THE INFLUENCE OF GENDER ON THE AETIOLOGY OF GASTRO-OESOPHAGEAL REFLUX, BARRETT'S OESOPHAGUS AND OESOPHAGEAL ADENOCARCINOMA

by

SHYAM SUNDAR MENON

Department of Gastroenterology, Sandwell General Hospital, West Bromwich

A Thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF MEDICINE

School of Cancer Sciences
The University of Birmingham
September 2011

Abstract

Symptoms of gastro-oesophageal reflux disease are equally common in both sexes and at all ages. However, complications of gastro-oesophageal reflux disease such as reflux oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma, although more common in men, increase sharply in older women, suggestive of a protective effect of female sex hormones in menstruating women. Oestrogen has anti-inflammatory properties, improves healing in oral and skin wounds and may therefore reduce the severity of reflux-induced oesophageal mucosal injury, consequently protecting women from developing severe reflux oesophagitis. Long-term oestrogen treatment with hormone replacement therapy seems to be additionally associated with a reduction in the risk of oesophageal cancer. Moreover, there are gender-specific genotypic differences in the response of oesophageal mucosa to chronic acid reflux suggestive of multiple factors that may play a role in explaining the male predominance of oesophageal adenocarcinoma.

Finally, oestrogen has no association with the severity of acid reflux once adjustment is made for the influence of increasing body mass index in women undergoing oesophageal pH monitoring.

The gender difference in the prevalence of gastro-oesophageal reflux disease and its complications may thus be related to the effect of female sex hormones, particularly oestrogen and its 'protective' effect in pre-menopausal women.

Dedication

I dedicate this thesis to my wife Shweta whose patience, support and advice made this work possible.

Acknowledgements

I am grateful to the following without whose help and support this thesis would not have been possible:

The patients who participated in the studies and who bore any inconvenience or discomfort with good humour.

My supervisor Nigel Trudgill for his patience, enthusiasm, guidance and invaluable advice.

Sandra Prew for her invaluable help with various projects.

Dr. Mark Anderson of the Department of Gastroenterology, City Hospital, Birmingham for his advice and assistance.

Dr. Chris Tselepis of The Institute for Cancer studies, University of Birmingham, for his advice and assistance.

Dr. John Arrand of The Institute for Cancer studies, University of Birmingham, for performing tissue microarray analysis as part of one the projects in the study.

Stephanie Evans of the Department of Gastroenterology, University Hospital of North Staffordshire for her assistance with recruiting patients, performing pH studies and collecting samples.

Gillian Parkes of the Department of Gastroenterology, Sandwell General Hospital and Nancy Moreton of Department of Gastroenterology, Princess Royal Hospital, Telford, for their assistance with recruiting patients and collecting samples.

Lynne Smith of the Department of Gastroenterology, Northern General Hospital, Sheffield, for her advice and assistance.

Peter Nightingale of Wolfson computer laboratories, University of Birmingham, for his statistical advice.

Alan Wall and Pervaz Mohammed of Clinical Biochemistry, Sandwell General Hospital and Mandy Donaldson of Clinical Biochemistry, Charing Cross Hospital, London, for their assistance with hormonal assays.

The staff at the Sandwell Day Unit, Sandwell General Hospital who helped me with my projects.

Contents

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Conclusions and Implications

Chapter 10

References

Chapter 11

Appendices

List of Illustrations

Figures

Chapter 1

Chapter 4

Chapter 5

Chapter 8

List of Tables

Chapter 4

Chapter 6

Chapter 7

Chapter 1

An introduction to the aetiology and pathophysiology of gastro-oesophageal reflux disease, its complications and relationship to gender.

1 Introduction

Gastro-oesophageal reflux, manifesting as heartburn and acid regurgitation affects nearly 30% of the UK population (Kennedy and Jones 2000). It is common during pregnancy and up to 70% of women develop acid reflux symptoms during pregnancy (Marrero et al. 1992). Gastro-oesophageal reflux disease (GORD) includes all individuals exposed to the risk of physical complications from gastro-oesophageal reflux or who experience clinically significant impairment of quality of life due to reflux related symptoms after reassurance of the benign nature of their symptoms (Dent 1998).

1.1 Epidemiology

Population surveys suggest a high degree of prevalence of acid reflux symptoms in the Western world. The prevalence of heartburn has been reported to be between 29-44% and between 10 and 20% report heartburn on a weekly basis (Haque et al. 2000; Kennedy et al. 2000; Locke, III et al. 1997). These estimates are largely based on the assumption that heartburn or acid regurgitation are indicators of GORD. However, patients with endoscopic oesophagitis or Barrett's oesophagus, which are objective markers of reflux-induced oesophageal injury do not always have heartburn. A recent systematic review suggested a prevalence of GORD of 10-20% in the West and about 5% in Asia (Dent et al. 2005). A population based survey in the United States reported that 6% and 3% of respondents had heartburn and acid regurgitation respectively occurring more than twice weekly (Camilleri et al. 2005). A working group concluded that mild symptoms occurring more than two days a week or moderate to severe symptoms occurring more than one day a week should constitute 'troublesome' acid reflux (Vakil et al. 2006). However, the group also concluded that ultimately, it is the patient who determines if their reflux symptoms are troublesome.

1.2 Clinical features of gastro-oesophageal reflux disease

Gastro-oesophageal reflux produces a range of clinical symptoms, the common ones being heartburn and acid regurgitation (Vakil et al. 2006). Other symptoms may include dysphagia, chest pain and water brash. Extra-oesophageal manifestations include chronic cough, laryngitis and bronchospasm (Koufman 1991).

Heartburn is typically described as a burning sensation in the retrosternal area and is often worsened by a meal. Acid regurgitation is experienced as an 'acid' taste in the mouth due to the flow of refluxed acidic gastric content into the mouth. Dysphagia typically occurs due to the development of an oesophageal stricture as a result of severe and longstanding acid reflux. However, it may also be a symptom of oesophageal cancer. Acid reflux-induced chest pain may be severe enough to mimic angina pectoris (Richter 1991). Water brash is acid reflux induced hyper-salivation in which patients may secrete as much as 10ml saliva per minute. Nausea, belching and odynophagia (pain during swallowing) may also occur as symptoms secondary to acid reflux. Laryngitis, bronchospasm and cough may occur due to aspiration of refluxed gastric content into the larynx and the lung.

Despite the variability of GORD-related symptoms, ambulatory oesophageal pH monitoring suggests that only heartburn and acid regurgitation symptoms vary significantly between patients with objective differences in acid reflux values (Klauser et al. 1990).

1.3 Complications of gastro-oesophageal reflux

Complications of gastro-oesophageal reflux can be divided broadly into two categories: oesophagitis and complications related to the reparative processes associated with oesophagitis, namely Barrett's oesophagus, peptic stricture and oesophageal adenocarcinoma.

1.3.1 Reflux oesophagitis

Reflux oesophagitis is the culmination of several processes that result in movement of gastric juice from the stomach into the oesophagus followed by impaired clearance of acid from the oesophagus. The dominant mechanisms that account for acid reflux into the oesophagus are transient lower oesophageal relaxations (TLOSR), a hypotensive lower oesophageal sphincter and anatomic disruption of the gastro-oesophageal junction as that occurs with a hiatus hernia.

Oesophageal acid clearance is important in emptying the refluxed gastric fluid from the oesophagus and is measured as the period that the oesophageal pH remains less than 4.0 following an episode of acid reflux. Acid is cleared by oesophageal peristalsis and is completed by dilution of the residual acid by swallowed saliva. Prolongation of oesophageal acid clearance occurs in around one-half of patients with reflux oesophagitis due to either oesophageal peristaltic dysfunction or 're-reflux' occurring with hiatal hernias.

Acid reflux results in hydrogen ion diffusion into the mucosa, leading to cellular acidification and necrosis. Acid and pepsin, more than bile acids in duodeno-gastric reflux, seem to be the principal factor in causing mucosal and cellular changes in reflux oesophagitis. Acid exposure results in loss of integrity of the epithelial barrier resulting in inability of the epithelial cells to maintain intracellular pH and consequent cellular oedema. Acid-reflux induces oesophageal mucosal inflammation leading to oesophagitis.

Upper gastro-intestinal endoscopy is important in assessing the severity of reflux oesophagitis, which is graded by the Savary-Miller classification (Monnier et al. 1984) or the more recent Los Angeles classification (Lundell et al. 1999). These are descriptive classifications that grade the severity of oesophagitis based on the endoscopic severity of reflux-related oesophageal mucosal injury.

1.3.2 Peptic stricture

Peptic strictures are caused as a result of the reparative processes secondary to ulcerative oesophagitis and occur due to collagen deposition, which result in luminal narrowing as the collagen fibres contract. These strictures typically occur around the gastro-oesophageal junction, are usually short in length and are associated with symptoms of dysphagia and infrequently, oesophageal obstruction.

1.3.3 Barrett's oesophagus

Norman Barrett's seminal paper in 1950 described a congenital short oesophagus with intra-thoracic gastric columnar lining and gastric heterotopia in the oesophagus with ulceration (Barrett 1950). This was corroborated by Allison in 1953, which provided an explanation as to why columnar lining could occur in the distal oesophagus in patients with gastro-oesophageal reflux (Allison et al. 1953). Further studies confirmed the development of a columnar lined oesophagus in an animal model (Bremner et al. 1970). Columnar lined oesophagus (termed Barrett's oesophagus) comprises of a histological spectrum of gastric fundic type epithelium and junctional type epithelium, possessing the ability to withstand acid-peptic damage and a type of intestinal metaplasia characterised by the presence of goblet cells (Paull et al. 1976). Its significance arises from its malignant potential to develop into oesophageal adenocarcinoma (Haggitt 1994).

Chronic GORD leads to oesophageal mucosal injury in the form of oesophagitis. The subsequent mucosal repair process results in columnar metaplasia of the oesophageal epithelium with the appearance of goblet cells. The extent of metaplasia is dependent on the duration and the severity of the injury, the nature of the cytokine response and the degree of oesophageal mucosal resistance to these restitutive processes. The length of columnarisation is proportional to the severity of acid reflux.

Barrett's oesophagus is present in 8 % of patients undergoing routine endoscopy and is present in around 10% of patients with GORD symptoms (Mann et al. 1989; Spechler 1999; Winters, Jr. et al. 1987). The median incidence of Barrett's oesophagus from ten studies has been shown to be 1.17 %, corresponding to approximately 30 new cases of Barrett's oesophagus per year in a population of 250,000 (British Society of Gastroenterology 2006; Cameron et al. 1992; Cooper et al. 1987; Macdonald et al. 1997; Nandurkar et al. 1997; Ovaska et al. 1989; Prach et al. 1997; Spechler et al. 1994). These studies also suggest an increase in the rate of Barrett's oesophagus at 0.08 % per year between 1980 and 1996.

Barrett's oesophagus was not associated with symptoms of GORD in 44 % of cases in a population based endoscopic screening study in Sweden (Ronkainen et al. 2005). Other studies have shown Barrett's oesophagus in 5-25 % of cases in which heartburn was not a symptom (Gerson et al. 2002; Rex et al. 2003). Moreover, physiological studies have shown that Barrett's oesophagus seems to be relatively insensitive to the effects of acid refluxate, judged by the high number of false negative Bernstein tests in a study (Sloan et al. 1992).

Barrett's oesophagus is clinically measured at endoscopy by the vertical distance between the squamo-columnar junction and the gastro-oesophageal junction. Traditionally, 'short segment Barrett's oesophagus' represents a length of Barrett's segment less than 3cm and 'long segment' denotes a segment of Barrett's oesophagus greater than 3 cm in length. Patients with long segment Barrett's oesophagus tend to have more severe degrees of acid reflux than those with short segment Barrett's oesophagus (Loughney et al. 1998).

In order to standardise inter-observer variation in the assessment of length of a segment of Barrett's oesophagus, an endoscopic grading system called the Prague C & M Criteria has been suggested, based on the assessment of the circumferential extent (C) and the maximum vertical length (M) in centimetres (Sharma et al. 2006). A validation study found that the criteria had good inter-observer reliability for Barrett's oesophagus ≥1 cm in length (Lee et al. 2010).

The presence of intestinal metaplasia within the segment of Barrett's oesophagus is pathognomonic for its pre-malignant potential and is distinct from Barrett's oesophagus with fundic (gastric) epithelium, which has no malignant potential (Reid et al. 1987). Intestinal metaplasia can exist in short and long segment Barrett's oesophagus and is not always apparent at endoscopy. The length of the segment of Barrett's oesophagus has been studied in relation to its overall risk of dysplasia. Patients with short-segment Barrett's oesophagus tend to have a lower incidence of dysplasia due to the involvement of a smaller area of mucosa (Hirota et al. 1999; Sharma et al. 1997; Weston et al. 1997). The risk of oesophageal adenocarcinoma has been estimated to be 2-15 times higher in

patients with long-segment Barrett's oesophagus (Rudolph et al. 2000; Weston et al. 1997).

Neither the endoscopic length of Barrett's oesophagus nor the histological identification of intestinal metaplasia is individually relevant to the malignant potential of Barrett's oesophagus. It is the combination of the two that characterises its malignant potential.

Dysplasia in Barrett's oesophagus develops in around 5% of patients (Katz et al. 1998; Miros et al. 1991; O'Connor et al. 1999). Dysplasia can progress sequentially from low to high-grade, finally culminating in invasive cancer. Low-grade dysplasia is seen frequently in prospective series and can persist, regress or progress to high-grade dysplasia and subsequently to oesophageal adenocarcinoma in 10-50 % of cases (Miros et al. 1991; Reid et al. 1992). High-grade dysplasia can regress to low grade dysplasia and may also progress rapidly to oesophageal adenocarcinoma but generally tends to persist for a number of years prior to progressing into invasive cancer.

1.3.4 Oesophageal adenocarcinoma

The incidence of oesophageal adenocarcinoma has increased exponentially in the last 15 years and has been as high as 4-10% per year making its incidence the fastest rising in the Western world (Sampliner 2002). Oesophageal adenocarcinoma now occurs with greater frequency than oesophageal squamous cell carcinoma, which used to be the commonest type of oesophageal cancer for many decades. It is five times as common among Whites compared to Blacks and six to eight times more common in men compared to women, for reasons that are unclear.

The prognosis of oesophageal adenocarcinoma is poor, with 5 year survival having improved from 5 % to only 13 % since 1973 (Eloubeidi et al. 2003). The 1 % annual conversion of Barrett's oesophagus to oesophageal adenocarcinoma in the United Kingdom is higher than that of the United States, which has an annual conversion rate of 0.4 % (Jankowski et al. 2002; Sampliner 2002).

There is debate regarding the role of chronic acid reflux as an independent risk factor for oesophageal adenocarcinoma as more than 50 % of cases of adenocarcinoma have no history of symptomatic reflux (Bytzer et al. 1999). However, a Scandinavian study found that reflux symptoms were associated with an odds ratio of 7.7 for adenocarcinoma of the oesophagus and 2.0 for adenocarcinoma of the gastric cardia (Lagergren et al. 1999) with the risk greatest among patients with long-standing acid reflux (greater than 20 years). Patients with severe acid reflux symptoms had an odds ratio of 43.5 for oesophageal adenocarcinoma (Lagergren et al.1999). A meta-analysis concluded that the odds of oesophageal adenocarcinoma were increased five-fold if symptoms of acid reflux occurred on a weekly basis while the odds increased seven-fold if acid reflux symptoms occurred daily (Rubenstein et al. 2010). The risk of oesophageal adenocarcinoma is increased at least 30-fold in patients with Barrett's oesophagus.

Helicobacter pylori (H.pylori) can colonise areas of gastric metaplasia in the oesophagus but studies have demonstrated that H.pylori is not more common in patients with Barrett's oesophagus and its distribution is no different among patients with Barrett's oesophagus than in controls (Csendes et al. 1997; Loffeld et al. 1992; Newton et al. 1997). H.pylori colonisation may however, be an important factor for inflammation and intestinal metaplasia of the cardia, a premalignant lesion for adenocarcinoma of the cardia (Goldblum et al. 1998). H.pylori plays a significant role in the development of

gastric adenocarcinoma (Crowe 2005). H.pylori is associated with a relative achlorhydric state and the absence of H.pylori could therefore be a risk factor for the development of oesophageal adenocarcinoma. There seems to also be an association between the presence of CagA positive strains of H.pylori and a reduced risk of oesophageal adenocarcinoma (Labenz et al. 1997; Vicari et al. 1998; Xia et al.1998). A meta-analysis concluded that there was an inverse association between the prevalence of H.pylori infection and Barrett's oesophagus and oesophageal adenocarcinoma but not oesophageal squamous cell carcinoma (Rokkas et al. 2007).

1.4 Gender differences in the prevalence of gastro-oesophageal reflux disease and its complications

Symptoms of gastro-oesophageal reflux disease (GORD) are equally common in both sexes and at all ages (Mohammed et al. 2005). In community surveys in the UK (Kennedy et al. 2000;Mohammed et al. 2005), USA (Locke III et al. 1997), Finland (Isolauri et al. 1995) and New Zealand (Haque et al. 2000), gender was not found to influence the prevalence of acid reflux symptoms. However, there is a distinct gender difference in the prevalence of complications of GORD such as Barrett's oesophagus and oesophageal adenocarcinoma. The prevalence of Barrett's oesophagus was found to be 1% in men and 0.5% in women in a retrospective study (Cameron et al. 1992). A post mortem study also noted a higher prevalence of Barrett's oesophagus in men (Cameron et al. 1990). However, the gender difference in the prevalence of oesophageal adenocarcinoma is more marked than that of Barrett's oesophagus. The annual incidence of oesophageal adenocarcinoma was noted to be 2.5 per 10000 in Caucasian males and 0.3 in Caucasian females in the USA (Blot 1991). More recent estimates suggest an incidence of 5.69 per 100,000 among Caucasian men and 0.74 in Caucasian women (Brown et al. 2008). This male predominance of oesophageal adenocarcinoma cannot be explained by sex-related differences in risk factors associated with oesophageal cancer such as obesity, ethnicity or smoking (Cooper et al. 2009;Gammon et al. 1997). A recent study reported a lag of 17 years among women developing oesophageal adenocarcinoma compared to men and this coincides with the average age of onset of menopause in women (Derakhshan et al. 2009; Soules et al. 2001).

1.5 Aetiology of gastro-oesophageal reflux disease

1.5.1 Age

The influence of increasing age in the prevalence of gastro-oesophageal reflux symptoms has been investigated in three large surveys, which did not find any relationship (Haque et al. 2000; Kennedy et al. 2000; Locke III et al. 1997). However, in a community survey in Finland, acid reflux symptoms were twice as common in subjects over the age of 60 compared with those aged 20-39 (Isolauri et al. 1995). Gastro-oesophageal reflux in the elderly may be associated with a different pattern of symptoms compared to those in younger individuals. A study found that 30% of elderly subjects with oesophagitis did not have heartburn or acid regurgitation and instead, their main symptoms were dysphagia, vomiting and respiratory symptoms (Raiha et al. 1991). A large retrospective study determined that the prevalence of Barrett's oesophagus increased from 0.2% in 20-29 year olds to 0.9% in subjects over the age of 60 years (Cameron et al. 1992).

1.5.2 Obesity

Body mass index (BMI), (weight in kilograms divided by height in metres squared) is a measure of obesity and has been shown to be an independent predictor of at least weekly acid reflux symptoms in a community study in the USA (Locke III et al. 1997). A population study from Finland reported similar findings with BMI greater than 28.5 associated with a doubling of the prevalence of acid reflux symptoms (Isolauri et al. 1995). An epidemiological study in the USA also found an increased risk of a hospital diagnosis of oesophagitis or hiatus hernia with obesity (Ruhl et al. 1999). In a prospective series of patients undergoing endoscopy, the presence of hiatus hernia and reflux oesophagitis correlated with increasing weight-for-height ratio (Stene-Larsen et al. 1988).

Obesity is a risk factor for oesophageal adenocarcinoma and adenocarcinoma of the gastric cardia. A meta-analysis showed that the pooled odds ratio of developing oesophageal adenocarcinoma for patients with a body mass index of 25-30 kg/m2 was 1.52 and the odds increased to 2.78 for patients with a body mass index of greater than 30 kg/m2 (Hampel et al. 2005). Obesity, particularly central adiposity, increases the risk of gastro-oesophageal reflux and is thus an indirect risk factor for Barrett's oesophagus (Cook et al. 2008; Corley et al. 2007; Edelstein et al. 2007).

1.5.3 Alcohol

Alcohol ingestion has been shown to adversely affect basal LOS pressures and oesophageal peristalsis but there are no epidemiological data to support an association with acid reflux (Haque et al. 2000; Locke III et al.1997).

Population-based studies have not found an association between alcohol consumption and oesophageal adenocarcinoma. One study found that alcohol consumption was not associated with an increased risk of reflux oesophagitis, Barrett's oesophagus or oesophageal adenocarcinoma and that wine consumption was associated with a reduced risk of complications of acid reflux (Anderson et al. 2009). Another population based-study found that increased alcohol consumption was associated with oesophageal squamous cell carcinoma but not oesophageal adenocarcinoma or junctional adenocarcinoma (Pandeya et al. 2009).

1.5.4 Smoking

Cigarette smoking has been shown to be associated with an increased frequency of acid reflux episodes but with no difference in total oesophageal acid exposure during 24-h pH monitoring (Schindlbeck et al. 1987). However, smoking was associated with an increased prevalence of acid reflux symptoms in a Finnish community study (Isolauri et

al. 1995). A similar trend of a higher prevalence of acid reflux symptoms in smokers (37%) compared with non-smokers (26%) was reported in a community study in the UK (Kennedy & Jones 2000). A study in New Zealand reported that both current and ex-smokers had an increased prevalence of acid reflux symptoms (Haque et al. 2000).

Smoking has been shown to be associated with risk of oesophageal adenocarcinoma, particularly in patients with Barrett's oesophagus (Gammon et al. 1997; Gray et al. 1993; Pandeya et al. 2008). In a pooled analysis of ten population-based case-control studies and two cohort studies, the risk of oesophageal adenocarcinoma was 2.1 times greater in smokers compared to controls (Cook et al. 2010). This risk increased with total pack-years of smoking and reduced after smoking cessation but not to the level of never-smokers.

1.5.5 Drug therapy

A number of drugs have been shown to reduce basal lower oesophageal sphincter pressures and may contribute to gastro-oesophageal reflux. Beta adrenergic agonists and theophylline have been shown to lower the LOS pressure and reduce contractile amplitudes in the smooth muscle of the oesophageal body in a dose dependent manner, thereby potentially aggravating gastro-oesophageal reflux (Crowell et al. 2001). Not only is there an increased prevalence of GORD in asthma, but also Barrett's oesophagus (Sontag et al. 1992). Drugs used to treat asthma, such as beta -adrenergic agonists; inhaled steroids and theophylline have also been shown to be associated with Barrett's oesophagus in a community case-control study (Corley et al. 2006). Furthermore, a Scandinavian study of hospital episode statistics demonstrated that patients who were hospitalized for asthma and who had concomitant acid reflux had a 50% excess risk of developing oesophageal adenocarcinoma (Ye et al. 2001).

1.6 Pathophysiology of gastro-oesophageal reflux disease

There are significant mechanistic differences between patients with GORD and asymptomatic patients. One of the principal abnormalities is abnormally frequent episodes of acid reflux in patients with GORD (Demeester et al. 1976).

1.6.1 Gastro-oesophageal junction

1.6.1.1 Lower oesophageal sphincter (LOS)

The lower oesophageal sphincter (LOS) acts as a crucial barrier in preventing gastric contents from filling the oesophagus.

Attempts at characterisation of the lower oesophageal pressures date back to 1883 when Kronecker and Meltzer used air-filled balloons to assess lower oesophageal pressures. However, reliable recordings of oesophageal and LOS pressures were produced only after the development of low-compliance infusion systems (Pope 1967). Liebermann-Meffert et al first described the detailed muscular anatomy of the LOS (Liebermann-Meffert et al. 1979). Their anatomic studies detailed a band of increased muscle thickness running obliquely from the lesser to the greater curve of the stomach, merging with the circular muscle of the oesophageal body proximally and inserting into the gastric sling fibres distally. Boyle et al demonstrated that the intrinsic LOS tone corresponds to end-expiratory manometric pressure suggesting that crural contraction during inspiration, causing the characteristic inspiratory rise in manometric LOS pressure, is the key contribution of the diaphragm to basal LOS pressure (Boyle et al. 1985).

The LOS is a tonically contracted segment of smooth muscle at the gastro-oesophageal junction, 3.5 ± 0.7cm (mean ± 1 SD) in length with an end-expiratory basal pressure of 15 ± 5mm Hg in asymptomatic subjects (Zaninotto et al. 1988). Tonic contraction is an intrinsic property of the LOS as the neural toxin tetradotoxin has little effect on basal LOS pressure (Goyal et al.1976) and this tonicity is thought to be due to specific

biochemical and neurophysiological properties of its circular muscle, which is distinct from that of the oesophageal body. Its resting membrane potential is lower (Decktor et al. 1982); it has increased permeability to potassium (Schulze et al. 1978) and a higher intra-cytosolic concentration of calcium (Schlippert et al. 1979). The parasympathetic nervous system has an excitatory effect on the LOS as vagal blockade reduces basal LOS pressures in cats (Reynolds et al. 1984) and atropine causes a reduction of LOS pressure in humans (Dodds et al. 1981). Sympathetic neurons have an excitatory effect through α receptors (Gonella et al. 1979). However, α antagonists have only a transient effect on basal LOS pressure and it seems unlikely that sympathetic neurons have a major influence (DiMarino et al.1973). The LOS also relaxes to the level of gastric pressure to allow the passage of a swallowed bolus or whilst venting from the fundus of the stomach (Wyman et al. 1990). Inhibitory neurons that release nitric oxide (Yamato et al. 1992) mediate LOS relaxation produced by vagal stimulation. Studies have shown a number of biological agents to influence basal LOS pressure. Gastrin (Giles et al. 1969), motilin (Meissner et al. 1976), substance P (Reynolds et al.1984) and histamine (Kravitz et al. 1978) have been shown to increase basal LOS pressure. Secretin (Cohen et al.1971), cholecystokinin (Fisher et al. 1975), glucagon (Behar et al. 1979), vasoactive intestinal polypeptide (Behar et al. 1979), progesterone (Van Thiel et al. 1976), dopamine (Rattan et al. 1976) and somatostatin (Bybee et al. 1979) reduce basal LOS pressure. Despite the association of these agents to LOS pressure, studies examining their effect on LOS pressure have used non-physiological doses and therefore, their physiological significance is unclear.

The importance of the LOS has been demonstrated in animal experiments where the LOS has been surgically disrupted, resulting in gastro-oesophageal reflux

(Bremner, Lynch, & Ellis, Jr. 1970). Studies have shown lower mean LOS pressure among patients with acid reflux symptoms compared to controls (Behar et al. 1976; Pope 1967). Patients with severe reflux oesophagitis have been shown to have lower basal LOS pressure compared to those with milder degrees of oesophagitis (Stein et al. 1990). Animal data has suggested that reduced basal LOS pressure may be a consequence of lower oesophageal acid exposure but the lack of improvement of basal LOS pressure following healing of oesophagitis suggests that it is either irreversible in patients with GORD or is a primary event (Eastwood et al. 1975; Eckardt 1988; Howard et al. 1994). Conversely, low basal pressure is not found among all patients with GORD. A basal LOS pressure of less than 10mm Hg has poor sensitivity (58%) but reasonable specificity (84%) in discriminating patients with GORD (Richter et al.1982). Patients with acid reflux symptoms have been shown to have an impaired LOS response to increased intra-abdominal pressure by the Valsalva manoeuvre, raising both legs or using an abdominal binder (Cohen et al.1971). Typically, in asymptomatic patients, these manoeuvres resulted in a greater rise in LOS pressure compared to the rise in gastric pressure, whilst in patients with acid reflux symptoms, the LOS pressure-rise was lesser than the rise in gastric pressure, suggesting that sudden increases in intra-abdominal pressure (e,g bending) might be responsible for some episodes of gastro-oesophageal reflux.

1.6.1.2 Transient lower oesophageal sphincter relaxation (TLOSR)

These are abrupt, physiological falls in LOS pressure to the level of gastric pressure that are not associated with a primary or secondary peristaltic sequence. They occur spontaneously following a normal swallow, during an incomplete peristaltic sequence or in association with multiple swallows. In asymptomatic controls, 73-100% of reflux episodes are associated with TLOSR, 13-27% are associated with swallow-related LOS relaxation, 0-2% related to persistently absent basal LOS pressure and 0-5% related to

deep inspiration, coughing or abdominal straining (Dent et al. 1988; Mittal et al. 1988; Schoeman et al.1995). In patients with GORD, 48-73% of reflux episodes are related to TLOSR, 9% associated with swallow-related LOS relaxation, 2-42% related to persistently absent basal LOS pressure and 1-18% related to deep inspiration, coughing or abdominal straining (Mittal et al.1988; Penagini et al. 1996).

1.6.1.3 Hiatus hernia

A hiatus hernia is a prolapse of part of the stomach through the diaphragmatic oesophageal hiatus into the thorax. The relationship between a hiatus hernia and oesophageal symptoms has been recognised since the 1920s and Allison in 1951 proposed that acid reflux was related to the presence of a hiatus hernia (Allison 1951). However, it became apparent that many patients with a hiatus hernia did not have symptoms suggestive of acid reflux and the presence of a hiatus hernia had no effect on basal LOS pressure and did not influence the increase in LOS pressure in response to raised intra-abdominal pressure. Detailed understanding of the sphincter mechanism at the gastro-oesophageal junction and the impact of a hiatus hernia has led to an increased awareness of the role played by hiatus hernia in acid reflux.

The oesophagus passes through the oesophageal hiatus in the diaphragm at the level of T10. The muscle of the diaphragm arising from the vertebral column forms two muscle bundles or crura on either side of the aorta, which encircle the oesophagus before fanning out to join the central tendon of the diaphragm. The right crus provides the majority of the muscle fibres and tends to compress the lower oesophagus. The fibro-elastic phreno-oesophageal ligament anchors the distal oesophagus to the diaphragm and maintains it within the oesophageal hiatus (Kahrilas 1999). During swallowing, the longitudinal muscle of the oesophagus shortens, (Dodds et al. 1973) causing tenting of the

cardia of the stomach into the oesophageal hiatus. The phreno-oesophageal ligament subsequently returns the lower oesophagus to the oesophageal hiatus. Hiatus hernia encompasses a spectrum of anatomical changes from an exaggeration of the physiological temporary herniation of the hiatus during swallowing to a permanent hernia that fails to reduce following swallowing (Kahrilas 1999).

There is a high prevalence of hiatus hernia among patients with GORD, with studies reporting a prevalence of up to 84% among patients with oesophagitis compared to 13% in controls (Wright et al. 1979). It has been hypothesized that loss of the elastic properties of the phreno-oesophageal membrane with age leads to increased herniation with swallowing and a failure to return the lower oesophagus and the cardia to their normal anatomical positions (Kahrilas 1999). Oesophageal shortening can be induced by acid perfusion of the oesophagus in animals; raising the possibility that acid reflux may contribute to the development of hiatus hernia (Paterson et al.1994).

The prevalence and severity of gastro-oesophageal reflux symptoms and reflux oesophagitis have been reported to increase with age (Collen et al. 1995; Johnson and Fennerty 2004; Lee et al. 2007; Pandolfino et al. 2008). Although basal LOS pressure has been reported to remain relatively normal with increasing age, there is an age-related decrease in the intra-abdominal length of the LOS (Lee et al., 2007). Furthermore, oesophageal acid exposure appears to increase by 1.1% for each decade of life (Lee et al 2007). Patients older than 65 years have been reported to be less likely to have normal peristalsis compared with younger patients (Achem et al. 2003). Other investigators have found that secondary peristalsis that aids in clearing the refluxate occurs less frequently and is often defective in older patients (Helm et al. 1984; Ren et al. 1995). Salivary

bicarbonate production also diminishes with age, prolonging oesophageal acid exposure in the elderly (Sonnenberg et al. 1982).

The prevalence of hiatus hernia seems to increase with age (Menon and Trudgill 2011) and this may be related to fibromuscular degeneration and the consequent loss of elasticity of the structures around the diaphragmatic hiatus. Pregnancy in women is another potential factor that may weaken fibromuscular structures around the gastro-esophageal junction through raised intra-abdominal pressures. Osteoporosis and kyphosis have been shown to be associated with hiatus hernia in elderly Japanese women (Furukawa et al. 1999; Yamaguchi et al. 2002). A kyphotic spine as a result of osteoporosis and vertebral fractures may result in the loss of the physiological effect of the lumbar spine in balancing the effects of increasing intra-abdominal pressure with the result that these intra-abdominal forces may be directed more vertically at the gastro-esophageal junction, contributing to the weakening and widening of the esophageal hiatus. Increasing gastro-oesophageal reflux and consequent oesophagitis has been shown in an animal model to result in oesophageal shortening due to fibrosis, and the formation of a hiatus hernia (Eastwood 1986).

There is an association between hiatus hernia, reflux oesophagitis and Barrett's oesophagus, which are all more common in men (Cameron 1999). Increased intra-abdominal pressure in occupations that involve lifting may result in an increase in the pressure gradient between the intra-thoracic and intra-abdominal pressures, thereby pushing the oesophago-gastric junction upwards and increasing the risk of developing a hiatus hernia. A prospective endoscopy study demonstrated that inguinal hernia was 2.5-times more likely to be associated with hiatus hernia and this association was stronger in men compared to women (odds ratio 2.86), (De et al. 2004).

The increasing prevalence of GORD has paralleled an increase in the prevalence of obesity. A high BMI has been shown to be associated with an increased prevalence of GORD symptoms and endoscopic oesophagitis (Diaz-Rubio et al. 2004; Fisher et al. 1999; Nilsson et al. 2002). Obese patients have been shown in population based studies to be 2.5 times as likely as those with normal BMI to have GORD symptoms (El-Serag et al. 2005). Mechanistically, an increase in abdominal girth due to obesity can cause an increase in intra-abdominal pressure, which in turn can increase intra-gastric pressure. Increased intra-gastric pressure facilitates gastro-oesophageal reflux and may promote the development of hiatus hernia due to axial pressure strain through the diaphragm. High resolution manometric evaluation of the morphology of the gastro-oesophageal junction in obese patients has shown that there is both disruption of the gastro-oesophageal junction and a change in the gastro-oesophageal pressure gradient; thereby facilitating reflux (Pandolfino et al. 2006).

1.7 The influence of gender on the pathophysiology of gastro-oesophageal reflux disease

Sex hormones may play an important role in GORD in a variety of clinical states, including pregnancy, obesity and the post-menopausal state. The effect of sex hormones, particularly oestrogen, on the pathophysiology of GORD is a complex and poorly characterised topic. Understanding the mechanisms behind the effects of sex hormones in these states is crucial to understanding the pathophysiology of GORD and its sequelae.

1.7.1 Female sex hormones

1.7.1.1 Oestrogen

Synthesis of oestrogen during the reproductive years occurs in the granulosa cells of the ovary by the aromatization of androstenedione in the theca follicular cells to oestrone (E1), which is then converted to oestradiol (E2) by 17-β hydroxysteroid reductase. The adrenal cortex is the principal source of androstenedione and dehydroepiandrosterone (DHEA). These C19 steroids are synthesized from cholesterol (Figure 1).

Cholesterol is initially converted to pregnenolone by cytochrome (CYP) P-450 11A1 (Simpson et al. 2000), which is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD) or to 17α-hydroxy-pregnenolone by CYP17A1. 17α-hydroxy-pregnenolone is further metabolised to 17α-hydroxy-progesterone by 3β-HSD. CYP17A1-mediated metabolism of 17α-hydroxy-pregnenolone results in DHEA which is converted to androstenedione by 3β-HSD-2 (Blair 2010). Androstenedione can additionally arise from CYP17A1-mediated oxidation of 17α-hydroxy-progesterone. In postmenopausal women, circulating DHEA and androstenedione can undergo further metabolism to androgens and oestrogens in the breast. DHEA is further metabolised to androstenediol by 17β-HSDs and to androstenedione by 3β- HSD-1 (Blair 2010). 3β-

HSD-1 converts androstenediol to testosterone. Aromatase (CYP19) converts androstenedione to oestrone and testosterone to oestradiol. Aromatase is found in many tissues including adipose tissue, brain, placenta, blood vessels, skin, bone, and endometrium. Aromatase can also be found in uterine fibroids, breast cancer, and endometrial cancer. Oestrone is converted to oestradiol in breast tissue by AKR1C3 (aldo-ketoreductase 1C3) together with 17β-HSD-1, 7, and 12 (Blair 2010; Jansson 2009).

More than 60% of circulating oestrogen in the pre-menopausal state is oestradiol and the remainder is oestrone, produced from the extra glandular conversion of androstenedione in adipose tissue (Siiteri et al.1973). In the post-menopausal state, oestrone replaces oestradiol as the predominant form of oestrogen due to a reduction in the ovarian contribution to oestrogen synthesis. Because adipose tissue is a major site of extra glandular oestrogen production, net oestrone production is greater in obese women. Total oestrogen production in massively obese post-menopausal women may even be greater than in pre-menopausal women (Edman et al.1978; Hemsell et al. 1974).

60% of circulating oestrogen is bound to serum albumin, 38% to sex-hormone binding globulin (SHBG), whilst 2-3% is free (Rosner W 1996). The protein bound form of oestrogen is inactive. SHBG concentrations are lowered in obesity through inhibition of hepatic synthesis of SHBG by insulin, which increases with increasing BMI, causing a resultant increase in the free, bioactive form of oestrogen (Key et al. 2001).

Figure 1: Adrenal steroid synthesis

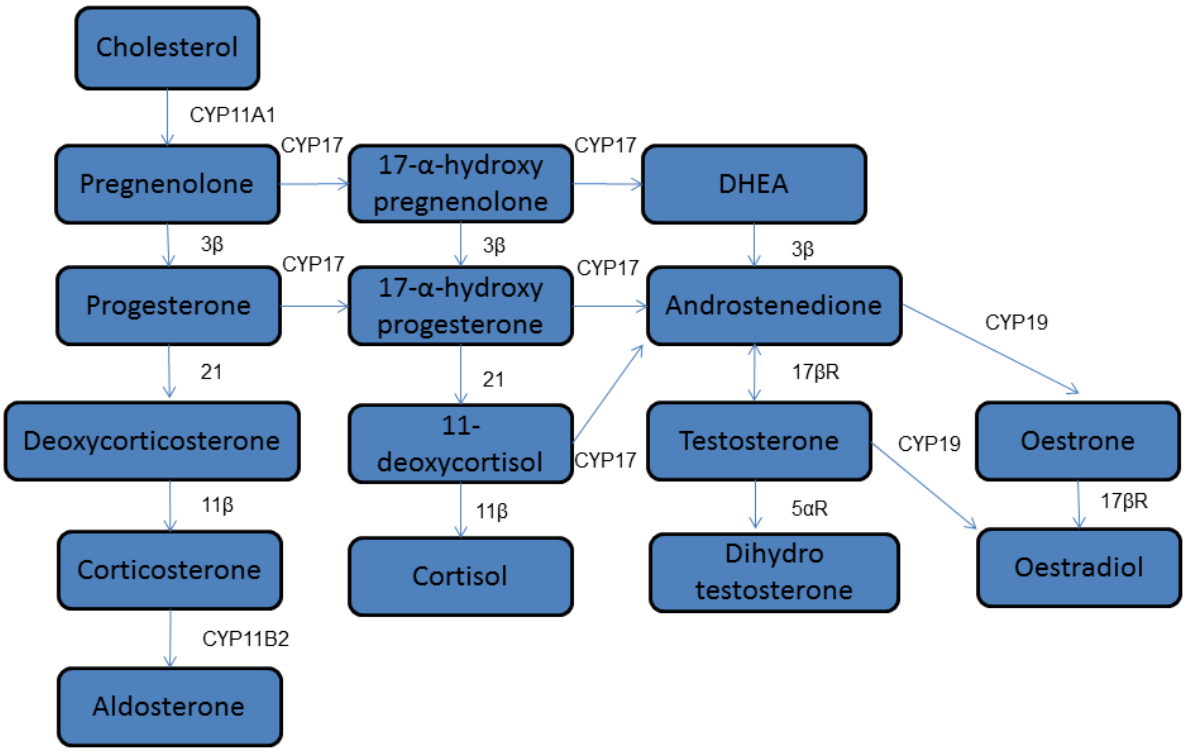


Figure 1: Adrenal steroid synthesis

The first step in adrenal steroid synthesis is the combination of acetyl CoA and squalene to form cholesterol, which is converted into pregnenolone by CYP 11A1. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD) or to 17α-hydroxy-pregnenolone by CYP17A1. 17α-hydroxy-pregnenolone is further metabolised to 17α-hydroxy-progesterone by 3β-HSD. CYP17A1-mediated metabolism of 17α-hydroxy-pregnenolone results in DHEA which is converted to androstenedione by 3β-HSD. Androstenedione can additionally arise from CYP17A1-mediated oxidation of 17α-hydroxy-progesterone. DHEA is further metabolised to androstenedione by 3β- HSD. 17β reductase (17βR) converts androstenedione to testosterone. Aromatase (CYP19) converts androstenedione to oestrone and testosterone to oestradiol. Deoxycorticosterone is converted to Aldosterone in the zona glomerulosa of the adrenal glands and cortisol, the androgens and oestrogen are synthesized in the zona fasciculata and zona reticularis.

