THE INFLUENCE OF DIETARY NITRATE AND ACID SUPPRESSION ON THE INTRAGASTRIC ENVIRONMENT AND THE RISK OF CARCINOGENESIS

by

CRAIG MOWAT, MBChB (Glasgow), MRCP (UK).

Thesis presented to the University of Glasgow

for the degree of Doctor of Medicine

from the

University Department of Medicine and Therapeutics

Western Infirmary

Glasgow

submitted May 2000

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

PREFACE

This body of work was produced over 2 years during which I had the fortune to work for Professor Kenneth McColl, a distinguished authority in gastroenterology and expert in the field of *Helicobacter pylori* infection. Initially I felt daunted and was unsure whether I had the qualities required for research. However, I received tremendous encouragement and quickly came to cherish the unique fellowship that is founded through the pursuit of a common research goal.

My work provided an opportunity to study basic science, perform laboratory bench work and engage in clinical studies. Some of the work has been published and reprints are submitted with this thesis. In all areas I have learned to appreciate the value of thorough preparation and attention to detail.

Collaboration with colleagues was necessary and this is described in the acknowledgements. Except where stated, the work presented was carried out by myself.

The writing of this thesis is entirely my own work.

ABSTRACT

In 1975, Correa's original hypothesis for gastric carcinogenesis proposed that atrophic gastritis leads to achlorhydria and secondary colonisation of the stomach with nitrosating bacteria. These organisms reduce dietary nitrate to nitrite, then catalyse the synthesis of potentially carcinogenic N-nitroso compounds (NOC) from nitrite and secondary amines present in gastric juice.

Since then, significant advances have been made in our understanding of gastric physiology which are also relevant to our understanding of gastric carcinogenesis. *Helicobacter pylori* (H. pylori) infection is now a recognised risk factor for gastric carcinogenesis. If it produces a pangastritis, there is an associated degree of gland-loss (atrophy) of the acid-secreting body of the stomach. Atrophic gastritis is a precursor of the development of gastric cancer. H. pylori infection also lowers levels of vitamin C, which is secreted into and concentrated within gastric juice. The synthesis of NOC is inhibited by vitamin C.

Advances in the treatment of acid-related disorders have resulted in the prescription of increasingly potent inhibitors of acid secretion. The proton pump inhibitors (PPI's) render H. pylori infected subjects achlorhydric from one dose to the next. They also promote the development of a pangastritis. Whether these effects are harmful in the long term remains hotly debated. Two recent reports suggest that long term PPI therapy does lead to development of moderate-severe atrophy of the gastric body in ~18% of H. pylori infected subjects, compared to 0 - 2% of no-infected subjects, thereby placing these patients at risk of gastric

cancer. There are great concerns that the falling incidence of gastric cancer in the developed world may be reversed by widespread prescription of these agents.

How PPI's produce gastric atrophy in H. pylori infected patients remains to be elucidated. This thesis postulates that, in the presence of co-existing H. pylori infection, drug-induced achlorhydria alters the intragastric environment in a way that will facilitate bacterial synthesis of carcinogenic NOC which in turn may damage gastric epithelial stem cell DNA.

The thesis begins by re-examining the roles of nitrate, nitrite and vitamin C in man. The enterosalivary recirculation of ingested nitrate is described, whereby dietary nitrate is reduced to nitrite in saliva to provide the main source of nitrite to the upper GI tract. The important chemical interactions between nitrite and ascorbic acid *in vitro* are described with reference to NOC synthesis. NOC synthesis is promoted by a high nitrite : ascorbic acid ratio. The *in vivo* interactions between saliva nitrite and gastric ascorbic acid are speculated upon and the potential effects of PPI-induced achlorhydria and H. pylori infection on these interactions are considered.

To begin, novel studies infused nitrite into the acid stomach to mimic salivary nitrite influx following a nitrate meal. These studies demonstrate for the first time *in vivo* the interaction between nitrite and vitamin C in the gastric lumen. Gastric juice ascorbic acid is depleted and in the process is converted to dehydroascorbic acid. Nitrite is lost from solution - presumably as nitric oxide. These novel studies were repeated, replacing the nitrite infusion with a solution of nitrate to mimic the

effects of ingesting a portion of lettuce. These studies confirm that ingestion of nitrate leads to elevation of saliva nitrite levels via the process of enterosalivary recirculation. They reveal that on meeting acid gastric juice, saliva nitrite is lost (presumably as nitric oxide gas) and ascorbic acid levels are depleted. This novel *in vivo* observation will occur where saliva and gastric juice first meet; namely the gastro-oesophageal junction. The role of nitric oxide at the gastro-oesophageal junction is speculated upon.

Studies focus on the process of enterosalivary recirculation of ingested nitrate. Oral bacteria generate nitrite from saliva nitrate within the mouth. Two groups are studied to examine whether the presence or absence of natural teeth is of importance to this process. The saliva nitrite levels generated in each group of subjects following the standard nitrate meal are similar, suggesting that bacteria resident in the dorsum of the tongue may be more important than those in gingival crevices. The effect of antibiotics on the enterosalivary recirculation of nitrate is then assessed. The antibiotics produced an effect in one subject only. Fasting nitrite levels in saliva and gastric juice became undetectable and did not rise following the nitrate meal. In the absence of nitrite, gastric juice ascorbic acid levels were preserved. It was concluded that oral bacteria are of prime importance in generation of nitrite. The effects of antibiotics on synthesis of nitric oxide within the gastric lumen are speculated upon.

The effect of prolonged acid inhibition on the chemistry of nitrite and vitamin C is then examined. Subjects are studied following a 4 week course of omeprazole 40mg daily. They are studied 2 hours following the last dose of the drug, when all

subjects are achlorhydric. Omeprazole raised median fasting gastric nitrite and lowered fasting gastric ascorbic acid to significant degrees. After the nitrate meal gastric nitrite levels were markedly increased. In H. pylori infected subjects, omeprazole also decreased total vitamin C levels in both gastric juice and serum. These changes in combination would facilitate bacterial synthesis of NOC. The reason for the elevated gastric nitrite is considered and that the chemical stability of nitrite at neutral pH is suggested as the main factor. In subjects with H. pylori infection, activated mucosal nitric oxide synthase is discounted as an additional source.

The studies conclude by examining whether the greater acid inhibition produced by PPI therapy in H. pylori +ve versus -ve subjects is indeed accompanied by more profound changes in the intragastric milieu which will facilitate bacterial synthesis of N-nitrosocompounds. During omeprazole, H. pylori +ve subjects recorded a higher intragastric pH and greater colonisation with non-H. pylori species. These bacteria included nitrosating species. H. pylori +ve subjects had higher intragastric nitrite levels following the nitrate meal. Omeprazole lowered intragastric vitamin C levels in H. pylori +ve subjects only. It is concluded that H. pylori +ve subjects on omeprazole experience disturbances in intragastric nitrite, vitamin C and bacterial colonisation that will facilitate bacterial N-nitrosation. This may place them at risk of future mutagenesis and carcinogenesis.

On the basis of these findings it is recommended that patients requiring long term PPI therapy have any co-existing H. pylori infection eradicated.

CONTENTS

	Page number
Preface	2
Abstract	3
Table of contents	7
List of figures	11
List of tables	12
Acknowledgements	13
Abbreviations	14
CHAPTER	
1. INTRODUCTION	15
1.1 Gastric carcinogenesis; the current hypothesis	16
1.2 Recent developments relevant to gastric carcinogenesis	17
1.3 The potential effects on gastric juice of drug-induced hypo/achlorhydria	21
1.4 Review of the properties of nitrate, nitrite and vitamin C	22
1.4.1 Nitrate	22
1.4.2 Nitrite	23
1.4.3 Vitamin C	25
1.5 The potential effects on gastric juice of drug-induced hypo/achlorhydria and co-existing H. pylori infection	27
1.5.1 PPI therapy, H. pylori infection and intragastric nitrite	27
1.5.2 PPI therapy, H. pylori infection and Vitamin C	29
1.5.3 PPI therapy, H. pylori infection and colonisation with nitrosating bacteria	29
1.6 Aims and hypothesis to be tested	30

2. METHODS 31

2.1 Study procedures 32

2.2 Analysis of nitrate, nitrite and vitamin C 34

2.3 Data analysis 39

3. NOVEL *IN VIVO* STUDIES OF THE INTERACTION BETWEEN NITRITE AND VITAMIN C IN GASTRIC JUICE AND THE INFLUENCE OF INTRAGASTRIC pH

3.1 Introduction 41

3.2 Subjects and Methods 43

3.3 Results 44

3.4 Figure legends and figures 47

3.5 Conclusions 50

4. THE EFFECTS OF DIETARY NITRATE ON LEVELS OF VITAMIN C AND NITRITE IN GASTRIC JUICE

4.1 Introduction 53

4.2 Subjects and Methods 53

4.3 Results 53

4.4 Figure legend and figure 56

4.5 Conclusions 58

5. THE EFFECTS OF DIETARY NITRATE AND PROLONGED OMEPRAZOLE THERAPY ON LEVELS OF VITAMIN C AND NITRITE IN GASTRIC JUICE

5.1 Introduction 62

5.2 Subjects and Methods 62

5.3 Results 62

5.3.1 *Helicobacter pylori* status and nitrate, nitrite and vitamin C 64

5.3.2 Studies of the contribution of mucosal nitric oxide synthase to intragastric nitrite concentrations 65

5.4 Figure legends and figures 68

5.5 Conclusions 74

6. STUDIES ON THE EFFECTS OF DENTAL STATUS, BACTERIA AND ANTIBIOTICS ON THE METABOLISM OF DIETARY NITRATE

6.1 Effect of dental status on the metabolism of dietary nitrate 82

6.1.1 Introduction 82

6.1.2 Subjects and Methods 82

6.1.3 Results 83

6.1.4 Figure legend and figure 84

6.1.5 Conclusions 86

6.2 Studies on effect of antibiotics on the metabolism of dietary nitrate in the presence of achlorhydria

6.2.1 Introduction 87

6.2.2 Subjects and Methods 87

6.2.3 Results 88

6.2.4 Figure legend and figure 91

6.2.5 Conclusions 93

7. WILL *HELICOBACTER PYLORI* INFECTION INCREASE THE RISK OF DEVELOPING GASTRIC CANCER DURING LONG TERM OMEPRAZOLE?

7.1 Introduction 96

7.2 Subjects and Methods 97

7.3 Results 100

7.4 Figure legends and figures and table 106

7.5 Conclusions 111

8. FINAL DISCUSSION AND CONCLUSIONS 117

REFERENCES 120

PUBLICATIONS AND COMMUNICATIONS 136

LIST OF FIGURES

Figure **Page number**

3.1 Effect of an intragastric infusion of nitrite on gastric juice nitrite levels in the acid stomach 48

3.2 Effect of an intragastric infusion of nitrite on gastric juice AA levels in the acid stomach 48

3.3 Effect of an intragastric infusion of nitrite on gastric juice TVC levels in the acid stomach 49

3.4 Effect of an intragastric infusion of nitrite on gastric juice nitrite levels at pH>4 49

4.1 Effect of standard nitrate meal on serum nitrate, saliva nitrite, gastric nitrite, TVC and AA 57

5.1 Effect of omeprazole on fasting gastric nitrite, AA and gastric nitrite levels post nitrate meal 69

5.2 Effect of standard nitrate meal, in the presence of omeprazole-induced hypochlorhydria, on serum nitrate, saliva nitrite, gastric nitrite, gastric TVC and gastric AA 71

5.3 Effect of standard nitrate meal on gastric TVC in H. pylori positive and negative subjects before and on omeprazole 72

5.4 Effect of 4 weeks treatment with omeprazole 40mg daily on fasting serum TVC levels in H. pylori positive and negative subjects 73

6.1 Effect of a nitrate meal on saliva nitrite levels in subjects with false teeth and natural teeth 85

6.2 Effects of amoxycillin on saliva nitrite, gastric nitrite and gastric vitamin C during drug-induced achlorhydria 92

7.1 The influence of H. pylori status on intragastric pH before and during omeprazole 106

7.2 The influence of H. pylori status on fasting gastric juice bacterial counts before and during omeprazole 107

7.3 The influence of H. pylori status on gastric juice nitrite levels following a standard nitrate meal before and during omeprazole 108

7.4 Influence of H. pylori status on fasting gastric juice TVC levels before and during omeprazole 109

LIST OF TABLES

Page number

Table

1.1 Progression to moderate-severe atrophy of gastric body on long term omeprazole 20

7.1 Bacterial species isolated from H. pylori positive and negative volunteers during omeprazole 110

ACKNOWLEDGEMENTS

The work presented in this thesis was only possible through the helpful co-operation of many colleagues. I was fortunate to have a supervisor with the enthusiasm and dedication of Professor McColl. He also proved to be a source of great leadership and friendship. Doctor Tom Preston provided expert knowledge of basic chemistry and Dr Craig Williams the advice for the bacteriology studies. The clinical studies would not have been possible without the technical assistance of Sister Angela Wirz. Their assistance is deeply appreciated.

I am grateful for the technical assistance of Mr Andrew Carswell who performed the vitamin C assay diligently. Mrs. Margaret Hossack produced the cultures of gastric juice bacteria. The solutions of nitrate and nitrite used in the experiments were produced by Kay Pollock of the Western Infirmary pharmacy. Kathy McFall of Medical Illustration produced some of the figures. Their efforts are greatly appreciated.

Finally, I would like to thank my wife Gillian for her patience, understanding and constant support throughout my career.

ABBREVIATIONS

Abbreviations used in the text:

NOC : N-nitroso compounds

PPI : proton pump inhibitor

ARS : anti-reflux surgery

AA : ascorbic acid

DHAA : dehydroascorbic acid

TVC : total vitamin C

HPLC : high pressure liquid chromatography

DTT : dithiothreitol

GORD : gastro-oesophageal reflux disease

CHAPTER 1

INTRODUCTION

1.1 Gastric carcinogenesis; the current hypothesis

Gastric cancer remains the second most common cancer in the world (1). Epidemiological studies have identified risk factors which include the presence of achlorhydria / hypochlorhydria (2,3) and diets which are high in nitrate or nitrite and low in vitamin C (4-10). More recently, *Helicobacter pylori* (H. pylori) infection has also been recognised as a major risk factor for cancer of the mid and distal stomach (11,12).

The mechanism by which the above risk factors predispose to gastric carcinogenesis is not fully understood. In 1975 Correa published his original hypothesis for the development of gastric cancer. It required some modification in the following years but remains unchallenged (13). He proposes that the development of H. pylori-related atrophic gastritis and the associated hypochlorhydria permit colonisation of the gastric lumen by oropharyngeal bacteria. Some species of bacteria have the ability to convert dietary nitrate to nitrite within the stomach and then facilitate the formation of N-nitroso compounds (NOC). These mutagenic compounds are generated in a nitrosation reaction between nitrite and other nitrogenous organic compounds (present in food and gastric juice). The levels of NOC generated are determined by both the amount of nitrite available (14,15) and the concentration of vitamin C, which inhibits nitrosation reactions (16-18). As the ratio of nitrite to vitamin C (ascorbic acid) increases, so the rate of nitrosation increases (19). NOC are recognised carcinogens in animals and can induce gastric carcinogenesis in rodents. To date there is no data directly incriminating NOC in gastric carcinogenesis in man (20).

1.2 Recent developments relevant to gastric carcinogenesis

Since Correa modified his hypothesis in 1992 there have been several developments in the understanding of H. pylori infection and its influence on gastric physiology that are relevant to gastric carcinogenesis. H. pylori infection is widespread in the West of Scotland, with a prevalence of 66% in the adult population, but the majority will remain unharmed. Infected subjects have an increased risk of developing peptic ulcers or cancer of the mid and distal stomach (11,21). It is now recognised that the risk of developing pathology is related to the changes induced by the infection in the histology and function of the stomach; these changes are in turn determined by the characteristics of the organism and the host subject. In most H. pylori infected subjects the associated gastric inflammation is largely confined to the antrum. These subjects have normal or increased acid secretion and a proportion may develop a duodenal ulcer. There is little or no increased risk of gastric cancer. In other subjects, the associated inflammation involves the body and fundus of the stomach. Degrees of mucosal atrophy and impairment of acid secretion usually accompany this pattern of inflammation. This group of subjects with atrophic gastritis and hypochlorhydria is recognised to have an increased risk of developing cancer of the mid and distal stomach (22-25). Recent studies have examined further the effects of H. pylori infection on gastric histology and function in a group of first-degree relatives of gastric cancer patients (26). It is well recognised that this group also has an increased risk of developing gastric cancer (27,28). These studies have shown that those first-degree relatives with current H. pylori infection have a much greater prevalence of acid hyposecretion and gastric atrophy than H. pylori infected controls. The prevalence of acid hyposecretion and gastric atrophy in

those first-degree relatives who were H. pylori negative was similar to the H. pylori negative control subjects. Therefore it appears that the combination of H.pylori gastritis and hypochlorhydria may act synergistically to induce carcinogenesis – as proposed by Correa. The genetic characteristics of the host subject also appear important. Additional effects produced by H. pylori infection within the stomach that are likely to be important for gastric carcinogenesis are:

1) Stimulation of increased epithelial DNA turnover
2) Stimulation of free radical formation
3) Depletion of gastric juice ascorbic acid:

Vitamin C is actively secreted into and concentrated within the gastric juice of the healthy stomach (29). In fasting gastric juice it is present mainly in the reduced form of ascorbic acid (AA) (30,31). In subjects with H. pylori infection the concentration of vitamin C in gastric juice is subnormal and a significant proportion is the inactive oxidised form of dehydroascorbic acid (DHAA) (32-34). Hence acquiring H.pylori infection results in depletion of a factor important in preventing intragastric NOC formation.

A further development in our understanding of gastric physiology, with relevance to gastric carcinogenesis, comes via the increasingly widespread prescription of proton pump inhibitors (PPI) for the treatment of acid related disorders. Compared to their predecessors the H_2 antagonists, PPI's suppress acid secretion more effectively and provide superior symptom control over a 24-hour period. Several studies have addressed the effects of PPI-induced hypochlorhydria on gastric physiology. It is now recognised that these drugs alter the natural history of H. pylori gastritis. H. pylori gastritis that is predominantly confined to the

gastric antrum in the acid stomach becomes more diffuse in the presence of acid suppression. The infection spreads proximally to involve the gastric body and fundus to generate a pangastritis (35,36). This widespread inflammation persists despite discontinuing the drug. The consequences of this appear to be two fold. Firstly, subjects prescribed omeprazole are converted from having an antral predominant gastritis with a low risk of gastric cancer to having a pangastritis with associated impaired acid secretion. As a consequence, the degree of acid suppression achieved with PPI's is greater with co-existing H. pylori infection compared to H. pylori negative subjects. Such subjects experience sustained acid inhibition which renders them achlorhydric from one dose to the next (37). This is believed to arise as a result of the inflammation spreading to involve the acid-secreting body of the stomach. Various inflammatory mediators have been shown *in vitro* to exert additional inhibitory effects on gastric parietal cells. Secondly, concerns have arisen that with long term use, PPI-induced chronic inflammation in the body of the stomach may result in gland loss (atrophy of the gastric body) with the development of irreversible hypochlorhydria and an increased risk of developing gastric cancer (38). Concerns regarding the safety of drug-induced acid inhibition are not new. In the management of dyspepsia, H_2 antagonists have been used to induce hypochlorhydria for more than 20 years, yet the feared rise in incidence of gastric cancer has not materialised. This may be explained by the fact that these agents are less potent and fail to achieve complete inhibition of acid secretion over a 24-hour period. Therefore these agents do not alter the natural history of H. pylori gastritis to the same degree.

To examine the safety of long term PPI therapy, workers have focused on whether it leads to the development of histological changes within the stomach that may predict progression to cancer. They have looked for the development of gastric atrophy - a recognised precursor to gastric cancer (22,23). Two studies have reported on the development of gastric body atrophy in patients with gastro-oesophageal reflux disease on long term PPI therapy and have stimulated great debate (39,40). Both studies used patients treated with anti-reflux surgery (ARS) as controls. Although the authors arrived at opposing conclusions, the study results were remarkably similar. **Table 1.1** indicates the proportion of patients progressing to moderate or severe gastric body atrophy in the arms of each study.

Table 1.1

	H. pylori negative	
	3 - 5 yrs post surgery	3 - 5 yrs on omeprazole
Kuipers et al	0%	2%
Lundell et al	0%	0%
	H. pylori positive	
	3 - 5 yrs post surgery	3 - 5 yrs on omeprazole
Kuipers et al	0%	18.6%
Lundell et al	4.5%	17.9%

Zero to 2% of H. pylori negative patients on long term omeprazole progressed to moderate-severe atrophy, which was similar to those treated with ARS. In contrast, approximately 18% of H. pylori positive subjects on long term omeprazole progressed to moderate-severe body atrophy, compared to approximately 4% of those treated with ARS. The exact mechanism for development of atrophy remains to be elucidated. However it is likely that the

inflammation of the gastric body mucosa and the hypochlorhydria are important because in combination they begin to fulfil the conditions for NOC formation and carcinogenesis required by Correa's hypothesis.

1.3 The potential effects on gastric juice of drug-induced hypo/achlorhydria

From previously published work we can predict that PPI-induced hypochlorhydria or achlorhydria may favour the intragastric formation of NOC in several ways. To enable secondary bacterial colonisation of the stomach, the intragastric pH must remain above 4 (41). When the intragastric pH is >4 colonisation of the stomach by bacteria capable of catalysing nitrosation can occur (42,43). In subjects with hypochlorhydria caused by atrophic gastritis (which is recognised to predispose to gastric cancer) levels of nitrite in gastric juice are elevated (44,45)and levels of ascorbic acid are depleted (31,45,46). Thus the nitrite:ascorbic acid ratio is altered to favour N-nitrosation; it is unclear to what extent this is due to the high pH or the gastritis.

PPI's are among the most frequently used drugs worldwide and will render many patients achlorhydric. The suggestion that long term use may lead to an increased risk of developing gastric cancer has stimulated a renewed interest in the other key risk factors for gastric cancer and their role in the intragastric environment. These risk factors will be discussed in turn.

1.4 Review of the properties of nitrate, nitrite and vitamin C

1.4.1 DIETARY NITRATE

Over recent decades, there have been concerns that exposure to increasing levels of nitrate within the environment may represent an increased risk of cancer through the production of N-nitroso compounds (47). Nitrate levels have increased as a result of fuel combustion and sewage recycling but mainly through the increased use of nitrate based fertilisers. As a result, there has been increased nitrate accumulation in some root and leafy vegetables and leaching of nitrate into drinking water, where levels are monitored closely. Nitrates are also regularly used as food preservatives (E251, E252 represent sodium nitrate and potassium nitrate respectively).

The concern about dietary nitrate (NO_3^-) is not related to the ion itself, but to the fact that through the process of enterosalivary recirculation it can be converted to nitrite (NO_2^-) as follows. Upon ingestion nitrate is rapidly absorbed from the upper small intestine and the majority excreted unchanged in the urine over the next 24 hours. However an estimated 25% of ingested nitrate undergoes enterosalivary recirculation (48). Nitrate is actively taken up from the circulation and concentrated within the salivary glands (49), then secreted within saliva. Upon entering the mouth, oral nitrate-reducing bacteria convert an estimated 20% of the nitrate to nitrite (50). Following a meal containing nitrate, salivary levels of nitrite will accumulate (51). With each post prandial swallow, therefore, large concentrations of nitrite are delivered within saliva to the

stomach. Nitrite (NO_2^-) represents a potential hazard as it can participate in nitrosation reactions with other dietary constituents (such as secondary amines) to generate potentially carcinogenic N-nitroso compounds (see below).

