STUDIES OF *IN SITU* NITROSATIVE STRESS FOLLOWING NITRATE INGESTION IN THE HUMAN UPPER GASTROINTESTINAL TRACT

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ABSTRACT

Nitrate ingestion leads to high luminal concentrations of nitric oxide being generated where saliva meets gastric acid. Nitric oxide generates *N*-nitrosative stress on reacting with oxygen at neutral pH. We aimed to ascertain if luminal nitric oxide exerts nitrosative stress in the upper GI tract, and to assess the influence of acid reflux. We utilised a specialised silicone tube as an epithelial model, inserting it into the upper gastro-intestinal tract of humans. Healthy volunteers were studied with and without ingestion of ^{15}N enriched nitrate and Barrett's oesophagus patients with and without stimulation of reflux. In volunteers, nitrate ingestion resulted in significantly higher concentrations of *N*-nitrosomorpholine in the tube sections exposed to acid. In Barrett's patients, generation of *N*-nitrosomorpholine shifted proximally, with most nitrosative stress occurring within the oesophagus during reflux episodes. This chemistry may be harmful to patients with erosive esophagitis whose epithelium will be more sensitive to chemical mutagenesis.

PREFACE

Over the past four years, I have been fortunate to know and work with Professor Kenneth McColl. I hope the work presented in this thesis will in some way repay his mentorship and trust, for both of which I have been very grateful.

In any complex research project, collaboration is essential. Where it has been required, it is described in the formal acknowledgements. Except where indicated, the work presented has been carried out by myself.

The writing of this thesis is entirely my own work.

ACKNOWLEDGEMENTS

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Combet E, Paterson S, Iijima K, Winter J, Mullen W, Crozier A, Preston T, McColl KEL. Fat transforms ascorbic acid from inhibiting to promoting acid-catalysed *N*-nitrosation. *Gut*; 56: 1678-1684

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LIST OF ABBREVIATIONS

GOJ	**GASTRO-OESOPHAGEAL JUNCTION**
NO	**NITRIC OXIDE**
NMOR	***N*-NITROSOMORPHOLINE**
NDBA	***N*-NITROSODIBUYLAMINE**
DCM	**DICHLOROMETHANE**
DEE	**DI-ETHYL ETHER**
GCMS	**GAS CHROMATOGRAPHY/ MASS SPECTROMETERY**
PPI	**PROTON PUMP INHIBITOR**
CLO	**CAMPYLOBACTER-LIKE ORGANISM**
DNA	**DE-OXYRIBONUCLEIC ACID**
NO^+	**NITROSONIUM ION**
NOSCN	**NITROSO-THIOCYANATE**
N_2O_3	**NITROUS ANHYDRIDE**
HNO_2	**NITROUS ACID**
NO_2^-	**NITRITE ION**

SUMMARY

Adenocarcinoma of the oesophagus and gastric cardia adenocarcinoma are collectively known as gastro-oesophageal junction cancer. The incidence of neoplasia at this location has increased substantially in the last 30 years, in contrast to the decline in incidence of non-cardia gastric cancer. The symptoms associated with cancer at this site manifest late and therefore the prognosis in the majority of patients remains poor. Although a number of risk factors have been associated with this disease, none explain the reason for the remarkable increase in incidence observed during the final quarter of the twentieth century, which suggests the involvement of an environmental mutagen.

A possible explanation for the rising incidence of this cancer is that it occurs at the site where saliva of neutral pH encounters gastric acid. The anatomical site where those two fluids meet may be very important as their interaction creates reactive nitrogen species which are potentially mutagenic. The rise in incidence of gastro-oesophageal cancer is associated epidemiologically with a substantial increase in nitrogen fertiliser use 20-30 years previously. Nitrate consumption has increased mainly as a result of higher concentrations of nitrate in vegetable crops.

Following ingestion, nitrate is rapidly absorbed from the small intestine and, within 30 minutes, 25% is re-secreted into the oral cavity by the salivary glands. 10-90% of this nitrate is then reduced to nitrite by buccal bacteria and

swallowed with in saliva. Ingested nitrite has been associated with development of gastric tumours through formation of *N*-nitroso compounds in the achlorhydric stomach which is heavily colonised with bacteria. However in the healthy, acid-secreting stomach, the nitrosating species formed by acidification of nitrite are rapidly reduced to nitric oxide where saliva meets gastric juice, which is rich in ascorbic acid. Very high concentrations of this lipid soluble gas are generated at the gastro-oesophageal junction in healthy subjects, or within the oesophagus when saliva meets regurgitated gastric juice in patients with gastro-oesophageal reflux disease.

This reaction can potentially lead to nitrosative stress by two separate mechanisms. Firstly, it has been demonstrated in humans that the luminal formation of nitric oxide depletes local ascorbic acid concentrations and leads to a high nitrite:ascorbate concentration where saliva meets acid. This creates suitable luminal conditions for the formation of *N*-nitroso compounds. Secondly, bench-top studies have shown that luminally generated nitric oxide can form *N*-nitroso compounds within the epithelial compartment of a stomach model as a result of diffusion of nitric oxide through the epithelial lipid membrane.

The aim of this thesis was to examine if the epithelial nitrosative stress measured in the laboratory bench-top model could be reproduced in human subjects following ingestion of nitrate, and to investigate the influence of gastro-oesophageal acid reflux on the maximal location of this stress. We utilised a segmented silastic tube containing phosphate buffer pH 7.4 and the secondary amine morpholine as an *in situ* epithelial model by placing it in the upper gastro-intestinal tract of seventeen healthy volunteers and eighteen patients with Barrett's oesophagus. The silicone wall of the tube possesses the same physical properties

as an epithelial lipid membrane, allowing passage of gases such as nitric oxide and oxygen but not that of hydrogen ions.

By studying healthy volunteers with and without ingestion of ^{15}N labelled nitrate we were able to demonstrate that dietary nitrate results in the generation of significantly higher concentrations of nitrite and *N*-nitrosomorpholine within our epithelial model, and that the proximal stomach was the site of greatest nitrosative stress. By studying patients with Barrett's oesophagus following ingestion of ^{15}N labelled nitrate with and without oesophageal acid reflux, we were able to demonstrate that during acid reflux, nitrosative stress occurs maximally within the oesophagus.

This thesis has demonstrated that nitrate ingestion exerts *in situ* nitrosative stress within an epithelial model situated in the human upper gastro-intestinal tract and that during acid reflux this nitrosative stress occurs predominantly within the oesophagus. Although we have not directly measured nitrosative stress within the epithelial cells of the human upper GI tract, the presence of substrates for nitrosative stress within these cells has previously been demonstrated. This chemistry may be particularly harmful to patients with erosive oesophagitis whose epithelium will be more sensitive to chemical mutagenesis.

CHAPTER ONE

A GENERAL INTRODUCTION ON THE INCIDENCE OF AND RISK FACTORS FOR ADENOCARCINOMA AT THE GASTRO-OESOPHAGEAL JUNCTION

1. ADENOCARCINOMA AT THE GASTRO-OESOPHAGEAL JUNCTION

1.1 Epidemiology

In developed countries, the incidence of adenocarcinoma at the gastro-oesophageal junction has risen markedly in the last twenty five years [1-3], and the increase does not appear to be explained by changes in diagnostic and reporting practices[4-6]. Gastro-oesophageal junction cancers comprise two different malignancies which may share a common pathogenesis: oesophageal adenocarcinoma and gastric cardia adenocarcinoma [1].

Scotland has the highest reported incidence of gastro-oesophageal junction cancer in the world.[1] Approximately 1,700 patients are diagnosed with oesophageal or gastric carcinoma in Scotland every year. Together, they constitute the 5th most common cancer, accounting for 6.5% of all new cancer diagnoses. Patients presenting with symptoms of these malignancies invariably have advanced disease, with median survival of 8.4 months from diagnosis. Only 40% of patients remain alive at one year. Five year survival for both these cancers remains poor at 10-18% [7], and as such these malignancies account for 9.4% of cancer deaths.

For most of the twentieth century, squamous carcinoma of the oesophagus was the most common oesophageal malignancy, comprising more than 90% of all oesophageal cancer in the United States of America. Over the last 30 years, the incidence of squamous carcinoma has remained stable, but the age adjusted incidence of adenocarcinoma has increased significantly, especially in white males [8, 9]. The changing incidences of both histological types of oesophageal carcinoma in Scottish males and females are shown in figure 1.1 and 1.2.

Gastric adenocarcinoma is one of the most common forms of cancer worldwide, and was the leading cause of cancer deaths until the 1980s when it was overtaken by lung cancer [10, 11]. Global incidence has declined rapidly in the last few decades [12], probably as a result of improved diet and living conditions, along with the recognition of risk factors such as *Helicobacter pylori*. However, in Western industrialised countries the incidence of adenocarcinoma of the gastric cardia, defined as malignancy with its epicentre between 1cm proximal and 2 cm distal to the gastro-oesophageal junction, has risen. The incidences of both anatomical subgroups of gastric cancer in Scottish males and females are demonstrated in figure 1.3 and figure 1.4. In 2002, 50% of gastric tumours occurred within the cardia, an area which represents only 5-10% of the total surface area of the stomach.

Figure 1.1: European age-adjusted standardised incidence rate for oesophageal carcinoma by histological type in Scottish males from 1980-2003.

Source: Scottish Cancer Registry, ISD Scotland.

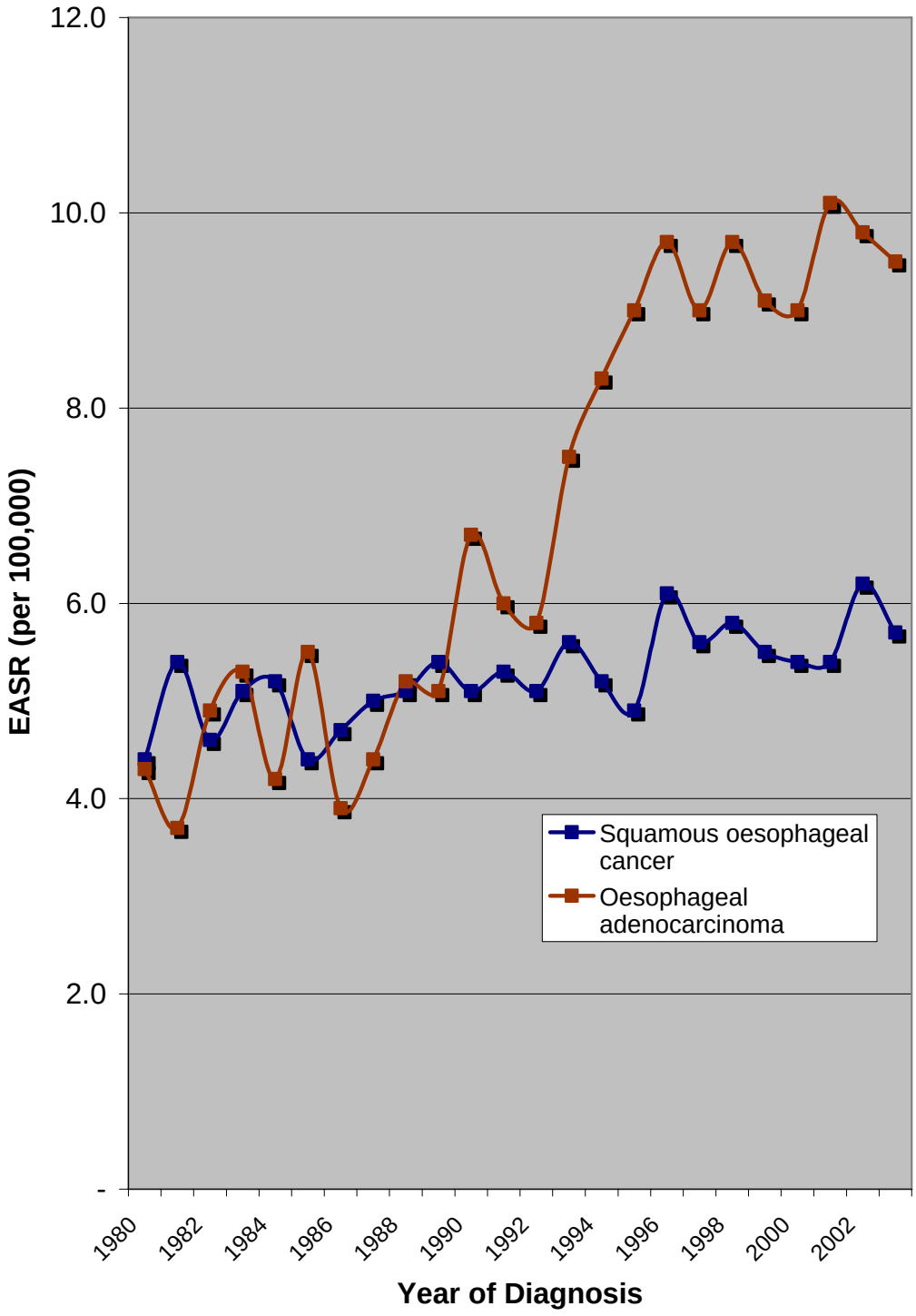


Figure 1.2: European age-adjusted standardised incidence rate for oesophageal carcinoma by histological type in Scottish females from 1980-2003.

Source: Scottish Cancer Registry, ISD Scotland.

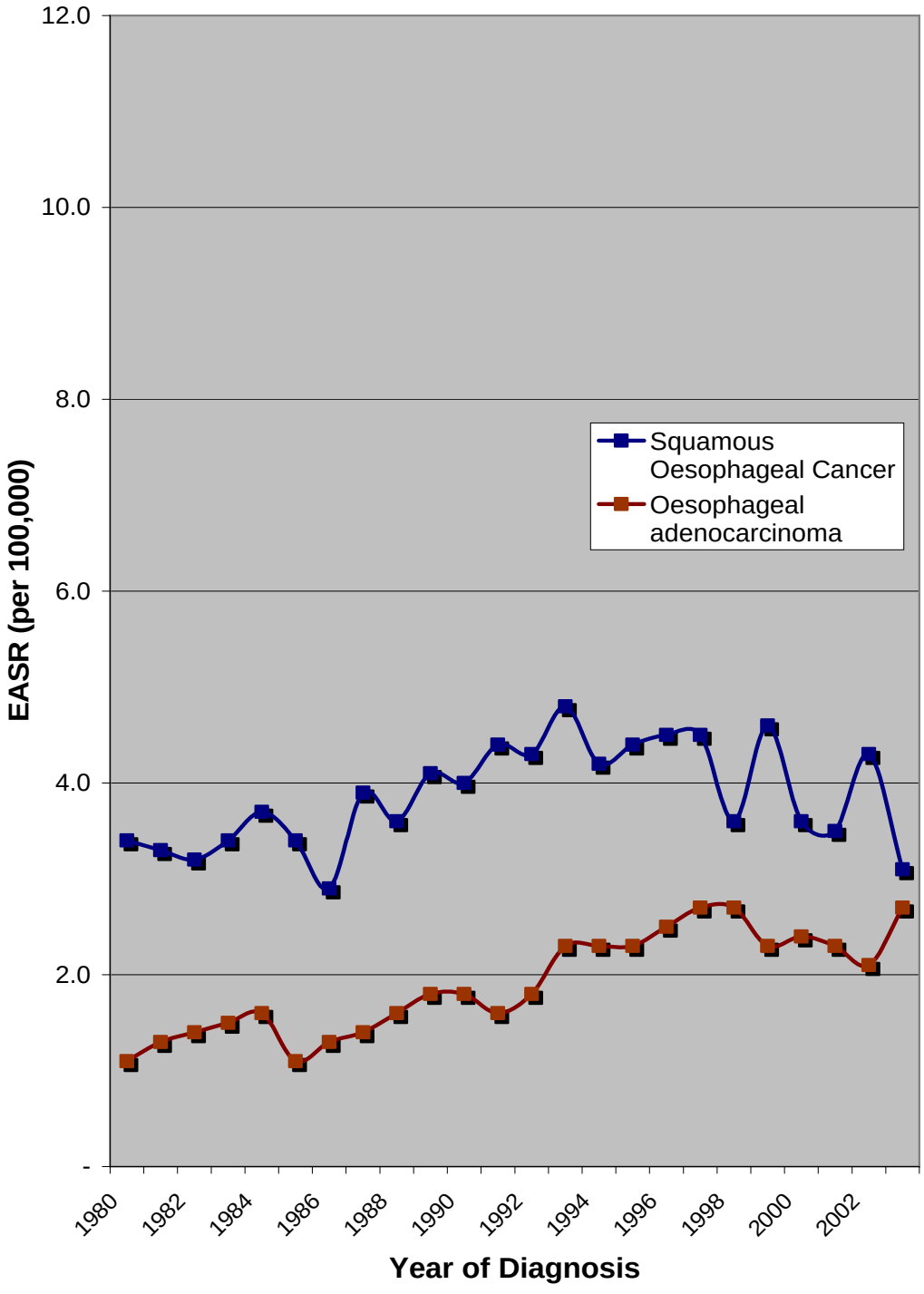


Figure 1.3: European age-adjusted standardised incidence rate for gastric carcinoma by anatomical site in Scottish males from 1980-2003.

Source: Scottish Cancer Registry, ISD Scotland

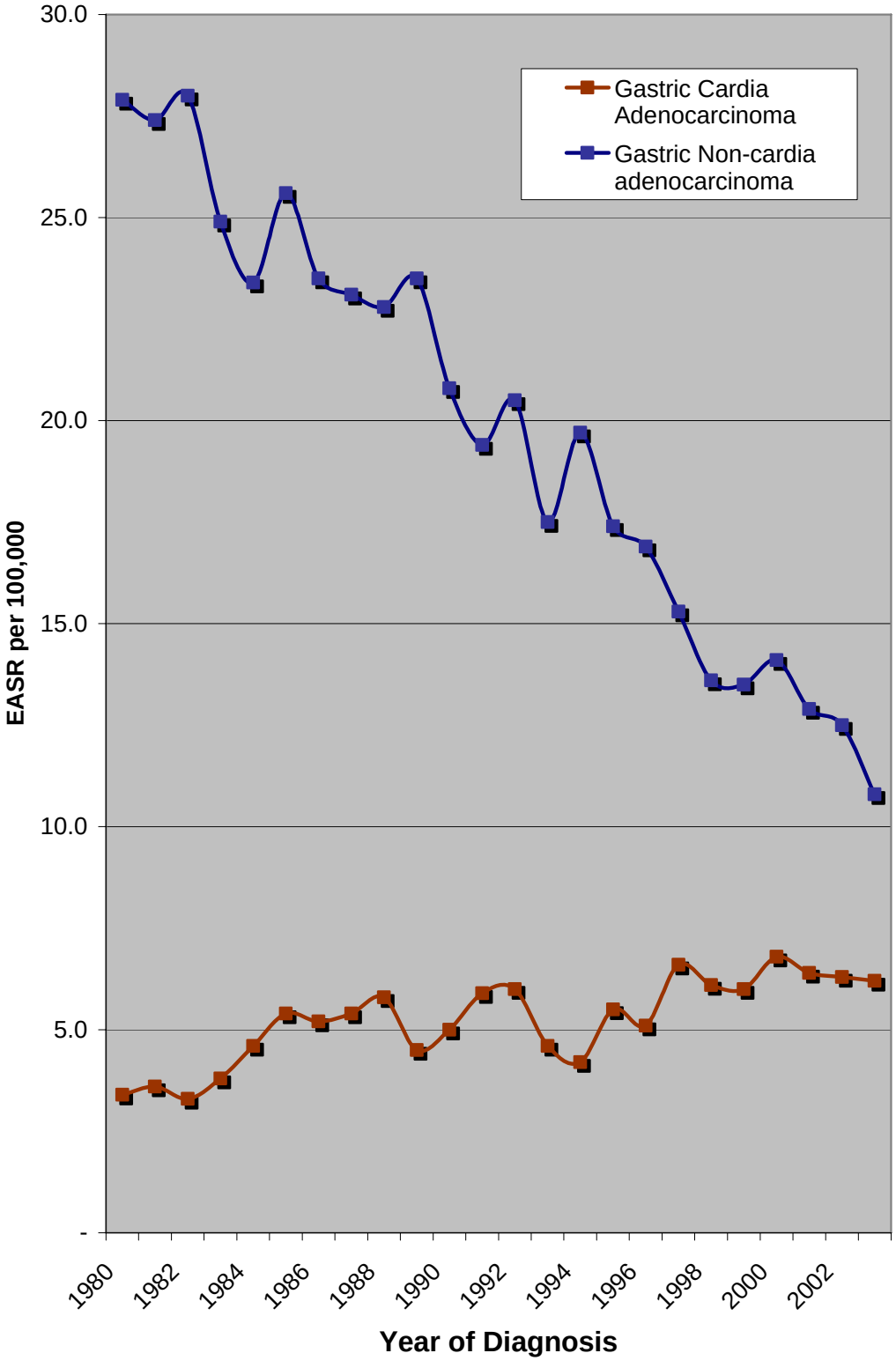
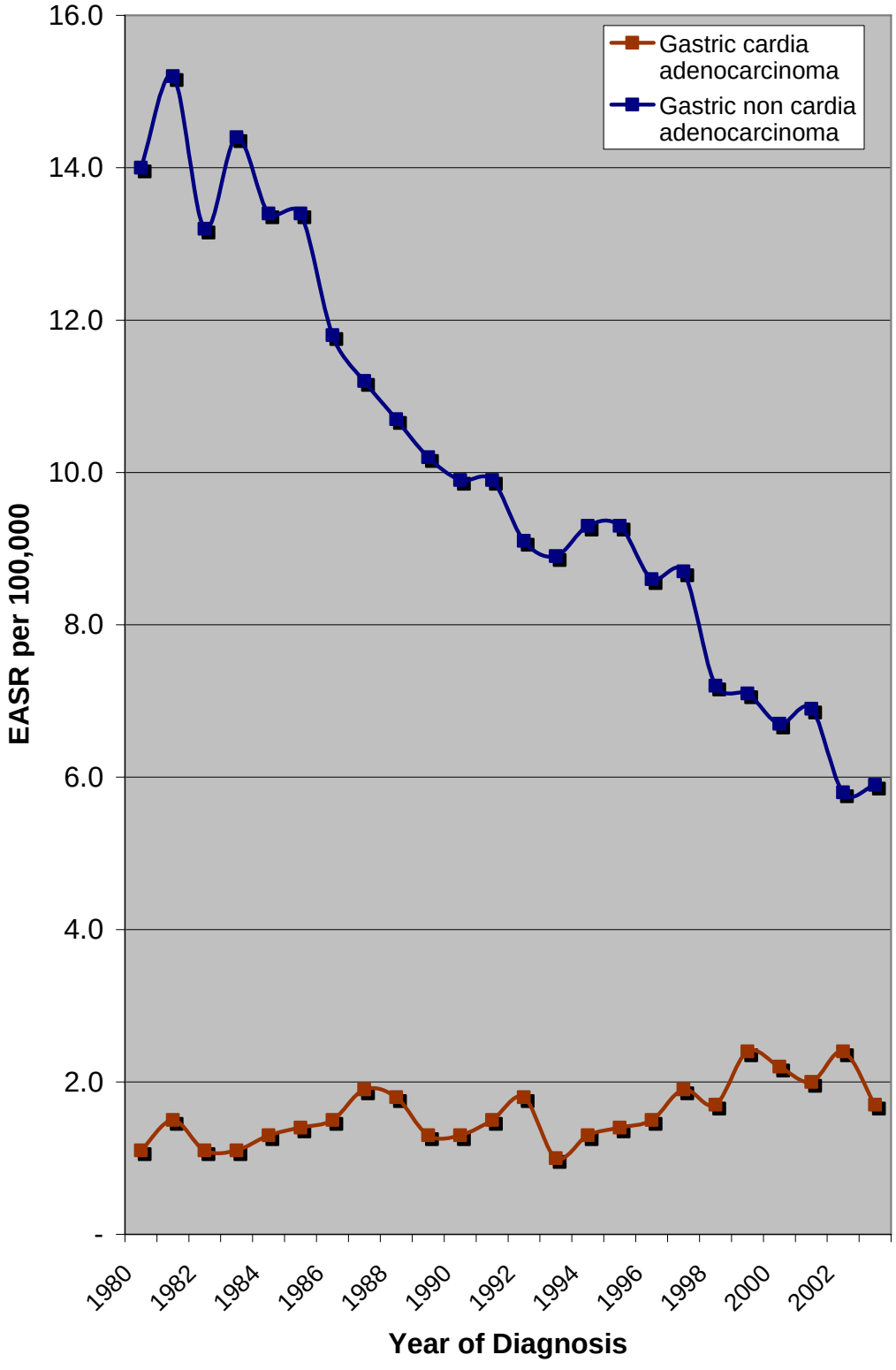


Figure 1.4: European age-adjusted standardised incidence rate for gastric carcinoma by anatomical site in Scottish females from 1980-2003.