CYP11A1: Cytochrome P450 11A1
CYP17 : Cytochrome P450 17A1
3β: 3β-hydroxysteroid dehydrogenase (HSD)
21: 21-hydroxylase (CYP21A2), 11β: CYP11B1 (11-β hydroxylase)
17βR: 17-β reductase, 5αR: 5-α reductase
CYP19: Cytochrome P450 19 (Aromatase)

The physiological effects of oestrogen are mediated through oestrogen receptors which are members of a family of nuclear hormone receptors that include receptors that bind other steroids, thyroid hormone and (Acevedo et al. 2004; McEwan 2004).

Two oestrogen receptor molecules have been identified: oestrogen receptor alpha (ER-α), and the oestrogen receptor beta (ER-β) (Kuiper et al. 1996). Their main components are the C or DNA-binding domain, which binds with high affinity and specificity to DNA sequences in the promoter regions of target genes (oestrogen response elements), and the E or ligand-binding domain, which binds oestrogens and oestrogen analogues. When an oestrogen or oestrogen analogue reaches the cell nucleus and binds to an oestrogen receptor, the conformation of the ligand-binding domain of the receptor is altered, allowing either interaction with co-activator molecules if the ligand is an agonist, but preventing this interaction if the ligand is an antagonist (Edwards 2000; Hewitt et al. 2005). The oestrogen receptor dimers bind to the response elements in target genes and induce transcription.

Oestrogen receptors such as epidermal growth factor and insulin-like growth factor-I can also function independently of oestrogen by acting through their extracellular membrane bound receptors (Curtis et al. 1996; Ignar-Trowbridge et al. 1995; Nelson et al. 1991).

Oestrogen also induces non-genomic effects such as the rapid induction of MAP kinase through direct action of oestrogen receptors that do not involve an ER-gene interaction (Coleman et al. 2001; Gray et al. 2001; Vasudevan et al. 2007). These responses are observed in diverse tissues, including the cardiovascular system, central nervous system, and in breast cancer cells (Pietras et al. 2007).

The mechanisms of oestrogen receptor-mediated regulation of target genes have been characterized using transgenic mice in which the oestrogen receptor genes or the gene for aromatase, the enzyme that converts androgens to oestrogens, have been knocked out (Bocchinfuso et al. 2000; Couse et al. 1999; Grumbach et al. 1999; Karas et al. 1999; Krege et al. 1998; Lubahn et al. 1993; Ogawa et al. 1998; Rubanyi et al. 1997). The main oestrogen-mediated effects are:

Female reproduction

In female mice, deletion of oestrogen receptor alpha or aromatase results in complete infertility, and deletion of oestrogen receptor beta results in subfertility.

Ovary

The ovaries of ER-α-deficient mice develop haemorrhagic cysts and lack mature follicles and corpora lutea, indicating the absence of ovulation, but they secrete increased amounts of oestrogen and testosterone (Krege et al. 1998; Lubahn et al. 1993), like the ovaries of transgenic mice that overexpress LH (Risma et al. 1995), indicating that the changes in the ovaries of the ER-α deficient mice are due to LH hypersecretion.

The ovaries of mice with ER-β deficiency are morphologically normal and gonadotropin administration leads to formation of fewer oocytes than are produced by similarly treated wild-type mice, indicating a role for oestrogen receptor beta in ovulation as well.

Uterus

Ovarian hormones are important for the initiation and maintenance of pregnancy. The pre-ovulatory peak in oestrogen secretion is crucial for proliferation of the uterine epithelium and prepares the epithelium for implantation.

Mammary tissue

The ductal tree of the mouse mammary gland elongates in response to oestrogen. In ER-α-deficient mice, the ductal rudiment fails to elongate (Bocchinfuso et al. 2000). However, in ER-β deficient mice the gland develops normally suggesting that ER-α is required for normal mammary gland maturation and development.

Non-reproductive tissues

The postmenopausal state is associated with an increase in risk of osteoporosis and cardiovascular disease indicating a role for oestrogen in bone and cardiovascular tissue. These tissues have been studied in oestrogen receptor- and aromatase-deficient mice.

Bone: Oestrogen receptor alpha and combined alpha/beta-deficiency is associated with sub-normal development of the femur in mice (Couse & Korach 1999).

Blood vessels: The protective effect of oestrogen on carotid injury has been studied in oestrogen-receptor deficient mice (Iafrati et al. 1997;Karas et al. 1999). ER-α deficient mice tend to have accelerated atherosclerosis which is not seen in oestrogen receptor beta-deficient mice (Pare et al. 2002).

Gastrointestinal Tract: Studies using the adenomatous polyposis coli (APC) Min/+ mouse, which bears a mutation in APC leading to intestinal adenomas, indicate that ovariectomy results in increased numbers of tumours, an effect that is reversed by

oestrogen replacement (Weyant et al. 2001). Studies in which ER-α or ER-β deleted mice were crossed to the APC Min/+ mouse suggest the protective effects of oestrogen are modulated by both oestrogen receptors (Cho et al. 2007).

1.7.1.2 Progesterone

Progesterone is a C-21 steroid hormone synthesized from pregnenolone and plays an important role in menstrual cycle, pregnancy and embryogenesis. Progesterone is produced in the corpus luteum of the ovaries following ovulation, the adrenals and in the placenta during pregnancy and is stored in adipose tissue.

Progesterone exerts its primary action through the intracellular progesterone receptor. The physiological effects of progesterone are amplified in the presence of oestrogen, which can upregulate the expression of progesterone receptors.

Progesterone effects are mediated by its nuclear receptor. Progesterone receptor (PR) proteins were originally characterised in the mammalian uterus and chick oviduct into two receptor proteins: PR-A and PR-B (Graham et al. 1997; Leavitt et al. 1972). These receptors form part of a family of ligand-activated nuclear transcription factors that include receptors for steroids, retinoids, thyroid hormones and vitamin D. Binding to the carboxyl-terminal ligand binding domain of PR results in the association of this dimer which is complexed with the progesterone receptor, with specific progestin response elements (PREs) in target genes (Graham et al. 1997). This, in turn results in modulation of transcription of these genes. The main effects of progesterone are on the uterus and ovaries, the mammary gland, the brain and the bone.

Progesterone levels decline post-menopausally and are very low in post-menopausal women.

Effects on the uterus and ovaries

Progesterone plays an important role in reproduction and is involved in ovulation, implantation, and pregnancy. Progesterone plays an important role in regulation of uterine function during the menstrual cycle by modulating the cyclical changes in endometrial proliferation and decidualization. High circulating progesterone levels are important for not only facilitating implantation, but also for maintaining pregnancy by stimulating uterine growth. Progesterone can both stimulate and inhibit cell proliferation in the uterus, in addition to a myriad of cellular functions such as stimulation of glycogenesis (Shapiro et al. 1980), nucleotide metabolism (Savouret et al. 1990) and cell cycle regulation (Clarke et al. 1990). Additionally, the proliferative effects in the uterus of Insulin-like growth factors (IGFs) are controlled by progesterone through the regulation of the IGF-binding protein 1 (IGFBP-1) (Graham et al. 1997).

Progesterone receptors are found in most follicular cell types and in the corpus luteum of the human ovary and studies on PR null mice have shown that there is failure of ovulation despite the presence of mature pre-ovulatory follicles, suggesting that progesterone plays an important role not only in ovulation but also the formation of the corpus luteum. Progesterone also increases relaxin in the endometrium of non-pregnant women and studies on relaxin in rat granulosa cells have suggested that an increase in relaxin may facilitate follicle rupture by increasing the secretion of plasminogen activator, collagenase, proteoglycanase and β-glucuronidase (Graham et al.1997).

Effects on the breast

Progesterone is responsible for the formation of the lobular and alveolar structures in the breast, particularly during pregnancy. Mammary glands in PR null mice develop ductal structures that are relatively similar to the wild type but fail to form an interductal lobular

structure on exposure to oestrogen and progesterone (Graham et al. 1997). Progesterone acts synergistically with oestrogen and prolactin during pregnancy to prepare for lactation by promoting lobuloalveolar development.

Effects on the brain

Both progesterone and oestrogen control specific brain functions involved in reproductive behaviour. The specific mechanisms by which progesterone acts within the brain are not fully defined but may be through the stimulation of γ- aminobutyric acid (GABA) signalling pathways (Graham et al. 1997).

Effects on bone

Expression of both PR in normal human osteoblast-like cells has been reported and evidence supports a role for steroid hormones in regulating the expression and function of matrix proteins and metalloproteinases involved in bone remodelling and resorption (Graham et al. 1997). Progesterone may have a role in bone matrix regulation through its effect on metalloproteinases.

1.7.2 The relationship between female sex hormones and acid reflux

Oestrogen increases nitric oxide synthesis in smooth muscle. Nitric oxide causes smooth muscle relaxation in animal models and in humans (De Man et al. 1991; Hirsch et al. 1998; Konturek et al. 1997). Oestrogen may therefore, directly promote acid reflux through its effects on nitric-oxide mediated relaxation of the lower oesophageal sphincter. The effects of oestrogen and progesterone on acid reflux have been studied mainly during pregnancy. Van Thiel et al analysed the serum levels of oestrone, oestradiol and progesterone during pregnancy in relation to lower oesophageal sphincter pressure changes and found a linear relationship between the reduction in lower oesophageal sphincter pressure and the progressive rise in hormonal levels, concluding that oestrogen and progesterone reduced lower oesophageal sphincter pressure (Van Thiel et al. 1977). Fisher et al similarly found that lower oesophageal sphincter function was reversibly inhibited during early pregnancy, when serum concentrations of oestrogen and progesterone were elevated (Fisher et al. 1978). Marrero et al suggested a direct relationship between urinary oestradiol and serum progesterone levels and the increase in the severity of GORD symptoms during the course of pregnancy, but the data on hormonal levels was obtained indirectly from published normal values during pregnancy (Klopper et al. 1963; Marrero et al. 1992).

1.7.3 The influence of age on the synthesis of female sex hormones

Ovarian follicular activity declines with age until menopause occurs, typically between the ages of 45-55 years and at an average age of 51 years (Belchetz 1994). Menopause is defined by twelve months of amenorrhoea after the final menstrual period. There is a concomitant reduction in oestrogen levels as ovarian function declines and a compensatory increase in the levels of follicle stimulating hormone from the anterior pituitary to maintain ovarian function. Menstruation finally ceases once the levels of oestrogen fall to levels suboptimal to promote endometrial development. Progesterone levels tend to decline precipitously as well.

A number of factors are thought to play a role in the age at which menopause occurs in women. Genome-wide association studies have identified 13 common single nucleotide polymorphisms (SNPs) associated with age at menopause, clustered in regions of chromosomes 5, 6, 19, and 20 (Stolk et al. 2009). In a study of 900 postmenopausal women, the mean age at menopause varied by 1.1 years among women with different alleles at the same locus of the oestrogen receptor gene (Weel et al. 1999). A family history of early menopause has also been linked to an earlier age at menopause. Studies on ethnicity have suggested there may be ethnic variation in the age of menopause. Two cohort studies suggested that menopause occurred earlier among Hispanic women and later among Japanese-American women when compared to Caucasian women (Gold et al. 2001; Henderson et al. 2008). The age of menopause is also reduced in women who smoke (Kaufman et al. 1980; Willett et al. 1983).

Cessation of ovarian function leads to various systemic manifestations. These include:

Vaginal bleeding

A rapid decline in ovarian progesterone production may result in unopposed oestrogen activity during the peri-menopausal period, which causes anovulatory bleeding and endometrial hyperplasia.

Hot flushes

Hot flushes occur in up to 75% of women and are most common during the late menopausal and early postmenopausal periods and are related to thermoregulatory dysfunction initiated at the level of the hypothalamus, precipitated by an oestrogen-withdrawal state. The core body temperature is normal at the onset of the hot flush, and falls below normal after it. Evidence for central mediation of the changes in temperature comes from studies demonstrating that hot flashes occur simultaneously with pulses of luteinizing hormone (Taffe et al. 2002). Hot flushes typically begin as the sudden sensation of heat centred on the upper chest and face that rapidly becomes generalized. The sensation of heat lasts from two to four minutes, is often associated with profuse sweating and occasionally palpitations, and is often followed by chills and shivering, and sometimes a feeling of anxiety.

Vaginal dryness

Lack of oestrogen leads to thinning of the vaginal epithelium, which is sensitive to oestrogen. The resultant vaginal atrophy causes symptoms of vaginal dryness, itching and often, dyspareunia (Dennerstein et al. 2000).

Osteoporosis

Bone loss secondary to lack of oestrogen begins prior to menopause as shown by prospective studies (Bainbridge et al. 2002; Chapurlat et al. 2000; Sowers et al. 1998). In a longitudinal cohort of women followed through the menopausal transition, there was little change in bone mineral density during the early peri-menopausal period but bone loss accelerated subsequently with annual rates of bone loss of 1.8 to 2.3 % in the spine and 1.0 to 1.4 % in the hip (Finkelstein et al. 2008). This confers to a significant risk of fractures in the post-menopausal period.

Cardiovascular disease

There exists historical evidence that menopause may be associated with a higher risk of cardiovascular events. However, a report from the Nurses' Health study suggested that bilateral oophorectomy and not menopause was associated with an excess risk of cardiovascular disease, when adjusted for age and smoking (Colditz et al. 1987). A subsequent analysis from the Women's Health Study showed that among women who were not taking hormone replacement therapy (HRT), those with low serum sex hormone binding globulin levels or a high free androgen index were at increased risk of cardiac events (Rexrode et al. 2003). However, this association was not significant after adjustment for BMI and other cardiovascular risk factors. Another factor suggestive of menopause not being directly responsible for the increase in cardiovascular disease risk is the lack of benefit of HRT in the Women's Health Initiative (primary prevention) and the HERS trials of secondary prevention (Grady et al. 2002; Hulley et al. 1998; Rossouw et al. 2002).

1.7.4 Hormone replacement therapy (HRT)

1.7.4.1 Indications and preparations

Hormone replacement therapy (HRT), comprising of either oestrogen-based replacement therapy or a combined (oestrogen and progesterone combination) was originally envisaged to treat post-menopausal symptoms such as hot flushes, vaginal dryness, urinary symptoms and emotional lability. However, its use extended to prevention of coronary heart disease and osteoporosis based on epidemiological data demonstrating a protective effect of oestrogen on the heart and bone. Data from the Women's Health Initiative study showed no cardiovascular benefit for unopposed oestrogen therapy and a small increase in risk with combined therapy. Follow-up analyses of this study suggested that the excess risk of coronary heart disease occurs mainly in older postmenopausal women (> 60 years) (Rossouw et al. 2007) and stroke risk appears to be independent of age with the absolute risk in younger postmenopausal women (50-60 years) being extremely small. There were also significant increases in the incidence of breast cancer and venous thromboembolism in patients on hormone therapy.

Following this study, postmenopausal hormone therapy prescriptions decreased significantly in the USA from 33-42% of women aged 50-74 years taking it between 1995-2001, to 28% by 2003 (Hersh et al. 2004). Current use of HRT is limited generally to patients with significant peri-menopausal symptoms such as hot flushes, vaginal dryness and urinary dysfunction.

1.7.4.2 Components of HRT

1.7.4.2.1 Oestrogen preparations

Oestrogen preparations are available in many forms: oral, transdermal, topical gels and lotions, intra-vaginal creams and tablets, and vaginal rings.

Systemic oestrogen

Systemic oestrogen is most often administered orally or transdermally. Oral oestrogen therapy has a first-pass effect leading to portal vein concentrations higher than those after transdermal application. Oral oestrogen therapy, therefore, increases the hepatic production of thyroxine-binding globulin, corticosteroid-binding globulin, sex hormone-binding globulin, triglycerides, high-density lipoprotein (HDL) cholesterol, and clotting factors, whereas their production is only minimally increased by transdermal oestrogen administration (Walsh et al. 1991).

A common oral oestrogen preparation is a mixture of sulpho-conjugated equine oestrogens (Premarin). Most of the other oral oestrogen preparations are derived from plant sources (soy and yams).

The potency and doses of oral oestrogen preparations are similar in efficacy. In general, 0.625 mg of conjugated oestrogens, esterified oestrogens or oestrone sulphate is considered equivalent to one mg of oestradiol, 0.05 mg of transdermal oestradiol, or 5 mcg of ethinyl oestradiol.

Transdermal oestrogen

Transdermal oestrogen is as effective as oral oestrogen in preserving bone density (Stevenson et al. 1990) and in treating menopausal symptoms. Transdermal oestrogen administration is associated with a lower risk of venous thromboembolism and stroke compared to oral oestrogen (Chetkowski et al. 1986).

There are many transdermal oestrogen preparations available that contain 17-beta oestradiol. There is a wide range of dosing options, from 25 mcg to100 mcg/day. A transdermal dose of 50 mcg/day is approximately equivalent to a 0.625 mg daily oral dose of conjugated oestrogens (Baker 1994).

Oestradiol is also available in topical preparations.

Vaginal preparations

Vaginal oestrogen is most commonly used in very low doses for the management of vaginal atrophy.

1.7.4.2.2 Progesterone preparations

Medroxyprogesterone acetate

The most commonly prescribed progestin is medroxyprogesterone acetate (MPA), typically given in a cyclic regimen (5 to 10 mg/day) or continuous regimen (2.5 mg/day).

Testosterone derivatives

Progesterone preparations can be either MPA or may be derived from testosterone (19-nortestestosterone), such as norethindrone acetate. These progestins are more commonly used in oral contraceptive preparations and have weak androgenic actions.

Drospirenone

Drospirenone, a newer progestin, is derived from 17 alpha-spironolactone and has progestogenic, antiandrogenic, and antimineralocorticoid activity. The first formulations to contain drospirenone were monophasic oral contraceptives containing 30 and 20 mcg of ethinyl oestradiol and 3 mg of drospirenone (Yasmin).

Intrauterine device

A levonorgestrel-containing intrauterine device (IUD) that releases 20 mcg of levonorgestrel per day is available for contraceptive use and has also been used for endometrial protection in peri-menopausal and postmenopausal women using oestrogen therapy.

1.7.4.3 HRT use and gastro-oesophageal reflux disease

HRT, principally containing oestrogen, has been reported to be associated with acid reflux in previous studies (Jacobson et al. 2008; Nilsson et al. 2002; Nilsson et al. 2003). The mechanism hypothesized to account for increased acid reflux is oestrogen-related relaxation of the lower oesophageal sphincter mediated through nitric oxide. However, the effect of HRT in increasing acid reflux symptoms in these studies was modified by BMI. A similar effect was seen with the use of HRT in post-menopausal female twins, in which the risk of acid reflux symptoms, which was found to be higher in the HRT group, was attenuated once corrected for BMI (Nordenstedt et al. 2008).

An association between HRT use and acid reflux symptoms has been described in the Nurses Health Study, with a similar modest increased risk in ever and current HRT users (Jacobson et al. 2006; Jacobson et al. 2008). Oestrogen-only HRT, combined HRT and selective oestrogen receptor modulators (SERM) such as Raloxifene were all associated with acid reflux symptoms in this study after adjusting for BMI (Jacobson et al. 2006).

1.7.4.4 HRT use and cardiovascular risks

The large women's health initiative (WHI) set of trials included two hormone trials using unopposed oestrogen (around 11,000 women) and continuous, combined oestrogen-progesterone therapy (around 16,000 women) in healthy postmenopausal women aged 50-70 years (Rossouw et al. 2002). These trials sought to determine the risk

of cardiovascular events in women on HRT. Both hormone based trials were discontinued in 2002 (combined oestrogen-progesterone based HRT trial) and in 2004 (oestrogen-only HRT trial) due to an increased risk of breast cancer, coronary heart disease, stroke, and venous thromboembolism with combined HRT used and an increased risk of stroke with oestrogen-only HRT.

1.7.4.5 HRT use and coronary events

The overall rate of coronary events increased with combined HRT use (39 versus 33 per 10,000 person-years for HRT and placebo respectively, hazard ratio 1.24 (95% CI 1.00-1.54). With oestrogen-only HRT, the incidence of coronary events did not markedly differ from placebo (hazard ratio 0.91 (0.75-1.12) HRT versus placebo) and despite a suggestion of a protective effect among women aged 50-59, the result was not statistically significant (Rossouw et al. 2002).

In a subsequent analysis that included 13 additional cases of coronary heart disease, the risk of coronary disease appeared to be lower among women less than 10 years since menopause. Coronary revascularization was significantly less frequent in women aged 50-59 years on oestrogen-only HRT compared to placebo (Rossouw et al. 2007). For the age groups of 50 to 59 years, 60 to 69 years, and 70 to 79 years, the hazard ratios for coronary heart disease were 0.93, 0.98, and 1.26, with absolute excess risks of -2, -1, and +19 per 10,000 person-years. This trend did not reach statistical significance. For women less than 10, 10 to 19 years or more than 20 years since menopause, hazard ratios for CHD were 0.76, 1.10, and 1.28, respectively, with absolute excess risks of -6, +4, and +17 per 10,000 person-years (p for trend = .02). A possible reduction in total mortality was also seen in women closer to menopause; HR for ages 50 to 50, 60 to 69, and 70 to 79 years were 0.70, 1.05, and 1.14, respectively.

1.7.4.6 HRT and stroke

A 31% increase in stroke risk was seen with combined HRT use compared to placebo (hazard ratio 1.31 (95% CI 1.02-1.68)) (Wassertheil-Smoller et al. 2003). Hazard ratios for ischaemic and haemorrhagic stroke were 1.44 (1.09-1.90) and 0.82 (0.43-1.56) respectively. The risk of stroke was seen in all age groups and was independent of other known risk factors for stroke. Stoke risk was similarly increased with oestrogen-only HRT compared to placebo (hazard ratio (1.39 (1.1-1.77)).

1.7.4.7 HRT and venous thromboembolism

The rate of venous thromboembolism in the women's health initiative trials increased with combined HRT use (34 versus 16 per 10,000 person-years, hazard ratio 2.06 (95% CI 1.6-2.7)) (Cushman et al. 2004). Risk of both deep vein thrombosis and pulmonary embolism increased similarly. Oestrogen-only HRT was also associated with increased risk of venous thromboembolism compared to placebo (hazard ratio 1.33 (0.99-1.79) but this result did not reach statistical significance.

1.7.4.8 HRT and breast cancer

The risk of invasive breast cancer significantly increased with combined HRT at an average follow-up of 5.6 years (hazard ratio 1.24 (1.01-1.54)). Combined HRT use was not only associated with larger primary cancers at the time of diagnosis compared to placebo, but also a higher percentage of women with positive lymph nodes. In contrast, with oestrogen-only HRT, a trend towards a slightly lower rate of breast cancer risk was seen (hazard ratio 0.77 for oestrogen-only HRT versus placebo, 95% CI (0.59 to 1.01) (Anderson et al. 2004). However, this was not statistically significant ($p = 0.06$).

1.7.4.9 HRT and osteoporosis

Combined HRT use in with women's health initiative trials was associated with a reduced risk of osteoporotic fracture at the hip (hazard ratio 0.67 (0.47-0.96)) and at the vertebrae and wrist (hazard ratio 0.65 (0.46-0.92) and hazard ratio 0.71 (0.59-0.85), respectively) (Cauley et al. 2003). Oestrogen-only HRT use was similarly associated with a reduced risk of hip fracture (0.61 (0.41-0.91) and vertebral fracture (0.62 (0.42-0.93)).

In a prospective cohort study of over one million women (the Million Women Study), current users of postmenopausal hormone therapy had a significantly lower risk of any fracture than nonusers (relative risk 0.62, 95% CI 0.58-0.66) (Banks et al. 2004). The protective effect was seen for all types of postmenopausal hormone therapy (oestrogen-only HRT, combined HRT, different routes of administration (oral versus transdermal), and different patterns of administration (cyclic versus continuous).

1.7.4.10 HRT and colorectal cancer

The risk of colorectal cancer in the women's health initiative study was reduced with combined HRT use (43 versus 72 cases in the HRT and placebo groups respectively, hazard ratio 0.56 (0.38-0.81)) (Chlebowski et al. 2004). In contrast, no significant risk reduction was noted with oestrogen-only HRT use (hazard ratio 1.08, 95% CI 0.75 to 1.55).

Large epidemiologic studies and a meta-analysis have shown a significant reduction in the risk of colorectal cancer with HRT use (Calle et al. 1995; Fernandez et al. 1998; Grodstein et al. 1999; Johnson et al. 2009; Rennert et al. 2009).

1.7.4.11 HRT and ovarian and endometrial cancers

In the women's health initiative trial, a non-significant increase in the risk of ovarian cancer was noted with combined HRT use (42 cases versus 27 per 100,000

person-years for HRT and placebo respectively, hazard ratio 1.6 (0.8-3.2)) (Anderson et al. 2003).

More women in the HRT group required assessment of vaginal bleeding with endometrial biopsies (33% versus 6% for placebo), but there was no significant difference in endometrial cancer risk between the HRT and placebo groups (hazard ratio 0.81 (0.48-1.36)).

1.8 The role of oestrogen in mucosal healing

1.8.1 Immune response in reflux oesophagitis

The inflammatory infiltrate in reflux oesophagitis is comprised of acute inflammatory cells with a preponderance of T lymphocytes in metaplastic foci. This can cause free radical formation, leading to the induction of cytokines, which modulate matrix degrading enzymes such as metalloproteinases. TNF-α, TGF-β, IL-1β and IFN-γ are all released as part of the inflammatory process (Jankowski et al. 2000). IL-8 released during oesophageal inflammation can mediate T-cell and neutrophil recruitment. This was shown in a study where reflux-mediated injury to squamous oesophageal tissue in a murine oesophago-duodenostomy model resulted in IL-8 secretion which caused an increase in the migration rates of both T cells and neutrophils (Souza et al. 2009). The addition of IL-8 blocking antibody significantly reduced the migration of neutrophils but did not affect T-cell migration.

1.8.2 Oestrogen and the immune response

There is evidence in the literature for a sexual dimorphism in the immune response (Grossman 1989). Women in their reproductive years have a more robust humoral and cellular immune response compared to males, reflected in the observations that females possess a more developed thymus, higher antibody concentrations and a greater capacity to reject tumours (Olsen et al. 1996). Physiologic levels of oestrogen stimulate the immune response whereas high levels, such as during pregnancy, down regulate cell-mediated immune responses. Oestrogen receptors are found in T cells, monocytes and macrophages and oestrogen mediated modulation of immune cell activity includes changes in cytokine production, cell activation and proliferation (Cocchiara et al. 1990; Gulshan et al. 1990; Mao et al. 2005; Weusten et al. 1986).

1.8.3 Role of oestrogen in wound repair

In vitro studies have suggested that neutrophil chemotaxis to a chemoattractant is inhibited in a dose-dependent manner by oestrogen (Ashcroft et al. 1999;Ito et al. 1995). Oestrogen also abolishes the L-selectin expressing subpopulation of neutrophils, which results in an inability of neutrophils to localise at sites of inflammation. This phenomenon has been primarily studied in cutaneous wound healing but may also explain other pathophysiological mechanisms, such as the inhibition of myocardial infiltration by neutrophils after reperfusion injury, by oestradiol (Delyani et al. 1996). The suppression of neutrophil chemotaxis to a wound results in reduced neutrophil elastase activity. Elastase activates matrix metalloproteinases (Ferry et al. 1997) and a reduction in neutrophil elastase activity results in better wound integrity during healing. Moreover, the suppression of neutrophil recruitment and accumulation at the site of injury results in reduced fibronectin degradation due to elastase. Fibronectin is an essential component of wound repair, influencing re-epithelisation, collagen deposition and wound contraction. The consequent increase in fibronectin enhances fibroblast influx and collagen deposition in the wound.

Oestrogen therefore influences wound healing by modulating the inflammatory response, cytokine expression and matrix deposition; by accelerating re-epithelialization; by stimulating angiogenesis and wound contraction; and by regulating proteolysis. This has been demonstrated in animal models where oestrogen deprivation in ovariectomized female mice results in markedly impaired wound healing with enhanced inflammation, delayed re-epithelialization, increased wound size and decreased collagen deposition (Ashcroft et al. 1997). Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine produced by monocytes, macrophages, neutrophils and endothelial

cells (Baumann et al. 2003; Calandra and Roger 2003; Hardman et al. 2005). Oestrogen has been shown to inhibit NF-κB and recent microarray work on a murine model of wound healing suggests that oestrogen mediated down-regulation of NF-κB reduces the transcription of MIF leading to a down-regulation of MIF and TNF-α expression in monocytes (Ashcroft et al. 2003; Evans et al. 2001). This leads to reduced inflammation and accelerated wound repair. Oestrogen may modulate angiogenesis by a direct effect on endothelial cells. Migration of oestradiol-treated endothelial cells into a wound occurs three-times faster than untreated cells and oestradiol enhances the ability of endothelial cells to form capillary-like structures when placed on a reconstituted basement membrane (Morales et al. 1995). These angiogenic effects can be blocked by oestrogen receptor antagonists (Ashcroft et al. 2003). Oestrogen stimulates the expression of platelet-derived growth factor (PDGF) by monocytes and macrophages (Shanker et al. 1995). PDGF is mitogenic and chemotactic for dermal fibroblasts and stimulates fibroblast-mediated wound contraction (Katz et al. 1991). An oestrogen-mediated increase in secretion of TGFβ-1 by fibroblasts inhibits the degradation of the extracellular matrix, stimulates the formation of granulation tissue, and promotes collagen deposition (Beck et al. 1993).

1.8.4 Oestrogen and wound repair in the elderly

There is evidence to support differential gene expression in wound healing in the elderly, which may be due to the relative lack of circulating oestrogen with age. Wound healing gene expression is altered in wounds from elderly subjects and has been shown to be regulated by oestrogen. Genes with attenuated expression in the elderly include the pro-healing growth factor TGFα (down-regulated) and genes linked to chronic wound healing such as arginase 1 (ARG1, down-regulated). L-arginine, an essential wound healing amino acid, is converted to nitric oxide, which acts to regulate inflammation. ARG1 metabolises L-arginine to generate proline, a substrate for collagen synthesis.

Hence, a down-regulation of ARG1 in the elderly due to lack of oestrogen results in an imbalance between tissue inflammation and matrix deposition in a wound, resulting in an increase in inflammation and decrease in matrix deposition in the aged (Hardman et al. 2008).

Topical application of oestrogen on wounds in elderly subjects has been shown to significantly increase wound collagen deposition and decrease fibronectin degradation (Ashcroft et al. 2003). A study on HRT-naïve post-menopausal women demonstrated this finding where wounds showed retarded re-epithelialization at 7 days whereas those who had taken HRT for at least 3 months displayed rates of re-epithelialization similar to those in the pre-menopausal women (Gilliver et al. 2007). Topical oestrogen application to wounds in another study resulted in improved wound healing in post- menopausal women compared to controls (Ashcroft et al. 1999).

1.8.5 Oestrogen and vaginal atrophy

The mucosa of the vagina is composed of stratified squamous non-keratinizing epithelium, similar to the oesophageal mucosa. Oestrogen helps to maintain the collagen content of vaginal epithelium, its glycogen content and helps maintains optimal vaginal blood flow (Castelo-Branco et al. 2005). Lack of oestrogen after menopause causes urogenital atrophy, characterized by vaginal dryness, pruritus, inflammation and dyspareunia. This is characterized by thinning of the top layer of epithelial cells, loss of elasticity of the vaginal epithelium, increased sub-epithelial connective tissue, loss of rugae and shortening and narrowing of the vaginal canal. Thinning of the vaginal epithelium exposes the layer below, which is more easily traumatized and more vulnerable to inflammation or infection, analogous to an injury model.

Oestrogen therapy leads to thickening of the epithelium, increased vaginal secretions and decreased vaginal dryness. These effects are mediated through oestrogen receptors localised to the basal and supra basal cells of the vaginal epithelium (Hodgins et al. 1998). Oestrogen receptors are also localised in the stromal cells, suggesting that oestrogen may mediate stromal-epithelial interactions. Oestrogen has also been shown to regulate vaginal Nitric oxide synthase (NOS) expression in murine models (Berman et al. 1998). NOS forms nitric oxide (NO), which is an important smooth-muscle relaxant and serves as a key molecule in host-defence mechanisms through its actions as a free-radical (Wink et al. 1993). NO may inhibit apoptosis in oestrogen-targeted tissues and a study on a mouse model demonstrated diminished apoptosis and vaginal atrophy on oestrogen replacement in oophorectomized mice with a significant increase in NOS expression (Chun et al. 1995).

These effects demonstrate the effects of oestrogen in maintaining epithelial integrity.

Summary of introduction to the aetiology and pathophysiology of gastro-oesophageal reflux disease, its complications and relationship to gender.

Gastro-oesophageal reflux disease occurs commonly and is characterised by a variety of clinical manifestations. Complications of chronic gastro-oesophageal reflux include reflux oesophagitis, oesophageal stricture, Barrett's oesophagus and oesophageal adenocarcinoma. There has been a marked increase in the incidence of oesophageal adenocarcinoma in the last decade and it has a marked male predominance. The reasons for this male predominance remain unclear and there may be a role for oestrogen offering 'protection' from reflux injury in menstruating women, possibly through its anti-inflammatory action, and preventing the development of severe oesophagitis and thus Barrett's oesophagus and oesophageal adenocarcinoma.

Chapter 2

Aims

Aims

1. To determine the influence of age and gender on endoscopic findings of gastro-oesophageal reflux.
2. To investigate the role of oestrogen in mucosal healing by creating an acute oral wound model in order to determine the effect of lack of systemic oestrogen using oophorectomized mice and subsequent oestrogen replacement on buccal mucosal healing.
3. To determine the influence of HRT on the complications of gastro-oesophageal reflux disease.
4. To determine the role of female sex hormones in excess acid exposure on oesophageal pH monitoring.
5. To evaluate gender-specific tissue responses in the oesophagus to acid reflux.

Chapter 3

Materials and Methods

3 Methods

3.1 Endoscopy database analysis

A retrospective analysis of the endoscopy databases of six hospitals near Birmingham (UK) was carried out: Birmingham Heartlands Hospital (BHH), City Hospital (CH), Good Hope Hospital (GHH), Princess Royal Hospital, Telford (PRH), Royal Shrewsbury Hospital (RSH) and Sandwell General Hospital (SGH). Permission was obtained from individual R&D departments and endoscopy lead clinicians to access the database.

Endoscribe™ (Unisoft Medical Systems®) was the endoscopy database in five hospitals (BHH, GH, CH, PRH, RSH) and an in-house endoscopy database was used in one hospital (SGH), which was subsequently changed to the ADAM™ (Fujinon®) system. Endoscribe™ has an embedded audit tool that allows searches based on separate and multiple search parameters. Data can be filtered as necessary during the search and files are exported from the database as Excel™ (Microsoft®) .csv files. Data from the original database from SGH was extracted from the hospital server as FoxPro (Microsoft Visual FoxPro®) files, which were then converted to Excel® (.xls) files using a DBF convertor. Data from the current ADAM™ (Fujinon®) system was extracted using built in user tools within the software. The extracted dataset was anonymised by not extracting name and address fields.

Each database was then examined to identify patients between 20-90 years, who underwent out-patient day-case gastro-intestinal endoscopy. Patients who had an endoscopic diagnosis of reflux oesophagitis, Barrett's oesophagus, hiatus hernia or benign oesophageal stricture were identified. Additionally, the SGH database contained data on proton pump inhibitor (PPI) use, as it had a tool to capture this data prior to endoscopy. Repeat endoscopies, surveillance endoscopies and planned therapeutic procedures (e.g percutaneous endoscopic gastrostomy (PEG)) were excluded. Each dataset was processed to remove entries with missing data, corrupted fields and multiple entries for a patient. The severity of reflux

oesophagitis was assessed on the basis of either the Savary-Miller classification or the Los Angeles (LA) classification of oesophagitis (Lundell et al. 1999; Monnier et al.1984). Savary-Miller grades I, II and III and LA grades A and B were classified as 'mild to moderate' reflux oesophagitis and Savary-Miller grades IV and V and LA grades C and D as 'severe' oesophagitis.

Data on the total number and recorded indication for endoscopies performed in the respective endoscopy units during the study period were also identified.

3.2 Preparation of acute oral mucosal wounds in mice

3.2.1 Experimental group

Female C57 mice kept on standard food and a well-lit and aired room were used. Female mice were divided into groups of five intact females (control group), five ovariectomized females (OVX) and five OVX females with oestrogen replacement (50µg oestradiol/day dorsal implants). A Home Office Project Licence was in place for this work (Faculty of Life Sciences, University of Manchester).

3.2.2 Bilateral oophorectomy

Female mice were anaesthetized in order to perform a bilateral oophorectomy. Following a 10mm incision in the lower part of the abdomen, blunt dissection was performed with incision of muscle fibres to isolate the oviduct and the uterus. A forceps was placed at the boundary of the oviduct and the uterus and the ovary and oviduct on either side was removed. The uterus was placed back in the abdominal cavity and the abdominal muscle layer and skin were closed with sutures.

3.2.3 Production of an acute buccal ulcer and determining the time course of healing

Under Isoflurane anaesthesia, buccal mucosa in mice between the jawbone and the inner aspect of the cheek was identified and a 1.5mm punch biopsy was performed with

sufficient pressure to create a depressed ulcer in the mucosa, similar to the technique described by Angelov et al (Angelov et al. 2004). Excess tissue was removed using a scalpel. In order to visualise the site of the buccal ulcer at different time-points, a black tattoo was placed at the base of the ulcer using a 0.2mm needle attached to an insulin syringe by injecting a black marking dye (Spot^{TM}, GI Supply, Camp Hill, PA, USA). This material is used routinely to mark lesions visualised at gastro-intestinal endoscopy due to its inert properties. Tissue was harvested at different time-points after sacrificing mice under anaesthesia by cervical dislocation and performing a 3mm full-thickness punch biopsy around the ulcer margins involving both buccal and skin surfaces. Wounds were created ten days after oophorectomy in female mice

3.2.4 Time-points

Tissue was harvested initially immediately after production of the buccal ulcer to create an initial time-point. Further 48h (2-day) and 96h (4-day) time-points were used to assess the time taken for complete re-epithelialization of the oral wound.

3.2.5 Histology and Immunohistochemistry

Tissue samples were kept in Z-fix^{TM} (Anatech Ltd, Battle Creek, MI, USA) for 48h, placed into cassettes and processed in an Automatic Tissue Processor. These were then embedded longitudinally in paraffin and sectioned at 5 microns, placed on treated slides (Vectabond^{TM}, Vector Laboratories, Peterborough, UK) and dried overnight in an incubator at 37°C. Sections were then deparaffinised and stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed to characterise cellular infiltrates and for matrix characterisation.

To characterise the epithelium, deparaffinised tissue sections were incubated for 1 hour with a 1:2000 dilution of rabbit anti-mouse cytokeratin-14 monoclonal antibody and a 1:500 dilution of rabbit anti-mouse cytokeratin-6 antibody.

