracheal epithelial cells after BA exposure is shown in Figure 4-12. BA exposure was shown to significantly increase TGF-β1 levels in comparison to the control. LCA at 1 μmol/L caused 243±25 pg/ml TGF-β1 to be produced and LCA at 10 μmol/L generated 344±31 pg/ml TGF-β1, showing a positive concentration dependency effect. DCA at 1 μmol/L caused 187±25 pg/ml TGF-β1 to be produced and DCA at 10 μmol/L generated 190±25 pg/ml TGF-β1, showing no concentration dependency effect. CA at 1 μmol/L caused 159±13 pg/ml TGF-β1 to be produced and CA at 10 μmol/L generated 177±33 pg/ml TGF-β1, showing no concentration dependency effect. CDCA at 1 μmol/L caused 218 ±22 pg/ml TGF-β1 to be produced and CDCA at 10 μmol/L generated 280±31 pg/ml TGF-β1, showing a positive concentration dependency effect. The mean level of TGF-β1 production in control cells was 23±8 pg/ml (Figure 4-12).

TGFβ1 levels produced by tracheal epithelial cells

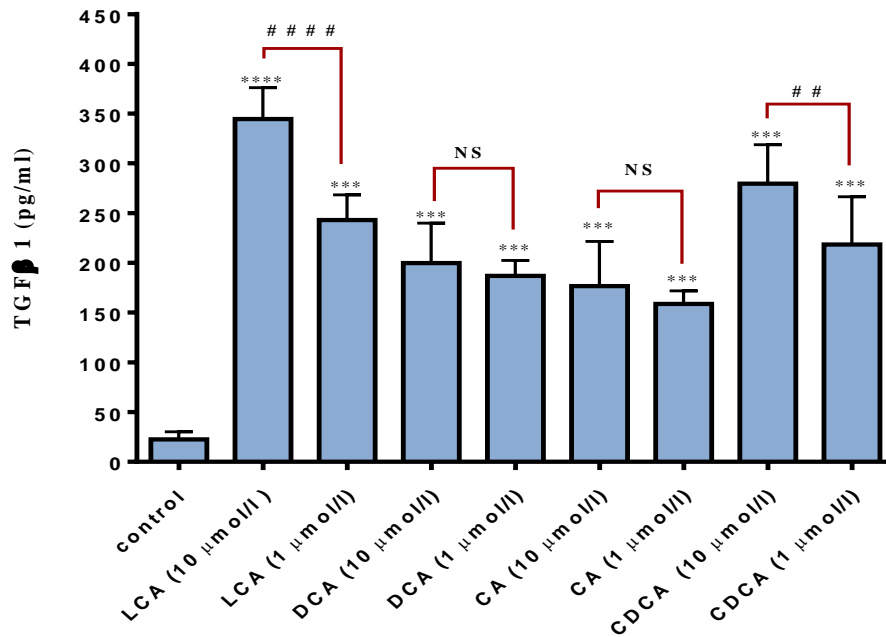


Figure 4-12: ELISA of TGFβ1 levels in culture medium produced by tracheal epithelial cells from the subglottic area, cells treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean ± S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ####: p<0.001 and #####: p<0.0001. (NS- not significant).

4.4.2.3 Measurement of TGFβ1 protein expression in BEAS-2B epithelial cell lines

TGF-β1 production by BEAS-2B cells after BA exposure is shown in Figure 4-13. By using Cell CellTiter-Blue to assess cell viability, chapter three illustrated the concentration of BAs concentration which did not have significant effect on the cell viability. Cell viability was confirmed at >90% (Figure 3-8 Figure 3-9) for this reason, cells were exposed for 48 hours to the following concentrations: LCA (1 and 5 μmol/L), DCA (15 and 30 μmol/L), CA and CDCA (30 and 50 μmol/L). TGF-β1 was measured using ELISA and analysis showed that TGF-β1 significantly increases following BAs stimulation as follows: LCA at 5 μmol/L caused 1844±40 pg/ml TGF-β1 to be produced and LCA at 1μmol/L generated 370±13pg/ml TGF-β1, showing a positive concentration dependency effect. DCA at 30μmol/L caused 1546±49 pg/ml TGF-β1 to be produced and DCA at 15μmol/L generated 969±5 pg/ml TGF-β1, showing a positive concentration dependency effect. CA at 50 μmol/L caused 1825±32 pg/ml TGF-β1 to be produced and CA at 30 μmol/L generated 738±17pg/ml TGF-β1, showing a positive concentration dependency effect. CDCA at 50 μmol/L caused 980±17 pg/ml TGF-β1 to be produced and CDCA at 30 μmol/L generated 1393±44 pg/ml TGF-β1, showing a negative concentration dependency effect. The mean level of TGF-β1 production in control cells was 84± 9 pg/ml (Figure 4-13).

TGFβ1 levels produced by BEAS-2B cells

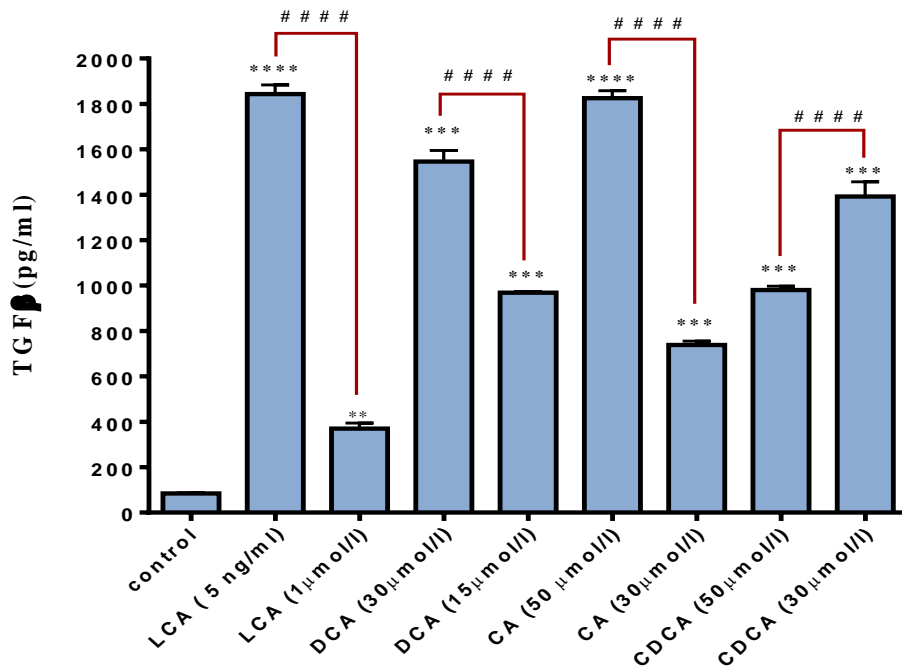


Figure 4-13: ELISA of TGFβ1 levels in culture medium produced by BEAS-2B treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean ± S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.2.4 Measurement of TGF β 1 protein expression in 16HBE14o- epithelial cell lines

TGF- β 1 production by 16HBE14o- cells after BA exposure is shown in Figure 4-14. By using Cell Titer-Blue to assess cell viability, chapter three illustrated the concentration of BAs concentration which did not have significant effects on the cell viability. Cell viability was confirmed at >90% (Figure 3-2 and Figure 3-3) for this reason, cells were exposed for 48 hours to the following concentrations: LCA (1 and 10 μ mol/L), DCA (15 and 30 μ mol/L), CA (30 and 75 μ mol/L) and CDCA (30 and 50 μ mol/L). TGF- β 1 was measured using ELISA and analysis showed that TGF- β 1 significantly increases following BA stimulation as follows: LCA at 10 μ mol/L caused 340 \pm 25 pg/ml TGF- β 1 to be produced and LCA at 1 μ mol/L generated 1460 \pm 36 pg/ml TGF- β 1, showing a negative concentration dependency effect. DCA at 30 μ mol/L caused 353 \pm 16 pg/ml TGF- β 1 to be produced and DCA at 15 μ mol/L generated 1083 \pm 63 pg/ml TGF- β 1, showing a negative concentration dependency effect. CA at 75 μ mol/l caused 325 \pm 11 pg/ml TGF- β 1 to be produced and CA at 50 μ mol/L generated 281 \pm 5pg/ml TGF- β 1, showing no concentration dependency effect. CDCA at 50 μ mol/L caused 1070 \pm 18 pg/ml TGF- β 1 to be produced and CDCA at 30 μ mol/L generated 256 \pm 14 pg/ml TGF- β 1, showing a positive concentration dependency effect. The mean level of TGF- β 1 production in control cells was 131 \pm 7 pg/ml (Figure 4-14).

TGFβ1 levels produced by 16HBE14o- cells

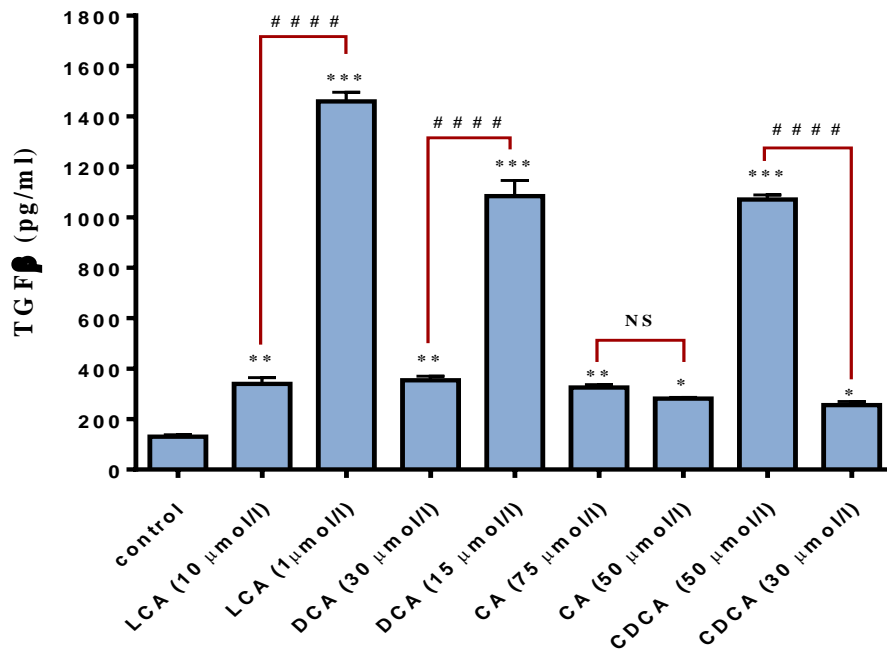


Figure 4-14: ELISA of TGFβ1 levels in culture medium produced by 16HBE14o- treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean ± S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.3 Measurement of MMP9 expression

4.4.3.1 Measurement of MMP-9 protein expression in primary human bronchial epithelial cell cultures from lung allografts (PHBEC)

MMP9 production by PHBECs following exposure to BAs at concentrations of 1 and 10 $\mu\text{mol/L}$ was measured using ELISA. These concentrations of the bile acids were the maximal concentration that did not cause high cell death based on cell viability. Cell viability was confirmed at $>90\%$ (Figure 4-1). Control cells produced little or no MMP9, but exposure to 5ng/ml TGF- β 1 caused a significant increase in MMP9 production. Exposure to BAs caused significant increases and these results are shown in Figure 4-15. 48h exposure of PHBECs to LCA, DCA, CA and CDCA at 1 and 10 $\mu\text{mol/L}$ have been shown to cause a significant increase in MMP9 production. MMP9 production was shown to increase as follows: TGF β 1 at 5ng/ml caused 1360 ± 56 pg/ml MMP-9 to be produced. LCA at 10 $\mu\text{mol/L}$ caused 1068 ± 32 pg/ml MMP-9 to be produced and LCA at 1 $\mu\text{mol/L}$ generated 480 ± 17 pg/ml MMP-9, showing a positive concentration dependency effect. DCA at 10 $\mu\text{mol/L}$ caused 298 ± 17 pg/ml MMP-9 to be produced and DCA at 1 $\mu\text{mol/L}$ generated 451 ± 11 pg/ml MMP-9, showing a negative concentration dependency effect. CA at 10 $\mu\text{mol/L}$ caused 538 ± 15 pg/ml MMP-9 to be produced and CA at 1 $\mu\text{mol/L}$ generated 444 ± 25 pg/ml MMP-9, showing no concentration dependency effect. CDCA at 10 $\mu\text{mol/L}$ caused 660 ± 22 pg/ml MMP-9 to be produced and CDCA at 1 $\mu\text{mol/L}$ generated 390 ± 19 pg/ml MMP-9, showing a positive concentration dependency effect. The mean level of MMP-9 production in control cells was 81 ± 7 pg/ml.

MMP-9 levels produced by PHBEC

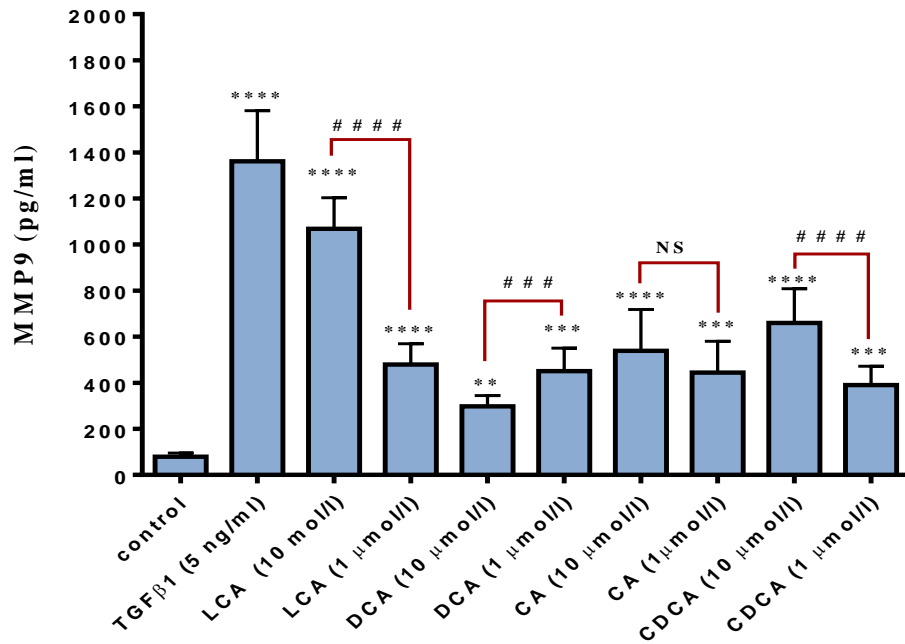


Figure 4-15: ELISA of MMP-9 production from primary human bronchial epithelial cell cultures from lung allografts (PHBEC) treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ####: p<0.001 and #####: p<0.0001. (NS- not significant).

4.4.3.2 Measurement of MMP-9 protein expression in in tracheal epithelial cells (upper airway cells) from the subglottic area

MMP9 production by tracheal epithelial cells following 48h exposure to BAs at concentrations of 1 and 10 $\mu\text{mol/L}$ was measured using ELISA. Maximal non-lethal conditions for bile acid stimulation were determined via cell viability assay. Cell viability was confirmed at >90% (Figure 4-4). Figure 4-16 shows that this treatment caused MMP9 levels to significantly increase as follows: TGF β 1 at 5 ng/ml caused 1800 \pm 19 pg/ml MMP-9 to be produced. LCA at 10 $\mu\text{mol/L}$ caused 518 \pm 9 pg/ml MMP-9 to be produced and LCA at 1 $\mu\text{mol/L}$ generated 1744 \pm 67 pg/ml MMP-9, showing a negative concentration dependency effect. DCA at 10 $\mu\text{mol/L}$ caused 850 \pm 32pg/ml MMP-9 to be produced and DCA at 1 $\mu\text{mol/L}$ generated 613 \pm 27 pg/ml MMP-9, showing a positive concentration dependency effect. CA at 10 $\mu\text{mol/L}$ caused 261 \pm 9 pg/ml MMP-9 to be produced and CA at 1 $\mu\text{mol/L}$ generated 121 \pm 4 pg/ml MMP-9 which did not increase significantly compared to control, showing a negative concentration dependency effect. CDCA at 10 $\mu\text{mol/L}$ caused 236 \pm 18 pg/ml MMP-9 to be produced and CDCA at 1 $\mu\text{mol/L}$ generated 298 \pm 18 pg/ml MMP-9, showing no concentration dependency effect. The mean level of MMP-9 production in control cells was 65 \pm 4 pg/ml.

MMP-9 levels produced by tracheal epithelial cells

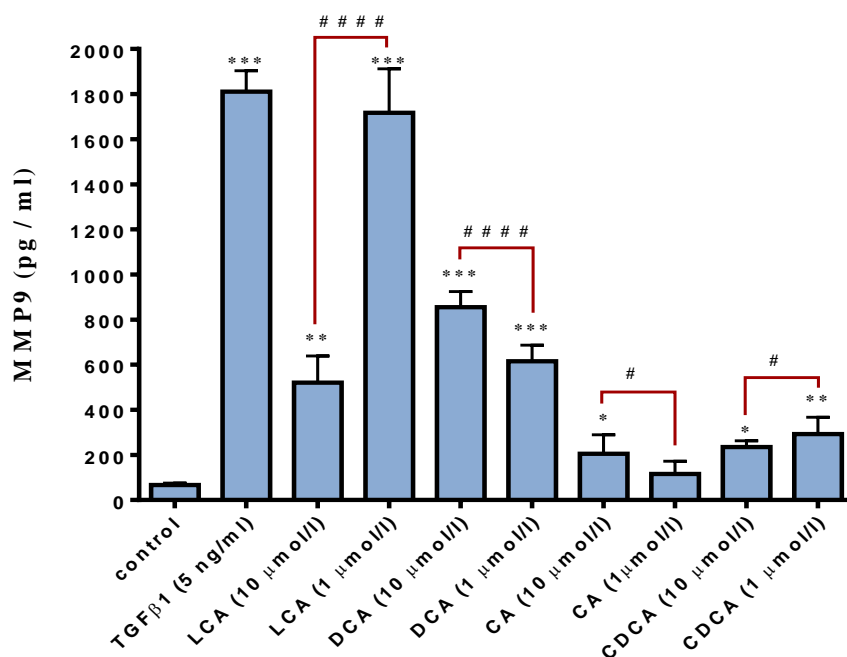


Figure 4-16: ELISA of MMP-9 production from tracheal epithelial cells from the subglottic area cell line treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS-not significant).

4.4.3.3 Measurement of MMP-9 protein expression in BEAS-2B epithelial cell lines

MMP9 production by BEAS-2B cells following 48h exposure to BAs at the following concentrations: LCA (1 and 5 $\mu\text{mol/L}$), DCA (15 and 30 $\mu\text{mol/L}$), CA (30 and 50 $\mu\text{mol/L}$) and CDCA (30 and 50 $\mu\text{mol/L}$), was measured using ELISA. These concentrations of the bile acids were the maximal concentration that did not cause high cell death based on cell viability (Figure 3-8 Figure 3-9). Cell viability was confirmed at >90%. Figure 4-17 shows that this treatment caused MMP9 levels to significantly increase as follows: TGF β 1 at 5 ng/ml caused 925 \pm 19 pg/ml MMP-9 to be produced. LCA at 5 $\mu\text{mol/L}$ caused 770 \pm 27 pg/ml MMP-9 to be produced and LCA at 1 $\mu\text{mol/L}$ generated 367 \pm 22 pg/ml MMP-9, showing a positive concentration dependency effect. DCA at 30 $\mu\text{mol/L}$ caused 431 \pm 43 pg/ml MMP-9 to be produced and DCA at 15 $\mu\text{mol/L}$ generated 246 \pm 8 pg/ml MMP-9, showing a positive concentration dependency effect. CA at 50 $\mu\text{mol/L}$ caused 558 \pm 12 pg/ml MMP-9 to be produced and CA at 30 $\mu\text{mol/L}$ generated 415 \pm 6 pg/ml MMP-9, showing a positive concentration dependency effect. CDCA at 50 $\mu\text{mol/L}$ caused 414 \pm 7 pg/ml MMP-9 to be produced and CDCA at 30 $\mu\text{mol/L}$ generated 384 \pm 11 pg/ml MMP-9, showing no concentration dependency effect. The mean level of MMP-9 production in control cells was 109 \pm 8pg/ml.

MMP-9 levels produced by BEAS-2B cells

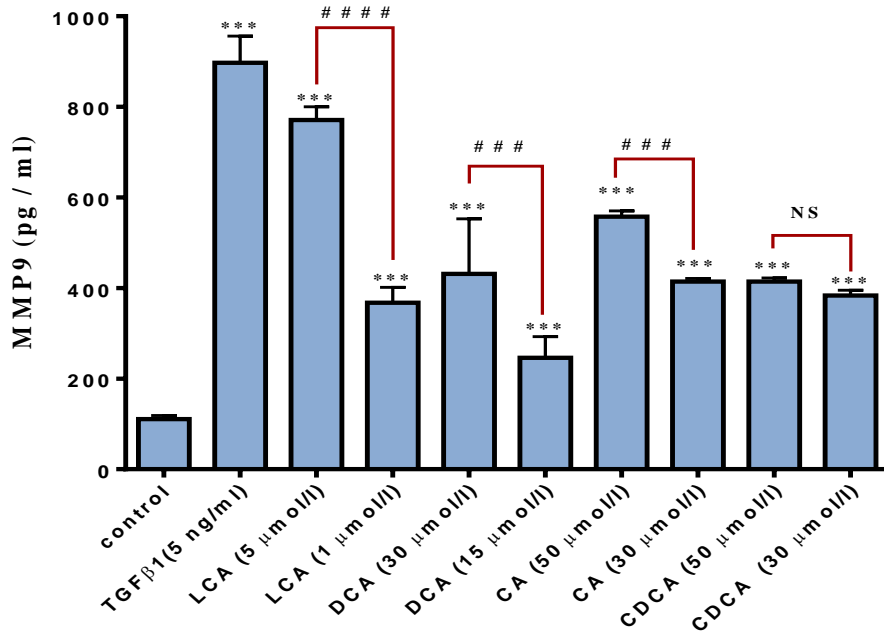


Figure 4-17: ELISA of MMP-9 production from BEAS-2B cell line treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean ± S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.3.4 Measurement of MMP-9 protein expression in 16HBE14o- epithelial cell lines

MMP9 production by 16HBE14o- cells following 48h exposure to BAs at the following concentrations: LCA (1 and 10 $\mu\text{mol/L}$), DCA (15 and 30 $\mu\text{mol/L}$), CA (50 and 75 $\mu\text{mol/l}$) and CDCA (30 and 50 $\mu\text{mol/L}$), was measured using ELISA. These concentrations of the bile acids were the maximal concentration that did not cause high cell death based on cell viability (Figure 3-2 Figure 3-3). Cell viability was confirmed at >90%. Figure 4-18 shows that this treatment caused MMP9 levels to significantly increase as follows: TGF β 1 at 5 ng/ml caused 1356 \pm 11pg/ml MMP-9 to be produced. LCA at 10 $\mu\text{mol/L}$ caused 155 \pm 7pg/ml MMP-9 to be produced and LCA at 1 $\mu\text{mol/L}$ generated 265 \pm 8pg/ml MMP-9, showing a negative concentration dependency effect. DCA at 30 $\mu\text{mol/L}$ caused 402 \pm 7pg/ml MMP-9 to be produced and DCA at 15 $\mu\text{mol/L}$ generated 269 \pm 4 pg/ml MMP-9, showing a positive concentration dependency effect. CA at 75 $\mu\text{mol/L}$ caused 307 \pm 7pg/ml MMP-9 to be produced and CA at 50 $\mu\text{mol/L}$ generated 254 \pm 12 pg/ml MMP-9, showing no concentration dependency effect. CDCA at 50 $\mu\text{mol/L}$ caused 475 \pm 7pg/ml MMP-9 to be produced and CDCA at 30 $\mu\text{mol/L}$ generated 577 \pm 10 pg/ml MMP-9, showing a negative concentration dependency effect. The mean level of MMP-9 production in control cells was 101 \pm 4pg/ml.

MMP-9 levels produced by 16HBE14o- cells

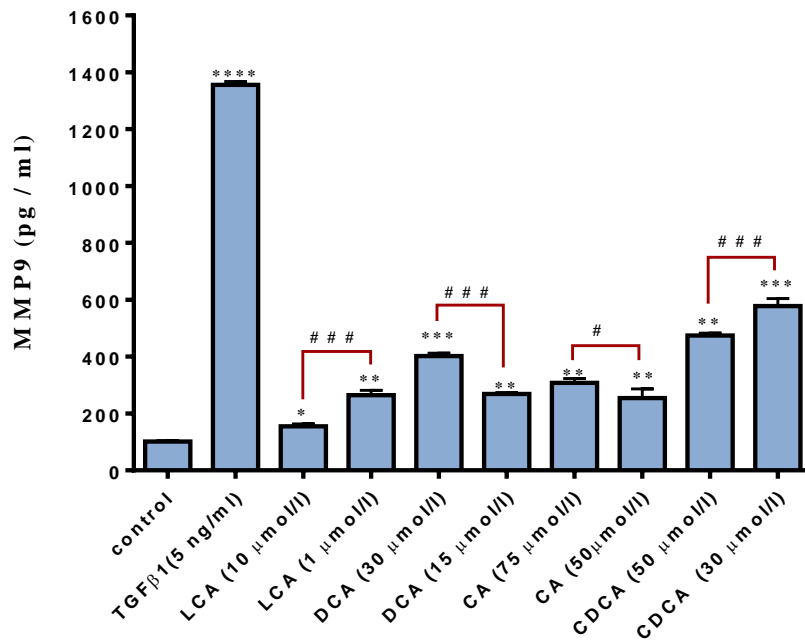


Figure 4-18: ELISA of MMP-9 production from 16HBE14o- cell line treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.4 Measurement of pro-collagen expression

4.4.4.1 Measurement of pro-collagen protein expression in primary human bronchial epithelial cell cultures from lung allografts (PHBEC)

Pro-collagen protein production by PHBECs following exposure to BAs at concentrations of 1 and 10 $\mu\text{mol/L}$ was measured using ELISA. Maximal non-lethal conditions for bile acid stimulation were determined via cell viability assay. Cell viability was confirmed at >90% (Figure 4-1). Control cells produced little or no pro-collagen, but exposure to 5ng/ml TGF- β 1 caused a significant increase in pro-collagen production. Exposure to BAs caused a significant increase and these results are shown in Figure 4-19. 48h exposure of PHBECs to LCA, DCA, CA and CDCA at 1 and 10 $\mu\text{mol/L}$ have been shown to cause a significant increase in pro-collagen production as follows: TGF β 1 at 5 ng/ml caused 1326 \pm 66 pg/ml pro-collagen protein to be produced. LCA at 10 $\mu\text{mol/L}$ caused 389 \pm 32 pg/ml of pro-collagen protein to be produced and LCA at 1 $\mu\text{mol/L}$ generated 203 \pm 28 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. DCA at 10 $\mu\text{mol/L}$ caused 214 \pm 11 pg/ml of pro-collagen protein to be produced and DCA at 1 $\mu\text{mol/L}$ generated 171 \pm 19 pg/ml of pro-collagen protein, showing no concentration dependency effect. CA at 10 $\mu\text{mol/L}$ caused 220 \pm 13 pg/ml of pro-collagen protein to be produced and CA at 1 $\mu\text{mol/L}$ generated 218 \pm 12 pg/ml of pro-collagen protein, showing no concentration dependency effect. CDCA at 10 $\mu\text{mol/L}$ caused 274 \pm 18 pg/ml of pro-collagen protein to be produced and CDCA at 1 $\mu\text{mol/L}$ generated 147 \pm 12 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. The mean level of pro-collagen protein production in control cells was 41 \pm 12 pg/ml.

Pro-collagen levels produced by PHBEC

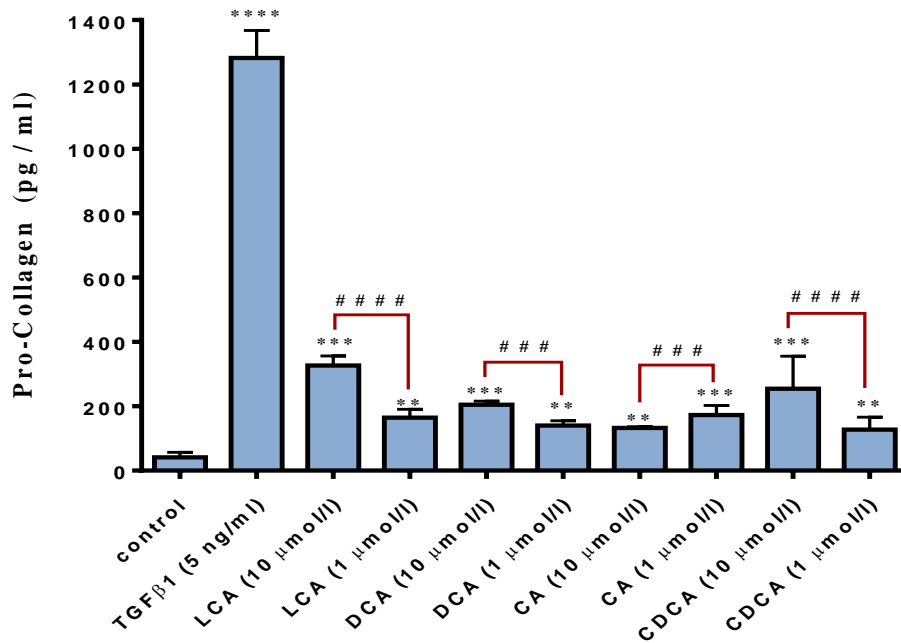


Figure 4-19: ELISA of Pro-collagen production from primary human bronchial epithelial cell cultures from lung allografts (PHBEC) treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.4.2 Measurement of pro-collagen protein expression in tracheal epithelial cells (upper airway cells) from the subglottic area

Pro-collagen protein production by tracheal epithelial cells following exposure to BAs at concentrations of 1 and 10 $\mu\text{mol/L}$ was measured using ELISA. These concentrations of the bile acids were the maximal concentration that did not cause high cell death based on cell viability. Cell viability was confirmed at $>90\%$ (Figure 4-4). Control cells produced little or no pro-collagen, but exposure to BAs caused a significant increase and these results are shown in Figure 4-20 48h exposure of damaged tracheal epithelial cells from the subglottic area to LCA at concentration 1 and 10 $\mu\text{mol/L}$ and 10 $\mu\text{mol/l}$ DCA have been shown to cause a significant increase in pro-collagen production as follows: TGF β 1 at 5 ng/ml caused 927 \pm 26 pg/ml pro-collagen protein to be produced. LCA at 10 $\mu\text{mol/L}$ caused 120 \pm 5 pg/ml of pro-collagen protein to be produced and LCA at 1 $\mu\text{mol/L}$ generated 116 \pm 12 pg/ml of pro-collagen protein, showing no concentration dependency effect. DCA at 10 $\mu\text{mol/L}$ caused 93 \pm 18 pg/ml of pro-collagen protein to be produced. Production of pro-collagen protein after stimulation with DCA at 1 $\mu\text{mol/L}$, CA at 1 and 10 $\mu\text{mol/L}$ and CDCA 1 and 10 $\mu\text{mol/L}$ did not increased pro-collagen protein significantly. DCA at 1 $\mu\text{mol/L}$ generated 74 \pm 6pg/ml of pro-collagen protein. CA at 10 $\mu\text{mol/L}$ caused 55 \pm 7 pg/ml of pro-collagen protein to be produced and CA at 1 $\mu\text{mol/L}$ generated 67 \pm 3 pg/ml of pro-collagen protein. CDCA at 1 $\mu\text{mol/L}$ caused 40 \pm 5 pg/ml of pro-collagen protein to be produced and CDCA at 10 $\mu\text{mol/L}$ generated 42 \pm 6 pg/ml of pro-collagen protein. The mean level of pro-collagen protein production in control cells was 28 \pm 26 pg/ml.

Pro-collagen levels produced by tracheal epithelial cells

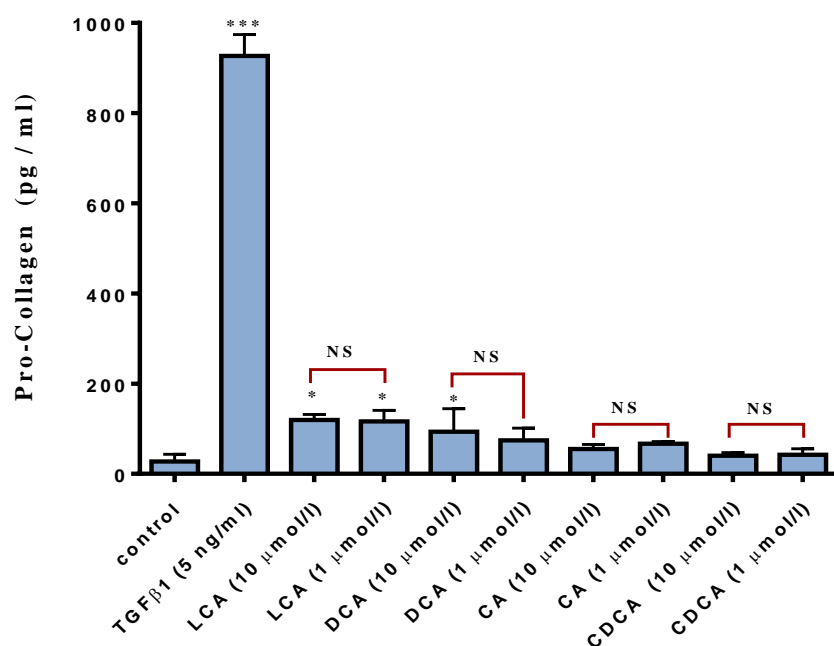


Figure 4-20: ELISA of Pro-collagen production from tracheal epithelial cells from the subglottic area treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's t-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.4.3 Measurement of pro-collagen protein expression in BEAS-2B epithelial cell lines

Pro-collagen protein production by BEAS-2B cells following exposure to BAs at the following concentrations: LCA (5 and 1 $\mu\text{mol/L}$), DCA (30 and 15 $\mu\text{mol/L}$), CA (50 and 30 $\mu\text{mol/L}$) and CDCA (50 and 30 $\mu\text{mol/L}$), which did not have significant effect on the cell viability (Figure 3-8 Figure 3-9), was measured using ELISA. Exposure to BAs caused a significant increase in pro-collagen protein production as shown in Figure 4-21. TGF β 1 at 5 ng/ml caused 520 \pm 6 pg/ml pro-collagen protein to be produced. LCA at 5 $\mu\text{mol/L}$ caused 344 \pm 18 pg/ml of pro-collagen protein to be produced and LCA at 1 $\mu\text{mol/L}$ generated 296 \pm 8 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. DCA at 30 $\mu\text{mol/L}$ caused 294 \pm 8 pg/ml of pro-collagen protein to be produced DCA at 15 $\mu\text{mol/L}$ generated 312 \pm 6 pg/ml of pro-collagen protein, showing no concentration dependency effect. CA at 50 $\mu\text{mol/L}$ caused 205 \pm 11 pg/ml of pro-collagen protein to be produced CA at 30 $\mu\text{mol/L}$ generated 218 \pm 6 pg/ml of pro-collagen protein, showing no concentration dependency effect. CDCA at 50 $\mu\text{mol/L}$ caused 208 \pm 8 pg/ml of pro-collagen protein to be produced CDCA at 30 $\mu\text{mol/L}$ generated 185 \pm 8 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. The mean level of pro-collagen protein production in control cells was 47 \pm 2 pg/ml.

