A ROLE FOR IRON IN OESOPHAGEAL ADENOCARCINOMA

by

JOHN KEITH ROBERTS

A thesis submitted to
The University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

School of Cancer Sciences
The University of Birmingham
October 2010

ABSTRACT

In Western populations the incidence of oesophageal adenocarcinoma (OAC) is increasing greater than any other malignant disease. Increasing epidemiological and experimental evidence associates iron with OAC. Our understanding of iron physiology has increased enormously in recent years due to the elucidation of transport proteins and regulatory pathways. Haem scavenging and metabolism remains to be clearly characterised although candidate import proteins have been identified. This project aimed to characterise the iron transport machinery in the progression of OAC and the effects of iron exposure *in-vitro*.

Overexpression of iron and haem import proteins, with a repression of iron export, was identified in the progression of Barrett's metaplasia to OAC suggesting a role for iron in disease progression. Interestingly expression of hepcidin, the hepatic peptide responsible for systemic control of iron, was identified in samples of OAC. Culturing OAC cells with iron or haem increased cell proliferation, migration and anchorage independent growth. These effects were inhibited by the addition of alginate, a naturally occurring chelator. Knockdown of LRP-1, the prime haem import candidate, confirmed the role of this transporter.

Understanding the differential expression of these proteins between benign and malignant tissue may permit novel therapeutic strategies in the future.

DEDICATION

This work is dedicated to my parents. Without their support and hard work (at times this cannot be overestimated) I would have never studied medicine. My career has followed from that point and, looking at the achievements and high points, it is clear that these have only been possible due the opportunity given to me by them.

ACKNOWLEDGEMENTS

I offer sincere thanks to Bob Spychal for approaching me before this project began for his support and direction during the period of research. The project would not have been possible without the effort, dedication and intellect of Chris Tselepis, my supervisor and friend. Thanks to those that offered intellectual support and friendship in the lab: Matt, Jess, John, Sharon and more recently Sam 'Flashman' Ford (in whose, more talented, hands this research continues).

Lewis Stevens completed his BSc during this project and contributed to some of the experiments using haem. His enthusiasm and hard work were an asset to the group.

Professor Derek Alderson and Mike Hallissey kindly offered support and guidance during this period.

FIGURES

TABLE

APPENDICES

ABBREVIATIONS

ABCG2	ATP binding cassette G2
APC	adenomatous polyposis coli
BCRP	breast cancer related protein
BM	Barrett's metaplasia
DCC	deleted in colorectal cancer
Dcytb	duodenal cytochrome B
DFO	desferoxamine
DMT1	divalent metal transporter
DNA	deoxyribonucleic acid
FLVCR	feline leukaemic virus receptor
FPN	ferroportin
GORD	gastro-oesophageal reflux disease
HCP-1	haem carrier protein 1
HGD	high grade dysplasia
HFE	haemochromatosis gene
HJV	haemojevulin
HH	hereditary haemochromatosis
HO-1	haem oxygenase 1
HP	haptoglobin
HPX	haemopexin
IRE	iron response element
IRP	iron responsive protein
kDa	kilo Dalton
LGD	low grade dysplasia
LRP-1	LDL receptor-related protein (CD91, LRP-1)
mRNA	messenger ribonucleic acid
OAC	oesophageal adenocarcinoma
OR	odds ratio
RNA	ribonucleic acid
ROS	reactive oxygen species
SSC	(oesophageal) stratified squamous carcinoma
Tf	transferrin
TfR1	transferrin receptor 1
TfR2	transferrin receptor 2
UTR	untranslated region
ZnPP	zinc protoporphyrin

CHAPTER 1: INTRODUCTION

1.1 Oesophageal adenocarcinoma

There are two histological forms of oesophageal cancer – stratified squamous carcinoma (SSC) and oesophageal adenocarcinoma (OAC). The incidence of OAC is increasing greater than any other malignant disease in Western populations[1] and is consequently the focus of intense research into disease aetiology and treatment[2]. Unfortunately the diagnosis is often made at a late stage when three quarters of patients cannot receive potentially curative treatment[1]. Of the minority that undergo surgery the 30 day mortality is 5-10% and less than 30% of this group or 10% of the total will survive five years[1] (Fig 1). Morbidity due to surgery is considerable with quality of life scores lower than pre-operative scores three years following surgery[3]. There have been improvements with overall 5-year survival improved from 3% 30 years ago to 10%-15% reported in recent series[4;5]. Advances in adjuvant therapies, in combination with potentially curative surgery, partly explain the slight improvement[1]. However the vast majority of patients still die from their disease and consequently it is of immense importance that the cellular events leading to progression of normal oesophageal mucosa to invasive adenocarcinoma are understood. It would be hoped that this would lead to novel therapeutic strategies where side effects, complications and mortality associated with existing forms of treatment can be avoided.

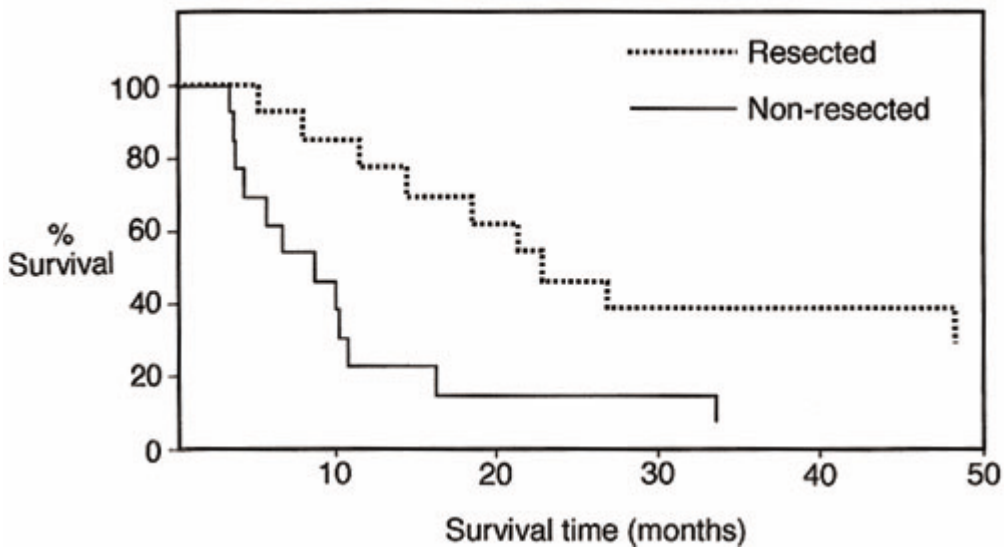


Figure 1. Medium term survival of patients with oesophageal adenocarcinoma (Park J R Coll Surg Ed; James IV lecture 2002[6])

Surgical resection offers the only chance of cure following a diagnosis of OAC. Patients with unresectable disease invariably die within three years of diagnosis although most within one year.

1.2 Epidemiology

The incidence of OAC is increasing at a rate greater than other malignant diseases in Western populations[7] (fig 2). This trend is a huge concern given the rate of increase, the poor prognosis and the lack of understanding behind this increase. The rising incidence contrasts with that of SSC which is decreasing in the same populations[8].

For the majority of the twentieth century OAC was a rare disease. Between 1926 and 1976 three large surgical series reported that 0.8-3.7% of oesophageal tumours were adenocarcinomas[9-11]. During the last quarter of the twentieth century there was approximately a 350% increase in OAC incidence[8]. This contrasts with the experience in the Far East where no increase in OAC has been observed and SSC continues to be the more common histological type[12]. In Western populations recent publications demonstrate that 30-70% of all oesophageal carcinomas are adenocarcinoma (United States[13;14], United Kingdom[15] and Switzerland[16]) with white males predominantly at risk[17]. The current rise in incidence of OAC is centred on the UK (5–8.7/100 000), over double that of the USA (3.7/100 0000). There are variations within Western populations with large rises in incidence, up to 30% per year, observed in West European white men, a rise not mirrored in Eastern Europe[18;19].

Worldwide the incidence of SSC varies considerably and also, to some extent, among ethnic groups within a common area. Some of the highest rates occur in northern China[20] and northern Iran[21], where incidence exceeds 100 in 100,000 individuals. In the U.K. (where the rate is the highest of any Western country), the incidence is less than 10 per 100,000. An individual's racial background appears important as SSC predominates in African Americans over Caucasians by a ratio of 6:1, and OAC has the opposite preponderance, occurring in Caucasians over African Americans at a ratio of 4:1[7].

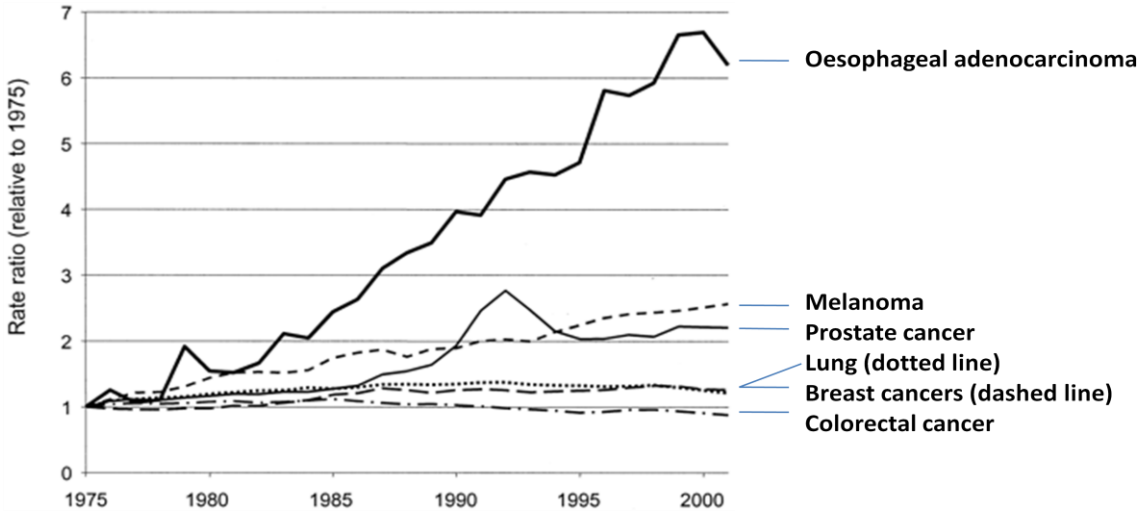


Figure 2. Change in the incidence of oesophageal adenocarcinoma relative to other malignant diseases (1975–2001) (adapted from Pohl *et al*. J Natl Cancer Inst 2005[22]).

Data from the US National Cancer Institute. The baseline was the average incidence between 1973 and 1975.

1.3 Risk factors for oesophageal tumours and the increasing incidence of oesophageal adenocarcinoma

Tobacco and alcohol, which have a synergistic effect, are very strong risk factors for SSC and moderate risk factors for OAC[23]. Tobacco exposure has been linked to a tenfold higher risk for SSC in heavy smokers relative to non-smokers with the risk related to the duration of exposure[24;25]. In contrast, smoking has been linked to only a two- to threefold greater risk for OAC in smokers relative to nonsmokers[8;26]. The relative risk of OAC remains high up to 30 years after smoking cessation, however, this contrasts to the significant decline in risk of SSC within a decade of smoking cessation[27]. There is a

greater than additive effect between smoking and alcohol consumption in SSC whilst the effect is additive in for OAC[28]. The exception to this may be the consumption of hard liquor, which has been found to have a stronger association with OAC than with SSC[29].

In Linxian, China, SSC is endemic and has been directly related to dietary nitrosamines and inversely related to consumption of riboflavin, nicotinic acid, magnesium, and zinc[30]. Nitrosamines are highly carcinogenic and are formed from certain food preservatives (nitrates and nitrites) widely used in the Far East. In the Western world there is less impact from dietary factors due to different food preservation techniques[30].

The most important epidemiological difference between SSC and OAC is the strong association between gastro-oesophageal reflux disease (GORD) and OAC[31]. The results of a population-based case controlled study suggest that symptomatic GORD is a risk factor for OAC[32]. The frequency, severity, and duration of reflux symptoms have been positively associated with increased risk of OAC[32-34].

Long-standing GORD predisposes to Barrett's metaplasia (BM)[31]. BM has been identified as the most significant risk factor for subsequent development of OAC[31].

1.4 Barrett's oesophagus and gastro-oesophageal reflux

Norman Barrett described the condition that bears his name in 1950 believing the oesophagus to be congenitally short[35]. The squamous epithelium of the distal oesophagus undergoes metaplastic change to become an intestinal type epithelium

with goblet cells. The goblet cells are different from those found in the stomach and on histological examination stain differently, allowing differentiation between BM and normal gastric epithelia[36]. BM is the most significant risk factor for the development of OAC[37] with an individual's relative risk of developing OAC 30 to 120 times greater when compared with those without BM[32;38;39]. Dysplasia in BM represents an alteration of the epithelium that heralds progression to invasive OAC[40].

Although the link between BM and OAC is clear the actual risk to the patient with BM is not as dramatic as one might expect: OAC develops in 0.5-1% of patients with BM per year[41-43]. A recent meta-analysis of the larger longitudinal studies identified an annual risk of 0.5% progression to OAC per year[44]. The authors of this study believe that publication bias may artificially elevate cancer risk with small studies being published due to the reported, eye catching, high estimates of cancer incidence[44].

It is likely that the metaplasia that characterises Barrett's oesophagus occurs in response to reflux of bile and acid into the oesophagus[32]. It is estimated that 5-15% of patients with GORD will have BM and that patients with long-standing GORD are at greatest risk for developing BM[32;45].

Due to the rare nature of OAC there are no prospective cohort studies of reflux patients to assess cancer risk. However, three case-control studies have examined reflux symptoms as a risk factor for the development of OAC. In Sweden, Lagergren *et al* performed the largest of these studies - a population based case-control study examining the relationship between reflux symptoms and OAC[32]. Control subjects were age and sex matched by using a national registry. Case and control subjects were

questioned about the frequency and severity of any reflux symptoms they experienced within the previous five years. After multiple risk factors for cancer were controlled for, subjects with OAC were eight times as likely to report at least weekly symptoms of reflux or regurgitation than were control subjects (95% confidence interval [CI], 5.3-11.4). Furthermore, a dose-response relationship existed. If an individual's reflux symptoms were long-term (>20 years) and severe (defined by a scale of frequency and severity of symptoms) the adjusted odds ratio (OR) for OAC was 43.5 (compared to subjects with short term and minor symptoms; 95% CI 18.3-103.5). As recall bias might explain the observed association between reflux symptoms and OAC a second group with SSC were also studied. No association was found between reflux symptoms and SSC risk[32].

A second study compared 196 patients with OAC from 1986 to 1992 with controls matched for age and sex[45]. Medical records were reviewed for a documented history of reflux disease, hiatal hernia, oesophagitis, and dysphagia. After relevant risk factors were adjusted for, patients with OAC were at least twice as likely as control subjects to have documentation of one of these conditions. A third population-based case-control study using United States tumour registries studied 293 patients with OAC and 695 control subjects[46]. This study also demonstrated a dose-response relationship between frequency of reflux symptoms and risk of OAC. The adjusted odds ratio (OR) for cancer of individuals experiencing at least daily reflux was 5.5 (95% CI, 3.2-9.3).

Although these studies report eye-catching results for relative risk (RR), it is the absolute risk that more accurately describes any individual's chance of getting cancer[44]. The most common clinical manifestation of GORD is heartburn; 44% of adults in the United States experience heartburn at least once a month.

As the number of individuals with reflux symptoms is high and because the incidence of OAC is so low, by necessity the absolute risk to the average person with reflux is low[44]. In a review of GORD and Barrett's disease Shaheen *et al* argues that this absolute risk is very low[44]. In the USA 14% of the over 50 population experience weekly symptoms of gastroesophageal reflux (approximately 10 million people)[47]. Of these 6500 will develop OAC per year and thus the annual cancer rate for patients aged over 50 is 0.00065. The cancer risk to any one given individual with reflux, therefore, would be very low[44].

Whilst the frequency of reflux symptoms relates to the development of OAC, conversely, a paucity of symptoms does not indicate lack of risk with many of those developing OAC never experiencing severe chronic reflux symptoms. In the study by Lagergren *et al*[32] 40% of those with OAC experienced reflux less frequently than once per week prior to development of cancer.

1.5 Clinical aspects of the Barrett's metaplasia – dysplasia – adenocarcinoma sequence

OAC is similar to other intestinal adenocarcinomas in that progression from normal to malignant epithelium can be observed through increasingly dysplastic changes with the risk of malignant change proportional to the degree of dysplasia[48]. The most important predictor of cancer risk in patients with BM is the presence and degree of dysplasia. Biopsy specimens are graded as negative, indefinite for dysplasia, low-grade dysplasia (LGD), and high-grade dysplasia (HGD)[49]. The progression from BM to OAC

is thought to occur stepwise from metaplasia to dysplasia to OAC[50]. However, the time for progression through these stages has not been well characterised[48].

A low degree of interobserver agreement between pathologists makes the diagnosis of LGD difficult to reach, thus studies of the natural history of LGD are highly variable and difficult to interpret[33]. A proportion of patients will progress to develop HGD[33]. Up to three quarters of patients may have non-dysplastic epithelium upon subsequent endoscopy[40;51] reflecting the difficulty in accurately diagnosing LGD.

The diagnosis of HGD upon histological assessment is more reliable[51]. In addition the natural history of HGD is more clear with progression to OAC a significant risk with up to one third of patients progressing to OAC within three years[51]. It has been identified that subclinical cancers may be present at the time of HGD diagnosis and missed by biopsy sampling error[52]. When these prevalent cases are excluded the proportion of patients with HGD who progress to OAC is lower. In support of this a recent large study of 1099 patients with BM reported a 16% progression from HGD to OAC over 7.3 years[52]. Management of patients with HGD is controversial. Some authors recommend prophylactic oesophagectomy due to the risk of disease progression and metastasis at an early stage[52]. As oeosphagectomy is associated with high morbidity and mortality[53;54] investigators have tried to identify subgroups of patients with HGD at particular risk. Positive indicators that HGD may rapidly progress to OAC include a high proportion of biopsy samples demonstrating HGD[51;55], nodular HGD[51], increased expression of cell cycle regulators such as proliferating cell nuclear antigen and Ki-67, abnormalities in the DNA content of metaplastic cells, and a loss of tumour suppressor genes such as p53[40;56]. Finally the development of less invasive management strategies may render these arguments

obsolete. Endoscopic submucosal resection [57] and radiofrequency ablation[58] can be curative though long term follow up in these patients is lacking.

1.6 A molecular and genetic basis for the evolution and progression of Barrett's metaplasia to adenocarcinoma

Accumulating molecular and genetic aberrations underlie the metaplastic change to specialised intestinal type epithelium that is characteristic of BM and subsequent dysplasia and adenocarcinoma formation. Pertinent aspects of the biochemical basis for the progression of BM to OAC are subsequently discussed. These changes are summarised diagrammatically (fig 3).

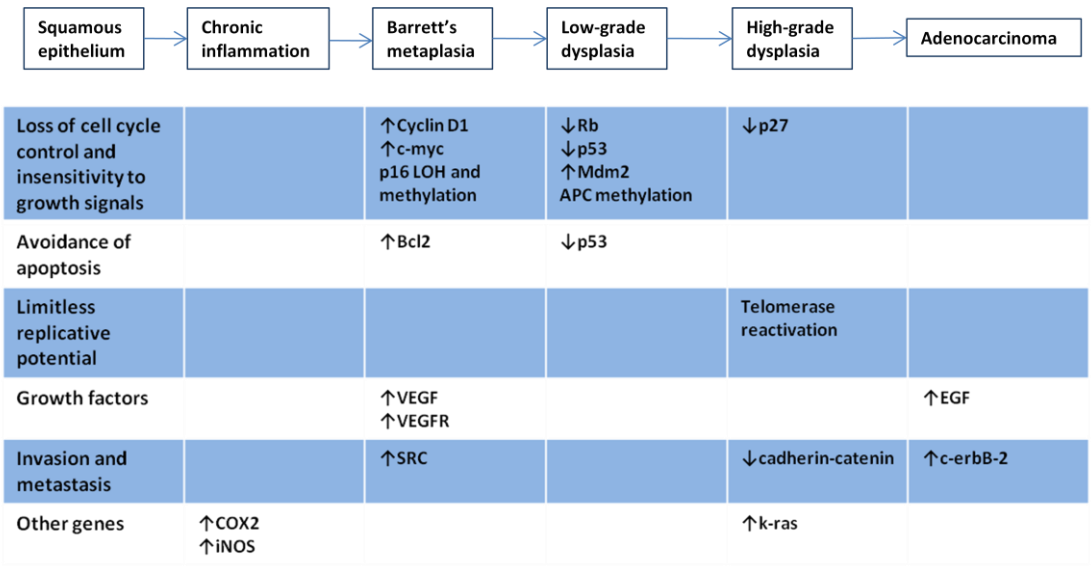


Figure 3. Summary of the genetic basis for progression through the Barrett's metaplasia-dysplasia-adenocarcinoma sequence

LOH - loss of heterozygosity; Rb - retinoblastoma gene; Mdm - murine double minute; APC - adenomatous polyposis coli gene; Bcl - B cell lymphoma; VEGF - vascular endothelial growth factor; VEGFR - vascular endothelial growth factor receptor; EGF - epidermal growth factor; COX - cycloxygenase; iNOS - inducible nitric oxide synthase; k-ras - Kirsten rat sarcoma

1.6.1 Control of the cell cycle and insensitivity to anti-growth signals in the progression of Barrett's metaplsia to oesophageal adenocarcinoma

Cell cycle progression is regulated by cyclin dependent kinases (CDK's) which in turn are influenced by numerous cell cycle control proteins[59]. A feature common to diverse malignant tissue types is the loss of activity of these cell cycle proteins[60].

p16. Methylation of the p16 gene represents one of the earliest changes in the progression of Barrett's metaplasia[61]. p16 is a CDK inhibitor and tumour suppressor gene. Increased expression of p16 reduces the proliferation of stem cells and acts as a protective mechanism against oncogene induced DNA replication providing a barrier to malignant progression[62]. p16 gene methylation has been demonstrated in 75% of BM[63] and 90% of dysplasia arising within BM[64]. Reid *et al*. consider p16 loss to be integral to the development and progression of BM[65]. It has been suggested that Barrett's cells with p16 loss have a survival advantage and undergo clonal expansion so that subsequent Barrett's mucosa is derived from the same population[65].

Adenomatous polyposis coli (APC). APC forms a complex with axin and glycogen synthase kinase-3β which is essential to the canonical Wnt pathway[66]. Extracellular Wnt proteins bind to cell surface receptors intiating intracellular pathways which, via the APC-axin-GSK complex, phosphorylates β-catenin resulting in its destruction. Loss of *APC* function, by methylation or mutation of the APC gene, therefore results in accumulation of nuclear β-catenin[67]. Downstream effects include increased expression of the oncogenes c-myc and cyclin D1[67]. In addition the APC protein binds to the ends of microtubules that form the spindle apparatus during chromosome segregation and so APC defects may mediate chromosomal instability during

metaphase[61;68]. APC loss occurs early in the metaplasia-dysplasia sequence[69;70] by methylation of the promoter region of its gene.

Cyclin D1. Cyclin D1 regulates the cell cycle by promoting the progression of cells from the G1 to S phase by inactivating the retinoblastoma protein[71;72]. The cyclin D1 proto-oncogene is overexpressed in 46% of Barrett's cases[73] and is associated with an increased risk (6-7 times greater compared to Barrett's patients without overexpression[74]) of progression to OAC.

Retinoblastoma gene, Rb. The Rb tumour suppressor gene product inhibits cell cycle progression and linked to apoptotic pathways mediated via p53[75]. The Rb gene locus is aberrant in 36-48%[70;76-78] of OAC and detectable in Barrett's epithelium[79].

p53. The p53 tumour suppressor protein halts cell cycle progression at G1 if a genetic abnormality is detected[80-83]. Efforts at repair are made, which if unsuccessful, leads to apoptosis*[81].* p53 mutations occur in up to 60% of Barrett's[80], 66% of dysplasia and up to 88% of adenocarcinomas[83]. An interesting observation is that p53 loss occurs after loss of p16 in almost all cases[84]. A second method of p53 inactivation is seen in adenocarcinomas overexpressing murine double minute-2 gene (Mdm2*)[85]*. Mdm2 stabilises wild type *p53* inactivating its tumour suppressor function[85].

P27. p27 is a further tumour suppressor that acts as a CDK inhibitor and thus helps control the cell cycle[86]. Repression of p27 is observed in 83% of OAC; in 50% of HGD the protein is relocalised to cellular locations where it is inactive[86].

K-ras. The K-ras oncogene encodes a highly conserved protein involved in signal transduction and promotes cell proliferation[87]; mutations have been detected in 40% of high grade dysplasias and 30% of adenocarcinomas[88]. These changes have not been detected in metaplastic tissue[89].

1.6.2 Avoidance of apoptosis in the malignant progression of oesophageal adenocarcinoma

Apoptosis or programmed cell death relies upon intact cellular control mechanisms. In early G1 p53 triggers a checkpoint halting cell cycle progression; damaged DNA undergoes attempts at repair prior to the cell entering S phase[82]. If the cell is committed to division p53 triggers programmed cell death[82]. Several other proteins controlling the cell cycle are dependent upon p53 or act upon it (fig 4). (The roles of MDM2, Cyclin D1 and Rb proteins in Barrett's and OAC are described above).

The Bcl2 oncogene inhibits apoptosis[90]. In all cases of LGD it is overexpressed but only in 20-40% of HGD cases or OAC suggesting that it plays role in the early progression of BM. Presumably OAC cells also avoid apoptosis by other mechanisms independent of Bcl2[91].

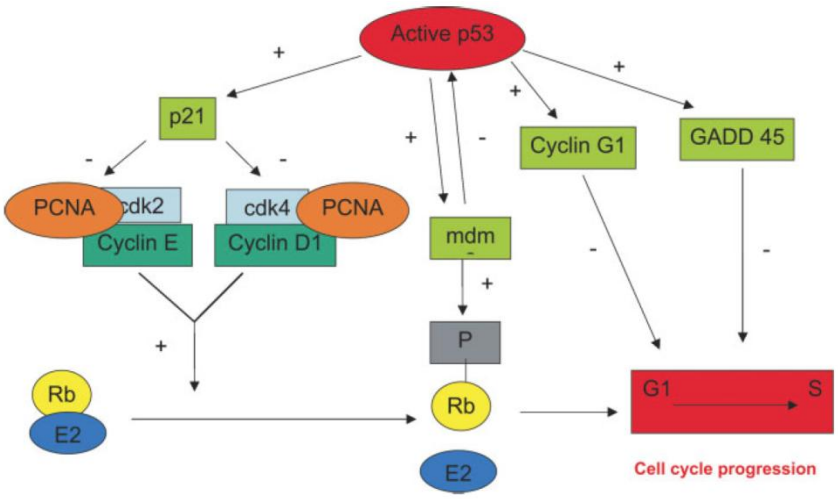


Figure 4. The role of p53 in cell cycle control (reproduced from Merola, 2006[82])

Passage from G1 to S phases of the cell cycle is controlled by p53. Following detection of DNA damage MDM2 inhibition results in accumulation of nuclear p53. Phosphorylation of the Rb gene product prevents cells from progressing through the restriction point late in G1[82].

1.6.3 Cell adhesion and signalling in the malignant progression of oesophageal adenocarcinoma

Local and distant growth of tumours is partly dependent upon the ability to modify adhesion between cells, the extracellular matrix and other cells[92]. Cell-cell adhesion is maintained by the adherens junction comprising of calcium dependent transmembrane glycoproteins termed cadherins[93]. Cadherins are anchored to the axin cytoskeleton. Cadherins are important in maintaining cell-cell contact by mediating signal transduction and cellular polarity[94]. The function of cadherins is dependent upon interaction with catenins[95;96]. Loss of E-cadherin leads to invasive properties of cells grown in culture[97] and proposed to be an important contributor of invasion[98] *in-vivo*. There is progressive loss of cadherin expression through the progression of Barrett's metaplasia to invasive OAC[92;99;100].

Catenins are involved in cell-cell adhesion in conjunction with cadherins. Secondly they interact with the APC tumour suppressor gene. As described above (1.6.1) loss of APC function results in β-catenin accumulation and nuclear translocation where it acts as a transcription factor of growth-promoting genes[98]. These genes, including c-myc and cyclin D1, consequently drive cell cycle progression[101].

The tyrosine kinase SRC has been reported to be overexpressed in BM[102]. This is implicated in deregulating cell adhesion[103] potentially by downstream activation of c-myc[103].

1.6.4 Growth factors and receptors in the progression of esophageal adenocarcinoma

Amplification of the epidermal growth factor gene is observed in the majority of OAC's[113;114] and represents a late event being associated with lymph node metastasis[115]. The c-erbB-2 gene codes for a transmembrane growth factor receptor[104;105] with ligands including Neu-differentiating factor and heregulin[106;107]. Overexpression is associated with metastasis in human tumours[108]. Amplifications appear in OAC and are late changes in the dysplasia – carcinoma sequence[109-111] being associated with a poor prognosis[112].

1.6.5 Immortalisation of cells in the progression of oesophageal adenocarcinoma

Normal somatic cells have progressive telomere shortening throughout life. In immortal cells, such as germ cells, telomerase utilises its own RNA template for the addition of sequences to chromosome ends to maintain telomeric length[116]. Telomerase re-expression confers immortalisation to cells facilitating accumulation of genetic defects[117]. In a study of telomerase expression all OAC and HGD samples were strongly positive as were 70% of Barrett's samples[116].

1.6.6 Other genes involved in the malignant progression of BM to OAC

Chronic inflammation of the lower oesophagus occurs due to repeated reflux of gastric acid and bile[32]. The resultant oxidative stress induces transcription of genes known to mediate malignant progression.

Inducible nitric oxide synthase (iNOS). Overexpression of iNOS is reported in 76% of patients with BM and 80% of those with OAC[118]. Nitric oxide is a potent reactive oxygen species capable of inducing mutations in DNA[119].

COX2. COX2 expression occurs in samples of BM following exposure to bile[120]. Downstream effects include expression of the oncogene Bcl2 (above), inhibition of immune surveillance and promotion of angiogenesis[120]. Thus COX-2 inhibitors may be useful in the treatment of patients with Barrett's oesophagus[121].

In the oesophagus accumulating genetic insults lead to dysplasia and finally a malignant epithelium. This is analogous to the colorectal adenoma-carcinoma sequence characterised by Fearon and Vogelstein[122]. Comparisons are made in fig5 and table1.

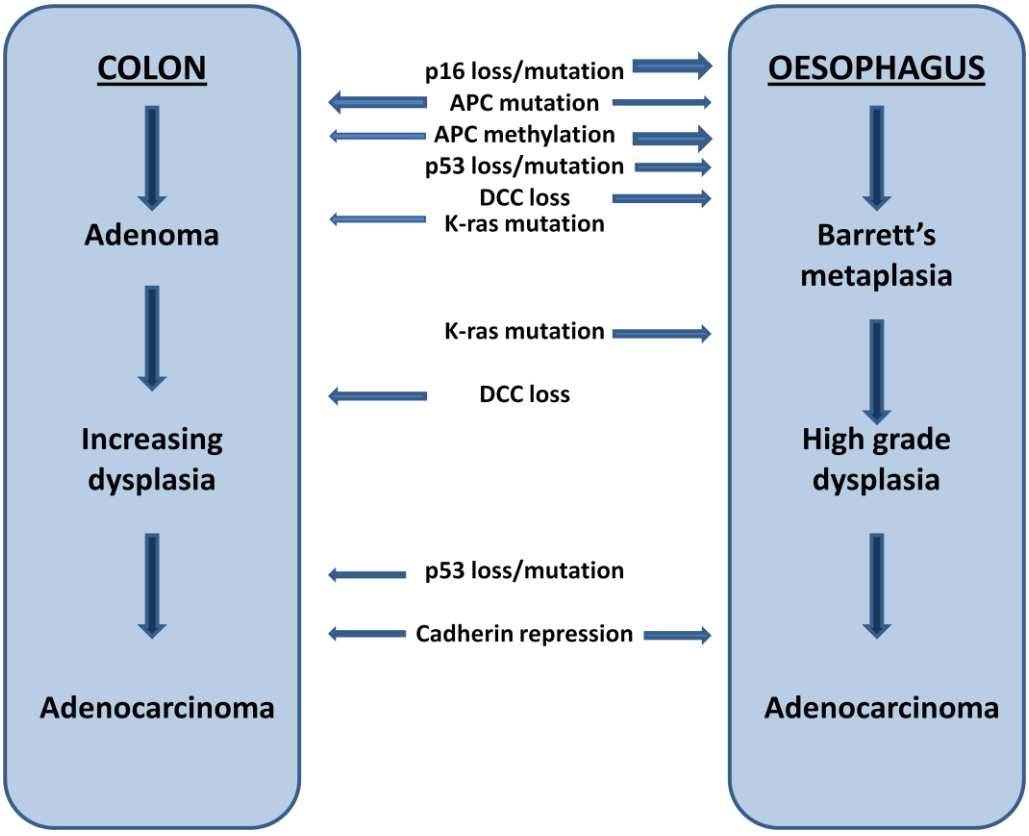


Figure 5. Key genetic defects in the progression of colorectal and oesophageal tissue in the progression of normal epithelium to dysplasia and adenocarcinoma.

APC - adenomatous polyposis coli gene; DCC - deleted in colorectal carcinoma; k-ras - Kirsten rat sarcoma

Mutation/ aberration	Frequency %				
	Colorectal		Oesophageal		
	Adenoma	Carcinoma	BM	Dysplasia	OAC
APC/5q loss	27-29	36-56	0-54 [79;123]	0[123]	55-66 [79;123]
APC methylation	59-82	52-60	36-85[69;124]	66[69]	42-93[69;124]
P53/17p loss	14	71-75	0 [79;123]	38-100[79;123]	79-100[79;123;125]
P53 mutation	4-26	71-75	60[126]	30-66[83;127;128]	40-88[83;127;128]
DCC/18q loss	11-70	73	41[79]		57[79]
β-catenin	2-12	1-17	66[92]		60[92]
k-ras	12-59	35-41	0-25[89;129]	25-40[88;129]	30-40[88;129;130]

Table 1: A comparison of genetic abnormalities between reported series of colonic adenomas/adenocarcinomas and oesophageal metaplasia, dysplasia and adenocarcinoma (colorectal data taken from the review by Leslie *et al*.[131])

APC - adenomatous polyposis coli gene; DCC - deleted in colorectal carcinoma; k-ras - Kirsten rat sarcoma

1.7 The role of dietary agents in the development of Barrett's metaplasia and oesophageal adenocarcinoma

Dietary habits of patients with BM and OAC have been studied to identify potential associations. Subjects with BM have a higher intake of red meat, fried food and lower intake of fruit and vegetables than subjects matched with similar body mass index and GORD symptoms[132]. In this study the odds ratio that a 'Western diet' (high meat and fast food intake) was associated with BM was 2.3 (95% CI 1.26-4.21) whilst in those with a 'health conscious diet' (high in fruit, vegetables and low in meat) it was 0.35 (95%CI 0.20-0.64; $p<0.001$)[132]. Other authors have observed that men and women with diets high in red meat have a greater risk of BM and OAC development[132-135].

Other studies have reviewed the potential role of antixodants. Dietary supplementation with vitamin C and beta carotene has been associated with a reduced incidence of BM (OR 0.48, 95%CI 0.26-0.90)[136]. The association of BM and OAC with diets low in antioxidants, fruit or vegetables has been made by others[26;137-139]. In support of this anti-inflammatory medication is associated with a protective effect against the development of BM and OAC. In a large population-based case-control study users of aspirin were at decreased risk of OAC (OR 0.37, 95%CI 0.24-0.58)[140]. Furthermore, aspirin use correlated with decreased rates of other upper GI malignancies (oesophageal squamous carcinoma: OR 0.49, 95%CI 0.28-0.87 and gastric adenocarcinoma: OR 0.46, 95%CI 0.31-0.68). Risk was similarly reduced among users of non-aspirin NSAIDs[140]. A meta-analysis reviewed 9 studies associating risk of OAC with aspirin use, all of which demonstrated a protective association[133;140-146].

1.8 The role of iron in oesophageal adenocarcinoma

The observation that diets high in red meat are associated with the development of BM and OAC suggest a potential role for iron in OAC[132;135;136;138]. It has been suggested that the substantial increase in dietary iron intake over the past few decades mirrors the rise in incidence of oesophageal adenocarcinoma[134]. As GORD predisposes to BM and subsequent development of OAC in humans animal models have been developed to explore these issues further. There is experimental evidence linking iron with reflux and progression to OAC. In a rat model of reflux an oesophago-duodenal anastomosis mimicks GORD[147]. In initial experiments using this model development of OAC was an infrequent event[147]. Furthermore, animals developed anaemia. This was corrected by administration of intra-peritoneal iron in subsequent experiments. It was then observed that animals receiving supplemental iron had a very high incidence of BM and OAC, 91% and 73% respectively, 31 weeks after surgery[148]. These animals demonstrated increasing deposition of iron within the lower oesophagus, cellular proliferation, hyperplasia and markers of oxidative damage (inducible nitric oxide synthase) when compared to animals not receiving supplemental iron[148].

The authors went on to explore the role of iron supplementation in animals with and without GORD[149]. Animals were randomly assigned to the following: group A (n=43) had oesophagogastric anastomosis as did group B (n=41) with this latter group receiving intra-peritoneal iron supplementation; group C (n=10) were non-operated controls as were group D (n=10) but this group had intra-peritoneal iron supplementation. After 40 weeks the animals were killed and the oesophagus inspected. 23 (53%) of the group A animals developed BM compared with 32 (78%) of

those in group B. OAC was present in 11 (26%) of group A animals compared with 22 (54%) of group B animals (all $p<0.05$). The tumour volume and degree of oesophagitis (measured histologically) were also greater in the group B animals. None of the controls developed metaplasia, dysplasia or adenocarcinoma. Iron staining of the oesophagus was assessed and found to be greatest in the animals with reflux and intra-peritoneal iron supplementation. Staining was also seen in the other model of reflux, group A, but was less obvious and no staining was observed in either control model. The authors conclude that iron, in the presence of GORD, was driving oxidative stress and thus responsible for the enhanced rate of metaplasia, dysplasia and adenocarcinoma[149].

1.9 Oxidative damage and iron in the development of oesophageal adenocarcinoma

Oxidative damage has been proposed to be closely related to reflux oesophagitis and a possible cause for BM[150]. In a study of oesophagitis reactive oxygen species increased with the grade of oesophagitis and were the highest in BM[151]. There are other sources of evidence to support this. B-carotene, an anti-oxidant, can prevent and even reverse BM[152]. In epidemiological studies intake of β-carotene has been inversely associated with risk of OAC[153]. Smoking which is known to stimulate production of reactive oxygen species and deplete endogenous antioxidant defence mechanisms is associated with OAC[154].

In an animal model of reflux Chen *et al* explored the role of iron, inflammation and OAC[155]. Iron staining was found only to be present in areas of inflammation, BM and adjacent to tumours of animals that had received supplemental iron[155]. At these

sites oxidative damage was observed in lipids, protein and DNA. Immunohistochemistry was used to locate cells affected by oxidative damage by using two genes known to be expressed following oxidative damage (haem oxygenase 1, HO-1 and metallothionine, MT)[155]. Strong expression of HO-1 and MT was observed in both BM and OAC. Chen *et al* suggest that elevated serum iron levels in rats receiving supplemental iron allowed, at sites of inflammation via increased capillary permeability, iron to leak into tissues[155]. This appears plausible as iron is taken up by macrophages and therefore 'carried' to sites of inflammation and deposited[156]. Chen *et al* argue this is the reason for extracellular iron being detected at sites of inflammation[155]. It was also noted that there was increased expression of transferrin receptor in BM, OAC and macrophages[155]. These cells are thus importing transferrin bound iron and this may present another mechanism of iron-related toxicity[157].

In addition to this murine evidence there is evidence linking iron with OAC in humans. In a study of subjects with the iron overload condition hereditary haemochromatosis the standardised incidence ratio of OAC was 42.9 (the 95% CI was broad [4.8-154.9] due to low numbers of affected adults in the study) [158].

1.10 Iron

Iron is essential for almost all organisms and is the most abundant metal in humans with the average adult containing 4g[159]. The reason for this abundance is that iron redox chemistry is facile and two way[160] with electron transfer easily accomplished between ferric and ferrous iron. This property permits diverse enzymatic reactions: iron

containing proteins catalyse key reactions involving energy metabolism, oxygen transport and DNA synthesis[160]. Iron plays a key role in the cell cycle where it is required by various control mechanisms such as p53, GADD45 and WAF1/p21 and without iron cells are unable to proceed from the G_1 to S phase[161;162].

The highly reactive nature of free iron, paradoxically, makes it a highly toxic compound. In the presence of oxidative stress ferrous iron can reduce oxygen to produce superoxide with subsequent hydroxyl radical formation (the classically described Fenton reaction, fig 6.). This hydroxyl free radical, possibly the most powerful oxidant encountered in organisms, can attack nucleic acids, proteins and initiate lipid peroxidation[160]. As a result any biological use of iron involves complexing iron with protein which allows access to the redox potential of iron but protects cells from free radical formation[163].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\bullet + HO^+$$

Figure 6. The Fenton reactions

In states of oxidative stress Fe^{2+} ferrous and Fe^{3+} ferric iron undergo electron transfer generating OH• hydroxyl and OOH• peroxide free radicals

1.11 Dietary iron

As iron is utilised by plants and animals it is readily available in the diet. It exists in two main forms. Inorganic iron is derived mainly from fruit, cereals and vegetables whilst organic iron is derived from meat mainly in the form of haem iron which is released following digestion of haemoglobin and myoglobin[163]. Haem (ferrous protoporphyrin IX) is a protein complex with a central iron atom surrounded by a porphyrin ring (fig 7.). A chronic lack of dietary iron will result in iron deficiency anaemia[163]. The proteins involved in the absorption of iron and haem from the gut are demonstrated in fig 8.

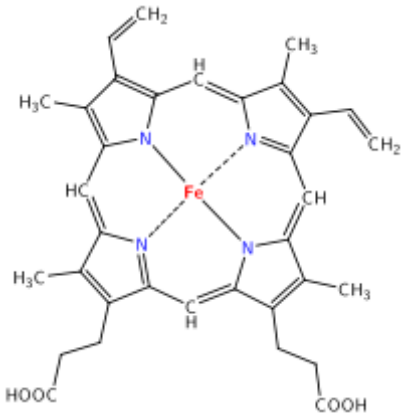


Figure 7. Haem - ferrous protoporphyrin IX

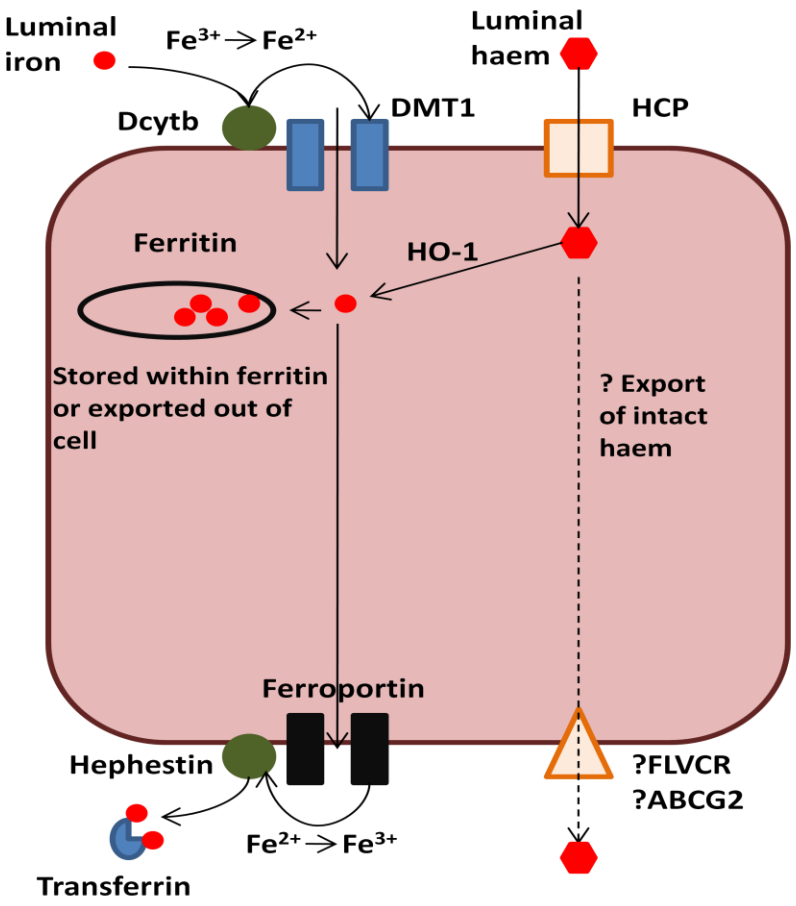


Figure 8. Cellular iron and haem transport proteins in the enterocyte

Dietary inorganic iron (Fe^{3+}) is reduced by a ferrireductase, duodenal cytochrome b (Dcytb), before transport into the enterocyte via the divalent metal transporter 1 (DMT1). Iron as haem is imported via the haem carrier protein (HCP) and metabolised by haem oxygenase (HO-1) releasing biliverdin, carbon monoxide and iron (Fe^{2+}). Cellular iron can be utilised by the cell, stored within ferritin or exported by the basolateral transporter ferroportin. Hephestin, a ferrioxidase, oxidises the iron prior to transport throughout the circulation bound to transferrin (Fe^{3+}). Transport of intact haem through the enterocyte into the circulation may occur. The feline leukaemic virus receptor (FLVCR) and ATP-binding cassette G2 (ABCG2) protein are the candidate haem export proteins responsible.

1.12 The structure, role and function of the cellular iron transport proteins

The following is a summary of the cellular and systemic iron transport machinery followed by a detailed description of each transport protein. Absorption of iron occurs almost entirely within the duodenum[164]. The majority of inorganic iron exists in the ferric (Fe^{3+}) state and must be reduced to the ferrous state (Fe^{2+}) before it can be absorbed[164]. This is achieved by the enterocyte brush border ferric reductase protein duodenal cytochrome B (Dcytb)[164]. Ferrous iron is imported via the divalent metal transporter (DMT1)[165]. Iron is then stored within the enterocyte bound to ferritin (where subsequent shedding of the cell into the GI lumen will result in loss of the iron) or exported through and out of the cell via the basolateral transporter ferroportin, a process facilitated by the ferroxidase activity of the ceruloplasmin homologue hephaestin[159]. This ferric iron is then free to bind to serum transferrin (Tf) and be transported throughout the body in a stable state[160]. Transferrin is secreted by hepatocytes and has two iron binding sites, each able to bind one iron atom. Cells requiring iron express the transferrin receptor (TfR1 or CD71) which selectively binds diferric Tf[160]. The TfR1 is a type 2 180kDa transmembrane glycoprotein consisting of two identical monomers joined via disulphide bonds[160]. Each monomer possesses a large extracellular C terminal domain with a binding site for Tf, a single pass transmembrane domain and a short intracellular N terminal domain[166]. Cell membrane expressing the receptor-ligand complexes invaginate to from clathrin coated endosomes[166]. Clathrin is removed and the endosome imports protons resulting in endosomal acidification; this leads to conformational changes in the Tf and TfR1 with iron release[160]. Ferric iron is reduced to the ferrous state via actions of

steap3[167], a ferrireductase, and transported out of the endosome via DMT1[165]. Iron can be utilised by the cell or stored in an inert form bound to ferritin.

1.12.1 Duodenal cytochrome b (Dcytb) is localised alongside DMT1 on the apical membrane of enterocytes and is upregulated in iron deficiency[168]. It acts as a ferrireductase reducing Fe^{3+} to Fe^{2+} prior to uptake into the enterocyte by DMT1[164;165]. This physiological role is debatable. In murine studies of Dcytb silencing animals do not become anaemic when fed a normal diet[169]. It is possible that dietary factors such as ascorbate are responsible for the reduction of Fe^{3+} rendering Dcytb non-essential[169].

1.12.2 Divalent metal transporter 1 (DMT1) mediates the transport of numerous divalent metals (Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}) though its prime role appears to be iron transport[165]. This function was identified in the *mk* mouse and Belgrade rat which have the same DMT1 mutation and both suffer iron deficiency anaemia[159;170]. Transcription of the DMT1 gene has four splice variants. The 5′ -end mRNA variant is spliced at two distinct transcription start sites at exon 1A or exon 1B[171;172]. Splice variant DMT1A is found within the gastrointestinal epithelium whilst DMT1B is expressed within the TfR1 endosome and responsible for the reduction of iron released from transferrin[173]. The two splice variants at the 3′ -end affect the UTR; one has an iron responsive element (IRE) (DMT1 IRE+ve) the other does not (DMT1 IRE-ve)[174]. The relevance is the ability of the transcribed mRNA to be sensitive to intracellular iron concentrations (DMT1 IRE+ve) and therefore under control of the IRE/IRP system (see below)[172].

1.12.3 Ferritins are highly conserved proteins with close similarity in structure and function between bacteria, plants and humans[160]. In humans ferritin is a heteropolymer of 24 subunits of H ('heavy' or 'heart', M_r=21,000) and L ('light' or 'liver', M_r=19,500) chains with a total mass approaching 450,000[160]. Although the ratio of H to L chains varies with cell type both are required in all ferritin polymers with knockout of the H chain being lethal in utero[175]. In the liver and spleen L chains predominate within the polymer whilst in myocardium and skeletal muscle the H type predominates[176;177]. Small quantities of ferritin is found within the serum; this is increased during times of stress such as inflammation or iron overload[178-180].

Ferritin acts as a store for cellular iron and serves to capture and compartmentalise free cellular iron[181]. This protects the cell from iron induced stress and subsequent cellular damage[182]. Two ferrous iron atoms are taken into the ferritin shell by H chain ferroxidase and undergo oxidation[183]. Ferric iron is freed and relocated to the inner surface of the ferritin shell probably by the action of the L chains[184]. An individual ferritin protein can store up to 4500 iron atoms though in most tissues approximately half this is most frequently observed[160].

Translational control of ferritin is mediated by the presence of its 5′ –IRE structure[185;186]. In the state of low cellular labile iron IRP binds to the 5′-IRE inhibiting mRNA translation[184]. Cellular control of iron through the IRE/IRP system is described in section 1.13.

1.12.4 Ferroportin is the major cellular iron export protein and is localised at the basolateral membrane of enterocytes[187]. It is also found within the liver, spleen,

macrophages, heart and placenta[188]. Ferroportin is a highly conserved protein and is essential for survival. Ferroportin knockout is a lethal condition in utero blocking placental iron transport[189].

Ferroportin function is influenced by the systemic iron regulator hepcidin and this is key to normal iron homeostasis[190] (below). In high iron states elevated hepcidin binds to ferroportin resulting in phosphorylation, internalisation and lysosomal degradation[190]. In this way iron export from the gut is inhibited[163;191]. Ferroportin mutations demonstrate the role of ferroportin in iron homeostasis. One mutation results in hepcidin insensitivity to ferroportin (the autosomal dominant 'ferroportin disease', see below for a description of the interaction between hepcidin and ferroportin) and mimics classic haemochromatosis[192]. Ferroportin remains at the cell surface exporting iron into the circulation regardless of the high systemic iron content and hepcidin levels[188]. This leads to hepatic and macrophage iron deposition with high serum transferrin saturation[188]. A second mutation results in ferroportin being unable to reach the basolateral cell surface and thus reduced iron uptake from the gut. This results in a low serum transferrin concentration[188]. Ferroportin also contains a 5′ -IRE and is thus influenced at the local level by the intracellular labile iron pool and hypoxia[187]. Interaction between iron regulatory proteins in a low iron state serves to reduce protein synthesis[187]. This observation requires further investigation as interaction between the IRP and IRE system is thought to regulate mRNA translation not transcription[187].

1.12.5 Hephaestin is co-localised on the basolateral cell border of enterocytes with ferroportin[193;194]. It is a member of the copper oxidase family similar to ceruloplasmin and has an iron binding site[195]. The cell location and ability to take part in redox

reactions indicate a likely function of oxidising ferrous to ferric iron for transport by transferrin. Sex linked anaemia in mice is associated with reduced intestinal iron absorption and a mutation of hephaestin[196]. Normal iron uptake into the enterocyte is observed but export is inhibited[196].

1.12.6 Transferrin receptor (TfR1). There are two transferrin receptors, TfR1 and TfR2. TfR1 is far more abundant and the most studied[160]. It is a type 2 transmembrane glycoprotein and a homodimer of two 90,000 kDa subunits joined by disulphide bonds[160]. The first 61 amino acids form a cytoplasmic domain followed by a hydrophobic sequence which traverse the cell membrane and then the extracellular transferrin binding site. The extracellular subunits contain a protease like domain, a β sheet and a helical domain[197]. Transferrin binds to the receptor with 2:2 stoichiometry[198]. TfR2 is found predominantly in the liver. TfR2 mediated uptake of Tf is 25 fold less than that by TfR1[199].

Diferric transferrin binds to TfR1 and is internalised via clathrin-coated pits into endosomes by receptor mediated endocytosis[199]. Protons are imported into the endosome and as the pH decreases Tf releases iron through conformational changes[200;201]. The TfR1 apo-transferrin complex is then recycled and relocated on the cell surface[199]. Ferric iron is reduced to ferrous iron by the actions of STEAP3[202] and exported out of the endosome via the divalent metal transporter (DMT1B)[170].

TfR1 expression is regulated at the post transcriptional level in response to intracellular iron levels[199;203]. At the 3′ -UTR TfR1 possesses 5 IRE's. During levels of low cellular iron IRP levels increase; the binding of IRP's with the 3′ -IRE stabilises the mRNA

protecting against degradation[203]. Consequently increased translation of TfR1 mRNA results in increased expression of membranous TfR1.

1.13 Cellular regulation of iron transport and storage proteins

Cellular iron is regulated via interaction between iron regulatory proteins 1 and 2 (IRP1, IRP2) and iron responsive elements (IRE). IRE's are RNA elements within mRNAs which regulate the expression of pertinent iron transport proteins[204]. They are stem loop structures at the 3′ or 5′ untranslated regions (UTR) of multiple genes[204]. The location at the 3′ or 5′ end is key to the function of the IRE/IRP system and cellular control[203;205].

In low iron conditions IRP's bind to IRE's[203;205]. This either inhibits translation or stabilises the mRNA dependent upon the binding site of the IRP with the IRE. Binding at the 3′ UTR stabilises the mRNA increasing protein translation whilst binding at the 5′ UTR marks the mRNA for degradation[204] (fig 9). Genes with 3′ IRE's include DMT1, TfR1[206] and the cell cycle protein CDC14A[207]. Those with a 5′ IRE include ferritin and ferroportin[187;208]. The relevant functions of IRP1 and 2 are dependent upon oxygen tension with IRP1 functioning in states of oxidative stress[209]. Under normal physiological oxygen tensions IPR2 is the dominant protein to bind with IRE's[210;211].

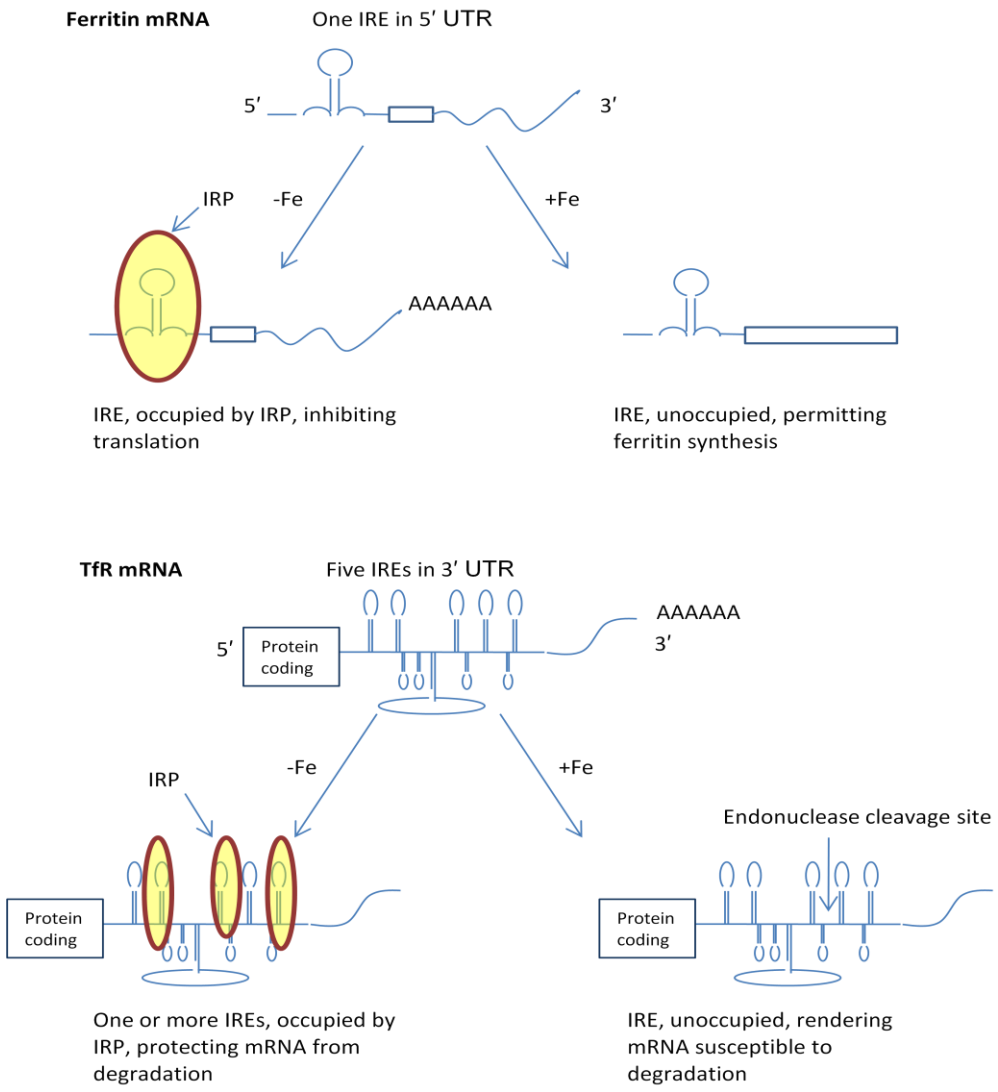


Figure 9. Cellular control of iron via interaction between IRP's with IRE's (reproduced from Roualt[204]).

In low iron states iron regulatory elements (IRE's) bind with iron regulatory proteins (IRP's). Binding at the 3′ untranslated region (UTR) stabilises TfR1 and DMT1 mRNA whilst binding at the 5′ UTR of ferritin and ferroportin mRNA inhibits translation. The effect is enhanced iron import with repressed storage and export. Ferritin and TfR1 mRNA are used as examples.

1.14 Systemic regulation of iron

The elegant control of iron at the cellular level by the IRE/IRP system has only recently been understood. This is also the case with the systemic control of iron homeostasis. Control of total body iron is essential with deficiency resulting in anaemia and excess in iron overload and the risks associated with this[160]. It is only with the identification of hepcidin, a 25-amino acid peptide, in 2000[212] that this system has been understood[213]. Hepcidin was discovered by chance in a murine knockout experiment. Knockout of a nearby gene, USF2, also knocked out hepcidin with mice developing severe haemochromatosis[214]. When USF2 was knocked out with sparing of the hepcidin gene the mice exhibited a normal phenotype[214]. Humans, homozygous for mutations of the hepcidin gene, were then noted to suffer severe juvenile haemochromatosis[215]. The role of hepcidin in iron homeostasis became more clear when mice overexpressing hepcidin were iron deficient with iron export inhibited at the small bowel[216].

