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Effects of cholecystectomy on gastric and oesophageal mucosa.

Syeda NS Gilani

Royal College of Surgeons in Ireland

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Effects of Cholecystectomy on Gastric and Oesophageal mucosa

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MD Thesis

February 2012



Royal College of Surgeons in Ireland

(Research conducted at the Department of Surgery, Connolly Hospital)

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To:

My beloved parents, for giving me the best possible start and for their unfailing love, support and guidance through life.

My life partner and the man in my life, for endless love, support and understanding, for cheering me up and reminding me of what life is really about.

My brothers, for being the best, most loving, caring, understanding and supportive brothers in the world.

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SUMMARY

Chronic exposure of oesophageal mucosa to bile has been implicated in the etiology of the oesophageal lesions which develop into Barrett's oesophagus. Loss of the gallbladder reservoir function at cholecystectomy may critically alter the dynamics of bile storage and release.

In our first study we aimed to investigate the prevalence of bile reflux in three patient populations; symptomatic controls, Barrett's patients and patients post oesophago-gastric resection for carcinoma. Augmentation of bile reflux was noted in patients who had previous history of cholecystectomy. A high proportion of patients remained symptomatic post-cholecystectomy and a high proportion of these still-symptomatic post-cholecystectomy patients had increased bile reflux index.

In our second retrospective study we examined the effects of cholecystectomy on gastric and oesophageal mucosa at molecular and histological level. There was an increase in bile reflux index, Ki67 and p53 in post-cholecystectomy

patients. This raised concern about development of pre-malignant changes in these patients.

In the third study, the effect of cholecystectomy on gastric and oesophageal microenvironment was evaluated prospectively. This study noted the histological and molecular changes precipitated by cholecystectomy are similar to those identified as precursors of Barrett's oesophagus. Such concerning changes suggest that options other than cholecystectomy be considered for patients with gallstones in a functioning gallbladder.

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ABBREVIATIONS

BMI	Body Mass Index
BRI	Bile Reflux Index
CCK	Cholecystokinin
COX-2	Cyclooxygenase-2
DGOR	Duodeno-gastro-oesophageal Reflux
DNA	Deoxyribonucleic acid
ESWL	Extracorporeal Shock Wave Lithotripsy
GI	Gastro-intestinal
GORD	Gastro-oesophageal Reflux Disease
HIDA Scan	Hepatobiliary Imino-Diacetic Acid Scan
IHC	Immunohistochemistry
LOS	Lower Oesophageal Sphincter
NF-kB cells	Nuclear factor kappa-light-chain-enhancer of activated B
NSAID	Non-Steroidal Anti-Inflammatory Drug
OGD	Oesophago-gastro-duodenoscopy
OGJ	Oesophago-gastric Junction
PCNA	Proliferating Cell Nuclear Antigen

PPI	Proton-pump Inhibitor
RGS	Reflux Gastritis Score
RNO	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TLOSRES episodes	Transient lower oesophageal sphincter relaxation
TP53	Tumour Protein 53

CHAPTER 1

INTRODUCTION

1.1 Background

The association of reflux and oesophageal carcinoma is well established but the specific component or components that cause most injury, or act as promoters for the metaplasia-dysplasia-neoplasia sequence, is less clear. Bile has long been suggested as a culprit. The most dramatic change to the dynamics of bile storage and release occurs following cholecystectomy when the bile storage reservoir during the inter-digestion period is lost. Instead of an orderly sequence of secretion, storage and release, bile trickles continuously into the duodenum where it is constantly available for reflux into the stomach and onwards into the oesophagus. There are other associations with bile reflux which are observed during endoscopy which may be cause, consequence or coincidence. In this Introduction I will discuss the current literature on bile reflux as it impacts on the stomach and lower oesophagus.

1.2 Normal Bile Storage And Release

1.2.1 Bile Storage

The gallbladder is a pear-shaped sac lying on the visceral surface of right lobe of liver in a fossa between the right and quadrate lobes (Figure 1).

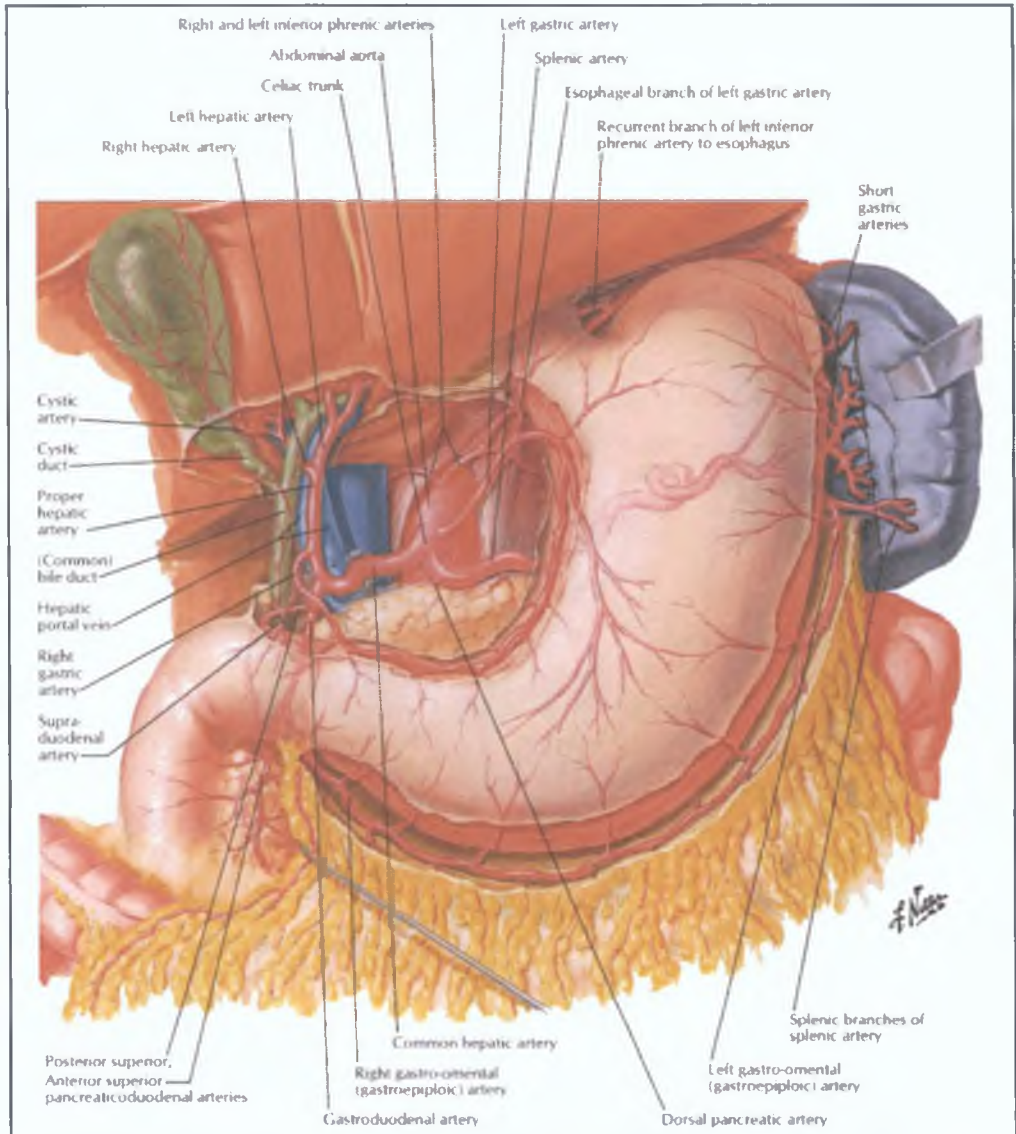


Figure 1 Gallbladder anatomy. Adapted from: Netter, Frank H. Atlas of human anatomy, 4th ed. Printed by Elsevier Inc. 2006 (Philadelphia, PA)

It has the capacity of 30-50ml. It has a rounded end, the fundus, which may project from the inferior border of the liver, where it comes in contact with the anterior abdominal wall at the level of the tip of the ninth costal cartilage. A major part in the fossa is the body of the gallbladder which may lie against the transverse colon and the first part of duodenum. The neck of the gallbladder is within the mucosal folds forming a spiral fold. The neck becomes continuous with the cystic duct which turns into the lesser omentum to join the common hepatic duct to form the common bile duct(1).

The gallbladder receives its blood supply from the cystic artery, a branch of the right hepatic artery. The cystic vein drains directly into the portal vein. The lymph drains into the cystic lymph node situated near the neck of gallbladder. The sympathetic and parasympathetic nerve supply is via vagal fibres from the coeliac plexus.

1.2.2 Normal Function Of The Gallbladder

The gallbladder receives, concentrates and stores bile from the liver. When digestion is not taking place, the sphincter of Oddi remains closed and the bile accumulates in the gallbladder. The gallbladder selectively absorbs bile salts, retains bile acid and secretes mucus. Active transport of sodium through the gallbladder epithelium is followed by secondary absorption of the chloride ions,

water and most other soluble constituents. Bile is normally concentrated about fivefold. To aid in these functions the mucous membrane is thrown into permanent folds that unite with each other giving the surface a honeycombed appearance and the columnar cells lining the surface have numerous microvilli on their free surface.

1.2.3 Bile Release

Bile is delivered to the duodenum as a result of contraction and partial emptying of the gallbladder. This mechanism is initiated by the entrance of fatty foods into the duodenum. The fat causes release of the hormone cholecystokinin from the "I" cells in the mucous membrane of the duodenum. The hormone then enters blood, causing the gallbladder to contract. At the same time the smooth muscle around the distal end of the bile duct and the ampulla relaxes, allowing the passage of concentrated bile into the duodenum(2).

1.2.4 CCK Pathway On Bile Release

During the interdigestive period the gallbladder is relaxed, the sphincter of Oddi is closed and the gallbladder fills with bile. Bile is stored in the gallbladder between meals and is expelled into the duodenum by contraction of the

gallbladder in response to meal stimulated CCK secretion from the duodenum (Figure 2).

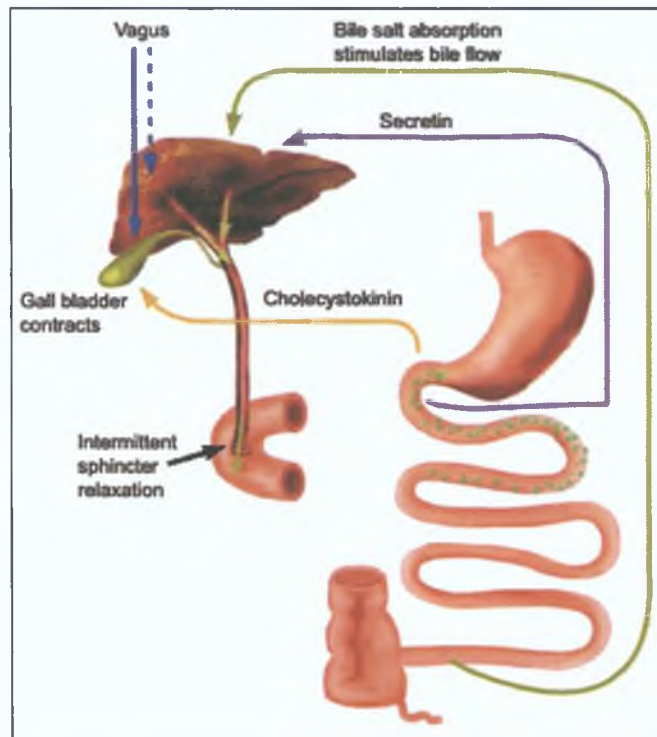


Figure 2 CCK released from duodenal mucosa causing the gallbladder to contract

CCK is released in response to small peptides and fatty acids in the duodenum. It causes contraction of the gallbladder and relaxation of the sphincter of Oddi. In addition CCK causes relaxation at the lower oesophageal sphincter (LOS)(2). CCK release is switched off by the negative feedback effect of the bile bolus in the duodenum(3). Thus, when the gallbladder is functioning normally bile is

mixed with food in the duodenum and little is free to enter the stomach or available for contact with the oesophageal mucosa (2, 4-6).

1.3 Composition of Bile

Bile contains bile salts, phospholipids, cholesterol and bile pigments (bilirubin).

It is produced continuously by hepatocytes, and drains into the hepatic ducts and is stored in the gallbladder for subsequent release.

1.3.1 Bile Salts

Bile salts are amphipathic molecules because they have both hydrophilic and hydrophobic portions. In aqueous solution bile salts orient themselves around droplets of lipid and keep the lipid droplets dispersed (emulsification). Bile salts aid in the intestinal digestion and absorption of lipids by emulsifying them and solubilising them in micelles. Bile salts are positioned on the outside of the micelle, with their hydrophilic portions dissolved in the aqueous solution of the intestinal lumen and their hydrophobic portions dissolved in the micelle interior. Free fatty acids and monoglycerides are present in the inside of the micelle essentially solubilised for subsequent absorption. The bile salts in the bile are important in emulsifying the fat in the intestine and in assisting with its digestion and absorption(3). Evidence is accumulating that bile salts are the noxious component in refluxed duodenal juice and that their ability to cause cellular injury is pH-dependant. For bile salts to enter mucosal cells and cause

injury they must be soluble and ionized. At a pH of 7.0 greater than 90% of bile salts are completely ionized and remain in solution. In a more alkaline gastric environment, however, as can occur with the use of acid-suppression medication, bile salts are partially dissociated. Non-ionized bile salts can rapidly cross mucosal cell membranes. Once inside the alkaline environment of the cell, they are converted back to ionized form. Consequently bile salts become trapped within the cell, accumulate and are ultimately toxic to the mitochondria(7). Bile salts in isolated rat hepatocytes have also been shown to activate protein kinase C, a calcium-activated phospholipid dependent enzyme which plays a key role in growth-signalling pathways (8).

1.3.2 Bile Acids

The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol by hepatocytes. In the intestine bacteria convert a portion of each of the primary bile acids to secondary bile acids, deoxycholic acid and lithocholic acid. Synthesis of new bile acids occurs as needed to replace those excreted in the faeces. The bile acids are conjugated with glycine or taurine to form their respective bile salts, which are named for the parent bile acid e.g. taurocholic acid is cholic acid conjugated with taurine. The primary bile acids cholate and chenodeoxycholate are conjugated with taurin or glycine at a ratio of 1:3. The conjugation greatly increases the solubility of the bile acids. Bile

acids can enter mucosal cells when in the lipophilic form. This occurs in a pH range of 2-5 for the conjugated bile acids and at neutral pH for unconjugated bile salts. This pH-dependency also influences precipitation. Conjugated bile acids precipitate, and are thereby rendered harmless, at a pH below 1.5, while unconjugated bile acids precipitate below a pH of 3-4. Bile acids are concentrated in the epithelial cells at levels as high as eight times the luminal concentration, probably by intracellular ionization and membrane entrapment(9). In an acidic, even weakly acidic, environment, even for brief periods, bile acids are associated with the development of dilated intercellular spaces, the most common change in the mucosa of patients with non-erosive reflux disease(10). The exposure of bile acids to the epithelium of the oesophagus initiates a series of reactions including increased cell permeability and dilated intercellular spaces, cell damage and repair, inflammation and activation of pro-inflammatory transcription factors and genes(11). And all of these reactions predispose the oesophagus to dysplasia and adenocarcinoma(12). Shirvani et al in 2000 suggested that bile acids stimulate COX-2 expression(13) which is associated with chronic inflammation and epithelial cell growth in Barrett's oesophagus and adenocarcinoma(14). Jenkins et al have shone light on another molecular target for bile acids in the oesophagus(15). In an in-vitro study they found that exposure of oesophageal mucosa to physiological levels of bile acids activates NF-kB which has been implicated in carcinogenesis in numerous tissue types (16, 17).

1.4 Reflux

1.4.1 Historical Perspective

Oesophagitis, one of the complications of gastro-oesophageal reflux disease, was first described by Galen in the second century AD(18). Some confusion as to the nature and causes of oesophagitis remained until the first half of the 20th century. In 1935 Winkelstein defined peptic oesophagitis clinically by recognizing “an oesophagitis resulting from the irritant action on the mucosa by free hydrochloric acid and pepsin”(19). In 1946 Allison equated the presence of oesophageal ulcer with that of a hiatus hernia(20). This last notion resulted in hiatus hernia being more or less synonymous with reflux oesophagitis well into the 1960’s when Palmer reported that many patients in a prospective study with hiatal hernias neither had reflux symptoms nor oesophagitis, and that many other patients had oesophagitis in the absence of hiatal hernia(21) (Figure 3).

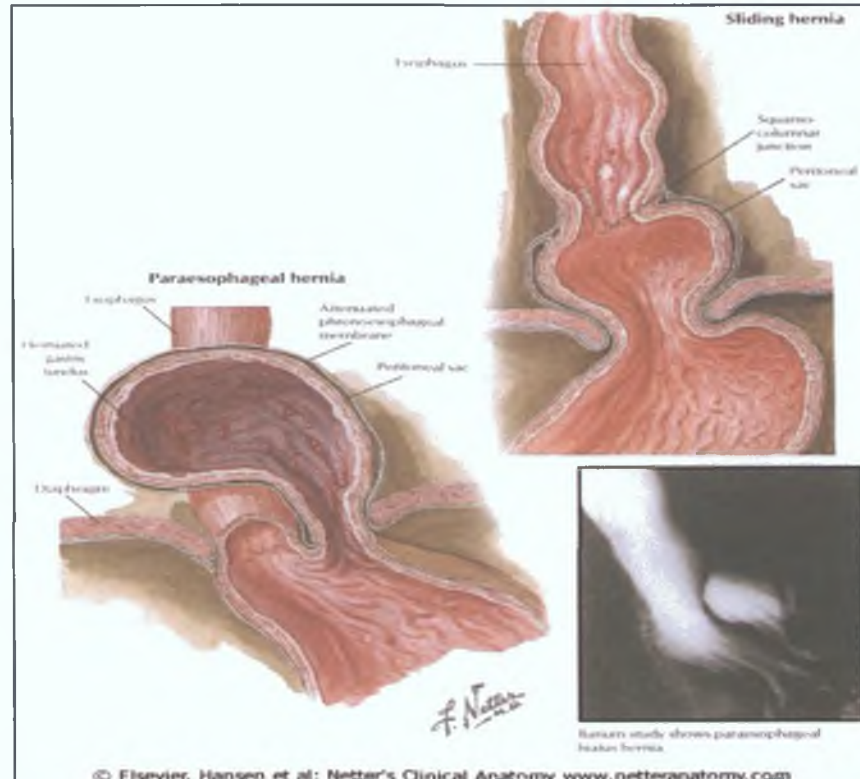


Figure 3 Rolling and Paraesophageal Hiatus Hernia

In 1950, Norman Barrett of Guy’s Hospital in London introduced the term “reflux oesophagitis” (22). Since then the interest in gastro-oesophageal reflux disease has increased exponentially and within the last decade there has been an explosion in the number of publications relating to gastro-oesophageal reflux disease. There is no universally accepted definition or classification of gastro-oesophageal reflux disease (GORD). In 2006 a consensus document was finally agreed upon, the Montreal Definition and Classification of Gastro-oesophageal Reflux Disease(23). This was developed by an international consensus group

consisting of 44 experts from 18 countries. According to the Montreal definition GORD is “a condition which develops when reflux of the stomach contents causes troublesome symptoms and/or complications”. These symptoms are further classified as troublesome “when they adversely affect an individual’s general well-being” and thus symptoms that are not troublesome “should not be diagnosed as GORD”. Furthermore, the characteristic symptoms of GORD are defined as heartburn and regurgitation.

1.4.2 Prevalence Of Symptoms

Estimates of the prevalence of GORD vary considerably from study to study, which is not surprising considering the previous lack of a consistent definition of GORD, but studies conclude that the prevalence is increasing. In population-based studies mild symptoms occurring two or more days a week, or moderate/severe symptoms occurring more than one day a week, are often considered troublesome by patients. The prevalence varies considerably from country to country. Studies from South America and Asia, predominantly China, indicate that the prevalence is considerably lower than in Europe and North America with prevalence of 2-3% of the population reporting at least one reflux episode weekly(24-28). The increasing prevalence in the Western world is mirrored by the increasing use of acid suppression medications and the rapidly rising incidence of oesophageal adenocarcinoma, the most feared complication of GORD (29-31).

Gastro-oesophageal reflux disease is one of the most common health problems in the western world today affecting up to 20% of the adult population at least weekly(32, 33). Less than half of these affected will see their primary care physician with their symptoms. Patients presenting with gastro-oesophageal reflux symptoms, nonetheless, may account for increasing primary care workload in Europe. Diseases associated with the reflux of duodenal and gastric contents into the oesophagus play an important role in the daily gastroenterological practice. The general practitioner is the first to be confronted with typical or atypical reflux complaints. Many patients without alarm symptoms such as weight loss or dysphagia, are treated with proton pump inhibitors (PPI's) for 4-6 weeks. An endoscopist or gastroenterologist only sees those patients who are refractory to therapy or experience recurrent symptoms after stopping therapy. Moreover, patients with frequent reflux symptoms experience difficulty sleeping, working and eating which contributes to their impaired quality of life. This produces substantial costs to patients as well as society in general. Duodeno-gastro-oesophageal reflux disease results in complications such as oesophagitis, oesophageal stricture, oesophageal haemorrhage, Barrett's oesophagus and oesophageal adenocarcinoma, a cancer with a poor prognosis and a rapidly increasing incidence in the western world.

1.4.3 Composition of Refluxate

The noxious agents responsible for injuring the oesophageal mucosa originate from both gastric and duodenal sources(34). The term duodeno-gastro-oesophageal reflux refers to regurgitation of duodenal contents through the pylorus into the stomach, with subsequent reflux into the oesophagus.

1.4.4 Importance of Acid Reflux

Substantial experimental and clinical evidence strongly supports the importance of acid and pepsin in causing oesophageal mucosal injury. Studies by DeMeester et al found that patients with Barrett's oesophagus have increased frequency and duration of oesophageal exposure to pH<4 than patients with oesophagitis, who had higher exposure times than healthy controls, suggesting a significant role for acid reflux in the development of oesophagitis and Barrett's oesophagus(35). Gotley et al found that oesophageal aspirates from patients with oesophagitis had significantly higher concentrations of acid and pepsin than aspirates from healthy controls(36).

1.4.5 Toxic Role of Duodenal Contents

Interest in this subject goes back a long time with pioneering work done as early as 1950 by Ferguson et al(37) in cats and dogs. Reflux of hydrochloric acid, which is generally accepted as the main cause of oesophagitis, can by no means

explain all cases of reflux disease. Oesophagitis is seen in patients after total and subtotal gastrectomy, where acid reflux is unlikely, and resolves after duodenal diversion(38-40). This indicates duodenal refluxate as an important factor. Resolution of oesophagitis with remaining abnormal acid reflux has also been described after duodenal diversion as an anti-reflux procedure, again indicating duodeno-gastro-oesophageal reflux as a pathogen(41). Modern proton pump inhibiting drugs effectively abolish gastric acid secretion but only heal 87-89% of the patients with oesophagitis(42). It is also known that 10-25% of patients with erosive oesophagitis have normal pH-studies, again indicating another explanation for injury(43, 44). In 1972 Gilison et al(45), working on primates, showed that duodenal juice causes severe oesophageal damage.

The role of duodenal contents, specifically bile acids and salts, in the development of oesophageal mucosal injury is the subject of many in vitro animal studies. Moffat and Berkas(46) showed that canine bile was capable of producing various degrees of erosive oesophagitis, confirming the earlier studies by Cross and Wangenstein(47).

The prevalence of duodeno-gastro-oesophageal reflux is even higher among patients who develop adenocarcinoma arising from the metaplastic epithelium (48-50). One mechanism could be the increase in epidermal growth factor seen

in the oesophageal mucosa with duodeno-gastro-oesophageal reflux, causing more immature cells to be displaced towards the epithelial surface where they are more susceptible to damage by biliary components(51, 52). This mechanism also seems to be mediated through cyclooxygenase-2 (COX2), and it has been found that bile acids are actively transported into the epithelial cell, thereby disrupting intra and extra-cellular membranes and junctions(53, 54).

The duration of exposure is also important. Bile acids have been shown in different animal studies to cause toxic damage to the oesophageal epithelium after a few hours of exposure at concentrations of 1-10mmol/l and after 5-8 days exposure at concentrations of 100-200 μ mol/l(55-57). The toxic mechanisms are not fully known and several theories have been put forth. It has also been shown, in animal studies, that cytotoxicity is mediated through mitochondrial dysfunction with depleted stores of ATP resulting in calcium influx and cell death (58-61).

1.5 Pathophysiology of Gastro-oesophageal Reflux Disease

Reflux episodes are very common in healthy individuals, occurring up to 50 times a day, usually during meals, in the postprandial state or due to gravitational influence such as bending forward(62). The LOS is formed by a

2.5-4.5cm segment of circular smooth muscle fibres in the distal oesophagus.

Almost all reflux episodes are related to transient lower oesophageal sphincter relaxation episodes (TLOSRES) in healthy individuals(63). In only a minority of individuals do these episodes cause symptoms or mucosal damage.

1.5.1 The Anti-reflux Mechanism

The physiological anti-reflux mechanism has three main components and it is only when this defence is overcome that reflux-induced damage ensues(64).

The first component of this anti-reflux mechanism is persistently high lower oesophageal sphincter pressure(65). The normal resting pressure is between 15 and 35 mmHg(66, 67). Even pressures of 5 to 10 mmHg seem to prevent reflux episodes efficiently and the normal pressure range should therefore present a generous pressure reserve.

The second component is the crural diaphragm which can be said to form an external sphincter, exerting pressure on the outside of the lower oesophageal sphincter(68). The oesophagus enters the abdomen through the crural diaphragm in the so-called "diaphragmatic hiatus". The diaphragmatic hiatus itself is approximately 2cm in length. The size of the hiatus is not fixed but it contracts whenever the intra-abdominal pressure increases, such as during

coughing or exercise. This mechanism can, therefore, be called a dynamic anti-reflux barrier.

The third component of the anti-reflux barrier is thought to be flap-valve mechanism of the lower oesophageal sphincter, formed by the sharp angle between the cardia of the stomach, commonly called the angle of His, the diaphragmal crurae, the phreno-oesophageal ligament, the intra abdominal segment of the oesophagus and the mucosal rosette(69).

1.5.2 Reflux Mechanisms

Throughout the last century there have been many different beliefs as to the mechanism of gastro-oesophageal reflux disease. During much of the first part of the 20th century the existence of a sphincter in the lower part of the oesophagus in humans was questioned, or as Ingelfinger put it: "The sphincter that is a sphinx"(70).

Dent and Freedman (71) with a study in asymptomatic healthy volunteers, showed that the episodes of reflux were due to transient relaxations of the lower oesophageal sphincter. The same research group (67), then showed that reflux episodes could occur by three different mechanisms; Transient relaxations of lower oesophageal sphincter, Transient increase in intra-

abdominal pressure, Spontaneous free reflux associated with a low resting pressure of the lower oesophageal sphincter.

Once a reflux episode has occurred, the degree of mucosal damage varies depending on a number of variables. These include the ability of the oesophagus to get rid of the refluxate, the contents of the reflux material and the degree of mucosal resistance, which is perceived to be maintained by the stratified squamous epithelial barrier(72). Additional protection is afforded by the swallowing of saliva, which both neutralizes and helps remove the acid(72). There is some correlation between the exposure of the oesophagus to acid and the severity of gastro-oesophageal reflux. Individuals with longer periods and more frequent episodes tend to suffer from more severe disease. Their oesophageal pH remains below 4 for significantly longer periods of time than in individuals with milder form of disease(73).