Pro-collagen levels produced by BEAS-2B cells

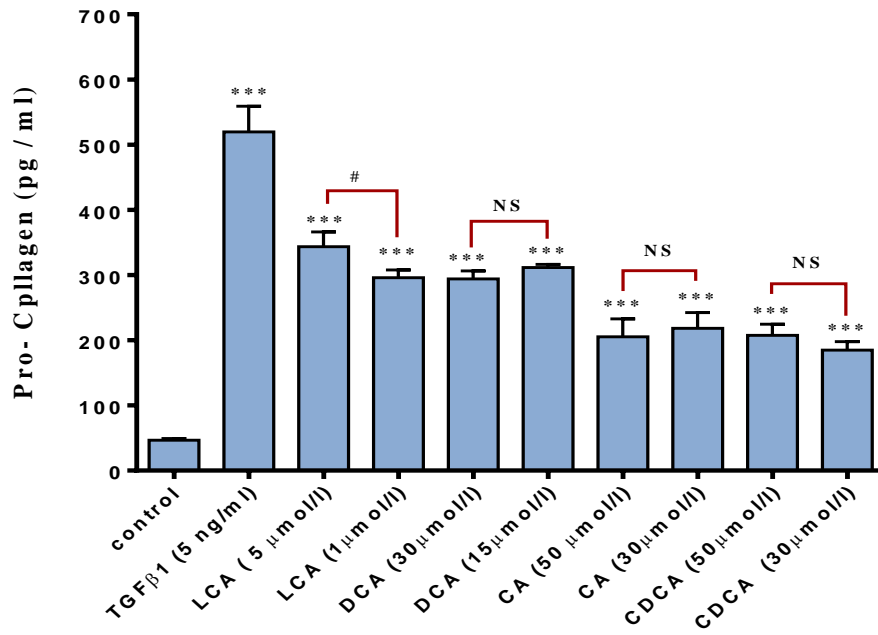


Figure 4-21: ELISA of Pro-collagen production from BEAS-2B cell line treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.4.4.4 Measurement of pro-collagen protein expression 16HBE14o- epithelial cell lines

Pro-collagen protein production by 16HBE14o- cells following exposure to BAs at the following concentrations: LCA (1 and 10 $\mu\text{mol/L}$), DCA (15 and 30 $\mu\text{mol/L}$), CA (50 and 75 $\mu\text{mol/L}$) and CDCA (30 and 50 $\mu\text{mol/L}$), which did not have significant effect on the cell viability (Figure 3-2 and Figure 3-3), was measured using ELISA. Exposure to BAs caused a significant increase in pro-collagen protein production as shown in Figure 4-22. TGF β 1 at 5 ng/ml caused 130 \pm 19 pg/ml pro-collagen protein to be produced. LCA at 10 $\mu\text{mol/L}$ caused 152 \pm 2 pg/ml of pro-collagen protein to be produced and LCA at 1 $\mu\text{mol/L}$ generated 9 \pm 4 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. DCA at 30 $\mu\text{mol/L}$ caused 128 \pm 4 pg/ml of pro-collagen protein to be produced and DCA at 15 $\mu\text{mol/L}$ generated 100 \pm 2 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. CA at 75 $\mu\text{mol/L}$ caused 79 \pm 6 pg/ml of pro-collagen protein to be produced and CA at 50 $\mu\text{mol/L}$ generated 85 \pm 5 pg/ml of pro-collagen protein, showing no concentration dependency effect. CDCA at 50 $\mu\text{mol/L}$ caused 128 \pm 2 pg/ml of pro-collagen protein to be produced and CDCA at 30 $\mu\text{mol/L}$ generated 77 \pm 6 pg/ml of pro-collagen protein, showing a positive concentration dependency effect. The mean level of pro-collagen protein production in control cells was 40 \pm 6 pg/ml.

Pro-collagen levels produced by 16HBE14o- cells

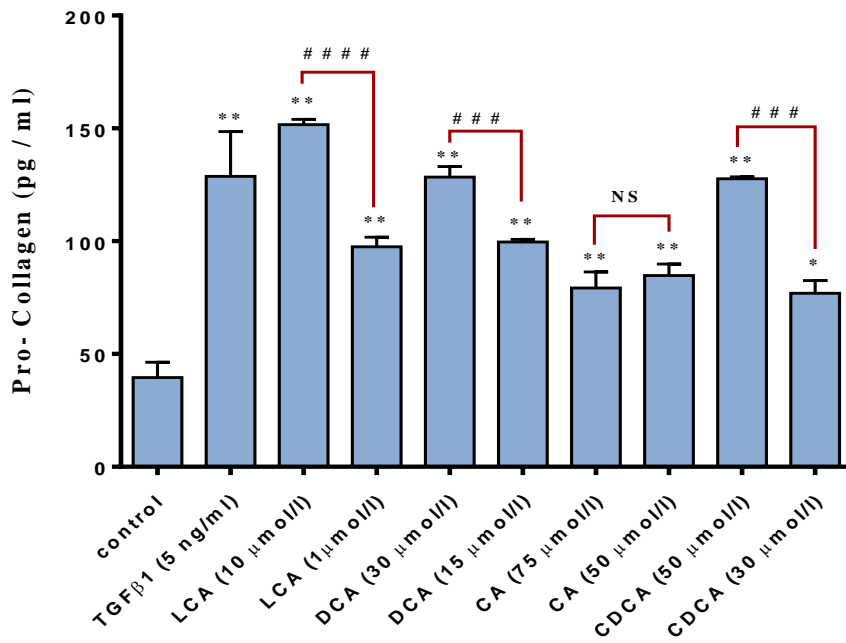


Figure 4-22: ELISA of Pro-collagen production from 16HBE14o- cell line treated with LCA, DCA, CA and CDCA. Statistical analysis was conducted by repeated-measures-paired-one-way ANOVA to compare to control. The data are representative of three independent experiments (each experiment n=6 wells). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. For pairwise analysis Student's *t*-test #: p<0.05, ##: p<0.01, ###: p<0.001 and ####: p<0.0001. (NS- not significant).

4.5 Discussion

The results in this chapter provide evidence for active epithelial-mesenchymal transition (EMT) in primary epithelial cell cultures from lung allograft, tracheal epithelial cells (upper airway cells) from the subglottic area, BEAS-2B cell lines and 16HBE14o- cell lines after stimulation with both primary and secondary BAs at varying concentrations.

Gastroesophageal reflux disease (GORD) and extraoesophageal reflux disease (EORD) can affect patients with a wide variety of airway diseases. A causative link has been hypothesised between respiratory disorders and GORD/EORD. This is based on the facts that airway epithelial cells produce fibroblast-promoting growth factors and that bile acid (BA) exposure leads to a fibrotic response. GOR symptoms and the pathologic reflux caused by COPD exacerbation (Casanova et al., 2004), are strongly correlated (Rascon-Aguilar et al., 2006), and GORD is also common among asthma sufferers (Hsu et al., 2005), especially in severe cases (Leggett et al., 2005). Idiopathic pulmonary fibrosis patients are especially prone to GORD (Tobin et al., 1998; Raghu et al., 2006) and may still aspirate duodenogastroesophageal refluxate even after undergoing a lung transplant. In this case, there is a correlation with bronchiolitis obliterans, which also is a fibrotic airway disorder (D'Ovidio et al., 2005).

This data supports the hypothesis that human airway epithelial cells exposed to BAs may undergo EMT, causing the formation of fibroblasts. To the best of my knowledge, this is the first time data of this type has been presented.

During embryo development, EMT is involved in organogenesis. It also plays a vital role in wound healing (Hay, 2005). When EMT becomes dysregulated however, it contributes to cancer progression and metastasis, and to the development of chronic degenerative fibrotic disorders in the lung as well as several other organs (Thiery, 2002; Wilson and Wynn, 2009). This can have several consequences, including COPD, pulmonary fibrosis and lung allografts rejection. Therefore, EMT is implicated in the pathogenesis of these conditions (Kalluri and Neilson, 2003; Lee *et al.*, 2009). In spite of an increasing evidence base that suggests a connection between EMT and fibrotic remodelling in the lungs and other organs, it has not been shown to play a role in the airway remodelling and fibrosis and there is little evidence that bronchial epithelial cells are affected (Doerner and Zuraw, 2009).

Patients with a range of airway diseases often exhibit gastroesophageal reflux (GORD). Cytokines and growth factors involved in fibroblast migration, proliferation, and myofibroblast differentiation in the lungs are stimulated by BAs, which can be aspirated during GORD and therefore GORD may play a prominent role in lung injury and airway fibrosis (Zhang *et al.*, 2001; Perng *et al.*, 2003). Airway fluids may contain a number of BAs including the unconjugated BAs used in this study (Legendre *et al.*, 2014). BALF has been shown to contain BAs at concentrations up to 240 $\mu\text{mol/L}$ (D'Ovidio *et al.*, 2005; Parikh *et al.*, 2013). The BAs used in this study therefore have profibrotic effects that are pathophysiologically relevant (Chen *et al.*, 2016).

The results of this study indicate that primary human bronchial epithelial cell cultures from lung allografts (PHBEC), when exposed to CA and CDCA at 1 and 10 $\mu\text{mol/L}$ significantly reduce E-cadherin expression. On the other hand E-cadherin gene expression levels did not decrease after stimulation with LCA and DCA. Moreover, fibronectin expression was significantly upregulated by CA and CDCA at 1 and 10 $\mu\text{mol/L}$, whereas fibronectin expression did not increase significantly with 1 and 10 $\mu\text{mol/L}$ LCA and DCA. These findings may indicate that CDCA and CA (primary bile acids) were more effective when compared to DCA and LCA (secondary bile acids).

Furthermore, in this study, tracheal epithelial cells (upper airway cells) from the subglottic area, which was derived from the subglottic airway and provide a physiological model of the upper airway *in vitro* (Powell J, 2015) was stimulated with BAs to assess E-cadherin and fibronectin expression. The most interesting finding was that the E-cadherin expression was significantly downregulated in 10 $\mu\text{mol/L}$ LCA, 1 and 10 $\mu\text{mol/L}$ CA and CDCA, whereas E-cadherin gene expression levels were similar to the control in 1 $\mu\text{mol/L}$ LCA and DCA treatment groups. Consequently, it would appear that upper airway cells are affected by BAs, which induce EMT.

These findings show that the upper airway cells and lower airway cells are affected by primary bile acids. On the other hand only LCA at 10 $\mu\text{mol/mL}$ caused downregulation in E-cadherin and increased fibronectin expression in upper airway cells but not lower airway cells. The secondary BAs may be more harmful and the concentration of LCA may be higher than other BAs in upper airway. LCA was identified in trachea at concentration of 2.8 ± 0.6 $\mu\text{mol/L}$ in patients with ventilator associated pneumonia (Wu *et al.*, 2009).

Polygonal epithelial cells adhere to form a tight mesh, creating the airway epithelial sheet (Sohal *et al.*, 2014). E-cadherin, a synthesis transmembrane glycoprotein found primarily in the adherens junctions between epithelial cells, has been implicated as a marker of differentiated epithelial phenotype (Arias, 2001; Zeisberg *et al.*, 2003). It is downregulated when epithelial cells transform (Yang and Liu, 2001), but plays a key role in maintaining epithelial polarity and consequently in tight junction development (Tunggal *et al.*, 2005). Epithelial-Mesenchymal Transition (EMT) in both cancerous and non-cancerous cells involves several steps, beginning with E-cadherin inhibition. Once the mesenchymal phenotype is fully realised, mesenchymal markers, such as fibronectin, proliferate. Fibronectin is involved in a range of processes, including cell adhesion and migration, and tissue repair (Miettinen *et al.*, 1994).

EMT like activity was induced in BEAS-2B and 16HBE14o- cells through BA exposure, indicating that the effect is not limited to primary cells, but that human bronchial epithelial cell lines (*in vitro*) are also vulnerable. I observed that the 16HBE14o- cells were more sensitive to BAs treatment and EMT induction than the BEAS-2B cells. For example in this study, immortalized cell line 16HBE14o-, which was derived from bronchial surface epithelial cells (Cozens *et al.*, 1994), were exposed to BAs at different concentration to assess E-cadherin and fibronectin expression. The results revealed a significant decrease in E-cadherin expression at all concentrations, also it was found that fibronectin expression increased at all concentrations of BAs.

On the other hand the E-cadherin expression in BEAS-2B, which significantly decreased in response to (15 and 30 $\mu\text{mol/l}$) DCA, (30 and 50 $\mu\text{mol/l}$) CDCA, (5 $\mu\text{mol/l}$) LCA and (50 $\mu\text{mol/l}$) CA, but there was no significant effect from 1 μmol LCA and 30 μmol CA exposure. Fibronectin expression, was significantly increased with (1 and 5 $\mu\text{mol/l}$) LCA, (15 $\mu\text{mol/l}$) DCA, (50 $\mu\text{mol/L}$) CA and (30 $\mu\text{mol/l}$) CDCA. However, (30 $\mu\text{mol/L}$ CA) and (50 $\mu\text{mol/l}$) CDCA had no significant effect.

BEAS-2B cells retain the morphology of an epithelial cell *in vitro* (Reddel *et al.*, 1988) and release IL-8 and IL-6 in addition to other cytokines. Under submerged conditions, BEAS-2B cells are commonly used as in the *in vitro* model of human airways epithelium- especially within studies on inhalation toxicity (Cha *et al.*, 2007; Petecchia *et al.*, 2009).

Furthermore, Cells were also stimulated with 5ng/ml TGF- β 1 as a positive control. An optimum concentration of TGF- β 1 (5 ng/ml) was used to stimulate primary cells and cell

lines. It was assumed that the same concentration was optimum to induce stress in cells as well. Published studies suggest that 5ng/ml of TGF- β 1 induces morphological changes in this immortalised cell lines (Doerner and Zuraw, 2009; Câmara and Jarai, 2010; Liu *et al.*, 2013).

An additional analysis was performed to detect TGF β 1, MMP9 and Pro-collagen secreted by primary bronchial human epithelial cell cultures from lung allografts (PHBECs), tracheal epithelial cells (upper airway cells) from the subglottic area and cell lines (BEAS-2B and 16HBE14o-) by using an ELISA. In this experiment, BAs stimulation of cells were associated with the release of TGF β 1, MMP9, but the most interesting finding was the production of pro-collagen protein after stimulation of human subglottic cells with DCA at 1 μ mol/L, CA at 1 and 10 μ mol /L and CDCA 1 and 10 μ mol/L did not increased significantly. A recent study suggested a significantly higher number of reflux events detected in laryngopharyngeal reflux (LPR) patients than patients with GERD (Hom and Vaezi, 2013). Moreover, patients with LPR may have a high concentration of bile acids and the concentration of BAs that reach the lower airway may be diluted (Sifrim, 2015). As a result the upper airway cells may become resistant. Furthermore, the secondary BAs can be more cytotoxic than primary BAs to upper airway cells.

Epithelial cells also generate matrix metalloproteinase 9 (MMP-9), a type IV collagenase, the presence of which can indicate EMT and epithelial damage. Col4 is the primary structural component of the true reticular basement membrane, which epithelial cells sit upon. When MMPs disrupt the airway epithelium, various forms of damage can occur, including translocation, inflammation and further EMT (Kalluri and Neilson, 2003).

A recent study has also shown that lung fibroblasts and alveolar epithelial cells (AECs) can be activated by BAs through both FXR-dependent and independent pathways. These findings have enabled a new perspective on the role of BA microaspiration on IPF development (Chen *et al.*, 2016). Mesenchymal trans-differentiation of AECs in pulmonary fibrosis is controlled by a number of key mechanisms. TGF β 1 exposure can induce EMT in both rat and human AECs in culture (Yao *et al.*, 2004; Willis *et al.*, 2005). Most of the EMT research in pulmonary fibrosis has focused on AECs.

The transcription factor, Farnesoid X receptor (FXR), contains both a DNA binding domain and a ligand binding domain, the latter of which is sensitive to BAs (Abu-Hayyeh *et al.*, 2013), and provides a mechanism for BAs to regulate their own synthesis and metabolism. It is interesting to note that apart from a role in BA homeostasis there is widespread expression

of FXR in tissues in humans and this includes expression in the airway and lung (Bishop-Bailey *et al.*, 2004) As a result, the lung's inflammatory response and its repair processes repair are critically dependent on the role of FXR (Zhang *et al.*, 2012).

There is evidence to suggest that FXR has a novel function in the regulation of hepatic inflammation and that its ligands might potentially serve a role in inflammatory liver disease treatment as well as the prevention of hepatocarcinogenesis. FXR is a known antagonist in the NF- κ B1 pathway, whose role in both the inflammatory response and in the mediation of tumor promotion therefore implicates FXR as playing a critical role in the inflammatory response (Greten *et al.*, 2004; Pikarsky *et al.*, 2004). The NF- κ B1 pathway is suppressed by FXR – irrespective of the presence of its exogenous ligand – in HepG2 cells at the gene transcription level. GW4064, an FXR agonist, inhibits the binding activity between NF- κ B1 and the DNA sequence, resulting in suppressed NF- κ B1 regulated gene expression in both HepG2 and mouse primary hepatocytes (Wang *et al.*, 2008).

Hepatocyte BA levels are decreased through the following mechanism: DNA binds with activated FXR to form a complex with the retinoid x receptor. In turn, this stimulates the transcription of various genes, which code for transporters (e.g. ABC transporters) that facilitate BA transfer from hepatocytes to the bile (Holt *et al.*, 2003). It was confirmed recently that the expression of CFTR, a member of the ABC transporter family, in epithelial cells might be regulated by activated FXR, thereby modulating chronic secretion (Mroz *et al.*, 2014). FXR has also been implicated in Cl^- secretion downregulation in the colonic epithelium and that Na^+/K^+ ATPase inhibition appears to regulate this process (Keating and Keely, 2009).

A study by Lafyatis and Farina explores the involvement of gut microbiota in toll-like receptor (TLR – primarily TLR2 and TLR4) signalling, which aggravates metabolic inflammation. TLRs play a significant role in the recognition of pathogen-associated molecular patterns, which are found on bacterial cell walls. For example, TLR4 is a ligand of lipopolysaccharide (LPS) (Lafyatis and Farina, 2012). Vavassori *et al.* found that an anti-inflammatory effect can be achieved by activation of FXR-modulated TLR4 (Vavassori *et al.*, 2009).

Bogdasarian and Olson first noted the association with reflux subglottic stenosis in 1980 (Bogdasarian and Olson, 1980) in their article on posterior glottis stenosis, which noted that gastric reflux should be added to the list of factors which cause posterior subglottic stenosis.

Other studies since then have also confirmed the relationship between acquired subglottic stenosis (ISS) and GORD (Little *et al.*, 1984). Koufman has conducted the largest study to date, using 197 patients with presumed GERD and a range of associated symptoms including laryngitis 48 patients, tracheal and subglottic stenosis 32 patients, tracheal and subglottic stenosis 32 patients, carcinoma of the larynx 31 patients, cough 25 patients, globus pharyngeus 24 patients, dysphagia 22 patients and miscellaneous 15 patients (Koufman, 1991).

Cells under submerged conditions have also been studied. Xie *et al.* investigated epithelial cells from asthmatic and non-asthmatic donors in two conditions: submerged and air-liquid interface conditions. It was found that EMT was induced by TGF- β 1 in both conditions (Xie *et al.*, 2004). These findings are supported by similar studies, which have found EMT-related changes in the polarized layer forming 16HBE-14o- cell line (Zhang *et al.*, 2009).

These findings have been supported by other studies, which have demonstrated that airway epithelial cells undergo EMT *in vitro* and that epithelial cells from obliterative bronchiolitis patients express mesenchymal cell markers, detected by immunohistochemistry (Borthwick *et al.*, 2009). Increased mesenchymal marker S100A4 expression in airway epithelial cells from lung transplant patients suggests that airway epithelial cells can differentiate into mesenchymal cells (Ward *et al.*, 2005).

Work for this thesis has shown that BA stimulation of airway epithelial cells causes TGF- β 1 production to increase. It has been suggested that in many tissues, including the lungs, TGF- β 1 plays the role of a ‘master switch’ in triggering fibrosis (Sime and O’Reilly, 2001; Borthwick *et al.*, 2012). IPF patients are found to exhibit upregulated TGF- β 1 levels in their lungs. Furthermore, active TGF- β 1 expression has been found to cause a ‘dramatic fibrosis response’ in rats’ lungs. In addition, the inability to respond to TGF- β 1 affords protection from bleomycin induced fibrosis (Zhao *et al.*, 2002). In the development, carcinogenesis and fibrosis of various isoforms, TGF- β 1 is a significant inducer of EMT (Nawshad *et al.*, 2005).

Given the significant spike in TGF- β 1 production in bronchial epithelial cells found by this study, it is suggested that these cells could become migratory during fibrosis development. A recent study using airway epithelial cells from asthmatics found these cells to be particularly sensitized to TGF- β 1 induced EMT (Hackett *et al.*, 2009). This, along with my study, builds to case for EMT being triggered in the epithelium as well as within the alveoli. Kuroishi

report has also shown that mouse tracheal epithelial cells *in vitro* can also exhibit TGF- β 1-induced EMT (Kuroishi *et al.*, 2009).

A549 alveolar epithelial cells exhibit an EMT-like process in response to TGF- β 1, which is thought to be due to initiation of the Smad2 signalling pathway. This further supports the hypothesis of EMT occurring in human lung epithelial cells, although further research is needed, especially in relation to the expression profile for EMT-induced alveolar cells. This research could reveal a deeper understanding of the role of EMT in pulmonary fibrogenesis.

The use of cultured cells in these EMT studies generates discussion around authenticity (true EMT), or if these experiments are simply causing a 'stress response' in cells. In light of the evidence to date, I believe that 'true' transition from epithelial to mesenchymal phenotypes can be better demonstrated through studying the functional properties of cells, not just their protein expression. Mesenchymal cells can be functionally characterised by their expression of MMP9 (Borthwick *et al.*, 2009) and my study found that epithelial cells treated with BAs are able to release this enzyme, as well as pro-collagen. These findings provide further evidence for the case that EMT may occur in airway cells, which could infiltrate the basement membrane and migrate into the subepithelial layer. From this point, cells would be in a position to generate disproportionate amounts of connective tissue.

A range of chronic airway disorders are associated with GORD symptoms. This study has shown that airway fibrosis development could be associated with the presence of BAs, which trigger excessive cytokine and growth factor production. Experiments in lung fibrosis have shown that these substances cause myofibroblasts as well as fibroblast migration and proliferation via EMT (Kim *et al.*, 2006; Kim *et al.*, 2009).

These findings may have translational significance in airways disease and suggest new therapeutic possibilities where modifying GORD may have positive effects on airway and lung disease. In summary, this study provides evidence of EMT markers in primary epithelial cell cultures from lung allograft, tracheal epithelial cells (upper airway cells) from the subglottic area, BEAS-2B cell lines and 16HBE14o- cell lines. The EMT procedure may instigate a pathway following injury in common diseases characterised by airway remodelling and fibrosis. These results confirm that EMT like changes were induced in BEAS-2B and 16HBE14o- cells through BAs exposure, indicating that the effect is not limited to primary human bronchial epithelial cells and BEAS-2B and 16HBE14O-cell lines are a sensitive and

useful model to study human respiratory processes and diseases related to BAs aspiration-
instigating EMT.

CHAPTER 5: THE ROLE OF DUODENO-GASTRO- OESOPHAGEAL REFLUXATE IN MUCUS PRODUCTION FROM NCI-H292 AND CALU-3 CELL LINES

5.1 Introduction

A glycoprotein-rich mucus layer lines the luminal epithelial surfaces of the respiratory tract, providing barrier protection from noxious and pathogenic agents. It is also involved in reactions to inflammation and infection, which result in upregulation mucus production. Between the trachea and the bronchioles, in healthy lungs, this mucus comprises secretions from both glandular and epithelial cells (mucus/goblet cells) (Sigurdsson *et al.*, 2013). Chronic inflammatory airway diseases, such as Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), bronchiectasis and* severe asthma, are characterised by the hypersecretion of mucus (Fahy, 2002; Kim *et al.*, 2008a); thereby leading to airway narrowing and impaired lung function (Aikawa *et al.*, 1992).

Mucin – part of a family of large (several thousand kilodaltons) glycoproteins – is a primary component of secreted mucus. These glycoproteins play a fundamental role in determining mucus viscoelasticity (Corfield, 2015). The principal glycoproteins in airway mucus secretions are expressed by the MUC5AC and MUC5B genes (Thornton *et al.*, 1997; Voynow, 2002). MUC5AC is secreted primarily by goblet cells in the surface airway epithelium (Suzaki *et al.*, 2016) and MUC5B mainly by mucous and serous cells in the submucosal glands (Zanin *et al.*, 2016).

It has been possible to maintain primary epithelial cells for 2-3 passages on a plastic surface, but the populations are heterogeneous, and the mucin expressing phenotype has not been observed. Furthermore, it is expensive to maintain rigorous culture conditions. Nevertheless, it has been possible to grow, maintain and passage carcinoma cell lines from epithelial samples and these have been used as investigative models for diseases including CF, COPD, asthma and inflammatory processes (Berger *et al.*, 1999).

Epithelial carcinoma cell lines have included Calu-3 and NCI H292 cells, which have provided useful models for *in vitro* study of gene regulation. Moreover, these cells carry the significant benefit of allowing both MUC5 mRNA and MUC5 glycoprotein regulation to be studied within the same model (Berger *et al.*, 1999; Rose *et al.*, 2000).

Bile acid (BA) is found commonly in the airways and bronchoalveolar lavage fluid of patients with chronic airway diseases, in addition to in murine models. Therefore, it is thought that BAs have some involvement in airway disease development (Cohen-Cymerknoh *et al.*, 2013). BAs may also cause airway obstruction by increasing mucus production (D'Ovidio and Keshavjee, 2006). However, further research is required on the role of BAs in the airway and their involvement with mucosal membranes.

Here, I hypothesise that due to the connection between mucus hypersecretion and gastroesophageal reflux in diseases such as CF, COPD and asthma, the presence of BAs in the lung could be a primary cause of this increased mucin secretion via epithelial cell stimulation.

Consequently, several cell lines originating from respiratory tract epithelial tissue were studied to identify those that expressed MUC genes similar to those displayed in upper and lower respiratory tract tissues (Berger, Voynow *et al.* 1999). In particular, the investigation aimed to determine whether MUC5 RNA expressing cell lines also secreted MUC5 mucins. These cell lines could then be used to determine the effect of BAs on mucin expression and mucus cell differentiation; hence, their role in airway diseases. Mucin gene expression was surveyed in two respiratory tract carcinoma cell lines: Calu-3 and NCI-H292.

5.2 Aims of the study

This chapter will explore the potential for using NCI-H292 and Calu-3 cell lines *in vitro* as models for investigating the hypothesis, which states that BAs in the lung could stimulate lung epithelial cells to synthesise or secrete mucin. Primary experimental aims are as follows:

- To measure the mucin content of the culture medium using a periodic acid-Schiff's (PAS) assay.
- To measure MUC5AC and MUC5B using indirect ELISA and to determine the concentration of BAs that caused maximum MUC5AC and MUC5B mucin production.
- To evaluate MUC5AC expression in NCI-H292 cells and Calu-3 cells stimulated with BAs, by quantitative real-time PCR.
- To examine the expression profile of MUC5B in NCI-H292 and Calu-3 cells stimulated with BAs via quantitative real-time PCR.

5.3 Methods

5.3.1 Cell line culture

NCI-H292 and Calu-3 were cultured in six-well plates as described in chapter 2, sections 2.2.3 and 2.2.4. At about 80-90% confluence, these were rested for 24 hours, then exposed to primary and secondary BAs at a range of concentrations

5.3.2 Stimulation of NCI-H292 and Calu-3 cells with BAs

NCI-H292 and Calu-3 cell cultures were exposed to LCA, DCA, CA and CDCA for 48 hours at concentrations of 1, 5, 10, 15, 20, 30, 50, 75 and 100 $\mu\text{mol/L}$ as described in chapter 2, section 2.6.

5.3.3 Detection of MUC5AC and MUC5B mucins

5.3.3.1 Determination of mucin production by Periodic Acid-Schiff (PAS) assay

Mucin production by NCI-H292 and Calu-3 cells was examined and measured using a PAS assay described in chapter 2, sections 2.8.4.

5.3.3.2 Quantification of MUC5AC and MUC5B mucin concentration by indirect ELISA

NCI-H292 and Calu-3 cells were cultured and stimulated (described above see chapter 2, sections 2.2.3 and 2.2.4). MUC5AC and MUC5B mucins were analysed using indirect ELISA as described in chapter 2, sections 2.8.2 and 2.8.3.

5.3.4 Molecular biology

RNA was isolated using the RNEasy Mini-kit (Qiagen) and reverse transcribed using a Tetro cDNA synthesis kit (Bioline). A quantitative PCR reaction was used to determine specific gene expression changes at a molecular level (see chapter 2, section 2.9).

In studying BAs effect on MUC5B and MUC5AC gene regulation, the cells were stimulated with BA concentrations that cause the highest levels of MUC5AC and MUC5B mucin production was measured by ELISA.

5.4 Results

5.4.1 Effect of BAs on mucin production in NCI-H292 cells, quantified using the PAS method

NCI-H292 is a mucous producing cell strain (Newland and Richter, 2008). Following 48-hour BA exposure, mucin production by NCI-H292 was examined using the PAS assay.

Figure 5-1 illustrates that high mucin levels were produced significantly by cells stimulated with LCA at 1, 5, 10, 15 and 20 $\mu\text{mol/L}$ concentrations. However, no difference was found between the five levels of LCA.

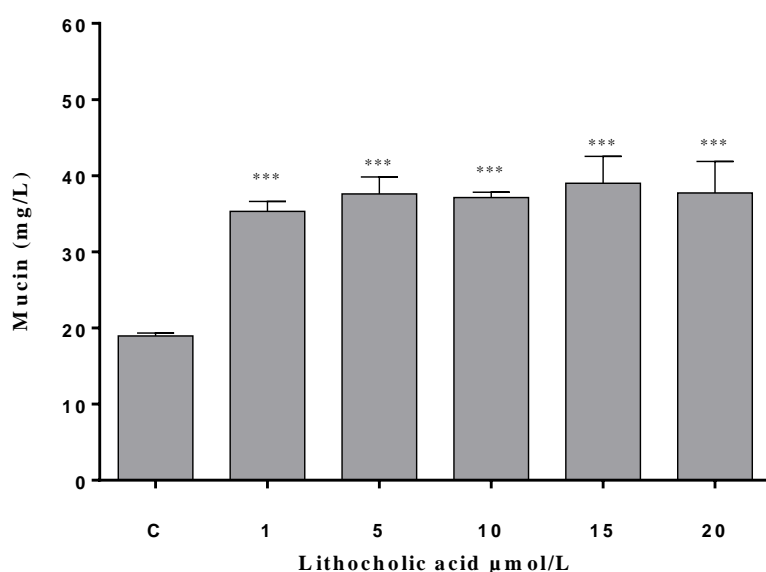


Figure 5-1: Mucin released by NCI-H292 cells following 48h LCA stimulation. PAS was used to measure mucin in cell supernatants, and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (***) = $p < 0.001$ compared with control, ANOVA).

Figure 5-2 shows a significant increase in mucin production by NCI-H292 cells challenged with 15, 30, 50 and 75 $\mu\text{mol/L}$ DCA in comparison with unchallenged cells. The highest level of mucin production was at concentration 15 $\mu\text{mol/L}$ of DCA. Moreover, there was an inverse dose response to DCA and 100 $\mu\text{mol/L}$ DCA did not induce mucin production.

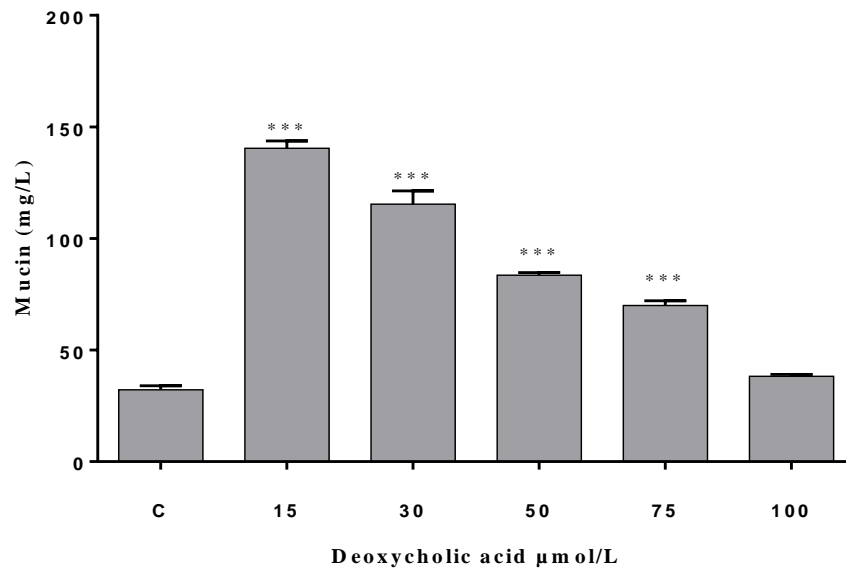


Figure 5-2: Mucin released by NCI-H292 cells following 48h DCA stimulation. PAS was used to measure mucin in cell supernatants, and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (***) = $p < 0.001$ compared with control, ANOVA).

Figure 5-3 presents a significant increase in mucin production by NCI-H292 cells challenged with 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CA in comparison with unchallenged cells. There was no difference in the level of mucin production by the NCI-H292 between any of the CA concentrations.

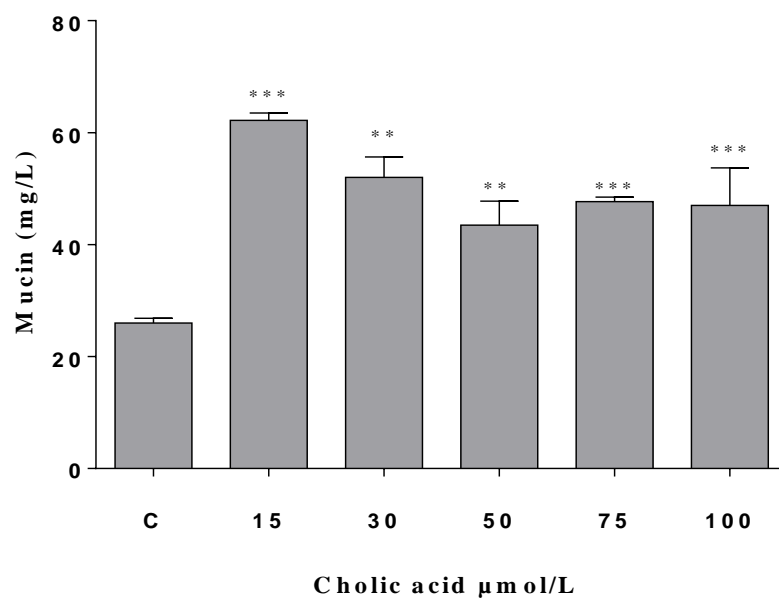


Figure 5-3: Mucin released by NCI-H292 cells following 48h CA stimulation. PAS was used to measure mucin in cell supernatants and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (** = $p < 0.01$, *** = $p < 0.001$ compared with control, ANOVA).

Figure 5-4 demonstrates a significant increase in mucin production by NCI-H292 cells challenged with 30, 50, 75 and 100 $\mu\text{mol/L}$ CDCA in comparison with unchallenged cells. The highest output of mucin was with 50 $\mu\text{mol/L}$. However, there was no significant increase at 15 $\mu\text{mol/L}$ CDCA.

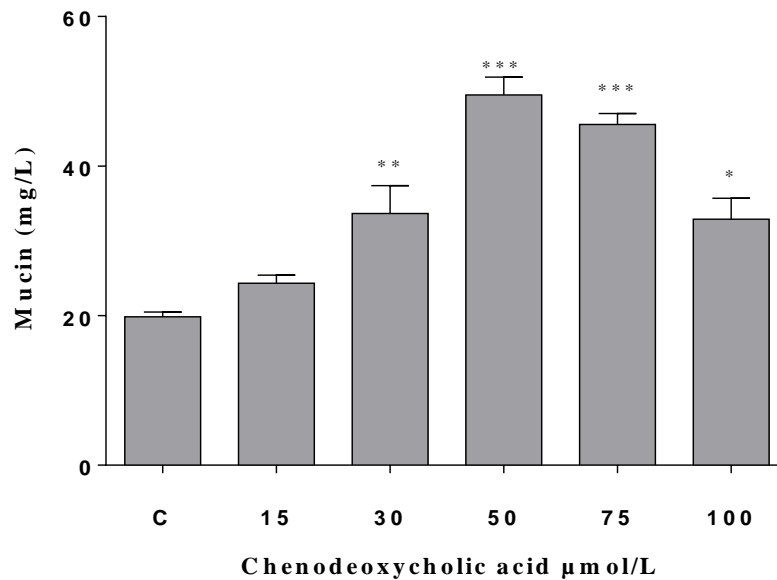


Figure 5-4: Mucin released by NCI-H292 cells following 48h CDCA stimulation. PAS was used to measure mucin in cell supernatants and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

In general, looking at mucin production by NCI-H292 cells measured by PAS assay, it can be seen that 15 and 30 $\mu\text{mol/L}$ of DCA had the highest level of mucin released. The second highest level of mucin production occurred with 15 $\mu\text{mol/L}$ of CA, 50 $\mu\text{mol/L}$ of CDCA also produced a similar level of mucin. Conversely, LCA caused the lowest level of mucin secretion due possibly to cell death (see Figure 3-14A), although mucin production by LCA remained significantly higher than the control (Figure 5-1 to Figure 5-4). The explanations for this fact are unclear yet but some possible explanations exist. Bile acids accumulate in cells and may inhibit intracellular function which does not kill the cell but reduces the rate of all cellular processes such as protein synthesis (Tabas, 2002). Moreover, extracellular matrix (ECM) may play a role in downregulation of mucin production. ECM component, type-IV collagen, inhibits MUC5AC production via integrin-signalling pathways, (Integrins are heterodimers composed of α 2- and β -subunit and are the main receptors involved in cell-cell and cell-ECM adhesion) (Iwashita et al., 2010).

5.4.2 Measurement of MUC5AC and MUC5B mucin in NCI-H292 cells

MUC5AC and MUC5B secretion by NCI-H292 cells is also affected by BA exposure. Levels of MUC5AC and MUC5B secreted by cells into the culture medium were measured using indirect ELISA

Figure 5-5 and Table 5-1 show that NCI-H292 exposure to LCA at concentrations of 1, 5 and 10 $\mu\text{mol/L}$ caused MUC5AC levels to increase significantly. There was an inverse dose-response to LCA. However, no significant increase was seen using concentrations of 15 and 20 $\mu\text{mol/L}$ LCA due to the cell death (see Figure 3-14A).