1.4.2 NITRITE

In contrast to nitrate, environmental levels of nitrite are low. Small quantities of nitrite can be found in food that has been smoked or treated with preservatives (E249, E250 represent potassium nitrite and sodium nitrite respectively) but the main source of nitrite to the upper GI tract is ingested nitrate (52). Nitrite levels in serum are negligible. This is because it is avidly taken up by haemoglobin to produce methaemoglobin. (This is the mechanism of death in subjects who ingest large quantities of nitrite). Normally, haemoglobin is regenerated enzymatically as nitrite is released back into the circulation as nitrate.

Whereas nitrate (NO_3^-) is a stable ion, nitrite (NO_2^-) is highly reactive at acid pH. It decomposes in acidic solution to exist in complex equilibria with several nitrosating agents (53). These equilibria are shown below with **nitrosating agents** highlighted. X represents a catalyst of N-nitrosation reactions (such as thiocyanate - SCN^- - which is abundant in saliva and gastric juice).

$$NO_2^- + H^+ \longleftrightarrow HNO_2$$

$$HNO_2 + H^+ \longleftrightarrow \mathbf{H_2NO_2^+} \longleftrightarrow H_20 + \mathbf{NO^+}$$

$$2HNO_2 \longleftrightarrow \mathbf{N_2O_3} + H_20$$

$$HNO_2 + HX \longleftrightarrow \mathbf{NOX} + H_2O$$

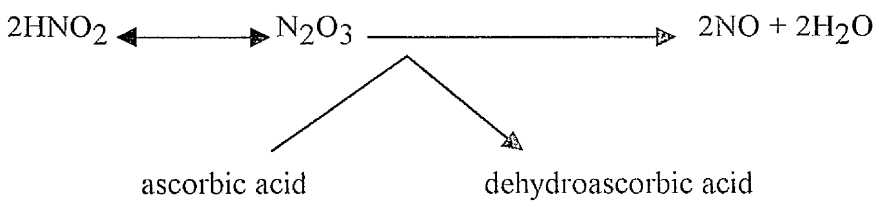
The relative importance of the various nitrosating agents is dependent on the ambient pH. Nitrosation reactions can proceed in the moderately hypochlorhydric stomach (pH 2 - 4) or in the profoundly achlorhydric stomach (pH>4) where they are catalysed by bacteria colonising the stomach (14,54). The maximal rate of nitrosation of amines occurs at pH 3.4 although in the presence of the catalyst thiocyanate the optimal pH is 2.5.

The nitrosating agents react with secondary amines to generate N-nitroso compounds (R_2NNO). Secondary amines are abundant in food and gastric juice. Examples of such reactions are illustrated below:

$$NO^+ + R_2NH \longrightarrow R_2NNO$$

$$N_2O_3 + R_2NH \longrightarrow R_2NNO$$

Ascorbic acid is a potent inhibitor of N-nitrosation reactions. If ascorbic acid is present it reacts with the nitrosating agents more rapidly than the nitrosating agents can react with the secondary amines, and converts them to non-nitrosating nitric oxide (NO). The ascorbic acid is oxidised to the inactive dehydroascorbic acid in the process e.g.:



It has previously been demonstrated that in the healthy acid stomach (which contains vitamin C), nitrite is largely undetectable (55). Our own *in vitro* studies have confirmed that when nitrite is added to acidic gastric juice it is rapidly lost - presumably as nitric oxide. However, it is well established that nitrite is readily detected in achlorhydria (55-57). Our own *in vitro* studies have shown that if the pH of the acidic gastric juice is elevated to > 4, nitrite is readily recovered. If nitrite is present, it is available for N-nitrosation reactions.

1.4.3 VITAMIN C

Vitamin C is a water-soluble vitamin that plays a fundamental role in mechanisms of immune response and tissue protection. Man cannot synthesise vitamin C and requires a regular intake to maintain tissue levels. The vitamin is abundant in fresh fruit and vegetables and it is recognised that diets low in vitamin C are associated with an increased risk of cancer (8-10). The vitamin takes two forms - ascorbic acid (AA) and dehydroascorbic acid (DHAA) which measured together constitute Total Vitamin C (TVC). In healthy subjects levels of vitamin C are greater within gastric juice than in serum. Once absorbed, vitamin C is actively secreted into and concentrated within the gastric juice of the healthy stomach (29). In fasting gastric juice it is present mainly in the reduced form of AA (30,31). Ascorbic acid is the active moiety of vitamin C. It is a strong reducing agent and at acid pH reacts rapidly with nitrite and other nitrosating agents. In the absence of oxygen, the reactions between ascorbic acid and the nitrosating agents proceed more quickly than the reactions between the nitrosating agents and secondary amines. Thereby, ascorbic acid can prevent formation of NOC within gastric juice by removing nitrite and the other nitrosating agents (58). As a consequence of these

reactions, AA is oxidised to DHAA while nitrite and nitrosating agents are converted to nitric oxide. Ascorbic acid also provides protection within the tissues by scavenging free radicals and preventing oxidative damage of DNA (59).

The efficacy of ascorbic acid is compromised in the presence of oxygen. The nitric oxide gas (NO) generated in the above reaction combines with O_2 to produce further nitrosating agents as follows (53):

$$2NO + O_2 \longrightarrow 2NO_2$$

$$NO_2 + NO \longrightarrow N_2O_3$$

$$NO_2 + NO_2 \longrightarrow N_2O_4$$

Thus the available supply of ascorbic acid is more likely to become exhausted. Once there is no further AA available, nitrosation reactions can proceed unhindered.

The efficacy of ascorbic acid is also affected by changes in pH. The molecule has 2 pKa values (4.17 and 11.57). As the pH increases above 4.17, AA in solution is more susceptible to oxidation to DHAA (60). Previous *in vitro* studies have observed that vitamin C is unstable in neutral gastric juice and is irreversibly degraded to inactive metabolites (31,46).

The efficacy of ascorbic acid is also affected by co-existing H. pylori infection. In subjects with H. pylori infection the concentration of vitamin C in gastric juice is

subnormal and a significant proportion is the inactive oxidised form of DHAA (32-34).

1.5 The potential effects on gastric juice of drug-induced hypo/achlorhydria and co-existing H. pylori infection

The incidence of cancer of the mid and distal stomach is falling in the developed world. However, the suggestion that one of the most widely prescribed acid suppressing agents may predispose H. pylori infected patients to developing gastric cancer means that the chemistry of the intragastric environment has a new found relevance. The interactions and reactions described above (which can result in NOC formation) will almost certainly be occurring *in vivo* within the stomach. These interactions may be the factors that facilitate the development of atrophic gastritis in the presence of drug-induced achlorhydria.

At present there is negligible information concerning the combined effect of H. pylori infection and PPI therapy on the factors recognised to be important in gastric carcinogenesis, namely 1) intragastric nitrite concentrations, 2) intragastric vitamin C concentrations and 3) colonisation of the stomach with nitrosating bacteria. There are theoretical reasons to believe that the interaction of H. pylori gastritis and profound acid suppression with PPI's will accentuate each one of these predisposing factors. These reasons will be discussed in turn:

1.5.1 PPI therapy, H. pylori infection and intragastric nitrite

Through the process of enterosalivary recirculation, there is a constant supply of nitrite to the stomach via saliva. Nitrite is highly reactive at acid pH. It is well

established from *in vitro* work that in the presence of ascorbic acid nitrite is converted to nitric oxide gas. This reaction should theoretically occur *in vivo* when saliva nitrite meets acid gastric juice containing ascorbic acid. This reaction has been observed indirectly in a study by Lundberg et al (61). Nitric oxide was shown to accumulate in the gastric head space following a nitrate meal. Previous *in vitro* studies have shown that at pH > 3 nitrite begins to remain in solution. In patients with achlorhydria, nitrite is readily detected (55). This suggests that gastric nitrite levels should theoretically increase following the prescription of omeprazole. Lundberg et al also observed this indirectly in the same study. After the prescription of omeprazole, Lundberg could no longer detect nitric oxide in the head space following the nitrate meal. This suggests that the nitrite derived from the nitrate meal had remained in the gastric juice. A further mechanism whereby omeprazole may theoretically raise gastric nitrite levels is by facilitating secondary bacterial overgrowth of the stomach with nitrate reducing bacteria, which can convert ingested nitrate to nitrite within the stomach.

In patients with H. pylori infection, the inflamed gastric mucosa is a theoretically important additional source of nitrite. The infection stimulates mucosal nitric oxide synthase activity (62)and the nitric oxide may be converted to nitrate and nitrite within the gastric lumen. Hence the combination of drug-induced achlorhydria and H. pylori infection could result in elevated gastric juice nitrite levels as a result of the chemical stability of nitrite at high pH. The nitrite may arise from saliva, the inflamed gastric mucosa or from bacterial reduction of ingested nitrate (as is traditionally believed).

1.5.2 PPI therapy, H. pylori infection and Vitamin C

Gastric juice ascorbic acid concentrations (the active form of vitamin C) are lowered in subjects with H. pylori infection (33)and also in patients with achlorhydria due to atrophic gastritis (45,46). In addition, it has been demonstrated that ascorbic acid is unstable in vitro at neutral pH hence it may no longer act as an efficient scavenger of nitrite in vivo (63). This suggests that the combination of PPI therapy and H. pylori infection is likely to result in profound depletion of gastric juice ascorbic acid concentrations. This may then facilitate accumulation of intragastric nitrite.

1.5.3 PPI therapy, H. pylori infection and colonisation with nitrosating bacteria

Colonisation of the human stomach with a mixed bacterial flora occurs with an intragastric pH>4. Recent studies have demonstrated that in those subjects with coexisting H. pylori infection the intragastric pH during PPI therapy is consistently higher (37). This higher intragastric pH will theoretically predispose them to greater colonisation with nitrosating bacteria.

In view of the above considerations it is important to document the effects of PPI therapy on intragastric nitrite, vitamin C and colonisation with nitrosating bacteria in subjects with H. pylori infection. If PPI therapy induces the changes in the intragastric environment predicted above, it may facilitate bacterial synthesis of N-nitrosocompounds.

Although there is a body of evidence supporting the safety of long term PPI therapy, it is important to consider that these toxicology studies were performed on laboratory animals not infected with H. pylori. Different results may arise in humans due to their high prevalence of H. pylori infection.

1.6 Aims and hypothesis to be tested

The hypothesis being tested is that PPI therapy in H. pylori infected patients will produce conditions predisposing to N-nitrosocompound formation and thus gastric carcinogenesis; in particular a) increased intragastric nitrite concentrations, b) low intragastric vitamin C concentrations and c) increased intragastric colonisation by nitrosating bacteria. We predict that these changes will be more profound with coexisting H. pylori infection. We also hypothesise that an important cause of the elevated nitrite concentration is induced nitric oxide synthase activity within the inflamed gastric mucosa. The hypothesis will be tested in the following way.

Volunteers will ingest a standard nitrate meal to mimic average dietary nitrate consumption and studies will:

1) Observe *in vivo* the interactions of nitrite and vitamin C
2) Study the effect of omeprazole therapy on the above interaction and record intragastric concentrations of nitrite, Vitamin C and bacterial colonisation in subjects with and without H. pylori infection.
3) Investigate the contribution of mucosal nitric oxide synthase activity to the production of intragastric nitrite in H. pylori positive and negative subjects.

CHAPTER 2

METHODS

2.1 STUDY PROCEDURES

For all the studies performed, the procedures were uniform and are described below.

Assessing H. pylori status

If required, the *Helicobacter pylori* status of volunteers was determined by a ^{14}C-urea breath test as previously described (64). Subjects presented after an overnight fast. They brushed their teeth and provided a baseline sample of breath for a CO_2 level. They ingested 0.4 MBq ^{14}C-urea in 20mls Ensure (to slow gastric emptying). A second breath sample was provided 30 minutes later. If H. pylori infection was present, the organism's urease enzyme converted the urea into ammonia and released the ^{14}C within CO_2. The enrichment of breath CO_2 with $^{14}CO_2$ was determined by liquid scintillation counting.

Dietary restrictions

In view of the influence of ingested nitrate on salivary and gastric juice nitrite levels, all subjects were asked to avoid consuming foods rich in nitrate (such as leafy vegetables) in the 24 hours leading up to a study day.

Obtaining gastric juice samples

All studies requiring samples of gastric juice followed the same initial procedure. The volunteers presented after an overnight fast. The nasal passages and pharynx were anaesthetised with lignocaine spray and a 16F-nasogastric tube (Anderson Inc., New York, NY) was passed into the most dependent part of the stomach.

The residual gastric juice was aspirated and discarded unless bacterial counts were required. To confirm that the tube lay in the most dependent part of the stomach a water recovery test was performed (65). Fifty mls of water were introduced via the tube. One minute later an attempt was made to recover the 50 mls.

Gastric juice pH measurements

The pH of gastric juice samples was determined immediately using a glass electrode.

Obtaining saliva samples

Subjects were asked to pool saliva within their mouths at intervals (dictated by each study protocol) then expectorate it into universal containers. It is recognised that insertion of a nasogastric tube stimulates salivary secretions. Therefore, in studies utilising a nasogastric tube, thirty minutes were allowed to elapse following tube insertion before any experiment commenced so that salivary flow could reach a steady state.

Obtaining blood samples

An intravenous cannula was sited in an antecubital fossa vein with a 3-way tap attached. This allowed repeated sample collection over the course of each study.

2.2 ANALYSIS OF NITRATE, NITRITE AND VITAMIN C

Nitrate analysis

It was originally planned to measure nitrate levels within samples of gastric juice, serum and saliva and observe the enterosalivary recirculation of the nitrate meal in its entirety. There were 3 options for sample analysis.

1. The method of nitrate analysis described by Tannenbaum et. al. (51) is based on High Pressure Liquid Chromatography (HPLC) of samples. Samples are injected into a stainless steel ion-exchange column containing cadmium. This reduces all nitrate (NO_3^-) in the sample to nitrite (NO_2^-). Thereafter, nitrite concentration is determined by the classical Griess reaction whereby the sample is mixed with sulphanilic acid and naphthylenediamine (the Griess reagents) in an acidic medium. This generates a colour change. The depth of colour produced is determined by the quantity of nitrite present and can be accurately measured with a colorimeter.
2. With advances in technology, a HPLC column designed specifically to detect nitrite and nitrate directly is now available.
3. A commercial kit is available which uses a bacterial nitrate reductase enzyme to convert nitrate (NO_3^-) in the sample to nitrite (NO_2^-). Thereafter the Greiss reagents are added (as above) to measure the nitrite for a final quantification.

Sample analysis using HPLC was attempted. Major difficulties were encountered in removing sediment and other impurities from the samples of saliva and gastric

juice. These impurities caused problems with blockage in the filters and column of the HPLC system. When samples of gastric juice were spiked with known quantities of nitrate and nitrite, several peaks were produced on chromatography. None of the peaks coincided with the aqueous nitrate and nitrite standards used for comparison. It was felt that further progress in developing our own HPLC assay of nitrate would be difficult and it was abandoned.

The kit assay for analysing nitrate utilised a bacterial nitrate reductase enzyme in place of a cadmium column to reduce the entire nitrate in the sample to nitrite. This had been validated for plasma, urine and tissue homogenates. It had never been used for measuring nitrate in saliva or gastric juice. The samples were prepared as follows. To clean the gastric juice, samples were spun in an ultracentrifuge at 30,000 rpm for 10 minutes. Saliva samples were spun in a minifuge at 7,000 rpm for 30 minutes. Serum collected for nitrate analysis was stored at -20^0C and prepared in accordance with the instructions of the kit manufacturer. On thawing, 400µl of sample was centrifuged through a 10kd ultrafilter ('Microcon 10', Millipore (UK) Ltd.) to remove high molecular weight proteins.

Samples were pipetted into a 96 well plate and mixed with a reaction buffer comprising bacterial nitrate reductase, enzyme co-factors (NADPH, FAD), phosphate buffer (pH 7.5). The plate was left to incubate in the dark for 3 hours at 37°C. This reduced all the nitrate (NO_3^-) to nitrite (NO_2^-). The samples were then mixed with the Griess reagents and colorometric analysis was performed 30 minutes later using a 540nm filter.

Major difficulties were encountered in measuring nitrate in samples of saliva and gastric juice. This was attributed to the poor performance of the bacterial enzyme in the stored samples of saliva and gastric juice. Prior to storage these samples were alkalinised (see below) to prevent loss of nitrite. Consequently it proved difficult to return the pH of the stored samples to pH7.5 to facilitate enzyme reduction of nitrate within the sample. Nitrate levels in serum, on the other hand, were accurately recorded as predicted by the kit manufacturer. The rise in serum levels of nitrate following ingestion of the standard nitrate meal could be monitored and thereby the first phase of enterosalivary recirculation observed. Levels of nitrate (NO_3^-) within saliva and gastric juice were of secondary importance to the studies compared to levels of nitrite (NO_2^-).

Nitrite analysis

In order to measure nitrite levels in saliva and gastric juice, it is necessary to elevate the pH of the sample to render it stable. Workers have traditionally added alkali to the samples immediately following collection to try to prevent loss of nitrite. Sobala et. al., for example, mixed 0.5ml of 0.5M NaOH with 1.5 ml gastric juice(45). In this series of studies, small volumes of concentrated alkali were added to the samples to minimise problems of sample dilution as follows: 1 ml of saliva was mixed with 10µl 5.0M NaOH. 2ml of gastric juice were mixed with 100µl 5.0M NaOH. Samples were stored at 4^0C and analysed on the same day. Nitrite levels in saliva and gastric juice were shown to remain stable for up to 1 week using this method.

Samples of saliva and gastric juice were prepared as described above and analysed on 96 well microplates by addition of the Griess reagents. Colorometric analysis was performed 30 minutes after the addition of the Griess reagents using a 540nm filter.

Analysis of ascorbic acid and total vitamin C

In order to assay vitamin C it was important to consider the highly reactive nature of ascorbic acid in the presence of nitrite and oxygen. This was especially relevant for assaying ascorbic acid in gastric juice. The methods previously reported were followed (29,66).

0.5 ml gastric juice was added to two test tubes. Each contained 0.5 ml equal volumes of 2 % metaphosphoric acid / 0.5 % sulfamic acid and one also contained 6 mg/ml dithiothreitol (DTT). Serum samples were prepared similarly except that 0.5 ml serum was added to two tubes containing 1.0ml of 2 % metaphosphoric acid only and one also contained 6mg/ml DTT. Upon collection samples were immediately placed in liquid nitrogen before transfer to storage at -80^{0}C.

Metaphosphoric acid denatures protein prior to HPLC, lowers the sample pH, and thus stabilises AA. Sulfamic acid removes any residual nitrite within the sample thus preventing it oxidising the ascorbic acid. The purpose of the DTT is to regenerate AA from any DHAA in the sample thereby allowing quantification of total vitamin C levels.

Samples were analysed within 4 weeks. Prior to analysis samples were thawed and centrifuged at 1000g. 0.3 ml of the supernatant was removed and further centrifuged in a minifuge at 9000g. Serum and gastric juice AA and TVC levels were measured by high performance liquid chromatography using a method similar to that first published by Schorah.C.J. (29,66). The instrumentation comprised a Waters 501 pump, a Gilson 231 automated sample injector and an Electrochemical detector (Shimadzu L-ECD-6A) set at 350 mV and 0.2 μA. Ascorbic acid was measured using reverse phase ion-pair chromotography on a Waters C18 ODS2 analytical column (4.6 x 160 mm); protected by an Uptight guard column (20 x 3 mm) hand-packed with LiChroprep RP-18. The mobile phase consisted of 0.1M sodium acetate containing 10mM octylamine. The final pH was adjusted to 4.3 with glacial acetic acid. The flow rate was 0.5 ml/min and the retention time of AA was 3 min. An aqueous stock standard solution (1 mg/ml) was prepared by adding 5mg AA to 5.0 ml DTT (3.5 mg/ml). Working standards of 5 and 10 μg/ml were prepared by taking aliquots of the stock standard solution (0.05, 0.1ml) and making the volume up to 10 ml. These standards were prepared daily. The autosampler was programmed to inject 150 μl aliquots of standards, quality controls and samples with the first injection being a DTT solution (3.5 mg/ml) to remove oxidising sites on the column. Subsequent standards and samples were processed with standards occurring every eighth sample. The rapid oxidation of AA to DHAA was largely overcome by keeping samples as cold as possible during assay procedure. The mobile phase was prepared fresh for each assay and the sample run was restricted to no more than fifty samples inclusive of standards and quality controls. This assay had a lower limit of detection of 0.2μg/ml.

CHEMICALS

All chemicals used in the assays were obtained from Sigma-Adrich Chemical Co. Ltd, Poole, U.K. except sodium acetate (Analar grade) which was obtained from BDH Ltd, Liverpool, U.K. and glacial acetic acid which was obtained from May & Baker Ltd, Dagenham, U.K. The water used throughout this work was distilled and deionised in a Fisons Fi-Streem still.

2.3 DATA ANALYSIS

The data were analysed using non-parametric statistical tests. The 1 sample Wilcoxon test was used for paired data. The Mann Whitney U test was used for non-paired data. The areas under the concentration - time profiles for increases in saliva nitrite and gastric nitrite were calculated using the trapezoid approximation and Spearman rank correlation coefficients were calculated as required.

CHAPTER 3

NOVEL *IN VIVO* STUDIES OF THE INTERACTION BETWEEN NITRITE AND VITAMIN C IN GASTRIC JUICE AND THE INFLUENCE OF INTRAGASTRIC pH

3.1 Introduction

The fate of dietary nitrate, ingested in foods such as leafy vegetables, has been well documented. Nitrate is absorbed in the upper intestine. 25% of the absorbed nitrate undergoes entero-salivary recirculation via the blood to the salivary glands where it is concentrated and secreted in saliva. In the mouth an estimated 20% of salivary nitrate is reduced to nitrite by the action of commensal bacteria resident on the dorsum of the tongue (50). With each swallow, therefore, nitrite is introduced to the acid environment of the stomach. Ingested nitrate is thereby the main source of nitrite to the upper GI tract (52). On entering the stomach saliva meets gastric juice containing ascorbic acid.

The chemical reactions described in chapter one dictate that when salivary nitrite meets acid gastric juice containing ascorbic acid, nitric oxide (NO) will be produced. Workers have recently identified and measured NO in the gastric head space of the acid stomach following ingestion of a portion of lettuce (61). The quantities encountered were far greater than those produced by vascular endothelium. It has previously been demonstrated that in the healthy acid stomach, nitrite is largely undetectable (55) and ascorbic acid is present in concentrations greater than those found in serum (46). These observations suggest that the presence of NO in the gastric head space is probably the result of the interaction between saliva nitrite and ascorbic acid in the acid stomach.

In the same study (61), Lundberg demonstrated that NO was undetectable in the gastric headspace following administration of omeprazole. It is well established that nitrite is readily detected in achlorhydric stomachs (55-57). This has been

attributed to bacterial over-growth of the stomach and bacterial reduction of ingested nitrate to nitrite. However, nitrite is chemically stable at high pH. Our own *in vitro* studies have shown that if the pH of the acidic gastric juice is elevated to > 4, nitrite is readily recovered. This accumulation of nitrite cannot be explained by the action of bacteria. This suggests an alternative, purely chemical, explanation for elevated gastric nitrite in achlorhydric patients. Furthermore, the pKa of ascorbic acid is 4.17 at low pH. At pH values above this it is less effective as a reducing agent and the reaction with nitrite proceeds slowly. Therefore omeprazole therapy could lead to elevation of gastric nitrite via 3 possible mechanisms; secondary bacterial overgrowth, the chemical stability of nitrite at high pH, or the impaired efficacy of AA at high pH.

In order to investigate the *in vivo* interactions of nitrite and vitamin C we aimed to simulate the conditions of the healthy stomach by infusing physiological quantities of nitrite into the stomach to mimic the delivery of saliva nitrite following a nitrate meal. In order to investigate the effect of intragastric pH on these parameters, we studied subjects before and after administration of the proton pump inhibitor omeprazole.