For characterization of cellular infiltrates, deparaffinised tissue sections were incubated overnight with a 1:100 dilution of rat anti-mouse Mac-3 (macrophage) monoclonal antibody and a 1:100 dilution of rat anti-mouse Ly-6G (neutrophil) antibody at 4°C. The sections were then washed and incubated for 30 minutes with biotin labelled rabbit anti-rat secondary antibody (1:300) or with biotin labelled goat anti-rabbit secondary antibody. All sections were treated with streptavidin peroxidase conjugate (NovaRed^{TM}, Vector Laboratories, Peterborough, UK) for 40-60 seconds, and then washed. Sections were then dehydrated and coverslipped.

Further assessment of the collagen content in the matrix was carried out by staining tissue sections with Sirius Red F3B diluted in 1.3% picric acid for 1 hour and washing in acidified water followed by dehydration of the sections. Stained sections were then examined under polarized light to characterise the type of matrix collagen.

Oral wounds were identified on each slide and wound images were taken with a digital camera (Eclipse E400^{TM}, Nikon Corporation Imaging Company, Tokyo, Japan) at low (4x) magnification. For characterising cellular infiltrates and the matrix, five non-overlapping images of the wound were taken at high magnification (20x). Image analysis and quantification of oral wound areas were performed using the Image pro-plus imaging system (Version 6.3.0, Media Cybernetics Inc, USA). Wound areas were outlined using the program by manual drawing of the wound margins from the edge of the wound and deeper into the mucosa around the sub-epithelial inflammatory infiltrate. Re-epithelisation was determined by drawing the rim of neo-epithelial granulation tissue from the wound margin onto the

centre of the wound separately on either edge of the wound. For assessment of the cellular infiltrate, stained cells in each field were counted and represented as cells/area.

3.3 General Practice Research Database (GPRD) database analysis

The UK GPRD is a large research database containing computerized information entered by general practitioners (GP) and covering a population in excess of 7 million and with more than 47 million person-years' worth of data (Garcia Rodriguez et al. 1998). All information is strictly anonymised and includes patient demographics, detailed prescription data, details of GP consultations, referrals and results of laboratory tests. Disease diagnoses are coded using a modification of the Oxford Medical Information System (OXMIS) and the Read classification systems and drugs are coded using a drug dictionary based on data from the Prescription Pricing Authority. Prescriptions are recorded directly from each participating GPs computer ensuring complete recording. Validation studies have demonstrated the high quality of data recorded in GPRD (Jick et al. 1991; Jick et al. 1992). Access to the dataset was acquired under the Medical Research Council licence. This licence restricts datasets to a maximum of 100,000 subjects. Ethical approval was obtained under the provisions of the ISAC (independent scientific advisory committee) approval process for GPRD.

3.4 Hormone assays

Blood tests were performed to determine serum levels of Oestrogen (Oestradiol (E2), Oestrone (E1)), progesterone, sex-hormone binding globulin (SHBG), testosterone, follicular stimulating hormone (FSH) and luteinizing hormone (LH). Samples were taken around the 10th day of the menstrual cycle (+/- 4 days) in menstruating women by scheduling their pH test appropriately. All samples were taken immediately after the pH test.

Electrochemiluminescence (ECLIA) immunoassays were carried out using the Roche® E170^{TM} immunoassay system platform. Coefficients of variation of the various assays used are summarised in Appendix 4.

3.5 Acid reflux questionnaire

Study subjects were asked to complete a previously validated reflux questionnaire (Mohammed et al. 2005). Questions on a number of factors that have been reported to be associated with GORD symptoms or oesophagitis including smoking, alcohol consumption, current medication, family history of GI disease, parity and educational attainment were included in the questionnaire.

3.6 Anthropometric measurements

Height and weight were recorded and BMI categorised into normal (20-24.9 kg/m2), underweight (<20), overweight (25-29.9), obese (30-34.9) and morbid obesity (>35 kg/m2). Waist-hip ratio and skin fold thickness were additionally measured. To calculate the waist-hip ratio, the waist circumference was measured in centimetres at a level midway between the lowest rib and the iliac crest, and the hip circumference at the level of the great trochanters, with the legs close together. The waist-hip ratio was calculated as the ratio of the waist circumference to the hip circumference. Triceps skin-fold thickness was measured in centimetres using a set of callipers, as the thickness of a fold of skin over the lower third of the left triceps muscle, with the measurement axis along a line connecting the left humerus to the left olecranon process.

3.7 Tissue microarray analysis

12 biopsy samples of paired normal oesophagus and Barrett's oesophagus (6 males, 6 females) were obtained from patients undergoing surveillance endoscopy for Barrett's oesophagus at City Hospital Birmingham (UK) and Sandwell General Hospital, West

Bromwich (UK), in addition to normal Barrett's surveillance biopsies. These samples were snap frozen in liquid nitrogen, transported to a laboratory in the Institute of Cancer studies, University of Birmingham (UK) and stored at -70°C.

Tissue microarray analysis was kindly performed by Dr. John Arrand at the Institute of Cancer studies, University of Birmingham. RNA was extracted by homogenising biopsies directly in Trizol. The resulting total RNA was then further purified using the Qiagen® RNeasy™ procedure. RNA quality was checked on an Agilent Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) and only RNA with a RIN of 6.0 or more was used. An Ambion WT Expression kit (Applied Biosystems, Austin, TX, USA) was used to work on 200 ng of total RNA which was then fragmented and labelled using an Affymetrix WT Terminal Labelling Kit (Affymetrix Inc. Santa Clara, CA, USA). Scanned images of microarry chips were analysed using the AffymetrixGeneChip® Command Console™ Software from Affymetrix, Inc. (Santa Clara, CA, USA) to generate CEL files, which were processed with the RMA-Sketch algorithm of Affymetrix Expression Console software to generate normalized relative expression values. Differentially expressed probe sets were identified using the limma (Smyth 2005) package of Bioconductor (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) (Gentleman et al. 2004) with a cut off p value of 0.01. No fold change criteria were imposed. Gene expression heat maps were generated using dChip (http://www.dchip.org) with default settings.

Chapter 4

The influence of age and gender on endoscopic findings of gastro-oesophageal reflux disease: an endoscopy database study.

4 Introduction

Symptoms of gastro-oesophageal reflux disease (GORD) are equally common in both sexes and at all ages (Mohammed et al. 2005). GORD is associated with the development of Barrett's oesophagus, a change from squamous to columnar morphology with intestinal metaplasia, of the native oesophageal epithelium and in turn the development of oesophageal adenocarcinoma (Fitzgerald 2006; Lagergren et al. 1999). Oesophageal adenocarcinoma has a striking male predominance (ratio 6:1) (Cooper et al. 2009). This difference in incidence by sex cannot be satisfactorily explained by differences in established risk factors for oesophageal adenocarcinoma, such as ethnicity, obesity and smoking (Gammon et al. 1997). Furthermore, oesophageal adenocarcinoma is observed overwhelmingly in post-menopausal women and women have a higher mean age of incidence than men (Cooper et al. 2009). This raises the possibility of a role for female sex hormones in delaying the onset of Barrett's oesophagus and oesophageal adenocarcinoma. Oestrogen may mitigate the severity of GORD-induced oesophagitis in menstruating women, thereby either delaying or preventing Barrett's oesophagus and oesophageal adenocarcinoma. Oestrogen has been shown to have anti-inflammatory effects and studies on cutaneous wound healing have demonstrated better healing with topical oestrogen therapy in ovariectomized mice (Ashcroft et al. 1997; Ashcroft et al.1999; Ito, Hayashi et al.1995).

Hypothesizing that a reduction in the levels of female sex hormones after the menopause may contribute to an increased incidence and severity of reflux oesophagitis and complications, a retrospective analysis of a large endoscopy database was performed in order to examine the incidence of reflux oesophagitis and its complications with increasing age in men and women.

Details of the methods used in this study are described in Chapter 3, section 3.1.

4.1 Statistical analysis

The incidence of reflux oesophagitis, Barrett's oesophagus, hiatus hernia and benign oesophageal stricture was determined as a percentage of the total number of upper gastro-intestinal endoscopies carried out during the same period. The difference in the incidence of oesophagitis, Barrett's oesophagus, hiatus hernia and oesophageal stricture above and below 50 years of age (the average age of the menopause) was compared using the Chi square test. Multivariate analysis was performed using logistic regression analysis for each endoscopic finding as a dependant variable and the association of age, sex, reflux oesophagitis, Barrett's oesophagus, hiatus hernia and benign oesophageal stricture as appropriate was examined and odds ratios and 95% confidence intervals for each of the variables examined were calculated.

4.2 Results

Demographics, indications and results of endoscopy by hospital

The details of the upper GI endoscopies included in the study by hospital are shown in tables 1 and 2. The sex ratio is remarkably consistent between hospitals (table 1). The incidence of different endoscopic findings was very similar in the different units with the exception of Birmingham Heartlands Hospital, which had a higher incidence of reflux oesophagitis and a lower incidence of Barrett's oesophagus.

There is some variation in the proportion of patients whose indication was reflux symptoms or dyspepsia, with City Hospital, Birmingham having a higher incidence and Sandwell General Hospital, a lower incidence but in the other hospitals, reflux symptoms or dyspepsia was a very consistent proportion of endoscopies (table 2). The proportion of patients with alarm symptoms (anaemia, dysphagia, weight loss) was very similar in the different units, with the exception of City Hospital, which had a significantly lower proportion.

Table 1

The total number of endoscopies, sex ratio and incidence of reflux oesophagitis, Barrett's oesophagus, hiatus hernia and benign oesophageal stricture in the different hospital endoscopy databases included in the study.

Hospital	Years included	Endoscopies (n)	M:F ratio	Incidence RO (%)	Incidence BO (%)	Incidence HH (%)	Incidence stricture (%)	Incidence oesophageal cancer (%)
BHH	1997-2008	45823	0.93	20.4	2.8	23.8	2.0	2.38
CH	1999-2008	32023	0.99	13.2	5.7	18.2	1.4	2.41
GHH	1996-2008	30184	0.93	12.8	4.8	24.8	1.3	2.19
PRH	2003-2008	11961	0.91	13.8	6.7	25.8	1.4	2.98
RSH	2001-2008	19316	0.94	15.5	4.6	23.2	2.1	3.35
SGH	2006-2009	15099	0.92	14.5	6.8	28.3	1.8	2.40
Total		154406	0.93	15.7	4.5	22.6	1.7	2.43

RO: reflux oesophagitis

BO: Barrett's oesophagus

HH: Hiatus hernia

BHH: Birmingham Heartlands Hospital, Birmingham

CH: City Hospital, Birmingham

GHH: Good Hope Hospital, Birmingham

PRH: Princess Royal Hospital, Birmingham

RSH: Royal Shrewsbury Hospital, Birmingham

SGH: Sandwell General Hospital, West Bromwich

Table 2

Indications for gastroscopy at each study site

Study site and Indication	Reflux symptoms / dyspepsia number, (%) Males	Females	Alarm features * number, (%) Males	Females	Other ¶ number, (%) Males	Males	Total endoscopies
BHH	8506 (18.6)	10679 (23.3)	7169 (15.6)	8415 (18.4)	5687 (12.4)	5367 (11.7)	45823
CH	7677 (24)	9281 (29)	3042 (9.5)	3557 (11.1)	4443 (13.9)	4023 (12.6)	32023
GHH	5439 (18)	6505 (21.6)	4457 (14.8)	5121 (17)	4188 (13.9)	4474 (14.8)	30184
PRH	2161 (18.1)	2509 (21)	1696 (14.2)	1981 (16.6)	1662 (13.9)	1952 (16.3)	11961
RSH	3569 (18.5)	4350 (22.5)	2756 (14.3)	3188 (16.5)	2674 (13.8)	2779 (14.4)	19316
SGH	2089 (13.8)	2185 (14.5)	2653 (17.6)	2758 (18.3)	3169 (21)	3295 (21.8)	15099

*anaemia, dysphagia, weight loss

¶ abdo pain, vomiting, chest pain, suspected GI bleed

BHH: Birmingham Heartlands Hospital, Birmingham

CH: City Hospital, Birmingham

GHH: Good Hope Hospital, Birmingham

PRH: Princess Royal Hospital, Birmingham

RSH: Royal Shrewsbury Hospital, Birmingham

SGH: Sandwell General Hospital, West Bromwich

4.3 Reflux oesophagitis

24,240 patients (15.7%) had an endoscopic diagnosis of reflux oesophagitis. The incidence of reflux oesophagitis by age and gender are shown in tables 3a and 3b. Both in absolute terms and relative to the number of endoscopies undertaken, the incidence of reflux oesophagitis increased with age (figures 1A and 1B). However, the mean age of men with reflux oesophagitis (59.7 years, standard deviation, SD (16.1)) was lower than that of women (64.4 years, (SD 15.1)). Analysing the incidence of reflux oesophagitis in subjects before and after 50 years of age (the mean age of the menopause in the UK), revealed that the incidence increased from 10.5% to 15.1% in women, ($p<0.001$), compared with a fall in men from 19.4% to 16.9%, ($p<0.001$). A similar pattern was seen in the incidence of mild-moderate reflux oesophagitis, which increased from 9.9% to 13.1% in women after 50 years of age ($p<0.001$), while in men it decreased from 17.8% to 14.2%, ($p<0.001$). In severe oesophagitis, the incidence in women increased from 0.6 to 2.0% ($p<0.001$) with a smaller but significant increase in men from 1.7% to 2.8%, ($p<0.001$).

On multivariate analysis, severe reflux oesophagitis was associated with age (odds ratio, OR 1.029 (95% CI 1.026-1.032), $p<0.001$), male gender (1.44 (1.33-1.57), $p<0.001$), Barrett's oesophagus (1.683 (1.437-1.971), $p<0.001$) and benign oesophageal stricture (3.166 (2.607-3.845), $p<0.001$). On examining the interaction of age and sex with the prevalence of severe reflux oesophagitis modelling the variables age*sex as a covariate, the increase in severe reflux oesophagitis in women was greater in women with increasing age than in men (1.013, (1.007-1.019), $p<0.001$). This is depicted as a graph that shows a greater acceleration in the predicted probability of developing severe reflux oesophagitis with age in women compared to men, with the curves converging at 90 years (figure 2). The ratio of the incidence of reflux oesophagitis in women compared to men shows a consistent increase with increasing age in both mild-moderate and severe oesophagitis (figure 3).

Table 3a

The incidence of reflux oesophagitis in women

Age (years)	Women with indication reflux symptoms (% endoscopies)	Incidence RO (%)	Incidence Mild-moderate RO (%)	Incidence Severe RO (%)	Total endoscopies performed
20-30	290 (9.0)	261 (8.1)	243 (7.5)	18 (0.6)	3224
30-40	846 (13.6)	605 (9.7)	565 (9.1)	40 (0.6)	6207
40-50	1629 (15.8)	1210 (11.7)	1142 (11.1)	68 (0.7)	10324
50-60	2479 (18.0)	2028 (14.7)	1893 (13.7)	135 (1.0)	13796
60-70	2708 (16.1)	2544 (15.2)	2282 (13.6)	262 (1.6)	16789
70-80	1816 (10.3)	2693 (15.3)	2292 (13.1)	401 (2.3)	17559
80-90	646 (5.6)	1751 (15.0)	1342 (11.5)	409 (3.5)	11639
Total	10414 (13.1)	11092 (13.9)	9759 (12.3)	1333 (1.7)	79538

RO: reflux oesophagitis

Table 3b

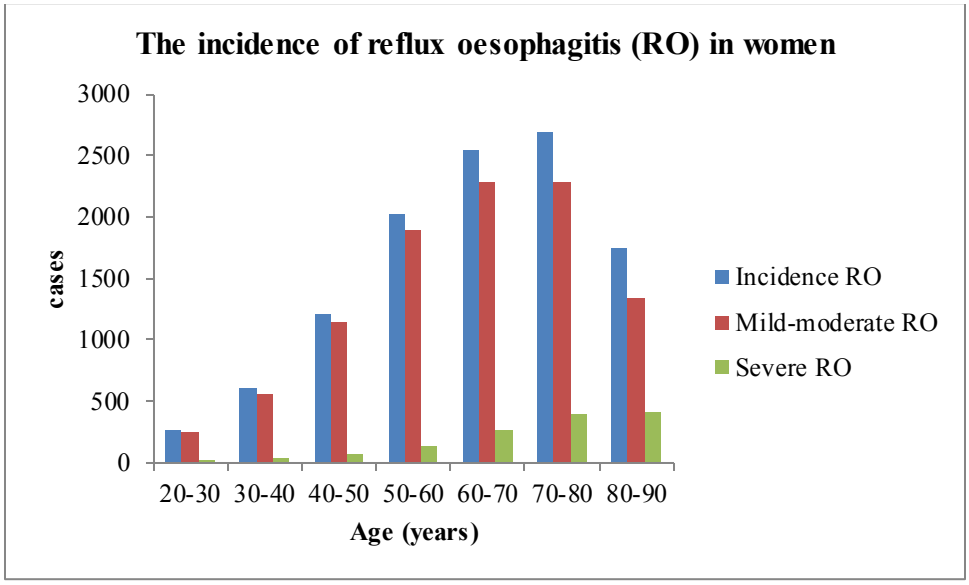
The incidence of reflux oesophagitis in men

Age (years)	Men indication reflux symptoms (% endoscopies)	Incidence RO (%)	Incidence Mild-moderate RO (%)	Incidence Severe RO (%)	Total endoscopies performed
20-30	433 (12.3)	571 (16.3)	526 (15.0)	45 (1.3)	3509
30-40	1100 (17.0)	1238 (19.2)	1155 (17.9)	83 (1.3)	6454
40-50	1429 (15.1)	1963 (20.7)	1767 (18.7)	196 (2.1)	9466
50-60	1851 (14.0)	2625 (19.9)	2296 (17.4)	329 (2.5)	13204
60-70	1796 (11.0)	2737 (16.8)	2386 (14.6)	351 (2.2)	16330
70-80	1223 (7.1)	2694 (15.5)	2149 (12.4)	545 (3.1)	17334
80-90	336 (3.9)	1320 (15.4)	1017 (11.9)	303 (3.5)	8571
Total	8168 (10.9)	13148 (17.6)	11296 (15.1)	1852 (2.5)	74868

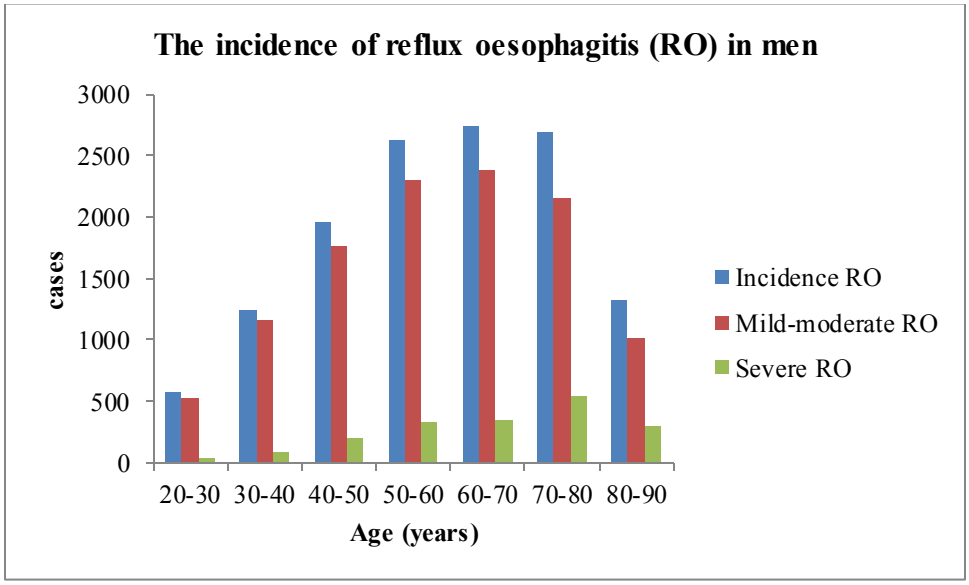
RO: reflux oesophagitis

Figure 1: The incidence of reflux oesophagitis

A



B



Figures 1A and 1B: Incidence of reflux oesophagitis (RO)

The absolute incidence of both mild-moderate and severe reflux oesophagitis (RO) increases with age in women (A) and men (B).

Figure 2

Probability of severe reflux esophagitis with increasing age

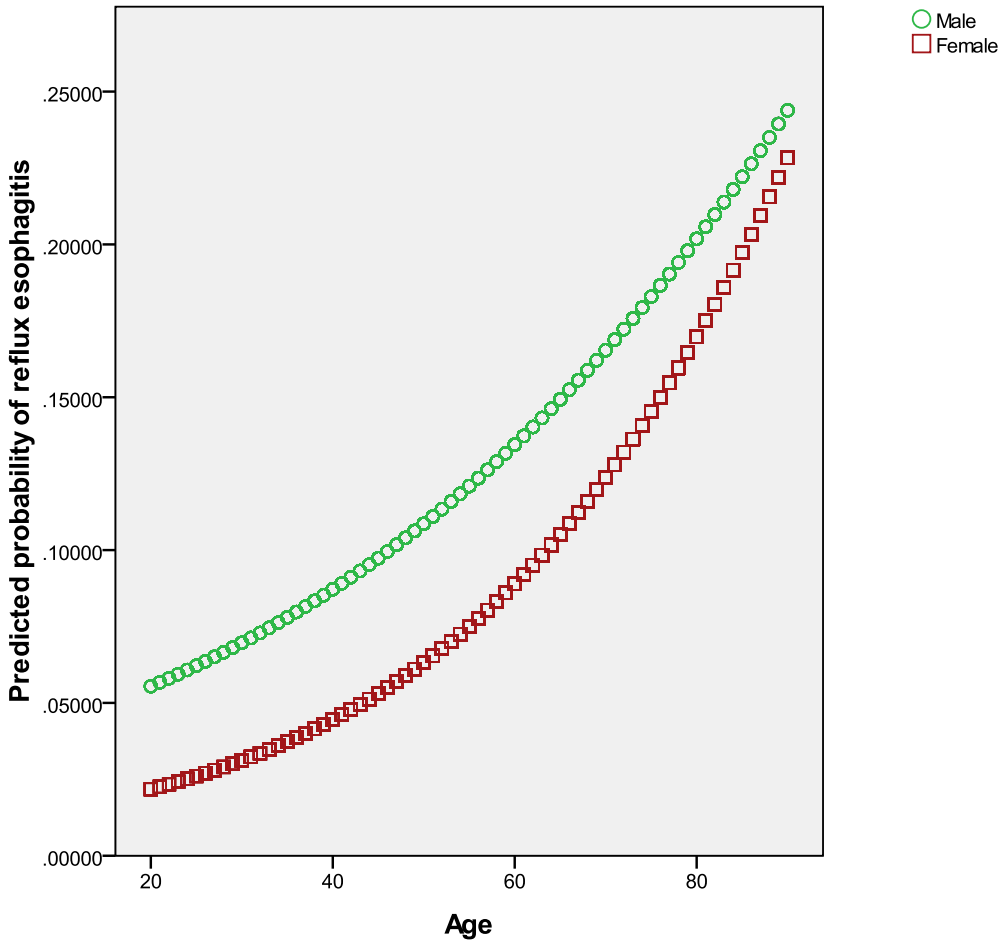


Figure 2: Probability of severe reflux esophagitis with increasing age by gender.

The slope of the curve for females is greater than that for males suggesting an acceleration in the incidence of severe reflux oesophagitis in older women compared to men.

Figure 3

Odds ratio of reflux oesophagitis with increasing age by gender

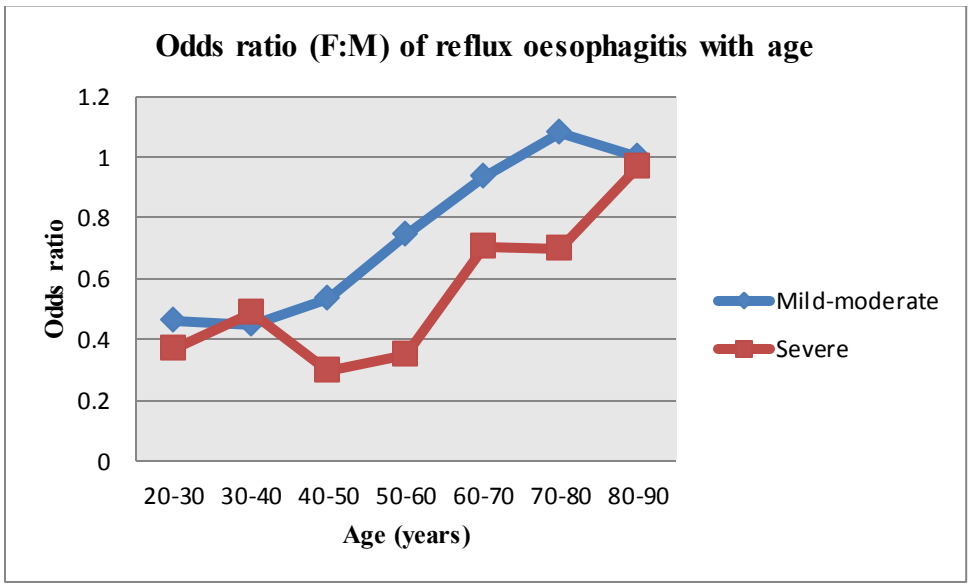


Figure 3: Odds ratio (incidence) of reflux oesophagitis with increasing age by gender (female: male).

The ratio of the incidence of both mild-moderate and severe reflux oesophagitis in women compared to men increases with age.

4.4 Barrett's oesophagus

The incidence of Barrett's oesophagus increased with age in men and women, (tables 4a and 4b). The mean age of men with Barrett's oesophagus (62.4 ± SD 14.5 years) was lower than that of women (68.4 ± SD 12.3 years). Although the incidence of Barrett's oesophagus increased in men and women (figure 4), the rate of increase of Barrett's oesophagus was not different between the sexes and Barrett's oesophagus remained more common among men at all ages. On multivariate analysis, Barrett's oesophagus was associated with age (odds ratio 1.02 (1.019-1.021), $p<0.001$), male sex (1.072 (1.004-1.074), $p=0.027$), reflux oesophagitis (3.459 (3.33-3.59), $p<0.001$), oesophageal stricture (1.199 (1.068-1.346), $p=0.002$) and hiatus hernia (1.217 (1.167-1.270), $p<0.001$).

Histopathology and endoscopy reports of all cases of Barrett's oesophagus from one unit (Sandwell General Hospital) were analysed. Short-segment Barrett's oesophagus (less than 3cm of columnar lined oesophagus) was more common (74.5%) than long-segment Barrett's oesophagus as expected. Intestinal metaplasia (IM) was present in 61% of all Barrett's oesophagus endoscopies. However, this was due to 88% of Barrett's oesophagus endoscopies negative for IM being short-segment Barrett's oesophagus. 95% of long-segment Barrett's oesophagus (> 3cm) had IM on biopsy. Furthermore, the mean number of biopsies taken in Barrett's oesophagus positive for IM (5.3) was greater than the number of biopsies taken in Barrett's oesophagus negative for IM (2.6). Insufficient sampling may therefore have underestimated the prevalence of IM in Barrett's oesophagus.

Table 4a

The incidence of Barrett's oesophagus, hiatus hernia and benign oesophageal strictures in women

Age (years)	BO (incidence (% all endoscopies))	HH (incidence (% all endoscopies))	BOS (incidence (% all endoscopies))	Total endoscopies performed
20-30	22 (0.7)	295 (9.2)	23 (0.7)	3224
30-40	82 (1.3)	896 (14.4)	65 (1.0)	6207
40-50	259 (2.5)	1894 (18.3)	110 (1.1)	10324
50-60	435 (3.2)	3226 (23.4)	179 (1.3)	13796
60-70	668 (4.0)	4550 (27.1)	284 (1.7)	16789
70-80	735 (4.2)	5125 (29.2)	363 (2.1)	17559
80-90	557 (4.8)	3543 (30.4)	354 (3.0)	11639
Total	2758 (3.5)	19529 (24.6)	1378 (1.7)	79538

BO: Barrett's oesophagus
HH: hiatus hernia
BOS: Benign oesophageal stricture

Table 4b

The incidence of Barrett's oesophagus, hiatus hernia and benign oesophageal strictures in men

Age (years)	BO (incidence (% all endoscopies))	HH (incidence (% all endoscopies))	BOS (incidence (% all endoscopies))	Total endoscopies performed
20-30	56 (1.6)	415 (11.8)	38 (1.1)	3509
30-40	184 (2.9)	1150 (17.8)	71 (1.1)	6454
40-50	504 (5.3)	1969 (20.8)	149 (1.6)	9466
50-60	984 (7.5)	3076 (23.3)	207 (1.6)	13204
60-70	1177 (7.2)	3747 (22.9)	378 (2.3)	16330
70-80	1132 (6.5)	4055 (23.4)	483 (2.8)	17334
80-90	503 (5.9)	2106 (24.6)	233 (2.7)	8571
Total	4540 (6.1)	16518 (22.1)	1559 (2.1)	74868

BO: Barrett's oesophagus
HH: hiatus hernia
BOS: Benign oesophageal stricture

Figure 4: The incidence of Barrett's oesophagus

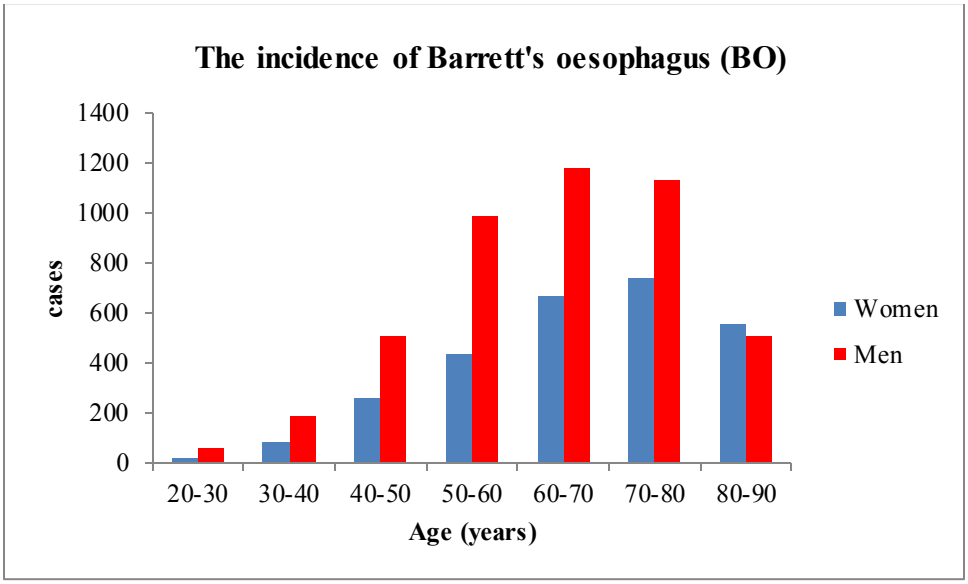


Figure 4: The incidence of Barrett's oesophagus

The absolute incidence of Barrett's oesophagus (BO) increases with age in both women and men. The incidence in women increases significantly after age 50.

Figure 5: The incidence of hiatus hernia

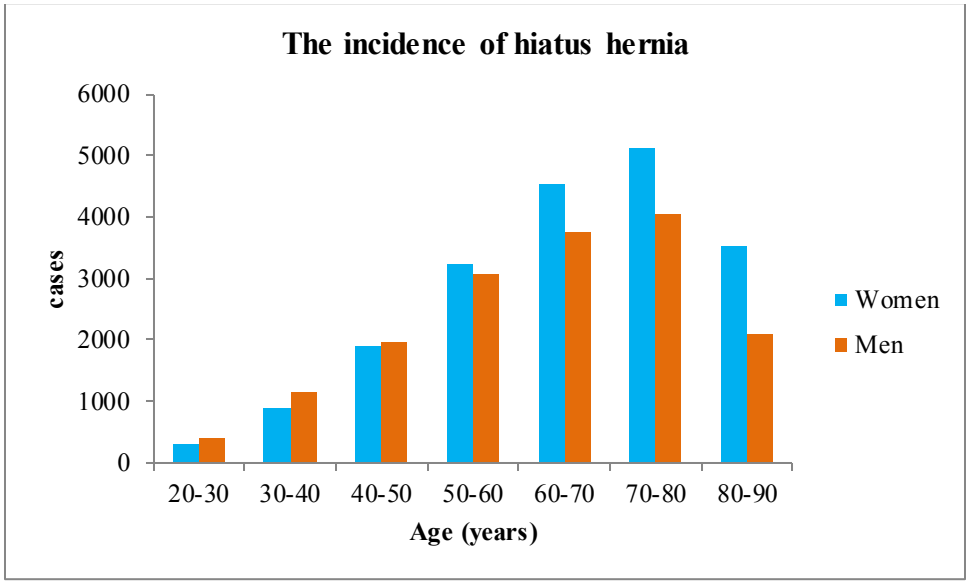


Figure 5: The incidence of hiatus hernia

The absolute incidence of hiatus hernia increases with age in both women and men. The rise in incidence with age is significantly greater in women compared to men.

Figure 6: The incidence of benign oesophageal strictures

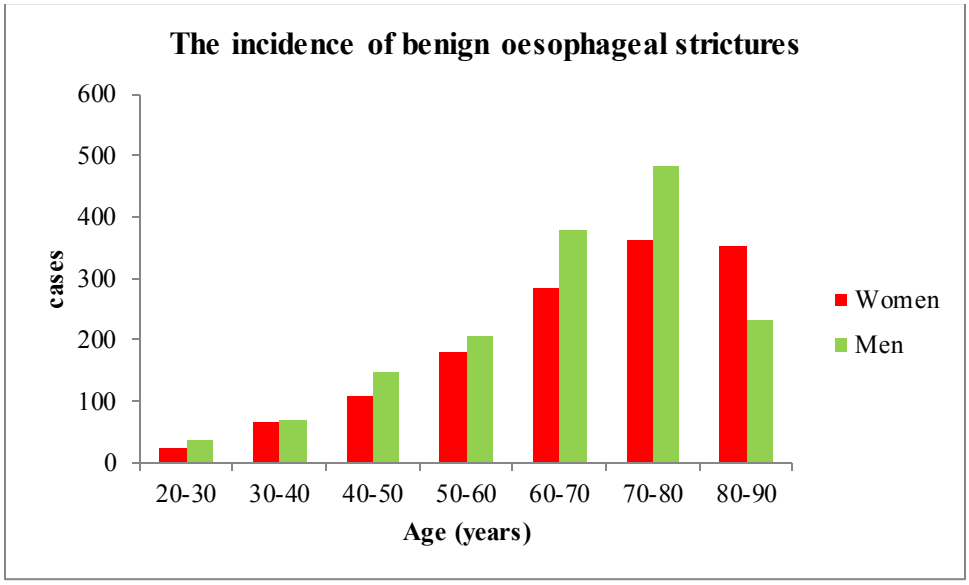


Figure 6: The incidence of benign oesophageal strictures

The absolute incidence of benign oesophageal strictures increases with age in both women and men. The rise in incidence with age is significantly greater in women compared to men and there is a crossover in the incidence in women and men at age 80-90 years.

4.5 Hiatus hernia

The incidence of hiatus hernia increased with age in men and women, (tables 4a and 4b). The mean age of men with hiatus hernia (61.4 ± SD 15.3 years) was lower than that of women (66.2 ± SD 14.1 years). The incidence of hiatus hernia increased with age in both men and women (figure 5) but the increase in incidence was greater in women with increasing age (table 5). On multivariate analysis, hiatus hernia was associated with age (1.009 (1.008-1.010), $p<0.001$), reflux oesophagitis (2.478 (2.394-2.564), $p<0.001$), Barrett's oesophagus (1.217 (1.167-1.270), $p<0.001$) and oesophageal stricture (1.817 (1.657-1.993), $p<0.001$). Interestingly, following adjustment for age, hiatus hernia was associated with female sex (1.259 (1.224-1.296), $p<0.001$). Reflux oesophagitis was still associated with increasing age (1.027 (1.025-1.030), $p<0.001$), despite controlling for the increasing incidence of hiatus hernia with age. This suggests that the increased incidence of hiatus hernia in women with age is not sufficient to explain the more rapid rise in the incidence of reflux oesophagitis in women.

Table 5

Age and sex-related differences in the incidence of Barrett's oesophagus, hiatus hernia and benign oesophageal stricture in men and women above and below 50 years of age

	Men <50 (% all endoscopies)	Women <50 (% all endoscopies)	$\chi 2$ test (2-tailed)	Men >50 (% all endoscopies)	Women >50 (% all endoscopies)	$\chi 2$ test (2-tailed)
Total number of endoscopies	19429	19755		55439	59783	
Barrett's oesophagus	744 (3.8)	363 (1.8)	p<0.001	3796 (6.8)	2395 (4.0)	p<0.001
Hiatus hernia	3534 (18.2)	3085 (15.6)	p<0.001	12984 (23.4)	16444 (27.5)	p<0.001
Benign stricture	258 (1.3)	198 (1.0)	p<0.001	1301 (2.3)	1180 (2.0)	p<0.001

4.6 Benign oesophageal stricture

The incidence of benign oesophageal stricture increased with age in men and women, (tables 4a and 4b). The mean age of men with benign oesophageal stricture (63.2 ± SD 15.7 years) was lower than that of women (66.9 ± SD 14.5 years). The prevalence of benign oesophageal stricture increased sharply in women after the age of 50 and charting the age and sex-specific incidence of benign oesophageal stricture reveals a crossover between the curves for prevalence in women compared to men between the ages of 80-90 years (figure 6). On multivariate analysis, benign oesophageal stricture was associated with age (1.02 (1.017-1.023)), male sex (1.196 (1.104-1.295), $p<0.001$), reflux oesophagitis (1.248 (1.128-1.382), $p<0.001$), BO (1.209 (1.078-1.357), $p=0.001$) and hiatus hernia (1.831 (1.669-2.007), $p<0.001$).

4.7 Proton pump inhibitor (PPI) use prior to endoscopy

Data on PPI use was available from one unit (Sandwell General Hospital). PPI data was available for 5937 subjects endoscoped at this unit. 1010 (17%) had received a PPI prior to endoscopy (493 (48.8%) were male, mean age 60.7 (SD 16.1) years). Of these 1010 patients, in 321 (31.8%), the indication for endoscopy was reflux symptoms and 47 (14.6%) had endoscopic oesophagitis. In comparison, 736 (14.9%) of 4927 patients not on PPI therapy prior to endoscopy had reflux symptoms as their indication and 195 (26.4%) had endoscopic oesophagitis, a significantly higher prevalence than patients with reflux symptoms on PPI prior to endoscopy ($p=0.032$).

4.8 Discussion

Community studies of GORD symptoms report equal prevalence in both sexes and at all ages (Mohammed et al. 2005). However, studies of endoscopic oesophagitis from endoscopy databases or population studies, consistently report an increased incidence with increasing age and that men are more likely to have oesophagitis than women (El-Serag and Sonnenberg 1997). This pattern is even more striking for more severe oesophagitis and the GORD complications of peptic stricture and Barrett's oesophagus (El-Serag et al. 1997).

In the present study, the incidence of reflux oesophagitis and its complications of Barrett's oesophagus and benign oesophageal stricture increased with age. The novel finding from the study is that with increasing age, the incidence of oesophagitis and benign oesophageal stricture increases more rapidly in women than men, so that the incidences are very similar over the age of 70 in both sexes. Barrett's oesophagus, the other complication of chronic GORD, increases with age in both sexes but there remains a male predominance at all ages.

The anti-inflammatory properties of oestrogen may lessen the severity of oesophageal mucosal injury secondary to acid and bile reflux, thereby either delaying or preventing the onset of metaplastic change of the ulcerated, inflamed oesophageal epithelium into Barrett's oesophagus. Conversely, in the post-menopausal state, chronic gastro-oesophageal reflux may lead to more severe oesophageal mucosal injury and a higher incidence of oesophagitis and its complications, Barrett's oesophagus and benign oesophageal stricture. Unfortunately, given the retrospective nature of this study, it was not possible to directly examine this hypothesis and data on precise age of menopause and hormone replacement therapy was not available.

One explanation for the more rapid increase of oesophagitis and its complications in elderly women may be the increase in hiatus hernia incidence seen particularly in women

with increasing age. This may lead to more severe gastro-oesophageal reflux, thereby leading to more severe oesophagitis and its complications. The increase in prevalence of hiatal hernia with age may be related to the loss of elasticity of the structures around the diaphragmatic hiatus. Pregnancy in women is another potential factor that may weaken fibromuscular structures around the gastro-oesophageal junction through raised intra-abdominal pressures. Osteoporosis and kyphosis have been shown to be associated with hiatal hernia in elderly Japanese women (Furukawa et al. 1999;Yamaguchi et al. 2002). However, in the present study, reflux oesophagitis was still associated with increasing age despite controlling statistically for the increase in incidence of hiatus hernia with age, suggesting this is not the only factor.