NCI-H292 cell line lung carcinoma cells also produce MUC5B. These cells were exposed to primary (CA and CDCA) and secondary (LCA and DCA) BAs. Figure 5-5 and Table 5-1 illustrate that exposure of NCI-H292 cells to LCA at concentrations of 1, 5, 10 and 15 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly. The highest response was at 1 $\mu\text{mol/L}$, but at 20 $\mu\text{mol/L}$ LCA there was no increase in MUC5B mucin due to cell viability, which is significantly decreased by LCA at 20 $\mu\text{mol/L}$ with $93\pm 3\%$ cell death (see Figure 3-14A).

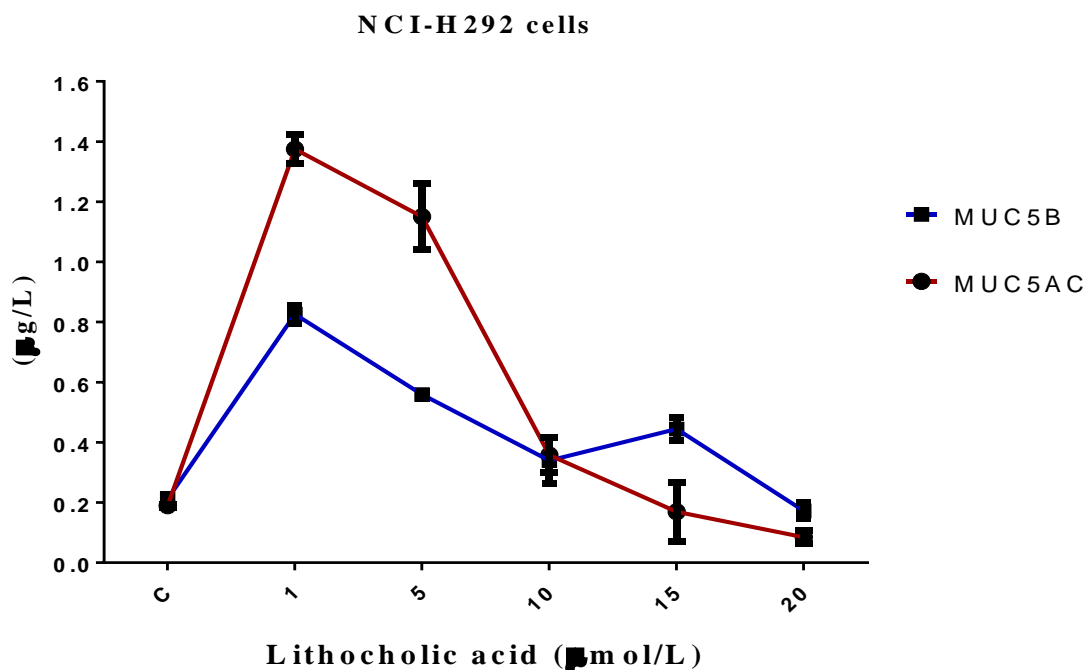


Figure 5-5: MUC5AC and MUC5B mucin release by NCI-H292 cells following exposure to LCA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-1). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	1	5	10	15	20
MUC5AC	↑***	↑***	↑*	_____	_____
MUC5B	↑****	↑***	↑**	↑**	_____

Table 5-1: The effect of different levels of Lithocholic acid on the NCL-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Figure 5-6 and Table 5-2 illustrate that exposure of NCI-H292 cells to DCA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5AC mucin levels to increase significantly. From the table, it is clear that MUC5AC mucin levels increased significantly at all concentrations. The highest level of MUC5AC mucin production was with 15 $\mu\text{mol/L}$ DCA and the level of MUC5AC mucin production then fell off due to cell death (see Figure 3-14B) but remained significantly higher than the control.

Furthermore, Figure 5-6 and Table 5-2 reveal that exposure of NCI-H292 cells to DCA at concentrations of 15 and 30 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly. The highest level of MUC5B mucin release occurred with 15 $\mu\text{mol/L}$ DCA. The MUC5B mucin levels reduced at 30 $\mu\text{mol/L}$ DCA but remained significantly above the control. There was no increase in MUC5B mucin due to cell viability being significantly affected by DCA at 50, 75 and 100 $\mu\text{mol/L}$ (see Figure 3-14B).

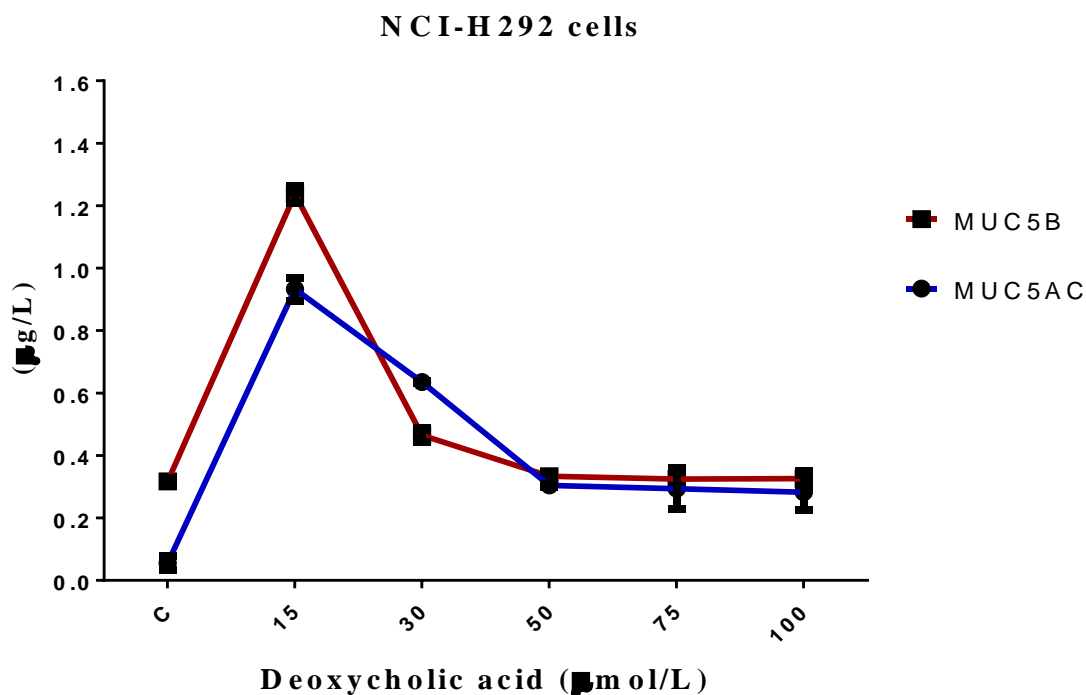


Figure 5-6: MUC5AC and MUC5B mucin release by NCI-H292 cells following exposure to DCA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-2). Bars represent mean \pm S.E.M values of each group.

	Deoxycholic acid				
Concentration μmol/L	15	30	50	75	100
MUC5AC	↑***	↑***	↑*	↑*	↑*
MUC5B	↑***	↑**	_____	_____	_____

Table 5-2: The effect of different levels of Deoxycholic acid on the NCL-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = p<0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared with control, ANOVA).

Figure 5-7 and Table 5-3 reveal a significant increase in MUC5AC mucin levels with CA at concentrations of 15, 30 and 50 $\mu\text{mol/L}$. The highest level of MUC5AC mucin secretion was obtained with 15 $\mu\text{mol/L}$ of CA. The MUC5AC mucin levels reduced at 50 $\mu\text{mol/L}$ CA but remained significantly above the control. The highest concentrations of CA (75 and 100 $\mu\text{mol/L}$) did not increase MUC5AC production significantly.

Figure 5-7 and Table 5-3 illustrates that exposure of NCI-H292 cells to CA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly. The highest level of MUC5B mucin production was with 15 $\mu\text{mol/L}$ CA and there was an inverse dose-response to CA.

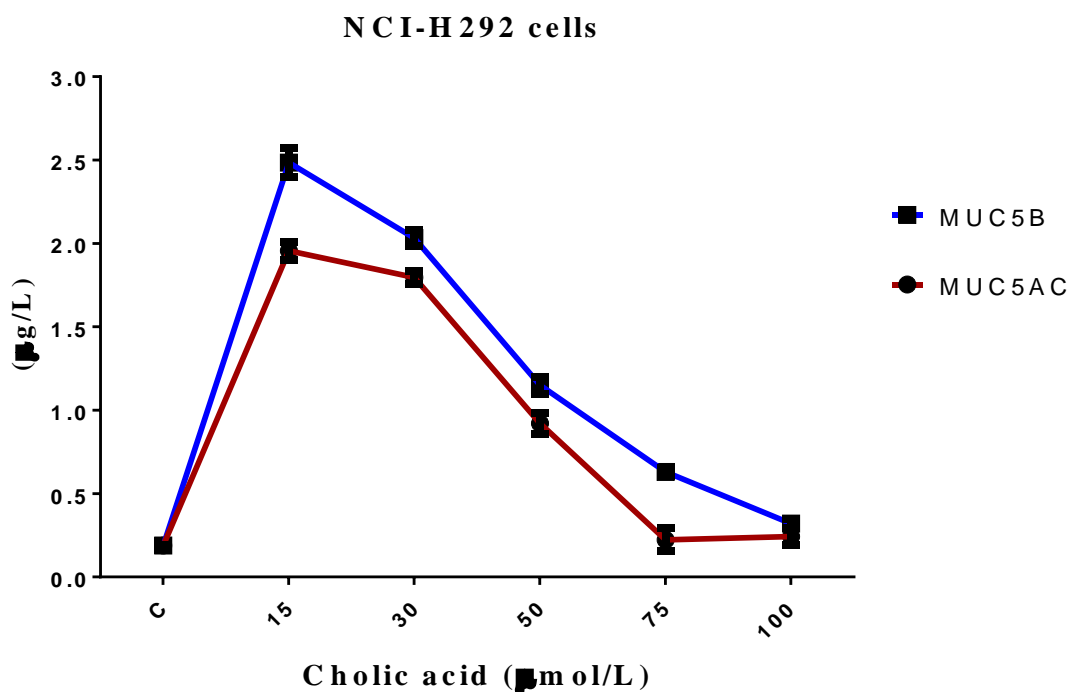


Figure 5-7: MUC5AC and MUC5B mucin release by NCI-H292 cells following exposure to CA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-3). Bars represent mean \pm S.E.M values of each group.

	Cholic acid				
Concentration μmol/L	15	30	50	75	100
MUC5AC	↑***	↑***	↑**	_____	_____
MUC5B	↑***	↑***	↑***	↑**	↑*

Table 5-3: The effect of different levels of Cholic acid on the NCL-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = p<0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared with control, ANOVA).

Figure 5-8 and Table 5-4 demonstrate a significant increase in MUC5AC mucin levels, with CDCA at concentrations of 50, 75 and 100 $\mu\text{mol/L}$, but not at 15 and 30 $\mu\text{mol/L}$. The highest level of MUC5AC mucin production was at 50 $\mu\text{mol/L}$ CDCA then declines as the concentration of CDCA increases up to 100 $\mu\text{mol/L}$.

Moreover, Figure 5-8 and Table 5-4 show that exposure of NCI-H292 cells to CDCA at concentrations of 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly, but this was not observed at concentrations of 15 $\mu\text{mol/L}$ CDCA. MUC5B mucin levels increased significantly at 30 $\mu\text{mol/L}$ CDCA and increased further to the maximum level of 75 $\mu\text{mol/L}$ CDCA. The level of MUC5B mucin production then fell off at 100 $\mu\text{mol/L}$ CDCA but remained significantly higher than the control.

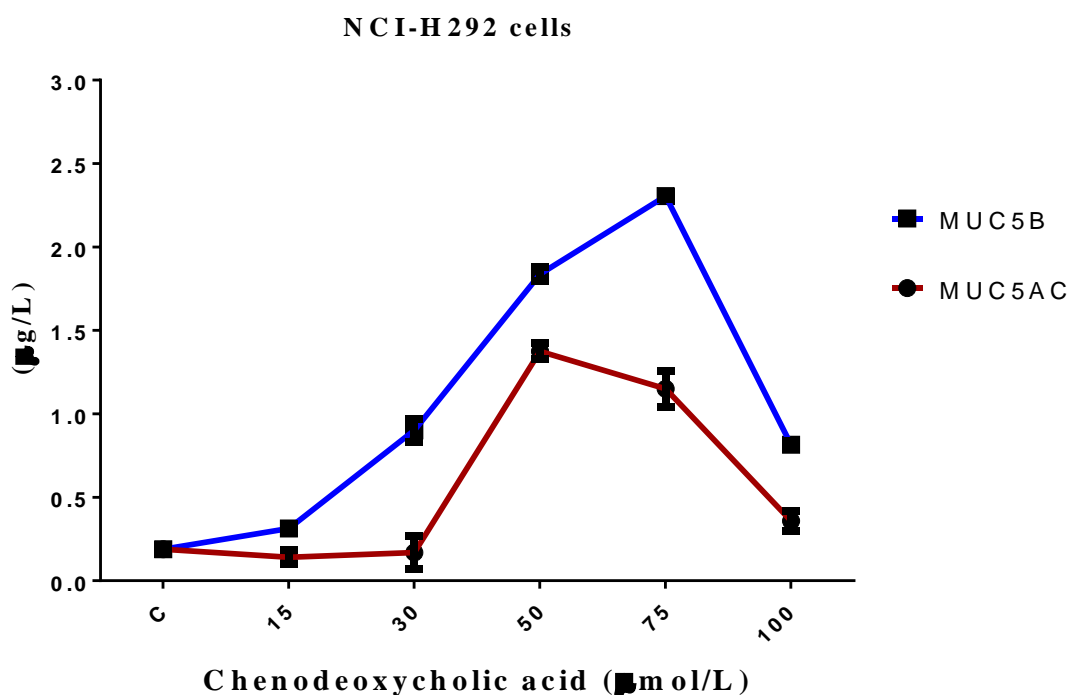


Figure 5-8: MUC5AC and MUC5B mucin release by NCI-H292 cells following exposure to CDCA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-4). Bars represent mean \pm S.E.M values of each group.

Concentration μmol/L	Chenodeoxycholic acid				
	15	30	50	75	100
MUC5AC	=====	=====	↑***	↑**	↑*
MUC5B	=====	↑**	↑***	↑***	↑**

Table 5-4: The effect of different levels of Chenodeoxycholic acid on the NCL-H292 cell line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Levels of MUC5AC secreted by NCI-H292 cells into the culture medium were measured using indirect ELISA. It is obvious from the results presented in Figure 5-5 to Figure 5-8 that there was an inverse dose-response to LCA, DCA and CA, but not with CDCA. Low concentrations of LCA, DCA and CA give the highest response. By contrast, the MUC5AC levels significantly increased following stimulation by 50 $\mu\text{mol/L}$ or above of CDCA. The CDCA showed no effect on cell viability, however it is possible that the assay was saturated and the signal out of the range of the absorbance measurement obscuring a change in cell number and that cell death accounted for the reduced MUC5AC levels, further experiments are necessary to clarify if this is the case (Figure 3-15 A).

Looking at MUC5B mucin production by NCI-H292 cells that were measured by indirect ELISA, it can be observed that primary BA (CA and CDCA) with 15 $\mu\text{mol/L}$ of CA and 75 $\mu\text{mol/L}$ of CDCA caused the highest level of MUC5B mucin released. The second highest level of MUC5B mucin production occurred with 15 $\mu\text{mol/L}$ of DCA; moreover, there was an inverse dose-response to DCA. Conversely, the LCA caused the lowest level of MUC5B mucin secretion due to cell death (see Figure 3-14A). Nevertheless, mucin production by LCA remains significantly higher than the control.

5.4.3 Effect of BA stimulation on the regulation of MUC5B and MUC5AC genes in NCI-H292 cells

NCI-H292 cells were exposed to BAs for 48 hours to determine the effect on MUC5AC and MUC5B gene expression. Based on the ELISA results, BA concentrations that caused the highest levels of mucin were as follows: 1 and 5 $\mu\text{mol/L}$ LCA; 15 and 30 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA; and 50 and 75 $\mu\text{mol/L}$ CDCA.

Figure 5-9 illustrates the MUC5AC mRNA levels determined using RT-PCR. All BA concentrations yielded increased MUC5AC expression. CA at 30 $\mu\text{mol/L}$ and 15 $\mu\text{mol/L}$ of DCA caused the highest level of upregulation with six-fold increase, and the lowest level of upregulation was with 75 $\mu\text{mol/L}$ CDCA, a five-fold increase.

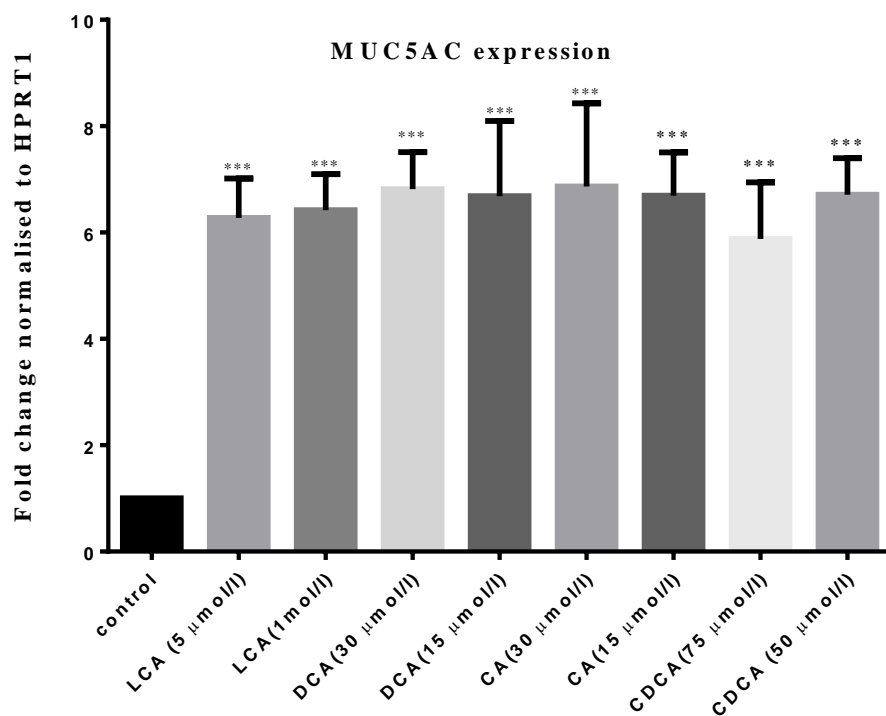


Figure 5-9: MUC5AC mRNA expression following BA exposure. Confluent NCI-H292 cells were cultured for 48 hours under the following conditions: no BA; 1 and 5 $\mu\text{mol/L}$ LCA; 15 and 30 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA and 50 and 75 $\mu\text{mol/L}$ CDCA. MUC5AC mRNA levels were normalised using HPRT1 mRNA and are presented as mean \pm SEM (n=3). All treatment groups exhibited significant upregulation of MUC5AC mRNA. (***) = $p < 0.001$ compared with control, ANOVA).

Figure 5-10 illustrates MUC5B mRNA levels determined using RT-PCR. All BA concentrations yielded increased MUC5B expression. The lowest level of upregulation was with DCA, 30 $\mu\text{mol/L}$ a three-fold increase and the highest was with CA, 30 $\mu\text{mol/L}$, a seven-fold increase over control.

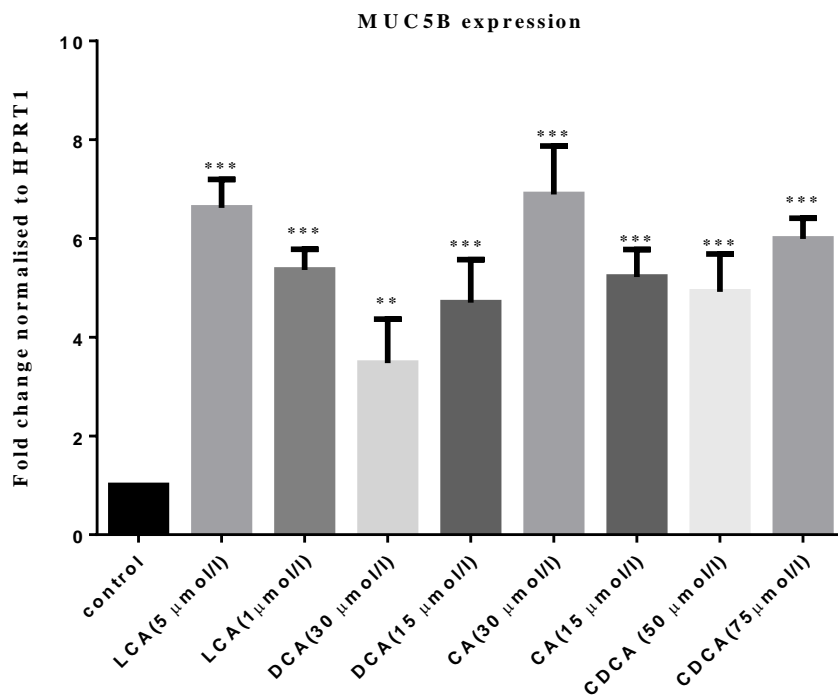


Figure 5-10: MUC5B mRNA expression following BA exposure. Confluent NCI-H292 cells were cultured for 48 hours under the following conditions: no BA; 1 and 5 $\mu\text{mol/L}$ LCA; 15 and 30 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA and 50 and 75 $\mu\text{mol/L}$ CDCA. MUC5B mRNA levels were normalised using HPRT1 mRNA and are presented as mean \pm SEM (n=3). All treatment groups exhibited significant upregulation of MUC5B mRNA. (** = $p < 0.01$, *** = $p < 0.001$ compared with control, ANOVA).

5.4.4 Effect of BA stimulation on mucin production in Calu-3 cells, quantified using the PAS method

Mucin secretion by Calu-3 cells was evaluated using the PAS assay, which enables a simple, direct assay in supernatants. Calu-3 cells were stimulated for 48 hours with BAs at concentrations between 15 and 100 μ mol/L.

Figure 5-11 illustrates a significant increase in mucin production by Calu-3 cells challenged with 15 and 30 μ mol/L LCA in comparison with unchallenged cells. However, 50, 75 and 100 μ mol/L LCA did not significantly induce mucin production.

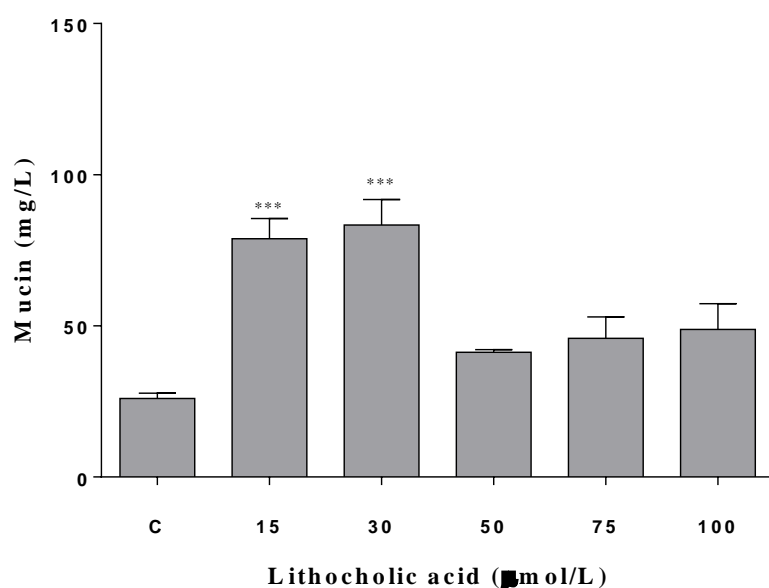


Figure 5-11: Mucin released by Calu-3 cells following 48h LCA stimulation. PAS was used to measure mucin in cell supernatants, and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (***) = $p < 0.001$ compared with control, ANOVA).

Figure 5-12 highlights a significant increase in mucin production by Calu-3 cells challenged with 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ DCA in comparison with unchallenged cells. Mucin production increased from 15 to 30 $\mu\text{mol/L}$ with the largest output produced by 30 $\mu\text{mol/L}$. There was a slow decline for 30 to 100 $\mu\text{mol/L}$ DCA.

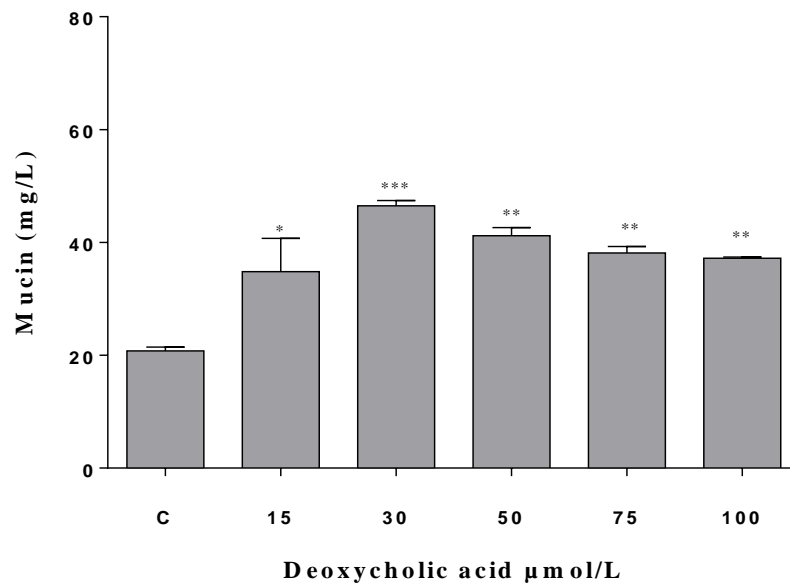


Figure 5-12: Mucin released by Calu-3 cells following 48h DCA stimulation. PAS was used to measure mucin in cell supernatants and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared with control, ANOVA).

Figure 5-13 illustrates a significant increase in mucin production by Calu-3 cells challenged with 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CA in comparison with unchallenged cells. The levels of mucin production were the same for all concentrations of CA.

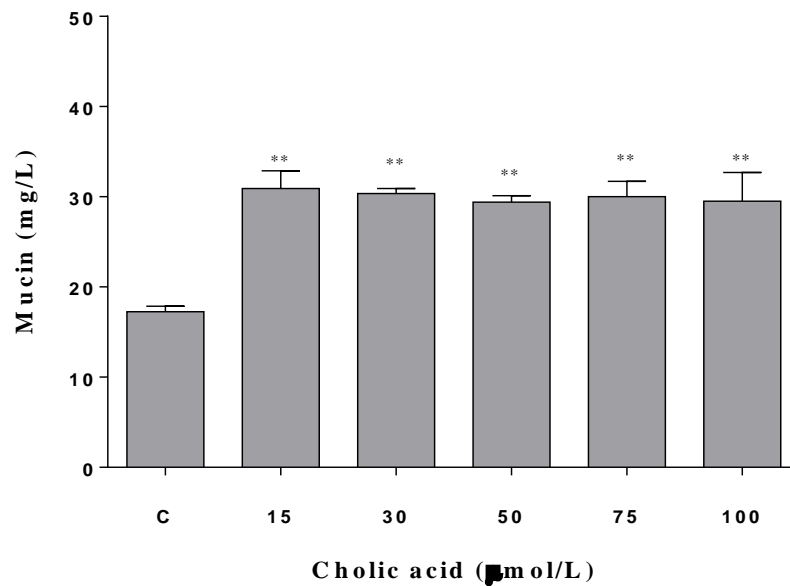


Figure 5-13: Mucin released by Calu-3 cells following 48h CA stimulation. PAS was used to measure mucin in cell supernatants, and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (** = $p < 0.01$ compared with control, ANOVA).

Figure 5-14 reveals a significant increase in mucin production by Calu-3 cells challenged with 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ CDCA in comparison with unchallenged cells. The highest effect occurred at 15 $\mu\text{mol/L}$ CDCA. The mucin level was then reduced at 50 and 75 $\mu\text{mol/L}$ CDCA, with 100 $\mu\text{mol/L}$ CDCA giving a similar response to 50 $\mu\text{mol/L}$ CDCA.

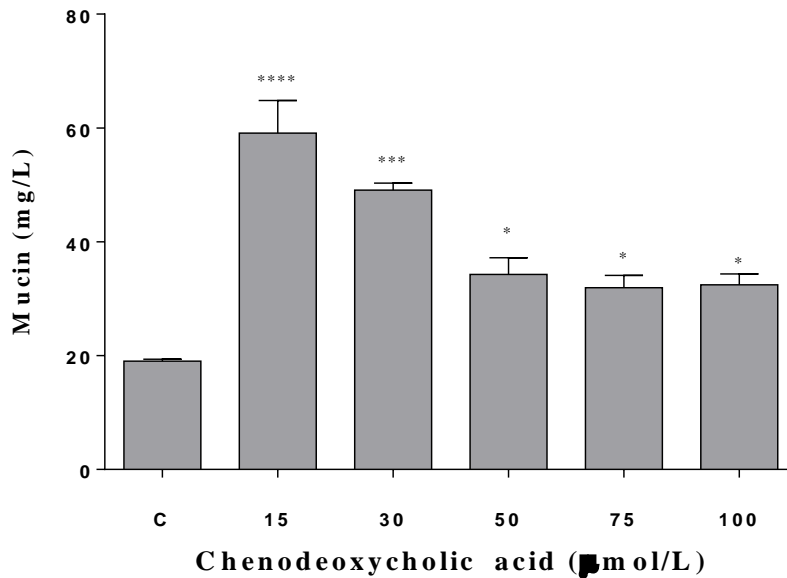


Figure 5-14: Mucin released by Calu-3 cells following 48h CDCA stimulation. PAS was used to measure mucin in cell supernatants, and this was statistically analysed using a one-way ANOVA test. P values of ≤ 0.05 were regarded as significant. These results are mean \pm S.E.M values from (n=6) experiments. (* = $p < 0.05$ and *** = $p < 0.001$ compared with control, ANOVA).

In general, looking at mucin production by Calu-3 cells measured by PAS assay, it can be observed that 15 and 30 $\mu\text{mol/L}$ of LCA had the highest level of mucin released; however, the higher concentrations did not cause mucin secretion due to cell death (see Figure 3-20A) . The second highest level of mucin production occurred with 15 $\mu\text{mol/L}$ of CDCA. Moreover, it is clear there was an inverse dose-response to CDCA. In contrast, CA caused the lowest level of mucin secretion with no difference in the level of mucin production by the Calu-3 cells between any of the CA concentrations (Figure 5-11 to Figure 5-14).

5.4.5 Measurement of MUC5AC and MUC5B mucin in Calu-3 cells

MUC5AC and MUC5B secretion by Calu-3 cells is also affected by BA exposure. Levels of MUC5AC secreted by cells into the culture medium were measured using indirect ELISA.

Figure 5-15 and Table 5-5 illustrate that Calu-3 cell exposure to LCA at concentrations of 15, 30 and 50 $\mu\text{mol/L}$ caused MUC5AC levels to increase significantly compared with the control. MUC5AC mucin levels increased significantly at 15 $\mu\text{mol/L}$ LCA and rose further to the maximum level at 30 $\mu\text{mol/L}$. The level of MUC5AC mucin production then fell off at 50 $\mu\text{mol/L}$ due to cell death with $51\pm 3\%$ but remained significantly higher than the control. However, there was no increase in MUC5AC mucin due to cell viability being greatly affected by LCA at 75 $\mu\text{mol/L}$ with $78\pm 3\%$ cell death and 100 $\mu\text{mol/L}$ with $98\pm 3\%$ cell death (see Figure 3-20A)

Calu-3 cells also produce MUC5B mucin. These cells were exposed to BAs for 48 hours at concentrations ranging from 15 to 100 $\mu\text{mol/L}$, revealing that exposure of Calu-3 cells to LCA at concentrations of 15, 30, 50 and 75 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly (Figure 5-15 and Table 5-5). The highest level of MUC5B mucin release occurred with 15 $\mu\text{mol/L}$ LCA. The levels reduced at 75 $\mu\text{mol/L}$ LCA but remained significantly above the control. There was no increase in MUC5B mucin due to cell viability being greatly affected by LCA at 100 $\mu\text{mol/L}$ with $95\pm 3\%$ cell death (see Figure 3-20A)

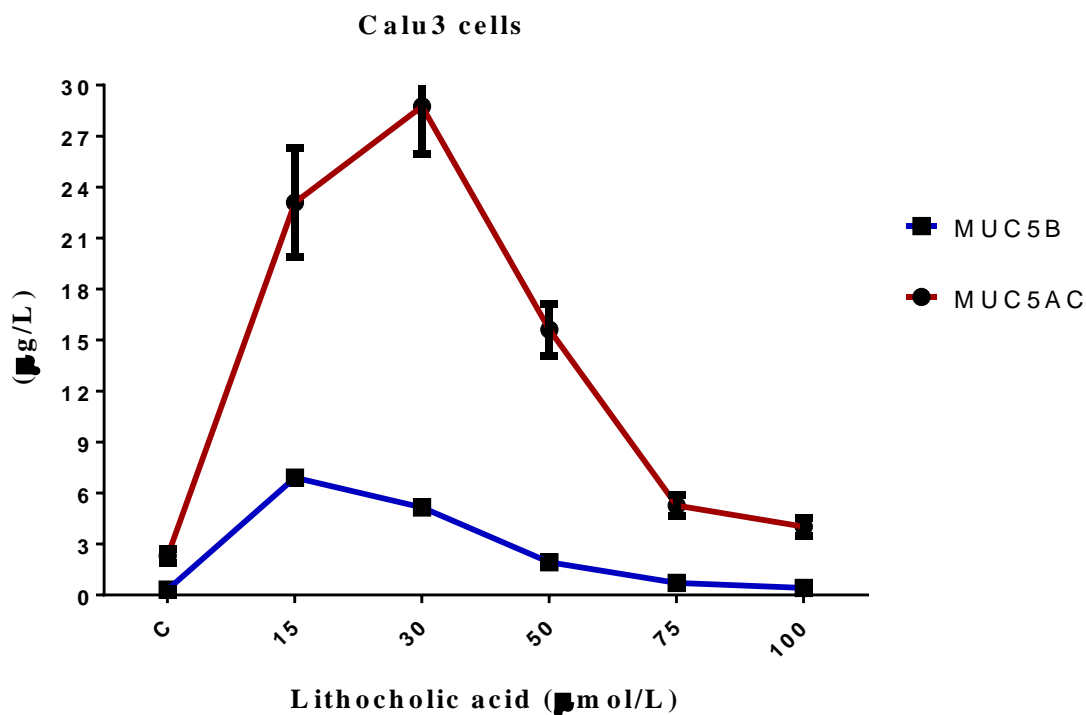


Figure 5-15: MUC5AC and MUC5B mucin release by Calu 3 cells following exposure to LCA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-5). Bars represent mean \pm S.E.M values of each group.

Concentration $\mu\text{mol/L}$	Lithocholic acid				
	15	30	50	75	100
MUC5AC	\uparrow ***	\uparrow ***	\uparrow ***	_____	_____
MUC5B	\uparrow ***	\uparrow ***	\uparrow **	\uparrow *	_____

Table 5-5: The effect of different levels of Lithocholic acid on the Calu 3 cells line following stimulation for 48 hours, \uparrow Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared with control, ANOVA).

Figure 5-16 and Table 5-6 reveal that Calu-3 cell exposure to DCA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5AC mucin levels to increase significantly compared with the control. The MUC5AC mucin levels increased significantly at 15 $\mu\text{mol/L}$ DCA and rose further to the highest level at 50 $\mu\text{mol/L}$ DCA. The level of MUC5AC mucin production then decreased at 75 and 100 $\mu\text{mol/L}$ DCA due to cell death (see Figure 3-21 B) while remaining significantly higher than the control.

Moreover, Figure 5-16 and Table 5-6 illustrate that exposure of Calu-3 cells to DCA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly though to a lesser extent than MUC5AC. MUC5B mucin levels increased significantly at 15 $\mu\text{mol/L}$ DCA and rose further to the maximum level at 50 $\mu\text{mol/L}$ DCA. The level of MUC5B mucin production then fell off at 75 and 100 $\mu\text{mol/L}$ DCA due to cell death (see Figure 3-21 B) while remaining significantly higher than the control.