3.2 Subjects and Methods

4 healthy asymptomatic volunteers were recruited. None had a previous history of gastrointestinal disease and none suffered from dyspepsia. This small number was chosen because we believed the elevation in gastric pH produced by omeprazole would produce a profound elevation in gastric juice nitrite, based on the findings of our *in vitro* experiments.

The study took place on two separate days. On day 1, basal samples of saliva and gastric juice were collected and the subjects fed potassium chloride (1ml of a 1mM solution) as a control solution. Further samples were collected over 40 minutes. A rest period of 30 minutes followed. Then subjects were fed a standard solution of sodium nitrite of a concentration similar to that previously recorded in human saliva (1ml of a 10mM solution). Further samples were collected at 1 minute, 5 minutes, 15 minutes and 30 minutes. Following this, the subjects were supplied with 2 capsules of omeprazole 40 mg to take at 12 hours and then 3 hours before their appointment for the second study day. This was to ensure an intragastric pH > 4. The above protocol was then repeated.

Safety

Overdose of nitrite leads to methaemoglobinaemia with cyanosis, dyspnoea and circulatory collapse. The concentration of nitrite administered was equivalent to that normally present in swallowed saliva following a standard meal: <u>total dose 0.69 mg.</u>

Toxic Dose (oral) Human: 14 mg / kg (RTECS, 1992)

Lethal Dose (oral) Human: 71 mg / kg (RTECS, 1992)

sodium nitrite solution (of concentration 10000μmol / litre)

690 mg in 1 litre = 10000μmol / litre

1 ml of the above contains 690/1000 mg of sodium nitrite

= 0.69 mg sodium nitrite

3.3 Results (all values expressed as medians with range in parenthesis)

Study day 1: Gastric pH was 1.2 (1.0 - 1.4) and remained unchanged following infusion of the solutions. Baseline saliva nitrite was 136μmol/L (31 - 262) and values remained similar for the duration of the experiment. Gastric juice nitrite was undetectable in all subjects at baseline and this remained the case after instillation of the control solution. Following instillation of the nitrite solution, levels rose markedly. At 1 minute post instillation, gastric nitrite concentration was 131μmol/L (70 - 229) **(figure 3.1)**. Thereafter, levels rapidly returned towards baseline such that at 15 minutes post instillation they were undetectable.

At baseline gastric juice total vitamin C (TVC) was 8.4μg/ml (3.3 - 11.8) and the ascorbic acid (AA) moiety was 7.1μg/ml (0.6 - 11.4). Thus gastric juice vitamin C was predominantly in the active form with an AA:TVC ratio of 0.84 (0.18 - 0.97). Following instillation of the control solution, these values remained similar such that at 30 minutes post instillation, gastric TVC was 7.5μg/ml (3.8 - 13.2) and gastric AA 6.8μg/ml (0.7 - 12.4). Immediately following instillation of the nitrite solution, gastric AA levels fell markedly to 0.9μg/ml (0.5 - 5.1) whereas gastric TVC remained similar at 6.6μg/ml (5.1 - 7.9). As a result, the AA:TVC ratio also fell to 0.14 (0.09 - 0.65) **(figures 3.2 and 3.3)**. Over the following 30 minutes, gastric AA levels recovered and gastric TVC levels remained unchanged. Consequently at 30 minutes post instillation of the nitrite solution, the AA:TVC ratio had returned towards baseline at 0.87 (0.19 - 0.98).

Study day 2: Baseline samples were collected without difficulty but the remainder of the protocol was difficult to follow due to problems obtaining gastric juice. This was of particular relevance to vitamin C analysis as it was difficult to collect samples rapidly, introducing the possibility of inaccuracies in measurement. Omeprazole elevated the intragastric pH in all subjects to 6.4 (4.1 - 7.2). Intragastric pH remained unchanged following instillation of the solutions and was 5.7 (5.1 - 7.2) at the end of the study. Saliva nitrite was 143μmol/L (63 - 157) at baseline and was unchanged over the duration of the study, measuring 94μmol/L (64 - 162) at the end of the study. These levels were similar to those recorded prior to omeprazole. At baseline, gastric nitrite levels were detectable in 3 of the 4 subjects at 25μmol/L (9 - 65). Thus in the presence of hypochlorhydria, gastric nitrite levels were readily measurable. This remained the case for the duration of the study. Levels of nitrite again increased markedly following instillation of the nitrite solution (**figure 3.4**). At 1 minute post instillation, gastric nitrite was 159μmol/L (19 - 456). Thereafter, levels returned towards baseline.

Gastric juice was collected for analysis of vitamin C in 3 of the 4 subjects. At baseline, gastric TVC was 8.6μg/ml (3.2 - 22.3) and gastric AA was 3.8μg/ml (0.2 - 6.0). Thus gastric TVC values were similar to those in the acid stomach. However, gastric AA values at this elevated pH were lower than those in the acid stomach. This is reflected in the lower baseline AA:TVC ratio of 0.27 (0.08 - 0.44). Thus in the presence of hypochlorhydria only 27% of the vitamin C was present in the active moiety compared to 87% in the acid stomach. Due to

problems with sample collection over the remainder of the study, the accuracy of subsequent vitamin C measurements was questionable.

3.4 Figure legends and figures

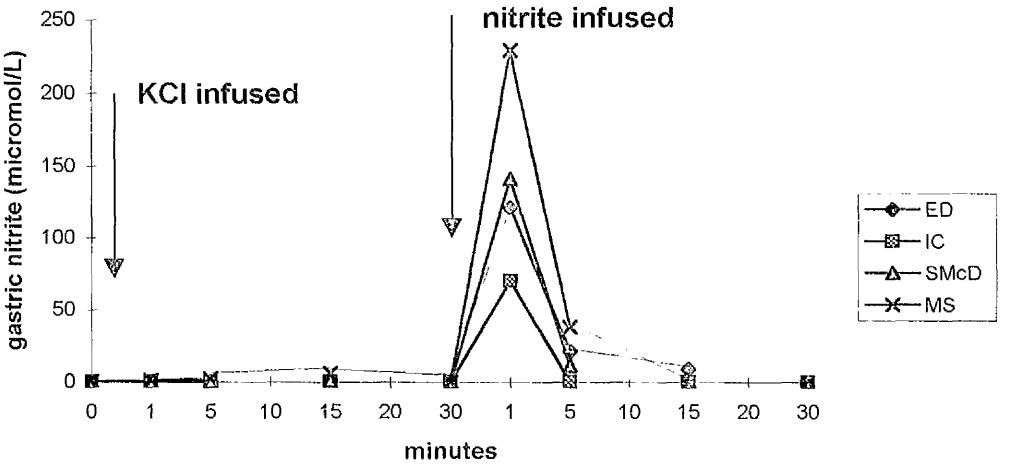
Figure 3.1: Effect of an intragastric infusion of nitrite on gastric juice nitrite levels in the acid stomach. 4 subjects were fed a standard solution of sodium nitrite of a concentration similar to that previously recorded in human saliva (1ml of a 10mM solution). A solution of potassium chloride (1ml of a 1mM solution) was used as a control solution as a control solution. Gastric nitrite levels were recorded over time.

Figure 3.2: Effect of an intragastric infusion of nitrite on gastric juice AA levels in the acid stomach. 4 subjects were fed a standard solution of sodium nitrite of a concentration similar to that previously recorded in human saliva (1ml of a 10mM solution). A solution of potassium chloride (1ml of a 1mM solution) was used as a control solution as a control solution. Gastric AA levels were recorded over time.

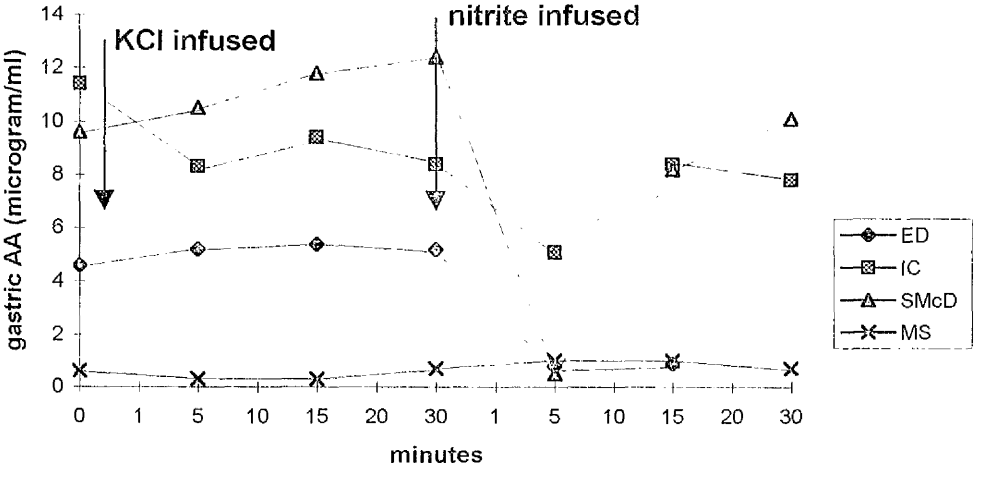
Figure 3.3: Effect of an intragastric infusion of nitrite on gastric juice TVC levels in the acid stomach. 4 subjects were fed a standard solution of sodium nitrite of a concentration similar to that previously recorded in human saliva (1ml of a 10mM solution). A solution of potassium chloride (1ml of a 1mM solution) was used as a control solution as a control solution. Gastric TVC levels were recorded over time.

Figure 3.4: Effect of an intragastric infusion of nitrite on gastric juice nitrite levels at pH>4. 4 subjects were fed a standard solution of sodium nitrite of a concentration similar to that previously recorded in human saliva (1ml of a 10mM solution). A solution of potassium chloride (1ml of a 1mM solution) was used as a control solution as a control solution. Gastric nitrite levels were recorded over time.

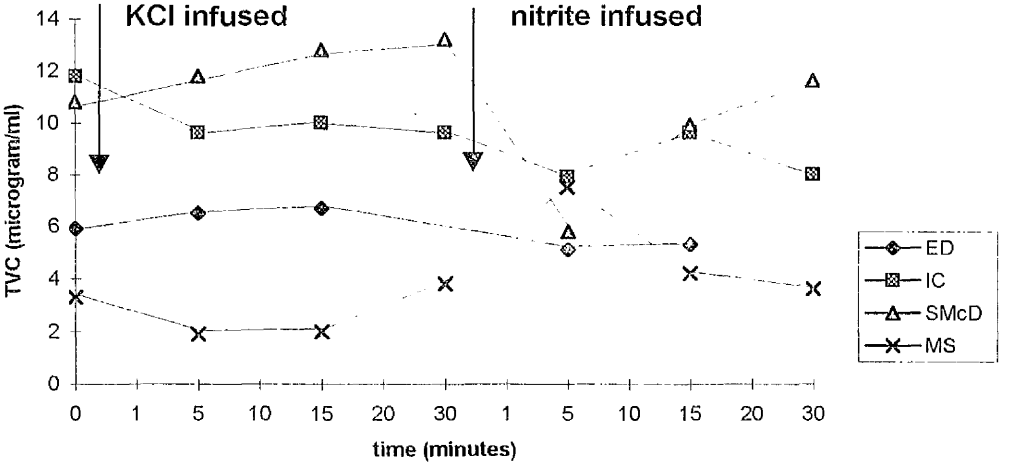
3.1 Effect of intragastric nitrite infusion on gastric juice nitrite at acid pH



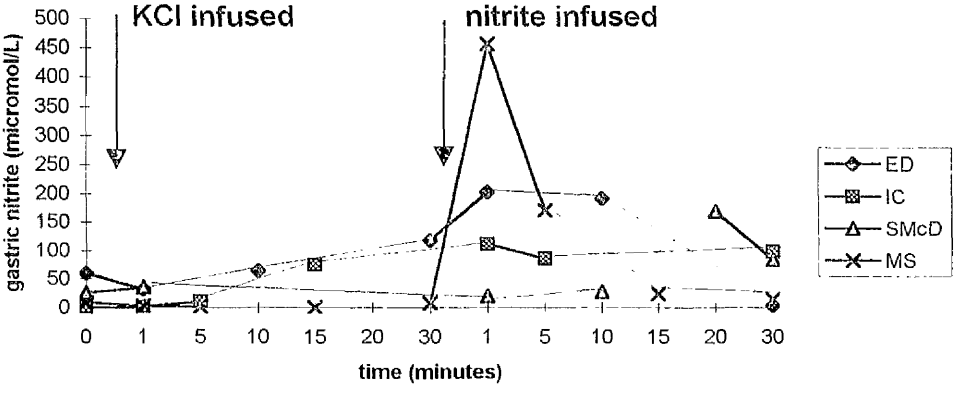
3.2 Effect of intragastric nitrite infusion on gastric juice ascorbic acid at acid pH



3.3 Effect of intragastric nitrite infusion on gastric juice total vitamin C at acid pH



3.4 Effect of intragastric nitrite infusion on gastric nitrite levels at pH >4



3.5 Conclusions

As anticipated, saliva nitrite levels were unaffected by intragastric instillation of chloride or nitrite. In the acid stomach, gastric nitrite was undetectable and vitamin C was predominantly in the form of ascorbic acid. These findings are in keeping with previous reports. Following intragastric instillation of the inert potassium chloride, levels of nitrite and vitamin C were unaffected. Following the instillation of nitrite (to mimic influx of saliva nitrite following a nitrate meal), a dramatic rise in gastric nitrite was observed. This coincided with a dramatic fall in gastric juice AA. Gastric TVC levels were unchanged and the predominant form of vitamin C was now dehydroascorbic acid (DHAA). This change in the composition of total vitamin C can be explained by the chemical interaction between nitrite and ascorbic acid which occurs when they meet at acid pH. Nitrite is converted to gaseous nitric oxide and ascorbic acid is oxidised to the inactive DHAA.

Over the following minutes, the ratio of AA:TVC recovered towards baseline. TVC levels were unchanged. This suggests that AA is being regenerated from the inactive DHAA. This would be in keeping with previous theories that the gastric mucosa is capable of recycling oxidised vitamin C.

Following omeprazole, intragastric pH was elevated in all subjects as anticipated. Unfortunately, due to the associated reduction in gastric juice volume that occurs in association with PPI's (67), it proved difficult to obtain all the required samples over a short period of time. From these limited observations on the effect of hypochlorhydria on gastric juice, we confirmed that gastric nitrite levels are

elevated and remained so for the duration of the experiment. In addition, ascorbic acid levels are depleted and vitamin C is predominantly in the oxidised form of DHAA.

The remainder of the samples collected in this protocol could not be analysed with any assurance of accuracy. In the presence of hypochlorhydria, sample collection times should be dispersed to allow accumulation of sufficient volume of gastric juice to facilitate speedy collection and processing of samples.

Numerous previous studies have examined gastric nitrite and vitamin C levels, but always independently of one another (44,45,57,68-70). All these studies have examined single samples of fasting gastric juice only. From the earlier work on the pharmacology of ingested nitrate (48) and the recognition that dietary nitrate provides the main source of nitrite to the upper GI tract (52) it is clear that measurements from fasting gastric juice do not reflect the true effects of exposure to nitrite. This novel dynamic study provides the first *in vivo* observation of the interaction between physiological doses of nitrite and vitamin C within the healthy stomach. This provides vital new information with respect to the gastric physiology of two factors recognised to play a key role in gastric carcinogenesis.

CHAPTER 4

THE EFFECTS OF DIETARY NITRATE ON LEVELS OF VITAMIN C AND NITRITE IN GASTRIC JUICE

4.1 Introduction

By infusing physiological quantities of nitrite into the acid stomach we have obtained evidence of an important and previously unrecognised reaction occurring *in vivo* between saliva nitrite and gastric juice vitamin C. In this study, we wished to recreate the conditions present following the ingestion of nitrate containing foods and repeat these observations in a larger sample.

4.2 Subjects and Methods

Twenty healthy volunteers were studied; (7 male, 13 female), mean age 30 years (range 20 to 47). None had a previous history of gastrointestinal disease and none suffered from dyspepsia. Their *Helicobacter pylori* status was determined by a ^{14}C-urea breath test as previously described (64).

Subjects avoided eating leafy vegetables for 24 hours before presenting fasted on the morning of the study day. Basal samples of blood, saliva and gastric juice were collected and then the subjects were fed 2 mmoL of potassium nitrate which is equivalent to a standard portion of lettuce (61). Further samples were collected at intervals over the next 2 hours.

4.3 Results

The median intragastric pH was 1.4 (1.1 - 3.2). The pH did not change following the intragastric infusion of nitrate.

The median fasting serum nitrate was 24μmol/L (12 - 67), saliva nitrite 44μmol/L (0 - 306) and gastric nitrite was undetectable in 16 subjects and at very low levels

in the other 4 (at 3, 5, 12, 12μmol/L respectively) . Following the intragastric administration of the nitrate meal, serum nitrate increased approximately 4 fold and peaked at 82μmol/L (49 - 131) and salivary nitrite increased approximately 6 fold, peaking at 262μmol/L (5 - 939) **(figure 4.1)**. Peak values for both parameters were recorded at 40 minutes post dosing. Despite this marked increase in salivary nitrite, the median gastric nitrite remained undetectable in 10 of the subjects. In 10 of the subjects studied, gastric nitrite levels increased following the nitrate meal. When compared with the 10 subjects in whom nitrite remained undetectable, these subjects had a higher salivary nitrite, as measured by the area under the concentration-time curve {saliva nitrite auc 22285μmol/L/min (10595 - 102000) vs. 5020 (3145 - 8650), $p < 0.001$}.

The median fasting serum AA was 3.2μg/ml (1.0 - 6.0) and serum TVC 3.6μg/ml (1.1 - 6.6). The fasting gastric juice AA was 3.8μg/ml (0.3 - 20.7) and gastric juice TVC 5.0μg/ml (1.2 - 21). Fasting AA and TVC levels were therefore higher within the gastric juice than the serum although this failed to reach significance. In the serum of each subject virtually all the vitamin C was in the form of AA, whereas in the fasting gastric juice the median ratio of AA to TVC (AA/TVC ratio) was 0.76 (range 0.25 - 1.0). Following the intragastric administration of nitrate, there was no change in the serum levels of AA and TVC. However, the gastric juice AA levels fell and this coincided with the rise in salivary nitrite **(figure 4.1)**. At 60 minutes post nitrate meal, gastric juice AA reached its nadir of 0.9μg/ml (0.4 - 31.8) compared with 3.8μg/ml (0.3 - 20.7) pre-dosing ($p<0.05$). By comparison, gastric juice TVC increased to 6.2μg/ml

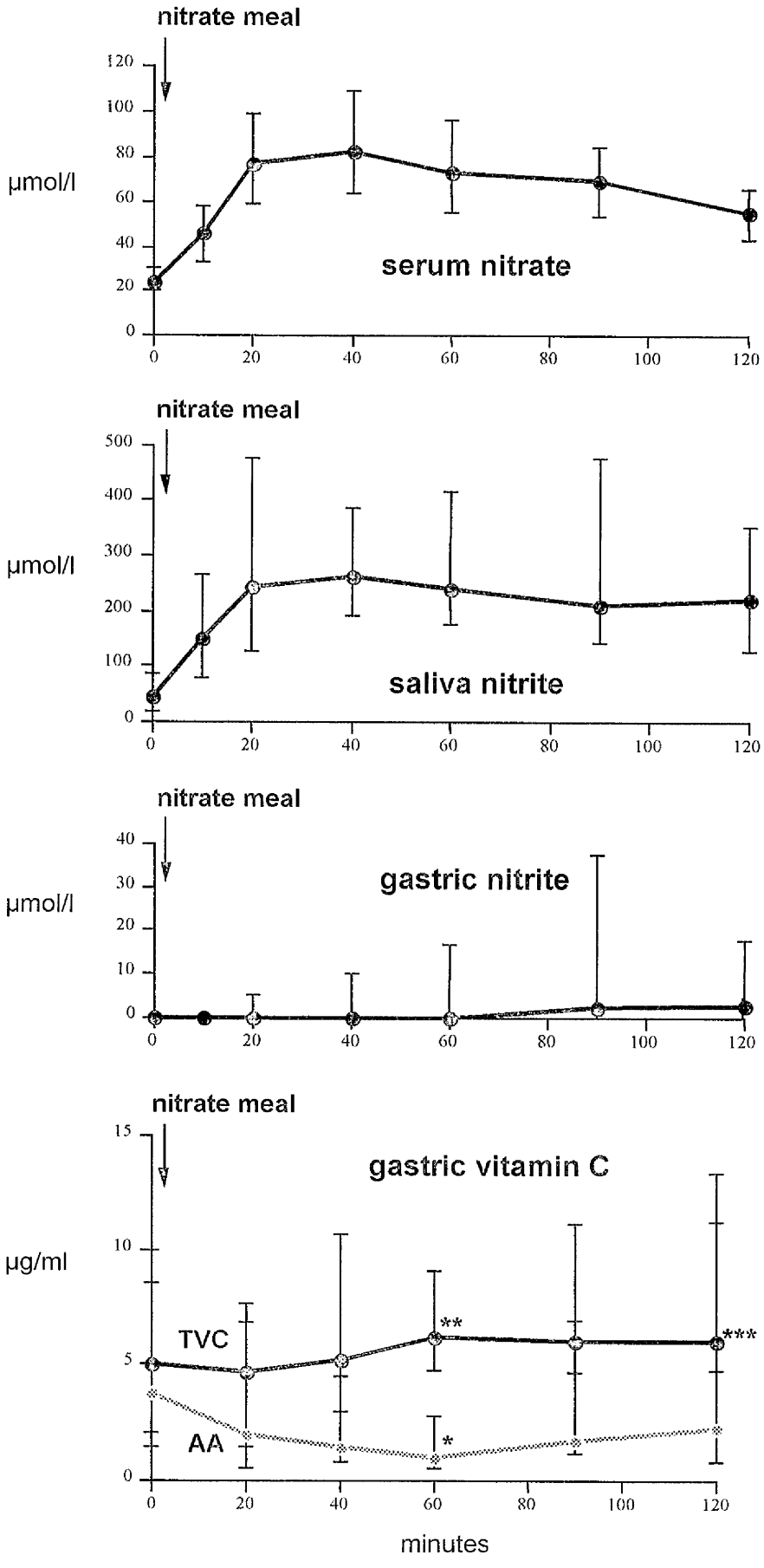
(2.0 - 32.0) at 60 minutes compared with 5.0μg/ml (1.2 - 21) pre-dosing ($p<0.05$). At 60 minutes after the nitrate meal, the gastric juice AA/TVC ratio was 0.2 (0.04 - 0.99) compared to 0.76 (0.25 - 1.0) pre-nitrate dosing ($p<0.001$). Over the subsequent 60 minutes the gastric juice AA concentration and gastric AA/TVC ratio gradually returned towards their original levels, and this coincided with the fall in saliva nitrite levels. At 120 minutes post dosing, gastric juice TVC was 6.0μg/ml (1.7 - 33.2) which was significantly higher than the fasting value of 5.0μg/ml (1.2 - 21) ($p=0.003$).

4.4 Figure legend and figure

Figure 4.1:

Effect of standard nitrate meal on serum nitrate, saliva nitrite, gastric nitrite, gastric total vitamin C (TVC) and gastric ascorbic acid (AA) in all 20 subjects. Intragastric bolus of 2 mmol nitrate in 50 ml H_2O introduced immediately following baseline (time zero) collections. Values are medians plus interquartile ranges. * indicates significantly different from value at time zero at $p<0.05$, ** $p<0.001$ and *** $p<0.01$.

Figure 4.1 : Effect of standard nitrate meal in the presence of acid



4.5 Conclusions

This study examined the effects on saliva nitrite, gastric nitrite and gastric vitamin C of ingesting a quantity of nitrate equivalent to that present in a salad meal. It sheds further light on the previously unrecognised interactions between salivary nitrite, gastric nitrite and vitamin C that occur in the acid stomach.

