PRECISION STAGING AND MANAGEMENT OF BARRETT'S OESOPHAGUS AND OESOPHAGEAL CANCER:

GENOMIC, IMAGING AND PATHOLOGICAL BIOMARKERS

John Mitchell Findlay

BMedSci (hons) BMBS (hons) MRCS

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ABSTRACT

Barrett's oesophagus and oesophageal cancer represent two of the most important and challenging oesophageal disease processes globally, combining increasing incidences with high morbidity treatments, often with poor clinical outcomes. A major contributory factor is that disease susceptibility, progression and response to therapy are largely unpredictable, due to inherent biological complexity and variability. At present, just staging groups are used routinely as thresholds for guiding the use of therapies such as ablation, resection, and oncological therapies. However, these represent blunt tools that do not necessarily reflect patients' experiences, or appropriately select from the range of treatments available. The aim of this thesis was to explore the potential of genomic, imaging, and pathological biomarkers in guiding more tailored and personalised therapy.

The first half of this thesis explores the role of genomic markers. The first results chapter describes the identification of new loci and gene pathways associated with susceptibility to Barrett's oesophagus, dysplasia and oesophageal adenocarcinoma, by further replication and analysis of a genome-wide association study. In addition, all reported genomic markers of these endpoints were identified and criticised by systematic review, and synthesised by meta-analysis. Validation of these was then attempted, and lessons for markers and future research drawn.

The second results chapter describes a similar appraisal and synthesis of genomic markers of oesophageal cancer prognosis, response to therapy, and stage.

The third describes the first next generation sequencing study performed in oesophageal adenocarcinoma (and indeed any gastrointestinal cancer as far as the author is aware), before and after neoadjuvant chemotherapy. Using whole exome sequencing a new model of genomic tumour response was developed, and the implications for biomarkers explored.

The second half of this thesis follows a large cohort of patients with oesophageal cancer, from nearly 1000 undergoing staging, to more than 300 undergoing neoadjuvant chemotherapy, restaging and resection. In the fourth results chapter, the first application of decision theory to cancer staging identified the potential for routine imaging data to personalise and optimise oesophageal cancer staging.

In the following chapter, positron emission tomography-computed tomography was found to be more sensitive for identifying disease progression during neoadjuvant chemotherapy than computed tomography alone. Two factors were identified that could stratify risk of progression to incurable disease, including that encountered at surgery. These included ^{18}F-FDG avid nodes, with new concepts of metabolic nodal stage and response developed in conjunction with predictive models.

Thereafter, a number of conventional and experimental metrics of metabolic tumour response were compared and refined as predictors of pathological response. Existing metrics of metabolic tumour response were found to be suboptimal, and these new concepts and classifications of metabolic nodal stage and response were found to have independent utility for clinical practice. Again, predictive models were generated.

Finally, the prognostic utilities of these markers were explored. Metabolic tumour response was found to be an imperfect surrogate of pathological response. However, metabolic nodal response demonstrated independent utility in identifying patients at high risk of early recurrence and death, both when used before surgery and afterwards. Indeed, a number of analyses demonstrated the additive utility of considering the primary tumour and nodal metastases as separate entities. Finally, prognostic models were generated, and a simple risk score was generated, using the four independent prognostic markers identified to stratify prognosis.

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CHAPTER 3

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CHAPTER 7

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CHAPTER 8

Predicting pathological response of esophageal cancer to neoadjuvant chemotherapy: the implications of metabolic nodal response for personalised therapy.

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Journal of Nuclear Medicine 2016 Sep 15 (epub ahead of print)

CHAPTER 9

Metabolic nodal response as a prognostic marker after neoadjuvant therapy for oesophageal cancer

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British Journal of Surgery (in press)

FOREWORD

The experiments described in this thesis were performed at the Wellcome Trust Centre for Human Genetics, University of Oxford under the supervision of Ian Tomlinson (Professor of Molecular and Population Genetics). The clinical studies described were performed at the Oxford OesophagoGastric Centre, Churchill Hospital and the Department of Oncology, University of Oxford under the supervision of Mark R Middleton (Professor of Experimental Cancer Medicine). This thesis overall was supervised by Mark R Middleton and Ian Tominson at the University of Oxford, and Mohammad Ilyas (Professor of Pathology) at the University of Nottingham.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AC	Adenocarcinoma
ANN	Artificial neural network
ASA	American Society of Anesthesiology
ASCO	American Society of Clinical Oncology
AspECT	Aspirin and Esomeprazole Chemoprevention Trial of Cancer in Barrett's Oesophagus
ATP	Adenosine triphosphate
AUC	Area under the curve
AUGIS	Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland
BEACON	Barrett's and Esophageal Adenocarcinoma Consortium
BMP	Bone morphogenic proteins
BO	Barrett's Oesophagus
bp	Base pair
C2orf43	Chromosome 2 open reading frame 43
CBP	C-bioportal
CDCV	Common disease-common variant
CF	Cisplatin and 5-FU
CGH	Comparative genomic hybridisation
ChoPIN	Chemoprevention Of Premalignant Intestinal Neoplasia
Chr	Chomrosome
CI	Confidence interval
CIN	Chromosomal instability
CMR	Complete metabolic response
CNV	Copy number variant
CoRGI	Colorectal Tumour Gene Identification
CT	Computed tomography
ctDNA	Circulating tumour DNA
dbSNP	The Single Nucleotide Polymorphisms Database
DCA	Decision curve analysis
DCR	Definitive chemoradiotherapy
DDR	DNA damage response
DFS	Disease-free survival
DNA	Deoxyribonucleic acid

DSS	Disease-specific survival
DTA	Decision tree analysis
EAGLE	Esophageal Adenocarcinoma Genetic LinkagE
ECF	Epirubicin, cisplatin and 5-FU
ECX	Epirubicin, cisplatin and capecitabine
EGFR	Epidermal growth factor receptor
EMR	Endoscopic mucosal resection
EOX	Epirubicin, oxaliplatin and capecitabine
eQTL	Expression quantitative trait locus
ER	Endoscopic resection
ESMO	European Society of Medical Oncology
EUS	Endoscopic ultrasound
FDG	Fluorine-18 fluorodeoxyglucose
FDR	False discovery rate
FISH	Fluorescent in situ hybridization
FNAC	Fine needle aspiration cytology
FPR	False positive rate
FRET	fluorescent resonance energy transfer
GDF7	Growth Differentiation Factor 7
GenGen	Genetic Genomics Analysis of Complex Data
GI	Genomic instability
GLACIER	Genetics of LobulAr Carcinoma In situ in EuRope
GO	Gene ontology
GOJ	Gastro-oesophageal junction
GORD	Gastro-oesophageal reflux disease
GSEA	Gene set enrichment analysis
gTR	Genomic tumour response
GWAS	Genome wide association studies
HER2	Human epidermal growth factor receptor 2
HGD	High-grade dysplasia
HR	Hazard ratio
HWE	Hardy-Weinberg equilibrium
ICGC	International Cancer Genetics Consortium
iGSEA	Improved Gene Set Enrichment Analysis for GWAS
IM	Intestinal metaplasia

Indels	Insertions/deletions
IQR	Interquartile range
KASPar	KBioscience competitive allele-specific PCR
LD	Linkage disequilibrium
LGD	Low-grade dysplasia
LOE	Level of evidence
LOH	Loss of heterozygosity
LR	Logistic regression
MAF	Minor allele frequency
Mb	Megabase
MDT	Multidisciplinary team
MHC	Major histocompatibility complex
MMR	Mismatch repair
MPG	Molecular and Population Genetics group
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
mN	Metabolic nodal stage
mNR	Metabolic nodal response
mRNA	Messenger ribonucleic acid
mTR	Metabolic tumour response
MTV	Metabolic tumour volume
NAC	Neoadjuvant chemotherapy
NACR	Neoadjuvant chemoradiotherapy
NCBI	National Centre for Biotechnology Information
NCCN	National Comprehensive Cancer Network
NER	Nucleotide excision repair
NGS	Next generation sequencing
NPV	Negative predictive value
OAC	Oesophageal adenocarcinoma
O:E	Observed:expected ratio
OGD	Oesophago-gastro-duodenoscopy
OF	Oxaliplatin 5-FU
OR	Odds ratio
OS	Overall survival

OUH	Oxford University Hospitals NHS Foundation Trust
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PERCIST	PET Response Criteria in Solid Tumours
PET-CT	Positron emission tomography-computed tomography
PFS	Progression-free survival
pL	Pathological lymphatic invasion
PMD	Progressive metabolic disease
PMR	Partial metabolic response
pN	Pathological nodal stage
PoBI	People of the British Isles
PPI	Proton pump inhibitor
PPV	Positive predictive value
pT	Pathological T stage
Pt	Probability threshold
pTR	Pathological tumour response
pV	Pathological vascular invasion
qPCR	Quantitative polymerase chain reaction
R	Pathological resection margin
RBM19	RNA-binding motif 19
RCT	Randomised controlled trial
REMARK	Reporting recommendations for tumour marker
RFA	Radiofrequency ablation
ROC	Receiver operator characteristic
rpm	Revolutions per minute
Rs	Reference SNP identification number
s	Seconds
SE	Standard error
SISH	Silver *in situ* hybridization
SMD	Stable metabolic disease
SMG	Significantly mutated gene
SNP	Single nucleotide polymorphisms
SSCP	Single strand conformation polymorphism
STR	Short tandem repeat

STS	Society of Thoracic Surgeons
SUV	Standardised uptake value
Taq	Thermus aquaticus polymerase
TCGA	The National Cancer Institute Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TGV	Tumour glycolytic volume
TNM	TNM Classification of Malignant Tumours
TP53	Tumour protein 53
TRG	Tumour regression grade
UK	United Kingdom
USA	United States of America
VAF	Variant allele frequency
WES	Whole exome sequencing
WGS	Whole genome sequencing
WTCCC	Wellcome Trust Case Control Consortium
WTCHG	Wellcome Trust Centre for Human Genetics
WTSI	Wellcome Trust Sanger Institute

CHAPTER I

INTRODUCTION

OESOPHAGEAL METAPLASIA, DYSPLASIA AND CANCER: STAGING, MANAGEMENT AND MARKERS

1. CHAPTER 1: INTRODUCTION: OESOPHAGEAL METAPLASIA, DYSPLASIA AND CANCER: STAGING, MANAGEMENT AND MARKERS

1.1. THE OESOPHAGUS AND GASTRO-OESOPHAGEAL REFLUX

The oesophagus is a unique structure, dependent upon the complex interplay of numerous anatomical and physiological factors. Its location, bridging the upper airway and digestive tracts spanning three body compartments, presents a number of challenges, particularly minimising epithelial exposure to stomach and duodenal contents, whilst allowing two-way transit. This is reflected in the clinical and biological complexity of its two most important diseases: Barrett's oesophagus (BO) and cancer.

The cervical oesophagus (5cm long) extends from cricopharyngeus to the thoracic inlet; the thoracic oesophagus (18cm) ends at the oesophageal hiatus; whilst the abdominal oesophagus comprises the final 1-2cm ending at the gastrooesophageal junction (GOJ) (Moore et al., 2002). These three compartments provide very different environments (particularly extrinsic pressure), reflected in anatomical and physiological differences. Its epithelium is non-keratinizing stratified squamous, abruptly becoming glandular columnar at or just above the level of the GOJ (Stevens, 2000), due to the injurious nature of gastric and duodenal contents.

1.1.1. Gastro-oesophageal reflux

Gastrooesophageal reflux disease (GORD) describes symptoms or mucosal damage caused by reflux of gastric contents into the oesophagus (Fox and Forgacs, 2006). Symptoms vary considerably and are often absent, however, it is undoubtedly one of the commonest health problems in the developed world, (Dent et al., 2005). Notably, its prevalence continues to increase, and drive concomitant increases in BO and oesophageal adenocarcinoma (OAC) (Lagergren et al., 1999).

1.1.2.2 Anti-reflux mechanisms and risk factors

A 10mmHg positive pressure gradient spans the stomach and oesophagus. That reflux is the exception rather than the rule is due to numerous anti-reflux factors, aberrations of which predispose to GORD, BO and OAC. These have been reviewed by the author previously but comprise mechanical barriers (such as the lower oesophageal sphincter), physiological defence mechanisms (such as peristaltic clearance) and epithelial resistance. Due to such complexity, numerous clinical risk factors in have been established, acting by anatomical (such as hiatus hernia), mechanical (obesity) and physiological (caffeine) mechanisms (Findlay, 2013b).

1.1.2.3 Genetic contribution

This complexity therefore affords numerous mechanisms for genetic variants to influence susceptibility, via anatomical and physiological variation predisposing to more potent and frequent reflux, and differential physiological and inflammatory epithelial responses. Indirect evidence for this is provided by a small number of studies demonstrating associations between family history and susceptibility (Mohammed et al., 2003)). Direct evidence is limited to a small number of linkage and candidate studies, implicating variants such as those in the collagen type III alpha 1 (*COL3A1*) (Asling et al., 2009), and the interleukin 1 (*IL1*) genes (Izakovicova Holla et al., 2013). By extension, these and may therefore predispose to metaplasia, dysplasia and neoplasia. However, it is likely that additional variants affect subsequent progression, via separate mechanisms.

1.2 BARRETT'S OESOPHAGUS

BO describes columnar metaplasia of the oesophagus, and is defined as replacement of any portion of normal oesophageal squamous epithelium with macroscopically visible columnar epithelium, of either intestinal (IM), cardiac or fundal subtypes (Watson, 2005).

1.2.1 Epidemiology

Adult prevalence of BO in the Western World is estimated at 0.5-2.0% (Powell and McConkey, 1992). Its incidence is increasing and strongly linked to GORD (van Soest et al., 2005): a recent meta-analysis of 26 cohort studies calculated GORD symptoms to confer an

odds ratio (OR) for BO of 2.90 (Taylor and Rubenstein, 2010), in addition to associations with symptom severity and duration (Eisen et al., 1997). However, a third of patients with BO are asymptomatic (Eisen et al., 1997), highlighting the need for markers to help identify patients at risk BO and ultimately OAC. BO increases the risk of developing OAC by 30-125 fold, with an annual incidence of 1% (Wild and Hardie, 2003), with the incidences of both increasing dramatically in recent decades, most likely due to GORD and obesity (Mistry et al., 2011).

1.2.2 Clinical risk factors

With GORD the primary risk factor, there is inevitable overlap with factors discussed in section 1.1.2, including Caucasian ethnicity (Spechler et al., 1994), obesity (Cook et al., 2008) smoking (Smith et al., 2005b) and hiatus hernia (Andrici et al., 2013). However, there are conspicuous disparities, indicative of BO's complicated pathogenesis, including positive associations with advancing age and male gender (Spechler et al., 1994) and negative associations with aspirin (Singh et al., 2013) and statin use (Nguyen et al., 2014).

1.2.3 Investigation and diagnosis

The presence of columnar epithelium is identified endoscopically and confirmed histopathologically. This combination is only moderately sensitive (81%) and specific (82%) (Eloubeidi and Provenzale, 1999), although may be improved by techniques such as chromoendoscopy (Canto et al., 1996). Endoscopic assessment includes determining extent using the Prague 'C and M' criteria: the maximal circumferential extent from the GOJ (C), and length (M) (Sharma et al., 2006). For histopathological confirmation quadrantic biopsies every 2cm are recommended, although there are differences as to the need to demonstrate IM; in contrast to most other countries (Sampliner, 1998), this is not required in the UK as whilst IM is the subtype predisposing to malignancy, sampling bias may result in false negatives. (Hellier, 2005).

1.2.4 Management

The primary goals of management are to reduce the risk of progression to OAC, whilst increasing the likelihood of detecting it. Although incompletely understood, the risk of progression to low-grade dysplasia (LGD) appears to be up to 18.6% over 5 years (Katz et

al., 1998). The majority of patients then remain stable or regress, with up to 26.4% progressing to high-grade dysplasia (HGD) (Lim et al., 2007). However, the subsequent risk of overt progression to OAC exceeds 50% over 2-5 years (Buttar et al., 2001).

1.2.4.1 Medical management and surveillance

Acid suppression using proton pump inhibitors (PPI) reduces the risk of malignant progression, although metaplastic regression tends to be incomplete (Singh et al., 2013). Whilst anti-reflux surgery appears comparable in observational studies (Corey et al., 2003), it lacks a comparison with PPIs by a randomised controlled trial (RCT). Surveillance endoscopies are recommended in the United Kingdom (UK) every 2 years, and every six months in the presence of LGD (Loft, 2005).

1.2.4.2 Management of dysplasia

A number of endoscopic techniques have been developed, including endoscopic mucosal resection (EMR), radiofrequency ablation (RFA), photodynamic therapy (PDT), argon plasma coagulation and multipolar electrocautery. Eradication of HGD using EMR or RFA is recommended (American Gastroenterological et al., 2011), but their role in in LGD (and even non-dysplastic BO) is contentious.

1.2.5 Pathophysiology

Normal oesophageal squamous and BO epithelia are in balance between proliferation, differentiation and shedding, the primary source being basal epithelial stem cells (Croagh et al., 2007, Barbera et al., 2014).

1.2.5.1 Stem cell theories and expansion

Four theories predominate, describing response to squamous stem cell damage: the *de novo* theory suggests that this instigates a change in phenotype to metaplastic stem cells (Jankowski et al., 2000), the translational zone theory that stem cells at the GOJ colonise the distal oesophagus (Quante et al., 2012), the duct cell theory from oesophageal ducts

(Schmidt et al., 1999), whilst a fourth suggests the haematogenous spread of bone marrow stem cells (Sarosi et al., 2008).

These colonising stem cells typically establish metaplasia within 3 years (Atherfold and Jankowski, 2006), but importantly this (and by extension possibly OAC) contains multiple genetically distinct and competing clones of independent origin (Leedham et al., 2008).

1.2.5.2 Molecular mechanisms of metaplasia

Traditionally GORD was considered a single entity, progressing from symptoms to oesophagitis and ultimately BO (Fox and Forgacs, 2006). However, these may be in some respects distinct processes: notably, progression from one end of the spectrum to the other is rare, and only a minority of patients with GORD develop BO, highlighting a complex and only partly understood process (Vela et al., 2001).

Refluxed acid is the primary factor driving inflammation, potentiated by pepsin and bile acids, with the latter also acting independently (Nehra et al., 1999). Sufficient epithelial exposure causes apoptosis and necrosis, with concomitant inflammatory infiltrate and T cell dysregulation (Oka et al., 1992). Elevated levels of reactive oxygen species perpetuate this inflammation (Wetscher et al., 1995), and in combination with an anti-oxidant imbalance damage DNA (Thanan et al., 2012). Elevated levels of pro-inflammatory cytokines (such as TNFα, IL-1β, IL-8 and NF-κB) are evident in oesophagitis and BO constituting an 'inflammatory gradient' (Fitzgerald et al., 2002a) and a T2 helper cell-specific profile (Fitzgerald et al., 2002b). These activate transcription factors, such as the caudal related homeobox homeotic regulators *CDX1* and *CDX2*, with consequent columnar epithelial gene expression (Stairs et al., 2008). Of urgent relevance is the association between BO and obesity/metabolic syndrome beyond dietary and mechanical factors: adipose tissue (primarily visceral) acts as a depot of circulating pro-inflammatory cytokines (Lysaght et al., 2011), whilst elevated leptin levels drive the metaplasia-dysplasia-adenocarcinoma sequence via cyclo-oxygenase-2 (COX2) and prostaglandin-dependent activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) (Ogunwobi et al., 2006). Other molecular pathways involved in promoting a columnar phenotype include the bone morphogenic proteins (BMP), members of the transforming growth factor-β (TGF-β) superfamily, in particular, BMP4 (Milano et al., 2007).

1.2.6 Genetic predisposition

As with GORD as discussed in section1.1.2.3, the complexity of BO pathogenesis affords huge potential for genetic variants to modulate susceptibility. Although most cases appear sporadic (Drovdlic et al., 2003), evidence for genetic predisposition is provided by concordance, linkage and candidate studies, and most recently genome wide association studies (GWAS). Such associations may have an important role to play in precision management, beyond clinical factors which are presently insufficient to guide selective screening.

1.2.6.1 Concordance and linkage studies of Barrett's oesophagus

Traditionally, estimates of disease-associated variance have been made using concordance studies of related individuals (ideally twins). One such study estimated 43% of the GORD phenotype to be heritable (Mohammed et al., 2003), although none have been performed for BO.

Linkage studies screen the genomes of cases and controls using a panel of polymorphic markers, to identify shared regions and variants (Risch, 1990). However, whilst such 'familial' BO has been reported in up to 20% of 1st or 2nd degree relatives, and an autosomal dominant mode of inheritance with 10% penetrance proposed (Sun et al., 2010) (possibly involving the *MSR1*, *ASCC1*, and *CTHRC1* genes (Orloff et al., 2011)), it is unlikely that this level of risk is representative of sporadic BO (Verbeek et al., 2014). In contrast to those with a single gene inheritance pattern, most diseases are thought possess a complex polygenic and environmental aetiology. Consequently, the low effect sizes of complex disease variants are often insufficient to be identified via co-segregation and linkage analysis (Visscher et al., 2012).

1.2.6.2 Candidate studies of susceptibility variants to Barrett's oesophagus

Candidate studies test pre-existing hypotheses, to identify functional variants in genes known or suspected to be involved in a disease. Whilst this hampers the identification of novel biology, it does allow studies to be conducted using relatively small numbers of unrelated cases and controls (Kwon and Goate, 2000). Such variants include single nucleotide polymorphisms (SNP), short insertion or deletions, short tandem repeats (STRs) and germline copy number variants (CNVs). Other than CNVs, these variants are curated by the

dbSNP database, which provides data and reference SNP (rs) identification numbers (National Centre for Biotechnology Information, 2014).

A number of statistically significant associations ($p<0.05$) with BO have been reported for genes involved in metaplasia. These include variants involved in endotoxin handling (such a glutathione s-transferase P1 [*GSTP1*] (Bull et al., 2009) and pregnane X receptor [*PXR*] (van de Winkel et al., 2011)), homeotic regulation (*CDX1* and *CDX2* (Ren et al., 2014)), inflammation (such as the *IL1* cluster (Izakovicova Holla et al., 2013)) and the growth factors (such as epidermal growth factor [EGF] (Menke et al., 2012a)). However, variable study methodology, quality, and external validation potentially limit their utility. An aim of Chapter 3 was to identify and evaluate reported DNA sequence markers of BO susceptibility, by performing the first systematic review and meta-analysis, and addition to *in silico* replication.

1.2.6.3 Genome wide association studies

GWAS use the principle of linkage disequilibrium (LD), the non-random association of alleles (Reich et al., 2001)), to genotype thousands of cases and controls using arrays of 100,000-1,000,000 SNPs. The 'half-life' of LD (the distance from a SNP at which its correlation coefficient D' is <0.5) in the human genome is approximately 60 kilobases (kb) (Reich et al., 2001). Typically a SNP is therefore redundant, with 3-10 perfect proxies. Therefore, genotyping 500,000 such 'tagging' SNPs is sufficiently representative of the 10 million common SNPs known to infer up to a half of additive variation (Lango Allen et al., 2010). Due to the enormous number of comparisons involved, statistical significance is by convention taken at $p<5.00\times10^{-8}$.

This *a posteriori*, rather than *a priori*, tenet has many advantages: variants can be identified even if the pathogenesis of the disease is unknown or prohibitively complex, unsuspected variants and processes can be identified, whilst genotyping provides ancestry information, facilitating quality control.

Disadvantages include the inevitable fact that variants identified will probably be proxies for unidentified functional variants, although fine mapping of chromosomal regions, imputation of genotypes, annotation and functional studies can help resolve this. In addition, as rare variants are by definition not included on arrays, these will not be identified. Furthermore, well in excess of 10,000 cases and controls is required (Gail et al., 2008), underlying the pragmatic multistage approach employed within Chapter 3: a subset of samples are

genotyped on the genome-wide platform (discovery phase), with SNPs with the best evidence of association taken forward for focussed genotyping in the remainder (replication phases). This, and meta-analysis with similar studies mitigates in part considerable expense and workload.

However, whilst a number of loci have been identified outside BO, cumulatively these explain only a minority of disease heritability (Visscher, 2008). The location and nature of this missing heritability is unclear; however candidates include rare variants of moderate effect, undiscovered common variants of weaker effect (which may account for 45%, by not being detected statistically) (Yang et al., 2010a)), variants not tagged on arrays, epigenetics and inflated heritability estimates (Manolio et al., 2009).

GWAS in general suggest that most disease-associated variants exert a weak effect on risk—typically an OR <1.5 per allele (Manolio et al., 2008)—and are present in >5% of individuals (Hindorff et al., 2009): the 'common disease-common variant' (CDCV) model.

1.2.6.4 Common disease common variant model

The CDCV model suggests that the majority of trait-associated variance occurs at frequently polymorphic and conserved loci. These variants demonstrate low penetrance and effect individually, but multiple variants combine to influence disease risk, subject to genomic, epigenomic, transcriptomic and proteomic regulation (Reich and Lander, 2001).

1.2.6.5 Gene set enrichment analysis

A further advantage of GWAS is Gene Set Enrichment Analysis (GSEA; the first such analysis undertaken in BO being performed in chapter 3). Advantages include the identification of new biological pathways associated with disease, and partially offsetting the limitations of considering genes and variant in isolation, including obscuring variants by correction for multiple comparisons, and recognition that genes and variants do not function in isolation. GSEA tests defined sets of genes in association with a disease, considering whether the members of these are randomly distributed throughout significance levels, or whether they deviate from than that predicted by chance (Subramanian et al., 2005). An enrichment score quantifies a set's significance level, with the resultant p value corrected by adjustment for the number of genes in the set, and its false discovery rate.

1.2.6.6 Genome wide association studies and Barrett's oesophagus

Two GWAS of BO have been performed to date: the first (and the basis for Chapter 3), exclusively in BO by the Wellcome Trust Case Control (WTCCC) consortium (Su et al., 2012), and the second by the Barrett's and Esophageal Adenocarcinoma Consortium (BEACON) in a mixed set of BO and OAC, (Ek et al., 2013). Two intriguing variants associated with BO were identified by the WTCCC GWAS, in addition to 3 for either BO or OAC by the BEACON GWAS, suggesting roles in thoracic and oesophageal development, and inflammation. The primary aim of Chapter 3 was to identify additional germline markers of BO susceptibility via further replication of the WTCCC GWAS.

The WTCCC study identified rs9257809 within the major histocompatibility complex (MHC) (OR 1.21 [1.13-1.28]; $p=4.09x10^{-9}$) and rs9936833, an intergenic SNP 141kb from *FOXF1* (OR 1.14 [1.10-1.19]; $p=2.74x10^{-10}$). Both were subsequently associated with OAC by a small candidate study (Dura et al., 2013b). The association with rs9257809 in the MHC at 6p21, is in keeping with the inflammatory model of BO, although attribution of more specific function is complicated by long-range linkage disequilibrium (LD) within this region. rs9936833 lies within a regulatory region of transcription factors affecting *FOXF1* (a forkhead family transcription factor). Whilst *FOXF1* has not previously been associated with BO, a role in thoracic development is evidenced by haploinsufficiency, which predisposes to oesophageal atresia and tracheo-oesophageal fistula (Mahlapuu et al., 2001).

The BEACON GWAS identified 3 variants associated with either BO or OAC, reinforcing the role of embryology: rs2687201 (*FOXP1*), rs11789015 (*BARX1*) and rs10419226 (*CRTC1*), although none reached the genome-wide threshold for BO alone (Levine et al., 2013). *FOXP1* encodes a transcription factor involved in oesophageal and pulmonary embryogenesis (Shu et al., 2007); *BARX1* similarly contributes to trachea-oesophageal embryogenesis via the Wnt pathway (Woo et al., 2011); *CRTC1* is a transcriptional co-activator, although its role is not clear. All 5 variants were validated in independent cohorts within both studies, although none have yet been externally validated. However, the BEACON study found supportive evidence for the WTCCC SNPs rs9257809 and rs9936833.

Both GWAS also assessed heritability of BO. The WTCCC GWAS used concordance of variants across individuals and a disease score analysis. Both approaches suggested > 1,000 variants to contribute to BO phenotype. The BEACON GWAS estimated heritability at 35% via similar analysis.

1.2.7 The genetics of progression

A number of molecular features characterise the metaplasia-dysplasia-carcinoma sequence. These include progressive aberrations in established processes driving metaplasia, in addition to new abnormalities. BO epithelium is characterised by progressive genomic instability (GI) and clonal diversity, both of which increase risk of progression (Maley et al., 2006).

1.2.7.1 Genomic instability and Barrett's oesophagus

GI describes a state of failure of coordinated progression through the cell cycle due to erroneous DNA replication, damage repair, and chromosomal segregation, and results in an accumulation of errors driving tumorigenesis (Shen, 2011) It comprises both chromosomal (CIN) and microsatellite instability (MSI).

1.2.7.2 Chromosomal instability

CIN is traditionally evidenced by an aberrant number of chromosomes (DNA ploidy) via flow cytometry, (Grady, 2004), and is apparent in a quarter of cases of BO (Bird-Lieberman et al., 2012). It is described by the oncogene-induced DNA replication stress model (Halazonetis et al., 2008): activation of oncogenes by amplification or mutation—such as the signalling factor *ras*, human epidermal growth factor receptor 2 (*HER2,* also known as *ERBB2*), vascular endothelial growth factor (*VEGF*), and cell cycle regulator cyclin D1 (*CCND1*)—first destabilizes entry into the cell cycle (Hartwell and Kastan, 1994). Dysregulated DNA replication causes replicative fork stalling and collapse; imperfect pathways are then recruited to complete replication, resulting in the formation of DNA double strand breaks, a DNA damage response (DDR) and large-scale chromosomal aberrations: loss of heterozygosity (LOH) and variable ploidy.

Normally, an intact DNA damage checkpoint recognises these, invoking cell cycle arrest and apoptosis (Bartek et al., 2004). When this checkpoint is breached—for example by tumour protein 53 (*TP53*) mutations or LOH—cells escape apoptosis and proliferate, beyond their usual telomere (Hayflick) limit, with progressive CIN (Wright and Shay, 1992). Dysplastic BO epithelium is associated with an accumulation of cells in G1, S and G2 phases, representing both increased proliferation and loss of cell cycle control (Reid et al., 1993).

1.2.7.3 Microsatellite instability

MSI represents a hyper-mutable phenotype, due primarily to failure of the DNA mismatch repair (MMR) system on account of mutations or LOH (Grady, 2004). The MMR comprises enzymes (such as *MLH1* and *PMS1*) that recognize DNA mismatch: either incorrect nucleotide incorporation by DNA polymerase, or 'slippage' during replication of short tandem repeats (STR), resulting in insertions/deletions (indels). MMR failure results in widespread mutations of such sequences: microsatellites (Boland and Goel, 2010). Genes containing STRs are vulnerable, including the MMR system itself, cell cycle and apoptotic regulators (such as Bax, Pten and the caspases) and growth factors (such as insulin-like growth factor-2) (Duval and Hamelin, 2002). In contrast to colorectal carcinoma, however, MSI appears less prevalent, and of uncertain clinical significance in OAC (Farris et al., 2011).

1.2.7.4 Progressive somatic aberrations and the hallmarks of cancer

The ubiquity of GI in human cancer suggests it to be a driver and hallmark of cancer (Hoeijmakers, 2001). Such increased propensity towards the accumulation of non-silent mutations, LOH and CNV of oncogenes and tumour suppressor genes—such *CCND1* and *TP53* respectively—is frequently observed in BO and OAC (Arber et al., 1996, Reid et al., 2001). Providing these mutational events permit cell viability, they may confer clones with a natural selection advantage by enhancing or suppressing these driver genes, and therefore cancer hallmarks. Six such functional hallmarks were described by Hanahan and Weinberg: self-sufficiency in growth signals, insensitivity to anti-growth signals, and the abilities to avoid apoptosis, sustain angiogenesis, invade and metastasize (Hanahan and Weinberg, 2000), as well as evade immune surveillance (Kroemer and Pouyssegur, 2008). Interrelated metabolic hallmarks, typified by cellular stress have subsequently been proposed: oxidative, metabolic, mitotic stress, DNA replicative, DNA damage and proteotoxic stress, as well as GI (Negrini et al., 2010).

1.2.7.5 Molecular processes of neoplastic progression

A large number of molecular processes have been elucidated in BO carcinogenesis. These include increasing inflammatory cytokine levels alone the metaplasia-dysplasia-carcinoma sequence (O'Riordan et al., 2005), which suppress tumour suppressor genes, such as E-cadherin by tumour necrosis factor-α (TNF-α) which alters cell adhesion (Tselepis et al.,

2002), and activate oncogenes. Receptor tyrosine kinase oncogenes are commonly induced via the MAP-kinase pathway (Paterson et al., 2013) with consequent over-expression of growth factors such as *ERBB2*/*HER2* (Rauser et al., 2007*).* Subsequent activation of further pathways—such as that of signal transducer and activator of transcription 3 (STAT3)—is a relatively late event, which further augments an evolving anti-apoptotic phenotype (Zhang et al., 2011a)

There is also strong evidence for increasing expression of COX2 and downstream mediators such as prostaglandin E2 (*PGE2*) with advancing inflammation and dysplasia (Taddei et al., 2014): a key contributor to the 'inflammogenesis' of cancer (Harris, 2007). In BO carcinogenesis, COX2 promotes a T-helper cell 2 (Th2) cytokine profile, exerts an anti-apoptotic phenotype via survivin and the caspases (Li et al., 2011).

1.2.7.6 Candidate genetic studies of Barrett's oesophagus progression

An urgent research need is the ability to stratify risk of progression. A number of clinical and histopathological markers have been identified: male gender, increasing age, a longer length of BO segment, (Menke-Pluymers et al., 1993)), dysplasia, smoking, alcohol and worse GORD symptoms (Jankowski, 2005). However, these are insufficient in isolation. As suggested by genes involved in progression, a number of candidate molecular biomarkers have been reported. These include somatic genomic variants (such as *TP53* mutations and LOH (Reid et al., 2001) and CIN (Reid et al., 2000)), aberrant expression (such as TP53, CCND1 and E-cadherin (Younes et al., 1997)) and serum interleukin levels (Thrift et al., 2013).

A recent advance in screening and risk stratification is the Cytosponge: a non-endoscopic sampling technique, retrieving oesophageal cells via a swallowed capsule. These are tested for a panel of such markers including trefoil factor (TFF), cyclin A and TP53 expression, and DNA ploidy, as currently being evaluated in the BEST2 trial (Benaglia et al., 2013).

However, experience is much less than for susceptibility. Relatively few studies and markers (germline or somatic) of progression have been described. As for germline markers of susceptibility, a primary aim of Chapter 3 was to perform the first identification of novel markers from GWAS data. Similarly, a secondary aim was to systematically identify, appraise and meta-analyse all previously reported markers.

1.3 OESOPHAGEAL CANCER

1.3.1 Epidemiology

Oesophageal cancer represents a formidable global health problem, coupling a common aggressive disease with a rapidly increasing incidence. It has an estimated annual incidence of 500,000 cases, being the eighth commonest cancer yet the fourth commonest cause of cancer death (Ferlay et al., 2010). After seemingly curative treatment, just 30-40% survive 5 years (Rouvelas et al., 2005), indicative of profound limitations in our understanding of its pathogenesis, and ability to stage and select patients for management.

Worldwide squamous cell carcinoma (SCC) predominates, particularly in Turkey, Iran and China, and in Southern and Eastern Africa (Kamangar et al., 2006), with subtypes such as small cell and neuroendocrine carcinomas, leimyosarcoma and melanoma rare (Enzinger and Mayer, 2003). However, in the USA, Europe and Australia the incidence of oesophageal adenocarcinoma (OAC) has outstripped that of SCC (Lepage et al., 2008, Lagergren et al., 1999), representing a profound transition from a rarely reported disease to one of the commonest malignancies. Indeed the incidence of OAC has increased by almost 500% in 30 years (Brown et al., 2008), similar to AC of the GOJ (El-Serag et al., 2002). Globally, the UK has the highest annual incidence at 7 per 100,000 (Bollschweiler et al., 2001).

1.3.2 Clinical risk factors

SCC and OAC, including GOJ adenocarcinoma (AC), whilst anatomically related represent distinct diseases. SCC is predisposed to by chronic squamous epithelial irritation by carcinogens (including tobacco, alcohol, viral infections, irradiation and achalasia) (Ribeiro et al., 1996). By contrast the primary risk factors for OAC are GORD, BO, male gender, age and obesity, low fruit and vegetable intake and to a lesser extent tobacco smoking (Anderson et al., 2007).

1.3.3 Pathophysiology

1.3.3.1 Molecular biology of oesophageal and gastrooesophageal junction adenocarcinoma

The molecular aetiology of OAC has been discussed in section 1.3.5, constituting aberrant activation and expression of oncogenes, with suppression and inactivation of tumour suppressor genes via a variety of molecular pathways. These driver genes modulate a wide range of processes, which result in the attainment of the functional hallmarks of cancer.

Clinically and pragmatically, oesophageal and GOJ AC are considered together. Teasing out their biological differences is difficult: tumours may be oesophageal and encroach into the GOJ, or conversely arise from the gastric cardia and extend into the oesophagus. Somewhat arbitrarily, GOJ tumours are classified endoscopically according to Siewert and Stein by their relation to the cardia: type I tumours arise from the oesophagus and extend into the GOJ (1-5cm from cardia); type II are centred on the GOJ/cardia; type III are gastric tumours arising 2-5cm distal to the cardia and extending to the GOJ (Siewert and Stein, 1998). The rationale for this is reported underlying epidemiological, histopathological and management differences. Type I tumours typically arise in patients with BO, metastasise to thoracic and abdominal nodes, and are resected by oesophagectomy; type III tumours are by contrast are associated with subcardial IM and *Helicobacter pylori* infection, largely metastasise to abdominal nodes and are resected via extended total gastrectomy. Type II tumours may be associated with either site of IM, representing malignant degeneration in a short BO segment, or gastric mucosa, but are typically resected by oesophagectomy (Siewert et al., 2005).

However, whilst pragmatic this classification may not reflect the subtle complexity of these tumours. The increasing incidence of oesophageal and GOJ AC, in contrast to the reducing incidence of non-cardia gastric AC suggests shared risk factors and pathogenesis, such as obesity and GORD (Dolan et al., 1999). Similarly, the precursor lesions for all are IM (Chandrasoma et al., 2007), and whilst there are immunohistochemical differences (Sarbia et al., 2004), treatment and outcomes are similar. Consequently, GOJ carcinomas are typically considered and managed along with OAC, as in this thesis.

1.3.3.2 Molecular biology of oesophageal squamous cell carcinoma

The mechanisms underpinning SCC are fundamentally similar to OAC: initiating events and cumulative genomic aberrations cause progressive dysplasia, culminating in acquisition of cancer hallmarks. However, the precise mechanisms differ, due to underlying epithelial differences and the role of exogenous carcinogens.

A number of molecular abnormalities have been identified, similar to OAC; these include amplification (copy number gain or over-expression) of autocrinous growth factors such as *VEGF*, *ERBB1-4*, *hst-1* and *int-2*, (Wong et al., 1994, Kitagawa et al., 1991, Ozawa et al., 1989) and cell cycle regulators such as *CCND1* (Nakagawa et al., 1995), *p15* and *p16* (Mori et al., 1994). As with OAC, *TP53* mutations and copy number loss are common; however, a different mutational spectrum highlights involvement of exogenous carcinogens. In OAC, mutations are typically dinucleotide transitions caused by cellular damage, whereas in SCC dinucleotide transversions predominate (Hollstein et al., 1990).

1.3.4 Genomics

Both germline and somatic variants influence susceptibility to SCC and OAC, in addition to tumour behaviour and outcomes. As with BO germline variants have typically been assessed by candidate studies and GWAS, with somatic variants assessed using either comparative genomic hybridisation (CGH; differences in gene copy number, assessed by polymorphism arrays) and next generation sequencing (NGS).

1.3.4.1 Germline genetic variants and oesophageal adenocarcinoma

Evidence for genetic predisposition to OAC is largely inseparable from that of BO, as discussed in section 1.2.6. However, as not all patients with BO progress, it is likely that as well as shared variants predisposing to OAC by increasing susceptibility to GORD and BO, other variants modulate susceptibility to progression, as discussed in 1.2.7.6.

Examples of shared variants include those in genes involved in endotoxin handling (such as *GTSP1* (Bull et al., 2009) and *PXR* (van de Winkel et al., 2011) pro-inflammatory cytokines (such as *TNFα* (Menke et al., 2012b) and IL-18 receptor-accessory protein [*IL-18RAP*] (Babar et al., 2012)), and growth factors (such as the insulin-like growth factor family (McElholm et

al., 2010)). Curiously, however, the OR of these variants whilst similar for BO and OAC are not the same. In addition, a large number of associations have been reported tested with OAC in isolation, as reviewed by Lao-Sirieix *et al* (Lao-Sirieix et al., 2010).

This overlap was demonstrated conclusively by the BEACON GWAS, considering as it did both BO and OAC in isolation and together. This demonstrated the genetic basis of both to be similarly polygenic, with SNPs typically exerting similar effects. In contrast to BO, OAC heritability was estimated at 25% rather than 35%, although there was strong correlation of variants between the two (Ek et al., 2013). A subsidiary aim of Chapter 3 was to identify new possible variants associated with OAC.

1.3.4.2 Germline genetic variants and oesophageal squamous cell carcinoma

Even more variants have been reported to be associated with oesophageal SCC, primarily in Chinese populations. A number of GWAS have reported associations in the DNA repair gene Thymine-DNA glycosylase (*TDA*), the acute phase reactant mannose-binding lectin (*MBL*), the pro-apoptotic caspase 8 (*CASP8*) and the cellular signalling initiator phospholipase CE1 (*PLCE1*) (Yang et al., 2015). Notably, some of these also conferred susceptibility to GOJ AC in Chinese populations (Duan et al., 2015, Mai et al., 2013), although notably not in Caucasians (Dura et al., 2013a).

1.3.4.3 Comparative genomic hybridization and oesophageal cancer

A recent study employing CGH in 70 SCCs and 189 ACs found similarities in the frequency of both copy number gains of well characterised genes such as the oncogene growth factors *VEGFA*, *EGFR*, and *ERBB2*, and losses of tumour suppressor genes such as fragile histidine triad (*FHIT*), and SMAD family member 4 (*SMAD4*). However, a number of regions of disparity were identified, including oncogene gains such as cell dependent kinase 2A (*CDKN2A*), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*), and *CCND1* being more common in SCC, whilst *TP53* loss was more common in OAC (Bandla et al., 2012).

1.3.4.4 Next generation sequencing

Rather than sequencing nucleotides base by base as for first generation Sanger sequencing, NGS techniques use massively parallel processes to sequence millions of fragments simultaneously, for selected regions, the whole exome (WES), whole genome (WGS) or transcriptome (Grada and Weinbrecht, 2013). Whilst a number of platforms exist, they have a number of generic steps, as reviewed extensively by Meyerson *et al* (Meyerson et al., 2010) and Quail *et al* (Quail et al., 2012).

Initially, a template library is generated: the genome is fragmented typically into fragments a few hundred bp long enzymatically, mechanically or chemically; in WES the exome is enriched, with targeted sequences captured by hybridization (binding to nucleotide baits with tags for isolation). The library is prepared by blunting and 5′ phosphorylating fragment ends, prior to amplification via the polymerase chain reaction (PCR). These fragments are then 'read' (i.e. their sequences determined) by a variety of methods, including sequential nucleotide flooding, pH changes and fluorescence.

