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Mitochondrial Dysfunction during Disease Progression in Barrett's Oesophagus

A dissertation submitted to the University of Dublin

for the degree of Doctor in Philosophy

by

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April 2014

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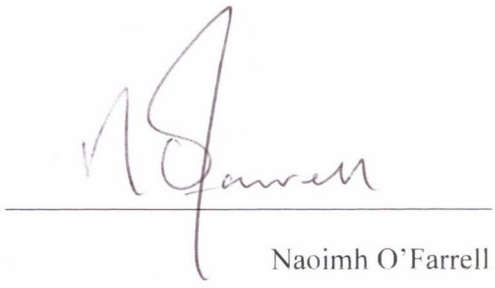

Naoimh O'Farrell

April 2014

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Thesis Abstract

Introduction: Barrett's oesophagus is the leading risk factor for oesophageal adenocarcinoma. However, it is difficult to identify patients at risk of disease progression. Mitochondria are highly susceptible to mutations due to high levels of reactive oxygen species (ROS) coupled with low levels of DNA repair. The timing and functional relevance of mitochondria mutagenesis and dysfunction during Barrett's oesophagus progression is unknown.

Methods: An *in-vitro* model representing the Barrett's oesophagus disease sequence of normal squamous epithelium (HET1A), intestinal metaplasia (QH), dysplasia (Go), and oesophageal adenocarcinoma (OE33) was used. Random mitochondrial mutations and mitochondrial function were assessed. Using *in-vivo* and *ex-vivo* models, random mutations, proxy markers of changes in mitochondrial biogenesis and the expression of the oxidative stress gene CYGB, and DNA repair genes, BRCA-1, BRCA-2 and ATM, were examined. The importance of 8-oxo-dG, an oxidative stress marker, was evaluated in patients undergoing Barrett's surveillance, to stratify cancer progressors.

Results: *In vitro;* Metaplasia had increased levels of ROS ($p<0.005$) and random mitochondrial mutations ($p<0.05$) compared with all other stages of the disease sequence. *Ex-vivo;* Metaplastic explants secreted higher levels of cytochrome c ($p=0.003$), SMAC/Diablo ($p=0.008$) and inflammatory cytokines ($p<0.05$) compared with surrounding normal tissue. *In-vivo;* Barrett's patients separated into groups with significantly lower and higher frequencies of random mutations ($p=0.0043$). CYGB expression was significantly increased in metaplasia compared with dysplasia ($p=0.01$) and adenocarcinoma ($p=0.02$). BRCA-1, BRCA-2 and ATM were significantly decreased in intestinal metaplasia compared with dysplasia and oesophageal adenocarcinoma ($p<0.05$). Longitudinal analysis demonstrated significantly ($p<0.05$) decreased levels of 8-oxo-dG in Barrett's progressing to cancer. Low levels of 8-oxo-dG significantly correlated with increased frequencies of random mitochondrial point mutations.

Conclusions: We have demonstrated increased random mitochondrial point mutations, oxidative stress and inflammation are an early event in the Barrett's disease sequence.

Patients with progressive disease demonstrated reduced oxidative stress levels. Reduced oxidative stress levels inversely correlated with random mutations. Perhaps, alterations in energy metabolism and DNA repair may enable the progression of Barrett's oesophagus, through prevention of normal mitochondrial apoptotic activity, initiated by increased ROS and mitochondrial instability in a normal cellular environment.

Abbreviations

A	adenine
ACG	American College of Gastroenterology
ADP	adenosine diphosphate
AFI	autofluorescence imaging
AGA	American Gastroenterological Association
AJCC	American Joint Commission on Cancer
AIF	human apoptosis inducing factor
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATM	ataxia telangiectasia mutated
ATP	adenosine 5′-triphosphate
BCP	bromo-3-chloro-propane
BEBM	bronchial epithelial cell basal media
bFGF	basic fibroblast growth factor
BRCA-1	breast cancer susceptibility gene-1
BRCA-2	breast cancer susceptibility gene-2
BSA	bovine serum albumin
BSG	British Society of Gastroenterology
C	cytosine
cDNA	complementary DNA
CD3	cluster of differentiation 3
CIN	cervical intraepithelial neoplasia
CK8	cytokeratin 8
CoA	acetyl co-enzyme A
CO_2	carbon dioxide
COX-1	cyclooxygenase-1
Ct	threshold cycle
CYGB	cytoglobin
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate

DCA	deoxycholic acid
dCTP	deoxycytidine triphosphate
DCF	2, 7 dichlorofluorescein
dGTP	deoxyguanosine triphosphate
D-loop	displacement loop
DMOG	dimethyloxalylglycine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetra acetic acid
EGCG	epigallocatechin-3-gallate
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMR	endoscopic mucosal resection
ER	endoplasmic reticulum
$FADH_2$	flavin adenine dinucleotide
FCS	foetal calf serum
FOX M1	forkhead box M1
G	guanine
GLRX2	glutaredoxin 2
Go	high grade dysplasia cell line
GORD	gastro-oesophageal reflux disease
H	hydrogen
HCl	hydrochloric acid
HER-2	human epidermal growth factor-2
HET1A	normal squamous epithelium oesophageal cell line
HGD	high grade dysplasia
HIF-1α	hypoxia-inducible factor 1α
HPP-1	hyperpigmentation, progressive-1 gene
HtrA2	human temperature requirement factor A2
H_2O	water
H_2O_2	hydrogen peroxide

H_2SO_4	sulphuric acid
IL-1β	interleukin-1beta
IL-6	interleukin-6
IL-8	interleukin-8
IM	intestinal metaplasia
IMC	intra-mucosal carcinoma
IND	indefinite for dysplasia
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
LGD	low-grade dysplasia
LOH	loss of heterozygosity
MAM	mitochondria-associated ER-membrane
$MgCl_2$	magnesium chloride
MMP	mitochondrial membrane potential
mRNA	messenger RNA
MUTYH	mutY homolog
NAC	N-acetylcysteine
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa beta
NO	nitric oxide
OE33	Barrett's derived oesophageal adenocarcinoma cell line
OGD	oesophagogastroduodenoscopy
OGG-1	8-oxoguanine DNA glycosylase
OH	hydroxyl
O_2	oxygen
P	phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
Pol-δ	polymerase-δ
Pol-ε	polymerase-ε

PP	pyrophosphate
PPI	proton pump inhibitor
PSA	prostate-specific antigen
QH	intestinal metaplasia cell line
qPCR	quantitative real-time PCR
RFA	radiofrequency ablation
RMC	random mutation capture
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
RUN X3	runt-related transcription factor 3 gene
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SIM	specialised intestinal metaplasia
SMAC/Diablo	second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
SOD	superoxide dismutase
T	thymine
TIM	translocase of the inner membrane
TMA	tissue microarray
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	tumour necrosis factor-α
VEGF	vascular endothelial growth factor
WT	wild type
4-HNE	4-hydroxy-2-nonenal
8-oxo-dG	8-oxo-7, 8-dihydro-2'-deoxyguanine

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Presentations

Oral presentations

International Oral Presentations (presenting author)

"Antioxidants play a potential role in reversing deoxycholic acid induced mitochondrial instability during the progression of Barrett's Oesophagus". October 2012, 13th World Congress of the International Society for Diseases of the Esophagus, Venice. Awarded **Best Abstract Oral Presentation**

"Deoxycholic acid drives mitochondrial instability during progression of Barrett's oesophagus to oesophageal adenocarcinoma". January 2012, Society of Academic and Research Surgery (SARS), Nottingham, United Kingdom

"The reliability of EUS in differentiating tumour stage in oesophageal carcinoma". January 2012, Society of Academic and Research Surgery (SARS), Nottingham, United Kingdom

"Deoxycholic acid drives mitochondrial instability during progression of Barrett's oesophagus to oesophageal adenocarcinoma". Prize session, November 2011, European Society of Esophagology, Newcastle, United Kingdom

National Oral Presentations (presenting author)

"Antioxidants play a potential role in reversing deoxycholic acid induced mitochondrial instability during the progression of Barrett's Oesophagus". Awarded the **William O'Keefe Prize**, October 2012, XXII Waterford Surgical October Meeting

"Antioxidants are a potential therapy for reversing deoxycholic acid induced mitochondrial instability in the progression of Barrett's oesophagus". September 2012, 3^{7th} Sir Peter Freyer Surgical Symposium, Galway

"Greater Prognostic Value of Actual Compared with Close (<1mm) Circumferential Resection Margin Involvement in the Pathological Assessment of Oesophageal and Junctional Cancer". September 2012, 37th Sir Peter Freyer Surgical Symposium, Galway

"Deoxycholic acid drives mitochondrial instability during progression of Barrett's oesophagus to oesophageal adenocarcinoma". Plenary session, March 2012, Sylvester O'Halloran meeting, Limerick

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Poster Presentations

International Poster Presentations (presenting author)

"Mitochondrial instability is elevated in Barrett's oesophagus and dimethyloxalylglycine has a potential role in reversing this dysfunction". June 2012, AUGIS, Liverpool, United Kingdom

"Superiority of actual compared with close (<1mm) circumferential resection margin involvement in the pathological staging of oesophageal and junctional cancer". June 2012, AUGIS, Liverpool, United Kingdom

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William O'Keefe Prize, October 2012, XXII Waterford Surgical October Meeting, Waterford, Ireland

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Chapter 1: Introduction

1.1 BARRETT'S OESOPHAGUS AND OESOPHAGEAL CANCER

1.1.1 Oesophageal cancer prognosis and changing epidemiological trends

Oesophageal cancer is one of the most rapidly increasing malignancies in the West (Jankowski, Perry et al. 2000), steadily increasing at a rate of 5-10% per annum since the mid-1970s (Blot, Devesa et al. 1991). Worldwide, the oesophagus is the eight most common site for cancer and oesophageal carcinoma is the sixth most common cause of cancer related mortality, emphasising the lethal nature of this disease (Ferlay, Shin et al. 2010). The overall 5-year survival rates are low at approximately 14% (Enzinger and Mayer 2003). However, in the past decade our Centre has demonstrated improved survival rates to approximately 40% to 50% in patients treated with curative intent (Reynolds, Donohoe et al. 2012); possibly reflecting an increase in early tumours, optimised staging and improved surgical and pathologic standards.

Oesophageal cancer has two main histological forms. Worldwide squamous cell carcinoma is the leading type, representing 70% of all cases (Parkin, Bray et al. 2001). However, the last several decades have seen a change in the histological trend with adenocarcinoma now the leading sub-type in the West (Blot, Devesa et al. 1991). This change in histology has been paralleled by a change in the location of these tumours, with distal oesophageal adenocarcinomas now forming the bulk of oesophageal malignancies in Western society. Oesophageal adenocarcinoma remains highest among white males, with a staggering increase of more than 800% in its incidence occurring over the last 40 years (DeMeester 2006). In the Irish population, data supplied by the National Cancer Registry of Ireland has shown a steady increase in the incidence of adenocarcinomas, particularly in males, while the incidence of squamous cell carcinomas has remained static (Figure 1.1).

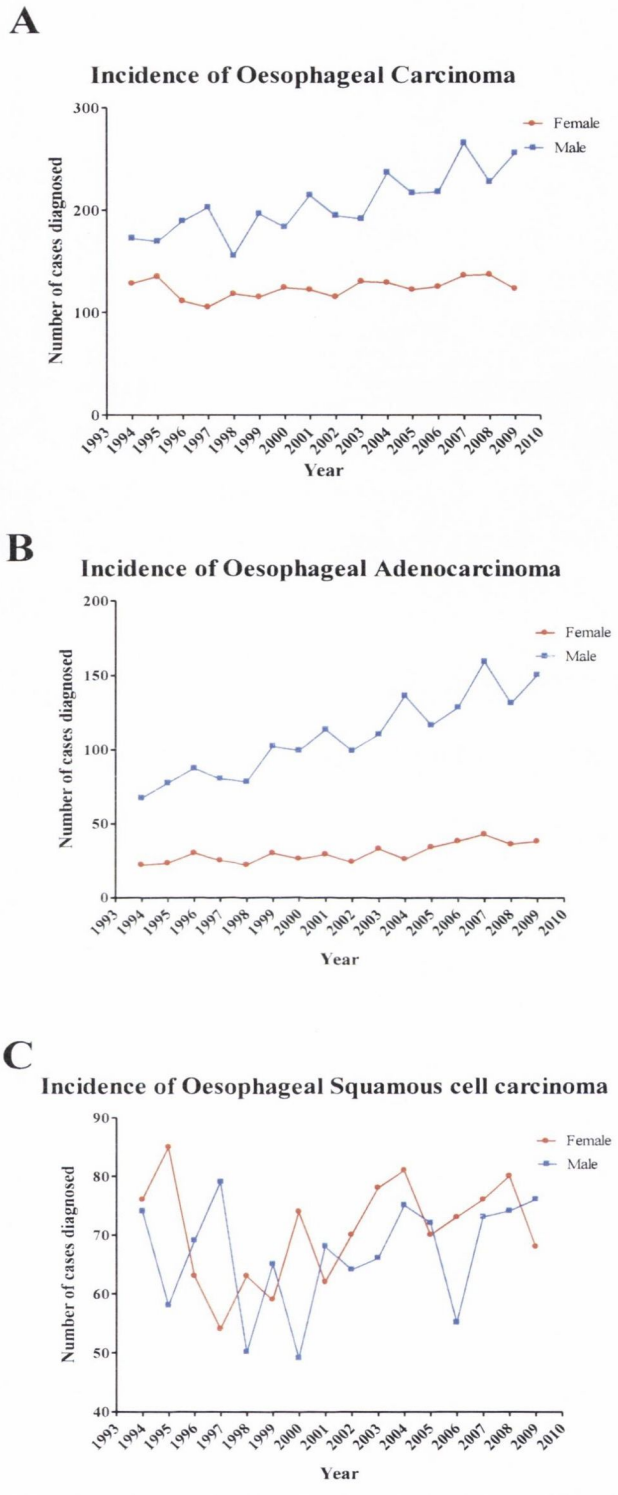


Figure 1.1: Changing trends in the incidence of oesophageal cancer in Ireland (1994-2009). (A) Overall the incidence of oesophageal cancer has increased in the Irish population. (B) The incidence of oesophageal adenocarcinoma has significantly increased since 1994, predominantly in males compared to females ($p<0.0001$). Overall the incidence of oesophageal adenocarcinoma has increased by 110% during this time period. (C) The rate of oesophageal squamous cell carcinoma has remained static in the Irish population throughout this time-frame. (Data provided by the National Cancer Registry of Ireland http://www.ncri.ie).

1.1.2 Barrett's oesophagus

Barrett's oesophagus is the leading risk factor for oesophageal adenocarcinoma (Cameron, Ott et al. 1985, Spechler and Goyal 1986). It is a premalignant condition, where normal squamous epithelium lining the distal oesophagus undergoes metaplastic transformation, typically in response to reflux disease. It is associated with a 30 to 50-fold increased risk of malignant conversion (Stein and Siewert 1993, O'Connor, Falk et al. 1999, Solaymani-Dodaran, Logan et al. 2004, Sharma, Falk et al. 2006) and follows the classic metaplasia-dysplasia-adenocarcinoma sequence. Overall, Barrett's oesophagus is associated with an annual risk of cancer progression of 0.12% to 1% (Sharma, Falk et al. 2006, Hvid-Jensen, Pedersen et al. 2011). Although many theories exist around this disease development (Figure 1.2), the exact pathogenesis remains unknown.

The current definition for Barrett's oesophagus proposed by the American Gastroenterological Association (AGA) is *"the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal oesophagus"* (Sharma, McQuaid et al. 2004). The AGA and the American College of Gastroenterology (ACG), agree that confirmation of intestinal metaplasia should be included as part of the Barrett's oesophagus standard definition; with the ACG defining Barrett's oesophagus as *"a change in the distal oesophageal epithelium of any length that can be recognised as columnar type mucosa at endoscopy and is confirmed to have intestinal metaplasia by biopsy of the tubular oesophagus"* (Wang, Sampliner et al. 2008). The British Society of Gastroenterology (BSG), however; defines Barrett's oesophagus as an apparent area above the oesophagogastric junction, supported by the finding of a columnar lined oesophagus on histology (Playford 2006). The British definition does not see intestinal metaplasia as mandatory for making a diagnosis of Barrett's oesophagus. While the BSG acknowledges that oesophageal adenocarcinoma usually originates from a segment containing intestinal metaplasia, its argument for excluding it from its definition is based on a study by Shepard *et al.* which demonstrated that *"if a sufficient number of biopsies are taken over an adequate period of time, intestinal metaplasia can usually be demonstrated (in the majority of these patients)"* (Shepard 1999). Thus, the risk of sampling errors at the initial endoscopy may result in missing

intestinal metaplasia, and therefore the confirmation of intestinal metaplasia is not seen as compulsory. However, it does appear that the BSG regard the intestinal metaplasia cohort as the at risk group that must not be missed. The leading gastroenterology groups in America recognise that the vast majority of adenocarcinomas of the oesophagus are accompanied by intestinal metaplasia and oesophageal adenocarcinoma is unlikely to occur in its absence (Skinner, Walther et al. 1983, Rosenberg, Budev et al. 1985, Paraf, Flejou et al. 1995, Wang, Sampliner et al. 2008). Three types of columnar epithelium may be found in the setting of Barrett's oesophagus: (1) gastric-fundic type, (2) cardiac type and (3) specialised intestinal type including goblet cells (Booth and Thompson 2012). However, it is, the third, specialised intestinal metaplasia (SIM), type which is associated with malignant potential (Yousef, Cardwell et al. 2008, Sikkema, de Jonge et al. 2010, American Gastroenterological, Spechler et al. 2011). It is precisely for this reason that both American societies currently recommend that while columnar-type mucosa may be identified during endoscopy, it is the combined presence of SIM verified with histology which equates to a diagnosis of Barrett's oesophagus (Wang, Sampliner et al. 2008, American Gastroenterological, Spechler et al. 2011, Booth and Thompson 2012). The BSG, AGA and ACG all acknowledge the at risk patient cohort is the SIM group, thus, for the purpose of this PhD, specialised intestinal metaplasia will be the variant of interest when describing the earliest stages of the Barrett's disease sequence.

The incidence of Barrett's oesophagus, like oesophageal adenocarcinoma, is steadily increasing, and is also more prevalent in the white, male population (Winters, Spurling et al. 1987, Johnston, Hammond et al. 1996). The initial metaplastic stage is the most common form of the Barrett's spectrum and diagnosis of this, as stated, is made by a combination of salmon-pink mucosa present at endoscopy and confirmation of specialised intestinal metaplasia containing goblet cells at histology. It is universally agreed that four quadrant biopsies must be taken every 2cm at oesophagogastroduodenoscopy (OGD) (Playford 2006). During endoscopic examination, the length of the Barrett's lesion is measured by the application of the standardised Prague classification system of circumferential and maximal length, and Barrett's is subsequently categorised as either short- or long-segment disease. Short segment Barrett's is the more prevalent phenotype and it defines specialised intestinal

metaplasia less than three centimetres long (Jankowski, Wright et al. 1999). Long-segment Barrett's oesophagus, $\geq$ 3cm, has greater malignant potential, with 65% of oesophageal adenocarcinomas arising from long-segment lesions (Jankowski, Wright et al. 1999).

While Barrett's oesophagus remains the leading known risk factor for oesophageal adenocarcinoma, with the exception of dysplastic disease, it is difficult to identify patients at greatest risk of cancer conversion. Therefore, the current standard of care for Barrett's oesophagus is to monitor all patients in endoscopic surveillance programmes.

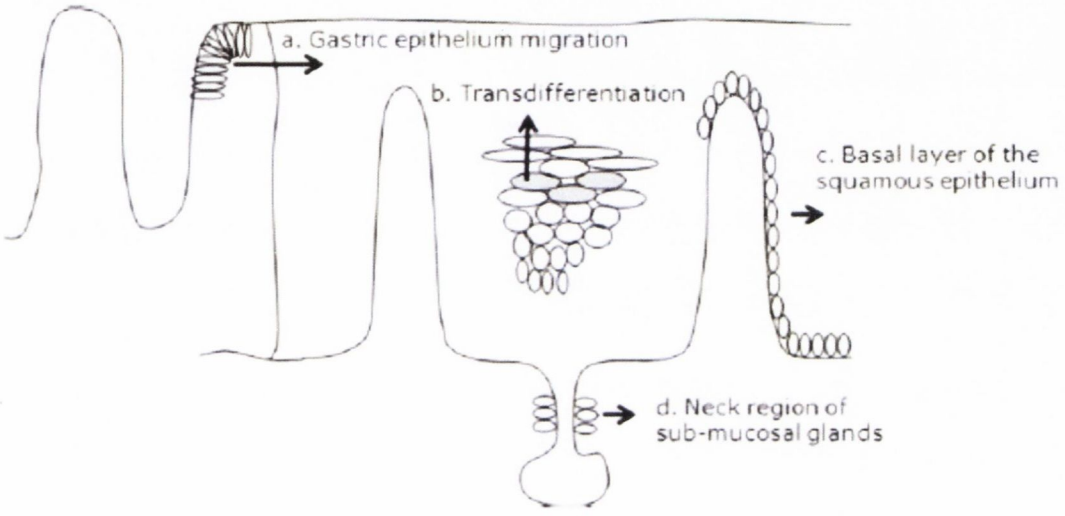


Figure 1.2: Proposed cellular origins of Barrett's oesophagus: (**a**) columnar cells in the gastro-oesophageal junction epithelium migrate upwards, colonising the distal oesophagus. (**b**) The process of transdifferentiation, where there is the irreversible switch in the phenotype of adult epithelial cells already committed to differentiation. Multipotent stem cells located either (**c**) in the basal layer of the normal squamous oesophagus or (**d**) in the neck of the oesophageal submucosal gland ducts are reprogrammed to differentiate in a columnar phenotype after chronic exposure to reflux. Image reproduced from Barbera *et al.* (Barbera and Fitzgerald 2010).

1.1.3 Dysplastic Barrett's oesophagus

1.1.3.1 Low grade dysplasia

Cancer development does not tend to occur directly from non-dysplastic disease (Jankowski, Wright et al. 1999). While it is difficult to predict adenocarcinoma progression in Barrett's metaplasia, low-grade dysplasia (LGD) carries a higher risk of cancer development (Curvers, ten Kate et al. 2010). Hvid-Jensen *et al.* demonstrated LGD had a 5-fold increased risk of progression to oesophageal adenocarcinoma compared to intestinal metaplasia (Figure 1.3), with the absolute and relative risk increasing substantially when dysplasia was present at the first surveillance endoscopy (Hvid-Jensen, Pedersen et al. 2011).

An initial diagnosis with LGD significantly increases the potential for malignant conversion; however, the incidence of cancer progression is very variable and ranges in the literature from less than 5% to 28% (Skacel, Petras et al. 2000, Basu, Pick et al. 2004, Sharma, Falk et al. 2006, Wani, Mathur et al. 2009). This variation is probably a reflection of inconsistencies observed in the diagnosis of LGD between specialist gastrointestinal pathologists (Reid, Haggitt et al. 1988, Skacel, Petras et al. 2000, Montgomery, Bronner et al. 2001). Studies focussing on the reproducibility of the grading criteria for dysplastic disease, confirmed high levels of inter-observer reproducibility at either ends of the Barrett's oesophagus spectrum (i.e. intestinal metaplasia versus high grade dysplasia [HGD]/intra-mucosal carcinoma [IMC]), but reported poorer reproducibility when distinguishing LGD (Montgomery, Bronner et al. 2001). The 1988 consensus criteria for grading dysplasia in Barrett's oesophagus defined LGD as the presence of architectural and cytologic changes that indicated neoplastic transformation of the columnar epithelium (Reid, Haggitt et al. 1988). These changes of glandular budding, branching and crowding or abnormal nuclear features such as varying shape and size, were less severe compared to HGD, and the quantity of these abnormalities stratified LGD and HGD from one another. These subtle changes were highly subjective (Montgomery, Bronner et al. 2001). Skacel *et al.* noted however, where a consensus agreement was reached by two specialised gastrointestinal pathologists on the diagnosis of LGD there was a definite increased risk of HGD/cancer progression, while lack of a consensus between two pathologists was not associated with any malignant change (Skacel, Petras et al. 2000). Regardless of these difficulties

in diagnosing early dysplasia, there is consistently higher malignant potential with early dysplastic changes compared to non-dysplastic disease, highlighting the importance for increased surveillance in this patient group.

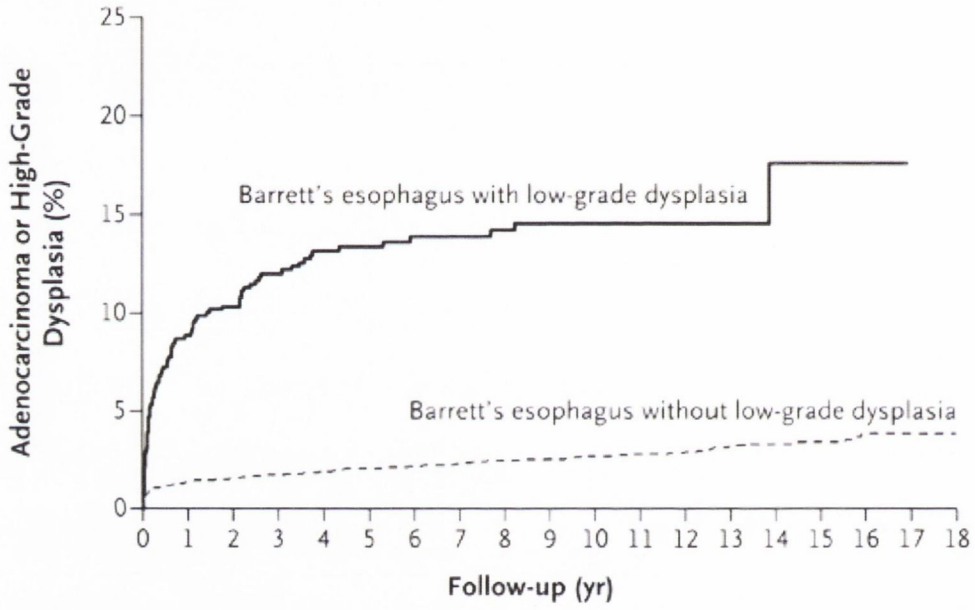


Figure 1.3: Risk of progression in SIM and LGD patients. The incidence of progression to HGD and oesophageal adenocarcinoma is significantly increased in patients with LGD compared to those with uncomplicated metaplastic Barrett's oesophagus. Image reproduced from Hvid-Jensen *et al.* (Hvid-Jensen, Pedersen et al. 2011)

1.1.3.2 High grade dysplasia

HGD is now classified as part of the cancer spectrum according to the AJCC 7th edition guidelines (Edge 2010) (Table 1.1). HGD and IMC are early malignant lesions and the introduction of Barrett's oesophagus surveillance programmes have enabled increased detection of such early tumours (Rice, Blackstone et al. 2001). In this Centre, we have shown an exponential increase in the incidence of early oesophageal cancer, in part probably related to increased Barrett's surveillance (**O'Farrell**, Reynolds et al. 2012) (Figure 1.4).

Table 1.1: American Joint Commission on Cancer (AJCC) staging system for oesophageal cancer: TNM definitions

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	**Carcinoma in situ/ High grade dysplasia**
T1	Tumour invades: T1a: invades lamina propria or muscularis mucosae (IMC) T1b: invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades adjacent structures 4a: invades resectable structures including pericardium, pleura or diaphragm 4b: Unresectable tumour invading other adjacent structures e.g. aorta, trachea, vertebrae etc.
Regional lymph nodes (N)	
NX	regional lymph node metastasis cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis: 1-2 nodes involved
N2	Regional lymph node metastasis: 3-6 nodes involved
N3	Regional lymph node metastasis: >7 nodes involved
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

HGD is now graded as an early oesophageal cancer and denoted by the abbreviation Tis (highlighted in bold) in the 7th edition AJCC guidelines.

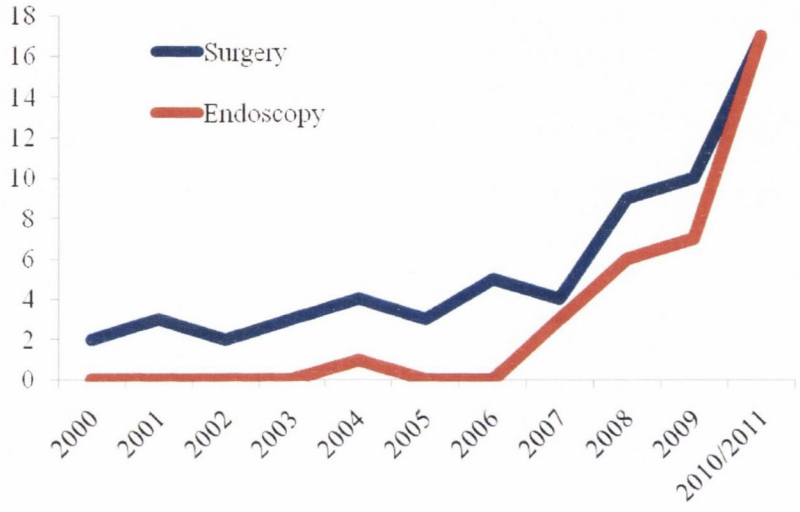


Figure 1.4: Changing trends in the incidence of early oesophageal cancer. Exponential rise in the incidence of early oesophageal malignancies (HGD and T1tumours) treated over the past decade by either oesophagectomy or endoscopic techniques at the Oesophageal and Gastric Centre at St. James's Hospital, Dublin, a high volume centre and the largest in Ireland (**O'Farrell**, Reynolds et al. 2012).

1.1.4 Barrett's oesophagus surveillance programmes

Barrett's oesophagus is an ideal condition to undergo interval surveillance, as it follows a stepwise progression of metaplasia-dysplasia-adenocarcinoma, and it is thought that cancer development does not tend to occur directly from non-dysplastic disease (Jankowski, Wright et al. 1999). For this reason Barrett's surveillance programmes have become established as common practice in Western society. Currently surveillance offers the best cancer-prevention strategy, enabling early cancer detection and cure (Peters, Clark et al. 1994, van Sandick, van Lanschot et al. 1998). Follow-up is dependent on histological findings, as non-dysplastic disease is associated with a low risk of cancer conversion, compared to patients with confirmed dysplasia (Table 1.2).

Recent reports, however, have questioned the necessity for rigorous surveillance, when the majority with non-dysplastic Barrett's oesophagus do not develop adenocarcinoma (Hvid-Jensen, Pedersen et al. 2011). Epidemiological studies have shown that up to 95% of oesophageal adenocarcinomas do not have a preceding diagnosis of Barrett's oesophagus (Cameron, Zinsmeister et al. 1990, Bytzer, Christensen et al. 1999, Corley, Levin et al. 2002, Dulai, Guha et al. 2002), thus posing the question does Barrett's lead to adenocarcinoma or is it merely a field effect of the cancer itself? Although, the conversion incidence is variable within the literature, most conclude that the malignant potential is less than 1% per annum (Shaheen, Crosby et al. 2000, Yousef, Cardwell et al. 2008), with the largest population-based study to date reporting annual conversion rates as low as 0.12% (Hvid-Jensen, Pedersen et al. 2011). With recent studies demonstrating the improved outcomes and survival in oesophageal adenocarcinoma patients diagnosed while on surveillance programmes compared to patients who were not, the validity of Barrett's oesophagus surveillance is still internationally accepted (Grant, Demeester et al. 2013). In fact, caution needs to be exerted when analysing recent publications, such as the Danish study, which does question the importance of Barrett's endoscopic surveillance; their study was not without flaws and as outlined in a recent Cochrane review (Bennett, Green et al. 2012), it included cases with only histologically confirmed Barrett's oesophagus. It did not analyse the concurrent endoscopic results. Therefore it may have included a number of non-Barrett's cases, such as metaplasia at the cardia.

Regardless, Barrett's oesophagus remains the leading known risk factor for oesophageal adenocarcinoma. The purpose of Barrett's oesophagus surveillance programmes is to ensure early cancer detection and offer the best opportunity for cure (Peters, Clark et al. 1994, van Sandick, van Lanschot et al. 1998). The clinical challenge we now face is to stratify patients with Barrett's into high and low cancer risk groups, eliminating the need for rigorous surveillance in the majority of patients unlikely ever to progress to malignancy.

Table 1.2: Follow-up recommendations for Barrett's oesophagus patients, categorised based on histologic findings (Booth and Thompson 2012)

Category	Histology		Follow-up
Negative for dysplasia	Architecture Cytology Maturation	Normal with well-spaced glands Regular nuclei Smooth membranes Complete	 Repeat OGD within 1 year OGD every 3 years
Indefinite for dysplasia	Architecture Cytology Maturation	Normal to mild distortion, often inflamed Hyperchromasia Overlapping nuclei Irregular nuclear borders Complete when intact surface epithelium is present	 Repeat OGD within 6 months, with follow-up as indicated by results PPI prior to repeat biopsy if significant inflammation is present
Low grade dysplasia	Architecture Cytology Maturation	Normal to mild distortion Gland crowding Minimal pleomorphism Maintained polarity Increased mitotic activity Minimal to none	Repeat OGD within 6 months to rule out HGD Expert pathologist confirmation Annual OGD until no dysplasia
High grade dysplasia	Architecture Cytology Maturation None	Mild to marked distortion Crowded glands Cribiform or budding glands Loss of polarity Markedly enlarged nuclei Prominent pleomorphism Atypical mitoses	 Repeat OGD in 3 months to rule out cancer Expert pathologist confirmation Individualised surveillance or treatment plan

1.1.5 Risk factors for Barrett's oesophagus

1.1.5.1 Gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) is a common condition in Western society, and prevalent in 20% to 30% of the population (Locke, Talley et al. 1997). Albeit a recognised precursor for the premalignant lesion Barrett's oesophagus, only approximately 10% of chronic refluxers develop this condition (Lord 2003). A link between these two conditions exists, with a study by Smith *et al.* demonstrating that successful reduction of reflux symptoms has led to regression of columnar epithelium (Smith, Kelly et al. 2010). Reflux control eliminates pathological changes associated with cancer. However, the exact pathogenesis underlying the disease progression from simple reflux to Barrett's metaplasia remains unclear. Other contributing factors, such as a higher frequency and a longer duration of reflux, have been associated with increased incidences of Barrett's oesophagus (Oberg, Peters et al. 2000, Conio, Filiberti et al. 2002). Cameron *et al.* identified an increased concordance for GORD in monozygotic and dizygotic twins (Cameron, Lagergren et al. 2002), while other studies have suggested an autosomal dominant trait was present on review of extended pedigrees with familial Barrett's oesophagus (Crabb, Berk et al. 1985, Jochem, Fuerst et al. 1992). The mechanism of any genetic component remains unknown.

1.1.5.2 Biliary reflux

Reflux and its composition play an important role in metaplastic change and cancer conversion (Harmon, Johnson et al. 1981, Smith, Kelly et al. 2010). Reflux is composed of oro-oesophageal, gastric, and duodenal components, in varying quantities (Kauer, Peters et al. 1995, Gutschow, Schroder et al. 2002). Acidic reflux is recognised as a major carcinogen, with acid suppression therapy used in Barrett's oesophagus treatments, however, despite reduction in gastric acidity, Barrett's cancer still develops (Jankowski, Harrison et al. 2000, Triadafilopoulos 2000), and for this reason, biliary reflux is another well-recognised carcinogen (Gillen, Keeling et al. 1988, Attwood, Smyrk et al. 1992, Stamp 2002). Following elimination of the gastric component, as is the case following a total gastrectomy, cases of Barrett's oesophagus have developed (Meyer, Vollmar et al. 1979). Stein *et al.* have also demonstrated higher concentrations of bile acids in Barrett's oesophagus patients compared to those with uncomplicated GORD (Stein, Feussner et al. 1994). While, Nehra *et al.* demonstrated that complicated

Barrett's oesophagus was associated with increased concentrations of more toxic bile acids such as deoxycholic acid (DCA) (Nehra, Howell et al. 1999). Studies have shown that Barrett's tissue is adapted to transport bile acids, expressing high levels of bile acid transporter proteins which are absent in normal squamous epithelium (Dvorak, Watts et al. 2009). In terms of the exact mechanisms of their carcinogenic actions, while unknown, bile acids have demonstrated damaging effects on cell function (Vaezi and Richter 2000), mitochondrial membranes (Palmeira and Rolo 2004) and cell membranes (Billington, Evans et al. 1980).

Unconjugated bile acids, in particular DCA, have been implicated in oesophageal cancer formation (Jankowski, Hopwood et al. 1993, Kauer, Peters et al. 1995, Jenkins, D'Souza et al. 2007), however; the exact cellular or molecular role of DCA in Barrett's development and progression remains unknown. Jenkins *et al.* demonstrated that DCA was the most genotoxic out of six examined bile acids, both at a neutral and acidic pH, through pathways inducing increased release of reactive oxygen species (ROS) in oesophageal cancer cells, although, these specific effects in relation to the entire Barrett's disease sequence have not been examined (Jenkins, D'Souza et al. 2007).

1.1.5.3 pH levels

While bile acids have been identified as a potential carcinogen in the development of Barrett's oesophagus, it is important to remember that bile reflux occurs in a complex environment with an acidic pH, hence, bile effects do need to be considered in a background of acidity. The rapid increase in the incidence in oesophageal cancer has occurred during a time of judicious use of Proton Pump Inhibitors (PPIs), H_2 antagonists, and over-the-counter antacids. All these medications act to increase the pH of the gastric content. Farrow *et al.* postulated in the case of H_2 antagonists, if such medications act to suppress gastric acid secretion, this results in a more alkaline gastric content (Farrow, Vaughan et al. 2000). Coupled with this is an alteration in gut flora, and possibly a rebound carcinogenic effect secondary to nitrosation of stomach content (Elder, Ganguli et al. 1979). Results from this large population-based case-control study, comprising of 293 patients with oesophageal adenocarcinoma and 695 controls, demonstrated an increased incidence of oesophageal adenocarcinoma in patients with severe GORD symptoms irrespective of the use of H_2 antagonists and over-the-counter

medications (Farrow, Vaughan et al. 2000). This study also demonstrated a moderately higher rate of oesophageal adenocarcinoma in those with long-term use of H_2 blockers and over-the-counter antacids. An increase in gastric pH could be associated with an increased risk of oesophageal adenocarcinoma. However, other possible explanations for the link between these medications and cancer development are that the indications for the medications, rather than their use, are responsible for the observed risk. In addition, possibly the increased use of PPIs, and similar drugs, may be suppressing reflux symptoms but not necessarily eliminating the underlying disease process, thus the carcinogenic event is only hidden, not cured.

Studies have demonstrated that at a neutral pH DCA is potentially more carcinogenic (Jenkins, Harries et al. 2004). Jenkins *et al.* in an *in-vitro* study, showed DCA treatment resulted in destabilisation of genes responsible for cell apoptosis, proliferation, differentiation, and invasion, such as nuclear factor kappa beta (NF-κB), β-catenin, E-cadherin, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), tumour necrosis factor-α (TNF-α), thus leading to abnormalities in their expression. This study demonstrated that alterations in gene expression occurred when DCA was at a neutral pH only. The authors concluded that their *in-vitro* findings had potential repercussions for patients taking PPIs, as previous studies have highlighted that acid-suppression therapies were associated with gastric bacterial overgrowth, a side-effect which caused the relative concentration of unconjugated bile acids to increase (Theisen, Nehra et al. 2000). As such, PPIs may increase the amount of unconjugated bile acids, such as DCA, in reflux resulting in a cascade of events initiating the Barrett's metaplastic pathway.

1.1.5.4 Obesity

The rise in oesophageal adenocarcinoma has been paralleled by an increase in the incidence of obesity in Western society (Enzinger and Mayer 2003). For this reason, obesity has been strongly implicated in the pathogenesis of oesophageal adenocarcinoma (Brown, Swanson et al. 1995, Vaughan, Davis et al. 1995, Chow, Blot et al. 1998, Lagergren, Bergstrom et al. 1999, Calle, Rodriguez et al. 2003, Engel, Chow et al. 2003, Ryan, Rowley et al. 2006, Renehan, Tyson et al. 2008). The

association is significantly stronger compared to most other solid tumours (Calle, Rodriguez et al. 2003, Renehan, Tyson et al. 2008) (Figure 1.5).

Visceral adipose tissue is largely comprised of omental adipose tissue but also includes other intra-abdominal fat sources such as mesenteric fat. One of the hypotheses driving the obesity and cancer link is that fat is metabolically active (Gutierrez, Puglisi et al. 2009) and visceral adipose tissue acts as a unique organ entity associated with the release of potent pro-inflammatory cytokines and growth factors, such as interleukin-6 (IL-6), TNF-α and VEGF (Cannon, Nerad et al. 1993, Lysaght, van der Stok et al. 2011). In the case of Barrett's oesophagus, this may potentially drive this inflammatory cancer pathway. In fact, increasing waist: hip ratio in Barrett's oesophagus patients is associated with an increased risk of cell cycle and genetic abnormalities (Vaughan, Kristal et al. 2002).

Another theory behind the role of visceral obesity and Barrett's oesophageal adenocarcinoma is the mechanical effects intra-abdominal fat exerts. Central obesity, where fat is predominantly stored in the abdomen, mainly occurs in males compared with females, where fat is typically stored in the lower part of the body such as the hips and thighs (Lemieux, Prud'homme et al. 1993). This is interesting, as males have a significantly increased incidence of oesophageal adenocarcinoma compared with females (Pera, Manterola et al. 2005, Bosetti, Levi et al. 2008). Central obesity has strongly been linked to increased levels of GORD (Lagergren, Bergstrom et al. 1999), possibly due to the increased pressure caused by a loss of intra-abdominal space, subsequently exerting increased levels of biliary and gastric reflux (Whiteman, Sadeghi et al. 2008, El-Serag, Hashmi et al. 2013).

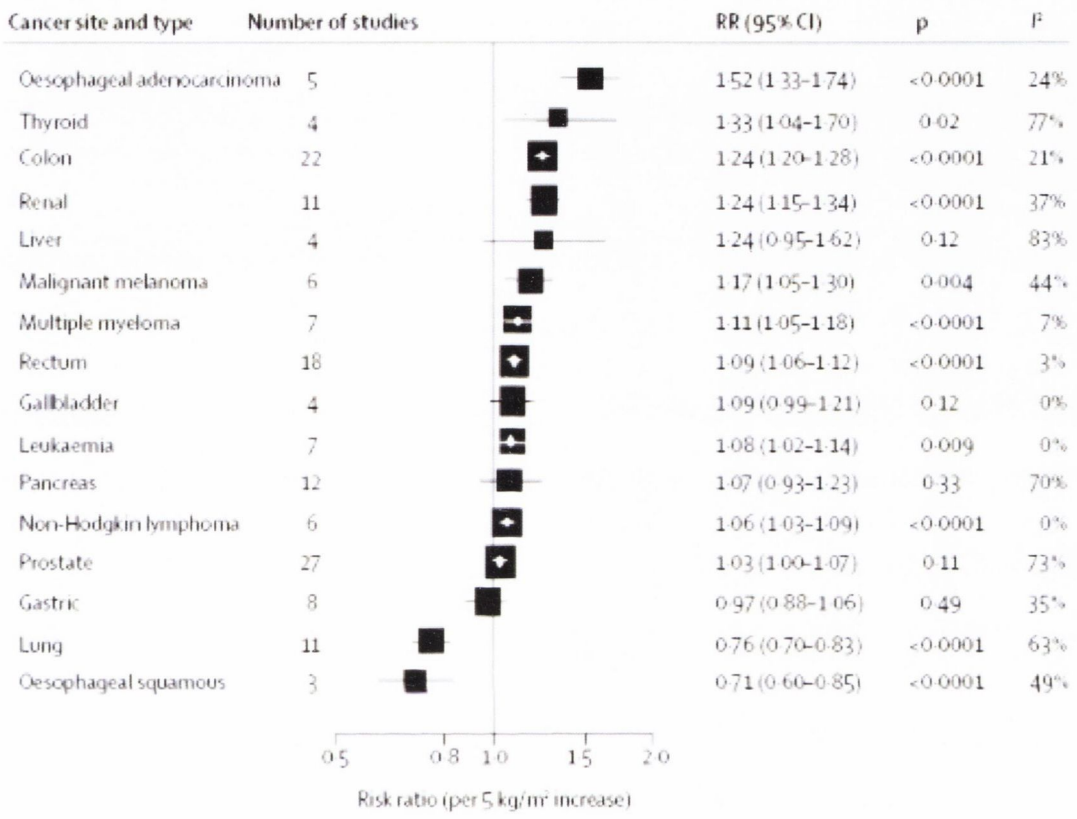


Figure 1.5: Association between body mass index and cancer incidence: Summary risk estimates by cancer sites for women (Renehan, Tyson et al. 2008).

1.1.6 Management of Barrett's oesophagus

1.1.6.1 Treatment of uncomplicated Barrett's oesophagus

There are no specific treatment options for Barrett's intestinal metaplasia. Once diagnosed patients enter a surveillance programme (Wang, Sampliner et al. 2008). Following initial histological confirmation of non-dysplastic Barrett's oesophagus, patients undergo a follow-up endoscopy at one year and then every three years in the presence of persistent metaplastic disease. The surveillance intervals suggested by the 2008 ACG guidelines are dependent on the pathology results (as outlined in Table 1.2).

Medical treatment options include PPIs and H_2 antagonists aimed at reducing acid secretion, primarily to relieve symptoms (Rees, Lao-Sirieix et al. 2010), but also to potentially dissipate the inflammatory cascade caused by acid exposure. In a select cohort of patients, with proven reflux disease refractory to medical treatment and possibly attributable to an incompetent lower oesophageal sphincter, anti-reflux surgery, typically Nissen fundoplication, may be offered. Studies have demonstrated that fundoplication surgery reduces reflux to normal levels and results in the regression of Barrett's columnar mucosa, with Smith *et al.* demonstrating that control of reflux reduced deleterious genomic changes associated with cancer (Smith, Kelly et al. 2010). Surgery has the added advantage of controlling both acid and biliary reflux, unlike medical therapy which alters gastric secretions and their acidity, without effect on the bilious component (Csendes, Bragheto et al. 2006, Lenglinger, Eisler et al. 2007). However, the benefits of fundoplication surgery over PPIs are not definitive, with conclusions in this area limited due to a lack of randomised control trials. In fact, a comprehensive meta-analysis, comparing the two treatment approaches, concluded fundoplication surgery did not reduce cancer development risk (Chang, Morris et al. 2007).

1.1.6.2 Treatment of LGD

An initial diagnosis with LGD significantly increases the potential for malignant conversion. It is for this reason that surveillance is more rigorous and frequent in this patient cohort. Typically patients are monitored at six monthly periods by way of OGD and biopsy, either until the disease progresses to HGD, at which point interventional

strategies are implemented, or disease regresses to metaplasia and surveillance is as per the protocol described in 1.1.6.1.

Current emerging concepts are the potential role for treatments, such as radiofrequency ablation (RFA) in the management of early dysplastic disease (Akiyama and Triadafilopoulos 2010, Fleischer, Odze et al. 2010, Caillol, Bories et al. 2012, Hur, Choi et al. 2012). Akiyama *et al.* (Akiyama and Triadafilopoulos 2010) advocates the use of RFA, given the difficulties of dysplasia assessment in mucosal biopsies. Dysplasia may also be missed during endoscopy as it may not be associated with any identifiable endoscopic abnormality. The authors concluded, with the safe and effective profiles of RFA, it should be considered as a treatment option for the whole Barrett's disease spectrum. However, the cost-effectiveness of this approach is questionable for metaplastic disease (Hur, Choi et al. 2012), and even in the case of early dysplasia still no standardised guidelines exist. In Ireland RFA is not routinely used in the management of LGD. In the future, a potential role for intervention in the earlier stages of Barrett's oesophagus, such as metaplasia and LGD, may prove cost-effective in combination with cancer-predicting biomarkers.

1.1.6.3 Treatment of HGD and IMC

The introduction of Barrett's oesophagus surveillance programmes have indeed led to increased detection of HGD and IMC (Rice, Blackstone et al. 2001). Until recently, the only definitive treatment strategy available for early oesophageal cancer was surgical resection. Oesophagectomy carries a great risk of morbidity and mortality (Roberts, Tang et al. 2012, Tapias, Muniappan et al. 2013). The advent of endoscopic therapies; endoscopic mucosal resection (EMR) and RFA, have provided alternative treatment options to surgery and its attendant risks (Ell, May et al. 2000, May, Gossner et al. 2002, Pacifico, Wang et al. 2003, Mino-Kenudson, Brugge et al. 2005, Peters, Kara et al. 2005). Endoscopic treatment is only appropriate for predicted node-negative disease where pathological assessment of the endoscopic resected specimen shows complete resection with no adverse pathological features (Stein and Feith 2005). Classically two situations are encountered: 1) smooth featureless Barrett's epithelium containing HGD (uni- or multifocal) is treated using RFA alone. 2) Visible early malignant lesions

within Barrett's mucosa are resected using EMR. The remaining smooth Barrett's epithelium is then eradicated using RFA.

The popularity of endoscopic management is increasing due to low morbidity and mortality rates relative to oesophagectomy (Fernando, Luketich et al. 2002, Menon, Stafinski et al. 2010). Studies estimate survival rates as high as 98%, in carefully selected cases of IMC (Ell, May et al. 2007). Rigorous assessment and follow-up is part of this treatment regime. This allows for further therapies, either repeat endoscopic treatments or oesophagectomy, in the event of disease progression or recurrence. However, the long term effectiveness of endoscopic therapy remains to be demonstrated. Concerns have been raised regarding residual cancer following treatment (Oh, Hagen et al. 2006, Pennathur, Farkas et al. 2009). There also remains the potential risk of under-staging in patients not managed surgically. With this, certain authors have thus maintained that oesophagectomy should be first-line treatment for early cancers in those fit for surgery (Ell, May et al. 2000, Rice, Murthy et al. 2011). In fact, significant differences in the management of early lesions are described between medical disciplines; Yackimski *et al.* (Yachimski, Nishioka et al. 2008) showed that 86% of these lesions were managed surgically if initially referred to a surgeon compared with 12% when initially referred to a gastroenterologist. In our Centre we advocate for an integrated programme model with specialist gastroenterologists, surgeons, and pathologists, in order to determine tailored approaches for individual patients (**O'Farrell**, Reynolds et al. 2013).

Over the last decade, we have seen a marked increase in patients presenting with early oesophageal cancers (perhaps due to improved Barrett's surveillance, greater awareness of oesophageal cancer, lower thresholds for endoscopy, and a referral bias to this centre), and we have demonstrated that with careful patient selection, EMR ± RFA appears to offer effective management in particular patterns of HGD and IMC (**O'Farrell**, Reynolds et al. 2013). More advanced and extensive disease still is managed surgically. Results showed that recurrences occur in patients with extensive long-segment Barrett's and multifocal changes. Such extensive disease patterns reinforce the concerns with using definitive endoscopic therapies for early oesophageal tumours, due to the potential of missing multifocal areas of disease. With some studies

demonstrating disseminated or more advanced disease in surgically resected specimens following oesophagectomy (Pennathur, Farkas et al. 2009, Zehetner, DeMeester et al. 2011) it emphasises the likely field effect associated with early cancers. It therefore appears that there are some patients in whom surgery must be considered the standard of care, despite the fact that both modalities could be an option for these patients.

1.1.6.4 Treatment of Barrett's oesophageal adenocarcinoma

Transthoracic oesophagectomy with lymphadenectomy represents the gold standard operative approach for oesophageal cancer (Altorki and Skinner 1990, Altorki, Girardi et al. 1997, Lerut, Coosemans et al. 1999, van de Ven, De Leyn et al. 1999, Peyre, Hagen et al. 2008, Rizzetto, DeMeester et al. 2008), with transhiatal oesophagectomy seen as a suitable option for patients with predicted node-negative cancers or those with resectable disease who are not candidates for the transthoracic approach because of co-morbidity (Donohoe, **O'Farrell** et al. 2012).

Neoadjuvant therapy is increasingly the standard of care in the management of locally advanced oesophageal cancer (Enzinger and Mayer 2003, Sjoquist, Burmeister et al. 2011). Pre- and postoperative chemotherapy represents current practice in the United Kingdom and in many European centres, based largely on outcomes from the OEO2 and MAGIC trials which showed superiority compared with surgery alone (2002, Cunningham, Allum et al. 2006). Multimodal therapy, combining chemotherapy and radiation therapy prior to surgery has been common practice in North America, and in some European and Asian countries (Sjoquist, Burmeister et al. 2011, Merkow, Bilimoria et al. 2012, Reynolds, Donohoe et al. 2012, van Hagen, Hulshof et al. 2012).

1.2 THE ROLE OF BIOMARKERS IN BARRETT'S OESOPHAGUS

Identifying an effective biomarker to enable stratification of Barrett's oesophagus patients into high and low cancer risk groups remains to be achieved. Di Pietro *et al.*, in an extensive review, reported Barrett's oesophagus as an ideal model to examine for biomarkers predicting cancer progression since patients with this condition are surveyed with endoscopic tissue sampling until the onset of HGD and oesophageal

adenocarcinoma (di Pietro and Fitzgerald 2009). However, no biomarker exists to date, highlighting that the identification of Barrett's biomarkers is less clear cut than Di Pietro implies. In fact, most patients with Barrett's oesophagus are unknown to the medical profession and present with advanced oesophageal cancer (Cameron, Zinsmeister et al. 1990, Bytzer, Christensen et al. 1999, Corley, Levin et al. 2002, Dulai, Guha et al. 2002). Among those with SIM picked up by endoscopy, most will not develop oesophageal adenocarcinoma or will die of other causes, so the condition is by no means 'ideal'. A recent literature review by Timmer *et al.* analysing potentially predictive biomarkers for Barrett's oesophagus did not identify a single biomarker, capable of predicting Barrett's oesophagus progression, which had advanced to a Phase 5 study, as outlined by the Early Detection Research Network recommendations for the development of a biomarker (Pepe, Etzioni et al. 2001, Timmer, Sun et al. 2013). There are five phases to biomarker validation in order to approve it for clinical use. Phase 1 is the exploratory phase which identifies potential biomarkers. This is followed by the development of a clinical assay which is then validated in a retrospective cohort, followed by a prospective cohort study (Phase 2, 3 and 4, respectively). Phase 5 then reviews the impact of the identified biomarker in the disease-burden population, assessing primary outcomes such as its cost effectiveness and its effect on outcome and survival.

One of the major challenges in the identification of Barrett's-progressor biomarkers has been the need for fresh tissue samples, difficulties with sample diagnosis (i.e. is the tissue truly Barrett's oesophagus or just adjacent inflamed tissue?) and the need for high patient numbers over long periods of surveillance. Emerging biomarkers have been identified in one of the largest studies to date; Bird-Lieberman *et al.* utilised data from a case-control study using archived samples from the Northern Ireland Barrett's oesophagus Register (1993-2005) (Bird-Lieberman, Dunn et al. 2012). It comprised of 89 patients with progressive Barrett's oesophagus, where HGD or oesophageal adenocarcinoma were the primary end-points, and 291 patients with non-progressive disease. A panel of biomarkers, comprising of established biomarkers (abnormal DNA content, p53, cyclin A expression) and new biomarkers (sialyl Lewis^{a}, Lewis^{x}, *Aspergillus oryzae* lectin and binding of wheat germ agglutinin) were measured in paraffin-embedded tissue samples taken from patients at their first Barrett's oesophagus

OGD. LGD had an 11-fold increased odds ratio for disease progression, while DNA ploidy and *Aspergillus oryzae* lectin were both associated with a 3-fold increased risk, and from the panel of biomarkers analysed, these three most accurately identified progressors from patients from non-progressive disease.

Recent additional work led by Professor Rebecca Fitzgerald, in a Dutch population, further evaluated the efficacy of both cyclin A and p53, in addition to methylation biomarkers; HPP-1, RUN X3 and p16, as biomarkers for the identification of early neoplasia in Barrett's oesophagus patients (Boerwinkel, Di Pietro et al. 2012). A diagnosis of Barrett's oesophagus is dependent on two parameters; macroscopic identification at endoscopy and histological confirmation. Therefore, the accuracy of endoscopic devices in differentiating metaplastic disease from dysplastic and oesophageal adenocarcinoma has been heavily investigated (Singh, Bansal et al. 2011, Bird-Lieberman, Neves et al. 2012, Sharma, Hawes et al. 2013). Autofluorescence imaging (AFI) has been previously shown to dramatically improve the sensitivity of endoscopic imaging for the detection of early neoplasia (Curvers, Singh et al. 2008). This imaging is based on endogenous substances called fluorophores, which emit fluorescent light when excited with short wavelengths of light. Kara *et al.* previously demonstrated that early oesophageal cancer had a different fluorescent spectrum compared with non-dysplastic Barrett's oesophagus (Kara, DaCosta et al. 2007). A direct consequence of this improved endoscopic sensitivity was the significant increase in false positive diagnoses of early oesophageal cancer. Therefore, the purpose of Professor Fitzgerald's study was to identify biomarkers that would aid the specificity of AFI in diagnosing the early stages of Barrett's cancer. Forty-eight Barrett's lesions were separated into four categories; true positives (AFI positive and histology positive), false positives (AFI positive and histology negative), true negatives (AFI negative and histology negative) and false negatives (AFI negative and histology positive). While p16, cyclin A and p53 were all significantly increased in the dysplastic positive patients, no biomarker successfully improved the specificity of AFI in diagnosing early cancer in Barrett's oesophagus. The authors hypothesised that this lack of difference in the expression of these biomarkers between the false positive and true positive cases and the false negative and true negative cases may be due to the investigated biomarkers corresponding to only subtle molecular changes. This group concluded that

other biomarkers related to malignant progression which were associated with gross cellular changes, such as aneuploidy and tetraploidy, may affect the AFI features of Barrett's oesophagus and thus warrant future investigation.

The Seattle Barrett's Oesophagus Study, established in 1983 and led by Dr Brian Reid, has provided extensive work in the field of Barrett's oesophagus biomarkers. A recent study based on the significant correlation between oesophageal adenocarcinoma and obesity, measured fasting glucose, insulin, leptin and adiponectin levels in 392 patients (Duggan, Onstad et al. 2013). Insulin resistance and leptin were associated with a significantly increased risk of oesophageal adenocarcinoma. While reduced high molecular weight adiponectin significantly increased the cancer conversion risk. Dr Reid's group have also identified clonal diversity as a potential biomarker for predicting Barrett's oesophagus progression (Maley, Galipeau et al. 2006, Merlo, Shah et al. 2010). Applying the hypothesis of clonal evolution; genetic heterogeneity within a neoplasm fuels cancer development, with natural selection allowing expansion of clones which promote survival and proliferation (Nowell 1976, Merlo, Pepper et al. 2006). Chromosome instability may be represented by a gain, loss or loss of heterozygosity (LOH) in large portions of a chromosome, such chromosomal instability has been implicated in the pathogenesis of oesophageal adenocarcinoma (Li, Galipeau et al. 2008, Paulson, Maley et al. 2009). This group has focussed on LOH at chromosome 9p and 17p, with models of clonal expansion assessing Barrett's oesophagus and adenocarcinoma sites from the same patients. 9p LOH was identified as the earlier event, predisposing Barrett's patients to DNA abnormalities along the pathway of progression to oesophageal cancer (Barrett, Sanchez et al. 1999). Their work has also demonstrated that increasing numbers of clones with 17p LOH, and its associated mutations in the corresponding TP_{53} gene, along with gross DNA abnormalities, tetraploidy and aneuploidy, are associated with increased progression to oesophageal adenocarcinoma in Barrett's oesophagus patients (Maley, Galipeau et al. 2004). The Seattle Barrett's Oesophagus Study group have consistently proven that measures of clonal diversity are strongly predictive of cancer development in Barrett's oesophagus (Merlo, Shah et al. 2010).

Previous studies have also analysed the tumour suppressor gene, p53, a transcription factor, responsible for controlling cell cycle, DNA repair and synthesis, cell differentiation and apoptosis. p53 is one of the most commonly mutated genes in human cancers (Hollstein, Metcalf et al. 1990) and mutations in p53 have been linked to VEGF and basic fibroblast growth factor (bFGF) expression (Rak, Mitsuhashi et al. 2000). Lord et al. (Lord, Park et al. 2003) demonstrated over-expression of VEGF and bFGF along the Barrett's disease pathway; from metaplasia to dysplasia to adenocarcinoma. However, while studies to date have shown increased p53 mutagenesis in oesophageal adenocarcinoma tissue and adjacent Barrett's epithelium from the same patients (Casson, Mukhopadhyay et al. 1991), in the pre-malignant setting, p53 mutations are rare, appearing only as a late event in the Barrett's disease sequence (Schneider, Casson et al. 1996), thus, it has not been validated as an effective biomarker for Barrett's progression identification.

Importantly, with respect to progressing Barrett's metaplasia, additional biomarkers still require identification, with translation into clinical practice. Until recently, studies have primarily focussed on finding differences between the various stages of the Barrett's disease sequence. However, currently the true challenge is to identify cancer-predisposing biomarkers, and enable segregation of progressors and non-progressors in the earliest stages of this disease process.

1.3 MITOCHONDRIA AND CANCER

1.3.1 Mitochondria structure

The mitochondrion contains outer and inner membranes composed of phospholipids. The two membranes, however, have different properties. Because of this double-membrane organisation, the mitochondria are composed of specialised regions that include the outer membrane, the intermembrane space, the inner membrane, and the cristae and matrix (Wilson and Brooks 2010). The main function of a mitochondrion is to provide energy for a range of activities, including movement, the regulation of signalling, cellular differentiation and cell death, and the control of cell cycle and cell growth (Shutt and Shadel 2010). The outer membrane is a relatively simple phospholipid bilayer, containing a large number of integral protein structures called

porins, which render the membrane permeable to molecules of about 10 kDa or less (i.e., ions, ATP, and some of the smallest proteins) (Roede and Jones 2010, Wilson and Brooks 2010). Disruption of the outer membrane permits proteins in the intermembrane space to leak into the cytosol, leading to certain cell death (Chipuk, Bouchier-Hayes et al. 2006). The mitochondrial outer membrane can associate with the endoplasmic reticulum (ER) membrane, via a structure called MAM (mitochondria-associated ER-membrane) (Hayashi, Rizzuto et al. 2009). This is important in ER-mitochondria calcium signalling and is involved in the transfer of lipids between the ER and mitochondria. The inner membrane, on the other hand, is freely permeable only to oxygen, carbon dioxide, and water and contains integral proteins for the active transport of specific metabolites across the membrane in a highly regulated manner. The inner mitochondrial membrane contains proteins with five types of functions. 1) The proteins that perform the redox reactions of oxidative phosphorylation, 2) the adenosine 5′-triphosphate (ATP) synthase, which generates ATP in the matrix, 3) the specific transport proteins that regulate metabolite passage into and out of the matrix, 4) the protein import machinery, and 5) the mitochondria fusion and fission protein.