Source: Scottish Cancer Registry, ISD Scotland



1.2 Risk factors

Adenocarcinoma of the oesophagus and gastric cardia share similar risk factor profiles in the developed world, but for the purpose of this review each will first be considered separately.

1.2.1 Oesophageal adenocarcinoma

The median age at diagnosis of oesophageal adenocarcinoma is 60 years, and incidence rises with increasing age. There is a striking male preponderance (4:1 in Scotland) which is similar in all populations studied [2, 13, 14], although the proportional increase in females is analogous [15]. It is more common in Caucasians than Afro-Caribbean Americans in the USA[2, 3].

The strongest risk factor for oesophageal adenocarcinoma is the presence of Barrett's oesophagus. Barrett's oesophagus is defined by the British Society of Gastroenterology as an oesophagus in which any portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium which is visible macroscopically[16]. In order to make a positive diagnosis of Barrett's oesophagus, a segment of columnar mucosa of any length must be visible endoscopically above the oesophago-gastric junction (the confluence of the proximal limit of the gastric folds, the distal limit of the linear oesophageal vessels and the point of flaring of the stomach from the tubular oesophagus when the lumen is deflated) and confirmed histologically. Barrett's oesophagus is an acquired condition secondary to long-standing gastro-oesophageal acid reflux, and confers a 30-60 fold excess relative risk of oesophageal adenocarcinoma compared to the general population[17-21].

Gastro-oesophageal reflux disease has been shown to be a strong and independent risk factor for adenocarcinoma of the oesophagus, probably acting via Barrett's metaplasia. Case control studies from Sweden[22] and from the USA[23] have demonstrated an association and dose-response relationship between frequent symptoms of acid reflux and risk of oesophageal adenocarcinoma. A further Swedish population based study used a cohort design to investigate the relationship between oesophageal reflux and adenocarcinoma. It revealed a nine-fold increased risk of adenocarcinoma among patients with endoscopically verified oesophagitis[24]. Acid suppressive therapy and anti-reflux surgery are highly effective in treating erosive oesophagitis, but there is no evidence that either can attenuate the risk of malignancy[22-27].

A further major risk factor is obesity. Although obesity appears to increase the risk of gastro-oesophageal reflux disease by increasing oesophageal acid exposure[28], there seems to be a true and dose dependent association between increased body mass index and risk of oesophageal adenocarcinoma which is independent of reflux symptoms [29-31]. Among persons with a historical BMI above 30, the relative risk has been found to be as high as 16 compared with the leanest.

There is a strong correlation between *Helicobacter pylori* infection and non cardia gastric adenocarcinoma [32]. However, there is accumulating evidence that *H. pylori* exerts a protective effect against development of adenocarcinoma of the oesophagus [33, 34], although the association is complex and the mechanism behind this protective effect is unclear. It has been postulated that the reduction in intragastric acidity as a result of pan-gastritis and atrophy confers protection by lowering the exposure of the oesophageal epithelium to refluxing gastric acid[35] and this is supported by a recently published retrospective cohort study from

Sweden, which reported that patients hospitalised for duodenal ulcer had a 70% increased risk of oesophageal adenocarcinoma compared with population controls but that patients hospitalised for gastric ulcer had no change in risk[36]. Patients with duodenal ulcer are likely to have antral predominant gastritis and subsequent high levels of acid secretion, which could exacerbate reflux disease. Patients with gastric ulcer are likely to constitute a mixture of individuals with antral and with body predominant gastritis who have varying levels of acid production. However, the relationship between *H pylori* and oesophageal adenocarcinoma may be more complex than the influence of the infection on acid secretion, as a further case control study found that despite a three-fold reduction in risk of oesophageal adenocarcinoma in patients with *H. pylori,* there was no association between gastric atrophy and oesophageal adenocarcinoma[34].

Smoking appears to exert only a moderate increase in the risk of oesophageal adenocarcinoma[17], with two population based case control studies from the USA finding an increase in odds ratio two to three-fold[30, 37], but a similar study from Sweden failing to demonstrate an association[38]. Although alcohol consumption is strongly associated with risk of squamous cancer of the oesophagus, it is not associated with adenocarcinoma, regardless of the type of alcoholic beverage consumed[30, 37, 38].

Many studies have examined the association between dietary factors and risk of oesophageal adenocarcinoma. However, data concerning dietary risk are susceptible to bias, particularly to the role of confounding by dietary variables[17]. The best established risk exposure is that of low intake of fruit and vegetables[39, 40], possibly as a result of reduced exposure to plant based antioxidants[41].

1.2.2 Gastric cardia adenocarcinoma

The gastric cardia is the most proximal region of the stomach adjoining the oesophagus. Its mucosa is similar to that of the gastric antrum. It is columnar in type, possessing mucus secreting glands but very few acid-secreting parietal cells or pepsin secreting chief cells. As with oesophageal adenocarcinoma, gastric cardia adenocarcinoma demonstrates a strong association with male gender[42, 43] but a similar proportional increase in both sexes[15] in recent years. A 3:1 male preponderance has been observed in Scotland.

Symptomatic gastro-oesophageal reflux disease is associated with adenocarcinoma of the gastric cardia, but the association is not as strong as that between reflux and oesophageal adenocarcinoma. Case control[22] and population based cohort studies[24] from Sweden have demonstrated a 2-3 fold increased risk of gastric cardia cancer for subjects with frequent reflux symptoms.

As with oesophageal carcinoma, obesity is a strong and independent risk factor for gastric cardia cancer. A large case control study from Sweden demonstrated that obesity conferred an increased risk of gastric cardia cancer, with odds ratio of 4.3 [29]. Being overweight is associated with increased risk of both oesophageal and cardia cancer, an association which does not exist for non-cardia gastric cancer[44].

The relationship between *Helicobacter pylori* and gastric cardia adenocarcinoma is complex. A recent meta-analysis showed that the prevalence of *H pylori* in patients with cardia cancer was the same as in controls[45]. However, the results appear to vary with the geographical region of the study. In Eastern countries, there is a trend for a positive association between *H pylori* and cardia cancer. However, in Western countries, there is a strong trend for a negative

association. A recently published nested case control study from Norway examined the relationship between *H. pylori* status, gastric atrophy and adenocarcinoma from different regions within the stomach[46]. As expected, non cardia cancer showed a positive association with *H. pylori* infection and gastric atrophy. Cardia cancer was negatively associated with *H. pylori*, but *H. pylori*-positive cardia cancer showed a strong relationship with gastric atrophy. These findings indicate that there are two distinct aetiologies of cardia cancer[47]. In Eastern countries, where *H. pylori* infection and non-cardia gastric cancer are common, cardia cancer is more likely to be related to *H. pylori* infection and atrophy. However in the West, where the incidence of oesophageal adenocarcinoma is rising and the prevalence of *H pylori* infection falling, cardia cancer is more likely to be associated with the oesophageal adenocarcinoma phenotype with no atrophy and normal gastric acid secretion. The increasing incidence of cardia and oesophageal adenocarcinoma in Western countries suggests a common pathogenesis.

Gastric cardia cancer, like oesophageal adenocarcinoma, is positively associated with cigarette smoking and is not associated with alcohol consumption[38]. Some studies have indicated that cardia cancer is associated with low consumption of fruit and vegetables[39, 40]. However, interestingly, one case control study from Sweden noted that, although incidence was lowest in the patients in the highest quartile for vegetable consumption, patients with moderate vegetable consumption had a higher risk of cardia cancer compared with the lowest quartile[40].

1.2.3 Effect of recognised risk factors on changing epidemiology

For any associated risk factor to be demonstrated as causal would require the incidence of the risk factor to be rising in a compatible epidemiological fashion to the incidence of the disease. None of the risk factors discussed above adequately explain the rapidly increasing incidence of oesophageal adenocarcinoma[17].

If gastro-oesophageal reflux were the main reason for the increasing incidence of these cancers then a similar increase in the incidence of reflux disease would have been expected in recent decades. The only available measure of the occurrence of reflux is from prevalence figures. Population based studies have shown no clear signs of increasing prevalence in later studies as compared with earlier ones[48-50]. If the incidence of reflux is rising, then it is likely to be due to a further, as yet unexplained, environmental factor.

Although the prevalence of obesity has undoubtedly increased in the Western world in recent years[51], the apparent sudden deflection in the incidence curve for adenocarcinoma, the rapidity of the increase, and the marked male preponderance are not consistent with obesity being the primary cause of these cancers[3, 52]. Although obesity is associated with an increased risk of many cancers, another interesting interpretation may be that as obese subjects consume more food *per se*, they are consuming greater quantities of an ingested mutagen than non obese subjects.

Tobacco smoking and lack of fruit and vegetable intake, although associated, are unlikely to be causal given the prevalence of smoking is falling

and the ingestion of fruit and vegetables increasing in the regions of the world with the highest incidence of these cancers.

An interesting observation is the association between social class and cancer risk. Whereas non cardia gastric cancer and squamous carcinoma of the oesophagus are correlated with increasing levels of deprivation, the association between social class and adenocarcinoma of the oesophagus and gastric cardia is more controversial. A case control study from Sweden found oesophageal adenocarcinoma to be correlated with declining socio-economic status, but the risk was attenuated by controlling for reflux symptoms and tobacco consumption[53]. In contrast, an analysis of population based cancer registry data from the West Midlands region of the UK showed that a higher proportion of male cases of adenocarcinoma of the oesophagus and gastric cardia were classified as higher social class (based on occupation) compared with other sites of gastric cancer, and to all sites of cancer combined[13]. In Scotland, the influence of social class was examined by using postcode as a marker. No correlation was found between risk of junctional adenocarcinoma and social class whereas non cardia gastric cancer and squamous oesophageal cancer were strongly associated with deprivation[43]. However, as this data was not adjusted for smoking (which is associated with deprivation in Scotland[54]) it is possible that the true risk is inversely associated with deprivation.

The suggestion that the increase in incidence of these cancers is secondary to the declining prevalence of *H. pylori* in the Western world is interesting but requires further study. If this explanation is correct, it could help to explain the tendency towards the lower incidence of adenocarcinoma of the gastric cardia and oesophagus among people living in more deprived communities in Scotland.

The next section will discuss pathophysiological similarities between oesophageal adenocarcinoma and gastric cardia cancer and also describe how these regions may be a ripe target for an ingested environmental mutagen.

1.3 Similarities between oesophageal adenocarcinoma and gastric cardia cancer

Gastro-oesophageal junction cancer appears to arise from foci of intestinal metaplasia that develop within the distal oesophagus or proximal stomach. The presence of intestinal metaplasia in the distal oesophagus is described as Barrett's oesophagus. As discussed above, Barrett's oesophagus is the strongest risk factor for oesophageal adenocarcinoma and develops in 6-12% of patients with longstanding reflux symptoms [55-57]. The length of the columnar segment is related to the severity of the reflux disease, which is typically present for 10-12 years before metaplasia develops. Long segment Barrett's oesophagus (>3cm) is present in 5% of patients with reflux symptoms undergoing upper GI endoscopy [58]. Interestingly, 17% of asymptomatic white male US veterans with age >50 were noted to have short segment Barrett's oesophagus (<3cm) on screening endoscopy [59].

Intestinal metaplasia originates from tissue that has been subject to inflammation. In the oesophagus, it occurs as a result of acid reflux. 90% of intestinal metaplasia in the oesophagus is termed histologically "incomplete", and defined as type II or type III on histochemical staining for mucin[60-62]. Intestinal metaplasia in this location, or Barrett's oesophagus, confers a 10-30 fold increased relative risk of neoplasia[63] and almost all oesophageal adenocarcinomas arise within tissue which has undergone a sequence of histological changes

initiated by inflammation – progressing from intestinal metaplasia to dysplasia and finally to adenocarcinoma[64].

In the distal stomach, intestinal metaplasia is associated with *H. pylori* infection. It is histologically "complete", possessing more features found in small intestinal epithelium and more likely be subtype I on histochemical staining for mucin. In this region, intestinal metaplasia confers a 2-3 fold increased relative risk of neoplasia [65], much lower than in the oesophagus. Histochemical staining of intestinal metaplasia at the gastric cardia has revealed "incomplete" metaplasia in 50% of cases[62]. This indicates that a proportion of metaplasia at this site is similar to the variety associated with *H pylori* infection found more distally within the stomach and a proportion is similar to the variety associated with gastro-oesophageal reflux which is found within the oesophagus.

In contrast to oesophageal adenocarcinoma, cancers of the distal stomach comprise two distinct histological groups. The first, or "intestinal type", develops via intestinal metaplasia and dysplasia in a similar fashion to oesophageal adenocarcinoma and contributes to approximately two thirds of non-cardia gastric cancers. Like oesophageal adenocarcinoma, incidence is strongly associated with increasing age, male sex and chronic inflammation (related to *H pylori* infection and gastric atrophy). The second histological subtype, "diffuse" gastric cancer, is relatively more common in females, has a weaker association with increasing age, and often has a strong familial tendency. Cardia cancer in the Western world is much more likely to be of the "intestinal" subtype[46] than the diffuse subtype. This observation, along with its negative association with *H pylori* infection and gastric atrophy in these geographical areas, indicates that the predominant phenotype of

cardia cancer in Europe and North America shares a similar pathogenesis to oesophageal adenocarcinoma.

It has been proposed that adenocarcinoma of the gastric cardia arises from two discrete and disparate aetiologies and should therefore be subdivided into two distinct phenotypes[47]. Type A comprises those cancers that are associated with *H pylori* infection, causing atrophy and intestinal metaplasia and is more prevalent in Eastern countries and the developing world. Type B comprises those cancers that are inversely associated with *H. pylori* infection and are aetiologically more similar to oesophageal adenocarcinoma, demonstrating a strong male preponderance and an association with reflux disease. This subtype is more prevalent in the Western world and its incidence is rising.

1.4 Influence of acid reflux on the location of junctional cancers

Oesophageal adenocarcinoma and a subtype of gastric cardia adenocarcinoma appear to be aetiologically related, with the former arising within intestinal metaplasia within the oesophagus (Barrett's oesophagus) and the latter arising at the oesophago-gastric junction. These sites of mutagenesis correspond with the anatomical site where the neutral pH of the oesophageal lumen meets with the acidic pH of the gastric lumen. In patients with gastro-oesophageal reflux, this interface occurs within the oesophagus and in patients without reflux it occurs within the most proximal cardia region of the stomach. This is consistent with the observation that both oesophageal adenocarcinoma and gastric cardia adenocarcinoma in the Western world are significantly associated with gastro-oesophageal reflux, although the association for gastric cardia cancer is weaker. Interestingly, it has been demonstrated that the most distal oesophageal mucosa is

frequently exposed to acidic gastric juice in healthy volunteers without reflux symptoms[66]. The proportion of cardia cancers arising from this short segment reflux may therefore be substantially higher than the association between these cancers and reflux symptoms would suggest.

A unifying concept explaining the location of all of these adenocarcinomas which are increasing in incidence is that they occur at the anatomical site where saliva of neutral pH meets acidic gastric juice. This hypothesis is strengthened by the fact that in areas of the world with the highest incidence of gastric cardia cancer (NW Iran, Linxian region of China), the great majority of cancers occur high on the right side of the cardia[67]. This is consistent with the preferential passage of swallowed fluids down the lesser curve, following the 'Magenstrasse' or 'canalis gastricus' formed by the longitudinal mucosal folds [68]. The gastric cardia is an important interface zone as it has been observed that an unbuffered pocket of highly acidic juice resides in this region in the post-prandial state, escaping the buffering effect of food[69]. In healthy volunteers, this area of unbuffered acid is only 2cm long, but in patients with gastro-oesophageal reflux disease it is significantly more extensive and extends more proximally[70].

The observation that adenocarcinoma of the oesophagus and gastric cardia occur where saliva meets gastric acid suggests that the increasing incidence of these malignancies in Western Europe and North America is occurring as a result of salivary carriage of increasing amounts of an ingested luminal mutagen.

CHAPTER TWO

THE POTENTIAL ROLE OF DIETARY NITRATE IN THE INCREASING INCIDENCE OF ADENOCARCINOMA AT THE GASTRO-OESOPHAGEAL JUNCTION

2.1 Dietary nitrate as a potential mutagen at the gastro-oesophageal junction

2.1.1 Trends in dietary nitrate consumption

Epidemiological observations have long indicated that 'diet' plays a major role in human carcinogenesis [71]. For many years, there has been interest in the potential to form carcinogenic *N*-nitrosocompounds within the human upper gastro-intestinal tract. There is an epidemiological association between the increased incidence of adenocarcinoma at the gastro-oesophageal junction and increased nitrate consumption. The modern diet contains substantial quantities of nitrate, mainly derived from nitrogenous fertilisers [72]. Nitrate is an essential plant nutrient and one of the main forms in which plants take up nitrogen from the soil. After uptake from the soil by plant roots, nitrate is reduced to the ammonium ion (NH_4^+) and then incorporated rapidly into organic compounds by the plant.

In cooler, temperate zones (such as the United Kingdom), the growing of vegetables in low light intensity/high temperature glass houses leads to much higher nitrate concentrations than in vegetables grown in natural sunlight [73]. Groundwater nitrate concentrations are gradually increased through leaching of fertiliser. After 1945, with increased intensive farming, nitrogen fertiliser use increased substantially. Figure 2.1 depicts nitrate fertiliser use in the UK from 1960 to 2002. The main sources of nitrate in the human diet are green leafy vegetables (such as lettuce and spinach), root vegetables (such as beetroot and potatoes) and water.

The established association between nitrate and infantile methaemoglobinaemia and the possible association between nitrate and gastric cancer led to governmental concern over the impact of increased population

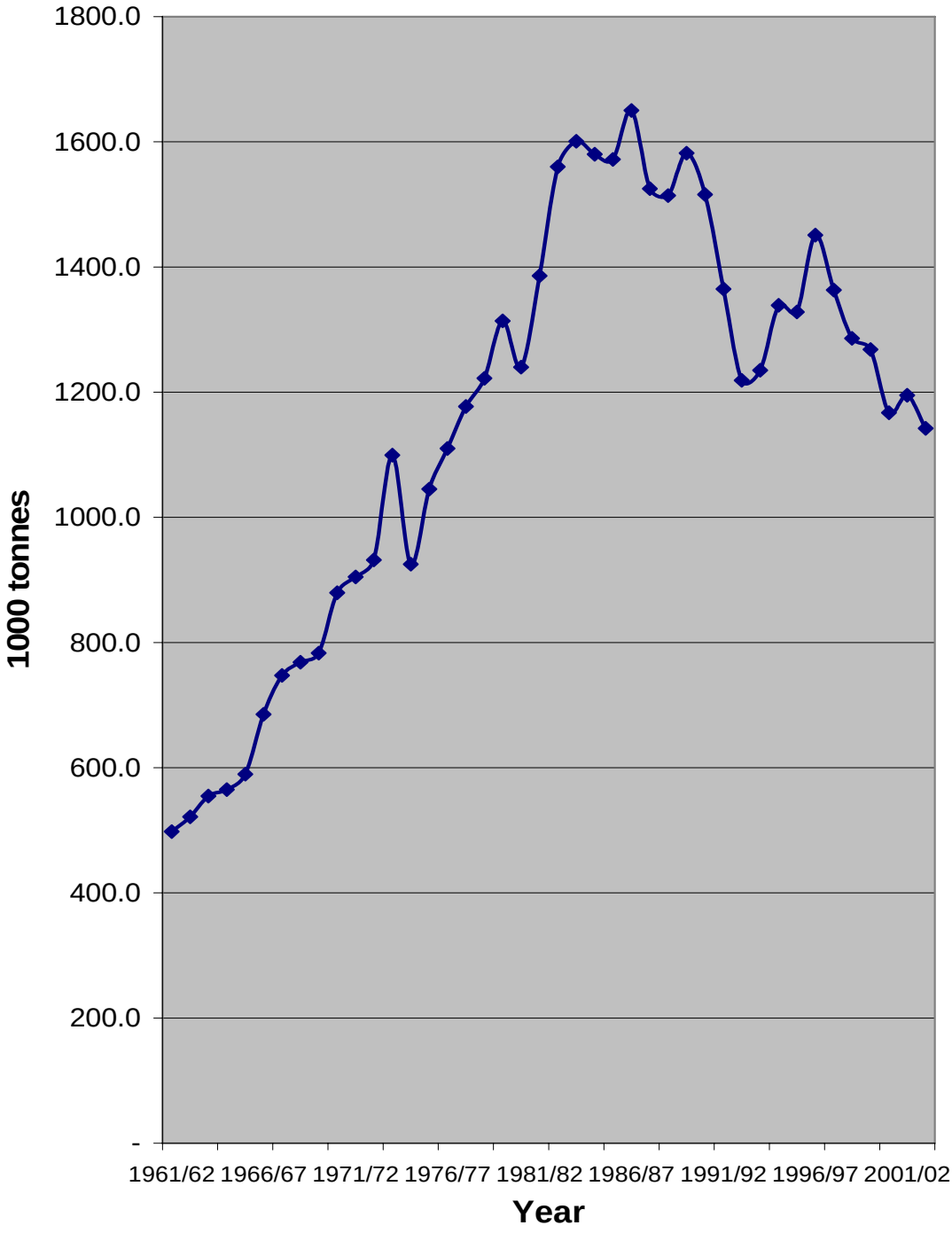
nitrate consumption. This resulted in a series of regulations being passed from 1970 through to 1991 aiming to reduce nitrogen fertiliser use and limit nitrate concentrations in food and water. The European Commission's (EC) Scientific Committee for Food (SCF) considered the implications for human health of nitrate and nitrite in food in 1990 and set an Acceptable Daily Intake (ADI) for the nitrate ion of 3.65 mg/kg body weight (equivalent to 219 mg/day for a 60 kg adult). [74]

Although nitrate fertiliser use has remained relatively stable since the early 1980s, the 30 year gap often observed between environmental mutagen and disease means that we may not yet have reached the peak incidence for any diseases whose incidences have been influenced by increased nitrate ingestion.

Figure 2.1: Nitrate fertiliser consumption in the UK 1961-2002

Source: International Fertiliser Industry Association

www.fertilizer.org/ifa/statistics/IFADATA/dataline.asp



2.1.2 Association between *N*-nitrosocompounds and carcinogenesis

For many years, luminal nitrite has been recognised as a potential carcinogen. This is due to its ability to be converted to carcinogenic *N*-nitrosocompounds under conditions present in the human upper GI tract. Interest in N-nitrosocompounds was limited to the chemical laboratory and to industrial applications, where secondary amines were used as a solvent in the rubber industry until, during investigation of the hepatotoxicity suffered by workers in the rubber industry[75], it was noticed that *N*-nitrosodimethylamine was a potent carcinogen in rodents[76]. Further studies revealed that while a wide range of *N*-nitrosocompounds were carcinogenic, the target organ varied depending on the type of *N*-nitrosamine and species of animal[77]. The discovery that this group of chemicals readily promoted carcinogenesis led many investigators to utilise *N*-nitrosocompounds for the induction of tumours in animals in studies of aetiology, rate of growth and treatment of neoplasia.

