Identification of clinically-informative biomarkers within the spectrum of gastro-oesophageal reflux disease in the South African population

CJ VAN RENSBURG

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Supervisor:  Dr M. J. Kotze
Co-supervisors:  Prof. C. Wright
                      Dr G. de Jong

April 2006
DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

Signature: ...........................................
Date: .............................................
SUMMARY

Patients with chronic gastro-oesophageal reflux disease are predisposed to Barrett’s metaplasia and oesophageal adenocarcinoma. The availability of molecular markers that can objectively identify patients with Barrett’s oesophagus at increased risk of carcinoma is highly desirable. A literature search was conducted to identify potentially useful biomarkers for genotype-phenotype correlation studies in South African patients with Barrett’s oesophagus.

The COX-2, c-myb and c-myc genes selected for mRNA expression analysis were analysed in 26 patients with Barrett’s metaplasia (BM) without dysplasia; 14 with Barrett’s oesophagus and dysplasia (BD); 2 patients with Barrett’s adenocarcinoma (BAC); 19 with erosive oesophagitis (ERD); 25 with non-erosive oesophagitis (NERD) and 19 control individuals with a normal gastroscopy and no gastro-oesophageal reflux disease (GORD) symptoms. In the BD/BAC group, 69% (11/16) showed increased c-myb mRNA expression compared with 35% (9/26) in the BM group (p = 0.03). A statistically significant difference (p = 0.002) in c-myb expression was also observed between Barrett’s patients (20/42, 48%) and the control groups (9/63, 14%). In the BD patients, 21% (3/14) had increased c-myc mRNA expression compared with none in those with BM (p < 0.05) and BAC. No significant differences in mRNA expression levels were observed between ethnic groups for the genes analysed.
In an attempt to determine whether the low expression level of c-myc in the study cohort may be related to possible gene-gene interaction, DNA samples of 199 individuals were subjected to genotyping of the functional GT-repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene. Both these genes are involved in iron metabolism and c-myc is known to repress NRAMP1/SLC11A1. Genotype and allele frequencies were similar in all the groups studied with the 3/3 genotype being the most common. However, none of the three above-mentioned BD patients with increased c-myc mRNA expression had the 3/3 genotype. Therefore, although small in number, c-myc-NRAMP1/SLC11A1 interaction may be of adverse significance in patients with allele 2.

TP53 mutation analysis was performed on 68 Barrett’s patients, and TP53 immuno-staining on oesophageal biopsy specimens of 55 subjects. Sporadic TP53 mutations were not identified in any of the patients with BM or dysplasia without BAC. Immuno-histochemistry staining of 2+ and 3+ intensity was similar in patients with metaplasia and dysplasia (58%). The low mutation frequency and relative non-specificity of TP53 immunostaining observed in Barrett’s patients seem to preclude its widespread use as a screening tool. TP53 mutation detection may however be useful for risk stratification once dysplasia has been diagnosed, as mutations G245R and D281Y were identified in two patients with BAC.
Of the genes studied in the South African population, c-myb represents the most useful marker for early detection of an increased cancer risk in Barrett’s patients. In future, patients with Barrett’s oesophagus may benefit from genetic assessment to complement existing cancer surveillance and treatment strategies.
OPSOMMING

Pasiënte met kroniese gastro-esofageale refluks se risiko vir Barrett se metaplasie (BM) en esofageale adenokarsinoom is verhoog. Daar is ’n behoefte vir molekulêre merkers wat objektiewe identifikasie van hoë-risiko pasiënte met Barrett esofagus moontlik sal maak. ’n Literatuurstudie is uitgevoer om potensieel bruikbare merkers te identifiseer vir genotipe-fenotipe studies in Suid-Afrikaanse pasiënte met Barrett esofagus.

Die COX-2, c-myb en c-myc gene wat geselekteer is vir mRNA uitdrukking analise is geanaliseer in 26 pasiënte met Barrett metaplasie (BM) sonder displasie; 14 met displasie (BD); 2 pasiënte met Barrett-geassosieerde adenokarsinoom (BAK); 19 met erosiewe esofagitis; 25 met nie-erosiewe esofagitis en 19 kontrole pasiënte met ‘n normale gastroskopie en sonder gastro-esofageale refluks simptome. In die BD/BAK groep het 69% (11/16) verhoogde c-myb mRNS uitdrukking getoon, vergeleke met 35% (9/26) in die BM groep (p = 0.03). ’n Statisties betekenisvolle verskil (p = 0.002) in c-myb mRNS uitdrukking is ook waargeneem tussen Barrett pasiënte (20/42, 48%) en die kontrole groepe (9/63, 14%). In die pasiënte met BD, het 21% (3/14) verhoogde c-myc mRNS uitdrukking getoon vergeleke met geen in die BM (p < 0.5) en BAK groep nie. Geen betekenisvolle verskille in geen mRNS uitdrukking is waargeneem tussen die verskillende etniese groepe nie.
In 'n poging om vas te stel of die lae vlak van c-myc uitdrukking wat waargeneem is verwant kan wees aan moontlike geen-geen interaksie, is die funksionele GT-herhaling polimorfisme in die promoter streek van die NRAMP1/SLC11A1 geen geanaliseer in DNS monsters van 199 individue. Beide gene is betrokke by yster metabolisme en c-myc opponeer die aksie van NRAMP1/SLC11A1. Die NRAMP1/SLC11A1 genotipe en alleel frekwensies was soortgelyk in alle groepe wat bestudeer is, met die 3/3 genotipe die algemeenste. Nie een van die pasiënte met BD en verhoogde c-myc mRNS-uitdrukking het 'n 3/3 genotipe gehad nie. Hoewel die getalle klein is, wil dit voorkom asof geen-geen interaksie tussen c-myc en NRAMP1/SLC11A1 moontlik kan bydra tot verhoogde risiko in pasiënte met alleel 2.

TP53 mutasieanalise is uitgevoer op 68 Barrett pasiënte en TP53 immuno-histochemie kleuring op die esofageale weefsel van 55 pasiënte. Sporadiese TP53 mutasies is nie in enige van die pasiënte met BM of displasie sonder BAK waargeneem nie. Immuno-histochemie kleuring van 2+ en 3+ intensiteit was dieselfde in pasiënte met metaplasie en displasie (58%). Die lae mutasie frekwensie en relatiewe nie-spesifisiteit van TP53 immunokleuring wat waargeneem is in Barrett se pasiënte beperk wye gebruik van hierdie merker as sifting hulpmiddel. Identifikasie van TP53 mutasies kan egter bruikbaar wees vir risiko stratifisering nadat displasie gediagnoseer is, want mutasies G245R en D281Y is waargeneem in twee pasiënte met BAK.
Van die gene wat bestudeer is in die Suid-Afrikaanse populasie, verteenwoordig c-myb die mees bruikbare merker vir vroeë waarneming van verhoogde kanker risiko in Barrett pasiënte. In die toekoms kan pasiënte met Barrett esofagus baat vind by genetiese bepalings wat bestaande opvolg vir kankerontwikkeling en behandeling strategieë komplimenteer.
DEDICATION

I would like to dedicate this work to all volunteers who participated in this study and without whom the clinical research would not have been possible and also to my family and friends for their encouragement.
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The University of Stellenbosch and AstraZeneca (SAGES 2004 scholarship) for financial support.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>OAC</td>
<td>Oesophageal adenocarcinoma</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta-actin</td>
</tr>
<tr>
<td>Bam H1</td>
<td>restriction endonuclease enzyme</td>
</tr>
<tr>
<td>BD</td>
<td>Barrett’s dysplasia</td>
</tr>
<tr>
<td>BM</td>
<td>Barrett’s metaplasia</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BO</td>
<td>Barrett’s oesophagus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>$X^2$</td>
<td>Chi-Square</td>
</tr>
<tr>
<td>CCK$_2$</td>
<td>cholecystokinin 2</td>
</tr>
<tr>
<td>CK</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase 2</td>
</tr>
<tr>
<td>CLO</td>
<td>Columnar-lined oesophagus</td>
</tr>
<tr>
<td>CLOtest®</td>
<td>rapid urease test</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>2’-deoxyadenosine-5’-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2’-deoxycytidine-5’-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2’-deoxyguanosine-5’-triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>2’-deoxyribonucleoside-5´-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2’-deoxythymidine-5´-triphosphate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>DNAase</td>
<td>modifying enzyme, deoxyribonuclease</td>
</tr>
<tr>
<td>ERD</td>
<td>Erosive reflux disease</td>
</tr>
<tr>
<td>g/dL</td>
<td>gram per decilitre</td>
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<tr>
<td>GORD</td>
<td>Gastro-oesophageal reflux disease</td>
</tr>
<tr>
<td>GOJ</td>
<td>Gastro-oesophageal junction</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HGD</td>
<td>High grade dysplasia</td>
</tr>
<tr>
<td>IGD</td>
<td>Intermediate grade dysplasia</td>
</tr>
<tr>
<td>LDA</td>
<td>Linear discriminant analysis</td>
</tr>
<tr>
<td>LGD</td>
<td>Low grade dysplasia</td>
</tr>
<tr>
<td>LOD</td>
<td>logarithm of odds</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LOS</td>
<td>Lower oesophageal sphincter</td>
</tr>
<tr>
<td>M</td>
<td>molar, moles per litre</td>
</tr>
<tr>
<td>Mg</td>
<td>magnesium</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>magnesium chloride</td>
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<tr>
<td>µg/L</td>
<td>microgram per litre</td>
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<tr>
<td>µL</td>
<td>microlitre</td>
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<tr>
<td>µmol/L</td>
<td>micro mole per litre</td>
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<tr>
<td>µM</td>
<td>micro molar</td>
</tr>
<tr>
<td>miz-1</td>
<td>c-myc-interacting zinc finger protein 1</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>NE</td>
<td>normal oesophagus</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>NERD</td>
<td>Non-erosive reflux disease</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO₃</td>
<td>nitrate</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>pmol</td>
<td>picomol</td>
</tr>
<tr>
<td>PNCA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton pump inhibitor</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAse</td>
<td>modifying enzyme, ribonuclease</td>
</tr>
<tr>
<td>Rsa 1</td>
<td>restriction endonuclease enzyme</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>solute carrier family 11 member 1</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>yrs</td>
<td>age in years</td>
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CHAPTER 1

INTRODUCTION
In the realm of gastro-oesophageal reflux disease (GORD), the most serious consequence is Barrett's metaplasia with the associated risk of oesophageal adenocarcinoma (OAC) (Cameron et al. 1995). Barrett's oesophagus is diagnosed in approximately 6–12% of patients undergoing endoscopy for symptoms of GORD (Sarr et al. 1985; Cameron and Lomboy 1992). It still remains unclear why some patients with GORD develop Barrett's oesophagus whereas others do not. The symptoms of Barrett's oesophagus may be no different than those of conventional GORD uncomplicated by columnar metaplasia (Bonelli 1993). Furthermore, there is a correlation between the length of Barrett's mucosa and the duration of oesophageal acid exposure (Loughney et al. 1998; Fass et al. 2001). The mechanism whereby injury triggers metaplasia, and why this occurs in some but not all individuals, is unknown.

Barrett's oesophagus is the condition in which columnar epithelium replaces the squamous epithelium that normally lines the distal oesophagus. Of the three histological types of columnar epithelia described in Barrett's oesophagus, specialised intestinal metaplasia is the most common. This is also the only type that has a clear malignant potential and therefore the term “specialised intestinal metaplasia” should preferably be used when referring to the increased cancer risk in Barrett’s oesophagus (Spechler 1996). Dysplasia in Barrett's mucosa is still the reference standard for assessing its pre-malignant potential, but histological classification is, unfortunately, subject to inter-observer error and interpretation problems (Alderson 2002; Levine 1997).
Oesophageal adenocarcinoma develops in approximately 0.5 percent of patients with Barrett's oesophagus per year (Shaheen et al. 2000). This is a tumor found predominantly in white men, among whom the frequency of oesophageal adenocarcinoma has inexplicably quadrupled over the past few decades (Devesa et al. 1998). Although GORD is the main recognised risk factor for this cancer (Lagergren et al. 1999), presumably because it causes Barrett's oesophagus, it is not clear whether the rising incidence of the tumor is due to an increasing frequency of GORD in the general population.

Oxidative damage has long been related to carcinogenesis in human cancers and animal cancer models. Various animal models (mainly rats) have been developed over recent years to study the possible involvement of iron in the pathogenesis of OAC. The majority of these reports are consistent in their observation that iron supplementation promotes epithelial cell proliferation and inflammation (Goldstein et al. 1998; Chen et al. 1999a). This enhances the production of reactive oxygen and nitrogen species in the oesophageal epithelium, which could lead to the formation of Barrett's oesophagus. Wetscher et al. (1997) found that reactive oxygen species (ROS), as measured by chemiluminescence and lipid peroxidation, increased with the grade of oesophagitis and were the highest in columnar-lined oesophagus (CLO). In a follow-up study by Chen et al. (2000), they suggested that according to their model, humans with gastro-oesophageal reflux and iron over-nutrition may be subject to the development of an oesophageal adenocarcinoma due to oxidative damage. Oxidative damage has been proposed to be closely related to reflux
oesophagitis, a possible cause for CLO and a driving force for adenocarcinogenesis (Reid et al. 2001).

The histological changes leading to adenocarcinoma are accompanied by alterations at the molecular level, including the accumulation of gene mutations and changes in gene expression. Many genes have been implicated as promising biomarkers for Barrett's oesophagus. Molecular markers of particular interest would be those that improve diagnostic reliability, risk stratification and/or provide molecular targets for intensified surveillance and treatment. Identification of genetic risk factors underlying the metaplasia-dysplasia-adenocarcinoma sequence of Barrett's oesophagus in relation to environmental factors, may allow the development of an individualised risk reduction intervention strategy. The selection of the genes analysed in our study population was based on the understanding that complex conditions such as Barrett's oesophagus may develop as a consequence of interaction between genetic risk factors triggered by environmental factors.

The cyclo-oxygenase 2 (COX-2) (Morris et al. 2001; Shirvani et al. 2000), c-myb (Brabender et al. 2001) and c-myc (Tselepsis et al. 2003) genes were included in the study based on the fact that their mRNA expression is known to increase early, progressively and significantly through the stages of Barrett’s metaplasia to OAC. Furthermore, inhibition of gene expression can decrease cell growth and increase apoptosis in vitro in oesophageal adenocarcinoma cell lines (Souza et al. 2000). The iron-related gene c-myc, and also the natural resistance-associated
macrophage protein-1 (NRAMP1 / SLC11A1), were included because of the role of iron in oxidative stress, implicated in the development of BO and OAC. c-Myc is regulated by iron and represses NRAMP1 / SLC11A1, which in turn modulates the cytoplasmic iron pool (Bowen et al. 2002). Analysis of TP53 was included as mutations resulting in protein over expression that is detectable by immuno-histochemistry (Lane and Benchimol 1990), may represent the most common genetic event in human malignancy. The likelihood that single biomarkers would be of limited predictive value in Barrett’s oesophagus led to the multi-gene approach. The COX-2, c-myb, c-myc, NRAMP1/SLC11A1 and TP53 genes selected for analysis this study have not previously been studied in the South African patient cohort for prognostication purposes.

Many questions remain unanswered about Barrett’s oesophagus. Will biomarkers indicating increased risk help us stratify patients by individual risk and will any treatment, be it medical, ablative, or surgical, have any effect on the natural history of this disease? Why does only a small subset of patients with GORD develop Barrett’s oesophagus and OAC, as experienced in the local population? Answers to these and other questions are eagerly awaited and some are provided in this study.
Barrett’s oesophagus is an intermediate lesion lying along the continuum linking normal epithelium to adenocarcinoma and is related to chronic gastro-oesophageal reflux disease. The malignant potential of Barrett’s oesophagus is specifically conferred by the presence of intestinal or specialised metaplasia as the defining hallmark. Much attention has been focused on understanding the molecular biology of the Barrett’s precursor lesion in the hope that this knowledge will eventually culminate in earlier detection and more effective treatments. Several new biomarkers that have been recently evaluated can potentially also help to identify a group of patients with an increased risk of developing high-grade dysplasia (HGD) and cancer. As yet, none of the molecular markers has shown to be a better predictor or more cost effective than the finding of dysplasia on biopsy.

2.1 Epidemiology and Clinical features

2.1.1 Epidemiology

Barrett’s oesophagus is found in approximately 6–12% of patients undergoing endoscopy for symptoms of chronic GORD (Sarr et al. 1985; Cameron et al. 1992). The mean age at the time of diagnosis is approximately 55 years (Spechler 1996). Although this affects children, it is rare before the age of five (Hassall 1997). This observation supports the contention that Barrett's oesophagus is an acquired and not a congenital condition. Barrett's oesophagus appears to be uncommon in blacks and Asians (Spechler et al. 2002). In a South African study with a 5:1 black to white
ratio in the population, only 5% of 216 consecutive patients diagnosed with Barrett’s oesophagus between 1970 and 1993 were black (Mason et al. 1998). In addition to white ethnicity, other established risk factors for adenocarcinoma in Barrett's oesophagus include male gender, obesity, advanced age and long duration of GORD symptoms (Sampliner 2002).