Under fasting conditions, nitrite was present in saliva but undetectable in gastric juice. Vitamin C was readily detectable in fasting gastric juice; its concentration was higher than in serum and it was mainly in the form of AA (AA/TVC ratio = 0.76). The higher concentration of vitamin C in gastric juice compared to serum is consistent with previous reports (29,30,46), and is thought to be the result of active secretion of the vitamin into the gastric lumen.

Following intragastric infusion of the nitrate solution, salivary nitrite concentrations increased markedly peaking at 40 minutes post dosing. This rise in salivary nitrite following ingestion of nitrate is well recognised and is due to enterosalivary recirculation of nitrate and its reduction to nitrite by bacteria in the mouth (50,51). The increase in salivary nitrite and consequent increased delivery of nitrite to the stomach in swallowed saliva resulted in a rise in gastric nitrite in only half of the subjects. The subjects who showed this rise in gastric nitrite had significantly higher salivary concentrations of nitrite.

In all subjects, the rise in salivary nitrite concentration was mirrored by a marked fall in gastric juice AA concentration but no change in gastric juice TVC. This fall in gastric juice AA and absence of a consistent rise in gastric juice nitrite

following the salivary nitrite load can be explained by the rapid reaction between saliva nitrite and gastric AA in the acidic stomach. At acid pH nitrite is unstable and it reacts with AA, with the former being converted to nitric oxide and the latter oxidised to DHAA (58). Nitric oxide has been shown to accumulate in the gastric head space following nitrate ingestion (61).

Following the nitrite induced conversion of gastric AA to DHAA, there was a later increase in the gastric juice TVC concentrations being most apparent at 2 hours post nitrate dosing. This rise may indicate increased secretion of the vitamin into the gastric juice. Little is known about the regulation of vitamin C secretion by the gastric mucosa but our observation would be consistent with stimulation of the process by either a low level of gastric juice AA or high level of DHAA.

These changes in gastric juice levels of nitrite and AA in the acid stomach following a nitrate meal may be relevant to the role of dietary nitrate and vitamin C in the aetiology of gastric cancer. In subjects with a diet high in nitrate and low in vitamin C the gastric juice will contain significant concentrations of nitrite following a meal. This will arise due to a high salivary nitrite load and inadequate gastric juice AA to convert it to nitric oxide. The high salivary nitrite load will also further deplete gastric juice AA, converting it to inactive DHAA. The resulting combination of high gastric juice nitrite plus low AA will favour the generation of potentially carcinogenic NOC (19). Chemical nitrosation in the acid stomach is accelerated by the presence of thiocyanate (15,71), which is also secreted in saliva and is present in high levels in smokers (72). Such chemical

nitrosation may be particularly important in the pathogenesis of cancers of the cardia or gastro-oesophageal junction which occur in the non-atrophic stomach (73) and are associated with smoking (74,75). Our studies have shown that saliva nitrite is the major source of gastric nitrite and therefore chemical nitrosation will occur predominantly where saliva meets acid gastric juice i.e. at the gastro-oesophageal junction and cardia.

CHAPTER 5

THE EFFECTS OF DIETARY NITRATE AND PROLONGED OMEPRAZOLE THERAPY ON LEVELS OF VITAMIN C AND NITRITE IN GASTRIC JUICE

5.1 Introduction

In chapter 3, we obtained preliminary data on the effects of drug-induced achlorhydria on gastric nitrite and vitamin C levels by infusing physiological quantities of nitrite into the stomach to mimic those conditions generated after a meal containing nitrate. In this study we aimed to collect further data on the interactions of nitrite and vitamin C in the presence of hypochlorhydria by examining the fate of a nitrate meal during a standard course of omeprazole (used to heal oesophagitis).

5.2 Subjects and Methods

The twenty healthy volunteers who participated in the previous study (in chapter 4) were recruited.

After completing the first study day, subjects were prescribed a 4 week course of omeprazole 40mg daily and returned 2 hours following the final dose. After collection of basal samples, the standard nitrate meal (2mmol potassium nitrate) was introduced into the stomach and a protocol identical to the first study day was followed. Measurements obtained on omeprazole were compared with those obtained pre-omeprazole.

5.3 Results

All values are expressed as medians (range).

Omeprazole produced a profound rise in intragastric pH in all subjects. The median intragastric pH increased from 1.4 pre-omeprazole to 7.2 (3.5 - 8.5) on

omeprazole, $p<0.001$. The median intragastric pH on omeprazole remained unchanged following infusion of the nitrate meal.

On omeprazole, the fasting serum nitrate and saliva nitrite were similar to the values pre-omeprazole at 27μmol/L (15 - 56) and 28μmol/L (6 - 297) respectively. However fasting gastric juice nitrite levels on omeprazole were elevated at 13 μmol/L (0 - 50) compared to the pre-omeprazole fasting levels of 0μmol/L (0 - 12) ($p= 0.001$) **(figure 5.1)**.

Following ingestion of the nitrate meal, the peak serum nitrate and saliva nitrite levels at 40 minutes post dosing were also similar to the pre-omeprazole values at 79μmol/L (46 - 165) and 295μmol/L (145 - 1041) respectively. However there was a profound rise in gastric nitrite levels **(figure 5.2)**. At 60 minutes post nitrate ingestion on omeprazole, gastric nitrite peaked at 154μmol/L (49 - 384) compared to 0μmol/L (0 - 111) at the 60 minute point pre-omeprazole ($p<0.001$) **(figure 5.1)**.

On omeprazole, the fasting serum levels of AA and TVC were similar to the values pre-omeprazole at 3.4μg/ml (1.1 - 6.4) and 3.4μg/ml (1.1 - 7.3) respectively. However, omeprazole produced a profound fall in fasting gastric juice AA levels from a median of 3.8μg/ml (0.3 - 20.7) pre-omeprazole to 0.7μg/ml (0.2 - 10.9) on omeprazole ($p<0.001$)**(figure 5.1)**, which was near the lower limit of detection. Gastric AA was undetectable in one subject. Fasting gastric juice TVC was also lower on omeprazole at 3.0μg/ml (0.8 - 11.3)

compared to 5.0μmol/L (1.2 - 21.0) pre-omeprazole ($p< 0.005$). On omeprazole the median fasting gastric AA/TVC ratio was 0.34 (0.06 - 1.0) compared with 0.76 (0.25 - 1.0) pre-omeprazole ($p=0.001$).

Following administration of the nitrate meal, there was no change in the serum levels of AA and TVC. The median gastric juice AA level remained low throughout **(figure 5.2)**. At 60 minutes post ingestion, gastric juice AA was 0.5μg/ml (0.2 - 18.7) which was similar to the fasting level. Gastric juice TVC increased following the nitrate meal. At 60 minutes post ingestion, gastric juice TVC had increased from the fasting value of 3.0 (0.8 - 10.9) to 3.6μg/ml (1.4 - 37.4) ($p=0.019$). Consequently at 60 minutes post ingestion the median gastric AA/TVC ratio was 0.18 (0.02 - 0.77) compared to a fasting ratio of 0.34 (0.06 - 1.0) ($p<0.001$).

5.3.1 *Helicobacter pylori* status and nitrate, nitrite and vitamin C

Of the 20 subjects who completed the study, 9 had a positive ^{14}C-urea breath test for H. pylori and 11 had a negative breath test. When the results of H. pylori positive subjects were compared with those of H. pylori negative subjects both pre and on omeprazole, no significant differences were found with respect to any of the parameters measured. However, when the effect of omeprazole therapy on the intragastric milieu and serum of each group was assessed, two key differences were observed between H. pylori positive and H. pylori negative subjects.

(1) The marked depletion of gastric TVC on omeprazole was confined to H. pylori positive subjects **(figure 5.3)**.

(2) In H. pylori positive subjects, fasting serum TVC levels fell on omeprazole by a median of 13% (range 0 - 33%) such that the median value of 3.2μg/ml (1.2 - 5.3) was significantly lower than the corresponding pre-omeprazole value of 4.3μg/ml (1.1 - 6.0) (p = 0.013) **(figure 5.4)**. In H. pylori negative subjects, omeprazole had no effect on serum TVC; median % change was 0% (range -28 to +45%). This difference between the two groups was significant (p=0.003).

5.3.2 Studies of the contribution of mucosal nitric oxide synthase to intragastric nitrite concentrations

On omeprazole, gastric nitrite levels are elevated. An additional mechanism behind the high levels of nitrite in H. pylori infected subjects may be the contribution of inducible Nitric Oxide Synthase (i-NOS). This enzyme is present in inflammatory cells such as neutrophils and macrophages and shows increased activity in the presence of H. pylori-associated inflammation (62). It generates nitric oxide in large quantities from the amino acid precursor L-arginine. The nitric oxide has a very short half-life and is rapidly oxidised to nitrite and nitrate within the tissues (76).

If any of the nitric oxide generated within the gastric mucosa were to diffuse into the gastric juice, at acid pH it may lead to further production of nitric oxide gas whereas at neutral pH it may give rise to further nitrite within the lumen.

Methods

To evaluate any possible contribution of mucosal i-NOS to intragastric levels of nitrite, a representative H. pylori positive subject was chosen along with a representative H. pylori negative subject to act as a control. Each subject was prescribed 10 days of omeprazole 40mg to elevate intragastric pH in order that any gastric juice nitrite arising from mucosal i-NOS would remain stable and detectable within the juice. The subjects presented 24 hours following the final dose of omeprazole. A protocol identical to the previous studies was followed. The subjects presented fasted and basal samples of saliva, gastric juice and serum were collected prior to infusion of the test solution. However, instead of nitrate, a 0.05mmol/kg L-arginine solution was infused down the nasogastric tube. The aim was to provide a quantity of precursor that could be utilised by mucosal i-NOS to generate nitric oxide and thereby give rise to nitrate and nitrite. Further samples were collected over a 90 minute period.

Results

The results for each subject are presented below in tabular form (units - μmol/L);

Subject PW. H. pylori positive. 88kg.

time (minutes)	0	10	20	40	60	90
saliva nitrite	33	24	37	29	31	28
gastric nitrite	3	0	2	3	3	7
plasma nitrate	17	17	17	17	17	16
gastric pH	7.9	7.5	7.8	7.5	7.7	7.2

Subject WH. H. pylori negative. 66.5kg.

time (minutes)	0	10	20	40	60	90
saliva nitrite	65	79	63	67	73	48
gastric nitrite	2	5	8		6	9
plasma nitrate	15	16	17	16	16	16
gastric pH	3.5	3.3	2.7	2.4	3.6	5.4

The gastric pH of the H. pylori positive subject on omeprazole remained neutral for the duration of the experiment. Following infusion of the L-arginine solution, there was no change in serum levels of nitrate. Consequently, the nitrate availability to the salivary glands was unchanged and saliva nitrite levels were unchanged. In addition, gastric nitrite levels were unaffected.

5.4 Figure legends and figures

Figure 5.1: Effect of omeprazole on fasting gastric nitrite, fasting gastric ascorbic acid and on gastric nitrite levels at 60 minutes following the intragastric administration of 2 mmol nitrate in 50ml H_2O. The results of all 20 subjects are included.

Figure 5.2: Effect of standard nitrate meal, in the presence of omeprazole-induced hypochlorhydria, on serum nitrate, saliva nitrite, gastric nitrite, gastric total vitamin C (TVC) and gastric ascorbic acid (AA) in all 20 subjects. Intragastric bolus of 2 mmol nitrate in 50 ml H_2O introduced immediately following baseline (time zero) collections. Values are medians plus interquartile ranges. * indicates significantly different compared to time zero at $p<0.05$.

Figure 5.3: Effect of standard nitrate meal on gastric total vitamin C (TVC) levels in 9 H. pylori positive and 11 H. pylori negative subjects before ——— and on ····· omeprazole. Intragastric bolus of 2 mmol nitrate in 50 ml H_2O introduced immediately following baseline (time zero) collections. Values are medians plus interquartile ranges. * indicates lower than corresponding pre-omeprazole value at $p<0.02$.

Figure 5.4: Effect of 4 weeks treatment with omeprazole 40mg daily on fasting serum total vitamin C (TVC) levels in 9 H. pylori positive and 11 H. pylori negative subjects.

Figure 5.1 : Effect of omeprazole on fasting gastric nitrite, fasting gastric ascorbic acid and meal stimulated gastric nitrite.

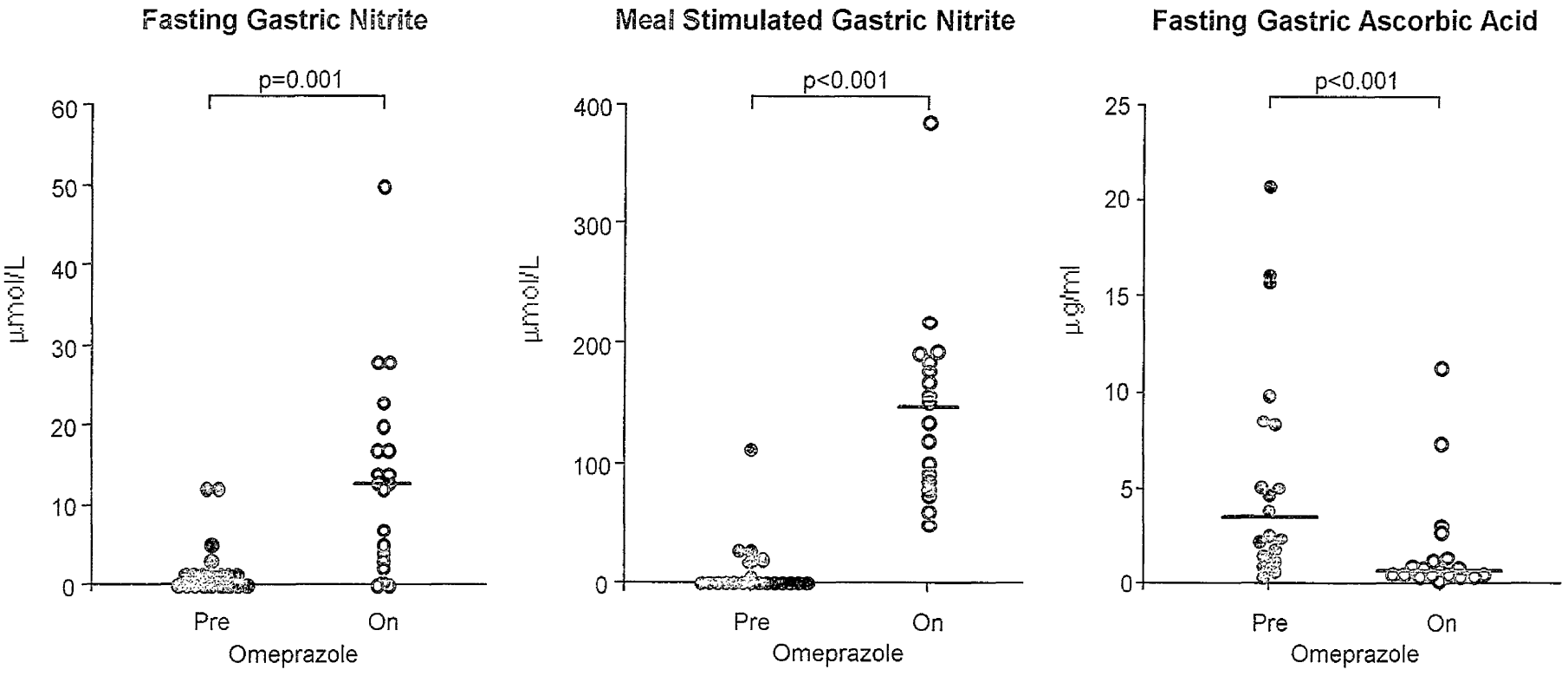


Figure 5.2 : Effect of standard nitrate meal in presence of achlorhydria

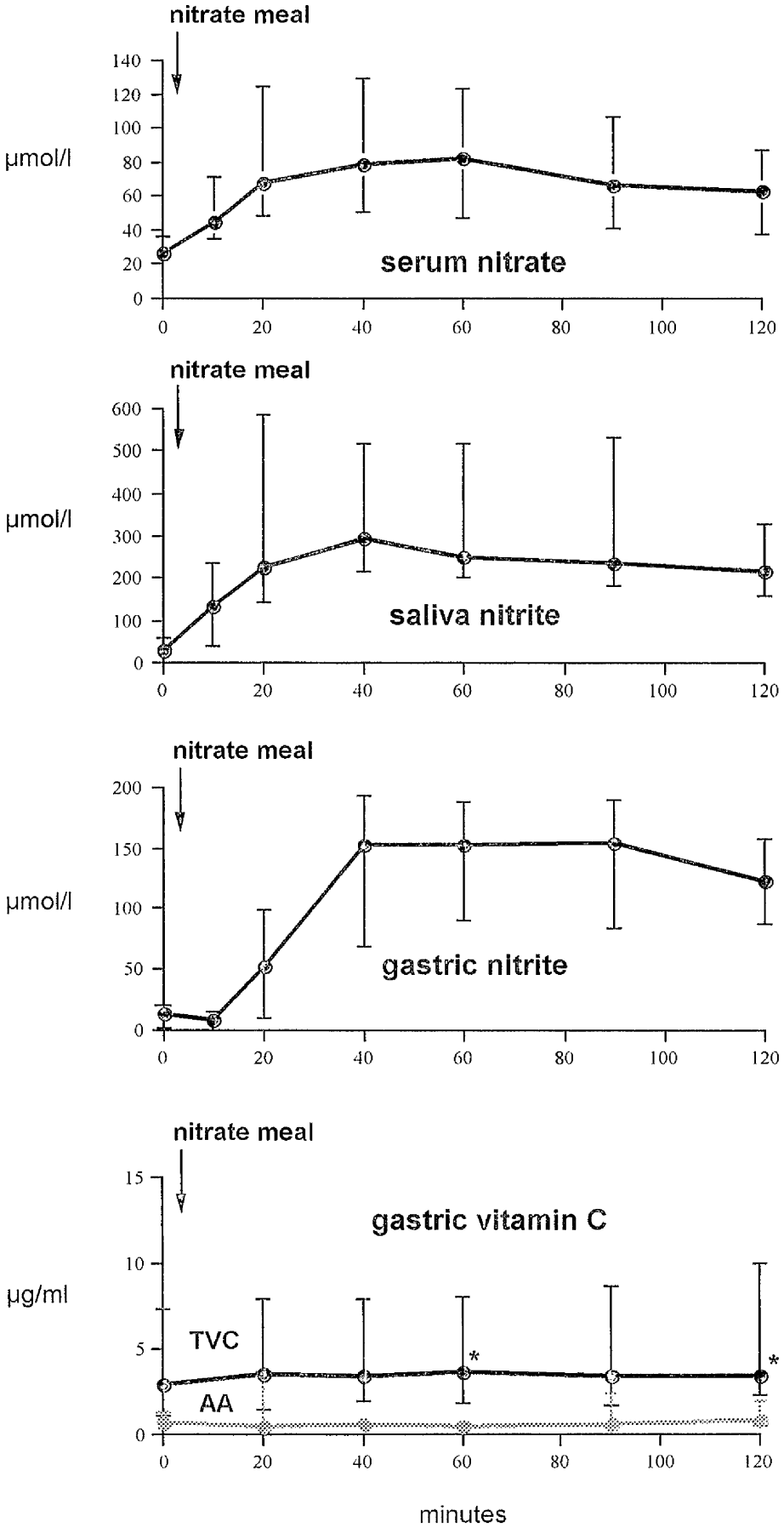


Figure 5.3 : Effect of omeprazole on gastric juice total vitamin C following a nitrate meal, according to H. pylori status

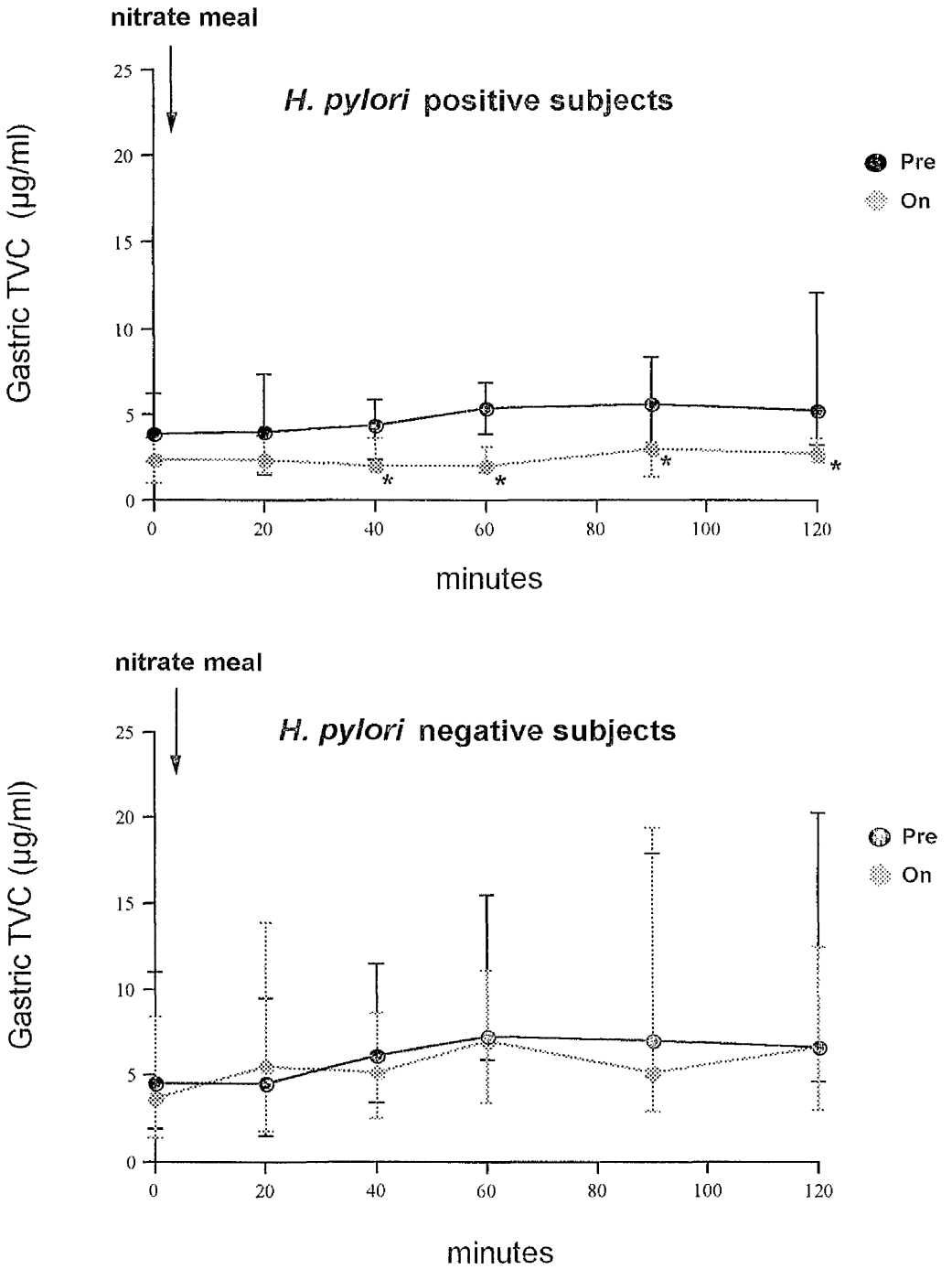
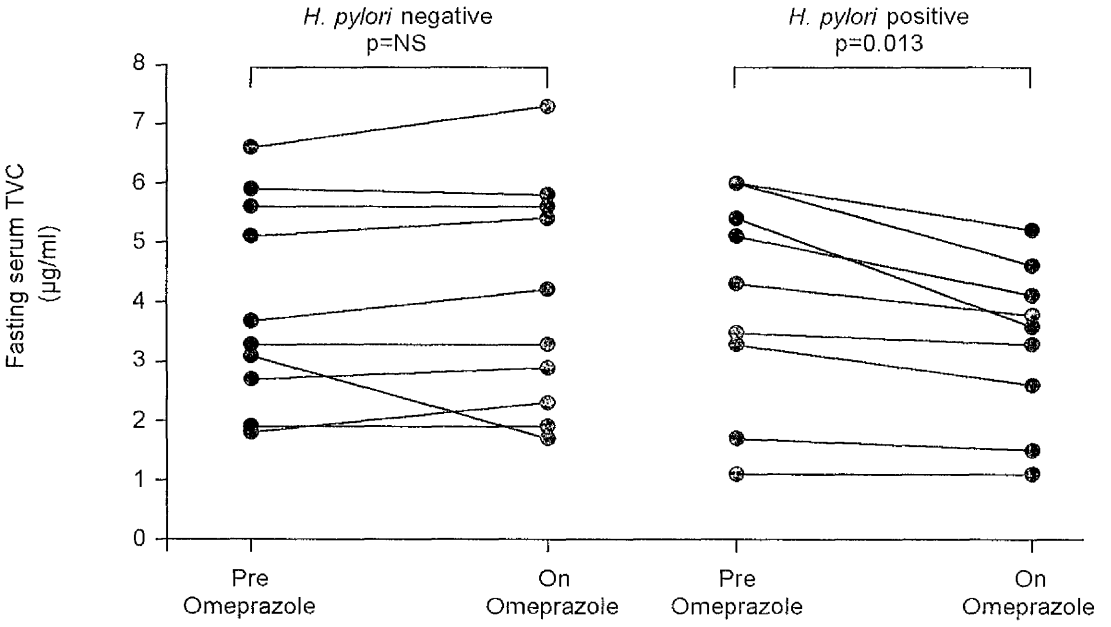


Figure 5.4: Effect of omeprazole on serum total vitamin C, according to H. pylori status



5.5 Conclusions

The present studies indicates that drug-induced profound hypochlorhydria depletes intragastric AA concentrations and increases intragastric nitrite concentrations. These changes are apparent in the fasting state but are even more marked following ingestion of nitrate. In H. pylori positive subjects, drug-induced hypochlorhydria also lowers both gastric and serum TVC concentrations. Furthermore, the elevation of gastric nitrite that occurs in H. pylori positive subjects during omeprazole does not appear to be enhanced by the activity of mucosal i-NOS.