Unfortunately, corroborative histological data was not available for the vast majority of patients with Barrett's oesophagus due to the anonymised format of the data. Detailed analysis of endoscopy and histology records for patients with Barrett's oesophagus from Sandwell General Hospital revealed that histological confirmation was almost universal in long segment (greater than 3cm) Barrett's oesophagus but much less common in the more common shorter Barrett's oesophagus segments. The lower number of biopsies (2-6 on average) in IM negative Barrett's oesophagus may explain this difference, as IM is well known to be patchy (Harrison et al. 2007). With one exception (Birmingham Heartlands Hospital), the prevalence of Barrett's oesophagus was similar within the different units, suggesting that observer bias is less likely to be a confounding factor in the Barrett's oesophagus findings, though not completely excluding it. Barrett's oesophagus, unlike reflux oesophagitis or benign oesophageal stricture, remained more common in men at all ages, suggesting that despite severe GORD and its complications being equally common in elderly men and women, men remain more prone to Barrett's oesophagus due to factors other than severe gastro-oesophageal reflux.

The study has several limitations. This was a retrospective database study and there was no information available on a number of potentially important aetiological factors in GORD and its complications, including BMI, smoking, H.pylori status and ethnicity. The most important of these risk factors is BMI, as increasing BMI is clearly associated with symptomatic GORD, oesophagitis and its complications (Lagergren et al. 1999; Mohammed et al. 2005; Nilsson et al. 2002). If the younger male patients in the study were more obese than the younger female patients and there was no BMI difference in older patients, this would potentially explain the study findings and this possibility cannot be excluded. Similarly, smoking is associated with symptomatic GORD and its complications. If younger men in the study were more likely to be smokers than younger women, this might potentially contribute to the study findings.

Although patients on a PPI were significantly less likely to be diagnosed with reflux oesophagitis, there was no difference in the number of male and female patients taking PPIs, suggesting that the increase in incidence of reflux oesophagitis in elderly women and the lower incidence in young women was not a consequence of gender differences in PPI therapy prior to endoscopy.

As a retrospective study with multiple endoscopists, it was not possible to standardise endoscopic findings. Observer (endoscopist) bias could therefore be another significant source of bias. There was some variation in the prevalence of reflux oesophagitis, Barrett's oesophagus and hiatus hernia among endoscopy units in a relatively small geographical area. This may be due to differing patient populations, observer bias or differences in indications for endoscopy in each unit. It is also possible that endoscopists may be biased in reporting endoscopic findings by gender and less likely to report pathology in younger female than younger male patients. These potential sources of bias are again difficult to exclude.

Selection bias may also be another potential source of bias in this study. Younger female

patients may seek endoscopy for more minor symptoms than younger male patients or male patients may be more likely to fail to attend for endoscopy despite their General Practitioner's recommendation. Female patients make more primary care visits and adhere better to screening programmes (Currie et al. 2003; von Euler-Chelpin et al. 2010). However, the increased incidence of oesophagitis in younger men was in both relative and absolute terms and reflux symptoms was a slightly less common indication for endoscopy in younger women, making this explanation for the study findings less likely. Since oesophagitis is more common in absolute terms in younger men, it would be necessary for younger women with oesophagitis to be less likely to undergo endoscopy than younger men with oesophagitis and women without oesophagitis for selection bias to be a significant source of bias, which seems implausible.

In conclusion, reflux oesophagitis and its complications, Barrett's oesophagus and benign oesophageal stricture, increase with age and are more common in absolute and relative terms among younger men than younger women. Reflux oesophagitis and stricture increase more rapidly in women so that the incidence in absolute and relative terms in elderly patients is similar in both genders. In part this may be due to the increase in the incidence of hiatus hernia in women with increasing age but hormonal factors, particularly the lack of oestrogen in post-menopausal women, may also potentially contribute.

Chapter 5

Does oestrogen reduce the inflammatory response to mucosal injury and promote epithelial healing?

5 Introduction

There appears to be a clear relationship between gastro-oesophageal reflux and oesophageal adenocarcinoma (Lagergren et al. 1999). Oesophageal adenocarcinoma is observed overwhelmingly in post-menopausal women and women have a higher mean age of oesophageal adenocarcinoma incidence than men, suggesting that female sex hormones (particularly oestrogen) may play a significant role in delaying the onset of Barrett's oesophagus and oesophageal adenocarcinoma. An analysis of endoscopy unit data reveals that more severe grades of oesophagitis and Barrett's oesophagus, while uncommon in younger men, are virtually never seen in pre-menopausal women (Menon et al. 2011). Oestrogen has anti-inflammatory properties and may play a role in preventing the development of severe reflux oesophagitis.

5.1 Animal models to replicate oesophageal injury

Animal models have been developed to mimic chronic duodeno-oesophageal reflux by creating a gastro-jejunostomy or an oesophago-duodenal anastomosis (Miwa et al. 1996; Oberg et al. 2000; Pera et al. 2000; Sato et al. 2002). However, not only is this technically difficult, but also has drawbacks in that it creates a non-physiological reflux model which may not accurately reflect the degree of distal oesophageal exposure to acid and bile. Injuring the oesophagus with mechanical trauma and acid or bile-mediated chemical injury is another possibility but this is technically difficult and repeated injury mimicking a chronic acid-reflux state is difficult to achieve. An alternative may be to create an ulcer/injury in the buccal or lingual mucosa, which has the advantage of accessibility.

The buccal mucosa is composed of stratified squamous keratinizing epithelium, similar to the epithelium of the oesophagus. Creating an injury model in the buccal mucosa may therefore allow easy access to study changes related to acute inflammation. Repeated chemical injury is also possible in order to create a model resembling chronic inflammation.

Experiments have previously been performed on buccal or lingual ulcers in mice. Okabe et al originally described a method of inducing experimental penetrating ulcers in rats (Okabe et al. 1971). Konturek et al described a modification of this technique for inducing oral ulcers in rats. These ulcers were created using a combination of mechanical injury with a stencil followed by chemical injury with 100% acetic acid applied to the mucosa for 60 seconds (Konturek SJ et al. 1993). Slomiany et al induced buccal ulcers in rats to study the effects of endothelin-1, peroxisome proliferator activated receptor-γ (PPAR- γ) and aspirin on healing of ulcers (Slomiany and Slomiany 2001; Slomiany and Slomiany 2002; Slomiany and Slomiany 2005). These ulcers were produced using acetic acid applied to the mucosa for 20 seconds without producing a mechanical injury. Oral ulceration in rats has also been induced by irradiating the mouth in order to replicate radiotherapy-related mucositis (Lee et al. 2007; Rezvani and Ross 2004). A similar model of mucositis involved a combination of mechanical injury by scoring the palatal mucosa with an 18G needle and treating with 5-fluorouracil (Lara et al. 2007). Angelov et al created buccal ulcers by using a 1.5mm dermal biopsy punch in order to study the effects of Secretory leukocyte protease inhibitor (SLPI) on healing of ulcers and also marked the ulcers locally by applying a black tissue dye (Angelov et al. 2004).

Machowska et al described the effects of testosterone and progesterone on the healing of induced lingual and gastric ulcers in mice and found that treatment with testosterone significantly delayed ulcer healing while producing a reduction in lingual blood flow at the margin of the ulcers (Machowska et al. 2004; Machowska et al. 2008). Progesterone reversed these changes completely. There is no data on the effect of oestrogen on healing of oral ulcers.

The aims of the study were to initially create an acute oral wound model in order to determine the effect of lack of systemic oestrogen (oophorectomized mice) and subsequent

oestrogen replacement on buccal mucosal healing. This work would subsequently lead on to creation of a chronic injury model with further experiments using acid and bile exposure to the wound in order to replicate acid-induced oesophageal mucosal injury. Characterising an injury model in the buccal mucosa will facilitate a study to determine the effects of repetitive acid and bile exposure to the buccal epithelium. Further studies could be then carried out to establish the phenotypic aspects of wound injury and assess the nature of the cellular infiltrate secondary to acid and bile exposure. Establishing a chronic buccal wound model would additionally enable us to determine the influence of oestrogen on wound healing and may provide an insight into the gender difference in the development of Barrett's metaplasia in the oesophagus.

A detailed description of the methods used in this study is presented in Chapter 3, section 3.2.

5.2 Statistical analysis

Data was expressed as mean and standard error. Differences between means were expressed using t-test. A p value of <0.05 was considered significant.

5.3 Results

5.3.1 Optimisation of the acute wound model

The wound model was as described by Angelov et al.(Angelov et al. 2004) Buccal mucosa of mice was exposed and a punch biopsy was performed with biopsied mucosa excised and the wound tattooed. As mentioned previously, female mice were divided into groups of five intact females (control group), five ovariectomized females (OVX) and five OVX females with oestrogen replacement (50µg oestradiol/day dorsal implants).

Following injury to the buccal mucosa and production of an oral ulcer, we found that there was no obvious change in food or water intake in the mice. Inspection of the ulcers suggested rapid healing, and by day 2 (48h) it was difficult to assess the margins of the ulcer due to significant re-epithelialisation. Initial tattooing of the ulcers (figure 1), therefore, made it possible to determine the wound site histologically at day 2 and 4 (96h). By day 4, there was near-total epithelialisation of the wound in all three groups.

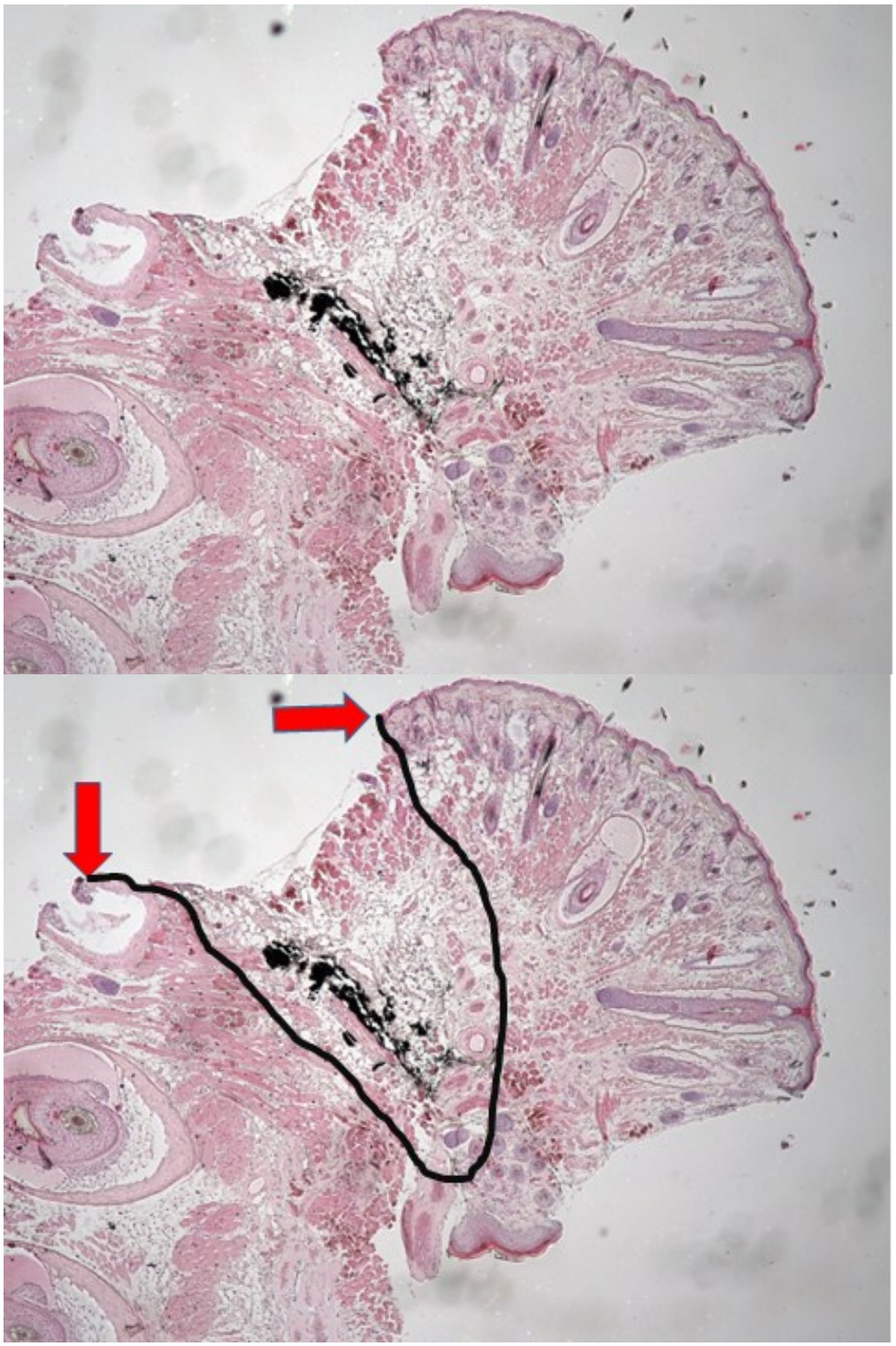


Figure 1: Images of an acute wound at Day 0 with a tattoo (black stippling within the wound) to define location in the buccal area (wound margins indicated by red arrows and wound area indicated by black line).

5.3.2 Wound measurements

5.3.2.1 Effect of oestrogen on wound diameter

Wound diameter was significantly greater at day 2 in the ovariectomized (OVX) mice (1.2 (SE 0.1) mm) compared to the control group (0.95 (SE 0.04) mm, p=0.04) and the OVX and oestrogen-treated group (0.87 (SE 0.03) mm, p=0.02, figure 2A, figure 4). However by day 4, wound diameter in the OVX group (0.84 (SE 0.05) mm, p=0.46), was similar to the control group (0.85 (SE (0.09) mm) and the oestrogen-treated group (0.81 (SE 0.05) mm, p=0.31), figure 3A, figure 5.

5.3.2.2 Effect of oestrogen on wound area

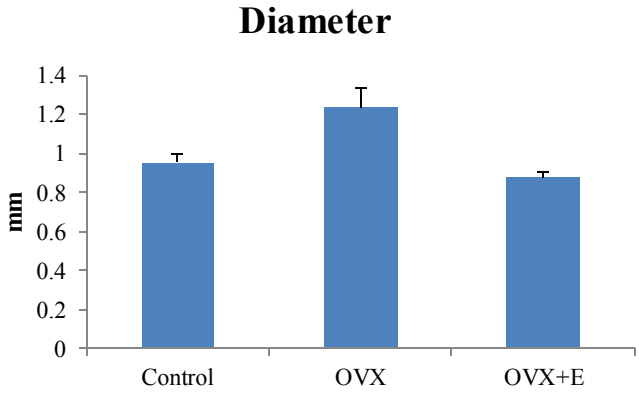
Wound area in the OVX group was significantly greater on day 2 compared to controls (3.32 (SE 0.2) mm, 0.53 (SE 0.05) mm, p<0.001) and the OVX and oestrogen-treated group (0.56 (SE 0.14) mm, p<0.001), (figure 2B). There was no difference in wound area between the OVX and control groups (0.52 (SE 0.04) mm, 0.60 (SE 0.1) mm, p=0.09) or the OVX and oestrogen-treated group on day 4 (0.46 (SE 0.05) mm, p=0.19), (figure 3B).

5.3.2.3 Effect of oestrogen on wound re-epithelialisation

Wound re-epithelialisation at day 2 was greater in the OVX and oestrogen-treated group compared to the OVX group (0.13 (SE 0.12) mm, 0.03 (SE 0.02), p=0.008), (figure 2C), but was similar to re-epithelialisation in controls (0.12 (SE 0.02) mm, p=0.47). At day 4, there was no difference in re-epithelialisation between the OVX (0.198mm), oestrogen-treated (0.214mm) or the control (0.211mm) groups (figure 3C).

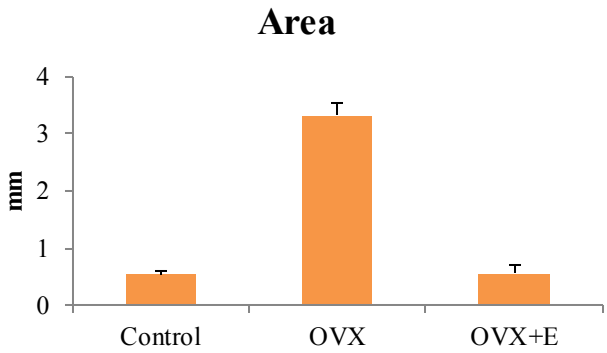
These results suggest that OVX wounds tend to be larger, with greater wound area, suggesting impaired healing and the differences in wound size noted at day 2 tend to diminish by day 4, when the wounds become comparable.

A



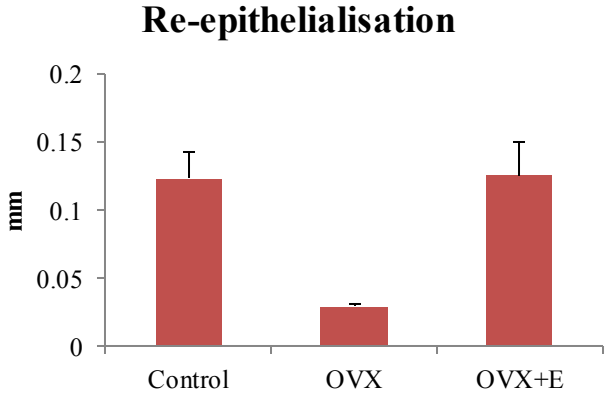
Mean (SE)
Control: 0.95 (0.04)
OVX: 1.23 (0.1)
OVX+E: 0.87 (0.03)

B



Mean (SE)
Control: 0.53 (0.05)
OVX: 3.32 (0.2)
OVX+E: 0.56 (0.14)

C

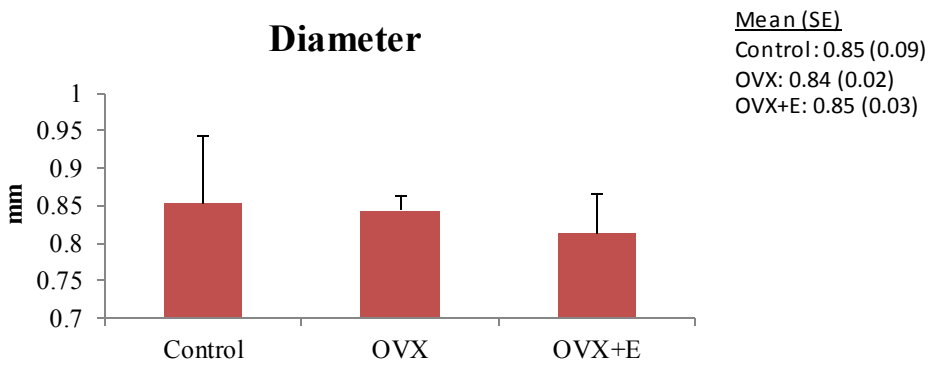


Mean(SE)
Control: 0.12 (0.02)
OVX: 0.03 (0.02)
OVX+E: 0.13 (0.12)

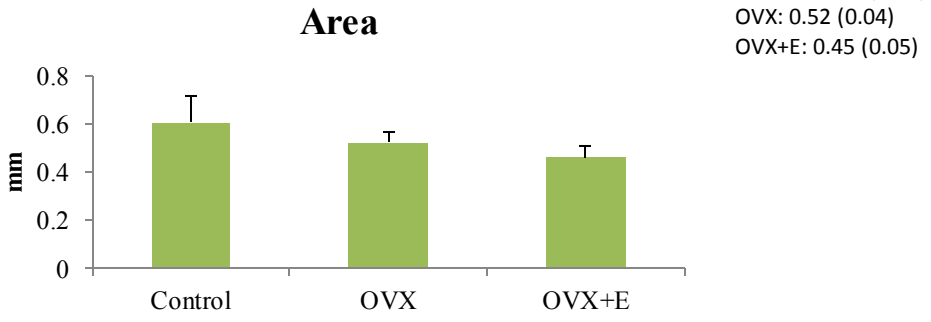
Figure 2: Wound measurements on day 2: A: Diameter, B: Area, C: Re-epithelialisation

Wound diameter, area and re-epithelialisation (mean (SE)) in mm are shown for the three groups (control, OVX and OVX with oestrogen treatment).

A



B



C

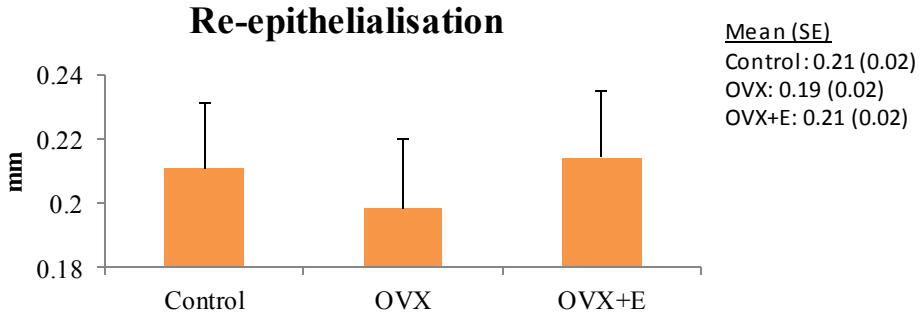
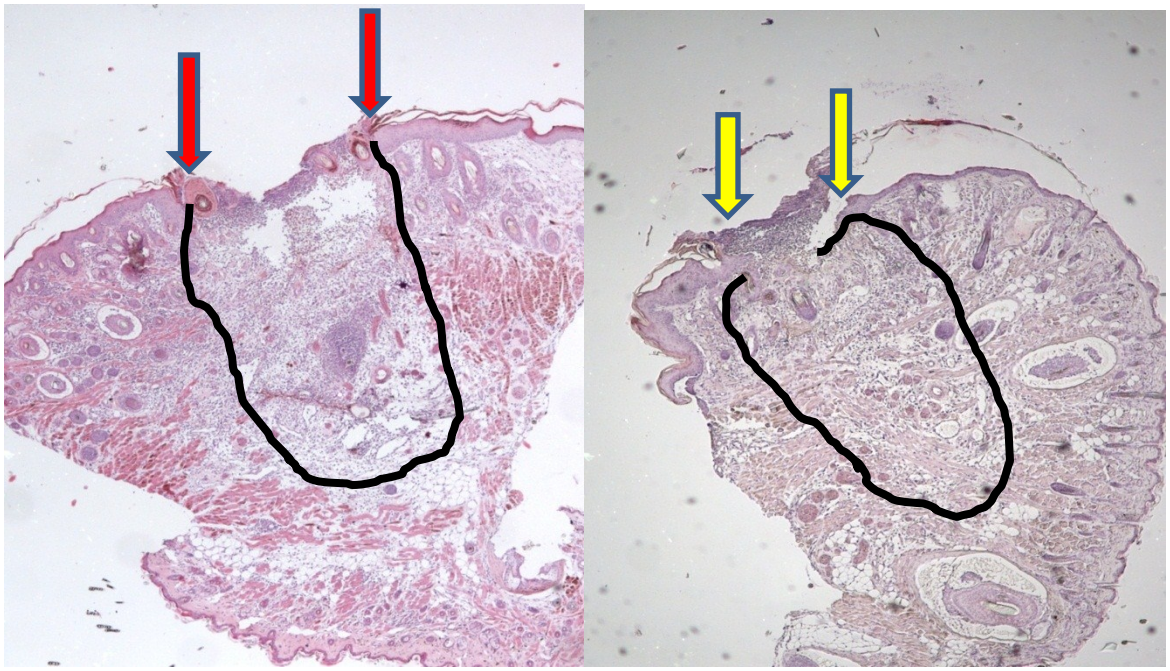


Figure 3: Wound measurements on day 4: A: Diameter, B: Area, C: Re-epithelialisation

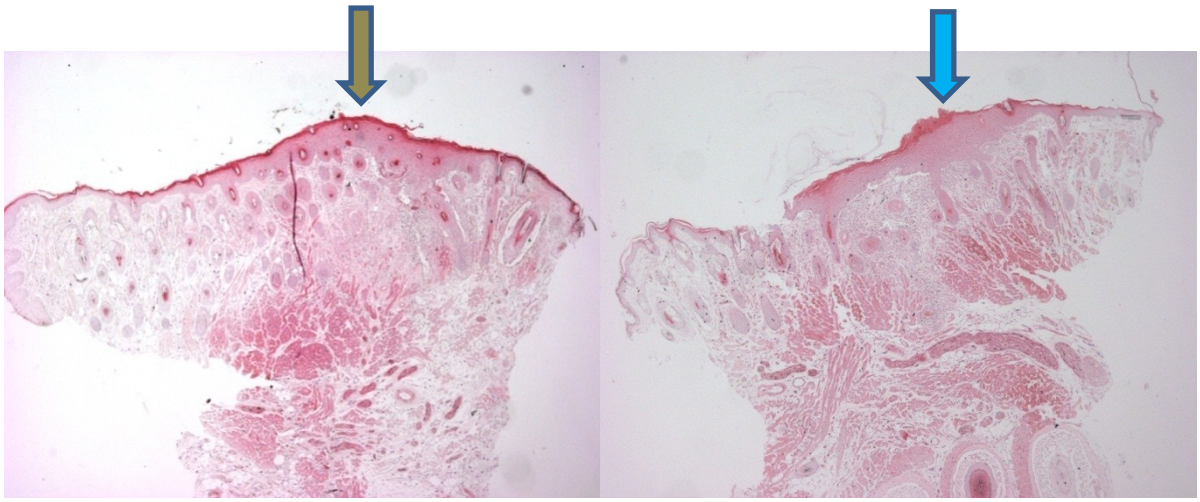
Wound diameter, area and re-epithelialisation (mean (SE)) in mm are shown for the three groups (control, OVX and OVX with oestrogen treatment).



OVX **OVX+E**

Figure 4: Comparison of OVX and oestrogen-treated (OVX+E) wound images on day 2 with wound edges and the area highlighted.

The OVX wound on the left is has a greater diameter, area and is less well re-epithelialized compared to the oestrogen-treated wound on the right. Wound edges are indicated by the red arrows in the OVX wound and the yellow arrows in the OVX+E wound. Wound area is marked in black.



OVX **OVX+E**

Figure 5: Comparison between OVX and oestrogen-treated (OVX+E) wounds on day 4.

The arrows highlight the wounds, which are almost completely re-epithelialized.

5.3.3 Effect of oestrogen on the cellular infiltrate

In addition to measuring the dimensions of the wound, we evaluated the nature of the inflammatory infiltrate in order to determine the effect of oestrogen on the infiltrate.

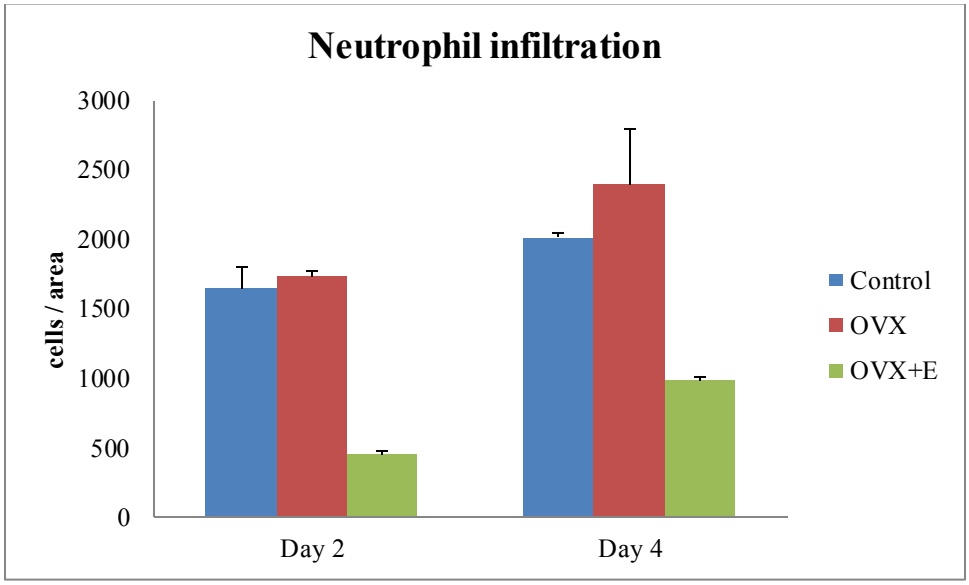
5.3.3.1 Effect on neutrophils

At day 2, neutrophils (cells/wound area) were greater in the OVX group (1735, SE (32.8)) compared to the oestrogen-treated group (448, SE (33.5), p=0.05) and controls, (figure 6A). At day 4, there was a significant excess of neutrophils in the OVX wounds compared to the OVX and oestrogen-treated wounds (2392, SE (397) versus 980 SE (31), p=0.05), (figure 6A).

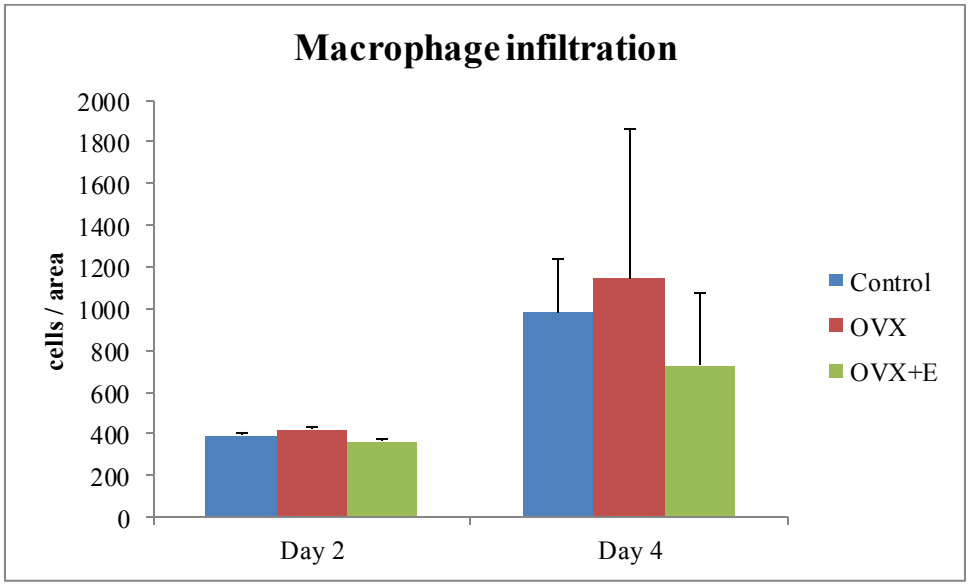
5.3.3.2 Effect on macrophages

At day 2, there was a non-significant increase in the number of macrophages in the OVX wounds compared to the oestrogen-treated wounds (419 SE (10) versus 363 SE (16), p=0.41) and controls, figure 6B. Day 4 macrophage counts were greater in the OVX group (1144, (SE (722)) compared to the oestrogen-treated group (726, SE (354)), but the result did not reach statistical significance (p=0.66), (figure 6B).

A



B



Figures 6A & 6B

6A: Neutrophil infiltration of the wound on days 2 and 4

6B: Macrophage infiltration of the wound on days 2 and 4

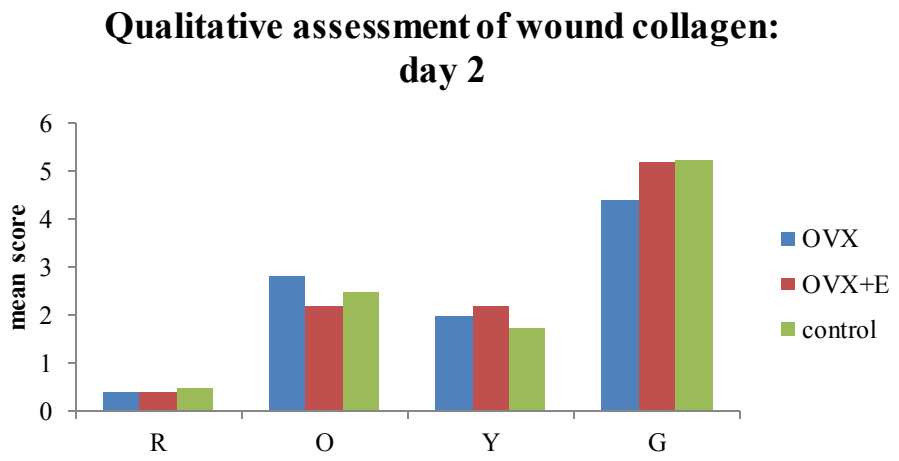
5.3.4 Qualitative assessment of the wound and matrix collagen

Images under polarized light were compared between groups in a blinded fashion and a qualitative assessment of the proportion of red, orange, yellow and green colours (representing decreasing order of thickness of collagen fibres) in each image was made using a visual analogue score. Each image was scored separately for the proportion of red, orange, yellow and green colours representing different types of collagen and a composite value representing the mean of scores for each colour was derived.

Images from day 2 and day 4 wounds were compared in this fashion. Day 2 wounds had an overall greater proportion of green (thin) collagen fibres (figure 8) compared to day 4 wounds (figure 7A, figure 8). There was an increase in the proportion of yellow and orange (thick) collagen fibres in the day 4 wounds (figure 7B, figure 8).

There was no significant difference in collagen fibres between groups.

A



B

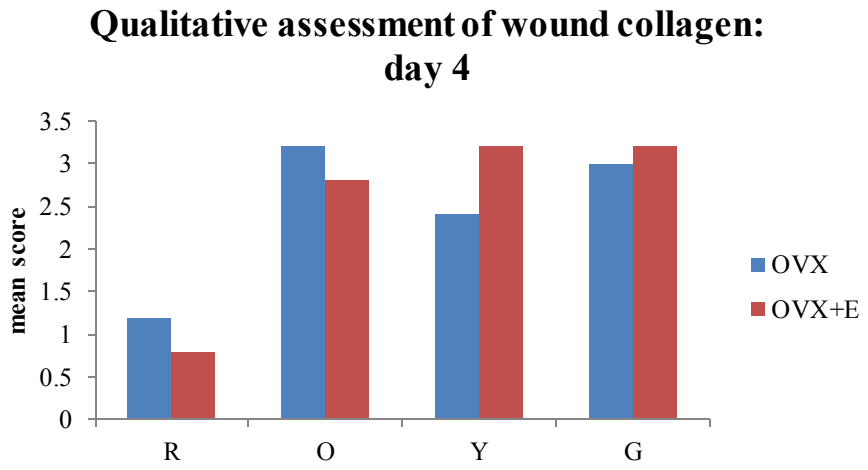
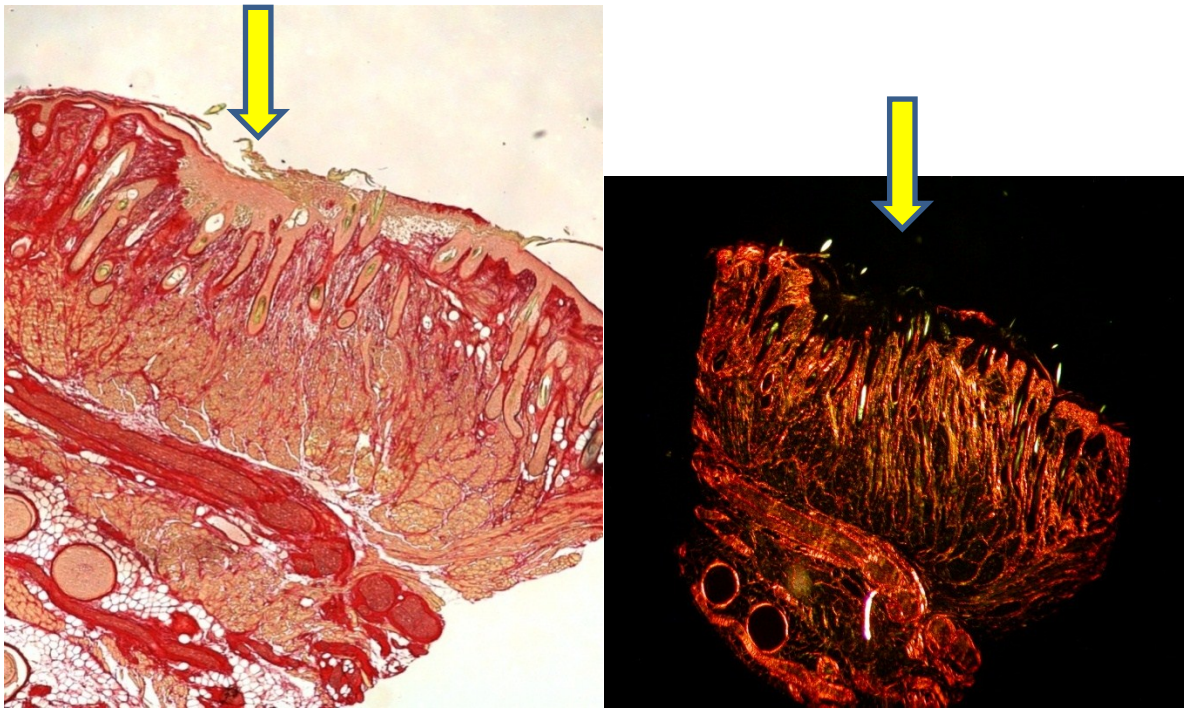


Figure 7A & 7B: Qualitative assessment of wound collagen at day 2 (7A) and day 4(7B)

Figure 7A: Assessment of the type of collagen present in the wound matrix on day 2 using a visual analogue scale between 0-10. (R: Red, O: Orange, Y: Yellow, G: Green: representing the types of collagen). The value on the y-axis represents the mean scores for each group (controls, OVX, oestrogen-treated (OVX+E)).

Figure 7B: Assessment of the type of collagen present in the wound matrix on day 4 using a visual analogue scale between 0-5. (R: Red, O: Orange, Y: Yellow, G: Green: representing the types of collagen). The value on the y-axis represents the mean scores for each group (OVX, oestrogen-treated (OVX+E)). A control group was not used in this instance.



Collagen staining (Picrosirius Red stain)

Collagen expression (polarizing light microscopy)

Figure 8: Day 2 Collagen expression on Picrosirius red stain (left) and under polarised light (right).

Collagen stains red with Picrosirius and under polarizing light, collagen fibres are visualised in red, orange, yellow and green colours, representing fibres of increasing thickness. The wound is indicated by the arrows.

5.4 Discussion

The purpose of the study was to assess the feasibility of producing and maintaining an acute buccal wound injury model. Data and experience generated from this pilot study would serve to design a further in depth study to assess the effect of oestrogen and also guide the creation of a chronic injury model with exposure of the wound to the acid and bile.

The data indicate that there is a distinct difference in wound diameter and area between oestrogen-deficient and oestrogen-replaced mice early in the healing process (day 2). This effect is less dramatic at the later day 4 time point, reflecting the speed of mucosal healing. This temporal information provides important reference time-points for further experiments. There were significant differences in the nature of the inflammatory infiltrate between the oestrogen-deficient and oestrogen-treated groups of mice in keeping with the putative anti-inflammatory effects of systemic oestrogen. Wound and matrix collagen expression was also assessed qualitatively, indicating that there was an overall change in collagen type between day 2 and day 4 wounds generally.

Angelov et al examined the effect of secretory leukocyte protease inhibitor (SLPI), an acid stable protein, found in a variety of mucosal fluids, including saliva (Angelov et al. 2004). SLPI inhibits serine proteinases, including proteinases from neutrophils, pancreatic acinar cells and mast cells (Hiemstra 2002). Endogenous generation of SLPI is an important regulator of the inflammatory response during an acute inflammatory episode, by suppressing NFκB activation with resultant inhibition of cytokines and chemokines (Ashcroft et al. 2000; Song et al. 1999; Ward and Lentsch 2002). Inhibition of serine proteases can prolong wound healing by maintaining high neutrophil elastase levels in wounds. SLPI-null mice were found to have markedly impaired buccal wound healing with reduction in the matrix deposition and an increase in matrix metalloproteinase (MMP) activity. SLPI-activity may confound the

current acute buccal wound model results and will be examined separately in a future chronic injury model.