A familial form of Barrett's oesophagus has been described in the setting of families with increased gastro-oesophageal reflux. Sometimes the latter appears to be inherited as a monogenetic trait, which increases the risk of developing Barrett's adenocarcinoma (Jochem et al. 1992).

2.1.2 Clinical Features

Clinically, the symptoms such as heartburn, regurgitation, and dysphagia of Barrett's oesophagus may be no different than those of conventional GORD uncomplicated by columnar metaplasia (Bonelli 1993). Among patients who have endoscopic examinations because of chronic GORD symptoms, long segment (> 3 cm) Barrett's oesophagus could be found in 3 to 5 percent, whereas 10 to 15 percent have short-segment (< 3 cm) Barrett's oesophagus (Winters et al. 1987; Spechler 2002).
2.2 Pathophysiology

2.2.1 The role of Acid and Bile

Acid and bile in the gastro-oesophageal refluxate play a key role in the pathogenesis of Barrett's oesophagus in a setting of chronic exposure of the oesophagus to noxious stimuli. There is a correlation between the length of Barrett's mucosa and the duration of oesophageal acid exposure (percent of total time that oesophageal pH is <4) and supine reflux (Fass et al. 2001; Spechler 1996). The pattern of acid secretion may be an important determinant in the neoplastic progression of Barrett's metaplasia. An ex vivo study demonstrated that pulsed acid exposure increased cell proliferation, but continuous acid exposure decreased cell proliferation (Fitzgerald et al. 1996). Other studies have demonstrated that patients with longstanding and severe reflux symptoms are at increased risk of adenocarcinoma of the oesophagus (Lagergren et al. 1999).

The response to chronic inflammation in a susceptible individual facilitates the transformation of the squamous oesophageal lining through the process of metaplasia, in which one kind of fully differentiated (adult) cell replaces another via key molecular alterations (Spechler et al. 1996). The metaplastic columnar cells of Barrett's oesophagus are, in some ways, a favourable adaptation to chronic reflux since they appear to be more resistant to reflux-induced injury than the native...
squamous cells. Unfortunately, oesophageal columnar metaplasia predisposes to the development of adenocarcinoma (Morales et al. 2002).

Given the propensity for severe GORD in patients with long segment Barrett's oesophagus, it was initially assumed that the metaplasia progressed in extent over the years, as columnar epithelium replaced more and more reflux-damaged squamous epithelium. However, for reasons that are unclear, such progression is observed only rarely (Cameron and Lomboy 1992). In most cases, Barrett's oesophagus appears to develop to its full extent over a short period of time (i.e. <1 year), with little or no subsequent progression. Why this occurs is not well understood.

Patients with short-segment Barrett's oesophagus often have few or no symptoms and signs of GORD. The development of intestinal metaplasia in patients with short-segment disease may be due to exposure to noxious agents that accumulate at the gastro-oesophageal junction (GOJ). After meals, there is a pocket of acid at the GOJ that escapes the buffering effects of ingested food (Fletcher et al. 2001). This postprandial acid pocket has a mean length of 2 cm, beginning in the most proximal stomach, and extending more than 1 cm above the squamo-columnar junction (Z-line) into the distal oesophagus. In healthy volunteers, the very distal oesophagus (5 mm above the Z-line) is exposed to acid for more than 10 percent of the day (Fletcher et al. 2004).
Potential consequences of such persistent acid exposure include not only acid-peptic injury, but also exposure to high concentrations of nitric oxide (NO) generated from dietary nitrates (NO$_3$) in green, leafy vegetables. Most ingested nitrate is absorbed by the small intestine and excreted unchanged in the urine, but approximately 25 percent is concentrated by the salivary glands and secreted into the mouth, where bacteria on the tongue reduce the recycled nitrate to nitrite (NO$_2$). When swallowed, nitrite encounters acidic gastric juice: the nitrite is converted rapidly to nitric oxide (NO). After nitrate ingestion, high levels of NO have been demonstrated at the GOJ (Iijima et al. 2002). NO can be genotoxic, and potentially carcinogenic. Thus, the GOJ is exposed repeatedly to acid, pepsin, NO, and other noxious agents in gastric juice that can lead to chronic inflammation and metaplasia.

2.2.2 The role of *Helicobacter pylori* infection

Gastric infection with *H. pylori* causes chronic inflammation that can result in intestinal metaplasia and cancer in the stomach (Chow et al. 1998). However, the organism does not infect the oesophagus and there is no positive association between *H. pylori* infection and GORD. Indeed, a number of studies suggest that *H. pylori* infection may protect the oesophagus from GORD and its complications like Barrett's oesophagus, perhaps by causing a chronic gastritis that interferes with acid production (Graham et al. 1998). *H. pylori* strains that express cytotoxin-associated gene A (cagA) appear to be especially damaging to the stomach, and especially protective towards the oesophagus (Vaezi et al. 2000).
Segal (2001) reviewed the epidemiological and clinical studies that have reported on GORD, Barrett’s oesophagus, OAC and *H. pylori* infection in sub-Saharan Africa. The data indicates that Barrett’s oesophagus is rare and OAC uncommon in all regions of sub-Saharan Africa studied (South Africa, Ethiopia, Nigeria, Zimbabwe, Kenya and Uganda). Similarly, hiatus hernia is also uncommon. The overwhelming majority of oesophageal cancers are squamous in type. *H. pylori* infection is ubiquitous with an overall prevalence of 61-100%. It was concluded that although urbanisation has resulted in an increase of risk factors associated with GORD, which would be expected to lead to an increase in this disease among Africans, this increase has not occurred. It is believed that the critical factor preventing GORD in black Africans is *H. pylori* infection, which is usually acquired in childhood, is for life and is probably protective for the oesophagus.

### 2.3 Morphology of Barrett’s oesophagus

#### 2.3.1 Macroscopic features

Glandular mucosa in the lower oesophagus presents as a red velvety mucosa over the gastro-oesophageal junction. It can extend either circumferentially or as one or several tongues, and in some cases as a mixture of these two patterns. Until recently, it was considered that this mucosa had to extend at least 30 mm above the gastro-oesophageal junction to diagnose Barrett’s oesophagus. But this definition has changed, owing to the recognition of short segment Barrett’s oesophagus measuring less than 30 mm (Spechler et al. 1996; Sharma and Sampliner 1998).
However, as it may be difficult to accurately measure a short segment Barrett’s oesophagus and to localise the metaplastic mucosa and the gastro-oesophageal junction, it is now well recognised that the major diagnostic criteria of Barrett’s oesophagus is histological. The significance of intestinal metaplasia discovered on biopsies taken from an endoscopically normal junction (sometimes considered as an "ultra short" Barrett’s oesophagus) remains controversial, and will not be discussed in this text.

2.3.2 Histological features

Traditionally, three types of columnar epithelia have been described in Barrett's oesophagus, namely cardiac, gastric fundic-type and specialised intestinal metaplasia (Paull et al. 1976). Specialised intestinal metaplasia is the most common and also the only type that specifically confers the malignant potential of Barrett’s oesophagus (Spechler 2002). Most authorities insist on the demonstration of specialised intestinal metaplasia to confirm an endoscopic diagnosis of Barrett’s oesophagus. Morphologically, it frequently shows a villiform pattern. The epithelium is composed mainly of goblet cells interspersed between intermediate mucous cells, both in the surface and glandular epithelium. Mature absorptive intestinal cells with a well defined brush border are rare. Paneth cells may be present, but they are as rare as in incomplete intestinal metaplasia of the gastric mucosa. Endocrine cells can be seen on special staining in the glands. On electron microscopy, the goblet cells have characteristic apical mucin granules, and the columnar mucin cells have features intermediate between gastric mucous cells and intestinal absorptive cells.
(Zwas et al. 1986). The presence of goblet cells is the most useful feature for distinguishing specialised intestinal metaplasia from gastric cardiac or fundic-type mucosa.

2.3.2.1 Mucin histochemistry

Mucins are produced by both columnar mucinous and goblet cells and can be characterised by mucin histochemistry. The columnar cells may produce neutral mucins, similarly to gastric surface epithelial cells, and/or acidic mucins, typical of intestinal mucosa. Therefore, these cells can stain red (neutral mucins), blue (acidic mucins), or magenta (neutral and acidic mucins) on a combined PAS–alcian blue stain (Peuchmaur et al. 1984; Rothery et al. 1986). Although it has been suggested that the presence of acidic mucins (blue on alcian blue) is a characteristic feature of Barrett’s oesophagus in the absence of typical goblet cells, this theory has been disputed by other studies that showed alcian blue positive columnar cells in gastric cardiac surface or neck cells in patients with neither metaplasia of the lower oesophagus nor gastro-oesophageal reflux disease (Genta et al. 1994; Chen et al. 1999b).

The presence of goblet cells is the only characteristic feature of intestinal Barrett’s mucosa. These cells produce in all cases acid mucins that are usually easily visualised on routinely stained sections. Therefore, routine staining of biopsies of the gastro-oesophageal junction with alcian blue may only be of value in demonstrating rare positive goblet cells, which may indicate short segment Barrett’s oesophagus. Acidic mucins can be divided into sialomucins and sulfomucins. On a combined high
iron diamine–alcian blue stain, sialomucins stain blue, and sulfomucins stain brown–black. In specialised Barrett’s mucosa, goblet cells usually contain both sulfomucins and sialomucins. The presence of sulfomucins in columnar cells is a characteristic feature of type III intestinal metaplasia of the stomach, a lesion with a premalignant potential. In Barrett’s oesophagus, it is very common to have sialomucin containing columnar cells, a feature that shows that this pattern cannot be used to delineate a population at high risk of malignancy (Peuchmaur et al. 1984; Rothery et al. 1986).

2.3.2.2 Immuno-histochemistry

For practical purposes, intestinal metaplasia of the oesophagus and the cardia can usually be distinguished by routine histology, but as immuno-histochemistry is now routinely used in almost all pathology departments, numerous studies have tried to find sensitive and specific markers of intestinal type mucosa in the oesophagus. These markers include the MUC antigens and other mucin components, and different cytokeratin (CK) subtypes.

Cytokeratins are the intermediate filaments characteristic of epithelial cells. There are 20 distinct subtypes, with the pattern of expression depending on the type and origin of the epithelium. As the characteristic pattern is conserved in most carcinomas, CK 20, a marker of intestinal differentiation, and CK 7, a marker of ductal differentiation, are routinely used in the diagnosis of poorly differentiated carcinomas. Immuno-histochemical studies, using monoclonal anti-bodies to CK 7 and 20, have been reported to make it possible to differentiate accurately between intestinal metaplasia originating from either the oesophagus or cardia of the stomach.
(Ormsby et al. 2000). In oesophageal intestinal metaplasia, CK 7 positivity is found in superficial and deep glands, whereas CK 20 positivity is limited to superficial glands only (Barrett’s CK 7/20 pattern). This sensitive and specific Barrett CK pattern has been observed in both long and short segment Barrett’s oesophagus, and even in ultra short segment Barrett’s oesophagus (Ormsby et al. 2000; Couvelard et al. 2001). In cardiac type intestinal metaplasia, CK 7 immuno-reactivity is absent (or weak or patchy), but CK 20 positivity is seen in superficial and deep glands. Although earlier reports suggest that CK 7/20 immuno-staining patterns are useful clinically, routine application of these immuno-histochemical techniques is controversial and should be used in conjunction with current endoscopic and histological findings (El-Zimaity et al. 2001; Mohammed et al. 2002). Similar results have been obtained with antibodies reacting with intestinal goblet cells, such as Das1 antibody (DeMeester et al. 2002).

Other antibodies have also been used to characterise intestinal metaplasia of Barrett’s oesophagus, directed against MUC mucin gene products, especially MUC1 and MUC2 (an intestinal mucin). These studies have demonstrated aberrant expression of MUC2 in Barrett’s intestinal mucosa which is lost when the cells become neoplastic. MUC1 was absent in metaplastic and dysplastic epithelium, but was expressed in carcinomas, which suggests that it could differentiate dysplasia from carcinoma in mucosal biopsies (Chinyama et al. 1999; Guillem et al. 2000). Molecular studies, in addition to the morphologic and immuno-histochemical features of intestinal metaplasia discussed above, further support the specialised cellular
differentiation of Barrett’s mucosa. Sucrase-isomaltase, an intestinal disaccharide, is expressed in 76% of Barrett’s mucosa, where it is localised to the apical membrane, and 82% of oesophageal adenocarcinomas, where immuno-histochemistry demonstrated a diffuse pattern (Wu et al. 1993).

2.3.2.3 Dysplasia

Cancers in Barrett's oesophagus evolve through a sequence of DNA alterations that give the cells certain growth advantages, and cause morphological changes in the tissue that the pathologist can recognise as dysplasia. Dysplasia has been defined by Riddell et al. (1983) as an unequivocal neoplastic epithelium strictly confined within the basement membrane of the gland from which it arises. Although this definition was initially proposed for premalignant changes developing in inflammatory bowel disease, it has been progressively extended to the entire gastrointestinal tract, including Barrett’s oesophagus (Schmidt et al. 1985). Dysplasia as a premalignant lesion is strictly synonymous to intraepithelial neoplasia, a term in use in most organs including the gynaecological tract, and has been recommended for use in Barrett’s oesophagus by the World Health Organization (Werner et al. 2000). Dysplasia has to be distinguished on both ends of the morphological spectrum of changes, from regenerative non-neoplastic modifications, often called atypia, and from invasive cancer, especially in its early or superficial form with invasion limited to the lamina propria.
Dysplasia is a constellation of histological abnormalities suggesting that one or more clones of cells have acquired genetic damage, rendering them neoplastic and predisposed to malignancy (Spechler 2001). Pathologists diagnose dysplasia when they recognise a constellation of characteristic cytological and architectural abnormalities in tissue biopsy specimens. Architectural changes include glandular distortion and crowding. Papillary extensions may be present in gland lumen, and villiform configuration of the mucosal surface can be observed. Cytological changes include nuclear alterations such as variation in size and shape, nuclear and/or nucleolar enlargement, increased nuclear to cytoplasmic ratio, hyperchromsia, and increased number or abnormal mitoses. Most authors consider that these changes have to involve the mucosal surface to warrant the diagnosis of dysplasia. (Geboes et al. 2000; Riddell et al. 1983;)

Based on the degree of alteration in nuclear morphology and glandular architecture, dysplasia is classified into grades of increasing severity. Although a three tiered classification (mild–moderate–severe) is still in use in some centres, most pathologists use a two tiered system that distinguishes between low grade dysplasia (LGD) and high grade dysplasia (HGD). In this two grade system, LGD includes the mild and moderate categories of the three grades’ system. Generally, low-grade dysplasia is distinguished from high-grade dysplasia based on nuclear localisation in relation to the luminal surface of the cell. The Riddell’s classification of dysplasia also includes a category of mucosa indefinite for dysplasia. The term carcinoma in situ (or intraepithelial carcinoma) is not used in the Riddell’s classification, as it is
considered indistinguishable from HGD. In intramucosal carcinoma, neoplastic cells have penetrated through the basement membrane and infiltrate into the lamina propria, leading to a small risk of regional lymph node metastasis.

Unfortunately, dysplasia is an imperfect marker for malignancy because of interobserver disagreement in grading its severity, biopsy sampling error and incomplete data on natural history. Among experienced pathologists, inter-observer agreement for the diagnosis of low-grade dysplasia in Barrett's oesophagus is less than 50 percent and for high-grade dysplasia approximately 85 percent (Reid et al. 1988; Skacel et al. 2000; Montgomery et al. 2001). Given the progressive and subtle changes that occur from non-dysplastic to LGD to HGD, it is not surprising that this variation exists. In the "expert" study by Montgomery et al. (2001), the diagnoses made by 12 senior gastrointestinal pathologists on 125 biopsies were compared. When a four grade system was employed (non-dysplastic/indefinite, low grade, high grade and cancer), the kappa index was low (0.43). Kappa improved (0.66) when a simplified classification was used (non-dysplastic/indefinite and low grade/high grade and cancer). In a study involving 20 general pathologists in the USA (Alikhan et al. 1999), there was very large variation in the diagnoses of non-dysplastic mucosa, LGD, and HGD. These results emphasise the need to obtain a second opinion on difficult cases, especially when a therapeutic decision has to be made. Furthermore, dysplastic changes should be interpreted with caution when atypical epithelial cells (arising from the background of active inflammation) are present.
2.3.2.4 Natural history of dysplasia

Barrett’s adenocarcinomas seem to develop by a multi-step process, recognised histologically as the metaplasia-dysplasia-adenocarcinoma sequence (Jankowski et al. 1999). When patients included in surveillance cohorts are considered, it has been well established that the presence of dysplasia indicates an increased risk of carcinoma. However, the natural history of this lesion is still very difficult to predict for one individual patient (Goldblum et al. 2002). HGD is the nearest precursor of adenocarcinoma, as shown by association with cancer in surgical specimens, and prior to the development of cancer in surveillance programmes. It must be remembered that dysplasia detected on endoscopic biopsies is also frequently a marker of synchronous carcinoma, as in most surgical series up to 40% of Barrett’s oesophagus resected for HGD have an occult adenocarcinoma (Heitmiller et al. 1996; Falk et al. 1999). The frequency of these unsuspected cancers varied upon the endoscopic and biopsy protocol, with very few cancers detected when patients were followed using the "Seattle" protocol (four quadrant biopsies at 1 cm intervals) at closely timed intervals (Levin et al. 1993).