Delayed gastric emptying, on the basis of retention at 4 hours, has been shown to occur in 26% of gastro-oesophageal reflux disease patients(74). It is hypothesized that delayed emptying could lead to gastro-oesophageal reflux disease due to increased gastric content that could increase the frequency of transient relaxations of the lower oesophageal sphincter and gastric acid secretion via a gastric distension mechanism(75). As delayed gastric emptying is

not very common, however, this is probably only an enhancing cofactor in some patients.

It is generally perceived that people with a hiatal hernia have more reflux than people without(76). There have been different suggestions as to why this is the case. The gastro-oesophageal junction must protect against reflux during both static and dynamic conditions. During sudden increases in intra-abdominal pressure, the crural diaphragm normally serves as a second sphincter(76). In individuals with a hiatal hernia this mechanism is substantially impaired. Since the diaphragmatic sphincter is anatomically distanced from the gastro-oesophageal junction, therefore, it loses its ability to function as an anti-reflux mechanism(77). Large hernias can also impair the process of oesophageal emptying thereby prolonging acid clearance time following a reflux event(78).

1.6 Disturbance Of Bile Storage And Release Leading To Bile Reflux

1.6.1 Gallbladder Disease

In the event of gallbladder disease bile storage capacity may be reduced. The most common condition is gallstones. Manifestation of symptomatic gallstones is right upper quadrant pain radiating to the tip of scapula demonstrated by

placing the hand behind the back with an upward reaching thumb, Collin's sign(79) (Figure 4).



Figure 4 Collins' sign demonstrated to Professor Paddy Collins by a patient with a hand behind the back and the thumb pointing upwards. This photograph was taken by a student on one of Prof Collins famous Sunday Morning teaching rounds.

Cholecystitis is another condition which is almost always associated with gallstones, and impaired gallbladder function. In these circumstances bile flows continuously into the duodenum and is free to reflux into the stomach where it is available for reflux into the oesophagus.

1.6.2 Upper Gastro-intestinal Surgery

Bile storage can also be disrupted by upper gastro-intestinal tract surgery.

Following surgery to the gallbladder, stomach, pylorus or duodenum, the orderly sequence of bile storage, release and progression down the gastro-intestinal tract is also disturbed.

An association between upper gastrointestinal surgery and reflux has been reported(80). Surgery on the upper gastrointestinal tract may cause reflux oesophagitis by reflux of juices backward into the oesophagus by damaging the gastro-oesophageal sphincter mechanism(81). Excessive duodeno-gastric reflux in humans after upper gastro-intestinal surgery has been implicated in the development of gastritis, gastric ulcer, cancer of stump and post-cholecystectomy dyspepsia (82-88). Sears et al studied 13 partial gastrectomy patients with reflux symptoms and found increased DGOR in 77% of them(89). This represents an excellent human model for increased DGOR because of the incompetent pylorus and free regurgitation of duodenal contents into the

stomach, resulting in gastric bile acid concentrations (0.5-3.0 mM) known to cause oesophageal mucosal injury(89).

We have previously shown that pathological gastro-oesophageal reflux occurs after cholecystectomy(90-92). Fountos et al, using scintigraphy, found that duodeno-gastro-oesophageal reflux is common after biliary surgery such as cholecystectomy or choledocho-duodenostomy(93). The mechanism for bile reflux after cholecystectomy seems to be an increased number of pyloric relaxations as suggested by a study on dogs(94).

Therapeutic biliary procedures that impair the function of sphincter of Oddi lead to uncontrolled flow of bile into the duodenum(95). These patients are prone to duodeno-gastro-oesophageal reflux. Postoperative reflux oesophagitis is described in 58.3% patients who had oesophagectomy(96).

Gallbladder disease has been clinically linked to truncal vagotomy(97), although the causal relationship remains unproven. Impaired gallbladder motor function leading to stasis and the accumulation of biliary sludge could result either directly from vagal denervation, creating a dilated atonic gallbladder, or indirectly from altered gastric emptying reducing cholecystokinin (CCK) release

and gallbladder contraction(98). Due to this impaired function of the gallbladder the storage and release of bile are disrupted and it continuously trickles into the duodenum where it is available for reflux.

Billroth I, or more formally Billroth's operation I, is an operation in which the pylorus is removed and the distal stomach is anastomosed directly to the duodenum. Billroth II, or more formally Billroth's operation II, is an operation in which the antrum of the stomach is removed and a loop of jejunum is brought up and joined in a side-to-side manner for drainage as a gastrojejunostomy. Billroth I and II procedures are commonly performed after distal gastrectomy (99, 100). After either reconstruction method the duodenal contents may reflux freely into the gastric remnant and oesophagus. In fact, duodeno-gastric and gastro-oesophageal reflux have been shown to be the most frequent complication in patients who had Billroth I reconstruction (101-103).

1.6.3 Factors Contributing To The Aetiology Of Reflux

While much is known about the aetiology of reflux and its consequences, much remains unclear. By increasing the knowledge base of duodeno-gastro-oesophageal reflux disease, including identifying risk factors, we can hopefully make our contribution to find a more effective treatment, improve quality of

life in gastro-oesophageal reflux patients, and help prevent duodeno-gastro-oesophageal reflux disease from developing in the first place.

1.6.3.1 Genetic Factors

Genetic factors have for many years been suspected as important in the aetiology of gastro-oesophageal reflux disease. Studies demonstrating familial aggregation of Barrett's oesophagus suggest that, in a minority of individuals with severe reflux, the liability to reflux disease is inherited in an autosomal-dominant manner(104, 105). Severe reflux with childhood onset has been shown to be inherited in an autosomal-dominant way and seems to be associated with a gene of undetermined function mapped to chromosome 13q14 (105). In 2002 Cameron et al, in a large population-based study of twins with reflux symptoms, demonstrated that genetic factors are important in the aetiology of gastro-oesophageal reflux disease and account for approximately 31% of the liability to reflux disease in the studied population(106). This was confirmed in another twin study which estimated heritability to account for approximately 43% of the propensity for reflux disease(107).

1.6.3.2 Obesity

During the last decades the prevalence of obesity has increased in almost epidemic manner in western population (108-110). Among physicians there is

by tradition a wide- spread belief that obesity causes reflux and patients are often recommended to lose weight in order to treat reflux(111-113). Four population-based studies, one cohort and three cross-sectional studies, estimating the risk of reflux symptoms associated with high body mass index concluded that obesity does increase the risk for reflux(114-117). One hospital based case-control study showed a strong association between increasing BMI and oesophagitis(118). Another study of an endoscopy case-series assessed BMI in relation to oesophagitis and hiatus hernia in which obesity was strongly associated with combined oesophagitis and hiatus hernia(119).

1.6.3.3 Female Sex Hormones

Female sex hormones are known to be the predominant cause of gastro-oesophageal reflux during early pregnancy(120). In the later stage of pregnancy there is some further contribution of the increased abdominal pressure (120-123). It has also been suggested that the association between obesity and gastro-oesophageal reflux disease in women might be mediated by oestrogen(111, 124). It has further been shown that the use of sequential oral contraceptives lowers the resting pressure of the lower oesophageal sphincter(125, 126).

1.6.3.4 Tobacco Smoking

Several studies using 24 hour oesophageal pH monitoring and manometry have presented convincing data indicating that tobacco smoking induces reflux episodes by lowering the lower oesophageal sphincter resting pressure(127-130) and this effect appears to be mediated by nicotine(131).

1.6.3.5 Alcohol

Alcoholic beverages have been shown to evoke reflux episodes detected by 24-hour oesophageal pH monitoring and this appears to be mediated by relaxing the lower oesophageal sphincter immediately after ingestion(132-134).

1.6.3.6 Dietary Factors

Dietary Fat: Several studies have demonstrated that meals with a high fat content increase the frequency of postprandial transient lower oesophageal sphincter relaxation and can induce reflux symptoms (63, 66).

Dietary Fibre: Fibre, especially of cereal origin, has been demonstrated to be associated with a reduced risk of oesophageal and gastric cardia adenocarcinoma (135, 136). A suggested mechanism by which dietary fibre protects against oesophageal and cardia cancers may be mediated by cereal fibre scavenging carcinogenic nitrosamines (137, 138).

Coffee: It is widely believed that coffee triggers reflux symptoms (139, 140) but three epidemiological studies, one population based cross-sectional, one population based cohort-study and an endoscopy-series of people going through endoscopy found no relationship between coffee drinking and reflux symptoms or oesophagitis (116, 117, 141).

1.6.3.7 Helicobacter Pylori

Infection of the stomach with *Helicobacter pylori* affects 25-50% of the population in the industrialized countries of Europe and North America and 70-90% of the population in the developing countries of Asia, Africa and South America (142, 143). During the last century the prevalence of *Helicobacter pylori* has decreased substantially in western population while it has remained high in developing countries (144). It has been noted that the prevalence of duodeno-gastro-oesophageal reflux disease seems to be substantially higher in populations with low prevalence of *Helicobacter pylori* infection than in populations where the *Helicobacter pylori* prevalence is still very high (24, 26, 145, 146).

1.6.3.8 Pharmaceutical Drugs

Nitroglycerines, β -receptor agonists, aminophyllines, anticholinergic drugs and benzodiazepines have all been reported to relax the smooth muscle of lower

oesophageal sphincter (147-150). In a cohort study of 92,986 adult patients hospitalized for asthma with an average follow-up of 8.5 years there was an overall 40% increase in risk of oesophageal adenocarcinoma(151). Aspirin (acetylsalicylic acid) and non-steroidal anti-inflammatory drugs (NSAID's) have been proposed to cause gastro-oesophageal reflux by damaging the oesophageal mucosa and making it more vulnerable to the influence of physiological reflux(152).

1.6.3.9 Physical Exercise

In cohort study by Ruhl et al(117) both recreational and professional physical exercise decreased the risk of hospitalization for reflux-related diagnosis. A reasonable hypothesis to explain the mechanism for such an association could be that frequent and regular exercise could strengthen the crural diaphragm, thereby reinforcing the dynamic striated muscle part of the antireflux barrier.

1.6.3.10 Psychological Factor

Many reflux patients report worsening of symptoms under the influence of psychological stress in their daily lives(153). Several studies in laboratory settings have evaluated the effect of psychological and cognitive stress on the occurrence of reflux episodes mainly yielding negative results(154). Johnson et al compared psychological profiles of symptomatic reflux patients, with and

without endoscopic or pH monitoring for evidence of gastro-oesophageal reflux disease, without finding any significant association(155, 156).

1.7 Detection of Bile Reflux

1.7.1 Oesophago-gastro-duodenoscopy (OGD)

In the evaluation of patients suspected of having reflux disease endoscopy is the first and most important investigation (Figure 5).

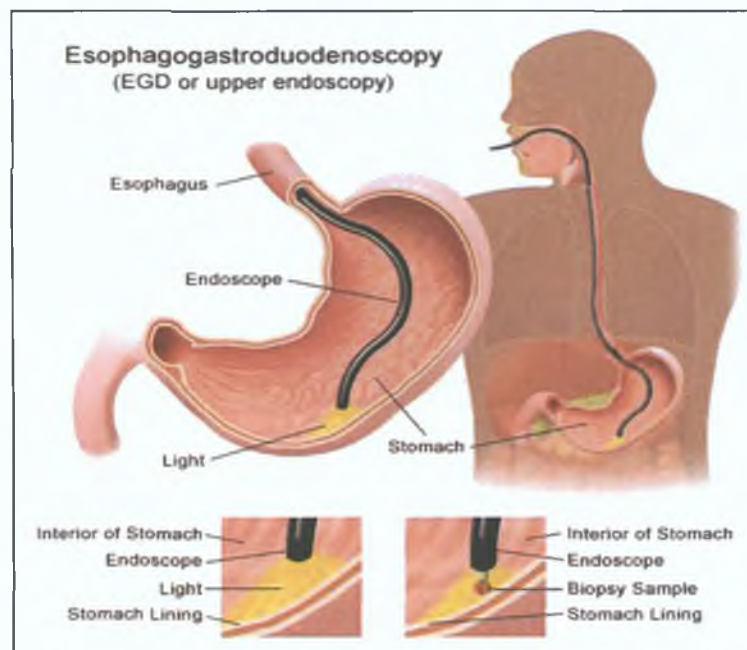
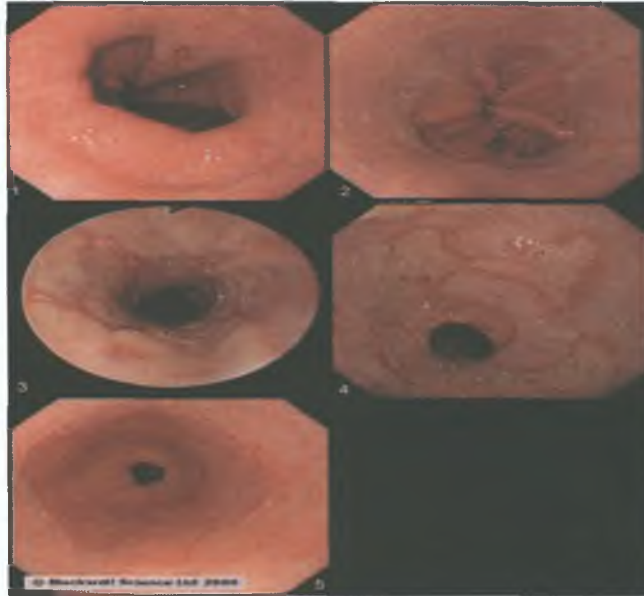


Figure 5 Oesophago-gastro-duodenoscopy OGD and biopsy

It is a quick and minimally invasive way to evaluate the presence of bile, to evaluate the grade of oesophagitis and to examine for complications of reflux such as Barrett's oesophagus, oesophageal stricture or carcinoma. The Savary

and Miller classification (Figure 6) and the Los Angeles criteria (Table 1) were developed to grade the severity of reflux induced oesophagitis(157, 158).



Grade 1	Single erosion above gastro-esophageal mucosal junction
Grade 2	Multiple, non-circumferential erosions above gastro-esophageal mucosal junction
Grade 3	Circumferential erosion above mucosal junction
Grade 4	Chronic change with esophageal ulceration and associated stricture
Grade 5	Barrett's esophagus with histologically confirmed intestinal differentiation within columnar epithelium.

Figure 6 Savary-Miller classification of reflux oesophagitis Grades 1-5

Table 1 The Los Angeles Classification System for the endoscopic assessment of reflux oesophagitis. Adapted from: Dent J et al: An evidence-based appraisal of reflux disease management - the Genval Workshop report. Gut, 1999, vol 44, suppl 2, 1 – 16

Grade A	One or more mucosal breaks no longer than 5 mm, none of which extends between the tops of the mucosal folds
Grade B	One or more mucosal breaks more than 5 mm long, none of which extends between the tops of two mucosal folds
Grade C	Mucosal breaks that extend between the tops of two or more mucosal folds, but which involve less than 75% of the oesophageal circumference
Grade D	Mucosal breaks which involve at least 75% of the oesophageal circumference

Endoscopic observations of a large bile lake in the stomach, antral mucosal erythema, friability, erosions and ulcers have been suggested as clinical indicators of excessive duodeno-gastric reflux(159).

1.7.2 Aspiration Techniques

Aspiration of the gastric and oesophageal content has been used and allows very accurate analysis of the components of refluxate(160, 161). It is resource intensive, however, and difficult to perform over extended periods of time.

Peaks in concentration may be lost because aliquots are collected. It is very difficult to perform in an ambulatory setting and its relation to normal physiology can be questioned.

1.7.3 Scintigraphic technique

This method is practical since it involves minimal discomfort for the patient, but it has several drawbacks. Tc^{99m}-N- (2,6-diethylphenylcarbomoylmethyl) iminodiacetic acid (HIDA) scintigraphy is limited by its relative insensitivity for oesophageal reflux and the use of radioactive isotopes(162). This technique is limited by the time span of the examination and cannot be used in ambulatory setting.

1.7.4 Histological Determination of Bile Reflux

Distinctive histological features of duodeno-gastric reflux include foveolar hyperplasia (FH), lamina propria vasodilatation and congestion (VC), oedema (Ede), and a paucity of acute (AI) and chronic (CI) inflammatory cells. Using grades (0-3) for each of these features, a composite "reflux gastritis score" (RGS) was devised by Dixon et al. in 1986(163). Many subjects with high RGS did not have bile reflux so a more accurate bile reflux index (BRI) was devised by stepwise logistic regression analysis of the histological grades used in RGS together with intestinal metaplasia (IM) and H. pylori (Hp) colonization found in

gastric biopsy(164). An index comprising (7xEde) + (3XIM) + (4XCI) - (6XHp) gave the best prediction of an elevated gastric-juice bile acid concentration. In this system, a histological bile reflux index (BRI) value above 14 indicates the presence of duodeno-gastric reflux (164).

1.7.5 Ambulatory Bilirubin Monitoring

In 1993 Bechi et al(165) validated a system for ambulatory detection of bile reflux by fibre optic spectrophotometer system (Bilitec® 2000, Synectics, Stockholm, Sweden) (Figure 7).



Figure 7 Bilitec 2000® monitor and probes for detection of bile reflux. Adapted from: www.saintluc.be/en/services/foregut/duodenogastric-reflux.

This system consisted of a fibreoptic probe of 5mm diameter with an open groove of 2mm across which two wavelengths of light are emitted and material

sampled. Bilirubin is detected between a mirror and the fibre optic tip at the end of the probe by light absorption at 453nm where bilirubin has an absorption peak. Data is collected in a portable data logger and later analyzed on computer. With a computer program (Synectics Medical, Stockholm, Sweden) the following parameters are measured: percentage total time absorbance units above >0.14 ; percentage upright time absorbance units >0.14 ; percentage recumbent time absorbance units >0.14 ; number of reflux episodes; number of reflux episodes with absorbance units >0.14 for five or more minutes and period of the longest single DGER exposure episode. This technique allows ambulatory detection of bile in the oesophagus in much the same manner as pH detection(166) (Figure 8).

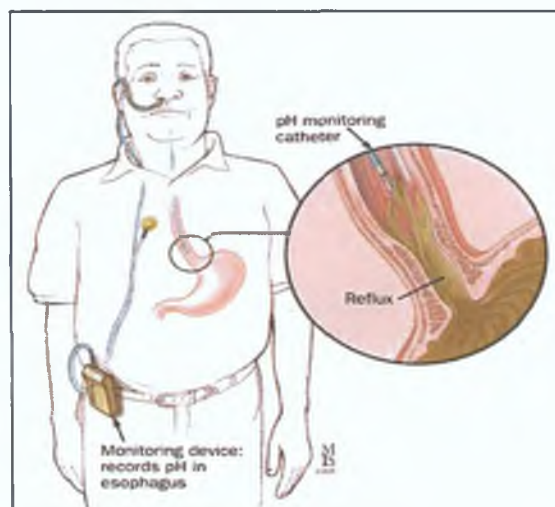


Figure 8 Ambulatory 24 hour pH monitoring. Adapted from: www.hopkinsgi.org.

Nevertheless there are some limitations. Some coloured foods may interfere with the measurements and small pieces of food may get stuck between the mirror and the tip of the fibre optic bundle. At low pH below 3.5 bilirubin forms dimers with different optic properties causing an *underestimation* of bilirubin concentration of at least 30 percent(167).

1.8 Treatment Options

The aim of the treatment in patients with gastro-oesophageal reflux is to relieve symptoms, to heal the complications of chronic reflux like oesophagitis and to prevent complications and recurrence of symptoms and lesions.

1.8.1 Lifestyle Measures

The cornerstone of the treatment of reflux disease is lifestyle modification. Although there are no controlled trials to prove its effectiveness, lifestyle modification is advised because of the inherent logic in contributory factors for gastro-oesophageal reflux disease. Patients are advised to stop smoking because chronic cigarette smoking compromises LES function (85-89). During bouts of coughing it has been observed that the lower oesophageal sphincter pressure is lower and that more sphincter relaxations take place (122). Dietary restrictions include avoiding eating or drinking alcoholic beverages 2-3 hours before sleeping (90-93). Patients with hiatus hernia are advised to elevate the

head of the bed by 15-20 cm, although no controlled study has been performed to prove the usefulness of this measure. Obese patients are advised to lose weight although it has not been proven that this will result in improvement in reflux.

1.8.2 Medical Therapy

Substantial progress has been made in the last two decades in the treatment of gastro-oesophageal reflux disease. Colloidal bismuth and sucralfate are effective against mild symptoms in clinical practice(168). They bind epidermal growth factor and elevates the concentration of peptide in ulcerated areas of gastrointestinal mucosa(169). The introduction of H2 blockers was a significant success and acid suppression brought great relief to the majority(170). Proton pump inhibitors (PPIs) have been shown to be more effective than H2 blockers in relieving symptoms and healing erosive oesophagitis. Results from 33 randomized trials with over 3000 patients showed that relief of symptoms could be anticipated in 83% of PPI treated patients compared with 60% of patients treated with H2 receptors, while oesophagitis healed in 78% and 50% of patients respectively(171). PPIs have been established as providing symptom relief and healing of lesions in up to 85% of patients(172). A trial has shown that a significantly higher percentage of patients remained in remission after 12 months treatment with omeprazole (72%) than with ranitidine (45%)(173). In some patients nocturnal acid breakthrough has been observed which is defined

as the occurrence of an intragastric pH lower than four during at least one hour at night(174). Additional suppression of nocturnal acid breakthrough can be achieved by adding an H₂-receptor antagonist to the optimal PPI dose twice daily, although rapid tolerance to the effect of the H₂-receptor antagonist may occur(175). Recent studies in patients with severe reflux have found that aggressive acid suppression with omeprazole decreased both acid and duodenal reflux (176). Gut et al (177)found that oesophageal acid and bile reflux were both significantly reduced ($p<0.02$) after 28 days of treatment with pantoprazole compared to pre-treatment values in patients. Marshall et al(178) studied oesophageal and gastric bile reflux in 23 patients with Barrett's oesophagus finding a significant reduction in both oesophageal and gastric acid and bile reflux after 6-10 weeks of treatment with omeprazole. These studies suggest that the decrease in duodeno-gastro-oesophageal reflux measured after the use of proton pump inhibitors is due to their inhibition of both gastric acidity and volume, making smaller quantity of the gastric content available to reflux into the oesophagus. Medical therapy has the advantage of avoiding a surgical procedure and its associated complications, an important consideration in the elderly and those with contraindications to surgery. However, in younger patients in whom long-term medical therapy is anticipated, anti-reflux surgery may be a more suitable and cost effective alternative.

1.8.3 The Role of Surgery

Although medical therapy is effective in the majority, in a subset of patients, reflux symptoms persist in spite of therapy with standard doses of PPIs. In about half of patients with persistent oesophagitis and reflux symptoms, despite standard dose PPI therapy, persisting acid reflux can be demonstrated(179). These may be considered for surgical control. Surgery should also be considered for young patients who do not want to take PPIs for the rest of their lives, patients with severe Barrett's oesophagus, patients who simply cannot afford the medication or patients who are allergic to PPI's. New local minimally invasive therapies have been developed like the endoscopic suturing device (Endocinch), the temperature-controlled radiofrequency energy system (Stretta)[®] and the local submucosal deposition of Ethylene-vinyl alcohol implantation (Enterix)(180). Despite initial enthusiasm the therapeutic role of these recently developed endoscopic anti-reflux procedures will require additional evaluation(181).

1.8.4 Anti-reflux Surgery

1.8.4.1 Roux-en-Y

Cesar Roux (1857-1934) began utilizing his "Loop-en-Y" procedure for gastric outlet obstruction(182)(Figure 8). This technique fell into disfavour due to a high incidence of postoperative stomal ulceration(183). It was revived with

additional vagotomy and was adapted for multiple applications(184). The greatest factor in its revival has been the treatment of duodeno-gastric reflux. Studies have underlined that Roux-en-Y diversion has low incidence of duodeno-gastric reflux and is an effective procedure to eliminate primary or secondary duodeno-gastric reflux(185) (Figure 9).

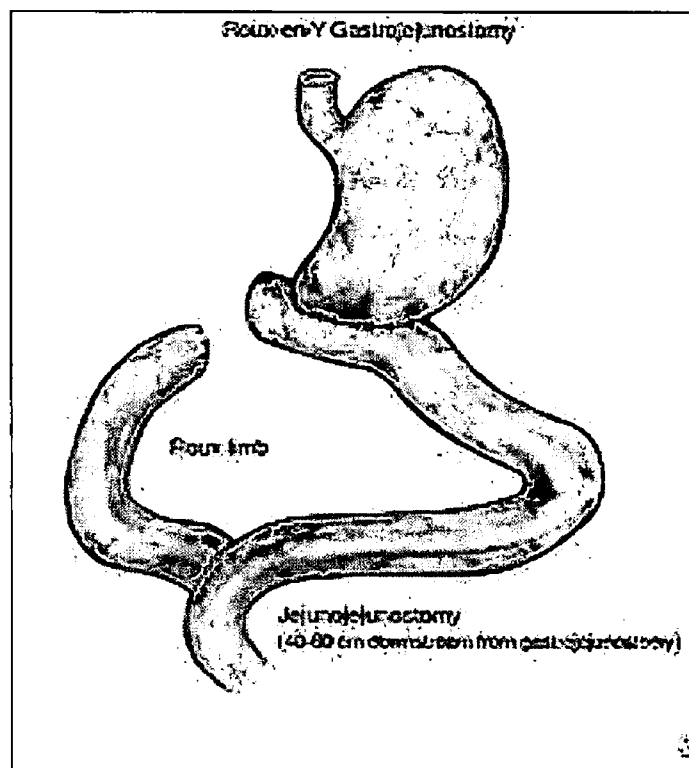


Figure 9 Roux-en-Y reconstruction

The conventional Roux-en-Y carries an increased risk of stomal ulceration which makes additional vagotomy necessary(186). The side effects of this include delayed gastric emptying, diminished gastric acid secretion, decreased size of

the gastric reservoir, interruption of neurohumoral relationships and feedback mechanisms of the stomach, duodenum, pancreas and the hepatobiliary system as well as reduced trophic effect of gastrin on gastric mucosa(187). Clinically this may result in epigastric fullness, nausea, intermittent or post-prandial vomiting or abdominal pain(188). Salminen et al(189) provided further insight for the importance of DGOR in GORD in their study where severe reflux disease was treated by Roux-en-Y procedure and selective vagotomy previously described by Fekete et al(186). All patients were relieved of their symptoms and oesophagitis healed, but post-operative pH analysis showed unaltered acid load in the oesophagus(186). Thus inhibition of biliary, but not acid, reflux promoted healing and symptom relief.

1.8.4.2 Nissen's Fundoplication

Antireflux surgery aims to prevent reflux by restoring a one way valve to the lower end of the oesophagus. This surgery can be performed laparoscopically through the abdomen (Figure 10).