Almost all ions and molecules require special membrane transporters to enter or exit the matrix. Proteins are transported into the matrix via the translocase of the inner membrane (TIM) complex or via Oxa1 (Herrmann and Neupert 2000). The inner membrane also harbours the components of the electron transport chain and the ATP synthetase complex, which are involved in cellular respiration and energy production, and is organised into internal folds called cristae to increase the surface area to accommodate the numerous structures above. The intermembrane space is the region between the inner and outer membranes. Its main function is oxidative phosphorylation which produces ATP as the result of transfer of electrons from NADH or FADH2 to O2 by a series of electron carriers located in the inner membrane (Wilson and Brooks 2010). The matrix contains the mitochondrial genome, the ribosomes and other components necessary for translation, and the enzymes responsible for the citric acid cycle reactions (Wilson and Brooks 2010).

1.3.2 Mitochondrial DNA and its susceptibility to damage

Mammalian cells typically contain hundreds to thousands of mitochondria, each containing two to ten copies of the mitochondrial genome (Bogenhagen and Clayton 1974). Sequenced in 1981 by Anderson and colleagues, mitochondrial DNA is composed of 16,569 base pairs held in a covalently closed-circular loop (Anderson, Bankier et al. 1981) (Figure 1.6). Mitochondrial DNA plays critical roles in respiration, oxidative phosphorylation, protein synthesis and cellular apoptosis. The proteins encoded by the mitochondria DNA are essential subunits in the electron transport chain and ATP synthase, and cells lacking mitochondria have been shown to be completely dependent on glycolysis for survival (Desjardins, de Muys et al. 1986). The close proximity of mitochondrial DNA to the ROS production site (i.e. the mitochondria themselves) and the low proficiency of mitochondrial DNA repair mechanisms make it more vulnerable to oxidative injury and mutations compared to nuclear DNA (Wallace 1992, Wallace 1992). Mitochondrial genomic instability has been identified in many human cancers (Woods and Dubuy 1945, Polyak, Li et al. 1998, Brandon, Baldi et al. 2006, Kroemer 2006, Putignani, Raffa et al. 2008). Specifically, for oesophageal carcinoma, defects in mitochondrial function, such as D-loop mutations, have been shown to be involved in this cancer development (Hibi, Nakayama et al. 2001, Abnet, Huppi et al. 2004). A recent study by Lee *et al.* showed the frequency of mitochondrial DNA mutations were significantly increased in tissue from Barrett's intestinal metaplasia compared to corresponding normal tissue from the same patient (Lee, Han et al. 2012). In turn, these mitochondrial mutations were associated with increased ROS levels, and the authors concluded that these findings may play a crucial role in Barrett's cancer development. However the exact relevance and timing of mitochondrial mutations during disease progression in Barrett's oesophagus is under-explored and is currently not understood.

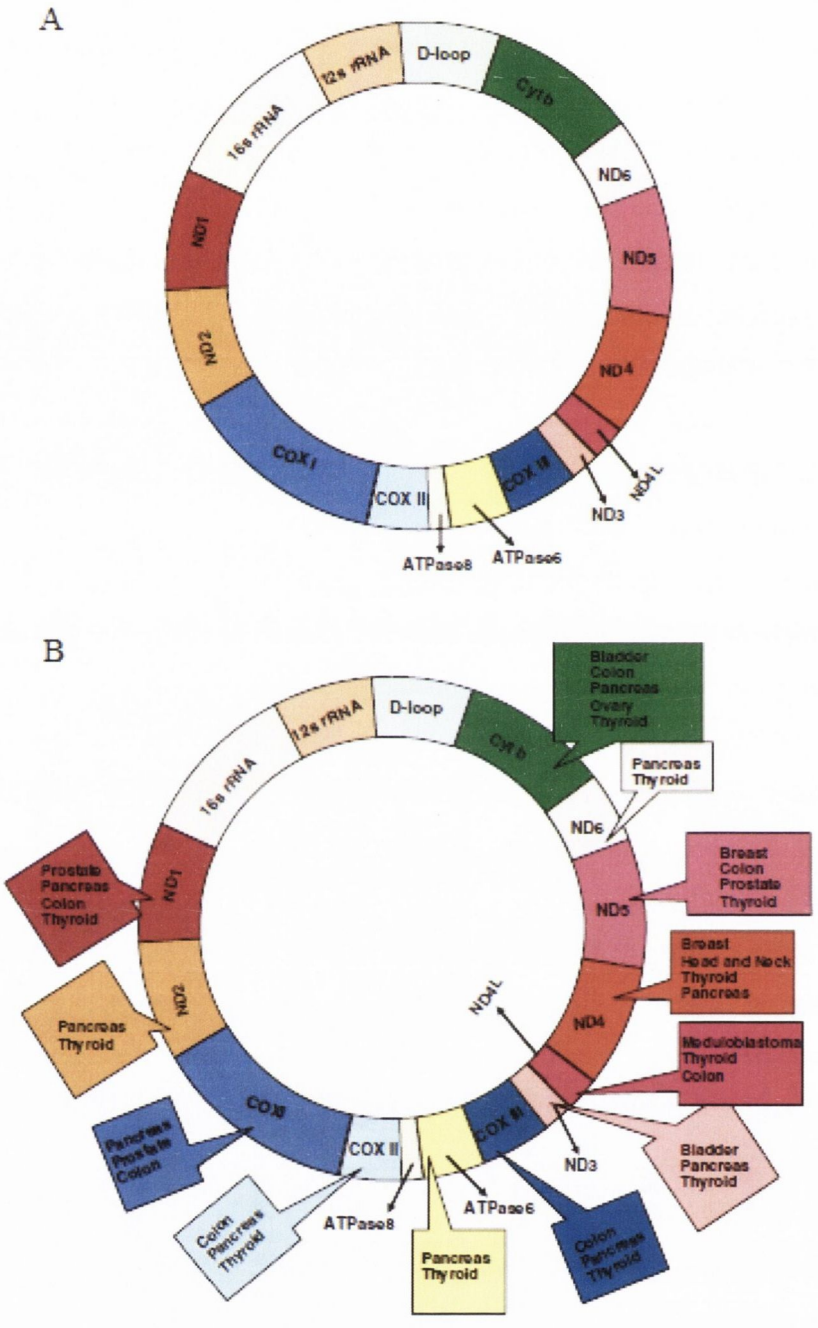


Figure 1.6: Mitochondrial DNA and cancer. (a) Schematic diagram of wild type mitochondrial DNA. Maternally inherited (Giles, Blanc et al. 1980), mitochondrial DNA is a double stranded circular molecule that consists of approximately 16,500 base pairs forming 37 genes, essential for normal mitochondrial function. (b) Different mitochondrial DNA sites associated with different cancer types. Image reproduced from Chatterjee *et al.* (Chatterjee, Mambo et al. 2006).

1.3.3 Mitochondria energy production sites and the Warburg effect

ATP acts as a free energy store (Knowles 1980, Tornroth-Horsefield and Neutze 2008). High energy bonds exist between phosphates and formation of these bonds are accompanied by decreases in free energy within cells. When the reverse occurs, ATP is hydrolysed to produce adenosine diphosphate (ADP) and phosphate (P); resulting in release of large amounts of free energy. ATP is an end product of energy producing processes such as glycolysis and oxidative phosphorylation and it is utilised in day-to-day processes such as biosynthetic reactions, motility and cellular divisions (Tornroth-Horsefield and Neutze 2008).

Glycolysis is the anaerobic breakdown of glucose, occurring in the cytoplasm, it results in the formation of two ATP and two NADH molecules (Figure 1.7) (Cooper 2000). In the absence of oxygen it is the predominant form of energy production, forming pyruvate as the end-product. In normal aerobic conditions, the generated pyruvate enters into the mitochondria to be metabolised during oxidative phosphorylation. However, in a persistent anaerobic environment the result is the generation of lactate and ethanol.

In aerobic conditions, pyruvate generated during glycolysis enters the mitochondria and undergoes oxidative phosphorylation (Cooper 2000). Initially, in the presence of oxygen, pyruvate undergoes oxidative decarboxylation and is converted to carbon dioxide (CO_2) and Acetyl Co-enzyme A (CoA). Acetyl CoA enters into the Citric acid cycle, also known as Kreb's cycle or oxidative phosphorylation (Figure 1.8), and undergoes oxidation to CO_2 and H_2O, generating the majority of ATP during the entire process of glucose breakdown. Also produced are NADH and flavin adenine dinucleotide ($FADH_2$), whose electrons are transferred along the electron transport chain, generating enough energy to synthesise ATP. Located in the inner mitochondrial membrane, the electron transport chain is crucial for energy production. The energy yield from this electron transfer is responsible for the synthesis of ATP, which is utilised in further energy production by re-entering back into glycolytic pathways or is utilised in biosynthetic processes in cells.

Altered cell metabolism is a defining feature of cancer biology. Normal cells demonstrate the Pasteur Effect, which is inhibition of lactate production in the presence of oxygen (Bensinger and Christofk 2012). In the 1920s Otto Warburg put forward his theory, called the Warburg effect (Warburg 1956, Warburg 1956). Oxygen consumption and lactate production were measured in thinly sliced sections of liver from rats with and without liver cancer. He discovered that cancer tissue had increased lactate generation, despite the presence of sufficient oxygen to power oxidative phosphorylation. He termed this aerobic glycolysis, and stated this was present in the cancer tissue and absent in normal rat liver (Koppenol, Bounds et al. 2011, Bensinger and Christofk 2012). It was subsequently hypothesised that cancer cells divert from normal energy metabolism as a protective mechanism. As outlined, during the Kreb's cycle free radicals are released along the electron transport chain, resulting in formation of ROS, with potential adverse consequences. If this pathway is avoided, and instead energy is metabolised via glycolysis, the result is less ROS generation and reduced cellular injury, thus enabling further propagation of cancer cells.

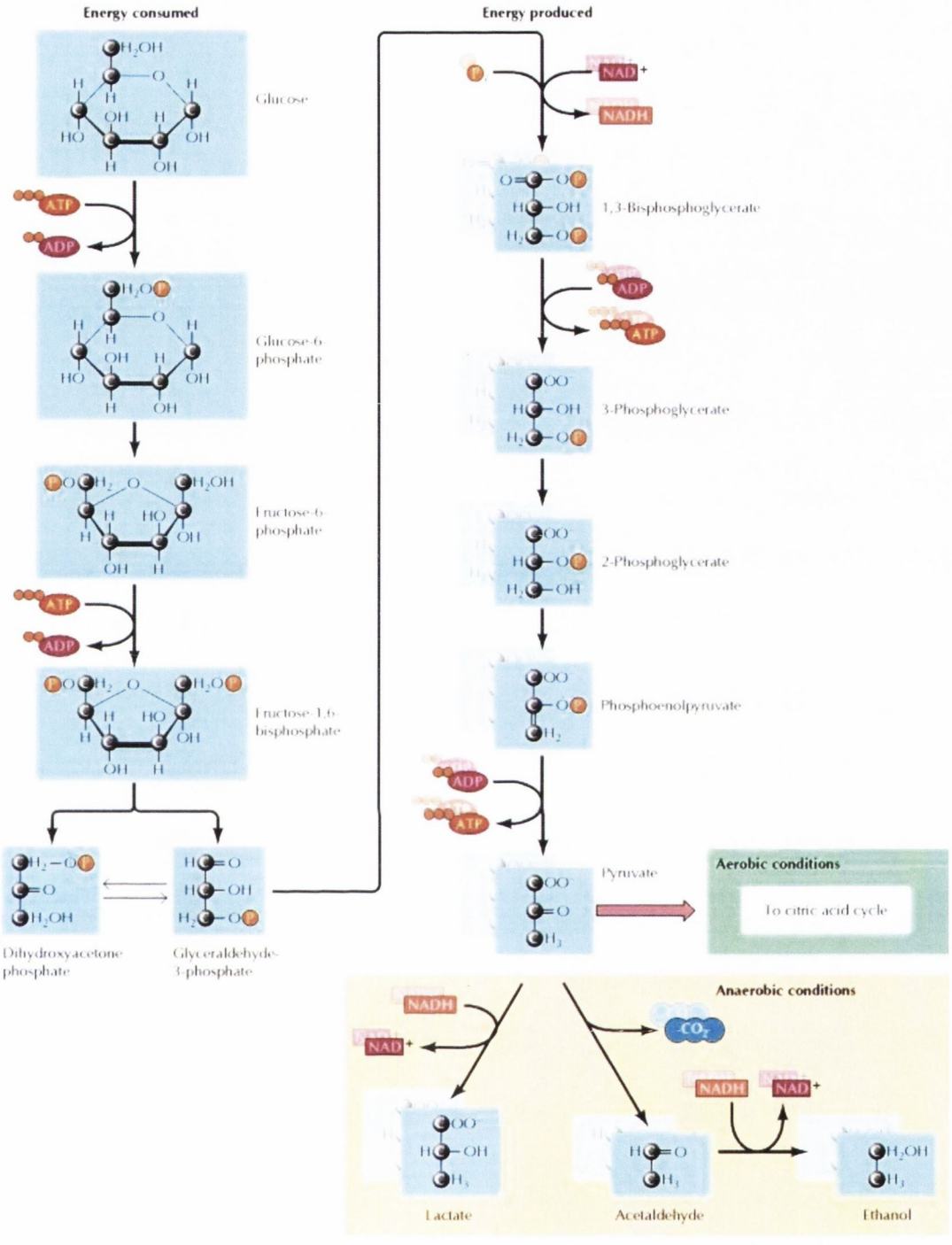


Figure 1.7: Pathway of Glycolysis. Glycolysis occurs in the absence of oxygen and is the process by which energy production occurs in the anaerobic environment. Glucose is broken down to Pyruvate with the production of two ATP and two NADH molecules. In the anaerobic setting, NADH is reoxidized by the conversion of pyruvate to ethanol or lactate, however, in an aerobic environment, pyruvate is enters the citric acid cycle and is further utilised in the production of free energy. (Downloaded from The Cell: A Molecular Approach. 2nd edition. Cooper GM. Sunderland (MA): Sinauer Associates; 2000. http://www.ncbi.nlm.nih.gov/books/NBK9903)

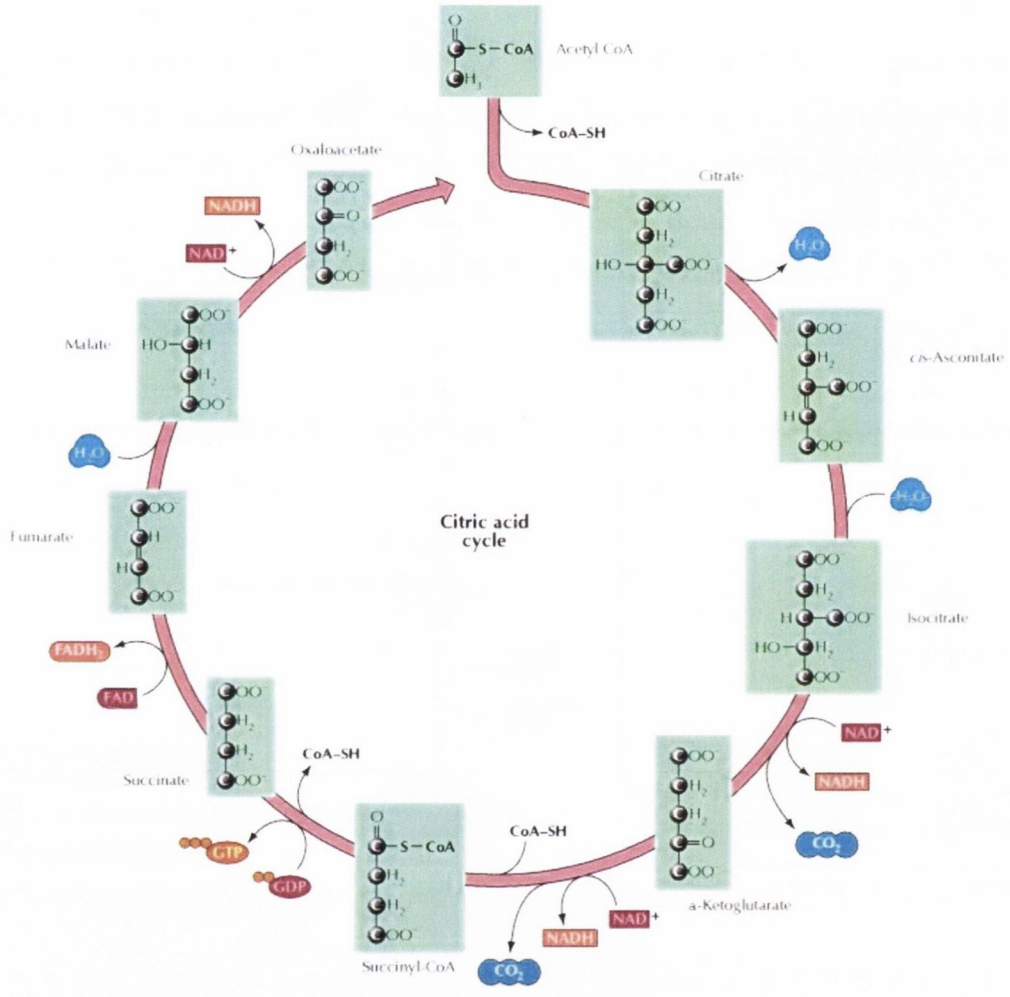


Figure 1.8: Kreb's cycle/Citric Acid Cycle. This process occurs in the aerobic setting and generates the majority of ATP molecules during glucose breakdown. (Downloaded from The Cell: A Molecular Approach. 2nd edition. Cooper GM. Sunderland (MA): Sinauer Associates; 2000. http://www.ncbi.nlm.nih.gov/books/NBK9903)

1.3.4 Mitochondria and the generation of Reactive Oxygen Species (ROS)

ROS, by-products of metabolism, have the potential to cause DNA damage. ROS are unstable reactive molecules, derived from reactions between oxygen (O_2) and free radicals (Turrens 2003). Free radicals are unpaired electrons that are produced predominantly within the electron transport chain located in the mitochondria, during oxidative phosphorylation (Figure 1.9). The mitochondria are the main sites of ROS production, generated by the escape of single electrons from the electron transport chain, and instability within the mitochondria as such may lead to increased production of ROS (Turrens 2003).

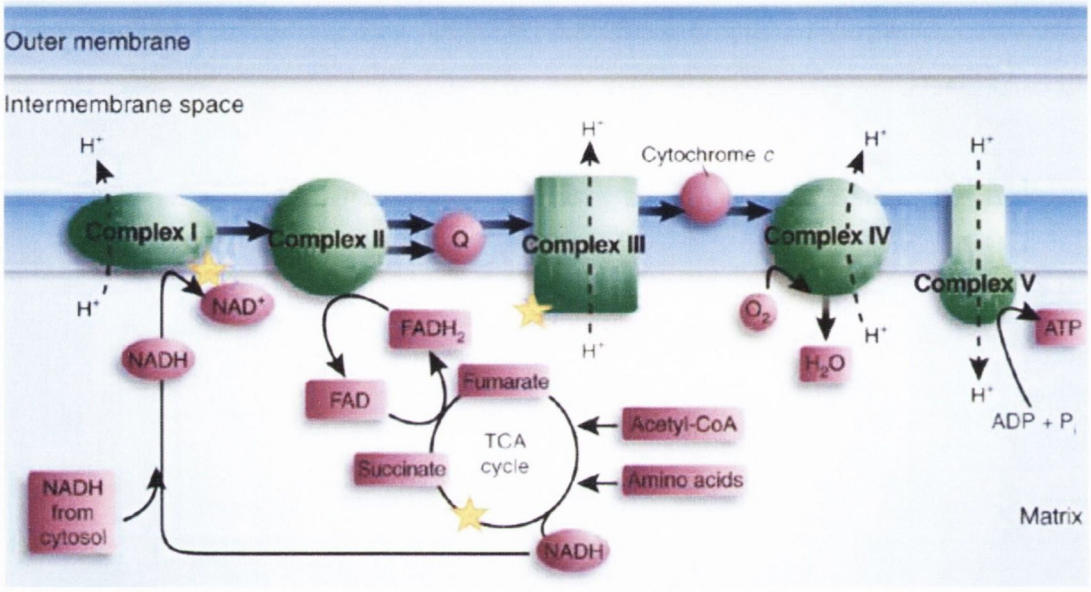


Figure 1.9: The electron transport chain located in the mitochondria. The electron transport chain is the main source of free radicals generated within the cell. These free radicals react with O_2 form ROS such as superoxides, peroxidases and nitric oxide. Image reproduced from Beal, 2005 (Beal 2005).

1.3.5 The Mutator Phenotype Hypothesis

As previously stated, the clinical challenge in Barrett's oesophagus is to be able to differentiate patients with intestinal metaplasia into low and high cancer risk groups, eliminating the need for rigorous surveillance in the majority of patients unlikely ever to progress to cancer. It is hypothesised that during tumour development, cancer cells exhibit a mutator phenotype (Loeb, Bielas et al. 2008). In an extensive review analysing the expression of this mutator phenotype in human cancers, Lawrence Loeb (Loeb 2011) referenced studies by Preston *et al.,* where the links between polymerase proofreading disruption and cancer development were explored (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009). Polymerase proofreading plays a critical role in mutation avoidance (Morrison, Johnson et al. 1993), and therefore it was speculated that defects in these mechanisms would be associated with increased rates of spontaneous mutations. To investigate the consequence of defective proofreading, Preston's group generated mutant mice, with an inactivating point mutation in the proofreading domain of DNA polymerase-δ (Pol-δ) and DNA polymerase-ε (Pol-ε). In the absence of any environmental insult, compared to the wild type mice, these mutant mice had a strong cancer predisposition and reduced survival which correlated with increased tumour incidence (Figure 1.10).

DNA replicates with enormous accuracy. The mutator phenotype hypothesis states that normal mutation rates are insufficient to account for the multiple mutations seen in human tumours; therefore, cancer cells must incur increased rates of mutagenesis during disease progression (Loeb, Springgate et al. 1974, Loeb, Bielas et al. 2008). This theory suggests that benign tumours with low levels of random (non-clonal) mutations would not have the ability to progress to malignancy. This theory maybe relevant to Barrett's oesophagus, however, it remains untested to date.

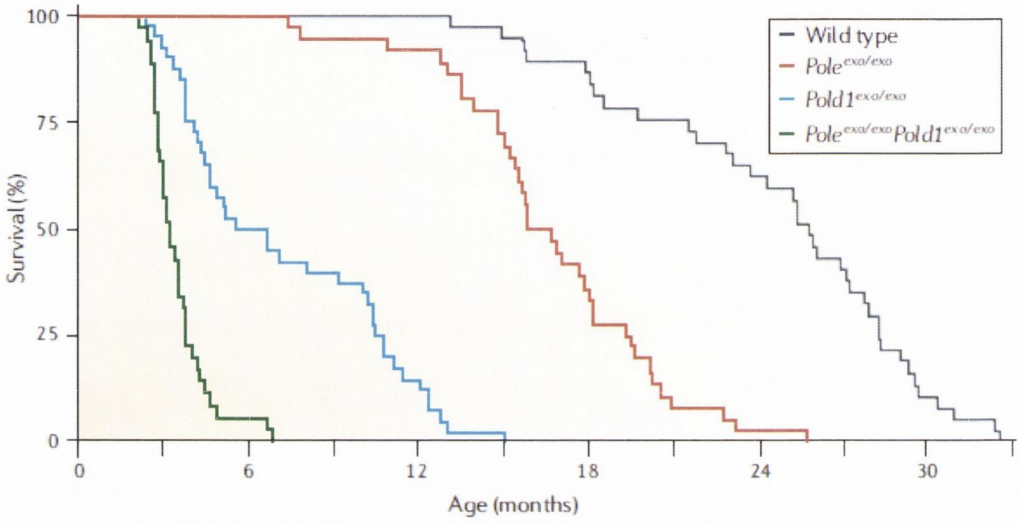


Figure 1.10: The incidence of cancer in mice expressing the mutator phenotype. Preston *et al* generated mutant mice ($Pole^{exo/exo}$ mice and $Pold1^{exo/exo}$ mice), in which the genes encoding in part the DNA polymerases Pol δ and Pol ε were substituted with genes that encoded for mutants for specific regions in these DNA polymerases. The wild type mice had the longest survival compared to the mutant mice. When both mutations were present, survival was shortest in these mutant mice, and survival was inversely proportional to the incidence of spontaneous tumours. Image reproduced from Loeb 2011 (Loeb 2011).

1.3.6 Mitochondria and random point mutations

The role of mitochondrial dysfunction in accelerated ageing is well established (Beal 2005, Trifunovic 2006). The free radical theory states that the steady accumulation of oxidative damage to macromolecules causes age-associated reductions in physiologic functions and reduces life expectancy. It is for this reason that the mitochondria have been implicated in accelerated ageing processes such as Alzheimer's disease and Parkinson's disease (Melov, Schneider et al. 1998, Beal 2004). Trifunovic *et al.* in an extensive review of mitochondria and ageing demonstrated increased ROS were associated with increased random mitochondrial mutations in the control setting. However, in an environment of enhanced mutagenesis the knock-on effect was disturbed respiratory chain function, subsequent defective oxidative phosphorylation, and no increase in the production of ROS (Trifunovic 2006). The resultant effect of this increased rate of mitochondrial mutagenesis was premature ageing seen in mitochondrial DNA mutator mice, all demonstrating accelerated ageing signs such as increased weight loss, loss of subcutaneous fat, alopecia, reduced fertility, osteoporosis and cardiomyopathy (Figure 1.11).

Telomere shortening and p53 activation are also recognised to play important roles in ageing (Balaban, Nemoto et al. 2005, Wallace 2005, Sahin, Colla et al. 2011, Sahin and DePinho 2012). Telomeres are nucleoprotein complexes at the ends of chromosomes, which maintain chromosome integrity and whose length is proportional to the expected cell age. Sahin *et al.* demonstrated telomere dysfunction was linked to mitochondrial compromise (Sahin, Colla et al. 2011, Moslehi, DePinho et al. 2012, Sahin and DePinho 2012). Telomere shortening was shown to repress master mitochondrial regulators, peroxisome proliferator-activated receptor gamma, co-activator 1 alpha and beta (PGC-1α and PGC-1β), and thus disrupt mitochondrial biogenesis. This was associated with decreased gluconeogenesis, cardiomyopathy, and increased ROS in mice models. Sahin *et al.* demonstrated that the telomere-mitochondrial connection was controlled by p53. Disrupted telomeres activated p53, which inactivated the master mitochondrial regulators, and a direct link between telomere length and mitochondrial function was effectively demonstrated (Sahin, Colla et al. 2011). These findings of

accelerated ageing and mitochondrial dysfunction are transferrable to cancer, which is in itself an accelerated ageing process.

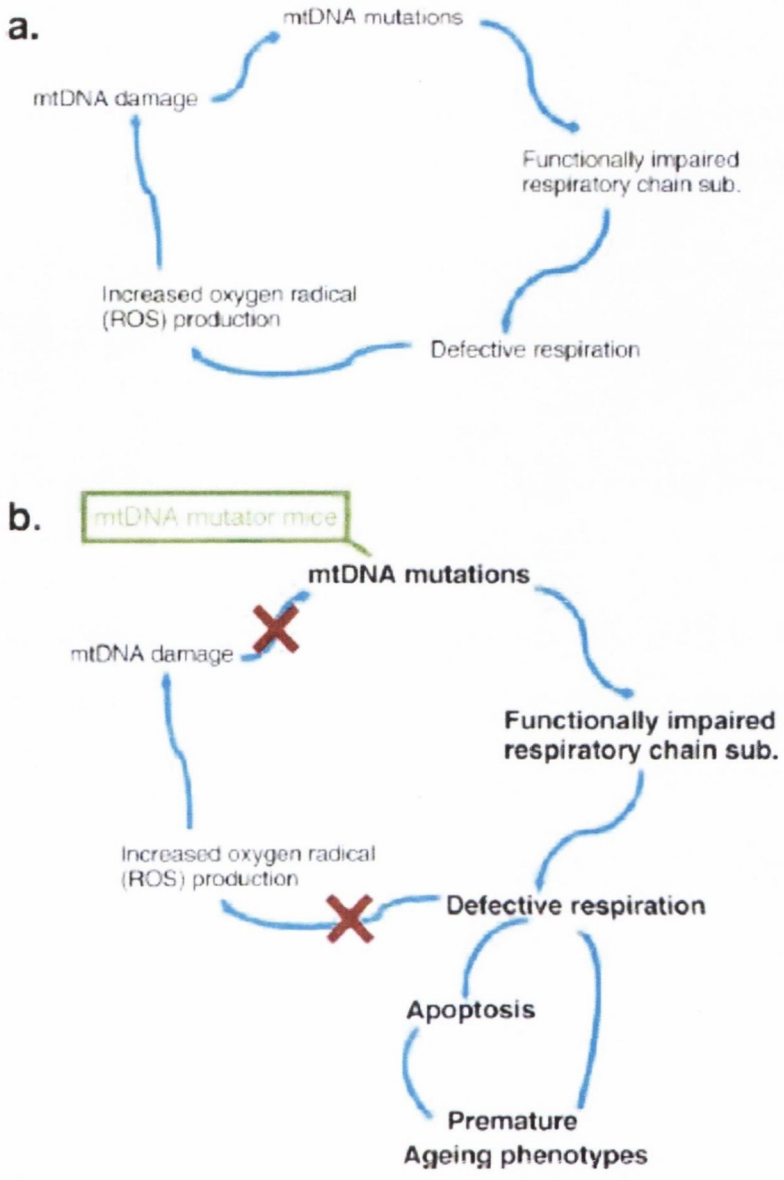


Figure 1.11: The effects of random mitochondrial point mutations in accelerated ageing. **(a)** Mitochondrial DNA mutations accumulate progressively during life and are responsible for defective oxidative phosphorylation, resulting in enhanced ROS production. This resultant increase in ROS leads to increased levels of mitochondrial DNA damage and mutations, thus causing a vicious cycle of increasing oxidative damage and dysfunction, resulting in ageing and ultimately cell death. **(b)** A 3- to 5-fold increase in the level of mitochondrial mutations in mitochondrial DNA mutator mice results in defective respiration and subsequent energy deficiency within the cells. This leads to increased apoptosis, a reduction in lifespan and the development of premature onset ageing-related phenotypes. Image reproduced from Trifunovic *et al.* (Trifunovic 2006).

In a collaborative study, the role of random mitochondrial point mutations were examined in adenomas and colorectal cancer *in-vivo* (Ericson, Kulawiec et al. 2012). Random mitochondrial mutations, at the COX-1 and 12s rRNA genes on the mitochondrial genome, were significantly decreased in colorectal carcinoma compared to adenomas. It was postulated that the decrease in random mutations in the cancer tissue was probably due to positive selection of a random mutation and subsequent clonal proliferation. Loeb *et al.* proposed that malignant cells, once established, undergo selection processes, where increased numbers of both lethal and neutral mutations would be expected; however, the neutral mutations would be more inclined to survive and capable of undergoing subsequent replication (Loeb, Springgate et al. 1974). Perhaps a clonal mutation and not random mutations supersede the initial catalyst for cancer development at this time point (Loeb, Loeb et al. 2003). In addition to the higher levels of random point mutations seen in the adenomas, there were significant differences in energy metabolism between the two different disease points. Tumour tissue had increased rates of glycolysis as represented by higher levels of lactate and lower levels of citrate. This altered energy metabolism at the cancer stage was consistent with the Warburg effect.

1.3.7 DNA repair processes

As previously stated by Preston and colleagues, random mutations may accumulate when there is an inability to repair DNA damage (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009). In their studies, defective proofreading by DNA polymerases, Pol-δ and Pol-ε, correlated with increased mutagenesis, stronger cancer predisposition and reduced lifespan (Figure 1.10). Shen *et al.* demonstrated that the increased oxidative damage seen in aging cells was in part attributable to reduced base excision repair and this played a significant role in aging (Shen, Galick et al. 2003). The mitochondria are especially important in DNA damage because the ROS produced in their electron transport chain can cause cellular injury and have been implicated in ageing and carcinogenic processes (Marnett 2000, Raha and Robinson 2000). Furthermore, the mitochondria are particularly vulnerable to DNA damage due to their close proximity to ROS, as up to 90% of free radicals within a cell are produced in the mitochondria themselves (Yakes and Van Houten 1997). The mitochondrial DNA also accumulates more DNA damage compared with nuclear DNA due to a combination of

factors such as lack of protective histones and less proficient DNA repair mechanisms (Wallace 1992, Wallace 1992). Thus, DNA repair mechanism may play an important role in preventing the propagation and accumulation of random mutations, and may be essential in maintaining genomic integrity and stability.

DNA repair mechanisms include base-excision, nucleotide excision, mismatch and double-strand break repair (Helleday, Petermann et al. 2008). ROS and oxidative stress result in the formation of large numbers of base lesions, the predominant type being the highly mutagenic 8-oxo-dG, which is capable of pairing with adenine during DNA replication, leading to GC→TA transversions (Moriya 1993). Specific to 8-oxo-dG damage, Anson *et al.* investigated damage caused by photo-activated methylene blue in human fibroblasts and demonstrated damage was removed by DNA repair processes rather than as a results of DNA replication, cell loss or cell degradation dampening the damaged DNA content (Anson, Croteau et al. 1998). Mitochondrial DNA has a deficient DNA repair capacity, in particular mitochondria completely lack nucleotide excision repair mechanisms (Clayton, Doda et al. 1974). It has been shown that mitochondrial DNA damage is primarily repaired by base excision (Sawyer and Van Houten 1999, Wallace 2002). Base excision repair follows three specific steps (Croteau, Stierum et al. 1999). (1) The process is initiated by DNA glycosylases, a groups of enzymes that identify specific sets of modified bases, such as 8-oxo-dG (Krokan, Standal et al. 1997), cleaving N-glycosylic bonds between the altered base and sugar. (2) Apyrimidinic/apurinic endonuclease incise the DNA backbone, generating a 3'-hydroxyl group, which is subsequently extended by polymerases. (3) DNA ligases complete the pathway, joining the free DNA ends.

1.4 OXIDATIVE STRESS, INFLAMMATION AND REDOX BALANCE

1.4.1 Inflammation and oxidative stress

Cancer development is viewed as an uncontrolled inflammatory response, with associated loss of control over mediators which promote tissue destruction, cell proliferation and angiogenesis. Almost one fifth of all cancers arise secondary to an infective process (Parkin 2006), while the pathogenesis of many other cancers have

been attributed to inflammatory conditions and chemical and physical irritants which work to evoke an inflammatory response (Ekbom, Helmick et al. 1990, Gulumian 1999). Barrett's is another inflammatory condition which leads to oesophageal adenocarcinoma, considered by many a malignancy of chronic inflammation (Picardo, Maher et al. 2012, Poehlmann, Kuester et al. 2012).

The redox system plays an important role in cell homeostasis. ROS are products of normal cellular metabolism, acting as both damaging and beneficial species. In normal physiological circumstances ROS maintain redox balance, however; overproduction results in loss of equilibrium between free radicals and antioxidants leading to oxidative damage and inflammation (Figure 1.12). Our group has previously investigated the effects of mitochondrial instability in inflammatory arthritis (Biniecka, Fox et al. 2011, Biniecka, Kennedy et al. 2011). In addition to excessive production of ROS generating pro-mutagenic DNA adducts such as 8-oxo-dG, ROS are lipid peroxidation-inducing agents, with 4-HNE formed during lipid peroxidation of 6-polyunsaturated fatty acids by superoxide (Poli, Biasi et al. 2008). In addition, it has been demonstrated that 4-HNE induces intercellular production of ROS (Nakamura, Miura et al. 2009). Our studies in inflammatory arthritis have shown hypoxia is associated with increased inflammation (Ng, Biniecka et al. 2010). A positive correlation between hypoxia and random mitochondrial point mutations was identified, and these correlated with increased ROS, 8-oxo-dG and 4-HNE (Biniecka, Kennedy et al. 2010). Furthermore, anti-TNF treatment was investigated, and patients with a good treatment response demonstrated reduced synovitis and reduction in inflammatory markers, C-reactive proteins, CD4 and CD68; this strongly correlated with increased oxygen tension in the arthritic joint (Kennedy, Ng et al. 2010). Thus, these studies effectively demonstrated a link between mitochondrial instability and inflammation. We showed in inflammatory arthritis mitochondrial instability was stabilised with antioxidant therapy (Biniecka, Kennedy et al. 2010). Antioxidants work to restore balance caused by increased ROS in the redox system, and may represent a novel therapeutic strategy in resolving this inflammation and oxidative damage.

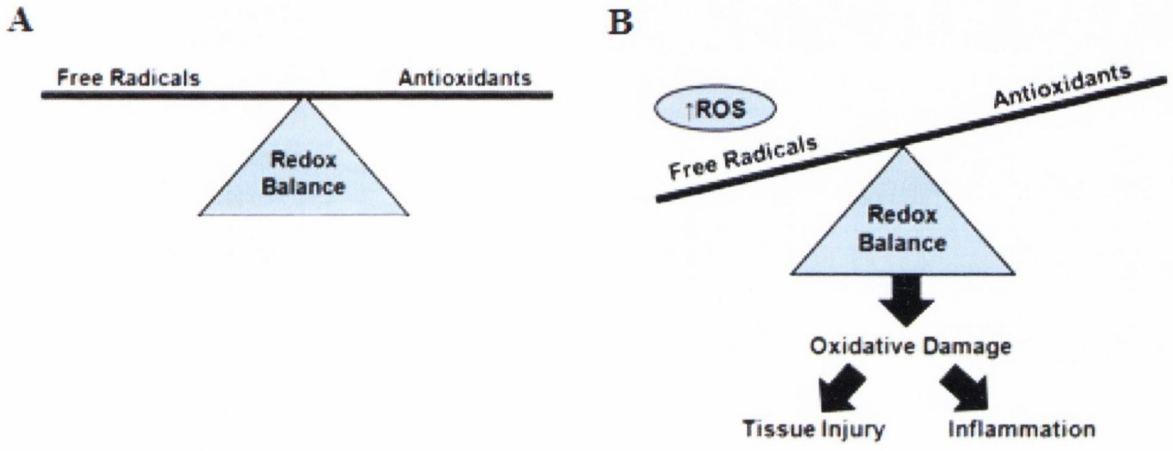


Figure 1.12: Redox system. (A) There is equilibrium between free radicals and antioxidants. **(B)** Loss of equilibrium between free radicals and antioxidants initiates a cascade of events resulting in inflammation and tissue damage (original image by NJ O'Farrell).

1.4.2 The potential role of antioxidants in the treatment of oxidative stress and inflammation

Previous studies performed by our group in the inflammatory condition, rheumatoid arthritis, have shown a direct correlation between increased random mitochondrial point mutations, oxidative stress and inflammation (Biniecka, Kennedy et al. 2011). Antioxidants inhibit or reduce oxidation of molecules and as a result reduce the production of ROS and oxidative stress. The applicability of antioxidants as a treatment for Barrett's oesophagus, an inflammatory process, therefore seems to be a reasonable therapeutic strategy requiring further exploration.

The therapeutic benefits of antioxidants have been seen in treatment of pulmonary fibrosis, cirrhosis and non-alcoholic steatohepatitis (Demedts, Behr et al. 2005, Baumgardner, Shankar et al. 2008, Vercelino, Crespo et al. 2010). N-acetylcysteine (NAC), an antioxidant commonly used in clinical practice to protect the kidneys from nephrotoxicity, has proven anti-inflammatory effects, reducing the synthesis of inflammatory cytokines, IL-6, IL-8 and TNF-α (Del Sorbo and Zhang 2004). Epidemiological evidence has demonstrated that consumption of green tea is associated with reduced incidences of cancer (Katiyar and Mukhtar 1996). We and others have shown that epigallocatechin-3-gallate (EGCG), a component of green tea, demonstrated antioxidant potential in prostate cancer (Morrissey, Brown et al. 2007), potentially due to its ability to decrease oxidative damage which has the well-established role in cancer initiation and progression (Guo, Zhao et al. 1999, Toschi, Bordoni et al. 2000). Dimethyloxalylglycine (DMOG), while not an antioxidant, is a hydroxylase inhibitor (which may affect antioxidant pathways), and has demonstrated protective properties reducing inflammation in colitis (Cummins, Seeballuck et al. 2008).

Specific to oesophageal adenocarcinoma, Jenkins *et al.* (Jenkins, D'Souza et al. 2007) examined the effects of the antioxidant vitamin C. *In-vitro*, pre-treatment with vitamin C significantly reduced the ROS load in DCA treated cells. Our group has previously published a pilot study examining the effects of dietary vitamin C on the expression of NF-κB and pro-inflammatory cytokines in Barrett's patients (Babar, Abdel-Latif et al. 2010). NF-κB is a central regulator of genes responsible for inflammation and carcinogenesis. We and others have shown NF-κB is progressively activated along the

Barrett's sequence, from oesophagitis to oesophageal adenocarcinoma (Abdel-Latif, O'Riordan et al. 2005, O'Riordan, Abdel-latif et al. 2005). In this study of 25 patients with Barrett's oesophagus, patients were administered 1000 mg/day of oral vitamin C. Pre- and post-vitamin C endoscopies and biopsies were performed and 35% of patients were found to experience down-regulation in the expression of NF-κB and pro-inflammatory cytokines.

In non-cancer and pre-malignant settings the role of antioxidants are seen to be beneficial. However, Das *et al.* highlighted the fine balance between antioxidants and ROS in a malignant setting (Das, Suman et al. 2013). The authors acknowledged that a moderate increase in ROS is seen to induce cell proliferation, while an excessive increase promotes cell apoptosis. In prostate cancer, the benefits of excessive ROS were analysed, through the treatment of prostate cancer cells with the pro-oxidant, dietary agent, 3, 9-dihydroxy-2-prenylcoumestan (psoralidin). Psoralidin showed it had the potential to be a specific target in prostate cancer cells, by inhibiting the migratory and invasive properties seen at baseline, inducing apoptosis and cell death. This pro-oxidant specifically altered mitochondrial function, leading to increased loss of mitochondrial membrane potential, cytochrome c release and cell apoptosis. Transfection with endogenous antioxidant enzymes, superoxide dismutase (SOD), and treatment with NAC inhibited ROS production *in-vitro*, and subsequently prevented the beneficial and inhibitory effects of psoralidin. Das *et al.* concluded that manipulation of ROS thresholds may be used in the treatment of prostate cancer, inhibiting cancer growth and metastasis.

The potential role of antioxidants warrants investigation along each stage of the Barrett's oesophagus disease sequence.

1.5 AIMS AND OBJECTIVES

1.5.1 Overall hypothesis

Mitochondrial instability is important in driving disease progression in Barrett's oesophagus, and random mutations and alterations in the oxidative stress environment contributes to this instability.

1.5.2 Overall aim

The overall aim of this thesis is to assess if levels of random mitochondrial mutations and mitochondrial instability can identify Barrett's oesophagus patients who progress to oesophageal adenocarcinoma.

1.5.3 Specific objectives

1. Investigate the frequency and spectrum of random mitochondrial mutations and mitochondrial function at baseline using a Barrett's oesophagus *in-vitro, in-vivo* and *ex-vivo* models.
2. Assess the effects of the bile acid, DCA, on the random mitochondrial mutations and mitochondrial function *in-vitro* and *ex-vivo*, and examine the role of antioxidants in rescuing mitochondrial instability.
3. Investigate the role of oxidative stress and T cell markers in the progression of Barrett's oesophagus using archived sequential samples and correlate levels of oxidative stress and T cell markers with levels of random mitochondrial point mutations.
4. Examine oxidative stress and DNA repair genes' expression along the Barrett's disease sequence *in-vitro* and *in-vivo*.

Chapter 2: Characterisation of mitochondrial function *in-vitro*, *in-vivo* and *ex-vivo* across the Barrett's disease sequence

2.1 INTRODUCTION

Barrett's oesophagus is the sole pathologic precursor of oesophageal adenocarcinoma. Following the classic metaplasia-dysplasia-adenocarcinoma sequence, it is speculated cancer development does not occur directly from intestinal metaplasia (Jankowski, Wright et al. 1999). As Barrett's oesophagus follows this natural stepwise progression, it makes the ideal candidate for interval surveillance. However, with incidences of cancer progression low, at less than 1% per annum, recent publications have questioned the relevance and timing of these surveillance programmes (Yousef, Cardwell et al. 2008, Hvid-Jensen, Pedersen et al. 2011). Ultimately the goal is to be able to stratify patients with metaplastic Barrett's oesophagus into high and low cancer risk groups, eliminating the need for rigorous life-long follow-up in the majority of patients unlikely ever to proceed to malignancy.

Excluding the nuclei, mitochondria are the only cellular organelles that contain their own DNA, with each cell containing hundreds of mitochondria and correspondingly thousands of strands of the mitochondrial genome. It is therefore possible for wild type and mutated mitochondrial DNA to co-exist (Chatterjee, Mambo et al. 2006). The rate of mutagenesis in mitochondrial DNA is approximately 10-times higher than mutation rates in the nuclear DNA. This is due to a combination of factors such as lack of protective histones, less proficient DNA repair mechanisms and high levels of exposure to ROS generated by the mitochondria during oxidative phosphorylation (Wallace 1992, Wallace 1992). These factors, along with evidence of altered energy metabolism in the cancer setting, i.e. aerobic glycolysis (Warburg 1956, Warburg 1956), have highlighted the potential role of the mitochondria in human cancer development (Chatterjee, Mambo et al. 2006, Kroemer 2006, Putignani, Raffa et al. 2008). Studies have predominantly focussed on clonal mitochondrial mutations and little data exists around the potential implications of random mitochondrial mutations in cancer development. Specific to oesophageal carcinoma, displacement-loop, or D-loop, mutations have been identified as playing a role in oesophageal cancer (Hibi, Nakayama et al. 2001, Miyazono, Schneider et al. 2002, Abnet, Huppi et al. 2004). The D-loop, is a major control site in mitochondrial genomic expression, involved in DNA replication and in the promotion of transcription (Taanman 1999). Miyazono *et al.*

(Miyazono, Schneider et al. 2002) demonstrated increased frequencies of D-loop mutations in Barrett's adenocarcinoma, predominantly unique to the tumour site and not present in the surrounding Barrett's tissue. The authors hypothesised that their findings supported a role for oxidative stress as a precursor for adenocarcinoma development given the relationship between mitochondrial mutations and high levels of free radicals. However, the exact role of these mitochondrial mutations along the metaplasia-dysplasia-adenocarcinoma sequence remains to be elucidated.

Random mutations offer an alternative strategy for cancer stratification. It is hypothesised that during tumour development, cancer cells exhibit the mutator phenotype and higher frequencies of random mutations which would not be supported by the rate of mutagenesis in normal cells (Loeb, Bielas et al. 2008). Loeb *et al.* therefore proposed that cancer cells must incur increased rates of mutagenesis during disease progression (Loeb, Springgate et al. 1974, Loeb, Bielas et al. 2008), and as such, this theory suggests that benign tumours or pre-cancerous inflammatory conditions with low levels of random mutations would not progress to malignancy. A recent publication by Lee *et al.* (Lee, Han et al. 2012), identified significantly increased random mitochondrial DNA mutations between Barrett's intestinal metaplasia compared to adjacent normal tissue, as well as increased free radicals in areas of metaplasia. The authors stated that this instability within the mitochondria may play a crucial role in Barrett's cancer development, however, no analysis compared intestinal metaplasia which progressed to oesophageal adenocarcinoma and non-progressive metaplasia, nor were these changes explored along the Barrett's disease sequence. In colorectal cancer, Ericson *et al.* (Ericson, Kulawiec et al. 2012) evaluated the implications of random mitochondrial point mutations in the pre-malignant setting of colonic adenomas. Interestingly, the level of random mutations were significantly increased in adenomas, and it was hypothesised that these random mutations became redundant once the cancer was established; potentially a clonal mutation, which expanded as a result of natural selection, superseded the triggering random mutagenesis which may be the potential instigator of this disease progression.

To date, detailed mitochondrial mechanisms involved in the progression of Barrett's oesophagus to oesophageal adenocarcinoma remains unknown. Understanding the

differences in mitochondrial function along the different stages of this disease spectrum remains to be elucidated.

2.2 HYPOTHESIS AND AIMS OF CHAPTER TWO

We hypothesise that Barrett's oesophagus patients with low levels of random mutations would not have the capabilities to progress to oesophageal adenocarcinoma, whereas those with high levels would have an inherent predisposition to undergo malignant conversion.

Specific aims of chapter two;

(1) Investigate the frequency of random mitochondrial point mutations and alterations in mitochondrial function in a cell line model representing the Barrett's oesophagus disease sequence.
(2) Determine if findings from the *in-vitro* model could be replicated in *in-vivo* patient samples.
(3) Using an *ex-vivo* Barrett's human explant model, assess the secretion levels of inflammatory and mitochondrial biomarkers.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

All laboratory chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (MO, USA) unless otherwise stated, and prepared and stored according to manufacturer's specifications. Solid reagents were weighed using a Scout Pro electronic balance (Ohaus Corporation, NJ, USA) or an Explorer Pro fine electronic balance (Ohaus Corporation, NJ, USA), and made up using double distilled water, unless otherwise stated. The pH of solutions was measured using a pH 211 microprocessor pH metre (Hanna instruments, RI, USA), calibrated with buffers at pH 4, pH 7, and, pH 10. Gilson pipettes (Gilson S.A., France), were used to transfer liquid volumes up to 1ml, electronic pipette aids (Drummond, PA, USA) and disposable Pasteur pipettes (Starstedt Ltd., Wexford, Ireland) were used for volumes greater than 1ml and graduated cylinders were used for volumes in excess of 10ml.

2.3.2. Cell culture

2.3.2.1 Barrett's sequence cell lines

All cell culture media was purchased from Lonza (MD, USA) and cell culture plastics were purchased from Starstedt Ltd. (Wexford, Ireland) unless otherwise stated. Four oesophageal cell lines were used; HET1A, QH, Go and OE33 cells representing the normal squamous epithelium-intestinal metaplasia-HGD-adenocarcinoma sequence, respectively (Figure 2.1). HET1A cells were obtained from American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK), and maintained in antibiotic-free bronchial epithelial cell basal media (BEBM) enhanced with hormonal cocktail BEGM® SingleQuots®. QH and Go cells were obtained from ATCC and cultured in BEBM enhanced with BEGM® SingleQuots® and supplemented with 10% foetal calf serum (FCS) (Lonza) and 1% penicillin/streptomycin (Lonza). OE33, Barrett's oesophageal adenocarcinoma, was sourced from the European Collection of Cell Cultures (Sailsbury, UK) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were incubated at 37°C in 95% humidified air containing 5% CO_2.

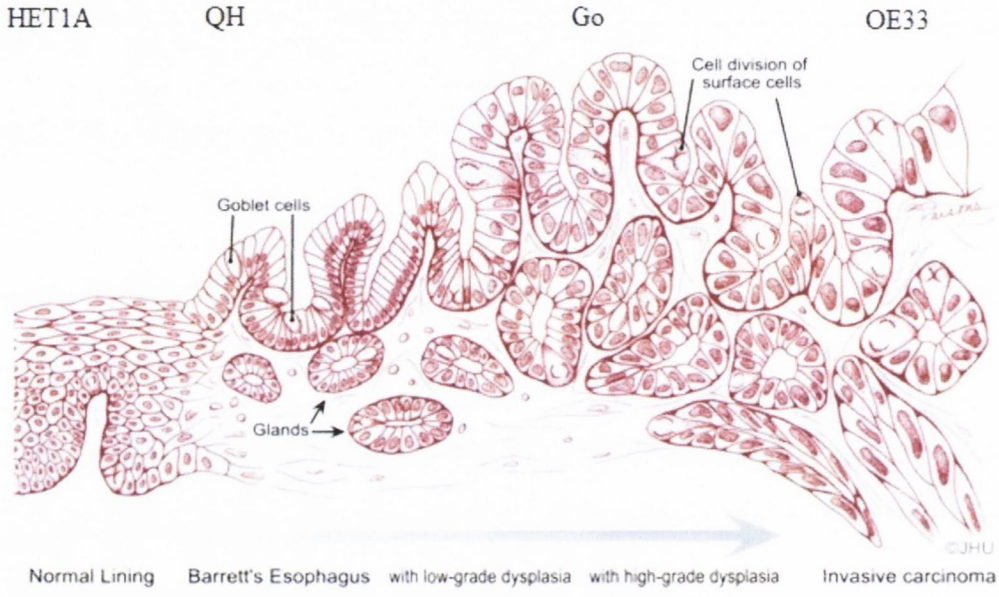


Figure 2.1: Barrett's oesophagus cell line sequence. HET1A cells are normal oesophageal squamous epithelium. QH cells are Barrett's oesophagus speclialised intestinal metaplasia. Go cells are derived from Barrett's oesophagus high grade dysplasia. OE33 cells are derived from invasive Barrett's oesophageal adenocarcinoma. (Image reproduced from http://pathology2.jhu.edu/beweb/definition.cfm)

2.3.2.2 Cell line subculture

All tissue culture was carried out using an aseptic technique in a grade II laminar air flow cabinet, cleaned and sterilised monthly. The unit was switched on at least 10 min before use. The unit, and all equipment and reagents used within the unit, were swabbed with industrial methylated spirits 70% (v/v) (Lennox Laboratory Supplies, Dublin, Ireland) before and after use.

Cell lines were examined daily using an inverted phase contrast Nikon microscope (Nikon Corporation, Tokyo, Japan) and subcultured upon reaching 80-90% confluency. 5ml of trypsin Ethylene Diamine Tetra Acetic Acid (EDTA) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the flask and incubated at 37°C for several minutes or until the cells detached from the surface of the flask. 10ml of complete media (containing 10% FCS) was added to inactivate the trypsin and cells were seeded at appropriate densities. For HET1A cells (whose media was not supplemented with FCS) the trypsinised cells were placed into 15ml tubes and centrifuged at 180 x g for 3min to produce a pellet. The media was poured off and HET1A BEBM media added to the pellet and mixed.

2.3.2.3 Preparation of frozen stocks

Frozen stocks were prepared from cell lines growing in the exponential growth phase and at 70-80% confluency. Cells were trypsinised as described above (section 2.3.2.2) and 10ml of maintenance media was added to inactivate the trypsin. Cells were centrifuged at 180 x g for 3min, the supernatant decanted, and pelleted cells were resuspended in FCS containing 10% dimethylsulfoxide (DMSO). The cell suspension was divided in 1ml aliqouts and cryovials were then transferred to a -80°C freezer for short term storage, or under liquid nitrogen for long term storage.

2.3.2.4 Reconstitution of frozen stocks

Frozen stocks were thawed rapidly at 37°C and added to 5ml of appropriate maintenance medium, and centrifuged at 180 x g for 3min. The supernatant was decanted and the cell pellet was resuspended in 5ml of maintenance media, transferred to a 25cm^2 flask and incubated overnight at 37°C and 5% CO_2. Media was replaced the

following day to remove any dead cells and subculture was continued as described (section 2.3).

2.3.2.5 Cell counting

Cells were counted using a Bright Line haemocytometer (Hausser Sceintific, PA, USA). Cells were trypsanised as described (section 2.3), added to 10ml of maintenance medium and centrifuged at 180 x g for 3 min. The supernatant was decanted and the cell pellet resuspended by repeated pipetting in 1ml maintenance media. 20µl of the cell suspension was added to 180µl trypan blue (0.4% w/v) solution and allowed to sit at room temperature for 1 min. Viable cells were unstained due to their exclusion of trypan blue, wheraes dead cells were stained blue, due to their disrupted membranes. 20µl of this cell suspension was added to the counting chamber of the haemocytometer and the number of viable cells in of of the four corners of the grid were counted. The number of cells per ml was calculated using the following equation:

$$(N/4) \times 10^4 \times 10 \text{ (dilution factor)} = \text{no. cells/ ml}$$

Where,

N = total cell number counted

4 = number of fields counted

10 = dilution factor

10^4 = constant

When necessary, all densities were adjusted using complete medium, unless otherwise stated.

2.3.2.6 Mycoplasma testing

Cell lines were routinely tested for mycoplasma contamination using a MycoAlert® Mycoplasma detection kit (Lonza). The MycoAlert® assay is a biochemical test that exploits the activity of certain mycoplasma enzymes. These enzymes react with the MycoAlert® substrate, catalysing the conversion of ADP to ATP. A ratio of the level of ATP in a sample before and after the addition of substrate indicates the presence or absence of mycoplasma. If mycoplasma enzymes are present, their reaction with the substrate results in elevated ATP levels. Cells were maintained and subcultured in antibiotic-free medium for a minimum of 2 passages. A 1 mL volume of culture

medium was taken from culture flasks and centrifuged at 180 × g for 3 min to pellet any cells and debris present. A 100 µL volume of supernatant was then added to a white-walled 96-well plate (Corning Incorporated, Corning, NY, USA). MycoAlert reconstitution buffer was used as a negative control, and supernatant from an in-house mycoplasma contaminated cell line provided a positive control. A 100 µL volume of reconstituted MycoAlert Reagent was added to each sample and incubated for 5 min at RT°C. A 1 s integrated luminescence reading was then taken (Reading A) using a Wallac Victor^2 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA). A 100 µL volume of MycoAlert substrate was then added to each sample and incubated for 10 min at RT°C. Luminescence was then measured as before (Reading B). The ratio of reading B to reading A was calculated, and a ratio of greater than 1 indicated mycoplasma infection.

2.3.3 Evaluation of mitochondrial function using mitochondrial assays for reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and mitochondrial mass

Cells were seeded in 96 well plates at density 2,500 to 8,000 cells/well, specific for each cell line. Seeding at different concentrations was necessary to compensate for the different growth rates between individual cell lines, in order to ensure the same degree of confluence at the initiation of treatments. Following 24hrs incubation, ROS production, MMP (mitochondrial membrane potential) and mitochondrial mass were assessed. To measure the level of ROS, cells were washed twice with a buffer (130mM NaCl, 5mM KCl, 1mM Na2HPO4, 1mM CaCl2, 1mM MgCl2 and 25mM Hepes, pH 7.4). DCFH-DA is a non-fluorescent molecule which diffuses into the cells where it is deacetylated and rapidly oxidized to highly fluorescent 2, 7 dichlorofluorescein (DCF) (Sigma-Aldrich) in the presence of the generated ROS. DCF emits a fluorescent signal of the product which is linearly related to the intracellular hydrogen peroxide concentration. To measure mitochondrial membrane potential, cells were washed using the above buffer and loaded with 5µM rhodamine-123 (Sigma-Aldrich) for 40min in the buffer at 37°C. Rhodamine-123 is taken up selectively by mitochondria, and its uptake is dependent on mitochondrial membrane potential. There is a linear relationship between the intensity of rhodamine-123 and MMP, as an indicator of polarisation of mitochondrial membrane. To measure mitochondrial mass, cells were washed using the

above buffer and loaded with mitotracker probe (Invitrogen) which is a cell-permeant mitochondrion-selective dye that contains a mildly thiol-reactive chloromethyl moiety (Invitrogen). The chloromethyl group appears to be responsible for keeping the dye associated with the mitochondria after fixation. Cells were loaded with 300nM of mitotracker for 40min in the buffer at 37°C. Following 40min incubation, ROS, MMP and mitochondrial mass probes were removed, cells were washed with the above buffer and analysed using the Spectra Max Gemini System. 2, 7 dichlorofluorescein diacetate and Rhodamine 123 were excited at 485nm, and fluorescence emission at 538nm was recorded. Mean fluorescence values for each condition were obtained from a minimum of three independent experiments.

2.3.4 Crystal violet assays

Crystal violet assays were performed at the same time as all functional assays to allow for normalisation to cell number between different cell lines and different treatments. Cells were seeded in 96 well plates at density 2,500 to 8,000 cells/well and 100µl of media was added and incubated for 24hrs. Seeding at different concentrations was necessary to compensate for the different growth rates between individual cell lines, in order to ensure the same degree of confluence at the initiation of treatments. Following 24hrs, the media was decanted. Cells were washed with PBS. Cells were fixed with 1% gluteraldehyde (Sigma-Aldrich) for 20min, 1% gluteraldehyde was discarded and 0.1% crystal violet solution was added for 30min and was removed by washing with water. Plates were blotted on tissue paper and were allowed to air dry on the bench overnight. Once dry, the cells were resuspended in 1% Triton X100 (Sigma-Aldrich) and incubated on a shaker for 15min. The absorbance was read at 550 nm using a Perkin Elmer Wallac 1420 Victor2 plate reader.

2.3.5 *In-vivo* and *ex-vivo* sample processing

These studies were approved by the St James's Hospital and Adelaide, Meath and National Children's Hospital Institutional Review Board. Biopsy specimens and blood samples were taken from all patients with informed consent. Patients were excluded from the study if they had another cancer at the time of sampling, or if they had a chronic infectious disease, such as HIV, Hepatitis B or Hepatitis C.

Fresh samples were snap frozen in liquid nitrogen and stored at -80°C for random capture assay experiments. Patients with histologically confirmed intestinal metaplasia (n=11) and Barrett's-associated oesophageal adenocarcinoma (n=9) were recruited while in attendance of Barrett's oesophagus surveillance endoscopy or oesophagectomy at the National Oesophageal and Gastric Centre at St. James's Hospital, Dublin. Barrett's biopsies and tumour tissue were snap-frozen prior to DNA extraction for use in the mitochondrial random mutation capture assay, outlined in section 2.3.6.2.