Hepcidin is produced by the liver, circulated in plasma and excreted in urine[212;213]. It is secreted as prohepcidin. Following cleavage the mature 25 amino acid β-sheet hairpin is stabilised by four disulfide bonds[190]. The mechanism by which total body iron influences hepcidin expression is unknown[217]. Hepcidin production is inhibited by hypoxia within hepatocytes but stimulated by inflammatory mediators with anaemia of chronic disease mediated by chronic hepcidin overexpression[218]. Levels of urinary hepcidin are elevated amongst patients with anaemia of chronic disease and correlates with the degree of inflammatory burden[219]. Animals undergoing trauma have increased levels of hepcidin mRNA within hepatocytes[220]. Infection increases hepcidin expression; fish infected with S*treptococcus iniae* have increased hepcidin

mRNA expression, up to 4500 fold greater than normal levels[221]. The effects of infection or inflammation upon hepcidin are indirect[218]. Exposure of hepatocytes to lipopolysaccharide mildly elevates hepcidin expression but in the presence of monocytes this effect is much greater[219]. It has been identified that interleukin 6 is the cytokine responsible[219] whilst other inflammatory mediators including interleukin 1 and tumour necrosis factor α have no effect. Injection of interleukin 6 into human volunteers resulted in an increase of urinary hepcidin (average 7.5 times greater than control) within several hours. This was associated with a 30% decrease in serum iron and transferrin saturation[219].

Hepcidin elegantly controls body iron stores. Hepcidin binds to ferroportin, the sole known cellular iron export protein, triggering tyrosine phosphorylation, internalisation and lysosomal degradation[222;223]. In this manner hepcidin acts upon the intestine sequestering iron within enterocytes and inhibiting absorption of iron from the gastrointestinal tract. The enterocytes are then shed into the GI tract and excreted with the sequestered iron[190]. Conversely, in states of anaemia and hypoxia, iron import is facilitated by suppressing hepcidin. Effects are also seen in the macrophage where the release of iron recovered from erythrocyte degradation is inhibited[209;220]. This function is likely to relate to inflammatory mediators stimulating iron sequestration within macrophages as part of the innate immune system[209;220]. By sequestering iron within the reticuloendothelial system during episodes of infection less iron is available to pathogens. Hepcidin was originally defined by its antimicrobial properties (*hep*atic bacteri*cid*al prote*in*)[218].

Disorders of hepcidin homeostasis result in hereditary haemochromatosis (HH). There are essentially three causes of HH that result in the final common clinical condition of

iron overload[163]: [1] A mutation of the hepcidin gene, HAMP, prevents production of functional hepcidin[215] [2] mutations of genes which up regulate hepcidin expression (haemochromatosis gene[224] [HFE] – most common cause of HH, transferrin receptor 2[225] [TfR2] and hemojevulin[226] [HJV]) [3] mutations of the ferroportin gene result in membranous ferroportin being insensitive to hepcidin thus control over iron import is lost[227]. There is great variance in the disease spectrum which is dependent upon the underlying cause, hetero and homozygosity and gene penetrance[163].

1.15 Absorption, transport and metabolism of haem

The acquisition, metabolism and recycling of haem has yet to be understood to the same degree as inorganic iron. Haem iron is the most bio-available form of iron from the gastrointestinal tract and is absorbed with approximately 10 times greater affinity than inorganic iron[191]. There is an increasing appreciation of systemic transport and uptake of haem from the circulation[228]. Recycling haem iron is essential due to the short life span of red blood cells (100-120 days) and the amount of iron within this pool. Daily recycling of approximately 20mg of iron from haemoglobin of senescent red blood cells is mediated by the action of macrophages[217]. However cellular transporters of haem iron have only very recently been characterised.

Haem iron provides the majority of iron from the GI tract in carnivores and is absorbed mainly from the duodenum[229]. The acidic environment of the stomach and upper duodenum prevents haem from forming insoluble aggregates[229]. Haem is taken into the enterocyte in an energy-dependent manner by the haem carrier protein 1 (HCP-1)[230]. Transport seems to be dependent upon the presence of the protoporphyrin

ring as zinc protoporphyrin (ZnPP), a haem analogue with a central zinc atom, is also taken up via HCP-1[230]. Anti-HCP-1 antibodies inhibit radiolabelled haem uptake strongly suggesting that HCP-1 has a direct role in intestinal haem absorbtion[230]. Within the enterocyte haem is likely to be degraded to release free iron, biliverdin and carbon monoxide by the actions of haem oxygenase 1 (HO-1)[231]. The iron atom enters the inorganic iron pathways described above being stored within ferritin or exported out of the enterocyte via ferroportin. Thus absorption of iron from the GI tract sourced from dietary haem remains under the control of hepcidin and ferroportin. Expression of HCP-1 mRNA is not affected by iron loading or iron deficiency but is positively influenced by hypoxia[230]. Cellular location of HCP-1 is however affected by exposure of the enterocyte to iron. Iron deficient mice strongly express HCP-1 at the brush border whilst iron replete mice demonstrate mixed membranous and cytoplasmic staining for HCP-1[230]. Following administration of oral iron to iron deficient rats the location of HCP-1 changes from being strongly membranous to cytoplasmic within hours[230]. Haem iron metabolism is linked to the IRE/IRP system. IRP2 binds haem with high affinity and when this occurs oxidation of cysteine results in conversion to alanine and subsequent marking of the IRP2-heam complex for degradation[232]. Thus cellular control of inorganic iron or haem iron are both mediated via a single mechanism, the IRE/IRP system[232].

There remains a final and huge potential source of haem iron: the circulating erythrocytes. One fate of senescent erythrocytes is to undergo degradation within the liver, spleen and marrow. Via the actions of HO-1 free iron is liberated[230]. Another fate of red cells, approximately 10-20%, is to undergo intravascular haemolysis[233].

Circulating haemoglobin can be filtered and excreted via the kidneys[234] but this is prevented by binding with the acute phase protein haptoglobin[235]. Haptoglobin bound haemoglobin is recovered by hepatocytes[236] and macrophages[237;238]. Free haem, as opposed to haemoglobin, is a highly reactive species facilitating oxidative damage and has been linked to neurodegenerative disorders[239]. In the circulation another acute phase protein, haemopexin, can bind haem. Haemopexin is a 60kDa protein with the highest affinity for haem of any protein[240]. There is a functional overlap of both haptoglobin and haemopexin indicating the importance of haem scavenging[241].

Fates of extracellular haem and haemoglobin are demonstrated in fig 10. Haem-haemopexin complexes are taken up via receptor mediated endocytosis. The receptor responsible was recently identified by ligand affinity as the CD91 or LDL receptor-related protein[242] (LRP-1). LRP-1 is a large (600kDa) transmembrane protein expressed by a wide variety of cell types including macrophages, hepatocytes and neurones[242]. Akin to other members of the LDL receptor group there are five structural units to the protein[243]. Over 30 ligands are recognised for the LRP-1 (table 2) which represent diverse families of proteins including lipoproteins, proteinases, proteinase-inhibitor complexes, extracellular matrix proteins, bacterial toxins, viruses and various intracellular proteins[243].

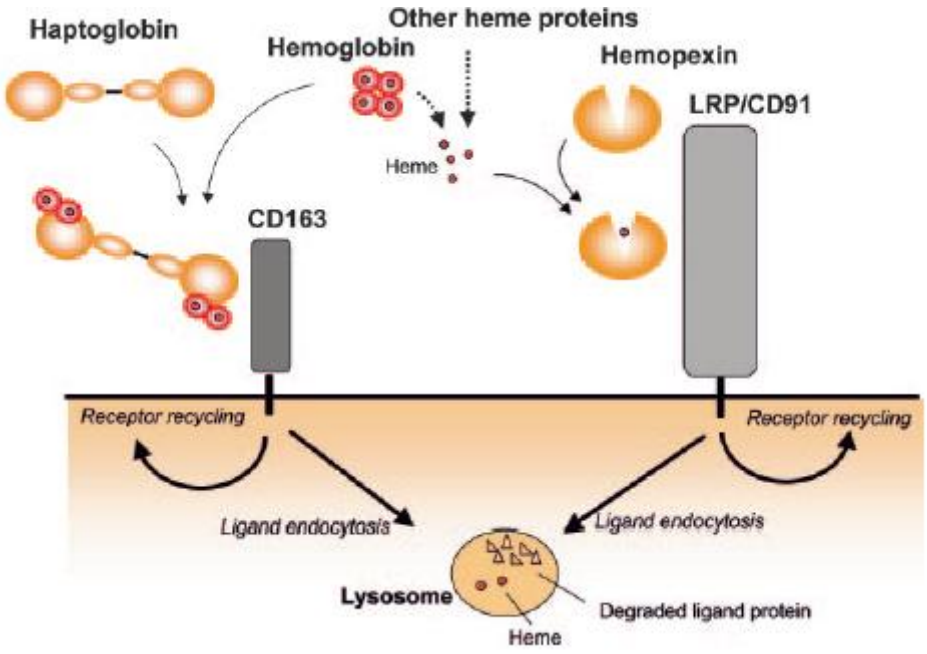


Figure 10. Receptor pathways for endocytosis of extracellular haem (Reproduced from Hvidberg[242])

CD163: macrophage haptoglobin receptor; LRP-1 LDL related protein

Scavenging of haemoglobin and haem in plasma exist to protect against oxidative damage. Haptoglobin-haemoglobin complexes are recycled via the hepatic CD163 receptor whist haemopexin-haem complexes are recycled via the CD91 or LRP-1 receptor.

Ligand	Ligand function
ApoE	Lipoprotein metabolism and transport
Lipoprotein lipase	
Hepatic lipase	
tPA	Fibrinolysis, signalling function in brain
uPA	Cell migration, wound healing
Factors IXa, VIIIa, VIIa/TFP1	Coagulation
MMP-13 and MMP-9	Angiogenesis and metastasis
Spingolipid activator protein (SAP)	
Pregnancy zone protein	Pan-proteinase inhibitors, infection
α2M	
Complement C3	Infection
PAI-1	Regulates tPA/uPA activity
C1 inhibitor	Regulates C1r/C1s activity
Antithrombin III	Regulates clotting
TFPI	
Heparin cofactor II	
α1-antitrypsin	Regulates neutrophil elastase
APP	Role unclear
Thrombospondin 1	TGF-β activation, matrix cell interactions
Thrombospondin 2	Collagen assembly, matrix cell interactions
Pseudomonas exotoxin A	
Lactoferrin	Antibacterial
Rhinovirus	
RAP	Chaperone
HSP-96	Chaperone
HIV-Tat protein	Transcriptional activation

Table 2. Diverse ligands for the LDL related protein (LRP-1)

LRP-1 is a large transmembrane protein expressed by a wide variety of cell types including macrophages, hepatocytes and neurones and has numerous diverse ligands. Haem bound haemopexin could be added to this list (Reproduced from Herz *et al*. LRP-1: a multifunctional scavenger and signalling receptor[243]).

1.16 Cellular haem export

Haem export from maturing erythroid cells occurs via the feline leukaemic virus receptor (FLVCR)[244] and presumably functions to protect the cell from the toxic effects of excess haem with repression seen during globin production and haemoglobinisation[244]. A second haem exporter has been identified as the ATP-binding cassette G2 protein[245] (ABCG2). It was first identified by its ability to export chemotherapeutic agents and hence its alternative name breast cancer resistance protein[246] (BCRP). ABCG2 is upregulated during oxidative stress and functions to export haem and protoporphyrin presumably protecting the cell from oxidative damage[247].

Expression of both FLVCR and ABCG2 has been identified within the GI tract[248] and Caco-2 cells have been shown to efflux haem[249]. This presents a possible mechanism of organic iron absorption from the gut and presumably bypasses the action of haem oxygenase 1.

1.17 The role of iron in cancer

Cancer cells have a high requirement for iron as it is essential for metabolism including energy production, DNA synthesis, cell proliferation and is required for numerous enzymes. Iron plays an essential role in cell cycle control and is required by a number of proteins that regulate cell cycle progression such as p53, WAF1/p21 and GADD45. In the absence of iron cells cannot proceed from G_1 to S phase[250].

An early observation highlighting the high iron requirements of neoplastic cells was that of the elevated levels of TfR1 expression in breast cancer tissue[250]. This has

since been observed in many malignant cell types: colorectal[251], glioma[252], non-Hodgkins lymphoma[253], chronic lymphoid leukaemia[254], adenocarcinoma of the lung[255], gynaecological tumours[256], transitional cell carcinomas[257], cervical carcinoma[258], melanoma[259], hepatocellular carcinoma[260] and hepatoblastoma[261]. Monoclonal antibodies to the TfR1 can block Tf uptake and inhibit cancer cell proliferation[262].

Intriguing epidemiological and animal studies strongly suggest iron acts as an initiator or promoter of carcinogenesis. Iron dextran intramuscular injections have been associated with sarcoma development in rats[263] and years later in humans[264]. A review of studies reviewing colorectal cancer risk and dietary iron intake, presented by Nelson, demonstrated a positive association in the majority of the 33 studies[265]. Variables assessed within the study included dietary intake, iron vitamin supplementation and gene studies for HH[265]. A prospective study of 14,407 Americans over 15 years identified iron intake and a higher Tf saturation to be associated with colorectal cancer development[266;267]. The incidence of pre-malignant colorectal adenomas was greater in individuals with higher serum ferritin concentrations[268]. The iron overload condition hereditary haemochromatosis is associated with the development of colorectal carcinoma[269;270] (risk in Hfe mutation homozygotes: OR 7.7, 95% CI = 1.0-59.9[269]; risk in heterozygotes: RR 1.27, 95% CI = 1.07-1.53[270]) as well as breast (OR 7.3, 95% CI = 0.8-65.7[269]) and hepatocellular tumours (RR 21, 95% CI 16-22[271]) . Accumulating iron levels within hepatocytes of HH patients leads to fibrosis, cirrhosis and eventually hepatocellular

carcinoma[272]. The risk of developing carcinoma once a patient has cirrhosis is 200 times greater than controls[273;274].

Free radical formation in the GI tract is observed following iron supplementation in healthy humans[275] and in animal models is associated with biochemical evidence of oxidative damage[276] and the development of aberrant crypt foci[277;278]. Patients with ulcerative colitis are at increased risk of developing colorectal cancer and frequently require dietary iron supplementation to treat chronic anaemia[279]. These two observations may not be causal as chronic inflammation is known to predispose to cancer risk. However, in mice given dextran sodium sulphate to induce a state similar to ulcerative colitis additional dietary supplementation with iron induced significantly more cancers than animals fed a normal diet[280;281].

Haem intake appears to be associated with an increased incidence of malignant diseases. A study of 494,036 elderly Americans identified higher rates of oesophageal (HR 1.51, 95% CI 1.09-2.08), colorectal (HR 1.24, 95% CI 1.12-1.36), hepatic (HR 1.61, 95% CI 1.12-2.31) and pulmonary cancers (HR 1.2, 95% CI 1.1-1.31) in subjects with the highest intake of meat versus those with the lowest[282]. In a recent study to explore the role of haem in colorectal carcinogenesis Sesink *et al* fed rats control diet or diet supplemented with haem, protoporphyrin IX, ferric citrate or bilirubin[283]. Proliferation of colonic epithelium was induced only in the haem group and faecal water from these animals was highly toxic to erythrocytes. Interestingly when haem was added to the faecal water of the control rats cytotoxicity was not observed indicating that dietary haem is modified and becomes toxic in the GI lumen. Haem intake is also associated with breast cancer with increased incidence amongst those with high intakes of fried[284] and red meat[285].

1.18 Iron chelation as a therapy against cancer

Understanding the dependence of cancer cells for iron and the differential expression of TfR1 between malignant and benign tissues leads to potential therapeutic strategies[286-293]. Antisense cDNA to the TfR1 results in decreased TfR1 mRNA expression and inhibition of growth in malignant breast cells[294]. Significantly reduced effects were also observed in benign cells[294]. Iron chelation has been used in numerous cell, animal and some human studies. Desferrioxamine (DFO) is a siderophore licensed for use in patients with iron overload secondary to hereditary haemochromatosis or multiple blood transfusions, typically, patients with β-thalassaemia. DFO forms a complex with Fe^{3+} in a 1:1 molar ratio. Breast cancer cells exposed to DFO exhibit decreased proliferation and increased apoptosis[295;296]. In nude mice DFO reduced growth of implanted hepatocellular carcinoma tumours by 60%[297]. There are several trials of iron chelation in humans: seven of nine children with neuroblastoma showed reduced bone marrow infiltration and one reduced tumour mass following a five day course of DFO[298]. In a further study of patients with unresectable neuroblastoma a complete response to chemotherapy, which included DFO, was observed in 3 patients, a partial response in 6 and a minor response in 1 patient[299]. Another study of multiple therapeutic agents including DFO, this time in patients with inoperable hepatocellular carcinoma, resulted in increased survival with decreased tumour burden when compared to five non randomised controls[300]. DFO has limitations, it is hydrophilic, must be given parenterally and has poor cell membrane permeability. More recent advances included the development of lipophilic iron chelators[301]. Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) is an iron chelator and a potent inhibitor of ribonucleotide reductase, an enzyme

required for DNA synthesis[302]. Several phase I and II studies have demonstrated no clinical benefit of Triapine in small cohorts of patients with advanced pancreatic adenocarcinoma[303], acute myeloid leukaemia[304], non-small-cell lung carcinoma[305] and renal cell carcinoma[306]. Methaemoglobinaemia stopped treatment of some patients in all studies. It had been suggested that Triapine could become the first widely used iron chelator in anticancer therapy[307]. The agent di-2-pyridylketone-4-4,-dimethyl-3-thisemicarbazone (Dp44mT) has shown promise *in-vitro* and in animal models. It inhibits iron uptake by transferrin and has a higher efficacy than DFO[308]. The Dp44mT-Fe complex induces ROS and subsequent apoptosis[308]. This effect is independent of p53 status which contrasts with some conventional therapeutic agents[293] as defective p53 inhibits cytotoxic mediated apoptosis.

When compared with 11 other chelators (DFO, Triapine, DOX, PKIH, PKTH, PKBH, PKBBH, Dp4aT, Dp4eT, Dp4mT, Dp4pT) Dp44mT was the most active against 28 tumour cell lines and significantly more toxic than established cytotoxic agents such as doxorubicin[293]. DFO was the least effective with an IC_{50} that ranged from 3 to >25 µmol compared to Dp44mT with the lowest IC_{50} range (0.005 to 0.4 µmol). The average IC_{50} for Dp44mT was 0.03 ± 0.01 µmol and significantly lower than triapine at 1.41 ± 0.37 µmol. Dp44mT demonstrates selective antitumour activity when compared with fibroblasts with an average IC_{50} of 0.03 ± 0.01 µmol vs. >25 µmol[308]. In a murine lung cancer model twice daily Dp44mT reduced tumour size to 47% of control after 5 days[308] and in a murine melanoma xenograft model daily use for seven weeks reduced tumour burden to 7% of control[293]. As mentioned above Dp44mT demonstrates activity against cells that have drug resistance to conventional chemotherapeutic agents. Resistance with traditional therapy is a marked clinical

problem[309]. As well as being effective against tumour cells, regardless of p53 status, Dp44mT was equally efficacious against cell line clones resistant and not resistant to etoposide and vinblastine[293]. However, concerns exist over the safety of these agents. Severe transient myelosuppression was observed in a trial of desferoxamine in children with unresectable neuroblastoma[299]. Triapine has also been associated with haematological suppression – anaemia, thrombocytopenia, leucopoenia – and met-haemoglobinaemia[310]. The development of met-haemoglobinaemia and haemolysis is severe in patients with glucose-6-dehydrogenase deficiency due to Triapines redox activity and the inability of these patients to reduce methaemoglobin to its ferrous state[311]. In murine models dose dependent cardiotoxicity was observed. Toxicity was manifested as ischaemic fibrosis and likely related to increased deposition of iron within myocardial cells[293]. Dp44mT remains to be trialled in humans.

1.19 Natural iron chelators

In 1969 Burtkitt associated intake of dietary fibre with a protective effect against the development of colorectal carcinoma[312] which has subsequently been observed by other authors[313-317]. Various explanations for this association have been made. The most simple is intuitive: increasing colonic fibre and stool bulk reduces transit time and therefore limits the duration to which a potential carcinogen would be exposed to colorectal mucosa[318]. Fibres have also been demonstrated to bind numerous toxic agents within the gastrointestinal tract (dioxins[319], heterocyclic amines[320], pentachlorobenzenes[321], binding of bile acids and in particular secondary bile acids[266;322-326]).

Despite enteric iron being strongly associated with colorectal carcinogenesis[266;267;275;277;278] there are few studies that directly look at modifying the levels of iron in the GI tract.

1.19.1 Phytic acid. Phytic acid is a major component of cereals and pulses[327] and it has been proposed that the ability of seeds to survive hundreds of years is due to the ability of phytate to maintain iron in the Fe^{3+} state obstructing the creation of ROS and oxidative damage[328;329]. In a rat model of colorectal adenocarcinoma addition of phytic acid to the diet decreased colonic cell proliferation[330]. In those given a carcinogen (azoxymethane) tumour burden was significantly decreased with the addition of phytic acid[331-333]. Nelson looked specifically at iron induced colorectal carcinogenesis in a rat model using 1,2 dimethylhydrazine to induce carcinogenesis. Tumour burden was significantly augmented by the addition of oral iron an effect which was inhibited by the simultaneous administration of phytic acid[334].

1.19.2 Seaweed and alginate. Another group of organic compounds known to bind various metals including heavy metals such as $Cadmium^{2+}$, $Copper^{2+}$, $Mercury^{2+}$ and $Lead^{2+}$ are alginates[335]. Alginates are salts of alginic acid, a component of the cell wall of algae of the Phaeophyta division or otherwise known as brown algae[335]. The cell wall consists of a fibrillar skeleton and amorphous matrix of alginic acid and smaller amount of polysaccharide, fig 11.

1.19.2.1 Seaweed. Epidemiological studies associate seaweed intake with decreased incidence of various tumours. High intake of seaweed amongst the Japanese was

associated with protective effects against the development of colorectal cancer and a relative risk of 0.2 (95%CI 0.0-0.9; p<0.01)[336]. Seaweed intake was associated with decreased incidence of oesophageal squamous carcinoma with an odds ratio of 0.42 (95%CI 0.29-0.6) in a second Japanese study[337]. In a third study intake of seaweed was associated with a protective effect against breast cancer[338;339]. *In-vitro* experiments demonstrated a seaweed extract, mekabu, to induce apoptosis in three breast cancer cell lines with higher efficacy than conventional therapeutic agents[338]. The ability of seaweeds to induce apoptosis has been observed by other authors: sensitivity of human malignant lymphoid cells to etopside was augmented two-fold by exposure to brown seaweed cell wall whilst seaweed on its own demonstrated no toxic effects[340]. In the gastric carcinoma cell line AGS seaweed extracts induced apoptosis and decreased expression of the Bcl-2 proteins and increased the caspase cascade[341].

As the artificial chelators DFO[342] and Dp44mT[308] augment apoptosis in the presence of conventional cytotoxic therapy it seems plausible to associate the iron binding properties of seaweeds with their anti-tumour effects.

1.19.2.2 Alginates. Brown algae are harvested for a wide range of commercial uses which include water holding for frozen food and syrups; gelling agents for instant puddings; dessert gels; explosives; emulsifying properties of polishes; stabilising properties in ceramics, welding rods and cleaners[335]. Alginate is one of the functional ingredients of the over the counter medicine Gaviscon^{TM}. Alginates are a group of linear polysaccharides containing various quantities of 1,4-linked β-D-mannuronic (M) and α-L-gluronic (G) acid residues arranged in an irregular pattern

along the chain[343] (fig 12). The salts with divalent or polyvalent metal ions are insoluble[344]. The affinity for divalent metals increases as the G content of the alginate increases, this is explained by the 'zigzag' structure which accommodates the divalent metals. Through dimerisation the chains interconnect promoting gel network formation[344]. The greater the linkage and interconnection the greater the viscosity of the gel[335]. *Sargassum fluitans* binds 0.99 mmolFe^{2+}/g alginate[345]. Alginate can also bind ferric iron sequestering 66 atoms of Fe^{3+} per chain[346].

In the gastrointestinal tract alginate binds iron rendering it unavailable for absorption. In a study of ileostomy patients receiving supplemental oral alginate the excreted content of iron via the ileostomy was greater than the oral intake at +12μM Fe compared to control (no alginate) at -8μM Fe[347].

The potential role of alginates in the treatment of various human cancers is yet to be anywhere near fully explored. Their attraction is increased enormously by the fact that they are non toxic, cheap, natural compounds and readily available.

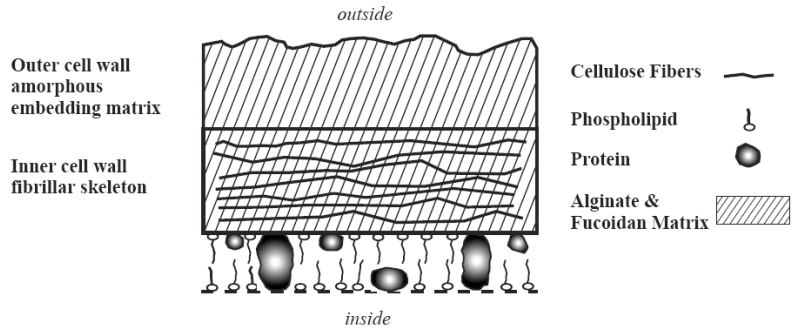
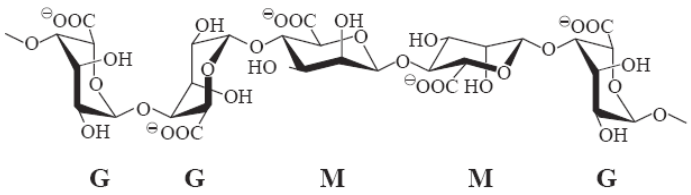


Figure 11. The cell wall of brown algae (reproduced from Davis *et al* 2003[335])

The alginate contained within the cell wall is responsible for the binding of divalent and toxic metals to seaweeds



M = 1,4-linked β-D-mannuronic acid residue; G = α-L-guluronic (G) acid residue

Figure 12. Alginate polymer (reproduced from Davis *et al* 2003[335])

Alginates bind divalent metals to become insoluble salts. In the gastrointestinal tract they sequester iron. Increasing the proportion of α-L-gluronic (G) acid residues increases the iron binding potential.

1.20 Hypothesis

Increasing evidence associates iron excess with the development of oesophageal adenocarcinoma. The expression of iron import and export proteins or haem import proteins in normal oesophagus, Barrett's oesophagus and oesophageal adenocarcinoma is unknown. This study hypothesises that expression of iron and haem transport proteins becomes modulated in the progression to oesophageal adenocarcinoma. Furthermore it is postulated that exposing oesophageal adenocarcinoma cells to iron or haem affect proliferation and a malignant phenotype. Finally chelating iron with alginates may provide a simple and non toxic way of inhibiting iron mediated effects.

1.21 Aims

The aims of this study are to:

1. Characterise the expression of iron and haem transport proteins in oesophageal adenocarcinoma compared to Barrett's and normal oesophageal epithelia

2. Identify the effects of exposing oesophageal adenocarcinoma cell lines to iron and haem-iron

3. Identify if the addition of alginate can rescue any iron or haem mediated effects

4. Identify the contribution of the main haem import protein LRP-1, the haemopexin receptor, to haem import by silencing its expression in oesophageal adenocarcinoma cells

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General, Cell Culture and Molecular Biology Reagents

General, cell culture and molecular biology reagents were obtained from the following sources:

Abcam, Cambridge UK

Anti CDC-14A (mouse IgG, clone DCS-291)

Amersham Pharmacia, Amersham, Buckinghamshire

ECL™ reagent; Hybond PVDF, Hyperfilm x-ray film, RPN800 rainbow ladder

Applied biosystems, Cheshire

96 well optical plate, optical adhesive covers, TaqMan ribosomal RNA control reagent

Appleton Woods, Selly Oak, Birmingham

10ml and 25ml pipettes

Boehringer Mannheim, Lewes, East Sussex

Ampicillin; Mycoplasma detection Kit

Cambrex Bio Science Wokingham, Ltd

ViaLight HS kit (ATP assay)

Chance Propper, Smethwick, West Midlands

Glass coverslips

Difco, West Moseley, Surrey

Bactoagar; Bacto Tryptone; Yeast Extract

Fisher Scientific, Loughborough, Leicestershire

BCA protein assay

FMC BioPolymer, Drammen, Norway

LFR 5/60 alginate, batch number 599345

Frontier Scientific, Logan, UT, USA

Zinc protoporphyrin

Geneflow, Southampton

Acrylamide

Genus pharmaceuticals, Newbury, Berkshire

Ciprofloxacin

Invitrogen, Paisley, Renfrewshire (incorporating Gibco BRL)

Dulbecco's Modified Eagles Medium (DMEM) with Glutamax-1, pyridoxine and 4500mg/L glucose; Foetal Calf Serum; Lipofectamine 2000 and Lipofectamine PLUS reagents; Normal goat serum; Optimem medium; Penicillin and Streptomysin solution; RPMI medium with 25mM Hepes buffer and L-glutamine; Trizol reagent; Trypsin EDTA

Iwaki, Stone, Staffordshire (Subsidiary of Barloworld scientific) 25, 75 and 150cm^2 tissue culture flasks

Mayne Pharma

Desferrioxamine mesilate 500mg

Pall Corporation, Newquay, Cornwall

0.2µm filters

Peptides international, Louisville, Kentucky, USA

Hepcidin peptide -1 0.1mg/ml

Promega, Chilworth Research Centre, Southampton

Dual-Luciferase Reporter Assay System; Nuclease free water; Reverse Transcription System

Radiolabelled haem^{55}Fe, kind gift Prof. A McKie, London

Roche Applied Science, Lewes, East Sussex

BrdU proliferation assay

Sigma Chemical Company Limited, Poole, Dorset

Ammonium acetate; ammonium persulphate; aprotinin; β-mercaptoethanol; calcium chloride; citric acid (tri-sodium citrate); DAB (3,3'-diaminobenzidene tetrahydrochloride) tablets; DAPI (4',6-diaminido-2-phenylindole); deoxynucleotide (dNTP) mix; dimethyl formamide; DNAse I; formalin; GenElute mammalian genomic DNA kit; glycerol; glycine; N-[2-Hydroxethyl]piperazine-n'-[2-ethanesulfonic acid] (HEPES); hydrochloric acid; hydrogen peroxide 30% w/v; hydroquinone (1,4-Benzenediol); immunofluorescence mounting media; isopropanol; lauryl sulphate; lithium chloride; Mayer's haematoxylin 0.1% solution; methanol; paraformaldehyde; polyoxyethylenesorbitan monolaurate (Tween 20); phosphate buffered saline; potassium acetate; potassium chloride; proteinase inhibitor cocktail; sodium acetate; sodium bicarbonate; sodium bisulfite; sodium carbonate; sodium chloride; sodium fluoride; TEMED; trichostatin A; tris hydrochloride; trizma base; Urea; xylene cyanol

TPP, Switzerland

6 well plates for tissue culture

2.1.2 Antibodies

2.1.2.1 Primary Antibodies. Primary antibodies, their source, class, positive controls and optimum working concentrations are described in table 2.1.

Target Antigen	Molecular weight	Source and clone	Antibody class	Positive control	Optimum Dilution
APC C terminus	312Kda	Santacruz 137	Rabbit IgG anti-human	RKO CELLS	WB: 4µg/ml (1/50)
Beta- catenin	95kDa	BD biosciences 13	Mouse IgG anti-human		IF: 10µ/ml WB: 1µl/ml (1/1000)
CDC-14A	67kDa	Abcam ab10536	Mouse IgG anti-human	A431 cell lysate	WB: 0.9µg /ml (1/2000)
Cytokeratin 19	45kDa	Calbiochem 9656	Mouse IgG anti-human		WB: 0.5µg/ml (1/2000)
Dmt1	95kDa	Sigma	Rabbit IgG anti-human	Duodenum	IH: 10µl/ml WB: 1µg/ml (1/2000)
DcytB	30kDa	Sigma 834	Rabbit IgG anti-human	Duodenum	IH: 1/100 WB: 1µg/ml (1/1000)
E-cadherin	120kDa	BD biosciences 34	Mouse IgG anti-human		IH and IF: 1.5 µg/ml WB: 0.1 µg/ml (1/2000)
Ferroportin	65kDa	Sigma 3566	Rabbit IgG anti-human	Duodenum	IH: 1/100 WB: 1µg/ml (1/1000)
Ferritin	40kDa dimer, 20kDa subunit	Sigma	Rabbit IgG anti-human	Liver	IH: 1/1000 WB: 0.5µg/ml (1/2000)
HCP	50kDa	Kind gift Prof. A McKie	Rabbit IgG anti-human	Duodenum	IH: 1/50 WB: (1/500)
Hepcidin-25	32kDa	Abcam ab31877	Rabbit IgG anti-human	Liver	WB: 0.5µg/ml (1/1000) IH: 1/100
Hephaestin	161kDa	Alpha diagnostics 11-A	Rabbit IgG anti-human	Duodenum	IH:20µl/ml WB: 5µg/ml (1/200)
LRP1	84kDa	Abcam ab694095	Mouse IgG anti-human	Liver	IH: 1/100; IF: 1/50 WB: (1/1000)
TfR-1	190kDa dimer, 95kDa subunit	Zymed 236-15375	Mouse IgG anti-human	Breast cancer	IH: 1/40 WB : 0.5 µg/ml

Table 2. 1. Primary antibodies

(IH= Immunohistochemistry; IF= Immunofluorescence; WB= Western Blotting).

2.1.2.2 Secondary Antibodies

Secondary antibodies were obtained from the following sources, and used at the given concentrations for appropriate applications (IF: Immunofluorescence; IH: Immunohistochemistry; WB: Western Blotting):

Amersham Pharmacia, Amersham, Buckinghamshire

Peroxidase conjugated anti-mouse IgG (WB: 1:10,000); Peroxidase conjugated anti-rabbit IgG (WB: 1:10,000)

Dako, Denmark House, Ely, Cambridgeshire

Dako REAL™ EnVision™ Detection System, Mouse/Rabbit kit (IH: all components used at 1:1000)

2.1.3 Oligonucleotides

The following oligonucleotides were used in qRT-PCR reactions (F=Forward, R=Reverse).

	Probe (5'FAM 3'TAMRA)	Forward Primer, 5'-3'	Reverse Primer, 5'-3'
DMT-1	CTGCATTCTGCCTTAGT CAAGTCTAGACAGCTAA ACC	AGCTGTCATGCCACACA AC	GCTTCTCGAACTTCCTGCTT ATTG
Ferroportin	AGGATTGACCAGTTAAC CAACATCTTAGCCCC	AGCAAATATGAATGCC ACAATACG-	CAAATGTCATAATCTGGCC AACAG
Ferritin H	CCAACGAGGTGGCCGA ATCTTCCTT	GGAACATGCTGAGAAA CTGATGAA	CATCACAGTCTGGTTTCTTG ATATCC
Hephaestin	ACA GTG ACA TAG TGG CTT CCA GCT TCT TAA AGT CTG	GGAAGAAATGTCATCA CGAACCA	TCC CCC TAT CCG GTT CTT G
TFR1	AAA GAC AGC GCT CAA AAC TCG GTG ATC ATA G	CGT GAT CAA CAT TTT GTT AAG ATT CA	CCA CAT AAC CCC CAG GAT TCT
Dcytb	CCAGGGCATCGCCATCA TCGT	CATGGTCACCGGCTTCG T	CAGGTCCACGGCAGTCTGT A
Hemopexin receptor CD 91	TGC CAT TTA CTC AGC CCG TTA CGA CG	TGG ATT GAC GCC GGT CAG	CCC GAA GCA CCT CCA TGT
HCP	ATG GGT TGC TTT TCC TGT CAT TAG TCA TCA CA	CTA TCA CGC CTC TCA TGT TCA CA	GGA GAG TTT AGC CCG GAT GAC
HO-1	TGA CCC GAG ACG GCT TCA AGC TG	CTG AGT TCA TGA GGA ACT TTC AGA AG	TGG TAC AGG GAG GCC ATC AC
Hepcidin	AGCTGCAACCCCAGGA	CCCACAACAGACGGGA CAA	TCTGGAACATGGGCATCC
IRP-1	ACTCCTATGGCTCCCGC CGAGG	CCTGCCTAACTCCACGA GAATT	TGCCATGACGGCGTCAT
IRP-2	TTTGACAAACAGAGGC CTTACCCC	AGGAATAGTGCTGCCG CTAAGT	TCGAGCTCCGTAAGAGTTG AATT
MYC	CAGCACAACTACGCAG CGCCTCC	TCAAGAGGTGCCACGT CTCC	TCTTGGCAGCAGGATAGTC CTT
E-cadherin	AAA TTC ACT CTG CCC AGG ACG CGG	GGC GCC ACC TCG AGA GA	TGT CGA CCG GTG CAA TCT T

Table 2. 2. Probes and primers used in qRT-PCR reactions

2.1.4 Tissue Samples

Selection of control tissue

For oesophageal adenocarcinoma the most suitable control tissue was considered to be matched Barrett's epithelium from the same patient. For benign Barrett's samples matched gastric and oesophageal epithelium were used as control tissues.

2.1.4.1 Fresh Material

Oesophageal cancer resection specimens. Samples (n=24) of oesophageal adenocarcinoma matched with normal gastric and oesophageal mucosa from the same subject were collected during surgery and stored at -80°C.

11 of these adenocarcinoma samples had Barrett's tissue surrounding part or all of the adenocarcinoma and sections of the Barrett's were also stored.

Normal duodenum, liver and breast carcinoma were used as positive controls where appropriate (see Table 2.1 above).

Biopsy samples. In order to obtain Barrett's tissue in patients without adenocarcinoma biopsies were obtained at endoscopy from patients undergoing endoscopic surveillance of Barrett's epithelium. Samples of Barrett's mucosa (n = 13) were confirmed on histological assessment in all cases.

2.1.4.2 Archived paraffin embedded material

Paraffin sections of oesophageal adenocarcinoma with associated Barrett's and normal mucosa present in the same section (n=16), Barrett's with low grade dysplasia (LGD) (n=10), Barrett's with high grade dysplasia (HGD) (n=20), and biopsy specimens of Barrett's with no evidence of dysplasia with normal mucosa in the same section (n=26)

were selected for immunohistochemistry. These sections were identified within the archived tissue bank, Dept of Pathology, Queen Elizabeth's Hospital Birmingham and processed for our use. Archive material was screened by Dr Phillipe Taniere, consultant histopathologist Queen Elizabeth's Hospital Birmingham, to confirm the samples contained the relevant tissue types.

2.1.4.3 Tissue microarray

76 oesophageal adenocarcinoma samples (from a separate cohort of patients to those above; kindly provided by Jonathan Bury, Sheffield) were presented on three slides for use in immunohistochemical experiments. These samples were used to evaluate the association between iron transporter expression and prognostic factors. The following variables were assessed: tumour differentiation (well, moderate, poor as assessed by the original reporting consultant pathologist), T stage of the tumour (T1: tumor invades lamina propria or submucosa; T2: tumor invades muscularis propria; T3: tumor invades adventitia; T4: tumor invades adjacent structures), serosal involvement, number of lymph nodes assessed (N0 – no lymph node metastases; N1 – 1 or more resected lymph nodes possess metastatic oesophageal adenocarcinoma), the presence of Barrett's metaplasia and vascular invasion.

To facilitate the screening of multiple tissue blocks from each patient, tissue microarrays were prepared containing 3 representative samples of tumour per patient. Sections were cut from each array block onto charged slides (Surgipath, UK) and heated for 1 hour at 60^0C followed by processing for immunohistochemistry and scored as described below.

2.1.5 Cell Lines

The following cell lines were used during the experiments:

OE21[348]: A human oesophageal squamous carcinoma cell line, also known as JROECL21, was established in 1993 from a squamous carcinoma of mid oesophagus of a 74 year-old male patient. The tumour was identified as pathological stage IIA(UICC) and showed moderate differentiation.

OE33[348]: A human oesophageal adenocarcinoma cell line, also known as JROECL33, was established from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year old female patient. The tumour was identified as pathological stage IIA (UICC) and showed poor differentiation

SEG1[349]: A human 'moderately differentiated oesophageal adenocarcinoma' (see below) cell line

OE19[348]: The cell line OE19, also known as JROECL19, was established in 1993 from an adenocarcinoma of gastric cardia/oesophageal gastric junction of a 72 year old male patient. The tumour was identified as pathological stage III (UICC) and showed moderate differentiation. The cell line OE19 expresses HLA-A, -B and -C antigens (MHC class I) constitutively. Treatment with interferon-gamma induced the expression of ICAM-1 (CD54). Expression of HLA-DR (MHC class II) on interferon-gamma addition was only measured in a sub-population of OE19. The cells express epithelial cytokeratins and are tumourigenic in nude mice.

MCF7: A human moderate-well differentiated breast carcinoma cell line.

THP1: Derived from a paediatric patient with acute monocytic leukaemia. Cells grow in suspension and were maintained at 2-9x10^5 cells/ml.

Hep G2: Derived from a 15year old male with well differentiated hepatocellular carcinoma.

OE19 and OE33 cell lines were used for experiments as a model of oesophageal adenocarcinoma. SEG1 cells were used for the initial 30 months of the project. Towards the end of the project it has been identified the line is a lung cancer derived cell line[349] despite being widely used in oesophageal adenocarcinoma research. It were felt that any data derived using this cell line would be inaccurate and misleading and was thus disregarded and removed from data interpretation, analysis and reporting. At this point the OE19 cell line was obtained and all previous experiments with SEG1 cells repeated. OE21 cells were used in some experiments for interest into the behaviour and expression profile of iron transport proteins in a squamous tumour but the data is not presented in this body of work.

MCF7 cells were used as positive controls for TfR1 expression. THP1 cells were used as positive controls for *in-vitro* experiments using hepcidin. HepG2 cells were used as positive controls for *in-vitro* experiments assessing haem transporter expression.

2.2 METHODS

2.2.1 Immunostaining of paraffin sections

2.2.1.1 Immunohistochemistry

Slide mounted sections were dewaxed by immersion in xylene twice followed by 100% ethanol twice, each for five minutes. The slides were placed in a hydrogen peroxide/methanol (1:10) mixture for 5 minutes in order to remove endogenous peroxidase activity. This was followed by 15 minutes microwave antigen retrieval using 0.01M Citric acid pH 6.0 (3x5 minutes with topping up of citric acid as required). Cooled sections were blocked with normal goat serum (20% v/v with antibody diluent) for 30 min and then incubated for 1 hr with appropriate primary antibody (above). Following extensive washing sections were then incubated with the appropriate peroxidase linked secondary antibody and immunoreactivity visualised using diaminobenzidine reagent followed by counterstaining with haematoxylin. Sections were then washed in tap water, taken through ethanol for 10 minutes and xylene for 10 minutes, then mounted in Depex. Controls included: duodenal mucosa as positive control for DMT1, DcytB, ferroportin, Hephaestin TfR and HCP, liver for ferritin, LRP1 and hepcidin, and omission of primary antibody as a negative control. Where the peptide was available a further control existed of antibody-peptide solution, pre mixed and left at room temperature for 30 minutes.

Images were visualised using a Nikon Eclipse E600 microscope and digital image taken using a Nikon DXM1200F camera (Surrey, UK). Nikon ACT-1 version 2.62 software was used for image acquisition (Surrey, UK).

2.2.1.2 DAB-enhanced Perls' Prussian blue staining of iron

Paraffin sections were dewaxed, washed in dH_20 and incubated in a 1:1 solution of 4% (v/v) HCl and 4% (w/v) ferrous cyanate for 30 minutes. Following incubation in PBS for 5 minutes, sections were incubated in DAB (Diaminobenzidine) Chromogen Solution 50X (Dako ChemMate)(1:200) for 15 minutes followed by a further incubation for 15 minutes in DAB (1:50) in substrate buffer (Dako ChemMate). Sections were either counterstained with haematoxylin for 30 seconds or processed for immunocytochemistry.

2.2.1.3 Immunofluorescence

Paraffin sections were dewaxed and dehydrated as in 2.2.1.1 then blocked in PBS with 5% normal goat serum and 1% bovine serum albumin (BSA) for 20 minutes. Primary antibodies were added to sections for 1 hour at room temperature. Sections were washed with PBS for 3 x 5 minutes and incubated for 1 hour with FITC or Texas Red conjugated secondary antibodies. Sections were washed with PBS (3 x 5 min) and nuclei were stained with DAPI (4,6-diaminido-2-phenylindole) at a concentration of 0.1mg/ml for 1 minute. Sections were washed in PBS (3 x 5 min), allowed to air dry, and mounted in immunofluorescence mounting medium (Sigma).

2.2.1.4 Evaluation of immunostained sections

Immunostained material was blindly scored by three independent observers – Keith Roberts, Chris Tselepis, Sharon Hughes. Phillipe Taniere, consultant histopathologist at Queen Elizabeth Hospital, also evaluated slides to ensure staining was specific and occurred at the known cellular location of protein of interest. Staining intensity was scored on two factors: [1] a scale of 0 to 3 (0 = absent, 3 = strong) and [2] percentage of cells within the microscopic field of view positively stained (0 = no cells, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of cells). These two scores were multiplied to give a total score of 0-12 (after Martino et al[350]).

2.2.2 Electrophoresis and western blotting

2.2.2.1 Sample preparation

Samples were derived from two sources: frozen tissue samples, and cultured cells.

Tissue specimens and cell pellets were lysed into radioimmunoprecipitation assay (RIPA) buffer (1%NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride], 2µg/ml aprotinin, 1µg/ml pepstatin and 10 µg/ml leupeptin). 1% (v/v) β-mercaptoethanol and 0.001% (v/v) bromophenol blue were added to the samples which were then heated to 100^{o}C for 5 minutes.

Cell lysates were prepared from cell culture dishes containing a confluent cell monlayer by initially washing with phosphate buffered saline then adding 0.5ml ice-cold RIPA buffer (0.1% (w/v) SDS, 0.5% (w/v) Deoxycholic Acid, 1% NP40, 10µg/ml aprotinin; 5 mM EDTA, 150 mM NaCl, 50 mM Tris HCl pH 7.5, 0.2mM PMSF). Cells were scraped into the RIPA buffer and the resultant suspension was aspirated into a 1.5 ml

Eppendorf tube. This was rotated on a wheel for 30 minutes at 4°C and then centrifuged at 11,000g for 10 minutes. The supernatant was decanted, protein content determined by BCA protein assay and again adjusted to 1.5 mg/ml of protein in Laemelli sample buffer and boiled for 5 minutes.

2.2.2.2 Protein normalisation

Protein content was determined by performing a western blot analysis using 10µl of each sample. Cytokeratin 19 antibody was used and protein content of each sample estimated. Further western blots were produced varying the amount of sample to achieve uniform band densitometry. The volume of each sample required to produce these bands was recorded and used in subsequent experiments.

2.2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

BioRad Protean–II mini-gel apparatus was used and the appropriate percentage resolving gel was poured (see Table 2.3 for recipe). The stacking gel was added, combs inserted and the wells equilibrated with electrophoresis running buffer (0.192 M glycine, 0.01% (w/v) SDS, 25 mM Tris HCl pH 8.3) prior to use. Appropriate volume of sample (see above) was loaded into each well, in addition to wide range kaleidoscopic pre-stained standards (5µl) to determine protein molecular weights. Electrophoresis was carried out at 200V until the dye front had reached the bottom of the gel.

Constituent	Stacking gel	Separating gels			
	4%	5%	8%	10%	12.5%
Water	3.7ml	6.75ml	4.8ml	3.5ml	1.9ml
Acrylamide	1.3ml	3.25ml	5.2ml	6.5ml	8.1ml
Buffer	5ml 0.2% SDS; 0.25M Tris HCl, pH6.8	10ml 0.2% SDS; 0.75M Tris HCl, pH 8.8			
Ammonium Persulphate	30mg	30mg			
TEMED	60µl	60µl			

Table 2. 3. SDS-Polyacrylamide Resolving Gel Composition

2.2.2.4 Western Blotting: Transfer of SDS-PAGE gels and protein detection

Proteins separated by SDS-PAGE were wet transferred directly onto Hybond PVDF membranes pre-soaked in methanol for 1 minute. SDS-PAGE gels were initially soaked in electrophoresis transfer buffer (48mM Tris HCl; 39mM glycine, 20% (v/v) methanol, 0.0375% (w/v) SDS) for 5 minutes. Transfer was performed at 100V for 1 hour, with constant cooling from an ice pack within the transfer apparatus.

Following transfer, the membrane was blocked for 30 minutes in 10% (w/v) dried skimmed milk in Tris-buffered Saline Tween (TBST, 10mM Tris-Cl pH 8.0, 150mM NaCl, 0.05% Tween-20). This was followed by incubation with the primary antibody for 1-2 hours (dependent upon antibody optimisation) at room temperature. Membranes were then washed in TBST (6 x 5 min), incubated with secondary peroxidase conjugated antibody for 30 min at room temperature, then washed again in TBST (6 x 5min). The membrane was then exposed to ECL^{TM} reagent for 1-240 minutes (dependent upon optimisation) prior to visualisation on Kodak X-OMAT AR film, which was subsequently developed using an X-OGRAPH X2 automatic developer.

2.2.3 Cell Culture

2.2.3.1 Routine cell culture

All cell culture procedures were performed in a laminar flow tissue culture cabinet using aseptic technique and cells were placed in an incubator at 37°C in a 5% CO_2 atmosphere. Cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) with Glutamax-1, pyridoxine and 4500mg/L glucose supplemented with 10% (v/v) Foetal Calf Serum (FCS), 50U/ml penicillin and 50µg/ml streptomycin (OE33, SEG1, OE19) or RPMI medium supplemented with FCS, penicillin and streptomycin at the same concentrations as in DMEM (OE21, MCF7, THP1). Adherent lines were passaged at ~90% confluence by aspirating the culture medium, washing in medium without FCS and incubating with 3 ml of 0.05% (w/v) trypsin EDTA until cells had detached. 5 ml of culture media was added and cells were disaggregated by trituration and then centrifuged at 250g. The cell pellet was either re-suspended in culture media and reseeded to typically a 30% confluence or was re-suspended in 1 ml of 10% (v/v) dimethyl sulphoxide (DMSO) in FCS and placed in a cryovial for cryopreservation. Vials were kept at –70^{o}C for 3 days before transfer to liquid nitrogen storage. Frozen cells were recultured by rapid warming of the cryovial in a 37^{o}C waterbath and the cells were then washed and suspended in pre-warmed culture media. Cells were then seeded into tissue culture flasks and grown in the standard manner.

2.2.3.2 Iron and haem loading of cell lines

Cells were plated out at $1x10^5$ cells per ml to achieve an initial 50-60% confluence into 6-well (3ml of medium per well) or 96-well plates (100µl per well) depending upon the experiment protocol. Cells were allowed to adhere to the plastic overnight and the next day the medium was replaced with fresh medium as follows:

Inorganic Iron: 0.14g iron sulphate was dissolved in 50ml dH_2O to give a stock solution concentration of 0.01M. A 100µM solution was used in experiments and thus 1µl of stock solution was added to each ml of medium in addition to ascorbate (0.1µl/ml). The ascorbate maintains iron in the reduced ferrous Fe^{2+} form.

Organic Iron stock haem solution (1.5mM) was prepared from 0.02g haem dissolved in just enough 0.1M NaOH (typically 0.5ml) and made up to 20ml with dH_2O. To achieve a 50µM solution 3.33 ml of stock haem was added to each 100ml of culture medium.

2.2.3.3 Iron chelation

Desferroxamine was used at 250µM in normal culture medium.

2.2.3.4 Supplementation with alginate or Gaviscon

LFR 5/60 alginate was used at a concentration of 1mg/ml. Gaviscon was used at 0.1% (v/v).

2.2.3.5 Zinc protoporphyrin uptake studies

Zinc protoporphyrin (ZnPP) is taken into cells via the haem carrier protein 1[351] and is used to demonstrate cellular uptake of haem. Under a fluorescent light ZnPP appears red.

A stock 0.3mM solution of ZnPP was produced with 1% ethanolamine (v/v) and 10 mg/ml of bovine serum albumin. This solution was buffered to pH 7.4 with 1.0M HCl and kept in the dark at 4°C.

Working solutions (20, 5 and 1µM) were made by diluting the stock with uptake solution (50mM HEPES, pH 7.4, 130mM NaCl, 10mM KCl, 1mM $CaCl_2$, and 1mM $MgSO_4$).

Tissue culture medium was aspirated off cultured cells which were then gently washed with uptake solution. All subsequent steps were performed in the dark or in a tissue culture hood that was as close to total darkness as practical. The working solution was placed on the cells and incubated for varying lengths of time (0.5, 2, 4, 24 hours) at 1, 5 and 20 µM. Control consisted of uptake solution only. To terminate the uptake process, the incubation solution was removed, and the cells were washed three times with ice-cold 5% bovine serum albumin (w/v) in uptake buffer followed by two more washes with uptake buffer. The bovine serum albumin incubation was done to remove surface bound ZnPP not internalised by the cells.

Cells were viewed under green light and images taken via a Zeiss AxioCam 5 attached to the microscope and stored using AxioVision V4.5 software. All fluorescence studies were performed with identical camera settings and a rhodamine-Texas red filter. All cells received the same fluorescence exposure, with each region of the cells being exposed to minimal fluorescence excitation only once, to ensure that the images

obtained from a given experiment were directly comparable. Control images were taken of the same field under normal light. Overlaying the two images demonstrated the cellular location of the ZnPP.

2.2.3.6 Radiolabelled haem^{55Fe} uptake

Haem^{55Fe} was a kind gift from Prof. AT McKie. Stock solution of 1.79mg haem^{55Fe} was dissolved in 100µl of methanol:cholorform:acetic acid (89:10:1); (specific activity of 0.28mCi/mg or 0.5mCi/100µl).

To maintain haem in a monomeric form and to avoid precipitation a working solution was made by a 1/100 dilution into stock haem solution described above. This was prepared fresh prior to each experiment. By dilution with culture medium a final solution of 50µM haem was used.

Cells were cultured in the presence of haem^{55Fe} for two hours following which the medium was aspirated and 0.5ml added to 4.5ml of scintillant. The adherent cells were gently washed with sterile PBS twice with subsequent removal by the addition of trypsin. 10% of this solution was kept for a protein assay whilst the remaining 90% was centrifuged at 3500g for 5 minutes. The cell pellet was then resuspended in 0.5ml 0.1% Triton X which was added to 4.5ml of scintillant.

2.2.3.7 Growth of cells with hepcidin

Cells were grown in medium with hepcidin at a final concentration of 1µM for 24 hours.

2.2.3.8 MTT cell viability assay

The MTT assay was used to quantify viable cells following a period of tissue culture. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme within viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The colour is quantified using a colorimetric assay and read on a multiwell scanning spectrophotometer (ELISA reader).

MTT was dissolved in PBS at 5mg/ml and filtered to sterilize and remove any insoluble residue present. 20µl of MTT was added to each 100µl well following a defined length of tissue culture (i.e. 24 or 48 hours). The plates were then incubated for 4 hours at 37 ^{o}C. Tissue culture media was removed and 80µl of DMSO were added. The plates were left for 20 minutes and read on a Molecular Devices VersaMax microplate reader at 490nm wavelength.

2.2.3.9 BrdU proliferation assay

During cell proliferation the pyrimidine analogue BrdU (5-bromo-2'-deoxyuridine) is incorporated into the cellular DNA in place of thymidine. Following a defined period of time allowing cell proliferation (2-24 hours) the cell DNA is denatured to improve the accessibility of the BrdU to antibody. Anti-BrdU binds to newly synthesized DNA and with the addition of substrate a colour change is observed – the intensity is

proportional to the number of cells proliferating. 10 µl of BrdU were added to cells cultured in 100 µl of media in a clear 96 well plate. Cells required 2 hours of exposure to BrdU. The media/BrdU were removed and 200µl of DNA denaturing solution added (100% ethanol) to each well with the plate incubated at room temperature for 30 minutes. The ethanol was removed, 100µl per well of anti-BrdU solution added and incubated for 90 minutes at room temperature. This solution was removed and 3 cycles of washing individual wells performed with the washing solution provided. The washing solution was replaced with 100µl/well of substrate and the plate incubated at room temperature for 5-30 minutes until a colour change was observed. The plates were read in a Berthold technologies Centro LB 960 luminometer at a wavelength of 490nm.

2.2.3.10 Ferrozine iron (Fe^{2+}) assay

Ferrozine assay was used to quantify inorganic iron within cells following tissue culture. Adherent cells were mobilised using trypsin and centrifuged at 225g for 5 minutes. Trypsin was aspirated off the cell pellet following which 100µl of Hepes buffered saline was used to resuspend the cells, 10µl of this solution was removed for protein quantification. 200µl of 20% (w/v) trichloroacetic acid in 4% (w/v) sodium pyrophosphate was added and the solution kept at 100°C for 10 minutes following which each ependorf was centrifuged at 13,000g for 5 minutes. The supernatant was removed and kept. The tissue pellet was again resuspended in 100µl of trichloroacetic acid sodium pyrophosphate and then kept at 100°C for 10 minutes followed by centrifuge at 10,000rpm for 5 minutes. The supernatant was removed and added to the previous supernatant. This process was repeated 2 more times. 200µl of the

supernatant was removed and added to 100µl of 0.23M sodium ascorbate, 80µl of 10mM ferrozine and 420µl of 2M sodium acetate. 200µl of this solution was added to a clear 96 well plate (each in triplicate). A blank set of 3 x 200µl wells and standard set of 3 x 200µl wells were used for control purposes. Blank = 200µl of trichloroacetic acid sodium pyrophosphate, 100µl of ascorbate, 80µl of ferrozine and 420µl of sodium acetate. Standard = 1µl of $FeCl_3$ with 200µl of trichloroacetic acid sodium pyrophosphate, 100µl of ascorbate, 80µl of ferrozine and 420µl of sodium acetate. Plates were read using Molecular Devices VersaMax microplate reader.

Protein assay. 10µl of cell suspension was obtained during the preparation of the ferrozine assay for protein assessment (see above). This was used to quantify and express the results derived from the ferrozine assay as nmol iron/mg protein.

Standard concentrations of protein (albumin) were prepared according to manufacturers instructions using the BCA™ protein assay kit (Pierce). These concentrations were 0.25, 0.5, 0.75, 1 1.5 and 2 mg/ml along with a blank control. The 10µl of protein available from cell samples was diluted 1 in 10 to achieve 100µl.

25µl of each standard and unknown sample were transferred into a microplate well in triplicate. 200µl of the working reagent was added to each well and the plate mixed thoroughly on a plate shaker for 30 seconds followed by incubation at 37°C for 30 minutes. After this period the plate was allowed to cool to room temperature following which absorbance was read at or near 562 nm on a Molecular Devices VersaMax microplate reader.

2.2.3.11 Soft agar colony forming assay

This assesses the anchorage independent ability of single cells to grow. Normal dividing cells require contact with neighbouring cells or basement membrane, without this they are programmed to undergo apoptosis. Anchorage independent growth correlates strongly with tumourigenicity and invasiveness. After preparing a base gel layer cells were prepared in a single cell gel suspension following trypsinisation and plated out at a concentration of $1x10^4$/ml. To ensure cells are single cells they were passed through a 50µm cell sieve. The base gel comprised 3.3% agar containing 5ml 2xDMEM, 10ml 1xDMEM and 2ml foetal calf serum and the superficial gel from 1.8% agar containing 6.7ml 2xDMEM, 3.6ml 1xDMEM and 1.7ml foetal calf serum. Each regime was conducted in 6 well plates.