Oral wound models have several advantages, of which the key advantage is the ease of access to the wound. Moreover, the stratified squamous epithelium of the buccal mucosa is of similar morphology to oesophageal epithelium, making the buccal mucosa an attractive surrogate for oesophageal mucosa.

Experiments that replicate oesophageal mucosal injury leading to Barrett's oesophagus and oesophageal adenocarcinoma have involved the creation of surgical bile reflux models in mice. These models have involved oesophageal and either duodenal or jejunal anastomosis in order to replicate acid and bile reflux. However, a crucial drawback is their significant postoperative morbidity and mortality of up to 30% (Miwa et al.1996; Oberg et al. 2000; Pera et al. 2000; Sato et al. 2002). These models of chronic reflux injury are associated with metaplasia of the oesophageal mucosa to a Barrett's type mucosa but the results of such models have been conflicting. Oesophageal adenocarcinomas induced by such surgical reflux models are not phenotypically similar to adenocarcinoma arising from Barrett's metaplasia in humans. A major limitation to an oral mucosal wound model is that changes within the oral wound model may not necessarily be truly reflective of cellular processes and mucosal injury within the oesophagus. The concentrations of saliva in the mouth and the oesophagus are also dissimilar, making it difficult to extrapolate findings from oral wounds to the oesophagus. Moreover, this is a small study with limited time-points due to the rapidity of healing of the buccal mucosa and the results at the time-points studied may not be generalizable to a chronic wound model.

Oestrogen receptors are distributed widely throughout the body including the skin, mouth and oesophagus. There are two different types of oestrogen receptors: oestrogen receptor α (ER α) and oestrogen receptor β (ER β). These receptors have been speculated to

play a role in the maturation and maintenance of oral epithelium and have also been demonstrated in oesophageal adenocarcinoma and Barrett's oesophagus. The role played by these receptors in mucosal injury is unclear.

In conclusion, there appears to be a differential response to acute oral wound injury in mice deprived of and replenished with systemic oestrogen. Further work on a chronic oral injury model will allow us to study the relationship between systemic oestrogen and chronic mucosal injury.

Chapter 6

Is hormone replacement therapy in post-menopausal women associated with a reduced risk of oesophageal cancer?

6 Introduction

Oesophageal cancer is more common among men. While the male predominance for oesophageal squamous cell carcinoma can be explained by differences in risk factors such as alcohol and tobacco consumption, the more marked male predominance for oesophageal adenocarcinoma remains unexplained by established risk factors such as reflux symptoms and obesity (Corley et al. 2001). A recent study of patients with oesophageal adenocarcinoma (OAC) found that uniquely among cancers the age-specific incidence was delayed by 17 years in women compared to men and one possible explanation is a potential protective effect of sex hormones in menstruating women (Derakhshan et al. 2009).

Studies on post-menopausal women have shown that systemic and topical oestrogen therapy are associated with accelerated cutaneous wound healing compared to age-matched controls (Ashcroft et al. 1997; Ashcroft et al. 1999). The same phenomenon may plausibly occur in oesophageal injury, raising the possibility that oestrogen may limit the severity of oesophageal injury in response to gastro-oesophageal reflux. Severe oesophageal reflux injury is thought to be a pre-requisite for the development of Barrett's oesophagus, which in turn is a pre-requisite for the development of oesophageal adenocarcinoma (Haggitt et al. 1978; Naef et al. 1975).

A previous nested case-control study examined the influence of hormone replacement therapy (HRT) among women with OAC and controls and found no association between oesophageal adenocarcinoma and HRT use but found a strong negative association between HRT and gastric cancer (Lindblad et al. 2006). The authors did not examine the use of HRT for prolonged periods or the influence of HRT on the related conditions of Barrett's oesophagus and reflux oesophagitis. This study therefore re-examined the influence of HRT on oesophageal cancer, Barrett's oesophagus and reflux oesophagitis in a matched cohort of post-menopausal women.

A detailed description of the methods used in this study is presented in Chapter 3, section 3.3.

6.1 Sample size estimation

The incidence of oesophageal adenocarcinoma in women over the age of 50 years is 14 per 100,000 per year (personal communication from West Midlands Cancer Intelligence Unit, Birmingham, UK), corresponding to 7 cases per year in a sample of 50,000 subjects and 35 cases in 5 years. Assuming that HRT is at most associated with a reduction of oesophageal cancer by 50%, a sample size of 50,000 women exposed to HRT and followed for at least 5 years would provide 80% power at the 5% significance level to detect this association.

6.2 Study design

A matched cohort study using data from GPRD between 1st January 1987 and 31st October 2008 was conducted. 50,000 women between 50-80 years of age with a prescription code for HRT at least twice per year for a minimum of 1 year and the same number of women without a record of having been prescribed HRT matched by age within 5 years, GP practice and duration on GPRD were identified. Subjects were excluded if they had a disease code for Barrett's oesophagus or oesophageal cancer before or six months after the first date of HRT prescription and if they had a code for reflux oesophagitis prior to starting HRT. Subjects with less than 1 year of follow up or those developing oesophageal cancers within 12 months of registration at the general practice were also excluded.

The cohort was followed longitudinally in the database until age 80 years, development of oesophageal cancer, death or exit from the database. Dates of diagnosis of reflux oesophagitis, Barrett's oesophagus and oesophageal cancer were extracted to analyse the temporal sequence of events. Data on use of lower-oesophageal sphincter (LOS) relaxing drugs, which may potentially promote acid reflux, and conditions such as asthma and chronic

obstructive lung disease (COPD), which have been associated with acid reflux, were also examined. Data were also extracted on hysterectomy, co-morbidities including diabetes mellitus, ischaemic heart disease and hypertension, frequency of GP consultations, rates of upper gastro-intestinal endoscopy, body mass index (BMI), alcohol consumption and smoking.

6.3 HRT exposure

All forms of HRT were considered for analysis, including oral, transdermal preparations and oestrogen implants. Duration of exposure was determined and the development of each outcome (reflux oesophagitis, Barrett's oesophagus and reflux oesophagitis) was examined in relation to the length of HRT use. "Current" users were defined as users who were on HRT during their time on the database, as opposed to 'ever' users, who had been exposed to HRT at some point in the database. A similar exposure window in each matched subject in the unexposed (control) group was defined. HRT use was further stratified by duration of exposure (in years) to exposure windows of 0-2 years, 2-5 years, 5-10 years and >10 years of HRT use. HRT prescriptions were grouped into oestrogen only; progesterone only, combined HRT and Tibolone. Subgroup analysis was carried out to determine the effect of each type of HRT on reflux oesophagitis, Barrett's oesophagus and oesophageal cancer.

6.4 Oesophageal cancer histology

Histology of each oesophageal cancer was sought by examining anonymised free text attached to each subject in GPRD searching for the following terms: oesophageal adenocarcinoma, oesophageal squamous cell carcinoma or squamous cell cancer, oesophageal cancer, oesophagectomy, chemotherapy, radiotherapy and neo-adjuvant. Oesophageal cancer was then divided into three groups: adenocarcinoma, squamous cell carcinoma and indeterminate (when histology was not available).

6.5 Statistical analysis

Skewed data were initially log transformed for analysis. Categorical data were compared between groups using the Chi-square test. Matched Cox-regression analysis was used to examine the relationship between HRT use and the risk of reflux oesophagitis, Barrett's oesophagus and oesophageal cancer and hazard ratios with 95% confidence intervals were calculated. Time-dependent covariates were additionally used to examine the effect of length of HRT use on the risk of reflux oesophagitis, Barrett's oesophagus and oesophageal cancer. Covariates examined included age, BMI (log transformed), smoking (categorised as never smoked ex-smoker and current smoker), alcohol consumption in units/week, hysterectomy, COPD, asthma, LOS-relaxing drugs, co-morbidities, frequency of GP consultations and rate of upper GI endoscopy. These covariates were examined independently initially in univariate models to understand their effect on the outcome variables (reflux oesophagitis, Barrett's oesophagus and oesophageal cancer). Covariates that had a significant association with the outcome variables were then incorporated into a multivariate model to determine the interaction between variables. Covariates that remained significant in multivariate analysis were included and adjusted for in the final Cox-regression model.

6.6 Results

6.6.1 Study cohort

51851 women over the age of 50 years with a prescription code for HRT and matched control subjects not exposed to HRT were identified. These 103702 subjects had 1,545,495 person-years of follow up recorded on the database. Baseline characteristics of the study cohort are listed in table 1. More subjects in the HRT group had never smoked but there was no difference in alcohol consumption between the groups. There were more subjects with a diagnosis of asthma, COPD and hypertension in the HRT-exposed group. Consequently, the HRT exposed group were more likely to take drugs that may affect the lower oesophageal sphincter. As expected, there were twice as many subjects who had undergone hysterectomy in the HRT-exposed group. There was slight excess of a recorded diagnosis of ischaemic heart disease in the HRT exposed group but not diabetes mellitus. Not surprisingly, the HRT exposed group had had more GP consultations per year and they were also more likely to have undergone endoscopy.

Table 1: The demographic characteristics of the study cohort

	HRT exposed group	Unexposed group	p value
Number of subjects	51851	51851	
Age (mean, SD)	55 (5.8)	55 (5.7)	
Body mass index (kg m^{-2}), mean (SD)	27.6 (5.3)	28.2 (6.2)	p=0.55
Smoking	Never 37542	Never 34148	p<0.001
	Current smoker 6411	Current smoker 7163	p<0.001
	Ex-smoker 7898	Ex-smoker 10540	p<0.001
Alcohol consumption (units/week), mean (SD)	8.3 (10.4)	7.8 (10.8)	p=0.60
HRT duration			
0-2 years	18660		
2-5 years	14808		
5-10 years	14164		
>10 years	4217		
Co-morbidity			
Diabetes Mellitus	5396	5503	p=0.334
Ischaemic heart disease	1366	1258	p=0.04
Hypertension	8380	7817	p<0.001
Asthma	3805	2725	p<0.001
COPD	1157	948	p<0.001
Hysterectomy	7621	3688	p<0.001
Type of HRT			
Oestrogen-only	16657		
Progesterone-only	45		
Combined HRT	27390		
Tibolone	7759		
Frequency of GP consultations (number / year), (SD)	10 (14)	1 (5)	p=0.01
Number of subjects endoscoped during the study period	4365	2768	p<0.001
Drug therapy			
Beta-2 agonists	11704	8424	p<0.001
Inhaled steroids	8825	6155	p<0.001
Anticholinergics	2330	1637	p<0.001
Theophylline	436	356	p=0.005

Table 2: Univariate analysis of risk factors associated with oesophageal cancer, Barrett's oesophagus and reflux oesophagitis in the study cohort

Variable	Oesophageal cancer (hazard ratio, 95% CI)	p value	Barrett's oesophagus (hazard ratio, 95% CI)	p value	Reflux oesophagitis (hazard ratio, 95% CI)	p value
Age	1.09 (1.06-1.13)	p<0.001	1.05 (1.03-1.08)	p<0.001	1.04 (1.03-1.04)	p<0.001
BMI	2.07 (0.52-8.23)	p=0.30	2.16 (0.94-4.99)	p=0.07	1.59 (1.33-1.91)	p<0.001
Smoking						
current	1.41 (0.63-3.16)	p-0.40	1.21 (0.70-2.19)	p=0.53	1.24 (1.14-1.34)	p<0.001
Ex	0.69 (0.32-1.45)	p=0.32	1.43 (0.97-2.01)	p=0.07	1.23 (1.12-1.35)	p<0.001
Never	0.38 (0.19-0.77)	p=0.01	1.16 (0.76-1.76)	p=0.50	0.71 (0.62-0.81)	p<0.001
Alcohol (units per week)	1.01 (0.99-1.03)	p=0.35	1.02 (1.01-1.02)	p<0.001	1.0 (0.99-1.01)	p=0.79
Asthma	3.33 (1.68-6.61)	p=0.001	3.41 (2.25-5.12)	p<0.001	3.45 (3.12-3.79)	p<0.001
COPD	10.3 (5.22-20.5)	p<0.001	6.12 (3.73-10.2)	p<0.001	3.25 (2.81-3.76)	p<0.001
Hysterectomy	2.83 (1.55-5.12)	p=0.001	3.79 (2.67-5.37)	p<0.001	3.45 (3.19-3.74)	p<0.001
Diabetes Mellitus	1.49 (0.73-3.73)	p=0.28	1.93 (1.30-2.90)	p=0.001	1.54 (1.40-1.70)	p<0.001
Ischaemic Heart Disease	1.15 (0.35-3.8)	p=0.82	3.78 (2.36-6.01)	p<0.001	2.62 (2.32-3.0)	p<0.001
Hypertension	3.54 (2.04-6.13)	p<0.001	3.29 (2.29-4.58)	p<0.001	3.59 (3.34-3.86)	p<0.001
Rates of endoscopy			1.37 (1.25-1.51)	p<0.001	1.53 (1.49-1.56)	p<0.001
GP consults			1.03 (1.00-1.05)	p=0.02	1.021 (1.02-1.024)	p<0.001
Beta-2 agonists	1.09 (0.98-1.21)	p=0.13	1.07 (1.01-1.13)	p=0.02	1.04 (1.02-1.05)	p<0.001
Inhaled steroids	0.97 (0.81-1.16)	p=0.74	1.07 (0.99-1.15)	p=0.08	1.04 (1.02-1.07)	p<0.001
Anticholinergics	0.97 (0.78-1.21)	p=0.79	0.98 (0.88-1.08)	p=0.63	0.98 (0.95-1.00)	p=0.08
Theophylline	0.96 (0.56-1.65)	p=0.88	0.94 (0.71-1.26)	p=0.70	0.99 (0.94-1.06)	p=0.97

Table 3: Multivariate analysis of risk factors associated with oesophageal cancer, Barrett's oesophagus and reflux oesophagitis in the study cohort

Multivariate analysis	Oesophageal cancer (hazard ratio, 95% CI)	p value	Barrett's oesophagus (hazard ratio, 95% CI)	p value	Reflux oesophagitis (hazard ratio, 95% CI)	p value
Age	1.07 (1.04-1.11)	p<0.001	1.03 (1.0-1.05)	p=0.02	1.05 (1.01-1.08)	p=0.02
Body mass index					1.05 (1.01-1.08)	p=0.02
Never smoker	0.38 (0.22-0.66)	p=0.001				
Ex-smoker					1.29 (1.19-1.39)	p<0.001
COPD	3.73 (1.66-8.41)	p=0.001	2.30 (1.30-4.05)	p=0.004	1.42 (1.20-1.68)	p<0.001
Asthma					2.22 (1.98-2.49)	p<0.001
Hysterectomy	1.90 (1.01-3.59)	p=0.05	2.26 (1.56-3.26)	p<0.001	2.18 (2.01-2.36)	p<0.001
Hypertension	2.39 (1.33-4.27)	p=0.003	2.27 (1.59-3.24)	p<0.001	2.27 (2.09-2.45)	p<0.001
Alcohol consumption			1.02 (1.01-1.02)	p<0.001		
Ischaemic Heart Disease			2.57 (1.58-4.12)	p<0.001	1.93 (1.70-2.18)	p<0.001
Frequency of GP consultations			1.03 (1.01-1.05)	p=0.004	1.01 (1.002-1.01)	p=0.02

Table 4: Hazard ratios for oesophageal cancer, Barrett's oesophagus and reflux oesophagitis for HRT exposed subjects relative to matched controls by duration of HRT exposure.

	Oesophageal cancer(hazard ratio, 95% CI)	Barrett's oesophagus(hazard ratio, 95% CI)	Reflux oesophagitis(hazard ratio, 95% CI)
HRT ever	0.81 (0.48-1.37), p=0.42	1.15 (0.81-1.63), p=0.45	1.57 (1.45-1.70), p<0.001
Current HRT	0.49 (0.17-1.42), p=0.19	1.30 (0.69-2.50), p=0.44	1.43 (1.24-1.65), p<0.001
Duration of HRT (0-2 years)	0.53 (0.23-1.26), p=0.15	0.66 (0.07-5.96), p=0.72	1.15 (0.74-1.79), p=0.53
Duration of HRT (2-5 years)	0.74 (0.1-5.49), p=0.77	0.90 (0.61-1.34), p=0.62	1.45 (1.12-1.83), p=0.02
Duration of HRT (5-10 years)	0.25 (0.07-0.95), p=0.04	1.67 (0.8-3.48), p=0.17	1.46 (1.24-1.71), p<0.001
Duration of HRT (>10 years)	No cases	0.65 (0.22-1.86), p=0.42	1.44 (1.07-1.95), p=0.02

Table 5: Hazard ratios for oesophageal cancer histological subtypes for HRT exposed subjects relative to matched controls

	Oesophageal adenocarcinoma	p value	Oesophageal squamous cell carcinoma	p value	Indeterminate oesophageal cancer	p value
Ever HRT	0.89 (0.28-2.82)	p=0.84	1.12 (0.32-4.2)	p=0.81	0.64 (0.32-1.29)	p=0.21
Current HRT	0.86 (0.27-2.76)	p=0.80	1.22 (0.32-4.65)	p=0.78	0.61 (0.30-1.23)	p=0.17
HRT 0-2 years	1.24 (0.20-7.70)	p=0.81	0.58 (0.05-6.42)	p=0.66	0.23 (0.06-0.88)	p=0.03
HRT 2-5 years	0.72 (0.17-3.02)	p=0.66	0.86 (0.12-6.31)	p=0.88	0.68 (0.29-1.58)	p=0.39
HRT 5-10 years	0.69 (0.20-2.32)	p=0.55	0.91 (0.23-3.57)	p=0.89	0.56 (0.27-1.15)	p=0.11

6.6.2 Oesophageal cancer

There were 25 cancers in the HRT exposed group compared to 31 in the unexposed group. Univariate analysis identified age, hysterectomy, asthma, COPD and hypertension as being positively associated and never smoking as negatively associated with risk of oesophageal cancer (table 2). Multivariate analysis revealed that age, hysterectomy, COPD and hypertension were positively associated and never smoking negatively associated with oesophageal cancer (table 3). These variables were controlled for in subsequent analyses.

Both 'ever' use of HRT (hazard ratio 0.69 (95% CI, 0.40-1.18, p=0.12)), and 'current' HRT use were not associated with oesophageal cancer (0.49 (0.17-1.42, p=0.19)). On examining the duration of HRT use, up to 5 years of HRT use was not associated with oesophageal cancer (table 4). However, when HRT was used for 5-10 years, there was an inverse association with risk of oesophageal cancer (0.25 (0.07-0.95, p=0.04)). This result was corroborated by applying time-dependent covariates to determine the association between length of HRT use and oesophageal cancer. Increasing duration of HRT use was associated with a reduced risk of oesophageal cancer (0.06 (0.01-0.43), p=0.005). Analysis by subtype of HRT revealed no significant association between subtype of HRT used and oesophageal cancer.

In the HRT exposed group the oesophageal cancers included 7 (28%) oesophageal adenocarcinoma, 6 (24%) oesophageal squamous cell carcinoma and 12 (48%) indeterminate oesophageal cancer. In the unexposed group, there were 6 (19%) oesophageal adenocarcinoma, 4 (13%) oesophageal squamous cell carcinoma and 21 (68%) indeterminate oesophageal cancer. The results of HRT exposure and increasing duration of HRT on the incidence of oesophageal adenocarcinoma, oesophageal squamous cell carcinoma and

indeterminate oesophageal cancer are shown in table 5. There was no significant association between HRT exposure and the subtypes examined (oesophageal adenocarcinoma and oesophageal squamous cell carcinoma) and no evidence of an association with increasing duration of HRT exposure in these smaller subsets of the study cohort.

6.6.3 Barrett's oesophagus

There were 78 Barrett's oesophagus cases in the HRT exposed group compared to 76 in the unexposed group. Univariate analysis identified that age, alcohol consumption, COPD, asthma, diabetes mellitus, hypertension, ischaemic heart disease, hysterectomy, frequency of endoscopy and GP consultations and beta-2 agonist therapy were associated with Barrett's oesophagus (table 2). Multivariate analysis revealed that age, alcohol consumption, COPD, hypertension, ischaemic heart disease, hysterectomy and frequency of GP consultations were associated with Barrett's oesophagus (table 3). These variables were adjusted for in subsequent analyses.

'Ever' HRT exposure (1.15 (0.81-1.63), p=0.45)) and current HRT (1.30 (0.69-2.50), p=0.44)) were not associated with Barrett's oesophagus (table 4). Increasing duration of HRT was also not associated with Barrett's oesophagus. Duration of HRT use was examined further by using time-dependent covariates and there was no association between length of HRT use and risk of Barrett's oesophagus (0.94 (0.58-1.55), p=0.82). Analysis by subtype of HRT did not reveal an association with Barrett's oesophagus (data not shown).

6.6.4 Reflux oesophagitis

There were 2221 cases of reflux oesophagitis in the HRT exposed group compared to 1467 in the unexposed group. Univariate analysis identified that age, BMI, smoking (current and ex-smokers positively associated and never smoked negatively associated), COPD, asthma, diabetes mellitus, hypertension, ischaemic heart disease, hysterectomy, frequency of

endoscopy and GP consultations, beta-2 agonist and inhaled steroid therapy were associated with reflux oesophagitis (table 2). Multivariate analysis identified age, BMI, ex-smoking status, COPD, asthma, hypertension, ischaemic heart disease, hysterectomy and frequency of GP consultations were associated with reflux oesophagitis (table 3). These variables were controlled for in subsequent analyses.

Both 'ever' HRT use (1.57 (1.45-1.70), $p<0.001$) and current HRT use (1.43 (1.24-1.65), $p<0.001$) were associated with reflux oesophagitis (table 4). Exclusion of patients who were on PPI therapy prior to their initial HRT prescription revealed that HRT was still associated with reflux oesophagitis (1.27 (1.12-1.43), $p<0.001$). However, on using time-dependent covariates to examine the effect of duration of HRT use on reflux oesophagitis, no association was found between length of HRT use and reflux oesophagitis (1.05 (0.97-1.14), $p=0.23$). Analysis by subtype of HRT suggested that 'ever' use of Tibolone use was associated with reflux oesophagitis (1.22 (1.06-1.41), $p=0.01$) but this effect was not seen with current use. Oestrogen, progesterone and combined HRT use were examined separately and no association was found between these subtypes of HRT and reflux oesophagitis.

6.7 Discussion

Oesophageal cancer has a striking male predominance, particularly oesophageal adenocarcinoma. The reasons for this male predominance are unclear and a role for hormonal factors in the aetiology of oesophageal adenocarcinoma is plausible. Derakshan et al reported a lag of 17 years among women developing oesophageal adenocarcinoma compared to men and this coincided with the onset of the menopause in women (Derakhshan et al. 2009). An acceleration in the incidence of endoscopic reflux oesophagitis (RO) in women after the age of 50, with the incidence of RO in women finally catching up with that in men by age 80 (Menon et al. 2011) was recently reported. It was hypothesized that a lack of systemic oestrogen in post-menopausal women may be responsible for this phenomenon.

Oestrogen has been shown to have anti-inflammatory properties with modulation of immune cell activity including changes in cytokine production, cell activation and proliferation (Benten et al. 2001; Cocchiara et al. 1990; Grossman 1989; Gulshan et al. 1990; Mao et al. 2005; Olsen et al.1996; Suzuki et al. 2007).

Oesophagitis and Barrett's metaplasia have been shown to be associated with a T-lymphocyte predominant inflammatory infiltrate (Jankowski et al. 2000). Oestrogen also abolishes the L-selectin expressing subpopulation of neutrophils, which results in an inability of neutrophils to localise at sites of inflammation leading to reduced wound neutrophil elastase activity (Ito et al. 1995). Reduction in neutrophil elastase activity results in better wound integrity during healing due to suppression of matrix metalloproteinases and reduced degradation of fibronectin (Ferry et al. 1997). Oestrogen therefore influences wound healing by modulating the inflammatory response, cytokine expression and matrix deposition but also by accelerating re-epithelialization, stimulating angiogenesis and wound contraction, and by regulating proteolysis. This has been demonstrated in animal models where oestrogen deprivation in ovariectomized female mice results in markedly impaired wound healing with

enhanced inflammation, delayed re-epithelialization, increased wound size and decreased collagen deposition (Ashcroft et al.1997). Oestrogen replacement in the form of HRT in post-menopausal women may therefore, confer a protective effect against oesophageal cancer by lessening oesophageal injury to acid reflux and therefore reducing the risk of Barrett's oesophagus and oesophageal adenocarcinoma.

In the present study 'ever' use of HRT was not associated with oesophageal cancer, as reported in a previous study of HRT and oesophageal cancer (Lindblad et al. 2006). However, examining prolonged HRT use in the present study revealed that HRT use for 5-10 years reduced the risks of oesophageal cancer four-fold. Time-dependent covariates were used separately to determine the effect of length of HRT use on the risk of oesophageal cancer and again using HRT for longer was associated with a significantly reduced risk of oesophageal cancer.

The study hypothesis would suggest that prolonged HRT use would be associated with a larger reduction in the incidence of oesophageal adenocarcinoma than oesophageal squamous cell carcinoma. Unfortunately, histological subtype data was only available on 41% of oesophageal cancers. Consequently, the number of cases was too small to identify significant associations with different oesophageal cancer histological subtypes.

HRT use was modestly associated with reflux oesophagitis. An association between HRT use and acid reflux symptoms has been described in a prospective cohort study among women enrolled in the Nurses Health Study, with a similar modest increased risk in ever and current HRT users (Jacobson et al. 2006;Jacobson et al. 2008). Oestrogen-only, combined HRT and selective oestrogen receptor modulators (SERM) such as Raloxifene were all associated with acid reflux symptoms despite adjusting for BMI (Jacobson et al.2006). A similar association between postmenopausal HRT use and acid reflux symptoms has been reported in a population based study (Nilsson et al. 2002; Nilsson et al. 2003). This group

also found an association between postmenopausal HRT use and endoscopic oesophagitis. Oestrogen increases nitric oxide (NO) synthesis in smooth muscle leading to smooth muscle relaxation in animal models and in humans and may consequently reduce lower oesophageal sphincter (LOS) pressure, which in turn, may potentially exacerbate gastro-oesophageal reflux (De Man et al.1991; Hirsch et al. 1998; Konturek et al.1997). There is however, a possibility of residual confounding in the association between HRT and reflux oesophagitis. Reflux oesophagitis was associated with the frequency of GP consultations and a number of co-morbidities (e.g hypertension and Ischaemic heart disease (IHD)) with no clear aetiological link with reflux oesophagitis. Although these associations were adjusted for, it remains possible that reflux oesophagitis is more likely to be diagnosed in subjects taking HRT; as such subjects are more likely to request investigation or treatment of symptoms, rather than HRT contributing to the development of oesophagitis. Furthermore, excluding subjects who had taken a proton pump inhibitor prior to HRT, suggesting a history of reflux symptoms, significantly weakened the strength of the association. Examining the effect of length of HRT exposure using time-dependent covariates on risk of reflux oesophagitis revealed no association between the length of HRT use and reflux oesophagitis, again casting some doubt on a causal association between HRT and reflux oesophagitis. Finally, HRT use was not associated with Barrett's oesophagus, suggesting that the association between HRT and reflux oesophagitis may be with more minor reflux oesophagitis rather than the severe reflux oesophagitis, which would be more likely to be associated with Barrett's oesophagus and oesophageal adenocarcinoma.

The strengths of the present study include the cohort design that allowed construction of a temporal sequence of events in relation to HRT use and thereby the examination of the effects of prolonged HRT use. The accuracy of recording of prescription data within GPRD are further strengths of the study design. However, the study has a number of potential

limitations. Data on important aetiological factors for oesophageal adenocarcinoma were not available. Longstanding, frequent acid reflux symptoms are associated with oesophageal adenocarcinoma (Ye et al. 2004). Although it was possible to control partly for the influence of pre-existing acid reflux in the association of HRT with reflux oesophagitis by adjusting for PPI therapy prior to starting HRT, it was not possible to accurately assess the influence of pre-existing acid reflux in the association of HRT with oesophageal cancer. Secondly, Helicobacter pylori is negatively associated with the development of oesophageal adenocarcinoma (Ye et al. 2004) but data on Helicobacter pylori status is not available within GPRD. Information on oesophageal cancer, Barrett's oesophagus and reflux oesophagitis and potential confounding factors were collected prospectively in GPRD, reducing the possibility of reporting bias. The study also attempted to control for the fact that subjects who seek HRT for menopausal symptoms may be more likely to seek endoscopy for reflux symptoms and therefore, more likely to be diagnosed with reflux oesophagitis. However, as noted above, despite controlling for the influence of endoscopy frequency and the frequency of GP consultations, this remains a plausible explanation for the association of reflux oesophagitis with HRT. The results of the associations with Barrett's oesophagus in the study population need to be interpreted cautiously. The prevalence of Barrett's oesophagus in the study is very low and similar to the prevalence of oesophageal cancer, strongly suggesting under-reporting of Barrett's oesophagus by endoscopists or general practitioners from endoscopy reports.

HRT use has been shown to reduce the incidence of colorectal cancer by 20-30% in randomised trials and observational data (Beral V et al. 2004; Beral et al. 2002; Rossouw et al. 2002). However, HRT use is associated with an increased risk of thromboembolic events and there is also an increased risk of breast, endometrial and ovarian cancers (Beral et al. 1999). HRT use has therefore declined significantly in the last decade. The role of oestrogen

as an anti-inflammatory agent remains intriguing and further study of the potential role of hormonal factors in the pathogenesis of oesophageal adenocarcinoma is merited to better understand the pronounced gender differences in this condition.

In conclusion, postmenopausal HRT use for more than 5 years was associated with a four-fold reduced risk of oesophageal cancer. HRT was also associated with a modest increased risk of reflux oesophagitis but not Barrett's oesophagus.

Chapter 7

Do differences in female sex hormones contribute to gastro-oesophageal reflux disease?

7 Introduction

A number of epidemiological studies have reported an association between increasing body mass index (BMI) and gastro-oesophageal reflux disease (GORD) and its complications (Dent et al. 2005; Lagergren et al.1999). It is commonly assumed that the increased intra-abdominal pressure seen in obese subjects is the mechanism behind this association through increasing the pressure gradient across the gastro-oesophageal junction (de Vries et al. 2008). However, when men and women have been examined separately, increasing BMI was associated with GORD symptoms or reflux oesophagitis in women but not in men (Nilsson et al. 2002; Nilsson et al. 2003). Furthermore, this association was noted to be stronger in pre-menopausal women and in post-menopausal women on hormone replacement therapy (HRT) (Nilsson et al. 2003), raising the possibility of a female sex-hormone mediated association.

Oestrogen increases nitric oxide (NO) synthesis in smooth muscle which has been shown to reduce lower oesophageal sphincter (LOS) pressure in animal models (Fisher et al. 1978; Hirsch et al. 1998; Konturek et al. 1997). LOS pressure has been reported to be lower in women taking sequential oral contraceptives during the phase when they take both progesterone and oestrogen, rather than oestrogen alone (Van Thiel et al.1976).

A case-control study was therefore undertaken of female sex-hormone levels in women undergoing ambulatory pH monitoring to establish whether elevated hormone levels are associated with increased acid exposure on pH monitoring.

7.1 Study design

Women aged 20-80 years with symptoms of GORD at least twice a week, a total time pH<4 of more than 5% on 24 hour ambulatory pH monitoring and a positive symptom-reflux correlation by symptom association probability (SAP) and symptom index (SI) (Aanen et al. 2008) were identified as ‘cases’. ‘Controls’ were women with a total time pH<4 of less than

5% on 24 hour pH monitoring and a negative symptom-reflux correlation. The cases and controls were matched by age within 5 years and BMI. BMI was categorised into five categories (normal (20-24.9 kg/m2), underweight (<20), overweight (25-29.9), obese (30-34.9) and morbid obesity (>35 kg/m2)) and cases and controls were matched within categories. Subjects with a history of anti-reflux surgery, on proton pump inhibitor (PPI) therapy during the pH study, on hormone therapy (oral contraceptive pill (OCP) or HRT), or with a history of ovarian surgery or polycystic ovarian syndrome (PCOS) were excluded. Menopausal status was verified by a detailed menstrual history during recruitment and cases and controls were stratified further by menopausal status into two groups (pre and post-menopausal). Written, informed consent was taken from subjects who were recruited prospectively from four study sites. The study was approved by the South Birmingham Research Ethics Committee (UK), ref: 09/H1207/5.

A detailed description of the methods used in this study is presented in Chapter 3, sections 3.4, 3.5 and 3.6.

7.2 Sample size estimation

Standard deviations corresponding to published reference ranges for the hormonal assays used in the study were determined (Appendix 2). A sample size of 100 patients (50 each in the pre and post-menopausal groups (25 cases and controls in each group)), was determined to be sufficient to detect differences in hormonal levels at 80% power and 5% significance (Appendix 3).

7.3 Statistical analysis

The association between BMI and both acid exposure and sex hormone levels was determined using Pearson's correlation. Non parametric factors including hormone levels and BMI were log transformed prior to analysis. Group comparisons on normally distributed

variables were carried out using the paired samples t-test. Univariate analysis was carried out to determine the association between each variable and increasing acid exposure. Significant associations were further examined in a multivariate model where these associations were adjusted for. Conditional logistic regression was used to calculate odds ratios with 95% confidence intervals. All analyses were performed using SPSS version 17.0 (IBM Corporation, Somers, NY, USA).

7.4 Results

7.4.1 Subjects

One hundred and twenty women were recruited, of whom 104 (54 pre-menopausal, 50 post-menopausal were matched for age and BMI. The baseline characteristics of the study groups are shown in table 1. Despite controlling for BMI, pre and post-menopausal cases had thicker skin-folds. There were no other significant differences between the groups.

7.4.2 Correlations with increasing acid exposure on pH monitoring

Increasing age did not correlate with increasing acid exposure in either pre-menopausal ($r = -0.07$, $p=0.58$) or post-menopausal subjects ($r = 0.19$, $p=0.18$).

Increasing BMI was associated with increasing acid exposure within groups ($r = 0.404$, $p=0.002$, Pearson's correlation, pre-menopausal and $r = 0.401$, $p=0.01$, post-menopausal). BMI was also correlated with increasing sex hormone levels (serum oestradiol in pre-menopausal women ($r = 0.52$, $p=0.004$ and oestrone in post-menopausal women ($r = 0.364$, $p=0.01$)).

On examining the correlation between sex hormones and increasing acid exposure, in the pre-menopausal group, there was no correlation between increasing acid exposure and oestradiol ($r = 0.26$, $p=0.06$), FSH ($r = 0.002$, $p=0.99$), LH ($r = 0.03$, $p=0.83$) and progesterone ($r = -0.14$, $p=0.33$). However, SHBG ($r = -0.27$, $p=0.05$) and testosterone levels correlated with increasing acid exposure ($r = 0.29$, $p=0.03$).

In the post-menopausal group, there was no correlation between increasing acid exposure and oestrone ($r = -0.103$, $p=0.48$), FSH ($r = 0.04$, $p=0.78$), LH ($r = -0.06$, $p=0.69$), progesterone ($r = -0.08$, $p=0.60$, SHBG ($r = -0.15$, $p=0.30$) or testosterone ($r = -0.09$, $p=0.55$).

The correlation between combined levels of oestradiol and oestrone on increasing acid exposure in both pre and post-menopausal groups was examined in order to reduce confounding by small levels of oestrone in pre-menopausal women (in whom oestradiol is the predominant type of oestrogen) and oestradiol in post-menopausal women (in whom oestrone is predominant). There was no correlation between combined levels of oestradiol and oestrone and increasing acid exposure in pre-menopausal ($r = 0.24$, $p=0.08$) and post-menopausal ($r = 0.03$, $p=0.85$) groups.

7.4.3 Correlations with lower oesophageal sphincter pressure

The correlation between LOS pressure and serum hormone levels was also examined. In the pre-menopausal group, there was no correlation between LOS pressure and oestradiol ($r = -0.09$, $p=0.53$), FSH ($r = 0.15$, $p=0.29$), LH ($r = 0.10$, $p=0.46$), progesterone ($r = -0.03$, $p=0.86$), SHBG ($r = 0.25$, $p=0.07$) and testosterone ($r = -0.18$, $p=0.19$. Additionally, there was no correlation between combined levels of oestradiol and oestrone and LOS pressure ($r = -0.08$, $p=0.59$).

In the post-menopausal group, there was significant correlation between oestrone and LOS pressure ($r = 0.34$, $p=0.02$). However, there was no correlation between LOS pressure and FSH ($r = -0.026$, $p=0.06$), LH ($r = -0.11$, $p=0.44$), progesterone ($r = -0.08$, $p=0.57$), SHBG ($r = -0.03$, $p=0.83$) and testosterone ($r = 0.16$, $p=0.27$. There was also no correlation between combined levels of oestradiol and oestrone and LOS pressure ($r = 0.28$, $p=0.052$).

Table 1: Baseline characteristics of the study groups

	Pre-menopausal Case (n=27)	Pre-menopausal Control (n=27)	p value	Post-menopausal Case (n=25)	Post-menopausal Control (n=25)	p value
Age (mean, SD)	43 (7)	42 (6)	0.19	61 (7)	58 (8)	0.12
BMI (mean, SD)	28 (4.4)	27 (4.5)	0.09	28 (4.4)	28 (4.7)	0.44
DeMeester score (mean, SD)	38 (22.5)	6.4 (4.8)	0.02	52.1 (26.9)	10.4 (5.7)	0.02
% time pH < 4 (mean, SD)	11.5 (7.1)	1.6 (1.5)	0.002	14.8 (7.5)	2.8(1.7)	0.01
Resting LOS pressure (mm Hg), (mean, SD)	12.5 (7)	13.2 (6)	0.36	10.8 (5.1)	14.5 (7)	0.15
Waist-hip ratio (mean, SD)	0.9 (0.1)	0.9 (0.1)	0.50	0.91 (0.1)	0.92 (0.1)	0.24
Skinfold thickness (mm),(mean, SD)	36.7 (8.9)	31 (8.2)	0.01	37.3 (18.5)	28.1 (12)	0.02
History of reflux during pregnancy (n)	7	8	0.12	4	14	0.02
Current smoking (n)	15	19	0.13	20	11	0.04
Current alcohol consumption (n)	10	9	0.14	7	10	0.23
Alcohol >14 units / week (n)	2	4		1	1	

7.4.4 Logistic regression analysis

Pre-menopausal group

On univariate analysis, only skinfold thickness (1.01 (1.002-1.02), p=0.02) was associated with increasing acid exposure (table 2). This was adjusted for in the multivariate analysis. Despite SHBG and testosterone correlating with increasing acid exposure, no association was found on logistic regression analysis with excess acid exposure.

Post-menopausal group

In the post-menopausal group, serum FSH levels (1.02 (1.00-1.05), p=0.05), smoking (5.09 (1.45-17.92), p=0.01) and a history of reflux during pregnancy (2.44 (1.01-5.93), p=0.05) were associated with excess acid exposure. These variables were adjusted for subsequently in the multivariate analysis.

Multivariate analyses

On multivariate analysis, none of the above factors were associated with excess acid exposure. There was no association between serum hormone levels and excess acid exposure.

Table 2: Logistic regression analysis of risk factors for excess acid exposure on 24 hour pH monitoring

	Pre-menopausal Odds ratio (95% CIs)	p-value	Post-menopausal Odds ratio (95% CIs)	p-value
Waist-hip ratio	1.05 (0.003-430.6)	p=0.99	11.02 (0.01-8796.8)	p=0.48
Skin fold thickness	1.01 (1.01-1.02)	p=0.02	1.01 (1.0-1.02)	p=0.06
Oestradiol	1.00 (0.99-1.001)	p=0.11	0.99 (0.99-1.002)	p=0.43
Oestrone	1.00 (0.99-1.00)	p=0.60	0.99 (0.99-1.00)	p=0.28
Oestradiol + oestrone	1.00 (1.0-1.001)	p=0.12	0.99 (0.997-1.001)	p=0.30
FSH	1.01 (0.96-1.03)	p=0.16	1.02 (1.00-1.05)	p=0.05
LH	1.04 (0.99-1.08)	p=0.09	1.03 (0.98-1.09)	p=0.21
SHBG	0.99 (0.97-1.00)	p=0.09	1.01 (0.98-1.04)	p=0.71
Progesterone	0.98 (0.92-1.03)	p=0.36	1.07 (0.88-1.33)	p=0.51
Testosterone	1.06 (0.54-2.09)	p=0.86	0.97 (0.36-2.59)	p=0.94
Smoking	1.90 (0.62-5.83)	p=0.26	5.10 (1.45-17.92)	p=0.01
Alcohol	0.67 (0.32-1.40)	p=0.28	0.69 (0.26-1.85)	p=0.46
History of reflux during pregnancy	1.59 (0.77-3.30)	p=0.21	2.40 (1.01-5.93)	p=0.05

7.5 Discussion

This is the first study to directly investigate the relationship between serum hormone levels and acid reflux in women. An association between obesity and acid reflux has also been reported by several investigators. Jacobson et al conducted a population-based questionnaire survey among women and found that increasing BMI was associated with GORD symptoms in both normal weight and overweight women (Jacobson et al. 2008). A meta-analysis reported a dose-response relationship between BMI and the risk of reporting GORD symptoms among both men and women (Hampel et al. 2005). Another survey found that there was a dose-response relationship between quartiles of BMI and daily frequency of acid reflux symptoms but this study lacked a control group (Oliveria et al. 1999).