Treatment with omeprazole produced a marked rise in fasting intragastric pH and this was accompanied by changes in intragastric nitrite and vitamin C concentrations in both fasting and post nitrate meal samples.

The salivary nitrite concentrations on omeprazole were similar to those pre-omeprazole. However in the fasting gastric juice, nitrite was detectable in 18 of the 20 subjects and its median concentration of 13μmol/L was significantly greater than the equivalent value of 0μmol/L pre-omeprazole. Following the nitrate meal gastric nitrite levels rose markedly and peaked at a median value of 154μmol/L at 60 minutes which was also significantly greater than the equivalent value of 0 μmol/L pre-omeprazole. Previous studies have failed to agree on whether omeprazole produces a rise in fasting gastric juice nitrite (57,70,77). This study is the first to show a profound rise post prandially.

What is the explanation for the marked increase in gastric nitrite levels on omeprazole? Workers have until now concluded that the high levels of gastric nitrite found in achlorhydric subjects are a consequence of the reduction of ingested nitrate within the stomach by bacteria colonising the achlorhydric stomach (69,78-80). This is possible. However, in this study the timing of the appearance of nitrite in the stomach following the nitrate load would indicate that the gastric nitrite was predominantly salivary in origin. Despite instilling the nitrate directly into the stomach there was no significant rise in gastric nitrite levels until nitrate had been absorbed, secreted into the saliva and converted to nitrite in the saliva. Thus the high levels of gastric juice nitrite on omeprazole can be explained by the nitrite in swallowed saliva remaining as nitrite within gastric juice. The persistence of swallowed nitrite in gastric juice during omeprazole but much less so pre-omeprazole can be explained by the fact that nitrite is stable at high pH but is converted to nitric oxide and other nitrogen oxides at low pH (15,61).

On omeprazole the great majority of vitamin C in fasting gastric juice was in the inactive oxidised form of DHAA. This is in keeping with a previous report (31). The reason why the great majority of the vitamin C in fasting gastric juice on omeprazole is in the inactive oxidised form is unclear. The pKa value of AA at this lower pH range is 4.17. As the pH increases above this value AA in solution is more susceptible to oxidation to DHAA (60). Other substances in gastric juice such as iron or copper may promote this oxidation of AA at neutral pH (63).

On omeprazole, the gastric juice TVC concentration was reduced by approximately 50%. This could be due to impaired secretion of the vitamin by the gastric mucosa and/or destruction of the vitamin in gastric juice of high pH. Previous *in vitro* studies have observed that vitamin C is unstable in neutral gastric juice and is irreversibly degraded to inactive metabolites (31,46). It is also possible that bacteria colonising the achlorhydria gastric juice could utilise the vitamin for their own metabolism (81).

Influence of *Helicobacter pylori* status

We studied subjects with and without H. pylori infection as the infection has been reported to impair the secretion of Vitamin C into gastric juice and to reduce the ratio of biologically active AA to inactive DHAA (32,33). Prior to commencing omeprazole, H. pylori positive subjects had a lower gastric TVC and a lower AA/TVC ratio but this did not reach significance, possibly due to the relatively small number of subjects in each group. The previously reported H. pylori-induced changes in gastric juice vitamin C have been most marked in subjects with H. pylori-induced atrophy where the hypochlorhydria will have contributed to the vitamin C abnormality (31). Our subjects were relatively young and unlikely to have atrophy.

On omeprazole the H. pylori positive and negative subjects were similar with respect to the depletion of gastric juice AA and elevation of gastric juice nitrite. However, our present study examined the subjects at 2 hours post dosing when gastric pH was markedly elevated in both groups of subjects. Previous studies have shown that pH on omeprazole remains markedly elevated over the entire 24

hour period only in H. pylori positive subjects (82). Due to the influence of intragastric pH on nitrite and AA concentrations, it is therefore likely that H. pylori positive subjects on omeprazole will over the entire 24h period have a more markedly lowered gastric juice AA concentration and higher nitrite level than H. pylori negative on omeprazole.

The observation that gastric juice nitrite levels were similar in the H. pylori positive and negative subjects on omeprazole (in the presence of similar gastric pH and saliva nitrite levels) is of interest. Within the mucosa of chronic gastritis the activity of inducible nitric oxide synthase is increased (62). The nitric oxide generated can undergo further oxidation to yield nitrate and nitrite (76). If the nitrite produced within the mucosa was contributing to gastric juice nitrite levels, it would remain stable and readily detectable in the gastric juice of H. pylori positive subjects at neutral pH. This was tested by infusing L-arginine (the precursor of nitric oxide) into the stomach of a representative H. pylori positive and negative subject rendered hypochlorhydric with omeprazole. The introduction of L-arginine did not result in elevation of serum nitrate, saliva nitrite or gastric nitrite in either subject. These experiments demonstrate that although gastric mucosal i-NOS may be induced in the presence of H. pylori infection (62), it does not contribute to concentrations of nitrate within plasma nor nitrite within gastric juice. Therefore, the mucosa in chronic gastritis is contributing little or no nitrite to gastric nitrite levels.

The H. pylori positive and negative subjects did differ with respect to the effect of omeprazole on gastric juice TVC levels. The lowering of gastric juice TVC by

omeprazole was confined to the H. pylori positive subjects. These differences in gastric juice vitamin C levels occurred despite a similar intragastric pH and similar gastric nitrite levels in each group and suggest that omeprazole has enhanced the negative effect of H. pylori infection on gastric mucosal secretion of vitamin C. This could be explained by the fact that on omeprazole the inflammation associated with H. pylori infection is more extensive, involving the body region of the stomach, thus disrupting the function of a greater area of mucosa.

The H. pylori positive and negative subjects also differed with respect to the effect of omeprazole on their serum TVC levels. In the H. pylori positive but not the negative subjects, omeprazole therapy resulted in a fall in fasting serum TVC levels. The reason for this is unclear but likely to be related to the observed depletion of vitamin C within the gastric juice of H. pylori positive subjects on omeprazole. The process depleting the fasting gastric juice vitamin C may also be destroying vitamin C ingested in the diet and thus reducing its bioavailability. Previous studies have observed that serum TVC is lower in subjects with gastric atrophy (44) and hypochlorhydria (45,46). This reduction in serum TVC in such subjects may be partly a consequence of reduced bioavailability of dietary vitamin C and could also contribute to progression of gastric atrophy which is known to be more common in states of vitamin C deficiency (13). Our observation that omeprazole therapy lowers both gastric juice and systemic vitamin C levels in H. pylori positive subjects may be relevant to the report of accelerated development of atrophic gastritis in H. pylori positive subjects on omeprazole (39). Vitamin C is known to provide protection against DNA damage by free radicals which are produced in excess by the H. pylori infected mucosa (59).

In summary, our studies indicate that drug-induced profound hypochlorhydria results in depletion of AA and accumulation of nitrite within the gastric juice and the elevation of nitrite is particularly marked following nitrate ingestion. This lowering of the ascorbic acid: nitrite ratio is likely to predispose to N-nitroso compound formation. At the high pH achieved with omeprazole, chemically-catalysed nitrosation is unlikely to occur but formation of NOC could occur in the presence of metabolically active nitrosating bacteria within the stomach. Previous studies have confirmed omeprazole leads to colonisation of the stomach with bacteria (57,70) They are mainly oropharyngeal in origin and such organisms have been shown to catalyse NOC formation *in vitro* (83). H. pylori itself has very weak nitrosating ability (83).

There have been conflicting reports concerning the presence of NOC in gastric juice on omeprazole (57,70,77). This may be due to the technical difficulties of measuring these compounds in gastric juice *in vivo* and because of the wide spectrum of such compounds that may be formed. In addition, these studies have either reported on only H. pylori negative subjects (70) or failed to take account of H. pylori status (57,77). None of the previous studies have been performed after a nitrate containing meal, which we show markedly increases the intragastric nitrite concentrations during omeprazole treatment. Further studies are required to examine the effect of pharmacologically-mediated hypochlorhydria on intragastric NOC concentrations. These studies should be performed in H. pylori positive as well as negative subjects, should include the effect of a nitrate

containing meal and should also consider the vitamin C status of the subjects being studied.

CHAPTER 6

STUDIES ON THE EFFECTS OF DENTAL STATUS, BACTERIA AND ANTIBIOTICS ON THE METABOLISM OF DIETARY NITRATE

6.1 Effect of dental status on the metabolism of dietary nitrate

6.1.1 Introduction

Endogenous formation of N-nitrosocompounds from nitrite is thought to be an important factor in the pathogenesis of oesophageal and gastric cancer. Saliva is the major source of the nitrite that reaches the oesophagus. The nitrite is generated within the mouth by the action of commensal bacteria upon dietary nitrate, which is absorbed and secreted in saliva. An important determinant of the number of bacteria in the mouth is the presence of natural teeth. Edentulous patients with dentures have fewer oral bacteria than subjects retaining their own teeth (84). The presence of natural teeth is therefore likely to exert a major influence on the salivary nitrite level. Over the past 20 years the prevalence of oesophageal adenocarcinoma and cancer of the gastric cardia has markedly increased (85). The prevalence of edentulous subjects has markedly fallen over the same period and this may explain the rise in prevalence of cancer through increasing concentrations of nitrite in saliva. The purpose of this study was to compare the salivary nitrite concentrations in subjects with and without their own teeth.

6.1.2 Subjects and Methods

Volunteers were excluded if they had taken antibiotic therapy within the previous 2 weeks or were taking any medication known to interfere with salivation.

20 volunteers were recruited. 11 subjects, mean age 70 (50 - 78), were edentulous and routinely placed their dentures in sterilising solution overnight. 9

subjects, mean age 59 (50 - 71), had retained all their teeth and cleaned them regularly.

The study involved one visit only. Volunteers presented after an overnight fast. The subjects with dentures placed them in their mouths at least 2 hours before attending. Those with their own teeth brushed them in their usual manner at least 2 hours before attending. A baseline sample of unstimulated saliva (for salivary nitrite) was collected. A standard nitrate meal of 2 mmoL potassium nitrate (equivalent to the nitrate content of a helping of lettuce) was then ingested. The mouth was rinsed out with water. Over the next 2 hours further samples of saliva were collected at 15 minute intervals.

6.1.3 Results

Baseline measurements of saliva nitrite were similar in the false teeth and natural teeth groups at 191μmol/L (34 - 414) and 60μmol/L (18 - 450) respectively.

Following ingestion of the standard nitrate meal, saliva nitrite levels increased markedly in all subjects to peak at 40 - 60 minutes post ingestion. Nitrite levels remained elevated for the remainder of the 2 hours of the study. The levels of nitrite recorded were particularly high as salivary flow was not stimulated. There was no difference between the levels of nitrite achieved following the standard nitrate meal in subjects with false teeth compared to those who retained their own teeth **(figure 6.1)**.

6.1.4 Figure legend and figure

Figure 6.1 The effect of a standard nitrate meal (2mmol potassium nitrate in 50 ml H_2O) on saliva nitrite levels in subjects with false teeth (n=11) and natural teeth (n=9). Median values and interquartile range over 120 minutes from the time of ingestion are recorded.

Figure 6.1a: Effect of nitrate meal on saliva nitrite levels (false teeth)

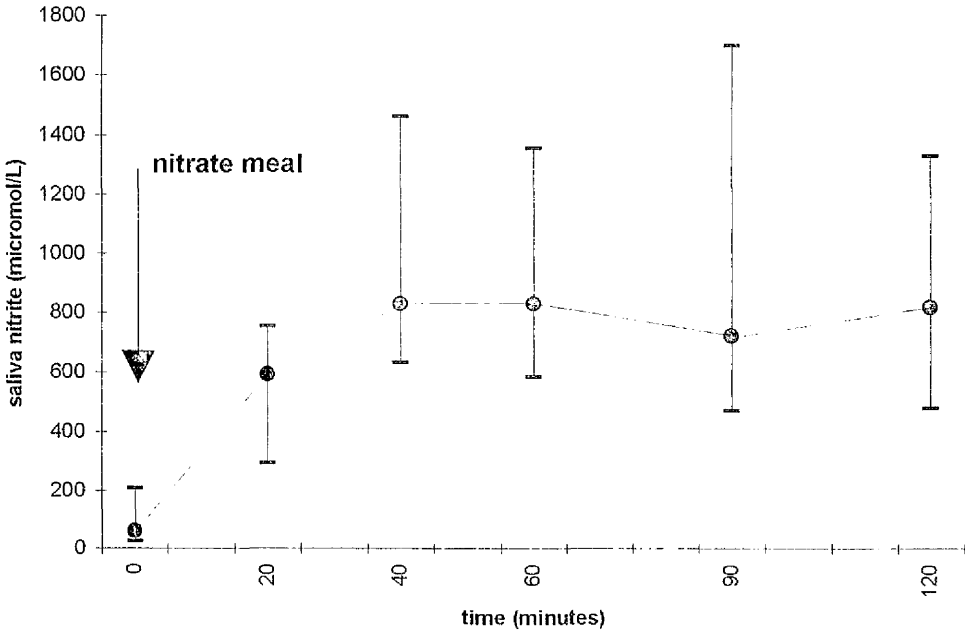
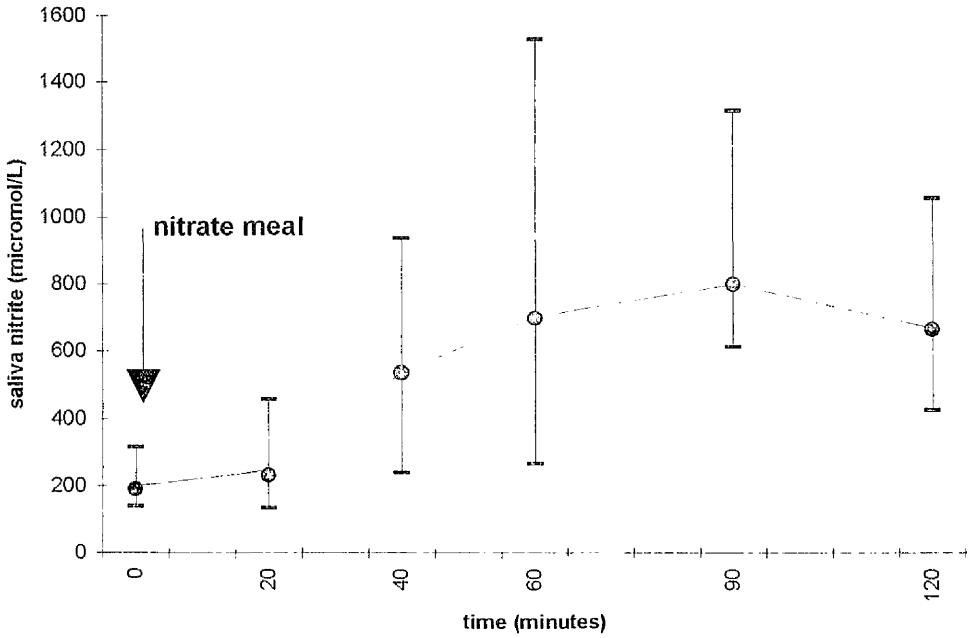


Figure 6.1b: Effect of a nitrate meal on saliva nitrite levels (natural teeth)



6.1.5 Conclusions

The presence of natural teeth does not appear to have a bearing on salivary nitrite levels. This is especially apparent in fasting salivary nitrite recordings but also seen following ingestion of the standard nitrate meal. This suggests that bacterial colonisation of the mouth does not depend on the presence of natural teeth. This would be in keeping with reports suggesting that bacteria resident within the sulci on the dorsum of the tongue are of prime importance in the production of nitrite from ingested nitrate (86).

6.2 Studies on effect of antibiotics on the metabolism of dietary nitrate in the presence of achlorhydria.

6.2.1 Introduction

In patients with achlorhydria, gastric nitrite levels are high. It is believed that denitrifying bacteria colonise the stomach and generate nitrite from dietary nitrate via a bacterial nitrate reductase enzyme. However, we have already demonstrated *in vitro* that levels of nitrite will accumulate in the absence of acid as a result of its chemical stability at high pH. Therefore the accumulation of nitrite in hypochlorhydric gastric juice may arise through two possible mechanisms. We sought to examine the relative contribution of bacteria to gastric nitrite levels in the presence of hypochlorhydria by prescribing a course of antibiotics.

6.2.2 Subjects and Methods

6 healthy volunteers who had no symptoms of dyspepsia were recruited. 3 were H. pylori positive and 3 were H. pylori negative. Subjects were excluded if they had received antibiotics within the previous month or were known to have a penicillin allergy.

Subjects received a 10 day course of omeprazole 40 mg each morning in order to elevate intragastric pH. They reported fasted 24 hours after the last dose. Baseline samples of saliva, blood and gastric juice were collected for saliva nitrite, serum nitrate and gastric juice pH, nitrite and vitamin C assays. A simulated salad meal, containing 2 mmoL potassium nitrate, was infused through the nasogastric tube. Over the next 2 hours further samples of saliva, gastric juice and blood were

taken. Following this, subjects were given 6 further days of omeprazole 40 mg along with amoxycillin 250 mg four times daily. Subjects returned after the first 24 hours of antibiotics and then after completing the course for further measurements. On each occasion the above protocol was followed.

6.2.3 Results

1. Fate of the nitrate meal during omeprazole

At 24 hours after completing the 10 day course of omeprazole, the median intragastric pH was 4.7 (1.3 - 8.0). The gastric pH of the 3 H. pylori infected subjects was 7.2, 7.4, 8.0 respectively; compared to 1.3, 1.9, 2.2 in the 3 H. pylori negative subjects.

Basal saliva nitrite on omeprazole was 54μmol/L (20 - 163) and gastric juice nitrite was 2μmol/L (0 - 19). Following the nitrate meal, saliva nitrite levels increased to a peak of 304μmol/L (243 - 1225) at 40 minutes post ingestion. Gastric nitrite levels also increased significantly in all subjects to a peak value of 64μmol/L (3 - 176) at 60 minutes post ingestion, $p=0.04$, and they remained elevated for the remainder of the experiment.

Basal gastric TVC on omeprazole was 3.4μg/ml (1.2 - 8.6) of which gastric AA comprised 2.9μg/ml (0.6 - 8.4). Following ingestion of the nitrate meal, gastric TVC values were largely unchanged. Gastric AA levels decreased and were low for the remainder of the experiment. The gastric AA:TVC fell in turn from a basal

value of 0.73 (0.33 - 1.0) to its nadir of 0.14 (0.08 - 0.87) at 60 minutes post ingestion, p=0.04.

2. Effect of antibiotic treatment on the fate of dietary nitrate during omeprazole

After completing the course of antibiotics, the study protocol was repeated and the results compared with those obtained prior to antibiotics. Basal saliva nitrite levels before and after completing the course of amoxycillin were similar at 54μmol/L (20 - 163) and 36μmol/L (24 - 144) respectively. The area under the saliva nitrite/time curve on omeprazole was calculated for each subject to compare saliva nitrite levels produced following the nitrate meal pre and post antibiotics. Following the course of antibiotics, the median saliva nitrite auc was lower but this failed to reach significance [26385μmol/L.min (340 - 58480) versus 30440μmol/L.min (23790 - 88255)]. Basal gastric nitrite levels before and after antibiotics were similar at 1.5μmol/L (0 - 19) and 3μmol/L (0 - 8) respectively. The area under the gastric nitrite/time curve on omeprazole was calculated for each subject to compare gastric nitrite levels produced following the nitrate meal pre and post antibiotics. Following the course of antibiotics, the median gastric nitrite auc was also lower but this too failed to reach significance [1385μmol/L.min (65 - 8320) versus 5340μmol/L.min (730 - 13520), p=0.93].

Gastric vitamin C levels did not change significantly following the course of antibiotics although they tended to be higher. Median gastric TVC increased from

3.4μg/ml (1.2 - 8.6) to 4.9μg/ml (2.0 - 10.3), p=0.29, and median gastric AA increased from 2.9μg/ml (0.6 - 8.4) to 3.6μg/ml (0.8 - 9.3), p=0.83. Fasting gastric AA:TVC ratio was unchanged by the antibiotics at 0.73.