The base gel was warmed to 45°C in a water bath. 2ml was plated into each well of a 6 well plate in a tissue culture hood (carefully to avoid bubbles in the solution). It was allowed to set and cool for 30 minutes. The superficial gel was kept warm at 37°C and the cells were added. 0.5ml of the superficial gel was placed on top of the base gel.

The six well plates were kept in a humidified incubator at 37°C for 14 days after which colonies are counted. Images were visualised using a Zeiss Axiovert 40 CFL microscope and camera with Axiovision Rel. 4.6 imaging software (N=6 for each well, 36 images total). Number of colonies and surface area of colonies were recorded using the above software.

2.2.3.12 Wound healing assay

When wounded or scratched, cell monolayers respond to the disruption of cell-cell contacts with an increased concentration of growth factors at the wound margin by healing the wound through a combination of proliferation and migration; these processes reflect the behaviour of individual cells as well as the properties of the cell sheet as a surrogate tissue. The wound heals in a stereotyped fashion – cells polarize toward the wound, initiate protrusion, migrate, and close the wound.

Cells were cultured on a 6 well plate until 100% confluent. A mark was made along the underside of each well (for orientating when measuring the wound size) and a 'wound' created in each well that crossed the mark. The wound was made using a 100µl pipette tip at 45° to the base. The cells were then washed with PBS and control or treatment media added. The wound was imaged using Zeiss Axiovert 40 CFL microscope at a 20x magnification and digital images recorded (N=12, two images per wound, 6 wells) with Axiovision Rel. 4.6 imaging software. The total wound surface was measured (N=12) at each time point. Mean wound surface area and SD were thus calculated for each experimental regime at each time point. The 6 well plates were replaced in the incubator and every subsequent 12 hours the wounds were photographed using the marks on each well to ensure the same part of the wound was being imaged. Measurement continued until the wound had healed.

2.2.4.1 Cell Transfection

Transient (48-72 hours) cell transfection was undertaken. This was carried out using lipofectamine PLUS reagents, a liposomal delivery system. Optimum number of cells seeded and plasmid DNA:lipofectamine:PLUS ratios for highest transfection efficiencies had previously been ascertained by workers in the lab for the cell lines used (J Boult, personal communication). 5 x 10^5 cells were trypsinised and plated into a 6-well dish and incubated overnight, such that they reached confluence of ~90%. Optimised quantities of plasmid DNA (2µg OE33, 4µg OE19) were diluted in 100µl of Optimem medium with PLUS reagent (8µl OE33, 16µl OE19). Lipofectamine (12µl OE33, 16µl OE19) was then diluted in 100µl Optimem and incubated at room temperature for 15 minutes. Both aliquots of Optimem were then combined, incubated at room temperature for 15 minutes and then added dropwise to wells containing cells (media removed, cells washed with PBS and 800µl Optimem added), with constant agitation.

2.2.4.2 Plasmid Preparation

Plasmids for transfection were grown in transformed *E. Coli* in Luria-Bertani (LB) medium (1% (w/v) Tryptone Peptone, 0.5% (w/v) Yeast Extract, 1% (w/v) NaCl) supplemented with ampicillin (50µg/ml)). Plasmids were stored in sterile glycerol (1:1 ratio) at –70^{o}C. Two culture sizes were grown for the purposes of the experiments outlined: (a) Cultures for 'mini' preps consisting of 10µl of glycerol plasmid stock in 5ml LB medium. (b) Cultures for 'maxi' preps consisting of 100µl of glycerol plasmid stock in 50ml LB medium. Both culture types were grown by shaking at 18g in a 37^{o}C incubator overnight. Purification of DNA from both 'mini' and 'maxi preps was achieved using an alkaline lysis method.

2.2.4.2.1 'Maxi' plasmid preparations

This purification method was used to produce large volumes of high purity plasmid for transfection purposes. After overnight culture bacteria were pelleted by centrifugation at 1,500g for 10 min at 4°C then resuspended in 4 ml GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl, pH 8) and incubated on ice for 5min. Cells were lysed by vigorous mixing with 8 ml 0.2M NaOH, 1% SDS then left on ice for 5 min, followed by the addition of 30% (w/v) potassium acetate in 11.5% (v/v) acetic acid, vortexing and leaving on ice for 10min. Bacterial cell debris was removed by centrifugation at 1,500g for 15 min at 4°C with the supernatant being filtered through a 0.45 µm filter. DNA was precipitated by the addition of 17ml isopropanol and chilling at –70°C for 15 minutes. DNA was then pelleted by centrifugation at 1,500g for 15 minutes. The supernatant was discarded and excess fluid removed by desiccation. The pellet was resuspended in 2 ml TE (0.1 mM EDTA, 10 mM Tris HCl, pH 7.5) buffer following which 2.5ml of 4.4M LiCl was added to precipitate DNA. This was incubated at 4°C for 1 hour and then centrifuged at 1,500g for 10 minutes. The supernatant was incubated with 20ml of ethanol at 4°C for 10 minutes and again centrifuged 1,500g for 10 minutes. The pellet was washed in 70% ethanol and allowed to air dry before resuspending in 1.5ml TE. 100µg of RNAse A, preheated to 37°C, was added. After incubation at 37°C for 15 minutes, 20µl of 10% SDS was added and the solution heated to 70°C for 10 minutes. An equal volume of phenol was added, vortexed and centrifuged at 2,000g for 1 minute. The upper aqueous layer containing DNA was aspirated and an equal volume of chloroform were added, vortexed and centrifuged at 2,000g for 1 minute. The upper aqueous layer was aspirated and two further phenol-chloroform extractions were

performed. Sodium acetate (10% of the volume of DNA) and ethanol (2.5x total volume of DNA plus sodium acetate) were added to precipitate DNA, which was pelleted by centrifugation at 8,500g. The DNA pellet was washed with 70% ethanol, allowed to air dry and then resuspended in nuclease free water.

2.2.4.2.2 'Mini' plasmid preparations

This purification method was used to produce small volumes of low purity plasmid for the purpose of restriction digest analysis of colonies derived from DNA sub-cloning, and is an abbreviated version of the protocol for 'maxi' preps (all solutions used are of the same composition as those used in 2.2.4.2.1). 1.5ml of an overnight culture was centrifuged at 1,500g for 1 minute, then resuspended in 100µl GET buffer and incubated on ice for 5 minutes. Bacteria were lysed by shaking in 200µl NaOH/SDS for 5 minutes then vortexed in 150µl potassium acetate and placed on ice for 5 minutes. Bacterial debris were pelleted by centrifugation at 1300rpm for 5minutes at 4^{o}C, then to the supernatant a phenol and chloroform extraction was performed as described in 2.2.4.2.1. 2 volumes of ethanol were added to precipitate DNA and the solution left to stand at room temp for 5 minutes. DNA was precipitated by centrifugation at 8,500g for 5 minutes, washed with 70% ethanol, allowed to air dry and then resuspended in 50µl nuclease free water containing RNAse A (20µg/ml). 10µl of resuspended DNA was used in each restriction, see 2.2.4.2.1.

2.2.4.3 Reporter plasmid transfections

OE19 and OE33 cells were optimised for transfection with lipofectamine PLUS as described above. The day following the transfection solution was removed by aspiration, and cells were either processed for reporter assay, or further stimulated with the appropriate media. Those cells which were further stimulated were cultured for a further 24 hours at 37°C with 1ml of stimulant media before extraction for the reporter assay.

Plasmid construct	Plasmid origin
Reporter plasmids	
pGL3/TOP	Dr Shiva Akbarzadeh, University of Birmingham, UK
pGL3/FOP	Dr Shiva Akbarzadeh, University of Birmingham, UK
Other plasmids	
pCDNA3/β-catenin-S37A	Dr Chris Tselepis
pCDNA3	Dr Jessica Boult, University of Birmingham, UK

Table 2. 4. Plasmids used during transfection and their origin

2.2.4.4 Measurement of promoter activity

The vector pGL3basic has a firefly Luciferase cDNA located downstream of a multiple cloning site. Promoter activity from the vectors (pGL3/TOP, pGL3/FOP or empty vectors) can be inferred by measuring firefly luciferase activity. Co-transfecting the vector pRLTK, in which a renilla luciferase cDNA is located downstream of a

constitutively active herpes simplex virus-thymidine kinase promoter, and measuring renilla luciferase activity in the same wells of cells that firefly luciferase is measured, allows normalisation of firefly luciferase activity for transfection efficiency. The dual-luciferase reporter activity system, in which firefly and renilla luciferase activities were sequentially measured, was used to analyse promoter activity of pGL3/TOP. Cells grown in 24-well plates were transfected as described above. Growth medium was removed, and cells were washed with 500µl of sterile PBS. The PBS was then aspirated and 100µl of passive lysis buffer (PLB) were added to the cells, then the cells were gentle shaken for 30 minutes. The suspension was aspirated and pipetted several times to obtain a homogenous solution. 20µl of these lysed cells were then added in triplicate into an opaque white 96-well plate. 100µl of luciferase assay reagent II were added to each well, and then mixed for 10 seconds by shaking. The luminescence was then measured for 10 seconds on a Wallac Victor 2 1420 multilabel counter, the final value representing relative luciferase activity (RLU) of the firefly luciferase. 100µl of Stop and Glo® reagent were added to each well and mixed for 10 seconds by shaking before the luminescence of renilla luciferase was measured for 10 seconds, the final value representing the RLU of the renilla luciferase. The RLU of firefly luciferase was then normalised to the RLU of the renilla luciferase to give a relative reporter activity value for each well. These could then be compared to control and a normalised RLU expressed as a percentage of this.

2.2.5 RNA interference

RNA interference (RNAi) is a technique of inhibiting gene function by inhibiting the translation of target mRNA. Short hairpin RNAs (shRNAs) are a method of RNAi where

the antisense nucleotide to the mRNA of interest is transcribed and, due to the construct design, a hairpin is created. The cell recognises this double strand RNA (dsRNA) as potential viral dsRNA. The shRNA is cleaved by the action of RNA-induced silencing complex (RISC) and the endonuclease DICER into short (20-25 base pair) dsRNA sequences. The anti guide strand is degraded leaving an antisense RNA sequence bound to the RISC complex which is free to bind to its complementary mRNA. This combination results in destruction of the complex or directly blocks the transcription of the related protein.

2.2.5.1 Lentiviral cell transfection

This technique uses a viral vector to overcome transfection resistance associated with conventional reagents. The transfection efficiency of OE33 cells never exceeded 1% of the cell population despite using several reagents (lipofectamine 2000, lipofectamine-plus, fugene, turbofect). As a consequence a GIPZ lentiviral vector was used to insert shRNA into the OE33 cell genome. There are several important aspects to using the viral vector: once incorporated into the genome of interest the viral genome cannot replicate; vectors other than for the shRNA of interest are included in the genome such as for green fluorescent protein, antibiotic resistance to help create a stable cell line and elements that enhance the stability and translation of transcripts.

2.2.5.2 LRP-1 Knockdown

LRP-1 knockdown was finally achieved by transfecting short hairpin RNA (shRNA) encoding bacterial plasmids into OE19 cells. shRNA consists of sense and anti-sense strands separated by a spacer region, and encodes a small portion of the mRNA of

interest, in this case LRP-1. These shRNA are cleaved cytoplasmically by the enzyme RNAase III Dicer to give double stranded short interfering RNA (siRNA), 21-23 nucleotides in length. This siRNA is then incorporated into RNAi-induced silencing complex (RISC) which mediates cleavage of mRNA bearing the same 21 nucleotide sequence as the guide siRNA. Thus there is repression of the protein expression due to post-transcriptional mRNA destruction. This reduced protein expression occurs stably as the shRNA encoding plasmid is retained within the cell and so shRNA production is maintained. Plasmids encoding three different shRNAs were used (Open Biosystems, UK): one encoded a non-silencing shRNA to act as a control while the other two encoded shRNAs corresponded to different sections of the LRP-1 (table 2.5) and should thus promote LRP-1 knockdown.

		Sequence		
Catalog No.	**Function**	**Sense**	**Anti-Sense**	**Loop**
RHS4346	Non-Silencing	Not disclosed but verified to produce siRNA with no match in mammalian genome		
RHS4430-98513013	LRP-1 Silencing	ACC CGC GAG GAC TAC ATT GAA T	ATT CAA TGT AGT CCT CGC GGG C	TAG TGA AGC CAC AGA TGT A
RHS4430-98841553	LRP-1 Silencing	AGG CCT GAC TGT GTT TGA GAA T	ATT CTC AAA CAC AGT CAG GCC G	TAG TGA AGC CAC AGA TGT A

Table 2. 5. Sense, anti-sense and loop sequences for shRNA used for LRP-1 knockdown.

2.2.5.4 Preparation of shRNA encoding plasmids

Plasmids were supplied in E.Coli bacteria which were amplified by initial culture on agar with 100µg/ml ampicillin as plasmids encoded ampicillin resistance. After overnight culture at 37°C one colony for each plasmid was transferred to 3ml LB broth for 8hr pre-culture at 37°C before transfer to 500ml LB broth (5g tryptone, 2.5g NaCl and 2.5g yeast extract in 500ml distilled water). Having thus amplified the cells, plasmids were extracted and purified using PureLink HiPure Plasmid Filter Purification Kit (Invitrogen, UK) as per instructions and using supplied solutions. Briefly, cultures in LB broth were spun at 4000g for 10 minute and supernatant discarded with pellet re-suspended in 20ml supplied resuspension buffer and transferred to 50ml centrifuge tube. 20ml of lysis buffer was added and cells were mixed and incubated at room temperature for 5 minutes. 20ml precipitation buffer was then added and mixed by inversion. The solution was passed through a maxi column with filtration cartridge and allowed to flow through by gravity until flow stopped. Columns were then rinsed with 10ml wash buffer and the filtration cartridge removed and discarded. The column was washed with 60ml wash buffer which flowed through by gravity. All flow through solutions were discarded and 15ml elution buffer were added to columns and allowed to flow by gravity collecting purified plasmids. To this 10.5ml Isopropanol was added and mixed by inversion. Tubes were centrifuged at 15000g for 30 minutes and supernatant discarded. The pellet was re-suspended in 70% ethanol before being re-spun at 15000g for 5 minutes with the supernatant discarded and the pellet air dried at room temperature for 20 minutes. The DNA pellet was then re-suspended in 500µl TE Buffer (0.1mM EDTA in 10mM Tris-HCl, pH 8).

2.2.5.5 Verification of purified plasmids

To ensure that purified DNA was that from the plasmids encoding the target shRNA, a restriction enzyme digest was carried out. DNA was diluted to 1µg/µl and 1µl was added to 1µl Sal-1 restriction enzyme, 2µl enzyme buffer and 16µl nuclease free water. This was incubated at 37°C for 1 hr then labelled using bromophenol blue. Electrophoresis at 50V for 1.5 hours was performed using a 0.7% agarose gel (700mg agarose, 100ml TAE buffer (80mM Tris, 1M Acetic acid, 1mM EDTA)) with ethidium bromide in TAE buffer. The gel was visualised using a GeneFlash UV Transilluminator (Syngene Bioimaging, UK). Using highranger and clonesizer DNA Ladders (Norgen Biotek, Canada) bands were seen at 5124bp, 4298bp and 2188bp for all three plasmids (figure 2.2) confirming these as being the shRNA encoding plasmids.

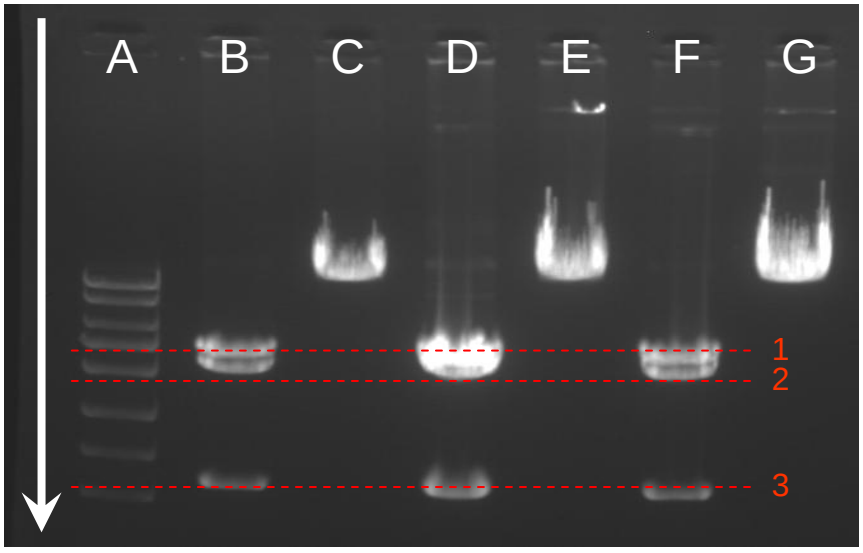


Figure 2. 1. Electrophoresis of plasmid digestion.

Lane A = Highranger ladder, Lane B = Control Plasmid, Lane D = C8 shRNA encoding plasmid, Lane F = H2 shRNA encoding plasmid. Bands labelled 1, 2 and 3 correspond to 5124bp, 4298bp and 2188bp of ladder. (White arrow indicates direction of flow, lanes C, E and G were undigested plasmids.)

2.2.5.6 Transfection of shRNA encoding plasmids

OE19 cells for transfection were plated in 500µl of antibiotic free DMEM with 10% FCS in 12 well plates at a confluence of 20%. These were then incubated at 37°C overnight to achieve 40% confluence, a cell density shown by optimisation to give best transfection efficiency. The shRNA encoding plasmids were then transfected into the cells using Lipofectamine Plus transfection system (Invitrogen, UK) as described above. Transfection efficiency was measured by microscopy of cells under blue light with transfected cells fluorescing green due to GFP expression.

2.2.5.7 Cell sorting of cells transfected with shRNA encoding plasmids

OE19 cells transfected with a low efficiency. To increase the proportion of transfected cells they were passed through a cell sorter [FACSVantage SE (Becton Dickinson, CA, USA) fitted with a 100 µm nozzle and using 488 nm laser light from an Enterprise II laser (Coherent, Santa Clara, CA, USA) for sample excitation]. FACSFlow (Becton Dickinson, CA, USA) was used as sheath fluid. Fluidic conditions were as follows; sheath pressure 8 PSI, sample differential pressure 0.8 PSI, giving a sample data rate of 500 events/second. Forward scattered laser light (FS) was collected through a 488/10 nm bandpass filter and neutral density filter. Side scattered laser light was collected through a 488/10 nm bandpass filter. Green fluorescence light (FL1) (GFP) was collected through a 530/30 nm bandpass filter CellQuest v 3.3 (Becton Dickinson, CA, USA) and was used for data acquisition, data analysis and to control the populations which were sorted. The population to be sorted was described by a sequential gating strategy. First single cells were defined by a single polygonal gate on a 2 dimensional dot plot of FS height (linear) v SS height (log). A second gate on a 1 dimensional

histogram of FL1 height (log) (GFP) was set so that the most fluorescent 5% (approximately) of the single cell population was defined as "GFP high". This is the population which was sorted and kept. The process was carried out under sterile conditions and the cells were reseeded into 24 well plates with 1ml of culture media. Cells were then left for 24hours before experiments were initiated.

2.2.6 Immunofluorescence on cultured cells

2.2.6.1 Preparation of cells for immunofluorescence

5 x 10^5 cells were seeded in their normal growth medium into tissue culture plates (24 well) or onto glass coverslips placed in a 6-well tissue culture dish. Coverslips had been sterilised by immersion in ethanol and then allowed to air dry immediately before use. When cells had reached confluence cell culture medium in the well was aspirated and cells were washed with PBS x 2 at room temperature. Cells were fixed in 2ml ice-cold methanol:acetone (1:1) at 4^{o}C for 20 minutes. Coverslips were then removed from wells and immunofluorescent analysis, as detailed below, was performed on cells.

2.2.6.2 Immunofluorescence protocol

Fixed coverslips were blocked in PBS containing 10% (v/v) goat serum and 1% (v/v) BSA for 30 minutes at room temperature. Coverslips were then incubated with primary antibody in PBS containing 1% BSA (PBS/BSA) for 1 hour at room temperature, then washed in PBS/BSA (3 x 5min) and incubated in FITC or Texas Red conjugated secondary antibody in PBS/BSA for 30 min at room temperature. Coverslips were then washed in PBS/BSA (3 x 5min), allowed to air dry, mounted in immunofluorescence

mounting medium and visualised on an Zeiss Axiovert 40 CFL microscope camera and images were captured with Axiovision Rel. 4.6 imaging software.

2.2.7 DNA extraction

2.2.7.1 Genomic DNA extraction from cells

Cells were cultured in 6-well dishes in normal or treatment growth medium. Following completion of the experiment culture medium was aspirated and the cells gently washed with PBS. This was removed and replaced with 1ml of lysis buffer (100mM NaCl, 10mM Tris-hydrochloride at pH 7.5, 5mM EDTA at pH 8, 0.5% sodium dodecyl sulphate (SDS) made up with distilled water. Just prior to lysis 1mg/ml of Proteinase K was added) for 60 minutes at 37 ^{o}C. 70µl of sodium acetate at pH 5.5 were added; the solution mixed, followed by the addition of 0.5ml phenol. This mixture was shaken and then centrifuged at 13,000g for 1minute. The upper aqueous layer was retained and 0.5ml phenol added; the solution shaken and centrifuged again. The upper layer was removed and added to 0.5ml of chloroform; the solution shaken and centrifuged. The upper layer was again retained and added to two times its volume of absolute ethanol. This mixture was shaken to precipitate DNA. It was kept at -20^{o}C for 30 minutes and shaken again before being centrifuged at 13,000g for 10 minutes. The liquid was removed leaving the pellet which was gently washed with 70% ethanol. The pellet was finally air dried before being dissolved in 10µl of nuclease free water.

2.2.8 RNA extraction from frozen tissue and cells

2.2.8.1 RNA extraction from frozen tissue

RNA was extracted from samples of (a) oesophageal adenocarcinoma with matched gastric and squamous oesophagus mucosa (n=24) and associated Barretts (n=11) and

(b) Barretts biopsy tissue (n=26) with matched gastric control (n=13). Tissue was thawed on ice and homogenised in 0.75ml Trizol using a PolyTurrax homogeniser on ice. Samples were incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.1ml of chloroform was added and the tube vortexed for 15 seconds, then incubated at room temperature for 15 min. Samples were then centrifuged at 12,000g for 15 min at 4^{o}C, and the upper aqueous phase harvested. RNA was precipitated by addition of 0.25ml isopropanol and incubation for 10 min at room temperature. RNA was pelleted by centrifugation at 12,000g at 4^{o}C for 10 min, washed with 75% ethanol (which was subsequently allowed to evaporate) and resuspended in 10µl nuclease free water. Optical Density of the resuspended RNA was measured at 260nm on a spectrophotometer and the concentration of RNA calculated using the equation 1 OD unit = 40µg/ml RNA. RNA was stored at -80 ^{o}C.

2.2.8.2 RNA extraction from cells

Cells were cultured in 6 well plates as per experimental protocol. At the end of the experiment medium was aspirated and cells were washed twice with filter sterilised PBS. 0.5ml Trizol reagent were added to cells and left at room temperature for 10 minutes following which the cells were non adherent and were aspirated; RNA extraction continued as in section 2.2.8.1.

2.2.9 cDNA Generation

cDNA was synthesised from RNA using a reverse transcription system utilising avian myelomatosis virus (AMV) reverse transcriptase. 0.5µg of total RNA were added to nuclease free water, made to a total volume of 4.5µl before the sample was denatured

at 70°C for 10 minutes, then held at 4°C for 5 minutes. 5.5µl of the reverse transcription mastermix containing, 2µl 25mM magnesium chloride, 1µl of reverse transcription buffer (10x), 1µl dNTP mix, 0.25µl RNAsin (1U/µl) 0.25µl AMV-reverse transcriptase (15U/µg), 0.5 µl random hexamer oligonucleotides (0.05µg/µl) and 0.5µl of nuclease free water per reaction, were then added to the denatured RNA. Each sample was subjected to the reverse transcription thermal cycle consisting of 10 minutes at 19°C, 50 minutes at 42°C, 5 minutes at 99°C and then held at 4°C. The synthesized cDNAs were subsequently stored at -20°C until required for qRT-PCR.

2.2.9.1 TaqMan quantitative real-time polymerase chain reaction (qRT-PCR)

The technique of qRT-PCR involves monitoring the PCR as it occurs with data collected in realtime. Primers were designed to allow the amplicon to span an exon/exon boundary in the mRNA of the gene of interest in order to eliminate contamination from genomic DNA. Fluorogenic probes incorporating a 5´ reporter dye and a 3´ quencher dye were designed to a sequence which lay between the two primer annealing sites, the sequences of these and the primers are given in Table 2.2. Cleavage of the probe, through a Taq DNA polymerase, separates the two dyes and permits reporter dye fluorescence. Further probing leads to cleavage with every cycle resulting in an increase in the intensity of the fluorescence which is proportional to amount of amplicon produced.

2.2.9.2 qRT- PCR technique

Newly synthesised cDNA was diluted five-fold and 1µl was added in triplicate into an optical 96-well reaction plate. The RT-PCR mastermix comprised: 12.5µl 2x PCR

reaction buffer 'SensiMix™' (containing dNTPS, DNA polymerase, magnesium chloride, uracil-N-glycosylase, stabilisers and ROX passive reference), 1.5µl to 3µl of 1.25pM gene specific flourogenic probe (optimised for volume and these are listed in Table 2.6), and 2.5µl of 9pM forward and reverse primers specific for the gene of interest, along with 0.125µl of 18s forward and reverse primers and VIC labelled probe, and nuclease free water (3.125µl to 4.625µl) to adjust final volume to 24µl for each reaction well. Once 24µl of this mastermix has been placed into each well the plate is sealed with an optical adhesive cover and loaded into the Applied Biosystems 7500 Fast Real-Time PCR detection system. The standard reaction protocol consists of 10 minutes at 95°C, then 40 cycles of 10 seconds at 95°C, and finally 1 minute at 60°C.

2.2.9.3 Probe optimisation

Each newly designed and synthesised gene-specific flourogenic probe required optimisation in order to maximize the acquired fluorescent signal. Using 1µl of positive control cDNA reactions were set up as described above but with varying volumes of 1.25pM probe from 0.5µl up to 4µl, the final volume adjusted to 25µl by varying the nuclease free water volume. Following the same thermal cycle real-time protocol the results were graphically analysed as cycle threshold against volume and the point at which the resultant curve levelled off was used as the optimum volume for subsequent reaction for each gene specific probe. All optimised specific probes are listed by volume for each reaction in Table 2.6.

2.2.9.4 Real-time PCR analysis

Samples from the protocol described in 2.2.9.2 were analysed using SDS software and a Microsoft Excel spreadsheet. Each reaction was associated with a fluorescence threshold at which the baseline was adjusted to. The cycle number at which fluorescence reaches this is termed the cycle threshold (C_T). This value is used for analysis, and a δC_T value calculated by subtracting the 18s (internal standard) C_T number from that of the target gene for each sample. Each δC_T value is now subtracted from the average control δC_T to give the $\delta\delta C_T$. The fold change can thus be calculated from this by using the equation $2^{-\delta\delta CT}$ Each sample was run in triplicate with an average of these giving the normalised fold change value.

Gene	Optimised Reaction Volume (µl)
CYBRD1 (Dcytb)	2.5
SLC11A2 (DMT1)	1.5
IREB2 (IRP2)	1.5
TFRC (TfR1)	2
FTH (H-ferritin)	2.5
SLC11A3 (ferroportin)	2.5
HEPH (hephaestin)	3
HCP1 (Haem carrier protein1)	2.5
HMOX1 (Haem Oxygenase1)	2
*LRP1 (*Haemopexin receptor)	2
*HAMP (*hepcidin*)*	3
CDH1 (E-cadherin)	2.5
MYC (c-myc)	3

Table 2. 6. Volumes of optimised fluorogenic probe used for TaqMan q-RT-PCR reactions

2.2.10 Statistical Analysis

Analyses were performed using SPSS version 10.0 (SPSS Inc, USA). Two-tailed assessments were performed with significance taken at the 5% level. Normal distribution of data was not assumed and therefore non-parametric tests were employed.

Mann Whitney test was employed to determine if statistical significance existed between the medians of two data sets. Comparison between categorical variables was made using a χ^2 analysis. Significance was accepted at $p \leq 0.05$ and all data are presented as means ± 2 standard error of the mean (SEM) which represents an estimation of the 95% confidence limits of a data set.

2.2.11 Ethical approval

This work has been carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was approved by the Solihull Local Research Ethics Committee (LREC 06/Q2706/65). All patients providing tissue samples gave informed written consent.

CHAPTER 3: CHARACTERISATION OF INORGANIC IRON TRANSPORT PROTEINS IN THE MALIGNANT PROGRESSION OF OESOPHAGEAL ADENOCARCINOMA

3.1 Introduction

Iron is implicated in the development and progression of various malignant diseases. It is required for DNA synthesis and progression through the cell cycle[250] and as such is essential for cancer cell proliferation and growth. Iron chelation blocks these effects demonstrating the essential requirement of iron for cancer cells [297;298;342]. Iron may also exacerbate or induce malignant change. Excess cellular iron facilitates production of reactive oxygen species in the presence of oxidative stress, which can subsequently induce damage to lipids, protein and DNA[150]. Iron deposition at sites of injury in the oesophagus has been demonstrated[148]. In an animal model of gastro-oesophageal reflux, supplemental iron increases the incidence of BM and OAC[149]. Animals without reflux but receiving supplemental iron did not develop either BM or OAC, demonstrating the synergistic effect of iron and chronic inflammation[149]. High iron requirements and TfR1 overexpression have been demonstrated in various tumours including breast[166], non-Hodgkin's lymphoma[253], glioma[252] and lung adenocarcinoma[255]. More recent work demonstrates that cancer cells also express the other iron import and export proteins involved in the absorption of iron from the GI tract[251]. In colorectal adenocarcinoma the expression of iron transport proteins is such that import exceeds export, with subsequent iron loading[251]. The expression of

these iron transport proteins is in the progression of Barrett's metaplasia to oesophageal adenocarcinoma is unknown.

Aims:

1. Examine the patterns of iron deposition within normal oesophagus, BM and OAC by immunocytochemistry and quantify this by ferrozine assay
2. Examine the expression of iron transport proteins (DMT1, Dcytb, TfR1, ferroportin, Hephaestin, Ferritin) in normal oesophagus, BM and OAC
3. Examine the expression of iron transport proteins (DMT1, Dcytb, TfR1, ferroportin, Hephaestin, Ferritin) in patients with advanced malignant disease

3.2 Intracellular quantification of iron content in oesophageal adenocarcinoma

3.2.1 Assessment of cellular iron by enhanced Perl's Prussian semiquantitative immunohistochemistry

Enhanced Perl's Prussian staining was used to identify the cellular location of intracellular iron stores in paraffin sections of oesophageal tissue (fig 3.1).

In normal squamous oesophagus there was no discernable staining other than in the occasional basal keratinocyte (fig 3.1 A, E). In sections of Barrett's metaplasia there was evidence of both diffuse cytoplasmic staining and also prominent apical surface staining (fig 3.1 B, F). In the majority of oesophageal adenocarcinoma specimens there were discrete areas of iron staining which appeared to be both nuclear and cytoplasmic in localisation (fig 3.1 C, G). The liver of a haemochromatosis patient is included as a positive control and demonstrates widespread iron deposition throughout the cell (fig 3.1 D, H).

3.2.2 Assessment of cellular iron content by ferrozine assay

This assay was used to quantify iron stores within tissue samples of oesophageal adenocarcinoma. Specimens of tissue were carefully prepared to remove connective tissue, muscle and deeper layers leaving mucosa only. These were homogenised and the assay performed. Significant increases in cellular iron content were seen in oesophageal adenocarcinoma when compared to matched samples of Barrett's metaplasia and squamous oesophagus ($n=5$, $p<0.02$) (fig 3.2).

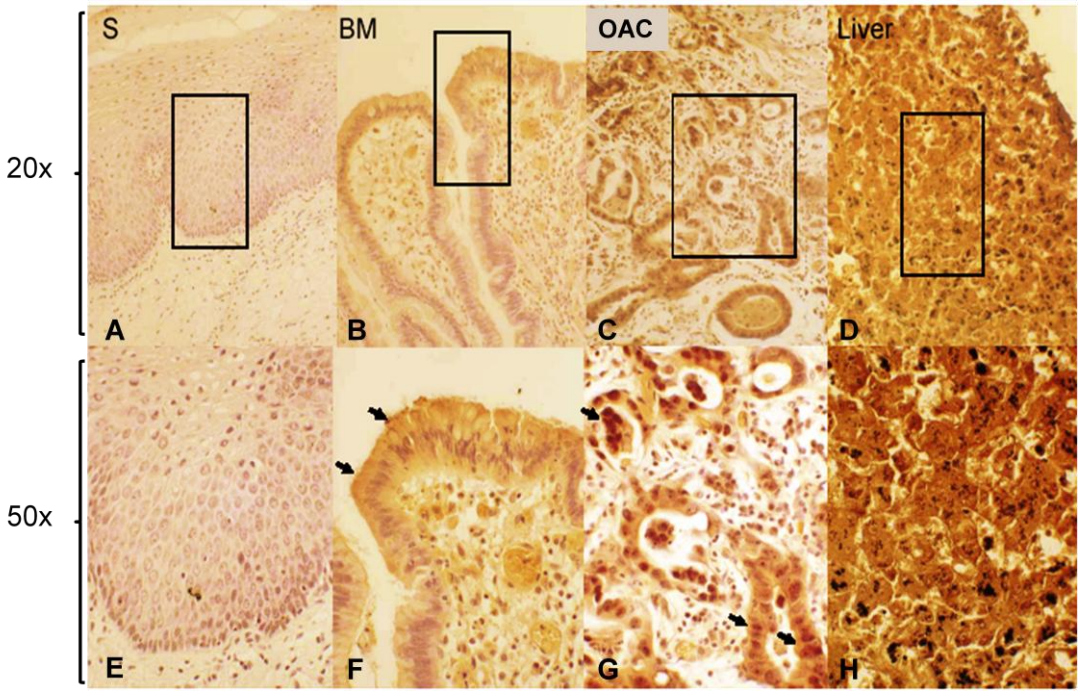


Figure 3. 1. **DAB-enhanced Prussian staining in the oesophagus**

Elevated diaminobenzidine (DAB) enhanced Prussian staining in oesophageal adenocarcinoma. Sections of normal oesophagus (n = 20; S), Barrett's metaplasia (n=20; BM) and oesophageal adenocarcinoma (n = 20; OAC) were subject to DAB enhanced Perl's Prussian staining. In normal squamous epithelium there was no detectable staining (**A** and **E**) while in Barrett's metaplasia there was marked staining at the luminal border and weak cytoplasmic staining (**B** and **F**) and in oesophageal adenocarcinoma diffuse cytoplasmic and nuclear staining was seen (**C** and **G**). Sections of liver from a patient with hereditary heamochromatosis, used as a positive control, demonstrate diffuse and dense staining for iron thoughout the hepatocyctes (**D** and **H**). (**A-D** original magnification x20; **E-F** original magnification x50).

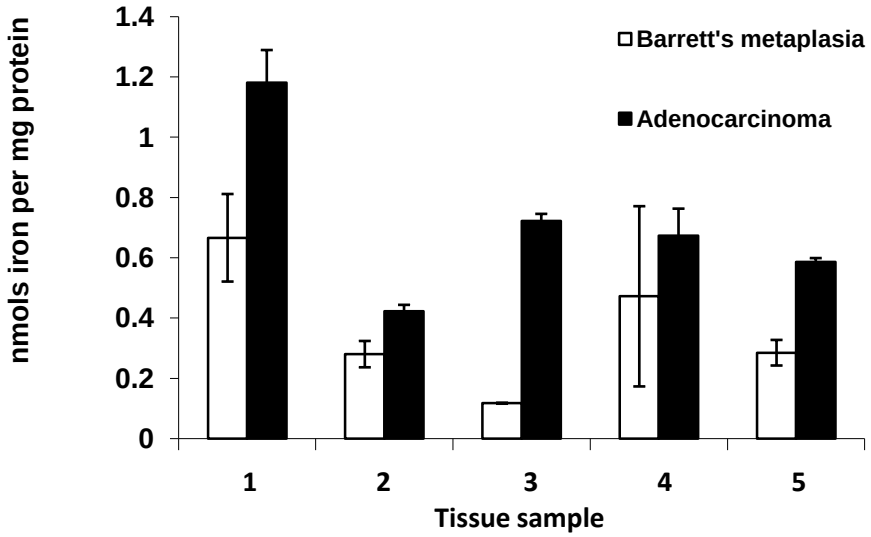


Figure 3. 2. **Ferrozine assay upon homogenised oesophageal adenocarcinoma and matched Barrett's metaplasia.**

Sections of oesophageal adenocarcinoma with matched Barrett's metaplasia (n=5) were processed in a ferrozine assay. The total quantity of iron in each tumour sample exceeded that of the matched Barrett's epithelium in every instance (this was statistically significant for the group, $p<0.02$). The average quantity of iron in oesophageal adenocarcinoma was 1.97 times greater than that in matched Barrett's epithelium (0.72 ± 0.05 vs 0.36 ± 0.11 nmol Fe/mg tissue protein).

3.3 Immunocytochemistry of cellular iron transport proteins in the progression of Barrett's metaplasia to oesophageal adenocarcinoma

Semiquantitative immunohistochemistry was used to elucidate the cellular localisation and to approximate the level of expression of the cellular iron transport proteins (Dcytb, DMT1, TfR1, ferritin, ferroportin, and hephaestin) in archived paraffin tissue specimens of normal oesophagus (n=10), Barretts metaplasia and matched samples of oesophageal adenocarcinoma (n=20).

3.3.1 Semiquantitative immunohistochemistry of Dcytb in the malignant progression of oesophageal adenocarcinoma

Strong immunoreacitvity for Dcytb was observed in the cytoplasm and on the plasma membrane of cells at the basal dividing layer of squamous oesophagus (Figure 3.3 Dcytb S). Weaker immunoreactivity was observed in cells within the stratified epithelium away from the basal layer. In Barretts metaplasia immunoreactivity was localised to the villous tips and decreased in cells lying deeper within crypts (Figure 3.3 Dcytb BM). Vesicular staining for Dcytb was observed throughout the cytoplasm. This pattern was observed in low and high grade dysplasia and oesophageal adenocarcinoma (Figure 3.3 Dcytb ADC). Dcytb immunoreactivity was significantly stronger in oesophageal adenocarcinoma and high grade dysplasia when compared to non dysplastic Barrett's metaplasia ($p = 0.0001$ and 0.045 respectively; Table 3.1).

3.3.2 Semiquantitative immunohistochemistry of DMT1 in the malignant progression of oesophageal adenocarcinoma

No immunoreactivity for DMT1 was observed within normal oesophagus (Figure 3.3 DMT1 S). In Barrett's metaplasia DMT1 was localised at the apical surface, a pattern similar to that of small bowel, whilst in cells deeper within crypts weaker immunoreactivity was observed (Figure 3.3. DMT1 BM). Immunoreactivity increased from low to high grade dysplasia. In oesophageal adenocarcinoma immunoreactivity was the strongest with dense localisation throughout the cytoplasm but with sparing of the nucleus (Figure 3.3. DMT1 ADC). Semiquantitative analysis demonstrated significantly increased DMT1 immunoreactivity in oesophageal adenocarcinoma compared to non dysplastic Barrett's metaplasia ($p = 0.001$; Table 3.1).

3.3.3 Semiquantitative immunohistochemistry of TfR in the malignant progression of oesophageal adenocarcinoma

No immunoreactivity for TfR was observed in squamous oesophagus (Figure 3.3. TfR S). In Barrett's metaplasia TfR was localised to cells at the apical border where dense immunoreactivity was observed throughout the cytoplasm (Figure 3.3. TfR BM). The strongest immunoreactivity was observed in oesophageal adenocarcinoma where TfR was abundantly expressed within the cytoplasm and plasma membrane (Figure 3.3. TfR ADC).

Semiquantitative analysis demonstrated significantly greater expression of TfR in oesophageal adenocarcinoma and high grade dysplasia when compared to non dysplastic Barrett's epithelium ($p = 0.0001$ and 0.0001; Table 3.1).

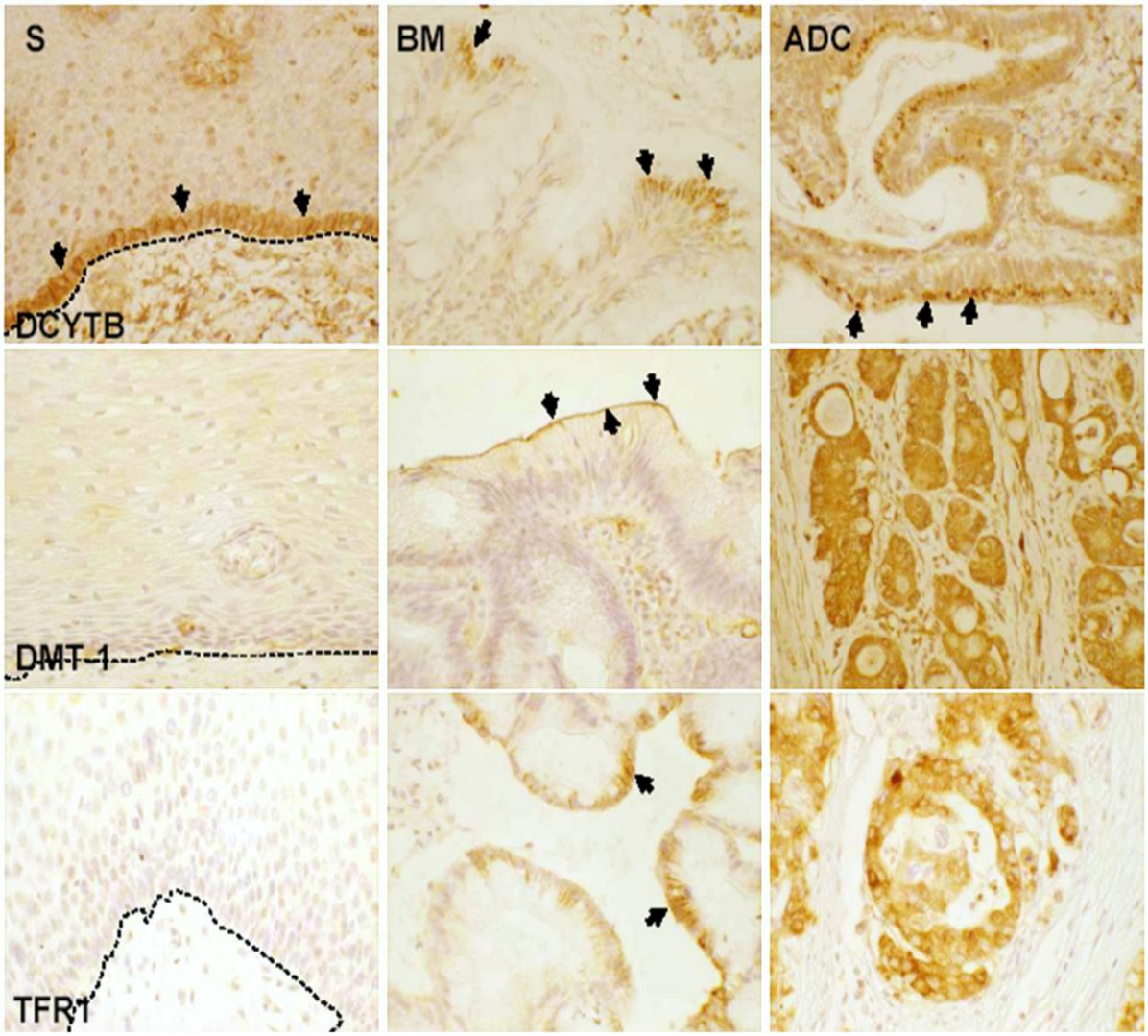


Figure 3. 3. Immunolocalisation of iron import proteins Dcytb, DMT1 and TfR in the malignant progression of oesophageal adenocarcinoma

Immunolocalisation of iron import proteins in paraffin embedded sections of normal squamous oesophagus (S; n=10), Barrett's metaplasia (BM; n=20) and matched oesophageal adenocarcinoma (ADC; n=20). Duodenal cytochrome B (**Dcytb**) was expressed in squamous oesophagus whilst no appreciable expression of either divalent metal transporter 1 (**DMT1**) or transferrin receptor (**TfR**) was observed. At the apical

membrane of Barrett's metaplasia staining for Dcytb, DMT1 and TfR was observed. In oesophageal adenocarcinoma Dcytb was expressed within vesicles whilst immunoreactivity for DMT1 and TfR was strongly expressed throughout the cytoplasm and at the cell membrane. Negative controls were performed during the experiments by omission of primary antibody followed by processing with the appropriate secondary antibody. Arrows denote areas of positive expression (original magnification x40). The dashed line represents the basement membrane.

3.3.4 Semiquantitative immunohistochemistry of the cellular iron storage protein ferritin in the malignant progression of oesophageal adenocarcinoma

H-ferritin expression was observed in normal oesophagus where immunoreactivity was strongest in the basal dividing cells with distribution throughout the cytoplasm (Figure 3.4 ferritin, S). Immunoreactivity was observed throughout the cells of the Barrett's metaplasia sections (Figure 3.4 ferritin BM). Stronger immunoreactivity was present in sections of oesophageal adenocarcinoma where H-ferritin was distributed throughout the cells with abundant cytoplasmic immunoreactivity (Figure 3.4 ferritin, ADC).

Semi-quantitative analysis revealed significantly increased immunoreactivity in oesophageal adenocarcinoma sections compared to non dysplastic Barrett's metaplasia ($p = 0.04$; Table 3.1).

3.3.5 Semiquantitative immunohistochemistry of the cellular iron export protein ferroportin in the malignant progression of oesophageal adenocarcinoma

No expression of ferroportin was observed in sections of normal oesophagus (Figure 3.4 FPN, S). Ferroportin immunoreactivity in Barrett's metaplasia was strongest in

apical cells being mainly supranuclear becoming weaker deeper in the crypts (Figure 3.4 FPN, BM). In adenocarcinoma immunoreactivity was stronger than in Barrett's with abundant ferroportin expression in cytoplasmic vesicles (Figure 3.4 FPN, ADC).

Semiquantitative analysis revealed significantly more expression of ferroportin in oesophageal adenocarcinoma compared with non dysplastic Barrett's metaplasia ($p = 0.002$; Table 3.1).

3.3.6 Semiquantitative immunohistochemistry of the cellular iron export protein hephaestin in the malignant progression of oesophageal adenocarcinoma

Weak immunoreactivity was observed in squamous oesophageal cytoplasm (Figure 3.4 HEPH, S). In sections of Barrett's metaplasia diffuse hephaestin immunoreactivity was observed in columnar cells being strongest at the basal pole (Figure 3.4 HEPH, BM). In oesophageal adenocarcinoma there was week immunoreactivity for hephaestin which was diffuse and not localised at the cell border (Figure 3.4 HEPH, ADC).

There was no significant difference in the expression of hephaestin between tissue type when performing semi-quantitative analysis (p=ns; Table 3.1).

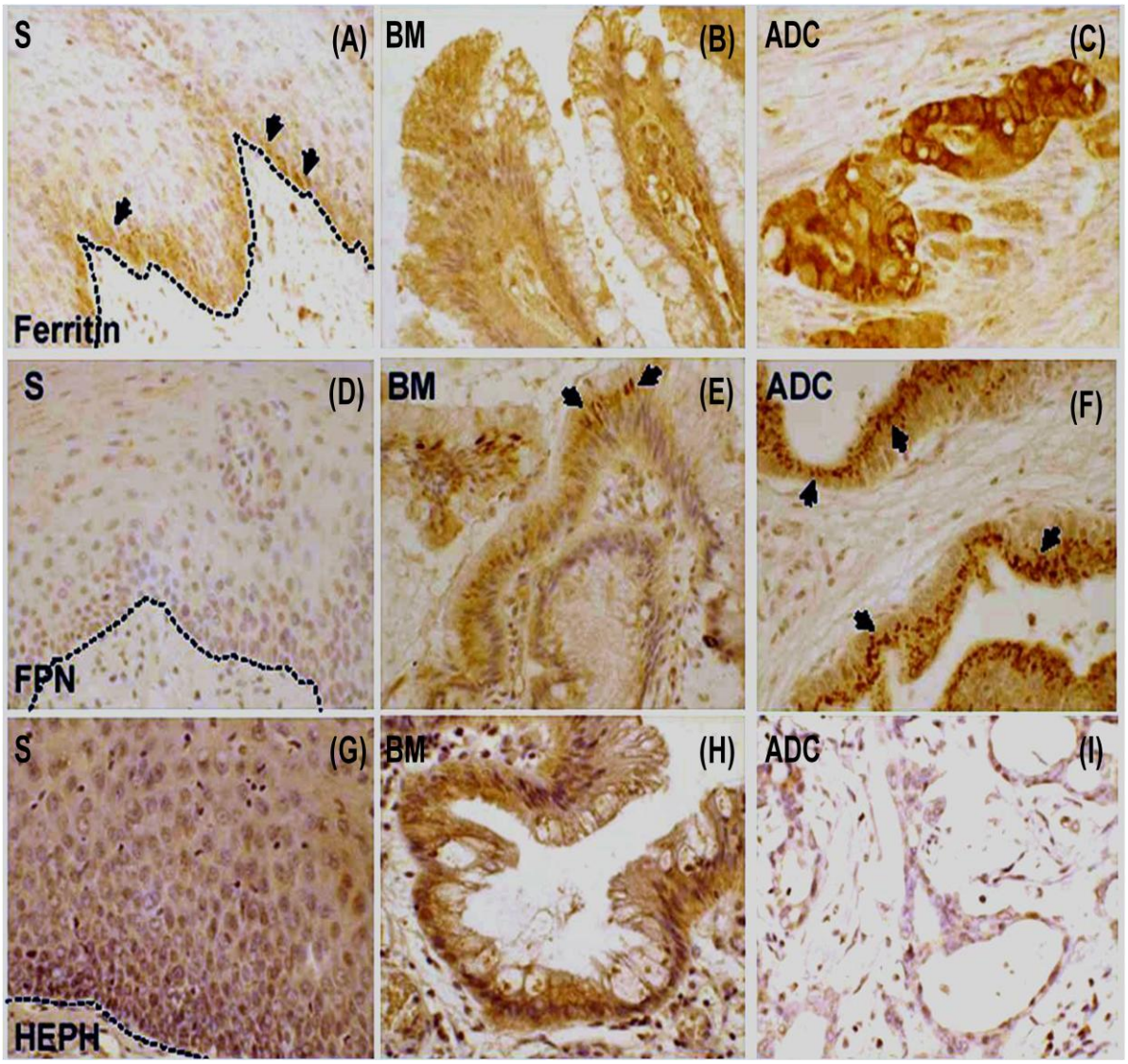


Figure 3. 4. Immunolocalisation of the iron storage and export proteins in the malignant progression of oesophageal adenocarcinoma

Immunolocalisation of iron export and storage proteins in paraffin embedded sections of normal squamous oesophagus (S; n=10), Barrett's metaplasia (BM; n=20) and matched oesophageal adenocarcinoma (ADC; n=20). Dashed line represents basal layer of squamous oesophagus.

Little immunoreactivity for ferroportin (FPN, D) or hephaestin (HEPH, G) in squamous epithelium whilst H-ferritin was localised mainly to the dividing layer of basal cells

(arrows, A). Cytoplasmic immunoreactivity for both ferroportin (arrows, E) and HEPH (H) was observed in apical cells in Barrett's metaplasia whilst H-ferritin was expressed throughout the cells (B). Extensive immunoreactivity for ferritin was observed throughout the oesophageal adenocarcinoma cells (C) whilst ferroportin was also abundantly expressed, mainly in vesicles (arrows, F). A repression of HEPH expression was observed in oesophageal adenocarcinoma (I).

Negative controls were performed during the experiments by omission of primary antibody followed by processing with the appropriate secondary antibody. Arrows denote areas of positive expression (original magnification x40).

	Mean difference in protein expression		
	LGD vs BM+ve	**HGD vs BM+ve**	**ADC vs BM+ve**
Dcytb	0.54	**1.65***	**7.72***
DMT1	1.67	1.72	**7.06***
TfR	0.98	**5.43***	**5.07***
H-ferritin	0.15	1.67	**3.38***
Ferroportin	1.17	1.08	**2.80***
Hephaestin	0.98	1.53	1.21

Table 3. 1. Semi-quantitative analysis of the immunoreactivity of iron transport proteins in the progression from Barrett's metaplasia through low and high grade dysplasia to oesophageal adenocarcinoma

Paraffin sections of Barrett's with low grade dysplasia (LGD) (n=10) and Barrett's with high grade dysplasia (HGD) (n=20) were compared to Barrett's metaplasia from sections adjacent to oesophageal adenocarcinoma (BM+ve) (n=20). Oesophageal adenocarcarinoma (ADC) sections were compared to the adjacent matched Barrett's metaplasia (BM+ve).

Samples were subject to immunohistochemistry with antibodies to duodenal cytochrome b (Dcytb), transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferroportin, hephaestin, and H-ferritin. Scoring of the slides is described in materials and methods; briefly 0 = no staining, 12 = strong staining of all epithelial cells within the slide. The mean score is presented and numbers in bold denote statistical significance (*$p<0.05$; Mann-Whitney-U test).

3.4 Correlation of cellular iron transport protein expression with prognostic variables using an oesophageal adenocarcinoma tissue microarray

Oesophageal adenocarcinoma samples (n=76, separate to those used elsewhere in this work) were prepared as a tissue microarray embedded in paraffin (kind gift from Jonathan Bury) and subjected to semiquantitative immunohistochemistry analysis. Expression of the iron transport proteins TfR, DMT1, Dcytb, ferritin, hephaestin and ferroportin was quantified and correlated with prognostic variables. These variables were T stage (T1-4), presence of nodal metastasis, vascular invasion, serosal involvement and differentiation (well, moderate or poor). There were 1 T1, 15 T2 and 60 T3 tumours; 3 well, 52 moderate and 21 poorly differentiated tumours; serosal involvement in 23 tumours; nodal metastasis in 52 tumours; vascular invasion in 19 tumours. The single T1 tumour and 3 tumours assessed as being well differentiated were excluded from comparison due to the low numbers. There were no T4 tumours (these are by definition not treated by surgical resection and therefore unavailable for assessment). Increased immunoreactivity for DMT1 was observed in tumours with nodal metastasis ($p = 0.0068$) compared to those without. There was no significant correlation between immunoreactivity for any other iron transport protein and prognostic variable (Table 3.2).

Protein	T stage		Differentiation		Vasular invasion		Nodal metastasis		Serosal involvement	
	T2	T3	Moder-ate	Poor	-ve	+ve	-ve	+ve	-ve	+ve
DMT1	2.5	3.02	3.02	2.38	2.77	3.24	**1.93***	**3.34***	2.58	3.59
Dcytb	4.97	4.44	4.85	4.43	4.78	4.87	4.9	4.79	5.11	4.51
TfR	5.96	9.36	9.04	7.4	7.84	9.00	4.56	9.88	8.16	9.56
H-ferritin	1.65	2.49	2.75	1.66	2.43	2.17	1.52	2.69	2.25	2.39
Ferroportin	2.99	2.6	2.66	2.33	2.7	2.38	2.33	2.71	2.77	2.36
Hephaestin	0.14	0.28	0.3	0.11	0.24	0.16	0.35	0.15	0.11	0.39

Table 3. 2. **Semiquantitative analysis of immunoreactivity of iron transport proteins in an oesophageal adenocarcinoma tissue microarray (n=76)**

Paraffin embedded oesophageal adenocarcinoma tissue array was used to correlate immunoreactivity of iron transport proteins (Dcytb, DMT1, TfR, H-ferritin, ferroportin and hephaestin) with prognostic variables (T stage, vascular invasion, lymph node metastasis, serosal involvement and degree of cellular differentiation). Assessment of immunostaining is described in section 2.2.1.3.

Increased immunoreactivity for DMT1 was observed in cases with lymph node metastasis. No further association between iron transporter immunoreactivity and prognostic variable was identified. The mean scores are presented and numbers in bold denote statistical significance (Mann-Whitney-U test with Bonferroni adjustment for multiple comparison; statistical significance accepted at $p \leq 0.0083$).

3.5 Quantitative analysis of iron transport mRNA expression in the malignant progression of oesophageal adenocarcinoma by qRT-PCR

To explore if the expression of iron transport proteins in normal oesophagus, Barrett's metaplasia and adenocarcinoma is regulated at a transcriptional level qRT-PCR was utilised. The mRNA expression of Dcytb, DMT1 IRE+ve isoform, TfR, H-ferritin, ferroportin and hephaestin was determined relative to 18s ribosomal mRNA internal standard. Non dysplastic Barrett's metaplasia (BM-ve) (over 3cm in length and confirmed as columnar epithelium with intestinal type goblet cells) with no evidence of adenocarcinoma or dysplasia was compared to matched samples of squamous oesophagus and gastric epithelium (n=14). Oesophageal adenocarcinoma samples (n=29) were compared to matched samples of normal squamous oesophagus and gastric epithelium and when present associated Barrett's metaplasia (BM+ve) (n=11).

3.5.1 mRNA expression of duodenal cytochrome B in the malignant progression of oesophageal adenocarcinoma

There was no significant difference in Dcytb mRNA expression between BM-ve and matched gastric epithelium with an average fold difference of 1.09±0.35 (mean±2SEM). There was significant upregulation when compared with matched squamous epithelium 2.91±0.74 ($p<0.05$). Expression of Dcytb mRNA was greater in all but one sample of oesophageal adenocarcinoma compared to BM+ve (4.05±0.78; $p=0.001$) (fig 3.5 A).

3.5.2 mRNA expression of DMT1 in the malignant progression of oesophageal adenocarcinoma

DMT1 IRE+ve mRNA expression was greater in BM-ve compared with matched gastric epithelium (1.79±0.56; $p<0.05$) and squamous epithelium (1.71±0.23; $p=0.006$). Expression in oesophageal adenocarcinoma was greater than BM+ve in every sample (6.51±1.8; $p=0.0002$) (fig 3.5 B).

3.5.3 mRNA expression of TfR1 in the malignant progression of oesophageal adenocarcinoma

Significantly greater expression of TfR was seen in BM-ve compared to matched squamous (1.75±0.37; $p<0.05$) but not gastric epithelium (1.87±0.96; $p=0.3$). All but one sample of oesophageal adenocarcinoma expressed more TfR than BM+ve (11.5±1.7; $p=0.003$) (fig 3.5 C).

3.5.4 mRNA expression of H-ferritin in the malignant progression of oesophageal adenocarcinoma

There was no significant difference in the expression of H-ferritin between BM-ve and matched gastric epithelium (2.37±0.6; $p=0.38$). 11/14 samples of BM-ve expressed more ferritin than matched squamous (3.97±0.93; $p<0.05$). Oesophageal adenocarcinoma expressed more ferritin than BM+ve (2.98±0.89; $p=0.02$) (fig 3.5 D).