Any association between sex hormones and GORD symptoms is therefore likely to be confounded by BMI. Patients were therefore matched on age and BMI to determine if there was a true association between serum hormone levels and acid reflux independent of the influence of BMI. Although increasing BMI correlated with acid exposure on 24 hour pH monitoring and sex hormone levels, once subjects were matched for age ad BMI, there was no association found between serum hormone levels and excess acid exposure. There does not seem to be an association between serum hormone levels and increasing acid exposure in women once matched for age and BMI. Serum hormones were also not associated with BMI when examined separately in pre and post-menopausal groups.

Previous studies have suggested a link between oestrogen and acid reflux symptoms. Nilsson et al demonstrated a dose-dependent association between postmenopausal hormone therapy and endoscopic oesophagitis (Nilsson et al. 2002) and a population-based study revealed that determined that there was a strong association between increasing BMI and

reflux symptoms in pre-menopausal women and post-menopausal women on hormone therapy (Nilsson et al. 2003). A large population based study evaluated GORD symptoms in post-menopausal women on HRT taking part in the Nurses Health Study (Jacobson et al. 2006; Jacobson et al.2008). This study concluded that HRT use was significantly associated with GORD symptoms and the increased risk of GORD symptoms did not differ between different strata of BMI, suggesting that this was an effect independent of BMI.

It has been suggested that oestrogen is associated with acid reflux through increased nitric oxide synthesis, which may in turn cause LOS relaxation (De Man et al.1991; Hirsch, et al. 1998; Konturek et al. 1997). GORD symptoms frequently begin for the first time in pregnancy and become progressively worse during the course of the pregnancy (Marrero et al.1992). LOS pressures have also been shown to decrease progressively through pregnancy, returning to normal after delivery (Nagler et al. 1961). Analysis of serum levels of oestrone, oestradiol and progesterone during pregnancy in relation to LOS pressure changes revealed a linear relationship between the reduction in LOS pressure and the progressive rise in sex hormone levels, suggesting that oestrogen and progesterone reduced LOS pressure (Van Thiel et al.1977). A direct relationship between urinary oestradiol and serum progesterone levels and the increase in the severity of GORD symptoms during the course of pregnancy has been suggested but the data on hormonal levels was obtained indirectly from published normal values during pregnancy (Johansson 1969; Klopper 1963; Ulmsten et al. 1978). Despite data suggesting a putative link between pregnancy, raised oestrogen and progesterone levels and low LOS resting pressures, there is no data to suggest that serum oestrogen and progesterone levels directly impact on the severity of acid exposure during pregnancy. Moreover, other factors such as increased intra-gastric pressure due to effect of the gravid uterus can exacerbate acid reflux. A reduction in the amplitude of distal oesophageal

contractions during pregnancy resulting in impaired oesophageal motility, has also been suggested to be a factor in promoting acid reflux in pregnancy by delaying acid clearance (Ulmsten et al.1978).

Although in post-menopausal subjects in the present study, oestrone levels correlated with LOS pressure, this finding is the opposite of the negative correlation that would be expected and unlikely to be a significant finding.

A weakness of the present study was the relatively small sample size. However, the sample size was sufficient to power the study. Moreover, the effect sizes (odds ratios) and narrow confidence intervals associated with hormonal levels suggest that there is unlikely to be any association between hormone levels and acid reflux once BMI is controlled for.

In conclusion, female sex hormone levels are not associated with excess acid exposure on pH monitoring once the effect of increasing BMI is controlled for.

Chapter 8

Acid reflux, gender and the oesophagus: Evaluation of gender-specific responses in the oesophagus

8 Background

The incidence of oesophageal adenocarcinoma has been continually rising in the last decade. The cancer arises from a background of Barrett's metaplasia (a mucin secreting, columnar epithelium that replaces the squamous epithelium of the distal oesophagus) which is a common consequence of reflux oesophagitis. The epidemic of this cancer is largely a male phenomenon with the ratio of male to female incidence rates standing at 5:1 in England and Wales. Data from the Five-Continents Database shows that this wide sex differential is seen globally, with the high male to female ratio of lifetime risk seen across the range of disparate socio-economic cultures (e.g 4:1 in Ecuador and 10:1 in Canada) (Corley et al. 2001). This implies that the disease is influenced by more than just environment and may be intrinsically linked to being male.

Studies on the incidence of Barrett's metaplasia have estimated the male to female differential to be at most 1.5:1 (Caygill et al. 1999). Similarly, gastro-oesophageal reflux disease occurs in 10 - 20% of the population and shows no significant preponderance for males (Mohammed et al. 2005). Therefore the adenocarcinoma sex differential cannot be explained simply by a higher prevalence of either reflux disease or Barrett's metaplasia.

An alternative hypothesis is that fundamental differences between male and female oesophageal tissue may exist. These differences lead to differential responses to acid reflux and the progression of Barrett's metaplasia. This study aimed to characterise the expression of biomarkers in male and female samples of normal oesophagus, oesophagitis, Barrett's metaplasia and adenocarcinoma, with particular focus on any qualitative or quantitative gender differences.

A detailed description of the methods used in this study is presented in Chapter 3, section 3.7.

8.1 Methods

The mRNA and protein expression of cdx-1 and 2, PCNA and cyclin-D1 in male and female biopsy samples of normal oesophagus, oesophagitis, Barrett's metaplasia and adenocarcinoma had been previously characterised (data unpublished). PCR results had suggested that there was up-regulation of cdx-1, cdx-2 and PCNA from squamous oesophagus to Barrett's metaplasia in both genders but was greater in males than females. In order to determine gender differences in gene expression secondary to acid reflux in patients with Barrett's oesophagus a pilot study involving tissue microarray analysis was carried out. Biopsies of normal oesophagus and Barrett's metaplasia in male and female subjects were used for this study. Regional ethical approval was obtained for this study and written, informed consent was taken.

Biopsies of Barrett's epithelium and squamous epithelium above the Barrett's segment were taken from six male and six female subjects attending surveillance endoscopy for Barrett's oesophagus. These biopsies were frozen immediately in liquid nitrogen and transported to a laboratory in the Institute of Cancer Sciences, University of Birmingham, where they were stored at -70°C until further analysis. Quality of each Barrett's biopsy was assessed by determining the presence of intestinal metaplasia on Haematoxylin & Eosin staining prior to subjecting each tissue (squamous oesophagus and Barrett's samples) to tissue microarray analysis.

8.2 Results

A Venn diagram filter was used to detect genes that were dysregulated in all three stratification pools. The central overlap (figure 1 A & B) shows the number of genes that were altered between male Barrett's compared with female Barrett's tissues, and between male squamous compared with female squamous tissues, and also between male squamous compared to male Barrett's tissues. Five genes were highlighted in this way (figure 2): DAZ-1, DAZ-2, CCL4, RPS4Y1 and USP9Y.

DAZ-1, DAZ-2, USP9Y and RPS4Y1 were over-expressed in male Barrett's biopsies (figure 2). CCL4 was significantly under-expressed in males compared to females.

A **B**

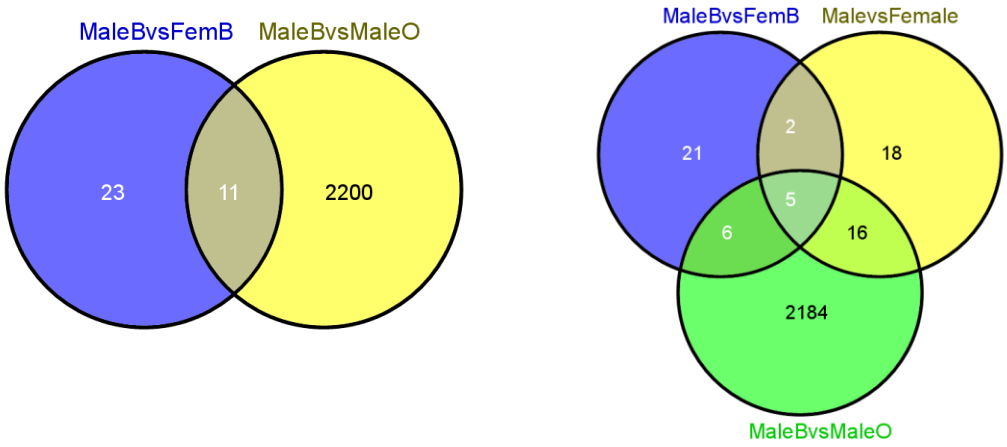


Figure 1: Venn diagram filters (A & B)

The Venn diagram filters show the number of genes commonly dysregulated in the different gene stratification pools. Specimens from Male and Female Barrett's oesophagus (blue circle) and male Barrett's and male squamous oesophagus (yellow), (Figure 1A) are compared. 11 genes (area of intersection) are common to these pools. In Figure 1B, three stratification pools (male and female Barrett's (blue), male and female squamous oesophagus (yellow) and male Barrett's and male oesophagus (green)) are compared. The area of intersection of the three pools shows that there are 5 genes that specific to male and female Barrett's tissues.

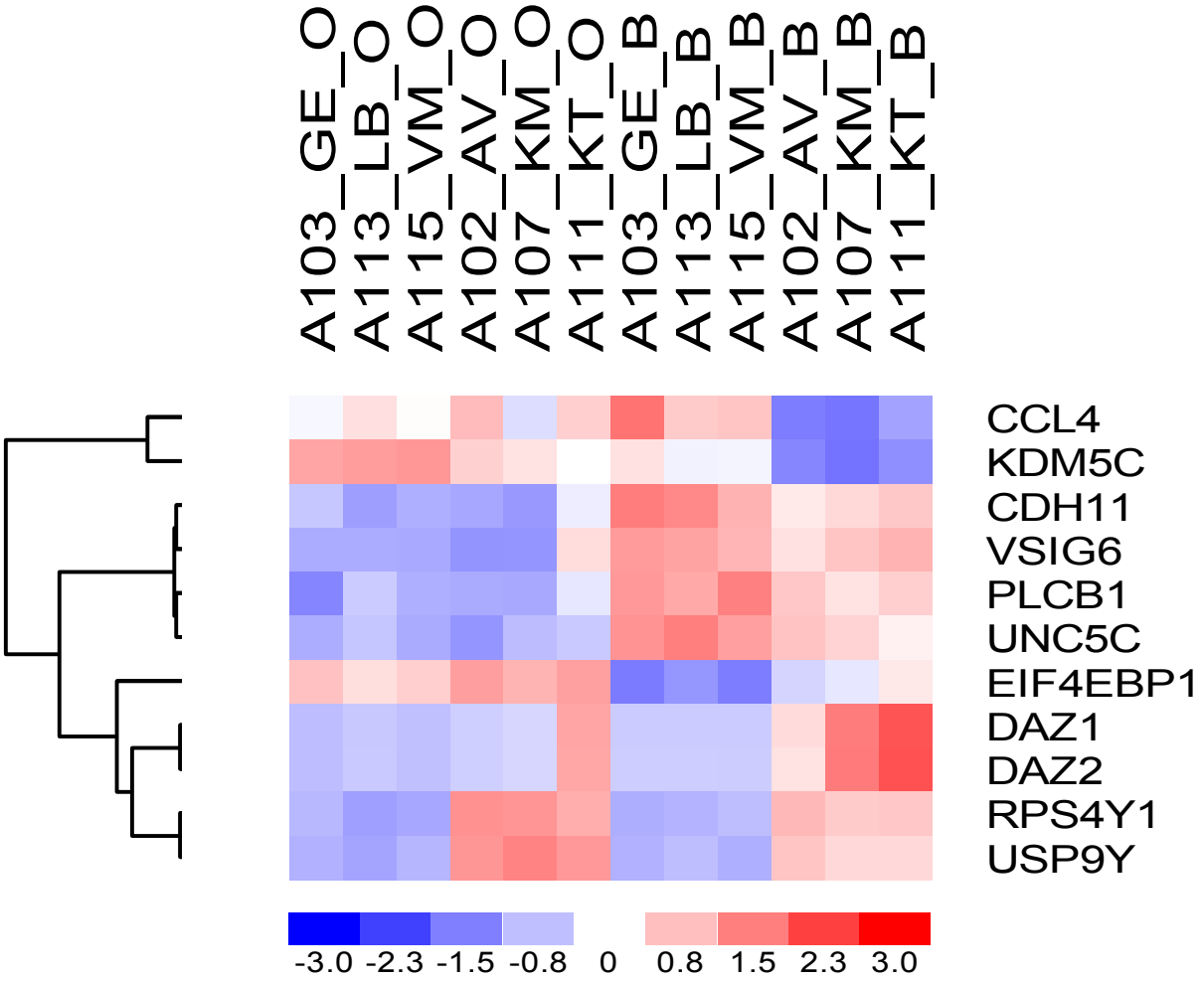


Figure 2: Heat map generated from tissue microarray analysis

Six squamous oesophageal specimens and six Barrett's specimens (3 males and females each for both sets) are compared. Candidate genes are listed in the column on the right. The colours in the heat map represent the degree of expression of the gene in the tissue with red indicating strong and blue, weak associations. The genes DAZ-1, DAZ-2, USP9Y and RPS4Y1 were strongly expressed in male Barrett's biopsies. CCL4 was weakly expressed in males compared to females.

8.3 Discussion

The male predominance of oesophageal adenocarcinoma cannot be explained by conventional risk factors such as smoking and alcohol (Gammon et al. 1997). An alternative hypothesis is that there may exist fundamental differences between male and female oesophageal tissue. These differences lead to differential responses to acid reflux and the progression of Barrett's metaplasia, and may explain why obesity and reflux disease have different consequences in men and women. This hypothesis implies that the male oesophagus responds in an intrinsically different manner to the female.

Inherited epigenetic imprints such as methylation are erased in embryogenesis and reset according to the sex of the embryo. Such sex specific imprinting has been shown to occur in the human foetus and therefore influence adult gene expression (Durcova-Hills et al. 2004). The human foetal oesophagus has columnar epithelium replaced by squamous epithelium at 20-25 weeks, when the differentiation of the foetal gonads has occurred and sex steroids are present (Menard et al. 1987). The developmental homeobox gene cdx-2 has been linked to the columnar differentiation pattern seen in adult metaplasia (Eda et al. 2003). It is hypothesised therefore that the sex of the individual can influence the lifelong dynamics of epithelial differentiation and the associated inflammatory responses.

Paradigms are emerging where female and male adults exhibit differential levels of gene expression following inflammatory stimuli that are not obvious consequences of sex-steroid hormones. In a study of dizygotic twins, females were found to produce less IL-10 than males in response to lipopolysaccharide (Reuss et al. 2002). In animal models of alcohol-induced liver injury, clear gender differences are seen with females displaying greater increases in IL-6 receptor expression than males (Gallucci et al. 2004). In anal squamous cell carcinoma, the apoptosis-regulating gene Bcl-2 is more likely to be up-regulated in female patients than males (Allal et al. 2005).

Patients with Barrett's metaplasia have greater exposures to gastric acid than control groups with oesophagitis. Expression profiling of an oesophageal cell line exposed to acid has identified an early up-regulation of genes associated with proliferation (PCNA), inflammatory cytokines and mitogen-activated protein kinase (MAPK) activity. Rapid increases in the activity of the MAPK proteins ERK and p38 have been demonstrated both in-vitro and in-vivo after acid perfusion of patient's distal oesophagus at endoscopy (Souza et al. 2002). Ex-vivo experiments using endoscopic biopsies of Barrett's metaplasia have shown increases in proliferation markers (PCNA), in expression of cyclo-oxygenase-2 (COX-2) and in subsequent release of prostaglandin E2 (PGE2) following pulses of acid exposure (Fitzgerald et al. 1996; Shirvani et al. 2000). In-vivo pharmacological acid suppression was shown to reverse the increase in cell cycle oncogenes cyclin D1 and cyclin E (Umansky et al. 2001). Assessment of the expression of developmental homeobox genes cdx-1 and cdx-2 shows that the cdx-2 gene is expressed in Barrett's epithelium and inflammatory oesophageal mucosa, implying that this is an early event in the development of metaplastic epithelium (Eda et al.2003).

This study has investigated qualitative differences in the responses between male and female oesophageal tissue. The study demonstrates that there is a gender-specific difference in biomarker expression in oesophageal tissue secondary to acid reflux. Of the five genes differentially expressed in males, DAZ (Deleted in Azoospermia) is located on the Y-chromosome and encodes an RNA-binding protein important for translation of mRNA. DAZ may therefore, have a role in cell maturation in columnar mucosa. CCL4 is macrophage inflammatory protein-1β and acts as chemokine attractant for NK cells and monocytes. Under-expression of CCL-4 in male tissues is further evidence that chronic inflammation may be modulated in a gender-specific fashion.

Limitations of the study include its small sample size and that only gene expression profiles in the tissues were analysed. A further study is being planned with future work needed to localise and characterise protein expression.

In conclusion, there appear to be gender-specific differences in the reflux-Barrett's pathway. A novel set of biomarkers in male Barrett's specimens have been identified in this study and further analysis of these genetic markers through immunohistochemistry and rt-PCR will be needed to characterise them.

Chapter 9

Conclusions and Implications

Conclusion and Implications

The aetiology of gastro-oesophageal reflux disease is multifactorial and is influenced by several variables. As detailed in the introduction, age, obesity, alcohol, smoking, drug therapy and gender are some of the important factors that play a role in perpetuating gastro-esophageal reflux and GORD-mediated complications. Whilst the role of most of these aetiological factors have been studied and characterised, the influence of gender is intriguing and has many aspects that are unexplained. The prevalence of GORD is increasing steadily and may be explained by several of the factors mentioned above but the astonishing rise in the incidence of oesophageal adenocarcinoma over the last two decades, virtually completely replacing oesophageal squamous cell carcinoma as the commonest type of oesophageal cancer, cannot be explained by risk factors detailed above which have traditionally been associated with oesophageal squamous cell carcinoma. Oesophageal adenocarcinoma is a complication of chronic gastro-oesophageal reflux and has a stark male predominance (6:1). Barrett's oesophagus, its premalignant precursor lesion which develops secondary to chronic gastro-oesophageal reflux, is also more common in males (1.5:1) but considerably less so in comparison to oesophageal adenocarcinoma. Gender is therefore a key influence in the aetiology of oesophageal adenocarcinoma. Examining the age-specific incidence of oesophageal adenocarcinoma in women reveals that the incidence of oesophageal adenocarcinoma peaks after the menopause at least 17 years later than the peak in incidence in men. This suggests that menopause is an important landmark for women in terms of their overall risk of oesophageal adenocarcinoma and that hormonal changes coinciding with menopause may play a crucial role in the aetiology and pathogenesis of oesophageal adenocarcinoma.

Results from examining six large endoscopy databases revealed that with increasing age, the incidence of severe reflux oesophagitis and benign oesophageal stricture in patients undergoing endoscopy for symptoms of GORD increased more rapidly in women than men and the incidences became very similar over the age of 70 in both sexes (Menon et al. 2011). This acceleration of the incidence of reflux oesophagitis, benign oesophageal stricture and Barrett's oesophagus was much more marked in women over the age of 50, with the peak in the acceleration between ages 60-70 years.

Why should older women have an increase in the incidence of severe reflux oesophagitis with age? An interesting hypothesis that could explain this phenomenon would be that oestrogen, with its anti-inflammatory properties, could potentially prevent severe oesophagitis from developing in women of menstrual age and once systemic oestrogen levels decline with age, the severity of acid-reflux induced oesophagitis increases. The anti-inflammatory properties of oestrogen have been studied in various tissues and topical oestrogen therapy has been shown to facilitate wound healing in human and animal models. Although surgical models of gastro-oesophageal reflux in animals have been studied, the primary aim of these models was to study the development of Barrett's oesophagus and oesophageal adenocarcinoma. These animal models are difficult to maintain and have high morbidity. Moreover, tissue acquisition is difficult and animals need to be sacrificed in order to obtain tissue. The animal study undertaken as part of this body of work was to create a chronic wound model in animals which could be accessible for experiments and tissue acquisition and would serve as a model for oesophagitis. Experiments on the acute oral wound model in mice suggested that lack of oestrogen delayed wound healing and changed the composition of the inflammatory infiltrate and were consistent with the above hypothesis.

If lack of oestrogen in the post-menopausal period resulted in an increased risk of severe complications of GORD in a subject with acid reflux, could oestrogen supplementation in the form of HRT in post-menopausal women reduce their risk of GORD-related complications? HRT has been studied previously in relation to colorectal cancer and there is data to suggest an inverse association between HRT use and the risk of colorectal cancer (Beral et al. 2004). The mechanisms of oestrogen-mediated protection against the risk of colorectal cancer have been postulated to be secondary to its modulation of the inflammatory response. HRT use in post-menopausal women has been previously studied in a case-control study, which did not show any association between HRT use and oesophageal cancer but showed a reduced risk for gastric cancer (Lindblad et al. 2006). The length of HRT treatment and its impact on the risk of oesophago-gastric cancer was not examined. A cohort study was therefore performed to determine if the length of HRT use had an impact on GORD-related complications. Although HRT use was related to an increased risk of reflux oesophagitis, there was no association between the duration of HRT use and the risk of reflux oesophagitis, suggesting there may be confounding in the association between HRT use and reflux oesophagitis with more women on HRT reporting reflux symptoms. HRT use was not related to the risk of Barrett's oesophagus but had an inverse association with duration of use and risk of oesophageal cancer. Prolonged use (> 5 years) of HRT was associated with a reduced risk of oesophageal cancer. Thus the gender difference in the prevalence of GORD complications may be due to hormonal factors. Despite its anti-inflammatory properties, which may protect against GORD-related complications in young women, oestrogen has LOS-relaxing properties through its action on nitric oxide. This may facilitate acid reflux and may even explain the association of HRT with reflux oesophagitis described above. A case-control study examining the relationship between serum sex hormones in women showed that oestrogen levels did not correlate with increasing acid exposure on 24 hour pH monitoring

once BMI was controlled for. There was also no correlation between LOS pressures and serum sex hormone levels. Serum sex hormone levels correlated as expected with BMI and this study confirmed again the critical role of increasing BMI in GORD.

Hormones clearly seem to play an important role in GORD and related complications. However, there may be an additional factor in that oesophageal tissue seems to respond in a gender-specific manner to acid reflux, with male Barrett's tissues expressing a distinct set of genes (DAZ-1, DAZ-2, CCL4, RPS4Y1 and USP9Y). DAZ and CCL4 have roles in cell maturation and chemotaxis and may serve an important role in modulating the overall inflammatory response to acid reflux.

The findings from these studies suggest that there may be a complex gender based genotypic response of oesophageal tissues to acid reflux, which may interact with phenotypic differences in systemic oestrogen levels. These are questions that need separate avenues of research. The acute oral wound model needs to be converted to a chronic wound model by application of acid and bile in order to examine the interaction between oestrogen and acid and bile induced mucosal inflammation. A further study is being planned to characterise the expression of the biomarkers detected at tissue microarray analysis using immunohistochemistry and PCR in male and female oesophageal biopsies. To examine the effect of HRT on subtypes of oesophageal cancer, a case-control study of post-menopausal women, powered to be able to achieve a significant result for each cancer (and hormonal) subtype will need to be planned.

A prospective study is currently underway called the Million women study (Beral et al. 2003, Beral et al. 2007), where more than a million women over the age of 50 years are

being followed up to understand the impact of reproductive and lifestyle factors (including HRT use) on their risk of cardiovascular disease, osteoporotic fractures and cancers among other health parameters. Initial data from the study do not suggest an association between HRT use and the risk of oesophageal cancer although there was a marked risk reduction in colorectal cancer with HRT use. Prospective long term data pertaining to histological subtype of oesophageal cancer may provide further information on the effect of HRT use and the overall risk of oesophageal cancer.

The data presented in this body of work has suggested that there is a distinct gender difference in the prevalence of gastro-oesophageal reflux disease and its complications. This difference is likely to be multifactorial and may be related to genotypic differences in oesophageal tissue in the response to acid reflux but seems to be also significantly influenced by female sex hormones, particularly oestrogen.

Chapter 10

References

References

Aanen, M.C., Bredenoord, A.J., Numans, M.E., Samson, M., & Smout, A.J. 2008. Reproducibility of symptom association analysis in ambulatory reflux monitoring. *Am.J Gastroenterol*, 103, (9) 2200-2208 available from: PM:18684174

Acevedo, M.L. & Kraus, W.L. 2004. Transcriptional activation by nuclear receptors. *Essays Biochem.*, 40, 73-88 available from: PM:15242340

Achem, A.C., Achem, S.R., Stark, M.E., & DeVault, K.R. 2003. Failure of esophageal peristalsis in older patients: association with esophageal acid exposure. *Am.J Gastroenterol*, 98, (1) 35-39 available from: PM:12526933

Allal, A.S., Brundler, M.A., & Gervaz, P. 2005. Differential expression of anti-apoptotic protein Bcl-2 in keratinizing versus non-keratinizing squamous cell carcinoma of the anus. *Int.J Colorectal Dis.*, 20, (2) 161-164 available from: PM:15688099

Allison, P.R. 1951. Reflux esophagitis, sliding hiatal hernia, and the anatomy of repair. *Surg.Gynecol.Obstet.*, 92, (4) 419-431 available from: PM:14835197

Allison, P.R. & Johnstone, A.S. 1953. The oesophagus lined with gastric mucous membrane. *Thorax*, 8, (2) 87-101 available from: PM:13077502

Anderson, G.L., Judd, H.L., Kaunitz, A.M., Barad, D.H., Beresford, S.A., Pettinger, M., Liu, J., McNeeley, S.G., & Lopez, A.M. 2003. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. *JAMA*, 290, (13) 1739-1748 available from: PM:14519708

Anderson, G.L., Limacher, M., Assaf, A.R., Bassford, T., Beresford, S.A., Black, H., Bonds, D., Brunner, R., Brzyski, R., Caan, B., Chlebowski, R., Curb, D., Gass, M., Hays, J., Heiss, G., Hendrix, S., Howard, B.V., Hsia, J., Hubbell, A., Jackson, R., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L., LaCroix, A.Z., Lane, D., Langer, R.D., Lasser, N., Lewis, C.E., Manson, J., Margolis, K., Ockene, J., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Ritenbaugh, C., Robbins, J., Rossouw, J.E., Sarto, G., Stefanick, M.L., Van, H.L., Wactawski-Wende, J., Wallace, R., & Wassertheil-Smoller, S. 2004. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA*, 291, (14) 1701-1712 available from: PM:15082697

Anderson, L.A., Cantwell, M.M., Watson, R.G., Johnston, B.T., Murphy, S.J., Ferguson, H.R., McGuigan, J., Comber, H., Reynolds, J.V., & Murray, L.J. 2009. The association between alcohol and reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma. *Gastroenterology*, 136, (3) 799-805 available from: PM:19162028

Angelov, N., Moutsopoulos, N., Jeong, M.J., Nares, S., Ashcroft, G., & Wahl, S.M. 2004. Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor. *Thromb.Haemost.*, 92, (2) 288-297 available from: PM:15269824

Ashcroft, G.S., Dodsworth, J., van, B.E., Tarnuzzer, R.W., Horan, M.A., Schultz, G.S., & Ferguson, M.W. 1997. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. *Nat.Med.*, 3, (11) 1209-1215 available from: PM:9359694

Ashcroft, G.S., Greenwell-Wild, T., Horan, M.A., Wahl, S.M., & Ferguson, M.W. 1999. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. *Am.J.Pathol.*, 155, (4) 1137-1146 available from: PM:10514397

Ashcroft, G.S., Lei, K., Jin, W., Longenecker, G., Kulkarni, A.B., Greenwell-Wild, T., Hale-Donze, H., McGrady, G., Song, X.Y., & Wahl, S.M. 2000. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat.Med.*, 6, (10) 1147-1153 available from: PM:11017147

Ashcroft, G.S., Mills, S.J., Flanders, K.C., Lyakh, L.A., Anzano, M.A., Gilliver, S.C., & Roberts, A.B. 2003. Role of Smad3 in the hormonal modulation of in vivo wound healing responses. *Wound.Repair Regen.*, 11, (6) 468-473 available from: PM:14617288

Ashcroft, G.S., Mills, S.J., Lei, K., Gibbons, L., Jeong, M.J., Taniguchi, M., Burow, M., Horan, M.A., Wahl, S.M., & Nakayama, T. 2003. Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. *J.Clin.Invest*, 111, (9) 1309-1318 available from: PM:12727922

Bainbridge, K.E., Sowers, M.F., Crutchfield, M., Lin, X., Jannausch, M., & Harlow, S.D. 2002. Natural history of bone loss over 6 years among premenopausal and early postmenopausal women. *Am.J Epidemiol.*, 156, (5) 410-417 available from: PM:12196310

Baker, V.L. 1994. Alternatives to oral estrogen replacement. Transdermal patches, percutaneous gels, vaginal creams and rings, implants, other methods of delivery. *Obstet.Gynecol.Clin.North Am.*, 21, (2) 271-297 available from: PM:7936545

Banks, E., Beral, V., Reeves, G., Balkwill, A., & Barnes, I. 2004. Fracture incidence in relation to the pattern of use of hormone therapy in postmenopausal women. *JAMA*, 291, (18) 2212-2220 available from: PM:15138243

Barrett, N.R. 1950. Chronic peptic ulcer of the oesophagus and 'oesophagitis'. *Br.J Surg.*, 38, (150) 175-182 available from: PM:14791960

Baumann, R., Casaulta, C., Simon, D., Conus, S., Yousefi, S., & Simon, H.U. 2003. Macrophage migration inhibitory factor delays apoptosis in neutrophils by inhibiting the mitochondria-dependent death pathway. *FASEB J*, 17, (15) 2221-2230 available from: PM:14656984

Beck, L.S., DeGuzman, L., Lee, W.P., Xu, Y., Siegel, M.W., & Amento, E.P. 1993. One systemic administration of transforming growth factor-beta 1 reverses age- or glucocorticoid-impaired wound healing. *J Clin.Invest*, 92, (6) 2841-2849 available from: PM:8254038

Behar, J., Biancani, P., & Sheahan, D.G. 1976. Evaluation of esophageal tests in the diagnosis of reflux esophagitis. *Gastroenterology*, 71, (1) 9-15 available from: PM:6361

Behar, J., Field, S., & Marin, C. 1979. Effect of glucagon, secretin, and vasoactive intestinal polypeptide on the feline lower esophageal sphincter: mechanisms of action. *Gastroenterology*, 77, (5) 1001-1007 available from: PM:226449

Belchetz, P.E. 1994. Hormonal treatment of postmenopausal women. *N.Engl.J Med.*, 330, (15) 1062-1071 available from: PM:8127335

Benten, W.P., Stephan, C., Lieberherr, M., & Wunderlich, F. 2001. Estradiol signaling via sequestrable surface receptors. *Endocrinology*, 142, (4) 1669-1677 available from: PM:11250949

Beral V, Banks, E., Reeves G, Green J, Gathani T, & Crossley B. The effect of hormone replacement therapy on breast and other cancers. Critchley H, Gebbie A, and Beral, V. Menopause and Hormone Replacement , 136-150. 2004. London, RCOG Press.

Beral, V. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, 362, (9382) 419-427 available from: PM:12927427

Beral, V., Banks, E., & Reeves, G. 2002. Evidence from randomised trials on the long-term effects of hormone replacement therapy. *Lancet*, 360, (9337) 942-944 available from: PM:12354487

Beral, V., Banks, E., Reeves, G., & Appleby, P. 1999. Use of HRT and the subsequent risk of cancer. *J Epidemiol.Biostat.*, 4, (3) 191-210 available from: PM:10695959

Beral, V., Bull, D., Green, J., & Reeves, G. 2007. Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet*, 369, (9574) 1703-1710 available from: PM:17512855

Berman, J.R., McCarthy, M.M., & Kyprianou, N. 1998. Effect of estrogen withdrawal on nitric oxide synthase expression and apoptosis in the rat vagina. *Urology*, 51, (4) 650-656 available from: PM:9586624

Blair, I.A. 2010. Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future. *Steroids*, 75, (4-5) 297-306 available from: PM:20109478

Bocchinfuso, W.P., Lindzey, J.K., Hewitt, S.C., Clark, J.A., Myers, P.H., Cooper, R., & Korach, K.S. 2000. Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology*, 141, (8) 2982-2994 available from: PM:10919287

Boyle, J.T., Altschuler, S.M., Nixon, T.E., Tuchman, D.N., Pack, A.I., & Cohen, S. 1985. Role of the diaphragm in the genesis of lower esophageal sphincter pressure in the cat. *Gastroenterology*, 88, (3) 723-730 available from: PM:3967808

Bremner, C.G., Lynch, V.P., & Ellis, F.H., Jr. 1970. Barrett's esophagus: congenital or acquired? An experimental study of esophageal mucosal regeneration in the dog. *Surgery*, 68, (1) 209-216 available from: PM:10483471

British Society of Gastroenterology. Guidelines for the diagnosis and management of Barrett's columnar lined oesophagus. A report of the working party of the British Society of Gastroenterology. 2006.

Brown, L.M., Devesa, S.S., & Chow, W.H. 2008. Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. *J.Natl.Cancer Inst.*, 100, (16) 1184-1187 available from: PM:18695138

Bybee, D.E., Brown, F.C., Georges, L.P., Castell, D.O., & McGuigan, J.E. 1979. Somatostatin effects on lower esophageal sphincter function. *Am.J Physiol*, 237, (1) E77-E81 available from: PM:111562

Bytzer, P., Christensen, P.B., Damkier, P., Vinding, K., & Seersholm, N. 1999. Adenocarcinoma of the esophagus and Barrett's esophagus: a population-based study. *Am.J.Gastroenterol.*, 94, (1) 86-91 available from: PM:9934736

Calandra, T. & Roger, T. 2003. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat.Rev.Immunol.*, 3, (10) 791-800 available from: PM:14502271

Calle, E.E., Miracle-McMahill, H.L., Thun, M.J., & Heath, C.W., Jr. 1995. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *J Natl.Cancer Inst.*, 87, (7) 517-523 available from: PM:7707438

Cameron, A.J. 1999. Barrett's esophagus: prevalence and size of hiatal hernia. *Am.J Gastroenterol*, 94, (8) 2054-2059 available from: PM:10445527

Cameron, A.J. & Lomboy, C.T. 1992. Barrett's esophagus: age, prevalence, and extent of columnar epithelium. *Gastroenterology*, 103, (4) 1241-1245 available from: PM:1397881

Cameron, A.J., Zinsmeister, A.R., Ballard, D.J., & Carney, J.A. 1990. Prevalence of columnar-lined (Barrett's) esophagus. Comparison of population-based clinical and autopsy findings. *Gastroenterology*, 99, (4) 918-922 available from: PM:2394347

Camilleri, M., Dubois, D., Coulie, B., Jones, M., Kahrilas, P.J., Rentz, A.M., Sonnenberg, A., Stanghellini, V., Stewart, W.F., Tack, J., Talley, N.J., Whitehead, W., & Revicki, D.A. 2005. Prevalence and socioeconomic impact of upper gastrointestinal disorders in the United States: results of the US Upper Gastrointestinal Study. *Clin.Gastroenterol.Hepatol.*, 3, (6) 543-552 available from: PM:15952096

Castelo-Branco, C., Cancelo, M.J., Villero, J., Nohales, F., & Julia, M.D. 2005. Management of post-menopausal vaginal atrophy and atrophic vaginitis. *Maturitas*, 52 Suppl 1, S46-S52 available from: PM:16139449

Cauley, J.A., Robbins, J., Chen, Z., Cummings, S.R., Jackson, R.D., LaCroix, A.Z., LeBoff, M., Lewis, C.E., McGowan, J., Neuner, J., Pettinger, M., Stefanick, M.L., Wactawski-Wende, J., & Watts, N.B. 2003. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial. *JAMA*, 290, (13) 1729-1738 available from: PM:14519707

Caygill, C.P., Reed, P.I., Hill, M.J., & Watson, A. 1999. An initial comparison of nine centres registering patients with the UK National Barrett's Oesophagus Registry (UKBOR). *Eur.J.Cancer Prev.*, 8, (6) 539-542 available from: PM:10643944

Chapurlat, R.D., Garnero, P., Sornay-Rendu, E., Arlot, M.E., Claustrat, B., & Delmas, P.D. 2000. Longitudinal study of bone loss in pre- and perimenopausal women: evidence for bone loss in perimenopausal women. *Osteoporos.Int.*, 11, (6) 493-498 available from: PM:10982164

Chetkowski, R.J., Meldrum, D.R., Steingold, K.A., Randle, D., Lu, J.K., Eggena, P., Hershman, J.M., Alkjaersig, N.K., Fletcher, A.P., & Judd, H.L. 1986. Biologic effects of transdermal estradiol. *N.Engl.J Med.*, 314, (25) 1615-1620 available from: PM:3012339

Chlebowski, R.T., Wactawski-Wende, J., Ritenbaugh, C., Hubbell, F.A., Ascensao, J., Rodabough, R.J., Rosenberg, C.A., Taylor, V.M., Harris, R., Chen, C., Adams-Campbell, L.L., & White, E. 2004. Estrogen plus progestin and colorectal cancer in postmenopausal women. *N.Engl.J Med.*, 350, (10) 991-1004 available from: PM:14999111

Cho, N.L., Javid, S.H., Carothers, A.M., Redston, M., & Bertagnolli, M.M. 2007. Estrogen receptors alpha and beta are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. *Cancer Res.*, 67, (5) 2366-2372 available from: PM:17332369

Chun, S.Y., Eisenhauer, K.M., Kubo, M., & Hsueh, A.J. 1995. Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology*, 136, (7) 3120-3127 available from: PM:7540548

Clarke, C.L. & Sutherland, R.L. 1990. Progestin regulation of cellular proliferation. *Endocr.Rev.*, 11, (2) 266-301 available from: PM:2114281

Cocchiara, R., Albeggiani, G., Di, T.G., Azzolina, A., Lampiasi, N., Rizzo, F., & Geraci, D. 1990. Modulation of rat peritoneal mast cell and human basophil histamine release by estrogens. *Int.Arch.Allergy Appl.Immunol.*, 93, (2-3) 192-197 available from: PM:1712002

Cohen, S. & Lipshutz, W. 1971. Hormonal regulation of human lower esophageal sphincter competence: interaction of gastrin and secretin. *J Clin.Invest*, 50, (2) 449-454 available from: PM:5540178

Colditz, G.A., Willett, W.C., Stampfer, M.J., Rosner, B., Speizer, F.E., & Hennekens, C.H. 1987. Menopause and the risk of coronary heart disease in women. *N.Engl.J Med.*, 316, (18) 1105-1110 available from: PM:3574358

Coleman, K.M. & Smith, C.L. 2001. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci.*, 6, D1379-D1391 available from: PM:11578956

Collen, M.J., Abdulian, J.D., & Chen, Y.K. 1995. Gastroesophageal reflux disease in the elderly: more severe disease that requires aggressive therapy. *Am.J Gastroenterol*, 90, (7) 1053-1057 available from: PM:7611195

Cook, M.B., Greenwood, D.C., Hardie, L.J., Wild, C.P., & Forman, D. 2008. A systematic review and meta-analysis of the risk of increasing adiposity on Barrett's esophagus. *Am.J.Gastroenterol.*, 103, (2) 292-300 available from: PM:17986313

