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Delineating the pro-Carcinogenic Pathways Activated by Obesity in Barrett's Oesophagus

A dissertation submitted to the University of Dublin

for the degree of Doctor of Philosophy

by

Rory Casey

Under the supervision of Dr. Graham Pidgeon

Departmental Head: Prof. John V. Reynolds

October 2011

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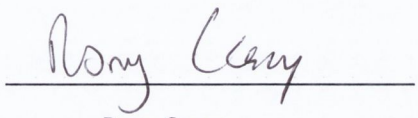

Rory Casey

October 2011

Summary

Incidences of obesity in the developed world represent a worrying epidemic. Obesity is associated with increased rates of diabetes, cardiovascular disease and cancer. Oesophageal adenocarcinoma rates have increased in the last 25 years hand in hand with obesity, and clear correlations between the two have been demonstrated previously. A major risk factor for oesophageal adenocarcinoma is Barrett's oesophagus, a consequence of prolonged exposure to gastro oesophageal reflux disease (GORD). However, only a fraction of Barrett's patients with GORD will ultimately progress to oesophageal adenocarcinoma indicating that there must be other factors at play in the neoplastic progression of Barrett's oesophagus independent of GORD. The aim of this thesis was to identify pro-carcinogenic pathways activated by obesity in Barrett's oesophagus independent of GORD.

Culture of the non-neoplastic BAR-T cell line, a cell line generated from a Barrett's metaplasia biopsy of a patient who later in life developed oesophageal adenocarcinoma, in adipose conditioned media (ACM) generated from omental tissue of viscerally obese oesophagectomy patients, activated angiogenic and metastatic pathways in the cells. Culture of the BAR-T cells in ACM also allowed for identification of five genes (MMP-1, TIMP-3, IL-8, MCAM and VEGF-A) whose expression showed significant changes in response to an obese microenvironment. The expression of these genes was analysed in Barrett's oesophagus biopsies from viscerally obese and non-obese patients, and it was determined that Barrett's metaplasia from viscerally obese patients expressed significantly higher levels of MMP-1 and IL-8, compared to their non-obese counterparts. Activation of pro-metastatic and pro-inflammatory pathways in Barrett's oesophagus by adipose derived factors is a viable mechanism that could underlie the neoplastic progression of Barrett's oesophagus.

MMP-1, TIMP-3, IL-8, MCAM and VEGF-A expression was investigated across a panel of oesophageal cell lines representing each stage of the metaplasia-dysplasia-adenocarcinoma sequence associated with the neoplastic progression of Barrett's oesophagus. Gene expression of 4/5 genes in response to ACM was highest in the HET-1A cell line and subsequently decreased as the Barrett's sequence progressed indicating that obesity mediated early activation of carcinogenic pathways, that ultimately lead to oesophageal adenocarcinoma, may be an event that precedes the onset of Barrett's oesophagus. Culture of the oesophageal cell lines in ACM and leptin, a prominent adipocytokine, also increased proliferative capacities of the cell lines, with greatest proliferation demonstrated later in the Barrett's sequence.

Alterations in telomere length in response to ACM treatment was observed with each stage of the metaplasia-dysplasia-adenocarcinoma sequence, with metaplastic Barrett's oesophagus QH cells displaying the shortest telomere length following ACM treatment. A novel association between obesity, Barrett's oesophagus and the shelterin complex (telomeric chromatin which regulates telomere length) was uncovered during the course of this thesis, with changes in shelterin expression following ACM treatment correlating with telomere length in the oesophageal cell lines. Chromosomal rearrangements that take place early in Barrett's oesophagus due to adipose derived factors may destine the metasplastic Barrett's epithelia to carcinogenesis.

Genomic instability (characterised by anaphase bridging and spindle assembly checkpoint dysregulation) was initiated at each stage of the metaplasia-dysplasia-adenocarcinoma sequence, following ACM treatment. Antibody neutralisation of VEGF and IL-8 from the ACM significantly decrease anaphase bridging in the HET-1A cell line, indicating that these abundant factors present in the ACM may be a critical component the of adipose tissue secretome in promoting anaphase bridging and genomic instability. Aberrant gene expression of members of the spindle assembly checkpoint, a mitotic checkpoint that ensure fidelity of chromosomal replication during anaphase, was also observed following ACM treatment in the panel of oesophageal cell lines.

Acknowledgements

First and foremost, many thanks to my supervisor Dr. Graham Pidgeon for giving me something to do for the last three years and for his guidance and help throughout the course of this project. A special thanks too to Dr. Jacintha O'Sullivan for over-seeing the genomic instability work conducted throughout the final year of my PhD research. I'd also like to thank Prof. John Reynolds for his direction and his surgical team for the adipose samples used over the course of this research. Thanks to Dr. Joanne Lysaght for all her help over the last three years and for the reams of corrections she did for me and thanks to Dr. Stephen Maher (roomie) for his help even though he didn't bother to correct my materials and methods that he's had since August! It would be remiss of me not to thank the CROSS charity for funding this research, so thank you, but you can find someone else to flog raffle tickets/Christmas decorations/ lollipops/ dress up as a Christmas cracker as of October 28th 2011.

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Thanks to my parents, my family and friends in Westport/Galway, who I haven't seen since July.

I could go on ad nauseum, pretty much thanks to everyone I've ever met.

Thanks again.

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Abbreviations

ACM	adipose conditioned media
ADP	adenosine diphosphate
APC/C	anaphase promoting complex/cyclosome
AR1	adiponectin receptor 1
AR2	adiponectin receptor 2
ATP	adenosine triphosphate
BCM	Barrett's conditioned media
BEBM	bronchial epithelial cell basal medium
B-F-B	breakage-fusion-bridge
BMI	body mass index
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta
cDNA	complimentary DNA
CDX	caudal related homeobox
CK	cytokeratin
COX-2	cyclooxygenase
CT	computerised tomography
Ct	cycle threshold
CDC20	cell-division cycle protein 20
CENPE	centromere protein E
DCA	deoxycholic acid
DKC1	dyskeratosis congenital 1
DMEM	Duldecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
ECM	extra cellular matrix

EDTA	ethylene diamine tetra acetic
ELISA	enzyme linked immunosorbant assay
ESPL1	extra spindle pole bodies homolog 1
FCS	foetal calf serum
GORD	gastro-oesophageal reflux disease
HDL	high density lipoprotein
IDF	International Diabetics Federation
IL	interleukin
KGM	keratinocyte growth media
LEPR	leptin receptor
LOH	loss of heterozygosity
M199	medium 199
MAD2L2	mitotic arrest deficient like 2
MCAM	melanoma cell adhesion molecule
MMP	matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MetS	metabolic syndrome
NF-κB	nuclear factor kappa beta
NHEJ	non-homologous end joining
NO	nitric oxide
NSAIDs	nonsteroidal anti-inflammatory drugs
OAD	oesophageal adenocarcinoma
PBS	phosphate buffered saline
PCR	polymerase chain reaction
POT-1	protector of telomeres 1
qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid

ROS	reactive oxygen species
RNase	ribonuclease
RPMI	Roswell park memorial institute medium
RT-PCR	reverse transcription polymerase chain reaction
SAC	spindle assembly checkpoint
SD	standard deviation
SEM	standard error of the mean
SFA	subcutaneous fat area
TEP1	telomerase protein component 1
TERF1	telomeric repeat-binding factor 1
TERF2	telomeric repeat-binding factor 2
TERF2IP	telomeric repeat-binding factor 2 interacting protein
TERT	telomerase reverse transcriptase
TERC	telomerase RNA component
TFF1	trefoil factor 1
TIMP	tissue inhibitor of metalloproteinase
TINF2	TERF1-interacting nuclear factor 2
TNF	tumour necrosis factor
TFA	total fat area
TMA	tissue microarray
TMB	tetramethyl benzidine
TPP1	adrenocortical dysplasia protein homolog
VEGF	vascular endothelial growth factor
VFA	visceral fat area
WC	waist circumference
WHO	world health organisation

Units

Bp	base pairs
^{o}C	degrees Celcius
g	gram
hr	hour
L	litre
M	molar
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
min	minutes
n	number size
ng	nanogram
nl	nanolitre
nM	nanomolar
pg	picogram
RPM	revolutions per minute
s	seconds
T/S	telomere to single copy gene
U	units
w/v	weight per volume
v/v	volume per volume

Publications and Presentations

Publications

Lysaght J., van der Stok E.P., Allott E.H., **Casey R.**, Donohoe C.L., Howard J.M., McGarrigle S.A., Ravi N., Reynolds J.V., Pidgeon G.P. (2011) 'Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue' *Cancer Lett.* Dec 15; 312(1):62-72

Casey R., Ravi N., Reynolds J.V., Pidgeon G.P. 'Pro inflammatory and pro metastatic pathways are activated by obesity in Barrett's metaplasia' (in preparation)

Casey R., Ravi N., Reynolds J.V., Pidgeon G.P., O'Sullivan, J.N. 'Adipose conditioned media promotes telomere shortening in the Barrett's metaplasia-dysplasia-adenocarcinoma disease sequence' (in preparation)

Casey R., Ravi N., Reynolds J.V., Pidgeon G.P., O'Sullivan, J.N. 'Obesity interrupts the regulation of the spindle assembly checkpoint and anaphase bridging in Barrett's oesophagus' (in preparation)

Oral presentations

'Genomic Instability, pro-inflammatory and pro-metastatic pathways are activated by obesity in Barrett's oesophagus' (National Barrett's Symposium, London, UK, April 2011)

'Pro inflammatory and pro metastatic pathways are activated by obesity in Barrett's metaplasia' (Society of Academic Research Surgery annual meeting, Dublin, January 2011)

'Adipose conditioned media from obese oesophagectomy patients activates several key oncogenes in cells derived from pre-malignant Barrett's oesophagus'. (World Organization for Specialized Studies on Diseases of the Oesophagus, Boston, USA, August 2010)

Poster presentations

'Obesity interrupts the regulation of the spindle assembly checkpoint and anaphase bridging in Barrett's oesophagus' (Irish Association of Cancer Research, Cork, March 2011)

'Adipose conditioned media from obese oesophagectomy patients activates several key oncogenes in cells derived from pre-malignant Barrett's oesophagus'. Ranked 2nd place in the poster category, selected for oral presentation. (World Organization for Specialized Studies on Diseases of the Oesophagus, Boston, USA, August 2010) (Irish Association of Cancer Research, Galway, March 2010)

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Chapter 1: General introduction

1.1 Barrett's oesophagus and oesophageal adenocarcinoma

The dominating histological type of oesophageal cancer is squamous cell carcinoma, however the last number of decades have witnessed a significant increase in the incidence of oesophageal adenocarcinoma (OAC), particularly in western countries (Rutegard, Lagergren et al. 2011).

The incidence of oesophageal adenocarcinoma is rising at a rate 5-10% per annum, greater than any other solid malignancy in the western world (O'Connor, Falk et al. 1999; Shaheen and Ransohoff 2002; Ryan, Rowley et al. 2006; Shaheen and Richter 2009). Many factors have been associated with this increase, including increased rates of obesity. In recent decades, people in the developed world have been leading more sedentary lifestyles due to advances in technology, and rates of obesity have risen hand in hand with incidences of all cancers (Hjartaker, Langseth et al. 2008). There is a substantial mortality rate associated with OAC, due to the fact that only 25% of cases are operable due to the late onset of symptoms and presentation at the clinic when cases are usually advanced (Dibb and Ang 2011). For the majority of these cases, the 5 years survival is only approximately 20% (Jankowski, Wright et al. 1999). There are multiple well defined risk factors associated with the development of OAC in addition to obesity. Caucasian ethnicity (Ye, Wang et al. 2008), male sex (Gillen, Feith et al. 2009), chronic gastro oesophageal reflux disease (GORD) (di Pietro, Peters et al. 2008), eradication of *Helicobacter pylori* from the gut (Queiroz, Guerra et al. 2004), and crucially, the presence of the pre-malignant lesion Barrett's oesophagus (Pace 2006), are all associated with the onset of OAC.

Barrett's oesophagus is a metaplastic condition in which the squamous cells, native to the oesophagus, are replaced with intestinal type columnar epithelial cells, following long term exposure to acid reflux (Jenkins, Doak et al. 2002), although no universal accepted definition for the condition currently exists (Reid, Li et al. 2010). This disease was first described by British surgeon Norman Barrett in 1950 (Barrett 1950) and was definitively linked with oesophageal adenocarcinoma in 1975 (Naef, Savary et al. 1975). Patients with Barrett's oesophagus have a 30-125 fold increased risk of developing OAC compared to the general public and also carry a 2-5% lifetime risk of their condition progressing to OAC (Jankowski, Wright et al. 1999). It is important to note that most patients suffering from Barrett's oesophagus are actually undiagnosed, with up to 90% of patients presenting with advanced OAC likely to have undiagnosed Barrett's oesophagus (Bird-Lieberman and Fitzgerald 2008; Bozio, Baulieux et al. 2011). A greater understanding of the molecular mechanisms behind the progression of Barrett's oesophagus to OAC is crucial as is argued that Barrett's oesophagus is currently the only clinically useful predicator of OAC (Armstrong 2008), as other risk factors such as GORD, smoking and obesity, are too common to be clinically useful.

Short-segment Barrett's oesophagus (SSBE), Barrett's metaplasia less than 3 cm in length, is found in 8–20% of adults, making it more prevalent than long-segment Barrett's oesophagus (LSBE) (1% adult prevalence) (Jankowski, Wright et al. 1999). Long segment Barrett's oesophagus is associated with an increased risk of the neoplastic progression of Barrett's oesophagus (Sharma, Morales et al. 1997)

The presence Barrett's oesophagus is typically identified via endoscopy by the presence of a pink salmon mucosa (Jankowski, Wright et al. 1999) and once established, this metaplasia appears to be irreversible without ablative treatments (Barr, Shepherd et al. 1996). Endoscopies displaying a healthy oesophagus, Barrett's oesophagus and invasive oesophageal adenocarcinoma are presented below in Figure 1.1.

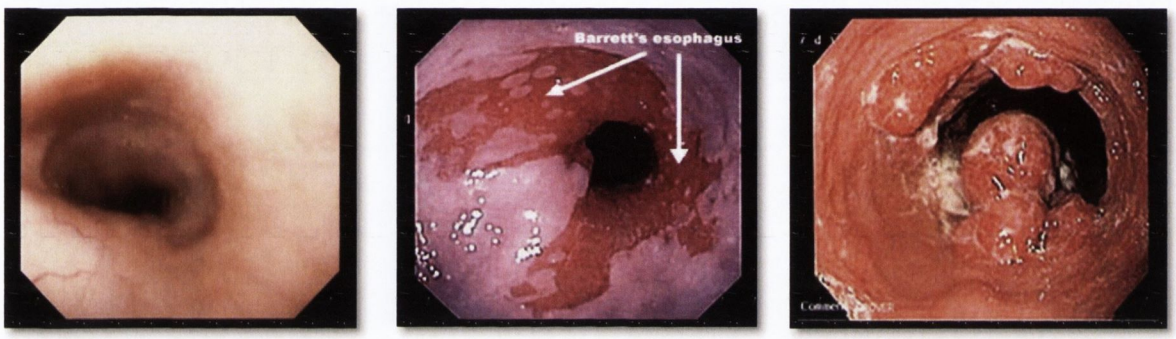

Figure 1.1. Representative images displaying (from left to right), healthy squamous oesophageal epithelium, Barrett's oesophagus, and invasive oesophageal adenocarcinoma. Taken from http://www.synecticsmedical.com/more.php?cat=7

1.1.1 Gastro-oesophageal reflux disease and Barrett's oesophagus

Barrett's oesophagus develops as a defence mechanism to long term exposure to (GORD). The exact aetiology for the metaplasia of the squamous cells to intestinal type epithelium has yet to be fully elucidated, but it is believed that GORD is heavily involved. It is generally understood that after insult from the components of the gastro-oesophageal refluxate, stem cells of squamous mucosa undergo an altered differentiation during repair, and the cells begin to display traits atypical from mucosal gastric stem cells (Jankowski, Wright et al. 1999). It is not known why only a minority of patients with GORD will develop Barrett's oesophagus though in recent years studies have suggested that key differences exist in the molecular mechanisms of repair in oesophageal squamous cells, determining whether the cells heal by squamous cell regeneration or through metaplasia, after insult by gastric oesophagitis. However, although GORD does seem to be crucial in the pathogenesis of Barrett's oesophagus and the subsequent progression to OAC (Lagergren, Bergstrom et al. 1999), only 10-15% of long term GORD patients will actually develop Barrett's oesophagus, and in those patients that do, only a subset progress to OAC (Edelstein, Farrow et al. 2007). Reflux symptoms themselves can prove a poor predicator of Barrett's oesophagus. In a recent endoscopic study of 1033 adults from 2

Italian villages, 46.2% of patients who presented with Barrett's oesophagus were asymptomatic (Zagari, Fuccio et al. 2008). Clinical and pathophysiological evidence indicates greater resistance of metaplastic Barrett's epithelium to reflux injury than normal squamous epithelium, and patients with Barrett's oesophagus usually lack reflux symptoms (Ostrowski, Mikula et al. 2007).

There is some evidence to support the hypothesis that there is a genetic aspect to the pathogenesis of Barrett's oesophagus and oesophageal adenocarcinoma. A case study of one family in the United Kingdom reports 3 brothers who suffered from oesophageal adenocarcinoma or high grade dysplasia, along with 6 children in the family who all presented with Barrett's oesophagus (Groves, Jankowski et al. 2005). High risk of malignancy in familial Barrett's oesophagus was also seen in a family in Spain. In this 24 member family, spanning 3 generations, 6 suffer from GORD, 4 from Barrett's oesophagus and there were a further 6 who were diagnosed with oesophageal adenocarcinoma (Munitiz, Parrilla et al. 2008).

GORD is relatively common in both sexes and across all ethnicities, but the risk of developing Barrett's oesophagus and the subsequent progression to OAC is far greater in older men of Caucasian ethnicity compared to any other group (Mitas, Almeida et al. 2005). This finding suggests that other factors besides GORD are involved in the pathogenesis of Barrett's oesophagus , which has led to the investigation of environmental factors such as obesity (Ryan, Healy et al. 2008) and molecular factors such as the activity of micro-RNAs (Yang, Gu et al. 2009).

1.1.2 Histologic characteristics of Barrett's oesophagus

Barrett's metaplasia secretes thick adherent mucus not present in normal squamous oesophageal cells (Allen, Dixon et al. 2001). The metaplastic columnar epithelium is composed of mucinous columnar epithelial cells on the surface and in crypt epithelia along with a mosaic of different cell types including goblet cells, enterocytes, paneth cells, endocrine cells and cells which display combined features of gastric, intestinal and squamous cells (Shields, Zwas et al. 1993) (Figure 1.2). The number of goblet cells present in a particular Barrett's epithelium varies according to patient age, the length of the lesion and the presence of dysplasia and neoplasia (Harrison, Perry et al. 2007). Goblet cells are more numerous in proximal regions of the Barrett's epithelia and are found in the crypt and at the surface of the affected oesophagus (Chandrasoma, Der et al. 2001). In areas of inflammation the epithelium shows mucin depletion, increased hyperchromicity and size of cell nuclei and cell stratification (Odze 2009).

A common feature of Barrett's mucosa is the presence of mucosal and submucosal mucous glands and their ducts, which can help pathologists identify biopsies as oesophageal in origin when the exact location (oesophageal versus gastric) is difficult to determine clinically (Srivastava, Odze et al.

2007).Architectural irregularities of the crypts are commonplace in the affected oesophagus; crypts frequently display a mild degree of budding, irregularity and branching —features often pronounced in areas of prominent goblet-cell metaplasia (Odze 2006).

Neovascularisation leading to increased number and size of thin-walled blood vessels are typically present in Barrett oesophagus segments, giving the lesion its ominous red colour (Auvinen, Sihvo et al. 2002). Other mesenchymal changes that occur in the affected oesophagus include splaying and duplication of the muscularis mucosa, in which the new (superficial) layer of muscularis mucosa attaches to the bases of the oesophageal crypts or glands (Hahn, Shahsafaei et al. 2008). Identification of the presence of a double muscularis mucosa is clinically and pathologically important, because tumours that penetrate through the new muscularis mucosa are still considered to be 'intramucosal' in relation to the risk of metastasis and subsequent treatment (Odze 2009).

Over 80% of cases of Barrett's oesophagus consist of specialised columnar epithelium with intestinal metaplasia and characteristic goblet cells. In the remaining cases, only cardia-type or gastric fundic-type is found (Guillem 2005). As only columnar epithelium with intestinal metaplasia predisposes Barrett's patients to adenocarcinoma (Hameeteman, Tytgat et al. 1989) a generally accepted definition of Barrett's oesophagus is the presence of metaplastic epithelium with goblet cells in the oesophagus, regardless of the length of the segment (Weinstein and Ippoliti 1996). The exact mechanisms which underlie Barrett's metaplasia are unknown but there is much debate in the literature to the nature of the progenitor cell from which Barrett's oesophagus arises.

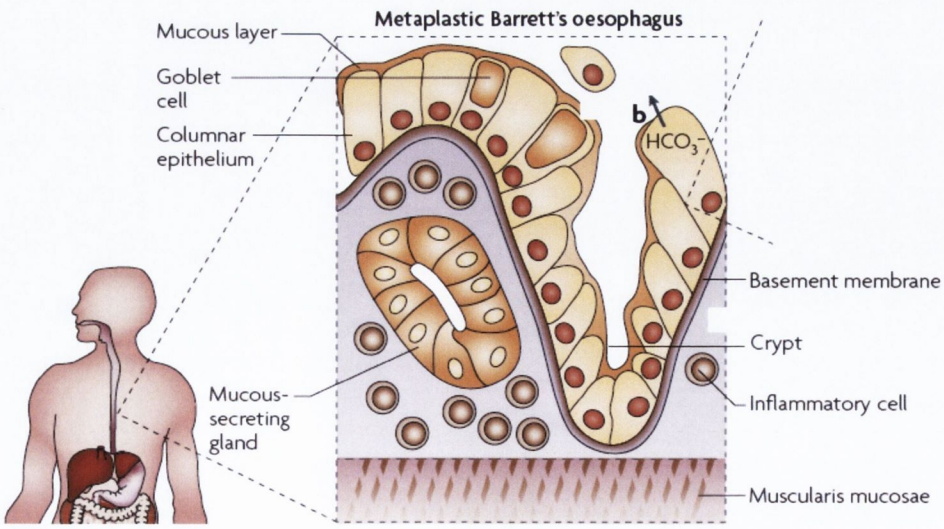

Figure 1.2. Barrett's specialised intestinal metaplasia (Reid, Li et al. 2010)

1.1.3 Progenitor cell of Barrett's oesophagus

The cellular and mechanistic processes behind the metaplastic transformation that occurs during Barrett's oesophagus are still poorly understood. Originally, it was believed that Barrett's oesophagus arose due to the upward cell migration of cells from the gastro-oesophageal junction and that these cells migrated and colonised the lower oesophagus in response to tissue damage from prolonged insult from GORD (Barbera and Fitzgerald 2010). Further studies investigating the distribution of oesophageal and gastric cardiac mucosae in oesophagectomy specimens suggested that mucus secreting cardiac glands exposed to the luminal surface become columnar epithelial islands that would clonally expand and give rise to Barrett's oesophagus (Nakanishi, Saka et al. 2007). However, even if the upward migration of gastric cells was a result of prolonged exposure to GORD, the various epithelial cell lineages in Barrett's oesophagus still need to be accounted for (Barbera and Fitzgerald 2010).

Aberrant differentiation of multi-potent stem cells in the squamous oesophagus, initiated by continual environmental stresses such as GORD and obesity, is another proposed mechanism by which Barrett's oesophagus arises. Tissue specific multi-potent stem cells have been associated with mechanisms underlying carcinogenesis (Pardal, Clarke et al. 2003) The stem cell theory would explain the clonal heterogeneity of Barrett's oesophagus (Robaszkiewicz, Volant et al. 1992), as well as providing a rationale for the regeneration of the squamous oesophageal epithelium after removal of Barrett's oesophagus via photothermal laser ablation (Barham, Jones et al. 1997). It should also be remembered that more than one population of progenitor cells could be present in the human oesophageal tissue, suggesting that the cellular origin of Barrett's oesophagus could be multiple (Barbera and Fitzgerald 2010).

Stem cell populations not originating in the oesophageal epithelia have also been suggested as a source of the Barrett's progenitor cell. Stem cells located in the glandular neck region of the oesophageal submucosal gland ducts are lined in their proximal two thirds by columnar cells and it has been suggested that these ducts are a source of the stem cells responsible for the origin of the columnar epithelium (Ahnen, Poulsom et al. 1994). A p16 mutation found in both the submucosal gland duct and adjacent Barrett's epithelia suggests a mutual genetic origin for these cells (Leedham, Preston et al. 2008). It has also been suggested that the hypothetical Barrett's oesophagus cell originates from bone marrow derived stem cells. In a study conducted by Sarosi *et al,* female rats were inoculated with bone marrow from male rats following an oesophagojejunostomy to stimulate GORD in the animals. Subsequent FISH analyses of the resected oesophagi showed that some of the

squamous epithelial cells and some of the columnar epithelial cells lining the glands of the intestinal metaplasia were positive for Y chromosome suggesting that multi-potential progenitor cells of bone marrow origin contribute to oesophageal regeneration and metaplasia in this rat model of Barrett's oesophagus (Sarosi, Brown et al. 2008).

Recent research has suggested residual embryonic cells as precursors of Barrett's oesophagus. Early in human embryogenesis, a single hollow endodermal tube gives rise to the respiratory tract and the oesophagus. The epithelium of this endodermal tube consists of two or three layers, each of which arise from a common progenitor which cell which expresses p63, a member of the p53 family (Daniely, Liao et al. 2004). As the endodermal tube matures during embryogenesis, the p63+ progenitor cells differentiate into columnar cells that do not express this marker. At approximately week 17 of development, squamous epithelium first appears in the mid-oesophagus and extends proximally and distally to replace the columnar epithelium (Johns 1952). Cells in the basal layer of stratified oesophageal squamous epithelium demonstrate expression of p63, whereas fully differentiated squamous cells in the upper layers show no p63 expression (Souza, Krishnan et al. 2008). A discrete population of these cells persist in mice and in humans and upon programmed damage to the squamous epithelium, these embryonic stem cells migrate towards specialised squamous cells in a process which may replicate early Barrett's oesophagus (Wang, Ouyang et al. 2011). This data in the literature suggests that embryonic oesophageal stem cells that express p63 have the capacity to give rise to both columnar and squamous epithelial cells and may be involved in the pathogenesis of Barrett's oesophagus.

1.1.4 Molecular events underlying the neoplastic progression of Barrett's oesophagus

The progression of Barrett's oesophagus to OAC follows a distinct metaplasia-dysplasia-adenocarcinoma sequence (Figure 1.3) with an accumulation of genetic traits conducive to carcinogenesis (Jankowski, Wright et al. 1999). Perhaps one of the earliest molecular events is the selection and propagation of the metaplastic clones with specialized intestinal metaplasia. Subsequently, loss of cell cycle check points and initiation of genomic instability may contribute to slow clonal expansion potentially through increased proliferation.

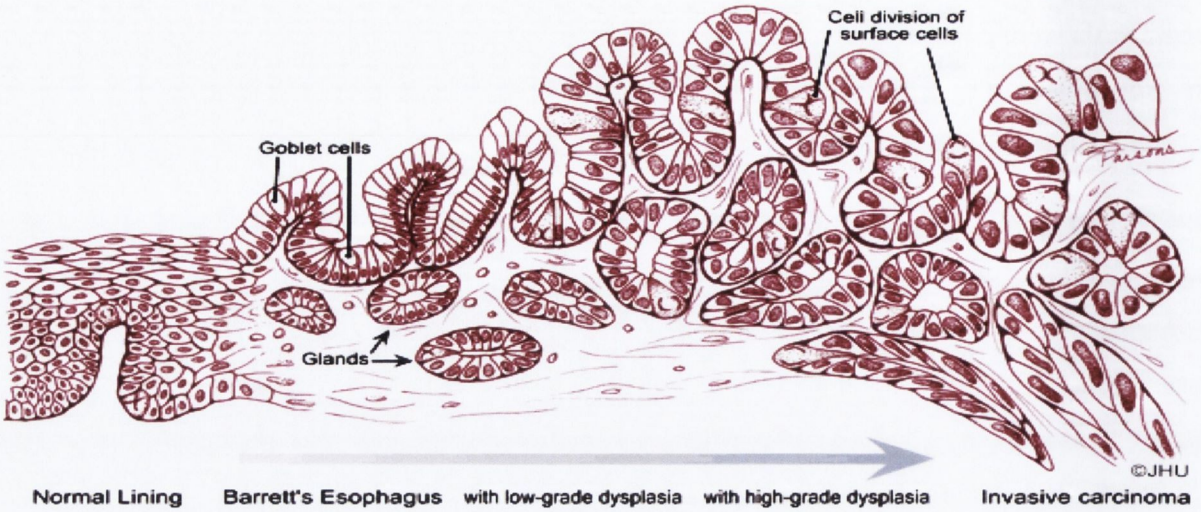

Figure 1.3. Pathological evolution displaying the metaplasia-dysplasia-adenocarcinoma sequence that characterises the pathogenesis and neoplastic progression of Barrett's oesophagus (taken from http://pathology2.jhu.edu/beweb/definition.cfm.

In contrast to healthy cells, Barrett's epithelia show evidence of a higher basal proliferative rate and a significantly reduced apoptotic index (Ogunwobi, Mutungi et al. 2006). The BCL-2 proto-oncogene, a gene which encodes for an anti-apoptotic protein, shows increased expression in reflux oesophagitis, non-dysplastic Barrett's oesophagus and low grade dysplastic Barrett's oesophagus inhibiting apoptosis at these disease stages, however expression is significantly lower in high grade dysplasia and invasive carcinoma (Wijnhoven, Tilanus et al. 2001). Elongation of cell survival by BCL-2 over expression early in the Barrett's sequence may promote carcinogenesis later in the sequence by allowing cells time to develop other ways to avoid the apoptosis.

Genomic instability appears to be associated with neoplastic progression, and oesophageal adenocarcinoma arising from Barrett's oesophagus is no exception. The literature suggests most cases of oesophageal adenocarcinoma arise in patients who show a gain or loss of large portions of chromosomes (or indeed whole chromosomes) (Reid, Li et al. 2010). Spatial data from Barrett's biopsies have shown that loss of heterozygosity (LOH) at 9p is an early event in Barrett's oesophagus, followed by 17p LOH and the mutation of the TP53 gene, along with DNA content aberrations (Barrett, Sanchez et al. 1999; Maley, Galipeau et al. 2004; Reid, Li et al. 2010). The mutant p53 protein has a prolonged half-life making intracellular p53 staining possible, with increased levels of intracellular dysfunctional p53 reported during the metaplasia-dysplasia-adenocarcinoma sequence (Rice, Goldblum et al. 1994).

Two independent studies show that methylation of the p16 promoter (with or without p16 loss of heterozygosity) is a common mechanism of p16 inactivation in dysplasia and adenocarcinoma arising

in Barrett's oesophagus (Wong, Barrett et al. 1997; Klump, Hsieh et al. 1998). Inactivation of this gene may be a worthwhile biomarker in predicting the risk of the neoplastic progression of Barrett's oesophagus. Accumulation of abnormal retinoblastoma protein in tandem with a significant overexpression of the apoptotic Fas protein during the progression of Barrett's metaplasia to carcinoma has also been reported (Karl, Coppola et al. 1999).

Most genetic changes that occur during the neoplastic progression of Barrett's oesophagus are involved with genes that regulate the cell cycle, leading to unchecked cellular proliferation (Flejou 2005). Studies investigating proliferating cell nuclear antigen (PCNA) and Ki67, two markers of cell proliferation, have proven useful in investigating cell cycle irregularities in Barrett's oesophagus. PCNA is an indicator of cell cycle progression at the G1/S transition, and Ki67 is expressed in proliferating cells (G1, S, G2, and M phases). Studies have reported an elevated number of cells stained by both antibodies parallel to the progression of histological changes from metaplasia to increasing grades of dysplasia (Flejou 2005).

Another reported molecular event in the progressive Barrett's sequence is the loss of cell adhesion. The E-cadherin-catenin complex is the key mediator of calcium-dependent cell–cell adhesion in healthy epithelial cells and is vital for the maintenance of adult tissue architecture (Bailey, Biddlestone et al. 1998). In non-malignant epithelia, E-cadherin and the catenins show a membranous localization at intercellular borders, reduced expression of these genes are reported in 60-80% of oesophageal adenocarcinomas (Krishnadath, Tilanus et al. 1997). Reduced expression of the E-cadherin-catenin complex was also shown to correlate with greater degrees of dysplasia and disease progression (Bailey, Biddlestone et al. 1998).

Increasing levels of telomerase RNA expression, a gene which acts to maintain telomere length and allows the cells to escape cellular senescence are reported accompanies the transition of metaplastic Barrett's tissue to high and low grade dysplasia and to oesophageal adenocarcinoma (Shay, Morales et al. 1998).

1.2 Obesity and cancer

Obesity is largely a consequence of over-nutrition and under-activity (Lawlor and Chaturvedi 2006) and is associated with a myriad of co-morbidities, including type II diabetes, coronary artery disease, obstructive sleep apnea, stroke, hypertension, osteoarthritis, and liver and biliary disease which collectively increase mortality (Valentino, Terzic et al. 2010). Since the late 70s, it has been documented that excess weight and obesity are major risk factors for the development and progression of many cancers (Calle, Rodriguez et al. 2003; Birmingham, Busik et al. 2009; Kim, Parks et al. 2009). Studies conducted by other groups have shown a clear association between obesity and incidences of cancers of the endometrium (Schindler 2009), kidney (Spyridopoulos, Petridou et al.

2009), gall bladder (Pan and DesMeules 2009), breast (Maruthur, Bolen et al. 2009), colon (Larsson and Wolk 2007), and oesophageal adenocarcinoma (Calle, Rodriguez et al. 2003). Of all cancers, oesophageal adenocarcinoma displays one of the strongest links with obesity (Renehan, Tyson et al. 2008). Overall, cancer death rates in men and women with BMIs >40kg/m^2 are 52% and 62% higher than in those in healthy weight ranges (Calle, Rodriguez et al. 2003). 14% of all cancers in men and 20% of all cancer in women over the age of 50 are directly attributable to obesity. Currently, smoking is the single leading risk factor for the development of cancer, however the WHO report of 2008 forecasts that by the year 2020, obesity will be the leading risk factor for cancer [http://www.who.int/cancer/publications/en/]. The increased incidence of obesity in the western world, may provide a rationalization for the rapidly increasing OAC rates we are seeing today (Corley 2007). The Republic of Ireland has seen a substantial increase in the prevalence of obesity since the early 1990s with 67% of men and 75% of women over the age of 51 classified as overweight or obese (McCarthy, Gibney et al. 2002), during which time incidences of oesophageal adenocarcinoma increased by 38% (Ryan, Rowley et al. 2006). Obesity rates in Ireland are amongst the highest in Europe with 23% of all adults in Ireland deemed obese, markedly higher than the European average of 15.5%, according to a study compiled in 2008 (Figure 1.4).

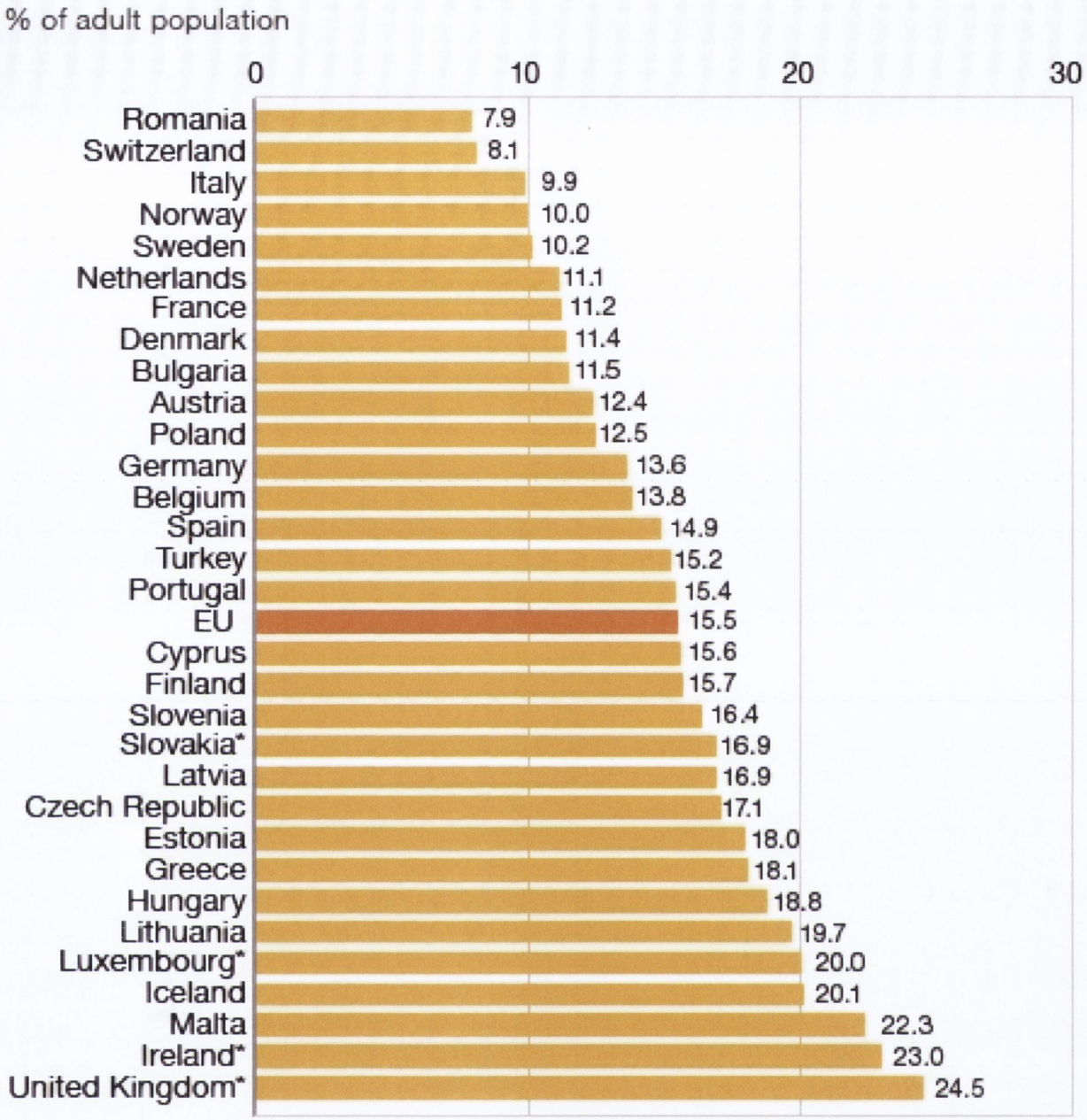

Figure 1.4. Obesity rates among adult population in Europe in 2008 (Source :OECD Health Data 2010)

1.2.1 Measuring obesity

Body mass index (BMI) is the classic measurement of obesity and the rationale for using BMI in determining obesity status is that it strongly correlates with hypertension, diabetes and overall mortality, and is also simple to calculate and inexpensive (Aronne and Segal 2002). It is calculated by dividing a patients mass by their height squared (kg/m^2).

Table 1.1. Classification of obesity status by body mass index (BMI) (Donohoe, Pidgeon et al. 2010)

Obesity Status	BMI (kg/m^2)
Underweight	≤18.5
Normal weight	18.5-22.5
Overweight	25-30
Obese class I	30-35
Obese class II	35-40
Obese class III	≥40

However, this anthropometric measurement does not take into account the composition of body mass or the distribution of adipose tissue in the body. BMI is a measure of overall adiposity and cannot distinguish between fat mass and lean body mass. Thus, it is inappropriate to use BMI as a measure of fatness in people who are very muscular (Hu 2007).

Visceral or abdominal fat shows a greater correlation with mortalities rather than just obesity alone (Eckel, Grundy et al. 2005) and it is this adipose tissue depot that is related to Barrett's oesophagus and oesophageal adenocarcinoma (Beddy, Howard et al. 2010). The cross sectional surface area of the visceral fat depot is measured by computerised tomography (CT) between vertebrae L3 and L4 to determine the visceral fat area (cm^2) (Figure 1.5). VFA calculations are considered the gold standard in measurement of visceral adiposity (Oka, Kobayashi et al. 2008; Hoenig, Cowin et al. 2010) with patients with a VFA of ≥130cm^2 considered viscerally obese (Beddy, Howard et al. 2010).

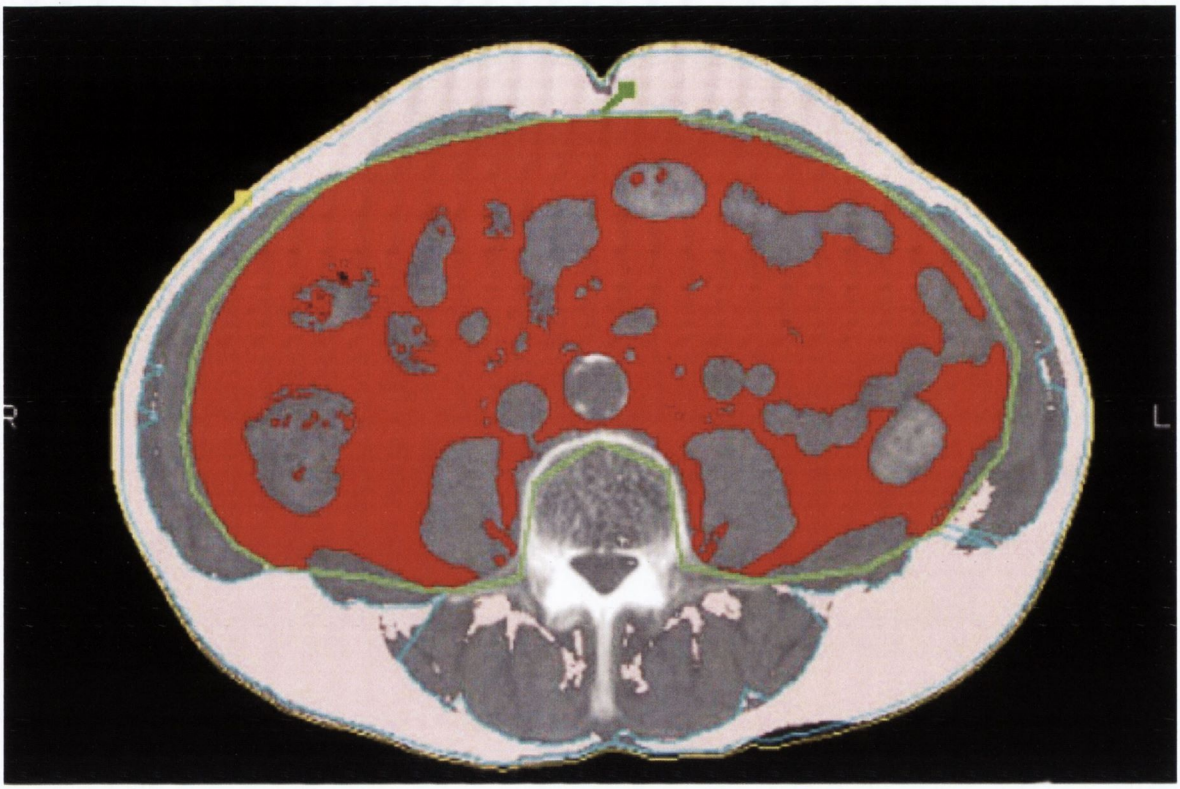

Figure 1.5. Representative CT scan displaying patient visceral fat area (red). The subcutaneous fat is displayed in the scan in pink (Makino, Kunisaki et al. 2008). The area inside the yellow line is the total fat area (TFA). The area inside the green line is the visceral fat area (VFA).

In the absence of suitable CT scanning facilities, patient waist circumference is a simple and reliable method for determining abdominal adiposity and correlates strongly with VFA, especially in men (Miyawaki, Abe et al. 2004). Determination of waist circumference requires only a tape measure, however it is difficult to standardize between patients as different studies measure the waist circumference at different locations (eg., at the level of the umbilicus, midway between lower rib and iliac crest, and at the right iliac crest) (Hu 2007). Waist circumference is reported to be a stronger predictor of life time risk of colon cancer than BMI (Moore, Bradlee et al. 2004), waist circumference has also demonstrated correlations with the presence of Barrett's oesophagus, correlating better with risk of developing Barrett's oesophagus than BMI (Corley, Kubo et al. 2007). A waist circumference cut off point of ≥94cm in male patients was used to differentiate between obese and non-obese patients in this thesis. This measurement was proposed by the International Diabetes Federation (IDF) (Alberti, Zimmet et al. 2005).

1.2.2 Obesity, Barrett's oesophagus and oesophageal adenocarcinoma

There is evidence in the literature to suggest that fat is metabolically active (Gutierrez, Puglisi et al. 2009) and this activity can pose a considerable cancer risk, particularly in OAC (Chow, Blaser et al. 1998). Visceral adipose tissue has been associated with increased serum levels of both IL-6 and TNFα (Cannon, Nerad et al. 1993; Xu, Barnes et al. 2003), two potent pro-inflammatory adipocytokines. Interestingly, adipose tissue may also lead to genetic instability in Barrett's oesophagus patients. Increasing waist: hip ratio seems to be associated with an increased risk of cell cycle and genetic abnormalities in patients with Barrett's oesophagus (Vaughan, Kristal et al. 2002).

A healthy diet and dietary intake of certain key vitamins have been associated with the prevention of Barrett's oesophagus progression to OAC. Vitamin C is an important scavenger molecule, protecting the body from oxidative stress and cancer arising from the damage to DNA molecules (Abdel-Latif, Kelleher et al. 2008). Reduced vitamin C plasma levels are observed in patients suffering from Barrett's oesophagus, compared with healthy controls (Fountoulakis, Martin et al. 2004).

Adipose tissue evolved over the course of evolution to act as an energy store for triglycerides (Gealekman, Guseva et al. 2011). Adipose tissue was initially found surrounding the viscera in the peritoneal cavity, but later in evolution, major depots of adipose tissue were located in subcutaneous sites. Development of large subcutaneous adipose tissue depots permits the compartmentalization of lipids, which prevents their build up in other tissues. Accumulation of lipids in non-adipose tissue is associated with co-morbidities such as insulin resistance, type II diabetes mellitus and cardiovascular disease (van Herpen and Schrauwen-Hinderling 2008).

There is increasing evidence that the location of adipose tissue contributes to obesity-related disease risk independent of overall adiposity (Hu 2007). The specific location of adipose tissue has been used to define two primary body shapes; gynecoid (pear shaped) where the adipose tissue is stored in the lower part of the body in the hips and the thighs and android (apple shaped) where adipose tissue is stored centrally in the abdomen (Figure 1.6). Visceral adipose tissue, adipose tissue that is stored in the abdomen, is more associated with male sex (Lemieux, Prud'homme et al. 1993) and male sex in turn shows a greater correlation with obesity related mortalities such as oesophageal adenocarcinoma compared to female sex (Lagergren 2011). Visceral adipose tissue also shows a strong correlation with GORD, the primary risk factor for Barrett's oesophagus and oesophageal adenocarcinoma (Lagergren, Bergstrom et al. 1999) .

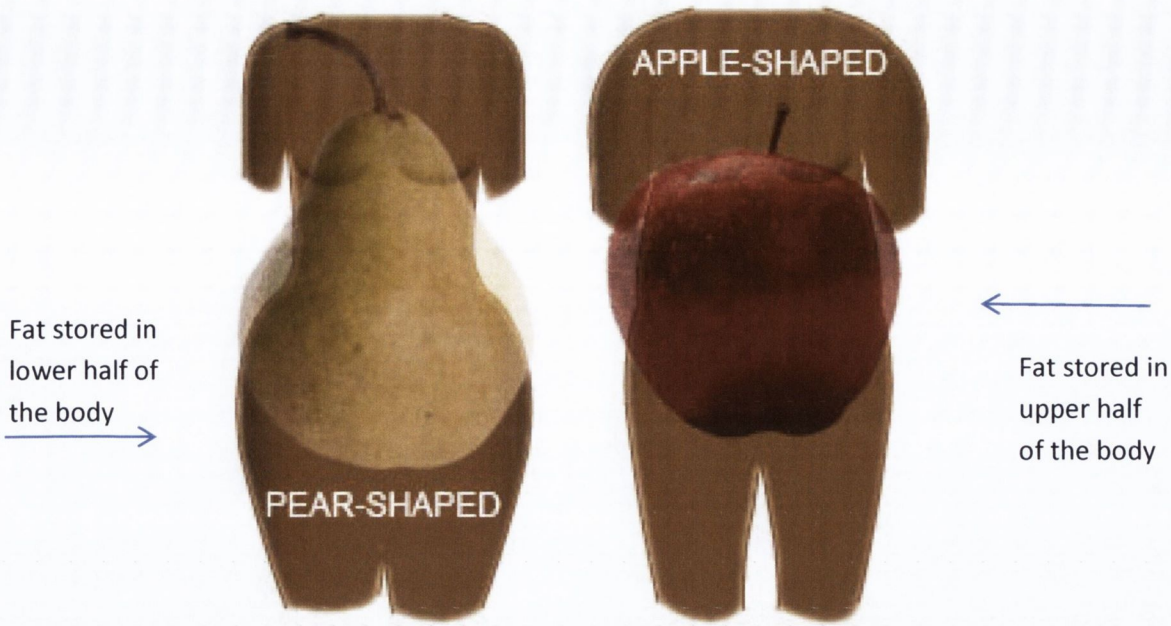

Figure 1.6. Typical distribution of body fat in men and women (taken from http://www.fitcommerce.com)

The risk of oesophageal adenocarcinoma development among patients with Barrett's oesophagus is equally distributed between the sexes (Solaymani-Dodaran, Logan et al. 2004), while prevalence of Barrett's oesophagus seems to be nearly doubled in men (Cook, Wild et al. 2005). This sex difference was found to be age-dependent, with an incidence four times greater in young and middle-aged men than in women (Rutegard, Lagergren et al. 2011) while overweight and obesity are slightly more frequent in men in Western societies. In a study conducted by our research department, it was noted that obese men had four times the risk of incident adenocarcinoma than lean men, while no such association was found in women (Ryan, Rowley et al. 2006). These findings might explain the predominance of oesophageal adenocarcinoma in men, which has a ratio of incidence of 7:1 between men and women (Lagergren 2011).

1.2.3 Metabolic syndrome and Barrett's oesophagus

The metabolic syndrome is a term used to describe a myriad of medical disorders associated with visceral obesity, blood lipid disorders, insulin resistance and Type II Diabetes Mellitus, and increased risk of cardiovascular disease (Despres and Lemieux 2006), while in recent years correlations have been made between the metabolic syndrome and various forms of cancer (Giovannucci 2007; Ryan, Duong et al. 2011). Various definitions of the metabolic syndrome have been adopted by different health authorities and in an attempt to unify criteria a joint scientific statement titled 'Harmonizing the Metabolic System' was issued which represents the outcome of a meeting between several

major organizations. It was agreed that there should not be an obligatory component, but that waist measurement would continue to be a useful preliminary screening tool (Alberti, Eckel et al. 2009).

Table 1.2. Definition of the metabolic syndrome

Clinical Measure	WHO[1] (1998)	EGIR[2] (1999)	ATP III[3] (2001)	IDF[4] (2005)	Harmonizing statement
	Impaired glucose regulation or hyperinsulinaemia plus any 2 of:	Insulin resistance plus 2 of:	3 or more of:	Waist circumference and plus any 2 of :	3 or more of:
Central Obesity	Men: WC>94cm Women: WC>80cm	Men: WC>94cm Women: WC>80cm	Men: WC≥102cm Women: WC≥88cm	Men: WC≥94cm Women:WC≥80cm WC ethnic specific differences	Elevated WC ethnic specific differences
Impaired glucose regulation	Fasting plasma ≥6.1mmol/L or 2hour post glucose load ≥7.8mmol/L	Fasting plasma ≥6.1mmol/L	6.1-7.0mmol/L	Fasting plasma ≥ 5.6mmol/L or previously diagnosed T2DM	Fasting plasma ≥ 5.6mmol/L or previously diagnosed T2DM
Hyperinsulinae-mia	Fasting serum insulin> third quartile for control group	Fasting serum insulin> third quartile for nondiabetic control group	Not included	Not included	Not included
Triglycerides	≥1.7mmol/l	≥2.0 mmol/L and /or HDL <1.0 mmol/l and/or treatment for dyslipidaemia	≥ 1.7 mmol/L	≥ 1.7mmol/L	≥ 1.7mmol/L
HDL concentration	Not included	Included with triglycerides	Men: < 1.04mmol/L Women: < 1.3mmol/L	Men: <1.03mmol/L Women: <1.29mmol/L	Men: <1.03mmol/L Women: <1.29mmol/L
Hypertension	≥140/90 mmHg	≥140/≥90 mmHg or treatment for same	≥130/≥80 mmHg	≥130/≥85 mmHg or treatment for same	≥130/≥85 mmHg or treatment for same
Microalbuminu-ria	Albumin/creatinine ratio >30mg/g	Not included	Not included	Not included	Not included

Key. WHO: World Health Organisation, EGIR: European Group for the Study of Insulin Resistance, ATPIII: Adult Treatment Panel III, IDF: International Diabetes Federation.

One key report in the literature has demonstrated an association between the metabolic syndrome and length of the Barrett's oesophagus segment (Ryan, Healy et al. 2008). In a study of 102 Barrett's oesophagus patients, 46% of patients had metabolic syndrome while 78% were centrally obese. Incidence of metabolic syndrome and central obesity were increased to 60% and 92% respectively, in patients with long segment Barrett's oesophagus obese compared with 23.8% and 62%, respectively in patients with short segment Barrett's (Figure 1.7). Long segment Barrett's oesophagus was also associated with hyper-insulinaemia and significantly increased levels of IL-6 compared with short segment Barrett's. The study postulated that metabolic syndrome may

potentially drive increased neoplastic progression of Barrett's oesophagus via increased length of the Barrett's oesophagus segment.

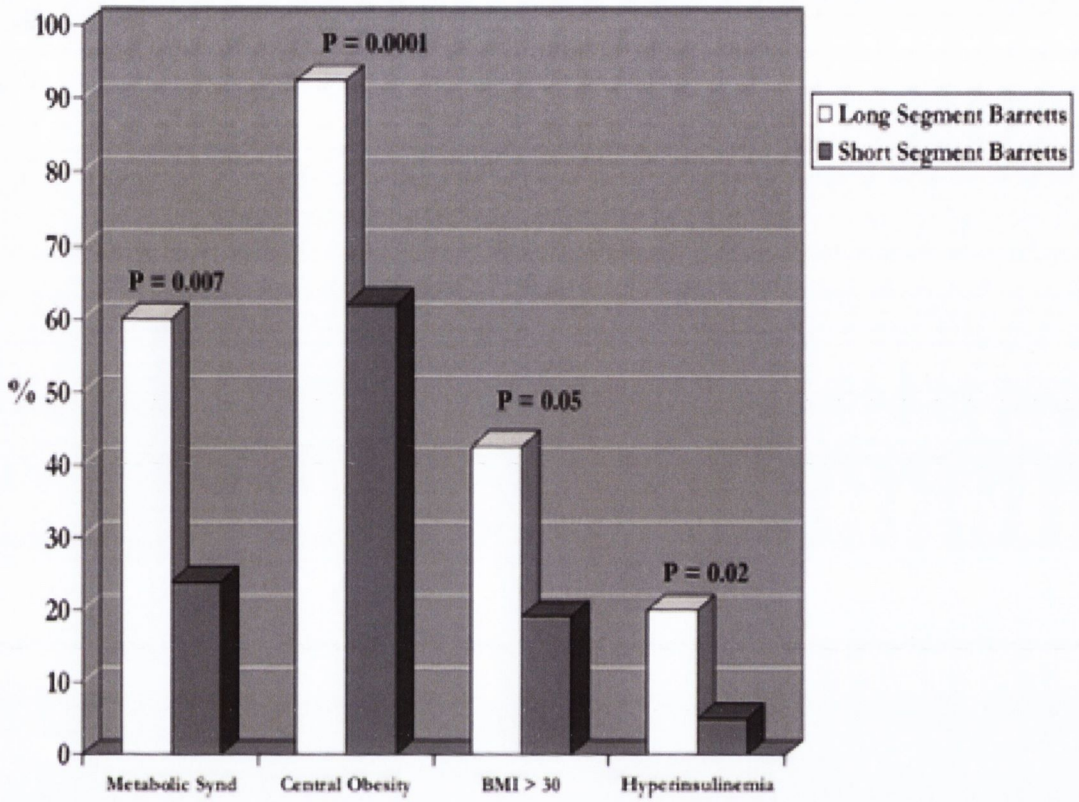

Figure 1.7. Long-segment Barrett (>3 cm) versus short-segment Barrett (<3 cm): features of the metabolic syndrome and anthropometry (Ryan, Healy et al. 2008)

1.2.4 Adipose derived hormones and obesity

Hormones associated with inflammation, healing and cell growth are all understood to be influenced by obesity (visceral fat in particular) and may represent GORD independent mechanisms in the development of oesophageal cancer from Barrett's oesophagus (Corley 2007). Indeed, certain molecules, such as adiponectin and leptin are produced by adipose tissue and can play major roles in signalling in the body (Zamboni, Di Francesco et al. 2007; Gandhi, Upaganlawar et al. 2010) (Figure 1.8).

1.2.4.1 Leptin

Leptin, a 16KDa protein, encoded by the *ob* gene, is synthesised and excreted principally by adipose tissue and is primarily involved with appetite regulation and energy expenditure (Yadav, Oury et al. 2011) , with levels of production directly proportional to obesity and BMI (Martin, Qasim et al. 2008). Leptin activity is modulated by the binding of leptin to one of six different receptors, all

spliced from the leptin receptor gene (Lee, Proenca et al. 1996). Leptin has many different functions in vivo, and has been implicated in wound healing, the immune system, renal and lung functions (Horiguchi, Sumitomo et al. 2006), angiogenesis (Sierra-Honigmann, Nath et al. 1998), and coronary atherosclerosis in type 2 diabetes (Reilly, Iqbal et al. 2004). Leptin has been shown by numerous researchers to act as a growth factor and has proliferative effects on a host of cell lines including breast, colon, pancreatic β and kidney cells (Ishikawa, Kitayama et al. 2004). However, leptin can have anti proliferative roles in certain cases, as evidenced by research carried in T24 bladder cancer cells (Yuan, Chung et al. 2004). Leptin is thought to be involved in inflammation due to its regulation of components of both the innate and adaptive immunity; both monocytes and lymphocytes can be regulated by leptin (Martin, Qasim et al. 2008). Leptin displays structural and functional similarities to pro inflammatory cytokine IL-6 (Zhang, Basinski et al. 1997), and may control C-reactive protein, a common marker of inflammation (Chen, Li et al. 2006).

Research indicates that leptin stimulates proliferation and inhibits apoptosis in the oesophageal adenocarcinoma OE33 cell line (Ogunwobi, Mutungi et al. 2006). The results reported suggest a definite link between obesity and OAC, through the production of leptin by adipose tissue that inhibits apoptosis and promotes proliferation. Leptin is also heavily involved in increasing COX-2 mRNA levels in the OE33 cells (Ogunwobi, Mutungi et al. 2006). Increased expression of the leptin receptor in Barrett's oesophagus and OAC has been reported previously (Francois, Roper et al. 2008; Reynolds, Howard et al. 2010). Increased expression of this receptor, sensitizes the cells to lower levels of leptin in vivo, making the cells responsive to ambient levels of leptin that would not usually elicit a response (Bodger 2005). Increased leptin production by adipose tissue along with increased sensitivity in the cells and their involvement in the Barrett's oesophagus-OAC progression may make this protein a potential target in the prevention of OAC.

1.2.4.2 Adiponectin

Adiponectin is an adipokine produced exclusively by adipose tissue, and has been shown to have many beneficial effects in numerous in vitro and in vivo studies, possessing anti diabetic, anti-inflammatory and anti-arthereosclerotic properties (Kadowaki, Yamauchi et al. 2003; Yamamoto, Kiyohara et al. 2005). Adiponectin levels are reported to be inversely proportional to obesity levels, as obesity levels increases, levels of adiponectin in vivo drop dramatically, leading to a condition known as hypoadiponectinaemia. This condition has been linked to considerably increased risk of colonic, breast, endometrial and prostate cancers (Konturek, Burnat et al. 2008).

Adiponectin and its association with Barrett's oesophagus and OAC have been investigated previously. Adiponectin enhanced dose dependant apoptosis in the Barrett's derived

adenocarcinoma cell line OE19, adiponectin also stimulated the expression of Bax, a pro apoptotic protein crucial in the intrinsic apoptotic pathway (Konturek, Burnat et al. 2008).

Adiponectin activity is mediated by two distinct receptors, Adipo-R1 and Adipo-R2 (Kadowaki, Yamauchi et al. 2006). Expression of these receptors is markedly lower in the Barrett's mucosa compared to oesophageal squamous mucosa (Konturek, Burnat et al. 2008). These observations highlight the two pronged effect hypoadiponectinaemia can have on the Barrett's oesophagus-OAC progression. Less adiponectin is produced by the body, and the adiponectin that is produced is less effective on cell signalling due to decreased receptor expression by the cells.

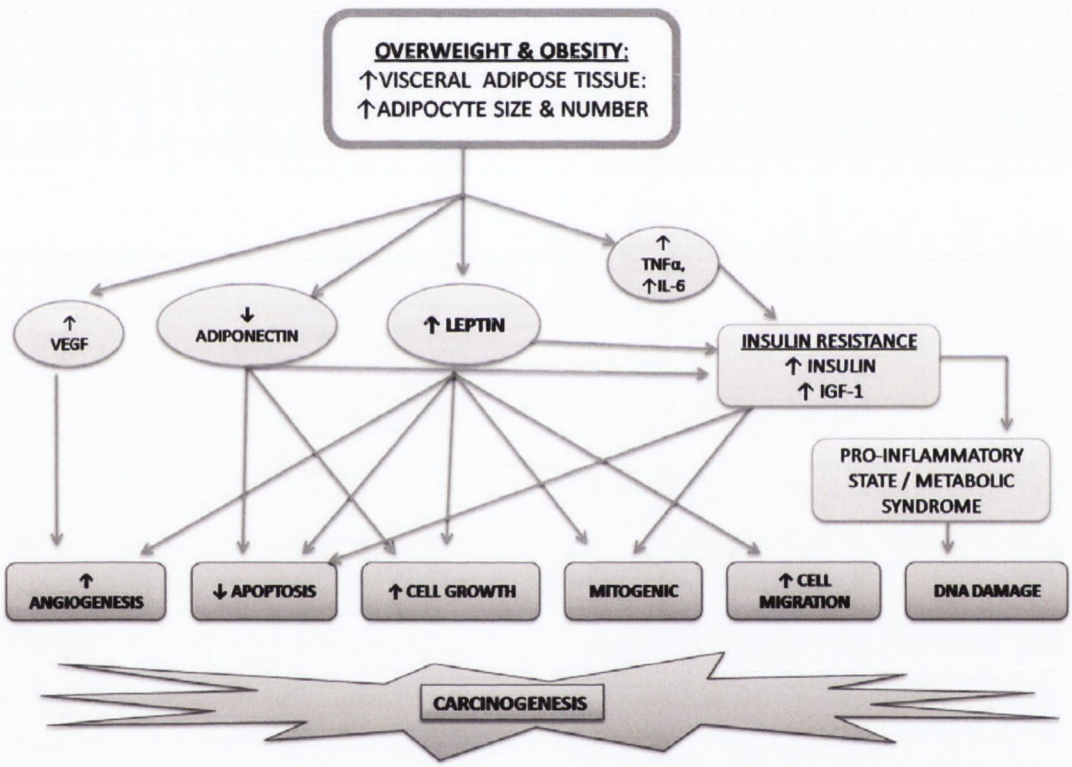

Figure 1.8. Mechanisms by which obesity can activate tumourigenic pathways (Howard, Pidgeon et al. 2010)

1.3 The Hallmarks of cancer and Barrett's derived carcinogenesis

It has been suggested by Hanahan and Weinberg that all cancerous cells possess 6 key variations in cell physiology that push the cell towards a malignant state (Hanahan and Weinberg 2000). These variations are self-sufficiency in growth signalling, resistance to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and finally, invasion and metastasis. The enumeration of these traits in Barrett's cells takes place during the progression of the disease to OAC.

1.3.1 Self-sufficiency in growth signalling

Self-sufficiency in growth signalling in OAC has been well documented in the literature; Increased or altered levels of these key cyclins are observed in OAC. Increased expression and activity of cyclin D1 has been reported in metaplastic Barrett's biopsies in non-dysplastic states (Arber, Lightdale et al. 1996), suggesting that this event occurs in early carcinogenesis. Contrary to these findings, a group observed that increased cyclin E activity in dysplastic Barrett's biopsies and in OAC itself, but that this was not seen in non-dysplastic states (Sarbia, Bektas et al. 1999). Dysregulation of cyclin B1 activity is seen in both dysplastic and non-dysplastic states in Barrett's oesophagus (Geddert, Heep et al. 2002), while increases in the expression of cyclin A mirrors the metaplasia-dysplasia-adenocarcinoma progression sequence in the evolution of the disease (Lao-Sirieix, Lovat et al. 2007).

While self-sufficiency in growth signalling is a key aspect in the tumour pathogenesis, cell surface receptors for key growth factors are targets of dysregulation in progression to a cancerous state (Hanahan and Weinberg 2000), generally through the over-expression of the receptor. This over expression may lead to hyper-responsiveness in the cells to ambient levels of growth factors, which under normal physiological circumstances, would not lead to proliferation and growth in the cells. This trend is seen in OAC, where the increased expression of epidermal growth factor (EGF) and its receptor (EGFR) along with increases in the expression of transforming growth factor alpha (TGF-α), has been reported by different groups studying OAC (Zhang, Spechler et al. 2008).

1.3.2 Resistance to anti-growth signalling

In healthy cells, various mechanisms operate to maintain homeostasis and cell quiescence, these mechanisms are regulated by soluble growth inhibitors (Zhang, Spechler et al. 2009). The genes involved in anti-growth signalling are generally termed tumour suppressor genes, and a number of them have been implicated in OAC. p16 a key tumour suppressor gene, in association with p53, a gene typically associated with the apoptotic machinery in the cell, blocks the cell cycle progression during cellular duress (Liggett and Sidransky 1998). Thus, deletion and mutation of p53 and p16 can lead to dysregulation of cell growth. This occurrence is seen in OAC, where allelic loss of 9P, the specific chromosomal location of the p16 gene fragment, and the methylation and subsequent silencing, of the p16 promoter, are seen in ~50% of all OAC cases (Barrett, Sanchez et al. 1996). Along with p16, p53 inactivation is seen in up to 90% of OAC, inactivation of these two key tumour suppressing genes contributes heavily to the tumour progression (Hamelin, Flejou et al. 1994).

Another important anti-growth factor associated with OAC is transforming growth factor β (TGF-β). This protein is a growth inhibitor for a host of epithelial cells, loss of growth inhibition mediated by TGF-β is seen in numerous neoplasms (Villanacci, Bellone et al. 2008). Villanacci *et al* reported that

TGF-β is suppressed in Barrett's oesophagus by Ski and SnoN, proteins that suppress TGF-β. Ski and SnoN are over expressed in Barrett's oesophagus, though this expression is down regulated through the metaplasia- dysplasia- adenocarcinoma progression.

1.3.3 Evasion of apoptosis

The ability of tumour cells to grow to unprecedented numbers, not only relies on an increased rate of cell proliferation, but also relies on the cells ability to evade the apoptotic mechanisms that keep cell numbers in check. The key gene involved in this process is p53, described by Lane as the "guardian of the genome" (Lane 1992). Differentiation, cell senescence, cell cycle arrest, and crucially, apoptosis are all controlled by p53 (Vousden and Lu 2002). Mutations of this gene are seen in 50% of all cancers (Soussi, Ishioka et al. 2006), including OAC, where a Japanese study saw mutations in the p53 gene in 34% of formalin-fixed, paraffin-embedded oesophageal cancer tissues (Uchino, Saito et al. 1996).

Aberrations in the activity of death receptors crucial for apoptosis have also been reported in OAC. An apoptotic response in the cell can be activated when death receptors on the cell surface bind with ligands such as FasL (Younes, Schwartz et al. 2000). Oesophageal adenocarcinoma cells have been found to express FasL, which can bind the Fas receptor on the surface of tumour-killing lymphocytes, thereby destroying the lymphocytes that might attack the cancer cells (Younes, Schwartz et al. 2000).

1.3.4 Limitless replicative potential

Telomerases are DNA polymerases whose function is to extend the length of the telomeres by adding long random repeats of the hexanucleotide 5'-TTAGGG-3' to the telomeres. Telomerase activity has been detected in 80% to 95% of cancers, and though activity of this enzyme is seen in certain stem cells and proliferative cells, normal tissue does not generally show telomerase activity (Shammas, Koley et al. 2004). Telomerase activity plays a significant role in cancer because it grants the cells unlimited replicative potential, and allows for evasion of senescence and critically, apoptosis. Again, this hallmark of cancer is observed in both Barrett's oesophagus and oesophageal adenocarcinoma (Lord, Salonga et al. 2000).