N-nitrosocompounds are potent inducers of oesophageal and gastric cancer in laboratory animals [78]. The effect of reflux on carcinogenesis associated with preformed *N*-nitrosocompounds was examined by Atwood and colleagues, who performed oesophago-gastroplasty in 20 rats and oesophago-duodenal anastomosis in a further 60 before exposing each group to *N*-nitrosamines[79]. Rats with duodeno-oesophageal reflux had an increased rate of oesophageal carcinoma. Furthermore, the type of refluxate altered the histological type of cancer, with duodenal reflux promoting development of adenocarcinomas as compared with squamous carcinomas in rats with the oesophagogastroplasty. These studies show that exposing the rat oesophagus to preformed *N*-nitrosamines and refluxed

duodenal contents promotes development of adenocarcinoma. This suggests that a similar mode of carcinogenesis may occur in humans with reflux of duodenal contents into the oesophagus. Bile and other duodenal juices have been detected in the oesophagus of patients with severe reflux disease and Barrett's oesophagus. Although ingestion of preformed *N*-nitroso compounds is rare, these compounds can be generated by reaction of secondary nitrosatable amino compounds with nitrite under the correct chemical conditions. The main source of nitrite in the human upper gastrointestinal tract is swallowed saliva, and its high nitrite content is derived from dietary nitrate.

Humans can be exposed to preformed *N*-nitrosocompounds and to *N*-nitrosocompounds produced *in vivo*. This *in vivo* formation can occur by acid catalysed and bacterial nitrosation in the stomach and via nitric oxide formation during inflammation [80]. Recently, a novel mechanism for the formation of *N*-nitrosocompounds via intra-luminal nitric oxide has been described, which may be of importance in the pathogenesis of gastro-oesophageal junction cancers [81].

N-nitrosocompounds have the potential to induce mutagenesis by their ability to alkylate human DNA. Tobacco specific *N*-nitrosocompounds have been implicated in the pathogenesis of cancers of the bronchus, oral mucosa and squamous oesophagus in tobacco users. Oral cancer is associated with *N*-nitrosamines in chewing tobacco[82, 83] and betel quids. The high incidence of bladder carcinoma in rubber factor workers has been associated with the high concentrations of volatile *N*-nitrosocompounds in the air of rubber factories [84].

2.1.3 Mechanisms of *in-vivo* Nitrosation

The first clear demonstration that humans can form *N*-nitrosocompounds *in vivo* was made in 1981, when it was shown that human ingestion of nitrate and L-proline resulted in measurement of significant concentrations of *N*-nitrosoproline in urine [85]. *N*-nitrosoproline was formed by gastric nitrosation of L-proline, with subsequent absorption and excretion in the urine. Formation of this non-carcinogenic *N*-nitrosamine was inhibited by ingestion of the anti-oxidants ascorbic acid and alpha-tocopherol.

Nitrite is an essential substrate for *in vivo* nitrosation. The main route of entry to the body is via the stomach, making this organ a prime candidate for *N*-nitroso compound mediated mutagenesis. 80% of nitrite entering the normal, acid producing stomach arises from the reduction of ingested or endogenous nitrate via the entero-salivary circulation. After dietary nitrate is absorbed, about 25% is actively secreted into the mouth by the salivary glands [86]. Bacteria on the dorsum of the tongue then convert 10-90% of this nitrate to nitrite [87, 88], producing high concentrations of nitrite in saliva. The remaining 20% of gastric nitrite arises from ingested nitrite in nitrite-preserved meat and fish and other foods [89].

Formation of *N*-nitrosocompounds may occur via acid nitrosation, bacterial nitrosation or via nitric oxide. The former two mechanisms are observed to occur following ingestion of exogenous nitrate and nitrite. Until recently, nitric oxide-mediated nitrosation was perceived to occur only from endogenous substrate in the context of inflammation.

Acidification of nitrite leads to the formation of nitrous acid, a precursor to the formation of nitrosating species. Nitrous acid dimerizes with loss of water to form N_2O_3 , and can be protonated to form $H_2NO_2^+$. Because of the acidification

step, these reactions are most likely to take place in the human stomach. A further nitrosating species in this organ is nitrosothiocyanate (NOSCN), formed by reaction of acidified nitrite with thiocyanate, which is present in high concentrations in saliva [90, 91] and increased by smoking.

Bacterial nitrosation proceeds at neutral pH and is most likely to take place within the achlorhydric stomach, as can occur in *Helicobacter pylori* mediated gastric atrophy. Ascorbic acid, an aqueous anti-oxidant, is actively secreted into gastric juice by healthy gastric mucosa and inhibits the potential for both acid and bacterially mediated nitrosation [92]. However, ascorbic acid levels are reduced in hypochlorhydric gastric juice [93], therefore increasing potential for nitrosation. Acid and bacterial [94] nitrosation have been implicated in the pathogenesis of intestinal type gastric carcinoma, which manifests in patients with hypochlorhydria, low ascorbic acid levels and chronic inflammation with intestinal metaplasia.

2.1.4 Nitric oxide-mediated nitrosation in inflammation

Endogenous nitric oxide (NO) is produced by the oxidation of an omega nitrogen of the amino acid arginine by nitric oxide synthase enzymes. This NO serves as a vasodilator and as a neurotransmitter but the concentrations produced for these functions are far less than those formed during the oxidative burst produced by macrophages and neutrophils during inflammation. NO reacts with dissolved oxygen to form nitrosating species. These then react with water at neutral pH to produce nitrite and with secondary amines and amides to form *N*-nitrosocompounds. These reactions occur near the site of NO production.

Chronic inflammation is associated with the pathogenesis of many malignancies, including colorectal carcinoma in ulcerative colitis, bladder carcinoma in bilharzia (schistosomiasis), hepatocellular carcinoma in chronic viral hepatitis and gastric carcinoma in chronic *H* pylori infection. These associations may be partially due to NO formation [95]. There are at least three mechanisms by which intracellular elevated NO could exert genotoxic affects after reacting with O_2. These include direct deamination of DNA bases, oxidation of DNA after formation of peroxynitrite and/or hydroxyl radicals and through generation of carcinogenic N-nitroso compounds. Synthesis of *N*-nitrosocompounds by activated human neutrophils via inducible nitric oxide synthesis has been demonstrated[96]. One or more of these mechanisms could, theoretically, explain why chronic infection increases the risk of certain cancers [97]

2.1.5 Nitric oxide-mediated nitrosation at the gastro-oesophageal junction

In recent years, it has been observed that the highest concentrations of nitric oxide occurring in the body are not the result of enzymatic synthesis but caused by chemical formation within the lumen of the stomach [98, 99]. This arises from the entero-salivary circulation of dietary nitrate. As described above, bacteria on the dorsum of the tongue reduce the nitrate actively secreted into the oral cavity to nitrite. This nitrite is then swallowed. On meeting gastric acid, nitrous acid is formed, which dissociates to form the nitrosating species.

Ingestion of a nitrate containing meal leads to elevated concentrations of nitric oxide within the gastric lumen [100-102], accompanied by a fall in gastric ascorbic acid concentration [103]. This can be explained by reduction of nitrosating species to nitric oxide by ascorbic acid, with its concurrent oxidation. *In vitro*

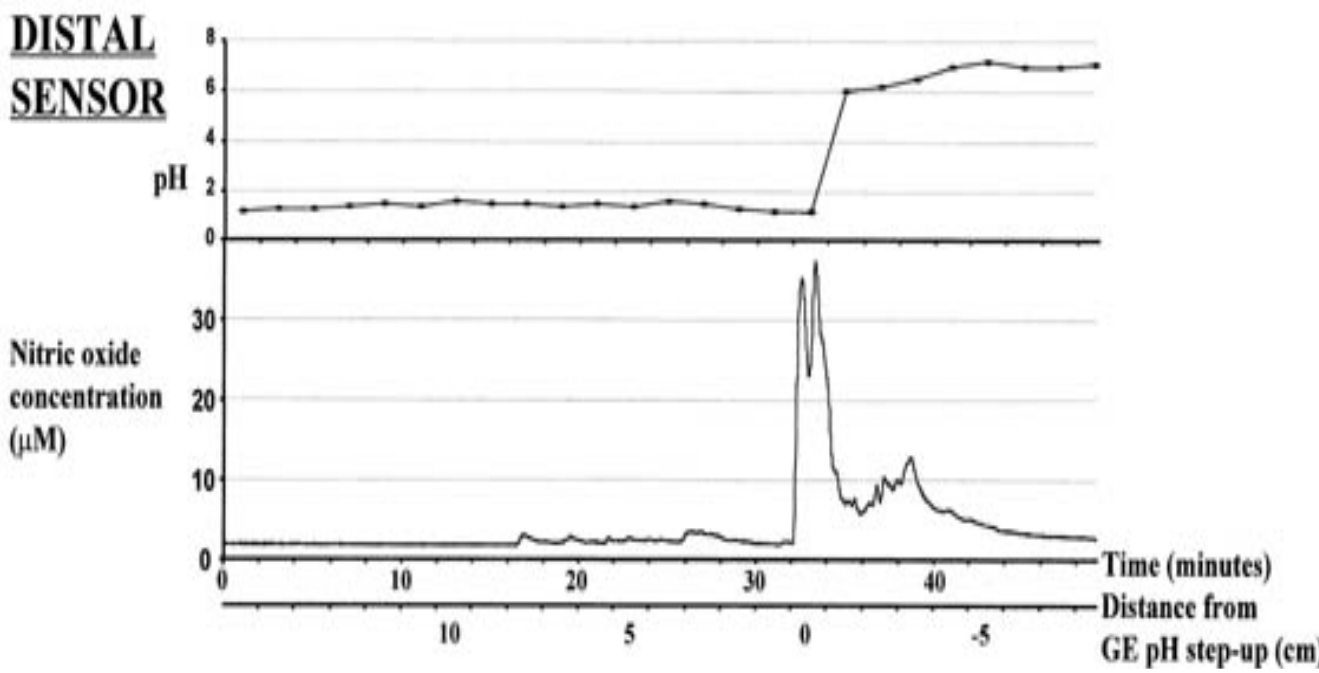
studies using a chemical stomach model have confirmed this mechanism [104], and have revealed that the reaction between the nitrite and ascorbic acid is extremely rapid, causing most of the nitrite to be converted to nitric oxide within a few seconds. This suggested that the production of nitric oxide from dietary nitrite would be most evident at the gastro-oesophageal junction, where saliva containing nitrite and gastric juice first meet.

This hypothesis was confirmed in 2002, when Iijima and colleagues [105] measured luminal pH and dissolved nitric oxide concentrations at 1cm intervals throughout the length of the stomach and distal oesophagus in 15 healthy human volunteers with and without ingestion of 2mmol potassium nitrate. Peak nitric oxide concentrations occurred within 1cm distal to the pH step up point. In 75% of subjects mean nitric oxide concentrations at the gastric cardia were significantly greater than at all other sites. A combined pH and nitric oxide tracing from one individual is shown in figure 2.2.

In patients with gastro-oesophageal reflux disease, the anatomical location where saliva meets gastric acid is different to normal subjects. In these individuals, acid and nitrite-rich saliva meet within the distal oesophagus and intra-luminal production of nitric oxide has been shown to occur at that site [106].

Figure 2.2: Tracing obtained in a single individual while a pH/nitric oxide sensor is withdrawn from the distal stomach into the oesophagus at 1cm increments every 2 minutes. A rise in nitric oxide concentration is seen on reaching the gastro-oesophageal pH step-up.

From : Iijima K, Henry E, Moriya A, Wirz A, Kelman AW, McColl KE.
Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastro-oesophageal junction. Gastroenterology 2002;122:1248-57.



2.1.6 Epidemiological associations between ingestion of nitrate and nitrate-rich foods and gastro-oesophageal cancer

Although the temporal association between increased nitrate consumption and the rising incidence of adenocarcinoma of the gastro-oesophageal junction is intriguing, there is currently no hard epidemiological evidence to substantiate this. Despite the observation that *N*-nitrosocompounds induce tumours in more than 40 animal species[107], there is no conclusive evidence that they are carcinogenic to humans. Two *N*-nitrosamines (*N*-nitrosodimethylamine and *N*-nitrosodiethylamine) are classified as probably carcinogenic by the International Agency for Research on Cancer[108]. Several studies have analysed the association between nitrates, nitrites and preformed *N*-nitrosocompound intake. One recent review found that available evidence supported a positive association between nitrite and nitrosamine intake and gastric cancer, but not conclusively so[108].

There are few epidemiological studies examining the association between nitrate intake and upper gastrointestinal cancer. Even fewer have controlled for the influence of *Helicobacter pylori* or have analysed oesophageal squamous carcinoma and adenocarcinoma separately. In the Shangdong Peninsula in north-eastern China, very high drinking water nitrate concentrations (from local wells) were correlated with the incidences of pre-malignant gastric lesions and gastric cancer[109]. In the Western world, where the principle source of ingested nitrate is from vegetables[110] and drinking water nitrate content is lower, no such association has been demonstrated between drinking water nitrate and upper gastrointestinal cancer incidence[111-114]. A large epidemiological study conducted across many countries used 24 hour urinary nitrate levels as a surrogate for nitrate intake and

demonstrated a strong correlation between urinary nitrate levels and mortality from gastric cancer[115]. However, it was suggested that salt intake was the rate limiting factor for stomach cancer mortality and that nitrate appeared to be only important with higher salt intake levels.

The association between nitrate intake and gastric cancer has been investigated by cohort studies from Western Europe[116, 117]. Taken together, these studies indicate a trend towards a protective effect with increasing nitrate intake and this appears to be attributable to nitrate intake from food rather than from drinking water. These findings are likely to reflect the protective effect of vegetable consumption against risk of non-cardia gastric cancer risk. The different subgroups of gastric cancer have not been studied separately and the influence of nitrate consumption on risk of oesophageal adenocarcinoma has not been investigated.

A further conceptual problem in associating the increasing incidence of junctional adenocarcinoma with nitrate consumption in the Western world is the fact that most nitrate ingested comes from dietary sources[110] and the greatest dietary source is vegetables, which are generally considered to have a protective effect against the occurrence of cancers of the aero-digestive tract[118]. However, recent epidemiological studies have cast doubt on this hypothesis. A comprehensive meta-analysis from 2003[119] included 1 cohort study (from China) and 12 case control studies which examined the association between fruit and vegetable intake and oesophageal carcinoma. The different histological subgroups (squamous and adenocarcinoma) were not differentiated. The case control studies from Europe and the USA indicated a significant protective effect for fruit but not for vegetables. 6 cohort studies and 14 case control studies were identified which

examined association between fruit and vegetable intake and gastric cancer incidence. The different regions of cancer occurrence (cardia and non-cardia) were not differentiated and there appeared to be a significant protective effect from higher fruit and vegetable intake, which was stronger for case control than for cohort studies.

Subsequent to the above meta-analysis, two large cohort studies have been published which have examined the association between fruit and vegetable intake and cancers of the oesophagus and stomach. The EPIC-EUROGAST study[120] collected dietary information from over 500,000 subjects in 10 European countries and data on oesophageal and gastric cancer incidence has now been reported after an average of 6.5 years follow up. This study has concentrated on oesophageal adenocarcinoma and has differentiated gastric cancer according to histological type (intestinal or diffuse) and region (cardia and non-cardia). Although there did appear to be a non-significant protective effect against oesophageal adenocarcinoma in those with the highest vegetable intake, overall there was no evidence of a significant association between vegetable intake and risk of gastric or oesophageal adenocarcinoma. This lack of association may be due to the relatively short period of follow up and to the small number of cancers identified in the follow up period (330 gastric cancers and 65 oesophageal cancers). The NIH-AARP Diet and Health study[121] examined dietary influences on oesophageal carcinoma in the USA following a similar design and provides the best prospective data on association currently. The subjects recruited were generally older (> 50 years at recruitment) and were followed up for a median of 5 years. A greater number of oesophageal cancers were identified in this study than in the European cohort (213 adenocarcinomas). Higher fruit intake, but not

vegetable intake, conferred a significant protective effect against development of oesophageal squamous cancer. No protective effect was seen for vegetables against development of either histological subgroup of cancer.

In summary, although there currently is a lack of epidemiological evidence suggesting that nitrate intake is associated with increased risk of adenocarcinoma of the gastro-oesophageal junction, recent prospective studies cast doubt on the previously held view that higher vegetable intake protects against development of these cancers in Western populations. These observations may be prejudiced by under-powering due to the relatively short follow up periods in the large cohort studies, resulting in small numbers of the cancers of interest being reported to date. However, as the published data currently stands, the potential influence of increasing nitrate consumption on the rising incidence of these tumours cannot presently be dismissed.

2.2. Detailed studies of nitrosation at the gastro-oesophageal junction

2.2.1 Bench-top model of luminal chemistry at gastro-oesophageal junction

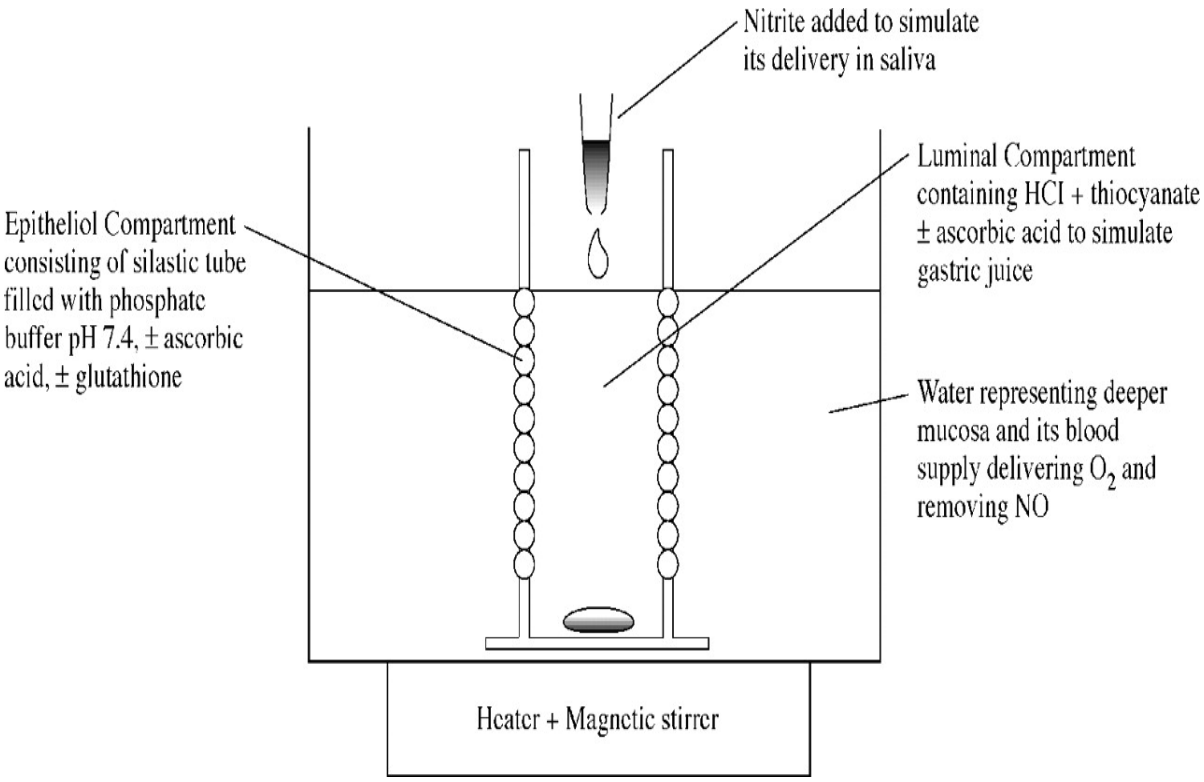
Given that the highest concentrations of nitric oxide measured anywhere in the human body are produced chemically where saliva meets gastric acid, it seems likely that this anatomical location is a target for nitric oxide-mediated nitrosative stress.

To investigate this further, our group developed a bench-top model re-creating the chemical conditions present at the human gastro-oesophageal junction[81]. A transparent plastic cylinder was filled with 20ml of hydrochloric acid pH 1.5 to represent the gastric lumen. Vertical fenestrations were cut into the walls of the cylinder and silastic tubing with internal diameter of 500 um and wall thickness 250um was wound around the cylinder in a single layer, completely occluding the fenestrations (figure 2.3). The silastic tube contained phosphate buffer pH 7.4 to represent the epithelial compartment. The chemical characteristics of silastic closely resemble those of lipid membranes, allowing the passage of gases such as nitric oxide and oxygen, but not that of hydrogen ions [122-124].

Figure 2.3: Diagrammatical cross-section of the bench-top model used to study nitrosative chemistry at the oesophago-gastric junction.

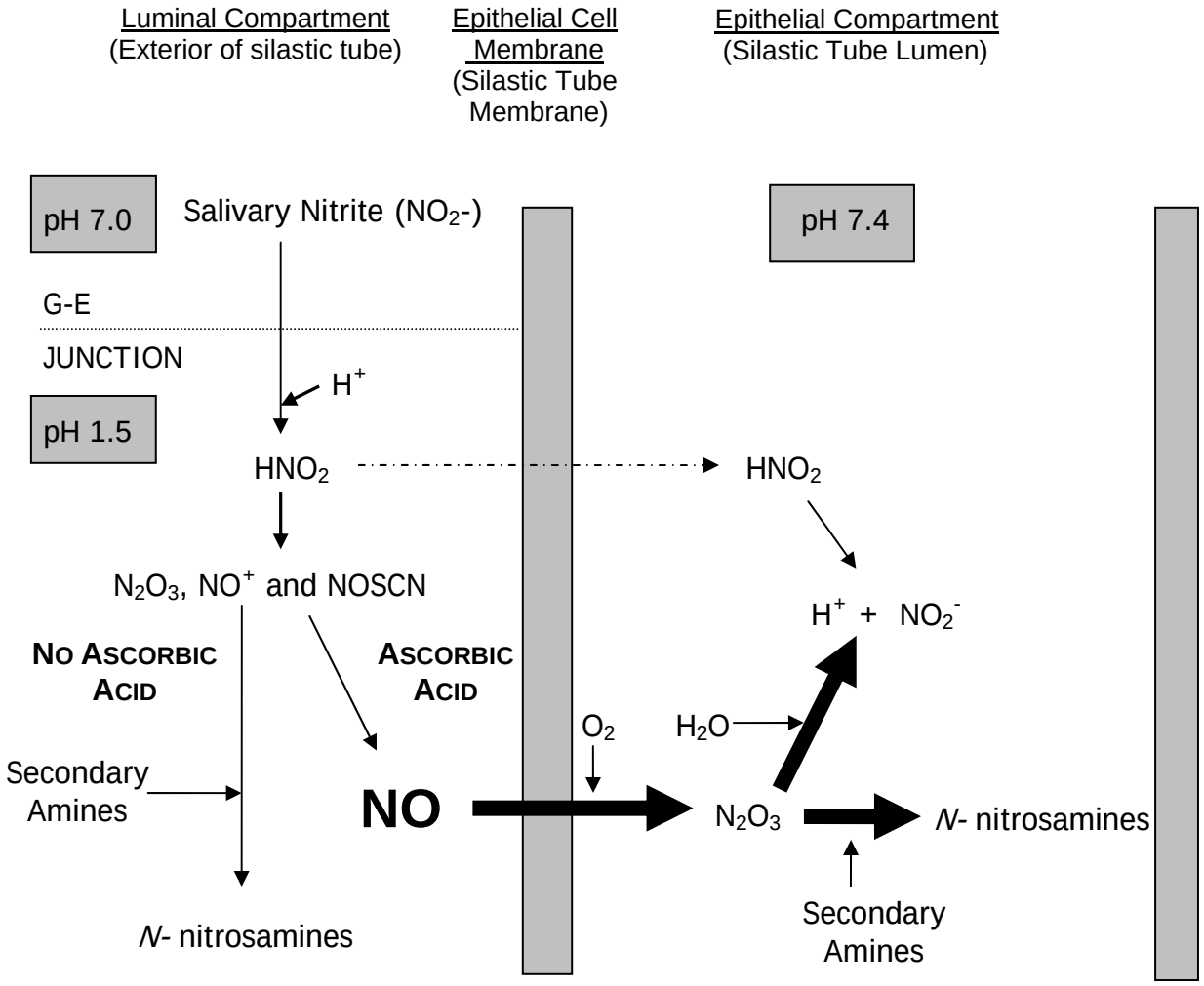
From: Iijima K, Grant J, McElroy K, Fyfe V, Preston T, McColl KE.