Barrett's oesophagus patients' biopsies (n=12), from areas of intestinal metaplasia and surrounding normal tissue, were also obtained for immediate *ex vivo* explant culture at 37°C. Matched-normal tissue biopsies were taken $\geq$ 5 centimetres from the most proximal border of macroscopic Barrett's. Biopsies were immediately placed on saline-soaked gauze and transported within 10 minutes to the laboratory. Barrett's and matched normal biopsies used for culture were placed into a well of a 24-well plate containing 1 mL of M199 media (Lonza) supplemented with 10% FCS, 1% penicillin/streptomycin and 1 µg/ml insulin. Tissues were cultured for 24hrs, and conditioned media stored at -80°C. Barrett's tissue was characterised by examining for the expression of the columnar epithelium molecular markers, cytokeratin 8 (Metabion, Martinsried, Germany) and villin (Metabion). Tissue viability following explant culture was confirmed using a lactate dehydrogenase (LDH) assay (Caymanchem, Michigan, USA).

2.3.6 Measurement of random mitochondrial point mutations

2.3.6.1 DNA extraction

In-vitro, HET1A, QH, Go and OE33 cells, of equal passage numbers (+/- 10) were grown to 70-80% confluence in 25cm^2 flasks. Cells were trypsinised as described in section 2.3.2.2, then neutralised with the appropriate media for each cell line and centrifuged at 180 g for 3min. Cell pellets were lysed in lysis buffer (10mM Tris-HCL, pH 8.0, 150mM NaCl, 20mM EDTA, 0.5% SDS buffer), vortexed and transferred to 1.5 ml eppendorf tubes, then digested with Proteinase K (Qiagen, West Sussex, UK) at a final concentration of 0.2 mg/ml. Following addition of Proteinase K, samples were gently mixed, (inverting eppendorf tubes 10 times) and incubated overnight at 56°C.

In-vivo, mitochondrial DNA was isolated from snap frozen biopsied or resected tissue (stored at -80°C) using methods described for cell line work, however; tissues required homogenisation in lysis buffer using the QIA tissue-lyser (Qiagen) at 25Hz for 5-10 mins. Proteinase K was added and samples were intubated over night at 56°C. Mitochondrial DNA was extracted using 25:24:1 phenol-chloroform-isoamyl alcohol (Sigma-Aldrich) added in a 1:1 ratio with lysed tissue, mixed by shaking, and centrifuged at 1,800 g for 10 minutes. The aqueous phase was removed from the top of the solution, without disturbing the interphase. The aqueous solution was mixed with 25:24:1 phenol-chloroform-isoamyl alcohol (Sigma-Aldrich) in a 1:1 ratio and re-extracted. The aqueous phase was removed and mixed with 99% chloroform in a 1:1 ratio, thoroughly mixed and centrifuged at 13,000 RPM x 10 mins. One-tenth volume of 3M sodium acetate was added to the extracted aqueous phase and isopropanolol (Sigma-Aldrich), samples were mixed and centrifuged at 13,000 RPM. The resultant pellet was precipitated with 2 to 2.5 volumes of ethanol (70% v/v). The DNA pellets were resuspended in 50µl 10mM TrisCl.

2.3.6.2 Random mutation capture assay

To characterise the frequencies of random mutations *in-vitro* and *in-vivo* samples we used the mitochondrial Random Mutation Capture (RMC) assay as previously published (Vermulst, Bielas et al. 2008) (see figures 2.2 & 2.3). This quantitative PCR-based approach allows for exact determination of mutation frequencies following the exhaustive digestion of all wild type (non-mutant) sequences by the restriction enzyme, *Taq*$^{\alpha}$I. This methodology allows for the exact determination of mutation frequencies in high-throughput screens that interrogate millions of base pairs simultaneously. This methodology screens and detects the presence of random mitochondrial point mutations in the gene encoding the 12S rRNA subunit (bp1215-1218). Single base pair changes in one of the 12S rRNA encoding genes may impair protein translation of some or all of the mitochondrial DNA encoded subunits of the electron transport chain. All biopsies were analysed in a blinded fashion. Ten micrograms of mitochondrial DNA were digested with 100 units of *Taq*$^{\alpha}$I restriction enzyme (New England BioLabs, Herts, United Kingdom), 1 x bovine serum albumin (BSA) (New England BioLabs, Herts, United Kingdom) and a *Taq*$^{\alpha}$I-specific digestion buffer (10mM Tris-HCl, 10mM

$MgCl_2$, 100mM NaCl, pH 8.4) (New England BioLabs, Herts, United Kingdom) for 10 hours; 100 units of *Taq*$^{\alpha}$I being added to the reaction mixture every hour.

Quantitative real-time PCR amplification was performed in 25µl reactions, containing 12.5µl 2 x SYBR Green Brilliant Mastermix (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA), 0.7µl of 10pM/µl forward and reverse primers (Integrated DNA Technologies, Inc., San Diego, CA, USA) and 6.7µl water. Samples were added to each well of a 96 well plate, as outlined in Figure 2.4. The reaction plate was sealed using an optical adhesive cover (Applied Biosystems), and the plate was centrifuged briefly to pool reagents and eliminate any bubbles. Real-time PCR detection was performed using an ABI Prism 7900HT real-time thermal cycler (Applied Biosystems). The samples were amplified using the following protocol: 37°C for 10 minutes and 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute. Samples were held at 72°C for 7 minutes and following melt curve analysis, immediately stored at -80°C. The primer sequences used for identifying random mitochondrial DNA mutations were 5'- ACAGTTTATGTAGCTTACCTCC -3' (forward) and 5'- TTGCTGCGTGCTTGATGCTTGT -3' (reverse); the primer sequences used for mtDNA copy number quantification were: 5'-ACAGTTTATGTAGCTTACCTCC-3' and 5'-TTGCTGCGTGCTTGATGCTTGT-3'. A no-template control using H_2O instead of DNA was also included.

Quantitative real-time PCR data was analysed using SDS 2.3 and SDS RQ Manager 1.2 relative quantification software. The threshold cycle (Ct) for each well was calculated and the expression levels were normalised to expression levels for the corresponding controls for each sample (see Figure 2.3). All PCR products were sequenced to identify the mutation at the *Taq*$^{\alpha}$I recognition site (High-Throughput Sequencing Facility, University of Washington and Source Bioscience sequencing, Dublin).

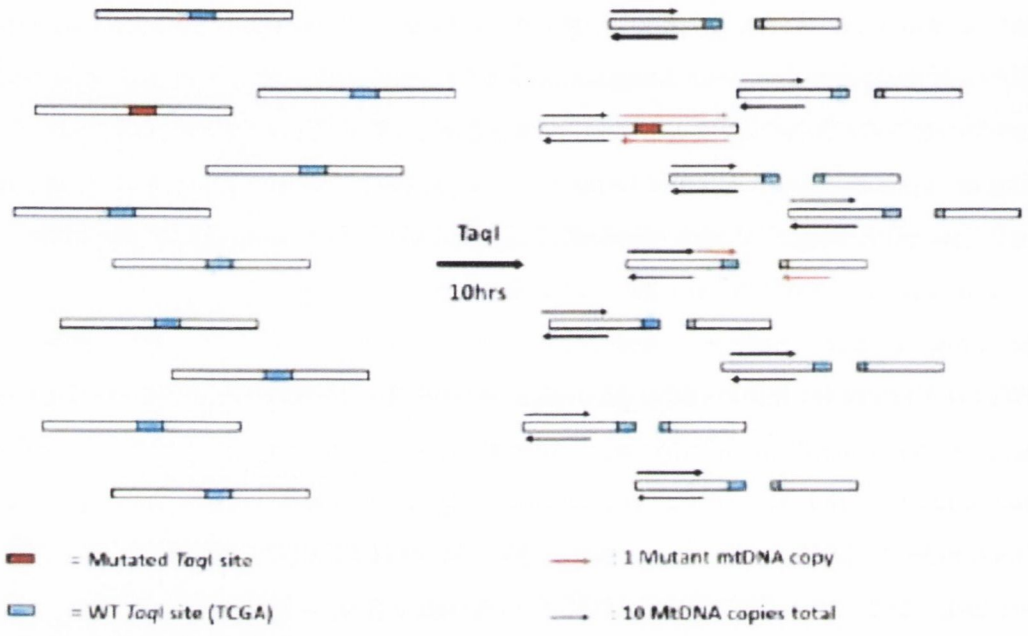


Figure 2.2: Concept of the random mutation capture assay. DNA molecules contain either a wild type (WT) or a mutant *Taq*$^{\alpha}$I restriction site. After mitochondrial DNA digestion with *Taq*$^{\alpha}$I, PCR is attempted across a *Taq*$^{\alpha}$I restriction site (red arrows). This PCR will amplify only DNA molecules that contain a mutation in the restriction site (red box), which rendered it resistant to cleavage. Amplicons with a WT restriction site (green box) are no longer a template for PCR amplification. The mutant molecules are then quantified by qPCR. A second qPCR, adjacent to the restriction site, quantifies every DNA molecule in a sample. The ratio of mutant molecules to the total number of molecules is a direct measurement of the mutant frequency, which can be used to calculate the mutation frequency per base pair Image reproduced from Vermulst *et al.* (Vermulst, Bielas et al. 2008).

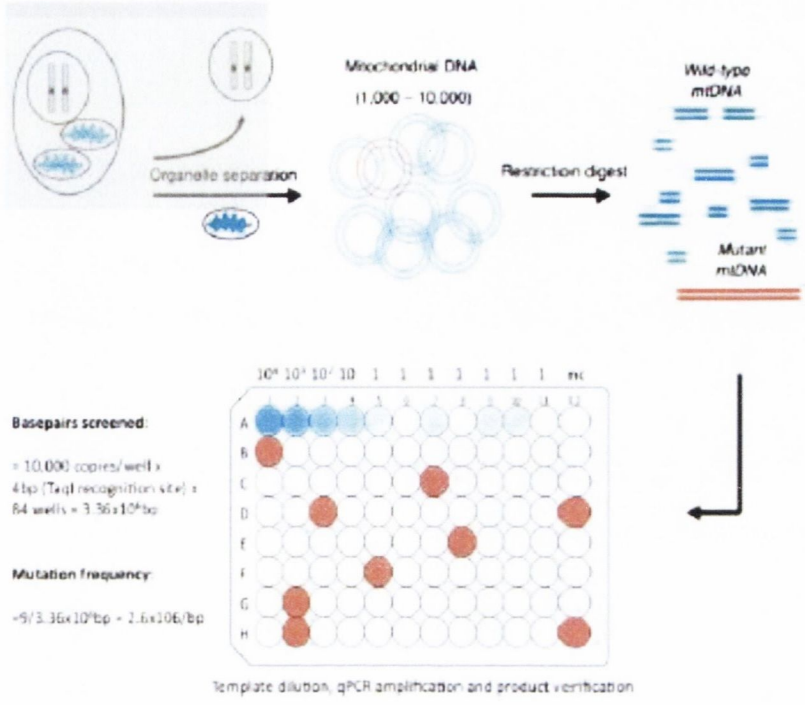


Figure 2.3: Schematic of random mutation capture assay. The RMC-assay consists of 4 steps, organelle separation, DNA extraction, DNA digestion and qPCR amplification. In order to calculate the mutation frequency mitochondrial DNA is displayed in a 96-well format. In the example presented above, 10,000 molecules of the mitochondrial genome are inserted into rows B-H. In each of these wells a PCR is attempted across the Taq1 restriction site. 9 wells displayed in red contained an amplified molecule. Sequencing of each of these PCRs confirmed that a mutation was present in the *Taq*$^{\alpha}$I restriction site. Serial dilutions from 10,000 copies 1, in row A, are used to confirm the copy number present in each well and provide an important control for PCR efficiency. At an estimated 1 copy per well (wells A5-A11), some wells do and some do not contain an amplified DNA molecule. The mutation frequency can be calculated as follows: if 10,000 copies of mtDNA are screened per well, that is equivalent to a screen of 40,000 base pairs per well, since the *Taq*$^{\alpha}$I site is 4 base pairs long and a mutation in any one of these base pairs will render it resistant to cleavage. 84-wells were screened in this experiment which amounts to 3.36x106 base pairs screened. Nine mutations were found yielding a mutation frequency of 2.6x10-6. Image reproduced from Vermulst *et al.* (Vermulst, Bielas et al. 2008).

2.3.7 Measurement of secreted mitochondrial proteins and inflammatory cytokines from *ex-vivo* Barrett's and matched normal explant tissue

Mitochondrial proteins, used as proxy measures of mitochondrial biogenesis and surrogates of mitochondrial instability were measured in cultured media from the Barrett's tissue and surrounding normal epithelium explant cultures. Concentrations of mitochondrial proteins; cytochrome c (R&D Systems, Minneapolis, MN, USA), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) (R&D Systems), Human Apoptosis Inducing Factor (AIF) (Abcam®, Cambridge, MA, USA), Endonuclease G (USCN Life Science Inc., Houston, TX, USA) and Human temperature requirement factor A2 (HtrA2) (RayBiotech, Inc., Norcross, GA, USA) were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits, protocols were followed as per manufactures' instructions. For all experiments, NUNC High binding ELISA plates (Cruinn Diagnostics, Dublin, Ireland) were coated with 50 µl capture antibody overnight at room temperature, and washed three times in wash buffer, except when a pre-coated plate was provided as part of the ELISA kit. 50 µl of sample was applied to each well in duplicate with the exception of blanks which contained only reagent diluent. A serial dilution was made from the standard supplied and 50 µl of each concentration were applied to the plate in duplicate. Samples were incubated for 2 hours at room temperature and the plate was washed three times in wash buffer. 50 µl of detection antibody was added and the plate incubated at room temperature for two hours followed by three washes in wash buffer. A 50 µl volume of streptavidin-HRP (R&D systems, Inc., MN, USA) (1:200 dilution in reagent diluent) was added to each well and incubated for 20 minutes at room temperature. 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich) or ELISA developing solution provided by the manufacturer was added to each well and covered with tinfoil until the colour developed (10-20 minutes). 50 µl of stop solution, 2N H_2SO_4, was added to each well and the plate was read immediately at 450nm using a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Protein concentrations were determined by interpolating from the standard curve of known concentrations.

Levels of cytokines released from the matched normal tissue-biopsies compared to the Barrett's biopsies were measured in the same patient sample conditioned media. A

multiplex assay for the cytokines interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) was obtained from Mesoscale Discovery® (Gaithersburg, MD, USA). Manufacturer pre-coated plates with cytokine capture antibodies were provided. Experiment was performed using the manufacturer's protocol. 25µl of sample was applied to each well in duplicate with the exception of blanks which contained only reagent diluent. A serial dilution was made from the standard supplied and 25µl of each concentration were applied to the plate in duplicate. Samples were incubated for 2 hours at room temperature and the plate was washed three times in wash buffer provided. 25µl of detection antibody was added and the plate incubated at room temperature for two hours followed by three washes in wash buffer. Then 25µl of Mesoscale Discovery® kit "readbuffer" was applied to each well for 20mins. Cytokines were quantified by reading on a specialised Mesoscale Discovery® multiplex plate reader at 450nm.

2.3.8 Statistical Analysis

Data were analysed with SPSS (PASW [Predictive Analytics Software] version 18) (IBM, Armonk, New York, USA) and Graph Pad Prism (Graph Pad Prism, San Diego, CA) software. Differences between HET1A, QH, Go and OE33 cell lines were calculated using unpaired Student's t-tests and Kruskal Wallis tests. Differences between continuous variables, for matched patient groups were calculated using Wilcoxon signed-rank tests, while in unmatched patient groups the Mann-Whitney U test was used. Statistical significance was defined by $p \leq 0.05$.

2.4 RESULTS

2.4.1 Baseline random mitochondrial point mutations in the Barrett's disease model

2.4.1.1 *In-vitro* assessment of random mitochondrial mutations

Levels of random mitochondrial point mutations were significantly higher in the QH, intestinal metaplasia cell line compared to the HET1A ($p=0.024$), Go ($p=0.008$) and OE33 ($p=0.006$) cells (Figure 2.4); showing that random mutations occurred at the earliest stages of the Barrett's sequence. There was no significant difference seen in the frequency of random mitochondrial point mutations between the HET1A, Go and OE33 cell lines ($p>0.05$).

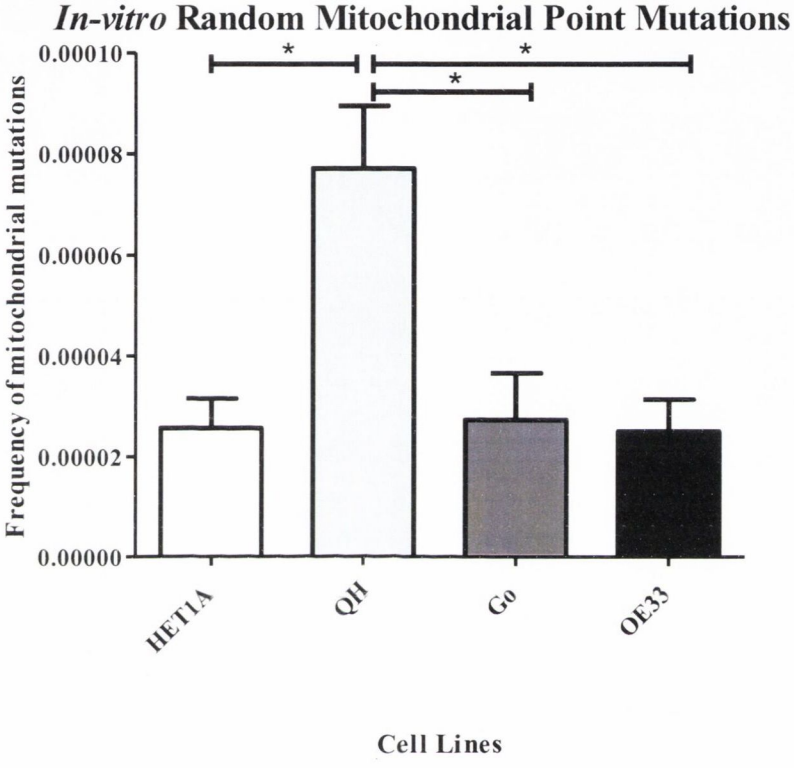


Figure 2.4: Random mitochondrial point mutations *in-vitro*. There was a significantly increased frequency of random mitochondrial DNA mutations in the QH cells (n=5) compared to HET1A (n=3), Go (n=7) and OE33 (n=5) cells. This demonstrated that random mutations were an early event in this *in-vitro* model of Barrett's progression.

2.4.1.2 *In-vivo* assessment of random mitochondrial mutations

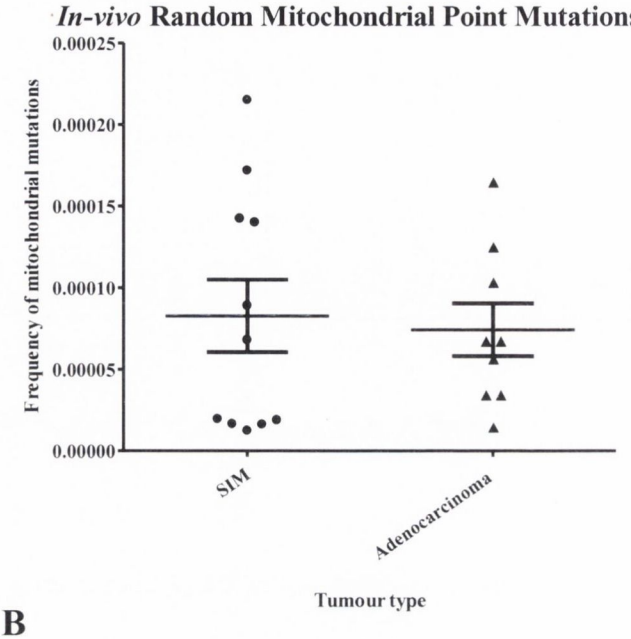
Initial analysis showed no difference between the frequency of random mitochondrial point mutations in intestinal SIM (n=11) (median=6.79 $x10^{-5}$) compared with oesophageal adenocarcinoma tissue (n=9) (median=6.73$x10^{-5}$) (p=1.00) (Figure 2.5A). However, a bimodal distribution of random mutations was evident in the SIM group. These patients were separated into two groups; a **low** group with mutation frequencies $\leq$ median (6.79$x10^{-5}$) and a **high** group with mutation frequencies greater than the median (>6.79$x10^{-5}$). The **low** group had a significantly lower frequency of random mutations compared to the **high** group (p=0.004) and adenocarcinoma biopsies (p=0.05) (Figure 2.5B). SIM patients in the **high** group had significantly higher mutations compared to those detected in the cancer patients (p=0.023).

Matched patient samples were compared between areas of SIM and surrounding normal epithelium (n=6) and areas of oesophageal adenocarcinoma and surrounding normal tissue (n=6) from the same patients (see Figures 2.6 and 2.7). Overall, no difference was seen in the level of random mitochondrial point mutations between the pathologically abnormal tissues and areas of squamous epithelium. Some patients demonstrated multiple mutations at the 12S RNA gene site which was analysed. In SIM patients, the types of random mutations were predominantly C>T and G>A transversions, while in the oesophageal adenocarcinoma patients, random mutations were predominantly C>T transversions (see Figures 2.6 and 2.7).

Deletion analysis was performed on all tissue samples and was validated in eight patients with SIM, one patient with HGD, one patient with IMC and five patients with oesophageal adenocarcinoma. Specific to SIM, matched normal tissue was available in six cases, and a significantly increased frequency of mitochondrial deletions was evident in the normal tissue compared to the metaplastic areas (p=0.031) (see Figure 2.8 A). For the purpose of statistical analysis, HGD, IMC and oesophageal adenocarcinoma samples were grouped together to enable comparative analysis between normal and cancerous tissue where paired tissue samples were available. While not significant, there was a trend of increased frequency of mitochondrial deletions in the normal tissue compared to cancerous tissue (p=0.063) (see Figure 2.8 B). Overall, the level of

deletions was significantly higher in SIM compared to HGD and oesophageal adenocarcinoma ($p=0.043$) (Figure 2.8 C).

A



B

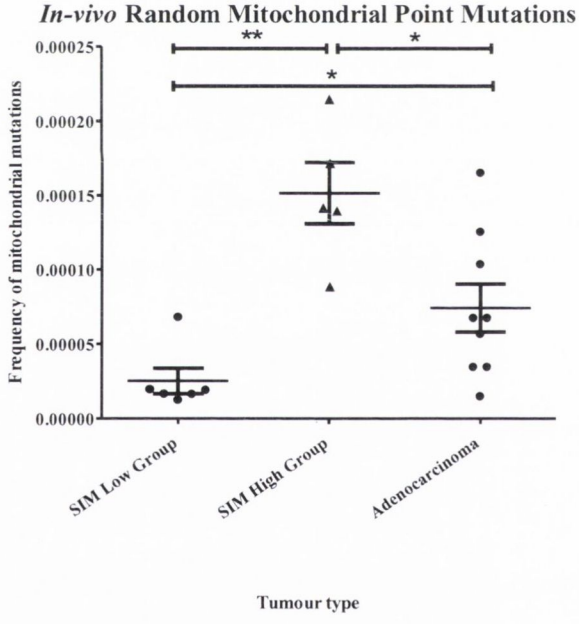
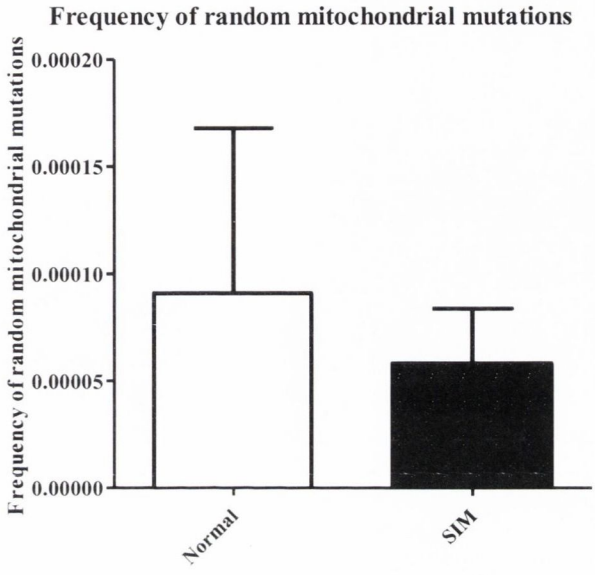


Figure 2.5: Random mitochondrial point mutations *in-vivo*. **(A)** Overall analysis, demonstrated no difference between levels of random mutations between SIM (n=11) and oesophageal adenocarcinoma patients (n=9) (p=1.00). **(B)** The SIM patients were divided into a **low** group (levels of random mutations ≤ median frequency for the overall Barrett's patient group) and a **high** group (mutations > median frequency). A Mann Whitney-U test showed significant differences in mutations between **low** and **high** groups, the **low** and oesophageal adenocarcinoma group and the **high** and oesophageal adenocarcinoma group. *$p \leq 0.05$, **$p \leq 0.005$

A



B

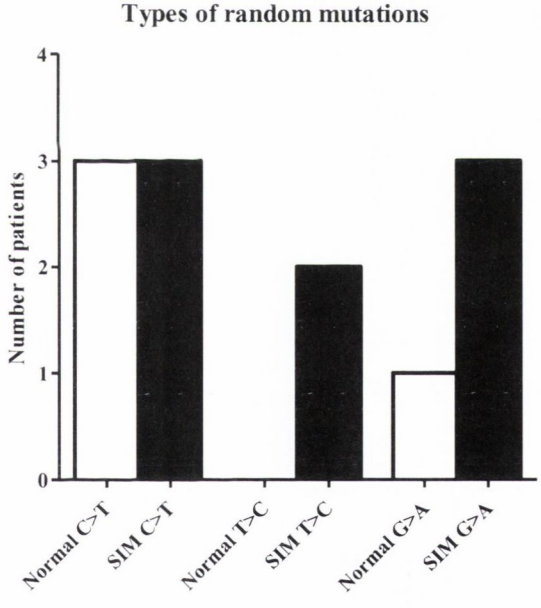


Figure 2.6: Random point mutations in SIM and matched normal tissue samples. (A) Wilcoxon matched-pairs signed rank tests demonstrated no significant difference in the frequency of random mitochondrial point mutations in areas of SIM and the surrounding matched normal squamous epithelium from the same patients (n=6) (p=0.844). **(B)** Sequence analysis showed that the types of random mutations were C>T and G>A transversions in the SIM biopsies compared to predominantly C>T transversions in the normal biopsies. Error bars represent SEM.

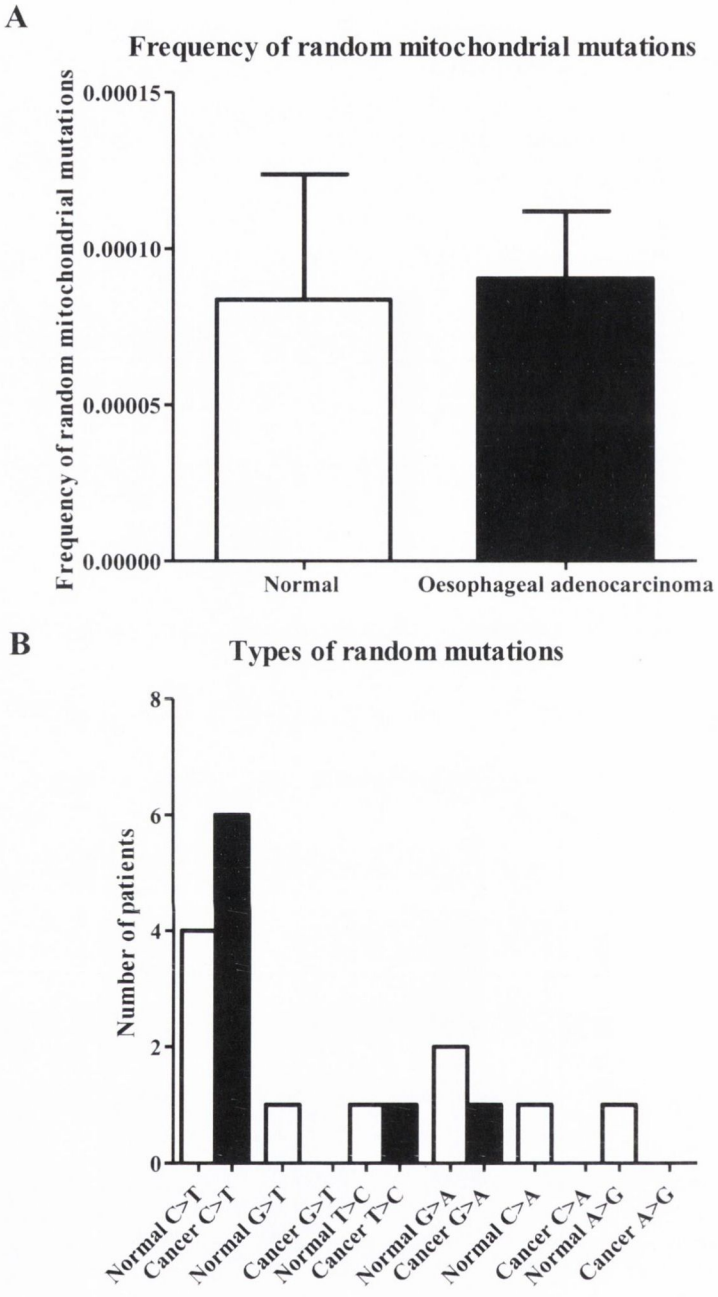


Figure 2.7: Random point mutations in oesophageal adenocarcinoma and surrounding normal tissue. (A) Wilcoxon matched-pairs signed rank tests demonstrated no significant differences in the frequency of random point mutations between oesophageal adenocarcinoma and surrounding matched normal tissue from the same patients (n=6) (p=0.688). **(B)** These mutations were predominantly C>T transversions in both the normal and cancer tissue. Error bars represent SEM.

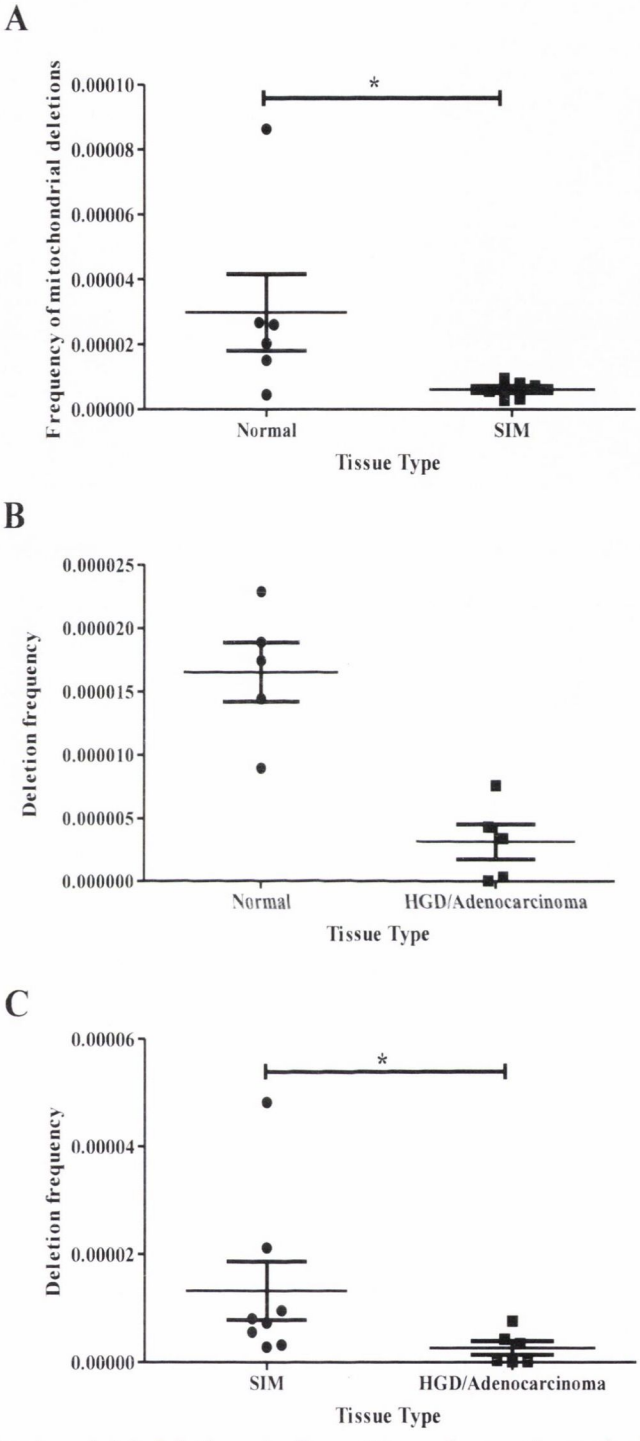


Figure 2.8: Mitochondrial deletions in Barrett's and oesophageal adenocarcinoma patient samples. (A) Wilcoxon matched-pairs signed rank tests demonstrated a significantly increased level of deletions in the matched normal tissue from patients with SIM. **(B)** There was a trend towards increased deletions in the matched normal tissue from oesophageal cancer patients. **(C)** Mann Whitney-U test demonstrated significantly increased frequencies of deletions in Barrett's tissue compared to patients with HGD and oesophageal adenocarcinoma. *$p \leq 0.05$

2.4.2 Baseline mitochondrial function across the Barrett's disease model

2.4.2.1 *In-vitro* mitochondrial functional assays

Differences in mitochondrial function were evident at each point along the cell line sequence (see Figures 2.9).

ROS release was significantly reduced in HET1A cells compared all other points along the Barrett's disease model. There was a 4.2-fold increase in ROS in the QH cells ($p<0.0001$), a 3.2-fold increase in the Go cells ($p<0.0001$) and a 2.5-fold increase in the OE33 cells ($p<0.0001$), relative to the HET1As. ROS release was highest in the QH cells, demonstrating significantly increased levels compared with Go ($p=0.003$) and OE33 ($p<0.0001$) cell lines. The release of ROS was 1.3 times higher in the Go cell line compared with the OE33s ($p=0.020$).

MMP significantly fluctuated across this cell line model. Unpaired t-tests demonstrated a significant 31% reduction in membrane potential in the QH cells ($p=0.037$) and a 30% reduction in the OE33 cells ($p=0.039$) compared with the HET1A cells. No significant difference was seen in the MMP between the HET1A and Go cell lines ($p=0.626$). The QH and OE33 cells had a significantly lower MMP compared to the Go cell line ($p=0.002$ and $p=0.001$, respectively). There was no difference in the MMP between the QH and OE33 cells ($p=0.720$).

There was a significant 1.5-fold increase in the mitochondrial mass in the QH cells compared with the HET1As ($p=0.026$). There was a trend towards a 1.5-fold increase in the Go cells compared with the normal cell line, although this was not statistically significant ($p=0.057$). The QH cell line demonstrated a 1.3-fold increase in mitochondrial mass compared with the cancer cell line ($p=0.014$). No significant difference was seen in the mass between QH and Go cells ($p=0.920$) and Go and OE33 cells ($p=0.080$).

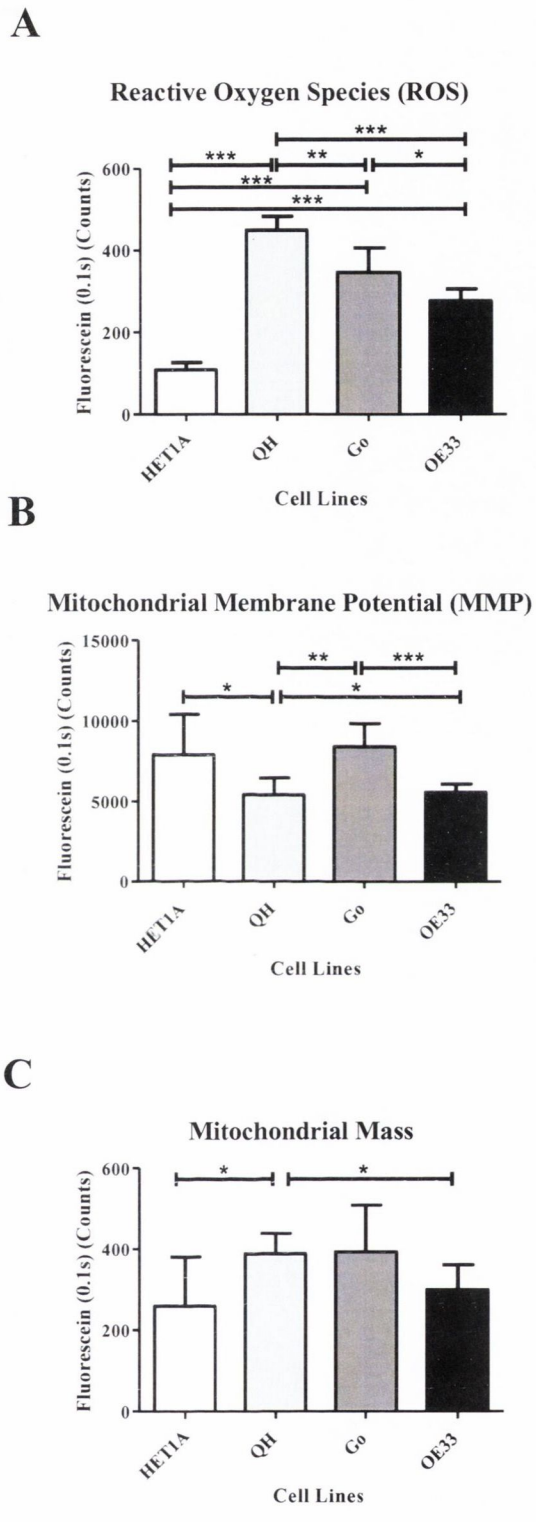


Figure 2.9: Mitochondrial function across the Barrett's disease sequence (n=5). **(A)** ROS was significantly lowest in the HET1A cells and highest in the QH cells. **(B)** MMP fluctuated across the disease sequence and was lowest in the QH cells. **(C)** Mitochondrial mass varied across the Barrett's disease sequence and was highest in the QH and Go cell lines.
*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$

2.4.2.2 *Ex-vivo* secretions of mitochondrial proteins and inflammatory cytokines from Barrett's and matched normal explants

Secreted cytochrome c was significantly higher from SIM tissue compared to normal tissue ($p=0.003$) (Figure 2.10 A). SMAC/Diablo was significantly higher in the Barrett's cultured media ($p=0.008$) compared to surrounding normal epithelium (Figure 2.10 B). There was no difference in the concentration of AIF ($p=0.342$), Endonuclease G ($p=0.077$) and HtrA2 ($p=0.098$) secretions from SIM and matched normal tissue (Figure 2.11).

Levels of pro-inflammatory cytokines were significantly higher in areas of SIM compared to matched normal tissue; IL-1β ($p=0.007$), IL-6 ($p=0.0005$), IL-8 ($p=0.002$) and TNF-α ($p=0.034$) (Figure 2.12).

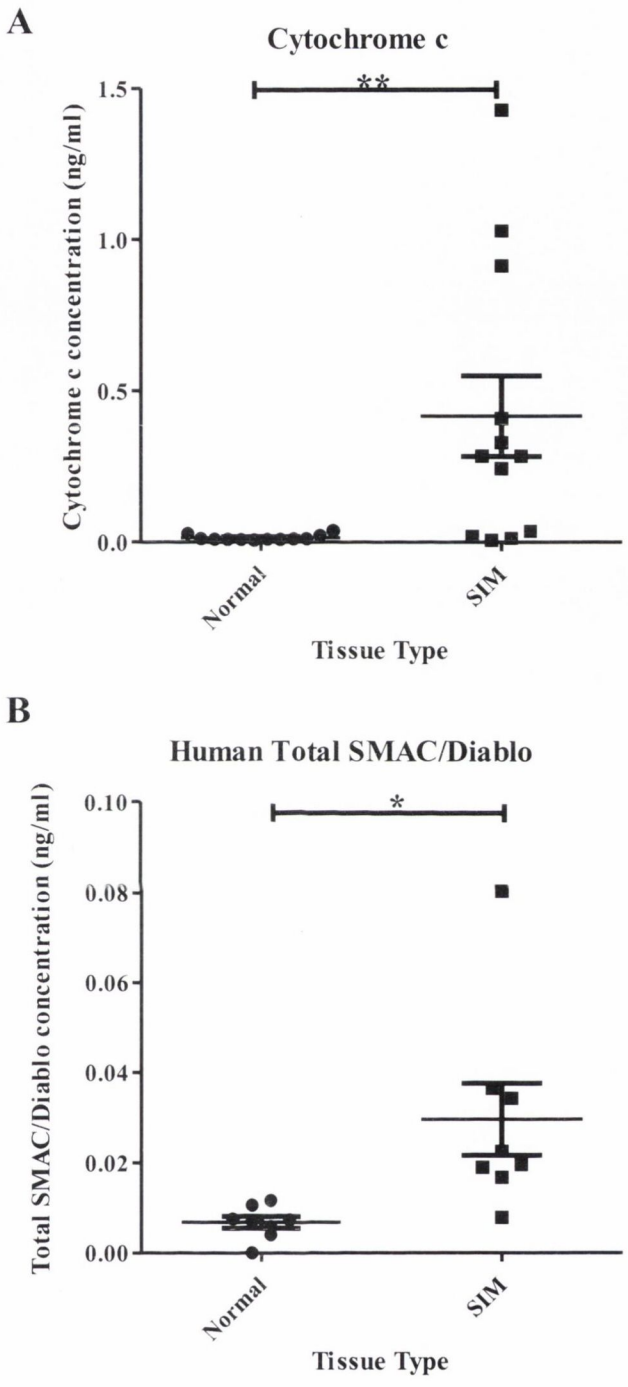


Figure 2.10: Mitochondrial proteins, cytochrome c and SMAC/Diablo, measured in areas of SIM and surrounding normal tissue. Wilcoxon matched-pairs signed rank tests analysed levels of **(A)** cytochrome c (n=12) and **(B)** SMAC/Diablo (n=8) and were significantly higher from SIM tissue compared to surrounding normal epithelium. *$p \leq 0.05$, **$p \leq 0.005$

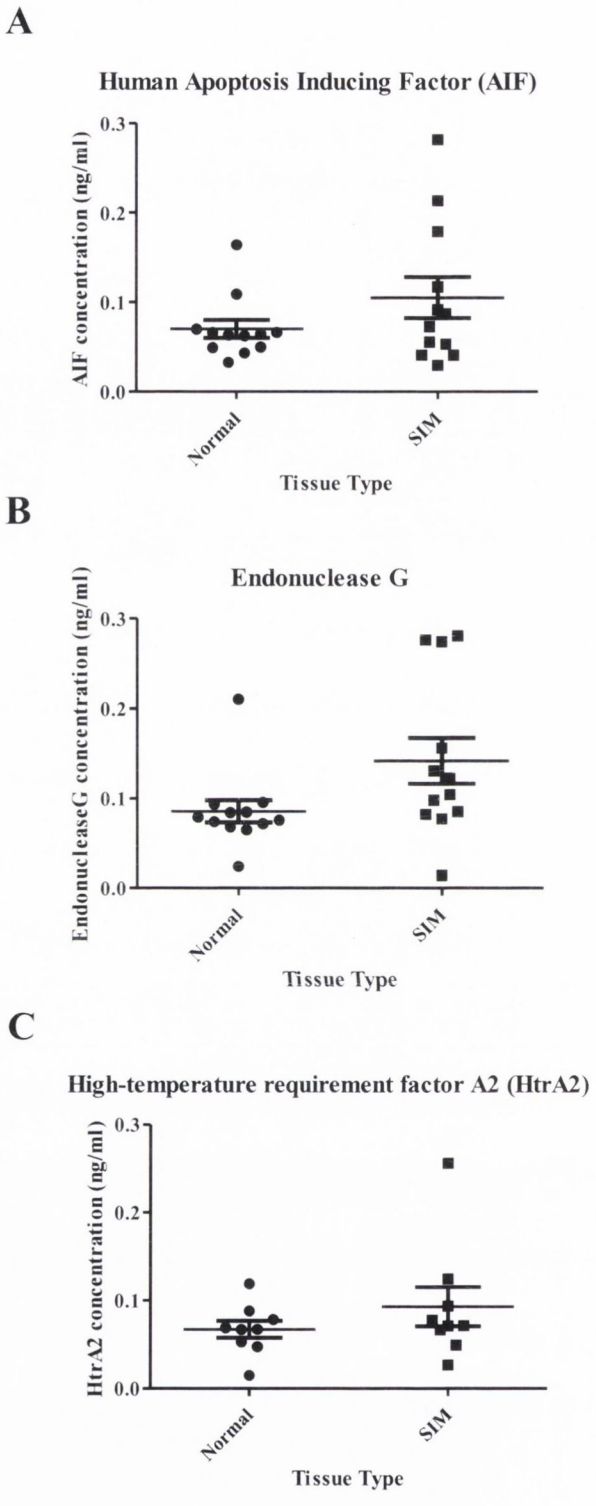


Figure 2.11: Mitochondrial proteins, AIF, Endonuclease G and HtrA2, were measured in explant cultured media from areas of SIM and surrounding normal tissue. Wilcoxon matched-pairs signed rank tests showed no significant difference in the levels of **(A)** AIF (n=12), **(B)** Endonuclease G (n=12), and **(C)** HtrA2 (n=9) between SIM tissue compared to surrounding normal epithelium.

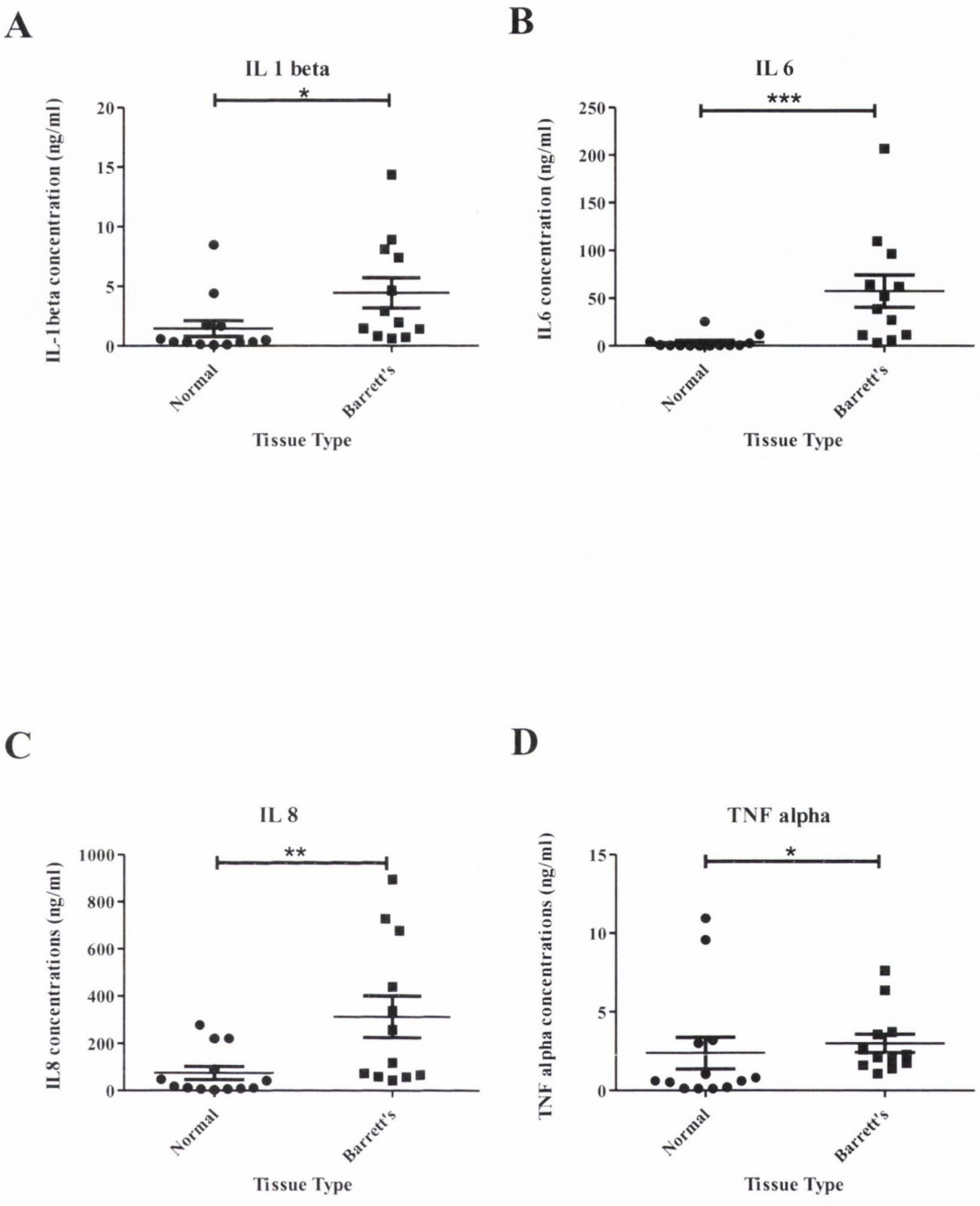


Figure 2.12: Pro-inflammatory cytokines were measured in explant cultured media from areas of SIM and surrounding normal tissue (n=12). Wilcoxon matched-pairs signed rank tests analysed levels of secreted **(A)** IL-1beta, **(B)** IL-6, **(C)** IL-8 and **(D)** TNF-alpha and were significantly higher from SIM tissue compared to surrounding normal epithelium. *$p \leq 0.05$, **$p \leq 0.005$ and ***$p \leq 0.0005$

2.5 DISCUSSION

The role of mitochondrial instability in progression of Barrett's oesophagus is poorly understood. Metabolic imbalances, such as reduced response to apoptosis and increased glycolysis are all features of cancer cells, and are tightly regulated by the mitochondria (King, Selak et al. 2006, Kroemer 2006, Robey and Hay 2006). Mutagenesis is a catalyst for cancer development, but to date, clonal gene mutations have been the main type of mitochondrial mutations analysed with respect to oesophageal carcinoma (Hibi, Nakayama et al. 2001, Abnet, Huppi et al. 2004). The mitochondrial genome is more vulnerable to random DNA mutations due to high ROS exposure and lower DNA repair mechanisms compared to nuclear DNA (Wallace 1992, Wallace 2005). This chapter examined if random mutations and mitochondrial instability was altered along the Barrett's disease sequence using *in-vitro, in-vivo* and *ex-vivo* models.

In this chapter mitochondrial instability was initially assessed through measurements of random mitochondrial point mutations using *in-vitro* and *in-vivo* Barrett's oesophagus models. Random mitochondrial mutations were significantly elevated in the intestinal metaplasia, QH, cells compared with all other points along the Barrett's disease sequence. *In-vivo,* we observed a bimodal distribution in the frequency of random mutations in SIM, and subsequently patients were arbitrarily divided into **low** and **high** mutation groups. We recognise that division of the Barrett's patients into these two groupings was an observational exercise applied to test the mutator-phenotype hypothesis in later chapters and it is important to acknowledge that this was performed on only a small number (n=11) of Barrett's patients. However, the RMC assay is a robust and reproducible experiment and we feel it is pertinent that we highlight this level of heterogeneity in our SIM cohort. Applying the mutator-phenotype hypothesis, it was hypothesised that high random mutations were associated with cancer predisposition (Loeb, Bielas et al. 2008). However, these samples would need to be followed prospectively in order to confirm this theory. This hypothesis is further tested in chapter four through the utilisation of archived biopsies from patients undergoing Barrett's surveillance since 1990 at St. James's Hospital.

A decline in the frequency of random mutations in patients with oesophageal adenocarcinoma compared with the high mutator SIM group was demonstrated,

suggesting random mutations may become redundant as the disease progresses. Ericson *et al.* found a similar relationship to exist in the case between adenomas and colorectal carcinoma, with significantly decreased random mitochondrial point mutations in cancer compared with adenomas (Ericson, Kulawiec et al. 2012). Our *in-vitro* findings also demonstrated mitochondrial mutations were significantly reduced in the OE33 cells compared to the QH cells. Malignant cells, once established, undergo selection processes, where increased numbers of both lethal and neutral mutations would be expected; however, the neutral mutations would be more inclined to survive and capable of undergoing subsequent replication (Loeb, Springgate et al. 1974). Perhaps a clonal mutation and not random mutations overtake the initial catalyst for cancer development at this time point (Loeb, Loeb et al. 2003).

In-vivo the frequency of mitochondrial deletions were significantly increased in SIM biopsies compared to biopsies from patients with HGD/IMC and oesophageal adenocarcinoma. As the seventh edition AJCC guidelines now recognise HGD to be part of the oesophageal cancer spectrum, for the purpose of comparative analysis between the pre-malignant and the malignant setting, it was deemed appropriate to combine HGD, IMC and adenocarcinoma. Significantly increased mitochondrial point deletions further support the environment of mitochondrial instability in SIM. Deletions are simply another form of rearrangement of the mitochondrial genome and are a recognised marker of mitochondrial instability, which have been highlighted in the natural ageing process (Tuppen, Blakely et al. 2010). Analysis of matched samples from normal and SIM areas and from normal and malignant sites demonstrated significantly increased deletions in the surrounding normal tissue. These findings substantiate the concept of a field effect in oesophageal cancer, which appears to be also present in the early stages of Barrett's oesophagus. Furthermore, no significant difference was evident in the level of random point mutations between the normal and SIM tissue and normal and cancer tissue, further supporting a field effect, which extends beyond the visible area of abnormality.

Mitochondrial instability was also evaluated through measurement of mitochondrial function *in-vitro* and measurement of proxy markers of altered mitochondrial biogenesis *ex-vivo*. The QHs demonstrated significantly increased levels of ROS

compared to all other points along the Barrett's disease sequence. These cells also demonstrated lower levels of MMP and higher mitochondrial mass compared with the other cell lines. This indicated that mitochondrial instability was an early event *in-vitro*. The Warburg effect theorises cancer cells reprogram energy metabolism, reducing oxidative phosphorylation and ROS production, potentially decreasing injury to mitochondrial DNA (Warburg 1956, Vander Heiden, Cantley et al. 2009); perhaps this may explain the significant reductions in ROS in our *in-vitro* model between the QH, intestinal metaplasia cell line, and the Go and OE33 cells. The role of ROS as a precursor for cancer progression has been studied in many cancers. In Helicobacter pylori-associated gastritis, excess ROS has been implicated as a gastric carcinogen (Drake, Mapstone et al. 1998). In breast cancer, BRCA-1, a tumour suppressor, has been shown to play a role in protecting against ROS damage; BRCA-1 mutations have subsequently been implicated in loss of redox balance with increased ROS, and this may potentially drive cancer development (Acharya, Das et al. 2010).

It was not possible to perform functional assays in tissue samples. Instead, secreted mitochondrial proteins were measured to assess alterations in the mitochondrial environment *ex-vivo*, which reflect changes in mitochondrial biogenesis. The explant model system is superior to monolayer cell cultures as it encompasses the tissue microenvironment (Gorman, Tosetto et al. 2009, Michielsen, Noonan et al. 2012, Michielsen, Ryan et al. 2012). The mitochondria play a critical role in cell apoptosis. Cytochrome c and SMAC/Diablo are apoptotic proteins, released into the cytosol in order to activate a series of caspases downstream. In this chapter, both of these proteins were significantly elevated in SIM compared with matched normal tissue, suggesting an increase in mitochondrial biogenesis in Barrett's metaplasia. Cytochrome c is located in the inter-membrane space and increased release of cytochrome c stores have been linked to increased permeability in the mitochondrial outer membrane (Mootha, Wei et al. 2001). In the cell line analysis, a significant decrease in MMP was present in the QH, intestinal metaplasia cells, compared to all other points along the disease sequence. Studies have demonstrated that a loss in membrane potential triggers the induction of the release of pro-apoptotic enzymes, such as cytochrome c, AIF and SMAC/Diablo (Creagh and Martin 2001, Ly, Grubb et al. 2003), and these findings would correlate with our *in-vitro* and *ex-vivo* results. With the mitochondria playing a central role in

cellular apoptosis (Desagher and Martinou 2000), decreases in membrane potential have been associated with loss of membrane permeability, with a subsequent release of pro-apoptotic factors into the cytosol (Zoratti and Szabo 1995, Kroemer and Reed 2000). In addition, increased membrane permeability has been associated with increased mitochondrial swelling due to electrolyte influx (Ly, Grubb et al. 2003), and this can be associated with increases in mitochondrial mass, which in our *in-vitro* analysis was significantly increased in the QH cells. These findings would support an environment of increased mitochondrial instability *in-vitro* and *ex-vivo*.

This work also showed that the inflammatory markers IL-1β, IL-6, IL-8 and TNF-α, were significantly increased in SIM compared with surrounding normal areas. Previous studies from our group have demonstrated significant positive correlations between oxidative damage and inflammation (Biniecka, Fox et al. 2011, Biniecka, Kennedy et al. 2011). Therefore, evidence of these increased pro-inflammatory cytokines in SIM suggests that such an environment may promote oxidative stress in Barrett's metaplasia. Overall our data demonstrated that the Barrett's environment was one of increased mitochondrial instability and altered inflammation *ex-vivo* which may contribute to disease progression.

We acknowledge certain limitations within this chapter. Ideally, *in-vitro* analysis would have been more robust if performed on more than one cell line representing each stage of the Barrett's disease sequence. However, HET1A and Go cell lines are the only commercially available cells established to represent normal oesophagus and HGD, respectively. While the BAR-T cells are a commercially available cell line derived from a Barrett's oesophagus biopsy, we were prevented from using this in our analyses, as it is grown on a feeder layer, which prohibits its use in all our functional studies. Several lines are established and available from oesophageal adenocarcinoma, however, the main focus of this study was to concentrate on the earlier stages of the Barrett's disease sequence, and therefore we were satisfied to use the OE33s as our sole cancer line in this research. In order to compensate for these limitations, analyses were repeated on *in-vivo* and *ex-vivo* samples, as human tissue is a superior model to *in-vitro* work (Gorman, Tosetto et al. 2009, Michielsen, Noonan et al. 2012, Michielsen, Ryan et al. 2012). However, we recognise our *in-vivo* samples were not without their own

limitations; in particular, Barrett's patients were recruited for the RMC assay and ELISA studies only if they had a pre-existing diagnosis of Barrett's oesophagus. As such, these patients were likely to be on regular PPIs to reduce acidic reflux and its resultant Barrett's inflammation, which in turn may have had effects on the mitochondrial and inflammatory environment. Fresh samples were necessary for these experiments, and in order to ensure the accuracy of our Barrett's oesophagus recruitment, it was not possible to use first time diagnostic (pre-PPI therapy) biopsies in this part of our analyses.

Allowing for these limitations, chapter two demonstrated that mitochondrial instability was an early event in the Barrett's disease sequence. Applying the mutator-phenotype hypothesis may enable stratification of SIM patients into low and high cancer risk groups, however; this theory needs further testing in future chapters. Once oesophageal adenocarcinoma was established, random mutations significantly decreased, possibly becoming redundant and maybe superseded by a clonal mutation. Overall, in this chapter we demonstrated increased mitochondrial instability in the early stages of Barrett's oesophagus, *in-vitro*, *in-vivo* and *ex-vivo*. This environment may play a key role in fuelling progression to oesophageal adenocarcinoma.

Chapter 3: Measurement of DCA and antioxidants effects on mitochondrial stability across the Barrett's disease sequence

3.1 INTRODUCTION

The pathogenesis of Barrett's oesophagus and factors fuelling progression are not fully understood. It is well recognised that duodenogastric reflux is a critical risk factor in Barrett's cancer development (Jankowski, Hopwood et al. 1993, Kauer, Peters et al. 1995, Smith, Kelly et al. 2010). While acid suppression therapies have demonstrated some effectiveness in controlling reflux symptoms, little evidence exists proving the impact of such treatments on the incidence of Barrett's oesophagus. This fuels the hypothesis that bile reflux may be a key carcinogen (Prach, MacDonald et al. 1997, Pohl and Welch 2005, Jenkins, Cronin et al. 2008). Indeed, concentrations of bile reflux are higher in patients with Barrett's oesophagus compared with uncomplicated GORD (Stein, Feussner et al. 1994).

Chapter two revealed a relationship between increased oxidative stress, mitochondrial instability and inflammation in SIM, with Barrett's oesophagus demonstrating increased ROS, mitochondrial biogenesis and inflammation *in-vitro* and *ex-vivo*. Excess ROS lead to a loss of equilibrium in a normal redox balanced system, where ROS begin to out-weigh antioxidants (Finkel and Holbrook 2000, Das, Suman et al. 2013). Counteracting this imbalance with antioxidants therefore seems to be a reasonable therapeutic strategy. This hypothesis has been previously applied by Chen *et al.*, who demonstrated antioxidants may arrest oesophageal tumour growth following treatment with selective inhibitors to inducible nitric oxide synthase (iNOS), a potent ROS (Chen, Nines et al. 2004, Chen, Hwang et al. 2006). Similarly, Jenkins *et al.* showed *in-vitro,* treatment with the antioxidant Vitamin C decreased ROS levels induced by DCA (Jenkins, D'Souza et al. 2007). *In-vivo*, our group has published a pilot study demonstrating dietary vitamin C resulted in down-regulation of NF-κB and pro-inflammatory cytokines in Barrett's patients (Babar, Abdel-Latif et al. 2010). NF-κB a central regulator of genes implicated in inflammatory pathways and cancer development, is known to be up-regulated along the Barrett's metaplasia-dysplasia-adenocarcinoma disease sequence (Abdel-Latif, O'Riordan et al. 2005, O'Riordan, Abdel-latif et al. 2005).

In this chapter we aim to explore whether the antioxidants, NAC, SOD and EGCG, and a hydroxylase inhibitor, DMOG, can rescue changes in mitochondrial function induced

by the bile acid, DCA. NAC, SOD and DMOG were selected, as previous studies from our group demonstrated that all reduced oxidative stress and random mitochondrial point mutations in inflammatory arthritis (Biniecka, Fox et al. 2011). DMOG, while strictly not an antioxidant, has proven protective properties reversing inflammation in inflammatory bowel disease, through the regulation of hypoxia-inducible factor 1α (HIF-1α) and NF-κB pathways (Cummins, Seeballuck et al. 2008). EGCG, a component of green tea, was selected due to its demonstrated affects in prostate cancer (Morrissey, Brown et al. 2007), potentially due to its ability to decrease oxidative damage (Guo, Zhao et al. 1999, Toschi, Bordoni et al. 2000).