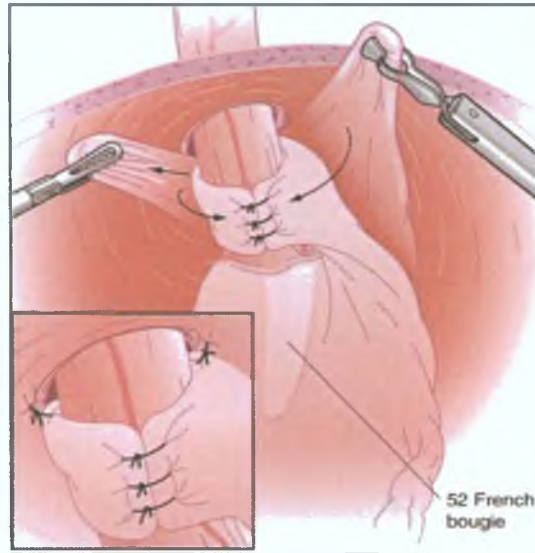


Figure 10 Nissen's Fundoplication. Adapted from: Sabastion Textbook of Surgery 17th Ed. 2004 Elsevier Pg 1160 by Courtney M. Townsend

Some patients will require conversion to an open operation as a result of abnormal anatomy, previous surgery with scarring or from complications from a short oesophagus, bleeding or injury to other organs. The success of surgery depends on the experience of the surgeon and the correct indication. The most frequently performed procedures are the Nissen-Rosetti (360°), or the Toupet (270°) operations. The Nissen fundoplication is the most popular procedure for patients with severe refractory reflux with a normally functioning oesophageal body but with proven pathological reflux, insufficient lower oesophageal sphincter or hiatus hernia. Fundoplication is well known to effectively inhibit gastro-oesophageal reflux and has been put forward as the treatment of choice

for patients with DGOR and Barrett's oesophagus(190). Nissen's fundoplication has been the main surgical treatment of patients with acid and bile reflux into the oesophagus. With this procedure reflux may be reduced but not completely abolished (191, 192).

1.8.4.3 Duodenal Switch

An alternative operation is diversion of duodenal juice such as by the duodenal switch procedure. This effectively inhibits duodenal juices reaching the stomach and oesophagus. The supra-papillary Roux-en-Y duodeno-jejunosotomy (Duodenal Switch), specifically designed to divert bile, without vagotomy and antrectomy seems to entail less drawbacks, and therefore might be better alternative than classical Roux-en-Y duodenal diversion(86). The introduction of duodenal switch procedure seems ideal to abolish completely alkaline reflux without disturbing gastric motility and emptying (86) (Figure 11).

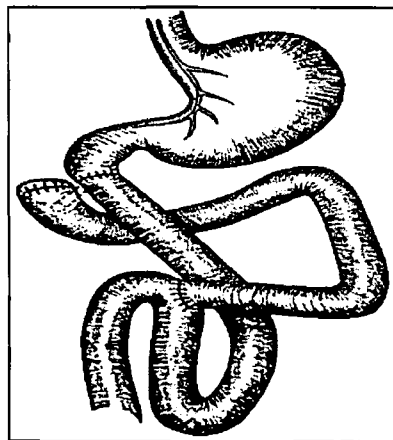


Figure 11 Duodenal Switch. Adapted from: Perdakis G, Saeki S. Alkaline gastroesophageal reflux disease. In: Hinder RA, e.

1.9 Clinical expression and Implications of reflux

The two most important typical symptoms of GORD are heartburn and acid regurgitation(193). Heartburn is a retrosternal burning sensation that radiates towards the throat or mouth. It may occur after meals, in supine position and on bending over or heavy lifting(194). Regurgitation is the effortless return of gastric content into the oesophagus and frequently into the mouth. This fluid can be acid or food and can occur while prone or supine(195, 196). It may result in acute dyspnoea, choking and coughing(197).

Gastro-oesophageal reflux disease may present with a number of atypical symptoms. Non-specific upper gastrointestinal symptoms are nausea, dyspepsia, bloating, belching or indigestion(198-200). Pulmonary symptoms attributed to reflux disease include chronic cough, wheezing, nocturnal dyspnoea, asthma, recurrent aspiration and pulmonary fibrosis(201, 202). Reflux related oto-rhino-laryngological and oral symptoms are hoarseness, dysphonia, postnasal drip, globus, halitosis and water brash(203, 204). These symptoms are probably caused by nocturnal reflux(205). Dental erosions are potential oral complications of reflux(206).

Oesophageal manifestations of reflux include erosive oesophagitis, oesophageal strictures and motility disturbances(193, 207). Overwhelming evidence

suggests Barrett's oesophagus (Figure 12) is an acquired condition secondary to chronic duodeno-gastro-oesophageal reflux and a known risk factor for oesophageal adenocarcinoma (30, 208-211).



Figure 12 Barrett's Oesophagus on Endoscopy Classic Barrett's Esophagus - Upper Endoscopy, Chapter 3 Barrett's esophagus and esophageal cancer, Fig 1 2003 Blackwell-2

1.10 Use of Molecular Markers

Biological markers as objective criteria may help to supplement and standardize histological diagnosis and improve efficacy of surveillance programs. Some of these molecular abnormalities may even become targets for new therapeutics.

In the following overview, two important biological markers are discussed in the context of their cellular and subcellular function.

1.10.1 p53

Transition of cells through different cell cycles and induction of apoptosis are crucial steps during tissue proliferation and several genes govern this. The transcription factor p53 induces cell cycle arrest, apoptosis, senescence, DNA repair and changes in metabolism by regulating target genes in response to a variety of cellular stresses (212). The p53 protein also appears to play a role in non-transcriptional cytoplasmic processes to induce apoptosis(213). A number of post-translational modifications and abundant isoforms of p53 modulate its activity(214). The p53 protein plays a major role in preventing tumour development(215). It responds to a range of potentially oncogenic stresses by activating protective mechanisms, most notably cell cycle arrest and apoptosis(216). DNA damage and other stress signals may trigger the increase of p53 proteins, which have three major functions: growth arrest, DNA repair and apoptosis (cell death)(217). Growth arrest stops the progression of cell cycle, preventing replication of damaged DNA. During growth arrest, p53 may activate the transcription of proteins involved in DNA repair(218). Apoptosis is the "last resort" to avoid proliferation of cells containing abnormal DNA(219). The cellular concentration of p53 must be tightly regulated. While it can

suppress tumours, high level of p53 may accelerate the aging process by excessive apoptosis. The mutated p-53 protein, because of its longer half-life, accumulates in the nuclei and is easily detectable by immunohistochemistry.

It is a mutation of the gene itself or loss of cell signalling, either upstream or downstream, that leads to the ubiquitous loss of p53 activity in human cancer cells (212, 213, 215). The p53 gene is found on the locus 17p13.1(220) and this locus is known for its involvement in the early stages of Barrett's oesophagus (221). A strong correlation between telomere length and alternations on chromosome 17 such as loss of the 17p arm signal has been demonstrated. Chromosomal instability can progress to DNA aneuploidy, shown to be a strong predictor of progression from Barrett's oesophagus to cancer (222).

The importance of p53 as a tumour suppressor is reflected by its high rate of mutation in human cancer, with >50% of adult human tumours specially adenocarcinoma of the oesophagus bearing inactivating mutations or deletions in the *TP53* gene (223). A subsequent multi-institutional study of ninety-eight cases illustrated TP53 mutations in dysplastic Barrett's metaplasia but not in low-grade or non-dysplastic Barrett's metaplasia (224). A more recent study showed that while all adenocarcinomas demonstrating TP53 mutations expressed p53 in Immunohistochemistry, a majority of cases of p53 expression

were without mutations(225). The findings of this study were that the vast majority of Barrett's associated oesophageal tumours expressed p53 accumulation (83%) whereas only 9.6% harboured the mutation(225). They concluded that p53 accumulation is a better marker for oesophageal adenocarcinoma than TP53 gene mutations (225).

The recognition of an association between p53 and Barrett's oesophagus and oesophageal adenocarcinoma dates to the early 1990s when a number of studies demonstrated positive IHC staining of dysplastic Barrett's metaplasia and adenocarcinoma with p53(217-219, 221, 226). These studies established that while carcinoma and dysplastic Barrett's oesophagus stain positively for p53 in a majority of cases, low grade dysplasia and Barrett's metaplasia without dysplasia did not exhibit p53 staining on IHC.

Since 2000 the degree of over-expression of p53 has been illustrated to be an indicator for advancement from low-grade dysplasia to high-grade dysplasia and adenocarcinoma by a number of studies (227-232). In 2009 a study by Hritz et al(233) concluded that quantification of p53 expression, as the percentage of positive nuclei, is predictive of the development of adenocarcinoma. Since 2007, studies have been directed at determining whether or not p53 expression is a predictor of response to therapies (234-236). In the last decade the use of

p53 as an indicator of Barrett's oesophagus and adenocarcinoma has become established as not only indicative of metaplasia and neoplasia but also predictive of progression from low-grade dysplasia to high grade or adenocarcinoma and response to therapy.

1.10.2 Ki-67

Cell proliferation is thought to be one of the earliest steps in the development of cancer and Barrett's oesophagus. Proliferation can be induced by chronic cell injury produced by reflux and may be assessed by immunohistochemistry with use of antibodies that recognize antigens, which are expressed in nucleus during proliferation. Ki-67 is a nuclear protein encoded by the MK167 gene. It was first discovered and labelled as an antigen in 1983 (Ki-67 antigen) in the city of Kiel, from which the protein derives its name. Gerdes et al(237) were the team that initially defined the protein and Gerdes has been prominent in the field of research surrounding the protein since. The antibody to the protein reacts with a certain nuclear structure present only in proliferating cells and so expression of the protein is strictly associated with cellular proliferation and the active phases of the cell cycle(238). This has lead to its use as an indicator of the growth fraction of a given cell population(239) that is, the fraction of cells born into the proliferative category. MIB-1 is the name given to the antibody used to stain the human Ki-67 protein which shows the proliferation activity of a group of cells. The marker has been used alongside proliferating cell nuclear antigen

(PCNA) in many cases but has also been shown to be easier than PCNA to interpret because it has less background staining and stronger, more uniform positive signals(240).

Much research has been conducted into the use of Ki-67 positive staining to reflect the presence of dysplasia or neoplastic growth. In the area of Barrett's oesophagus, it has been shown that the number of Ki-67-positive cells was significantly greater in Barrett's epithelial mucosa when compared to normal oesophageal mucosa(241). This indicates that certain Ki-67 staining patterns may demonstrate the presence of intestinal metaplasia.

It has been demonstrated that abnormally persistent expression of Ki-67 is associated with premalignant dysplasia in oesophageal squamous epithelium and Barrett's mucosa(242). The case has also been made that there may be a predictive value in the Ki-67 levels present in gastric carcinoma(243). A Japanese study showed that patients with lymph node metastases had higher Ki-67 labelling percentages than those without(244).

CHAPTER 2

AIMS

We hypothesize that the internal micro-environment holds the key to the development of oesophageal adenocarcinoma. Changes in this environment may have a major impact on the potential for neoplasia of the lower oesophagus or the stomach.

We further hypothesized that events which impact on this micro-environment, such as removal of the gallbladder and thus the storage facility for secreted bile, or upper gastrointestinal surgery may critically alter the dynamics of bile release and reproduce the conditions conducive to the development of oesophageal and gastric cancer.

The aims of this thesis were to conduct a series of studies to examine changes at a clinical, pathological and molecular level and to examine the impact of these changes on the mucosa of stomach and of the lower oesophagus. The first study was conducted to look at the prevalence of bile reflux in three distinct patient groups: Patients presenting with upper abdominal symptoms, patients on Barrett's surveillance and patients post oesophago-gastrectomy. The second study was retrospective and conducted to specifically look at the histological and molecular changes brought about in patients with a remote history of cholecystectomy. The third study was prospective and designed to look at the early and short-term effects of cholecystectomy in paired population.

CHAPTER 3

GENERAL METHODOLOGY

3.1 Study Approval

These studies were approved by the ethics committee of Connolly Hospital, Dublin and conducted in accordance with their guidelines. All participants gave their consent to participate in the study. No economic reimbursement was given for participation.

3.2 Upper GI Endoscopy

Upper gastrointestinal endoscopy was performed on all patients by both the primary researcher, who had 16 training sessions with the supervisor before the study, and the supervisor (professor of GI surgery) in Connolly Hospital. In order to achieve endoscopic assessment reliability the two endoscopists had a consensus meeting and reviewed common macroscopic findings and standardized criteria for inflammation, Barrett's oesophagus, gastric and duodenal ulceration. Multiple biopsies, at least 4, were taken from the gastric antrum, the lower oesophagus or the anastomotic site.

3.3 Tissue Processing and Staining

The Bile Reflux Index (BRI) was obtained by histological examination of sections of the specimens that were stained with haematoxylin and eosin (H&E).

Haematoxylin stains nuclei a dark blue or navy colour and eosin stains

cytoplasm pink. The slides with tissue slices 4µm thin are placed into the Tissue Tek® Prisma™ Automated slide stainer for staining. The machine first melts the wax by chemically dissolving it in xylene. It then immerses the baskets of slides into solution reservoirs of ethanol advancing from higher grade to lower finally into a reservoir of water. The process of staining for haematoxylin is regressive staining. The slides are placed in a reservoir of haematoxylin and then into a 1% hydrochloric acid solution, which removes all of the staining except at the nuclei. Staining for eosin is then performed by initially immersing the slides in 95% ethanol and then eosin. The slides then proceed to the Tissue Tek® Glass coverslipper for automated coverslipping. The coverslips form a secure bond with the slide by applying the Thermo Scientific Shandon Consul-Mont, a xylene based glue. The coverslipped slides are then dried in the machine by two drying fans above and below the slides. The dried slides are then ready for examination under light microscopy.

3.4 Histological Assessment

The biopsy specimens were oriented on filter paper and immediately fixed in formalin. Paraffin processed less than 5µM sections were cut at three levels, stained by haematoxylin and eosin and an additional section at the second level was stained with alcian blue, pH 2.5 and periodic acid Schiff (AB/PAS) to demonstrate intestinal metaplasia.

The biopsies were assessed by the senior consultant histopathologist who was blind to the clinical details of the patients and the endoscopy findings.

Histological features of bile reflux were calculated by using the Bile Reflux Index (BRI) devised by Sobala et al (164). In this system an index is derived based on the presence and/or severity of certain histological parameters: oedema of the lamina propria (E), intestinal metaplasia (IM), chronic inflammation (CI), and *Helicobacter pylori* colonization in the stomach (Figure 13).

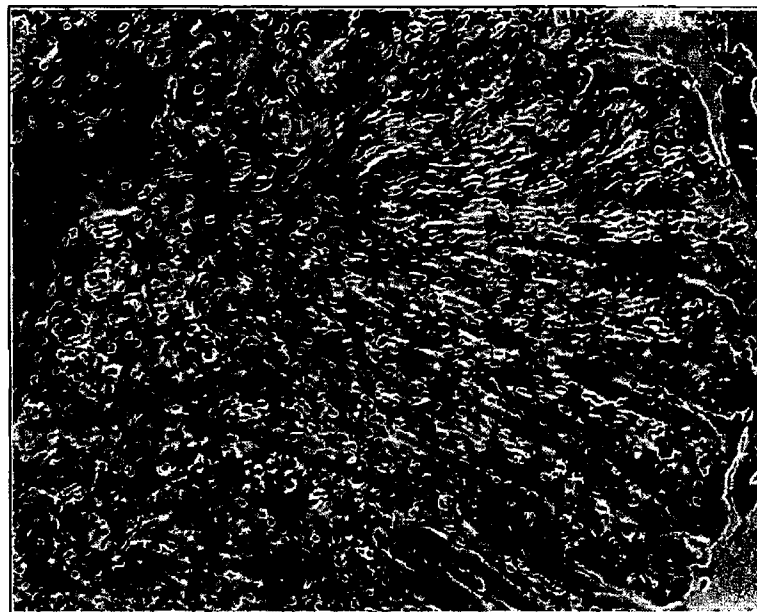


Figure 13 Severe reflux gastritis as shown by H & E stain

For every specimen the pathologist assigned a grade from 0 to 3 representing absent, mild, moderate or marked respectively based on severity. An index value was then calculated using a formula derived from stepwise logistic regression analysis:

$$\text{BRI} = (7 \times \text{E}) + (3 \times \text{IM}) + (4 \times \text{CI}) - (6 \times \text{Hp})$$

A BRI above 14 indicates duodeno-gastric reflux (defined as bile acid level >1mmol/L, the upper limit of physiological reflux) with 70% sensitivity and 85% specificity. For analysis all specimens with a BRI above 14 were identified as BRI (+). The diagnosis of Barrett's epithelium was made if specialized intestinal metaplasia, characterized by goblet cells, was identified.

3.5 Immunohistochemistry

The biopsy specimens were placed into cassettes, packed into embedding sets and placed in the Tissue-Tek® 5 Vacuum Infiltration Processor (VIP) overnight.

The VIP allows promoting penetration of paraffin wax into tissues by applying a vacuum and warming up the embedding sets from 40°C to 60 °C in a stepwise fashion. The dehydration process is performed in the VIP by administration of ethanol. The sets go through two changes of 70%, 94% and 100% ethanol.

After having dehydrated the tissues, the ethanol is removed by applying three changes of xylene. Finally, the wax is applied in four cycles and the cassettes are removed from VIP and the tissues are embedded in paraffin wax using the

Tissue-Tek® TEC® 5 Tissue Embedding Console System. In a process called microtomy, the blocks are then sliced into sections 4µm thin. The slices are placed into a hot water bath set at 50°C before being placed onto pre-cleaned coated slides. The fully automated IHC and ISH Leica Bond-Max technology was used for the Immunohistochemistry (IHC) staining of the slides to demonstrate expression of Ki-67 (Figure 14) and p53 (Figure 15).

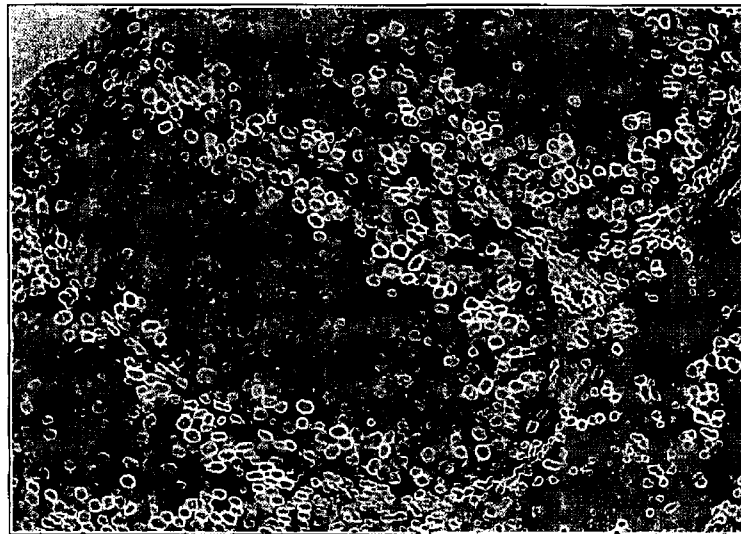


Figure 14 15-20% Ki-67 proliferation index at 5cm above the OGJ

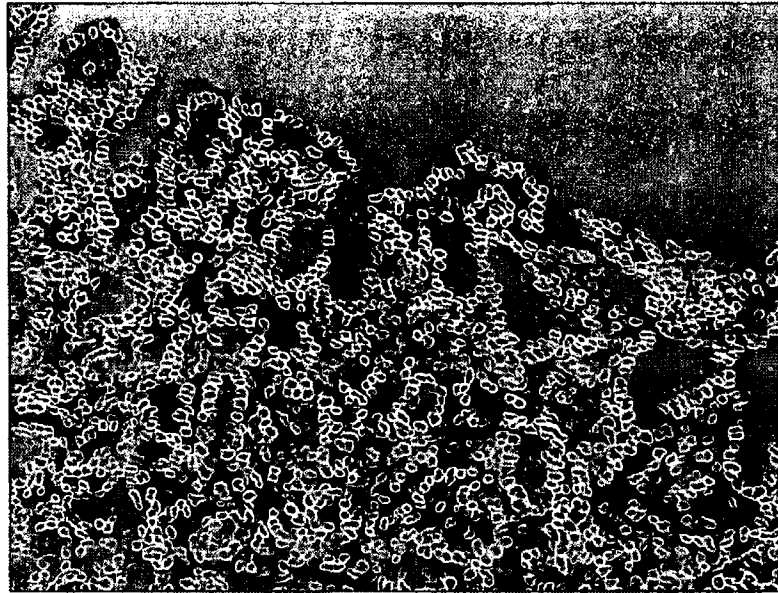


Figure 15 30% positive expression of p53 nuclei in the gastric antrum

The Bond Covertile™ is placed on top of the slides before they are placed into the machine. The capillary network of covertile allows maximum penetration into the sample using only 150µL of reagent. The reagents used were the Bond™ Ready-to-use Primary Antibody p53 (DO-7) and Dako Ready-to-use N-Series Primary Monoclonal Mouse Anti-Human Ki-67 Antigen, clone MIB-1. Each of these reagents is optimally diluted. Therefore, reconstitution, mixing, dilution or titration is not required.

While it is possible to use antibodies to p53, this is not possible for Ki-67 because the epitope recognized by anti-Ki-67 antibody is highly labile and

processing of the tissues leads to the recombination of the Ki-67 molecule. To detect recombinant fragments of the Ki-67 molecule, MIB-1, a more robust agent was produced. MIB-1 is the reagent used in this study to detect Ki-67. All reagents are mouse anti-human monoclonal antibodies indicated for examination via light microscopy of formalin-fixed, paraffin-embedded tissue.

Once the BondMax Staining Run is set up, the machine moves the slides through a number of automated steps. The slides are deparaffinised and heated on a hot plate to retrieve the masked antigens. The primary antibodies or reagents are then applied. A secondary antibody is then applied which binds to the primary antibody and a tertiary reagent, the chromogen, is the final step before coverslipping. The secondary antibody is an anti-mouse IgG horseradish peroxidase polymer. It amplifies the signal from the chromogen, allowing smaller amounts of antigen to be detected via light microscopy. Coverslipping for IHC slides was performed using the same automated system as the H&E slides.

CHAPTER 4

STUDY – 1

The Prevalence of Gastric Bile Reflux in Three Patient Cohorts

ABSTRACT

Background: Duodeno-gastro-oesophageal reflux (DGOR) has been implicated as the cause of increased oesophageal pathology. In this study we examined the causes and consequences of bile reflux, defined as injury to the gastric mucosa, consequent upon DGOR.

Aims: The aim of this study was to examine the incidence and contributing factors to duodeno-gastric bile reflux and the effects of the refluxate on the gastric and oesophageal mucosa in three defined patient cohorts.

Patients and Methods: We prospectively studied 150 consecutive patients who presented to the surgical endoscopy suite for investigation and who fitted one of three patient profiles; Group 1 (n = 98) were patients who presented for upper gastrointestinal endoscopy for investigation of upper abdominal symptoms. Group 2 (n = 20) were patients with Barrett's oesophagus. Group 3 (n = 32) were patients on surveillance post-oesophago-gastrectomy. These groups were compared with respect to parameters including age, gender, BMI, use of PPI, smoking, history of cholecystectomy and bile reflux index (BRI).

Results: There was no difference in age or BMI between the groups but Group 2 and 3 had significantly higher number of male patients compared to Group 1 (p=0.005). Ratio of BRI (+) to BRI (-) biopsy specimens in Group 3 was higher when compared with Group 1 (p=0.05). 25% patients had history of cholecystectomy and of these 76% were BRI (+) as compared to 66% BRI (+) in

non-cholecystectomy group. The odds ratio was 1.37 and the number need to be harmed by cholecystectomy was 15.2. Previous history of cholecystectomy was noted in 30% of symptomatic patients and of these 72% were BRI(+). Within Group 2 patients 91% of all PPI users had BRI (+) on biopsies while only 50% of patients not on PPI therapy were BRI (+) (p=0.035).

Conclusion: It is concerning the way post-cholecystectomy patients are predisposed to iatrogenic injury induced by bile reflux. High proportion (30%) of patients remain symptomatic post-cholecystectomy and 72% of these patients were BRI (+). Increase in BRI positivity was noted in patients on PPI therapy who were on surveillance of Barrett's oesophagus. This suggests a possible role of DGOR in pathogenesis of Barrett's and symptoms of dyspepsia.

4.1 INTRODUCTION

Gastro-oesophageal reflux disease (GERD), with its symptoms of heartburn and regurgitation, is one of the most common conditions affecting the oesophagus. The prevalence of GERD is greatest in North America and Europe, with the highest rates seen in the USA (13–29%), Sweden (17%), UK (10%), and Spain (10%)(245). Estimates indicate that approximately 24% of the population will experience heartburn daily or more often, whilst 43% experience heartburn once or twice a week(246). The symptomology of GERD patients is complex and extends to extra-oesophageal manifestations (247).

Duodeno-gastro-oesophageal reflux, as a component of gastro-oesophageal reflux disease, has been implicated as the cause of increased oesophageal pathology(15). Reflux of alkaline duodenal contents through the pylorus and into the stomach occurs during the early morning and postprandial periods and can be observed during endoscopy(248). Excessive duodeno-gastric reflux can cause chronic gastritis, gastric ulcers, reflux oesophagitis and Barrett's oesophagus and an increased risk of gastric and oesophageal cancer(249, 250). The more duodeno-gastric refluxate that is present in the stomach the more that reaches the oesophagus. A positive relationship between duodeno-gastric bile reflux and oesophagitis has been illustrated in a number of studies(251, 252). Barrett's oesophagus is a known complication of reflux(253). A study on patients that were investigated with manometry, gastric and oesophageal 24-h

pH studies and simultaneous bile monitoring compared healthy controls and groups with oesophagitis and Barrett's oesophagus. This study revealed a trend in increasing bile levels present in the oesophagus of patients with Barrett's oesophagus (254), with a median of 0% oesophageal bile reflux episode found in control group compared to 3.5% in patients with oesophagitis and 7.8% in patients with Barrett's oesophagus (254).

Studies either directly measuring bile acid content of oesophageal aspirates (160), or using spectro-photometric monitoring of bilirubin concentration as a surrogate marker for bile (255), showed that bile exposure in the oesophagus increased in proportion to the severity of oesophagitis, with the greatest exposure in patients with Barrett's oesophagus(256). In a study by Vaezi et al increased oesophageal bilirubin exposure was observed in patients with dysplasia and oesophageal adenocarcinoma(257). This may explain the disturbing increase in prevalence of Barrett's oesophagus and oesophageal adenocarcinoma noted in the Western countries(258, 259).

The potential detrimental effects of the refluxed duodenal contents on the oesophageal mucosa have been shown in animal models (260). In one animal model oesophageal exposure to duodenal contents was a key factor in the development of oesophageal adenocarcinoma(261). Another animal study

suggest that DGOR, at concentrations of > 1mmol/L, usually causes the most oesophageal damage in synergy with acid(262, 263). Early studies by Cross and Wangensteen (47) suggested a role for bile and its constituents (namely, bile acids) in oesophageal mucosal damage. Using a dog model with biliary diversion and a jejunal conduit anastomosing directly to the oesophagus, Moffat and Berkas(46) showed that canine bile was capable of producing various degrees of erosive oesophagitis, thereby confirming earlier studies by Cross and Wangensteen(47). Bile salts in isolated rat hepatocytes have been shown to activate protein kinase C, a calcium-activated phospholipid-dependent enzyme which plays a key role in growth-signalling pathways (8).