Novel mechanism of nitrosative stress from dietary nitrate with relevance to gastro-oesophageal junction cancers. Carcinogenesis 2003;24:1951-60.



The exterior surface of the silastic tube was exposed to the chemistry occurring in the lumen of the gastro-esophageal junction when saliva meets acidic gastric juice, and this we refer to as the luminal compartment. The secondary amine, morpholine, was added to both the luminal and epithelial compartments. When the luminal compartment contained nitrite and hydrochloric acid, there was formation of *N*-nitrosomorpholine only in the luminal compartment. The further addition of ascorbic acid to the luminal compartment transformed the chemistry in both compartments - preventing *N*-nitrosomorpholine generation in the luminal compartment, generating high concentrations of nitric oxide in the luminal compartment and high concentrations of *N*-nitrosomorpholine in the adjacent epithelial compartment. The findings are explained by the nitric oxide generated by the reaction between acidified nitrite and ascorbic acid diffusing into the epithelial compartment and within it reacting with oxygen to form N_2O_3, which was nitrosating the morpholine. Nitrite was formed within the epithelial compartment by two mechanisms. The first and major mechanism is via nitric oxide. The N_2O_3, formed by nitric oxide within the membrane reacts with water to form nitrite ($N_2O_3 + H_2O \dashrightarrow 2\ NO_2^- + 2\ H^+$). The second mechanism by which nitrite may accumulate within the tube is due to nitrous acid diffusing through the wall of the tube (Figure 7). The addition of ascorbic acid or glutathione to the epithelial compartment could only reduce the generation of *N*-nitrosomorpholine by a maximum of 40%. The silastic tube filled with phosphate buffer thus provides a means of measuring *in-situ N*-nitrosation arising from luminally generated nitric oxide and within a chemical environment resembling epithelial cells.

Figure 2.4: Chemical reactions occurring when nitrite enters acidic gastric juice. Nitrite is converted to nitrous acid and nitrosating species (N_2O_3, NO+ and NOSCN). In the absence of ascorbic acid, these can react with nitrosatable species to form *N*-nitrosocompounds in the luminal compartment. Ascorbic acid in the gastric juice prevents this luminal nitrosation by converting the nitrosating species to nitric oxide. However, nitric oxide can diffuse into the epithelial compartment, reacting with oxygen to form the nitrosating species N_2O_3 which exerts nitrosative stress within the epithelial compartment. Nitrite is formed in the epithelial compartment predominantly by reaction of N_2O_3 with water, but also by diffusion of nitrous acid (NO_2).



2.2.2 Two potential mechanisms for nitrosative stress at the gastro-oesophageal junction

Delivery of nitrite-rich saliva to the oesophago-gastric junction can potentially lead to mutagenesis at this site by two distinct mechanisms: via acid nitrosation in the lumen and via nitric oxide-mediated nitrosation in the epithelium

2.2.2.1 Acid Nitrosation in the Lumen

A validated technique using microdialysis probes[125] was used to measure chemicals involved in nitrosation within the oesophagus, cardia and proximal and distal stomach of 17 healthy volunteers before and after administration of 2mmoles nitrate intra-gastrically[126]. Before nitrate administration, the lowest concentrations of ascorbic acid were detected at the gastric cardia. The reason for this is unclear. However, ascorbic present within fasting gastric juice is believed to be actively secreted by the gastric mucosa[93, 127] and, although the cells responsible for secreting ascorbic acid are unknown, biopsies from different regions of the stomach have shown that mucosal levels of vitamin C are highest in the antrum[128].

Following nitrate administration, luminal nitrite concentrations increased significantly within the cardia but not within the proximal and distal stomach. The lowest ascorbic acid concentrations were again measured at the cardia. This region had the lowest median ascorbate: nitrite ratio before and after administration of nitrate and the ratio fell after nitrate administration. Therefore the most favourable conditions for luminal acid nitrosation were present at the gastric cardia.

Gastro-oesophageal reflux was stimulated in ten patients with Barrett's oesophagus, who were then studied in identical fashion[106]. During acid reflux, the Barrett's segment of the oesophagus had the lowest ascorbate:nitrite ratio and therefore was the region with greatest potential for acid mediated nitrosation within the lumen.

It seems likely that, in the absence of reflux, the proximal cardia region of the stomach possesses the lowest ascorbic acid concentrations because it is the region furthest from the site of ascorbic acid secretion. When nitrite present in swallowed saliva meets gastric acid, the nitrite concentration falls rapidly due to reduction of nitrosating species by ascorbic acid to nitric oxide. The cardia therefore has the lowest ascorbate:nitrite ratio in the upper gastrointestinal tract because local ascorbic acid is consumed and some nitrite remains. During reflux, the nitrite meets gastric acid more proximally, within the distal oesophagus, and this region then becomes the site with lowest ascorbate:nitrite ratio. With ingestion of increasing amounts of nitrate, an even lower ascorbate:nitrite ratio is anticipated as higher concentrations of luminal nitrite will remain once the local ascorbic acid has been exhausted.

2.2.2.2 Nitric Oxide-Mediated Nitrosation within the Epithelium

In the bench-top model described by Iijima[81], generation of nitric oxide can lead to nitrosative stress occurring within the epithelium as a result of the nitric oxide diffusing into the epithelial lipid membrane and forming nitrosating species at this site. Epithelial cells contain many compounds (secondary amines, polyamines, amino acids, peptides and amides) which can be *N*-nitrosated to mutagenic products[129-134].

The aim of this thesis is to translate the bench-top studies of nitric oxide nitrosative stress to human subjects, by examining the potential for nitrate to generate *N*-nitroso compounds in the epithelium of the human upper GI tract.

CHAPTER 3

PLAN OF INVESTIGATION

3.1 Hypothesis

Our *in vitro* and *in vivo* studies have generated the hypothesis that:

"The high luminal concentration of nitric oxide generated at the anatomical site where nitrite-laden saliva first encounters acidic gastric juice is exerting nitrosative stress on the adjacent epithelium and thereby contributing to the high incidence of mutagenesis at this location".

This hypothesis is consistent with the epidemiological characteristics of cancer at the gastro-oesophageal junction.

3.2 Aims of the thesis

To test our hypothesis, we aimed to determine whether nitric oxide generated within the human upper gastrointestinal tract from dietary nitrate can lead to *in-situ* generation of nitrosating species and *N*-nitroso compounds within the adjacent epithelial compartment.

The main research questions to be addressed were:

(1) Does the luminal generation of nitric oxide from dietary nitrate exert nitrosative stress and lead to *in-situ* generation of *N*-nitrosocompounds within the adjacent epithelial compartment?

(2) Does the anatomical location of maximal nitrate-induced epithelial nitrosative stress correspond to the locations of high cancer incidence i.e. at the gastric cardia in subjects without acid reflux and within the distal oesophagus in patients with acid reflux?

3.3 Description of proposed investigative method

We proposed to employ a similar epithelial model as in our *in vitro* studies [81]. This epithelial model was to be inserted in the upper gastro-intestinal tract of human subjects, and used to ascertain if nitric oxide derived from ingested nitrate resulted in the *in situ* generation of nitrite and *N*-nitrosocompounds.

The epithelial model was to consist of a silastic tube containing the secondary amine morpholine, buffered at pH 7.4. As described above, the chemical characteristics of silastic are similar to that of the epithelial lipid membrane, allowing passage of small, non-ionic lipid soluble molecules such as nitric oxide (NO) and oxygen (O_2), but not that of hydrogen ions (H^+). To provide information on localised chemistry in the human upper GI tract, the tube required to be partitioned into short segments. In order that we were able to confirm the location of the tube in the upper gastro-intestinal tract, it required to be attached to a multi-channel pH catheter.

We aimed to recruit 20 *Helicobacter pylori*-negative healthy volunteers and 20 *Helicobacter pylori*-negative patients with severe gastro-oesophageal reflux and associated Barrett's oesophagus. The size of the study was based upon results of earlier studies of luminal nitrosative stress performed in our unit, in which nitric oxide production was found to be maximal at the gastro-oesophageal junction using 12 patients at p=0.02. All subjects would attend for investigation on two separate study days. On each visit, the tube was to be inserted into the stomach by the nasal or oral route and remain *in-situ* for two hours after administration of a study drink. Blood and saliva samples were to be collected at

regular intervals for measurement of serum nitrate and salivary nitrite concentrations.

The healthy volunteers were to ingest water containing 2 mmoles of ^{15}N enriched potassium nitrate (50% of the Acceptable Daily Intake for a 70kg human) on one day and water only on the other visit as a control. The patients with Barrett's oesophagus were to ingest water containing 2 mmoles of ^{15}N enriched potassium nitrate on each study day. On one day, we attempted to stimulate acid reflux in the patients with Barrett's oesophagus.

The purpose of this study was to determine (a) whether *N*-nitrosomorpholine is generated within the tube *in situ*; (b) whether the concentration *N*-nitrosomorpholine is higher after nitrate; (c) whether there is ^{15}N enrichment of the N-nitrosomorpholine further confirming that it originated from the ingested nitrate; (d) whether the concentrations of *N*-nitrosomorpholine and nitrite generated within the tube are maximal at the gastric cardia in patients without reflux and within the oesophagus in those with reflux.

Before commencing our studies in humans, we required to perform a number of laboratory experiments to confirm that our apparatus was suitable and our experimental and analytical methods were correct.

CHAPTER 4

DESIGN OF EXPERIMENTAL METHOD FOR STUDIES OF NITROSATIVE CHEMISTRY IN HUMAN SUBJECTS

4.1 Silastic Tube

4.1.1 Appropriate dimensions of silastic tube

We required to adapt the silastic tube which had been employed in our previously published *in vitro* studies[81] for use in human subjects. Before, a long tube with narrow internal diameter (0.5mm) had been used, containing a total fluid volume of 250ul. As we planned to investigate localised *in situ* chemical change in our human studies, we required to partition the tube into multiple sections of equal length and of adequate volume to permit analysis of nitrite and *N*-nitrosomorpholine concentration. To permit analyses of the silastic tube contents, a minimum segmental volume of 50ul was required. This provided 10ul of fluid for measurement of nitrite concentration and 40ul of fluid for analysis of *N*-nitrosomorpholine concentration. We therefore chose to employ silastic tubing (Altec Corporation, Cornwall, UK) with internal diameter of 2.5mm and wall thickness 0.5mm. When partitioned into sections of 15mm length, each section contained a maximum volume of 74ul of fluid, which was adequate for our analyses.

4.1.2 Compartmentalisation of silastic tube

To study localised chemical change in the human upper gastro-intestinal tract, it was important that our tube was divided into multiple compartments of equal size and good integrity. Initially it was realised that in order to obtain compartments with no leakage through to other neighbouring compartments we had to twist the tube along its longitudinal axis at the appropriate length, creating a compressed area of tube of 3-5mm in length. Suture material was then tied at

proximal margin of the compressed section, wrapped around the segment ten times and tied again at the distal margin. Several different materials were tried to tie a suitable knot. Of those tried, 4-0 vicryl suture (Ethicon Corporation, Somerville, New Jersey, USA) was the most suitable as it provided required strength while remaining soft enough to avoid cutting through the silicone wall.

Inter-compartmental integrity was tested both subjectively and objectively: subjectively, by noting that there was no contribution to aspirated volume from adjacent segments when each was drained using a glass syringe; and objectively, by partitioning fluid of varying pH between different sections and observing that pH was maintained after being left for a period of twelve hours.

4.2 Design of experimental method for *N*-nitrosomorpholine sample preparation

4.2.1 Choice of substrate secondary amine

Morpholine is a heterocyclic secondary amine with the chemical formula C_4H_9NO. It is a weak base with pKa of 8.3. It reacts with nitrosating species to form *N*-nitrosomorpholine, a volatile *N*-nitrosamine which can be detected by gas-chromatography and mass spectrometry. Morpholine is a suitable substrate to assess nitrosation in our *in-situ* studies as neither it nor *N*-nitrosomorpholine occur naturally within biological systems. Therefore, any *N*-nitrosomorpholine measured in our analysis is derived from nitrosation of the provided substrate and the amount measured is therefore not contributed to via contamination by substrate or product.

4.2.2 Choice of internal standard

Our silastic tube sections contained morpholine dissolved in aqueous solution and buffered at pH 7.4 using 0.02M phosphate buffered saline. In order to analyse *N*-nitrosomorpholine formed within the silastic tube solution we had to transfer it from the aqueous to the organic solvent phase. Analysis also required addition of an internal standard. *N*-nitrosodibutylamine was chosen as an internal standard. It is an aliphatic *N*-nitrosamine with a high boiling point and a similar polarity to *N*-nitrosomorpholine, and therefore should partition between the aqueous and organic solvent phases in similar proportions to *N*-nitrosomorpholine.

4.2.3 Choice of extraction solvent mix

We measured *N*-nitrosamine concentrations by gas chromatography: tandem mass spectrometry using the Finnigan Polaris*Q* GC/MS^n Bench top Ion Trap Mass Spectrometer (Thermo Electron Corporation, Austin, Texas, USA). The analytical method required *N*-nitrosamines to be dissolved in the organic solvent dichloromethane. Both *N*-nitrosomorpholine and *N*-nitrosodibutylamine preferentially move into the organic solvent phase, and this can be enhanced by increasing the ionic content of the aqueous phase by adding a concentrated ionic solution such as 2M sodium chloride. Dichloromethane (CH_2Cl_2) is an organic solvent with density higher than water ($1.33g/cm^3$), and therefore forms a layer below the aqueous layer on mixing. As we were working with small volumes, removal of the organic solvent layer would be simpler if it formed a layer above the aqueous phase. To facilitate this, we mixed the dichloromethane (DCM) with diethyl ether ($C_4H_{10}O$) in a ratio of 45:55. Diethyl ether (DEE) is a solvent with

low density (0.71 g/cm^3) which is infinitely miscible with DCM. Mixing in the above proportions creates an organic solvent phase which is less dense than the aqueous phase and therefore lies above it, making removal easier. Following its removal, the organic solvent layer can be concentrated by blowing down in a gentle stream of nitrogen gas.

4.2.4 Artefact formation during sample preparation

If the sample or analytical agents contain nitrite then formation of *N*-nitroso compounds may occur by artefact during the analytical process. This should only occur in aqueous solutions below pH 7, as the nitrite ion is ineffective as a nitrosating agent in the absence of catalysts such as mineral acids [135]. In acidic aqueous solutions, sulphamic acid [136, 137] is often employed to remove nitrite by reacting with it to produce nitrogen gas. Both nitrite and *N*-nitrosomorpholine are likely to be formed within our silastic tube sections by reaction of nitrosating species generated via nitric oxide with water and morpholine respectively. Providing the pH remains 7.4 then nitrosation should not occur once the tube is removed from a luminal environment containing large amounts of nitric oxide, as the nitrite ion is ineffective as a nitrosating agent in the absence of mineral acids [135]. Sulphamic acid had been added in the analyte preparation in our previously published work [81], but we had been measuring *N*-nitrosamines in acid solution as well as pH neutral solution in that circumstance. Under less acidic solutions sulphamic acid is very much less effective[135], and there were concerns that its presence may result in hydrolysis of *N*-nitrosamines at neutral pH. Additionally, it has been reported that at pH 4.0 sulphamic acid can actually enhance *N*-nitrosation [138].

We suspected that artefactual *N*-nitrosation of morpholine would not occur by exposure of morpholine to nitrite at pH 7.4 and that using sulphamic acid in the processing of our samples was therefore unnecessary and possibly deleterious. We required to perform a few experiments to investigate this.

4.2.4.1 Artefact formation from exposure of morpholine to nitrite at pH 7.4

We designed an experiment to confirm whether further nitrosation occurred when morpholine was exposed to nitrite at neutral pH in the absence of exposure of the surface of the tube to nitric oxide. 50ml of 0.02M phosphate buffered saline containing 25mM morpholine (pH 7.4) was added to a glass beaker. Earlier laboratory studies carried out by my colleague Dr Stuart Paterson had ascertained that a morpholine concentration of 25mM was optimal for the assessment of nitrosation.

Sodium nitrite was then added to give a final nitrite concentration of 100uM. The solution was then left to stand at room temperature for 30 minutes, representing the time delay from removal of the silastic tube from the human subject to its transfer to the laboratory for analysis. After 30 minutes, five 40ul aliquots were processed and analysed for *N*-nitrosomorpholine. As a control, five 40ul aliquots of the beaker solution were removed prior to addition of nitrite. No *N*-nitrosomorpholine was detected in any of the 5 samples or 5 controls, confirming that further *N*-nitrosation does not occur when morpholine is exposed to nitrite at pH 7.4. This finding confirmed that we did not need to remove the nitrite from the pH 7.4 solution when preparing for *N*-nitrosamine analysis.

4.2.4.2 Evaluation of efficiency of the extraction process with and without sulphamic acid

We planned to assess the efficiency of our *N*-nitrosamine extraction process and the effect of sulphamic acid on this. We designed an experiment to analyse the correlation between expected and measured *N*-nitrosomorpholine concentrations following extraction in a series of aqueous *N*-nitrosomorpholine solutions of varying strength.

Eight solutions of *N*-nitrosomorpholine were prepared in 0.02M phosphate buffered saline. Concentrations were 0 uM, 1.7uM, 8.6uM, 17.2uM, 43 uM, 86 uM, 129 uM and 172 uM. 40ul aliquots of each of these solutions was added to a polypropylene vial along with 10ul *N*-nitrosodibutylamine (internal standard) and 50ul of either 5% sulphamic acid solution in 6M sodium chloride (as previously used [81]) or 2M sodium chloride. The nitrosamines were then extracted into organic solvent using a 45:55 mix of DCM: DEE. The extraction procedure was repeated to optimise recovery. *N*-nitrosomorpholine concentration was then measured by GCMS/MS.

The expected and measured concentrations of *N*-nitrosomorpholine (NMOR) when sulphamic acid was used in sample processing are shown Table 4.1 and in Figure 4.1. The expected and measured concentrations of *N*-nitrosomorpholine (NMOR) when 2M sodium chloride was used in sample processing are shown Table 4.2 and in Figure 4.2. The data represented in both tables is from a single experiment.

Table 4.1: Comparison between expected and measured *N*-nitrosomorpholine concentrations in a series of aqueous standards when 5% sulphamic acid was used in the sample processing.

Vial Number	Expected NMOR conc. (uM)	Measured NMOR conc. (uM)	Percentage Error
1	172	91.7	47%
2	129	65	50%
3	86	25.9	70%
4	43	13	70%
5	17.2	3.7	78%
6	8.6	1.3	85%
7	1.7	0.3	82%
8	0	0	0%

Figure 4.1: Comparison between expected and measured *N*-nitrosomorpholine concentrations in a series of aqueous standards when 5% sulphamic acid used in the sample processing.

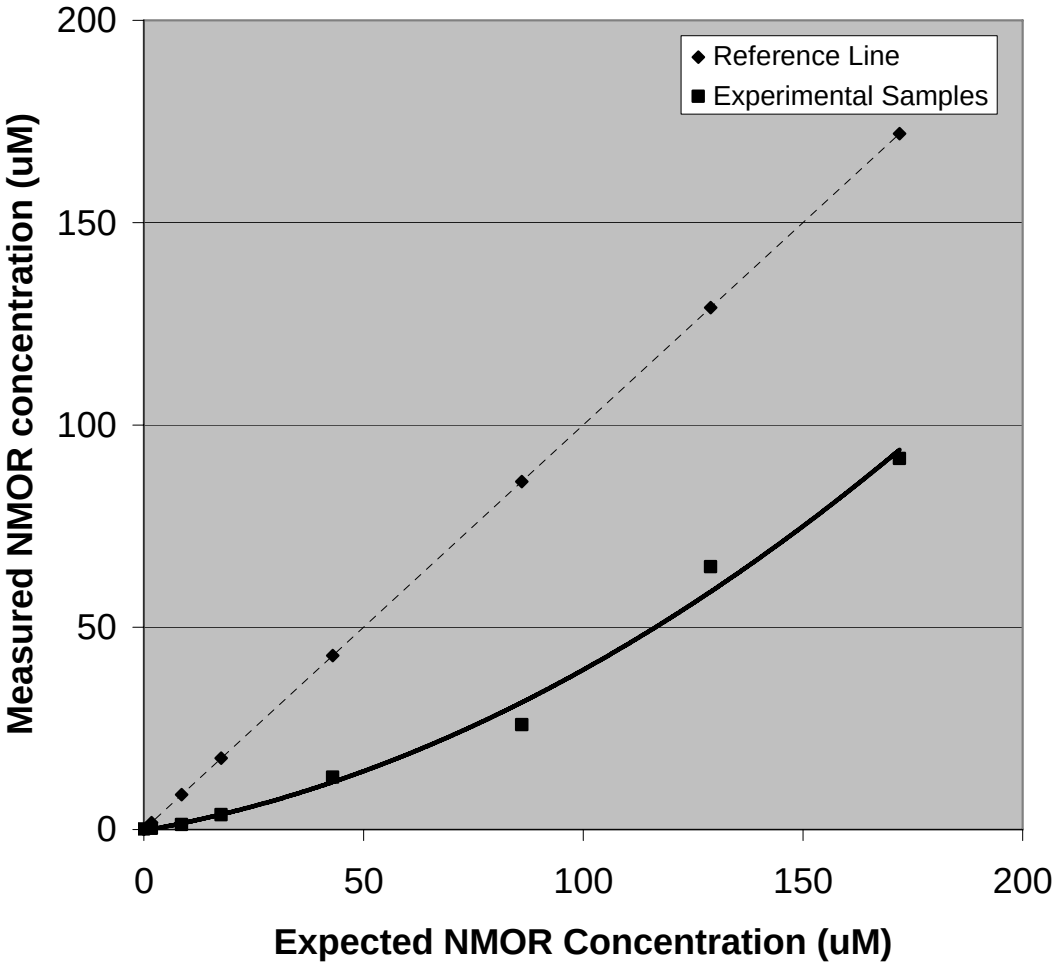


Table 4.2: Comparison between expected and measured *N*-nitrosomorpholine concentrations in a series of aqueous standards when 2M sodium chloride was used in the sample processing.