3.5.5 mRNA expression of ferroportin in the malignant progression of oesophageal adenocarcinoma

Significantly greater expression of ferroportin was seen in BM-ve compared to matched squamous (6.91±2.6; $p<0.05$) but not gastric epithelium (2.95±0.85; $p=0.16$). Ferroportin expression was greater in oesophageal adenocarcinoma than BM+ve (14.8±2.4; $p=0.01$) (fig 3.5 E).

3.5.6 mRNA expression of hephaestin in the malignant progression of oesophageal adenocarcinoma

Significantly greater expression of hephaestin was seen in BM-ve compared to matched squamous (14.95±0.26; $p<0.05$) but not gastric epithelium (1.96±0.28; $p=0.17$). There was no significant difference in expression between oesophageal adenocarcinoma and BM+ve (1.2±0.69; $p=0.11$) (fig 3.5 F).

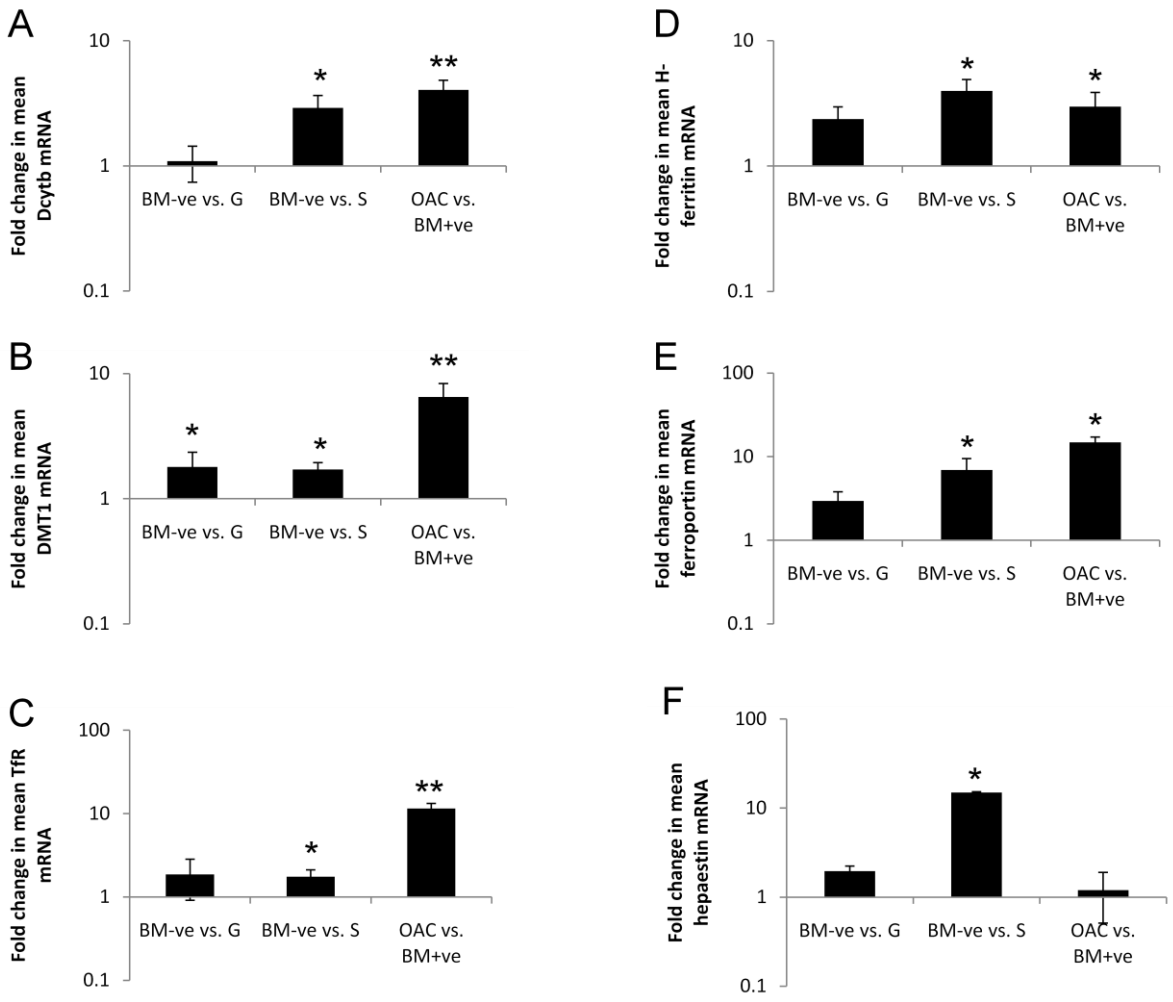


Figure 3. 5. Mean expression of iron import (A – Dcytb; B – DMT1; C – TfR), storage (D – ferritin) and export (E – ferroportin; F – hephaestin) proteins.

There was an increased expression of DMT1 mRNA in non dysplastic Barrett's epithelium (BM-ve, n=14) compared with matched gastric epithelium (G). Increased expression of each iron transport protein was observed in non dysplastic Barrett's epithelium compared with matched squamous epithelium (S). Significantly greater expression of each transport protein, with the exception of hephaestin, in oesophageal adenocarcinoma (OAC, n=11) compared with matched Barrett's epithelium (BM+ve). Dcytb – duodenal cytochrome b; DMT1 divalent metal transporter 1; TfR – transferrin receptor; ferritin – H-ferritin. 2SEM shown; * $p<0.05$; ** $p<0.01$

3.6 Quantitative expression of iron transport proteins in the malignant progression of oesophageal adenocarcinoma

The expression of iron transport proteins (transferrin receptor, H-ferritin and ferroportin) was determined by western blotting using optimised antibodies. Normal oesophagus was compared with matched non dysplastic Barrett's epithelium (n=15) and oesophageal adenocarcinoma with matched dysplastic Barrett's epithelium (n=10).

3.6.1 Expression of TfR1 protein in the malignant progression of oesophageal adenocarcinoma

Expression of TfR1 was greater in oesophageal adenocarcinoma than in matched Barrett's (32.4±14.3 vs. 1±0.44; $p<0.01$) whilst there was no significant difference between squamous oesophagus and non dysplastic Barrett's epithelium (1±0.1 vs 1.2±0.3; p=ns) (fig 3.6A).

3.6.2 Expression of ferritin protein in the malignant progression of oesophageal adenocarcinoma

There was no significant difference in expression of H-ferritin between the tissue samples (sqaumous oesophagus 1±0.1; non dysplastic Barrett's 1.3±0.4; dysplastic Barrett's 1±0.5; oesophageal adenocarcinoma 0.7±0.3; all p=ns) (fig 3.6B).

3.6.3 Expression of ferroportin protein in the malignant progression of oesophageal adenocarcinoma

An overexpression of ferroportin protein was observed in oesophageal adenocarcinoma versus matched Barrett's epithelium (11.3±4.0 vs 1±0.4; $p<0.05$). A

non significant increase in expression was observed between non dysplastic Barrett's epithelium versus matched squamous oesophagus (3.1±2.5 vs 1±0.1; p=ns) (fig 3.6C).

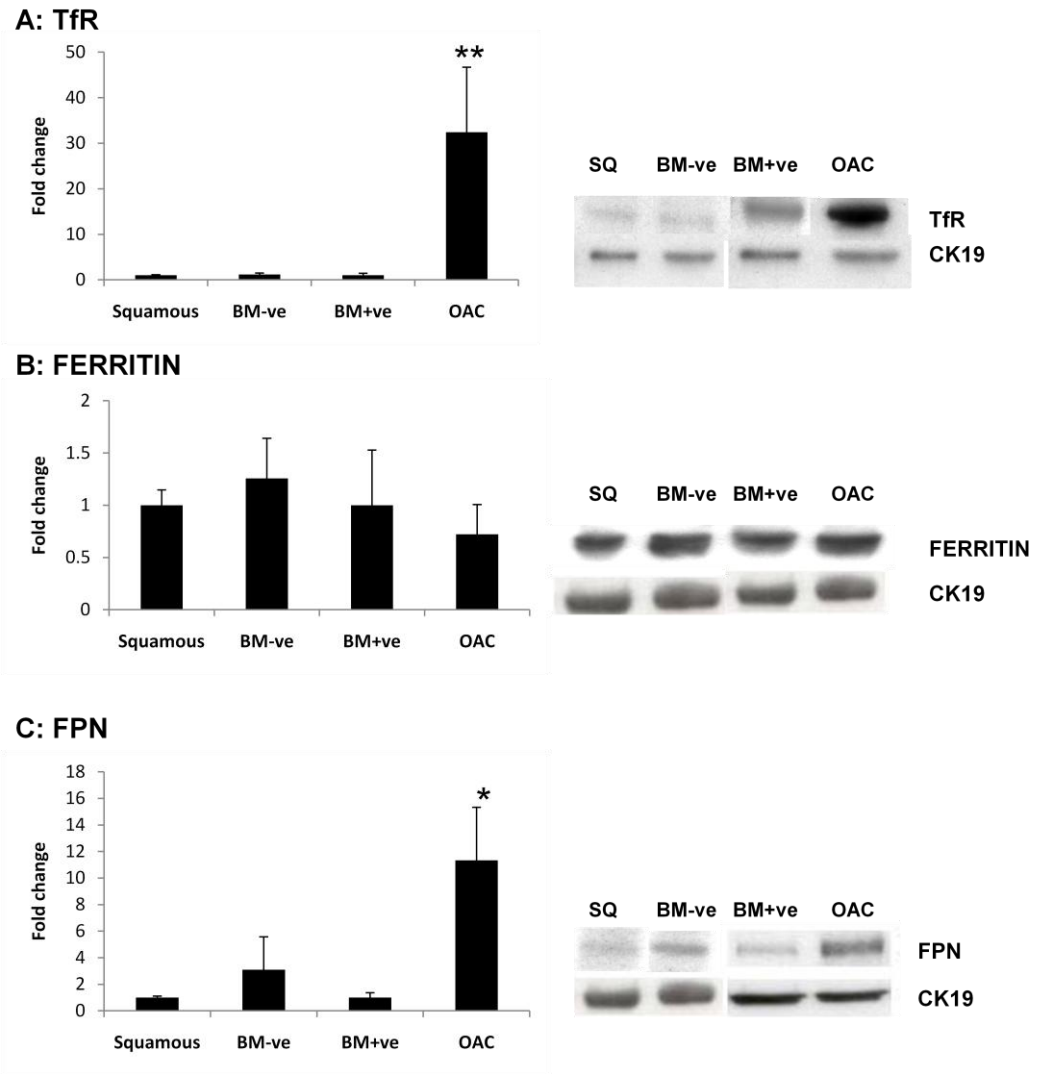


Figure 3. 6. Analysis of iron storage and export proteins in normal oesophagus, Barrett's metaplasia and oesophageal adenocarcinoma

Western blotting was utilised to determine the protein expression of iron storage and export proteins in squamous compared with matched BM-ve (n=15). OAC was

compared with matched BM+ve (n=10). A mouse monoclonal antibody to TfR (90kDa), rabbit polyclonal antibodies to H-ferritin (21kDa) and ferroportin (64kDa) were used. Densitometry was used to quantify immunoreactive bands and normalised to cytokeratin 19 (CK19; 40kDa) as a loading control. A significant increase in TfR and ferroportin protein expression but not ferritin was observed in OAC versus matched BM+ve whilst no significant change was seen against BM-ve with squamous.

Squamous - normal oesophagus; BM-ve - non dysplastic Barrett's metaplasia; BM+ve - dysplastic Barrett's metaplasia; OAC - oesophageal adenocarcinoma; TfR – transferrin receptor; FPN – ferroportin; ferritin – H-ferritin; mean values ± 2SEM shown; * $p<0.05$; ** $p<0.01$

3.7 Expression of iron regulatory protein 2 in the malignant progression of oesophageal adenocarcinoma

The expression of TfR, ferritin, ferroportin and DMT1 (IRE+ve isoform) is influenced by interaction between their iron regulatory elements and iron regulatory protein 2. Expression of IRP2 mRNA was assessed by qRT-PCR in samples of oesophageal adenocarcinoma with matched Barrett's metaplasia (n=11) and in samples of squamous and gastric epithelium with matched benign Barrett's metaplasia (n=14) (fig 3.7).

There was a non significant decrease in IRP2 mRNA expression between BM-ve and matched squamous epithelium with an average fold difference of 0.61±0.38 ($p=0.07$) (mean±2SEM) there was however a significant reduction when compared with matched gastric epithelium with an average fold difference of 0.56±0.34 ($p=0.03$).

There was a non significant upregulation of IRP2 when oesophageal adenocarcinoma was compared to BM+ve (4.6±3.1; p=0.07) (fig 3.7).

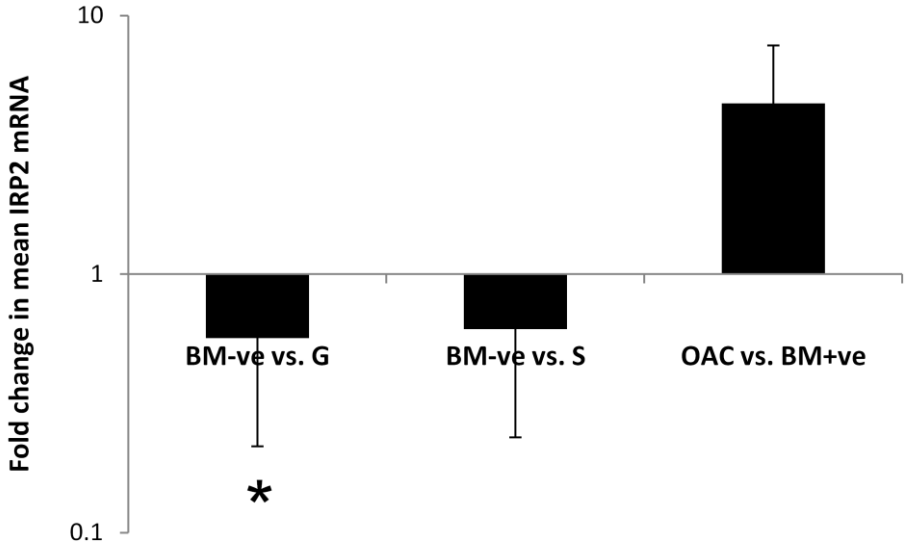


Figure 3. 7. Mean expression of IRP2 mRNA in the malignant progression of oesophageal adenocarcinoma

Fig 3.7 demonstrates decreased IRP2 mRNA expression in non dysplastic Barrett's epithelium (BM-ve, n=14) compared with matched gastric epithelium (G) (p=0.03). Non significant decrease when compared to matched squamous epithelium (p=0.07) and non significant increase in IRP2 expression in OAC versus matched Barrett's epithelium (BM+ve) (p=0.07).

IRP – iron regulatory protein. Mean values ± 2SEM shown; * p<0.05

3.8 Expression of the iron regulatory protein hepcidin in oesophageal adenocarcinoma samples

Hepcidin expression in oesophageal adenocarcinoma tissue was assessed by qRT-PCR in samples of adenocarcinoma with matched Barrett's metaplasia (figs 3.8 A) and a larger group of adenocarcinoma samples without matched Barrett's (n=11) (fig 3.8 B). Immunohistochemistry was used to assess the cellular location of hepcidin expression (fig 3.9).

3.8.1 Hepcidin mRNA expression in the malignant progression of oesophageal adenocarcinoma

Hepcidin mRNA expression was observed in the majority of tumours (6 of 8) with matched Barrett's metaplasia (mean 1/dCt expression of hepcidin mRNA in these tumour samples was 0.035 ± 0.002). Only one sample of Barrett's metaplasia expressed hepcidin mRNA (fig 3.8 A) (Fishers exact test p=0.02). 11 of 19 oesophageal adenocarcinoma samples without matched Barrett's metaplasia expressed hepcidin mRNA (mean 1/dCt expression of hepcidin mRNA in these tumour samples was 0.027 ± 0.001) (fig 3.8 B).

3.8.2 Semiquantitative immunohistochemistry of hepcidin expression in the malignant progression of oesophageal adenocarcinoma

Immunohistochemistry revealed weak immunoreactivity for hepcidin which was predominantly localised to the basal membrane samples of Barrett's metaplasia (fig 3.9 B). Dense cytoplasmic immunoreactivity for hepcidin with sparing of the nucleus was observed in oesophageal adenocarcinoma samples (fig 3.9 C,E,D,F).

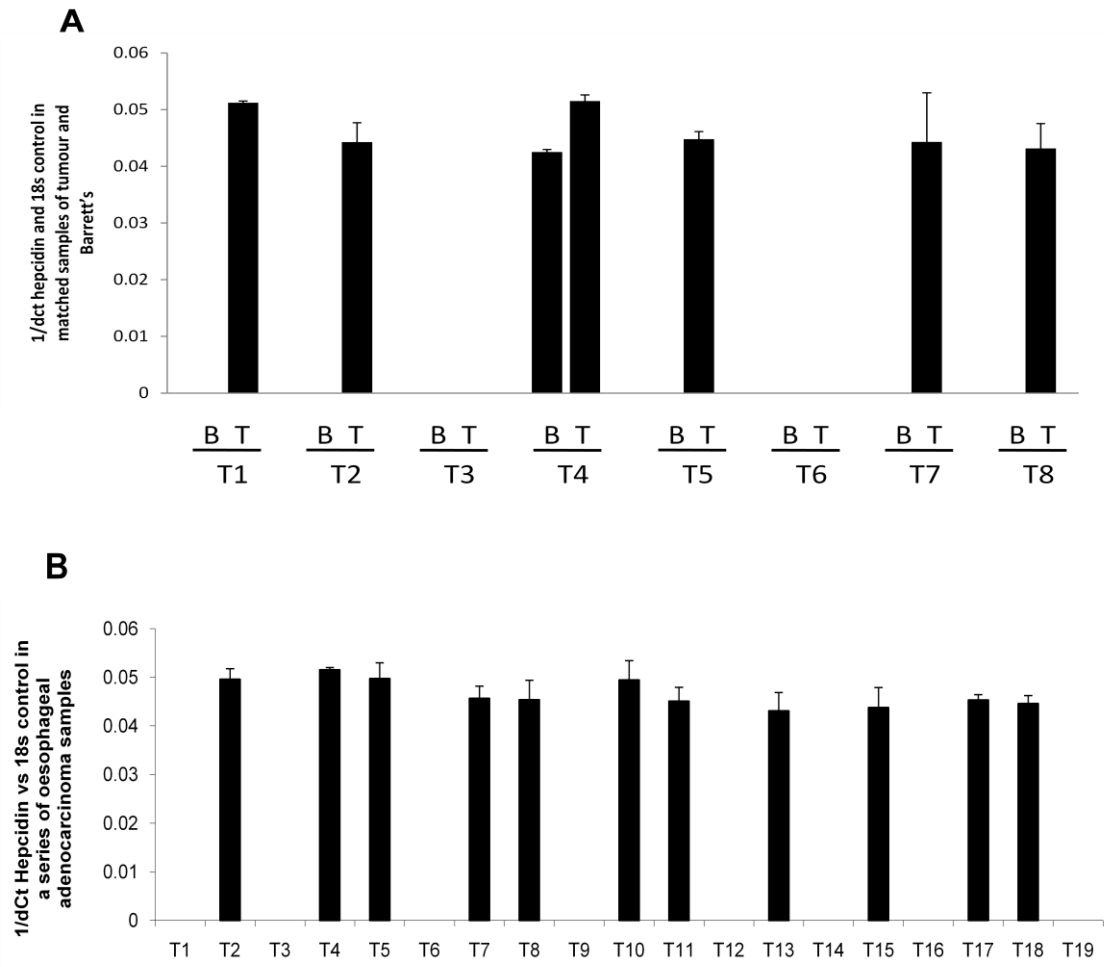


Figure 3. 8. **Hepcidin mRNA expression in samples of oesophageal adenocarcinoma**

Samples of oesophageal adenocarcinoma with matched Barrett's metaplasia (fig 3.8 A; B-Barrett's metaplasia, T – matched tumour, TN – sample number) and in a larger group of oesophageal adenocarcinoma samples without matched Barrett's (fig 3.8 B). Results are presented as 1/dct between hepcidin and 18s internal control as the majority of Barrett's and some of the adenocarcinoma samples expressed no hepcidin. Oesophageal adenocarcinoma samples were more likely than matched Barrett's to express hepcidin mRNA (p=0.02) (fig 3.8A). The majority of adenocarcinoma samples expressed hepcidin mRNA (fig 3.8B); for each sample matched squamous oesophagus and gastric mucosa were screened for hepcidin expression but every sample was negative (results not shown).

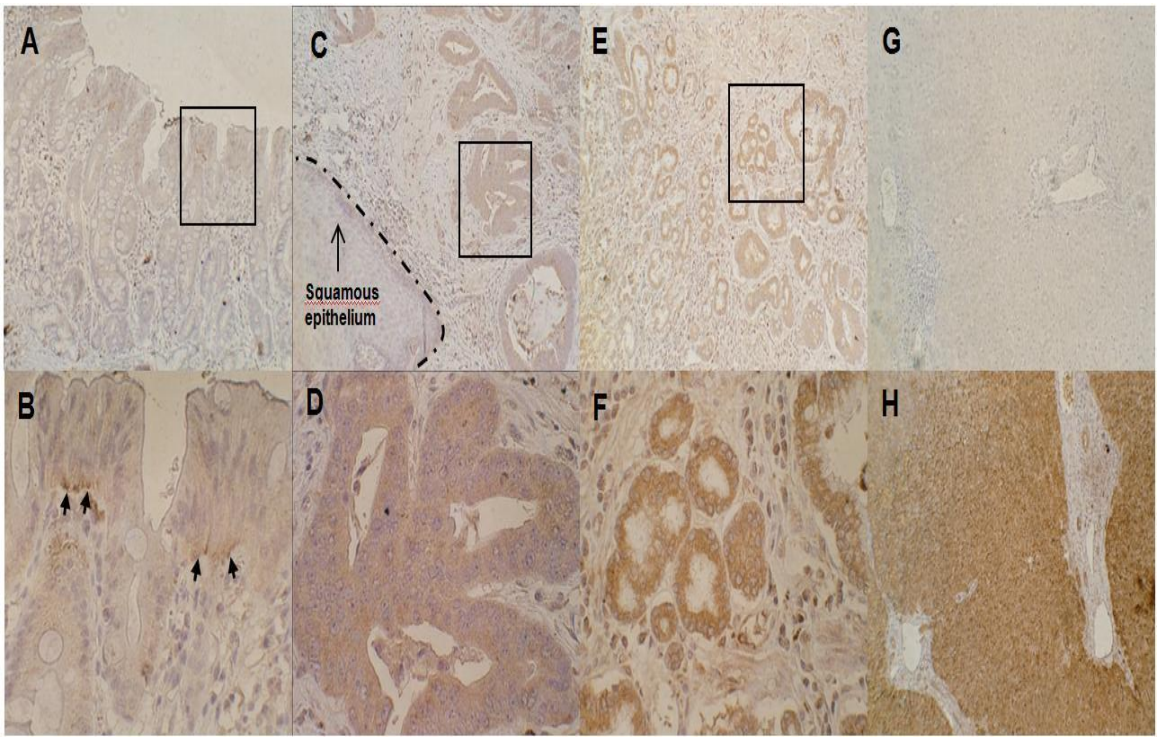


Figure 3. 9. Hepcidin immunohistochemistry in the malignant progression of oesophageal adenocarcinoma

Weak immunoreactivity for hepcidin was observed in Barrett's metaplasia at the basal cell border (figs A and B, 40x and 100x magnification respectively). Dense immunoreactivity for hepcidin was observed throughout the cytoplasm of all oesophageal adenocarcinoma cells (figs C, E and D, F, 40x and 100x magnification respectively). No hepcidin immunoreactivity was seen in neighbouring normal squamous epithelium adjacent to invasive adenocarcinoma (fig 3.9C). Fig G and H are sections of liver; G is a negative control with no anti-hepcidin but incubation with goat serum and secondary antibody; H is a positive control with addition of anti-hepcidin antibody.

3.9 Conclusions

This is the first time that iron transport proteins have been investigated in oesophageal adenocarcinoma. Other tumours have been associated with overexpression of transferrin receptor[166;252;253;255] and perhaps it is not surprising to identify this in OAC. Cancer cells have requirements for iron over and above that of benign cells; iron is required by cell division proteins such as cyclin dependent kinases[250] and is essential for cell metabolism. This work is more complete and novel not only because it is the first to observe the expression of transferrin receptor in oesophageal adenocarcinoma but also evaluates other cellular import, storage and export proteins. Furthermore, we have observed the expression of the proteins in the progression of normal oesophagus to Barrett's metaplasia and oesophageal adenocarcinoma.

The expression of these iron transport proteins within the proximal upper gastrointestinal tract seems very unlikely to be related to the absorption of intestinal iron. The expression profile and cellular location indicate that iron acquisition occurs mainly via transferrin receptor endocytosis. The role of Dcytb is however unclear. In the duodenum it acts as a luminal ferrireductase aiding import via DMT1[164;165]. We have observed cytoplasic immunoreactivity within adenocarcinoma and weak staining within the basal layer of proliferating normal squamous oesophagus. Whether Dcytb has other roles or its expression is constitutive within these cells being lost as they develop is unclear.

The widespread deposition of iron seen throughout OAC tissue sections is presumably a phenotype resulting from increased iron import and dysregulation of storage/export. mRNA expression of the iron export protein ferroportin was greater in adenocarcinoma than in associated Barrett's metaplasia. Its cytoplasmic location however, as revealed by immunocytochemistry, suggests a non functional role[190]. The cause of the re-localisation of ferroportin is unclear. It seems logical that a prime candidate is the hepatic anti-microbial peptide hepcidin[217;218] which we have shown to be expressed by the majority of oesophageal adenocarcinoma samples. This would mediate a loss of membranous ferroportin expression (which we observed in samples of oesophageal adenocarcinoma tissue). It could be postulated that this results in iron sequestration within tumour cells. Possible effects would include cells becoming more aggressive since increasing cellular iron can drive cellular proliferation[250], modulate cell adhesion and through Fenton reactions mediate free radical production[160]. The last may be a method of creating further cell aberrations such as DNA adducts/mutation with evolution of the cancer[160]. Why tumour cells express hepcidin whilst native oesophageal and metaplastic Barrett's tissue do not is unclear though. The major trigger for hepatic hepcidin production is inflammatory mediators[218], whereas hypoxia inhibits hepcidin production. The effects of infection or inflammation upon hepcidin are indirect[218], with interleukin 6 being identified as the cytokine responsible[219]. Overexpression of interleukin 6 has been observed in the malignant progression of Barrett's by other authors, with levels increasing as Barrett's becomes dysplastic[352] and highest levels being observed within oesophageal adenocarinoma[353], however the association with hepcidin expression has not previously been reported. Overexpression of interleukin 6 is a feature common

to gastrointestinal malignancies including gastric adenocarcinoma[354], pancreatic adenocarcinoma[355] and colonic adenocarcinoma[356]. The expression of hepcidin within these tumour types has not yet been characterised, though we have demonstrated hepcidin expression in colorectal adenocarcinoma tissue samples[357]. The stimulus for tumour cells to express hepcidin is unclear. Potential autocrine effects could result in iron loading of cells that express ferroportin.

The mechanism of cellular control in the expression of iron transport proteins in the malignant progression of oesophageal adenocarcinoma is unclear. On the basis of increased cellular iron in oesophageal adenocarcinoma (as demonstrated by immunohistochemistry and quantified by ferrozine assay) one could predict the expression profile of the pertinent cellular transport proteins if the IRE/IRP system was functioning. This should result in decreased transferrin receptor, DMT1 and increased ferritin and ferroportin expression[204]. This is not the case, with transferrin receptor, DMT1 and ferritin being overexpressed. Ferroportin overexpression is seen in adenocarcinoma but the cytoplasmic location away from basal cell border implies a non-function role[217;218]. Auto- or paracrine expression of hepcidin could explain the relocalisation of ferroportin but other mechanisms of cellular control must play a role, though they are as yet uncharacterised.

In the presence of repeated reflux of acid/bile into the oesophagus and consequent inflammation it is likely that these OAC cells are at risk of oxidative stress and iron mediated free radical damage[148;149;155;307;358]. This may drive carcinogenesis

and possibly explain the rapid progression and poor prognosis of the majority of OAC's[307]. The lack of correlation in iron transport expression and known prognostic markers identified by the tissue microarray (with the exception of increased expression of DMT1 with nodal metastasis) indicates that accumulation of iron and cellular damage are likely to occur early in the development of OAC. The change in iron transporter expression was most obvious between OAC and matched samples of BM. Immunocytochemistry and mRNA assessment associated increased expression of Dcytb, DMT1, TfR1, ferritin and ferroportin with the development of OAC. When high grade dysplasia was compared with Barrett's from patients with OAC, an increase in TfR1 and a mild increase in Dcytb was observed. There was no difference between low grade dysplasia and BM.

The second striking difference in the progression of BM and OAC from squamous oesophagus was between the normal squamous epithelium and BM. The expression of every iron transport mRNA and protein was different. The cumulative effect appears to be associated with increased iron deposition as seen by Perls Prussian staining in BM sections compared with normal squamous epithelia. This may in part reflect the characteristics of the different epithelia, however, it is interesting to observe iron deposition in benign Barrett's epithelium. Whether this reflects the normal state for this epithelium it is not clear. In the presence of repeated inflammation, mediated by repeated episodes of gastroesophageal reflux, oxidative damage and highly reactive oxygen species may be exacerbated in the presence of increased cellular iron. This may provide a mechanism by which Barrett's epithelium becomes increasingly dysplastic and finally into invasive adenocarcinoma.

An interesting aspect of oesophageal adenocarcinoma is the relatively infrequent development of OAC within pre-existing BM (approximately 1% per year). It would be interesting to quantify iron transport protein, interleukin-6 and hepcidin expression in a cohort of patients with benign BM. If this cohort underwent endoscopic surveillance tissue samples could be used to observe changes in protein expression profile with time and more interestingly amongst the few subjects that progress to dysplasia and adenocarcinoma. Unfortunately such a study would require a large number of participants due to the low rate of progression.

CHAPTER 4: EFFECTS OF IRON, WITH OR WITHOUT ALGINATE, IN OESOPHAGEAL ADENOCARCINOMA

4.1 Introduction

Iron is implicated in the development of Barrett's metaplasia and progression to oesophageal adenocarcinoma. Epidemiological studies of dietary patterns associate iron intake with Barrett's metaplasia[132] and oesophageal adenocarcinoma[26;134;135;137-139]. Patients with the iron overload disease hereditary haemochromatosis have an increased risk of oesophageal adenocarcinoma[158]. In murine models of gastroesophageal reflux supplemental iron increases iron deposition at sites of inflammation, dysplasia and adenocarcinoma formation[148;149;155].

The consequences of iron loading upon oesophageal adenocarcinoma cells are unknown. Indirect evidence suggests this has the potential to modify cell behaviour. Iron is essential for proteins that regulate cell cycle and proliferation. Iron is required for the activity of ribonucleotide reductase (the enzyme responsible for the synthesis of deoxyribonucleotides) and iron deprivation inhibits its function[359-362]. The cell division cycle 14A protein controls progression of cells through the cell cycle. It has recently been identified to possess an iron responsive element within its mRNA 3′ - untranslated region, linking intracellular iron levels, the IRE/IRP system and control of cell cycle[207]. Iron is also required by other cell cycle proteins, cyclins and cyclin dependent kinases[363-365].

In colorectal carcinoma iron induces Wnt signalling and increases β-catenin[366], an effect which is abrogated by restoration of APC function. Iron also suppresses expression of the cell-cell adhesion molecule E-cadherin[251;367]. Loss of APC function occurs in almost all cases of oesophageal adenocarcinoma. Futhermore suppression of E-cadherin is observed in the development of oesophageal adenocarcinoma and subsequent metastasis[92;100;365;368], suggesting a potential role for iron mediated effects in oesophageal adenocarcinoma through an APC deficient route.

N-myc downstream regulated gene (Ndrg-1) is a tumour suppressor gene that acts to control cell proliferation[369;370] and protects cells from developing an invasive and metastatic phenotype[370;371]. Ndrg-1 expression is inhibited by iron[372;373] and provides another mechanism by which the migratory potential of malignant cells is promoted by iron.

Researchers have utilised the high requirement of cancer cells for iron to develop potential therapeutic strategies. Iron chelation has been used as a therapy in the treatment of different cancer types. Desferroxamine decreased tumour mass in children with neuroblastoma[298;299] and increased length of survival and decreased tumour burden in patients with inoperable hepatocellular carcinoma[300]. *In-vitro* experiments demonstrate that novel iron chelators increase sensitivity of tumour cells to conventional chemotherapeutics, even amongst cells that express natural resistance[293], increasing apoptosis[295;296]. Toxic complications associated with these therapies detract from and limit their use[299;310;311]. However, natural iron chelators such as alginates are non toxic, cheap and readily available[328;347]. In epidemiological studies diets high in alginates are associated with a protective effect against various malignant processes[336-339] though the underlying protective

mechanisms are unclear. In a murine model of colorectal dysplasia natural chelation was associated with decreased cell proliferation and tumour burden[330]. The role of alginates as a therapy against the development of oesophageal adenocarcinoma has not been identified.

The increases in cellular iron content and dysregulated expression of iron transport proteins in the progression of oesophageal adenocarcinoma described in chapter 3 indicate a role for iron in the malignant progression of oesophageal adenocarcinoma. Furthermore the role of iron binding by alginates is unclear but may inhibit iron mediated effects.

Thus the aims of this chapter are to:

1. Determine the ability of oesophageal adenocarcinoma cells to import iron and the effects upon expression of iron transport proteins
2. Determine the effects of iron exposure in an in-vitro model of oesophageal adenocarcinoma with respect to cellular proliferation, viability, migration and anchorage independent growth
3. Determine whether effects observed in 2. above can be modulated, by the natural iron chelator, alginate

4.2 Quantification of cellular iron content in oesophageal adenocarcinoma cell lines: effects of iron, desferroxamine and alginate

4.2.1 Iron loading of oesophageal adenocarcinoma cell lines

A ferrozine assay was used to quantify the iron content of oesophageal adenocarcinoma cell lines OE33 and OE19 (fig 4.1A) following culture with iron. In OE33 cells the cellular iron content increased with increasing duration of exposure to iron (100μM ferrous sulphate). Following 12 hours of culture little difference in iron content was observed but after 24 hours a significant increase was observed with a further increase observed in cells cultured for 48 hours ($p<0.001$). In OE19 cells following 12 hours of cell culture a non significant increase in cellular iron content was observed which became strongly significant after 24 and 48 hours of culture ($p<0.001$).

4.2.2 Cellular iron loading is inhibited by the addition of the iron chelator desferroxamine to the media

To determine the ability of iron chelation to inhibit iron uptake by oesophageal adenocarcinoma cells desferroxamine was added to the culture media with and without the addition of supplemental iron. A ferrozine assay was used to measure intracellular iron content following 48 hours of culture (fig 4.1B).

In OE33 cells cultured with supplemental iron there was 9.3 fold increase in iron content relative to control (7.4 ± 1.3 vs. 0.8 ± 0.7 nmols iron per mg cell protein; $p<0.01$). There was non-significant increase in cellular iron content when cells were cultured with iron and desferroxamine relative to control cells (1.9 ± .9 vs. 0.8 ± 0.7 nmols iron per mg cell protein). There was no difference in iron content between

control cells and cells cultured in normal growth media with desferroxamine (0.8 ± 0.7 vs. 0.9 ± 0.3 nmols iron per mg cell protein).

In OE19 cells cultured with supplemental iron there was 7.6 fold increase in iron content compared to control (6.3 ± 1.4 vs. 0.8 ± 0.1 nmols iron per mg cell protein; $p<0.01$). There was a non-significant decrease in cellular iron content when cells were cultured with iron and desferroxamine relative to control cells (0.8 ± 0.7 vs. 0.6 ± 0.1nmols iron per mg cell protein). There was also a non significant difference in iron content between control cells and cells cultured in normal growth media with desferroxamine (0.8 ± 0.7 vs. 0.6 ± 0.1nmols iron per mg cell protein). Following culture with desferroxamine for 5 days cell death occurred with both OE33 and OE19 cells.

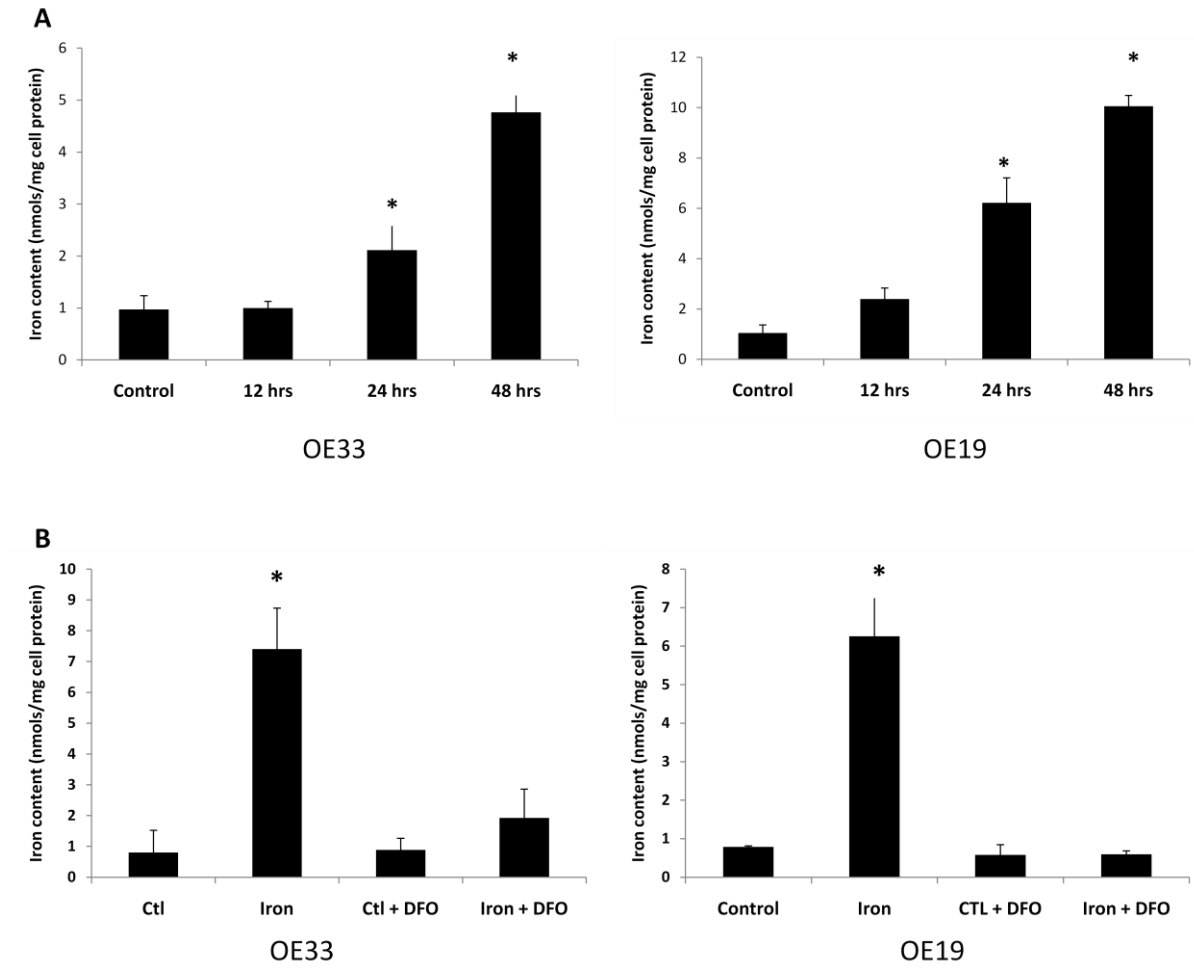


Figure 4. 1. **Iron loading of oesophageal adenocarcinoma cell lines OE33 and OE19 increases cellular iron content, an effect inhibited by iron chelation**

OE33 and OE19 cell lines were exposed to iron (100µM ferrous sulphate) for increasing lengths of time (fig 4.1 A). In both lines prolonging exposure increased cellular iron content. Non-significant increases were observed after 12 hours which became significantly greater after 24 and 48 hours of culture ($p<0.01$). Addition of the iron chelator desferroxamine (250µM) to the culture media inhibited cellular uptake of iron after 48 hours of culture (fig 4.1 B). There was no significant increase in iron content when cells were co-cultured with iron and desferroxamine. After increasing the duration of co-culture cell death occurred in both control and iron loaded cells in the presence of desferroxamine (data not shown).

Ctl – Control; DFO – desferroxamine; *$p<0.01$ relative to control.

4.2.3 Cellular iron loading is inhibited by the addition of alginate to culture media

Having demonstrated that oesophageal adenocarcinoma cells internalise iron following exposure to ferrous sulphate the ability of alginates to bind iron in solution was explored. A 1% w/v solution of LFR5/60 alginate or a 0.1% v/v solution containing the over the counter medicine Gaviscon was added to culture media with or without supplemental ferrous sulphate. A ferrozine assay was used to measure intracellular iron content following 48 hours of cell culture (fig 4.2A – OE33 and fig 4.2B – OE19).

In OE33 cells cultured with supplemental iron there was 3.2 fold increase in iron content compared to control (5.2 ± 0.6 vs. 1.6±0.2 nmols iron per mg cell protein; $p<0.0001$). The addition of alginate to media resulted in a decrease in iron content relative to control (0.9±0.2 vs. 1.6±0.2 nmol/mg cell protein $p<0.01$) whilst Gaviscon also decreased cellular iron content (1.2±0.1 vs. 1.6±0.2 nmol/mg cell protein $p<0.01$). Addition of alginate to iron loaded media resulted in a decrease in iron content relative to cells cultured with iron loaded media (3.3±0.2 vs. 5.2±0.6 nmol/mg cell protein $p<0.01$) as did Gaviscon (1.7±0.1 vs. 5.2±0.6 nmol/mg cell protein; $p<0.001$).

In OE19 cells cultured with supplemental iron there was 8.4 fold increase in iron content compared to control cells (22.5 ± 1.7 vs. 2.7 ± 0.3 nmols iron per mg cell protein; $p<0.0001$). The addition of alginate to control media resulted in a decrease in iron content relative to control cells (1.9±0.5 vs. 2.7±0.3 nmol/mg cell protein; $p<0.01$) whilst Gaviscon had no effect (2.6±1.7 vs. 2.7±0.3 nmol/mg cell protein; p=ns). Addition of alginate to iron loaded media resulted in a decrease in iron content relative to cells cultured with supplemental iron (8.5±0.6 vs. 22.5±1.7 nmol/mg cell protein; $p<0.001$) as did Gaviscon (14.9±0.5 vs. 22.5±1.7 nmol/mg cell protein; $p<0.001$).

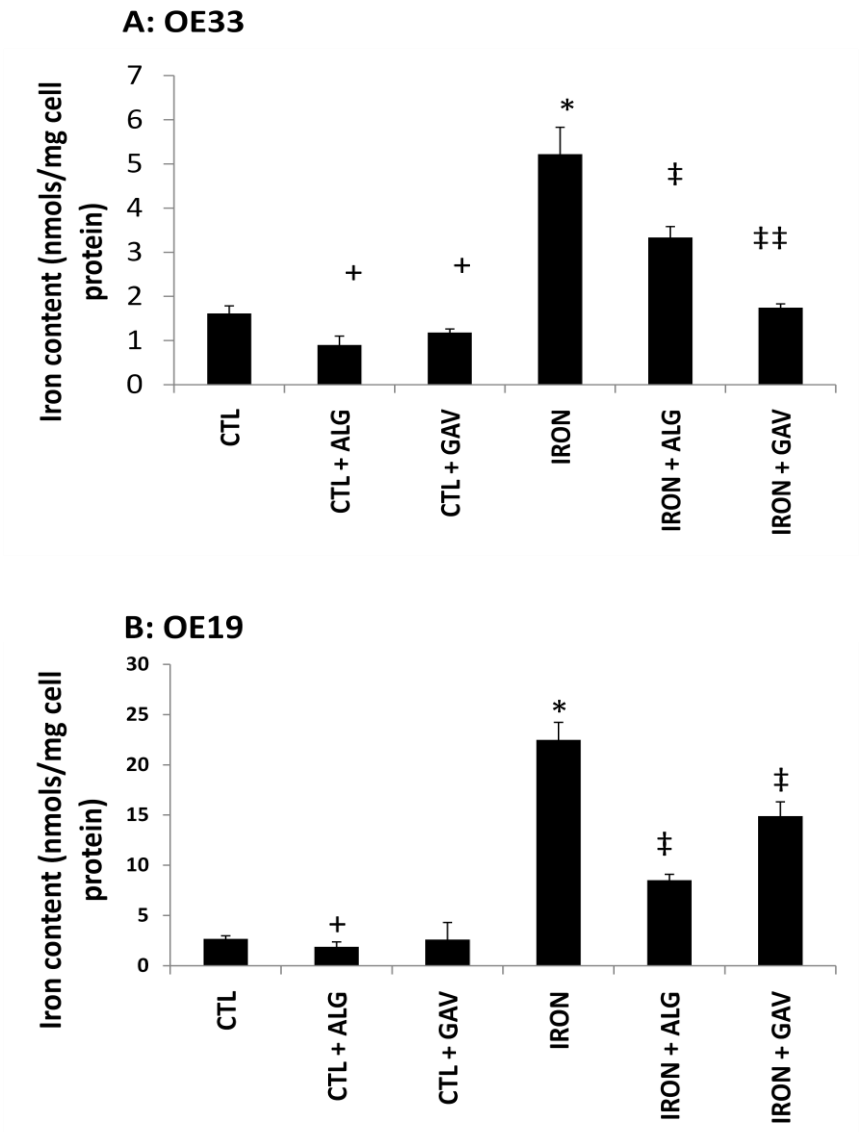


Figure 4. 2. **Alginates suppress iron uptake by oesophageal adenocarcinoma cells**

Cells were cultured in the presence of alginate (1% w/v) or Gaviscon (0.1% v/v) ± iron (100µM ferrous sulphate) for 48 hours. Addition of alginate to control media resulted in a significant reduction of iron content in both cell lines. The addition of Gaviscon to control media resulted in a reduction of iron content in OE33 cells. Addition of alginate or Gaviscon to media loaded with iron resulted in a reduction of iron content in both cell lines compared to cells cultured in iron alone.

+ $p<0.01$ relative to control cells; * $p<0.00001$ relative to control cells; ‡ $p<0.01$ relative to cells exposed to iron; ‡‡ $p<0.001$ relative to cells exposed to iron. CTL: control; ALG: alginate; GAV: Gaviscon; IRON: ferrous sulphate

4.3 Iron loading of oesophageal adenocarcinoma cell lines increases the cell population

To indirectly determine the effects of iron upon proliferation a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify the number of viable cells following culture with or without iron (n=12). In addition alginate or Gaviscon were added to control and iron loaded media to determine effects of alginates upon cell viability (fig 4.2) (n=12). Results are demonstrated relative to control cells normalised to a value of 1.

In OE33 cells a significantly greater number of viable cells were present following 48 hours of cell culture with iron (1.55 fold greater, 2SEM 0.02; $p<0.001$). The addition of alginate to control media resulted in a decrease in viability (0.56 ± 0.09 vs 1 ± 0.07; $p<0.01$) as it did to iron loaded media (iron alone 1.55 ± 0.02 vs. 0.9 ± 0.17; $p<0.001$). Gaviscon produced similar effects in addition to control media (0.68 ± 0.1 vs 1 ± 0.07; $p<0.01$) and iron loaded media (iron alone 1.55 ± 0.02 vs. 1.1 ± 0.11; $p<0.001$), fig 4.2A. In OE19 cells iron also increased the number of viable cells following 48 hours of cell culture (1.49 fold greater, 2SEM 0.05 vs. 1 ± 0.01; $p<0.001$). The addition of alginate to control media resulted in a decrease in viability (0.83 ± 0.09 vs. 1 ± 0.01; $p<0.01$) as it did to iron loaded media (iron alone 1.49 ± 0.05 vs. 0.9 ± 0.17; $p<0.001$). Gaviscon did not effect the cell viability when added to control media (0.99 ± 0.1 vs. 1 ± 0.01; p=ns) but when added to iron loaded media resulted in a significant decrease (iron alone 1.49 ± 0.05 vs. 0.99 ± 0.05; $p<0.001$), fig 4.2B.

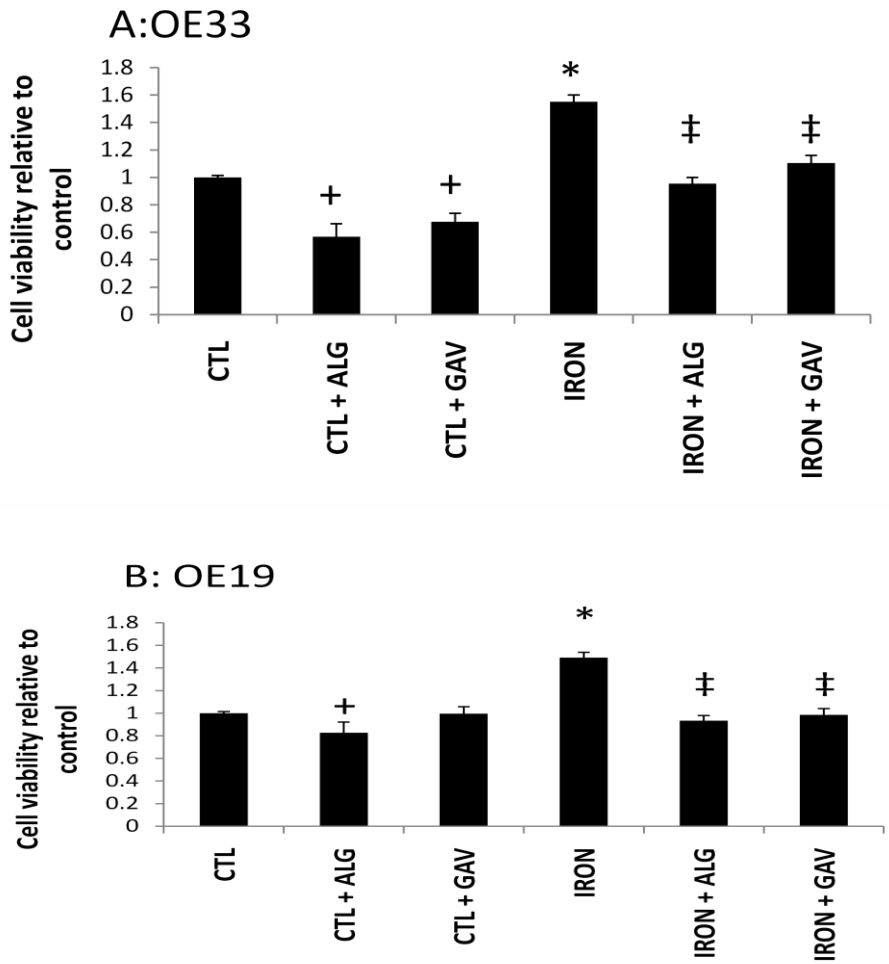


Figure 4. 3. **Alginates inhibit iron mediated increase in cell population**

Cells were cultured in the presence of alginate (1% w/v) or Gaviscon (0.1% v/v) ± iron (100µM ferrous sulphate) for 48 hours (for each regime n=12). Iron significantly increased the viable cells in both cell lines. Addition of alginate to control media or iron loaded media resulted in a significant reduction in cell viabiltiy in both cell lines relative to control or iron loaded media respectively. Gaviscon reduced cell population when added to control media of OE33 cells but not OE19 cells. When added to iron loaded media Gaviscon reduced the cell population of both cell lines.

+ $p<0.01$ relative to control cells; * $p<0.0001$ relative to control cells; ‡ $p<0.001$ relative to cells exposed to iron. CTL: control; ALG: alginate; GAV: Gaviscon; IRON: ferrous sulphate

4.4 Iron loading oesophageal adenocarcinoma cell lines increases cell proliferation

A bromodeoxyuradine (BrdU) assay was used as an indirect measure of cellular proliferation following culture with or without iron (fig 4.4). To further identify effects of iron upon proliferation and the cell cycle analysis of the expression of the cell cycle control protein CDC14A was performed by western blotting (fig 4.5). To determine the effects of alginate or Gaviscon upon proliferation of oesophageal adenocarcinoma cells control and iron loaded cells were exposed to either alginate or Gaviscon.

In OE33 cells the addition of alginate had no effect upon cellular proliferation compared to control (0.96 ± 0.04 vs. 1 ± 0.04; p=ns) whilst Gaviscon was associated with a decrease in BrdU uptake (0.88 ± 0.08 vs. 1 ± 0.04; $p<0.05$). Iron induced proliferation relative to control (1.76 ± 0.07 vs. 1 ± 0.04; $p<0.0001$), an effect partially inhibited by the addition of alginate (1.36 ± 0.19 vs. 1.76 ± 0.07; $p<0.05$) or Gaviscon (1.41±0.04 vs. 1.76±0.07; $p<0.001$), fig 4.3A.

CDC14A is a cell cycle control protein involved in the exit of cell mitosis and initiation of DNA replication. Assessment by western blot analysis demonstrated a 3.3 fold increase in protein expression of CDC14A following iron exposure, fig 4.5.

In OE19 cells the addition of alginate decreased cellular proliferation compared to control (0.59 ± 0.12 vs. 1 ± 0.07; $p<0.001$) whilst Gaviscon was associated with no significant difference in BrdU uptake (1.05 ± 0.06 vs. 1 ± 0.07; p=ns). Iron induced proliferation relative to control (1.36 ± 0.06 vs. 1 ± 0.07; $p<0.0001$), an effect completely inhibited by the addition of alginate (0.88 ± 0.07 vs. 1.36 ± 0.06; $p<0.001$) and partially inhibited by the addition of Gaviscon (1.14 ± 0.03 vs. 1.36 ± 0.06; $p<0.05$), fig 4.4B.

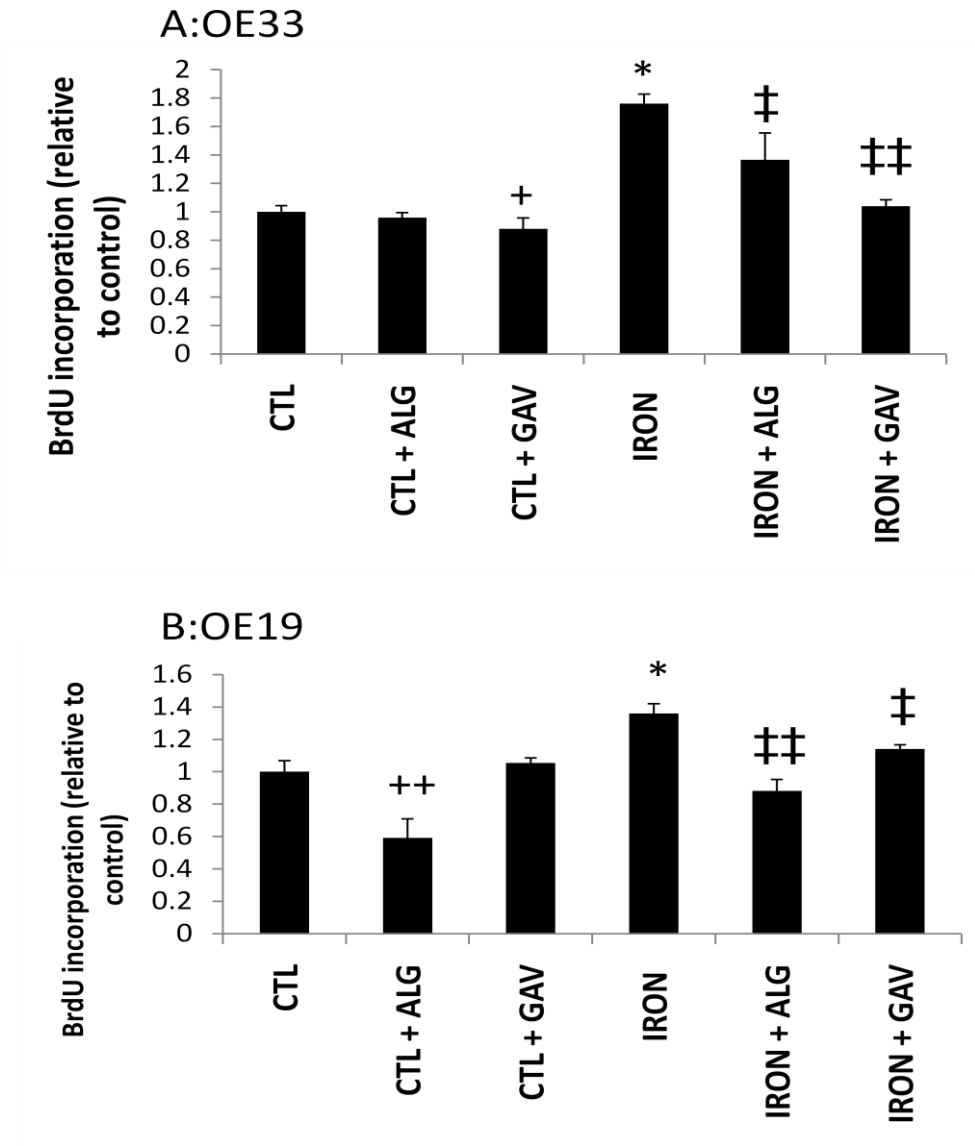


Figure 4. 4. **Iron loading oesophageal adenocarcinoma cell lines increases cell proliferation whilst addition of alginate or Gaviscon inhibits proliferation following 48 hours of cell culture**

Cells were cultured in the presence of alginate (1% w/v) or Gaviscon (0.1% v/v) ± iron (100µM ferrous sulphate) for 48 hours (for each regime n=12). Iron significantly increased cell proliferation in both cell lines. Addition of alginate to control media resulted in a repression of proliferation in OE19 but not OE33. Gaviscon repressed proliferation in OE33 but not OE19 cells. The addition of alginate or Gaviscon

decreased cell proliferation when added to iron loaded media in both lines relative to cells cultured with iron loaded media.

+ $p<0.05$ relative to control cells; ++ $p<0.001$ relative to control cells * $p<0.0001$ relative to control cells; ‡ $p<0.05$ relative to cells exposed to iron; ‡‡ $p<0.001$ relative to cells exposed to iron

CTL: control; ALG: alginate; GAV: Gaviscon; IRON: ferrous sulphate

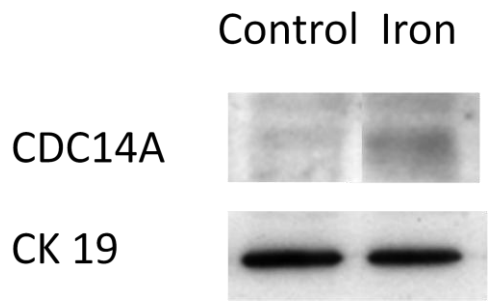


Figure 4. 5. **Iron loading OE33 cells increases expression of the cell cycle control protein CDC14A**

A 3.3 fold increase in expression of the cell cycle control protein CDC14A was observed in OE33 cells following 24 hours of culture with 100µM ferrous sulphate.

4.5 The effect of iron and alginate upon cell migration

Since it has been previously demonstrated that iron can influence E-cadherin expression, a transmembrane cell adhesion molecule involved in cellular migration and invasion, the effect of iron on oesophageal adenocarcinoma cell migration was assessed by wound migration assay (fig 4.6). The effects of supplemental alginate or Gaviscon upon cells cultured with control media or iron loaded media were also explored (figs 4.7 and 4.8 respectively).