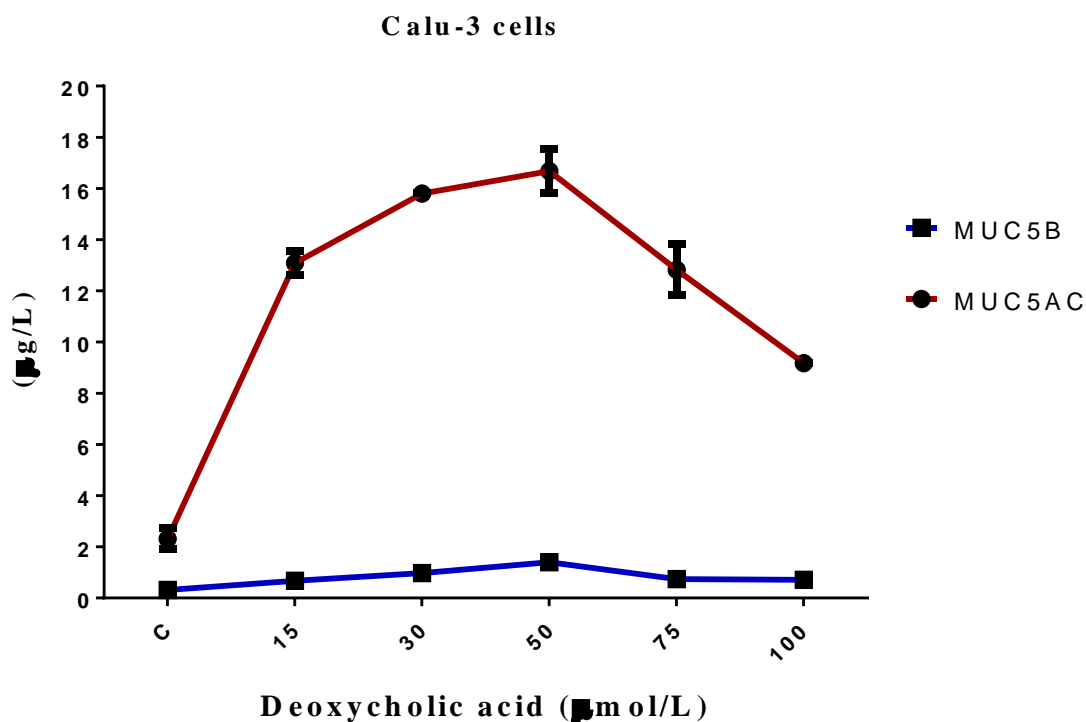


Figure 5-16: MUC5AC and MUC5B mucin release by Calu 3 cells following exposure to DCA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-6). Bars represent mean \pm S.E.M values of each group.

	Deoxycholic acid				
Concentration μmol/L	15	30	50	75	100
MUC5AC	↑***	↑***	↑*****	↑***	↑***
MUC5B	↑**	↑***	↑***	↑*	↑*

Table 5-6: The effect of different levels of Deoxycholic acid on the Calu-3 cells line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = p<0.05, ** = p < 0.01, *** = p < 0.001, ***** = p < 0.0001 compared with control, ANOVA).

Figure 5-17 and Table 5-7 highlight that Calu-3 cell exposure to CA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5AC mucin levels to increase significantly compared with the control. The highest level of MUC5AC mucin secretion was with 15 $\mu\text{mol/L}$ of CA and the mucin level then reduced to 50 $\mu\text{mol/L}$ CA. This decreased further to the lowest level at 75, 100 $\mu\text{mol/L}$ CA but no difference was found in the level of mucin production between 75 and 100 $\mu\text{mol/L}$ CA.

Figure 5-17 and Table 5-7 highlight that exposure of Calu-3 cells to CA at concentrations of 15, 30 and 50 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly, again to a much lesser extent than seen with MUC5AC. The highest level of MUC5B mucin release occurred with 15 $\mu\text{mol/L}$ CA. The levels reduced to 50 $\mu\text{mol/L}$ CA but remained significantly above the control. There was no increase in MUC5B mucin due to cell viability being significantly affected by CA at 75 and 100 $\mu\text{mol/L}$ (see Figure 3-21A).

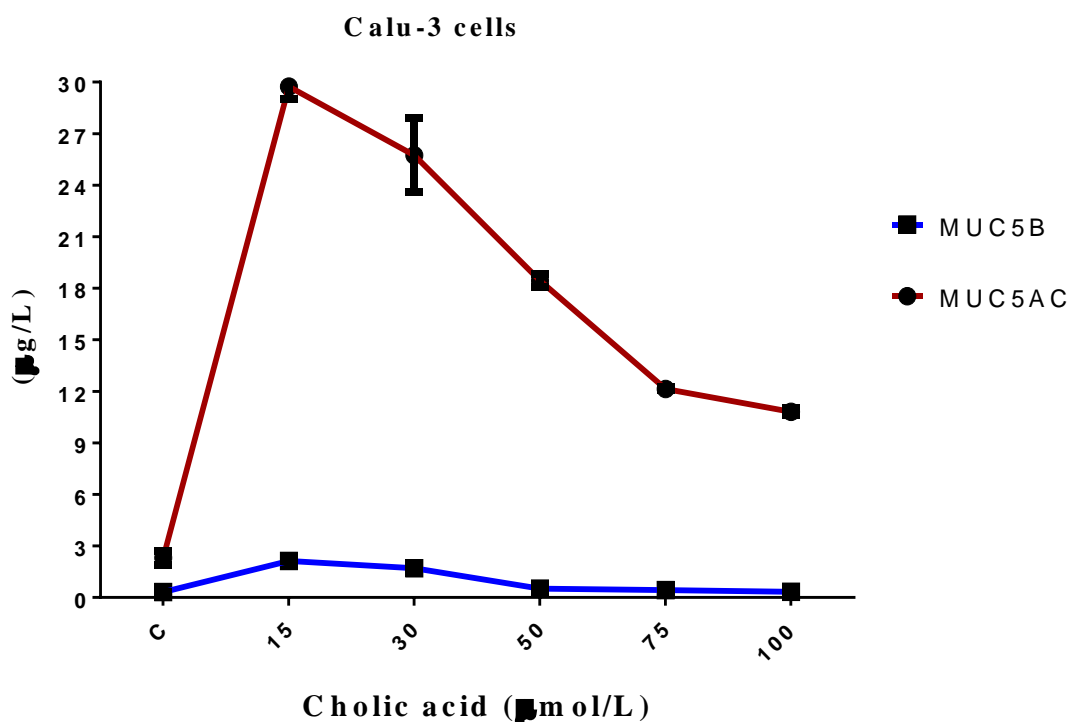


Figure 5-17: MUC5AC and MUC5B mucin release by Calu 3 cells following exposure to CA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-7). Bars represent mean \pm S.E.M values of each group.

	Cholic acid				
Concentration μmol/L	15	30	50	75	100
MUC5AC	↑***	↑***	↑***	↑**	↑**
MUC5B	↑***	↑***	↑*	_____	_____

Table 5-7: The effect of different levels of Cholic acid on the Calu-3 cells line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = p<0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared with control, ANOVA).

Figure 5-18 and Table 5-8 illustrate that Calu-3 cell exposure to CDCA at concentrations of 15, 30, 50, 75 and 100 $\mu\text{mol/L}$ caused MUC5AC mucin levels to increase significantly compared with the control. The highest effect occurred at 15 $\mu\text{mol/L}$ CDCA. The MUC5AC mucin level then reduced to 30 $\mu\text{mol/L}$ CDCA, followed by a slow decline for 30 to 100 $\mu\text{mol/L}$ CDCA.

Figure 5-18 and Table 5-8 demonstrate that exposure of Calu-3 cells to CDCA at concentrations of 15, 30, 50 and 75 $\mu\text{mol/L}$ caused MUC5B mucin levels to increase significantly. The highest level of MUC5B mucin release occurred with 15 $\mu\text{mol/L}$ CDCA. The levels MUC5B mucin reduced to 75 $\mu\text{mol/L}$ CDCA but remained significantly above the control. However, no significant increase was observed using concentrations of 100 $\mu\text{mol/L}$ CDCA due to cytotoxicity (see Figure 3-21 B).

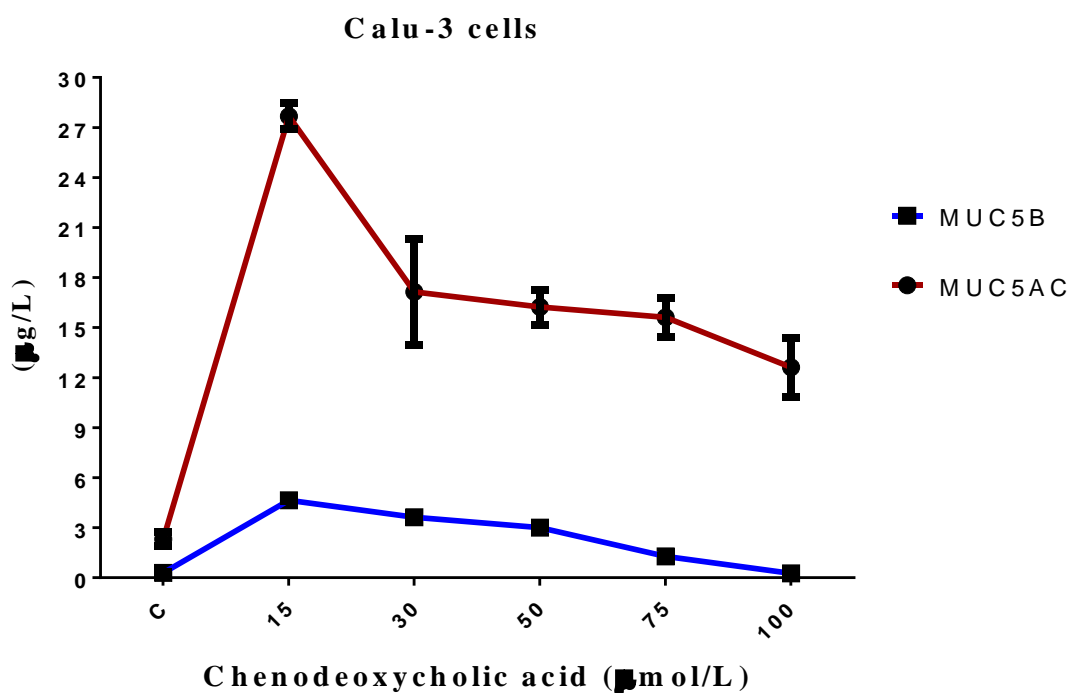


Figure 5-18: MUC5AC and MUC5B mucin release by Calu 3 cells following exposure to CA for 48 hours. Indirect ELISA was used to measure MUC5AC and MUC5B levels in the supernatant. Statistical analysis was conducted using a one-way ANOVA test. The data are representative of three independent experiments (n=6) (see table 5-8). Bars represent mean \pm S.E.M values of each group.

	Chenodeoxycholic acid				
Concentration μmol/L	15	30	50	75	100
MUC5AC	↑****	↑***	↑***	↑***	↑***
MUC5B	↑*	↑*	↑*	↑*	_____

Table 5-8: The effect of different levels of Chenodeoxycholic acid on the Calu-3 cells line following stimulation for 48 hours, ↑ Increase demonstrated. * Significant stimulation of MUC5AC and MUC5B release. (* = p<0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 compared with control, ANOVA).

Levels of MUC5AC secreted by Calu-3 cells into the culture medium were measured using indirect ELISA. It is evident from results outlined in Figure 5-15 to Figure 5-18 that 15 and 30 $\mu\text{mol/L}$ (low concentration) of LCA, CA and CDCA had the highest level of mucin released. Conversely, 50 $\mu\text{mol/L}$ of DCA produced the highest response.

Looking at MUC5B mucin production by Calu-3 cells measured by indirect ELISA, we can observe that it was much lower than MUC5AC. The highest level of MUC5B mucin production occurred with 15 $\mu\text{mol/L}$ of LCA with 7 mg/L, while the highest level of MUC5AC mucin production at 15 $\mu\text{mol/L}$ of LCA was 25 mg/L. The 50 $\mu\text{mol/L}$ of DCA had the highest effect on MUC5B and MUC5AC mucin production between all DCA concentrations. Moreover, there was an inverse dose-response to LCA, CA and CDCA.

5.4.6 Effect of BAs on regulation of MUC5B and MUC5AC Genes in Calu-3 cells

Hypersecretion of mucus can also be detected through changes in gene expression, in particular, an increased expression of the genes for MUC5AC and MUC5B. Under investigation, Calu-3 cells were exposed to BAs for 48 hours to determine the effect on MUC5AC and MUC5B gene expression. Based on the previous ELISA results, BA concentrations that caused the highest levels of mucin production were used, as follows: 15 and 30 $\mu\text{mol/L}$ LCA; 30 and 50 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA; and 15 and 30 $\mu\text{mol/L}$ CDCA.

Figure 5-19 illustrates the MUC5AC mRNA levels determined using RT-PCR. All BA concentrations yielded increased MUC5AC expression. LCA at concentration 30 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$ of DCA caused the highest level of upregulation with eight-fold increase, and the lowest level of upregulation was with DCA 30 $\mu\text{mol/L}$ and LCA 15, a six-fold increase.

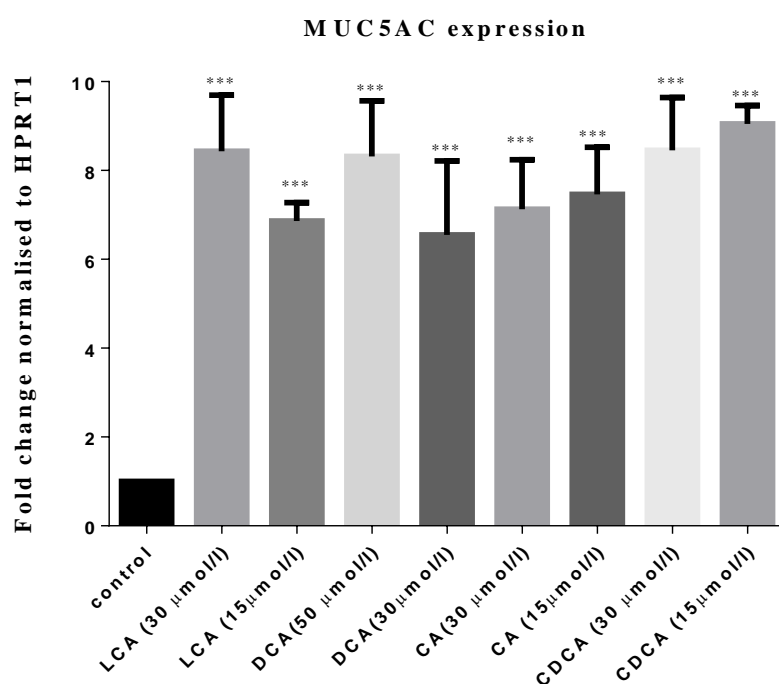


Figure 5-19: MUC5AC RNA expression following BA exposure. Confluent Calu-3 cells were cultured for 48 hours under the following conditions: no BA; 15 and 30 $\mu\text{mol/L}$ LCA; 30- and 50 $\mu\text{mol/L}$ DCA; 30 and 75 $\mu\text{mol/L}$ CA and 15 and 30 $\mu\text{mol/L}$ CDCA. MUC5AC mRNA levels were normalised using HPRT1 mRNA and are presented as mean \pm SEM ($n=3$). All treatment groups exhibited significant upregulation of MUC5AC mRNA. (***) = $p < 0.001$ compared with control, ANOVA).

Figure 5-20 reveals the MUC5B mRNA levels determined using RT-PCR. All BA concentrations yielded increased MUC5B expression. LCA and DCA at concentration 30 $\mu\text{mol/L}$ induced nine-fold increases, and the lowest level of upregulation was with DCA 50 $\mu\text{mol/L}$, a six-fold increase.

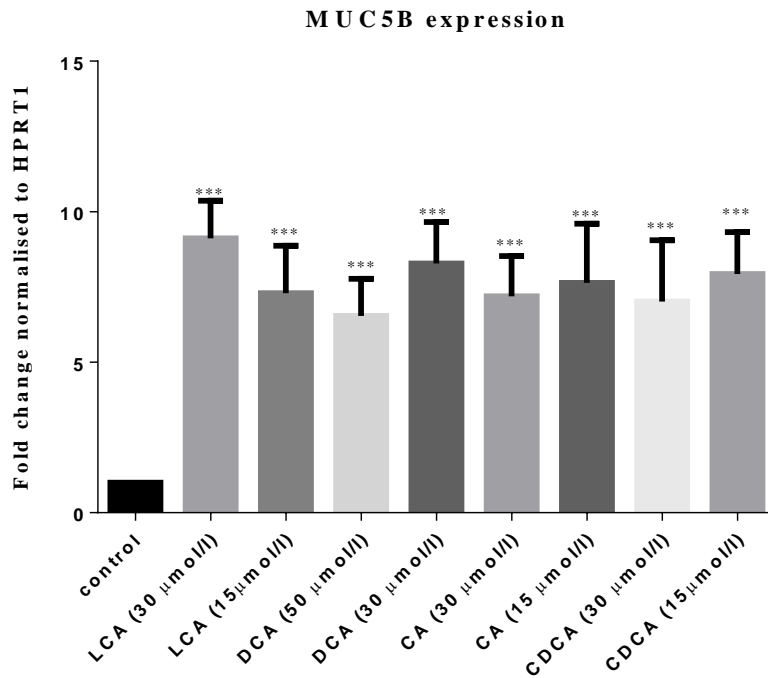


Figure 5-20: MUC5B RNA expression following BA exposure. Confluent Calu-3 cells were cultured for 48 hours under the following conditions: no BA; 15 and 30 $\mu\text{mol/L}$ LCA; 30 and 50 $\mu\text{mol/L}$ DCA; 30 and 75 $\mu\text{mol/L}$ CA and 15 and 30 $\mu\text{mol/L}$ CDCA. MUC5B mRNA levels were normalised using HPRT1 mRNA and are presented as mean \pm SEM (n=3). All treatment groups exhibited significant upregulation of MUC5B mRNA. (***) = $p < 0.001$ compared with control, ANOVA).

5.5 Discussion

Mucus is a critical factor in protecting the lungs and the rest of the airway system against foreign objects, toxins and pathogenic agents. Mucus hypersecretion is associated with various airway disorders, including COPD, CF and asthma (Lange and Vestbo, 2016; Theilmann *et al.*, 2016). When gastric contents, including BAs, make their way into the airway, the mucosa may incur damage, resulting in symptoms that include overproduction of mucous (Fox and Forgacs, 2006). Such pathologies are often caused by aspiration of gastric reflux contents (Brownlee *et al.*, 2016). Previous research, for example, has found a positive correlation between the presence of pepsin in the airway and mucus production.

Stovold *et al.* noted that exposure of goblet cells (cell line HT29-MTX) to pepsin caused MUC5AC mucin levels to increase significantly. In this study, HT29-MTX cell lines were stimulated with 50 µg/ml pepsin at pH 7.4 and 50 µg/ml pepsin at pH 7.0. The level of MUC5AC production was measured by indirect ELISA. This study found that pepsin at pH 7.0 significantly increased HT29-MTX cell mucin production from a baseline of 18µg/ml to 26µg/ml at 72h (Stovold *et al.*, 2008).

The present study seeks to explore the production of MUC5AC mucin and MUC5B mucin in NCI-H292 and Calu-3 cells exposed to BAs at a range of concentrations. The toxic effect of BAs on the colonic mucosa (Turjman and Nair, 1981; Owen *et al.*, 1984), and gastric mucosa (Gadacz and Zuidema, 1978; Gillen *et al.*, 1988) is well researched, but less is known about the effect of BAs on oesophageal mucosa (Nasr *et al.*, 2013). This study builds on the strong literature base linking oesophageal acid exposure to both mucosal damage and Barrett's oesophagus, with its attendant complications (Legrand *et al.*, 1990; Iftikhar *et al.*, 1993; Takahashi *et al.*, 2011).

It is not yet known exactly how bile acids damage mucosal cells. The most likely mechanisms are either a) by detergent action or b) as free acids. BAs are capable of intracellular accumulation, causing mitochondrial toxicity and intramucosal damage. The BAs are able to breach the mucosal barrier in the stomach. Moreover, their detergent action may be far more damaging in the lungs, by disturbing the mucus layer and disrupting the surfactant-based lipids (Iftikhar *et al.*, 1993; Jolly *et al.*, 2004).

BA concentrations greater than 1mmol/l are toxic to oesophageal mucosa *in vivo* (Gotley *et al.*, 1991). Furthermore, the damaging effect of some BAs is significantly increased by the

presence of H⁺ ions, which renders them more hydrophobic (Hopwood et al., 1981; Gotley et al., 1991; Kauer et al., 1997). Levels of BA hydrophobicity are correlated with, for example, oxygen free radical production, which in turn influences their disruptive detergent effect (Toohill and Kuhn, 1997). The induction of apoptosis (Iftikhar et al., 1993) the induction of mucosal ornithine decarboxylase activity followed by stimulation of proliferation, and mitochondrial membrane disruption. Intracellular ionisation may be partially responsible for the accumulation of BAs in the oesophageal mucosa, which can reach up to seven times that of luminal BA concentrations. It is also thought that intracellular BA accumulation might be related to their ability to induce pathophysiological alteration in mucosal permeability and disrupt the mucosal barrier to diffusion (Schweitzer et al., 1986; Kauer et al., 1997).

Due to the lack of reliable assay techniques there is considerable variability in the levels of detection of bile acids in healthy people and patients with respiratory disease which makes comparisons difficult. Indeed, BAL analysis has been the most popular form of study so far. This method has inherent problems, as it involves perfusion of the airways with 100-200 ml of fluid (180ml common), resulting in BAL appearing more dilute in terms of BAs (Perng et al., 2008; Zecca et al., 2008; Aseeri et al., 2012). BOS patients who have also received a lung transplant have been reported to exhibit greatly increased BA levels in BAL fluid, up to 32 µmol/L compared to normal level which is from 0 to 16 µmol/L (D'Ovidio et al., 2005). Oesophageal bile acid levels which are considered normal – even in Barrett's oesophagus – are between 0 and 200µmol/L and very rarely exceed 1000µmol/L. No bile acids are detected at all in about 25% of patients with reflux (Gotley et al., 1991). Patients with respiratory disease had higher level of bile acids in sputum. For example, the concentration of BAs found in sputum of patients with CF was 3.3µmol/l and with chronic cough patients 0.72µmol/l. A study of 38 patients was performed and only 16 who had DF508 (Delta-F508) homozygote genotype proved to have bile acids in sputum. DF508 (Delta-F508) is a common mutation within the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. This mutation is the most common cause of cystic fibrosis (CF) (Stanke, Ballmann et al. 2008). On the other hand, bile acids were found in sputum of 4/10 chronic unexplained cough patients. No bile acids were found in healthy controls (Blondeau et al., 2008b). The effect of combinations of bile acids on the cell lines used in this study would be something for future research to address. Our own research group investigated the effect of combinations of bile acids on PBECs. Cells were stimulated with a bile acid mixture. The results showed that there was no significant changes in the levels of IL-8, IL-6 and GM-CSF from PBECs after

stimulation with bile acid mixture (Aseeri, 2012). It is possible that the lack of stimulating effect of the mixture is because the conjugated bile acids are having an inhibitory effect (Pearson et al., 2011).

Understanding the mechanisms inherent to chronic airway diseases relies on in vitro models, using primary epithelial cells and cell lines (Stewart et al., 2012). Primary cells are frequently considered the 'gold standard' for such research, but they do carry some disadvantages; for example, limited lifespan, variability between donors, passage number and cost. Consequently, further experiments using cell lines are required for validation.

Calu-3 and NCI H292 cells are used commonly in vitro for gene regulation studies. These epithelial cell lines are derived from carcinomas and have the significant advantage of allowing the study of both MUC5 mRNA and MUC5 glycoprotein in the same model (Berger et al., 1999; Rose et al., 2000).

This study found primarily that stimulation by BAs caused NCI-H292 and Calu-3 cells to secrete MUC5AC mucin and MUC5B mucin. It is, to the best of my knowledge, the only study that determines the effects of BA exposure on mucin expression and mucus overproduction by airway epithelial cells. MUC5B and MUC5AC are predominant in airway secretion and were, therefore, chosen as markers for screening.

Berger et al. identified three carcinoma cell lines of respiratory tract tissue origin. Of these, Calu-3 and NCI-H292 both expressed MUC1, MUC4, MUC5AC, and MUC5B genes in varying combinations. Currently, it is held that MUC1, MUC4, MUC5AC and MUC5B are well expressed in both a healthy respiratory tract and lung cancer tumour tissues. MUC2 is less well expressed, suggesting that both normal and tumour respiratory epithelium Calu-3 and NCI-H292 cell lines present similar mucin gene profiles (Berger et al., 1999).

In this study, the supernatant was analysed for mucin, using a PAS assay which showed that the glycoprotein (mucin) content of NCI-H292 and Calu-3 cells secretions increased significantly. PAS assay is used routinely to detect mucin production by cells. The results of this study indicate that LCA, DCA, CA and DCA caused a significant increase in mucin production from NCI-H292 cells at all concentration, except 100µmol/l of CDCA and 15µmol/l of DCA. Moreover, this experiment detected the significant level of mucin after stimulating Calu-3 cells with DCA, CA and CDCA at all concentrations. LCA caused a significant increase at concentrations 15 and 30 µmol/l, but the results did not reveal any increase at concentrations 50, 75 and 100 µmol/l due possibly to cell death.

However, this technique was not sensitive enough to identify mucin, so an indirect ELISA was therefore used for additional analysis. These findings revealed that NCI-H292 cells produced maximal levels of MUC5AC and MUC5B following stimulation with the subsequent BA for 48 hours at concentrations: 1 and 5 $\mu\text{mol/L}$ LCA; 15 and 30 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA; and 50 and 75 $\mu\text{mol/L}$ CDCA. Similarly, Calu-3 cells produced maximal levels of MUC5AC and MUC5B following stimulation with the following BA for 48 hours at concentrations: 15 and 30 $\mu\text{mol/L}$ LCA; 30 and 50 $\mu\text{mol/L}$ DCA; 15 and 30 $\mu\text{mol/L}$ CA; and 15 and 30 $\mu\text{mol/L}$ CDCA, returning to basal levels.

The results in this chapter demonstrated that low concentrations of bile acids stimulate significant MUC5AC and MUC5B production in NCI-H292 and Calu-3 cells, although higher bile acid concentrations yielded no significant increase in production. High concentrations of LCA affect cell viability (see Figure 3-14A and Figure 3-20A), and this was associated with no increase in MUC5AC and MUC5B mucins (see Figures 5-21 and 5-22). It was found however, that CA in high concentrations downregulated production of MUC5AC and MUC5B (see Figures 5-23 and 5-24), whilst having no effect on cell viability (see Figure 3-14A and Figure 3-20A). It can be inferred from these findings that the negative correlation between BA concentration and mucin production must be caused by factors other than cell viability. These factors are as yet unknown, but the following explanations are possible. First, downregulation of mucin production may be caused by extracellular matrix (ECM). Iwashita *et al.* (2010) found that type-IV collagen, found in ECM, inhibits MUC5AC production via integrin-signalling pathways. Integrins are the primary receptors involved in cell-cell and cell-ECM adhesion. These heterodimers comprise of α 2- and β -subunits, which bind cells to collagen, activating integrin signalling pathways, which in turn, can activate multiple types of gene expression. The researchers suggest that this process is involved in a previously unknown type of MUC5AC regulation involving integrins and ECM components. My study found that BA exposure stimulated ECM (Pro-collagen and fibronectin) expression (see chapter 4), further confirming the findings by Iwashita *et al.* (2010).

Secondly, *in vitro* studies reveal that MUC5AC and MUC5B expression are stimulated by IL-8 and IL-6 (Smirnova *et al.*, 2002; Smirnova *et al.*, 2003). On the other hand it has also been found that mucin genes and mucin secretion can be downregulated by certain inflammatory mediators, including IL-4 and IL-13 (Jayawickreme *et al.*, 1999; Kim *et al.*, 2002a). NHTBE cell cultures, when exposed to IL-4 exhibited a reduction in mucin secretion, and MUC5AC and MUC5B gene expression (Jayawickreme *et al.*, 1999). Further to this, Kim *et al.*

confirmed the link between inflammatory mediators and mucin production by studying mucin gene expression and mucin secretion in human nasal epithelial cells when exposed to IL-13. Using an immunoblotting assay, they found that total mucin secretion and MUC5AC gene expression substantially decreased after exposure to IL-13 (Kim *et al.*, 2002a). Consequently, it could be the case that high enough concentrations of bile acids can lead to an inflammatory release involving IL-4 and IL-13, thereby significantly affecting MUC5 regulation. Third, as bile acids accumulate they inhibit intracellular function which does not kill the cell but reduces the rate of all cellular processes such as protein synthesis (Tabas, 2002).

In one study, Gosalia *et al.* demonstrated that mucus was produced by Calu-3 cells. The purpose of this study was to identify the regulatory mechanisms that control the expression of the genes within the gel-forming mucin gene cluster located at chromosome 11p15.5. In order to compare the individual mucin genes within a cell type, absolute quantification was used to measure mucin gene expression. Standard curves generated on cloned cDNAs for each gene were used to calculate the gene expression for the four gel-forming mucin genes (MUC2, MUC6, MUC5B and MUC5AC). In picomolar amounts, absolute expression was calculated in LS180 cDNA. It was demonstrated by these results that the lung carcinoma cell lines Calu-3 and A549 both express MUC5AC mRNA, but that MUC5B mRNA is only expressed by A549 cells. Moreover, the significance of the CCCTC-binding factor (CTCF) sites to mucin gene expression was also demonstrated by this study when Calu-3 lung carcinoma cells were exposed to lipopolysaccharide (LPS). In this experiment, it was found that the LPS up-regulation of MUC5AC and MUC2, and the repression of MUC5B transcription is accompanied by a depletion in the binding of CTCF at specific sites. Further, the treatment with LPS significantly reduced the expression of MUC5B, which would suggest a coordinated regulatory mechanism for the gene cluster, whereby the up-regulation of one or several gel-forming mucins is followed by the down-regulation of others. The mechanism whereby the expression of MUC5B is reduced may result in repressive histone modifications being established, which is consistent with previous observations on MUC5B regulation (Gosalia *et al.*, 2013).

Both surface epithelial cells and submucosal glands produce MUC5AC (Audie *et al.*, 1993), but MUC5B tends only to be detected in submucosal glands (Berger *et al.*, 1999). Consequently, it was expected that this study would find higher MUC5B levels in Calu-3 cells, which are derived from submucosal glands (Kreda *et al.*, 2007), where MUC5B is relatively greater than MUC5AC (Grainger *et al.*, 2009). The ELISA assay in this study found

that both NCI-H292 and Calu-3 cells expressed MUC5AC and MUC5B. Kreda *et al.* (2007) used gel electrophoresis to quantify MUC5AC and MUC5B levels in Calu-3 cells, finding relatively low MUC5B levels in comparison with MUC5AC. The present study's findings, therefore, concur with the literature (Kreda *et al.*, 2007).

Moreover, the lack of MUC5B induction by agonists of mucin synthesis and secretion suggests that its resulting mucin may be constitutively expressed. Ordinarily, basal mucin synthesis and secretion levels are maintained by MUC5B levels in airways. However, in cases of chronic airway diseases, enlargement of the submucosal glands may cause increased mucin secretion due to more cells being involved in MUC5B synthesis. In such situations, there is an advantage in having an alternative inducible mucin, MUC5AC, which can respond rapidly to irritants. Furthermore, such diseases often cause biochemical changes in airway mucus (Davies *et al.*, 1996; Berger *et al.*, 1999). This may be caused in part by differential regulation of airway mucins, both inducible and non-inducible.

In this study, the total mucin was measured using a PAS assay. PAS assay is used routinely to detect mucin production by cells. However, this technique was not sensitive enough to identify mucin (Mantle and Allen, 1978). The differences between specific ELISA methods and the PAS method may be accounted for by the fact that the PAS assay is not solely specific to mucin. The PAS assay measures all carbohydrate containing molecules and is interfered with by protein and lipid to some extent.

The most physiologically relevant cell type to evaluate airways damage was arguably primary human bronchial epithelial cells (PHBECs). However, these are costly and not easy to culture (Stewart *et al.*, 2012). Moreover my results showed that it was possible to maintain primary epithelial cells for 2-3 passages on a plastic surface, but the populations are heterogeneous, and the mucin expressing phenotype has not been observed.

Conversely, Berger *et al.* (1999) have found that carcinoma cell lines can be grown, maintained and passaged from epithelial samples. Our understanding of diseases such as COPD, CF, asthma and other inflammatory processes have benefited from such investigative models. Early models used submerged monolayer cell cultures on a plastic substrate. However, Sachs *et al.* (2003) found that using a defined medium and an air-liquid interface (ALI) to culture primary human bronchial epithelial cells (HBECs), enabled a more physiological model, thereby driving a differentiated phenotype. Studies using this model have observed a pseudostratified polarised phenotype, which incorporates both ciliated and

goblet cells, and exhibits high transepithelial electrical resistance (TEER) (Jiang *et al.*, 2001; Chan *et al.*, 2010).

The formation of tight junctions can be measured indirectly via TEER, which can indicate epithelial layer disruption (Pedemonte, 1995), although it must be noted that ALI models exhibit limited capacity for cell differentiation. Calu-3 cells, for example, are similar to primary cells in terms of features (Stewart *et al.*, 2012), but are also able to express apical MUC5, although they cannot form cilia (Grainger *et al.*, 2006).

Several studies have been carried out to investigate MUC gene regulation in NCI-H292 cells *in vitro*. Factors implicated in the upregulation of MUC5AC expression include: Cigarette smoke (Baginski *et al.*, 2006), Interleukin-1 β (Kim *et al.*, 2002b), IL-4 (Dabbagh *et al.*, 1999) and IL-17 (Chen *et al.*, 2003). Borchers *et al.* (1999) studied the effects of TNF- α on MUC5AC and MUC5B steady-state mRNA levels in NCI-H292 cells, finding that TNF- α regulates MUC5AC but not MUC5B. They concluded that the pathogenesis of obstructive airway disease is caused partly by direct exposure to irritants, as well as inflammatory mediators, which increase the synthesis of mucin mRNA in airway epithelial cells (Borchers *et al.*, 1999). NCI-H292 cells also produce mucus glycoconjugates. IL-6 expression in NCI-H292 cells has also been investigated and found to increase mucin gene expression as well as mucin production (Louahed *et al.*, 2000).

Mucin upregulation is believed to be caused by the binding of these factors to specific surface cell receptors, such as P2Y2 and toll-like receptors (Voynow *et al.*, 2006). Furthermore, mucin gene regulation is influenced by stimulation of the epidermal growth factor receptor (Takeyama *et al.*, 1999). Once these receptors are bound (through a variety of stimulants), mitogen-activated protein kinase (MAPK) pathways are activated, triggering the activation of nuclear factor kappa B (NF κ B), a transcription factor that also regulates mucin gene expression (Rose and Voynow, 2006). To elucidate the role of MUC5AC as a treatment target in bile duct inflammation, recent research has focused on the effect of lipopolysaccharide (LPS) stimulation on MUC5AC mRNA and protein expression. They further explored the role of p38 MAPK regulating MUC5AC in bile duct inflammation. Their results demonstrated that p38 MAPK can affect airway inflammation and gastric epithelial cell apoptosis by the influence of LPS induced bile duct inflammation by regulating MUC5AC expression (Xiong *et al.*, 2016).

In vitro studies reveal that MUC5AC and MUC5B expression are also stimulated by IL-8 (a mucin secretagogue), IL-6 and GM-CSF (Smirnova *et al.*, 2002; Smirnova *et al.*, 2003). The connection between BAs and mucus secretion is, therefore, further elucidated by findings around the effects of BA exposure on IL-8, IL-6 and GM-CSF production, as described in chapter three. The epithelium produced IL-8 to combat toxins or pathogens that have made their way into the lung. The present study found that BA stimulation significantly increased IL-8 production in both NCI-H292 and Calu-3 cell lines.

Stimulation of PHBECs by BAs was also found to stimulate GM-CSF production. GM-CSF is a growth factor involved in the regulation and control of neutrophils. The findings from this study (see Chapter 3) showed that aspiration of BAs can lead to neutrophilic inflammation, tissue remodelling (Walters *et al.*, 2008) and mucus hypersecretion (Smirnova *et al.*, 2002; Smirnova *et al.*, 2003), causing potential lung injury.

Other cytokines may also influence mucin production and secretion. Chen *et al.* (2003) exposed differentiated human primary tracheobronchial epithelial cells to various cytokines, including interleukins-1 α & β , 13 and TNF- α , in an air-liquid interface culture. The study found that IL-6 and IL-17 stimulation resulted in elevated MUC5AC. Further investigation explored IL-17 stimulation of IL-6 production in bronchial epithelial cells, to determine if mucin expression might be mediated through an IL-6 paracrine/autocrine loop. MUC5 expression mediated by IL-17 was shown to decrease significantly when a neutralising IL-6 antibody was introduced (Chen *et al.*, 2003). Airway epithelial cells also produce IL-6, so it is possible that BA stimulation of NCI-H292 and Calu-3 cells may, therefore, promote increased mucin production via a cytokine induction mechanism, resulting in further obstruction of previously injured airways (see Chapter 3).

BA aspiration can also damage the pulmonary system (el-Serag and Sonnenberg, 1997; Wilshire *et al.*, 2013) and cell membranes are especially vulnerable to bile damage, as shown by studies on Type II pneumocytes *in vitro* (D'Ovidio *et al.*, 2005; D'Ovidio and Keshavjee, 2006). Type II pneumocytes in the lungs are responsible for surfactant protein and phospholipid production and are therefore especially vulnerable to lipid disruption as a result of mucus barrier damage.