Vial Number	Expected NMOR conc. (uM)	Measured NMOR conc. (uM)	Percentage Error
1	172	193.7	13.%
2	129	140.4	9%
3	86	97.3	13%
4	43	51	19%
5	17.2	19.1	11%
6	8.6	10.6	23%
7	1.7	0.6	65%
8	0	0	0%

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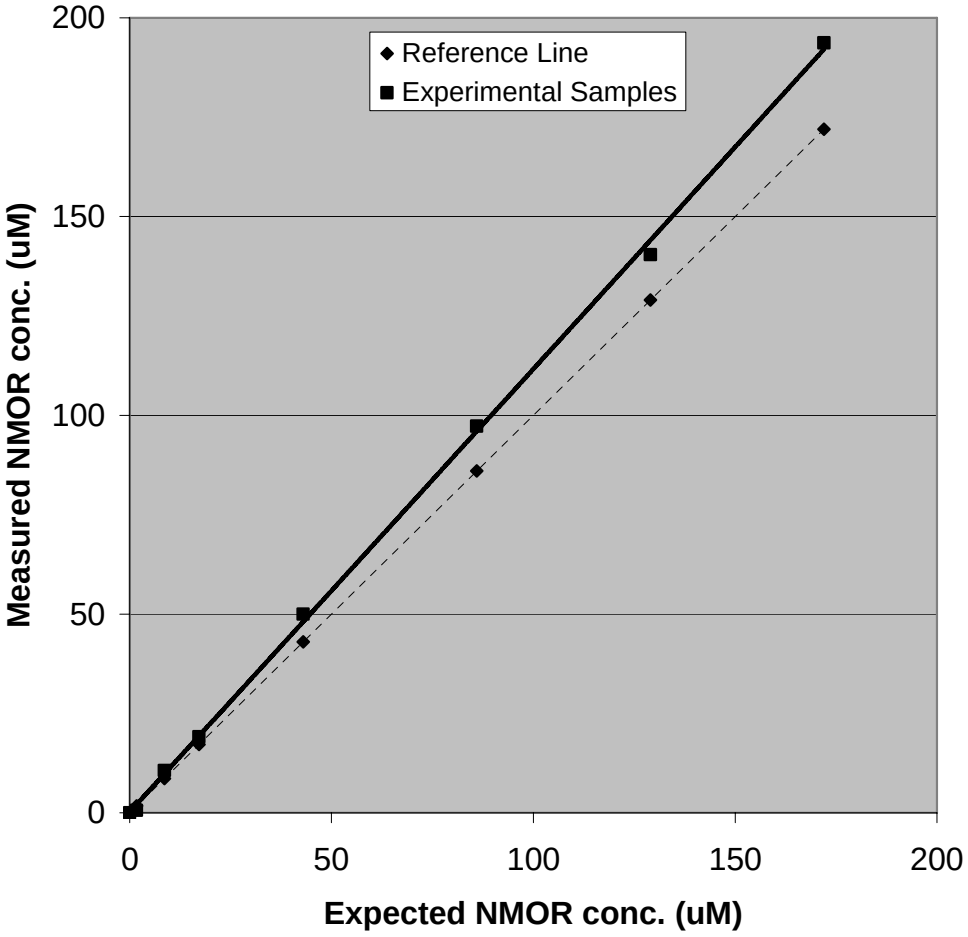
Figure 4.2: Comparison between expected and measured *N*-nitrosomorpholine concentrations in a series of aqueous standards when 2M sodium chloride used in the sample processing.



Use of 5% sulphamic acid in a concentrated sodium chloride solution during the processing of the aqueous samples resulted in significantly lower levels of *N*-nitrosomorpholine being measured than would have been expected. Substitution of 2M sodium choride for sulphamic acid solution improved the accuracy of our analysis. A linear relationship existed between expected and measured concentrations of *N*-nitrosomorpholine, with satisfactory accuracy (10-23% expected) at all concentrations above the lowest (1.7uM). No false positive *N*-nitrosomorpholine detection occurred. Use of 2M sodium chloride therefore appeared superior to sulphamic acid in the sample processing.

4.2.5 Method for *N*-nitrosomorpholine sample preparation

Following the above considerations, we were able to devise a method for *N*-nitrosomorpholine analysis:

1. 40ul of fluid from each silastic tube section was added to a 1.5ml polypropylene vial (Eppendorf, Hamburg, Germany) containing 50ul of 2M sodium chloride and 10ul of 0.01% *N*-nitrosodibutylamine in methanol as internal standard. The vial contents were then mixed vigorously.

2. 250ul of extraction solvent mix (45:55 dichloromethane:di-ethylether) was added to each vial and well mixed. To further assist separation, samples were then centrifuged at 3000rpm for 3 minutes.

3. The upper (organic solvent) layer was then aspirated and transferred to a 1.1ml flat bottomed glass vial with a tapered insert (Chromacol, Hertfordshire, UK). A further 250ul of fresh solvent was then added to the original vial and the extraction process repeated.

4. The resulting 300ul of organic solvent with dissolved nitrosamine was then concentrated to 50ul by blowing down with a gentle stream of nitrogen gas.

5. The tapered vials were then crimp sealed and stored at -20C until analysis by gas chromatography: tandem mass spectrometry using the Finnigan Polaris*Q* GC/MS^n Bench top Ion Trap Mass Spectrometer (Thermo Electron Corporation, Austin, Texas, USA).

4.3 Accuracy and Precision of *N*-nitrosomorpholine Analysis

Analytical considerations in the measurement of *N*-nitrosamines include minimising sample contamination during analysis and avoiding artefact formation. We carefully considered limiting these risks by choosing a secondary amine (morpholine) which does not occur naturally and by evaluating the risk of nitrosation during the analytical process.

A number of factors are likely to contribute to recovery losses including inefficient extraction procedures and vapour loss. By measuring the concentration of a number of known *N*-nitrosomorpholine solutions following extraction from aqueous to organic phase we were able to confirm that the efficiency of our extraction process was satisfactory. Inaccuracies can also manifest within the GCMS/MS due to build up of debris within the column or column liner, resulting in poorer chromatographic peak sensitivities.

We observed subjectively within many of our earlier GCMS analyses that disproportionate differences in peak height manifested over time between the target analyte (*N*-nitrosomorpholine) and the internal standard (*N*-nitrosodibutylamine). This was despite routine changes of the column liner and inspection of the GCMS/MS column between every run. In order to minimise

error, we elected to simultaneously analyse a series of known concentrations of *N*-nitrosomorpholine which had undergone aqueous-organic solvent extraction along with every set of samples from our human studies on the GCMS/MS machine. We utilised the same series of concentrations (0 uM, 1.7uM, 8.6uM, 17.2uM, 43uM, 86uM, 129uM, 186uM) as we had used to assess the efficiency of the extraction process. This allowed us to develop a standard curve following GCMS/MS analysis and apply a correction factor for our unknowns.

Table 4.3 depicts the degree of variability in analysis of the extracted *N*-nitrosomorpholine standards when these standards were analysed along with the samples from 25 of the human subjects in the study. The variability seen was due to variations in the chromatography performed by the GCMS/MS machine. Using these standards to draw a curve in each case helped correct for this.

Table 4.3. Degree of variability in analysis of the extracted *N*-nitrosomorpholine standards when analysed along with samples from study subjects

Expected NMOR concentration (uM)	Median Measured NMOR concentration (uM)	Interquartile range (uM)
172	187.7	143.3-245.3
129	145.2	114.6-177.8
86	83.9	57.1-127
43	39.6	25.8-74.7
17.2	13.5	8.2-22.3
8.6	5.8	3.7-9.5
1.7	1.2	0.8-1.8
0	0	0

4.4 Nitric oxide-mediated nitrosation within the epithelial compartment *in vitro*

Before commencing our studies in humans, we wished to confirm that we were able to reproduce the results achieved in our previously published bench-top studies[81]. We wished to confirm:

1. The ability to reproduce the previously observed nitric oxide-diated nitrosation in the epithelial compartment using our wider calibre silastic tube.
2. Efficacy of our new method for processing and analysing *N*-nitrosamines
3. Ability of the GCMS/MS machine to confirm generation of ^{15}N enriched *N*-nitrosomorpholine via nitric oxide following administration of ^{15}N enriched nitrite to the luminal compartment.

We utilised a segmented silastic tube (internal diameter 25mm, wall thickness 5mm) to represent the epithelial cell membrane. The tube contained phosphate buffered saline at pH 7.4 to represent the epithelial compartment. This compartment also contained 25mM morpholine as a substrate secondary amine. The gastric lumen was represented by a glass beaker containing de-ionised water, 0.01M hydrochloric acid, sodium thiocyanate (to final concentration 1mM), EDTA (to final concentration (1mM) and ascorbic acid (to final concentration 1mM). The final pH of the gastric luminal compartment was 1.5.

Two glass beakers (beaker A and beaker B) containing 100ml of the gastric lumen solution were placed in a water bath at 37 degrees Celsius. Within each beaker was immersed a silastic tube partitioned into 15mm segments. Each tube consisted of 14 sections and was positioned such that the most peripheral 4 sections on either side of the tube lay out-with the gastric lumen compartment and the central six compartments were immersed within it (figure 4.3). The sections of the tube in beaker A were labelled 1-14 and the sections of the tube in beaker B were labelled 15-28.

At a specified time, 1ml 10mM sodium nitrite was added to the solutions in beakers A and B simultaneously, creating a final concentration of 100uM nitrite in each. The sodium nitrite added to beaker A was 99% enriched with ^{15}N whereas the sodium nitrite added to beaker B was not. The apparatus is depicted in figure 10. The reaction was allowed to proceed for 30 minutes, after which the silastic tube was removed from each beaker and the contents of each analysed for nitrite and *N*-nitrosomorpholine.

Sections 1-4 and 11-14 of the tube in beaker A and sections 15-18 and 25-28 of the tube in beaker B were not immersed in the gastric lumen solution and nitrite and *N*-nitrosomorpholine were not detected within these sections. Sections 5-10 and 19-24 were immersed in the beaker solutions and nitrite and *N*-nitrosomorpholine concentrations measured in each are shown in table 4.4.

Generation of nitric oxide in each beaker following administration of nitrite was confirmed using a nitric oxide sensor (ISO:NO Mark II: World Precision Instruments Inc., Sarasota, Florida).

Figure 4.3: Experiment to confirm nitric oxide-mediated nitrosation within the epithelial compartment of segmented silastic tube with wall thickness 5mm and internal diameter 25mm. Two tubes comprising 15 segments are partially immersed into beakers A and B, which comprise a chemical stomach model. 15N enriched sodium nitrite is added to beaker A and standard sodium nitrite is added to beaker B.

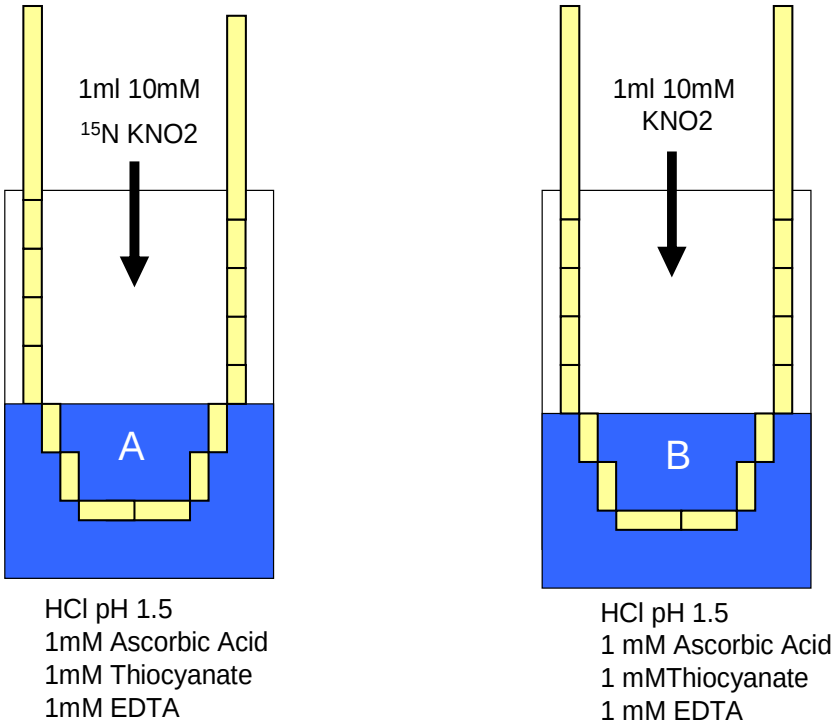


Table 4.4: Silastic tube fluid concentrations of nitrite, *N*-nitrosomorpholine and ^{15}N enrichment of *N*-nitrosomorpholine for the sections exposed to acidified nitrite and ascorbic acid. Sections 5-10 were exposed to ^{15}N enriched nitrite and sections 19-24 to standard nitrite.

Section Number	Nitrite conc. (uM)	NMOR conc. (uM)	NMOR ^{15}N enrichment	NMOR: Nitrite ratio
5	378	55.3	99%	0.15
6	498	91.7	99%	0.18
7	522	88.4	99%	0.17
8	618	84.5	99%	0.14
9	654	76.9	99%	0.12
10	392	40.1	99%	0.1
19	316	52.8	0%	0.17
20	588	94.8	0%	0.16
21	438	82.4	0%	0.19
22	502	83.9	0%	0.17
23	558	108.1	0%	0.19
24	420	72.5	0%	0.17

Only sections 6-9 and sections 20-23 were constantly exposed to acidified nitrite during the experiment, as sections 5, 10, 19 and 24 were only partially submerged. Median nitrite concentration for those sections constantly exposed to acidified nitrite and ascorbic acid was 570uM and median *N*-nitrosomorpholine for those sections was 86.4uM. The sections exposed to ^{15}N enriched nitrite were 99% enriched with 15N and the sections exposed to standard nitrite were not enriched. In this experiment, both ascorbic acid and dissolved oxygen were present in excess of nitrite, therefore the vast majority of nitrite entering the epithelial compartment will be in the form of nitric oxide. The median *N*-nitrosomorpholine ratio, an expression of the proportion of nitrosation occurring as a result of nitric oxide versus nitrous acid, was 0.17.

This experiment has confirmed that our wider bore silastic tube can be used to determine nitrite and *N*-nitrosomorpholine generation within an epithelial model exposed to nitric oxide formed by reaction of acidified nitrite and ascorbic acid and that, using GCMS/MS, we are able to determine if measured *N*-nitrosomorpholine is enriched with ^{15}N.

CHAPTER FIVE

IN SITU GENERATION OF *N*-NITROSO COMPOUNDS IN THE UPPER GASTROINTESTINAL TRACT OF HUMAN HEALTHY VOLUNTEERS FOLLOWING INGESTION OF NITRATE

5.1 Introduction

This study was designed to investigate if the ingestion of nitrate generated *in situ* nitrosative stress within our epithelial model located in the human upper gastro-intestinal tract, and to ascertain the maximal location of any stress measured. To examine this we studied healthy human volunteers after ingestion of nitrate and after ingestion of de-ionised water as a control drink. The nitrate administered was 99% enriched with the heavy, stable nitrogen isotope 15N, which accounts for only 0.366% of all nitrogen occurring naturally

This chapter documents the methods and results for this study in healthy human volunteers. The results will be discussed along with the results from the study in patients with Barrett's oesophagus in Chapter Seven.

5.2 Materials and Methods

5.2.1 Subjects studied

17 healthy volunteers (11 males, median age 27) with no history of reflux disease were studied on 2 separate days following an overnight fast. All tested negative for *Helicobacter pylori*

H. pylori status was determined using the ^{14}C urea breath test. This test has previously been validated in our unit and, using a cut off value of 30 (kg %dose $mmol^{-1}$) for the 20 minute results, has a sensitivity of 98% and specificity of 100%.

5.2.2 Methods

As described earlier, we employed silastic tubing (Altec, Cornwall, UK), with internal diameter 2.5mm and wall thickness 0.5mm for our *in-situ* epithelial model. A 100cm section of tubing was filled with the secondary amine morpholine, which was buffered at intracellular pH (7.4) using 2mM phosphate buffer. The tubing was then partitioned into 21 sections, each 15mm long, by twisting twice and then tying with 4-0 vicryl suture (Ethicon Corporation, Somerville, New Jersey, USA). Every section contained approximately 74ul. This length of tubing was attached to a customized 4 channel pH catheter (Synectics Medical, Middlesex, UK) in order that we could simultaneously monitor luminal acidity. The pH sensors were aligned to centre of the 19^{th}, 14^{th}, 13^{th} and 8^{th} sections of the silastic tube respectively, and attached by tying with vicryl suture and securing with Sleek® waterproof tape (figure 5.1). A 5cm length of 10F

gastric tube (Vygon, Ecouen, France) containing a radio-opaque marker was secured to the distal end of the pH catheter to assist passage.

Subjects attended the gastro-intestinal investigation unit on 2 separate days following an overnight fast. On each study day, the tube was passed naso or oro-gastrically. The tube was positioned such that sections 1-13 lay within the oesophagus and sections 14-21 lay within the stomach. This was achieved by monitoring the pH step-up and confirmed by X-ray (Figure 5.2). 30 minutes after insertion of the tube, the subjects were administered the study drink. On one day, the subjects drank 60 ml of water containing 2 mmoles potassium nitrate and on the other 60ml of water without nitrate. 8 of the subjects received nitrate which was 99% enriched with the heavy, stable isotope ^{15}N (Sercon Ltd, Nantwich, UK). The order in which the different drinks were administered was random.

The tube remained *in-situ* for 2 hours after the drink, with pH being monitored at each site every 4 seconds. Immediately prior to the drink, and at 15 minute intervals following it, 10 ml blood and 1ml saliva samples were collected for measurement of serum nitrate and salivary nitrite concentrations. The tube was then removed and each section carefully aspirated using a 100ul glass syringe **(**SGE, Milton Keynes, UK**)**. 10ul was used for measurement of silastic tube nitrite concentration and 40ul for analysis of silastic tube *N*-nitrosomorpholine concentration.

Figure 5.1: Photograph of region of segmented silastic tube attached to pH catheter. Each section contains morpholine 25mM buffered at pH 7.4 and is 15mm in length. Section 10 is apposed to probe 1 and sections 13 and 14 to probes 2 and 3 respectively.

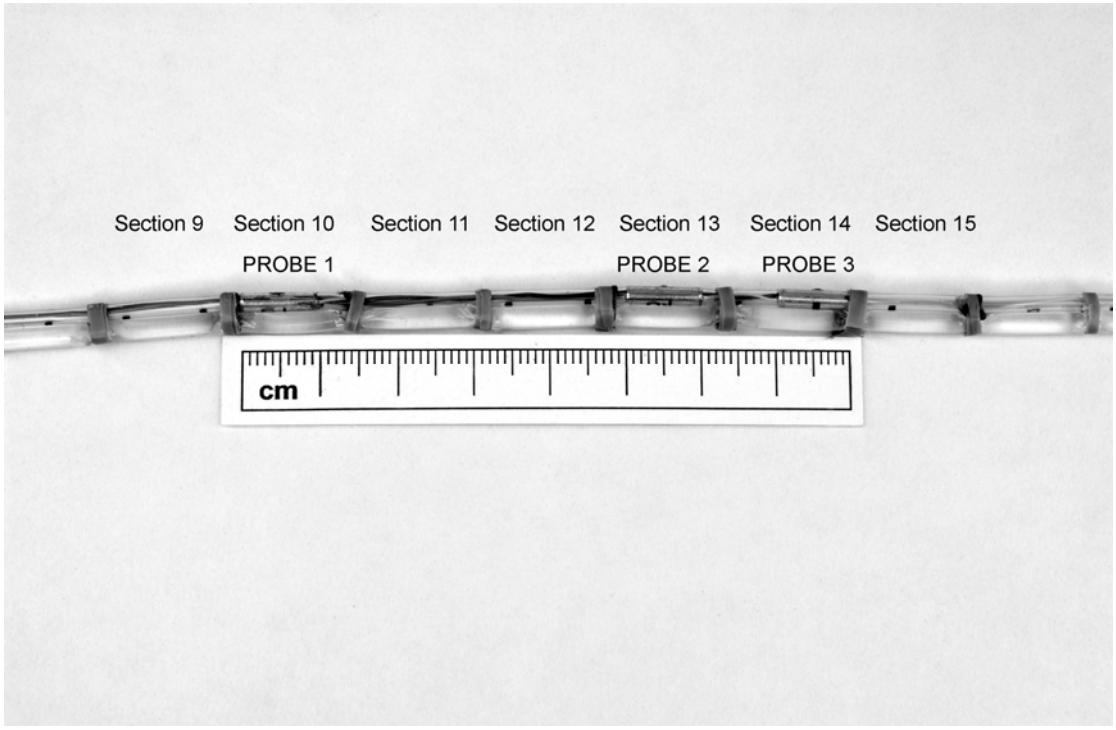
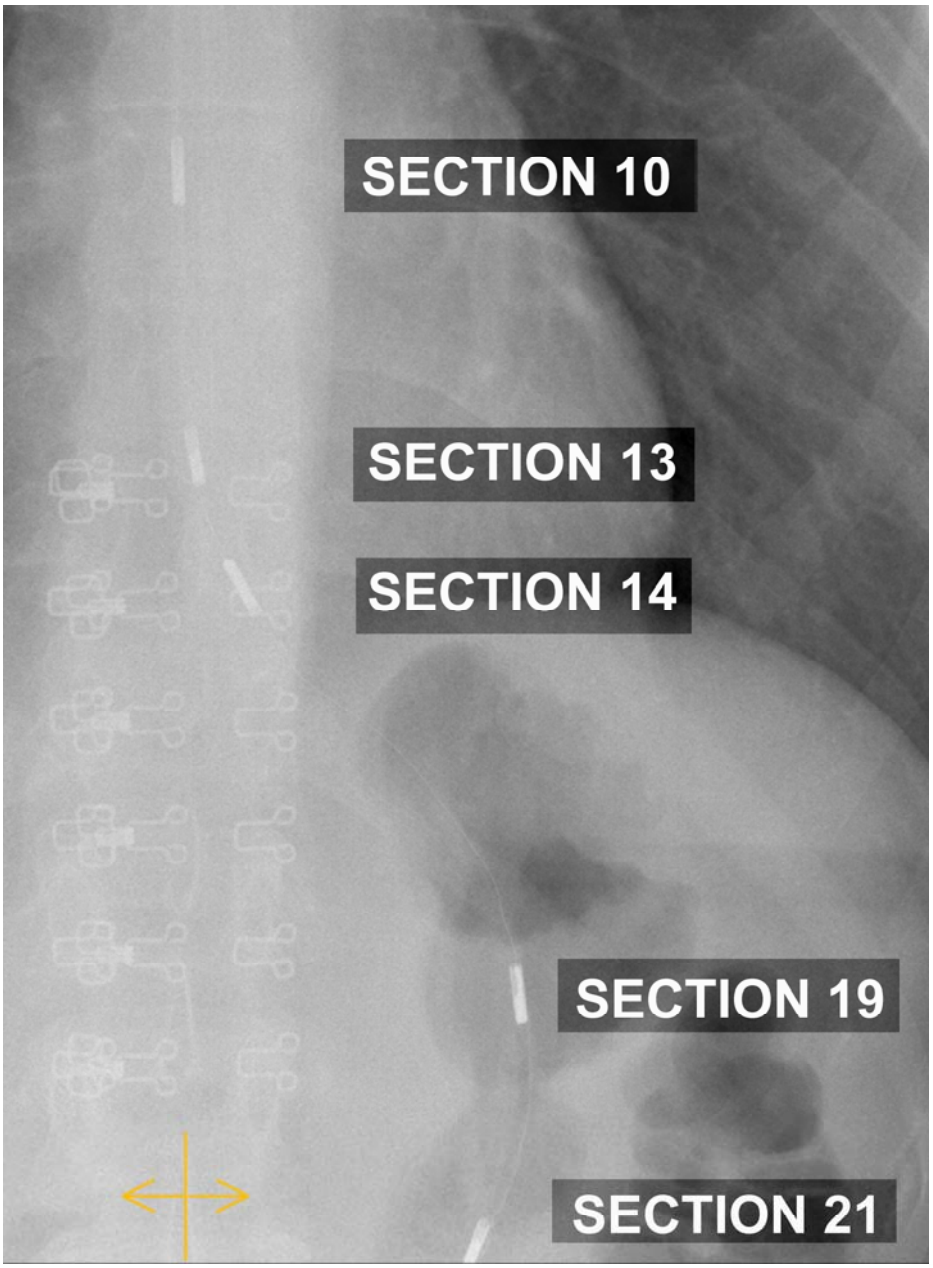


Figure 5.2: Plain postero-anterior chest X ray of specialized oesophago-gastric tube *in-situ* within the upper gastro-intestinal tract of a human healthy volunteer. Sections 13 and 14 are apposed to probes 2 and 3 and lie on either side of the pH step up point. Section 10 is apposed to probe 1 and positioned 5cm proximal to the pH step up point. Section 19 is apposed to probe 4 and section 21 lies 12cm inside the stomach, apposed to a radio-opaque marker.