Data are then pre-processed: adapter sequences and low quality reads are removed, and fragments aligned to reconstitute a continuous sequence. By comparison with a reference genome (both generic and from 'normal' patient germline DNA, variants are identified. Inevitably, errors occur, but are offset by increasing the 'read depth': the number of fragments covering each base, as opposed to coverage—the proportion of bases covered at least once. The functional significance of variants is predicted, and significantly mutated genes of likely functional or driver effect identified by a variety of software tools, in addition to metrics such as mutational signature and copy number.

WGS currently costs approximately £500-£1,000 per sample. Consequently, WES is often performed, for the 180,000 protein coding regions constituting 1% of the genome (30Mb)(Ng et al., 2009). This 1% is greatly enriched for non-synonymous functional variants: 20% of these result in loss of function, 53% are mildly-deleterious, whilst just 27% are apparently neutral. Whilst WES does not identify variants in the non-coding portion of the genome (other than those immediately flanking genes), which may exert subtle regulatory effects, it allows 20 times as many samples to be sequenced for the same resources as WGS (Kryukov et al., 2007).

1.3.4.5 Next generation sequencing in oesophageal cancer

The first NGS study in oesophageal cancer was performed for 11 OACs and 12 SCCs by Agrawal *et al* from the USA and China in 2012. Using WES, the authors reported differences in mutational signature: A:T>C:G substitutions were more common in OACs, whilst C:G>G:C transversions and indels in SCCs. *TP53* was frequently mutated in both; 6 other genes were mutated in at least 25% of SCCs including the cell signalling oncogenes *NOTCH1* and *NOTCH3* and the cyclin-binding ubiquitin ligase *FBXW7* (Agrawal et al., 2012).

This was followed by Dulak *et al* who performed WES in 145 microsatellite stable OACs from the USA, with 15 also undergoing WGS (Dulak et al., 2013). 26 potential significantly mutated driver genes were identified, including 5 previously described (*TP53*, *CDKN2A*, *SMAD4*, AT Rich Interactive Domain 1A [*ARID1A*] involved in chromatin remodelling and *PIK3CA*) and four not: *PG20*, *TLR4*, *ELMO1* and *DOCK2*). The overall non-silent mutation rate was estimated to be 3.51 per Mb, with a mean 104 such mutations per tumour. A trend ($p=0.076$) towards A>C transversions at AA dinucleotides was noted in distal oesophageal versus GOJ AC, possibly indicating acid-induced mutagenesis (Dulak et al., 2013).

Chong *et al,* also in 2013, performed WES of 8 OACs, reporting predominant C>T and A>G transversions. Although no statistical analysis was performed, they identified recurrent mutations in driver genes such as *TP53*, *ARID1A*, *ATM*, *BRCA2*, *ERBB2*, *PIK3CA*, *PTEN*, *KRAS*, and *MSH6*). Lin *et al* performed WES of 139 oesophageal SCCs in 2014, similarly identifying previously implicated genes (*TP53*, *PIK3CA* and *NOTCH1*) and novel candidates (*FAT1*, *FAT2*, *ZNF750* and *KMT2D*) (Lin et al., 2014).

However, whilst heterogeneity between tumours and cell types has been assessed by these studies, none have assessed temporal heterogeneity: the evolution of cancers between different time points. Indeed, no studies involving solid organ cancers have assessed temporal heterogeneity before and after neoadjuvant therapy by NGS, or attempted to elucidate how these cancers respond to genotoxic therapies. This was the primary aim of Chapter 5.

1.3.5 Staging

The poor prognosis and high recurrence rates of oesophageal cancer highlight its aggressive and metastatic behaviour. Pre-treatment staging is therefore crucial in reaching the most appropriate management decisions: selecting patients with apparently 'curable' disease for radical treatment, whilst identifying those with 'incurable' disease to prevent such inappropriate and high-risk treatment.

Of those with seemingly curable disease in the UK, the primary role of staging is to identify patients amenable to resection—either endoscopic or surgical—without neoadjuvant therapy, and those with incurable disease. The latter may be due to direct invasion into unresectable structures, lymphatic metastasis outside a radical resection field (suggesting disseminated metastases), haematogenous and peritoneal metastases. This is reflected in the Union for International Cancer Control-American Joint Committee on Cancer (UICC-AJCC) tumour, node and metastasis (TNM) staging system. These were recently updated from the 6th to the 7th edition (Table 1.1). Most notably, T stage now distinguishes between mucosal and submucosal tumours, and those invading resectable and unresectable structures, whilst N stage incorporates number of involved nodes.

Table 1.1 Comparison of the 6th and 7th editions of the UICC-AJCC TNM anatomical staging systems

Component	TNM 6th Edition (Sobin, 2003)	TNM 7th Edition(Edge and Compton, 2010)
T stage	T0 not assessable T1 invading submucosa T2 invading muscularis propria T3 invading adventitia T4 invading adjacent structures	Tis Carcinoma *in situ* / HGD T1a invading lamina propria / muscularis mucosae T1b invading submucosa T2 invading muscularis propria T3 invading adventitia T4a invading pleura, pericardium, diaphragm, adjacent peritoneum T4b invading other adjacent structures, e.g. aorta, vertebral body, trachea
N stage	N0 no involved lymph nodes N1 involved nodes	N0 no involved lymph nodes N1 1-2 N2 3-6 N3 >6
M stage	*For upper third tumours* M0 no distant metastases M1a involved cervical nodes M1b other distant metastases For middle third tumours M0 no distant metastases M1a involved coeliac nodes M1b other distant metastases *For lower third tumours* M0 – no distant metastases M1 – distant metastases	M1 distant metastases
Stage groupings	I T1N0 IIA T2N0 or T3N0 IIB T1N1 or T2N1 III T3N1 or T4N0 or T4N1 IV M1	IA T1N0 IB T2N0 IIA T3N0 IIB T1N1 or T2N1 IIIA T4aN0 or T3N1 or T1N2 or T2N2 IIIB T3N2 IIIC T4aN1 or T4aN2 or T1-3N3 IV M1

1.3.5.1 Modalities for tumour thickness

Endoscopic ultrasound (EUS), using a linear echoendoscope, is the current gold standard modality. Puli *et al* demonstrated by meta-analysis of 49 studies sensitivity to range from 81.6% (CI 77.8-84.9) for T1 disease, to 92.4% (89.2-95.0) for T4 disease, with specificities 99.4% (99.0-99.7) and 97.4% (96.6-98.0) respectively (Puli et al., 2008a). EUS has limited utility in impassable tumours, although mini-probe EUS can determine T stage with 60% accuracy in such patients (Meister et al., 2013).

Multi-detector computed tomography (CT) using gas granules, oral and intravenous contrast generates a resolution of 2.5mm, although a lack of soft tissue contrast results in inferior accuracy (Onbas et al., 2006). No comparable CT meta-analyses have been performed, however, in a series of 75 patients Lowe *et al* reported a diagnostic accuracy of 43.0% (18.0-71.0%), compared with 71.0% (42.0-92.0) for EUS. Due to technical challenges (primarily motion artefact) magnetic resonance imaging (MRI) remains largely of research interest (van Rossum et al., 2015).

1.3.5.2 Modalities for nodal disease

EUS again represents the most accurate modality for determining local nodal involvement, using four criteria: size >1cm, hypoechoity, demarcation and round shape, (British Society of Gastroenterology 2004). In the Puli meta-analysis, EUS was 84.7% (82.9-86.4) sensitive and 84.6% (83.2-85.9) specific for TNM 6th Edition disease. However, the addition of fine needle aspiration cytology improves performance (Puli et al., 2008a).

Nodes are evaluated arbitrarily on CT by size (>1cm); consequently, CT again performs relatively poorly: a meta-analysis of 48 studies (although all performed before 2006) reported just 50.0% (41.0-60.0%) sensitivity and 83.0% (77.0-89.0) specificity (van Vliet et al., 2008). MRI again represents an evolving technology, with metrics of 38.0-62.0% and 68.0-85.0% respectively (Alper et al., 2011). However, there is evidence that positron emission tomography (PET) may have an important role, particularly when fused with CT (PET-CT).

1.3.5.3 Positron emission tomography

PET is a functional imaging technique, quantifying the biodistribution of a tracer, as a surrogate of biochemical processes (Phelps, 2000). A radioisotope is combined with a biologically active molecule, most commonly fluorodeoxyglucose (^{18}F-FDG), injected intravenously and taken up by tissues. Resultant avidity is detected by beta decay and usually quantified by standardised uptake value (SUV) (Porenta, 1994). This is then combined with anatomical data such as that from CT or MRI.

^{18}F-FDG is taken up by glucose-using cells and phosphorylated by hexokinase. The replacement of an oxygen atom by ^{18}F prevents further metabolism, and the molecule accumulates in the most functionally-active tissues. Cancer tissues are disproportionately avid, with up-regulated glycolysis due to loss of the Krebs cycle, and rapid cellular

proliferation (Weber, 1977). This is Warberg hypothesis: that unlike normal cells, cancer cells generate adenosine triphosphate (ATP) via non-oxidative glycolysis, even in the presence of normoxia (Warburg, 1956).

Consequently, PET-CT has considerable utility in oesophageal staging. For N stage PET-CT is considered inferior to EUS, as metastases may be non-avid, not visualised on CT or obscured by FDG uptake in adjacent tumour, whilst inflammation may cause false positive results (Bruzzi et al., 2007a). In the van Vliet meta-analysis, sensitivity was 57.0% (43.0-70.0) and specificity 84.0% (76.0-95.0) (van Vliet et al., 2008). However, PET may provide valuable functional information, and assess nodes too distant to be visualised by EUS.

1.3.5.4 Metastatic stage

Overt distant metastases are present in a significant minority of patients at presentation, most commonly nodal (54.0%), hepatic (35.0%), pulmonary (20.0%), skeletal (9.00%) and adrenal (5.00%) in one pre-PET analysis (Quint et al., 1995). Traditionally, these estimates were based on CT findings correlated with unresectable disease at surgery, estimated to be 46.1% sensitive and 73.6% specific (Luketich et al., 1999). However, high 'recurrence' rates most likely represent occult metastases that were present but undetected before treatment.

Beyond CT, EUS occasionally identifies hepatic metastases and peritoneal spread. However, PET-CT is the modality of choice: in the direct but historical comparison by Luketich *et al* in 1999, sensitivity was 69.0% and specificity 93.4% (Luketich et al., 1999). PET-CT can also assess equivocal lesions; overall Blencowe *et al* reported PET-CT to alter management in 38.2% of 238 patients, although definitions of what constitutes a 'change' in management are contentious (Blencowe et al., 2013), and more modest results have been reported (Noble et al., 2009, Gillies et al., 2011a).

Small peritoneal metastases are often poorly visualised radiologically, and so laparoscopy is routinely performed for tumours extending beneath the diaphragm and/or involving the GOJ. Its utility is less well quantified, but a recent review of 5 series (661 patients) reported the identification of occult metastases in 5% (Richardson and Khan, 2012). Unresolved questions are whether the lesser sac should be inspected, and lavage and cytology performed routinely.

1.3.5.5 Staging recommendations

There is consensus internationally as to staging algorithms: the Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland (AUGIS), the USA Society of Thoracic Surgeons (STS) and National Comprehensive Cancer Network, (NCCN) and European Society of Medical Oncology (ESMO) recommend CT and EUS for patients in whom potentially curative treatment may be both feasible and appropriate, followed by PET-CT for T1b-T4 disease (Varghese et al., 2013, National Comprehensive Cancer Guidelines, 2014, Stahl et al., 2010, Allum et al., 2011). In the USA and Europe, laparoscopy is reserved for T3/T4 disease involving the GOJ; in the UK it is also recommended for distal oesophageal tumours.

However, using all these modalities by default consumes resources, delays treatment, confers small but clinically significant risks, whilst in practice potentially not altering management decisions. The primary aim of staging is to partition patients into optimal management groups—for example, endoscopic versus surgical resection, neoadjuvant therapy versus resection alone, and curable versus incurable disease—rather than achieve optimal precision without altering management. It is not known whether patient subgroups exist for whom test utilities differ. Similarly, decision analytic metrics are not available to guide staging (i.e. whether a test's utility justifies its risk). Exploring these uncertainties was the aim of Chapter 6, to determine via decision theory whether this staging algorithm could be pragmatised on an individual patient basis, to retain optimal decision-making whilst improving efficiency and safety.

1.3.5.6 Restaging after neoadjuvant therapy

After neoadjuvant therapy re-staging before surgery is required to identify disease progression to distant metastases, and invasive unresectable disease, although such contemporary rates of progression are unclear (Jamil et al., 2008). Historically, most experience has been limited to CT alone, although intuitively, one would expect the staging superiority of PET-CT to translate to re-staging. Indeed, this is supported by a small study (n=48) in which PET-CT identified M1 disease marginally more frequently than either CT or EUS (Cerfolio et al., 2005).

However, this lack of evidence precludes recommendations, which are absent from the AUGIS and STS guidelines, whilst both ESMO and NCCN suggest that either CT or PET-CT

can be used (National Comprehensive Cancer Guidelines, 2014) (Stahl et al., 2010). The goals of Chapter 7 were firstly to quantify the rate of progression during NAC to unresectable disease, and to compare indirectly the utilities of CT and PET-CT in this regard within the context of decision theory. Secondly, to identify predictive factors for disease progression and use these to determine whether high or low risk patients could be identified.

1.3.6 Curative management

Curative management can be attempted either by resection—with or without neoadjuvant or adjuvant therapy—or by radical/definitive chemoradiotherapy.

1.3.6.1 Endoscopic resection

EMR is established for T1aN0 disease, in conjunction with surveillance and EMR or ablation of residual BO segments. The largest series reported to date (n=1,000) demonstrated complete resection plus a negative subsequent endoscopy in 96.3%. 14.5% developed local recurrence, in 85.5% of whom repeat EMR was successful. Overall EMR was successful in 93.8%, with minimal but significant morbidity in 1.5%, mainly due to bleeding or perforation (Pech et al., 2013).

Submucosal ER by contrast is not established in the UK, facilitating *en bloc* rather than piecemeal resection, and resecting T1bN0 disease. A recent meta-analysis (21 studies; 1,159 patients) reported a complete resection rate (R0) of 85.0% (95% CI 80.0-90.0%), associated with a minimal locoregional recurrence rate of 0.43%, but major complications in 6% (5% stenosis, 1% perforation) (Sun et al., 2014).

1.3.6.2 Surgical resection

The principles of surgical resection comprise resection of the primary tumour, lymphadenectomy and reconstruction. A number of approaches have been described: open procedures broadly include transhiatal (laparotomy with a cervical anastomosis) and transthoracic approaches. The latter include left thoracoabdominal oesophagectomy, and laparotomy combined with right thoracotomy (Ivor-Lewis oesophagectomy), with or

without neck dissection for lymphadenectomy and anastomosis (three stage McKeown oesophagectomy) (Pennathur et al., 2013).

Approach is guided by anatomical location of the tumour and nodal metastases, and surgeon preference. Due to the latter, direct comparisons are difficult, however, RCTs have compared transhiatal with left and right chest approaches, generally reporting more post-operative complications after thoracotomy, but trends towards improved long-term survival (Omloo et al., 2007).

Over recent decades, minimally invasive techniques have become increasingly used, as previously reviewed by the author (Findlay, 2013a). Overall, such techniques appear at least comparable to open surgery, with the sole RCT reporting fewer complications without oncological compromise (Biere et al., 2012). However, the dramatic differences seen in survival between R0 and R1/2 resections (microscopic or macroscopic residual tumour at the resection margin) suggest that the overriding goal of resection is to ensure an R0 margin.

1.3.6.3 Lymphadenectomy

The presence of nodal metastases is a major determinant of worse prognosis following resection (Mariette et al., 2008). Options for lymphadenectomy range from none, single field, two field (abdominal and thoracic stations) and three-field lymphadenectomy (plus cervical). Whether radical lymphadenectomy improves survival this has been challenged, however a recent analysis of 4,267 resections found more radical lymphadenectomy to be associated with improved survival of both node positive and negative cancers; indeed, all other than TisN0M0 and N3M0 disease (Rizk et al., 2010). In Europe and the USA, where distal oesophageal/GOJ AC predominates, two-field approaches are typical (Allum et al., 2011).

1.3.6.4 Neoadjuvant chemotherapy

The aims of neoadjuvant therapy are to downstage the primary tumour, thereby increasing the likelihood of a complete R0 resection, and to treat nodal and micrometastases outside the resection field. This is performed either by neoadjuvant chemotherapy (NAC) or chemoradiotherapy (NACR). However, not all patients will benefit; when considered as a cohort rather than individually, neoadjuvant therapy confers a 2-year survival benefit (Sjoquist et al., 2011). However, approximately half of tumours show either minimal or no

pathological tumour response (pTR) (Urschel et al., 2002, Campbell and Villaflor, 2010). For these patients, a period of ultimately futile therapy delays surgery, and may therefore allow disease progression and possibly confer a worse prognosis (Kelsen et al., 1998). In the absence of reliable mechanisms to identify these patients, a significant number pay the physiological and symptomatic cost of therapy at best deriving no benefit, and at worst progressing to incurable disease.

Whilst an in depth discussion of the evidence base is beyond this thesis, a recent meta-analysis of 9 RCTs and one 2x2 factorial study (n=2,062 patients) reported improved overall survival compared with surgery alone: HR 0.87 (79-0.96) (Sjoquist et al., 2011). Inevitably, however, there has been considerable heterogeneity as regards regimens and inclusion criteria. And notably, a significant benefit was demonstrated only for OAC, rather than SCC.

Largely on the basis of the MRC OEO2 trial, NAC has become the UK standard of care (Allum et al., 2011). This demonstrated significant benefit to two cycles of preoperative cisplatin and 5-FU (CF) (n=390), compared with surgery alone (n=397): overall survival (OS) hazard ratio (HR) 0.79 (0.63-0.93); disease-free survival (DFS) HR 0.75 (0.63-0.89) (Medical Research Council Oesophageal Cancer Working, 2002).

The subsequent MRC MAGIC (STO2) trial of gastrooesophageal AC randomised 250 patients to 3 cycles of pre- and post-operative epirubicin, cisplatin and 5-FU (ECF), and 253 to surgery alone. Twenty-six percent had either distal oesophageal or GOJ AC. Significant improvements in OS and DFS were seen (Cunningham et al., 2006), establishing the role of neoadjuvant ECF for GOJ tumours, with 3 further cycles post-operatively for type II and III tumours (Allum et al., 2011). The efficacy of epirubicin, cisplatin and capecitabine (ECX) compared with CF was explored for OAC in the as yet unpublished MRC OEO5 trial (MRC OE05 Clinical Trials Team, 2008).

However, there have been persistent disparities in stage inclusion in these trials: the MRC OEO2 trial had no restrictions (with not even CT mandated), whilst the STO2 trial included only T2N0 disease and above, and the OEO5 trial included T1N1 but not T2N0 tumours. This highlights an unresolved but crucial question: the stage threshold at which cohort benefit is derived, particularly whether patients with T2N0 disease benefit (Stahl et al., 2010). In general, as EUS is only 84% sensitive for nodal disease, however, NAC is typically considered for both T1N1 and T2N0 disease in most centres, including Oxford University Hospitals NHS Foundation Trust (OUH). However, the inadequate reflection of biological

behaviour by T and N stages suggests the role for functional makers, including PET-CT and genetic.

1.3.6.5 Neoadjuvant chemoradiotherapy

In contrast to the UK, NACR is the standard of care in the USA and Europe. In contrast to NAC, Sjoquist *et al* found significant survival benefits for both SCC and AC compared with surgery alone: OS HR 0.80 (0.68-0.93) and 0.75 (0.59-0.95) (Sjoquist et al., 2011). The recent CROSS trial also demonstrated similar benefits (van Hagen et al., 2012). However, there have been no comparisons between the two by a phase III RCT, although the Sjoquist meta-analysis reported a trend towards improved survival with NACR via an indirect comparison. A recent meta-analysis of 23 studies found no difference in post-operative morbidity or mortality (Kumagai et al., 2014). Again, there is no consensus on the utility of NACR in patients with T2N0 disease (Stahl et al., 2010). This is reflected in the on-going UK NeoSCOPE trial of two phase II NACR regimens (carboplatin plus paclitaxel, versus oxaliplatin plus capecitabine) for OAC, which excludes T2N0 disease. Ultimately, the optimal regimen will be taken forward to a RCT for comparison with NAC (Trials, 2014). Similarly, the NeoAEGIS multicentre phase III open-labelled RCT (University of Southampton Clinical Trials Unit, 2015) aims to compare CROSS NACR with MAGIC NAC for AC of the oesophagus and GOJ .

1.3.6.6 Definitive chemoradiotherapy

Although resection remains the mainstay of treatment in Europe and the USA, definitive chemoradiotherapy (DCR) represents a valid alternative, certainly for SCC (Smit et al., 2013). There have been few comparisons with surgery plus neoadjuvant therapy. Stahl *et al* randomised 172 patients to receive either NACR followed by surgery (n=86), or DCR (n=86) (Stahl et al., 2005). Progression-free survival (PFS) was improved in the surgery arm (HR 0.47 [0.29-0.77]), but at the expense of worse treatment mortality (12.8-v-3.5%), although entirely unrepresentative of current practice. In the UK DCR is recommended for proximal third SCC, and as an alternative for mid and distal third SCC (Allum et al., 2011).

1.3.6.7 Platinum chemotherapy agents

A full discussion of agents is beyond this thesis, and has been extensively reviewed by Klelland (Kelland, 2007). As well as cisplatin, a number of second-generation analogues have been described, including oxaliplatin. Cisplatin undergoes intracellular activation, before binding DNA to cause crosslinking, typically between adjacent nucleotides resulting in intra-strand adducts, but also between strands (Fichtinger-Schepman et al., 1985). The resultant DNA distortions are recognised by the DDR pathway, triggering activation of signalling pathways which shield adducts from replication, and promote recognition by cell cycle checkpoints (Jordan and Carmo-Fonseca, 2000). Nucleotide excision repair (NER) is attempted, but if unsuccessful apoptosis ensues. Analogues function similarly, but may possess greater antineoplastic activity and preferable toxicity profiles.

1.3.6.8 Anti-metabolites

5-fluorouracil (5-FU) is a fluoropyrimidine anti-metabolite in extensive use, acting synergistically with platinum cytotoxics, also administered orally as the pro-drug capecitabine (Schuller et al., 2000). 5-FU is activated by thymidine phosphorylase, inactivated by dihydropyrimidine dehydrogenase and has a number disruptive anti-metabolic effects (Longley et al., 2003).

1.3.6.9 Other cytotoxic agents

Other agents in use or evaluation include the anthracyclines doxorubicin and epirubicin, which damage DNA directly and indirectly via reactive oxygen species (Wang et al., 2001), the taxanes paclitaxel and docetaxel which bind cellular microtubules, disrupting cell cycle progression and inducing TP53 and apoptosis (Chien and Moasser, 2008), and the DNA topoisomerase II inhibitor etoposide which causes widespread DNA strand breaks, GI and apoptosis.

1.3.6.10 Targeted and novel immunotherapies

By contrast, targeted therapies interact selectively with molecular target(s) involved in neoplasia. Experience in oesophageal cancer has been limited, due to the lack of such

'smoking gun' driver genes or pathways. However, more than 130 targeted therapies exist, either in clinical use or development (Garnett et al., 2012), with many tested in early phase trials involving oesophagogastric cancer (Lin and Papadopoulos, 2007).

Most prominent are those inhibiting growth factors. Trastuzumab (Herceptin), an ERBB2 monoclonal antibody is the only targeted therapy recommended for oesophageal cancer as yet, improving PFS (Bang et al., 2010). The ongoing ST03 trial is currently assessing the efficacy of bevacizumab, a VEGF monoclonal antibody. (Chen et al., 2012). In addition, there is considerable interest in the use of immune checkpoint inhibitors, such as the PD-1 inhibitor Nivolumab, although their use has been confined to early phase trials of metastatic disease only (ONO Pharmaceuticals Lts, 2015).

1.3.6.11 Chemotherapy resistance

Again, whilst a full discussion is beyond the scope of this thesis most tumours demonstrate varying degrees of resistance, which is complex and multifactorial involving numerous levels of agents' mechanisms (Siddik, 2003). These may be macroscopic, for example tumour perfusion or proliferation rate, or molecular, modifying genetic, epigenetic or expression levels.

1.3.7 Assessing response to therapy

1.3.7.1 Histopathological response to neoadjuvant therapy

After surgical resection, neoadjuvant therapy response is assessed histopathologically. The original classification was described by Mandard *et al* in 1994, with pTR of oesophageal SCC to cisplatin-based NACR qualified by 5 tumour regression grades (TRG):

1 Complete regression: absence of tumour cells; extensive fibrosis
2 Incomplete regression: rare (<10%) tumour cells; predominant fibrosis
3 Partial regression: significant residual cells, but >50% fibrosis
4 Minimal regression: residual cancer outgrowing fibrosis
5 No regression: no fibrosis

DFS was independently and negatively associated with pTR, defined as TRG 1-3 (Mandard et al., 1994). Thereafter, the Mandard TRG has become established for a range of solid tumours. Similar classifications have been developed, primarily for rectal cancer but also used in oesophageal tumours as reviewed by MacGregor *et al* (MacGregor et al., 2012). These include the Royal College of Pathologists (Williams, 2007), and the Dworak classifications (Dworak et al., 1997).

However, classifying pTR remains relatively subjective and tempered by inter-observer variability. Response may also be heterogeneous spatially within tumours for unclear reasons, rendering TRG prone to sampling bias (Chetty et al., 2012). As well, nodal tumour response is not assessed. The significance of this is not completely clear: persistent nodal metastases has been reported as an independent marker of survival by some (Verlato et al., 2010, Mehta et al., 2013), but not all studies (Mandard et al., 1994, Hermann et al., 2006).

Unsurprisingly due to differences in patients, regimens and TRG, reported response rates vary considerably. Overall rates seem higher for NACR (Mandard TRG 1-3 approximately 15-20% each (Reynolds et al., 2007, Schneider et al., 2005)) compared with 7% Mandard TRG 1 and 33% TRG2/3 rates for NAC (Davies et al., 2014a).

Overall, survival associations of pTR have been variable; whilst most studies have unsurprisingly demonstrated Mandard TRG 1 to independently predict better survival, a number (although typically underpowered) have failed to demonstrate associations for TRG2-5 (Hermann et al., 2006, MacGregor et al., 2012). A further caveat is that the precise genetic and clonal mechanisms of pTR are unknown, as are those occurring between therapy and surgery (temporal heterogeneity). These questions were assessed in Chapters 5 and 7.

1.3.7.2 Radiological response to neoadjuvant and definitive therapy

Radiological surrogates are used for DCR, palliative therapies and interval response during or after neoadjuvant therapy. This is usually qualified using the Response Evaluation Criteria in Solid Tumours 1.1 for both primary and nodal tumour (Eisenhauer et al., 2009). For oesophageal cancer this is usually assessed by CT, but limited by the delay between tumour cell death and a visible reduction in volume. A meta-analysis of 3 studies calculated the pooled accuracy of CT for response to be just 54.0% (31.0-77.0%) (Westerterp et al., 2005). EUS has little utility, as it struggles to distinguish between tumour and fibrosis, and also determine resectability (Beseth et al., 2000).

PET-CT improves accuracy considerably, reported to be 86.0% (80.0-93.0%) by the above meta-analysis (n=4 studies). However, these studies used different thresholds of metabolic tumour response (mTR) reduction in maximum ^{18}F-FDG SUV (SUVmax). Consequently, the European Organization for Research and Treatment of Cancer (Young et al., 1999), and subsequently PET Response Criteria in Solid Tumours (PERCIST) classification were described (Wahl et al., 2009):

- Complete metabolic response (CMR): complete resolution of SUVmax
- Partial metabolic response (PMR): ≥30% reduction after ≥two cycles NAC
- Stable metabolic disease (SMD): change in SUVmax between +30% and -30%
- Progressive metabolic disease (PMD): ≥30% increase in SUVmax

As SUVmax represents only the maximal tumour focus in isolation, additional metrics have been used, including metabolic tumour volume and tumour glycolytic volume; however, SUVmax remains most commonly used (Tan et al., 2013).

1.3.8 Predicting therapy response and survival

A crucial goal of precision oncology is to tailor therapies to tumours. At present there are no reliable means to do so, other than tumour status of molecular target genes, and a greater likelihood of complete pTR with SCC (Merkow et al., 2012).

1.3.8.1 Genetic markers of therapy response

Many candidate markers have been reported, mainly of gene expression for oesophageal cancer, either in isolation or panels, although with limited utility (Schauer et al., 2010, Shimokuni et al., 2006). By contrast, DNA sequence markers have been less extensively investigated.

Potential candidates include germline and somatic variants modulating drug delivery, metabolism, activation and effect (Uemura and Kondo, 2014). Some studies have reported pTR to NAC, NACR or DCR to be less likely in *TP53* mutant tumours (Ribeiro et al., 1998, Yamasaki et al., 2010), although this has been refuted (Ito et al., 2001, Gibson et al., 2003). Similarly, germline variants in the NER gene *ERCC1* have been variably reported (Warnecke-Eberz et al., 2009). However, no systematic reviews or meta-analyses have been performed

to collate and prioritise markers. Consequently, the aim of Chapter 4 was to review, appraise, and meta-analyse the existing evidence base for DNA sequence biomarkers of stage, survival, recurrence, therapy response and treatment complications.

1.3.8.2 Radiological markers of therapy response

As discussed in section 1.3.7.2, mTR seems a reasonable surrogate of pTR. A series of papers from Munich demonstrated that interval SUVmax reduction after one cycle of CF NAC in 40 patients with OAC and GOJ AC was strongly associated with pTR. Receiver operator characteristic (ROC) analyses demonstrated that a threshold reduction of 35% was 93% sensitive and 95% specific for response (defined as Mandard TRG 1-2, but notably not 3) (Weber et al., 2001). This was partially validated in 65 further patients (80.0% sensitive, 78.3% specific (Ott et al., 2006)), and used in MUNICON phase II trial: 119 patients received one cycle of cisplatin/oxaliplatin plus 5-FU prior to interval PET-CT assessment. Non-responders proceeded to surgery, whilst responders continued with NAC. A dramatically worse DFS was evident in non-responders: HR 2.18 (1.32-3.62). mTR was 100.0% sensitive and 72.0% specific for Mandard TRG 1-2 (Lordick et al., 2007). However, this arguably represents a self-fulfilling prophecy, with inevitable misclassification, bias and worse individual outcomes, with some sensitive tumours not receiving NAC. No controlled trials have been performed, and the trial was notable by excluding Mandard TRG3. As well, no studies have refined or explored this SUVmax threshold. This was a primary aim of Chapter 8: to explore different thresholds and different PET-CT metrics of mTR in a much larger series, in addition to the novel concept of nodal response (mNR) developed in Chapter 7.

1.3.8.3 Genetic markers of prognosis

That only 30-40% of patients undergoing seemingly curative treatment (neoadjuvant therapy and resection) survive 5 years, and 50% suffer recurrence lays bare profound limitations in our ability to stage, identify micrometastatic disease and select patients for their most appropriate management (Rutegard et al., 2012, Rouvelas et al., 2005, Davies et al., 2014a). Half of patients therefore receive well-intentioned but futile radical treatment, which might prolong life but risks major medical complications or death (approximately 8% for NAC, and operative mortality and morbidity of 3% and 50% respectively) (Medical Research Council Oesophageal Cancer Working, 2002, Findlay et al., 2014d)), with often profound consequences for quality of life in at least the medium term (Scarpa et al., 2013, Djarv et al., 2014). Conversely, and less commonly, anecdotal evidence suggests a subset of patients with

seemingly incurable disease which is exquisitely chemosensitive and may thereafter be resected. There is therefore an urgent need for markers to predict survival and recurrence. Numerous DNA, mRNA, protein, epigenetic and metabolomic markers have been reported.

A range of germline polymorphisms have been reported, typically affecting the activity and expression of genes in a range of ethnicities. These include *TP53* and its suppressor *MDM2* (Cescon et al., 2009), *VEGFA* (Tamura et al., 2012), cell cycle regulators such as *AURKA* and *CCND1* (Pan et al., 2012), and inflammatory mediators such as *IL2* and *IL6* (Motoyama et al., 2012). Polymorphisms have also been associated with survival within the context of chemotherapy, such as those affecting *ERCC1*, *TYMS*, and *ABCB1* (Bradbury et al., 2009, Kaneko et al., 2011).

Fewer somatic variants have been reported, although coding sequence alterations in exons 5-8 of *TP53* have been associated with worse survival (Casson et al., 2003, Schneider et al., 2000), in addition to growth factor CNV such as *ERBB2* and *EGFR* (Prins et al., 2013, Marx et al., 2010). However, as with markers of pTR the majority derive from retrospective univariate candidate studies, few have been validated and none have entered clinical practice, and no reviews or meta-analyses have been performed: a consequent goal of Chapter 4.

1.3.8.4 Radiological markers of prognosis

As discussed in section 1.3.5, burden of disease at baseline is quantified using the TNM classification, in part to stratify prognosis. However, this was developed using pathological rather than radiological stage, and may be confounded by neoadjuvant therapy, in which subsequent pTR or downstaging is in reality the primary determinant of outcome (Rice et al., 2010b, Davies et al., 2014a).

However, PET-CT may have additional utility. A previous study of 121 patients by Gillies *et al* from the author's centre staged at baseline with PET-CT (n=103 undergoing resection) reported that whilst primary tumour SUVmax and FDG-avid length did not predict outcome, the presence of FDG-avid nodes did (Gillies et al., 2012). This lack of association with baseline SUVmax was corroborated, (Al-Taan et al., 2014, Brown et al., 2012), but by contrast SUVmax after neoadjuvant therapy has been shown to negatively associate with survival (Swisher et al., 2004). However, no studies have assessed the impact of FDG-avid nodes after therapy. Notably, whilst mTR dramatically influences survival, it is unclear whether this represents a surrogate of pTR or has independent utility (Ott et al., 2006).

1.3.8.5 Pathological markers of prognosis

The prognostic value of pathological stage after resection has been much more extensively investigated; progressive pT, pN and pM stage plus grade have all been associated with worse survival in the absence of neoadjuvant therapy (Talsma et al., 2012), as well as a positive resection margin (R1, both circumferential and longitudinal) (Pultrum et al., 2010). However, as discussed above after neoadjuvant therapy a major pathological marker of survival is pTR, with some studies also demonstrating pN stage (but not pT) to be independent (Davies et al., 2014b). A recent large series from London by Davies *et al* identified tumour downstaging (a reduction from clinical to pathological TNM 6th edition stage) following NAC to be the primary determinant of survival, as well as resection margin status; however, notably this was not compared with pTR (Davies et al., 2014a).

The aims of Chapter 9 were to better delineate and assess these radiological and pathological markers. It remains unclear whether mTR to NAC is an independent prognostic factor, and if so which metric is optimal; similarly, whilst pN stage is established, no studies have assessed the role of FDG-avidity after neoadjuvant therapy.

1.4 BIOMARKERS

A common theme of this thesis is the identification of biomarkers to guide precision oncology: DNA sequence markers of BO susceptibility and progression, and oesophageal cancer outcome and stage, radiological and pathological markers of staging and restaging outcomes, as well as those of NAC response, survival and recurrence.

1.4.1 Classification

Biomarkers are classified by the risk they quantify: exposure (such as toxins), susceptibility (germline polymorphisms), or effect (disease endpoints) (World Health Organization, 2001). In precision oncology markers must reliably, reproducibly and accurately predict a useful endpoint (Strimbu and Tavel, 2010). Effect biomarkers are classified further as 'prognostic' and 'predictive': 'prognostic' predict a disease's natural history irrespective of treatment, whilst 'predictive' predict response to a specific treatment (Buyse et al., 2010). However, in reality these characteristics are often inseparable: for example, a predictive marker for neoadjuvant therapy therefore inevitably also having prognostic utility.

1.4.2 Performance, validity and utility

Validation refers to establishing the effectiveness of a biomarker (its accuracy, reliability and generalizability) as a surrogate endpoint (Strimbu and Tavel, 2010). However, this implies that a biomarker is either valid or invalid, when in reality is a spectrum and context dependent. Evaluation may therefore be a better description (World Health Organization, 2001), although validation is used by convention throughout this thesis.

1.4.2.1 Sensitivity, specificity and receiver operating characteristic

It is important to determine whether a marker predicts an outcome reliably. Internal validation considers this within a particular study's population and context. External validation considers generalizability to other populations and contexts. A number of metrics exist, based on completeness of association with an outcome; the 'attributable portion'. For example, is a marker a pre-requisite, or does it contribute incompletely to risk? Sensitivity is the ratio of true to total positives detected, whilst specificity is the ratio of true to total negatives. In reality, a compromise between the two is best, and the most useful threshold or marker for this depends on the relative consequences of false positives or negatives (i.e. the clinical context).

This compromise can be visualized and quantified by ROC analysis. ROC curves plot the true positive rate (sensitivity) against the false positive rate (1 minus specificity) for a range of values. The area under this (AUC), also known as the *c* statistic is a measure of discrimination between cases with an endpoint, and those without (Cook, 2007). A completely accurate marker has an AUC of 1.00; one with no discriminatory ability 0.5; whilst one which is usually wrong has <0.5. By arbitrary convention, 0.75 is often used to define a 'useful' marker (Cook, 2007).

1.4.2.2 Positive and negative predictive value

Other metrics include positive (PPV) and negative predictive values (NPV). PPV is equal to the number of true positives divided by the total true and false positives. NPV, the number of true negatives divided by the total true and false negatives. When the pre-test probability of an outcome is equal, PPV and NPV can be interpreted as the post-test probability of and endpoint (Altman and Bland, 1994).

1.4.2.3 Statistical validation

The performance of a marker described in a study is its 'apparent performance'. However, markers, thresholds and models are inevitably biased by sampling: optimism bias or over-fitting (Bleeker et al., 2003). This can be quantified by internal, temporal and external statistical validation.

Internal validation can be performed in a number of ways. In split sample validation, a dataset is randomly divided into partitions. The marker is assessed in a 'development' set, before being tested in the 'validation' set (Picard, 1990). However, this means that the 'test' data cannot contribute to development, and is therefore lost.

To mitigate this, in cross validation data are partitioned as for split sample validation, with apparent and test performance metrics calculated and then repeated and averaged using each set in turn (for example, 10 times) (Steyerberg et al., 2001). However, the most accurate method, although usually applied to models with multiple biomarkers, is bootstrapping; an extension of cross-validation. A bootstrapped sample is equal in size to the original dataset, but composed of a variable portion of samples with duplicates (sampling with resampling). In regular bootstrapping, performance is calculated in the bootstrap dataset, evaluated in the complete dataset, and an average taken over a number of iterations, typically 1000-2000. In the 0.632 variant the bootstrap consists of 63.2% of cases (with resampling to reach the size of the complete set); the marker or model is assessed in the bootstrap and then tested in the un-sampled 36.8% by iteration. The validated performance is the mean of the estimated performance for a given iteration (0.632 x apparent performance + 0.368 x test performance) (Steyerberg et al., 2003).

Temporal validation is an extension of split sample validation; apparent performance is assessed in the development set, and tested in a subsequent cohort of patients. However, whilst independent, this cohort may still be subject to sampling and situational bias specific. The generalizability of a biomarker to other centres, populations and contexts can only be assessed by external validation, whereby the marker is assessed in a completely independent dataset (Altman, 2000).

1.4.2.4 Biomarker evidence quality

In 2001, Pepe *et al* described the 5 stages of biomarker development (Pepe et al., 2001), which can be used to assess levels of evidence (LOE). Systems include that of the American Society of Clinical Oncology (ASCO), which stratifies LOE from I-V on the basis of study quality and validation (Hayes et al., 1996). This was revised by Simon *et al* to recognise to value of retrospective analysis of data and tissue archived from prospective trials (Simon et al., 2009), and was used in Chapter 4. Greater criticism is afforded by classifications such as the REMARK guidelines used in Chapter 3 (McShane et al., 2005b).

1.5 AIMS OF THIS THESIS

This introduction has outlined the molecular pathogenesis of BO and oesophageal cancer, and highlighted the rationale and limitations of staging and management of BO and oesophageal cancer. The overall aim of this thesis was to explore the potential of genomic, imaging and pathological markers, to improve precision staging and management of these important conditions.

The first half of this thesis (Chapters 3-5) describes the role of genetics. This includes the identification and evaluation of variants and pathways associated with susceptibility to BO, dysplasia and OAC, as well as clinical endpoints such as pTR, treatment complications and prognosis. In addition, the effect of neoadjuvant chemotherapy on the OAC genome was assessed, to provide a model of genomic tumour response (gTR) and assess the implications for markers and targeted therapies. These aims were accomplished using further replication and analysis of a GWAS, a number of meta-analyses of existing data, and WES.

The second half of this thesis describes the role of imaging and pathological markers, including new concepts of metabolic nodal stage and response. Initially, a cohort of patients with oesophageal cancer before treatment was generated to ascertain the utilities of imaging

modalities in staging and restaging, and identify markers and models to generate pragmatic and more precise staging and restaging algorithms. Finally, those patients proceeding the NAC and surgery were analysed to comprehensively re-evaluate pathological and imaging markers of pTR, and their utility in predicting survival and recurrence after treatment.

CHAPTER 2

MATERIALS AND METHODS

2. CHAPTER 2: MATERIALS AND METHODS

2.1 CHAPTER 3: Genetic markers of susceptibility to Barrett's oesophagus, dysplasia and oesophageal adenocarcinoma

2.1.1 Patients and samples

Three association analyses were performed for: (1) susceptibility to BO, (2) progression to dysplasia/OAC, and (3) OAC irrespective of preceding BO. As described in 1.2.6.3 a number of cohorts were analysed: discovery (genome-wide), replication phases, and meta-analysis with the BEACON GWAS.

2.1.1.1. Susceptibility analysis cases and controls

Discovery cases were drawn the phase III Aspirin and Esomeprazole Chemoprevention Trial of Cancer in Barrett's Oesophagus (AspECT) RCT (table 2.1.1) (Oncology Clinical Trials Office, 2009). Controls: non-BO cohort of the WTCCC GWAS collaboration involving approximately 120,000 patients (Wellcome Trust Case Control Consortium, 2008).

Table 2.1.1: AspECT trial inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Age ≥18 years	Presence of HGD/OAC at enrolment
Performance status 0 or 1	Significant medical comorbidities: Ischaemic heart disease, respiratory disease, inflammatory bowel disease, recent severe stroke
Circumferential Barrett's metaplasia: ≥C1M1, ≥C0M2	Frequent use of non-steroidal drugs
Histological confirmation (not IM)	Contraindications to PPI / aspirin

Replication phase cases: epidemiological CHemoprevention of Premalignant Intestinal Neoplasia (ChoPIN) (Jankowski, 2009) and Esophageal Adenocarcinoma Genetic LinkagE (EAGLE) consortia (Jankowski et al., 2010), and three prospective observational registries: one Belgian (University Hospital Gathuisberg, Leuven) and two Dutch (Radboud University Nijmegen Medical Centre, Nijmegen; Academic Medical Centre Amsterdam, Amsterdam). All samples from the Leuven collection had confirmed IM.
Replication controls: 1958 Birth Cohort control data set (Winney et al., 2012), People of the British Isles (PoBI) collection (Power and Elliott, 2006), a prospective Irish Biobank of 2000

healthy volunteers (Trinity College Dublin) (Biobank), the CoRGI Consortium of colorectal carcinoma/adenomata (Tomlinson et al., 2007), and observational Genetics of LobulAr Carcinoma In situ in EuRope (GLACIER) study (Roylance, 2013).

Meta-analysed case control data: the BEACON GWAS (Levine et al., 2013).

2.1.1.2. Ethical approval

Ethical approval was provided by local ethical review boards/committees for all donating studies. The GWAS overall was approved by the East London and the City Research Ethics Committee (8th June 2004, 04/Q0603/1).