4.5.1 The effect of elevated intracellular iron upon cell migration

In OE33 cell monolayers cells rapidly closed a large wound (fig 4.6A). This process was augmented when cells were cultured in the presence of 100μM ferrous sulphate. There was no difference in the initial wound size between the groups ($p=0.3$). After 12 hours of culture 56.9 ± 8.9% of the control wounds remained vs. 43.8 ± 2.4% of wounds exposed to iron ($p<0.05$). After 24 hours of culture 26.0 ± 3.4% of control wound remained vs. 10.8 ± 2.2% of wounds cultured in iron ($p<0.05$).

In OE19 cells wounds were slower to heal than for OE33 cells but again the addition of 100μM ferrous sulphate increased the rate of wound closure (fig 4.6B). There was no difference in the initial wound size between the different groups ($p=0.9$). After 24 hours of culture 68.0 ± 5.0% of control wound remained vs. 48.4 ± 0.7% of wounds exposed to iron ($p<0.001$). After 48 hours 52.1 ± 5.5% of control wounds remained vs. 31.6 ± 2.4% of wounds cultured in iron ($p=0.01$). After 96 hours 23.1 ± 8.5% of control wounds remained vs. 7.2 ± 1.6% of wounds cultured in iron ($p<0.01$; all control wounds remained open while 4/6 wounds exposed to iron had closed). All wounds had closed after 120 hours.

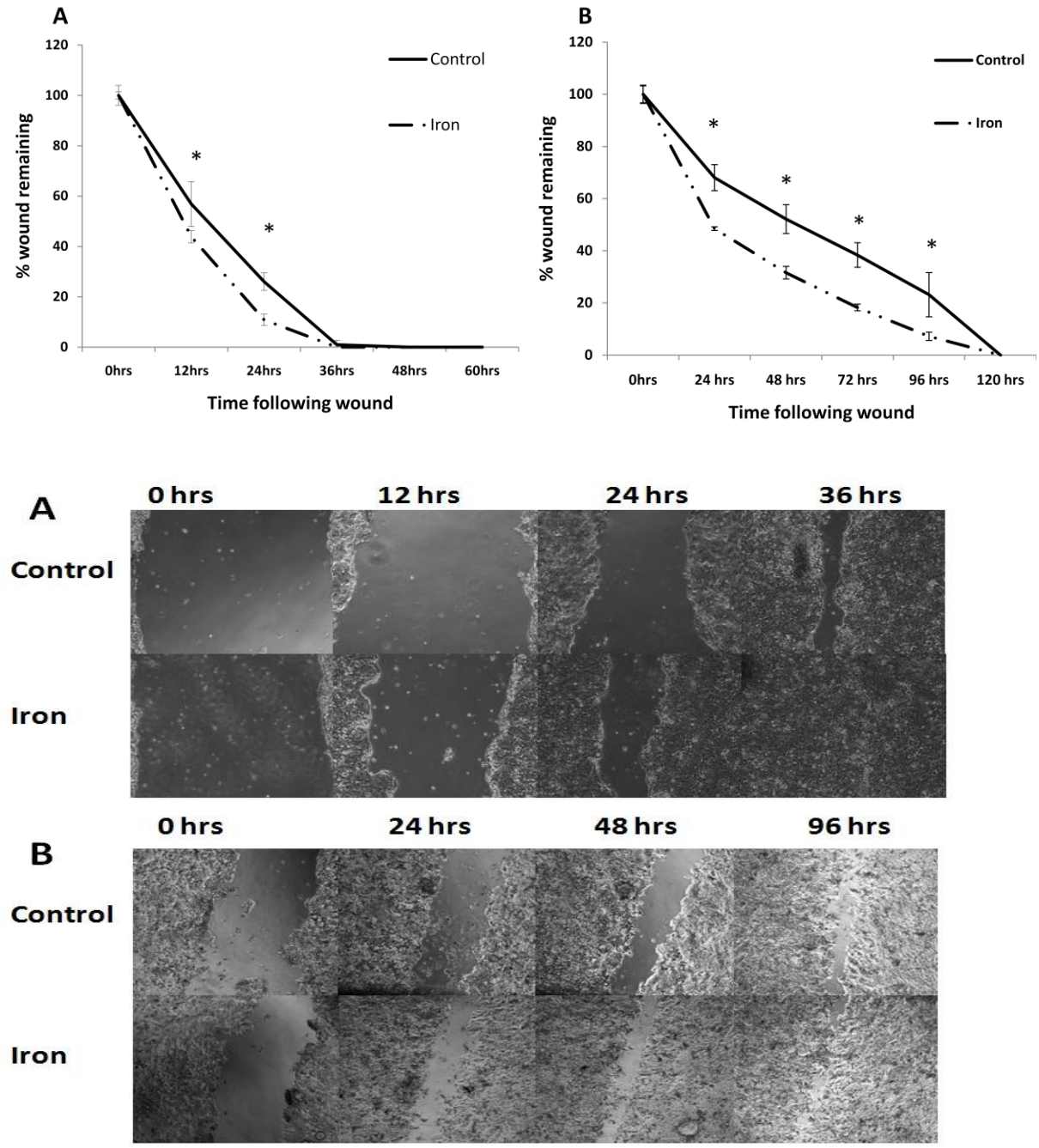


Figure 4. 6. Exposing oesophageal adenocarcinoma cells to iron increases cell migration

Confluent monlayers of OE33 (fig A) and OE19 (fig B) were wounded and cultured in control media (control) or media supplemented with 100µM ferrous sulphate (iron). Cell migration and the rate of wound closure in both cell lines was greater when exposed to iron (* $p<0.05$; N = 6 for each regime)

4.5.2 The addition of alginate or Gaviscon to media does not affect cell migration

Alginate or Gaviscon inhibits iron mediated affects upon cell phenotype including cell migration. To identify effects of alginate or Gaviscon upon cell migration amongst cells cultured in normal culture media a wound healing assay was performed with or without these agents (fig 4.7).

In OE33 cells there appeared to be inhibition of cell migration in cells cultured in the presence of Gaviscon but not alginate (fig 4.7A). There was a significant repression at a single time point: at 24 hours of culture 26.0 ± 3.4% of control wound remained vs. 45.1 ± 13% of wounds cultured with Gaviscon ($p<0.05$). There was no significant difference when alginate was added at any time point.

In OE19 cells there was no difference in cell migration between cells cultured with or without alginate/Gaviscon at any time point (fig 4.7B).

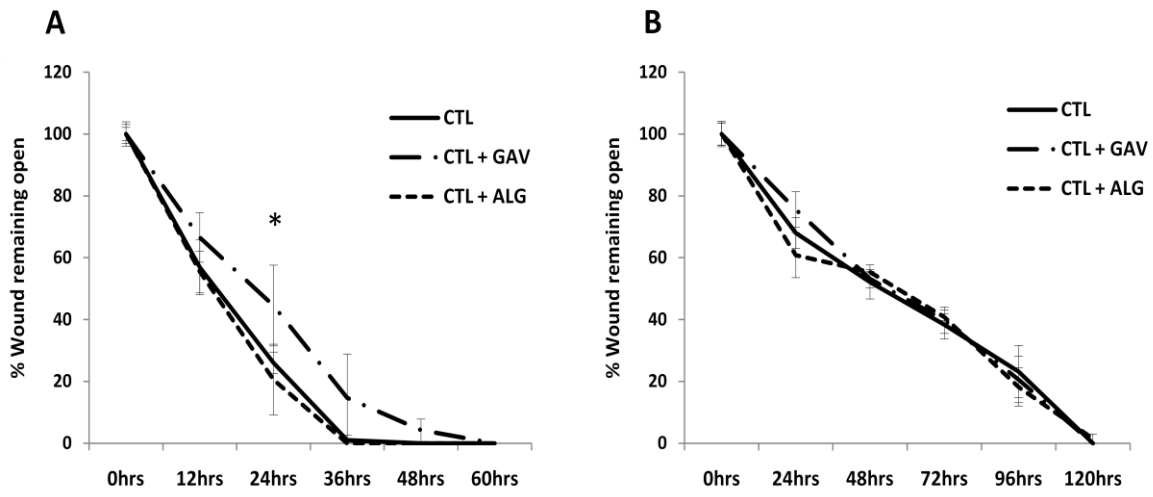


Figure 4. 7. The addition of alginate or Gaviscon to normal culture media has minimal effects upon cell migration in a wound healing assay

Confluent monlayers of OE33 (fig A) and OE19 (fig B) were wounded and cultured in control media (control) or media supplemented with 1% alginate or 0.1% Gaviscon. Cell migration and the rate of wound closure were similar in both lines when Gaviscon or alginate were added to control cells with the exception of Gaviscon at 24 hours in OE33 cell (* $p<0.05$; n = 6 for each regime).

4.5.3 The effect of elevated intracellular iron upon cell migration is inhibited by addition of alginate or Gaviscon

Elevated intracellular iron induces a greater rate of cell migration in oesophageal adenocarcinoma cell lines. To assess whether alginate or Gaviscon can affect this observation the wound healing assay was performed with cells grown in media stimulated with 100μM ferrous sulphate ± alginate (1% w/v LFR5/60 alginate) or Gaviscon (0.1% v/v) (fig 4.8).

In OE33 cells the addition of either alginate or Gaviscon to culture media with iron resulted in a reduced rate of wound closure (fig 4.8A). After 12 hours of culture 43.8 ± 2.4% of wounds exposed to iron remained vs. 58.8 ± 7.1% iron + alginate ($p<0.001$) and vs. 55.9 ± 13.0% iron + Gaviscon ($p<0.05$). After 24 hours of culture 10.8 ± 2.2% of wounds cultured in iron remained vs. 39.5 ± 7.9% iron + alginate ($p<0.05$) and vs. 36.7 ± 9.5 iron + Gaviscon ($p<0.05$). After 36 hours all wounds cultured in the presence of iron alone had closed. At this point 18.4 ± 9.9% of iron + alginate wounds remained as did 12.5 ± 13.6% of iron + Gaviscon (both $p<0.0001$); fig 4.8A

In OE19 cells again the addition of either alginate or Gaviscon to culture media with iron resulted in a reduced rate of wound closure (fig 4.8B). There was no difference in the initial wound size (iron vs iron +alginate $p=0.27$, vs. iron + Gaviscon $p=0.24$). After 24 hours of culture 48.5 ± 0.7% of wounds exposed to iron remained vs. 72.0 ± 6.8% iron + alginate ($p<0.05$) and vs. 87.7 ± 3.7% iron + Gaviscon ($p<0.0001$). After 48 hours of culture 31.6 ± 2.4% of wounds cultured in iron remained vs. 58.3 ± 6.6% iron + alginate ($p=0.02$) and vs. 64.8 ± 7.6 iron + Gaviscon ($p<0.01$). After 72 hours of culture

18.3 ± 1.3% of wounds exposed to iron remained vs. 48.5 ± 9.1% iron + alginate ($p=0.02$) and vs. 43.8 ± 8.1% iron + Gaviscon ($p=0.02$). After 96 hours of culture 7.2±1.6% of wounds cultured in iron remained vs 27.2±6.3% iron + alginate ($p=0.02$) and vs. 30.4±4.5 iron + Gaviscon ($p<0.01$). After 120 hours of culture all wounds cultured in iron had closed whilst 12.9±5.8% iron + alginate remained ($p=0.07$) and vs. 4.5±4.5% iron + Gaviscon ($p=0.36$); fig 4.8B and fig 4.9.

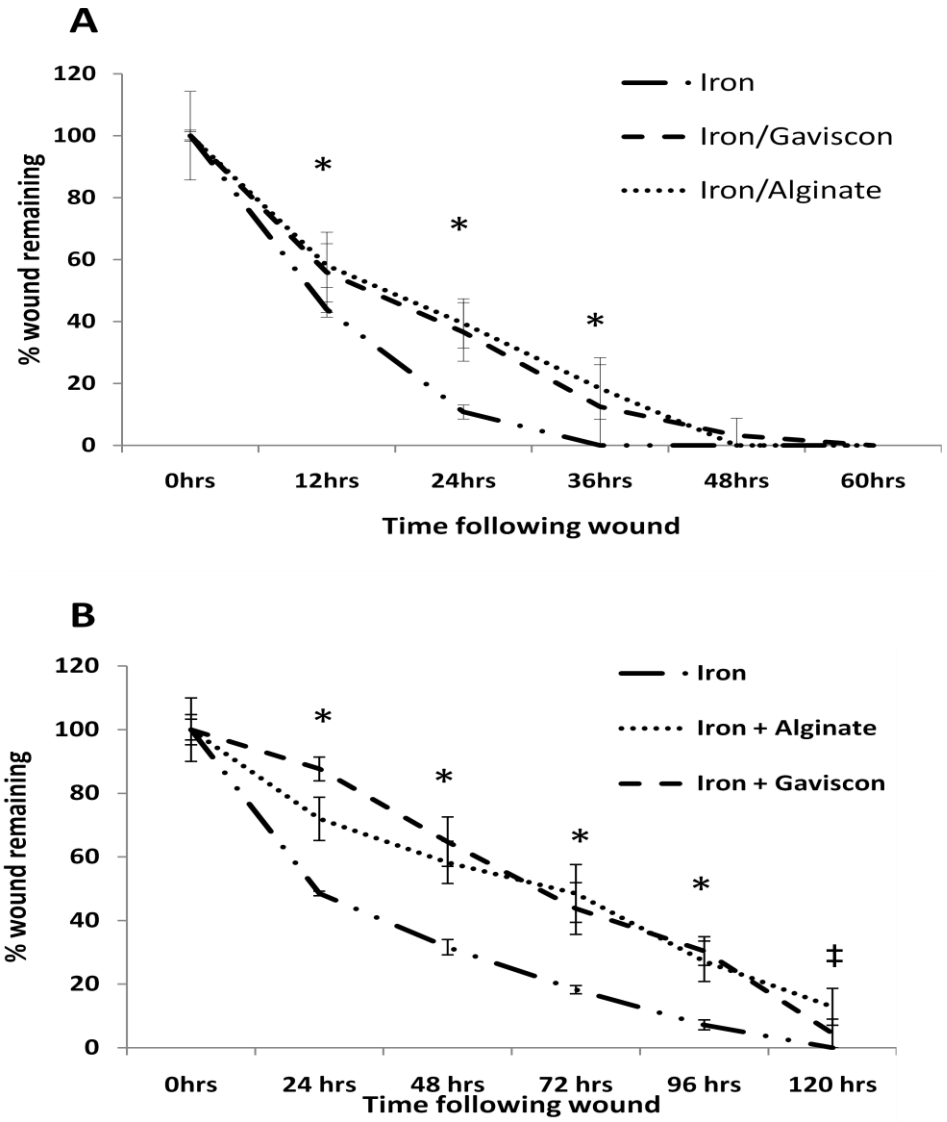


Figure 4. 8. Addition of alginate or Gaviscon to iron loaded media decreases wound migration by oesophageal adenocarcinoma cells

Confluent monlayers of OE33 (fig 4.7A) and OE19 (fig 4.7B) were wounded and cultured in media supplemented with 100µM ferrous sulphate (iron) or with additional alginate (1% W/V) or Gaviscon (0.1% V/V). Cell migration and the rate of wound closure in both cell lines was greater when exposed to iron (* $p<0.05$ iron vs both iron + alginate and iron + gaviscon, ‡ <0.05 iron vs. Iron + alginate; N = 6 for each regime)

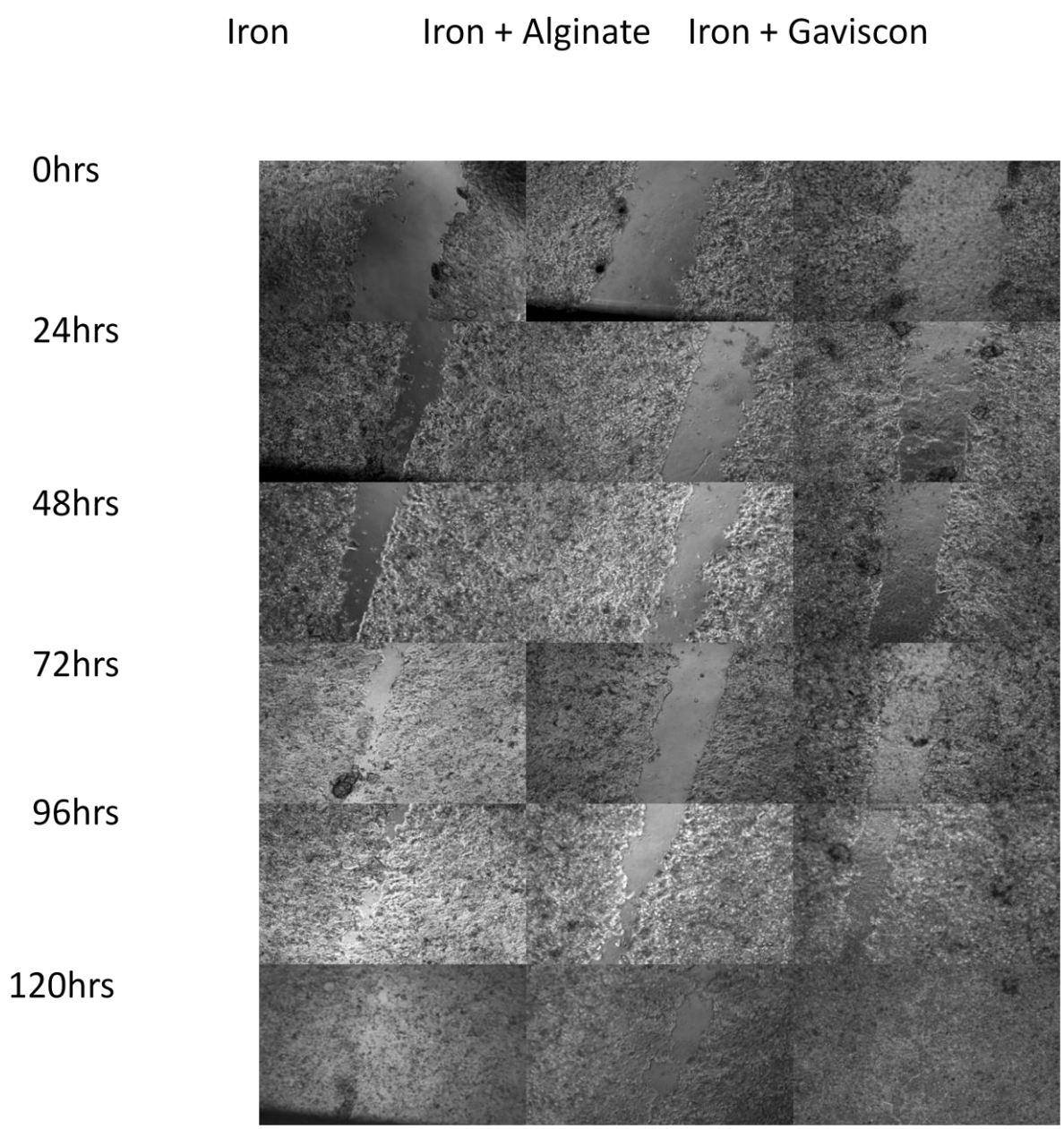


Figure 4. 9. Photo-montage of OE19 wound healing assay

Cells were cultured in media supplemented with 100µM ferrous sulphate (iron) or with additional alginate (1% w/v) or Gaviscon (0.1% v/v).

4.6 The effect of iron and alginate upon anchorage independent growth of oesophageal adenocarcinoma cells

Anchorage independent growth is an important process in carcinogenesis. Benign cells require cell – cell contact and exogenous growth factors for cell division whilst malignant cells have a much greater potential to divide independently. To further explore effects of iron upon cell phenotype colony forming assay was performed (figs 4.10 and 4.11). The effects of alginate to inhibit colony formation were also explored. Single cell agar suspension was seeded in 6 well plates (N=6 each regime) and cultured for 14 days. 3 photographs at random were taken of each well and colonies measured within each photograph.

In OE33 cells iron increased the average size of colonies compared to control (7491 ± 1097 vs. 5564 ± 1038 μM^2, p=0.05). The addition of alginate or Gaviscon decreased the size of colonies relative to control (2514 ± 1014 and 2250 ± 840 respectively vs. 5564 ± 1038 μM^2, both p<0.0001). The addition of alginate or Gaviscon to iron loaded media resulted in a decrease in colony size relative to both iron loaded and control media (1731 ± 414 and 1565 ± 373 μM^2 for iron + alginate and iron and Gaviscon vs. iron [7491 ± 1097 μM^2] both p<0.0001 and vs. control [5564 ± 1038 μM^2] both p<0.0001. There was no significant difference between cells cultured with aliginate ± iron (p=0.15) or with Gaviscon ± iron (p=0.16). Fig 4.10 A and B.

In OE19 cells iron increased the average size of colonies compared to control (14179 ± 2878 vs. 5556 ± 1413 μM^2, p=0.00002). The addition of alginate or Gaviscon had no significant effect upon the size of colonies relative to control (5281 ± 2180 and 6241 ± 2457 respectively vs. 5556 ± 1413 μM^2, both p=ns). The addition of alginate or Gaviscon to iron loaded media resulted in a decrease in colony size relative to iron loaded media (5504 ± 2160 and 4670 ± 1578 μM^2 for iron + alginate and iron and Gaviscon vs. 14179 ± 2878 μM^2 both p<0.0001). This was comparable to colonies grown in control media ± alginate or Gaviscon (both p=NS). Fig 4.11 A and B.

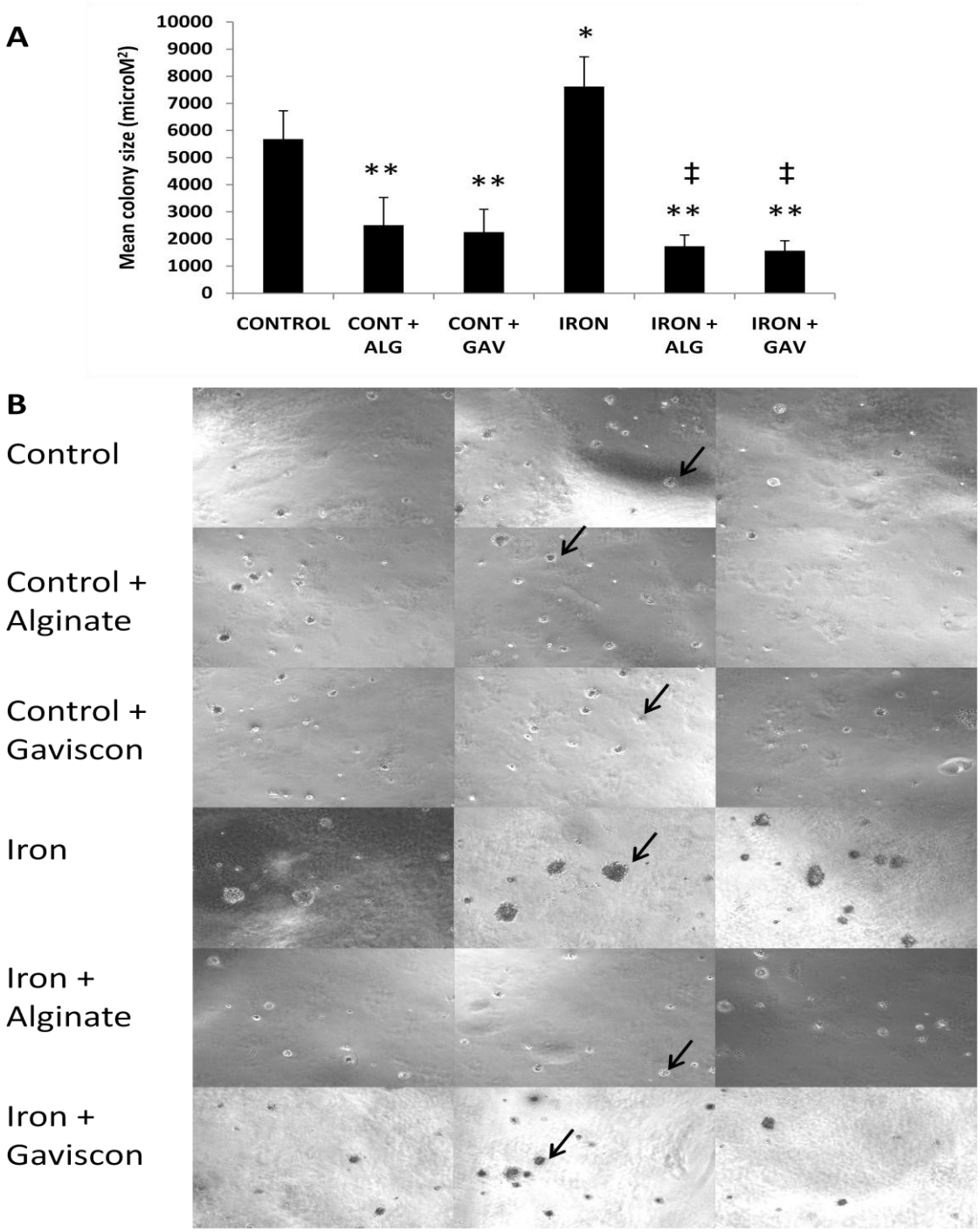


Figure 4. 10. **Colony forming assay OE33 adenocarcinoma cell line**

Cells were cultured in control media or media supplemented with 100µM ferrous sulphate (iron) ± additional alginate (1% w/v) or Gaviscon (0.1% v/v). Colonies were measured (fig A) and photographed (fig B, typical colonies are indicated with arrows) 14 days after seeding a single cell suspension. * $p<0.05$; ** $p<0.0001$ vs control; ‡ $p<0.0001$ vs iron.

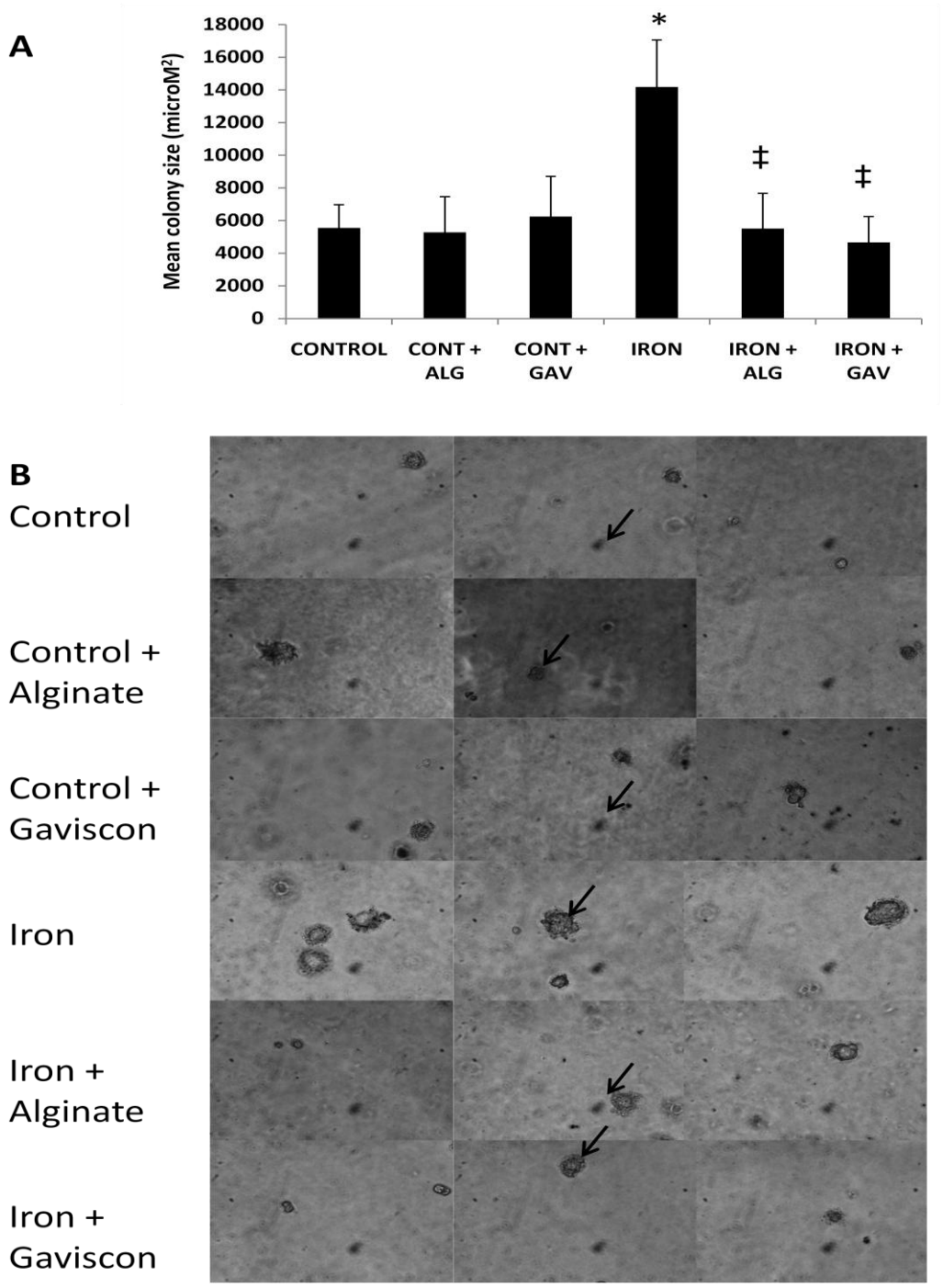


Figure 4. 11. **Colony forming assay OE19 cell line**

Cells were cultured in control media or media supplemented with 100µM ferrous sulphate (iron) ± additional alginate (1% w/v) or Gaviscon (0.1% v/v). Colonies were measured (fig A) and photographed (fig B, typical colonies are indicated with arrows) 14 days after seeding a single cell suspension. * $p<0.05$; ‡ $p<0.0001$ vs iron.

4.7 The effect of iron on the expression of the cellular iron transport proteins in oesophageal adenocarcinoma

To identify how the exposure of oesophageal adenocarcinoma cells to iron affects the expression profile of the iron transporters TfR, DMT1, ferritin, and ferroportin, cells were cultured with or without supplemental iron. OE33 and OE19 cells were exposed to iron and the optimum time period for observing changes in mRNA expression was identified at 12 hours (other periods observed were 3, 6, 9, 24 and 48 hours, data not shown).

Significant induction of TfR and DMT1 mRNA was observed when cultured with iron in both OE33 and OE19 cell lines. There was no significant effect upon the expression of ferritin or ferroportin mRNA in either cell line (table 4.1).

	OE33			OE19		
	CONTROL	IRON LOAD	P	CONTROL	IRON LOAD	P
TfR	1±0.19	2.02±0.26	<0.01	1±0.03	3.36±1.01	0.02
DMT1	1±0.23	1.9±0.2	0.02	1±0.36	2.71±0.16	<0.01
Ferritin	1±0.28	1.35±.032	ns	1±0.19	0.59±0.32	ns
Ferroportin	1±0.05	0.97±0.13	ns	1±0.2	0.9±0.37	ns

Table 4. 1. Change in mRNA expression of TfR, DMT1, Ferritin and ferroportin following culture with or without supplemental iron.

Data are the mean fold change in expression ± 2SEM standardised to control expression. Students T test.

4.8 Elevated expression of TfR and DMT1 mRNA is potentially due to aberrant Wnt signalling

On the basis that increased deposition of iron was observed in samples of oesophageal adenocarcinoma following an iron challenge one could predict a reduction in TfR1 and DMT1 mRNA expression mediated via the IRP/IRE mechanism. This is not occurring as demonstrated by increased TfR1 and DMT1 mRNA expression following culture with iron in 4.7 above suggesting an aberration in IRP/IRE sensing or an alternative mechanism of control. In colorectal adenocarcinoma iron mediates upregulated expression of TfR and DMT1 through positive feedback in cells with an APC gene mutation[251;366]. The mechanism is due to aberrant Wnt signalling with accumulation of beta catenin and downstream upregulation of the oncogene c-myc. This in turn increases IRP2 and thus the increased expression of TfR and DMT1. To explore if similar biochemical events occur in oesophageal adenocarcinoma mRNA from oesophageal adenocarcinoma cell lines was analysed for c-myc and IRP2. Anti beta-catenin was used upon cell lysate to identify expression of beta catenin. Finally an assessment of APC status was performed upon the cell lines used in these experiments by gene sequencing and Western blotting.

4.8.1 Expression of IRP2, c-myc and beta-catenin following iron loading oesophageal adenocarcinoma cells

In OE33 cells cultured with iron an increased expression of IRP2 (2.71 ± 0.16, $P<0.01$) and c-myc (4.12 ± 0.45, $P<0.05$) mRNA was observed relative to control (1 ± 0.66, IRP2 and 1 ± 0.32, c-myc) (fig 4.12). This was associated with an increased expression of beta-catenin protein expression (1.8 ± 0.1 vs 1 ± 0.13, $P<0.001$) (fig 4.13).

In OE19 cells cultured with supplemental iron similar changes were observed with increased expression of IRP2 (2.43 ± 0.67, p<0.05) and c-myc (2.58 ± 0.33, P<0.05) mRNA relative to control (1 ± 0.45, IRP2 and 1 ± 0.32, c-myc) (fig 4.11). This was associated with an increased expression of beta-catenin protein expression (1.6 ± 0.1 vs 1 ± 0.24, P<0.001) (fig 4.13).

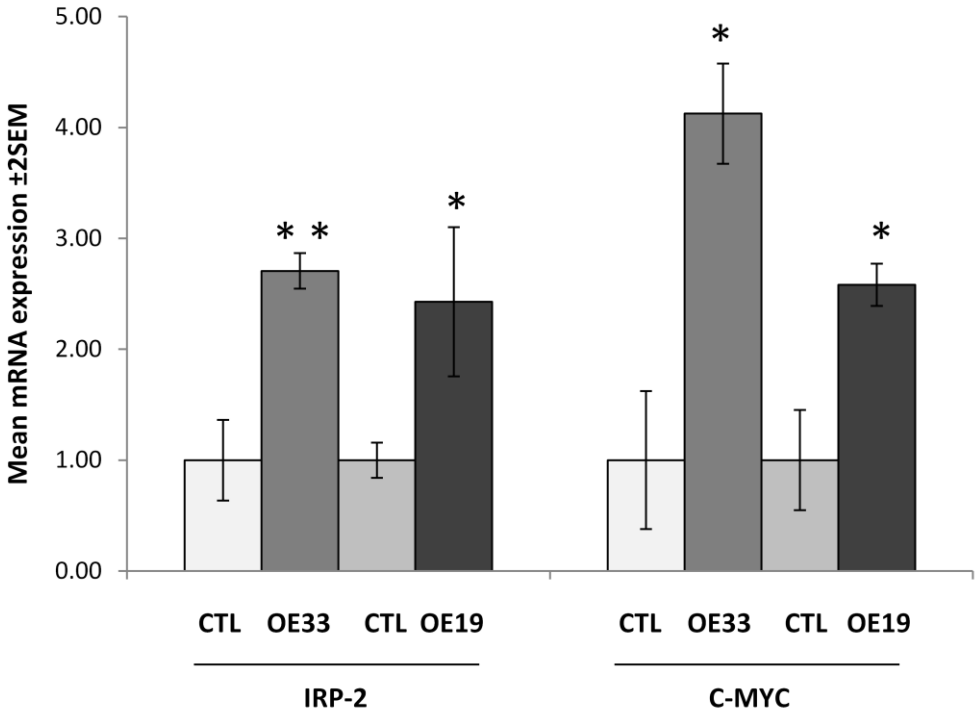
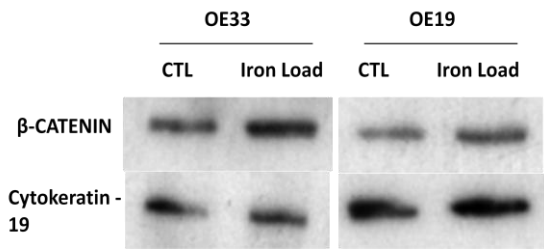


Figure 4. 12. **mRNA expression of IRP-2 and c-myc following exposure to iron in OE33 and OE19 cell lines**

After 12 hours of culture in normal media (control) or media with supplemental iron there was significant upregulation of both IPR-2 and c-myc mRNA expression in both cell lines.

* p<0.05; ** p<0.01



	Mean expression	2SEM	P value
CTL	1.00	0.13	
OE33	1.80	0.09	<0.001
CTL	1.00	0.08	
OE19	1.56	0.15	<0.001

Figure 4. 13. β-catenin protein expression in OE33 and OE19 cell lines following exposure to iron

Increased β-catenin expression was observed in both cells lines following culture with iron (n=3 each regime; $p<0.001$ both cell lines vs control; Student's T test).

4.8.2 APC status of OE33 and OE19 cell lines

APC function is lost in most if not all cases of oesophageal adenocarcinoma[79;124]. Wnt signalling via APC together with axin and GSK-3β acts to phosphorylate beta-catenin. A loss of APC function increases beta-catenin with downstream effects including the transcription of the oncogene c-myc. In colorectal carcinoma this is associated with an increase in iron regulatory protein 2 with subsequent increased iron import via TfR1 and DMT1[366]. The common mechanism of loss of APC function in oesophageal adenocarcinoma is APC gene methylation[124], though APC mutation can occur[79]. To investigate the APC status of the OE19 and OE33 cells used in this body of work gene analysis and assessment of protein function were performed.

4.8.2.1 APC gene sequencing of OE33 and OE19 cell lines

Both cell lines were prepared according to protocol (see methods). PCR demonstrated successful isolation of the APC fragments G, H and I in both cell lines. There was no major gene mutation of the gene multiple cluster region fragments G, H or I in either cell line. A shift mutation occurred at locus 1493 in both cell lines. OE33 cells are homozygous for the A allele (fig 4.14A) whilst OE19 are homozygous for the G allele (fig 4.14B). There is no functional significance as threonine remains threonine in both lines.

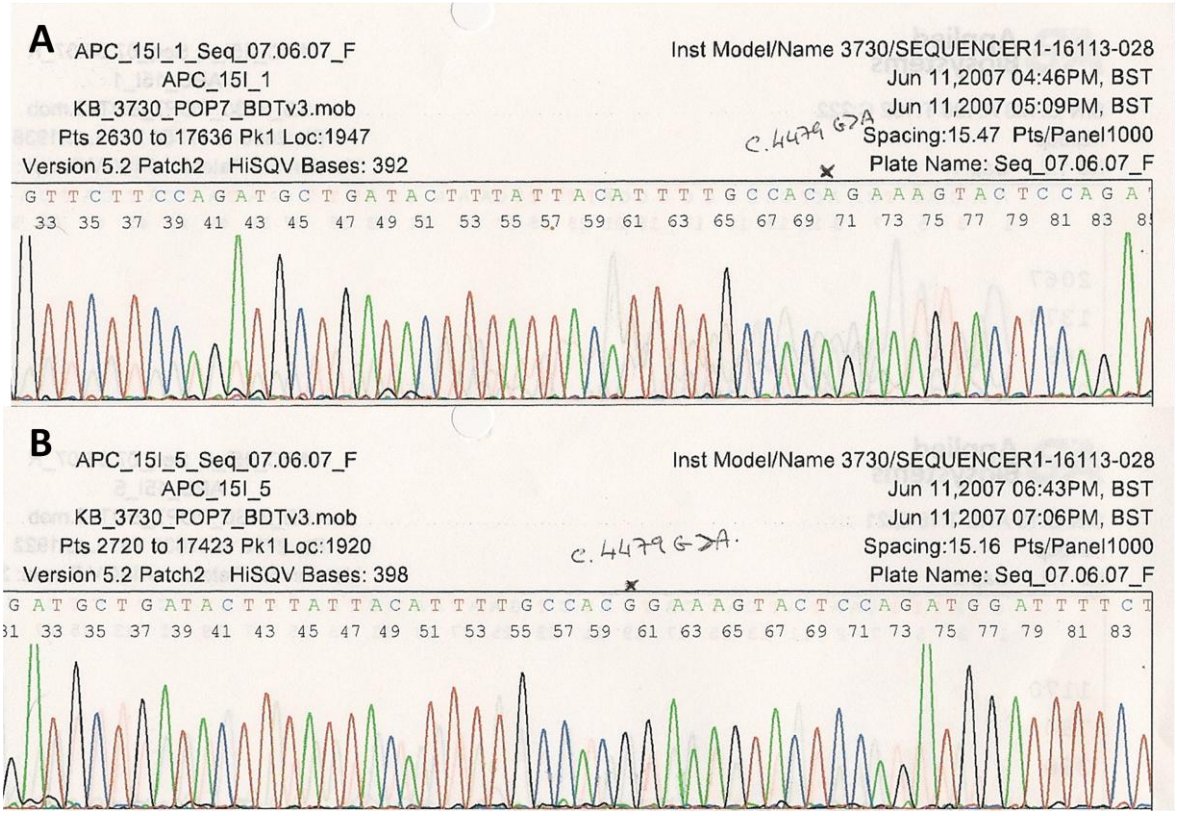


Figure 4. 14. **APC gene analysis of OE33 and OE19 cell lines**

A polymorphism was present in both OE33 and OE19 cell lines in the APC fragment 15I at locus 1493. OE33 cells are homozygous for A allele (fig A) whilst OE19 are homozygous for the G allele (fig B).

4.8.2.2 APC protein function of OE33 and OE19 cell lines

Western blot analysis to the C terminus of the APC gene was performed using RKO cells (wild type for APC) as a positive control. In neither OE19 or OE33 cell lines was functional APC observed (fig 4.15). This is in keeping with findings from other authors[66].

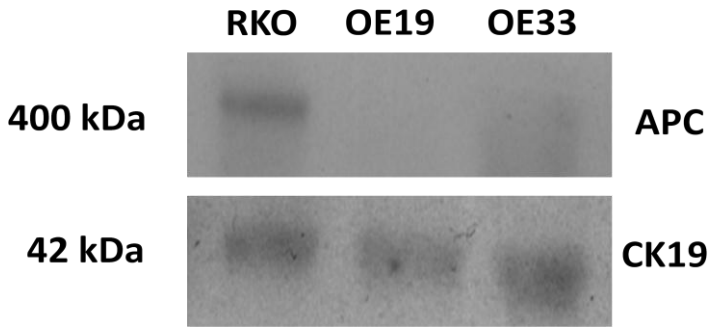


Figure 4. 15. APC analysis of OE19 and OE33

In neither OAC cell line was APC protein expression observed. RKO cells wild type for APC provide a positive control

4.9 Conclusions

Having observed the aberrant expression of iron transport proteins in the progression of oesophageal adenocarcinoma, the *in-vitro* results demonstrated in this chapter further implicate iron metabolism as a key component of this disease process.

Iron is essential for the control of cell cycling through proteins such as p53, p21 and GADD45[250]. In addition iron is essential for cell metabolism, enzymatic reactions, energy production and DNA synthesis[160], with cells, such as tumour cells that are metabolically highly active and rapidly dividing, clearly requiring an elevated level of iron over less active cells[250]. At first glance the overexpression of iron import and repression of export proteins observed in chapter 3 appears likely to reflect the natural metabolism of oesophageal adenocarcinoma cells i.e. the profile of a metabolically active and rapidly dividing cell type. However, following exposure to iron, *in-vitro*, there appears to be positive feedback, with cells demonstrating increasing proliferation and thus the intriguing assumption that iron may actually drive carcinogenesis. Iron exposure of oesophageal adenocarcinoma cells is associated with an increase in expression of transferrin receptor, divalent metal transporter and relocalisation of the iron export protein ferroportin to the cytoplasm where it is non functional. Presumably this profile of iron transport proteins increases the quantity of iron within the cell generating positive feedback. Direct effects of this broadly induce a phenotype one would expect of a malignant cell i.e. increased proliferation, cell migration and anchorage independent growth. Direct evidence that iron induces proliferation is demonstrated with increased expression of the cell cycle control protein CDC14A. The observation in this chapter that exposure of both OAC cell lines OE33 and OE19 to iron

increases expression of the oncogene c-myc, which is accompanied by increased β-catenin, implicates iron with aberrant Wnt signalling in oesophageal adenocarcinoma. This observation has been demonstrated within colorectal adenocarcinoma, where APC mutant cells have increased TOPFLASH reporter activity following culture with iron loaded media[366]. The consequence of this enhanced TCF signalling is activation of Wnt target genes, including the oncogene c-myc[366]. C-myc can induce expression of TfR1 and IRP2 whilst repressing expression of ferritin[374-376]. In APC deficient cells positive feedback is clearly demonstrated in that iron exposure results in elevated expression of TfR1. The physiological effects of this elevated cellular iron load is augmented by increased expression of IRP2. It would be expected that elevated IRP2 would induce the cell to behave in an iron deficient manner, despite the actual elevated iron load, with further induction of TfR1 and DMT1[366]. Whilst the mechanism of APC inactivation is different in oesophageal adenocarcinoma compared to colorectal adenocarcinoma, gene methylation as opposed to mutation, the consequences of iron exposure appear the same with elevated expression of c-myc and TfR1, DMT1 and repression of ferritin. TOPFLASH assays were performed with both OE33 and OE19 cell lines to directly observe the effects of iron exposure upon Wnt signalling in oesophageal adenocarcinoma. Despite numerous attempts and with various transfection mechanisms/strategies it was not possible to reliably and efficiently transfect either cell line with the TOPFLASH reporter. Despite this other authors have demonstrated that OE33[377] and OE19[66] cells have aberrant Wnt signalling pathways and thus it would appear that iron mediated effects within

oesophageal adenocarcinoma replicate those seen in other APC deficient tumours[366] and are likely to reflect pathological Wnt signalling.

CHAPTER 5: CHARACTERISATION OF HAEM IMPORT PROTEINS AND EFFECTS OF HAEM, WITH OR WITHOUT ALGINATE, IN THE MALIGNANT PROGRESSION OF OEOSPHAGEAL ADENOCARCINOMA

5.1 Introduction

Epidemiological evidence associates diets high in iron content with Barrett's metaplasia[132] and oesophageal adenocarcinoma[132-134]. In a rat model of oesophageal reflux iron deposition occurs at sites of oesophagitis which mirrors the cellular location of iron deposition seen in human tissue samples[155-157]. In the animal model metaplasia and adenocarcinoma only occurred in animals receiving supplemental iron[147-149]. Cancer cells have a high requirement for iron which is likely to give them a selective advantage through effects upon cell cycle control[250], tumour suppressor[162;163] and oncogene function[366].

It is unclear whether these effects are mediated by inorganic iron, haem or both. The potential for haem as a causal agent has, to date, been largely neglected. This is presumably due to a lack of understanding of mechanisms of haem absorption from the gut with cellular transport proteins only recently being characterised. The evidence that haem plays an important role in oesophageal adenocarcinoma is summarised as follows:

(1) The association of diets high in red meat with oesophageal adenocarcinoma the majority of epidemiological evidence associates haem, not inorganic iron, with this disease[132-135].

(2) For omnivors haem is both more abundant in the diet and absorbed with greater affinity[230] than elemental iron, thus providing the majority of dietary iron[191].

(3) Body stores of haem and elemental iron should be considered together, as iron can pass from one pool to the other, depending upon body and cell requirements.

(4) Haem absorbed by enterocytes can undergo metabolism to release iron which is exported via ferroportin and transported bound to transferrin. However increasing evidence demonstrates that haem can be exported intact (candidates include the feline leukaemic virus receptor [FLVCR[244]] and the ATP-binding cassette G2 protein [ABCG2[245]] which have both been identified within enterocytes and are known haem exporters within the reticuloendithelial system).

(5) Elaborate control of cellular iron content is affected by the IRE/IRP system. It is interesting to note that haem binds IRP2 with high affinity[232] with subsequent degradation. Depleting IRP2 levels decreases cellular inorganic iron import and facilitates storage via ferritin[232]. Thus haem and inorganic iron within cells are linked via a common control mechanism.

(6) Erythrocytes are a significant store of body iron, in the form of haem, and given the rate of red blood cell turnover recycling of this iron is essential. The fate of the majority of red cells is degradation within liver, spleen and bone marrow with release of free iron. However approximately 20% of cells undergo intravascular haemolysis with release of free haem[378] – a potent oxidant. Scavenging and recycling of this haem occurs via binding with haemopexin.

(7) Haem is a potent oxidant[239] and thus oxidative damage in the malignant progression of Barrett's metaplasia may be mediated via haem if native oesophagus,

metaplastic or adenocarcinoma cells possess haem transporters. This appears plausible as inflammation from gastro-oesophageal reflux, the trigger for Barrett's metaplasia, is associated with increased expression of haemoxygenase-1, an enzyme which catalyses the degradation of haem in response to oxidative stress[155;379].

It is likely that as well as inorganic iron, haem may play a role in the malignant progression of oesophageal adenocarincoma. Therefore it is possible that chelation of haem may also provide a platform for therapeutic intervention. Whether alginates, which others have shown to be natural dietary iron chelators[345;346], are able to comparably bind haem is currently not known.

Thus the aims of this chapter are to:

1. Examine the expression of haem import proteins (HCP-1 and LRP-1) in normal oesophagus, BM and OAC
2. Determine the ability of in-vitro cultured oesophageal adenocarcinoma cells to import haem and the effects upon expression of haem and iron transport proteins
3. Determine the effects of haem exposure in an *in-vitro* model of oesophageal adenocarcinoma with respect to cellular proliferation, viability, migration and anchorage independent growth
4. Determine whether effects observed in 3 above can be modulated, by the natural chelator, alginate
5. Perform knockdown of LRP-1 expression by oesophageal adenocarcinoma cells and determine effects upon haem transport and cell phenotype

5.2 Semiquantitative immunohistochemistry of haem transport proteins in the malignant progression of oesophageal adenocarcinoma

Semiquantitative immunohistochemistry was used to elucidate the cellular localisation and to approximate the expression of haem transport proteins haemopexin receptor (LRP-1) and haem carrier protein (HCP-1) in archived paraffin tissue specimens of normal oesophagus, benign Barrett's metaplasia, Barrett's metaplasia from sections adjacent to matched oesophageal adenocarcinoma and oesophageal adenocarcinoma.

5.2.1 Immunolocalisation of LRP-1 in oesophageal tissue

Weak immunoreactivity was observed in squamous oesophageal cells as they progress towards the luminal border. Immunoreactivity was present in the cytoplasm with no localisation at the cell border. No immunoreactivity was seen in cells in the basal layer (Figure 5.1 S – image A and B). In benign Barrett's metaplasia there was weak immunoreactivity again seen in the cytoplasm of cells at the luminal border but not within the deeper crypts (figure 5.1 BM – images D and E). Dense immunoreactivity was observed in the nucleus and at the apical border of Barrett's samples adjacent to oesophageal adenocarcinoma (Figure 5.1 BM with OAC – images F and G). Dense immunoreactivity was observed in oesophageal adenocarcinoma where LRP-1 was localised to vesicles near the apical border and along the apical membrane (Figure 5.1 OAC images H and I).

5.2.2 Immunolocalisation of HCP-1 in oesophageal tissue

No expression of HCP-1 was observed in squamous oesophagus (Figure 5.2 S – image A). In benign Barrett's metaplasia there was very little immunoreactivity observed (figure 5.2 BM – images C and D). In samples of Barrett's epithelium adjacent to oesophageal adenocarinoma expression was seen throughout the cytoplasm though staining was weak (figure 5.2 BM WITH OAC – images E and F). Strong immunoreactivity was observed in oesophageal adenocarcinoma where HCP-1 was found diffusely throughout the cytoplasm of invasive adenocarcinoma (Figure 5.2 OAC images G and H).

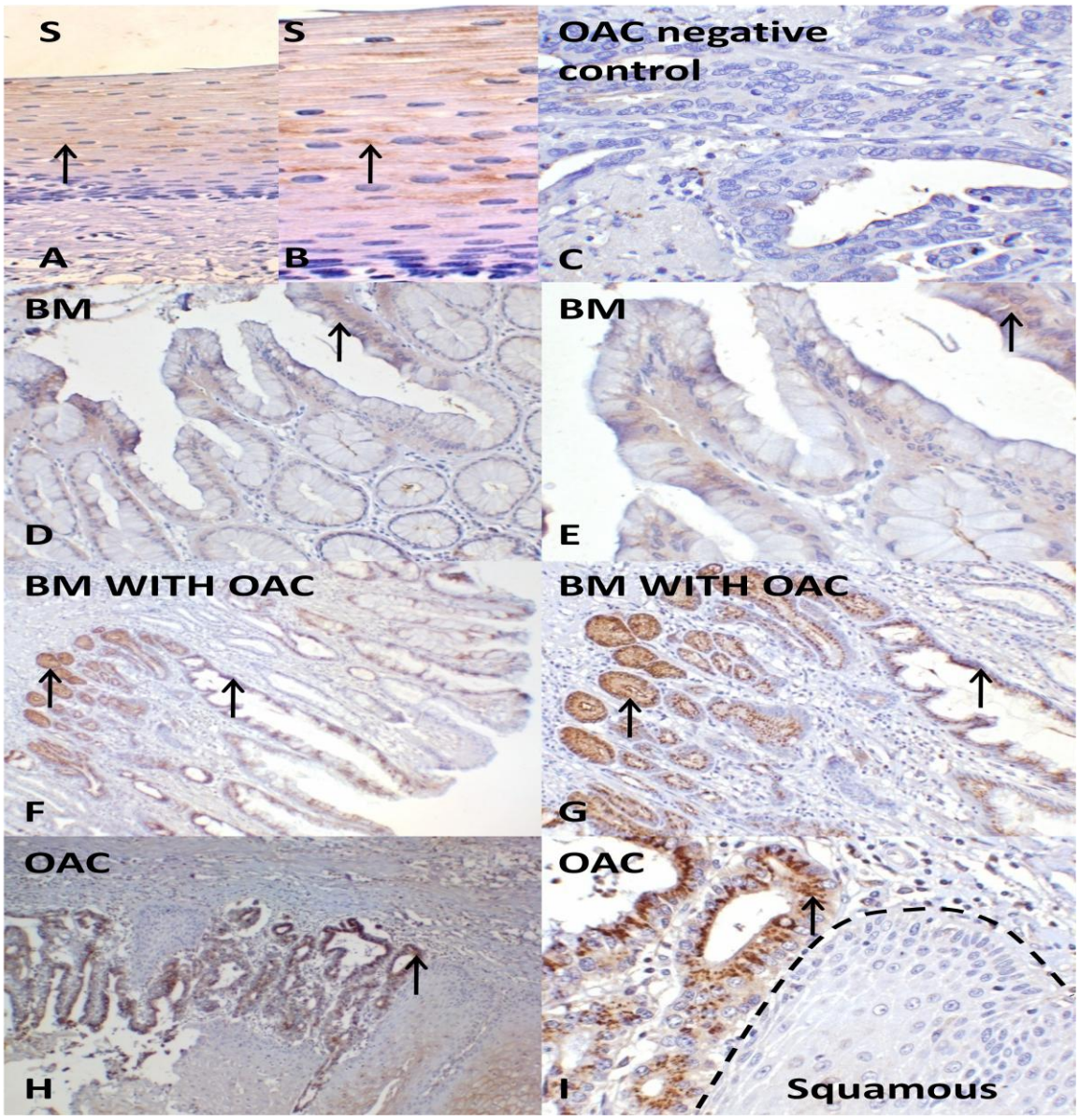


Figure 5. 1. **Immunolocalisation of the LRP1 receptor in oesophageal adenocarcinoma**

LRP-1 immunolocalisation in sections of normal squamous oesophagus (S: A,B n=10), Barrett's metaplasia (BM: D,E n=20), Barrett's metaplasia with matched oesophageal adenocarcinoma (BM with OAC: F,G n=20) and oesophageal adenocarcinoma (OAC: H,I n=20). Weak staining for LRP-1 was observed in squamous oesophagus (S) and benign Barrett's epithelium (BM). Staining was stronger in both Barrett's associated with adenocarcinoma (BM with OAC) and adenocarcinoma (OAC). Negative controls (OAC: C) were performed during the experiments by omission of primary antibody followed by processing with the appropriate secondary antibody. Arrows denote areas of positive expression (original magnification A, D, F, H x20; B, C, E, G, I X40).

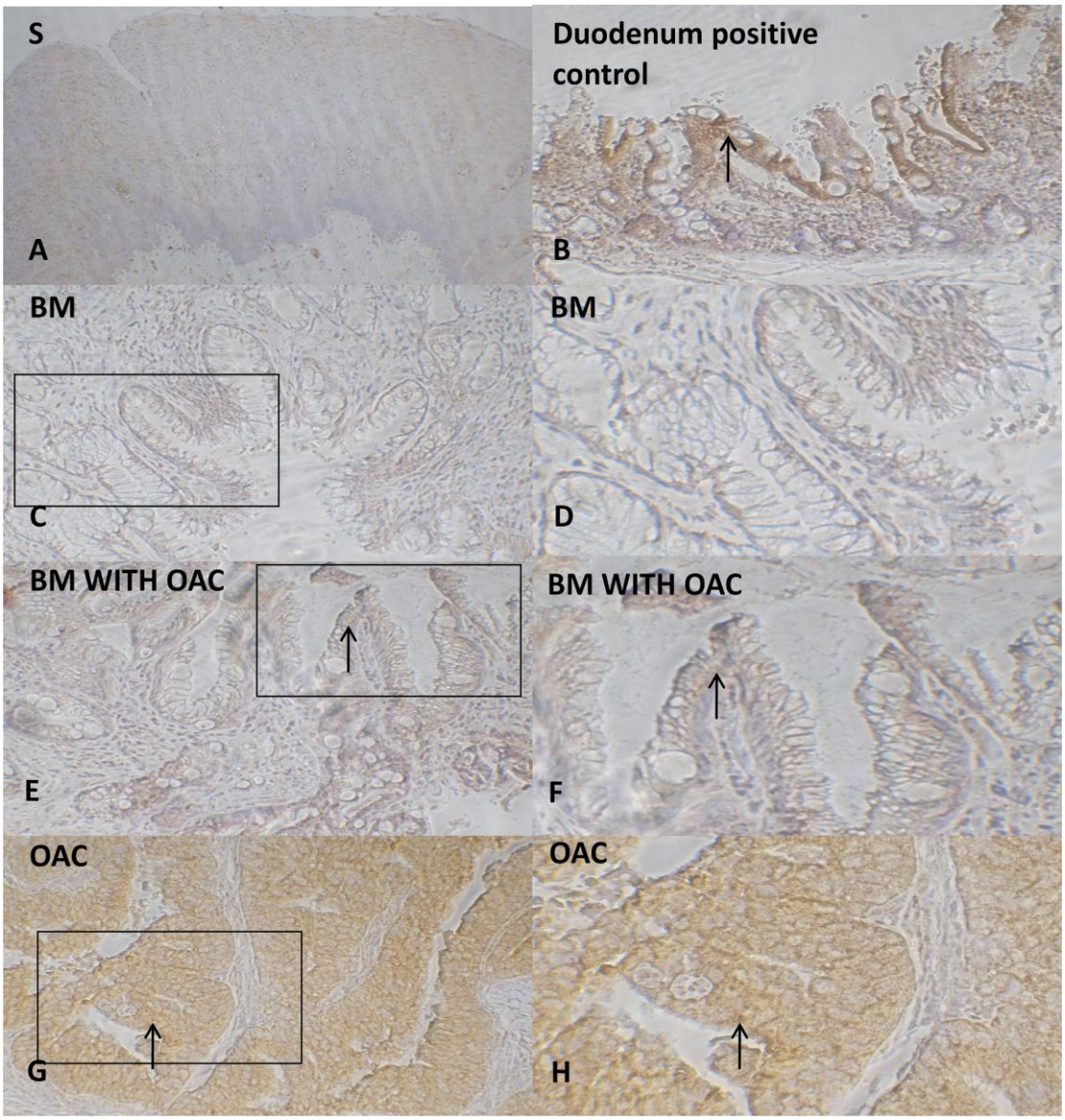


Figure 5. 2. **Immunolocalisation of the HCP1 protein in oesophageal adenocarcinoma**

Immunolocalisation of HCP-1 in sections of normal squamous oesophagus (S – A; n=10), Barrett's metaplasia (BM – C and D; n=20), Barrett's metaplasia from subjects with oesophageal adenocarcinoma (BM WITH OAC – E and F, n=20) and oesophageal adenocarcinoma (OAC – G and H; n=20). No HCP-1 expression was observed in squamous oesophagus. In Barrett's epithelium HCP-1 expression was minimal but in Barrett's adjacent to OAC expression was seen weakly through the cytoplasm. Staining was strong and diffusely spread throughout invasive adenocarcinoma. Negative controls were performed by omission of primary antibody followed by processing with the appropriate secondary antibody, positive control was human duodenum (B) (original magnification A, B, C, E, G x20; D, F, H X40).