3.2 HYPOTHESIS AND AIMS

We have demonstrated in chapter two that mitochondrial instability is most pronounced in SIM. We hypothesise that DCA may induce mitochondrial changes and treatment with antioxidants may rescue this induced mitochondrial dysfunction.

The specific aims of chapter three are;

(1) Investigate the effects of DCA on the frequency of random mitochondrial point mutations and mitochondrial function *in-vitro* and *ex-vivo*.

(2) Determine the effects of antioxidants, NAC, SOD, EGCG, and the hydroxylase inhibitor, DMOG, on DCA induced mitochondrial changes *in-vitro*.

3.3 MATERIALS AND METHODS

3.3.1 Evaluation of ROS, MMP and mitochondrial mass response to treatment with DCA, antioxidants and DMOG using mitochondrial assays

Reagents, cell lines and cell subculture methods as described in 2.3.2 were followed. HET1A, QH, Go and OE33 cells were seeded in 96 well plates at densities ranging from 2,500 to 8,000 cells/well, depending on the cell line. Seeding at different concentrations was necessary to compensate for the different growth rates between individual cell lines, in order to ensure the same degree of confluence at the initiation of treatments. Following 24hrs incubation, cells were treated with DCA (Sigma-Aldrich)

at 0, 10, 20, 50, 100, 200 and 300μM DCA concentrations for 24 hours. At the same time, cells were treated with 75μg/ml of SOD (Sigma-Aldrich) or 5mM NAC (Sigma-Aldrich) or 40μM EGCG (Sigma-Aldrich) or 1mM DMOG (Cambridge Biosciences) at 37°C for 24hrs +/- DCA. Following 24hrs, ROS production, MMP and mitochondrial mass were assessed, following methods outlined in 2.3.3. Mean fluorescence values for each condition were obtained and compared to the relative controls for each cell line. All experiments were repeated a minimum of three times with a minimum of three replicates within each independent run. Due to the complexity of the plate set-up, each experimental run did not necessarily always provide three sufficient replicates for analysis. The n value provided for each of the functional experiments represents the total sum of the independent runs and replicates available for study, however, importantly data was only analysed when there were results available from a minimum of three independent runs. All mitochondrial functional data were normalised to cell number using crystal violet assays as described in section 2.3.4.

3.3.2 Measurement of the effects of DCA, antioxidants and DMOG on random mitochondrial point mutations

QH, Go and OE33 cells were grown to 60-70% confluence in 25cm^2 flasks. HET1A cells were grown to 60-70% confluence in 75cm^2 flasks in order to obtain an adequate cell yield for DNA extraction. Following 24hrs incubation, cells were treated with 100μM DCA +/- antioxidants (75μg/ml of SOD +/- 5mM NAC +/- 40μM EGCG) +/- 1mM DMOG at 37°C for 24hrs. Cells were used for the random mutation capture assay, outlined in 2.3.6.

3.3.3 Effects of DCA on mitochondrial proteins and pro-inflammatory cytokine secretions from *ex-vivo* explant Barrett's tissue

As previously described in section 2.3.7, mitochondrial proteins and pro-inflammatory cytokines were measured in cultured media taken from cultured Barrett's explant tissue and matched normal tissue. Concentrations of mitochondrial proteins; cytochrome c, SMAC/Diablo, AIF and HtrA2 were determined using commercially available ELISA kits following the manufactures' instructions. Levels of cytokines released from the matched normal tissue-biopsies compared to the Barrett's biopsies were measured in the same samples. IL-1β, IL-6, IL-8 and TNF-α concentrations were measured using a

commercially available multiplexed immunoassay following the manufacturer's instructions.

Two normal and two Barrett's biopsies were used from each patient. In conjunction with incubating one normal and one Barrett's oesophagus biopsy in media alone, as previously described, one normal and one Barrett's biopsy from each patient was also incubated in media supplemented with 100μM DCA for 24 hours. Conditioned media was stored at -80°C. Matched cultured biopsies were stored in RNA Later solution. Barrett's tissue was confirmed both by histology on simultaneously sampled diagnostic biopsies (performed by an independent histopathologist). Barrett's tissue was characterised using methods outlined in section 2.3.5. Tissue viability following explant culture was confirmed using a commercially available lactate LDH assay (Caymanchem, Michigan, USA), which demonstrated that DCA treatment did not affect tissue viability following 24hr culture.

3.3.4 Measurement of the effects of DMOG on cell cycle using Flo-cytometry

Cell cycle analysis was performed by Propidium iodide (PI) staining and flow cytometry[464]. Cells were seeded in 6-well plates, and allowed to adhere overnight at 37°C in 5% CO_2/95% humidified air. Cells were treated with 1mM DMOG for 24hrs. Following treatment, cells were collected by trypsinisation as described previously (section 2.3.2.2) and transferred to 5 mL falcon tubes (BD Biosciences). Cells were centrifuged at 180 × g for 3 min, and the supernatant decanted. The cell pellet was washed with 1 mL PBS and centrifuged as before. The cell pellet was fixed and permeabilised by drop-wise addition of 4.5 mL ice-cold ethanol (70% v/v in PBS) (Merck, Darmstadt, Germany), and incubated at 4^0C for a minimum of 2 hours. The fixative was decanted after centrifugation at 180g for 3 min. Cells were washed with 1 mL PBS and centrifuged as before. Each sample was resuspended in 0.5 mL PI staining solution [PI (0.02 mg/mL)/Triton X- 100 (0.1%)/RNase A (0.2 mg/mL)], except for appropriate controls. Samples were incubated at 37°C for 30 min and then at room temperature for 1.5 hours in staining solution. Unstained control samples used for instrumental setup were incubated in 1 mL PBS. Flow cytometry was performed using a FACSCalibur™ flow cytometer (Becton- Dickson, San Jose, CA), data acquired using Summit v4.3 software (Dako, Glostrup, Denmark) and data analysed by histogram plot

using CELLQuest™ software. A minimum of 10,000 events were collected, and doublets were excluded from analysis using doublet discrimination. The X-axis of the histogram plot represents PI fluorescence or DNA content, whilst the Y-axis represents cell number.

3.3.5 Statistical Analysis

Data were analysed with Graph Pad Prism (Graph Pad Prism, San Diego, CA) software. Differences between continuous variables between an individual cell line control and treatment group were calculated using Paired Student's t-tests and one-way ANOVA tests. Differences between different cell lines were calculated using unpaired Student's t-tests. Differences between continuous variables, for matched patient groups were calculated using Wilcoxon signed-rank tests. Statistical significance was defined by $p \leq 0.05$.

3.4 RESULTS

3.4.1 Mitochondria functional response to DCA *in-vitro*

There was no significant decrease in cell number in QH, Go and OE33 cells following 24hrs treatment with 100μM DCA (see figure 3.1), however; 100μM DCA caused a significant 29.8% growth inhibition in HET1A cells (p=0.016).

In the QH cells, membrane potential and mitochondrial mass were significantly decreased following 100μM DCA (p=0.002 and p=0.002, respectively) (Figure 3.2). However, ROS did not alter significantly following 24hrs treatment (p=0.586). In the Go cell line, 100μM DCA caused a significant 21.8% increase in the release of ROS (p=0.011) and 44.9% increase in MMP (p=0.021). No change was seen in mitochondrial mass following DCA treatment in Go cells (p=0.843). Treatment with 100μM DCA caused a significant 41.4% increase in ROS (p=0.001), however no significant change was demonstrated in MMP (p=0.142) and mass (p=0.260) following 24hrs exposure. Mitochondrial dysfunction was induced at 100μM DCA in the QH, Go and OE33 cells, without associated cell death (see Figure 3.2). In the HET1A cell line, 100μM DCA caused a 14.1% increase in ROS (p=0.042), a 20.9% increase in membrane potential (p=0.004) and a 32.2% increase in mass (p=0.0004). 100μM DCA was selected as the treatment dose for all future experiments, as it was the maximum dose that stressed the mitochondria without inducing cell death in the Barrett's cell lines. Mitochondria functional results were normalised to cell number for all experiments.

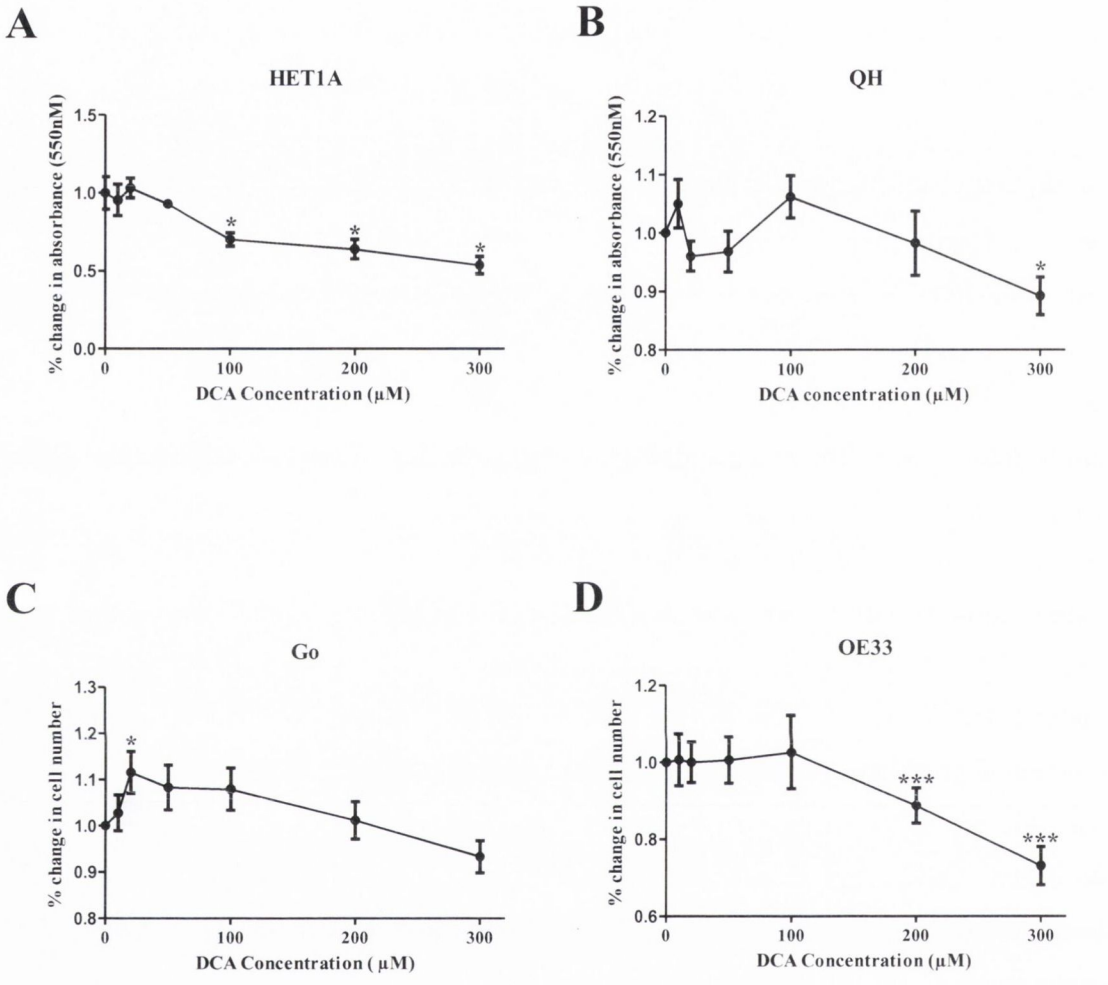


Figure 3.1: Cell density changes following treatment with 0, 10, 20, 50, 100, 200, 300μM DCA for 24hrs (n=12). (A) There was a significant decrease in cell density following treatment with ≥100μM DCA in the HET1A, normal squamous epithelium, cell line. **(B)** 300μM DCA caused a significant decrease in the QH, intestinal metaplasia cells. **(C)** 100μM DCA did not change cell density in the HGD, Go, cell line. **(D)** OE33 cells began to significantly decrease at concentrations >100μM DCA. *$p \leq 0.05$, ***$p \leq 0.0005$

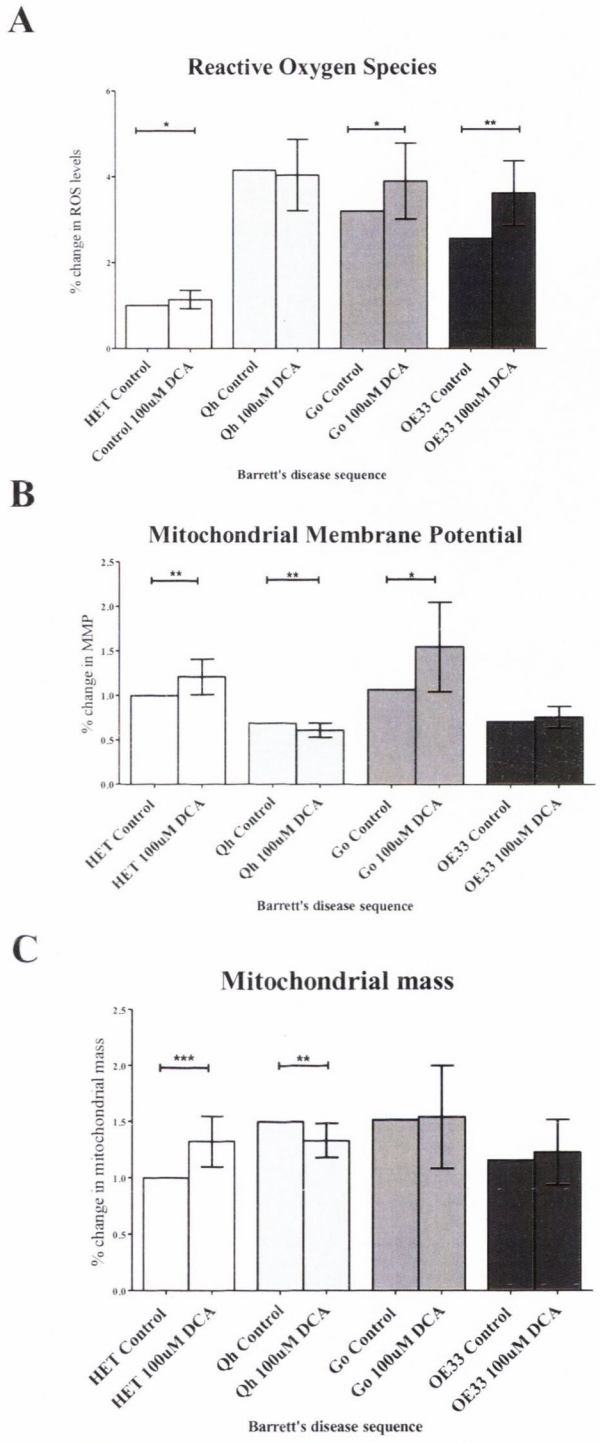


Figure 3.2: The effects of DCA on mitochondrial function along the Barrett's *in-vitro* cell line model. (A) There was a significant increase in ROS release in the HET1A (n=12), Go (n=14) and OE33 (n=11) cell lines. No change was demonstrated in the QH cells (n=16) **(B)** MMP was significantly reduced in the QHs (n=16) but increased in the HET1A (n=12) and Go (n=14) cells. **(C)** Mitochondrial mass was initially significantly increased following DCA exposure in the HET1As (n=12) and then significantly reduced in the QH cells (n=13). No change was demonstrated in the Go (n=14) and OE33 (n=21) cells. Error bars represent standard deviation. *p≤0.05, **p≤0.005, ***p≤0.0005

3.4.2 Effects of DCA in the Barrett's explant *ex-vivo* model

3.4.2.1 Measurement of mitochondrial secreted proteins following DCA treatment

As outlined in section 2.4.2.2, mitochondrial protein secretions; cytochrome c, SMAC/Diablo, endonuclease G, AIF and HtrA2, were used as surrogate markers of mitochondrial function *ex-vivo*. Explant cultured SIM and squamous epithelium biopsies from matched patients were treated with 100μM DCA for 24hrs in order to determine if DCA altered mitochondrial function *ex-vivo*.

Treatment with DCA did not significantly change the secretion of cytochrome c from the normal squamous epithelium ($p=0.636$) or SIM ($p=0.733$) (see Figure 3.3 A). DCA did not alter the release of SMAC/Diablo from the normal ($p=0.734$) or SIM ($p=0.959$) biopsies (see Figure 3.3 B). AIF secretions were unchanged from both normal ($p=0.542$) and SIM ($p=0.622$) tissue following DCA treatment. Endonuclease G release was also unaffected in the normal ($p=0.455$) and SIM ($p=0.569$) cultured media. Finally, 100μM DCA for 24hrs did not alter the release of HtrA2 from normal squamous epithelium ($p=0.695$) or SIM ($p=0.250$) cultured biopsies (see Figure 3.4).

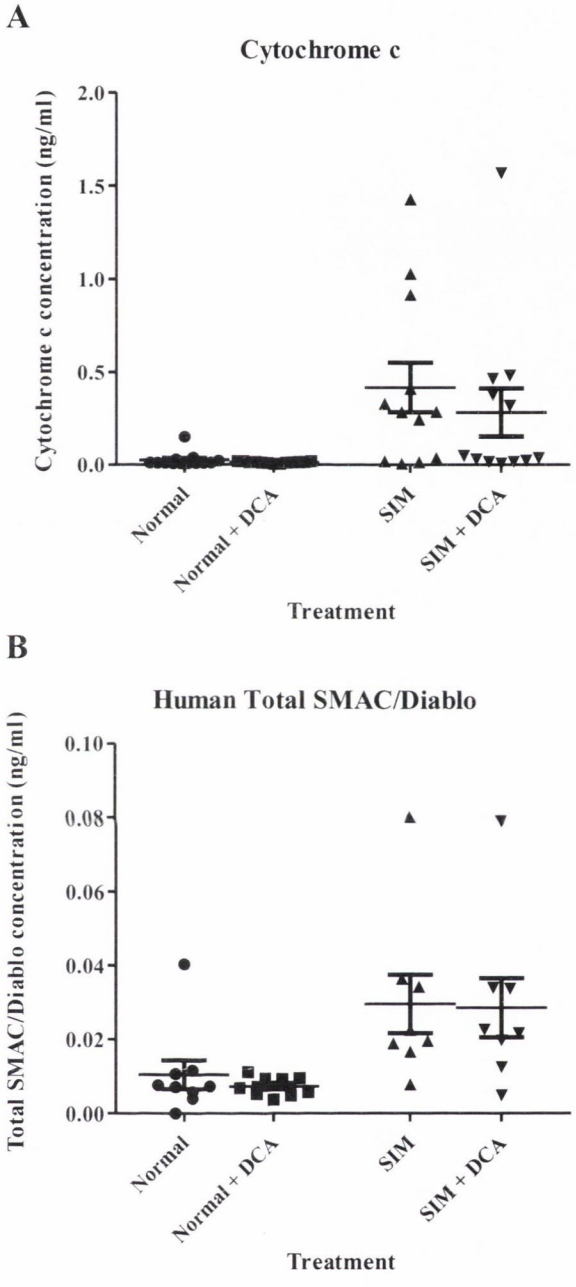


Figure 3.3: Measurement of the release of cytochrome c and SMAC/Diablo from explant cultured SIM biopsies following 24hrs treatment with 100μM DCA. Wilcoxon matched-pairs signed rank test showed no significant difference in the release of **(A)** cytochrome c (n=12) and **(B)** SMAC/Diablo (n=8) following 24hrs DCA treatment.

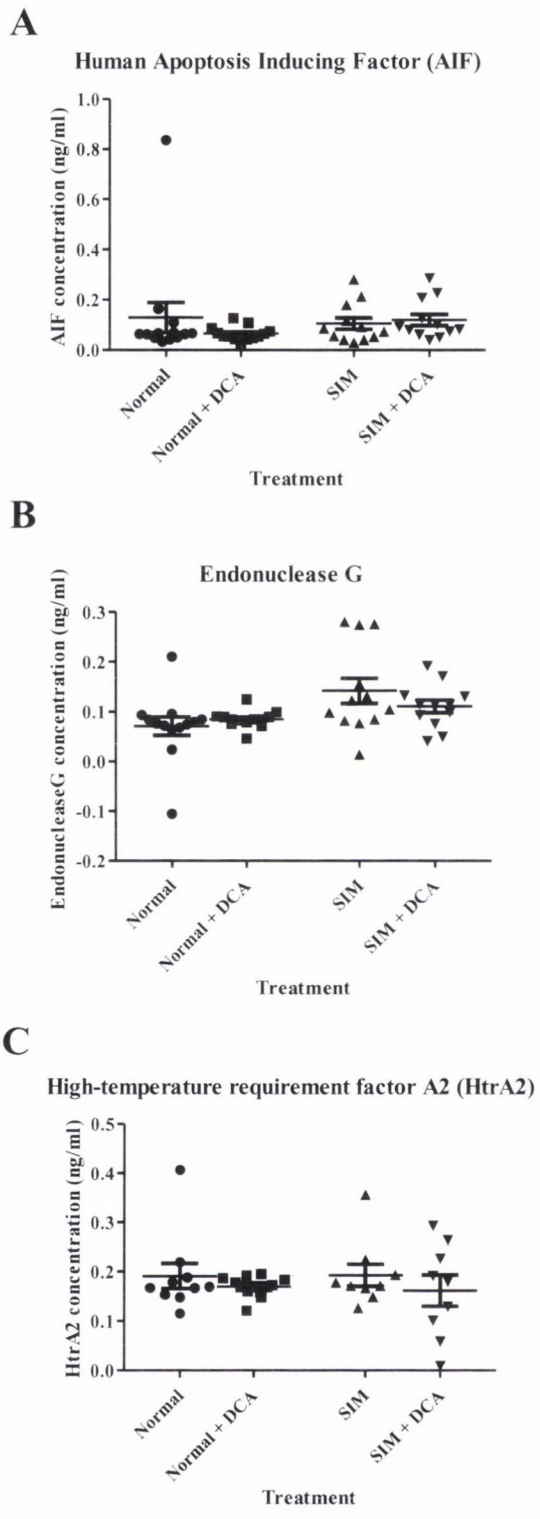


Figure 3.4: Measurement of mitochondrial proteins from explant cultured SIM biopsies following 24hrs treatment with 100μM DCA. Wilcoxon matched-pairs signed rank test showed no significant difference in the release of **(A)** AIF (n=12), **(B)** Endonuclease G (n=12) and **(C)** HtrA2 (n=9) following 24hrs treatment.

3.4.2.2 Measurement of cytokines following treatment with DCA

As outlined in section 2.4.2.2, pro-inflammatory cytokines; IL-1β, IL-6, IL-8 and TNF-α, were measured due to the known relationship between inflammation and oxidative stress from previous studies performed by our group (Biniecka, Kennedy et al. 2011). Explant cultured SIM and squamous epithelium biopsies from matched patients were treated with 100μM DCA for 24hrs. Treatment did not significantly change the secretion of IL-1β, IL-6, IL-8 or TNF-α in the normal squamous epithelium or the matched SIM biopsies (Figure 3.5 A-D).

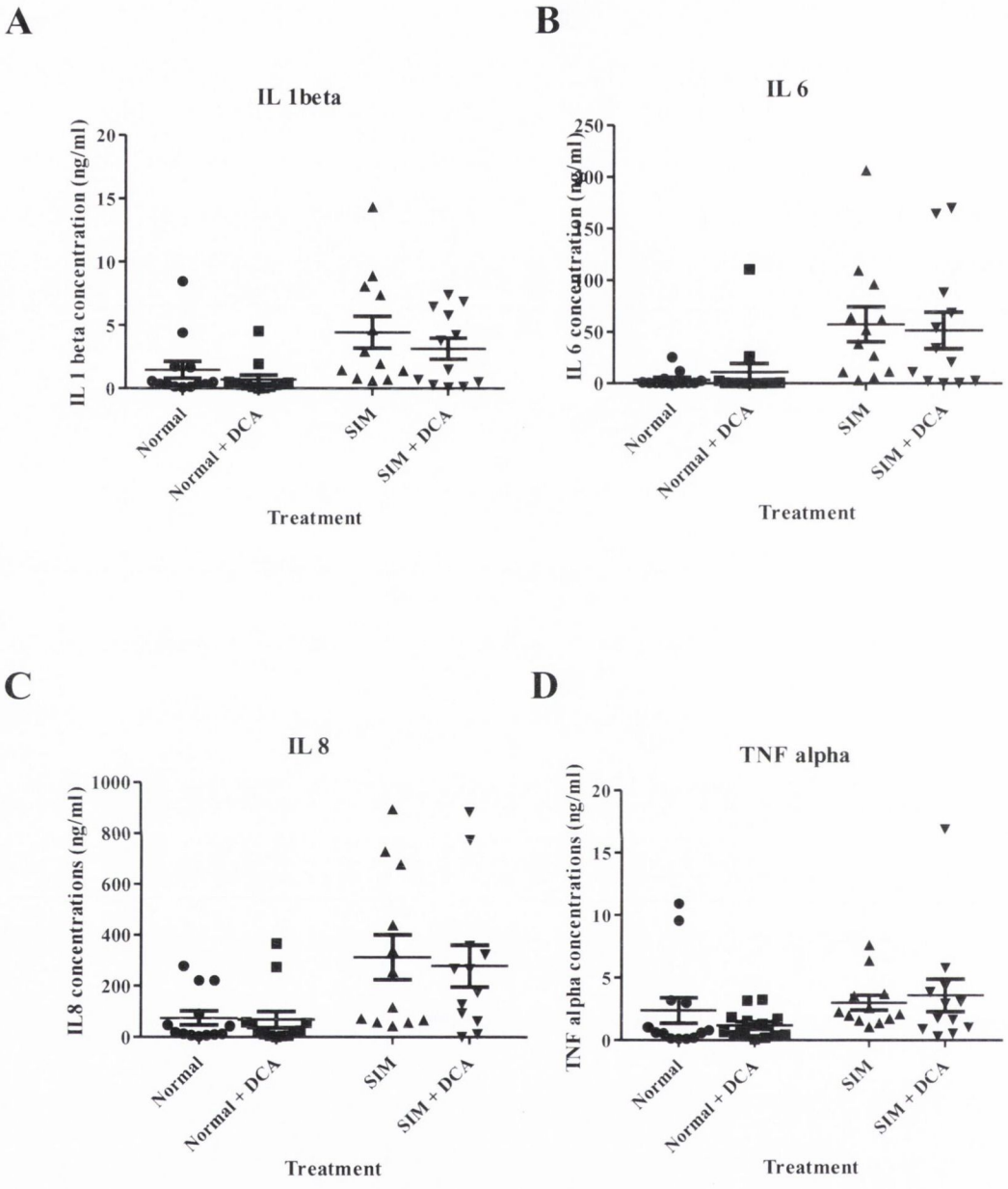


Figure 3.5: Measurement of pro-inflammatory cytokines from explant cultured SIM biopsies following 24hrs treatment with 100μM DCA (n=12). Wilcoxon matched-pairs signed rank test showed no significant difference in the release of **(A)** IL-1β in the normal (p=0.191) or SIM (p=0.301) biopsies. **(B)** IL-6 was unchanged in the normal (p=0.787) and SIM (p=0.970) tissue. **(C)** DCA did not alter the secretion of IL-8 in either squamous epithelium (p=0.893) or SIM (p=0.569) cultured biopsies. **(D)** There was no difference in the release of TNF-α in the normal (p=0.685) and SIM (p=0.910) biopsies.

3.4.3 Random mitochondrial point mutations in response to DCA *in-vitro*

Treatment with 100μM DCA for 24 hours induced changes in mitochondrial function in all four cell lines and therefore this concentration was selected for further analysis, as it induced changes in the mitochondrial function without inducing concurrent cell death in the intestinal metaplasia, HGD and adenocarcinoma cell lines. It was important to evaluate DCA as a catalyst for mitochondrial dysfunction and not cell death, as the purpose of this study was to determine if dysfunction was a trigger for disease progression. Following 24hrs, 100μM DCA caused no effect on the production of random mitochondrial point mutations in the HET1A ($p=0.561$), QH ($p=0.162$), Go ($p=0.456$) and OE33 ($p=0.222$) cells (see Figure 3.6).

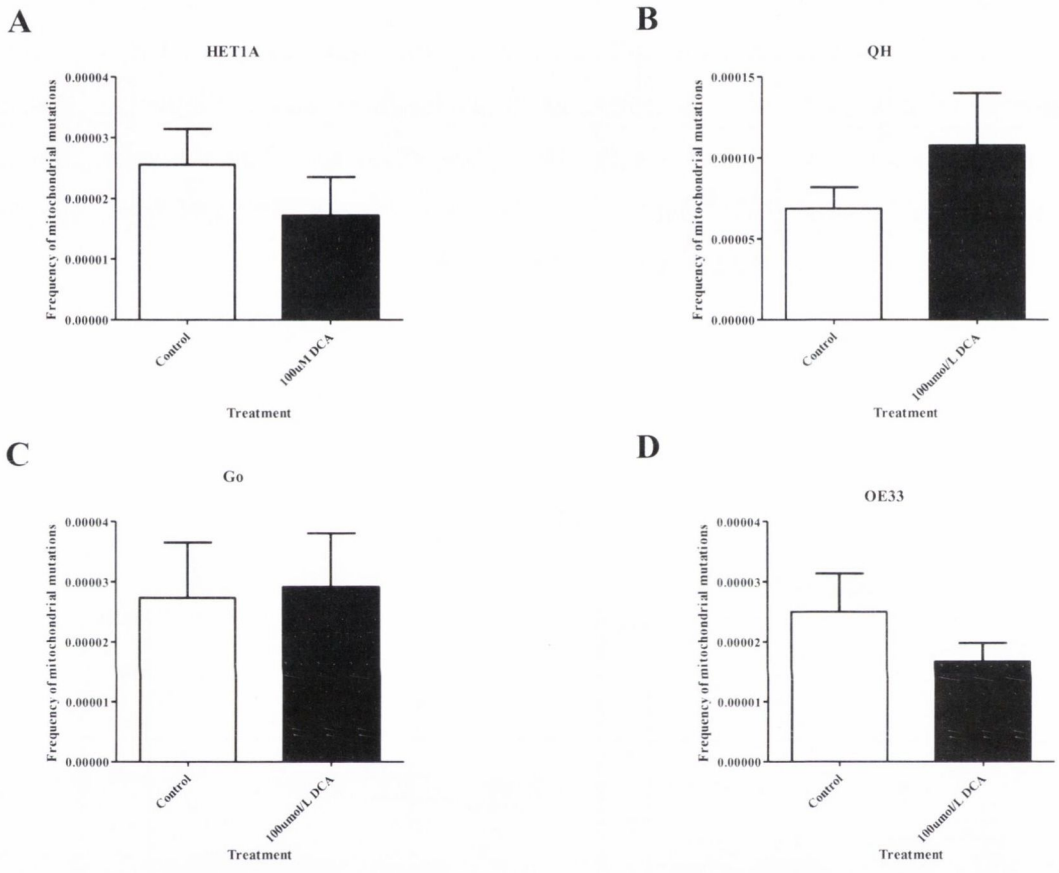


Figure 3.6: Frequency of random mitochondrial point mutations following treatment with 100μM DCA for 24hrs. Paired Student's t-tests demonstrated no significant difference in the frequency of random mitochondrial point mutations between the **(A)** HET1A (n=3) controls (SD 1.02 x 10^{-5}) and DCA treated cells (SD 1.04 x 10^{-5}) (p=0.561), **(B)** QH (n=6) controls (SD 3.25 x 10^{-5}) and DCA treated cells (SD 7.87 x 10^{-5}) (p=0.162), **(C)** Go (n=7) controls (SD 2.44 x 10^{-5}) and DCA treated cells (SD 2.34 x 10^{-5}) (p=0.456) and **(D)** OE33 (n=6) controls (1.43 x 10^{-5}) and DCA treated cells (SD 7.65 x 10^{-5}) (p=0.222).

3.4.4 Effects of antioxidants and DMOG on DCA induced mitochondrial dysfunction *in-vitro*

As demonstrated in section 3.4.1, treatment with 100μM DCA for 24hrs significantly altered ROS, MMP and mitochondrial mass at varying stages along the *in-vitro* model. Combined treatment with antioxidants and DMOG were analysed to determine if DCA induced mitochondrial dysfunction could be rescued.

DCA caused significant increases in ROS in the HET1A, Go and OE33 cells. ROS release returned to baseline levels following treatment with NAC in HET1A ($p<0.0001$) and OE33 ($p=0.0009$) cells. DMOG caused a significant reduction in ROS in the Go ($p=0.001$) and OE33 ($p<0.0001$) cells. EGCG reduced ROS in the OE33 cells ($p=0.002$) (Figure 3.7).

MMP was returned to baseline potentials following treatment with SOD ($p=0.013$) and DMOG ($p=0.045$) the QH cells. Treatment with SOD and DMOG reversed changes in membrane potential induced by DCA in the Go cell line ($p=0.039$ and $p=0.050$, respectively). ANOVA did not demonstrate any resolution of DCA induced increases in MMP in the HET1As ($p=0.650$) (Figure 3.8).

Mitochondrial mass returned to pre-DCA treatment levels only in the HET1A cells but not the QH cells, following treatment with EGCG. NAC, SOD and DMOG did not rescue any DCA induced changes in either cell lines (Figure 3.9).

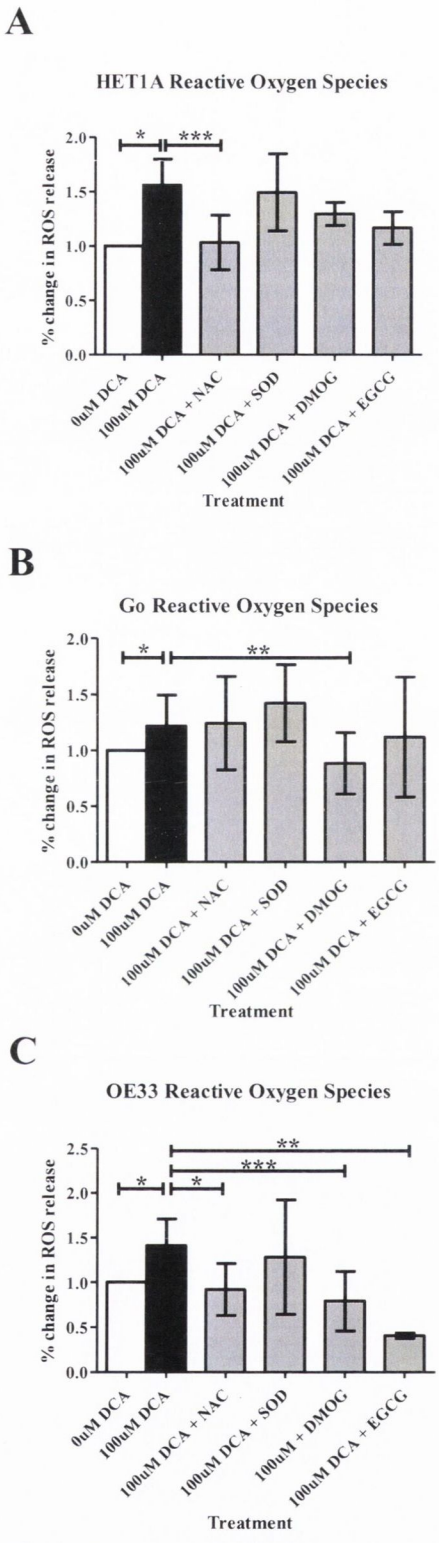


Figure 3.7: Measurement of ROS release following treatment with DCA, antioxidants (NAC, SOD, and EGCG) and DMOG. (A) In the HET1A cell line (n=15), ANOVA showed that NAC significantly reduced ROS increases induced by DCA. **(B)** There was a significant reduction in ROS release following treatment with DMOG in the Go cells (n=14). **(C)** ROS was significantly reduced below DCA induced increases in the OE33 cell line (n=11) by NAC, DMOG and EGCG. Errors bars represent standard deviation. *p<0.05, **p<0.005, ***p<0.0005

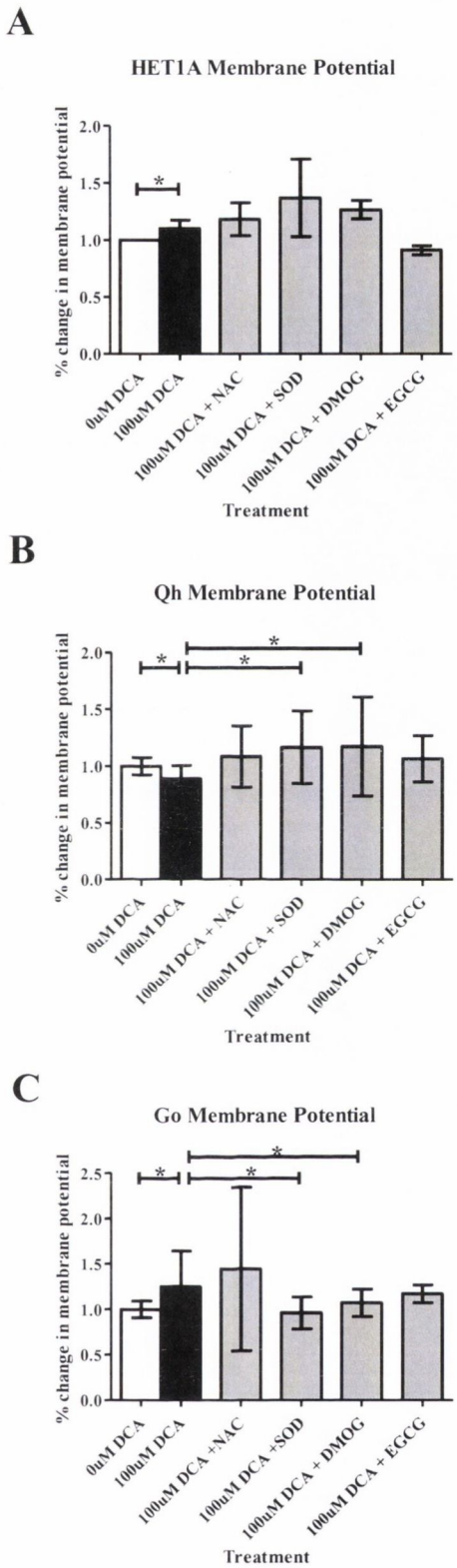


Figure 3.8: Measurement of MMP following treatment with DCA, antioxidants (NAC, SOD, EGCG) and DMOG. (A) In the HET1A cell line (n=15), ANOVA showed no improvement. **(B)** There was a significant increase in MMP following treatment with SOD and DMOG in the QH cells (n=16). **(C)** SOD and DMOG significantly reduced MMP below DCA induced increases in the Go cell line (n=9). Error bars represent standard deviation. *p<0.05, **p<0.005, ***p<0.0005

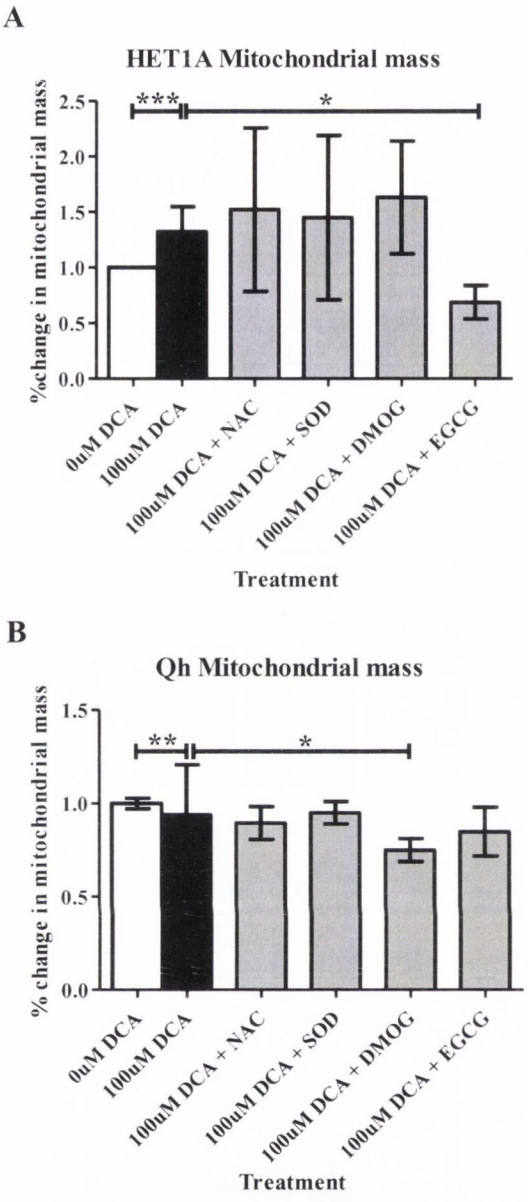


Figure 3.9: Measurement of mitochondrial mass following treatment with DCA, antioxidants (NAC, SOD, EGCG) and DMOG. (A) In the HET1A cell line (n=12), EGCG significantly reduced mitochondrial mass. **(B)** DMOG caused a significant decrease in mitochondrial mass in the QH cells (n=10), further reducing DCA induced changes. Error bars represent standard deviation. *$p<0.05$, **$p<0.005$, ***$p<0.0005$

3.4.5 Effects of antioxidants and DMOG on mitochondrial function at baseline in the Barrett's oesophagus *in-vitro* model

Simultaneously, while analysing the effects of DCA on the different parameters of mitochondrial function, the effects of NAC, SOD, DMOG and EGCG were compared to the untreated cells.

In the HET1A cell line, SOD resulted in a 21% increase in cell density (p=0.0206), while treatment with DMOG and EGCG caused a significant reduction in cell density by 20% (p=0.0005) and 10% (p=0.040), respectively (see Figure 3.10). Microscopically there was no evidence of increased cell death (i.e. floating cells) following 24hrs treatment with DMOG, despite the reduced cell number. ROS levels were significantly decreased by 23% with NAC treatment (p=0.030). EGCG was associated with 35% increase in the release of ROS (p=0.048). Similarly to DCA, DMOG (p=0.006) caused an increase in MMP. NAC and DMOG caused an increase in mitochondrial mass (p=0.004 and p=0.0005, respectively). EGCG significantly reduced mitochondrial mass (p=0.0001).

Treatment with DMOG caused a significant 25% reduction in cell density in the QH cell line (p=0.003) (see Figure 3.11). Once again, microscopically this reduction in cell number was not associated with any evidence of increased dead cells. DMOG caused a significant 31% reduction in the levels of ROS (p=0.002). NAC, SOD and DMOG caused a 16% (p=0.005), 14% (p=0.011) and 84% (p=0.027) increase in membrane potential, respectively. Mitochondrial mass was reduced by 13% following treatment with EGCG (p=0.027).

At baseline, treatment with SOD resulted in a 22% increase (p=0.029) in cell density, while DMOG was associated with a 24% decrease (p=0.035) in cell number in the Go cell line (Figure 3.12). DMOG treatment resulted in a significant reduction in ROS release in the control setting (p=0.020). MMP decreased following treatment with SOD (p=0.010).

Antioxidants and DMOG did not significantly affect cell number in the OE33 cell line ($p=0.915$) (Figure 3.13). ROS release was reduced by 40% with DMOG ($p=0.0001$) and by 61% with EGCG ($p<0.0001$) treatments. Mitochondrial mass decreased with EGCG treatment ($p=0.002$). Membrane potential did not significantly change following 24hrs treatments ($p=0.519$).

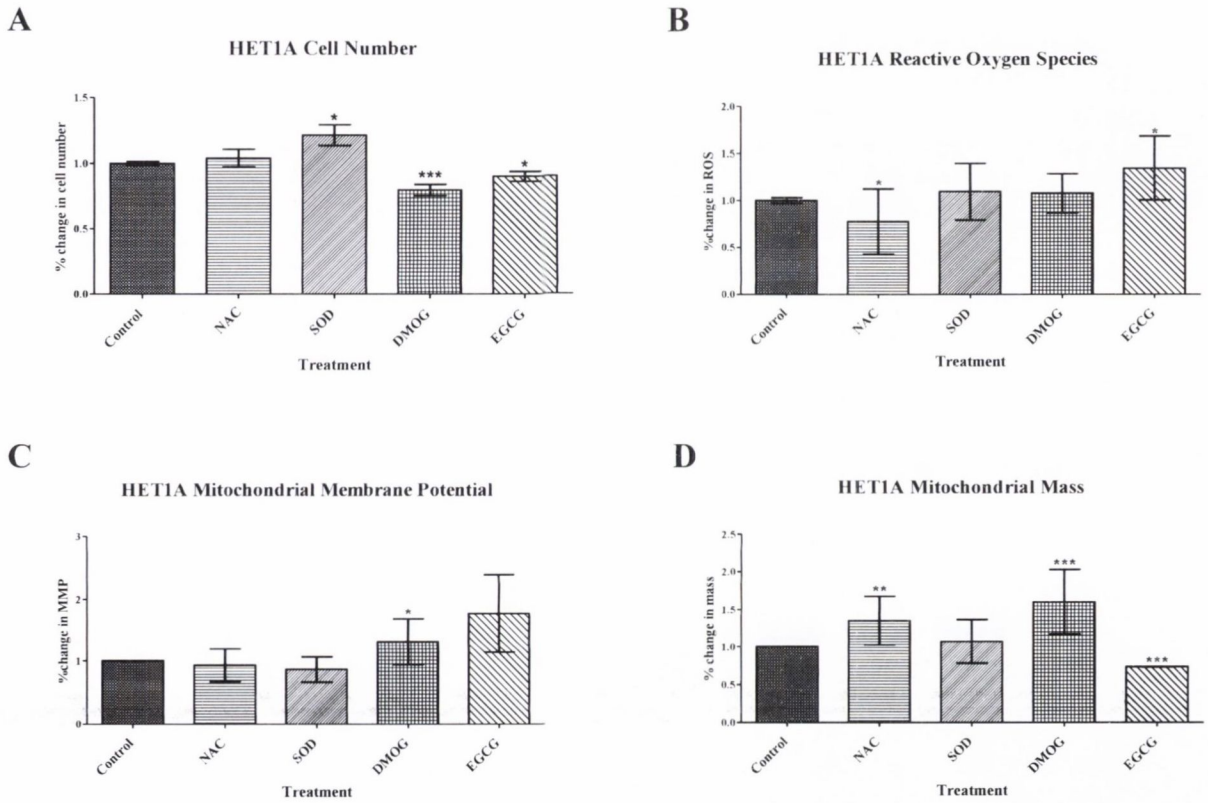


Figure 3.10: Effects of antioxidants and DMOG on mitochondrial function in HET1As at baseline. (A) SOD caused a significant increase in cell number, while DMOG and EGCG demonstrated a significant decrease in cell number (n=21). Importantly, there was no evidence of increased cell death (i.e. floating cells) following treatment with DMOG, but there was suspected evidence of a slower growth rate. **(B)** NAC significantly decreased ROS and EGCG treatment was associated with increased levels of ROS (n=18). **(C)** DMOG and EGCG treatment was associated with a significant increase in MMP (n=15). **(D)** Mass was significantly increased following treatment with NAC and DMOG; EGCG reduced mass (n=12). Error bars represent standard deviation. *$p \leq 0.05$, ***$p \leq 0.0005$

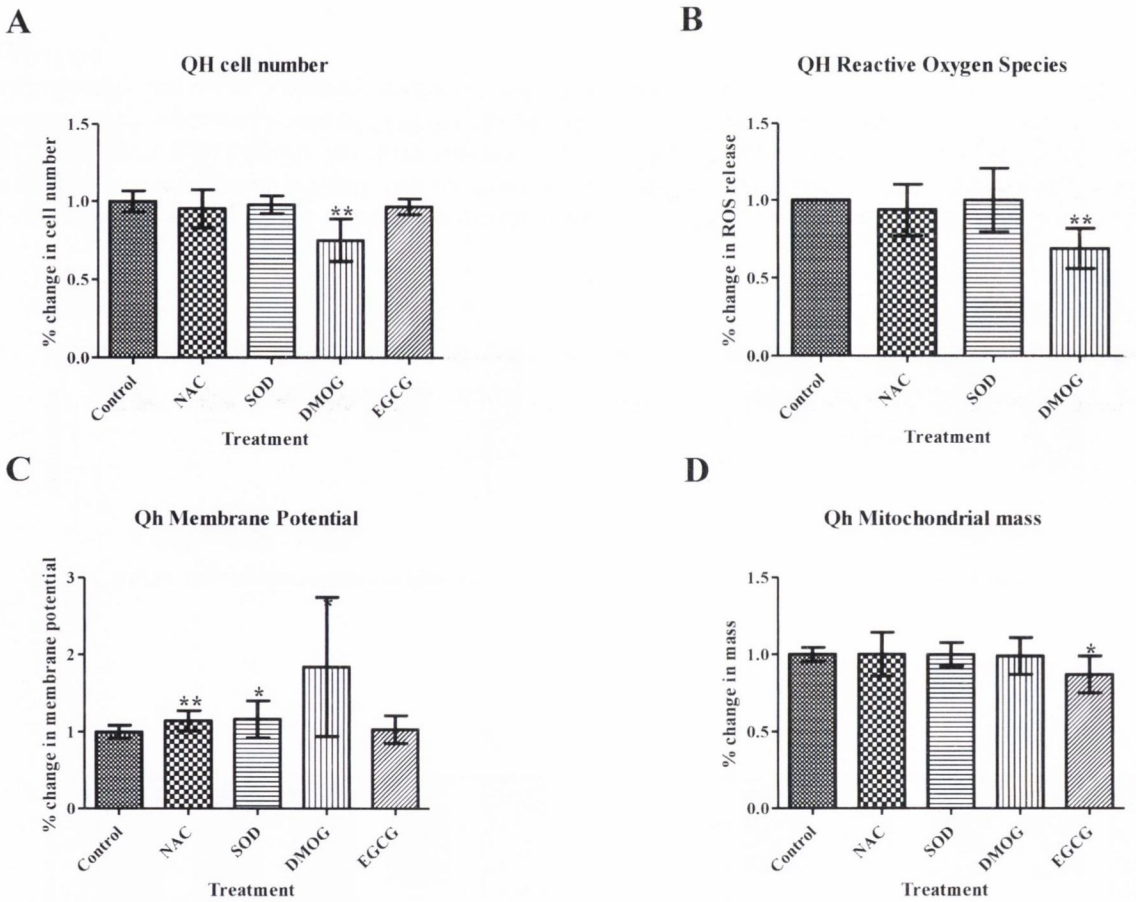


Figure 3.11: Effects of antioxidants and DMOG on mitochondrial function in the QH cell line at baseline. (A) DMOG caused a significant reduction in cell density, however; this was not associated with increased number of floating dead cells microscopically (n=6). **(B)** ROS were significantly reduced by DMOG treatment for 24hrs (n=8). **(C)** NAC, SOD and DMOG all were associated with an increase in MMP (n=12). **(D)** EGCG caused a reduction in mass (n=8). Error bars represent standard deviation. *p<0.05, **p<0.005

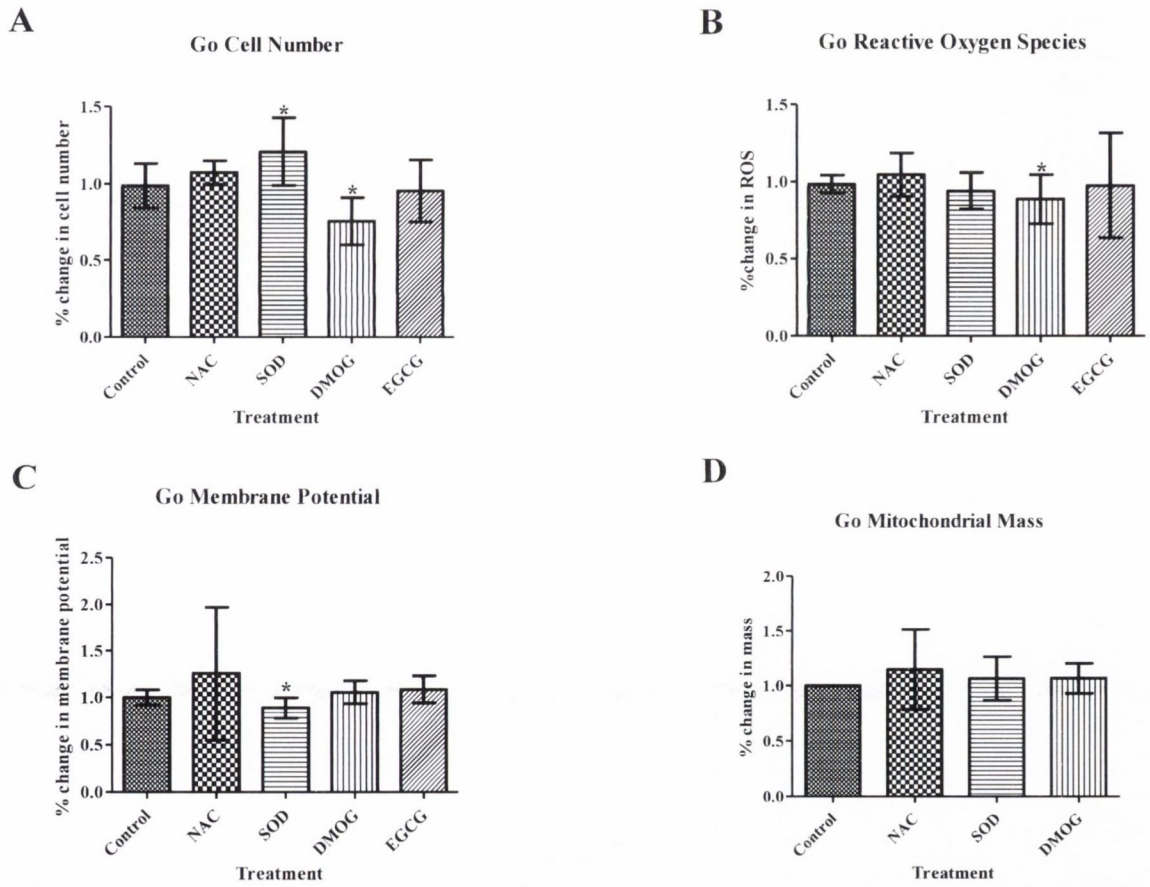


Figure 3.12: Go cell line response to antioxidant and DMOG treatment at baseline. (A) SOD induced a significant increase and DMOG induced a decrease in cell density (n=9). **(B)** DMOG caused an 11% reduction in the release of ROS in the GO cells (n=14). **(C)** SOD treatment resulted in a loss of MMP (n=11). **(D)** Treatments did not affect mitochondrial mass at baseline (n=9). Error bars represent standard deviation. *$p<0.05$

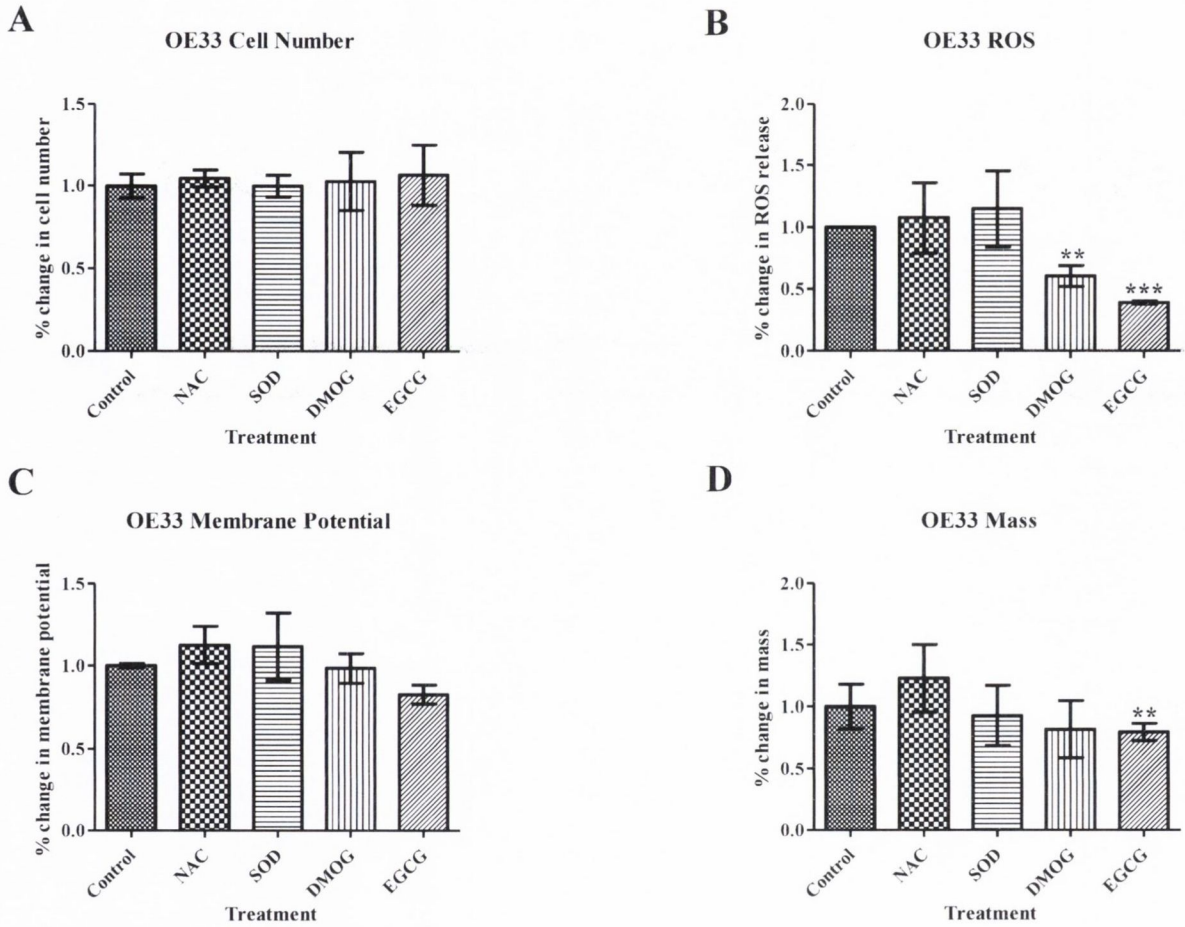


Figure 3.13: OE33 cell line response to antioxidant and DMOG treatment at baseline. (A) There was no significant change in the cell density following 24hrs treatment with NAC, SOD, DMOG or EGCG (n=9) (ANOVA p=0.9151). **(B)** Treatment with DMOG and EGCG significantly reduced ROS release in the OE33 cell line (n=16). **(C)** MMP did not significantly change (n=16) (ANOVA p=0.5189). **(D)** Mitochondrial mass decreased following EGCG treatment (n=11). Error bars represent standard deviation. **p<0.005, ***p<0.0005.

3.4.6 Effects of DMOG on random mitochondrial point mutations *in-vitro*

100μM DCA at 24hrs did not result in any change in the frequency of random mitochondrial point mutations; experiments were therefore not repeated in the presence of NAC, SOD, DMOG or EGCG. However, selecting DMOG, the treatment with the most pronounced effects on stabilising DCA induced functional changes, with significantly reduced release of ROS in QH, Go and OE33 cells, we analysed its effects on random mitochondrial point mutation frequencies in all cell lines (Figure 3.14). DMOG did not significantly alter the frequency of random mitochondrial point mutations in the HET1A ($p=0.723$), QH ($p=0.511$), Go ($p=0.495$) and OE33 ($p=0.677$) cell lines.

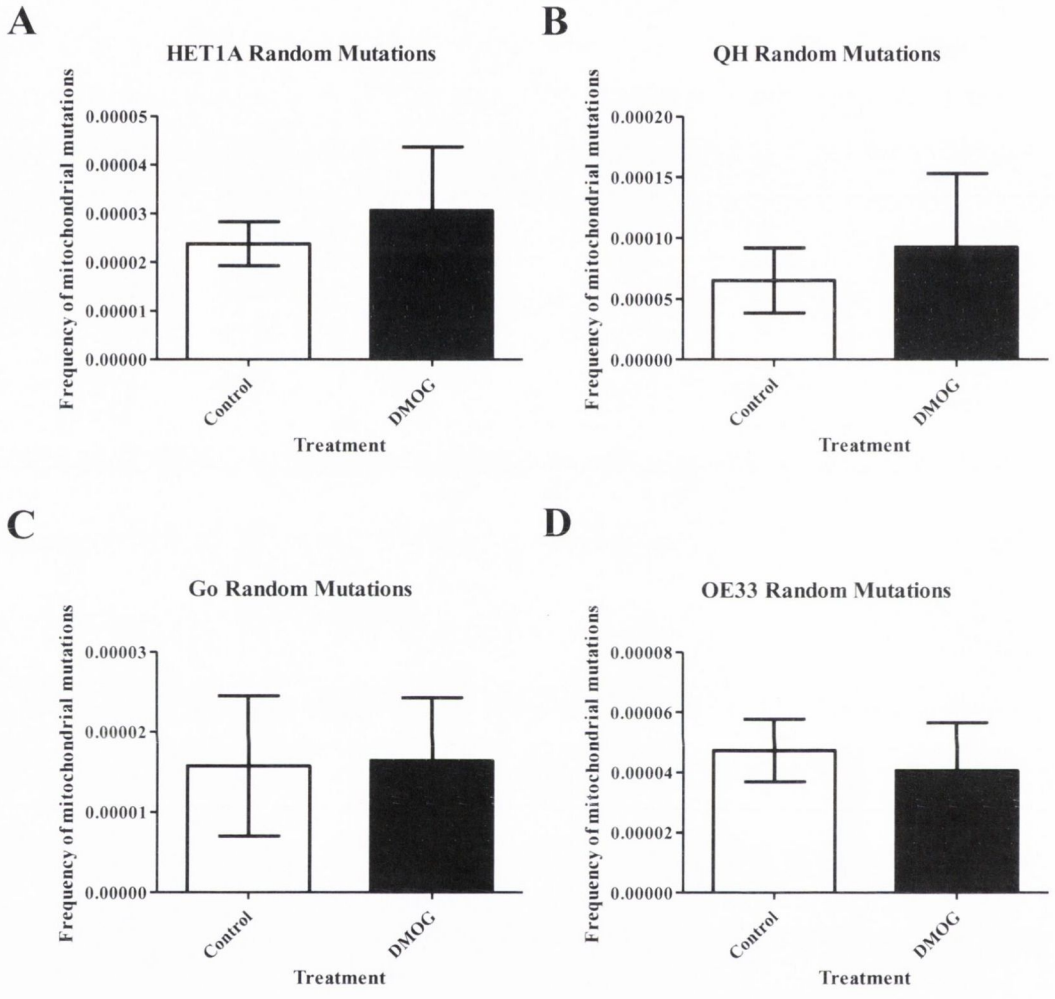


Figure 3.14: Frequency of random mitochondrial point mutations following treatment with 1mM DMOG *in-vitro*. There was no significant change in the frequency of random mutations in the **(A)** HET1A (n=4), **(B)** QH (n=3), **(C)** Go (n=4) and **(D)** OE33 (n=3) cell lines. Error bars represent standard deviation.