In conclusion, patients with advanced lung disease, particularly CF (Blondeau *et al.*, 2008a; Pauwels *et al.*, 2012), COPD (Rascon-Aguilar *et al.*, 2006), and ventilator-associated pneumonia (Wu *et al.*, 2009), often also present with gastroesophageal reflux (GERD)

(Legendre *et al.*, 2014). Many of these diseases also display mucin hypersecretion. The current study's findings make a strong contribution to the argument that claims exposure to BAs strongly influences mucus production, potentially leading to – or furthering – airway injury. The present study focussed on Calu-3 and NCI H292 cells, exposing these to varying BA concentrations. The findings established Calu-3 and NCI H292 cells as good models for gene regulation studies *in vitro*. The broader study also found that NCI-H292 and Calu-3 production of IL-8, IL6 and GM-CSF is heavily influenced by BA stimulation (see Chapter 3). A potential connection, therefore, exists between increased mucus secretion and IL-8, IL6 and GM-CSF production.

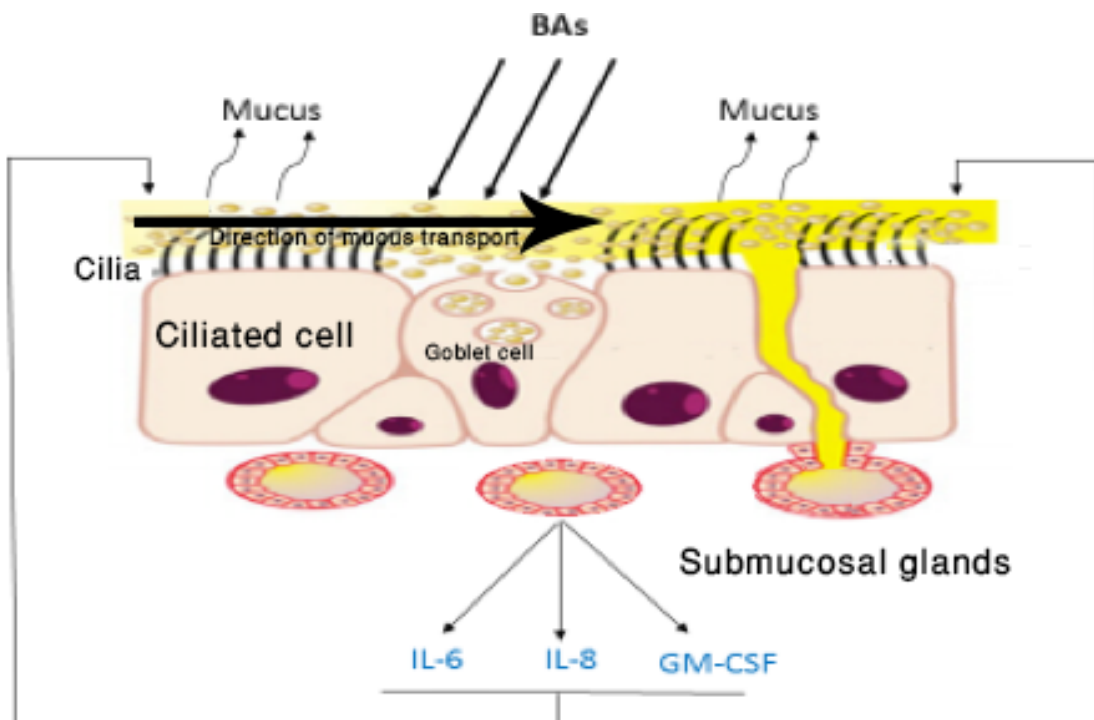


Figure 5-25: diagram showing proposed influences of BAs in mucus production (Adapted from Varielle., 2011 (Vareille *et al.*, 2011))

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

Lung disease is one of the world's greatest causes of mortality and morbidity, and these cases are on the rise. As an example, COPD is the fifth largest cause of death worldwide, while interstitial lung disease (ILD) and cystic fibrosis (CF) are also prevalent with negative consequences such as a low life-quality and death. All these diseases are to some extent influenced by either chronic or acute inflammation, (Rincon and Irvin, 2012). For some considerable time, the connection between GOR and lung disease has come under suspicion (Pearson and Wilson, 1971; Belsey, 1979; D'Ovidio *et al.*, 2005).

In patients suffering from lung disease of varying types, the prevalence of GOR has in fact been recorded: for example abnormal levels of GOR have been identified in patients with idiopathic pulmonary fibrosis, cystic fibrosis (CF) and asthma (Feigelson *et al.*, 1987; Tobin *et al.*, 1998; Prather *et al.*, 2014). Additionally, the development of pneumonia and bronchiolitis obliterans has also been linked to GOR (Liu *et al.*, 2015).

For chronic respiratory diseases such as the chronic cough (Sifrim *et al.*, 2005; Houghton *et al.*, 2016), cystic fibrosis (Blondeau *et al.*, 2008a; Brodlie *et al.*, 2015), and asthma (Harding, 2001; Rosztóczy *et al.*, 2008; Houghton *et al.*, 2016), it has been identified by pathophysiological studies that gastroesophageal reflux (GOR) could be a common instigator. Moreover, an increased GOR following a lung transplantation (LTx) can also lead to bronchiolitis obliterans syndrome (BOS) or chronic rejection (Palmer *et al.*, 2000; Young *et al.*, 2003; D'Ovidio and Keshavjee, 2006; Blondeau *et al.*, 2008a). Exposure to gastric contents can also damage both nonciliated and ciliated epithelial cells within the airway (Wynne *et al.*, 1981; Ohru *et al.*, 1997; Harding, 2001; Blondeau *et al.*, 2008b).

Furthermore, gastric contents that are aspirated impair mucociliary transport and facilitate the growth of bacteria within the airway tract (Zeiber and Hornick, 1996; D'Ovidio and Keshavjee, 2006). It is increasingly accepted that rather than being sterile as once taught that even in normal subjects there is a microbiome associated with the human respiratory system.

Research into respiratory biomes is often concerned with detecting signature indicators of Cystic Fibrosis (CF) (Surette, 2014), Chronic Obstructive Pulmonary Disease (COPD) (Han *et al.*, 2012) and, more recently, asthma and non-CF bronchiectasis (Boyton *et al.*, 2013; Huang and Boushey, 2015). In particular, the pathogen *Pseudomonas aeruginosa* is being

increasingly associated with COPD and is strongly linked with intense airway inflammation and worse outcomes for COPD patients (Lin *et al.*, 2007; Murphy *et al.*, 2008).

Bacterial enzyme action aids primary BA deconjugation into free bile acids. More specifically, the hydroxyl (OH) group is detached from the seventh carbon atom by the enzyme 7 α -dehydroxylase. Gastro-oesophageal reflux disease (GORD) patients undergoing therapy which targets acid suppression can exhibit overgrowth of bacteria in the intestine. In turn, this causes BAs to deconjugate, resulting in a greater number of unconjugated BAs in circulation that are more toxic (Solaas *et al.*, 2000).

For patients with such respiratory diseases, BA aspiration into the lungs has been found to be a significant co-morbidity factor (Aseeri *et al.*, 2012; Reen *et al.*, 2014). For patients with CF, there is a correlation between BA aspiration and both reduced biodiversity as well as increased levels of dominant Proteobacterial pathogens (Reen *et al.*, 2012; Reen *et al.*, 2014). In general, the presence of BAs in the respiratory tract is associated with increased inflammation (D'Ovidio *et al.*, 2005).

Among other findings, our own research group demonstrated that BA concentrations similar to those found in the stomach could have a negative impact on *P. aeruginosa* growth. Furthermore, *P. aeruginosa* behaviour was also altered when exposed to different BAs in different concentrations. The study found that *P. aeruginosa* exposure to BAs resulted in a tendency to form a biofilm, and the different BAs at different concentrations affected the type of biofilm formed. Moreover, this study confirms the ability of bacteria to survive in the stomach, constituting a potential reservoir of viable pathogens relevant to CF disease (Al-Momani *et al.*, 2016).

Reen *et al.* (2016) carried out a systems-based analysis on *P. aeruginosa* – which is primarily associated with morbidity and mortality in CF patients – and other pathogens, to gain an understanding of their chronic response and the response of host cells to BA aspiration. An integrated approach was taken, drawing on transcriptomics, functional genomics and immunochemistry, which found that the bile response in both pathogen and host cells was underpinned by a network of genes linked to virulence, adaptive metabolism, and antibiotic tolerance. A chronic response could be elicited by individual BAs and chenodeoxycholic acid (CDCA) was found to signal across both pathogen and host cell interfaces. Distinct profiles were found for BA aspiration and non-aspiration through longitudinal analysis of the paediatric CF microbiome. Isolates from these patients underwent phenotypic analysis, which

found responses mostly conserved to physiologically relevant BA concentrations, although the study did find evidence of phenotypic heterogeneity (Reen *et al.*, 2016). This study potentially significantly increases the understanding around the molecular mechanisms by which respiratory pathogens are stimulated by BAs to convert from an acute virulent lifestyle to a chronic infection phase, in particular *P. aeruginosa*. Antibiotic challenge has little effect on such chronic biofilm infections, which is noteworthy in the context of increased tolerance to the polymyxin and macrolide classes of antibiotics observed in the presence of bile.

My overall hypothesis would be that the presence of BAs in the lungs of patients with respiratory disease – usually through aspiration – plays a significant role in the deterioration of lung function leading to increased morbidity and that the interaction of BAs with the respiratory microbiome could be a significant factor in this.

A recent study has also shown that aspiration of non-sterile gastric juice may be a source of microbiological challenge in patients with CF. Aside from injuring the lung directly, aspirated acid also leads to release of the inflammatory cytokine IL-8, as measured in alveolar and bronchoalveolar lavage fluid (Zeiber and Hornick, 1996).

Additionally, unusually high reflux levels are also experienced by children and adults suffering from CF (Ledson *et al.*, 1998; Blondeau *et al.*, 2008b; Aseeri *et al.*, 2012; Brodlie *et al.*, 2015). Furthermore, there is a growing consensus that non-acidic and clinically hidden, aspiration and reflux may be a substantial cause of lung injury (Blondeau *et al.*, 2008b) and include a microbiological challenge to the lung (Al-Momani *et al.*, 2016). Because of the neutralising effects present in duodenal contents, a duodeno-gastro-oesophageal refluxate has a higher tendency to be non-acidic, and also has the potential of containing bile acids which have associated biological effects. Levels of BA up to the millimolar level have been found in refluxate obtained from GORD patients (Lefebvre *et al.*, 2009). Moreover, BAs with a broad range of biological implications may be present in duodeno-gastro-oesophageal refluxate (Lefebvre *et al.*, 2009). Blondeau *et al.*, as well as a range of other studies from the Leuven group found that bile acids were identified in 60% of BAL samples from individuals with CF who had a lung transplant procedure. This indicates a potential role for the aspiration of bile acid in the pathophysiology of BOS (Blondeau *et al.*, 2008a). Moreover, Brodlie *et al.*, have recently demonstrated that, when measured by tandem mass spectrometry, bile acids can be detected in advanced CF lung disease within the lower airway, and that this possible cause of injury persists even after the lung has been transplanted (Brodlie *et al.*, 2015).

Though the role BAs play in inflammation and respiratory infection is becoming subject to increasing and wider study, the underlying mechanisms of BA mediated injuries relevant to lung diseases are still poorly understood. In humans, the majority of BAs exist in their conjugated forms (Hofmann, 1999b). In this project, the first aim was to explore the possible association between damage to the epithelium - which may enable inflammatory airway disease to develop - and the presence of bile acids in the airway. I investigated the impact of CDCA, CD, LCA and DCA on cell viability and the expression of inflammatory markers (IL-6, IL-8 and GM-CSF) present in inflammatory lung disease that play a significant role.

An influx of inflammatory cells into the lung, which is chronic and fails to resolve is a primary characteristic of airway disease (Boehler et al. 1998) and chemokines such as (IL-6) interleukin 6, and (IL-8) interleukin 8 and the colony-stimulating factor of granulocyte-macrophage (GM-CSF) have all been demonstrated as key in inflammatory cell recruitment. In addition to being a neutrophilic chemoattractant, IL-8 is also an angiogenic factor. In order to identify the localisation of IL-8 within peribronchial lesions, immunostaining has been used (Zheng *et al.*, 2000). The functions of IL-6 are to differentiate T-cells, to mature B-cells and to activate monocytes. Furthermore, when an infection is present, fever is also induced by the pyrogenic effect (Rincon and Irvin, 2012). On the other hand, the role of GM-CSF is in the activation of macrophages and for neutrophils it is both a powerful chemoattractant and also increases neutrophil life span. As a result, this stimulates phagocytotic and microbicidal activity as well as oxidative metabolism, and also enhances the cytotoxicity of both neutrophils (Katano *et al.*, 2010) and macrophages (Sorgi *et al.*, 2012).

For inflammatory mediator expression to be compared, both inducible and basal, before and after bile acid stimulation, four cell types were investigated in this study. The cell type that was the most physiologically relevant to assess damage in the airways were arguably primary human bronchial epithelial cells (PHBECs). However, these are difficult and expensive to culture (Stewart *et al.*, 2012). As a consequence, the ability to provide a useful model to complement the use of PHBECs was a prime, important reason for the use of cell lines in my work. The human bronchial epithelial cell line, BEAS-2B was identified as able to secrete other cytokines in addition to IL-8 and IL-6 while maintaining the morphology of the epithelial cell *in vitro*. Due to these qualities, BEAS-2B is a popular cell line for functional studies which rely on the airway epithelial structure (Swanson *et al.*, 2009). The mucoepidermoid NCI-H292, derived from the cell line carcinoma, reacts to the presence of cigarette smoke in a similar way to PHBECs and presents a useful model for studies on the

production of mucus (Binker *et al.*, 2009). Furthermore, 16HBE14o-, the virally transformed bronchial epithelial line, is used often in research on barrier function and transportation of ions (Gruenert *et al.*, 1988; Cozens *et al.*, 1994). Lastly, the Calu-3 cell line derived from human bronchial submucosal glands presents a model for studying liquids on the airway's surface, mucins as well as substances that are similarly immunologically active (Shen *et al.*, 1994), alongside pro-inflammatory cytokines that are involved in epithelial damage and inflammation of the airway tract (Hamid *et al.*, 1999).

It has been previously reported that the conjugation of BAs results in the loss of their damaging effects (Debruyne *et al.*, 2002; Blau *et al.*, 2007). However, it should be mentioned that most BAs that reside in humans are found in conjugated forms (Hofmann, 1999b). Following treatment using proton pump inhibitors and acid suppression therapies, commonly used in the treatment of GORD, BAs can deconjugate (Theisen *et al.*, 2000), which means that there may be an increased ratio of deconjugated to conjugated BAs for patients undertaking GORD treatment procedures (Theisen *et al.*, 2000). One possible explanation for this could be that an overgrowth of bacteria within the patient's stomach may arise as a result of suppression therapies, and this increased number of microbes deconjugate more of the patient's bile acids (Dettmar *et al.*, 2011). CDCA, DCA, and LCA have all been identified in their unconjugated forms within secretions of the airway (Wu *et al.*, 2009); implying that, for patients with GORD, the effect of these may be of a particular relevance.

As per the current view in this area of research, the understanding of the impact bile acids have in the airways is currently limited by a lack of reports that identify and quantify specific bile acid quantities within the lungs using accurate methodology. This is mainly because of difficulties with the sampling procedure and the low reliability of the bile acid assays. Until now, studies on BAL have been the primary subject of research. The highest bile acid concentration in BAL was reported to be 100 $\mu\text{mol/L}$ (D'Ovidio *et al.*, 2005; Parikh *et al.*, 2013); as BAL samples are diluted at an estimated 100 to 200 fold in practice this implies that bile acid concentrations may be substantially more dilute in the BAL than those that the real-life airways of the patient are actually exposed to. Because of this, the bile acid concentrations used in the current study ranged from 1 to 100 $\mu\text{mol/L}$, which more closely emulated the physiological bile acid content in gastric refluxate estimated to reach a patient in a real-life scenario.

Increased levels of GM-CSF, IL-6 and IL-8 following bile acid challenge have been demonstrated by my research, and these are frequently found in airways in lung disease where the injury had been impacted by reflux and aspiration. My findings reveal that BEAS-2B, NCI-H292, 16HBE14O and Calu-3 cell lines are useful and sensitively efficient models for studying respiratory processes in humans and for studying diseases related to lung injury induced by the aspiration of BAs. These cell lines provide data that are complimentary to the rarer and harder to obtain PHBECs. Overall my data in cell lines and PHBECs indicate that bile acid aspiration could be linked to airway inflammation via the production of inflammatory cytokines.

This study's second primary aim was to explore the link between the epithelial–mesenchymal transition (EMT), that may be causing airway fibrosis; and the presence of bile acids that may be injuring epithelial cells within the airway that enables this to occur. In embryonic development EMT is known to play a role in organogenesis, and also plays a vital role in the body's wound healing process (Hay, 2005). However, when EMT becomes unregulated, it can also facilitate the progression of metastasis and cancer. EMT may also play a role in chronic degenerative fibrotic disorders in the lung as well as in other organs within the body (Thiery, 2002; Wilson and Wynn, 2009). These effects have been implicated in a number of airway and lung pathophysiologies, including pulmonary fibrosis, COPD and the rejection of lung allografts. Despite the growing evidence that shows an association between fibrotic remodelling within organs and the lungs, and the presence of EMT, its role in fibrosis and airway remodelling has not been definitively demonstrated and there is little evidence to suggest this has any impact on bronchial epithelial cells (Doerner and Zuraw, 2009).

E-cadherin has been demonstrated by various studies as a marker of differentiated epithelial phenotype (Arias, 2001; Zeisberg *et al.*, 2003). Also, it is critical in maintaining epithelial polarity and tight junction development (Tunggal *et al.*, 2005). EMT comprises a number of stages, the first of these is E-cadherin downregulation. An increase in mesenchymal markers defines the acquisition of the mesenchymal phenotype, this includes fibronectin production, a protein that holds a function of repairing tissue and is involved in the adhesion and migration of cells (Miettinen *et al.*, 1994). The production of matrix metalloproteinase (MMP) 9 by epithelial cells also characterises epithelial damage and EMT. The true reticular basement membrane, on which the epithelial cells are layered, contains Collagen type IV and MMP-9 is a type IV collagenase. By disrupting the airway epithelium's basement membrane, MMPs can

cause inflammation, translocation, further EMT and other damage (Kalluri and Neilson, 2003).

Consisting of over 30 different types and all with significant biological roles, the transforming Growth Factor (TGF)- β 1 family of growth factors is a well-known and especially effective inducer of EMT in either pathological or normal conditions. TGF- β 1 is a pleiotropic growth factor and regulates both proliferation and epithelial cell plasticity, which on one hand inhibits the usual proliferation of epithelial cells (which thereby opposes the development of cancer), but on the other hand stimulates fibroblast characteristics.

I have demonstrated in this study that the development of airway fibrosis may be associated with the presence of BAs, which may involve EMT. My study showed that BA's triggered the production of excessive levels of growth factor and cytokines, as well as leading to the migration and proliferation of fibroblasts. It has been demonstrated by experiments in lung fibrosis that the growth factors and cytokines produced following BA stimulation result in the formation of myofibroblasts (Kim *et al.*, 2006; Kim *et al.*, 2009). The myofibroblast is defined as possessing a phenotype between that of a fibroblast and a smooth muscle cell, (Gabbiani, 1992). They can be found in all tissues in the body and play a vital role in remodelling after inflammation or injury (Powell *et al.*, 1999). Collagenous and noncollagenous matrix molecules are produced by myofibroblasts, which may cause thickening and stiffening of the basement membrane. Consequently the myofibroblasts may be involved in the development of airway diseases (Singh and Hall, 2008).

This study has provided evidence of EMT markers in primary epithelial cell cultures from lung allograft, tracheal epithelial cells (upper airway cells) from the subglottic area, BEAS-2B cell lines and 16HBE14o- cell lines. The process of EMT may provide an incentive for a pathway following injury in common diseases that is characterised by fibrosis and airway remodelling

One interesting point to note is that these results confirm that, through exposure to BAs, EMT was induced in 16HBE14o- and BEAS-2B cells, which indicates that this effect is not constrained to the primary bronchial epithelial cells found in humans. This indicates that 16HBE14O- and BEAS-2B cell lines are a useful and efficient model to study the processes of human diseases linked to the aspiration of BAs and the possible links with EMT. These findings may have translational significance and may present new opportunities in therapy. My results indicate that modification of GORD could have a positive impact on the airways in

patients with existing lung disease. The process of EMT may represent a final common pathway following airway injury in diseases that leads to the characteristic fibrosis and airway remodelling.

The final goal of the study was to investigate the impact BAs have on the expression of MUC5B and MUC5AC from Calu-3 and NCI-H292 cells. The production of mucus is an important part of the body's immune response to pathogens within the respiratory tract. Mucus hyper-secretion is also an important contribution to the pathophysiology of common airways diseases such as COPD and asthma. Within the human airway, cells that produce mucin comprise of secretory (serous and mucous) cells present in the submucosal glands and the goblet cells that are found on the surface of the airway epithelium. Neither mucous cells nor goblet cells have proven easy to isolate, nor have terminally differentiated cells from healthy epithelia been maintained as cell lines or established as a culture (Rose *et al.*, 2000). When grown on plastic, primary epithelial cells that are isolated from tissues in the airway or the lungs can be conserved for two to three passages, but do not possess the phenotype associated with mucin-expressing cells. Additional problems are that culture conditions are rigorous, the populations of the cells are heterogeneous and the components required for the process are expensive. Conversely, carcinoma-derived cell lines of epithelial origin grow quickly, are passaged with ease and so these cell lines have been used for the study of epithelial-specific diseases such as asthma, COPD, CF and processes of inflammation (Berger *et al.*, 1999). NCI H292 and Calu-3 cells are both epithelial carcinoma-derived cell lines. As an *in vitro* model system, they have been used frequently for research into regulation and present major advantages as the regulation of both the glycoprotein MUC5AC and MUC5B can be studied under this same framework (Berger *et al.*, 1999; Rose *et al.*, 2000).

Within the process of mucus secretion, mucins are one of the primary components. Mucins themselves belong to a group of large glycoproteins and possess a molecular mass of several thousand kilodaltons. In mucus secretions, the mucin composition is the a major determinant of viscoelasticity (Corfield, 2015). MUC5B and MUC5AC appear to be the genes most prevalently expressed, and their glycoprotein products are the most abundantly identified in secretions of mucus (Thornton *et al.*, 1997; Voynow, 2002). Furthermore, MUC5AC appears to be mostly manufactured by goblet cells within the airway epithelium (Suzaki *et al.*, 2016), while MUC5B is mostly manufactured within the submucosal glands that lie underneath (Zanin *et al.*, 2016).

For the colonic mucosa and gastric mucosa (Gadacz and Zuidema, 1978; Gillen *et al.*, 1988), bile acids have a toxic impact (Turjman and Nair, 1981; Owen *et al.*, 1984). Their toxic impact on the oesophageal mucosa however has undergone less significant research (Nasr *et al.*, 2013). A much stronger link has been suggested between the exposure to oesophageal acid (OAE) and the presence of mucosal damage e.g. Barrett's oesophagus, along with the complications that come with it (Legrand *et al.*, 1990; Iftikhar *et al.*, 1993; Takahashi *et al.*, 2011)

In the case of many common, important airway diseases such as asthma and COPD, mucin is hyper secreted and reflux and aspiration has been implicated in the overall pathophysiology. In my project, the production of mucus was significantly increased over the course of this study by the NCI H292 and Calu-3, when the cells were exposed to varying levels of BAs. As a whole, the data from this study therefore strongly supports the hypothesis that exposure to BAs may have a substantial effect on mucus upregulation. This may therefore lead to injury in the airway and airflow limitation. My results also confirmed that within *in vitro* model system, NCI H292 and Calu-3 cells can be used for further research into the processes of mucin gene regulation.

6.1 Summary

It has long been suspected that there is a significant association between lung disease and GOR. A high prevalence of GOR has been found amongst patients with cystic fibrosis, idiopathic pulmonary fibrosis and asthma (Feigelson *et al.*, 1987; Tobin *et al.*, 1998). GORD patients' refluxate can contain bile acids in concentrations up to millimolar levels.

To conclude the work done in this project has extended this research area by showing:

- Cell death was encountered after stimulation of Calu-3, BEAS-2B and 16HBE14O-cell lines with primary BAs (CA - CDCA) and secondary BAs (LCA- DCA). In contrast NCI-H292 cell viability was affected by secondary BAs (LCA- DCA) only. This suggests aspiration may be an important cause of injury to airway epithelium cells and the secondary BAs are more cytotoxic than primary BAs.
- The cells used in this study increased the levels of IL-8, IL-6 and GMCSF released after challenge by primary and secondary bile acids. IL-8, IL-6 and GM-CSF are commonly implicated in airways and lung disease where reflux and aspiration have been implicated as a possible injury.
- This study confirmed that NCI-H292, Calu-3, BEAS-2B and 16HBE14O-cell lines are a useful model to study human respiratory processes and diseases related to BAs aspiration-induced lung injury.
- E-cadherin (an epithelial marker) expression was downregulated and Fibronectin (a mesenchymal marker) was upregulated by BA treatment in primary human bronchial epithelial cell cultures from lung allografts (PHBEC). These findings were also confirmed in tracheal epithelial cells (upper airway cells) from the subglottic area, BEAS-2B and 16HBE14O-cell lines
- The level of TGF- β 1, MMP9 and pro-collagen were increased from primary human bronchial epithelial cell cultures from lung allografts (PHBEC), tracheal epithelial cells from the subglottic area, BEAS-2B and 16HBE14O-cell lines after challenge with BAs.
- Primary human bronchial epithelial cell cultures from lung allografts (PHBEC), tracheal epithelial cells from the subglottic area, BEAS-2B and 16HBE14O-cell lines underwent EMT after stimulation with BAs.

- The EMT process may represent a pathway following airway injury in diseases characterised by airway remodelling and fibrosis where GOR and aspiration are also implicated.
- MUC5AC and MUC5B levels were induced in response to stimulation of Calu-3 and NCI H292 cells with BAs. This suggests that aspiration may lead to mucus hypersecretion as seen in the disease.
- The Calu-3 and NCI H292 cells can be used as an in vitro model system to study MUC gene regulation.

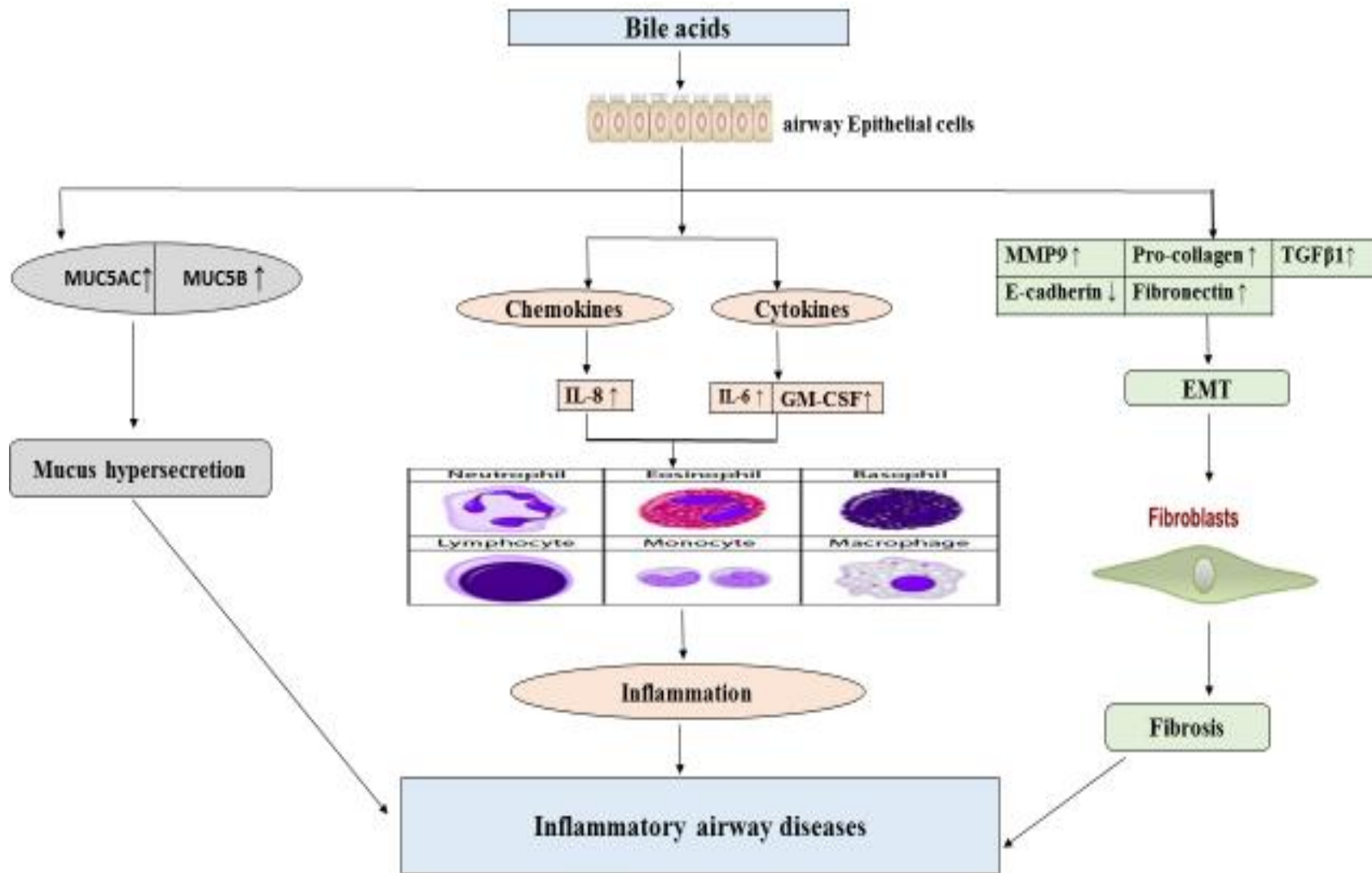


Figure 6-1: A proposed sequence of events following epithelial cells injury after exposure to bile acids

Future directions

- In this study, submerged culture systems were used to study the association between airways diseases and BAs. Future work could include establishing an air-liquid interface of the primary bronchial epithelial cells. The cells will differentiate into ciliated epithelial cells. The BAs stimulation would then be repeated and a number of cytokine and mucins could be measured.
- There is evidence that bile acids may be taken up by lung epithelia cells. It may therefore be of interest to stain biopsies and epithelial cells taken from lung transplant recipients for BAs, in an attempt to further understand what happens to the bile acids once they have been aspirated into the lungs.
- Bile acids can be taken up by laryngeal epithelial cells (extra-oesophageal reflux disease); for this reason the impact of bile acids on a pharyngeal cell line should be investigated.
- In this study cells were stimulated with BAs for 48 hours, future studies could evaluate the effect of longer term stimulation with bile acids.
- My results showed that PHBEC, tracheal epithelial cells from the subglottic area, BEAS-2B and 16HBE14O-cell lines underwent EMT after stimulation with BAs. These results should be confirmed by immunohistochemistry.
- A study should be set up to investigate sensitive methods that can be used to measure bile acids in the airways.
- Mucus gel removed from the lungs of patient undergoing bronchoscopy with airway disease should be studied. Mucus gels consist of mucin IgA, protein, lipid, and nucleic acid. Studying the rheological properties of mucus gels is fundamental to understand the role of these non-mucin components.
- The airway cancer cell line models could be used to provide additional insight into the differential regulation of EMT-associated phenotypes. For example, flow cytometry could be used to count epithelial and mesenchymal cell types within cultured cell samples. Flow cytometers separate different cell types suspended within a fluid stream through hydrodynamic focussing. One or more lasers are then pointed at the fluid stream and photomultipliers detect the resultant fluorescent and scattered light. Fluorophores on or within the cells are detected using optical filters and their emission spectra peaks can be used to establish a reliable cell-type count (O'Neill et al., 2013). Flow cytometry remains

the preferred method for sorting viable cells by focussing on multiple cell surface proteins and their quantitative, simultaneous analysis (Strauss et al., 2013). Flow based methods have been used for the quantification of EMT in the setting of lung transplantation. Flow cytometry may have several advantages including the fact that several markers can be detected at once and large numbers of cells can be counted. This could be a useful future development of EMT research in my future studies.

Mesenchymal marker expression, specifically α SMA, S100A4 and ED-A FN and HLA-DR, has been assessed using flow cytometry, whilst TGF β 1 and HGF expression has been measured in Bronchoalveolar Lavage (BAL). Patients with Bronchiolitis Obliterans Syndrome (BOS) exhibited increased expression of all three mesenchymal markers as well as HLA-DR. BAL HGF was also seen to increase, but TGF β 1 was not. Analysing bronchial brushing samples using flow cytometry has been shown to be safe, fast, simple and quantitative (Hodge et al., 2009). On this basis, the method is proposed as an important additional means for EMT development studies.

- Discovery and quantification of transcripts can be carried out in a single experiment using the high-throughput assay RNA-Seq, or ‘whole transcriptome sequencing’ (Cloonan et al., 2008; Guttman et al., 2010). For experiments that involve a broader dynamic range of gene expression, it has been shown that RNA-Seq provides more accuracy than expression microarrays (Marioni et al., 2008; Fu et al., 2009). Such approaches may be useful for future studies of EMT.

RNA-Seq is now a standard tool in many large-scale genomic analyses, which seek to establish connections between genetic variation and epigenetic state, and transcriptional and post-transcriptional regulation. A number of these studies have indicated that cell type and specificity are determined by alternative splicing and isoform selection (Pan et al., 2008; Graveley et al., 2011). Furthermore, a large number of genes are characterised by having a large genomic ‘footprint’, containing a range of splice variants, promoters and proteins. Measuring changes in the way individual transcripts are expressed allows investigation into the regulation of isoform selection and diversity. In airway diseases, RNA-Seq may be employed to identify airway transcriptome and gene expression alterations that are linked with exposure to BAs and inflammatory diseases. This assay is likely to enable better understandings of the molecular mechanisms involved in EMT therefore and could represent a future direction for this area of work.