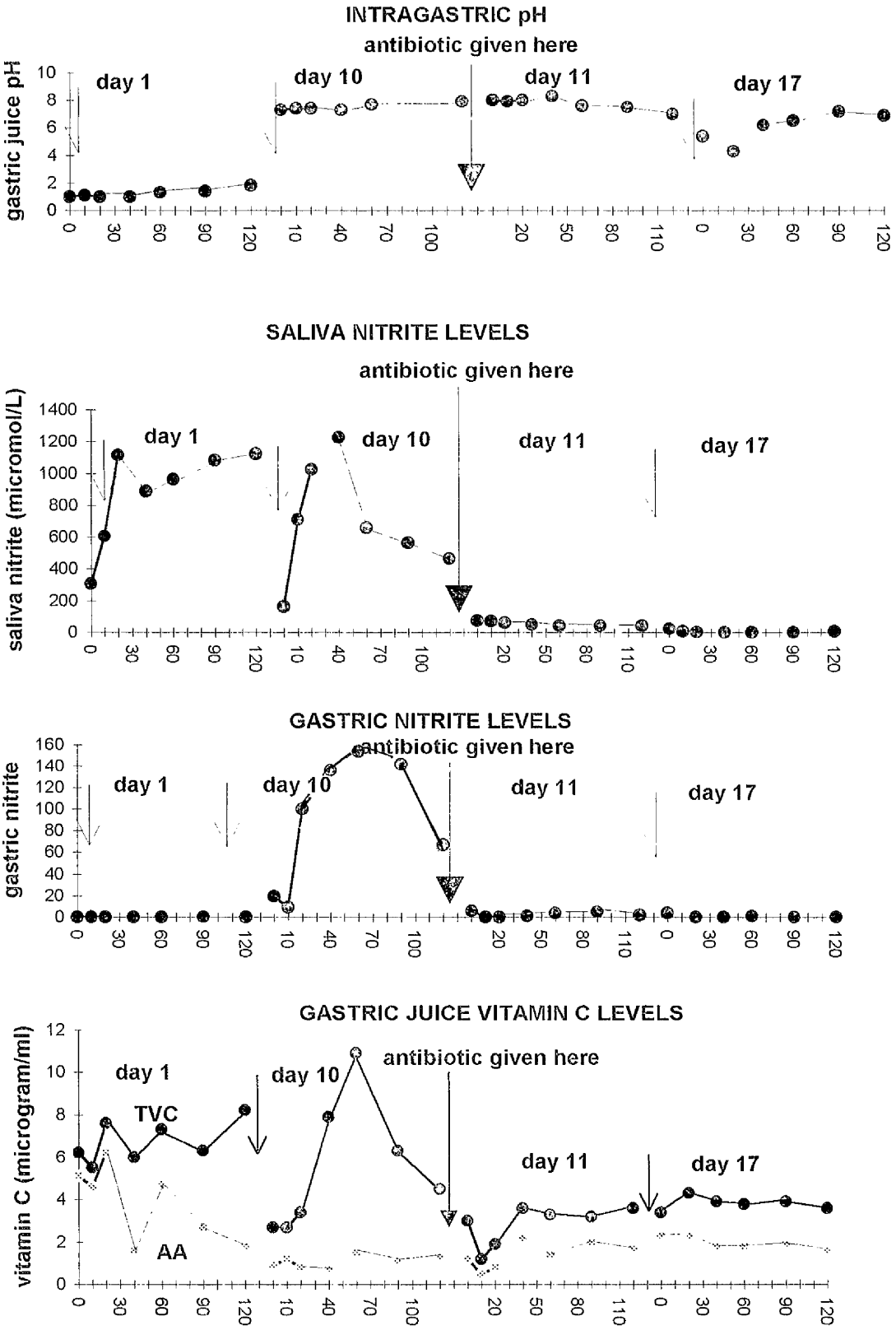
In the 6 individuals studied, the course of amoxycillin altered nitrite levels in only one subject (Subject X); but the effect was substantial **(figure 6.2)**. Within 24 hours of beginning the antibiotics, basal saliva nitrite levels were approximately 50% lower. More significantly, levels did not rise following ingestion of the nitrate meal. Basal gastric nitrite levels were also more than 50% lower. Following ingestion of the nitrate meal, gastric nitrite levels did not rise. These effects were greater still on day 17 after completing the full course of antibiotics; nitrite was undetectable in both saliva and gastric juice.

The course of antibiotics also had a bearing on gastric vitamin C levels following the nitrate meal as illustrated **(figure 6.2)**. In the 2 previous studies, gastric TVC increased markedly following ingestion of nitrate. This rise in gastric TVC coincided with the marked influx of saliva nitrite. Following the course of antibiotics and depletion of saliva nitrite, gastric TVC levels remained steady following the nitrate meal. Gastric AA levels were low in the presence of achlorhydria and were not significantly changed following the course of antibiotics.

6.2.4 Figure legend and figure

Figure 6.2: Summary of the effects of amoxycillin on saliva nitrite, gastric nitrite and gastric vitamin C in Subject X during drug-induced achlorhydria. Data from all 4 study days are displayed. On each study day, 2mmol potassium nitrate is ingested at time zero and measurements taken over the following 2 hours. Day 1 is prior to omeprazole. Day 10 is during omeprazole. Day 11 is following 24hrs of amoxycillin during omeprazole. Day 17 is following 1 week of amoxycillin during omeprazole.

Figure 6.2: Effects of amoxycillin on saliva nitrite, gastric nitrite and gastric vitamin C during drug-induced achlorhydria.



6.2.5 Conclusions

The effect of the course of amoxycillin on nitrite levels was disappointing with respect to the group as a whole. The great diversity of bacteria present in the oropharynx and the presence of antibiotic resistant strains can explain this. However in one subject studied the amoxycillin clearly destroyed all the nitrate reducing bacteria in the oropharynx. This can be inferred from the dramatic absence of saliva nitrite within basal and post nitrate meal samples after a week of antibiotics. The antibiotics also effectively prevented the accumulation of nitrite within the achlorhydric stomach. However, this does not give any further clues as to the origin of nitrite in the achlorhydric stomach. The oropharyngeal bacteria colonise the stomach during achlorhydria therefore antibiotics will destroy these bacteria regardless of where they lie. The results do emphasise the role of oral bacteria in generating a constant supply of nitrite to the upper GI tract.

In the absence on a saliva nitrite influx following the nitrate meal, gastric TVC and the gastric AA:TVC ratio were unchanged for the duration of the experiment. This contrasts with the rise in gastric TVC observed in the previous studies. This reinforces the effect of saliva nitrite on gastric juice AA levels and suggests that the influx of saliva nitrite may be a factor regulating gastric secretion of vitamin C.

These observations of the effect of amoxycillin on saliva nitrite may be of importance in the individual with a healthy acid stomach. If antibiotics reduce or prevent saliva nitrite production, then this will reduce or prevent nitric oxide

generated at the gastro-oesophageal junction when saliva nitrite meets acid gastric juice. Nitric oxide is toxic to bacteria. It has been proposed that the nitric oxide production at the gastro-oesophageal junction is part of the host defences against bacterial infection (87). Therefore this side effect of antibiotics may partly explain the problems of GI infection secondary to antibiotic use.

A further key observation in this study was that at 23 hours after the last dose of omeprazole, the degree of acid suppression was greater in the 3 H. pylori infected subjects. They all remained achlorhydric, whereas the 3 H. pylori negative subjects had near normal gastric pH values. This is in keeping with previous reports (37). This suggests that any effects on the intragastric milieu created by drug-induced achlorhydria are likely to be more sustained in H. pylori-infected subjects.

CHAPTER 7

WILL *HELICOBACTER PYLORI* INFECTION INCREASE THE RISK OF DEVELOPING GASTRIC CANCER DURING LONG TERM OMEPRAZOLE?

7.1 Introduction

In chapter 5 we observed that during omeprazole treatment gastric juice vitamin C concentrations fell in H. pylori positive subjects but not in H. pylori negative subjects. Gastric nitrite levels were markedly elevated in both groups. Thus the ascorbic acid : nitrite ratios were lowered in both groups of subjects, increasing the nitrosating potential of the gastric juice. In that study we examined all subjects at 2 hours following their last dose of omeprazole, when the effects of the drug on acid secretion are maximal, and intragastric pH was >4 in both the H. pylori positive and negative subjects. It is now recognised that over a 24 hour period PPI therapy is much more likely to maintain an intragastric pH>4 in H. pylori positive subjects (82). We observed this first hand in chapter 6. It is likely that H. pylori infected subjects taking a PPI will have a greater nitrosating potential. Any differences in the intragastric milieu (and nitrosating potential) that may exist between H. pylori positive and negative subjects arising as a consequence of drug-induced hypochlorhydria will therefore be most apparent 24 hours after the last dose.

The clinical relevance of this is already apparent. There is growing concern that long term PPI therapy with co-existing H. pylori infection might increase the risk of gastric cancer. Kuipers et al reported that long term omeprazole therapy prescribed for GORD resulted in an increased prevalence of corpus atrophy in H. pylori infected subjects (39). The methodology of this study has since received criticism. Lundell et al recently re-examined this question and concluded that omeprazole produced no significant increase in corpus atrophy (40). However both studies showed a similar degree of accelerated progression to moderate or

severe corpus atrophy in H. pylori positive subjects on omeprazole; 19% after 5 years in the Kuipers study and 18% after 3 years in the Lundell study. Development of moderate or severe atrophy in H. pylori negative subjects on omeprazole and in H. pylori positives not given omeprazole was low in both studies at approximately 2%. The mechanism of the development of atrophic gastritis in H. pylori positive subjects on omeprazole remains to be elucidated.

We propose that the environment created within the lumen of the stomach may be responsible. In the present study we have examined intragastric pH, nitrite, vitamin C and bacterial counts in H. pylori positive and negative subjects before and during a course of omeprazole 40mg daily.

7.2 Subjects and Methods

To facilitate the collection and immediate processing of samples, different groups of subjects were used for the bacteriology and biochemical studies.

29 healthy volunteers were recruited to determine the effect of H. pylori status on bacterial colonisation during omeprazole therapy. The mean age of the H. pylori negative group was 27 (range 20 - 45) and that of the positive group 29 (21 - 45). 32 different subjects (18 male, 14 female), mean age 35 (range 18 - 65) were recruited to examine the effects of H. pylori status on gastric nitrite and vitamin C during omeprazole. 5 of these subjects had dyspepsia and were recruited following upper GI endoscopy with CLO test and histology; 27 were healthy volunteers. All volunteers had their *Helicobacter pylori* status determined by a ^{14}C-urea breath test as previously described (64).

Design.

For the bacteriology study, subjects presented fasted on the morning of each study day. On day 1, gastric juice was sampled. Subjects were then prescribed a 6 week course of omeprazole 40 mg daily. They returned 24 hours after the final dose for a repeat study. For the biochemical study, subjects avoided eating leafy vegetables for 24 hours before presenting fasted on the morning of each study day. On day 1, basal samples of blood, saliva and gastric juice were collected and then the subjects were fed 2 mmol of potassium nitrate, which is equivalent to a standard portion of lettuce (61). Further samples were collected at intervals over the next 2 hours. Subjects were then prescribed a 10-day course of omeprazole 40 mg daily (it is recognised that the degree of acid suppression reaches steady state after 10 days) and returned 24 hours after the final dose for a repeat study. Subjects were asked to avoid antibiotic use for the duration of the studies.

Bacterial analysis

Ten ml of gastric aspirate was immediately inoculated into an anaerobic Bactec Blood Culture bottle (Beckton Dickinson, Oxford, UK) and transported to the laboratory. All samples were processed within 4 hours. The Bacteriologist was unaware of the treatment and H. pylori status of the individual. On receipt, serial decimal dilutions up to 10^{-6} of the Blood Culture medium were made in Brain Heart Infusion broth. 5µl of each dilution was inoculated onto:

Bacitracin agar: blood agar base No. 2 (Oxoid Ltd, Basingstoke, UK) with 5% (v/v) defibrinated horse blood Haemophilus selectatab (Mast MS27A, Liverpool, UK) for isolation of *Haemophilus spp.*

5% blood agar: blood agar base No. 2 (Oxoid), with 5% (v/v) defibrinated horse blood and:

Cysteine Lactose Electrolyte deficient (C.L.E.D.) agar (Oxoid) for determination of aerobic bacterial Total Viable Count (BTVC.)

Gonococcus agar (Oxoid), with 5% (v/v) lysed horse blood, freeze dried antibiotic supplement for *Neisseria gonorrhoea* (Oxoid), yeast autolysate supplement (Oxoid) for isolation of *Neisseria spp.*

5% blood agar: blood agar base No. 2 (Oxoid), 5% (v/v) defibrinated horse blood with Skirrows Selective supplement for isolation of *Helicobacter pylori*

Rogosa agar: rogosa agar (Oxoid), 1.32ml/l glacial acetic acid (Sigma-Aldrich Chemical Co. Ltd., Poole, England) for isolation of *Lactobacillus spp.*

Schaedler agar: schaedler agar base (Oxoid), with 5% (v/v) defibrinated horse blood for anaerobic BTVC and isolation of gram positive anaerobes.

Neomycin agar: schaedler agar base (Oxoid CM 437) with Neomycin supplement (Prolab Diagnostics, Neston, Cheshire, UK), 5% (v/v) defibrinated horse blood for gram negative anaerobes.

Veillonella agar: Bacto Veillonella agar (Difco, East Molsey, UK), Vancomycin supplement (Prolab) for isolation of *Veillonella spp.*

Sabourauds agar (Oxoid) for isolation of Yeasts.

The aerobic plates were incubated for 48 hours at 37°C in air, the anaerobic plates incubated for 72 h at 37°C in an anaerobic jar (BBL anaerobic systems, Oxford, UK). The Rogosa agar was incubated at 37°C in 5% CO_2 for 5 days. Plates for the isolation of *H. pylori* were incubated microaerophilically at 37^0 C for 5 days (BBL Campypak). Bacteria were identified by standard methods to

species or genus level as appropriate. Total viable count was determined by a total colony count of all bacterial types on blood and anaerobic blood agar.

7.3 Results

Of the 29 subjects recruited for the bacteriology study, 18 had a negative ^{14}C-urea breath test and 11 had a positive breath test. All completed 6 weeks of omeprazole.

Of the 32 subjects recruited for the biochemical study, 5 were excluded after incomplete water recovery tests and a further 5 failed to return for the second study day. Thus 22 subjects completed the 10 day course of omeprazole; 20 were healthy volunteers and 2 had dyspepsia. 10 had a positive ^{14}C-urea breath test or positive CLO test plus H. pylori gastritis, 12 had a negative breath test. All values are expressed as medians with range in parentheses.

Intragastric pH (figure 7.1)

Before commencing omeprazole, the intragastric pH of all the H. pylori positive and negative subjects from both studies was similar at 1.6(1.2 - 2.6) and 1.6(1.2 - 5.4) respectively. During omeprazole treatment, median intragastric pH was elevated in both groups. However, it was elevated to a significantly greater degree in the H. pylori positive subjects at 7.8(1.8 - 8.2) compared to 3.0(1.3 - 8.1) in the H. pylori negative subjects, $p<0.00001$. At 24 hours following the final dose of omeprazole, 95% of the H. pylori positive subjects had a pH>4 compared with 40% of the H. pylori negative subjects.

Gastric juice bacterial counts (figure 7.2)

Before commencing omeprazole, the non-H. pylori flora were not significantly different in the H. pylori positive and negative subjects. During omeprazole the bacterial count increased in both groups. There were a significantly greater number of bacteria, expressed as total viable count (BTVC), in the H. pylori positive group compared to the H. pylori negative group [6×10^7 colony forming units (cfu) / ml ($0 - 5 \times 10^8$) versus 5×10^5 cfu / ml ($0 - 6 \times 10^8$), $p<0.05$]. The subjects with the highest intragastric pH during omeprazole therapy had the greatest intragastric bacterial counts {(n=28), Spearman correlation coefficient = 0.846, $p<0.002$}.

During omeprazole treatment a wide variety of bacterial species were identified in both H. pylori positive and negative groups **(table 7.1)**. The most common aerobic isolates in both groups were the α-haemolytic or viridans streptococci, which predominate in the oral flora. Most of the other aerobic bacteria isolated, including *Neisseria spp. Haemophilus spp*, are typical of the oro-pharyngeal flora as opposed to only three isolates of coliforms, which predominate in the aerobic colonic flora. In the anaerobic group the most common isolates were *Veillonella spp and Bacteroides spp* which are part of the anaerobic flora of the entire gastrointestinal tract. Both H. pylori positive and negative groups had organisms that have been shown to have the ability to reduce nitrate to nitrite (illustrated with an asterisk in **table 7.1)**.

Nitrite

Before omeprazole treatment, the median fasting values for saliva nitrite were similar in the H.pylori positive and H.pylori negative groups at 29μmol/L (13 - 306μmol/L) and 57μmol/L (20-169μmol/L) respectively. Similarly, fasting gastric nitrite was largely undetectable at 0μmol/L (0 - 10μmol/L) and 0μmol/L (0 - 13μmol/L) respectively. Following the intragastric administration of the nitrate meal salivary nitrite levels rose significantly in all subjects, peaking at 20-40 minutes post ingestion. In H.pylori positive subjects, median salivary nitrite peaked at 286μmol/L (126 - 1113μmol/L) compared to 498μmol/L (107 - 774μmol/L) in the H.pylori negative subjects. When the areas under the concentration/time curves for saliva nitrite were calculated for each subject, there was no difference in the salivary nitrite load between the H.pylori positive and H.pylori negative groups [26862μmol/L/min (12085 - 115365) vs. 36325μmol/L/min (16625 - 75010) respectively]. Despite this marked rise in salivary nitrite following the nitrate meal, and therefore increased nitrite delivery to the stomach, median gastric nitrite levels remained low. There was no difference between the H.pylori positive and negative subjects in post-meal gastric nitrite levels, as calculated by the median area under the concentration/time curve [670μmol/L/min (9 - 4900) vs. 1237 μmol/L/min (0 - 12945), p=NS].

During omeprazole treatment, the salivary nitrite load delivered to the stomach following the nitrate meal was similar in the H. pylori positive and negative subjects – as measured by the area under the saliva nitrite concentration/time

curve – at 37247 μmol/L/min (14315 - 87079 μmol/L/min) and 39637 μmol/L/min (14630 - 112610 μmol/L/min) respectively. The fasting gastric nitrite levels in the H. pylori positive and negative groups during omeprazole were similar at 7.5μmol/L (0 – 20μmol/L) and 8.5μmol/L (0 – 24μmol/L) respectively and were greater than pre-omeprazole levels in both groups. Following the nitrate meal, however, the H. pylori positive subjects showed a significant rise in gastric nitrite compared to pre-omeprazole levels, as calculated by the area under the gastric nitrite concentration/time curve { median auc: 12450 μmol/L.min (1454 - 17495) vs. 670 μmol/L.min (9 - 4900) pre-omeprazole, p = 0.006}. In contrast in the H. pylori negative subjects there was a positive trend towards an increase in gastric nitrite but this failed to reach significance {median auc: 4708μmol/L.min (680 - 13515) vs. 1237μmol/L.min (0 - 12945) pre-omeprazole, p=0.13}. Consequently on omeprazole the gastric nitrite levels following the nitrate meal were greater in the H. pylori positive subjects compared to the H. pylori negative subjects {12450 μmol/L.min (1454 - 17495) vs. 4708μmol/L.min (680 - 13515), p=0.04}**(figure 7.3)**. Taking all the subjects together there was a significant positive correlation between intragastric pH during omeprazole and gastric nitrite levels following the nitrate meal {(n = 22) Spearman correlation coefficient = 0.48, $p<0.05$}.

Ascorbic acid and total vitamin C

Before commencing omeprazole, the median fasting serum AA in the H.pylori positives was 3.1μg/ml (0.9 - 5.0μg/ml) and serum TVC was 3.4μg/ml (0.9 - 5.4μg/ml). In the H.pylori negative subjects, the values were similar at 3.6μg/ml

(1.4 - 5.4μg/ml) and 3.6μg/ml (1.4 - 6.5μg/ml) respectively. The fasting gastric juice AA in the H.pylori positives was 2.0μg/ml (1.3 - 5.1μg/ml) and gastric TVC was 3.7μg/ml (1.7 - 6.2μg/ml) and the values were similar in the H.pylori negative subjects at 2.6μg/ml (0.8 - 18.6μg/ml) and 4.2μg/ml (1.8 - 19.5μg/ml) respectively. Following the intragastric administration of the nitrate meal, the gastric AA levels fell in both groups and this coincided with the rise in salivary nitrite. Gastric TVC levels remained unchanged. There was no difference between the H. pylori positive and negative subjects following the nitrate meal with respect to AA or TVC.

During omeprazole treatment, fasting gastric TVC levels in the H. pylori positive subjects fell from 3.7μg/ml (1.7 - 6.2μg/ml) pre-omeprazole to 1.8μg/ml (0.7-3.4μg/ml), $p<0.009$, but were unchanged in the H. pylori negative subjects at 3.4μg/ml (1.3 - 14.8μg/ml) vs. 4.2μg/ml (1.8 - 19.5μg/ml) pre-omeprazole. Consequently during omeprazole treatment gastric juice TVC was significantly lower in the H. pylori positives at 1.8 μg/ml (0.7 - 3.4μg/ml) vs. 3.4 (1.3 - 14.8μg/ml) in the H. pylori negatives, $p = 0.02$ **(figure 7.4)**. Following the nitrate meal, TVC levels rose over the 2 hours and at 120 minutes post dosing were significantly greater than fasting levels in both groups. However, gastric TVC levels in H. pylori positive group remained significantly lower than the H. pylori negative group throughout.

Fasting gastric AA levels also fell significantly in the H. pylori positive group on omeprazole from 2.0μg/ml (1.3 - 5.1μg/ml) pre-omeprazole to 1.2μg/ml (0 -

2.4μg/ml), p=0.018, such that AA became undetectable in 5 of the 10 subjects. In the H. pylori negative subjects the fall in fasting gastric AA was less marked, from 2.6μg/ml (0.8 - 18.6μg/ml) pre-omeprazole to 1.6 μg/ml (0.9 - 8.4μg/ml) and was not statistically significant (p=0.13). In this group AA became undetectable in 2 of the 12 subjects. Following administration of the nitrate meal, gastric AA levels were undetectable at the 60 minute time point in 6 of the 10 H. pylori positive subjects versus 3 of the 12 H. pylori negative subjects (p=NS).

7.4Figure legends, figures and table

Figure 7.1: The influence of H. pylori status on intragastric pH before and during omeprazole. Subjects from the bacteriology and biochemical studies are presented together.

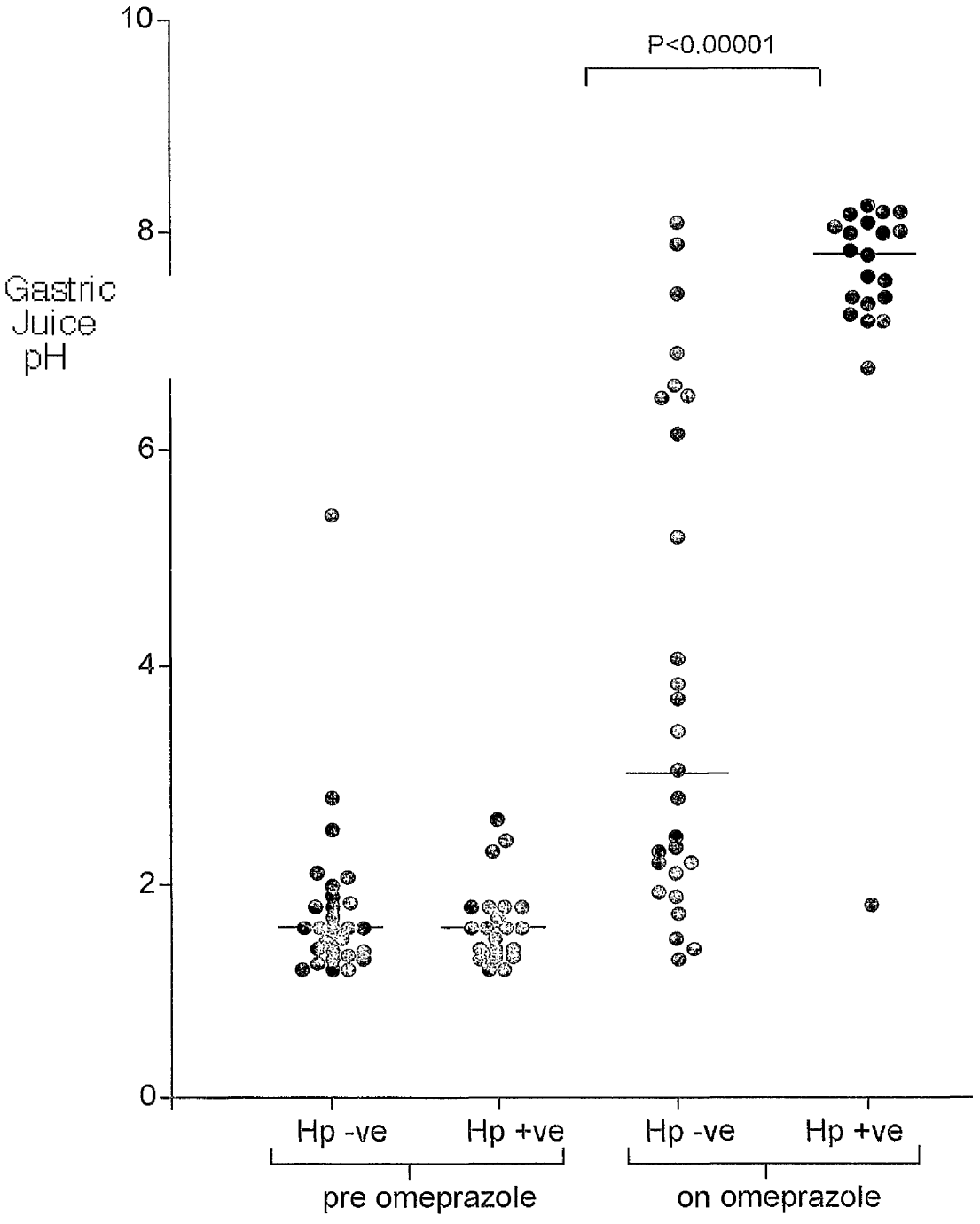


Figure 7.2: The influence of H. pylori status on fasting gastric juice bacterial counts (expressed as colony forming units per millilitre) before and during omeprazole.