2.1.1.3. Progression analysis cases and controls

Data on progression were available for two cohorts, with limitations; as a primary endpoint of the AspECT trial, only progression status to dysplasia/OAC as of January 2013 could be obtained (analysis February 2013). For the CHoPIN study, progression status to OAC only.

2.1.1.4. Cancer analysis cases and controls

A cancer analysis (non-genome wide) was performed using cases (distal OAC/Siewert type 1) from cohorts with OAC cases: ChOPIN and Dutch cohorts. Population controls from the 1958 Birth, POBI, and Dutch cohorts.

2.1.2 DNA extraction and genotyping

2.1.2.1. DNA extraction

Extraction from whole blood was performed using the Maxwell 16 MDx elution buffer and cartridges and Maxwell 16 Clinical Instrument (Promega, Southampton, UK):

1. 300μL whole blood (thawed from -80°C) added to well 1 (of 7) of cartridge containing lysis buffer.
2. Nucleic acids purified by transfer through 6 wash cycles bound to magnetised silica particles.
3. Transferred to 300μL elution buffer in magnetic tube rack (to retain particles)
4. DNA solution extracted.

2.1.2.2. DNA quantification and quality assessment

DNA was quantified by spectrophotometry using the NanoDrop 2000 Instrument (Thermo Scientific, Wilmington, DE, USA), according to the manufacturer's instructions using 1μl DNA and 1μl 1xbuffer TE (blanking agent). Purity was assessed using the 260/280 (ratio ~1.8) and 260/230 absorbance ratios (2.0-2.2) and spectral curves.

2.1.2.3. Genotyping

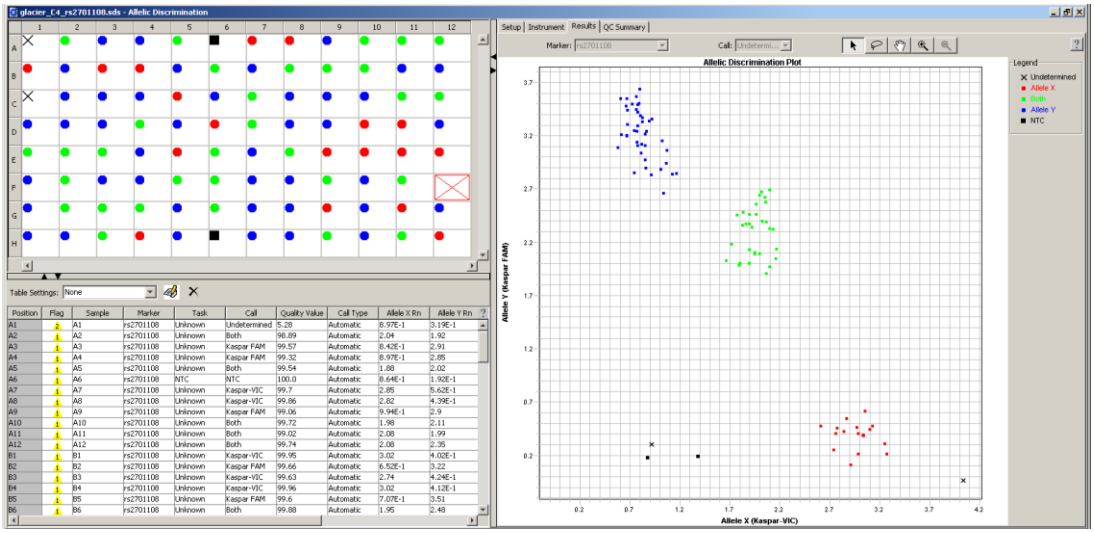
Genotyping was performed using techniques most appropriate to study stage.

Discovery samples: Illumina Human 660W-Quad and custom Human 1.2M–Duo arrays at the WTSI. Replication stage 1 and 2: Illumina Immunochip (a collaborative array comprising 195,524 SNPs, 2,400 contributed by discovery phase of this GWAS (Cortes and Brown, 2011)) or Illumina custom Human 1.2M-Duo array. Stage 3: at the WTSI using the Illumina Immunochip, Illumina Hap550, or three custom Sequenom iPLEXs designed using Assay Design Suite version 1.0 (Sequenom, San Diego, California, USA). BEACON samples: at the Fred Hutchinson Cancer Research Center (Seattle, Washington, USA) on the Illumina Omni1M Quad array.

2.1.2.4. KASPar genotyping

Manual genotyping was performed using KASPar (KBiosciences, LGC Genomics, Hoddeston, Herts, UK), a homogeneous fluorescent endpoint allele specific system based on fluorescent resonance energy transfer (FRET) with two components: a SNP-specific assay mix incorporating 2 competitive allele-specific forward primers, one common reverse primer, and reaction mix (two universal FRET reporting cassettes, and Taq polymerase) (KBioscience, 2013). Each forward primer (one for each allele) contains a tail sequence corresponding with one of the FRET cassettes. Resultant PCR results in corresponding emission is detected by a plate reader, following excitation and balancing using ROX™ passive reference. For heterozygous samples, an imbalance is detected, for homozygous samples a balance. Samples are plotted with clustering of genotypes. >99% concordance between Immunochip and KASPar assays were previously demonstrated (Su et al., 2012).

Figure 2.1 – A typical KASPar genotyping plot
Each plot represents an individual sample, with clustering in genotypes (blue common homozygous; green heterozygous, red rare homozygous; grey, no template controls).



PCR was performed using G-Storm Thermocyclers (G-Storm, Somerton, Somerset, UK), and 96 well semi-skirted PCR plates (Fisher Scientific [Thermo Fisher Scientific], Loughborough, Leicestershire, UK). KASPar was performed using the HT7900 Real Time PCR system and SDSv2.3 software (Applied Biosystems [Life Technologies™, Thermo Fisher Scientific], Paisley, UK). Signals were plotted using SDSv2.3; genotype clusters called automatically were confirmed graphically.

2.1.2.5. Primer design

Custom primers were designed using Primer Picker software (KBioscience, LGC Genomics, Hoddeston, Herts, UK), using Fasta DNA sequences from dbSNP (National Centre for Biotechnology Information, 2014), synthesized by Sigma-Aldrich (Gillingham, Dorset, UK) and Invitrogen (Life Technologies, Thermo Fisher Scientific, Paisley, UK) purified to 25nm desalt (salt-free solution; table 2.1.2).

Table 2.12. Primers used for KASpar genotyping

SNP	Allele	Sequence
rs6926118	A	GAAGGTGACCAAGTTCATGCTTCCTGAGGGTCAGTAGCTGAT
	G	GAAGGTCGGAGTCAACGGATTCCTGAGGGTCAGTAGCTGAC
	C1	CTCTGTAGAGTCTGATGAATTCTCATGAT
	C2	CTGACAACATTCAGTGTTTTGAAATGGAAT
rs41341748	A	GAAGGTGACCAAGTTCATGCTCCCACTGGAGAAAGTGGTCCAT
	G	GAAGGTCGGAGTCAACGGATTCCACTGGAGAAAGTGGTCCAC
	C1	CCTGGATGTATATCATCTATCTTCACAGTA
	C2	CTAAAATTACCATACCTATTGGACCTGGAA
rs6032951	G	GAAGGTGACCAAGTTCATGCTAAAATAAAAGCAATAGGCAGTTTTCGAAG
	T	GAAGGTCGGAGTCAACGGATTCAAAATAAAAGCAATAGGCAGTTTTCGAAT
	C1	CCTTCCATTAAATATTTCAATGGATTCCAT
	C2	GGATTCCATATTTTTGCTTAGGTGAAGAAT
rs2902637	C	GAAGGTGACCAAGTTCATGCTCAACCCATTCCCAGAATCATTCTC
	T	GAAGGTCGGAGTCAACGGATTCCAACCCATTCCCAGAATCATTCTT
	C1	AAAGGAAGCCCGGATCAAATATTTGCTTT
	C2	CCGGATCAAATATTTGCTTTCAAGGACTT
rs12985909	C	GAAGGTGACCAAGTTCATGCTGGTGGCCAATCAGCGTTCCC
	T	GAAGGTCGGAGTCAACGGATTGGTGGCCAATCAGCGTTCCT
	C1	GTGCTCTTTCTTTAACCAATCCCTGTA
	C2	CAATCCCTGTAGCCAGGGGGAT
rs9941024	C	GAAGGTGACCAAGTTCATGCTGGCAGTCAGAGCGACAGCAG
	T	GAAGGTCGGAGTCAACGGATTAAGGCAGTCAGAGCGACAGCAA
	C1	TTCTGGGTTCCCGCAGCACCTT
	C2	CCGCAGCACCTTGAACCCGTTT
rs7816766	C	GAAGGTGACCAAGTTCATGCTAAGAAATAAATTACAATATGCATTTACATTACC
	T	GAAGGTCGGAGTCAACGGATTAAGAAATAAATTACAATATGCATTTACATTACT
	C1	CTGTTGAAAGGTATCCATTTCCCTGATAA
	C2	CCCTGATAAATAAATAGGTAGCACATGTTT
rs189247	C	GAAGGTGACCAAGTTCATGCTCAGATGCCCATCAGAAAACCCAG
	T	GAAGGTCGGAGTCAACGGATTCAGATGCCCATCAGAAAACCCAA
	C1	CCTGACACTCCACAGATGGAAACAA
	C2	CAGATGGAAACAAACCTGGGAACCTT
rs2178146	A	GAAGGTGACCAAGTTCATGCTAATAAACAGGTAAGCAGGAAGGCCA
	G	GAAGGTCGGAGTCAACGGATTAAACAGGTAAGCAGGAAGGCCG
	C1	GCCCAGGAGAGCTGCCGTGA
	C2	GAGCTGCCGTGAGTGTGGTCAA
rs2701108	A	GAAGGTGACCAAGTTCATGCTGGCAGGAGAAAATGTGTACTCTCAT
	G	GAAGGTCGGAGTCAACGGATTGCAGGAGAAAATGTGTACTCTCAC
	C1	CCTCCCTGCCCTGCCTCCTA
	C2	CTGCCTCCTAGGTGACTCTGGAA
rs3784262	A	GAAGGTGACCAAGTTCATGCTCTGAGCACATAATCTGACTGGCAT
	G	GAAGGTCGGAGTCAACGGATTGAGCACATAATCTGACTGGCAC
	C1	CCTCTGGAGGAAAGGAATTTAAAAATAAAA
	C2	CAATTTTTTCCTCTGGAGGAAAGGAATTTA
rs4523255	C	GAAGGTGACCAAGTTCATGCTCATGAATCAGGATTAAAGAATCTGTGG
	T	GAAGGTCGGAGTCAACGGATTCATGAATCAGGATTAAAGAATCTGTGA

	C1	ACTAATGTCTCCATCCTGCACAATGAAA
	C2	CCATCCTGCACAATGAAAATGAACGTTAT
rs11771429	C	GAAGGTGACCAAGTTCATGCTCAAAGTTTATCGTAAAACTACAGGAGG
	T	GAAGGTCGGAGTCAACGGATTACAAAGTTTATCGTAAAACTACAGGAGA
	C1	AAAGCCAAAGGGCCTTGATAACATTAGTT
	C2	GGGCCTTGATAACATTAGTTTGGAAGATT
rs254348	A	GAAGGTGACCAAGTTCATGCTACAAGAAGGAGAGGATAAGGAGACA
	G	GAAGGTCGGAGTCAACGGATTAAGAAGGAGAGGATAAGGAGACG
	C1	CATGAGGGTCAGGCTGGGGAAT
	C2	CAGGCTGGGGAATGACAGAGGAT
rs1497205	A	GAAGGTGACCAAGTTCATGCTAGCCTATCGTCCCTCTGGTGT
	G	GAAGGTCGGAGTCAACGGATTGCCTATCGTCCCTCTGGTGC
	C1	CCATTATGAAGCACAGAGCACCTGA
	C2	CACAGAGCACCTGAGCCTATAAACAA
rs2342002	C	GAAGGTGACCAAGTTCATGCTGCAAATGTACCATCATAAAGAGTAGATG
	T	GAAGGTCGGAGTCAACGGATTAAGCAAATGTACCATCATAAAGAGTAGATA
	C1	AGATGAATGCACACATCATGAAGCAGTTT
	C2	ACATCATGAAGCAGTTTGTCAGATAGGTT
rs4800353	A	GAAGGTGACCAAGTTCATGCTAAAAGAAGACAGACCACAAAGACTGA
	G	GAAGGTCGGAGTCAACGGATTAAAGAAGACAGACCACAAAGACTGG
	C1	GCAATTTGCCCTGGGTCATTACCAA
	C2	CATTACCAAGCCACAGAATGGGGAT
rs6898743	C	GAAGGTGACCAAGTTCATGCTCAGTTTAGATCTGAGTACATACATACAG
	G	GAAGGTCGGAGTCAACGGATTCAGTTTAGATCTGAGTACATACATACAC
	C1	GGGCCGTACCATTTGCATGGAATAT
	C2	CATGGAATATCTTTTTCCATCACTTACCTT
rs2715425	C	GAAGGTGACCAAGTTCATGCTCATCACTCCAAATAGAAAACCTATCG
	T	GAAGGTCGGAGTCAACGGATTTTCATCACTCCAAATAGAAAACCTATCA
	C1	ATAGAGTGTGGAGGGGAATGGGAA
	C2	GTGGAGGGGAATGGGAATTATTCCAT

C1=common 1 primer; C2=common 2 primer

The KASPar protocol was as follows:

1. Assay mixes made using 12μl each allele-specific primers, 30μl common primer and 46μl H_20.
2. 1-2μl DNA mixed with 9μl master mix (comprising 5μl 2x KASPar reagent, 3.86μl H_2O, 0.14μl assay mix) and plated
3. Plates centrifuged (1000rpm for 15s)
4. PCR: initial 15 minute 94°C hot start Taq activation, followed by 20 cycles (94°C for 10 seconds, 57°C for 5 seconds, 72°C for 10 seconds
5. 10 extra cycles if required to maximise allele clustering.
6. At least one non-template control (1-2μl H_2O) included for all plates
7. For rare SNPs 2 positive controls were included
8. Optimisation using control DNA performed to identify optimal primer, PCR protocol and DNA volume for each SNP.

2.1.3 Analysis endpoints, inclusion criteria and quality control

2.1.3.1. Barrett's oesophagus susceptibility, progression and adenocarcinoma analyses

The analysis endpoint was cases with BO, versus controls without. Sample inclusion criteria were self-reported Caucasian ancestry, endoscopic evidence of BO, and age≥18 years. Sample exclusion criteria were, evidence of gender or ethnicity disparity in manifests or ChOPIN replication samples recruited to AspECT (i.e. duplicates). SNP exclusion criteria were: genotyping call rate<0.95, evidence of deviation ($p<1x10^{-5}$) from Hardy-Weinberg equilibrium (HWE) in controls, minor allele frequency (MAF) <1% or significantly different ($p<0.001$) between relevant cohorts, or information score<0.975. Identical quality control was used for the progression and OAC analyses

2.1.4 Association testing and meta-analysis

2.1.4.1. Principal component analysis

Principal component analysis (PCA) for the discovery phase has been previously described (Su et al., 2012), and is a multivariate technique for complex datasets with numerous measurements. It reduces these measurements or dimensions to mathematical components/combinations which best explain the data. In GWAS these represent useful measures of shared variance (such as ethnicity). The first principal component (PC; the best such explanatory combination) was significant when comparing cases and controls (suggestive of variance) and so was included as a covariate in the frequentist model used for association testing in discovery phase. The genomic control lambda (inflation due this variance (λ_{GC}) was 1.077 before and 1.068 after adjustment.

2.1.4.2. Association testing and meta-analysis

Case-control analysis was performed using frequentist tests under a missing data logistic regression model using SNPTEST v2.4.1 (University of Oxford, UK) (Wellcome Trust Case Control Consortium, 2007). Each SNP genotype was tested as a discrete variable, coded as

0, or 2 to allow a multiplicative model for allelic risk. Case status was the binary response. The first PC was included for BO susceptibility analyses, but not for progression (as no significant differences in PC were identified), or for the OAC analysis (as based on Immunochip rather than genome-wide data).

For meta-analysis across stages, inverse variance-weighted fixed-effect meta-analysis was performed using Genome Wide Association Meta-Analysis (GWAMA) v2.1 (WTCHG) (Magi and Morris, 2010). Missing data logistic regression models were applied. Two-sided p-values were calculated; normal distribution was assumed for combined data z-scores. Pruning of SNPs for LD was performed by interrogating the BROAD Institute SNP Annotation and Proxy (SNAP) database (Johnson et al., 2008). SNPs with $r^2>0.4$ were excluded; if<0.4 multi-SNP association testing (stepwise linear regression) was performed within chromosomes.

2.1.5 Fine mapping and functional annotation

2.1.5.1. Fine mapping and imputation

Fine mapping of SNPs was performed by Dr Claire Palles using IMPUTE2 (University of Oxford) (Howie et al., 2009) and discovery data (HapMap3 release 2 and 1000 Genomes Project 2009 release as reference panels) to impute the 1Mb flanking regions surrounding each SNP.

2.1.5.2. eQTL annotation

Annotation was performed (by the author) for identified and imputed SNPs and those in moderate LD ($r^2>0.4$) identified using the BROAD SNAP database and 1000 Genomes Pilot 1 eQTL browsers. Three databases were used: HaploRegv2(Ward and Kellis, 2012), Regulome DB (Boyle et al., 2012), and GENevar (Yang et al., 2010b).

For each candidate expression quantitative trait locus (eQTL) and additional variants in strong LD ($r^2>0.8$) HaploReg returns information regarding: sequence conservation (GERP and SiPhy scores); effects on regional promoter and enhancer chromatin state; effects on overlapping regulatory motifs; regional hypersensitivity to DNAase; protein-binding; and tissue-specific eQTL data, based on the Encyclopaedia of DNA Elements Consortium

(ENCODE) database (Consortium et al., 2012). GERP and SiPhy identify conserved areas by a disproportionate lack of nucleotide substitutions (Garber et al., 2009).

RegulomeDB annotates SNPs with known and predicted regulatory elements within intergenic regions. This is used to qualify likely functional effects, and quantify the strength of evidence for a variant affecting protein binding (table 2.1.3). GENevar (Yang et al., 2010b) provides 4 databases with eQTL data covering adipose, skin, fibroblasts, lymphblastoid, and T-cells tissue.

Table 2.1.3. RegulomeDB score: how likely a variant is to affect protein binding

Score	Supporting data
1a	eQTL+TF binding+matched motif+matched DNase footprint+Dnase peak
1b	eQTL+TF binding+any motif+Dnase footprint+Dnase peak
1c	eQTL+TF binding+matched motif+Dnase peak
1d	eQTL+TF binding+any motif+Dnase peak
1e	eQTL+TF binding+matched motif
1f	eQTL+TF binding/Dnase peak
2a	TF binding+matched motif+matched Dnase footprint+Dnase peak
2b	TF binding+any motif+Dnase footprint+Dnase peak
2c	TF binding+matched motif+Dnase peak
3a	TF binding+any motif+Dnase peak
3b	TF binding+matched motif
4	TF binding+Dnase peak
5	TF binding/Dnase peak
6	Other

TF=transcription factor

2.1.5.3. Gene expression and function data

The Human Protein Atlas v12 (Uhlen et al., 2010) was interrogated for expression data for genes flanking SNPs, containing immunohistochemical and mRNA expression data in cell types. Protein function was ascertained from the NCBI Gene (National Center for Biotechnology Information, 2014a) and Protein (National Center for Biotechnology Information, 2014b) databases.

2.1.6 Systematic review and meta-analysis of reported DNA sequence biomarkers of Barrett's oesophagus

2.1.6.1. Inclusion criteria

Studies eligible were those testing an association between a DNA sequence marker (germline or somatic) and diagnosis/progression of BO. Diagnosis was defined as endoscopic and histopathological evidence of BO (with or without IM); progression as subsequent progression from LGD to HGD/OAC, HGD to OAC, or recurrence of the above following ablative therapy. DNA sequence markers included SNPs, SNVs mutations, CNV, LOH, CIN (alterations in ploidy) and MSI.

2.1.6.2. Exclusion criteria

Non-English studies and those comparing metaplasia/dysplasia/malignancy within samples or between patients at one time point were excluded. Studies using cell line, epigenetic and expression data were excluded unless discrete patient or DNA-specific data could be extracted.

2.1.6.3. Literature search

A search was performed on 1st May 2014 of the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and EMBASE (www.embase.com) databases in accordance with MOOSE (Meta-analysis Of Observational Studies in Epidemiology) and PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines) (Stroup et al., 2000).

The following search term was used for all fields: (((((esophageal OR esophagus OR gastrooesophageal)) AND (Barretts OR metaplasia OR columnar)) AND (genomic OR genetic OR genome OR pharmacogenetic OR pharmacogenomic OR amplification OR copy OR mutation OR polymorphism OR polymorphic OR variant OR deletion OR insertion OR locus OR loci OR allele OR ploidy OR instability OR biomarker))). The references cited by retrieved articles were also assessed.

2.1.6.4. Study data

Data extracted were: methodology; variant(s) and gene(s); outcome measures; population; effect size (OR/HR) and variance (SE/CI). For studies not presenting OR, these were calculated using provided allele/variant frequencies. Gene names were standardized (HUGO Gene Nomenclature Committee) (Gray et al., 2013). Variants were mapped to dbSNP rs numbers (National Centre for Biotechnology Information, 2014) by searching referenced methodology, *in vitro* PCR using the UCSC Genome Browser (Kent et al., 2002) with specialized nucleotide flank (BLAST) Basic Local Alignment Search Tool (National Center for Biotechnology Information, 2013)); or New England Biocutter (NEBcutter v2.0 (Vincze et al., 2003))

Association data for SNPs identified were obtained from all GWAS phases possible. If not genotyped, the SNAP database was used to identify the best proxy SNP.

2.1.6.5. Evidence quality

Study quality and LOE was evaluated using the revised ASCO scale for biomarker research (Simon et al., 2009). Methodological quality was appraised using REMARK guidelines (McShane et al., 2005a) modified by the author (table 2.1.4)

For associations it was determined whether correction for multiple comparisons had been performed (Bonferroni method, false discovery rate [FDR], multivariate analysis including all variables) (Bland and Altman, 1995). If none had made, *post hoc* Bonferroni correction was performed. For GWAS variants significance was assumed at $p<5x10^{-8}$. Genomic quality criteria included: genotyping call rate (or providing adequate data to allow calculation) and assessment of HWE (germline variants) (Graffelman, 2013).

Table 2.1.4 Modified REMARK guidelines for study quality assessment

Criterion	1 point for each; 0.5 points if all sub-criteria not fulfilled
Introduction	
1	State: marker(s) examined; objectives; hypotheses.
Methods: patients	
2	Describe: characteristics of patients; their source, inclusion and exclusion criteria
3	Describe: treatment(s) and how chosen
Methods: specimen characteristics	
4	Describe: type of material used (including controls); methods of preservation and storage.
Methods: assay(s)	
5	Specify: assay method and provide/reference detailed protocol; whether and how assays were performed; and whether blinded to end point.
6	Specify: quality control procedures (e.g. HWE, call rate) if applicable; reproducibility assessments, quantitation methods, scoring and reporting protocols
Methods: study design	
7	State: method of case selection and whether prospective or retrospective; stratification or matching
8	Define all clinical end points.
9	List all candidate variables examined/considered
10	Give rationale for sample size, power and effect size.
Methods: statistical analysis	
11	Specify all methods, including adjustment for multiple comparisons
12	Clarify how marker values were handled; if relevant, describe methods for threshold determination.
Results – data	
13	Describe: flow and numbers of patients, including reasons for dropout Report: numbers of patients and events for subgroups
14	Report: distributions of basic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and marker, including missing values.
Analysis and presentation	
15	Show: relationship to standard prognostic variables.
16	Present: univariate analyses showing relationship between marker and outcome, with estimated effect. Preferably provide similar analyses for all other variables. For effect of marker on a time-to-event, provide Kaplan–Meier plot
17	For key multivariable analyses, report: estimated effects with CI; and, at least for final model all other variables.
18	Among reported results, provide estimated effects with CI from an analysis in which the marker and standard prognostic variables are included
Discussion	
19	Interpret results in context of the hypotheses and relevant studies; include discussion of limitations
20	Discuss implications for future research and clinical value.

2.1.6.6. Meta-analysis

Meta-analysis was performed using natural logarithms of OR/HR and SE using RevMan v5.2 (The Nordic Cochrane Centre, 2014) for markers assessed by more than one study (except variants assessed by both GWAS and non-GWAS studies due to major methodological differences). For SNPs, analysis was performed using the major common allele as reference using genotype permutations shared by all studies. In the case of A/T and C/G substitutions, the minor variant was confirmed from study allele frequencies. Heterogeneity was quantified using I^2 and Chi square estimates. For moderate heterogeneity ($I^2 \geq 50\%$) a random-effects rather than fixed-effects model was used. Heterogeneity and bias were also assessed by funnel plot asymmetry; (Sterne et al., 2011) visually for analyses ≥5 studies. Statistical assessment using Begg's and Egger's tests was not performed due to low number

of studies (Sterne et al., 2011). After considering alternative, potential publication bias was corrected using the 'trim and fill' method. (Sutton et al., 2000). Sensitivity analyses were performed on the basis of IM-only versus non-IM studies. In the case of studies reporting updated results using cohorts previously reported (i.e. population overlap), the most recent study was used.

2.1.7 Literature review to identify obesity-associated SNPs

2.1.7.1. Inclusion and exclusion criteria

All GWAS reporting variants associated with body mass index, waist:hip ratio and body fat percentage in a European Caucasian population were included. Studies/variants previously assessed were excluded (Su et al., 2012).

2.1.7.2. Literature search

The National Human Genome Research Institute (NHGRI) Catalog of Published Genome–Wide Association Studies (https://www.genome.gov/26525384) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) databases were searched on 3rd February 2014 using the search parameters (obesity) and (GWAS OR genome-wide association).

2.1.7.3. Data extraction and analysis

OR and p values were extracted from studies and the discovery dataset. If not directly genotyped, the best genotyped proxy was used (r^2>0.8, within <250kbp). For each, the forward strand minor variant (European population) was determined via dbSNP, for comparison of the concordance of risk allele effect with the sign test.

2.1.8 Gene set enrichment analysis

2.1.8.1. Pathway analysis, samples and endpoints

GSEA was performed for the BO phenotype (discovery genome-wide data), and sub-phenotypes of progression to dysplasia/carcinoma (discovery), and OAC+/-BO (replication stage 1, ImmunoChip). The latter included 481 cases (305 UK, 176 Dutch) with OAC (oesophageal and Siewert type 1) without BO genotyped but excluded from BO meta-analysis.

2.1.8.2. Gene set enrichment analysis platforms

Two platforms were used, using SNP-based and phenotype-based permutation respectively: iGSEA (Zhang et al., 2010)) and GenGen (Wang et al., 2007)). Input data for iGSEA were negative $\log_{10}$ p-values (for association) and rs identifiers; SNPs mapping within 500kb up or down-stream gene were assigned to that gene. Inputs for GenGen were Chi square statistics for discovery, generated by association testing using calculate_gsea.pl (http://gengen.openbioinformatics.org/en/latest/), plus Chi square statistics for 1000 phenotype permutations (calculated similarly). FDR<0.05 indicated significant, and FDR<0.25 possible, enrichment.

2.1.8.3. Gene sets and confirmatory set based testing

Sets were curated by the MSigDBv2.5 (BROAD Institute Molecular Signature Database; http://www.broadinstitute.org/gsea/msigdb/archived_releases/msigdb_march_2008/index.html): both canonical pathways and Gene Ontology (GO) biological processes. For pathways identified by both platforms (FDR<0.05/0.25), confirmatory set-based tests were performed with Plink v1.07 (Purcell et al., 2007). Each set of SNPs related to a gene set was tested in a case-control analysis; LD pruning was performed using default settings (r^2>0.5); all independently significant SNPs were retained with a mean set p value generated, repeated for 10,000 phenotype permutations. The resultant empirical p value represents the number of times the permuted set-statistic exceeds the real one; i.e. if p<0.05 corrected for multiple comparisons, the variants in the set are significantly associated with the phenotype.

2.2 CHAPTER 4: A systematic review and meta-analysis of DNA sequence biomarkers of oesophageal cancer survival, therapy response and stage

2.2.1 Literature search and data

The literature search, inclusion and exclusion criteria, outcomes and data-extraction techniques are described fully within section 4.2.

2.2.2 Evidence quality

Quality was appraised using the revised ASCO LOE (Simon et al., 2009).

2.2.3 Meta-analysis, heterogeneity and bias

Meta-analysis was performed as described in sections 2.1.6.6, and 4.3.1. Sensitivity analyses were performed for all analyses including 5 studies or more, whereby studies were omitted one by one.

2.3 CHAPTER 5: Whole exome sequencing of oesophageal adenocarcinoma before and after neoadjuvant chemotherapy: a new model of genomic response and its implications for precision oncology

2.3.1 Phase II study

This is described in Chapter 5.2.1. Ethical approval was provided by Oxfordshire Regional Ethics Committee (EudraCT number 2005-000834-34). All patients provided written informed consent, including for molecular analyses.

2.3.2 Tissue sampling and pathological assessment

This is described in Chapter 5.2.2.

2.3.3 DNA extraction and quality control

2.3.3.1. DNA extraction

DNA was extracted using DNeasy Blood and Tissue Kit (Quiagen, Manchester, UK) and stored at -80°C by Mr Richard Gillies (n=22 samples) and the author (n=8):

1. Lysis using 20μL 600 mili-Ansen Units (AU)/ml proteinase K in 1.5ml centrifuge tube; vortexed for 15s; incubated for 48 hours at 56°C, with intermittent vortexing. Further 20μL proteinase K added and cycle repeated.
2. 200μL buffer AL added; vortexed
3. 200μL 100% ethanol added; vortexed
4. Mixture transferred to mini spin column within 2ml collection tube, and centrifuged (8000rpm 1 minute).
5. Column inserted into new tube, 500μl Buffer AW1 added and centrifuged (8000rpm 1 minute).
6. Column inserted into new tube, 500μl Buffer AW2 added, and centrifuged (14,000rpm 3 minutes).

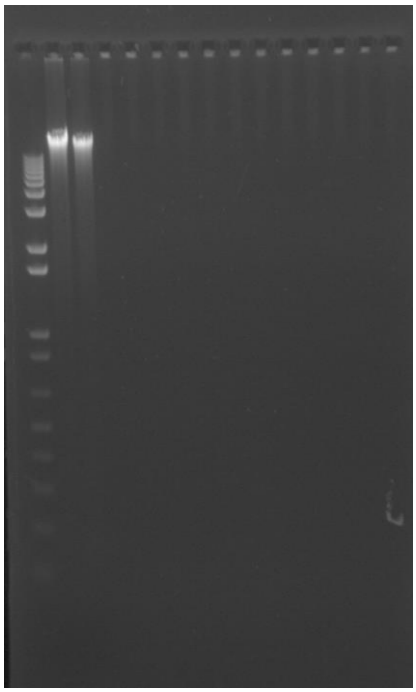
7. Column transferred to 1.5 ml microcentrifuge tube, 200μl Buffer AE added onto membrane, incubated (room temperature 1 minute) and centrifuged (8000 rpm 1 minute).

2.3.3.2. DNA electrophoresis

Quality was assessed using gel electrophoresis and spectrophotometry absorbance ratios (section 2.1.2.1). 14 well 1cm 0.7% agarose gels were prepared by dissolving 3.5 g agarose (all materials unless stated, Sigma-Aldrich, Gillingham, Dorset, UK) in 0.5L 1xTBE buffer; this was heated in a 800 Watt microwave for 3 mins, mixed, and heated for a further 2 minutes until completely clear. 8.5μl ethidium bromide was added and mixed, and the resultant gel was stored at 56°C. Gels were poured to size and allowed to set for 20mins.

2μl DNA was added to 8μl Orange G loading buffer. 10μl 1kb plus reference ladder was used. Electrophoresis was performed at 140V for 60 minutes, in 1xTBE buffer. Gel product was visualized using a ChemiDoc MP Universal Hood III (BioRad, Hercules, Ca, USA). Adequate quality was assessed by non-smearing of bands (figure 2.1).

Figure 2.1 Typical electrophoresis quality assessment of DNA for exome sequencing: 1kb plus reference ladder in left well, 1μL DNA in adjacent wells.



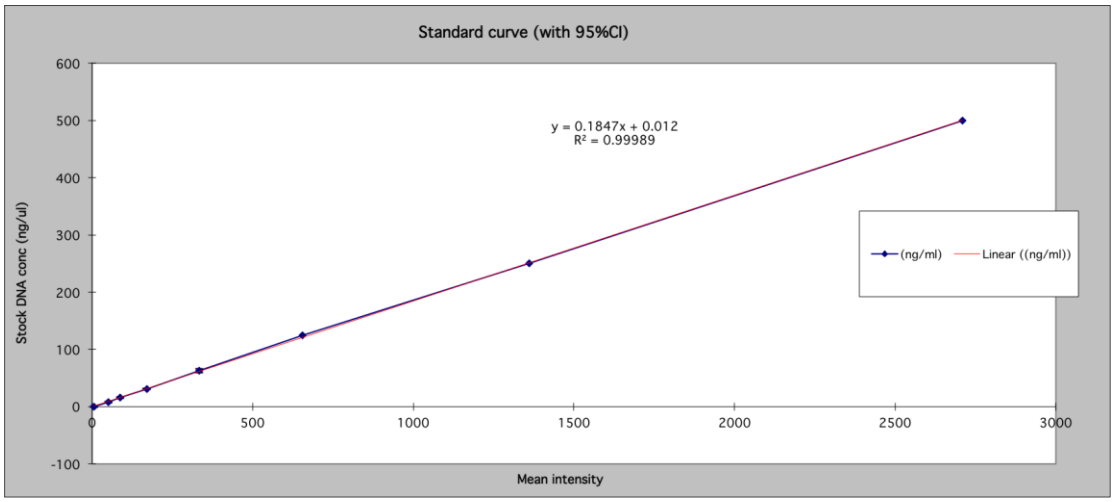
DNA was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen [Life Technologies, Thermo Fisher Scientific], Paisley, UK), containing 20xTE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5), dsDNA reagent (in DMSO) and lamda DNA standard (100µg/ml in TE), using modified manufacturer's instructions:

1. Reference curve generated by sequential dilution of DNA standard.
    - Seven 1.5ml Eppendorf tubes (Fisher Scientific, Loughborough, UK) labelled
    - 594µl 1X TE buffer added to tube 1; 300µl to tubes 2-7
    - 6µl DNA standard added to tube 1 (100x dilution: 1ng/µl) and mixed by vortex.
    - 300µl DNA dilution transferred from tube 1 to 2 and vortexed; repeated for the remaining tubes.
    - 50µl 1X TE added to wells A1 and A2 of a 96-well black fluorometer plate (Thermo Scientific, Wilmington, DE, USA), with 50µl of each tube 7 to 1 (ascending order of concentration) in remaining columns (e.g. tube 7 to B1 and B2, tube 6 to C1 and C2 etc.)

2. DNA sample preparation:
    - 1µl DNA mixed with 3µl 1X TE (4X dilution)
    - 3µl mixed with 42µl 1X TE (15X dilution)
    - 6µL of this mixed with 114µL 1X TE (20X dilution) to create 1200X dilution
    - 50µl added to black plate in duplicate for each sample (e.g. sample 1 in wells A3 and A4, sample 2 in wells B3 and B4, etc.)

3. PicoGreen
    - 1:200 dilution of PicoGreen reagent with 1X TE; 50ul added to each well
    - Plate covered with aluminium foil and incubated (room temperature, 5 minutes)
    - Read at 480nm excitation and 520nm emission using SPECTRAFluor Plus plate reader (Tecan, Mannedorf, Switzerland), including confirmation of standard DNA reference curve (fig 2.2)

Figure 2.2: Typical PicoGreen standard DNA reference curve



2.3.4 Exome sequencing

2.3.4.1. Library preparation, enrichment and sequencing

Sequencing was performed in two rounds of 20 and 10 patients (patients 1-20; 21-30). Library preparation, enrichment and sequencing were performed by the High-Throughput Genomics Group at the WTCHG. Libraries were constructed using the Illumina TruSeq and Nextera preparation kits respectively; exome enrichment was performed using TruSeq and Nextera exome enrichment kits (Illumina, San Diego, CA, USA) as per manufacturer's specifications. The concentration of each multiplexed library was determined by real-time PCR using Agilent qPCR Library Quantification Kit and a MX3005P instrument (Agilent, Santa Clara, CA, USA). Sequencing was performed on an Illumina® HiSeq2000™ instrument using 1000bp paired end reads.

2.3.4.2. Quality control, mapping and variant calling

These steps were performed by Dr Francesc Castro-Giner at the WTCHG, other than *post hoc* confirmation of sample identify (performed by the author). Reads were trimmed to the first base with quality score ≥20 at both ends and mapped to the Genome Reference Consortium human genome (GRCh) build 37 (Genome Reference Consortium, 2009) using Stampy (v1.0.21) (Lunter and Goodson, 2011) and BWA (v0.7.3a) (Li and Durbin, 2009). Duplicate reads were removed using Picard Mark Duplicates v1.86 (Institute, 2014a) and

local realignment around indels performed using Genome Analysis Toolkit (v2.5.2) (McKenna et al., 2010). Sample contamination with normal tissue was estimated using PurBayes v1.3 (Larson and Fridley, 2013). SNVs and short indels were called using Platypus (v0.1.0) (Rimmer et al., 2014) and MuTect (Cibulskis et al., 2013), with a threshold of either 5 reads, or 5% total reads for a sequence. Somatic variants were confirmed by comparison with normal. Variants within low mappability regions (Genome Multitool Mapper (Derrien et al., 2012)), within genome duplications (>1000 bases) or with a read depth $\leq$10 in normal samples were removed. Functional annotation was performed using SnpEff (GRCh37.70, v3.3h)[47].

CNV were called using Control-FREEC v6.0 (Boeva et al., 2011). For comparison of CNV between time points and tumours, a threshold p value of certainty of <0.01 was used. For exploratory comparison in candidate genes before and after treatment, $p<0.05$ was used.

Post hoc confirmation of sample identity was performed by assessing genotype concordance between normal, pre- and post-NAC tumour samples for a 22 SNP panel (Pengelly et al., 2013). Probability of discordance was calculated using 1000 genome allele frequencies. For all patients this was $<3.85\times10^{-31}$.

2.3.4.3. Driver mutation and pathway analysis

Candidate drivers were assessed using IntOGen Mutations v2.3.0 (Gonzalez-Perez et al., 2013) and MutSigCV v1.3.01(Lawrence et al., 2013). Functional annotation of genes was performed using the NCBI Gene database (National Center for Biotechnology Information, 2014a). The frequency of mutations in other studies was determined from The National Cancer Institute Cancer Genome Atlas (TCGA; n=184 WES OAC) (Institute, 2014b, Gao et al., 2013)), and International Cancer Genetics Consortium (ICGC; n=92 WES OAC) (Zhang et al., 2011b). Pathway analysis was performed using IntOGen OncodriveFM, for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Alterations in mutation and pathway frequencies were compared using Fisher's exact test with FDR values (fdrtool v1.2.12 (Klaus, 2014)).

2.3.4.4. Identification of biomarkers and therapeutic targets

Biomarker genes for OAC survival, recurrence and therapy sensitivity were identified from Chapter 4. Genes related to targeted therapies currently approved for clinical/trial use were identified from a 2012 systematic evaluation (Garnett et al., 2012).

2.3.4.5. Statistical analysis

All analysis was performed using R v3.0.2 (R Core Team, 2013). Median variant allele frequencies (VAF) were compared using the Mann Witney U test. Individual mutation frequencies were compared using Fisher's exact test. Exome-wide mutational frequencies and pathway enrichment were compared using a logistic regression model.

2.4 CHAPTER 6: Precision staging of oesophageal cancer using decision theory: selective endoscopic ultrasound, positron emission tomography and laparoscopy

CHAPTER 7: Restaging oesophageal cancer after neoadjuvant chemotherapy with ^{18}F-FDG PET-CT: utility, novel classifications of FDG-avid nodal stage and metabolic response, and predicting metastatic and unresectable disease

CHAPTER 8: Predicting response of oesophageal cancer to neoadjuvant chemotherapy using clinical, pathological and ^{18}F-FDG PET-CT biomarkers

CHAPTER 9: Predicting survival and recurrence of oesophageal cancer after surgery using clinical, pathological and ^{18}F-FDG PET-CT biomarkers

2.4.1 Study population and data collection

Consecutive patients with oesophageal/GOJ cancer staged beyond CT in OUH from May 2006-July 2014 were identified from 4 parallel prospective databases: a multidisciplinary cancer database (InfoFlex, Chameleon Information Management Services Ltd, Rickmansworth, UK), a departmental patient and operations database (Janus Clinical Audit and Research Database, OUH), and trust radiology database (Webpacs, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and laboratory database (Casenotes v5.0, OUH). Unless otherwise stated, all data were obtained by the author from these databases, and were those recorded contemporaneously. Non-routine data were generated/collected by colleagues were stated.

2.4.2 Clinical governance approval

Approval was provided by the OUH NHS Trust Clinical Governance Department (Service evaluation of oesophageal cancer staging; Clinical Audit Number 2516).

2.4.3 Staging

All investigations were reported and reviewed by a specialist multidisciplinary team (MDT) comprising consultant oesophagogastric surgeons, oncologists, radiologists, pathologists and gastroenterologists, in accordance with the contemporary AJCC TNM staging manual (6th (Greene, 2002) or 7th edition (Rice et al., 2010a, Gillies et al., 2011a) .

Patients without unequivocal metastases on CT were staged sequentially with ^{18}F-FDG PET-CT, EUS and laparoscopy of the greater sac (without routine peritoneal lavage cytology) plus OGD for GOJ tumours and distal oesophageal tumours extending below the diaphragm. NAC was considered for disease beyond T1N0; ER from 2008 for possible T1a tumours.

2.4.3.1. CT

CT was performed locally (n=367) or regionally (586) at 4 referring hospital trusts: Buckinghamshire Healthcare NHS Trust, Great Western Hospitals NHS Trust, Milton Keynes Hospital NHS Foundation Trust, and Heatherwood and Wexham Hospitals NHS

Foundation Trust using a standardized protocol (n=11 hospitals outside this usual referring region) involving16-128 slice systems, 0.5-0.675mm slice image acquisition, volumetric multi-planar reformatting with ≤2.5mm reconstruction from the neck to symphysis pubis. Examinations were performed prone following oral gas granules and contrast, with 100 ml of 300 mg/ml iodine portal phase intravenous contrast.

2.4.3.2. PET-CT

^{18}F-FDG PET-CT was performed in OUH using 2 scanners: before November 2009 using a General Electric Discovery STE 16-slice (scanner 1; 60 minutes post 400MBq ^{18}F-fluorodeoxyglucose [FDG]), and Discovery 690 64-slice system after (scanner 2; 90 minutes post 4MBq/Kg FDG), without intravenous contrast using standard iterative reconstruction. Examinations were reported independently by two dedicated PET-CT radiologists (Dr Kevin M Bradley, Professor Fergus V Gleeson.

2.4.3.3. EUS and ER

EUS and ER were performed in OUH using 5–10 MHz radial (n=800) or linear (2) echoscopes (Hitachi, Wellingborough, UK), by 1 of 3 consultants (Dr Barbara Braden, Dr Jane Phillips-Hughes, Mr Robert Marshall) and reported using the contemporary TNM edition and British Society of Gastroenterology guidelines (British Society of Gastroenterology 2004). Mini-probe was variably available. ER was performed using multiband ligation mucosectomy (Duette, Cook Medical, Limerick, Ireland). Nodal stage was determined using EUS, with fine needle aspiration cytology (FNAC) considered if the result would alter management.