3.4.7 Effects of DMOG on cell cycle

In section 3.4.5 DMOG was seen to cause a significant reduction in cell number, without microscopic evidence of increased cell death. Here we demonstrated that DMOG caused a significant alteration in the cell growth cycle (Figure 3.15). In the HET1A cells, DMOG caused a significant increase in the G1 phase ($p=0.0005$), while significantly reducing the percentage of cells entering the S phase ($p=0.045$) and G2-M phase ($p=0.0004$). DMOG treatment in the QH cells significantly increased the percentage of cells in the sub G0 phase ($p=0.029$) and the G1 phase ($p=0.001$). There was a significant decrease in the percentage of QH cells entering into the S phase ($p=0.001$) and the G2-M phase ($p=0.0003$). In the Go cell line, DMOG caused a significant increase in the percent of cells in the sub G0 phase ($p=0.0003$) and G1 phase ($p=0.002$). There was a significant reduction in the percentage of Go cells entering into the S phase ($p=0.0001$) and G2-M phase ($p=0.003$). DMOG treatment caused a significant increase in the number of OE33 cells in the sub G0 phase ($p=0.045$) and G1 phase ($p=0.003$). DMOG treatment significantly reduced the percent of OE33 cells entering into the S phase ($p=0.004$) and G2-M phase ($p=0.007$).

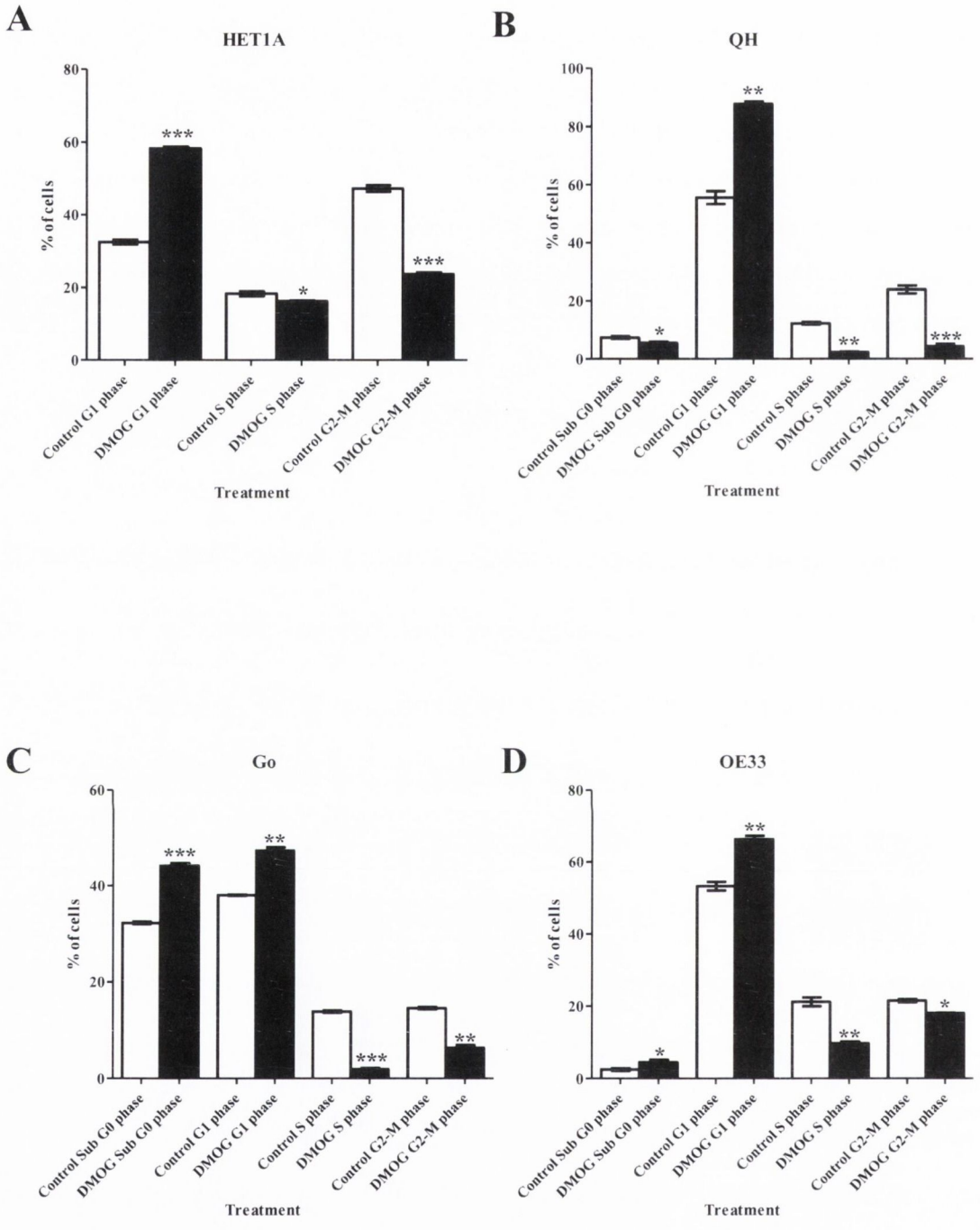


Figure 3.15: The effects of DMOG treatment on cell growth cycle (n=3). (A) DMOG treatment caused a significant increase in the percent of cells in the G1 phase, while reducing the percent entering the S and G2-M phases. DMOG caused a significant increase in the percent of cell in the sub G0 and G1 phase, while causing a significant decrease in the percent entering the S and G2-M phases in the **(B)** QH, **(C)** Go and **(D)** OE33 cell lines. Error bars represent standard deviation. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$

3.5 DISCUSSION

Bile reflux is a well-recognised precursor in the development and progression of Barrett's oesophagus (Jankowski, Hopwood et al. 1993, Stein, Feussner et al. 1994, Kauer, Peters et al. 1995, Jenkins, D'Souza et al. 2007, Looby, Abdel-Latif et al. 2009). While bile is formed by many different components, unconjugated bile acids, such as DCA, have been identified as perhaps having an increased carcinogenic potential (Jankowski, Hopwood et al. 1993, Kauer, Peters et al. 1995, Jenkins, D'Souza et al. 2007). However, the exact biological mechanisms by which DCA may be a precursor for Barrett's oesophagus are poorly understood. Based on findings in chapter two, DCA was selected to determine if it effected mitochondrial function along the Barrett's disease sequence.

Of all the bile acids, we selected DCA based on findings from work by Jenkins *et al.* which demonstrated DCA was the most genotoxic of examined bile acids, both at a neutral and acidic pH, inducing increased ROS release in two oesophageal cancer cell lines, one of those being OE33s (Jenkins, D'Souza et al. 2007). However, the effects of DCA in the premalignant stages of Barrett's oesophagus were not examined in their study. Further supporting the examination of DCA as a carcinogenic factor in Barrett's oesophagus was McAdam *et al.* who demonstrated that DCA caused an increase in the expression of iNOS and subsequently the production of NO (McAdam, Haboubi et al. 2012). Over-expression of iNOS has been implicated in diseases such as, rheumatoid arthritis (McInnes, Leung et al. 1996, Grabowski, Wright et al. 1997), colon (Kojima, Morisaki et al. 1999) and breast (Vakkala, Kahlos et al. 2000) cancer, and early stages of Barrett's oesophagus (Wilson, Fu et al. 1998). Our results demonstrated treatment with DCA induced some significant changes in mitochondrial function along the cell-line sequence. The most significant results were demonstrated in the HET1A cell line, indicating that DCA may play a role in Barrett's oesophagus propagation through the alteration of all mitochondrial function parameters which we examined. However, the effects of DCA on the letter stages of the disease process were less pronounced. DCA treatment caused significant ROS increases in HET1A, Go and OE33 cells. No change was demonstrated in ROS levels in the QH cell lines, although in chapter two, this cell line had significantly increased ROS at baseline, and DCAs effects may have therefore

been less obvious. DCA caused varying changes in the membrane potential and mass at different points along the Barrett's disease sequence. Our *in-vitro* findings showed treatment with 100μM DCA did not cause any significant increases in the frequency of random mitochondrial point mutations. Although, it is recognised that mitochondrial dysfunction may occur independent of mutagenesis (Trifunovic 2006).

Ex-vivo, treatment with DCA for 24hrs did not lead to increased secretion of mitochondrial proteins; cytochrome c, SMAC/Diablo, AIF, endonuclease G and HtrA2, or pro-inflammatory cytokines; IL-1β, IL-6, IL-8 and TNF-α. The lack of response to DCA in the *ex-vivo* analysis may be due to the heterogeneity of the explant environment making it less sensitive to DCA effects compared with the monolayer cell lines, and therefore for effects to be demonstrated in the *ex-vivo* setting higher doses or longer durations of treatment may be required. Overall, the effects of 100μM DCA for 24hrs on mitochondrial function and mutagenesis were found to be minimal. The most significant finding was in the HET1As, which experienced the most predominant changes in mitochondrial function, but, no increase in the frequency of random mitochondrial point mutations.

We acknowledge certain limitations within our study. Mutagenesis may take longer than 24hrs to be induced or increased DCA concentrations may be required. Jolly *et al.* showed DCA caused DNA damage in a dose-dependent manner (between concentrations of 10μM to 300μM) after 3hrs treatment in HET1A cells, through an iNOS and NF-κB linked process (Jolly, Wild et al. 2009). Perhaps higher concentrations of DCA may be required, however, in this study, we aimed to use a concentration of DCA which did not induce confounding elements such as cell death, and from our dose response analysis it was treatment with 100μM DCA for 24hrs which caused our most significant changes in mitochondrial function. In an ideal setting, it would have been interesting to explore the effects of DCA, in the explant studies, at varying time points, however, we must acknowledge that fresh Barrett's tissue is a limited resource and for this reason only one time-point (i.e. 24 hours) was selected. Similarly, while Jenkins *et al.* demonstrated DCA was most toxic at a neutral pH *in-vitro* (Jenkins, D'Souza et al. 2007), *in-vivo* refluxate is derived from both an acidic gastric, as well as a bile component. We acknowledge concentrating solely on the

bile element, at a neutral pH, may explain the lack of significant results, and perhaps it is the reflux environment in its entirety which may alter mitochondrial function and mutations.

In a redox balanced environment there is equilibrium between free radicals and antioxidants. In chapter two, SIM displayed increased mitochondrial instability with increased secretion of inflammatory proteins. Mitochondrial instability, leading to increased ROS, may induce increased random mutations, and disturbances in metabolism may arise as a consequence (Trifunovic 2006). Therefore the premise for attempting to stabilise this mitochondrial disruption, particularly at the early disease stages, is logical, and increasing antioxidant levels to restore redox balance maybe a reasonable treatment approach (Babar, Abdel-Latif et al. 2010). Our results showed all antioxidants and DMOG reduced DCA induced mitochondrial dysfunction, when it occurred, *in-vitro*. DMOG had the most significant stabilising effects; reducing DCA induced ROS increases back to baseline levels in Go and OE33 cells, while altering membrane potential in QH and Go cells. It was for this reason it was selected to determine its effects on random mitochondrial point mutations. However, DMOG did not reduce the frequency of mutations in any cell line in the Barrett's disease sequence.

The effects of all antioxidants and DMOG on each cell line at baseline were analysed. Consistent with the results in section 3.4.4; where DCA/antioxidant combinations rectified DCA induced changes, NAC, SOD, DMOG and EGCG tended to produce similar changes when used as a single therapy. At baseline, DMOG caused the significant reduction in release of ROS in the QH, Go and OE33 cell lines, further supporting our choice for selecting it for analysis in all future experiments. However, interestingly, the effects of these treatments in the HET1A, normal squamous epithelium cell lines, paralleled DCA induced mitochondrial dysfunction. EGCG caused a significant increase in ROS, DMOG and EGCG significantly increased MMP and NAC and DMOG significantly increased mitochondrial mass. These findings may be explained by an extensive Cochrane review by Bjelakovic *et al.* which assessed the effects of antioxidant supplementation for the prevention of mortality in a healthy population and in patients with various diseases (Bjelakovic, Nikolova et al. 2012). Interestingly, the authors found that antioxidant supplementations with beta-carotene,

vitamin A and vitamin E, in a healthy population, was associated with a significant increase in mortality. While they were unable to determine the specific biochemical mechanisms behind these detrimental effects, it referenced studies by Hercberg *et al.* which stated that antioxidants show interdependency and may have effects when only given in combination with other agents, in this case, DCA (Hercberg, Galan et al. 1998). While Ristow *et al.* stated that antioxidants may reduce the lifespan of organisms, and in a healthy environment ROS promote health and longevity (Ristow and Zarse 2010). In fact, Bjelakovic hypothesised while oxidative stress and dysfunction is implicated in many chronic diseases, in the normal healthy environment, if we disrupt the redox balanced system, we interfere with some essential defensive mechanism like apoptosis and detoxification, which in turn may explain increased mortality in the healthy population and in the normal HET1A cell line.

This chapter has shown that DCA had a limited effect on the mitochondrial environment. It most significantly altered function in the *normal* cell line. These findings were not replicated in our explant analysis, and the complexity of the tissue micro-environment may explain this lack of a response; longer durations and higher concentrations in pulsatile release formats, as opposed to continuous 24hrs treatment, may be required over many years to induce mitochondrial dysfunction. Treatment with antioxidants and DMOG reduced DCA induced instability *in-vitro*. Interestingly, antioxidants and DMOG in the control setting appeared to induce dysfunction, similar to DCA, in the normal cell line. This may reflect the importance of a balanced redox environment in the control setting, which is required for normal cellular function. These pathways present a target for further study and potential therapeutic interventions.

Chapter 4: Examination of oxidative stress, lipoperoxidation and inflammatory markers across the Barrett's oesophagus sequence, identifying different patterns in patients with cancer progression

4.1 INTRODUCTION

The rise in incidence of Barrett's has paralleled the rising incidence of oesophageal adenocarcinoma (Blot, Devesa et al. 1991, Jankowski, Perry et al. 2000, van Soest, Dieleman et al. 2005). While it has been speculated increased Barrett's rates may be in part a consequence of reduced thresholds for performing upper endoscopies in this modern treatment era; figures from the Netherlands indicate Barrett's oesophagus has increased from 14.3/100,000 to 23.1/100,000 person years between 1997 and 2002, irrespective of the number of endoscopies performed (van Soest, Dieleman et al. 2005). Barrett's oesophagus is the leading known risk factor for oesophageal adenocarcinoma (Cameron, Ott et al. 1985, Spechler and Goyal 1986), and surveillance programmes have been established in an attempt to ensure early cancer detection, allowing for earlier treatment and improved outcome and survival (Peters, Clark et al. 1994, van Sandick, van Lanschot et al. 1998). A recent retrospective analysis by Grant *et al.* reviewed records of 224 patients treated with HGD or oesophageal adenocarcinoma; 36 cases were detected while on a Barrett's oesophagus surveillance programme (Grant, Demeester et al. 2013). The authors demonstrated a significantly improved overall disease-free survival, lower nodal burden and reduced need for surgical resection in the surveillance cohort compared to patients with a primary diagnosis of oesophageal cancer.

However, despite the increasing incidence of oesophageal adenocarcinoma, data indicates the cancer risk associated with Barrett's oesophagus may not be as high as originally documented, and for many patients the lifetime risk of progression to cancer is minimal (O'Connor, Falk et al. 1999, Ryan, Rowley et al. 2006, Hvid-Jensen, Pedersen et al. 2011). As such, the cost-effectiveness of surveying a non-dysplastic Barrett's cohort is debatable, and the clear challenge for translational science is to both understand the drivers of tumourigenesis and also find biomarkers with a high sensitivity and specificity for identifying Barrett's patients at greatest risk of cancer progression.

Potential biomarkers have been identified in one of the largest studies to date, which utilised archived samples from the Northern Ireland Barrett's oesophagus Register

(Bird-Lieberman, Dunn et al. 2012). This population based study compared 89 patients with progressive Barrett's oesophagus with 291 patients with non-progressive disease. LGD had an 11-fold increased odds ratio for disease progression, while DNA ploidy and *Aspergillus oryzae* lectin were both associated with a 3-fold increased risk. It was acknowledged, however, that the odds ratios' confidence intervals were wide, with sensitivities and specificities of these individual biomarkers low.

Work by the Seattle Barrett's Oesophagus Study group, has identified clonal diversity as a biomarker for Barrett's oesophagus progression (Maley, Galipeau et al. 2006, Merlo, Shah et al. 2010). More recently, they have demonstrated that insulin resistance and leptin is associated with a significant 2.5-fold increased risk of oesophageal adenocarcinoma progression (Duggan, Onstad et al. 2013). Low levels of high molecular weight adiponectin were also significantly associated with increased risk of cancer progression (hazard ratio 0.34). This group believe that these biomarkers may be used to determine the risk of cancer in Barrett's oesophagus patients. However, despite these findings, a single biomarker capable of predicting Barrett's progression, has yet to be identified. To date, no biomarker has advanced to a Phase 5 study for the development of such a biomarker (Pepe, Etzioni et al. 2001, Timmer, Sun et al. 2013).

In inflammation to cancer pathways, oxidative stress and altered energy metabolism have attracted increased attention, with a particular focus on the mitochondria (Kroemer 2006, Wallace 2012). Increased oxidative stress is a precursor for increased rates of mitochondrial mutations and dysfunction, which in turn carries an inherent predisposition for cancer development (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009, Loeb 2010). With respect to Barrett's oesophagus, applying the mutator phenotype hypothesis would suggest that Barrett's lesions with low levels of mutations would not have the capability to undergo cancer conversion, and therefore these may potentially serve as biomarkers for the stratification of SIM. The goal of this chapter was to examine levels of oxidative stress markers, 8-oxo-dG and 4-HNE, and inflammation markers, CD3, across the Barrett's disease sequence. A second goal was to determine if there were differences in oxidative stress, a surrogate marker for mitochondrial instability, between patients who progressed to HGD/oesophageal adenocarcinoma and non-progressors.

4.2 HYPOTHESIS AND AIMS

We hypothesise that levels of DNA adducts, lipid peroxidation and T cell levels alter during disease progression and may segregate non-progressive and progressive Barrett's oesophagus.

Specific aims of chapter four:

(1) Investigate the expression of DNA adducts (8-oxo-dG), lipid peroxidation (4-HNE) and T cell (CD3) levels in tissue samples across the Barrett's oesophagus disease sequence.

(2) Evaluate the expression of these markers in patients with progressive and non-progressive Barrett's intestinal metaplasia, where HGD and oesophageal adenocarcinoma are the primary end-points.

(3) Correlate the expression of 8-oxo-dG, 4-HNE and CD3 with the frequency of random mitochondrial point mutations from chapter two.

(4) Quantify the levels of 8-oxo-dG and 4-HNE in serum samples from patients representing the different stages of the Barrett's oesophagus disease sequence.

4.3 MATERIALS AND METHODS

4.3.1 Barrett's oesophagus registry

Data were collected from a Barrett's oesophagus registry at St. James's Hospital, Dublin, which is maintained by a dedicated database manager. A committee was set up for the Barrett's registry to establish protocol for patient eligibility, data collection and patient consent. Data were collected retrospectively from the charts of 940 patients with histologically confirmed Barrett's oesophagus attending our Centre for routine surveillance endoscopy. The data manager also attended a dedicated Barrett's clinic and obtained information from patients at this time. A data record form was prepared and completed for each patient and entered into the registry based on their first visit and each subsequent visit; this data was maintained in a computer database (Dendrite Clinical Systems Ltd., London, UK).

4.3.2 Patient samples

Barrett's oesophagus surveillance patients with disease progression, regression or static Barrett's disease were selected for tissue microarray (TMA) construction. Patients were selected from the Barrett's oesophagus and St. James's hospital pathology databases. Normal oesophageal biopsies (n=15) were used from patients with no evidence of oesophageal, gastric or duodenal pathology at OGD. Following an initial diagnostic biopsy, follow-up biopsies were available for all cases of metaplasia (n=26) and LGD (n=13), which either remained stable, regressed or progressed to HGD, IMC or oesophageal adenocarcinoma. First-time and sequential biopsies were used to construct TMAs from theses Barrett's patients. Diagnoses of metaplasia, LGD, HGD, IMC and oesophageal adenocarcinoma were all previously made by a specialist upper gastrointestinal consultant pathologist. All selected samples were subsequently marked and reviewed on two separate occasions (pre- and post-TMA construction) by a gastrointestinal pathologist, in order to ensure the accuracy of histology samples selected.

4.3.3 Barrett's oesophagus TMAs

Haematoxylin- and eosin- stained slides from formalin-fixed, paraffin-embedded tissue blocks were used to identify specific areas of normal squamous epithelium, esophagitis, intestinal metaplasia, LGD, HGD, IMC and oesophageal adenocarcinoma. The areas of interest were marked by a pathologist and 0.6mm cores were taken from the blocks and tissue microarrays were constructed. Several representative cores (mean 2, range 1 to 6) were taken from diagnostic biopsies to construct the TMAs. 4μm sections were sectioned onto Superfrost Plus poly-L-lysine coated glass slides (Thermo Fisher Scientific, IL, USA), and baked overnight at 37°C in a tissue drying oven (Binder, Tuttlingen, Germany). Slides were then stored at 4°C until stained.

4.3.4 8-oxo-dG, 4-HNE and CD3 immunohistochemistry

A mouse anti-8-oxo-dG monoclonal antibody (Genox, Maryland, USA) was used to stain for 8-oxo-dG, a marker of oxidative stress. This antibody has a specificity for 19 analogues of 8-oxodG (guanosine, 7-methyl-G, 6-SH-G, 8-bromo-G, dA, dC, dT, dI, dU, dG, O6-methyl-dG, 8-OHdA, guanine, O6-methyl-Gua, 8-OHGua, uric acid, ur-

ea, creatine, creatinine). A mouse anti-4-HNE monoclonal antibody (Genox, Maryland, USA) was used to stain for 4-HNE, a marker of lipid peroxidation. This antibody is specific for 4-HNE-lysine, 4-HNE-histidine and 4-HNE cysteine adduct. A polyclonal rabbit anti-human antibody (Dako, Glostrup, Denmark) was used to stain for CD3, a T cell marker.

Antigen retrieval was carried out using Triology™ (Cell Marque™ Corporation, CA, USA) which combines three pre-treatment steps: deparaffinisation, rehydration and unmasking. Sections were incubated in Triology™ (1/20 dilution in distilled water) in a Princess DYB350 programmable pressure cooker on low pressure for 10mins. Vectastain Elite kits (Vector Labs, CA, USA) were used for all immunohistochemical staining. Tissue sections were intubated in 3% H_2O_2 in methanol (Sigma-Aldrich) for 30 mins to quench endogenous peroxidase activity. Sections were washed three times for 5 mins each in PBS and blocked for 30 mins with a 1:66 dilution of normal serum. Sections were incubated in primary antibody (1:40 for 8-oxo-dG, 1:40 for 4-HNE and 1:100 for CD3) for 2hrs at room temperature. A control slide (full-face section) was incubated for the same time with PBS and no antibody. Sections were washed three times for 5 mins each in PBS. Sections were incubated for 30 mins in a 1:400 dilution of biotinylated secondary antibody and washed again three times for 5 mins each in PBS. Sections were incubated for 30 mins in avidin-biotin complex reagent, followed by three washes for 5 mins each in PBS, followed be incubation for 1-5 mins (depending on the level of protein expression in the tissue) in the dark in diaminobenzidine (DAB) peroxidase (Sigma-Aldrich) solution. Sections were rinsed in tap water and counterstained in Harris's haematoxylin (Sigma-Aldrich) for 30secs. Sections were placed in a PBS bath for 5 mins and subsequently rinsed in gently running tap water for 5 mins. Sections were dipped in two separate baths of 100% methanol (Sigma-Aldrich) (up and down 10 times), then transferred into two separate baths of xylene (Sigma-Aldrich, MO, USA) for 5 mins each, before being placed in a bath of xylene overnight. Coverslips were mounted using DPX mountant (BDH Ltd., Dorset, UK) and left to dry in a fume hood. Images were then taken using Aperio Scanscope XT digital scanner (University College Dublin).

Immunohistochemistry was assessed at 40X magnification in a semi-quantitative manner for each marker by two observers who were blinded to clinical outcome during scoring. For 8-oxo-dG and 4-HNE; epithelial and stroma cells were assessed for percentage of nuclear and cytoplasmic cells with positive staining and the associated intensity of nuclear and cytoplasmic staining. Intensity was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). For CD3; the percentage of epithelial and stroma cells staining positive were recorded, CD3 stains only the nucleus within the cells. The average and maximum value of each parameter was calculated for each patient.

4.3.5 Ki67 immunohistochemistry

Ki67 (Dako, Glostrup, Denmark) immunohistochemistry staining was performed using a Bond III automated immunostainer (Leica Microsystems, Wetzlar, Germany) in collaboration with Dr Tony O'Grady at Beaumont Hospital, Dublin. 4µm sections were loaded onto the system and automated staining was carried out using Ki67 antibody. Antibody staining was detected using DAB solution and sections were counterstained lightly with haematoxylin. Coverslips were mounted onto the slides using DPX mountant and left to dry in a fume hood. Images were taken using Aperio Scanscope XT digital scanner. The percentage of epithelial and stroma cells with positive staining were then quantified. The average and maximum value of each parameter was calculated and averaged from the results of the cores and scores from two blinded observers.

4.3.6 Immunohistochemical full face staining for correlation with frequency of random mitochondrial point mutations

Patients with intestinal metaplasia and oesophageal adenocarcinoma, in whom the frequency of random mitochondrial point mutations were previously calculated in section 2.4.1.2, were used for the purpose of correlation analysis in this chapter. Corresponding paraffin-embedded diagnostic biopsies taken at the same time as Barrett's oesophagus surveillance biopsies or oesophagectomy were obtained from St. James's Hospital pathology archives. 5µm sections were cut using a Microm HM325 microtome (Thermo Fisher Scientific, IL, USA) and floated on to Superfrost Plus poly-L-lysine coated glass slides. Cut sections were baked overnight at 37°C in a tissue drying oven, stored at 4°C. These full face sections were subsequently stained with 8-

oxo-dG, 4-HNE and CD3. For each antibody, all slides were processed and stained on the same day. Staining was as per the protocol outlined in section 4.3.4. Grading was performed by two independent observers and scores were averaged.

4.3.7 Measurement of 8-oxo-dG and 4-HNE in patient serum

Serum samples from patients encompassing the Barrett's disease spectrum; intestinal metaplasia (n=24), LGD (n=15), HGD (n=16), IMC (n=9) and invasive adenocarcinoma (n=10), were used. The 8-OHdG ELISA kit (JaICA, Shizuoka, Japan) was used to determine the concentration of 8-oxo-dG in patient serum. The Hexanoyl-Lys adduct (HEL) ELISA kit (Gentaur Ltd., London, UK) was used to determine the concentration of 4-HNE in patient serum. ELISAs were performed following the same principles outlined in section 2.3.7 with modifications made as per manufacturers' specific instructions.

4.3.8 Statistical analysis

Statistical analysis was performed using SPSS® (version 18.0) software (SPSS, Chicago, IL, USA). Differences between continuous variables in the different histological groups were calculated using Mann-Whitney U and Kruskal-Wallis tests. Differences between categorical variables were analysed using Chi-squares tests. Categorical differences between Barrett's metaplasia progressors and non-progressors were calculated using Chi-square tests, while Mann-Whitney U tests were used to determine differences in percentage positivity. Correlations between variables were investigated using the Spearman rho correlation coefficient; a value >0.50 was taken to strongly correlate, <0.30 was seen as a poor correlation and values between were viewed as correlations of medium strength. Statistical consultation was provided by Dr Ricardo Segurado, from C-star (University College Dublin, http://www.cstar.ie). Statistical significance was defined by $p \leq 0.05$.

4.4 RESULTS

4.4.1 Differences in proliferation (Ki67), oxidative stress (8-oxo-dG and 4-HNE) and T cell (CD3) markers in the different Barrett's oesophagus histology groups

4.4.1.1 Aged-matched histology groups

The median age of the overall patient cohort was 62 years (range 29-84 years). To enable comparative analysis in the levels of oxidative stress between different histology groups representing the Barrett's oesophagus disease sequence, all groups were age matched (p=0.117) (Figure 4.1).

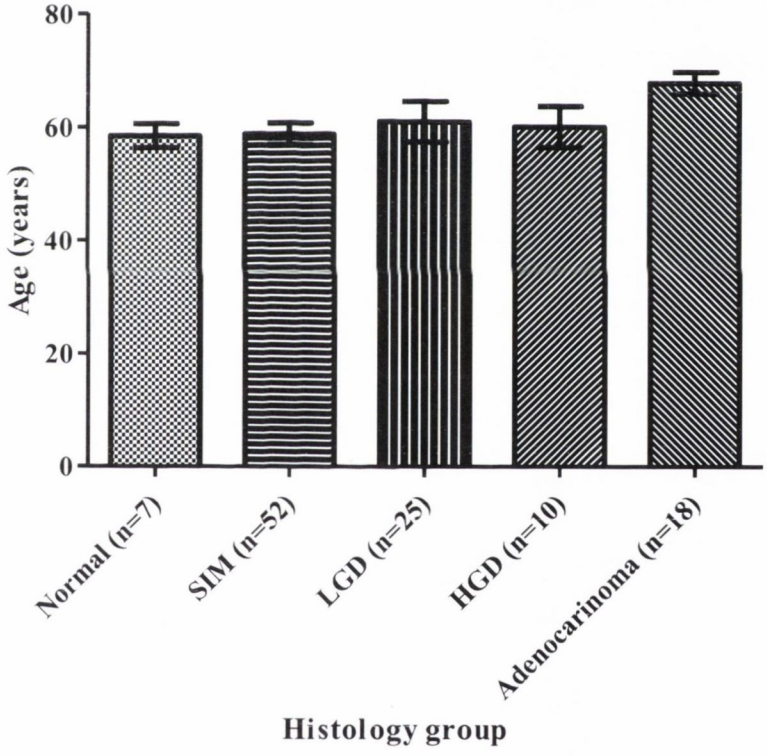


Figure 4.1: Age distribution between the different histology groups representing the Barrett's oesophagus disease sequence. Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated no significant difference in the ages of patients analysed between the different histology groups (p=0.117). Error bar represents SEM.

4.4.1.2 Ki67 expression along the Barrett's oesophagus disease sequence

In aged matched histology groups, maximum stromal Ki67 percent positivity was 3.1-fold higher in oesophageal adenocarcinoma compared with intestinal metaplasia (p=0.035). However, no differences were demonstrated in maximum Ki67 stroma percent positivity between the remaining histological groups. The maximum epithelial Ki67 was significantly 1.6-fold higher in adenocarcinoma biopsies compared with metaplasia tissue (p=0.012) (Figure 4.2). No significant difference was demonstrated between intestinal metaplasia and normal (p=1.000), LGD (p=0.260) and HGD (p=0.152) tissue. There was no difference seen between LGD and HGD (p=0.600) or oesophageal adenocarcinoma (p=0.143). No difference in Ki67 was demonstrated between HGD and adenocarcinoma (p=0.308).

The mean Ki67 expression in epithelial and stroma cells followed the same trends as seen with maximum values and are presented in appendix two.

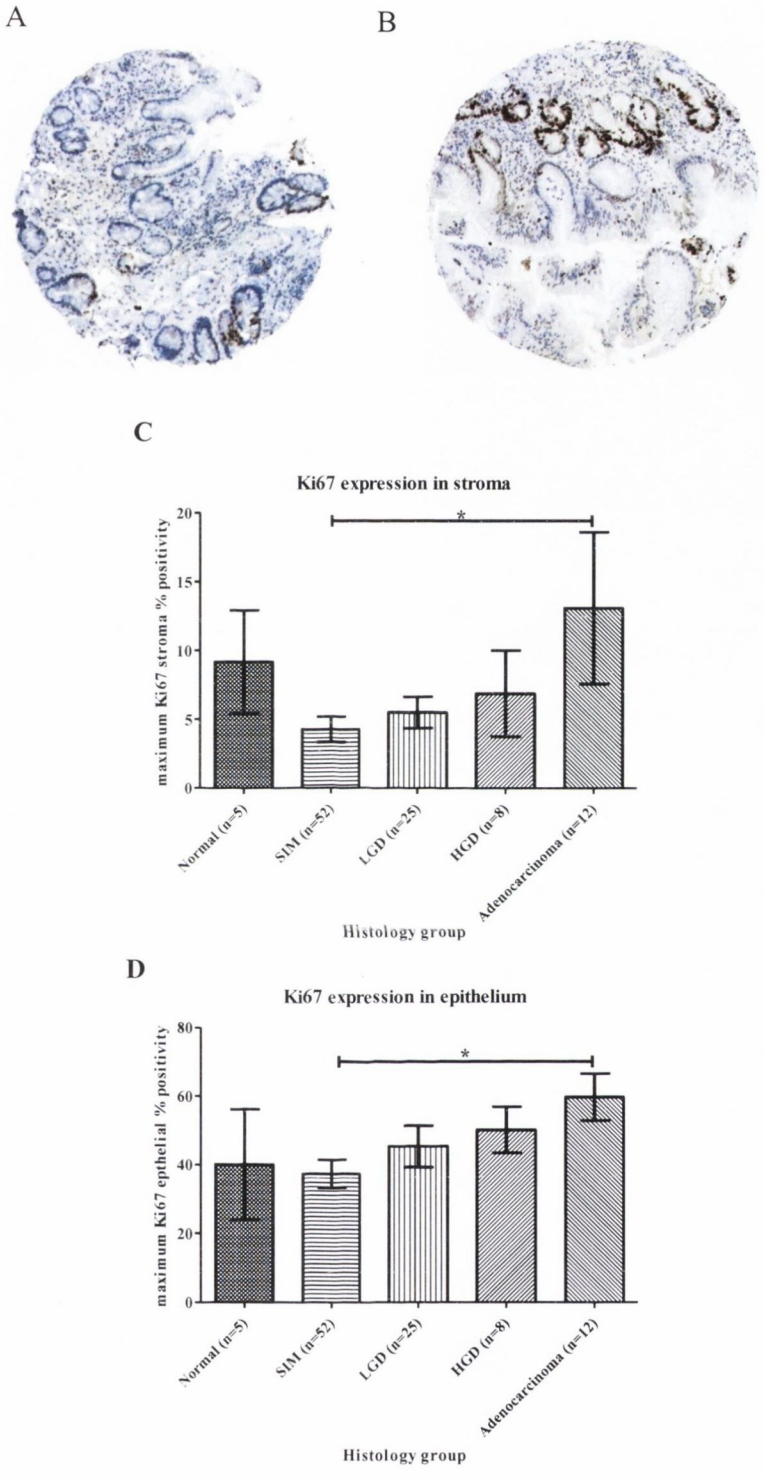


Figure 4.2: Maximum Ki67 staining in the Barrett's oesophagus disease spectrum *in-vivo*. **(A)** Image of Ki67 staining in intestinal metaplasia. (B) Image of increased Ki67 epithelial staining in oesophageal adenocarcinoma. **(C)** There was a significant increase in the percent of oesophageal adenocarcinoma stroma cells positive for Ki67 compared with intestinal metaplasia. **(D)** The percentage of positive Ki67 epithelial cells was significantly decreased in intestinal metaplasia compared with oesophageal adenocarcinoma groups. Error bars represent SEM. *$p \leq 0.05$

4.4.1.3 8-oxo-dG expression along the Barrett's oesophagus disease sequence

Representative images of 8-oxo-dG staining are shown in Figure 4.3. There was a significant difference in the maximum percent of cells positive for 8-oxo-dG in the stroma cytoplasm between normal and oesophageal adenocarcinoma ($p=0.016$) and between HGD and adenocarcinoma ($p=0.035$) histology groups (Figure 4.4). There was a trend towards reduced 8-oxo-dG levels in stroma cytoplasm between SIM and adenocarcinoma, however, this was not significant ($p=0.079$). Initial analysis demonstrated no significant difference in maximum percent of cells positive for 8-oxo-dG in the stroma nuclei ($p=0.741$). However, as there was a significant difference in maximum Ki67 between SIM and oesophageal adenocarcinoma stroma (section 4.4.1.3), 8-oxo-dG stroma percentage positivity results were normalised to Ki67 stroma values to correct for differences in proliferation. Following normalisation, a significant increased percentage of SIM stroma cytoplasm ($p=0.010$) and stroma nuclei ($p<0.0001$) were positive for 8-oxo-dG compared with oesophageal adenocarcinoma (Figure 4.5). Chi-square tests demonstrated no significant difference in the maximum intensity of 8-oxo-dG in the stroma cytoplasm ($p=0.910$) and stroma nuclei ($p=0.692$) across the Barrett's disease spectrum.

Initial Kruskal-Wallis tests demonstrated no significant differences in percentage positivity of epithelial cytoplasm ($p=0.633$) and epithelial nuclei ($p=0.573$) for 8-oxo-dG across the Barrett's disease spectrum (Figure 4.6). However, as there was a significant difference in maximum Ki67 percentage positivity between SIM and adenocarcinoma (section 4.4.1.2), maximum 8-oxo-dG epithelium percentage positivity results were normalised to Ki67 epithelial values to correct for differences in proliferation. Following normalisation, a significant increase in epithelial cytoplasm percentage positivity was demonstrated in SIM compared with adenocarcinoma ($p=0.009$) (Figure 4.7). 8-oxo-dG levels in the epithelial nuclei were not significantly different between SIM and adenocarcinoma tissue following adjustment for proliferation ($p=0.465$). Chi-square tests demonstrated no significant difference in the maximum intensity of 8-oxo-dG in the epithelial cytoplasm ($p=0.262$) and epithelial nuclei ($p=0.569$) across the different Barrett's oesophagus histology groups.

Mean results analysis are outlined in appendix two.

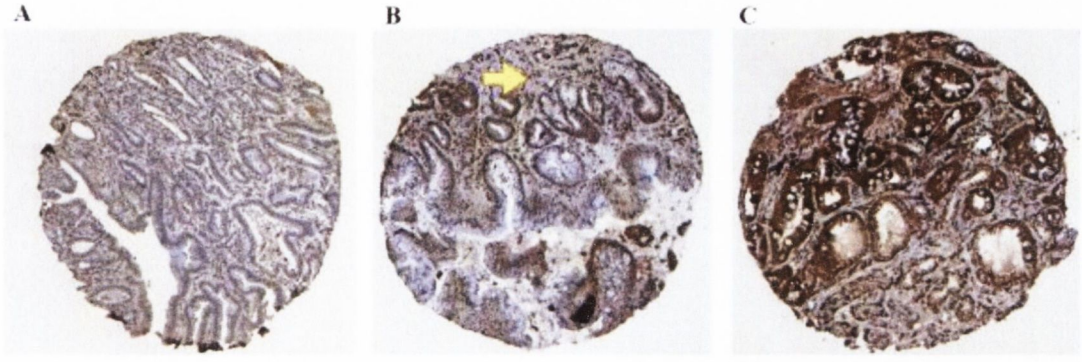


Figure 4.3: Representative images of 8-oxo-dG staining in Barrett's disease sequence samples *in-vivo*. **(A)** Image representing overall low percent positivity and weak staining for 8-oxo-dG in the epithelium. **(B)** Image representing overall moderate percent positivity and moderate 8-oxo-dG staining in the stromal tissue (yellow arrow). **(C)** Image representing overall high percent positivity and strong staining for 8-oxo-dG in the epithelium.

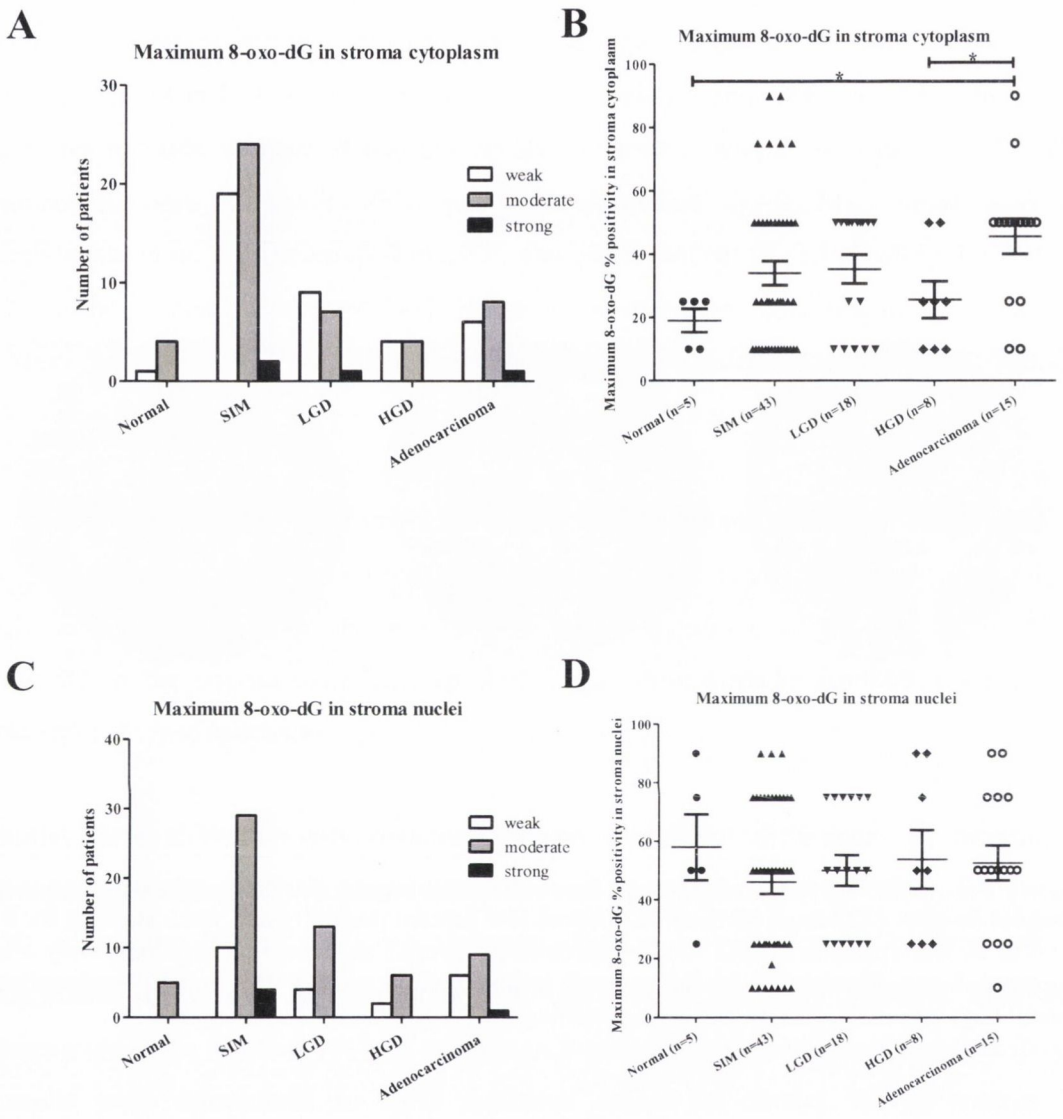


Figure 4.4: Maximum stroma 8-oxo-dG in aged-matched histology groups encompassing the Barrett's oesophagus disease spectrum. Chi square test demonstrated no significant difference in intensity of 8-oxo-dG in **(A)** stroma cytoplasm and **(C)** stroma nuclei. **(B)** Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated a significant increase in the levels of cytoplasm 8-oxo-dG in adenocarcinoma compared with normal and HGD patients. **(D)** There was no significant difference in levels of 8-oxo-dG in stroma nuclei. *$p \leq 0.05$

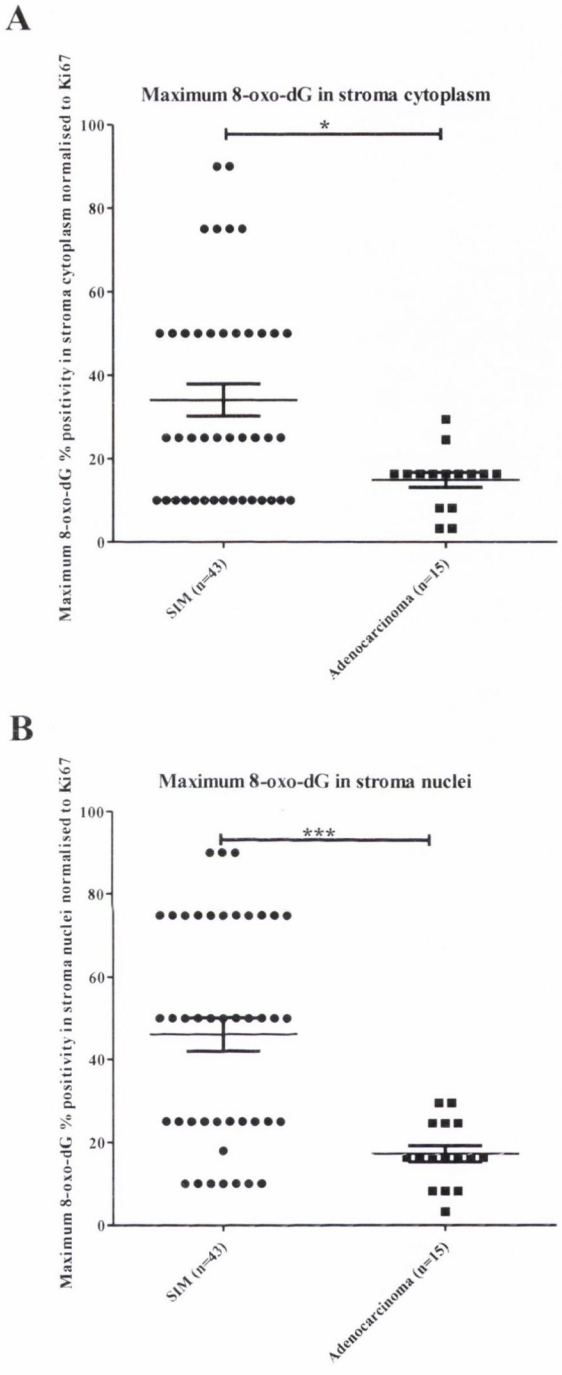


Figure 4.5: Maximum stroma cells positive for 8-oxo-dG, following normalisation with Ki67 percent positivity. There was significantly increased levels of 8-oxo-dG in **(A)** stroma cytoplasm and **(B)** stroma nuclei in SIM compared with oesophageal adenocarcinoma. Error bars represent SEM. *$p<0.05$, ***$p<0.0005$

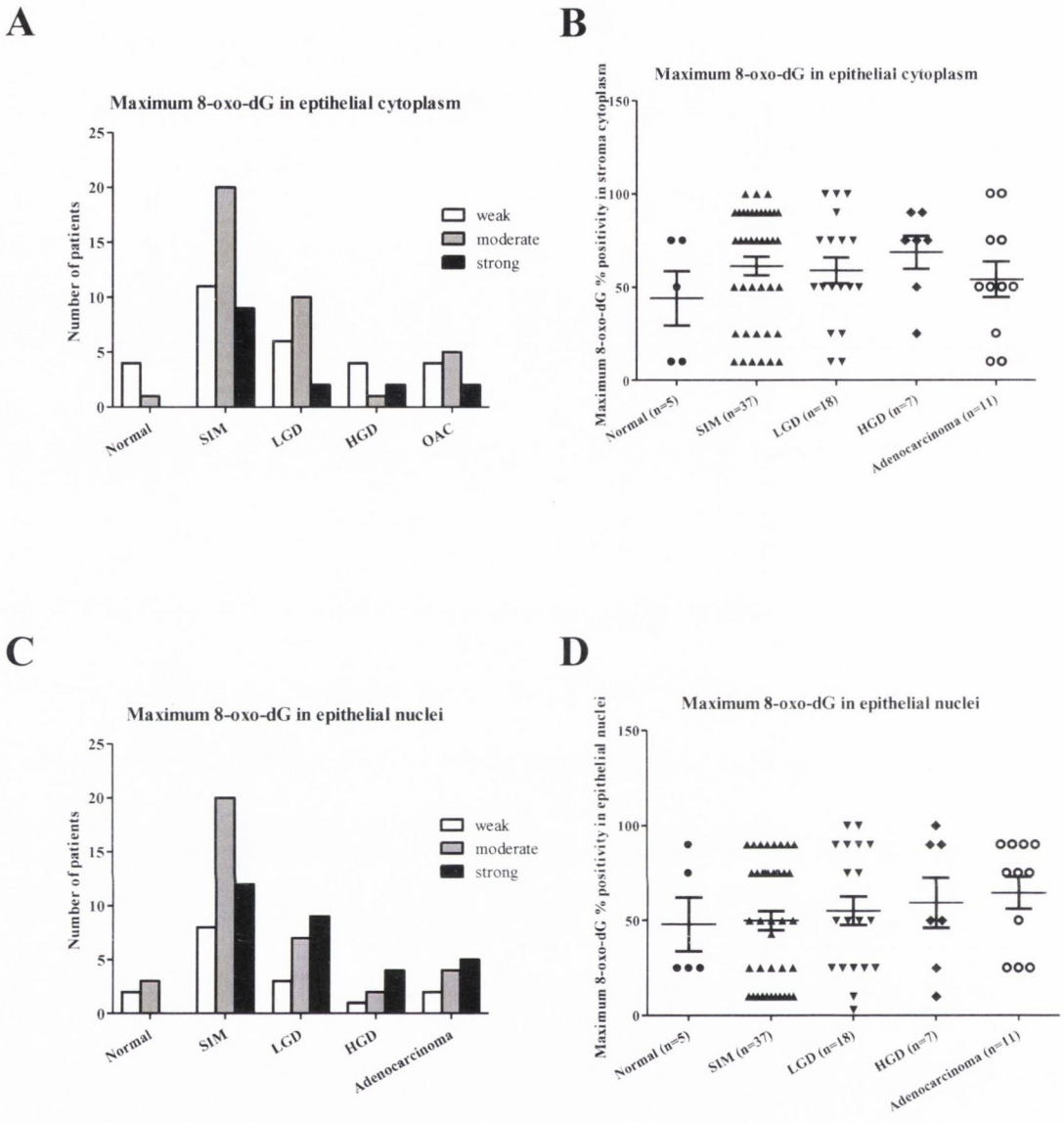
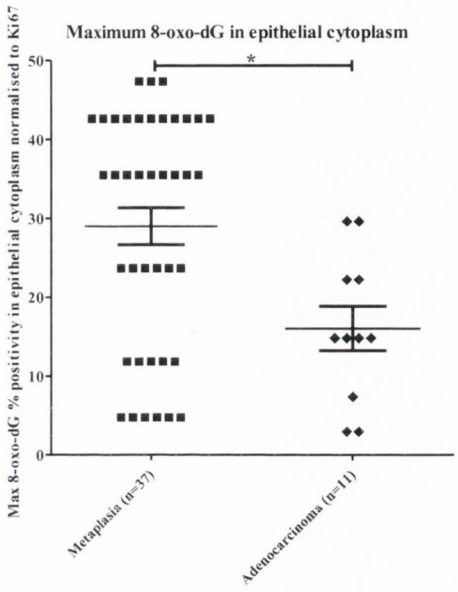


Figure 4.6: Maximum epithelial 8-oxo-dG in aged-matched histology groups encompassing the Barrett's oesophagus disease spectrum. Chi square test demonstrated no significant difference in intensity of 8-oxo-dG in **(A)** epithelial cytoplasm (p=0.262) and **(C)** epithelial nuclei (0.569). Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated no significant difference in 8-oxo-dG percentage positivity in **(B)** the cytoplasm (p=0.763) and **(D)** nuclei (p=0.697).

A



B

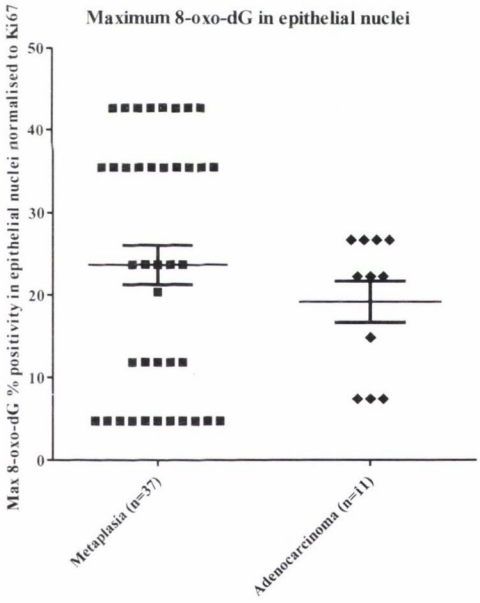


Figure 4.7: Maximum epithelial cells positive for 8-oxo-dG, following normalisation with Ki67 percent positivity. There was significantly increased levels of 8-oxo-dG in **(A)** epithelial cytoplasm in SIM tissue compared with oesophageal adenocarcinoma. **(B)** No differences were demonstrated in 8-oxo-dG levels in epithelial nuclei ($p=0.465$). *$p<0.05$,

4.4.1.4 4-HNE expression along the Barrett's oesophagus disease sequence

Representative images of 4-HNE staining are shown in figure 4.8. There was a significant difference between maximum percent of stroma nuclei positive for 4-HNE between LGD and oesophageal adenocarcinoma ($p=0.035$) (Figure 4.9). No difference was demonstrated between oesophageal adenocarcinoma and normal tissue ($p=0.582$), SIM ($p=0.096$) and HGD ($p=0.099$). Kruskal-Wallis tests demonstrated no significant difference maximum 4-HNE percentage positivity in stroma cytoplasm ($p=0.309$), although there was a trend towards an increased level in oesophageal adenocarcinoma compared with HGD ($p=0.056$). As there was a significant difference in maximum Ki67 between SIM and oesophageal adenocarcinoma stroma (section 4.4.1.2), 4-HNE stroma percentage positivity results were normalised to Ki67 stroma values to correct for differences in proliferation. Following normalisation, a significant increased percentage of metaplastic stroma cytoplasm ($p<0.0001$) and stroma nuclei ($p=0.007$) were positive for 4-HNE compared with oesophageal adenocarcinoma (Figure 4.10). Chi-square tests showed no significant difference in the maximum intensity of stroma cytoplasm ($p=0.646$) and stroma nuclei ($p=0.924$).

Chi-square tests demonstrated no significant difference in 4-HNE intensity in epithelial cytoplasm ($p=0.790$) and epithelial nuclei ($p=0.653$) (Figure 4.11). A significantly increased percentage of epithelial cytoplasm was positive for 4-HNE in oesophageal adenocarcinoma compared with SIM ($p=0.004$), LGD ($p=0.003$) and HGD ($p=0.003$). A significant increase in percentage of epithelial nuclei positive for 4-HNE were seen in oesophageal adenocarcinoma compared with LGD ($p=0.041$) and HGD ($p=0.022$), with a trend towards reduced levels in SIM compared with invasive cancer ($p=0.066$). As there was a significant difference in maximum epithelial Ki67 percentage positivity between SIM and oesophageal adenocarcinoma (section 4.4.1.2), maximum 4-HNE epithelium percentage positivity results were normalised to Ki67 epithelial values to correct for differences in proliferation (Figure 4.12). Following normalisation, no significant difference was seen in 4-HNE levels between SIM and adenocarcinoma in the epithelial cytoplasm or nuclei ($p=0.200$ and $p=0.974$, respectively).

Mean results analysis are outlined in appendix two.

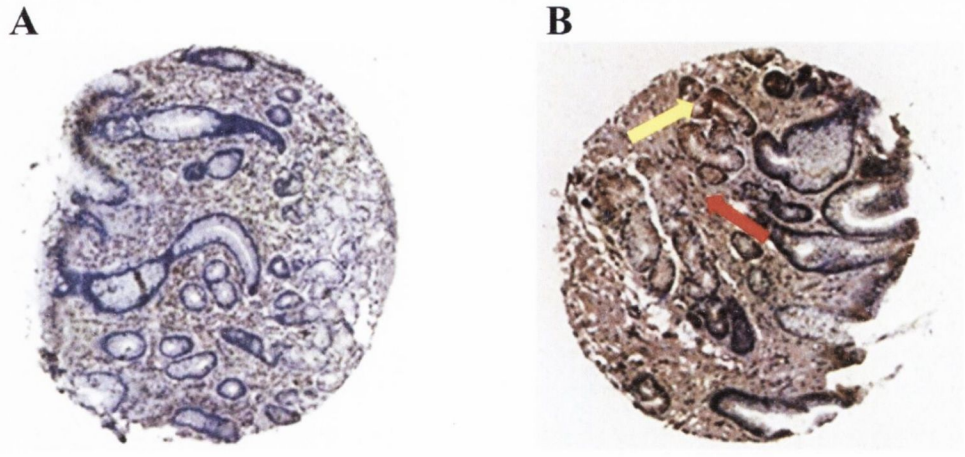


Figure 4.8: Representative images of 4-HNE staining in Barrett's disease sequence samples *in-vivo*. **(A)** Image representing a low percentage of 4-HNE positive epithelial cells, but moderate stroma percent positivity and weak staining intensity. **(B)** Image representing a moderate percentage of 4-HNE positive stroma cells of moderate intensity (red arrow), and a strong epithelial staining intensity (yellow arrow).