Work carried out by Morales (Morales, Gandia et al. 2003) shows that there is very low telomerase activity in healthy squamous cells that line the oesophagus, this activity however is increased in Barrett's oesophagus, and further increased during OAC (Morales, Gandia et al. 2003). It has been put forward that the immortalized clones produced by increased telomerase activity in Barrett's oesophagus may progress from a metaplasia to dysplasia and ultimately to OAC, through the accumulation of other traits (Shammas, Koley et al. 2004) Levels of telomerase expression and

activity above normal are found in OAC, and activity of these enzymes increase in the progression of the dysplasia from low grade to high grade in Barrett's oesophagus (Lord, Salonga et al. 2000).

1.3.5 Sustained angiogenesis

Like any cells in the body, tumour cells require oxygen and nutrients for survival, all cells must be within 100μm of a blood vessel, in order to supply the necessary nutrients and oxygen, and remove metabolic waste products (Fidler, Yano et al. 2002). Vascular endothelial growth factor (VEGF) is the gene most associated with angiogenesis and cancer (Hanahan and Weinberg 2000).

VEGF expression has been shown to increase significantly during the metaplasia–dysplasia–adenocarcinoma sequence in Barrett's oesophagus patient samples using immunohistochemistry, leading to significant neovascularisation (Mobius, Stein et al. 2003). In this study, VEGF expression was increased before progression to invasive carcinoma, this finding may potentially offer a strategy for the detection and prevention of the malignant progression of Barrett's oesophagus.

Barrett's epithelia show increased expression of VEGF-A compared to normal oesophagus, likewise, the expression of VEGF-A receptor, VEGFR-2, is also increased (Auvinen, Sihvo et al. 2002). In Barrett's epithelium, increased VEGF-A expression may also be able to sustain new blood vessels in the absence of pericytes and smooth muscle cells (Auvinen, Sihvo et al. 2002), factors usually crucial to the formation and function of blood vessels (Bergers and Song 2005).

Angiogenesis may also be involved in the malignant progression of Barrett's oesophagus due to oxidative stress. One group demonstrated increased angiogenesis, due to oxidative stress, in the metaplasia- dysplasia- adenocarcinoma progression of the disease (Sihvo, Ruohtula et al. 2003).

1.3.6 Metastasis and invasion

The final hallmark of cancer, and perhaps the most lethal, is invasion and metastasis. The distal tumour sites formed by the metastasising cells are reported to be the cause of 90% of all cancer death (Sporn 1996). The molecular biology behind metastasis is remarkably complex and is amongst the least understood in the field of molecular oncology.

Mutations in cell-cell adhesion molecules called cadherins are believed to be involved in metastasis. These proteins anchor cells to the extracellular matrix (ECM) and adjacent cells, loss of this function in cancerous cells enables the cells to escape the primary tumour and travel to distal sites in the body and form secondary tumours (Jiang 1996). In normal gastric epithelium, anti E-cadherin antibodies stain with high intensity, revealing a high expression of this molecule, however reduced or absent staining is observed in 25% of intestinal type Barrett's epithelium (Jankowski JA and M 1994) and as Barrett's oesophagus progress to dysplasia, the staining of E-cadherin reduces further

(Bongiorno, al-Kasspooles et al. 1995). E-cadherin levels on the cell membrane decreases during neoplastic progression of Barrett's oesophagus while catenins, necessary for the function of E-cadherin, showed reduced expression and aberrant nuclear localisation (Bailey, Biddlestone et al. 1998).

Matrix metalloproteinases (MMPs) are also implicated in metastasis. This protein family breaks down the ECM and play an important role in biological processes such parturition. However, the activities of MMP7 (Masaki, Matsuoka et al. 2001) and MMP9 (van Kempen and Coussens 2002) have been shown to allow for tumour metastasis, by breaking down the ECM and freeing tumour cells from the primary mass (Crawford and Matrisian 1994). Increased expression of MMP9 has been seen in the metaplasia-dysplasia-adenocarcinoma sequence compared to healthy normal tissue, using paraffin-embedded tissue samples analysed by immunohistochemistry (Herszenyi, Hritz et al. 2007).

Interestingly, the involvement of MMPs in cancer and metastasis could further reinforce the links between cancer and inflammation. Pro-inflammatory protein TNFα, a key effector in the inflammatory response, has been shown to stimulate the expression of members of matrix metalloproteinase family. TNFα, also blocks the production of tissue inhibitors of matrix metalloproteinases, proteins which negatively regulate matrix metalloproteinases (Keelan, Blumenstein et al. 2003).

1.3.7 Updated hallmarks of cancer

The authors of the original 'Hallmarks of Cancer', manuscript proposed an update recently to the six already established (Figure 1.9). Dysregulation of cellular energetics and evasion of the immune system were proposed as additional hallmarks of cancer, while tumour promoting inflammation and genome instability and mutation were proposed as enabling characteristics of cancer cells (Hanahan and Weinberg 2011).

Dysregulation of cellular energetics has been reported previously in Barrett's oesophagus. Barrett's oesophagus displays a strong increase in glycolytic metabolism compared to normal squamous epithelium with Barrett's epithelia displaying increased expression of enzymes associated with glycolysis (van Baal, Milano et al. 2005; van Baal, Diks et al. 2006), a common characteristic of carcinogenesis (Gatenby and Gillies 2004). Evolutionary models show that clonal expansion of pre-malignant tumour populations is ultimately limited by substrate availability (Gatenby and Vincent 2003), due to unchecked cell growth and proliferation carrying the cell population ever further from its blood supply, necessitating a change in cellular metabolism to anaerobic metabolism, allowing for maintenance of the metabolic activities of the cell in absence of oxygen from blood supply (Gatenby

and Gillies 2004). It has been proposed that persistent metabolism of glucose to lactate, a key feature of anaerobic metabolism (Huckabee 1958), even in aerobic conditions is a cellular adaptation to intermittent hypoxia in pre-malignant conditions. Subsequent cell populations capable of utilising both aerobic and anaerobic metabolism have a potent growth advantage, promoting unconstrained cell proliferation and invasion (Gatenby and Gillies 2004), mechanisms characteristic of malignant cancer cells. This mechanism may contribute to neoplastic transformation in Barrett's oesophagus.

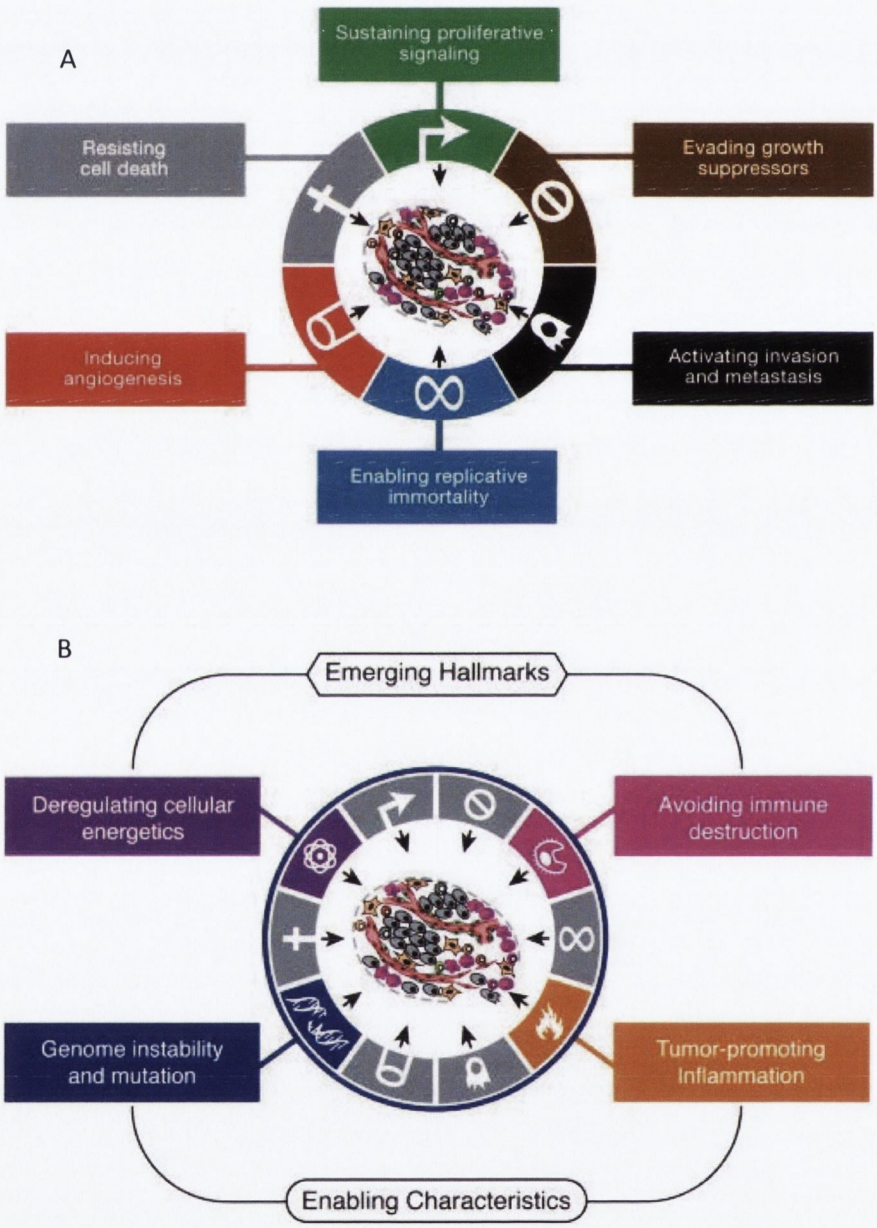

Figure 1.9. The six original Hallmarks of Cancer (A) and the updated hallmarks and enabling characteristics (B) (Hanahan and Weinberg 2011).

1.4 Inflammation and Barrett's oesophagus

Obesity may be defined as a state of chronic systemic low grade inflammation. It is linked to a chronic inflammatory response, evidenced by modifications in cytokine production, activation of inflammatory signalling pathways, and increased synthesis of acute phase reactants (Tilg and Moschen 2008). Further evidence linking inflammation to obesity reports increased levels of C-reactive protein, a sensitive marker of systemic inflammation, in both obese men and women (Visser, Bouter et al. 1999). Due to chronic low grade inflammation in obese patients, they are at a higher risk of developing cancer due to the well-established links between inflammation and cancer (Aggarwal, Vijayalekshmi et al. 2009). Some of the carcinogenic aspects of the inflammatory response, and their relationship to Barrett's oesophagus and OAC, are expanded in more detail in the following sections.

1.4.1 Pro-inflammatory cytokines

Increased expression of pro-inflammatory cytokines are observed in numerous cancers including, but not exclusive to, breast, lung, ovary and pancreatic cancers (Serefoglou, Yapijakis et al. 2008). Members of the tumour necrosis factor (TNF) super family are associated with numerous malignancies and increased expression of TNFα has been reported in the progression of Barrett's oesophagus to OAC (Tselepis, Perry et al. 2002). The two key pro-inflammatory cytokines, TNFα and IL1β, regulate their own synthesis through feedback loops involving the transcriptional factor NF-κB(Sosic, Richardson et al. 2003), and dysregulation of these processes may lead to DNA damage, and stimulate processes including angiogenesis and tumour growth (Serefoglou, Yapijakis et al. 2008).

Mutations and alterations in cytokine signalling are of particular interest in this study due to the fact that adipose tissue has been shown to secrete cytokines such as TNFα and IL-6 (Kern, Ranganathan et al. 2001). IL-6 in particular may be of significant importance in the progression of Barrett's oesophagus to OAC. IL-6 has been shown to stimulate VEGF in stroma cells and granulocytes (Ferrara 2000), and an increase in the expression of this key angiogenic factor may contribute to neovascularisation in Barrett's oesophagus and the subsequent progression to OAC.

COX-2, a key inflammatory protein has been linked to Barrett's oesophagus and OAC (Oyama, Fujimura et al. 2005). COX-2 is an isozyme of COX and is involved in inflammatory signalling. Barrett's oesophagus and OAC patients demonstrate significant increases in COX-2 expression, and may contribute to carcinogenesis by promoting cell proliferation, blocking apoptosis and stimulating angiogenesis (Oyama, Fujimura et al. 2005). Work on animal models demonstrates that acid reflux and GORD leads to significant increases in COX-2 expression, as the disease progresses from

Barrett's oesophagus to OAC. Administration of celecobix, a specific COX-2 inhibitor, prevented the development of Barrett's oesophagus and OAC in rats, by suppressing oesophagitis (Oyama, Fujimura et al. 2005). Research has shown that administration of aspirin, another COX-2 inhibitor, may prevent the progression of Barrett's oesophagus to OAC, and is associated with a lower risk of myocardial infarction (Hur, Broughton et al. 2008).

COX-2 has been implicated in the metaplasia-dysplasia-adenocarcinoma progression in Barrett's patients. COX-2 expression is up-regulated in Barrett's oesophagus and OAC but it is not associated with an inflammatory response in the cells. Expression of COX-2 is increased in Barrett's oesophagus near the gastro-oesophageal junction but away from sites of maximal inflammation. Distribution of COX-2 in oesophageal adenocarcinoma tissue is concentrated in the cancerous cells and is not related to the distribution of the inflammatory infiltrate, indicating that COX-2 expression in the oesophagus may be more directly associated with signalling pathways involved with cancer progression than inflammation (Abdalla, Sanderson et al. 2005).

1.4.2 NF-κB

NF-κB, a transcription factor, regulates the expression of genes controlling inflammation, cell survival and apoptosis (Lawrence 2009). Biopsies of healthy oesophageal tissue show no detectable NF-κB activity, while biopsies from oesophageal adenocarcinoma tissue, showed significant levels of NF-κB activity and expression, as well as low levels of IκB, the inhibitory molecules of NF-κB (Abdel-Latif, Kelleher et al. 2008). It is hypothesised that NF-κB is activated in Barrett's mucosa by an inflammatory response to the insult caused to the oesophageal epithelium by gastric oesophagitis (Lee, Oh et al. 2001). NF-κB may also contribute to carcinogenesis in Barrett's epithelium by activating COX-2, which is heavily implicated in the Barrett's oesophagus-OAC progression as previously discussed (Oyama, Fujimura et al. 2005). Other research has illustrated a stepwise increase in NF-κB expression as reflux oesophagitis progressed through Barrett's oesophagus to OAC, in fresh biopsy patient samples (O'Riordan, Abdel-latif et al. 2005). In this same study, NF-κB was responsible for an increase in expression of pro-inflammatory cytokines IL-8 and IL-1β, and the expression of these cytokines coincided with the stage of the disease (O'Riordan, Abdel-latif et al. 2005).

Another mechanism whereby this master transcription factor can contribute to the pathogenesis of Barrett's oesophagus is through its regulation of the caudal homeobox genes (CDX) transcription factors, CDX1 and CDX2 (Colleypriest, Ward et al. 2009). These transcriptional factors direct the development of intestinal cells with the gastrointestinal tract (Souza, Krishnan et al. 2008) and may be responsible for the switch of squamous epithelium in healthy oesophageal tissue, to the

columnar intestinal like cells characteristic of Barrett's oesophagus. NF-κB may bind to one of two distinct binding sites in the CDX promoter region, activating the transcription of CDX1 and CDX2 (Kazumori, Ishihara et al. 2006). Increased expression of CDX1 and CDX2 has been seen in biopsies from patients with Barrett's oesophagus. CDX2 expression levels have been reported up to 400 times higher in Barrett's metaplasia biopsies compared to healthy squamous epithelia (Vallbohmer, DeMeester et al. 2006), in which levels of this transcriptional factor were barely detectable.

1.5 Genomic instability and carcinogenesis

The human body contains in excess of 10 trillion cells, and remarkably almost every cell contains an identical set of chromosomes, even following successive rounds of cell replication. In order to achieve a high level of chromosomal fidelity and integrity, cells have developed sophisticated and controlled pathways to replicate, repair and segregate chromosomal material (Chan and Hickson 2011). Dysfunction of these mechanisms can result in genomic instability, which is an established feature of most forms of cancer (Tlsty, Briot et al. 1995; Negrini, Gorgoulis et al. 2010), and has recently been described as an emerging hallmark of cancer (Hanahan and Weinberg 2011). Genomic instability leads to myriad of molecular alterations in the genome of the cancer cell. These alterations include (but are not limited to) mutations of single genes, amplifications, deletions or rearrangements of chromosomal material, and gain or loss of entire chromosomes (Shen 2011). Accumulation of these genomic alterations in cells may lead to cell growth and division going unchecked, evasion of normal cell death pathways, and ultimately, cancer. Genomic instability can lead to both the loss of tumour suppressor gene expression through deletions and chromosome loss, or over-expression of oncogenes due to gene activation (Schwab 1999; Katoh and Katoh 2003).

Maintenance of genomic stability and integrity are essential for an organism to function and propagate successfully, and telomeres are one of the key elements in regulating this stability (Blackburn 2001; Blackburn and Chan 2004). Seminal work by McClintock and by Muller (McClintock 1941; Muller and Herskowitz 1954) demonstrated that the ends of normal chromosomes must be protected somehow as they lack the 'stickiness' of broken chromosomes and will not fuse together (Figure 1.10). McClintock demonstrated that if a broken chromosome lacking a telomere is replicated, it may fuse with another forming a dicentric chromosome.

1.5.1 Telomeres and genomic instability

DNA in humans and all eukaryotes is linear (Reddel and Cesare 2010)and these linear chromosomes pose a fundamentally important problem, their ends cannot be completely replicated by the organism's DNA replication machinery, leading to telomere shortening with each cell replication (Olovnikov 1973), termed the 'end replication problem'. Telomere length shortens usually between 50-200bp at each replication (Wright, Huffman et al. 2000). These telomeres are composed of tandem repeats of TTAGGG and are associated with a wide variety of telomere binding proteins which mediate their function (Wai 2004; Martinez and Blasco 2010). The gradual shortening of these telomeres due to cell proliferation generates critically short telomeres which elicits a DNA damage response, ultimately leading to permanent growth arrest and cellular senescence (Harley, Futcher et al. 1990).

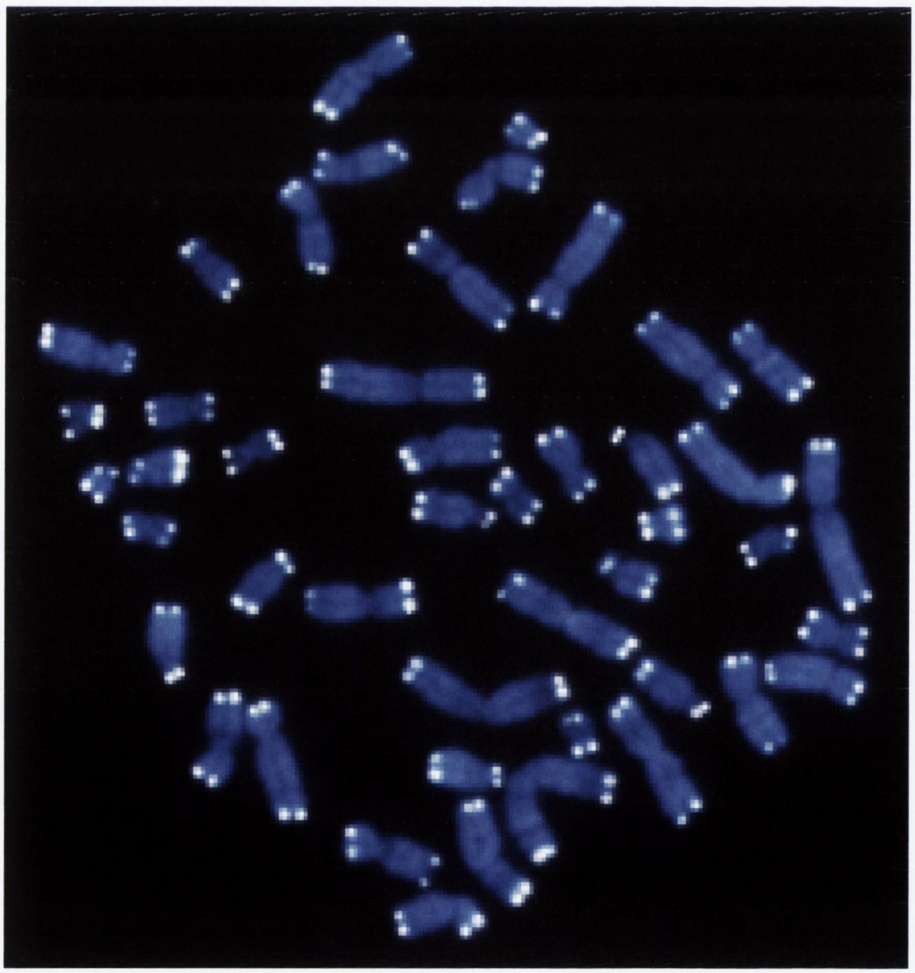

Figure 1.10. The 46 human chromosomes, shown in blue, with telomeres appearing as white pinpoints (taken from http://www.nih.gov).

Structural analysis of telomeres from a variety of species has revealed that the telomeres consist of a double stranded region comprised of telomeric repeats followed by a much shorted single stranded overhang on the 3' G-rich strand (Price and Wei 2003). The length of this overhang varies between species and ranges from as short as 14 nucleotides in some ciliates to as long as 200 nucleotides in vertebrates (Price, Jacob et al. 2003). In some organisms, including humans, this G-rich strand folds back in a loop into the duplex region of the telomere tract to form the t-loop found at the end of the telomeres (Griffith, Comeau et al. 1999). Formation of the t-loop (Figure 1.12A) is essential to protect the telomeres from degradation and for recruiting telomeric chromatin involved with telomere maintenance (Price, Jacob et al. 2003).

All organisms with linear chromosomes need to protect the chromosomal terminus from end-to-end joining and degradation. They must also overcome the end-replication problem that arises from the inability of DNA polymerase to replicate the extreme 5' end of a linear DNA molecule (Price and Wei 2003). The discovery of the ribonuceloprotein telomerase complex in 1985 solved the riddle of the end replication problem in eukaryotes (Greider and Blackburn 1985), research which ultimately resulted in the Nobel prize for medicine for the authors in 2009. The telomerase complex consists of the telomerase reverse transcriptase (TERT) and the telomerase RNA template component (TERC) (Figure 1.11). The telomerase complex synthesises one strand of telomere DNA, the 5' to 3' strand, by copying the TERC template sequence (Greider and Blackburn 1989). Synthesis of the complementary strand of the telomeric repeats is presumed to occur through lagging stand synthesis of the C rich strand by the cell's DNA replication machinery (Chan and Blackburn 2004).

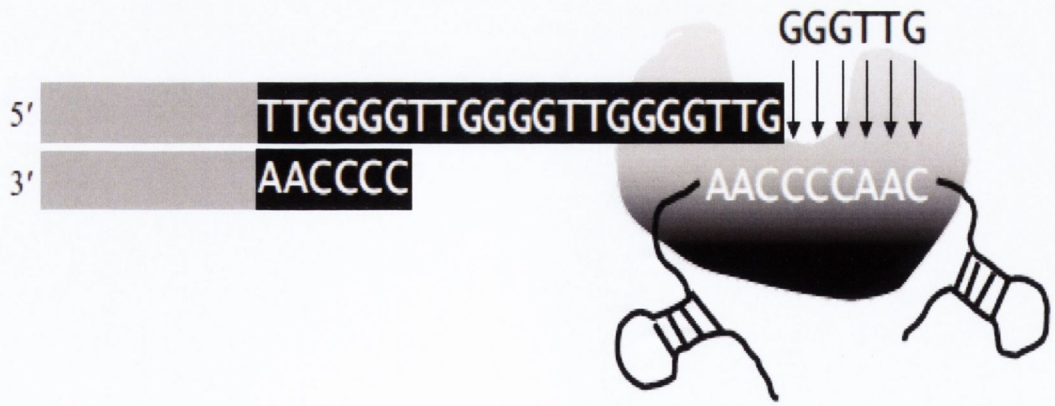

Figure 1.11. DNA elongation action of telomerase (Chan and Blackburn 2004).

Somatic adult cells display very little telomerase expression and activity (Vaziri and Benchimol 1998), with the exception of stem cells, however telomerase activity has been reported in virtually all human tumours, but not in adjacent normal cells (Kim, Piatyszek et al. 1994; Shay and Bacchetti 1997). It is hypothesised that maintenance of telomere length is essential for the long term proliferation of tumours (Holt and Shay 1999). Thus, increases in telomerase expression and activity and maintenance of telomere length, allows cells to escape cellular senescence and represents an important step in oncogenesis that most tumours necessitate for the proliferation (Shay, Zou et al. 2001).

A group of proteins termed the shelterin complex believed to mediate telomerase activity and the proximal ends of telomeres (Palm and de Lange 2008) (Figure 1.12B). This complex consists of six members; telomeric repeat binding factor 1 (TERF1), telomeric repeat binding factor 2 (TERF2), TERF1 interacting nuclear factor 2 (TINF2), protector of telomeres 1 (POT1), telomeric repeat-binding factor 2-interacting protein (TERF2IP) and TPP1 (Blasco and Martinez 2011). TERF1 and TERF2 bind telomeric double stranded DNA directly, while POT1 binds to the 3' singled stranded G rich overhang.

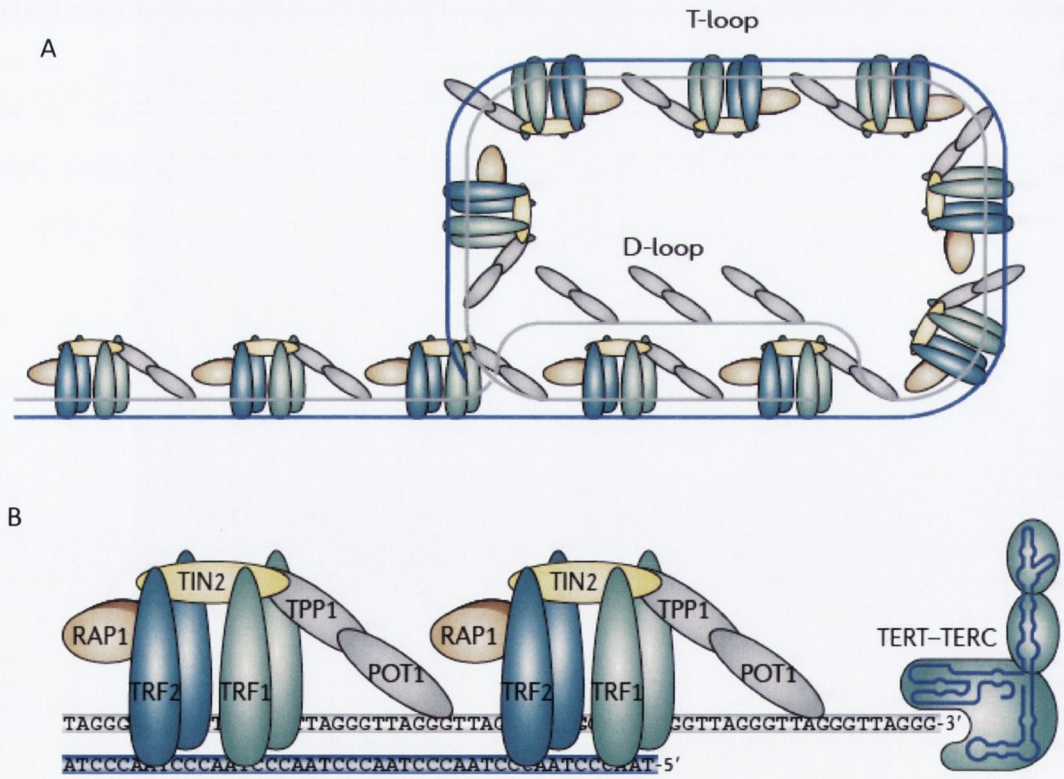

Figure 1.12. Telomere structure (A) and functional roles of the telomeric proteins (B)(Blasco and Martinez 2011)

1.5.2 Bridge breakage fusion cycles

Dicentric chromosomes form when two broken chromosomes fuse at their ends after severe telomere shortening, absence or low numbers of telomere repeats have been observed at the junctions of dicentric chromosomes in ovarian cancer cells (Wan, Martens et al. 1999). During anaphase, the two centromeres of a dicentric chromosome are pulled to the opposite poles of the mitotic spindle, forming an anaphase bridge which eventually breaks. The site of breakage does not occur exactly at the site of fusion, resulting in one daughter cell acquiring a chromosome with a duplication on its end in the form of an inverted repeated, while the chromosome sequestered to the other daughter cell contains a terminal deletion (Murnane 2006). Since neither of these chromosomes contain a telomere, the cycle will be repeated the next time the cell replicates and this cycle continues, causing extensive DNA amplification and terminal deletions, until the chromosome eventually gains a new telomere and becomes stable (Lo, Sabatier et al. 2002) (Figure 1.13). Successive breakage fusion bridge (B/F/B) cycles in human cancer results in chromosomal rearrangement and gene amplification in human cancers (Schwab 1999). The overall consequences of successive B/F/B cycles depends on whether chromosome fusions occur between different chromosomes or between sister chromatids (Murnane 2011).

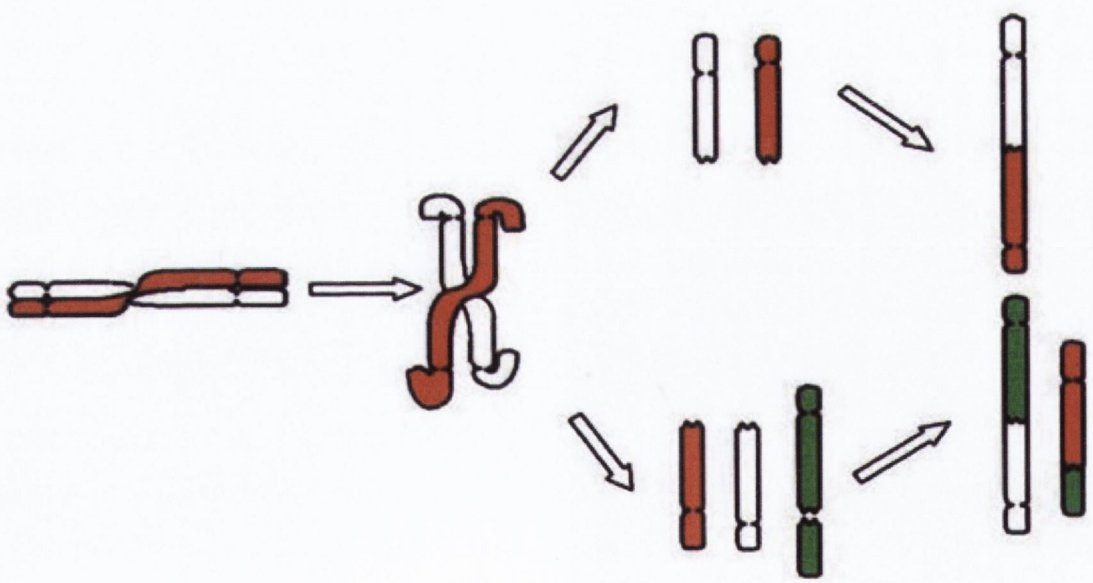

Figure 1.13. Dicentric chromosomes and chromosomes involved in telomeric associations can also form bridges at anaphase. The broken ends may re-join subsequently or rearrange with other broken chromosomes, forming new variants of dicentric chromosomes (Gisselsson, Pettersson et al. 2000).

Fusion of chromosomes can occur through various mechanisms depending on cell type and mechanism for telomere loss. The majority of chromosome fusions occur due to double strand break (DSB) repair involving nonhomologous end joining (NHEJ), of which there are two categories classical nonhomologous end joining (C-NHEJ) and alternative nonhomologous end joining (A-NHEJ) (Murnane 2011). C-NHEJ is characterised by microhomology and the fusion sites and is associated with significant deletions of chromosomal rearrangement (Haber 2008). C-NHEJ is the more prominent form of NHEJ and A-NHEJ is only seen in cells which are deficient in C-NHEJ , as C-NHEJ acts as a suppressor of A-NHEJ (Bennardo, Cheng et al. 2008; Jasin and Simsek 2010). The activity of NHEJ may lead to the deletion of large sections of a chromosome resulting in inactivation of tumour suppressor genes while amplification of chromosome regions could result in activation of proto-oncogenes (Vogelstein 1999).

Telomere dysfunction has been investigated in Barrett's oesophagus previously. Chromosome instability driven by telomere shortening has been reported in Barrett's oesophagus patients with loss of the short (p) arm of chromosome 17 and 11q arm gains correlating with telomere length (Rabinovitch, Finley et al. 2006); telomere shortening and chromosome instability was reported to be an early event in Barrett's oesophagus neoplastic progression and occurred prior to dysplasia. This result correlates well with another that reported a major increase in telomerase activity occurring after the Barrett's oesophagus stage in oesophageal carcinogenesis (Nwokolo, Barclay et al. 2005). Telomere shortening and the subsequent bridge breakage fusion events leading to chromosomal rearrangement followed by an increase in telomerase activity to maintain telomere length in the now mutant cells may provide a rationale for carcinogenesis arising in Barrett's oesophagus. In this thesis, these events will be investigated in a panel of oesophageal cell lines representing the metaplasia-dysplasia-adenocarcinoma Barrett's disease sequence, in response to culture in adipose conditioned media.

1.5.3 The spindle assembly checkpoint

A growing body of evidence suggests that defects in the spindle assembly checkpoint (SAC), a surveillance mechanism that ensures the fidelity of chromosome replication during mitosis (Figure 1.14), is responsible for an increased rate of aneuploidy and subsequent tumourigenesis (Roychoudhury, Mondal et al. 2007; Foulkes, Frio et al. 2010).

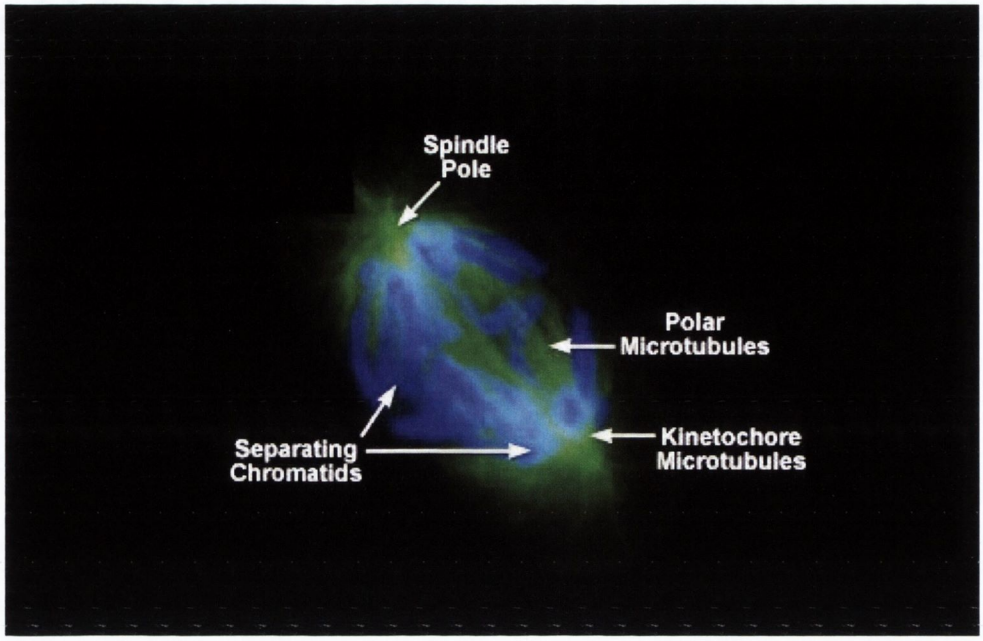

Figure 1.14. The mitotic spindle

(taken from http://micro.magnet.fsu.edu/cells/fluorescencemitosis/anaphase1large.html).

The SAC controls the metaphase-anaphase transition during cell replication in mitosis (Sunkel, Malmanche et al. 2006). The SAC controls this complex by inhibiting CDC20, a co-factor of the anaphase promoting complex/cyclosome (APC/C), resulting in checkpoint arrest until all chromosomes are attached to the mitotic spindle (Doncic, Ben-Jacob et al. 2009) (Figure 1.15). While it is universally accepted that the SAC controls the metaphase to anaphase transition, the exact transduction pathways involved are the subject of intense scrutiny. While the existence of a control mechanism for the metaphase to anaphase transition had been postulated in the early 1970s (Zirkle 1970), the SAC was first identified in budding yeast and revealed the MAD1,-2,-3 (mitotic arrest deficient) and BUB1,-2,-3 (budding uninhibited by benomyl) genes which have since been shown to be conserved throughout all metazoans (Rudner and Murray 1996). The MAD and BUB proteins, along with CDC20 are localised at the kinetochores, a protein complex that forms at the centromeres during cell division at the prometaphase stage of mitosis, and this localisation of SAC proteins generates a diffusible 'wait anaphase' stop signal (Hardwick and Musacchio 2002).

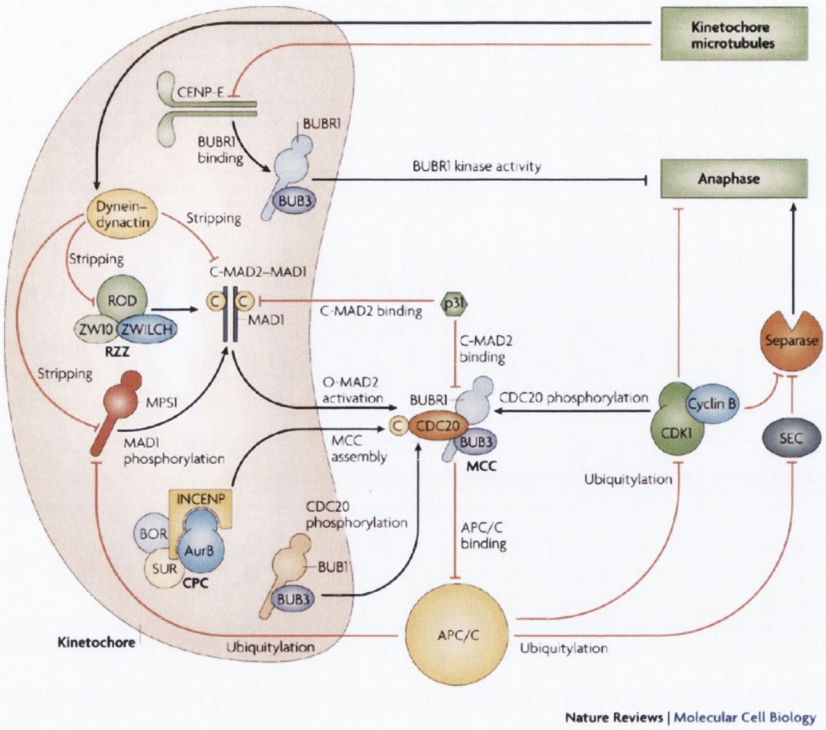

Figure 1.15. Components of the spindle assembly checkpoint (SAC) (Musacchio and Salmon 2007)

In healthy cells a single unattached kinetochore can stop cells entering anaphase (Rieder, Cole et al. 1995; Chan and Yen 2003), however in cancer cells with a compromised SAC, this signal is not sufficient to generate the 'wait anaphase' signal (Kops, Weaver et al. 2005). Dysregulations in the spindle assembly checkpoint have been documented in a number of human cancers (Thirthagiri, Robinson et al. 2007). In ovarian cancer cell lines, restoration of MAD2 in cells that failed to arrest in the G(2)-M phase of the cell cycle in response to microtubule disruption, led to a successful mitotic checkpoint response and cell cycle was arrested, preventing further chromosomal instability (Wang, Jin et al. 2002). Significant mutations have also been reported in the BUB genes correlating with complex chromosomal abnormalities in Adult T-cell leukemia/lymphoma (ATLL) (Ohshima, Haraoka et al. 2000). While dysregulations in the spindle assembly checkpoint due to obesity or in the Barrett's neoplastic sequence have never previously been investigated, aberrant expression of spindle assembly proteins conducive to carcinogenesis has been characterised in pre malignant conditions. BUB1B was significantly associated with the progressive morphological changes in patients with ulcerative colitis, a precursor lesion of colorectal cancer, with increasing aneuploidy and cell proliferation occurring in tandem with up-regulations in BUB1B expression (Burum-Auensen, Deangelis et al. 2007).

1.6 Aims and objectives

1.6.1 General aim

The overall aim of this thesis is to delineate the pro-carcinogenic pathways and cellular instability profiles activated by obesity and excess adiposity in Barrett's oesophagus, independent of gastro-oesophageal reflux disease.

1.6.2 Specific objectives

The overall aim of this thesis will be investigated by

a) Identification of a pro-tumour gene expression signature activated by adipose conditioned media in a non-neoplastic Barrett's oesophagus cell line model and to investigate the expression of these genes in Barrett's oesophagus biopsies from obese and non-obese patients

b) Determining the effect of obesity on the invasive capacity and the proliferative capacity on a panel of oesophageal cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence associated with the neoplastic progression of Barrett's oesophagus

c) Examination of telomere length in response to adipose conditioned media in the panel of oesophageal cell lines

d) Investigation of the effect of adipose conditioned media on the anaphase bridging and dysregulation of the spindle assembly checkpoint complex on the panel of oesophageal cell lines

Chapter 2: Materials and methods

2.1 Reagents

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

2.2 Cell culture

All cell culture media was purchased from Lonza (Basel, Switzerland) and all cell culture plastics were purchased from Starstedt Ltd. (Wexford, Ireland) unless otherwise stated. All cell lines were purchased from the American Collection of Cell Cultures (ATCC, Virginia, USA) unless otherwise stated. Cell culture was carried out in a dedicated cell culture room which was cleaned and sterilised monthly. Cell culture was carried out in a Grade II laminar hood using aseptic technique while wearing a clean lab coat with elasticated cuffs and disposable latex gloves. The cabinet was cleaned with 70% (v/v) ethanol before and after each use and all reagents, media, and plastics were taken into the cabinet and cleaned in this manner.

2.2.1 Generation of 3T3 feeder layer

3T3 cells were grown to 90% confluency in T-75 flasks in *Dulbecco's Modification of Eagle's Medium (*DMEM) media supplemented with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin (pen/strep). The maintenance media was removed, cells washed with PBS, and then treated for 2hr with 20ml of DMEM media containing 0.5mg/ml of mitomycin C (Sigma Aldrich, Ireland). After 2hr, the media was removed, cells washed, and trypsinised and centrifuged to generate a cell pellet. This pellet was washed with PBS, re-centrifuged, and then re-suspended in freezing media consisting of 90% FCS and 10% dimethyl sulfoxide (DMSO). One mitomycin-c treated T-75 flask was sufficient to generate 8 vials of feeder layers for freezing. To prepare a feeder layer for BAR-T cells, feeder layers were thawed 24hr before sub-culturing of BAR-T cells, in DMEM containing 10% FCS and 1% Pen/Strep, in 10cm cell culture

dishes. Plates that were at least 30% confluent after 24hr were suitable for growth of the BAR-T cell line.

2.2.2 BAR-T cell line

The BAR-T cell line, derived from a Barrett's oesophagus patient biopsy, was a kind gift from Dr. Rhonda F. Souza of South-western University, Dallas, Texas, U.S.A and was maintained in keratinocyte growth media (KGM-2) containing 5% FCS and 1% pen/strep. The media was also supplemented with 400ng/ml hydrocortisone, 20ng/ml epidermal growth factor, 0.1nM cholera toxin, 20μg/ml adenine, 5μg/ml insulin, 5μg/ml transferrin, and 70μg/ml of bovine pituitary extract. This cell line was grown on a mitomycin-c treated 3T3 feeder layer in $10cm^2$ petri dishes and incubated at 37°C and 5% CO_2.

2.2.3 HET-1A cell line

The HET-1A cell line, which represents healthy squamous oesophageal tissue was purchased from the American Collection of Cell Cultures (ATCC, Virginia, USA). The cell line was maintained in bronchial epithelial cell basal media (BEBM) enhanced with hormonal cocktail BEGM® SingleQuots®. This supplemented the media with BEGM singlequots include bovine pituitary extract (BPE), insulin, hydrocortisone, GA-1000, transferrin, tri-iodo thyronine (T3), epinephrine and human epidermal growth factor.

2.2.4 Barrett's oesophagus cell lines

The QH and GO cell lines, representing metaplastic Barrett's oesophagus and high grade dysplastic Barrett's oesophagus, respectively, were a kind gift from Dr. Shane Duggan of Trinity College, Dublin. These cell lines were maintained in bronchial epithelial cell basal media (BEBM) enhanced with hormonal cocktail BEGM® SingleQuots®. This supplemented the media with BEGM singlequots include bovine pituitary extract (BPE), insulin, hydrocortisone, GA-1000, transferrin, tri-iodo thyronine (T3), epinephrine and human epidermal growth factor. Additionally, QH and GO cell line media were supplemented with 10% FCS and 1% pen/strep. The cell lines were incubated at 37°C and 5% CO_2.

The QH cell line is used as a substitute for the BAR-T cell line in functional studies as the BAR-T cell line cannot be used because of the 3T3 necessary for growth of the cell line.

2.2.5 Oesophageal cancer cell line

The OE33 cell line, derived from oesophageal adenocarcinoma was purchased from the American Collection of Cell Cultures (ATCC) and was maintained in Roswell Park Memorial

Institute (RPMI) 1640 medium supplimented with 10% FCS and 1% pen/strep. The cell line was incubated at 37°C and 5% CO_2.

2.2.6 Cell subculture

The cell lines were examined daily using an inverted phase contrast Nikon microscope (Nikon Corporation, Tokyo, Japan) and subcultured upon reaching 80-90% confluency. 5ml of trypsin Ethylene Diamine Tetra Acetic Acid (EDTA) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the flask and incubated at 37°C for several minutes or until the cells detached from the surface of the flask. 10ml of complete media (containing 10% FCS) was then added to inactivate the trypsin and cells were seeded at appropriate densities in new flasks.

2.2.6.1 Subculture of BAR-T cell line

Growth media was extracted and cells were washed with phosphate buffer saline (PBS) to remove residual media. The cells were washed in 10ml of 0.5M Ethylene Diamine Tetra Acetic Acid (EDTA) at a 45° angle. Half the plate was washed 10 times and then the dish was rotated 180° and the other half of the plate was washed. The EDTA wash was repeated and then the cells were washed with PBS. The dish was then inspected under a microscope to ensure complete removal of the feeder layer. The remaining BAR-T cells were treated with 3ml of trypsin EDTA for 2min at room temperature. 2.5ml of trypsin EDTA was then removed and the dish was then incubated at 37°C for 5min. 10ml of KGM-2 media was then added to inactivate the trypsin and cells were seeded in appropriate densities in pre-prepared dishes containing the 3T3 feeder layer.

2.2.7 Preperation of frozen stocks

Frozen stocks were prepared from cell lines growing in the exponential phase and at 70-80% confluency. Cells were trypsinised as described above (section 2.2.6) and 10ml of maintenance media was added to inactivate the trypsin. Cells were centrifuged at 180 x g for 3min, the supernatant decanted, and pelleted cells were resuspended in FCS containing 10% DMSO. The cell suspension was divided in 1ml aliqouts and cryovials were then transferred to a -80°C freezer for short term storage, or under liquid nitrogen for long term storage.

2.2.8 Reconstitution of frozen cells

Frozen stocks were thawed rapidly at 37°C and added to 5ml of appropriate maintenance medium, and centrifuged at 180 x g for 3min. The supernatant was decanted and the cell pellet was resuspended in 5ml of maintenance media, transferred to a $25cm^2$ flask and incubated overnight at 37°C and 5% CO_2. Media was replaced the following day to remove any dead cells and subculture was continued as described (section 2.2.6).

2.2.9 Cell counting

Cells were counted using a Bright Line haemocytometer (Hausser Sceintific, PA, USA). Cells were trypsanised as described (section 2.2.6), added to 10ml of maintenance medium and centrifuged at 180 x g for 3 min. The supernatant was decanted and the cell pellet resuspended by repeated pipetting in 1ml maintenance media. 20µl of the cell suspension was added to 180µl trypan blue (0.4% w/v) solution and allowed to sit at room temperature for 1 min. Viable cells were unstained due to their exclusion of trypan blue, wheraes dead cells were stained blue, due to their disrupted membranes. 20µl of this cell suspension was added to the counting chamber of the haemocytometer and the number of viable cells in of of the four corners of the grid were counted. The number of cells per ml was calculated using the following equation:

(total number of cells/4) x 10^4 x 10 (dilution factor) = no. cells/ ml

2.2.10 Mycoplasma testing

Upon receipt of a new cell line, and every six months thereafter, cells were tested for mycoplasma infection using the MycoAlert™ myoplasma detection system (Cambrex BioScience, Rockland, ME, USA). Mycoplasmal enzymes react with the MycoAlert™ substrate resulting in the conversion of ADP to ATP. The level of ATP in the sample before and after the addition of this substrate therefore indicates whether the sample is contaminated with mycoplasma. Cell lines to be tested were passaged twice in antibiotic free medium before a 1ml sample of supernatant was taken. The supernatant was centrifuged at 180 x g for 3 min to pellet any floating cells and 100µl of supernatant was transferred to a luminescence compatible plate. 100µl of MycoAlert™ reagent was added and incubated for 5 min before a one second intergrated reading ('Reading A') was taken on a luminometer (Wallac Victor 2 1420, Perkin Elmer, Ballymount, Dublin). 100µl of MycoAlert™ substrate was then added to the sample and incubated for 10 min before a second reading ('Reading B') was taken. A 'Reading B : Reading A' ratio <1 indicated cells were free of mycoplasma and all cell lines remained cell of mycoplasma infection throughout the course of this work (representative test results shown in Appendix I).

2.3 Adipose tissue biobank

An adipose biobank was established in the Department of Surgery in July 2007 and standard operating procedures were optimised from a previously published study (Alvarez-Llamas et al., 2007).

2.3.1 Patient recruitment and classification

Male patients (>40 years old) undergoing resective surgery for oesophageal adenocarcinoma were recruited to the adipose tissue biobank. All recruited patients gave informed consent for use of their adipose tissue in this study, and the study obtained ethical approval for St James's Hospital Review Board. Patients were seperated into viscerally obese and non obese categories based on waist circumference cut off measurement of 94cm as specified by the International Diabetes Federation (IDF). Waist circumference was measured at the midpoint between superior iliac crest and subcostal margin, height and weight was recorded and used to determine body mass index (BMI) while computed tomography (CT) at the L3-L4 intervertabral space was used to calculate the visceral, subcutaneous and total fat area.

2.3.2 Biobanking

Two 3ml fasting blood samples were taken in serum collection tubes on the morning of surgery. The samples were centrifuged at 750 x g for 10 min to seperate the serum which was then stored at -80°C. An omental and subcutaneous adipose tissue specimen were excised at the beginning of the surgical procedure and immediately transported in sterile transport buffer (glucose (0.15%), gentamycin (0.05mg/ml) in PBS) for processing in a Grade II laminar air flow cabinet. A thumbnail sized sample of both subcutaneous and omental adipose tissue was preserved in 10% paraformaldehyde for future immunohistochemical analysis. The remaining adipose tissue was minced with a pair of scissors and washed with sterile PBS. Aliquots of 300mg of tissue were placed in seperate cryovials, snap frozen in liquid nitrogen and stored at -80°C for RNA extraction, while the remainder was cultured to produce adipose conditioned media (ACM). This was carried out as followed: in a tissue culture dish, 5g samples of minced and washed adipose tissue were incubated in 10ml of serum free M199 media for 72hr at 37°C and 5% CO_2. ACM was then filtered (BD Biosciences, Bedford, MA, USA) to remove adipose tissue fragments and the supernatant was stored at -80°C.

2.3.3 Culture of cell lines with ACM

Cells were seeded in 6 well plates at a concentration of 1.5 x 10^5 cells/well and allowed to adhere for a minimum of 6 hr before serum starving (media containing 0.5% FCS) overnight. The following day 2ml of ACM or 2ml of M199 control media was added to the wells and the plate was incubated at 37°C and 5% CO_2 for 24hr or 48hr depending on the experiment. RNA was then harvested from the cells using the TriReagent protocol and supernatant was stored at -80°C.

2.4 Barrett's oesophagus biobank

Male patients (>40 years old) attending an out patient endoscopy clinic were recruited to the Barrett's oesophagus biobank. All recruited patients gave informed consent for use of their adipose tissue in this study, and the study obtained ethical approval for St James's Hospital Review Board. Patients were seperated into viscerally obese and non obese categories based on waist circumference cut off measurement of 94cm as specified by the International Diabetes Federation (IDF). Waist circumference was measured at the midpoint between superior iliac crest and subcostal margin, height and weight was recorded and used to determine BMI.

2.4.1 Biobanking

Four biopsies of Barrett's metaplasia were taken from patients, attending an outpatient endoscopy clinic, at 2cm intervals using a disposable biopsy forceps. Samples were briefly stored on saline gauze at room temperature before transfer to the lab. A single biopsy of Barrett's metaplasia from each patient was placed in RNAlater immediately upon receipt. A single Barrett's metaplasia biopsy was also cultured in 1ml of serum free M199 media for 24hr at 37°C and 5% CO_2 to produce Barrett's conditioned media (BCM). This media was collected and stored at -80°C. Two 3ml fasting blood samples were taken in serum collection tubes during the clinic. The samples were centrifuged at 750 x g for 10 min to seperate the serum which was then stored at -80°C.

Serums taken as part of the Chemoprevention of Premalignant Intestinal Neoplasia (ChOPIN) study, a multicentre trial in Ireland and the U.K which aims to validate putative cancer biomarkers in Barrett's oesophagus, were also included in the biobank for screening.

2.5 Investigation of RNA expression

2.5.1 RNA isolation from Barrett's metaplasia biopsies and cell lines using Qiagen Rneasy kit

RNA was extracted using Qiagen Rneasy Mini kit (Qiagen Inc., CA, USA). Barrett's metaplasia biopsies were homogenised in 600µl Buffer RLT using a Qiagen TissueLyser (Retsch GmbH & Co., Haan, Germany) at 250rpm for 6 min with one 5mm stainless steal bead (Qiagen Inc., CA, USA) added to each sample to aid homogenisation. The bead was removed and samples were centrifuged at 12000 x g for 3 min at 4°C, and supernatant was transferred to a new tube by pipetting. 600µl of ethanol (70% v/v) was added to the supernatant and mixed immediately by

pipetting and transferred in volumes of 700µl to an Rneasy spin column placed in a 2ml collection tube. The spin column was centrifuged at 12000 x g for 15s and the flow through was discarded. The spin column was washed by first applying 700µl buffer RW1, then 500µl buffer RPE with centrifugation at 12000 x g for 15s at room temperature between each step. A final wash step in 500µl buffer RPW was centrifuged for 2 min in order to dry the membrane and ensure no ethanol was carried over. RNA was eluted in 30 µl of Rnase free water by centrifugation for 1 min at at 12000 x g at room temperature.

For Human Cancer Pathway Profiler Arrays, RNA was isolated from BAR-T and HET-1A cells using Qiagen Rneasy kit as described, omitting the homogenisation step.

2.5.2 RNA isolation from cell lines using TriReagent method

RNA to be used for reverse transcription polymerase chain raction (RT-PCR) and quantitative real time polymerase chain reaction (qPCR) was isolated from cell lines using the TriReagent method. RNA was isolated from cell lines using 1ml of TriReagent (Molecular Research Center, Montgomery Road, OH, USA), containing a mixture of guanidine thiocyanate and phenol in monophasic solution. Samples lysed in TriReagent were stored at -80°C unti RNA extraction. Total RNA was isolated as follows: samples were thawed and stored at room temperature for 5 min following which 100µl of Bromo-3-chloro-propane (BCP) was added. Samples were then vortexed for 15s and allowed to stand at room temperature for 15 min. Following centrifugation at 12000 x g at 4°C for 15 min, the upper aqueous phase was carefully removed to a new tube and subsequent steps were carried out at room temperature. 500µl of isopropanol was added to the tube and incubated for 10 min followed by centrifugation at 12000 x g for 8 min. The supernatant was decanted and the pellet washed in 1ml ethanol (75% v/v) by brief vortexing followed by centrifugation at 12000 x g for 5 min. The ethanol was decanted and the pellet allowed to air dry for 3-5 min. The pellet was resuspended in 40µl Rnase free water.

2.5.3 RNA quantification and purity analysis

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

relatively pure RNA yield, while a 260:230 ratio greater than 1.7 indicated the sample was free of phenol contamination.

2.5.4 cDNA synthesis

The reverse transcriptase enzyme and buffer were purchased from Bioline (Bioline, Kilkenny, Ireland), all other reagents were purchased from Invitrogen (Invitrogen Corp., CA, USA). cDNA was synthesised by adding random primers (0.5 μg/μl) to 250ng total RNA and heating the sample to 70°C for 10 min in order to denature the RNA, and then immediately chilled on ice for at least 1 min. A master mix containing reverse transcription components was then added to each sample containing: RNaseOUT recombinant ribonuclease inhibitor (1 Unit/ μl), dNTPs (10mM, prepared as a 1:1:1:1 ratio of dATP, dGTP, dTTP and dCTP), Bioscript reverse transcriptase (200units/μl) in 5X Bioscript reaction buffer. This mixture was them incubated at 37°C for 1 hr and 70°C for 10 min. The resulting cDNA was stored at -20°C and 1μl of cDNA template was used to investigate gene expression.

Table 2.1. Reagents and volumes used in cDNA synthesis

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

For Human Cancer Profiler Arrays, cDNA synthesis was generated using First Strand cDNA synthesis kit (Super Array BioScience Corporation, Frederick, MD, USA). 250ng of each RNA sample was combined with 1ml of random primers in a sterile PCR tube and made up to a final volume of 10μl with RNase free water. The contents were vortexed briefly and centrifuged before being placed in a thermal cycler at 70°C for 3 min, followed by 37°C for 10 min. 10μl of reverse transcription cocktail (4μl of 5x RT-Buffer, 4μl of RNase free water, 1μl of RNase inhibitor and 1μl of reverse transcriptase) was added to each sample, briefly vortexed and centrifuged before incubating at 37°C for 1 hr. Samples were then heated to 95°C for 5 min to

degrade the RNA and deactivate the reverse transcriptase. 80µl of RNase free water was added to each sample and mixed well to give a final volume of 100µl. Samples were stored overnight and run on PCR arrays the following day (section 2.5.10).

2.5.5 Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was used as a template for PCR. The following PCR master mix was added to each sample: Mangomix® Master mix (Bioline, Kilkenny, Ireland) forward primer (20µM), reverse primer (20µM) and RNase free water. 1µl cDNA was used per reaction and a non template control was included for each primer set by replacing cDNA with 1µl of RNase free water. Template cDNA was denatured at 94°C for 5 min, and each cycle consisted of primer annealing (55-62°C for 1 min, optimised for each target) and extension (72°C for 1 min). This was followed by an elongation step to complete the amplification cycle (72°C for 5 min).

Table 2.2. Reagents and volumes used in RT-PCR

Reagent	**Volume (µl) (per sample)**
Mangomix® Master Mix	10
Forward Primer	2
Reverse Primer	2
H_2O	6
cDNA	1
Total Volume	**21**

All reverse transcription primers were purchased in lyophilised form from Metabion (Metabion International, Martinsried, Germany) and rehydrated to a stock concentration of 400µM in RNase free water. Primer sequences and number of amplifaction cycles used in PCR are outlined in the below table.

Table 2.3. Primer sequences in RT-PCR

Gene	Forward Primer	Reverse Primer	Cycle
GAPDH	5'-GCAGGGGGGAGCCAAAAGGG-3'	5'-TGCCAGCCCCAGCGTCAAAG-3'	30
Villin	5'-AATGGCCACCATGGAGAACA-3'	5'-ACCACAATTCTGTCTTTCACGG-3'	35
CK8	5'- AACAACCTTAGGCGGCAGCT -3'	5'- GCCTGAGGAAGTTGATCTCG -3'	35

2.5.6 Agarose gel electrophoresis

Agarose gels were prepared in Tris-Acetate-EDTA (TAE) buffer (40mM Tris HCl (pH8.3), 20mM acetic acid, 1mM EDTA). A 1.4% agarose solution was heated in a microwave for 90s to allow the agarose to dissolve. It was allowed to cool to approximately 55°C before adding SYBR® safe DNA gel stain (10000 x concentration in DMSO, Invitrogen corporation, CA, USA) to a final concentration of 1µg/ml. The solution was poured into a gel tray with a comb to a depth of 3-5mm and allowed to set.

The DNA samples, containing loading buffer present in the Mangomix®reagent were loaded into the agarose gel. Electrophoresis of the samples was carried out at 100 volts in an EC-360 Maxicell gel system (EC Apparatus Corp., FL, USA), using 1x TAE as a running buffer. The bands were visualised and photographed under UV light using a Biospectrum imaging system (Ultra Violet Products, Cambridge, UK).

2.5.7 Quantitative real time PCR (qPCR)

Quantitative PCR was used to quantify mRNA expression in samples relative to the 18s ribosomal RNA endogenous control. This was carried out using a probe based method, which works as follows: sequence specific probes are labelled with a flourescent reporter molecule at the 5' end, and a flourescence quencher molecule at the 3' end. The close proximity of the reporter to the quencher prevents detection of flourescence. Upon transcription, the 5-3 exonunclease activity of the Taq polymerase removes the quencher molecule and allows fluorescence emission, detected following laser excitation of the sample. All reagents were purchased from ABI Biosystems (CA, USA). A master mix containing primer/probe and Taqman® Gene Expression Master Mix, was added to 1µl of cDNA template (see table below). A final volume of 20µl was pipetted into a well of a MicroAmp™ Optical 96 well reaction plate in duplicate(ABO Biosystems, CA, USA) and a real time PCR was performed on the ABI Prism 7500 (ABI Biosystems, CA, USA) real time thermal cycler. The plate was heated to 95°C for 15 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The threshold cycle (Ct) for each well was calculated using the instrument software and data analysis was carried out using Microsoft Excel. Data analysis was based on the ΔΔCt method with raw data normalised by the 18s housekeeping gene included on the plate.

Table 2.4. Reagents and volumes used for qPCR

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

2.5.8 Human Cancer Profiler array validations and gene expression in Barrett's metaplasia biopsies

Real time PCR was carried out to validate gene targets identified on the Human Cancer Profiler Array in the HET-1A, BAR-T, QH, GO and OE33 cell lines, and Barrett's metaplasia biospies from consented patients. Real time PCR of the gene targets was also carried out on the murine 3T3 feeder layer to ensure potential carry through of murine RNA in BAR-T RNA extractions did not influence results.

Taqman fluorogenic gene expression probe sets (ABI Biosystems, CA, USA) were used for all gene expression experiments. All probe sets used were FAM labelled: MMP1 Assay ID: Hs00899658_m1 TIMP3 Assay ID: Hs00165919_m1. IL8 Assay ID: Hs01567912_m1: MCAM Assay ID: Hs001744838_m1: VEGFA Assay ID: Hs00900055_m1. Quantitative normalisation of cDNA in each sample was performed using the ribosomal RNA subunit 18s (Assay ID: Hs 99999901_s1) as an endogenous control.

2.5.9 Real time PCR of spindle assembly checkpoint genes and telomere binding proteins

Real time PCR was carried out in the in the HET-1A, QH, GO and OE33 cell lines to investigate gene expression of spindle assembly checkpoint genes (SAC) and telomere binding proteins after ACM treatment of these lines. Taqman fluorogenic gene expression probe sets (ABI Biosystems, CA, USA) were used for all gene expression experiments. All probe sets used were FAM labelled: BUB1B Assay ID: Hs01084828_m1: CENPE Assay ID: Hs01068241_m1: CDC20 Assay ID: Hs00415851_g1: ESPL1 Assay ID: Hs00202246_m1: MAD2L2 Assay ID: Hs01057448_m1: DKC1 Assay ID: Hs00154737_m1: TPP1 Assay ID: Hs00166099_m1: TERF1 Assay ID: Hs00744634_m1: TERF2 Assay ID: Hs00194619_m1: TERT Assay ID: Hs00972656_m1:

POT1 Assay ID: Hs00209984_m1: TEP1 Assay ID: Hs00200091_m1: TINF2 Assay ID: Hs00173291_m1: TERF2IP Assay ID: Hs00430292_m1: TRC Assay ID: Hs03297287_s1. Quantitative normalisation of cDNA in each sample was performed using the ribosomal RNA subunit 18s (Assay ID: Hs 99999901_s1) as an endogenous control.

2.5.10 Human Cancer Pathway Finder Arrays

Changes in the expression profile of a panel of genes representative of molecular pathways of tumourigenesis were examined using RT^2 profiler™ Human Cancer Pathway Finder PCR Array technology (Super Array Bioscience Corporation, Frederick, MD, USA). This array is based on a 96 well plate format, containing primer sets for 84 genes involved in six biological pathways of tumourigenesis: apoptosis, cell cycle, angiogenesis, invasion and metastasis, signal transduction and adhesion, as previously described (Hanahan and Weinberg, 2000). Five housekeeping genes were included to normalise the data and two negative controls were included to estimate the level of genomic DNA contamination in the PCR system. A full list of genes measured on this array are listed in Appendix III.

Samples were prepared for loading onto the qPCR array by adding 1225µl of 2X SuperArray PCR master mix and 1127µl RNase free water to 98µl of the diluted cDNA synthesis reaction to give a final volume of 1225µl. 25µl of reaction mix was added to each well except the non template control, to which the same mix was added with RNase free water instead of cDNA. Real time PCR detection was performed on an ABI Prism 7500 (ABI Biosystems, CA, USA) real time thermal cycler. The plate was heated to 95°C for 15 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The threshold cycle (Ct) for each well was calculated using the instrument software and data analysis was carried out using a Microsoft Excel based data analysis template. Data analysis was baed on the ΔΔCt method with raw data normalised by the house keeping genes included on the plate. Fold changes in gene expression observed in the RT-PCR array in the analysis were validated using qPCR using the protocols outlined previously in section 2.4.8. Gene changes were only reported if the transcript was amplified before 35 cycles.

2.6 Telomere Length

2.6.1 DNA extraction

Cells were seeded in 12 well plates at a concentration of 3×10^4 cells/ml and allowed to adhere to the plate for at least 6 hr. Cells were then serum starved in media containing 0.5% FCS and subsequently grown in ACM generated from surgically excised visceral adipose tissue from

both obese and non obese male oesophageal adenocarcinoma patients, for 24hr and 48hr. Genomic DNA was extracted by adding 1ml DNA lysis buffer directly to the well. The constituents of the lysis buffer are listed below in Table 2.5

Table 2.5. Constituents of DNA lysis buffer

Constituent	Volume (ml)
10mM Tris HCl pH 8.0	5
0.5M EDTA	0.5
10% SDS	1
5M NaCl	2
20mg/ml proteinase K	0.25
Deionised H_20	41.25
Final Volume	**50**

Cells were scraped with cell scrapers (Sarstedt, Wexford, Ireland), transferred to 2ml eppendorfs and were incubated at 37°C for 3 hr. 1ml of isopropanol was added to the lysate and samples were left to stand at room temperature for 30 min before centrifugation at 12000 x g at 4°C. The supernatant was decanted and 0.5 ml of 70% (V/V) ethanol was added to the DNA pellet. The sample was subsequently centrifued at 3500 x g for 5 min and the pellet was resuspended in 30µl of DNase free water.

2.6.2 DNA quantification and purity analysis

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

2.6.3 Telomere Length PCR

Telomere length was measured by Quantitative-PCR using a method adapted from the one origanally described by Cawthon (Cawthon 2002) . For each sample, two PCRs were performed: the first one to amplify the telomeric DNA and the second one to amplify a single-copy control gene (36B4, acidic ribosomal phosphoprotein PO). This provided an internal

control to normalize the starting amount of DNA. A five-point standard curve (2-fold serial dilutions from 10 to 0.625ng of DNA) was included in all PCRs to allow the transformation of Ct (cycle threshold) into nanograms of DNA. All samples were run in triplicate and the median was used for subsequent calculations. The amount of telomeric DNA was divided by the amount of control-gene DNA, producing a relative measurement of the telomere length of the sample. Two control samples were run in each experiment to allow for normalization between experiments and periodical reproducibility experiments were performed to guarantee correct measurements.

All telomere length PCR reactions were run in the ABI Prism 7500 (ABI Biosystems, CA, USA) real time thermal cycler. 2ng of genomic DNA was run per sample per well and all samples were run in duplicate in a final volume of 20µl. Each reaction included 2X Quantifast ™ SYBR green PCR master mix (Qiagen Inc., CA, USA), 1µl of forward and reverse primer (Metabion, Germany) and 7µl of DNase free water. The telomere PCR used 300nM of each primer (tel1b: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT; tel2b: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT). and 30 cycles of amplification at 95°C for 15s and at 56°C for 60s. The control gene PCR used 300nM of forward primer (36B4u: CAGCAAGTGGGAAGGTGTAATCC) and 500nM (36B4d: CCCATTCTATCATCAACGGGTACAA) of reverse primer and 35 cycles of amplification at 95°C for 15s and at 56°C for 20s and 72°C for 20 s. Both PCR reactions had an initial denaturation step at 95°C for 15 min. Ct values for each sample were converted into nanograms of DNA using standard curves.