The natural history of HGD is still a matter of debate. In two large series that included 145 patients with HGD, although the risk of malignant transformation was relatively high, the majority of patients did not progress to adenocarcinoma after several years of follow up (Schnell et al. 2001; Levine et al. 1996). In these two studies, after the initial diagnosis of HGD, 25% and 16% of patients developed
carcinoma after a mean surveillance period of 2.5 years and 7.3 years, respectively. When considering these series, it can be concluded that HGD does not progress to adenocarcinoma in the majority of patients within some years, and that non-surgical procedures (surveillance and endoscopic treatments) can be considered as reasonable options in those patients - a statement that is still very much debated in the literature (Goldblum et al. 2002). It is interesting to note that in one of these two series, there was an unusually high proportion of Barrett's oesophagus patients with LGD (737 of 1099, 67%), and the histological diagnoses were made during a period of 20 years by one experienced pathologist (Schnell et al. 2001).

Recently, the distinction between unifocal and multifocal HGD has been emphasised by Weston et al. (2000), with a high rate of progression from unifocal to multifocal HGD or invasive carcinoma (8 of 15 patients within a mean follow up period of 37 months). Similarly, another study demonstrated that the risk of malignant progression increased by 3.7 when diffuse HGD was present (Buttar et al. 2001). These findings have recently been challenged by Dar et al. (2003). It has also been shown that the presence of endoscopic polypoid lesions [an equivalent of DALM (Dysplasia Associated Lesion or Mass) in inflammatory bowel disease] was an indicator of high risk of cancer (Thurberg et al. 1999).

The natural history of LGD is even less defined. This could be at least partially due to the poor diagnostic reproducibility of this lesion. It was considered traditionally that LGD was a very slowly progressing lesion. In most series, there was even a
high rate of apparent regression from LGD to non-dysplastic mucosa reported. This last phenomenon has several potential explanations: initial over diagnosis of LGD, due to the difficulty in differentiating reactive from dysplastic changes; sampling variability; or real neoplastic regression. However, this opinion about the benign course of LGD has been challenged in some recent studies. In a study based on a multi centre pathological recruitment of 26 cases with a diagnosis of LGD, 4 patients (15%) developed HGD and 4 (15%) an adenocarcinoma, 2 – 65 months after the initial diagnosis of LGD. In another study, 7 patients (28%) developed HGD (5 patients) or an adenocarcinoma (2 patients) after a mean follow up of 26 months (range 2 – 43 months) after the diagnosis of LGD (Skacel et al. 2000). Very interestingly, in this latter study all cases were reviewed blindly by 3 gastrointestinal pathologists. When all 3 pathologists agreed on the initial diagnosis of LGD, 4 of 5 patients progressed to a more severe lesion, while none of the 8 patients with no agreement for the initial diagnosis progressed.

2.3.3 Adenocarcinoma arising from Barrett’s oesophagus

Barrett’s oesophagus is thought to be the precursor of adenocarcinoma of the oesophagus and of the gastro-oesophageal junction (GOJ). Adenocarcinomas that straddle the GOJ are approximately twice as common as adenocarcinomas that clearly arise from the oesophagus (Cameron et al. 1995). With straddling tumours, it can be difficult to determine whether the neoplasm arose from the columnar epithelium in the distal oesophagus or in the proximal stomach (the gastric cardia).
These tumours cannot be distinguished from one another morphologically, and they share a number of epidemiologic features including an association with GORD, a strong predilection for white males, and a rapidly rising incidence in Western countries (Cameron et al. 1995).

Biochemical studies are also consistent with the hypothesis that Barrett's oesophagus is the precursor for most GOJ tumours. In one series, for example, similar profiles of intestinal-type proteins were detected by immuno-fluorescence microscopy in Barrett's oesophagus and in 26 cases of adenocarcinoma with or without obvious Barrett's in tumours from both oesophagus and cardia (Mendes de Almeida et al. 1997). These profiles were not seen in normal stomach or oesophageal mucosa, reflux oesophagitis or squamous cell carcinoma.

2.4 Mechanisms of malignant transformation

Human tumours are thought to arise as a multi-step process, modulated by genetic and environmental factors. The accumulation of genetic alterations leads to genomic instability and through complex interactions between stimulatory oncogenes and regulatory tumour-suppressor genes, results in widespread clonal outgrowth of cells exhibiting aberrant cell cycle regulation, with the capacity for invasion (Hanahan and Weinberg 2000). Generally, genomic instability precedes the appearance of histological changes. More specifically, cancers in Barrett's oesophagus evolve through a sequence of genetic alterations in which the metaplastic cells acquire the ability to proliferate without exogenous stimulation, to resist growth-inhibitory signals,
and to avoid triggering the programmed death mechanism (apoptosis) that ordinarily destroys cells that acquire extensive genetic damage. Many of the genetic changes that endow cancer cells with these growth advantages do so by affecting components of the cell cycle clock apparatus, the pivotal molecular machinery in the cell nucleus that controls whether a cell will proliferate, differentiate, become quiescent or die.

2.4.1 Cell-cycle kinetics

Neoplasia is the ultimate result of disruption of the normal cell cycle. The normal cell cycle comprises several well-defined stages. Progression through each stage is modulated by complex interactions between stimulatory and inhibitory signals mediated by oncogenes and tumour-suppressor genes respectively. Quiescent cells (stage G0) enter the cell cycle in the G1 (first gap) phase and progress under external mitogenic stimulation to S phase, in which DNA synthesis occurs. The cell now is committed to divide irrespective of exogenous factors, and after proceeding through G2 (second gap), enters M phase were mitoses occurs. The RNA and proteins needed for DNA replication are synthesized during the G1 phase. There is a critical point, late in the G1 phase, where a decision is made either to continue and complete the cell cycle, or to exit the cycle. This critical juncture is called the 'restriction point' (R-point) (Pardee 1974). DNA replication does not proceed until the S phase, during which the cell's DNA content doubles, increasing from the diploid value of 2n to the fully replicated, tetraploid value of 4n. During the G2 phase, the tetraploid cell prepares for the upcoming mitotic division. Finally, in M phase the cell
divides into two daughter cells, each containing a diploid (2n) complement of DNA. After mitosis, cells may withdraw from the cell cycle to enter a quiescent state termed G0. Under certain conditions, such cells can be stimulated to leave the G0 phase and re-enter the cell cycle.

The cell cycle is regulated at key checkpoints (G1/S and G2/M) by cyclins and cyclin-dependent kinases (cdks), which interact with cellular proteins to activate transcriptional factors having positive and negative regulatory effects. For example, the cyclin D1/cdk-4 (or cdk-6) complex regulates the early to mid-G1 phase, after phosphorylation of the retinoblastoma (Rb) tumour-suppressor protein, the E2F transcription factor activates genes required for DNA synthesis in S phase, driving the cell to G1/S transition. Whereas the p16 tumour-suppressor gene inhibits association of cdk-4 (and cdk-6) with cyclin G1, mutational activation of ras oncogenes induces cyclin D1 expression (Filmus et al. 1994). This regulatory process is modulated further by interactions with other upstream and downstream genes. The TP53 tumour-suppressor gene may induce cell-cycle arrest by transcriptional activation of p21 (WAF-1), which sequesters various cdks (El-Deiry et al. 1993; Kirsch et al. 1998). Therefore, progression through the G1/S checkpoint may result from the loss of Rb, p16, or TP53 or by over expression of ras or cyclin D1.

A reciprocal relation among Rb, cyclin D1 and p16 expression has been reported in oesophageal cancer. In general, tumours that retain Rb expression typically exhibit
over expression of cyclin D1, p16 inactivation, or both, often in the context of TP53 mutations (Schrump et al. 1996).

2.4.2 Increased proliferation

Immuno-histochemistry and flow cytometry have been used to study cell proliferation in oesophageal tissues, evaluating distribution of proliferating cell nuclear antigen (PNCA) and Ki67 (Hong et al. 1995; Jankowski et al. 1992; Whittles et al. 1999). PCNA is an indicator of cell cycle progression at the G1/S transition, and Ki67 is expressed in proliferating cells (G1, S, G2, and M phases). Whereas PCNA immunostaining normally is seen in the basal layer of metaplastic Barrett’s epithelium, the immunoreactivity is seen to extend superficially with high-grade dysplasia. Immuno-histochemical studies with the monoclonal antibody MIBI-1 (against Ki67) demonstrated a higher percentage of proliferating cells in metaplastic Barrett’s mucosa compared with normal gastric epithelium. Staining patterns for low- and high-grade dysplasia were similar to PCNA, suggesting a greater turn-over of differentiated cells in the surface epithelium by immature proliferating cells arising from basal layers.

Increased proliferative activity and the altered cell-cycle kinetics also have been shown with flow cytometry in Barrett’s epithelium. An increased G1 fraction seems the earliest finding, progressing to increased S-phase fractions with aneuploidy, high-grade dysplasia, and carcinoma (Chanvitan et al. 1995; Reid et al. 1992; Reid
et al. 1996). These findings suggest a functional instability of Barrett’s mucosa, predisposing to increasing dysplasia and malignancy.

2.4.3 DNA content

2.4.3.1 Changes in DNA content

Systematic flow cytometry can identify patients with increased 4n or aneuploidy and has also been used in recent studies. Abnormalities in DNA content occur early and often in Barrett’s epithelium. Initial studies laying the groundwork for molecular studies in Barrett’s oesophagus focused on abnormal DNA content known as aneuploidy. Flow cytometry has proven valuable in this context and aids in detecting clonality within tissues and cells which in turn characterises early development of genomic instability (Nowell et al. 1976). This paradigm has been validated in earlier studies showing that aneuploidy or increased tetraploidy (4n) populations occur in more than 90% to 95% of Barrett’s-associated cancers, arise in premalignant Barrett’s epithelium, and predict progression (Reid et al. 1992; Reid et al. 1987; Galipeau et al. 1996, Barrett et al. 1999). One earlier study suggested that alterations in ploidy correlated with dysplasia in Barrett’s oesophagus (James et al. 1989). Some patients with questionable dysplasia in this study were also found to have altered DNA ploidy (Reid et al. 1987). In another series of patients, the presence of aneuploid cells on flow cytometric analyses of histologically equivocal biopsies allowed identification of areas of mild dysplasia. Furthermore, aneuploidy
was always associated with some morphological abnormality; varying from mild dysplasia to frank carcinoma (James et al. 1989). Further studies subsequently emerged asserting that aneuploidy may serve as an adjunct in identifying patients with Barrett’s oesophagus who were more likely to progress to dysplasia (Reid et al. 1992, Fennerty et al. 1989). A 28% 5-years’ cumulative oesophageal cancer incidence was found in those Barrett’s patients with either aneuploidy or increased 4n compared to a 0% 5-year cumulative oesophageal cancer incidence in patients with neither aneuploidy nor increased 4n fractions (Reid et al. 2000).

Others found that aneuploidy and dysplasia can occur discordantly (Fennerty et al. 1989). In a seminal study of DNA ploidy in systematically mapped Barrett’s epithelium, clonal growth similar to that seen in fully developed cancer was present in metaplastic Barrett’s mucosa (Raskind et al. 1992).

2.4.3.2 Chromosomal Abnormalities

In several studies that have addressed specific chromosomal alterations in Barrett’s oesophagus, diverse karotypic abnormalities have been documented. This includes Y chromosome loss, trisomies, and translocations of chromosome 7 and 11, as well as over presentation of chromosome 8 and loss of chromosome 17 (Garewal et al. 1989, 1990; Haung et al. 1992). A report evaluating chromosomal abnormalities in oesophageal adenocarcinoma noted gains of chromosomes 12 (eight cases), 6
(seven cases), and 11 (six cases). The total number of chromosomal abnormalities varied from 0 to 10, with an average of 4.6 per case (Persons et al. 1981).

2.4.3.3 Loss of Heterozygosity

During the process of cancer evolution, molecular events that lead to a growth or survival advantage result in clonal expansion. Moreover, normal DNA repair mechanisms check replication fidelity before permitting progression through subsequent stages of the cell cycle. Deletions and other DNA alterations permit unhampered progress through the cell cycle, leading to proliferation and expansion of abnormal or mutated clones of cells.

Studies have reported varying frequencies of LOH involving multiple chromosomal loci in Barrett’s oesophagus. One of the earliest regions investigated was chromosome 17p13.1, the locus of the TP53 tumour suppressor gene. The 17p LOH was detected in 52% to 93% of Barrett’s associated oesophageal adenocarcinoma (Blount et al. 1991). Furthermore, 17p allelic loss was found to precode 5q allelic loss in patients with Barrett’s and high-grade dysplasia or adenocarcinoma (Blount et al. 1993).

Evidence has been presented that allelic loss of 9p21 and point mutations of the p16 gene develops as early lesions during neoplastic progression in Barrett’s oesophagus (Barrett et al. 1996). However, one study showed that deletions of loci
harbouring the TP53, p16, and APC genes were infrequent in patients with Barrett’s oesophagus without dysplasia but were evident in adenocarcinomas arising in Barrett’s oesophagus (Gonzales et al. 1997). Particularly interesting is a recent report suggesting that deletion of a locus (31-32.1) on chromosome 14q can be used to differentiate adenocarcinomas of the oesophagus versus gastric cardia origin (Van Dekken et al. 1999). These findings suggest that deletions of these important tumour genesis genes may constitute later neoplastic events.

2.4.4 Tumour-suppressor genes

The products of tumour-suppressor genes prevent the acquisition of the transformed phenotype in vivo. Loss of function results in tumour development.

2.4.4.1 TP53

The tumour-suppressor gene TP53 encodes a 53-kd polypeptide that regulates cell-cycle progression, DNA repair, apoptosis, and neo-vascularisation in normal and malignant cells by means of highly complex DNA and protein interactions (Prives and Hall 1999). TP53 mediates cell-cycle arrest in part by inducing the expression of p21 (WAF-1), which sequesters various cdks facilitating G1and G2/M arrest (El-Deiry et al. 1993). When the DNA of normal cells sustains damage during G1, TP53 protein rapidly accumulates to halt any further progression of the cell cycle (Giaccia and Kastan 1998). This period of cell cycle arrest allows time for the intrinsic DNA
repair mechanisms to correct the genomic damage before the mutation can be perpetuated by DNA replication during S phase. One means by which the tumour suppressor TP53 halts cell cycle progression is by inhibiting the actions of cyclins D1 and E, thereby preventing the phosphorylation of Rb protein that allows advancement to S phase. If the cell is able to repair its damaged genome, TP53 levels return to baseline values, cyclins D1 and E are no longer inactivated, and the cell can proceed through the R-point (Kastan et al. 1991). If a cell loses its normal TP53 function, then DNA damage might not be repaired in G1, and the mutation can be perpetuated. If the mutation endows the cell with growth advantages, carcinogenesis may result.

Frequent point mutations of TP53 were shown to occur in Barrett’s-associated adenocarcinoma and adjacent dysplasia or metaplasia (Casson et al. 1991), and therefore make TP53 perhaps the key tumour-suppressor gene in oesophageal adenocarcinoma. Up to 70% of oesophageal cancers carry TP53 mutation and/or deletion (Wang et al. 1993). Frequently LOH of the TP53 locus on 17p13.1 is seen not only in adenocarcinoma, but also in Barrett’s metaplasia and dysplasia (Barrett et al. 1999). Recent studies have found that in individuals, who progressed from Barrett’s oesophagus to oesophageal adenocarcinoma, one of two normal TP53 alleles was inactivated by mutation and the second was lost by a mechanism termed as loss of heterozygosity (LOH). Reid and colleagues (2001) followed 256 patients with Barrett’s oesophagus and TP53 LOH data at baseline for up to 5 years and found TP53 LOH to be a strong predictor of progression to oesophageal
adenocarcinoma (relative risk 16; 95% CI 6.2 to 39; p<0.001). However, the prevalence of TP53 alteration in non-dysplastic Barrett’s mucosa is relatively low (Gonzalez et al. 1997). In most patients with high-grade dysplasia, the Barrett’s mucosa contains a mosaic of clones and sub clones with differing patterns of 17p LOH (Galipeau et al. 1999).