2.4.4 Neoadjuvant chemotherapy

NAC was considered for disease beyond T1N0. Individual regimens varied with departmental practice and clinical trials at the time. Patients with oesophageal and GOJ type 1/2 tumours received either: CF (2 cycles; (Medical Research Council Oesophageal Cancer Working, 2002)), OF (2 cycles; (EU Clinical Trials Register, 2006)), ECF (3 cycles), ECX (3 or 4 cycles; (MRC OE05 Clinical Trials Team, 2008)), or EOX (3 cycles). Patients with type 3 GOJ tumours received ECX/EOX/ECF (3 cycles). Some patients (distal oesophageal/GOJ)

received 3 cycles of ECX pre-and post-operatively+/-bevacizumab (MRC Clinical Trials Unit, 2008), or 3 cycles of ECF pre- and post-operatively (Cunningham et al., 2006). For analysis, NAC variables comprised: regimen (grouped pragmatically as dual/triple therapy due to the number of regimens and small patient groups), and days between scans, and re-staging scan/operation.

2.4.5 Re-staging examinations

Patients were re-staged routinely in OUH using CT before 1st January 2008 and PET-CT afterwards (although some underwent CT due to clinical trial protocols), typically 4-6 weeks after completion of NAC. Metastatic disease was defined as that outside a radical lymphadenectomy field, either definitively identified on imaging with consensus of the MDT or requiring confirmatory biopsy/imaging.

2.4.6 Operations

Resections were performed open typically 2 weeks after re-staging, via left thoracoabdominal, Ivor-Lewis (right thoracotomy plus laparotomy), transhiatal and three-stage approaches (laparotomy, thoracotomy plus neck dissection) as previously described (Gillies et al., 2011b). A minimum two-field lymphadenectomy was performed; for mid/distal oesophageal/type 1 GOJ tumours this included subcarinal, aortopulmonary window and left gastric nodes; for distal/GOJ tumours left gastric and common hepatic artery nodes. Metastatic disease was confirmed via frozen-section histopathology. Unresectable disease due to local invasion or extent was confirmed by two consultant surgeons.

2.4.7 Pathological staging

Pathological staging data was converted to the 7th edition AJCC TNM staging if originally staged using the 6th. pTR was qualified using the Mandard TRG (Mandard et al., 1994). If pTR had been determined using an alternative TRG, Mandard TRG was determined retrospectively by Dr Lai Mun Wang using H&E sections.

2.4.8 Patient follow up

Patients were reviewed clinically at 2 and 6 weeks after surgery, 3 monthly for a year and 6 monthly thereafter. No routine imaging was performed.

2.4.9 Statistical analysis

Analysis was performed using R v3.0.2 (R Core Team, 2013). $p<0.05$ was corrected for multiple comparisons (Bonferroni method (Bland and Altman, 1995)) using individual tests and packages as described.

2.4.9.1. Regression

Multivariate logistic and linear regression included all variables following exclusion of perfect separators unless otherwise stated (final models). Continuous variables were assessed using kernel density plots and transformed to a normal distribution if required as follows: age^2; log: SUVmax, SUVmean, time to surgery, time to re-staging). Categorical variables were dummy-coded.

2.4.9.2. Decision analytic measures

Probability thresholds (Pt) were calculated to inform decision theory and models (Pauker and Kassirer, 1980). Test probability thresholds were calculated using sensitivity, false positive rate (FPR), treatment risk, net benefit and test risk for each investigation. These were taken from this study, or the highest level of evidence in the literature if not available. Decision curve analysis (DCA) was performed to calculate the effect of varying Pt reflecting variations in risk (Vickers and Elkin, 2006).

2.4.9.3. Predictive model development and tuning

Three modelling techniques were used: logistic regression (LR), decision tree analysis (DTA; recursive partitioning) and artificial neural networks (ANN). Recursive and partitioning trees partition data use dependent variables and misclassification costs to generate intuitive decision trees, and identify important interactions and subgroups of risk; however, they are

prone to over-fitting, and under-fitting ('ignoring') clinically plausible partitions (Nelson et al., 1998). LR, whilst less able to identify plausible subgroups, is optimal for linear associations. By contrast, ANNs can identify more subtle interactions between factors and multiple non-linear complex associations and subgroups, but at the risk of over-fitting (Tu, 1996). LR models were generated using backwards stepwise binary logistic regression (threshold $p<0.100$; Regression Modeling Strategies v4.1-0 (Harrell, 2013)). DTA was performed using Recursive Partitioning (rpart v4.1-3) (Therneau, 2013) incorporating loss matrices for misclassification. Feed forward back-propagation multilayer perceptron ANNs were developed using nnet v7.3-7 (Venables, 2002).

Model tuning to minimize over-fitting was performed for DTA by using the optimal complexity parameter (cp). For Chapters 6 and 7 (based on decision theory) manual pruning was performed if required for terminal nodes/partitions not resulting in a clinically meaningful split (defined as reducing the probability of the outcome of interest to within the 95^{th} percentile of the estimated P_t). ANNs were refined using tuning, variable dimension reduction, and overall model performance. Tuning determined the optimal hidden layer size and weight decay via 1000 bootstrapped samples (caret v6.0-24). Variable selection was performed by backwards stepwise elimination: variable importance was estimated for 1000 averaged ANNs (Gevrey, 2003); mean metrics were calculated for 1000 iterations of an ANN comprising all variables, repeated with sequential omission of the least important variable. When omission resulted in a significant reduction in either sensitivity or specificity (McNemar's t test; DTComPair v1.0-3 (Stock, 2014)), the optimal inputs were those comprising the preceding model).

2.4.9.4. Model validation and performance assessment

LR models were internally validated using bootstrapping (1000 iterations, 0.632 sampling and replacement (Steyerberg et al., 2001); Regression Modeling Strategies v4.1-0 (Harrell, 2013)). DTA was internally validated using Breiman and Cutler's Random Forests (randomForest v4.6-7)(Liaw, 2002), using 500 iteration trees with 0.632 bootstrapping. ANNs were validated using 0.632 bootstrapping with 1000 iterations (caret v6.0-24(Kuhn, 2014)). Temporal validation was performed in the validation datasets described.

Performance was quantified using: calibration (observed:expected [OE] ratio); accuracy (Brier and Kappa scores); discrimination (ROC AUC (Robin et al., 2011)); utility (sensitivity,

specificity, PPV, NPV], and Peirce's net benefit (Peirce, 1884)). Sensitivities and specificities were compared using McNemar's t-test (DTComPair v1.0.3 (Stock, 2014)).

2.4.10 Chapter 6-specific analyses

2.4.10.1. Data

Data collected were: patient (age at diagnosis, gender), endoscopic (impassable at OGD) radiological (CT, EUS, PET-CT TNM staging; PET-CT SUVmax, length, presence of avid nodes; additional PET-CT findings, subsequent investigations), pathological (pre-treatment cell type, grade (Compton, 2005); pathological stage, grade) and MDT management decision. Tumour anatomical location was determined endoscopically, or by PET-CT if impassable. CT T stage was grouped pragmatically as Tx/possible T1 (early), definite T2-T4a (locally advanced) and possible T4b; FDG-avid local nodes as separate from the tumour within a standard lymphadenectomy territory (SUVmax >2.5 /subjectively >background activity). For FDG-negative tumours, a background SUVmax of 2.5 normalised to mediastinal blood pool and length of 0cm were assigned; for sub-1cm foci, a length of 1cm.

Two TNM editions were used within this period; N and overall stage were classified using the 6th (insufficient data being available for conversion to the 7th). Management was uniform for both (for example, patients with 6th edition M1a nodes were considered for radical treatment).

2.4.10.2. Primary test utilities for decision theory calculations: EUS T1N0 disease

Sensitivity and FPR for pT1N0 disease were those calculated within this study (as no meta-analyses have reported metrics for T1N0 disease); treatment risk was defined as risk of major complications of EMR (1.5% (Pech et al., 2013)); test risk as perforation rate from this study (0.14%); net benefit of detection as the probability of EUS T1aN0 disease (50.0% in this study) x that of avoiding surgery (93.8% (Pech et al., 2013)), plus the probability of EUS T1bN0 disease x that of avoiding major complications of NAC (7.75%(Medical Research Council Oesophageal Cancer Working, 2002)).

2.4.10.3. EUS T4b disease

As patients with T4b disease do not undergo resection, T4 was used as a surrogate (Puli et al., 2008a): sensitivity (100.0%) and FPR (2.5%); treatment risk (palliative intent) and test benefit were assumed by necessity to be equal (100%).

2.4.10.4. PET-CT

Risk of whole body ^{18}F-FDG PET-CT was the additional lifetime risk of cancer for a 60-year old exposed to a 13mSv radiation dose (0.067%)(Radiation, 2006), adjusted for the approximate probability of 5 year survival (total=0.033%)(Rouvelas et al., 2005, Jamieson et al., 2004); FPR for metastases was assumed by necessity to be 0.00%, as PET-CT+/-additional confirmatory tests represents the gold standard for and no contemporary data are available for estimation; similarly, sensitivity could not be meaningfully estimated and assumed to be 100.0%); net treatment benefit was that of approximate 2 year survival following NAC and surgery (40.0% (Gebski et al., 2007)) treatment risk was risk of in-hospital operative mortality within OUH (2.45%), previously calculated for a subset of these patients by the author (Findlay et al., 2014c)).

2.4.10.5. Laparoscopy

Risk was assumed to be the major complication rate of staging laparoscopy without feeding jejunostomy from our series (0.29%), FPR 0.00% (assuming all metastases are histopathologically confirmed), sensitivity 75.7% (de Graaf et al., 2007), and net benefit of detecting metastases 100%; net risk of treatment (palliative management) was not relevant (as FPR=0.00%).

2.4.10.6. Decision curve analysis

DCA was performed to calculate the effect of varying P_t (0.496-2.719%) reflecting EUS perforation risk (0.02-1.00%)(Vickers and Elkin, 2006).

2.4.10.7. Cost analysis

Relative costs of algorithms were calculated using 2013-2014 NHS departmental tariffs for each potential component (NAC with 2 cycles CF), as for P_t (Department of Health, 2013).

2.4.10.8. PET-CT metric threshold calculation and comparisons

ROC and optimal thresholds were calculated with pROC (Robin et al., 2011); 95% confidence intervals were calculated using 200 iterations of 0.632 bootstrapping. Comparative sensitivities and specificities were compared using McNemar's test (DTComPair v1.0.3.(Stock, 2014))

2.4.11 Chapter 7-specific analyses

2.4.11.1. Data

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Pre-treatment variables were as described in section 2.4.8.1. PET-CT variables for all patients comprised: primary tumour FDG-avidity (SUVmax, length [cm]), number of FDG-avid local nodes (visible separately from the tumour, within a standard lymphadenectomy territory, with SUVmax>2.5 or background), involved body compartments (neck, thorax, abdomen), and SUVmax of the most avid node.

For examinations using the second PET-CT scanner, additional variables were generated retrospectively (by Dr Jamie M Franklin and Dr Eugene J Teoh): metabolic tumour volume (MTV), SUVmean, SUVpeak, and tumour glycolytic volume (TGV) mean/max. MTV was measured using a fixed threshold technique (SUV$\geq$4). TGVmean was calculated as the product of MTV and SUVmean. TGVmax was calculated as the product of MTV and SUVmax. mTR was quantified using percentage reduction in avidity (ΔSUVmax, length) and PERCIST criteria (SUVmax)(Wahl et al., 2009). mN stage and response were developed and defined as described within Chapter 7. When considering dynamic PET-CT variables, only patients staged and re-staged using the same scanner were considered.

2.4.11.2. Decision analytic measures and cost analysis

Restaging PET-CT Pt and cost-analysis were calculated using sensitivity for metastatic disease in this study (overt metastases plus unsuspected unresectable disease 39.5%); test risk (lifetime attributable cancer risk, as in section 2.4.8.4); net treatment benefit of metastases (avoidance of futile surgery;100%); treatment risk: inappropriate palliative management (similarly 100%); and FPR (assumed by necessity to be 0.00%). For LR models for which a test P_t was not applicable (predicting unresectable disease), the optimal classification threshold was determined using pROC (Robin et al., 2011). No P_t for additional investigations to identify unresectable disease or inform models could be calculated, as a number of options for re-staging exist and vary on an individual patient basis.

2.4.11.3. Predictive modelling for interval progression to incurable disease

Models were derived for progression to metastases on restaging examination, or the finding of unresectable disease at surgery. Models were developed using patients staged and re-staged using the more recent PET-CT scanner, validated internally, and independently using patients staged and re-staged using the earlier scanner.

2.4.12 Chapter 8-specific analyses

2.4.12.1. Data

Pre- and post-treatment variables were as described in section 2.4. Mandard TRG≤3 constituted pTR, following validation of this threshold as described in Chapter 9. Down-staging response was that as defined by Davies *et al*: a reduction from clinical to pathological TNM 6th edition stage (Davies et al., 2014a).

2.4.12.2. Models predicting pathological tumour response

LR, DTA and ANN were tuned, generated and validated internally (bootstrapping) using the development group (patients staged and re-staged using the more PET-CT recent scanner), and validated independently (patients staged and re-staged using the earlier scanner; validation group).

2.4.13 Chapter 9-specific analyses

2.4.13.1. Survival analysis

Recurrence was that evident either on imaging or histopathology. Survival data were obtained from patient contact episodes in hospital databases. Censoring was performed from the most recent date at which the patient was known to be alive. DFS was defined as being alive without evidence of recurrent disease. Cox proportional hazards models were generated using Survival v2.37-7 (Therneau, 2014), with censoring on the 1st June 2015. Three full multivariate models were generated after exclusion of perfect separators; (1) pre-NAC adjusting for age, gender, T and N stage, ASA grade, grade, cell type, tumour site, PET-CT metrics and scanner used; (2) post-NAC this also included re-staging PET-CT and NAC data; (3) post-operatively, this included operative and pathological data. Final (restricted) models were then generated using independently predictive factors identified; for (2) this included scanner and cell type (both influencing tumour avidity, as demonstrated in Chapter 6); for (3) also pathological variables. For additional PET-CT metrics, overlapping metrics were excluded. Survival metrics were calculated using the Kaplain-Meier method. Kaplan-Meier curves were plotted using survMiscv0.4.2 (Dardis, 2014) and gg2tool (Wickham, 2009). ROC AUC were calculated and compared with pROC (Robin et al., 2011) (200 iterations of 0.632). Sensitivities and specificities were compared using McNemar's test (DTComPair v1.0.3) (Stock, 2014).

CHAPTER 3

GENETIC MARKERS OF SUSCEPTIBILITY TO BARRETT'S OESOPHAGUS, DYSPLASIA AND ADENOCARCINOMA

IDENTIFICATION OF SUSCEPTIBILITY VARIANTS AND PATHWAYS BY FURTHER REPLICATION OF A GENOME WIDE ASSOCIATION STUDY, GENE SET ENRICHMENT ANALYSIS, SYSTEMATIC REVIEW AND META-ANALYSIS

3. CHAPTER 3: GENETIC MARKERS OF SUSCEPTIBILITY TO BARRETT'S OESOPHAGUS, DYSPLASIA AND ADENOCARCINOMA

3.1 SYNOPSIS

3.1.1 Introduction

The potential benefits to identifying patients at risk of developing BO, and subsequent progression to dysplasia/OAC are numerous. However, no molecular biomarkers (genetic or otherwise) are currently in clinical use. This study aimed firstly to identify novel variants associated with BO via a further replication phase (2) of the original WTCCC GWAS. Secondly, to identify variants associated with progression (to dysplasia/OAC), and OAC alone via similar analyses. Thirdly, to perform GSEA to identify biological pathways associated with BO/progression. And finally, to undertake the first systematic review and meta-analysis of genetic markers of BO and progression susceptibility, to provide context and attempt their validation.

3.1.2 Methods

68 SNPs underwent replication in 2,010 cases and 2,059 controls (stage 3b). The top 7 underwent further replication in 1,423 additional cases and 2,026 controls, followed by meta-analysis with 3,295 cases and 3,204 controls from the BEACON GWAS (stage 4). Association testing was also performed for progression and OAC using existing GWAS and replication data. GSEA was performed using 2 platforms (iGSEA and GenGen) and corroborated using gene set tests (PLINK). Variants identified by systematic review underwent meta-analysis and *in silico* validation using genome-wide discovery SNP data.

3.1.3 Results

Two intergenic SNPs associated with BO were identified: rs3072 (2p24.1; OR 1.14; 95% CI 1.10-1.17; $p=1.75\times10^{-11}$) and rs2701108 (12q24.21; OR 0.90; 95% CI 0.86-0.93; $p=7.48\times10^{-9}$. *In silico* annotation using eQTL databases suggested rs3072 to regulate *GDF7* (potentially involved in development and metaplastic tissue response), and rs2701108 to tag another variant (rs1920562, potentially acting via *TBX5* and development).

26 independent variants with evidence of association with progression ($p<1.0x10^{-4}$) were identified; 13 with OAC irrespective of BO. Two canonical pathways were strongly associated with susceptibility: the Type 1 Diabetes Mellitus and Antigen Processing and Presentation pathways.

251 full text articles were reviewed; 52 were included. 33 germline markers of susceptibility were identified; 17 were included. 5 somatic markers of progression were identified; meta-analysis demonstrated a significant association for CIN. One somatic marker of progression/relapse following photodynamic therapy was identified. A number of failings of methodology and reporting were identified; evidence for *in silico* validation was poor.

3.1.4 Conclusions

Two novel GWAS loci associated with BO were identified. A number of plausible and intriguing candidate variants for progression were identified. GSEA identified novel biological processes involved in BO and subsequent progression. Limitations of study quality temper the utility of the markers identified by meta-analysis, although some—in particular CIN for progression—appear plausible, although robust validation is required.

3.2 INTRODUCTION

As discussed in section 1.2, BO presents a considerable number of challenges to the clinician. Other than general associations with GORD and broad clinical risk factors, it is not currently possible to identify patients particularly susceptible to developing BO for targeted screening. Similarly, other than broad yet imprecise stratification on the basis of dysplasia, it is not possible to reliably predict which patients will progress to OAC. The molecular and genetic complexity of BO and progression suggests a number of candidate such markers; however, none are currently in clinical use.

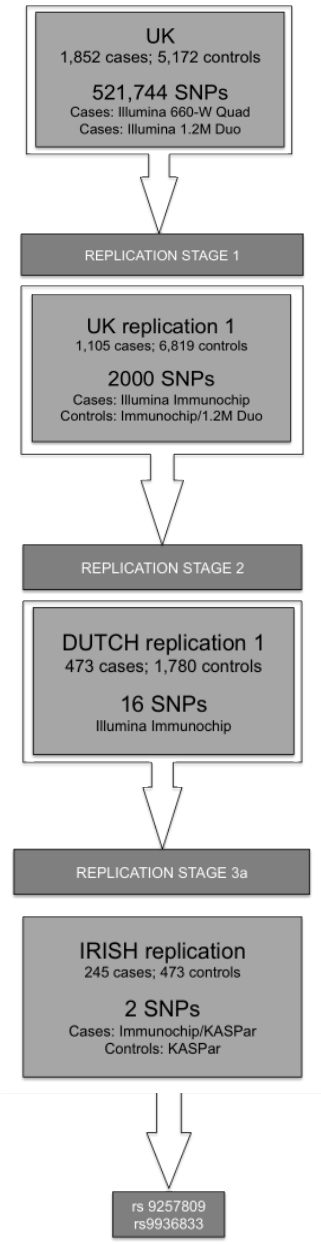
The aims of this chapter were: firstly to identify novel variants associated with BO via a further replication phase of the original WTCCC GWAS. Secondly, to identify variants associated with progression (to dysplasia/OAC), and OAC alone via similar analyses. Thirdly, to undertake the first systematic review and meta-analysis of genetic markers of BO and progression susceptibility, to provide context and attempt their validation. And finally, to perform the first GSEA for BO to identify biological pathways associated with susceptibility and progression.

3.3 RESULTS

3.3.1 Structure of original WTCCC GWAS and replication phase 1

The original GWAS comprised genome-wide discovery plus replication phase 1. It was performed by the MPG Group at the WTCHG, University of Oxford prior to the author's involvement, in conjunction with the Jankowski group at Queen Mary University and University College, London (figure 3.1). Its structure and methodology are described in brief, but have been extensively described previously (Su et al., 2012).

Figure 3.1: Structure of original GWAS and Replication Phase 1



3.3.1.1. Discovery phase

The discovery phase comprised 1,852 (AspECT) cases and 5,172 controls (non-BO WTCCC). 521,744 SNPs were genotyped following quality control. 100 SNPs with evidence of moderate association with BO ($p < 5 \times 10^{-4}$) were identified.

3.3.1.2. Replication phase 1

Replication Phase 1 comprised three individual stages (1-3a), plus meta-analysed data from a separate GWAS (BEACON).

For stage 1, the top 2,400 SNPs were genotyped in 1,105 UK cases (drawn from the ChOPIN and EAGLE studies) and 6,829 controls (n=4,421 1958 Birth Cohort, n=2,578 PoBI), using the Illumina Immunochip for all other than PoBI (Illumina custom Human 1.2M-Duo array). Following this, 16 SNPs had further evidence of association (meta-analysed $p<5x10^{-5}$).

In stage 2, these 16 SNPs were genotyped in the Dutch (Nijmegen) cohort (473 cases and 1,780 controls) using the Immunochip. This identified 2 SNPs with combined $p<5x10^{-8}$: rs9257809 and rs9936833.

In stage 3a both SNPs underwent replication in separate Irish and UK (UK replication 2) cohorts, plus meta-analysis with BEACON. Irish cohort: 245 cases and 473 controls (Trinity Biobank). 168 cases were genotyped on the Immunochip; 77 cases and all controls using competitive allele specific PCR (KASPar). The UK replication 2 cohort comprised 1,765 cases (AspECT), and 1,586 controls (CorGI). Data were extracted from BEACON GWAS study (2,398 cases and 2,167 controls from Australia, England, Ireland, Sweden, Canada and the United States of America).

3.3.2 Present study: GWAS replication phase 2

The present study (replication phase 2) was performed by the author and Dr Claire Palles at the WTCHG, and the Jankowski group (based initially at University College London, and subsequently Plymouth University). It comprised two replication stages (3b and 4, the latter involving an additional BEACON meta-analysis; figure 3.2; table 3.1). At least 50% of replication stage 3 KASPar genotyping, 33% of stage 4, and all analysis in this chapter (other than replication stage 4) was performed by the author.

Figure 3.2 – Structure of the current GWAS study

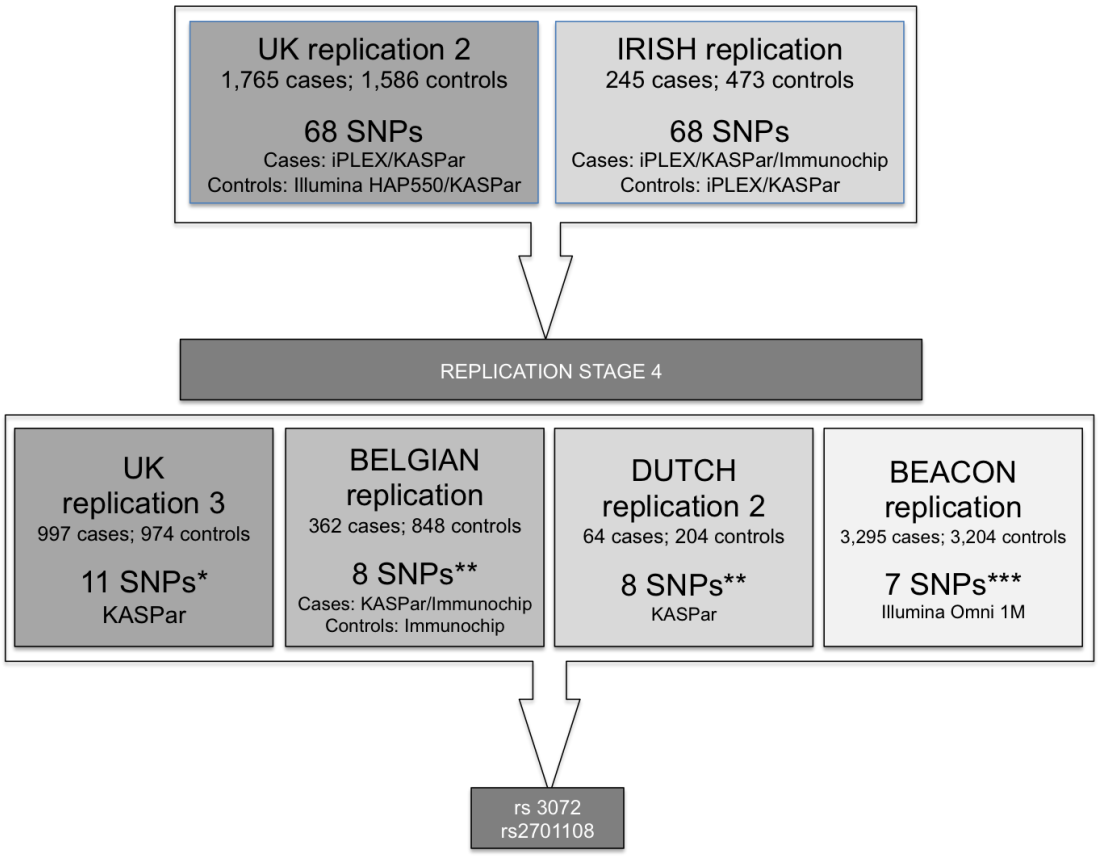


Table 3.1: Patient details for cases and controls used replication phase 2

	UK replication 3		Belgian Replication		Dutch 2		BEACON	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
	n=997	n=947	n=341	n=848	n=64	n=206	n=3295	n=3204
Gender								
Males	707	0	225	404	18	127	2489	2325
Females	274	947	74	444	2	79	806	879
Unknown	16	0	42	0	44		0	0
Age (at diagnosis)								
<=40	16	0	0	0	0	0	0	0
41-50	73	0	0	0	1	0	0	0
51-60	200	0	0	0	5	0	0	0
61-70	397	0	0	0	7	0	0	0
70+	286	0	0	0	7	0	0	0
Not reported	25	947	341	848	44	206	3295	3204
Ethnicity								
White Caucasian	934	947	341	848	64	206	3295	3204
Other	0	0	0	0	0	0	0	0
Not reported	63	0	0	0	0	0	0	0
Diagnostic criteria								
Intestinal Metaplasia	603	NA	341	NA	4	NA	3295	NA
Not specified	394	NA	0	NA	60	NA	0	NA

3.3.2.1. Replication stage 3B

Sixty-eight SNPs were replicated in the Irish (245 cases. 473 controls) and UK replication 2 (1,865 cases, 1,586 controls) cohorts used in Stage 3a (section 3.2.1.2; table 3.1).

SNPs were selected due to: (a) $p<1x10^{-4}$ in phase 1 without reaching genome-wide significance, (b) $p<1x10^{-4}$ in discovery phase, but not included in phase 1, or (c) evidence ($p<1x10^{-4}$) of gender-specific effects. 80 SNPs met these criteria: 63, 12 and 5 respectively (supplementary table 3.1). In addition, 3 candidate SNPs reported to be associated with BO/OAC not included in discovery were tested: rs41341748 missense variant in *MSR1* (Orloff et al., 2011), and two with non-significant association with OAC: rs6898743 (*GHR*) and rs2715425 (*IGF1-R*) (McElholm et al., 2010).

Genotyping was performed using custom-designed Sequenom iPLEX, genotype extraction from prior rounds, and KASPar at the WTCHG. iPLEXs could be designed for 65/83 SNPs. 62 were successfully genotyped (call rate >0.95); 52 were independent following pruning for linkage disequilibrium (LD; $r^2<0.4$). Manual genotyping by KASPar was performed for 3 of the 18 SNPs for which iPLEX could not be designed (those in the 40 with the lowest p values), and were genotyped using KASPar.

For the Irish cohort, 77 cases and all 473 controls were genotyped using Sequenom iPLEX/KASPar. 168 cases were previously genotyped on the Illumina Immunochip; the genotypes for 47 SNPs could be extracted. KASPar genotyping for the remaining 18 SNPs was not performed in this cohort due to the low number of cases. The UK replication 2 cases were genotyped using iPLEX and KASPar. Controls were genotyped using extraction from Illumina Hap550 arrays (n=59 SNPs) or by KASPar (n=6).

In total, 55 independent SNPs were successfully genotyped in the UK replication 2 (1,765 cases; 1,586 controls) and Irish replication (245 cases; 473 controls) cohorts. Following meta-analysis with preceding phases (discovery and replication phase 1, total 5,440 cases and15,830 controls), 7 SNPs showed further evidence of association with BO (combined $p<1x10^{-6}$; table 3.2) These underwent further replication in replication stage 4.

The 3 candidate SNPs included (rs6898743, rs41341748 and rs2715425) were genotyped for the UK replication 2 cohort only; table 3.2). None demonstrated evidence of association, although power was limited for the rare rs41341748 SNP (MAF=0.78%). A further round of

genotyping was therefore performed using UK replication cohort 3 (using KASPar), again without demonstrating an association (meta-analysed OR=1.07, 95%CI 0.70-1.43, p=0.79).

3.3.2.2. Replication stage 4

Three cohorts were used: UK replication 3 (997 ChOPIN cases, 974 GLACIER controls), Belgian (362 cases, 848 controls, Leuven) and Dutch replication 2 (64 cases, 204 controls, Nijmegen). An additional 480 cases were obtained from another Dutch registry, however, DNA failed optimisation of KASPar genotyping (call rate~80%), and so were excluded. Whilst the UK replication 3 and Belgian cohorts were analysed in isolation, due to the small size of the Dutch 2 cohort it was analysed in association with the Dutch 1 cohort from Phase 1.

7/68 SNPs were selected on the basis of progressive association in replication stage 3B ($p<1.00x10^{-6}$; table 3.3). In addition, 4 SNPs were identified ($p<1x10^{-5}$) following meta-analysis of data from both BEACON with WTCCC discovery GWAS data: rs3784262, rs1497205, rs254348 and rs4523255.

All UK replication 3 and Dutch 2 cases and controls were genotyped at the WTCHG using KASPar. 145 Belgian cases and all Belgian controls had been genotyped using the Immunochip (genotypes extracted). 196 more recent cases were genotyped using KASPar. One of the BEACON SNPs (rs3784262) was also genotyped in these additional 196 cases. For the 145 cases genotyped on the Immunochip, rs3784263 was used as a proxy for rs3784262 (pairwise LD $r^2=1$, D'=1). A further round of *in silico* replication was performed for these 7 SNPs, using genotypes from complete BEACON GWAS (3,295 cases and 3,204 controls). Two SNPs were not genotyped directly by BEACON, but for one (rs6751791) a perfect proxy (pairwise $r^2=1$; rs7598399) was available. For the other (rs189247), 4 SNPs genotyped by BEACON were used to impute genotypes (accuracy=98.2%).

Following meta-analysis of this final replication stage of 4,718 cases and 5,230 controls with prior stages (total 10,158 cases and 21,062 controls), 2 SNPs demonstrated a significant association with BO (table 3.3): rs3072 (OR 1.14; 95% CI 1.10-1.17; $p=1.75x10^{-11}$) and rs2701108 (OR 0.90; 95% CI 0.86-0.93; $p=7.48x10^{-9}$).

3.3.2.3. Assessment of intestinal metaplasia and gender associations

Subgroup analysis by gender did not demonstrate significant heterogeneity for either SNP: rs3072 p=0.180; rs2701108 p=0.690). When the analysis was restricted to cases with IM (n=8,521) the association persisted for rs3072 (OR=1.13; 95% CI 1.09-1.17; $p=1.25 \times 10^{-9}$). That of rs2701108 demonstrated borderline significance (OR 0.90; 95% CI 0.86-0.94; $p=6.20 \times 10^{-8}$).

3.3.2.4. Assessment of four additional SNPs

A further 4 SNPs of interest were identified following meta-analysis of discovery data with BEACON data, and were genotyped in stage 4 by KASPar (table 3.4). Following meta-analysis with both WTCCC discovery and BEACON data, non-significant associations were demonstrated: rs1497205 (OR=0.89 [0.85-0.94]; $p=4.18 \times 10^{-6}$), rs254348 (OR=0.92 [0.88-0.96]; $p=4.70 \times 10^{-5}$), rs3784262 (OR=0.90 [0.87-0.94]; $p=1.19 \times 10^{-6}$), rs4523255 (OR=1.10 [1.05-1.15]; $p=3.71 \times 10^{-5}$)

Table 3.2. Meta-analysed associations of seven SNPs with evidence of association with Barrett's Oesophagus following replication stage 3b

Variant				Meta-analysis			Discovery	UK replication 1	Dutch (1&2)	Irish	UK replication 2
SNP	Chr	Position (build 37)	Minor/ Major allele	OR (95% CI) p value	I^2	n	OR (95% CI) p value MAF	OR (95% CI) p value MAF	OR (95% CI) p value MAF	OR (95% CI) p value MAF	OR (95% CI) p value MAF
rs3072	2	20878406	G/A	1.15 (1.11-1.20) 4.24×10^{-9}	0.423	5	1.23 (1.15-1.31) 2.64×10^{-7} 0.41/0.36	1.06 (0.97-1.15) 0.222 0.38/0.37	1.19 (1.05-1.34) 0.018 0.41/0.36	1.02 (0.78-1.26) 0.847 0.35/0.35	1.15 (1.05-1.25) 4.79×10^{-3} 0.41/0.37
rs6751791	2	35581997	G/A	1.13 (1.08-1.17) 1.88×10^{-7}	0.367	5	1.14 (1.07-1.22) 5.03×10^{-4} 0.51/0.48	1.18 (1.09-1.27) 3.05×10^{-4} 0.52/0.48	1.13 (0.99-1.28) 0.090 0.5/0.47	1.30 (1.08-1.53) 0.022 0.45/0.51	1.03 (0.93-1.12) 0.597 0.48/0.48
rs2731672	5	176842474	A/G	1.14 (1.07-1.21) 1.04×10^{-6}	0.000	5	1.18 (1.09-1.27) 1.64×10^{-4} 0.27/0.24	1.15 (1.05-1.25) 8.03×10^{-3} 0.27/0.25	1.09 (0.93-1.26) 0.289 0.27/0.25	1.05 (0.8-1.3) 0.683 0.28/0.27	1.10 (0.99-1.21) 0.085 0.27/0.25
rs2701108	12	114674261	G/A	0.88 (0.83-0.93 2.10×10^{-7}	0.340	5	0.88 (0.80-0.96) 0.00100 0.38/0.41	0.86 (0.76-0.95) 0.000976 0.37/0.4	0.94 (0.79-1.08) 0.383 0.37/0.39	0.71 (0.48-0.93) 2.86×10^{-3} 0.34/0.42	0.93 (0.83-1.03) 0.146 0.39/0.41
rs189247	15	97586630	A/G	1.14 (1.09-1.19)) 4.33×10^{-8}	0.000	5	1.17 (1.09-1.25) 5.67×10^{-5} 0.41/0.37	1.10 (1.01-1.19) 0.0437 0.4/0.38	1.26 (1.11-1.41) 2.86×10^{-3} 0.42/0.36	1.03 (0.79-1.26) 0.821 0.41/0.4	1.11 (1.01-1.21) 0.040 0.39/0.37
rs2043633	16	5819274	C/A	0.87 (0.82-0.91) 4.24×10^{-9}	0.000	5	0.85 (0.78-0.93) 6.04×10^{-5} 0.37/0.41	0.90 (0.81-0.99) 0.021 0.38/0.4	0.84 (0.70-0.98) 0.018 0.37/0.42	0.87 (0.65-1.09) 0.228 0.35/0.39	0.88 (0.78-0.98) 0.0121 0.38/0.41
rs12985909	19	18439383	G/A	1.12 (1.07-1.17) 5.62×10^{-7}	0.000	5	1.12 (1.05-1.20) 2.94×10^{-3} 0.48/0.45	1.11 (1.02-1.21) 0.019 0.47/0.44	1.11 (0.97-1.25) 0.156 0.49/0.47	1.09 (0.86-1.32) 0.444 0.46/0.44	1.11 (1.01-1.21) 0.038 0.47/0.45

Table 3.3. Final meta-analysed associations of seven SNPs with evidence of association with Barrett's Oesophagus following stage 4

Variant				Final meta-analysis			Discovery	Stage1	Stage 2	Stage 3b		Stage 4		BEACON
SNP	Chr	Position (build 37)	Minor/ Major allele	OR (95% CI) p value	I^2	N	OR (95% CI) p MAF	OR (95% CI) p MAF	OR (95% CI) p MAF	OR (95% CI) p MAF		OR (95% CI) p MAF		OR (95% CI) p MAF
							Discovery	UKREP1	Dutch(1+2)	UKREP2	Irish	UKREP3	Belgian	BEACON
rs3072	2	20878406	G/A	1.14 (1.10-1.17) 1.75×10^{-11}	0.42	8	1.23 (1.15-1.31) 2.64×10^{-7} 0.41/0.36	1.06 (0.97-1.15) 0.222 0.38/0.37	1.19 (1.05-1.34) 0.018 0.41/0.36	1.15 (1.05-1.25) 4.79×10^{-3} 0.41/0.37	1.02 (0.78-1.26) 0.847 0.35/0.35	1.18 (1.05-1.31) 0.012 0.4/0.36	0.95 (0.77-1.13) 0.558 0.36/0.38	1.11 (1.03-1.19) 6.64×10^{-3}
rs6751791*	2	35581997	G/A	1.08 (1.04-1.11) 7.65×10^{-5}	0.60	8	1.14 (1.07-1.22) 5.03×10^{-4} 0.51/0.48	1.18 (1.09-1.27) 3.05×10^{-4} 0.52/0.48	1.13 (0.99-1.28) 0.090 0.5/0.47	1.03 (0.93-1.12) 0.597 0.48/0.48	1.30 (1.08-1.53) 0.022 0.45/0.51	0.98 (0.81-1.15) 0.804 0.48/0.49	0.96 (0.78-1.14) 0.629 0.48/0.49	1.00 (0.94-1.08) 0.892
rs2731672	5	176842474	A/G	1.07 (1.03-1.11) 0.00166	0.62	8	1.18 (1.09-1.27) 1.64×10^{-4} 0.27/0.24	1.15 (1.05-1.25) 8.03×10^{-3} 0.27/0.25	1.09 (0.93-1.26) 0.289 0.27/0.25	1.10 (0.99-1.21) 0.085 0.27/0.25	1.05 (0.8-1.3) 0.683 0.28/0.27	1.02 (0.83-1.21) 0.813 0.26/0.25	0.90 (0.70-1.10) 0.298 0.25/0.27	0.95 (0.88-1.03) 0.209
rs2701108	12	114674261	G/A	0.90 (0.86-0.93) 7.48×10^{-9}	0.14	8	0.88 (0.80-0.96) 1.00×10^{-3} 0.38/0.41	0.86 (0.76-0.95) 9.76×10^{-4} 0.37/0.4	0.94 (0.79-1.08) 0.383 0.37/0.39	0.93 (0.83-1.03) 0.146 0.39/0.41	0.71 (0.48-0.93) 2.86×10^{-3} 0.34/0.42	0.97 (0.85-1.10) 0.669 0.4/0.4	0.95 (0.78-1.13) 0.604 0.39/0.41	0.91 (0.85-0.98) 0.0143
rs189247**	15	97586630	A/G	1.10 (1.06-1.14) 3.55×10^{-7}	0.28	8	1.17 (1.09-1.25) 5.67×10^{-5} 0.41/0.37	1.10 (1.01-1.19) 0.044 0.4/0.38	1.26 (1.11-1.41) 2.86×10^{-3} 0.42/0.36	1.11 (1.01-1.21) 0.040 0.39/0.37	1.03 (0.79-1.26) 0.821 0.41/0.4	1.07 (0.94-1.2) 0.280 0.4/0.38	1.00 (0.82-1.18) 0.989 0.41/0.41	1.04 (0.97-1.12) 0.310
rs2043633	16	5819274	C/A	0.92 (0.88-0.95) 2.25×10^{-6}	0.58	8	0.85 (0.78-0.93) 6.04×10^{-5} 0.37/0.41	0.90 (0.81-0.99) 0.021 0.38/0.4	0.84 (0.70-0.98) 0.017 0.37/0.42	0.88 (0.78-0.98) 0.0121 0.38/0.41	0.87 (0.65-1.09) 0.228 0.35/0.39	1.03 (0.90-1.15) 0.683 0.39/0.38	0.84 (0.65-1.02) 0.056 0.38/0.42	0.99 (0.92-1.06) 0.787
rs12985909	19	18439383	G/A	1.10 (1.06-1.13) 3.28×10^{-7}	0.00	8	1.12 (1.05-1.20) 2.94×10^{-3} 0.48/0.45	1.11 (1.02-1.21) 0.019 0.47/0.44	1.11 (0.97-1.25) 0.156 0.49/0.47	1.11 (1.01-1.21) 0.038 0.47/0.45	1.09 (0.86-1.32) 0.444 0.46/0.44	1.03 (0.91-1.15) 0.647 0.46/0.46	1.14 (0.96-1.32) 0.151 0.5/0.47	1.07 (1.00-1.15) 0.052

*Perfect proxy rs7598399 (r^2=1) used for BEACON, as rs6751791 not genotyped; **4 genotyped SNPS used to impute BEACON rs189247 (imputation accuracy=98.2%)

Table 3.4 Replication of 4 SNPs identified through meta-analysis of BEACON and discovery data

Variant						BEACON meta-analysis		Discovery		Overall meta-analysis			
SNP	Chr	Position	Gene	Minor/ major allele	Phenotype	OR (95%CI)	p value	OR (95%CI)	p value	Study stages	OR (95% CI)	p value	I^2
rs1497205	4	76169067	*PARM1, RCHY1*	C/T	BO	0.86(0.80-0.92)	2.86×10^{-5}	1.09 (1.02-1.15)	7.28×10^{-3}	Discovery, phase 1 (UK rep 2), phase 2 (UK rep 3, Belgian, Dutch)	0.89 (0.85-0.94)	4.18×10^{-6}	0.00
					BO/OAC	0.87(0.82-0.93)	1.28×10^{-5}	NA	NA		0.89 (0.86-0.93)	5.71×10^{-7}	0.00
rs254348	16	65980789	Intergenic	T/C	BO	0.88(0.83-0.94)	1.15×10^{-4}	1.07 (1.02-1.13)	0.0120	Discovery, phase 1 (UK rep 2), phase 2 (UK rep 3, Belgian, Dutch)	0.92 (0.88-0.96)	4.70×10^{-5}	0.26
					BO/OAC	0.89(0.84-0.94)	1.40×10^{-5}	NA	NA		0.91 (0.88-0.95)	3.32×10^{-6}	0.22
rs3784262	15	58253106	*ALDH1A2*	G/A	BO	0.85(0.80-0.90)	3.62×10^{-7}	0.94 (0.89-1.00)	0.0368	Discovery, phase 1 (UK rep 2), phase 2 (UK rep 3, Belgian, Dutch)	0.90 (0.87-0.94)	1.19×10^{-6}	0.45
					BO/OAC	0.88(0.83-0.92)	6.72×10^{-7}	NA	NA		0.91 (0.88-0.95)	1.28×10^{-6}	0.35
rs4523255	8	8713038	*MFHAS1*	A/G	BO	1.13(1.06-1.21)	2.46×10^{-4}	1.08 (1.03-1.14)	3.68×10^{-3}	Discovery, phase 1 (UK rep 2), phase 2 (UK rep 3, Belgian, Dutch)	1.10 (1.05-1.15)	3.71×10^{-5}	0.16
					BO/OAC	1.13(1.07-1.20)	4.15×10^{-5}	NA	NA		1.11 (1.06-1.15)	1.02×10^{-6}	0.35

3.3.4 Fine mapping and functional annotation

3.3.4.1. Fine mapping and imputation

Imputation was performed by Dr Claire Palles of 1Mb regions surrounding each SNP, using the August 2012 version of the 1000 genomes project for the discovery phase data. This confirmed rs3072 to be the strongest variant associated with BO within chr2p24. However, analysis of chr12q24 identified a neighbouring SNP (rs1920562) to demonstrate a more significant association with BO than rs2701108: OR=0.84, p= 1.42×10^{-5}, versus OR=0.88, p=1.39×10^{-3})

rs3072 is located 7.5kb downstream of *GDF7/BMP12* and 6.5kb downstream of *C2orf43*. rs2701108 is located 270kb upstream of *RBM12* and 117kb downstream of *TBX5*. The imputed 12q24 SNP rs1920562 is located 131kb downstream of *TBX5*, and 256kb upstream of *RBM19*, and is in moderate LD with rs2701108 (r2=0.608; D'=0.837).