Ct values from the telomere assay were normalized to the single gene reference. The telomere length (x) from each sample was based on the telomere to single copy gene ratio (T/S ratio) and was based on the calculation of the ΔC_T [C_T(telomere)/C_T(single gene)]. Telomere length was expressed as a relative T/S ratio, which was normalized to the average T/S ratio of the reference sample [$2^{-(\Delta CT_x \cdot \Delta CT_r)} = 2^{-\Delta\Delta C_T}$].

2.7 Anaphase Bridging

2.7.1 Staining of Anaphase Bridges

Cells were seeded in 12 well plates at a concentration of 3 x 10^4 cells/ml for a 24hr ACM treatment and 1.5 x 10^4 cells/ml for a 48hr ACM treatment and allowed to adhere to the plate for at least 6 hr. Cells were then serum starved in media containing 0.5% FCS and subsequently grown in ACM generated from excised ommental adipose tissue from both obese and non obese male oesophageal adenocarcinoma patients, or M199 control, for 24 and 48 hr at 37°C . The cells were then washed with PBS and fixed to wells with 3.7% paraformaldeyde at room

temperature for 15 min. The cells were then stained with a 1 in 2 dilution of haemotoxylin in PBS for 30 min at room temperature. The fixed cells were then washed once with tap water to remove excess stain, and then washed in 50% and 100% ethanol before drying on the bench for 1 hr. Anaphase bridges were counted using a Olympus CKX41 (Toyko, Japan) microscope at 100x magnification. Number of anaphase bridges counted were normalised for cell number with a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and expressed as a percentage of the M199 control.

2.7.1.1 IL-8 and VEGF quenching in ACM

Cells were seeded in 12 well plates and grown in ACM and M199 control as described previously (section 2.7.1). At t=0 these cells were then treated with either anti VEGF neutralising antibodies (R&D Systems) to a final concentration of 100ng/ml/well or anti-IL8 neutralising antibodies (R&D systems) to a final concentration of 1µg/ml/well and grown for 24 hr at 37°C in 5% CO_2. Cells were subsequently fixed and stained as described previously (section 2.7.1).

2.7.2 MTT assay

Cells were seeded in 12 well plates at a concentration of 3 x 10^4 cells/ml for a 24hr ACM treatment and 1.5 x 10^4 cells/ml for a 48hr ACM treatment and allowed to adhere to the plate for at least 6 hr. Cells were then serum starved in media containing 0.5% FCS and subsequently grown in ACM generated from excised ommental adipose tissue from both obese and non obese male oesophageal adenocarcinoma patients, or M199 control, for 24 and 48 hr. MTT reagent was dissolved in M199 media was added to the wells to a final concentration of 0.5mg/ml and incubated for 2 hr at 37°C. The media was subsequently removed, the cells washed with PBS, and 600µl of DMSO was added to each well to lyse the cells. The 12 well plate was incubated in the dark for 5 min to allow colour development and contents of the wells were transferred to a 96 well plate and read immediately at 450nm using an Alpha Fluor Plus Spectrophotometer (Tescan Trading AG, Switzerland) to determine cell number.

2.7.1.2 IL-8 and VEGF quenching in ACM

Cells were seeded in 12 well plates and grown in ACM and M199 control as described previously (section 2.7.2). At t=0 these cells were then treated with either anti VEGFA neutralising antibodies (R&D Systems) to a final concentration of 100ng/ml/well or anti-IL8 neutralising antibodies (R&D systems) to a final concentration of 1µg/ml/well and grown for 24 hr at 37°C in 5% CO_2. Cells were subsequently treated with MTT agent as described previously (section 2.7.2) and cell number calculated.

2.8 ELISA

Enzyme linked immunosorbent assays (ELISAs)(R&D systems, Inc., MN, USA) were used to determine IL8, IL-6 and VEGF levels in ACM. ELISA plates (Greiner-Bio, Frickenhausen, Germany) were coated with with 50µl capture antibody at room temperature overnight, and washed three times in PBS (with 0.05% Tween 20). Samples were diluted to appropriate concentrations in 1% BSA, as determined by optimisation steps and 50µl of sample was loaded to each well in triplicate, with the exception of blanks which contained only 1% BSA. For the VEGF ELISA, the patient ACM samples were loaded neat onto the plate. For the IL8 ELISA, the patient serum samples were loaded neat onto the plate but the ACM samples required a 1/200 dilution as determined by optimisation. A serial dilution was made from the standard supplied and 50µl of each concentration of the standard was loaded onto the plate in triplicate. Samples were incubated at room temperature overnight and the plate was washed three times in PBS (with 0.05% Tween 20). 50µl of detection antibody was added to each well and the plate was incubated for 2 hr at room temperature. The plate was washed three times in PBS (with 0.05% Tween 20) and 50µl of streptavidin-HRP (1/200 dilution in 1% BSA) was added to each well and incubated at room temperature for 20 min. 50µl of substrate tetramethylbenzidine (TMB) was added to each well and placed in the dark until the colour developed (2-10 min). 25µl of H_2SO_4 was added to each well to stop the reaction and the plate was read immediately at 450nm using an Alpha Fluor Plus Spectrophotometer (Tescan Trading AG, Switzerland). Protein concentrations were determined by interpolating from a standard curve of known concentrations (Appendix).

2.9 BrdU cell proliferation assay

Cells were seeded at a dilution of 5×10^3 cells per well in appropriate maintenance medium in 96 well plates and allowed to adhere for a minimum of 6hr. They were then serum starved (media containing 0.5% FCS) overnight. The following day they were incubated with 100µl of ACM or the required treatment for a period of 24 hr and cell proliferation was assessed using BrdU (5-bromo-2'- deoxyuridine) cell proliferation ELISA (Roche Diagnostics Ltd., Sussex, UK). The pyrimidine analogue BrdU is incorporated in the place of thymine in the newly synthesised DNA of proliferating cells, and is bound by the BrdU anti-POD antibody. The antibody complexes are then detected by a subsequent substrate reaction.

BrdU label (1/1000 dilution in appropriate cell culture media) was added to each well (except no BrdU label control) for 3 hr at 37°C, following which the media was tapped and flicked off. Cells were fixed by adding 200µl of fixative solution (Roche Diagnostics Ltd., Sussex, UK) for 30

min at room temperature. 100µl anti BrdU-POD (mouse monoclonal antibody, peroxidase conjugated) was added to each well and the plate was incubated for 90 min at room temperature. Cells were then washed in PBS and 100µl of substrate solution was added to each well. The plate was incubated at room temperature for 5-10 min or until colour development was sufficient for photometric detection. 25µl H_2SO_4 was then added to stop the reaction and the plate absorbance was read at 450nm on an Alpha Fluor Plus spectrophotometer (Tescan Trading AG, Switzerland). Wells containing cells but no BrdU label were used to subtract back ground absorbances and percentage increase/ decrease in proliferation was calculated relative to untreated cells.

2.10 Cell Invasion Assay

Migration assays were carried out using the QCM well Cell Invasion assay (Millipore, Watford, UK). This assay is performed in a 96 well plate, each well containing a chamber lined with an 8µm pore size polycarbonate membrane. The membrane pores are occluded by a thin coating of extra cellular matrix (ECM), blocking non invasive cells from moving through. Cells with invasive properties, on the other hand, can move through the ECM layer and porous membrane and cling to the bottom of the chamber. These invasive cells are dislodged following incubation in detachment buffer, lysed and detected with a fluorescent dye.

The assay was performed in a Grade II laminar hood. 100µl of prewarmed serum free media was added to the chambers of the invasion assay and incubated at room temperature for 90 min to rehydrate the ECM. Adipose conditioned media for viscerally obese male oesophageal adenocarcinoma patients, and control M199 media, was place in the feeder wells beneath the chambers in triplicate. Cells were grown to 70% confluency and serum starved (media containing 0.5% FCS) overnight. They were then trypsinised and seeded at a dilution of 1×10^5 cells per chamber. Following an incubation period of 24 hr at 37°C in 5% CO_2, non invasive cells which had remained in the chamber were flicked and tapped off. The chambers were rinsed by placing the plate in a feeder tray of PBS for 1 min. The PBS was replaced by cell detachment solution and the chamber plate was incubated in this solution for 30 min at 37°C. Cells were dislodged by gently tilting the chamber plate back and forth several times during the incubation period. A lysis buffer containing fluorescent dye was made up for all samples by diluting CyQuant fluorescent dye 1:75 with 4x lysis buffer. 50µl of this solution was added to each well of the feeder tray containing the cells which had invaded through the membrane. The tray was incubated at room temperature for 15 min and 150µl of this mixture was transferred to a fluorescence compatible plate and the proportion of cells which had invaded

through the chamber was determined using a fluorimeter (Wallac Victor 2 1420, Perkin Elmer, Ballymount, Dublin) at the 485nm/ 535nm filter set. Control wells, containing all components but no cells, were used as blanks and these fluorescent values were subtracted from all others in order to interpret the data. Results were calculated as a percentage increase in invasive capacity relative to untreated cells.

2.11 Statistical Analysis

Statistical analysis was computed using Graphpad Prism 5 software. All data are expressed as mean ± SEM. SEM is calculated as the SD of the original sample divided by the square root of the sample size. Student's t-tests were used to compare the means of two groups. In cases were data were paired (ie tumour/normal matched samples from the same patient) a paired t-test was used for statistical analysis. Otherwise an unpaired t-test was used. For all statistical analysis, a probability of (p) of ≤ 0.05 was considered to represent significant difference between groups.

Chapter 3: Identification of a pro-tumour gene expression signature activated by adipose conditioned media in a non-neoplastic Barrett's oesophagus cell line model

3.1 Introduction

Gastro-oesophageal reflux disease (GORD) has long been established as a major risk factor for Barrett's oesophagus and oesophageal adenocarcinoma (Lagergren, Bergstrom et al. 1999), causing inflammation and destruction of the oesophageal squamous mucosa and subsequent regeneration with columnar epithelium (Jenkins, Doak et al. 2002). However, it has been demonstrated that despite long term exposure of the oesophageal epithelium to acid reflux insult, some patients will never develop glandular metaplasia whilst others have a particular propensity to do so (Vaughan, Edelstein et al. 2007), and in patients that do develop Barrett's oesophagus, only a minute fraction subsequently develop oesophageal adenocarcinoma (Spechler, Robbins et al. 1984; Cameron, Ott et al. 1985). GORD is relatively common in both sexes and across all ethnicities, but the risk of developing Barrett's oesophagus and the subsequent progression to OAC is far greater in men of Caucasian ethnicity compared to any other group (Mitas, Almeida et al. 2005), indicating that other factors besides GORD are involved in Barrett's derived carcinogenesis. Reflux symptoms themselves can prove a poor predicator of Barrett's oesophagus. In a recent endoscopic study of 1033 adults from 2 Italian villages, 46.2% of patients who presented with Barrett's oesophagus were asymptomatic (Zagari, Fuccio et al. 2008).

It is generally understood that following insult from the components of GORD, stem cells of squamous mucosa undergo an altered differentiation during repair, and the cells begin to display traits atypical from mucosal gastric stem cells (Jankowski, Wright et al. 1999). Deoxycholic acid, a major constituent of gastro refluxate (Nehra, Howell et al. 1999), has been demonstrated to activate tumourigenic pathways by a number of authors. DCA can activate NF-κB pathways allowing cells to evade apoptosis in Barrett's oesophagus (Souza, Huo et al. 2011) and has also been demonstrated to promote reactive oxygen species genotoxicty in Barett's oesophagus (Jenkins, D'Souza et al. 2007). DCA may also promote carcinogenesis through the suppression of p53 by stimulating proteasome mediated p53 protein degradation in colon cancer (Martinez, Qiao et al. 2001).

Obesity, in addition to GORD, is a well-defined causal factor in Barrett's oesophagus and OAC. Obese patients suffer from increased intra-abdominal and intra-gastric pressures compared to their non-obese counterparts (La Vecchia, Negri et al. 2002) and have a more relaxed lower oesophageal sphincter (Orlando 2001). Merry *et al* reported direct correlations between BMI, a standard measure of obesity, in early adult life and incidences of OAC (Merry, Schouten et al.

2007). They found that a high baseline and subsequent increases in BMI, above the age of 20 but not before, are strongly associated with the risk of a patient developing OAC (Merry, Schouten et al. 2007). Interestingly, other groups reported that it is the particular deposition of fatty tissue, independent of overall BMI that is a risk factor for Barrett's oesophagus (Corley, Kubo et al. 2008). A study involving 36 Barrett's oesophagus patients suggested that increased abdominal or visceral fat was more important in influencing Barrett's oesophagus neoplastic progression rather than overall BMI itself (Corley, Kubo et al. 2008). This observation may go some way to explaining the increased rates of Barrett's oesophagus in men, as visceral obesity is more common in men than women. In a retrospective cross sectional study of 65 cases of Barrett's oesophagus and 385 cases without Barrett's oesophagus, each 10-pound increase in weight or 5 point increase in BMI was associated with 10% and 35% increase in risk of Barrett's oesophagus, respectively (Stein, El-Serag et al. 2005). Visceral fat (fat that is stored centrally) is more metabolically active than fat that is stored subcutaneously, and evidence demonstrates that this fat has increased production of adipokines such as adiponectin, leptin, visfatin and resistin and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Corley, Kubo et al. 2008).

There is much evidence in the literature to suggest that fat is metabolically active (Gallagher, Visser et al. 1996; Gutierrez, Puglisi et al. 2009; Mohan, Indulekha et al. 2011) and this activity can pose a considerable cancer risk, particularly in oesophageal adenocarcinoma (Chow, Blaser et al. 1998). Visceral adipose tissue has been associated with increased serum levels of both IL-6 and TNFα, two potent pro-inflammatory adipocytokines (Cannon, Nerad et al. 1993; Xu, Barnes et al. 2003), and VEGFA, an angiogenic mediator (Takahashi, Miyazawa-Hoshimoto et al. 2003).

Along with obesity and GORD, it has been tentatively suggested that hypoxia may also promote carcinogenesis in Barrett's oesophagus. Hypoxia inducible proteins erythropoietin, erythropoietin receptor and VEGF increase in expression in the metaplasia-dysplasia-adenocarinoma sequence associated with neoplastic Barrett's oesophagus progression (Griffiths, Pritchard et al. 2007), although the association of hypoxia and Barrett's has not been studied in depth. Hypoxia has previously been shown to drive carcinogenesis in prostate cancer and endometrial cancer (Zhong, Semenza et al. 2004; van Diest, Horree et al. 2007) and thus may also play a role in Barrett's derived carcinogenesis.

An influential manuscript was published in Cell in 2000 entitled "The Hallmarks of Cancer " (Hanahan and Weinberg 2000), where the authors identified six fundamental characteristics

that are applicable across the constellation of cancers. These hallmarks were identified as: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis. In this chapter we aimed to identify genes and pathways associated with these hallmarks activated by obesity in a Barrett's oesophagus cell model, the BAR-T cell line.

The BAR-T cell line used in this project developed from a biopsy from a Barrett's oesophagus patient who later in life went on to develop oesophageal adenocarcinoma. This cell line is ideal for use in this study, as is "pre-programmed", with all the molecular mechanisms related to carcinogenesis arising in Barrett's oesophagus. The BAR-T cells exhibit non-neoplastic properties, such as contact inhibition and anchorage-dependent growth. The cells maintain a diploid chromosome number, have histological differentiation markers characteristic of benign Barrett's epithelium such as cytokeratin 8 and villin, and also maintain appropriate expression of p21 and p53 (Jaiswal, Morales et al. 2007). This cell line is proposed to be a useful model for the study of the early events in carcinogenesis in Barrett's oesophagus (Jaiswal, Morales et al. 2007; Das, Kong et al. 2011) and it has been successfully used to explore the molecular mechanisms by which environmental factors (e.g. acid and bile exposure) promote cancer development in Barrett's oesophagus (Souza, Zhang et al. 2007; Bajpai, Liu et al. 2008; Zhang, Zhang et al. 2009).

Dysregulation of genes associated with the hallmarks of cancer have all been previously associated to carcinogenesis arising in Barrett's oesophagus (Zhang, Spechler et al. 2009) but have not been related directly to obesity previously. Obese Barrett's oesophagus patients show increased incidences of progression to oesophageal adenocarcinoma (Chow, Blot et al. 1998; Holmes and Vaughan 2007; Corley, Kubo et al. 2008) and the principle aim of this chapter was to identify the carcinogenic pathways and specific genes activated by obesity in Barrett's oesophagus.

3.2 Aims and Objectives

The overall aim of this chapter was to investigate how obesity and excess adiposity is related to activation of oncogenic pathways in a Barrett's oesophagus cell model and to compare activation of these pathways with other reported causal factors of Barrett's oesophagus. Gene expression changes in the BAR-T cell line were examined in response to culture of the BAR-T cell line in adipose conditioned media generated from surgically excised omental adipose tissue from consenting oesophagectomy patients.

Specific Objectives

- Investigation of the pro-tumourigenic properties of adipose conditioned media generated from subcutaneous adipose tissue and visceral adipose tissue
- Identification of cancer related genes activated by adipose conditioned media generated from viscerally obese oesophageal adenocarcinoma patients
- Evaluate efficacy of gene activation between adipose conditioned media generated from viscerally obese and non-obese oesophageal adenocarcinoma patients
- Compare and contrast activation of genes by adipose conditioned media with other known mechanisms associated with neoplastic progression in Barrett's oesophagus

3.3 Results

3.3.1 Pro-tumourigenic properties of subcutaneous adipose conditioned media (sACM) and visceral adipose conditioned media (vACM)

Key to the research conducted over the course of this PhD was the establishment and maintenance of an adipose tissue biobank. Subcutaneous and visceral fat was surgically excised from consented gastrointestinal cancer patients undergoing surgical oesophageal and colorectal resection. Adipose conditioned media (ACM) was generated from culturing both subcutaneous and omental adipose tissue in M199 for 72hr (section 2.3.2) and it was this media that was used to mimic an obese microenvironment in vitro for investigate the effects of obesity on a Barrett's oesophagus cell model. The biobank was established in 2007 and at the time of submission of this thesis, contains ACM from 281 patients (Table 3.1). Patients had a mean age of 66.4 years, and 66% were male. Over one third of patients (36.6%) met the International Diabetes Federation definition for the Metabolic syndrome. Approximately 55% of these patients were centrally obese by waist circumference (>80 cm females, >94 cm males) and CT measurements (VFA > 130 cm^2), while just over 16% were deemed to be obese by BMI. This observation underlines the importance of adequate assessment of obesity status in patients, particularly central obesity, which is linked to metabolic abnormalities and obesity associated comorbidities. Only ACM generated from oesophageal adenocarcinoma patients undergoing surgical oesophageal resection was used in this thesis.

Table 3.1. Patient demographics of the adipose tissue biobank

n = 281		*n*	%
Age (years)	Mean + SEM	66.4 ± 0.6	
	Range	31–96	
Gender	Male	185	66
Cancer type	Oesophageal adenocarcinoma	119	42.40
	Oesophageal squamous cell carcinoma	28	9.90
	Gastric cancer	21	7.50
	Colorectal adenocarcinoma	113	40.20
Body mass index[a]	Mean + SEM	25.3 ± 0.24	
	Range	15.43–41.02	
	Underweight (<19.99)	18	7.30
	Normal (20–24.99)	90	36.40
	Overweight (25–29.99)	99	40.10
	Obese (>30)	40	16.20
Central waist circumference (cm)[b]	Mean + SEM	92.45 ± 0.72	
	Range	61–130	
	Normal	114	43.70
	Central obesity (>80 cm female, >94 cm male)	147	56.30
CT visceral fat area (cm^2)[c]	Mean ± SEM	142.97 ± 8.2	
	Range	5.8–338.5	
	Normal	72	44.70
	CT viscerally obese (VFA > 130 cm^2)	89	55.30
CT subcutaneous fat area (cm^2)	Mean ± SEM (cm^2)	176.0 ± 7.88	
	Range	5.4–479.7	
Metabolic syndrome status[d]	Meets criteria (IDF 2006)	89	36.60

IDF: International Diabetes Federation Definition 2006
[a] BMI was available for 247 patients only
[b] Waist circumference was available for 261 patients only
[c] CT scans were available for 161 patients only
[d] Metabolic syndrome status available for 243 patients only

The proliferative capacity and the protein concentration of VEGF and IL-6 was determined in ACM generated from both subcutaneous adipose tissue and visceral adipose tissue in order to determine which ACM subtype would elicit greater pro-cancer responses in the oesophageal

cell models utilised in this study. Increased cellular proliferation is a well-established feature of carcinogenesis (Moolgavkar 1993) while increased levels of VEGF (Talar-Wojnarowska, Gasiorowska et al. 2010) and IL-6 (Chen and Huang 2009) have also been associated with carcinogenesis.

OE33 cells, representative of oesophageal adenocarcinoma, were cultured in the presence of subcutaneous ACM (sACM) or visceral ACM (vACM) generated from either centrally obese or non-obese patients, and a BrdU ELISA was used to determine cellular proliferation. Overall, higher levels of cellular proliferation were observed following culture of cancer cells with vACM compared with sACM. Significantly higher levels of cellular proliferation were demonstrated in response to ACM generated from centrally obese patients following culture with both sACM ($p=0.015$) and vACM ($p=0.0004$) compared to the untreated control. vACM generated from obese patients elicited significantly higher cellular proliferation ($p=0.006$) compared to vACM generated from non-obese patients, a result that was not replicated following culture with sACM (Figure 3.1A-B). While a significant increase ($p=0.011$) was observed in cell proliferation following culture with vACM from non-obese patients over that of media alone, there was no significant increase in proliferation demonstrated following culture with sACM from non-obese patients over that of media alone.

The protein concentration of two well-defined adipose derived factors, IL-6 (Kanaya, Harris et al. 2004) and VEGF (Garcia de la Torre, Rubio et al. 2008)were assessed in sACM and vACM using commercially available ELISAs (section 2.8). Significantly elevated levels of IL-6 ($p=0.012$) and VEGF ($p=0.001$) were observed in vACM compared to sACM (Figure 3.1C-D). Visceral adipose tissue is known to be more metabolically active than subcutaneous adipose tissue (Smith, Lovejoy et al. 2001; Fox, Massaro et al. 2007) and increased production of these two adipose derived factors by visceral adipose tissue in tandem with increased cellular proliferation may provide a rationale for the increased metabolic activity and cancer burden associated with visceral adiposity (Donohoe, Doyle et al. 2011). ACM from viscerally obese patients was thus selected for investigating the effects of obesity on carcinogenesis in Barrett's oesophagus for the remainder of this project.

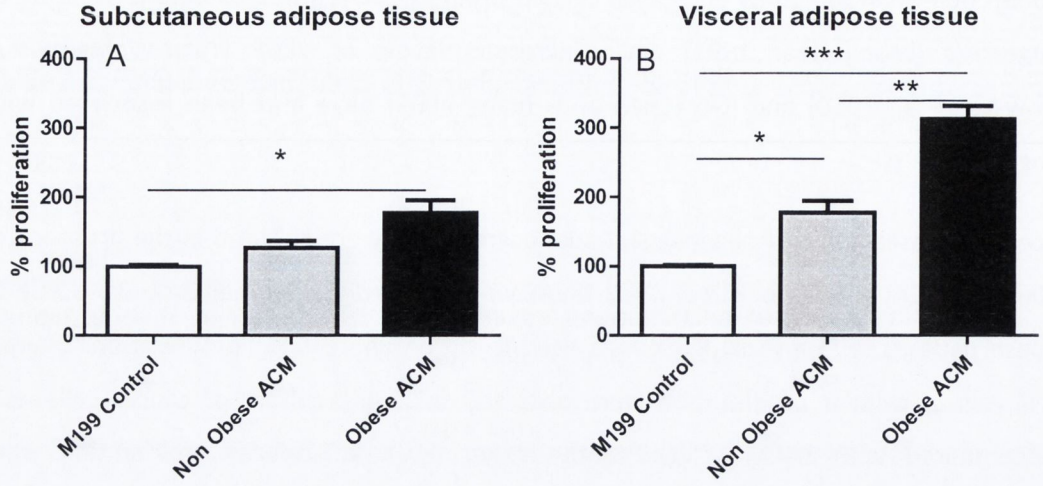

Figure 3.1. Proliferative response of OE33 cells to subcutaneous adipose conditioned media (sACM) and visceral adipose conditioned media (vACM) (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using a paired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

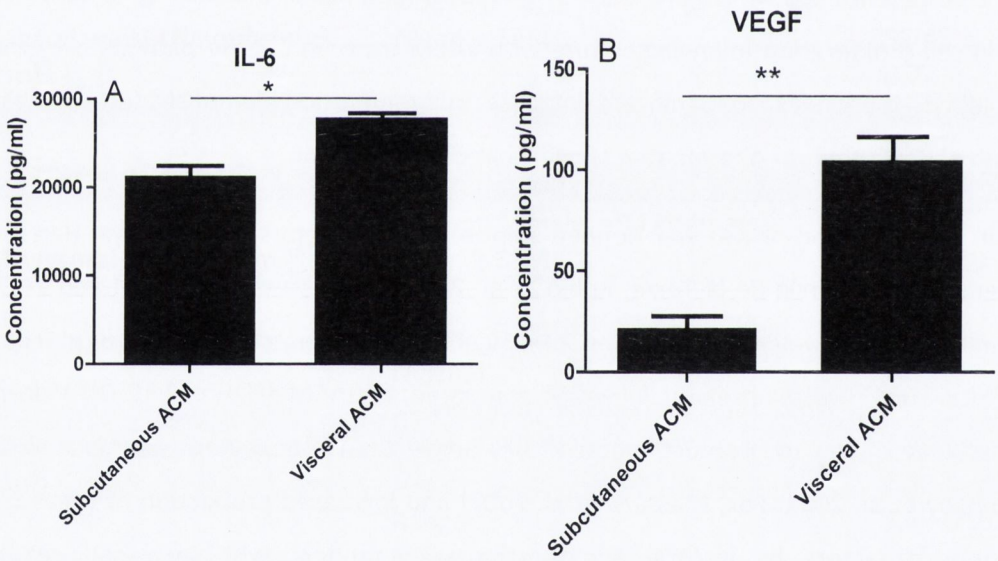

Figure 3.2. IL-6 and VEGF concentrations of subcutaneous adipose conditioned media (sACM) and visceral adipose conditioned media (vACM) (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using a paired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

3.3.2 Characterisation of the BAR-T cell line

The BAR-T cell line was characterised by looking at the expression of specific Barrett's oesophagus markers by semi quantitative RT-PCR (Figure 3.3). Investigation of the expression of cytokeratin 8 and villin is a reliable method used to identify Barrett's metaplasia. These genes were used to characterise both the cell lines and primary biopsies throughout this project. Cytokeratin 8 is a key biomarker of metaplastic transformation in Barrett's oesophagus and can be used distinguish Barrett's metaplasia from healthy squamous epithelia (Hage, Siersema et al. 2005) while villin is a sensitive marker for the detection of Barrett's oesophagus and early intestinal metaplasia (Leong, Shi et al. 2008).

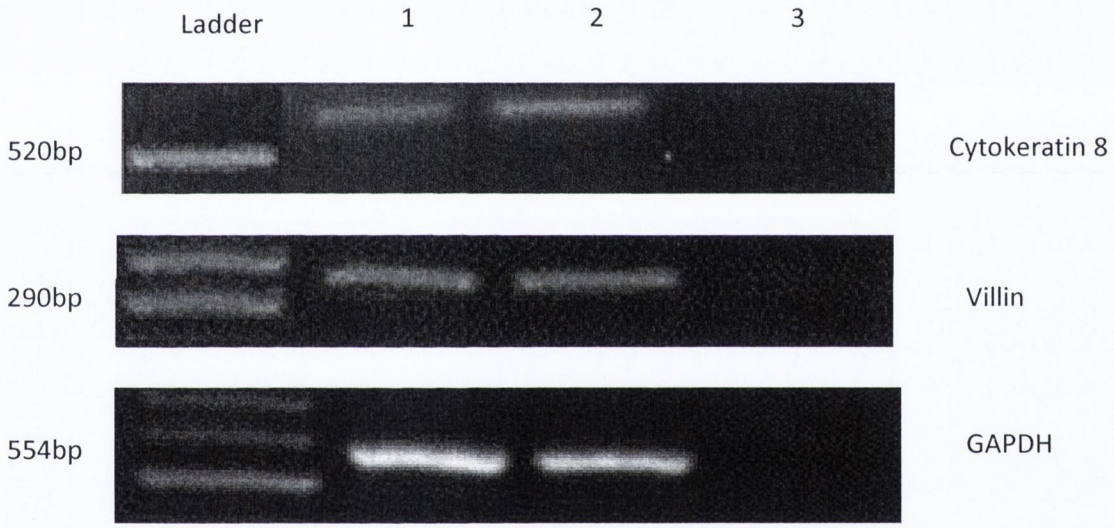

Figure 3.3. BAR-T cells express differentiation Barrett's epithelial markers cytokeratin 8 and villin. The non neoplastic BAR-T cell line was cultured in maintenance media (lanes 1 and 2) for 24hr before gene expression was examined using RT-PCR. Lane 3 is the no-template control, controlling for contamination in the PCR reaction.

The cell line requires a feeder layer for growth, so this feeder layer had to be generated before culture of the BAR-T cell line could commence. This feeder layer was generated through a 24hr *Streptomyces caespitosus* derived 0.5mg/ml mitomycin-c treatment of the murine 3T3 cell line and was adapted from a method developed by Rheinwald (Rheinwald 1980) (section 2.2.1). Feeder cells must have their proliferation arrested to allow for selective expansion of co-culture target cells, but must remain metabolically active so as to allow for synthesis of ligands or cytokines necessary to promote the growth of normal human cells (Roy, Krzykwa et al. 2001). Use of a murine feeder layer, rather than a human feeder layer is advantageous, due to the fact that specific human real time primer-probe sets will not detect or amplify murine sequences.

As the BAR-T cell line was cultured from primary tissue, a number of optimizations had to be carried out to ensure successful culturing (Table 3.2). Once mitomycin-c treatment of the feeder layer was complete the cells were frozen in media consisting of 90% FBS and 10% DMSO and stored short term at -80^{o}C until required for use. Many of these frozen aliquots could not be used after they were broken out, as the mitomycin-c treatment significantly reduced the viability of the cells, rather than just rendering them incapable of proliferating. Selection of feeder layers that were stable after they were broken out from frozen stocks was paramount to successful culturing of the BAR-T cell line. These feeder layers were returned to culture in 10cm cell culture dishes and BAR-T cells were added to these feeder layers, once the feeder layers were viable and had a confluency of approximately 30%. Culture of the BAR-T cells required a complicated media that required many components. The media contained low concentrations of pen-strep (0.5%) and was prone to bacterial contamination in the 10cm tissue culture dishes. Acidification of the culture media in the incubator was also a factor in the culture of the BAR-T cells. Wrapping the cell culture dishes in parafilm while the BAR-T cells were in culture to delay media acidification, promoted the growth and proliferation of these cells compared to unwrapped flasks. The cells also entered growth arrest and senescence due to contact inhibition, if the cells reached confluency levels close to 100%, so careful monitoring of the cells every day was required. The BAR-T cell line also demonstrated sensitivity to trypsin. Subculture of the cell line with 5ml of trypsin for 5 min resulted in cell death; trypsin volume was reduced to 3ml during subculture and removed after 2 min followed by a 5 min incubation at 37^{o}C, resulting in a significant increase in BAR-T cell viability following subculture.

Optimising the culture of the BAR-T cell line to ensure there was sufficient numbers of healthy viable cells to carry out experiments took approximately 4 months.

Table 3.2. Optimisation of conditions for growth of BAR-T cell line

Issue	Resolution
Poor viability of feeder layer	All aliquots of cell feeder layer were discarded and a new feeder layer was generated.
Acidification of media leading to cell death	Wrapping the cell culture dishes in parafilm while the BAR-T cells were in culture to delay media acidification.
Cellular senescence	Subculture of cells when confluency >80%.
Bacterial contamination	Wrapping the cell culture dishes in parafilm while the BAR-T cells were in culture.
Trypsin cytoxicity	Decrease volume of trypsin and incubation time during subculture.

3.3.3 Culture of BAR-T cell line with adipose conditioned media alters the expression of genes involved with pro-tumour pathways

The human cancer profiler array was used to identifiy gene targets associated with obesity and carcinogenesis arising in Barrett's oesophagus. The 96 well PCR array contains 84 gene targets plus endogenous and negative controls. Genes that were amplified later than 35 cycles were excluded from plate analysis and genes displaying significant up-regulation in response to ACM treatment and association with obesity in the literature, were selected for further analysis and validation using specific primer-probe sets.

Culture of the non-neoplastic BAR-T cell line with ACM generated from the omental tissue of obese oesophagectomy patients altered gene expression in each of the pro-cancer pathways represented on the plate, with 27 genes in total showing a two fold up-regulation/ down-regulation in gene expression. The greatest gene expression changes were observed in the pathways related to angiogenesis (26%) and invasion and metastasis (26%), while changes were also observed in pathways regulating signal transduction and transcription factors (20%), cell cycle control and DNA repair (14%), apoptosis and cell senescence (7%) and adhesion (7%) (Figure 3.4).

Following ACM treatment, the greatest fold-increase in gene expression was observed in the pro-metastatic regulator matrix metalloproteinase-1 (MMP-1), expression of which increased 10.8 fold in the BAR-T cell line, while expression of the MMP-1 inhibitory molecule tissue inhibitor of matrix metalloproteinases-3 (TIMP-3) was down regulated 5.4 fold. Pro-inflammatory and pro-angiogenic factor interluekin-8 (IL-8) was up regulated 4.3 fold. There was also a 8.4 fold up-regulation in gene expression of retinoblastoma protein-1 (RB1), a cell cycle control molecule. Expression of pro-angiogenic vascular endothelial growth factor-A (VEGFA) increased 2.7 fold following ACM treatment, while expression of melanoma cell adhesion molecule, a protein involved with cell adhesion, was decreased 4.4 fold (Table 3.4).

Table 3.3. Anthropometric data for patient ACM used in cancer pathway profiler

Patient	Sex	Age	Diagnosis	WC (cm)	BMI	VFA (cm^2)	MetS?	Neoadj?
1	M	60	OAC	101	25.4	172.1	1	1
2	M	62	OAC	126	33.8	383.8	1	0
3	M	62	OAC	96	28.2	155.2	0	0

The Internation Diabetes Federation (IDF) cut off of WC> 94cm was used to define presence of visercal obesity. (WC= waist circumference, BMI= body mass index, VFA= visceral fat area, MetS= metabolic syndrome, Neoadj= neoadjuvent therapy.

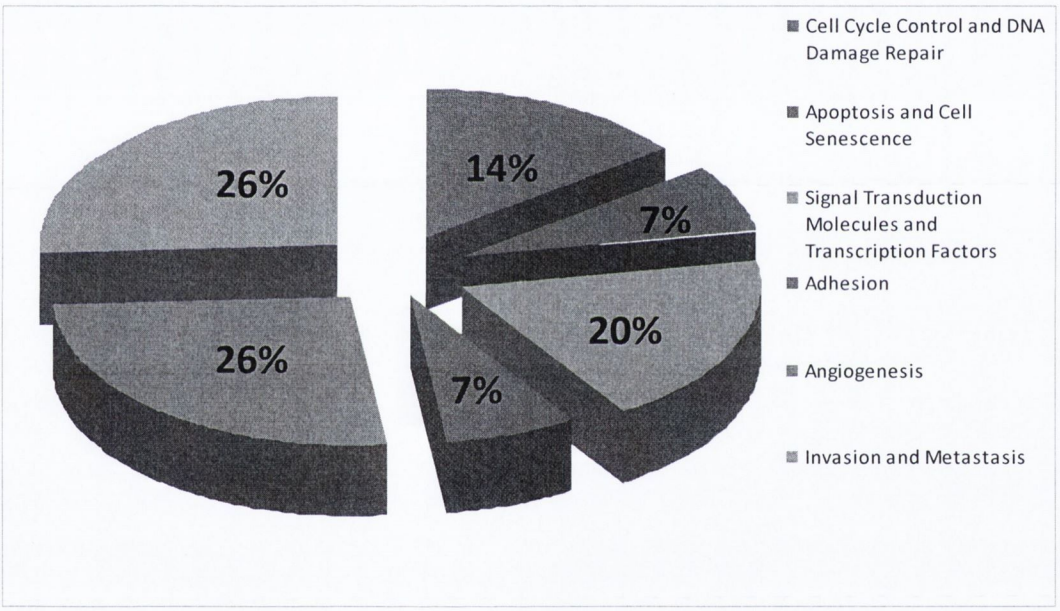

Figure 3.4. ACM treatment of BAR-T cell line activates well defined cancer pathways (n=3). Overall pathway changes were calculated based on the number of gene changes, two fold or greater, in each of the six pathways of carcinogenesis, measured by Human Cancer Pathway Array.

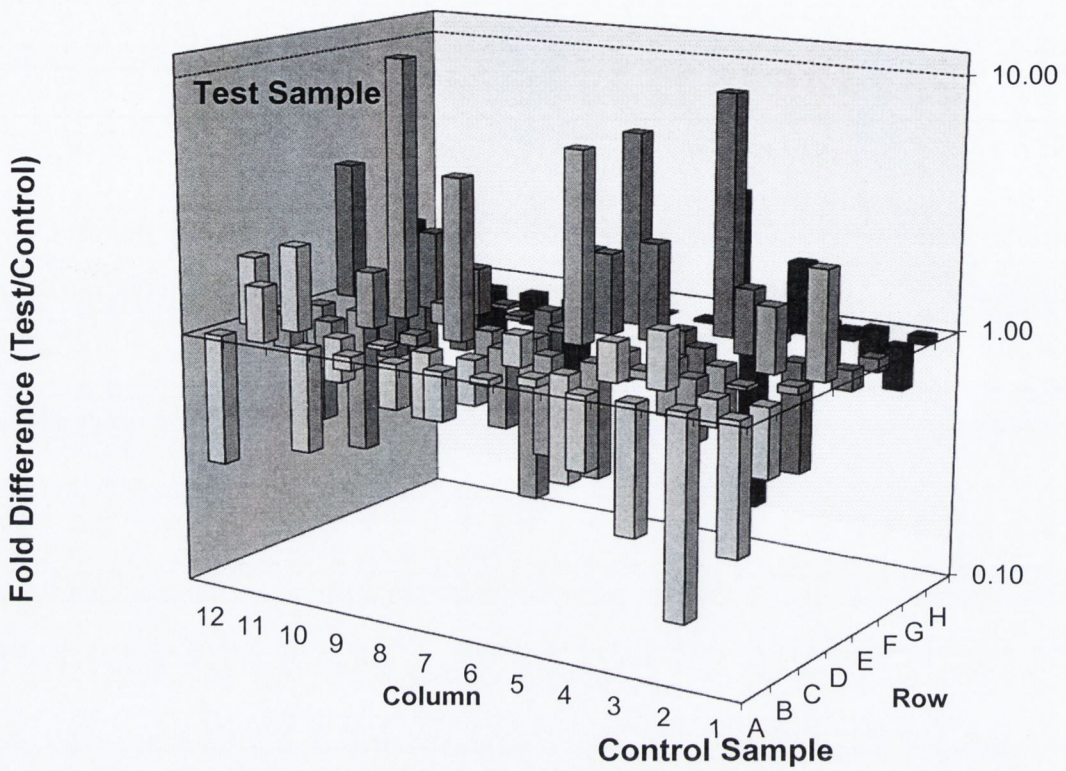

Figure 3.5. Changes in gene expression profiles in BAR-T cell line following 24hr ACM treatment, analysed using Human Cancer Pathway Finder Arrays (n=3). Fold changes in gene expression were calculated using the Ct method.

Table 3.4. Fold change in expression of genes altered in BAR-T cell line following cultured with ACM generated from visceral adipose tissue from obese OAC patients.

Genes highlighted with boxes were selected for validation via qPCR with specific primer-probes

Gene	Function	Up/Down Regulation
AKT1	Signal Transduction Molecule	-3.19
ANGPT1	Angiogenesis	-4.05
ANGPT2	Angiogenesis	-3.15
BRCA1	Cell Cycle Control	-2.55
CCNE1	Cell Cycle Control	-3.28
CDKN2A	Cell Cycle Control	-2.60
ETS2	Signal Transduction Molecule	2.18
IFNA1	Angiogenesis	-2.79
IFNB1	Angiogenesis	-2.46
IGF1	Angiogenesis	-2.05
IL-8	Angiogenesis	4.34
ITGAV	Adhesion	2.62
MAP2K1	Signal Transduction Molecule	5.68
MCAM	Adhesion	-4.42
MMP-1	Invasion and Metastasis	10.84
MMP2	Invasion and Metastasis	-3.73
MMP9	Invasion and Metastasis	-3.00
MTA2	Invasion and Metastasis	-2.69
NFKB1	Signal Transduction Molecule	2.41
NFKBIA	Signal Transduction Molecule	2.08
PDGFB	Angiogenesis	2.03
PLAU	Invasion and Metastasis	3.50
RB1	Cell Cycle Control	8.87
SERPINB5	Invasion and Metastasis	5.74
TERT	Apoptosis/ Cell Senescence	-2.93
TIMP-3	Invasion and Metastasis	-5.46
TNFRSF25	Apoptosis/ Cell Senescence	-2.06
VEGFA	Angiogenesis	2.74

3.3.4 Validations of Human Cancer Profiler Arrays

Based on altered gene regulation in response to ACM treatment, and known association with obesity in the literature, MMP-1, TIMP-3, RB1, VEGFA, MCAM, and IL-8 were selected for validation and the expression of these genes in the BAR-T cell line in response to ACM generated from OAC patients was analysed. Following treatment, RNA was extracted, and gene expression was analsyed via quantitative qPCR, using specific primer-probe sets (Figure 3.6).

Investigation of the gene targets induced by ACM, confirmed results to the qPCR human cancer array for each of the gene targets, except RB1. ACM treatment of the BAR-T cell line increased MMP-1 expression 3.5 fold ($p<0.05$), while expression of MMP-1 inhibitory molecule TIMP-3 decreased 6.0 fold ($p<0.001$). Expression of IL-8 and VEGFA were increased 2.6 fold ($p<0.05$) and 3.8 fold ($p<0.05$) respectively, while MCAM expression decreased 3.2 fold ($p<0.05$). RB1, the expression of which was increased 8.4 fold in the BAR-T cell line following ACM treatment as determined by the human cancer profiler arrays, could not be validated following ACM treatment in the larger sample cohort and was thus omited from further analysis.

Expression of these genes was also investigated in the mitomycin-c treated murine 3T3 cell line to ensure that potential carry through of mRNA from this feeder layer did not contribute to real time results. Only TIMP-3 mRNA was detected in this cell line via qPCR at cycle 38 (Figure 5B), with expression levels too low to contribute to gene expression analysis in the BAR-T cell line.

Table 3.5. Anthropometric data of patient ACM used for validation of the cancer profiler array plate results.

No. Subjects	**10**
Sex (male), n(%)	10 (100%)
Age at surgery, mean (range)	62.5 (55-72)
Diagnosis	OAC (100%)
Waist Circumference (cm), mean range	107.2 (96-126)
BMI, mean (range)	29.3 (25-35)
VFA (cm^2) mean (range)	269.1 (155.2- 383.3)
Meets criteria for the metabolic syndrome, n(%)	7 (7%)
Received neoadjuvant therapy, n(%)	5 (50%)

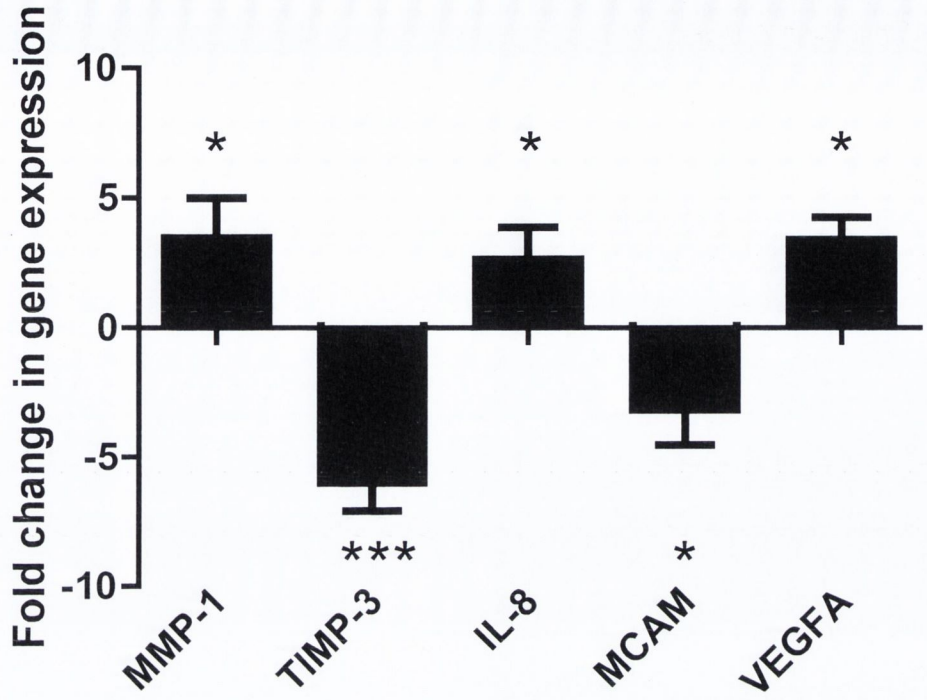

Figure 3.6. Fold change in gene expression of MMP-1, TIMP-3, IL-8, MCAM and VEGFA in non-neoplastic BAR-T cell line after 24hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using a paired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

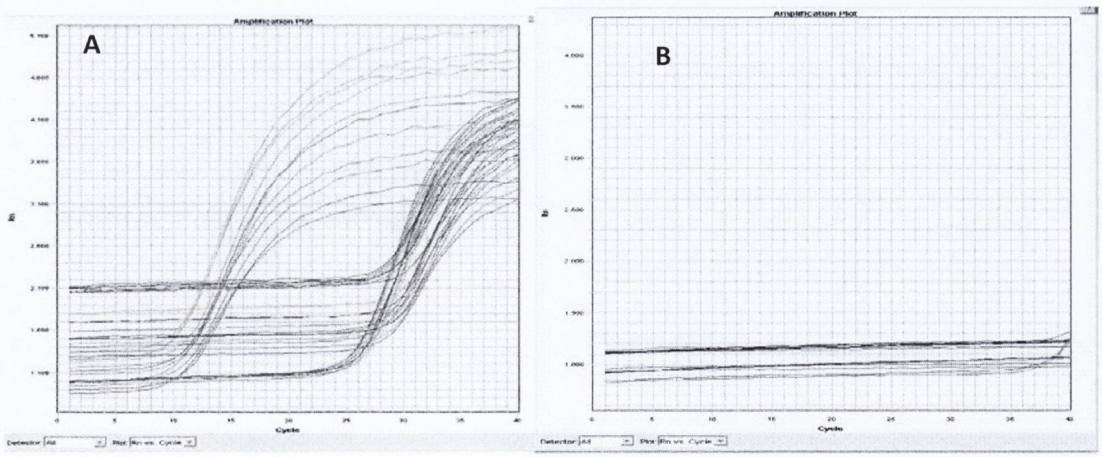

Figure 3.7. Representative amplification plots of identified genes in (A) BAR-T cells and (B) 3T3 cell line. In the non-neoplastic BAR-T cell line, all genes were detected at <35 cycles, indicating reliable detection of target sequences. Only TIMP-3 was detectable in the 3T3 cell line, albeit at negligibly low levels.

3.3.5 Obesity status of oesophagectomy patients influences gene alterations in BAR-T cells following culture with ACM.

The expression of the five genes identified by the human cancer profiler array, were analysed in the BAR-T cells following treatment with ACM generated from obese and non-obese OAC patients to investigate whether visceral adiposity or just adipose tissue alone affects the expression of these genes in Barrett's oesophagus. The anthropometric data of the patients used in this study are presented below in Table 3.6. Obesity status was determined using waist circumference; patients with a waist circumference greater than 94cm were deemed viscerally obese.

Table 3.6. Anthropometric data for patient ACM for comparison of dysregulation of gene expression by ACM generated by viscerally obese and non-obese OAC patients.

	Non Obese	Obese
No. Subjects	10	10
Diagnosis	OAC	OAC
Sex (male) n (100%)	10	10
Age at surgery, mean (range)	62.2 (44-77)	62.5 (55-72)
Waist circumference (cm), mean (range)	88.6 (80-94)	107.2 (96-126) ***
BMI, mean (range)	25.2 (20.7-29.2)	29.3 (25.0-35.0) **
VFA (cm^2), mean (range)	119.2 (34.1-116.1)	269.1 (155.2-383.3) **
Metabolic Syndrome, n (%)	1 (10%)	5 (50%)
Neoadjuvant therapy, n (%)	1 (10%)	3 (30%)

Statistical analysis was carried out using an unpaired student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (BMI= Body mass index, VFA= Visceral fat area).

ACM was generated from omental tissue surgically excised from two distinct sets of oesophagectomy patients with significantly different anthropometric status. Age and sex matched obese and non-obese patients were used in this study. Viscerally obese patients had significantly larger waist circumferences ($p<0.001$), BMIs ($p<0.01$) and visceral fat areas ($p<0.01$). Patient ACMs were selected based on waist circumferences with a waist circumference of 94cm a cut of point for visceral obesity.

The expression of 3 out of the 5 identified genes were significantly different in the BAR-T cell line following treatment with ACM generated from obese patients versus non-obese patients (Figure 3.8). There was a significant increase ($p=0.038$) in IL-8 expression in the BAR-T cell line following treatment with ACM generated from obese compared to a non-obese ACM treatment (Figure 3.8C). VEGFA expression also showed significant up-regulation ($p=0.025$) in the BAR-T cell line in response to ACM from obese patients versus non-obese (Figure 3.8E).

Interestingly, while MCAM gene expression was modestly up-regulated in response to ACM generated from non-obese OAC patients, expression was decreased in response to ACM generated from obese OAC patients, representing a significant decrease between the two treatment types ($p=0.025$) (Figure 3.8D). No significant difference was seen in MMP-1 or TIMP-3 gene expression in response to obese ACM versus non-obese ACM, in the BAR-T cell line, though ACM generated from non-obese patients did elicit a slighter higher level of MMP-1 gene expression in the BAR-T cell line.

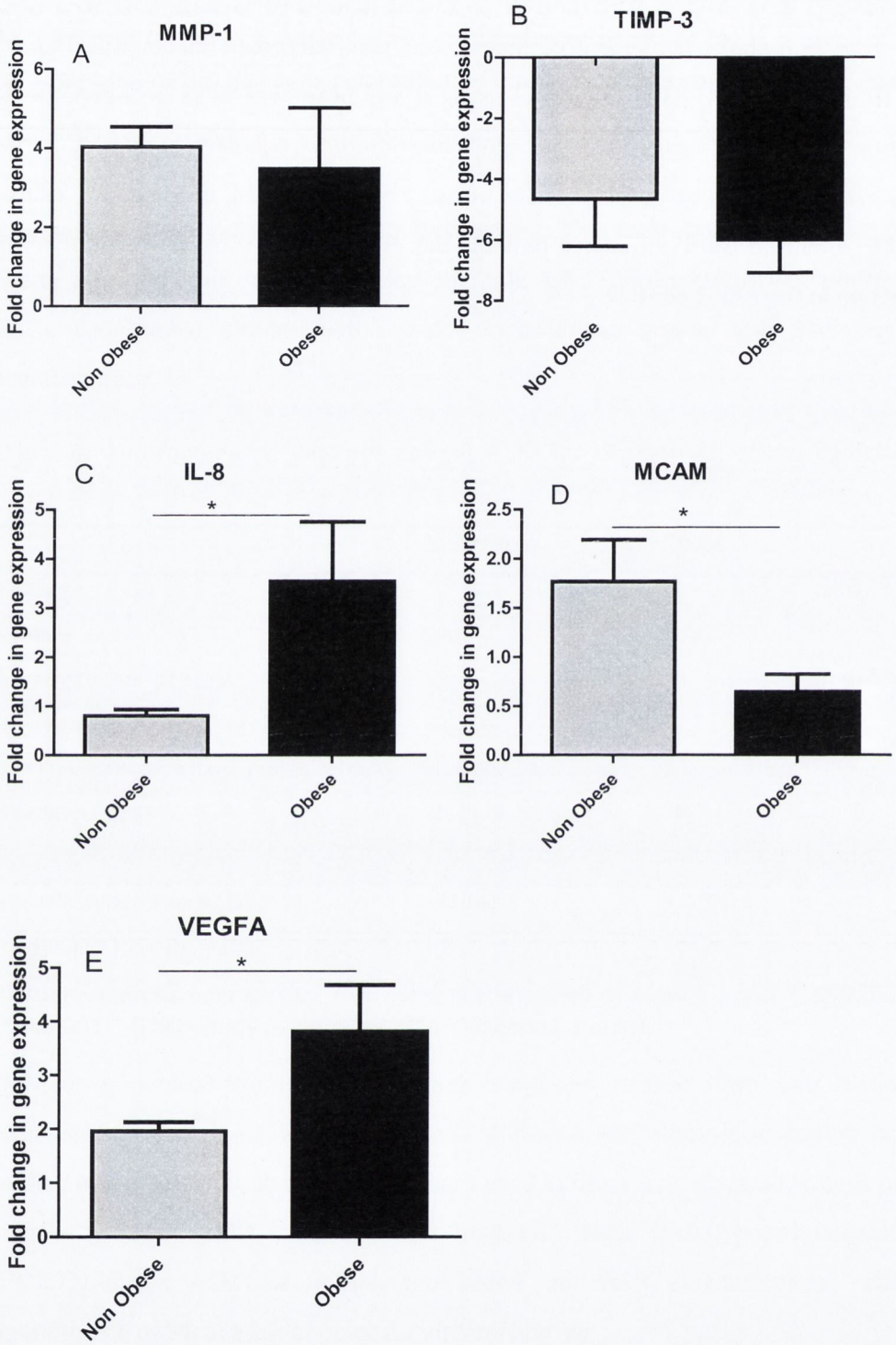

Figure 3.8. Gene expression of MMP-1 (A), TIMP-3 (B), IL-8 (C), MCAM (D), and VEGFA (E) in BAR-T cells following treatment with ACM generated from non-obese (n=10) and obese (n=10) patients. Data are expressed as mean ± SEM. Statistical analysis was carried out using an unpaired student's t-test (*$p<0.05$).

3.3.6 MMP-1 and IL-8 are significantly up regulated in Barrett's metaplasia from obese patients compared to non-obese patients.

The expression of the 5 gene signature of MMP-1, TIMP-3, IL-8, MCAM and VEGFA was investigated in Barrett's metaplasia biopsies from obese and non-obese patients to investigate whether there was a difference in gene expression between the two patient cohorts.

Barrett's oesophagus biopsies were collected from patients attending an endoscopy clinic as detailed in full in section 2.4.1. Biopsies consist of a heterogeneous cell population; they may contain epithelia and stromal cells, along with inflammatory factors, and in the case of Barrett's oesophagus, goblet cells. To ensure the biopsies were in fact Barrett's metaplasia, it was necessary to characterise the biopsies by RT-PCR for a specific Barrett's oesophagus marker. CK8 and villin are expressed in Barrett's metaplastic tissue but not in healthy squamous oesophageal tissue (Mitas, Almeida et al. 2005; Anders, Sarbia et al. 2008), and their expression was investigated in the Barrett's biopsies. All biopsies displayed high levels of CK8 and villin expression confirming that the biopsies were metaplastic Barrett's oesophagus in origin and not squamous oesophageal epithelium (Figure 3.9).

MMP-1 mRNA levels in Barrett's metaplasia biopsies were 3.5 fold higher ($p=0.041$) in viscerally obese Barrett's oesophagus patients than compared to age and sex matched non obese patients. Similarly, the mRNA expression of pro-angiogenic and pro inflammatory marker IL-8 was 3.8 fold higher ($p=0.036$) in Barrett's metaplasia biopsies from viscerally obese Barrett's oesophagus patients than compared to age and sex matched non obese patients. There were slight variations in levels of VEGFA, MCAM and TIMP-3 in Barrett's metaplasia in viscerally obese patients versus non obese patients but these levels failed to reach statistical significance (Figure 3.10).

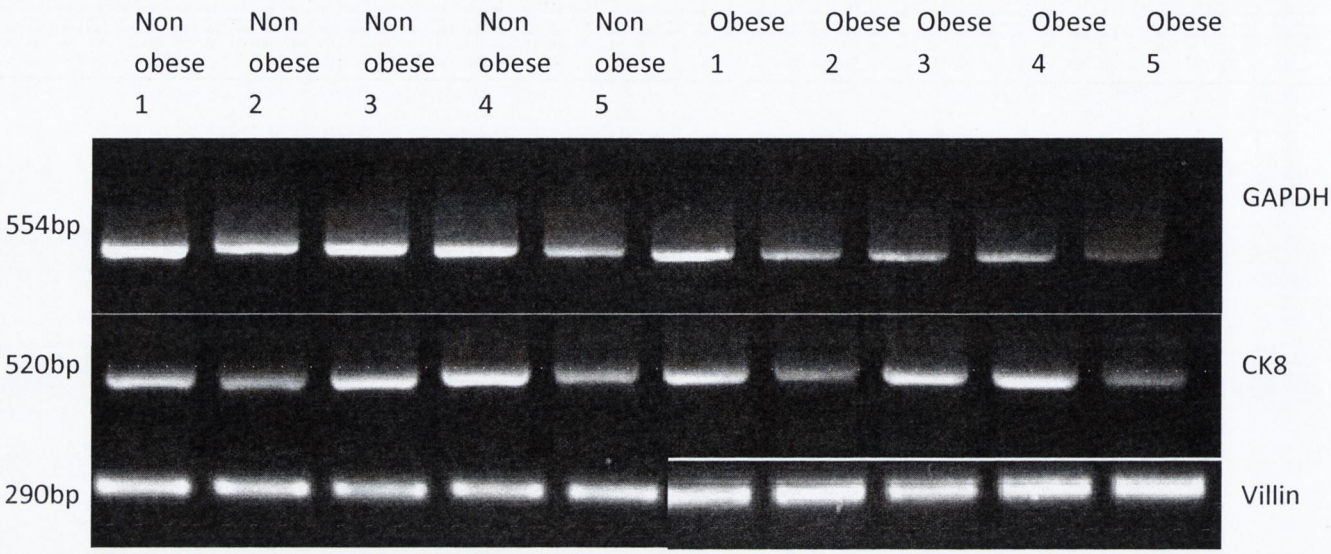

Figure 3.9. Representative RT-PCR gel picture of GAPDH, CK8 and villin expression in Barrett's metaplasia biopsies from both obese and non-obese patients

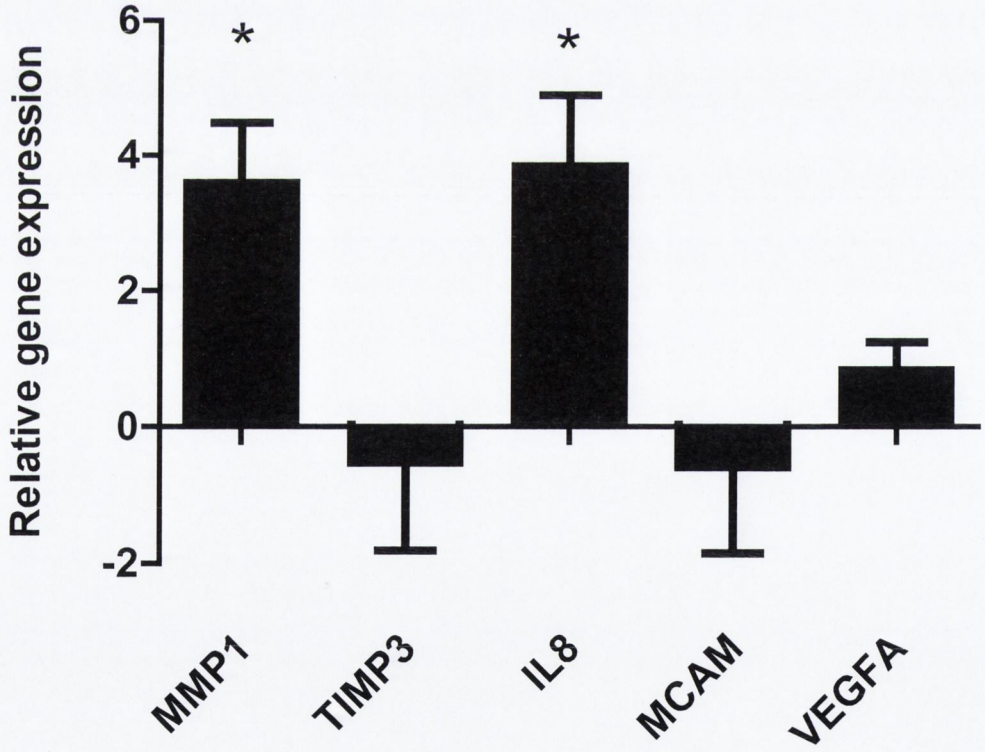

Figure 3.10. Expression of cancer related genes in Barrett's metaplasia biopsies in obese patients (n=10) versus non obese patients (n=10). Data are expressed as mean ± SEM. Statistical analysis was carried out using a paired student's t-test (*$p<0.05$).

3.3.7 Deoxycholic acid deregulates gene expression in BAR-T cell line

DCA is a major component of bile acid, and prolonged exposure to these bile acids is a major risk factor in the progression of Barrett's derived oesophageal adenocarcinoma (Menges, Muller et al. 2001). The effect of a 300μM pulsed DCA treatment and a 150 μM continuous DCA treatment on gene expression in the BAR-T cell line was investigated via qPCR (Figure 3.11). These DCA concentrations and length of treatments were used as they are similar to the physiological conditions observed in GORD patients (Tselepis, Morris et al. 2003; Jenkins, Harries et al. 2004).

Gene alterations were observed in the BAR-T cell line in response to a 3hr 300μM pulsed DCA treatment. MMP-1 expression was increased 89 fold ($p=0.002$) and VEGFA expression was increased 5.8 fold ($p=0.047$), compared to control cells grown in maintenance media. TIMP-3 expression decreased 3.9 fold but did not reach statistical significance, while there was no change in either MCAM or IL-8 expression (Figure 3.11A).

A similar pattern of gene expression was demonstrated in the BAR-T cell line following a 24hr continuous 150μM DCA treatment (Figure 3.11B). MMP-1, IL-8 and VEGFA all demonstrated increases in gene expression though failed to reach statistical significance. Both TIMP-3 and MCAM expression was decreased following a 24hr continuous 150μM DCA treatment but levels failed to achieve statistical significance. A 300μM pulsed DCA treatment is more representative of the average gastro-oesophageal reflux episode *in vivo* (Tselepis, Morris et al. 2003) and this treatment elicited a greater increase in MMP-1 and VEGFA expression than a 150 μM continuous DCA treatment. Changes in gene expression in response to both DCA treatments in the BAR-T cell line showed the same pattern of expression as seen in the cell line in response to an ACM treatment. In both cases mRNA expression of MMP-1, IL-8 and VEGFA were up regulated, while TIMP-3 and MCAM were down regulated. IL-8, MMP-1, VEGFA, leptin and various other adipocytokines may be exacerbating the damage caused by GORD, by activating key tumourigenic pathways, in metaplastic Barrett's oesophagus

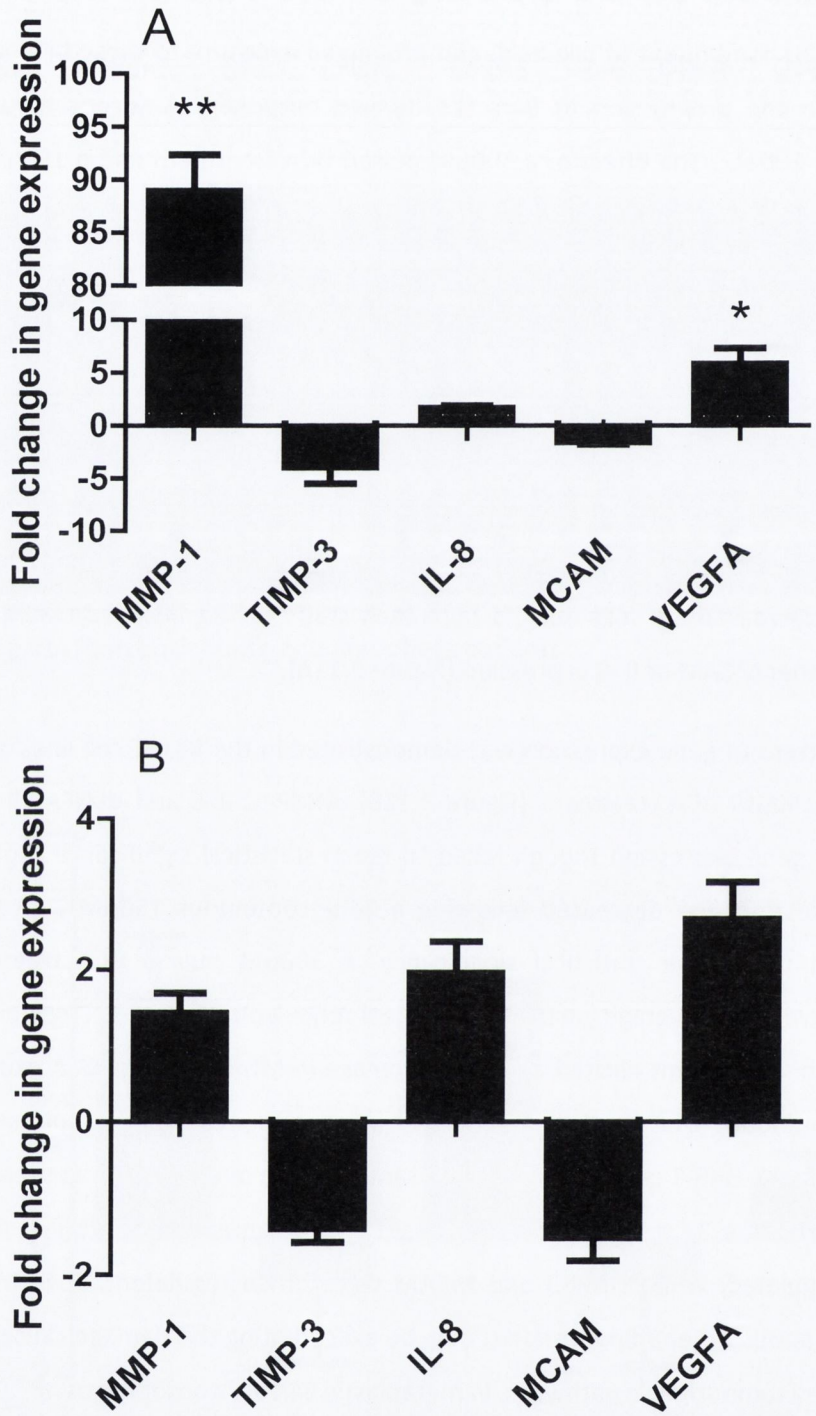

Figure 3.11. Fold change in gene expression of MMP-1, TIMP-3, IL-8, MCAM and VEGFA in non-neoplastic BAR-T cell line following (A) 3hr pulsed 300μM DCA treatment (n=5) and (B) 24hr continuous 150μM DCA treatment (n=5). Data are expressed as mean ± SEM. Statistical analysis was performed using a paired student's t-test (*$p<0.05$, **$p<0.01$).

3.3.8 Hypoxia deregulates gene expression in BAR-T cell line

The non-neoplastic BAR-T cell line was cultured in a hypoxic environment at 37^{0}C for 24hr in a 0.5% O_2 atmosphere in order to determine the effects of hypoxia on gene expression in Barrett's oesophagus. Gene expression was assessed in the cell lines via qPCR using specific primer-probe sets. Increases in hypoxia related proteins Epo-R and Ki-67 have been reported in the metaplasia-dysplasia-adenocarcinoma sequence associated with Barrett's carcinogenesis (Griffiths, Pritchard et al. 2007) indicating that hypoxia may play a role in the neoplastic progression of Barrett's oesophagus. Expression of HIF-1α, the oxygen-regulated transcription factor, also has been shown to correlate with environmental inflammatory reactions in Barrett's metaplasia (Schneider, Ling et al. 2009).

Culture of the BAR-T cell line in a hypoxic environment for 24hr significantly increased the expression of pro-metastatic MMP-1 7.8 fold (p=0.046). In contrast, mRNA expression of IL-8 was significantly decreased 18.3 fold (p=0.007) following 24hr culture in a hypoxic environment, contrary to results correlating inflammatory factors with hypoxia by other authors (Ling, Khochfar et al. 2009). A significant 1.9 fold decrease in gene expression was seen in MCAM (p=0.010), while angiogenic factor VEGFA showed a significant increase in expression of 1.9 fold (p=0.037). TIMP-3 also showed increases in expression; however levels failed to reach statistical significance (Figure 3.12).