In a recent study by Dolan et al. (2003) TP53 mutation analysis was performed on premalignant and malignant tissue from 30 patients undergoing oesophagectomy for adenocarcinoma, and on premalignant biopsies from 48 patients participating in a Barrett’s surveillance program. The mean follow-up for the patients in the surveillance program was 5 years. TP53 mutations were detected in a third of the patients with oesophageal adenocarcinomas, and were more common in well-differentiated carcinomas. An identical TP53 mutation was detected in carcinoma and adjacent dysplasia. Two patients with premalignant Barrett's esophagus had TP53 mutations and one of these patients developed adenocarcinoma on follow-up whilst the other has not yet progressed beyond metaplasia at the time of publication of their study. No patient without TP53 mutation developed high-grade dysplasia or adenocarcinoma. Similarly, in a study by Klump et al. (1999), TP53 mutations were detected in 33% of oesophageal adenocarcinomas and in 4% of premalignant Barrett's oesophagus in patients undergoing endoscopic surveillance.

The role of TP53 in the malignant progression of Barrett's oesophagus has been studied primarily using immuno-histochemistry. Wild-type TP53 protein is labile, has
a very short half-life, and normally is not demonstrable by immunostaining. In contrast, mutant TP53 is often more stable, and can accumulate within the cell to the point that it is detectable by immunostaining. Immunostaining for TP53 has been found in the non-dysplastic, specialised intestinal metaplasia of Barrett's oesophagus, and such staining is found with increasing frequency as dysplasia progresses in severity (Ramel et al. 1992; Younes et al. 1993; Flejou et al. 1993; Hamelin et al. 1994). In Barrett’s oesophagus, frequencies of TP53 over expression correlate with the degree of dysplasia (Galipeau et al. 1999). Furthermore, early dysplastic lesions with TP53 expression have been associated with a higher likelihood of progression to high-grade dysplasia, suggesting a value to determine TP53 status in patients with low-grade dysplasia (Glimenez et al. 1999).

In summary, TP53 mutations can be detected before the development of high-grade dysplasia or carcinoma, and may be useful in stratifying the risk of adenocarcinoma in patients with Barrett’s oesophagus. However, low frequencies of elevated TP53 expression in Barrett’s oesophagus without dysplasia seem to preclude its widespread use as a screening tool. Furthermore, immunohistochemistry can give false negative results if the TP53 mutations lead to protein truncation not detectable by the antibody (Fitzgerald 2005).
2.4.4.2 Retinoblastoma

The retinoblastoma (Rb) gene, located on 13q14, encodes a 105-kd nuclear phosphoprotein that ultimately is involved in regulation of the G\textsubscript{1} restriction point (Chen et al. 1995b). Loss or inactivation of both alleles of the Rb gene is the primary mechanism underlying retinoblastoma (Knudson et al. 1971). Moreover, a high incidence of second primary tumours among patients inheriting one inactive Rb allele suggested that this cancer gene plays a key role in the aetiology of several other primary malignancies (Murphee et al. 1984). Altered retinoblastoma mRNA transcript size and quantity have been demonstrated in both dysplasia and cancerous Barrett’s tissues (Huang et al. 1992).

Oesophageal cancer cells show decreased expression of Rb protein (Coppola et al. 1999). Most importantly, LOH and/or abnormal mRNA transcripts involving Rb have been demonstrated in 36% to 67% of oesophageal tumours and, in one study, were associated with unfavourable survival (Wang et al. 1993). Finally, accumulation of abnormal Rb protein during the progression of Barrett’s metaplasia to carcinoma leading to unsuppressed tumour growth has been demonstrated (Coppola et al. 1999).
2.4.4.3 p16

The tumour-suppressor gene p16, located on chromosome 9p21, is an inhibitor of the cyclin dependent kinase cdk-4 and cdk-6, preventing cyclin D-dependent phosphorylation of Rb protein (Grana and Reddy 1995). A paradigm emerging from studies of oesophageal squamous cell carcinoma is that either cyclin D₁, over expression or p16 inactivation seems to be required to accelerate the cell through G₁ (Klump et al. 1998). The p16 locus undergoes frequent LOH in oesophageal cancer. However, relatively low frequencies of p16 inactivating mutations have been documented in oesophageal cancers (Esteve et al. 1996). More recently, p16 promoter hypermethylation has been reported in oesophageal carcinoma (Wong et al. 1997), suggesting that p16 (and possibly p15 as well) are the genes targeted on 9p21.

2.4.5 Proto-oncogenes

Typically, oncogenes are either genes that encode a normal cellular protein that is expressed at inappropriately high levels or mutated genes that produce a structurally altered protein that exhibits inappropriate function. The normal cellular genes from which the oncogenes derive are designated proto-oncogenes or cellular oncogenes. The protein encoded by the oncogenes comprise at least four distinct groups: peptide growth factors, that may be excreted in the extra cellular milieu, protein kinases, signal transducing proteins associated with the inner cell membrane surface
(membrane-associated G proteins), and transcriptional regulatory protein located in the nucleus (Haubruck and McCormick 1991). Proto-oncogenes were the first major class of cancer-related genes to be studied.

2.4.5.1 Signal transduction-related oncogenes – membrane-associated G proteins

The ras family of proto-oncogenes was evaluated in small series of Barrett’s oesophagus-associated dysplastic lesions. The first of these studies that measured expression of the proto-oncogene c-Ha-ras, showed that there was no detectable expression of c-Ha-ras mRNA in normal or metaplastic oesophageal mucosa (Meltzer et al. 1989). Further analysis confirmed this finding and demonstrated that mutations of the ras family of genes were indeed infrequent in Barrett’s oesophagus-associated neoplasia (Trautmann et al. 1996).

2.4.5.2 Protein kinase related oncogenes

Relatively few studies assessed proto-oncogene abnormalities in Barrett’s oesophagus per se. Jancowski and co-workers (1992) elegantly described positive c-erb-B2 immunostaining in 9 of 15 patients with Barrett’s metaplasia as well as 11 of 15 additional patients with oesophageal adenocarcinoma. Less frequent immunopositivity for c-src, c-ras, c-jun and c-fox was seen in both metaplastic and frankly cancerous epithelium (Jankowski et al. 1992). In another study, c-erb-B2
oncoprotein over expression was observed in 7 (11%) of 66 cases of Barrett’s adenocarcinoma, but not in surrounding dysplastic and non-dysplastic epithelium (Flejou et al. 1994). In this latter report c-erb-B2 expression was associated with poor survival. More recently, results of studies of other proto-oncogenes have appeared in the literature, including the important positive cell cycle regulator, cyclin D1 (Arber et al. 1996); the powerful apoptosis inhibitor, Bcl-2 (Goldblum and Rice 1995); and the prototypical tyrosine kinase proto-oncogene, c-src (Kumble et al. 1997). An important study of cyclin D1, expression demonstrated positive nuclear staining in 38% of men and 25% of women with Barrett’s oesophagus (Arber et al. 1996). In another study, biopsy specimens from 11 of 12 patients with oesophageal adenocarcinoma stained positive for cyclin D1 and a statistically significant risk for progression to adenocarcinoma (OR 6.85; 95% CI 1.57 to 29.91, p 0.0106) was found in the patients who stained positively for this biomarker (Bani-Hani et al. 2000).

2.4.5.3 Nuclear oncogenes

The role of nuclear oncogenes that encode transcriptional regulatory proteins and that are involved in protein-protein interaction is exemplified by the myc family. The c-myc protein product is involved in critical cellular functions such as proliferation, differentiation, apoptosis, transformation, and transcriptional activation of key genes (Luscher and Eisenman 1990). The c-myc gene is located on chromosome 8q24 and encodes a nuclear protein thought to regulate the transcription of other genes important for cell growth (Dang et al. 1999). Activation of the c-myc gene may contribute to tumour progression by preventing the cells from entering the G0 resting
phase. Studies suggest that c-myc is the target gene of the chromosome 8q high-level amplifications found in oesophageal adenocarcinomas (Persons et al. 1998; van Dekken et al. 1999). Using in situ hybridization, Abdelatif et al. (1991) found enhanced c-myc expression in dysplastic Barrett’s epithelium and adenocarcinomas, but not in non-dysplastic Barrett’s mucosa. In contrast, c-myc could not be detected immuno-histochemically in oesophageal adenocarcinomas or BO (Jankowski et al. 1992). It is unclear whether amplification or mutation of c-myc plays a significant role in the malignant progression to BO, but it appears to have limited prognostic value in human oesophageal carcinomas (Miyazaki et al. 1992).

The transcription factor c-myb has a well-defined role in the differentiation and proliferation of immature haemopoietic cells. Although c-myb expression was initially thought to be restricted to the haemopoietic system, elevated levels of c-myb mRNA and protein expression have subsequently been detected in human colonic carcinomas and pre-malignant adenomatous polyps, suggesting that up-regulated c-myb expression may lead to hyperproliferation of colonic mucosa and, therefore, plays an important role in the early neoplastic progression of colonic carcinogenesis (Ramsay et al. 1992).

The role of c-myb mRNA expression in Barrett’s oesophagus has been elucidated by Brabender et al. (2001). This study demonstrated that c-myb expression levels increased progressively and significantly in histopathologically worse tissue types, with an increase from normal squamous oesophagus mucosa to Barrett's intestinal metaplasia, and from Barrett's intestinal metaplasia to adenocarcinoma of the
oesophagus (p = 0.002). Median c-myb expression levels were also significantly higher in histologically normal squamous oesophagus tissues from cancer patients compared to normal oesophagus tissues from patients without cancer (p < 0.001) and a control group without evidence of Barrett's oesophagus or gastro-oesophageal reflux disease (p = 0.003). Very high c-myb mRNA expression levels were found only in patients with cancer.

2.4.6 Prostaglandins

Cyclo-oxygenases are membrane-associated proteins that catalyze the rate-limiting step in the prostaglandin (PG) production pathway (Dubois et al. 1998; Forman et al. 1996). There are two different isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed and is involved, for example, in cytoprotection of gastric mucosa. In contrast, COX-2 is normally absent in most tissues, but is transiently induced by pro-inflammatory cytokines and growth factors and is involved in inflammation and mutagenesis. They can promote angiogenesis, inhibit immune surveillance, increase cell proliferation, reduce apoptosis and cell adhesion, and bind to the nuclear peroxisome proliferation activator receptors that act directly as transcription factors on ligand binding (Tsujii et al. 1998; Gupta and Dubois 2001; Souza et al. 2001). However, inhibition of expression can decrease cell growth and increase apoptosis in vitro in oesophageal adenocarcinoma cell lines (Guo et al. 2002).
Gastrin has been shown to induce proliferation and COX-2 expression in gastric and intestinal cells through the activation of the cholecystokinin 2 (CCK\textsubscript{2}) receptor (Konturek et al. 2003; Abdalla et al. 2004). Abdalla et al. (2004) found that COX-2 expression was significantly increased in all Barrett’s oesophagus before the development of dysplasia. Expression was highly variable within Barrett’s dysplasia and adenocarcinoma samples independent of differentiation status. In a longitudinal case-control study, the expression levels within patients increased over time, regardless of the degree of malignant progression. Biopsies from non-dysplastic Barrett’s esophagus expressed increased gastrin mRNA levels compared with other biopsies. Gastrin significantly induced COX-2, prostaglandin E\textsubscript{2}, and cell proliferation in biopsies and cell lines. They concluded that COX-2 is up-regulated early in the Barrett’s metaplasia sequence and that during carcinogenesis; gastrin is a significant determinant of COX-2 activity levels via the CCK\textsubscript{2} receptor.

2.5 Oxidative stress and iron

Iron overload could enhance the production of reactive oxygen and nitrogen species in the oesophageal epithelium, which could lead to the formation of Barrett’s oesophagus. Oxidative damage has been proposed to be closely related to reflux oesophagitis as a driving force for adenocarcinogenesis (Fitzgerald et al. 1996). c-Myc is regulated by iron and represses the natural resistance-associated macrophage protein-1 (NRAMP1/SLC11A1) gene, which in turn modulates the cytoplasmic iron pool (Bowen et al. 2002).
2.5.1 NRAMP1/SLC11A1 (solute carrier family 11 member a1)

The natural resistance-associated macrophage protein (NRAMP1/SLC11A1) family consists of Nramp1, Nramp2, and yeast proteins Smf1 and Smf2. The NRAMP/SLC11A family is a novel family of functional related proteins defined by a conserved hydrophobic core of ten transmembrane domains (Govoni and Gros 1998). NRAMP1/SLC11A1 is an integral membrane protein expressed exclusively in cells of the immune system and is recruited to the membrane of a phagosome upon phagocytosis (Pinner et al. 1997). By controlling divalent cation concentrations NRAMP1/SLC11A1 may regulate the interphagosomal replication of bacteria. Mutations in NRAMP1/SLC11A1 may genetically predispose an individual to susceptibility to infectious diseases including leprosy and tuberculosis. NRAMP1/SLC11A1 regulates macrophage functions that are of potential importance in the induction and/or maintenance of autoimmune diseases such as rheumatoid arthritis, type I diabetes and Crohn's disease. NRAMP2 is a multiple divalent cation transporter for Fe2+, Mn2+ and Zn2+ amongst others it is expressed at high levels in the intestine; and is major transferrin-independent iron uptake system in mammals (Pinner et al. 1997). The yeast proteins Smf1 and Smf2 may also transport divalent cations (Lapham et al. 2004).

By depleting the macrophage cytosol of iron, the NRAMP1/SLC11A1 gene affects iron-regulated gene expression and iron-dependent processes such as cell growth for which iron is essential. NRAMP1/SLC11A1 expression is repressed and
activated by the proto-oncogene c-myc and miz-1 (c-myc-interacting zinc finger protein 1) respectively (Yeung et al. 2004). These authors recently reported a study in which they investigated if c-myc regulates NRAMP1/SLC11A1 expression. They were able to show an inverse correlation with cell growth, and in co-transfection experiments c-myc repressed the NRAMP1/SLC11A1 promoter. The NRAMP1/SLC11A1 gene has been linked to various infectious (allele 2) and autoimmune (allele 3) diseases, which has been ascribed largely to the effect of the functional 5’-[GT] repeat polymorphism in the promoter region of the gene (Searle and Blackwell 1998).

### 2.6 The significance of gene expression analysis in Barrett’s Oesophagus

Recent advances in biotechnology have revolutionised the large-scale analysis of gene expression. In particular, the development of high-throughput quantitative real-time polymerase chain reaction (PCR) (Heid et al. 1996) and c-DNA micro-array technologies has made it possible to analyse the expression of a large panel of genes simultaneously. These techniques, in combination with advances in bioinformatics, promise more accurate disease classification, earlier detection and higher efficiency in the field of cancer diagnosis (Perou et al. 2000).

In order to identify genes or combinations of genes that have the power to discriminate between pre-malignant Barrett's oesophagus and Barrett's associated adenocarcinoma, Brabender et al. (2004) analysed a panel of 23 genes using
quantitative real-time PCR (qRT-PCR) and bio-informatics tools. The genes chosen were either known to be associated with Barrett's carcinogenesis or were filtered from a previous cDNA micro array study on Barrett's adenocarcinoma. A total of 98 tissues, obtained from 19 patients with Barrett's oesophagus (BO group) and 20 patients with Barrett's associated oesophageal adenocarcinoma (OAC group), were studied. Analysis of the 23 candidate genes together with an internal control gene was effected using all samples, for a total of more than 9 016 single PCR reactions. It was found that distinct classes of gene expression patterns exist in the different types of tissues. The most informative genes clustered in six different classes and had significantly different expression levels in Barrett's oesophagus tissues compared to adenocarcinoma tissues. Linear discriminant analysis (LDA) distinguished four genetically different groups. The normal squamous oesophagus tissues from patients with BO or OAC were not distinguishable from one another, but Barrett's oesophagus tissues could be distinguished from adenocarcinoma tissues. Using the most informative genes, obtained from a logistic regression analysis, it was possible to completely distinguish between benign Barrett's and Barrett's adenocarcinomas. A combination of three very informative genes (BFT, TSPAN and TP) was found to be the most powerful combination of discriminating between BO and Barrett's associated adenocarcinoma. This study provided the first non-array parallel mRNA quantitation analysis of a panel of genes in the Barrett's oesophagus model of multistage carcinogenesis. The results suggested that mRNA expression quantitation of a panel of genes can discriminate between pre-malignant and malignant Barrett's disease.
Similarly, Mitas et al. (2005) recently reported on using a quantitative three-tiered algorithm and multi-marker real-time reverse transcription-PCR to accurately discriminate between Barrett's oesophagus and OAC. They showed that OAC can be distinguished from normal oesophagus (NE) and oesophageal squamous cell carcinoma by plotting expression values for EpCam, TFF1, and SBEM in three-dimensional Euclidean space. They developed a highly sensitive assay for monitoring progression of Barrett's oesophagus to OAC whereby 50 ng of RNA are first converted to cDNA using 16 gene-specific primers. A novel quantitative three-tiered algorithm using only 3 genes allowed for accurate (overall accuracy = 61/63, 97%) discrimination of Barrett's oesophagus versus OAC tissues. The gene used in the first tier of the algorithm is TSPAN: samples not diagnosed as BO or OAC by TSPAN in the first tier were then subjected to a second-tier analysis using ECGF1, followed by a third-tier analysis using SPARC. A five-gene marker panel by adding TFF1 and SBEM to the first tier increased the overall accuracy of their assay to 98% (62/63) and resulted in mean molecular diagnostic scores (± SD) that were significantly different between OAC and Barrett's oesophagus samples (3.19 ± 1.07 versus –2.74 ± 1.73, respectively). The data generated by this study suggests that relatively few genes can be used to monitor progression of Barrett's oesophagus to oesophageal adenocarcinoma.
2.7 Aims of the Study

The overall objective of the present study was to define a panel of clinically useful biomarkers for genomic and/or transcriptional profiling in South African patients with Barrett’s oesophagus. Although the prevalence of Barrett’s oesophagus in the local population is similar to that reported in the literature, there is a distinct difference amongst ethnic groups.