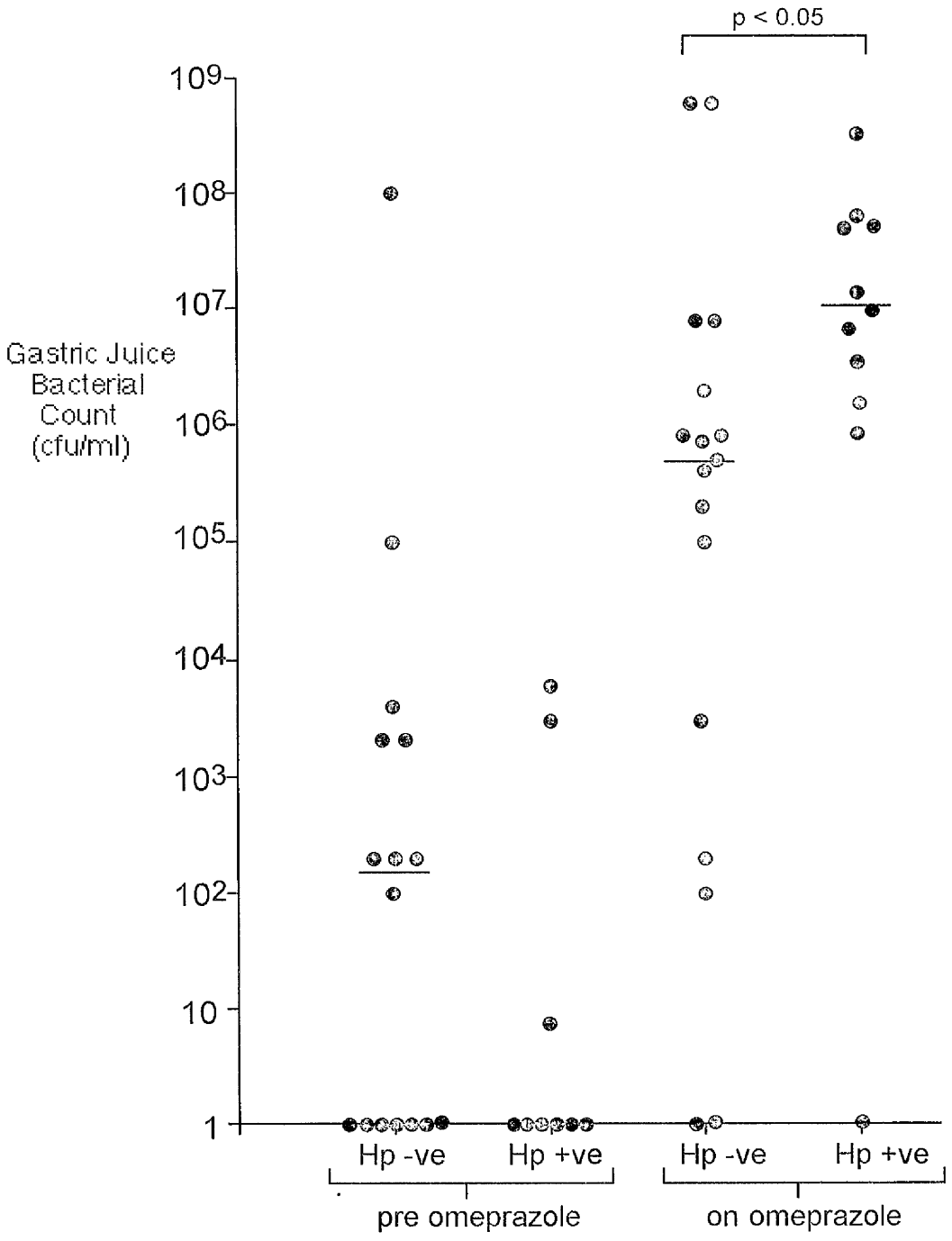


Figure 7.3: The influence of H. pylori status on gastric juice nitrite levels following ingestion of a standard nitrate meal before and during omeprazole. Nitrite levels were recorded in each subject over a 2 hour period following ingestion of the meal and a nitrite concentration/time curve was plotted. Nitrite levels are presented as the area under the concentration/time curve (auc).

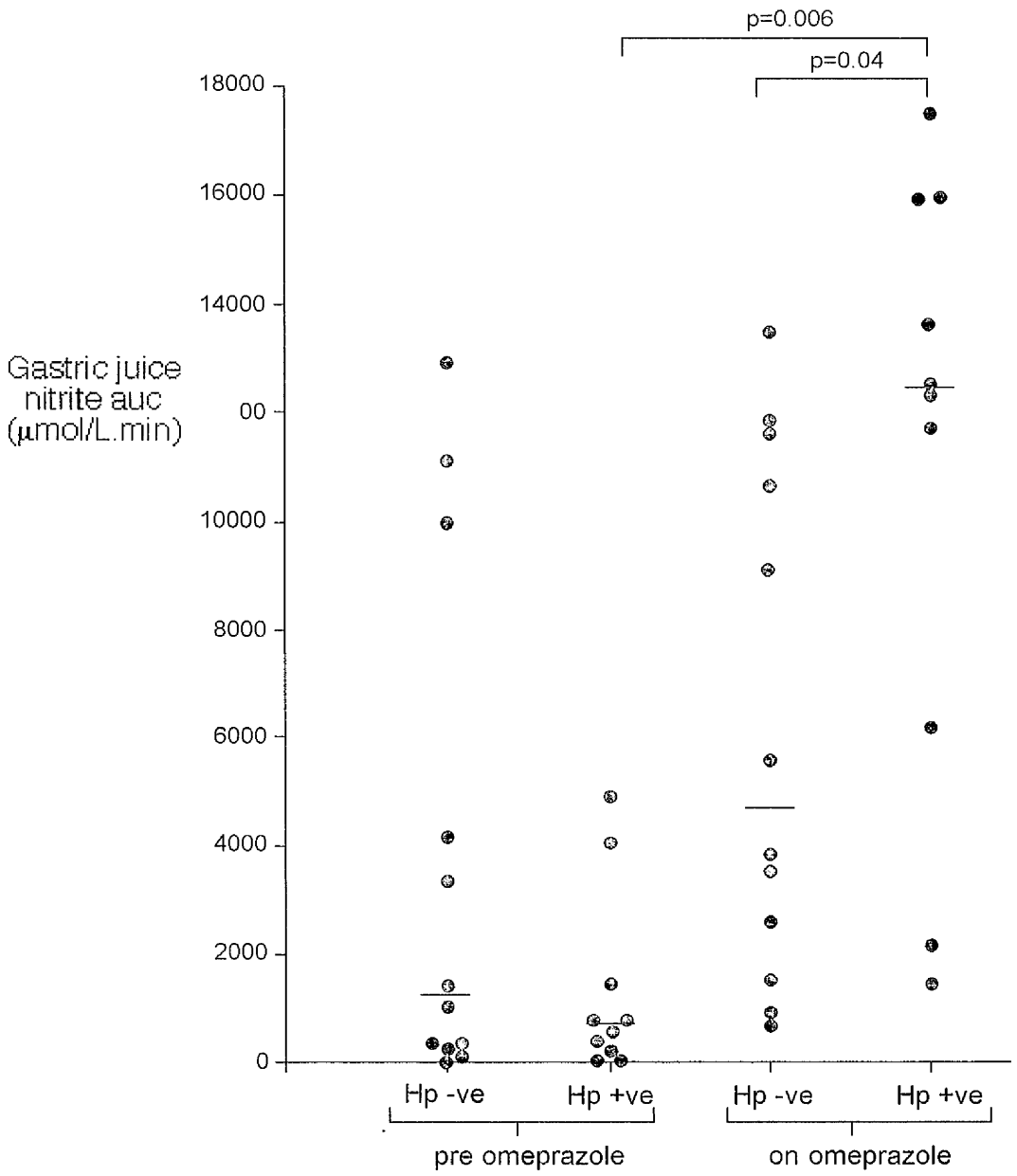


Figure 7.4: Influence of H. pylori status on fasting gastric juice total vitamin C levels before and during omeprazole.

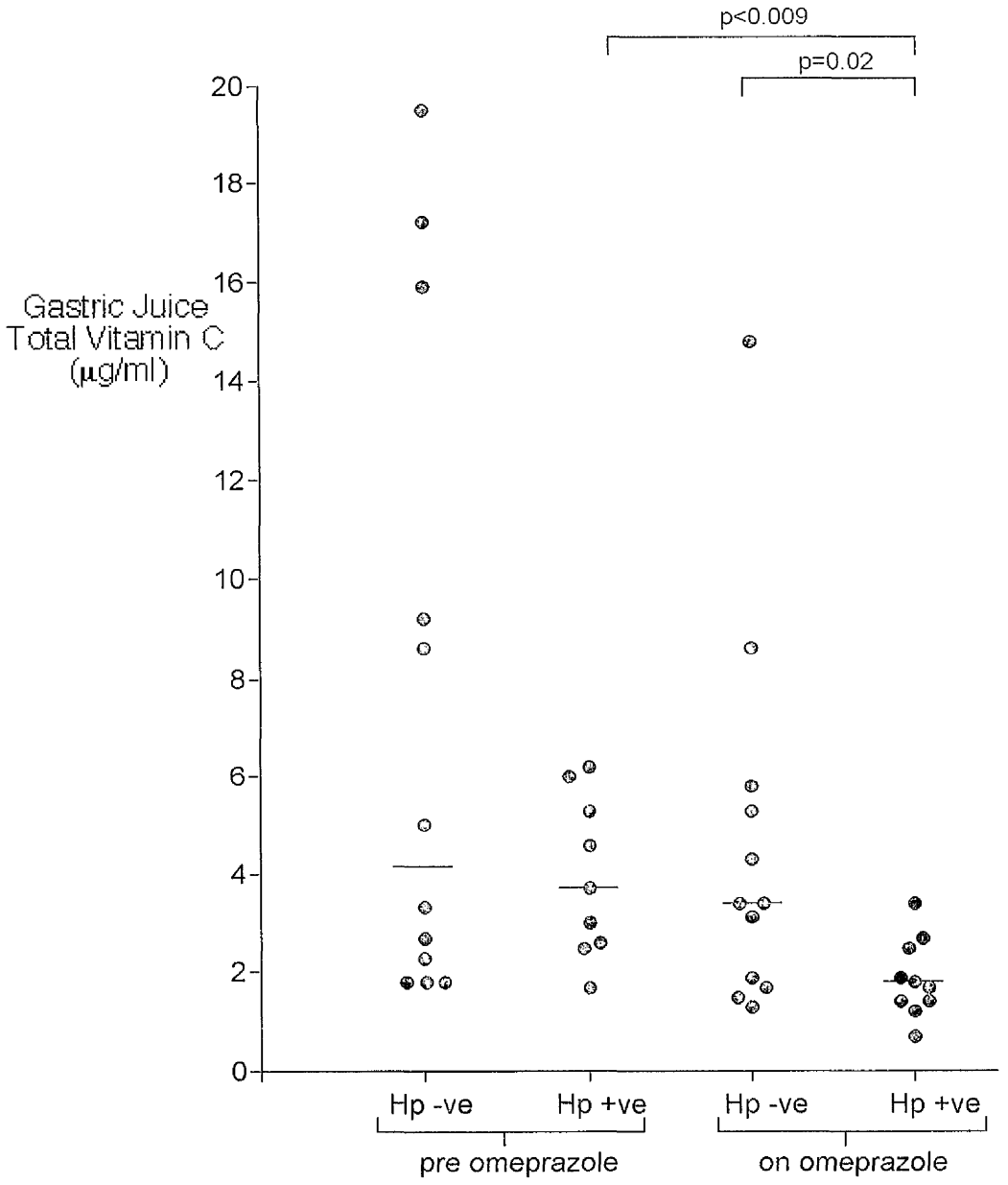


Table 7.1 : Bacterial species isolated from H. pylori positive and negative volunteers during omeprazole.

	H. pylori -ve (n = 18)	H. pylori +ve (n =11)
Non-haemolytic *streptococcus*	3	2
α - haemolytic *streptococcus*	14	9
β - haemolytic *streptococcus*	2	2
Staphylococcus aureus *	3	2
Coagulase negative *Staph.* *	6	5
H. influenzae *	1	1
Haemophilus spp. *	3	4
Branhamella cattharralis *	1	1
Neisseria spp. *	7	8
Lactobacillus spp.	5	1
Corynebacterium spp. *	2	1
Lactose Fermenting Coliform *	2	-
Non Lactose Fermenting Coliform *	1	-
Yeast	1	-
Bacteroides spp.	4	7
Veillonella spp. *	3	6
Clostridium spp *	4	2
Helicobacter pylori	-	2

*** = can reduce nitrate to nitrite**

7.5 Conclusions

This study confirms previous reports (82) that subjects infected with H. pylori achieve greater elevation of intragastric pH during omeprazole treatment than non-infected subjects. It extends previous work (88) by demonstrating that the bacterial colonisation, elevation of intragastric nitrite and depletion of intragastric vitamin C each associated with proton pump inhibitor therapy are more pronounced in or confined to H. pylori infected subjects. During proton pump inhibitor therapy the alteration of the intragastric milieu to favour bacterial synthesis of NOC is therefore more pronounced in H. pylori positive subjects. This may be of relevance to the observed trend of increased moderate to severe corpus atrophy in H. pylori positive subjects during long term PPI therapy(39,40).

The mechanism by which omeprazole produces more profound elevation of intragastric pH in H. pylori infected subjects is incompletely understood. Recent studies have indicated that it is related to greater inhibition of gastric acid secretion rather than the simple neutralisation of the acid by ammonia produced by H. pylori (37). PPI therapy causes H. pylori gastritis to extend into the acid secreting body region of the stomach and this may impair the functioning of the oxyntic mucosa.

It has been known for some time that intragastric pH is the principal determinant of the presence and amount of the non-H. pylori gastric bacterial flora (41). A major role of gastric acid is to destroy bacteria or other micro-organisms which

are constantly being ingested. Achlorhydric patients can be infected by a smaller inoculum of pathogens such as Salmonella spp (89) and Listeria monocytogenes (90) than those with normal levels of acid. In our present study we found that the sustained hypochlorhydria produced by omeprazole in 95% of H. pylori positive subjects was accompanied by a non-H. pylori bacterial overgrowth which was 100 fold greater in density than that observed in the H. pylori negative subjects. This can be explained by the fact that at 24 hours post dose 60% of the H. pylori negative subjects had an intragastric pH<4 at which bacteria would no longer survive.

Previous studies have examined bacterial colonisation of the stomach during PPI therapy. Sharma et al (57) showed that following 14 days of omeprazole 30mg daily the total number of gastric bacteria was $58.6x10^9$/l. This study was not controlled for H. pylori status but the bacterial counts recorded are even higher than our H. pylori positive group. Verdu et al (70) examined the effect of omeprazole 20mg daily on H. pylori negative healthy volunteers and showed a median total viable count of $3.1x10^5$ ($1.0x10^4$-$5.2x10^7$). These results are comparable with our H. pylori negative group and underline the importance that H. pylori status has on bacterial counts.

The species of bacteria colonising the stomach during omeprazole treatment were similar in the H. pylori positive and negative subjects and were consistent with being mainly of oropharyngeal origin. The types of bacteria found in this study are consistent with earlier studies that indicate the predominant flora of the achlorhydric stomach are gram positive facultative streptococci. Our study does

differ in some respects from earlier work in that we found the most common anaerobes isolated were Bacteroides spp and not Veillonella spp. and we isolated more Haemophilus spp (91,92). Some of the bacterial species isolated from the stomach on omeprazole have been demonstrated previously to be able to convert nitrate to nitrite (93) and to synthesise NOC (83,94,95).

We found that the rise in intragastric nitrite levels during omeprazole was significantly greater in H. pylori positive individuals. This was most apparent after the nitrate meal. The increase in nitrite levels on omeprazole may occur by two mechanisms. The first mechanism is related to the enterosalivary recirculation of nitrate and its conversion to nitrite by oral bacteria. 25% of absorbed dietary nitrate is secreted into saliva and 6% converted to nitrite by bacteria on the dorsum of the tongue. This nitrite is then swallowed in the saliva. If the gastric juice is acidic and contains ascorbic acid then the nitrite is rapidly removed by conversion to NO (58). In the process of converting nitrite to NO, the AA is oxidised to DHAA and this explains the fall in AA in gastric juice following the nitrate meal and increased delivery of nitrite in saliva. Elevation of the intragastric pH by omeprazole markedly slows this reaction allowing persistence and accumulation of the swallowed nitrite in the stomach. The higher pH in the H. pylori positive subjects on omeprazole will impair the removal of nitrite swallowed in saliva from gastric juice to a greater degree. The second possible mechanism for the elevation of intragastric nitrite on omeprazole is the presence of oropharyngeal organisms in the stomach and their ability to convert nitrate swallowed in saliva to nitrite within the stomach. Again, the increased density of

colonisation of the stomach in the H. pylori positive subjects on omeprazole will increase the formation of nitrite within the stomach by this additional mechanism.

Changes in gastric juice vitamin C and AA on omeprazole were also more marked in H. pylori positive versus negative subjects. Omeprazole treatment significantly lowered the TVC concentration in the H. pylori positive but not negative subjects. Consequently, during omeprazole treatment the intragastric concentration of TVC both fasting and following the nitrate meal was lower in the H. pylori positive versus negative subjects. The fall in AA on omeprazole both fasting and following the nitrate meal was again more marked in the H. pylori positive subjects. Following the nitrate meal, gastric juice AA became undetectable in 60% of the H. pylori positive subjects versus only 25% of the H. pylori negative subjects.

Several reasons may explain the more marked change in vitamin C and AA in the H. pylori positive versus negative subjects on omeprazole. During omeprazole treatment the inflammation of the gastric mucosa induced by the infection is not confined to the antral region of the stomach but extends to the rest of the stomach producing a pangastritis. This more extensive inflammation of the body mucosa may impair the ability of the mucosa to secrete AA into the gastric juice. In addition the free radicals produced by the inflamed mucosa will oxidise AA to DHAA and the latter to irreversible metabolites of the vitamin (59). The more marked elevation of intragastric pH on omeprazole in the H. pylori positive subjects may also contribute to the greater depletion of TVC as DHAA is less stable at this pH and can be converted to irreversible metabolites.

The more marked depletion of AA in the H. pylori positive subjects on omeprazole may be an additional reason for their significantly greater accumulation of nitrite. The removal of nitrite from gastric juice by converting it to NO is dependent on the presence of AA in gastric juice as well as the acidity of gastric juice. The fact that the H. pylori positive subjects have both a higher intragastric pH and lower intragastric AA will predispose to greater accumulation of nitrite.

In summary, during omeprazole treatment H. pylori positive subjects had a higher intragastric pH, greater secondary bacterial colonisation, a greater rise in intragastric nitrite and a more marked depletion of gastric juice vitamin C than H. pylori negative subjects. These more profound changes may all be a consequence of the more marked elevation of intragastric pH observed in the H. pylori positive subjects. However, in an earlier study (88) we examined H. pylori positive and negative subjects at 2 hours following completion of a 4 week course of omeprazole when the intragastric pH of both groups was similar at pH > 7. On that occasion an equivalent rise in gastric nitrite was observed in both groups. However, total vitamin C concentrations fell in the H. pylori positive subjects only. This suggests that the changes in vitamin C cannot be explained by the change in pH alone but are more likely due to the associated pan-gastritis.

Our studies therefore indicate that changes in the intragastric milieu during PPI therapy which predispose to intragastric NOC formation are more marked in H. pylori positive versus negative subjects. During omeprazole treatment the H.

pylori infected subjects had a greater degree of colonisation by bacteria with the potential to form NOC, a greater rise in intragastric nitrite and more marked depletion of gastric juice TVC. These changes in the intragastric milieu observed in H. pylori positive subjects on omeprazole are identical to changes observed in patients with H. pylori-induced atrophic gastritis; which is now recognised to be a major condition predisposing to gastric cancer (22). The latter subjects have elevated intragastric pH, bacterial colonisation, elevated nitrite and depletion of vitamin C (29,44-46,96,97).

At present it is unclear whether the development of cancer in patients with H. pylori-induced atrophic gastritis is due to the altered intragastric milieu permitting intragastric bacterial synthesis of NOC or due to the more direct effects of the inflamed mucosa or a combination of the two. It is known that H. pylori infection in animals increases their susceptibility to NOC induced cancers (98). H. pylori positive subjects on omeprazole have both the altered intragastric milieu associated with gastric cancer and the inflamed mucosa and considerable concern must exist about the potential long term adverse effects of this combination.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

Achlorhydria is a recognised risk factor for gastric cancer. Correa's original hypothesis for gastric carcinogenesis proposed that the development of atrophic gastritis produced achlorhydria and secondary colonisation of the stomach with nitrosating bacteria. These organisms reduce dietary nitrate to nitrite, then catalyse the synthesis of potentially carcinogenic N-nitroso compounds (NOC) from nitrite and secondary amines present in gastric juice. Vitamin C inhibits NOC synthesis. Subsequent work has identified that H. pylori infection can produce atrophic gastritis and the World Health Organisation has declared it a definite carcinogen.

The development of proton pump inhibitors has greatly enhanced the treatment of acid related disorders. They achieve greater acid suppression than H_2 antagonists. In the presence of co-existing H. pylori infection, they render subjects achlorhydric from one dose to the next. This is associated with the development of a pangastritis, which in turn is associated with a degree of atrophy. Whether this is harmful in the long term remains hotly debated. Two recent reports have suggested that long term PPI therapy leads to the development of moderate-severe atrophic gastritis. This would place patients at an increased risk of gastric cancer.

This thesis examines the intragastric environment produced by PPI's, paying particular attention to the key parameters of dietary nitrate, nitrite and vitamin C that are relevant to NOC synthesis. Using novel methodology this work ultimately demonstrates that, during omeprazole therapy, subjects infected with H. pylori experience greater acid suppression, greater bacterial colonisation of the

stomach, greater gastric nitrite levels than non-infected subjects and also suffer depletion of gastric vitamin C levels. In combination, these changes to the intragastric environment will facilitate intragastric bacterial NOC synthesis. Further studies are required to confirm that NOC synthesis occurs. Measurement of NOC *in vivo* has been attempted by others and is technically difficult due to their volatile nature. Creation of an artificial stomach in which the conditions can be manipulated to mimic the *in vivo* situation is a possible solution. In the interim, any patients requiring long term PPI therapy should have any co-existing H. pylori infection eradicated.