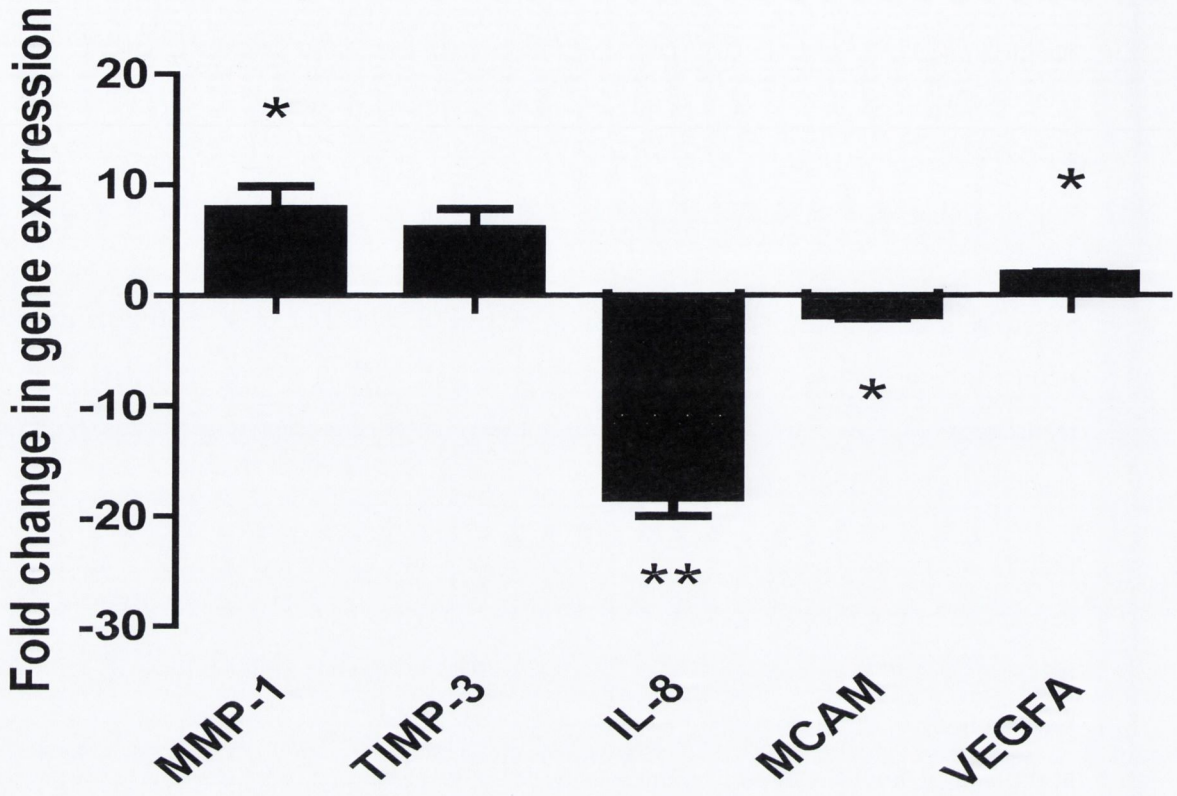

Figure 3.12. Fold change in gene expression of MMP-1, TIMP-3, IL-8, MCAM and VEGFA in non-neoplastic BAR-T cell line following 24hr 0.5% hypoxic treatment (n=5). Fold changes in gene expression are compared to an untreated control grown under normoxic conditions. Statistical analysis was performed using a paired student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3.4 Discussion

Obesity is understood to be a major risk factor in Barrett's oesophagus and the progression of this malignancy to invasive adenocarcinoma (Stein, El-Serag et al. 2005; Merry, Schouten et al. 2007; Reynolds, Ryan et al. 2008). The aim of this chapter was to determine what tumourigenic pathways were activated by obesity in a Barrett's oesophagus cell model, independent of GORD.

The location of this excess adipose tissue, rather than obesity alone is thought to be an important causative factor in the pathogenesis of Barrett's oesophagus and oesophageal adenocarcinoma (Corley, Kubo et al. 2008). Culture of the oesophageal adenocarcinoma OE33 cell line with vACM and sACM showed notably different proliferative capabilities and alluded to the pro-tumourigenic pathways activated by visercal adipose tissue. The greater proliferation demonstrated in the OE33 cell line in response vACM compared to culture with sACM may due to the greater levels of VEGF in the vACM. VEGF is a well-defined pro-angiogenic and survival factor for a diverse range of cancer types (Donovan and Kummar 2006) and its expression in the visceral adipose tissue may have an effect on cancer development (Ferrara 2009). Omental adipose tissue has also been reported as a site for seeding and growth of peritoneal metastasis from both ovarian (Gerber, Rybalko et al. 2006) and endometrial cancer (Fujiwara, Saga et al. 2008). These observations in tandem with the increased proliferative capacity and VEGF content of vACM compared to sACM suggests that omental adipose rather than subcutaneous adipose tissue may be crucial in driving pro-tumourigenic pathways in centrally obese patients. Based on this hypothesis, ACM generated from visceral adipose tissue, rather than subcutaneous adipose tissue was used to investigate the effects of obesity on driving the neoplastic progression of Barrett's oesophagus in the remainder of this thesis.

Visceral ACM was used in this study to simulate the soluble factors present in the obese microenvironment *in vitro*. ACM generated from visceral adipose tissue, surgically excised from obese oesophagectomy patients, activated 27 well defined cancer-related genes in the non-neoplastic BAR-T cell line, with each gene associated with at least one pathway of carcinogenesis.

Pathways associated with angiogenesis, and invasion and metastasis showed greatest activation within the BAR-T cell line in response to ACM treatment, and these pathways relate directly to two of the hallmarks of cancer identified previously by Hanahan and Weinberg (Hanahan and Weinberg 2000). Pro-angiogenic pathways have been associated with obesity

related carcinogenesis previously in breast cancer (Christiani, Wu et al. 2011; Gu, Young et al. 2011), while obesity has also been show to activate pro-invasive pathways in renal cell carcinoma (Horiguchi, Sumitomo et al. 2006).

The Human cancer pathway profiler enabled the identification of a signature of five cancer related genes whose gene expression was altered by ACM treatment in the BAR-T cell line. MMP-1, IL-8 and VEGFA demonstrated up regulation while, TIMP-3 and MCAM were down regulated in expression. Each of these genes has showed an association with carcinogenesis previously.

MMP-1 mRNA expression showed the greatest up-regulation by ACM in the BAR-T cell line following a 24hr treatment, as determined by both the Human cancer pathway finder arrays and validation by qPCR using a specific primer-probe set. MMP-1 also showed significantly higher levels of expression in Barrett's oesophagus biopsies from obese patients compared to non-obese patients. This pro-invasive factor has been associated with a myriad of cancers including gastric (Yang, Bao et al. 2010), breast (Poola, DeWitty et al. 2005), and thyroid (Mizrachi, Koren et al. 2011) carcinoma. MMP-1 levels, as determined by immunohistochemistry have been associated with a particularly poor prognosis in oesophageal adenocarcinoma. Survival analysis demonstrated that the presence of MMP-1 in oesophageal adenocarcinoma specimens correlated with a more aggressive cancer phenotype with all patients whose tumours contained MMP-1 were dead by 29 months, while nine patients out of 11 whose tumours were MMP-1-negative remained alive at 60 months (Murray, Duncan et al. 1998). MMP-1 and other members of this proteinase family are involved in extracellular matrix remodelling, an event that takes place during obesity mediated adipose tissue formation. Increased MMP-2, MMP-3, MMP-12, MMP-14 and MMP-19 activity in obese adipose tissue and modulate adipogenesis (Tartare-Deckert, Chavey et al. 2003), while treating pre-adipocytes with MMP-2 and MMP-9 neutralizing antibodies significantly decreases adipocyte differentiation (Bouloumie, Sengenes et al. 2001). Production of adipose derived factors by newly synthesized adipose tissue due to MMP activity may ultimately promote neoplastic progression in Barrett's oesophagus. Members of the MMP family can also promote carcinogenesis independent of their role in extra cellular matrix remodelling (Sternlicht and Werb 2001). MMP-7 has been shown to be involved with Fas ligand, and may interfere with the Fas mediated apoptotic pathway, promoting carcinogenesis (Fingleton, Vargo-Gogola et al. 2001).

The activity of MMPs is regulated via inhibition of their activity by endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) (Pardo and Selman 2006). The MMP/TIMP balance is shifted towards increased extracellular matrix remodelling and degradation in obesity (Chavey, Mari et al. 2003). Following ACM treatment of the BAR-T cell line, TIMP-3 mRNA expression, a tissue inhibitor of MMP-1, and a gene previously associated with colorectal adenocarcinoma (Powe, Brough et al. 1997), is significantly down regulated as MMP-1 expression increases. The down regulation of TIMP-3 levels in obese adipose tissues compared to lean adipose tissues has been reported previously (Chavey, Mari et al. 2003). Obese patients may have lower levels of TIMP-3 in their adipose tissues compared to non-obese patients, which may further exacerbate the effect of MMP-1 *in vitro* and in obese Barrett's oesophagus patients, with increased activity of MMP-1 and extra cellular matrix remodelling conducive to angiogenesis, invasion and metastasis (Rao and Pulukuri 2008).

The expression of the pro-inflammatory chemokine IL-8 was significantly up regulated in the BAR-T cell line following a 24hr ACM treatment. IL-8 is produced and released by adipocytes and adipose tissue (Bruun, Lihn et al. 2004) and expression of IL-8 has been demonstrated to correlate with the metastatic potential of human melanoma cells in nude mice with IL-8 inducing proliferation of low metastatic A375P cells in a dose dependant manner (Singh, Gutman et al. 1994). Obesity has been linked to a chronic inflammatory response, evidenced by modifications in cytokine and chemokine production (such as IL-8), activation of inflammatory signalling pathways, and increased synthesis of acute phase reactants (Wellen and Hotamisligil 2003). ACM generated from viscerally obese patients led to a significant increase in IL-8 expression in the BAR-T cell line compared to ACM generated from non-obese patients, while IL-8 mRNA expression was significantly higher in Barrett's metaplasia biopsies from obese patients compared to age matched non-obese patients. Circulating IL-8 levels have been shown to correlate significantly with obesity-related parameters such as BMI and waist circumference (Kim, Park et al. 2006) and this excess adipose tissue derived IL-8 may help drive carcinogenesis through both angiogenic and pro-inflammatory pathways in obese Barrett's oesophagus patients. It has recently been suggested that an inflammatory microenvironment is an additional hallmark of cancer (Colotta, Allavena et al. 2009; Hanahan and Weinberg 2011), adding to the six defined previously by Hanahan and Weinberg (Hanahan and Weinberg 2000).

VEGFA expression is significantly increased in the BAR-T cell line in response to ACM treatment. The link between angiogenesis, VEGFA signalling and Barrett's oesophagus has been reported and examined extensively by previous authors (Auvinen, Sihvo et al. 2002; Lord,

Park et al. 2003; Mobius, Stein et al. 2003). Indeed the ominous salmon pink colour that is characteristic of Barrett's oesophagus is believed to be due to increased neovascularisation in the Barrett's epithelium (Auvinen, Sihvo et al. 2002). Leptin, a hormone produced and secreted by adipose tissue, has been linked to VEGF and increased neovascularisation with leptin promoting expression of VEGF and its receptor VEGF-R2 in breast cancer (Gonzalez, Cherfils et al. 2006), thus adipose derived leptin in obese Barrett's oesophagus patients may be driving angiogenic events mediated by VEGFA in Barrett's oesophagus. High levels of neovascularisation have been reported in Barrett's oesophagus previously (Couvelard, Paraf et al. 2000) with acquisition of angiogenic properties thought to be a pre-cancerous event. This 'angiogenic switch' has been proposed by other authors (Mobius, Stein et al. 2003) who report that the neovascularisation phase in Barrett's oesophagus may precede tumour growth and metastasis. VEGFA expression was significantly increased in the BAR-T cells in response to ACM generated from obese patients compared to non-obese patients. Increased circulating VEGF levels have been shown to correlate positively with patient obesity status (as defined by BMI) in the literature (Loebig, Klement et al. 2010). VEGF has been linked to adipogenesis and it has been postulated that the coupling of VEGF mediated angiogenesis with adipogenesis is essential for the differentiation of precursor cells into adipocytes in obesity (Sugiura, Nishimura et al. 2007). The adipogenic properties of VEGF coupled with increased production of VEGF by the new synthesised adipose tissue in obese patients, may generate a positive feedback loop increasing angiogenesis and leading to a greater chance of neoplastic events arising in Barrett's oesophagus in obese patients. Abnormal VEGFA signalling may work in tandem with increased MMP-1 activity in obese Barrett's oesophagus patients, to promote angiogenesis and extracellular matrix remodelling, conducive to invasion and eventual metastasis (Couvelard, Paraf et al. 2000). The pie chart data in the chapter lends further credence to this theory. Genes involved with both invasion and metastasis and adhesion showed the greatest changes in gene expression following 24hr incubation in ACM generated from viscerally obese OAC patients.

The expression of MCAM was significantly down regulated in the BAR-T cell line following ACM treatment. This adhesion factor is present on both vascular endothelial cells and melanoma cells and enhances melanoma metastasis (Braeuer, Zigler et al. 2011). MCAM has also been associated with poor prognosis with breast cancer, with increased expression contributing to tumour aggressiveness by promoting malignant cell motility (Zabouo, Imbert et al. 2009). Serum adiponectin, an adipocytokine released by adipose tissue and a component of the ACM, has been linked with MCAM expression and diabetic nephropathy in diabetic patients but the

exact role of MCAM in this process has yet to be elucidated (Saito, Saito et al. 2007). MCAM expression was significantly down regulated in the BAR-T cells in response to ACM generated from obese OAC patients compared to non-obese OAC patients. Down regulations of such cell adhesion molecules has been linked to node positive breast cancer, shedding of these molecules is seen in cases with nodal metastasis which may enable the tumour cells to escape the host defence mechanism (Madhavan, Srinivas et al. 2002). Similar events may be occurring in obese Barrett's patients, leading to increased invasion and metastasis arising in the pre-malignant tissue due to adipose tissue mediated down regulation of MCAM and similar adhesion molecules.

Genes activated in the BAR-T cell line in response to ACM treatment were subsequently assayed in response to a 300μM pulsed DCA treatment and a 150μM continuous DCA treatment, to mimic the effect of GORD in the Barrett's oesophagus cell model, and to see whether changes in gene expression are complimentary to those seen following ACM treatment. A 150 μM pulsed DCA treatment elicited significantly higher levels of expression of MMP-1 and VEGFA than a 300μM continuous ACM treatment. A short pulsed treatment is more representative of GORD *in vivo*, and these results indicate the potential metastatic and angiogenic consequences of GORD in Barrett's patients. Following both DCA treatments , up regulations of MMP-1, VEGFA were seen in the BAR-T cell line, with the pattern of expression of the other obesity related genes, TIMP-3, IL-8 and MCAM, identical to that seen in the BAR-T cell line following ACM treatment with several of these genes showing a strong association with GORD and Barrett's oesophagus previously. IL-8 is expressed at significantly higher levels in oesophageal mucosa of GORD patients compared to asymptomatic controls (Isomoto, Saenko et al. 2004). It has also been demonstrated that elimination of reflux with Nissen fundoplication significantly reduces IL-8 expression in both squamous and Barrett's mucosa (Oh, DeMeester et al. 2007). DCA, a major component of GORD, was shown to induce significantly higher levels of VEGFA in Barrett's mucosa compared to healthy oesophageal tissue. DCA treatment also increases VEGFA expression in the OE33 oesophageal adenocarcinoma cell line (Konturek, Burnat et al. 2007); these observations highlight a pro-angiogenic and pro-tumourigenic mechanism activated by GORD in the oesophagus. While GORD is an important risk factor for Barrett's oesophagus, GORD alone cannot account for the incidences of Barrett's oesophagus and the neoplastic progression of the condition (Edelstein, Farrow et al. 2007). The tumourigenic burden of excess adipose tissue in obese Barrett's oesophagus patients may contribute to carcinogenesis due to the adipose derived factors secreted by excess adipose tissue. IL-8, MMP-1, VEGFA, leptin and various other

adipocytokines may be exacerbating the damage caused by GORD, by activating key pro-cancer pathways in metaplastic Barrett's oesophagus. DCA may also promote carcinogenesis in Barrett's through NF-κB activation; DCA induces reactive oxygen species production, which causes genotoxic injury and simultaneous activation of the NF-κB pathway, which enables cells with DNA damage to resist apoptosis (Souza, Huo et al. 2011).

Gene expression in the BAR-T cell line in response to hypoxia was also investigated in this study. Significant increases in MMP-1 and VEGFA expression along with significant decreases in both IL-8 and MCAM were revealed in the BAR-T cell line following culture in a hypoxic for 24hr. Hypoxia and adipose tissue have been shown to work hand in hand to promote adipocytokine dysregulation previously; induction of hypoxia in the adipose tissue of mice was shown to down regulate the production of adiponectin (Hosogai, Fukuhara et al. 2007), a potent anti-cancer hormone (Konturek, Burnat et al. 2008). Hypoxia may contribute towards neoplastic progression in Barrett's oesophagus through key angiogenic pathways. HIF-1α,the primary transcription factor which controls hypoxic processes in the cell, has been shown to be involved in VEGFA activation (Forsythe, Jiang et al. 1996). HIF-2α, VEGF, Epo-R and Ki67, genes inducible by hypoxia showed increased expression with progression along the Barrett's carcinogenesis sequence (Griffiths, Pritchard et al. 2007). Culture of the BAR-T cell line in a hypoxic environment in this study, significantly up regulated VEGFA expression compared to a passage matched control grown under normoxic conditions.

While there is limited data in the literature on the association of Barrett's oesophagus with hypoxia, there is a wealth of literature linking hypoxia with cancer progression (Hockel, Schlenger et al. 1996; Anastasiadis, Stisser et al. 2002; Zhou, Schmid et al. 2006). HIF-1α, a major subunit of the HIF-1 heterodimer, has been shown to be up-regulated in pre-neoplastic lesions. Both high-grade prostate intraepithelial neoplasia (Zhong, Semenza et al. 2004) and endometrial intra-epithelial neoplasia (van Diest, Horree et al. 2007), pre-malignant lesions of prostatic adenocarcinoma and endometrioid adenocarcinoma, respectively, showed over expression in mRNA levels of this transcription factor. Further examination of the role HIF-1α plays in the neoplastic progression of Barrett's oesophagus is warranted, but is beyond the scope and focus of this thesis.

While the cancer burden of excess adiposity and obesity in Barrett's carcinogenesis is well established, the exact mechanisms have yet to be elucidated. This series of experiments demonstrate that in a Barrett's oesophagus cell model, pathways related to angiogenesis and invasion and metastasis, two of the six well defined hallmarks of cancer (Hanahan and

Weinberg 2000), are activated by obesity, demonstrating the pro-tumourigenic potential of adipose derived factors in Barrett's oesophagus. Adipose derived factors in the ACM also regulate the expression genes intrinsically linked to cancer, identifying a five gene signature of MMP-1, TIMP-3, IL-8, MCAM, and VEGFA. Treatment of the BAR-T cell line with a pulsed 300μM and continuous 150μM DCA treatments, to mimic the effects of GORD in vitro, regulated the expression of the same five genes, in the same expression pattern. MMP-1 and IL-8 also showed significant up regulation in Barrett's metaplasia biopsies from obese patients compared to non-obese patients. This study establishes that visceral obesity may activate pro-cancer pathways in Barrett's oesophagus, not only through the mechanical mechanisms of excess adipose tissue and increased intra-abdominal pressure, but through the activation of neoplastic pathways by adipose derived factors produced and secreted by excess adipose tissue. The oncogenic burden of excess adiposity and the activation of the pathways and genes associated with tumourigenesis may explain why some Barrett's patients exposed to prolonged GORD progress to oesophageal adenocarcinoma.

Chapter 4: Functional role of obesity in the metaplasia-dysplasia-adenocarcinoma disease sequence

4.1 Introduction

Barrett's oesophagus develops in healthy squamous epithelium and progresses through low and high grade dysplasia to invasive oesophageal adenocarcinoma through a well characterised, though poorly understood mechanism (Jankowski, Wright et al. 1999). Barrett's epithelium is characterised by the presence of goblet cells and expression of intestinal markers such as MUC2, alkaline phosphatase and villin (Flejou 2005). An improved understanding of the molecular aetiology of this disease may allow for improved diagnosis, therapy, and prognosis. Surveillance studies have demonstrated that progression from Barrett's oesophagus to adenocarcinoma develops through a multistep morphological pathway and that this process is characterised by increasing grades of dysplasia, the precursor of invasive adenocarcinoma (Montgomery, Goldblum et al. 2001). Concurrent to the metaplasia–dysplasia–adenocarcinoma sequence, studies have demonstrated the accumulation of genetic abnormalities in cells from normal squamous epithelia to invasive malignant adenocarcinoma (Flejou 2005).

Recent research has shed some light on the various histologic stages associated with the neoplastic progression of Barrett's oesophagus. The neoplastic progression of Barrett's oesophagus is associated with changes in gene structure, gene expression and protein structure (Jankowski, Wright et al. 1999). Concurrent to the morphological stages that are evident in the neoplastic progression of Barrett's oesophagus, chromosomal and genetic changes occur, with ensuing irregularities in gene expression and cell cycle regulation. Loss of cell cycle control occurs early in the sequence and increases as the disease progresses, with increased S phase fractions occurring in a subset of biopsy specimens at more advanced stages of neoplastic progression and often in association with the development of aneuploidy (Reid, Sanchez et al. 1993). p53 mutations have reflected histological progression in Barrett's patients (Jenkins, Doak et al. 2003). Cell adhesion proteins E-cadherin and β-catenin also show a decreased expression as the sequence progresses (Bailey, Biddlestone et al. 1998); expression of these proteins is key to tissue architecture and loss of expression is conducive to tumour invasion and metastasis (Mareel, Vleminckx et al. 1992).

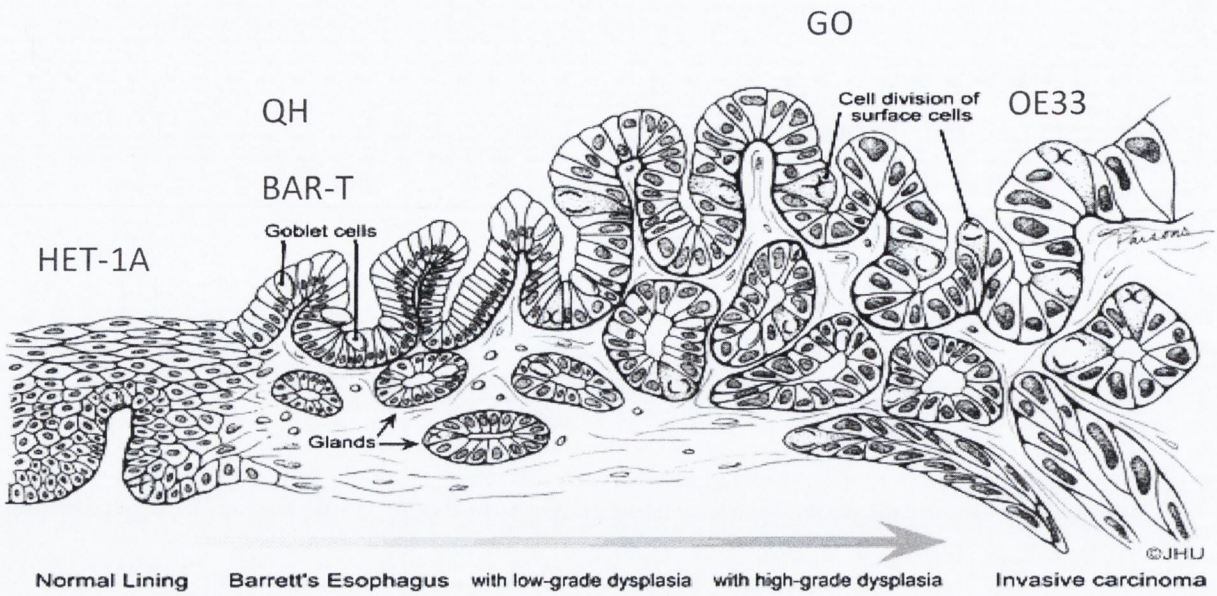

Figure 4.1 .Pathological evolution displaying the metaplasia-dysplasia-adenocarcinoma sequence that characterises the pathogenesis and neoplastic progression of Barrett's oesophagus (taken from http://pathology2.jhu.edu/beweb/definition.cfm. Cell lines which represent each stage of the metaplasia-dysplasia-adenocarcinoma sequence are also displayed on the figure in red.

The hallmarks of cancer proposed in 2000 (Hanahan and Weinberg 2000); self-sufficiency in growth signalling, insensitivity to anti-growth signalling, evasion of apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis have all been observed in carcinogenesis arising in Barrett's oesophagus (Morales, Lee et al. 1998; Shureiqi, Xu et al. 2001; Auvinen, Sihvo et al. 2002; Morales, Souza et al. 2002). As benign, metaplastic Barrett's cells advance to invasive carcinoma, they accumulate a number of genetic alterations that provide them with certain growth advantages. These alterations eventually endow the cells with the essential physiological hallmarks of cancer cells (Zhang, Spechler et al. 2009) and may promote carcinogenesis arising in a background of Barrett's oesophagus.

Activation of pro-invasive pathways has been reported previously in Barrett's oesophagus along the metaplasia-dysplasia-adenocarinoma sequence in both patient explant cultures, and cell culture models (Lagorce-Pages, Paraf et al. 1998; Clemons, Shannon et al. 2010) while adipose tissue derived factors have been shown to be a potent stimulator of cell invasion in breast cancer (Gillespie, Sorbero et al. 2010). It has also been suggested that adiponectin, an adipocytokine whose circulating levels are inversely related to body mass index, may inhibit invasion and metastasis in gastric cancers (Ishikawa, Kitayama et al. 2005). Plasma adiponectin levels have also been shown to be significantly lower in prostate cancer patients than in age and sex matched patients with benign prostatic hyperplasia and healthy controls (Goktas, Yilmaz et al. 2005). Adiponectin has also been associated with leukaemia by suppressing the

growth of myelomonocyte cells lines, and inducing apoptosis in myelomonocytic progenitor cells in vitro (Yokota, Oritani et al. 2000). Leptin, an adipocytokine whose circulating levels are directly proportional to body mass index, has been shown to induce invasion and migration of glioma cells through the activation of MMP-13 (Fu, Yeh et al. 2009), and leptin stimulated anchorage independent growth has been demonstrated in the T47D breast cancer cell line (Hu, Juneja et al. 2002). It has also been reported that freshly prepared leukemic cells from some acute myeloid leukemia patients exhibit a proliferative response to leptin alone and this response is amplified in the presence of hematopoietic cytokines such as IL-3 (Konopleva, Mikhail et al. 1999).

Adipose tissue derived factors have also been shown to be potent stimulators of cellular proliferation in numerous cancers. TNFα, an inflammatory cytokine linked to the neoplastic progression of Barrett's oesophagus (Tselepis, Perry et al. 2002), enhances proliferation of mammary tumours and the human breast cancer cell line T47D (Rivas, Carnevale et al. 2008). Visfatin, a pre B-cell enhancer, which also plays a role mediating insulin secretion from pancreatic beta cells, is another adipocytokine which has demonstrated proliferative effects on cancer cells, such as PC3 human prostate cancer cells (Patel, Mistry et al. 2010). Visfatin also increases matrix metalloproteinase expression and activity, and increased expression of these pro-invasive factors may be a mechanism by which adipose derived visfatin promotes neoplastic progression (Patel, Mistry et al. 2010). Obesity regulated factors IGF-1 and estrogen have also been shown to enhance cell proliferation and early tumourigenesis of breast cancer cells (Lautenbach, Budde et al. 2009).

In this chapter, changes in expression of genes related to tumourigenesis and pro-invasive and proliferative pathways in these cell lines in response to ACM were investigated in cell lines which represent each stage of the metaplasia-dysplasia-adenocarcinoma sequence.

4.2 Aims and Objectives

The specific aim of this chapter was to investigate the molecular and functional mechanisms by which visceral adiposity drives carcinogenesis at different stages of the metaplasia-dysplasia-adenocarcinoma sequence associated with the neoplastic progression of Barrett's oesophagus.

Specific Objectives

- Identification of oncogenic pathways activated by ACM generated from viscerally obese oesophageal adenocarcinoma patients in the HET-1A cell line
- Tracking of the expression of the five gene signature of MMP-1, TIMP-3, IL-8, MCAM and VEGFA in the HET-1A, QH, GO and OE33 cell lines, in response to ACM treatment
- Investigate the proliferative and invasive capacities of the oesophageal cell lines in response to ACM treatment

4.3 Results

4.3.1 Characterisation of the oesophageal cell models

In this study four oesophageal cell lines were used with each cell line representing a different stage of the metaplasia-dysplasia-adenocarcinoma sequence (Jankowski, Wright et al. 1999). The HET-1A cell line represents normal squamous oesophageal epithelium, the QH cell line represents benign Barrett's oesophagus, the GO cell line represents high grade dysplastic Barrett's oesophagus, while the OE33 is a cell line model for oesophageal adenocarcinoma arising in Barrett's oesophagus. The oesophageal cell models were characterised by examining specific markers of columnar epithelium, cytokeratin 8 and villin. Cytokeratin 8 is a key biomarker of metaplastic transformation in Barrett's oesophagus and can be used distinguish Barrett's metaplasia from healthy squamous epithelia (Hage, Siersema et al. 2005), while villin is an important columnar cell differentiation marker (Rahman, Kadowaki et al. 2010). The oesophageal cell lines QH, GO and OE33 showed high levels of expression of Barrett's oesophagus molecular markers villin and cytokeratin 8. The HET-1A cell line showed no expression of these molecular markers indicating that this cell line is representative of squamous oesophageal epithelium without metaplastic transition to a columnar phenotype (Figure 4.2).

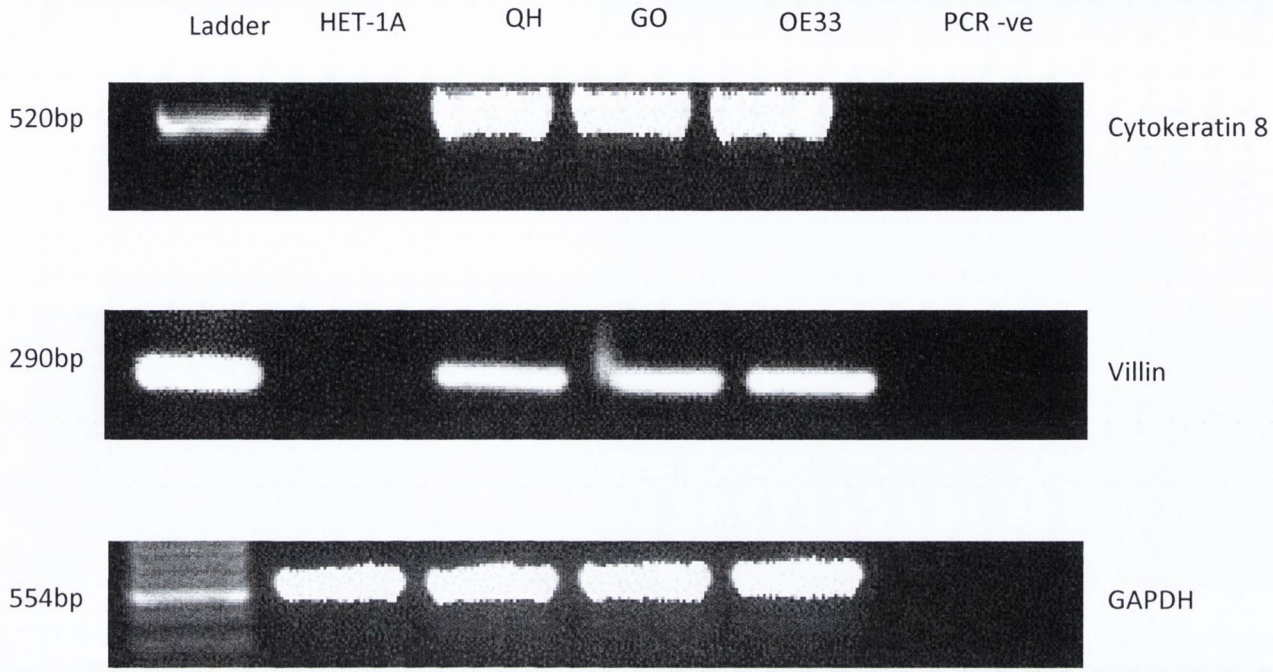

Figure 4.2. QH, GO and OE33 cells express differentiation Barrett's epithelial markers cytokeratin 8 and villin, while HET-1A cells do not. The cell lines were cultured in maintenance media for 24hr before gene expression was examined using RT-PCR. The PCR –ve lane contains sterile water instead of cDNA, controlling for contamination in the PCR reaction.

4.3.2 Culture of HET-1A cell line with adipose conditioned media alters the expression of genes involved with pro tumour pathways

The human cancer profiler array was used to identify pathways associated with obesity and carcinogenesis activated in the HET-1A cell line. The HET-1A cells were cultured in the same patient ACM as the BAR-T cells (section 3.3.3) in this assay in order to limit the effects of inter-patient variability on the results of the Human cancer profiler array.

Culture of the non-neoplastic HET-1A cell line with ACM generated from the omental tissue of obese oesophagectomy patients altered gene expression in each of the oncogenic pathways represented on the plate, with 40 genes in total showing a fold up-regulation/ down-regulation in gene expression, with a change of at least 2-fold classified as an up-regulation/ down-regulation (Table 4.2). The greatest gene expression changes were observed in the pathways related to invasion and metastasis (25%) and angiogenesis (22.5%), while changes were also observed in pathways related to adhesion (17.5%), apoptosis and cell senescence (17.5%) cell cycle control and DNA repair (12.5%), and signal transduction and transcription factors (5%) (Figure 4.3).

Following ACM treatment, the greatest increase in gene expression was seen in cyclin dependant kinase 4 (CDK4), a member of a protein kinase family that is critical for cell cycle G1 phase progression. Expression of this gene increased 26.6 fold in the HET-1A cell line following ACM treatment. Caspase 8 (CASP8) also showed a marked increase in expression in the HET-1A cell line following ACM treatment, with expression of this pro-apoptotic regulator increased 18.3 fold. Serpine 1, also known as plasminogen activator inhibitor 1, which acts as an inhibitor of matrix metalloproteinases also demonstrated a substantial increase in expression in the HET-1A cell line following ACM treatment, with gene expression levels increased 17.1 fold. MMP-1, IL-8 and VEGF, genes associated with tumourigenesis that showed increases in expression in the BAR-T cell line following ACM treatment, also showed increases in expression in the HET-1A cell line following an identical treatment, with gene expression increased 5.5 fold, 3.4 fold, and 10.2 fold respectively (Table 4.2).

Table 4.1. Anthropometric data for patient ACM used in cancer pathway profiler

Patient	Sex	Age	Diagnosis	WC (cm)	BMI	VFA (cm^2)	MetS?	Neoadj?
1	M	60	OAC	101	25.4	172.1	1	1
2	M	62	OAC	126	33.8	383.8	1	0
3	M	62	OAC	96	28.2	155.2	0	0

The Internation Diabetes Federation (IDF) cut off of WC> 94cm was used to define presence of visercal obesity. (WC= waist circumference, BMI= body mass index, VFA= visceral fat area, MetS= metabolic syndrome, Neoadj= neoadjuvent therapy.

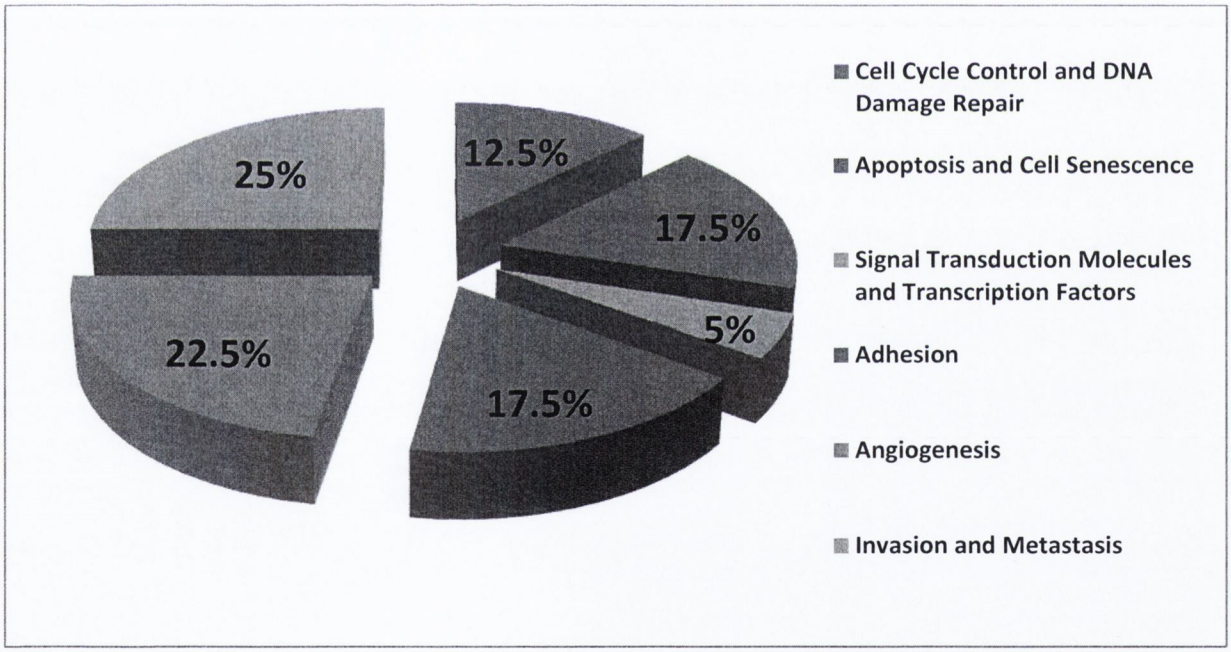

Figure 4.3. ACM treatment (n=3) of HET-1A cell line activates well defined cancer pathways. Oncogenic pathway dysregulation in the HET-1A cell line following ACM treatment mirrors results seen in the BAR-T cell line, with genes involved in the angiogenic pathway and the metastatic and invasive pathways underlying carcinogenesis, showing the greatest number of changes.

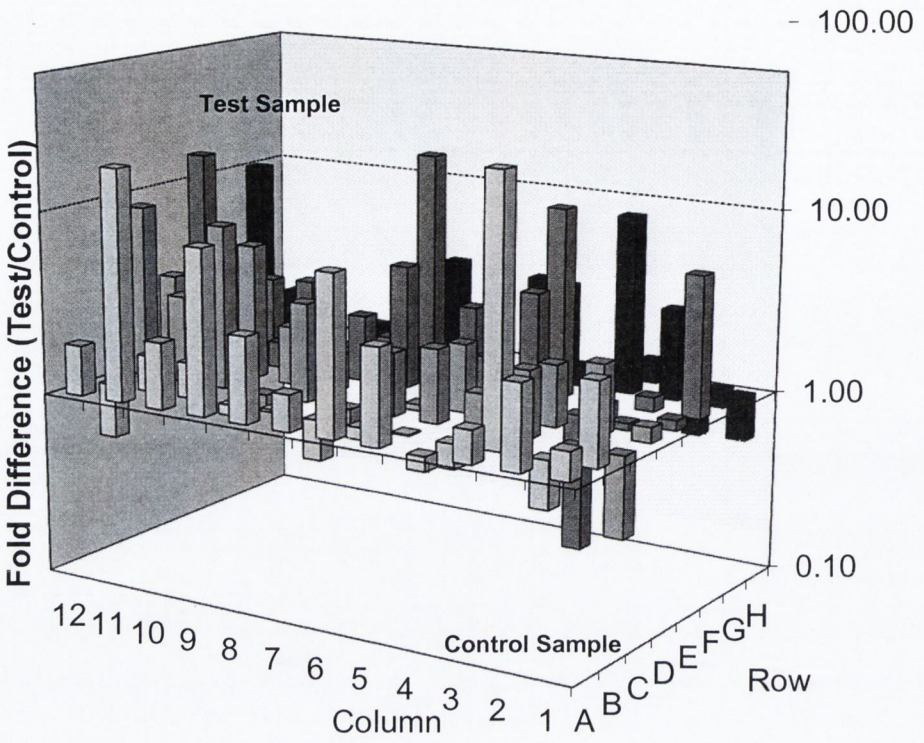

Figure 4.4. Changes in gene expression profiles in HET-1A cell line following 24hr ACM treatment (n=3), analysed using Human Cancer Pathway Finder Arrays. Fold changes in gene expression were calculated using the Ct method.

Table 4.2. Fold change in expression of genes altered in the HET-1A cell line following culture with ACM generated from visceral adipose tissue of obese OAC patients (n=3).

Gene	Pathway	Fold Change
ANGPT1	Angiogenesis	**2.86**
ATM	Cell Cycle Control	**3.33**
BAD	Apoptosis and Cell Senescence	**7.36**
BCL2	Apoptosis and Cell Senescence	**2.95**
BCL2L1	Apoptosis and Cell Senescence	**7.97**
BRCA1	Cell Cycle Control	**2.31**
CASP8	Apoptosis and Cell Senescence	**18.31**
CDC25A	Cell Cycle Control	**2.79**
CDK4	Cell Cycle Control	**26.56**
FGFR2	Angiogenesis	**-2.73**
GZMA	Apoptosis and Cell Senescence	**2.30**
IFNA1	Angiogenesis	**2.51**
IFNB1	Angiogenesis	**2.20**
IL8	Angiogenesis	**3.44**
ITGA2	Adhesion	**7.83**
ITGA3	Adhesion	**2.97**
ITGA4	Adhesion	**8.72**
ITGB1	Adhesion	**2.37**
ITGB3	Adhesion	**2.15**
JUN	Signal Transduction Molecule	**2.34**
MET	Invasion and Metastasis	**2.08**
MMP1	Invasion and Metastasis	**5.47**
MMP9	Invasion and Metastasis	**3.21**
MTSS1	Adhesion	**-5.44**
MYC	Signal Transduction Molecule	**4.18**
NME1	Invasion and Metastasis	**4.67**
NME4	Invasion and Metastasis	**2.30**
PLAU	Invasion and Metastasis	**14.20**
PLAUR	Invasion and Metastasis	**5.80**
RB1	Cell Cycle Control	**10.63**
SERPINB5	Invasion and Metastasis	**2.65**
SERPINE1	Invasion and Metastasis	**17.15**
TEK	Angiogenesis	**2.76**
TERT	Apoptosis and Cell Senescence	**2.65**
THBS1	Angiogenesis	**3.10**
TIMP1	Invasion and Metastasis	**9.29**
TNF	Angiogenesis	**3.69**
TNFRSF1A	Apoptosis/ Cell Senescence	**3.99**
EPDR1	Adhesion	**2.01**
VEGF	Angiogenesis	**10.19**

4.3.3 Tracking of MMP-1, TIMP-3, IL-8, MCAM and VEGFA gene expression in response to ACM treatment across metaplasia-dysplasia-adenocarcinoma sequence

The expression of MMP-1, TIMP-3, IL-8, MCAM and VEGFA was analysed via qPCR in the HET-1A, QH, GO and OE33 cell lines in response to ACM generated from the omental tissue of viscerally obese oesophageal adenocarcinoma patients. This five gene signature had been identified in the BAR-T cell line using the human cancer profiler array and subsequently validated using specific primer-probe sets.

MMP-1 gene expression was up regulated 36 fold in the HET-1A cell line following treatment with ACM generated from the omental tissue of viscerally obese oesophageal adenocarcinoma patients (Figure 4.5A). Expression of this pro-metastatic factor was also increased in the QH, GO and OE33 cell lines, with expression increased 3.1 fold, 2.9 fold and 1.3 fold respectively following ACM treatment. The up regulation of MMP-1 expression was significantly higher in the HET-1A cell line compared to the QH ($p=0.004$), GO ($p=0.012$) and OE33 ($p=0.029$) cell lines.

Expression of TIMP-3, a tissue inhibitor of MMP-1, was up regulated 1.3 fold in the HET-1A cell line following ACM treatment (Figure 4.5B). Though this represents a modest increase, the expression of this TIMP-3 is decreased in the QH, GO and OE33 cell lines with expression decreased 1.2 fold, 5.6 fold, and 7.5 fold, respectively, following ACM treatment. mRNA expression levels of TIMP-3 along the metaplasia-dysplasia adenocarcinoma sequence were significantly lower in the QH ($p=0.008$), GO ($p=0.006$) and OE33 ($p=0.002$) cell lines, compared to the HET-1A cell line.

The expression of pro-inflammatory chemokine IL-8 showed flucatations in response to ACM treatment across the metaplasia-dysplasia-adenocarcinoma sequence (Figure 4.5C). Expression of IL-8 was increased 13.8 fold in the HET-1A cell line following ACM treatment, which represented significantly higher increase in gene expression compared to the 3.5 fold increase seen in the QH cell line ($p=0.080$) and the 4.4 fold increase in OE33 cell line ($p=0.050$), following identical ACM treatments. While the HET-1A cell line demonstrated higher levels of IL-8 gene upregulation than the GO cell line in response to ACM treatment, the levels in gene expression did not reach statistical significance ($p=0.4$).

MCAM, a cell adhesion molecule, demonstrated the greatest up-regulation of gene expression in the dysplastic GO cell line, following ACM treatment. MCAM expression increased 9.2 fold in this cell line, while gene expression was decreased in each the HET-1A (1.6 fold), QH (1.2 fold)

and OE33 (2.1 fold) cell lines, compared to the M199 control post ACM treatment. Expression of MCAM in the GO cell line was signifiantly higher than HET-1A (p=0.01), QH (p=0.031) and OE33 (p=0.02) cell lines, following ACM treatment (Figure 4.5D).

VEGFA mRNA increases in expression in response to ACM, were highest in the HET-1A cell line, and demonstrated an overall decrease across the metaplasia- dysplasia- adenocarcinoma sequence associated with Barrett's derived adenocarcinoma (Figure 4.5E). VEGFA mRNA expression was up-regulated 7.2 fold in the HET-1A cell line following ACM treatment, with up-regulations of 4.7 fold, 3.6 fold, and 1.9 fold seen in the QH, GO, and OE33 cell lines respectively, following idenitcal ACM treatments. VEGFA mRNA expression levels in the HET-1A cell line failed to reach statisical significance over the other cell lines.

Early increases in expression of cancer related genes in the epithelia of pre-neoplastic conditions has been demonstrated previously in ovarian (Choi, Auersperg et al. 2003) and colorectal cancer (Kou, Marusawa et al. 2007) and a similar mechanism conducive to carcinogenesis may be taking place in squamous oesophageal epithelium in obese patients.

Table 4.3. Significance values of genes related to tumourigenesis between oesophageal cell lines following ACM treatment (n=5). Statistical analysis was performed using an unpaired student's t-test. Data are expressed as mean fold change in gene expression following ACM treatment.

Cell Line	MMP-1 Fold change	TIMP-3 Fold change	IL-8 Fold Change	MCAM Fold Change	VEGFA Fold Change
HET-1A	36.0	1.3	13.2	-1.6	7.5
p value	-	-	-	*0.01*	-
QH	3.1	-1.2	3.5	-1.2	4.7
p value	*0.042*	*0.008*	*0.050*	*0.031*	*0.16*
GO	2.9	-5.6	10.0	9.2	3.7
p value	*0.01*	*0.006*	*0.4*	-	*0.13*
OE33	1.3	-7.5	4.4	-2.1	1.9
p value	*0.02*	*0.002*	*0.05*	*0.02*	*0.06*

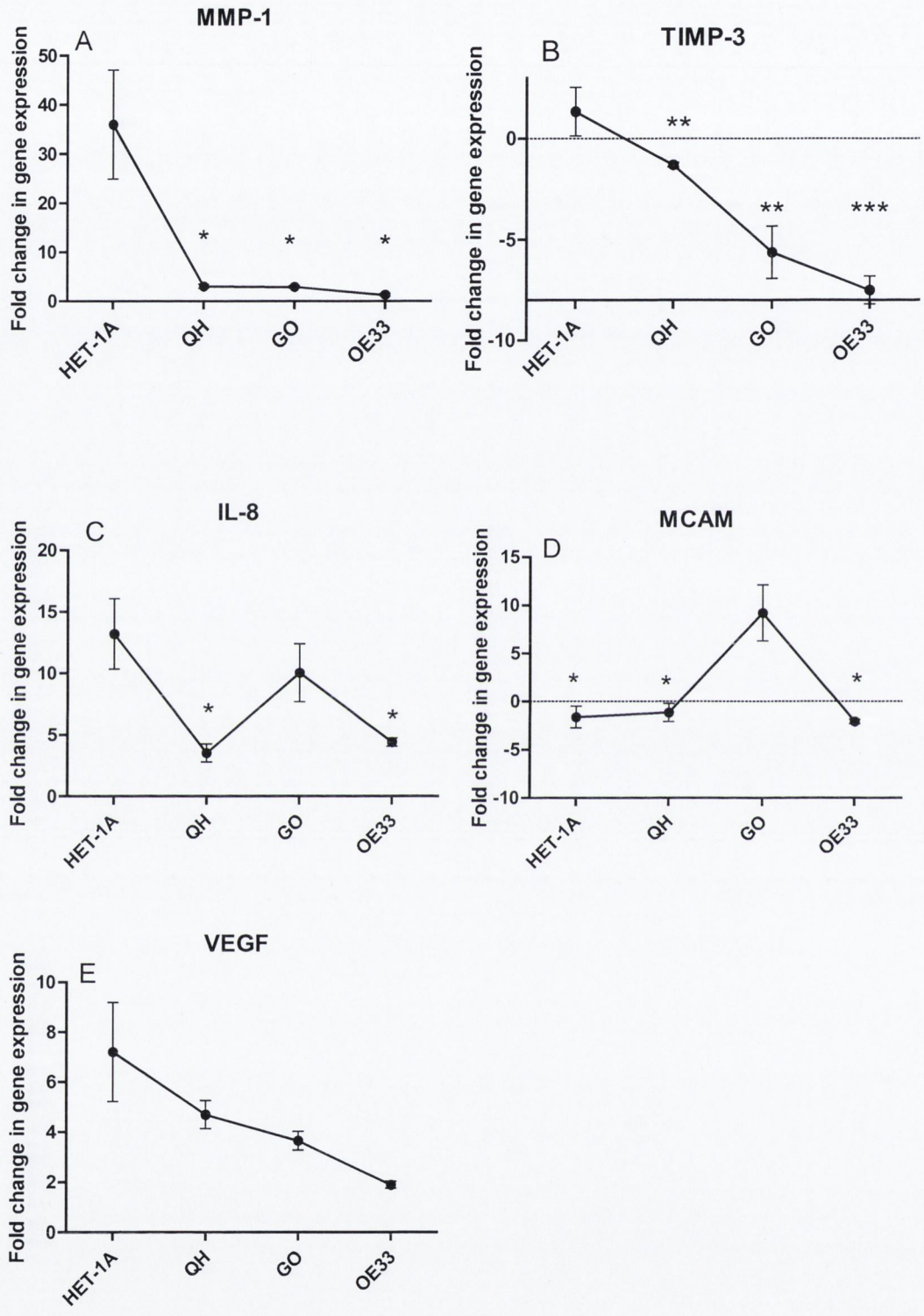

Figure 4.5. Expression of MMP-1 (A), TIMP-3 (B) , IL-8 (C), MCAM (D) and VEGFA (E) in oesophageal cell models in response to ACM generated from the omental tissue of viscerally obese oesophageal adenocarcinoma patients (n=5). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

4.3.4 Adipocytokines adiponectin and leptin alter cell proliferation in dysplastic Barrett's oesophagus (GO) and oesophageal adenocarcinoma (OE33) cell lines in a dose dependant manner

Cell proliferation, determined by BrdU ELISA, in response to leptin and adiponectin, well-defined adipocytokines, was investigated in the oesophageal cell lines. The QH cell line was used to represent metaplastic Barrett's oesophagus in place of the BAR-T cell line in this assay and all functional assays. The BAR-T cell line was unsuitable for use in this assay due the mitomycin-c treated 3T3 feeder layer required for growth of the cell line

In the HET-1A cell line, leptin treatment resulted in a modest decrease in cell proliferation at all concentrations, though this decrease did not follow a clear dose dependant pattern (Figure 4.6A). A similar result was demonstrated in the benign Barrett's oesophagus QH cell line, with leptin treatment eliciting a modest decrease in cell proliferation; however this decrease in proliferation was not dose dependant (Figure 4.6B). A significant dose dependant response was however exhibited by the GO, and OE33 cell lines in response to leptin. In the GO cell line a 10ng/ml leptin treatment increased proliferation 120% compared to the untreated control, representing a significant increase in proliferation ($p=0.047$). A further significant increase in proliferation compared to untreated control was demonstrated in response to 100ng/ml ($p=0.015$) and a 500ng/ml ($p=0.018$) leptin treatment with proliferation increasing 155% and 162%, respectively (Figure 4.6C). A similar pattern of leptin dose dependant increases proliferation was demonstrated in the OE33 cell line (Figure 4.6D). A 10ng/ml leptin treatment increased proliferation 116% compared to the untreated control, representing a significant increase in proliferation ($p=0.017$). A significant increase of cell proliferation of 134% ($p=0.054$) and 150% ($p=0.013$) was also observed following 100ng/ml and 500ng/ml treatments, respectively, compared to the untreated control.

Cell proliferation, determined by BrdU ELISA, in response to adiponectin, an adipocytokine with anti-cancer properties, was also investigated in the panel of oesophageal cell lines (Figure 4.7). Adiponectin treatment elicited no response in the HET-1A cells (Figure 4.7A). An identical treatment of both the QH (Figure 4.7B) and GO (Figure 4.7C) cell lines demonstrated a modest decrease in cell proliferation in a dose dependant manner, though these decreases did not reach statistical significance. However, the OE33 cell line, which is representative of invasive oesophageal adenocarcinoma, showed significant decreases in cell proliferation in a dose dependant manner (Figure 4.7D). Significant decreased in cell proliferation compared to the untreated control were demonstrated in each treatment. A 0.1μg/ml treatment decreased proliferation to 81% of the control ($p=0.014$), a 1μg/ml treatment decrease proliferation to

73% of the control (p=0.001), a 10µg/ml treatment decrease proliferation to 62% of the control (p=0.0007) and a 50µg/ml treatment decrease proliferation to 53% of the control (p=0.0002).

Increased tumour cell proliferation by increased levels of circulating leptin in obese individuals represents a mechanism by which obese patients may be at greater risk of carcinogenesis (Lautenbach, Budde et al. 2009), a similar association can also be made with the risk of increased carcinogenesis in obese people due to decreased levels of circulating leptin (Arditi, Venihaki et al. 2007).

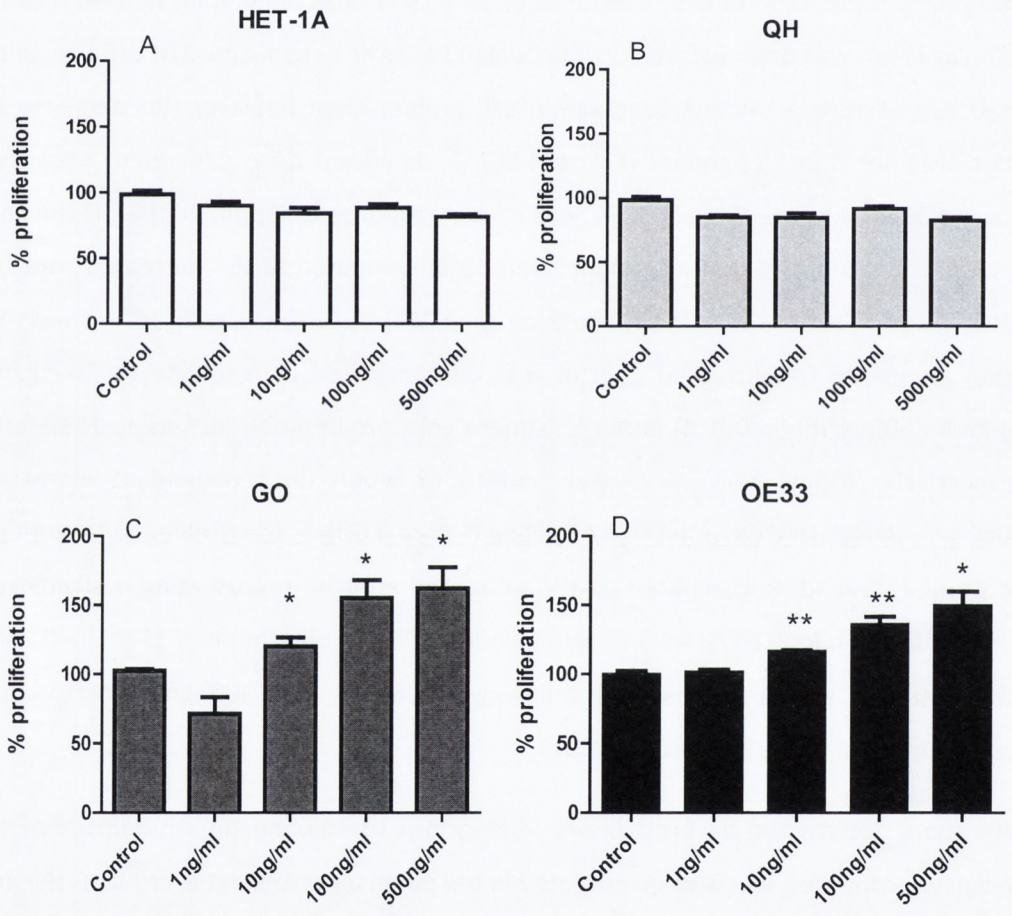

Figure 4.6. Leptin dose response in oesophageal cell lines following 24hr treatment as determined by BrdU ELISA (n=5). Data are expressed as mean ± SEM. Cells treated with media alone were used to normalise the results. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01).

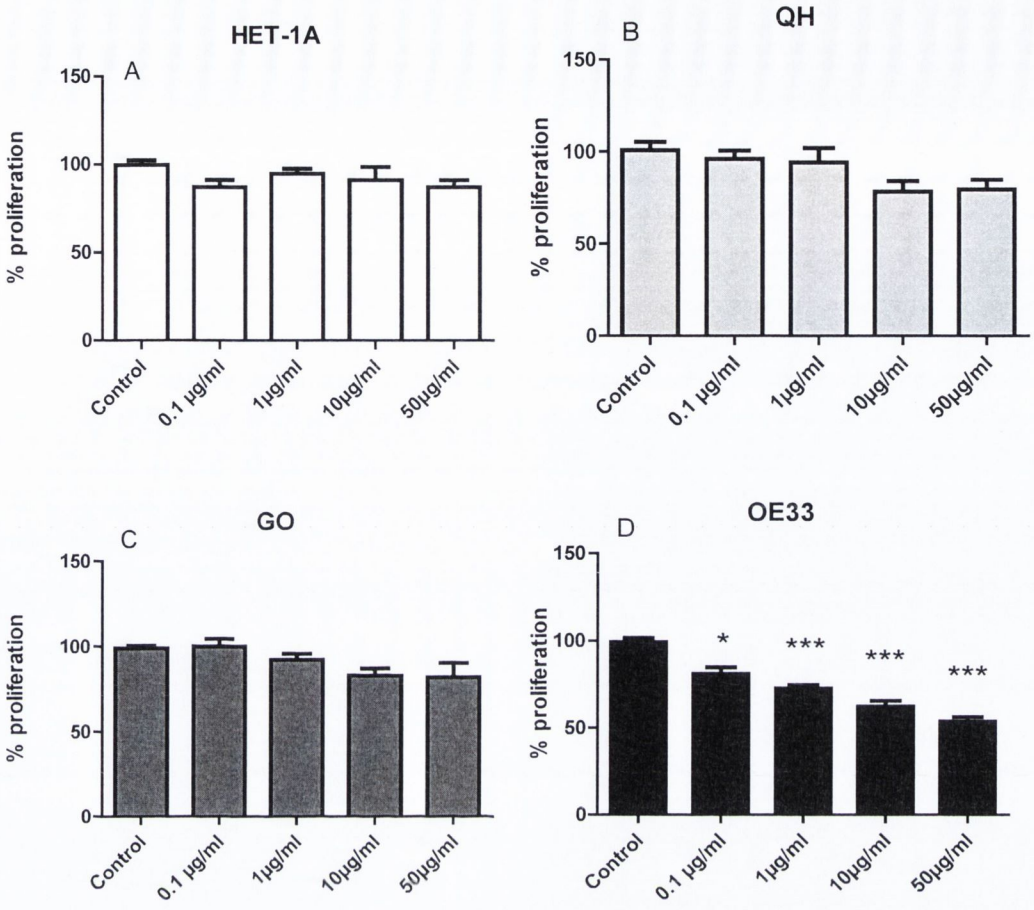

Figure 4.7. Adiponectin dose response in oesophageal cell lines following 24hr treatment as determined by BrdU ELISA (n=5). Data are expressed as mean ± SEM. Cells treated with media alone was used to normalise the results. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.3.6 Adipocytokine receptor expression in Barrett's oesophagus cell models

The expression of adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), and the expression of leptin receptor (LEPR), were assessed via qPCR in the panel of cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence (Figure 4.8). Expression of these receptors modulate the sensitivity of the cells to adiponectin and leptin and increases of expression of these receptors has been demonstrated in Barrett's oesophageal adenocarcinoma previously (Reynolds, Howard et al. 2010).

Expression of AdipoR1 was significantly higher in the OE33 cell line compared to the HET-1A ($p=0.0001$), QH ($p=0.0006$) and GO ($p=0.0001$) cell lines (Figure 4.8A). A similar trend was observed in AdipoR2 expression in the Barrett's oesophagus cell lines, with significantly higher expression of this receptor exhibited by the OE33 cell line compared to the HET-1A ($p=0.002$), QH ($p=0.005$) and GO ($p=0.002$) cell lines (Figure 4.8B). LEPR showed greatest mRNA expression in the OE33 cell line, with levels significantly higher than those seen in the HET-1A ($p=0.025$) and QH ($p=0.024$) cell lines. The OE33 cell line also demonstrated greater LEPR mRNA expression levels than the GO cell line, though these levels did not reach statistical significance (Figure 4.8C).

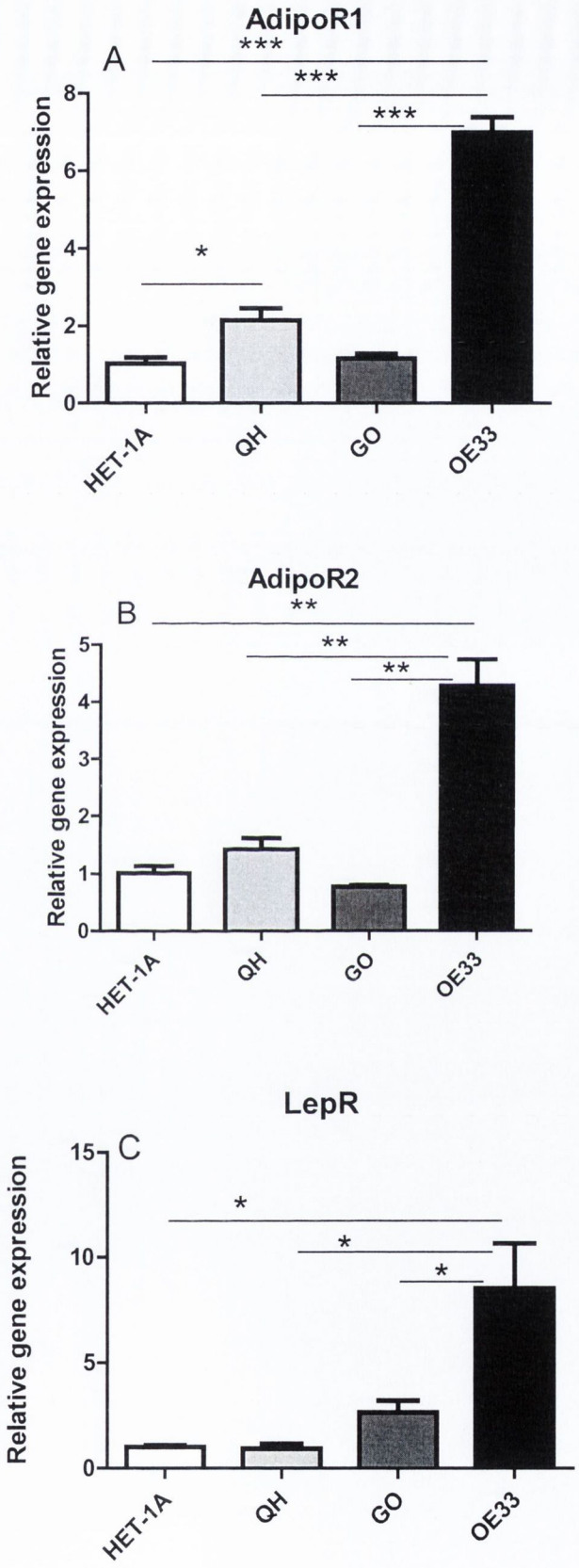

Figure 4.8. Relative expression of adipocytokine receptors in oesophageal cell line models (n=3). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

4.3.7 ACM increases the invasive capacity of oesophageal cell lines

The invasive capacity of the oesophageal cell lines following ACM treatment was investigated using a cell invasion assay based on the Boyden chamber method (Figure 4.9). Invasive capacity was determined by measuring the ability of the cells to move out of a porous chamber, coated with extra cellular matrix (ECM), into a reservoir containing ACM from viscerally obese patients following 24hr ACM treatment (as described in section 2.10).

Each of the oesophageal cell lines showed increases in invasive capacity in response to culture with ACM generated from viscerally obese OAC patients. The QH cell line demonstrated the greatest increase in cell invasion, with invasion levels of 331% compared to untreated control QH cells ($p<0.0001$). A marked increase in invasion compared to untreated controls was also observed in the HET-1A (231% of control, $p=0.01$) and OE33 cell lines (168% of control, $p=0.003$). Cell invasion was increased to 135% of the untreated control in the GO cell line following ACM treatment, but levels failed to achieve statistical significance.

ACM was shown to alter the expression of genes involved in the invasion pathway in this chapter and in chapter 3, and the results of the cell invasion assay back up this finding with functional data. Early increases in invasion has been reported previously in Barrett's derived carcinoma (Stolte, Kirtil et al. 2010) and the data presented here reinforces that theory.

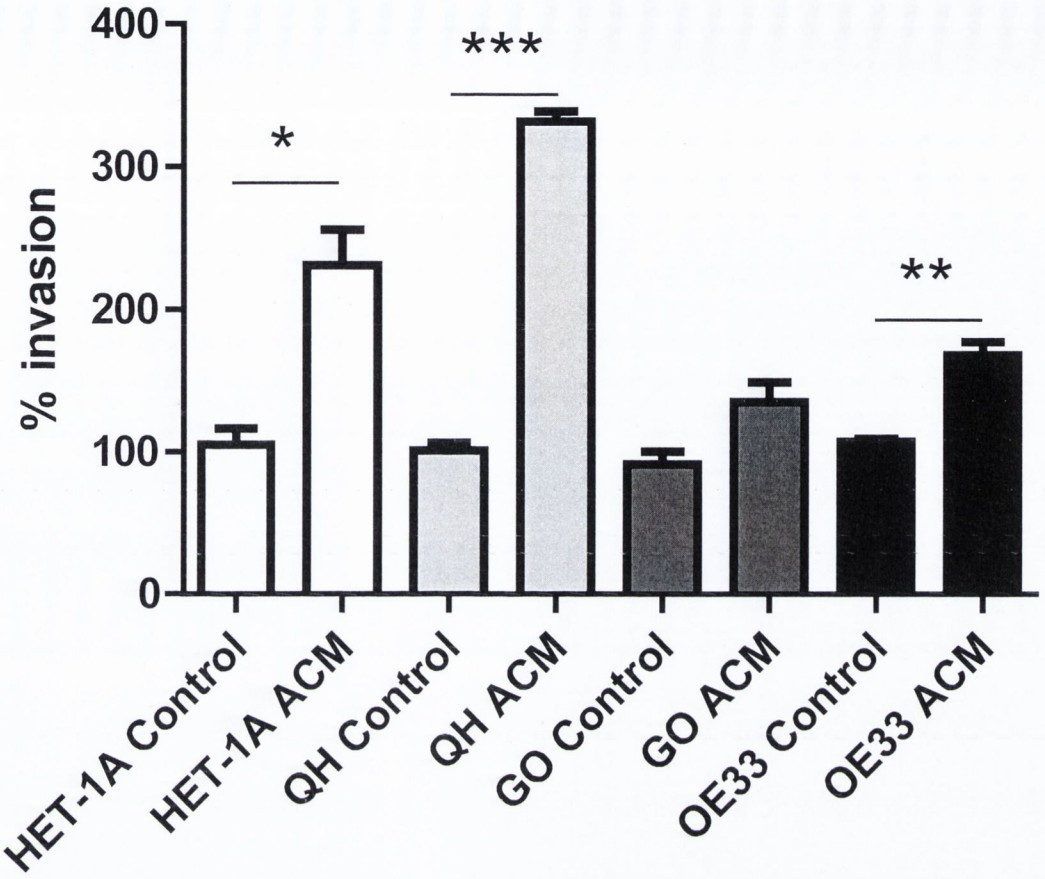

Figure 4.9. ACM stimulates cell invasion in oesophageal cell line models as determined by a Boyden chamber based cell invasion assay (n=5). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

4.3.8 ACM induces proliferation in oesophageal cell lines

The oesophageal cell lines were treated with ACM or M199 control media for 24hr and proliferation of these cell lines in response to ACM treatment was assessed by a BrdU cell proliferation ELISA (described in section 2.8).