The selection of the COX-2, c-myc and c-myb genes for inclusion in the study was based on literature reports indicating progressively increased expression at an early stage through the metaplasia-dysplasia-adenocarcinoma sequence. The NRAMP1/SLC11A1 gene was studied due to its role in iron dysregulation and interaction with c-myb, also involved in iron regulation. In addition, TP53 was included because protein over expression has consistently been reported in Barrett’s oesophagus and is considered to be the commonest genetic event in human malignancy. Availability of a genetic assay that includes different risk markers that are evaluated within the context of environmental risk factors, may lead to effective risk reduction intervention tailored to the needs of the individual. One could envision lengthening endoscopic surveillance intervals for patients at low risk for developing adenocarcinoma, and intensifying endoscopic surveillance intervals for patients at increased risk of developing adenocarcinoma.
The specific aims were:

1) mRNA expression analysis of the COX2, c-myb and c-myc genes to identify differential expression patterns in patients with varying degrees of gastro-oesophageal disease severity;

2) Analysis of the GT-repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene to determine whether c-myc mRNA expression patterns are confined to certain genotypes due to possible gene-gene interaction;

3) Analysis of TP53 by using mutation detection and immuno-histochemistry to define the role of this gene in the metaplasia-dysplasia-adenocarcinoma sequence in the study population.

It is envisaged that molecular studies performed in the genetically diverse South African population will provide a better understanding of the disease mechanisms underlying the progression from BM to BAC.
CHAPTER 3

PATIENTS AND METHODS
The Ethics Committee of the Tygerberg Academic Hospital and the University of Stellenbosch reviewed and approved the study protocol. The informed consent form used for analysis of the role of iron in health and disease, and particularly the iron-related genes NRAMP1/SLC11A1 and c-myc studied in the context of several other genes, is provided in the Addendum.

3.1 Patients

The study population was recruited from outpatients who routinely attended the Gastroenterology Clinic at Tygerberg Academic Hospital during a 3-year period starting June 2001. This included Caucasians of European ancestry, individuals of mixed ancestry (coloured population of San, Khoi, African Negro, Madagascar, Javanese and Western European origin) and black Africans. Written informed consent was obtained from each patient. Particular attention was given to the ethical aspects of genetic testing. The patients were given the assurance of confidentiality, and were informed of the possible benefits that could be derived from participation. It was also explained to the study participants that the meaning of genetic alterations that may be detected and its association with health or disease may be uncertain, and that they may not be informed of the findings.

In total 105 patients were recruited for determination of mRNA expression (COX-2, c-myb and c-myc) in oesophageal biopsy specimens. The study population for the
determination of mRNA expression consisted of 26 individuals with Barrett’s metaplasia (BM) without dysplasia; 14 with Barrett’s oesophagus and dysplasia (BD); 2 patients with Barrett’s adenocarcinoma (BAC); 19 with erosive oesophagitis; 25 with non-erosive oesophagitis and 19 patients with a normal gastroscopy and no gastro-oesophageal reflux (GORD) symptoms used as controls. Matching squamous oesophageal tissue were also collected from all the BM and BD/BAC patients (n=42). Analysis of the functional GT-repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene was performed in 199 patients. Finally, the TP53 gene was studied in 68 Barrett’s patients including 2 with BAC, 12 with BD and 54 with BM, and protein immunostaining was performed in 36 BM and 19 low-grade BD specimens.

Both male and female patients more than 18 years of age were included in the study. Relevant demographic data was collected that included age, gender, race, smoking history and alcohol consumption, age of onset and duration of reflux disease. A questionnaire (see addendum) was used to denote personal details (including lifestyle and dietary factors), family history and clinical characteristics. All the patients, with the exception of the controls, suffered from chronic gastro-oesophageal reflux disease. This was defined as the presence of one or more of the typical reflux symptoms (i.e. heartburn, acid regurgitation and dysphagia/odynophagia) for at least 6 months, experienced on a regular basis (i.e. at least 3 times per week). The prior use of proton pump inhibitors and non-steroidal anti-inflammatory drugs were recorded. Body mass index (BMI = weight in kg/height
m²) was calculated for each patient. The data was computerised for statistical comparisons.

3.2 Methods

3.2.1 Endoscopic procedure

Upper endoscopy was performed as described previously (Weston et al. 1999). During endoscopy the presence of a hiatus hernia and any mucosal abnormalities in the oesophagus were recorded. Non-erosive reflux disease (NERD) was diagnosed when a patient displayed typical reflux symptoms (heartburn, acid regurgitation and dysphagia/odynophagia) with a normal endoscopy. In the case of erosive reflux oesophagitis, the severity and extent of mucosal breaks (erosions) were recorded and classified according to the Los Angeles System (Lundell et al. 1999). Barrett’s oesophagus (BO) was diagnosed by the typical endoscopic appearance of the columnar epithelium having a reddish colour and velvet-like texture compared to squamous oesophageal epithelium with a pale, glossy appearance. The length of the Barrett’s epithelium was determined in the absence of erosive oesophagitis as this might impair the recognition and extent thereof (Sharma 2004). The oesophagogastric junction (OGJ) was defined as where the tubular oesophagus joined the saccular stomach (Haggit 1994), and for patients with hiatus hernias as the point where the proximal margins of the gastric folds met the slight circumferential, band-like narrowing representing the lower oesophageal sphincter (LOS).
Oesophageal biopsies were obtained utilising direct endoscopic vision and the turn and suction method using large, fenestrated, spiked 9-mm open span jumbo biopsy forceps (FK-13K-1 Olympus). Short segment BO was defined as Barrett’s epithelium length of < 3 cm above the OGJ. Four-quadrant biopsies of the Barrett’s epithelium were obtained at intervals of ≤ 2 cm from the OGJ to the squamo-columnar junction. In addition matching biopsies of normal appearing squamous oesophageal tissue were taken from the Barrett’s patients and oesophageal biopsies from all non-Barrett’s patients.

3.2.2 Histological procedure

The specimens for histology were fixed in 10% buffered formalin, stepped serially sectioned and then stained with hematoxylin and eosin (H&E). Goblet cells within metaplastic epithelium were confirmed by staining with alcian blue at pH 2.5 i.e. histological evidence of oesophageal intestinal metaplasia (confirmed/histological BO, Figure 1) (Sampliner 2002). The diagnosis of low/intermediate grade dysplasia (LGD/IGD) and high grade dysplasia (HGD) was according to previously established criteria (Haggit 1994). The histological diagnosis of dysplasia was reviewed seperately and agreed on by 2 experienced gastrointestinal pathologists.
To determine whether TP53 immunostaining might assist in identifying dysplasias, especially low-grade dysplasia, histological specimens of 55 patients (36 BM and 19 BD) were stained with a rabbit polyclonal antibody (NCL-p53-CM1, Novocastra Laboratories) Ltd. This antibody is specific for human wild type and mutant forms under denaturing and non-denaturing conditions. A semi-quantitative scale was used from 0 to 3+ scored as follows: 0, fewer than 1% of cells staining; 1, up to 10% of cells staining, primarily in the lower third of the crypts; 2, more than 10% but fewer than 50% staining; and 3, more than 50% of cells staining.

3.2.3 DNA and RNA analysis

DNA and RNA were extracted using standard techniques to perform mutation screening and expression studies using polymerase chain reaction based (PCR)-based methods. Molecular studies performed at a private genetic laboratory,
included analysis of the NRAMP1/SLC11A1 (susceptibility gene), COX-2, c-myb and c-myc genes and TP53 (prognostic marker).

3.2.3.1 DNA extraction and restriction enzyme analysis

Genomic DNA was isolated from whole blood using a salting-out method (Miller et al. 1988). The PCR primers used to amplify a 116-bp fragment of the NRAMP1/SLC11A1 promoter were 5′-GGGGTCTTGGAACTCCAGAT-3′ (forward) and 5′- TACCCCATGACCACACCCC-3′ (reverse) (Kotze et al. 2001). The positions of the primers in relation to the NRAMP1/SLC11A1 promoter sequence are shown below (forward primer sequence indicated in blue and reverse primer sequence indicated in red).

DNA amplification was performed in 25 μl reaction volumes containing ~20 ng of genomic DNA, 5 mM each deoxyribonucleoside triphosphates (dNTPs), 15 pmol of each PCR primer, Mg²⁺-containing reaction buffer and 0.375 units of DNA Taq polymerase (Roche Diagnostics, Switzerland). A step-down PCR amplification procedure was applied with one cycle initial denaturation for 3 minutes at 95°C; ten cycles of denaturation at 95°C for 45 seconds; annealing at 65°C for 30 seconds; extension at 72°C for 45 seconds; followed by twenty-five cycles of denaturation at 95 °C for 45 seconds; annealing at 60°C for 30 seconds; extension at 72°C for 45
seconds and final extension at 72°C for 10 minutes. PCR amplification products were visualised in 2% agarose (Seakem) by ethidium bromide staining and ultraviolet transillumination to confirm successful DNA amplification. Alleles of the SLC11A1 5'[GT]n promoter polymorphism were distinguished by RsaI (Promega Inc., UK) restriction enzyme analysis (0.5 units per reaction) as previously described (Graham et al. 2000). Digested PCR products were electrophoresed at room temperature in 5% cross-linked polyacrylamide gels at 300 V for 2 hours. DNA fragments in the respective polyacrylamide gels were visualised by ethidium bromide staining and ultraviolet transillumination.

3.2.3.2 RNA extraction and expression analysis

Collection of the samples for RNA extraction was standardised to ensure reliable expression data. The biopsy specimens used in the study were obtained at endoscopy or operation and were immediately frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted using the Qiagen RNeasy kit. Aliquots of 8.5 µl were immediately used in a RT-PCR reaction in a total volume of 20 µl (Roche, First strand cDNA synthesis kit, Mannheim, Germany). Three complementary primer sets were designed (Table 1) (LightCycler probe design, Roche, Mannheim, Germany) from Genbank sequences for application of real-time PCR.
Table 1. Primer sets designed across the exon-intron boundaries of the COX-2, c-myb, c-myc and β-actin (ACTB) genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>5’- GCTGACTATGGCTACAAAAG-3’</td>
<td>5’- GATGCGTGAAGTGCTG-3’</td>
</tr>
<tr>
<td>c-myb</td>
<td>5’- TTCATAGAGACCCAGACTGTG-3’</td>
<td>5’- ACTCAGCAACAAATCCAGA-3’</td>
</tr>
<tr>
<td>c-myc</td>
<td>5’- GTGGAAGAAATTGCGAGCT-3’</td>
<td>5’- CCACATACAGCTCCCTGGAT-3’</td>
</tr>
<tr>
<td>ACTB</td>
<td>5’- AAGAGCTACGAGGCTGC-3’</td>
<td>5’- CACTGTGTGGCGGTACA-3’</td>
</tr>
</tbody>
</table>

c-DNA synthesised from RNA extracted from normal and abnormal tissue of patients with BM were used to optimise the multi-gene assay for expression analysis of the COX-2, c-myb and c-myc genes (van Rensburg et al. 2004). The diagram below illustrates the process from sample collection to obtaining the mRNA expression results.

The RT-PCR was performed using the Roche LightCycler (software version 3.5) (Figure 3).
The reaction mixture consisted of cDNA (1 µl), 10 pmol of each primer, 1 µl LightCycler FastStart DNA Master SYBR Green 1 Mix (Roche, Mannheim, Germany), 500 ng BSA (Gibco, BRL, Gaithersburg, MD) and 3 mM MgCl₂ in a total volume of 10 µl. The FastStart polymerase was activated and cDNA denatured by a pre-incubation of 10 min at 95°C. The template was amplified for 40 cycles of denaturation at 95°C for 0 s, annealing at 60°C for 8 s and extension at 72°C for 12 s. Standard curves for the housekeeping gene β Actin (ACTB) and target (COX-2, c-myb, c-myc) genes were generated from series diluted cDNAs and analysed using the LightCycler quantification software set on the second derivative maximum. The genes of interest and the reference gene were amplified in separate reactions. Coff files were generated when the quality of the standard curve and PCR efficiency (E=10^{(-1/slope)}=2) was optimum (Figure 4). Reference controls from the standard curve were included in each run.
Figure 4. The fit coefficients or Coff files are created from a dilution series and describes the kinetics during the PCR reaction. The PCR efficiency can be calculated using the slope of the standard curve. A sample from this dilution series is included in every run and acts as an external control.

3.2.4 c-DNA sequencing of the TP53 gene

Due to limited c-DNA quantity a nested PCR method was used to amplify the coding region of the TP53 gene in two fragments for mutation analysis. The primer pairs are indicated in Table 2.

Table 2. Primer sets for nested PCR designed for amplification of two fragments spanning the TP53 coding sequence.

<table>
<thead>
<tr>
<th></th>
<th>FORWARD PRIMERS</th>
<th>REVERSE PRIMERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside</td>
<td>5’-GTGCTTTCCACGAGGTTGAC-3’</td>
<td>5’-CAAATGAAGTCCTGCTGGTGCTT-3’</td>
</tr>
<tr>
<td>Fragment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>5’-GACTGCCTTCCGCGTGACT-3’</td>
<td>5’-TCCACACGCAAATTCCTTCCTCCACT-3’</td>
</tr>
<tr>
<td>Fragment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>5’-GCAGTCACACAGCAGTGAGCGG-3’</td>
<td>5’-TGACGCACACCTATTGCAAGCAAG-3’</td>
</tr>
</tbody>
</table>
The positions of the primers are shown below in relation to the TP53 coding sequence. The initiation codon (ATG) and termination codon (TGA) are underlined. The forward primers are indicated in blue and the reverse primers in red.

The FastStart polymerase was activated and cDNA denatured by a pre-incubation of 10 min at 95°C. The template was amplified for 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 45s and extension at 72°C for 60s, with a final extension of 10 minutes. The PCR products were sequenced bidirectional using an ABI Prism 3130 Genetic Analyser (Applied Biosystems) according to the manufacturer’s
instructions. Sequence alignment and analyses were performed using the GAP4 genome assembly program (Bonfield et al. 1995).

3.2.5 Statistical analysis

One-way Analysis of Variance (ANOVA) was used to identify significant differences in mRNA expression levels among the histopathological groups for the COX-2, c-myb, and c-myc genes. The chi-square test was used to determine dependencies between the groups and whether the patient samples showed increased mRNA expression or not. A 5% significance level (p<0.05) was used as guideline for determining significant differences. NRAMP1/SLC11A1 genotype and allele frequencies were analysed by STATISTICA 7 for Windows (StatSoft. Inc. 1984 – 2005), which offers different methods to determine significant associations in the study population. The Chi-square ($\chi^2$) test and/or Fischer-exact test were performed to assess significant associations with NRAMP1/SLC11A1 in the study population.
CHAPTER 4

RESULTS
4.1 Prevalence of Barrett’s oesophagus

The prevalence of Barrett’s oesophagus (Figure 1) was determined in 1606 consecutive patients with chronic gastro-oesophageal reflux disease that attended the Tygerberg Hospital Gastroenterology Clinic over a 6 months’ period. The overall prevalence of Barrett’s oesophagus was 3% (Table 1). White males had the highest prevalence (8%), followed by white females (5%) and males of mixed ancestry (4%). Only one percent of the females of mixed ancestry presented with Barrett’s oesophagus, while one black male had Barrett’s oesophagus.