5.3 Quantitative analysis of haem transport protein mRNA by real time qRT-PCR

To explore if the expression of haem transport proteins in normal oesophagus, Barrett's metaplasia and adenocarcinoma is regulated at a transcriptional level qRT-PCR was utilised. The expression of LRP-1 and HCP-1 mRNAs was determined relative to 18s ribosomal mRNA internal standard. Non dysplastic Barrett's metaplasia (BM-ve) (over 3cm in length and confirmed as columnar epithelium with intestinal type goblet cells) with no evidence of adenocarcinoma or dysplasia was compared to matched samples of squamous oesophagus and gastric epithelium (n=10). Oesophageal adenocarcinoma samples were compared to matched samples of associated Barrett's metaplasia (BM+ve) (n=12).

5.3.1 Assessment of HCP-1 expression in oesophageal tissue by qRT-PCR

There was no significant difference in HCP-1 mRNA expression between BM-ve and matched gastric epithelium (0.84 ± 0.40, mean ± 2SEM) or with matched squamous epithelium (1.61 ± 0.4). Expression of HCP-1 mRNA was greater in 11/12 samples of oesophageal adenocarcinoma compared to BM+ve (7.90 ± 3.97; p=0.035); fig 5.3A.

5.3.2 Assessment of LRP-1 expression in oesophageal tissue by qRT-PCR

There was no significant difference in LRP-1 mRNA expression between BM-ve and matched gastric epithelium with an average fold difference of 1.66 ± 0.67 (mean ± 2SEM) or when compared with matched squamous epithelium 1.81 ± 1. Expression of LRP-1 mRNA was greater in every sample of oesophageal adenocarcinoma compared to BM+ve (6.20 ± 0.60; p=0.02); fig 5.3B.

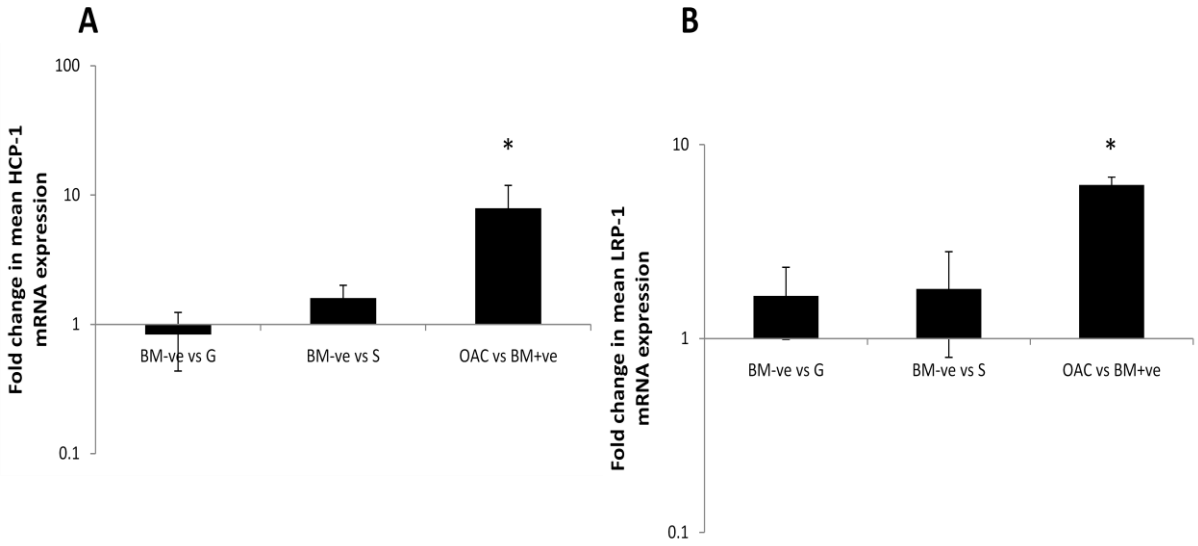


Figure 5. 3. **Mean mRNA expression of haem transport proteins in the progression of oesophageal adenocarcinoma**

No significant difference in expression of HCP-1 (fig A) or LRP-1 (fig B) when comparing BM-ve with gastric or squamous oesophagus. Significant upregulation of HCP-1 and LRP-1 mRNA expression in oesophageal adenocarcinoma versus associated Barrett's epithelium (n=12; $p<0.05$).

HCP-1 – haem carrier protein; BM-ve - -benign Barrett's epithelium; G - gastric epithelium; S - squamous oesophagus; OAC - oesophageal adenocarcinoma; BM+ve - Barrett's epithelium associated with oesophageal adenocarcinoma ; * $p<0.05$

5.4 Determination of haem import by oesophageal adenocarcinoma cell lines

To determine whether oesophageal adenocarcinoma cells are able to import haem the following assays were performed using oesophageal adenocarcinoma cell lines:

(1) Cell culture in the presence of the fluorescent haem analogue, zinc protoporphyrin (ZnPP)

(2) Cultured in the presence of haem followed by a ferrozine assay to assess cellular iron content

(3) Cultured in the presence of radiolabelled haem^{55}Fe

5.4.1 Determination of fluorescence following culture with ZnPP

After culture in the presence of ZnPP adherent cells were thoroughly washed with 5% albumin (w/v) solution to remove surface ZnPP. Cells were excited by light at 415 nm and observed at 630 nm. Both OE33 and OE19 cells showed evidence of fluorescence as demonstrated in figs 5.4 A and B. Most cells demonstrated moderate diffuse fluorescence whilst a few cells demonstrated marked fluorescence.

5.4.2 Determination of cellular iron content by ferrozine assay following culture with haem

After 24 hours of cell culture in the presence of 50μM haem a ferrozine assay was performed on both OE19 and OE33 cell lines (fig 5.5 A and B). Exposure to haem increased the iron content from 2.7 ± 0.3 to 14.6 ± 0.5 nmol Fe/mg cell protein ($p<0.0001$) in OE19 cells and from 4.0 ± 0.4 to 16.9 ± 0.9 nmol Fe/mg cell protein ($p<0.0001$) in OE33 cells.

5.4.3 Quantification of radiolabelled haem^{55}Fe uptake in oesophageal adenocarcinoma cell lines

OE19 cells were cultured in the presence of a 50μM haem solution containing 1% haem^{55}Fe for two hours. The same volume of haem^{55}Fe media was added to empty wells for the same duration. A further set of control wells contained OE19 cells cultured in media containing a 50μM haem solution without supplemental haem^{55}Fe. Culture media were aspirated and added to scintillant. Adherent cells were washed following which they were trypsinised and resuspended in scintillant fluid. 91.0±2.4% of cell counts remained in the culture fluid from wells containing OE19 cells (fig 5.6 A). In the cell pellets little background radioactivity was evident in cells cultured in normal media, 57 ± 25 DPM^{-1}, but in those cultured with haem^{55}Fe there were 20600 ± 163 DPM^{-1} (fig 5.6 B, $p<0.00001$).

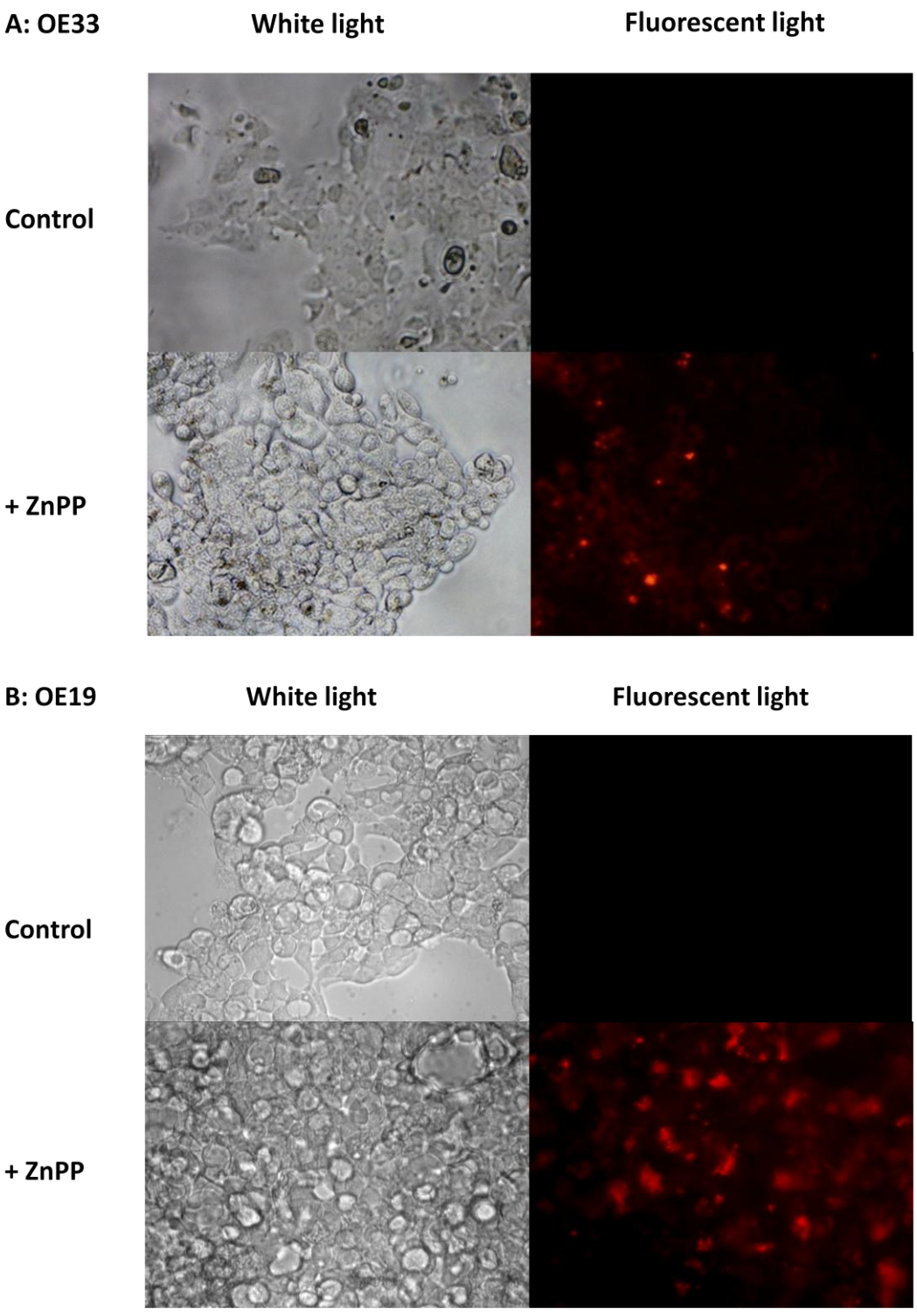


Figure 5. 4. Uptake of zinc protoporphyrin by OE33 (fig 5.4A x40) and OE19 (fig 5.4B x40) cells

Both cell lines internalised ZnPP following 4 hours of culture.

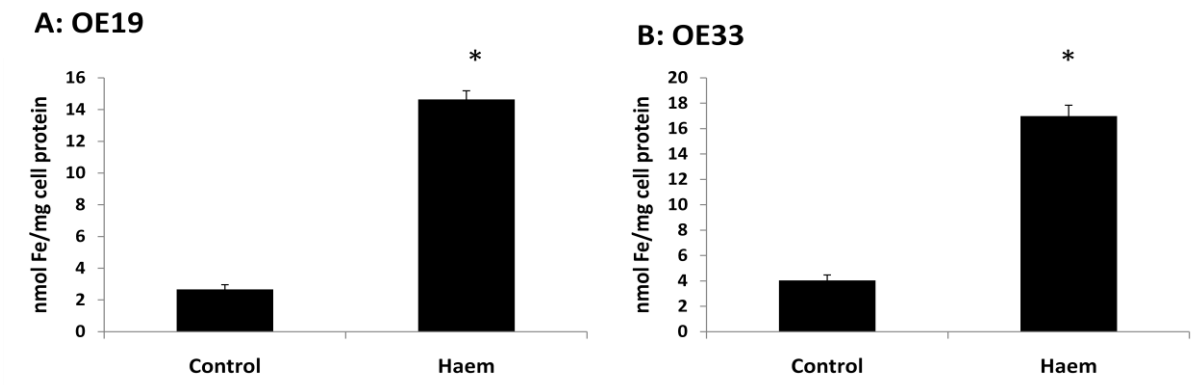


Figure 5. 5. **Ferrozine assay upon oesophageal adenocarcinoma cells following culture with haem**

OE19 (fig A) and OE33 (fig B) cells were cultured in a 50µM solution of haem or control media for 24 hours. Following this cells were washed, trypsinised with the subsequent cell pellet washed three times. Ferrozine assay demonstrated increased intracellular iron content of both OE19 and OE33 cells (both p<0.0001 *)

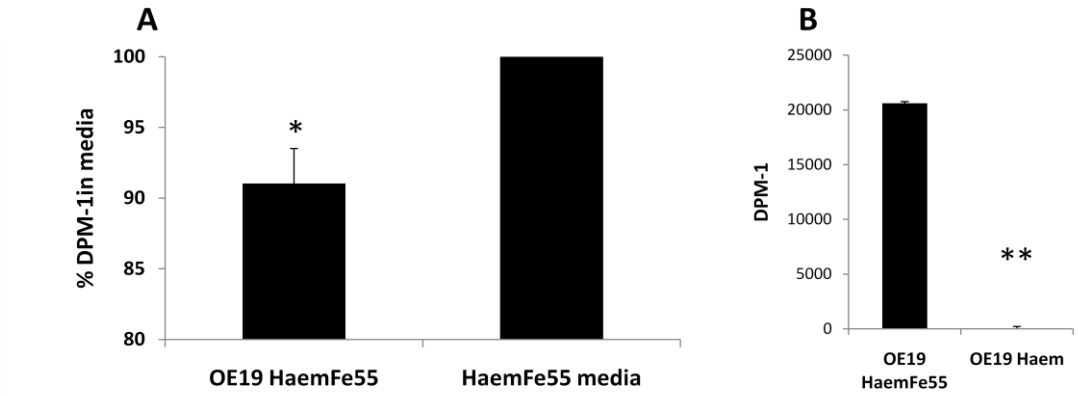


Figure 5. 6. **Uptake of radiolabelled haem^{55}Fe by OE19 cells**

haem^{55}Fe loaded media (3ml with 50µM haem [99% haem, 1% haem^{55}Fe] was added to wells containing OE19 cells or empty wells (fig A); a further control consisted of OE19 cells cultured with haem with no radioisotope (fig B). After two hours the media was

aspirated and the disintegrations per minute (DPM^{-1}) were measured. Media from wells with OE19 cells expressed 91 ± 2.4% of the disintegrations that media from empty wells expressed (fig 5.6 A, n=3, *p<0.001). Fig 5.6B demonstrates little background radioactivity in control cells but the OE19 cell pellet from the wells with haem^{55}Fe had significantly greater radioactivity (n=3,** p<0.0001).

5.5 Cellular iron loading of cells cultured in haem is inhibited by the addition of alginates to culture

Oesophageal adenocarcinoma cells cultured in haem have elevated intracellular iron content. Alginate suppresses iron loading of cells cultured in supplemental iron sulphate and thus the effects of alginate to abrogate the haem loading of cell lines exposed to haem with or without supplemental alginate was explored. A 1% (w/v) solution of LFR 5/60 alginate or a 0.1% (v/v) solution of Gaviscon was added to the culture media with or without supplemental haem (for each regime n=6). A ferrozine assay was used to quantify intracellular iron content (fig 5.7A – OE33 and fig 5.7B – OE19).

In OE33 cells, after 48 hours of cell culture with 50µM haem solution, there was a 1.6 fold increase in iron content compared to control (2.52 ± 0.3 vs. 1.62 ± 0.17 nmol Fe/mg cell protein, p=0.014). Addition of alginate to control media reduced iron content (0.90 ± 0.2 vs. 1.62 ± 0.17 nmol Fe/mg cell protein, p=0.02) as did Gaviscon (1.19 ± 0.07 vs. 1.62 ± 0.17 nmol Fe/mg cell protein, p=0.0001). Addition of alginate to haem loaded media resulted in a non significant decrease in iron content (1.87 ± 0.18

vs. 2.52 ± 0.3 nmol Fe/mg cell protein, p=0.26) whilst the addition of Gaviscon reduced the iron content to that similar to control + Gaviscon (0.82 ± 0.18 vs 2.52 ± 0.3 nmol Fe/mg cell protein, p=0.0001).

In OE19 cells, after 48 hours of cell culture with haem solution, there was a 5.5 fold increase in cellular iron content compared to cells grown in control media alone (14.6 ± 0.31 vs. 2.67 ± 0.2 nmol Fe/mg cell protein, p=0.0001). Addition of alginate to control media resulted in a slight decrease in iron content (1.89 ± 0.34 vs. 2.67 ± 0.2 nmol Fe/mg cell protein, p=0.002) whilst Gavison had no significant effect (2.60 ± 0.13 vs. 2.67 ± 0.2 nmol Fe/mg cell protein, p=0.4). The addition of alginate to haem loaded media resulted in a significant decrease in iron content compared to cells culture in haem loaded media alone (12.39 ± 0.61 vs. 14.6 ± 0.31 nmol Fe/mg cell protein, p=0.001) as did Gaviscon (10.81 ± 0.31 vs. 14.6 ± 0.31 nmol Fe/mg cell protein, p=0.0001).

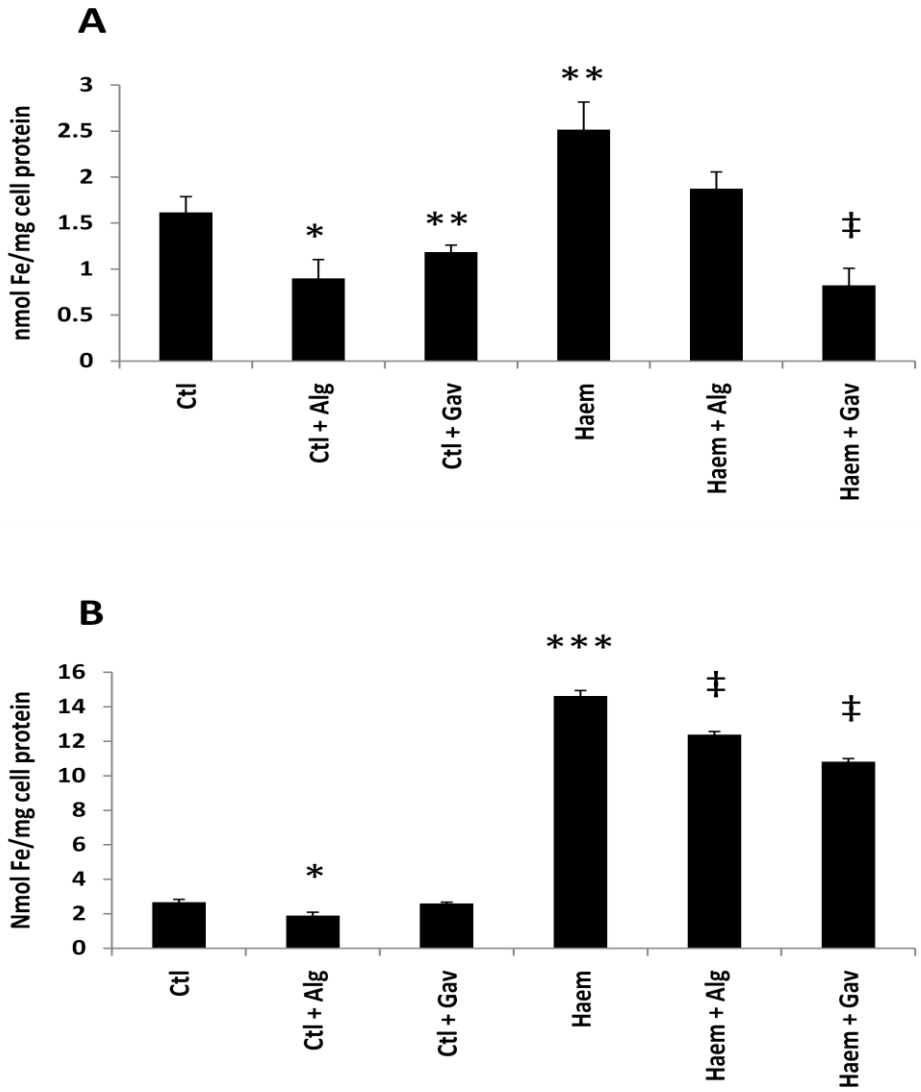


Figure 5. 7. **Addition of alginate or Gaviscon to haem loaded media decreases cellular iron content in OE33 cells (fig 5.7A) and OE19 cells (fig 5.7B)**

Cells were cultured for 48 hours in the presence of control media or a 1% alginate solution or 0.1% Gaviscon solution ± 50µM Haem (for each regime n=6). Addition of alginate to control media resulted in a significant decrease in iron content in both cell lines ($p<0.05$) whilst Gaviscon decreased the iron content in OE33 cells ($p<0.05$). Haem

significantly increased the iron content of both cell lines ($p<0.0001$), an effect significantly suppressed by the addition of Gaviscon to either cell line ($p<0.001$). The addition of alginate to haem media decreased the iron content of OE19 cells ($p<0.001$) but not in OE33 cells.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control cells; ‡ $p<0.001$ vs haem loaded cells

Ctl: control; Alg: 1% w/v alginate; Gav: 1% Gaviscon; Haem: 50µM haem solution

5.6 Haem loading oesophageal adenocarcinoma cell lines increases the cell viability an effect suppressed by the addition of alginate

Culturing oesophageal adenocarcinoma cell lines with supplemental iron increased cell viability. To explore whether haem exposure would produce similar effects an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to quantify the viable cells following 48 hours of culture with and without haem added to culture media. Given that the addition of alginates to media decreases the cellular iron content of cells cultured in the presence of iron the effect of adding alginate (1% w/v) or Gaviscon (0.1% v/v) to control and haem loaded media was also explored (fig 5.8) (for each regime n=12).

In OE33 cells, following 48 hours of cell culture, significantly greater numbers of viable cells were present when cultured in the presence of media supplemented with 50µM haem (1.55 ± 0.02 vs 1.0 ± 0.07, $p=0.0001$). The addition of alginate to control media significantly decreased the cell viability relative to control (0.57 ± 0.09 vs. 1 ± 0.07,

p=0.001) as did Gaviscon (0.68 ± 0.11 vs. 1 ± 0.07, p=0.0009). Supplementing haem loaded media with alginate decreased the cell viability relative to haem loaded media alone (0.95 ± 0.16 vs. 1.55 ± 0.02, p=0.0003) with Gaviscon having a similar effect (1.1 ± 0.1 vs. 1.55 ± 0.02, p=0.0003), fig 5.8A.

In OE19 cells haem also increased the population of viable cells following 48 hours of cell culture compared to control (1.47 ± 0.19 vs 1.0 ± 0.12, p=0.01). The addition of alginate to control media non-significantly decreased cell viability relative to control (0.83 ± 0.12 vs. 1 ± 0.12, p=0.15) as did Gaviscon (0.78 ± 0.19 vs. 1 ± 0.12, p=0.06). Supplementing haem loaded media with alginate non-significantly decreased cell viability relative to haem loaded media alone (1.20 ± 0.13 vs. 1.47 ± 0.19, p=0.1) with Gaviscon having a more marked and significant effect (0.82 ± 0.13 vs. 1.47 ± 0.19, p=0.002), fig 5.8B.

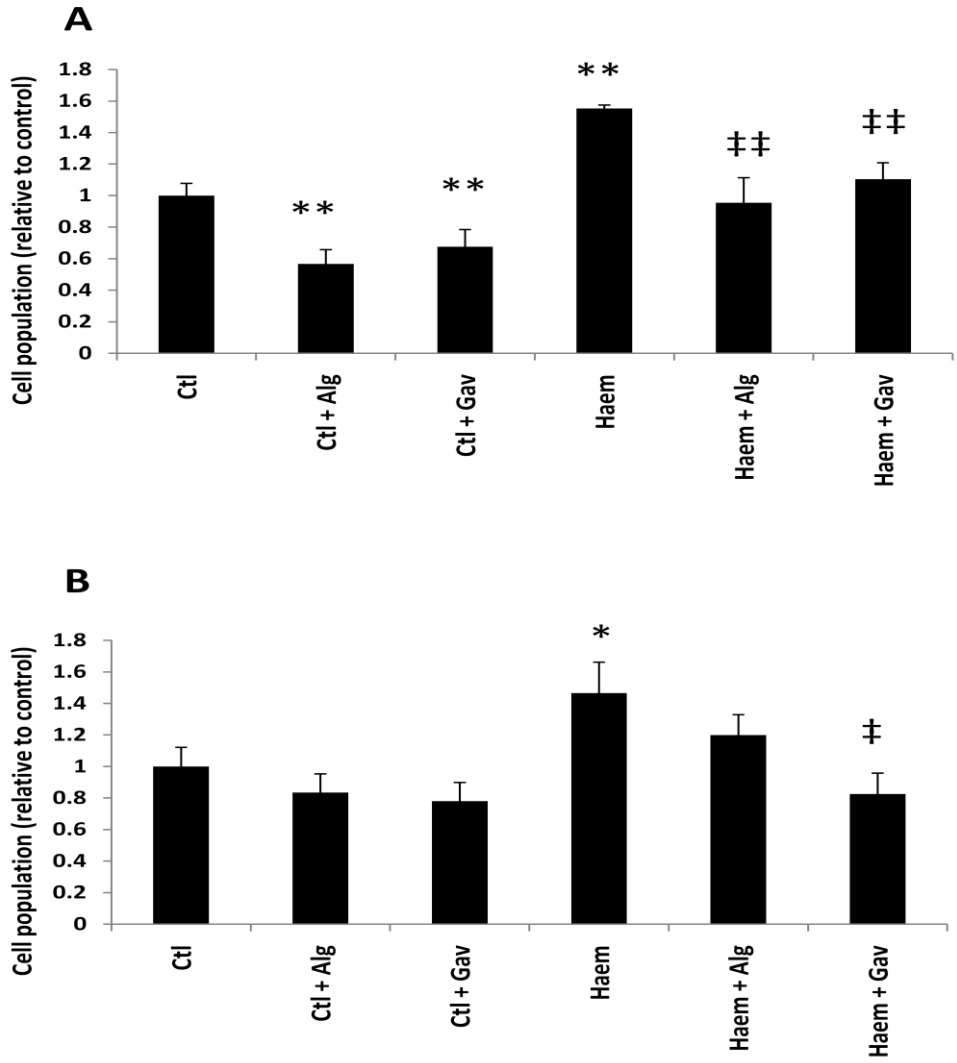


Figure 5. 8. **Haem increases the viable cell population, an effect which is inhibited by the addition of alginate or Gaviscon in OE33 cells (fig 5.8A) and OE19 cells (fig 5.8B)**

Cells were cultured for 48 hours in the presence of control media or a 1% alginate solution or 0.1% Gaviscon solution ± 50µM Haem (for each regime n=12). Haem significantly increased cell population in both cell lines. The addition of Gaviscon to cells cultured in the presence of haem decreased the cell population significantly in both lines whilst the addition of alginate led to a significant decrease in OE33 and non significant decrease in OE19 cells lines. Alginate and Gaviscon led to significant decreases in viable cells compared to a control cell population in OE33 cells and non significant differences in OE19 cells.

* p=0.01, ** p=0.001 vs. control cells; ‡ p=0.01, ‡‡ p=0.003 vs haem loaded cells

Ctl: control; Alg: 1% w/v alginate; Gav: 1% Gaviscon; Haem: 50µM haem solution

5.7 Haem loading oesophageal adenocarcinoma cell lines increases BrdU incorporation an effect suppressed by the addition of alginate

To further explore the effects of haem upon oesophageal adenocarcinoma cell phenotype a bromodeoxyuradine (BrdU) assay was used as an indicator of cell proliferation following culture with or without haem (fig 5.9). To further explore the effects of alginate and Gaviscon the experiment included the addition of these agents to control and haem loaded media (for each regime n=12).

In OE33 cells the addition of haem significantly increased cellular BrdU incorporation relative to control (2.06 ± 0.08 vs. 1 ± 0.04; p=0.001). The addition of alginate resulted in non significant decreases in BrdU incorporation when added to control (0.96 ± 0.03 vs. 1 ± 0.04; p=0.2) and haem loaded media (1.84 ± 0.16 vs. 2.06 ± 0.08; p=0.55). Addition of Gaviscon reduced BrdU incorporation relative to control (0.88 ± 0.08 vs. 1 ± 0.04; p=0.04) and when added to haem loaded media (1.02 ± 0.1 vs. 2.06 ± 0.08; p=0.004), fig 5.9A.

In OE19 cells again haem significantly increased cell BrdU incorporation relative to control cells (1.60 ± 0.12 vs. 1 ± 0.07; p=0.0001) whilst Gaviscon was associated with no significant difference in BrdU uptake compared to control cells (1.05 ± 0.06 vs. 1 ± 0.07; p=ns). When added to haem loaded media Gaviscon resulted in a significant decrease in BrdU incorporation (1.17 ± 0.04 vs. 1.6 ± 0.12; p=0.0001). Addition of alginate decreased BrdU incorporation compared to control (0.59 ± 0.12 vs. 1 ± 0.07; p=0.001) and to haem loaded media (0.73 ± 0.09 vs. 1.6 ± 0.12; p=0.00001), fig 5.9B.

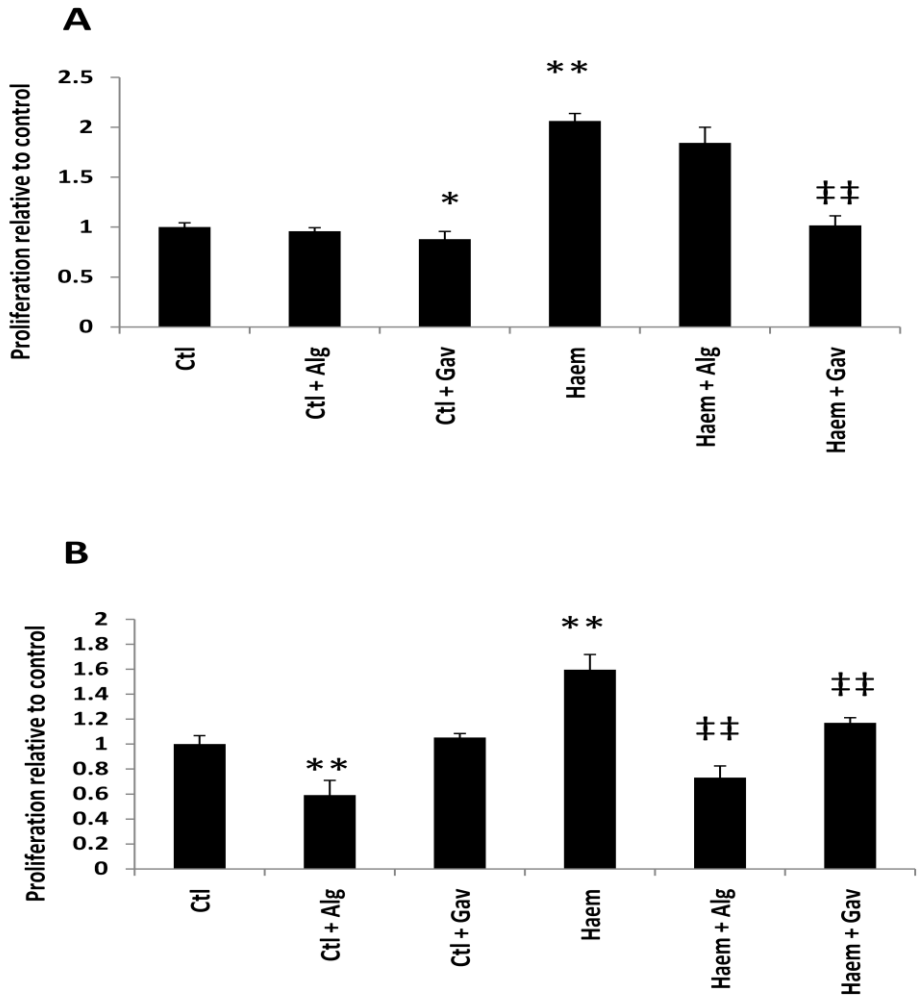


Figure 5. 9. **Haem increases BrdU incorporation in OE33 cells (A) and OE19 cells (B), an effect largely inhibited by the addition of alginate or Gaviscon**

Cells were cultured for 48 hours in the presence of control media or a 1% alginate solution or 0.1% Gaviscon solution ± 50µM Haem (for each regime n=12). Haem significantly increased BrdU incorporation in both cell lines. The addition of Gaviscon to cells cultured in the presence of haem decreased BrdU incorporation significantly in both lines whilst the addition of alginate led to a significant decrease in OE19 and non significant decrease in OE33 cells lines.

* p=0.04, ** p=0.001 vs. control cells; ‡‡ p<0.0001 vs haem loaded cells

Ctl: control; Alg: 1% w/v alginate; Gav: 1% Gaviscon; Haem: 50µM haem solution

5.8 The effect of haem exposure upon the rate of cellular migration in oesophageal adenocarcinoma cell lines

Elevated intracellular iron stimulates oesophageal adenocarcinoma cell proliferation, cell cycling and migration. We have observed that oesophageal adenocarcinoma cells import haem increasing cellular iron content. To further explore effects of haem upon cell phenotype migration was assessed by utilising a wound healing assay (fig 5.10).

OE33 cells that were cultured in the presence of supplemental haem rapidly closed a large wound (fig 5.10A) at a significantly greater rate than control. There was no difference in the initial wound size ($p=0.07$). After 12 hours of culture 56.9 ± 8.9% of the control wound surface area remained vs. 29.9 ± 5.3% of wound surface area exposed to haem ($p=0.007$). After 24 hours of culture 26.0 ± 3.4% of control wound surface area remained vs. 3.0 ± 5.3% of wound surface area cultured in haem ($p=0.002$).

In OE19 cells again the addition of haem resulted in significantly greater cell migration (fig 5.10B). There was no difference in the initial wound surface area between groups ($p=0.28$). After 24 hours of culture 68.0 ± 5.0% of control wound surface area remained vs. 45.5 ± 8.5% of wound surface area exposed to haem ($p=0.04$). After 48 hours 52.1 ± 5.5% of control wound surface area remained vs. 33.8 ± 8.8% of wound surface area cultured in haem ($p=0.03$). After 96 hours 23.1 ± 8.5% of control wound surface area remained vs. 8.6 ± 2.4% of wound surface area cultured in haem ($p=0.03$). All wounds had closed after 120 hours.

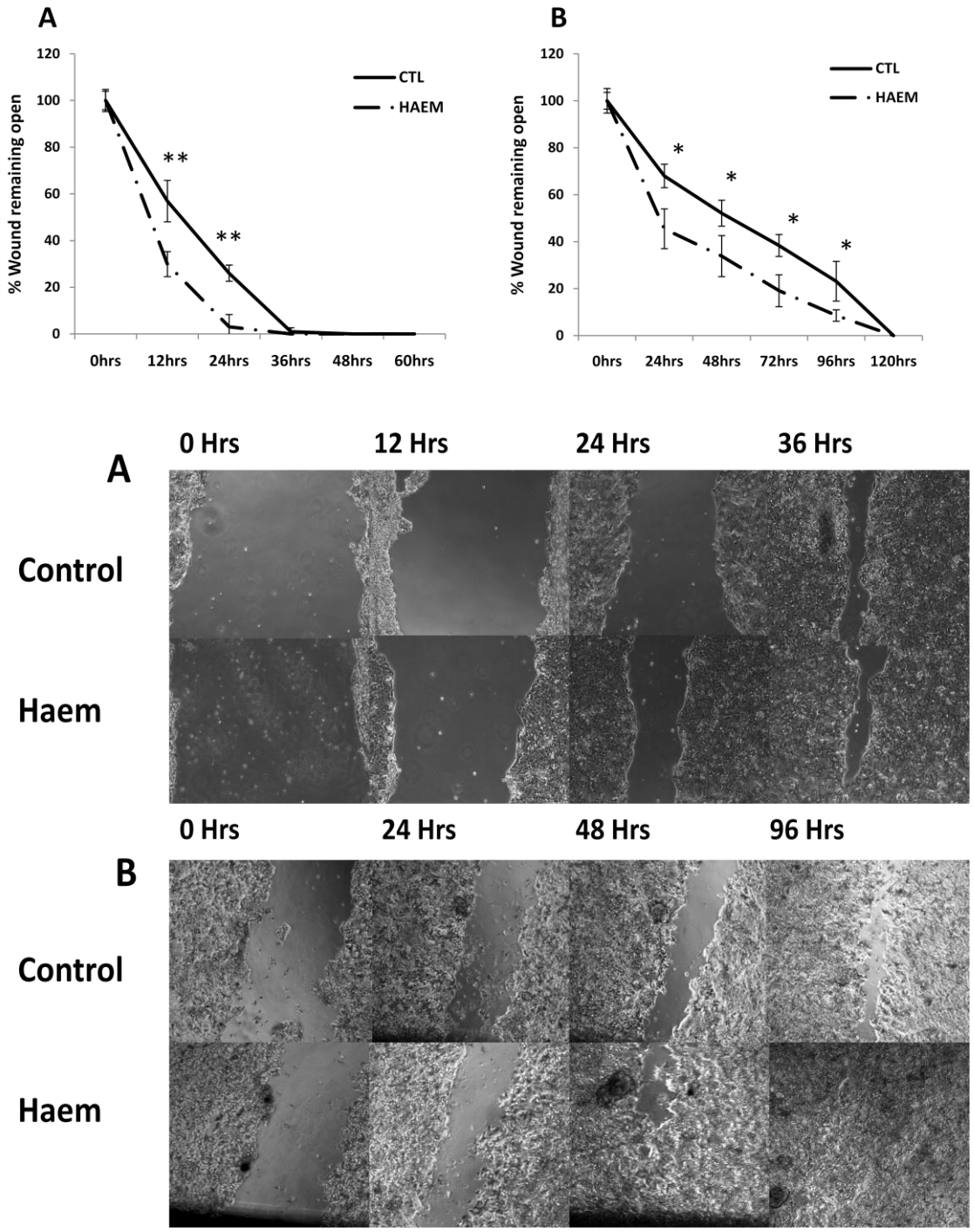


Figure 5. 10. **Haem increases migration in oesophageal adenocarcinoma cells**

Confluent monolayers of OE33 (fig A) and OE19 (fig B) were wounded and cultured in control media (control) or media supplemented with 50µM haem. Cell migration and the rate of wound closure in both cell lines was greater when exposed to haem (* $p<0.05$, ** $p<0.01$; N = 6 for each regime)

5.9 The effect of haem exposure upon cell migration is inhibited by addition of alginate or Gaviscon

Exposure of oesophageal adenocarcinoma cell lines to haem induces a greater rate of cell migration. To assess whether alginate or Gaviscon can affect this observation the wound healing assay was performed with cells grown in media stimulated with 50μM haem ± alginate (1% w/v LFR5/60 alginate) or Gaviscon (0.1% v/v) (fig 5.11).

In OE33 cells the addition of alginate or Gaviscon to media supplemented with haem had no significant effect upon the rate of wound closure relative to cells cultured in media with supplemental haem alone (fig 5.11A). After 12 hours of culture 29.9 ± 4.8% of wounds exposed to haem remained vs 36.0 ± 5.4% haem + alginate (p=0.3) and vs. 42.8 ± 9.8% haem + Gaviscon (p=0.27). After 24 hours of culture 3.1 ± 5.3% of wounds cultured in haem remained vs 9.6 ± 4.5% haem + alginate (p=0.78) and vs. 5.1 ± 6.4% haem + Gaviscon (p=0.37). After 36 hours all wounds cultured in the presence of haem alone had closed. At this point 2.4 ± 2.4% of haem + alginate wounds remained as did 2.5 ± 4.6% of haem + Gaviscon; fig 5.11A.

In OE19 cells the addition of either alginate or Gaviscon to culture media with haem resulted in a reduced rate of wound closure (fig 5.11B and fig 5.12). There was no difference in the initial wound size (haem vs haem + alginate p=0.27, vs. haem + Gaviscon p=0.24). After 24 hours of culture 45.5 ± 4.3% of wounds exposed to haem remained vs. 70.9 ± 4.3% haem + alginate (p=0.04) and vs. 76.1 ± 10.5% haem + Gaviscon (p=0.002). After 48 hours of culture 33.9 ± 8.5% of wounds cultured in haem

remained vs 55.4 ± 6.9% haem + alginate (p=0.08) and vs. 62.7 ± 4.6 haem + Gaviscon (p=0.006). After 72 hours of culture 19.1 ± 6.8% of wounds exposed to haem remained vs. 33.7 ± 7.6% haem + alginate (p=0.03) and vs. 46.9 ± 1.3% haem + Gaviscon (p=0.001). After 96 hours of culture 8.6 ± 2.4% of wounds cultured in haem remained vs 33.7 ± 5.5% haem + alginate (p=0.003) and vs. 20.9 ± 5.6 haem + Gaviscon (p=0.03). After 120 hours of culture all wounds cultured in haem had closed whilst 15.9 ± 8.0% haem + alginate remained (p=0.04) and vs. 6.15 ± 3.7% haem + Gaviscon (p=0.07); fig 5.11B.

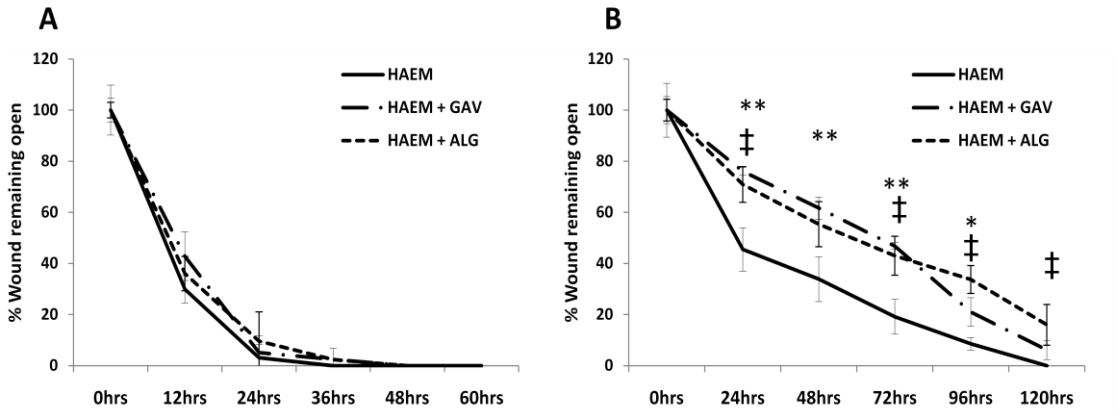


Figure 5. 11. **The effect of adding alginate or Gaviscon to haem loaded media upon cell migration of oesophageal adenocarcinoma cells**

Confluent monolayers of OE33 (fig 5.11A) and OE19 (fig 5.11B) were cultured and subsequently wounded in media supplemented with 50µM haem or with supplemental LFR 50 alginate (1%w/v) or Gaviscon (0.1%v/v). The addition of alginate or Gaviscon reduced the rate of cell migration and thus wound closure in OE19 cells but not in OE33 cells (*/‡ $p<0.05$, ** $p<0.01$; */** = haem + Gaviscon vs. haem; ‡ = haem + alginate vs. haem; N = 6 for each regime).

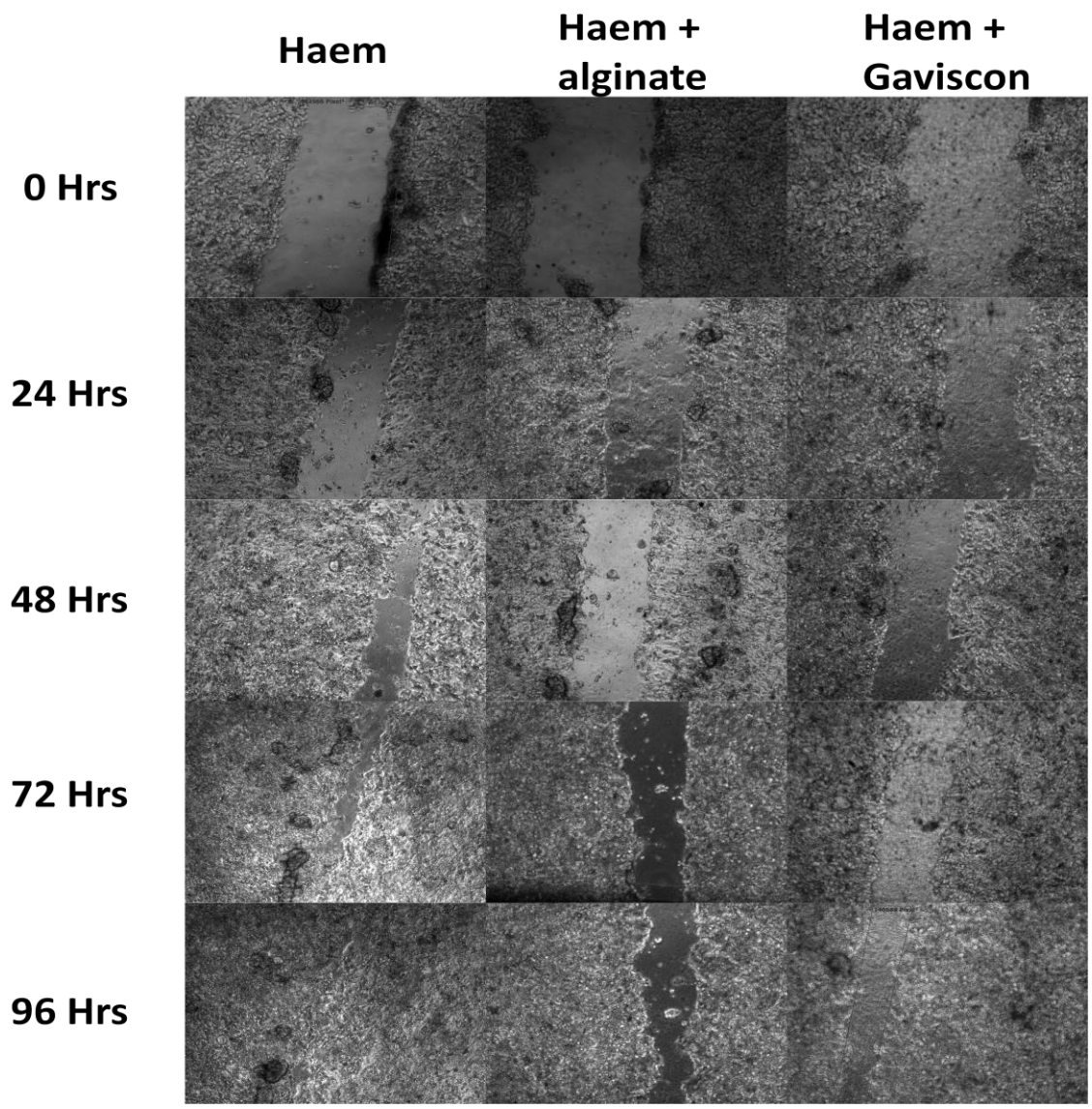


Figure 5. 12. **Representative images of wound healing in a confluent monolayer of OE19 cells with haem +/- supplemental alginate or Gaviscon**

Cells were cultured in media supplemented with 50μM haem or with supplemental LFR50 alginate (1% w/v) or Gaviscon (0.1% v/v).

5.10 The effect of elevated haem exposure upon anchorage independent growth of oesophageal adenocarcinoma cells

To further explore effects of haem upon cell phenotype a colony forming assay was performed (figs 5.13 and 5.14). Single cell agar suspension ($1x10^5$/ml) was seeded in 6 well plates (N=6 each regime) and cultured for 14 days. 3 photographs at random were taken of each well and the surface area of colonies measured within each photograph. Results are expressed as the mean surface area ± 2SEM.

In OE33 cells haem exposure increased the average size of colonies compared to control (12084 ± 3329 vs. 5564 ± 1038 μM^2, p=0.05). The addition of alginate or Gaviscon decreased colony size relative to control (2514 ± 1014 and 2250 ± 840 vs. 5564 ± 1038 μM^2 respectively, both p<0.0001). The addition of alginate or Gaviscon to haem loaded media decreased colony size relative to both haem loaded and control media (2526 ± 1699 and 2594 ± 1798 μM^2 for haem + alginate and haem and Gaviscon vs. haem [12084 ± 3329 μM^2] both p<0.00001 and vs control [5564 ± 1038 μM^2] both p<0.0001. Fig 5.13 A and B.

In OE19 cells haem exposure increased the average surface area of colonies compared to control (13481 ± 1745 vs. 5556 ± 1413 μM^2, p=0.000005). The addition of alginate or Gaviscon had no significant effect upon the size of colonies relative to control (5281 ± 2180 and 6241 ± 2457 respectively vs. 5556 ± 1413 μM^2, both p=NS). The addition of alginate or Gaviscon to haem loaded media resulted in a decrease in colony size relative to haem loaded media (5978 ± 2169 and 5013 ± 2598 μM^2 for haem + alginate and haem + Gaviscon vs. 14179 ± 2878 μM^2 both p<0.0001). This was comparable to colonies grown in control media ± alginate or Gaviscon (both p=NS). Fig 5.14 A and B.

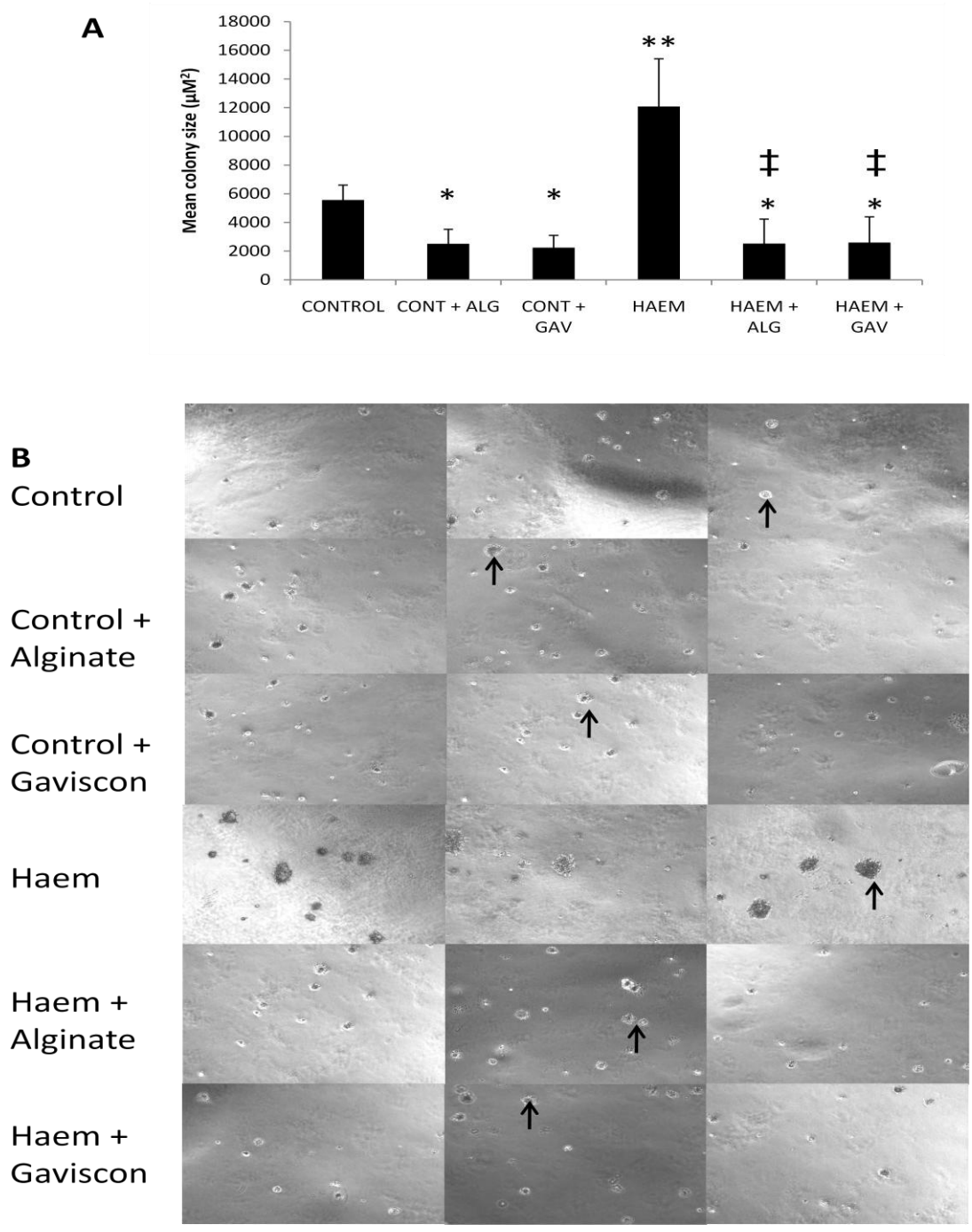


Figure 5. 13. **Colony forming assay with OE33 adenocarcinoma cells**

Cells were cultured in control media or media supplemented with 50μM haem ± additional alginate (1% W/V) or Gaviscon (0.1% V/V). Colonies were measured (fig 5.13A) and photographed (fig 5.13B, representative colonies demonstrated by arrows) 14 days after seeding a single cell suspension.

* $p<0.01$; ** $p<0.00001$ vs. control; ‡ $p<0.00001$ vs. haem.

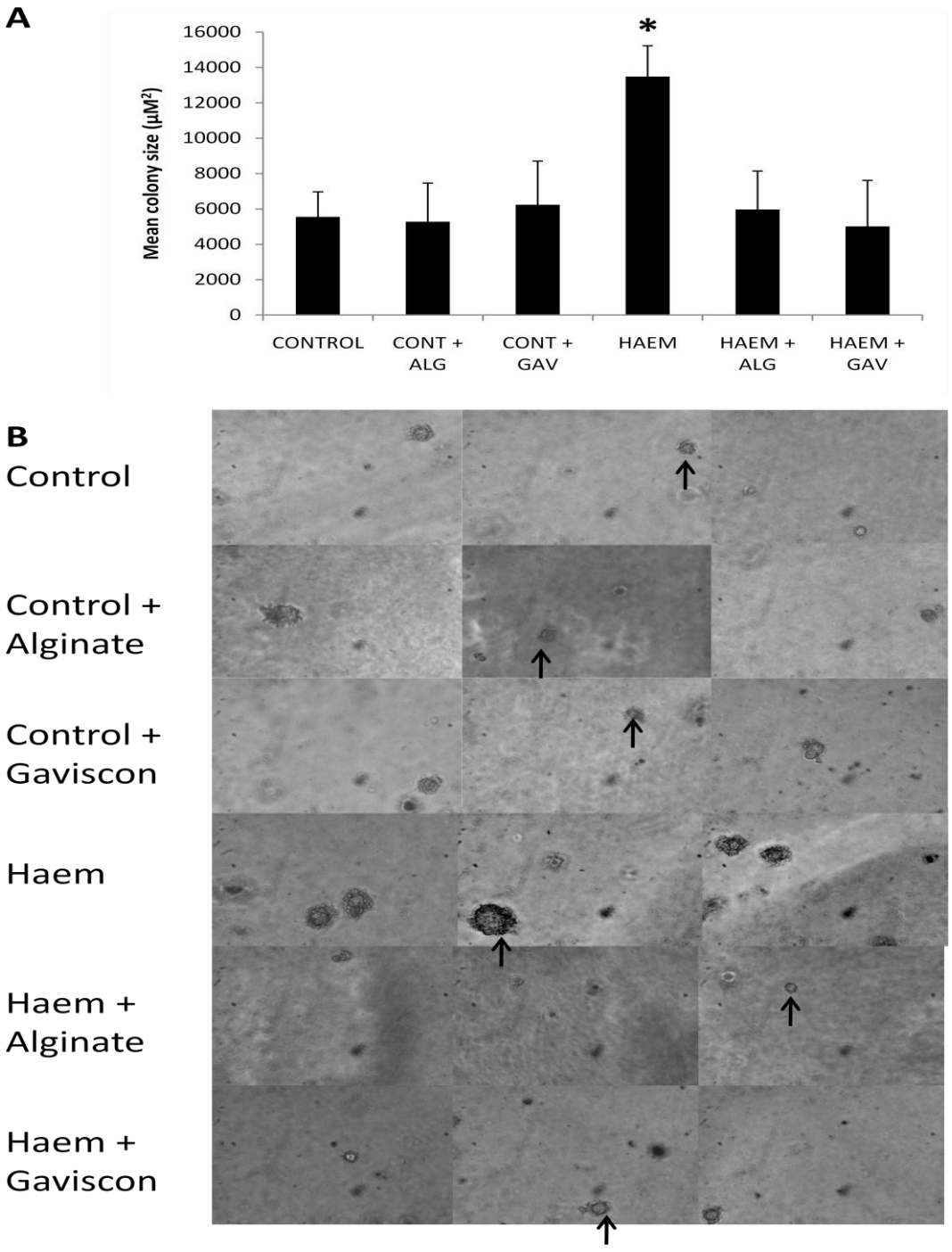


Figure 5. 14. **Colony forming assay OE19 adenocarcinoma cell line**

Cells were cultured in control media or media supplemented with 50µM haem ± additional alginate (1% w/v) or Gaviscon (0.1% v/v). Colony surface areas were measured (fig 5.14A) photographed (fig 5.14B, representative colonies demonstrated by arrows) 14 days after seeding a single cell suspension.

* p=0.00005 vs control.

5.11 The effect of haem exposure upon haem and iron transport proteins in oesophageal adenocarcinoma cell lines

Since haem appears to alter cellular iron metabolism and cell phenotype, the effect of haem on the expression of the pertinent cellular iron and haem transport proteins was assessed by qRT-PCR in the cell lines OE19 and OE33. (N=3 for each regime and then run in triplicate. Results are expressed as the mean fold change ± 2SEM).

5.11.1 Expression of haem transport proteins following exposure to haem

A significant reduction in LRP-1 mRNA expression was observed in both cell lines following culture with haem supplemented media compared to cells cultured in control media. There was a significant reduction in the expression of HCP-1 mRNA in OE33 but a non-significant decrease was seen in OE19 cells (data expressed in table 5.1 and as a figure in 5.15A).

5.11.2 Expression of pertinent iron transport proteins following exposure to haem

Significant increases in both TfR and DMT1 mRNA expression was observed in both OE33 and OE19 cell lines. There was no significant difference in ferritin expression between either line. A significant decrease in ferroportin expression was observed in OE19 cells though there was no significant decrease in OE33 cells (data expressed in table 5.1 and as a figure in 5.15B).

5.11.3 c-myc expression in oesophageal adenocarcinoma cells following exposure to haem

Induction of c-myc mRNA expression was observed when cell lines were cultured in media with supplemental iron. To explore whether haem exposure would alter c-myc expression qRT-PCR was performed on OE19 and OE33 cells following culture with haem. Significant increase in c-myc mRNA expression was observed in both cell lines following culture with haem (data expressed in table 5.1 and as a figure in 5.15C).