5.2.3 Analyses

5.2.3.1 Salivary and silastic tube nitrite concentration

It was important that any fluid to be analysed for nitrite concentration was not at acid pH, avoiding loss as nitric oxide following pH dependent conversion to nitrous acid and nitrosating species. 10ul of silastic tube solution (pH 7.4) was diluted to 1:10 prior to colorimetric analysis by adding 90ul of de-ionised water. 1ml of saliva was alkalinised and diluted to 10:11 by addition of 1M sodium hydroxide.

Samples were analysed against known concentrations of nitrite in a 96 microwell plate, using the modified Greiss reaction for nitrite analysis, as previously described [103, 126]. Greiss reagent consists of sulphanilamide, hydrochloric acid and NEDD (N-1-Napthylethylenediaminedihydrocholide). Presence of nitrite in an acidic environment leads to production of an azo dye that is detected via its absorbance on a plate reader at 540nm.

5.2.3.2 Serum nitrate concentration

Thirty minutes after collection, blood samples were centrifuged at 3000rpm for 3 minutes. Serum was removed and stored at -20 degrees Celsius until nitrate analysis. After thawing, serum samples were filtered through a 10kDa microfilter (Microcon 10; Millipore UK Ltd, Watford, UK) to remove high molecular weight substances. Nitrate was reduced to nitrite using bacterial nitrate reductase, as previously described [103]. Nitrite was then measured by Greiss reaction as stated above.

5.2.3.3 Silastic tube *N*-nitrosomorpholine concentration

10ul of silastic tube solution was utilised for measurement of *N*-nitrosomorpholine concentration as described on page 72. To facilitate direct comparison between *N*-nitrosomorpholine concentrations from the same silastic tube section on the two study days, the auto-sampler carousel was arranged such that the corresponding sections were analysed in sequence. As described on page 74, a series of extracted samples of known un-extracted concentrations of *N*-nitrosomorpholine were analysed on the same run as each subject's samples. This allowed calculation of a standard curve against which to compare the concentrations from the subject's samples.

5.2.4 Statistical analyses

All data is presented as medians (Interquartile range). Paired data were analysed using a one sample Wilcoxon test, and unpaired data by Mann-Whitney U test**.**

Total nitrite and *N*-nitrosomorpholine within the silastic tube were calculated from the area under the curve on the graph comparing concentration of each compound by location. Area under the curve was estimated using the trapezoid method, and a volume correction factor (x 0.074) applied.

Calculations were performed using Minitab ® Release 14 statistical software.

5.2.5 Ethical approval

The study was approved by North Glasgow NHS Trust Ethics Committee and each subject gave written, informed consent.

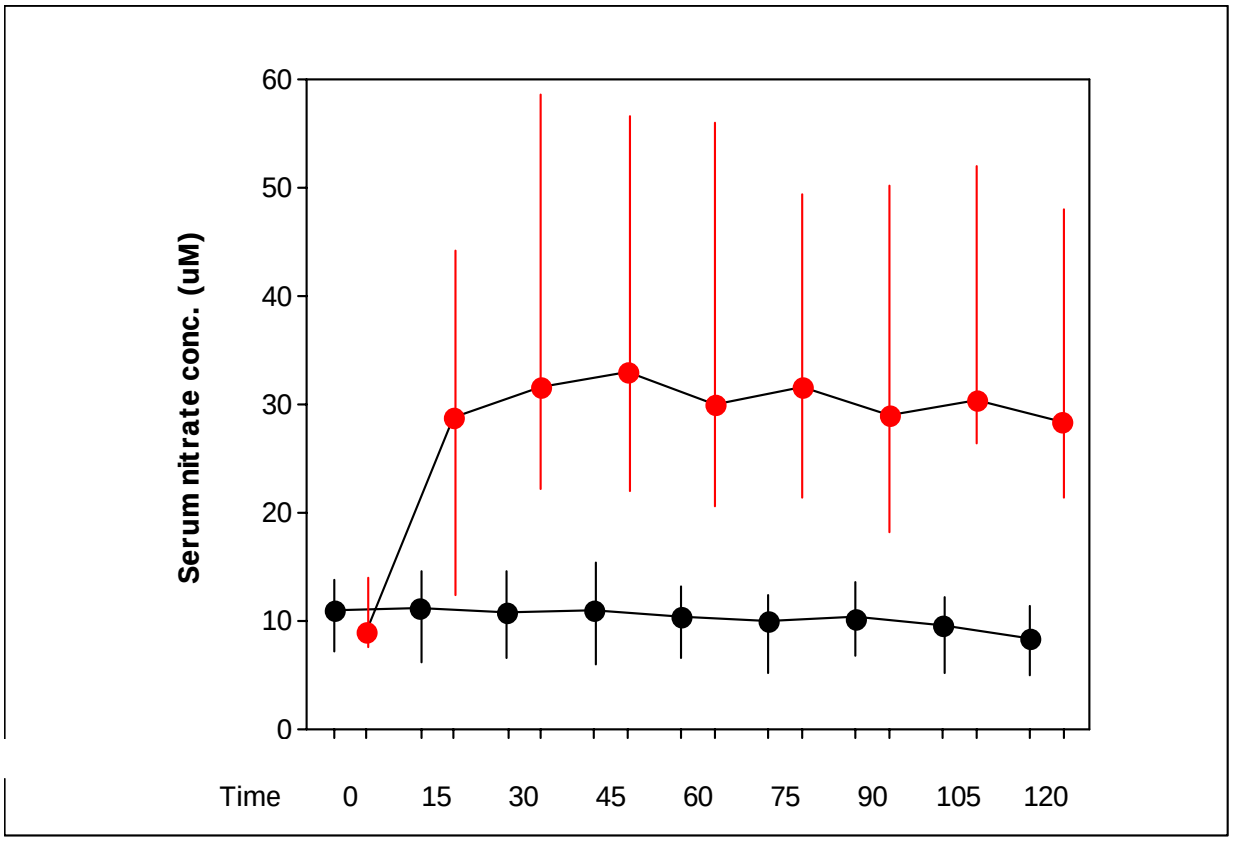
5.3 RESULTS

16 of the 17 subjects completed both arms of the study. One subject was achlorhydric (intragastric pH >4.0), and was later confirmed to be *H. pylori*-positive by serological testing after an initial negative breath test.

5.3.1 Serum nitrate

Prior to administration of the study drink, median serum nitrate concentration was 11.0 (7.2-13.8) uM on the day when the control drink was administered, not significantly different from 9.1 (7.5-13.9) uM on the day when the nitrate drink was administered. Following administration of 2 mmoles potassium nitrate, there was a large and significant rise in serum nitrate concentration (p=0.001), which peaked at 33.1(21.9-56.5) uM after 45 minutes and remained at this level for the duration of the study. Following administration of the control drink, serum nitrate concentration did not change from basal, remaining at 11.1(6.0-15.3) uM after 45 minutes (Figure 5.3)

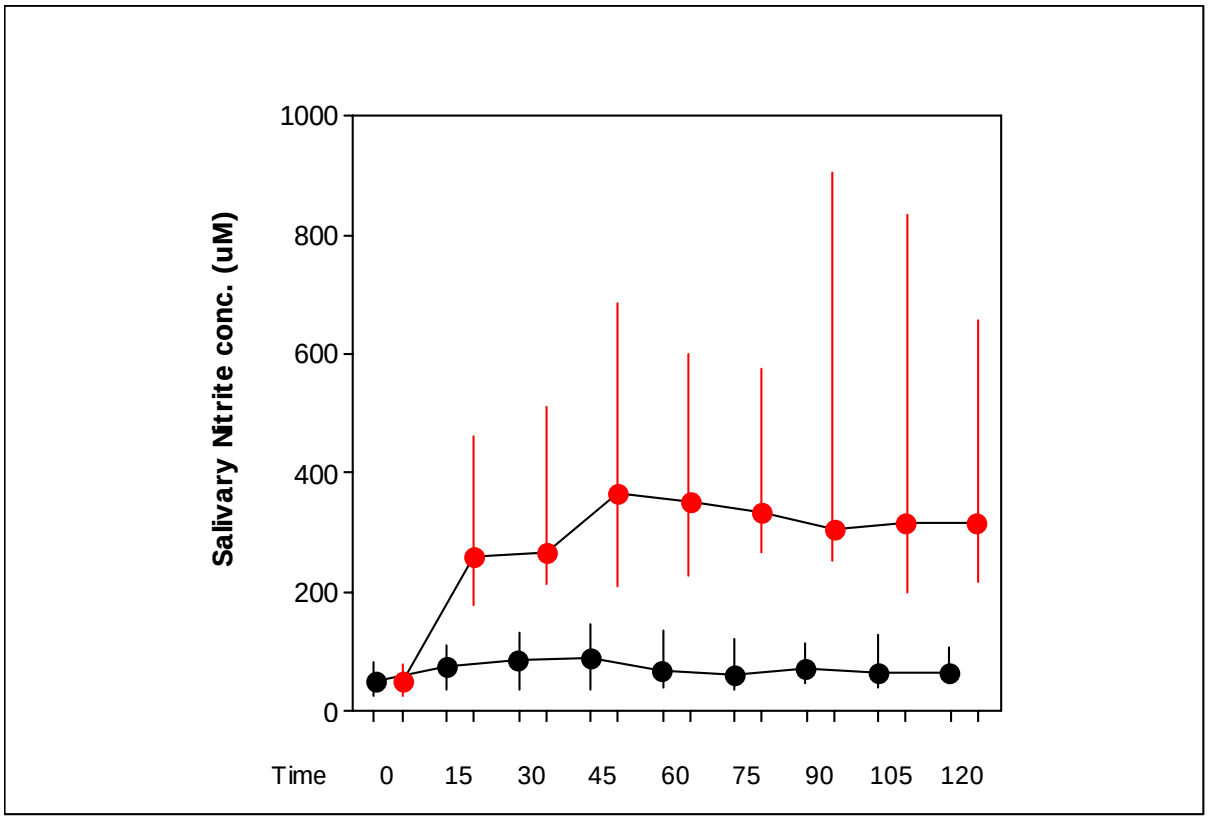
Figure 5.3: Median serum nitrate concentration with time (in minutes) for 15 healthy volunteers. Black dots represent concentrations following the control drink and red dots represent concentrations following the nitrate drink. Whiskers depict interquartile ranges.



5.3.2 Salivary nitrite

Prior to administration of the study drink, median salivary nitrite concentration was 51.4 (24.7-79.4) uM on the day when the control drink was administered, not significantly different from 48.7 (24.8-77.3) uM on the day when the nitrate drink was administered. Following administration of 2mmoles potassium nitrate, there was a large and significant rise in salivary nitrite concentration ($p=0.001$), which peaked at 331.8 (265.5-575) uM after 75 minutes and remained at almost this level for the duration of the study. After administration of the control drink, salivary nitrite concentration changed little from basal, remaining at 59 (32-122.3) uM at 75 minutes (Figure 5.4).

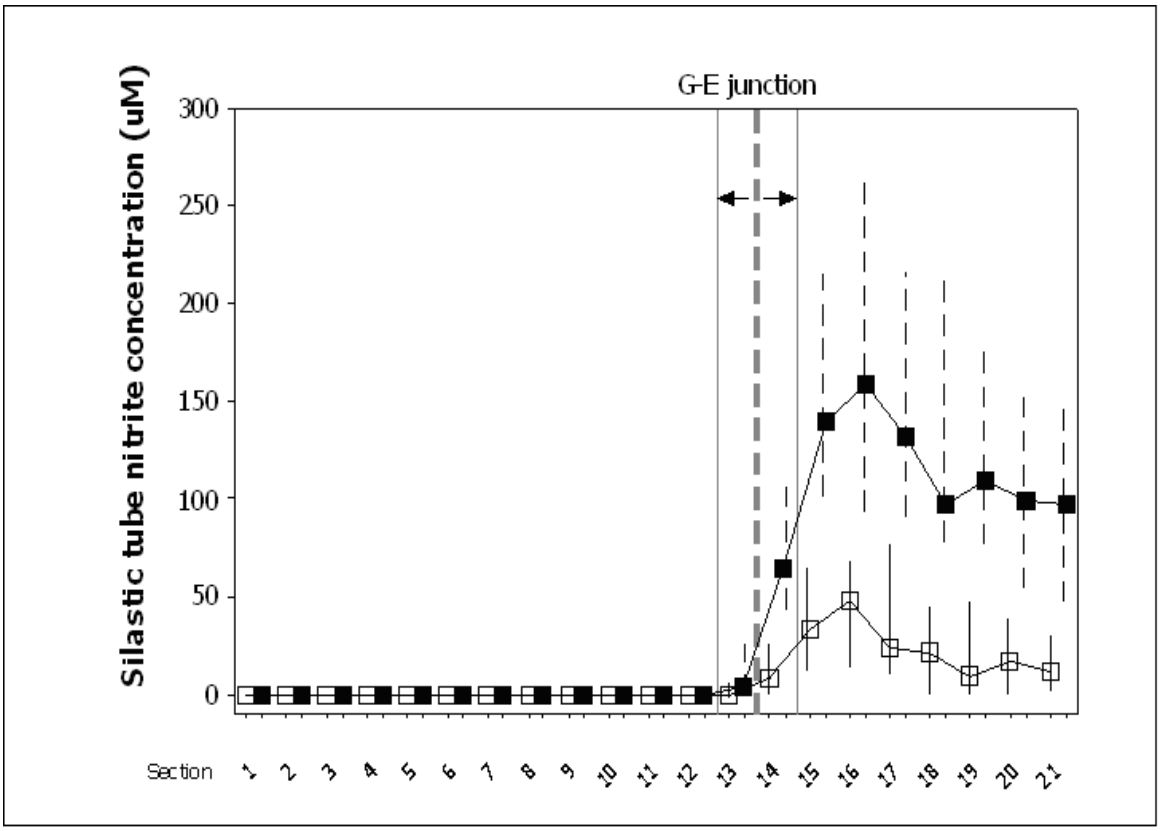
Figure 5.4: Median salivary nitrite concentration with time (in minutes) for 15 healthy volunteers. Black dots represent concentrations following the control drink and red dots represent concentrations following the nitrate drink. Whiskers depict interquartile ranges.



5.3.3 Silastic tube nitrite

The diffusion of either nitric oxide or nitrous acid through the wall of the tube forms nitrite within the tube. Nitrite was not detected within the tube sections lying within the oesophagus. However, nitrite was present in the sections exposed to gastric acid on both study days. The median total nitrite formed within the silastic tube was 17.3 (7.9-28.5) nmoles on the control day and 62.1 (40.8-110.3) nmoles on the day when potassium nitrate was administered (p=0.001). The concentration profile along the gastric sections of the tube was similar on both study days, with the highest concentrations being detected in sections 15 and 16 (figure 5.5), which are the first sections likely to be constantly exposed to intragastric acidity. We have observed using fluoroscopic screening that the excursion of the oesophago-gastric junction with normal respiration is 3-4cm. The probe at section 13 showed pH <2 for 7% of the time and the probe at section 14 showed pH <2 for 63% of the time. Peak concentrations of nitrite were detected in section 16, being 48 (13.8-68.3) uM after the control drink and increasing over three-fold to 159 (93-264) uM following nitrite. Our one subject who was achlorhydric had no detectable nitrite in any of the silastic tube sections.

Figure 5.5: Median silastic tube nitrite concentration by location for 15 healthy volunteers. Sections 1-13 lie above the pH step up point and 14-21 below the pH step up point. The grey broken vertical line between sections 13 and 14 represents the pH step up point, and the surrounding rectangle represents the range of excursion of the pH step up point with normal respiration. Results following administration of the control drink are represented by (□) and following administration of 2 mmoles nitrate by (■). Whiskers represent interquartile ranges.

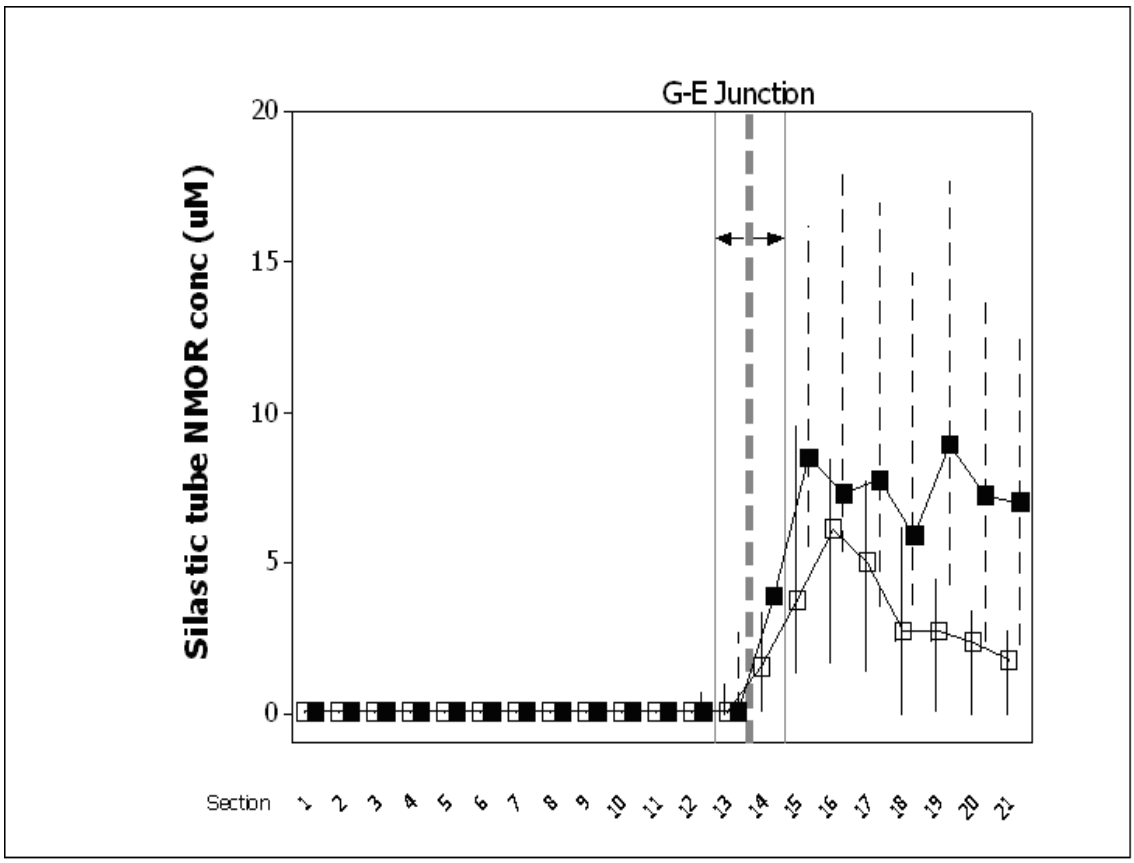


5.3.4 Silastic tube *N*-nitrosomorpholine

N-nitrosomorpholine was not detected within the oesophageal sections of the silastic tube. However, it was detected in the sections exposed to gastric acid when subjects had taken either the control drink or the nitrate drink. The median total *N*-nitrosomorpholine formed within the silastic tube was 2.3 (0.8-3.2) nmoles on the control day and 4.5 (2.7-7.9) nmoles on the day potassium nitrate was administered (p=0.008). On the control day, peak *N*-nitrosomorpholine concentration was 6.1 (1.7-8.5) uM, measured in section 16. However, following the nitrate the *N*-nitrosomorpholine concentration profile was different, producing a plateau rather than the peak concentration recorded without nitrate (Figure 5.6). *N*-nitrosomorpholine concentration in section 16 was 7.3 (5.3-12.7) uM following nitrate, not much greater than after the control drink. Peak *N*-nitrosomorpholine concentration after nitrate was measured in section 19, where the concentration of 8.9 (4.2-17.7) uM was over three-fold greater than 2.7 (0-4.5) uM detected in this section following the control drink. Our one subject who was achlorhydric had no detectable *N*-nitrosomorpholine in any of the silastic tube sections.

Eight of the healthy volunteers were administered nitrate which was 99% enriched with the heavy nitrogen isotope ^{15}N. The *N*-nitrosomorpholine in these volunteers had a median enrichment of 77.4% (64.4-87.3) after administration of nitrate, compared with no enrichment when the control drink was administered. This confirmed that the excess *N*-nitrosomorpholine formed following administration of nitrate originated from the ingested nitrate.

Figure 5.6: Median silastic tube *N*-nitrosomorpholine (NMOR) concentration by location for 15 healthy volunteers. Sections 1-13 lie above the pH step up point and 14-21 below the pH step up point. The grey broken vertical line between sections 13 and 14 represents the pH step up point, and the surrounding rectangle represents the range of excursion of the pH step up point with normal respiration. Results following administration of the control drink are represented by (□) and following administration of 2 mmoles nitrate by (■).



5.3.5 Correlation of silastic tube nitrite versus *N*-nitrosomorpholine

The ratio of *N*-nitrosomorpholine: nitrite within the silastic tube is an expression of nitric oxide: nitrite in the lumen of the gastro-intestinal tract. *N*-nitrosomorpholine can only be formed within the epithelial compartment by the diffusion of nitric oxide, whereas nitrite can be formed via the diffusion of either nitric oxide or nitrous acid into the tube [81].

Administration of nitrate resulted in a three-fold increase in peak silastic tube nitrite concentrations, but only a 50% increase in peak *N*-nitrosomorpholine concentrations. We calculated the ratio of *N*-nitrosomorpholine: nitrite concentrations for each silastic tube section in all of the healthy volunteers. The median of all ratios of *N*-nitrosomorpholine: nitrite concentration following nitrate ingestion was 0.064 (0.045-0.099), significantly lower than 0.11 (0.052 – 0.43) following the control drink ($p = 0.02$).

CHAPTER SIX

THE INFLUENCE OF GASTRO-OESOPHAGEAL ACID REFLUX ON THE MAXIMAL LOCATION OF NITROSATIVE STRESS IN SUBJECTS WITH BARRETT'S OESOPHAGUS

6.1 INTRODUCTION

This study was designed to investigate the influence of acid reflux on the maximal location of *in situ* nitrosative stress following ingestion of nitrate within an epithelial model positioned in the human upper gastrointestinal tract.

To facilitate this aim, we elected to study patients with Barrett's oesophagus, as these are individuals who have significant and measurable gastro-oesophageal reflux disease[139] in whom reflux can be readily stimulated[106]. All subjects ingested 2 mmoles of potassium nitrate and were studied on two separate days. On one day we attempted to stimulate gastro-oesophageal acid reflux and on the other day we did not.

This chapter documents the methods and results for this study in patients with Barrett's oesophagus. The results will be discussed along with the results from the study in healthy volunteers in Chapter Seven.

6.2 MATERIALS AND METHODS

6.2.1 Subjects studied

18 *H. pylori*-negative patients with Barrett's oesophagus (13 males, mean age 61) were recruited from our gastroenterology clinic. The median length of the Barrett's mucosa was 6cm (range 2cm – 20cm). In some patients, *H. pylori* status had already been confirmed by negative ^{14}C urea breath test, serology, histology or CLO test. In other patients in whom *H pylori* status was unknown, ^{14}C urea breath test was performed after a 7 day cessation of proton pump inhibitor therapy.