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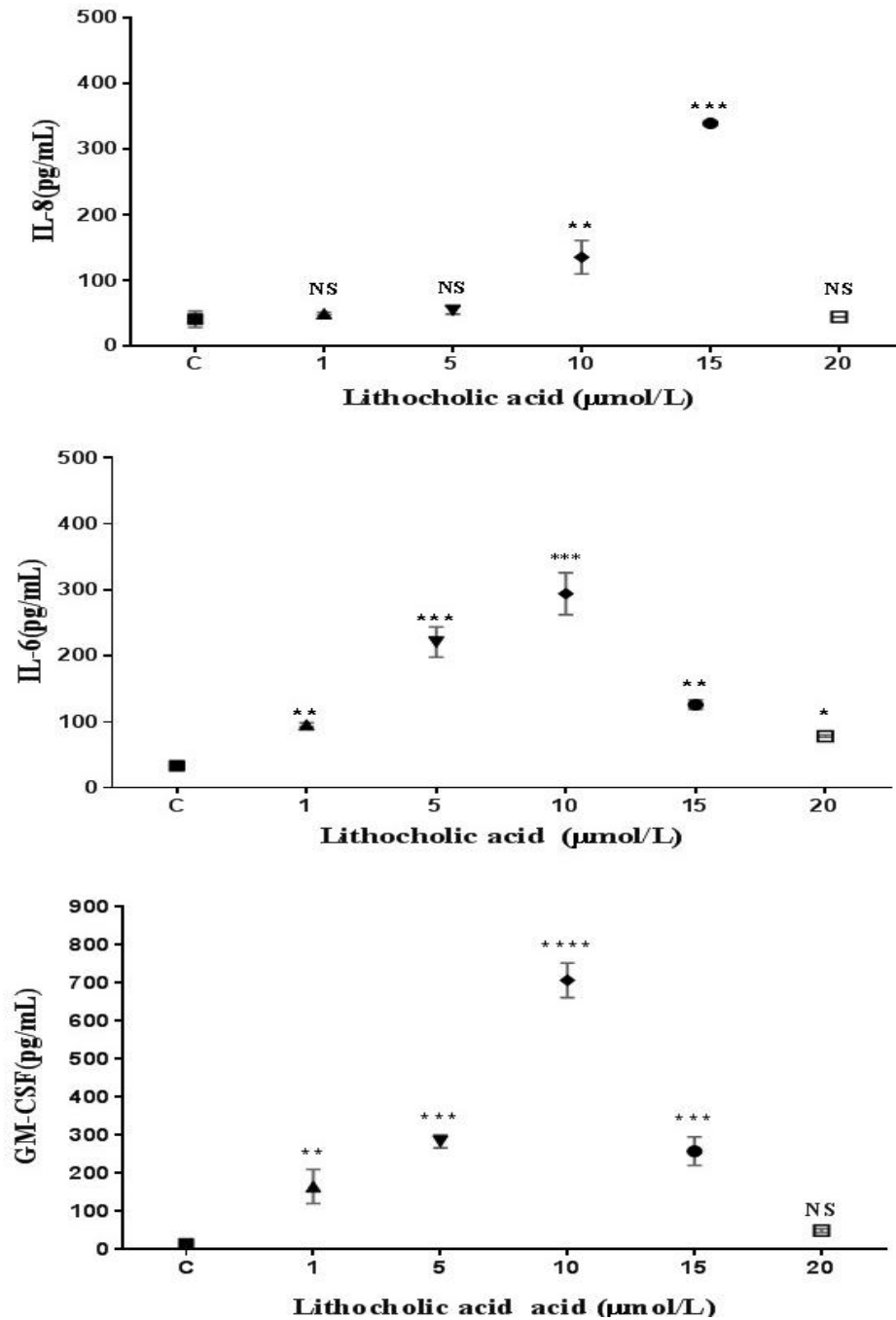
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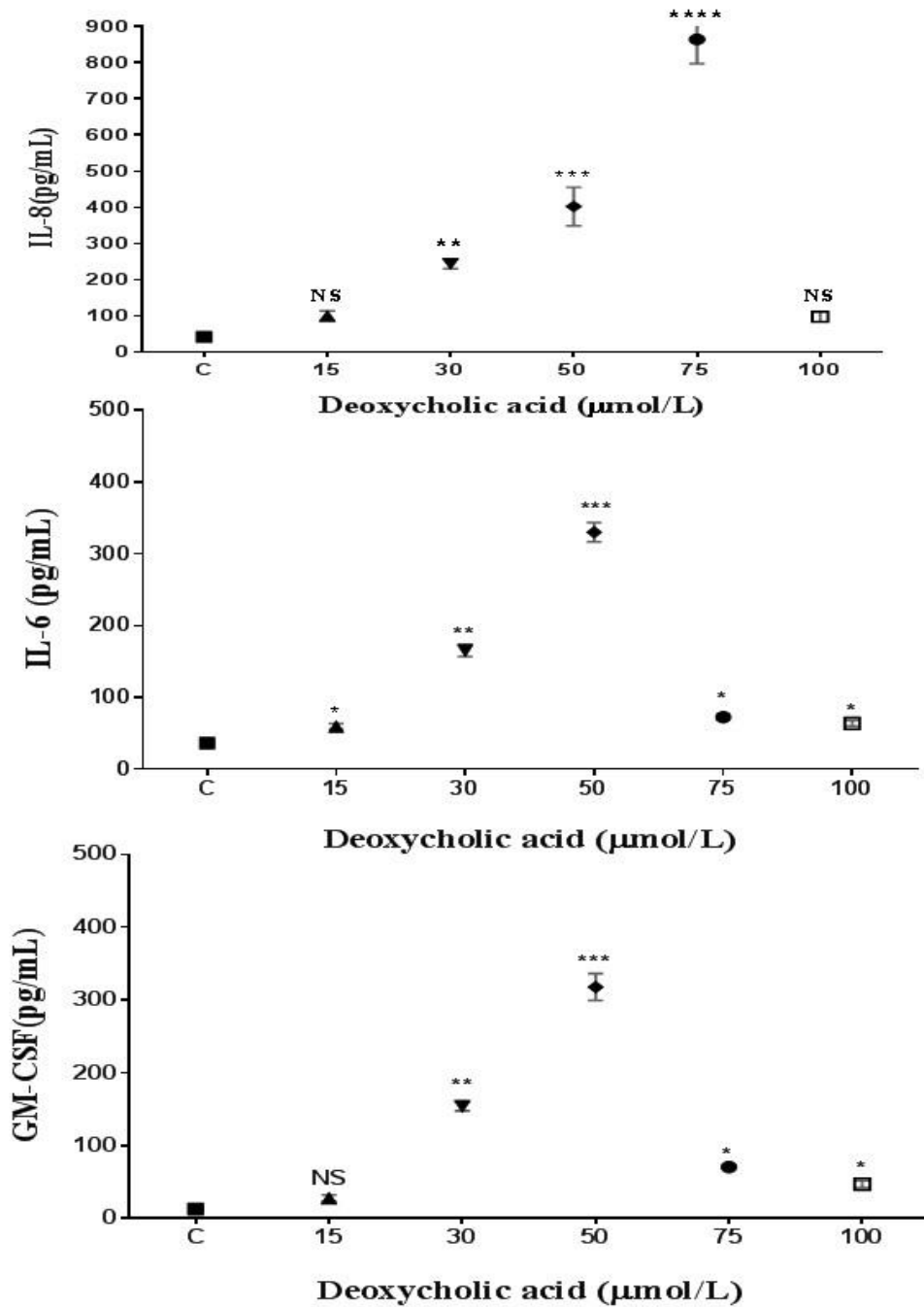
APPENDICES

APPENDIX 1

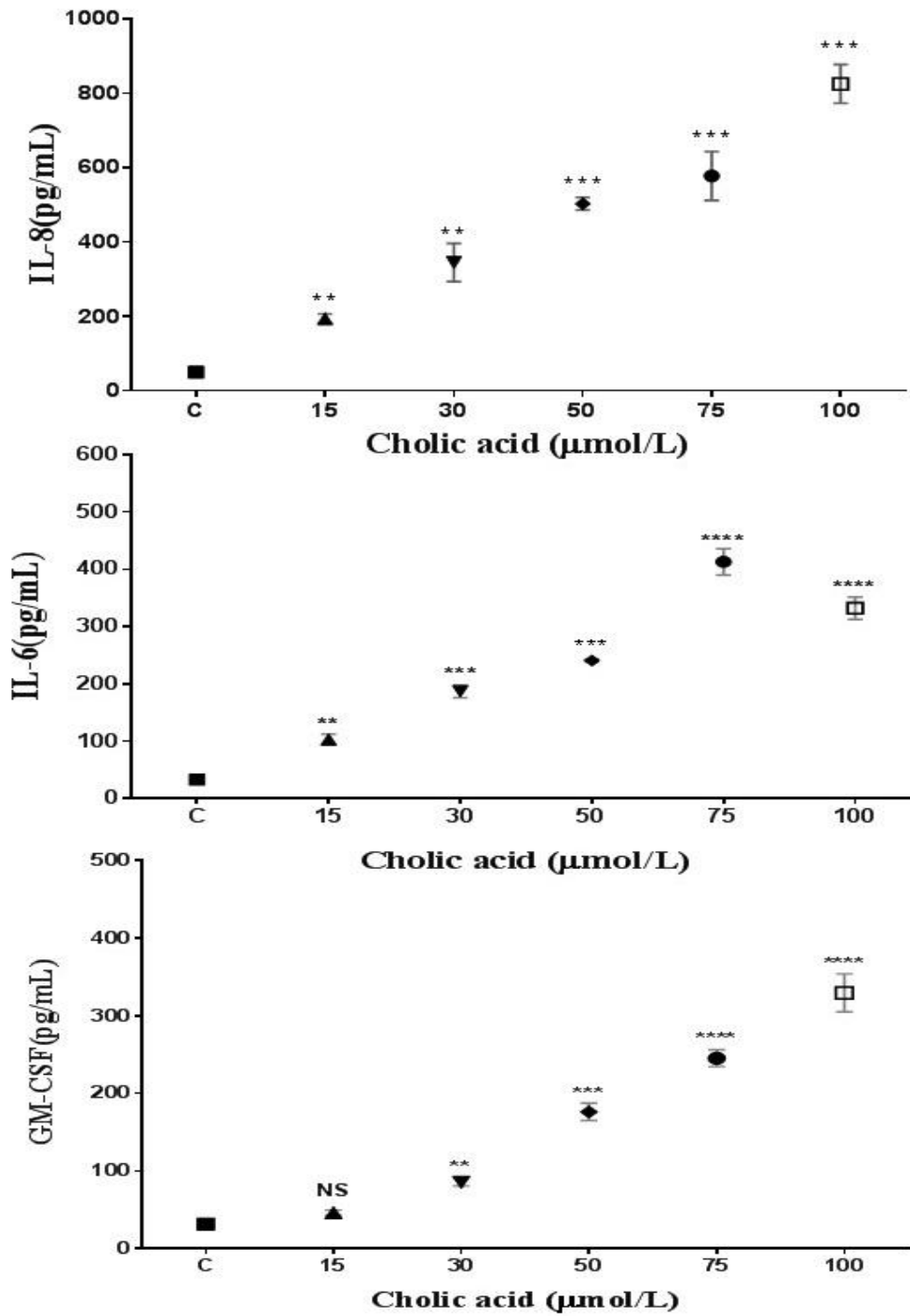
Influence of individual bile acid on 16HBE14o- cell viability and inflammatory cytokine (IL-8, IL-6 and GM-CSF) release



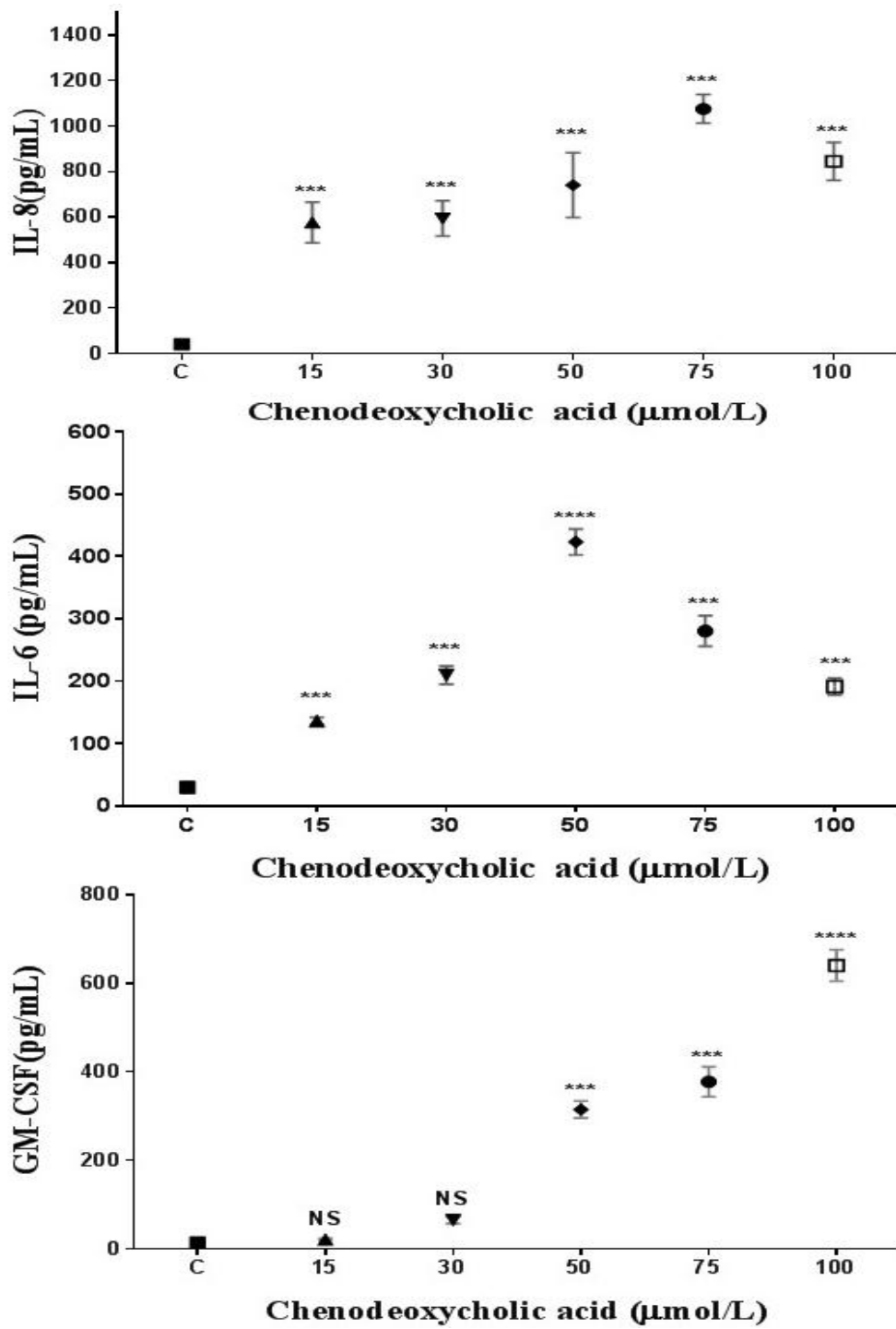
Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. (NS-not significant).



Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with DCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



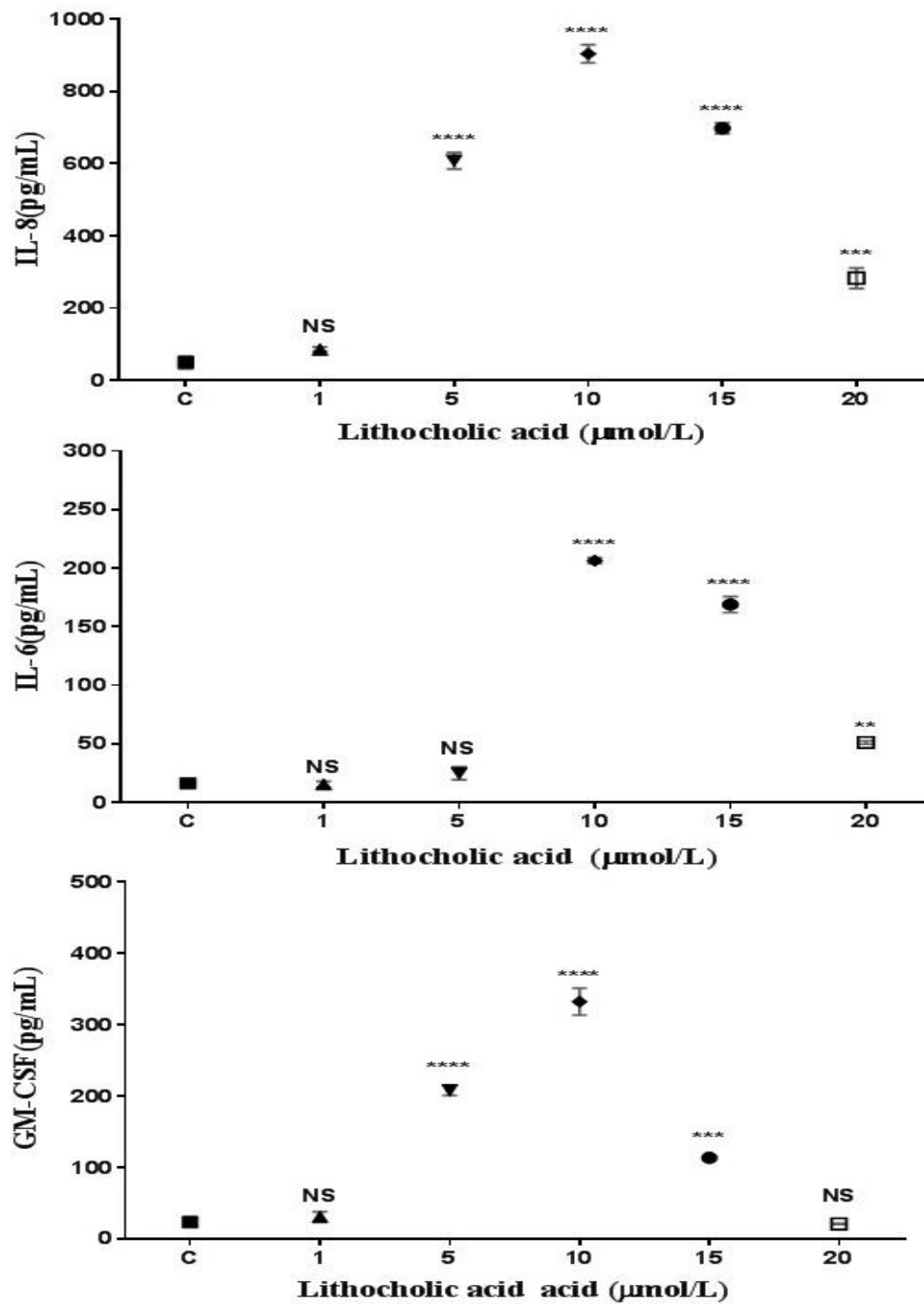
Measurement of IL-8, IL-6 and GM-CSF production from 16HBE14o- cells treated with CA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. (NS- not significant).



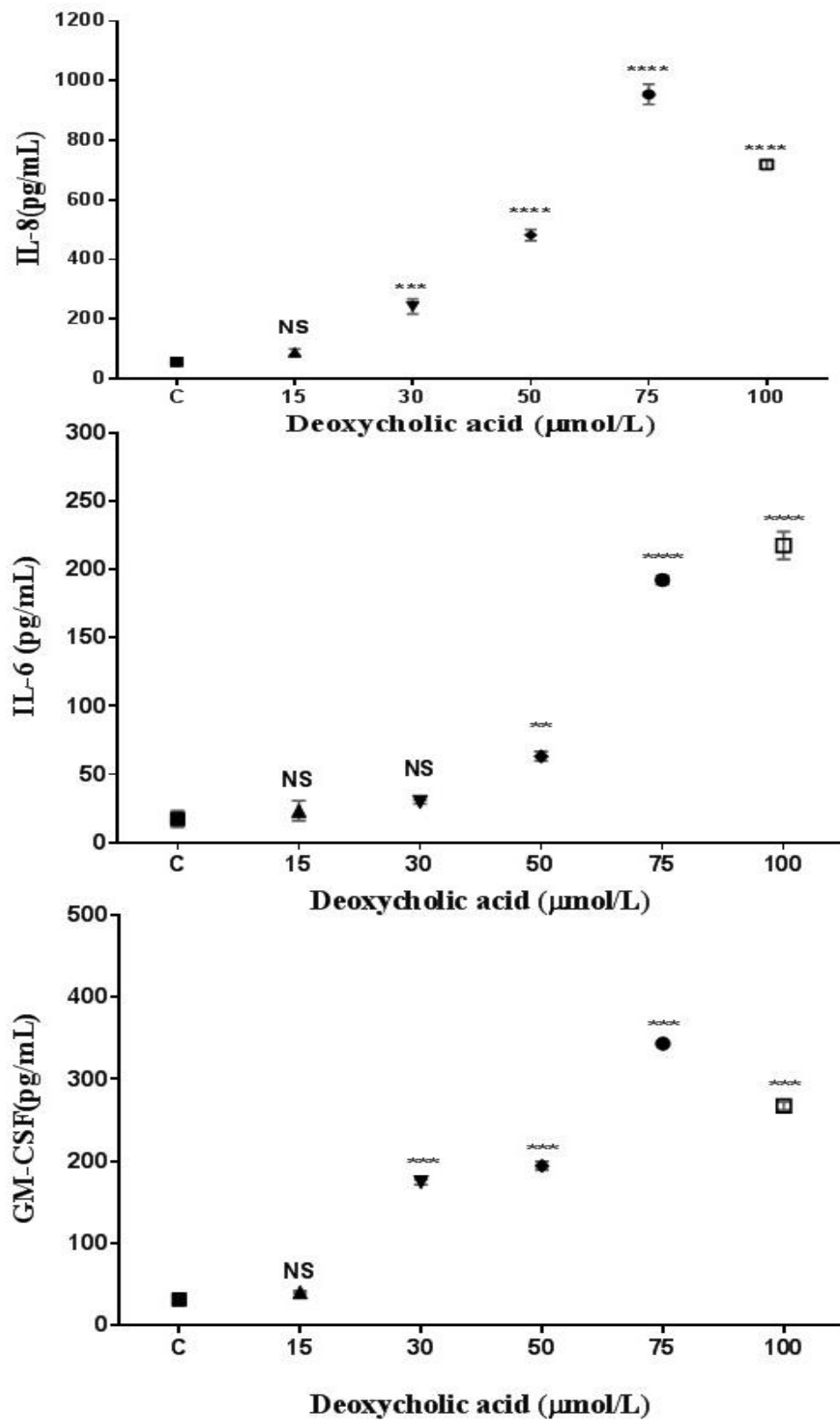
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APPENDIX 2

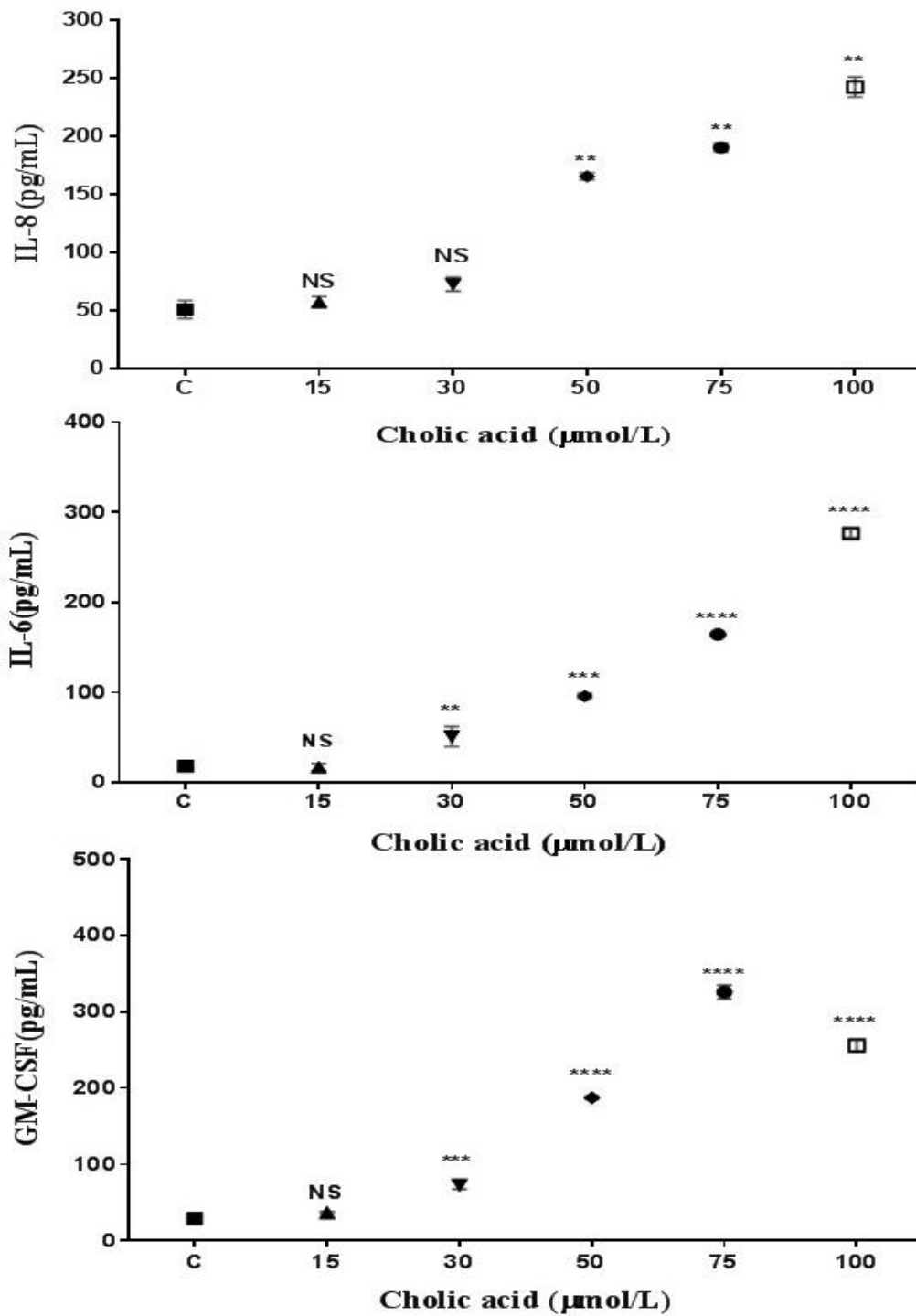
Influence of individual bile acid on BEAS-2B cell viability and inflammatory cytokine (IL-8, IL-6 and GM-CSF) release



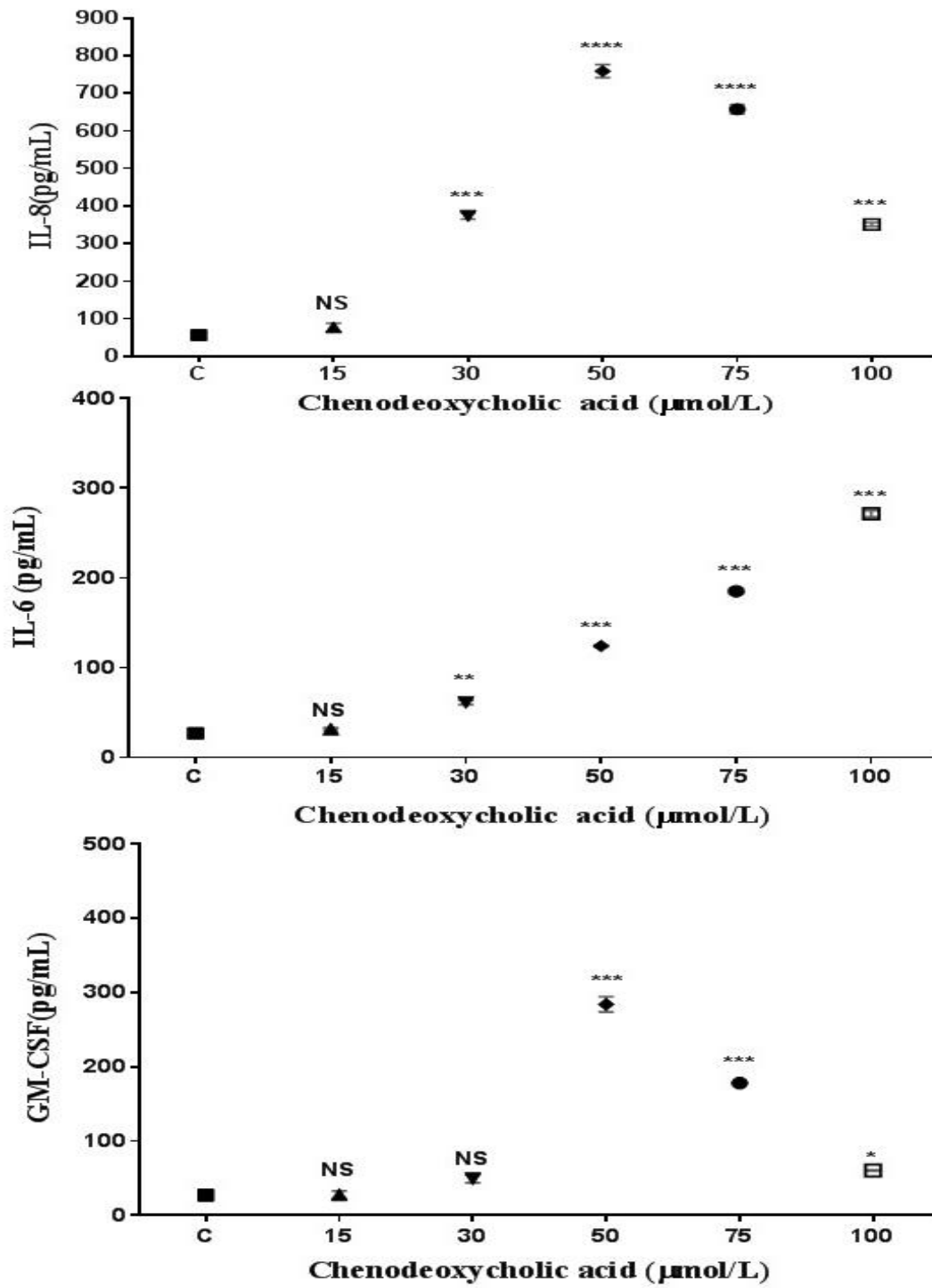
Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with LCA. ELISA was used to measure cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with DCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



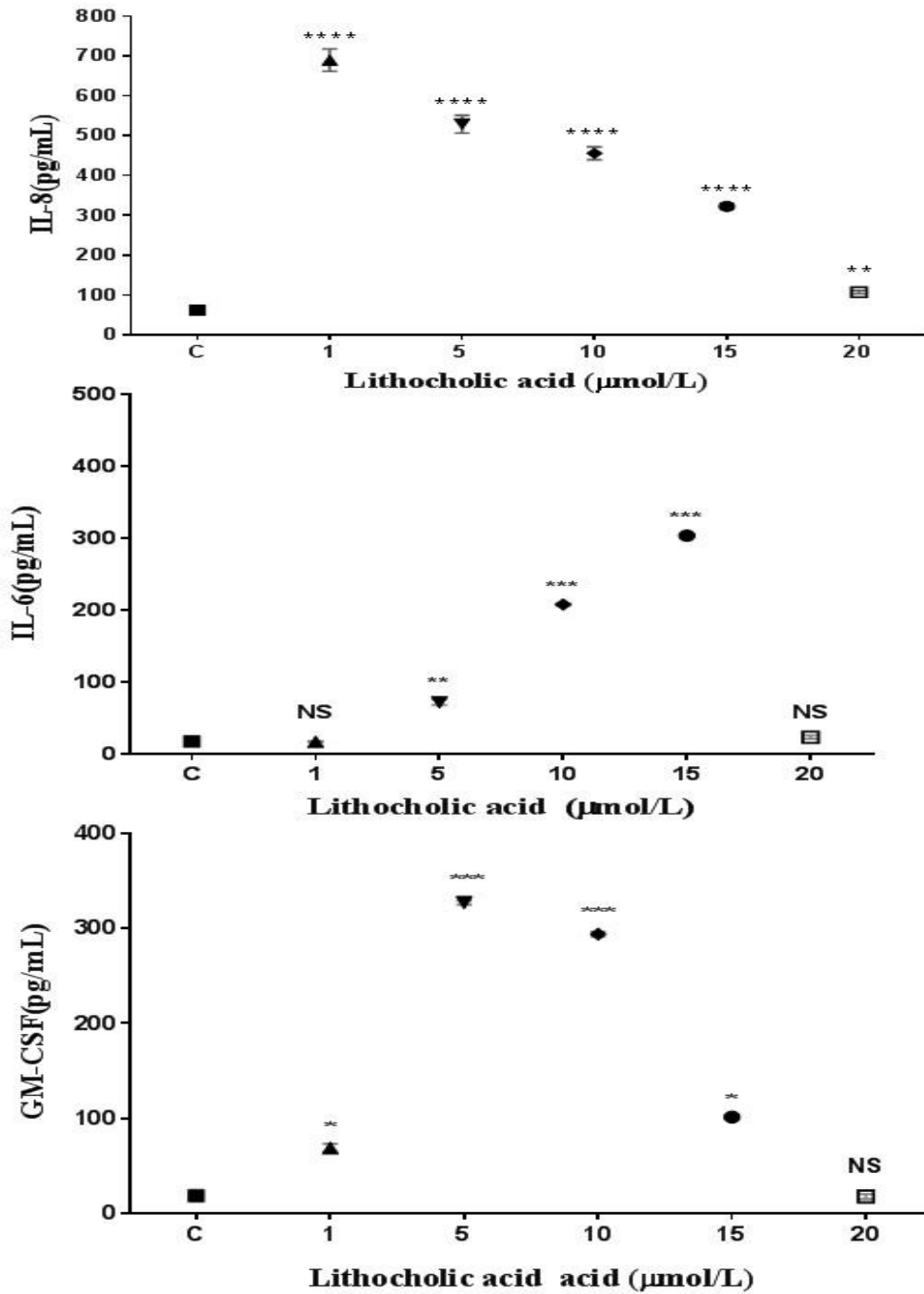
Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with CA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01and, ***: p<0.001. (NS- not significant).



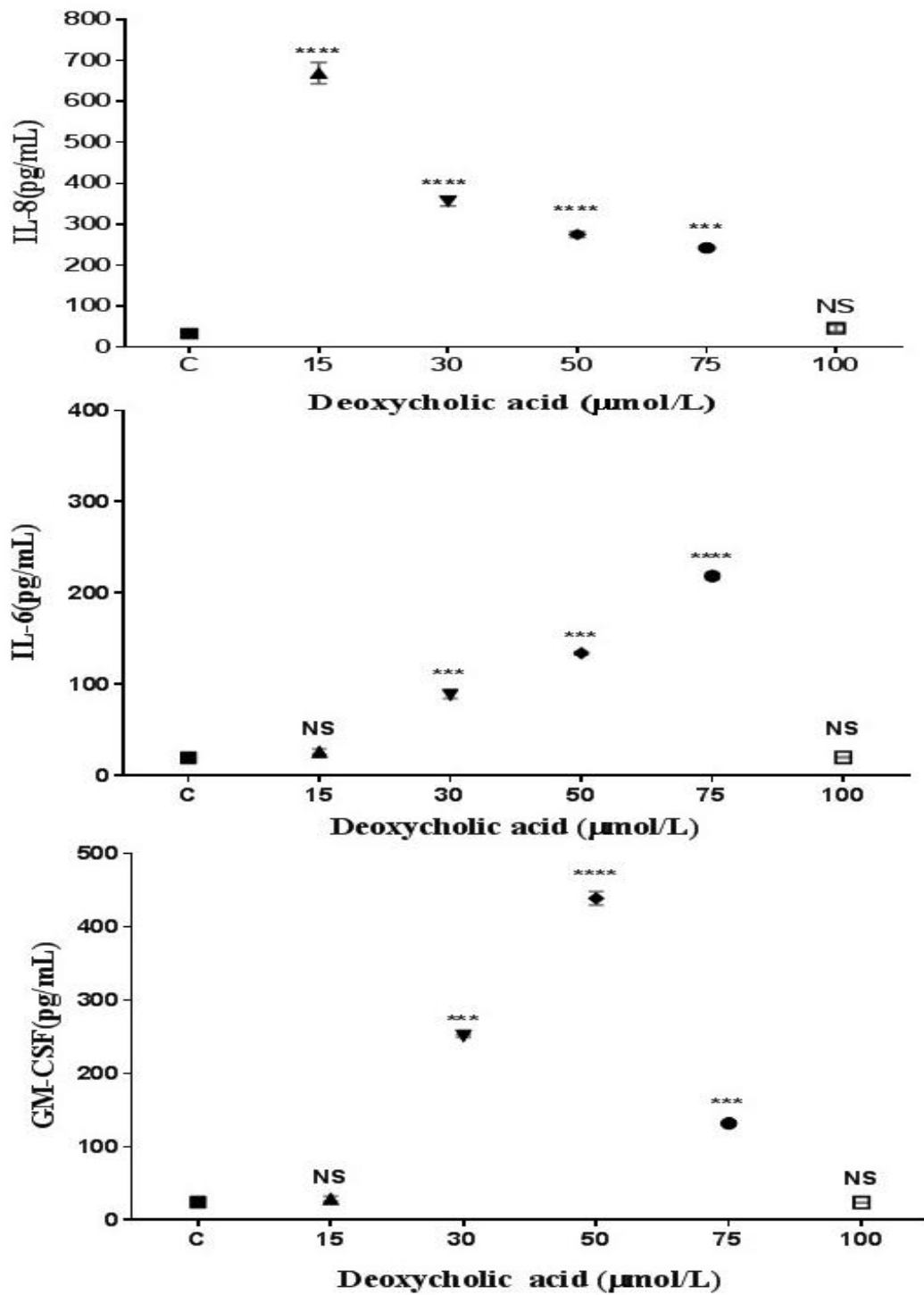
Measurement of IL-8, IL-6 and GM-CSF production from BEAS-2B cells treated with CDCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).