	OE33			OE19		
	CONTROL	HAEM LOAD	P	CONTROL	HAEM LOAD	P
LRP-1	1±0.18	0.27±0.13	<0.001	1±0.15	0.6±0.20	0.01
HCP-1	1±0.18	0.64±0.11	0.02	1±0.07	0.7±0.22	ns
TfR	1±0.03	1.9±0.19	<0.01	1±0.12	2.28±0.31	<0.01
DMT1	1±0.36	1.75±0.23	0.02	1±0.26	1.76±0.18	0.01
Ferritin	1±0.24	0.65±0.28	ns	1±0.14	1.33±0.57	ns
Ferroportin	1±0.20	0.82±0.05	ns	1±0.12	0.37±0.22	0.02
c-Myc	1±0.32	2.25±0.11	<0.01	1±0.32	4.12±0.58	<0.001

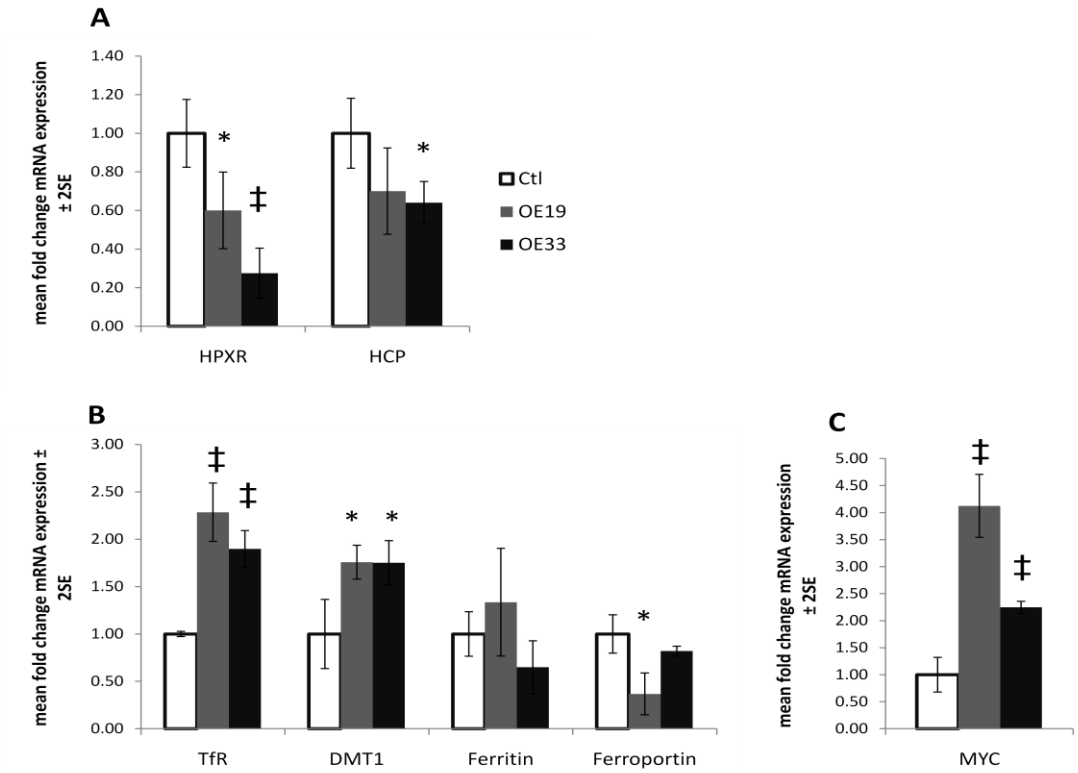


Table 5. 1 and Figure 5. 15. mRNA expression of pertinent iron and haem transport proteins and c-myc in OE33 and OE19 cell lines following culture with haem

Cells were cultured in control media or media supplemented with 50µM haem for 3 hours. Significant repression of LRP-1 (OE33, OE19), HCP-1 (OE33) and ferroportin (OE19) and induction of TfR, DMT1 and c-myc (OE33, OE19) mRNA was observed. Values are mean expression ± 2SEM * p<0.05, ‡ p<0.01

5.12 Modulating LRP-1 expression affects haem and iron metabolism and cell phenotype in oesophageal adenocarcinoma

Having observed the novel expression of LRP-1 in OAC tissue and cell lines suppression of the functional protein was performed to identify effects upon haem transport, cellular iron storage and cell behaviour. Suppression of expression was achieved by transfection with shRNA. The OE33 line would not transfect using conventional transfection reagent kits or with electroporation techniques and thus OE19 cells were used for the majority of the following experiments. Knockdown of LRP-1 expression was performed by transiently transfecting cells with shRNA plasmids (3 potential plasmids were assessed and the plasmid producing the most effective knockdown was used in subsequent experiments). As the OE19 cell populations typically achieved a maximum transfection efficiency of 5-10% the population was first passed through a sterile cell sorting process using FACS assessment of GFP expression with subsequent selection of only cells that had been successfully transfected with plasmid. Control cells were transfected with a plasmid encoding expression of GFP in addition to a scrambled non-functional LRP-1 sequence.

5.12.1 Evidence of successful LRP-1 knockdown

OE19 cells transfected with LRP-1 shRNA or scrambled LRP-1 shRNA were analysed for LRP-1 expression. Haem oxygenase-1 mRNA expression was also quantified as a measure of haem import and subsequent metabolism. qRT-PCR analysis demonstrated no significant effect upon LRP-1 mRNA expression (1 ± 0.06 vs 1.20 ± 0.12, p=ns) but a significant reduction of HO-1 mRNA expression (1 ± 0.07 vs 0.43 ± 0.05, p=0.006) (fig

5.16). Assessment of LRP-1 protein expression by western blotting demonstrated successful knockdown of LRP-1 protein (table 5.2 and fig 5.17).

Further analysis of LRP-1 function was assessed by zinc protoporphyrin uptake. A mixed population of non-transfected cells and cells transfected and with LRP-1 shRNA were exposed to zinc protoporphyrin (ZnPP) for 1 hour. Images were taken of the cells demonstrating transfection (with successfully transfected cells demonstrating green fluorescent protein expression). This was followed by images of the same field of view demonstrating ZnPP uptake (with cells internalising ZnPP demonstrating red fluorescence) and compared (fig 5.18). Transfected cells with LRP-1 knockdown imported less ZnPP than non-transfected cells.

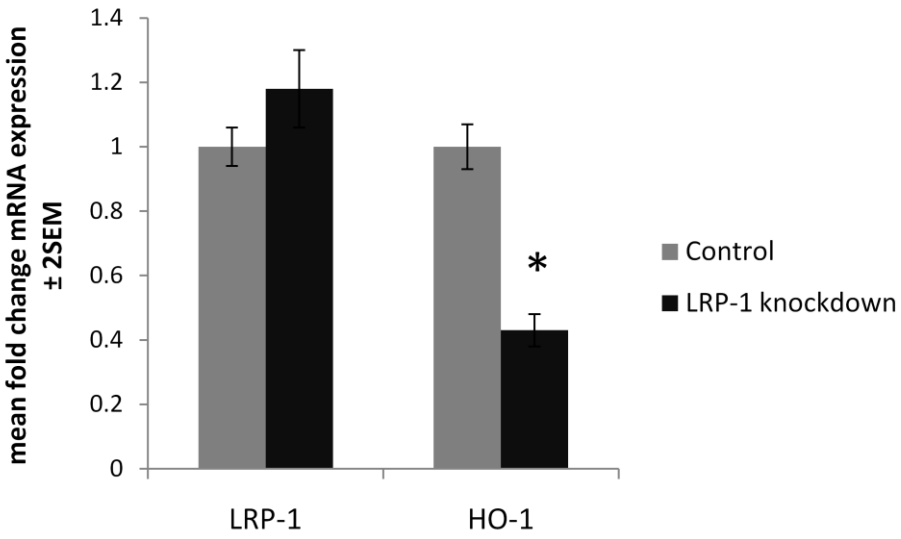


Figure 5. 16. mRNA expression following LRP-1 knockdown in OE19 cells

OE19 cells were transiently transfected with a plasmid to knockdown LRP-1 expression. No significant effect upon LRP-1 mRNA expression was observed but a significant repression of HO-1 was observed (*p =0.006).

	Normalised protein expression	p value
Control	1 ± 0.23	0.002
LRP-1 knockdown	0.42 ± 0.17	

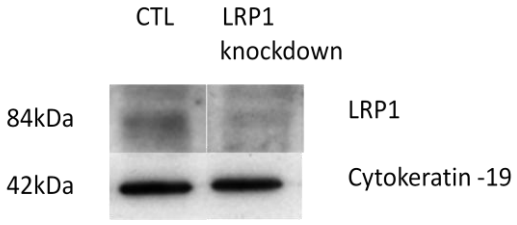


Table 5. 2 and Figure 5. 17. LRP-1 western blot analysis following LRP-1 shRNA knockdown in OE19 cells

A significant repression of LRP-1 protein expression was observed following LRP-1 knockdown (n=3, p=0.002).

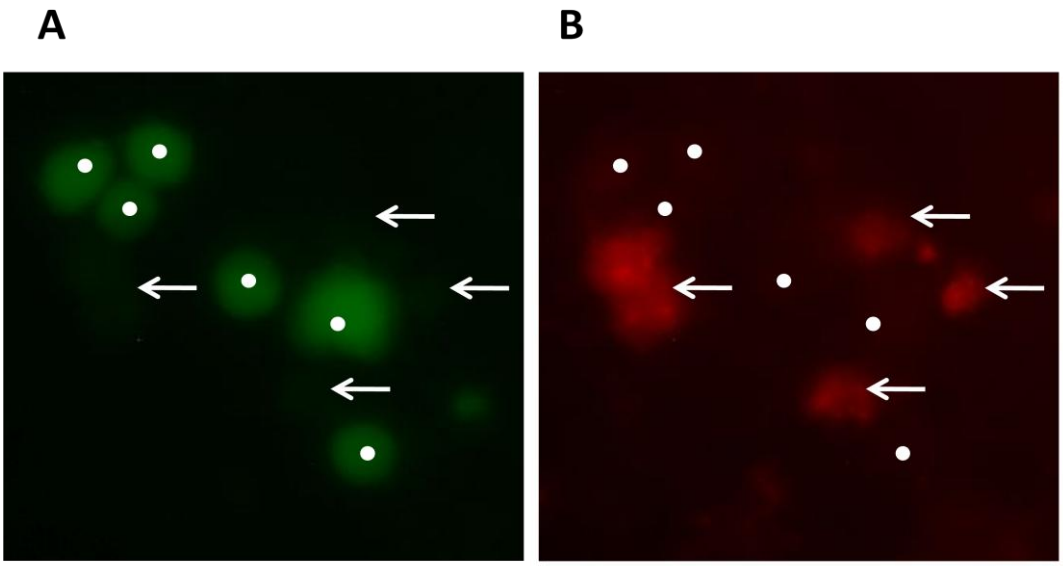


Figure 5. 18. Images of a mixed population of LRP-1 knockdown and untransfected OE19 cells following exposure to the haem analogue ZnPP

Images were taken under light microscopy (x40). Successfully transfected cells are observed fluorescing green (white dots) (fig 5.18 A). Cells that had not been successfully transfected with LRP-1 knockdown would not fluoresce green. Figure 5.18 B demonstrates ZnPP fluorescence and it can be observed that the successfully transfected cells demonstrate little ZnPP fluorescence whilst non-transfected cells (white arrows) demonstrate marked ZnPP fluorescence.

5.12.2 LRP-1 knockdown inhibits cellular uptake of haem^{55}Fe

Successfully transfected control and LRP-1 shRNA OE19 cells were passed through a sterile cell sorter and, reserving only those strongly expressing GFP (top 5% of cells expressing GFP), placed into a 12 well plate. After 24 hours of cell culture a 50μM haem solution containing 1% haem^{55}Fe was added to the wells. After 2 hours the media was aspirated and kept. The cells were washed, trypsinised and the cell pellet resuspended in scintillant. Both media and cells were independently analysed by radiation scintigraphy.

Within the cell pellets of LRP-1 knockdown cells there was significantly lower radioisotope activity relative to sham knockdown cells (mean sum of disintegrations per minute (DPM^{-1}) was 1201 ± 51 vs. 1755 ± 48 DPM^{-1} respectively, p=0.001) (fig 5.19A). This data was normalised to the protein content of the wells as assessed by BCA protein assay. Correspondingly media aspirated from wells containing LRP-1 knockdown cells had a significantly higher DPM^{-1} count than media taken from control cells (28045 ± 2563 vs. 20600 ± 364 DPM^{-1}, p=0.001) (fig 5.19B).

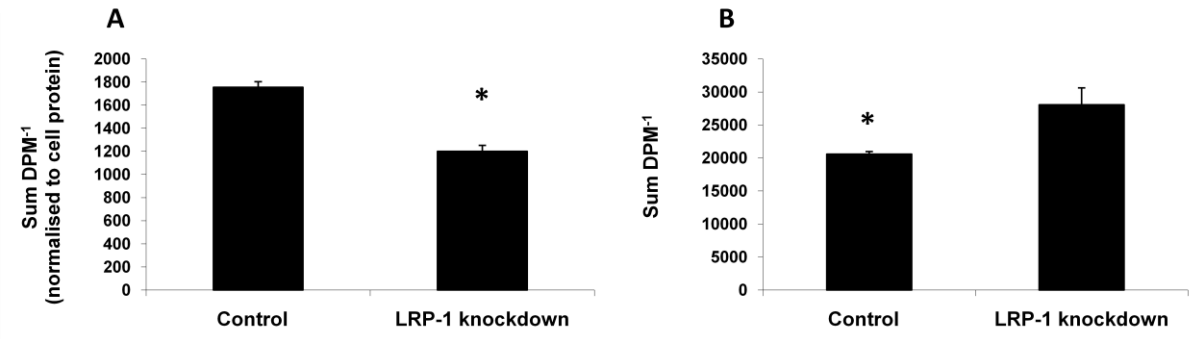


Figure 5. 19. **Knockdown of LRP-1 in OE19 cells inhibits uptake of haem^{55}Fe**

LRP-1 shRNA knockdown resulted in a significant reduction of radiolabelled haem uptake by cells (fig A) and correspondingly a significantly greater level of haem^{55}Fe in aspirated culture media (fig B) compared to cells transfected with sham shRNA.

DPM^{-1} - disintegrations per minute * p=0.001

5.12.3 LRP-1 knockdown decreases cellular iron content and proliferation in oesophageal adenocarcinoma cells

To identify effects of repressing LRP-1 expression upon cellular iron content and proliferation ferrozine and BrdU assays were performed respectively. Due to the numbers of cells required for these experiments it was necessary to use alternative cell lines as OE33 cells would not transfect and the highest yield of transfection using OE19 cells was 10%. It was not financially viable to pass such a volume of cells through a cell sorter. These experiments were performed upon the mixed populations of non-transfected and transfected OE19 cells but demonstrated no significant difference, presumably, due to the majority of untransfected cells.

The oesophageal squamous cancer cell line OE21 and the line Seg-1 were used in the following experiments. At the time of writing this thesis it was reported that the cell

line Seg-1 is in fact a lung cancer derived cell line[349] and not derived from oesophageal adenocarcinoma as was previously thought. Following transient transfection successful repression of LRP-1 was demonstrated by western blotting (data not shown).

5.12.3.1 LRP-1 knockdown decreases cell iron content in oesophageal adenocarcinoma cells

24 hours following cell transfection media was supplemented with a 50μM solution of haem. After a further 24 hours of cell culture a ferrozine assay demonstrated a significant reduction in iron content in both cell lines (Seg-1: LRP-1 knockdown 1563 ± 70 vs control 2245 ± 121 ng Fe/μg protein, N=6, $p<0.0001$; OE21: LRP-1 knockdown 420 ± 7 vs control 848 ± 97 ng Fe/μg protein, N=6, $p=0.001$) (fig 5.20 A).

5.12.3.2 LRP-1 knockdown decreases cell proliferation in oesophageal adenocarcinoma cells

24 hours following cell transfection media was supplemented with a 50μM solution of haem. After a further 6 hours of cell culture a BrdU assay demonstrated a significant reduction in proliferation in both cell lines (Seg-1: LRP-1 knockdown 0.82 ± 0.06 vs control 1 ± 0.09, N=6, $p=0.05$; OE21: LRP-1 knockdown 0.52 ± 0.08 vs control 1 ± 0.13, N=6, $p<0.0001$) (fig 5.20 B).

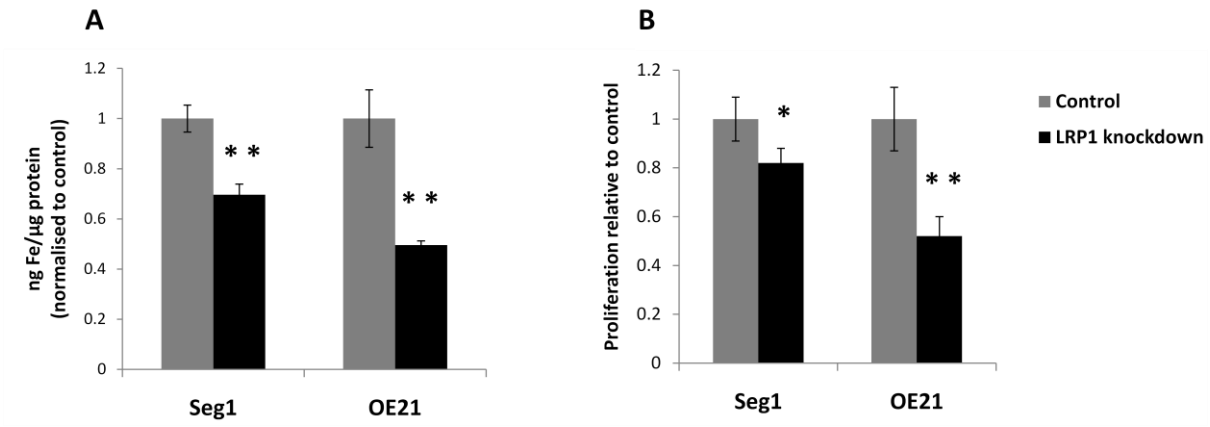


Figure 5. 20. **Knockdown of LRP-1 in tumour cell lines decreases cellular iron content and inhibits proliferation**

OE21 and Seg-1 cell lines were transiently transfected with LRP-1 shRNA resulting in a significant decrease in cell iron content (31 ± 4% vs control in Seg-1 cells, p=0.001 and 51 ± 2% vs control in OE21 cells, p<0.0001; fig 5.20 A).

A significant reduction in cell proliferation was also observed (18 ± 6% vs control in Seg-1 cells, p=0.05 and 48 ± 8% vs control in OE21 cells, p<0.0001; fig 5.20 B). *p=0.05, **P≤0.001

5.13 Conclusions

The finding that oesophageal adenocarcinoma tissue expresses haem import proteins HCP-1 and LRP-1 is novel. There is little background expression of these proteins in native squamous oesophagus or Barrett's metaplasia from samples of non-dysplastic tissue. There appears to be a stage in the progression of early Barrett's metaplasia to Barrett's tissue associated with oesophageal adenocarcinoma where the expression of these proteins increases. The occurrence of this observed increase in expression is interesting as the triggers for stable Barrett's epithelium to become dysplastic and give rise to adenocarcinoma are not known. In oesophageal adenocarcinoma expression of the import proteins is strongest and it is likely they mediate haem import which could provide an alternative source of iron[228;242] to inorganic iron captured by transferrin receptor overexpression. Iron can be utilised by cancer cells for energy production, cell cycle control and DNA synthesis[160;250]. Presumably liberated iron is also responsible, directly or indirectly, for the increased rates of proliferation, migration and anchorage independent growth that have been observed in this body of work. There are other potential candidates that may mediate this effect following haem exposure. Culturing oesophageal adenocarcinoma cells with haem induces haem oxygenase 1 (HO-1) expression to metabolise the degradation of haem to liberate free iron. HO-1 induction has been identified in various tumours where overexpression of the protein is associated with increased tumour burden[380-382] and conversely repression decreases tumour growth[381;383-385]. HO-1 levels influence cell proliferation directly in animal and *in-vitro* models and may represent an alternative mechanism of inducing a malignant phenotype following haem exposure. In pancreatic

adenocarcinoma inhibiting HO-1 via siRNA decreases tumour cell proliferation[383] whilst overexpressing HO-1 mRNA increases proliferation[382]. HO-1 can repress the function of the cyclin dependent kinase inhibitor p21[382;386;387] and is associated with an increased production of epidermal growth factor[382]. HO-1 expression is associated with angiogensis *in-vitro* and *in-vivo*. HO-1 knockout is associated with decreased expression of vascular endothelial growth factor (VEGF) compared to wild type endothelial cells[388]. Overexpression of HO-1 however is associated with increased VEGF expression and enhanced capillary formation in collagen gel[389]. In a rat ischaemic limb model HO-1 gene transfer was associated with augmented levels of VEGF, angiogenesis and improved muscle blood flow[99].

The LRP-1 receptor has diverse ligands[243] but from the experiments presented here including shRNA knockdown of protein expression it is clearly demonstrated that LRP-1 has an important role in oesophageal adenocarcinoma cells acquiring haem. It is important to appreciate the complex activity of the LRP-1 receptor given that it captures up to 30 other ligands[243]. Thus the relevance of LRP-1 overexpression in oesophageal adenocarcinoma is complex but one might hypothesise that it represents a mechanism of cellular haem capture and aids progression of malignant disease. More broadly speaking the role of LRP-1 in cancer is unclear with evidence that LRP-1 expression has pro- or anti-tumour activity. LRP-1 regulates extracellular proteolysis via its actions upon serine proteases and matrix metalloproteinases[390]. Increased expression of LRP-1 has been associated with a less invasive phenotype[391] whilst anti-LRP-1 IgG increases levels of matrix metalloproteinase-2[392]. Others, however, have associated LRP-1 repression with decreased tumour invasiveness[393;394]. LRP-1

silencing in thyroid follicular cells prevented invasion despite increases in proteolytic enzymes[395] suggesting LRP-1 mediated affects tumour invasion are in part due to as yet uncharacterised mechanisms. Haem could be a candidate having observed increased expression of LRP-1 in oesophageal adenocarcinoma and increased migration and anchorage independent growth. What is clear is that the role of LRP-1 had diverse functions, many of which remain to be fully understood.

The role of HCP-1 is also unclear. Initial evidence pointed to a role in haem import by intestinal cells[230]. However knockout studies had little effect upon haem import suggesting alternative pathways of uptake[230], presumably via LRP-1, and further work demonstrates a more likely role as a folate transporter[396]. For these reasons LRP-1 is considered the most important haem import protein and thus the focus of attention of knockout studies performed here. Knockout of HCP-1 expression would have been desirable but was not performed due to a lack of time and financial resource.

It is interesting to observe an increase in transferrin receptor and divalent metal transport protein mRNA expression following exposure to haem. Intracellular haem binds to iron regulatory proteins marking the complex for degradation. This would result in repression of iron import protein expression and thus an alternative mechanism of control over their expression must be present.

It is interesting that alginates inhibit haem mediated effects upon cell phenotype. The ability of alginates to bind divalent metals[335] is described as is the binding of toxins

including dioxins, heterocyclic amines and bile acids to fibres[319-323]. Prevention of haem mediated effects via presumed binding with alginate provides further evidence of the potential therapeutic platform of this compound.

CHAPTER 6: DISCUSSION

6.1 Conclusions

From results presented in this thesis it is clear that the expression of iron transport proteins and requirements for iron by oesophageal adenocarcinoma differs to that of native benign tissue. This is an exciting though not surprising observation as iron is essential for cell cycling, enzyme production, energy generation, proliferation and growth[160-162]. The observation of transferrin receptor overexpression in oesophageal adenocarcinoma has not been published previously though authors have observed this in other tumours (breast[166;250], colorectal adenocarcinoma[251], glioma[252], non-Hodgkins lymphoma[253], chronic lymphoid leukaemia[254], lung adenocarcinoma[255], gynaecological tumours[256], renal transitional cell carcinoma[257], cervical carcinoma[258], melanoma[259], hepatocellular carcinoma[260] and hepatoblastoma[261]).

A more complete description of the remaining iron transport machinery given here allows greater insight into how tumour cells acquire and utilise iron. In addition to changes in the expression of transferrin receptor it is apparent that several of the other iron transport proteins are also altered in the process of tumorigenesis. Most notably our studies have suggested that ferroportin, which is normally located at the basal cell border (for its appropriate export function), is relocalised to a cytoplasmic location. This is likely to render the protein inactive. The process of ferroportin relocalisation has previously been reported in colorectal adenocarcinoma[251]. However the exact trigger for this event remains elusive. The most likely candidate for

mediating this relocalisation is the anti-microbial peptide hepcidin[213;357]. The finding that oesophageal adenocarcinoma cells express hepcidin is exciting. In health hepcidin is produced by the liver and controls systemic levels of iron. Local production of hecidin by tumour cells may be an autrocrine mechanism of cellular iron loading by preventing cellular efflux on the background of trasnferrin receptor and LRP-1 overexpression.

A further interesting observation is the paradoxical increased expression of transferrin receptor and divalent metal transport protein-1 following culture in the presence of supplemental iron or haem. This contradicts how cells should respond to an iron load via an appropriate IRE/IRP response. Following iron loading IRP2 levels would be expected to decrease with a subsequent decrease in TfR1 and DMT1 mRNA expression through increased degradation[204;397] which was not observed. In these experiments this is associated with an increased expression of the oncogene c-myc. In an elegant series of experiments[366;397] my laboratory co-workers were able to demonstrate that overexpressing c-myc reproduces the expression profile of elevated TfR1 and DMT1 seen following iron exposure in colorectal adenocarcinoma. Furthermore they associated c-myc overexpression with loss of APC function. Using an inducible APC plasmid in a colorectal cell line with truncated APC (SW480), elevated levels of TOPFLASH reporter assay indicated activation of the β-catenin/TCF signalling pathway following exposure to iron or haem. This occurred only in the native cell type but no activation was observed following restoration of APC function. An associated increase in expression of c-myc was observed within mutant APC cells but not those with reinstated APC function. In this work we have observed that both OE33 and OE19

cell lines possess inactive APC and thus it seems plausible that iron and haem mediated effects in oesophageal adenocarcinoma may be in part via β-catenin/TCF signalling.

The expression of HCP-1 and LRP-1 in Barrett's metaplasia and oesophageal adenocarcinoma is a novel finding. This is a complex issue given the paucity of clear evidence as to how cells handle haem in health[191;228-230;242] let alone in malignant disease. By acquiring iron through haem import pathways cells can access a significant pool of circulating iron[242]. Characterising their expression in malignant disease and identifying that oesophageal adenocarcinoma cells can metabolise haem adds to the plethora of evidence implicating iron with carcinogenesis but also brings many unanswered questions. What is the functional significance and relative contribution of iron acquired via haem pathways rather than conventional transferrin bound iron? Is haem transport overexpression a feature of other tumour types? Are there other roles of HCP-1 and LRP-1? HCP-1 takes up folate with great affinity from the intestine and may play a more important role in folate, rather than haem, transport. LRP-1 has many ligands and functions. By knockdown studies we have observed that it does play an important role in haem transport but does LRP-1 overexpression mediate other, non iron, pathways in oesophageal adenocarcinoma?

The trigger for HCP-1 and LRP-1 expression is not clear. There appears to be a stage between non-dysplastic Barrett's epithelium and Barrett's epithelium associated with oesophageal adenocarcinoma where expression of both transporters becomes

apparent. Very little background expression of either transporter was evident in samples of benign BM whilst samples of BM adjacent to OAC demonstrated strong expression whilst OAC cells themselves express most strongly. There appears to be a similar change in expression profile of inorganic iron transport proteins at the same stage in the metaplasia-adenocarcinoma sequence. Key genetic events known to occur with high frequency at this stage in the progression of BM to OAC include APC methylation[69;70], loss of p53 function[80;83], p16 methylation[63-65;398], loss of p27 function[86] and overexpression of cyclin D1[71;72]. Loss of APC function would be a good candidate. Inactivation of tumour suppressor genes p53, p27 and p16 are less likely candidates. They perform house-keeping of the cells genome triggering apoptosis[81] or control progression of cell cycle inhibiting clonal expansion of stem cells or by acting as a CDK inhibitor[65;86] . APC inactivation, however, is known to have downstream effects mediated via dysregulation of β-catenin/TCF signalling pathway resulting in overexpression of oncogenes such cyclin D1 and the oncogene c-myc. Cyclin D1 regulates the cell cycle by inactivating the retinoblastoma protein and is thus an unlikely candidate. Overexpression of c-myc is however a possible downstream candidate and has been discussed above.

Although imported via independent routes exposure to transferrin bound iron or haem iron yield remarkably similar responses by the cells. In epidemiological studies haem iron is more strongly associated with oesophageal adenocarcinoma[282] in addition to other epithelial tumours such as colorectal[282] and breast[284;285]. Furthermore recent evidence suggests that organic iron is absorbed more avidly from the

gastrointestinal tract than inorganic iron[228]. There is also an inherent protective effect with some foods high in inorganic iron; vegetables are relatively high in antioxidants which would counteract the actions of reactive oxygen species and the associated fibre could bind iron within the intestinal lumen and increase transit time[312;318]. Alginates are an example of a polysaccharide which can strongly bind iron within the GI tract and result in its excretion[347]. Diets high in alginate are associated with decreased incidence of colorectal[336], oesophageal[337] and breast cancers[338]. Furthermore being natural products, cheap and readily available they have commercial value. We have observed that alginate or Gaviscon inhibit both iron and haem mediated effects upon proliferation, migration and anchorage independent growth. This lends weight to the rational for iron chelation as a therapy against malignant disease. However in what form or at which stage of disease alginates could provide benefit in treating oesophageal adenocarcinoma is less clear. We have observed that iron mediates increased proliferation and oncogene expression in oesophageal adenocarcinoma. It would seem likely that the addition of dietary alginate at this stage of disease would provide little clinical benefit as patients have advanced disease and a poor prognosis. Systemic chelation may provide a temporary benefit for example using desferroxamine but it is difficult to hypothesise that alginate would have a beneficial effect such as that seen in the epidemiological studies above. Perhaps alginates can provide protection at an earlier stage in the progression of oesophageal adenocarcinoma. In rat models of oesophageal adenocarcinoma gastroesophageal reflux alone did not result in metaplastic change and subsequent development of OAC. Only when animals were given supplemental iron did this

occur[147-149]. Thus in patients with reflux disease a potential therapeutic study could involve the randomised administration of dietary alginate or placebo and an observation of the rates of progression to Barrett's epithelium and oesophageal adenocarcinoma.

Oesophageal adenocarcinoma incidence is increasing at a rate greater than any other malignant disease in Western populations. Obesity and gastroesophageal reflux disease are strongly linked. Accumulating evidence defines the genetic defects associated with the disease but oesophageal adenocarcarinoma remains a challenge to science and modern medicine. Understanding the differential expression of iron and haem transporters allows an appreciation of the importance iron metabolism plays in oesophageal adenocarcinoma. Utilising the differential expression of these transporters between malignant and benign tissue may pave the way for novel therapeutic strategies to deliver chemotherapeutic agents. A more simple approach would be to bind iron within the gastrointestinal tract by the use of alginates. This strategy may benefit subjects with high risk lifestyle factors.

6.2 Future work

6.2.1 Laboratory experimental work

To clearly understand the role of iron and aberrant β-catenin/TCF signalling in oesophageal adenocarcinoma cells it would be desirable to overexpress c-myc, IRP2 or to restore APC function in appropriate cell lineages. This would require an oesophageal adenocarcinoma cell line whose population would successfully undergo a high rate of transient transfection or to develop an alternative strategy for transfection of either OE19 or OE33 cell lines.

Utilising singly or in combination the overexpression of the transferrin, HCP-1 and or LRP-1 receptors to deliver targeted chemotherapeutic agents to OAC cells and populations of benign cells to calculate the IC_{50} and LD_{50} would provide evidence to support the wider application of agents to include human phase 1 and 2 clinical trials.

6.2.2 Clinical experimental work

A randomised trial of alginate use in subjects with risk factors for developing Barrett's metaplasia and oesophageal adenocarcinoma would be exciting. Being naturally occurring compounds they would not need to undergo as extensive testing prior to use. Furthermore they are cheap and readily available. If benefit could be proven they would likely be well tolerated by people who prefer natural compounds to pharmaceutical ones.

There is no reason to limit the focus of iron to oesophageal adenocarcinoma. Increasing evidence associates iron with breast and hepatocellular cancer.

REFERENCE LIST

1. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 2002;359:1727-1733.

2. Bird-Lieberman EL, Fitzgerald RC: Early diagnosis of oesophageal cancer. Br.J Cancer 2009;101:1-6.

3. Djarv T, Lagergren J, Blazeby JM, Lagergren P: Long-term health-related quality of life following surgery for oesophageal cancer. Br.J Surg. 2008;95:1121-1126.

4. Swisher SG, Hunt KK, Holmes EC, Zinner MJ, McFadden DW: Changes in the surgical management of esophageal cancer from 1970 to 1993. Am.J Surg. 1995;169:609-614.

5. Parker SL, Tong T, Bolden S, Wingo PA: Cancer statistics, 1996. CA Cancer J Clin. 1996;46:5-27.

6. Park KG: Gastro-oesophageal cancer: facts, myths and surgical folk lore. J R.Coll.Surg.Edinb. 2002;47:716-730.

7. Blot WJ, Fraumeni JF, Jr.: Trends in esophageal cancer mortality among US blacks and whites. Am.J Public Health 1987;77:296-298.

8. Devesa SS, Blot WJ, Fraumeni JF, Jr.: Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 1998;83:2049-2053.

9. Cederqvist C, Nielsen J, Berthelsen A, Hansen HS: Adenocarcinoma of the oesophagus. Acta Chir Scand. 1980;146:411-415.

10. Raphael HA, Ellis FH, Jr., Dockerty MB: Primary adenocarcinoma of the esophagus: 18-year review and review of literature. Ann.Surg. 1966;164:785-796.

11. Turnbull AD, Goodner JT: Primary adenocarcinoma of the esophagus. Cancer 1968;22:915-918.

12. Law S, Wong J: Changing disease burden and management issues for esophageal cancer in the Asia-Pacific region. J Gastroenterol.Hepatol. 2002;17:374-381.

13. Blot WJ, Devesa SS, Kneller RW, Fraumeni JF, Jr.: Rising incidence of adenocarcinoma of the esophagus and gastric cardia. JAMA 1991;265:1287-1289.

14. Pera M, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR: Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. Gastroenterology 1993;104:510-513.

15. Powell J, McConkey CC: The rising trend in oesophageal adenocarcinoma and gastric cardia. Eur.J Cancer Prev. 1992;1:265-269.

16. Levi F, La Vecchia C: Adenocarcinoma of the esophagus in Switzerland. JAMA 1991;265:2960.

17. Anikin VA, McManus KG, Graham AN, McGuigan JA: Total thoracic esophagectomy for esophageal cancer. J Am.Coll.Surg. 1997;185:525-529.

18. el Serag HB: The epidemic of esophageal adenocarcinoma. Gastroenterol.Clin.North Am. 2002;31:421-40, viii.

19. Wei JT, Shaheen N: The changing epidemiology of esophageal adenocarcinoma. Semin.Gastrointest.Dis. 2003;14:112-127.

20. He YT, Hou J, Chen ZF, Qiao CY, Song GH, Meng FS, Jin HX, Chen C: Trends in incidence of esophageal and gastric cardia cancer in high-risk areas in China. Eur.J Cancer Prev. 2008;17:71-76.

21. Kamangar F, Strickland PT, Pourshams A, Malekzadeh R, Boffetta P, Roth MJ, Abnet CC, Saadatian-Elahi M, Rakhshani N, Brennan P, Etemadi A, Dawsey SM: High exposure to polycyclic aromatic hydrocarbons may contribute to high risk of esophageal cancer in northeastern Iran. Anticancer Res. 2005;25:425-428.

22. Pohl H, Welch HG: The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. J Natl.Cancer Inst. 2005;97:142-146.

23. Blot WJ: Esophageal cancer trends and risk factors. Semin.Oncol. 1994;21:403-410.

24. Blot WJ, McLaughlin JK: The changing epidemiology of esophageal cancer. Semin.Oncol. 1999;26:2-8.

25. Doll R, Peto R: Mortality in relation to smoking: 20 years' observations on male British doctors. Br.Med J 1976;2:1525-1536.

26. Zhang ZF, Kurtz RC, Sun M, Karpeh M, Jr., Yu GP, Gargon N, Fein JS, Georgopoulos SK, Harlap S: Adenocarcinomas of the esophagus and gastric cardia: medical conditions, tobacco, alcohol, and socioeconomic factors. Cancer Epidemiol.Biomarkers Prev. 1996;5:761-768.

27. Heath EI, Limburg PJ, Hawk ET, Forastiere AA: Adenocarcinoma of the esophagus: risk factors and prevention. Oncology (Williston.Park) 2000;14:507-514.

28. Kabat GC, Ng SK, Wynder EL: Tobacco, alcohol intake, and diet in relation to adenocarcinoma of the esophagus and gastric cardia. Cancer Causes Control 1993;4:123-132.

29. Vaughan TL, Davis S, Kristal A, Thomas DB: Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: adenocarcinoma versus squamous cell carcinoma. Cancer Epidemiol.Biomarkers Prev. 1995;4:85-92.

30. Cheng KK: The etiology of esophageal cancer in Chinese. Semin.Oncol. 1994;21:411-415.

31. Spechler SJ, Goyal RK: The columnar-lined esophagus, intestinal metaplasia, and Norman Barrett. Gastroenterology 1996;110:614-621.

32. Lagergren J, Bergstrom R, Lindgren A, Nyren O: Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. N.Engl.J Med 1999;340:825-831.

33. Skacel M, Petras RE, Gramlich TL, Sigel JE, Richter JE, Goldblum JR: The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. Am.J Gastroenterol. 2000;95:3383-3387.

34. Wijnhoven BP, Tilanus HW, Dinjens WN: Molecular biology of Barrett's adenocarcinoma. Ann.Surg. 2001;233:322-337.

35. BARRETT NR: Chronic peptic ulcer of the oesophagus and 'oesophagitis'. Br.J Surg. 1950;38:175-182.

36. Ishizuka I, Andoh A, Koyama S, Moritani S, Kushima R, Hattori T, Fujiyama Y: Immunohistochemical analysis of short-segment Barrett's esophagus. J Gastroenterol.Hepatol. 2004;19:1410-1416.

37. O'Riordan JM, Byrne PJ, Ravi N, Keeling PW, Reynolds JV: Long-term clinical and pathologic response of Barrett's esophagus after antireflux surgery. Am.J Surg. 2004;188:27-33.

38. Cameron AJ, Ott BJ, Payne WS: The incidence of adenocarcinoma in columnar-lined (Barrett's) esophagus. N.Engl.J Med 1985;313:857-859.

39. Miros M, Kerlin P, Walker N: Only patients with dysplasia progress to adenocarcinoma in Barrett's oesophagus. Gut 1991;32:1441-1446.

40. Reid BJ, Blount PL, Rubin CE, Levine DS, Haggitt RC, Rabinovitch PS: Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective endoscopic surveillance of a cohort. Gastroenterology 1992;102:1212-1219.

41. Drewitz DJ, Sampliner RE, Garewal HS: The incidence of adenocarcinoma in Barrett's esophagus: a prospective study of 170 patients followed 4.8 years. Am.J Gastroenterol. 1997;92:212-215.

42. O'Connor JB, Falk GW, Richter JE: The incidence of adenocarcinoma and dysplasia in Barrett's esophagus: report on the Cleveland Clinic Barrett's Esophagus Registry. Am.J Gastroenterol. 1999;94:2037-2042.

43. Spechler SJ, Lee E, Ahnen D, Goyal RK, Hirano I, Ramirez F, Raufman JP, Sampliner R, Schnell T, Sontag S, Vlahcevic ZR, Young R, Williford W: Long-term outcome of medical and surgical therapies for gastroesophageal reflux disease: follow-up of a randomized controlled trial. JAMA 2001;285:2331-2338.

44. Shaheen NJ, Crosby MA, Bozymski EM, Sandler RS: Is there publication bias in the reporting of cancer risk in Barrett's esophagus? Gastroenterology 2000;119:333-338.

45. Chow WH, Finkle WD, McLaughlin JK, Frankl H, Ziel HK, Fraumeni JF, Jr.: The relation of gastroesophageal reflux disease and its treatment to adenocarcinomas of the esophagus and gastric cardia. JAMA 1995;274:474-477.

46. Farrow DC, Vaughan TL, Sweeney C, Gammon MD, Chow WH, Risch HA, Stanford JL, Hansten PD, Mayne ST, Schoenberg JB, Rotterdam H, Ahsan H, West AB, Dubrow R, Fraumeni JF, Jr., Blot WJ: Gastroesophageal reflux disease, use of H2 receptor antagonists, and risk of esophageal and gastric cancer. Cancer Causes Control 2000;11:231-238.

47. Nebel OT, Fornes MF, Castell DO: Symptomatic gastroesophageal reflux: incidence and precipitating factors. Am.J Dig.Dis. 1976;21:953-956.

48. Shaheen NJ: Advances in Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 2005;128:1554-1566.

49. Reid BJ, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H, .: Observer variation in the diagnosis of dysplasia in Barrett's esophagus. Hum.Pathol. 1988;19:166-178.

50. Badreddine RJ, Wang KK: Barrett's Esophagus: Pathogenesis, Treatment, and Prevention. Gastrointest.Endosc.Clin.N.Am. 2008;18:495-512.

51. Buttar NS, Wang KK, Sebo TJ, Riehle DM, Krishnadath KK, Lutzke LS, Anderson MA, Petterson TM, Burgart LJ: Extent of high-grade dysplasia in Barrett's esophagus correlates with risk of adenocarcinoma. Gastroenterology 2001;120:1630-1639.

52. Schnell TG, Sontag SJ, Chejfec G, Aranha G, Metz A, O'Connell S, Seidel UJ, Sonnenberg A: Long-term nonsurgical management of Barrett's esophagus with high-grade dysplasia. Gastroenterology 2001;120:1607-1619.

53. Heitmiller RF, Redmond M, Hamilton SR: Barrett's esophagus with high-grade dysplasia. An indication for prophylactic esophagectomy. Ann.Surg. 1996;224:66-71.

54. Swisher SG, Deford L, Merriman KW, Walsh GL, Smythe R, Vaporicyan A, Ajani JA, Brown T, Komaki R, Roth JA, Putnam JB: Effect of operative volume on morbidity, mortality, and hospital use after esophagectomy for cancer. J Thorac.Cardiovasc.Surg. 2000;119:1126-1132.

55. Weston AP, Badr AS, Hassanein RS: Prospective multivariate analysis of factors predictive of complete regression of Barrett's esophagus. Am.J Gastroenterol. 1999;94:3420-3426.

56. Beilstein M, Silberg D: Cellular and molecular mechanisms responsible for progression of Barrett's metaplasia to esophageal carcinoma. Gastroenterol.Clin.North Am. 2002;31:461-79, ix.

57. Ahmadi A, Draganov P: Endoscopic mucosal resection in the upper gastrointestinal tract. World J Gastroenterol. 2008;14:1984-1989.

58. Ganz RA, Overholt BF, Sharma VK, Fleischer DE, Shaheen NJ, Lightdale CJ, Freeman SR, Pruitt RE, Urayama SM, Gress F, Pavey DA, Branch MS, Savides TJ, Chang KJ, Muthusamy VR, Bohorfoush AG, Pace SC, DeMeester SR, Eysselein VE, Panjehpour M, Triadafilopoulos G: Circumferential ablation of Barrett's esophagus that contains high-grade dysplasia: a U.S. Multicenter Registry. Gastrointest.Endosc. 2008;68:35-40.

59. Sherr CJ: Cancer cell cycles. Science 1996;274:1672-1677.

60. DelSal G, Loda M, Pagano M: Cell cycle and cancer: critical events at the G1 restriction point. Crit Rev.Oncog. 1996;7:127-142.

61. Jenkins GJ, Doak SH, Parry JM, D'Souza FR, Griffiths AP, Baxter JN: Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. Br.J Surg. 2002;89:824-837.

62. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Orntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006;444:633-637.

63. Galipeau PC, Prevo LJ, Sanchez CA, Longton GM, Reid BJ: Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl.Cancer Inst. 1999;91:2087-2095.

64. Klump B, Hsieh CJ, Holzmann K, Gregor M, Porschen R: Hypermethylation of the CDKN2/p16 promoter during neoplastic progression in Barrett's esophagus. Gastroenterology 1998;115:1381-1386.

65. Maley CC, Reid BJ: Natural selection in neoplastic progression of Barrett's esophagus. Semin.Cancer Biol 2005;15:474-483.

66. Clement G, Braunschweig R, Pasquier N, Bosman FT, Benhattar J: Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. Oncogene 2006;25:3084-3092.

67. Schneikert J, Behrens J: The canonical Wnt signalling pathway and its APC partner in colon cancer development. Gut 2007;56:417-425.

68. Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS: Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. Curr.Biol 2001;11:44-49.

69. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PW, Skinner KA: Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res. 2000;60:5021-5026.

70. Kawakami K, Brabender J, Lord RV, Groshen S, Greenwald BD, Krasna MJ, Yin J, Fleisher AS, Abraham JM, Beer DG, Sidransky D, Huss HT, DeMeester TR, Eads C, Laird PW, Ilson DH, Kelsen DP, Harpole D, Moore MB, Danenberg KD, Danenberg PV, Meltzer SJ: Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J Natl.Cancer Inst. 2000;92:1805-1811.

71. Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ: Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. 1993;7:331-342.

72. Lundberg AS, Weinberg RA: Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol.Cell Biol 1998;18:753-761.

73. Arber N, Lightdale C, Rotterdam H, Han KH, Sgambato A, Yap E, Ahsan H, Finegold J, Stevens PD, Green PH, Hibshoosh H, Neugut AI, Holt PR, Weinstein IB: Increased expression of the cyclin D1 gene in Barrett's esophagus. Cancer Epidemiol.Biomarkers Prev. 1996;5:457-459.

74. Bani-Hani K, Martin IG, Hardie LJ, Mapstone N, Briggs JA, Forman D, Wild CP: Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma. J Natl.Cancer Inst. 2000;92:1316-1321.

75. Alao JP: The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol.Cancer 2007;6:24.

76. Boynton RF, Huang Y, Blount PL, Reid BJ, Raskind WH, Haggitt RC, Newkirk C, Resau JH, Yin J, McDaniel T, .: Frequent loss of heterozygosity at the retinoblastoma locus in human esophageal cancers. Cancer Res. 1991;51:5766-5769.

77. Huang Y, Meltzer SJ, Yin J, Tong Y, Chang EH, Srivastava S, McDaniel T, Boynton RF, Zou ZQ: Altered messenger RNA and unique mutational profiles of p53 and Rb in human esophageal carcinomas. Cancer Res. 1993;53:1889-1894.

78. Reid BJ, Barrett MT, Galipeau PC, Sanchez CA, Neshat K, Cowan DS, Levine DS: Barrett's esophagus: ordering the events that lead to cancer. Eur.J Cancer Prev. 1996;5 Suppl 2:57-65.

79. Barrett MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ: Evolution of neoplastic cell lineages in Barrett oesophagus. Nat.Genet. 1999;22:106-109.

80. Casson AG, Kerkvliet N, O'Malley F, Inculet R, Troster M: p53 immunoreactivity in carcinosarcoma of the esophagus. J Surg.Oncol. 1994;56:132-135.

81. Kokontis JM, Wagner AJ, O'Leary M, Liao S, Hay N: A transcriptional activation function of p53 is dispensable for and inhibitory of its apoptotic function. Oncogene 2001;20:659-668.

82. Merola E, Claudio PP, Giordano A: p53 and the malignant progression of Barrett's esophagus. J Cell Physiol 2006;206:574-577.

83. Schneider PM, Casson AG, Levin B, Garewal HS, Hoelscher AH, Becker K, Dittler HJ, Cleary KR, Troster M, Siewert JR, Roth JA: Mutations of p53 in Barrett's esophagus and Barrett's cancer: a prospective study of ninety-eight cases. J Thorac.Cardiovasc.Surg. 1996;111:323-331.

84. Maley CC, Galipeau PC, Li X, Sanchez CA, Paulson TG, Reid BJ: Selectively advantageous mutations and hitchhikers in neoplasms: p16 lesions are selected in Barrett's esophagus. Cancer Res. 2004;64:3414-3427.

85. Soslow RA, Altorki NK, Yang G, Xie D, Yang CS: mdm-2 expression correlates with wild-type p53 status in esophageal adenocarcinoma. Mod.Pathol. 1999;12:580-586.

86. Singh SP, Lipman J, Goldman H, Ellis FH, Jr., Aizenman L, Cangi MG, Signoretti S, Chiaur DS, Pagano M, Loda M: Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Res. 1998;58:1730-1735.

87. Mu DQ, Peng YS, Xu QJ: Values of mutations of K-ras oncogene at codon 12 in detection of pancreatic cancer: 15-year experience. World J Gastroenterol. 2004;10:471-475.

88. Lord RV, O'Grady R, Sheehan C, Field AF, Ward RL: K-ras codon 12 mutations in Barrett's oesophagus and adenocarcinomas of the oesophagus and oesophagogastric junction. J Gastroenterol.Hepatol. 2000;15:730-736.

89. Campomenosi P, Conio M, Bogliolo M, Urbini S, Assereto P, Aprile A, Monti P, Aste H, Lapertosa G, Inga A, Abbondandolo A, Fronza G: p53 is frequently mutated in Barrett's metaplasia of the intestinal type. Cancer Epidemiol.Biomarkers Prev. 1996;5:559-565.

90. Zinkel S, Gross A, Yang E: BCL2 family in DNA damage and cell cycle control. Cell Death.Differ. 2006;13:1351-1359.

91. Katada N, Hinder RA, Smyrk TC, Hirabayashi N, Perdikis G, Lund RJ, Woodward T, Klingler PJ: Apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus. Arch.Surg. 1997;132:728-733.

92. Bailey T, Biddlestone L, Shepherd N, Barr H, Warner P, Jankowski J: Altered cadherin and catenin complexes in the Barrett's esophagus-dysplasia-adenocarcinoma sequence: correlation with disease progression and dedifferentiation. Am.J Pathol. 1998;152:135-144.

93. Tsukita S, Itoh M, Nagafuchi A, Yonemura S, Tsukita S: Submembranous junctional plaque proteins include potential tumor suppressor molecules. J Cell Biol 1993;123:1049-1053.

94. McNeill H, Ozawa M, Kemler R, Nelson WJ: Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. Cell 1990;62:309-316.

95. Ozawa M, Ringwald M, Kemler R: Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc.Natl.Acad.Sci U.S.A 1990;87:4246-4250.

96. Nagafuchi A, Takeichi M: Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J 1988;7:3679-3684.

97. Behrens J, Mareel MM, Van Roy FM, Birchmeier W: Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 1989;108:2435-2447.

98. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, Kerr D, Young LS: Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am.J Pathol. 1999;154:965-973.

99. Suzuki M, Iso-o N, Takeshita S, Tsukamoto K, Mori I, Sato T, Ohno M, Nagai R, Ishizaka N: Facilitated angiogenesis induced by heme oxygenase-1 gene transfer in a rat model of hindlimb ischemia. Biochem.Biophys.Res.Commun. 2003;302:138-143.

100. Tselepis C, Perry I, Jankowski J: Barrett's esophagus: disregulation of cell cycling and intercellular adhesion in the metaplasia-dysplasia-carcinoma sequence. Digestion 2000;61:1-5.

101. Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422-426.

102. Kumble S, Omary MB, Cartwright CA, Triadafilopoulos G: Src activation in malignant and premalignant epithelia of Barrett's esophagus. Gastroenterology 1997;112:348-356.

103. Pagano M, Draetta G: Cyclin A, cell cycle control and oncogenesis. Prog.Growth Factor Res. 1991;3:267-277.

104. Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T: The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 1986;232:1644-1646.

105. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA: The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature 1984;312:513-516.

106. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, .: Identification of heregulin, a specific activator of p185erbB2. Science 1992;256:1205-1210.

107. Wen D, Peles E, Cupples R, Suggs SV, Bacus SS, Luo Y, Trail G, Hu S, Silbiger SM, Levy RB, .: Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell 1992;69:559-572.

108. Yokota J, Yamamoto T, Toyoshima K, Terada M, Sugimura T, Battifora H, Cline MJ: Amplification of c-erbB-2 oncogene in human adenocarcinomas in vivo. Lancet 1986;1:765-767.

109. Hardwick RH, Shepherd NA, Moorghen M, Newcomb PV, Alderson D: c-erbB-2 overexpression in the dysplasia/carcinoma sequence of Barrett's oesophagus. J Clin.Pathol. 1995;48:129-132.

110. Moskaluk CA, Hu J, Perlman EJ: Comparative genomic hybridization of esophageal and gastroesophageal adenocarcinomas shows consensus areas of DNA gain and loss. Genes Chromosomes.Cancer 1998;22:305-311.

111. Nakamura T, Nekarda H, Hoelscher AH, Bollschweiler E, Harbeck N, Becker K, Siewert JR, Harbec N: Prognostic value of DNA ploidy and c-

erbB-2 oncoprotein overexpression in adenocarcinoma of Barrett's esophagus. Cancer 1994;73:1785-1794.

112. Brien TP, Odze RD, Sheehan CE, McKenna BJ, Ross JS: HER-2/neu gene amplification by FISH predicts poor survival in Barrett's esophagus-associated adenocarcinoma. Hum.Pathol. 2000;31:35-39.

113. al Kasspooles M, Moore JH, Orringer MB, Beer DG: Amplification and over-expression of the EGFR and erbB-2 genes in human esophageal adenocarcinomas. Int.J Cancer 1993;54:213-219.

114. Yacoub L, Goldman H, Odze RD: Transforming growth factor-alpha, epidermal growth factor receptor, and MiB-1 expression in Barrett's-associated neoplasia: correlation with prognosis. Mod.Pathol. 1997;10:105-112.

115. Fitzgerald RC, Triadafilopoulos G: Recent developments in the molecular characterization of Barrett's esophagus. Dig.Dis. 1998;16:63-80.

116. Morales CP, Lee EL, Shay JW: In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer 1998;83:652-659.

117. Shay JW, Bacchetti S: A survey of telomerase activity in human cancer. Eur.J Cancer 1997;33:787-791.

118. Wilson KT, Fu S, Ramanujam KS, Meltzer SJ: Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res. 1998;58:2929-2934.

119. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC: Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl.Cancer Inst. 1999;91:86-88.

120. Zhang F, Altorki NK, Wu YC, Soslow RA, Subbaramaiah K, Dannenberg AJ: Duodenal reflux induces cyclooxygenase-2 in the esophageal mucosa of rats: evidence for involvement of bile acids. Gastroenterology 2001;121:1391-1399.

121. Souza RF, Shewmake K, Beer DG, Cryer B, Spechler SJ: Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. Cancer Res. 2000;60:5767-5772.

122. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990;61:759-767.

123. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation

analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin.Pathol. 1997;50:212-217.

124. Clement G, Braunschweig R, Pasquier N, Bosman FT, Benhattar J: Methylation of APC, TIMP3, and TERT: a new predictive marker to distinguish Barrett's oesophagus patients at risk for malignant transformation. J Pathol. 2006;208:100-107.

125. Gleeson CM, Sloan JM, McManus DT, Maxwell P, Arthur K, McGuigan JA, Ritchie AJ, Russell SE: Comparison of p53 and DNA content abnormalities in adenocarcinoma of the oesophagus and gastric cardia. Br.J Cancer 1998;77:277-286.

126. Casson AG, Manolopoulos B, Troster M, Kerkvliet N, O'Malley F, Inculet R, Finley R, Roth JA: Clinical implications of p53 gene mutation in the progression of Barrett's epithelium to invasive esophageal cancer. Am.J Surg. 1994;167:52-57.

127. Hamelin R, Flejou JF, Muzeau F, Potet F, Laurent-Puig P, Fekete F, Thomas G: TP53 gene mutations and p53 protein immunoreactivity in malignant and premalignant Barrett's esophagus. Gastroenterology 1994;107:1012-1018.

128. Neshat K, Sanchez CA, Galipeau PC, Blount PL, Levine DS, Joslyn G, Reid BJ: p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. Gastroenterology 1994;106:1589-1595.

129. Wada R, Yamaguchi T: K-ras codon 12 mutations of the super-minute dysplasia in Barrett's esophagus by DNA extraction using a microdissection method. Dis.Esophagus. 2003;16:214-217.

130. Galiana C, Lozano JC, Bancel B, Nakazawa H, Yamasaki H: High frequency of Ki-ras amplification and p53 gene mutations in adenocarcinomas of the human esophagus. Mol.Carcinog. 1995;14:286-293.

131. Leslie A, Carey FA, Pratt NR, Steele RJ: The colorectal adenoma-carcinoma sequence. Br.J Surg. 2002;89:845-860.

132. Kubo A, Levin TR, Block G, Rumore GJ, Quesenberry CP, Jr., Buffler P, Corley DA: Dietary patterns and the risk of Barrett's esophagus. Am.J Epidemiol. 2008;167:839-846.

133. Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW, Jr.: Aspirin use and risk of fatal cancer. Cancer Res. 1993;53:1322-1327.

134. US government printing office, Washington DC. FEBS Life sciences research office. Third report on nutrition monitoring in the United States: Volume 1. 1995.
Ref Type: Report

135. Lee DH, Anderson KE, Folsom AR, Jacobs DR, Jr.: Heme iron, zinc and upper digestive tract cancer: the Iowa Women's Health Study. Int.J Cancer 2005;117:643-647.

136. Kubo A, Levin TR, Block G, Rumore GJ, Quesenberry CP, Jr., Buffler P, Corley DA: Dietary antioxidants, fruits, and vegetables and the risk of Barrett's esophagus. Am.J Gastroenterol. 2008;103:1614-1623.

137. Anderson LA, Watson RG, Murphy SJ, Johnston BT, Comber H, Mc GJ, Reynolds JV, Murray LJ: Risk factors for Barrett's oesophagus and oesophageal adenocarcinoma: results from the FINBAR study. World J Gastroenterol. 2007;13:1585-1594.

138. Dong LM, Kristal AR, Peters U, Schenk JM, Sanchez CA, Rabinovitch PS, Blount PL, Odze RD, Ayub K, Reid BJ, Vaughan TL: Dietary supplement use and risk of neoplastic progression in esophageal adenocarcinoma: a prospective study. Nutr.Cancer 2008;60:39-48.

139. Wolfgarten E, Rosendahl U, Nowroth T, Leers J, Metzger R, Holscher AH, Bollschweiler E: Coincidence of nutritional habits and esophageal cancer in Germany. Onkologie. 2001;24:546-551.

140. Farrow DC, Vaughan TL, Hansten PD, Stanford JL, Risch HA, Gammon MD, Chow WH, Dubrow R, Ahsan H, Mayne ST, Schoenberg JB, West AB, Rotterdam H, Fraumeni JF, Jr., Blot WJ: Use of aspirin and other nonsteroidal anti-inflammatory drugs and risk of esophageal and gastric cancer. Cancer Epidemiol.Biomarkers Prev. 1998;7:97-102.

141. Cheng KK, Sharp L, McKinney PA, Logan RF, Chilvers CE, Cook-Mozaffari P, Ahmed A, Day NE: A case-control study of oesophageal adenocarcinoma in women: a preventable disease. Br.J Cancer 2000;83:127-132.