In an acidic, even weakly acidic, environments, even for brief periods, bile acids are associated with the development of dilated intercellular spaces, the most common change in the mucosa of patients with non-erosive reflux disease (10). More recent studies show that oesophageal mucosal damage by bile acids is dependent on the conjugation state of the bile acids and the pH of the refluxate(57). Using net acid flux (NAF) across the oesophageal lumen as an index of mucosal injury, Harmon et al. (57) showed that taurine-conjugated bile salts (1-5 mmol/L), taurodeoxycholate and taurocholate (both with pKa of 1.9) increased NAF at pH 2, whereas the unconjugated forms (1-5mmol/L) increased NAF at pH 7 but not at pH 2. Hence, conjugated bile acids are more injurious to the oesophageal mucosa at acidic pH, whereas unconjugated bile acids are

more harmful at pH 5-8(57). The exposure of bile acids to the epithelium of the oesophagus initiates a series of reactions including increased cell permeability and dilated intercellular spaces, cell damage and repair, inflammation and activation of pro-inflammatory transcription factors and genes(11). All of these reactions predispose the oesophagus to dysplasia and adenocarcinoma(12).

Shirvani et al in 2000 suggested that bile acids stimulate COX-2 expression(13) which is associated with chronic inflammation and epithelial cell growth in Barrett's oesophagus and adenocarcinoma(14). Jenkins et al shone light on another molecular target for bile acids in the oesophagus(15) when they reported that exposure of oesophageal mucosa to physiological levels of bile acids activates NF-kB, which has been implicated in carcinogenesis in numerous tissue types (16, 17).

While the role of acid reflux in gastro-oesophageal reflux disease has been well established the role of bile reflux, its incidence, its clinical impact and its role in the aetiology and pathogenesis of gastric and oesophageal mucosal injury have yet to be clearly established.

4.2 Aims

The aim of this study was to examine the factors contributing to reflux and its effects on the gastric and oesophageal mucosa in three cohorts of patients.

4.3 PATIENTS AND METHODS

4.3.1 Study Approval

This study was approved by the ethics committee of Connolly Hospital in Dublin and conducted in accordance with their guidelines. All participants gave their consent to participate in the study. No economic reimbursement was given for participation.

4.3.2 Study Groups

We prospectively recruited 150 consecutive patients who presented to the surgical endoscopy unit and belonged to one of three identifiable groups:

Group 1 (n = 98) were patients who presented for upper gastro-intestinal endoscopy for investigation of upper abdominal symptoms. None of these patients had Barrett's or adenocarcinoma of the oesophagus or had previous gastric or oesophageal surgery.

Group 2 (n = 20) were patients on surveillance for Barrett's oesophagus, and had never undergone gastric or oesophageal surgery.

Group 3 (n = 32) were patients on surveillance following oesophago-gastrectomy for carcinoma of the oesophagus.

4.3.3 Upper GI Endoscopy

Upper gastrointestinal endoscopy was performed on all patients by both the primary researcher, who had 16 training sessions with the supervisor before the study, and the supervisor (professor of GI surgery) in Connolly Hospital. In order to achieve endoscopic assessment reliability the two endoscopists had a consensus meeting and reviewed common macroscopic findings and standardized criteria for inflammation, Barrett's oesophagus, gastric and duodenal ulceration. Multiple biopsies, at least 4, were taken from the gastric antrum or the anastomotic site.

4.3.4 Histological Assessment

The biopsy specimens were oriented on filter paper and immediately fixed in formalin. Paraffin processed less than 5 μ M sections were cut at three levels, stained by haematoxylin and eosin and an additional section at the second level was stained with alcian blue, pH 2.5 and periodic acid Schiff (AB/PAS) to demonstrate intestinal metaplasia. A senior consultant histopathologist, who was blind to the clinical details of the patients and the endoscopy findings, assessed the biopsies for histological features of bile reflux in the gastric antrum or anastomotic site, which were calculated by using the BRI devised by Sobala et al(164). This has been explained in detail in chapter 3. A BRI above 14 indicates duodeno-gastric reflux (defined as bile acid level >1mmol/L, the upper limit of

physiological reflux). Patients deemed BRI (+) were bile refluxers and BRI (-) were non-bile refluxers.

4.3.5 Study Protocol

The groups were compared with respect to the following parameters: age, gender, BMI, history of smoking, use of proton pump inhibitors and previous history of cholecystectomy.

4.3.6 Statistical Analysis

P-values were all two tailed and the alpha level of significance was set at 0.05.

The prevalence is shown as percentage. Multiple comparisons between and within groups were performed using ANOVA with post-hoc correlations calculated with Dunnett's T3 test. Data was analyzed using SPSS version 18.

4.4 RESULTS

4.4.1 Bile Reflux Index

Across all patient groups, 69% (103/150) of patients had biopsies which were BRI (+) on histopathological analysis. The bile reflux index at the gastric antrum was positive in 63% (62) patients under endoscopy for upper GI (Group 1), a 2:1 ratio, in 75% (15) of patients undergoing Barrett's surveillance (Group 2), a 3:1 ratio, and in 81% (26) of patients undergoing endoscopy following oesophago-gastrectomy for oesophageal malignancy (Group 3), a 4:1 ratio. The ratio of BRI positive: BRI negative biopsy specimens in Group 3 was statistically-significantly higher when compared with Group 1 ($p=0.05$) but not when compared with group 2 ($p=0.441$) (Figure 16).

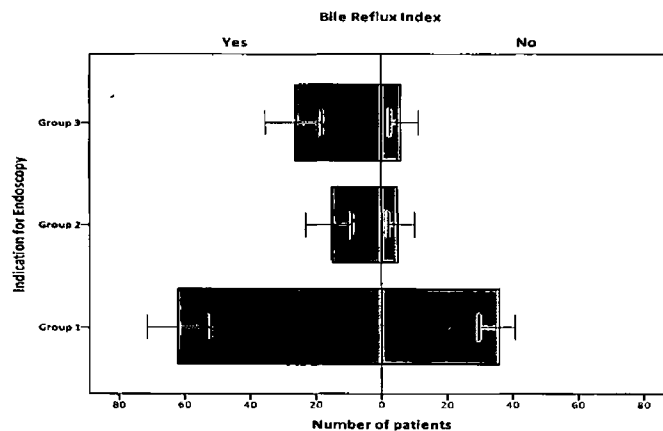


Figure 16 Incidence of Bile Reflux Index in three groups. Group1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett's surveillance. Group 3: Patients on surveillance post oesophago-gastrectomy.

4.4.2 BRI Positivity and Cholecystectomy

Thirty-seven of 150 patients studied (24.6%) were found to have had previously undergone cholecystectomy. Of these 37 patients, 76% (28) were found to be BRI (+) on endoscopic biopsy while in the 75.4% (113) of patients who had not previously undergone cholecystectomy, 66% (75) were BRI positive ($p=0.290$) (Figure 17). This gave an odds ratio of 1.37 for patients with a history of cholecystectomy to express BRI-positivity on biopsy, compared with patients who had never undergone cholecystectomy. The number of patients 'needed to be treated' with cholecystectomy in order to provoke bile-mediated cellular change (demonstrated as BRI-positivity), was thus calculated as 15.2.

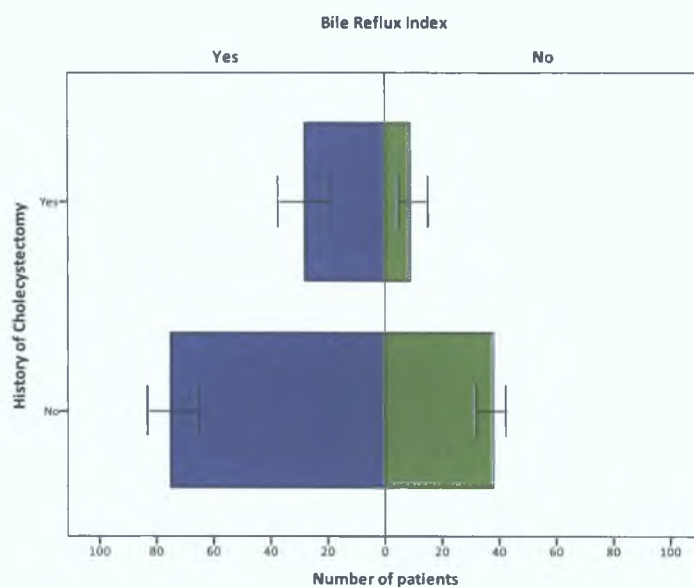


Figure 17 Overall history of cholecystectomy and incidence of Bile Reflux Index.

Notably, only 9% (3) of patients in Group 3 had a history of cholecystectomy and of these 67% (2) were bile refluxers ($p=0.497$). In stark contrast, in the symptomatic patients (Group 1), 30% (29) of patients had a previous history of cholecystectomy and of these 72% (21) were bile refluxers, while of the 70% who did not have cholecystectomy, just 59% were bile refluxers ($p=0.258$). Similarly, in Group 2, 25% (5) of Barrett's patients were post-cholecystectomy and of these 100% (5) were bile refluxers, while of the 75% who had not undergone cholecystectomy, 66% had bile reflux, a noteworthy trend despite not achieving statistical significance ($p=0.316$)(Figure 18).

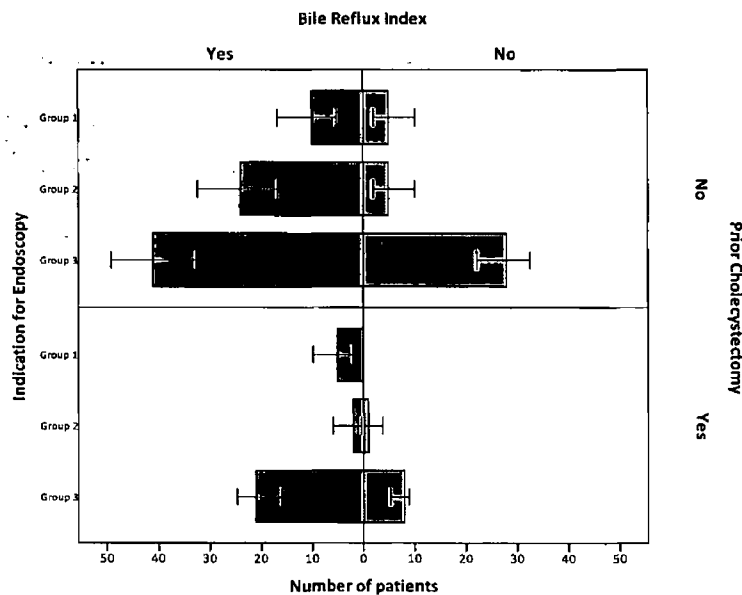


Figure 18 Incidence of bile reflux index in patients with history of cholecystectomy in the three groups. Group 1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett's surveillance. Group 3: Patients on surveillance post oesophago-gastrectomy.

4.4.3 Age

The mean age of patients in Group 1 was 56, Group 2 was 64 and Group 3 was 60 years; the mean patient age in Group 1 did not differ significantly from that of Group 3 ($p=0.589$), nor did Groups 2 and 3 differ ($p=0.628$). The mean age of patients with BRI+ biopsies was 58 years, compared with a mean of 57 years in BRI- patients ($p=0.607$) (Figure 19). The mean age of patients with a past surgical history of cholecystectomy was 57 years did not differ from the mean age of 60 years in those who had never undergone cholecystectomy ($p=0.763$).

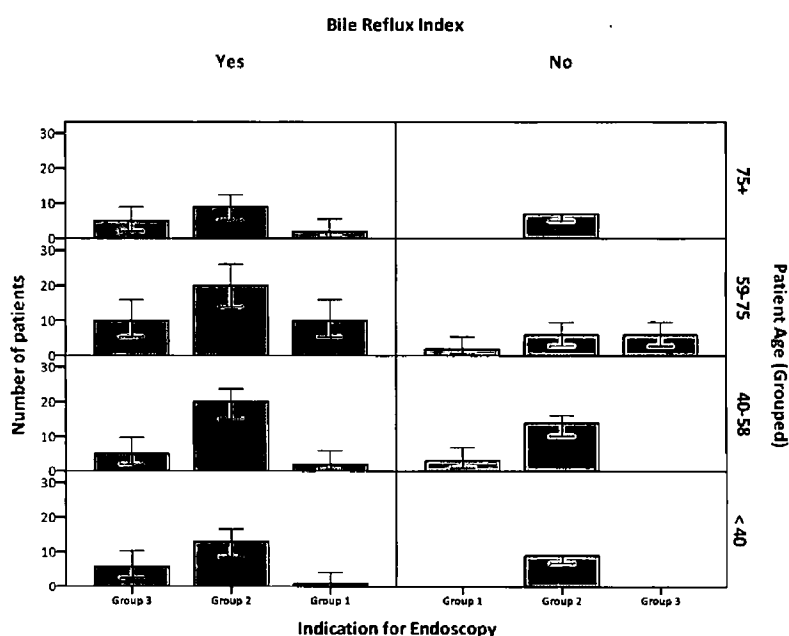


Figure 19 Age Distribution in three groups and incidence of Bile Reflux Index.

Group 1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett’s surveillance. Group 3: Patients on surveillance post oesophago-gastrectomy.

4.4.4 Gender

An almost-equal gender ratio existed across the entire study cohort, mimicking the general population, with 73/150 male patients (48.7%) and 77/150 female patients (51.3%). However, within groups, the male:female ratio varied greatly. There were 55% (11) males in Group 2, 78% (25) in Group 3 and 39% (37) in Group 1. Groups 2 and 3 had significantly more male patients as compared to Group 1 ($p=0.005$)(Figure 20).

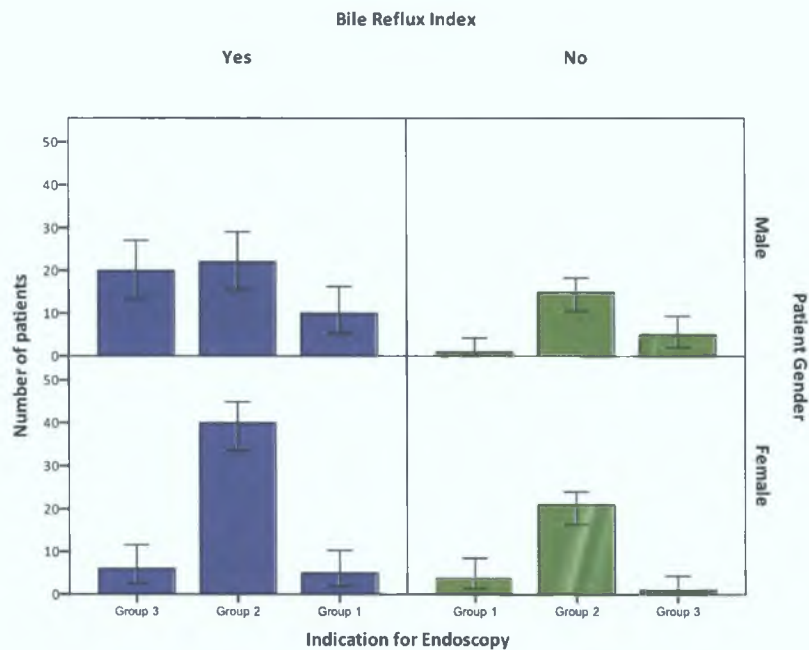


Figure 20 Gender Distribution in three groups and incidence of Bile Reflux Index. Group1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett’s surveillance. Group 3: Patients on surveillance post oesophago-gastrectomy.

4.4.5 Gender, Bile Reflux Index and Cholecystectomy

Despite a near-equal gender ratio, 27 of the 37 patients (73%) in our study who had previously undergone cholecystectomy were female, representing 35% of all female patients (27/77), compared with only 10/73 males (13.7%) ($p=0.002$).

Furthermore, of the 103/150 (68%) patients with BRI-positive biopsies, 28/103 (27%) of these patients had previously undergone cholecystectomy (accounting for 75% of all reported cholecystectomies), and of those, 21/28 (75%) were female ($p=0.002$). In contrast, among the 47/150 (32%) patients with BRI-negative biopsies, only 9/47 (19%) of these patients had previously undergone cholecystectomy (accounting for the remaining 25% of reported cholecystectomies), and of those, 6/9 (66%) were female.

4.4.6 BMI

There was no difference in BMI between groups ($p=0.359$). Group 1 had 85% (83), Group 2 had 95% (19) and Group 3 had 91% (29) patients with a BMI < 30 (Figure 21). Eight of the 37 patients (21%) who had previously undergone cholecystectomy were found to be obese, with a BMI greater than 30, compared with only 9.7% (11/103) of those who had never undergone cholecystectomy ($p=0.059$).

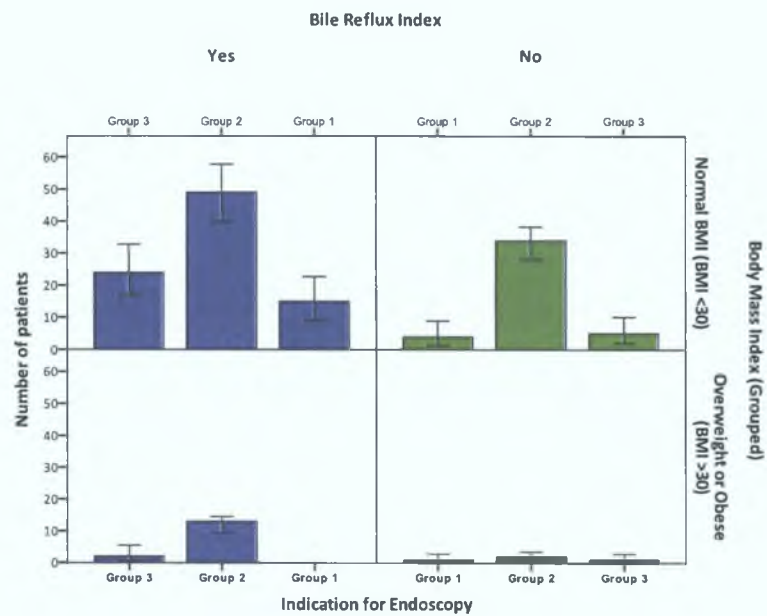


Figure 21 Body Mass Index (BMI) in three groups and incidence of Bile Reflux Index. Group1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett's surveillance. Group 3: Patients on surveillance post-oesophago-gastrectomy.

4.4.7 PPI Usage

One hundred and four of the 150 study patients (69%) were taking a proton-pump inhibitor at the time of study enrolment. In Group 1, 70% (69) patients were on PPI, 60% (12) in Group 2, and 72% (23) in Group 3, with no statistically-significant difference in the use of PPI observed between the groups ($p=0.650$). Within Group 2 (patients undergoing Barrett's surveillance), 91% (11/12) of all patients on PPI therapy had BRI-positive biopsies, while only 50% (4/8) of patients not on PPI therapy had BRI-positive biopsies. This correlation between chronic bile-mediated cellular change, as demonstrated by BRI-positivity, and the requirement to take a PPI was found to be statistically significant ($p=0.035$).

While not achieving statistical significance, a similar trend was noted amongst patients undergoing surveillance following oesophagogastrectomy for oesophageal malignancy (Group 3). In these patients, 87% (20/23) of those taking a PPI had biopsy-demonstrated BRI-positivity, compared with BRI-positivity in only 66% (6/9) of patients not on PPI therapy ($p=0.186$) (Figure 22).

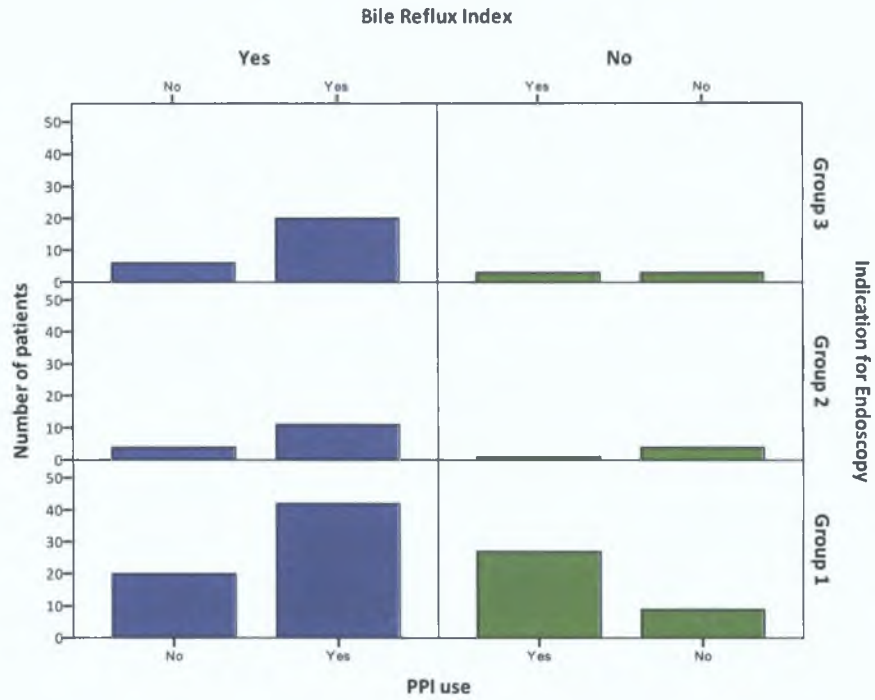


Figure 22 Use of Proton-Pump Inhibitors (PPI) in three groups and incidence of Bile Reflux Index. Group1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett’s surveillance. Group 3: Patients on surveillance post-oesophago-gastrectomy.

4.4.8 Smoking

In Group 1, 44% (43) patients had a history of smoking compared with 55% (11) in Group 2 and 34% (11) in Group 3. Smoking rates were not statistically significantly different between the groups ($p=0.143$) (Figure 23).

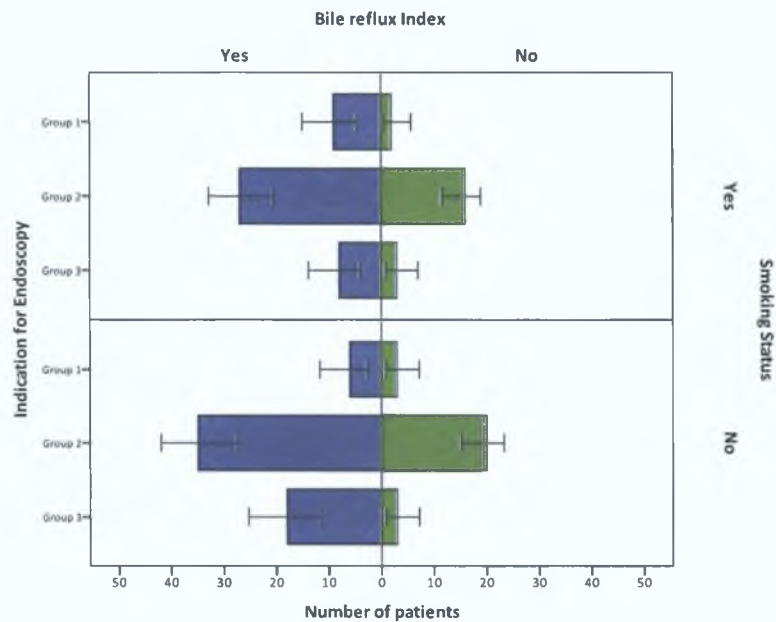


Figure 23 History of smoking in three groups and incidence of Bile Reflux Index.

Group 1: Patients with upper abdominal symptoms. Group 2: Patients on Barrett's surveillance. Group 3: Patients on surveillance post oesophago-gastrectomy.

4.5 DISCUSSION

In this study we based our definition of duodeno-gastric reflux on the bile reflux index BRI (164) as a histological marker for injury inflicted by bile on the mucosa rather than attempting to measure the refluxed bile using techniques such as aspiration(264) or 24-hr Bilitec® monitoring(265). We felt that for this study a metric of the direct impact of bile on the mucosa was preferable to the mere measurement of bilirubin, on which the Bilitec is based. We have previously published on the deficiencies noted in the use of the Bilitec® (266, 267) which is currently the most commonly used methods for monitoring bilirubin levels. The Bilitec® is useful in determining the percentage time that bilirubin is refluxing into an empty stomach during the monitoring period. It has its limitations, however, as it's *in-vivo* sensitivity is not as good as its *in-vitro* sensitivity(268), it is not reliable for monitoring the amount and concentration of bile in the stomach(269) and it is convincingly argued that as bilirubin is not the toxic agent its assessment may be a poor surrogate of the impact of bile reflux on the gastric and oesophageal mucosa (270). Bilirubin absorbance is also affected by diet(271). Foods with an absorbance between 400-450 nm can result in false positive results (e.g. coffee, coke, carrot, tomato etc)(271). In clinical practice what is needed is either an accurate measurement of the duration of exposure to the toxic agent or agents in the duodeno-gastric refluxate or information on the pathological changes produced in the mucosa and the molecular marker disturbances. Since we are unclear as to the exact nature of the toxic agent(s),

and since some are thought to be volatile and unstable, neither the Bilitec® nor aspiration studies have been shown to be superior to the histologically derived bile reflux index which has an acceptable 70% sensitivity and 85% specificity (164). Considering all the limitations to *in vivo* and *in vitro* monitoring of bile, we preferred the BRI as a histological marker for injury inflicted by bile on the gastric mucosa.

Across all 150 consecutive patients 69% had positive bile reflux index on the histopathological analysis of biopsies at the gastric antrum. Increase in bile reflux was noted in patients on surveillance for Barrett's oesophagus when compared to the patients who presented for investigations of upper abdominal symptoms. A statistically significant surge occurred in bile reflux index in patients who were post oesophago-gastrectomy when compared with controls ($p=0.05$), consequent upon the mechanical alterations to upper gastro-intestinal anatomy which facilitates DGOR.

Both duodeno-gastric reflux(272) and gastro-oesophageal reflux are well described in patients who undergo oesophago-gastrectomy followed by gastric tube reconstruction (273). This is attributable to the alteration in the dynamics of bile flow as a result of vagotomy (274) and the anatomical distortion

subsequent to partial gastrectomy(275). The disturbance of the function consequent upon vagotomy probably impairs the ability of the antro-pyloric mechanism to prevent duodeno-gastric reflux(96). There is a considerable body of literature about the consequences of surgical destruction of the pylorus after distal gastrectomy(276) or pyloroplasty(277) which probably result in increased duodeno-gastro-oesophageal reflux due to mechanical disruption of the pylorus(278). But as none of the post-oesophagectomy patients in our study had a pyloroplasty the cause of the bile reflux may reflect the effect of vagotomy on the function of the antro-pyloric unit and on gallbladder contractile function. We have previously shown the patients with oesophageal carcinoma have impaired gallbladder function and this may have had an aetiological role in the development of carcinoma in this cohort(4).

The development of gastric remnant carcinoma as a result of bile reflux after gastric resection has also been well documented (273). Yumiba et al studied 30 patients that had undergone total gastrectomy with 24hr oesophageal pH and Bilitec[®] monitoring and found that even in the absence of gastric acid, individuals whose oesophagus was exposed to bile were more likely to suffer from reflux oesophagitis(279). Marshall et al demonstrated that duodeno-gastro-oesophageal reflux was associated with erosive oesophagitis after partial or total gastrectomy(280). Several recent studies have shown that severe reflux oesophagitis or Barrett's oesophagus can occur after total gastrectomy(281).

Roux-en-Y anastomosis diverts the bile and therefore duodeno-gastro-oesophageal reflux after distal gastrectomy is noted to be less frequent in these patients than in patients with Billroth 1(gastro-duodenostomy) or Billroth 2 (gastro-jejunostomy) reconstruction(282).