Cook, M.B., Kamangar, F., Whiteman, D.C., Freedman, N.D., Gammon, M.D., Bernstein, L., Brown, L.M., Risch, H.A., Ye, W., Sharp, L., Pandeya, N., Webb, P.M., Wu, A.H., Ward, M.H., Giffen, C., Casson, A.G., Abnet, C.C., Murray, L.J., Corley, D.A., Nyren, O., Vaughan, T.L., & Chow, W.H. 2010. Cigarette smoking and adenocarcinomas of the esophagus and esophagogastric junction: a pooled analysis from the international BEACON consortium. *J Natl.Cancer Inst.*, 102, (17) 1344-1353 available from: PM:20716718

Cooper, B.T. & Barbezat, G.O. 1987. Barrett's oesophagus: a clinical study of 52 patients. *Q.J.Med.*, 62, (238) 97-108 available from: PM:3498962

Cooper, S.C., Day, R., Brooks, C., Livings, C., Thomson, C.S., & Trudgill, N.J. 2009. The influence of deprivation and ethnicity on the incidence of esophageal cancer in England. *Cancer Causes Control*, 20, (8) 1459-1467 available from: PM:19533393

Corley, D.A. & Buffler, P.A. 2001. Oesophageal and gastric cardia adenocarcinomas: analysis of regional variation using the Cancer Incidence in Five Continents database. *Int.J.Epidemiol.*, 30, (6) 1415-1425 available from: PM:11821356

Corley, D.A., Kubo, A., Levin, T.R., Block, G., Habel, L., Zhao, W., Leighton, P., Quesenberry, C., Rumore, G.J., & Buffler, P.A. 2007. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. *Gastroenterology*, 133, (1) 34-41 available from: PM:17631128

Corley, D.A., Levin, T.R., Habel, L.A., & Buffler, P.A. 2006. Barrett's esophagus and medications that relax the lower esophageal sphincter. *Am.J Gastroenterol*, 101, (5) 937-944 available from: PM:16573773

Couse, J.F. & Korach, K.S. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr.Rev.*, 20, (3) 358-417 available from: PM:10368776

Crowe, S.E. 2005. Helicobacter infection, chronic inflammation, and the development of malignancy. *Curr.Opin.Gastroenterol*, 21, (1) 32-38 available from: PM:15687882

Crowell, M.D., Zayat, E.N., Lacy, B.E., Schettler-Duncan, A., & Liu, M.C. 2001. The effects of an inhaled beta(2)-adrenergic agonist on lower esophageal function: a dose-response study. *Chest*, 120, (4) 1184-1189 available from: PM:11591558

Csendes, A., Smok, G., Cerda, G., Burdiles, P., Mazza, D., & Csendes, P. 1997. Prevalence of Helicobacter pylori infection in 190 control subjects and in 236 patients with gastroesophageal reflux, erosive esophagitis or Barrett's esophagus. *Dis.Esophagus.*, 10, (1) 38-42 available from: PM:9079272

Currie, D. & Wiesenberg, S. 2003. Promoting women's health-seeking behavior: research and the empowerment of women. *Health Care Women Int.*, 24, (10) 880-899 available from: PM:14742127

Curtis, S.W., Washburn, T., Sewall, C., DiAugustine, R., Lindzey, J., Couse, J.F., & Korach, K.S. 1996. Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc.Natl.Acad.Sci.U.S.A*, 93, (22) 12626-12630 available from: PM:8901633

Cushman, M., Kuller, L.H., Prentice, R., Rodabough, R.J., Psaty, B.M., Stafford, R.S., Sidney, S., & Rosendaal, F.R. 2004. Estrogen plus progestin and risk of venous thrombosis. *JAMA*, 292, (13) 1573-1580 available from: PM:15467059

De Man, J.G., Pelckmans, P.A., Boeckxstaens, G.E., Bult, H., Oosterbosch, L., Herman, A.G., & Van Maercke, Y.M. 1991. The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter. *Br.J Pharmacol.*, 103, (1) 1092-1096 available from: PM:1652336

de Vries, D.R., van Herwaarden, M.A., Smout, A.J., & Samsom, M. 2008. Gastroesophageal pressure gradients in gastroesophageal reflux disease: relations with hiatal hernia, body mass index, and esophageal acid exposure. *Am.J Gastroenterol*, 103, (6) 1349-1354 available from: PM:18510603

De, L.L., Di, G.P., Signoriello, G., Sorrentino, E., Rivellini, G., D' Amore, E., De, L.B., & Murray, J.A. 2004. Relationship between hiatal hernia and inguinal hernia. *Dig.Dis.Sci.*, 49, (2) 243-247 available from: PM:15104364

Decktor, D.L. & Ryan, J.P. 1982. Transmembrane voltage of opossum esophageal smooth muscle and its response to electrical stimulation of intrinsic nerves. *Gastroenterology*, 82, (2) 301-308 available from: PM:7054030

Delyani, J.A., Murohara, T., Nossuli, T.O., & Lefer, A.M. 1996. Protection from myocardial reperfusion injury by acute administration of 17 beta-estradiol. *J Mol.Cell Cardiol.*, 28, (5) 1001-1008 available from: PM:8762038

Demeester, T.R., Johnson, L.F., Joseph, G.J., Toscano, M.S., Hall, A.W., & Skinner, D.B. 1976. Patterns of gastroesophageal reflux in health and disease. *Ann.Surg.*, 184, (4) 459-470 available from: PM:13747

Dennerstein, L., Dudley, E.C., Hopper, J.L., Guthrie, J.R., & Burger, H.G. 2000. A prospective population-based study of menopausal symptoms. *Obstet.Gynecol.*, 96, (3) 351-358 available from: PM:10960625

Dent, J. 1998. Gastro-oesophageal reflux disease. *Digestion*, 59, (5) 433-445 available from: PM:9705529

Dent, J., El-Serag, H.B., Wallander, M.A., & Johansson, S. 2005. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut*, 54, (5) 710-717 available from: PM:15831922

Dent, J., Holloway, R.H., Toouli, J., & Dodds, W.J. 1988. Mechanisms of lower oesophageal sphincter incompetence in patients with symptomatic gastrooesophageal reflux. *Gut*, 29, (8) 1020-1028 available from: PM:3410327

Derakhshan, M.H., Liptrot, S., Paul, J., Brown, I.L., Morrison, D., & McColl, K.E. 2009. Oesophageal and gastric intestinal-type adenocarcinomas show the same male predominance due to a 17 year delayed development in females. *Gut*, 58, (1) 16-23 available from: PM:18838486

Diaz-Rubio, M., Moreno-Elola-Olaso, C., Rey, E., Locke, G.R., III, & Rodriguez-Artalejo, F. 2004. Symptoms of gastro-oesophageal reflux: prevalence, severity, duration and associated factors in a Spanish population. *Aliment.Pharmacol.Ther.*, 19, (1) 95-105 available from: PM:14687171

DiMarino, A.J. & Cohen, S. 1973. The adrenergic control of lower esophageal sphincter function. An experimental model of denervation supersensitivity. *J Clin.Invest*, 52, (9) 2264-2271 available from: PM:4727458

Dodds, W.J., Dent, J., Hogan, W.J., & Arndorfer, R.C. 1981. Effect of atropine on esophageal motor function in humans. *Am.J Physiol*, 240, (4) G290-G296 available from: PM:6784581

Dodds, W.J., Stewart, E.T., Hodges, D., & Zboralske, F.F. 1973. Movement of the feline esophagus associated with respiration and peristalsis. An evaluation using tantalum markers. *J Clin.Invest*, 52, (1) 1-13 available from: PM:4682383

Durcova-Hills, G., Burgoyne, P., & McLaren, A. 2004. Analysis of sex differences in EGC imprinting. *Dev.Biol.*, 268, (1) 105-110 available from: PM:15031108

Eastwood, G.L. 1986. Histologic changes in gastroesophageal reflux. *J Clin.Gastroenterol*, 8 Suppl 1, 45-51 available from: PM:3734375

Eastwood, G.L., Castell, D.O., & Higgs, R.H. 1975. Experimental esophagitis in cats impairs lower esophageal sphincter pressure. *Gastroenterology*, 69, (1) 146-153 available from: PM:1150019

Eckardt, V.F. 1988. Does healing of esophagitis improve esophageal motor function? *Dig.Dis.Sci.*, 33, (2) 161-165 available from: PM:3338364

Eda, A., Osawa, H., Satoh, K., Yanaka, I., Kihira, K., Ishino, Y., Mutoh, H., & Sugano, K. 2003. Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J Gastroenterol*, 38, (1) 14-22 available from: PM:12560917

Edelstein, Z.R., Farrow, D.C., Bronner, M.P., Rosen, S.N., & Vaughan, T.L. 2007. Central adiposity and risk of Barrett's esophagus. *Gastroenterology*, 133, (2) 403-411 available from: PM:17681161

Edman, C.D. & MacDonald, P.C. 1978. Effect of obesity on conversion of plasma androstenedione to estrone in ovulatory and anovulator young women. *Am.J Obstet.Gynecol.*, 130, (4) 456-461 available from: PM:629290

Edwards, D.P. 2000. The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. *J Mammary.Gland.Biol.Neoplasia.*, 5, (3) 307-324 available from: PM:14973393

El-Serag, H.B., Graham, D.Y., Satia, J.A., & Rabeneck, L. 2005. Obesity is an independent risk factor for GERD symptoms and erosive esophagitis. *Am.J Gastroenterol*, 100, (6) 1243-1250 available from: PM:15929752

El-Serag, H.B. & Sonnenberg, A. 1997. Associations between different forms of gastro-oesophageal reflux disease. *Gut*, 41, (5) 594-599 available from: PM:9414963

Eloubeidi, M.A., Mason, A.C., Desmond, R.A., & El-Serag, H.B. 2003. Temporal trends (1973-1997) in survival of patients with esophageal adenocarcinoma in the United States: a glimmer of hope? *Am.J Gastroenterol*, 98, (7) 1627-1633 available from: PM:12873590

Evans, M.J., Eckert, A., Lai, K., Adelman, S.J., & Harnish, D.C. 2001. Reciprocal antagonism between estrogen receptor and NF-kappaB activity in vivo. *Circ.Res.*, 89, (9) 823-830 available from: PM:11679413

Fernandez, E., La, V.C., Braga, C., Talamini, R., Negri, E., Parazzini, F., & Franceschi, S. 1998. Hormone replacement therapy and risk of colon and rectal cancer. *Cancer Epidemiol.Biomarkers Prev.*, 7, (4) 329-333 available from: PM:9568789

Ferry, G., Lonchampt, M., Pennel, L., de, N.G., Canet, E., & Tucker, G.C. 1997. Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. *FEBS Lett.*, 402, (2-3) 111-115 available from: PM:9037177

Finkelstein, J.S., Brockwell, S.E., Mehta, V., Greendale, G.A., Sowers, M.R., Ettinger, B., Lo, J.C., Johnston, J.M., Cauley, J.A., Danielson, M.E., & Neer, R.M. 2008. Bone mineral density changes during the menopause transition in a multiethnic cohort of women. *J Clin.Endocrinol.Metab*, 93, (3) 861-868 available from: PM:18160467

Fisher, B.L., Pennathur, A., Mutnick, J.L., & Little, A.G. 1999. Obesity correlates with gastroesophageal reflux. *Dig.Dis.Sci.*, 44, (11) 2290-2294 available from: PM:10573376

Fisher, R.S., DiMarino, A.J., & Cohen, S. 1975. Mechanism of cholecystokinin inhibition of lower esophageal sphincter pressure. *Am.J Physiol*, 228, (5) 1469-1473 available from: PM:165733

Fisher, R.S., Roberts, G.S., Grabowski, C.J., & Cohen, S. 1978. Altered lower esophageal sphincter function during early pregnancy. *Gastroenterology*, 74, (6) 1233-1237 available from: PM:648814

Fitzgerald, R.C. 2006. Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. *Gut*, 55, (12) 1810-1820 available from: PM:17124160

Fitzgerald, R.C., Omary, M.B., & Triadafilopoulos, G. 1996. Dynamic effects of acid on Barrett's esophagus. An ex vivo proliferation and differentiation model. *J Clin.Invest*, 98, (9) 2120-2128 available from: PM:8903332

Furukawa, N., Iwakiri, R., Koyama, T., Okamoto, K., Yoshida, T., Kashiwagi, Y., Ohyama, T., Noda, T., Sakata, H., & Fujimoto, K. 1999. Proportion of reflux esophagitis in 6010 Japanese adults: prospective evaluation by endoscopy. *J Gastroenterol*, 34, (4) 441-444 available from: PM:10452674

Gallucci, R.M., Sloan, D.K., O'Dell, S.J., & Reinke, L.A. 2004. Differential expression of liver interleukin-6 receptor-alpha in female versus male ethanol-consuming rats. *Alcohol Clin.Exp.Res.*, 28, (3) 365-373 available from: PM:15084893

Gammon, M.D., Schoenberg, J.B., Ahsan, H., Risch, H.A., Vaughan, T.L., Chow, W.H., Rotterdam, H., West, A.B., Dubrow, R., Stanford, J.L., Mayne, S.T., Farrow, D.C., Niwa, S., Blot, W.J., & Fraumeni, J.F., Jr. 1997. Tobacco, alcohol, and socioeconomic status and adenocarcinomas of the esophagus and gastric cardia. *J Natl.Cancer Inst.*, 89, (17) 1277-1284 available from: PM:9293918

Garcia Rodriguez, L.A. & Perez, G.S. 1998. Use of the UK General Practice Research Database for pharmacoepidemiology. *Br.J Clin.Pharmacol.*, 45, (5) 419-425 available from: PM:9643612

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R.,

Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., & Zhang, J. 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.*, 5, (10) R80 available from: PM:15461798

Gerson, L.B., Shetler, K., & Triadafilopoulos, G. 2002. Prevalence of Barrett's esophagus in asymptomatic individuals. *Gastroenterology*, 123, (2) 461-467 available from: PM:12145799

Giles, G.R., Mason, M.C., Humphries, C., & Clark, C.G. 1969. Action of gastrin on the lower oesophageal sphincter in man. *Gut*, 10, (9) 730-734 available from: PM:5386630

Gilliver, S.C. & Ashcroft, G.S. 2007. Sex steroids and cutaneous wound healing: the contrasting influences of estrogens and androgens. *Climacteric.*, 10, (4) 276-288 available from: PM:17653954

Gold, E.B., Bromberger, J., Crawford, S., Samuels, S., Greendale, G.A., Harlow, S.D., & Skurnick, J. 2001. Factors associated with age at natural menopause in a multiethnic sample of midlife women. *Am.J Epidemiol.*, 153, (9) 865-874 available from: PM:11323317

Goldblum, J.R., Vicari, J.J., Falk, G.W., Rice, T.W., Peek, R.M., Easley, K., & Richter, J.E. 1998. Inflammation and intestinal metaplasia of the gastric cardia: the role of gastroesophageal reflux and H. pylori infection. *Gastroenterology*, 114, (4) 633-639 available from: PM:9516382

Gonella, J., Niel, J.P., & Roman, C. 1979. Sympathetic control of lower oesophageal sphincter motility in the cat. *J Physiol*, 287, 177-190 available from: PM:430393

Goyal, R.K. & Rattan, S. 1976. Genesis of basal sphincter pressure: effect of tetrodotoxin on lower esophageal sphincter pressure in opossum in vivo. *Gastroenterology*, 71, (1) 62-67 available from: PM:1278651

Grady, D., Herrington, D., Bittner, V., Blumenthal, R., Davidson, M., Hlatky, M., Hsia, J., Hulley, S., Herd, A., Khan, S., Newby, L.K., Waters, D., Vittinghoff, E., & Wenger, N. 2002. Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II). *JAMA*, 288, (1) 49-57 available from: PM:12090862

Graham, J.D. & Clarke, C.L. 1997. Physiological action of progesterone in target tissues. *Endocr.Rev.*, 18, (4) 502-519 available from: PM:9267762

Gray, G.A., Sharif, I., Webb, D.J., & Seckl, J.R. 2001. Oestrogen and the cardiovascular system: the good, the bad and the puzzling. *Trends Pharmacol.Sci.*, 22, (3) 152-156 available from: PM:11239579

Gray, M.R., Donnelly, R.J., & Kingsnorth, A.N. 1993. The role of smoking and alcohol in metaplasia and cancer risk in Barrett's columnar lined oesophagus. *Gut*, 34, (6) 727-731 available from: PM:8314502

Grodstein, F., Newcomb, P.A., & Stampfer, M.J. 1999. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *Am.J Med.*, 106, (5) 574-582 available from: PM:10335731

Grossman, C. 1989. Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. *J Steroid Biochem.*, 34, (1-6) 241-251 available from: PM:2696846

Grumbach, M.M. & Auchus, R.J. 1999. Estrogen: consequences and implications of human mutations in synthesis and action. *J Clin.Endocrinol.Metab*, 84, (12) 4677-4694 available from: PM:10599737

Gulshan, S., McCruden, A.B., & Stimson, W.H. 1990. Oestrogen receptors in macrophages. *Scand J Immunol.*, 31, (6) 691-697 available from: PM:2356436

Haggitt, R.C. 1994. Barrett's esophagus, dysplasia, and adenocarcinoma. *Hum.Pathol.*, 25, (10) 982-993 available from: PM:7927321

Haggitt, R.C., Tryzelaar, J., Ellis, F.H., & Colcher, H. 1978. Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. *Am.J Clin.Pathol.*, 70, (1) 1-5 available from: PM:696666

Hampel, H., Abraham, N.S., & El-Serag, H.B. 2005. Meta-analysis: obesity and the risk for gastroesophageal reflux disease and its complications. *Ann.Intern.Med.*, 143, (3) 199-211 available from: PM:16061918

Haque, M., Wyeth, J.W., Stace, N.H., Talley, N.J., & Green, R. 2000. Prevalence, severity and associated features of gastro-oesophageal reflux and dyspepsia: a population-based study. *N.Z.Med.J*, 113, (1110) 178-181 available from: PM:10917077

Hardman, M.J. & Ashcroft, G.S. 2008. Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly. *Genome Biol.*, 9, (5) R80 available from: PM:18477406

Hardman, M.J., Waite, A., Zeef, L., Burow, M., Nakayama, T., & Ashcroft, G.S. 2005. Macrophage migration inhibitory factor: a central regulator of wound healing. *Am.J.Pathol.*, 167, (6) 1561-1574 available from: PM:16314470

Harrison, R., Perry, I., Haddadin, W., McDonald, S., Bryan, R., Abrams, K., Sampliner, R., Talley, N.J., Moayyedi, P., & Jankowski, J.A. 2007. Detection of intestinal metaplasia in Barrett's esophagus: an observational comparator study suggests the need for a minimum of eight biopsies. *Am.J Gastroenterol*, 102, (6) 1154-1161 available from: PM:17433019

Helm, J.F., Dodds, W.J., Pelc, L.R., Palmer, D.W., Hogan, W.J., & Teeter, B.C. 1984. Effect of esophageal emptying and saliva on clearance of acid from the esophagus. *N.Engl.J Med.*, 310, (5) 284-288 available from: PM:6690951

Hemsell, D.L., Grodin, J.M., Brenner, P.F., Siiteri, P.K., & MacDonald, P.C. 1974. Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. *J Clin.Endocrinol.Metab*, 38, (3) 476-479 available from: PM:4815174

Henderson, K.D., Bernstein, L., Henderson, B., Kolonel, L., & Pike, M.C. 2008. Predictors of the timing of natural menopause in the Multiethnic Cohort Study. *Am.J Epidemiol.*, 167, (11) 1287-1294 available from: PM:18359953

Hersh, A.L., Stefanick, M.L., & Stafford, R.S. 2004. National use of postmenopausal hormone therapy: annual trends and response to recent evidence. *JAMA*, 291, (1) 47-53 available from: PM:14709575

Hewitt, S.C., Harrell, J.C., & Korach, K.S. 2005. Lessons in estrogen biology from knockout and transgenic animals. *Annu.Rev.Physiol*, 67, 285-308 available from: PM:15709960

Hiemstra, P.S. 2002. Novel roles of protease inhibitors in infection and inflammation. *Biochem.Soc.Trans.*, 30, (2) 116-120 available from: PM:12023837

Hirota, W.K., Loughney, T.M., Lazas, D.J., Maydonovitch, C.L., Rholl, V., & Wong, R.K. 1999. Specialized intestinal metaplasia, dysplasia, and cancer of the esophagus and esophagogastric junction: prevalence and clinical data. *Gastroenterology*, 116, (2) 277-285 available from: PM:9922307

Hirsch, D.P., Holloway, R.H., Tytgat, G.N., & Boeckxstaens, G.E. 1998. Involvement of nitric oxide in human transient lower esophageal sphincter relaxations and esophageal primary peristalsis. *Gastroenterology*, 115, (6) 1374-1380 available from: PM:9834264

Hodgins, M.B., Spike, R.C., Mackie, R.M., & MacLean, A.B. 1998. An immunohistochemical study of androgen, oestrogen and progesterone receptors in the vulva and vagina. *Br.J Obstet.Gynaecol.*, 105, (2) 216-222 available from: PM:9501790

Howard, J.M., Reynolds, R.P., Frei, J.V., Flowers, M.A., McDonald, T.J., Tilbe, K., & Bondy, D.C. 1994. Macroscopic healing of esophagitis does not improve esophageal motility. *Dig.Dis.Sci.*, 39, (3) 648-654 available from: PM:8131704

Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., & Vittinghoff, E. 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA*, 280, (7) 605-613 available from: PM:9718051

Iafrati, M.D., Karas, R.H., Aronovitz, M., Kim, S., Sullivan, T.R., Jr., Lubahn, D.B., O'Donnell, T.F., Jr., Korach, K.S., & Mendelsohn, M.E. 1997. Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. *Nat.Med.*, 3, (5) 545-548 available from: PM:9142124

Ignar-Trowbridge, D.M., Pimentel, M., Teng, C.T., Korach, K.S., & McLachlan, J.A. 1995. Cross talk between peptide growth factor and estrogen receptor signaling systems. *Environ.Health Perspect.*, 103 Suppl 7, 35-38 available from: PM:8593872

Isolauri, J. & Laippala, P. 1995. Prevalence of symptoms suggestive of gastro-oesophageal reflux disease in an adult population. *Ann.Med.*, 27, (1) 67-70 available from: PM:7742002

Ito, I., Hayashi, T., Yamada, K., Kuzuya, M., Naito, M., & Iguchi, A. 1995. Physiological concentration of estradiol inhibits polymorphonuclear leukocyte chemotaxis via a receptor mediated system. *Life Sci.*, 56, (25) 2247-2253 available from: PM:7791512

Jacobson, B.C., Moy, B., Colditz, G.A., & Fuchs, C.S. 2008. Postmenopausal hormone use and symptoms of gastroesophageal reflux. *Arch.Intern.Med.*, 168, (16) 1798-1804 available from: PM:18779468

Jacobson, B.C., Somers, S.C., Fuchs, C.S., Kelly, C.P., & Camargo, C.A., Jr. 2006. Body-mass index and symptoms of gastroesophageal reflux in women. *N.Engl.J Med.*, 354, (22) 2340-2348 available from: PM:16738270

Jankowski, J.A., Harrison, R.F., Perry, I., Balkwill, F., & Tselepis, C. 2000. Barrett's metaplasia. *Lancet*, 356, (9247) 2079-2085 available from: PM:11145505

Jankowski, J.A., Provenzale, D., & Moayyedi, P. 2002. Esophageal adenocarcinoma arising from Barrett's metaplasia has regional variations in the west. *Gastroenterology*, 122, (2) 588-590 available from: PM:11845805

Jansson, A. 2009. 17Beta-hydroxysteroid dehydrogenase enzymes and breast cancer. *J Steroid Biochem.Mol.Biol.*, 114, (1-2) 64-67 available from: PM:19167496

Jick, H., Jick, S.S., & Derby, L.E. 1991. Validation of information recorded on general practitioner based computerised data resource in the United Kingdom. *BMJ*, 302, (6779) 766-768 available from: PM:2021768

Jick, H., Terris, B.Z., Derby, L.E., & Jick, S.S. 1992. Further validation of information recorded on a general practitioner based computerized data resource in the united kingdom. *Pharmacoepidemiology and Drug Safety*, 1, (6) 347-349

Johansson, E.D. 1969. Plasma levels of progesterone in pregnancy measured by a rapid competitive protein binding technique. *Acta Endocrinol.(Copenh)*, 61, (4) 607-617 available from: PM:5409083

Johnson, D.A. & Fennerty, M.B. 2004. Heartburn severity underestimates erosive esophagitis severity in elderly patients with gastroesophageal reflux disease. *Gastroenterology*, 126, (3) 660-664 available from: PM:14988819

Johnson, J.R., Lacey, J.V., Jr., Lazovich, D., Geller, M.A., Schairer, C., Schatzkin, A., & Flood, A. 2009. Menopausal hormone therapy and risk of colorectal cancer. *Cancer Epidemiol.Biomarkers Prev.*, 18, (1) 196-203 available from: PM:19124498

Kahrilas, P.J. 1999. The role of hiatus hernia in GERD. *Yale J.Biol.Med.*, 72, (2-3) 101-111 available from: PM:10780571

Karas, R.H., Hodgin, J.B., Kwoun, M., Krege, J.H., Aronovitz, M., Mackey, W., Gustafsson, J.A., Korach, K.S., Smithies, O., & Mendelsohn, M.E. 1999. Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice. *Proc.Natl.Acad.Sci.U.S.A*, 96, (26) 15133-15136 available from: PM:10611350

Katz, D., Rothstein, R., Schned, A., Dunn, J., Seaver, K., & Antonioli, D. 1998. The development of dysplasia and adenocarcinoma during endoscopic surveillance of Barrett's esophagus. *Am.J.Gastroenterol.*, 93, (4) 536-541 available from: PM:9576444

Katz, M.H., Alvarez, A.F., Kirsner, R.S., Eaglstein, W.H., & Falanga, V. 1991. Human wound fluid from acute wounds stimulates fibroblast and endothelial cell growth. *J Am.Acad.Dermatol.*, 25, (6 Pt 1) 1054-1058 available from: PM:1810982

Kaufman, D.W., Slone, D., Rosenberg, L., Miettinen, O.S., & Shapiro, S. 1980. Cigarette smoking and age at natural menopause. *Am.J Public Health*, 70, (4) 420-422 available from: PM:7361965

Kennedy, T. & Jones, R. 2000. The prevalence of gastro-oesophageal reflux symptoms in a UK population and the consultation behaviour of patients with these symptoms. *Aliment.Pharmacol.Ther.*, 14, (12) 1589-1594 available from: PM:11121906

Key, T.J., Allen, N.E., Verkasalo, P.K., & Banks, E. 2001. Energy balance and cancer: the role of sex hormones. *Proc.Nutr.Soc.*, 60, (1) 81-89 available from: PM:11310427

Klauser, A.G., Schindlbeck, N.E., & Muller-Lissner, S.A. 1990. Symptoms in gastro-oesophageal reflux disease. *Lancet*, 335, (8683) 205-208 available from: PM:1967675

Klopper, A. & Billewicz, W. 1963. Urinary Excretion Of Oestriol And Pregnanediol During Normal Pregnancy. *J Obstet.Gynaecol.Br.Commonw.*, 70, 1024-1033 available from: PM:14100063

Konturek SJ, Pytko-Polonczyk J, & Brzozowski T. Healing of oral and gastric ulcers: effects of blood flow, epidermal growth factor and sensory innervation. Eur.J Gastroenterol Hepatol. 5, S45-S52. 1993.

Konturek, J.W., Thor, P., Lukaszyk, A., Gabryelewicz, A., Konturek, S.J., & Domschke, W. 1997. Endogenous nitric oxide in the control of esophageal motility in humans. *J Physiol Pharmacol.*, 48, (2) 201-209 available from: PM:9223025

Koufman, J.A. 1991. The otolaryngologic manifestations of gastroesophageal reflux disease (GERD): a clinical investigation of 225 patients using ambulatory 24-hour pH monitoring and an experimental investigation of the role of acid and pepsin in the development of laryngeal injury. *Laryngoscope*, 101, (4 Pt 2 Suppl 53) 1-78 available from: PM:1895864

Kravitz, J.J., Snape, W.J., Jr., & Cohen, S. 1978. Effect of histamine and histamine antagonists on human lower esophageal sphincter function. *Gastroenterology*, 74, (2 Pt 2) 435-440 available from: PM:620912

Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.A., & Smithies, O. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc.Natl.Acad.Sci.U.S.A*, 95, (26) 15677-15682 available from: PM:9861029

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J.A. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc.Natl.Acad.Sci.U.S.A*, 93, (12) 5925-5930 available from: PM:8650195

Labenz, J., Blum, A.L., Bayerdorffer, E., Meining, A., Stolte, M., & Borsch, G. 1997. Curing Helicobacter pylori infection in patients with duodenal ulcer may provoke reflux esophagitis. *Gastroenterology*, 112, (5) 1442-1447 available from: PM:9136820

Lagergren, J., Bergstrom, R., Lindgren, A., & Nyren, O. 1999. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N.Engl.J.Med.*, 340, (11) 825-831 available from: PM:10080844

Lara, R.N., da Guerra, E.N., & de Melo, N.S. 2007. Macroscopic and microscopic effects of GaAlAs diode laser and dexamethasone therapies on oral mucositis induced by fluorouracil in rats. *Oral Health Prev.Dent.*, 5, (1) 63-71 available from: PM:17366763

Leavitt, W.W. & Blaha, G.C. 1972. An estrogen-stimulated, progesterone-binding system in the hamster uterus and vagina. *Steroids*, 19, (2) 263-274 available from: PM:4552110

Lee, J., Anggiansah, A., Anggiansah, R., Young, A., Wong, T., & Fox, M. 2007. Effects of age on the gastroesophageal junction, esophageal motility, and reflux disease. *Clin.Gastroenterol Hepatol.*, 5, (12) 1392-1398 available from: PM:17936081

Lee, S.W., Jung, K.I., Kim, Y.W., Jung, H.D., Kim, H.S., & Hong, J.P. 2007. Effect of epidermal growth factor against radiotherapy-induced oral mucositis in rats. *Int.J Radiat.Oncol.Biol.Phys.*, 67, (4) 1172-1178 available from: PM:17336218

Lee, Y.C., Cook, M.B., Bhatia, S., Chow, W.H., El-Omar, E.M., Goto, H., Lin, J.T., Li, Y.Q., Rhee, P.L., Sharma, P., Sung, J.J., Wong, J.Y., Wu, J.C., & Ho, K.Y. 2010. Interobserver reliability in the endoscopic diagnosis and grading of Barrett's esophagus: an Asian multinational study. *Endoscopy*, 42, (9) 699-704 available from: PM:20806154

Liebermann-Meffert, D., Allgower, M., Schmid, P., & Blum, A.L. 1979. Muscular equivalent of the lower esophageal sphincter. *Gastroenterology*, 76, (1) 31-38 available from: PM:81791

Lindblad, M., Garcia Rodriguez, L.A., Chandanos, E., & Lagergren, J. 2006. Hormone replacement therapy and risks of oesophageal and gastric adenocarcinomas. *Br.J.Cancer*, 94, (1) 136-141 available from: PM:16404367

Locke, G.R., III, Talley, N.J., Fett, S.L., Zinsmeister, A.R., & Melton, L.J., III 1997. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology*, 112, (5) 1448-1456 available from: PM:9136821

Loffeld, R.J., Ten Tije, B.J., & Arends, J.W. 1992. Prevalence and significance of Helicobacter pylori in patients with Barrett's esophagus. *Am.J.Gastroenterol.*, 87, (11) 1598-1600 available from: PM:1442681

Loughney, T., Maydonovitch, C.L., & Wong, R.K. 1998. Esophageal manometry and ambulatory 24-hour pH monitoring in patients with short and long segment Barrett's esophagus. *Am.J.Gastroenterol.*, 93, (6) 916-919 available from: PM:9647018

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., & Smithies, O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc.Natl.Acad.Sci.U.S.A*, 90, (23) 11162-11166 available from: PM:8248223

Lundell, L.R., Dent, J., Bennett, J.R., Blum, A.L., Armstrong, D., Galmiche, J.P., Johnson, F., Hongo, M., Richter, J.E., Spechler, S.J., Tytgat, G.N., & Wallin, L. 1999. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut*, 45, (2) 172-180 available from: PM:10403727

Macdonald, C.E., Wicks, A.C., & Playford, R.J. 1997. Ten years' experience of screening patients with Barrett's oesophagus in a university teaching hospital. *Gut*, 41, (3) 303-307 available from: PM:9378382

Machowska, A., Brzozowski, T., Sliwowski, Z., Pawlik, M., Konturek, P.C., Pajdo, R., Szlachcic, A., Drozdowicz, D., Schwarz, M., Stachura, J., Konturek, S.J., & Pawlik, W.W. 2008. Gastric secretion, proinflammatory cytokines and epidermal growth factor (EGF) in the delayed healing of lingual and gastric ulcerations by testosterone. *Inflammopharmacology.*, 16, (1) 40-47 available from: PM:18046513

Machowska, A., Szlachcic, A., Pawlik, M., Brzozowski, T., Konturek, S.J., & Pawlik, W.W. 2004. The role of female and male sex hormones in the healing process of preexisting lingual and gastric ulcerations. *J Physiol Pharmacol.*, 55 Suppl 2, 91-104 available from: PM:15608364

Mann, N.S., Tsai, M.F., & Nair, P.K. 1989. Barrett's esophagus in patients with symptomatic reflux esophagitis. *Am.J Gastroenterol*, 84, (12) 1494-1496 available from: PM:2596449

Mao, A., Paharkova-Vatchkova, V., Hardy, J., Miller, M.M., & Kovats, S. 2005. Estrogen selectively promotes the differentiation of dendritic cells with characteristics of Langerhans cells. *J Immunol.*, 175, (8) 5146-5151 available from: PM:16210618

Marrero, J.M., Goggin, P.M., de Caestecker, J.S., Pearce, J.M., & Maxwell, J.D. 1992. Determinants of pregnancy heartburn. *Br.J.Obstet.Gynaecol.*, 99, (9) 731-734 available from: PM:1420011

McEwan, I.J. 2004. Sex, drugs and gene expression: signalling by members of the nuclear receptor superfamily. *Essays Biochem.*, 40, 1-10 available from: PM:15242335

Meissner, A.J., Bowes, K.L., Zwick, R., & Daniel, E.E. 1976. Effect of motilin on the lower oesophageal sphincter. *Gut*, 17, (12) 925-932 available from: PM:1017712

Menard, D. & Arsenault, P. 1987. Maturation of human fetal esophagus maintained in organ culture. *Anat.Rec.*, 217, (4) 348-354 available from: PM:3592260

Menon, S., Jayasena, H., Nightingale, P., & Trudgill, N.J. 2011. Influence of age and sex on endoscopic findings of gastrooesophageal reflux disease: an endoscopy database study. *Eur.J Gastroenterol Hepatol.*, 23, (5) 389-395 available from: PM:21448069

Menon, S. & Trudgill, N. 2011. Risk factors in the aetiology of hiatus hernia: a meta-analysis. *Eur.J Gastroenterol Hepatol.*, 23, (2) 133-138 available from: PM:21178776

Miros, M., Kerlin, P., & Walker, N. 1991. Only patients with dysplasia progress to adenocarcinoma in Barrett's oesophagus. *Gut*, 32, (12) 1441-1446 available from: PM:1773946

Mittal, R.K. & McCallum, R.W. 1988. Characteristics and frequency of transient relaxations of the lower esophageal sphincter in patients with reflux esophagitis. *Gastroenterology*, 95, (3) 593-599 available from: PM:3396810

Miwa, K., Sahara, H., Segawa, M., Kinami, S., Sato, T., Miyazaki, I., & Hattori, T. 1996. Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. *Int.J Cancer*, 67, (2) 269-274 available from: PM:8760598

Mohammed, I., Nightingale, P., & Trudgill, N.J. 2005. Risk factors for gastro-oesophageal reflux disease symptoms: a community study. *Aliment.Pharmacol.Ther.*, 21, (7) 821-827 available from: PM:15801917

Monnier, P. & Savary, M. Contribution of endoscopy to gastroesophageal reflux disease. Scand J Gastroenterol 19, 26. 1984.