Proton pump inhibitors (PPIs) were stopped 7 days prior to investigation to allow time for recovery of gastric acid secretion. Our choice of 7 days post PPI treatment in these *H. pylori*-negative subjects was based upon our previous published work showing that this is the time point most representative of the pre-PPI acid secretion. At an earlier time point, inhibitory effects may persist and at a later time rebound secretion becomes more marked [140, 141].

6.2.2 Methods

As described earlier, we employed silastic tubing (Altec, Cornwall, UK), with internal diameter 2.5mm and wall thickness 0.5mm for our *in-situ* epithelial model. The silastic tube was partitioned into sections of 15mm length and attached to a 4 channel pH catheter as described on page 82.

Patients attended the gastro-intestinal investigation unit on two occasions after an overnight fast. At the first visit, gastro-intestinal endoscopy was performed either consciously using lignocaine local anaesthetic spray or under conscious sedation using intravenous midazolam. The extent of the Barrett's

mucosa was inspected and two stainless steel mucosal clips (MD-59; Olympus, Southend-on-Sea, UK) were placed at the proximal margin of the gastric folds to mark the anatomical gastro-oesophageal mucosal junction (figure 6.1) Unlike in healthy volunteers, where the pH step-up point is a reliable indicator of the squamo-columnar junction [69], in the patients with Barrett's oesophagus we required a radiological marker to demonstrate this anatomical site. In the first few patients with Barrett's oesophagus, clips were also deployed at the proximal margin of the segment of columnar lined mucosa. However, this was abandoned laterally as it prolonged the time of the endoscopic procedure and was uncomfortable for patients with very long Barrett's segments.

One hour following endoscopy, the specialised silastic tube was passed naso or oro-gastrically. It was positioned such that sections 1-13 were proximal and 14-21 distal to the gastro-oesophageal junction, as demonstrated by the radio-opaque clips on lateral chest X-ray (figure 6.2). The probe was placed in an identical position on the second study day, again using the radio-opaque clips which remained attached.

The study days were designed to maximize and minimize acid reflux. On one day, we studied the patients in the sitting position without attempt to stimulate reflux. On the other visit, we attempted to stimulate acid reflux by placing the subjects in the right lateral decubitus position, with or without the head down position, and infusing a low dose of pentagastrin (0.06ug/kg/h) (Cambridge Laboratories, Wallsend, UK) intravenously to stimulate physiological levels of acid secretion. On each study day, once the tube was satisfactorily positioned, subjects drank 60ml water containing 2mmoles potassium nitrate. Twelve subjects received nitrate which was 99% enriched with ^{15}N on both study days. The probe

remained *in-situ* for 2 hours following the drink, with blood and saliva samples for nitrate and nitrite collected at 15 minute intervals. Luminal pH adjacent to the 4 probes was recorded every 4 seconds for the duration of the study. The position of the tube was confirmed radiologically immediately prior to its removal. Each section of the tube was aspirated for analysis of nitrite and *N*-nitrosomorpholine concentration as discussed earlier.

Figure 6.1: Endoscopic view of stainless steel mucosal clips deployed at the top of the gastric folds in a patient with columnar lined oesophagus.

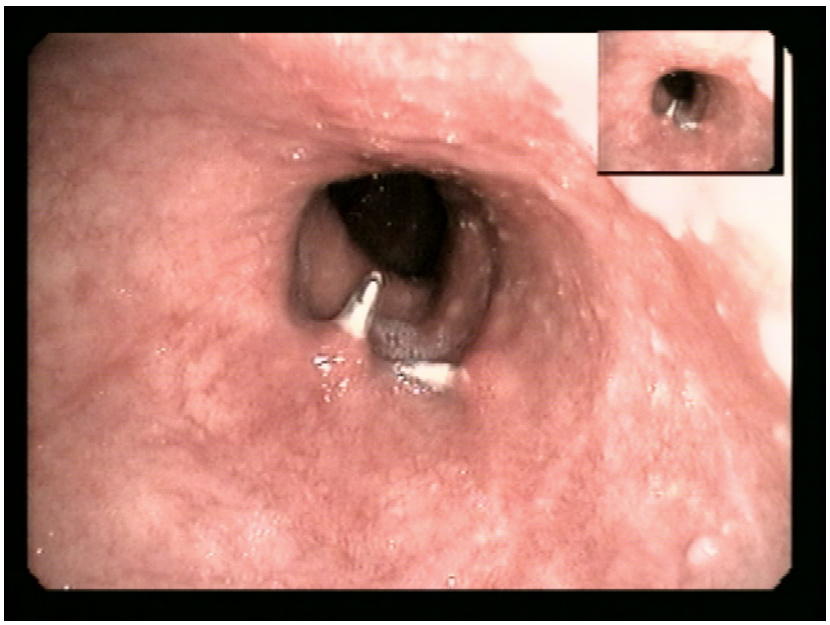
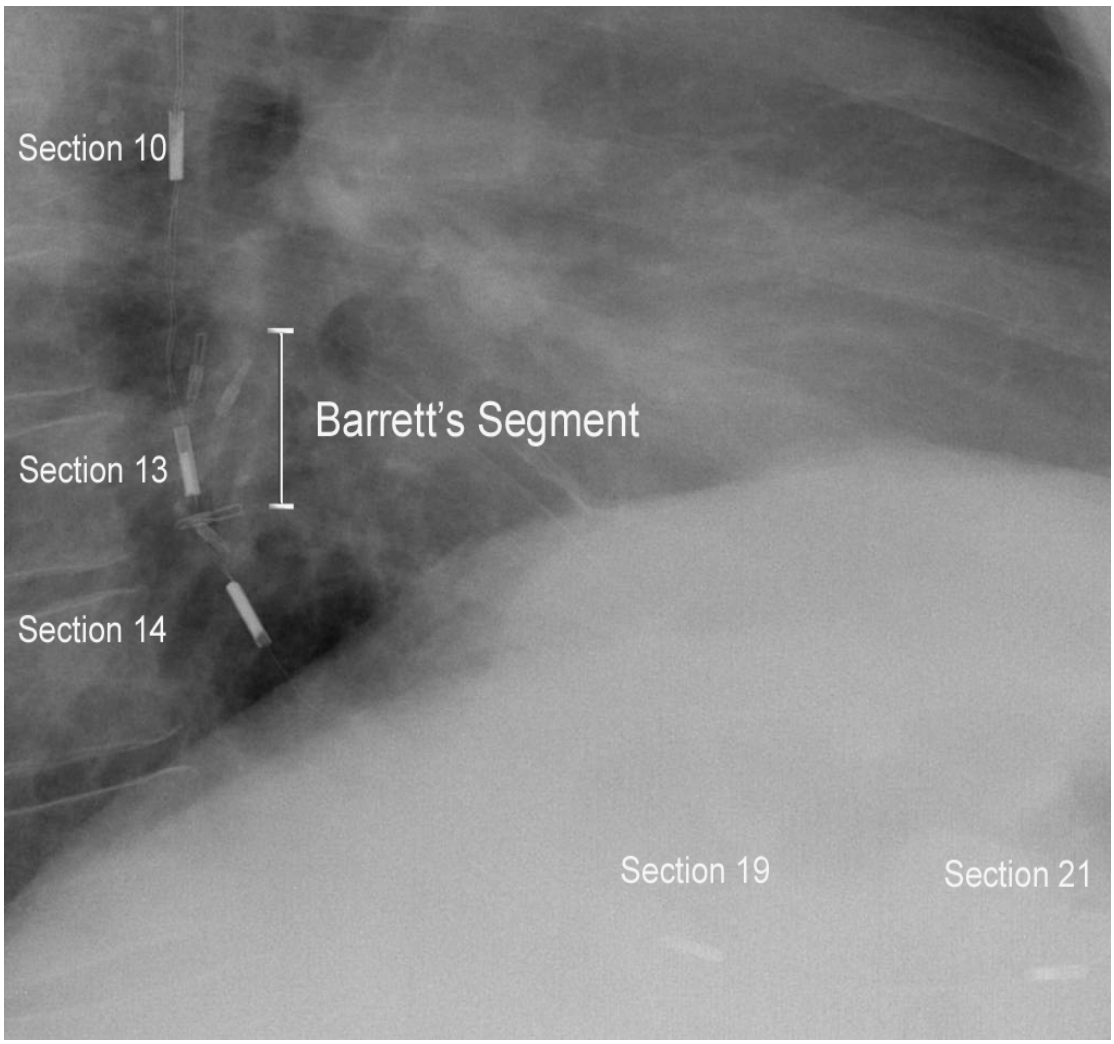


Figure 6.2: Lateral chest X ray depicting proximity of specialized oesophago-gastric tube to mucosal clips marking the gastro-oesophageal junction in a patient with 3cm of columnar lined oesophagus.



6.2.3 Chemical analyses

As in the healthy volunteers, we measured serum nitrate, salivary nitrite, silastic tube nitrite and silastic tube *N*-nitrosomorpholine. The analytical methods are as described in chapter 5.

6.2.4 Statistical analyses

All data is presented as medians (Interquartile range). Paired data were analysed using a one sample Wilcoxon test, and unpaired data by Mann-Whitney U test**.** Spearman's rank test was employed to assess correlation when examining the relationship between oesophageal acid reflux and nitrosative stress in that region.

Calculations were performed using Minitab ® Release 14 statistical software.

6.2.5 Ethical approval

The study was approved by North Glasgow NHS Trust Ethics Committee and each subject gave written, informed consent.

6.3 RESULTS

Eighteen patients with Barrett's oesophagus were recruited. One patient was unable to tolerate the oesophago-gastric tube and withdrew from the study. One further patient completed both arms of the study but produced very little saliva. This was probably as a results of the patient having psoriatic arthropathy, which has a recognized association with reduced salivary flow[142]. Results from 16 patients were included in the analysis.

6.3.1 Gastro-oesophageal acid reflux

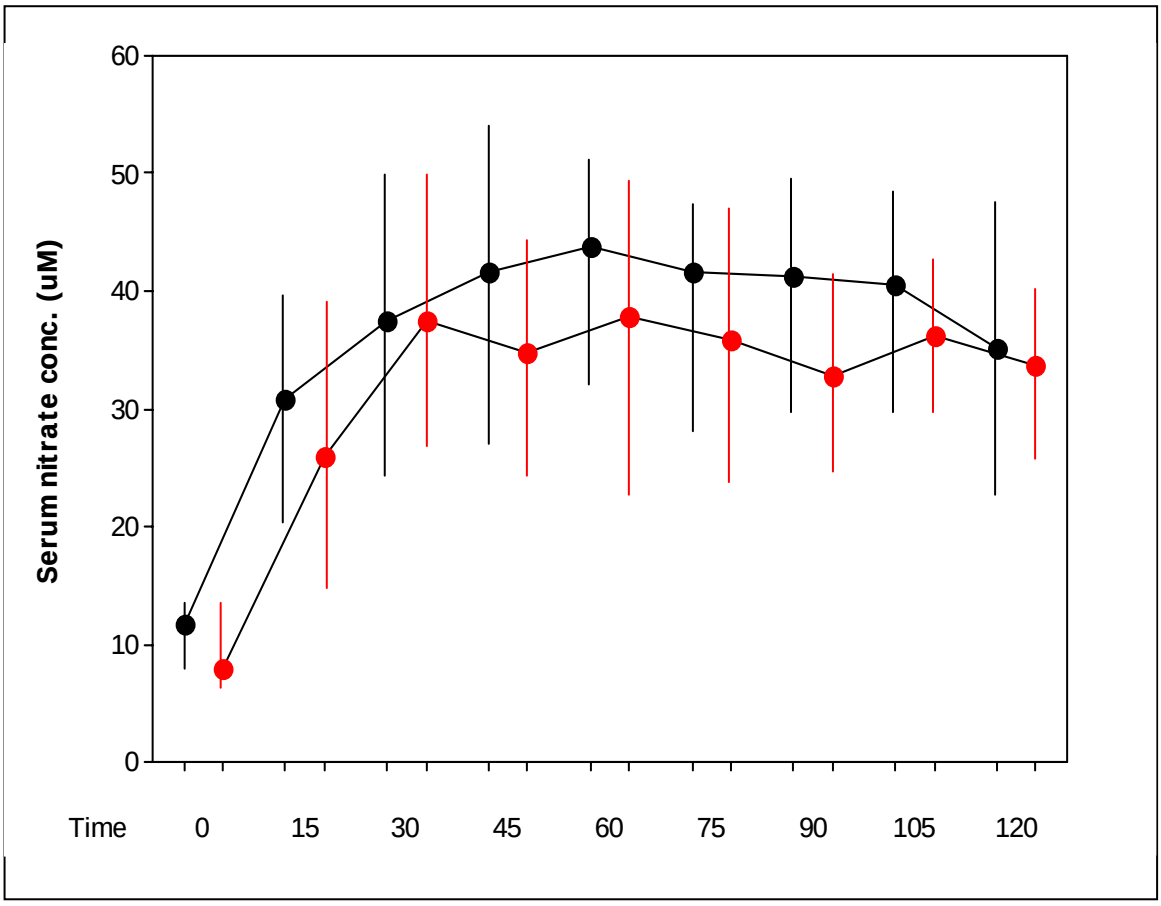
Gastro-oesophageal acid reflux was defined as pH <4 detected 5cm proximal to the clips marking the top of the gastric folds (the anatomical gastro-oesophageal junction). pH was <4 at this location for a median of 14.5% (IQ range 2.6-48.7%) of 32 study days. On the 16 study days when reflux was stimulated by postural and pharmacological methods, pH was less than 4 at this location for a median of 40% (IQ range 14.3-63.6%). This was significantly higher than the 3.6% (0.1-19%) when reflux was not stimulated (p=0.003) which was in turn significantly higher than 0.1% (0-1.0%) in the healthy volunteers who received nitrate (p = 0.03).

6.3.2 Serum nitrate

Prior to administration of the study drink, median serum nitrate concentration was 11.3 (7-13.4) uM on the day when reflux was not provoked, not significantly different from 7.8 (5.8-13.4) uM on the day we provoked reflux. Following administration of 2mmoles of potassium nitrate, serum nitrate concentrations

increased almost four fold on both days, peaking after 60 minutes at 43.1 (30.4-50.6) uM on the day when reflux was not provoked and 37.5 (24-48.6) uM on the day we provoked reflux (Figure 6.3). Basal and peak nitrate concentrations in patients with Barrett's oesophagus were not significantly different from the healthy volunteers.

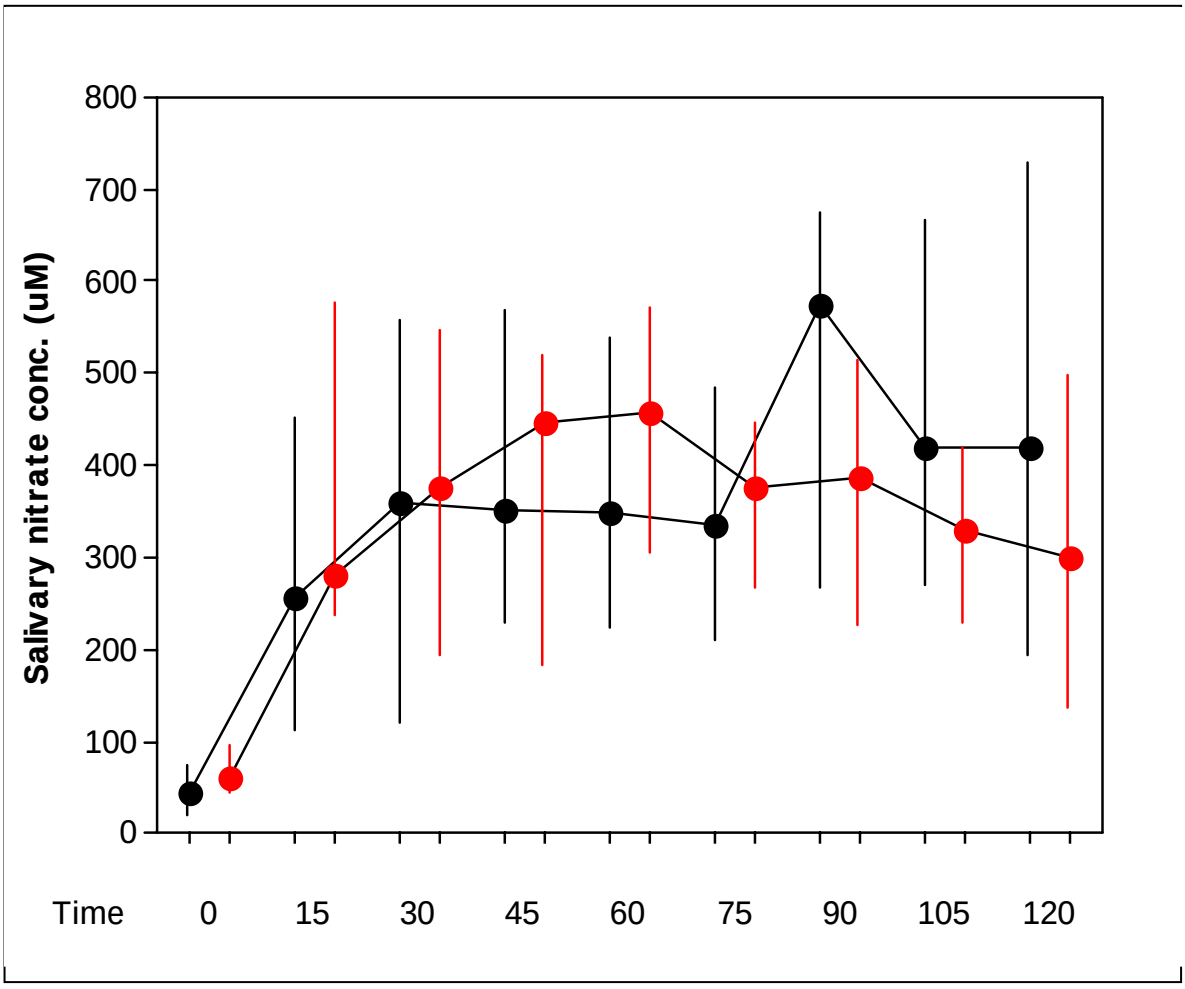
Figure 6.3: Median serum nitrate concentration with time for 16 Barrett's oesophagus patients. Black dots represent concentrations on the day reflux was not provoked and red dots represent concentrations on the day we provoked reflux. Whiskers depict interquartile ranges.



6.3.3 Salivary nitrite

Prior to administration of the study drink, median salivary nitrite concentration was 38.2 (16.4-70.7) uM on the day when reflux was not provoked, not significantly different from 59.2 (41 – 90.1) uM on the day we provoked reflux. Following administration of 2mmoles of potassium nitrate, salivary nitrite concentrations increased significantly on both days, peaking at 578 (271-666) uM after 90 minutes on the day when reflux was not provoked and at 457.7 (307-572.6) uM after 60 minutes on the day we provoked reflux (figure 6.4). Basal and peak salivary nitrite concentrations in patients with Barrett's oesophagus were not significantly different from the healthy volunteers.

Figure 6.4: Median salivary nitrite concentration with time for 16 Barrett's oesophagus patients. Black dots represent concentrations on the day reflux was not provoked and red dots represent concentrations on the day we provoked reflux. Whiskers depict interquartile ranges.

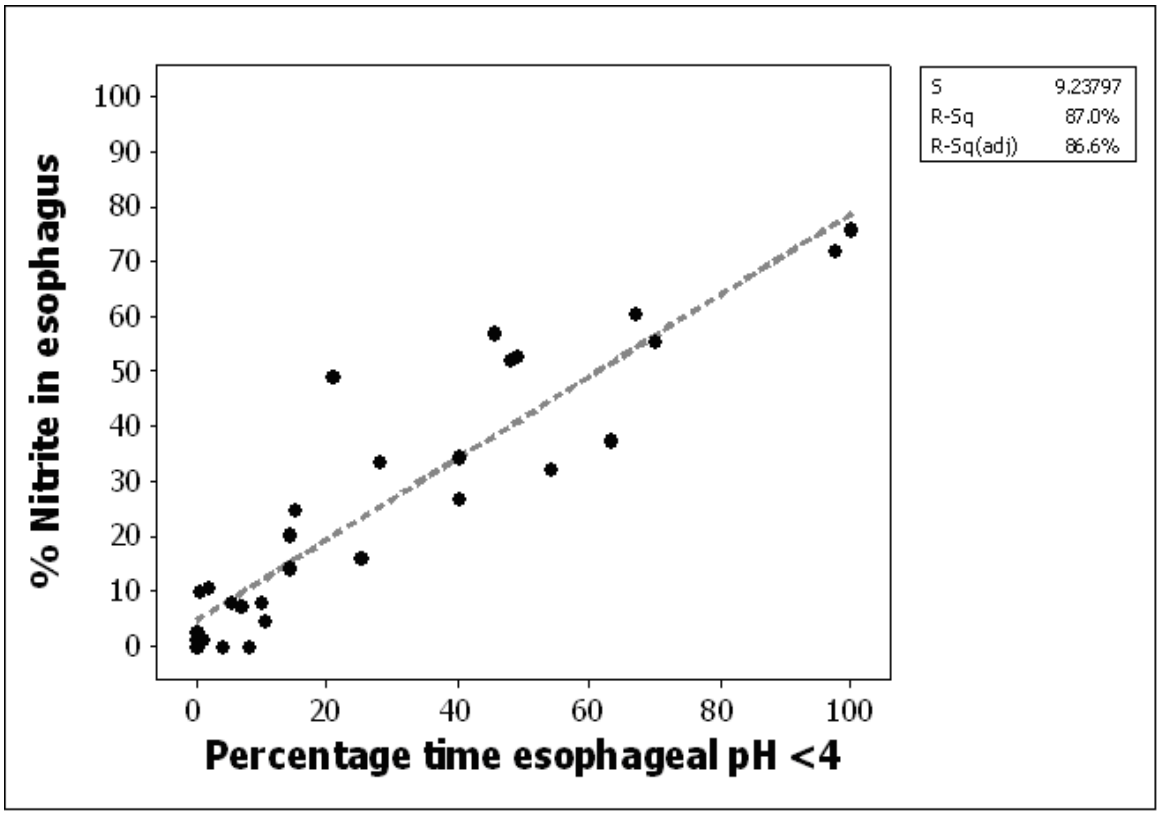


6.3.4 Silastic tube nitrite

On the day that reflux was stimulated in the patients with Barrett's oesophagus, a median of 33% (17.13 – 54.9%) of the total nitrite formed within the silastic tube was formed within the oesophageal sections. This was a significantly greater proportion than 8.1% (1.5 – 31.8%) when reflux was not stimulated (p= 0.01). In the healthy volunteers who received nitrate, the proportion of nitrite present within the oesophagus was 0.7 % (0-2.5%), which was significantly lower than the Barrett's patients in whom reflux was not stimulated (p=0.007).