Augmentation of bile reflux was noted in patients who had previous history of cholecystectomy (76% BRI+) when compared with the patients who had no history of cholecystectomy (66% BRI+). Conspicuously 30% of patients who presented for investigations of upper abdominal symptoms had a previous history of cholecystectomy. Strikingly 72% of these patients had BRI positivity in antral biopsies. This was a noteworthy discovery which revealed that a high proportion of still-symptomatic patients were post-cholecystectomy and they had increased bile reflux index. This, however, suggests that the symptoms of these patients were not biliary in origin, or that their biliary problem was unsuccessfully addressed or that new symptoms emerged as a consequence of cholecystectomy. A further explanation is that this cohort of patients developed new symptoms as a result of cholecystectomy induced bile reflux. The "post-cholecystectomy syndrome" was a term coined to describe these symptoms(283) and it is best defined as gastrointestinal symptoms that develop subsequent to or persist despite cholecystectomy. Post-operative symptoms include abdominal pain, nausea, distension, bloating, belching and heartburn(92). The mechanism of this possible association is unclear, but may

be related to a variety of processes involving impaired gastric motility(284, 285), lower oesophageal sphincter relaxations(286), increased frequency of pyloric relaxations(94) with subsequent pyloric incompetence (287-289) or loss of reservoir function of the gallbladder in patients resulting in duodeno-gastric reflux following cholecystectomy(290). There is much experimental and clinical evidence that gallbladder disease and cholecystectomy can increase duodeno-gastric reflux(290, 291). Previous studies have suggested that gastro-oesophageal reflux worsens after cholecystectomy (80, 90, 292-295). Bhat et al (ref) used Tc-99m mebrofenin hepatobiliary scanning to measure duodeno-gastric reflux prior to cholecystectomy and post-cholecystectomy. There was significantly increased duodeno-gastric reflux in patients after cholecystectomy increasing from 23.3 percent preoperatively to 46.6 percent postoperatively(296).

Cholecystectomy is the most commonly performed surgical procedure. Approximately 500,000 people in USA undergo cholecystectomy every year(297). We know that cholecystectomy increases DGOR. Our results revealed an odds ratio of 1.37, which means 37% patients with a previous history of cholecystectomy, will express BRI positivity on biopsy compared with patients who had never undergone cholecystectomy. This translates into the number of patients 'needed to be treated' with cholecystectomy in order to provoke bile-mediated cellular damage demonstrated as BRI positive as 15.2.

Extrapolating that to the 500,000 cholecystectomies performed in USA, we can estimate that of these, approximately 33,000 patients may have iatrogenic bile induced mucosal injury to the gastric and oesophageal mucosa bringing them to risk of Barrett's oesophagus and oesophageal adenocarcinoma.

The mean age did not differ in patients with upper abdominal symptoms, patients on Barrett's surveillance and patients post-oesophago-gastrectomy. There was no difference in age with respect to bile reflux. The mean age of the post-cholecystectomy patients was 57 years which was similar to those who had no previous history of cholecystectomy. The mean age of patients with Barrett's oesophagus was 64 years compared with 60 years in the post oesophago-gastrectomy group. This is consistent with previous literature and Cameron et al have suggested that prevalence of Barrett's begins only after the age of 50 years(298).

An almost-equal gender ratio existed across the entire study cohort, mimicking the general population. However, within groups there was great variance noted with respect to patient gender. There were statistically-significantly more males noted amongst patients with Barrett's oesophagus and patients post oesophago-gastrectomy. The gender effect in these two groups may be due to differences in parietal cell mass between males and females(299) or may also

be due to the action of testosterone (300). As expected when looked at the patients who had a previous history of cholecystectomy, 73% were females. The link of female gender and gallstone disease has been extensively reported in literature and recently revalidated as well(301).

There was no difference in BMI within the groups but, as predicted, patients post-cholecystectomy had a significantly greater BMI as compared to those who had never undergone cholecystectomy ($p=0.059$). We already know that increase in BMI is a risk factor for the development of gallstone disease(301).

Proton pump inhibitors are prescribed, in general, to symptomatic patients. In our study we found that 70% of patients who presented for investigations of upper abdominal symptoms were on PPI therapy and 61% of these were BRI positive compared with 30% who were not on PPIs and only 30% of these were BRI positive. We noticed that a significant number of patients had persistent upper abdominal symptoms despite being on PPI therapy when they presented for investigations of their symptoms. A study carried out in our lab by Nasr et al found that gastric acid suppression, in the presence of duodenal refluxate, was associated with increased rates of chronic inflammatory changes, intestinal metaplasia and molecular proliferative activity in a rodent model(302). Another study showed that there is a further potential danger with this

approach in patients with bile reflux, as continuous medication with proton pump inhibitors may lead to deconjugation of bile acids at a neutral pH which may become more harmful (303). This may suggest that while PPI's can suppress the acidic element contributing to their symptoms, bile and its toxic effects persist. This became even clearer when we looked at our groups individually. Patients in group 2 who were on surveillance for Barrett's oesophagus had increased BRI and increased incidence of PPI use was noted in these patients as well. The presence of increased DGOR in Barrett's patients taking a PPI suggests a possible role for DGOR both in the pathogenesis of Barrett's and in the symptoms of dyspepsia. It is clear that bile plays an important role in the pathogenesis of gastric and oesophageal pathology.

It is concerning the way post-cholecystectomy patients are predisposed to this iatrogenic injury induced by bile reflux. Provoked by this we designed our next study on the effects of cholecystectomy on gastric and oesophageal mucosa at molecular and histological level.

CHAPTER 5

STUDY – 2

Effects of Cholecystectomy on the Gastric And Oesophageal Microenvironment

ABSTRACT

Background: Cholecystectomy, the definitive treatment for gallstone disease, removes the bile reservoir and may critically alter the dynamics of bile storage and release.

Aims: To examine the effects of cholecystectomy on the internal micro-environment of the upper gastrointestinal tract at a histological and molecular level.

Patients and Methods: Group 1(n=26) were gallstone-free controls. Group 2(n=25) were pre-cholecystectomy patients and Group 3(n=29) were patients who had undergone cholecystectomy over 1 year previously. All patients underwent oesophago-gastro-duodenal (OGD) endoscopy and had biopsies of the gastric antrum, oesophago-gastric junction (OGJ) and 5cm above the OGJ. The bile reflux index (BRI) was calculated and immunohistochemistry performed for p53 and Ki-67.

Results: The positive BRI rate at the gastric antrum was 11% in Group 1, 20% in Group 2 and 69% in Group 3 (Group 1 v Group 3, $p=0.001$); at the OGJ it was 19% in Group 1, 12% in Group 2 ($p=0.708$) and 41% in Group 3 (Group 3 vs Group 1, $p=0.032$). At 5cm above OGJ there was no difference between groups($p=0.258$). Expression of p53 was higher at all sites in Groups 2 and 3 compared to Group1 ($p=0.001$); Ki-67 was greater in Group 3 than Group 1 at all three sites ($p=0.001$).

Conclusions: Increase in BRI in post-cholecystectomy patients reflects inflammatory change induced by bile reflux. Increased expression of Ki-67 and p53 in post-cholecystectomy patients raises concerns about development of premalignant changes in these patients and suggests that options other than cholecystectomy should be considered in patients with gallstones in functioning gallbladder.

5.1 INTRODUCTION

Cholecystectomy has remained the definitive treatment for gallstone disease for over 100 years. Since the introduction of the laparoscopic approach; surgeons and patients have lowered the threshold for proceeding to cholecystectomy(304) so that approximately 500,000 cholecystectomies are performed each year in the USA and up to 50,000 in the UK(305). In Ireland the overall cholecystectomy rate has continued to rise with laparoscopic cholecystectomy rates rising by 45% according to a study performed in 60 Irish public hospitals over a period of seven years(304).

But cholecystectomy is not innocuous. As it removes the bile reservoir it may cause a dramatic alteration in the internal milieu by compromise of bile storage and release during the inter-digestive period (90). Bile is normally stored in the gallbladder during the interdigestive period, to be expelled in response to meal stimulated CCK secretion from the "I" cells in the proximal duodenum. CCK release is in turn switched off by the negative feedback effect of the bile bolus in the duodenum(4). Thus, bile is mixed with food in the duodenum and little is free to enter the stomach or available for contact with the oesophageal mucosa (306, 307).

This orderly sequence of bile storage and release is dramatically disturbed by cholecystectomy (90, 308-310). When the gallbladder is removed the facility for

storage between meals is destroyed and bile flows continuously into the duodenum promoting retrograde reflux into the stomach (311-313) leading to duodeno-gastric reflux. Furthermore, as no bile bolus enters the duodenum there is no mechanism for switching off CCK secretion. CCK levels remain elevated after meals(2) and CCK may further contribute by reducing the lower oesophageal pressure and altered cardia function(314-317) facilitating gastro-oesophageal reflux. The exaggerated CCK response is directly involved in transient lower oesophageal sphincter relaxations, further facilitating duodeno-gastro-oesophageal reflux(318).

Clinical and epidemiological data suggest an association between cholecystectomy and augmented bile reflux(319). Duodeno-gastric reflux is common in patients with previous cholecystectomy (320, 321). Lorusso et al (322) showed increased duodeno-gastric reflux of bile acids after cholecystectomy with a mild morphologic alteration of the gastric mucosa. This resulted in an elevation of gastric pH in post-cholecystectomy patients, which in turn resulted in increased parietal cell density and increased gastrin levels(322). Cholecystectomy has appeared to be a critical factor in the pathogenesis of bile gastritis in patients who have not had prior gastric surgery(85).

Freedman et al(309) suggested an association between cholecystectomy and an increased risk of adenocarcinoma of the oesophagus, possibly due to the toxic

effect of refluxed bile on oesophageal mucosa. Over the last three decades the incidence of oesophageal adenocarcinoma has increased 463% among white males and 335% among white women(323). The rate of this increase is far greater than any other epithelial malignancy(324). Since the reasons for this change in incidence have not been fully explained and the 5 year overall survival for symptomatic adenocarcinoma remains less than 15%(325), there is great pathological concern about the understanding of this rise.

Recent advances in immunohistochemistry have enabled better characterization of premalignant conditions of the oesophagus and stomach, by detecting different proteins related to apoptosis and proliferation like transcription factor p53 which plays a major role in preventing tumour development(213). It responds to a range of potentially oncogenic stresses by activating protective mechanisms, most notably cell cycle arrest and apoptosis (212). Ki-67 expression is associated with cellular proliferation and the active phases of the cell cycle(238). Abnormally persistent expression of Ki-67 is associated with premalignant dysplasia in oesophageal squamous epithelium and Barrett's mucosa (242).

In Study 1 we found that a clinically significant number of patients (30%) being investigated for upper GI symptoms had a previous history of cholecystectomy.

We also found that patients with previous history of cholecystectomy had increased bile reflux index suggesting that cholecystectomy increases pathologic bile reflux leading to changes at histological and molecular level. The aim of this retrospective study was to examine the effects of cholecystectomy on the internal milieu of the upper gastrointestinal tract, at histological and molecular level.

5.2 PATIENTS AND METHODS

5.2.1 Study Groups

Three groups of patients were recruited for this study:

Group 1: The Control Group (n = 26) were patients who presented with upper abdominal pain and required upper GI endoscopy. None had symptoms of gastro-oesophageal reflux disease and all had absence of gallstone disease confirmed by ultrasound scan.

Group 2: The Pre-cholecystectomy Group (n = 25) were patients who had a confirmed diagnosis of gallstones and were awaiting cholecystectomy. Upper GI endoscopy was carried out as a part of pre-operative investigations.

Group 3: The Post-cholecystectomy Group (n = 29) were patients that had undergone cholecystectomy at least one year previously.

5.2.2 Exclusion Criteria

Participants with a previous history of surgery either oesophageal, gastric, duodenal, hepatic or pancreatic surgery were excluded; patients with a history of carcinoma such as gastric and oesophageal adenocarcinoma, or with an oesophageal stent in situ, or who were receiving or had received chemotherapy or radiotherapy were also excluded; patients with choledocholithiasis, surgical obstructive jaundice, cholangitis, known peptic ulcer disease or biliary tract

disease other than cholelithiasis were excluded from the study; participants with conditions known to affect gallbladder motility, including patients with diabetes or chronic liver disease were excluded; and finally patients being prescribed pharmacological agents known to affect acid secretion or gastrointestinal or gallbladder motility were excluded.

5.2.3 Patient Information and Consent

The study was discussed with all patients recruited. An information booklet was given to all patients who had the opportunity to ask questions related to this study. They were aware that they had the right to withdraw from the study without obligation, or compromise to their treatment. Patients were reassured that their individual privacy would be maintained in all published and written material resulting from this study. Informed consent was signed by each patient prior to recruitment to the study (Appendix 1 and 2).

5.2.4 OGD And Collection Of Biopsy Samples

Upper gastrointestinal endoscopy was performed on all patients by the primary researcher. Multiple biopsies, at least 4, were taken from gastric antrum, oesophago-gastric junction and 5cm above the oesophago-gastric junction. The biopsy specimens were oriented on filter paper and immediately fixed in formalin.

5.2.5 Tissue Processing and Staining

The Bile Reflux Index was obtained by histological examination of sections of the specimens that were stained with haematoxylin and eosin (H&E) as described in Chapter 3. Briefly, the haematoxylin stains nuclei a dark blue or navy colour and eosin stains cytoplasm pink. The slides with tissue slices 4µm thin were placed into the Tissue Tek® Prisma™ automated slide stainer for staining. The machine has oven which melts the wax at 63°C and then the remaining wax is melted chemically with xylene. It then immerses the baskets of slides into solution reservoirs of ethanol advancing from higher grade to lower finally into a reservoir of water. The process of staining for haematoxylin is regressive staining. The slides are placed in a reservoir of haematoxylin and then into a 1% hydrochloric acid solution, which removes all of the staining except at the nuclei. Staining for eosin is then performed by initially immersing the slides in 95% ethanol and then eosin.

The slides then proceed to the Tissue Tek® Glass coverslipper for automated coverslipping. The coverslips form a secure bond with the slide by applying the Thermo Scientific Shandon Consul-Mont, a xylene based glue. The coverslipped slides are then dried in the machine by two drying fans above and below the slides. The dried slides are then ready for examination under light microscopy.

5.2.6 Bile Reflux Index (BRI)

A senior consultant histopathologist, who was blind to the clinical details of the patients and the endoscopy findings, assessed the biopsies for histological features of bile reflux in the gastric antrum and oesophago-gastric junction (OGJ) which were calculated by using the Bile Reflux Index (BRI) devised by Sobala et al(164). This has been explained in detail in the chapter 3. A BRI above 14 indicates duodeno-gastric reflux (defined as bile acid level >1mmol/L, the upper limit of physiological reflux). The biopsies from 5cm above the OGJ were assessed for bile reflux by grading 1-3 according to the basal cell layer proliferation in the squamous oesophageal mucosa and also by the height of the papillae within the epithelium.

5.2.7 Immunohistochemistry

The details of this procedure have been described in the chapter 3. Briefly it consisted of fixing, embedding, cutting, mounting, deparaffinisation, rehydration, staining, counterstaining, dehydrating and stabilizing with mounting medium and viewing the staining under the microscope. We used this method for demonstrating the presence and location of p53, and Ki-67 proteins in tissue sections at the gastric antrum, oesophago-gastric junction and 5cm above the oesophago-gastric junction.

5.2.8 Statistical Analysis

P-values were all two tailed and the alpha level of significance was set at 0.05.

The prevalence is shown as a percentage. Chi-2 test was using for testing comparison in univariate analysis. ANOVA was used for multivariate analysis.

Post hoc correlations were calculated with Dunett's T3 test. Data was analyzed using SPSS version 18.

5.2.9 Ethics approval

The certificate of approval for this study was granted before enrolment by the Research Ethics Committee, Connolly Hospital, Blanchardstown, Dublin. All participants gave their consent to participate in the study. No economic reimbursement was given for participation.

5.3 RESULTS

5.3.1 Patient Demographics

The mean age (SEM) across all patient groups was 57.3(1.5) years and there was no significant difference ($p=0.626$) between the cohorts. The mean age of patients was 58(1.7) years in Group 1, 54.2(2.6) years in Group 2 and 59.2(3.1) years in Group 3.

There were significantly fewer female patients in the control group than in the pre- and post- cholecystectomy groups ($p=0.005$). In Group 1, 35% of patients were female compared with 76% in Group 2 and 69% in Group 3.

The mean (SEM) BMI across all groups was 27.8 (0.54). The mean BMI of patients was 26.8(4.3) in Group 1, 29.1(4.5) in Group 2 and 28(5.4) in Group 3. There was no significant difference in BMI between or within groups ($p = 0.706$). Patients were further divided into normal (BMI < 25), overweight (BMI 25-30) and obese (BMI >30), to increase the statistical power. The distribution of normal BMI patients between the groups was 39% in Group 1, 20% in Group 2 and 31% in Group 3 ($p=0.532$). Of overweight patients there were 16 (62%) in Group 1, 20 (80%) in Group 2 and 20 (69%) in Group 3 ($p=0.918$). Of obese patients there were 4(15%) in Group 1, 7(28%) patients in Group 2 and 6(21%) in Group 3 ($p=0.687$).

5.3.2 Bile Reflux Index (BRI)

Gastric Antrum

When biopsies of the gastric antrum that had a positive BRI were compared there were 11% (3) in Group 1, 20% (5) in Group 2 and 69% (20) in Group 3 (p=0.001)(Figure 24).

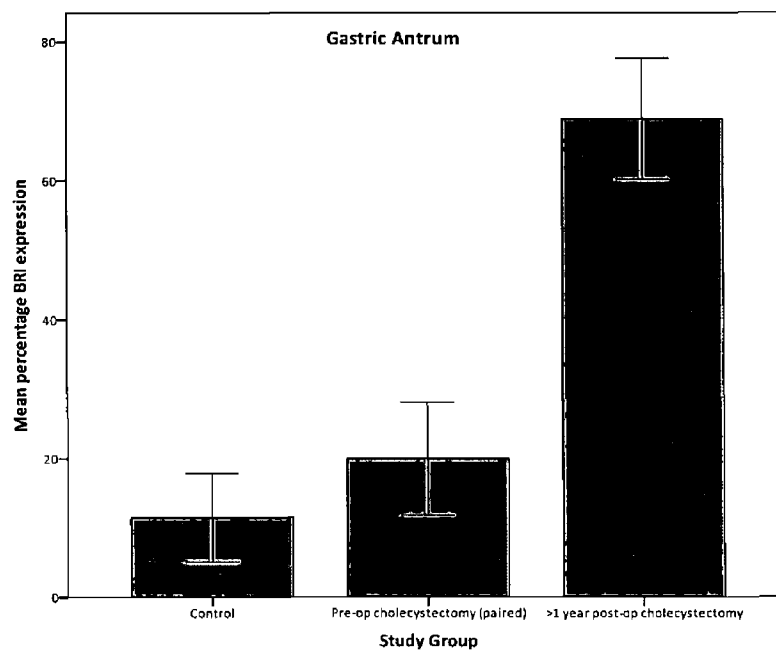


Figure 24 Positive Bile Reflux Index (BRI) at the gastric antrum in three groups.

Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group

3: Patients with history of cholecystectomy more than a year previously.

Oesophago-gastric Junction (OGJ)

When biopsies of the OGJ were compared there were 19% (5) of patients with a positive BRI in Group 1, 12% (3) in Group 2 and 41% (12) in Group 3. The BRI positivity in Group 3 was significantly higher than Group 1 ($p=0.032$) while Group 2 did not differ significantly from Group 1 ($p=0.708$)(Figure 25).

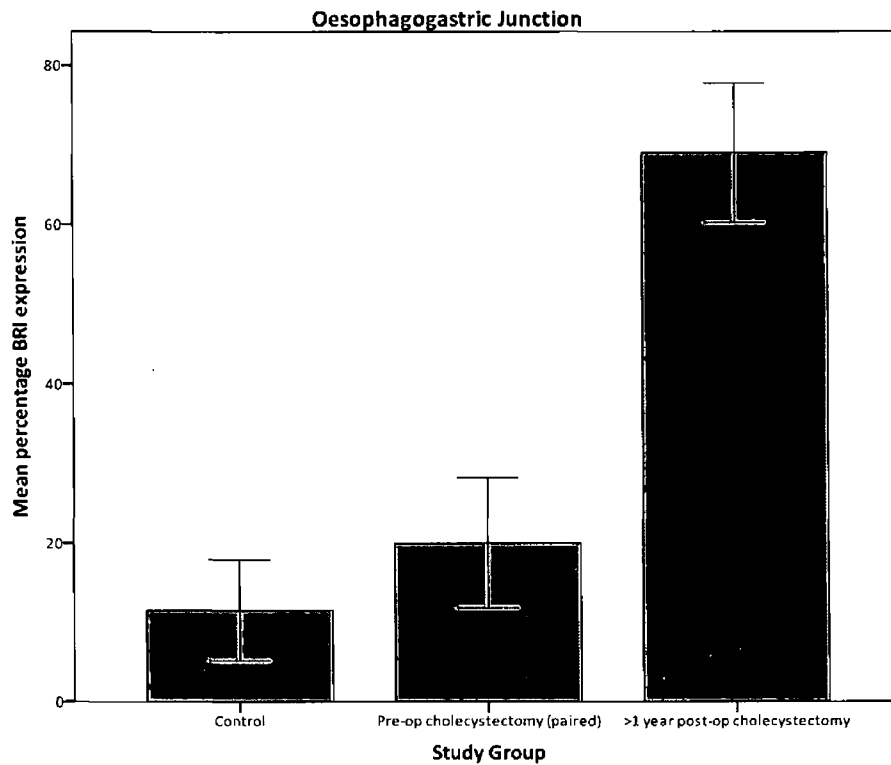


Figure 25 Positive Bile Reflux Index (BRI) at the oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

Changes 5cm Above OGJ

When biopsies taken 5cm above the OG junction were compared only 8% (2) of patients in Group 1, were positive for BRI, compared with 24% (6) in Group 2 and 14% (4) patients Group 3. There was no difference between the groups ($p=0.258$)(Figure 26).

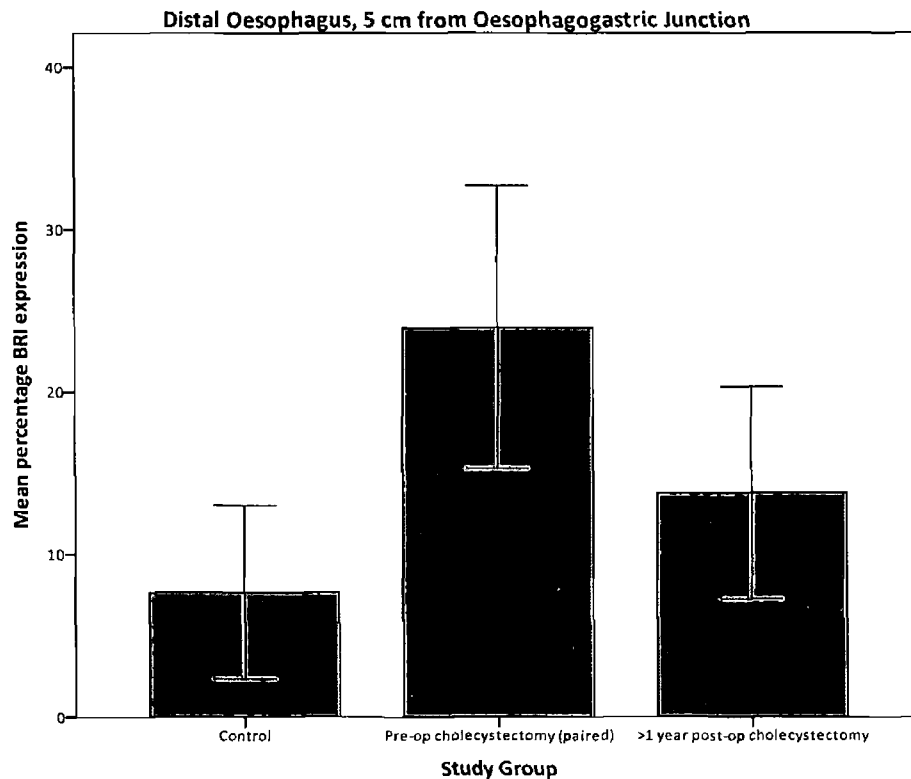


Figure 26 Positive Bile Reflux Index (BRI) at 5 cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

5.3.3 Expression of p53

Gastric Antrum

p53 was expressed in only 4% (1) of Group 1 subjects compared with 44% (11) of Group 2 and 52% (15) of patients in Group 3. Expression of p53 was significantly higher in the gastric antrum in Groups 2 and 3 compared to Group 1 ($p=0.001$) (Figure 27).

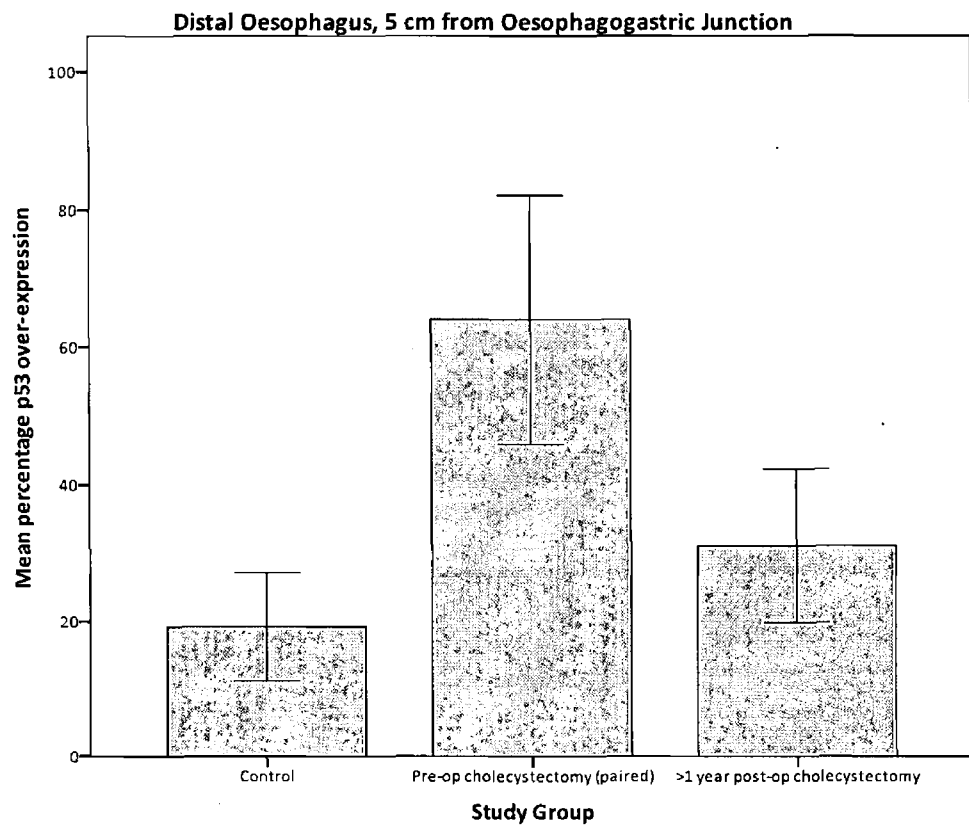


Figure 27 Expression of p53 protein at gastric antrum in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

Oesophago-Gastric Junction (OGJ)

p53 expression was significantly higher at the OGJ in Groups 2 and 3 compared to Group 1 ($p=0.001$). Expression of p53 increased from 19% (5) in Group 1, to 48% (12) in Group 2 and 66% (19) in Group 3 (Figure 28).