The HET-1A cell line showed no response to ACM generated from omental tissue from either viscerally obese or non-obese OAC patients. Cell proliferation was significantly increased in the QH cell line in response to ACM generated from non-obese patients compared to the untreated control ($p=0.014$) and there was also significantly more proliferation in this cell line in response to ACM generated from obese patients versus non-obese patients ($p=0.036$). This pattern of increased proliferation was observed in both the GO and OE33 cell lines, with non-obese ACM increasing cell proliferation compared to the untreated control ($p=0.007$ and $p=0.01$, respectively) and obese ACM stimulating significantly more proliferation than non-obese ACM ($p=0.04$ and $p=0.006$, respectively). Increased proliferation in response to adipose derived factors has been demonstrated in pre malignant endometrial epithelial cells (Villavicencio, Aguilar et al. 2010) and specifically to ACM in oesophageal and colorectal cancer cell lines (Lysaght, van der Stok et al. 2011).

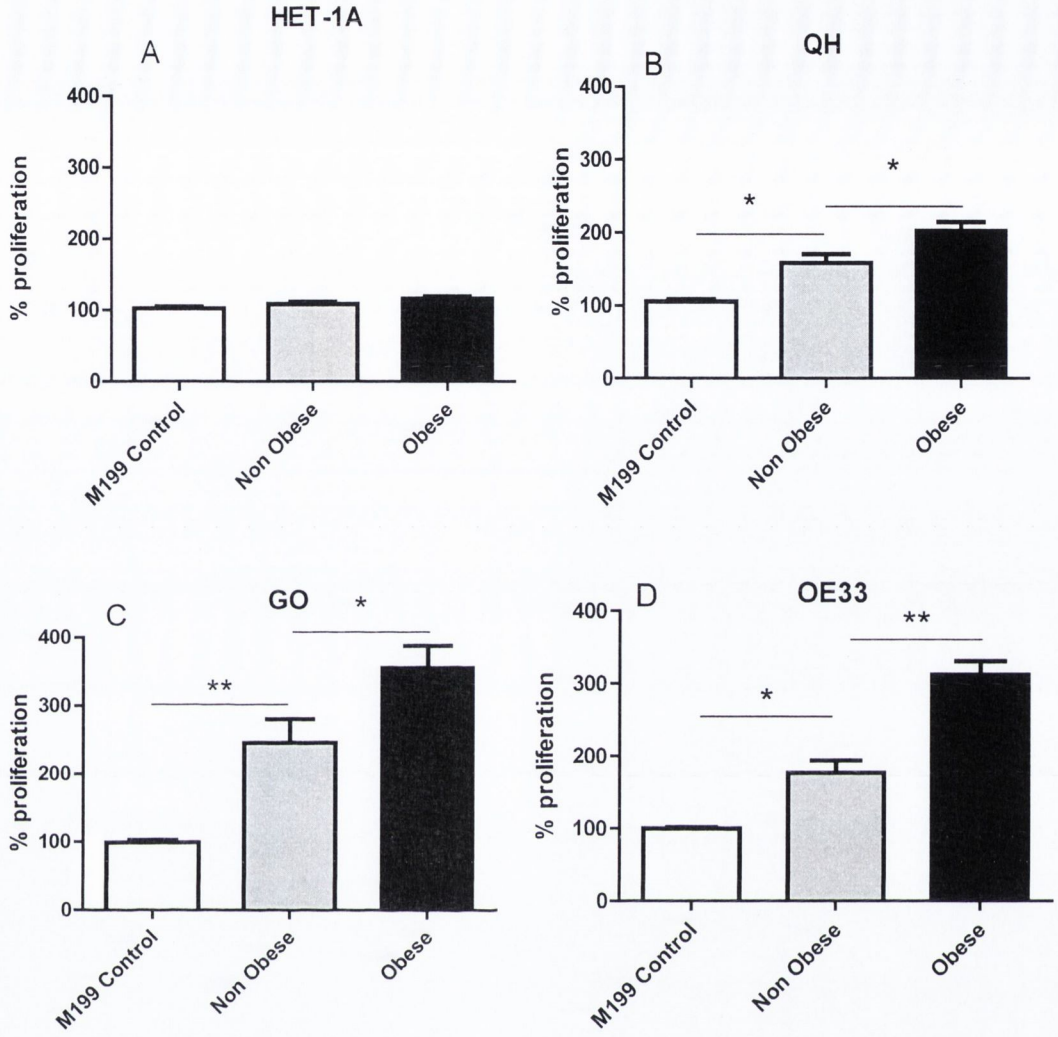

Figure 4.10. Culture of QH (B), GO (B) and OE33 (C) cell lines with ACM for 24hr significantly increased cell proliferation, as determined by BrdU ELISA (n=5). No effect of ACM on proliferation of the HET-1A cell line was observed (A). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired students t-test (*$p<0.05$, **$p<0.01$).

4.4 Discussion

Obesity is growing at an alarming rate in the western hemisphere (Ford and Mokdad 2008) with incidences of Barrett's oesophagus in the general population (van Soest, Dieleman et al. 2005) and the neoplastic progression of this premalignant lesion, also increasing in frequency (Brown, Devesa et al. 2008). This chapter demonstrates that dysregulation of genes by obesity involved in well-defined oncogenic processes may actually precede the pathogenesis of Barrett's oesophagus. Genes intrinsically linked to cancer show greatest increases in gene expression when cultured with ACM in the HET-1A cell line and the degree of gene expression drops as the metaplasia-dysplasia-adenocarcinoma sequence progresses. Cell proliferation in response to ACM was demonstrated in all cell cells except the HET-1A cell line, however activation of cell invasion, a tumourgenic mechanism associated with invasive carcinoma, by ACM is greatest in the HET-1A and QH cell lines, cells representative of healthy squamous epithelium and benign metaplastic Barrett's oesophagus respectively, indicating that obesity related malignancy in Barrett's oesophagus may be an early event.

ACM treatment altered pro tumour pathways in the squamous epithelial HET-1A cell line as determined by the human cancer profiler array. In total, the expression of 40 genes related to carcinogenesis were altered following ACM treatment, with each of these genes falling into a least one of the six tumorigenic pathways represented on the human cancer profiler array. Similar to results observed in the BAR-T cell line following ACM treatment (section 3.3.3), molecular pathways related to angiogenesis, and invasion and metastasis showed the greatest dysregulation, with these two pathways directly related to the cancer hallmarks of sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). While the dysregulation of these pathways may be responsible for the neoplastic progression of Barrett's oesophagus, it may also be implicated with the pathogenesis of the Barrett's oesophagus arising in healthy squamous epithelium. While the association of visceral obesity with GORD and incidences of Barrett's oesophagus has been reported by numerous authors previously (El-Serag, Kvapil et al. 2005; Corley, Kubo et al. 2007; Abrams 2009), this study demonstrates that well defined pro-cancer pathways are activated in oesophageal epithelial cells by adipose derived factors, independent of cellular insult from GORD. Activation of these pathways, either in tandem or independent of GORD, may potentially result in the *transition* of oesophageal *squamous* mucosa into a *columnar* epithelium, and the subsequent formation of goblet cells characteristic of Barrett's oesophagus (Vallbohmer, DeMeester et al. 2006).

Expression of MMP-1, TIMP-3, IL-8, MCAM and VEGFA, identified as genes associated with obesity mediated carcinogenesis in the BAR-T cell model of Barrett's oesophagus, were analysed across cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence associated with Barrett's derived carcinogenesis (Jankowski, Wright et al. 1999). MMP-1, TIMP-3, IL-8 and VEGFA showed greatest increases in mRNA expression in the HET-1A cell line in response to culture in ACM, and the trend observed in the data was that the degree of increase in gene expression following ACM treatment diminished along the metaplasia-dysplasia-adenocarcinoma sequence. Interestingly, MCAM, a gene expressed on primary and metastatic melanoma cells, showed greatest increases in gene expression in the GO cell line, which represents dysplastic Barrett's oesophagus. MCAM (also known as MUC-18) is a member of the mucin family of complex high molecular weight glycoproteins, and these family members have been demonstrated to be deregulated along the metaplasia-dysplasia-adenocarcinoma sequence previously (Arul, Moorghen et al. 2000). This study reported up-regulation of MUC1 and MUC4 in dysplastic and neoplastic tissues taken from patients, compared to biopsies taken from normal oesophageal epithelium and Barrett's oesophagus. Up-regulation of MCAM may follow a similar pattern as its related family members MUC1 and MUC4 in Barrett's oesophagus in response to adipose derived factors conferring pro-tumourigenic properties onto the cells.

Dysregulation of the expression of these cancer related genes and activation of pro-tumour pathways in the oesophagus by obesity, independent of insult from GORD, are an early event in Barrett's derived carcinogenesis and may even precede Barrett's oesophagus itself. The same pathways that are involved in neoplastic progression of Barrett's oesophagus may also be responsible for driving the squamous epithelial-columnar epithelial metaplastic switch associated with Barrett's oesophagus, as evidenced by the data in Figure 3.3 and Figure 3.4. Genotypic and phenotypic damage/ mutations due to obesity early in the metaplasia-dysplasia-adenocarcinoma sequence may select for clones with a neoplastic phenotype, which is conducive to dysplasia and eventual carcinogenesis.

Cell proliferation in response to adiponectin and leptin at physiological levels was investigated in the four oesophageal cell lines. Leptin, a well-defined adipocytokine showing increased expression in obese patients (Somasundar, Riggs et al. 2003; Ogunwobi and Beales 2008; Reynolds, Howard et al. 2010), significantly increased cell proliferation in the GO and OE33 cell lines in a dose dependant manner at physiological levels. Leptin has been shown by other groups to promote significant cancer cell proliferation at physiological levels (Ogunwobi, Mutungi et al. 2006) a result that was replicated in this study. The OE33 cell line displayed the

greatest levels of leptin receptor (LepR) mRNA expression, significantly higher than those seen in both the HET-1A and QH cell lines. Studies from our research department in the past have correlated obesity with significantly higher levels of LepR in oesophageal adenocarcinoma (Reynolds, Howard et al. 2010). Increased LepR expression would confer increased leptin sensitivity in this oesophageal adenocarcinoma and the results presented here corroborate that hypothesis. Increased leptin expression has been demonstrated to correlate with increased expression of leptin receptor in gastric cancer (Ishikawa, Kitayama et al. 2006) and expression of both leptin and leptin receptor significantly increase hand in hand with levels of tumour invasion. Increased tissue leptin expression and leptin receptor expression was reported in Barrett's mucosa compared to normal oesophageal mucosa (Francois, Roper et al. 2008), with increased leptin receptor expression conferring hypersensitivity to moderate serum levels of leptin. These findings, along with increased serum leptin levels in obese patients (Rosicka, Krsek et al. 2003), suggest that obese Barrett's patients may be at a greater risk of adipose derived leptin mediated carcinogenesis.

Adiponectin has displayed anti-proliferative effects in a host of cancer cell lines (Wang, Lam et al. 2005; Dieudonne, Bussiere et al. 2006; Arditi, Venihaki et al. 2007; Giudicelli, Dos Santos et al. 2008) and increases apoptosis in the OE19 Barrett's adenocarcinoma cell line (Konturek, Burnat et al. 2008). In this study adiponectin elicited a significant decrease in cell proliferation in a dose dependant manner in the OE33 cell line, at physiological levels. The OE33 cell line had significantly higher expression of both AdipoR1 and AdipoR2 receptors, compared to the HET-1A, QH and GO cell lines, conferring greater sensitivity to adiponectin and explaining in part the dose dependant decrease in cell proliferation observed in the cell line.

Up-regulation of AdipoR2 has been correlated significantly with obesity status and lymph node metastasis in oesophageal adenocarcinoma previously (Reynolds, Howard et al. 2010). It may seem counter-intuitive that AdipoR2 correlates with a more aggressive tumour type considering the well documented anti-cancer properties of adiponectin (Giudicelli, Dieudonne et al. 2006; Cong, Li et al. 2011) however, neovascularisation and sustained angiogenesis, one of the hallmarks of cancer central to malignancy, can be stimulated by adiponectin by promoting cross-talk between AMP-activated protein kinase and Akt signaling within endothelial cells (Walsh, Ouchi et al. 2004). The decrease in proliferation in response to adiponectin was demonstrated in a dose-dependent manner and perhaps more importantly at levels of adiponectin similar to those that have been reported in the non-obese population (Arikan, Bahceci et al. 2010). This provides evidence that elevated levels of circulating adiponectin may deter the growth of oesophageal adenocarcinoma tumour tissue.

In order to determine the potential proliferative effect of these adipose derived factors in visceral obesity, the various oesophageal cell lines were grown in ACM from both obese and non-obese patients. ACM contains not only adiponectin and leptin, but also a host of other tumourigenic factors such as VEGF, IL-8, TNFα and IFNγ. Adipose derived factors other than leptin may be driving proliferation in these cell lines as ACM generated from both obese and non-obese patients significantly up-regulated proliferation in the QH, GO and OE33 cell lines, while leptin treatment at physiological levels only promoted cell proliferation in the GO and OE33 cell lines. Increased cellular proliferation is evident in the later stages of the metaplasia-dysplasia-adenocarcinoma sequence (Whittles, Biddlestone et al. 1999).

Obese Barrett's patients may be at a greater risk of unchecked cell proliferation, a familiar feature of tumourigenesis (Deveraux, Aza-Blanc et al. 2003), compared to their non-obese counterparts, and this event may partially explain the increased incidences of oesophageal adenocarcinoma arising in obese patients (Ryan, Duong et al. 2011) compared to non-obese patients. An obese phenotype was been associated with increased proliferation and tumourigenesis in breast cancer (Lautenbach, Budde et al. 2009) and endometrial cancer (Villavicencio, Aguilar et al. 2010). The proliferative capacity of ACM has been demonstrated before (Lysaght, van der Stok et al. 2011) in OE33 and the HCT-15 colorectal cancer cell lines. Other factors such as acid exposure and bile salts can drive cell proliferation in Barrett's oesophagus (Kaur, Ouatu-Lascar et al. 2000; Fitzgerald 2005) and adipose derived factors such as adipocytokines may be exacerbating the effect of both acid and bile exposure in driving cell proliferation in pre-neoplastic Barrett's oesophagus. Activation of this pro-tumourigenic mechanism by obesity in Barrett's oesophagus may represent a viable route to carcinogenesis.

There was no proliferation seen in the HET-1A cell line in response to ACM from obese and non-obese patients or leptin. The Barrett's oesophagus and oesophageal adenocarcinoma cell lines show greater proliferative responses to these adipose derived factors, and thus it may be later in the metaplasia-dysplasia-adenocarcinoma sequence that visceral adipose tissue derived factors exhibit their proliferative effects in the Barrett's progressive sequence.

The invasive potential of the cell lines was also investigated in response to a 24hr ACM treatment. Pro invasive pathways showed the greatest activation following ACM treatment in both the HET-1A and BAR-T cell lines and activation of these pathways is a noted hallmark of cancer (Hanahan and Weinberg 2000). Similarly, MMP-1 showed significant increases in gene expression in both the HET-1A and BAR-T cell lines following ACM treatment, and this matrix metalloproteinase is one of the primary genes shown to enhance tumour invasion (Shiozawa,

Ito et al. 2000). Dysregulation of MMP-1 and TIMP-3 in the HET-1A and QH cell lines would explain why these cells show the greatest invasive potential following ACM treatment. The OE33 cell line also showed significant increases in invasion, and increases in the invasive capacity of oesophageal adenocarcinoma cell models in tandem with dysregulation of MMP-1 and TIMP-3 expression has been reported before in OE33 cells in response to a nitric oxide treatment (Fitzgerald, Clemons et al. 2010). Dysregulation of the normal balance between MMPs and TIMPs can induce changes in tumour activity as well as defining the overall proteolytic, invasive and proliferative capacity of the tumour (Lopez-Otin, Folgueras et al. 2004). The GO cell line, which is representative of high grade dysplastic Barrett's oesophagus showed the least invasive capacity of all cell lines in response to ACM. The results presented here are counter intuitive as the GO cell line also displays up regulation of both MCAM and IL-8 following ACM treatment, two invasive factors which have been shown to up regulate tumour invasion and metastasis in melanoma (Bar-Eli and Melnikova 2006).

In conclusion, adipose derived factors present in ACM, show greatest dysregulation of pathways related to angiogenesis and invasion and metastasis in the squamous oesophageal epithelium HET-1A cell line, and it is these pathways that also show the greatest dysregulation in an identical treatment of a Barrett's oesophagus cell model (BAR-T). Obesity was also shown to deregulate the gene expression of five genes intrinsically linked to cancer in four distinct oesophageal cell lines representing each stage of the metaplasia-dysplasia-adenocarcinoma sequence, and the degree of gene expression dropped across sequence. Dysregulation of these genes and oncogenic pathways by obesity may be an early event in Barrett's derived carcinogenesis and select for clones which display phenotypic and genotypic alterations conducive to neovascularisation and metastasis. Adipose derived factors present in the ACM also conferred pro-invasive and proliferative abilities to the cell lines, with greatest increases in these mechanisms seen in pre-neoplastic states. Obesity as a causative factor in Barrett's derived carcinogenesis elicits cellular dysfunction before the onset of malignancy, and with the global obesity pandemic showing no signs of abating (Swinburn, Sacks et al. 2011), increased incidences of Barrett's oesophagus, and carcinogenesis arising in this malady, are to be expected.

Chapter 5: Adipose conditioned media promotes telomere shortening in the Barrett's metaplasia-dysplasia-adenocarcinoma disease sequence

5.1 Introduction

Genomic instability is an established feature of most forms of cancer (Tlsty, Briot et al. 1995; Negrini, Gorgoulis et al. 2010), and has been described as an emerging hallmark of cancer (Hanahan and Weinberg 2011). Genomic instability leads to alterations in the genome of the cancer cell including, but not limited to, mutations of single genes, amplifications, deletions or rearrangements of chromosomal material, and gain or loss of entire chromosomes (Shen 2011). Accumulation of these genomic alterations in cells may lead to unchecked cell growth and division, evasion of normal cell death pathways, and ultimately, cancer. While genomic instability is a feature of most cancers, it has been associated with pre-malignant conditions such as ulcerative colitis (Waldman, Willenbucher et al. 1999; O'Sullivan, Bronner et al. 2002) and pre malignant breast disease (O'Connell, Pekkel et al. 1998). The role of genomic instability in carcinogenesis arising in Barrett's oesophagus has been studied since the late 1980s (Rabinovitch, Reid et al. 1989), however the exact molecular mechanisms involved remain elusive.

The ends of chromosomes are capped at both ends by protective structures termed telomeres. Telomeres are composed of tandem repeats of TTAGGG and are associated with a wide variety of telomere binding proteins which mediate their function (Wai 2004; Martinez and Blasco 2010). These chromosomal caps were first proposed seventy years ago in maize when McClintock demonstrated that the ends of chromosomes did not fuse following chromosomal breakage and therefore must somehow be "capped" (McClintock 1941). Telomere length varies from species to species and telomere length is also dictated by cell type and developmental stage (Flores, Canela et al. 2008). Telomere lengths in humans typically span 10-15kb in length while in mice, telomeres span 25-50kb (Blasco 2005). The ends of telomeres are characterised by a G-rich 150-200 nucleotide long 3'overhang, termed the G-strand overhang, which can fold back onto the double stranded telomeric DNA to from the T loop (Martinez and Blasco 2010), which is suggested to protect chromosome ends from degradation, DNA repair machinery and elongation by telomerase (Griffith, Comeau et al. 1999).

Telomeres protect the chromosomes from end to end fusion, recombination and degradation. During cell replication, telomere DNA cannot be completely replicated, leading to progressive telomere shortening over time (Levy, Allsopp et al. 1992). Eventually, telomere shortening will reach a threshold, and cell replication is prohibited as the cells undergo morphologic and biochemical changes and enter replicative senescence (Wai 2004). Cancer cells however

demonstrate an ability to escape this cell senescence by increasing telomerase expression and activity. Telomerase is an enzyme complex which maintains and elongates telomere length and gives cells immorality (Murnane 2011).The telomerase complex compensates for telomere attrition during replication. It consists of two main components which elongate telomeres through the *de novo* addition of TTAGGG repeats by TERT onto chromosome ends using an associated RNA component (TERC) as a template (Martinez and Blasco 2011).

Telomerase is active during development and while normal somatic cells display almost no telomerase activity (Liu, Schoonmaker et al. 1999), a vast array of cancers report telomerase activity. Significant telomerase activity has been reported in oral carcinoma, lung cancer, prostate cancer, liver cancer, breast cancer, neuroblastoma, colorectal cancer and bladder cancer (Martinez and Blasco 2011), however it is yet to be established whether telomerase activity may represent a sensitive and reliable biomarker for diagnosis and malignant transformation in cancer (Belair, Yeager et al. 1997). Telomerase activity is mediated by transcription, mRNA splicing and modification of TERC and TERT, though TERT expression seems to be the most important factor in the regulation of the activity of this complex. VEGF, a well characterised angiogenic factor and an abundant factor of ACM, is known to regulate telomerase activity in ovarian cancer through binding to proximal regions of the TERT promoter (Bennudez, Yang et al. 2007). The oncogene *cMYC* is also reported to be a positive regulator of TERT expression and telomerase activity, promoting cell proliferation (Dalla-Favera, Wu et al. 1999), however, MAD1, a key member of the spindle assembly checkpoint which ensures mitotic fidelity during chromosomal replication, is a negative regulator of telomerase expression (Englert, Gunes et al. 2000), showing a direct link between the spindle assembly checkpoint and maintenance of telomere length.

While telomeres protect the end of chromosomes from degradation, the telomeres themselves are protected from degradation by a six protein complex termed the shelterin complex (Zhong, Shiue et al. 1992; Chong, Vansteensel et al. 1995). This complex consists of three proteins which bind telomeres directly; TERF1, TERF2, and POT1, and these are interconnected by three additional proteins; TINF2, TPP1, and TERF2IP. Formation of the shelterin complex allows the cells to distinguish telomeres from sites of DNA damage. Loss of this complex leads to processing of the telomere ends by the cell's DNA damage machinery (de Lange 2005) and, in contrast to the telomerase complex, shelterin complex proteins are negative regulators of telomere elongation (de Lange and Donigian 2007). Three separate effects of shelterin on telomeres have been documented to date. Shelterin controls the

structure of the telomeric terminus, it is responsible for the generation of T-loops, and it mediates the recruitment and synthesis of telomeric repeats by telomerase (de Lange 2005).

Dysfunction of the shelterin complex has not been reported in Barrett's oesophagus nor has it been associated with obesity; however there is a wealth of data in the literature associating this complex with carcinogenesis. TERF1 deficiency in mice has been shown to lead to degenerative pathologies and increased cancer incidence (Blasco, Martinez et al. 2009). Loss of TERF1 expression is also reported in kidney cancer and TERF1 levels are negatively correlated with malignancy of the cancer (Shi, Ding et al. 2004). Dysregulation of TERF2 has been demonstrated in cervical and breast cancer cell lines (Tahara, Shin-Ya et al. 2006) however loss of POT1 by RNA interference leads to decreased cell proliferation and induction of apoptosis and senescence in human gastric cancer cells (Ning, Yang et al. 2010).

Along with members of the telomerase complex and the shelterin complex, TEP1 and DKC1 are known to bind telomeres and are involved with regulation of telomere length (Martinez and Blasco 2011). DKC1, a gene linked to dyskeratosis congenita, a severe bone marrow failure syndrome in which patients also show signs of premature aging, has been shown to closely associate with TERC and promote telomere shortening and cancer (Marrone and Dokal 2004). TEP1, interacts with TERC and is associated with telomerase activity in vivo (Harrington, McPhail et al. 1997) however unlike DKC1, is not essential for telomerase function (Harrington, Liu et al. 2000; Watanabe, Koyanagi et al. 2000).

Obesity and telomere length has been associated with morbidities other than cancer. Leukocyte telomere length, as expressed by mean terminal restriction fragment (TRF) length, has shown significant or borderline correlations with incidence of diabetes, fasting glucose level, and fasting incidence levels, all markers of obesity, in cardiovascular patients (Fitzpatrick, Kronmal et al. 2007).

Telomere shortening has been investigated in Barrett's oesophagus previously, with telomere shortening in Barrett's patient leukocytes associated with increased oesophageal adenocarcinoma risk (Risques, Vaughan et al. 2007), independent of smoking, obesity and nonsteroidal anti-inflammatory drug (NSAID) use. Telomere length has been investigated in obesity with excess adiposity conducive to telomere shortening in adults but not children (Zannolli, Mohn et al. 2008), while a study in Sweden reported an association with telomere shortening and an "obese phenotype", however this was only present in women (Nordfjall, Eliasson et al. 2008). Buxton *et al* reported significantly higher incidences of telomere shortening in obese children (aged 2-17 years) compared to their non-obese counterparts

(Buxton, Walters et al. 2011) however there was no difference in telomere length observed between sexes in either the controls or the obese subjects. Interestingly, telomere length has also been shown to be significantly shorter in the subcutaneous adipose tissue of formerly obese subjects compared to never-obese subjects of similar age, sex and BMI (Fernandez-Real, Moreno-Navarrete et al. 2010). Shorter telomere length in formerly obese patients suggests that telomere shorting by adipose derived factors may be an irreversible consequence of obesity and may ultimately contribute to disease progression morbidity (Moreno-Navarrete, Ortega et al. 2010).

5.2 Aims and objectives

The specific aim of this chapter was to investigate the effect of adipose conditioned media from obese and non-obese patients on telomere length, telomerase complex and shelterin complex expression across the metaplasia-dysplasia-adenocarcinoma sequence associated with Barrett's carcinogenesis.

Specific Objectives

- Investigate telomere length in the HET-1A, QH, GO and OE33 cell lines following ACM treatment from obese and non-obese patients
- Determine the effect adipose conditioned media on the expression of telomerase complex
- Evaluate expression of telomere associated proteins DKC1 and TEP1 following adipose conditioned media treatment in the HET-1A, QH, GO and OE33 cell lines
- Examine expression of members of the shelterin complex in the above cell lines following adipose conditioned media treatment from obese and non-obese patients
- Correlate telomere length, telomerase complex expression, shelterin complex expression and telomere associated proteins expression with BMI, VFA and waist circumference from patients which ACM was derived.

5.3 Results

5.3.1 ACM treatment of oesophageal cell lines significantly decreases telomere length

Telomere length was assessed via q-PCR (section 2.6.3) in the HET-1A, QH, GO and OE33 cell lines which represent the metaplasia-dysplasia-adenocarcinoma sequences associated with the neoplastic progression of Barrett's oesophagus following 24hr and 48hr treatment with ACM generated from obese and non-obese oesophagectomy patients. The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length (section 2.6.3).

Table 5.1. Anthropometric data for patient ACM used.

	Non Obese	Obese
No. Subjects	5	5
Diagnosis	OAC	OAC
Sex (male) n (100%)	5	5
Age at surgery, mean (range)	58.2 (40-79)	57.8 (42-68)
Waist circumference (cm), mean (range)	86.5 (79-94)	109.4 (100-127)**
BMI, mean (range)	24.3 (20.7-29.2)	30.3 (30.0-35.6)***
VFA (cm^2), mean (range)	103.2 (34.1-116.1)	209.6 (172.0-216.8) **
Metabolic Syndrome, n (%)	1 (20%)	3 (60%)
Neoadjuvant therapy, n (%)	1 (20%)	1 (20%)

Statistical analysis was carried out using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001). (BMI= Body mass index, VFA= Visceral fat area).

Basal telomere length was assessed in the HET-1A, QH, GO and OE33 cell lines (Figure 5.1). The OE33 cell line, representative of invasive oesophageal adenocarcinoma, demonstrated the greatest basal telomere length, with a T/S ratio of 22.4. This telomere length was significantly higher compared to the HET-1A (p=0.035) and QH (p=0.008) cell lines, where T/S ratios of 10.9 and 6.9 were observed, respectively. A T/S ratio of 17.7 was observed in the GO cell line, representing a significantly greater telomere length than the QH cell line (p=0.047)

Telomere shortening in response to a 24hr and a 48hr ACM treatment was assessed in the cells lines representing the metaplasia-dysplasia-adenocarcinoma sequence (Figure 5.2). Telomere shortening was demonstrated in the HET-1A, with the T/S ratio decreasing from 10.9 in untreated cells to 2.3 (p<0.0001) and 3.2 (p=0.004) following 24hr and 48hr ACM treatment respectively (Figure 5.2A). Significant telomere shortening following ACM treatment was observed in the QH cell line, where the T/S ratio decreased from 6.9 in the untreated cells to 0.4 (p<0.0001) and 0.7 (p<0.0001) following 24hr and 48hr ACM treatment respectively (Figure

5.2B). There was no significant difference in telomere length between 24hr and 48hr ACM treatment. Significant telomere shortening was also observed in the GO cell line where the T/S ratio of 17.7 in the untreated cells decreased to 0.9 ($p<0.0001$) and 2.8 ($p<0.0001$) following 24hr and 48hr ACM treatment, respectively (Figure 5.2C). Significant telomere elongation was detected following 48hr ACM treatment compared to a match 24hr treatment ($p=0.015$). In the OE33 cell line, the T/S ratio decreased significantly from 22.4 in the untreated cells to 5.5 ($p=0.001$) following 24hr ACM treatment and 0.75 following 48hr ACM treatment ($p<0.0001$) (Figure 5.2D). Telomere shortening was also significantly shorter following 48hr ACM treatment compared to a matched 24hr treatment ($p=0.006$).

Next the effect of obese ACM compared to non-obese ACM on telomere length was assessed in the HET-1A, QH, GO and OE33 cell lines (Figure 5.3). Telomere length in all cell lines was significantly down regulated in response to ACM generated from both viscerally obese and non-obese patients at both time points. While ACM treatment showed significant decrease in telomere lengths in all cell lines, there was no significant difference in telomere shortening in the HET-1A, QH or OE33 cell lines when the effect of obese ACM versus non obese ACM was examined. Significant changes in telomere length were demonstrated in the GO cell line is response to 24hr treatment with ACM generated from obese and non-obese patients (Figure 5.3C). A T/S ratio of 17.7 was calculated in the untreated GO cells, this was significantly decreased to 1.6 ($p=0.002$) and 0.5 ($p<0.0001$) following 24hr treatment generated from non-obese and viscerally obese patients, respectively. This additional decrease in T/S ratio in the GO cells treated with obese ACM represented a significantly shorter telomere length than the GO cells treated with non-obese ACM ($p=0.008$). However this additional increase in telomere shortening in the GO cells treated with obese ACM compared to non-obese ACM was not observed following 48hr ACM treatment.

Telomere length in response to 24hr and 48hr ACM treatment was compared in the panel of oesophageal cell lines representing the Barrett's neoplastic sequence (Figure 5.4).

Following both 24hr and 48hr ACM treatment, telomere length in the QH cell line was significantly shorter than the HET-1A, GO and OE33 cell lines (Figure 5.4A) following 24hr ACM treatment. A T/S ratio of 0.4 was observed in the QH cell line, representing a significantly shorter telomere length compared to the HET-1A cell line (T/S ratio= 2.3, $p<0.0001$), the GO cell line (T/S ratio= 0.9, $p=0.038$) and the OE33 cell line (T/S ratio=5.5, $p=0.009$). A similar pattern of telomere length was observed following 48hr ACM treatment (Figure 5.4B). The QH cell line had a T/S ratio of 0.6, which was significantly shorter than the HET-1A cell line (T/S

ratio=3.2, p=0.001), the GO cell line (T/S ratio=2.8, p=0.005) and the OE33 cell line (T/S ratio=2.2 fold, p=0.034).

Correlations between patient VFA and telomere length was investigated in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.5). Patient ACM VFA correlated with telomere length in the GO and OE33 cell lines following 48hr ACM treatment. VFA negatively correlated with telomere length in the OE33 cell line, however this correlation was statistically significant (p=0.04, R=0.74) (Figure 5.5A). Following 48hr ACM treatment in the GO cell line, patient ACM VFA negatively correlated with telomere length, but again reached statistical significance (p=0.04, R=0.82) (Figure 5.5B).

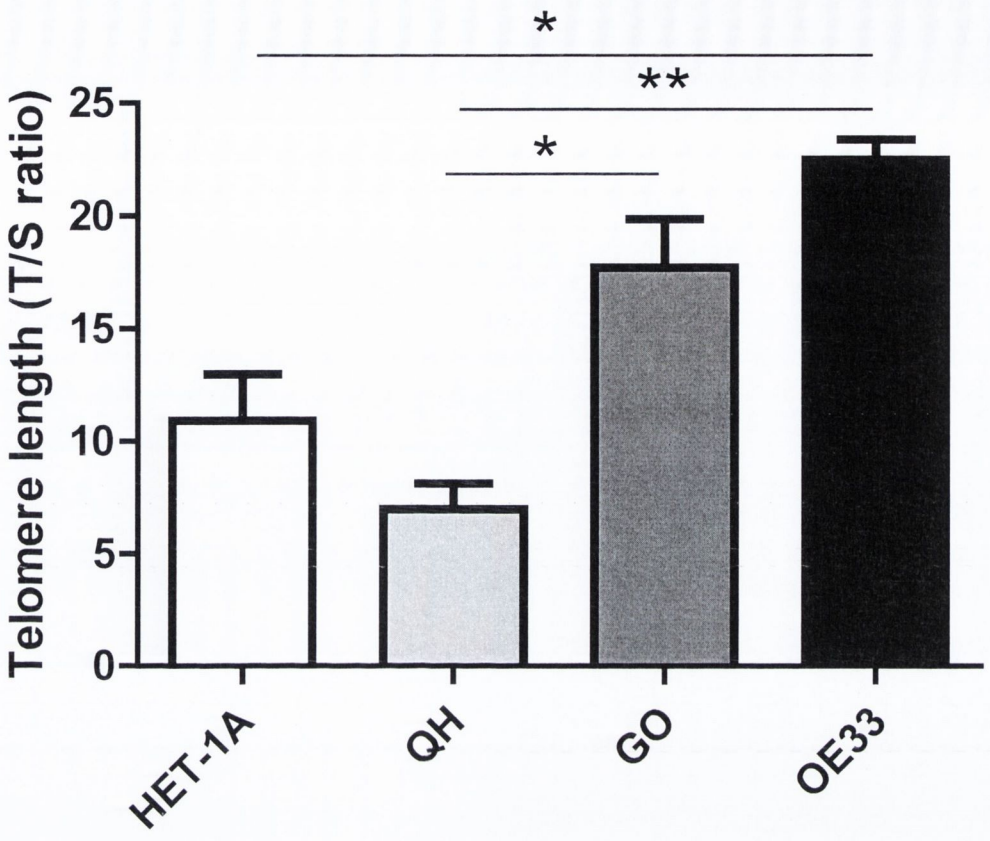

Figure 5.1. Basal telomere length in oesophageal cell lines (n=3). The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

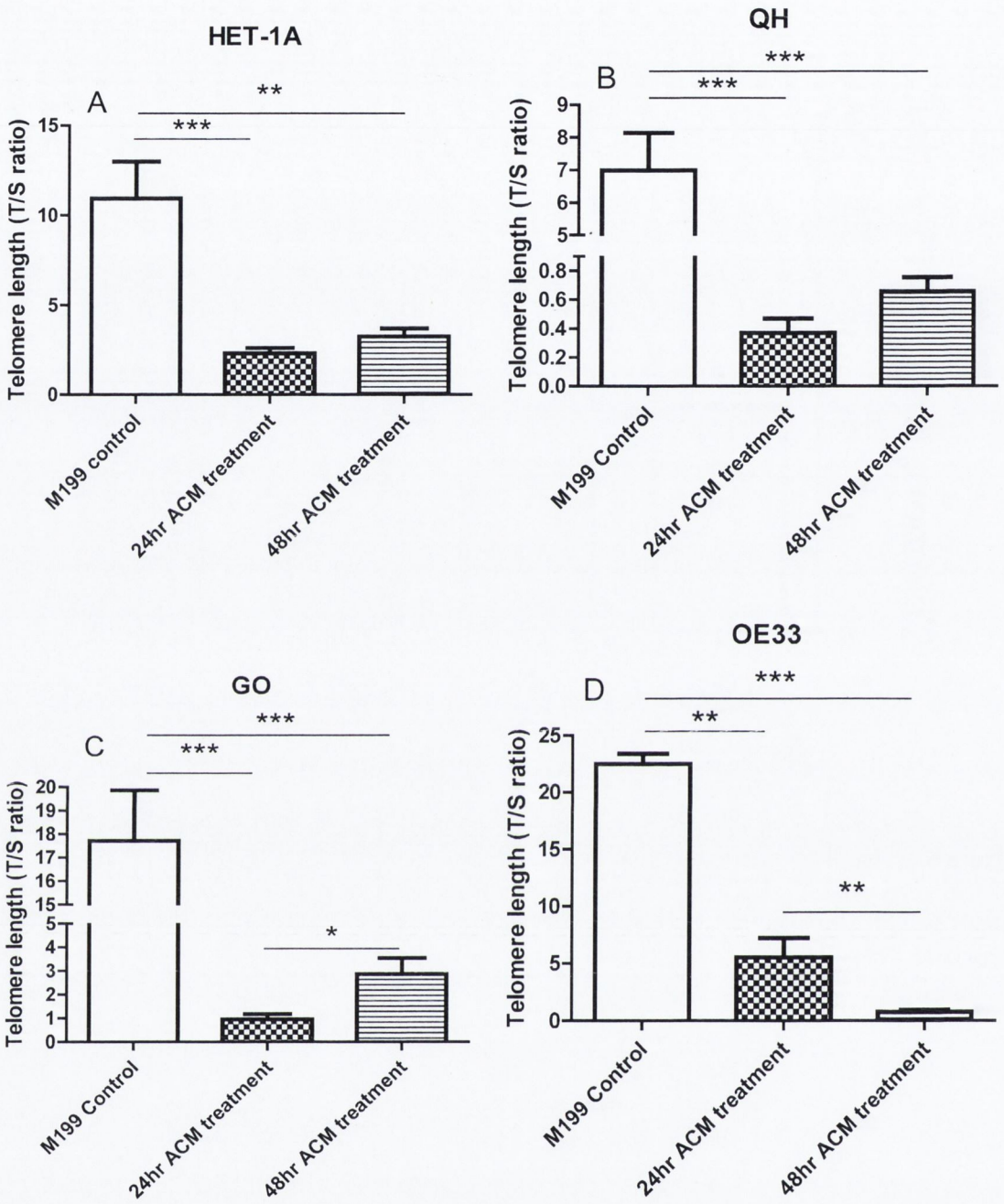

Figure 5.2. Telomere length in oesophageal cell lines representative of the metaplasia-dysplasia-adenocarcinoma sequence following 24hr and 48hr ACM treatment (n=10). The average T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$).

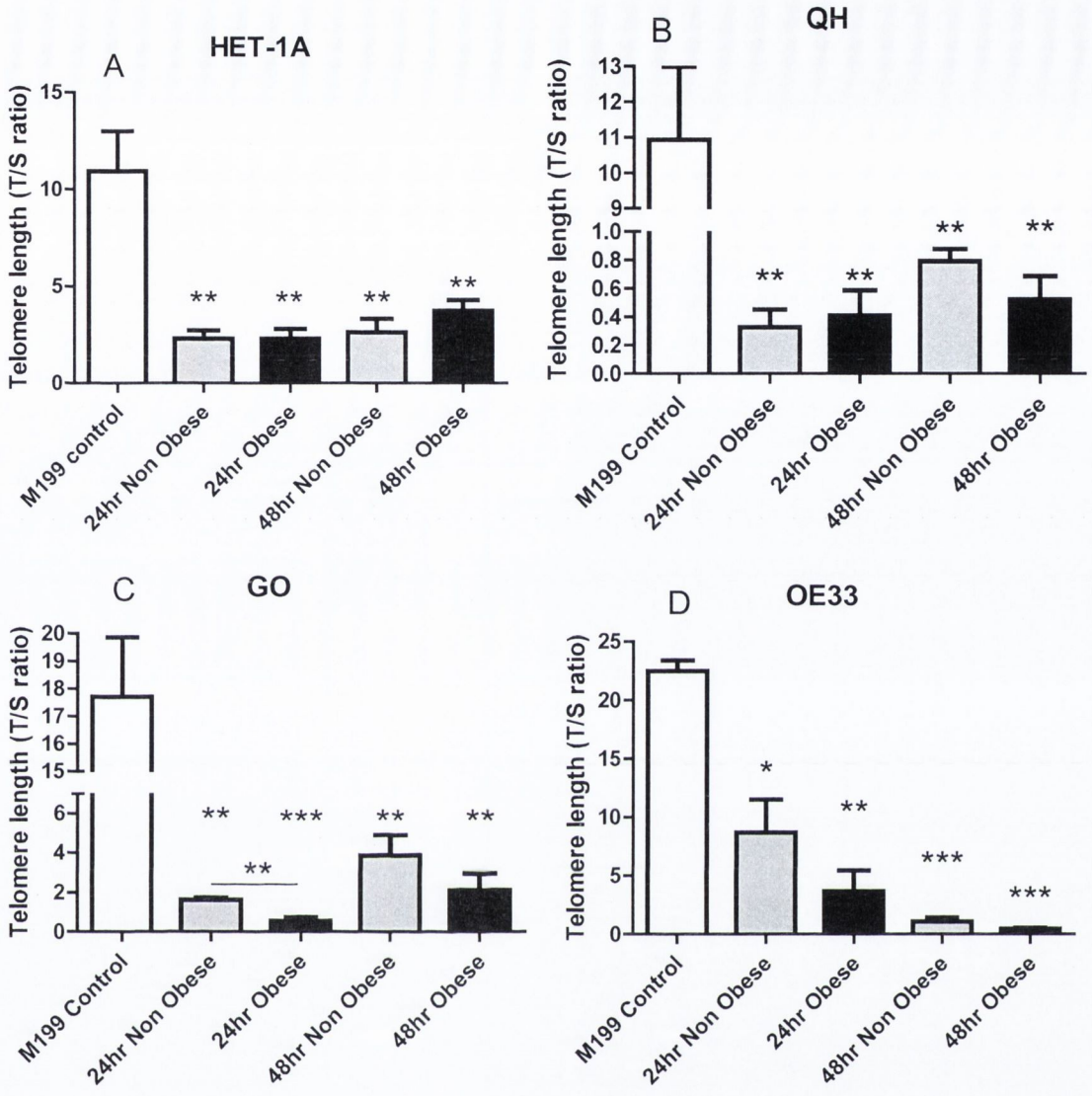

Figure 5.3. Telomere shortening in the oesophageal cell lines in response to ACM generated from viscerally obese (n=5) and non-obese (n=5) patients. The average T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$).

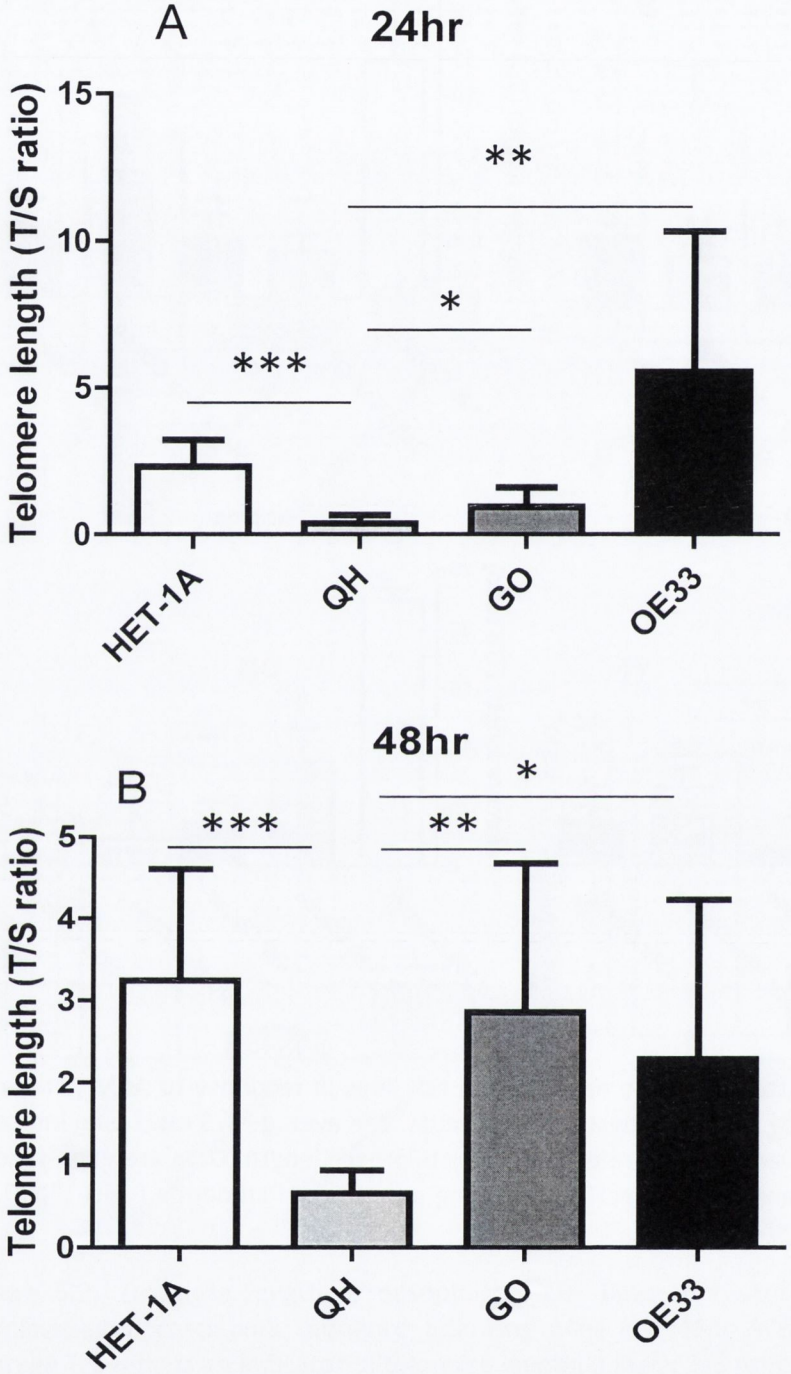

Figure 5.4. Benign QH Barrett's oesophagus cell line displays the shortest telomere length following ACM treatment (n=10). The average T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

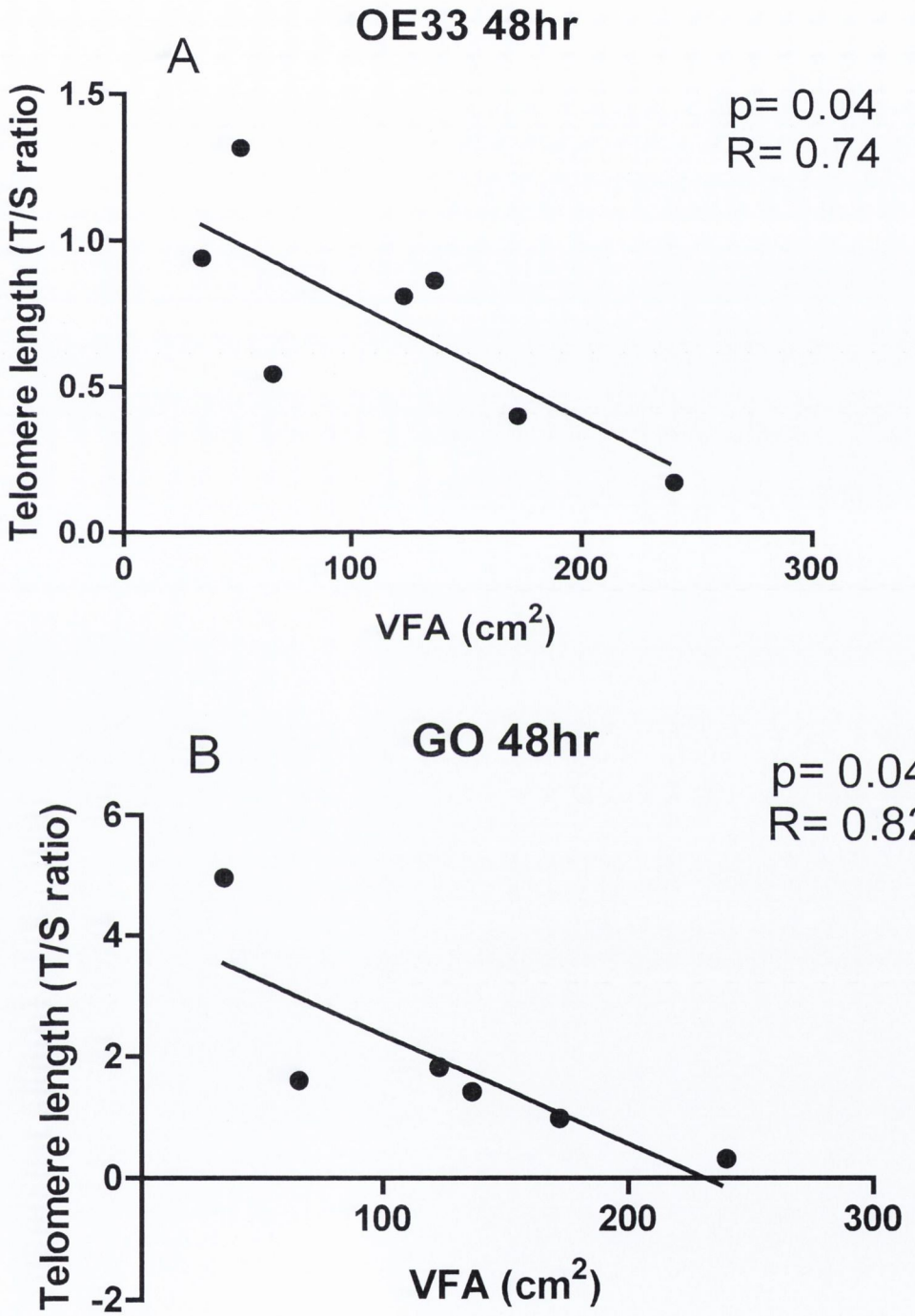

Figure 5.5. Patient VFA negatively correlates with telomere length in GO and OE33 cell lines following 48hr ACM treatment (n=7). The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Statistical analysis was performed using linear regression analysis.

5.3.2 Dysregulation of telomerase complex gene expression following ACM treatment in the oesophageal cell lines

Telomerase complex gene expression was analysed in the panel of oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.6). Expression of telomerase complex protein TERC was significantly higher in the HET-1A cell line compared to the QH ($p=0.04$) and OE33 cell lines ($p=0.02$) following a 24hr ACM treatment (Figure 5.6A) but showed no difference between cell lines following 48hr ACM treatment (Figure 5.6B). Expression of telomerase complex protein TERT was significantly higher in the QH cell line compared to the HET-1A ($p=0.0002$), GO ($p=0.0002$) and OE33 ($p=0.0001$) cell lines, following 48hr ACM treatment (Figure 5.6B). TERC expression was not significantly different between the cell lines following 48hr ACM treatment (Figure 5.6C). Expression of TERT was highest in the HET-1A cell line and decreased significantly in the QH ($p=0.01$), GO ($p=0.005$) and OE33 ($p<0.0001$) cell lines (Figure 5.6D).

Levels of telomerase complex expression following 24hr and 48hr ACM treatment was compared in all cell lines (Figure 5.7). TERC expression was significantly higher in the QH, GO and OE33 cell line following 48hr ACM treatment compared to a 24hr ACM treatment ($p=0.0004$, $p=0.018$ and $p=0.009$ respectively) (Figure 5.7A). Significantly higher levels of TERT gene expression were seen in the HET-1A cell line ($p=0.0002$) following a 48hr ACM treatment versus 24hr ACM treatment. The opposite effect was seen in the QH cell line where a 24hr ACM treatment had significantly higher expression at 24hr compared to a 48hr treatment ($p=0.003$) (Figure 5.7B).

The effect of ACM generated from obese patients compared to non-obese patients on telomerase complex gene expression was analysed in the oesophageal cell lines following both 24hr and 48hr ACM treatment (Figure 5.8).There were significant differences observed between TERC expression in the QH cell line in response to ACM generated from both obese and non-obese patients, with obese ACM eliciting significantly greater gene expression ($p=0.005$), following a 48hr ACM treatment. No other significant difference in telomerase complex gene expression were observed between treatment with ACM from obese patients compared to non-obese patients in the oesophageal cell lines at either time point.

Correlations between patient VFA and telomerase complex gene expression were investigated in all cell lines following 24hr and 48hr ACM treatment (Figure 5.9). Expression of telomerase complex proteins TERT and TERC correlated significantly with patient VFA following 24hr ACM treatment of the QH cell line ($p=0.02$, $R=0.83$ and $p=0.03$, $R=0.79$). Expression of telomerase

complex proteins TERT and TERC also correlated significantly with patient visceral fat in the HET-1A cell line following a 48hr ACM treatment ($p=0.03$, $R=0.65$ and $p=0.05$, $R=0.72$). There were no correlations between telomerase complex gene expression with patient VFA following 48hr ACM treatment.

Expression of telomerase complex protein TERC correlated significantly with telomere length, in the QH cell line following a 48hr ACM treatment ($p=0.01$, $R=0.74$). TERC expression also correlated significantly with telomere length ($p=0.0009$, $R=0.76$) in the OE33 cell line following a 48hr ACM treatment (Figure 5.10). Neither patient BMI nor waist circumference correlated with telomere length or telomerase complex expression in this study.

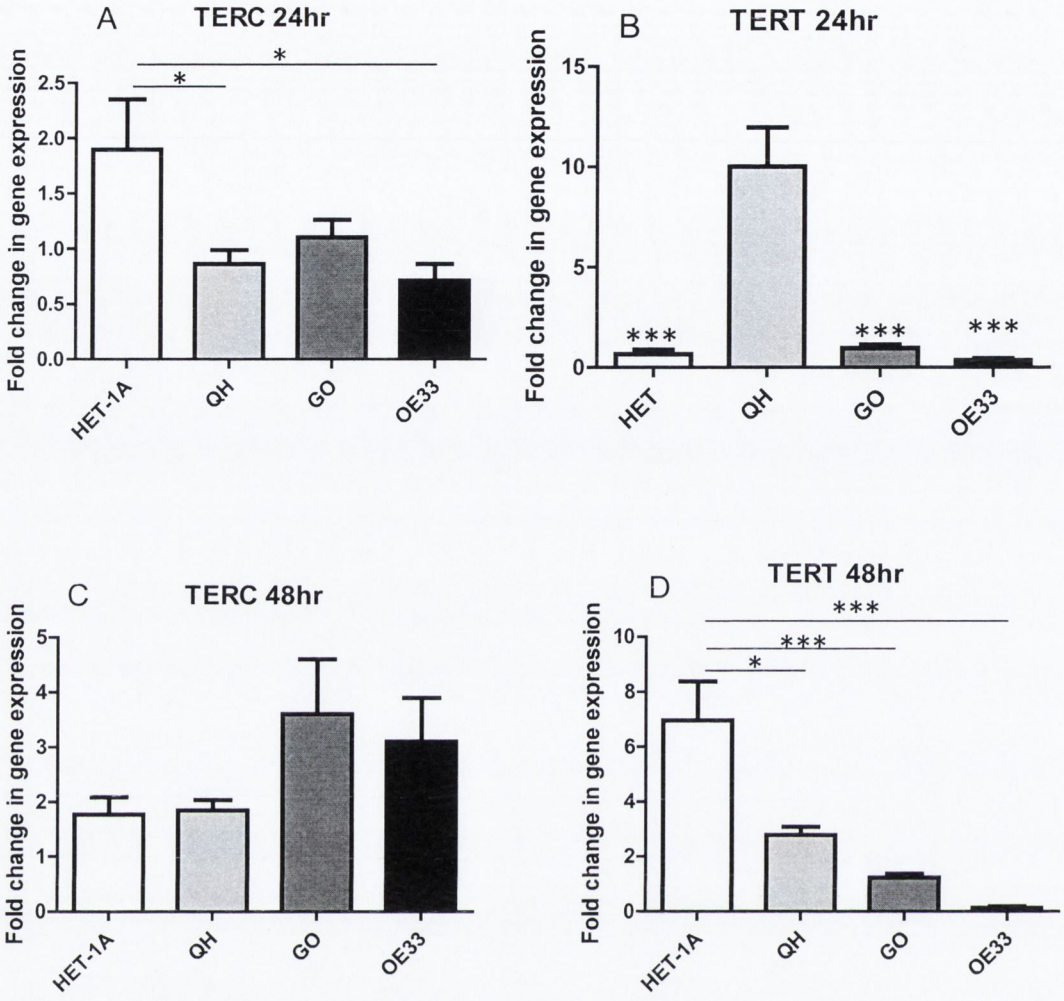

Figure 5.6. Expression of telomerase complex members TERC and TERT in the oesophageal cell lines following 24hr and 48hr ACM treatment (n=10) . Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

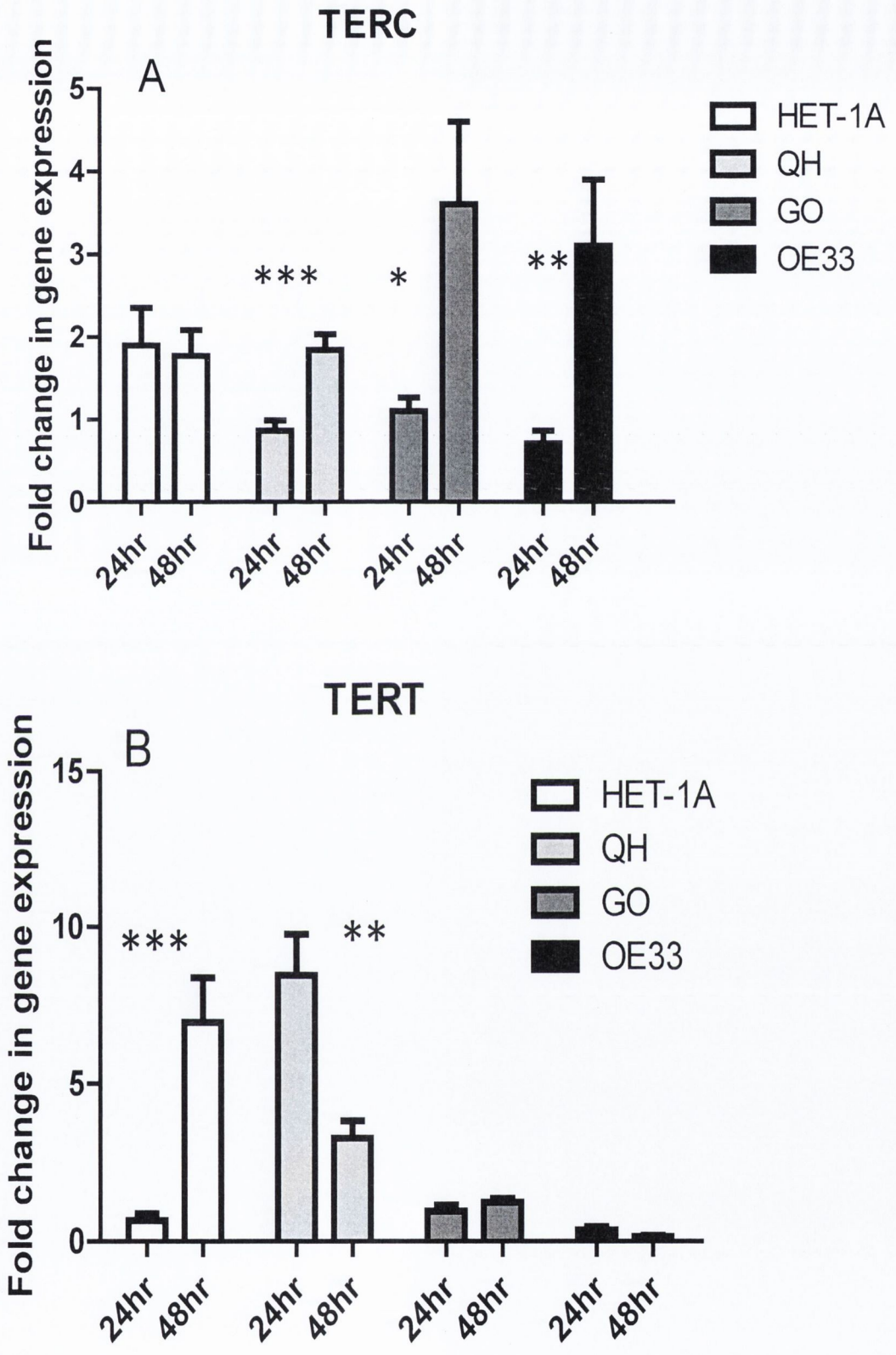

Figure 5.7 Expression of telomerase complex members in the oesophageal cell lines following ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

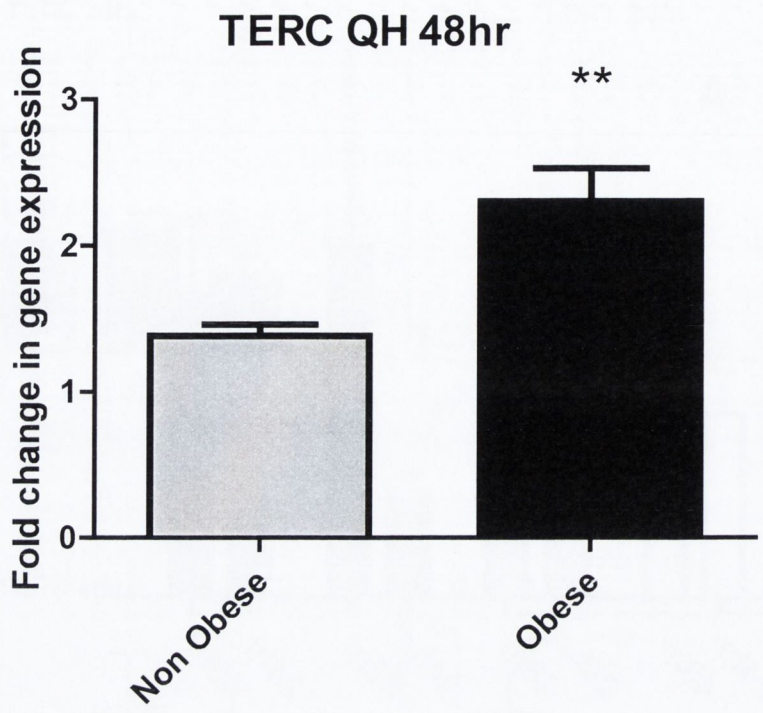

Figure 5.8. ACM from obese patients (n=5) elicited significantly higher gene expression of telomerase complex protein TERC, than ACM from non-obese patients (n=5) in QH cell line, following 48hr ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01)

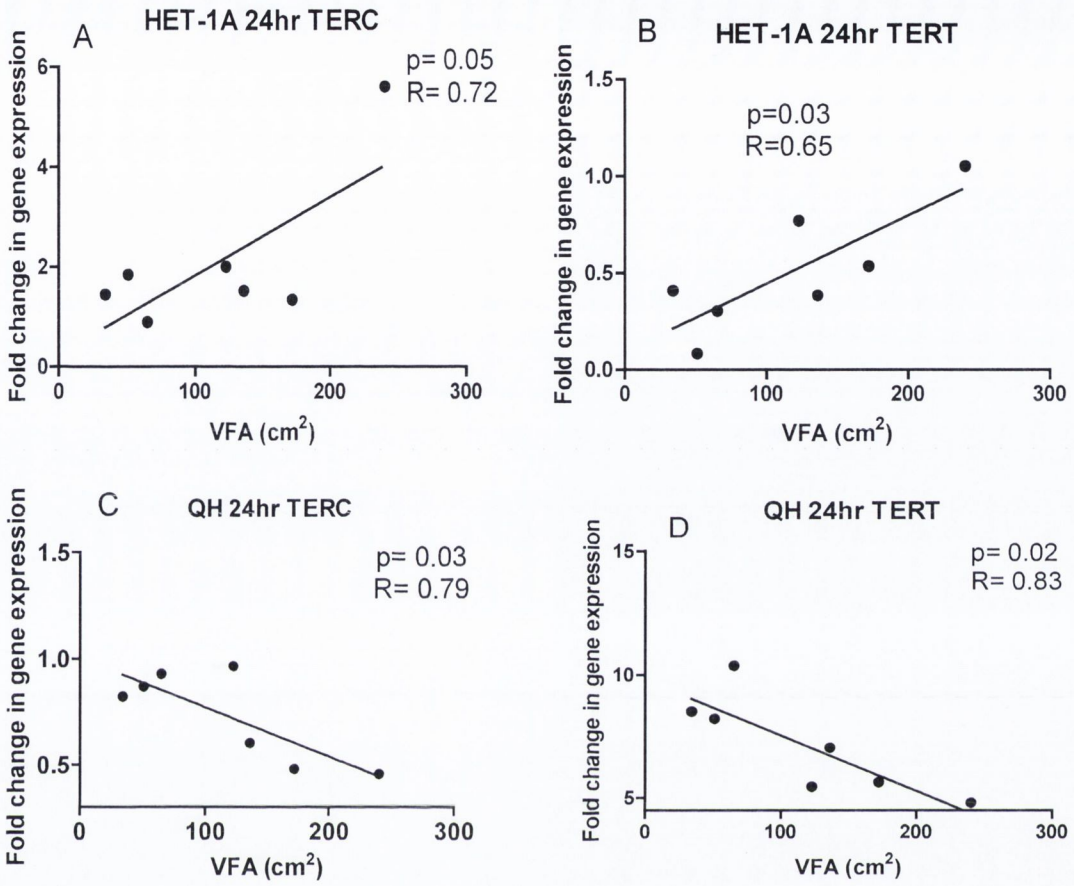

Figure 5.9. Patient ACM visceral fat area (n=7) correlates with gene expression of members of the telomerase complex in pre-malignant oesophageal cell lines following 24hr ACM treatment. Statistical analysis was carried out using linear regression analysis.

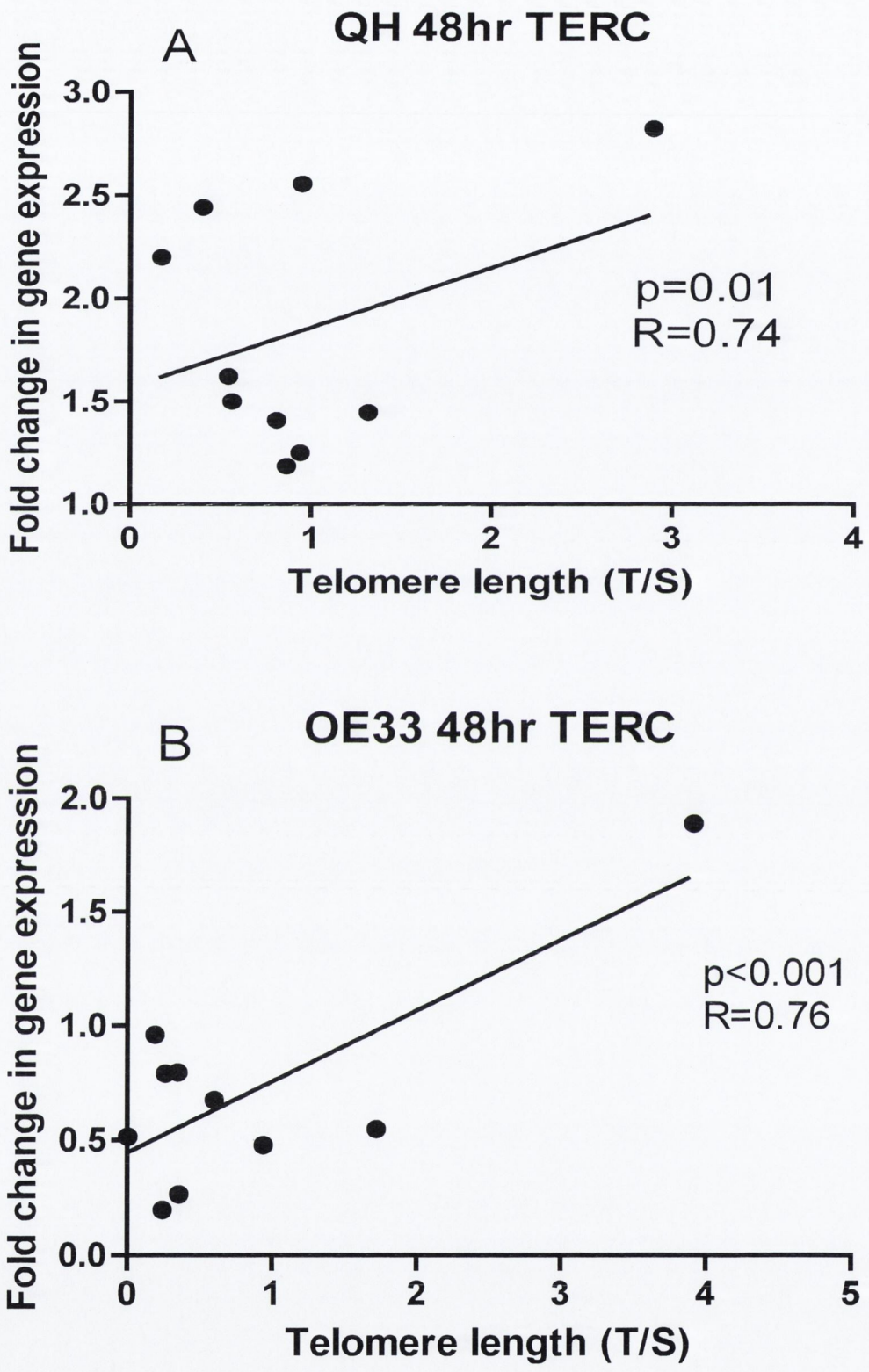

Figure 5.10. Telomere length correlates with the expression of TERC in the QH cell line following 24hr and 48hr ACM treatment (n=10). The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Statistical analysis was carried out using linear regression analysis.