This work also describes the previously unrecognised chemical reaction that occurs *in vivo* when saliva nitrite meets gastric juice ascorbic acid. This finding may be of benefit in understanding the pathophysiology of diseases of the upper GI tract, and the gastro-oesophageal junction in particular. Further studies should be directed towards measurement of the nitric oxide produced by this reaction and its effects. Further studies may also be directed towards examining the chemistry within the lumen in other disorders of the GI tract, such as inflammatory bowel disease, where understanding of the pathophysiology remains elusive.

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993;54:594-606.

2. Hastrup Svendsen J, Dahl C, Bo Svendsen L, Christiansen PM. Gastric cancer in achlorhydric patients; a long-term follow-up study. Scandinavian journal of Gastroenterology 1986;21:16-20.

3. Lechago J, Correa P. Prolonged Achlorhydria and Gastric Neoplasia: Is there a Causal Relationship? Gastroenterology 1993;104:1554-7.

4. Cuello C, Correa P, Haenszel W, Gordillo G, Brown C, Archer MC, Tannenbaum SR. Gastric cancer in Columbia.1. Cancer risk and suspect environmental agents. JNCI 1976;57:1015-20.

5. Tannenbaum SR, Archer MC, Wishnok JS, Correa P, Cuello C, Haenszel W. Nitrate and the etiology of gastric cancer. In: Hiatt HH, Watson JD, Winsten J, eds. Origins of Human Cancer. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1977:1609-1625.

6. Haenszel W, Kurihara M, Sigi M, Lee RKC. Stomach cancer among Japanese in Hawaii. JNCI 1972;49:969-88.

7. Graham S, Schotz W, Martino P. Alimentary factors in the epidemiology of gastric cancer. Cancer 1972;30:927-38.

8. Bjelke E. Epidemiological studies of cancer of the stomach, colon and rectum, with special emphasis on the role of diet. Scandinavian journal of Gastroenterology 1979;9 (suppl.):1-235.

9. Block G. Epidemiologic evidence regarding vitamin C and cancer. Am J Clin Nutr 1991;54:1310s-4s.

10. Palli D. Review in depth: gastric carcinogenesis; dietary factors. European Journal of Gastroenterology and Hepatology 1994;6:1076-82.

11. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman J, Orentreich N, Sibley RL. *Helicobacter pylori* infection and the risk of gastric carcinoma. New England Journal Of Medicine. 1991;325:1127-31.

12. International Agency for Research on Cancer. Schistosomes, Liver Flukes and *Helicobacter pylori*. Lyon, France: IARC, 1994: 61.

13. Correa P. Human Gastric Carcinogenesis: A Multistep and Multifactorial Process - First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Research 1992;52:6735-40.

14. Xu GP, Reed PI. N-Nitroso compounds in fresh gastric juice and their relation to intragastric pH and nitrite employing an improved analytical method. Carcinogenesis 1993;14(12):2547-51.

15. Fan T-Y, Tannenbaum SR. Factors Influencing the Rate of Formation of Nitrosomorpholine from Morpholine and Nitrite: Acceleration by Thiocyanate and Other Anions. J.Agric.Food Chem. 1973;21(2):237-40.

16. Mirvish SS, Wallcave L, Eagan M, Shubik P. Ascorbate - nitrite reaction: possible means of blocking the formation of carcinogenic N-nitroso compounds. Science 1972;177:65-8.

17. Ohshima H, Bartsch H. Quantitative Estimation of Endogenous Nitrosation in Humans by Monitoring N-Nitrosoproline Excreted in the Urine. Cancer Research 1981;41:3658-62.

18. Mackerness CW, Leach SA, Thompson MH, Hill MJ. The inhibition of bacterially mediated N-nitrosation by vitamin C; relevance to inhibition of endogenous N-nitrosation in the achlorhydric stomach. Carcinogenesis 1989;10(2):397-9.

19. Archer MC, Tannenbaum SR, Fan T-Y, Weisman M. Reaction of nitrite with ascorbate and its relation to nitrosamine formation. JNCI 1975;34(5):1203-5.

20. Magee PN. Nitrosamines and human cancer: introduction and overview. European Journal of Cancer Prevention 1996;5(suppl. 1):7-10.

21. Forman D, Newell DG, Fullerton F, Yarnell JWG, Stacey AR, Wald N, Sitas F. Association between infection with *Helicobacter pylori* and risk

of gastric cancer: evidence from a prospective investigation. British Medical Journal 1991;302:1302-5.

22. Sipponen P, Kekki M, Haapakoski J, Ihamaki T, Siurala M. Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross sectional data. Int J Cancer 1985;35:173-7.

23. Siurala M, Ihamaki T, Lehtola M. Atrophic gastritis and its sequelae. Scandinavian journal of Gastroenterology 1974;9:441-6.

24. Tatsuta M, Lishi H, Nakaizumi A, Okuda S, Taniguchi H, Hiyama T, Tsukuma H, Oshima A. Fundal atrophic gastritis as a risk factor for gastric cancer. Int J Cancer 1993;53:70-4.

25. Testoni PA, Masci E, Marchi R, Guslandi M, Rouchi G, Titobello A. Gastric cancer in chronic atrophic gastritis. Associated gastric ulcer adds no further risk. J Clin Gastroenterol 1987;9:298-302.

26. El-Omar EM, Oien K, Murray LS, El-Nujumi A, Wirz A, Gillen D, Williams C, Fullarton G, McColl KEL. Increased prevalence of precancerous changes in relatives of gastric cancer patients: critical role of *H. pylori*. Gastroenterology 2000;118:22-30.

27. La Vecchia C, Negri E, Franceschi S, Gentile A. Family history and the risk of stomach and colorectal cancer. Cancer 1992;70:50-5.

28. Zanghieri G, Di Gregorio C, Sacchetti C, Fante R, Sassatelli R, Cannizzo G, Carriero A, Deleon MP. Familial occurrence of gastric cancer in the 2-year experience of a population-based registry. Cancer 1990;66:2047-51.

29. Sobala GM, Schorah CJ, Sanderson M, Dixon MF, Tompkins DS, Godwin P, Axon ATR. Ascorbic Acid in the Human Stomach. Gastroenterology 1989;97:357-63.

30. Rathbone BJ, Johnson AW, Wyatt JI, Kelleher J, Heatley RV, Losowsky MS. Ascorbic acid: a factor concentrated in human gastric juice. Clin Sci 1989;76:237-41.

31. Schorah CJ, Sobala GM, Sanderson M, Collis N, Primrose JN. Gastric juice ascorbic acid: effects of disease and implications for gastric carcinogenesis. Am J Clin Nutr 1991;53:287-293s.

32. Sobala GM, Schorah CJ, Shires S, Lynch DAF, Gallacher B, Dixon MF, Axon ATR. Effect of eradication of Helicobacter pylori on gastric juice ascorbic acid concentrations. Gut 1993;34:1038-41.

33. Banerjee S, Hawksby C, Miller S, Dahill S, Beattie AD, McColl KEL. Effect of *Helicobacter pylori* and its eradication on gastric juice ascorbic acid. Gut 1994;35:317-22.

34. Ruiz B, Rood JC, Fontham ETH, Malcolm GT, Hunter FM, Sobhan M, Johnson WD, Correa P. Vitamin C Concentration in Gastric juice before and after Anti-*Helicobacter pylori* Treatment. The American Journal of Gastroenterology 1994;89(No.4):533-8.

35. Logan RPH, Walker MM, Misciewicz JJ, Gummett PA, Karim QN, Baron JH. Changes in the intragastric distribution of *Helicobacter pylori* during treatment with omeprazole. Gut 1995;36:12-6.

36. Stolte M, Meining A, Schmitz JM, Alexandridis T, Seifert E. Changes in *Helicobacter-pylori*-induced gastritis in the antrum and corpus during 12 months of treatment with omeprazole and lansoprazole in patients with gastro-oesophageal reflux disease. Aliment Pharmacol Ther 1998;12:247-53.

37. Gillen D, Wirz AA, Neithercut WD, Ardill JES, McColl KEL. *Helicobacter pylori* infection potentiates the inhibition of gastric acid secretion by Omeprazole. Gut 1999;44:468-75.

38. Kuipers EJ, Lee A, Klinkenberg-Knol EC, Meuwissen S. Review article: the development of atrophic gastritis - *Helicobacter pylori* and the effects of acid suppressive therapy. Aliment Pharmacol Ther 1995;9:331-40.

39. Kuipers EJ, Lundell L, Klinkenberg-Knol EC, Havu N, Festen HPM, Liedman B, Lamers CBHW, Jansen JBMJ, Dalenback J, Snel P, Nelis GF,

Meuwissen S. Atrophic gastritis and *Helicobacter pylori* infection in patients with reflux oesophagitis treated with omeprazole or fundoplication. NEJM 1996;334(No.16):1018-22.

40. Lundell L, Miettinen P, Myrvold HE, Pederson SA, Thor K, Andersson A, Hattlebakk J, Havu N, Janatuinen E, Levander K, Liedman B, Nystrom P, and the Nordic GERD study group. Lack of effect of acid suppression therapy on gastric atrophy. Gastroenterology 1999;117:319-26.

41. Gray JDA, Shiner M. Influence of gastric pH on gastric and duodenal flora. Gut 1967;8:574-81.

42. Stockbruegger RW, Cotton PB, Menon CG, Beilby JO, Bartholomew BA, Hill MJ, Walters CL. Pernicious Anaemia, intragastric bacterial overgrowth and possible consequences. Scandinavian journal of Gastroenterology 1984;19:355-64.

43. Stockbruegger RW. Bacterial overgrowth as a consequence of reduced gastric acidity. Scandinavian journal of Gastroenterology 1985;suppl. 111:7-16.

44. Jaskiewicz K, Van Helden PD, Wiid IJF, Steenkamp HJ, Van Wyk MJ. Chronic atrophic gastritis, Gastric pH, Nitrites and Micronutrient Levels in a Population at Risk for Gastric Carcinoma. Anticancer Research 1990;10:833-6.

45. Sobala GM, Pignatelli B, Schorah CJ, Bartsch H, Sanderson M, Dixon MF, Shires S, King RFG, Axon ATR. Levels of nitrite, nitrate, N-nitroso compounds, ascorbic acid and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach. Carcinogenesis 1991;12(2):193-8.

46. O'Connor HJ, Schorah CJ, Habibzedah N, Axon ATR, Cockel R. Vitamin C in the human stomach: relation to gastric pH, gastroduodenal disease, and possible sources. Gut 1989;30:436-42.

47. Forman D, Al-Dabbagh S, Doll R. Nitrates, nitrites and gastric cancer in Great Britain. Nature 1985;313:620-5.

48. Bartholomew BA, Hill MJ. The pharmacology of dietary nitrate and the origin of urinary nitrate. Fd Chem.Toxicol. 1984;22(10):789-95.

49. Burgen ASV, Emmelin G. In: Burgen ASV, Emmelin G, eds. Physiology of the Salivary Glands. Baltimore, MD: Williams & Wilkins, 1961: 140.

50. Spiegelhalder B, Eisenbrand G, Preussman R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to the *in vivo* formation of N-nitroso compounds. Fd Cosmet.Toxicol. 1976;14:545-8.

51. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. Fd Cosmet.Toxicol. 1976;14:549-52.

52. White JW. Relative significance of dietary sources of nitrate and nitrite. J.Agric.Food Chem. 1975;23:886-91.

53. Young-Kyung K, Tannenbaum SR, Wishnok JS. Effects of Ascorbic acid on the Nitrosation of Dialkyl Amines. Advances in Chemistry 1982;200:571-85.

54. Mirvish SS. Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. Cancer Letters 1995;93:17-48.

55. Ruddell WSJ, Bone ES, Hill MJ, Blendis LM, Walters CL. Gastric juice nitrite: a risk factor for cancer in the hypochlorhydric stomach. The Lancet 1976;ii:1037-9.

56. Jones S, Davies P, Savage A. Gastric juice nitrite and gastric cancer. Lancet 1978;ii:1355

57. Sharma BK, Santana IA, Wood EC, Walt RP, Pereira M, Noone P, Smith PLR, Walters CL, Pounder RE. Intragastric bacterial activity and nitrosation before, during, and after treatment with omeprazole. British Medical Journal 1984;289:717-9.

58. Licht WR, Tannenbaum SR, Deen WM. Use of ascorbic acid to inhibit nitrosation: kinetic and mass transfer considerations for an *in vitro* system. Carcinogenesis 1988;9(no.3):365-72.

59. Rose RC, Bode AM. Biology of free radical scavengers: an evaluation of ascorbate. FASEB J 1993;7:1135-42.

60. Washko PW, Welch RW, Dhariwal KR, Wang Y, Levine M. Ascorbic acid and Dehydroascorbic acid Analyses in Biological Samples. Analytical Biochemistry 1992;204:1-14.

61. Lundberg JON, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. Gut 1994;35:1543-6.

62. Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang X-J, Ruiz B, Fontham ETH, Mera R, Miller MJS, Correa P. Inducible Nitric Oxide Synthase, Nitrotyrosine, and Apoptosis in *Helicobacter pylori* Gastritis: Effect of Antibiotics and Antioxidants. Cancer Research 1996;56:3238-43.

63. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radical Biology and Medicine 1994;17(4):333-49.

64. El-Omar EM, Oien K, El-Nujumi A, Gillen D, Wirz A, Dahill S, Williams C, Ardill JES, McColl KEL. Helicobacter pylori infection and Chronic Gastric Acid Hyposecretion. Gastroenterology 1997;113:15-24.

65. Hassan HA, Hobsley H. Positioning of subject and of nasogastric tube during a gastric secretion study. British Medical Journal 1970;1:458-60.

66. Sanderson MJ, Schorah CJ. Measurement of ascorbic acid and dehydroascorbic acid in gastric juice by high pressure liquid chromatography. Biomed.Chromatogr. 1987;2:197-202.

67. Nishana K, Mikawa K, Takao Y, Shiga M, Maekawa N, Obara H. A comparison of rabeprazole, lansoprazole, and ranitidine for improving preoperative gastric fluid properties in adults undergoing elective surgery. Anesth Analg 2000;90(3):717-21.

68. Milton-Thompson GJ, Lightfoot NF, Ahmet Z, Hunt RH, Barnard J, Bavin PMG, Brimblecombe RW, Darkin DW, Moore PJ, Viney N. Intragastric acidity, Bacteria, Nitrite, and N-Nitroso compounds before, during, and after cimetidine treatment. The Lancet 1982;i:1091-5.

69. Stockbruegger RW, Cotton PB, Eugenides N, Bartholomew BA, Hill MJ, Walters CL. Intragastric nitrites, nitrosamines, and bacterial overgrowth during cimetidine treatment. Gut 1982;23:1048-54.

70. Verdu E, Viani F, Armstrong D, Fraser R, Siegrist HH, Pignatelli B, Idstrom J-P, Cederberg C, Blum AL, Fried M. Effect of omeprazole on intragastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. Gut 1994;35:455-60.

71. Boyland E, Nice E, Williams K. The Catalysis of Nitrosation by Thiocyanate from Saliva. Fd Cosmet.Toxicol. 1971;9:639-43.

72. Schievelbein H, Werle E, Schulz EK, Baumeister R. The influence of tobacco smoke and nicotine on thiocyanate metabolism. Naunyn-Schmiedebergs Arch.exp.Path.Pharmak. 1969;262:358

73. McColl KEL, Melby K, Aase S, Jellun E, Fyfe V, Ferguson J, Vollset SE, Hansen S. Gastric mucosal atrophy is a strong predictor of non-cardia gastric cancer but not of cardia cancer. Gut 1998;42(suppl. 1):A52

74. Wu-Williams AH, Yu MC, Mack TM. Life-style, workplace, and stomach cancer by sub-site in young men of Los Angeles County. Cancer Research 1990;50:2569

75. Unakami M, Hara M, Fukuchi S, Akiyama H. Cancer of the gastric cardia and the habit of smoking. Acta Pathol.Jpn. 1989;39:420

76. Moncada S, Higgs A. Mechanisms of disease: The L-Arginine - Nitric Oxide Pathway. NEJM 1993;329:2002-12.

77. Thorens J, Froelich F, Schwizer W, Saraga E, Bille J, Gyr K, Duroux P, Nicolet M, Pignatelli B, Blum AL, Gonvers JJ, Fried M. Bacterial overgrowth during treatment with omeprazole compared with cimetidine: a prospective randomised double blind study. Gut 1996;39:54-9.

78. Reed PI, Smith PLR, Haines K, House FR, Walters CL. Effect of cimetidine on gastric juice N-nitrosamine concentration. The Lancet 1981;(ii):553-5.

79. Mirvish SS. The Etiology of gastric cancer; intragastric nitrosamide formation and other theories. Journal of the National Cancer Institute 1983;71(3):629-47.

80. Correa P. A Human Model of Gastric Carcinogenesis. Cancer Research 1988;48:3554-60.

81. Odum L, Andersen LP. Investigation of *Helicobacter pylori* ascorbic acid oxidating activity. FEMS Immunology and Medical Microbiology 1995;10:289-94.

82. Labenz J, Tillenberg B, Peitz U, Idstrom J-P, Verdu EF, Stolte M, Borsch G, Blum AL. Helicobacter pylori augments the pH-increasing effect of omeprazole in patients with duodenal ulcer. Gastroenterology 1996;110:725-32.

83. Ziebarth D, Spiegelhalder B, Bartsch H. N-nitrosation of medicinal drugs catalysed by bacteria from human saliva and gastro-intestinal tract, including *Helicobacter pylori*. Carcinogenesis 1997;18(2):383-9.

84. Socransky SS, Manganiello SD. The oral microbiota of man from birth to senility. J.Periodontol 1971;42:485-96.

85. Blot W, Devesa SS, Kneller RW, Fraumeni Jr JF. Rising incidence of adenocarcinoma of the oesophagus and gastric cardia. JAMA 1991;13(265):1287-9.

86. Li H, Duncan C, Townend J, Killham K, Smith LM, Johnston P, Dykhuisen R, Kelly D, Golden M, Benjamin N, Liefert C. Nitrate-reducing bacteria on rat tongues. Appl.Environ Microbiol 1997;63(3):924-30.

87. Dougall H, Smith L, Duncan C, Benjamin N. The effect of amoxycillin on salivary nitrite concentrations: an important mechanism of adverse reactions? British Journal of Clinical Pharmacology 1995;39:460-2.

88. Mowat C, Carswell A, Wirz A, McColl KEL. Omeprazole and dietary nitrate independently affect levels of Vitamin C and nitrate in gastric juice. Gastroenterology 1999;116:813-22.

89. Gianella RA, Brontman SA, Zamacheck N. Salmonella enteritis 1: role of reduced gastric acid secretion in pathogenesis. Dig Dis 1972;16:1000-6.

90. Glupczynski Y. Prolonged gastric acid suppression therapy: a significant risk factor for *Listeria monocytogenes* infection? European Journal of Gastroenterology and Hepatology 1996;8(11):1063-6.

91. Borrelio SP, Reed PI, Dolby JM, et al. Microbial and metabolic profile of achlorhydric stomach: comparison of pernicious anaemia and hypogammaglobulinaemia. J.Clin.Pathol. 1985;38:946-53.

92. Husebye E, Skar V, Hoverstad T, Melby K. Fasting hypochlorhydria with gram positive flora is highly prevalent in healthy old people. Gut 1992;33:1331-7.

93. Forsythe SJ, Dolby JM, Webster ADB. Nitrate and nitrite reducing bacteria in the achlorhydric stomach. J.Med Microbiol 1988;25:253-9.

94. Calmels S, Ohshima H, Vinert P, Gounot AM, Bartsch H. Isolation of an enzyme catalysing nitrosamine formation in *Ps aeruginosa* and *N. mucosae*. Carcinogenesis 1985;6(6):911-5.

95. Mills AL, Alexander M. N-nitrosamine formation by cultures of several microorganisms. Applied and Environmental Microbiology 1976;31(6):892-5.

96. Rood JC, Ruiz B, Fontham ETH, Malcolm GT, Hunter FM, Sobhan M, Johnson WD, Correa P. *Helicobacter pylori* -associated gastritis and the

ascorbic acid concentration in gastric juice. Nutrition and Cancer 1994;22(1):65-72.

97. Houben GMP, Stockbruegger RW. Bacteria in the aetio-pathogenesis of gastric cancer: A review. Scandinavian journal of Gastroenterology 1995;30(suppl 212):13-8.

98. Sugiyama A, Maruta F, Ikeno T, Ishida K, Kawasaki S, Katsuyama T, Shimizu N, Tatematsu M. *Helicobacter pylori* infection enhances N-Methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. Cancer Research 1998;58:2067-9.

PUBLICATIONS AND COMMUNICATIONS

PAPERS

Omeprazole and dietary nitrate independently affect levels of vitamin C and nitrite in gastric juice. Mowat C, Carswell A, Wirz A, McColl KEL. Gastroenterology 1999; 116:813-822.

Omeprazole, *Helicobacter pylori* status and alterations in the intragastric milieu facilitating bacterial N-nitrosation. Mowat C, Williams C, Gillen D, Hossack M, Gilmour D, Carswell A, Wirz A, McColl KEL (Gastroenterology; in press).

In vitro studies indicate that nitrosation in acid stomach will be maximal at the gastroesophageal junction and cardia. A Moriya, J Grant, C Mowat, C Williams, A Carswell, T Preston, S Anderson, K E L McColl. (submitted)

ABSTRACTS

Omeprazole lowers gastric juice ascorbic acid and elevates gastric juice nitrite concentrations. Gut 1998: 42; suppl. 1: A67. Mowat C, Carswell A, McColl KEL.

Omeprazole lowers gastric juice ascorbic acid and elevates gastric juice nitrite concentrations. Gastroenterology 1998: 114; No.4; A236. Mowat C, Carswell A, McColl KEL.

Dietary nitrate lowers gastric juice ascorbic acid concentrations.

Gastroenterology 1998: 114; No.4; A648. Mowat C, Carswell A, McColl KEL.

Omeprazole induced changes in gastric nitrite and vitamin C are greater in H. pylori positive subjects. Gut 1999:44; suppl. 1: A81. Mowat C, Carswell A, McColl KEL.

Reaction of salivary nitrite with gastric acid and vitamin C relevant to proximal gastric cancer and Barrett's oesophagus. Gastroenterology 1999: 116; No.4; A414. Moriya A, Grant J, Williams C, Mowat C et al.

Oxygen facilitates nitrosamine synthesis by depleting ascorbic acid in acid stomach:

Relevance to proximal gastric cancer. Gastroenterology 1999: 116; No.4; A469. Moriya A, Grant J, Williams C, Mowat C et al.

ORAL PRESENTATIONS

Will long term proton pump inhibitor therapy increase the risk of gastric cancer? Scottish Society of Physicians, Stirling, September 1997

Effect of omeprazole therapy on intragastric concentrations of nitrite and ascorbic acid. European Gastro Club, Munster, October 1997

Dietary nitrate lowers gastric juice ascorbic acid concentrations.

American Gastroenterology Association, New Orleans, May 1998

Omeprazole lowers gastric juice ascorbic acid and elevates gastric juice nitrite concentrations. Caledonian Society of Gastroenterology, Dundee, June 1998

Omeprazole will facilitate intragastric bacterial synthesis of N-nitrosocompounds in *Helicobacter pylori* infected subjects. Caledonian Society of Gastroenterology, Livingston, November 1999

POSTER PRESENTATIONS

Omeprazole lowers gastric juice ascorbic acid and elevates gastric juice nitrite concentrations. British Society of Gastroenterology, Harrogate, March 1998

Omeprazole lowers gastric juice ascorbic acid and elevates gastric juice nitrite concentrations. American Gastroenterology Association, New Orleans, May 1998

Omeprazole induced changes in gastric nitrite and vitamin C are greater in H. pylori positive subjects. British Society of Gastroenterology, Glasgow, March 1999

Omeprazole, *Helicobacter pylori* status and alterations in the intragastric milieu facilitating bacterial N-nitrosation. American Gastroenterology Association, San Diego, May 2000