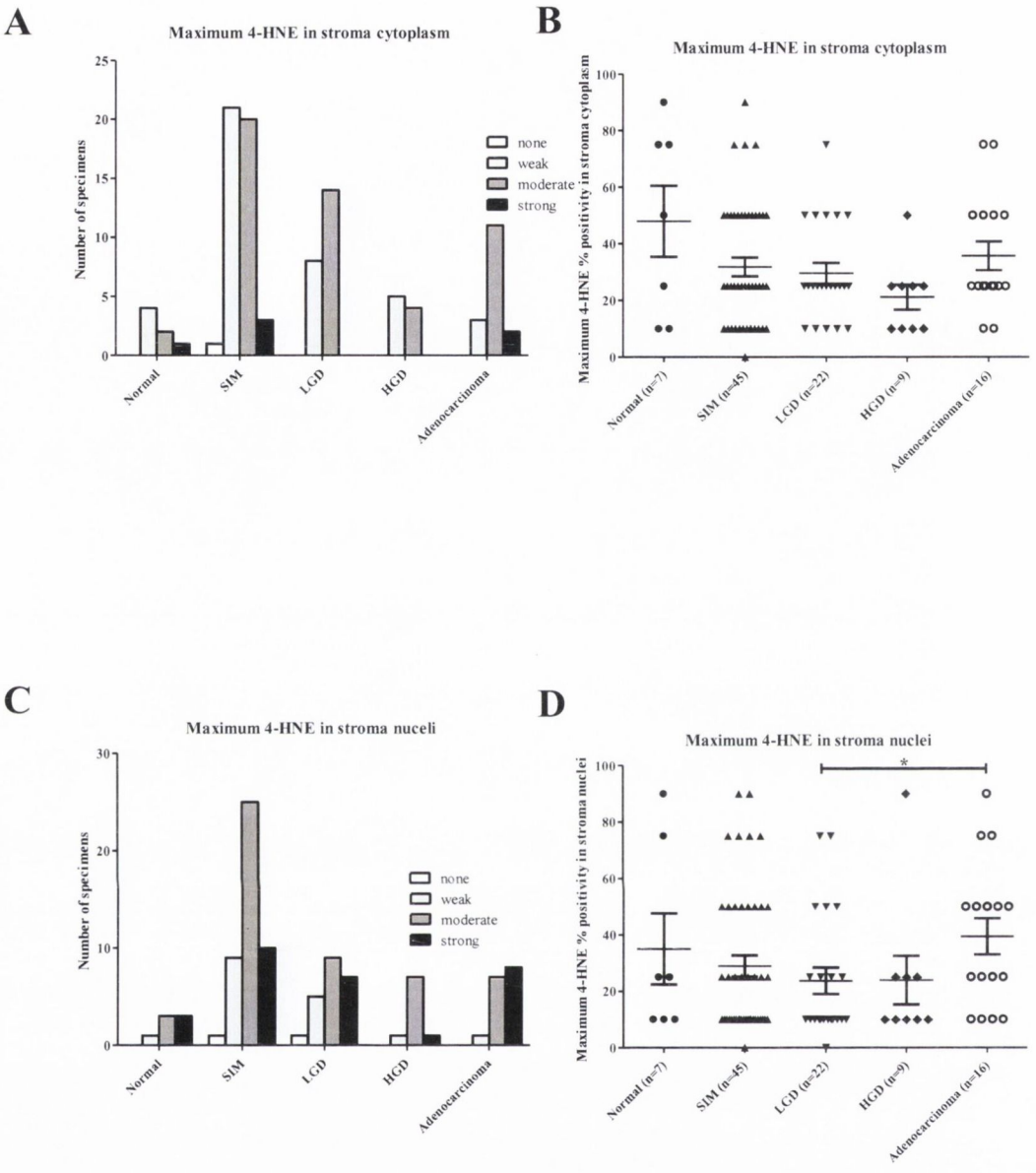


Figure 4.9: Maximum stroma 4-HNE in aged-matched histology groups encompassing the Barrett's oesophagus disease spectrum. Chi square test demonstrated no significant difference in intensity of 4-HNE in **(A)** stroma cytoplasm and **(C)** stroma nuclei. **(B)** Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated no significant difference in the levels of 4-HNE in the stroma cytoplasm. **(D)** There was a significant difference in levels of 4-HNE in stroma nuclei between LGD and oesophageal adenocarcinoma. *$p \leq 0.05$

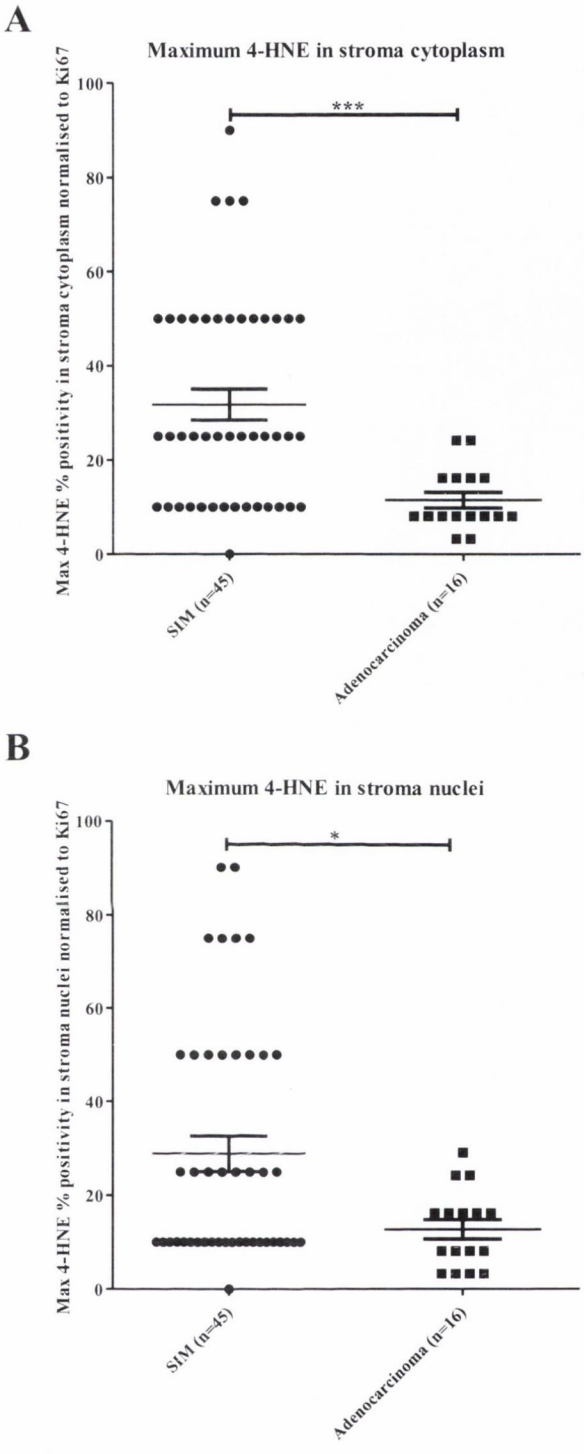


Figure 4.10: Maximum stroma cells positive for 4-HNE, following normalisation with Ki67 percent positivity. There was significantly increased levels of 4-HNE in **(A)** stroma cytoplasm and **(B)** stroma nuclei in SIM compared with oesophageal adenocarcinoma. *$p \leq 0.05$, ***$p \leq 0.0005$

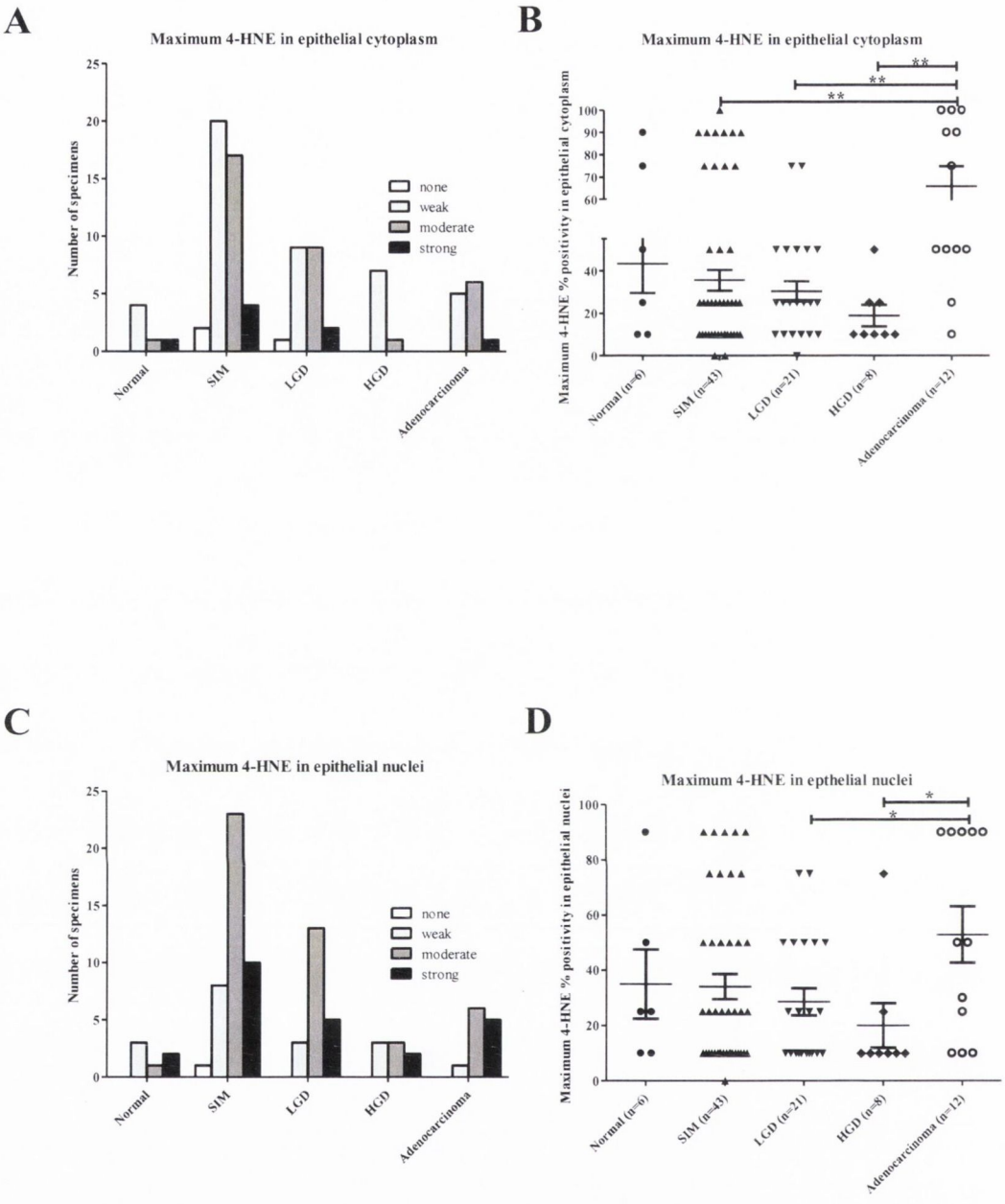


Figure 4.11: Maximum epithelial 4-HNE in aged-matched histology groups encompassing the Barrett's oesophagus disease spectrum. Chi square test demonstrated no significant difference in intensity of 4-HNE in **(A)** epithelial cytoplasm (p=0.790) and **(C)** epithelial nuclei (0.653). Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated significant differences in 4-HNE levels in **(B)** the cytoplasm and **(D)** nuclei between oesophageal adenocarcinoma and earlier stages of the Barrett's disease spectrum. *p≤0.05, **p≤0.005

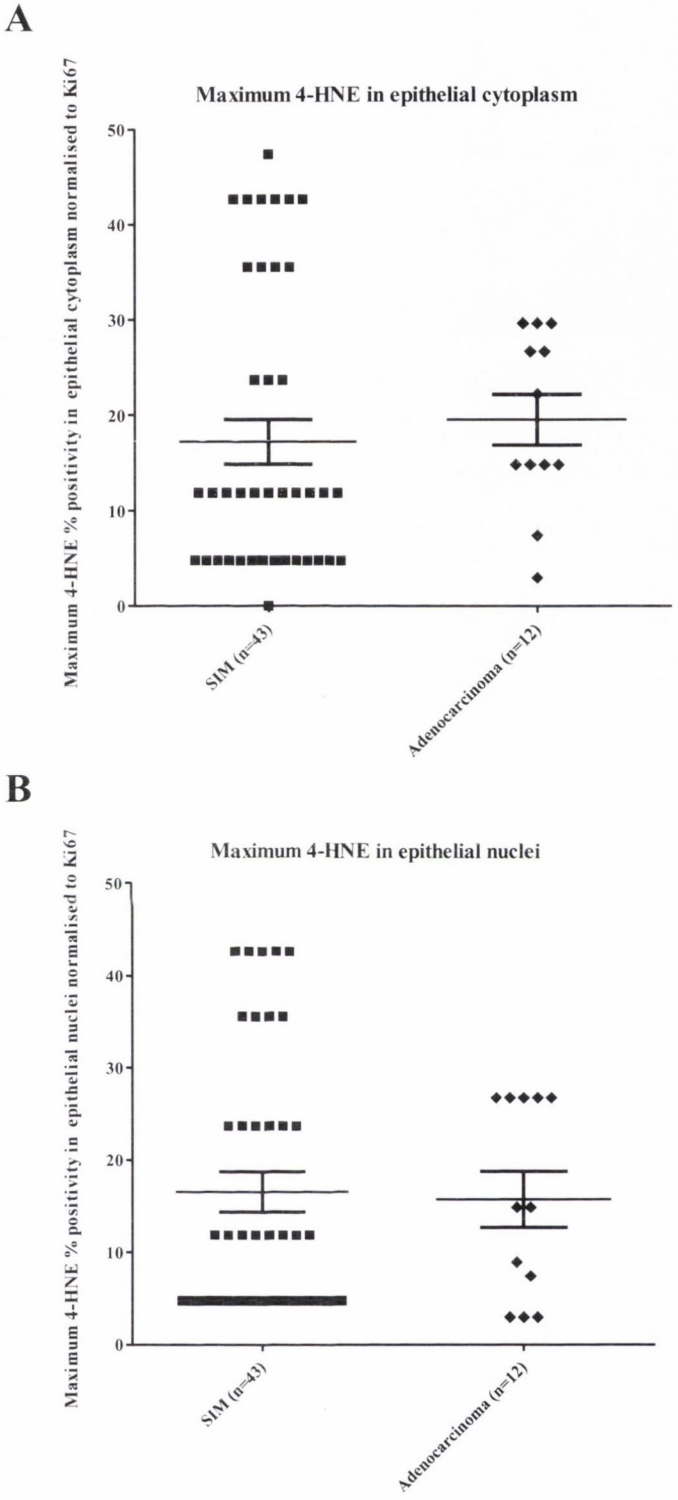


Figure 4.12: Maximum epithelial cells positive for 4-HNE, following normalisation with Ki67 percent positivity. There was no significant difference in 4-HNE in **(A)** epithelial cytoplasm (p=0.200) and **(B)** epithelial nuclei (p=0.974).

4.4.1.5 CD3 expression along the Barrett's oesophagus disease sequence

Kruskal-Wallis analysis with Dunn's multiple comparison test demonstrated no significant differences in maximum CD3 levels in the stroma ($p=0.163$) and epithelia ($p=0.832$) (Figure 4.13). As there was a significant difference in maximum Ki67 between SIM and oesophageal adenocarcinoma in the stroma and epithelium (section 4.4.1.2), CD3 results were normalised to Ki67 values to correct for differences in proliferation. Following normalisation, there was a significantly increased level of CD3 in stroma cells ($p<0.0001$) and in epithelial cells ($p=0.007$) in SIM compared with oesophageal adenocarcinoma (Figure 4.14).

Mean results analysis are outlined in appendix two.

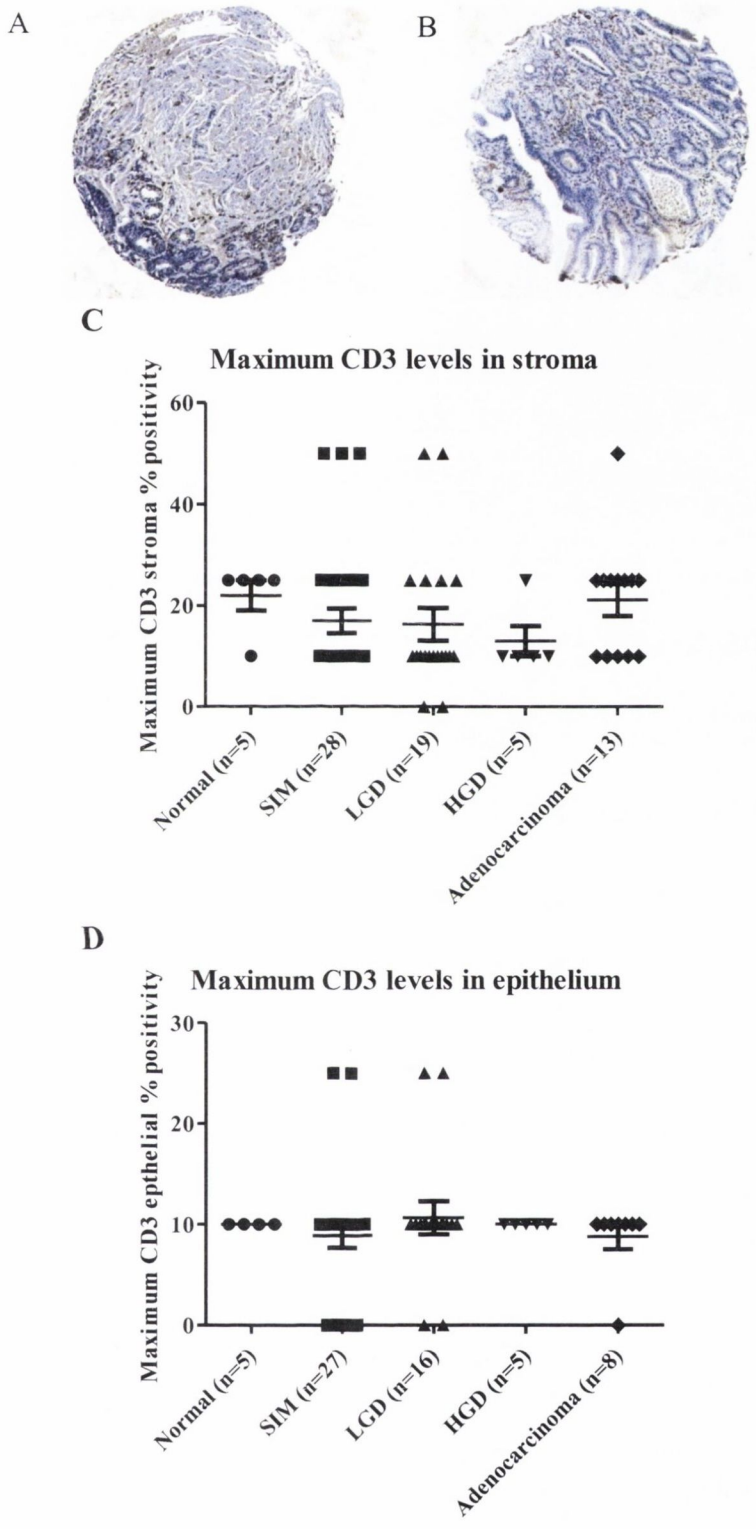


Figure 4.13: Maximum CD3 levels in stroma and epithelia across the Barrett's disease spectrum. (A) A representative image of 10% CD3 stroma staining in SIM. **(B)** A representative image of 25% CD3 stroma staining in oesophageal adenocarcinoma. There was no significant difference in levels of CD3 in **(C)** stroma (p=0.163) and **(D)** epithelia (p=0.832).

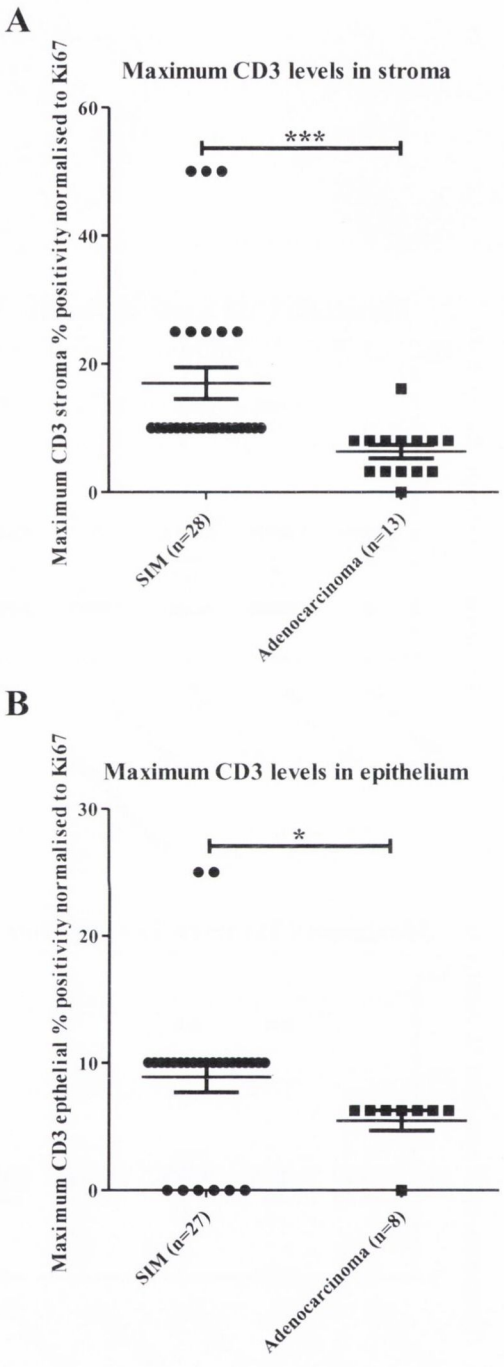


Figure 4.14: Maximum CD3 levels, following normalisation with Ki67 percent positivity. There was a significantly increased level of CD3 in SIM **(A)** stroma ($p<0.0001$) and **(B)** epithelia ($p=0.007$). *$p\leq0.05$, ***$p\leq0.0005$

4.4.2 Differences in proliferation (Ki67), oxidative stress (8-oxo-dG), lipoperoxidation (4-HNE) and inflammatory (CD3) markers between Barrett's oesophagus progressors and non-progressors

4.4.2.1 Demographics of Barrett's oesophagus progressors and non-progressors

Focussing on patients with a primary diagnosis of SIM on first surveillance endoscopy; progressors (n=13) and non-progressors (n=10) were separated, with the primary end-point being progression to HGD and/or oesophageal adenocarcinoma. The median age of patients with SIM was 59 years (26-84 years), and there was a 2.4-fold male predominance. There was no significant difference in age (p=0.8504) and gender (p=0.873) between progressors and non-progressors. Median time of progression to cancer was 2 years (Range 1-16 years). The non-progressor group was followed for a median of 7.5 years (Range 2-17 years) and had no evidence of conversion to HGD and/or oesophageal adenocarcinoma.

4.4.2.2 Ki67 expression in Barrett's oesophagus patients

No difference was seen in the maximum percent of stroma cells positive for Ki67 between progressive (mean 3.3%, SEM 1.67%) and non-progressive (mean 5.2%, SEM 1.63%) (p=0.278) patients (Figure 4.15). There was no significant difference in the maximum percent of epithelial cells positive for Ki67 between progressive (mean 24.4%, standard error of mean [SEM] 7.04%) and non-progressive (mean 40.5%, SEM 8.83%) patients (p=0.270). Mean data outlined in appendix two.

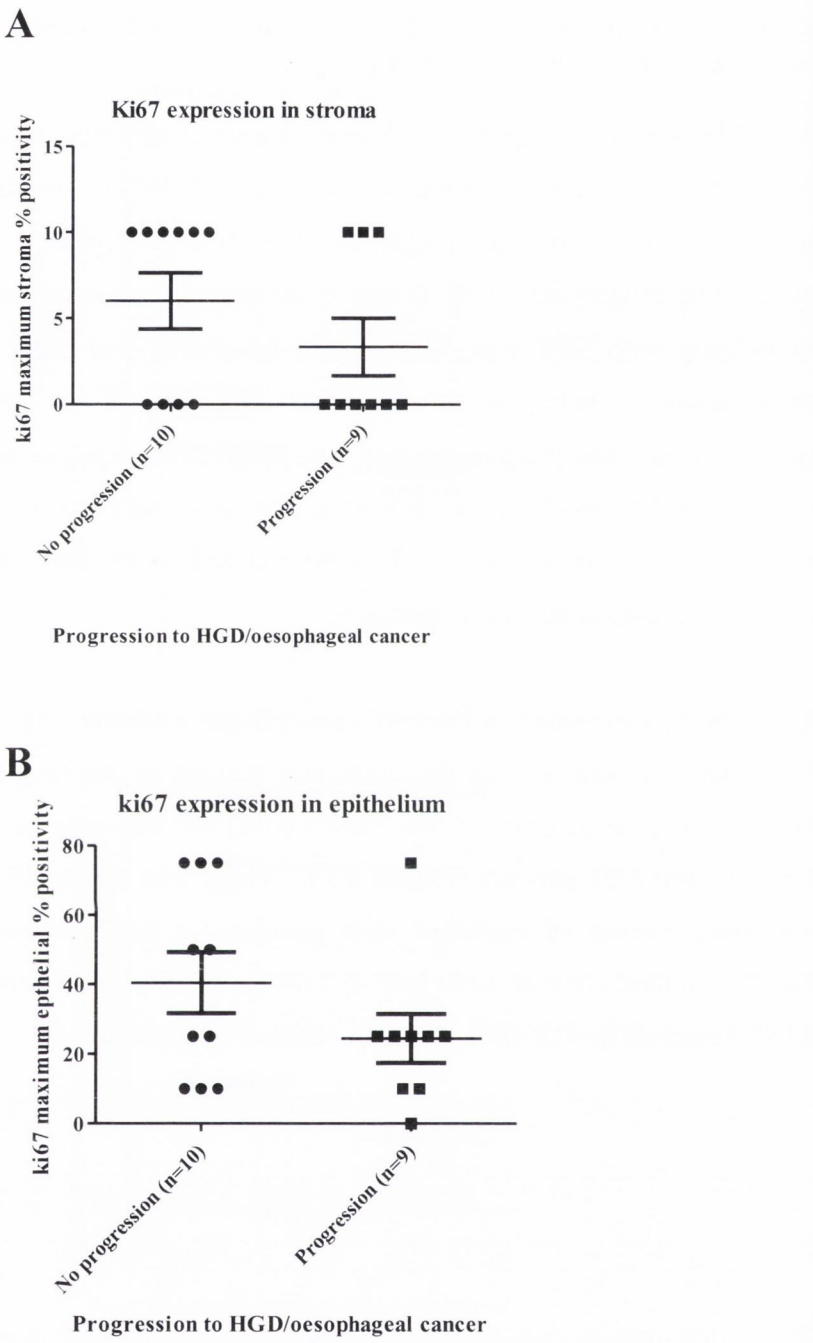


Figure 4.15: Maximum expression of Ki67 between Barrett's oesophagus progressors and non-progressors. Mann-Whitney U test demonstrated no significant difference in **(A)** stroma (p=0.278) and **(B)** epithelia (p=0.270).

4.4.2.3 8-oxo-dG expression in Barrett's oesophagus patients

The maximum intensity of 8-oxo-dG in the stroma cytoplasm and stroma nuclei were significantly reduced in patients progressing to HGD and/or oesophageal adenocarcinoma (p=0.0075 and p=0.0209, respectively) (Figure 4.16 A and C). The maximum percentage positivity of 8-oxo-dG in the stroma nuclei was significantly decreased in patients with progressive disease (mean 29.5%, standard error of mean [SEM] 7.3) compared with non-progressors (mean 55.0%, SEM 8.6) (p=0.039) (Figure 4.19 B and D). There was a significantly lower percent of epithelial cytoplasm staining positive for 8-oxo-dG in progressive Barrett's patients (mean 35.6%, SEM 8.8) compared with non-progressors (mean 68%, SEM 9.1) (p=0.0295) (Figure 4.17). The mean differences between progressors and non-progressors are outlined in appendix two.

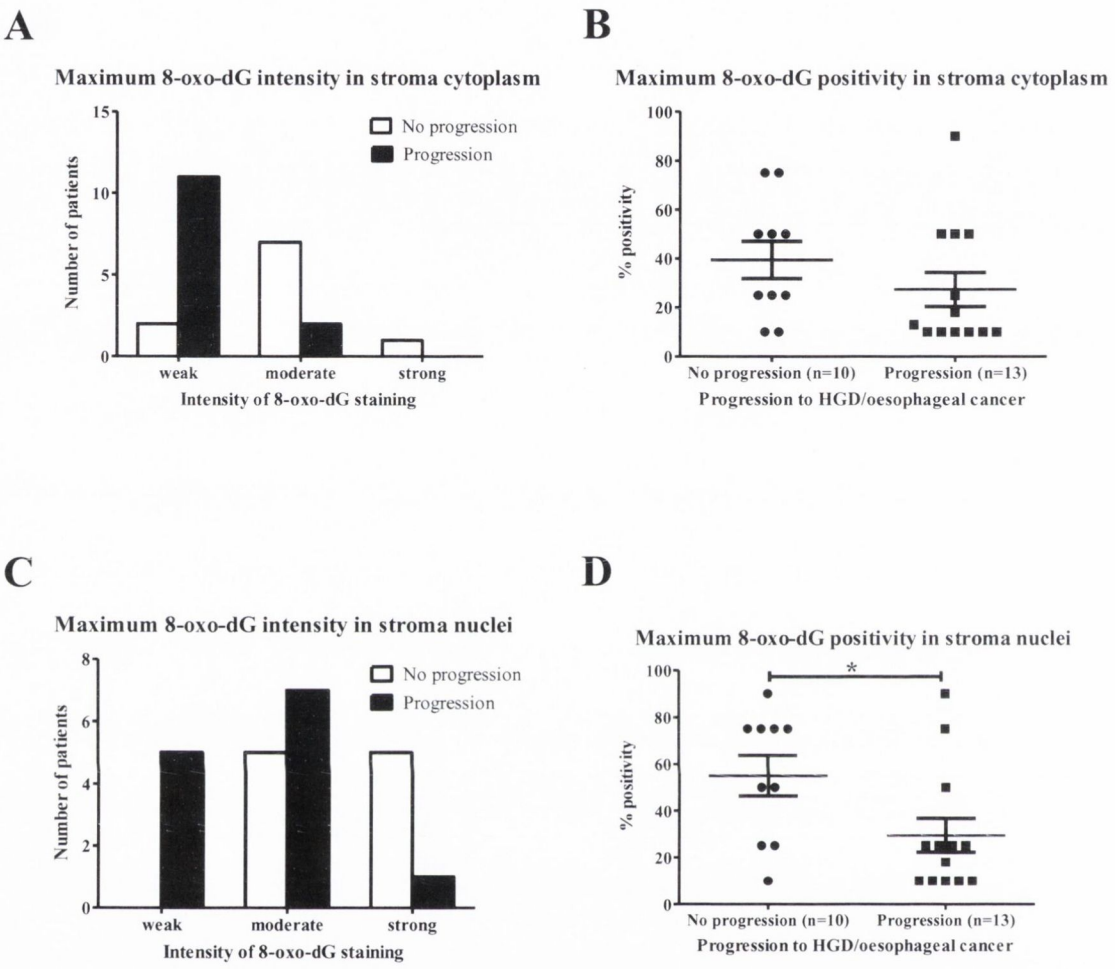


Figure 4.16: 8-oxo-dG staining in stroma cytoplasm and nuclei in SIM progressors and non-progressors. (A) Chi-square test demonstrated a significantly higher rate of weaker staining in patients with progressive disease compared to patients with non-progressive disease ($p=0.008$). **(B)** A Mann-Whitney U test showed no difference in the percentage of stroma cytoplasm positive for 8-oxo-dG ($p=0.168$). **(C)** Chi-square test showed an increased incidence of weaker 8-oxo-dG staining in the progressors ($p=0.0201$). **(D)** Non-progressive Barrett's oesophagus was associated with an increased percentage of positive stroma nuclei ($p=0.039$). *$p \leq 0.05$

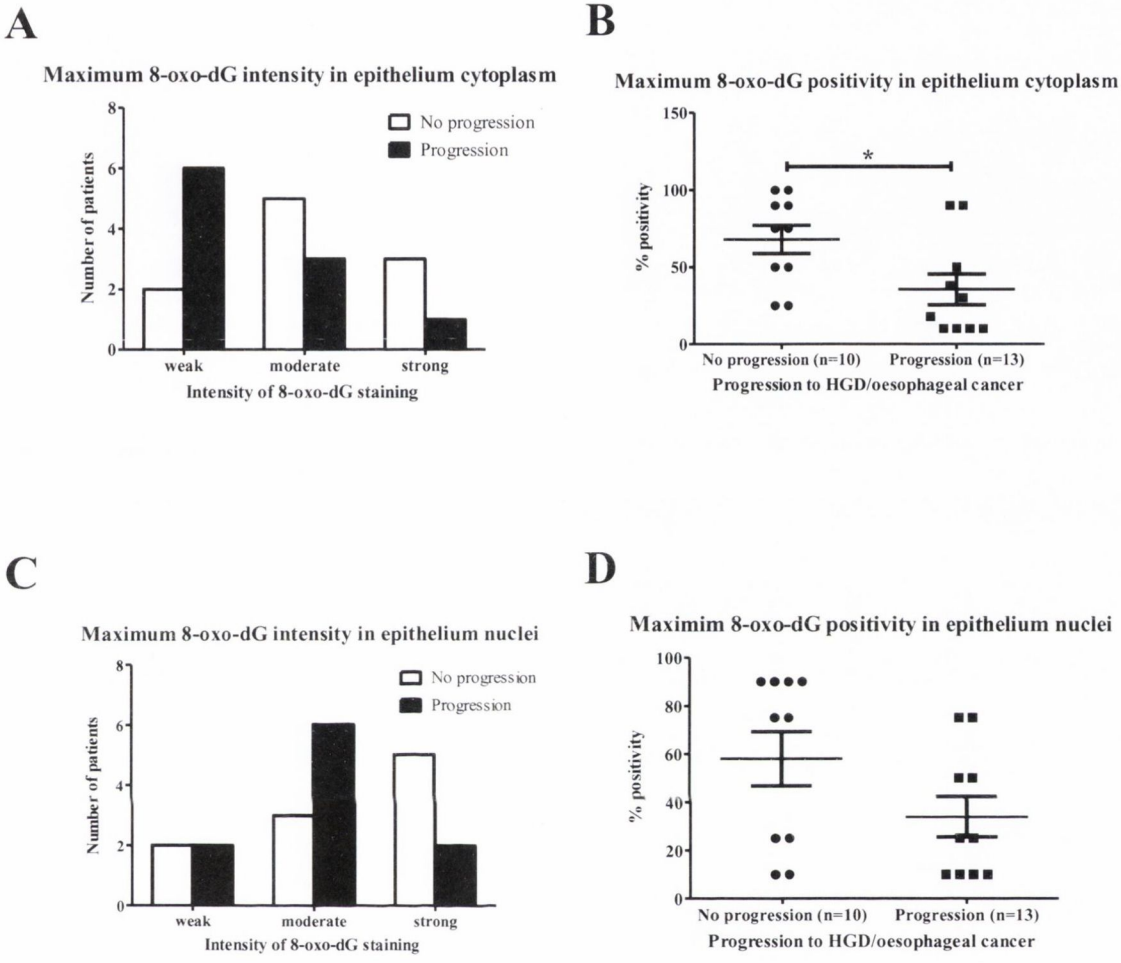


Figure 4.17: 8-oxo-dG staining in epithelial cytoplasm and nuclei in SIM progressors and non-progressors. (A) Chi-square test showed no difference in the intensity of staining in the epithelial cytoplasm ($p=0.174$). **(B)** The maximum percentage of positive epithelial cytoplasm was significantly higher in the non-progressing group ($p=0.030$). **(C)** Chi-square test demonstrated no significant difference in the intensity of 8-oxo-dG staining in the epithelium nuclei between both groups ($p=0.320$). **(D)** Mann Whitney U test showed no difference in the overall percentage of 8-oxo-dG epithelial nuclei staining positive ($p=0.100$). *$p<0.05$

4.4.2.4 4-HNE expression in Barrett's oesophagus patients

There was no difference in the maximum expression of 4-HNE between patients progressing to HGD and/or oesophageal adenocarcinoma and patients with non-progressive Barrett's oesophagus (Figures 4.18 and 4.19). Mean 4-HNE results are presented in appendix two.

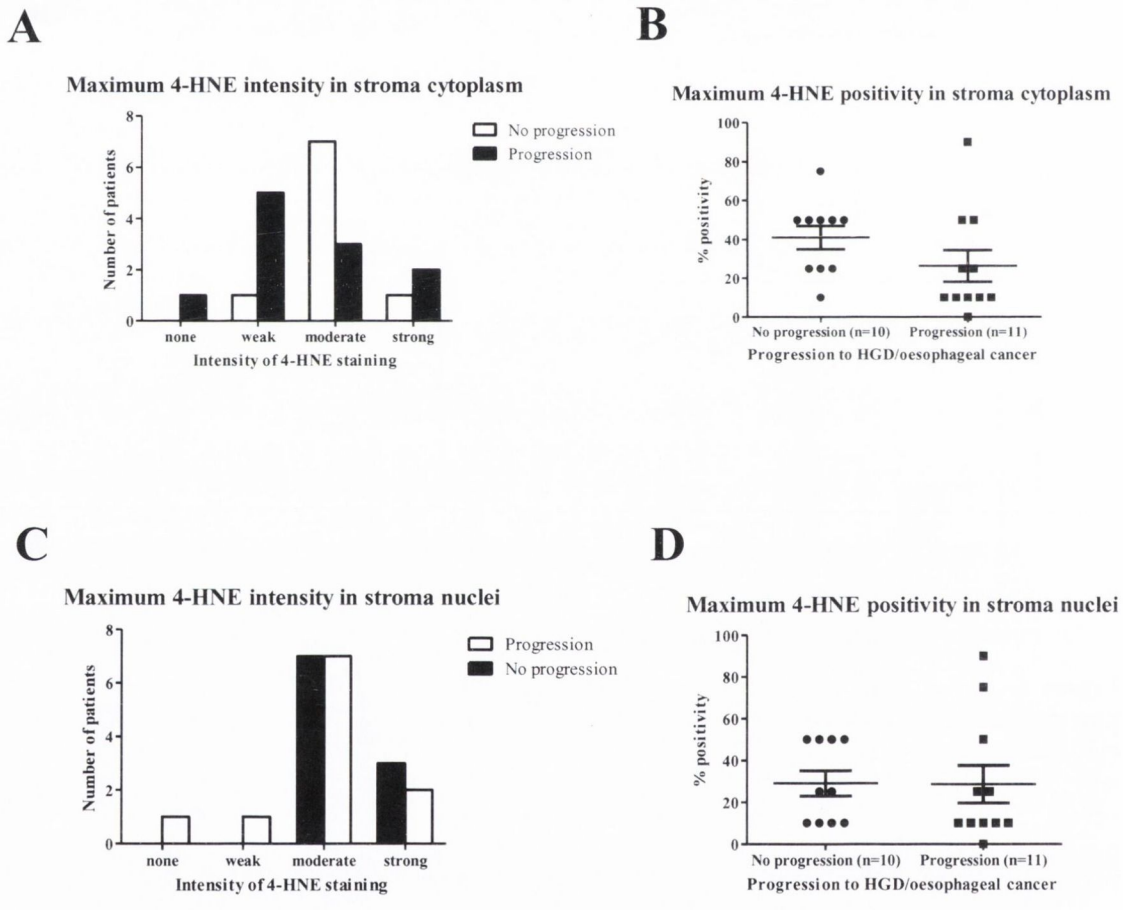


Figure 4.18: 4-HNE staining in stroma cytoplasm and nuclei in SIM progressors and non-progressors. Chi-square test demonstrated no significant difference in **(A)** 4-HNE intensity in stroma cytoplasm (p=0.141) and **(C)** 4-HNE intensity in stroma nuclei (p=0.540). Mann-Whitney U tests showed no difference in the percentage of stroma **(B)** cytoplasm and **(D)** nuclei positive for 4-HNE (p=0.079 and p=0.630, respectively).

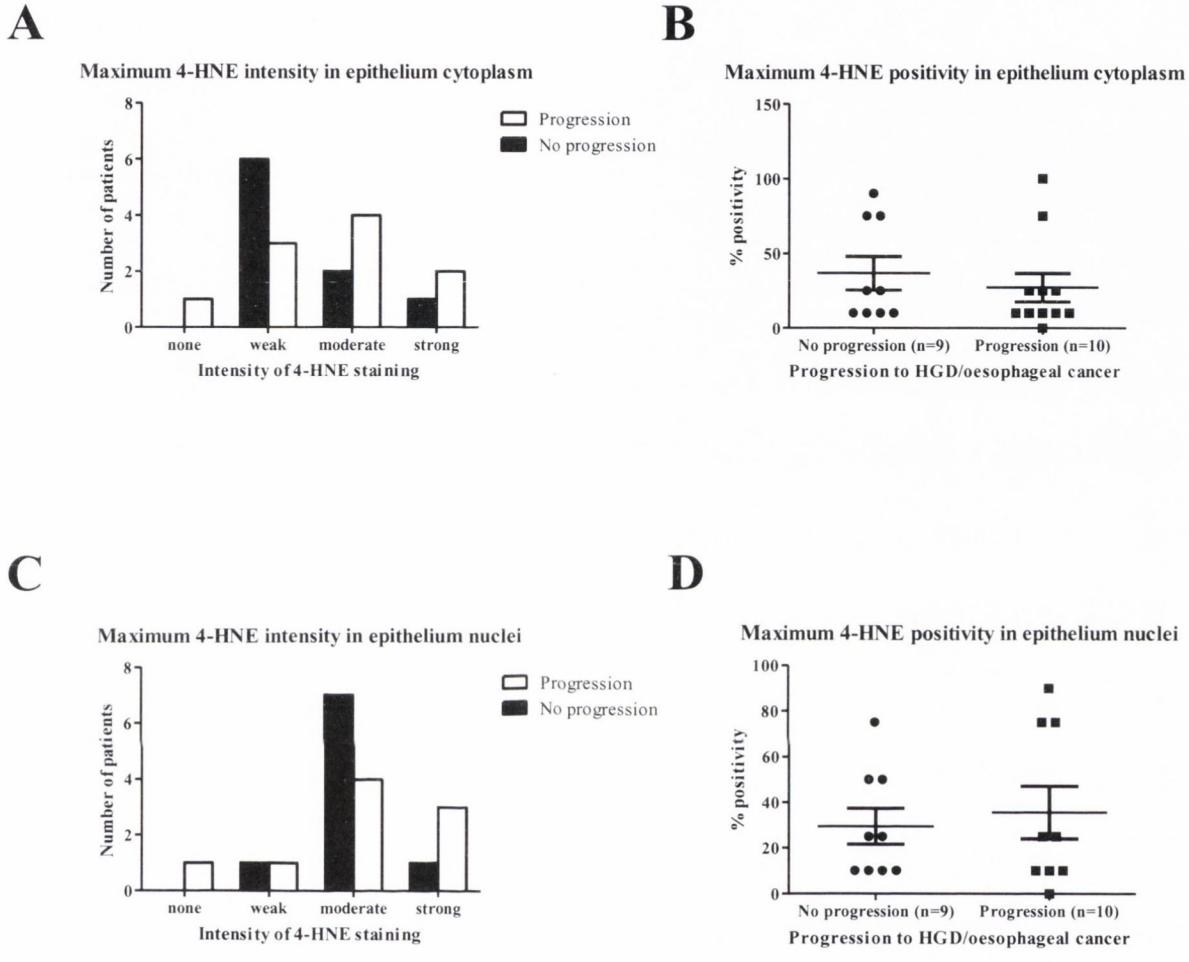


Figure 4.19: 4-HNE staining in epithelium cytoplasm and nuclei in SIM progressors and non-progressors. Chi-square test demonstrated no significant difference in **(A)** 4-HNE intensity in epithelial cytoplasm (p=0.399) and **(C)** 4-HNE intensity in epithelial nuclei (p=0.421). Mann-Whitney U tests showed no difference in the percentage of **(B)** epithelial cytoplasm and **(D)** epithelial nuclei positive for 4-HNE (p=0.520 and p=0.923, respectively).

4.4.3.5 CD3 expression in Barrett's oesophagus patients

Mann Whitney U tests demonstrated no significant difference in maximum CD3 levels in the stroma (p=0.382) and epithelium (p=0.535) between Barrett's patients with progressive and non-progressive disease (Figure 4.20). Mean CD3 results are reported in appendix two.

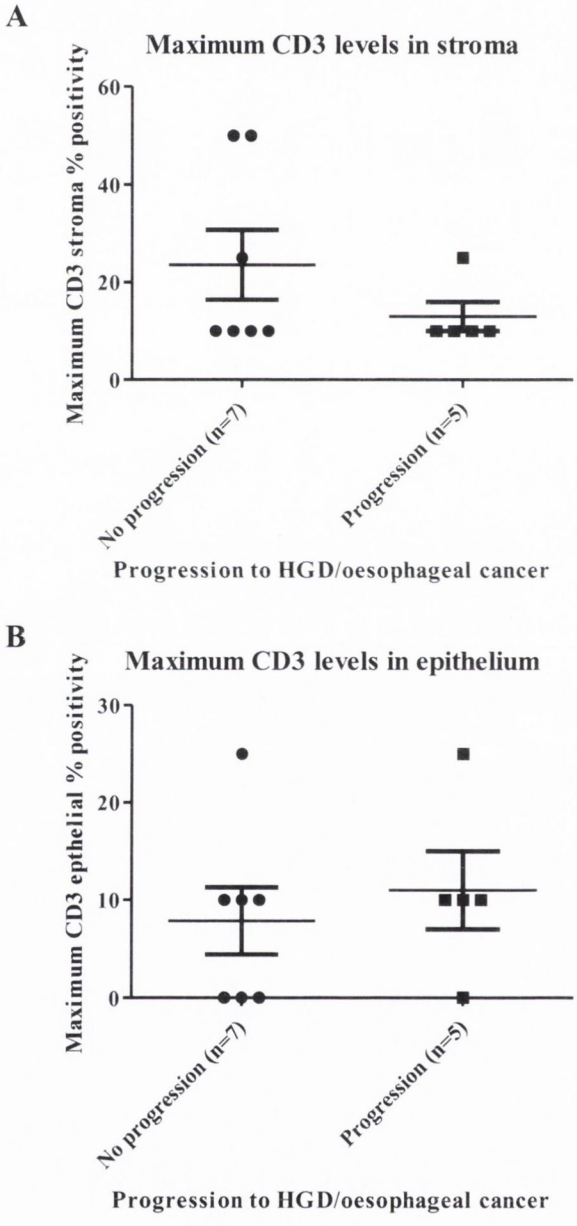


Figure 4.20: Maximum expression of CD3 between Barrett's oesophagus progressors and non-progressors. Mann-Whitney U test demonstrated no significant difference in **(A)** stroma (p=0.382) and **(B)** epithelium (p=0.535).

4.4.3 Analysis of the relationship between oxidative stress (8-oxo-dG), lipoperoxidation (4-HNE) and inflammatory (CD3) markers and the frequency of random mitochondrial point mutations

4.4.3.1 Correlation between 8-oxo-dG and random mitochondrial point mutations

We previously investigated the levels of random mitochondrial point mutations in SIM and oesophageal adenocarcinoma patients in chapter two. To determine if levels of the oxidative stress marker, 8-oxo-dG, correlated with random mitochondrial point mutations, the matched paraffin embedded specimens from patients studied in chapter two were stained with 8-ox-dG and the results were correlated.

There was a significant negative correlation between the frequency of random mutations and maximum percentage of positive stroma cytoplasm for 8-oxo-dG in SIM biopsies (Spearman Rho= -0.730, p=0.031) (Figure 4.21). There was no determined relationship between the other parameters analysed (Table 4.1).

There was no significant relationship seen between 8-oxo-dG and mutagenesis in the oesophageal cancer cohort (Table 4.2). Mean 8-oxo-dG results are outlined in appendix two.

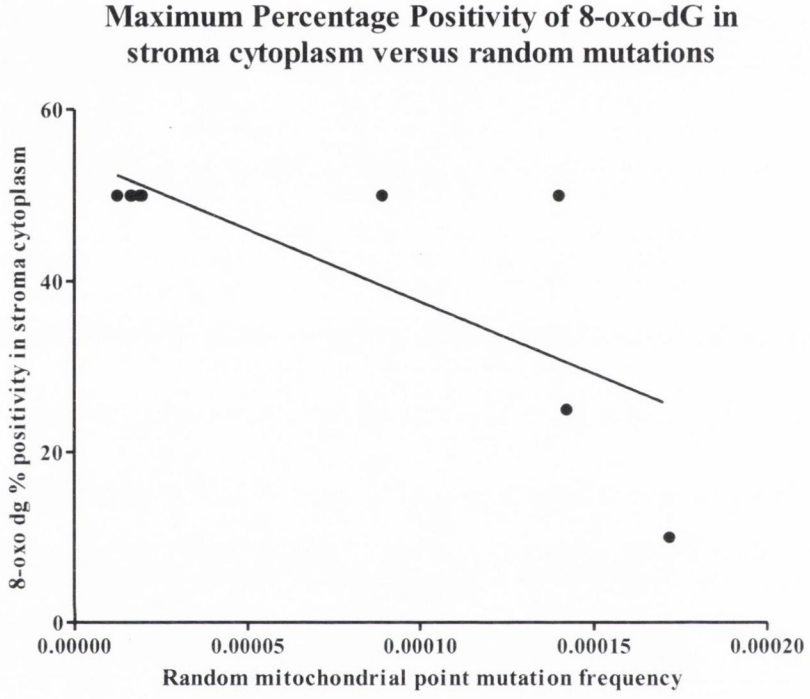


Figure 4.21: Correlation between 8-oxo-dG and frequency of random mitochondrial point mutations in patients with SIM. A strong negative correlation between random mitochondrial mutations and the percent of stroma cytoplasm positive for 8-oxo-dG was detected (Spearman rho= -0.730, p=0.031).

Table 4.1: Correlations between maximum 8-oxo-dG expression and frequency of random mitochondrial point mutations in intestinal SIM patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.433	0.250
Nuclear intensity	0.104	0.810
Cytoplasm % positivity	**-0.730**	**0.031**
Cytoplasm intensity	0.087	0.843
Epithelium		
Nuclear % positivity	-0.065	0.880
Nuclear intensity	-0.176	0.514
Cytoplasm % positivity	0.129	0.744
Cytoplasm intensity	0.473	0.198

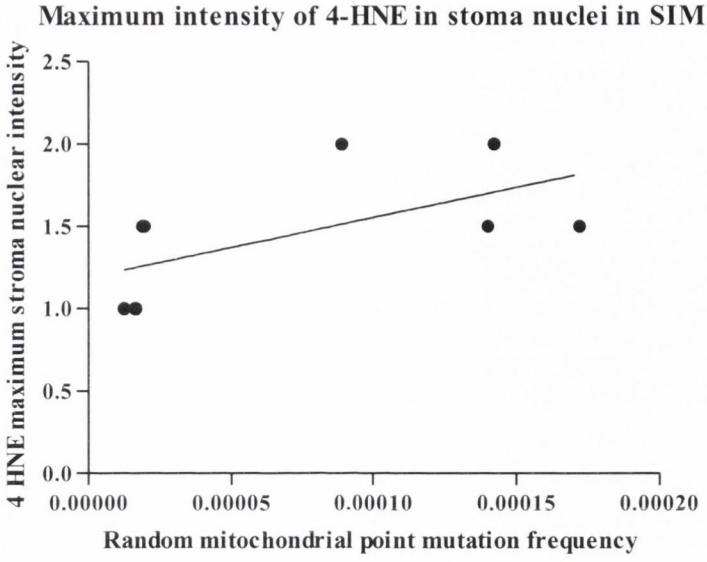
Table 4.2: Correlations between maximum 8-oxo-dG expression and frequency of random mitochondrial point mutations in oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.151	0.783
Nuclear intensity	0.503	0.267
Cytoplasm % positivity	-0.123	0.783
Cytoplasm intensity	0.389	0.396
Epithelium		
Nuclear % positivity	0.248	0.595
Nuclear intensity	0.334	0.444
Cytoplasm % positivity	0.537	0.236
Cytoplasm intensity	0.510	0.236

4.4.3.2 Correlation between 4-HNE and random mitochondrial point mutations

Although no significant difference was demonstrated in 4-HNE levels between Barrett's patients with or without progression to HGD and/or oesophageal adenocarcinoma, the relationship between random mutations and 4-HNE expression was analysed in SIM and oesophageal cancer tissue. In SIM, there was a strong positive correlation detected between the frequency of random mitochondrial mutations and maximum intensity of 4-HNE staining in stroma nuclei (Spearman rho=0.775, p=0.017). A similar relationship was demonstrated in the oesophageal adenocarcinoma tumour tissue (Spearman rho=0.973, p=0.017) (Figure 4.22). No other significant relationships were demonstrated between the remaining staining parameters analysed (Table 4.3 and 4.4). Mean 4-HNE results are reported in appendix two.

A



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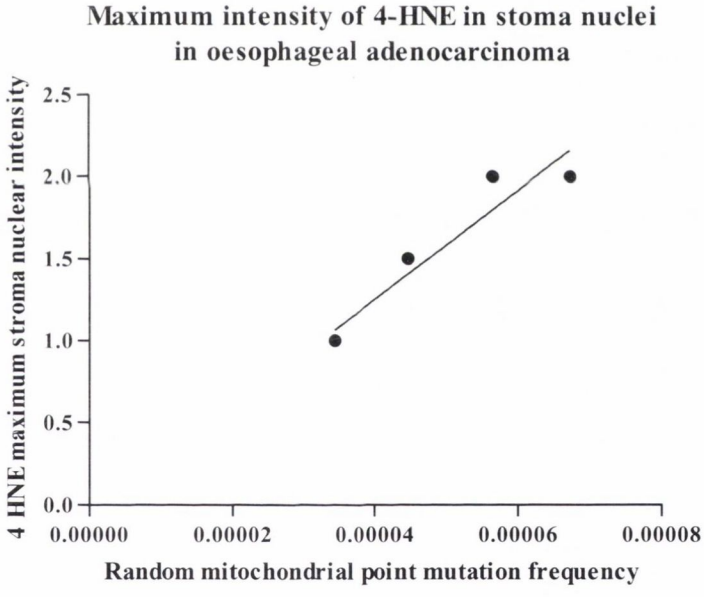


Figure 4.22: Correlation between 4-HNE and frequency of random mitochondrial point mutations in patients with SIM and oesophageal cancer. A strong positive correlation was seen between random mitochondrial mutations and the intensity of stroma nuclei staining for 4-HNE in **(A)** SIM (Spearman rho=0.7751, p=0.0172) and **(B)** oesophageal adenocarcinoma (Spearman rho=0.9733, p=0.0167) tissue.

Table 4.3: Correlations between maximum 4-HNE expression and frequency of random mitochondrial point mutations in SIM patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.249	0.521
Nuclear intensity	**0.775**	**0.017**
Cytoplasm % positivity	0.485	0.194
Cytoplasm intensity	0.488	0.178
Epithelium		
Nuclear % positivity	0.018	0.982
Nuclear intensity	-0.211	0.581
Cytoplasm % positivity	0.069	0.880
Cytoplasm intensity	0.146	0.708

Table 4.4: Correlations between maximum 4-HNE expression and frequency of random mitochondrial point mutations in oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	-0.444	0.450
Nuclear intensity	**0.973**	**0.017**
Cytoplasm % positivity	-0.132	0.783
Cytoplasm intensity	-0.688	0.233
Epithelium		
Nuclear % positivity	-0.344	0.517
Nuclear intensity	-0.444	0.450
Cytoplasm % positivity	0.054	0.950
Cytoplasm intensity	-0.352	0.517

4.4.3.3 Correlation between CD3 and random mitochondrial point mutations

There was no significant relationship identified between the mutation frequencies and maximum CD3 expression in either patients with SIM or oesophageal adenocarcinoma (Table 4.5). Mean CD3 results are outlined in appendix two.

Table 4.5: Correlation between maximum CD3 expression and frequency of random mitochondrial point mutations in intestinal metaplasia and oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Intestinal Metaplasia		
Stroma % positivity	0.0598	0.9063
Epithelium % positivity	-0.1443	0.7825
Oesophageal adenocarcinoma		
Stroma % positivity	-0.2236	0.7833
Epithelium % positivity	0.0000	1.0000

4.4.4 Analysis of circulating oxidative stress (8-oxo-dG) and lipoperoxidation (4-HNE) markers across the Barrett's disease sequence

The levels of circulating 8-oxo-dG did not change across the Barrett's disease spectrum ($p=0.965$). No significant difference was detected in the circulating levels of 4-HNE along the metaplasia-dysplasia-adenocarcinoma disease sequence ($p=0.838$) (Figure 4.23).

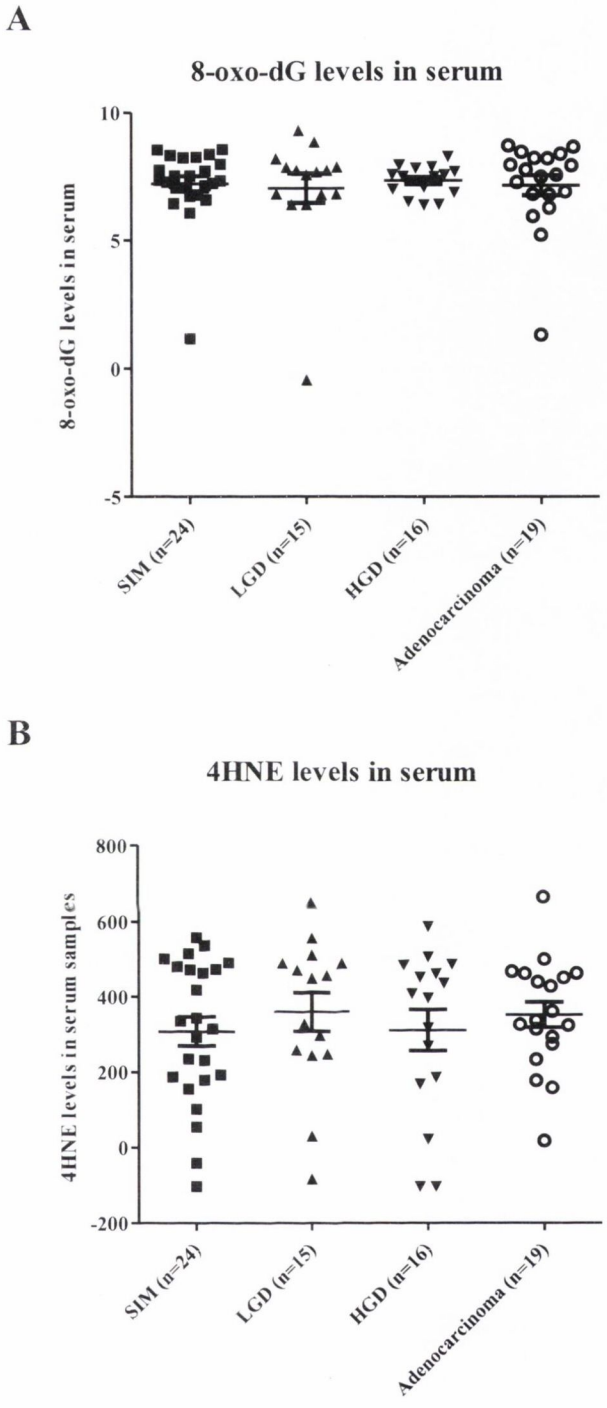


Figure 4.23: Circulating levels of 8-oxo-dG and 4-HNE in serum samples from patients representing the Barrett's oesophagus disease sequence. Kruskal Wallis tests demonstrated no significant difference in circulating levels of **(A)** 8-oxo-dG ($p=0.965$) and **(B)** 4-HNE ($p=0.838$) along the Barrett's metaplasia-dysplasia-adenocarcinoma disease sequence.

4.5 DISCUSSION

The role of mitochondrial instability as a predictor for Barrett's oesophagus progression is unknown. Our group has previously demonstrated a strong relationship between oxidative stress and inflammation in rheumatoid arthritis (Biniecka, Kennedy et al. 2010, Biniecka, Fox et al. 2011). Thus far, in this Ph.D., we has shown that SIM is an environment of increased inflammation and oxidative stress, however, the changes across the Barrett's metaplasia-dysplasia-oesophageal adenocarcinoma sequence remained to be further elucidated *in-vivo*. The potential role of mitochondrial instability as an instigator for disease progression was therefore investigated in this chapter.

We chose to examine 8-oxo-dG in Barrett's oesophagus, because 8-oxo-dG is a marker of oxidative stress formed in the presence of excessive ROS production and associated with increased levels of random mitochondrial point mutations and inflammation (Moriya 1993, Biniecka, Kennedy et al. 2010). This form of oxidative stress has been shown to occur in oesophageal tissue following treatment with bile acid and low pH, both composites of reflux and recognised precursors of Barrett's oesophagus (Dvorak, Payne et al. 2007). 8-oxo-dG is a pro-mutagenic lesion, formed by the reaction of hydroxyl radicals with DNA guanine, resulting in GC→TA transversions (Moriya 1993). Our initial analysis showed no significant difference in 8-oxo-dG levels between SIM and the other stages of the Barrett's disease sequence. Since increased cellular proliferation may increase 8-oxo-dG levels, results were normalised to Ki67 values to eliminate proliferation differences as a confounding variable. Subsequently, results demonstrated increased levels of 8-oxo-dG in SIM compared with oesophageal adenocarcinoma.

Similar results were also found with 4-HNE levels in the stroma cells. We selected to measure 4-HNE as another recognised marker of oxidative stress (Poli, Biasi et al. 2008). It has been shown to induce intercellular production of ROS (Nakamura, Miura et al. 2009), and it too has correlated with increased inflammation and mitochondrial mutations (Biniecka, Kennedy et al. 2010, Ng, Biniecka et al. 2010). We also detected a significantly increased level of CD3, a marker for inflammation, in SIM compared with adenocarcinoma. These results were consistent with findings from chapter two, which

showed SIM was an environment of increased oxidative stress, mitochondrial instability and inflammation.

In chapter two, we observed a bimodal distribution in the frequency of random mitochondrial point mutations in Barrett's oesophagus patients, enabling us to separate SIM patients into low and high mutation groups. While these samples would need to be followed prospectively in order to confirm the theory that patients with increased random mitochondrial mutations have a cancer predisposition (Loeb 2010), our archived biopsies enabled a longitudinal element be applied to this study. While no difference was seen in the levels of Ki67, 4-HNE and CD3 in SIM patients with HGD and/or oesophageal adenocarcinoma progression, 8-oxo-dG percentage positivity and intensity in the stroma and epithelium were significantly reduced in patients with disease progression. We aimed to identify differences between progressive and non-progressive disease at the earliest stages of the Barrett's disease sequence, and therefore biopsies taken at the first surveillance endoscopy were analysed. As stated, 8-oxo-dG, is a by-product of oxidative damage to DNA (Krahn, Beard et al. 2003). The mitochondria are exposed to significantly increased levels of ROS and therefore the mitochondrial DNA is more vulnerable to this damage (Wallace 2002). This oxidative damage can result in disruption to mitochondrial DNA and the genes which encode for respiratory chain complexes (Taylor and Turnbull 2005).

As such, 8-oxo-dG represents a marker of oxidative stress and mitochondrial instability that may result in respiratory chain disruption and dysfunction (Doudican, Song et al. 2005, Ott, Gogvadze et al. 2007). ROS are a recognised precursor for cancer development (Marnett 2000, Raha and Robinson 2000), with loss of balance in the redox system implicated in oesophageal cancer (Kelsell, Risk et al. 1996, Risk, Evans et al. 2002, Langan, Cole et al. 2004, McRonald, Liloglou et al. 2006). Trifunovic *et al.* demonstrated increased ROS were associated with mitochondrial mutagenesis in the control setting, however, in an environment of enhanced mutagenesis the knock-on effect was disturbed respiratory chain function, defective oxidative phosphorylation and no subsequent increase in the production of ROS (Trifunovic 2006). Thus, it may be explained that progressive disease is associated with significantly increased frequencies of random mitochondrial point mutations, but at this point defunct oxidative

phosphorylation occurs and therefore this may explain why significantly reduced levels of 8-oxo-dG may be a predictor of disease progression. As with the Warburg theory, cancer cells reprogram their energy metabolism, reducing oxidative phosphorylation and ROS production, potentially decreasing injury to mitochondrial DNA (Warburg 1956, Vander Heiden, Cantley et al. 2009), and perhaps this explains the significant reductions in 8-oxo-dG in the progressing Barrett's oesophagus patients.

A strong negative correlation was detected between 8-oxo-dG and the frequency of random mitochondrial point mutations, with low levels of 8-oxo-dG percentage positivity in stroma cytoplasm associated with significantly increased mutations in the SIM group (Spearman rho= -0.730, p=0.031). This relationship did not persist in the oesophageal cancer tissue, indicating that the metaplastic and adenocarcinoma tissue may behave differently. As Barrett's patients with cancer progression displayed significantly reduced 8-oxo-dG expression, perhaps, by association high levels of random mutations may predict cancer conversion.