5.3.3 Expression of telomerase associated proteins DKC1 and TEP1 is altered by ACM treatment in the oesophageal cell lines

The expression of telomerase associated proteins DKC1 and TEP1 was analysed in the panel of oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.11).

DKC1 gene expression was highest in the QH cell line following 24hr ACM treatment, and gene expression levels were significantly higher than those in the GO ($p=0.006$) and OE33 ($p=0.003$) cell lines (Figure 5.11A). TEP1 showed greatest increases in gene expression following 24hr ACM treatment in the HET-1A cell line, and levels were significantly higher compared to those observed in the GO ($p=0.003$) and OE33 ($p=0.009$) cell lines (Figure 5.11B).

DKC1 expression was highest in the QH cell line following 48hr ACM treatment (Figure 5.11C) with expression levels significantly higher than those seen in the GO ($p=0.002$) and OE33 ($p<0.0001$) cell lines. Similar levels of DKC1 expression were also seen in the HET-1A cell line compared to the QH cell line. TEP-1 expression was highest in the QH cell line following 48hr ACM treatment (Figure 5.11D) and was significantly higher compared to the HET-1A ($p<0.0001$), GO ($p<0.0001$) and OE33 ($p<0.0001$) cell lines.

The expression of these telomerase complex associated proteins was compared in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.12). The expression of DKC1 was significantly higher in the GO cell line following a 48hr ACM treatment ($p=0.003$), compared to a matched 24hr ACM treatment (Figure 5.12A). TEP1 gene expression was significantly higher ($p=0.045$) following 24hr ACM treatment compared to a 48hr ACM treatment in the HET-1A cell line. However, in the GO cell line significantly higher expression of TEP1 ($p=0.0003$) following 48hr ACM treatment compared to a matched 24hr ACM treatment (Figure 5.12B).

Expression of DKC1 and TEP1 was investigated in response to ACM generated from viscerally obese and non-obese patients in the oesophageal cell lines following 24hr and 48hr ACM treatment; however no significant difference between gene expression elicited between the ACM subtypes was demonstrated. Neither patient BMI nor waist circumference correlated with DKC1 or TEP1 gene expression at either time point in this study.

Correlations between patient VFA and DKC1 and TEP1 gene expression were investigated in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.13). Following 24hr ACM treatment expression of TEP1 correlated significantly with patient ACM visceral fat area in the HET-1A cell line, representative of healthy squamous epithelium ($p=0.01$, $R=0.74$).

Significant correlations were also detected between DKC1 and TEP1 gene expression with telomere length in the oesophageal cell lines following 48hr ACM treatment (Figure 5.14). In the GO cells, TEP1 expression correlated with telomere length ($p=0.04$, $R=0.68$) following a 48hr ACM treatment. Following a 48hr ACM treatment in the OE33 cell line a significant correlation was observed between between DKC1 expression and telomere length ($p=0.02$, $R=0.72$).

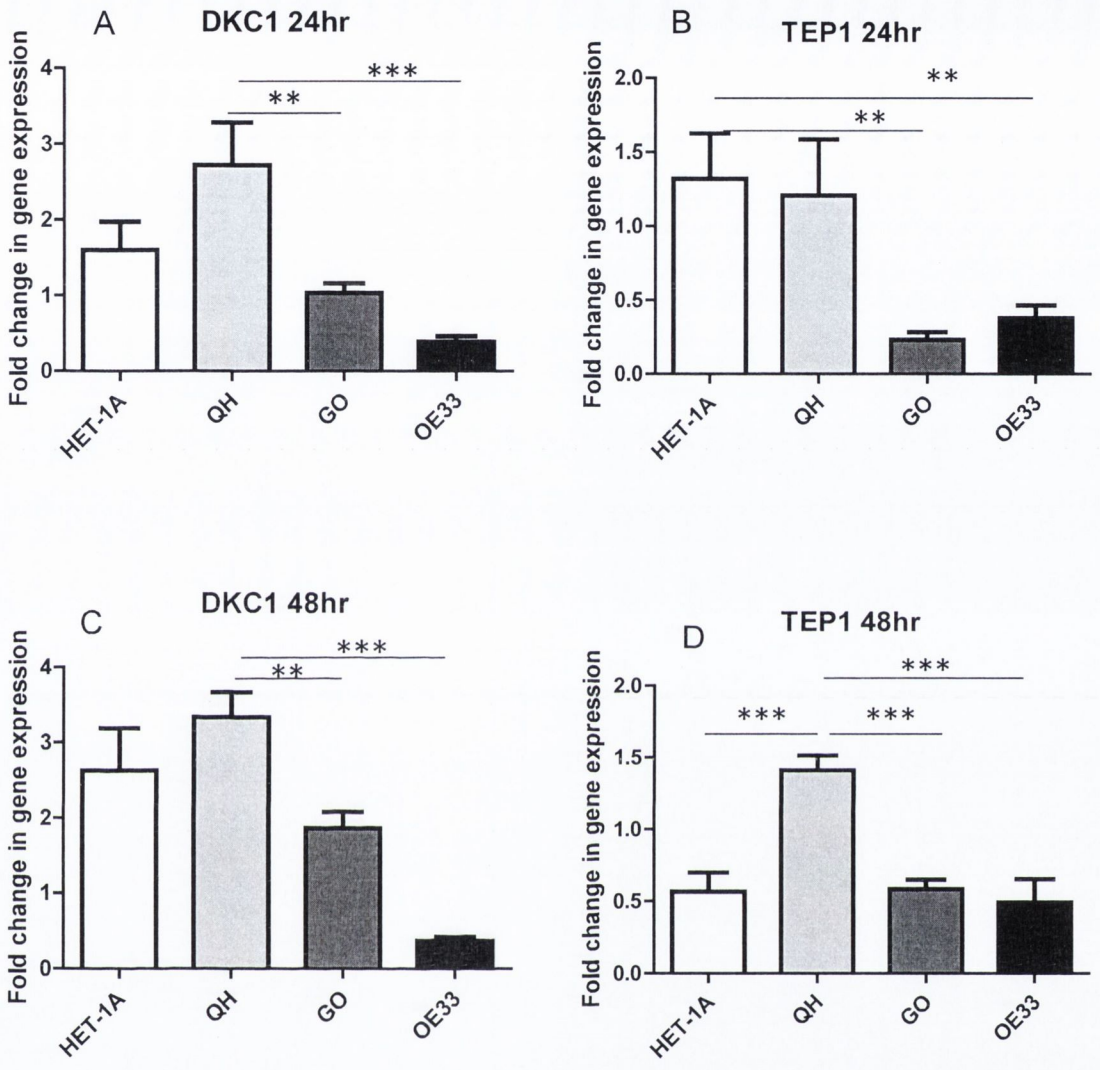

Figure 5.11. Expression of telomerase associated proteins DKC1 and TEP1 in the oesophageal cell lines following 24hr and 48hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

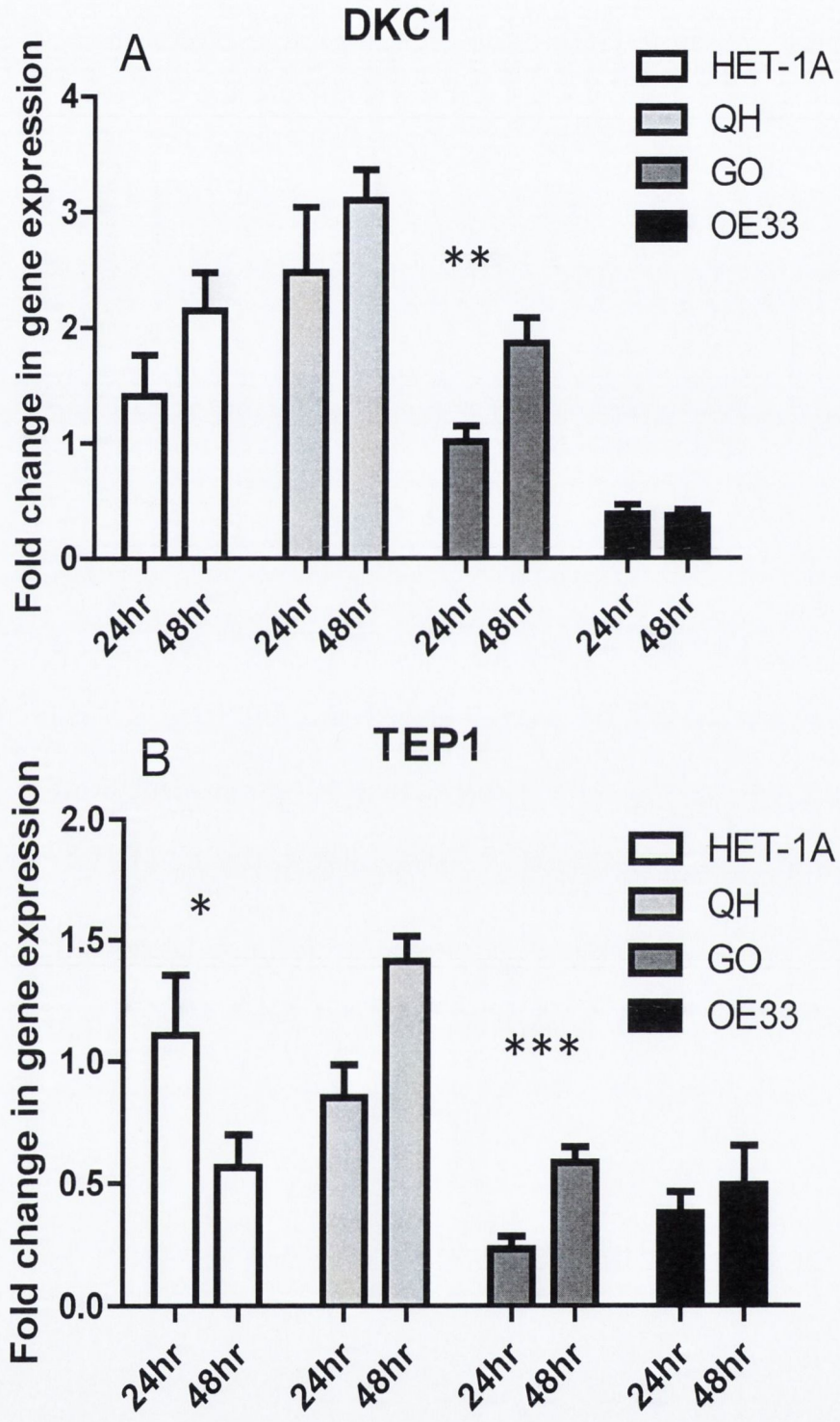

Figure 5.12. Expression of telomerase associated proteins DKC1 and TEP1 in the oesophageal cell lines following 24hr and 48hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

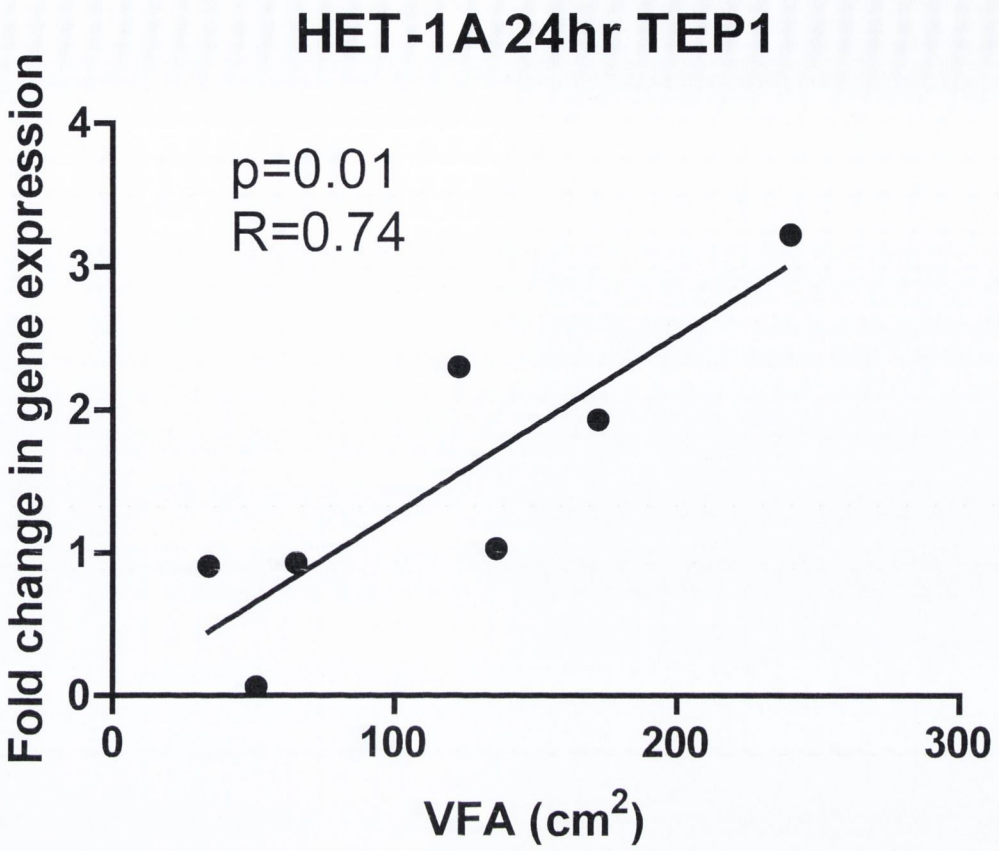

Figure 5.13. Patient ACM visceral fat area (n=7) correlates with TEP-1 expression in the HET-1A cell line following 24hr ACM treatment. Statistical analysis was carried out using linear regression analysis.

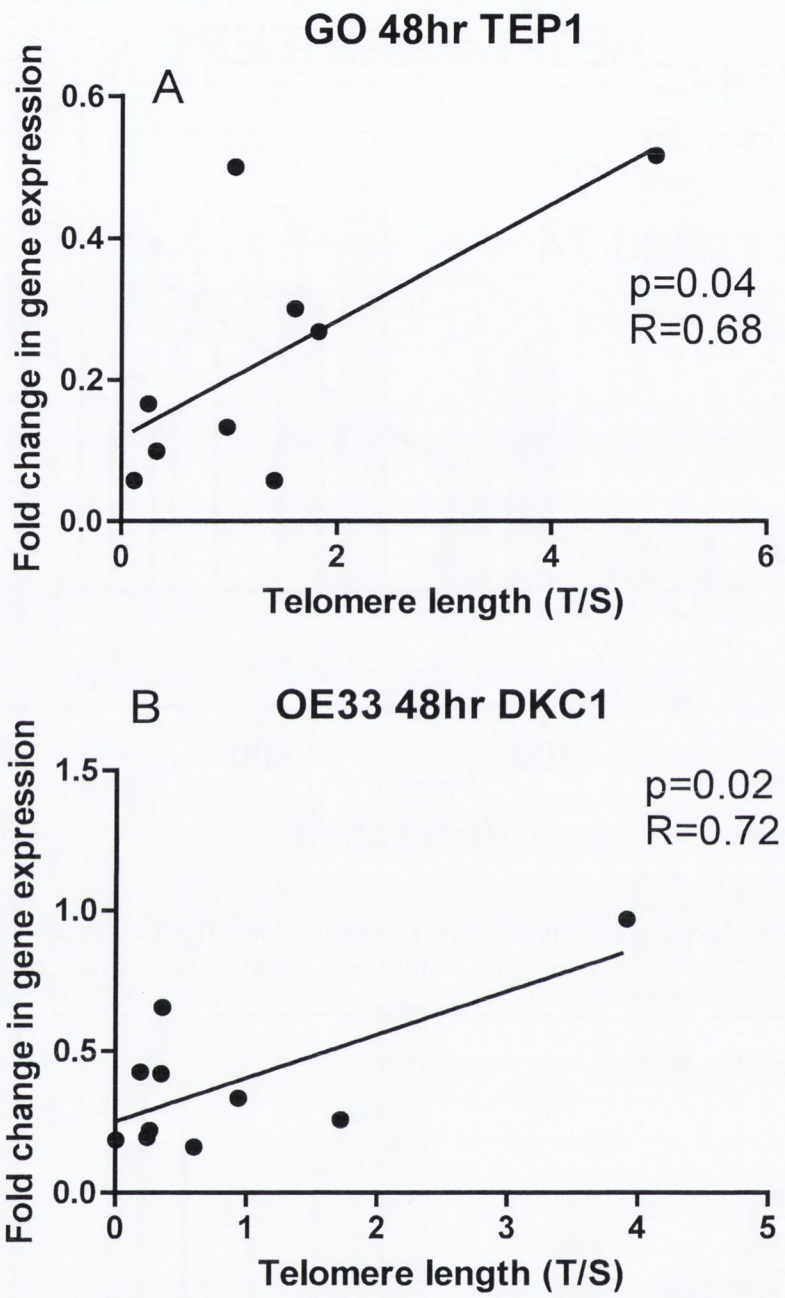

Figure 5.14. Telomere length correlates with the expression of TEP1 in the GO cell line following 24hr ACM treatment (n=10) and DKC1 in the OE33 cell line following 48hr ACM treatment. The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Statistical analysis was carried out using linear regression analysis.

5.3.4 Shelterin complex expression deregulated by ACM treatment in oesophageal cell lines

The expression of shelterin complex genes were investigated in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.15). TERF2 expression was significantly higher in the QH cell compared to expression seen in the OE33 cell line ($p=0.03$) following 24hr ACM treatment (Figure 5.15B). Similarly, TINF2 gene expression was significantly higher in the QH cell line following 24hr ACM treatment, compared to the OE33 cell line ($p=0.04$) (Figure 5.15D). POT1 gene expression levels were highest in the GO cell line following a 24hr ACM treatment (Figure 5.15C), these levels were significantly higher compared to those seen in the HET-1A cell line ($p=0.0008$). No significant difference was observed in the TPP1, TERF1 and TERF2IP expression in in the cell lines following ACM treatment.

TERF1 expression in in the oesophageal cell lines following a 48hr treatment was significantly higher in the QH cell line compared to HET-1A ($p=0.017$), GO ($p<0.0001$) and the OE33 ($p<0.0001$) cells (Figure 5.16A). TERF2 showed greatest gene expression in the QH cell line post 48hr ACM treatment, with significantly higher levels than both the GO ($p<0.0001$) and OE33 ($p<0.0001$) cell lines (Figure 5.16B). Expression of POT-1 was highest in the QH cell line following 48hr ACM treatment (Figure 5.15C) reaching significance over expression levels seen in the HET-1A ($p=0.002$), GO ($p<0.0001$) and OE33 ($p<0.0001$) cell lines. Following 48hr ACM treatment TINF2 expression was highest in the QH cell line (Figure 5.16D) and reached statistical significance over each of the other oesophageal cell lines ($p<0.0001$). The QH cell line also demonstrated significantly higher expression of TPP1 following 48hr ACM treatment compared to the HET-1A ($p<0.0001$), GO ($p<0.0001$) and OE33 ($p=0.0004$) cell lines (Figure 5.16E). TERF2IP gene expression was highest in the QH cell line post 48hr ACM treatment (Figure 5.16F) and were statistically higher than the HET-1A, GO and OE33 cell line ($p<0.0001$).

Shelterin complex gene expression was compared following 24hr and 48hr ACM treatment, in the oesophageal cell lines (Figure 5.17). TERF1 gene expression was significantly higher in both the QH ($p=0.02$) and GO ($p=0.01$) following 48hr ACM treatment compared to 24hr treatment (Figure 5.17A), with no significant difference observed in either the HET-1A and OE33 cell lines. A 48hr ACM treatment significantly increased TERF2 gene expression ($p=0.003$) versus a 24hr ACM treatment (Figure 5.17B), however there was no difference in gene expression between the two time points. In the HET-1A cell line, following a 48hr ACM treatment, POT-1 gene expression was significantly higher compared to a 24hr ACM treatment ($p<0.0001$), however

there was no significance differences in gene expression observed in the other oesophageal cell lines (Figure 5.17C). Only the QH cell line demonstrated a significant difference in TPP1 expression between 24hr and 48hr ACM treatment ($p<0.0001$) (Figure 5.17D). There was significantly higher TINF2 gene expression ($p=0.04$) in the OE33 cell line following a 48hr ACM treatment compared to a 24hr ACM treatment however there were no significant differences observed in the other oesophageal cell lines (Figure 5.17E). There was no significant difference observed in TERF2IP expression between either time point in each of the four oesophageal cell lines treated with ACM (Figure 5.17F).

Expression of shelterin complex proteins was investigated in response to ACM generated from viscerally obese and non-obese patients in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.18). While a 24hr ACM treatment down-regulated the expression shelterin proteins TPP1 (Figure 5.18A) and TERF2 in the GO cell line (Figure 5.18B), ACM generated from non-obese patients elicited significantly more down regulation of both TPP1 ($p=0.02$) and TERF2 ($p=0.05$) (Figure 5.18C). No other shelterin complex genes showed significant differences in gene expression following treatment with ACM generated from obese patients, compared to ACM generated from non-obese patients, at either time point in any of the oesophageal cell lines.

Correlations between patient VFA and shelterin complex gene expression were investigated in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 5.19). Following 24hr ACM treatment expression of shelterin complex proteins TEP1 and TERF2 correlated significantly with patient ACM visceral fat area in the HET-1A cell line ($p=0.01$, $R=0.74$ and $p=0.05$, $R=0.57$, respectively). Expression of shelterin complex proteins TERF1, TERF2 and POT1 correlated significantly with patient ACM visceral fat area in the HET-1A cell line ($p=0.01$ $R=0.74$, $p=0.01$ $R=0.85$, and $p=0.03$ $R=0.63$, respectively) following 48hr ACM treatment. In the QH cell line, TPP1 expression correlated significantly with patient visceral fat area following a 48hr ACM treatment ($p=0.05$, $R=0.56$). Neither patient BMI nor waist circumference correlated with shelterin complex gene expression at either time point in this study.

Expression of shelterin complex proteins was also correlated with telomere length following 24hr and 48hr ACM treatment (Figure 5.20). In the QH cell line, a negative correlation was found between expression of shelterin complex proteins TERF2 and TERF2IP and telomere length which reached significance ($p=0.05$, $R=0.70$ and $p=0.04$, $R=0.72$, respectively) following a 24hr ACM treatment. A 48hr ACM treatment in the OE33 cell line elicited a significant

correlation between shelterin complex members TERF1, and TINF2 and telomere length (p=0.05, R=0.62 and p= 0.71, R=0.76; respectively).

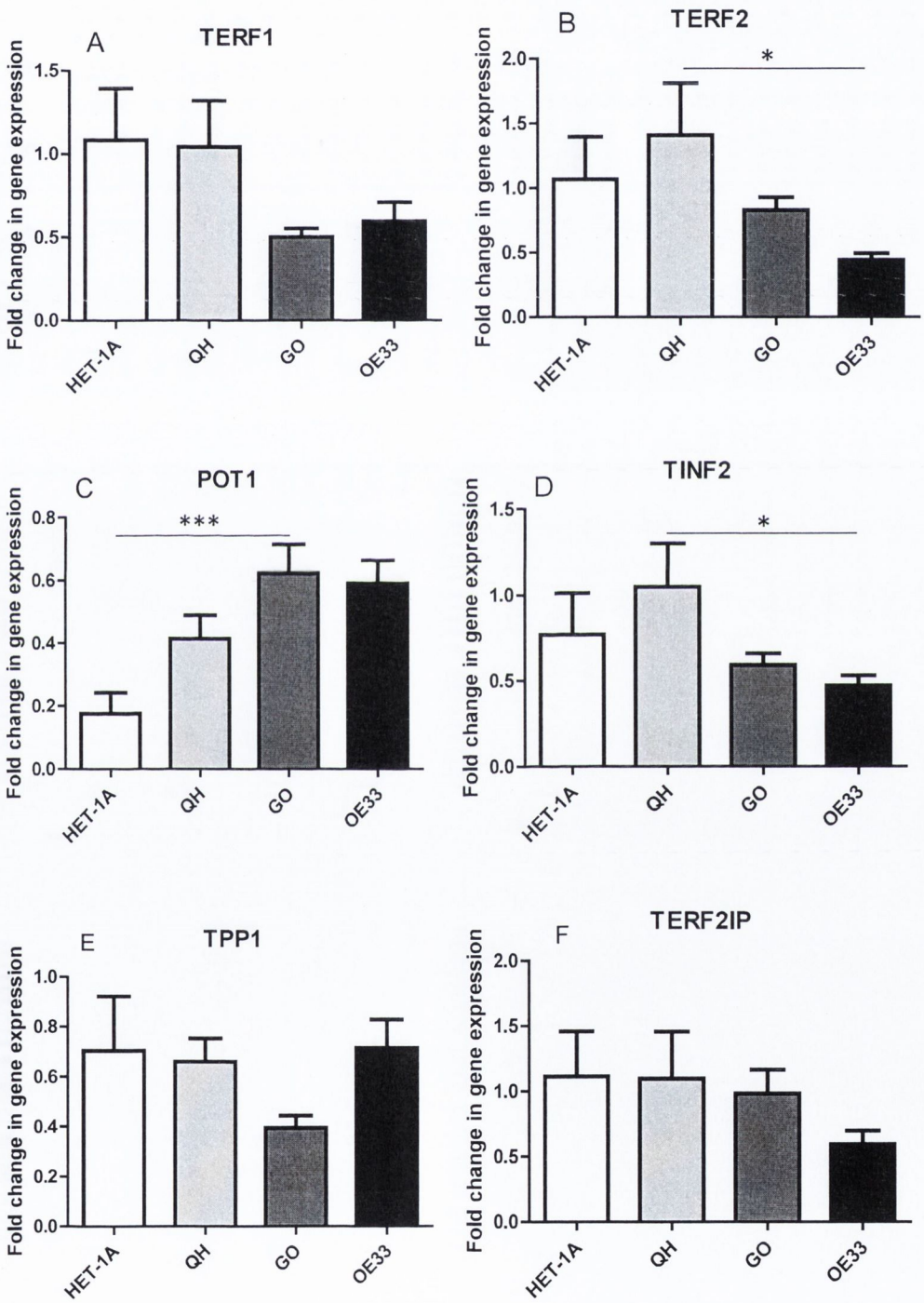

Figure 5.15. Expression of shelterin complex proteins in the oesophageal cell lines following 24hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05).

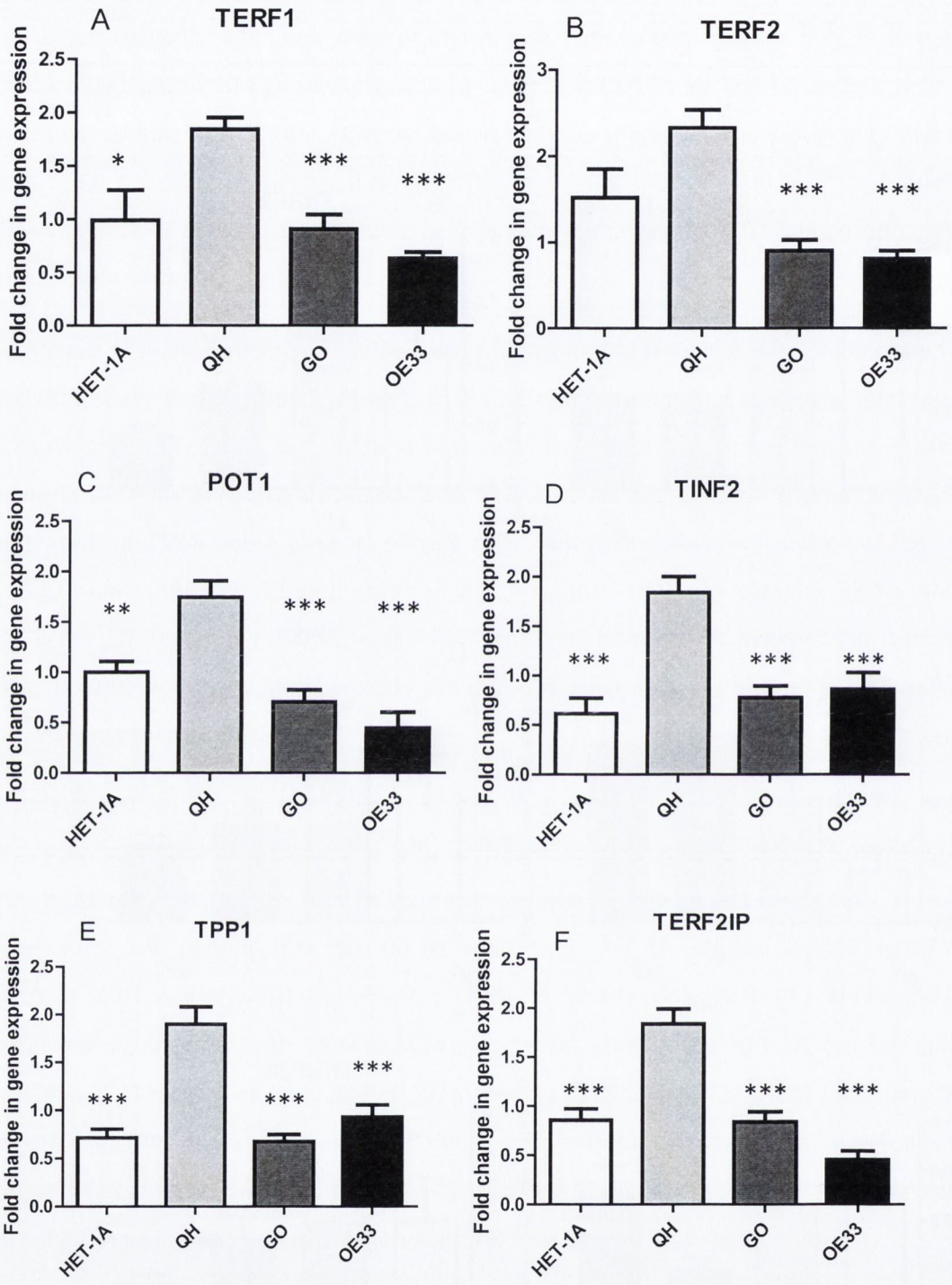

Figure 5.16. Expression of shelterin complex proteins in the oesophageal cell lines following 48hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

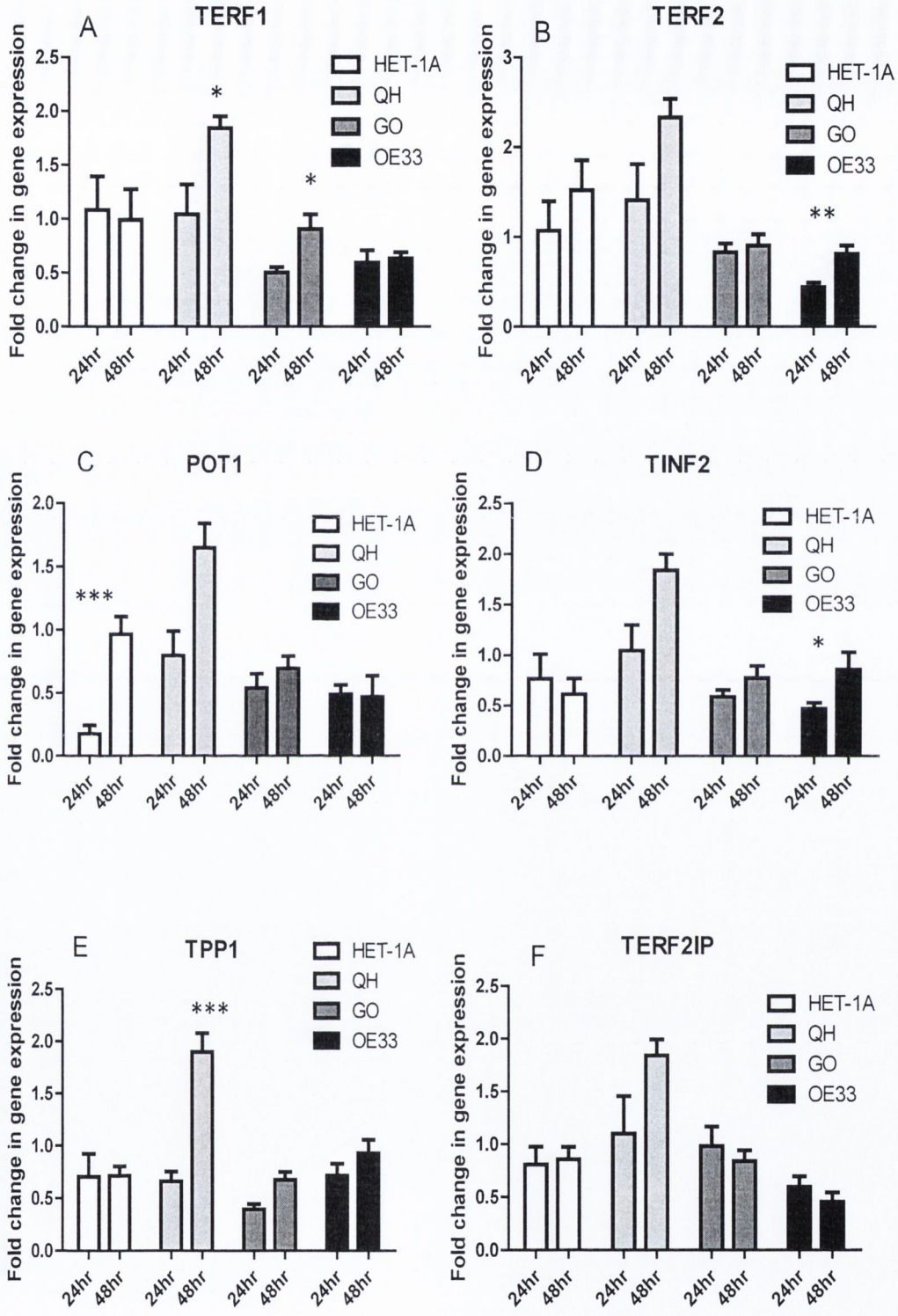

Figure 5.17. Expression of shelterin complex proteins in the oesophageal cell lines following 24hr and 48hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

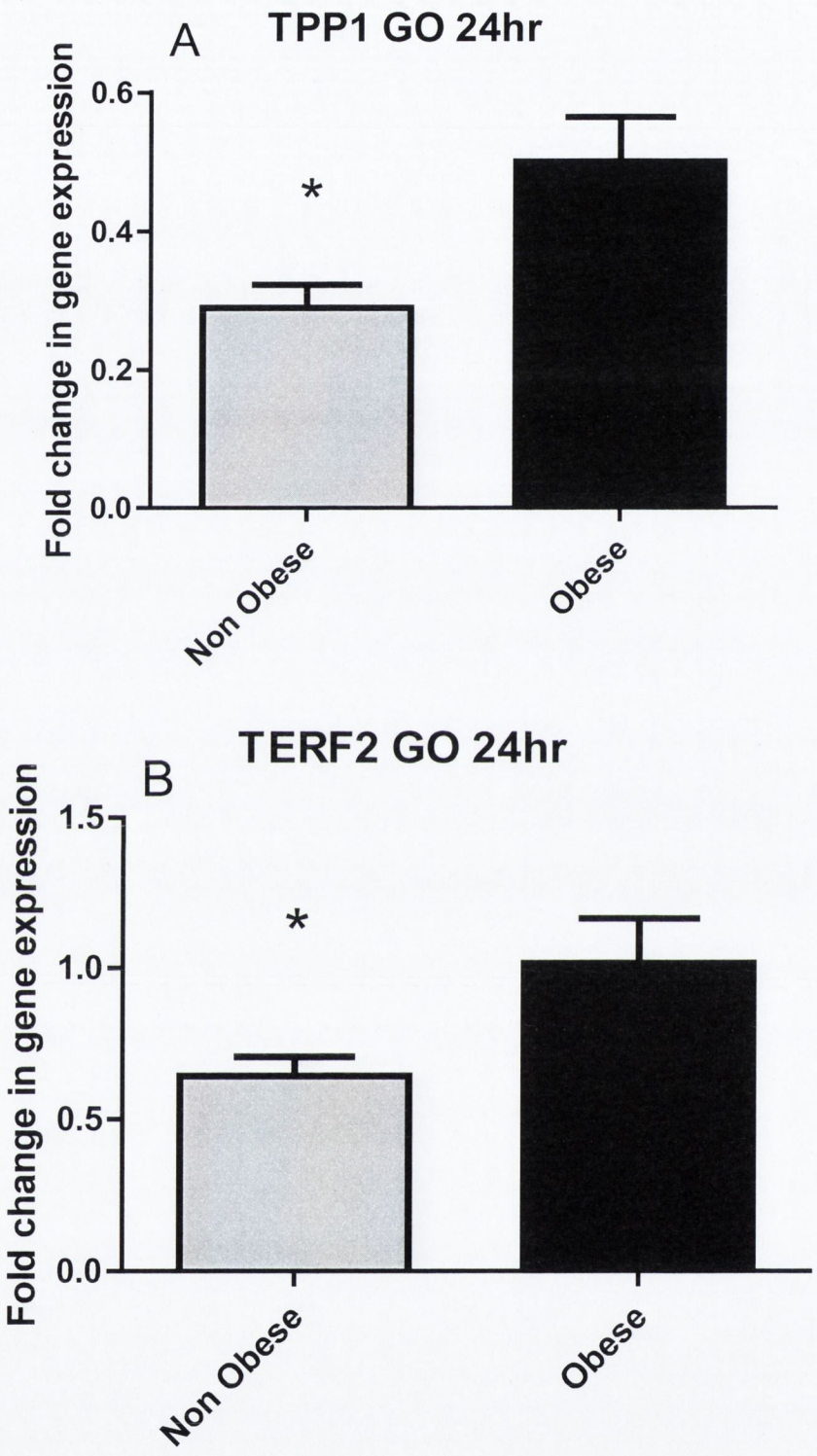

Figure 5.18. ACM from obese patients (n=5) elicited significantly higher gene expression of shelterin proteins TPP1 and TERF2, than ACM from non-obese patients (n=5) in GO cell line, following 24hr ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$)

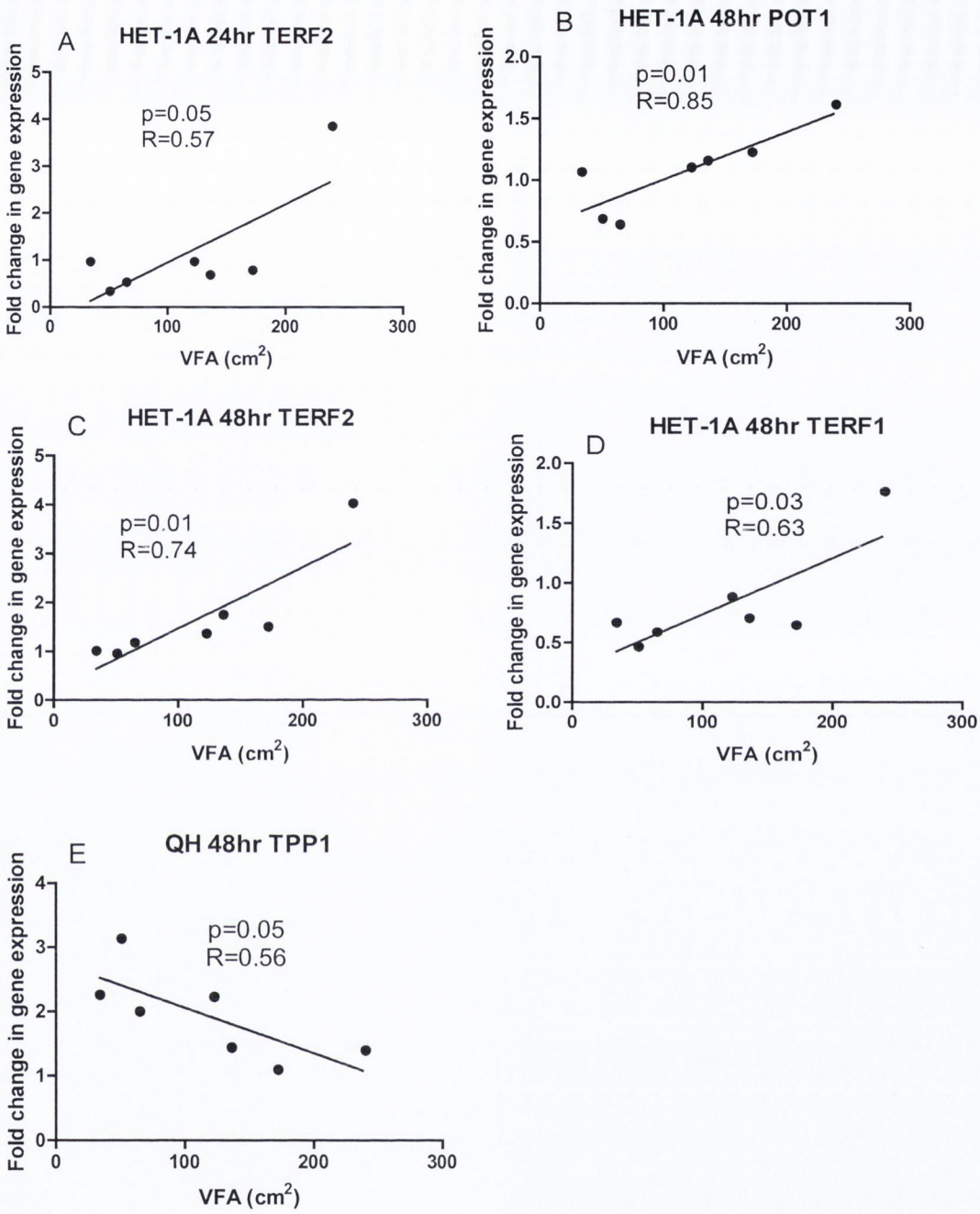

Figure 5.19. Patient ACM visceral fat area (n=7) correlates with the expression of members of the shelterin complex in oesophageal cell lines following 24hr and 48hr ACM treatment. Statistical analysis was carried out using linear regression analysis.

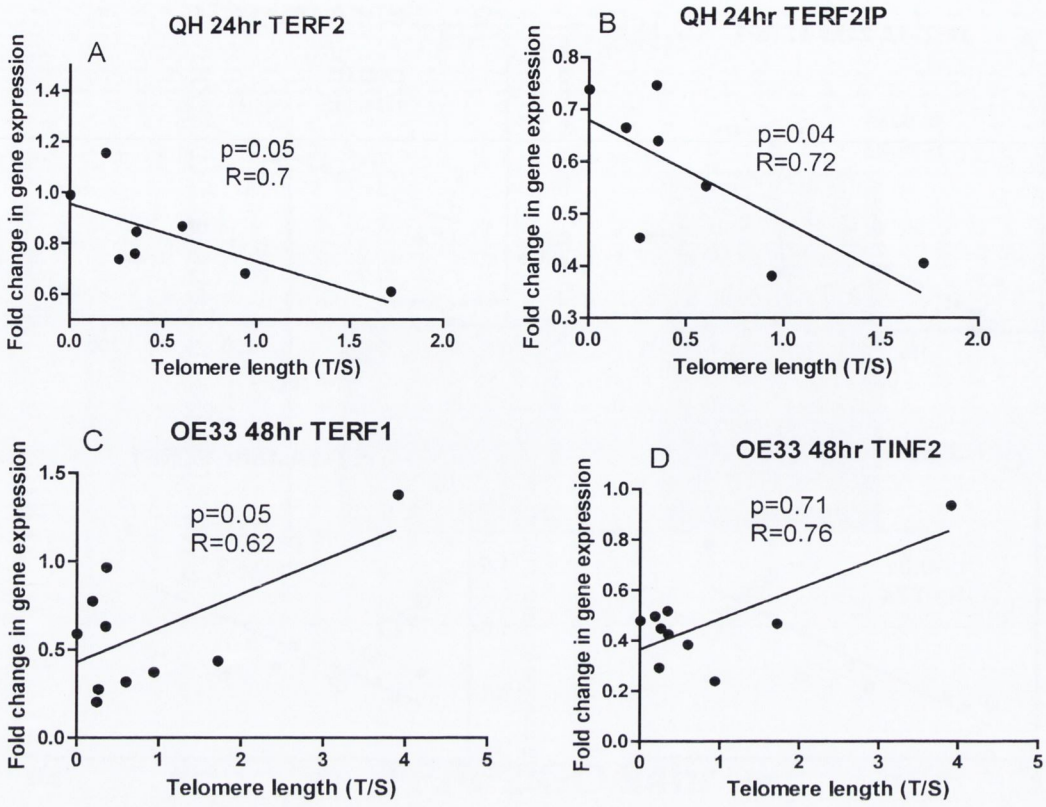

Figure 5.20. Telomere length correlates with the expression of members of the shelterin complex in the QH cell line following 24hr ACM treatment (n=10) and OE33 cell line following 48hr ACM treatment. The T/S ratio is an indicator of telomere length; a lower T/S ratio reflects shorter telomere length. Statistical analysis was carried out using linear regression analysis.

5.4 Discussion

Telomere shortening and dysregulation of both the telomerase and shelterin complexes is a common feature of cancer (Harley 2008; Bilsland, Cairney et al. 2010; Shammas 2011). This chapter demonstrates that dysregulation of telomere length and the associated telomeric chromatin is altered by ACM in a panel of oesophageal cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence associated with Barrett's carcinogenesis. While there is a wealth of data in the literature linking telomerase activity and telomere length in pre-neoplastic diseases and cancer, this is the first study to assess the effects of ACM from obese and non-obese patients on telomere length, telomerase dysfunction, and expression of shelterin proteins.

All oesophageal cell lines demonstrated significant telomere shortening following ACM treatment at 24hr and 48hr. Greatest telomere shortening in response to ACM treatment was detected in the QH cell line, representative of metaplastic Barrett's oesophagus. Previous research examining telomere length in Barrett's oesophagus biopsies of advancing histologic grade showed that telomere length was shortest in metaplastic Barrett's oesophagus and increases as the disease progresses (Rabinovitch, Finley et al. 2006) indicating that telomere shortening may be an early event in Barrett's derived adenocarcinoma. This has also previously been documented in other pre-neoplastic gastrointestinal diseases (O'Sullivan, Bronner et al. 2002; Kuhn, Meeker et al. 2011).

Telomere length in response to obese ACM was significantly lower compared to non-obese ACM in the GO cell line following 24hr ACM treatment. Telomere length following 48hr ACM treatment also displayed a significant inverse correlation with patient ACM VFA in the GO and OE33 cell lines. Obese patients may be susceptible to telomere shortening due to prolonged exposure in the oesophagus to factors secreted by adipose tissue and this may drive bridge breakage fusion, chromosomal rearrangement and eventual tumourigenesis (Murnane 2011). Telomere shortening has also been associated with obesity and weight gain in the elderly, where telomere length was shown to negatively associate with percentage body fat and subcutaneous fat (Njajou, Cawthon et al. 2011). In this chapter, ACM generated from visceral adipose tissue was shown to negatively regulate telomere length in a Barrett's metaplasia-dysplasia-adenocarcinoma cell model while research conducted by Njajou *et al* demonstrates that adipose tissue from fat depots other than visceral fat can also promote telomere shortening, particularly in elderly patients. Telomere shortening is a natural consequence of aging (Stewart and Weinberg 2002) and considering the continuous increase in age specific incidences of oesophageal adenocarcinoma (Corley and Buffler 2001), telomere shortening

related to an obese phenotype maybe an underlying mechanism related to increased rates of oesophageal adenocarcinoma in obese elderly patients.

Expression of telomerase complex proteins (TERT and TERC) are crucial for the maintenance of telomere length (Blasco, Gasser et al. 1999) and thus investigation of this complex was critical in determining telomere dysfunction in the oesophageal cell lines, in response to ACM treatment. The oesophageal cell lines demonstrated no clear pattern of TERC expression across the metaplasia-dysplasia-adenocarcinoma sequence, with ACM showing a trend towards down regulation following a 24hr treatment, then up-regulation following a 48hr treatment. This suggests that telomere length may be independent of TERC expression and activity. ACM derived from obese patients significantly down regulated TERC expression in the QH cell line following 48hr treatment, compared to ACM generated from their non-obese counterparts. There are contrasting reports in the literature on TERC expression across the metaplasia-dysplasia-adenocarcinoma sequence; increasing expression of TERC has been reported to accompany the transition from low grade to high grade dysplasia (Morales, Lee et al. 1998) however Gertler *et al* showed no difference in TERC expression between Barrett's carcinoma mucosa and non-cancerous mucosa (Gertler, Doll et al. 2008). However our study is the first to demonstrate dysregulation of TERC expression in response to ACM.

Reports of the expression of TERT across the metaplasia-dysplasia-adenocarcinoma sequence in Barrett's oesophagus are also controversial in the literature with either significant increases (Lord, Salonga et al. 2000), or no change in expression (Nwokolo, Barclay et al. 2005). In this study, a 24hr ACM treatment significantly up regulated TERT expression in the QH cell line compared to the HET-1A, GO and OE33 cell lines, which demonstrated modest decreases in TERT expression. Following 48hr ACM treatment, TERT expression was significantly increased in each HET-1A and QH cell lines; however the levels of TERT mRNA up regulation decrease across the metaplasia-dysplasia-adenocarcinoma sequence. ACM contains components such as leptin which is known to promote TERT expression and activity in cancer (Ren, Zhao et al. 2010; Rahmati-Yamchi, Zarghami et al. 2011) while VEGFA, an abundant component of the ACM, has been demonstrated to induce TERT expression and telomerase activity in ovarian cancer cells (Bennudez, Yang et al. 2007) and human umbilical vein endothelial cells (Trivier, Kurz et al. 2004).

There were significant inverse correlations between patient VFA and TERC and TERT expression in the QH cell line following 24hr ACM treatment, and a significant correlation between VFA and TERC expression in the HET-1A cell line following 24hr ACM treatment.

Factors present in the adipose tissue from obese patients may downregulate TERT and TERC expression in the QH cell line, causing shorter telomeres and subsequently initiate bridge-breakage-fusion cycles and genomic instability (Selvarajah, Yoshimoto et al. 2006). Ren *et al*, has shown that leptin activates telomerase in a dose-dependent manner and up-regulates the expression of TERT at mRNA and protein levels in MCF-7 breast cancer cells (Ren, Zhao et al. 2010). Zarghami et al reported a significant correlation between leptin levels and TERT expression in breast cancer biopsies from obese patients (Rahmati-Yamchi, Zarghami et al. 2011). However, there is some evidence in the literature to suggest that TERT and TERC mRNA expression do not regulate the activity of the telomerase complex and it is the post-transcriptional regulation of these genes or the presence of additional proteins that may moderate telomerase complex activity (Swiggers, Nibbeling et al. 2004). The work presented in this chapter supports this theory as TERC expression only significantly correlated with telomere length following a 48hr ACM treatment in the QH and OE33 cell lines, while TERT expression did not correlate with telomere length in any cell line at either time point tested.

We have demonstrated that ACM mediated dysregulation of TERT and TERC expression is independent of telomere length, however dysregulation of these genes may still may drive carcinogenesis in Barrett's oesophagus as telomerase complex proteins can play roles other than regulating telomere length (Blasco and Martinez 2011). Targeting of TERC in the HCT116 human colon cancer cell line down regulated the expression of genes linked to invasion and metastasis, and also led to cancer cell growth inhibition (Li, Crothers et al. 2005). TERT, independent of TERC, has been shown to stimulate epithelial cell proliferation through transcriptional control of Myc and Wnt, two proteins associated with stem cell function and cancer (Artandi, Choi et al. 2008). TERT, in tandem with TERC, has also been reported to stimulate skin tumourigenesis in mice (Blasco, Cayuela et al. 2005). TERT has also been associated with both the promotion of apoptosis via localization of TERT to the mitochondria, rendering cells more susceptible to oxidative stress (Santos, Meyer et al. 2006) and evasion of apoptosis where it was demonstrated that fibroblasts expressing TERT were more resistant to stress-induced apoptosis and necrosis (Gorbunova, Seluanov et al. 2002). In our study, TERT expression was significantly up-regulated in the QH cell line following ACM treatment, these cells also showed the greatest telomere shortening. TERT may be involved in initiating aberrant cellular processes at this stage of the disease sequence, independent of telomere maintenance.

The expression of telomerase associated proteins also showed dysfunction in the oesophageal cell lines following ACM treatment. Dyskerin (DKC1) is associated with the telomerase complex

(Gu, Bessler et al. 2009) and showed greatest up regulation in the QH cell line and a subsequent decrease in expression along the metaplasia-dysplasia-adenocarcinoma sequence, following 24hr and 48hr ACM treatments. DKC1 expression only displayed a significant correlation with telomere length in the OE33 cell line following 48hr ACM treatment. A direct correlation has been shown previously with DKC1 expression and telomere length in different malignancies (Pandolfi, Ruggero et al. 2003; Artandi, Batista et al. 2011). Decreased DKC1 levels have been linked with telomere shortening in X-linked dyskeratosis congenital (Armanios, Parry et al. 2011) while DKC1 is down regulated in sporadic chronic lymphocytic leukaemia (Gilson, Poncet et al. 2008). DKC1 has also been associated with tumourigenesis through means other than telomere maintenance with both compromised function and over expression of DKC1 promoting cancer. siRNA knockdown of DKC1 expression diminished cell proliferation in a prostate cancer cell line however over expression of DKC1 is demonstrated in prostate cancer tissues (Schulz, Sieron et al. 2009). We observed significant increases in expression of DKC1 following ACM treatment QH cells, in tandem with telomere shortening. DKC1 may be driving tumourigenesis in the QH cell line through mechanisms other than telomere dysfunction. Impairment of DKC1 function has been associated with p53 inactivation in a subset of human cancers through defective mRNA translation (Montanaro, Calienni et al. 2010). Down regulation of DKC1 due to obesity and subsequent p53 inactivation in Barrett's oesophagus, may be a mechanism by which obesity promotes carcinogenesis in Barrett's oesophagus; mutations in p53 have been linked to Barrett's progression previously (Rice, Schneider et al. 1996).

TEP1 expression was also investigated in the oesophageal cell lines. The TEP1 gene encodes for telomerase protein component 1, an enzyme that is associated with telomerase activity, and it specifically interacts with TERC, however it is not essential to telomerase activity or maintenance of telomere length (Harrington, Liu et al. 2000). TEP1 expression was significantly higher in the QH cell line following ACM treatment, and decreases in expression are detected later in the metaplasia-dysplasia-adenocarcinoma sequence. TEP1 expression levels have been shown to predict overall survival in breast cancer (Mokbel, Salhab et al. 2008), while it has also been investigated in both skin (Pang, Hu et al. 2002) and cervical (Kyo, Takakura et al. 1998) cancers but showed no association with malignancy. In our study, TEP1 expression did however significantly correlate with patient VFA in the HET-1A cell line following 24hr ACM treatment, and a significant correlation was seen between TEP1 expression and telomere length in the GO cell line following 48hr ACM treatment.

The expression of shelterin complex proteins was investigated along with telomere length and telomerase complex expression in this study. This complex consists of six key proteins that are involved in mediating telomerase complex activity at telomere ends (de Lange 2005; Palm and de Lange 2008). A review of the literature on the shelterin complex reveals that expression of this complex has not been studied in relation to obesity or Barrett's oesophagus. It has however been investigated in ulcerative colitis, a similar premalignant condition that progresses though various histologic grades to invasive carcinoma (Thorsteinsdottir, Gudjonsson et al. 2011). Shelterin complex proteins TERF2 and TERF2IP showed a down regulation in whole blood leukocytes in patients with Crohn's disease and ulcerative colitis compared to healthy subjects. Telomere uncapping due to the dysregulation of the complex has also been demonstrated in the early stages of chronic lymphocytic leukaemia where telomere dysfunction correlated with down regulation of TPP1 and TINF2 (Gilson, Augereau et al. 2011).

TERF1 expression significantly correlated with patient VFA in the HET-1A cell line following 48hr ACM treatment. An up-regulation of this shelterin complex protein in response to ACM treatment was conducive to telomere shortening as TERF1 is regarded as a negative regulator of telomere length (Gilson, Ancelin et al. 2002). TERF1 expression correlated significantly to telomere length in the OE33 cell line. Interestingly, TERF1 has been shown to associate with SAC proteins BUBR1 and MAD2 at mouse telomeres resulting in aberrant mitosis, indicating a link between the shelterin complex, the SAC and mitotic regulation (Blasco, Munoz et al. 2009). Dysregulation of TERF1 expression has also been shown to cause severe telomere damage in the absence of telomere shortening and promote the pathogenesis of pre-neoplastic lesions such as epithelial dysplasia (Blasco, Martinez et al. 2009). Dysregulation of TERF1 expression in Barrett's oesophagus by obesity may also promote early neoplastic events, as TERF1 expression is significantly increased following 48hr ACM treatment in the QH cells.

TERF2 expression displayed a significant inverse correlation with telomere length in the QH cell line following 48hr ACM treatment, a result which coincided with significant telomere shortening. This may potentially lead to bridge breakage fusion, genomic instability and carcinogenesis in Barrett's oesophagus. TERF2 has been linked to tumourigenesis previously, with elevated expression of the shelterin complex protein associated with human skin carcinomas (Blasco, Munoz et al. 2005). Cells deficient in functional TERF2 demonstrate extreme genomic instability due to telomere fusion but do not display telomere shortening (van Steensel, Smogorzewska et al. 1998). TERF2 has also been associated with the repair of

DNA damage caused by dysfunctional telomeres and other mechanisms (Meyn, Bradshaw et al. 2005). TERF2 expression was significantly higher in response to ACM derived from obese patients versus non-obese patients in the GO cell line following 48hr ACM treatment. Increased telomere shortening due to increased TERF2 activation in obese Barrett's patients due to prolonged exposure to factors secreted by adipose tissue may be a mechanism by which genomic instability may drive disease progression in Barrett's oesophagus.

In the HET1A cell line, POT-1 expression correlates with visceral fat area. This may be favourable to telomeric stability. Yasui *et al*, have shown that inhibition of POT-1 can cause an increase in the incidences of anaphase bridging in human gastric cancer cell lines, thus increasing genomic instability. In the same study POT-1 expression was shown to negatively correlate with gastric tumour stage, with expression decreasing during disease progression (Yasui, Kondo et al. 2004). Decreased POT-1 expression was demonstrated later in the metaplasia-dysplasia-adenocarcinoma sequence in the GO and OE33 cell lines following 48hr ACM treatment.

TPP1 has been demonstrated to regulate telomere elongation as TPP1binding results in decreased TERT binding to telomeres and accelerated telomere shortening in mouse embryonic fibroblasts (Blasco, Tejera et al. 2010). Loss of TPP1 expression and telomere de-protection has also been linked to carcinogenesis arising in adrenocortical dysplasia (Else, Trovato et al. 2009), the data in this chapter suggests a similar event could occur in Barrett's oesophagus. Increased TPP1 expression following 48hr treatment with ACM generated from obese patients, compared to non-obese patients, was observed in the GO cell line. Dysregulation of TPP1 in obese patients may represent a path to carcinogenesis in Barrett's oesophagus due to telomere uncapping and subsequent breakage fusion bridge cycles leading to chromosomal rearrangement.

TINF2 expression also significantly correlated with telomere length in the OE33 cell line following 48hr ACM treatment. TINF2, a TERF1 interacting protein essential for TERF1 function, is responsible for bridging of shelterin complexes through the recruiting of TPP1 and POT1 (Martinez and Blasco 2010). The binding of TINF2 to TERF1 causes a conformational change in TERF1, making the telomere inaccessible to the telomerase complex. Over expression of TINF2 inhibits telomere elongation in various human cell lines, whereas deletion of TINF2 causes unrestricted telomere elongation (Kim, Kaminker et al. 1999). TERF2IP was the final member of the shelterin complex investigated in this study. TERF2IP does not bind telomeres directly, but is recruited by TERF2 and is a negative regulator of telomere length (Li and de Lange 2003). In

the QH cell line, following 24hr ACM treatment, TERF2IP expression displays a significant inverse correlation with telomere length, reinforcing the theory of TERF2IP as a negative regulator of telomere length. Similar to the telomerase complex proteins, TERF2IP has demonstrated roles in cells independent of telomere maintenance. TERF2IP can mediate transcriptional changes in genes related to cell adhesion, and metabolism through binding to extra-telomeric TTAGGG sites in the genome (Martinez, Thanasoula et al. 2010). TERF2IP forms a complex with IKKs (IκB kinases), and is crucial for the ability of IKKs to be recruited to, and phosphorylate, the p65 subunit of NF-κB to make it transcriptionally competent, regulating an important signalling cascade (Tergaonkar, Teo et al. 2010). Thus, through its role in telomere shortening and NF-κB regulation, dysregulation of TERF2IP expression in Barrett's oesophagus may be driving carcinogenesis through two independent pathways.

Patient VFAs correlated with telomere length and telomere binding protein expression in the oesophageal cell lines following ACM treatment of the HET-1A, QH and OE33 cell lines while other established anthropometric measurements of obesity, BMI and waist circumference, showed no correlation. In investigating the oncogenic burden of obesity on telomere dysfunction and tumourigenesis arising in Barrett's oesophagus, patient visceral fat area seems to be the most clinically useful measurement of patient obesity status. The ACM from ten patients was used in investigating telomere dysfunction in this study, however the VFAs from three of the patients from which the ACM was derived were not available, hence correlations in this study are for 7 patients. Changes in expression of a wide range of telomere binding proteins by ACM in a metaplasia-dysplasia-adenocarcinoma cell model have been demonstrated in this study. Dysregulation of the telomerase complex and telomere length has been demonstrated previously in Barrett's oesophagus, however this is the first study to provide evidence of the effect of ACM in cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence on telomere and telomerase complex dysfunction. This study is also the first to investigate dysregulation of the shelterin complex in obesity and Barrett's oesophagus. Alterations in telomere length in response to ACM is associated with each stage of the metaplasia-dysplasia-adenocarcinoma sequence, with significant telomere shortening demonstrated at each histologic stage of Barrett's carcinogenesis, represented by the HET-1A, QH, GO and OE33 cell lines. Chromosomal rearrangements may take place early in Barrett's oesophagus as a result of telomere shortening, and while telomere elongation is observed later in the disease sequence (Rabinovitch, Finley et al. 2006), the cellular dysfunction caused by early genomic instability due to adipose derived factors may predestine Barrett's metaplasia to carcinogenesis. Targeting telomeres, the shelterin complex and telomerase

complex has been tentatively suggested as an anti-cancer therapy (Bilsland, Cairney et al. 2010). These events may play an important role in pre-neoplastic diseases such as Barrett's oesophagus. We are particularly interested in the downstream events driven by telomere dysfunction; this is the focus of the next chapter.

Chapter 6: Obesity interrupts the regulation of the spindle assembly checkpoint and anaphase bridging in Barrett's oesophagus

6.1 Introduction

A common cause and indicator of chromosomal instability is the formation of anaphase bridges (Hoffelder, Luo et al. 2004), first described by McClintock in maize (McClintock 1941). She hypothesised that these bridges were a consequence of the fusion of telomeres, leading to the formation of dicentric chromosomes, with each centromere of the chromosome pulled to opposite spindle poles during mitosis. These bridges can break under stress from the mitotic apparatus which triggers complex chromosomal rearrangements with a net gain of chromosomal material in one daughter cell, and a corresponding net loss in the other daughter cell (Gagos and Irminger-Finger 2005). In addition to broken chromosomes, anaphase bridges can also occur due to telomere shortening and by persistent chromatid cohesion (Hoffelder, Luo et al. 2004). Telomere shortening followed by anaphase bridge formation and chromosomal instability conducive to carcinogenesis has been observed in ulcerative colitis, a pre-neoplastic lesion of colorectal cancer (O'Sullivan, Bronner et al. 2002). Anaphase bridging may influence disease progression in Barrett's oesophagus through the accumulation of clonal diversity which is conducive to tumourigenesis (Maley, Galipeau et al. 2006) and has been strongly linked to chromosomal instability in human pre-neoplastic diseases (O'Sullivan, Bronner et al. 2002; Stewenius, Gorunova et al. 2005; O'Sullivan, Risques et al. 2006).

Chromosome instability may lead to a cellular phenotype displaying aneuploidy. Aneuploidy can be defined as (hyperdiploid) or (hypodiploid) with cells displaying more or less chromosome numbers, respectively. This aneuploidy is thought to be responsible for deactivation of tumour suppressor genes (Sugai, Nakamura et al. 1999). Higher levels of aneuploidy in tumours have been correlated with poorer prognosis for cancer patients (Owainati, Robins et al. 1987; Draviam, Xie et al. 2004). Over 90% of high grade dysplasias and adenocarcinomas developed in Barrett's oesophagus are DNA aneuploid, and there is a significant relation between the presence of DNA aneuploid population and the progression from non-dysplastic Barrett's intestinal mucosa to dysplasia and adenocarcinoma (Rabinovitch, Reid et al. 1989; Flejou 2005).

To maintain genetic stability in the cell, precise apportioning of chromosomes from one mother cell to two daughter cells occurs during mitosis. When an error occurs in this process, or any spindle damage is detected, a mitotic metaphase-to-anaphase checkpoint is activated, which arrests cell cycle progression until all kinetochores (the protein complexes assembled at

each centromere) are attached to both poles of the mitotic spindle, ensuring equal allocation of the chromosomes between daughter cells (Mondal, Sengupta et al. 2007). Unattached kinetochores are also the signal generators for a mitotic checkpoint, which arrests mitosis until all kinetochores have correctly attached to spindle microtubules. This represents the major cell cycle control mechanism protecting against aneuploidy (Cleveland, Mao et al. 2003). This checkpoint is known as the spindle assembly checkpoint (SAC). Evidence in the literature suggests that defects in the spindle assembly checkpoint may drive aneuploidy during carcinogenesis (Malmanche, Maia et al. 2006; Li and Zhang 2009). Cells with an impaired spindle assembly checkpoint enter anaphase prematurely before all kinetochores are attached and can suffer chromosomal rearrangement which may ultimately drive tumourigenesis (Musacchio and Salmon 2007). The genes associated with this process are BUB1B, MAD2, CDC20, CENPE and ESPL1. These checkpoint proteins are recruited onto unattached kinetochores where they generate a diffusible signal to prevent anaphase onset during mitosis (Cleveland, Mao et al. 2005).

Mutations in BUB1B, and related family members BUBR1 and BUB3, have been identified in numerous cancers, though at a low frequency (Cahill, Lengauer et al. 1998). In the SAC, BUB1B acts as a principal regulator in terms of recruitment and assembly of the checkpoint proteins at the kinetochores during cell division. Once chromosome condensation occurs, BUB1B localizes to the kinetochores immediately and is responsible for efficient kinetochore localization of CENPF, CENPE and MAD2L2 (Taylor, Johnson et al. 2004). BUB1B is thought to be essential for precise regulation of the SAC and expression of a mutant BUB1B protein leads to a compromised checkpoint, facilitating genomic instability (Hunt, Geley et al. 2001).

MAD2L2 is an essential component of the SAC, inactivation or hyperactivation of this protein drives tumourigenesis in mice (Sotillo, Hernando et al. 2007). It is an unusual two state protein and conformational switches between its two known states are assisted by MAD2L1, an upstream regulator of MAD2L2 (Englander, Skinner et al. 2008). MAD2L2 acts in a closely regulated complex with MAD2L1 to correct errors in unattached kinetochores before anaphase. MAD2L2 binds directly to CDC20 during mitosis and is essential for checkpoint dependant arrest of the anaphase promoting complex cyclosome (APC/C). When all kinetochores have successfully attached, anaphase is initiated via the dissociation of MAD2L2 from the APC/CDC20 complex (Herbert, Heriche et al. 2005; Yu 2006).

CDC20 is a co-factor of the APC/C and is targeted and controlled by the SAC (Musacchio and Salmon 2007) and dysregulation of CDC20 is reported in many forms of cancer (Kidokoro,

Tanikawa et al. 2008). During mitosis, the attachment of sister kinetochore pairs and the correct alignment of chromosomes negatively regulates the SAC signal. Once this signal is quenched, CDC20 is released from MAD2L2 and can then activate the APC/C (Kramer, Gieffers et al. 1998). The SAC negatively regulates the ability of CDC20 to activate the APC/C mediated polyubiquitylation of cyclin B and securin, ultimately averting their destruction by the proteasome. Securin is an inhibitor of a protease known as seperase (encoded by the ESPL1 gene in humans), which is required to cleave the cohesin complex that holds chromatids together. Cleavage of this cohesion complex is necessary to initiate anaphase.