APPENDIX 3

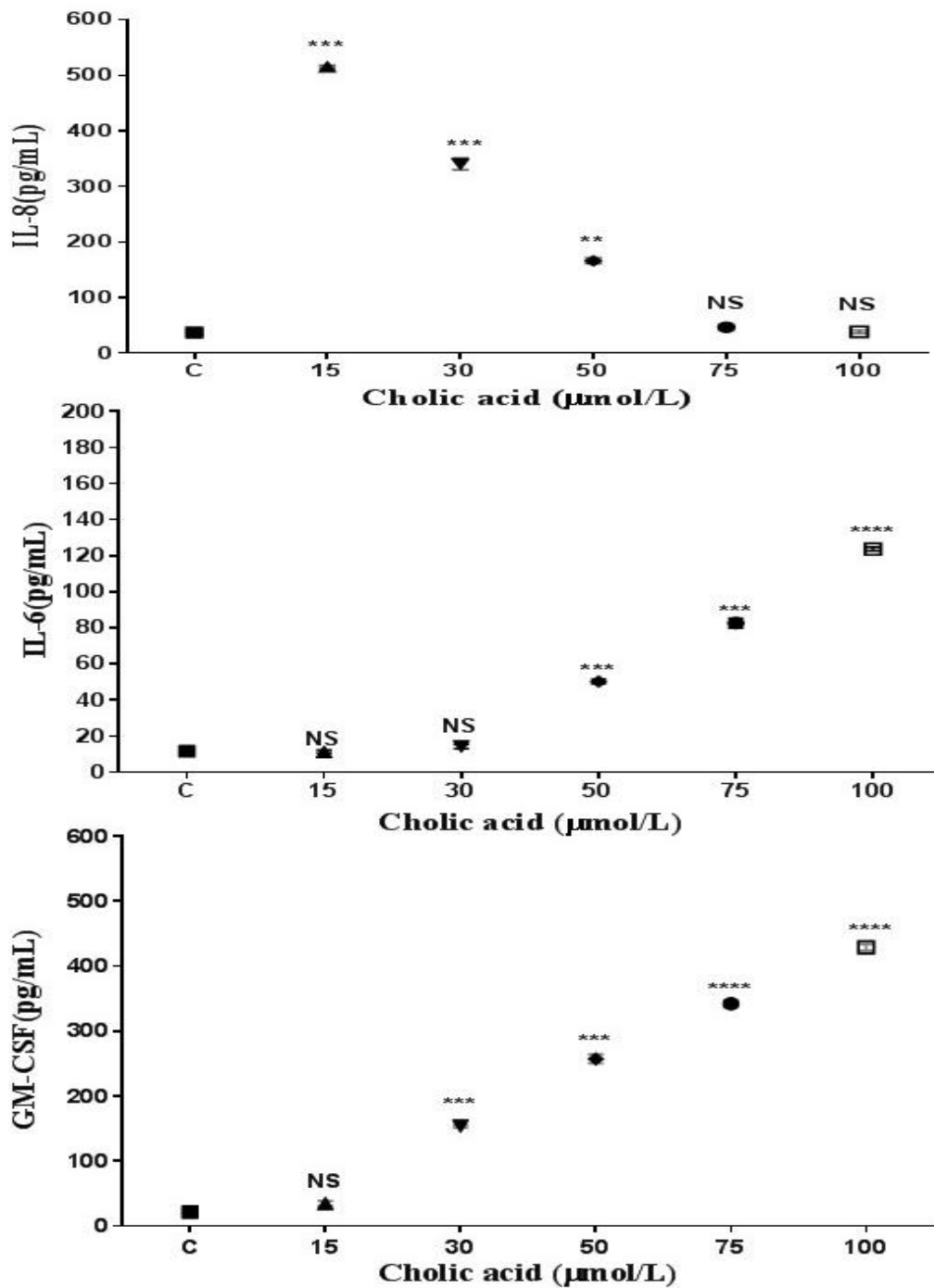
Influence of individual bile acid on NCI-H292 cell viability and inflammatory cytokine (IL-8, IL-6 and GM-CSF) release



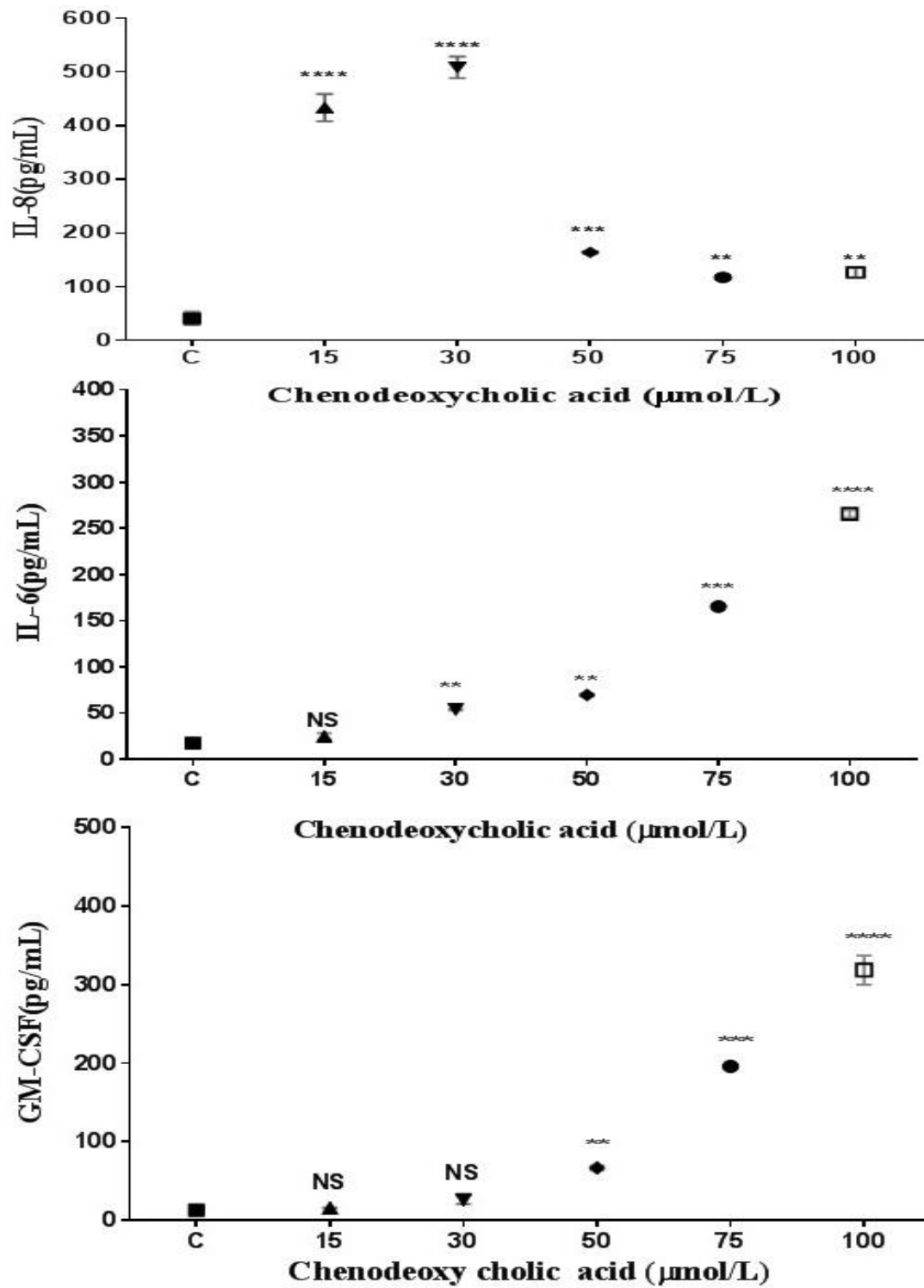
Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with LCA. ELISA was used measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with DCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



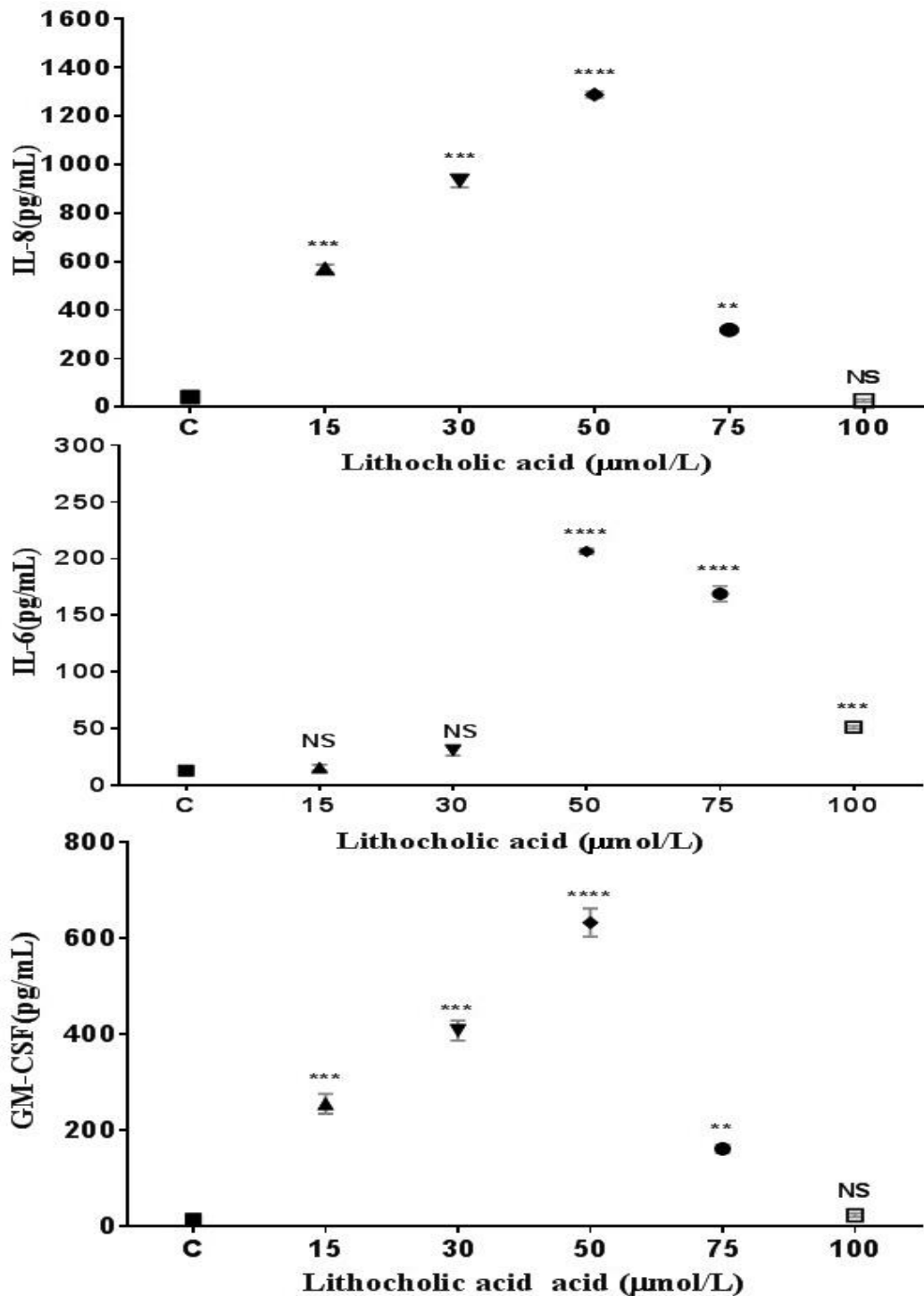
Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with CA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



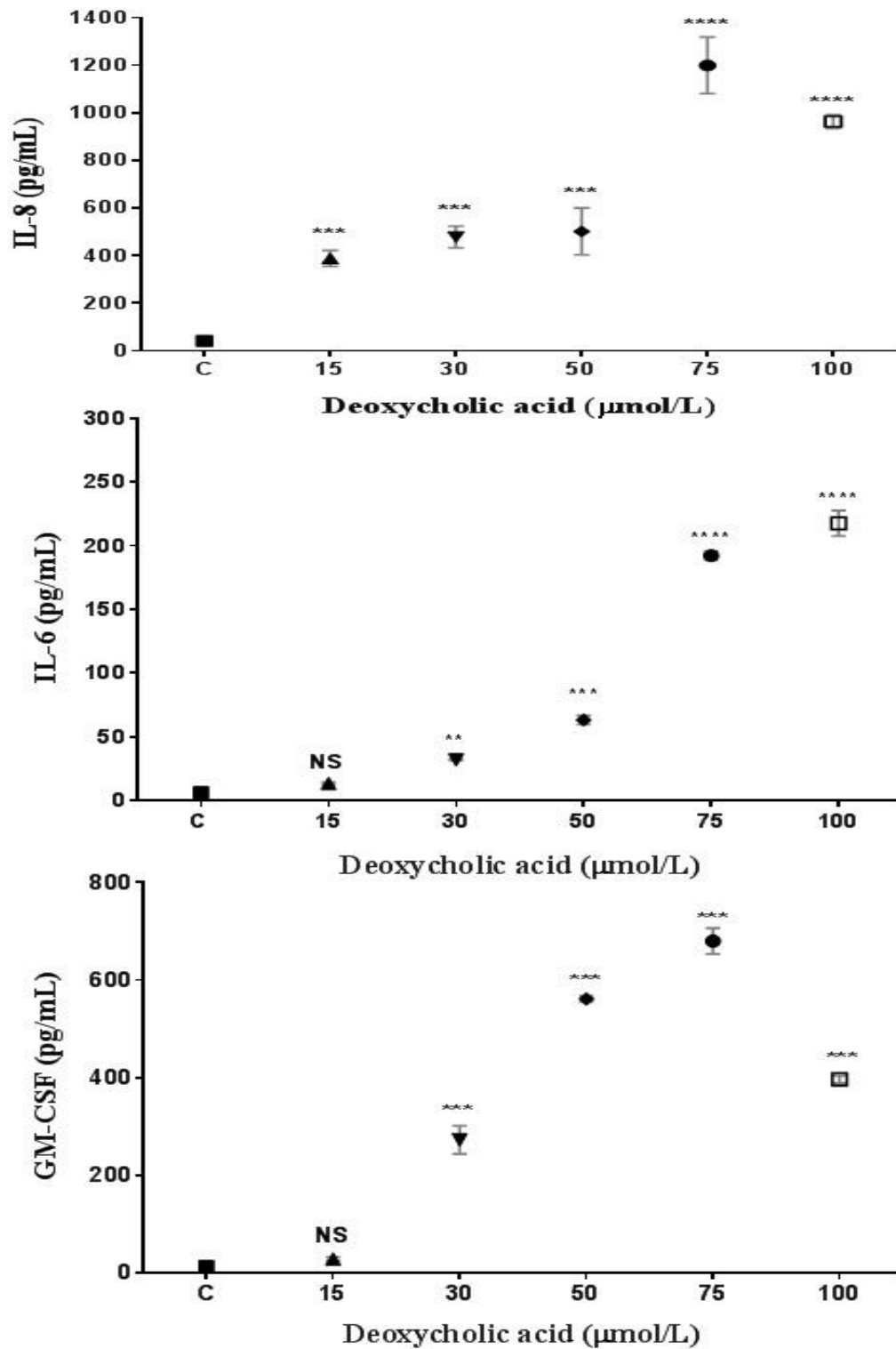
Measurement of IL-8, IL-6 and GM-CSF production from NCI-H292 cells treated with CDCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).

APPENDIX 4

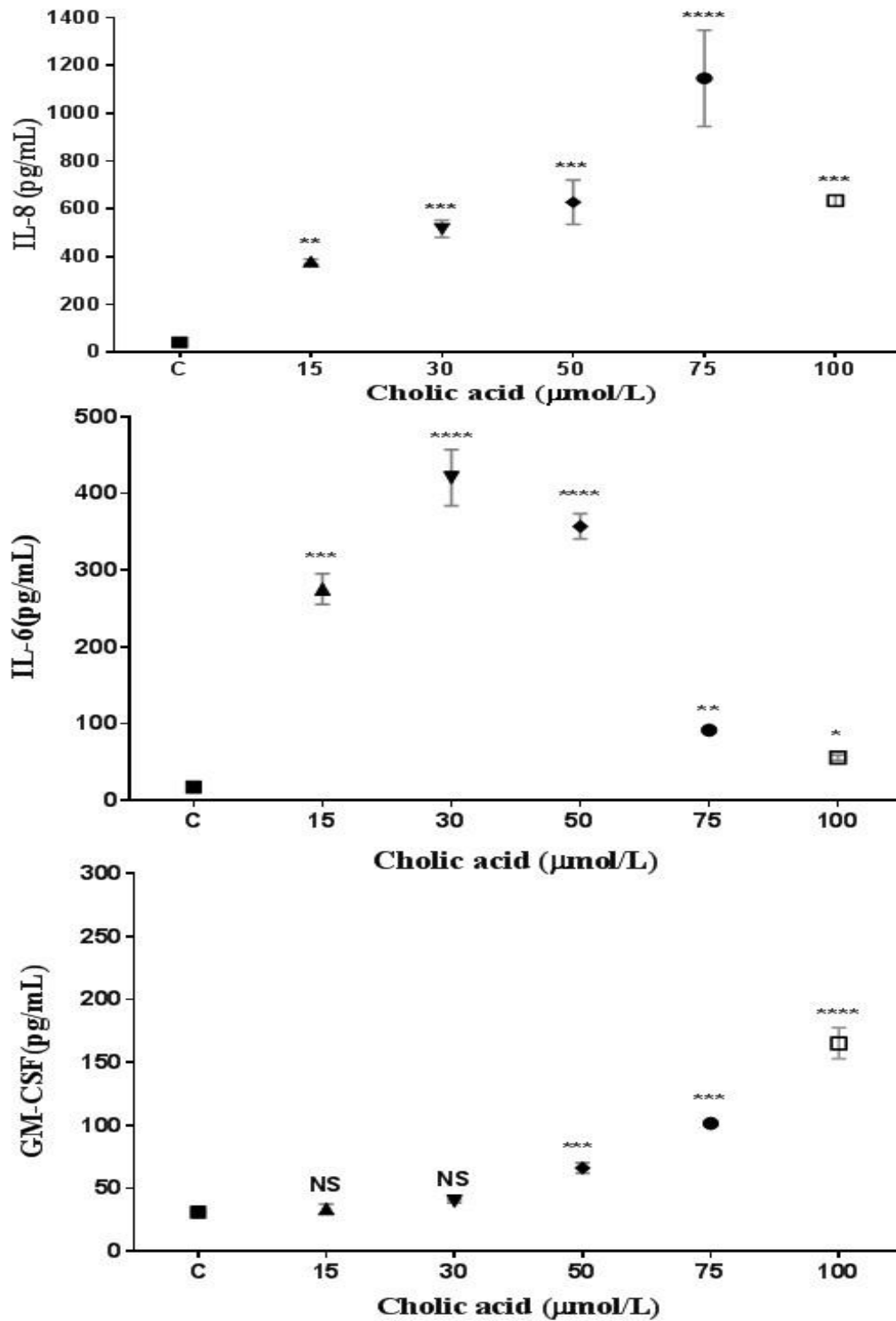
Influence of individual bile acid on Calu-3 cell viability and inflammatory cytokine (IL-8, IL-6 and GM-CSF) release



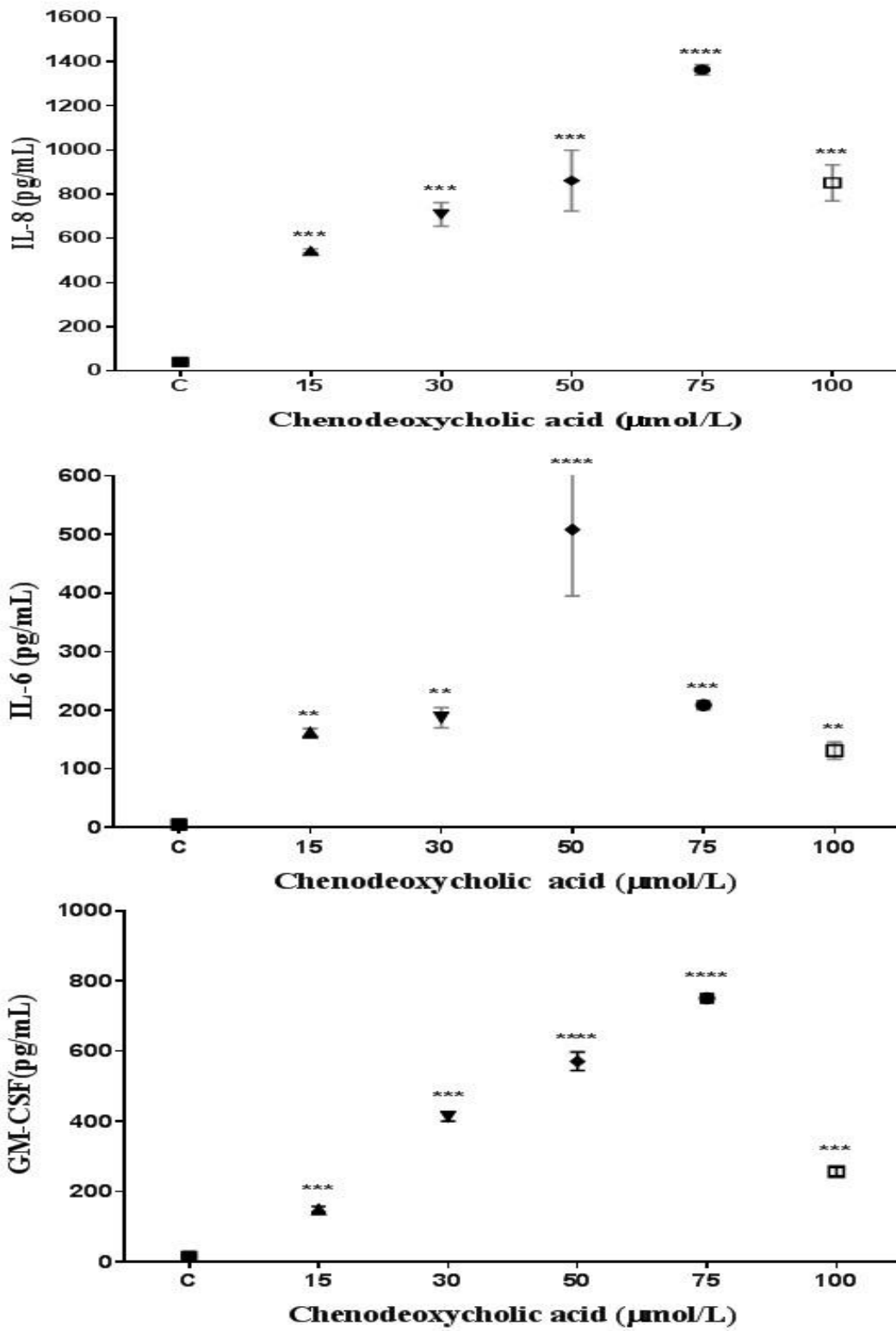
Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with LCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with DCA. ELISA was used measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: $p < 0.05$, **: $p < 0.01$ and, ***: $p < 0.001$. (NS- not significant).



Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with CA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01 and, ***: p<0.001. (NS- not significant).



Measurement of IL-8, IL-6 and GM-CSF production from Calu-3 cells treated with CDCA. ELISA was used to measured cytokine secretion. Statistical analysis was conducted using repeated-measures-paired-one-way ANOVA (n=6). Bars represent mean \pm S.E.M values of each group, *: p<0.05, **: p<0.01and, ***: p<0.001.(NS- not significant).

ORIGINAL ARTICLE
LUNG BIOLOGY

Effects of bile acids on human airway epithelial cells: implications for aerodigestive diseases

Adil Aldhahrani¹, Bernard Verdon¹, Chris Ward^{1,2,3} and Jeffery Pearson^{1,3}

Affiliations: ¹Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK. ²Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK. ³These authors contributed equally.

Correspondence: Chris Ward, Institute for Cellular Medicine, The Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. E-mail: chris.ward@ncl.ac.uk

ABSTRACT Gastro-oesophageal reflux and aspiration have been associated with chronic and end-stage lung disease and with allograft injury following lung transplantation. This raises the possibility that bile acids may cause lung injury by damaging airway epithelium. The aim of this study was to investigate the effect of bile acid challenge using the immortalised human bronchial epithelial cell line (BEAS-2B).

The immortalised human bronchial epithelial cell line (BEAS-2B) was cultured. A 48-h challenge evaluated the effect of individual primary and secondary bile acids. Post-challenge concentrations of interleukin (IL)-8, IL-6 and granulocyte-macrophage colony-stimulating factor were measured using commercial ELISA kits. The viability of the BEAS-2B cells was measured using CellTiter-Blue and MTT assays.

Lithocholic acid, deoxycholic acid, chenodeoxycholic acid and cholic acid were successfully used to stimulate cultured BEAS-2B cells at different concentrations. A concentration of lithocholic acid above $10 \mu\text{mol}\cdot\text{L}^{-1}$ causes cell death, whereas deoxycholic acid, chenodeoxycholic acid and cholic acid above $30 \mu\text{mol}\cdot\text{L}^{-1}$ was required for cell death. Challenge with bile acids at physiological levels also led to a significant increase in the release of IL-8 and IL6 from BEAS-2B.

Aspiration of bile acids could potentially cause cell damage, cell death and inflammation *in vivo*. This is relevant to an integrated gastrointestinal and lung physiological paradigm of chronic lung disease, where reflux and aspiration are described in both chronic lung diseases and allograft injury.



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Bile acid exposure has a significant effect on cytokine production which could contribute to airway injury <http://ow.ly/wfa309Rqu>

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Introduction

The airway epithelium plays a critical role in diseased as well as non-diseased lungs, primarily by providing a physical barrier to foreign particles such as particulates and smoke, as well as viruses and bacteria. In addition, it enables a range of pro-inflammatory, anti-microbial and regulatory functions. Epithelial regulatory functions can involve generating and disseminating a range of mediators for various responses, including chemotaxis, inflammatory cell differentiation and activation in both diseased and non-diseased conditions [1].

Many chronic degenerative diseases, including lung allograft dysfunction, are characterised by epithelial damage related to pro-inflammatory cytokines. This process commonly involves bronchial cell injury and necrosis, as well as leukocyte infiltration, leading to inflammatory lung disease [2].

When the lower oesophageal sphincter becomes relaxed, gastric contents may pass into the oesophagus by retrograde flow. Gastro-oesophageal reflux (GOR) is not uncommon and generally may not be harmful [3]. However, certain conditions may lead to GOR becoming pathological, resulting in gastro-oesophageal reflux disease (GORD) [1, 2]. GORD symptoms can be relieved by proton pump inhibitors (PPIs). PPIs work by binding covalently to gastric H⁺ K⁺-ATPase pumps, causing irreversible inhibition of the final step in gastric acid secretion. The use of PPIs therefore causes gastric pH to be elevated [4]. PPI treatment can reduce gastric acid secretion, but does not prevent overall gastric aspiration, which includes non-acid components [5]. This is one reason why acid suppression alone may not be entirely effective in preventing GOR [6], and in cases where symptoms are unresponsive to PPI treatment, it is thought that these continued symptoms may be caused by reflux of agents other than gastric acid [7].

Bile acids (BAs) are sparingly water-soluble steroids produced by hepatocytes. Despite being strongly cytotoxic and associated with gastrointestinal malignancy [8], BAs are an essential component of the digestive process, aiding lipid digestion in the small intestine. "Primary BAs" are synthesised by conversion of cholesterol into cholic acid (CA) and chenodeoxycholic acid (CDCA), which are then conjugated to glycine or taurine. These are deconjugated by bacterial enzyme action in the colon into "free BAs". The action of 7 α -dehydroxylase converts free BAs into "secondary BAs" (CDCA into lithocholic acid (LCA) and CA into deoxycholic acid (DCA)).

Gastric mucosa [8, 9], colonic mucosa [9, 10] and hepatocytes [11] are all affected by BAs. However, their toxicity to airway mucosa is less well understood [12]. Patients with a range of airway diseases have been shown to exhibit higher BA concentrations in their bronchoalveolar lavage (BAL) [13]. Gastric aspiration has been revealed to lead to severe pneumonitis [14]. Most therapeutic interventions for GORD and associated inflammatory disease have sought to inhibit acid production by gastric parietal cells. However, a study conducted by KAUER *et al.* [15] measured both acid (using standard pH tests) and duodenal juice (bilirubin monitoring) reflux in 53 GORD patients. Both types of reflux were found to be present in two-thirds of the patients. Furthermore, higher levels of bilirubin were identified in the refluxate of patients with Barrett's oesophagus. These findings may explain the limited success of PPI therapy in this setting and perhaps why some patients appear more prone to developing Barrett's oesophagus.

The most physiologically relevant cell type to evaluate airways damage is arguably the primary human bronchial epithelial cell (PBEC), but these cells are costly and difficult to culture [16]. Consequently, cell lines have been investigated regarding their ability to provide a useful model to complement the use of PBECs. The human bronchial epithelial cell line, BEAS-2B, for example, is able to secrete interleukin (IL)-6, IL-8 and other cytokines, while maintaining epithelial cell morphology *in vitro*. These qualities make BEAS-2B a favoured cell line for airway epithelial structure and functional studies [17].

This study hypothesised that BAs, described previously as being present in the lung, can cause damage to the airway epithelium, which may be a mechanism for lung injury. The study aimed to establish a model for airway epithelial cell injury that is relevant to reflux-associated chronic degenerative airways diseases. BEAS-2B cells were challenged with primary and secondary Bas, and pro-inflammatory cytokine levels (IL-8, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) were measured by ELISA. These cytokine markers were chosen on the basis of prior studies, which have found these factors to be released by damaged cells, and because they are associated with airway pathophysiology [17].

Materials and methods

Cell culture

BEAS-2B culture

BEAS-2B (obtained from ATCC; LGC Standards, Teddington, UK) is a human bronchial epithelial cell line derived from normal human epithelial cells immortalised using a hybrid of adenovirus 12 and simian virus 40 [18]. BEAS-2B cells were seeded in 24-well plates at a density of 6 to 7 $\times 10^5$ cells mL⁻¹ and were grown in 500 μ L bronchial epithelial growth medium (BEGM) (Lonza, Cambridge, MA, USA) at 5% CO₂

and 37°C. This was supplemented with 2 mL bovine pituitary extract (0.004 mL·mL⁻¹), 0.5 mL insulin (5 µg·mL⁻¹), 0.5 mL hydrocortisone (0.5 µg·mL⁻¹), 0.5 mL retinoic acid (0.1 ng·mL⁻¹), 0.5 mL transferrin (10 µg·mL⁻¹), 0.5 mL tri-iodothyronine (6.7 ng·mL⁻¹), 0.5 mL adrenaline (0.5 µg·mL⁻¹), 0.5 mL recombinant epidermal growth factor human (10 ng·mL⁻¹), 100 U·mL⁻¹ L-glutamine and 100 U·mL⁻¹ penicillin/streptomycin (Sigma, Gillingham, UK).

BEAS-2B cell stimulation

At about 80% confluence, BEAS-2B cells were rested for 24 h in serum-free medium (BEBM, 50 mg·mL⁻¹ gentamycin, 50 µg·mL⁻¹ amphotericin B (Lonza), 100 U·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin (Sigma)). BAs were dissolved in methanol to prepare stock solutions (100 Mmol·L⁻¹). The solution was diluted with resting medium to achieve a range of experimental concentrations. A total of three experiments with five replicate wells (n=15) were conducted. The following BA concentrations were used: 1, 5, 10, 15, 20, 30, 50, 75 and 100 µmol·L⁻¹. After 48 h incubation, cell culture supernatants were collected to measure cytokine concentrations. Commercial IL-8, IL-6 and GM-CSF ELISA kits were used (R&D Systems, Abingdon, UK).

Cell viability assays

CellTiter-Blue assay

Cell viability was assessed using the CellTiter-Blue assay (Promega, Madison, WI, USA). Known ratios of living to dead cells were used to generate a standard curve (with the dead cell control prepared by exposure to ice-cold methanol for 5 min) and to provide cell mixtures. The standard curve comprised 100% live cells (resting media); 25/75%, 50/50%, 75/25% dead/live cells; and 100% dead cells. This was used to determine the percentages of viable cells. Wavelengths of 560 and 600 nm were used to measure absorbance and determine signal strength.

Thiazolyl blue tetrazolium bromide

The MTT (Sigma, St Louis, MO, USA) assay, which measures mitochondrial function, was used for several experiments. For the MTT assay, cells were cultured and stimulated with BAs. After 48 h, the medium was removed and replaced with 100 µL MTT solution (5 mg·mL⁻¹ in 3 mL of fresh medium without BAs). The plate was incubated for a further 4 h at 37 °C. Formazan crystals developed, and were dissolved in MTT solubilisation solution equal to the original culture medium volume. A gyratory shaker was used to accelerate dissolution. The standard curve comprised 100% live cells (resting media); 25/75%; 50/50%; 75/25% dead/live cells; and 100% dead cells. This was used to determine the percentages of viable cells. Spectrophotometric analysis, with reference and background wavelengths of 570 and 690 nm, was used to measure well absorbance.

Statistical analysis

Analyses were performed using GraphPad Prism v6 for Windows. All data are represented as mean±SEM and n indicates the number of repeat experiments performed. For experiments involving comparison of three or more matched groups, repeated measures ANOVA was used followed by the Bonferroni *post hoc* test, which compares all pairs of groups. In line with convention, p-values of ≤0.05 were considered significant.

Results

Effect of BA on BEAS-2B cell viability

Both the CellTiter-Blue and MTT assays were used to calculate viability. The results revealed no significant difference between the two methods of assessment.

The results showed that cell viability reduced significantly in response to exposure to 5 µmol·L⁻¹ concentrations of LCA (to 78±3%) and then decreased with 10 µmol·L⁻¹ to 46±3%. This decreased further at 20 µmol·L⁻¹ (to 7±3%) (figure 1a).

Cell viability was also reduced significantly by DCA at concentration 50 µmol·L⁻¹ (94±3%). However, 15 and 30 µmol·L⁻¹ DCA did not affect cell viability (figure 1b).

Cell viability also reduced significantly in response to exposure to 50 µmol·L⁻¹ concentrations of CA to 88±3%. This decreased further to the lowest cell viability level at 100 µmol·L⁻¹ (53±3%). The lowest concentration (15 and 30 µmol·L⁻¹) of CA did not affect cell viability (figure 1c).

Cell viability was also diminished significantly by CDCA at concentration 50 µmol·L⁻¹ to 78±3%. The cell viability greatly reduced to 100 µmol·L⁻¹ of CDCA with 23±3%. No difference was found in the cell viability at lowest concentration 15 and 30 µmol·L⁻¹ compared to control (figure 1d).

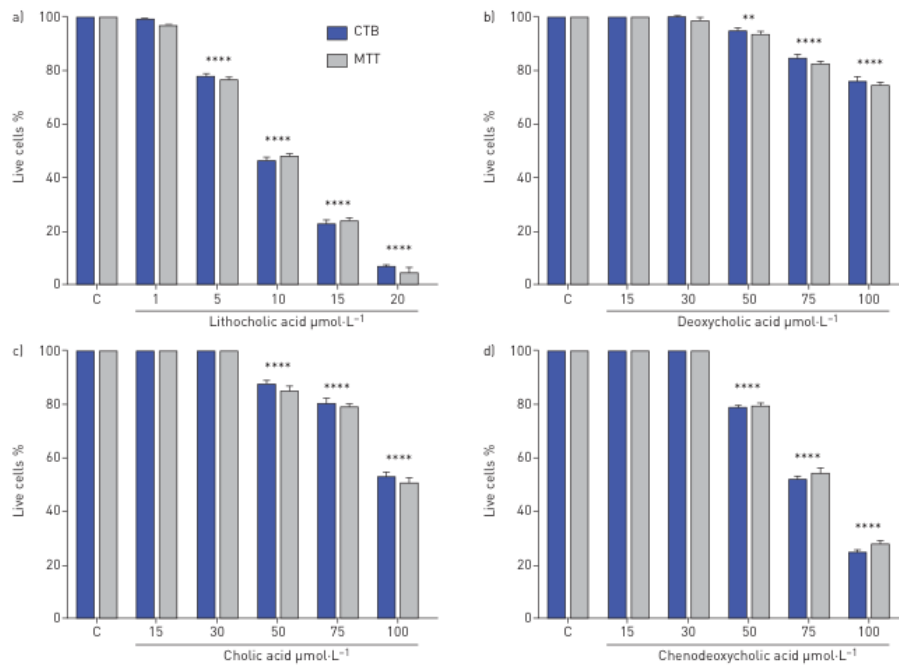


FIGURE 1 Effects of BAs on BEAS-2B cell viability. BEAS-2B cell viability following stimulation with a) lithocholic, b) deoxycholic, c) cholic and d) chenodeoxycholic acids at varying concentrations. Viability is measured as a percentage of vehicle control after 48 h exposure. Data are presented as mean±SEM. Negative controls were measured by fixing the cells in methanol before adding the TiterBlue and MTT reagent (n=6). C: control; CTB: CellTiter-Blue assay; MTT: thiazolyl blue tetrazolium bromide. **: p=0.01; ****: p<0.0001 versus controls.

Release of pro-inflammatory markers by BEAS-2B cells following exposure to BA

Lithocholic acid

IL-8, IL-6 and GM-CSF secretion were all effected in BEAS-2B cells by exposure to lithocholic acid (LCA) at concentrations from 1 to 20 $\mu\text{mol}\cdot\text{L}^{-1}$. IL-8 levels increased significantly at 5 $\mu\text{mol}\cdot\text{L}^{-1}$ and increased further up to the maximum level at 10 $\mu\text{mol}\cdot\text{L}^{-1}$. The level of IL-8 production then fell off at 20 $\mu\text{mol}\cdot\text{L}^{-1}$ but was still significantly higher than the control (figure 2a). Moreover, there was a significant increase in IL-6 levels with LCA at concentrations of 10, 15 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$, but not at 1 and 5 $\mu\text{mol}\cdot\text{L}^{-1}$. The result revealed that the highest level of IL-6 production was at 10 $\mu\text{mol}\cdot\text{L}^{-1}$ and then declined as the concentration of LCA increased to 20 $\mu\text{mol}\cdot\text{L}^{-1}$ (figure 2b). The GM-CSF levels increased significantly at 5 $\mu\text{mol}\cdot\text{L}^{-1}$ LCA, and rose further to the maximum level at 10 $\mu\text{mol}\cdot\text{L}^{-1}$. The level of GM-CSF production then fell off at 20 $\mu\text{mol}\cdot\text{L}^{-1}$ (figure 2c).