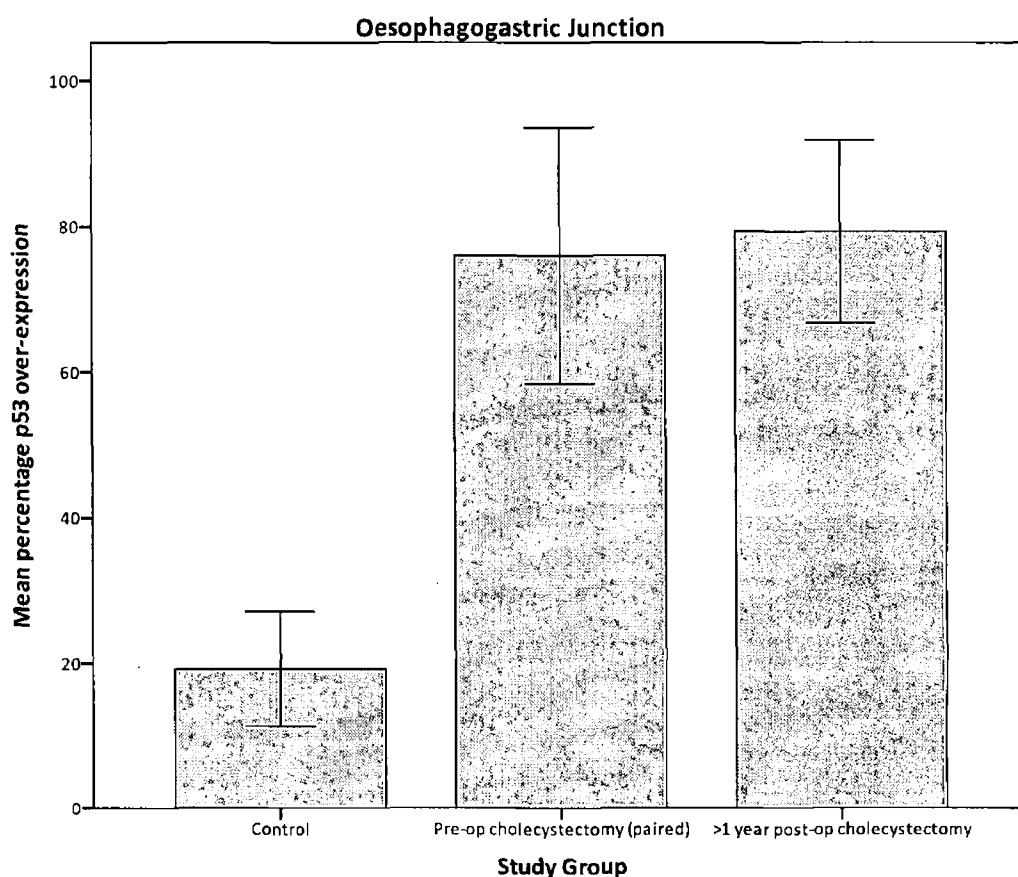


Figure 28 Expression of p53 protein at oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

Oesophagus 5cm above OGJ

Expression of p53 occurred in 19% (5) of Group 1 patients compared with 36% (9) patients in Group 2 and 24% (7) patients in Group 3. Group1 did not differ from any other group ($p = 0.363, 0.999, 0.945$ respectively). There were no other significant interactions within or between groups. (Figure 29).

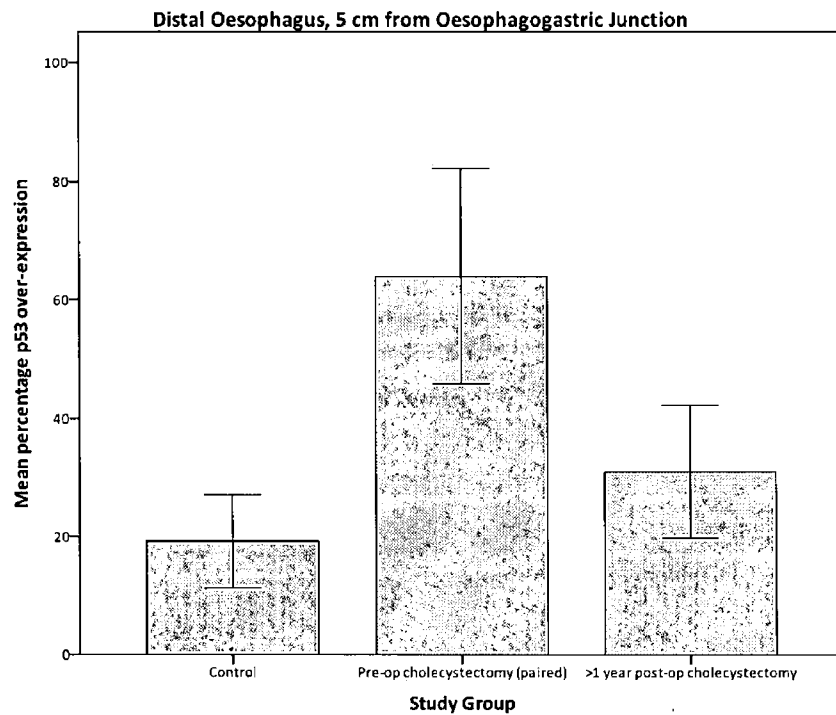


Figure 29 Expression of p53 protein at 5cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

5.3.4 Expression of Ki-67

Gastric Antrum

Expression of Ki-67 at the gastric antrum was significantly higher in Group 3 as compared to Groups 1 and 2 ($p = 0.001$). Ki-67 expression was positive in 23% (6) of Group 1 patients compared with 4% (1) of Group 2 and 59% (17) of Group 3 patients (Figure 30).

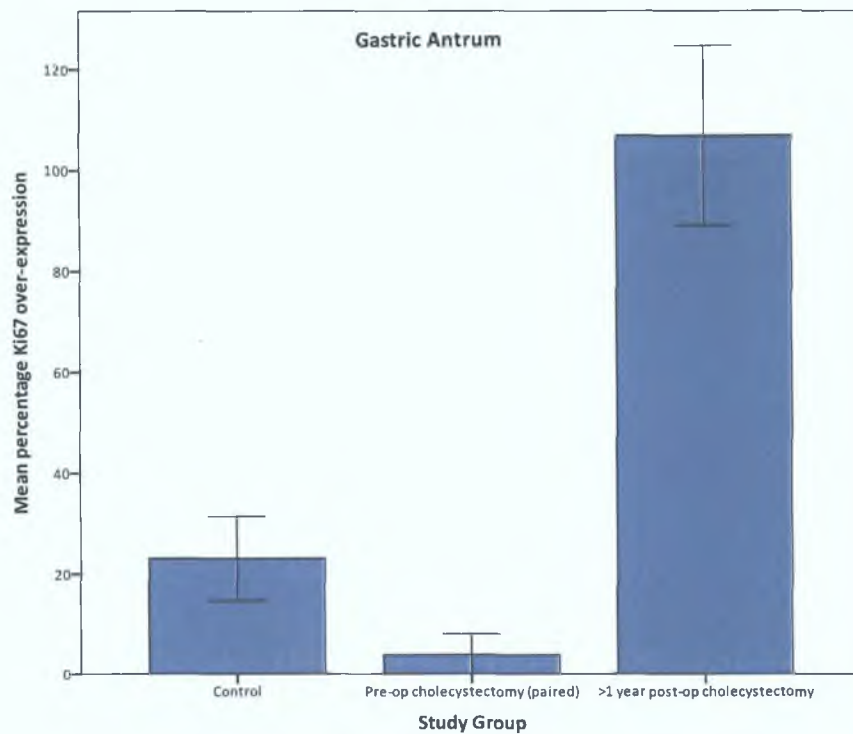


Figure 30 Expression of Ki-67 at gastric antrum in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

Oesophago-gastric Junction (OGJ)

Ki-67 expression was significantly higher at the OGJ in Group 3 compared to Groups 1 and 2 ($p = 0.001$). Ki-67 expression was positive in 19% (5) of Group 1 patients compared with 12% (3) Group 2 patients and 62% (18) Group 3 patients (Figure 31).

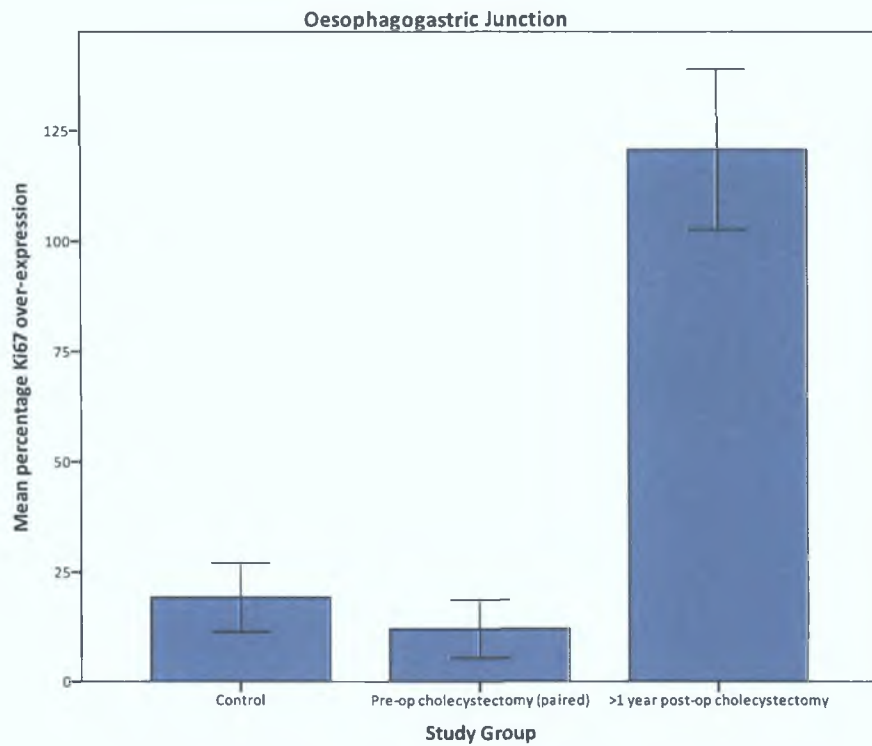


Figure 31 Expression of Ki-67 at oesophago-gastric junction in three groups.

Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group

3: Patients with history of cholecystectomy more than a year previously.

Oesophagus 5cm above OGJ

Ki-67 expression was positive in 12% (3) Group 1 patients compared to 4% (1) Group 2 and 69% (20) in Group 3. Ki-67 expression was significantly higher 5cm above the OGJ in Group 3 compared to Groups 1 and 2 ($p = 0.001$). (Figure 32).

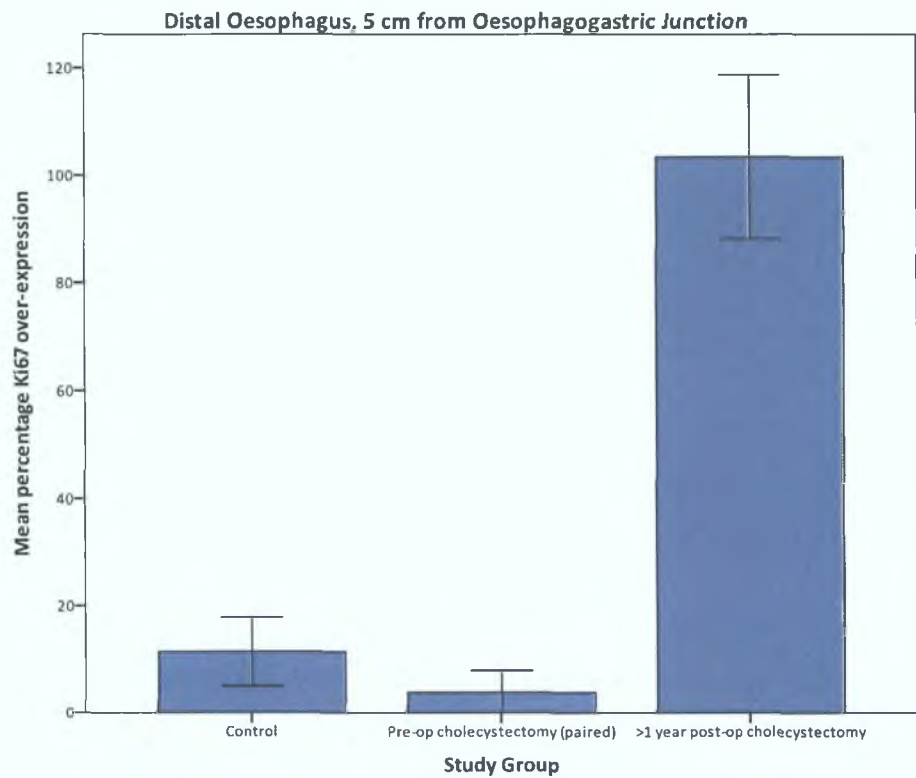


Figure 32 Expression of Ki-67 at 5 cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy more than a year previously.

5.4 DISCUSSION

In this study we examined the changes to the gastric antral mucosa, the OGJ mucosa and the mucosa 5 cm above the OGJ caused by cholecystectomy using histological change and molecular markers.

This study on patients with a remote history of cholecystectomy found that the bile reflux index was increased at the gastric antrum and oesophago-gastric junction in post-cholecystectomy patients compared with patients who were pre-cholecystectomy and with controls. This was associated with a highly significant increase in Ki-67 expression between controls and post-cholecystectomy patients at all three sites. Ki-67, a marker of cellular proliferation(326-330), is present only in the mitotic phases of cell cycle(237) and is an indicator of regenerating and reactive epithelium. The expression was marked at the antrum and OGJ and at 5cm above the OGJ. p53 expression was also increased in patients with a history of cholecystectomy at the gastric antrum and oesophago-gastric junction. The p53 protein plays a major role in preventing tumour development and is a known tumour suppressor protein(213). Higher expressions of p53 indicate apoptosis and cell death (212). The expression of both molecular markers is consistent with the distribution of refluxed bile.

Patients with duodeno-gastro-oesophageal reflux post-cholecystectomy have increased bile acids in the stomach and in the oesophagus (319). Different theories have been presented on the detrimental effects of bile acids and salts and their pathophysiological mechanisms. Toxic effects may be mediated through cyclooxygenase 2 and it has been found that blocking this enzyme decreases cancer growth *ex-vivo*(331). Bile acids have also been shown to disrupt intra and extra-cellular membranes and junctions(9). Duodenal refluxate has not only been found to induce adenocarcinoma of the oesophagus in an animal model(332, 333) but also in patients who develop adenocarcinoma arising from metaplastic epithelium(190).

Cholecystectomy has been previously associated with increased risk of adenocarcinoma of the oesophagus by toxic effects of duodenal refluxate(334). This is the first study to identify increased expression of the molecular markers p53 and Ki-67 in post-cholecystectomy patients and demonstrate a possible association between duodeno-gastro-oesophageal reflux and the early stages of carcinogenesis. Ki-67 over-expression has been suggested to have a potential value in identifying patients at increased risk of progression towards high grade dysplasia and oesophageal adenocarcinoma(231). The positivity of Ki-67 at the gastric antrum, OGJ and 5cm above OGJ suggests that the proliferation step in mucosa is actively induced by exposure to bile reflux. It is inevitable that the activation of cell cycle regulating proteins and increasing Ki-67 positive cells

occur to repair oxidative DNA damage, which is thought to develop as a result of bile reflux(335). Therefore, the rapid turnover process in the epithelial cells probably leads to p53 mutations and p53 protein accumulation. Previous studies have demonstrated that positive rates of p53 protein expression are gradually increased from normal to reflux oesophagitis and furthermore to Barrett's metaplasia and dysplasia(232). In a study by Shaheen and Ransohoff (336) patients with oesophagitis have been reported to have five-fold increased risk of oesophageal adenocarcinoma. Therefore, these molecular markers are depictees of these early carcinogenic changes that bile acids and salts induce in the oesophageal mucosa as a result of cholecystectomy predisposing to increased risk of oesophageal adenocarcinoma.

Our study suggests that bile reflux is increased in patients with a remote history of cholecystectomy. Bile reflux may have a carcinogenic effect on the upper GI mucosa of these patients, which is indicated by extremely high expression of Ki-67 and p53. This high expression of Ki-67 and p53 takes place as a consequence of increased proliferation, reactive and regenerating epithelium, oncogenic stress, apoptosis and cell death. It is important for surgeons to understand that these changes occur in post-cholecystectomy patients. What is unclear is whether these changes are brought about by cholecystectomy or whether

oesophageal and gastric changes occur in a population of patients also at risk for gallstones. This will require resolution in a prospective study.

CHAPTER 6

STUDY – 3

Effects of Cholecystectomy on the Gastric And Oesophageal Microenvironment: A Prospective Study

ABSTRACT

Background: Cholecystectomy is not innocuous. Loss of the reservoir function of the gallbladder results in a continuous flow of bile into the duodenum which predisposes to bile reflux into the stomach and oesophagus.

Aims: To examine the effects of cholecystectomy-induced bile reflux on the gastric and oesophageal mucosa in a paired population using the bile reflux index (BRI) and the molecular markers p53 and Ki-67.

Patients and Methods: Three study groups were compared: Group 1 (n=26) were gallstone-free patients who presented with upper abdominal pain and required upper GI endoscopy. Group 2 (n=25) were patients with gallstones awaiting cholecystectomy. Group 3 (n=25) were the same patients that had undergone cholecystectomy, studied at 10 to 12 months following surgery.

Results: The cohorts were matched for age and BMI but Group 1 had fewer females ($p = 0.002$). BRI positivity was higher at the gastric antrum and OGJ in Group 3 compared with Group 1 ($p=0.040$). There was no significant difference in BRI between the groups at 5cm above OGJ. There was a significant increase in p53 expression at the antrum and OGJ in Group 2 ($p=0.002$) and Group 3 ($p=0.008$) compared to Group 1, but expression did not differ between the groups at 5cm above the OGJ. Ki-67 expression was increased in the antrum, OGJ and 5cm above OGJ in Group 3 compared to Group 1 ($p<0.05$).

Conclusion: Changes occur in the mucosa of the stomach and oesophagus following cholecystectomy which are attributable to bile reflux. The molecular changes precipitated by cholecystectomy are similar to those identified as precursors of Barrett's oesophagus which is a risk factor for oesophageal adenocarcinoma. It may be time to reconsider our approach to patients with gallstones; to consider methods of preserving the functioning gallbladder and, in patients with a strong family history of gallstone disease, to consider methods of altering the composition of secreted bile.

6.1 INTRODUCTION

Cholecystectomy is the standard of care for symptomatic cholelithiasis however, not all patients are relieved of their symptoms following surgery. Up to 50% of patients have persistent or new symptoms (337) and up to one quarter of patients may consult their physician for persistent abdominal pain(338). This suggests that either their original symptoms were not biliary in origin, that their biliary problem was unsuccessfully addressed or that new symptoms emerged as a consequence of cholecystectomy. Post-operative symptoms include abdominal pain, nausea, abdominal distension, bloating, belching and heartburn(92). The term "post-cholecystectomy syndrome" was coined to describe these symptoms; best defined as gastrointestinal symptoms that develop subsequent to, or persist in spite of, cholecystectomy. Pibram was the first to define it but attributed all cases to residual stones(283). We have previously shown that cholecystectomy is associated with increased gastro-oesophageal reflux (80, 90, 91, 293). With the loss of the reservoir function of the gallbladder there is a continuous flow of bile into the duodenum which may predispose to bile reflux into the stomach. Cholecystectomy results in increased plasma cholecystokinin (CCK) production which reduces lower oesophageal sphincter pressure(2) and increases transient lower oesophageal sphincter relaxation episodes, further exposing the lower oesophagus to the refluxed bile(318).

Bile in the stomach is noxious and excessive bile reflux may result in erythema of gastric mucosa and ulceration(339). The refluxed bile is believed to contribute to damage to the gastric and oesophageal lining(340). The data linking bile reflux to gastric and oesophageal cancer is persuasive. Evidence has accumulated that exposure of the cells of the gastro-intestinal tract to repeated high physiologic levels of bile acids is an important risk factor for upper gastrointestinal cancer(341). High exposure of bile leads to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) through multiple pathways involving disruptions of the cell membrane and mitochondria(342). Increased production of ROS/RNS, can lead to increased DNA damage and then increased mutation(342).

Barrett's oesophagus is a metaplastic lesion of the distal oesophagus, characterized by the replacement of the normal squamous epithelium by columnar intestinal epithelium containing goblet cells(343). It is an important predisposing condition for the development of oesophageal adenocarcinoma(343). Barrett's oesophagus is associated with increased duodeno-gastro-oesophageal reflux (344, 345). Expression of bile acid transporter proteins is increased in Barrett's oesophagus suggesting that Barrett's metaplasia may be an adaption to protect cells from bile acids(346).

In our previous study we found an increase in the bile reflux index (BRI) in patients with a previous history of cholecystectomy compared to the non-operated gallstone patients and to controls. It was unclear from this study whether these findings were truly attributable to cholecystectomy or whether other environmental factors could have contributed to both conditions as it was an unpaired study. For this reason we embarked on a prospective study of paired patients to compare subjects before and after a significant surgical intervention. In this study, in the same group of patients, we evaluated the mucosal expression of bile reflux injury using the bile reflux index based on histological markers and markers of apoptosis and proliferation based on immunohistochemical methods.

6.2 PATIENTS AND METHODS

6.2.1 Study Groups

Three study groups were compared:

Group 1: Controls (n=26) were patients who presented with upper abdominal pain and required upper GI endoscopy. None had symptoms of gastro-oesophageal reflux disease and all patients had absence of gallstone disease confirmed by ultrasound scan.

Group 2: Pre-cholecystectomy (n=25) were patients who had a confirmed diagnosis of gallstones and were awaiting cholecystectomy. Upper GI endoscopy was carried out as a part of pre-operative investigations.

Group 3: Post-cholecystectomy < 1 year (n=25) were the same patients in Group 2 that had undergone cholecystectomy and who were studied at 10 to 12 months following surgery.

6.2.2 Exclusion Criteria

Participants with a previous history of oesophageal, gastric, duodenal, hepatic or pancreatic surgery were excluded; patients with a history of gastric and oesophageal adenocarcinoma, or with an oesophageal stent in situ, or who were receiving or had received chemotherapy or radiotherapy were also excluded; patients with choledocholithiasis, surgical obstructive jaundice,

cholangitis, known peptic ulcer disease or biliary tract disease other than cholelithiasis were excluded from the study; participants with conditions known to affect gallbladder motility, including patients with diabetes or chronic liver disease were excluded; and finally patients being prescribed pharmacological agents known to affect acid secretion or gastrointestinal or gallbladder motility were excluded.

6.2.3 Patient Information and Consent

The project was discussed with all patients recruited. An information booklet was given to all patients who had the opportunity to ask questions related to this study. They were aware that they had the right to withdraw from the study without obligation, or compromise to their treatment. Patients were reassured that their individual privacy would be maintained in all published and written material resulting from this study. Informed consent was signed by each patient prior to recruitment to the study (Appendix 1 and 2).

6.2.4 OGD And Collection Of Biopsy Samples

Upper gastrointestinal endoscopy was performed on all patients by the primary researcher. Multiple biopsies, at least 4, were taken from gastric antrum, oesophago-gastric junction and 5cm above the oesophago-gastric junction. The

biopsy specimens were oriented on filter paper and immediately fixed in formalin.

6.2.5 Tissue Processing and Staining

The protocol for this has already been explained in detail in chapter 3 on for general methodology. Briefly, however, it consisted of deparaffinising and rehydrating the tissue blocks, staining with haematoxylin solution, rinsing with an HCl solution and counterstaining with xanthene dye e.g eosin.

6.2.6 Bile Reflux Index (BRI)

This has been described in detail in chapter 3 and performed by a senior consultant histopathologist, who was blind to the clinical details of the patients and the endoscopy findings. He assessed the biopsies for histological features of bile reflux in the gastric antrum and oesophago-gastric junction (OGJ) which were calculated by using the BRI devised by Sobala et al(164). A BRI above 14 indicated duodeno-gastric reflux (defined as bile acid level >1mmol/L, the upper limit of physiological reflux). The biopsies from 5cm above the OGJ were assessed for bile reflux by grading 1-3 according to the basal cell layer proliferation in the squamous oesophageal mucosa and also by the height of the papillae within the epithelium.

6.2.7 Immunohistochemistry

The details of this procedure have been described in chapter 3. Briefly, however, it consisted of fixing, embedding, cutting, mounting, deparaffinising, rehydrating, staining, counterstaining, dehydrating and stabilizing with mounting medium and viewing the staining under the microscope. We used this method for demonstrating the presence and location of p53 and Ki-67 proteins in tissue sections at the gastric antrum, oesophago-gastric junction and 5cm above the oesophago-gastric junction.

6.2.8 Ethical Approval

The certificate of approval for this study was granted before enrolment by the Research Ethics Committee, Connolly Hospital, Blanchardstown, Dublin. All participants gave their consent to participate in the study. No economic reimbursement was given for participation.

6.2.9 Statistical Analysis

P-values were all two tailed and the alpha level of significance was set at 0.05. The prevalence is shown as a percentage. Chi-2 paired test was used for testing comparison in paired group. ANOVA was used to test multivariate analysis between and within groups. Post hoc correlations were calculated with Dunnett's T3 test. Data was analyzed using SPSS version 18.

6.3 RESULTS

6.3.1 Age

The mean age (SEM) across all patients was 57.3(1.5) years. The mean age of patients in Group 1 was 58(1.7) years and 54.2(2.6) years in Group 2/3 respectively. ANOVA between and within groups did not find any significant differences (between groups $p=0.626$, no significant interactions within groups).

6.3.2 Gender

There were significantly fewer female patients 9/26(35%) in Group 1 as compared to Groups 2/3, with 19 of 25 patients being female (76%), ($p = 0.002$).

6.3.3 BMI

The mean BMI was similar in all groups. Twelve of 26 (46%) patients in Group 1 were overweight having a BMI 26-30 compared with 13(52%) overweight patients in Group 2 and 3 ($p = 0.676$) while 4(15%) patients were obese in Group 1 with BMI > 30, compared with 7(28%) in Group 2 and 3 ($p = 0.274$).

6.3.4 Bile Reflux Index

Gastric Antrum

The BRI at the gastric antrum was positive in 11% (6) of Group 1, compared with 20% (5) in Group 2 and 36% (9) in Group 3. There was no significant difference between groups 1 and 2 ($p=0.406$) or groups 2 and 3 ($p = 0.208$) but there was a difference between groups 1 and 3 ($p=0.04$) (Figure 33).