The concentration profile of nitrite along the length of the tube in the 32 studies from 16 Barrett's patients correlated with the frequency of reflux. A linear relationship existed between the duration of acid reflux (proportion of the study that pH was <4 at 5cm above the gastro-oesophageal junction) and the proportion of the total nitrite that was formed within the oesophageal sections (Spearman ρ 0.927, $p < 0.01$), with 80% of the nitrite being formed within the oesophagus during reflux. (figure 6.5)

Figure 6.5: Fitted line plot comparing percentage of time pH <4 5cm above the gastro-oesophageal junction (marked by the clips at the top of the gastric folds) with the proportion of total nitrite formed within the oesophageal sections



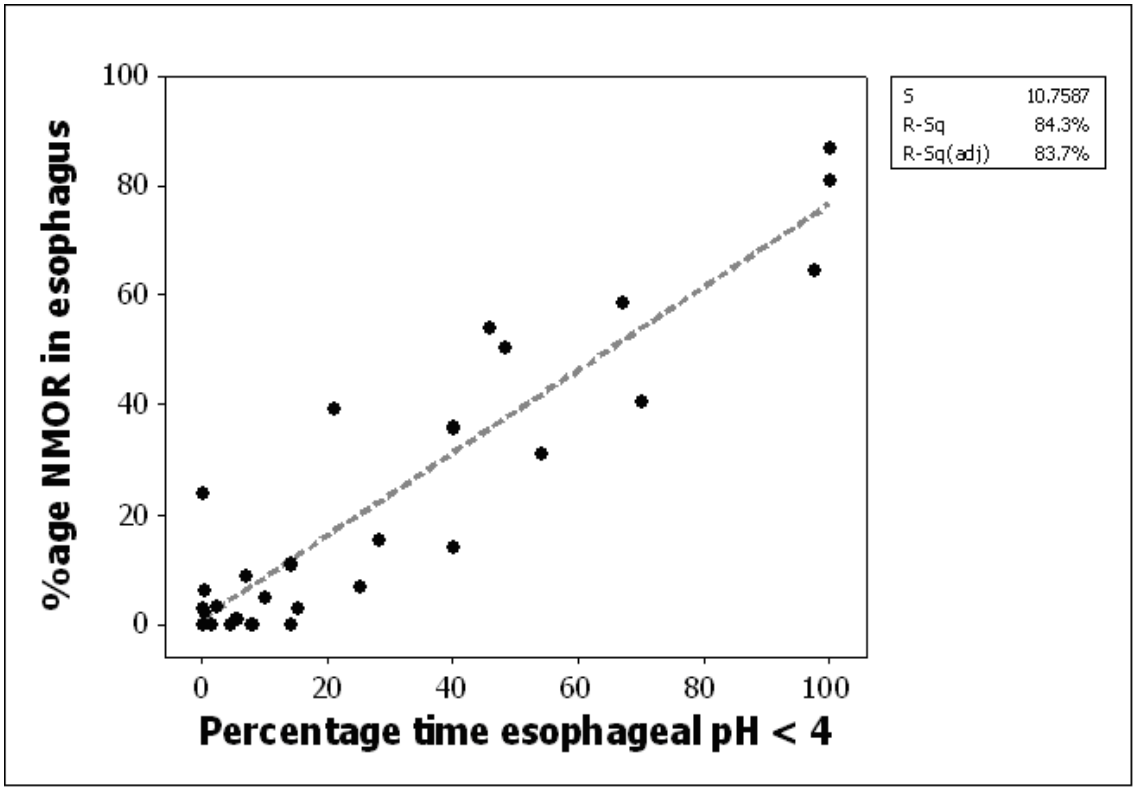
6.3.5 Silastic tube *N*-nitrosomorpholine

On the day that reflux was stimulated in the patients with Barrett's oesophagus, a median of 29% (6.9– 50.5%) of the total *N* –nitrosomorpholine formed within the silastic tube was formed within the oesophageal sections. This was significantly greater than 5.1% (0 – 24%) on the day when reflux was not stimulated ($p<0.001$). In the healthy volunteers who received nitrate, the proportion of *N* –nitrosomorpholine present within the oesophagus was 1.6 % (0-5.1%), which was lower than the Barrett's patients in whom reflux was not stimulated.

From 28 studies in 14 Barrett's patients, a linear relationship existed between the duration of acid reflux and the proportion of the total *N*-nitrosomorpholine that was formed within the oesophageal sections (Spearman's ρ 0.75, $p<0.01$), with 80% being formed in the oesophagus during reflux. (figure 6.6)

Twelve patients with Barrett's oesophagus were administered nitrate that was 99% enriched with the heavy nitrogen isotope ^{15}N on each study day. Median enrichment was 74.6% (59-83.1), confirming that the *N*-nitrosomorpholine formed was derived from the nitrate ingested.

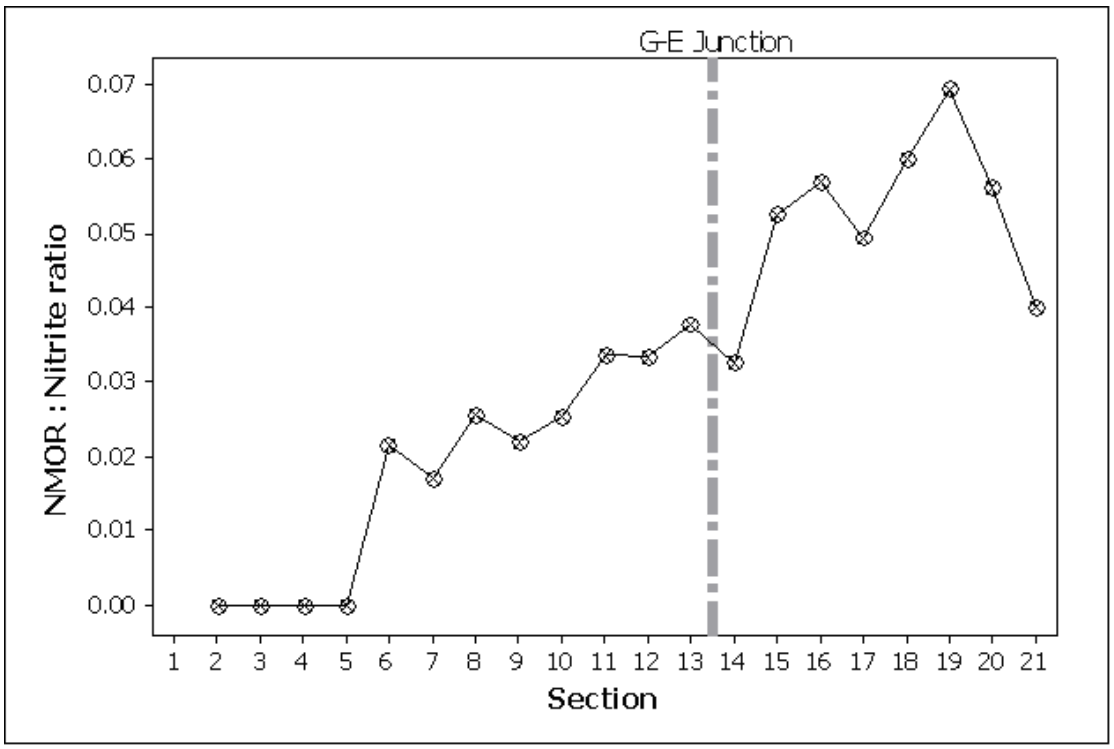
Figure 6.4: Fitted line plot comparing percentage of time pH <4 5cm above the gastro-oesophageal junction (marked by the clips at the top of the gastric folds) with the proportion of total *N*-nitrosomorpholine formed within the oesophageal sections.



6.3.6 Correlation of silastic tube nitrite versus *N*-nitrosomorpholine

We calculated the ratio of *N*-nitrosomorpholine: nitrite concentrations for each silastic tube section in all of the subjects with Barrett's oesophagus. The median of all ratios of *N*-nitrosomorpholine: nitrite concentration was 0.053 (0.026-0.089) for the gastric sections. This was significantly higher than 0.028(0-0.06) for the oesophageal sections ($p<0.001$), but not significantly different from 0.064 (0.045 - 0.099) for the gastric sections of the healthy volunteers who had received nitrate. This finding indicates that a greater proportion of the nitrite measured within the oesophagus was generated via nitrous acid compared with the stomach. Median *N*-nitrosomorpholine concentration as a percentage of nitrite concentration by location is depicted in figure 6.7.

Figure 6.7: Median ratio of *N*-nitrosomorpholine: nitrite by location in the upper GI tract in patients with Barrett's oesophagus.



CHAPTER SEVEN

DISCUSSION

Our studies demonstrate a novel mechanism by which dietary nitrate may induce nitrosative stress and generate *N*-nitroso compounds within the upper gastrointestinal tract of subjects with healthy acid-secreting stomachs. This mechanism is via the generation of nitric oxide formed when nitrite in saliva encounters acidic gastric juice containing reducing agents such as ascorbic acid. The nitric oxide formed in this way can diffuse from the lumen into an adjacent compartment of neutral pH and, within it, combine with oxygen to form the nitrosating species N_2O_3.

The potential for generation of *N*-nitroso compounds within the lumen of the acidic stomach has been recognised for many years [95, 143]. This is due to the large amounts of nitrite constantly delivered into the stomach in swallowed saliva [72, 87]. The nitrite in saliva is derived from the entero-salivary recirculation of dietary nitrate and its reduction to nitrite by buccal bacteria [86, 87, 144]. When the nitrite encounters the acidic pH of gastric juice at the gastric oesophageal junction, it is immediately converted to nitrous acid and nitrosating species such as NO+, NOSCN and N_2O_3. The latter can react with secondary amines to form potentially carcinogenic *N*-nitroso amines. The main factor protecting against this acid catalysed *N*-nitrosation is ascorbic acid [93] which effectively competes with secondary amines for the nitrosating species. In this reaction between the nitrosating species and ascorbic acid, the former is reduced to nitric oxide and the latter oxidized to dehydroascorbic acid. In this previously recognised pathway of acid catalysed nitrosation, the reaction between the nitrosating species and ascorbic acid has been regarded as entirely protective by preventing the generation of *N*-nitroso compounds within the acidic lumen. However, our current studies demonstrate that the nitric oxide formed from the reaction between ascorbic acid

and acidified nitrite can induce nitrosative stress and the generation of *N*-nitroso compounds within an adjacent compartment of neutral pH.

How do we know that the *N*-nitrosomorpholine generated *in-situ* within the epithelial compartment was formed via nitric oxide and not by the direct reaction between morpholine and the nitrosating species formed by the acidifications of salivary nitrite? In our previously published bench top studies [81], we examined the generation of *N*-nitrosomorpholine within the silastic tube when its exterior surface was exposed to the chemical conditions occurring within the lumen of the human proximal stomach. The amount of *N*-nitrosomorpholine formed inside the silastic tube was directly proportional to the nitric oxide concentration generated in the luminal compartment to which the external surface of the tube was exposed. If the luminal compartment contained only nitrite and hydrochloric acid then the nitrosating species formed in this solution only generated N-nitrosomorpholine in the same luminal compartment and not in the epithelial compartment. However, the further addition of ascorbic acid to the luminal compartment prevented nitrosation in this compartment and produced high concentrations of nitric oxide in the luminal compartment and high concentrations of N-nitrosomorpholine in the epithelial compartment. This, therefore, indicates that the *N*-nitrosomorpholine generated within the silastic tube is derived from nitric oxide entering the tube and not from acid catalysed nitrosating species entering the tube.

How do we know that the *N*-nitrosomorpholine generated within the tube was derived from the administered nitrate? The administration of nitrate increased the concentration of *N*- nitrosomorpholine formed in the silastic tube in the healthy volunteers. Further confirmation of the origin of this *N*-

nitrosomorpholine was obtained by administering ^{15}N nitrate to 8 of our healthy volunteers and 12 of our patients with Barrett's oesophagus. In these subjects, we found that 75% of the *N*-nitrosomorpholine formed within the tube was enriched with ^{15}N. This demonstrates that the administered nitrate had been absorbed from the gastrointestinal tract, taken up the salivary glands and secreted into the mouth, reduced to nitrite by buccal bacteria, swallowed in saliva, converted to nitrous acid and nitrosating species when encountering acidic gastric pH and converted to nitric oxide by ascorbic acid or other reducing agents within the stomach. The nitric oxide generated was then able to pass through the gas permeable silastic wall of the tube and, within that neutral pH environment, react with oxygen to form N_2O_3 which in turn reacted with the secondary amine to form *N*-nitrosomorpholine.

In addition to *N*-nitrosomorpholine, we also detected substantial amounts of nitrite within the silastic tube exposed to gastric acidity. Our previous studies have indicated two mechanisms by which nitrite can accumulate within the silastic tube exposed to conditions occurring within the lumen of the human upper gastrointestinal tract. The first and major mechanism is by nitric oxide formed from the reaction between nitrosating species and ascorbic acid, diffusing through the wall of the tube and then reacting with O_2 to form the nitrosating species N_2O_3. The latter can then react either with the secondary amines to form *N*-nitrosamines as described above or can react with water to form nitrite ($N_2O_3 + H_2O \dashrightarrow 2\ NO_2^- + 2\ H^+$). The second mechanism by which nitrite may accumulate within the tube is due to nitrous acid diffusing through the wall of the tube. Nitrous acid is a weak acid ($HNO_2 \leftrightarrow H^+ + NO_2^-$: pKa = 3.34) and at intragastric pH it exists predominantly as the relatively non-polar molecule HNO_2

which will be able to pass through the wall of the silastic tube. Nitrous acid is a much larger molecule than nitric oxide and thus passes through the silastic tube wall much more slowly. When the nitrous acid passes through the wall of the silastic tube and encounters the solution of neutral pH within the silastic tube, it is immediately converted to nitrite. Unlike nitric oxide, nitrous acid does not form N_2O_3 or other nitrosating species required for the formation of *N*-nitroso compounds within the neutral pH environment of the tube. In addition, when nitrous acid diffuses through the wall of the tube, it does not result in the generation of *N*-nitrosocompounds within the tube. This is due to the fact that nitrous acid only forms nitrosating species at acidic pH. The ratio of *N*-nitrosomorpholine to nitrite formed within the tube indicates the extent to which the nitrite added to the acidic luminal compartment has been converted to nitrous acid and nitrosating species versus nitric oxide. When ascorbic acid is present in abundance in the luminal compartment causing all the added nitrite to be converted to nitric oxide, then the ratio is 0.1- 0.2 (median of 0.17 in our bench-top studies from this thesis [chapter 4, page 67]) . In contrast, when there is no ascorbic acid in the surrounding medium and any nitrite added is in the form of nitrous acid or nitrosating species, then the ratio is less than 0.01 as almost no *N*-nitrosomorpholine is formed [81].

In the present study, administration of nitrate to the healthy volunteers not only increased the production of *N*-nitrosomorpholine and nitrite within the tube but also reduced the ratio of *N*-nitrosomorpholine to nitrite within the tube. This can be explained by the increase in nitrite in swallowed saliva following nitrate administration not all being converted to nitric oxide in the proximal stomach but some remaining in the form of nitrous acid and nitrosating species. This will

happen if the increased nitrite load overwhelms the gastric juice concentrations of ascorbic acid and its ability to reduce all the nitrous acid and nitrosating species to nitric oxide. This means that following the nitrate administration, there were nitrosating species within the lumen of the gastrointestinal tract and thus the potential for generation of *N*-nitroso compounds by the previously well recognised acid-catalysed luminal chemistry.

The design of our silastic tube also enabled us to investigate the anatomical location of the nitrosative chemistry occurring throughout the upper gastrointestinal tract and its correlation with luminal pH. In the healthy volunteers with or without nitrate administration, no nitrite or *N*-nitrosomorpholine was formed in the segments located in the oesophagus and not exposed to gastric acid. Without nitrate administration both nitrite and *N*-nitrosomorpholine were formed in the segments exposed to the acidic gastric environment and the concentrations of both were highest in the most proximal segments constantly exposed to intragastric acidity. Following nitrate administration, the peak nitrite concentration was again evident in the proximal stomach but the *N*-nitrosomorpholine formed more of the plateau. There was therefore a tendency for the ratio of nitrite to *N*-nitrosomorpholine to be lower in the proximal stomach following the administration of nitrate. This may be explained by the higher amounts of nitrite delivered into the proximal stomach following the nitrate, exhausting the ascorbic acid concentration in the most proximal stomach and allowing nitrite to enter as nitrous acid rather than as nitric oxide. We have previously observed that the ratio of nitrite to ascorbic acid is highest in the most proximal stomach [126].

One of the healthy volunteers was found to be achlorhydric. In this subject, no nitrite and no *N*-nitrosomorpholine was detected in any of the tube segments. This highlights the key role of acid in forming nitrosating species within the lumen and subsequently in generating nitric oxide which may form *N*-nitrosomorpholine within the silastic tube. There is of course a third mechanism of *N*-nitrosation and which may occur in the achlorhydric stomach. This is when the stomach becomes colonised by bacteria which can both reduce nitrate to nitrite and nitrite to nitrosating species and *N*-nitroso compounds. This mechanism which occurs in the achlorhydric stomach is thought to be relevant in patients who develop non-cardia gastric cancer secondary to atrophic gastritis [94, 145].

We were able to investigate the effect of acidic gastro-oesophageal reflux on the location of the nitrosative chemistry. In order to do this, we studied patients with Barrett's oesophagus as they usually have severe reflux disease. In these subjects, we found that nitrite and *N*-nitrosomorpholine was detected in the oesophageal, as well as gastric, segments. In addition, the extent to which the chemistry was detected in the oesophagus versus stomach was directly related to the amount of reflux occurring while the probe was *in-situ*. From this correlation, it is apparent that during reflux 80% of the nitrosating chemistry was occurring within the oesophageal segments. Normally, the nitrosative chemistry occurs due to the swallowed saliva encountering acid on entering the stomach and occurs predominantly within the proximal stomach. In contrast, during reflux, the chemistry occurs in the oesophagus due to the acid entering the oesophagus and reacting with the nitrite within the oesophageal lumen. The impaired oesophageal clearance characteristic of severe reflux disease is likely to contribute to the degree of nitrosative chemistry occurring within the oesophagus.

We were also able to study the ratio of nitrite to nitrosomorpholine occurring within the oesophageal segments versus the gastric segments. The ratio of nitrite to *N*-nitrosomorpholine observed in the stomach of the reflux patients following nitrate was similar to that observed in the healthy volunteers following nitrate. However, the ratio in the oesophageal sections of the silastic tube was significantly lower than in the stomach, indicating that in the oesophagus a higher proportion of the luminal nitrite was in the form of nitrous acid and nitrosating species versus nitric oxide than in the stomach. A similar trend was observed in the proximal versus the distal stomach of the healthy volunteers following nitrate as discussed above. This indicates that, during acid reflux, nitrate induces nitrosative stress within the oesophagus by two distinct mechanisms. The first mechanism involves generation of high concentrations of luminal nitric oxide arising from reaction between salivary nitrite and acidic gastric juice containing ascorbic acid and this will be the sole mechanism when nitrite is present in excess of ascorbic acid. This nitric oxide can diffuse into the epithelial cells, exerting nitrosative stress. The second mechanism involves the generation of nitrosating species within the lumen due the acidification of nitrite. This mechanism occurs when luminal supplies of ascorbic acid have been exhausted. Both mechanisms of nitrosative stress are also apparent in the proximal stomach following nitrate.

Currently available analytical methods do not allow the measurement of *N*-nitroso compounds within endoscopic epithelial biopsies and we therefore studied their generation using an *in situ* compartment of neutral pH containing the *N*-nitrosatable compound morpholine. Our current studies do not allow accurate prediction of the extent to which the luminal nitrosative chemistry is generating *N*-nitroso compounds within the actual epithelial cells. However, both of these

mechanisms of nitrosative stress could result in damage to oesophageal epithelial DNA in humans. Epithelial cells contain an abundant and wide range of compounds (secondary amines, polyamines, amino acids, peptides and amides) which can be *N*-nitrosated to mutagenic products[129-134] and therefore provide potential substrates for nitric oxide-mediated *N*-nitrosation. Additionally, during reflux of gastro-duodenal contents into the oesophagus, the oesophageal lumen contains nitrosatable compounds such as bile acid conjugates, which are potential substrates for luminal, acid-mediated *N*-nitrosation to form *N*-nitrosamines such as *N*-nitrosoglycocholic acid[129, 130]. In animal models, oesophageal epithelium subject to duodeno-oesophageal reflux is more susceptible to the mutagenic effects of luminal *N*-nitrosocompounds[79, 146, 147].

Our use of morpholine within the silastic tube provides a measure of nitric oxide-mediated nitrosative stress *per se*, and not just of its ability to form *N*-nitroso compounds. Nitric oxide-induced nitrosative stress can also induce DNA damage by other mechanisms. These include directly de-aminating DNA and inactivating DNA repair enzymes such as O_6-alkylguanine DNA alkyl transferase[148-150] and DNA repair proteins such as formamidopyrimidine DNA-glycosylase that repairs 8-oxodeoxyguanine residues[146, 151, 152].

A synergistic action may therefore occur during reflux of gastro-duodenal contents into the oesophagus. Erosion of the oesophageal epithelial surface by acidic reflux will make the basal stem cells more exposed to the luminal nitrosative chemistry. In addition, acid reflux stimulates basal cell proliferation and replicating DNA will therefore be more sensitive to the mutagenic effects of nitric oxide and of luminally and epithelially generated *N*-nitroso compounds, all produced by the same acid reflux[153-156].

Further studies are required to determine if the nitrosative stress demonstrated in our *in situ* epithelial model translates into cellular damage and mutagenesis and identification of a suitable biomarker is needed. This could be investigated by looking for specific or non-specific markers of DNA damage in oesophageal and gastric epithelial cells following exposure to high luminal concentrations of nitric oxide.

N-nitrosocompounds result in specific damage to the composition of DNA. *In vitro* studies have detected the presence of the *N*-nitroso compound specific adduct O(6)-carboxymethylguanine in human blood DNA following reaction of nitric oxide with glycine[157]. Further studies into the effect of diet on colon cancer have demonstrated that volunteers on a red meat diet had a significant increase in the endogenous formation of *N*-nitrosocompounds compared with vegetarians as measured by apparent total *N*-nitroso compound in faeces. This rise in colonic *N*-nitroso compounds was associated with significantly higher numbers of exfoliated colonic cells staining positive for the DNA adduct O(6)-carboxymethyl guanine[158].

Non specific DNA damage following nitric oxide exposure could potentially be assessed using the comet assay, which measures strand breaks and alkali-labile sites in DNA. This assay has confirmed higher levels of DNA damage within Barrett's mucosa than squamous oesophageal mucosa in Barrett's oesophagus patients, with the highest levels of DNA damage being associated with increased risk of development of high grade dysplasia and malignancy[159]. It has also been used to highlight potentially mutagenic consequences of employing chromo-endoscopy with methylene blue in Barrett's surveillance programmes[160].

Further studies are also required to ascertain the stage at which *N*-nitrosative stress may influence mutagenesis. In preliminary studies, malignant and pre-malignant oesophageal cell lines have been exposed to physiological levels of nitric oxide. Nitric oxide exposure increased the ability of cell lines containing adenocarcinoma or high grade dysplasia to invade through Matrigel, but did not affect the behaviour of non-dysplastic Barrett's oesophagus cell lines. Nitric oxide induced invasive behaviour correlated with the induction of matrix metalloproteinases (MMPs) 1 and 9 and tissue inhibitor of MMP 1 (TIMP1), all of which have previously been implicated in invasion[161]. Maximum expression of genes for these proteins was observed 1-3 hours after the addition of nitric oxide. These findings indicate that nitric oxide may promote disease progression in the later stages of carcinogenesis by increasing invasive potential through the regulation of genes known to be involved in invasion. Although the cell lines were exposed to concentrations of nitric oxide similar to those found where saliva meets gastric acid, it is unclear if they were subjected to the same rapid rate of delivery as occurs physiologically and further investigations in this area are warranted.

In summary, our studies indicate that nitric oxide generated by the reaction between salivary nitrite and acidic gastric juice may induce nitrosative stress and generation of *N*-nitroso compounds within adjacent compartments of neutral pH such as the epithelial cells. Our studies also demonstrate that during acid reflux, this potentially mutagenic chemistry occurs predominantly or entirely within the lumen of the oesophagus. This chemistry may be particularly harmful to patients with erosive esophagitis whose epithelium will be more sensitive to chemical mutagenesis.

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