Figure 1. Endoscopic view (left panel) of an adenocarcinoma (arrow) developing in a Barrett’s segment). The right panel depicts the histological confirmation (H & E) of the adenocarcinoma.
Table 1. Prevalence of Barrett’s oesophagus in 1606 patients with chronic reflux disease assessed over a six months’ period.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Race</th>
<th>Total</th>
<th>Barrett’s</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Mixed Ancestry</td>
<td>900</td>
<td>7</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>55</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>215</td>
<td>11</td>
<td>5%</td>
</tr>
<tr>
<td>Male</td>
<td>Mixed Ancestry</td>
<td>291</td>
<td>13</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>27</td>
<td>1</td>
<td>0.03%</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>118</td>
<td>10</td>
<td>8%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1606</td>
<td>41</td>
<td>3%</td>
</tr>
</tbody>
</table>

4.2 Demographic, Endoscopic and Histological Data

The demographic data of the patients selected for gene expression analysis is summarised in Table 2. Of note is that in the Barrett’s study population 14/26 patients with metaplasia only, and 7/16 patients with dysplasia and/or adenocarcinoma (Figure 2) had a short segment (< 3cm). A hiatus hernia was present in more than 60% of patients. The mean reflux duration was significantly more in the Barrett’s dysplasia / adenocarcinoma group (28.3 ± 22 years). There was only one patient with severe dysplasia in the short segment Barrett’s group.
Table 2. Demographic, Endoscopic and Histological Features of the Study Population.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=19)</th>
<th>NERD (n=25)</th>
<th>ERD (n=19)</th>
<th>BM (n=26)</th>
<th>BD/BAC (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean ± SD, yr)</strong></td>
<td>41.3± 12.9</td>
<td>41.8 ± 9.8</td>
<td>44.0 ± 8.3</td>
<td>62 ± 16</td>
<td>67 ± 13</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>14</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Mixed</td>
<td>16</td>
<td>20</td>
<td>18</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Black</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Smoking – mean pack years</strong></td>
<td>17.1 ± 13.4 (n=10)</td>
<td>12.0 ± 9.3 (n=12)</td>
<td>6.36 ± 5.1 (n=13)</td>
<td>47.1 ± 46.2 (n = 13)</td>
<td>31.7 ± 26.4 (n = 9)</td>
</tr>
<tr>
<td><strong>Mean age (yrs) of reflux onset</strong></td>
<td>31.3 ± 12.5</td>
<td>37.4 ± 10.1</td>
<td>41.0 ± 22</td>
<td>37 ± 16</td>
<td></td>
</tr>
<tr>
<td><strong>Mean reflux duration (yrs)</strong></td>
<td>11.2 ± 12.9</td>
<td>6.7 ± 7.0</td>
<td>12.4 ± 9.6</td>
<td>28.3 ± 22</td>
<td></td>
</tr>
<tr>
<td><strong>Barrett’s ≤ 3 cm ≥ 3 cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>23</td>
<td>16</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No dysplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGD/AC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27.6 ± 6.3</td>
<td>26.8 ± 5.5</td>
<td>28.9 ± 9.2</td>
<td>21.4 ± 13.0</td>
<td>27.4 ± 9.6</td>
</tr>
</tbody>
</table>

NERD, non-erosive oesophagitis; ERD, erosive oesophagitis; BM, Barrett’s metaplasia; BD, Barrett’s dysplasia; BAC, Barrett’s adenocarcinoma; LGD, low grade dysplasia; HGD, high grade dysplasia; AC, adenocarcinoma; BMI, body mass index.

### 4.3 COX-2, c-myb and c-myc mRNA expression

mRNA expression levels were determined for COX-2, c-myb and c-myc in control samples and Barrett’s patients (Figure 2). In the normal, non-erosive (NERD) and erosive reflux oesophagitis (ERD) groups 14% (9/63) had marginally increased expression of c-myb mRNA, compared with 48% (20/42) in the Barrett’s patients (p = 0.002). Increased c-myb mRNA expression was also found in the matching
squamous cell epithelium (MSE) of 14% (6/42) of the Barrett’s patients studied. COX-2 and c-myc mRNA levels were not increased in MSE, NERD and control samples, while only a few individuals showed increased levels in the ERD and Barrett’s groups.

![Barrett's Er osive Non-erosive Control]

**Figure 2.** Percentage of patients with increased mRNA expression (> 1.0) for COX-2, c-myc and c-myb in control individuals with a normal gastroscopy (n=19), erosive oesophagitis (n=19), non-erosive oesophagitis (n=25) and Barrett’s oesophagus (n=42).
Eleven patients in the BD/BAC group 69% (11/16) showed increased c-myb mRNA expression compared with 35% (9/26) in the BM group (p = 0.03) (Figure 3). c-Myb (1,02 and 2,59) and COX-2 (6,7 and 1,52) but not c-myc (0,24 and 0,42) mRNA levels were increased in the adenocarcinoma tissue of the two patients with BAC. A similar expression pattern was seen for c-myb in the dysplastic tissue of these patients (1,75 and 2,59), while one also showed COX-2 overexpression (0,58 and 1,52). In comparison, none of the three genes showed increased mRNA expression in the matching normal tissue of the patients diagnosed with BAC.

**Figure 3.** c-Myb over expression in Barrett’s patients with and without dysplasia.

c-Myb mRNA over expression occurred equally in patients with short and long segment Barrett’s oesophagus. In the BD group 21% (3/14 patients) had increased c-myc mRNA expression compared to none with BM (p < 0.05) and BAC. No significant associations were observed for COX-2 in any of the study groups.
groups. Mean expression levels for mRNA in the Barrett’s patients and non-Barrett’s control groups combined (normal, ERD, NERD) are depicted in Table 3. The mean c-myb mRNA expression was significantly higher \((p < 0.01)\) in Barrett’s patients \((1.40 \pm 1.43)\), compared with those without Barrett’s \((0.62 \pm 0.44)\). There was a significant progressive elevation of c-myb expression through the stages of Barrett’s intestinal metaplasia to dysplasia/adenocarcinoma. No significant differences in mRNA expression levels were observed between ethnic groups for the genes analysed (Figure 4).

Table 3. Mean mRNA levels of patients with Barrett’s metaplasia with and without dysplasia or adenocarcinoma in the abnormal oesophageal tissue.

<table>
<thead>
<tr>
<th></th>
<th>c-myb mRNA (mean ± SD)</th>
<th>Percentiles (25th-75th)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB (n=63)</td>
<td>0.62 ± 0.44*</td>
<td>0.31-0.83</td>
</tr>
<tr>
<td>BM (n=26)</td>
<td>1.24 ± 1.62#</td>
<td>0.4-1.25</td>
</tr>
<tr>
<td>BD/BAC (n=16)</td>
<td>1.68 ± 1.03*</td>
<td>0.69-2.29</td>
</tr>
<tr>
<td>Barrett’s total (n=42)</td>
<td>1.40 ± 1.43*</td>
<td>0.49-1.97</td>
</tr>
</tbody>
</table>

*Non-Barrett’s vs Barrett’s total group: \(p < 0.01\)
#BM vs BD/BAC: \(p = 0.04\) (this p-value was obtained by using the Mann-Whitney test and is considered to be only marginally significant, because the ANOVA p-value was found to be 0.34). NB, non-Barrett’s group including 19 controls, 25 non-erosives and 19 erosives; BM, Barrett’s metaplasia; BD, Barrett’s dysplasia.

Figure 4. Ethnic comparison of mean levels of c-myb mRNA expression.
None of the patients used non-steroidal anti-inflammatory drugs. In the Barrett’s groups only three patients took aspirin as cardiovascular protection on a regular basis. Two of these patients had increased c-myb mRNA expression and one increased c-myc mRNA expression. Of the 39 patients with no such history, 18 had increased c-myb mRNA expression, two c-myc mRNA expression and two COX-2 mRNA expression. Thirteen of the Barrett’s patients were on maintenance proton pump inhibition. Of these patients, 6 had increased c-myb mRNA expression and one increased c-myc mRNA expression. Twenty-nine patients were not on maintenance proton pump inhibition. Fourteen of these patients had increased c-myb expression and two each increased c-myc and COX-2 expression.

4.4. Genotyping of the 5’-[GT]n repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene

The DNA samples extracted from blood samples of 199 patients were genotyped for the NRAMP1/SLC11A1 promoter polymorphism. Figure 5 shows a representative gel electrophoresis pattern following restriction enzyme analysis using Rsa I digestion.
Figure 5. Restriction enzyme analysis using Rsal digestion and polyacrylamide gel electrophoresis. Heterozygotes with both alleles 2 and 3 show two DNA fragments of 57 bp and 48 bp respectively, homozygotes for allele 2 have two upper bands and homozygotes for allele 3 have two lower bands (see arrows).

The genotype distribution and allele frequencies of the NRAMP1/SLC11A1 5′[GT]n repeat polymorphism in controls and patients subdivided according to histology and ethnic background are summarised in Table 4.
Table 4. Genotype distribution and allele frequencies of the NRAMP1/SLC11A1 5'([GT])n repeat polymorphism in controls and patients subdivided according to histology and ethnic background (numbers observed are indicated with percentages in brackets).

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>NRAMP</th>
<th>CONTROLS</th>
<th>NERD</th>
<th>ERD</th>
<th>BM</th>
<th>BD#</th>
</tr>
</thead>
<tbody>
<tr>
<td>White (n=47)</td>
<td>Genotype</td>
<td>2/2</td>
<td>2/3</td>
<td>3/3</td>
<td>Allele</td>
<td>n=8</td>
</tr>
<tr>
<td>2/2</td>
<td>0(0%)</td>
<td>3(50%)</td>
<td>1(11%)</td>
<td>4(14%)</td>
<td>1(8%)</td>
<td>2</td>
</tr>
<tr>
<td>2/3</td>
<td>1(25%)</td>
<td>2(33%)</td>
<td>3(33%)</td>
<td>10(36%)</td>
<td>3(25%)</td>
<td>7</td>
</tr>
<tr>
<td>3/3</td>
<td>3(75%)</td>
<td>1(17%)</td>
<td>5(56%)</td>
<td>14(50%)</td>
<td>8(67%)</td>
<td>30</td>
</tr>
<tr>
<td>Mixed (n=150)</td>
<td>Genotype</td>
<td>2/2</td>
<td>2/3</td>
<td>3/3</td>
<td>Allele</td>
<td>n=40</td>
</tr>
<tr>
<td>2/2</td>
<td>1(5%)</td>
<td>6(9%)</td>
<td>5(10%)</td>
<td>1(6%)</td>
<td>0(0%)</td>
<td>10</td>
</tr>
<tr>
<td>2/3</td>
<td>8(40%)</td>
<td>27(42%)</td>
<td>16(33%)</td>
<td>5(28%)</td>
<td>1(20%)</td>
<td>30</td>
</tr>
<tr>
<td>3/3</td>
<td>11(55%)</td>
<td>31(48%)</td>
<td>27(56%)</td>
<td>12(67%)</td>
<td>4(80%)</td>
<td>15</td>
</tr>
<tr>
<td>Black (n=2)</td>
<td>Genotype</td>
<td>2/2</td>
<td>2/3</td>
<td>3/3</td>
<td>Allele</td>
<td>n=2</td>
</tr>
<tr>
<td>2/2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2/3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>3/3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (n=199)</td>
<td>Genotype</td>
<td>2/2</td>
<td>2/3</td>
<td>3/3</td>
<td>Allele</td>
<td>n=50</td>
</tr>
<tr>
<td>2/2</td>
<td>1(4%)</td>
<td>9(13%)</td>
<td>6(10%)</td>
<td>5(11%)</td>
<td>1(6%)</td>
<td>11</td>
</tr>
<tr>
<td>2/3</td>
<td>9(36%)</td>
<td>29(41%)</td>
<td>19(33%)</td>
<td>15(33%)</td>
<td>4(24%)</td>
<td>39</td>
</tr>
</tbody>
</table>

#Numbers for BD and OAC are combined for statistical comparisons

The 3/3 genotype was most common (108/199, 54%) and the 2/2 the least (22/199, 11%) in the patient groups studied. Statistically, there was no significant difference in the genotype distribution between the ethnic groups. Similarly, there was no statistical significant difference in the allele frequencies in the different patient groups (Barrett’s oesophagus, ERD, NERD and the controls, p=0.40) studied. Likewise, there was no difference in the genotype distribution or allele frequencies in the Barrett’s group with or without dysplasia (p=0.57).
4.5 Analysis of NRAMP1/SLC11A1 in relation to mRNA expression

In the Barrett’s patient group, only 3 patients with dysplasia had increased c-myc mRNA expression. None of these patients demonstrated the most common 3/3 genotype. One of these patients had a 2/2 and the other two a 2/3 genotype. The genotype distribution of the patients with Barrett’s oesophagus that showed an increase in c-myb mRNA expression (Figure 6), was similar to the patients with no increase and matched the distribution pattern of the patient group as a whole, with the 3/3 genotype being the most common. Only 5 patients with Barrett’s oesophagus had an increased COX-2 expression, 2 each of the 2/3 and 3/3 genotypes, respectively.

![Categorized Histogram: c-myb abnormal overexpressed x NRAMP1 RsaI result](image)

Chi-square test: \( p = .44932 \)

Figure 6. NRAMP1/SLC11A1 allele frequencies of Barrett’s patients with and without increased c-myb mRNA expression.
4.6 c-DNA sequencing and Immuno-staining of TP53

The possible significance of TP53 in the malignant progression of Barrett’s oesophagus was studied in South African Barrett’s patients by using both immuno-histochemistry and mutation detection.

4.6.1 TP53 mutation analysis

Direct sequencing of the coding region of the TP53 gene in 68 patients with Barrett’s oesophagus revealed two common polymorphisms and two rare mutations (Figure 7). Sporadic mutations, G245R (patient no. 563) and D281Y (patient no. 573), were detected in two patients with BAC, both in the adenocarcinoma and dysplastic tissue. Mutation D281X was also detected in the normal tissue of the patient. Notably, increased c-myb mRNA expression was detected in both TP53 mutation-positive patients with BAC.
P36P heterozygote CCG-CCA at nucleotide position 108

P36P homozygote CCG-CCA at nucleotide position 108

R72P normal CGC-CCC at nucleotide position 215
R72P heterozygote CGC-CCC at nucleotide position 215

R72P homozygote CGC-CCC at nucleotide position 215
G245R normal GGC-CGC at nucleotide position 733

G245R homozygote GGC-CGC at nucleotide position 733
Figure 7. Detection of one silent (P36P) and three missense mutations (R72P, D281Y and G245R) by direct sequencing of TP53 c-DNA samples. *Base changes; normal, base change absent; heterozygote, one copy; homozygote, two copies.
The TP53 mutations identified in the study population is summarised in Table 5. Similar to previous studies in other populations, polymorphisms R72P and P36P were identified at a relatively high frequency in the Barrett’s patients. These missense (R72P) and silent (P36P) mutations are germline mutations since they were also identified in the normal tissue of the patients. The patient with mutation D281Y was homozygous for the codon 36 and 72 polymorphisms, and all 3 base changes were also present in the matching normal tissue of this patient. The allele frequencies of the wild-type and mutant alleles for R72P are 46% (50/108) and 54% (58/108), respectively, in patients with BM. The mutated allele was detected at a lower frequency in the 12 patients with dysplasia (9/24, 37.5%).

Table 5. TP53 gene variations detected in Barrett’s epithelial/adenocarcinoma tissue for 68 patients analysed

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Barrett’s Metaplasia (n=54)</th>
<th>Barrett’s Dysplasia (n=12)</th>
<th>Adenocarcinoma (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P36P Exon 4</td>
<td>4 heterozygotes</td>
<td>2 homozygotes</td>
<td></td>
</tr>
<tr>
<td>R72P Exon 5</td>
<td>26 heterozygotes</td>
<td>16 homozygotes</td>
<td>5 heterozygotes*</td>
</tr>
<tr>
<td>G245R Exon 7</td>
<td>1 homozygote*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D281Y Exon 8</td>
<td>1 heterozygote*#</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mutations G245R and D281Y were also detected in the matching dysplasia tissue.

#Two copies each of variants P36P and R72P were detected in the same patient. Heterozygote, one copy of the mutation, Homozygote, two copies of the mutation P, proline; R, arginine; G, glycine; E, glutamate; D, aspartate; Y, tyrosine
4.6.2 TP53 Immuno-histochemical staining

Immuno-staining using a rabbit polyclonal antibody was performed in 36 BM and 19 low-grade BD specimens to determine whether TP53 immunoreactivity could help with the identification of low-grade dysplasias. Although more specimens of patients with mild BD stained positive for TP53 compared with BM (64 vs. 84%), staining of 2+ and 3+ intensity was similar in both groups (58%). As shown in Table 6, TP53 immuno-staining does not differentiate accurately between mild dysplasia and BM (p=0.05) in the South African study group.