Correlation analysis also showed a strong significant positive correlation between 4-HNE in the stroma nuclei and the frequency of random mitochondrial point mutations. This positive correlation is consistent with previous work performed by our group (Biniecka, Fox et al. 2011). 4-HNE and 8-oxo-dG represent different markers of mitochondrial disturbance. While it was originally suspected that both would behave in a similar fashion, theoretically a positive correlation between 4-HNE and random mutations and a negative correlation between 8-oxo-dG and random mutations may reflect the different mechanisms by which these markers are generated. However, 8-oxo-dG was the marker which emerged as significantly different between the SIM patients with progressive and non-progressive disease. 4-HNE did not between these two groups; perhaps a larger sample size may be needed to prove any consistent and strong relationship between random mutations and these markers of oxidative damage and lipoperoxidation. Consistent with immunohistochemistry results, circulating levels of 4-HNE and 8-oxo-dG were not significantly different across the Barrett's oesophagus disease spectrum, indicating that differences in 8-oxo-dG and 4-HNE levels are unique to the oesophageal tissue and their differences are too subtle to be detected systemically.

We acknowledge certain limitations of this work. Firstly, our sample size is small. However, our findings of increased oxidative stress and inflammation in the SIM cohort were consistent with results from previous chapters. First time surveillance biopsies performed at St. James's Hospital were used for all SIM samples in order to provide a consistent baseline and overcome the different times to progression seen between patients. The patients in the non-progressive group did not progress to HGD or oesophageal cancer after a median follow-up of 7.5 years, thereby satisfying the criteria for non-progressive disease, given recent findings by Hvid-Jensen *et al.* in the largest Barrett's oesophagus study to date (Hvid-Jensen, Pedersen et al. 2011). Analysing 11,028 patients with histologically confirmed Barrett's oesophagus, they showed that greater than two-thirds of Barrett's-cancers were diagnosed within the first year of follow-up. Maximum intensity and percentage positivity values were analysed, as several representative cores (mean 2, range 1 to 6) were taken from diagnostic biopsies to construct the TMAs. It was felt, selecting the site of maximum expression of a particular marker was more representative. The heterogeneity of Barrett's oesophagus lesions was reflected by variation in the intensity and percentage positivity from the same patient biopsies, taken from areas with the same confirmed histology at the same surveillance procedure. Following consultation with statisticians, it was realised that this variation was affecting mean values, with the mean value also dependent on the number of available biopsies taken on the day of the surveillance OGD, thus for consistency the maximum values were analysed.

Indeed the role of the stromal and epithelial cells within a tissue microenvironment may play different roles and the cross talk between these cells could also influence their function. When we scored the staining positivity and intensity of our different markers in both cell compartments, we did not identify the specific stromal subset; endothelial, fibroblasts and immune cells. Also, we examined the entire epithelial cell together. If one was to either identify specific immune cells in the stroma or mature surface cells, we would have had to perform dual immunofluorescence which is more difficult to quantitate and would require additional TMA sections which were not available for our study. At this point it is very hard to predict if the mitochondrial dysfunction induced in the Barrett's progression sequence is a driving force or a secondary effect of inflammation. Detailed mechanistic studies would be required to prove causality.

Thus far, this study has demonstrated that mitochondrial instability is an early event in the Barrett's disease sequence. This chapter supports earlier findings of Barrett's oesophagus as an environment of increased oxidative stress and mitochondrial instability. Progressing Barrett's oesophagus patients demonstrated significantly reduced 8-oxo-dG, and in SIM low levels of 8-oxo-dG strongly correlated with high levels of random mutations. Significantly reduced 8-oxo-dG may be representative of defunct respiratory function in the Barrett's progressors, and may aid in the differentiation of those with greatest malignant potential. Overall, we have demonstrated that the mitochondrial environment may play a key role in fuelling progression in Barrett's oesophagus, and mechanisms which maybe controlling these pathways may present a target for further study.

Chapter 5: Examination of oxidative stress and DNA repair genes along the Barrett's oesophagus disease sequence

5.1 INTRODUCTION

Thus far, we have demonstrated the Barrett's metaplastic environment is one of increased oxidative stress compared with other points along the Barrett's disease sequence. In chapter three mitochondrial instability was rescued following antioxidants and DMOG therapy, with DMOG demonstrating the greatest propensity to stabilise DCA induced disruption. However, the mechanisms behind DCA's role in Barrett's oesophagus development, and the potential rescuing properties of DMOG still remain unclear. Mitochondrial outputs, such as the release of ROS, mitochondrial protein secretions, inflammatory secretions, 8-oxo-dG and 4-HNE, have been examined, while the potential genes regulating the Barrett's environment are unknown. Our group has consistently demonstrated the anti-inflammatory effects of DMOG, which has reduced ROS, oxidative stress markers and frequencies of random mitochondrial point mutations (Biniecka, Kennedy et al. 2010, Biniecka, Fox et al. 2011). With ROS seen as recognised precursors for cancer development, a natural extension of this thesis was to investigate genes regulating ROS. Supporting this approach are studies which have implicated ROS in oesophageal cancer development (Kelsell, Risk et al. 1996, Risk, Evans et al. 2002, Langan, Cole et al. 2004, McRonald, Liloglou et al. 2006), with over-expression of the cytoglobin gene (CYGB), a protective gene against oxidative stress, demonstrating detoxifying effects against ROS in oesophageal squamous cell carcinoma *in-vitro* (McRonald, Risk et al. 2012).

The mechanisms behind Barrett's oesophagus development and progression are still poorly understood. The role of DNA repair genes in Barrett's cancer development appeared logical to investigate, given such findings from Preston and colleagues, which have shown direct links between defective DNA repair, increased mutations and carcinogenesis (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009). In addition, mitochondrial DNA is more vulnerable to injury by carcinogens, with many DNA damaging agents preferentially binding to the mitochondrial genome (Wunderlich, Schutt et al. 1970, Wunderlich, Tetzlaff et al. 1972, Wilkinson, Hawks et al. 1975, Singh, Sharkey et al. 1992). With mitochondrial DNA mutations implicated in the development of many human cancers (Kroemer 2006), the maintenance of mitochondrial genomic stability and integrity is imperative. With it known that

mitochondrial DNA accumulates more DNA damage compared with nuclear DNA, (Wallace 1992, Wallace 1992), there is a definite need to understand the genes controlling DNA repair.

ROS and oxidative stress result in the formation of large numbers of base lesions, predominantly the pro-mutagenic 8-oxo-dG (Moriya 1993). Studies have shown that 8-oxo-dG damage is managed by DNA repair mechanisms, rather than removal via apoptotic pathways (Anson, Croteau et al. 1998). Mitochondrial DNA has a deficient DNA repair capacity, in particular mitochondria completely lack nucleotide excision repair mechanisms (Clayton, Doda et al. 1974). It has been shown that mitochondrial DNA damage is primarily repaired by base excision (Sawyer and Van Houten 1999, Wallace 2002). In ageing cells, increased oxidative damage is partly attributable to reduced base excision repair (Shen, Galick et al. 2003). With mitochondrial DNA exhibiting increased vulnerability to mutagenesis, we therefore hypothesise that efficient DNA repair processes may play an important role in the prevention of cancer development (Goldsby, Lawrence et al. 2001).

Until now, we have focussed primarily on the role of random mitochondrial point mutations and mitochondrial dysfunction in Barrett's oesophageal cancer development. The potential genes controlling the mitochondrial environment have not been explored. In this chapter we investigate clonal oxidative stress and DNA repair genes' expression across the Barrett's disease sequence.

5.2 HYPOTHESIS AND AIMS

We have shown Barrett's oesophagus is an environment of oxidative stress and mitochondrial DNA damage. We therefore hypothesise that oxidative stress and DNA repair genes will be altered along the disease sequence. We also hypothesise that treatment with DCA will damage oxidative stress genes, while DMOG treatment will rescue this injury.

Specific aims of chapter five;

(1) Investigate the changes in the expression of oxidative stress and antioxidant genes across the Barrett's oesophagus disease sequence and elucidate the effects of DCA and DMOG on gene expression.
(2) Investigate changes in DNA repair genes across the Barrett's oesophagus disease sequence.
(3) Evaluate the risk of progression associated with LGD using data extracted from the St. James's Hospital Barrett's registry.

5.3 MATERIALS AND METHODS

5.3.1 RNA extraction

5.3.1.1 RNA isolation from SIM biopsies and cell lines using Qiagen Rneasy kit

In-vitro, HET1A, QH, Go and OE33 cells were grown to 60-70% confluence in 25cm^2 flasks over a 24hr period. Cell culture was performed using protocols previously outlined in section 2.3.2. Cells subsequently underwent no treatment (control) or were treated with 100μM DCA, 1mM DMOG or with 100μM DCA + 1mM DMOG for 24hrs as previously described in section 3.3.3. For antioxidant and oxidative stress gene arrays and DNA repair gene arrays, RNA was isolated using Qiagen Rneasy Mini kit (Qiagen Inc., CA, USA). Cells were trypsinised and transferred to 1.5 mL eppendorf tubes. Samples were centrifuged at 180 x g for 3 min at 4°C, the supernatant was decanted and 600μl of lysis buffer, Buffer RLT (supplied), was added to the pellet.

Lysates were homogenised using a 2mL syringe and needle to mix the tube contents up and down. 600µl of ethanol (70% v/v) was added to the supernatant, mixed immediately and transferred in volumes of 700µl to an RNeasy spin column placed in a 2ml collection tube. The spin column was centrifuged at 12,000 × *g* for 15 seconds and the flow through was discarded. The spin column was washed by first applying 700 µl buffer RW1 (supplied), then 500 µl buffer RPE (supplied) with centrifugation at 12,000 × *g* for 15 seconds at room temperature between each step. In a final wash step, 500 µl of buffer RPE was added and samples were centrifuged for 2 minutes at 12,000 × *g* in order to dry the membrane and ensure no ethanol was carried over. RNA was eluted in 30 µl of RNase free water by centrifugation for 1 minute at 12,000 × *g* at room temperature.

For all patient samples, RNA was extracted using this method. Biopsies were homogenised in 600µl Buffer RLT using a Qiagen TissueLyser (Retsch GmbH & Co., Haan, Germany) at 250 RPM for 6 min with one 5mm stainless steal bead (Qiagen Inc., CA, USA) added to each sample to aid homogenisation. The bead was removed and samples were centrifuged at 12,000 x g for 3 min at 4°C, and supernatant was transferred to a new tube by pipetting. RNA extraction was performed using the same steps as described for cell lines.

5.3.1.1 RNA isolation from cell lines using TRI-Reagent method

For validation of genes, RNA was isolated from cell lines using TRI-reagent® RNA isolation reagent (Molecular Research Centre Inc., Cincinnati, OH, USA). TRI-reagent combines phenol and guanidine thiocyanate to facilitate the inhibition of RNase activity. Samples were lysed in TRI-reagent, and addition of bromochloropropane (BCP) separated the lysate into organic and aqueous phases. RNA partitions to the upper aqueous phase, DNA to the interphase, and protein to the lower organic phase. RNA was be precipitated from the aqueous phase.

Medium was removed from cells and the cells lysed directly by addition of 1 mL or 0.5 mL TRI-reagent into 75 cm^2 or 25 cm^2 flasks, respectively. Cells were scraped from the surface of the culture vessel using a plastic cell scraper, passed through a pipette tip three times to aid lysing of cells, and transferred to a 1.5 mL eppendorf tube. Samples

were stored immediately at -80°C. To isolate total RNA, samples were thawed and left to stand at room temperature for 10 min, to allow the complete dissociation of nucleoprotein complexes. A volume of 100 µL BCP per 1 mL TRI-reagent used, was added to the lysate, which was vigorously mixed for 15 secs, and stored for 10 min at room temperature. Samples were centrifuged at 13,400 × g for 15 min at 4°C, separating the sample into a lower red phenol-chloroform phase, white interphase, and upper colourless aqueous phase. The aqueous phase was transferred to a 1.5 mL eppendorf and RNA was precipitated by addition of 0.5 mL isopropanol per 1 mL TRI-reagent used. Samples were stored for 5 min at room temperature and centrifuged at 13,400 × g for 8 min at 4°C. The supernatant was removed and the RNA pellet was washed by vortexing with 1 mL ethanol (75% v/v) (Merck) per 1 mL TRI-reagent used. Samples were then centrifuged at 7,500 × g for 5 min at 4°C. The ethanol wash was removed and the pellet was air dried for ~4 min. The RNA pellet was resuspended in 30 µL RNase-free, molecular-grade H_2O. Isolated RNA was stored at -80°C.

RNA quantity and quality was determined spectrophotometrically using a Nanodrop 1000 spectrophotometer (version 3.1.0, Nanodrop technologies, DE, USA). 1µl Rnase free water was used to blank the instrument prior to RNA analysis. 1µl of each sample of isolated RNA was loaded onto the instrument and concentration was measured in ng/µl. 260:280 and 260:230 purity ratios were also recorded. A 260:280 ratio greater than 1.65 was indicative of a relatively pure RNA yield, while a 260:230 ratio greater than 1.7 indicated the sample was free of phenol contamination.

5.3.2 cDNA synthesis

The reverse transcriptase enzyme and buffer were purchased from Bioline (Bioline, Kilkenny, Ireland), all other reagents were purchased from Invitrogen (Invitrogen Corp., CA, USA). High quality total RNA was reverse transcribed to cDNA using random hexamer oligodeoxyribonucleotides that prime mRNA for cDNA synthesis. A volume of 1 µL of random hexamers was added to 400ng to 1.0µg total RNA, and the volume adjusted to 11µL with RNase-free H_20. RNA concentrations remained consistent between specific experiments. The reaction mixture was incubated at 70°C for 10 min to denature the RNA and was placed on ice. A master mix of reverse-

transcription (RT) components was made up to contain (per RT reaction): RNaseOUT recombinant ribonuclease inhibitor (1 Unit/ µl), dNTPs (10mM, prepared as a 1:1:1:1 ratio of dATP, dGTP, dTTP and dCTP), Bioscript reverse transcriptase (200units/µl) in 5X Bioscript reaction buffer. This mixture was incubated at 37°C for 1 hr and 70°C for 10 min. The resulting cDNA was stored at -20°C and 1µl of cDNA template was used to investigate gene expression.

Table 5.1: Reagents and volumes used in cDNA synthesis

Reagent	Volume (µl) (per sample)
Random Primers	1
250ng of RNA + RNase free H_2O	11
Heated for 10 min at 70°C and cooled on ice for 1 min then following mix added:	
5X Bioscript reaction buffer	4
H_2O	2
DNTPs	1
RNase out	0.5
Bioscript Reverse Transcriptase	0.5
Total Volume	**20**

5.3.3 Quantitative real-time PCR (qPCR)

Quantitative PCR was used to quantify mRNA expression in samples relative to the 18s ribosomal RNA endogenous control. This was carried out using a probe based method, which works as follows: sequence specific probes are labelled with a flourescent reporter molecule at the 5' end, and a flourescence quencher molecule at the 3' end. The close proximity of the reporter to the quencher prevents detection of flourescence. Upon transcription, the 5-3 exonunclease activity of the Taq polymerase removes the quencher molecule and allows fluorescence emission, detected following laser excitation of the sample. All reagents were purchased from ABI Biosystems (CA, USA) or Roche Products Ltd. (Dublin, Ireland). A master mix containing primer/probe and Taqman® Gene Expression Master Mix, was added to 1µl of cDNA template (see table below). A final volume of 20µl was pipetted into a well of a MicroAmp™ Optical 96 well reaction plate in duplicate (ABI Biosystems, CA, USA). The reaction plate was

sealed using an optical adhesive cover (Applied Biosystems), and the plate was centrifuged briefly to pool reagents and eliminate any bubbles. Real-time PCR detection was performed using an ABI Prism 7900HT real-time thermal cycler (Applied Biosystems), using the thermal cycling program outlined in table 5.3. Gene expression data was analysed as described below in section 5.4, 18S ribosomal RNA was used as endogenous controls for data normalisation.

Table 5.2: Reagents and volumes used for qPCR

Reagent	Volume (µl)
Master Mix	10
Forward Primer	1
Reverse Primer	
Probe	
RNase Free Water	8
cDNA	1
Final Volume	**20**

Table 5.3: Thermal cycling conditions for Quantitative real-time PCR

Number of Cycles	Time (Min)	Temperature (°C)
1	2	50
1	10	95
40	0.25	95
	1	60

5.3.4 Quantitative real-time PCR data analysis

Quantitative real-time PCR data was analysed using SDS 2.3 and SDS RQ Manager 1.2 relative quantification software. A visual inspection of duplicate expression values was initially performed to identify any abnormalities and exclusion was performed. The threshold cycle (Ct) for each well was calculated and the expression levels of target genes were normalised to expression levels of specific endogenous control genes. Analysis of gene expression data was then carried out using the $2^{-\Delta\Delta Ct}$ relative

quantification method, which describes the change in expression of a target gene relative to the expression of a reference group, such as an untreated control.

5.3.5 Oxidative stress and antioxidant gene analysis

cDNA samples were applied to Human Oxidative Stress and Antioxidant Defence RT^2 Profiler™ PCR Arrays (SA Biosciences) and cycled on a 7900 Real Time PCR system (Applied Biosystems Inc.) as per the manufacturer's instructions. The Human Oxidative Stress and Antioxidant Defence RT^2 Profiler™ PCR Arrays contain 84 genes (listed in appendix two) related to oxidative stress and antioxidant defence. The purpose of this analysis was to determine the mechanisms by which DCA and DMOG may affect the expression of these genes across the Barrett's oesophagus disease model. The QH cell line was selected for initial analysis +/- 100μM DCA +/- 1mM DMOG +/- 100μM DCA and 1mM DMOG for 24hrs, in duplicate. The 24hr time-point was selected for consistency with the analyses performed in chapter three.

Genes were selected for validation across the Barrett's disease sequence *in-vitro* +/- *in-vivo* where there was a $\geq$ 3.5-fold up-regulation or down-regulation of a specific gene and following literature review to determine relevance to this study. *In-vitro*, selected gene primers (SA Biosciences) were validated across the cell line sequence in the HET1A, QH, Go and OE33 cells +/- 100M DCA +/- 1mM DMOG +/- 100M DCA + 1mM DMOG for 24hrs ($n \geq 3$), using the standard protocol described in sections 5.3.3 and 5.3.4. The HET1A cell line was used as the control cell line, and changes in gene expression were calculated in the remaining cell lines relative to the HET1As. For evaluation of the effects of DCA and DMOG treatment, within each cell line changes were measured relative the control, untreated cells. All experiments were performed a three times.

Genes with significant changes across the entire disease sequence were selected for further validation in patient samples encompassing the entire Barrett's oesophagus disease spectrum; normal squamous epithelium (n=6), SIM (n=30), LGD (n=6), HGD (n=12) and invasive oesophageal adenocarcinoma (n=8). Normal biopsies were taken from patients undergoing elective OGDs, with no evidence of any pathology in the oesophagus, stomach and duodenum. All OGDs were performed by two consultant

gastroenterologists; Professor Dermot O'Toole and Dr Finbar McCarthy (St. James's Hospital, Dublin).

5.3.6 DNA repair gene analysis

cDNA samples were applied to DNA repair gene PCR Arrays (Roche Products Ltd., Dublin, Ireland). These arrays were cycled on a Real Time PCR system (Roche LightCycler® 480) as per the manufacturer's instructions. The arrays contained 84 genes (listed in appendix two) encoding for the enzymes that repair damaged DNA. The array represented genes involved in base excision, nucleotide excision, mismatch, double stranded break and other repair processes. The purpose of this study was to determine alterations in the DNA repair mechanisms across the Barrett's oesophagus disease sequence. Initial analysis was performed on each of the Barrett's cell lines, HET1A, QH, Go and OE33 cells, in duplicate. Genes were selected for further validations across the Barrett's disease sequence *in-vivo* where there was a $\geq$ 3.5-fold up-regulation or down-regulation of a specific gene relative to the control cell line and following literature review to determine relevance to this study. *In-vivo,* specific gene primers (Roche Products Ltd.) were validated using an ABI Prism 7900HT real-time thermal cycler as outlined in section 5.3.3.

5.3.7 Statistical analysis

Data were analysed with Graph Pad Prism (Graph Pad Prism, San Diego, CA) software. Differences between continuous variables in cell lines' control and treatment groups were calculated using Paired Student's t-tests and one-way ANOVA tests. Differences between different cell lines were calculated using unpaired Student's t-tests. Differences between continuous variables, within different histology patients groups were calculated using Mann-Whitney U tests. Progression statistics, in the LGD cohort, were calculated using the Kaplan-Meier method and the log rank test was used to assess differences between groups. Statistical significance was defined by $p \leq 0.05$.

5.4 RESULTS

5.4.1 Expression of antioxidant and oxidative stress genes across the Barrett's oesophagus disease sequence

5.4.1.1 Global antioxidant and oxidative stress gene analysis

Using gene arrays, the expression of the genes listed in appendix two were analysed in the QH, intestinal metaplasia cell line +/- 100μM DCA +/- 1mM DMOG. Preliminary analysis demonstrated DMOG caused a 13-fold reduction in cytoglobin (CYGB) gene expression, a 6.4-fold reduction in forkhead box M1 (FOXM1) gene expression and a 3.9-fold decrease in the expression of glutaredoxin 2 (GLRX2) gene. Following treatment with DCA + DMOG for 24hrs, similar reductions in CYGB, FOX M1 and GLRX2 gene expression were also demonstrated (results shown in appendix two). Preliminary analysis did not identify a strong change in antioxidant and oxidative stress gene expression in the panel of genes analysed following treatment with 100μM DCA for 24hrs.

From these preliminary findings, CYGB, FOX M1 and GLRX2 were selected for validation *in-vitro* and *in-vivo*.

5.4.1.2 Antioxidant and oxidative stress genes validation *in-vitro*

5.4.1.2.1 Expression of genes across the Barrett's disease sequence

Unpaired t-tests demonstrated a significant 35% decrease in the expression of FOX M1 in the QH cells compared with the HET1A cell line (p=0.009) (Figure 5.1). FOX M1 was significantly 76% reduced in the OE33 cells compared with HET1A cells (p=0.001). The QH cells demonstrated a significantly increased expression of FOX M1 compared with the OE33 cell line (p=0.019). There was a 357% increase observed in the expression of FOX M1 in the Go cells compared with the HET1As, however, this was not significant (p=0.104). There was no significant difference in the expression of FOX M1 in the Go cells compared with the QH (p=0.083) and OE33 cells (0.064).

CYGB was significantly altered along the Barrett's oesophagus cell line sequence (p=0.0004). The HET1A cell line was selected as the control cell line and the change in the expression of CYGB was calculated relative to the HET1As. There was 60-fold increase in the expression of CYGB in the QH cells relative to the HET1As (p<0.0001). There was a 72-fold increase in the expression of CYGB in the Go cells compared with the HET1As (p<0.013). A 96-fold reduction in the expression of CYGB in the OE33 cells was seen (p<0.0001). The expression of CYGB was significantly less in the OE33s in comparison with the QHs (p<0.0001) and the Go cells (p=0.012). No difference was identified between the QH and Go cells (p=0.502).

Unpaired t-tests were used to evaluate the differences in the expression of GLRX2 along the *in-vitro* Barrett's oesophagus model. There was a significant 17-fold increased expression of GLRX2 in the QH (p=0.036) compared with the HET1A cells. No significant difference was demonstrated between the Go and HET1A cells (p=0.073). The QH cells demonstrated an increase in the expression of GLRX2 when compared with the OE33 cells (p=0.042). There was no significant difference in GLRX2 expression between the Go cells and the OE33s (p=0.074).

Overall, a similar trend in the expression of FOX M1, CYGB and GLRX2 was demonstrated along the Barrett's oesophagus cell line model. Significant reductions were identified in the OE33 cancer cell lines, compared with overexpression of these genes in the QH and Go cells.

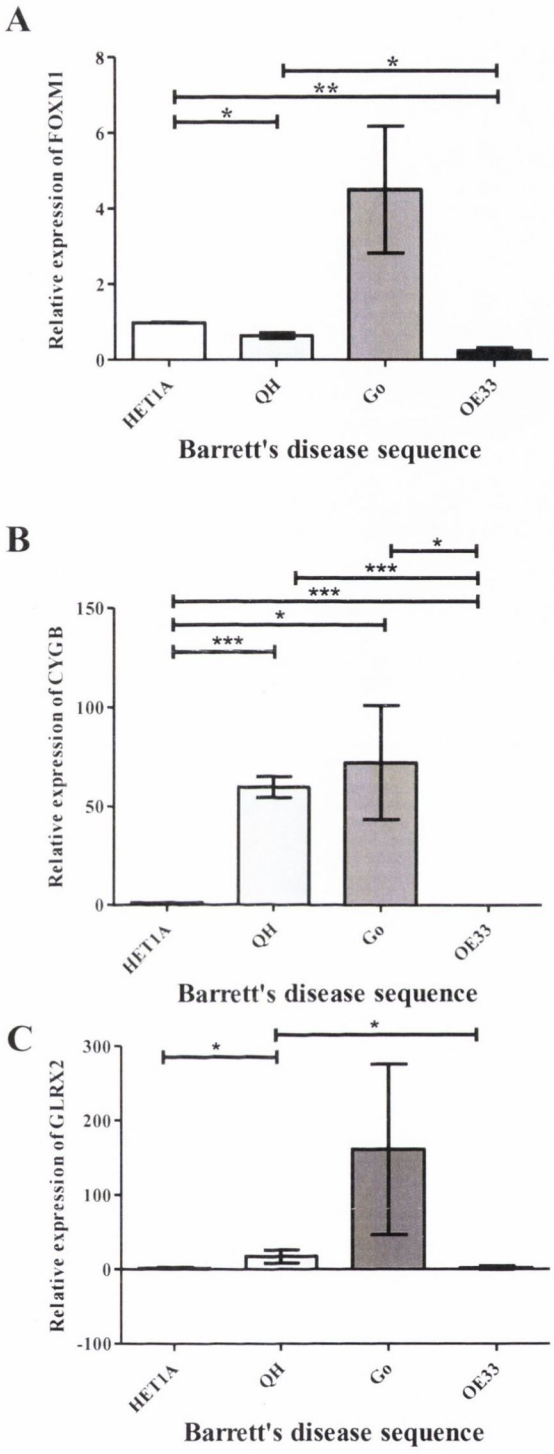


Figure 5.1: Expression of antioxidant and oxidative stress genes across the Barrett's disease sequence *in-vitro* (n=3). (A) There relative expression of FOXM1 was significantly higher in the HET1As compared with the QH and OE33s. There was a reduction in the expression of FOX M1 in the OE33s compared with the QHs. **(B)** ANOVA demonstrated a significant difference in the expression of CYGB along the disease sequence (p=0.0004). **(C)** GLRX2 was significantly increased in the QHs compared to the HET1A and OE33 cells. Error bars represent standard deviation. *p<0.05, ***p<0.0005.

5.4.1.2.2 Gene expression following DCA +/- DMOG treatment *in-vitro*

In the HET1A cell line, treatment with DCA +/- DMOG did not significantly alter the expression of FOX M1 (p=0.692) and GLRX2 (p=0.323). CYGB expression was significantly reduced following 100μM DCA treatment for 24hrs (p=0.011) (Figure 5.2).

In the QH cell line, treatment with DCA +/- DMOG did not significantly alter the expression of FOX M1 (p=0.580), CYGB (p=0.510) or GLRX2 (p=0.613) (Figure 5.3).

In the Go cell line, treatment with DCA +/- DMOG did not significantly alter the expression of FOX M1 (p=0.899), CYGB (p=0.602) or GLRX2 (p=0.743) following 24hrs treatment (Figure 5.4).

In the OE33 cell line, treatment with DCA +/- DMOG did not significantly alter the expression of FOX M1 (p=0.344) or GLRX2 (p=0.862). CYGB was unexpressed in the OE33 cells and this did not change following treatment with 100μM DCA +/- 1mM DMOG (Figure 5.5).

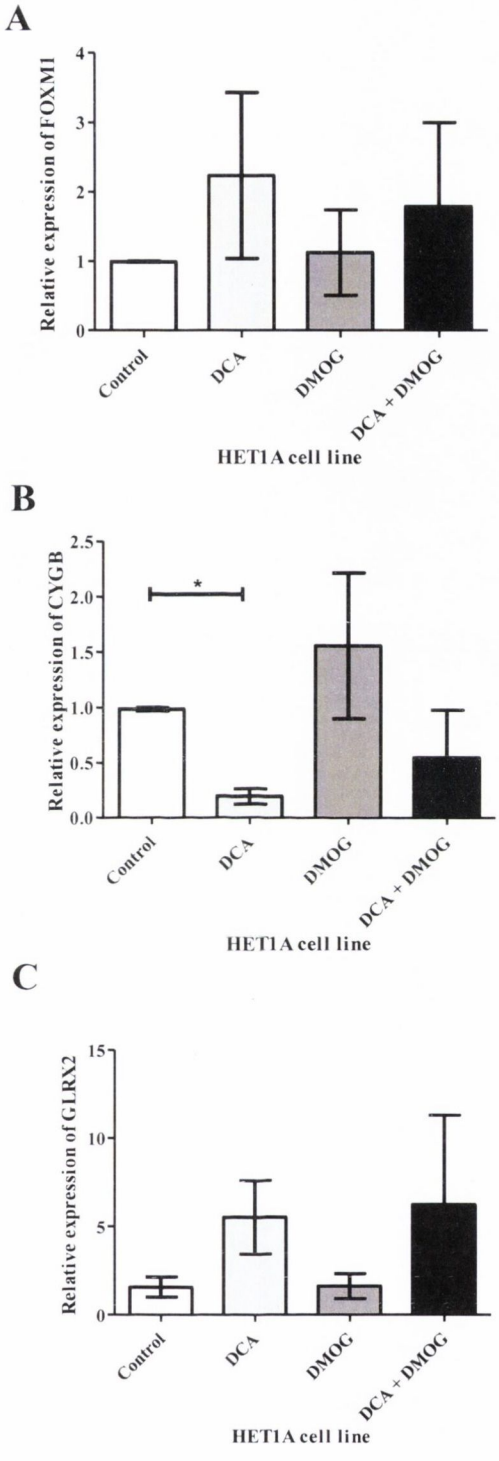


Figure 5.2: Relative expression of FOXM1, CYGB and GLRX2 in HET1A cells +/- DCA +/- DMOG +/- DCA & DMOG (n=3). (A) There was no significant change in the expression of FOXM1. **(B)** DCA induced a significant decrease in the relative expression of CYGB, however, DMOG did not alter CYGB expression. **(C)** There was no difference in the expression of GLRX2 following treatment. Error bars represent standard deviation. *$p<0.05$

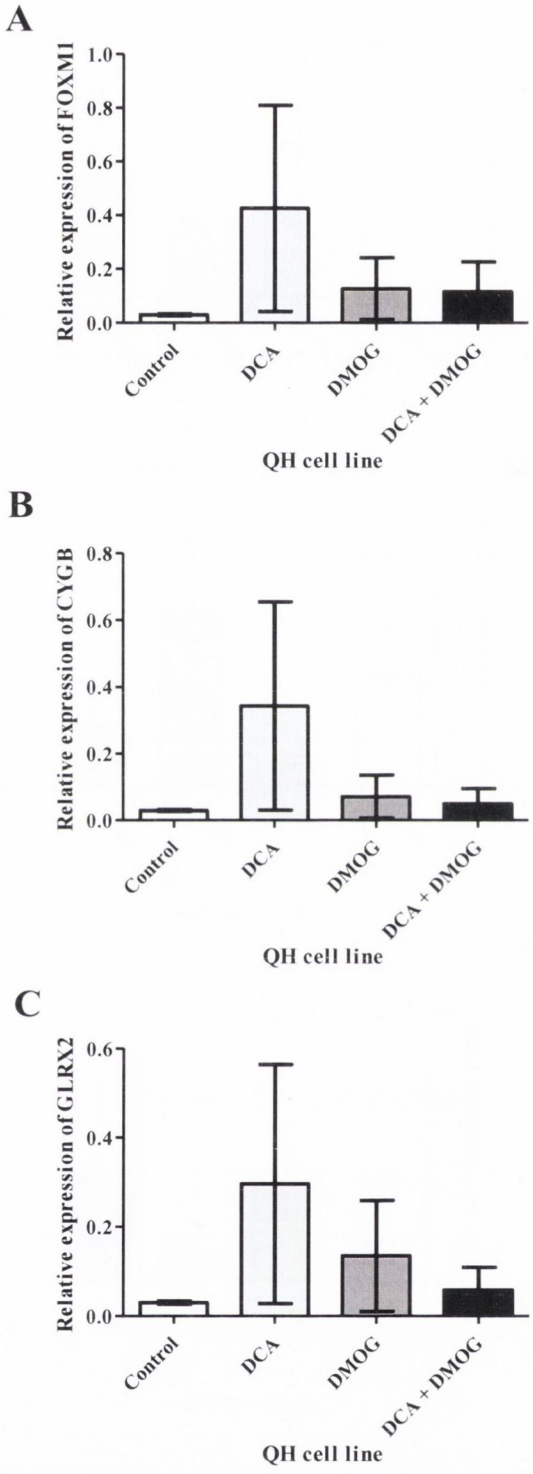


Figure 5.3: Relative expression of FOXM1, CYGB and GLRX2 in QH cells +/- DCA +/- DMOG +/- DCA & DMOG (n=3). There was no difference in the relative expression of **(A)** FOXM1 (p=0.580), **(B)** CYGB (p=0.510) and **(C)** GLRX2 (p=0.613) following 24hrs treatment with 100μM DCA +/- 1mM DMOG. Error bars represent standard deviation.

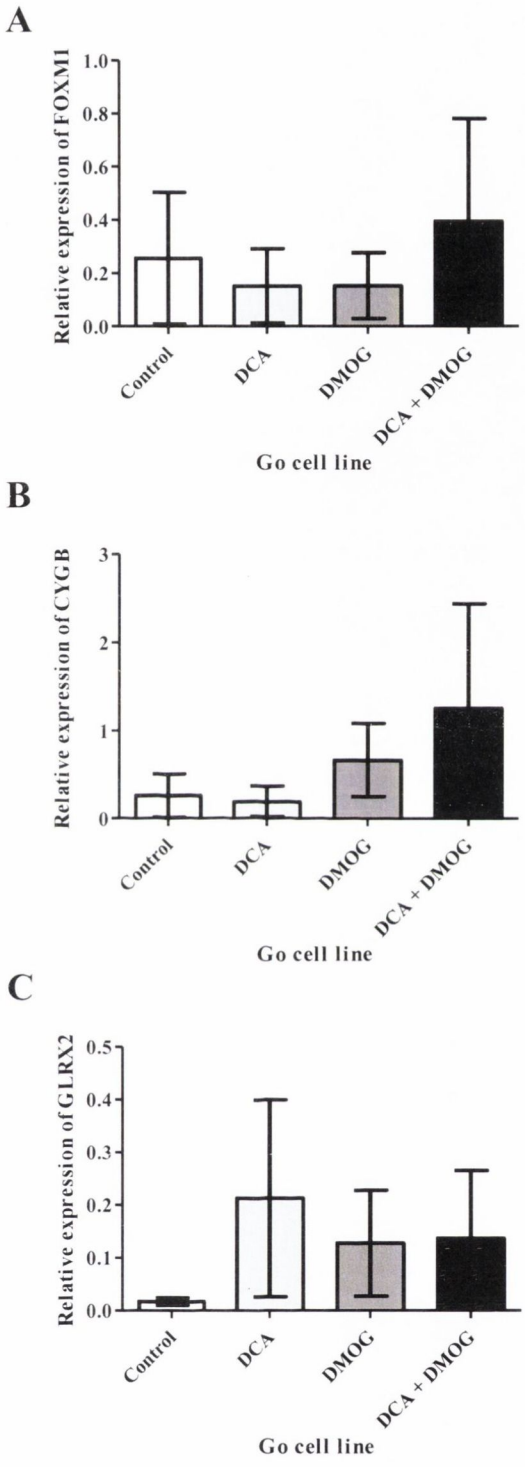


Figure 5.4: Relative expression of FOXM1, CYGB and GLRX2 in Go cells +/- DCA +/- DMOG +/- DCA & DMOG (n=3). There was no difference in the relative expression of **(A)** FOXM1 (p=0.899), **(B)** CYGB (p=0.602) and **(C)** GLRX2 (p=0.743) following 24hrs treatment with 100μM DCA +/- 1mM DMOG. Error bars represent standard deviation.

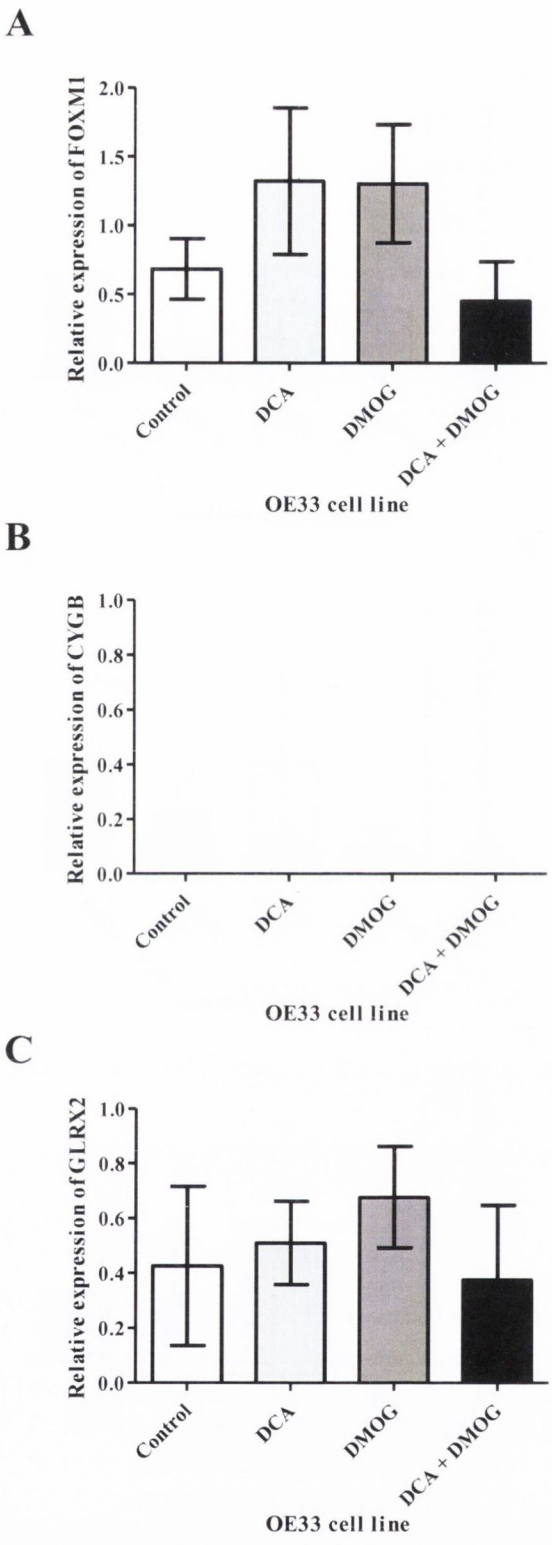


Figure 5.5: Relative expression of FOXM1, CYGB and GLRX2 in OE33 cells +/- DCA +/- DMOG +/- DCA & DMOG (n=3). There was no difference in the relative expression of **(A)** FOX M1 (p=0.344), **(B)** CYGB (unexpressed) and **(C)** GLRX2 (p=0.862) following treatment with 100μM DCA +/- 1mM DMOG. Error bars represent standard deviation.

5.4.1.2 CYGB validation in an *in-vivo* Barrett's oesophagus model

In our *in-vitro* model, CYGB was the gene most significantly altered along the cell line disease sequence. It was therefore selected to be validated *in-vivo*. Tissue biopsies from patients with no evidence of oesophageal or gastric pathology at OGD (n=6) were compared with biopsies from patients with SIM (n=31), LGD (n=7), HGD (n=11) and oesophageal adenocarcinoma (n=6).

There was a 10.6-fold increase in the expression of CYGB in SIM when compared with normal tissue (p=0.013). CYGB expression was significantly increased in LGD (p=0.001), HGD (p=0.018) and oesophageal adenocarcinoma (p=0.002) compared with normal tissue. Similar to our *in-vitro* analysis, SIM over-expressed the CYGB gene compared with LGD and invasive adenocarcinoma (p=0.010 and p=0.022, respectively) (Figure 5.6). In patients samples analysed, no significant difference was demonstrated between SIM and HGD (p=0.567), HGD and adenocarcinoma (p=0.097) and LGD and oesophageal adenocarcinoma (p=0.628). There was a trend towards a decrease in the expression of CYGB seen in the HGD patients compared with the LGD cohort, however this was not significant (p=0.057).

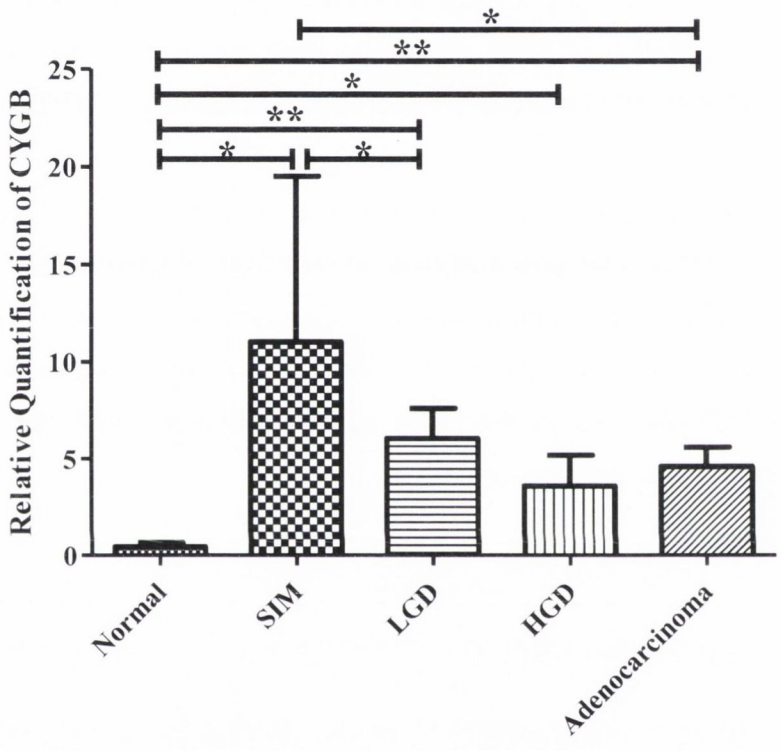


Figure 5.6: Expression of CYGB along the Barrett's oesophagus disease sequence *in-vivo*. SIM over-expressed CYGB relative to LGD and invasive cancer. There was a significant increase in CYGB in SIM, LGD, HGD and oesophageal adenocarcinoma samples when compared with normal squamous epithelium. Error bars represent SEM. *$p<0.05$, **$p<0.005$.

5.4.2 Expression of DNA repair genes along the Barrett's oesophagus sequence

5.4.2.1 Global DNA repair gene analysis

Using DNA repair gene arrays, the expression of the genes listed in appendix two were analysed across the Barrett's oesophagus *in-vitro* model. Preliminary analysis demonstrated a step-wise increase in the expression of BRCA-1 in the QH (> 900-fold) and Go (>15 x 10^6-fold) cell lines. BRCA-2 decreased in the QH (> -85-fold), Go (> -37-fold) and OE33 (> -47-fold) cells relative to the HET1A cell line. The expression of MUTYH relative to the normal cell line, decreased in the QH (> -7-fold), Go (> -23-fold) and OE33 (> -7-fold) cells.

From these preliminary findings, BRCA-1 (breast cancer susceptibility gene-1), BRCA-2 (breast cancer susceptibility gene-2) and MUTYH (mutY homolog), ATM (ataxia telangiectasia mutated) and OGG-1 (8-oxoguanine DNA glycosylase) were selected for further analysis in an *in-vivo* study.

5.4.2.2 BRCA-1 expression across the Barrett's oesophagus sequence *in-vivo*

The expression of BRCA-1 significantly increased in LGD (p=0.015), HGD (p=0.055) and oesophageal adenocarcinoma (p=0.001) compared with normal tissue samples (Figure 5.7). There was no significant difference in the expression of BRCA-1 between the normal and SIM tissue (p=0.572). BRCA-1 was significantly lower in SIM compared with LGD (p=0.009), HGD (p=0.021) and oesophageal adenocarcinoma (p<0.0001). No significant difference was seen in the expression of BRCA-1 between LGD and HGD (p=0.282). Although not significantly different there was a trend towards increased expression of the gene in invasive cancer compared with LGD (p=0.059). There was an increase in expression of BRCA-1 in adenocarcinoma biopsies compared with HGD (p=0.004).

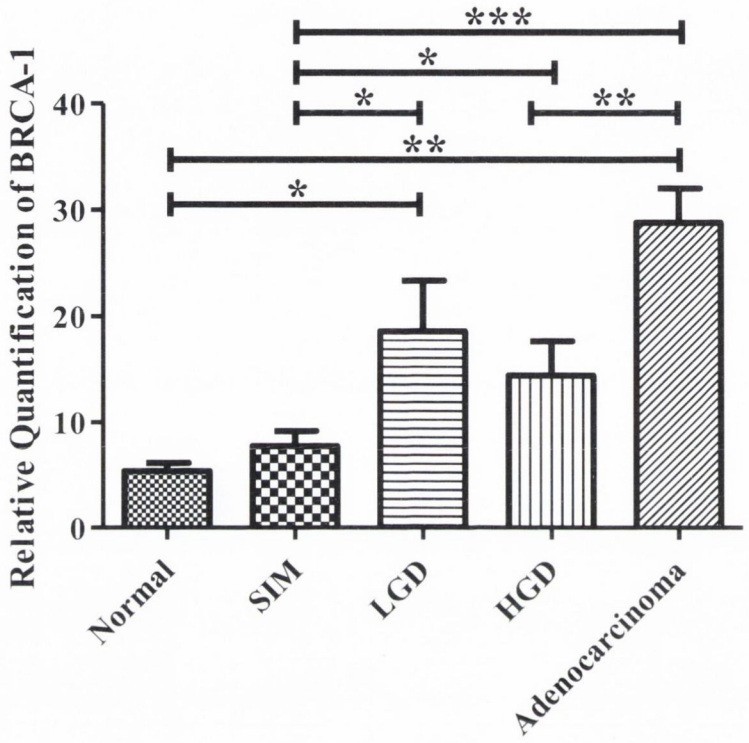


Figure 5.7: Expression of BRCA-1 across the Barrett's oesophagus disease sequence *in-vivo*. There was a stepwise increase in the expression of BRCA-1 seen in patients with normal oesophageal and gastric findings (n=6), with SIM (n=30), LGD (n=6), HGD (n=12) and oesophageal adenocarcinoma (n=8). Error bars represent SEM. *p<0.05, **p<0.005, ***p<0.0005.

5.4.2.3 BRCA-2 expression across the Barrett's oesophagus sequence *in-vivo*

The expression of BRCA-2 increased along the Barrett's oesophagus disease sequence (Figure 5.8). BRCA-2 gene expression was significantly increased in oesophageal adenocarcinoma compared with SIM (p=0.001). There was no significant difference in the expression of BRCA-2 between SIM and normal tissue (p=0.259). The expression of BRCA-2 was significantly increased in LGD (p=0.015), HGD (p=0.044) and adenocarcinoma (p=0.001) compared with normal tissue samples. No difference was identified between SIM and LGD (p=0.090) or HGD (0.144). No difference was identified between LGD and HGD (p=0.815) or between HGD and adenocarcinoma (p=0.097). Although not significantly different, there was a trend towards an increased expression of BRCA-2 in the cancer tissue compared with LGD (p=0.059).

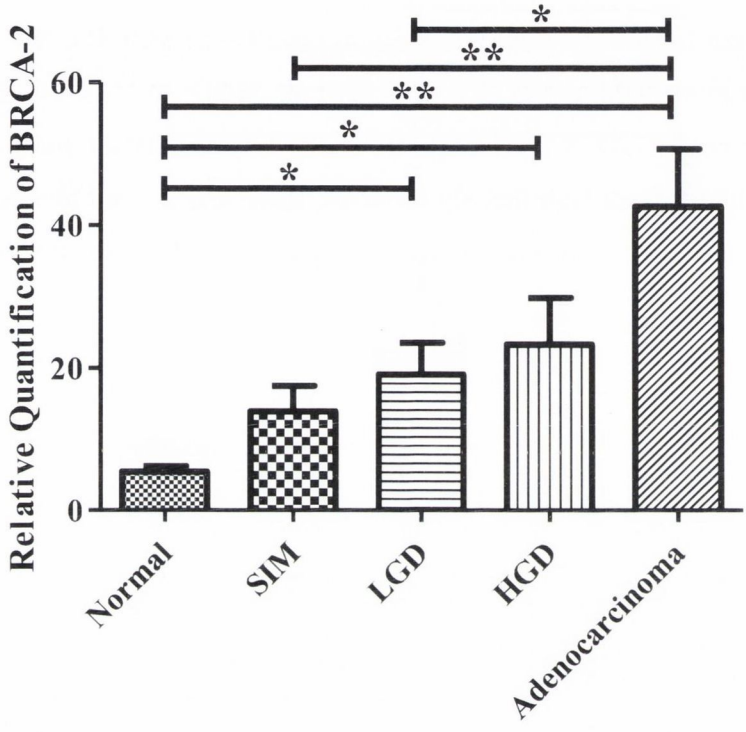


Figure 5.8: Expression of BRCA-2 across the Barrett's oesophagus disease sequence *in-vivo*. There was a stepwise increase in the expression of BRCA-2 seen in patients with normal oesophageal and gastric findings (n=6), with SIM (n=30), LGD (n=6), HGD (n=12) and oesophageal adenocarcinoma (n=8). Error bars represent SEM. *$p<0.05$, **$p<0.005$.

5.4.2.4 ATM expression across the Barrett's oesophagus sequence *in-vivo*

There was a trend towards a stepwise increase in the expression of ATM along the Barrett's oesophagus disease sequence (Figure 5.9). ATM was significantly decreased in the normal epithelium compared with HGD (0.028) and oesophageal adenocarcinoma (p=0.003). There was no significant difference in ATM expression in normal tissue compared with SIM (p=0.408) and LGD (p=0.065). There was a trend towards decreased expression in SIM compared with LGD (p=0.053). Significantly increased levels of ATM were demonstrated in HGD (p=0.029) and adenocarcinoma (p=0.001) compared with SIM. A difference in the expression of the gene was demonstrated between HGD and oesophageal adenocarcinoma (p=0.049); however, there was no difference seen between LGD and HGD (p=0.815) and LGD and cancer (p=0.414).

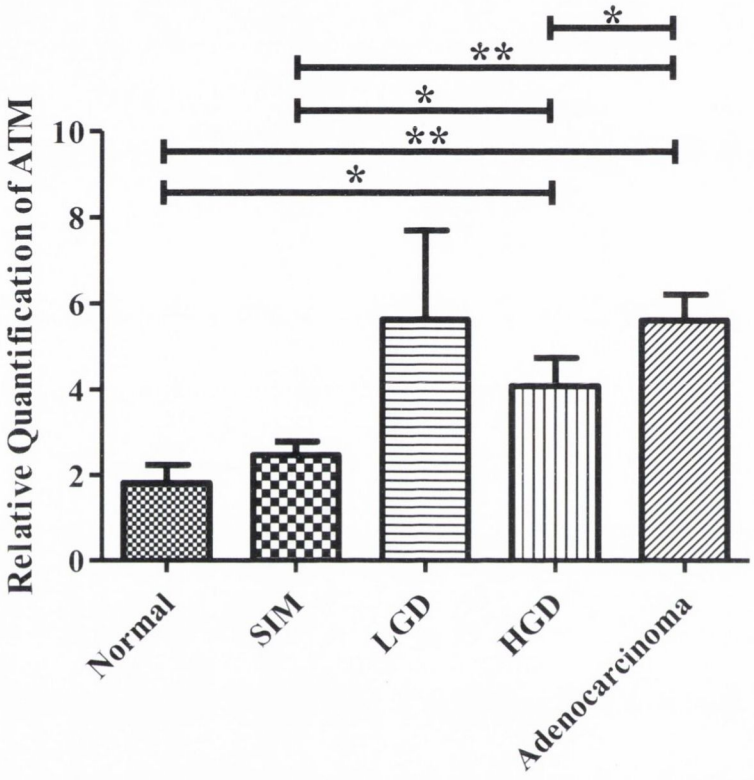


Figure 5.9: Expression of ATM across the Barrett's oesophagus disease sequence *in-vivo*. There was a stepwise increase in the expression of ATM seen in patients with normal oesophageal and gastric findings (n=6), with SIM (n=30), LGD (n=6), HGD (n=12) and oesophageal adenocarcinoma (n=8). *p<0.05, **p<0.005.

5.4.2.5 OGG-1 expression across the Barrett's oesophagus sequence *in-vivo*

In the patient samples analysed, the expression of OGG-1 was significantly increased in SIM ($p=0.005$), LGD ($p=0.041$), HGD ($p=0.0002$) and oesophageal adenocarcinoma ($p=0.001$) compared with normal squamous epithelium (Figure 5.10). However, there was no demonstrated difference in the expression of OGG-1 between the varying pathologies within the Barrett's disease sequence. SIM showed no significant difference compared with LGD ($p=0.484$), HGD ($p=0.405$) and adenocarcinoma ($p=0.702$). LGD did not demonstrate any difference when compared with HGD ($p=0.958$) and invasive cancer ($p=0.628$). No difference was identified between HGD and adenocarcinoma ($p=0.475$) in the patients analysed.

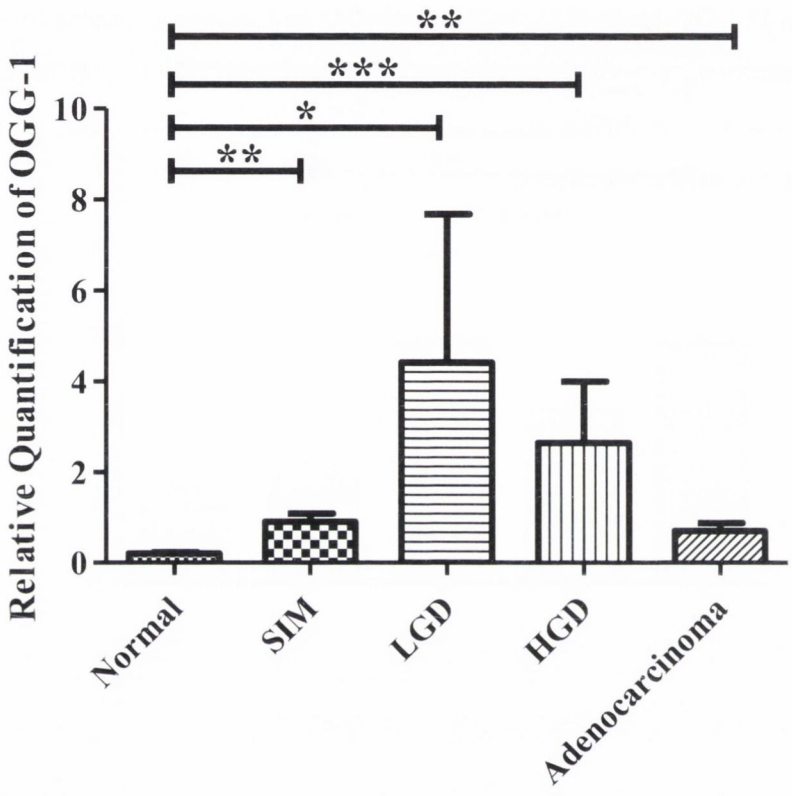


Figure 5.10: Expression of OGG-1 across the Barrett's oesophagus disease sequence *in-vivo*. There was a significant increase in the expression of OGG-1 seen in patients with SIM (n=22) (p=0.005), LGD (n=6) (p=0.041), HGD (n=10) (p=0.0002) and oesophageal adenocarcinoma (n=7) (p=0.001) when compared with patients with normal squamous epithelium lining the distal oesophagus (n=6). Error bars represent SEM.*p<0.05, **p<0.005, ***p<0.0005.

5.4.2.6 MUTYH expression across the Barrett's oesophagus sequence *in-vivo*

The expression of MUTYH was not significantly different between SIM, LGD, HGD and oesophageal adenocarcinoma (Figure 5.11). However, it was significantly decreased expression of MUTYH in normal squamous epithelium compared with SIM ($p=0.047$), HGD ($p=0.023$) and oesophageal adenocarcinoma ($p=0.008$). There was no significant difference in the expression of MUTYH between normal tissue and LGD ($p=0.065$).

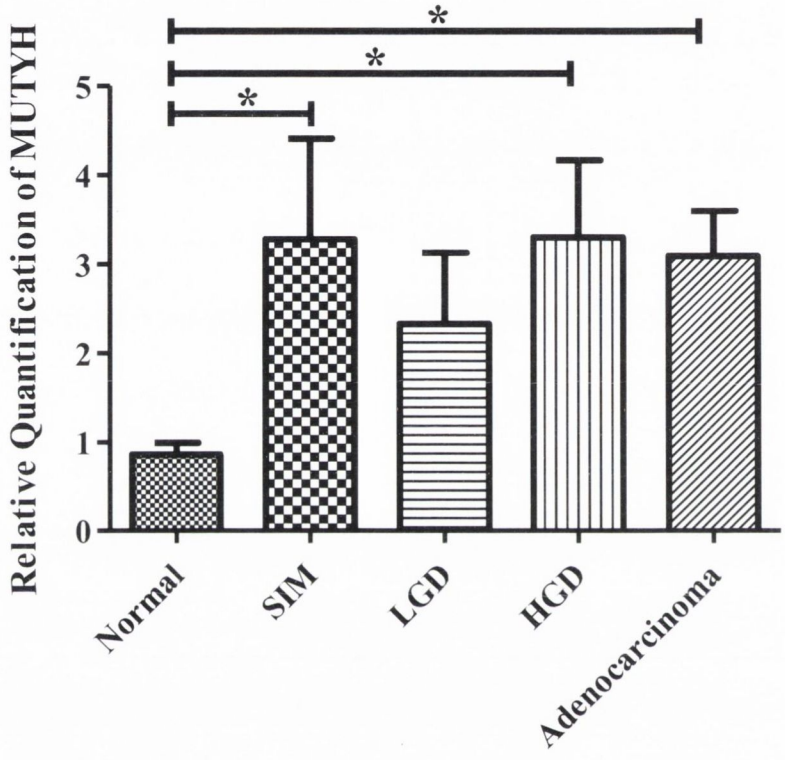


Figure 5.11: Expression of MUTYH across the Barrett's oesophagus disease sequence *in-vivo*. There was a significant increase in the expression of MUTYH seen in patients with SIM (n=22), HGD (n=10) and oesophageal adenocarcinoma (n=7) when compared with patients with normal squamous epithelium lining the distal oesophagus (n=6). Error bars represent SEM. *$p<0.05$.

5.5 DISCUSSION

The role of oxidative stress and DNA repair genes in the progression of Barrett's oesophagus is unknown. As previously shown, the Barrett's metaplastic environment is one of increased oxidative stress and mitochondrial instability, relative to all other points along the disease sequence. However, the mechanisms altering this redox balance in Barrett's oesophagus remain undetermined. DNA repair defects have equally been implicated in mutatgenesis and cancer development (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009), with impaired DNA repair associated with accelerated ageing and carcinogenesis (Marnett 2000, Raha and Robinson 2000). DNA repair is less proficient in the mitochondrial genome (Clayton, Doda et al. 1974), and studies have demonstrated that defects in the primary mitochondrial DNA repair pathways have been associated with increased oxidative damage in the ageing cell (Sawyer and Van Houten 1999, Wallace 2002). Therefore, there is a need to understand the variation in the expression of these genes along the Barrett's disease sequence.

Thus far, we have focussed on the role of random mitochondrial point mutations in the development and progression of Barrett's oesophagus. While these random mutations may be the initial catalyst in tumour progression, it has been speculated once established, random mutations undergo selection processes, with clonal mutations likely superseding the initial catalyst for cancer development (Loeb, Loeb et al. 2003). In this chapter, we examined if genes regulating oxidative stress and DNA repair mechanisms altered along the Barrett's disease sequence *in-vitro* and *in-vivo*.

Oxidative stress and antioxidant gene arrays were used to evaluate changes in gene expression following DCA and DMOG treatment. In chapter three, we showed DMOG therapy had the greatest propensity to rescue mitochondrial changes using the *in-vitro* Barrett's disease model. However, the exact cellular and molecular mechanisms by which these changes were achieved are unknown. In this chapter preliminary analysis was conducted using the QH cells; we were interested in the response at the Barrett's metaplastic stage, given that once HGD is established, cancer development is deemed inevitable (Edge 2010). We wished to determine mechanisms controlling mitochondrial

responses to DCA and DMOG and to assess alterations in these genes along the Barrett's disease sequence.

Based on gene arrays data, CYGB, FOX M1 and GLRX2 were selected for validation in the *in-vitro* cell line model. All three genes were over-expressed in the HGD, Go cell line, although this was not significant for the FOX M1 gene. FOX M1, a transcription factor located at the 12p13 chromosome, is involved in regulation of cell proliferation, differentiation and transformation (Liu, Zhang et al. 2013). Studies have found it to be over-expressed in pre-malignant conditions such as cervical intraepithelial neoplasia (CIN) and others have shown it stimulates proliferation of tumour cells during progression of non-small cell lung cancer (NSCLC) (Kim, Ackerson et al. 2006, Chan, Yu et al. 2008). Our analysis demonstrated a reduction in expression of FOX M1 in the OE33 cells compared with the normal and intestinal metaplasia cell line, and its expression was not significantly altered following DCA +/- DMOG treatment.

GLRX2 is located at chromosome 1q31, and has been linked to redox homeostasis and protection against oxidative stress induced apoptosis (Enoksson, Fernandes et al. 2005, Murphy 2009). Studies have shown that deletions of the gene are associated with reduced tolerance to oxidative damage, while stable cells overexpressing the gene display enhanced proliferation and decreased ROS (Wu, Lin et al. 2011, Kim, Jung et al. 2012). Our findings demonstrated the expression of this gene was significantly increased in the QH cells, 17-fold, compared with the HET1As, and was significantly increased in the QH cells compared with OE33s. However, treatment with DCA +/- DMOG did not significantly alter this gene expression.