Deoxycholic acid

Cells were treated with deoxycholic acid (DCA) at concentrations from 15 to 100 $\mu\text{mol}\cdot\text{L}^{-1}$. IL-8 levels increased significantly at 30 $\mu\text{mol}\cdot\text{L}^{-1}$ and rose further to the maximum level at 75 $\mu\text{mol}\cdot\text{L}^{-1}$. The level of IL-8 production then fell off at 100 $\mu\text{mol}\cdot\text{L}^{-1}$ but was still significantly higher than the control. However, no significant increase was observed using concentrations of 15 $\mu\text{mol}\cdot\text{L}^{-1}$ DCA (figure 3a). IL-6 levels rose considerably with DCA at concentrations of 50, 75 and 100 $\mu\text{mol}\cdot\text{L}^{-1}$, but not at 15 and 30 $\mu\text{mol}\cdot\text{L}^{-1}$. The highest level of IL-6 secretion was with 100 $\mu\text{mol}\cdot\text{L}^{-1}$. The levels reduced to 50 $\mu\text{mol}\cdot\text{L}^{-1}$ but remained significantly above the control (figure 3b). Furthermore, the GM-CSF levels increased significantly at 30 $\mu\text{mol}\cdot\text{L}^{-1}$ and increased further up to the maximum level at 75 $\mu\text{mol}\cdot\text{L}^{-1}$. The level of GM-CSF production then decreased at 100 $\mu\text{mol}\cdot\text{L}^{-1}$ but remained significantly higher than the control. However, no significant increase was observed using a concentration of 15 $\mu\text{mol}\cdot\text{L}^{-1}$ DCA (figure 3c).

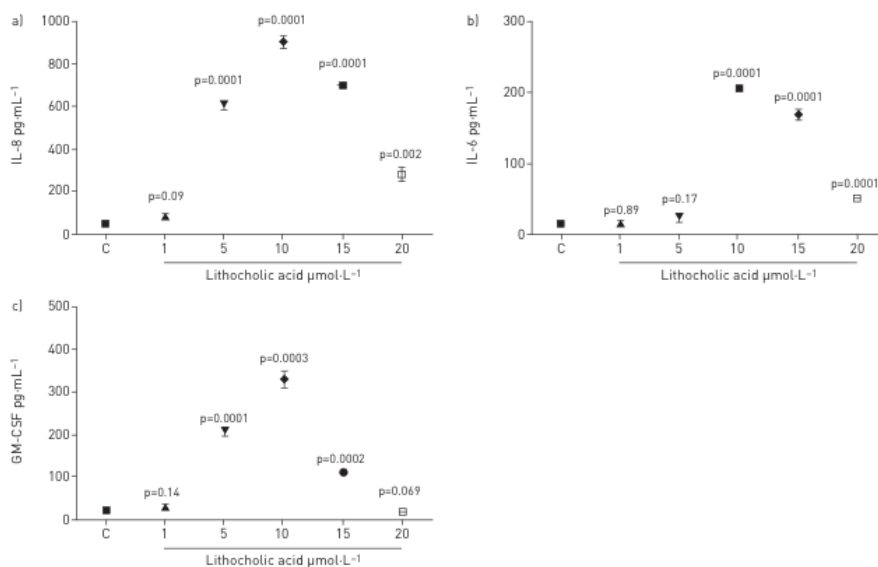


FIGURE 2 Measurement of a) IL-8, b) IL-6 and c) GM-CSF release by BEAS-2B cells stimulated with lithocholic acid for 48 h. Data are presented as mean \pm SEM ($n=6$). ELISA measured cytokine secretion in cell supernatants. Statistical analysis was performed using one-way ANOVA test. p-values of 0.05 or less were regarded as significant. C: control; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor.

Cholic acid

ELISA analysis detected IL-8, IL-6 and GM-CSF in cell culture supernatants following cholic acid (CA) stimulation of BEAS-2B. IL-8 levels increased significantly with CA at concentrations of 50, 75 and 100 $\mu\text{mol L}^{-1}$, but not at 15 and 30 $\mu\text{mol L}^{-1}$ (figure 4a). The highest level of IL-8 secretion was with 100 $\mu\text{mol L}^{-1}$. The levels reduced to 50 $\mu\text{mol L}^{-1}$ but remained considerably above the control. All CA concentrations significantly elevated IL-6 levels except 15 $\mu\text{mol L}^{-1}$. IL-6 production increased in a dose-dependent manner (figure 4b). Furthermore, the GM-CSF levels increased significantly at 30 $\mu\text{mol L}^{-1}$ and rose further to the maximum level at 75 $\mu\text{mol L}^{-1}$. The level of GM-CSF production then dropped at 100 $\mu\text{mol L}^{-1}$ but was still significantly higher than the control (figure 4c).

Chenodeoxycholic acid

Concentrations of chenodeoxycholic acid (CDCA) from 15 to 100 $\mu\text{mol L}^{-1}$ were tested. IL-8 levels increased significantly, but this was not evident at concentrations of 15 $\mu\text{mol L}^{-1}$ (figure 5a). It is clear that IL-8 levels increased significantly at 30 $\mu\text{mol L}^{-1}$ and rose further to the maximum level at 50 $\mu\text{mol L}^{-1}$. The level of IL-8 production then fell off at 100 $\mu\text{mol L}^{-1}$ but was still significantly higher than the control. The IL-6 levels were increased with CDCA at concentrations of 30, 50, 75 and 100 $\mu\text{mol L}^{-1}$, but not at 15 $\mu\text{mol L}^{-1}$ (figure 5b). The highest level of IL-6 secretion was with 100 $\mu\text{mol L}^{-1}$. The levels reduced at 30 $\mu\text{mol L}^{-1}$ but remained significantly above the control. ELISA detected GM-CSF following CDCA stimulation of BEAS-2B cells. The CDCA significantly upregulated GM-CSF production at 30, 50, 75 and 100 $\mu\text{mol L}^{-1}$ (figure 5c). It was apparent that GM-CSF production increased significantly at 30 $\mu\text{mol L}^{-1}$ and rose further to a maximum level at 50 $\mu\text{mol L}^{-1}$. The level of GM-CSF production then decreased at 100 $\mu\text{mol L}^{-1}$ but was still significantly higher than the control.

Discussion

Diseases of the lung are a major source of mortality and morbidity worldwide and are becoming increasingly prevalent. Chronic obstructive pulmonary disease (COPD), for example, is the fifth biggest

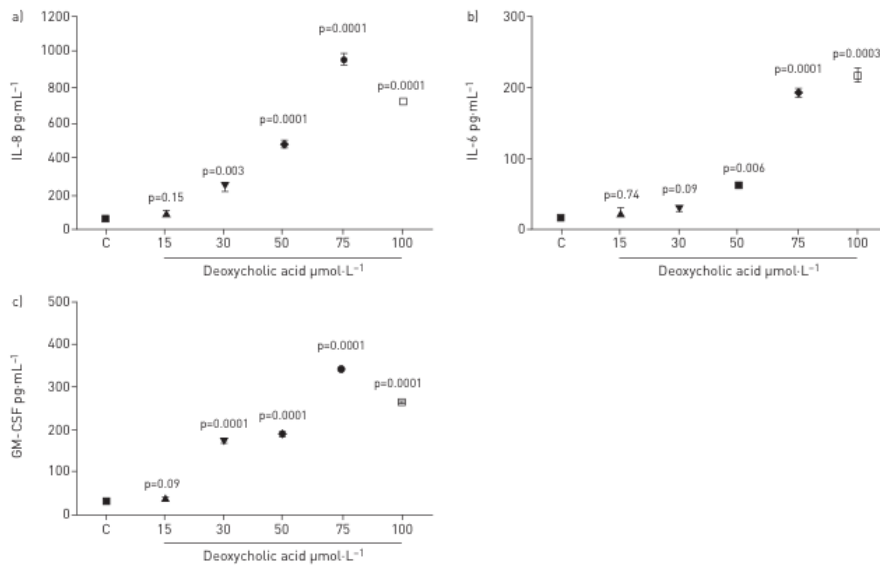


FIGURE 3 Measurement of a) IL-8, b) IL-6 and c) GM-CSF release by BEAS-2B cells stimulated with deoxycholic acid for 48 h. Data are presented as mean \pm SEM (n=6). ELISA measured cytokine secretion in cell supernatants. Statistical analysis was performed using one-way ANOVA test. p-values of 0.05 or less were regarded as significant. C: control; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor.

cause of death globally, while cystic fibrosis (CF) and interstitial lung disease are not rare and have a poor prognosis, including low quality of life and premature death. A connection between lung disease and GORD has long been suspected [15, 19] and high incidences of GORD are known to affect sufferers of idiopathic pulmonary fibrosis, asthma, CF and COPD [20, 21]. It is of interest that in COPD it has been revealed that the second most powerful predictor of exacerbations is a history of heartburn, a symptom associated with GOR [22]. Refluxate from GORD patients has been found to contain BA levels up to a millimolar level [23]. We have previously described high levels of BAs in the lungs of people with CF, both before and after transplantation [13].

The potential role of BAs in respiratory infection and inflammation is becoming increasingly recognised, although the mechanisms are still not well understood. Most BAs in humans exist in their conjugated forms [24]. Treatment for GORD, including the use of PPIs and acid suppression therapies can result in deconjugation of BAs [25], producing a higher ratio of deconjugated to conjugated BAs in patients undergoing GORD treatment [25]. CDCA, DCA and LCA have all been found in their unconjugated forms in airway secretions [26], so their effect may be of particular relevance to patients with GORD.

The effects of both primary and secondary BAs on BEAS-2B viability were studied. Cell viability was found to be affected depending on BA concentration. There was an inverse relationship between BA concentration and measured cell viability, with increasing concentration and decreasing cell viability in a dose-dependent manner. LCA at $5 \mu\text{mol}\cdot\text{L}^{-1}$ caused a decrease in cell viability and $20 \mu\text{mol}\cdot\text{L}^{-1}$ killed nearly all cells. These results are supported by data provided by Wu *et al.* [18], whose study exposed human epithelial cells to BAs. The precise mechanism by which BA exposure affects the viability of BEAS-2B cells remains unclear. It is known that cell membranes and cationic permeability are disrupted by BAs in type II pneumocytes *in vitro* [27], which may provide a mechanistic explanation for our study findings. *In vivo*, it is possible that this detergent effect may cause mucosal barrier breakdown, disrupting the mucus layer and surfactant function in the lungs, consequently damaging type II pneumocytes, which produce both surfactant protein and phospholipids [27, 28].

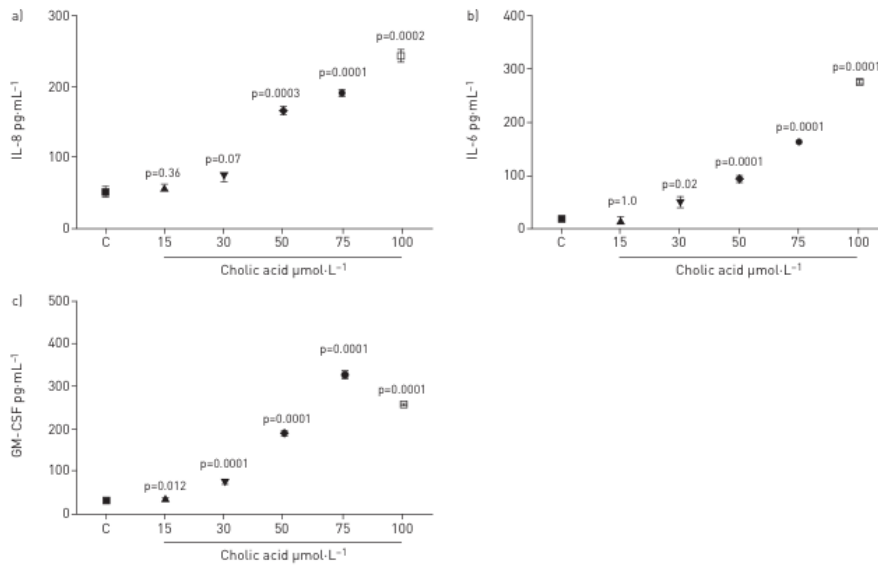


FIGURE 4 Measurement of a) IL-8, b) IL-6 and c) GM-CSF release by BEAS-2B cells stimulated with cholic acid for 48 h. Data are presented as mean \pm SEM (n=6). ELISA measured cytokine secretion in cell supernatants. Statistical analysis was performed using one-way ANOVA test. p-values of 0.05 or less were regarded as significant. C: control; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor.

BEAS-2B cells retain the morphology of an epithelial cell *in vitro* [18] and release IL-8 and IL-6 in addition to other cytokines [29]. Under submerged conditions, BEAS-2B cells are commonly used as the *in vitro* model of human airways epithelium, especially within studies on inhalation toxicity.

Our work demonstrates that cell viability decreased depending on BA concentration. There was a dose response to BA concentration, where increasing levels of BAs were associated with decreased cell viability. Our data demonstrated that low levels of LCA ($5 \mu\text{mol}\cdot\text{L}^{-1}$) affected viability and inflammatory cytokine outputs from human bronchial epithelial cells. Moreover, LCA effects occurred at five- to 10-fold lower concentrations compared to the other BAs.

Alongside the potential effect on cell viability, our data show that BA stimulation of epithelial cells is associated with a release of pro-inflammatory cytokines. The study found an increase in the expression of IL-8, IL-6 and GM-CSF following BA challenge.

The lung diseases in which reflux and aspiration are implicated all involve a contribution from acute or chronic inflammation, leading frequently to the use of anti-inflammatory medication, such as steroids [17]. The data gathered in this study support, overall, the hypothesis that BA exposure may have a significant effect on cytokine production and subsequently contribute to airway injury.

IL-8 is a neutrophilic chemoattractant and an angiogenic factor. Immunostaining has been used to identify IL-8 localisation in peribronchial lesions [30]. IL-6 plays a role in the activation of monocytes, T-cell differentiation and B-cell maturation. Moreover, it serves as a pyrogen, inducing fever in response to infection [31]. GM-CSF is involved in macrophage activation and is a strong chemoattractant for neutrophils. It stimulates microbicidal and phagocytotic activity, oxidative metabolism, and improves the cytotoxicity of both macrophages [32] and neutrophils [33]. Increased levels of IL-8, IL-6 and GM-CSF are a common finding in airways and lung disease where reflux and aspiration have been implicated as a possible injury [17, 31, 34].

A strength of our study, which is complementary to the limited amount of previous work available, was that the use of BEAS-2B allowed a comprehensive evaluation of a range of primary and secondary BAs, cytotoxicity and inflammatory cytokine production. This was possible because the available cell numbers did not limit the experiments.

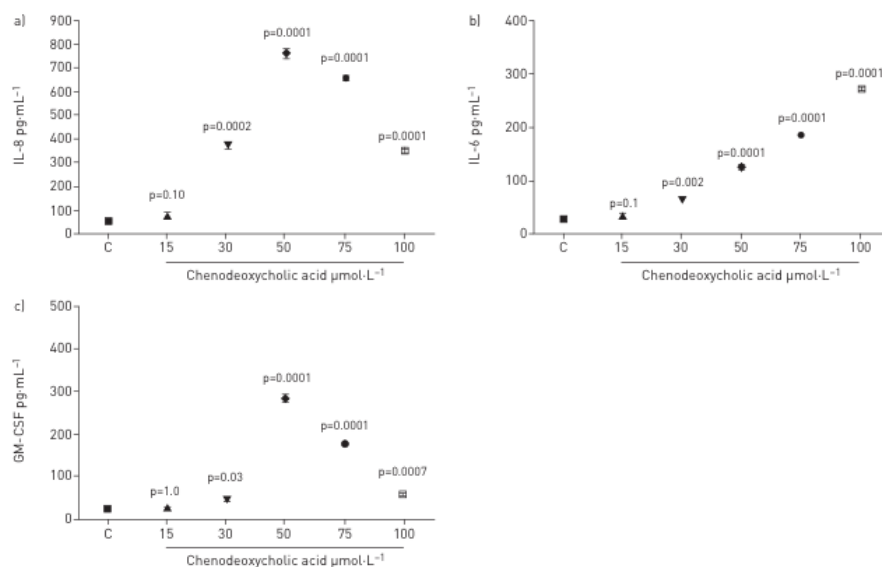


FIGURE 5 Measurement of a) IL-8, b) IL-6 and c) GM-CSF release by BEAS-2B cells stimulated with chenodeoxycholic acid for 48 h. Data are presented as mean \pm SEM (n=6). ELISA measured cytokine secretion in cell supernates. Statistical analysis was performed using one-way ANOVA test. p-values of 0.05 or less were regarded as significant. C: control; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor.

Our work could be strengthened by studies involving PBECs. In this study, submerged culture systems were used to examine the association between airways diseases and BAs. Future work could include establishing PBECs at an air-liquid interface, where cells are differentiated into ciliated epithelial cells. The present BA stimulation could then usefully be repeated in differentiated cells.

Conclusions

Our model data, revealing cytotoxicity and increased levels of inflammatory cytokines associated with BA stimulation of BEAS-2B epithelial cells, may explain the decreased lung function and chronic inflammation connected with aspiration in respiratory disease patients. These findings and further studies may contribute to an integrated "aerodigestive paradigm" regarding the understanding of reflux-related chronic lung diseases. Increased knowledge in this field may represent an opportunity to consider new approaches to patient therapy.

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Bile acids: a potential role in the pathogenesis of pharyngeal malignancy

Shellman, Z.,* Aldahrani, A.,[†] Verdon, B.,[†] Mather, M.,* Paleri, V.,^{‡§} Wilson, J.,^{§¶} Pearson, J.,[†] Ward, C.*[¶] & Powell, J.*^{§¶}

*Institute of Cellular Medicine, [†]Institute for Cell and Molecular Biosciences, [‡]Northern Institute for Cancer Research, Newcastle University, [§]Department of Otolaryngology, Head and Neck Surgery, Freeman Hospital, [¶]Institute of Health and Society, Newcastle University, Newcastle Upon Tyne, UK

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Objective: Gastro-oesophageal reflux disease is thought to be a risk factor for head and neck malignancies. Bile acids are one of the principle components of gastric refluxate and have previously been implicated in the development of oesophageal and bowel malignancies. There is clear evidence that bile acids reflux into the laryngopharynx. Despite this, the carcinogenic properties of bile acids in this area are yet to be fully identified. We therefore investigated the potential role of bile acids in pharyngeal malignancy, through the highly conserved process of epithelial–mesenchymal transition (EMT). EMT occurs in invasion and metastasis and is a central process in the development of epithelial carcinoma.

Design: Translational research study.

Methods: Human hypopharyngeal squamous carcinoma FaDu cells were challenged with primary (cholic or chenodeoxycholic) and secondary (deoxycholic or lithocholic)

bile acids. EMT-relevant proteins TGF- β 1 and MMP-9 were measured in the cell culture supernates at 48 h via ELISA. Cell viability was confirmed >95% via CellTiter-Blue assay.

Results: Significantly greater concentrations of TGF- β 1 were measured in the culture supernates of cells treated with cholic acid, deoxycholic acid and chenodeoxycholic acid. MMP-9 levels were increased in deoxycholic acid and lithocholic acid stimulations when compared to control ($P < 0.05$).

Conclusion: This is the first demonstration that bile acids induce TGF- β 1 and MMP-9 in pharyngeal cells. TGF- β 1 is considered a master switch for EMT, while MMP-9 is a part of the EMT proteome which degrades basement membranes. This implies a potential role for bile acids in pharyngeal carcinogenesis through the mechanism of EMT and suggests potential novel therapeutic targets.

Globally, there are 600 000 new cases and 300 000 deaths from head and neck cancer (HNC) worldwide per year.¹ Despite improvements in treatment modalities, HNC still carries a poor prognosis and has a substantial impact on quality of life.² While tobacco smoking, alcohol and high-risk papilloma viruses are well-recognised etiologic agents, gastro-oesophageal reflux disease (GORD) is being increasingly implicated as a risk factor in the development of HNC, particularly pharyngeal and laryngeal subsites.^{3,4}

Gastro-oesophageal reflux disease is highly prevalent in the general population.⁵ There is clear evidence that duodeno-gastric contents are refluxed beyond the oesophagus in the so-called extra-oesophageal or laryngopharyngeal reflux (LPR) episodes.⁶ Gastric refluxate contains gastric

acid, pepsin and bile acids. Evidence of all three components has been found in upper airway samples.^{7–9}

Increasing evidence is emerging for the role of pepsin and acidic environments in inducing laryngopharyngeal carcinogenesis.^{10–14} The carcinogenic properties of bile acids in this area are yet to be fully identified. This is despite the potent carcinogenic properties of bile acids identified in other digestive subsites, such as gastro-oesophageal^{15,16} and colonic.^{17,18}

We therefore investigated the impact of bile acids on a head and neck squamous cell line, specifically the highly conserved process of epithelial–mesenchymal transition (EMT). EMT is a central process in the development of carcinoma and contributes to cancer invasion and metastasis.¹⁹ Transforming growth factor beta 1 (TGF- β 1) is the recognised ‘master switch’ that promotes EMT, while matrix metalloproteinase 9 (MMP-9) is a part of the EMT proteome which degrades basement membranes. Both TGF- β 1 and MMP-9 have been shown to play an important role in EMT in HNC.^{20,21}

Correspondence: J. Powell, Otolaryngology–Head and Neck Surgery, Freeman Hospital, Newcastle Upon Tyne, NE7 7DN, UK. Tel.: (+44) 191 223 1086; Fax: (+44) 191 223 1246; e-mail: jason.powell@doctors.org.uk
[¶]Equal contribution made.

Materials and methods

Cell culture

Human hypopharyngeal squamous cell carcinoma FaDu cells (ATCC, Manassas, Virginia, USA) were cultured on collagen-coated flasks in Eagle's minimum essential medium supplemented with foetal calf serum 10%, non-essential amino acids 0.1 mM, penicillin/streptomycin 100 U/100 µg/mL and L-glutamine 2 mM (Sigma-Aldrich, St. Louis, Missouri, USA), incubated at 37°C in a 5% CO₂ incubator. Medium was changed every 2–3 days. Upon near confluence, cells were trypsinised (Sigma, USA), diluted in equal volume medium, centrifuged at 200 g for 7 min and seeded in a new container.

Bile acid preparation and challenge

The four predominant bile acids in the human digestive tract are cholic acid (CA), lithocholic acid (LA), deoxycholic acid (DA) and chenodeoxycholic acid (CDA). Each stock bile acid (Sigma, USA) was prepared in methanol. Controls contained methanol alone. Bile acids or methanol controls were diluted in resting medium for all experiments. Resting medium comprised standard culture media excluding foetal calf serum. Cells were placed in resting medium for a minimum of 3 h prior to bile acid challenges in order to promote the resting phase of the growth cycle.

Enzyme-linked immunosorbent assays (ELISAs)

After 48-h challenge, cell culture supernates were collected and stored in a –80°C freezer until use. Human TGF-β1 and Human MMP-9 DuoSet ELISA kits (R&D, Minneapolis, Minnesota, USA) were used according to the manufacturer's instructions. Samples were diluted to optimum concentrations and absorbance measured at 450 nm with a plate reader (M200, Männedorf, Switzerland) and compared against a known concentration standard curve to calculate unknown concentrations.

CellTiter-Blue viability assay

Cell viability was assessed using CellTiter-Blue viability assay (Promega, Madison, Wisconsin, USA). After the 48-h challenge period, the supernate was removed and a CellTiter-Blue reagent was added to the cells for 3 h. No-cell controls were prepared in triplicate to determine the background absorbance. Dead cell controls were also set up in triplicate by adding 100% methanol to cells. Live unchallenged cell controls were also set up in triplicate. Fluorescence excitation and emission ratio were measured at 560 and

590 nm, respectively. Percentage viability was calculated from fluorescence of challenged cells against controls.

Statistical analysis

Data from each experiment comprised four biological replicates, each with two technical replicates. Analysis was performed on PRISM 5 (GraphPad, San Diego, CA, USA) using Mann–Whitney *U*-tests. Comparison was made between each experimental condition and control results. Data were expressed as mean ± the standard error of the mean (SEM). Significance was taken as $P < 0.05$.

Results

We demonstrated a significant ($P < 0.05$, $n = 4$) increase in the expression of TGF-β1 (via ELISA of cell culture supernates) with exposure to concentrations of CDA 100 µM, CA 600 µM and DA 100 µM, 75 µM compared to control samples. LA at concentrations up to 20 µM did not produce a significant increase in TGF-β1 levels (Fig. 1). MMP-9 ELISA of cell supernate showed a significant ($P < 0.05$, $n = 4$) increase in MMP-9 in challenged human FaDu cells compared to controls at concentrations of DA 100 µM and LA 100 µM. CA at concentrations up to 600 µM and CDA at concentrations up to 100 µM produced no significant increase in MMP-9 concentrations compared to controls (Fig. 2). Cell viability was confirmed at >95% in all experimental conditions by CellTiter-blue viability assay.

Discussion

Synopsis of key findings

We have demonstrated for the first time a potential link between bile acids and upregulation of proteins implicated in EMT and cancer. We investigated proteins associated with EMT that are recognised to have a substantial role in the development of epithelial tumours and are of significant relevance in HNC.^{19–21} TGF-β1 is considered the 'master switch' for EMT and can induce changes in epithelial architecture and phenotype accompanied by an EMT signature proteome, ultimately resulting in the epithelial cells transitioning to a mesenchymal phenotype.²¹ MMP-9 is a type IV collagenase and is a potent basement membrane-degrading enzyme which has been closely tied to epithelial cells being able to invade into local structures.²¹

Significantly greater concentrations of TGF-β1 were measured in the culture supernates of cells treated with cholic acid (CA), deoxycholic acid (DA), chenodeoxycholic acid (CDA) and of MMP-9 in the cell culture supernates of cells challenged with deoxycholic acid (DA) and lithocholic

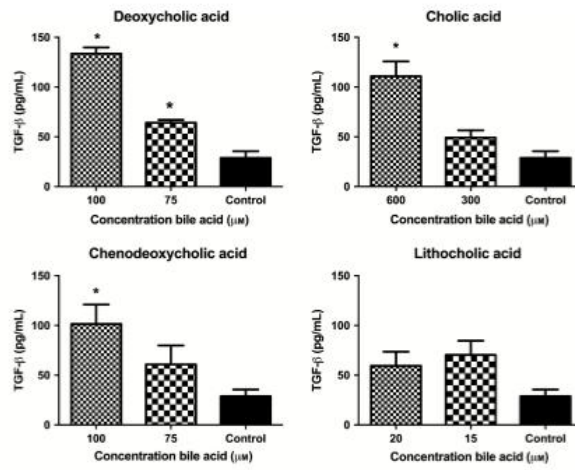


Fig. 1. Elevation of TGF-β1 in cell culture supernates after 48-h challenge with bile acids: FaDu cells were challenged with bile acids; after 48 h, TGF-β1 concentrations were measured in the cell culture supernates ($n = 4$), $*P < 0.05$. CellTiter-Blue viability assay showed >95% cell viability in each experimental condition.

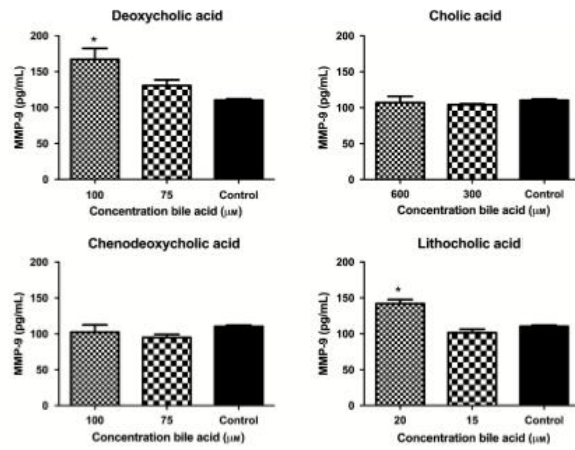


Fig. 2. Effect of bile acids on MMP-9 concentration in cell culture supernates of FaDu cells: FaDu cells were challenged with bile acids; after 48 h, MMP-9 concentrations were measured in the cell culture supernates ($n = 4$), $*P < 0.05$. CellTiter-Blue viability assay showed >95% cell viability in each experimental condition.

acid (LA), when compared to controls ($P < 0.05$). We used concentrations of the differing bile acids up to the maximal concentration that did not induce high cell death based on our optimisation work. CellTiter-blue viability assay confirmed >95% cell viability in all conditions; hence, release of these EMT markers was from cell expression and not cell death. The variable role of the different bile acids on EMT markers was a surprising finding. TGF- β 1 is considered an earlier EMT marker and may promote MMP-9 production.²² Therefore, as DA was the most potent TGF- β 1 stimulator, it could be hypothesised that this leads to downstream production of MMP-9. The finding that LA only produced significant increased amounts of MMP-9, and not TGF- β 1, requires further investigation into alternative EMT pathways. E-cadherin and fibronectin are then next two most relevant EMT markers, and future study of their expression would be of benefit.

Strengths and weaknesses of the study

A limitation of this study is absence of evidence quantifying the concentrations of each bile acid in the laryngopharynx during or after reflux events. Therefore, extrapolation of these current results to the *in vivo* environment is difficult. In humans, CA, CDA and DA are predominantly found in the digestive tract, with only small amounts of LA present (<10%).²³ The total concentrations of bile acids in gastric juice, derived from the stomach, are quoted as between 10 and 10 000 μM .²⁴ We used a range of between 15 and 600 μM for each individual bile acid, so are well within the physiological range expected in gastric refluxate. However, further investigation into the dose–response relationship over a broader range of concentrations would be of interest in future studies. The use of a cell line carries numerous limitations and further studies in primary cells is now required. The FaDu hypopharyngeal squamous cell line is, however, a well-established and characterised cell line in reflux and head and neck cancer research.¹⁴

Comparisons with other studies

There is a large body of evidence demonstrating an association between GERD and oesophageal malignancy.²⁵ The relationship between GERD and head and neck malignancies is less clearly established. A recent meta-analysis by Zhang *et al.*³ summarised the conflicting findings of recent population-based studies. They highlighted the difficulty in demonstrating an association due to the substantial confounding smoking and alcohol history in the cancer groups.

Several *in vitro* studies have demonstrated a potential role of reflux constituents, pepsin and acid, in prompting laryngopharyngeal carcinogenesis.^{10–14} However, Galli

*et al.*²⁶ in a series of 40 achlorhydric gastrectomised patients demonstrated that 15% developed premalignant or malignant laryngeal tumours compared to 2.5% in a control group of dyspeptic patients, indicating a potentially substantial role of bile acids in laryngopharyngeal carcinogenesis. Further to this, there is an increasing body of *in vitro* evidence demonstrating a potential link between bile acids and other digestive subsites malignancies, such as gastro-oesophageal^{15,16} and colonic.^{17,18}

We could identify only two other studies, which investigated cellular mechanistic relationships between bile acids and head and neck squamous cells. Sung *et al.*²⁷ demonstrated increased expression of cyclo-oxygenase-2 (COX-2) by pharyngeal cells after challenge with CDA. In a more recent study, Sasaki *et al.*²⁸ demonstrated increased expression of nuclear factor-kappaB (NF- κ B) by hypopharyngeal cells after challenge with a cocktail of bile acids. Our study has, however, for the first time, been able to demonstrate differing potencies and mechanistic relationships between each of the four main human bile acids on EMT pathways in head and neck squamous cells.

Clinical applicability of the study

This is a preliminary *in vitro* study, which demonstrates a potentially important link between bile acids found in duodeno-gastric refluxate and HNC.

The importance in identifying gastro-duodenal refluxate as a potential co-factor in HNC is the potential for preventative interventions, such as counteracting reflux in individuals at high risk of HNC. The other essential consideration of bile acids as a specific causative agent in HNC is the need to move away from acid neutralisation agents alone. Agents such as proton pump inhibitors do not reduce biliary secretions or impede bile acid function. Other agents such as alginates may have an effect in physically blocking biliary reflux into the laryngopharynx.

Keypoints

- Gastro-oesophageal reflux disease is suspected to be a risk factor for head and neck malignancies.
- Bile acids are one of the principle components of gastric refluxate and have previously been implicated in the development of oesophageal and bowel malignancies.
- We investigated proteins associated with epithelial–mesenchymal transition (EMT) that are recognised to have a substantial role in the development of epithelial tumours and are of significant relevance in head and neck malignancies.
- We demonstrate that bile acids induce EMT markers TGF- β 1 and MMP-9 in pharyngeal cells *in vitro*.

- This implies a potential role for bile acids in pharyngeal carcinogenesis through the mechanism of EMT and suggests potential novel therapeutic targets.

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Conflict of interest

None to declare.

Financial disclosures

None.

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APPENDIX 6

- RPMI Medium

Component	R0883 [1x] g/L	R1145 [10x] g/L	R1383 g/L	R1780 [1x] g/L	R2405 [1x] g/L	R6504 g/L	R8758 [1x] g/L
Inorganic Salts							
Calcium Nitrate • 4H ₂ O	0.1	1	0.1	0.1	0.1	0.1	0.1
Magnesium Sulfate (anhydrous)	0.04884	0.4884	0.04884	0.04884	0.04884	0.04884	0.04884
Potassium Chloride	0.4	4	0.4	0.4	0.4	0.4	0.4
Sodium Bicarbonate	2	—	—	2	2	—	2
Sodium Chloride	6	60	6	6	6	6	6
Sodium Phosphate Dibasic (anhydrous)	0.8	8	0.8	0.8	0.8	0.8	0.8
Amino Acids							
L-Alanyl-L-Glutamine	—	—	—	—	0.4344	—	—
L-Arginine	0.2	2	0.2	—	0.2	0.2	0.2
L-Asparagine (anhydrous)	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Aspartic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Cystine • 2HCl	0.0652	0.652	0.0652	0.0652	0.0652	0.0652	0.0652
L-Glutamic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Glutamine	—	—	0.3	0.3	—	0.3	0.3
Glycine	0.01	0.1	0.01	0.01	0.01	0.01	0.01
L-Histidine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
Hydroxy-L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Isoleucine	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Leucine	0.05	0.5	0.05	—	0.05	0.05	0.05
L-Lysine • HCl	0.04	0.4	0.04	—	0.04	0.04	0.04
L-Methionine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Phenylalanine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Serine	0.03	0.3	0.03	0.03	0.03	0.03	0.03
L-Threonine	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Tryptophan	0.005	0.05	0.005	0.005	0.005	0.005	0.005
L-Tyrosine • 2Na • 2H ₂ O	0.02883	0.2883	0.02883	0.02883	0.02883	0.02883	0.02883
L-Valine	0.02	0.2	0.02	0.02	0.02	0.02	0.02
Vitamins							
D-Biotin	0.0002	0.002	0.0002	0.002	0.002	0.0002	0.0002
Choline Chloride	0.003	0.03	0.003	0.003	0.003	0.003	0.003
Folic Acid	0.001	—	0.001	0.001	0.001	0.001	0.001
myo-Inositol	0.035	0.35	0.035	0.035	0.035	0.035	0.035
Niacinamide	0.001	0.01	0.001	0.001	0.001	0.001	0.001
p-Aminobenzoic Acid	0.001	0.01	0.001	0.001	0.001	0.001	0.001
D-Pantothenic Acid (hemicalcium)	0.00025	0.0025	0.00025	0.00025	0.00025	0.00025	0.00025
Pyridoxine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Riboflavin	0.0002	0.002	0.0002	0.0002	0.0002	0.0002	0.0002
Thiamine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Vitamin B ₁₂	0.000005	0.00005	0.000005	0.000005	0.000005	0.000005	0.000005



Formulation for Eagle's Minimum Essential Medium (EMEM) ATCC® 30-2003

Inorganic Salts (g/liter)

CaCl ₂ (anhydrous)	0.20000
MgSO ₄ (anhydrous)	0.09767
KCl	0.40000
NaHCO ₃	1.50000
NaCl	6.80000
NaH ₂ PO ₄ ·H ₂ O	0.14000

Amino Acids (g/liter)

L-Alanine	0.00890
L-Arginine·HCl	0.12640
L-Asparagine·H ₂ O	0.01500
L-Aspartic Acid	0.01330
L-Cystine·2HCl	0.03120
L-Glutamic Acid	0.01470
L-Glutamine	0.29200
Glycine	0.00750
L-Histidine·HCl·H ₂ O	0.04190
L-Isoleucine	0.05250
L-Leucine	0.05250
L-Lysine·HCl	0.07250
L-Methionine	0.01500
L-Phenylalanine	0.03250
L-Proline	0.01150
L-Serine	0.01050
L-Threonine	0.04760
L-Tryptophan	0.01000
L-Tyrosine·2Na·2H ₂ O	0.05190
L-Valine	0.04680

Vitamins (g/liter)

Choline Chloride	0.00100
Folic Acid	0.00100
myo-Inositol	0.00200
Nicotinamide	0.00100
D-Pantothenic Acid (hemicalcium)	0.00100
Pyridoxine·HCl	0.00100
Riboflavin	0.00010
Thiamine·HCl	0.00100

Other (g/liter)

D-Glucose	1.00000
Phenol Red, Sodium Salt	0.01000
Sodium Pyruvate	0.11000