<table>
<thead>
<tr>
<th>Consensus Histological Category</th>
<th>Immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Barrett’s Metaplasia</td>
<td>13 (36%)</td>
</tr>
<tr>
<td>Low Grade Dysplasia</td>
<td>3 (16%)</td>
</tr>
</tbody>
</table>

*Data are given in numbers (percentage). Scoring are as follows: 0, fewer than 1% of cells staining; 1, up to 10% of cells staining, primarily in the lower third of the crypts; 2, more than 10% but fewer than 50% staining; and 3, more than 50% of cells staining.
CHAPTER 5

DISCUSSION AND CONCLUSION
5.1 Epidemiology of Barrett’s oesophagus

Autopsy data provided evidence that the majority of cases of Barrett's oesophagus are not detected in the general population, and it is estimated that for every known case of Barrett's oesophagus, 20 additional cases go unrecognised (Spechler 1999; Eckardt et al. 2001). Barrett's oesophagus predominates in middle-aged white men (Spechler et al. 1994). Environmental factors play an important role in the development of Barrett's oesophagus and it is suggested that the increased prevalence may be related to the decline of *Helicobacter pylori* infection (Guanrei et al. 1988; Macdonald et al. 2000). Since obesity is an important risk factor for oesophageal adenocarcinoma, lifestyle factors may affect the incidence of Barrett's oesophagus. A better understanding of interaction between the genetic and lifestyle factors that predisposes an individual to the development and progression of Barrett's oesophagus will be crucial for the implementation of effective screening and prevention programs in future.

The overall prevalence of Barrett's oesophagus in our clinic was 3% over a 6-month period in 1606 patients assessed for chronic reflux disease. The findings in Caucasians and males of mixed ancestry are in keeping with the reported prevalence of approximately 6–12% in patients undergoing endoscopy for symptoms of chronic GORD (Sarr et al. 1985; Cameron and Lomboy 1992). Of the 82 black patients with chronic reflux disease, Barrett's oesophagus was diagnosed in one individual, which support the evidence that this condition is uncommon in blacks
(Spechler et al. 1998, 2002). In a previous South African study of 216 consecutive patients diagnosed with Barrett’s oesophagus between 1970 and 1993, only 5% were black (Mason et al 1998). Segal (2001) reviewed data on the epidemiology of GORD, Barrett’s oesophagus, OAC and H. pylori infection in sub-Saharan Africa and reported similar findings. Barrett’s oesophagus is rare and OAC uncommon in all regions of sub-Saharan Africa studied (South Africa, Ethiopia, Nigeria, Zimbabwe, Kenya and Uganda). Urbanisation, despite an increase in risk factors associated with GORD, has not led to an increase in this disease among Africans, as anticipated. It is believed that the critical factor preventing GORD in black Africans is H. pylori infection (with an overall prevalence of 61 – 100%), which is usually acquired in childhood, is for life and is probably protective for the oesophagus.

Oesophageal cancer is one of the least studied and deadliest cancers worldwide. During the past three decades, important changes have occurred in the epidemiologic patterns associated with this disease. Persons with recurring symptoms of reflux have an eightfold increase in the risk of oesophageal adenocarcinoma (Lagergren et al. 1999). Despite the dramatic increase in the incidence of oesophageal adenocarcinoma over recent years with a reported incidence of 0.5% per year in Barrett’s oesophagus patients (Alderson 2002), we did not see a similar trend in our local population. During the past 5 years only two patients presented with an adenocarcinoma that developed in a Barrett’s segment, while not a single patient known with Barrett’s oesophagus developed this complication over a 5-year surveillance period.
5.2 Dysplasia

Dysplasia is a histological diagnosis suggesting that one or more clones of epithelial cells have acquired genetic alterations rendering them neoplastic and prone to malignancy. Endoscopic surveillance is performed primarily to identify dysplasia, but it is an imperfect predictor of malignancy. The histological abnormalities of low-grade dysplasia are not specific for neoplasia, because similar changes can occur in normal tissue in response to injury. Even among experienced pathologists, the extent of inter observer agreement for the diagnosis of low-grade dysplasia in Barrett's oesophagus may be less than 50 percent (Skacel et al. 2000; Montgomery et al. 2001). It is not clear whether low grade dysplasia has the same pathogenesis and natural history as high grade dysplasia, or whether short-segment disease progresses to long-segment disease. Although logic and indirect evidence suggest that the risk of cancer should vary with the extent of oesophageal metaplasia, this contention has not been proven (Eckardt et al. 2001).

In the South African study cohort low grade dysplasia was as prevalent in the short segment (<3cm) as in long segment Barrett’s (7/14 and 6/14 respectively). Only one of the patients studied had high grade dysplasia.
5.3 Genetic studies

Molecular-genetic changes underlying the development of Barrett’s oesophageal adenocarcinoma remain poorly understood. Chromosomal changes are frequent and include losses, gains or amplifications. Results of studies performed to match specific chromosomal aberrations with particular genes were inconsistent. Several genes appear to have a central role in cancer development, including COX-2, Bcl-2, TP53, p16, p27, cyclin D1, retinoblastoma protein, epidermal growth factor (and its receptor), erb-b2, and E-cadherin and the cadherin-catenin complex (Wijnhoven et al. 2001; Singh et al. 1998; Shirvani et al. 2000; Katada et al. 1997; Arber et al. 1996; Yacoub et al. 1997; Polkowski et al. 1999; Krishnadath et al. 1997).

5.3.1 mRNA expression analysis

Mitas et al. (2005) recently reported on using a quantitative three-tiered algorithm and multi-marker real-time reverse transcription-PCR to accurately discriminate between Barrett's oesophagus and OAC, and to monitor progression of Barrett's oesophagus to OAC. In their algorithm, a five-gene panel (TSPAN, ECGF1, SPARC, TFF1 and SBEM) increased the overall accuracy of discrimination to 98% (62/63) and resulted in mean molecular diagnostic scores (± SD) that were significantly different between OAC and Barrett’s oesophagus samples (3.19 ± 1.07 versus -2.74 ± 1.73, respectively). Their results suggested that relatively few genes can be used to monitor progression of Barrett’s oesophagus to oesophageal adenocarcinoma.
Once OAC has developed the survival rate is extremely poor. Therefore, one of the main objectives of the present study was to measure c-myb, c-myc and COX-2 mRNA expression levels to determine whether one or more of these genes have the power to improve discrimination between Barrett’s metaplasia without dysplasia and Barrett’s associated dysplasia and/or adenocarcinoma in South African patients. The genes for mRNA expression were selected on the basis of the previously-reported increasing levels of expression as relatively early events through the metaplasia-dysplasia-adenocarcinoma sequence.

Our study population included patients and controls from different ethnic groups in South Africa. Although the numbers in each subgroup were relatively small, it was clear that ethnic differences did not affect the results. Increased c-myb mRNA expression was demonstrated in 69% of patients with Barrett’s associated dysplasia or adenocarcinoma compared with 35% in the Barrett’s metaplasia group. These values were significantly higher compared with the control groups, showing increased c-myb mRNA expression in only 14% of cases. These findings are in keeping with those reported by Brabender et al. (2001) who used a multi-gene panel to distinguish between benign Barrett's and BAC. Their results have shown that up-regulation of c-myb mRNA is an early event in the development of Barrett’s oesophagus and associated adenocarcinoma. High c-myb mRNA expression levels therefore seem to be a useful biomarker for the prediction and detection of Barrett’s oesophagus. Once the environmental triggers responsible for cancer development
in patients with increased c-myb mRNA expression are known, this marker could also serve as a molecular target for implementation of preventative measures.

COX-2 expression has been reported to play an important role in the metaplasia-dysplasia-carcinoma sequence in Barrett's oesophagus. This leads to a higher proliferation potential, a risk which may be thwarted by non-steroidal anti-inflammatory drugs administration, inhibits apoptosis and promote tumour angiogenesis (Wilson et al. 1998; Amano et al. 2004; Wen et al. 2002). Expression of COX-2 protein has been reported in both dysplastic and non-dysplastic Barrett's epithelium (Michael et al. 2003). In a recent article by Amano et al. (2004), COX-2 expression was reported in 71% of Barrett's epithelium biopsy samples. Surprisingly, in our study only two patients with Barrett’s oesophagus had increased expression of COX-2 mRNA levels. There was no prior non-steroidal anti-inflammatory drug-intake in our patients and none of the three patients on aspirin prophylaxis had increased COX-2 expression.

The importance of c-myc in cellular proliferation and its role in sensitising cells to apoptotic triggers is well established. Tselepsis et al (2003) demonstrated up-regulation of c-myc mRNA in 33% of Barrett’s metaplasia compared with 84% in adenocarcinoma samples. Comparatively, only 21% (3/14) of our study population with Barrett’s associated dysplasia had increased c-myc mRNA expression, but none with Barrett’s metaplasia and Barrett’s associated adenocarcinoma. Failure to show similar increases in c-myc mRNA expression through the metaplasia-dysplasia-
adenocarcinoma sequence might relate to the low overall incidence of Barrett’s dysplasia and OAC in the local population. This finding highlights the potential significance of external influences on c-myc expression, including acidified bile acid and cellular iron status (Goldstein and Yang 1998; Chen et al. 1999).

5.3.2 5'-[GT]n repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene

In a complementary study the 5'-[GT]n repeat polymorphism in the promoter region of the NRAMP1/SLC11A1 gene was analysed to determine whether increased c-myc mRNA expression patterns in the realm of gastro-oesophageal reflux disease are confined to certain genotypes due to possible gene-gene interaction. In our population group a similar pattern of c-myc mRNA expression in the metaplasia-dysplasia-adenocarcinoma sequence in Barrett’s oesophagus could not be demonstrated as was previously reported by Tselepsis et al. (2003).

The NRAMP1/SLC11A1 gene is involved in the release of iron acquired by phagocytic iron uptake. The basis for the reported inhibition of NRAMP1/SLC11A1 gene expression by c-myc has still to be clarified (Bowen et al. 2002). NRAMP1/SLC11A1 can be induced by pro-inflammatory cytokines, iron and redox stress caused by chronic inflammation and as such may have a protective effect. c-Myc positively and negatively regulates genes that increase and decrease, respectively, the labile iron pool (Wu et al. 1999). The rationale for c-myc increasing
the iron content within the cytosol, is to provide iron for enzymes such as ribonucleotide reductase. It has been shown that iron chelation leads to growth arrest. NRAMP1/SLC11A1-mediated divalent cation flux may deplete the levels within the cytosol.

In the patient group as a whole we were unable to demonstrate any statistically significant differences in the NRAMP1/SLC11A1 genotype distribution and allele frequencies amongst the controls, the patients with NERD and ERD, and the patients with Barrett’s with and without dysplasia. The 3/3 genotype was the most common in all study groups, which was in accordance to NRAMP1/SLC11A1 genotyping in various other population groups, including the genetically distinct population groups of South Africa (Kotze et al. 2001).

This study failed to show any significant differences in allele frequencies and genotype distribution of the 5΄-(GT)n NRAMP1/SLC11A1 polymorphism in patients with Barrett’s oesophagus with and without dysplasia. However, of the three Barrett’s dysplasia patients with increased c-myc mRNA expression none had the common 3/3 genotype. The number of patients with allele 2 is very small, but this finding raises the possibility that the presence of allele 2 of NRAMP1/SLC11A1 increases the risk of high c-myc expression previously implicated in BD and cancer development. It seems possible that unique gene-gene interaction patterns may explain differences in mRNA expression profiles and BAC incidences reported in populations with diverse genetic backgrounds.
5.3.3 TP53 analysis

TP53 appears to have both a function as an oncoprotein, perhaps by complexing in its mutant form with wild type TP53, and as tumour suppressor gene in its normal state (Lane and Benchimol 1990). Similar to the study performed by Schneider et al. (1996), none of the tissue samples from South African patients with Barrett's esophagus alone and no dysplasia/adenocarcinoma had any sporadic TP53 mutations. These authors performed mutation detection in 50 patients with Barrett's adenocarcinoma, of which 23 (46%) had TP53 mutations in Barrett's epithelium, tumors, or both. Twenty of these patients had TP53 mutations in the tumor only (n = 16) or in both tumor and Barrett’s epithelium (n = 4), suggesting that the mutation plays a direct role in carcinogenesis. In three patients with cancer, mutations occurred only in Barrett's epithelium, suggesting that such mutations may also be a marker for genomic instability. Mutations were predominantly found in exons 5, 7, and 8, the hotspot region of the TP53 gene. The missense mutations identified in the South African patients with BAC were also located in these gene regions.

In the present study, both patients with BAC had mutations in the TP53 gene, and none of the patients with BM or BD alone. To our knowledge, mutations D281Y and G245R were detected for the first time in patients with BAC, while mutation G245R has previously been identified in colorectal tumours (Hao et al. 2002). More than half the patients with BM and BD tested positive for the relatively common R72P
germline polymorphism. Two copies of the mutated allele were also detected in the
patient with the D281Y mutation.

A recent study in Chinese patients with hepatocellular carcinoma has demonstrated
the potential significance of the R72P polymorphism in the TP53 gene as important
risk factors for cancer development (Zhu et al. 2005). The presence of two copies of
the mutant form (proline) of this genetic variant is associated with the highest risk of
cancer development and seems to be a useful prognostic marker in patients with
breast cancer. In the study performed by Tommiska et al. (2005) in 939 Finnish
familial and 888 unselected breast cancer patients, as well as 736 healthy population
controls, the genotype distribution was similar in all groups studied, suggesting no
association with breast cancer risk. However, unselected breast cancer patients with
the 72P homozygous genotype presented significantly more often with lobular
carcinoma, whereas R72 allele carriers had a significantly higher frequency of ductal
carcinomas (P = 0.004). Survival analysis showed that unselected breast cancer
patients with 72P homozygous genotype had significantly poorer survival than
patients with other genotypes (P = 0.003). This effect on survival was independent
of TP53 expression in the tumors and multivariate analysis showed that 72P
homozygous genotype was overall an independent prognostic factor. This finding of
codon 72 genotype as an independent prognostic marker for breast cancer warrants
further studies in patients with other cancer types such as BAC.
Regarding TP53 immuno-histochemistry analysis, there was a positive correlation between epithelial TP53 immunoreactivity and increasing degrees from epithelial metaplasia to dysplasia. This finding confirms those of previous studies examining TP53 immunostaining in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett esophagus (Coggi et al. 1997; Glickman et al. 2001; Weston et al. 2001). It is of interest that the correlation between TP53 protein accumulation detected by immuno-histochemical analysis and genetic mutations of the TP53 gene is poor, as determined in the present and previous studies (Coggi et al. 1997). In a recent study by Robert et al. (2005) that represents the largest examination of the immunostaining of TP53 in relation to dysplasia by a multi-institutional panel of gastrointestinal pathologists, they were unable to define a practical use of immuno-histochemical staining for TP53 in the most difficult areas of diagnosis, i.e., negative, indefinite, and low-grade dysplasia. The present study supported the potential, but undefined role of previous studies of immunostaining for TP53 in the histological assessment of dysplasia in biopsy specimens of Barrett's oesophagus.

Mutations of TP53 are clearly involved in the pathogenesis of Barrett's cancer for a subset of patients (~50%). The fact that mutations could be detected in both the dysplastic and adenocarcinoma biopsies of the two South African BAC patients studied supports the hypothesis that TP53 mutations may be a useful marker for patients at increased risk for development of invasive cancer. Absence of TP53
mutations in the BM and BD (without BAC) patients, suggest that mutations occur as relative late events in the metaplasia-dysplasia-adenocarcinoma sequence.

5.4 Management options

Early diagnosis of patients who are progressing toward adenocarcinoma at a curable asymptomatic phase has become an important clinical goal. Only about 5% of patients with Barrett’s oesophagus are diagnosed and 0.5-1% of patients with this condition per year will actually develop adenocarcinoma (Murray et al. 2003). Even though the overall incidence of BAC is low compared with most other cancers, the incidence is increasing rapidly in the Western world and the 5-year survival rate is less than 20%.

Despite the fact that endoscopic screening and surveillance for Barrett’s oesophagus is the current standard of practice, there is no conclusive evidence that the death rate from oesophageal cancer is decreased. Recommendations for screening and surveillance are based on conjecture and questionable models. Furthermore, it has not been proven that any of the treatment options, including aggressive acid suppression, anti-reflux surgery, chemoprevention and ablation therapy, affect the risk of BAC in chronic GORD.

Currently dysplasia is considered the best predictor of malignancy. However, it represents an imperfect biomarker due to sampling error and interpretation.
differences among pathologists. Data on the safety and efficacy of intensive endoscopic surveillance of patients with high-grade dysplasia is also limited (Enzinger and Mayer 2003). It is therefore envisaged that targeted genetic testing in combination with conventional methods of screening and surveillance may significantly improve management of Barrett’s oesophagus patients.

Reliable identification of low-grade dysplasia remains problematic and accurate molecular markers of low-grade dysplasia have not previously been identified. In this study c-myb analysis enhanced the diagnostic assessment of low-grade dysplasia in Barrett’s oesophagus as almost 70% of BD patients showed mRNA over expression. The role of this gene in risk assessment may be comparable to that of many functional polymorphisms that occur in the general population, but at an increased frequency in affected patients. Polymorphic variants occurring in more than 1% of the general population may have a relatively large effect on population risk but small effect on individual risk, depending on the effect on protein function, if any.