142. Coogan PF, Rosenberg L, Palmer JR, Strom BL, Zauber AG, Stolley PD, Shapiro S: Nonsteroidal anti-inflammatory drugs and risk of digestive cancers at sites other than the large bowel. Cancer Epidemiol.Biomarkers Prev. 2000;9:119-123.

143. Funkhouser EM, Sharp GB: Aspirin and reduced risk of esophageal carcinoma. Cancer 1995;76:1116-1119.

144. Peleg II, Maibach HT, Brown SH, Wilcox CM: Aspirin and nonsteroidal anti-inflammatory drug use and the risk of subsequent colorectal cancer. Arch.Intern.Med 1994;154:394-399.

145. Sharp L, Chilvers CE, Cheng KK, McKinney PA, Logan RF, Cook-Mozaffari P, Ahmed A, Day NE: Risk factors for squamous cell carcinoma of the oesophagus in women: a case-control study. Br.J Cancer 2001;85:1667-1670.

146. Suleiman UL, Harrison M, Britton A, McPherson K, Bates T: H2-receptor antagonists may increase the risk of cardio-oesophageal adenocarcinoma: a case-control study. Eur.J Cancer Prev. 2000;9:185-191.

147. Goldstein SR, Yang GY, Curtis SK, Reuhl KR, Liu BC, Mirvish SS, Newmark HL, Yang CS: Development of esophageal metaplasia and adenocarcinoma in a rat surgical model without the use of a carcinogen. Carcinogenesis 1997;18:2265-2270.

148. Goldstein SR, Yang GY, Chen X, Curtis SK, Yang CS: Studies of iron deposits, inducible nitric oxide synthase and nitrotyrosine in a rat model for esophageal adenocarcinoma. Carcinogenesis 1998;19:1445-1449.

149. Chen X, Yang G, Ding WY, Bondoc F, Curtis SK, Yang CS: An esophagogastroduodenal anastomosis model for esophageal adenocarcinogenesis in rats and enhancement by iron overload. Carcinogenesis 1999;20:1801-1808.

150. Olyaee M, Sontag S, Salman W, Schnell T, Mobarhan S, Eiznhamer D, Keshavarzian A: Mucosal reactive oxygen species production in oesophagitis and Barrett's oesophagus. Gut 1995;37:168-173.

151. Wetscher GJ, Hinder RA, Klingler P, Gadenstatter M, Perdikis G, Hinder PR: Reflux esophagitis in humans is a free radical event. Dis.Esophagus. 1997;10:29-32.

152. Fleisher LN, Ferrell JB, McGahan MC: Synergistic uveitic effects of tumor necrosis factor-alpha and interleukin-1 beta. Invest Ophthalmol.Vis.Sci 1992;33:2120-2127.

153. Tavani A, Negri E, Franceschi S, La Vecchia C: Risk factors for esophageal cancer in lifelong nonsmokers. Cancer Epidemiol.Biomarkers Prev. 1994;3:387-392.

154. Zhang ZF, Kurtz RC, Marshall JR: Cigarette smoking and esophageal and gastric cardia adenocarcinoma. J Natl.Cancer Inst. 1997;89:1247-1249.

155. Chen X, Ding YW, Yang G, Bondoc F, Lee MJ, Yang CS: Oxidative damage in an esophageal adenocarcinoma model with rats. Carcinogenesis 2000;21:257-263.

156. Birgegard G, Caro J: Increased ferritin synthesis and iron uptake in inflammatory mouse macrophages. Scand.J Haematol. 1984;33:43-48.

157. Aust SD: Ferritin as a source of iron and protection from iron-induced toxicities. Toxicol.Lett 1995;82-83:941-944.

158. Hsing AW, McLaughlin JK, Olsen JH, Mellemkjar L, Wacholder S, Fraumeni JF, Jr.: Cancer risk following primary hemochromatosis: a population-based cohort study in Denmark. Int.J Cancer 1995;60:160-162.

159. Fleming RE, Bacon BR: Orchestration of iron homeostasis. N.Engl.J Med 2005;352:1741-1744.

160. Aisen P, Enns C, Wessling-Resnick M: Chemistry and biology of eukaryotic iron metabolism. Int.J Biochem.Cell Biol 2001;33:940-959.

161. Darnell G, Richardson DR: The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: the effect of the ligands on molecular targets involved in proliferation. Blood 1999;94:781-792.

162. Gao J, Lovejoy D, Richardson DR: Effect of iron chelators with potent anti-proliferative activity on the expression of molecules involved in cell cycle progression and growth. Redox.Rep. 1999;4:311-312.

163. Andrews NC: Forging a field: the golden age of iron biology. Blood 2008;112:219-230.

164. McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ: An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 2001;291:1755-1759.

165. Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA: Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 1997;388:482-488.

166. Shindelman JE, Ortmeyer AE, Sussman HH: Demonstration of the transferrin receptor in human breast cancer tissue. Potential marker for identifying dividing cells. Int.J Cancer 1981;27:329-334.

167. Ohgami RS, Campagna DR, Greer EL, Antiochos B, McDonald A, Chen J, Sharp JJ, Fujiwara Y, Barker JE, Fleming MD: Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat.Genet. 2005;37:1264-1269.

168. Collins JF, Franck CA, Kowdley KV, Ghishan FK: Identification of differentially expressed genes in response to dietary iron deprivation in rat duodenum. Am.J Physiol Gastrointest.Liver Physiol 2005;288:G964-G971.

169. Gunshin H, Starr CN, Direnzo C, Fleming MD, Jin J, Greer EL, Sellers VM, Galica SM, Andrews NC: Cybrd1 (duodenal cytochrome b) is not necessary for dietary iron absorption in mice. Blood 2005;106:2879-2883.

170. Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC: Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. Proc.Natl.Acad.Sci U.S.A 1998;95:1148-1153.

171. Hubert N, Hentze MW: Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. Proc.Natl.Acad.Sci U.S.A 2002;99:12345-12350.

172. Mackenzie B, Takanaga H, Hubert N, Rolfs A, Hediger MA: Functional properties of multiple isoforms of human divalent metal-ion transporter 1 (DMT1). Biochem.J 2007;403:59-69.

173. Garrick MD, Kuo HC, Vargas F, Singleton S, Zhao L, Smith JJ, Paradkar P, Roth JA, Garrick LM: Comparison of mammalian cell lines expressing distinct isoforms of divalent metal transporter 1 in a tetracycline-regulated fashion. Biochem.J 2006;398:539-546.

174. Lee PL, Gelbart T, West C, Halloran C, Beutler E: The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Mol.Dis. 1998;24:199-215.

175. Ferreira C, Bucchini D, Martin ME, Levi S, Arosio P, Grandchamp B, Beaumont C: Early embryonic lethality of H ferritin gene deletion in mice. J Biol Chem 2000;275:3021-3024.

176. Caskey JH, Jones C, Miller YE, Seligman PA: Human ferritin gene is assigned to chromosome 19. Proc.Natl.Acad.Sci U.S.A 1983;80:482-486.

177. Worwood M, Brook JD, Cragg SJ, Hellkuhl B, Jones BM, Perera P, Roberts SH, Shaw DJ: Assignment of human ferritin genes to chromosomes 11 and 19q13.3----19qter. Hum.Genet. 1985;69:371-374.

178. Koziol JA, Ho NJ, Felitti VJ, Beutler E: Reference centiles for serum ferritin and percentage of transferrin saturation, with application to mutations of the HFE gene. Clin.Chem 2001;47:1804-1810.

179. Lipschitz DA, Cook JD, Finch CA: A clinical evaluation of serum ferritin as an index of iron stores. N.Engl.J Med 1974;290:1213-1216.

180. Lipschitz DA, Cook JD, Finch CA: A clinical evaluation of serum ferritin as an index of iron stores. 1974. Nutrition 1992;8:443-447.

181. Torti FM, Torti SV: Regulation of ferritin genes and protein. Blood 2002;99:3505-3516.

182. Ames BN, Shigenaga MK, Hagen TM: Oxidants, antioxidants, and the degenerative diseases of aging. Proc.Natl.Acad.Sci U.S.A 1993;90:7915-7922.

183. Bauminger ER, Harrison PM, Hechel D, Hodson NW, Nowik I, Treffry A, Yewdall SJ: Iron (II) oxidation and early intermediates of iron-core formation in recombinant human H-chain ferritin. Biochem.J 1993;296 (Pt 3):709-719.

184. Hintze KJ, Theil EC: Cellular regulation and molecular interactions of the ferritins. Cell Mol.Life Sci 2006;63:591-600.

185. Sierzputowska-Gracz H, McKenzie RA, Theil EC: The importance of a single G in the hairpin loop of the iron responsive element (IRE) in ferritin mRNA for structure: an NMR spectroscopy study. Nucleic Acids Res. 1995;23:146-153.

186. Addess KJ, Basilion JP, Klausner RD, Rouault TA, Pardi A: Structure and dynamics of the iron responsive element RNA: implications for binding of the RNA by iron regulatory binding proteins. J Mol.Biol 1997;274:72-83.

187. McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ: A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol.Cell 2000;5:299-309.

188. Wessling-Resnick M: Iron imports. III. Transfer of iron from the mucosa into circulation. Am.J Physiol Gastrointest.Liver Physiol 2006;290:G1-G6.

189. Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC: The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metab 2005;1:191-200.

190. Ganz T: Hepcidin--a peptide hormone at the interface of innate immunity and iron metabolism. Curr.Top.Microbiol.Immunol. 2006;306:183-198.

191. Zimmermann MB, Hurrell RF: Nutritional iron deficiency. Lancet 2007;370:511-520.

192. Gordeuk VR, Caleffi A, Corradini E, Ferrara F, Jones RA, Castro O, Onyekwere O, Kittles R, Pignatti E, Montosi G, Garuti C, Gangaidzo IT, Gomo ZA, Moyo VM, Rouault TA, MacPhail P, Pietrangelo A: Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. Blood Cells Mol.Dis. 2003;31:299-304.

193. Han O, Kim EY: Colocalization of ferroportin-1 with hephaestin on the basolateral membrane of human intestinal absorptive cells. J Cell Biochem. 2007;101:1000-1010.

194. Kuo YM, Su T, Chen H, Attieh Z, Syed BA, McKie AT, Anderson GJ, Gitschier J, Vulpe CD: Mislocalisation of hephaestin, a multicopper ferroxidase involved in basolateral intestinal iron transport, in the sex linked anaemia mouse. Gut 2004;53:201-206.

195. Syed BA, Beaumont NJ, Patel A, Naylor CE, Bayele HK, Joannou CL, Rowe PS, Evans RW, Srai SK: Analysis of the human hephaestin gene and protein: comparative modelling of the N-terminus ecto-domain based upon ceruloplasmin. Protein Eng 2002;15:205-214.

196. Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ: Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat.Genet. 1999;21:195-199.

197. Lawrence CM, Ray S, Babyonyshev M, Galluser R, Borhani DW, Harrison SC: Crystal structure of the ectodomain of human transferrin receptor. Science 1999;286:779-782.

198. Enns CA, Sussman HH: Physical characterization of the transferrin receptor in human placentae. J Biol Chem 1981;256:9820-9823.

199. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML: The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin.Immunol. 2006;121:144-158.

200. Bali PK, Zak O, Aisen P: A new role for the transferrin receptor in the release of iron from transferrin. Biochemistry 1991;30:324-328.

201. Sipe DM, Murphy RF: Binding to cellular receptors results in increased iron release from transferrin at mildly acidic pH. J Biol Chem 1991;266:8002-8007.

202. Ohgami RS, Campagna DR, McDonald A, Fleming MD: The Steap proteins are metalloreductases. Blood 2006;108:1388-1394.

203. Pantopoulos K: Iron metabolism and the IRE/IRP regulatory system: an update. Ann.N.Y.Acad.Sci 2004;1012:1-13.

204. Rouault TA: The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nat.Chem Biol 2006;2:406-414.

205. Rouault T, Klausner R: Regulation of iron metabolism in eukaryotes. Curr.Top.Cell Regul. 1997;35:1-19.

206. Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger MA: Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett 2001;509:309-316.

207. Sanchez M, Galy B, Dandekar T, Bengert P, Vainshtein Y, Stolte J, Muckenthaler MU, Hentze MW: Iron regulation and the cell cycle: identification of an iron-responsive element in the 3'-untranslated region of human cell division cycle 14A mRNA by a refined microarray-based screening strategy. J Biol Chem 2006;281:22865-22874.

208. Lymboussaki A, Pignatti E, Montosi G, Garuti C, Haile DJ, Pietrangelo A: The role of the iron responsive element in the control of ferroportin1/IREG1/MTP1 gene expression. J Hepatol. 2003;39:710-715.

209. Wallander ML, Leibold EA, Eisenstein RS: Molecular control of vertebrate iron homeostasis by iron regulatory proteins. Biochim.Biophys.Acta 2006;1763:668-689.

210. Erlitzki R, Long JC, Theil EC: Multiple, conserved iron-responsive elements in the 3'-untranslated region of transferrin receptor mRNA enhance binding of iron regulatory protein 2. J Biol Chem 2002;277:42579-42587.

211. Meyron-Holtz EG, Ghosh MC, Iwai K, LaVaute T, Brazzolotto X, Berger UV, Land W, Ollivierre-Wilson H, Grinberg A, Love P, Rouault TA: Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. EMBO J 2004;23:386-395.

212. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K: LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147-150.

213. Park CH, Valore EV, Waring AJ, Ganz T: Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806-7810.

214. Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S: Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc.Natl.Acad.Sci U.S.A 2001;98:8780-8785.

215. Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C: Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat.Genet. 2003;33:21-22.

216. Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirito M, Sawadogo M, Kahn A, Vaulont S: Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc.Natl.Acad.Sci U.S.A 2002;99:4596-4601.

217. Ganz T: Hepcidin and its role in regulating systemic iron metabolism. Hematology.Am.Soc.Hematol.Educ.Program. 2006;29-35, 507.

218. Ganz T: Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 2003;102:783-788.

219. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T: Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003;101:2461-2463.

220. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S: The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin.Invest 2002;110:1037-1044.

221. Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Shimizu C, Bulet P, Burns JC: Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur.J Biochem. 2002;269:2232-2237.

222. De D, I, Ward DM, Langelier C, Vaughn MB, Nemeth E, Sundquist WI, Ganz T, Musci G, Kaplan J: The molecular mechanism of hepcidin-mediated ferroportin down-regulation. Mol.Biol Cell 2007;18:2569-2578.

223. Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T: The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. Blood 2006;107:328-333.

224. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A: The discovery of the new haemochromatosis gene. 1996. J Hepatol. 2003;38:704-709.

225. Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C: Hepcidin is decreased in TFR2 hemochromatosis. Blood 2005;105:1803-1806.

226. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M, Nemeth E, Thompson J, Risler JK, Zaborowska C, Babakaiff R, Radomski CC, Pape TD, Davidas O, Christakis J, Brissot P, Lockitch G, Ganz T, Hayden MR, Goldberg YP: Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat.Genet. 2004;36:77-82.

227. Schimanski LM, Drakesmith H, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR: In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. Blood 2005;105:4096-4102.

228. Latunde-Dada GO, Simpson RJ, McKie AT: Recent advances in mammalian haem transport. Trends Biochem.Sci 2006;31:182-188.

229. Hurrell RF: Fortification: overcoming technical and practical barriers. J Nutr. 2002;132:806S-812S.

230. Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, Halliday N, Khan Y, Warley A, McCann FE, Hider RC, Frazer DM, Anderson GJ, Vulpe CD, Simpson RJ, McKie AT: Identification of an intestinal heme transporter. Cell 2005;122:789-801.

231. Raffin SB, Woo CH, Roost KT, Price DC, Schmid R: Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. J Clin.Invest 1974;54:1344-1352.

232. Jeong J, Rouault TA, Levine RL: Identification of a heme-sensing domain in iron regulatory protein 2. J Biol Chem 2004;279:45450-45454.

233. Kino K, Tsunoo H, Higa Y, Takami M, Nakajima H: Kinetic aspects of hemoglobin.haptoglobin-receptor interaction in rat liver plasma membranes, isolated liver cells, and liver cells in primary culture. J Biol Chem 1982;257:4828-4833.

234. Fagoonee S, Gburek J, Hirsch E, Marro S, Moestrup SK, Laurberg JM, Christensen EI, Silengo L, Altruda F, Tolosano E: Plasma protein haptoglobin modulates renal iron loading. Am.J Pathol. 2005;166:973-983.

235. Ascenzi P, Bocedi A, Visca P, Altruda F, Tolosano E, Beringhelli T, Fasano M: Hemoglobin and heme scavenging. IUBMB.Life 2005;57:749-759.

236. Higa Y, Oshiro S, Kino K, Tsunoo H, Nakajima H: Catabolism of globin-haptoglobin in liver cells after intravenous administration of hemoglobin-haptoglobin to rats. J Biol Chem 1981;256:12322-12328.

237. Moestrup SK, Moller HJ: CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann.Med 2004;36:347-354.

238. Maniecki MB, Moller HJ, Moestrup SK, Moller BK: CD163 positive subsets of blood dendritic cells: the scavenging macrophage receptors CD163 and CD91 are coexpressed on human dendritic cells and monocytes. Immunobiology 2006;211:407-417.

239. Smith MA, Hirai K, Hsiao K, Pappolla MA, Harris PL, Siedlak SL, Tabaton M, Perry G: Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress. J Neurochem. 1998;70:2212-2215.

240. Hrkal Z, Vodrazka Z, Kalousek I: Transfer of heme from ferrihemoglobin and ferrihemoglobin isolated chains to hemopexin. Eur.J Biochem. 1974;43:73-78.

241. Tolosano E, Fagoonee S, Hirsch E, Berger FG, Baumann H, Silengo L, Altruda F: Enhanced splenomegaly and severe liver inflammation in haptoglobin/hemopexin double-null mice after acute hemolysis. Blood 2002;100:4201-4208.

242. Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ, Moestrup SK: Identification of the receptor scavenging hemopexin-heme complexes. Blood 2005;106:2572-2579.

243. Herz J, Strickland DK: LRP: a multifunctional scavenger and signaling receptor. J Clin.Invest 2001;108:779-784.

244. Quigley JG, Yang Z, Worthington MT, Phillips JD, Sabo KM, Sabath DE, Berg CL, Sassa S, Wood BL, Abkowitz JL: Identification of a human heme exporter that is essential for erythropoiesis. Cell 2004;118:757-766.

245. Krishnamurthy P, Schuetz JD: The ABC transporter Abcg2/Bcrp: role in hypoxia mediated survival. Biometals 2005;18:349-358.

246. Dean M, Hamon Y, Chimini G: The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res. 2001;42:1007-1017.

247. Antolin I, Uria H, Tolivia D, Rodriguez-Colunga MJ, Rodriguez C, Kotler ML, Menendez-Pelaez A: Porphyrin accumulation in the harderian glands of female Syrian hamster results in mitochondrial damage and cell death. Anat.Rec. 1994;239:349-359.

248. Keppler D, Konig J: Hepatic secretion of conjugated drugs and endogenous substances. Semin.Liver Dis. 2000;20:265-272.

249. Uc A, Stokes JB, Britigan BE: Heme transport exhibits polarity in Caco-2 cells: evidence for an active and membrane protein-mediated process. Am.J Physiol Gastrointest.Liver Physiol 2004;287:G1150-G1157.

250. Kwok JC, Richardson DR: The iron metabolism of neoplastic cells: alterations that facilitate proliferation? Crit Rev.Oncol.Hematol. 2002;42:65-78.

251. Brookes MJ, Hughes S, Turner FE, Reynolds G, Sharma N, Ismail T, Berx G, McKie AT, Hotchin N, Anderson GJ, Iqbal T, Tselepis C: Modulation of iron transport proteins in human colorectal carcinogenesis. Gut 2006;55:1449-1460.

252. Recht LD, Griffin TW, Raso V, Salimi AR: Potent cytotoxicity of an antihuman transferrin receptor-ricin A-chain immunotoxin on human glioma cells in vitro. Cancer Res. 1990;50:6696-6700.

253. Habeshaw JA, Lister TA, Stansfeld AG, Greaves MF: Correlation of transferrin receptor expression with histological class and outcome in non-Hodgkin lymphoma. Lancet 1983;1:498-501.

254. Beguin Y, Lampertz S, De Groote D, Igot D, Malaise M, Fillet G: Soluble CD23 and other receptors (CD4, CD8, CD25, CD71) in serum of patients with chronic lymphocytic leukemia. Leukemia 1993;7:2019-2025.

255. Kondo K, Noguchi M, Mukai K, Matsuno Y, Sato Y, Shimosato Y, Monden Y: Transferrin receptor expression in adenocarcinoma of the lung as a histopathologic indicator of prognosis. Chest 1990;97:1367-1371.

256. Buckshee K, Saha A: Relevance of transferrin receptors to gynaecological malignancy. Indian J Med Res. 1986;84:292-296.

257. Seymour GJ, Walsh MD, Lavin MF, Strutton G, Gardiner RA: Transferrin receptor expression by human bladder transitional cell carcinomas. Urol.Res. 1987;15:341-344.

258. Lloyd JM, O'Dowd T, Driver M, Tee DE: Demonstration of an epitope of the transferrin receptor in human cervical epithelium--a potentially useful cell marker. J Clin.Pathol. 1984;37:131-135.

259. Soyer HP, Smolle J, Torne R, Kerl H: Transferrin receptor expression in normal skin and in various cutaneous tumors. J Cutan.Pathol. 1987;14:1-5.

260. Sciot R, Paterson AC, van Eyken P, Callea F, Kew MC, Desmet VJ: Transferrin receptor expression in human hepatocellular carcinoma: an immunohistochemical study of 34 cases. Histopathology 1988;12:53-63.

261. Sciot R, van Eyken P, Desmet VJ: Transferrin receptor expression in benign tumours and in hepatoblastoma of the liver. Histopathology 1990;16:59-62.

262. Trowbridge IS, Lopez F: Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro. Proc.Natl.Acad.Sci U.S.A 1982;79:1175-1179.

263. RICHMOND HG: Induction of sarcoma in the rat by iron-dextran complex. Br.Med J 1959;1:947-949.

264. Greenberg G: Sarcoma after intramuscular iron injection. Br.Med J 1976;1:1508-1509.

265. Nelson RL: Iron and colorectal cancer risk: human studies. Nutr.Rev. 2001;59:140-148.

266. Stevens RG, Jones DY, Micozzi MS, Taylor PR: Body iron stores and the risk of cancer. N.Engl.J Med 1988;319:1047-1052.

267. Wurzelmann JI, Silver A, Schreinemachers DM, Sandler RS, Everson RB: Iron intake and the risk of colorectal cancer. Cancer Epidemiol.Biomarkers Prev. 1996;5:503-507.

268. Bird CL, Witte JS, Swendseid ME, Shikany JM, Hunt IF, Frankl HD, Lee ER, Longnecker MP, Haile RW: Plasma ferritin, iron intake, and the risk of colorectal polyps. Am.J Epidemiol. 1996;144:34-41.

269. Beckman LE, Van Landeghem GF, Sikstrom C, Wahlin A, Markevarn B, Hallmans G, Lenner P, Athlin L, Stenling R, Beckman L: Interaction between haemochromatosis and transferrin receptor genes in different neoplastic disorders. Carcinogenesis 1999;20:1231-1233.

270. Nelson RL, Davis FG, Persky V, Becker E: Risk of neoplastic and other diseases among people with heterozygosity for hereditary hemochromatosis. Cancer 1995;76:875-879.

271. Elmberg M, Hultcrantz R, Ekbom A, Brandt L, Olsson S, Olsson R, Lindgren S, Loof L, Stal P, Wallerstedt S, Almer S, Sandberg-Gertzen H, Askling J: Cancer risk in patients with hereditary hemochromatosis and in their first-degree relatives. Gastroenterology 2003;125:1733-1741.

272. Bonkovsky HL: Iron and the liver. Am.J Med Sci 1991;301:32-43.

273. Bradbear RA, Bain C, Siskind V, Schofield FD, Webb S, Axelsen EM, Halliday JW, Bassett ML, Powell LW: Cohort study of internal malignancy in

genetic hemochromatosis and other chronic nonalcoholic liver diseases. J Natl.Cancer Inst. 1985;75:81-84.

274. Niederau C, Fischer R, Sonnenberg A, Stremmel W, Trampisch HJ, Strohmeyer G: Survival and causes of death in cirrhotic and in noncirrhotic patients with primary hemochromatosis. N.Engl.J Med 1985;313:1256-1262.

275. Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT: Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am.J Clin.Nutr. 1999;69:250-255.

276. Lund EK, Fairweather-Tait SJ, Wharf SG, Johnson IT: Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. J Nutr. 2001;131:2928-2931.

277. Davis CD, Feng Y: Dietary copper, manganese and iron affect the formation of aberrant crypts in colon of rats administered 3,2'-dimethyl-4-aminobiphenyl. J Nutr. 1999;129:1060-1067.

278. Liu Z, Tomotake H, Wan G, Watanabe H, Kato N: Combined effect of dietary calcium and iron on colonic aberrant crypt foci, cell proliferation and apoptosis, and fecal bile acids in 1,2-dimethylhydrazine-treated rats. Oncol.Rep. 2001;8:893-897.

279. Campbell S, Ghosh S: Ulcerative colitis and colon cancer: strategies for cancer prevention. Dig.Dis. 2002;20:38-48.

280. Seril DN, Liao J, Ho KL, Yang CS, Yang GY: Inhibition of chronic ulcerative colitis-associated colorectal adenocarcinoma development in a murine model by N-acetylcysteine. Carcinogenesis 2002;23:993-1001.

281. Seril DN, Liao J, Ho KL, Warsi A, Yang CS, Yang GY: Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice. Dig.Dis.Sci 2002;47:1266-1278.

282. Cross AJ, Leitzmann MF, Gail MH, Hollenbeck AR, Schatzkin A, Sinha R: A prospective study of red and processed meat intake in relation to cancer risk. PLoS.Med 2007;4:e325.

283. Sesink AL, Termont DS, Kleibeuker JH, Van der MR: Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. Cancer Res. 1999;59:5704-5709.

284. Knekt P, Steineck G, Jarvinen R, Hakulinen T, Aromaa A: Intake of fried meat and risk of cancer: a follow-up study in Finland. Int.J Cancer 1994;59:756-760.

285. Egeberg R, Olsen A, Autrup H, Christensen J, Stripp C, Tetens I, Overvad K, Tjonneland A: Meat consumption, N-acetyl transferase 1 and 2

polymorphism and risk of breast cancer in Danish postmenopausal women. Eur.J Cancer Prev. 2008;17:39-47.

286. Daniels TR, Delgado T, Helguera G, Penichet ML: The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin.Immunol. 2006;121:159-176.

287. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML: The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin.Immunol. 2006;121:144-158.

288. Daniels TR, Ng PP, Delgado T, Lynch MR, Schiller G, Helguera G, Penichet ML: Conjugation of an anti transferrin receptor IgG3-avidin fusion protein with biotinylated saporin results in significant enhancement of its cytotoxicity against malignant hematopoietic cells. Mol.Cancer Ther. 2007;6:2995-3008.

289. Ng PP, Dela Cruz JS, Sorour DN, Stinebaugh JM, Shin SU, Shin DS, Morrison SL, Penichet ML: An anti-transferrin receptor-avidin fusion protein exhibits both strong proapoptotic activity and the ability to deliver various molecules into cancer cells. Proc.Natl.Acad.Sci U.S.A 2002;99:10706-10711.

290. Ng PP, Helguera G, Daniels TR, Lomas SZ, Rodriguez JA, Schiller G, Bonavida B, Morrison SL, Penichet ML: Molecular events contributing to cell death in malignant human hematopoietic cells elicited by an IgG3-avidin fusion protein targeting the transferrin receptor. Blood 2006;108:2745-2754.

291. Penichet ML, Kang YS, Pardridge WM, Morrison SL, Shin SU: An antibody-avidin fusion protein specific for the transferrin receptor serves as a delivery vehicle for effective brain targeting: initial applications in anti-HIV antisense drug delivery to the brain. J Immunol. 1999;163:4421-4426.

292. Rodriguez JA, Helguera G, Daniels TR, Neacato II, Lopez-Valdes HE, Charles AC, Penichet ML: Binding specificity and internalization properties of an antibody-avidin fusion protein targeting the human transferrin receptor. J Control Release 2007;124:35-42.

293. Whitnall M, Howard J, Ponka P, Richardson DR: A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. Proc.Natl.Acad.Sci U.S.A 2006;103:14901-14906.

294. Yang DC, Jiang XP, Elliott RL, Head JF: Inhibition of growth of human breast carcinoma cells by an antisense oligonucleotide targeted to the transferrin receptor gene. Anticancer Res. 2001;21:1777-1787.

295. Thompson HJ, Kennedy K, Witt M, Juzefyk J: Effect of dietary iron deficiency or excess on the induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. Carcinogenesis 1991;12:111-114.

296. Wang F, Elliott RL, Head JF: Inhibitory effect of deferoxamine mesylate and low iron diet on the 13762NF rat mammary adenocarcinoma. Anticancer Res. 1999;19:445-450.

297. Hann HW, Stahlhut MW, Rubin R, Maddrey WC: Antitumor effect of deferoxamine on human hepatocellular carcinoma growing in athymic nude mice. Cancer 1992;70:2051-2056.

298. Donfrancesco A, Deb G, Dominici C, Pileggi D, Castello MA, Helson L: Effects of a single course of deferoxamine in neuroblastoma patients. Cancer Res. 1990;50:4929-4930.

299. Donfrancesco A, Deb G, Dominici C, De Sio L, Inserra A, Boglino C, Takahashi M, Uchino J, Helson L: D-CECaT as preoperative chemotherapy for unresectable neuroblastoma in children over one year of age. Anticancer Res. 1995;15:2347-2350.

300. Kountouras J, Boura P, Karolides A, Zaharioudaki E, Tsapas G: Recombinant a2 interferon (a-IFN) with chemo-hormonal therapy in patients with hepatocellular carcinoma (HCC). Hepatogastroenterology 1995;42:31-36.

301. Yu Y, Wong J, Lovejoy DB, Kalinowski DS, Richardson DR: Chelators at the cancer coalface: desferrioxamine to Triapine and beyond. Clin.Cancer Res. 2006;12:6876-6883.

302. Le NT, Richardson DR: The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim.Biophys.Acta 2002;1603:31-46.

303. Attia S, Kolesar J, Mahoney MR, Pitot HC, Laheru D, Heun J, Huang W, Eickhoff J, Erlichman C, Holen KD: A phase 2 consortium (P2C) trial of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) for advanced adenocarcinoma of the pancreas. Invest New Drugs 2008;26:369-379.

304. Odenike OM, Larson RA, Gajria D, Dolan ME, Delaney SM, Karrison TG, Ratain MJ, Stock W: Phase I study of the ribonucleotide reductase inhibitor 3-aminopyridine-2-carboxaldehyde-thiosemicarbazone (3-AP) in combination with high dose cytarabine in patients with advanced myeloid leukemia. Invest New Drugs 2008;26:233-239.

305. Ma B, Goh BC, Tan EH, Lam KC, Soo R, Leong SS, Wang LZ, Mo F, Chan AT, Zee B, Mok T: A multicenter phase II trial of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine) and gemcitabine in advanced non-small-cell lung cancer with pharmacokinetic evaluation using peripheral blood mononuclear cells. Invest New Drugs 2008;26:169-173.

306. Knox JJ, Hotte SJ, Kollmannsberger C, Winquist E, Fisher B, Eisenhauer EA: Phase II study of Triapine in patients with metastatic renal cell carcinoma: a trial of the National Cancer Institute of Canada Clinical Trials Group (NCIC IND.161). Invest New Drugs 2007;25:471-477.

307. Huang X: Iron overload and its association with cancer risk in humans: evidence for iron as a carcinogenic metal. Mutat.Res. 2003;533:153-171.

308. Yuan J, Lovejoy DB, Richardson DR: Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment. Blood 2004;104:1450-1458.

309. Aouali N, Eddabra L, Macadre J, Morjani H: Immunosuppressors and reversion of multidrug-resistance. Crit Rev.Oncol.Hematol. 2005;56:61-70.

310. Wadler S, Makower D, Clairmont C, Lambert P, Fehn K, Sznol M: Phase I and pharmacokinetic study of the ribonucleotide reductase inhibitor, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone, administered by 96-hour intravenous continuous infusion. J Clin.Oncol. 2004;22:1553-1563.

311. Foltz LM, Dalal BI, Wadsworth LD, Broady R, Chi K, Eisenhauer E, Kobayashi K, Kollmannsburger C: Recognition and management of methemoglobinemia and hemolysis in a G6PD-deficient patient on experimental anticancer drug Triapine. Am.J Hematol. 2006;81:210-211.

312. Burkitt DP: Related disease--related cause? Lancet 1969;2:1229-1231.

313. Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Stampfer MJ, Rosner B, Speizer FE, Willett WC: Dietary fiber and the risk of colorectal cancer and adenoma in women. N.Engl.J Med 1999;340:169-176.

314. Howe GR, Benito E, Castelleto R, Cornee J, Esteve J, Gallagher RP, Iscovich JM, Deng-ao J, Kaaks R, Kune GA, .: Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. J Natl.Cancer Inst. 1992;84:1887-1896.

315. Jensen OM, MacLennan R, Wahrendorf J: Diet, bowel function, fecal characteristics, and large bowel cancer in Denmark and Finland. Nutr.Cancer 1982;4:5-19.

316. McKeown-Eyssen GE, Bright-See E: Dietary factors in colon cancer: international relationships. Nutr.Cancer 1984;6:160-170.

317. Reddy BS, Ekelund G, Bohe M, Engle A, Domellof L: Metabolic epidemiology of colon cancer: dietary pattern and fecal sterol concentrations of three populations. Nutr.Cancer 1983;5:34-40.

318. Lewis SJ, Heaton KW: Roughage revisited: the effect on intestinal function of inert plastic particles of different sizes and shape. Dig.Dis.Sci 1999;44:744-748.

319. Aozasa O, Ohta S, Nakao T, Miyata H, Nomura T: Enhancement in fecal excretion of dioxin isomer in mice by several dietary fibers. Chemosphere 2001;45:195-200.

320. Nishiyama C, Nagai T, Tano T: Adsorption of mutagens in distilled water by dietary-fibres. Agric Biol Chem 1991;55:797-802.

321. Ikegami S, Umegaki K, Kawashima Y, Ichikawa T: Viscous indigestible polysaccharides reduce accumulation of pentachlorobenzene in rats. J Nutr. 1994;124:754-760.

322. Debruyne PR, Bruyneel EA, Li X, Zimber A, Gespach C, Mareel MM: The role of bile acids in carcinogenesis. Mutat.Res. 2001;480-481:359-369.

323. Ikegami S, Harada H, Tsuchihashi N, Nagayama S, Nishide E, Innami S: Effect of indigestible polysaccharides on pancreatic exocrine secretion and biliary output. J Nutr.Sci Vitaminol.(Tokyo) 1984;30:515-523.

324. Ochsenkuhn T, Bayerdorffer E, Meining A, Schinkel M, Thiede C, Nussler V, Sackmann M, Hatz R, Neubauer A, Paumgartner G: Colonic mucosal proliferation is related to serum deoxycholic acid levels. Cancer 1999;85:1664-1669.

325. Owen RW: Faecal steroids and colorectal carcinogenesis. Scand.J Gastroenterol.Suppl 1997;222:76-82.

326. Wang W: Binding of bile salts to soluble and insoluble dietary fibers of seaweed. Fish Sci 2001;67:1169-1173.

327. Maga JA: Phytate; its chemistry, occurrence, food interactions, nutritional significance and methods of analysis. J Agric Food Chem 1982;30:1-9.

328. Graf E, Empson KL, Eaton JW: Phytic acid. A natural antioxidant. J Biol Chem 1987;262:11647-11650.

329. Graf E, Eaton JW: Antioxidant functions of phytic acid. Free Radic.Biol Med 1990;8:61-69.

330. Nielsen BK, Thompson LU, Bird RP: Effect of phytic acid on colonic epithelial cell proliferation. Cancer Lett 1987;37:317-325.

331. Shamsuddin AM, Elsayed AM, Ullah A: Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis 1988;9:577-580.

332. Shamsuddin AM, Ullah A: Inositol hexaphosphate inhibits large intestinal cancer in F344 rats 5 months after induction by azoxymethane. Carcinogenesis 1989;10:625-626.

333. Ullah A, Shamsuddin AM: Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. Carcinogenesis 1990;11:2219-2222.

334. Nelson RL, Yoo SJ, Tanure JC, Andrianopoulos G, Misumi A: The effect of iron on experimental colorectal carcinogenesis. Anticancer Res. 1989;9:1477-1482.

335. Davis TA, Volesky B, Mucci A: A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 2003;37:4311-4330.

336. Hoshiyama Y, Sekine T, Sasaba T: A case-control study of colorectal cancer and its relation to diet, cigarettes, and alcohol consumption in Saitama Prefecture, Japan. Tohoku J Exp.Med 1993;171:153-165.

337. Nakachi K, Imai K, Hoshiyama Y, Sasaba T: The joint effects of two factors in the aetiology of oesophageal cancer in Japan. J Epidemiol.Community Health 1988;42:355-364.

338. Funahashi H, Imai T, Mase T, Sekiya M, Yokoi K, Hayashi H, Shibata A, Hayashi T, Nishikawa M, Suda N, Hibi Y, Mizuno Y, Tsukamura K, Hayakawa A, Tanuma S: Seaweed prevents breast cancer? Jpn.J Cancer Res. 2001;92:483-487.

339. Teas J: The consumption of seaweed as a protective factor in the etiology of breast cancer. Med Hypotheses 1981;7:601-613.

340. Philchenkov A, Zavelevich M, Imbs T, Zvyagintseva T, Zaporozhets T: Sensitization of human malignant lymphoid cells to etoposide by fucoidan, a brown seaweed polysaccharide. Exp.Oncol. 2007;29:181-185.

341. Kwon MJ, Nam TJ: A polysaccharide of the marine alga Capsosiphon fulvescens induces apoptosis in AGS gastric cancer cells via an IGF-IR-mediated PI3K/Akt pathway. Cell Biol Int. 2007;31:768-775.

342. Buss JL, Torti FM, Torti SV: The role of iron chelation in cancer therapy. Curr.Med Chem 2003;10:1021-1034.

343. Haug A, Larsen B, Smidsrod O: A study of the constitution of alginic acid by partial acid hydrolysis. Acta Chem Scand 1996;20:183-190.

344. Morris ER, Rees DA, Robinson G, Young GA: Competitive inhibition of interchain interactions in polysaccharide systems. J Mol.Biol 1980;138:363-374.

345. Figueira MM, Volesky B, Ciminelli VS: Assessment of interference in biosorption of a heavy metal. Biotechnol.Bioeng. 1997;54:344-350.

346. Sreeram KJ, Yamini SH, Nair BU: Studies on the nature of interaction of iron(III) with alginates. Biochim.Biophys.Acta 2004;1670:121-125.

347. Sandberg AS, Andersson H, Bosaeus I, Carlsson NG, Hasselblad K, Harrod M: Alginate, small bowel sterol excretion, and absorption of nutrients in ileostomy subjects. Am.J Clin.Nutr. 1994;60:751-756.

348. Rockett JC, Larkin K, Darnton SJ, Morris AG, Matthews HR: Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. Br.J Cancer 1997;75:258-263.

349. Boonstra JJ, van Marion R, Beer DG, Lin L, Chaves P, Ribeiro C, Pereira AD, Roque L, Darnton SJ, Altorki NK, Schrump DS, Klimstra DS, Tang LH, Eshleman JR, Alvarez H, Shimada Y, van Dekken H, Tilanus HW, Dinjens WN: Verification and unmasking of widely used human esophageal adenocarcinoma cell lines. J Natl.Cancer Inst. 2010;102:271-274.

350. Di Martino E, Wild CP, Rotimi O, Darnton JS, Olliver RJ, Hardie LJ: IGFBP-3 and IGFBP-10 (CYR61) up-regulation during the development of Barrett's oesophagus and associated oesophageal adenocarcinoma: potential biomarkers of disease risk. Biomarkers 2006;11:547-561.

351. Worthington MT, Cohn SM, Miller SK, Luo RQ, Berg CL: Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. Am.J Physiol Gastrointest.Liver Physiol 2001;280:G1172-G1177.

352. Dvorakova K, Payne CM, Ramsey L, Holubec H, Sampliner R, Dominguez J, Dvorak B, Bernstein H, Bernstein C, Prasad A, Fass R, Cui H, Garewal H: Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. Clin.Cancer Res. 2004;10:2020-2028.

353. Deans DA, Wigmore SJ, Gilmour H, Paterson-Brown S, Ross JA, Fearon KC: Elevated tumour interleukin-1beta is associated with systemic inflammation: A marker of reduced survival in gastro-oesophageal cancer. Br.J Cancer 2006;95:1568-1575.

354. Lee SA, Choi SR, Jang JS, Lee JH, Roh MH, Kim SO, Kim MC, Kim SJ, Jeong JS: Expression of VEGF, EGFR, and IL-6 in Gastric Adenomas and Adenocarcinomas by Endoscopic Submucosal Dissection. Dig.Dis.Sci 2009.

355. Talar-Wojnarowska R, Gasiorowska A, Smolarz B, Romanowicz-Makowska H, Kulig A, Malecka-Panas E: Clinical significance of interleukin-6 (IL-6) gene polymorphism and IL-6 serum level in pancreatic adenocarcinoma and chronic pancreatitis. Dig.Dis.Sci 2009;54:683-689.

356. Nikiteas NI, Tzanakis N, Gazouli M, Rallis G, Daniilidis K, Theodoropoulos G, Kostakis A, Peros G: Serum IL-6, TNFalpha and CRP levels in Greek colorectal cancer patients: prognostic implications. World J Gastroenterol. 2005;11:1639-1643.

357. Ward DG, Roberts K, Brookes MJ, Joy H, Martin A, Ismail T, Spychal R, Iqbal T, Tselepis C: Increased hepcidin expression in colorectal carcinogenesis. World J Gastroenterol. 2008;14:1339-1345.

358. Chen X, Yang CS: Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention. Carcinogenesis 2001;22:1119-1129.

359. Chaston TB, Lovejoy DB, Watts RN, Richardson DR: Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311. Clin.Cancer Res. 2003;9:402-414.

360. Green DA, Antholine WE, Wong SJ, Richardson DR, Chitambar CR: Inhibition of malignant cell growth by 311, a novel iron chelator of the pyridoxal isonicotinoyl hydrazone class: effect on the R2 subunit of ribonucleotide reductase. Clin.Cancer Res. 2001;7:3574-3579.

361. Nyholm S, Mann GJ, Johansson AG, Bergeron RJ, Graslund A, Thelander L: Role of ribonucleotide reductase in inhibition of mammalian cell growth by potent iron chelators. J Biol Chem 1993;268:26200-26205.

362. Thelander L, Reichard P: Reduction of ribonucleotides. Annu.Rev.Biochem. 1979;48:133-158.

363. Kulp KS, Green SL, Vulliet PR: Iron deprivation inhibits cyclin-dependent kinase activity and decreases cyclin D/CDK4 protein levels in asynchronous MDA-MB-453 human breast cancer cells. Exp.Cell Res. 1996;229:60-68.

364. Nurtjahja-Tjendraputra E, Fu D, Phang JM, Richardson DR: Iron chelation regulates cyclin D1 expression via the proteasome: a link to iron deficiency-mediated growth suppression. Blood 2007;109:4045-4054.

365. Yu Y, Kovacevic Z, Richardson DR: Tuning cell cycle regulation with an iron key. Cell Cycle 2007;6:1982-1994.

366. Brookes MJ, Boult J, Roberts K, Cooper BT, Hotchin NA, Matthews G, Iqbal T, Tselepis C: A role for iron in Wnt signalling. Oncogene 2008;27:966-975.

367. Bilello JP, Cable EE, Isom HC: Expression of E-cadherin and other paracellular junction genes is decreased in iron-loaded hepatocytes. Am.J Pathol. 2003;162:1323-1338.

368. Swami S, Kumble S, Triadafilopoulos G: E-cadherin expression in gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma: an immunohistochemical and immunoblot study. Am.J Gastroenterol. 1995;90:1808-1813.

369. Guan RJ, Ford HL, Fu Y, Li Y, Shaw LM, Pardee AB: Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res. 2000;60:749-755.

370. Kovacevic Z, Richardson DR: The metastasis suppressor, Ndrg-1: a new ally in the fight against cancer. Carcinogenesis 2006;27:2355-2366.

371. Bandyopadhyay S, Pai SK, Gross SC, Hirota S, Hosobe S, Miura K, Saito K, Commes T, Hayashi S, Watabe M, Watabe K: The Drg-1 gene suppresses tumor metastasis in prostate cancer. Cancer Res. 2003;63:1731-1736.

372. Dong Z, Arnold RJ, Yang Y, Park MH, Hrncirova P, Mechref Y, Novotny MV, Zhang JT: Modulation of differentiation-related gene 1 expression by cell cycle blocker mimosine, revealed by proteomic analysis. Mol.Cell Proteomics. 2005;4:993-1001.

373. Le NT, Richardson DR: Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: a link between iron metabolism and proliferation. Blood 2004;104:2967-2975.

374. Habel ME, Jung D: c-Myc over-expression in Ramos Burkitt's lymphoma cell line predisposes to iron homeostasis disruption in vitro. Biochem.Biophys.Res.Commun. 2006;341:1309-1316.

375. O'Donnell KA, Yu D, Zeller KI, Kim JW, Racke F, Thomas-Tikhonenko A, Dang CV: Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis. Mol.Cell Biol 2006;26:2373-2386.

376. Wu KJ, Polack A, Dalla-Favera R: Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. Science 1999;283:676-679.

377. Anderson MR, Harrison R, Atherfold PA, Campbell MJ, Darnton SJ, Obszynska J, Jankowski JA: Met receptor signaling: a key effector in esophageal adenocarcinoma. Clin.Cancer Res. 2006;12:5936-5943.

378. Kino K, Tsunoo H, Higa Y, Takami M, Nakajima H: Kinetic aspects of hemoglobin.haptoglobin-receptor interaction in rat liver plasma membranes, isolated liver cells, and liver cells in primary culture. J Biol Chem 1982;257:4828-4833.

379. Stocker R: Induction of haem oxygenase as a defence against oxidative stress. Free Radic.Res.Commun. 1990;9:101-112.

380. Marinissen MJ, Tanos T, Bolos M, de Sagarra MR, Coso OA, Cuadrado A: Inhibition of heme oxygenase-1 interferes with the transforming activity of the Kaposi sarcoma herpesvirus-encoded G protein-coupled receptor. J Biol Chem 2006;281:11332-11346.

381. Sunamura M, Duda DG, Ghattas MH, Lozonschi L, Motoi F, Yamauchi J, Matsuno S, Shibahara S, Abraham NG: Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer. Angiogenesis. 2003;6:15-24.

382. Was H, Cichon T, Smolarczyk R, Rudnicka D, Stopa M, Chevalier C, Leger JJ, Lackowska B, Grochot A, Bojkowska K, Ratajska A, Kieda C, Szala S, Dulak J, Jozkowicz A: Overexpression of heme oxygenase-1 in murine

melanoma: increased proliferation and viability of tumor cells, decreased survival of mice. Am.J Pathol. 2006;169:2181-2198.

383. Berberat PO, Dambrauskas Z, Gulbinas A, Giese T, Giese N, Kunzli B, Autschbach F, Meuer S, Buchler MW, Friess H: Inhibition of heme oxygenase-1 increases responsiveness of pancreatic cancer cells to anticancer treatment. Clin.Cancer Res. 2005;11:3790-3798.

384. Doi K, Akaike T, Fujii S, Tanaka S, Ikebe N, Beppu T, Shibahara S, Ogawa M, Maeda H: Induction of haem oxygenase-1 nitric oxide and ischaemia in experimental solid tumours and implications for tumour growth. Br.J Cancer 1999;80:1945-1954.

385. Hirai K, Sasahira T, Ohmori H, Fujii K, Kuniyasu H: Inhibition of heme oxygenase-1 by zinc protoporphyrin IX reduces tumor growth of LL/2 lung cancer in C57BL mice. Int.J Cancer 2007;120:500-505.

386. Busserolles J, Megias J, Terencio MC, Alcaraz MJ: Heme oxygenase-1 inhibits apoptosis in Caco-2 cells via activation of Akt pathway. Int.J Biochem.Cell Biol 2006;38:1510-1517.

387. Li VG, Sacerdoti D, Sangras B, Vanella A, Mezentsev A, Scapagnini G, Falck JR, Abraham NG: Carbon monoxide signaling in promoting angiogenesis in human microvessel endothelial cells. Antioxid.Redox.Signal. 2005;7:704-710.

388. Cisowski J, Loboda A, Jozkowicz A, Chen S, Agarwal A, Dulak J: Role of heme oxygenase-1 in hydrogen peroxide-induced VEGF synthesis: effect of HO-1 knockout. Biochem.Biophys.Res.Commun. 2005;326:670-676.

389. Jozkowicz A, Huk I, Nigisch A, Weigel G, Dietrich W, Motterlini R, Dulak J: Heme oxygenase and angiogenic activity of endothelial cells: stimulation by carbon monoxide and inhibition by tin protoporphyrin-IX. Antioxid.Redox.Signal. 2003;5:155-162.

390. Barmina OY, Walling HW, Fiacco GJ, Freije JM, Lopez-Otin C, Jeffrey JJ, Partridge NC: Collagenase-3 binds to a specific receptor and requires the low density lipoprotein receptor-related protein for internalization. J Biol Chem 1999;274:30087-30093.

391. Kancha RK, Stearns ME, Hussain MM: Decreased expression of the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor in invasive cell clones derived from human prostate and breast tumor cells. Oncol.Res. 1994;6:365-372.

392. Yang Z, Strickland DK, Bornstein P: Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. J Biol Chem 2001;276:8403-8408.

393. Chazaud B, Bonavaud S, Plonquet A, Pouchelet M, Gherardi RK, Barlovatz-Meimon G: Involvement of the [uPAR:uPA:PAI-1:LRP] complex in human myogenic cell motility. Exp.Cell Res. 2000;258:237-244.

394. Li Y, Wood N, Grimsley P, Yellowlees D, Donnelly PK: In vitro invasiveness of human breast cancer cells is promoted by low density lipoprotein receptor-related protein. Invasion Metastasis 1998;18:240-251.

395. Dedieu S, Langlois B, Devy J, Sid B, Henriet P, Sartelet H, Bellon G, Emonard H, Martiny L: LRP-1 silencing prevents malignant cell invasion despite increased pericellular proteolytic activities. Mol.Cell Biol 2008;28:2980-2995.

396. Yuasa H, Inoue K, Hayashi Y: Molecular and functional characteristics of proton-coupled folate transporter. J Pharm.Sci 2009;98:1608-1616.

397. Price MA: CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. Genes Dev. 2006;20:399-410.

398. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Orntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006;444:633-637.

PUBLICATIONS AND PRESENTATIONS FROM EXPERIMENTAL WORK DURING THIS PERIOD OF RESEARCH

Peer reviewed publications

Boult J, **Roberts K**, Brookes MJ, Hughes S, Bury JP, Cross SS, Anderson GJ, Spychal R, Iqbal T, Tselepis C. Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma. **Clin Can Research** 2008; 14:379-87

Brookes M, Boult J, **Roberts K**, Cooper C, Hotchin N, Matthews G, Iqbal T, Tselepis C. A role for iron in Wnt signalling. **Oncogene** 2008; 27:966-75

Ward DG, **Roberts K**, Stonelake P, Goon P, Zampronio C, Martin A, Johnson PJ, Iqbal T, Tselepis C. SELDI-TOF-MS determination of hepcidin in clinical samples using stable isotope labelled hepcidin as an internal standard. **Proteome Sci** 2008;6:28

Ward DG, **Roberts K**, Brookes MJ, Joy H, Martin A, Ismail T, Spychal R, Iqbal T, Tselepis C. Increased hepcidin expression in colorectal carcinogenesis. **World J Gastroenterol** 2008;14:1139-45

Roberts K, Harper E, Alderson D, Hallissey M. Long term survival and cost analysis of an annual Barrett's surveillance programme. **Eur J Gastroenterol Hepatol**. 2010 Apr;22(4):399-403

Published abstracts

Roberts K, Harper E, Alderson D, Hallissey M. Cost effectiveness of annual surveillance for Barrett's oesophagus. **Br J Surg** 2008;95(S7):12

Roberts K, Spychal R, Brookes M, Strugala V, Dettmar P, Johnstone L, Jolliffe I, Tselepis C. Potential therapeutic role of alginates in the management of oesophageal adenocarcinoma. **Gastroenterology** 2008;134(S1):M2016

Roberts K, Brookes M, Spychal R, Iqbal T, Tselepis C. Increasing evidence for haem metabolism in oesophageal adenocarcinoma. **Gastroenterology** 2008;134(S1):M2015

Roberts K, Ward D, Brookes M, Naqvi M, Ismail T, Iqbal T, Tselepis C. Hepcidin expression relates to colorectal tumour stage. **Gastroenterology** 2008;134(S1):M1996

Roberts K, Harper E, Hallissey M. Five year survival of patients with oesophageal adenocarcinoma identified during an annual surveillance programme. **Gastroenterology** 2008;134(S1):M1594

Brookes MJ, **Roberts K**, Iqbal T, Tselepis C. Cellular iron drives in vitro proliferation and migration in colorectal adenocarcinoma. **Gastroenterology** 2008;134(S1):A37

Brookes MJ, **Roberts K**, Iqbal T, Tselepis C. Cellular iron regulation is defective in colorectal adenocarcinoma. **Gastroenterology** 2008;134(S1):S2029

Roberts K, Spychal R, Brookes M, Strugala V, Dettmar P, Johnstone L, Jolliffe I, Tselepis C. Alginates inhibit iron induced proliferation and migration in a model of oesophageal adenocarcinoma. **Br J Surg** 2008;95(S3):5

Roberts K, Brookes M, Spychal R, Iqbal T, Tselepis C. Haem induces proliferation and migration in oesophageal adenocarcinoma. **Br J Surg** 2008;95(S3):4

Roberts K, Harper E, Hallissey M. Five year survival of patients with oesophageal adenocarcinoma identified during an annual surveillance programme. **Br J Surg** 2008;95(S3):71

Roberts K, Boult J, Brookes M, Iqbal T, Spychal R, Tselepis C. Deregulated iron metabolism in oesophageal carcinogenesis. **Ann Surg** 2008;90:353

Roberts K, Spychal R, Brookes M, Strugala V, Dettmar P, Johnstone L, Jolliffe I, Tselepis C. Alginates inhibit the effects of iron in an in-vitro model of oesophageal adenocarcinoma. **Gut** 2008;57(S1):A1-A172

Brookes MJ, **Roberts K,** Iqbal T, Tselepis C. Iron mediated cellular proliferation, migration and colony formation is dependent on adenomatous polyposis coli status in colorectal adenocarcinoma. **Gut** 2008;57(S1):A1-A172

Brookes MJ, **Roberts K**, Iqbal T, Tselepis C. The regulation of cellular iron is defective in APC mutant colorectal carcinogenesis. **Gut** 2008;57(S1):A1-A172

Roberts K, Brookes MJ, Boult J, McKie AT, Iqbal T, Spychal R, Tselepis C. Expression of HCP-1 in Oesophageal Carcinogenesis. **Gastroenterology** 2007;132(S2):A303

Roberts K, Brookes MJ, Boult J, Anderson GJ, Spychal R, Iqbal T, Tselepis C. A modulation of iron transport proteins in oeosphageal adenocarcinoma. **Gastroenterology** 2007;132(S2):A305

Roberts K, Brookes M, Boult J, McKie A, Spychal R, Iqbal T, Tselepis C. Uptake of haem iron in oesophageal adenocarcinoma:a possible mechanism of carcinogenesis. **Br J Surg** 2007;94(S2):89

Roberts K, Brookes M, Boult J, Anderson G, Spychal R, Iqbal T, Tselepis C. A role for iron in oesophageal adenocarcinoma. **Br J Surg** 2007;94(S2):89

Boult J, Hughes S, Brookes MJ, **Roberts K**, Taniere P, Iqbal T, Tselepis C. A role for iron transporters in oesophageal adenocarcinoma. **Gut** 2006;55(S2): A338

Oral presentations

Haem induces proliferation and migration in oesophageal adenocarcinoma. Association of Surgeons of Great Britain and Ireland, Bournemouth 2008 (Moynihan Prize Session)

Alginates inhibit iron induced proliferation and migration in a model of oesophageal adenocarcinoma. Association of Surgeons of Great Britain and Ireland, Bournemouth 2008

Cost effectiveness of a Barrett's surveillance programme. Association of Upper Gastrointestinal Surgeons, Liverpool 2008

Cost effectiveness and long term survival of patients in a Barrett's surveillance program. West Midlands Surgical Society, November 2008

Degregulated iron metabolism in oesophageal adenocarcinoma. West Midlands Surgical Society – Registrars prize, November 2007

Poster presentations

Roberts K, Spychal R, Brookes M, Strugala V, Dettmar P, Johnstone L, Jolliffe I, Tselepis C. Potential therapeutic role of alginates in the management of oesophageal adenocarcinoma. AGA DDW 2008

Roberts K, Brookes M, Spychal R, Iqbal T, Tselepis C. Increasing evidence for haem metabolism in oesophageal adenocarcinoma. AGA DDW 2008

Roberts K, Ward D, Brookes M, Naqvi M, Ismail T, Iqbal T, Tselepis C. Hepcidin expression realtes to colorectal tumour stage. AGA DDW 2008

Roberts K, Harper E, Hallissey M. Five year survival of patients with oesophageal adenocarcinoma identified during an annual surveillance programme. AGA DDW 2008

Roberts K, Brookes MJ, Boult J, McKie AT, Iqbal T, Spychal R, Tselepis C. Expression of HCP-1 in Oesophageal Carcinogenesis. AGA DDW 2007

Roberts K, Brookes MJ, Boult J, Anderson GJ, Spychal R, Iqbal T, Tselepis C. A modulation of iron transport proteins in oeosphageal adenocarcinoma. AGA DDW 2007

Roberts K,O'Neil, Iqbal T, Spychal R, Tselepis C. The role of LRP1 in oesophageal adenocarcinoma. NCRI 2008

Roberts K, Harper E, Hallissey M. Five year survival of patients with oesophageal adenocarcinoma identified during an annual surveillance programme. ASGBI 2008 (poster of distinction)

Roberts K, Spychal R, Brookes M, Strugala V, Dettmar P, Johnstone L, Jolliffe I, Tselepis C. Alginates inhibit the effects of iron in an in-vitro model of oesophageal adenocarcinoma. BSG 2008

Roberts K, Brookes MJ, Boult J, Anderson GJ, Spychal R, Iqbal T, Tselepis C. Deregulated iron metabolism in oesophageal carcinogensis. ASiT 2008

Roberts K, Brookes M, Boult J, McKie A, Spychal R, Iqbal T, Tselepis C. Uptake of heme iron in oesophageal adenocarcinoma: a possible mechanism of carcinogenesis. ASGBI 2007

Roberts K, Brookes M, Boult J, Anderson G, Spychal R, Iqbal T, Tselepis C. A role for iron in oesophageal adenocarcinoma. ASGBI 2007

Boult J, Hughes S, Brookes MJ, Roberts K, Taniere P, Iqbal T, Tselepis C. A role for iron transporters in oesophageal adenocarcinoma. BSG 2006

Brookes MJ, Roberts K, Boult J, Taniere P, Iqbal T, Spychal R, Tselepis C. Expression of a novel heme transporter (HCP-1) in oesophageal and colorectal adenocarcinoma. MGS. June 2006