To investigate whether these SNPs might be statistically associated by tagging coding SNPs in their flanking genes, the BROAD SNAP database was interrogated. There was no evidence of LD (r^2>0.4) between rs3072, rs2701108 and rs1920562 and coding SNPs in any gene. This suggests that these SNPs (or unidentified functional they tag) exert regulatory rather than protein coding effects. To explore this, 3 eQTL databases were interrogated. eQTLs are loci regulating gene expression of mRNA/proteins, evidenced by comparing genomic and transcriptomic/proteomic data (Cookson et al., 2009). The Haploregv2, RegulomeDB and Genevar databases were interrogated, including all additional SNPs in moderate LD (r^2>0.4; supplementary tables 3.2-3.4). The Human Protein Atlas v12 was interrogated for expression data (immunohistochemical and mRNA expression). Protein and gene functions were obtained from the NCBI Protein and Gene databases.

3.3.4.2. rs3072

rs3072 lies within an intergenic region containing histone modifications, suggesting enhancer regions (evidenced within the GM12878 (B-lymphocyte/lymphblastoid cell line). rs3072 itself lies within two regulatory motifs binding the transcription factor GATA, and transcription repressor Gfi1. GATA is primarily expressed in erythrocytes and involved in erythropoiesis (Zeng et al., 2012); Gfi1 causes target gene promoter suppression; it is primarily a

haematopoietic factor, but also modulates the inflammatory response (Sharif-Askari et al., 2010), and developmental processes via cell cycle progression (Liu et al., 2010). There was weak evidence (score 5) in the RegulomeDB database that rs3072 is involved in binding the transcription factor RFX3, involved embryogenesis via left-right symmetry and cilia. It is expressed at moderate levels in both oesophageal and gastric epithelia (Jolma et al., 2010).

Further evidence for involvement of this region is suggested by the fact that 2 additional SNPs in almost complete LD with rs3072 are found within this same region: rs9306894 (r^2=0.97; D'=0.99) and rs9306895 (r^2=0.97; D'=1.00); a further SNP rs7255 is in moderate LD (r^2=0.60; D'=0.95). rs930684 is a cis-eQTL, linked to *C2orf43* expression in monocytes (Zeller et al., 2010).

Whilst no oesophageal cell line data were available within the GENevar database, interrogation of the Nica *et al* dataset of lymphoblastoid and adipose cells (Nica et al., 2011), demonstrated both rs3072 ($p=1x10^{-6}$) and 9306894 ($p=7x10^{-4}$) to be associated with *C2orf43* expression *in vivo*. There were no associations with *GDF7*.

3.3.4.3. C2orf43

C2orf43 (Chromosome 2 open reading frame 43) encodes a hypothetical protein product LOC60526. Its function is unknown, but is highly conserved across eukaryotes, and variations have been linked to prostate cancer susceptibility in both Caucasian (Christensen et al., 2010) and Chinese (Yang et al., 2010b) populations. Interrogation of the Human Protein Atlas demonstrated it to be expressed by both oesophageal squamous epithelium and gastric glandular cells (moderate antibody staining).

3.3.4.4. GDF7

GDF7/BMP12 (Growth Differentiation Factor 7/Bone Morphogenic Protein 12) encodes a member of the TGF-β super-family, and is similarly conserved and involved in embryonic development and tissue repair. *GDF7* has yet to be assessed by immunohistochemistry in the Human Protein Atlas, but there was evidence of mRNA expression within oesophageal squamous epithelium.

More specifically, the TGF-β pathway (involved in functions including differentiation, growth, homeostasis and apoptosis) has been implicated as a 'master switch' gene family involved in

metaplasia of injured oesophageal squamous epithelium (Barnard et al., 1990). This is evidenced by increased expression of *BMP4* in BO epithelium (Castillo et al., 2012) upregulating intestinal-specific gene expression (Milano et al., 2007), and reduced expression of other mediators (SMAD2, SMAD4 and TβR11) (Onwuegbusi et al., 2006), and impaired TGF-β pathway function causing columnar GOJ metaplasia in a knockout mouse model (Crawford et al., 1998).

3.3.4.5. rs2701108 and rs1920562

rs2701108, whilst lying within 4 potential regulatory motifs (TBX5, BDP1, Evi-1, and ZEB1) was not associated with eQTL function. However, rs1920562 demonstrated a number of potentially functional effects. As with rs3072, rs1920562 maps to an intergenic enhancer region (as evidence by histone modifications within H1 embryonic stem cell and NH lung fibroblast cell lines). This sequence was considered highly conserved by GERP, with moderate (3a) evidence of affecting protein binding (IKZF1) in RegulomeDB. IKZF1 is a DNA-binding protein associated with chromatin remodelling; whilst evidence of expression in oesophageal epithelium is limited to RNA (protein not detected), it was detected at low levels within gastric epithelium.

An additional variant, rs1247938, although only in moderate LD (r^2=0.41, D'=0.8) with rs1920562, was identified as being of interest (due to its strongest regional association with protein binding, score 2a). It is predicted to affect binding of two proteins highly expressed in oesophageal and gastric epithelia: CTCF (a transcriptional regulator) and RAD21 (a nuclear phosphoprotein, involved in DNA double strand repair, and chromatid cohesion).

3.3.4.6. TBX5

TBX5 (T-box5) is a member of the highly conserved T-box transcription factors, involved in transcriptional regulation of mesodermal development. It is expressed in moderate levels in both oesophageal and gastric epithelia, and is linked to thoracic embryogenesis (cardiac, pulmonary, tracheal), including formation of diaphragmatic skeletal muscle (Rallis et al., 2003).

3.3.4.7. RBM19

RBM19 (RNA-binding motif 19) is involved in ribosomal biogenesis, and as with *TBX5* is expressed at moderate levels in oesophageal and gastric epithelia. However, there was no evidence of eQTLs for either gene or any of the 3 SNPs.

3.3.5 Assessment of shared susceptibility variants with obesity

3.3.5.1. Prior findings

As part of the original GWAS, it was reported that 29/40 SNPs associated with obesity in European populations (as evidenced by body mass index, waist:hip ratio or body fat percentage) by GWAS, demonstrated concordant effects: i.e. SNPs shared the same risk alleles for both BO and obesity phenotypes (Su et al., 2012). This analysis was updated to include all SNPs associated with obesity subsequent to the prior analysis (in July 2012).

3.3.5.2. New variants identified

The National Human Genome Research Institute (NHGRI) Catalog of Published Genome–Wide Association Studies and PubMed databases were searched on 3rd February 2014 using the search parameters (obesity) and (GWAS OR genome-wide association).

An additional 47 SNPs were identified. However, following exclusion of those in LD (r^2>0.3; the threshold used in the above analysis) 9 novel SNPs were shown to be independent (table 3.5).

Five had been directly genotyped in the discovery phase; 4 had valid proxies (r^2>0.8) that had been genotyped. Combining the two analyses, for 32 (65.3%) of the 49 SNPs associated with obesity by GWAS, risk alleles were concordant (p=0.044, sign test).

Table 3.5: Concordance between reported obesity GWAS SNPs and BO phenotype in the discovery phase

Variant				Proxy	Phenotype	Risk allele	Gene	Reference	Discovery risk allele	Discovery p value
Chr	rs ID	Position (build 37)	SNP	SNP						
SNPs genotyped directly										
3	rs7638110	138903985	G/T	NA	BMI	T	*MRPS22*	(Melka MG, 2012)	G	0.913
3	rs7647305	185834290	C/T	NA	BMI	C	*SFRS10-ETV5-DGKG*	(Thorleifsson et al., 2009)	T	0.405
13	rs534870	79857208	A/G	NA	Fat %	G	*SPRY2*	(Kilpelainen et al., 2011)	G	0.687
16	rs6499640	53769677	A/G	NA	BMI	A	*FTO*	(Thorleifsson et al., 2009)	G	0.622
17	rs7503807	76205706	A/C	NA	BMI	C	*RPTOR*	(Berndt et al., 2013)	C	0.117
SNPs genotyped via proxy										
1	rs2820464	219693220	G/A	rs2785990	WHR	G	*LYPLAL1*	(Berndt et al., 2013)	A	0.873
2	rs2943650	227105921	C/T	rs2972143	Total fat mass	C	*IRS1*	(Kilpelainen et al., 2011)	T	0.520
8	rs4735692	76615663	A/G	rs2060604	BMI	A	*HNF4G*	(Berndt et al., 2013)	G	0.003
9	rs16933812	36969205	G/T	rs10814489	Total Fat mass	G	*PAX5*	(Melka MG, 2012)	G	0.546

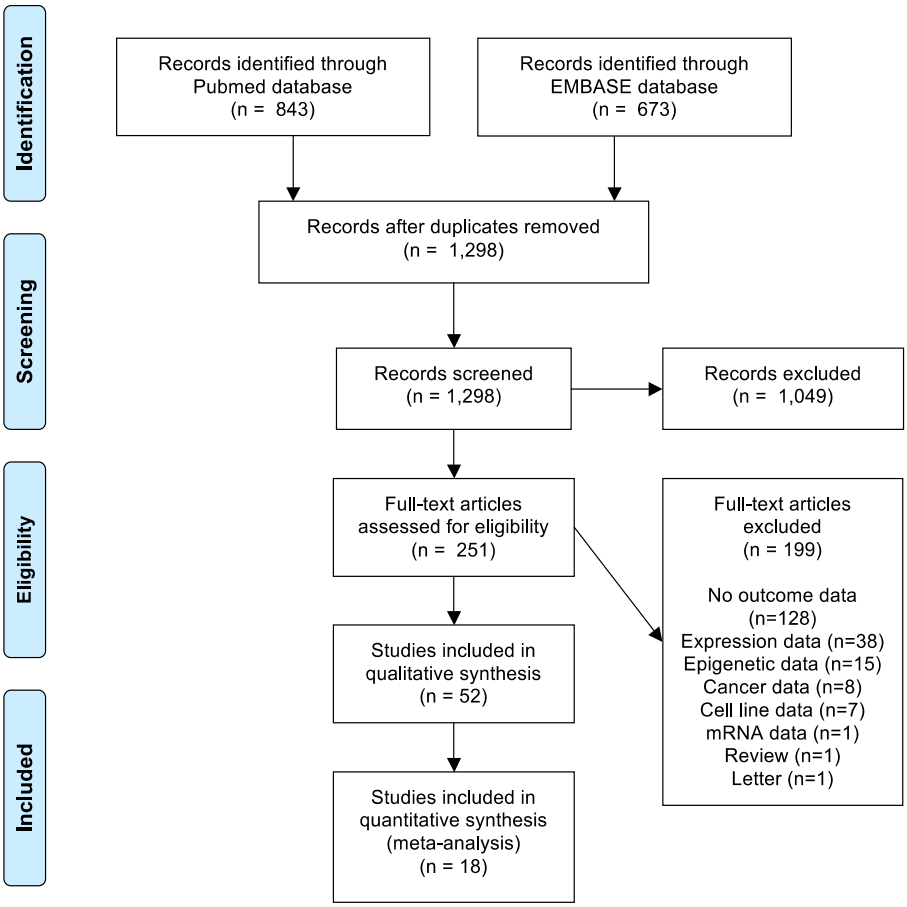
BMI=Body mass index; WHR=waist-hip rati

3.3.6 Systematic review and meta-analysis of germline and somatic genetic biomarkers of susceptibility to Barrett's Oesophagus and progression

3.3.6.1. Literature search

A search was performed on 1st May 2014 of the PubMed and EMBASE databases. 1,516 articles, of which 218 were duplicates. 1,298 articles were therefore appraised on the basis of their title and abstract. 251 full text articles were appraised in further detail, of which 52 met the inclusion criteria (figure 3.3).

Figure 3.3: PRISMA diagram for systematic review of genomic markers of Barrett's oesophagus



3.3.6.2. Studies assessing germline markers of susceptibility to Barrett's oesophagus

32 studies were included: 2 GWAS (the original WTCCC and BEACON studies), 29 candidate studies, and 1 meta-analysis of four included candidate studies (supplementary tables 4 and 5). To comply with the world limit for this thesis, supplementary tables 4-10 are available online at http://link.springer.com/article/10.1007%2Fs10620-015-3884-5.

Methodological quality was appraised using revised ASCO LOE criteria (A-D; tables 2.14-2.15), and the REMARK guidelines modified by the author (table 2.16). All original studies were LOE C (indicating prospective observational registries, without standardized treatment and follow-up), published between 1999-2014. Median modified REMARK score was 15/17 for both GWAS; 13/18 (range 7-16) for candidate studies (supplementary table 6).

A number of frequent methodological issues were identified. The most common of these included failure to: report sample storage conditions (18/31; 58.1%), blind investigators to (18; 58.1%), fully perform/report quality control procedures (19; 61.3%) and match cases and controls (23; 74.2%). Similarly, a number of recurrent reporting issues were identified. The most common of these were: full comparison of marker(s) with established risk factors (30; 96.8%); appropriate adjustment for multiple comparisons (22; 71.0%), perform power calculations (n=15; 48.4%), perform multivariate adjustment for confounding factors (20; 64.5%), and report multivariate effect sizes for these factors (30; 96.8%). Of the 27 studies assessing more than one variant, 24 did not perform multivariate analysis including all tested variants (77.4%). For GWAS, mean numbers of subjects were 5506.5 (cases) and 14,158.5 (controls); for candidate studies, mean numbers were 133.5 (cases) and 196.2 (controls). 13 studies reported on subjects drawn from one of 3 study populations.

3.3.6.3. Variants associated with Barrett's oesophagus susceptibility

187 candidate variants/haplotypes were tested in association with BO, with 29 statistical associations reported. Following *post hoc* Bonferroni correction, 13 variants were therefore associated with BO (table 3.6; p values $<5x10^{-8}$ for GWAS or appropriately corrected $\propto$); 16 variants were excluded (table 3.7). However, for 2 included variants (rs6785049 and rs9344) precise p values were not provided to allow for Bonferroni correction.

The rs1695 variant (*GSTP1*) was assessed by 4 studies, which underwent meta-analysis by Bull and colleagues (Bull et al., 2009). This calculated an OR of 1.50 (95% CI 1.16-1.95 p=not presented; LOE II). Whilst derived from a large total cohort (434 cases and 738 controls) none of the four studies adjusted for risk factors; indeed the sole study finding a significant association had only 22 cases (Kala et al., 2007). The *GSTM1* null genotype was also assessed by 4 studies and underwent updated meta-analysis in this study. Overall, no association was demonstrated. On sensitivity analysis, a significant negative association was apparent for the 2 studies not requiring IM (Kala et al., 2007, van Lieshout et al., 1999) although the relevance of this is unclear. Meta-analysis was performed for 5 other variants, none of which demonstrated associations (table 3.8).

An additional 5 markers were identified reporting statistically significant associations with BO/OAC (table 3.9): those previously identified by Levine *et al* in the BEACON GWAS (Levine et al., 2013) and Orloff *et al (Orloff et al., 2011)*.

3.3.6.4. In silico replication of markers using genome-wide data

Of the 27 markers reported (other than the 2 identified in the original phase of this study), Thirteen were genotyped directly, whilst 7 had valid proxies; for 7 neither the marker nor a proxy had been genotyped during the discovery phase.

Supportive evidence was demonstrated for 2 SNPs with $p<0.05$ in the discovery/meta-analysis phases: rs3776083 in *CDX1* (via perfect proxy rs3776084; $r^2=1.00$, D'=1.00), discovery OR=1.09 (1.01-1.18; p=0.025); and rs909253 in *TNFB* (via the proxy rs1041981; $r^2=0.930$, D'=1.00) meta-analysis OR 1.07 (1.01-1.13); p=0.016. A trend towards significance was identified for rs11209026 in *IL23R*, meta-analysis OR 1.10 (0.99-1.23); p=0.073 (table 3.10).

Of the additional 5 variants associated with BO/OAC first 4 (identified by BEACON) were examined within discovery GWAS data; rs41341748 was included in replication stage 3b respectively. There was supportive ($p<0.05$) evidence of association for 2: rs2687201 in *FOXP1* and rs10423674 in *CRTC1*; there was non-significant evidence of association for rs11789015 in *BARX1*, however, there was no evidence of association for rs41341748 (table 3.11).

Table 3.6: Reported germline markers of Barrett's oesophagus susceptibility

Marker			Individual studies							Association (meta-analysis / individual study)				
LOE	Variant	Gene	Association			EV	LOE	Ethnicity	Methodology					
			Variant	Wild-type	None					Phenotype and OR (95% CI)	I^2	Chi	p	n
C	rs9257809 rs9936833	MHC *FOXF1*	Su 2012			A C	C	Caucasian	Multistage GWAS Pro/retro archived	BO^A 1.21(1.13-1.28) BO^A 1.14 (1.10-1.19)	NA	NA	$4.09x10^{-9}$ $2.74x10^{-10}$	1
C	rs3776082	*CDX1*	Ren 2014			AA	C	Caucasian	Candidate Prospective	BO-v-RO 4.02 (1.80-9.00)	NA	NA	<0.005	1
C	rs2237091	*CDX1*		Ren 2014		AA	C	Caucasian	Candidate Prospective	BO-v-RO 0.28 (0.13-0.59)	NA	NA	<0.005	1
C	rs717746	*CDX1*	Ren 2014			GG	C	Caucasian	Candidate Prospective	BO-v-RO 3.65 (1.73-7.69	NA	NA	<0.005	1
C	rs4444903	*EGFR*	Menke 2012a			GG	C	Caucasian*	Candidate Retro archived	BO^A 3.00 (1.50-6.20)	NA	NA	0.002	1
C	rs917997	*IL18RAP*		Babar 2012		CC	C	Caucasian**	Candidate Retro archived	BO^A 0.59 (0.43-0.80)	NA	NA	$6.00x10^{-4}$	1
C	rs6785049	*PXR*	van de Winkel 2011			G	C	Caucasian*	Candidate Retro archived	BO 1.36 (1.03-1.79)	NA	NA	<0.05	1
C	rs6214	*IGF1*		McElholm 2010		AA	C	Caucasian**	Candidate retro archived	BO^A 0.43 (0.24-0.75)	NA	NA	$3.00x10^{-3}$	1
C	rs2229765 + BMI>30	*IGF1R*	Macdonald 2009			A	C	Caucasian	Candidate Retro archived	BO^A 3.11 (1.12-8.63)	NA	NA	<0.05 (NS?)	1
C	rs3212227	*IL12B*	Moons 2008			C	C	Caucasian*	Candidate retro archived	BO^A 1.82 (1.17-2.69)	NA	NA	$7.00x10^{-3}$	1
C	rs1695	*GSTP*	Bull 2009 Kala 2007		 Murphy 2007 Casson 2006 van Lieshout 1997	G G G G G	C	Caucasian Caucasian Caucasian NP Caucasian	Meta-analysis Candidate (Pro) Candidate (Retro) Candidate (pro) Candidate (pro)	BO 1.50 (1.16-1.95)	NA	NA	<0.01	4
C	rs9344	*CCND1*	Casson 2005a			AA	C	NP	Candidate (pro)	BO^A 3.69 (1.46-9.29)	NA	NA	<0.05	1
C	rs25487	*XRCC1*	Casson 2005b			AA	C	NP	Candidate (pro)	BO^A 0.65 (0.25-1.68)	71%	3.42	0.370	2

EV=effect variant; NS=non-significant; NP=not presented; A=adjusted OR; pro=prospective; retro=retrospective; * / ** overlapping study populations; RO=reflux oesophagitis; NA=not applicable;

Table 3.7: Reported germline markers of Barrett's oesophagus excluded on the basis of multiple comparisons

Marker			Individual studies						Association (meta-analysis / individual study)		
LOE	Variant	Gene	Association		EV	LOE	Ethnicity	Methodology	Phenotype and OR (95% CI)	p	n
			Variant	Wild-type							
C	rs3776083	*CDX1*	Ren 2014		AG	C	Caucasian	Candidate pro	BO-v-RO 1.91 (1.09-3.33)	0.02	1
C	rs4769585	*CDX2*	Ren 2014		CT	C	Caucasian	Candidate pro	BO-v-RO 2.68 (1.20-5.98)	0.02	1
C	rs3812863	*CDX2*	Ren 2014		AG	C	Caucasian	Candidate pro	BO-v-RO 2.53 (1.24-5.14)	0.01	1
C	rs1143634	*IL1B*		Izakovicova Holla 2013	T		Caucasian (Czeck)	Candidate pro	BO -v-RO 0.56 (0.33-0.93)	0.016	1
C	VNTR polymorphism	*IL1RN*		Izakovicova Holla 2013	1/2		Caucasian (Czeck)	Candidate pro	BO 0.62 (0.42-0.94)	0.015	1
C	rs1800587/rs16944/ rs1143634/IL1RN	*IL1B/ILRN*		Izakovicova Holla 2013	C/T/C/long		Caucasian (Czeck)	Candidate pro	BO 1.95 (1.09-1.30)	0.03	1
C	rs1800587/rs16944/rs 1143634/IL1RN	*IL1B/ILRN*		Izakovicova Holla 2013	T/C/C/long		Caucasian (Czeck)	Candidate pro	BO 2.67 (1.15-6.24)	0.02	1
C	rs909253	*TNFB*	Menke 2012b		AA	C	Caucasian (Netherlands)	Candidate retro archived	BO^{A} 1.98 (1.03-3.81)	0.04	1
C	rs2305764	*MyoPb*	Menke 2012c		GG	C	Candidate Retro archived	Candidate retro archived	BO^{A} 2.96 (1.10-7.99)	0.032	1
C	rs1946518	*IL18*	Babar 2012		CC		Caucasian (Irish)	Candidate retro archived	BO^{A} 1.45 (1.07-1.98)	0.02	1
C	rs1799750	*MMP1*	Bradbury 2009		GG		Caucasian (USA)	Candidate retro archived	BO^{A} 2.21 (1.08-4.53)	0.03	1
C	rs11209026	*IL23R*	Gaj 2008		A		Caucasians (Polish)	Candidate pro	BO 3.49 (1.37-8.90)	0.011	1
C	rs1800566	*NQO1*	di Martino 2007		T		NP (UK)	Candidate pro	BO^{A}-v-RO 0.22 (0.07-0.76)	0.016	1
C	B7	*HLA*	Rajendra 2005		B7		Asian (India)	Candidate pro	BO^{Ex} 21.4 (1.22-372	0.036	1
C	rs415958 / rs16944	*IL1RA/IL1B*	Gough 2005		CC/A		NS (UK)	Candidate pro	BO-v-RO 9.5 (1.19-75.9)	0.010	1
C	rs1800896	*IL10*	Gough 2005		CC		NS (UK)	Candidate pro	BO-v-RO 1.84 (1.04-3.28)	0.035	1

EV=effect variant; NP=not presented; A=adjusted OR; pro=prospective; retro=retrospective; NA=not applicable; RO=reflux oesophagitis; Ex=extrapolated

Table 3.8: Meta-analysed markers assessed in association with BO susceptibility

Marker			Individual study			Meta-analysis				
			Association							
Variant	Gene	EV	Variant	Wild-type	No association	OR (95% CI)	I^2	Chi	p	n
Null	*GSTM1*	Null			Kadioglu 2010; Kala 2007; Casson 2006 van Lieshout 1999	OR 0.83 (0.61-1.12)	27%	NA	0.220	4
rs4880	*SOD2*	T			Kadioglu 2010 ; di Martino 2007; Murphy 2007	0.90 (0.65-1.24)	0.00	0.07	0.520	3
rs16944	*IL1B*	AA			Izakovicova Holla 2013; Gough 2005	OR 1.13 (0.63-2.03)	0.00	0.00	0.680	2
rs1052133	*OGG1*	G			Kadioglu 2010; Ferguson 2008b	OR^A 1.34 (0.59-3.09)	67%	3.01	0.480	2
rs25487	*XRCC1*	TT		Casson 2005b	Ferguson 2008b	OR^A 0.65 (0.25-1.68)	71%	3.42	0.370	2
rs13181	*ERCC2*	CC			Casson 2005b; Fergusons 2008b	OR^A 0.92 (0.57-1.48)	31%	1.44	0.730	2

EV=effect variant; OR^A=adjusted OR

Table 3.9: Reported germline markers of either Barrett's oesophagus or oesophageal adenocarcinoma

Marker			Individual studies						Association (meta-analysis / individual study)	
LOE	Variant	Gene	Association		EV	LOE	Ethnicity	Methodology		
			Variant	Wild-type					Phenotype and OR (95% CI)	p
C	rs2687201	*FOXP1*	Levine 2013		T	C	Caucasian	Multistage GWAS Pro/retro archived	BO/OAC: 1.18 (1.12–1.25)	5.47×10^{-9}
C	rs11789015	*BARX1*		Levine 2013	G	C	Caucasian	Multistage GWAS Pro/retro archived	BO/OAC: 0.83 (0.79–0.88)	1.02×10^{-9}
C	rs10419226	*CRTC1*	Levine 2013		A	C	Caucasian	Multistage GWAS Pro/retro archived	BO/OAC: 1.18 (1.12–1.24)	3.55×10^{-10}
C	rs10423674	*CRTC1*	Levine 2013		T	C	Caucasian	Multistage GWAS Pro/retro archived	BO/OAC 0.84 (0.80–0.89)	1.75×10^{-9}
C	rs41341748	*MSR1*	Orloff 2011		T		NS (USA)	Candidate (linkage analysis) Retro archived	BO/OAC: 6.9-v-0.00% OR^{Ex} 16.8 (0.98-289)	<0.01 0.052 ^{Ex}

LOE=level of evidence; NP=not presented; A=adjusted OR; pro=prospective; retro=retrospective; Ex=extrapolated

Table 3.10 Association metrics of reported germline biomarkers of susceptibility in current study

Marker		Individual studies		Association						
Variant	Gene	Association		EV	Original study / meta-analysis		Discovery		Meta-analysis	
		Variant	Wild-type		OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
rs3776082	*CDX1*	Ren 2014		AA	BO-v-RO 4.02 (1.80–9.00)	<0.005	0.99 (0.91-1.06)	0.716	0.99 (0.91-1.06)	0.716
rs2237091 (proxy=rs717746; r^2=1.00, D'=1.00	*CDX1*		Ren 2014	AA	BO-v-RO 0.28 (0.13–0.59)	<0.005	1.01 (0.94-1.09)	0.732	1.01 (0.94-1.09)	0.732
rs717746	*CDX1*	Ren 2014		GG	BO-v-RO 3.65 (1.73–7.69	<0.005	1.01 (0.94-1.09)	0.732	1.01 (0.94-1.09)	0.732
rs3776083 (proxy=rs3776084; r^2=1.00, D'=1.00	*CDX1*	Ren 2014		AG	BO-v-RO 1.91 (1.09–3.33)	0.020	1.09 (1.01-1.18)	0.025	1.09 (1.01-1.18)	0.025
rs4769585 (proxy=rs4614573; r^2=0.967, D'=1.00)	*CDX2*	Ren 2014		CT	BO-v-RO 2.68 (1.20–5.98)	0.020	1.00 (0.93-1.08)	0.928	1.00 (0.93-1.08)	0.928
rs3812863	*CDX2*	Ren 2014		AG	BO-v-RO 2.53 (1.24–5.14)	0.010	NA	NA	NA	NA
rs1143634	*IL1B*		Izakovicova Holla 2013	T	BO-v-RO 0.56 (0.33-0.93)	0.016	1.01 (0.92-1.10)	0.893	1.01 (0.95-1.08)	0.739
VNTR polymorphism	*IL1RN*		Izakovicova Holla 2013	1/2	BO 0.62 (0.42–0.94)	0.015	NA	NA	NA	NA
rs1800587/rs16944/ rs1143634/IL1RN	*IL1B/ILRN*		Izakovicova Holla 2013	C/T/C/ long	BO 1.95 (1.09-1.30)	0.030	NA	NA	NA	NA
rs1800587/rs16944/rs1143634/IL1RN	*IL1B/ILRN*		Izakovicova Holla 2013	T/C/C/ long	BO 2.67 (1.15-6.24)	0.020	NA	NA	NA	NA
rs4444903 (proxy=rs12640600; r^2=0.965, D'=1.00)	*EGFR*	Menke 2012a		GG	BO[A] 3.00 (1.50-6.20)	0.002	0.97 (0.90-1.05)	0.434	0.97 (0.90-1.05)	0.434
rs909253 (proxy rs1041981; r^2=0.930, D'=1.00)	*TNFB*	Menke 2012b		AA	BO[A] 1.98 (1.03–3.81)	0.040	1.12 (1.03-1.21)	0.005	1.07 (1.01-1.13)	0.016
rs2305764 proxy=rs8108998; r^2=1.00; D'=1.00)	*MyoPb*	Menke 2012c		GG	BO[A] 2.96 (1.10–7.99)	0.032	0.97 (0.90-1.04)	0.391	0.97 (0.90-1.04)	0.391
rs917997	*IL18RAP*		Babar 2012	CC	BO[A] 0.59 (0.43–0.80)	$6.00x10^{-4}$	1.06 (0.96-1.16)	0.233	1.06 (0.96-1.16)	0.233
rs6785049	*PXR*	van de Winkel		G	BO 1.36 (1.03-1.79)	<0.05	1.05 (0.97-1.14)	0.201	1.05 (0.97-1.14)	0.201

		2011								
rs6214	*IGF1*		McElholm 2010	AA	BO[A] 0.43 (0.24-0.75)	$3.00x10^{-3}$	1.01 (0.93-1.09)	0.802	1.01 (0.93-1.09)	0.802
rs1799750	*MMP1*	Bradbury 2009		GG	BO[A] 2.21 (1.08–4.53)	0.03	NA	NA	NA	NA
rs2229765 + BMI>30	*IGF1R*	Macdonald 2009		A	BO[A] 3.11 (1.12-8.63)	<0.05	0.98 (0.91-1.05)	0.546	0.98 (0.91-1.05)	0.546
rs3212227 (proxy=rs3213094; r^2=1.00; D'=1.00)	*IL12B*	Moons 2008		C	BO[A] 1.82 (1.17-2.69)	$7.00x10^{-3}$	0.96 (0.87-1.06)	0.428	0.97 (0.90-1.04)	0.372
rs11209026	*IL23R*	Gaj 2008		A	BO 3.49 (1.37-8.90)	0.011	1.03 (0.87-1.17)	0.662	1.10 (0.99-1.23)	0.073
rs1695	*GSTP*	Bull 2009		G	BO 1.50 (1.16-1.95)	<0.01	1.02 (0.95-1.11)	0.556	1.02 (0.95-1.11)	0.556
rs1800566	*NQO1*	di Martino 2007		T	BO[A]-v-RO 0.22 (0.07-0.76)	0.016	0.98 (0.88-1.08)	0.637	0.98 (0.88-1.08)	0.637
B7	HLA	Rajendra 2005		B7	BO[Ex] 21.4 (1.22-372	0.036	NA	NA	NA	NA
rs415958 / rs16944	*IL1RA/IL1B*	Gough 2005		CC/A	BO-v-RO 9.5 (1.19-75.9)	0.010	NA	NA	NA	NA
rs1800896	*IL10*	Gough 2005		CC	BO-v-RO 1.84 (1.04-3.28)	0.035	0.99 (0.91-1.06)	0.725	1.03 (0.97-1.09)	0.373
rs9344 (proxy=rs602652; r^2=0.934, D'=0.966)	*CCND1*	Casson 2005a		AA	BO[A] 3.69 (1.46–9.29)	<0.05 (NS?)	0.99 (0.91-1.07)	0.756	0.99 (0.91-1.07)	0.756
rs25487	*XRCC1*	Casson 2005b		AA	BO[A] 0.65 (0.25-1.68)	0.370	1.01 (0.91-1.11)	0.868	1.01 (0.91-1.11)	0.868

HLA=human leukocyte antigen; VNTR=variable number tandem repeat

Table 3.11 Attempted replication of variants associated with Barrett's oesophagus/adenocarcinoma

Marker		Studies		Association									
Variant	Gene	Association	EV	Original study / meta-analysis		Discovery		Replication 3b		Replication 4		Meta-analysis	
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR	p	OR (95% CI)	p
rs2687201	*FOXP1*	Levine 2013	T	1.18 (1.12–1.25)	5.47×10^{-9}	1.14 (1.03-1.27)	0.010	NA	NA	NA		1.14 (1.03-1.27)	0.012
rs11789015	*BARX1*	Levine 2013	G	0.83 (0.79–0.88)	1.02×10^{-9}	0.90 (0.81-1.01)	0.070	NA	NA	NA		0.90 (0.81-1.01)	0.066
rs10419226	*CRTC1*	Levine 2013	A	1.18 (1.12–1.24)	3.55×10^{-10}	1.01 (0.91-1.11)	0.860	NA	NA	NA		1.01 (0.91-1.11)	0.865
rs10432674	*CRTC1*	Levine 2013	T	0.84 (0.80–0.89)	$1.75x10^{-9}$	0.94 (0.88-1.00)	0.050	NA	NA	NA		0.94 (0.88-1.00)	0.050
rs4134748	*MSR1*	Orloff 2011	T	[Ex] 16.8 (0.98-289)	0.052	NA	NA	1.09 (0.64-1.54)	0.701	NA		NA	NA

EV=effect variant; [Ex]=extrapolatedw

3.3.6.5. Studies assessing germline variants associated with progression

One study was identified, published in 2007 (supplementary table 7). LOE was C; modified REMARK 13.5/19. This assessed 4 variants, none of which were associated with progression.

3.3.6.6. Studies assessing somatic variants associated with progression

Sixteen studies were identified, published between 2012-1989 (supplementary table 8). ASCO LOE was C for 12 and D for 4. Mean modified REMARK score was 13.2 (range 10.5-18.5/19; supplementary table 9. Five qualified progression as HGD/OAC; 10 as OAC; 1 as OAC/CIN.

The primary methodological deficiencies identified were: failure to report sample storage procedures (9; 56.3%), failure to blind investigators to phenotype (12; 75.0%), failure to perform appropriate quality control/reproducibility (9; 56.3%), failure to match controls (14; 87.5%), and failure to perform appropriate power calculations (14; 87.5%). The primary reporting deficiencies were: failure to report univariate association effects (9; 60.0%), failure to adjust for appropriate risk factors (including the presence of dysplasia at baseline; 14; 87.5%) and failure to fully report all constituent coefficients of multivariate models (13; 81.3%).

3.3.6.7. Somatic variants associated with progression

Of 7 variants assessed, 5 were reported to be associated with progression (table 3.12). Meta-analysis was possible for one variant: CIN, assessed by 11 studies (defined as aneuploidy by 4, tetraploidy by 1, aneuploidy/tetraploidy by 5, and aneuploidy/tetraploidy/hypodiploidy by 1). Six of these studies were derived from one sample archive; for 4 it was not apparent that individual study populations were separate. All studies reported statistically significant associations with progression, measured by OR (n=6) and HR (n=4). Only 2 studies adjusted for confounding variables including the presence of dysplasia (Bird-Lieberman et al., 2012, Sikkema et al., 2009); a further 2 whilst not adjusting for dysplasia adjusted for length of Barrett's segment (Galipeau et al., 2007, Maley et al., 2004). CIN was independently associated with progression in all instances.

Meta-analysis was performed for both OR and HR of progression to HGD/EAC. Significant associations were demonstrated for both (table 3.12; figure 3.3; figure 3.4). All studies included patients with IM only. Meta-analysed OR was 5.98 (2.10-17.1; $p=8.00x10^{-4}$; n=5 studies; following exclusion of overlapping studies and correction for publication bias). However, only one of these studies adjusted for the presence of dysplasia. Meta-analysed HR was 1.36 (1.26-1.47; n=2 studies; $p<1.00x10^{-5}$; following exclusion of one overlapping study). Overall LOE for CIN was II.

Figure 3.3 Forrest plot for CIN and OR and HR of progression

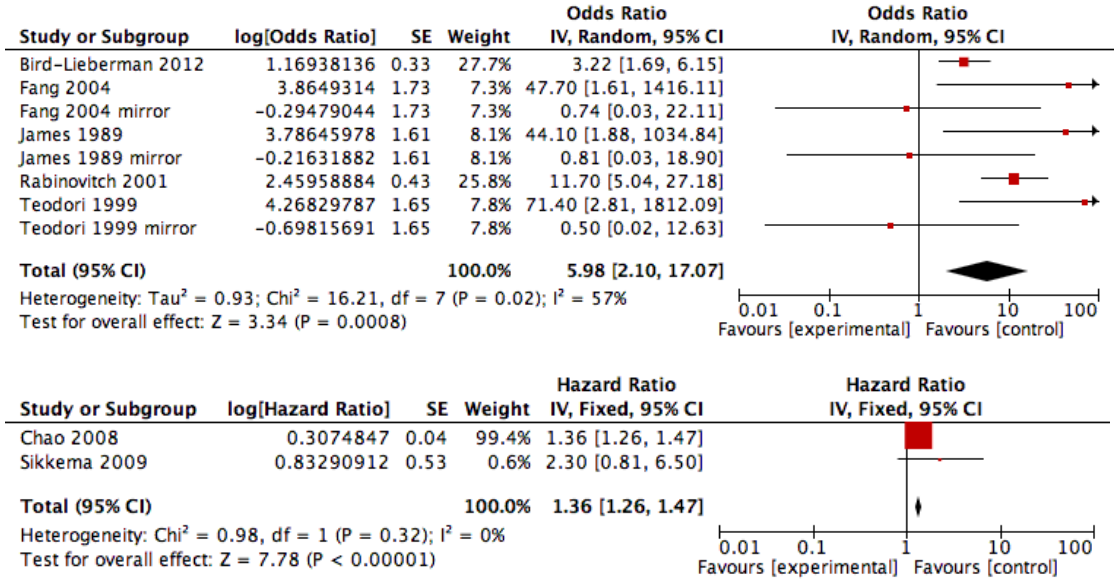
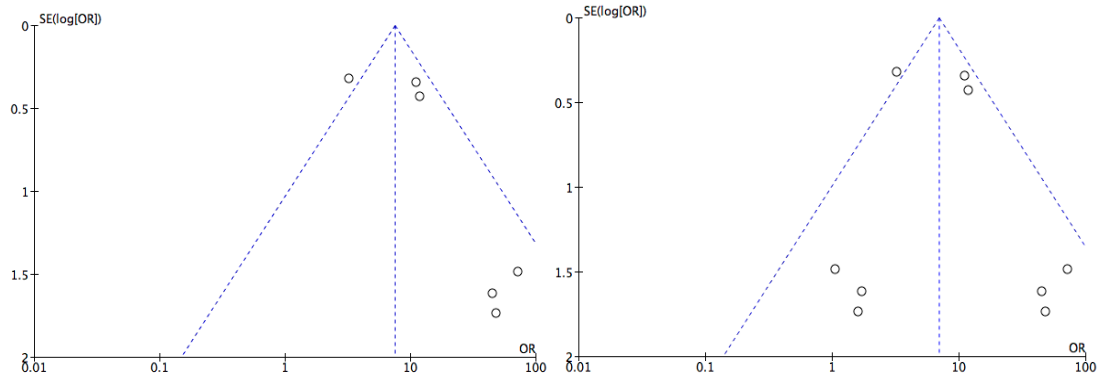


Figure 3.4 Uncorrected and corrected funnel plots for CIN and OR of progression



Whilst more than one study assessed mutant *TP53* (n=3) and LOH *TP53* (n=2), meta-analysis was not possible for either. Two studies for each were derived from the same populations, and the third *TP53* study used a different measure of effect size.

3.3.6.8. Variants associated with progression following photodynamic therapy

3 studies assessed markers of progression to HGD/OAC following PDT (either aminolaevulinic acid or porfimer sodium; supplementary table 10). LOE was C for 2 studies, and D for 1. Mean modified REMARK score was 16.2/20 (range 14.5-19; supplementary table 7). For two studies, EMR was variably performed, but only controlled for in one study.

A total of 6 variants were assessed; whilst 5 were assessed by 2 studies, meta-analysis could not be performed due to study population overlap. One variant (CIN; at both 4 and 12 months) was reported to be associated with risk of progression to HGD/OAC after 12 months (table 3.13).

Table 3.12: Reported somatic markers of Barrett's oesophagus progression to dysplasia / adenocarcinoma

Marker			Individual study							Association (meta-analysis / individual study)				
LOE	Variant	Gene	Association: Variant	Association: None	EV	LOE	Ethnicity	Phenotype	Adjusted for confounders?	Phenotype and OR / HR (95% CI)	I^2	Chi	p	n
II	CIN	NA	Bird-Lieberman 2012		CIN	C	NP	HGD/OAC	Yes (length, dys)	HGD/OAC $OR^{(A)\ IM\ PB}$ 5.98 (2.10-17.1)	57	16.2	$8.00x10^{-4}$	5
			Sikkema 2009		CIN	D	Cau	HGD/OAC	Yes (plus dys)	HGD/OAC $HR^{(A)\ IM}$ 1.36 (1.26-1.47)	0	0.98	$<1.00x10^{-5}$	2
			Chao 2008*		CIN	C	NP	OAC	No					
			Galipeau 2007*		CIN	C	NP	OAC	Yes (length not dys)	PB- adjusted for publication bias Fang				
			Fang 2004		CIN	C	NP	OAC	No	2004, Rabinovitch 2001, Teodori 1999				
			Maley 2004*		CIN	C	NP	OAC	Yes (length not dys)					
			Rabinovitch 2001*		CIN	C	NP	OAC	Yes (length not dys)	***Overlapping populations				
			Reid 2000**		CIN	D	NP	OAC	No					
			Teodori 1999		CIN	C	NP	HGD/OAC	No					
			Reid 1992**		CIN	C	NP	OAC	No					
			James 1989		CIN	D	NP	OAC	No					
III	CNV	NA	Paulson 2009		>70 Mbp	C	NP	OAC/CIN	No	OAC/CIN HR 4.90 (1.60-14.8)	NA	NA	$4.70x10^{-3}$	1
III	LOH	*TP53*	Galipeau 2007*		LOH	C	NP	OAC	Yes (length, not dys)	OAC HR^{A} 5.4 (2.5–12.0)	NA	NA	$<1x10^{-3}$	1
			Maley 2004*		LOH	C								
			Reid 2001*		LOH	C								
III	LOH	*P16*	Galipeau 2007*		LOH	C	NP	OAC	Yes (length, not dys)	OAC^{A} HR 2.4 (1.0–5.5)	NA	NA	0.045	1
				Maley 2004*	LOH	C	NP	OAC	Yes (length, not dys)					
III	Mutant	*TP53*	Maley 2004*		Mutant	C	NP	OAC	No	OAC HR^{A} 1.27 (1.07-1.50)	NA	NA	<0.01 (NS?)	1
			Dolan 2003b			C	NP	OAC	Yes (length, not dys)	No meta possible as study population overlap and differing effect measures				
				Galipeau 2007*		C	NP	OAC	No					

Table 3.13: Reported somatic biomarkers of Barrett's oesophagus progression to dysplasia / adenocarcinoma following photodynamic therapy

Marker			Individual study							Association (meta-analysis / individual study)				
LOE	Variant	Gene	Association: Variant	Association: None	EV	LOE	Ethnicity	Phenotype	Adjusted for confounders?	Phenotype HR (95% CI)	I^2	Chi	p	n
D	CIN	NA	Dunn 2010		CIN 4m-post PDT	D	NS	HGD	Yes	OAC/HGD HR^{A} 4.10 (1.30-13.0	NA	NA	$9.00x10^{-3}$	1
					CIN 12m-post PDT					OAC/HGD HR^{A} 3.60 (1.05-12.3)			$2.90x10^{-3}$	

3.3.7 Identification of novel germline variants associated with progression to dysplasia, and adenocarcinoma

3.3.7.1. Endpoints

Limited association testing was performed for two sub-phenotypes: progression to dysplasia/OAC, and a diagnosis of OAC (with or without previous BO). This information was only available for two cohorts: the AspECT cohort from Discovery and UK replication 2 (genome-wide), and ChOPIN and Dutch 1 cohorts of Replication Stage 1 respectively (non-genome-wide). Data on progression were limited; as a primary endpoint of the AspECT trial, only progression status to dysplasia/carcinoma (January 2013) could be obtained. Data were available for cases progressing to OAC only (rather than dysplasia) on the CHoPIN trial. However, as this cohort was genotyped on the non-genome-wide Immunochip (a SNP panel selected for immunologically-mediated phenotypes), this analysis was performed with the caveat of significant potential for bias.