Although COX-2 and c-myc over-expression are known to occur early and progressively increasingly in the metaplasia-dysplasia-carcinoma sequence, the low rate of mRNA over-expression limits the use of these genes as biomarkers for risk stratification in the South African population. Differences in genetic background and environmental exposure may underlie these discrepancies between different population groups studied. The expression profiles of cancer-related genes may
also be influenced by functional polymorphisms such as NRAMP1/SLC11A1 analysed in this context.

The detection of pre-neoplastic changes relies almost solely on the careful histological evaluation of oesophageal biopsy specimens. However experts in the field agree that the diagnosis of low-grade dysplasia in particular is a subjective assessment, with considerable variation even among trained observers. Consequently, significant effort has been made to elucidate more objective biomarkers in the dysplasia-adenocarcinoma sequence so as to identify patients at increased risk for developing cancer. Attention has been given almost exclusively to changes in nuclear programming, especially TP53 mutations or loss of heterozygosity, p16 mutations, DNA ploidy, and G1/S DNA content in higher grade lesions. Despite promising results, reliable identification of low-grade dysplasia remains problematic.

Identification of high-grade dysplasia, intramucosal and invasive carcinoma is not the area of interpretive difficulty in the histological diagnoses of Barrett’s oesophagus. A study by Montgomery et al (2001) showed that consensus histological diagnoses of high-grade dysplasia, intra-mucosal carcinoma, or invasive carcinoma correlated with high intensity TP53 staining in mucosa. However, immuno-staining for TP53 in the histological assessment of dysplasia in Barrett’s oesophagus, is still undefined. Similarly, Robert et al (2005) reported recently on strong positive correlation of TP53 immuno-reactivity with increasing degrees of epithelial dysplasia and carcinoma. In
this study by using a collapsed dysplasia scale of 4 categories, only 4 of 19 cases (21%) of dysplasia stained positively at the +3 level.

The progression of metaplastic epithelium through dysplasia to adenocarcinoma is attributable to changes in nuclear regulation. However, histological identification of dysplasia involves assessment of cytoplasmic and nuclear alterations, such as epithelial polarity and nuclear/cytoplasmic ratio. The possibility that markers of cellular polarity such as Rab11a might assist in identifying dysplasia, especially low-grade dysplasia, has been explored only recently (Robert et al 2005). Although the identification of molecular markers of altered cellular polarity is an attractive alternative to nuclear markers of low-grade dysplasia the authors were unable to distinguish the varying degrees of dysplasia by Rab 11a immuno-histochemical analysis. Therefore it remains an attractive alternative to use c-myb mRNA over expression as adjunct to the histological diagnosis in order to define low grade dysplasia more accurately in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett’s oesophagus. This practice can simplify the differentiation of low-grade dysplasia from inflammatory atypia as well as modify the endoscopic surveillance programs of these individuals.

Based on the insight gained through this study, it is proposed that the screening of an epidemiological high-risk patient should be combined with genetic testing to more accurately identify individuals for intensified surveillance. Different genetic risk factors triggered by environmental factors may contribute to the same disease
process. Therefore the identification of the both genetic and environmental risk factors may individualise management in future. c-Myb mRNA expression was confirmed to be a valuable biomarker, as it was increasingly over expressed in the metaplasia-dysplasia-carcinoma sequence. The fact that almost 70% of BO patients with low-grade dysplasia showed c-myb over expression, allows for the identification of a subset of BO necessitating intensified surveillance and timeous intervention. Therefore, the patients with high c-myb mRNA expression included in this study will be followed up within the setting of an endoscopic surveillance program. An improved understanding of the molecular biology of Barrett’s oesophagus would allow improved diagnosis, prognosis and therapy.

A proposed model for future screening and surveillance of Barrett’s oesophagus in the local patient population is depicted in the flow diagram below. A subset of patients with high c-myb mRNA expression could also be subjected to TP53 mutation detection for improved risk stratification and prognostication.
In recent years it became evident that biologic markers of propensity to progress at discrete stages of carcinogenesis could help in selecting therapeutic modalities. It is envisaged that genetic studies will in future form an important part of treatment choice, similar to assessment of HER2/neu now routinely used to pre-determine response to Herceptin treatment in breast cancer (Montemurro et al. 2005).

In conclusion, this study contributed to a much greater understanding of the molecular alterations accompanying the metaplasia-dysplasia-adenocarcinoma sequence in Barrett’s oesophagus in our study population. c-Myb mRNA overexpression has shown to be an important biomarker in this context and can serve as an adjunct to the standard practice of regular endoscopic surveillance for patients with Barrett’s metaplasia, as it increases progressively through the metaplasia-
dysplasia-adenocarcinoma sequence. Data to support the efficacy of endoscopic surveillance is lacking worldwide and even more so in the South African context. A combined approach may contribute to more targeted screening, aimed at high-risk sub-groups, thereby improving the effectiveness of this practice. It may also prove to be useful in differentiating between an adenocarcinoma arising from the OGJ and a true Barrett’s-associated adenocarcinoma. Given the considerable inter-observer variation in histopathology, the finding of increased c-myb mRNA in Barrett’s tissue may enhance the efficacy of dysplasia as a biomarker with increased risk of progression to adenocarcinoma and lead to more intensified surveillance strategies.

The highly significant over expression of COX-2 and c-myc mRNA in the metaplasia-dysplasia-adenocarcinoma sequence as reported in the literature, could not be reproduced in the local population. However, when over expressed this may contribute to more effective management strategies. Similarly, detection of TP53 mutations is indicative of a poor prognosis and DNA sequencing may be useful to identify Barrett’s patients with low-grade dysplasia who are at high risk of progression to carcinoma.

It is well known that different genetic backgrounds, gene-gene and gene-environment interaction may explain different disease patterns in diverse populations. With the established role of iron in the pathogenesis of oesophageal adenocarcinoma, patients with a 2/2 and 2/3 genotype of the iron-transporter gene NRAMP1/SLC11A1 [5´-(GT)_n repeat polymorphism in the promoter region] combined
with over expression of c-myc mRNA may be at increased risk of oesophageal adenocarcinoma due to gene-gene interaction.

5.6 Future Prospects

The current practice of endoscopic screening and surveillance of patients with Barrett's oesophagus has clear limitations. In the future, it is likely that patients who have Barrett's oesophagus will be stratified further according to their risk for progression to invasive carcinoma, allowing for the development of more rational surveillance programmes. Models incorporating objective scores of epidemiologic risk factors (i.e. age, sex, and body-mass index), reflux symptoms and endoscopic and histological findings, would likely include panels of biomarkers for further stratification of patients in low, intermediate, or high risk.

No biomarker has yet emerged that is superior to the histological identification of dysplasia and it remains to be seen whether other novel approaches, such as high-throughput hypermethylation analyses or transcriptional profiling using microarray technology can prospectively identify molecularly distinct, but histologically indistinguishable high-risk groups of BM patients. The real-time PCR technology applied in this study, displaying high inter- and intra-assay reproducibility, is considered the ideal method for mRNA expression analysis of highly selected clinically useful biomarkers, as it provides statistically confident results.
In the South African context, it was confirmed by real-time PCR that c-myb mRNA expression in Barrett’s mucosa may be a valuable adjuvant to the screening and surveillance of these patients. c-Myb can be considered a clinically useful biomarker for the diagnosis of malignancy in patients with Barrett’s oesophagus (Brabender et al. 2001), as upregulation is not a simply a function of generalised inflammation but a specific effect leading to hyperproliferation (Ramsay et al. 1992). Additionally, TP53 mutation detection may help to identify a subset of Barrett’s patients will progress to cancer development. c-Myb mRNA expression needs to be studied prospectively in Barrett’s oesophagus patients to determine the impact of genetic testing on clinical outcome. In future analysis of TP53 mutation status should be combined with c-myb mRNA expression to evaluate the predictive value in terms of progression. Ultimately, the use of molecular-genetic tests that can objectively identify patients with Barrett’s oesophagus at increased risk of carcinoma may lead to improved survival rates.

Genetic testing could possibly be combined with improved sampling techniques during endoscopic surveillance programmes, such as the use of brush cytology. Although not freely advocated, the latter has the potential to sample tissues more widely than endoscopic biopsy and may prove to be more cost-effective.

Finally, biomarker discovery programs are increasingly inextricably linked to the search for novel targeted treatments and chemoprevention in Barrett’s patients. It
seems unlikely that future applications of genetic testing would replace existing clinical management protocols relying on subjective histological evaluation of mucosal biopsy. However, application of clinically useful makers such as c-myb could serve as an important adjunct to identify patients with low-grade dysplasia for intensified surveillance intervals.
References


177. van Rensburg CJ, de Villiers JNP and Kotze MJ. Development of a multi-gene real-time (RT-) PCR assay to study and monitor disease progression in the Barrett’s metaplasia (BM)-dysplasia carcinoma sequence. SAMJ 2004;94:680 [abstract].


181. Weston AP, Badr AS, Hassanenein RS. Prospective multivariate analysis of clinical, endoscopic and histologic factors predictive of the development of


ADDENDUM:

Abstracts of this work were presented at the following meetings:

1) The Congress of the South African Gastroenterology Society, August 2004, Cape Town:


   b) van Rensburg CJ, de Villiers JNP and Kotze MJ. Development of a multi-gene real-time (RT-) PCR assay to study and monitor disease progression in the Barrett’s metaplasia (BM)-dysplasia carcinoma sequence. SAMJ 2004 Aug;94(8)675.

2) Digestive Disease Week® and the 106th Annual Meeting of the American Gastroenterology Association, 14 – 19 May 2005, Chicago, IL:


3) World Congress of Gastroenterology, 10 – 14 September 2005, Montreal, Canada:
Identification of clinically-informative biomarkers for risk stratification within the spectrum of gastro-oesophageal reflux disease in the South African population.

4) 13th United European Gastroenterology Week, 15 – 19 October 2005, Copenhagen, Denmark:


BARRETT’S DATA SHEET

PATIENT DEMOGRAPHICS
ENTRY DATE: ..............................................
STUDY NUMBER ...........................................
SUBJECT INITIALS:........................................
FOLDER NUMBER: ........................................ D.O.B…………AGE:....................
RACE:................................................................SEX: ....................

PATIENT CHARACTERISTICS:
HEIGHT: .......................... WEIGHT:............. BMI:........
SMOKING:Y/N NO. OF CIGARETTES/DAY: .................. DURATION:........................
ALCOHOL: Y/N UNITS/WEEK:.................. DURATION: ..............................

PATIENT HISTORY
CO-MORBID DISEASE:  ......................................................................................................
CONCOMMITANT MEDS: ......................................................................................................
FAMILY HISTORY OF CA: ........................................................................................................
HRT: Y/N TYPE:.............................. DURATION..................................................
CONTRACEPTION/HYSTERECTOMY: ....................................................................................
SELF-INDUCED VOMITING:Y/N
METHOD:.......................... FREQUENCY:.................. DURATION..................................
MINERAL SUPPLEMENTS: Y/N TYPE:.......................... DURATION..................................
BLOOD DONATION: ............................................. DURATION................................

PATIENT SYMPTOMS
REFLUX: Y/N AGE OF ONSET .......................... DURATION............................
DYSPHAGIA: Y/N ODYNOPHAGIA: Y/N

SEVERITY FREQUENCY OF HEARTBURN
NONE NONE
MILD OCCASIONAL BRIEF EPISODES
MODERATE FREQUENT > 2/WEEK
SEVERE DAILY, PAIN INTERFERES WITH SLEEP, WORK, SOCIAL ACTIVITIES

ENDOSCOPY/OESOPHAGITIS
NORMAL
OESOPHAGITIS..........................................................................................................
BARRETT'S: Y/N
LENGTH OF BARRETT'S..................................................................................
HISTOLOGY:........................................................................................................
FOLLOW-UP:.......................................................................................................
KONSEP INLIGTINGS- EN TOESTEMMING DOKUMENT

PROJEKTITEL
Molekulêre ondersoek na die rol van yster in gesondheid en siektetoestande

VERKLARING DEUR DEELNEMER
Ek/my verteenwoordiger, die ondergetekende,.................................ID nommer............... Adres................................................................................................................................. Tel nommer...........................................(w).................................................................(h) Gesondheidstoestand.........................._______________________________________________________________________________

A  **Ek/my verteenwoordiger bevestig dat:**
1. Ek uitgenooi is om deel te neem aan bogemelde navorsingsprojek wat deur navorsers verbone aan die Universiteit van Stellenbosch onderneem word.
2. Daar aan my verduidelik is dat
   • Die projek onderneem word om die die rol van yster (gebrek- of -oormaat) te ondersoek in algemene gesondheidsprobleme soos kanker, hartsiekte, neurologiese siektes en infektiewe siektes. Die hoofdoel van die ondersoek is om molekulêre toetse te ontwikkel vir risikobepaling t.o.v. siektes in die algemeen, waar ysterstatus 'n rol mag speel.
   • Indien ek deelneem aan die projek, 20-50 ml bloed (2-3 eetlepels) versamel sal word vir analise van DNA/RNA en plasma/serum. In geselekteerde gevalle mag addisionele navorsingsmateriaal geneem word, nadat die rede hiervoor verduidelik is en die nodige toestemming verkry is.
   • Ek gewaarsku is dat die proses van bloedtrek effense ongemak mag meebring, wat gepaard gaan met bloeding waar die naald die vel binnedring.
   • Daar verder aan my verduidelik is dat deelname aan die projek sal bydra tot die uitbouing van mediese kennis. Die ontwikkeling van 'n molekulêre toets kan lei tot vroeë opsporing en voorkomende behandeling van algemene siektetoestande waar yster 'n rol mag speel.
   • Ek meegedeel is dat die inligting wat ingewin word as vertroulik beskou sal word, maar wel aangewend sal word vir publikasies in vaktydskrifte en tesisse.
   • Ek meegedeel is dat ek mag weier om deel te neem aan hierdie projek, asook dat ek te enige tyd deelname daaraan mag staak sonder benadeling van toekomstige behandeling.
   • Ek verstaan dat, aangesien hierdie navorsing is, daar onsekerheid kan wees oor die betekenis van genetiese veranderinge wat opgespoor mag word en die assosiasie daarvan met siekte- of gesondheidstoestande, en daarom sal ek nie in kennis gestel word van die bevindings nie. Indien die verandering egter voorheen beskryf is of aanvaar kan word as siekte-verwant, wil ek ingelig word en raadgewing ontvang van 'n gekwalifiseerde persoon.

JA  NEE

B  **Ek/verteenwoordiger stem hiermee vrywillig in** om deel te neem aan die bogemelde projek

Geteken/bevestig te ..............................................................op..................................................

.............................................................. ..............................................................
Handtekening van deelnemer / Getuie
verteenwoordiger
SUBJECT INFORMATION AND CONSENT FORM

TITLE OF PROJECT:
Molecular investigation into the role of iron in health and disease

DECLARATION BY PARTICIPANT
I/my representative, the undersigned, ………………………..……ID……………………..
Address…………………………………………………………………………….………..
Tel number……………………………………(w)…………………………………………(h)

Health status………………………………………………………………………………

A. I/my representative declare that
1. I was invited to participate in the above mentioned research project
2. It has been explained to me that
   • The project is being undertaken to investigate the role of iron (overload or deficiency) in common health problems such as cancer, heart disease, neurological diseases and infectious diseases. The overall aim of the investigation is to develop molecular tests for risk assessment for diseases where iron status may be involved.
   • If I participate in the project, 20-50 ml of blood (approximately 2-3 Tablespoons) may be collected from the arm for analysis of DNA/RNA and plasma/serum. Additional research material may be obtained in selected cases, after an explanation why this is necessary and consent have been given.
   • I have been warned that the drawing of blood may result in slight discomfort, which can be coupled with bleeding where the needle pierces the skin.
   • It has been explained to me that participation in this project will result in the broadening of medical knowledge. The development of a molecular test can facilitate early detection and preventive treatment of common diseases involving iron.
   • I have been informed that all information collected will be treated confidentially. The results will be used for publication in subject matter magazines and for a thesis, without revealing the identity of any individual.
   • I have been told that participation is voluntary and that I may refuse to participate in this project, and that I may also at any time withdraw my participation from the project without penalising future treatment.
   • I understand that, this being research, the meaning of genetic alterations that may be detected and its association with health or disease may be uncertain and I will not be informed of such findings. However, if the alteration has been reported or is generally accepted as disease-causing, I want to be informed and would like to be counselled by a qualified person.

YES ☐ NO ☐

B. I/my representative voluntarily agree(s) to participate in the above mentioned project

Signed/affirmed at …………………………………………… on ……………………………...

…………………………………………………………………………………………………
Signature of participant / Witness
representative