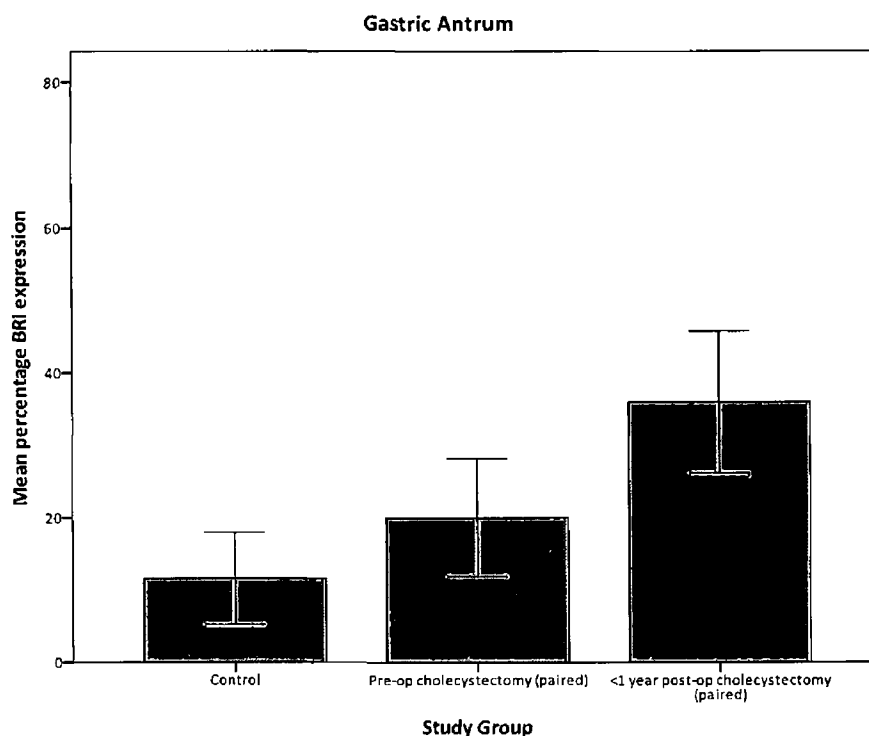


Figure 33 Positive Bile Reflux Index (BRI) at the gastric antrum in three groups.

Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Oesophago-gastric Junction (OGJ)

Nineteen percent (5) of patients had a positive BRI at the OG junction in Group 1, compared with 12%(3) of Group 2 and 20% (5) of Group 3. There was no significant difference between any of the groups [Group 1 vs Group 2 (p=0.478); Group 1 vs Group 3 (p=0.803) or Group 2 vs Group 3 (p=0.625)](Figure 34).

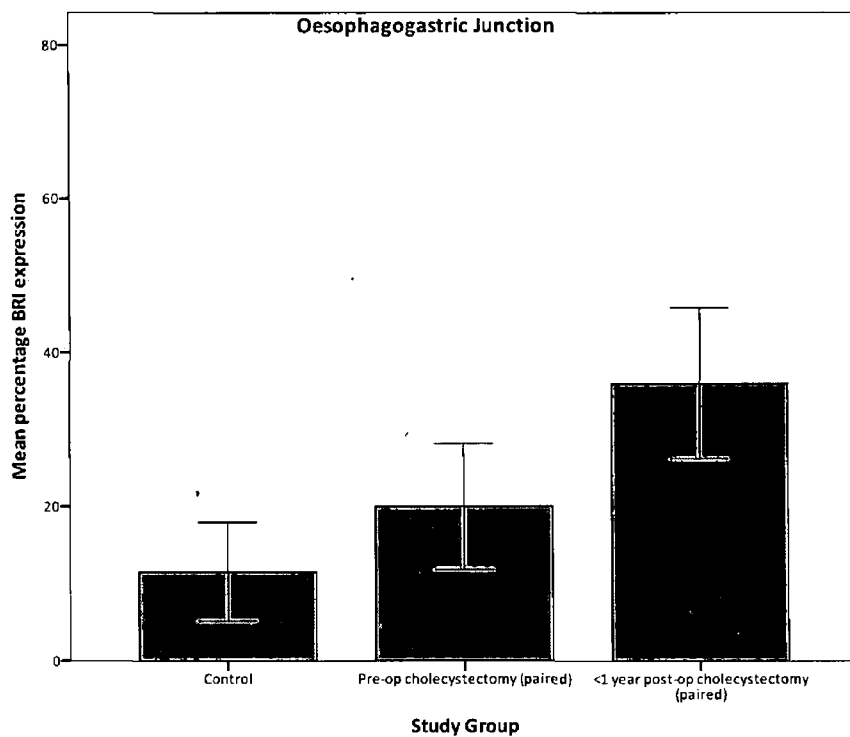


Figure 34 Positive Bile Reflux Index (BRI) at the oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Changes 5 cm above OGJ

Only 8% (2) of patients in Group 1 had a positive BRI 5cm above the OGJ compared with 24% (6) of Group 2 and 12% (3) of Group 3 patients. There was no significant difference between any of the groups. [Group 1 vs Group 2 ($p = 0.109$); Group 1 vs Group 3 ($p = 0.605$); Group 2 vs Group 3 ($p = 0.269$)](Figure 35).

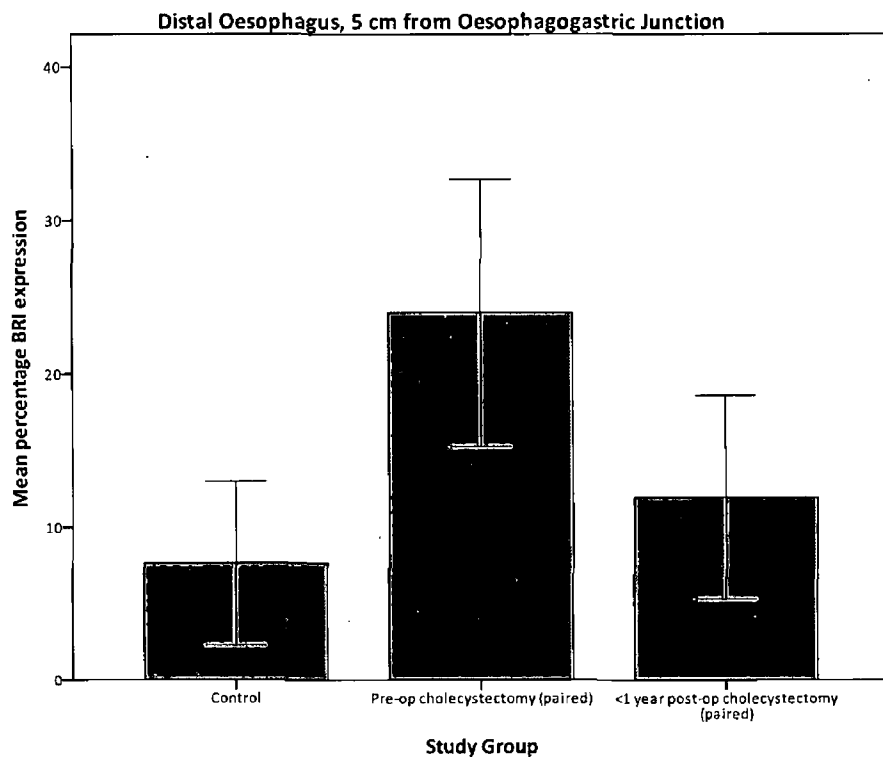


Figure 35 Positive Bile Reflux Index (BRI) at 5 cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

6.3.5 Expression of p53

Gastric Antrum

P53 was expressed in 4% (1) of control (Group 1) patients, in 44% (11) of Group 2 patients and in 32% (8) of Group 3 patients. There was a significant increase in expression seen in Group 3, compared with Group 1 ($p=0.008$). The incidence of p53 overexpression did not differ between Groups 2 and 3 ($p=0.597$)(Figure 36).

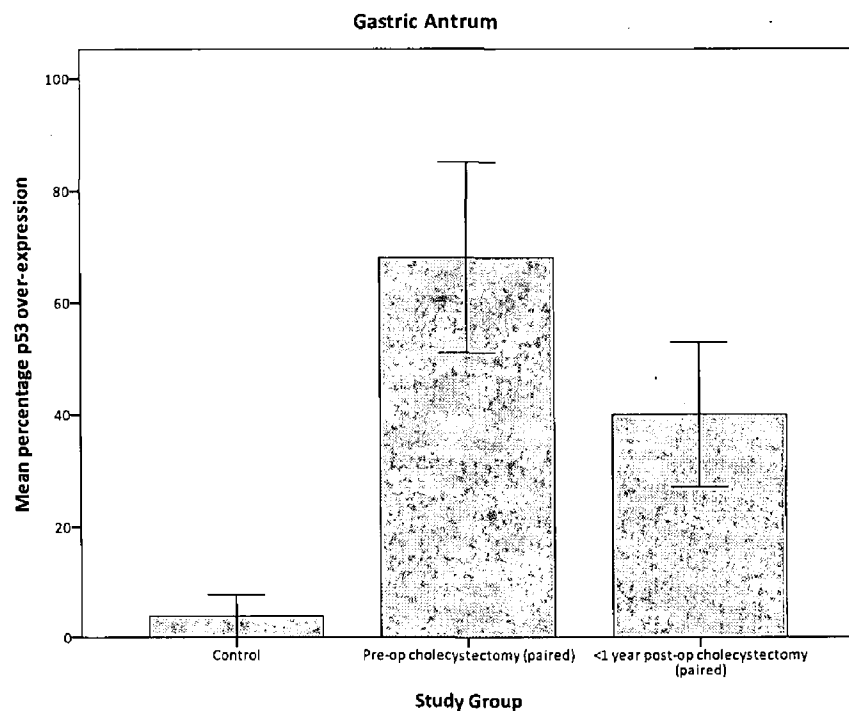


Figure 36 Expression of p53 protein at the gastric antrum in three groups.

Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Oesophago-gastric Junction (OGJ)

Expression of p53 was identified in 19% (5) of Group 1, increasing significantly to 48% (12) in Group 2 ($p=0.029$) and remained elevated at 32% (8) in Group 3, ($p=0.296$)(Figure 37).

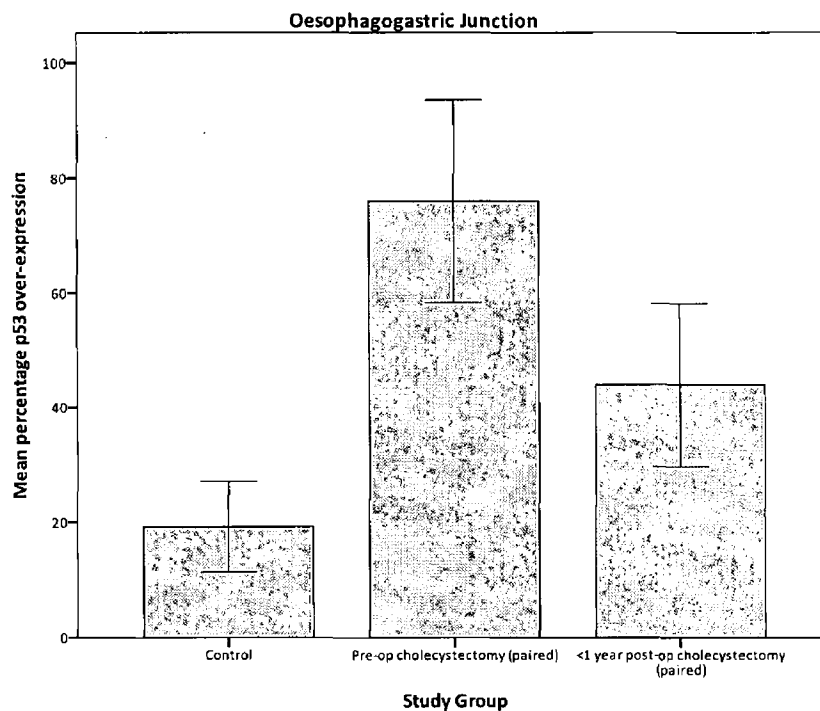


Figure 37 Expression of p53 protein at the oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Oesophagus 5cm above OGJ

The expression of p53 5cm above the OGJ was 19%(5) in Group 1, 36%(9) in Group 2, and 20% (7) of Group 3(p=0.180). P53 expression did not differ significantly between groups at this anatomic level (Group 1 vs Group 2 p=0.22; Group 2 vs Group 3 p=0.384; Group 1 vs Group 3 p=0.751)(Figure 38).

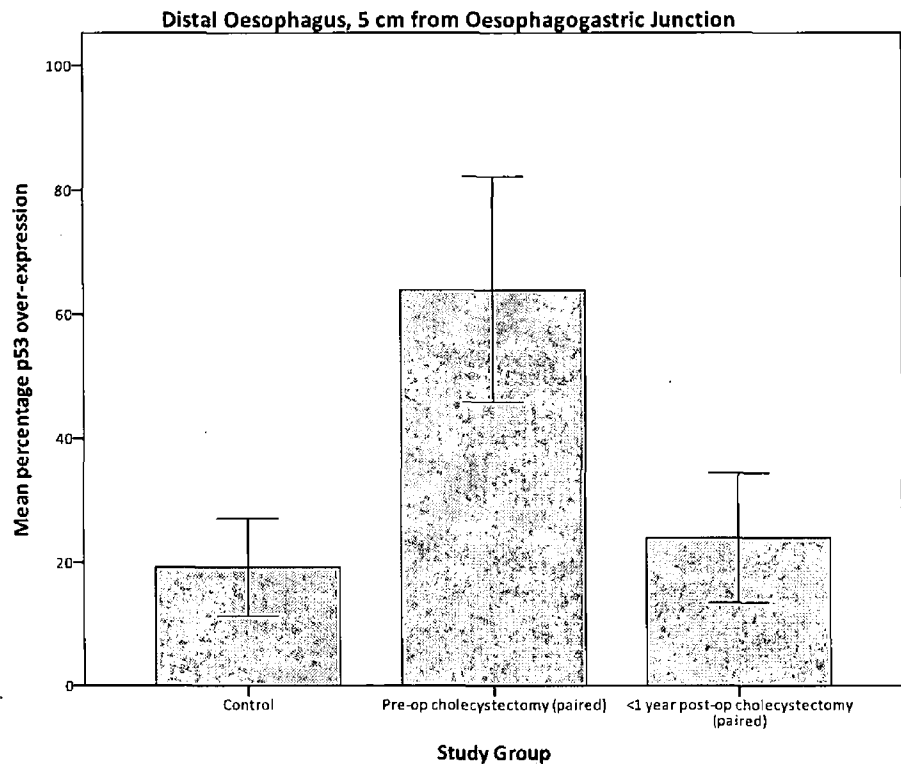


Figure 38 Expression of p53 protein at 5 cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

6.3.6 Ki-67 Expression

Gastric Antrum

Ki-67 expression was positive at the gastric antrum in 23% (6) of patients in Group 1 compared with 4% (1) in Group 2 ($p=0.048$) which increased to 44% (11) in Group 3 after cholecystectomy (Group 1 vs Group 3; $p=0.144$. Group 2 vs Group 3 $p = 0.002$) (Figure 39).

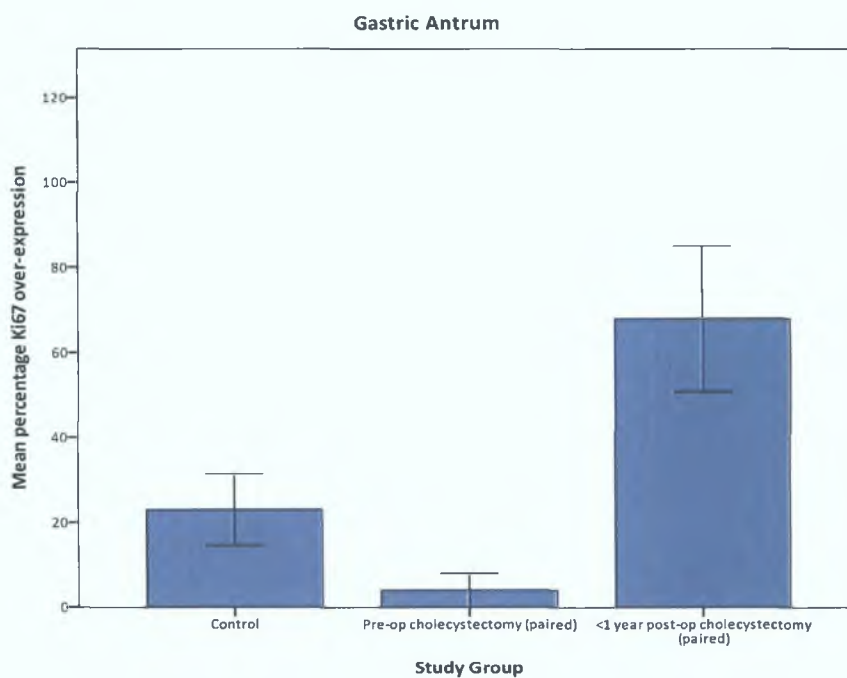


Figure 39 Expression of Ki-67 at the gastric antrum in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Oesophago-gastric Junction (OGJ)

Ki-67 expression was positive at the OGJ in 19% (5) of patients in Group 1 as compared to 12%(3) of Group 2, ($p=0.703$) and 48%(11)of Group 3 patients , ($p=0.044$). The increase in Group 3 compared with Group 2 was significant ($p = 0.012$)(Figure 40).

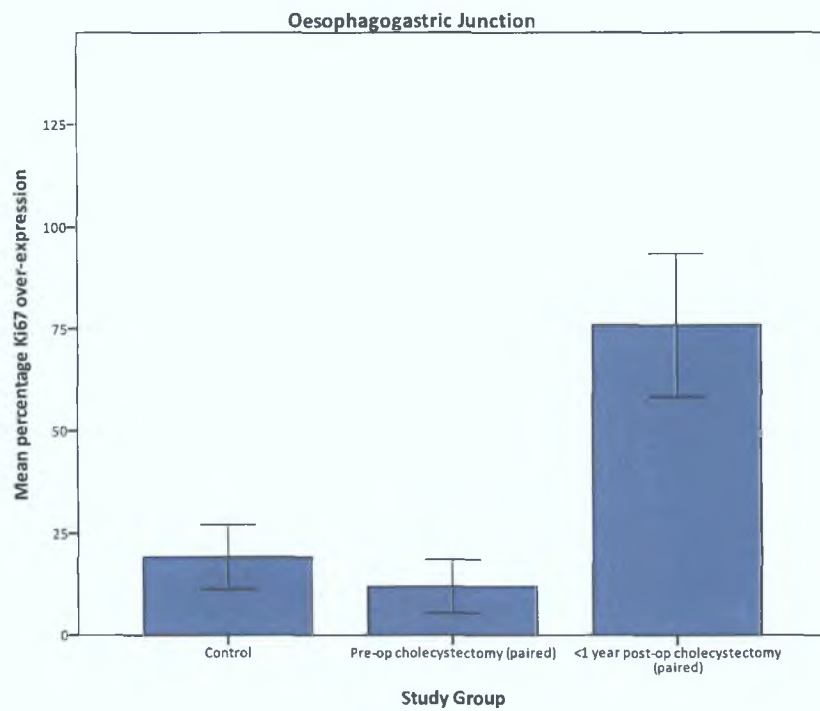


Figure 40 Expression of Ki-67 at the oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

Oesophagus 5cm above OGJ

Ki-67 expression was positive 5cm above the OGJ in Group 1 in 11% (3) compared to 4% (1) in Group 2 ($p=0.277$) and 36% (9) in Group 3 respectively ($p=0.025$). There was a significant difference between group 3 and Group 2 ($p=0.005$)(Figure 41).

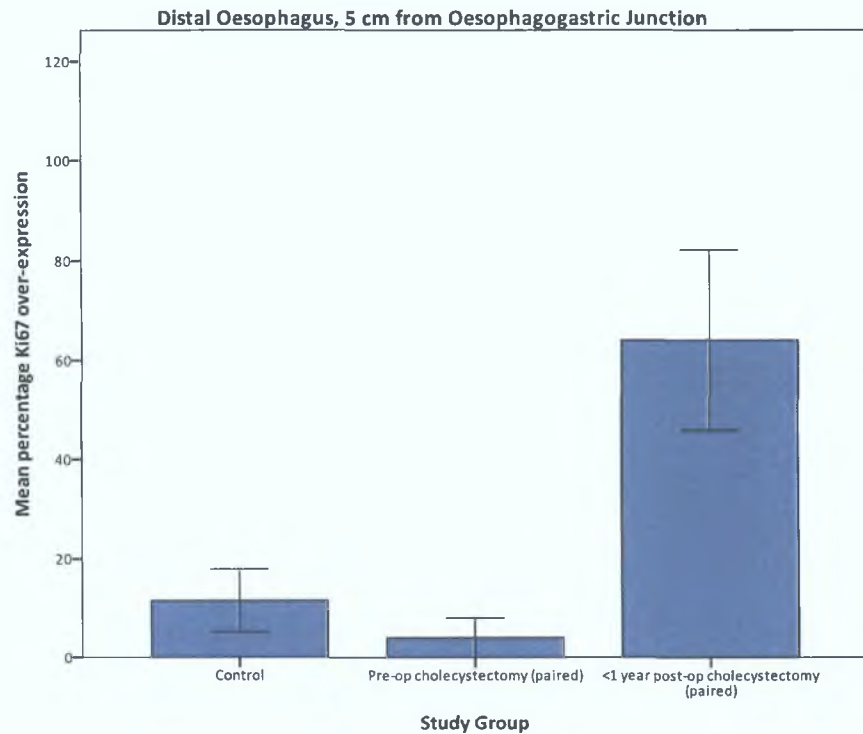


Figure 41 Expression of Ki-67 at 5 cm from oesophago-gastric junction in three groups. Group 1: Gallstone-free controls. Group 2: Pre-cholecystectomy patients. Group 3: Patients with history of cholecystectomy less than one year previously (Paired Study).

6.4 DISCUSSION

In this paired study we found an increased incidence of bile reflux injury in patients with gallstones and this injury increased following cholecystectomy. It supports an association between bile reflux into the stomach and histological and molecular changes. The increased incidence of bile reflux injury in patients with gallstones before surgery probably reflects the fact that some of these will have non-functioning or poorly functioning gallbladders due to the presence of gallstones in the gallbladder or because of a stone impacted in the neck of the gallbladder or cystic duct. These patients will have been functionally cholecystectomised by their disease and will not have a bile reservoir. Little physiological change will result for these patients after cholecystectomy.

The increased BRI was associated with increased expression of the molecular marker Ki-67 within one year of cholecystectomy. Immunohistochemical staining for MIB-1, the Ki-67 proliferation antigen, has been shown to increase expression in the Barrett's oesophagus-dysplasia-adenocarcinoma sequence(241, 347). The increased expression of Ki-67 may reflect chronic mucosal inflammation and repetitive cycles of epithelial regeneration (347).

We found a low expression of p53. This may reflect mutation of the p53 gene, or accumulation of wild type p53 protein due to inflammatory process undetectable by immunohistochemistry. It may also be because studies have

shown p53 over expression at later stages of disease progression. Studies have demonstrated that rates of p53 protein expression are increased gradually from normal to reflux oesophagitis to Barrett's metaplasia and to dysplasia. Expression of p53 has been shown beyond the stage of chronic active gastritis and intestinal metaplasia formation.

Several studies have suggested that bile can damage DNA directly (348, 349). Lithocholic acid has specifically has been identified as a promoter in experimental carcinogenesis by causing cell transformation and DNA strand breaks (350-352). Duodeno-gastric reflux is thus considered the main cause of various symptoms after gastrectomy that adversely affect quality of life(353). The role of duodeno-gastro-oesophageal reflux in the pathogenesis of Barrett's oesophagus is well established (344, 354, 355). And Barrett's oesophagus is a known premalignant precursor lesion for oesophageal adenocarcinoma (356). It has been suggested that prevalence of gallstone disease is increased in Barrett's oesophagus (357). We have shown previously that gallbladder function is impaired in patients with Barrett's oesophagus and oesophageal adenocarcinoma(4). The impaired motility and function of the gallbladder may be a contributing factor for development of gallstones(358). The decreased motility and formation of stones all predispose to duodeno-gastro-oesophageal reflux as the function of the gallbladder with stones becomes progressively impaired or completely non-functional (359). Cholecystectomy will worsen

duodeno-gastro-oesophageal reflux in patients with a functioning gallbladder due to the immediate loss of a bile reservoir(360).

A large study with a long and complete follow-up indicated an association between cholecystectomy and increased risk of oesophageal adenocarcinoma(361). This was a retrospective population-based cohort study calculating the risk of oesophageal adenocarcinoma in the entire Swedish population. A study by Katja et al in 2007 revealed an overall 11% increase in risk of gastric cancer in patients who had undergone cholecystectomy(362). A further study found an increased risk of oesophageal adenocarcinoma after cholecystectomy and the carcinogenic effect of refluxed bile was proposed as an explanation in 2001 but the study size was too small to prove the risk statistically (309).

In the era of peptic ulcer surgery partial gastrectomy was identified as a predisposing cause of gastric cancer after an interval of 20 or more years (363). Duodeno-gastric reflux has been reported as a cause for changes in the mucosa of post-operative stomach leading to stump carcinogenesis (364, 365). Billroth II patients have been reported to be at higher risk for gastric carcinogenesis than those treated with Billroth I(366, 367) because of increased bile in gastric stump(368). The putative link with the development of cancer in these patients

was variously attributed to nitrosamines(369) and to bile(275, 363). Roux-en-Y reconstruction is effective in preventing bile reflux into the gastric remnant and reduces the risk of carcinogenesis in the gastric remnant(370, 371), 47). Endoscopic findings of remnant gastritis and histologic alteration of the stump is significantly lower with Roux-en-Y anastomosis than with Billroth I or II(372, 373).

This study further validates the findings of our previous study that cholecystectomy predisposes to increased bile reflux and predisposes to a change in the internal micro-environment which are similar to Barrett's oesophagus and which may further lead to metaplasia-dysplasia-neoplasia sequence. The findings of this study have major potential implications for the management of gallstone disease. If loss of the storage reservoir results in potentially damaging mucosal injury other approaches must be reconsidered. In patients with a non-functioning gallbladder there is little option but to proceed to cholecystectomy. For patients with a functioning gallbladder there is an option of gallstone removal or dissolution. There was a vogue in the early 1990s to conserve the gallbladder with a range of alternatives such as percutaneous removal of gallstones or percutaneous cystolithotomy (374), percutaneous intubation and direct gallstone dissolution(375), extracorporeal shock wave lithotripsy(376), bile acid treatment(377) and ESWL with a combination of lithotripsy and oral bile salts(378-381). But these approaches

appear to have run out of steam, partly due to the introduction of laparoscopic cholecystectomy and partly because of the high rate of gallstone recurrence of up to 60% at ten years in some studies(382-384). With the findings of these studies conservative management options should be revisited.

Prevention of gallstone formation is a further option. Gallstone pathogenesis has been linked to cholesterol supersaturated bile(385). Impaired gallbladder motility leading with stagnant bile also contributes to stone formation(386). The prevention of stone formation in animals(387) and sludge in humans(388) by promoting gallbladder emptying raises the possibility that oral prokinetic agents may prevent stone formation in humans specially in high risk groups(389). The ability to medically alter the concentration of secreted bile to reduce the concentration of the lithogenic elements would also be a major advance.

Our study suggests that patients with gallstones have increased indicators of reflux-induced injury and these increase further following cholecystectomy. The molecular changes that occur as a result of cholecystectomy are similar to those identified as precursors of Barrett's oesophagus which itself is a major risk factor for oesophageal adenocarcinoma. Therefore this study provides further evidence for bile reflux as a precursor for gastric and oesophageal malignant

transformation. It raises further concerns about the long term safety of cholecystectomy and supports the decisions to avoid cholecystectomy in patients with gallstones where indications for the surgery are uncertain. It further suggests that alternatives to cholecystectomy be further explored in patients with gallstones in a functioning gallbladder. Preventative measures should be explored in patients with a strong family history who are at considerable risk for gallstone development.