CENPE is required for the maintenance of chromosomal stability through stabilization of microtubule capture at kinetochores. CENPE acts as a microtubule motor that facilitates chromosomal movement during mitosis (Putkey, Cramer et al. 2002). Without the kinetochore-associated microtubule motor protein CENPE, the checkpoint cannot be established or maintained (Cleveland, Mao et al. 2005). Studies have shown an interaction between CENPE with SAC genes BUB1B and MAD2L2, which are important in kinetochore attachment to the chromosomes at the end of metaphase (Yao, Anderson et al. 1997). It has also been suggested that CENPE generates the tension required for successful chromosome and kinetochore stabilisation (Yen, Schaar et al. 1997) and full chromosome alignment is prohibited by disruption of CENPE function in *Xenopus* egg extracts (Wood, Sakowicz et al. 1997). Dysregulation of CENPE function drives aneuploidy both in vitro and in an age dependant manner in mice (Cleveland, Weaver et al. 2007).

ESPL1 (the human homolog of seperase), regulated by securin, is involved in the separation of sister chromatids, which takes place at the metaphase-to-anaphase transition. Initiation of the APC/C signal by CDC20 leads to the degradation of securin, initiating ESPL1 activity (Musacchio and Salmon 2007). The correct timing of sister chromatid separation is crucial for accurate chromosomal segregation into daughter cells, both premature and delayed separation of sister chromatid can result in extensive chromosomal remodelling (Cohen-Fix and Agarwal 2002). ESPL1 over expression has been shown to drive aneuploidy and tumourigenesis in mice (Mukherjee, Ge et al. 2011).

6.2 Aims and Objectives

The overall aim of this chapter was to assess if ACM drives anaphase bridging and dysregulation of the SAC complex.

Specific Objectives

- To examine if ACM increases anaphase bridging in the cell lines which represent different stages of the metaplasia-dysplasia-adenocarcinoma sequence
- Investigate if anaphase bridge formation is increased following treatment with obese ACM compared to non-obese ACM
- Examine whether anaphase bridge formation correlates with obesity status of patients from which the ACM was generated
- Identify if abundant factors expressed in adipose tissue (VEGF and IL-8), play a functional role in regulating bridge formation
- Determine the effect of ACM on expression of the spindle assembly complex in the cell lines and compare effects of ACM generated from obese patients versus ACM generated from non-obese patients
- Correlate telomere length, telomerase complex expression, shelterin complex expression and telomere associated proteins expression with anaphase bridging in the HET-1A, QH, GO and OE33 cell lines

6.3 Results

6.3.1 ACM stimulates anaphase bridge formation in oesophageal cell lines following 24hr and 48hr culture

Anaphase bridging was assessed in the four cell lines that represent the different stages of the metaplasia-dysplasia-adenocarcinoma sequence associated with Barrett's carcinogenesis in response to ACM treatment (method described in section 2.7). Anaphase bridges were defined as 1 or more interconnected strands between daughter nuclei, cells with no interconnecting strands were scored as negative. Anaphase bridges represent lagging chromosomes that do not fully resolve during anaphase (Figure 6.1). The HET-1A, QH, GO and OE33 cell lines were treated with ACM for 24hr and 48hr in order to investigate if ACM drives anaphase bridging. The same patient ACM cohort was used as in the previous chapter to allow for comparison of results.

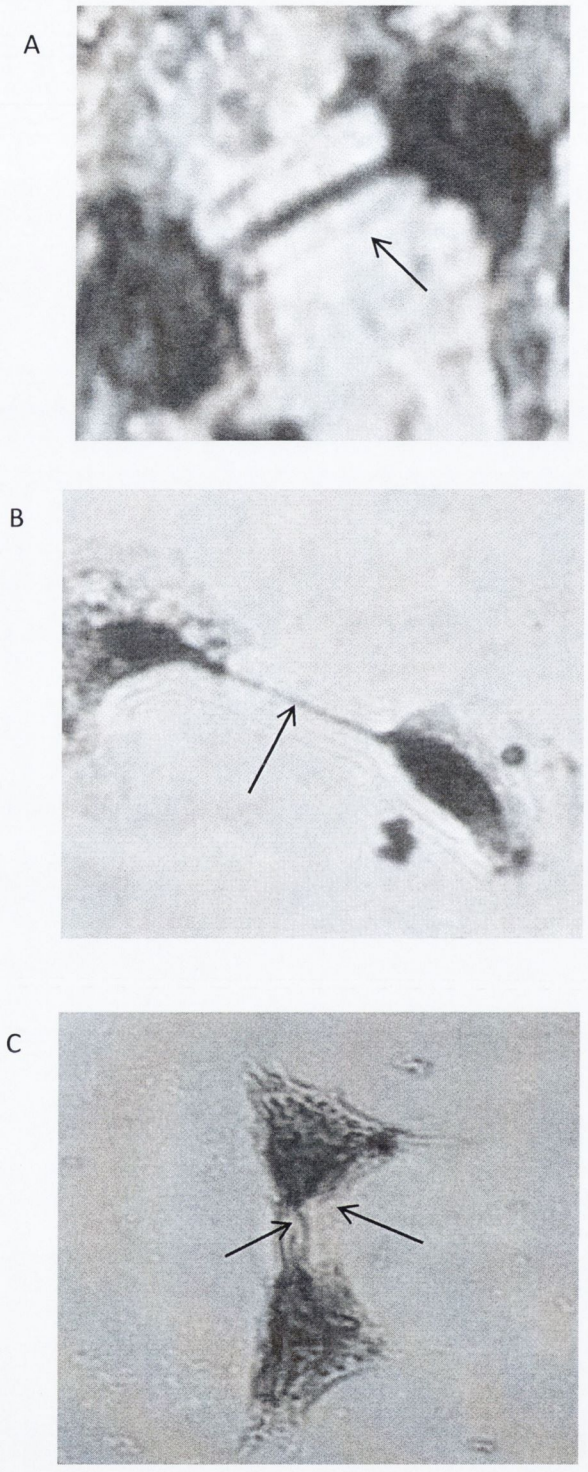

Figure 6.1. Representative anaphase bridges in the oesophageal cell lines.

6.3.1.1 Effect of ACM on cell number in the HET-1A, QH, GO and OE33 cell lines

Cells with greater proliferation would display greater numbers of anaphase bridges therefore cell number was assessed in the cells following ACM treatment via MTT assay to normalise bridging counts to cell number (Figures 6.2 and 6.3).

There was no significant increase in cell number following 24hr ACM treatment compared to the untreated control in the HET-1A, QH and GO cell lines (Figure 6.2 A,C,E). However in the OE33 cell line (Figure 6.2H), a 24hr ACM treatment significantly increased cell number ($p=0.025$). Cell number was also assessed in response to ACM generated from viscerally obese and non-obese patients in the oesophageal cell lines. While no significant increases in cell number were observed in the HET-1A, QH and GO cell lines by ACM generated from non-obese or obese patients (Figure 6.2 B,D,F) the OE33 cell line demonstrated significantly increased cell number in response to both non obese ($p=0.043$) and obese ($p=0.048$) ACM, respectively (Figure 6.2G).

Following 48hr ACM treatment, only the HET-1A cell line demonstrated significantly increased cell number (Figure 6.3A), compared to the untreated control ($p=0.007$). There was no significant increase in cell number observed in the QH, GO and OE33 cell lines (Figure 6.3 C,E,F). Cell number was subsequently compared in response to ACM generated from viscerally obese and non-obese patients in the oesophageal cell lines. In the HET-1A cell line, ACM generated from non-obese patients elicited a significant increase in cell (Figure 6.3B) ($p=0.009$) while ACM from obese patients also significantly increased cell number ($p=0.049$). The QH cell line demonstrated a significant decrease in cell number following 48hr treatment with ACM generated from non-obese patients with cell number decreasing to 54% (Figure 6.3D) of the untreated control ($p=0.020$). Cell number in response to a 48hr treatment with ACM generated from obese patients elicited no increase compared to the untreated control in the QH cell line, however a significant increase in cell number was observed compared to 48hr treatment with non-obese ACM ($p=0.003$) (Figure 6.3D). In the GO cell line, cell number was significantly increased ($p=0.018$) in response to ACM generated from obese patients (Figure 6.3F). Cell number in response to 48hr treatment with ACM generated from obese patients was also significantly higher compared to cells treated with ACM generated from non-obese patients ($p=0.002$). No difference in cell number was observed in the OE33 cell line following 48hr treatment with ACM generated from either obese or non-obese patients (Figure 6.3H). There was no significant difference in cell number between 24hr and 48hr ACM treatment in any of the cell lines.

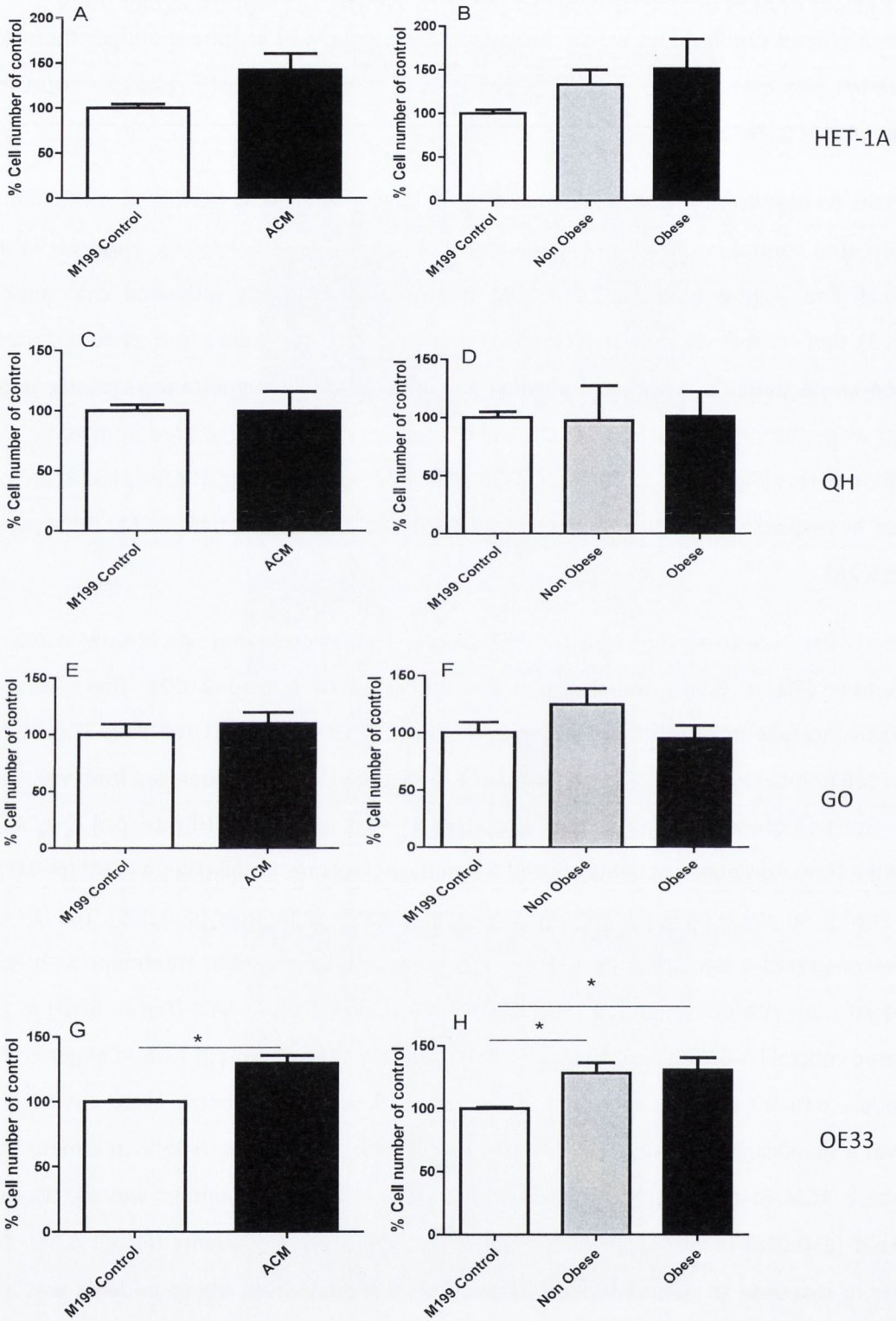

Figure 6.2. Relative cell number in the oesophageal cell lines 24hr following treatment with ACM generated from viscerally obese (n=5) and non-obese (n=5) patients. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05)

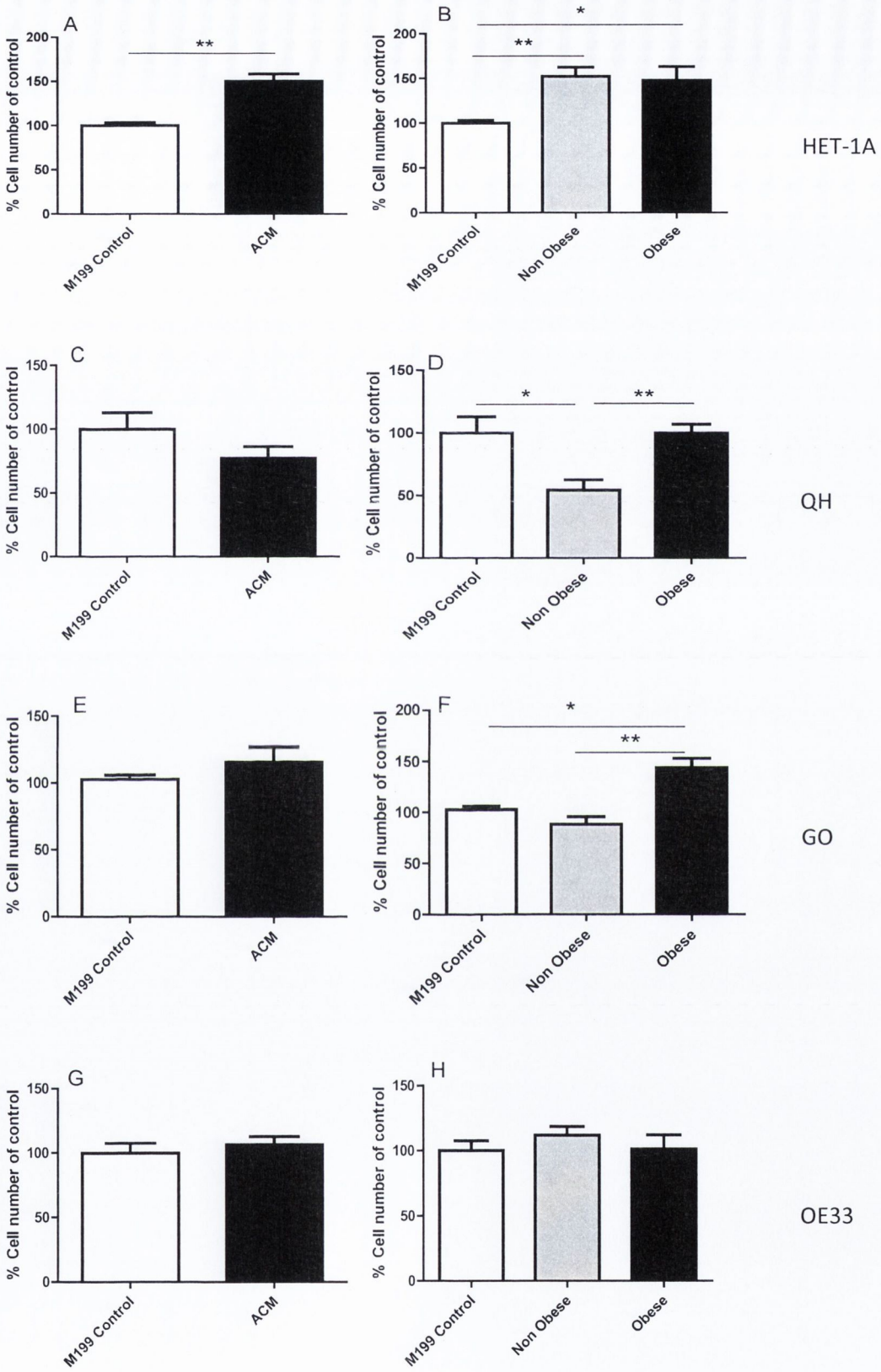

Figure 6.3. Relative cell number in the oesophageal cell lines 48hr following treatment with ACM generated from viscerally obese (n=5) and non-obese (n=5) patients. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01).

6.3.1.2 ACM treatment promotes anaphase bridging in the HET-1A, QH, GO and OE33 cell lines

Basal anaphase bridging was investigated in the oesophageal cell lines (Figure 6.4). There was a significant increase in basal anaphase bridging across as the cells models progressed from normal squamous epithelium to metaplasia (QH) and dysplasia (GO). The GO cell line, which is representative of dysplastic Barrett's oesophagus, showed significantly higher levels of basal bridging compared to the HET-1A ($p=0.012$), QH ($p=0.046$) and OE33 ($p=0.010$) cell lines. The OE33 cells, derived from an oesophageal adenocarcinoma patient showed the lowest levels of basal anaphase bridging, significantly lower than those observed in the GO cell line ($p=0.002$).

While we have shown that ACM can affect anaphase bridging, we next wanted to determine if ACM treatments at 24hr and 48hr elicited different levels of anaphase bridging (Figure 6.5). In the HET-1A cell line, anaphase bridging was significantly increased following 24hr ACM treatment compared both the untreated control ($p=0.044$) and compared to a 48hr ACM treatment ($p=0.005$) (Figure 6.5A). In the QH cell line, anaphase bridging was significantly increased following 48hr ACM treatment (Figure 6.5B), compared to basal levels ($p=0.005$) and following 24hr ACM treatment ($p<0.0001$). In the dysplastic GO cell line, anaphase bridging was significantly decreased compared to basal levels following 24hr ACM treatment ($p=0.002$) while 48hr ACM treatment elicited significantly higher levels of anaphase bridging compared to a 24hr ACM treatment ($p<0.0001$) but not the basal levels (Figure 6.5C). Anaphase bridging in the OE33 cell line was significantly increased following 48hr ACM treatment (Figure 6.5D) and increases in anaphase bridging reached statistical significance compared to both basal levels ($p=0.002$) and a 24hr ACM treatment ($p<0.0001$).

Anaphase bridging was subsequently assessed in the cell lines in response to ACM generated from viscerally obese and non-obese patients at both 24hr and 48hr ACM treatment (Figure 6.6). In the HET-1A cell line following a 24hr treatment, ACM generated from viscerally obese patients generated significantly more anaphase bridging compared to both the basal levels ($p<0.0001$) and ACM generated from non-obese patients ($p<0.0001$) (Figure 6.6A). Increased anaphase bridging was also observed following 48hr treatment with ACM generated from viscerally obese patients compared to ACM generated from non-obese patients in the HET-1A cell line ($p=0.012$) (Figure 6.6A).

Anaphase bridging was significantly higher in QH cell line following 24hr treatment (Figure 6.6B) in cell treated with ACM generated from viscerally obese patients compared to non-obese patients ($p=0.050$). A decrease in anaphase bridging was observed following 24hr ACM treatment with ACM generated from non-obese patients compared to basal levels, however

this was not significant (p=0.07). Following 48hr ACM treatment, significantly increased anaphase bridging was observed in cells treated with non-obese (p=0.016) and obese ACM (p=0.021) compared to basal levels of anaphase bridging. Significantly higher levels of anaphase bridging were also demonstrated between cells treated with non-obese ACM for 24hr compared to cell treated with obese ACM for 48hr (p=0.007) (Figure 6.6B).

Significant decreases in anaphase bridging were demonstrated in the GO cell line following 24hr treatment with ACM generated from both non-obese (p=0.001) and viscerally obese patients (p=0.034). Significantly increased anaphase bridging was also observed following 24hr treatment with obese ACM compared to non-obese ACM (p=0.002) (Figure 6.6C). Differences in anaphase bridging observed following 48hr treatment did not reach statistical significance between obese and non-obese ACM or basal levels of anaphase bridging (Figure 6.6C). Significantly higher levels of anaphase bridging were also observed between cells treated with non-obese ACM for 24hr compared to cells treated with obese ACM for 48hr (p=0.005).

In the OE33 cell line, there were no significant differences demonstrated in anaphase bridging levels observed between non obese and obese ACM or basal levels, following 24hr treatment (Figure 6.6D). However following 48hr ACM treatment, significantly higher anaphase bridging was demonstrated following treatment with both non obese (p=0.003) and obese ACM (p=0.021) compared to basal levels (Figure 6.6D). Significantly higher levels of anaphase bridging were also documented between cells treated with non-obese ACM for 24hr compared to cells treated with obese ACM for 48hr (p<0.0001).

Levels of anaphase bridging were compared across the cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence, following both 24hr and 48hr ACM treatment (Figure 6.7). Following 24hr ACM treatment, anaphase bridging was greatest in the HET-1A cell line and was significantly higher than levels observed in the QH (p=0.003), GO (p=0.004) and OE33 (p=0.0004) cell lines (Figure 6.7A). Anaphase bridging levels were also significantly lower in the OE33 cell line compared to the GO cell line (p=0.0009). Following 48hr ACM treatment however (Figure 6.7B), highest levels of anaphase bridging were observed in the GO cell line, significantly higher than those demonstrated in both the HET-1A (p<0.0001) and OE33 (p<0.0001) cell lines. The QH cell line also demonstrated significantly higher levels of anaphase bridging in response to a 48hr ACM treatment, compared to the HET-1A (p<0.0001) and OE33 (p<0.0001) cell lines.

Patient waist circumference was correlated with anaphase bridging in the oesophageal cell lines (Figure 6.8). Significant correlations were observed between patient waist circumference

and levels of anaphase bridging in the HET-1A cell line in response to both 24hr and 48hr ACM treatment (p=0.0002, R=0.91 and p=0.0018. R=0.87, respectively). Significant correlations were similarly observed between patient waist circumference and levels of anaphase bridging in the QH cell line (p=0.008, R=0.71) and the GO cell line (p=0.0002, R=0.91) following 24hr ACM treatment.

Significant correlations were also demonstrated between the VFA of the patients from which the ACM was derived and anaphase bridging in the GO cell line (p=0.0002, R=0.91) and the HET-1A cell line following 48hr ACM treatment (p=0.0017, R=0.84) (Figure 6.9).

No significant correlations were observed between patient BMI and anaphase bridging in the panel of oesophageal cell lines, at either time point.

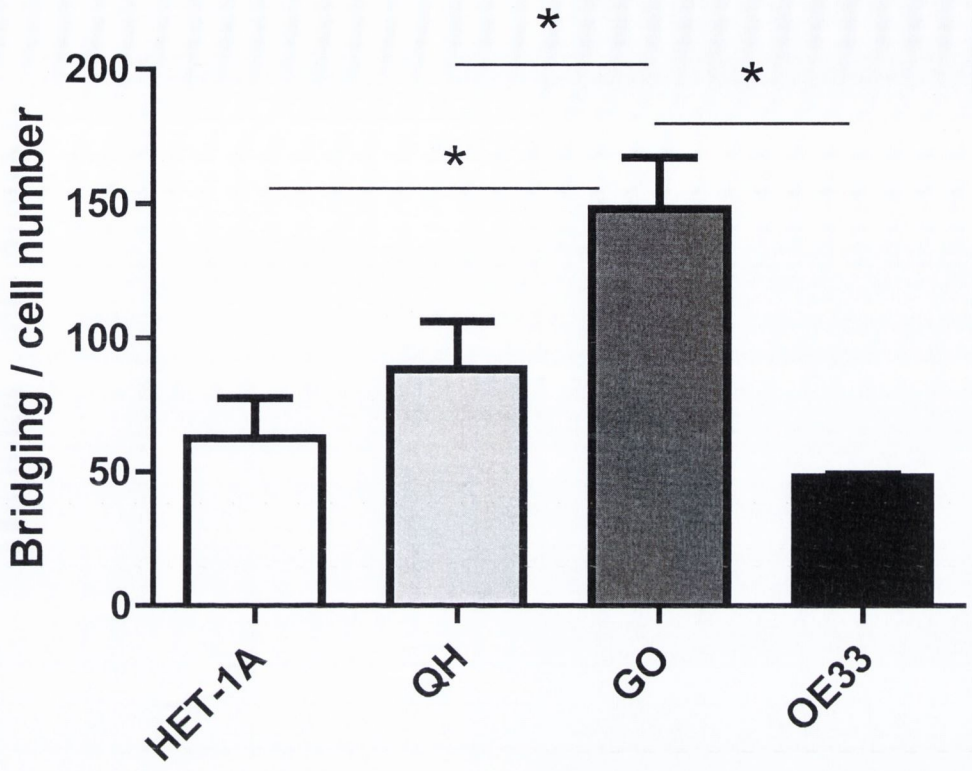

Figure 6.4. Basal anaphase bridging in the oesophageal cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence (n=3). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$).

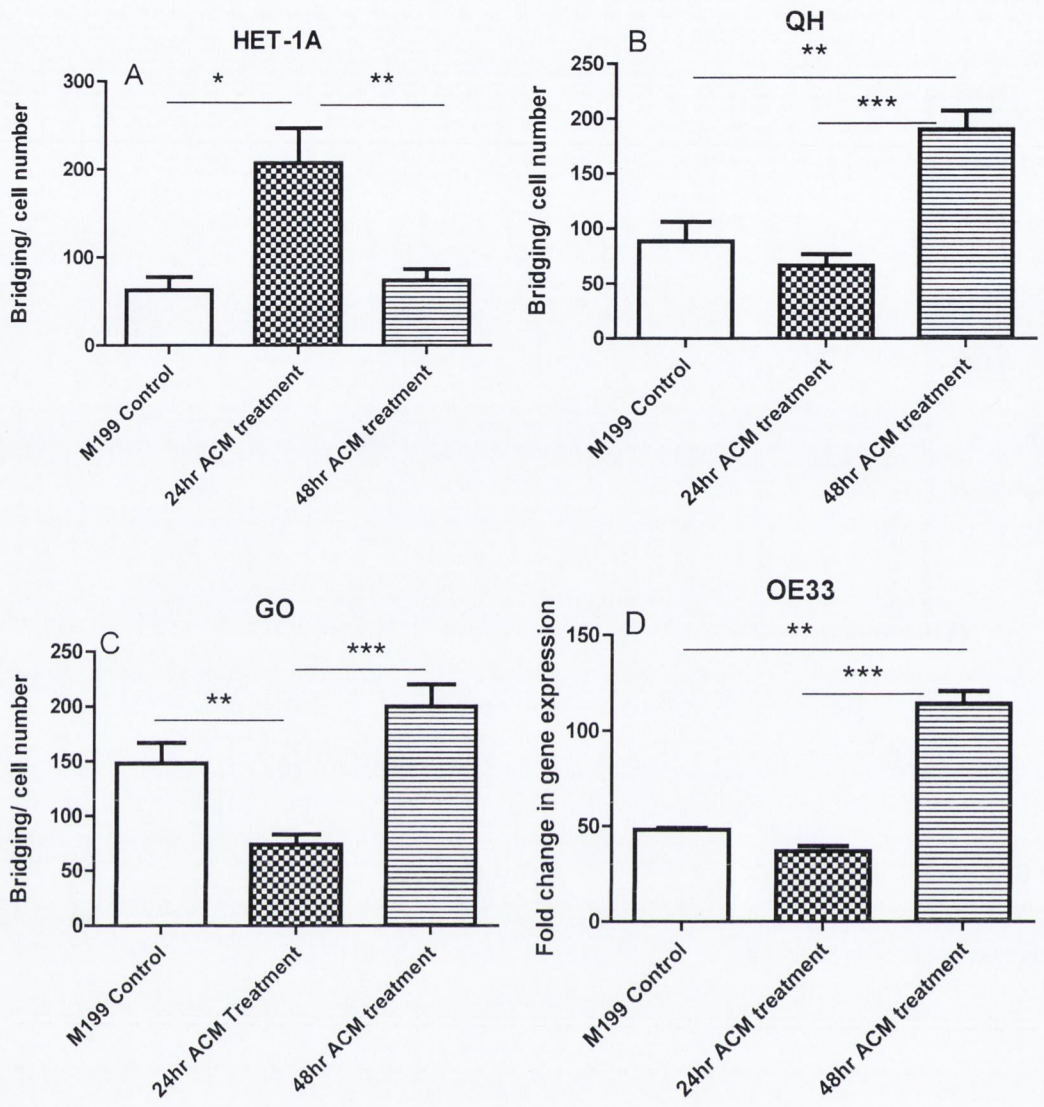

Figure 6.5. Anaphase bridging in oesophageal cell lines representative of the metaplasia-dysplasia-adenocarcinoma sequence following 24hr (n=10) and 48hr (n=10) ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

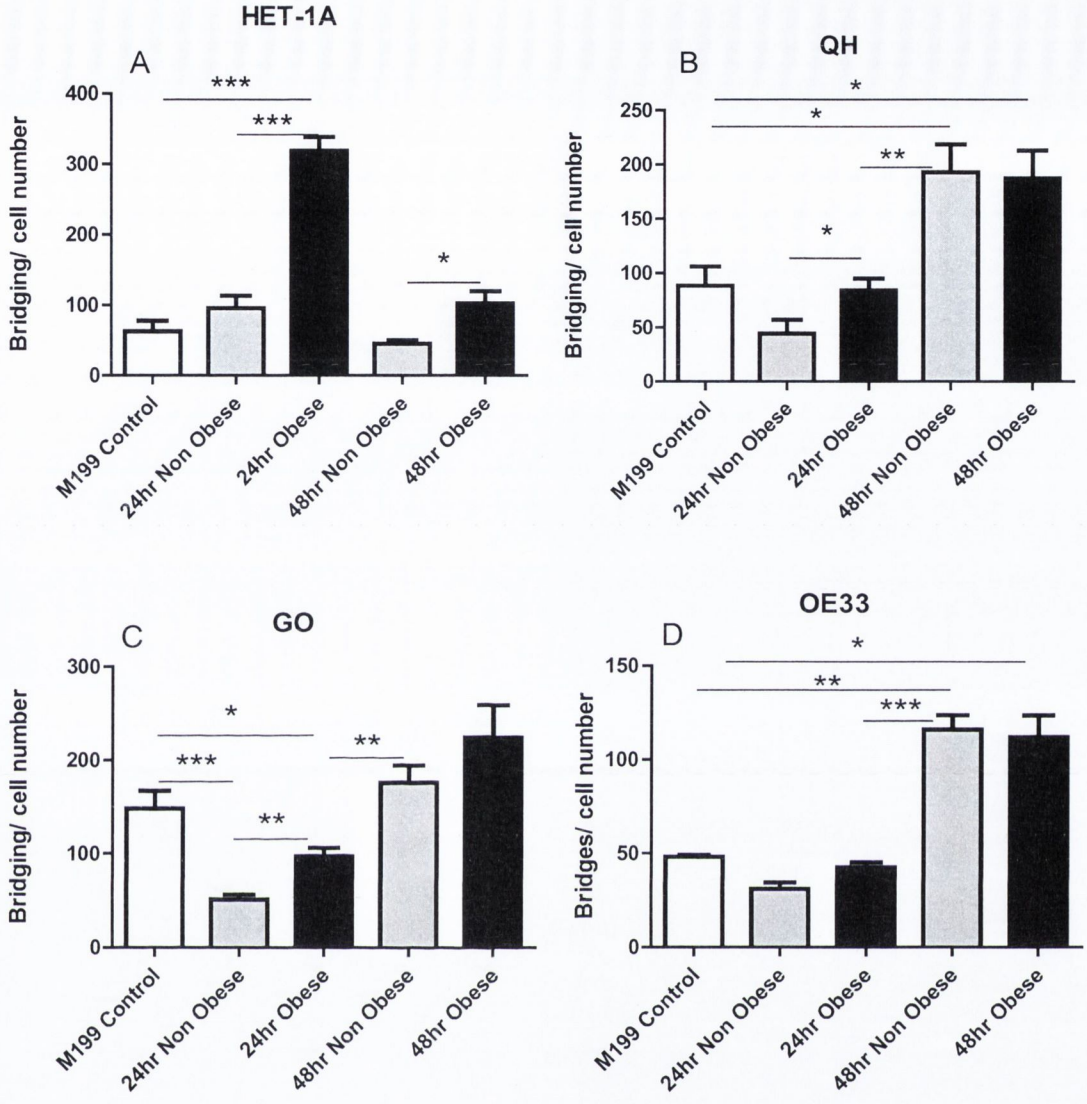

Figure 6.6. Anaphase bridging in the oesophageal cell lines in response to ACM generated from viscerally obese (n=5) and non-obese (n=5) patients following 24hr and 48hr ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

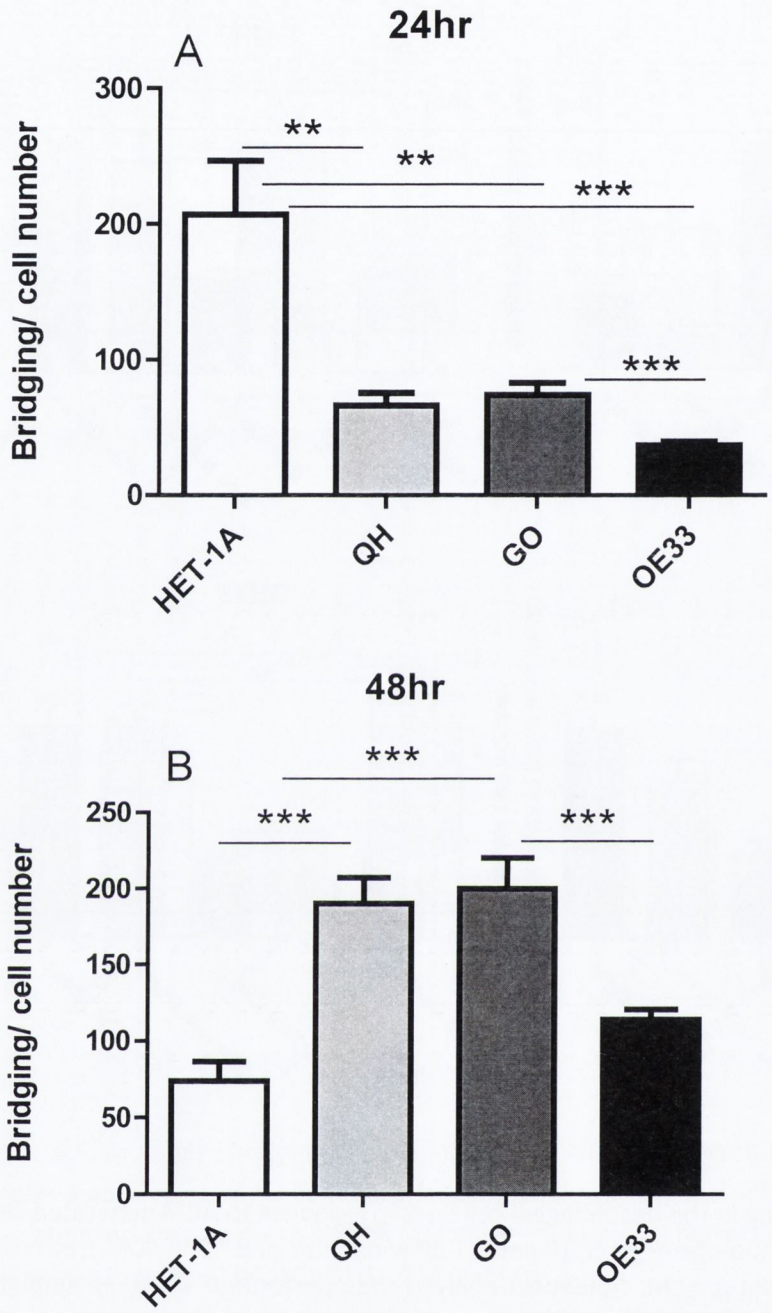

Figure 6.7. Anaphase bridging in response to 24hr and 48hr ACM treatment (n=10) across the cell lines which represent the metaplasia-dysplasia-adenocarcinoma sequence. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

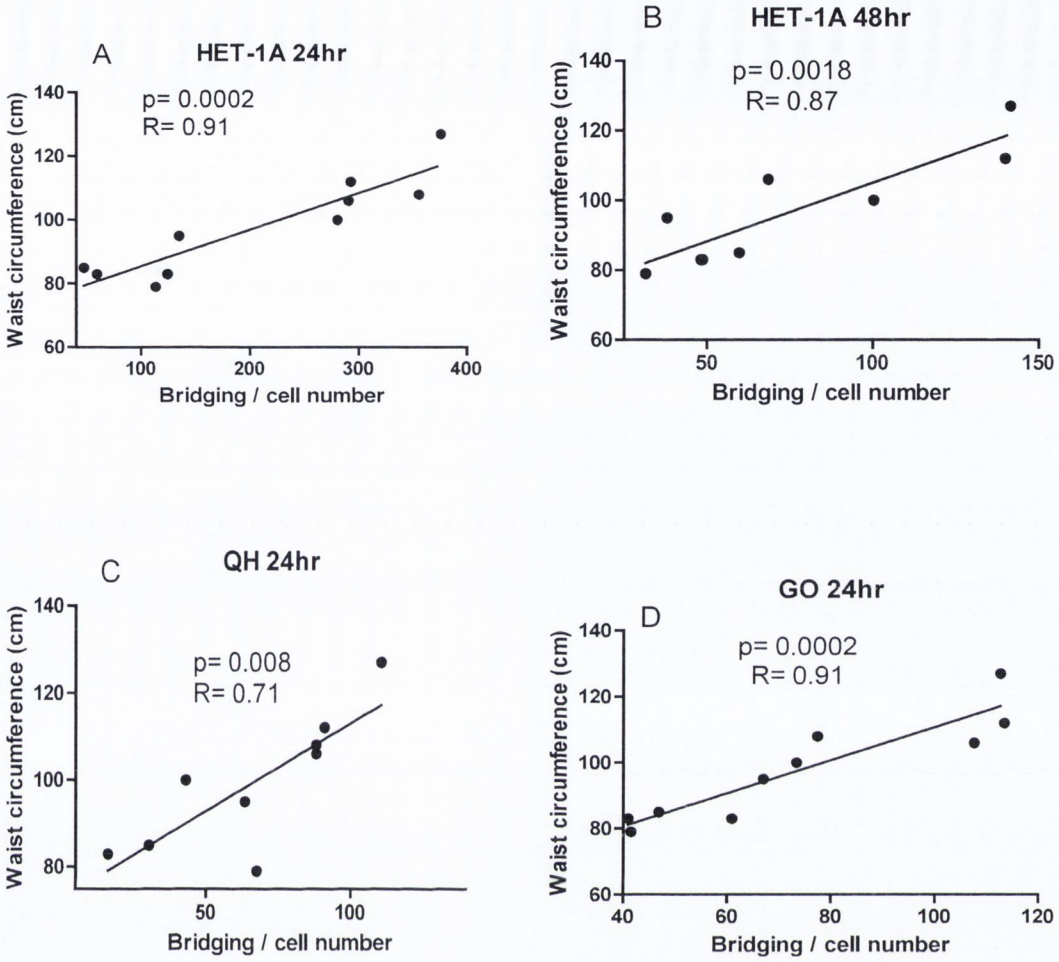

Figure 6.8. Patient waist circumference correlates significantly with anaphase bridging in the GO cell line following 24hr ACM (n=7) treatment and the HET-1A cell line following 48hr ACM treatment (n=7). Statistical analysis was carried out using linear regression analysis.

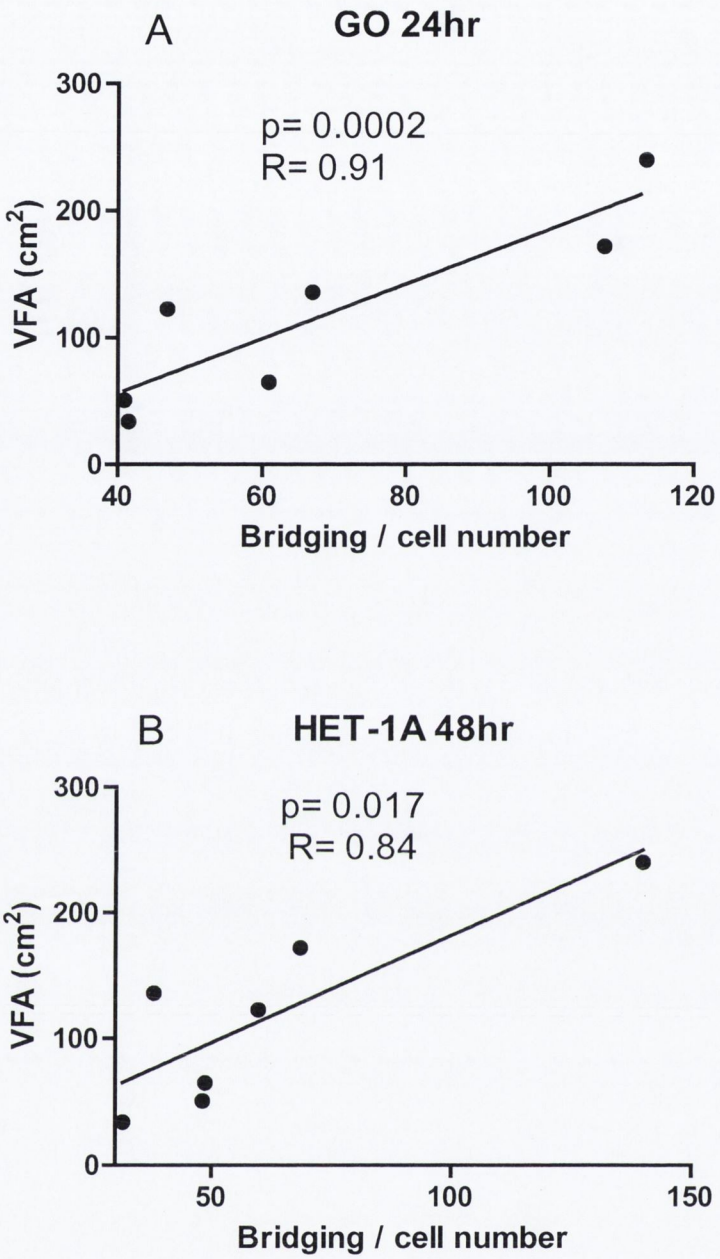

Figure 6.9. Patient VFA correlates significantly with anaphase bridging in the GO cell line following 24hr ACM (n=7) treatment and the HET-1A cell line following 48hr ACM treatment (n=7). Statistical analysis was carried out using linear regression analysis.

6.3.2 Anaphase bridging correlates with expression of telomeric chromatin

Expression of telomere binding proteins examined in chapter 5 were correlated with levels of anaphase bridging in the HET-1A, QH, GO and OE33 cell lines following 24hr and 48hr ACM treatments (Figure 6.10).

Significant correlations were also demonstrated between anaphase bridging and expression of TERT and TERC (Figure 6.10A-C). Following 24hr ACM treatment, TERT expression correlated significantly with anaphase bridging in the GO cell line ($p=0.002$, $R=0.83$) and TERC expression correlated significantly with anaphase bridging in the OE33 cell line ($p=0.02$, $R=0.68$). Following 48hr ACM treatment, TERC expression correlated significantly with anaphase bridging in the HET-1A cell line ($p=0.01$, $R=0.79$)

Significant correlations were demonstrated between DKC1 expression and anaphase bridging in the cells lines following 24hr ACM treatment (Figure 6.10D-E). DKC1 expression correlated with anaphase bridging in both the GO cell line ($p=0.005$, $R=0.79$) and the OE33 cell line ($p=0.04$, $R=0.79$).

A significant correlation was also demonstrated between expression of TERF2IP and anaphase bridging in the OE33 cell line following 24hr ACM treatment ($p=0.04$, $R=0.66$) (Figure 6.10F).

No correlation was observed between telomere length and anaphase bridging in the cell lines at either time point.

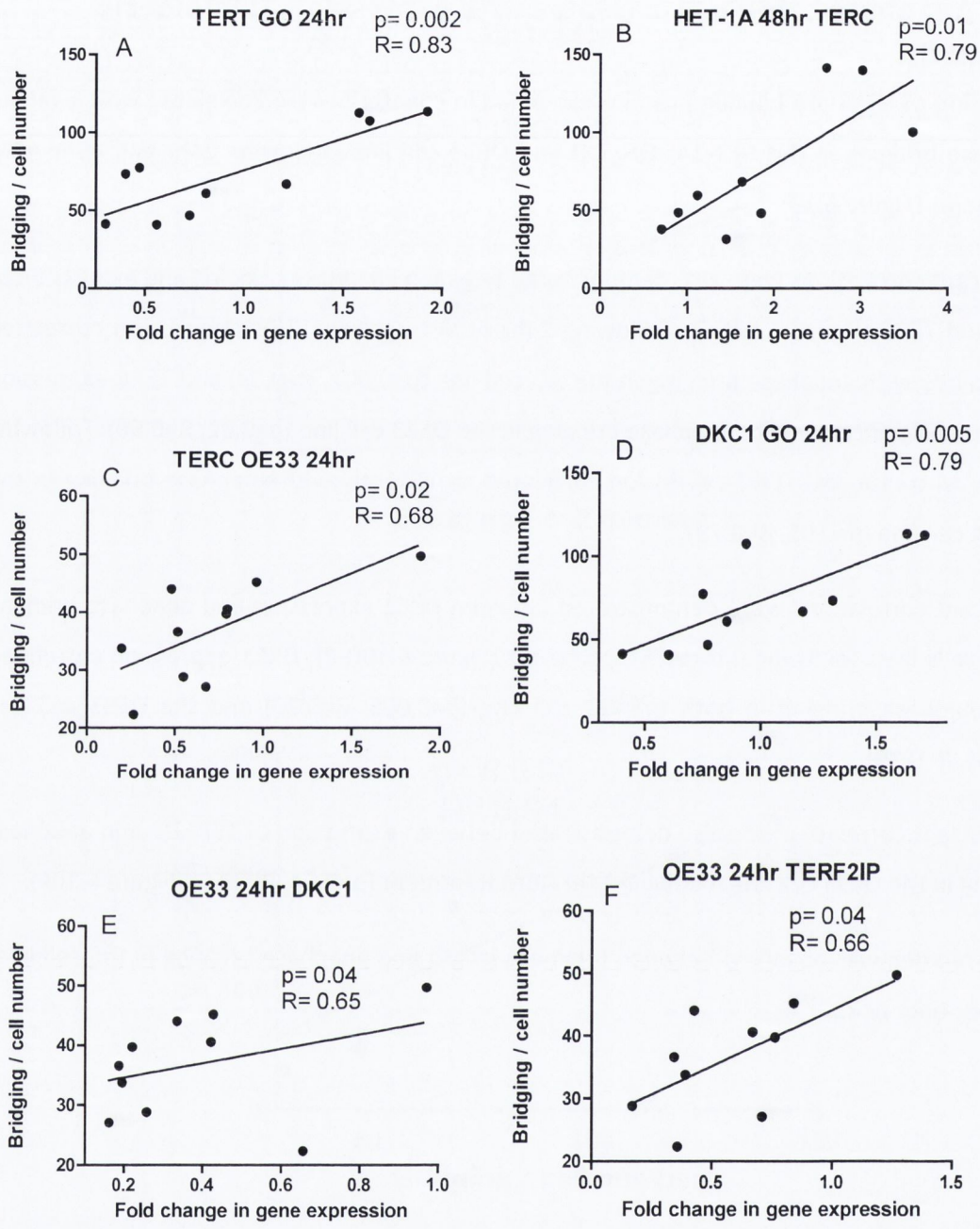

Figure 6.10. Telomerase complex expression correlates significantly with anaphase bridging following 24hr (n=10) and 48hr ACM (n=10) treatment, in the oesophageal cell lines. Statistical analysis was carried out using linear regression analysis.

6.3.3 Neutralising VEGF and IL-8 in ACM significantly down regulates anaphase bridging in HET-1A cells following ACM treatment

In chapter 3 it was established that IL-8 is significantly up-regulated in a Barrett's oesophagus cell model following ACM treatment, and that Barrett's metaplasia biopsies from obese patients express significantly higher levels of IL-8 compared to non-obese patients. Also in chapter 3, it was established that VEGF is one of the factors abundantly expressed in ACM, and is significantly up- regulated in a Barrett's oesophagus cell model following ACM treatment. We therefore wanted to investigate whether these two factors where important in driving anaphase bridge formation. These two factors were neutralised in the ACM to functionally examine if these factors promote anaphase bridging in the HET-1A cell line. The HET-1A cell line was selected to investigate the effect of VEGF and IL-8 neutralisation in ACM because this cell line showed to greatest levels of anaphase bridging following 24hr ACM treatment. As we hypothesise that anaphase bridging are a driving force in neoplastic progression it would be of interest to assess if we could decrease anaphase bridging in the normal squamous epithelia stage of the Barrett's sequence, represented by the HET-1A cell line.

Protein concentrations of IL-8 and VEGF in ACM generated from viscerally obese and non-obese oesophagectomy patients were assessed. Levels of VEGF were significantly higher ($p=0.035$) in ACM generated from viscerally obese patients than levels present in ACM from non-obese patients (Figure 6.11A). IL-8 levels were increased in ACM generated from viscerally obese patients compared to levels present in ACM from non-obese patients though these levels did not achieve statistical significance ($p=0.08$) (Figure 6.11B).

Neutralizing antibodies were used to deplete VEGF or IL-8 from the ACM, in order to determine their contribution to anaphase bridging. HET-1A cells were treated with M199 media alone, M199 media with neutralizing antibodies, or ACM treated with neutralizing antibodies for 24 hours, after which time anaphase bridge formation was assessed.

Neutralisation of VEGF in the ACM significantly down regulated anaphase bridging compared to matched ACM without VEGF neutralisation ($p=0.007$). Anaphase bridging levels were significantly decreased to levels observed in the untreated controls (Figure 6.11C). A similar result was demonstrated following IL-8 neutralisation of the ACM, with levels of anaphase bridging significantly down regulated ($p=0.001$) compared to matched ACM without IL-8 neutralisation, with anaphase bridging levels significantly decreased to levels observed in the untreated controls (Figure 6.11D).

Levels of anaphase bridging in response to both obese and non-obese ACM in the presence of neutralising VEGF and IL-8 antibodies was also assessed however, obese ACM did not demonstrate significantly more bridging that non obese ACM, following both VEGF and IL-8 neutralisation (Figure 6.11E and Figure 6.11F).

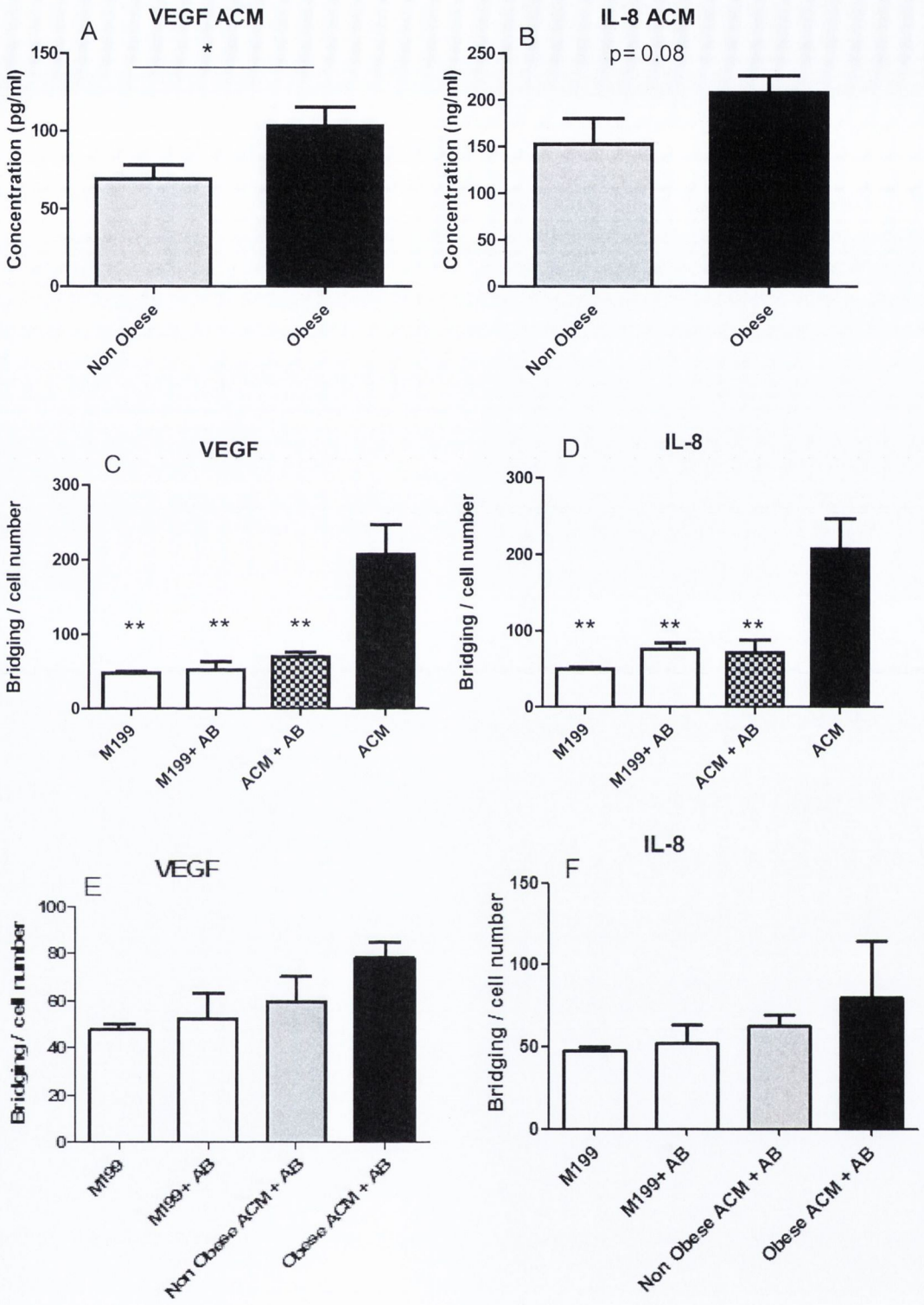

Figure 6.11. Neutralising VEGF and IL-8 in ACM significantly down regulates anaphase bridging in HET-1A cell line (n=10) following 24hr ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

6.3.4 Spindle assembly checkpoint family member gene expression is dysregulated by ACM

Expression of the SAC markers was investigated following ACM treatment in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 6.12).

Following 24hr ACM treatment (Figure 6.12A), BUB1B showed no changes in expression in the HET-1A cell line, however, there was a significant decrease in the expression of this SAC family member in the QH ($p=0.003$), GO ($p=0.002$) and OE33 ($p=0.002$) cell lines, compared to HET-1A, following identical ACM treatments. CDC20 displayed a similar pattern of expression following an identical 24hr ACM treatment in the oesophageal cell lines (Figure 6.12B). While there was little change in CDC20 expression in the HET-1A cell line, the QH ($p=0.009$), GO ($p=0.003$) and OE33 ($p=0.0001$) cell lines demonstrated significant decreases in CDC20 expression, compared to the HET-1A cell line. Similarly, CENPE expression showed no change in expression in the HET-1A cell line following a 24hr ACM treatment, and a 24hr ACM treatment in the QH ($p=0.0006$), GO ($p=0.001$) and OE33 ($p=0.0007$) cell lines, displayed significant decreases in CENPE expression compared to the HET-1A cell line (Figure 6.12C). A 24hr ACM treatment elicited no change in ESPL1 expression in the HET-1A cell line, however a 24hr ACM treatment in the QH ($p=0.004$), GO ($p=0.001$) and OE33 ($p=0.032$) cell lines (Figure 6.12D) caused significant decreases in CENPE expression compared to the HET-1A cell line. MAD2L2 demonstrated no increase in expression in the HET-1A cell line following 24hr ACM treatment, and while there was no change in expression in the GO and OE33 cell lines, MAD2L2 expression was significantly down regulated ($p=0.013$) in the QH cell line compared to the HET-1A cell line (Figure 6.12E).

Following 48hr ACM treatment (Figure 6.13), the QH cell line displayed 3 fold increase in BUB1B gene expression, representing a significant increase compared to the HET-1A ($p<0.0001$), GO ($p=0.009$) and OE33 ($p<0.0001$) cell lines (Figure 6.13A). The GO cell line also displayed significantly higher expression levels ($p<0.0001$) of BUB1B expression compared to the OE33 cell line following ACM treatment CDC20 expression has highest in the GO cell line following 48hr ACM treatment (Figure 6.13B). The GO cell line displayed significantly higher CDC20 expression than the HET-1A ($p=0.008$), QH ($p=0.045$) and OE33 ($p=0.029$) cell lines. CDC20 expression was also significantly higher in the QH cell line compared to the HET-1A cell line following ACM treatment ($p=0.0008$). Expression of CENPE was highest in the QH cell line following 48hr ACM treatment (Figure 6.13C), with significantly higher levels compared to the HET-1A ($p=0.0001$), GO ($p=0.0005$) and OE33 ($p=0.011$) cell lines. The QH cell line displayed significantly higher levels of ESPL1 (Figure 6.12D) compared to both the HET-1A ($p<0.0001$) and

OE33 ($p<0.0001$) cell lines following 48hr ACM treatment. The GO also cell line expressed significantly higher levels of ESPL1 compared to both the HET-1A ($p<0.0001$) and OE33 ($p<0.0001$) cell lines. Following 48hr ACM treatment MAD2L2 expression was significantly higher in the QH cell line (Figure 6.13E) compared to the HET-1A ($p<0.0001$), GO ($p=0.001$) and OE33 ($p=0.006$) cell lines.

Expression of the SAC markers at 24hr and 48hr were compared for all cell lines (Figure 6.14). BUB1B expression was significantly higher following 48hr ACM treatment compared to 24hr ACM treatment in the QH ($p<0.0001$), GO ($p<0.0001$) and OE33 ($p=0.043$) cell lines (Figure 6.14A). BUB1B expression was however significantly higher in the HET-1A cell line following 24hr ACM treatment ($p=0.004$), compared to a matched 48hr ACM treatment. CDC20 displayed a similar pattern of expression with levels higher following 48hr treatment in the QH, GO and OE33 cell lines (Figure 6.14B), significance was detected in the QH ($p=0.029$) and GO ($p=0.002$) cell lines. Expression of CDC20 was significantly higher ($p<0.0001$) following 24hr ACM treatment in the HET-1A cell line compared to an identical 24hr treatment. CENPE expression was significantly higher following 48hr ACM treatment in the QH ($p<0.0001$), GO ($p<0.0001$) and OE33 ($p<0.0001$) cell lines compared to a 24hr ACM treatment (Figure 6.14C). Again, expression of this SAC marker was significantly higher ($p<0.0001$) following 24hr ACM treatment compared to a 48hr ACM treatment. Expression of ESPL1 was significantly higher following 48hr ACM treatment compared to a 24hr treatment in the QH ($p<0.0001$) and GO ($p<0.0001$) cell lines (Figure 6.14D). ESPL1 was also higher following 48hr ACM treatment in the GO cell line but levels failed to reach statistical significance. Significantly higher levels of ESPL1 were demonstrated in the HET-1A cell line following 24hr ACM treatment compared to a matched 48hr treatment ($p<0.0001$). Finally, MAD2L2 expression was significantly higher following 48hr ACM treatment compared to a 24hr treatment in the QH ($p<0.0001$) and GO ($p<0.0001$) cell lines (Figure 6.14E), with levels failing to reach statistical significance in the OE33 cell line. MAD2L2 levels were also significantly higher following 24hr ACM treatment in the HET-1A cell line, compared to a 48hr ACM treatment ($p=0.011$).

Expression of the SAC markers was also compared in response to treatment with ACM generated from both obese and non-obese patients in all oesophageal cell lines at both 24hr and 48hr time points. There was no significant difference in SAC gene expression observed in any of the oesophageal cell lines following treatment with ACM generated from obese and non-obese patients following a 24hr treatment, however there was significance observed following a 48hr treatment (Figure 6.15). CDC20 expression was significantly lower in the HET-1A cell line following culture in ACM generated from obese patients, compared to non-obese

patients (p=0.026). Similarly CENPE expression in the HET-1A cell line was significantly lower following 48hr culture with ACM generated from obese patients compared to non-obese patients (p=0.002), however a 48hr ACM treatment in the QH cell line demonstrated significantly higher levels in ACM generated from obese patients compared to non-obese patients (p=0.002). All other genes showed no difference in gene expression elicited by non-obese and obese ACM following 24hr and 48hr ACM treatment.

Significant correlations were observed between expression of SAC members and anaphase bridging early in metaplastic-dysplastic-adenocarcinoma sequence following 48hr ACM treatment (Figure 6.16). BUB1B expression demonstrated a significant inverse correlation with anaphase bridging in the HET-1A cell line following 48hr ACM treatment (p=0.03, R=0.74). In the same cell line, CENPE expression displayed a significant inverse correlation with anaphase bridging (p=0.019, R=0.75). CENPE also demonstrated a significant inverse correlation with anaphase bridging in the QH cell line following 28hr ACM treatment (p=0.02, R=0.71).

Patient waist cirumference was correlated with SAC gene expression in the oesophageal cell lines following 24hr and 48hr ACM treatment (Figure 6.17). In the QH cell line, following 24hr ACM treatment, significant correlations were observed between patient waist circumference and BUB1B (p=0.05, R=0.63) and ESPL1 (p=0.04, R=0.65) gene expression. In the HET-1A cell line, following 48hr ACM treatment, there was a significant inverse correlations demonstrated between BUB1B (p=0.04, R=0.64), CDC20 (p=0.03, R=0.67) and CENPE (p=0.04, R=0.64). Patient VFA or BMI did not correlate with SAC gene expression in the oesophageal cell lines.

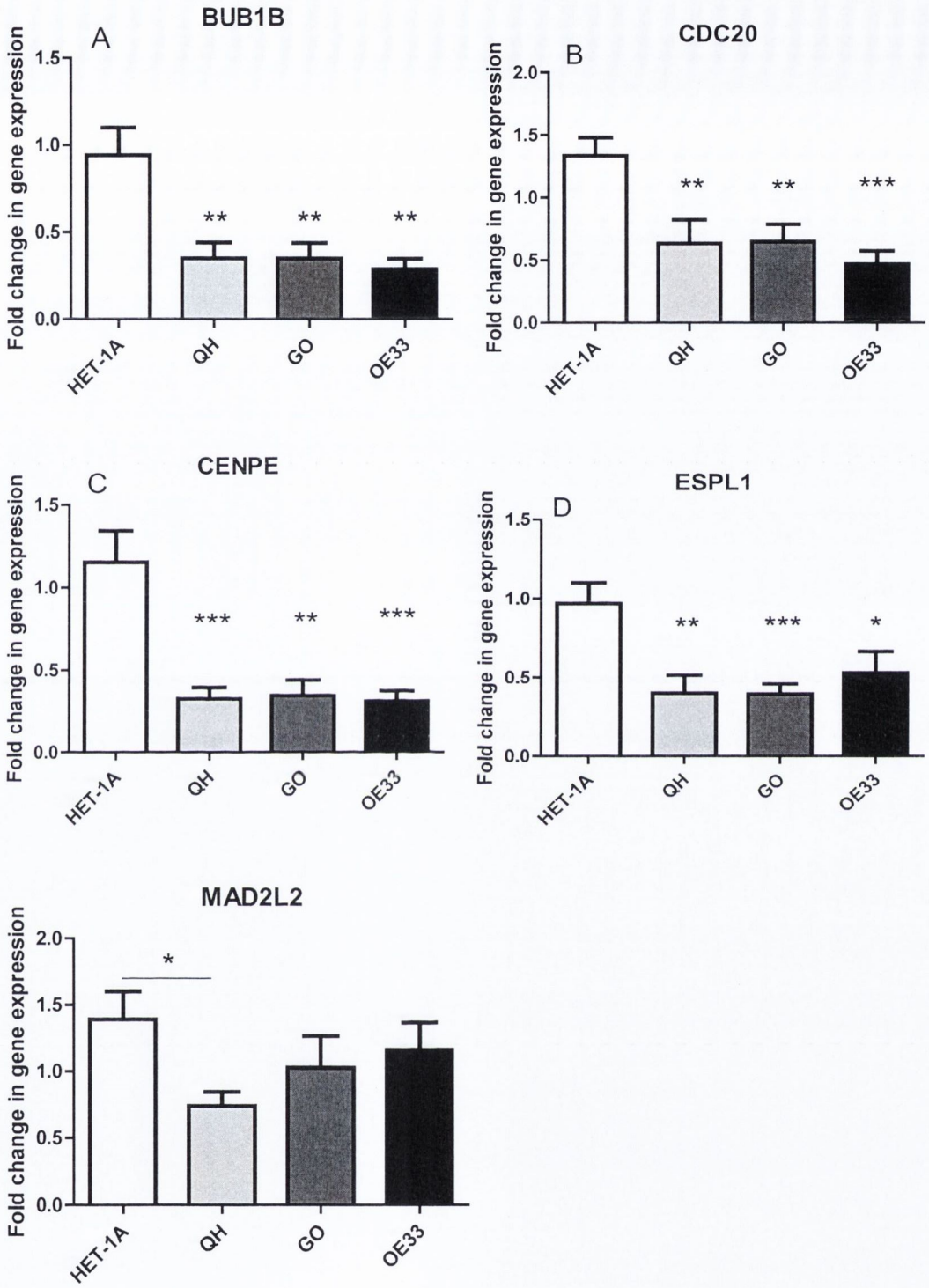

Figure 6.12. Expression of SAC family members in the oesophageal cell lines following 24hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

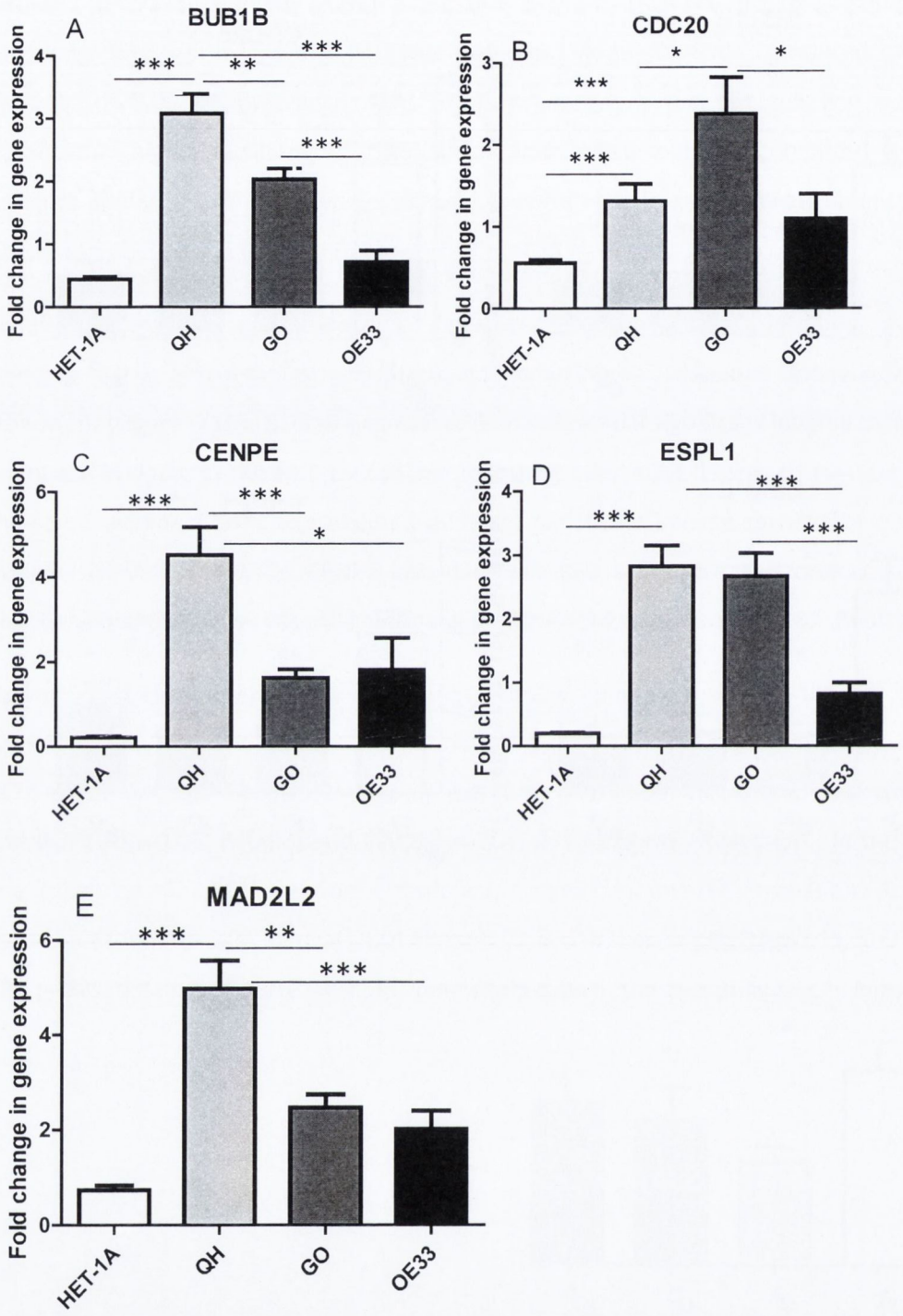

Figure 6.13. Expression of SAC family members in the oesophageal cell lines following 48hr ACM treatment (n=10). Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

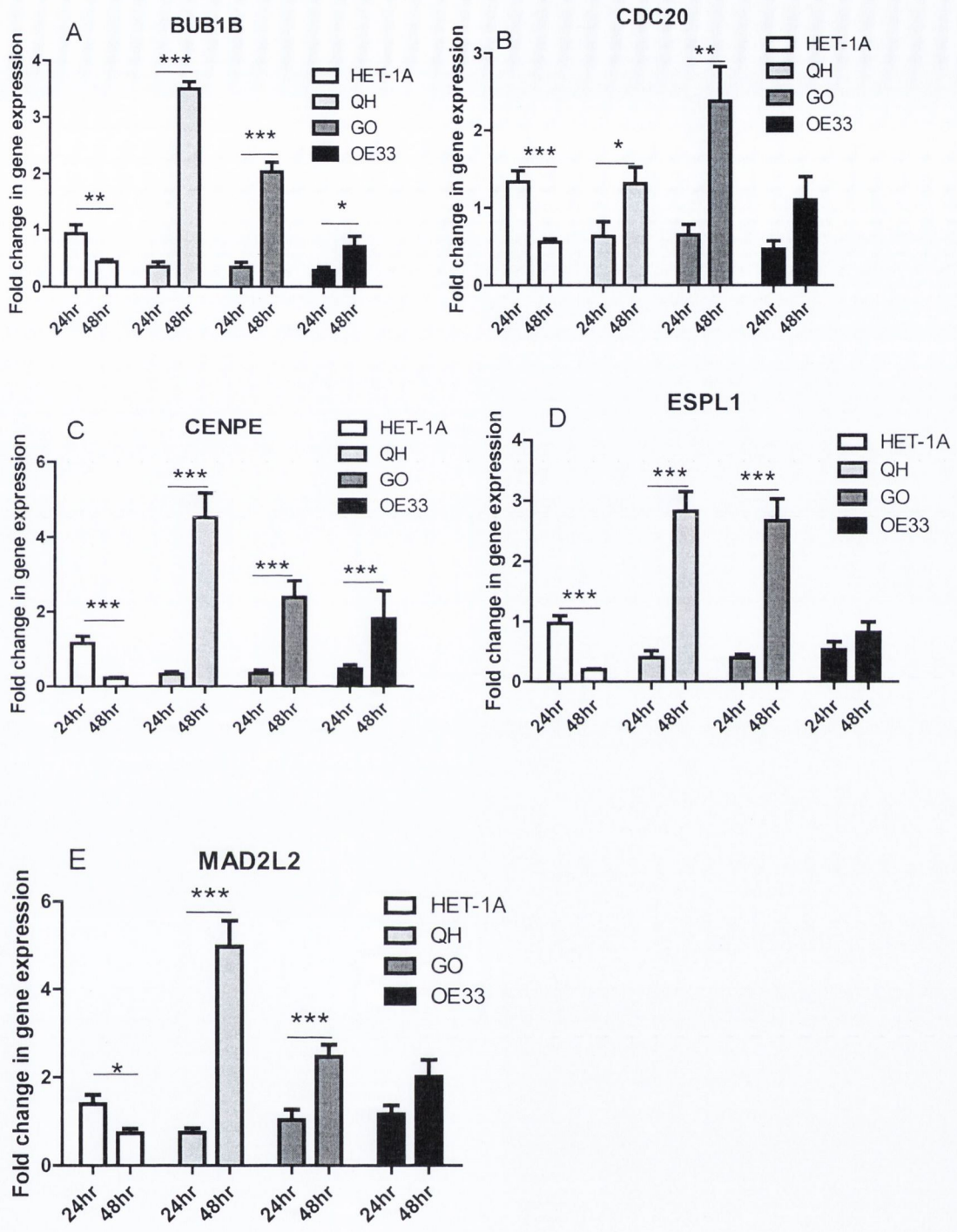

Figure 6.14. Comparison of expression of SAC family members in the oesophageal cell lines following 24hr (n=10) and 48hr (n=10) ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001).