3.3.7.2. Progression to dysplasia or oesophageal adenocarcinoma (genome-wide)

The progression phenotype was defined (by necessity) as pathological diagnosis of dysplasia (indefinite, low grade and high grade) or OAC (oesophageal and Siewert type 1). Within the discovery cohort 155 (8.37%) progressed, 1,697 did not. Association testing was performed with non-progressors as controls. A 'replication' analysis for SNPs also included on the Immunochip was performed for the ChOPIN cohort (of whom 445 [29.5%] progressed, 1,062 [70.5%] did not). Results are presented in table 3.14.

511,623 SNPs passed QC in the discovery data (λ=1.01); 128,767 passed QC for UK replication 1 (ChOPIN) data (λ=0.994; figure 3.3). Meta-analysis was performed for the 22,915 SNPs passing QC in both sets of data. 29 SNPs were identified with $p<1\times10^{-4}$ in either discovery alone, replication alone or in meta-analysis. Only 6 had information from both discovery and UK replication. 3 SNPs were removed by LD pruning ($r^2>0.4$); a further 2 on the basis of significant heterogeneity, leaving a total of 26 SNPs with evidence of association with progression ($p<0.05$). Variants were then annotated as for BO-associations (supplementary table 3.4)

Only one of these (rs6716108, OR 1.10; $p=4.49x10^{-5}$) demonstrated concurrent association ($p<0.05$) with BO (OR=1.09; p=0.045). rs6716108 is an intergenic SNP, 20kb upstream of *SH2D6*, and 198kb downstream of *MAT2A*. It lies in an intergenic enhancer region (in mammary epithelial and keratinocyte cell lines), and alters the regulatory motifs of PPAR and NRSF. Interrogation of the GENevar database did not demonstrate any associations with gene expression.

The function of *SH2D6* is unknown; whilst not expressed in the squamous oesophagus, it is moderately expressed (protein) in the stomach. *MAT2A* encodes methionine adenosyltransferase, involved in cysteine and methionine metabolism and therefore widely expressed (at moderate levels in the oesophagus, and low levels in the stomach); any potential role in progression is therefore similarly unclear.

There were two notable associations in chromosomal regions identified by both previous GWAS, those of *C2orf43* (this study) and *FOXP1* (the BEACON GWAS). The most significantly associated SNP for the progression sub-phenotype (rs587139; OR 2.22 [1.53-3.21]; $p=2.29x10^{-5}$) lies within an intron of *C2orf43* (identified as a potential functional target of rs3072). Whilst there was no evidence for association of this SNP with BO susceptibility by itself, this supports the involvement of this region (and potentially *C2orf43*) with BO/progression. It lies within an enhancer region, although there was no evidence of association with *C2orf43* expression in Genevar. As previously discussed, the function of *C2orf43* is unknown, although it is expressed (protein and RNA in oesophageal and gastric tissue). There is minimal LD between rs3072 and rs587139 (r^2 0.017; D' 0.459) suggesting these associations to be independent, and therefore even more intriguing.

The second most significant SNP (rs9880789) lies 456kp 5' of *FOXP1*, implicated by the rs2687201 SNP described by the BEACON GWAS. Both are independent (r2=0.00; D'=0.00), but there was no evidence of association between rs9880789 and *FOXP1* expression in the available databases. However, again this is again tentatively supportive of the role of this region in BO/OAC pathogenesis.

The variant with best *in silico* evidence was rs1631576 (discovery OR 0.61; $p=4.00 x10^{-5}$); an intronic variant within *SMAD2* (a transcriptional regulator and integral component of the TGF-β pathway, which includes *GDF7* [which flanks rs3072], and is heavily implicated in BO metaplasia). rs1631576 is predicted to bind the DNA-binding and transcriptional regulatory CTCF (by both RegulomeDB and HaploReg), and is a *cis*-eQTL associated with *SMAD2*

expression in all 4 Genevar databases ($p<1x10^{-4}$). The latter is expressed (moderate protein levels) in both oesophageal and gastric mucosa.

Other plausible associations include that of rs283741 and *POU5F1B*, and rs786994 and death-associated protein kinase 1 (*DAPK1*). POU5F1B is a transcriptional regulator expressed in both gastric and oesophageal tissue and linked (via protein over-expression) with prostate cancer) (Kastler et al., 2010). *DAPK1* is a pro-apoptotic tumour suppressor gene, expressed in gastric rather than oesophageal tissue). However, there was no *in silico* evidence of association with expression for either SNP, although rs283741 lies within an enhancer region.

3.3.7.3. Oesophageal adenocarcinoma susceptibility (Immunochip analysis)

There were 388 cases of OAC in UK replication 1, and 401 in the Dutch cohort. Association testing was performed using the cohort-specific controls (4,421 BC58 and 2.578 PoBI; 633 and 1147 respectively). 126,306 SNPs passed QC in UK data (λ=1.16); 91,426 passed QC in Dutch data (λ =1.21). Meta-analysis was performed for the 88,427 SNPs passing QC in both sets of data.

39 SNPs demonstrated $p<1x10^{-4}$, pruned to 14 following removal of SNPs in LD (pairwise $r^2>0.5$); table 3.15. A further SNP was removed for significant heterogeneity (; $I^2=0.86$). Of the remaining 13 SNPs, only rs2801539 demonstrated a p-value <0.05 in the susceptibility meta-analysis below 0.05 (OR=1.12; p=0.02091; table 3.15).

Variants were then annotated as for BO-associations (supplementary table 3.5) rs2801539 is an intergenic SNP, 15kb downstream of MIR4679 (a microRNA, involved in post-transcriptional modification); it lies within enhancer (3 cell types) and DNAase (7 cell types) regions, and alters GATA1 and GATA2 binding sites. However, there were no relevant eQTL data in the GENevar database. Similarly, any potential role is unclear.

However, a number of biologically-plausible associations with cell adhesion molecules (candidate tumour suppressor genes) were demonstrated: *CDH6*, *CADM1* and *ANK3*. rs12658568 (90kbp upstream of *CDH6*) lies within 12 regulatory motifs, and is associated with *CDH6* expression in adipocytes (Grundberg 2012 database; $p<1.00x10^{-5}$). rs4867305 (a SNP in near-perfect LD: r^2 0.99 and D' 1.00) is similarly a *cis*-eQTL, but lies within a highly conserved enhancer region, and binds FOXA1.

rs1048932 (OR 1.23. $p=7.24x10^{-5}$) lies within a highly conserved region of *CADM1* (intronic); whilst there was minimal evidence of protein binding, this variant lies within 5 regulatory motifs, and is an eQTL of *CADM1* within lymphoblastoid and adipose tissues (Nica, 2011, $p=1.1x10^{-11}$; Grundberg, 2012, $p<1x10^{-9}$) databases.

rs7923097 is an intronic SNP within *ANK3*, and 569Kbp upstream of *CDK1* (cyclin dependent kinase 1), although there was no evidence of significant regulatory effect on either gene.

Other interesting potential associations include rs12515934, lying within an enhancer region 303kbp upstream of *PTGE4R* (prostaglandin E4 receptor). This location is involved in PU1 binding, and a range of transcription factor motifs, although there was no evidence of eQTL activity in the available databases.

4 variants were identified in close proximity at 6p21 (near the MHC); however, no obvious mechanistic hypotheses of evidence of regulatory effect were apparent. The association of this region may be representative of the bias towards immunological variants genotyped on the Immunochip in this cohort.

Table 3.14: Variants with evidence of association progression to dysplasia/adenocarcinoma in discovery/UK rep 2 cohorts

Variant				Meta-analysis					Discovery		UK replication 2	
SNP	Chr	Position	Effect variant (MAF)	OR (95% CI)	p	N	I^2	FDR	OR (95% CI)	p	OR (95% CI)	p
rs587139	2	20812160	C/A 0.11	2.22 (1.53-3.21)	2.29×10^{-5}	1	NA	1	2.22 (1.53-3.21)	2.29×10^{-5}	NA	NA
rs9880789	3	72069948	G/A 0.10	1.57 (1.27-1.94)	2.47×10^{-5}	2	0.466	0.171	1.97 (1.34-2.89	5.61×10^{-4}	1.43 (1.11-1.84)	5.29×10^{-3}
rs16964327	15	35003879	A/G 0.04	0.22 (0.11-0.45)	2.68×10^{-5}	1	NA	1	0.22 (0.11-0.45)	2.68×10^{-5}	NA	NA
rs7367706	1	66480444	A/C 0.04	0.45 (0.31-0.65)	2.75×10^{-5}	1	NA	1	NA	NA	0.45 (0.31-0.65)	2.72×10^{-5}
rs283741	8	128322175	A/G 0.41	1.32 (1.16-1.51)	2.91×10^{-5}	2	0.511	0.153	1.52 (1.21-1.92)	3.71×10^{-4}	1.24 (1.06-1.85)	8.77×10^{-3}
rs1631576	18	43657481	A/G 0.42	0.61 (0.48-0.77)	4.00×10^{-5}	1	NA	1	0.61(0.48-0.77)	4.00×10^{-5}	NA	NA
rs6716108	2	85537721	A/G 0.52	0.69 (0.57-0.82)	4.49×10^{-5}	2	0.869	5.56×10^{-3}	0.46 (0.33-0.65)	5.45×10^{-6}	0.81 (0.65-1.00)	5.59×10^{-2}
rs7869944	9	89422222	A/G 0.05	3.35 (1.87-5.98)	4.59×10^{-5}	1	NA	1	3.35 (1.87-5.98)	4.59×10^{-5}	NA	NA
rs997185	4	67351175	A/G 0.15	0.54 (0.40-0.72)	4.75×10^{-5}	1	NA	1	0.54 (0.40-0.72)	4.75×10^{-5}	NA	NA
rs12351988	9	129053122	A/C 0.45	0.75 (0.66-0.86)	4.83×10^{-5}	2	0	0.977	0.76 (0.59-0.97))	2.50×10^{-2}	0.75 (0.64-0.89)	6.92×10^{-4}
rs10753834	1	196945716	A/C 0.292	1.39 (1.19-1.63)	5.12×10^{-5}	1	NA	1	NA	NA	1.39 (1.19-1.63)	5.12×10^{-5}
rs7722480	5	149762613	A/C 0.07	0.59 (0.46-0.76)	5.33×10^{-5}	2	0.764	3.94×10^{-2}	0.41 (0.26-0.63)	6.19×10^{-5}	0.71 (0.53-0.97)	3.30×10^{-2}
rs220137	21	42406163	A/G 0.16	0.55 (0.41-0.73)	5.60×10^{-5}	1	NA	1	0.55 (0.41-0.73)	5.60×10^{-5}	NA	NA
rs1203578	7	40864663	A/G 0.52	0.71 (0.59-0.84)	5.99×10^{-5}	1	NA	1	NA	NA	0.71 (0.59-0.84)	5.99×10^{-5}
rs9525688	13	43393917	A/C 0.27	0.29 (0.16-0.53)	6.17×10^{-5}	1	NA	1	NA	NA	0.29 (0.16-0.53)	6.17×10^{-5}
rs2937952	15	84141529	A/G 0.22	0.51 (0.36-0.71)	6.37×10^{-5}	1	NA	1	0.51 (0.36-0.71)	6.37×10^{-5}	NA	NA
rs2763321	10	28498908	A/G 0.22	0.73 (0.62-0.85)	6.76×10^{-5}	2	0	0.490	0.67 (0.51-0.89)	4.72×10^{-5}	0.76(0.63-0.91)	3.77×10^{-3}
rs6718783	2	11686323	A/C 0.07	0.36 (0.22-0.59)	7.33×10^{-5}	1	NA	1	0.36 (0.22-0.59)	7.33×10^{-5}	NA	NA
rs9544021	13	75151355	A/G 0.48	0.77 (0.68-0.88)	7.85×10^{-5}	2	0.327	0.223	0.68 (0.54-0.86)	1.20×10^{-5}	0.81(0.70-0.95)	1.01×10^{-2}
rs7679085	4	83883231	A/G 0.37	1.61 (1.27-2.05)	7.98×10^{-5}	1	0	1	1.61 (1.27-2.05)	7.98×10^{-5}	NA	NA
rs12406216	1	34420965	A/G 0.63	1.61 (1.27-2.04)	8.82×10^{-5}	1	NA	1	1.61 (1.27-2.04)	8.82×10^{-5}	NA	NA
rs284541	2	217077084	A/G 0.32	0.76 (0.66-0.87)	8.83×10^{-5}	2	0	0.851	0.78 (0.61-0.99)	4.15×10^{-2}	0.75 (0.64-0.89)	7.87×10^{-4}

rs17595975	14	34610515	A/G 0.11	0.47 (0.32-0.68)	8.96×10^{-5}	1	NA	1	0.47 (0.32-0.68)	8.88×10^{-5}	NA	NA
rs1346139	15	67824893	A/G 0.44	1.58 (1.26-2.00)	9.16×10^{-5}	1	NA	1	1.58 (1.26-2.00)	9.16×10^{-5}	NA	NA
rs1936688	1	190681611	A/C 0.38	0.72 (0.62-0.85)	9.27×10^{-5}	1	NA	1	NA	NA	0.72 (0.62-0.85)	9.27×10^{-5}
rs4638779	2	65008776	A/G 0.27	0.57 (0.44-0.76)	9.62×10^{-5}	1	NA	1	0.57 (0.44-0.76)	9.62×10^{-5}	NA	NA

Table 3.15: Variants with evidence of association with OAC in ChOPIN/Dutch cohorts (non-genome-wide Immunochip analysis)

Variant				Meta-analysis				UK replication 1		Dutch	
SNP	Chr	Position	EV (MAF)	OR (95% CI)	p	I^2	q p	OR (95% CI) MAF cases/controls	p	OR (95% CI)	p
rs35272229	2	197654637	G/A (0.07)	1.68 (1.36-2.09)	$6.95X10^{-6}$	0.00	0.57	1.55 (1.11-2.17) 0.07/0.05	$9.93X10^{-3}$	1.77 (1.32-2.23) 0.10/0.06	$1.91X10^{-4}$
rs13070425	3	144329972	A/G 0.34	0.79 (0.70-0.88)	$1.63X10^{-5}$	0.00	0.53	0.81 (0.69-0.95) 0.30/0.35	$6.78X10^{-3}$	0.76 (0.65-0.88) 0.32/0.38	$6.35X10^{-4}$
rs12658568	5	31139623	G/A 0.14	1.39 (1.19-1.63)	$1.43X10^{-5}$	0.86	0.01	1.15 (0.95-1.40) 0.17/0.15	$1.65x10^{-1}$	1.73 (1.40-2.15) 0.20/0.13	$9.6x10^{-7}$
rs12515934	5	40412687	G/A 0.12	1.45 (1.21-1.73)	$4.79X10^{-5}$	0.00	0.81	1.25 (1.02-1.52) 0.14/0.10	$3.31X10^{-2}$	1.42 (1.08-1.87) 0.12/0.09	$1.1x10^{-7}$
rs6601062	5	178998248	G/A 0.14	1.34 (1.17-1.53)	$9.43X10^{-5}$	0.00	0.32	1.23 (1.05-1.44) 0.18/0.15	$6.78X10^{-3}$	1.45 (1.17-1.80) 0.19/0.14	$6.1x10^{-7}$
rs3734262	6	34946555	G/A 0.10	1.40 (1.18-1.68)	$8.95X10^{-5}$	0.53	0.15	1.26 (1.01-1.56) 0.13/0.11	$4.30X10^{-2}$	1.63 (1.27-2.11) 0.13/0.09	$2.8x10^{-7}$
rs1737727	6	35012466	A/G 0.23	1.31 (1.14-1.50)	$5.63X10^{-5}$	0.00	0.72	1.28 (1.08-1.53) 0.24/0.19	$5.93X10^{-3}$	1.34 (1.10-1.63) 0.22/0.18	3.5x10-7
rs1996913	6	35108431	A/G 0.10	1.45 (1.21-1.73)	$5.55X10^{-5}$	0.00	0.45	1.36 (1.08-1.72) 0.12/0.09	$9.58X10^{-3}$	1.57 (1.17-2.10) 0.10/0.07	$1.8x10^{-7}$
rs73411720	6	35295138	G/A 0.07	1.65 (1.30-2.09)	$5.53X10^{-5}$	0.58	0.12	1.43 (1.05-1.96) 0.07/0.05	$2.51X10^{-2}$	2.16 (1.43-3.26) 0.06/0.03	$2.7x10^{-7}$
rs4750290	10	13045926	G/A 0.08	1.67 (1.32-2.11)	$4.00X10^{-5}$	0.71	0.06	1.35 (0.97-1.88) 0.07/0.05	$7.25X10^{-2}$	2.16 (1.52-3.07) 0.07/0.04	$3.4x10^{-7}$
rs7923097	10	61634552	G/A 0.13	1.31 (1.14-1.50)	$8.11X10^{-5}$	0.00	0.88	1.30 (1.09-1.55) 0.23/0.18	$4.14X10^{-3}$	1.32 (1.07-1.64) 0.19/0.15	$8.0x10^{-7}$
rs2801539	10	90830632	A/G 0.28	1.32 (1.15-1.52)	$2.26X10^{-5}$	0.00	0.45	1.38 (1.15-1.64) 0.29/0.23	2.82X10-4	1.25 (1.02-1.52) 0.24/0.20	$2.0x10^{-7}$
rs1048932	11	114550060	G/A 0.48	0.79 (0.71-0.87)	$1.31X10^{-5}$	0.00	0.87	0.78 (0.68-0.89) 0.35/0.41	$9.98X10^{-4}$	0.79 (0.68-0.93) 0.39/0.44	$4.1x10^{-7}$
rs7132203	12	38787992	A/C 0.48	1.23 (1.12-1.36)	$7.24X10^{-5}$	0.00	0.40	1.30 (1.13-1.49) 0.44/0.50	5.38X10-4	1.19 (1.01-1.39) 0.51/0.47	$3.37X10^{-2}$

3.3.8 Gene set enrichment (pathway) analysis

3.3.8.1. Susceptibility to Barrett's oesophagus

Two GSEA software platforms were used (iGSEA and GenGen), to identify significantly 'enriched' pathways (those whose constituent genes contain significant differences in variants between cases and controls). Analysis was restricted to the genome-wide discovery GWAS data. For pathways identified (FDR<0.25) by both, confirmatory set-based tests were performed using PLINK.

3.3.8.2. Canonical pathways

Four significantly enriched canonical pathways were identified by iGSEA (FDR<0.05). A further five pathways demonstrated possible association (FDR<0.25). Fifteen significantly enriched pathways were identified by GenGen; of these, 7 demonstrated significant set tests. 28 possibly associated pathways were identified (supplementary tables 3.7-8).

No canonical pathways were significantly enriched by both approaches, but 3 were significantly enriched by iGSEA, and possibly enriched within GenGen: HSA04940 Type I Diabetes (iGSEA FDR=0.007, p<0.001; GenGen FDR = 0.07, p=0.0013), HSA04612 antigen processing and presentation (iGSEA FDR=0.0055, p=0.001; GenGen FDR=0.183, p=0.01500) and HSA00230 purine metabolism (iGSEA FDR=0.2222, p=0.002; GenGen FDR=0.001, p=<0.0001).

Confirmatory gene set testing was performed. Following correction for multiple comparisons (α=0.017), one remained statistically significant: of 1223 SNPs contributing to the KEGG HSA04940 Type I Diabetes pathway, 126 were significant and 73 independent; $p=4.60 \times 10^{-3}$. The HSA04612 antigen processing and presentation pathway demonstrated borderline significance (p=0.042; of 734 SNPs contributing, 107 were significant and 63 independent). No evidence of association was demonstrated for the HSA00230 Purine metabolism pathway (p=0.205; 5114 SNPs, 342 significant, 220 independent).

The KEGG Type I Diabetes pathway involves antigen presentation via the MHC I pathway, CD4+ T helper cell recruitment (predominantly Th1) and inflammatory cytokines. The KEGG antigen processing and presentation pathway is similar to the Type I Diabetes

pathway; it is downstream of both the MHC 1 and MHC 2 pathways, and involves inflammatory cytokine mediated endoplasmic and endosomal processing, and subsequent activation of CD4 (T helper), CD8 and NK (cytotoxic) cells.

12 genes are shared by both pathways (supplementary table 3.9): *TNFA* and 12 human leukocyte antigens (HLA). Both associations (and their overlap) are therefore consistent with the immunological and inflammatory mechanisms of BO.

3.3.8.3. Gene ontology biological processes

Two significantly enriched (and 12 possibly enriched) GO processes were identified by iGSEA. Sixteen and 67 were identified by GenGen (supplementary tables 3.10-11). However, no processes were enriched (either significantly or possibly) by both GenGen and iGSEA.

3.3.8.4. Relationship of GWAS-associated genes to pathways

GDF7 (associated with rs3072) is found in the TGF-beta signalling pathway, although this pathway was not significant following correction for multiple comparisons (GenGen canonical pathway FDR=0.186; p=0.02400). *C2orf43* (rs3072), *TBX5* and *RBM19* (rs2701108) are not found in any of the assessed pathways.

3.3.8.5. Progression to dysplasia and adenocarcinoma

Similar pathway analysis was performed for AspECT cases (from the discovery phase) progressing to dysplasia/adenocarcinoma. Sixteen significantly enriched canonical pathways were identified by iGSEA, plus 28 with FDR between 0.05 and 0.25 (supplementary table 3.12); none were identified by GenGen. 5 (plus 55) GO biological processes were identified by iGSEA (supplementary table 3.13); again none were identified by GenGen.

The 16 canonical pathways implicated by iGSEA (FDR<0.05) were predominantly those related to cell death, apoptosis and immunological signalling, entirely consistent with existing models of neoplastic progression.

Three of the 5 GO processes implicated pertain to cellular responses to extrinsic stimuli (other organisms, or microorganisms). These sets include a number of interleukins, tumour necrosis factors, chemokines and pro-apoptotic regulators. There was considerable overlap, with 30 genes shared these 3 pathways (supplementary table 3.14); these include pro-apoptotic factors (including *NOD1*, *NOD2*, *Bcl10*), the mucosal defence and differentiation gene *DMBT1* (a candidate tumour suppressor gene in oesophageal cancer, as evidenced by reduced expression(Mori et al., 1999))

Whilst the exocytosis and positive regulation of developmental processes GO processes share a number of gene families (including chemokines), only one gene (scinderin, *SCIN*, an actin-severing protein) was shared.

3.3.8.6. Relationship of possible susceptibility loci/genes to pathways

Of the 9 plausible gene associations identified, *SMAD2* again demonstrated the best supportive evidence. Although the TGF-β pathway was not enriched, *SMAD2* is present in: one significantly enriched canonical pathway (HSA0452 Adherens Junction, FDR=0.019), one possibly enriched canonical pathway (HSA05212 Pancreatic Cancer, FDR=0.172) and 9 possibly enriched GO biological processes (positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, FDR=0.079; pattern specification process, FDR=0.083; positive regulation of transcription, FDR=0.094; positive regulation of transcription DNA dependent, FDR=0.095; positive regulation of RNA metabolic process, FDR=0.123; negative regulation of RNA metabolic progress, FDR=0.165; negative regulation of transcription DNA dependent, FDR=0.165; negative regulation of transcription, FDR=0.172; and positive regulation of transcription from RNA polymerase II promoter, FDR=0.210).

There was minimal supportive evidence for other genes, although *CDH6* and *CADM1* are present in the significantly enriched canonical HSA04520 Adherens Junction pathway (FDR=0.019), the latter is present in the GO process Secretion by Cell (FDR=0.171), *DAPK1* is present in the GO process Positive Regulation of Developmental Process (FDR=0.04), and *ANK3* in the canonical HSA04360 Axon Guidance (FDR=0.034). *C2orf43*, *FOXP1*, *Pou5f1b* and *PTGER4* are not present in any curated pathways.

3.4 DISCUSSION

3.4.4 Summary of findings

Firstly, by further replication of the WTCCC GWAS, two new SNPs associated with BO susceptibility were identified by meta-analysis of 10,158 cases and 21,062 controls: rs3072 at 2p24, and rs2701108 at 12q24. Fine mapping and annotation was performed, with some success in generating functional hypotheses. It seems likely that both SNPs/loci exert a regulatory effect on neighbouring genes, potentially involved in thoracic and oesophageal development, and therefore increasing propensity to gastrooesophageal reflux.

Secondly, by systematic review and meta-analysis 27 germline variants (in addition to those previously described by the WTCCC GWAS) reported to be associated with BO susceptibility were identified, most commonly those involved in the inflammatory response to reflux. In addition, 5 associated with a combined BO/OAC phenotype were identified. However, only 13 survived *post hoc* correction for multiple comparisons, none have been validated, and overall evidence was poor. Replication using discovery data supported just one variant. Five somatic variants associated with progression were identified, although again study quality was poor. This notwithstanding, meta-analysis demonstrated a significant association with progression for CIN.

Thirdly, via systematic review an additional 9 GWAS variants associated with obesity were identified and combined with an existing analysis; concordance was seen for 32 (65.3%) of 49 SNPs (p=0.044).

Fourthly, exploratory analysis was performed for progression and OAC: GWAS (n=155 dysplasia/OAC and 1,697 controls) plus replication, and Immunochip alone (n=789 OAC and 8,809 controls). Twenty-six and 13 SNPs respectively were identified with $p<1x10^{-4}$. The genome-wide analysis identified one highly plausible association (rs1631576 and *SMAD2*) and two striking associations: rs587139 and *C2orf43*, and rs9880789 and *FOXP1*, both regions and genes previously identified as associated with BO, but via independent SNPs The Immunochip analysis identified 3 plausible associations with cell adhesion molecules.

Finally, using two GSEA platforms, 3 enriched pathways potentially associated with BO susceptibility were identified. Of these one, the KEGG Type 1 Diabetes pathway demonstrated strong evidence of association. Whilst a number of pathways associated with

progression were identified by one platform (GenGen), none were identified by the other iGSEA. These enriched pathways, however, provided strong evidence for the association of *SMAD2* with progression. Overall, GSEA suggested a strong role for the inflammatory and remodelling responses to reflux in both susceptibility to BO, and subsequent progression.

3.4.5 Discussion of new GWAS susceptibility variants

3.4.6 Variants identified

Both variants identified are common (minor allele frequencies 30.8% and 31.3% respectively) and intergenic, therefore either functional and regulatory rather than non-coding, or non-functional and 'tagging' another SNP.

Imputation, fine mapping and annotation suggested the locus at 2p24 to be functional. The lead SNP rs3072 lies within an enhancer region, between two genes: *GDF7* and *C2orf43*. The former is a more plausible target, being a member of the BMP pathway, implicated as a 'master switch' family promoting transition of injured oesophageal squamous epithelium to BO (Castillo et al., 2012, Milano et al., 2007). A viable antibody to detect protein expression is not available, although mRNA is expressed within oesophageal squamous epithelium. In addition, 2 local SNPs (rs9306894 and rs7255) demonstrated possible eQTL activity (although less strongly associated with BO using imputation).

However, *in silico* eQTL analysis demonstrated no evidence of association of rs3072 with *GDF7* expression, rather highlighting *C2orf43* as a functional target of all three SNPs. This encodes a 325 amino acid protein UPF0554; whilst its function is unknown, it contains motifs suggesting enzymatic activity. Intriguingly, a SNP located within *C2orf43* (rs13385191) was identified previously by GWAS as associated with prostate cancer (Christensen et al., 2010), whilst another (independent) intronic *C2orf43* SNP, rs587139, was strongly associated with progression to dysplasia/OAC in the AspECT GWAS progression analysis.

Whilst the lead SNP at 12q24 identified was rs2701108, this mapped to a region with little obvious regulatory effect. However, imputation of the GWAS discovery data suggested rs1920562 to have the strongest association signal, and therefore in fact be functional. This was supported by its mapping to a highly-conserved intergenic enhancer region, and binding of the DNA-binding protein IKZF1, the RNA of which is expressed in gastric epithelium. *TBX5* appears the likely functional target, although there are no data currently supporting

interaction with either SNP; it is expressed in both oesophageal and gastric epithelia, and regulates mesodermal and thoracic embryogenesis, including formation of diaphragmatic skeletal muscle (Rallis et al., 2003).

The simplest functional explanations of both SNPs are an increased propensity to metaplastic gene expression and phenotype via the BMP pathway in response to reflux-induced inflammation for rs3072, and greater predisposition to this via diaphragmatic/oesophageal anatomical variance for rs2701108/rs1920562. Assuming these to be the case, this is supportive of the inflammatory and embryological hypotheses drawn from prior GWAS loci: rs9257809 within the MHC, and SNPs potentially related to *FOXF1* (rs9936833) *FOXP1* (rs2687201) and *BARX1* (rs11789015) respectively.

3.4.6.1. Limitations of this study and GWAS

The hypothesis-free tenet of GWAS confers a number of advantages over candidate studies, primarily the ability to identify new biological processes (Mohlke et al., 2008), but with considerable implications for cost and resources. Furthermore, the difficulties seen in unravelling the mechanisms of GWAS 'hits', and the disparity between the number of these and overall heritability estimates, have led to the value of GWAS being questioned (Visscher et al., 2012).

Firstly, an estimated 1000 cases and controls are required to identify variants with OR between 1.7-2 (Eberle et al., 2007, Gail et al., 2008). Consequently, much larger numbers are required to detect most CDCV-predicted variants, underlying the multistage approach within this study to achieve comparable power and type 1 error rates to single phase GWAS (Thomas et al., 2009). However, whilst variants are usually taken forward to replication stages on the basis of their p values (as in this study), other methods have been suggested; including false positive report probability and population-attributable risk, and Bayesian modelling (Hunter and Kraft, 2007, Wakefield, 2007).

For multi-stage replication, it is crucial to minimise measurement and population heterogeneity (i.e. diagnostic criteria for BO and ethnicity). To obtain sufficient samples in this study required the use of a number of prospective registries. Whilst the latter was minimised using self-reported ethnicity and PCA, the former varied in line with international practices. However, 8,521 of 10,158 cases had IM, and when the analysis was restricted to these, significance persisted for rs3072, and was borderline for rs2701108.

As discussed in Chapter 1.2.6.3, GWAS thus far have accounted for only a small minority of common trait heritability. This missing heritability may be due to common variants as yet unidentified, or variants presently beyond the reach of GWAS. The failure of GWAS to identify the former may be due to insufficient power and/or sampling bias, due to variable allele frequencies across populations. GWAS typically account for approximately 10-20% of trait phenotype (Franke et al., 2010). A recent model, however, suggested that less of this heritability is actually missing; rather, that the stringent statistical thresholds used in GWAS mean that although contributory variants are often tagged, their effects are not statistically detected (Yang et al., 2010a).

Further explanations include undiscovered rare variants (as predicted by the common-disease-rare variant model (Do et al., 2012)) which by definition are not included on GWAS arrays (Zeggini et al., 2005), structural variance (McCarroll, 2008), incomplete LD, gene-gene and variant-variant interaction, transcriptional and post-transcriptional modulation, epigenetic regulation and environmental interaction. As NGS and bioinformatics technologies advance, and costs reduce, large-scale sequencing may mitigate some of these.

3.4.6.2. Further exploration of GWAS variants

Elucidating the mechanisms of GWAS 'hits' is challenging, particularly as the variant is unlikely to be functional, but rather tagging another. This can be addressed using fine mapping via imputation, which uses local LD structure to infer 'hidden' genotypes from apparent genotypes (those included on arrays), using haplotypes on a reference dataset. This assumes that the reference and genotyped populations are the same (Halperin and Stephan, 2009). However, strong LD between variants may still obscure functional relevance. It may be possible to compare phenotypes with fine mapping in different ancestral populations, although this is confounded by strong association of BO with Caucasian ancestry. Further difficulties include the fact that a variant may demonstrate the greatest statistical association in GWAS by tagging multiple true risk alleles of weaker effect. Whilst stepwise logistic regression could theoretically be used within a group of such candidates, in reality the strength of LD and weakness of effect sizes would require a study population several orders of magnitude greater than a typical GWAS (McCarthy and Hirschhorn, 2008).

If a variant lies within a coding sequence, its functional consequences can be predicted using techniques such as PolyPhen (Adzhubei et al., 2010) and evaluated *in vivo*. The function of intronic and intergenic variants can be explored *in silico* as in this study, using eQTL, RNA

and protein expression databases. Again, as with this study, these may identify additional candidate variants via LD. However, public data are often limited as regards variants and tissue expression, and the lack of an antibody (such as for GDF7) may limit analysis. Despite this, eQTL databases allow for mechanistic hypotheses, which can be assessed by techniques such as transgenic models (for example using gene 'knock-out', or more subtle genome-editing for variants to assess phenotype) (Heckl et al., 2014). However, as the biological impact of such variants is unknown, determining which is the tissue of interest may be difficult (particularly if, as in this study, there is an embryological hypothesis).

3.4.7 Attempted replication of variants reported in the literature

The 4 BO/OAC GWAS variants described by Levine *et al* were evaluated within the discovery GWAS data; whilst the evidence was supportive for rs2687201 ($p=1.18x10^{-2}$), rs10423674 ($p=4.96x10^{-2}$) and rs11789015 ($p=6.63x10^{-2}$), no replication was evident for rs10419226 (p=0.865). The disparity between GWAS questions their generalizability, although may more likely reflect sampling bias and variance of effect sizes between both populations.

Of the 27 reported non-GWAS variants in the literature, 20 underwent replication by analysing discovery GWAS data. Supportive evidence was demonstrated for 2 SNPs with $p<0.05$ in the discovery/meta-analysis phases: rs3776083 in *CDX1* (p=0.025); and rs909253 in *TNFB* (p=0.016), and one with $p>0.05$: rs11209026 in *IL23R* (p=0.073). However, whilst nominally significant, these p values are 5 orders of magnitude below the GWAS threshold. Whilst this, as discussed above, does not preclude weak (and combination) associations, the disparity seen between the original studies and discovery data suggests that the original associations were due to sampling bias.

Further sources of bias are evident in study methodology. Whilst the comprehensive REMARK guidelines used for a 'gold standard' were devised for tumour markers of outcome, these were modified by the author for the context of BO susceptibility and progression. Most studies did not blind researchers to phenotype/outcome, match cases and controls for confounding risk factors, and report quality control steps. There was also heterogeneity as to phenotypes, with studies either comparing BO (with variable diagnostic criteria) to healthy controls, or those with reflux oesophagitis. Most did not adjust appropriately for multiple comparisons, and when performing multivariate analysis did not adjust for clinically-relevant confounding variables, and only 7 assessing more than one

marker assessed their interactions. Ultimately, whilst perhaps plausible, few of the numerous variants of susceptibility described are supported by quality evidence, and as none have been independently validated do not have clinical utility. Whilst CIN as a somatic marker of progression was supported by meta-analysis, this was performed within the context of considerably heterogeneity.

There are number of methodological limitations of this review. Whilst searching of two major databases was performed with a comprehensive search term, it is possible that relevant publications were not identified. In addition non-English articles were excluded. Only one meta-analysis (CIN and OR of progression) involved more than 5 studies, but evidence of funnel plot asymmetry (likely publication bias) was corrected using the 'trim-and-fill' method. The small number of studies involved in each analysis precluded meta-regression to explore potential confounding factors (Thompson and Higgins, 2002). There are also limitations to the use of the revised ASCO guidelines. Whilst these do not capture the complexity inherent in data quality, we sought to do so using custom REMARK guidelines. However, determination of overall LOE may be difficult; evidence can be upgraded by validation studies, yet disagreement of effect size and direction is not always reflected. In particular, they do not allow for differences in methodology, for example GWAS versus candidate studies.

Ultimately, whilst plausible, no markers (other than CIN) have been independently validated and therefore lack clinical utility. Whilst CIN appears promising, precise definitions are required in addition to more generic validation. Consequently, a number of lessons can be drawn for future studies: primarily the need for much larger populations powered for variants of more modest and plausible effect sizes, robust adjustment for established (and readily available) clinical risk factors, and appropriate statistical techniques to adjust for any multiple comparisons.

3.4.8 Variant concordance for obesity and Barrett's oesophagus

An additional 9 GWAS variants associated with obesity were identified and combined with an existing analysis; concordance was seen for 32 (65.3%) of 49 independent SNPs ($p=0.044$).

More than 60 GWAS SNPs predisposing to obesity have been reported. Obesity is as a strong risk factor for BO, likely mediated by abdominal obesity overwhelming the basal tone

of the lower oesophageal sphincter, the ability of the diaphragmatic sphincter to compensate, and also anatomical restraints to hiatus hernia (Mercer et al., 1987, Drahos et al., 2014). However, additional mechanisms have been suggested, related to the systemic effects of obesity and the metabolic syndrome as reviewed in Chapter 1.2.5.2.

Such concordance of phenotypes has been suggested to allow 'reverse genetics'; a reverse GWAS approach would take an established variant, and look for associations with a large number of phenotypes. Fine-mapping and imputation of phenotypes might be performed (McCarthy and Hirschhorn, 2008), to identify concordance between phenotypes (comparable to LD) and combinations (analogous to genetic haplotypes). This combined phenotype may then be found to predispose to another (such as metabolic syndrome and Parkinson's disease) (Zhang and Tian, 2014).

3.4.9 Variants associated with progression and adenocarcinoma

Two analyses were performed, one genome-wide for progression to dysplasia/OAC, and one for OAC with or without preceding BO. These identified a number of intriguing SNPs with non-significant associations (as expected due to the low number of progression/OAC events), although within the context of a number of caveats.

The different definitions of progression and lack of specific information for the AspECT cohort were due to information constraints for the relevant studies. Overall, power for the GWAS analysis was limited with only 155 progressors, although there were ample controls. Although patients underwent prospective surveillance, there were no data available on the time to event to allow a proportional hazards analysis. Similarly, no adjustment/control could be made for important confounding variables (such as length of metaplasia). There were also important differences in genotyping; for the GWAS analysis, 1,852 AspECT cases had genome-wide genotypes (from discovery), however, the entire ChoPIN and Dutch cohorts (the Immunochip OAC analysis and GWAS 'replication') were genotyped on the Immunochip (196,524 SNPs with possible immunological/inflammatory effects), with consequent bias. A further limitation of the latter analysis was the use of population controls, rather than BO non-progressors as in the former.

Consequently, the associations identified from the Immunochip (ChOPIN/Dutch) analysis should be viewed with caution, exemplified by the identification of 4 variants (without obvious mechanistic hypotheses) at 6p21: the MHC. This notwithstanding, a number of

plausible associations were identified ($p<1.00x10^{-4}$). The strongest was that of the cell adhesion molecules, with 3 variants (of which rs12658568 and rs4867305, its perfect proxy, an eQTL within a likely enhancer for *CDH6*, appears most plausible). Aberrant cell adhesion molecules are near universal within carcinogenesis (Nair et al., 2005). Cadherin 6 (*CDH6*, a class II K-Cadherin promoting epithelial embryogenesis) is upregulated in both renal and ovarian carcinoma (Paul et al., 1997, Kobel et al., 2008). Whilst *CDH1* is a component of the TGF-β pathway, *CDH6* has recently been identified as a downstream target of this pathway in thyroid cancer (Sancisi et al., 2013), adding further weight to the importance of this pathway in both BO metaplasia and progression.

Similarly, rs1048932was identified as an eQTL associated with Adhesion Molecule 1 (*CADM1*) in lymphoblastoid and adipose tissues; epigenetic inactivation of *CADM1* is associated with a worse prognosis of oesophageal SCC (Lee et al., 2013), and this gene is suspected to be a common non-oesophageal tumour suppressor (Michels et al., 2008). Another potential association (although without eQTL evidence) was rs12515934, lying within an enhancer region 303kbp upstream of *PTGE4R* (prostaglandin E4 receptor), which in addition to molecular evidence of BO progression promotes endometrial tumour proliferation (Catalano et al., 2011). Furthermore, the rs13361707 variant (in minimal LD with rs12515934) has been identified as a risk SNP for non-cardia gastric cancer by a Chinese GWAS (Shi et al., 2011).

Thirteen SNPs with strong evidence of association ($p<1.00x10^{-4}$) with progression were identified by the GWAS analysis. That with the best mechanistic hypothesis was the *SMAD2* intronic variant rs1631576 (discovery OR 0.61; $p=4.00 x10^{-5}$), associated with *SMAD2* expression in all Genevar databases, and expressed in both oesophageal and gastric mucosa. Progressively reduced SMAD2 mRNA and protein expression (along with other members of the TGF-β pathway) have been demonstrated within BO metaplastic, dysplastic and OAC cells (Onwuegbusi et al., 2006). LOH of 18q21.1 is a frequent finding in metaplasia (32-46%), and again progressively more common with dysplasia and neoplasia (69%)(Wu et al., 1998, Barrett et al., 1996). Further supportive evidence for the role of *SMAD2* in progression was provided by GSEA, present in one significantly enriched and 10 possibly enriched pathways. Therefore, an aberrant TGF-β pathway may both promote metaplasia, and as a tumour-suppressor pathway subsequent progression (Dumont and Arteaga, 2003).
There were two notable associations in chromosomal regions identified by both previous GWAS, those of *C2orf43* and *FOXP1*. rs587139 (OR progression 2.22 [1.53-3.21]; $p=2.29x10^{-5}$) is an intronic variant of *C2orf43* independent of rs3072, lying within a putative

enhancer region (similar to that of the prostate risk SNP rs13385191 (Long et al., 2012)). Although there was no evidence of an eQTL within the available databases, this provides strong supportive evidence of this region (and potentially *C2orf43*) with both predisposition metaplasia and subsequent progression, although the role of the encoded UPF0554 protein is unclear.

The other striking association was that of rs9880789, downstream of *FOXP1*, independent of the rs2687201 SNP described by the BEACON GWAS. Although there was no evidence of eQTL activity, this again supports the potential role of this region/gene in OAC pathogenesis (particularly as rs268702 was associated with a combination BO/OAC phenotype, rather than BO alone).