CHAPTER 7

General Discussion And Conclusions

GENERAL DISCUSSION

The incidence of proximal gastric(31), cardia(390) and oesophageal adenocarcinoma has risen three-fold in the Western world over last two decades (391-394). This dramatic increase cannot be explained by genetic factors alone (391, 395, 396). Environmental factors must be involved. The lower oesophagus is constantly exposed to the contents of the internal micro-environment of the gastric lumen. Changes in this micro-environment may have a major impact on the mucosa of the cardia and lower oesophagus. The causes of these changes and their impact remain unclear. Barrett's oesophagus, which results from reflux of the duodeno-gastric contents into the oesophagus, is the only risk factor with a proven association with adenocarcinoma (397-401). Epidemiologic data suggests that the incidence of Barrett's oesophagus has increased significantly over the past three decades(402, 403). Until recently it was believed that 0.5-1% of these patients would progress to oesophageal adenocarcinoma each year(404). In a recent nationwide population based, cohort study, however, the annual risk of oesophageal adenocarcinoma in patients with Barrett's oesophagus was found as low as 0.12%(405).

The exact component or components which promote intestinal metaplasia is/are unknown. Hitherto the vast majority of studies focused on acid reflux. This was due to the fact that the development of the pH probe was the first

sophisticated investigative tool available to the researcher. And while acid may be involved in injury, acid reflux may only be one of, or a minor component of, the toxic chemicals bathing the lower oesophageal lumen. Duodeno-gastric reflux of bile, pancreatic and duodenal secretions may have an equally important role(406) and chronic exposure of oesophageal mucosa to bile has been implicated in the etiology of the oesophageal lesions which develop into Barrett's oesophagus(407).

There are many triggers of changes in the internal milieu of the upper GI tract but the most direct is due to altered gallbladder function. Gallbladder function may be impaired either spontaneously, in the absence of injury (4), or by the development of gallstones, empyema, mucocele or chronic inflammation, leading to impaired bile storage and release. This compromise of bile storage and release is most dramatically seen in patients after cholecystectomy(90). Since its introduction into the surgeon's armamentarium, laparoscopic cholecystectomy has become the operation of choice for patients with gallstones as it is associated with less postoperative pain and discomfort, better cosmesis, a shorter hospital stay and a chance for early return to work. After cholecystectomy, however, bile transport is distorted. Instead of bile being stored in the gallbladder, to be released intermittently to mix with meals, bile is continuously produced and secreted by the liver into the duodenum leading to duodeno-gastric reflux and consequent gastro-oesophageal reflux(310).

In our first study we aimed to investigate the incidence, effects and contributing factors to duodeno-gastric bile reflux in three patient populations; symptomatic controls, Barrett's patients and patients post oesophago-gastric resection for carcinoma. As anticipated increase in bile reflux was noted in patients with Barrett's oesophagus and post oesophago-gastrectomy when compared with controls. The mean age of patients did not differ in the three groups. There were statistically-significantly more males noted in the Barrett's oesophagus and post oesophago-gastrectomy cohorts. There was no difference in BMI within the groups. However, when looking at patients who had previous history of cholecystectomy we noted the increased BRI was seen in association with the constellation of demographic risk-factors for cholelithiasis/gallbladder dysfunction that in turn increased the likelihood of requiring cholecystectomy (age, BMI, gender). We also noticed that 70% patients who presented for investigations of upper abdominal symptoms were on PPI therapy and 61% of these had BRI positivity. A significant number of patients had persistent upper abdominal symptoms despite being on PPI therapy when they presented for investigations of their symptoms. This may suggest that while PPI's can suppress the acidic element contributing to their symptoms, bile and its toxic effects persist. Nasr and colleagues (302) have shown that while PPIs have a dramatic effect on acute inflammatory changes, chronic inflammation persists. This became even clearer when we looked at our groups individually. Patients in Group 2 who were on surveillance for Barrett's oesophagus had increased BRI

and increased incidence of PPI use was noted in these patients as well. The failure of PPI therapy (which targets acid) to resolve symptoms associated with DGOR in Barrett's patients gives us an insight which unmasks the chronic inflammation that was hidden from scrutiny by the previous interest in acid-mediated damage.

Augmentation of bile reflux was noted in patients who had previous history of cholecystectomy when compared with the patients who had no history of cholecystectomy. The most unexpected finding of this study, however, was that one third of patients who presented for investigations of upper abdominal symptoms were noted to have had a previous history of cholecystectomy, and that their symptoms had persisted despite their surgical history. A high proportion of these still-symptomatic post-cholecystectomy patients had increased bile reflux index suggesting that the symptoms of these patients were not biliary in origin, that their biliary problem was unsuccessfully addressed or that new symptoms emerged as a consequence of cholecystectomy. A further explanation is that this cohort of patients developed new symptoms as a result of cholecystectomy induced bile reflux. When looking at the odds ratio in patients who undergo cholecystectomy, 37% are more likely to develop bile reflux than those who do not. This translates into the number of patients 'needed to be harmed' with cholecystectomy in order to provoke bile-mediated cellular damage (demonstrated as BRI positive) as 15.2 placing them at

increased risk of developing Barrett's oesophagus and oesophageal adenocarcinoma. This quantifiable excess DGOR-mediated mucosal injury secondary to cholecystectomy can thus be linked to increased risk of Upper gastro-intestinal carcinogenesis. It is concerning the way post-cholecystectomy patients are exposed to this iatrogenic injury induced by bile reflux. Provoked by this we went on to perform our next study on the effects of cholecystectomy on gastric and oesophageal mucosa at molecular and histological level.

In our *retrospective* study we compared patients who had had a cholecystectomy from one to 30 years previously with patients diagnosed with gallstones who were awaiting surgery and also with patients who were confirmed gallstone-free by ultrasonography. We examined the effects of cholecystectomy on the mucosa of the stomach and oesophagus using BRI as the histological marker of injury, along with the molecular markers p53 and Ki-67, at three anatomical sites; gastric antrum, OGJ and 5cm above OGJ.

The BRI was elevated at all three sites in post-cholecystectomy patients compared with patients pre-cholecystectomy and with normal controls. The severity of bile-induced histological changes was demonstrated by the presence and severity of glandular atrophy, chronic inflammation, lamina propria oedema and foveolar hyperplasia. These histological changes show the implication of

duodeno-gastric reflux that develops in patients with history of cholecystectomy, Barrett's and post-oesophago-gastrectomy. The step-wise increase in the proportion of BRI-positive patients from controls to patients with intact, dysfunctional/dyskinetic gallbladders to those with a remote history of cholecystectomy was noted.

We observed extensive expression of Ki-67 at all three sites in post-cholecystectomy patients compared to the other two groups. This expression of Ki-67 indicates proliferation of cells which occurs as a result of bile-induced inflammation in the gastric and oesophageal mucosa. Proliferation of cells is a common pre-neoplastic response to chronic inflammation. This may be the reason why the expression of Ki-67 is suppressed in the patients with intact non-functional gallbladder with gallstones. When the gallbladder is removed the bile exposure of gastric and oesophageal mucosa increases leading to the chronic inflammatory changes which are expressed by the over-expression of Ki-67 as years pass by after cholecystectomy indicating cumulative DGOR mediated cellular damage.

Chronic inflammation, repetitive cycles of epithelial regeneration and aberrant growth lead to over-expression of Ki-67 and these changes activate p53 by post-translational modifications, such as phosphorylation, in response to cellular

stress or/and DNA damage. The most common responses to oncogenic stress induced by p53 are cell cycle arrest and DNA repair. If the DNA damage cannot be repaired, p53 induces apoptosis (408, 409). The expression of p53 increased significantly in gallstone patients. There was a three-fold over-expression of p53 at all 3 sampling locations (antrum, OGJ, distal oesophagus) in patients with non-functional intact gallbladders compared with controls. It may be due to the effects of loco-regional inflammatory response that these patients developed as many of them would have been recruited to the study on their presentation with acute cholecystitis. This over-expression at the gastric antrum and OGJ remains high years after the gallbladder is removed, though not to the same extent at 5cm above OGJ.

It was unclear from this retrospective study whether these findings were truly attributable to cholecystectomy or whether other environmental factors could have contributed to both conditions as it was an unpaired study. For this reason we performed a prospective study to compare subjects before and after cholecystectomy. There was an increase in our histological measure of DGOR-mediated gastric antral and oesophago-gastric junctional mucosal damage directly attributable to cholecystectomy as expressed by increased BRI within one year of cholecystectomy. Ki-67 expression mimics the retrospective study and was suppressed in the acute setting (gallstone patients), but was markedly upregulated in the first year post-cholecystectomy in the same patients,

suggesting that bile-induced proliferative changes occur early in patients post-cholecystectomy. The p53 over-expression was seen pre-operatively in response to acute inflammation and though still present it seems to start resolving in the first year following removal of the gallbladder. As suggested by Andreas et al it is possible that positive p53 immunostaining pre-operatively in our patients is related to accumulation of inactivated p53 protein due to mucosal inflammation. This increase in p53 expression in these paired patients when followed within one year of cholecystectomy persists especially when compared with controls suggesting initiation of DNA damage, repair and apoptosis. This trend is clearly evident in the antrum and OGJ. As seen in the retrospective unpaired study in the distal oesophagus the p53 levels are almost comparable to controls post-operatively.

When all groups are compared across all studies it becomes clear that the changes at the antrum are progressive from controls, to patients with gallstones (some of whom may have a non-functioning or poorly functioning gallbladder and who are probably refluxing bile), to patients shortly post-cholecystectomy and to patients who are 30 years post-cholecystectomy. "Time heals all wounds"; except those to the mucosa caused by DGOR; in fact, time (i.e. chronic bile exposure) makes it worse. Over-exposure to bile leads to histological damage which is best seen at the antrum and OGJ and not at 5cm above OGJ(Figure 42).

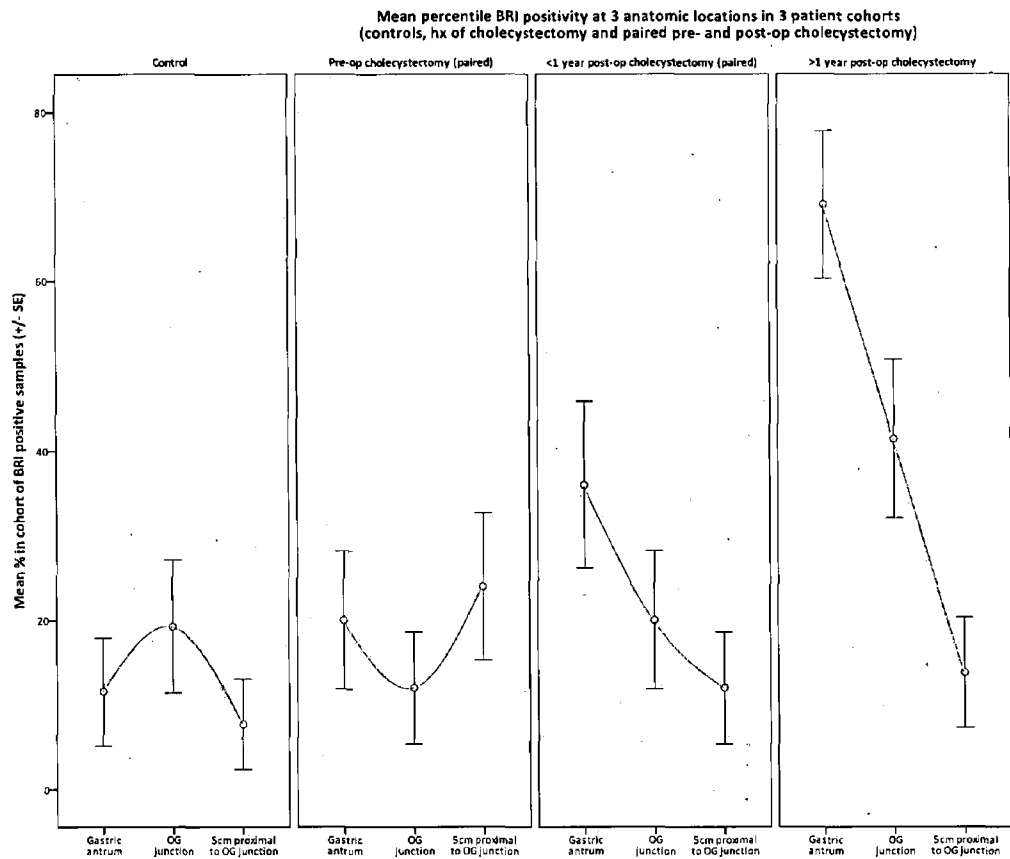


Figure 42 Incidence of Bile reflux index at the gastric antrum, OGJ and 5cm above OGJ in Control group, pre-cholecystectomy patients, post-cholecystectomy < 1 year patients (paired), post-cholecystectomy > 1 year patients.

Changes arise at molecular level as a result of bile-induced injury. These histological changes paralleled the findings at molecular level as identified by Ki-67. The over-expression of Ki-67 post-cholecystectomy, within one year and persisting up to 30 years post-cholecystectomy is concerning (Figure 43).

Mean percentile Ki67 overexpression at 3 anatomic locations in 3 patient cohorts (controls, hx of cholecystectomy and paired pre- and post-op cholecystectomy)

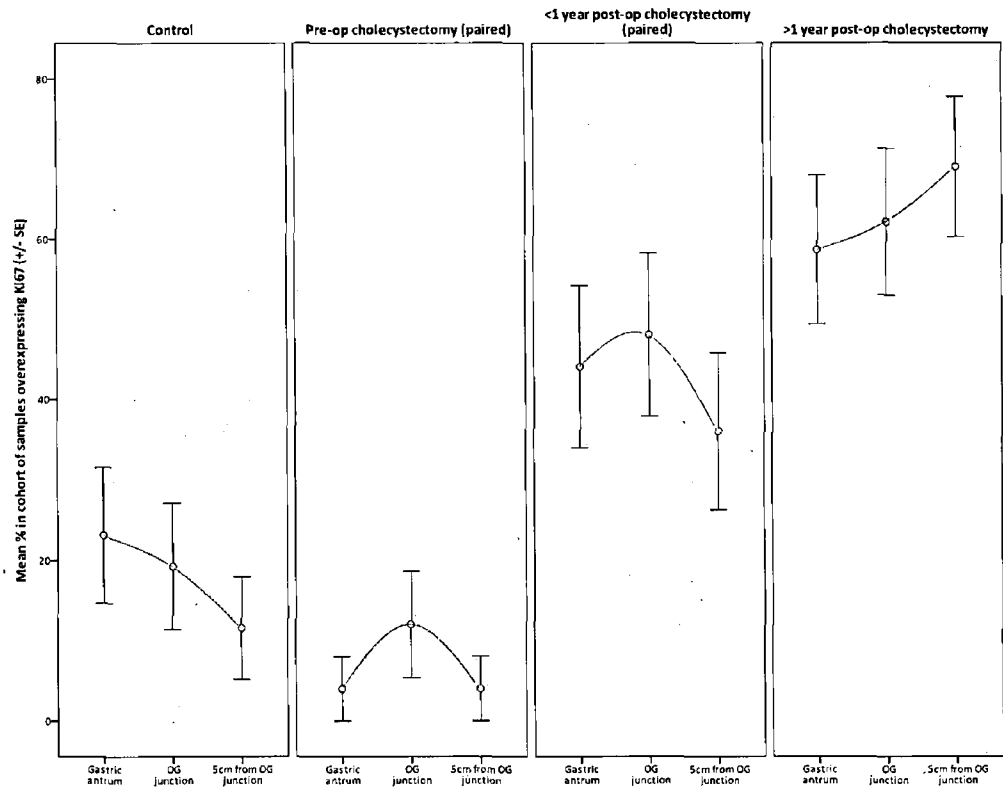


Figure 43 Expression of Ki-67 protein at the gastric antrum, OGJ and 5cm above OGJ in control group, pre-cholecystectomy patients, < 1 year post-cholecystectomy (paired) patients and >1year post cholecystectomy.

Cell proliferation has been reported to be one of the first steps in the development of adenocarcinoma in Barrett's oesophagus, and it can be induced by chronic cell damage caused by gastro-oesophageal reflux(410). Proliferation of tissue cells correlates with the development of carcinoma by tumour

promotion and rate of random mutations. The immunohistochemistry identified expression of Ki-67 in Barrett's oesophagus has been found to be enhanced in adenocarcinoma arising in Barrett's oesophagus and in high degree dysplasia, less expressed in low degree dysplasia and Barrett's oesophagus without dysplasia(411). The cell proliferation index, as revealed by immunohistochemistry tests for Ki-67(412-415), have been extensively reported in literature, with the aim of identifying the evolution of Barrett's oesophagus to adenocarcinoma (411, 416, 417).

The expression of p53 when compared across all our studies shows a surge in pre-cholecystectomy gallstone patients, decreasing within one year of cholecystectomy and then re-expressing in patients with remote history of cholecystectomy (Figure 44).

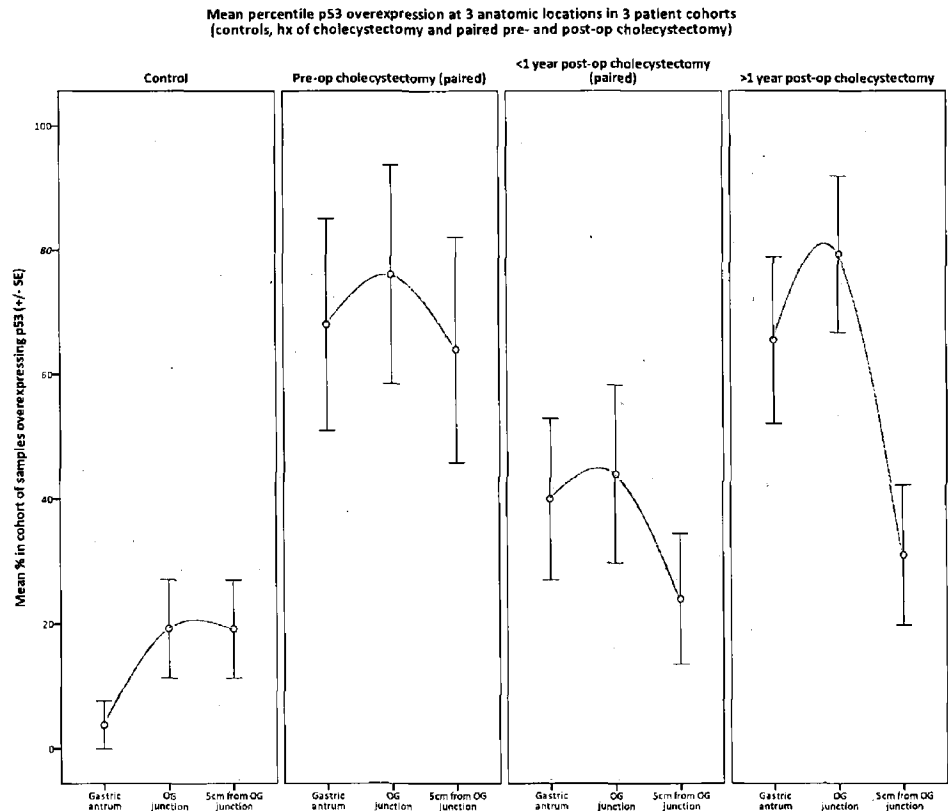


Figure 44 Expression of p53 protein at the gastric antrum, OGJ and 5cm above OGJ in control group, pre-cholecystectomy patients, < 1 year post-cholecystectomy (paired) patients and >1year post cholecystectomy.

The sudden surge in p53 expression in pre-operative gallstone patients may be the result of accumulation of inactivated p53 protein as a result of mucosal inflammation. The drop in p53 within one year of cholecystectomy as compared to patients with remote history of cholecystectomy can be explained by accumulation of wild-type p53 protein, probably due to an acute inflammatory process which occurs as a result of bile reflux. Inflammation, DNA

damage, and other cellular stresses can up-regulate wild-type p53 protein (223, 418). The wild-type p53 has a short half life in the cell and is usually present at levels below the threshold of detection by immunohistochemistry(419). Some p53 mutations can also produce a truncated protein that is not detectable by immunohistochemistry(420-423). Increase in p53 expression is again noted in patients with remote history of cholecystectomy as a result of chronic inflammation. This late expression of p53 in our patients with a remote history of cholecystectomy is also compatible with Craanen et al's study who described an absence of p53 expression in early benign gastric lesions(424). Flejou et al identified that p53 mutations occur relatively late in metaplasia-dysplasia-neoplasia sequence in Barrett's oesophagus (425). Other studies have also demonstrated that positive rates of p53 protein expression were gradually increased from normal to reflux oesophagitis and furthermore to Barrett's metaplasia and dysplasia(232, 425, 426). It has been shown that p53 protein accumulation occurs beyond the stage of chronic active gastritis and intestinal metaplasia formation(424). We have demonstrated similar late over-expression of p53 in patients with remote history of cholecystectomy who had increased exposure to bile leading to chronic inflammatory changes.

These changes in BRI, Ki-67 and p53 were noted at the gastric antrum and the OGJ, but were not remarkably seen in the distal oesophagus (above the "high-tide mark"), suggesting that the attentions of previous investigators at this

location were misplaced, resulting in missed or delayed diagnosis of DGOR-mediated injury. This is a significant finding suggesting that our traditional focus of reflux changes at 5cm above the LOS(427) defining the presence or absence of reflux may have been misplaced. Reflux has traditionally been defined in terms of the pH detected by a pH probe placed 5 cm above the proximal margin of the lower oesophageal sphincter (LOS) (428, 429). The 5 cm distance from the LOS was adopted to ensure placement of the tip of the pH probe within the oesophageal body, but sufficiently close to the oesophago-gastric junction to reflect the degree of reflux(430). The hope was that it would allow detection of reflux events and avoids inadvertent advancement of the probe into the stomach during swallowing when the oesophagus shortens. However, the average length of the lower oesophageal sphincter is 4 cm and thus the pH probe is placed approximately 7 cm above the distal margin of the oesophagus. As a result, in an average oesophagus, which measures approximately 25 cm in length, the pH probe is located more closely to the mid-oesophagus than to the end of the distal oesophagus. While adequate for the assessment of symptomatic reflux, where acid reflux is the major contributor to symptoms, current pH and Bilitec[®] probe findings may not reflect the true picture when studying the factors contributing to oncological change. In this context the most significant site to assess the impact of duodeno-gastric reflux is at the gastric antrum and OGJ where most mucosal injury occurs.

The majority of patients with adenocarcinoma develop cancer at the lower oesophagus. Intestinal metaplasia at the cardia has been reported in 15-22% of dyspeptic patients with endoscopically normal lower oesophagus and is considered to be a premalignant condition(431). Intestinal metaplasia, which is a recognized pre-neoplastic lesion for adenocarcinoma in Barrett's oesophagus is a common finding in the mucosa adjacent to cancer of gastric cardia(432). It has been shown that early stage adenocarcinoma in Barrett's oesophagus and gastric cardia have very similar epidemiologic, clinical and pathologic characteristics and may represent same disease(432). Another large study showed an association between adenocarcinoma of oesophagus and gastric cardia regardless of the presence of Barrett's(433). There is increasing evidence that duodeno-gastric reflux is responsible for this change(434). With the increasing incidence of more proximal gastric tumours and also adenocarcinoma at OGJ, these findings raise serious concerns.

This series of studies provided further evidence that cholecystectomy induced bile reflux causes changes to the gastric and oesophageal mucosa which may act as a precursor for gastric and oesophageal malignant transformation. The molecular/epigenetic changes and early carcinogenesis as shown by the increased expression of p53 and Ki-67 that occur as a result of cholecystectomy are similar to those identified as precursors of Barrett's oesophagus which itself is a major risk factor for oesophageal adenocarcinoma.

Iatrogenic DGOR-mediated mucosal injury caused by cholecystectomy and its implications for the projected future incidence of upper gastrointestinal tumorigenesis raise serious concerns. Of note, the prevalence of cholecystectomy in the population of the state of Ontario rose by 30% from 1989-1996(435), while the incidence of oesophageal adenocarcinoma rose by 4% year-on-year over the same time-period, with further accelerated growth-rate noted in the last decade(436). While rate-changes in cancer incidence always lags behind changes in population exposure-rates to carcinogens, and while the aetiology of adenocarcinoma is multifactorial and involves many environmental causes (smoking, alcohol etc.), it must be extrapolated that cholecystectomy-induced DGOR may play at least some part in this increase.

These observations raise concerns about the long term safety of cholecystectomy and support the decisions to avoid cholecystectomy in patients with gallstones where indications for the surgery are uncertain. It is important for surgeons to understand the changes brought about by cholecystectomy. It may be time to reconsider alternatives to cholecystectomy in patients with gallstones in a functioning gallbladder. The ability to medically alter the concentration and composition of secreted bile to reduce the lithogenic elements in patients with a family history of gallstones would also be a major advance. We believe that the studies presented in this thesis have

provided new insights into the risk factors for upper gastro-intestinal adenocarcinomas.

CHAPTER 8

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Appendix 1

Patient Information Leaflet

Patient information leaflet

Effects of Cholecystectomy on Oesophageal and gastric mucosa

Investigators:

Dr. Syeda Nadia Shah Gilani, Professor Thomas Noel Walsh.

Introduction:

You are being asked to take part in a clinical research project taking place at Connolly Hospital. Before you decide whether you wish to take part in this research, you should read the following information carefully and if you wish to discuss it with your family, friends or GP please do so prior to agreeing to participate. You should clearly understand what is required during this study so that you can make a decision that is right for you. You may change your mind at any time without having to justify your decision and without impact on the care you receive.

This study is approved by an independent body that safeguards the rights, safety and well-being of people in these kinds of research studies – Connolly Hospital Research Ethics Committee.

Background:

You are attending Connolly Hospital for upper G.I endoscopy (camera test). At the time of endoscopy, it is very common to take biopsy (a small sample of tissue from gullet, stomach or small intestine). This biopsy is then sent for

further examination under the microscope (histology) to confirm the presence or absence of a certain disease process. The remainder of tissue obtained at endoscopy is then saved in histology lab in Connolly Hospital. In our study we will use the biopsies taken for histology examination. We will study different markers like p53, Ki-67 and Bcl-2 on the cells obtained from biopsy during endoscopy. Please do not hesitate to contact me if you would like to know more about the markers or this study project.

Safety:

Patients taking part in this study will not be exposed to any additional test for this research project. No additional risk will be added to the risks of having an endoscopy (camera test) as a result of this study.

Finally:

My name is Dr. S. Nadia S. Gilani and I will be pleased to answer any questions you may have about this study. I can be contacted at the department of Surgical Research, Academic Centre, Connolly Hospital, Blanchardstown, Dublin-15.

Tel: 6465659

Email: syedagilani@rcsi.ie

Appendix 2

Patient Consent of Participation

Patient Consent of Participation

Project title: Effect of cholecystectomy on gastric and oesophageal mucosa

I _____ of _____

Here by consent to participate in the above named study. I have read the information presented in the patient information leaflet and I had the opportunity to ask questions related to this study, receive satisfactory answers to my questions, and any additional details I wanted.

I fully understand the objectives of this study, which is to study epigenetic changes in mucosal biopsies of oesophagus and stomach after cholecystectomy.

I am aware that I may withdraw from the study without any obligation or compromise to my treatment in this hospital. I am also fully aware that my individual privacy will be maintained in all published and written data resulting from this study. With full knowledge of all foregoing, I agree of my own free will to participate in this study.

Print Name

Signature of Participant

Date

Witness signature