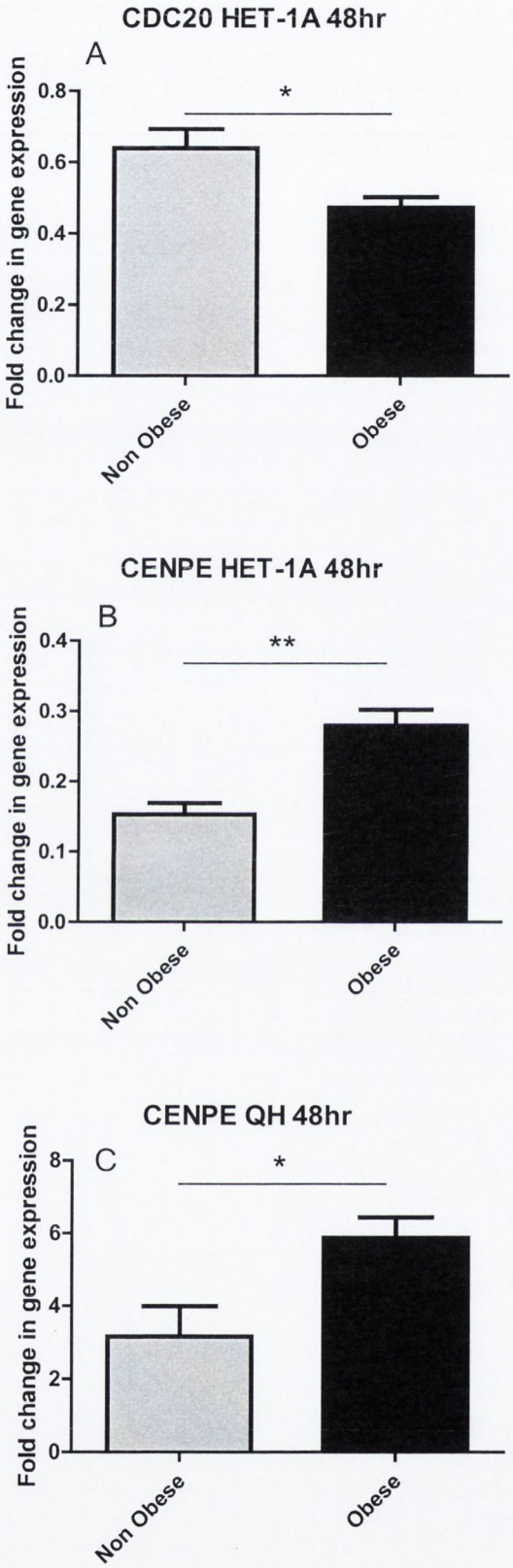

Figure 6.15. Expression of CDC20 and CENPE in response to ACM generated from viscerally obese (n=5) and non-obese patients (n=5) following 48hr ACM treatment. Data are expressed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test (*$p<0.05$, **$p<0.01$).

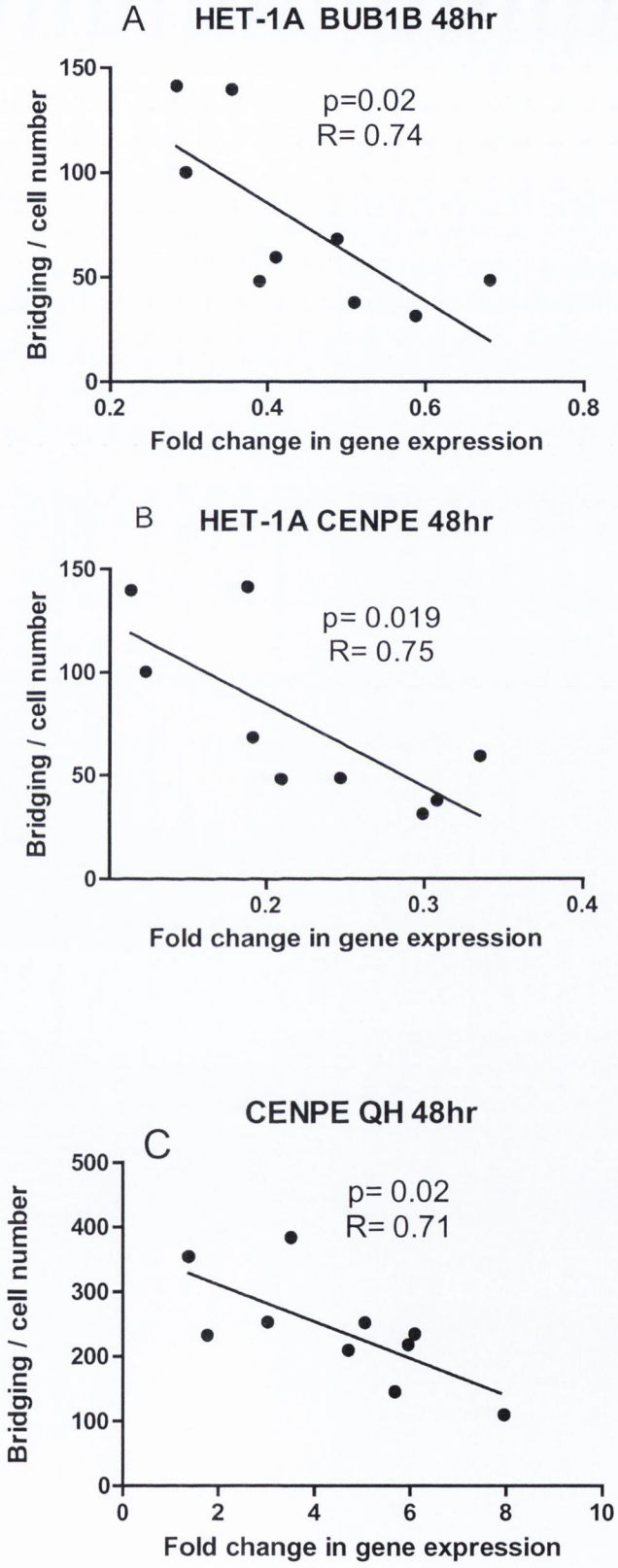

Figure 6.16. Anaphase bridging correlates with expression of members of the SAC following 48hr ACM treatment (n=10) in HET-1A and QH cell lines. Statistical analysis was carried out using linear regression analysis.

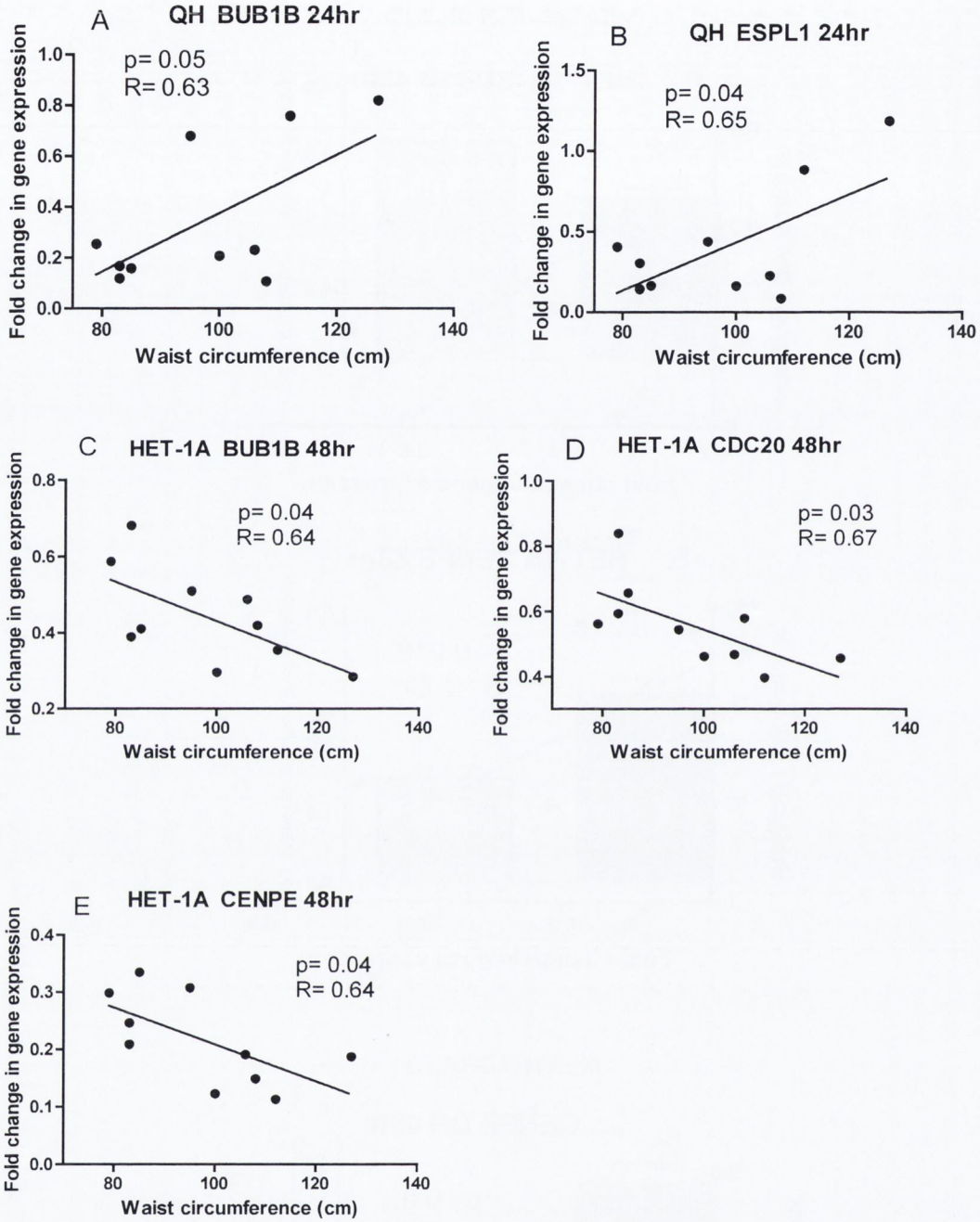

Figure 6.17. Patient ACM waist circumference correlates with SAC family gene expression in the oesophageal cell lines following 24hr and 48hr ACM treatment (n=10). Statistical analysis was carried out using linear regression analysis.

6.4 Discussion

The work presented in this chapter demonstrates for the first time a mechanism by which obesity can drive genomic instability in Barrett's oesophagus through anaphase bridging and dysregulation of the SAC complex proteins. Anaphase bridging, a functional marker of genomic instability (Boukamp, Popp et al. 2005) was increased in all each cell line representing the metaplasia-dysplasia-adenocarcinoma sequence following ACM treatment. Extensive genomic instability during premalignant neoplastic progression has been demonstrated in Barrett's oesophagus with loss of heterozygosity of CDKN2A and TP53 genes as early events in neoplastic progression (Lai, Paulson et al. 2007). Obesity has been shown to drive carcinogenesis previously (Bianchini, Kaaks et al. 2002) and has also been implicated with Barrett's oesophagus (Merry, Schouten et al. 2007; Corley, Kubo et al. 2008; Ryan, Healy et al. 2008).

Following 24hr ACM treatment, anaphase bridging was significantly increased in the HET-1A cell line compared to the other cell lines. The enhanced sensitivity of the HET-1A cells to the ACM indicates that this normal oesophageal squamous epithelium maybe most susceptible to obesity driven genomic instability events. Genomic instability events initiated by obesity in squamous columnar epithelium may be involved in the squamous-columnar transition associated with the onset of Barrett's oesophagus and these same events may also be involved in the neoplastic progression of Barrett's oesophagus. In our study, the QH and GO cell lines, which represent metaplastic and dysplastic Barrett's oesophagus respectively, show significant increases in anaphase bridging following 48hr ACM treatment. Carcinogenesis arising in dysplasia due to genomic instability has been observed in ulcerative colitis; losses of chromosome 18 or 18q and chromosome 5 or 5q in patient biopsies were shown to be common to both dysplastic ulcerative colitis and colorectal cancer (Willenbucher, Aust et al. 1999). A similar mechanism to the one reported by Willenbucher *et al* may play a role in the neoplastic progression of dysplastic Barrett's oesophagus. Chromosomal losses at 17p and 9p and chromosomal gains at 11q have been shown to correlate with telomere shortening in Barrett's oesophagus and may be involved in the early stages of Barrett's oesophagus neoplasia (Finley, Reid et al. 2006).

Anaphase bridging in the HET-1A cell line was significantly higher in response to obese ACM compared to non-obese ACM. Sensitivity of the cellular machinery that regulate genomic stability in the HET-1A cell line to factors such as adiponectin and leptin may explain this result. Significant correlations were observed between patient waist circumference and the levels of

anaphase bridging elicited by the ACM in the HET-1A, QH and GO cell lines. These data suggest that increased viscerally adiposity in patients is associated increased genomic instability. These correlations are more pronounced in the HET-1A cells further suggesting that obesity mediated genomic instability may be an early event in the Barrett's oesophagus progressive sequence. Increased genomic instability has been correlated with obesity status previously in other studies; women with polycystic ovary syndrome displayed increased genomic instability characterised by a significantly higher number of bi-nucleated lymphocytes, and this increase was related to an obese phenotype (Moran, Noakes et al. 2008). Increased genomic stability and subsequent chromosomal rearrangement due to anaphase bridging elicited by obesity observed in this chapter represents a novel mechanism by which oesophageal adenocarcinoma may arise in Barrett's oesophagus.

Significant correlations were demonstrated between expression of telomeric chromatin and anaphase bridging following 24hr ACM treatment in the cell lines. DKC1 expression significantly correlated with anaphase bridging in the GO and OE33 cell lines following 24hr ACM treatment. Decreases in DKC1 expression are known to drive telomere shortening in X-linked dyskeratosis congenital (Parry, Alder et al. 2011). Decreased telomere lengths would be conducive to increased anaphase bridging due to the initiation of breakage-fusion-bridge cycles (Lo, Sabatier et al. 2002). TERC expression significantly correlated with levels of anaphase bridging in the OE33 cell line following 24hr ACM treatment and following 48hr ACM treatment in the HET-1A cell line. TERC and DKC1 regulate telomere length (Martinez and Blasco 2011) and increases in expression of these proteins would be expected to correlate to increased telomere length and decreased anaphase bridging. However our study has shown that in the cell lines representing the metaplasia-dysplasia-adenocarcinoma sequence, telomere shortening in response to ACM is independent of expression of telomerase complex proteins. Overexpression of shelterin proteins have been shown to be negative regulators of telomere length due to the inability of the telomerase complex to bind to the proximal ends of telomeres (de Lange 2005) and this mechanism may play a role in telomere shortening and anaphase bridging in Barrett's derived adenocarcinoma. Telomere maintenance independent of the telomerase complex has been reported in the literature (Stewart 2005). This alternative form of telomere maintenance is termed the ALT mechanism and accounts for 7-10% of telomere maintenance in carcinogenesis (Stewart 2005). Due to dysregulations in telomere length independent of telomerase complex expression in our study, it is likely that the ALT mechanism is involved in telomere maintenance and subsequent breakage fusion bridging events in the cell lines following ACM treatment.

We have previously shown that VEGF neutralisation of ACM leads to significant decreases in cancer cell proliferation (Lysaght, van der Stok et al. 2011) demonstrating the pro-tumourigenic capacity of VEGF in ACM. Neutralisation of IL-8 has also been associated with inhibition of angiogenesis and metastasis, two well-defined hallmarks of cancer, in melanoma cells (Huang, Mills et al. 2002). Neutralising both VEGF and IL-8 in the ACM significantly decreased anaphase bridging in the HET-1A cell line to levels similar to the untreated control suggesting that these factors may be critical component of visceral adipose tissue secretome in driving genomic and chromosomal instability. Sensitivity of the HET-1A cells to these adipose derived factors may explain why greatest anaphase bridging is observed in this cell line compared to the others in response to ACM treatment. A relationship between these two potent angiogenic factors has been demonstrated previously in the literature; IL-8 and VEGF share a common NF-κB mediated pathway of activation (Bancroft, Chen et al. 2001), while IL-8 has been shown to stimulate VEGF production and the activation of VEGF receptors through NF-κB (Martin, Galisteo et al. 2009). Pro-inflammatory molecules such as IL-8 have been shown to play important roles in genomic instability and cancer previously (Colotta, Allavena et al. 2009), while VEGF has been associated with increased angiogenesis and genomic instability in breast cancer (Mironchik, Winnard et al. 2005).

The differences in levels of anaphase bridging in the cell lines at 24hr compared to 48hr may be explained by the expression of key SAC genes at these time points. Dysregulation of this pathway has been implicated in carcinogenesis previously with both over expression (Sotillo, Hernando et al. 2007; Ando, Kakeji et al. 2010) and under expression (Musio, Montagna et al. 2003) of SAC genes affecting neoplastic events. Members of the SAC have also been correlated with anaphase bridging previously in cancer studies; depletion of MAD2 increases levels of anaphase bridging in the MCF-7 breast cancer cell line (Prencipe, Fitzpatrick et al. 2009). Following a 24hr ACM treatment, the fold changes in the expression of SAC genes are not significantly different from the control in the HET-1A cell line, but are significantly decreased in the QH, GO and OE33 cell lines, cell lines which display low levels of anaphase bridging at this time point. Following 48hr ACM treatment, expression of SAC genes reduces significantly in tandem with a significant decrease in bridging in the HET-1A cell line while the QH, GO and OE33 cell lines demonstrate an increase in SAC expression and an associated increase in anaphase bridging. Decreased expression of SAC markers in response to ACM confers genomic stability in the oesophageal cell lines, represented by lower anaphase bridging levels. Our study is the first to demonstrate the role that obesity plays in SAC regulation and genomic instability in a Barrett's oesophagus model. Over expression of SAC genes (Sotillo, Hernando et

al. 2007; Ando, Kakeji et al. 2010) has been associated with neoplastic events previously and prolonged exposure of patients suffering from metaplastic Barrett's oesophagus and high grade dysplasia to adipose derived factors, may drive increased SAC expression leading to genomic instability and carcinogenesis arising in Barrett's oesophagus.

Expression of CDC20 in the HET-1A cell line also significantly inversely correlated with patient waist circumference at 48hr ACM treatment. Significant decreases in CDC20 expression were also observed in the HET-1A cell line following 48hr treatment with ACM derived from obese patients, and this decrease in CDC20 expression corresponded to a significant increase in anaphase bridging in the cells treated with obese ACM. This gene is the overall target of the SAC (Musacchio and Salmon 2007), and inhibition of this gene has been proposed as an important tumour suppressing mechanism (Li, Fang et al. 2009), the SAC also negatively regulates the stability of CDC20 by targeting it for proteasome-dependent degradation (Ge, Skaar et al. 2009). This relationship between CDC20 expression and genomic instability was observed in the oesophageal cell lines following ACM treatment, with greatest levels of genomic stability occurring in the oesophageal cell lines when CDC20 expression is decreased. Correlations with patient waist circumference and SAC marker gene expression levels were observed in the cell lines in pre neoplastic states. At 24hr, expression of SAC markers BUB1B and ESPL1 correlated significantly with patient ACM waist circumference in the metaplastic QH Barrett's oesophagus cell line, while at 48hr, significant correlations were observed between gene expression of BUB1B, CDC20, and CENPE with ACM waist circumference in the HET-1A cell line. Adipose derived factors may be deregulating this essential mechanism that ensures the fidelity of chromosomal replication during mitosis (Musacchio and Salmon 2007), due to cells entering into anaphase before all kinetochores are attached and chromosomal alignment is complete, and this mechanism may be at play in the pathogenesis of Barrett's oesophagus.

CENPE showed significant increases in expression in response to ACM generated from obese patients, compared to non-obese patients, in the HET-1A and QH cell lines following 48hr ACM treatment. Deletion of CENPE has been shown to induce genomic instability in a murine model (Putkey, Cramer et al. 2002), however in the work presented in this chapter, an increase in CENPE expression following treatment with obese ACM compared to non-obese ACM, coincided with an increase in genomic instability in the HET-1A cell line, and no change in the QH cell line. Lung adenocarcinomas and squamous cell carcinomas show 5-fold and 20-fold increases in expression over normal lung tissue, respectively, indicating that increases in CENPE expression due to obesity, similar to those demonstrated in the QH cell lines following 48hr ACM treatment, may be associated with carcinogenesis (Wood, Chua et al. 2008).

Other cell checkpoint proteins besides SAC have been demonstrated to play a role in genomic instability. Mutations in well-established tumour suppressor gene p53 (Lane 1992) have been linked to genomic instability in the literature (Kahlenberg, Stoler et al. 1996). p53 has been directly associated with expression of SAC genes CDC20 (Roychoudhury, Banerjee et al. 2009) and MAD2L2 (Li, Li et al. 2003) previously; p53 inhibits tumour cell growth through the indirect regulation of CDC20 (Kidokoro, Tanikawa et al. 2008) and MAD2L2 (Benezra, Schvartzman et al. 2011). With mutations of the p53 protein reported along the metaplasia-dysplasia-adenocarcinoma sequence (Schneider, Casson et al. 1996), unchecked CDC20/MAD2L2 mediated genomic instability and tumour proliferation in the absence of a functional p53 protein may be a mechanism by which oesophageal adenocarcinoma arises in Barrett's oesophagus. In chapter 3 ACM treatment of the BAR-T cell line and investigation of gene and pathway changes via the Human cancer profiler qPCR array identified 4 genes related to cell cycle control deregulated by obesity; BRCA1, CCNE1, CDKN2A and RB1 (table 3.2). BRCA1 was down regulated in the BAR-T cell line following ACM treatment and this tumour suppressor gene is essential for functional spindle assembly checkpoint activation in mouse oocytes (Xiong, Li et al. 2008), disruption of BRCA1 functioning has also been shown to increase anaphase bridge formation in the MCF-7 breast cancer cell line (French, Dunn et al. 2006). Loss of BRCA1 expression by obesity in Barrett's oesophagus may lead to a compromised SAC and anaphase bridge formation which are conducive to aneuploidy and carcinogenesis. Over expression of CENPA, a related centromere protein of CENPE, has been associated with an increase in genomic stability a process controlled by RB1 (Amato, Schillaci et al. 2009). Loss of this cell cycle control has been shown to promote aneuploidy mainly through the induction of CENPA expression affecting the correct attachment of spindle microtubules to kinetochores (Amato, Schillaci et al. 2009). Dysregulations in RB1 expression were demonstrated in the BAR-T cell line following ACM treatment in chapter 3, and loss of expression in tandem with an increase in CENPA expression may be involved in driving genomic instability in Barrett's oesophagus.

In conclusion, by replicating an obese microenvironment in-vitro via ACM treatment in a panel of oesophageal cell lines, genomic instability (characterised by anaphase bridging and SAC dysregulation) was initiated at the different stages of the metaplasia-dysplasia-adenocarcinoma sequence. The non-neoplastic HET-1A cell line exhibited the greatest levels of anaphase bridging following 24hr ACM treatment and we have shown that neutralising IL-8 and VEGF levels in the ACM significantly decreased anaphase bridging in the HET-1A cell line suggesting they are key components of the adipose tissue secretome involved in regulating

genomic instability. The SAC is essential for cell viability (Kops, Weaver et al. 2005) and while dysregulation of the SAC represents a route to genomic instability and carcinogenesis in the oesophageal cell lines, pharmacologic abrogation of the SAC has been proposed as a novel promising strategy for anticancer treatment. Indolocarbazole compound Gö6976 potently overrides the spindle checkpoint–mediated mitotic arrest by abrogating the phosphorylation and kinetochore localization of several spindle checkpoint proteins, resulting in an aberrant progression of mitosis, leading to apoptosis in various human cancer cell lines (Stolz, Vogel et al. 2009). Targeting high grade dysplastic Barrett's oesophagus tissue via this mechanism may represent a viable treatment for patients at increased risk of carcinogenesis. Obesity has long been associated with carcinogenesis arising in Barrett's oesophagus without the exact molecular mechanisms fully understood. Activation of genomic instability by excess adiposity in Barrett's oesophagus patients, leading to loss of an efficient spindle assembly checkpoint and chromosome remodelling due to anaphase bridging may represent the driving forces in the neoplastic progression of Barrett's oesophagus.

General Discussion

7.1 General discussion

Incidences of obesity in the developed world represent a worrying epidemic (Hjartaker, Langseth et al. 2008). Obesity is associated with increased rates of diabetes, cardiovascular disease and cancer. Oesophageal adenocarcinoma rates have increased in the last 25 years hand in hand with obesity, and clear correlations between the two have been demonstrated previously (Ryan, Rowley et al. 2006; Corley 2007). A major risk factor for oesophageal adenocarcinoma is Barrett's oesophagus, a consequence of prolonged exposure to GORD. However, only a fraction of Barrett's patients with prolonged GORD will ultimately progress to oesophageal adenocarcinoma indicating that there must be other factors at play in the neoplastic progression of Barrett's oesophagus independent of GORD (Edelstein, Farrow et al. 2007). The aim of this thesis was to identify pro-carcinogenic pathways activated by obesity in Barrett's oesophagus independent of GORD.

Following ACM treatment, the HET-1A, QH, GO and OE33 cell lines, which represent the metaplasia-dysplasia-adenocarcinoma sequence associated with the neoplastic progression of Barrett's oesophagus, each demonstrated aberrant cellular dysfunction conducive to tumourigenesis following ACM treatment. The HET-1A and BAR-T/QH cell lines demonstrated the greatest amount of cellular dysfunction following treatment with adipose conditioned media generated from both obese and non-obese oesophogectomy patients indicating that the pro-tumourigenic pathways activated by obesity in the metaplasia-dysplasia-adenocarcinoma sequence are an early event in the disease sequence. The pro-tumourigenic cellular dysfunctions observed in the panel of oesophageal cell lines following ACM treatment are displayed below in Figure 7.1

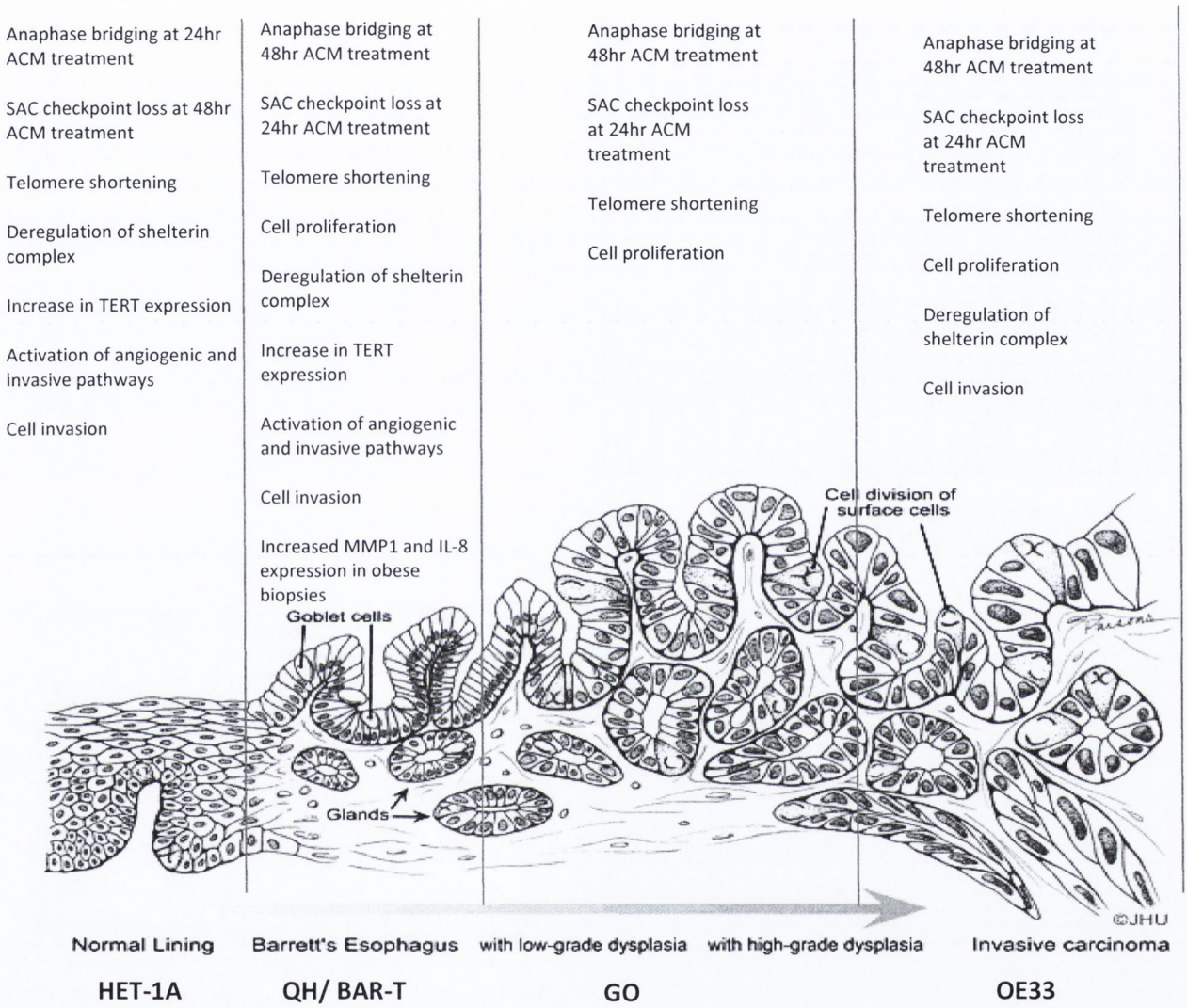

Figure 7.1. Pro-tumourigenic cellular dysfunction elicited by ACM treatment in panel of oesophageal cell lines representing the metaplasia-dysplasia-adenocarcinoma associated with the neoplastic progression of Barrett's oesophagus

HET-1A/ Normal oesophageal squamous epithelium

Upon treatment with ACM the HET-1A cell line demonstrated the greatest expression of MMP-1, TIMP-3, IL-8, and VEGF gene expression of all the cell lines used in this study. Early increases in expression of cancer related genes in the epithelia of pre-neoplastic conditions has been demonstrated previously in ovarian (Choi, Auersperg et al. 2003) and colorectal cancer (Kou, Marusawa et al. 2007). In human ovarian squamous epithelia, cells activate MAPK and

proliferate in response to extracellular calcium (Wright, Toth-Fejel et al. 2002) while in colonic epithelial cells proinflammatory cytokine-mediated aberrant expression of activation-induced cytidine deaminase is a genotoxic factor linking inflammation, somatic mutations, and colorectal cancer development (Endo, Marusawa et al. 2008).

ACM treatment altered pro tumour pathways in the squamous epithelial HET-1A cell line as determined by the human cancer profiler array. Molecular pathways related to angiogenesis, and invasion and metastasis showed the greatest dysregulation in the HET-1A cell line, with these two pathways directly related to the cancer hallmarks of sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). Activation of these pathways, either in tandem or independent of GORD, may potentially result in the *transition* of oesophageal *squamous* mucosa into a *columnar* epithelium. There were also significant increases in TERT expression in this cell line following ACM treatment; expression of TERT has been linked to malignant transformation in pancreatic tumours independent of telomere maintenance (Hashimoto, Murakami et al. 2008), and TERT expression could similarly be involved in the squamous to columnar transition in the pathogenesis of Barrett's oesophagus independent of telomere maintenance.

While the HET-1A cell line showed no proliferative response to leptin or ACM derived from obese and non-obese patients, there was a significant increase in cell invasion observed following ACM treatment. This increase in cell invasion can be explained by increased of MMP-1 expression and the activation of pro-invasive pathways following ACM treatment, increases in cell invasion in tandem with increased MMP expression has been reported in cell lines previously (Cheng, Chou et al. 2006; Kuang, Wang et al. 2011). Early expression of MMPs conducive to invasion and carcinogenesis in pre neoplastic lesions has been reported in ovarian tumourigenesis (Cai, Yang et al. 2007) and Barrett's oesophagus (Grimm, Lazariotou et al. 2010). Activation of this mechanism in healthy squamous epithelium by obesity may be critical in the early stages of the Barrett's disease progression.

Genomic instability, characterised by anaphase bridging, dysregulation of the expression of the SAC complex, telomere shortening and dysregulation of the expression of telomeric chromatin was evident in the HET-1A cell line following ACM treatment. Significant telomere shortening was also elicited by a 24hr and a 48hr ACM treatment in the HET-1A cell line and this may explain both the increased levels of anaphase bridging (Shimizu, Shingaki et al. 2005) and the gene activation in the HET-1A cell line (Lo, Sabatier et al. 2002). Genomic instability in the pathogenesis of Barrett's oesophagus has been investigated previously and may underlie the

squamous-columnar transition in Barrett's oesophagus. Molecular alterations including LOH, allelic imbalance, and microsatellite instability could be detected in all types of metaplastic changes in Barrett's metaplasia biospies and sporadically in the squamous epithelium adjacent to the metaplastic tissue (Romagnoli, Roncalli et al. 2001). In a study conducted by Romagnoli *et al*, a *p53* mutation was observed in squamous epithelium and in nonintestinal metaplasia, a D5S82 allelic imbalance in squamous epithelium and D5S82 LOH in nonintestinal metaplasia, and a D17S513 allelic instability in squamous epithelium and intestinal metaplasia with low-grade dysplasia, D17S513 LOH in squamous epithelium, and nonintestinal metaplasia. Some of these molecular alterations precede the development of intestinal metaplasia, suggesting that genetic alterations take place early in the Barrett's sequence (Romagnoli, Roncalli et al. 2001), and the results presented in this thesis suggests that these genetic alterations may be a consequence of obesity.

BAR-T/ QH/ Metaplastic Barrett's oesophagus

Pathways associated with angiogenesis, and invasion and metastasis showed greatest activation within the BAR-T cell line in response to ACM treatment, and these pathways relate directly to two of the hallmarks of cancer identified previously by Hanahan and Weinberg (Hanahan and Weinberg 2000). Pro-angiogenic pathways have been associated with obesity related carcinogenesis previously in breast cancer (Christiani, Wu et al. 2011; Gu, Young et al. 2011), while obesity has also been shown to activate pro-invasive pathways in renal cell carcinoma (Horiguchi, Sumitomo et al. 2006).

ACM treatment of the BAR-T cell lines followed by gene expression analysis via the Human cancer pathway profiler enabled the identification of a signature of five cancer related genes whose gene expression was altered by obesity; MMP-1, TIMP-3, IL-8, MCAM and VEGFA. These genes have all been associated with carcinogenesis previously and expression of 4/5 of these genes, MCAM excluded, showed greatest increases in expression early in the metaplasia-dysplasia-adenocarcinoma sequence with expression levels decreasing in response to ACM and the disease sequence progressed. Treatment of the BAR-T cell line with a pulsed 300μM and continuous 150μM DCA treatments, to mimic the effects of GORD in vitro, regulated the expression of the same five genes, in an identical expression pattern. MMP-1 and IL-8 also showed significant up regulation in Barrett's metaplasia biopsies from obese patients compared to non-obese patients. While GORD is an important risk factor for Barrett's oesophagus, GORD alone cannot account for the incidences of Barrett's oesophagus and the neoplastic progression of the condition (Edelstein, Farrow et al. 2007). The tumourigeneic

burden of excess adipose tissue in obese Barrett's oesophagus patients may contribute to carcinogenesis due to the adipose derived factors secreted by excess adipose tissue. IL-8, MMP-1, VEGFA, leptin and various other adipocytokines may be exacerbating the damage caused by GORD, by activating key pro-cancer pathways in metaplastic Barrett's oesophagus.

Expression of these genes was also investigated in Barrett's metaplasia biopsies obtained from consenting patients attending an outpatient Barrett's oesophagus clinic in St. James's Hospital. IL-8 and MMP-1 were expressed at significantly higher levels in biopsies from obese patients compared to age and sex matched non obese patients. IL-8 also known as CXCL-8, has been shown to regulate pathological angiogenesis, tumour growth, and metastasis (Li, Dubey et al. 2003). Circulating IL-8 levels have been shown to correlate significantly with obesity-related parameters such as BMI and waist circumference (Kim, Park et al. 2006) and this excess adipose tissue derived IL-8 may promote carcinogenesis through both angiogenic and pro-inflammatory pathways in obese Barrett's oesophagus patients. It has recently been suggested that an inflammatory microenvironment is an additional hallmark of cancer (Colotta, Allavena et al. 2009; Hanahan and Weinberg 2011). Increased inflammatory microenvironments have been demonstrated within pre-malignant lesions previously in colonic adenomas (McLean, Murray et al. 2011) and it has been suggested that a phenotypic and genotypic 'switch' occurs early in the disease sequence, with expression of inflammatory cytokines and chemokines deregulated in the transition from normal mucosa to adenomatous polyp, rather than at the polyp to invasive disease transition (McLean, Murray et al. 2011); a similar mechanism may be at play in Barrett's derived adenocarcinoma. MMP-1 is a key metastatic regulator in cancer (Sunami, Tsuno et al. 2000; Jiang, Dutton et al. 2003) and expression of MMP-1 in proliferating Barrett's oesophagus and oesophageal adenocarcinoma has been linked to malignant proliferation following the clonal expansion (Grimm, Lazariotou et al. 2010). Interestingly, IL-8 has shown to up-regulate the expression and activity of MMPs. MMP-2 and MMP-9 expression and activity in endothelial cells, are enhanced by recombinant human IL-8, suggesting that IL-8 is involved in the degradation of extracellular matrix, leading to endothelial cell migration, invasion, and capillary tube organization (Li, Dubey et al. 2003). MMP-1 and IL-8 may have similar relationship in Barrett's oesophagus, promoting neoplastic progression in obese patients.

There was considerable genomic instability demonstrated in the QH cell line following ACM treatment in this study. Telomere shortening leading to genomic instability has been studied extensive in Barrett's oesophagus (Finley, Reid et al. 2006; Shiraishi, Mikami et al. 2009) while significantly shorter telomeres have also been demonstrated in obese adults compared to their

non-obese counterparts (Zannolli, Mohn et al. 2008). The QH cell line demonstrated significant telomere shortening following ACM treatment and demonstrated dysregulation of the shelterin complex, a form of telomeric chromatin which regulates telomerase recruitment and telomere length (Abreu, Aritonovska et al. 2010). Over expression of shelterin complex proteins, particularly POT-1, is conducive to telomere shortening as over expression of POT-1 on telomere increases the chance that telomerase will be blocked, resulting in telomere shortening (de Lange 2005). In the QH cell line there was significant increases in POT-1 expression in tandem with telomere shortening, following ACM treatment. This is a novel finding in this thesis as the shelterin complex has never previously been studied with respect to Barrett's oesophagus or obesity. Significant anaphase bridging was also demonstrated in the metaplastic QH cells following 48hr ACM treatment; bridge breakage fusion events driven by obesity at this stage of the disease sequence may be a potential source of carcinogenesis in Barrett's oesophagus.

The QH cell line showed no proliferative response to a leptin treatment however, cell proliferation, as determined by a BrdU incorporation ELISA, showed that cell proliferation was significantly increased by both non obese and obese ACM indicating that adipose derived factors independent of leptin are responsible for cell proliferation in a Barrett's oesophagus cell model. Barrett's oesophagus cells show an increased proliferative index in tandem with dysregulation of cell cycling compared to normal squamous cells (Fitzgerald 2005). An obese phenotype has been associated with increased endometrial cell proliferation in women with endometrial cancer (Villavicencio, Aguilar et al. 2010), while increased colorectal epithelial cell proliferation has also demonstrated a link with obesity, with morbidly obese colorectal patients showing significantly increased proliferation compared with age and sex matched normal weight individuals (Sainsbury, Goodlad et al. 2008). Increased proliferation induced by obesity in Barrett's oesophagus may be a mechanism through which carcinogenesis is initiated at this stage in the disease sequence. Increases in cell proliferation can be explained by the increase in TERT expression in this cell line following ACM treatment. TERT has been shown to stimulate epithelial cell proliferation through transcriptional control of Myc and Wnt (Choi, Southworth et al. 2008) and confer resistance to apoptosis in human fibroblasts(Gorbunova, Seluanov et al. 2002). These tumourigenic properties of TERT, independent of telomere maintenance, may be active in Barrett's oesophagus due to excess obesity.

The QH cell line also showed significant increases in cell invasion following ACM treatment; the QH cell line demonstrated greatest increases in cell proliferation following ACM treatment than the HET-1A, QH and GO cell lines used in this study. Increases in cell invasion following

ACM treatment at this stage of the Barrett's sequence can be explained by the increases in MMP-1 expression and decrease in TIMP-3 expression elicited by ACM in this cell line. Dysregulation of the normal balance between MMPs and TIMPs can induce changes in invasive and proliferative capacities of cells (Lopez-Otin, Folgueras et al. 2004) and these same pathways may be active in Barrett's oesophagus in response to an obese microenvironment.

GO/High grade dysplastic Barrett's oesophagus

Significant cell proliferation was evident at this stage of the disease sequence, in response to leptin and ACM generated from both viscerally obese and non-obese patients, Increased proliferation is a common feature of dysplastic states of leukaemia (Sun, Medeiros et al. 2003) and gastric cancer (Miracco, Spina et al. 1995). High grade dysplastic Barrett's oesophagus demonstrates significantly greater rates of cell proliferation in vivo, compared to metaplastic Barrett's oesophagus (Whittles, Biddlestone et al. 1999). Activation of the serine-threonine protein kinase and proto-oncogene Akt leading to increased proliferation (Beales, Ogunwobi et al. 2007) has been reported in high grade dysplastic Barrett's oesophagus. Akt activity is promoted by adipose derived insulin-like growth factor I, leptin, TNF-α and IL-6, leading to increased cell survival and cell growth, and promoted cell cycle in obesity-associated colon cancer (Huang and Chen 2009). Activation of this pathway in Barrett's oesophagus by obesity represents a potential source of carcinogenesis that warrants further investigation.

Similar to the QH cell line, there was significant genomic instability demonstrated in the GO cells following ACM treatment. The GO cells demonstrated significant telomere shortening following 24hr and 48hr ACM treatment, and a significant increase in anaphase bridging following 48hr ACM treatment. Anaphase bridging leads to increased rates of chromosomal rearrangement and aneuploidy (Stewenius, Gorunova et al. 2005) and this increased rate of aneuploidy has been associated with high grade dysplastic Barrett's oesophagus (Menke-Pluymers, Mulder et al. 1994). Aneuploidy in high grade dysplastic Barrett's oesophagus has been linked to loss of heterozygosity of 17p (p53) and increased rates of progression to cancer (Reid, Prevo et al. 2001). The GO cell line also demonstrates loss of an efficient SAC response following 24hr ACM treatment, then a significant increase following 48hr ACM treatment. Both decreases (Rao, Yamada et al. 2009) and increases in SAC member expression, particularly MAD2L2 and BUB1B (Wada, Yoshida et al. 2008) are conducive to carcinogenesis, and this aberrant expression of the SAC in high grade dysplastic Barrett's oesophagus, as demonstrated in this thesis, may be involved with neoplastic progression.

OE33/ Invasive oesophageal adenocarcinoma

Similar to the GO cell line, there were significant increases in proliferation in response to both leptin and ACM derived from both viscerally obese and non-obese patients, in the OE33 cell line, representative of invasive oesophageal adenocarcinoma. Unchecked cell proliferation is a well-documented feature of cancer (Jones and Thompson 2009), and increases in OE33 cell proliferation in response to both leptin (Ogunwobi and Beales 2008) and ACM (Lysaght, van der Stok et al. 2011) have been reported previously, and these results were replicated in this thesis.

There were significant increases in cellular invasion elicited by ACM in the OE33 cell lines. Exogenous stimuli have been observed to drive cellular invasion in this cell line previously, nitric oxide at physiological levels significantly increased the invasive capacity of this cell line (Clemons, Shannon et al. 2010). An obese phenotype has been reported to be associated with the presence of angiolymphatic invasion in breast cancer (Gillespie, Sorbero et al. 2010), and increases the risk for the growth and dissemination of pancreatic cancer (Zyromski, Mathur et al. 2009). The metastatic spread of oesophageal adenocarcinoma is associated with brain metastases (Vaquero, Abreu et al. 1982), lymph node metastases (Shigemitsu, Naomoto et al. 2002) and the predominant forms are liver metastases (Enzinger, Ilson et al. 1999) and bone (Goodner and Turnbull 1971). Invasion and metastasis stimulated by obesity may further drive the metastatic spread of oesophageal adenocarcinoma.

Significant dysfunction of the SAC complex and anaphase bridging was seen at this stage of the disease progression following 24hr and 48hr ACM treatment, respectively. A compromised SAC has been reported in gastro-intestinal cancers previously (Scully 2010) while it has recently been demonstrated that manipulation of this checkpoint through the up-regulation of MAD2L2 in human oesophageal squamous cell carcinoma may be a viable therapy (Wang, Guo et al. 2012).

Expression of members of the shelterin complex demonstrated significant down regulation in tandem with significant decreases in telomere length following both 24hr and 48hr ACM treatment of the OE33 cell line. An obese phenotype is conducive to telomere shortening (Zannolli, Mohn et al. 2008), and this association was seen in the OE33 cell line. The shelterin complex has shown dysregulations in carcinogenesis previously (Bilsland, Cairney et al. 2011) but this thesis is the first study to associate the shelterin complex with obesity, Barrett's oesophagus and oesophageal adenocarcinoma.

In conclusion, activation of pro-tumourigenic pathways by ACM is evident at each step in the metaplasia-dysplasia-adenocarcinoma sequence associated with the neoplastic progression of Barrett's oesophagus. The HET-1A and BAR-T/QH cell lines, representative of squamous oesophageal epithelium and metaplastic Barrett's oesophagus, respectively, show the greatest number of cellular dysfunctions following ACM treatment, indicating that the activation of pro-tumourigenic pathways is an early event in Barrett's oesophagus. Cellular damage elicited by adipose derived factors at this stage of the disease sequence may lead to activation of angiogenic and metastatic pathways, cellular proliferation, telomere shortening in tandem with shelterin complex dysregulation and loss of an efficient spindle assembly checkpoint. Carcinogenesis can be defined as an accumulation genetic abnormalities leading to cellular dysfunction (Jones 2003). Cellular dysfunctions mediated in Barrett's oesophagus by adipose derived factors as demonstrated in this thesis, may underlie the neoplastic progression to oesophageal adenocarcinoma, independent of insult from GORD.

7.2 Future direction

Tissue micro arrays currently are currently under construction in the department that encompass biopsies from each stage of the metaplastia-dysplasia-adenocarcinoma sequence associated with disease progression in Barrett's oesophagus to further evaluate and validate potential biomarkers at the protein level. This will allow the tracking of the expression of specific protein biomarkers across the progressive sequence of this disease.

Future direction of this work will include investigation of the effects of microRNAs in obesity and Barrett's oesophagus. MicroRNAs are associated with many cell processes and are responsible for up to 30% of all post transcriptional mRNA modification (Lewis, Burge et al. 2005). These biomolecules have previously been associated with Barrett's oesophagus during the transdifferentiation of the cells during metaplasia (Slack 2007). A wide range of microRNAs have been defined in Barrett's oesophagus but have not been studied extensively with regards to obesity. Investigation of the expression of these microRNAs in Barrett's metaplasia biopsies from obese and non-obese patients and determination whether obesity status reflects microRNA expression/activity is planned.

Dublin, October 2011

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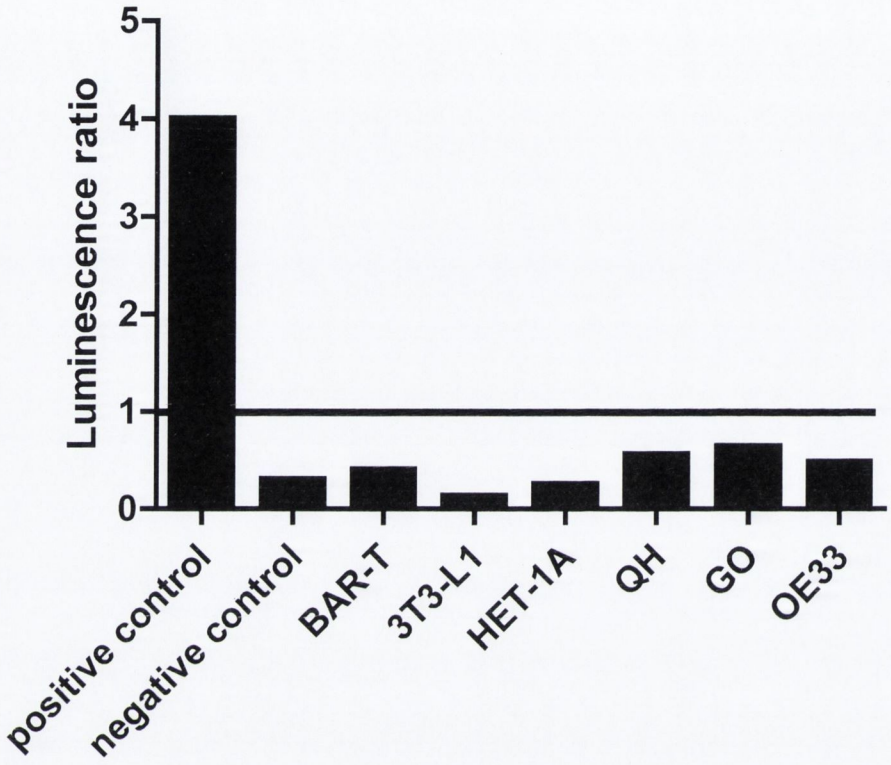
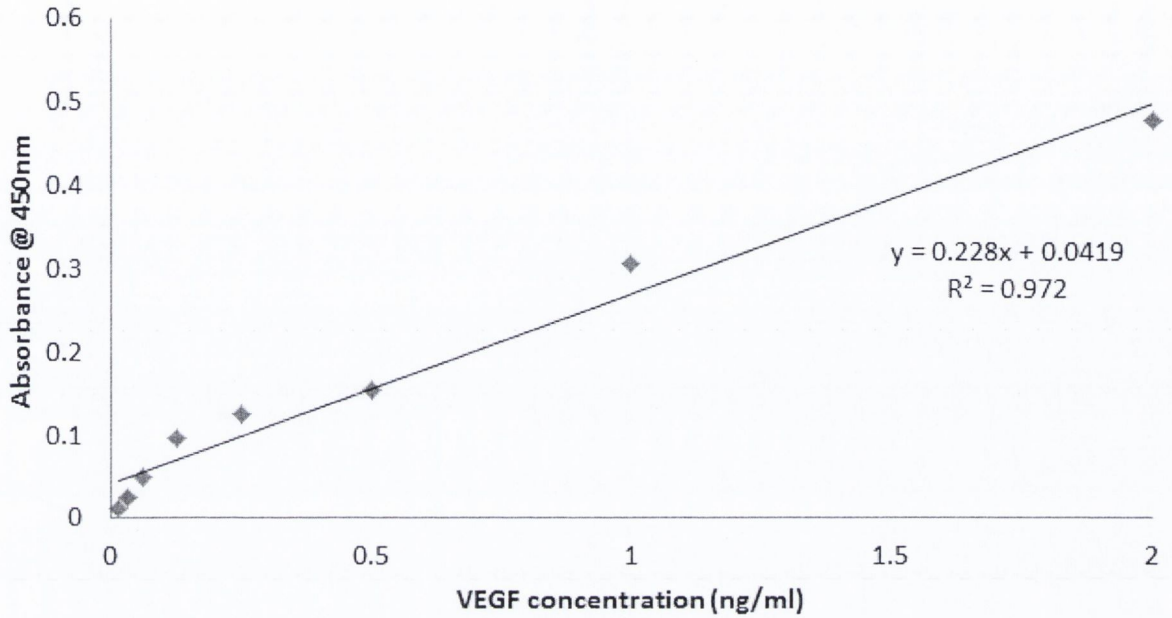
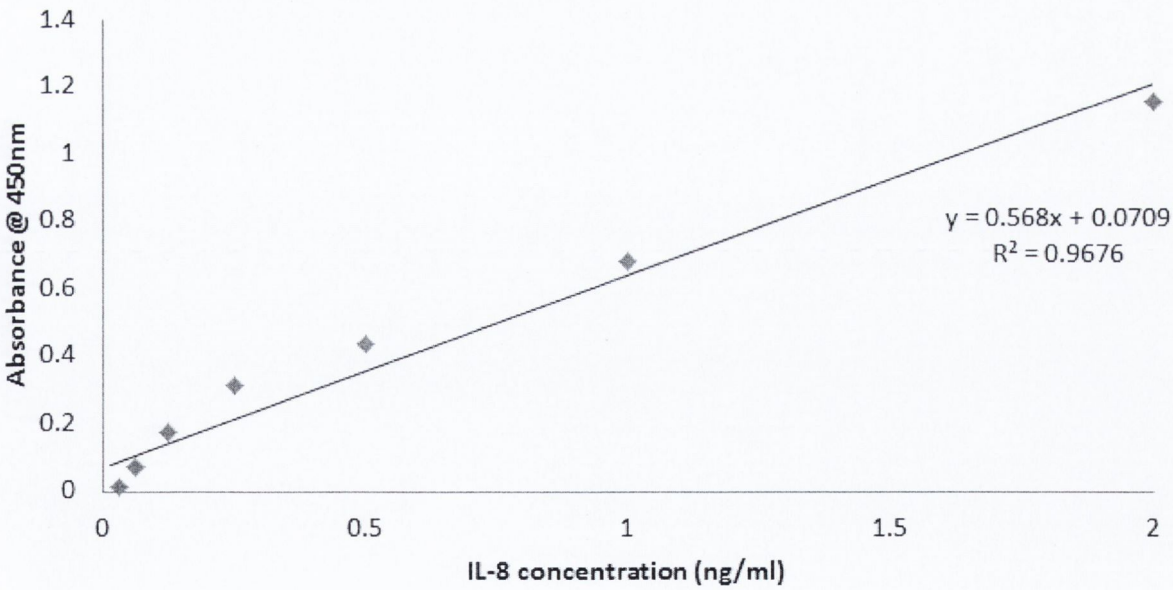


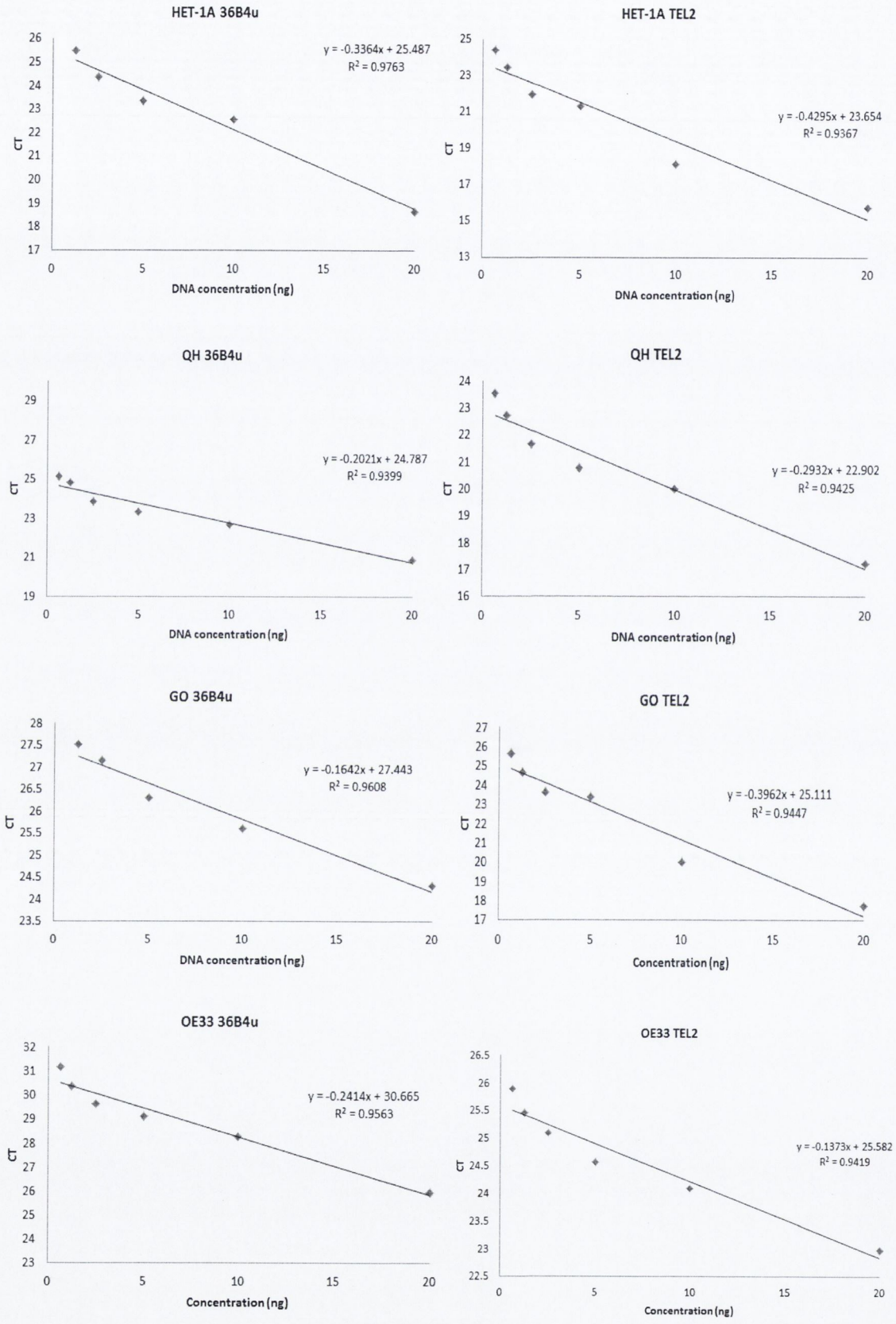
Cell culture supernatants were collected from all cells lines on a regular basis and tested for mycoplasma contamination. The presence of mycoplasma in these cells is determined by a luminescence ration of >1. All cell lines were negative for the presence of mycoplasma throughout the course of this work.

Appendix II: Standard curves





Representative standard curves for VEGF and IL-8 ELISAs. ELISAs were used to quantify protein levels present in adipose conditioned media (ACM). (R^2= regression coefficient).



Representative standard curves for 38B4u and TEL2 expression in the HET-1A, QH, GO and OE33 cell line

Appendix III: Gene list on Human Cancer Profiler qPCR Array plates (SABiosciences)

Position	UniGene	RefSeq	Symbol	Gene Name
A01	Hs.525622	NM_005163	AKT1	PKB/PRKBA
A02	Hs.369675	NM_001146	ANGPT1	AGP1/AGPT
A03	Hs.553484	NM_001147	ANGPT2	AGPT2/ANG2
A04	Hs.552567	NM_001160	APAF1	CED4
A05	Hs.435561	NM_000051	ATM	AT1/ATA
A06	Hs.370254	NM_004322	BAD	BBC2/BCL2L8
A07	Hs.159428	NM_004324	BAX	Bax zeta
A08	Hs.150749	NM_000633	BCL2	Bcl-2
A09	Hs.516966	NM_138578	BCL2L1	BCL-XL/S
A10	Hs.194143	NM_007294	BRCA1	BRCAI/BRCC1
A11	Hs.369736	NM_001228	CASP8	FLICE/MACH
A12	Hs.244723	NM_001238	CCNE1	CCNE
B01	Hs.1634	NM_001789	CDC25A	CDC25A2
B02	Hs.19192	NM_001798	CDK2	p33(CDK2)
B03	Hs.95577	NM_000075	CDK4	CMM3/PSK-J3
B04	Hs.370771	NM_000389	CDKN1A	CAP20/CDKN1
B05	Hs.512599	NM_000077	CDKN2A	ARF/CDK4I
B06	Hs.390736	NM_003879	CFLAR	CASH/CASP8AP1
B07	Hs.291363	NM_007194	CHEK2	CDS1/CHK2
B08	Hs.517356	NM_030582	COL18A1	KNO
B09	Hs.96055	NM_005225	E2F1	E2F-1/RBBP3
B10	Hs.446352	NM_004448	ERBB2	HER-2/HER-2
B11	Hs.517296	NM_005239	ETS2	c-Ets2
B12	Hs.244139	NM_000043	FAS	ALPS1A/APO-1
C01	Hs.533683	NM_000141	FGFR2	BEK/BFR-1
C02	Hs.25647	NM_005252	FOS	c-fos
C03	Hs.90708	NM_006144	GZMA	CTLA3/HFSP
C04	Hs.90753	NM_006410	HTATIP2	CC3/TIP30
C05	Hs.37026	NM_024013	IFNA1	IFL/IFN
C06	Hs.93177	NM_002176	IFNB1	IFB/IFF
C07	Hs.160562	NM_000618	IGF1	IGFI
C08	Hs.624	NM_000584	IL8	3-10C/AMCF-I
C09	Hs.519304	NM_181501	ITGA1	CD49a/VLA1
C10	Hs.482077	NM_002203	ITGA2	BR/CD49B
C11	Hs.265829	NM_002204	ITGA3	CD49C/GAP-B3
C12	Hs.553495	NM_000885	ITGA4	CD49D/IA4
D01	Hs.436873	NM_002210	ITGAV	CD51/MSK8
D02	Hs.429052	NM_002211	ITGB1	CD29/FNRB
D03	Hs.218040	NM_000212	ITGB3	CD61/GP3A
D04	Hs.13155	NM_002213	ITGB5	Integrin b5
D05	Hs.525704	NM_002228	JUN	AP1
D06	Hs.145442	NM_002755	MAP2K1	MAPKK1/MEK1
D07	Hs.511397	NM_006500	MCAM	CD146/MUC18
D08	Hs.567303	NM_002392	MDM2	hdm2
D09	Hs.132966	NM_000245	MET	HGFR/RCCP2
D10	Hs.83169	NM_002421	MMP1	CLG/CLGN
D11	Hs.513617	NM_004530	MMP2	CLG4/CLG4A
D12	Hs.297413	NM_004994	MMP9	CLG4B/GELB
E01	Hs.525629	NM_004689	MTA1	Mta-1
E02	Hs.173043	NM_004739	MTA2	DKFZp686F2281/MTA1L1
E03	Hs.336994	NM_014751	MTSS1	MIM/MIMA
E04	Hs.202453	NM_002467	MYC	c-Myc
E05	Hs.431926	NM_003998	NFKB1	DKFZp686C01211/EBP-1
E06	Hs.81328	NM_020529	NFKBIA	IKBA/MAD-3
E07	Hs.118638	NM_000269	NME1	AWD/GAAD
E08	Hs.9235	NM_005009	NME4	NM23H4/nm23-H4
E09	Hs.376032	NM_002607	PDGFA	PDGF-A/PDGF1
E10	Hs.1976	NM_002608	PDGFB	PDGF2/SIS
E11	Hs.132225	NM_181504	PIK3R1	GRB1/p85-ALPHA
E12	Hs.77274	NM_002658	PLAU	ATF/UPA
F01	Hs.466871	NM_002659	PLAUR	CD87/UPAR
F02	Hs.409965	NM_002687	PNN	DRS/SDK3
F03	Hs.159130	NM_002880	RAF1	CRAF/Raf-1
F04	Hs.408528	NM_000321	RB1	OSRC/RB
F05	Hs.81256	NM_002961	S100A4	18A2/42A
F06	Hs.55279	NM_002639	SERPINB5	PI5/maspin
F07	Hs.414795	NM_000602	SERPINE1	PAI/PAI-1
F08	Hs.349470	NM_003087	SNCG	BCSG1/SR
F09	Hs.371720	NM_003177	SYK	Syk
F10	Hs.89640	NM_000459	TEK	CD202B/TIE-2
F11	Hs.492203	NM_003219	TERT	EST2/TCS1
F12	Hs.1103	NM_000660	TGFB1	CED/DPD1
G01	Hs.494622	NM_004612	TGFBR1	ACVRLK4/ALK-5
G02	Hs.164226	NM_003246	THBS1	THBS/TSP
G03	Hs.522632	NM_003254	TIMP1	CLGI/EPA
G04	Hs.297324	NM_000362	TIMP3	HSMRK222/K222
G05	Hs.241570	NM_000594	TNF	DIF/TNF-alpha
G06	Hs.521456	NM_003842	TNFRSF10B	DR5/KILLER
G07	Hs.279594	NM_001065	TNFRSF1A	CD120a/FPF
G08	Hs.462529	NM_003790	TNFRSF25	APO-3/DDR3
G09	Hs.408312	NM_000546	TP53	LFS1/TRP53
G10	Hs.66744	NM_000474	TWIST1	ACS3/BPES2
G11	Hs.416007	NM_017549	EPDR1	MERP-1/MERP1
G12	Hs.73793	NM_003376	VEGF	VEGFA/VPF
H01	Hs.534255	NM_004048	B2M	B2M
H02	Hs.412707	NM_000194	HPRT1	HGPRT/HPRT
H03	Hs.546356	NM_012423	RPL13A	RPL13A
H04	Hs.544577	NM_002046	GAPDH	G3PD/GAPD
H05	Hs.520640	NM_001101	ACTB	b-Actin
H06	N/A	N/A	GDC	GDC
H07	N/A	N/A	RTC	RTC
H08	N/A	N/A	RTC	RTC
H09	N/A	N/A	RTC	RTC
H10	N/A	N/A	PPC	PPC
H11	N/A	N/A	PPC	PPC
H12	N/A	N/A	PPC	PPC