The lack of concordance seen between susceptibility and progression variants is unsurprising, as it is plausible that both discrete and overlapping processes are involved. However, the concordance of the *C2orf43* region via independent variants is particularly intriguing; this principle has not been previously demonstrated, and increases the likelihood that *C2orf43* is functional, rather than or in addition to *GDF7*. That of *FOXP1* is less surprising, as it was previously described within the context of BO/OAC. This generates the new hypothesis that variants in the region of susceptibility variants may independently predispose to progression. This hypothesis was considered *post hoc*: by convention 1 megabase pairs (Mbp) is considered a surrounding region; 2,256 variants in the 1Mbp surrounding the 8 previously identified GWAS variants were present on the discovery arrays, of which 1,550 are independent ($r^2<0.8$). The resultant Bonferroni-corrected p value would be 3.24×10^{-5}, greater than the p value demonstrated for both such variants.

3.4.10 Gene set enrichment analysis

GSEA of discovery phase data for susceptibility, using two platforms, identified 56 canonical pathways and gene ontology biological processes overall. A number of related platforms are available, but the few comparative validations performed have failed to determine the optimal method, advising instead the use of complementary software (Fehringer et al., 2012).

Two contrasting approaches were therefore performed and compared, and to further minimise false positive associations, *post hoc* set-based tests were performed. Both platforms, whilst retaining the same gene set classifications and input parameters have important differences: iGSEA uses SNP-label rather than phenotype-label permutation, and

weights gene sets towards those with greater proportions of significant genes. By contrast, GenGen uses 1000 phenotype permutations (Wang et al., 2007). Neither prune for LD. Whilst GenGen incorporates only the most significantly associated SNP within a gene within the analysis, iGSEA includes all, which may improve sensitivity, but at the expense of introducing bias towards gene sets with larger genes (Fehringer et al., 2012). GenGen is perhaps the most widely used method in the literature, with arguably a more robust design limiting the number of significant pathways identified (Wang et al., 2009, Fehringer et al., 2012).

Three pathways were supported by evidence from both platforms. One was corroborated by highly significant set-based tests (HSA04940 Type 1 Diabetes pathway), and another with nominal significance (HSA04612 antigen processing and presentation pathway, p=0.042). Both are supportive of the role of the inflammatory response to reflux in susceptibility to BO, and their identification provides a context for the most likely contributory genes and variants.

3.4.11 Conclusions and priorities for future research

Two novel susceptibility variants and loci were identified: rs3072 and rs2701108. The former appears functional but its target gene(s) unclear: whilst *GDF7* is a member of the TGF-β pathway and therefore the most plausible, *in silico* expression analysis suggests *C2orf43* (encoding a hydrolase of unknown function) to be most likely. By contrast, rs2701108 may be tagging rs1920562; the most plausible target gene being *TBX5* (involved in thoracic and diaphragmatic development), although there are a lack of supportive expression data. Preliminary progression analysis also highlighted an independent *C2orf43* variant (in addition to *FOXP1*) to be associated with progression, an entirely novel observation. A further variant, an eQTL of *SMAD2* (another member of the TGF-β pathway) was associated with progression, and strongly supported by GSEA. These, and other variants arising from both analysis (and the two pathways strongly associated with susceptibility) provide priority candidates for analysis in future genetic and expression analyses.

CHAPTER 4

A SYSTEMATIC REVIEW AND META-ANALYSIS OF SOMATIC AND GERMLINE DNA SEQUENCE BIOMARKERS OF OESOPHAGEAL CANCER SURVIVAL, THERAPY RESPONSE AND STAGE

4. CHAPTER 4: A SYSTEMATIC REVIEW AND META-ANALYSIS OF SOMATIC AND GERMLINE DNA SEQUENCE BIOMARKERS OF OESOPHAGEAL CANCER SURVIVAL, THERAPY RESPONSE AND STAGE

4.1 SYNOPSIS

4.1.1 Introduction

There is an urgent need for biomarkers to help predict prognosis and guide management of oesophageal cancer. Genetic markers represent an important class of potential markers, however, no systematic reviews or meta-analyses have been performed to date. The aim of this chapter was to review identify, evaluate and meta-analyse the evidence for reported somatic and germline DNA sequence biomarkers of outcome and stage.

4.1.2 Methods

A systematic review was performed of the PubMed, EMBASE and Cochrane databases (20th August 2014), in conjunction with the ASCO level of evidence scale for biomarker research. Meta-analyses were performed for all reported markers associated with outcome measures by more than one study.

4.1.3 Results

4004 articles were identified, 762 retrieved and 182 studies included. There were 65 reported markers of survival or recurrence; 12 (18.5%) were excluded due to multiple comparisons. Following meta-analysis, significant associations were seen for 6 tumour variants (mutant *TP53* and *PIK3CA*, copy number gain of *ERBB2*/*HER2*, *CCND1* and *FGF3*, and CIN/ploidy) and 7 germline polymorphisms: *ERCC1* rs3212986, *ERCC2* rs1799793, *TP53* rs1042522, *MDM2* rs2279744, *TYMS* rs34743033, *ABCB1* rs1045642 and *MTHFR* rs1801133. Twelve germline markers of treatment complications were reported; 10 were excluded. Two tumour and 15 germline markers of chemo(radio)therapy response were reported. Following meta-analysis, associations were demonstrated for mutant *TP53*, *ERCC1* rs11615

and *XRCC1* rs25487. There were 41 tumour/germline reported markers of stage; 27 (65.9%) were excluded.

4.1.4 Conclusions

In conclusion, numerous DNA markers of outcome and stage have been reported, yet few are backed by high quality evidence. Despite this, a small number of variants appear reliable. These merit evaluation in prospective trials, within the context of high-throughput sequencing and gene expression.

4.2 INTRODUCTION

As discussed in Chapter 1.3 there is an urgent need for biomarkers to help guide management of oesophageal and GOJ cancer. A poor prognosis despite what often appears to be curative treatment highlights limitations in our biological understanding, and our urgent need for biomarkers to predict prognosis, recurrence and sensitivity to therapy, and ultimately better personalize care.

Most experience with oesophageal molecular biomarkers has involved protein expression with; whilst such markers are used to select patients for early phase trials, the sole tumour marker in routine use is *ERBB2*/*HER2* status (Allum et al., 2011, Chan et al., 2012). However, rapid advances in NGS have highlighted the potential role of somatic DNA sequence markers. These may function as independent markers, serve to refine or explore existing expression markers, or constitute novel therapeutic targets (Dulak et al., 2013, Agrawal et al., 2012, Streppel et al., 2014). Similarly, advances in custom and genome-wide SNP arrays have emphasized the role of germline variants in modulating cancer and treatment outcome (Kim et al., 2011, Patel et al., 2013).

The aim of this chapter therefore was to undertake the first systematic review of DNA sequence biomarkers of oesophageal cancer, to systematically identify and evaluate all candidate somatic and germline DNA sequence markers of outcome (survival, recurrence, therapy response and treatment complications) and stage. Meta-analysis was then performed for all markers with a nominally statistical association in at least one study.

4.3 RESULTS

4.3.1 Literature search, inclusion criteria, data, and meta-analysis

A systematic review was performed of the PubMed, EMBASE and Cochrane databases on the 20th August 2014. Studies eligible were those testing an association between a DNA sequence marker (germline/somatic) and outcome (clinical, radiological, pathological) or stage of oesophageal/GOJ cancer. Clinical outcomes comprised survival (any measure), recurrence, disease progression and treatment complications. Radiological outcomes comprised therapy response. Histopathological outcomes comprised pTR (as defined by the individual study) and incomplete resection (R1/2). Stage comprised clinical and pathological TNM and grade (Rice et al., 2010a). Studies using cell lines or expression data were excluded unless discrete tumour or DNA-specific data could be extracted. Non-English articles were excluded.

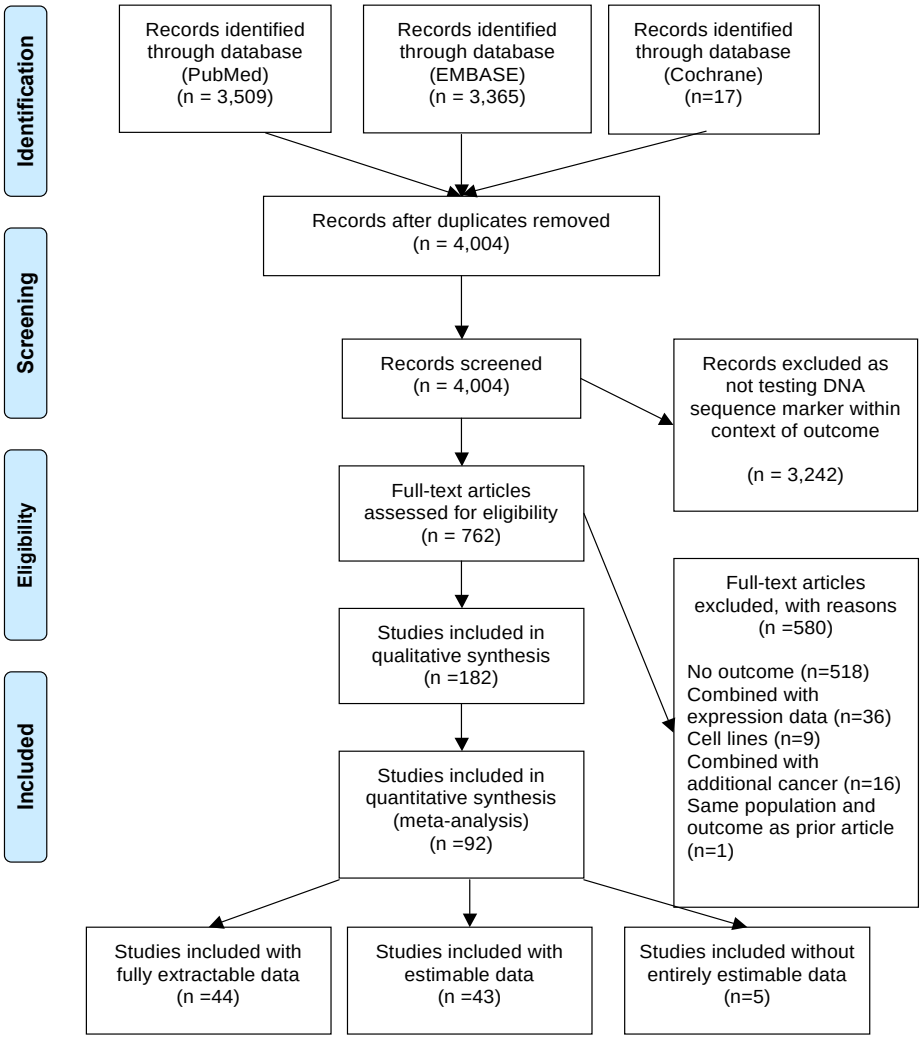
For associations it was determined whether correction for multiple comparisons had been performed (Bonferroni method, FDR, multivariate analysis including all variables) (Bland and Altman, 1995). If none had made, *post hoc* Bonferroni correction was performed. For markers with p>corrected α, effect metrics were calculated but the marker excluded and presented separately (supplementary tables 1 and 2), unless assessed by more than one study (in which case meta-analysis was performed. In studies not presenting HR, OR and SE, these were estimated using the methods of Parmar, or extracted from magnified Kaplan–Meier survival curves: HR and SE were estimated at constant time points, with censored patients allocated to the appropriate time interval (Parmar et al., 1998).

Separate meta-analyses were performed where possible for major methodological differences such as adjusted/unadjusted HR, genotyping methods, treatment, cell type and ethnicity (International HapMap, 2003), as described in Section 2.1.6.6. In 6 meta-analysed studies (all non-significant results) (Miller et al., 2003, Uchino et al., 1996, Chikuba et al., 1995, Itakura et al., 1994, Wang et al., 1999, Warnecke-Eberz et al., 2009) it was not possible to extrapolate statistics for all variants; an lnHR of 0 (HR of 1) and SE of the most closely matched study (cell type, size and methodology) were used.

4.3.2 Study characteristics

4004 articles were identified, 762 retrieved for evaluation, 580 excluded (figure 4.1) and 182 included, published between 1989 and 2014. 73 assessed markers of clinical outcome, 80 clinical outcome and stage, and 29 stage alone. To comply with the word limit of this thesis, individual study details are available in the supplementary data (supplementary tables 1-23) accompanying the published online version of this study (http://annonc.oxfordjournals.org/content/26/4/624) (Findlay et al., 2014b).

Figure 4.1: Literature search



Survival measures were OS (n=133), DFS (n=20), recurrence (n=19), PFS (n=4), and DSS (n=4). Twenty-nine studies assessed response to therapy (chemo+/-radiotherapy, or biological). Eleven assessed treatment complications. Treatment intent was curative (n=156), palliative (n=5), mixed (n=21) and unspecified in 1. Curative modalities were resection alone

(n=111), resection+/-neoadjuvant (n=33) or adjuvant (n=2) therapy, or DCR (n=9). All chemotherapy regimens involved platinum agents with or without 5-fluorouracil, except 3 (bleomycin, gefitinib, irinotecan). 176 studies were candidate based, and 6 genome-wide (1 SNP, 5 CNV). 117 studies assessed tumour variants, 64 germline, and 1 both. Cell types assessed were SCC (n=117), AC (n=40), both (n=3) and unspecified (n=22).

4.3.3 Methodological quality

LOE was B for 5 studies (2.72%), C for 105 (57.1%), and D for 74 (40.0%). Median number of subjects was 90 (range 10-2932), although 48 studies included fewer than 50. 46 (25.0%) studies were prospective; 137 (74.5%) were retrospective; 1 (0.55%) had both components. Multivariate adjustment of effect sizes was performed by 57 studies (31.0%).

4.3.4 Molecular quality

Just 37 (56.9%) of 65 studies assessing germline variants assessed HWE; 59 (90.8%) reported genotyping success rate (or provided data allowing its calculation).

4.3.5 Markers of survival and recurrence

There were 65 reported markers of survival or recurrence: 24 tumour (table 4.1; 3 mutations, 16 CNV, 2 LOH regions, 1 telomere length ratio, CIN, heterogeneous ploidy) and 40 germline polymorphisms (table 4.2).

4.3.5.1. Tumour mutations

Three mutant genes were reported to be associated with outcome: *TP53*, *PIK3CA* and *NRF2* (LOE IV). *TP53* (n=21) and *PIK3CA* (n=3, SCC) underwent meta-analysis.

TP53 status was variably defined and genotyped, although all studies assessed exons 5-8 as a minimum, by single strand conformation polymorphism (SSCP) analysis with or without sequencing (n=13), or Sanger sequencing alone (n=8). Following correction for likely publication bias, a significant negative survival association was demonstrated for mutant *TP53* tumours: HR 1.27 (1.01-1.59; p=0.04; n=21 studies; figures 4.2-4.4). Significant associations

were demonstrated on subgroup meta-analysis of: genotyping technique (SCCP), AC and SCC cell types, and treatment (resection only). Directions of effect were consistent but non-significant for adjusted HR alone (n=6) and use NACR (n=7). An association with DFS was demonstrated for mutant *PIK3CA* SCC tumours (HR 0.42 [0.21-0.85]; n=2; p=0.02), but not OS.

Figure 4.2: Uncorrected Forrest plot: *TP53* mutation and survival (all cell types)

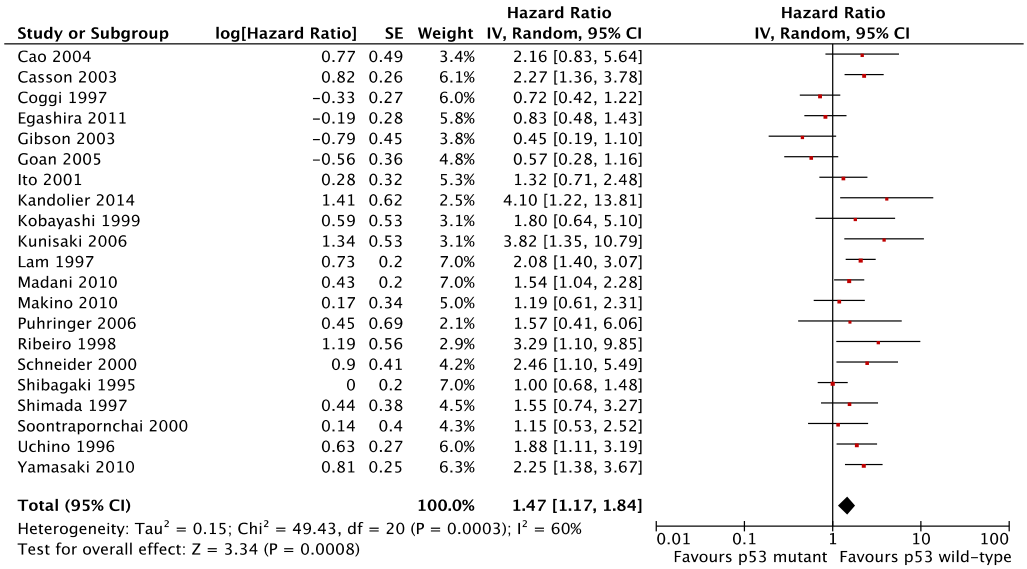


Figure 4.3: Uncorrected Forrest plot: *TP53* mutation and overall survival (adenocarcinoma)

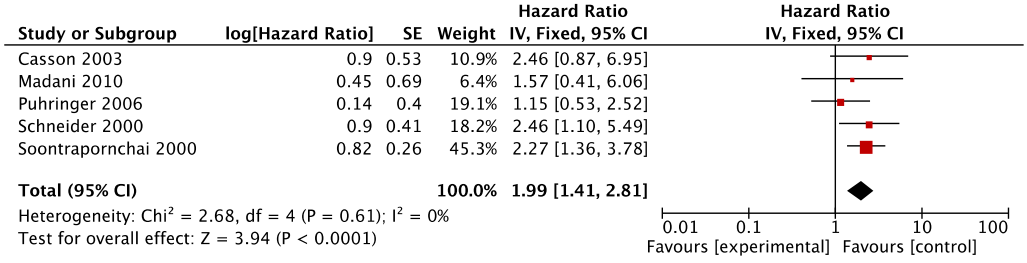
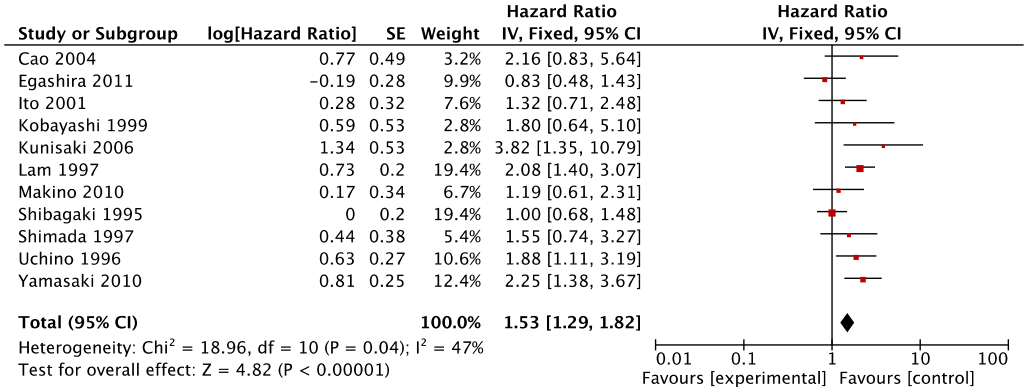


Figure 4.4: Uncorrected Forrest plot: *TP53* mutation and overall survival (squamous cell carcinoma)



4.3.5.2. Tumour copy number variants

16 tumour CNVs had previously reported associations with prognosis; 3 were excluded due multiple comparisons. For the remaining 13 LOE were II (1), III (7) and IV (5). Four markers underwent meta-analysis: associations with worse survival were demonstrated for gains in *ERBB2* (*HER2*; LOE III), *CCND1* (LOE III) and *FGF3* (LOE IV), but not *EGFR* (LOE II). For all 4 there was heterogeneity regarding definition of CNV (absolute copy numbers, or ratio to normal), and genotyping technique (fluorescent/silver *in situ* hybridisation [FISH/SISH], quantitative PCT [qPCR], and slot/Southern blot).

ERBB2/*HER2* analysis was restricted to 11 studies performing FISH/SISH; 3 (using qPCR (Miller et al., 2003), or slot (Ikeda et al., 1996)/Southern (Suzuki et al., 1997) blot) were excluded. Worse OS was demonstrated for *ERBB2*/*HER2* gain overall: HR 1.63 (1.20-2.21; $p=2x10^{-4}$, n=11). Significance persisted for adjusted HR (2.32 [1.64-2.58]; $p<1x10^{-5}$; n=3), and was borderline for cell type (SCC; AC p=0.06). Treatment regimens were resection alone for all studies, except one (Lennerz et al., 2011) including mixed regimens involving the c-MET-GFR inhibitor Crizotinib.

Two meta-analyses were performed for *CCND1*: studies using qPCR (n=4), and slot blot/FISH (n=2). Worse OS was demonstrated for qPCR (HR=2.09 [1.27-3.42]; p=0.004), with a concordant but non-significant trend for FISH/blot. All 4 studies assessing *FGF3* used slot/Southern blot; an association with worse OS was demonstrated (HR 1.83 [1.18-2.83]; p=0.006). *EGFR* meta-analysis was performed for FISH and blot techniques (excluding 2 studies using anti-EGFR therapy, (Luber et al., 2011, Janmaat et al., 2006) and one performing qPCR) (Miller et al., 2003). No associations were demonstrated.

4.3.5.3. Loss of heterozygosity

LOH (6 regions in total) was associated with outcome in 4 studies; one study (6p and 13q) was excluded due to multiple comparisons; (Shibagaki et al., 1994) for another (2p, 3p and 12p) whilst 3 year survival rates were significant the extrapolated HR was not.(Ikeguchi et al., 1999) 1q22-23 LOH was associated with worse OS in one study (LOE IV).

4.3.5.4. Telomere length ratio

One study reported worse OS with a tumour:normal telomere length >1.17 (LOE III).

4.3.5.5. Genomic instability

CIN was assessed by 6 studies. Following exclusion of one study including intratumoural heterogeneous ploidy (Edwards et al., 1989), an association with worse OS was demonstrated (HR 1.63 [1.25-2.11]; n=4; $p=2.00x10^{-4}$). One study assessed intratumoural heterogeneity alone, reporting better survival than with homogeneity (Deguchi et al., 1993). There were no associations between MSI and survival.

4.3.5.6. Germline polymorphisms

29 reported associations were identified (following exclusion of 12 due to multiple comparisons). Cumulative LOE was II (n=3), III (n=22) and IV (n=4). 15 variants underwent meta-analysis (table 4.2). Significant associations were demonstrated for 6 SNPs: *ERCC1* rs3212986 (cisplatin treatment; LOE II; Caucasian ethnicity), *ERCC2* rs1799793 (cisplatin; Caucasian), *TP53* rs1042522 (Caucasian), *MDM2* rs2279744 (Caucasian), *TYMS* rs34743033 (Japanese; LOE III) *ABCB1* rs1045642 (Caucasian and Japanese; LOE IV). An association was demonstrated for *VEGFA* rs2010963, but combining 2 studies with East Asian ethnicities (Taiwanese and Japanese). One association with recurrence was demonstrated: *MTHFR* rs1801133 (Caucasian; LOE III).

4.3.6 Markers of treatment complications

Twelve reported germline associations (8 studies) were identified; 10 were excluded due to multiple comparisons. One marker, *TNFA* rs1800629 (Azim et al., 2007), underwent meta-analysis (non-significant). The remaining variant, *ACE* rs4646994 (LOE III), was associated with post-operative pulmonary complications by one study (table 4.3).

4.3.7 Markers of response to chemo(radio)therapy

Two tumour variants (mutant *TP53* and CIN) and 15 germline polymorphisms were reported to be associated with clinical or pathological response to chemo+/-radio-therapy (table 4.3); 11 polymorphisms were excluded due to multiple comparisons).

Mutant *TP53* was assessed by 6 studies; 3 for pathological and 3 for clinical response. A lower OR of pTR was demonstrated (OR 0.24 [0.06-0.95], n=3, p=0.04); effect direction for clinical response was concordant but non-significant (OR 0.43 [0.08-2.24]; p=0.32).

Following meta-analysis, 2 polymorphisms were associated with a major pTR to platinum-based chemo/radiotherapy in Caucasians: wild type *XRCC1* rs25487 (GG genotype, LOE III; OR 1.91 [1.30-2.81], n=3, p=0.001), and variant *ERCC1* rs11615 (TT/CT; OR 4.57 [3.01-6.94; n=3; $p<1x10^{-5}$). The AA variant of *ERCC1* rs3212986 (LOE III) was associated with radiological response to palliative cisplatin-based chemotherapy in one study (Chinese ethnicity), but not major pTR to neoadjuvant chemotherapy in two (Caucasian).

4.3.8 Markers of stage

24 tumour markers were reported: 2 mutations, 12 CNV, 7 LOH, 2 MSI and CIN; 15 were excluded.

4.3.8.1. Tumour mutations

Following exclusion of *PIK3CA*, the sole tumour mutation with a reported association was *TP53* (n=19; LOE III; table 4.4). Following meta-analysis, mutant *TP53* tumours were associated with more advanced T (T3/T4) and N (≥N1) stages, but not overall TNM stage (III/IV), grade (G3/4) or positive resection margin (R1).

4.3.8.2. Copy number variants

Twelve tumour CNVs were identified; 8 were excluded due to multiple comparisons (table 4.4). Meta-analysis was possible for 2 markers (*ERBB2* and *EGFR*), with 1 significant association demonstrated: *EGFR* (T1/2 stage; LOE IV).

4.3.8.3. Loss of heterozygosity

Seven LOH variants were identified; 5 were excluded (table 4.4). Meta-analysis was possible for 1 marker: a significant association was demonstrated for LOH 13p and ≥N1 stage.

4.3.8.4. Genomic instability

Following meta-analysis CIN (LOE III; table 4.4) was associated with overall TNM 6th edition stage (III/IV: OR 2.68 [1.10-6.54]; p=0.03; n=2 studies) and nodal stage (OR 2.18 [1.06-4.47]; p=0.03; n=7 studies), but not T stage or grade. MSI (using the 5 Bethesda markers (Boland et al., 1998), and 10 markers at 17q24-25) was associated with more advanced stage; another measure was excluded.

4.3.8.5. Germline polymorphisms

17 polymorphisms were identified; 12 were excluded due to multiple comparisons. One marker (GNAS1 *rs7172*) underwent meta-analysis, without significance.

4.3.9 Funnel plot asymmetry, heterogeneity and publication bias

Begg's and Egger's tests were non-significant for all meta-analyses. Visual inspection of plots identified asymmetry for 9 outcome analyses: mutant *TP53* (OS overall, adjusted HR, SCC and unspecified cell types, neoadjuvant therapy and SSCP/direct sequencing analyses; figures 4.4 and 4.5), *ERRBB2*/*HER2* (OS), and *FGF3* (OS), and 3 stage analyses: *EGFR* (overall) and *ERBB2*/*HER2* (N and grade). These were interpreted as likely publication bias and corrected (without affecting any conclusions). All sensitivity analyses were negative.

Figure 4.4: Funnel plot (unadjusted) for *TP53* mutant tumours and overall survival

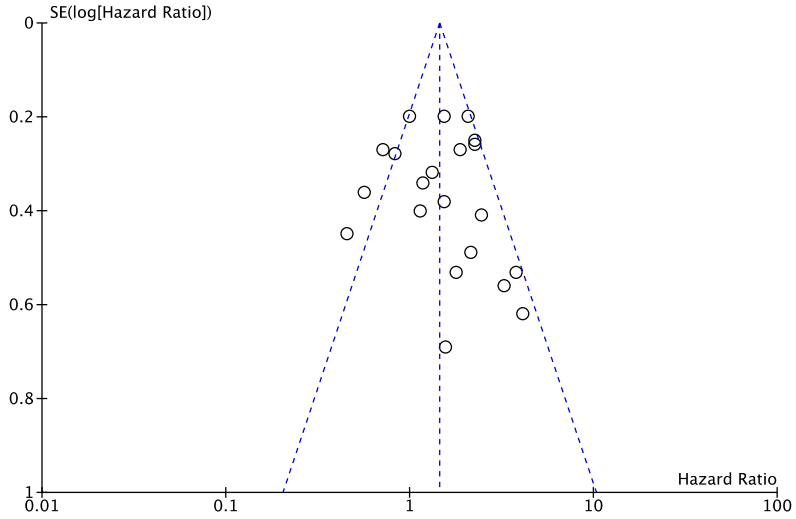


Figure 4.5: Funnel plot (adjusted) for *TP53* mutant tumours and overall survival

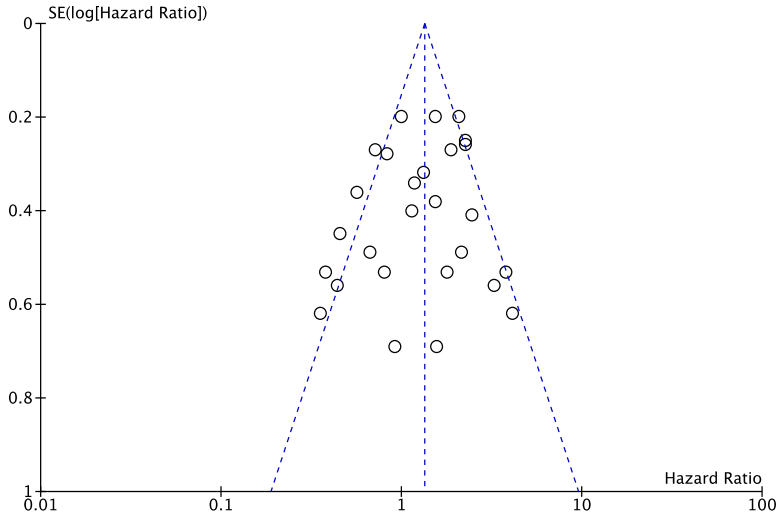


Table 4.1: Reported tumour markers (mutations, copy number variants, and chromosomal instability) associated with survival and recurrence following treatment

LOE	Variant	Gene *function*	Association: minor variant	Association : wild type	No association	Cell type	Population	LOE	Meta-HR [effect variant]	Chi	I^2	n	p
MUTATIONS													
III	Exon	*TP53* Apoptotic / DNA repair regulator	OS&DFS Casson 2003			AC	Res	C	$OS^{(A)PB}$1.27 (1.01-1.59)	68.1	62	21	0.040
			OS^{A} Schneider 2000			AC	Res	C	$OS^{A\ PB}$ 1.19 (0.63-2.27)	34.5	80	6	0.590
			OS&DFS Madani 2010			AC	Res	C	$AC^{(A)}$ 1.99 (1.44-2.81)	2.68	0	5	<0.001
					OS Puhringer 2006	AC	Res+/-NAC(R) (CF)	B	$SCC^{(A)PB}$1.47 (1.24-1.73)	22.7	43	11	<0.001
					OS Soontrapornchai 1999	AC	Res	D	$US^{(A)PB}$ 0.77 (0.41-1.47)	21.3	72	5	0.440
			OS^{A} Yamasaki 2010			SCC	Mixed	D					
			OS^{A} Kunisaki 2006			SCC	Res+NAC	C	$DFS^{(A)}$ 2.67 (1.38-5.15)	7.44	73	3	0.003
			OS Kobayashi 1999			SCC	Res+NAC	D					
					OS Makino 2010	SCC	Res+NACR (CF)	D	Res $OS^{(A)PB}$1.35 (1.04-1.76)	27.0	56	13	0.030
					OS Shimada 1997	SCC	Res	D	$NAC/R^{A(PB)}$1.23 (0.81-1.87)				
			OS Uchino 1996			SCC	Res	C		32.5	66	7	0.320
					OS Egashira 2011	SCC	Res	C	SSCP analysis				
					OS Ito 2001	SCC	DCRT	C	$OS^{(A)PB:}$ 1.56 (1.33-1.82)	27.8	44	13	<0.001
					OS Lam 1997	SCC	Res	D	Direct sequencing only				
					OS Shibagaki 1995	SCC	Res	C	$OS^{(A)PB}$ 0.96 (0.62-1.46)				
					OS Goan 2005	SCC	Res	D	SCCP band only	28.3	65	8	0.830
					OS Cao 2004	SCC	Res	C					
					OS^{A} Gibson 2003	US	Res+NACR	C					
					OS^{A} Coggi 1997	US	Res	D					
			OS&DFS Ribeiro 1998			US	NACR (CF+IFN)	D					
			OS&DSS Kandolier 2014			US	Res+NAC (CF)	C					
III	rs9344	*CCND1* Cell cycle kinase	OS^{A} Izzo 2007			AC	Res	D	OS^{A}: 3.48 (1.94-6.23) [AA]	NA	NA	1	<0.001
IV	Exon 9/20	*PIK3CA* Cell signalling		OS^{A}&DFS^{A} Shigaki 2013	Rec Shigaki 2013	SCC	Res+/-NACR(CF+/- tax)	D	$OS^{(A)}$ 0.63 (0.26-1.56)	6.13	67	3	0.320
					OS^{A}&Rec Wang 2014	SCC		D	$DFS^{(A)}$ 0.42 (0.21-0.85)	0.26	0	2	0.020
					OS&DFS Hou 2014	SCC	Res+/-NACR	C	Rec^{Ex} 0.64 (0.23-1.75)	2.81	64	2	0.390
							Res						
IV	Exon	*NRF2 / BIRC2* Transcription factor	OS&Rec Shibata 2011			SCC	Res+NACR (F)	D	OS^{Ex} 3.54 (1.60-7.88)	NA	NA	1	0.005
									Rec NP				0.046

COPY NUMBER VARIANTS													
II	Gain	*EGFR* Epidermal growth factor receptor	OS Marx 2010 OS Kitagawa 1996 OS Lennerz 2011	 OS[A] Luber 2011	 OS Miller 2003 OS Janmaat 2006 OS Chikuba 1995 OS Itakura 1994 OS Sunpaweravong 2005	AC SCC AC SCC SCC SCC US AC SCC	Res Res Res Palliative gefitinib Res+ACRT (C) Res Mixed PC (OFL+cetuximab Res	D D C B D D D B D	Gain assessed by FISH/CISH OS: 2.43 (0.75-7.84) Gain assessed by slot/Southern blot OS: 1.63 (0.63-4.22) Excluding Miller 2003 (qPCR)	23.9 18.1	92 8	3 3	0.140 0.320
III	Gain	*ERBB2/HER2* Growth factor receptor	OS Prins 2013 DFS[A] Rauser 2007 OS[A] Brien 2000 OS Zhan 2012 OS Sato-Kuwabara 2009 OS[A] Mimura 2005		DSS&OS[A] Yoon 2012 OS[A] Rauser 2007 OS Thompson 2011 OS Miller 2003 OS Konig 2013 OS Sunpawerayong 05 OS Suzuki 1997 OS&DSS Ikeda 1996 OS Lennerz 2011	AC AC AC AC AC AC Both SCC SCC SCC SCC SCC SCC US	Res Res Res Res Res Res Res Res Res Res Res Res Res Mixed	D C D D D C D D D D C D D D	OS[(A)] 1.63 (1.20-2.21) OS[A] 2.31 (1.64-3.24) OS[(A)] (AC) 1.59 (0.99-2.56) OS[(A)] (SCC) 1.92 (1.12-3.29) DFS[A] 2.1 (1.06-4.26) Gain assessed via FISH/SISH/IHC; Miller 2003, Ideka 1996, Suzuki 1997 excluded	31.2 0.72 15.4 27.7 NA	62 0 68 82 NA	11 3 6 5 1	0.002 <0.001 0.060 0.020 0.033
III	Gain	*ERBB2/HER2* Growth factor receptor	DSS[A], OS[A] Yoon 2012 (heterogeneous amplification)			AC	Res	C	OS: 2.02 (1.09-3.74) DSS: 2.04 (1.09-3.79)	NA NA	NA NA	1 1	0.026 0.025
III	Gain	*CCND1* Cell cycle kinase	OS[A] Wang 2012b OS[A] Miller 2003 OS[A] Takeshita 2010 OS Shimada 1997 OS Shinozaki 1996 Rec[A] Komatsu 2014 (ctDNA)		 OS Sunpawerayong 2005 OS Gramlich 1994	SCC AC SCC SCC SCC SCC SCC	Res Res Res Res Res Res Res	C C C D C D D	Gain: qPCR only OS[(A)]: 2.09 (1.27-3.42) Gain: FISH/IHC OS[(A)] 1.54 (0.93-2.57) Gain: slot blot OS 4.29 (2.47-7.45)	3.17 3.94 NA	5 75 NA	4 2 1	0.004 0.100 <0.001
III	Gain	1p36.32	OS[A] Carneiro 2008			SCC	Res	C	OS[A] 19.6 (2.5-153.9)	NA	NA	1	0.005
III	Gain	19p13.3	OS[A] Carneiro 2008			SCC	Res	C	OS[A] 7.0 (1.5-31.9)	NA	NA	1	0.011
III	Gain	*MDM2* Ubiquitin ligase	OS Shibagaki 1995			SCC	Res	C	OS[Ex] 3.82 (1.81-8.07)	NA	NA	1	5.3×10^{-3}
IV	Gain	*FGF3/INT2* Fibroblast growth factor	OS Ikeda 1996		OS Mori 1992 OS Shimada 1997	AC SCC SCC	Res Res Res	D D D	OS[PB] 1.83 (1.18-2.83) PB – Corrected for Ikeda 1996	5.65	29%	4	0.006

					OS Suzuki 1997	SCC	Res	D					
IV	Gain	*FGF4/HST1* Fibroblast growth factor	OS Chikuba 1995		Rec Chikuba 1995	SCC SCC	Res+ACR (C) Res	D D	Median survival different but: OS^{Ex} 1.4 (0.86-2.30) Rec^{Ex} 1.09 (0.659-1.80)	NA	NA	1	>0.05
IV	Gain	*TERC* Telomerase	OS Wang 2013			SCC	Res	D	OS^{Ex} 7.87 (3.32-18.7)	NA	NA	1	0.010
IV	Gain	*MET* Growth factor	OS Lennerz 2011			US	Mixed	D	OS^{Ex} 3.72 (2.56-5.39)	NA	NA	1	<0.001
IV	Gain	*CPT1A* Mitochondrial oxidation	OS^{A} Shi 2011			SCC	Res	D	OS^{A} 4.39 (1.34-14.14)	NA	NA	1	0.015
TELOMERE LENGTH													
III	Telomere length ratio (>1.17)		OS^{A} Gertler 2008			AC	Res	C	OS^{A} 3.40 (1.3-8.9)	NA	NA	1	<0.02
LOH													
III	One of 2p, 3p, 17p		OS Ikeguchi 1999			SCC	Res	C	2 loci: OS (3yr) 48%-v-75% OS^{Ex} 1.81 (0.53-6.25)	NA	NA	1	0.048 >0.05
IV	1q21-23		OS Maru 2009			AC	Res	D	OS^{Ex} 3.90 (1.13-13.5)	NA	NA	1	0.030
CHROMOSOMAL INSTABILITY													
III	Aneuploid /polyploidy		OS Doki 1993 Rec Tsutsui 1992 Rec Kaketani 1989 OS Ohno 1989 OS Kuwano 1995		 OS^{A} Wang 1999 OS Edwards 1989	SCC SCC SCC SCC SCC SCC SCC	Res Res Res Res+NACR Res Res Res	D D D C C D D	$^{PB}OS^{(A)}$: 1.63 (1.25-2.11) Rec: 5.41 (0.87-33.8)	2.99 2.11	25 53	4 2	$2x10^{-4}$ 0.070
IV	Ploidy heterogeneity			OS Deguchi 1993	DFS Deguchi 1993	SCC	Res	D	OS: 0.10 (0.03-0.36) DFS: 0.34 (0.08-1.42)	NA NA	NA	1	<0.05

rec=recurrence; A=adjusted; (A)=including adjusted; PB=adjusted for publication bias; Ex=extrapolated; NP=not presented; NA=not applicable; Res=resection; C=cisplatin; F=5FU; CFL=cisplatin-5FU-leucovorin; OFL=cisplatin-5FU-leucovorin; tax=taxane; ACR=adjuvant chemoradiotherapy

Table 4.2: Reported germline markers (polymorphisms) associated with survival and recurrence following treatment

LOE	SNP (major/ minor allele)	Gene *function*	Association: minor variant	Association: wild type	No association	Cell type	Population	LOE	Meta-HR [variant allele / genotype] –v- wild-type' [Ethnicity]	Chi	I^2	n	p
II	rs3212986 (C/A)	*ERCC1* DNA NER		OS^A Bradbury 2009b DFS^A Wang 2011 OS^A Rumiato 2013	 OS Warnecke 2009	US SCC US US	NAC (CF) PC (CF) Res+/-NAC (CF) Res+NACR (CF)	B C C C	$OS^{(A/Ex)}$ 0.63 (0.42-0.93) [TT/CT, cisplatin, Caucasian] $DFS^{(A)}$ 1.98 (1.19-3.03) [AA/CA+cis,,Chinese]	4.12 NA	51 NA	3 1	0.02 0.001
II	rs1799793 (G/A)	*ERCC2* DNA NER		OS^A Bradbury 2009b	O &Rec Ott 2011 OS Rumiato 2013	US AC US	Res+NAC(CF) NAC(C/OF+/-tax) Res+/-NAC (CF)	B B C	$OS^{(A/Ex)}$ 0.71 (0.54-0.94) [GA/AA; cisplatin; Caucasian] DFS 0.33 (0.20–0.07) [AA]	3.88 NA	48 NA	3 1	0.020 0.002
II	rs13181 (T/G)	*ERCC2* DNA NER		OS^A & DFS^A Bradbury 2009b	OS&Rec Ott 2011 OS Rumiato 2013 OS^A&Rec^A Wu 2006	US AC US US	Res+NAC(CF) NAC(C/OF+/-tax) Res+/-NA (CF) NACR(CF+/-tax)	B B C D	$OS^{(A/Ex)}$ 0.82 (0.65-1.05) [TG/GG, Caucasian] DFS^A 0.32 (0.20-0.60 $Rec^{(A)}$ 0.94 (0.30-2.81)	5.14 NA 0.03	42 NA 0	4 1 2	0.110 0.002 0.91
III	rs11614913	*MIR196A2* Micro RNA		OS^A Wu 2014	 OS^A Yang 2014b	SCC SCC	Pall chemo Mixed	C C	OS^A: 1.26 (0.72-2.21) [TT; Chinese/Taiwanese] PFS^A: 1.01 (0.54–1.88)	2.17 NA	54 NA	2 1	0.420 0.972
III	CA-SSR-1 (DNR)	*EGFR* Epidermal growth factor receptor	OS&Rec Vashist 2014			Both	Res	C	OS (AC) 1.70 (1.20-2.80) [LL; Caucasian] Rec (AC) 2.70 (1.70-4.30) OS (SCC) 3.50 (2.10-6.00) Rec (SCC) 2.50 (1.30-4.80)	NA	NA	1	0.010 <0.001 <0.001 0.005
III	rs1800796 (C/G)	*IL6* Interleukin	OS Motoyama 2012b		DSS Motoyama 2012b	SCC	Res+/-AC	C	OS: HR^A 3.40 (CI NP) [GG/GC; Japanese] OS: $HR^{A\ Ex}$ 3.49 (1.28-9.45)	NA	NA	1	5.9 $x10^{-3}$ <0.05
III	rs238406 (G/T)	*ERCC2* NER repair		OS^A & DFS^A Lee 2011		SCC	Res + NACRT (CF/C+tax)	C	OS^A: 0.61 (0.40–0.93) [CC; Taiwanese] DFS^A: 0.57 (0.38-0.85)	NA	NA	1	0.02 0.007
III	rs1800975 (G/A)	*XPA* DNA NER repair	OS^A Yang 2013		DFS Yang 2013	SCC	Mixed	C	OS^A: 1.36 (1.06–1.76) [AG; [Taiwanese] DFS: 1.20 (0.95-1.51) [AG]	NA	NA	1	0.014 0.126
III	rs34743033 (STR 2/3/4)	*TYMS* DNA repair/ replication	OS^A Kaneko 2011		 OS Okuno 2007 OS Sarbia 2006 OS Rumiato 2013	SCC SCC SCC US	DCR (CF) NACR (CF) NACR (CF+E) Res+NAC (CF)	C C C C	$OS^{(A)}$ 1.54 (1.00-2.38) [≥2/3; Caucasian/Japanese] $OS^{(A)}$ 2.47(1.20, 5.06) [Japanese] $OS^{(A)}$ 1.18 (0.69, 2.03) [Caucasian]	5.78 0.45 0.18	65 0 0	3 2 2	0.61 0.010 0.550