CYGB, an antioxidant gene, located at chromosome 17q25, has demonstrated protection during oxidative stress (Li, Hemann et al. 2012). Our results showed that CYGB was significantly over-expressed in the QH and Go cell lines. CYGB was not expressed in the OE33 cells. CYGB encodes for the ubiquitously expressed protein cytoglobin. Cytoglobin protein occurs in response to oxidative stress in prostate cancer, and increased expression suggests an environment of oxidative damage (Mogal, Watson et al. 2012). In fact, in tylosis, a rare autosomal skin disorder associated with early onset oesophageal squamous cell carcinoma, a 70% reduction in CYGB expression was

demonstrated in oesophageal biopsies from tylosis patients compared with normal patients (McRonald, Liloglou et al. 2006). We speculate that loss of CYGB expression renders oesophageal cells susceptible to ROS damage. Our findings of reduced CYGB expression in the HET1A and OE33 cells correlates with the levels of ROS measured *in-vitro,* seen in chapter two. ROS levels were lowest in these two cell lines, indicating that response to oxidative damage may not be necessary at these stages. However, ROS levels were significantly greatest in the QH cells, and this was coupled by a significant increase in the expression of CYGB. Treatment with 100μM DCA for 24hrs caused a significant reduction in CYGB expression in the HET1A cells. DCA induced an increase in ROS in the HET1As in chapter three, and therefore, it was expected that CYGB should be over-expressed following DCA exposure. Perhaps, it is an impaired CYGB response in the normal cell line which is associated with initiation of the Barrett's disease process. CYGB has detoxifying effects against free-radicals and down-regulation of this gene has been associated with sporadic cases of oesophageal and lung cancer (McRonald, Liloglou et al. 2006, Xinarianos, McRonald et al. 2006). It has been speculated that CYGB is a candidate tumour suppressor gene (Lv, Wang et al. 2008, Fang, Ma et al. 2011), and changes in normal CYGB response to oxidative stress may initiate a cascade of events responsible for the development of Barrett's oesophagus.

Our *in-vitro* studies were based on treatment of all cell-lines with DCA and DMOG for 24hrs. As explained in the methods, this time-point was selected for consistency purposes with experiments performed in chapter three. The purpose of this chapter was to determine the mechanism by which these treatments may alter the previously demonstrated changes in mitochondrial function. However, as explained in chapter three, a fixed time-point of 24hrs and a fixed-dose of 100μM DCA is not without its limitations. The *in-vivo* setting is a much more complex model; and perhaps higher concentrations of DCA for longer durations may be required to induce changes in expression of the genes measured. Also, given DCA was examined only at a neutral pH, we acknowledge concentrating solely on this single element of reflux may affect our outcomes.

CYGB was subsequently selected for validation using *in-vivo* patient oesophageal biopsies. Findings were relatively consistent with our *in-vitro* results. CYGB was over-expressed in SIM compared with normal, LGD and oesophageal adenocarcinoma tissue, supporting our findings that SIM is an environment of oxidative stress (Mogal, Watson et al. 2012). Over-expression of CYGB in the metaplastic stage may reflect the response to the increased mitochondrial biogenesis and inflammation in SIM demonstrated in chapter two. Loss of CYGB expression in the latter stages of the disease potentially may reflect the inability to regulate oxidative stress, and loss of protection once tumour growth is established (McRonald, Liloglou et al. 2006, McRonald, Risk et al. 2012).

The role of DNA repair mechanisms in the development and progression of Barrett's oesophagus is unknown. We were interested in evaluating alterations in DNA repair genes along the Barrett's disease sequence, due to an association between impaired repair processes, mutagenesis and oxidative stress (Goldsby, Lawrence et al. 2001, Albertson, Ogawa et al. 2009). Preliminary analysis was conducted using DNA repair gene arrays, measuring changes in these genes' expression across the Barrett's cell line model. BRCA-1, BRCA-2, OGG-1, ATM and MUTYH were subsequently selected for validation using *in-vivo* oesophageal biopsies encompassing the entire Barrett's disease spectrum.

BRCA-1, a tumour suppressor gene, is a master regulator of DNA integrity through co-ordination and involvement in DNA repair (Kennedy, Quinn et al. 2002, Matsuoka, Ballif et al. 2007, Huen, Sy et al. 2010). Best known for its conferred genetic predisposition to early onset breast and ovarian cancer (Kennedy, Quinn et al. 2002), its role in Barrett's oesophagus to the best of our knowledge has never been explored. Our *in-vivo* results demonstrated a significant increase in the expression of BRCA-1 along the metaplasia-dysplasia-adenocarcinoma sequence. BRCA-1 was significantly increased in the dysplastic tissue compared with SIM. BRCA-1 was also over-expressed in oesophageal adenocarcinoma compared with SIM and HGD. In oesophageal squamous cell cancer, studies have shown reduced expression of BRCA-1 was associated with good response rates and increased overall survival to cisplatin, with up regulation of the gene thought to be associated with DNA repair mediated resistance to

the DNA damaging effects of cisplatin (Gao, Zhu et al. 2013). Studies of BRCA-1 deficient mice have demonstrated increased ROS and sensitivity to oxidative stress, along with increased incidences of oesophageal and stomach tumours (Cao, Xu et al. 2007). BRCA-1 deficiencies have also been linked to genomic instability, premature ageing and tumourigenesis (Cao, Li et al. 2003, Cao, Kim et al. 2006). In breast cancer, BRCA-1 plays an important role in protecting against ROS damage and maintaining redox balance (Acharya, Das et al. 2010). BRCA-1 was significantly reduced in our SIM cohort, the environment with increased oxidative stress. BRCA-1 expression was inversely proportional to the level of ROS and other markers of oxidative damage demonstrated in chapter two, and perhaps Barrett's oesophagus development and progression may occur due to deficiencies in BRCA-1 which are required to regulate and repair oxidative damage at the earlier stages of the Barrett's disease sequence.

BRCA-2 demonstrated a stepwise increase in its expression across the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. BRCA-2 was significantly over-expressed in the oesophageal adenocarcinoma biopsies compared with SIM and LGD. Similar to BRCA-1, BRCA-2 is a tumour suppressor gene, involved in the maintenance of genomic stability, through processes of double-strand DNA repair (Foulkes and Shuen 2013). Its role in oesophageal cancer has not been studied before in Barrett's oesophagus. A similar trend of a stepwise increase in gene expression was also repeated for ATM; significantly upregulated in oesophageal adenocarcinoma tissue compared with normal, metaplastic and dysplastic patient biopsies. ATM was also significantly decreased in SIM compared with HGD. ATM encodes for protein kinases and is a regulator of a wide variety of downstream proteins such as p53 and BRCA-1, and is activated in response to double strand breaks (Shiloh and Ziv 2013). Studies have demonstrated that activation of the ATM–signalling pathway has contributed to the suppression of neoplastic transformation in oesophageal and gastric tumours (Cao, Kim et al. 2006). As impaired DNA repair mechanisms are associated with increased mutagenesis, it is fitting that our results from chapter two demonstrate SIM has increased rates of random mutations, while our results in this chapter show SIM is associated with significantly reduced expression of DNA repair genes. Perhaps down-regulation of BRCA-1, BRCA-2 and ATM in the early stages of the disease process may play a role in driving increased mutagenesis.

The expression of OGG-1 and MUTYH were not significantly different across the Barrett's disease sequence. Although the expression of these genes were significantly reduced in the normal squamous epithelium. OGG-1 is involved in base excision repair of 8-oxo-dG (Janssen, Schlink et al. 2001, Elahi, Zheng et al. 2002). MUTYH encodes for a DNA glycosylase involved in oxidative DNA damage repair, and is linked to colorectal cancer (Venesio, Balsamo et al. 2013). Within the Barrett's disease sequence, these genes appear not to impact on cancer progression, although, reduced expression in normal tissue may indicate that Barrett's oesophagus is an environment of genomic instability which requires activation of DNA repair mechanisms, at the earliest stages and throughout the disease sequence.

Once again, we acknowledge certain limitations of our *in-vivo* work. Firstly, controversy surrounds the subjectivity in making a diagnosis of LGD. However, it is accepted that an initial diagnosis with early dysplasia significantly increases the potential for malignant conversion; although, the incidence of cancer progression is variable and ranges in the literature from less than 5% to 28% (Skacel, Petras et al. 2000, Basu, Pick et al. 2004, Sharma, Falk et al. 2006, Wani, Mathur et al. 2009). This variation is probably a reflection of inconsistencies observed in the diagnosis of LGD between specialist gastrointestinal pathologists (Reid, Haggitt et al. 1988, Skacel, Petras et al. 2000, Montgomery, Bronner et al. 2001). Regardless of these difficulties, we have published data, from our Barrett's oesophagus registry, which demonstrates a significantly higher cancer risk ($p<0.0001$) in patients diagnosed with LGD at our centre compared with SIM (Picardo, O'Brien et al. 2014), satisfying us that there are indeed histological differences between patients diagnosed with LGD and SIM at our centre. Interestingly, differences were seen in the expression of BRCA-1, BRCA-2 and ATM between the LGD patients and patients with SIM, HGD and oesophageal adenocarcinoma. There may be a future role for these genes in aiding a specific diagnosis along the Barrett's oesophagus disease spectrum. Secondly, while significant differences were demonstrated in gene expression across the disease sequence, the significance of these results will need to be explored in larger sample sizes in order to determine if any clinical relevance may be extrapolated from these results.

In summary, this chapter has demonstrated that oxidative stress is significantly higher in SIM, with over-expression of CYGB both *in-vitro* and *in-vivo*. DCA caused a significant down-regulation of the CYGB gene in the HET1A cell line, perhaps indicating that DCA initiates Barrett's oesophagus by disturbances in this tumour suppressor gene, impeding a normal reaction to oxidative stress. The expression of CYGB across the Barrett's disease model mirrors the findings of increased ROS, mitochondrial biogenesis and mutagenesis highlighted in the preceding chapters. DNA repair genes, BRCA-1, BRCA-2 and ATM significantly increased in a step-wise manner across the disease sequence. Down-regulation of these genes mirror increased oxidative stress in SIM, and potentially reduced DNA repair correlates with impaired responses to oxidative damage. These findings may play a key role in the development and progression of Barrett's oesophagus, and present a target for further study in risk stratification and therapeutic intervention.

Chapter 6: General Discussion

The mitochondria is an intriguing target in the study of cancer, particularly in inflammation associated cancer, where oxidative stress, cytokines, and energy metabolism are recognised as important emerging hallmarks of cancer (Hanahan and Weinberg 2011). Moreover, metabolic imbalances, such as reduced response to apoptosis and increased glycolysis are all features of cancer cells, and are tightly regulated by the mitochondria (King, Selak et al. 2006, Kroemer 2006, Robey and Hay 2006). The Barrett's disease sequence is a paradigm example of an inflammation associated cancer, ideal for exploring the putative role of the mitochondria and characteristics distinguishing SIM, dysplasia and oesophageal adenocarcinoma. In this Ph.D. thesis, we investigated the role of mitochondria in the development and progression of Barrett's oesophagus. Using *in-vitro*, *in-vivo* and *ex-vivo* models, we measured mitochondrial function, random mitochondrial point mutations, oxidative stress, inflammation and DNA repair genes in order to understand the mechanisms controlling mitochondrial instability across the Barrett's disease sequence.

During tumour development cancer cells are understood to exhibit a mutator phenotype with increased rates of mutagenesis during disease progression (Loeb, Springgate et al. 1974, Goldsby, Lawrence et al. 2001, Loeb, Bielas et al. 2008, Albertson, Ogawa et al. 2009, Loeb 2010). In this theory, benign tumours with low levels of random defects will not progress to malignancy, and the mutator frequency will influence risk. Most studies have focused on nuclear instability, however, mitochondria are more susceptible to instability from oxidative stress and inflammation (Brown, George et al. 1979, Wallace 1992, Wallace 1992, Mecocci, MacGarvey et al. 1993). In the Barrett's oesophagus model, increased random mitochondrial mutations have been reported in SIM compared with adjacent normal tissue (Lee, Han et al. 2012). In chapter two, using a novel RMC assay, mitochondrial deletions, a form of rearrangement of the mitochondrial genome and a recognised marker of mitochondrial instability (Tuppen, Blakely et al. 2010), were significantly increased in SIM compared with HGD/oesophageal adenocarcinoma. An increased frequency of deletions in SIM compared with HGD/oesophageal adenocarcinoma is mirrored in colorectal polyp/cancer studies (Ericson, Kulawiec et al. 2012), both supporting the hypothesis that random mutations/deletions may become redundant as disease progresses. In our study, surrounding normal tissue demonstrated increased deletions compared with areas

of SIM or HGD/adenocarcinoma, suggesting mitochondrial instability is not just confined to the visible site of abnormality in the oesophagus with Barrett's disease, but exerts a field effect, which has been previously demonstrated in colorectal tumours (Ericson, Kulawiec et al. 2012).

Chapter two also demonstrated a significant increase in the frequency of random mitochondrial point mutations in QH cells compared with the latter stages of the Barrett's disease sequence. However, these findings were not reproduced *in-vivo*. We acknowledge limitations with our cell line model. A single line was selected to represent each stage of the Barrett's disease process. Particularly for the SIM studies, we recognise more than one cell line would be more informative, however, only the QHs fit the criteria for functional analyses, while the other available SIM cell line (BAR-T) required feeder layers which prohibited their use in such experiments. In order to overcome this shortcoming, all experiments were repeated using a comparable tissue model from human recruits. *In-vivo*, no difference was demonstrated in random mutations between SIM and oesophageal adenocarcinoma. Instead we merely observed a wide range of heterogeneity in the SIM group, and separated these patients into groups with low and high random mutations for further analysis in chapter four.

Mitochondrial genomic instability was mirrored by functional changes. *In-vitro*, the QH cells demonstrated increased mitochondrial instability through measurements of ROS, mitochondrial membrane potential and mitochondrial mass. *Ex-vivo* studies demonstrated a significant increase in SIM compared with matched normal tissue of cytochrome c and SMAC/Diablo, pro-apoptotic mitochondrial proteins, previously demonstrated in oesophageal cancer cell lines (Aggarwal, Taneja et al. 2000). This suggested an increase in mitochondrial biogenesis. There was a parallel increase in inflammatory cytokines, IL-1β, IL-6, IL-8 and TNF-α, with previous observations from our group demonstrating associations between inflammation and mitochondrial instability (Biniecka, Kennedy et al. 2011). These data reinforce the finding that mitochondrial instability and aligned functional and inflammatory changes are early events in the Barrett's disease sequence.

Chapter three focussed on the role of DCA as a precursor for mitochondrial instability in the Barrett's condition. Minimal positive findings were produced. While 100μM DCA was carefully selected as our treatment dose, the effects were most pronounced in the HET1A cell lines. However, *in-vivo*, 100μM DCA was not associated with any change in mitochondrial biogenesis following 24hr treatment. Also, DCA caused no change in the frequency of random mitochondrial point mutations in any of our Barrett's cell lines. We do recognise that a fixed dose of DCA, for a fixed time of 24hrs, does not ideally represent the *in-vivo* reflux environment. Perhaps, it is DCA at an acidic pH, and at higher concentrations for longer durations, which may affect mitochondrial stability. However, for the purpose of this study, we aimed to use a concentration of DCA which did not cause concurrent cell death, and it was for this reason that this dose was carefully selected. Future experimental designs should take these factors into consideration.

Chapter four measured levels of 8-oxo-dG, 4-HNE and CD3 in tissue samples encompassing the entire Barrett's disease spectrum. 8-oxo-dG demonstrated the most significant results. A marker of oxidative stress, 8-oxo-dG is formed in the presence of excessive ROS production and is associated with increased levels of random mitochondrial point mutations and inflammation (Moriya 1993, Biniecka, Kennedy et al. 2010). Since increased cellular proliferation may increase 8-oxo-dG levels, results were normalised to Ki67 values to eliminate proliferation differences as a confounding variable. Results demonstrated increased levels of 8-oxo-dG in SIM compared with oesophageal adenocarcinoma. Similar findings were demonstrated with 4-HNE and CD3.

Using 8-oxo-dG expression as a marker of oxidative stress, and studying original biopsies from patients diagnosed with Barrett's oesophagus, patients who subsequently progressed to HGD/oesophageal adenocarcinoma were compared with non-progressors. In progressors, 8-oxo-dG was significantly decreased, suggesting that cancer progressors display a mitochondria environment of reduced oxidative stress. This finding, that a marker of mitochondrial instability was decreased in SIM with greater malignant potential, appeared counterintuitive, yet the loss of ROS with disease progression is also consistent with this observation. An explanation may be provided in

a study by Trifunovic *et al.* of mitochondria and ageing, where increased ROS were associated with mitochondrial mutagenesis in the control setting, however, in an environment of enhanced mutagenesis the knock-on effect was disturbed respiratory chain function, defective oxidative phosphorylation and no subsequent increase in the production of ROS (Trifunovic, Wredenberg et al. 2004, Trifunovic 2006). Moreover, the Warburg effect theorizes cancer cells reprogramme their energy metabolism, reducing oxidative phosphorylation and ROS production, potentially decreasing injury to mitochondrial DNA (Warburg 1956, Vander Heiden, Cantley et al. 2009). In this context, the lack of mitochondrial disruption may be a precursor for cancer development, as the normal mitochondrial response, including increased apoptosis, is inhibited, and even at the earliest stages of Barrett's oesophagus, progressors are displaying similarities to the HGD/adenocarcinoma phenotype. Thus, we would suggest, and this requires further study, that progressive disease may be associated with defunct oxidative phosphorylation and likely associated reduced levels of 8-oxo-dG and other oxidative stress markers.

In order to study any potentially predictive value of the previously calculated random mitochondrial point mutations, we correlated the frequency of these mutations with 8-oxo-dG, 4-HNE and CD3. We acknowledge the study numbers were low, however, there was a significant link between high levels of random mutations and decreased levels of 8-oxo-dG (8-oxo-dG was previously shown to be significantly reduced in patients with progressive disease). While only preliminary analysis, this is interesting and may lend itself to future biomarker studies in large patient cohorts.

Chapter five measured changes in gene expression involved in oxidative stress and DNA repair. The oesophagus is redox-sensitive in response to refluxate (Jenkins, D'Souza et al. 2007), but the role of oxidative stress across the Barrett's spectrum is unknown. Mitochondria are the main source of ROS production, with excess levels of ROS associated with oxidative damage (Moriya 1993, Marnett 2000, Raha and Robinson 2000). CYGB, a marker of oxidative stress, was over-expressed in SIM compared with normal, LGD and oesophageal adenocarcinoma, supporting the concept that Barrett's metaplasia is an environment of oxidative stress (Mogal, Watson et al. 2012), likely reflecting the response to the increased mitochondrial biogenesis seen in

SIM. Loss of CYGB expression in the latter stages of the disease potentially may reflect the inability to regulate oxidative stress, and loss of protection once tumour growth is firmly established (McRonald, Liloglou et al. 2006, McRonald, Risk et al. 2012).

DNA repair genes, BRCA-1, BRCA-2 and ATM were seen to significantly increase along the metaplastic-dysplastic-adenocarcinoma sequence. The role of DNA repair genes in the development and progression of Barrett's oesophagus is unknown. BRCA-1 deficiencies have been linked to genomic instability and tumourigenesis (Cao, Li et al. 2003, Cao, Kim et al. 2006), and plays an important role in protecting against ROS damage (Acharya, Das et al. 2010). We demonstrated BRCA-1 was significantly reduced in SIM, the stage with maximum oxidative stress. BRCA-1 expression was inversely proportional to the level of ROS and other markers of oxidative damage demonstrated in chapter two. Perhaps Barrett's oesophagus development and progression may be in part due to deficiencies in BRCA-1 which are required to regulate and repair oxidative damage. By applying a similar rationale, this may explain why an equal trend was seen in the expression of BRCA-2 and ATM in our Barrett's model. However, these findings warrant further study, in order to determine if they play an actual role in disease progression.

In conclusion, this thesis has shown that increased mitochondrial dysfunction, random mitochondrial deletions, mitochondrial protein production, inflammatory cytokines, and oxidative stress, are early events in this inflammation to tumour model, using *in-vitro*, *ex-vivo* and *in-vivo* studies. Although unclear whether mitochondrial dysfunction is the cause or consequence of these events, the studies show for the first time that SIM occurs in an environment of increased oxidative stress and mitochondrial instability. Perhaps the oxidative stress gene, CYGB, and DNA repair genes, BRCA-1, BRCA-2 and ATM play a role in regulating these changes in the Barrett's mitochondrial environment. The paradoxical reduction in a marker of oxidative stress in progressors may represent redundancy of these pathways, and this novel observation requires further testing in large prospective series ideally utilising high quality well characterised bio-resourced biopsy material.

FUTURE DIRECTIONS

A logical extension of this research would be to examine the effects of the other risk factors, such as acidic reflux and obesity, on mitochondrial function. Barrett's oesophagus is a complex entity, whose exact pathogenesis remains unknown. Biliary reflux has been identified as a major risk factor for its development, however, it is unlikely to be the sole instigator of this disease process (Gillen, Keeling et al. 1988, Attwood, Smyrk et al. 1992, Stein, Feussner et al. 1994, Stamp 2002).

The promising findings relating to increased mitochondrial instability in SIM has resulted in a separate four year research project analysing the role of energy metabolism in fuelling Barrett's cancer development (currently been undertaken by James Phelan). This initial work has demonstrated a switch to increased aerobic glycolysis in oesophageal adenocarcinoma compared with increased oxidative phosphorylation and citrate production in SIM, thus supporting the theory of the Warburg effect (Warburg 1956, Warburg 1956). Perhaps the reduced 8-oxo-dG levels demonstrated in this study between Barrett's progressors and non-progressors may be driven by altered energy metabolism, leading to reduced ROS production, however, this remains to be determined.

With respect to the role of random mitochondrial point mutations as a biomarker for predicting Barrett's oesophagus cancer progression (Loeb 2010), a collaborative effort like that led by Professor Rebecca Fitzgerald with the Belfast archived patient samples, will likely to be needed to fully determine the predictive value of random mutations (Bird-Lieberman, Dunn et al. 2012). Only then can the oxidative stress markers, 8-oxo-dG and 4-HNE, along with random mutations be considered for study in a prospective trial.

Our findings of significant alterations in DNA repair genes across the metaplasia-dysplasia-adenocarcinoma sequence offers a new area of exploration in Barrett's oesophagus. To the best of our knowledge, this is one of the first studies to examine the role of BRCA-1 and BRCA-2 in the Barrett's condition. Future directions include analysing the adjacent tissue to determine if these alterations in gene expression are

unique to the pathological tissue or if they are ubiquitously expressed. Other directions include serum analysis, to calculate gene expression across the disease spectrum, as to date, no serum biomarker is available to segregate the different stages of the Barrett's disease sequence.

Appendix One

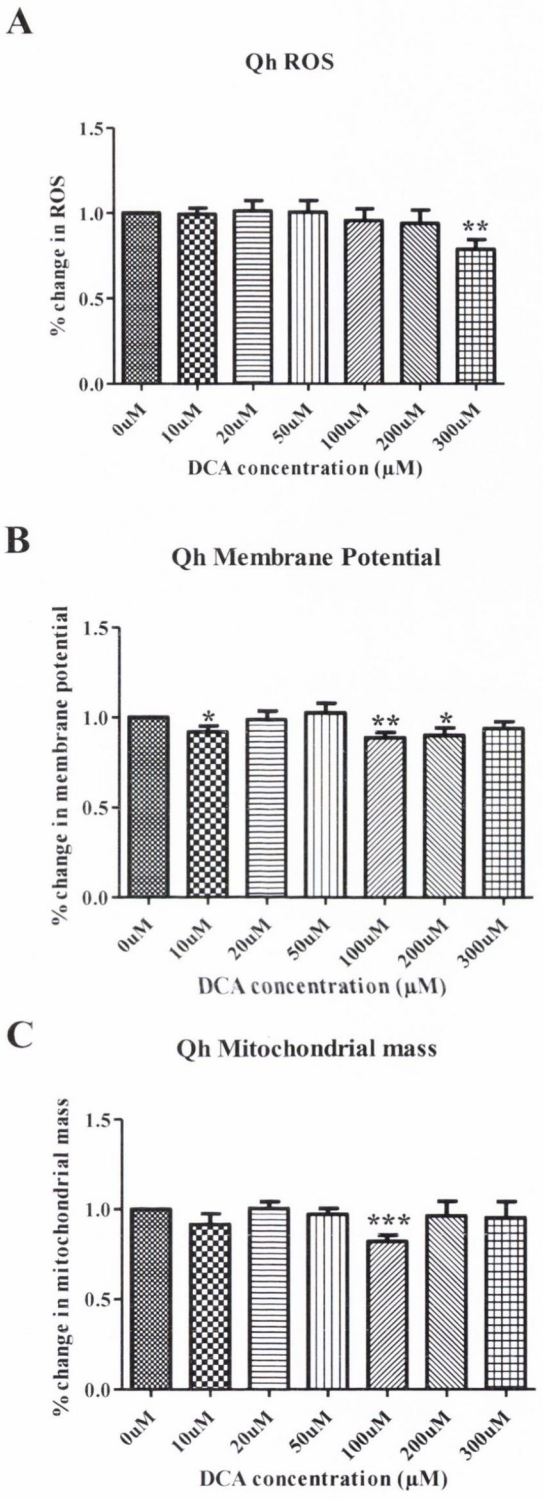


Figure A1.1: Mitochondrial function response to DCA treatment in the QH cell line. (A) ROS significantly decreased following 300µM DCA treatment for 24hrs, this corresponded with concurrent cell death (n=11). (B) MMP significantly decreased following treatment with 10µM, 100µM and 200µM DCA (n=16). (C) Mass significantly reduced following treatment with 100µM DCA (n=16). *p<0.05, **p<0.005, ***p<0.0005

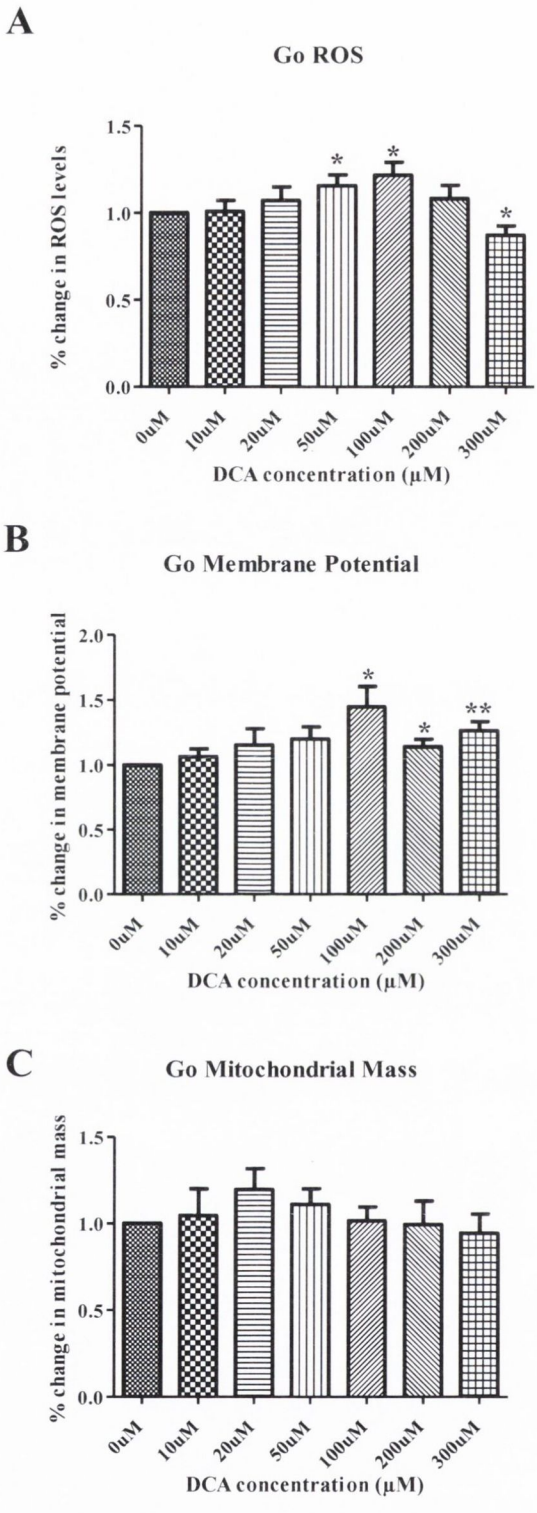


Figure A1.2: Mitochondrial function response to DCA treatment in the Go cell line. (A) ROS significantly increased following 50μM and 100μM DCA treatment, and significantly decreased at 300μM DCA which corresponded with concurrent cell death (n=14). (B) MMP significantly increased following treatment with ≥100μM DCA (n=9). (C) Mass did not change following treatment with DCA (ANOVA p=0.6966) (n=14). *p<0.05, **p<0.005

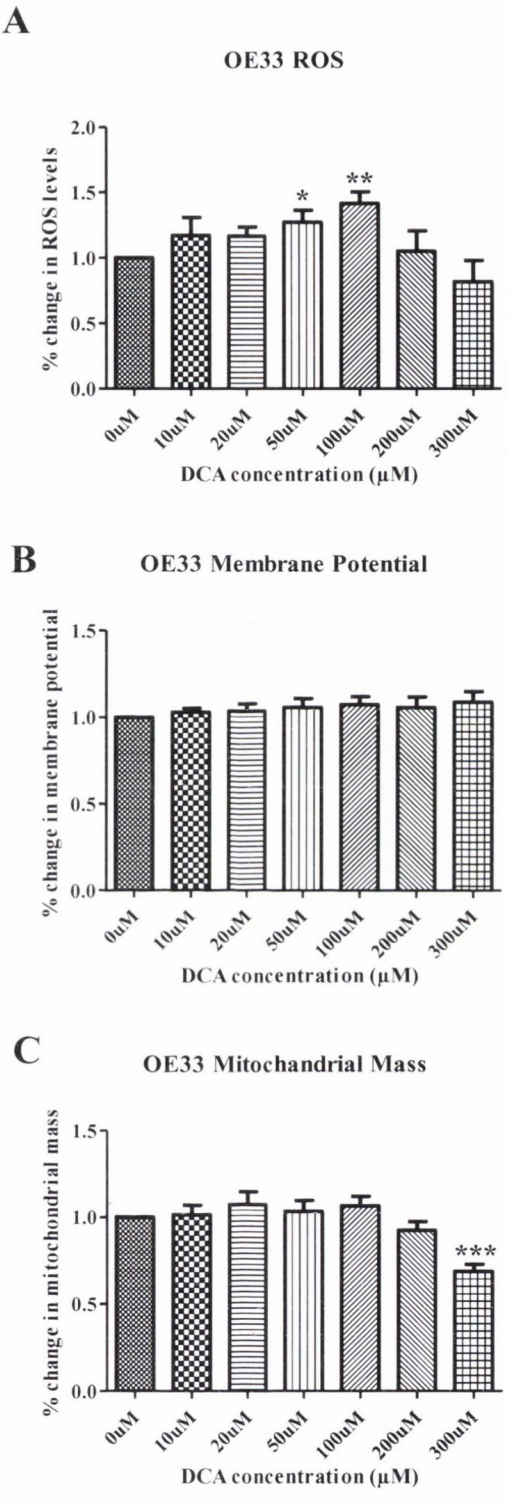
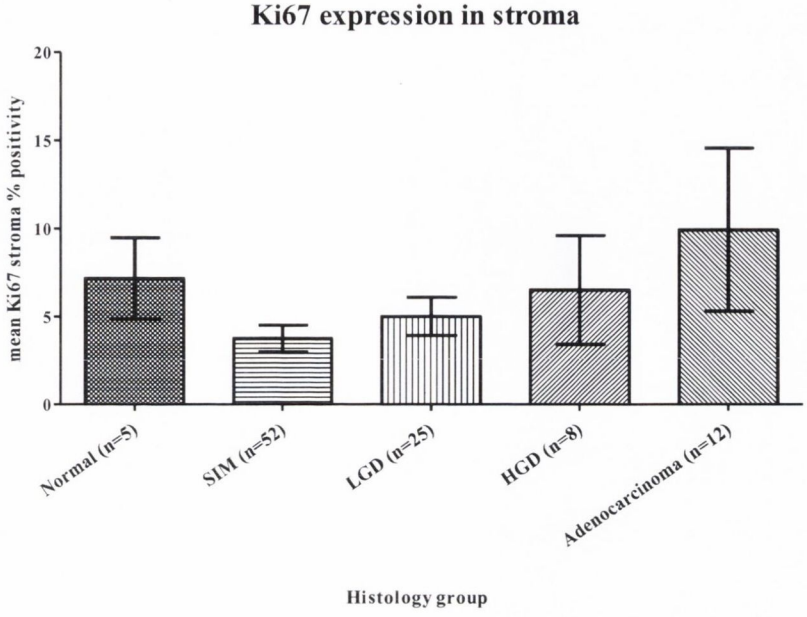


Figure A1.3: Mitochondrial function response to DCA treatment in the OE33 cell line. (A) ROS significantly increased following 50μM and 100μM DCA treatment (n=11). (B) MMP did not significantly change following treatment with DCA (ANOVA p=0.8717) (n=12). (C) Mass reduced with 300μM DCA (n=16). *p<0.05, **p<0.005, ***p<0.0005

Appendix Two

A



B

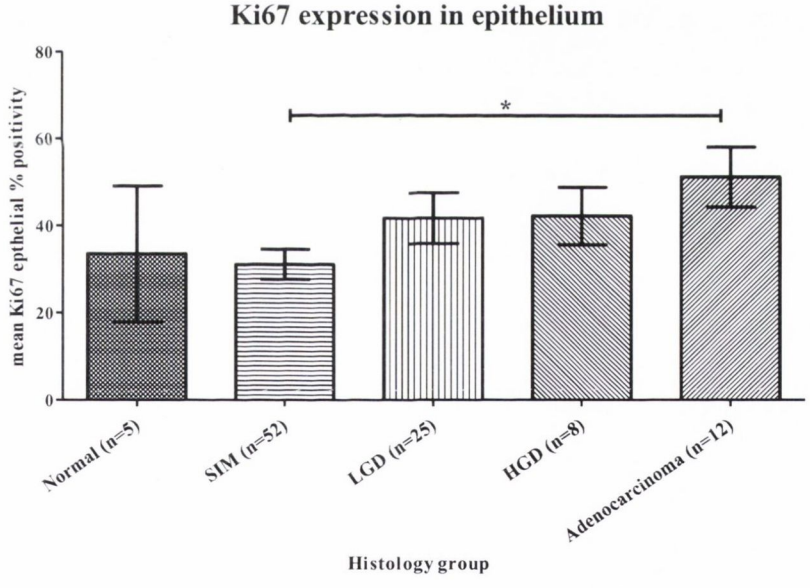


Figure A2.1: Mean Ki67 staining in the Barrett's oesophagus disease spectrum *in-vivo*.
(A) There was no significant difference in the percent of stroma cells positive for Ki67 (p=0.344). However, there was a trend towards a 2.6-fold increase in stroma staining in adenocarcinoma tissue compared with intestinal metaplasia (p=0.093). **(B)** The percentage of positive Ki67 epithelial cells was significantly decreased in intestinal metaplasia compared with oesophageal adenocarcinoma groups. Error bars represent SEM. *$p \leq 0.05$

Mean 8-oxo-dG expression along the Barrett's oesophagus disease sequence

No significant difference was seen in mean 8-oxo-dG percentage positivity or intensity in the stroma or epithelium (Table A2.1). However, as there was a significant difference in mean epithelial Ki67 percentage positivity between SIM and oesophageal adenocarcinoma, mean 8-oxo-dG epithelium percentage positivity results were normalised to mean Ki67 epithelium values to correct for significant differences in proliferation. Following normalisation, mean 8-oxo-dG percentage positivity in epithelial cytoplasm was significantly increased in SIM compared with adenocarcinoma (p=0.01) (Figure A2.2).

Table A2.1: Differences in the mean 8-oxo-dG levels in aged-matched histology groups encompassing the Barrett's disease spectrum

Variable	p value
Stroma	
Cytoplasm intensity	0.562
Cytoplasm % positivity	0.165
Nuclear intensity	0.692
Nuclear % positivity	0.102
Epithelium	
Cytoplasm intensity	0.656
Cytoplasm % positivity	0.586
Nuclear intensity	0.290
Nuclear % positivity	0.791

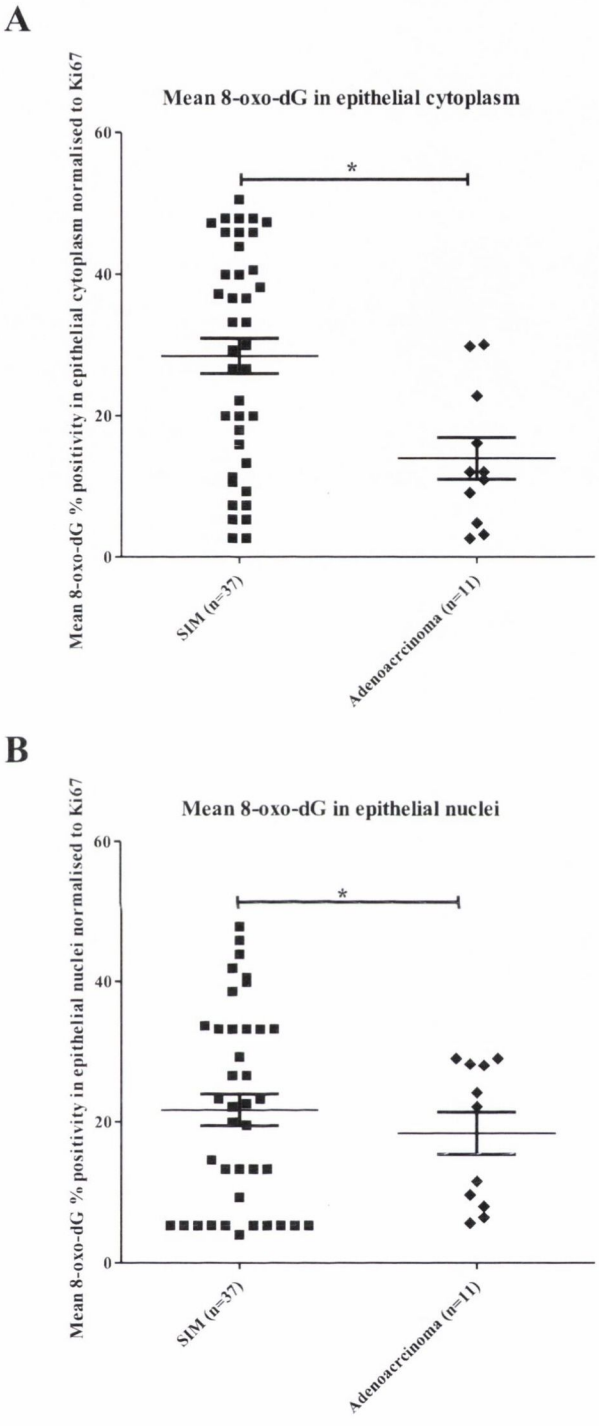


Figure A2.2: Mean epithelial cells positive for 8-oxo-dG, following normalisation with Ki67 percent positivity. There was significantly increased 8-oxo-dG levels in epithelial cytoplasm in in metaplasia compared with adenocarcinoma tissue. *$p<0.05$

Mean 4-HNE expression along the Barrett's oesophagus disease sequence

Results of mean 4-HNE percentage positivity and intensity in the stroma and epithelium are outlined in Table A2.2. Kruskal-Wallis tests demonstrated a significant difference in maximum 4-HNE percentage positivity in epithelium cytoplasm (p=0.018). This followed a similar trend for the maximum 4-HNE results, with significantly increased levels of 4-HNE in oesophageal adenocarcinoma compared with SIM (p=0.010), LGD (p=0.007) and HGD (p=0.009). As there was a significant difference in mean epithelial Ki67 percentage positivity between SIM and oesophageal adenocarcinoma (Figure A2.1), mean 4-HNE epithelium percentage positivity results were normalised to mean Ki67 epithelium values to correct for significant differences in proliferation. Normalisation showed no significant difference in mean 4-HNE percentage positivity in epithelial cytoplasm and nuclei between SIM and oesophageal adenocarcinoma (p=0.210 and p=0.826, respectively) (Figure A2.3).

Table A2.2: Differences in the mean 4-HNE levels in aged-matched histology groups encompassing the Barrett's disease spectrum

Variable	p value
Stroma	
Cytoplasm intensity	0.950
Cytoplasm % positivity	0.224
Nuclear intensity	0.367
Nuclear % positivity	0.349
Epithelium	
Cytoplasm intensity	0.807
Cytoplasm % positivity	**0.018**
Nuclear intensity	0.894
Nuclear % positivity	0.313

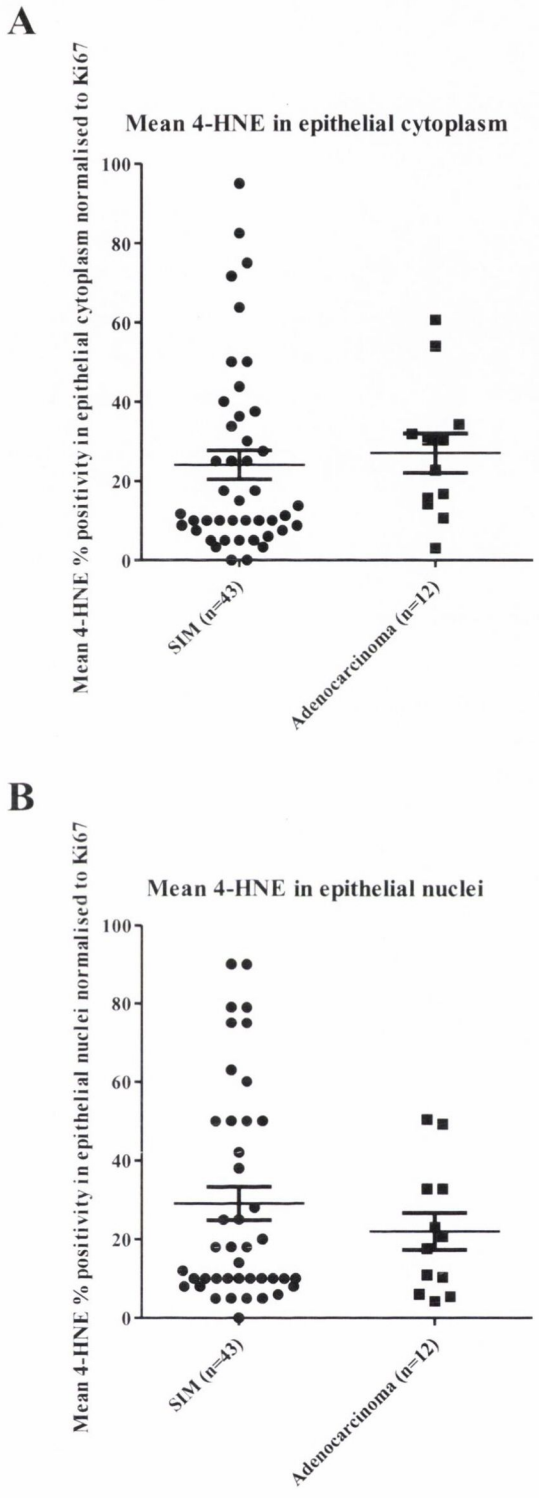


Figure A2.3: Mean epithelial cells positive for 4-HNE, following normalisation with Ki67 percent positivity. There was no significant difference in levels of 4-HNE in **(A) epithelial** cytoplasm (p=0.210) and **(B)** epithelial nuclei (p=0.826).

Mean CD3 expression along the Barrett's oesophagus disease sequence

There was no significant difference in mean CD3 values in the stroma and epithelia across the Barrett's disease sequence (p=0.220 and p=0.432, respectively). Normalisation of mean CD3 epithelial levels demonstrated significantly increased CD3 levels in SIM tissue compared with oesophageal adenocarcinoma (p=0.007).

Differences in mean proliferation (Ki67) between Barrett's oesophagus progressors and non-progressors

There was no significant difference in the mean percent of stroma cells (p=0.186) and epithelial cells (p=0.605) positive for Ki67 between SIM progressors and non-progressors.

Differences in mean 8-oxo-dG expression between Barrett's oesophagus progressors and non-progressors

Differences in mean 8-oxo-dG levels are outlined in Table A2.3.

Table A2.3: Differences in the mean 8-oxo-dG levels between Barrett's oesophagus progressors and non-progressors

Variable	p value
Stroma	
Cytoplasm intensity	0.162
Cytoplasm % positivity	0.208
Nuclear intensity	0.265
Nuclear % positivity	0.101
Epithelium	
Cytoplasm intensity	**0.025***
Cytoplasm % positivity	0.063
Nuclear intensity	0.584
Nuclear % positivity	0.190

*30% of non-progressors had weak staining compared with 80% of progressors

Differences in mean 4-HNE expression between Barrett's oesophagus progressors and non-progressors

There was no difference in the mean expression of 4-HNE seen between progressors and non-progressors (Table A2.4).

Table A2.4: Differences in the mean 4-HNE levels between Barrett's oesophagus progressors and non-progressors

Variable	p value
Stroma	
Cytoplasm intensity	0.250
Cytoplasm % positivity	0.769
Nuclear intensity	0.113
Nuclear % positivity	0.936
Epithelium	
Cytoplasm intensity	0.610
Cytoplasm % positivity	0.880
Nuclear intensity	0.328
Nuclear % positivity	0.381

Differences in mean CD3 expression between Barrett's oesophagus progressors and non-progressors

No difference was demonstrated in the mean CD3 levels in the stroma (p=0.440) and epithelium (p=0.480) between progressive and non-progressive patients.

Correlation between mean 8-oxo-dG and random mitochondrial point mutations

There was no significant correlations demonstrated between the mean 8-oxo-dG values and the frequency of random mitochondrial point mutations (Table A2.5 and A2.6).

Table A2.5: Correlations between 8-oxo-dG mean values and random mitochondrial point mutations in patients with SIM.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.393	0.219
Nuclear intensity	0.207	0.581
Cytoplasm % positivity	-0.350	0.389
Cytoplasm intensity	0.207	0.581
Epithelium		
Nuclear % positivity	0.087	0.843
Nuclear intensity	-0.275	0.474
Cytoplasm % positivity	0.227	0.552
Cytoplasm intensity	0.040	0.912

Table A2.6: Correlations between mean 8-oxo-dG expression and frequency of random mitochondrial point mutations in oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.151	0.783
Nuclear intensity	0.060	0.906
Cytoplasm % positivity	-0.075	0.906
Cytoplasm intensity	-0.180	0.713
Epithelium		
Nuclear % positivity	0.248	0.595
Nuclear intensity	-0.139	0.609
Cytoplasm % positivity	0.569	0.200
Cytoplasm intensity	0.502	0.267

Correlation between mean 4-HNE and random mitochondrial point mutations

The relationship between these two variables are outlined in Table A2.7 and A2.8.

Table A2.7: Correlation between mean 4-HNE expression and frequency of random mitochondrial point mutations in intestinal metaplasia patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	0.281	0.436
Nuclear intensity	**0.780**	**0.017**
Cytoplasm % positivity	0.490	0.178
Cytoplasm intensity	0.486	0.194
Epithelium		
Nuclear % positivity	0.086	0.843
Nuclear intensity	-0.060	0.880
Cytoplasm % positivity	0.102	0.810
Cytoplasm intensity	0.158	0.678

Table A2.8: Correlation between mean 4-HNE expression and frequency of random mitochondrial point mutations in oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Stroma		
Nuclear % positivity	-0.395	0.517
Nuclear intensity	0.553	0.350
Cytoplasm % positivity	-0.0205	0.783
Cytoplasm intensity	-0.369	0.517
Epithelium		
Nuclear % positivity	-0.344	0.517
Nuclear intensity	-0.444	0.450
Cytoplasm % positivity	-0.026	0.950
Cytoplasm intensity	-0.290	0.683

Correlation between mean CD3 and random mitochondrial point mutations

There was no significant relationship identified between the mutation frequencies and mean CD3 expression in either patients with SIM or oesophageal adenocarcinoma (Table A2.9).

Table A2.9: Correlation between mean CD3 expression and frequency of random mitochondrial point mutations in intestinal metaplasia and oesophageal adenocarcinoma patients.

Variable	Correlation (Spearman rho)	p value
Intestinal Metaplasia		
Stroma % positivity	0.2684	0.5008
Epithelium % positivity	0.1764	0.6646
Oesophageal adenocarcinoma		
Stroma % positivity	-0.2582	0.7500
Epithelium % positivity	0.0000	1.0000

Appendix Three

Table A3.1: List of antioxidant and oxidative stress genes analysed and the expression of these genes following treatment with 100μM DCA in the QH cells, relative to the untreated cells.

Target gene	Fold change following DCA treatment
GSR	-1.1908
GPX3	-1.1899
PRDX2	-1.1324
PTGS1	-1.1203
SCARA3	-1.1027
B2M	-1.0915
CYGB	-1.0905
CSDE1	-1.0777
PRDX3	-1.072
GLRX2	-1.0655
NUDT1	-1.065
GPX1	-1.0533
GSTZ1	-1.053
RNF7	-1.0456
HPRT1	-1.0438
TXNRD2	-1.0435
MPV17	-1.0334
SOD1	-1.0263
SRXN1	-1.0147
CCS	-1.01
GTF2I	-1.0085
PRDX1	-1.0065
CAT	-1.0028
PPC	1.0072
MTL5	1.0143
ATOX1	1.015
PDLIM1	1.0176
TTN	1.026
ACTB	1.0309
NME5	1.0318
PRDX4	1.0326
RTC	1.0382
GAPDH	1.0407
FOXM1	1.0483
SIRT2	1.0484
CYBA	1.051
STK25	1.0512
PRNP	1.058
RTC	1.0591
PNKP	1.0722
PRDX6	1.0735
PRDX5	1.0778
DUOX1	1.0789

DUSP1	1.0794
MGST3	1.0857
RPL13A	1.0915
PRG3	1.0917
GSS	1.0942
OXSR1	1.0957
ALOX12	1.1
NCF2	1.1122
PPC	1.1126
RTC	1.1138
AOX1	1.1219
TPO	1.1241
GPX4	1.1244
OXR1	1.1244
GPX2	1.1246
TXNRD1	1.1255
MSRA	1.1298
PTGS2	1.1303
SELS	1.1337
PPC	1.1381
PXDNL	1.1414
SOD2	1.151
ALB	1.1752
HGDC	1.1768
PREX1	1.1913
GPX6	1.1932
ANGPTL7	1.2
GPR156	1.2037
CCL5	1.2082
MT3	1.2295
EPHX2	1.2312
LPO	1.2547
NOX5	1.2547
SOD3	1.267
MBL2	1.2671
BNIP3	1.2688
PXDN	1.2725
NCF1	1.2907
GPX5	1.2988
EPX	1.3157
DHCR24	1.3168
SEPP1	1.3176
DUOX2	1.3201
APOE	1.3264
SGK2	1.37
KRT1	1.3785
MPO	1.4068

TXNDC2	1.4091
SFTPD	1.4127
GPX7	1.4373
NOS2	1.4991
DGKK	1.5083
IPCEF1	1.5733

Table A3.2: List of antioxidant and oxidative stress genes analysed and the expression of these genes following treatment with 1mM DMOG in the QH cells, relative to the untreated cells.

Target gene	Fold change following DMOG treatment
CYGB	-13.0836
FOXM1	-6.3656
DHCR24	-4.08
HPRT1	-3.8858
GLRX2	-3.7873
PRDX3	-3.2817
PRNP	-2.8043
PRDX1	-2.7412
GTF2I	-2.7097
RTC	-2.547
ACTB	-2.5445
RTC	-2.4477
RTC	-2.4284
GSS	-2.3882
SRXN1	-2.3616
ATOX1	-2.3013
AOX1	-2.2934
MTL5	-2.2896
NUDT1	-2.2782
PNKP	-2.2151
TXNRD2	-2.0699
PRDX6	-2.0487
CSDE1	-1.9466
SELS	-1.9428
STK25	-1.8707
MPV17	-1.8521
PRDX2	-1.8349
SOD2	-1.8273
PRDX4	-1.8101
CYBA	-1.774
NCF2	-1.6327
OXSR1	-1.6228
SOD1	-1.5615
PTGS2	-1.4961
GPX1	-1.4771
PRDX5	-1.4402
RNF7	-1.3426
GSR	-1.3285
OXR1	-1.3023
SIRT2	-1.2937
GPX3	-1.2346
CCS	-1.2101
SCARA3	-1.1736

PDLIM1	-1.1322
PPC	-1.1281
SFTPD	-1.119
DUOX1	-1.0945
B2M	-1.064
MSRA	-1.0261
PPC	-1.0243
CAT	1.0261
GPR156	1.0294
PPC	1.0343
GPX2	1.0429
ALOX12	1.0491
RPL13A	1.064
GSTZ1	1.0777
GPX4	1.1755
TXNRD1	1.1756
NOX5	1.1991
PREX1	1.2101
PRG3	1.2427
IPCEF1	1.2571
NME5	1.2654
ANGPTL7	1.2676
SEPP1	1.2679
BNIP3	1.293
TXNDC2	1.3048
APOE	1.3199
NCF1	1.3351
TTN	1.3374
PXDNL	1.3538
MGST3	1.3675
GPX5	1.4385
GPX6	1.4547
TPO	1.5069
MT3	1.5584
KRT1	1.5831
CCL5	1.6294
DUOX2	1.6679
ALB	1.7147
SOD3	1.7521
NOS2	1.7593
SGK2	1.8474
EPX	1.8776
GPX7	2.108
EPHX2	2.1366
HGDC	2.137
LPO	2.1679
PXDN	2.2375

MBL2	2.3074
GAPDH	2.4547
DGKK	2.6065
MPO	2.7122
DUSP1	3.9252
PTGS1	6.6937

Table A3.3: List of antioxidant and oxidative stress genes analysed and the expression of these genes following treatment with 100μM DCA + 1mM DMOG in the QH cells, relative to the untreated cells.

Target gene	Fold change following DCA+DMOG treatment
FOXM1	-14.1088
CYGB	-12.4882
GLRX2	-5.3556
HPRT1	-4.819
PRDX3	-4.6452
NUDT1	-4.0484
PRDX1	-3.8872
ATOX1	-3.8407
DHCR24	-3.4113
PRDX2	-3.2455
MTL5	-3.0165
PRNP	-3.0151
GSS	-2.9804
ACTB	-2.8873
SOD1	-2.8287
SRXN1	-2.7148
PRDX4	-2.6921
PRDX6	-2.6507
TXNRD2	-2.6305
AOX1	-2.6289
GTF2I	-2.5367
MPV17	-2.4934
PNKP	-2.3442
STK25	-2.3061
GPX3	-2.2739
PRDX5	-2.1796
NCF2	-2.0387
CYBA	-1.7274
CSDE1	-1.72
PTGS2	-1.6993
SELS	-1.6489
GPX1	-1.6273
RNF7	-1.6087
CCS	-1.5056
OXSR1	-1.4736
GSR	-1.4224
SOD2	-1.3728
OXR1	-1.363
SIRT2	-1.3377
RTC	-1.2818
PPC	-1.2595
RTC	-1.2347

PPC	-1.1885
PPC	-1.1808
RTC	-1.1805
PDLIM1	-1.1572
MSRA	-1.0757
B2M	-1.0595
GSTZ1	-1.0473
GPX4	-1.0375
SFTPD	-1.0294
DUOX1	-1.0257
TXNRD1	1.0166
CAT	1.02
MGST3	1.0355
RPL13A	1.0595
NME5	1.0784
GPX2	1.0922
NOX5	1.2797
GPR156	1.2981
SCARA3	1.3241
ANGPTL7	1.3506
TTN	1.3644
ALOX12	1.4175
BNIP3	1.4716
EPX	1.4815
SOD3	1.5379
NCF1	1.5666
APOE	1.5843
MBL2	1.5851
TXNDC2	1.6015
PRG3	1.6329
PREX1	1.6834
PXDNL	1.6856
LPO	1.6953
SEPP1	1.715
KRT1	1.7343
HGDC	1.7385
TPO	1.74
MT3	1.7446
IPCEF1	1.7616
GPX6	1.8189
GPX5	1.8559
PXDN	1.858
EPHX2	1.8619
ALB	1.8662
GAPDH	1.8745
MPO	1.8773
DGKK	1.9644

NOS2	1.9862
GPX7	2.0409
CCL5	2.1181
DUOX2	2.2074
SGK2	2.2946
DUSP1	3.9104
PTGS1	6.2851

Table A3.4: List of DNA repair genes analysed and the expression of these genes across the Barrett's oesophagus cell line model, relative to the Het1a, control, cell line.

Target Genes	HET1a (control)	QH	Go	OE33
MGMT	1	0	-5.479115479	0
MSH4	1	0	0	0
BRCA2	1	-85.36040609	-37.83127109	-47.67115521
MSH6	1	-55.98866052	-19.0821256	-9.717097171
MPG	1	-9.772727273	-5.472727273	-1.36199095
ERCC3	1	-9.567357486	0	-10.75667262
RAD21	1	-9.461697723	-17.50957854	-3.067114094
XAB2	1	-9.362373737	-11.80732484	-3.861979167
ATXN3	1	-8.406862745	-5.220700152	-27.85221275
NEIL1	1	-8.358208955	-1.914529915	1.225446429
MUTYH	1	-7.346534653	-23.73134328	-7.753396029
CCNO	1	-6.442996743	-58.52071006	1.246208291
TOP3B	1	-6.343355965	-1.37394958	-2.43575419
MLH3	1	-6.325242718	-11.25215889	-8.9124487
RAD54L	1	-5.199216199	-1.950980392	-3.146245059
UNG	1	-4.841201717	-1.662082515	-1.755186722
MSH2	1	-4.449458484	-3.390646492	-5.135416667
NTHL1	1	-4.440965187	-7.534375	10.86395686
FEN1	1	-4.268551237	-2.745454545	-2.885350318
LIG1	1	-4.217118998	-7.426470588	-1.582245431
OGG1	1	-4.108416548	-1.694117647	1.236111111
ERCC4	1	-3.712374582	-5.170807453	-7.10021322
ATM	1	-3.711180124	-1.639231824	-5.322939866
TOP3A	1	-3.512915129	-3.260273973	-2.45994832
XRCC3	1	-3.242857143	-3.58044164	-1.769290725
POLD3	1	-2.994241843	-1.30726257	-1.864541833
MRE11A	1	-2.869897959	-2.205882353	-1.949740035
BRIP1	1	-2.775449102	-1.519672131	-3.213171577
PARP2	1	-2.639344262	1.178571429	-1.98459168
CCNH	1	-2.568946797	-2.770491803	-3.840909091
NEIL2	1	-2.429961089	-1.565162907	11.78542834
RPA1	1	-2.408872902	1.244732039	-8.336099585
RAD51C	1	-2.314211213	1.067042254	-2.085781434
MSH3	1	-2.253505933	-6.368902439	-4.726244344
ATR	1	-2.167689162	-2.161060143	-2.267379679
RAD51L3	1	-2.053370787	-2.030555556	1.253077975
PMS1	1	-2.04109589	1.845637584	-1.087591241
PARP1	1	-2.022485207	-1.067457839	-3.507439713
EXO1	1	-1.97242842	-3.821917808	-1.897959184

ERCC2	1	-1.961038961	-1.808383234	-1.455421687
MLH1	1	-1.959150327	-1.561197917	-1.2002002
AC103686.3	1	-1.951919627	-1.622911695	-3.087400681
RAD18	1	-1.933993399	-2.413509061	-1.883033419
TREX1	1	-1.884758364	-1.377717391	-2.181583477
NEIL3	1	-1.780392157	-2.764920828	-7.604690117
DMC1	1	-1.752332485	3.388189739	-1.038190955
RAD23A	1	-1.676171079	-1.19622093	1.309842041
XPA	1	-1.629310345	-1.442748092	-1.58490566
XRCC6BP1	1	-1.584444444	2.266479663	-1.233564014
ERCC8	1	-1.578947368	-2.003610108	-3.384146341
PNKP	1	-1.553398058	-2.081784387	2.183035714
RPA3	1	-1.535471698	-1.699665831	-1.425218914
SLK	1	-1.503009458	-2.765822785	-1.39061257
RAD50	1	-1.482021886	-1.037956204	-4.601941748
RFC1	1	-1.282002535	-2.396919431	-2.948979592
XRCC2	1	-1.279620853	2.185185185	-2.151394422
XRCC5	1	-1.186674217	-0.650155119	-1.693239968
TDG	1	-1.174338086	-1.826996198	-1.696880518
RAD51L1	1	-1.170896785	-1.123376623	-1.541202673
XRCC6	1	-1.135072909	-1.322898032	1.452332657
XPC	1	-1.105341246	-2.604895105	-2.577854671
XRCC4	1	-1.009940358	-1.668856767	2.082677165
POLB	1	1.005773672	2.193995381	4.792147806
RAD52	1	1.008888889	1.008888889	1.017777778
POLL	1	1.034090909	-2.784810127	-1.205479452
APEX2	1	1.111888112	1.477855478	-1.245283019
LIG3	1	1.15008881	1.03374778	3.090586146
RAD23B	1	1.15434986	-1.122309711	-1.529874776
XRCC1	1	1.223396999	0.847885402	-1.060014461
DDB1	1	1.2402746	1.764975098	1.016287522
LIG4	1	1.361179361	-3.815625	-4.710648148
ERCC6	1	1.404181185	-1.470286885	-1.551351351
MMS19	1	1.44461305	-1.176785714	1.153262519
CDK7	1	1.457749879	-1.683739837	1.60791888
ERCC1	1	1.559177533	-2.250564334	-1.4212402
MSH5	1	1.614237288	-1.407442748	-1.038001407
PARP3	1	1.623348018	3.348017621	2.577092511
ERCC5	1	1.77259887	2.556497175	1.408898305
RAD51	1	2.007005254	4.746059545	3.893169877
SMUG	1	2.395833333	1.510416667	2.3125

PMS2	1	3.041700736	2.273098937	1.100572363
APEX1	1	6.376805613	11.46512588	8.377631036
DDB2	1	12.83659379	-1.206944444	3.970080552
BRCA1	1	913.4313725	15032679.74	0

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