Morales, D.E., McGowan, K.A., Grant, D.S., Maheshwari, S., Bhartiya, D., Cid, M.C., Kleinman, H.K., & Schnaper, H.W. 1995. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation*, 91, (3) 755-763 available from: PM:7530174

Naef, A.P., Savary, M., & Ozzello, L. 1975. Columnar-lined lower esophagus: an acquired lesion with malignant predisposition. Report on 140 cases of Barrett's esophagus with 12 adenocarcinomas. *J Thorac.Cardiovasc.Surg.*, 70, (5) 826-835 available from: PM:1186274

Nagler, R. & Spiro, H.M. 1961. Heartburn in late pregnancy. Manometric studies of esophageal motor function. *J Clin.Invest*, 40, 954-970 available from: PM:13727455

Nandurkar, S., Talley, N.J., Martin, C.J., Ng, T.H., & Adams, S. 1997. Short segment Barrett's oesophagus: prevalence, diagnosis and associations. *Gut*, 40, (6) 710-715 available from: PM:9245922

Nelson, K.G., Takahashi, T., Bossert, N.L., Walmer, D.K., & McLachlan, J.A. 1991. Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. *Proc.Natl.Acad.Sci.U.S.A*, 88, (1) 21-25 available from: PM:1986369

Newton, M., Bryan, R., Burnham, W.R., & Kamm, M.A. 1997. Evaluation of Helicobacter pylori in reflux oesophagitis and Barrett's oesophagus. *Gut*, 40, (1) 9-13 available from: PM:9155568

Nilsson, M., Johnsen, R., Ye, W., Hveem, K., & Lagergren, J. 2003. Obesity and estrogen as risk factors for gastroesophageal reflux symptoms. *JAMA*, 290, (1) 66-72 available from: PM:12837713

Nilsson, M., Lundegardh, G., Carling, L., Ye, W., & Lagergren, J. 2002. Body mass and reflux oesophagitis: an oestrogen-dependent association? *Scand.J.Gastroenterol.*, 37, (6) 626-630 available from: PM:12126237

Nordenstedt, H., Zheng, Z., Cameron, A.J., Ye, W., Pedersen, N.L., & Lagergren, J. 2008. Postmenopausal hormone therapy as a risk factor for gastroesophageal reflux symptoms among female twins. *Gastroenterology*, 134, (4) 921-928 available from: PM:18294635

O'Connor, J.B., Falk, G.W., & Richter, J.E. 1999. The incidence of adenocarcinoma and dysplasia in Barrett's esophagus: report on the Cleveland Clinic Barrett's Esophagus Registry. *Am.J.Gastroenterol.*, 94, (8) 2037-2042 available from: PM:10445525

Oberg, S., Lord, R.V., Peters, J.H., Chandrasoma, P., Theisen, J., Hagen, J.A., Demeester, S.R., Bremner, C.G., & Demeester, T.R. 2000. Is adenocarcinoma following esophagoduodenostomy without carcinogen in the rat reflux-induced? *J Surg.Res.*, 91, (2) 111-117 available from: PM:10839958

Ogawa, S., Eng, V., Taylor, J., Lubahn, D.B., Korach, K.S., & Pfaff, D.W. 1998. Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice. *Endocrinology*, 139, (12) 5070-5081 available from: PM:9832446

Okabe, S., Roth, J.L., & Pfeiffer, C.J. 1971. A method for experimental, penetrating gastric and duodenal ulcers in rats. Observations on normal healing. *Am.J Dig.Dis.*, 16, (3) 277-284 available from: PM:5554507

Oliveria, S.A., Christos, P.J., Talley, N.J., & Dannenberg, A.J. 1999. Heartburn risk factors, knowledge, and prevention strategies: a population-based survey of individuals with heartburn. *Arch.Intern.Med.*, 159, (14) 1592-1598 available from: PM:10421282

Olsen, N.J. & Kovacs, W.J. 1996. Gonadal steroids and immunity. *Endocr.Rev.*, 17, (4) 369-384 available from: PM:8854050

Ovaska, J., Miettinen, M., & Kivilaakso, E. 1989. Adenocarcinoma arising in Barrett's esophagus. *Dig.Dis.Sci.*, 34, (9) 1336-1339 available from: PM:2766899

Pandeya, N., Williams, G., Green, A.C., Webb, P.M., & Whiteman, D.C. 2009. Alcohol consumption and the risks of adenocarcinoma and squamous cell carcinoma of the esophagus. *Gastroenterology*, 136, (4) 1215-2 available from: PM:19250648

Pandeya, N., Williams, G.M., Sadhegi, S., Green, A.C., Webb, P.M., & Whiteman, D.C. 2008. Associations of duration, intensity, and quantity of smoking with adenocarcinoma and squamous cell carcinoma of the esophagus. *Am.J Epidemiol.*, 168, (1) 105-114 available from: PM:18483122

Pandolfino, J.E., El-Serag, H.B., Zhang, Q., Shah, N., Ghosh, S.K., & Kahrilas, P.J. 2006. Obesity: a challenge to esophagogastric junction integrity. *Gastroenterology*, 130, (3) 639-649 available from: PM:16530504

Pandolfino, J.E., Kwiatek, M.A., & Kahrilas, P.J. 2008. The pathophysiologic basis for epidemiologic trends in gastroesophageal reflux disease. *Gastroenterol Clin.North Am.*, 37, (4) 827-43, viii available from: PM:19028320

Pare, G., Krust, A., Karas, R.H., Dupont, S., Aronovitz, M., Chambon, P., & Mendelsohn, M.E. 2002. Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury. *Circ.Res.*, 90, (10) 1087-1092 available from: PM:12039798

Paterson, W.G. & Kolyn, D.M. 1994. Esophageal shortening induced by short-term intraluminal acid perfusion in opossum: a cause for hiatus hernia? *Gastroenterology*, 107, (6) 1736-1740 available from: PM:7958685

Paull, A., Trier, J.S., Dalton, M.D., Camp, R.C., Loeb, P., & Goyal, R.K. 1976. The histologic spectrum of Barrett's esophagus. *N.Engl.J Med.*, 295, (9) 476-480 available from: PM:940579

Penagini, R., Schoeman, M.N., Dent, J., Tippett, M.D., & Holloway, R.H. 1996. Motor events underlying gastro-oesophageal reflux in ambulant patients with reflux oesophagitis. *Neurogastroenterol.Motil.*, 8, (2) 131-141 available from: PM:8784797

Pera, M., Brito, M.J., Poulsom, R., Riera, E., Grande, L., Hanby, A., & Wright, N.A. 2000. Duodenal-content reflux esophagitis induces the development of glandular metaplasia and adenosquamous carcinoma in rats. *Carcinogenesis*, 21, (8) 1587-1591 available from: PM:10910963

Pietras, R.J. & Marquez-Garban, D.C. 2007. Membrane-associated estrogen receptor signaling pathways in human cancers. *Clin.Cancer Res.*, 13, (16) 4672-4676 available from: PM:17699844

Pope, C.E. 1967. A dynamic test of sphincter strength: its application to the lower esophageal sphincter. *Gastroenterology*, 52, (5) 779-786 available from: PM:6067283

Prach, A.T., MacDonald, T.A., Hopwood, D.A., & Johnston, D.A. 1997. Increasing incidence of Barrett's oesophagus: education, enthusiasm, or epidemiology? *Lancet*, 350, (9082) 933 available from: PM:9314878

Raiha, I., Hietanen, E., & Sourander, L. 1991. Symptoms of gastro-oesophageal reflux disease in elderly people. *Age Ageing*, 20, (5) 365-370 available from: PM:1755393

Rattan, S. & Goyal, R.K. 1976. Effect of dopamine on the esophageal smooth muscle in vivo. *Gastroenterology*, 70, (3) 377-381 available from: PM:942933

Reid, B.J., Blount, P.L., Rubin, C.E., Levine, D.S., Haggitt, R.C., & Rabinovitch, P.S. 1992. Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective endoscopic surveillance of a cohort. *Gastroenterology*, 102, (4 Pt 1) 1212-1219 available from: PM:1551528

Reid, B.J., Haggitt, R.C., Rubin, C.E., & Rabinovitch, P.S. 1987. Barrett's esophagus. Correlation between flow cytometry and histology in detection of patients at risk for adenocarcinoma. *Gastroenterology*, 93, (1) 1-11 available from: PM:3582897

Ren, J., Shaker, R., Kusano, M., Podvrsan, B., Metwally, N., Dua, K.S., & Sui, Z. 1995. Effect of aging on the secondary esophageal peristalsis: presbyesophagus revisited. *Am.J Physiol*, 268, (5 Pt 1) G772-G779 available from: PM:7762661

Rennert, G., Rennert, H.S., Pinchev, M., Lavie, O., & Gruber, S.B. 2009. Use of hormone replacement therapy and the risk of colorectal cancer. *J Clin.Oncol.*, 27, (27) 4542-4547 available from: PM:19704062

Reuss, E., Fimmers, R., Kruger, A., Becker, C., Rittner, C., & Hohler, T. 2002. Differential regulation of interleukin-10 production by genetic and environmental factors--a twin study. *Genes Immun.*, 3, (7) 407-413 available from: PM:12424622

Rex, D.K., Cummings, O.W., Shaw, M., Cumings, M.D., Wong, R.K., Vasudeva, R.S., Dunne, D., Rahmani, E.Y., & Helper, D.J. 2003. Screening for Barrett's esophagus in colonoscopy patients with and without heartburn. *Gastroenterology*, 125, (6) 1670-1677 available from: PM:14724819

Rexrode, K.M., Manson, J.E., Lee, I.M., Ridker, P.M., Sluss, P.M., Cook, N.R., & Buring, J.E. 2003. Sex hormone levels and risk of cardiovascular events in postmenopausal women. *Circulation*, 108, (14) 1688-1693 available from: PM:12975257

Reynolds, J.C., Ouyang, A., & Cohen, S. 1984. A lower esophageal sphincter reflex involving substance P. *Am.J Physiol*, 246, (4 Pt 1) G346-G354 available from: PM:6202152

Rezvani, M. & Ross, G.A. 2004. Modification of radiation-induced acute oral mucositis in the rat. *Int.J Radiat.Biol.*, 80, (2) 177-182 available from: PM:15164799

Richter, J.E. 1991. Gastroesophageal reflux disease as a cause of chest pain. *Med.Clin.North Am.*, 75, (5) 1065-1080 available from: PM:1895806

Richter, J.E. & Castell, D.O. 1982. Gastroesophageal reflux. Pathogenesis, diagnosis, and therapy. *Ann.Intern.Med.*, 97, (1) 93-103 available from: PM:6124198

Risma, K.A., Clay, C.M., Nett, T.M., Wagner, T., Yun, J., & Nilson, J.H. 1995. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc.Natl.Acad.Sci.U.S.A*, 92, (5) 1322-1326 available from: PM:7877975

Rokkas, T., Pistiolas, D., Sechopoulos, P., Robotis, I., & Margantinis, G. 2007. Relationship between Helicobacter pylori infection and esophageal neoplasia: a meta-analysis. *Clin.Gastroenterol Hepatol.*, 5, (12) 1413-7, 1417 available from: PM:17997357

Ronkainen, J., Aro, P., Storskrubb, T., Johansson, S.E., Lind, T., Bolling-Sternevald, E., Vieth, M., Stolte, M., Talley, N.J., & Agreus, L. 2005. Prevalence of Barrett's esophagus in the general population: an endoscopic study. *Gastroenterology*, 129, (6) 1825-1831 available from: PM:16344051

Rosner W. Sex steroid transport binding proteins. Reproductive Endocrine Surgery, Technology , 605-626. 1996. Philadelphia, Lippincott-Raven.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., & Ockene, J. 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA*, 288, (3) 321-333 available from: PM:12117397

Rossouw, J.E., Prentice, R.L., Manson, J.E., Wu, L., Barad, D., Barnabei, V.M., Ko, M., LaCroix, A.Z., Margolis, K.L., & Stefanick, M.L. 2007. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA*, 297, (13) 1465-1477 available from: PM:17405972

Rubanyi, G.M., Freay, A.D., Kauser, K., Sukovich, D., Burton, G., Lubahn, D.B., Couse, J.F., Curtis, S.W., & Korach, K.S. 1997. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *J Clin.Invest*, 99, (10) 2429-2437 available from: PM:9153286

Rubenstein, J.H. & Taylor, J.B. 2010. Meta-analysis: the association of oesophageal adenocarcinoma with symptoms of gastro-oesophageal reflux. *Aliment.Pharmacol.Ther.*, 32, (10) 1222-1227 available from: PM:20955441

Rudolph, R.E., Vaughan, T.L., Storer, B.E., Haggitt, R.C., Rabinovitch, P.S., Levine, D.S., & Reid, B.J. 2000. Effect of segment length on risk for neoplastic progression in patients with Barrett esophagus. *Ann.Intern.Med.*, 132, (8) 612-620 available from: PM:10766679

Ruhl, C.E. & Everhart, J.E. 1999. Overweight, but not high dietary fat intake, increases risk of gastroesophageal reflux disease hospitalization: the NHANES I Epidemiologic Followup Study. First National Health and Nutrition Examination Survey. *Ann.Epidemiol.*, 9, (7) 424-435 available from: PM:10501410

Sampliner, R.E. 2002. Updated guidelines for the diagnosis, surveillance, and therapy of Barrett's esophagus. *Am.J.Gastroenterol.*, 97, (8) 1888-1895 available from: PM:12190150

Sato, T., Miwa, K., Sahara, H., Segawa, M., & Hattori, T. 2002. The sequential model of Barrett's esophagus and adenocarcinoma induced by duodeno-esophageal reflux without exogenous carcinogens. *Anticancer Res.*, 22, (1A) 39-44 available from: PM:12017320

Savouret, J.F., Misrahi, M., & Milgrom, E. 1990. Molecular action of progesterone. *Int.J Biochem.*, 22, (6) 579-594 available from: PM:2199252

Schindlbeck, N.E., Heinrich, C., Dendorfer, A., Pace, F., & Muller-Lissner, S.A. 1987. Influence of smoking and esophageal intubation on esophageal pH-metry. *Gastroenterology*, 92, (6) 1994-1997 available from: PM:3569773

Schlippert, W., Schulze, K., & Forker, E.L. 1979. Calcium in smooth muscle from the opossum esophagus. *Proc.Soc.Exp.Biol.Med.*, 162, (2) 354-358 available from: PM:515013

Schoeman, M.N. & Holloway, R.H. 1995. Integrity and characteristics of secondary oesophageal peristalsis in patients with gastro-oesophageal reflux disease. *Gut*, 36, (4) 499-504 available from: PM:7737553

Schulze, K., Hajjar, J.J., & Christensen, J. 1978. Regional differences in potassium content of smooth muscle from opossum esophagus. *Am.J Physiol*, 235, (6) E709-E713 available from: PM:736131

Shanker, G., Sorci-Thomas, M., & Adams, M.R. 1995. Estrogen modulates the inducible expression of platelet-derived growth factor mRNA by monocyte/macrophages. *Life Sci.*, 56, (7) 499-507 available from: PM:7869830

Shapiro, S.S., Dyer, S.D., & Colas, A.E. 1980. Progesterone-induced glycogen accumulation in human endometrium during organ culture. *Am.J Obstet.Gynecol.*, 136, (4) 419-425 available from: PM:7355921

Sharma, P., Dent, J., Armstrong, D., Bergman, J.J., Gossner, L., Hoshihara, Y., Jankowski, J.A., Junghard, O., Lundell, L., Tytgat, G.N., & Vieth, M. 2006. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. *Gastroenterology*, 131, (5) 1392-1399 available from: PM:17101315

Sharma, P., Morales, T.G., Bhattacharyya, A., Garewal, H.S., & Sampliner, R.E. 1997. Dysplasia in short-segment Barrett's esophagus: a prospective 3-year follow-up. *Am.J.Gastroenterol.*, 92, (11) 2012-2016 available from: PM:9362182

Shirvani, V.N., Ouatu-Lascar, R., Kaur, B.S., Omary, M.B., & Triadafilopoulos, G. 2000. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure. *Gastroenterology*, 118, (3) 487-496 available from: PM:10702199

Siiteri PK & MacDonald PC. Role of extra glandular oestrogen in human endocrinology. Handbook of physiology.Sect 7: Endocrinology, Vol II.Female reproductive system. II, 615-630. 1973.

Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M., & Davis, S. 2000. The role of local estrogen biosynthesis in males and females. *Trends Endocrinol.Metab*, 11, (5) 184-188 available from: PM:10856920

Sloan, S., Rademaker, A.W., & Kahrilas, P.J. 1992. Determinants of gastroesophageal junction incompetence: hiatal hernia, lower esophageal sphincter, or both? *Ann.Intern.Med.*, 117, (12) 977-982 available from: PM:1443984

Slomiany, B.L. & Slomiany, A. 2001. Delay in oral mucosal ulcer healing by aspirin is linked to the disturbances in p38 mitogen-activated protein kinase activation. *J Physiol Pharmacol.*, 52, (2) 185-194 available from: PM:11453099

Slomiany, B.L. & Slomiany, A. 2002. Activation of peroxisome proliferator-activated receptor gamma suppresses inducible cyclooxygenase and nitric oxide synthase during oral mucosal ulcer healing. *J Physiol Pharmacol.*, 53, (2) 159-169 available from: PM:12120893

Slomiany, B.L. & Slomiany, A. 2005. Role of endothelin-1-dependent up-regulation of leptin in oral mucosal repair. *J Physiol Pharmacol.*, 56, (4) 531-541 available from: PM:16391412

Smyth, G. Limma: linear models for microarray data. Gentleman, R, Carey V, Dudoit, S, Irizarry, R, and Huber, W. Bioinformatics and Computational Biology Solutions using R and Bioconductor. 397-420. 2005. New York, Springer.

Song, X., Zeng, L., Jin, W., Thompson, J., Mizel, D.E., Lei, K., Billinghurst, R.C., Poole, A.R., & Wahl, S.M. 1999. Secretory leukocyte protease inhibitor suppresses the inflammation and joint damage of bacterial cell wall-induced arthritis. *J Exp.Med.*, 190, (4) 535-542 available from: PM:10449524

Sonnenberg, A., Steinkamp, U., Weise, A., Berges, W., Wienbeck, M., Rohner, H.G., & Peter, P. 1982. Salivary secretion in reflux esophagitis. *Gastroenterology*, 83, (4) 889-895 available from: PM:7106518

Sontag, S.J., Schnell, T.G., Miller, T.Q., Khandelwal, S., O'Connell, S., Chejfec, G., Greenlee, H., Seidel, U.J., & Brand, L. 1992. Prevalence of oesophagitis in asthmatics. *Gut*, 33, (7) 872-876 available from: PM:1644324

Soules, M.R., Sherman, S., Parrott, E., Rebar, R., Santoro, N., Utian, W., & Woods, N. 2001. Executive summary: Stages of Reproductive Aging Workshop (STRAW). *Fertil.Steril.*, 76, (5) 874-878 available from: PM:11704104

Souza, R.F., Huo, X., Mittal, V., Schuler, C.M., Carmack, S.W., Zhang, H.Y., Zhang, X., Yu, C., Hormi-Carver, K., Genta, R.M., & Spechler, S.J. 2009. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology*, 137, (5) 1776-1784 available from: PM:19660463

Souza, R.F., Shewmake, K., Terada, L.S., & Spechler, S.J. 2002. Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. *Gastroenterology*, 122, (2) 299-307 available from: PM:11832445

Sowers, M., Crutchfield, M., Bandekar, R., Randolph, J.F., Shapiro, B., Schork, M.A., & Jannausch, M. 1998. Bone mineral density and its change in pre-and perimenopausal white women: the Michigan Bone Health Study. *J Bone Miner.Res.*, 13, (7) 1134-1140 available from: PM:9661077

Spechler, S.J. 1999. The role of gastric carditis in metaplasia and neoplasia at the gastroesophageal junction. *Gastroenterology*, 117, (1) 218-228 available from: PM:10381931

Spechler, S.J., Zeroogian, J.M., Antonioli, D.A., Wang, H.H., & Goyal, R.K. 1994. Prevalence of metaplasia at the gastro-oesophageal junction. *Lancet*, 344, (8936) 1533-1536 available from: PM:7983953

Stein, H.J., Eypasch, E.P., Demeester, T.R., Smyrk, T.C., & Attwood, S.E. 1990. Circadian esophageal motor function in patients with gastroesophageal reflux disease. *Surgery*, 108, (4) 769-777 available from: PM:2218890

Stene-Larsen, G., Weberg, R., Froyshov, L., I, Bjortuft, O., Hoel, B., & Berstad, A. 1988. Relationship of overweight to hiatus hernia and reflux oesophagitis. *Scand.J.Gastroenterol.*, 23, (4) 427-432 available from: PM:3381064

Stevenson, J.C., Cust, M.P., Gangar, K.F., Hillard, T.C., Lees, B., & Whitehead, M.I. 1990. Effects of transdermal versus oral hormone replacement therapy on bone density in spine and proximal femur in postmenopausal women. *Lancet*, 336, (8710) 265-269 available from: PM:1973969

Stolk, L., Zhai, G., van Meurs, J.B., Verbiest, M.M., Visser, J.A., Estrada, K., Rivadeneira, F., Williams, F.M., Cherkas, L., Deloukas, P., Soranzo, N., de Keyzer, J.J., Pop, V.J., Lips, P., Lebrun, C.E., van der Schouw, Y.T., Grobbee, D.E., Witteman, J., Hofman, A., Pols, H.A., Laven, J.S., Spector, T.D., & Uitterlinden, A.G. 2009. Loci at chromosomes 13, 19 and 20 influence age at natural menopause. *Nat.Genet.*, 41, (6) 645-647 available from: PM:19448619

Suzuki, T., Shimizu, T., Yu, H.P., Hsieh, Y.C., Choudhry, M.A., & Chaudry, I.H. 2007. Salutary effects of 17beta-estradiol on T-cell signaling and cytokine production after trauma-hemorrhage are mediated primarily via estrogen receptor-alpha. *Am.J Physiol Cell Physiol*, 292, (6) C2103-C2111 available from: PM:17287365

Taffe, J.R. & Dennerstein, L. 2002. Menstrual patterns leading to the final menstrual period. *Menopause.*, 9, (1) 32-40 available from: PM:11791084

Ulmsten, U. & Sundstrom, G. 1978. Esophageal manometry in pregnant and nonpregnant women. *Am.J Obstet.Gynecol.*, 132, (3) 260-264 available from: PM:707565

Umansky, M., Yasui, W., Hallak, A., Brill, S., Shapira, I., Halpern, Z., Hibshoosh, H., Rattan, J., Meltzer, S., Tahara, E., & Arber, N. 2001. Proton pump inhibitors reduce cell cycle abnormalities in Barrett's esophagus. *Oncogene*, 20, (55) 7987-7991 available from: PM:11753681

Vakil, N., van Zanten, S.V., Kahrilas, P., Dent, J., & Jones, R. 2006. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am.J.Gastroenterol.*, 101, (8) 1900-1920 available from: PM:16928254

Van Thiel, D.H., Gavaler, J.S., Joshi, S.N., Sara, R.K., & Stremple, J. 1977. Heartburn of pregnancy. *Gastroenterology*, 72, (4 Pt 1) 666-668 available from: PM:14050

Van Thiel, D.H., Gavaler, J.S., & Stremple, J. 1976. Lower esophageal sphincter pressure in women using sequential oral contraceptives. *Gastroenterology*, 71, (2) 232-234 available from: PM:939383

Vasudevan, N. & Pfaff, D.W. 2007. Membrane-initiated actions of estrogens in neuroendocrinology: emerging principles. *Endocr.Rev.*, 28, (1) 1-19 available from: PM:17018839

Vicari, J.J., Peek, R.M., Falk, G.W., Goldblum, J.R., Easley, K.A., Schnell, J., Perez-Perez, G.I., Halter, S.A., Rice, T.W., Blaser, M.J., & Richter, J.E. 1998. The seroprevalence of cagA-positive Helicobacter pylori strains in the spectrum of gastroesophageal reflux disease. *Gastroenterology*, 115, (1) 50-57 available from: PM:9649458

von Euler-Chelpin, M., Brasso, K., & Lynge, E. 2010. Determinants of participation in colorectal cancer screening with faecal occult blood testing. *J Public Health (Oxf)*, 32, (3) 395-405 available from: PM:20015868

Walsh, B.W., Schiff, I., Rosner, B., Greenberg, L., Ravnikar, V., & Sacks, F.M. 1991. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *N.Engl.J Med.*, 325, (17) 1196-1204 available from: PM:1922206

Ward, P.A. & Lentsch, A.B. 2002. Endogenous regulation of the acute inflammatory response. *Mol.Cell Biochem.*, 234-235, (1-2) 225-228 available from: PM:12162438

Wassertheil-Smoller, S., Hendrix, S.L., Limacher, M., Heiss, G., Kooperberg, C., Baird, A., Kotchen, T., Curb, J.D., Black, H., Rossouw, J.E., Aragaki, A., Safford, M., Stein, E., Laowattana, S., & Mysiw, W.J. 2003. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. *JAMA*, 289, (20) 2673-2684 available from: PM:12771114

Weel, A.E., Uitterlinden, A.G., Westendorp, I.C., Burger, H., Schuit, S.C., Hofman, A., Helmerhorst, T.J., van Leeuwen, J.P., & Pols, H.A. 1999. Estrogen receptor polymorphism predicts the onset of natural and surgical menopause. *J Clin.Endocrinol.Metab*, 84, (9) 3146-3150 available from: PM:10487678

Weston, A.P., Krmpotich, P.T., Cherian, R., Dixon, A., & Topalosvki, M. 1997. Prospective long-term endoscopic and histological follow-up of short segment Barrett's esophagus:

comparison with traditional long segment Barrett's esophagus. *Am.J.Gastroenterol.*, 92, (3) 407-413 available from: PM:9068459

Weusten, J.J., Blankenstein, M.A., Gmelig-Meyling, F.H., Schuurman, H.J., Kater, L., & Thijssen, J.H. 1986. Presence of oestrogen receptors in human blood mononuclear cells and thymocytes. *Acta Endocrinol.(Copenh)*, 112, (3) 409-414 available from: PM:3489342

Weyant, M.J., Carothers, A.M., Mahmoud, N.N., Bradlow, H.L., Remotti, H., Bilinski, R.T., & Bertagnolli, M.M. 2001. Reciprocal expression of ERalpha and ERbeta is associated with estrogen-mediated modulation of intestinal tumorigenesis. *Cancer Res.*, 61, (6) 2547-2551 available from: PM:11289129

Willett, W., Stampfer, M.J., Bain, C., Lipnick, R., Speizer, F.E., Rosner, B., Cramer, D., & Hennekens, C.H. 1983. Cigarette smoking, relative weight, and menopause. *Am.J Epidemiol.*, 117, (6) 651-658 available from: PM:6859020

Wink, D.A., Hanbauer, I., Krishna, M.C., DeGraff, W., Gamson, J., & Mitchell, J.B. 1993. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc.Natl.Acad.Sci.U.S.A*, 90, (21) 9813-9817 available from: PM:8234317

Winters, C., Jr., Spurling, T.J., Chobanian, S.J., Curtis, D.J., Esposito, R.L., Hacker, J.F., III, Johnson, D.A., Cruess, D.F., Cotelingam, J.D., Gurney, M.S., & . 1987. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. *Gastroenterology*, 92, (1) 118-124 available from: PM:3781178

Wright, R.A. & Hurwitz, A.L. 1979. Relationship of hiatal hernia to endoscopically proved reflux esophagitis. *Dig.Dis.Sci.*, 24, (4) 311-313 available from: PM:456217

Wyman, J.B., Dent, J., Heddle, R., Dodds, W.J., Toouli, J., & Downton, J. 1990. Control of belching by the lower oesophageal sphincter. *Gut*, 31, (6) 639-646 available from: PM:2379867

Xia, H.H. & Talley, N.J. 1998. Helicobacter pylori infection, reflux esophagitis, and atrophic gastritis: an unexplored triangle. *Am.J Gastroenterol*, 93, (3) 394-400 available from: PM:9517647

Yamaguchi, T., Sugimoto, T., Yamada, H., Kanzawa, M., Yano, S., Yamauchi, M., & Chihara, K. 2002. The presence and severity of vertebral fractures is associated with the presence of esophageal hiatal hernia in postmenopausal women. *Osteoporos.Int.*, 13, (4) 331-336 available from: PM:12030548

Yamato, S., Spechler, S.J., & Goyal, R.K. 1992. Role of nitric oxide in esophageal peristalsis in the opossum. *Gastroenterology*, 103, (1) 197-204 available from: PM:1612326

Ye, W., Chow, W.H., Lagergren, J., Boffetta, P., Boman, G., Adami, H.O., & Nyren, O. 2001. Risk of adenocarcinomas of the oesophagus and gastric cardia in patients hospitalized for asthma. *Br.J.Cancer*, 85, (9) 1317-1321 available from: PM:11720467

Ye, W., Held, M., Lagergren, J., Engstrand, L., Blot, W.J., McLaughlin, J.K., & Nyren, O. 2004. Helicobacter pylori infection and gastric atrophy: risk of adenocarcinoma and squamous-cell carcinoma of the esophagus and adenocarcinoma of the gastric cardia. *J.Natl.Cancer Inst.*, 96, (5) 388-396 available from: PM:14996860

Zaninotto, G., Demeester, T.R., Schwizer, W., Johansson, K.E., & Cheng, S.C. 1988. The lower esophageal sphincter in health and disease. *Am.J Surg.*, 155, (1) 104-111 available from: PM:3341525

Chapter 11

Appendices

Appendix 1: Acid reflux questionnaire

QUESTIONNAIRE ON GASTRO-OESOPHAGEAL REFLUX

Please answer all questions. If you are uncertain, please write down your best guess.

It is easy to miss questions, so please check that you have not left any out as you go.

If you wish to comment on any questions or qualify your answers, use the space in the margins; these comments will be read and taken into account.

All information provided will be kept completely confidential.

Thank you for your help.

First we would like to ask you some questions about HEARTBURN in the last year. Heartburn is a burning pain or discomfort behind the breast bone or in the top part of the stomach. Please do not count pain from heart trouble.

1. Have you had heartburn in the last year? *(Tick one)*

1 □ No → Please go to question 3

2 □ Yes → Please answer the following question

↓

2. How many times have you had heartburn in the last year? *(Tick one)*

1 □ Less than once a month
2 □ About once a month
3 □ About once a week
4 □ Several times a week
5 □ Every day

We would now like to ask you some questions about ACID REGURGITATION in the last year. Acid regurgitation is a fluid coming up into your throat or mouth, which may be burning or have a sour or bitter taste.

3. Have you had acid regurgitation in the last year? *(Tick one)*

1 □ No → Please go to question 5

2 □ Yes → Please answer the following question

↓

4. How many times have you had acid regurgitation in the last year? *(Tick one)*

1 □ Less than once a month
2 □ About once a month
3 □ About once a week
4 □ Several times a week
5 □ Every day

5. If you have had heartburn or acid regurgitation in the past year, when did it first begin? *(Tick one)*
If you have had neither and have answered "No" to question 1 and "No" to question 3, please go to question 8 on page 3.

1 □ In the last year
2 □ More than 1 year to 2 years ago
3 □ More than 2 years to 5 years ago
4 □ More than 5 years to 10 years ago

5 □ More than 10 years ago

NOTE: when we say "often", we mean more than 25% of the time in the last year

6 Is your heartburn or acid regurgitation often made better by taking antacids (likeAludrox, Andrews, Asilone, Bisodol, Gaviscon, Maalox, Milk of Magnesia, Rap-eze, Remegel, Rennies or Settlers)? *(Tick one)*

1 □ No
2 □ Yes
3 □ I have never taken antacids for heartburn

7. How many times have you taken antacids (like Aludrox, Andrews, Asilone, Bisodol, Gaviscon, Maalox, Milk of Magnesia, Rap-eze, Remegel, Rennies or Settlers) in the last year? *(Tick one)*

0 □ Never
1 □ Less than once a month
2 □ About once a month
3 □ About once a week
4 □ Several times a week
5 □ Every day

Which brands of antacids have you used in the last year? ……
……

We would now like to ask you about other complaints you may have had in the last year

8. Have you had an ache or pain in the stomach or belly in the last year? (Please donot count pain with menstrual periods, heartburn or chest pain) *(Tick one)*

1 □ No → Please go to question 26 on PAGE 6

2 □ Yes→ Please answer the following questions

↓

9. How many times have you had this ache or pain in the last year? *(Tick one)*

1 □ Less than once a month
2 □ About once a month
3 □ About once a week
4 □ Several times a week
5 □ Every day

10. When did this ache or pain first begin? *(Tick one)*

1 □ In the last six months

2 □ Seven months to 1 year ago
3 □ More than 1 year to 2 years ago
4 □ More than 2 years to 5 years ago
5 □ More than 5 years to 10 years ago
6 □ More than 10 years ago

NOTE: when we say "often", we mean more than 25% of the time in the last year

11. Is the ache or pain often made BETTER by having a bowel movement or passing wind? *(Tick one)*

1 □ No
2 □ Yes

12. Do you often have MORE bowel movements when the ache or pain begins? *(Tick one)*

1 □ No
2 □ Yes

13. Do you often have LESS bowel movements when the ache or pain begins? *(Tick one)*

1 □ No
2 □ Yes

14. Do you often have LOOSER bowel movements when the ache or pain begins? *(Tick one)*

1 □ No
2 □ Yes

15. Do you often have HARDER bowel movements when the ache or pain begins? *(Tick one)*

1 □ No
2 □ Yes

16. Do you often have less than three bowel movements each week? *(Tick one)*

1 □ No
2 □ Yes

17. Do you often have more than three bowel movements each day? *(Tick one)*

1 □ No
2 □ Yes

18. Do you often STRAIN to have a bowel movement? *(Tick one)*

1 □ No
2 □ Yes

19. Have you often seen MUCUS in your stools in the last year (that is white or green slimy material)? *(Tick one)*

1 □ No
2 □ Yes

20. Are your stools often LOOSE or WATERY? *(Tick one)*

1 □ No
2 □ Yes

21. Are your stools often HARD? *(Tick one)*

1 □ No
2 □ Yes

22. After finishing a bowel movement, do you often feel there is still stool that needs to be passed? *(Tick one)*

1 □ No
2 □ Yes

23. Do you often experience an URGENT need to open your bowels that makes you rush to a toilet? *(Tick one)*

1 □ No
2 □ Yes

24. Do you often feel bloated and actually see your belly swell up? *(Tick one)*

1 □ No
2 □ Yes

25. Have you often felt as if there is a "lump in your throat" when NOT swallowing in the last year? *(Tick one)*

1 □ No
2 □ Yes

Please check that all questions that apply to you have been answered

26. For each of the complaints or problems listed below, please indicate how often it occurred and how much it bothered you in the last year.

Please write down a number from 0 to 4 for all sixteen questions below in both of the columns.

How often?	How much does it bother you?
0 – Never or less than once a month 1 – Occurs about once a month 2 – Occurs about once a week 3 – Occurs several times a week 4 – Occurs daily	0 – Not a problem 1 – It bothers me slightly 2 – It bothers me moderately 3 – It bothers me severely 4 – It bothers me extremely

		How often? (0 - 4)	How much does it bother you? (0 – 4)
A.	Headaches	. ……	……..
B.	Backaches	…….	……..
C.	Asthma	…….	……..
D.	Trouble breathing	…….	……..
E.	Insomnia (trouble sleeping)	…….	……..
F.	Fatigue (tiredness)	…….	……..
G.	Depression (feeling sad)	…….	……..
H.	General stiffness	…….	……..
I.	Heart palpitation (racing or pounding)	…….	……..
J.	Joint pains	…….	……..
K.	Eye pain with reading	…….	……..
L.	Dizziness	…….	……..
M.	Weakness	…….	……..
N.	Nervousness or shakiness	…….	……..
O.	Hot or cold spells	…….	……..
P.	High blood pressure	…….	……..

Please check that you have written two numbers by each complaint or problem – one in the "how often?" and one in the "how much does it bother you?" section

27. At what age did you start having periods? ……. Years

28. Have you ever been pregnant? *(Tick one)*

1 □ No → Please go to question 34

2 □ Yes → Please answer the following questions

29. Have you been pregnant in the last year? *(Tick one)*

1 □ No
2 □ Yes

If you have been pregnant and have had heartburn or acid regurgitation in the past year, did the heartburn or acid regurgitation only occur while you were pregnant? *(Tick one)*

1 □ No
2 □ Yes

30. How many times have you been pregnant? ……... times

31. How many children have you had? ……... children

32. Did you have heartburn or acid regurgitation when you were pregnant? *(Tick one)*

1 □ No
2 □ Yes

33. Did you breast or bottle feed your children? *(Tick one)*

1 □ Bottle
2 □ Breast
3 □ Bottle and breast

If you breast fed your children, how long did you do this for on average? *(Tick one)*

1 □ Less than three months
2 □ Three to six months
3 □ Six to nine months
4 □ More than nine months

34. Have you taken any of the following medicines in the last year?

	No	Yes	If yes, how many days?
Axid (nizatidine)	1 □	2 □	…….
Losec (omeprazole)	1 □	2 □	…….
Nexium (esomeprazole)	1 □	2 □	…….
Pariet (rabeprazole)	1 □	2 □	…….
Pepcid (famotidine)	1 □	2 □	…….
Protium (pantoprazole)	1 □	2 □	…….
Tagamet (cimetidine)	1 □	2 □	…….
Zantac (ranitidine)	1 □	2 □	…….
Zoton (lansoprazole)	1 □	2 □	…….

Please write down the health problems for which you have taken these medicines in the last year ……..
……..…

35. Have you taken any tablets, capsules or medicines (including birth control pills) regularly in the last year? *(Tick one)*

1 □ No
2 □ Yes

If "Yes", please write down the names of ALL of these medicines ……...
……..
……..

36. Has your husband/partner had heartburn or a disease of the oesophagus (gullet) or stomach? *(Tick one)*

1 □ No
2 □ Yes

What problem? ……

37. Have any of your immediate family members (mother, father, brothers, sisters, children) had heartburn or a disease of the oesophagus or stomach? *(Tick one)*

1 □ No
2 □ Yes

If "Yes", who? …….
What problem? ……

38. Have you ever had a dilatation (stretching) of your oesophagus? *(Tick one)*
1 □ No
2 □ Yes

If "Yes", when? …….

39. Have you ever had an operation on your oesophagus or stomach? *(Tick one)*

1 □ No
2 □ Yes

If "Yes", when? …….
What operation? …….

40. Have you ever had a disease of the bowels or intestine? *(Tick one)*

1 □ No
2 □ Yes

If "Yes", what disease? …….
When? …….

Please check that all questions that apply to you have been answered

To help us interpret the results of this survey, we would like to ask some general questions about you. Please be assured that all information will be kept strictly confidential.

41. Have you ever smoked cigarettes regularly (at least one cigarette per day for at least 30 days)? *(Tick one)*

1 □ No
2 □ Yes

If "Yes", at what age did you start? ……. years
When smoking the heaviest, how many cigarettes did you smoke per day? ……..
How many cigarettes do you currently smoke? ……
If you have stopped smoking, at what age did you stop? ……

Next there is a question about drinks that contain alcohol (that is beer, wine or spirits such as whisky, vodka, gin or brandy). One drink is equal to a can or half a pint of beer, a glass of wine or a single measure of spirits.

42. How many alcoholic drinks a week have you had on average in the last year? *(Tick one)*

0 □ None or less than 1 drink a week
1 □ 1 to 10 drinks a week
2 □ 11 to 20 drinks a week
3 □ 21 to 30 drinks a week
4 □ 31 to 40 drinks a week
5 □ More than 40 drinks a week

43. How many days a week do you have an alcoholic drink on? *(Tick one)*

0 □ None
1 □ 1 day
2 □ 2 days
3 □ 3 days
4 □ 4 or 5 days
5 □ 6 or 7 days

44. What is (or was) the name and title of your main job?

Occupation ……

Industry ……

45. How old were you when you left full-time education? _____ years

46. Do you have any educational qualifications? *(Tick one)*

1 □ None
2 □ City and Guilds
3 □ O levels/School Certificate
4 □ A levels/Higher School Certificate
5 □ University degree
6 □ Other – please specify _____

47. How old are you? …… years

48. What is your height? ……. feet ……. inches

49. What is your weight (without your clothes)? …….stones ……pounds

50. How much did you weigh at the age of 18? …….stones ……pounds

51. How would you describe your ethnic or racial origin? *(Tick one)*

□ White □ Black (Caribbean)

□ Black (African) □ Black (Other)

□ Indian □ Pakistani

□ Bangladeshi □ Chinese

□ Other – please specify ……..

52. If your ethnic or racial origin is not white, were you born in the UK?

1 □ No

2 □ Yes

If "Yes", which generation of your family first came to live in the UK?

1 □ My parents
2 □ My grandparents
3 □ My great-grandparents
4 □ Other – please specify ……..

Thank you for your help with our study. Please check that you have answered all the questions that apply to you.

Is there anything else you would like to tell us about your health problems? If so, please use this space for this purpose.

Also, any comments that may help us understand these problems better would be appreciated, either here or in a separate letter.

Appendix 2: Reference ranges and standard deviations for hormonal assays

Reference ranges and standard deviations for the hormonal assays used in the study in pre and post-menopausal women

Hormone	Pre-menopausal group (follicular phase)	Standard Deviation	Post-menopausal group	Standard Deviation
Oestradiol (E2)	60-300pmol/L	60	50-150pmol/L	25
Oestrone (E1)	200-800pmol/L	150	<250pmol/L	50
SHBG	40-80nmol/L	10	40-80nmol/L	10
Testosterone (T)	0.51-1.89nmol/L	0.35	0.4-0.86nmol/L	0.12
FSH	2.8-14.4 IU/L	2.96	21.7-153 IU/L	33.49
LH	1.1-11.6 IU/L	2.68	11.3-39.8 IU/L	7.27

Appendix 3: Minimum detectable differences in hormones

Minimum detectable differences for a sample size of 100 patients, at 80% power and 5% significance, in each of the groups:

	Pre-menopausal	Post-menopausal
E2	49 (pmol/l)	20 (pmol/l)
E1	122 (pmol/l)	41 (pmol/l)
SHBG	8 (nmol/l)	8 (nmol/l)
T	0.28 (nmol/l)	0.1 (nmol/l)
FSH	2.4 (IU/L)	27 (IU/L)
LH	2.2 (IU/L)	5.9 (IU/L)

Appendix 4: Hormone assays and coefficients of variation

Analyte	Method	Platform	(n)	Mean level	CV%
FSH	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	5.33 IU/L	3.6
LH	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	5.81 IU/L	2.0
Testosterone	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	1.67 nmol/L	5.6
Progesterone	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	10.0 nmol/L	2.8
Oestradiol	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	472 pmol/L	2.5
SHBG	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	42.0 nmol/L	2.1
Oestrone	Electrochemiluminescence immunoassay (ECLIA)	Roche E170 immunoassay system	21	150nmol/L	10

Appendix 5: Publications and presentations arising from work in this thesis

Publications

1. Menon S, Jayasena H, Nightingale P, Trudgill N. The influence of age and gender on endoscopic findings of gastro-oesophageal reflux disease: an endoscopy database study. European Journal of Gastroenterology and Hepatology. 2011 May;23(5):389-95.

2. Menon S, Trudgill NJ. Risk factors in the aetiology of hiatus hernia: a meta-analysis. . European Journal of Gastroenterology and Hepatology. 2011 Feb;23(2):133-8.

Presentations

Oral presentation

The influence of gender on gastro-oesophageal reflux and its complications. Midlands Gastroenterology Society (MGS) research prize, presented in October 2010, MGS meeting, West Bromwich.

Posters

1. Menon S, Tselepis C, Anderson MR. Is there a gender specific response of oesophageal mucosa to acid reflux? Gastroenterology 2011; Sa1704.

2. Menon S, Tselepis C, Anderson MR. Is there a gender specific response of oesophageal mucosa to acid reflux? Gut 2011;60:A176.

3. Menon S, Trudgill NJ. Is hormone replacement therapy associated with reflux oesophagitis, Barrett's oesophagus and oesophageal cancer? Gut 2010; 59 (Suppl III) A117.

4. Menon S, Trudgill NJ. The influence of age, sex and obesity on the prevalence of hiatus hernia: a meta-analysis. Gut 2010; 59 (Suppl III) A117.

5. Menon S, Nightingale P, Butterworth J, Trudgill N. Severe oesophagitis and its complications are more common in women after the menopause. Gut 2009; **58** (Suppl I):A145.