III	rs2279744 (T/G)	*MDM2* Ubiquitin ligase	OS[A] Renouf 2013	DFS[A] Boonstra 2011 OS[A]&DFS[A] Cescon 2009		Both Both AC	Res Mixed	D C	(SCC) DFS[A] 2.78 (0.24-31.9) [GG; Caucasian] (AC) DFS[A] 0.92 (0.65-1.29) [GG] (AC) OS[A] 2.01 (1.38-2.95) (SCC) OS[A]: 7.89 (2.40-26.0)	9.36 0.04 0.48 NA	89 0 0 NA	2 2 2 1	0.410 0.620 <0.001 <0.001
III	rs2273535 (A/T)	*AURKA* Cell cycle kinase	OS&DFS[A] Pan 2012		DFS[A] Boonstra 2011	US Both	NACR (CF+tax) Res	C D	OS[Ex] 0.30 (0.10-0.92) [TT; Caucasian] DFS[A] 0.55 (0.17-1.74)	NA 2.92	NA 66	1 2	<0.05 0.310
III	rs2010963 (G/C)	*VEGFA* Epithelial mitogen		OS Tamura 2012 OS Yang 2014		SCC SCC	DCR (CF) Res+/-NACRT (CF)	C	OS HR[(Ex)] 0.68 (0.50-0.92) [CC]	0.16	0	2	0.010
III	rs3025039 (C/T)	*VEGFA* Epithelial mitogen	DFS[A] Lorenzen 2011	OS[A] Bradbury 2009	OS Tamura 2012	AC AC SCC	Res+NAC (CF) Mixed DCR (CF)	C C C	OS[(A)] 0.75 (0.54-1.03) [CT; Caucasian/Japanese] DFS[A] 1.8 (1.04-3.09) [CT/TT; Caucasian]	0.79 NA	0 NA	2 1	0.080 0.040
III	rs1042522 (C/G)	*TP53* Apoptotic / DNA repair regulator	OS[A]&PFS[A] Renouf 2013 OS[A]&DFS[A] Cescon 2009		OS[A]&Rec[A] Wu 2006	AC AC US	Mixed Mixed NACR (CF+/-tax)	C C D	OS[A]: 1,84 (1.34-2.53) [GG; Caucasian] DFS[A]: 2.03 (1.29-3.18) [GG] Rec[A]: 1.29 (0.24-7.14) [GG]	0.44 NA NA	0 NA NA	3 1 1	<0.001 0.002 >0.05
III	rs2069762 (A/C)	*IL2* Cytokine	DSS[A] Motoyama 2011			SCC	Res	C	DSS: 3.54 1(1.69-7.39) [C; Japanese] DSS[A]: 3.36 (NA)	NA	NA	1	0.023 0.014
III	rs1800471 (C/G)	*TGFB1* Growth factor regulator	OS Tang 2013			SCC	Mixed	C	OS: 3.51 (2.18–5.67) [CG/GG; Chinese]	NA	NA	1	<0.001
III	rs1050631 (G/A)	*SLC39A6* Zinc transporter	OS[A] Wu 2013			SCC	Mixed	C	OS[A]: 1.3 (1.19-1.43) [AA; Chinese]	NA	NA	1	3.77×10^{-8}
III	rs41458645 (C/T)	Mitochondrial D loop	OS[A] Zhang 2010			SCC	Mixed	C	OS[A]: 3.00 (1.03-8.76) [CT; Chinese]	NA	NA	1	0.044
III	rs139001869 (A/G)	Mitochondrial D loop	OS[A] Zhang 2010			SCC	Mixed	C	OS[A]: 3.48 (1.07-11.36) [AG; Chinese]	NA	NA	1	0.039
III	rs3769818 (G/A)	*CASP8* Caspase	OS[A] Umar 2011			SCC	Mixed	C	OS[A]: 3.36 (1.07-10.61) [AA; Indian]	NA	NA	1	0.039
III	rs1695 (A/G)	*GSTP1* Detoxification enzyme	OS[A] Lee 2005		OS Okuno 2007 OS Warnecke 2009 OS[A]&Rec[A] Wu 2006 OS[A] Rumiato 2013	SCC SCC US US US	Res+NAC (C+F/tax) Res+NACR (CF) Res+NACR (CF) Res+NACR (CF+/-tax) Res+NAC (CF)	C C C D C	OS[(A)]: 1.29 (1.03-1.61) [TG/GG; Caucasian/Taiwanese/Japanese] OS([A]) 1.15 (0.78-1.70) [TG/GG; Caucasian] Rec[A] 0.50 (0.16-1.58) [Cauc]	2.36 1.84 NA	0 0 NA	5 3 1	0.030 0.490 >0.05

III	rs72214039 (CA ins)	*EGFR* Epidermal growth factor receptor	OS^{A} Lee 2011b			SCC	Res+NACR (CF/C+tax)	C	OS^{A}: 1.88 (1.02–3.49) [Short/Short; Taiwanese]	NA	NA	I	0.045
III	rs7121 (T/C)	*GNAS* G protein subunit		OS^{A} Alakus 2014 OS^{A} & DFS^{A} Vashist 2011	 OS Alakus 2009	US US US	Res Res Res+NACR (CF)	D C B	$OS^{(A)}$ 0.73 (0.46-1.16) [CC; Caucasian] DFS^{A} 0.55 (0.34-0.89	9.08 NA	67 NA 	3 I	0.180 0.025
III	rs111509018 (STR)	*ECRG2/SPINK7* Serpin-inhibitor	OS^{A}&DFS^{A} Kaifi 2007			US	Res	C	OS^{A} 2.56 (1.53-4.29) [TCA_4/TCA_4; Caucasian] DFS^{A}: 2.30 (1.37-3.87)	NA	NA	I I	<0.001 <0.001
IV	rs1801133 (G/A)	*MTHFR* Folate metabolism		Rec^{A} Wu 2006	OS^{A} Wu 2006 OS^{A}&Rec^{A} Ott 2011 OS Lu 2011 OS Umar 2010 OS Sarbia 2006 OS Warnecke 2009	US AC SCC SCC SCC SCC	Res+NACR (CF+/-tax) Res+NAC (C/OF+/-tax) Res only Mixed Res+NACR (CF, ,E) Res+NACR (CF)	D B C C C C	$OS^{(A)}$ 0.93 (0.67-1.29)[AA; Caucasian/Chinese/Indian] $OS^{(A)}$ 0.92 (0.63-1.33) [Caucasian] Rec^{A} 0.40 (0.21-0.78) [Caucasian]	3.06 0.69 1.54	0 0 35	6 4 2	0.660 0.640 0.007
IV	rs1801131 (A/C)	*MTHFR* Folate metabolism		OS^{A} Wu 2006	Rec^{A} Wu 2006 OS&Rec Ott 2011 OS Warnecke 2009	US AC SCC	Res+NACR (CF+/-tax) Res+NAC (C/OF+/-tax) Res+NACR (CF)	D B C	$OS^{(A)}$ 0.99 (0.64-1.55) [AA; Caucasian] $Rec^{(A)}$0.80 (0.22-2.95)	0.67 2.38	0 54	3 2 	0.970 0.740
IV	rs1045642 (C/T)	*ABCB1* Drug efflux		 OS^{A} & Rec^{A} Wu 2006	OS Narumiya 2011 OS Okuno 2007 OS Warnecke 2009	US SCC US US	Res+NACR (CF) Res+NACR (CF) Res+NACR (CF) Res+NACR (CF+/-tax)	D C C D	$OS^{(A)}$ 0.57 (0.37-0.87) [TT; Caucasian/Japanese] $OS^{(A)}$ 0.51 (0.32-0.81) [Caucasian] Rec 0.26 (0.09-0.81)	1.87 0.68 NA	0 0 NA	4 3 I	0.009 0.004 <0.05
IV	rs11267092 (DEL/INS)	*F2R* Angiogenesis	OS^{A}&Rec^{A} Lurje 2011			AC	Res	D	OS^{Ex} 1.70 (1.16-2.48) [INS/INS / INS/DEL; Caucasian] Rec 2.41 (1.25-4.65) [INS/INS]	NA	NA	I I	<0.001 0.003

rec=recurrence; A=adjusted; (A)=including adjusted; PB=adjusted for publication bias; Ex=extrapolated; NP=not presented; US=unspecified; Res=resection; C/Cis=cisplatin; O=oxaliplatin; tax=taxane; F=5FU; E=etoposide; Res=resection; Ex=Extrapolated; INS=insertion; DEL=deletion; NA=not applicable; Cauc=Caucasian

Table 4.3: Reported tumour variants and germline polymorphisms associated with treatment complications and response to chemo(radio)therapy

LOE	Variant	Gene	Association: mutant	Association: wild type	No association	Cell type	Population	LOE	Meta OR [effect allele / genotype / haplotype]	Chi	I^2	n	P
TREATMENT COMPLICATIONS													
III	rs4646994 (INS/DEL)	*ACE* Vasodilator	Post-op pulmonary[A] Lee 2005b			US	Res	C	OR[A] 3.12 (1.01–9.65) [DEL/DEL; Taiwanese]	NA	NA	1	0.049
III	rs1800629 (G/A)	*TNFA* Cytokine	Post-op infection[A] Azim 2007		Motoyama 2009	US SCC	Res+/-NAC(R) Res	C D	OR[A] 4.02 (0.00-18347) [GG; Caucasian/Japanese]	0	0	2	0.750
RESPONSE TO CHEMO(RADIO)THERAPY													
Tumour													
III	High DNA ploidy	-	pTR – HCR-v- CR Ohno 1989			SCC	Res+NACR–v-HNACRT (bleomycin)	C	pTR 13.18 (5.30-32.7) [High ploidy]	NA	NA	1	<0.001
IV	Mutant (exon)	*TP53*		pTR Ribeiro 1998 cTR Yamasaki 2010 pTR Makino 2010 cTR Kunisaki 2006	 cTR Ito 2001 cTR Gibson 2003	US SCC SCC SCC SCC US	Res+NACR (CF+IFN) Res+NAC Res+NACR/DCR (CF) DCRT Res+NACR (CF) Res+NACR (CF)	D D D C C C	pTR 0.24 (0.06-0.95) cTR 0.43 (0.08-2.24)	2.87 14.2	30 86	3 3	0.040 0.320
Germline													
II	rs7121 (T/C)	*GNAS1* G protein subunit	pTR[A] Alakus 2009			US	Res+NACR (CF)	B	pTR[A] 7.25 (1.30-40.62) [CC; Caucasian]	NA	NA	1	<0.05
III	rs3212986 (C/A)	*ERCC1* DNA NER	cTR[A] Wang 2011		 pTR Warnecke 2009 pTR Rumiato 2013	SCC US US	Palliative chemo (CF) Res+NAC (CF) Res+NAC (CF)	C B C	cTR OR[A] 2.62 (1.11-6.23) [AA/CA; Chinese] pTR OR 1.52 (0.73-3.20) [CT/CC; Caucasian]	NA 0.76	NA 0	1 2	<0.05 0.260
III	rs11615[MC] (A/G)	*ERCC1* DNA NER	pTR Metzger 2012 pTR Warnecke 2009		pTR Rumiato 2013	AC US US	Res+NACR (CF) Res+NACR (CF) Res+NAC (CF)	D C C	pTR OR 4.57 (3.01-6.94) [TT/CT; Caucasian]	3.48	43	3	<0.001
IV	rs25487 (G/C)	XRCC1 *DNA* repair	pTR Wu 2006		pTR Ott 2011 pTR Warnecke 2009	US AC US	Res+NACR (CF+/-tax) Res+NAC (C/OF+/-tax)) Res+NACR (CF)	D B C	pTR 1.91 (1.30-2.81) [GG; Caucasian]	3.13	36	3	0.001

rec=recurrence; A=adjusted; HNACRT=hyperthermic neoadjuvant chemoradiotherapy; CF=cisplatin-5FU; DEL=deletion; OF=cisplatin-5FU; Res=resection cTR=major clinical response; IFN=interferon; NA=not applicable

Table 4.4: Reported tumour markers (mutations, copy number variants, genomic and chromosomal instability) associated with stage

LOE	Variant	Gene	Association – mutant	No association	Cell type	Population	LOE	Meta OR [effect allele / genotype / haplotype]	Chi	I^2	n	p
MUTATIONS												
III	Mutant	*TP53*	T & N Madani 2010	R Madani 2010	AC	Res	C	O[PB] 1.28 (0.71-2.31)	32.4	63	12	0.410
			N Cao 2004	T Cao 2004	SCC	Res	D	T: 1.40 (1.12-1.74)	17.5	20	15	0.003
		Apoptosis /	N Hattori 2003		SCC	Res	C	N: 1.39 (1.07-1.81)	18.1	17	15	0.010
		DNA repair	O Ribeiro 1998		US	Res+NACR	D	M: 1.21 (0.72-2.03)	2.99	0	5	0.480
		regulator		O, T, N Schneider 2000	AC	Res	C	G: 1.46 (0.83-2.58)	19.1	53	10	0.190
				T,N,G Soontrapornchai 1999	AC	Mixed	D	R: 2.10 (0.470-9.35)	3.45	71	2	0.330
				T,N,G Egashira 2011	SCC	Res	C					
				T,N,M,O Yamasaki 2010	SCC	NAC(CF)+/-Res	D	O: B p=0.540; E p=0.275				
				T,M,G Ito 2001	SCC	Res+NACR(CF)	C	T: B p=0.393; E p=0.071				
				O Kobayashi 1999	SCC	Res	D	N: B p=0.765; E p=0.443				
				T,N. M G Uchino 1996	SCC	Res	D	M: B p=0.207; E=0.492				
				O Coggi 1997	US	Res	D	G: B p=0.719; p=0.543				
			O, T, N, G-Casson 2003		AC	Res	C					
				O, T, N Goan 2005	SCC	Res	D	[PB] Corrected for Ribeiro 1998				
				O, G Lam 1997	SCC	Res	D					
				O, T, N Shibagaki 1995	SCC	Res	C					
				T, N Puhringer 2006	AC	Res+/-NACR (CF)	B					
			N - Makino 2010	T, M, G Makino 2010	SCC	NACR/DCR (CF)	D					
COPY NUMBER VARIANTS												
III	Gain	*SPK2*	N,O Wang 2009		SCC	Res	C	O: 8.00 (2.25-28.5)	NA	NA	1	1.30×10^{-3}
		Protein kinase						N: 8.10 (2.28-28.8)			1	1.20×10^{-2}
III	Gain	*PRKC1*	T Yang 2008	N, O G Yang 2008	SCC	Res	C	O: 4.64 (1.71-12.4)	NA	NA	1	0.002
		Serpin						N: 3.12 (1.21-8.02)	NA	NA	1	0.019
								T: 2.63 (0.78-8.81)	NA	NA	1	0.118
III	Gain	*HER2/ERBB2*	T, G Yoon 2012		AC	Res	C	O: 1.13 (0.83-1.54)	14.0	43	10	0.510
		Epidermal	G,O Zhan 2012	T,M,L Zhan 2012	SCC	Res	D	T: 0.84 (0.55-1.27)	17.5	48	10	0.400
		growth	O, G Lennerz 2011		US	Mixed	D	N[PB]: 0.96 (0.69-1.35)	20.2	31	11	0.510
		factor		O, T, N Suzuki 1997	SCC	Res	C	M: 1.77 (0.69-4.56)	3.85	22	4	0.240
		receptor	N Ikeda 1996		SCC	Res	D	G[PB]: 0.61 (0.34-1.09)	40.0	72	10	0.100
				O, T,N,M,R,G Reichelt 2007	US	Res	C					
				O, N Brien 2000	AC	Res	D	[PB] – Corrected for Ikeda 1996, Al-Kasspooles 1993, Suzuki 1997, Minmura 2005				
				O, N, G Mimura 2005	SCC	Res	D					
				O, T, N, G, R Sato-Kuwabara 2009	SCC	Res	D					
				O, G Sunpaweravong 2005	SCC	Res	D	[PB] – Corrected for Sunpaweravong 2005, Zhan 2012				
				O, T, N, G, M, R Thompson 2011	AC	Res	D					
				O, T, N, G, M – Al-Kasspooles	AC	Res	C					
				T, N, G – Prins 2013	AC	Res	D					

IV	Gain	*EGFR* Epidermal growth factor receptor	T,N Marx 2010 G Lennerz 2011 N Kitagawa 1996 N Yang 2012	M,G Marx 2010 O Lennerz 2011 O, T Kitagawa O,G Yang 2012 O, T,N,M Miller 2003 O, T,N,M,G Al-Kasspooles 1993 O,T,N,G Itakura 1994	AC US SCC SCC AC AC SCC	Res Mixed Res Res Res Res Res	D D D D C C D	O^{PB}: 0.96 (0.6-1.53) T: 0.51 (0.37-0.71) N: 0.95 (0.41-2.16) M: 0.91 (0.29-2.87) G: 1.24 (0.70-2.20) [PB] Corrected for Kitagawa 96	9.27 2.31 8.44 1.38 3.36	35 0 41 0 8	6 5 6 3 3	0.850 <0.001 0.890 0.870 0.460
LOSS OF HETEROZYGOSITY												
III	LOH	3p14.2	T^{A} Qin 2008	N, M, G Qin 2008	SCC	Res	C	T: 5.67 (1.77-18.2)	NA	NA	1	0.003
III	LOH	13q	T, G Huang 2002 N Harada 1999 N Shibagaki 1994	N Huang 2002 T,M,H,O Harada 1999	SCC SCC SCC	Res Res Res	C C C	T: 3.08 (0.17-60.8) N: 4.17 (1.84-9.47)	5.04 3.06	80 35	2 3	0.440 $6x10^{-4}$
CHROMOSOMAL INSTABILITY												
III	CIN		T,N,G Yu 1989 G Doki 1993 T,N Kuwano 1995 N Ohno 1989	 T & N Doki 1993 N,V,G Kuwano 1995 O,T Ohno 1989 T,N,G Tsutsui 1992 T,L,G Edwards 1989 O, T, N G Wang 1999 T Kaketani 1989	SCC SCC SCC SCC SCC SCC SCC SCC	Res Res Res Res+/-NACR Res Res Res+/-AR Res	C D C C D D D D	O: 2.68 (1.10-6.54) T: 1.41 (0.97-2.05) N: 2.18 (1.06-4.47) G: 1.51 (0.99-2.31)	2.01 7.21 18.2 5.29	0 0 57 5	2 9 7 6	0.030 0.070 0.030 0.060
MICROSATELLITE INSTABILITY												
IV	MSI	17q24-25	T Matsumoto 2007	N,G,O Matsumoto 2007	SCC	Res	D	T: 0.325 (0.11-0.96)	NA	NA	1	0.043

=T stage (III/IV-v-I/II); N=nodal stage(N0-v-≥N1); M=metastatic stage (M0-v-M1); G=cell grade (III/IV-v-I/II); O=overall stage (III/IV-v-I/II); R=resection stage (R1-v-R0); L=L stage (L1-v-L0); V=venous invasion (V1-v-V0); Res=resection; PB=corrected for publication bias;; NA=not applicable

Table 4.5: Reported germline markers (polymorphisms) associated with stage

LOE	Variant	Gene	Association–variant	Association–wild type	No association	Cell type	Population	LOE	Meta OR [effect allele / genotype/haplotype]	Chi	I^2	n	p
II	rs6573 (C/A)	*RAP1A* RAS oncogene		O[A] Wang 2012		SCC	Mixed	B	O[A]: 1.89 (1.06–3.36) [CA/AA; Chinese]	NA	NA	1	0.030
II	rs1800471 (G/C)	*TGFB1* Growth factor regulator	O,G Tang 2013			SCC	Mixed	B	O: OR[A] 2.71 (1.44–5.09) [GC/CC; Chinese] G: OR[A] 2.65 (1.44–4.87)	NA NA	NA NA	1 1	<0.001 0.002
III	rs353163 (T/C)	*TMPRSS11A* Serine peptidase	N Umar 2013b			SCC	RT/DCR (CF)	C	N: 3.27 (1.68–6.39) [CC; Indian]	NA	NA	1	<0.001
III	rs2273535 (A/T)	*AURKA* Cell cycle kinase	O Miao 2004			SCC	Res	C	O: 2.13 (1.04-4.39 [TT; Chinese]	NA	NA	1	<0.05
III	rs7121 (C/T)	*GNAS* G protein subunit	O,N Vashist 2011		T, M, G Vashist T, N, R Alakus 2009	US US	Res Res+NACR (CF)	C B	O: 2.10 1.17-3.76) [T; Caucasian] N: 1.16 (0.76-1.77) [T]	NA 3.94	NA 49	1 3	0.013 0.500

T=T stage (III/IV-v-I/II); N=nodal stage (≥N1-v-N0); M=metastatic stage (M0-v-M1); G=cell grade (III/IV-v-I/II); O=overall stage (III/IV-v-I/II); R=resection stage; L=L stage; CF=cisplatin-5FU; Res=resection; NA=not applicable

4.4 DISCUSSION

4.4.1 Summary of findings

This study identified 182 studies, which assessed a total of 165 candidate genomic markers. Overall, 91 markers were reported to have significant associations with oesophageal cancer outcome, and 41 with stage. Overall study quality was poor: most studies were retrospective with small sample sizes, and all except 5 (2.75%) were of level C or D quality. There was considerable heterogeneity in patient selection, treatment approach, genotyping techniques and definitions used. Common areas of weakness were failure to perform subgroup analysis for AC and SCC; failure of quality control such as reporting call rates and HWE; failure to perform/report multivariate adjustment of hazard ratios; and failure to adjust for multiple comparisons. Furthermore, just 30.2% of reported markers subsequently had attempted validation data published.

Despite these limitations, sufficient data were available for appropriate meta-analyses. These demonstrated a small number of associations of DNA sequence markers with worse survival (mutant *TP53*, *HER2*, *CCND1* and *FGF3* copy number gain, and CIN) and resistance to chemo+/-radiotherapy (*TP53*).

4.4.2 Limitations

As far as the author is aware, this is the first attempt to collate and evaluate all evidence of DNA sequence markers and oesophageal cancer, and to demonstrate the above associations by meta-analysis. As such it has a number of generic and specific strengths and weaknesses. A comprehensive search strategy was used to minimize identification and selection bias (requiring detailed appraisal of studies including gastric cancer, cell lines and expression data), it is possible that studies were not identified. For those included, methodological heterogeneity and small sample sizes introduce potential for bias. Although there was no statistical evidence of funnel plot asymmetry using Begg's and Egger's tests, these are underpowered in meta-analyses of fewer than 25 studies (Begg and Mazumdar, 1994); all funnel plots were therefore inspected, and the reasons for any apparent asymmetry explored, and corrected 8 analyses for likely publication bias (without altering overall effects). Whilst the small number of studies involved in each analysis precluded meaningful meta-regression to explore additional potential confounding factors (Thompson and Higgins,

2002), potential bias was addressed by performing subgroup analyses, including cell type genotyping techniques and ethnicity. There are also limitations to the revised ASCO guidelines in this context; firstly, regarding capture of the complexity inherent in data quality, and secondly determination of LOE: evidence can be upgraded by validation studies, yet disagreement of effect size and direction between studies is not always reflected in the ultimate LOE.

4.4.3 Discussion of markers identified

The strongest evidence found for an outcome marker was tumour *TP53* mutation. Association with worse survival was demonstrated for both AC and SCC. Whether this is truly independent of the association demonstrated with T and N stage was not conclusively demonstrated, and indeed only assessed by 6 studies. Although 4 reported significant adjusted HR, the resultant meta-analysed direction of effect was concordant but not significant due to the use of a random-effects model. *TP53* mutant tumours were also found to be less chemo(radio)sensitive,

As other recent meta-analyses have reported similar findings in breast and colorectal carcinoma (Olivier et al., 2006, Smith et al., 2005a) this is of particular translatable relevance. *TP53* is one of the most frequently mutated and studied genes in human cancer (Xu et al., 2011), with resultant attempts to develop targeted therapies (Lehmann et al., 2012). 95% of functional mutations occur within exons 5-9, which encode the DNA binding domain, and typically cause loss of efficacy either directly by disrupting DNA contact, or indirectly by aberrant protein folding (Bellini et al., 2012). Subsequently cell cycle, DNA repair and apoptotic regulation may fail (Lai et al., 2007), although oncogenic gains of function are occasionally seen. The most characterized variant is the germline rs1042522 G>C substitution, itself conferring a worse HR for both OS and DFS in this meta-analysis.

TP53 as an oesophageal tumour biomarker is often considered in terms of TP53 status: aberrant expression, with or without mutation. An association with expression alone and worse outcome has been demonstrated on meta-analysis for SCC (Chen et al., 2013), as has aberrant status (increased expression [28 studies] with or without mutation [3 studies]) and reduced likelihood of response to chemotherapy (Zhang et al., 2013). However, *TP53* mutational and expression statuses may be discordant (Eguchi et al., 2000) particularly in the case of high impact mutations precluding expression, or dramatically reducing half-life. The

ability to predict this from sequencing data reinforces the need to explore the interaction of these aspects of *TP53* status, in parallel (Sim et al., 2012, Adzhubei et al., 2010).

Typically, resection specimens are used to assess associations between tumour markers and pathological response to chemotherapy. However, by definition these comprise clonal populations selected for chemo/radio-resistance. Whilst such tumours appear to be disproportionately *TP53* mutated, deep re-sequencing and clonal studies comparing the prevalence and associations of pre- and post-treatment tumour are required to establish the true pre-treatment predictive utility of mutations in this regard.

Three associations between tumour copy number gain (albeit variably quantified) were demonstrated by meta-analysis: *ERBB2*/*HER2*, *CCND1* and *FGF3* gain. *ERBB2*/*HER2* is particularly relevant; a proto-oncogene, it is the sole molecular marker in clinical use for gastrooesophageal cancer, guiding the use of targeted therapies (Allum et al., 2011). This study builds on a recent meta-analysis of *HER2* status, defining positivity by overexpression or amplification, including 6 of the studies included in this meta-analysis (Chan et al., 2012). Gain was found to confer a worse prognosis for both AC and SCC, independent of stage. Interestingly, all patients in 10/11 studies underwent radical treatment with resection; whilst palliative monoclonal antibody therapy for *HER2*-positive gastrooesophageal AC is effective in prolonging survival (Bang et al., 2010), an urgent unanswered question is therefore whether it has a role in curative treatment.

Similarly, regarding the cell cycle regulator *CCND1*, phase I and II data have suggested a possible role for cyclin-dependent kinase inhibitors in non-gastrooesophageal cancer (Bible et al., 2012, Luke et al., 2012). These findings therefore suggest the need to assess their effect in oesophageal tumours with *CCND1* gain. *EGFR*, a tyrosine kinase receptor, has also been extensively investigated within gastrooesophageal cancer; phase II data support targeted therapy (antibodies and tyrosine kinase inhibitors) for metastatic disease (Lorenzen et al., 2009, Rodriguez et al., 2010), although not yet neoadjuvant regimens (Idelevich et al., 2012, De Vita et al., 2011). Whilst no association with outcome using the requisite random-effects model was found, significant effects were evident with a fixed model; consequently, there may be an undetected association. CIN was also found to be associated with worse outcome, in keeping with a previous colorectal cancer meta-analysis (Walther et al., 2008), although whether it modulates chemo-sensitivity is unclear.

Survival associations were demonstrated for 6 common germline polymorphisms by meta-analysis: *ERCC1* rs3212986 (for cisplatin-treatment and Caucasian ethnicity), *ERCC2* rs1799793 (cisplatin and Caucasian), *TP53* rs1042522 (Caucasian), *MDM2* rs2279744 (Caucasian), *TYMS* rs34743033 (Japanese) *ABCB1* rs1045642 (both Caucasian and Japanese). The association of *VEGFA* rs2010963 was evident only on combining Taiwanese and Japanese study populations. *MTHFR* rs1801133 was associated with recurrence in Caucasians. *XRCC1* rs25487 and *ERCC1* rs11615 were associated with response to chemotherapy in Caucasians. These associations are likely to be due to aberrant protein expression or function.

rs3212986 modifies *ERCC1* mRNA stability (Zhou et al., 2004), a component of the NER pathway, variants of which are associated with platinum-sensitivity and survival in pancreatic, gastric, colorectal and lung cancers (Yin et al., 2011, Bradbury et al., 2009, Giovannetti et al., 2011). The missense rs1799793 SNPs results in an aspartate-asparagine substitution at codon 312 of the *ERCC2* component of the NER pathway, and has been similarly associated with survival in gastric and other cancers (Li et al., 2013). The rs10456402 SNP in exon 26 of *ABCB1* (Multi Drug Resistance 1) reduces expression (and consequent platinum-analogue membrane transportation)(Hoffmeyer et al., 2000), and is similarly associated with colorectal cancer prognosis (Wu et al., 2013). rs2279744 increases mRNA expression of *MDM2*, which suppresses *TP53* activity (Bond et al., 2004), and is associated with increased susceptibility to a number of cancers (including gastric)(Ma et al., 2013). rs34743033 is a 28bp variable number tandem repeat in TYMS (thymidylate synthetase), with enhancer function correlating with increased TYMS expression (Horie and Takeishi, 1995), and survival in platinum-treatment non-small cell lung carcinoma (Lima et al., 2014). The rs1801133 missense SNP induces an alanine-valine substitution at codon 222, with reduced activity of methylenetetrahydrofolate reductase (Frosst et al., 1995), and increased susceptibility to gastric cancer (Boccia et al., 2008). rs25487 induces a glutamine-arginine substation in codon 399, with resultant reduction in function of the DNA repair enzyme *XRCC1* (Abdel-Rahman and El-Zein, 2000), and an association with survival of lung cancer (Cui et al., 2012). rs11615 reduces *ERCC1* expression (Yu et al., 2000), and increases likelihood of response to platinum chemotherapy in gastric and colorectal cancer (Yin et al., 2011).

4.4.4 Limitations of genomic biomarkers

Biomarkers themselves carry a number of limitations. Typically, they are classified as 'prognostic' or 'predictive', however, in reality these are not mutually exclusive, and such a classification was therefore not attempted. Biomarker development culminates in

demonstration of clinical, requiring at least multi-centre prospective validation for prognosis, and incorporation into interaction randomized controlled trials for prediction. These challenges reinforce the utility of retrospectively analysing samples archived during prospective trials. Other challenges include the use of pre-treatment biopsies. Firstly, analysis may be impaired by inclusion of non-cancerous tissue; whilst this can be mitigated by techniques such as laser-capture micro-dissection and higher-depth sequencing, these are time and cost-intensive. More profound is the challenge of intra-tumoural heterogeneity and clonality: a single biopsy is representative of just 34% of the mutational burden of a 'single' cancer (Gerlinger et al., 2012), and will not include metastatic sub-clones. How to surmount this is not yet clear.

Finally, whilst it may be pragmatic to consider DNA sequence variants in isolation, their effects (and therefore utility) are subject to complicated modulation by the other 'omics', (epigenomics, transcriptomics, metabolomics and proteomics), genes, and clinical and environmental covariates (Baylin and Ohm, 2006, Simon and Roychowdhury, 2013). Whilst a discrete variable might provide useful complementary information of itself, this complexity at present precludes its use to dichotomize decision-making. Consequently, a robust approach to personalized cancer medicine must incorporate parallel processing of DNA, RNA, proteins and metabolites.

4.4.5 Conclusions and future research

In conclusion, numerous DNA sequence markers have been described for oesophageal cancer. However, as with complementary fields within personalized cancer research (and the similar analysis performed in Chapter 3), the underlying research is largely poor in quality and disparate in methodology, with a lack of robust validation of markers and incorporation into trials. Whilst a number of promising candidates have been identified the data required to incorporate these into prognostic/predictive models do not yet exist; future validation will require larger studies, with improvements in the standardized collection of samples for analysis, parallel assessment of expression, and the incorporation of parallel biomarkers within high-quality clinical trials, robust adjustment for confounding variables, and sharing of resultant data with multicentre collaboration.

CHAPTER 5

WHOLE EXOME SEQUENCING OF OESOPHAGEAL ADENOCARCINOMA BEFORE AND AFTER NEOADJUVANT CHEMOTHERAPY: A NEW MODEL OF GENOMIC RESPONSE AND IMPLICATIONS FOR PRECISION ONCOLOGY

5. CHAPTER 5: WHOLE EXOME SEQUENCING OF OESOPHAGEAL ADENOCARCINOMA BEFORE AND AFTER NEOADJUVANT CHEMOTHERAPY: A NEW MODEL OF GENOMIC RESPONSE AND IMPLICATIONS FOR PRECISION ONCOLOGY

5.1 SYNOPSIS

5.1.1 Introduction

The genetic pathogenesis of OAC remains incompletely understood. In particular, the basis for variability and unpredictability of tumour chemosensitivity represents a major area of interest. Recently, 4 studies have reported results from NGS (WGS or WES), comparing normal tissue to OAC. This chapter reports the first comparison of normal, pre-treatment and post-treatment following neoadjuvant therapy of any gastrointestinal cancer, performed in patients undergoing resection of OAC after oxaliplatin and 5-FU, to identify candidate driver genes, markers and pathways of chemosensitivity and prognosis, in addition to exploring genomic response to such therapy and the implications for biomarkers and precision oncology.

5.1.2 Methods

DNA was extracted from fresh frozen triplet samples (normal tissue, tumour tissue before and tumour after treatment) for 30 patients. WES was performed using a standard pipeline for clinical samples at the WTCHG. In brief, the Illumina TruSeq and Nextera Exome Capture platforms was used for WES, with variant calling performed using two platforms to maximise sensitivity: Platypus and Mutect (assisted by Dr Francesc Castro-Giner). Subsequent analysis was performed by the author. SMGs were identified using three complementary techniques: IntOGen FM (over-representation of functional mutations) and OncoCLUST (over-representation with respect to background mutation rate), and MutSigCV; over-represented pathways were identified by IntOGen. Temporal heterogeneity was assessed by absolute alterations in gene and pathway mutation states, as well as alterations in VAF (potentially representing tumour clonality).

5.1.3 Results

In addition to corroborating established and recently described driver genes, two possible novel associations were identified (*STAG2* and *NRP2*), as well as novel candidate driver pathways for carcinogenesis overall and pTR. No such associations were demonstrated for mTR, mNR, stage, and recurrence and survival at 2 and 5 years. There was evidence that responding and non-responding tumours differed fundamentally at a genetic level, although there were no statistically significant differences at the level of individual genes or pathways. There was considerable evidence of temporal heterogeneity as regards mutated genes and their VAF, pathways, DNA sequence biomarkers identified in Chapter 4 and therapeutic targets. In particular, *TP53* mutations (identified as a marker of lack of pTR) before NAC did not predict pTR; rather it was the persistence of a mutation and greater VAF after NAC that was strongly associated with a lack of response.

This indicated a new model of 'genomic response' (gTR) to NAC: some tumours demonstrated no response, with evidence that the major clone was intrinsically resistant and consolidating its position, whilst others passed through a genetic bottleneck, typified by (counterintuitive) loss of driver gene mutations (primarily *TP53*) and a reduction in VAF, with the resultant clone (perhaps a minor relatively indolent clone unobserved before NAC) often bearing little resemblance to the tumour before NAC. Typically, gTR and pTR were concordant; however, in a limited number of cases discordance suggested that some tumours responded at a genomic level (corroborated by mTR on PET-CT), but then the residual clone regrew rapidly to obliterate evidence of pTR. Tumours with a greater number of mutations and VAF had a worse prognosis.

5.1.4 Discussion

This chapter has added further evidence for previously identified driver genes, and identified a number of candidate genes and pathways. Further exploration of these by targeted resequencing will help determine their role as predictive and prognostic biomarkers to help guide precision medicine of OAC. However, the most novel and important findings are that evidence of considerable temporal heterogeneity that NAC may instigate profound changes in the OAC genome. This means that biomarkers and therapeutic target status may change (mandating re-assessment after intervention), and indicates a new model of gTR. Discordance between gTR, pTR (and mTR) in a small number of cases suggests that serial

genomic monitoring (i.e. assessing gTR as well as mTR during or after NAC) may help personalise management.

5.2 INTRODUCTION

As discussed in Chapters 1.3 and 4, the management difficulties evident for oesophageal cancer both exemplify limitations in our understanding of OAC biology, and demonstrate an urgent need for precision OAC oncology: biomarkers of prognosis, recurrence and sensitivity to therapy, and targeted therapies. In Chapter 4, a range of markers was identified. Some, for example those constituting a tumour's 'status' as regards a gene target for which a therapy exists, provide a basis for entry into clinical trials, or treatment of metastatic disease (Allum et al., 2011).

However, these associations (based on inter-tumour heterogeneity) are confounded by intra-tumour heterogeneity (the potential existence of multiple clones within a cancer). Indeed, targeted studies using sequencing, CGH or SNP arrays have suggested OAC and BO to be oligoclonal, both within primary tumours (Barrett et al., 1999, Leedham et al., 2008) and between its nodal and distant metastases (Konig et al., 2013, Hjortland et al., 2011). Temporal heterogeneity has also been suggested, on the basis of possible clonal evolution/selection during therapy (Zeki et al., 2013, Kostadinov et al., 2013). The fact that the biomarker status of a tumour may change with time has major implications for clinical practice. This is exemplified by worse survival seen for patients with HER2-negative breast cancer metastases, who are treated with anti-HER2 therapy on the basis of an initially positive primary tumour (Niikura et al., 2012).

As discussed in section 1.3.4.5, to date, 3 WGS/WES studies of oesophageal SCC (Gao et al., 2014, Lin et al., 2014, Song et al., 2014), 4 of AC (Chong et al., 2013, Dulak et al., 2013, Streppel et al., 2014, Weaver et al., 2014), and one of both (Agrawal et al., 2012) have been published. These have concentrated on identifying and corroborating a number of candidate driver genes. Overall assessment of spatial heterogeneity has been minimal; Weaver and colleagues assessed the clonality of 15 genes in 22 cases of OAC (Weaver et al., 2014), Lin and colleagues demonstrated multi-clonal signatures within WES of SCC (Lin et al., 2014), Agrawal and colleagues reported variable variant allele frequence (VAF) of *TP53* mutations across SCC and OAC (Agrawal et al., 2012) and Chong and colleagues identified very low

VAF of *HGF* suggestive of a sub-clone in OAC (Chong et al., 2013). However, none of these studies have assessed temporal heterogeneity.

As discussed in Chapter 1.3.7, oesophageal tumours vary considerably in their response to NAC. Whilst some tumours appear completely resistant or sensitive to NAC, others appear incompletely resistant/sensitive. The latter maybe either true incomplete sensitivity, or rather that the tumour comprises a mixed population, with some clones sensitive and some resistant. Complete pTR is rare (and indeed, resistant metastases may occasional persist), so it is likely that almost all cancers in reality contain at least some chemoresistant cells, mandating subsequent surgical resection. However, no oesophageal (or indeed any solid cancer studies) have assessed such response on a genomic level. The only studies to do so have involved haematological tumours such as leukaemia, demonstrating differences at the level of individual genes between disease at presentation and at relapse (Bachas et al., 2012, Bachas et al., 2010). These suggest oligoclonality, with selection and expansion of a pre-existing chemoresistant subclone. NGS of such tumours relapsing after chemotherapy has suggested relapsing cells appear fundamentally related to the 'original' tumour cells, but with less mutational burden or complexity, and a number of unique mutations (Ding et al., 2012, Walter et al., 2012).

Experience has been much less with solid organ tumours; a recent study of *TP53* and *PIK3CA* driver mutations in breast carcinoma samples before and after paclitaxel and carboplatin-based NAC identified surprising alterations, with approximately 50% of tumours 'reverting' from mutant to wild-type, with an associated reduction in VAF and a survival benefit. In addition, a limited number of NGS studies of gliomas following radiotherapy and chemotherapy, suggested considerable evolutionary clonal shifts (Johnson et al., 2014, Kim et al., 2015).

In this study, WES was performed for 30 patients with OAC, within the context of a phase II trial of oxaliplatin and 5-FU NAC, clinical stage and outcomes. Normal, pre-chemotherapy tumour and post-chemotherapy tumour were compared. The primary aims of this study were, firstly, to identify possible candidate driver genes and pathways of carcinogenesis, response to chemotherapy (pTR/mTR), and prognosis. Secondly, to assess temporal heterogeneity and genomic evolution as a response to NAC. And finally, to explore the implications for established biomarkers of outcome and precision treatments as regards spatial and temporal heterogeneity.

5.3 RESULTS

5.3.1 Phase II trial

Samples were drawn from a phase II trial of oxaliplatin 5-FU NAC, conducted prior to the author's involvement at OUH by Mr Richard Gillies and Prof Mark Middleton. Recruitment occurred from May 2006-February 2010. Inclusion criteria were: age>18, histologically-proven invasive cancer of the oesophagus/GOJ (excluding type II tumours), >T1N0 UICC-AJCC TNM 6th edition disease (Greene, 2002)), World Health Organisation performance status 0-2 and adequate haematological, renal and hepatic function. Exclusion criteria were: pregnancy/lactation, evidence of unresectable disease, dihydropyrimidine dehydrogenase deficiency or a clinically significant disease likely to interfere with study evaluations.

Sequentially staging was performed with CT, ^{18}F-FDG PET-CT, EUS and laparoscopy for tumours extending below the diaphragm. Patients received two cycles, each lasting 21 days: 130 mg/m^2 intravenous oxaliplatin on day 1, followed by 1000 mg/m^2/day intravenous 5-FU on days 1-4. Restaging was performed by PET-CT approximately 4 weeks after the second cycle, with surgery as soon as possible thereafter.

5.3.2 Patient clinical and pathological characteristics

Forty of 51 patients had OAC. Random biopsies of the primary cancer and normal oesophageal squamous epithelium >5cm proximally were taken at EUS within 3 weeks of diagnosis and before NAC (by Mr Richard Gillies). Some were snap-frozen; other adjacent samples fixed in formalin and paraffin embedded for confirmation of malignant cells. Thirty-six patients proceeded to surgery 12-14 weeks after diagnosis (one died during chemotherapy, one did not proceed due to performance status, and two developed metastases on re-staging). Successful resection was performed in 34 patients (2 having unresectable disease). Of these, the tumour specimen underwent histopathological assessment within one hour, with macrodissected samples (approximately 5mm^3) taken randomly and frozen for molecular analysis; again adjacent samples were fixed for pathological confirmation. One tumour underwent complete pTR, leaving 33 potentially suitable, of which 30 were chosen for WES. Normal tissue biopsies >5cm proximally were again taken and frozen. Mandard TRGs were performed by Dr Lai Mun Wang.

Characteristics of the 30 patients are presented in table 5.1. Median age was 66 (29-78); 24 patients (80.0%) were male. Fifteen patients (50%) demonstrated a pTR (Mandard TRG 2-3); 20 (66.7%) demonstrated an mTR (either CMR or PMR using PERCIST criteria (Wahl et al., 2009)). Recurrence occurred in 11 patients (36.7%) at 2 years, and 16 (53.3%) at 5. Twenty (66.7%) and 13 (43.3%) patients were alive at 2 and 5 years respectively.

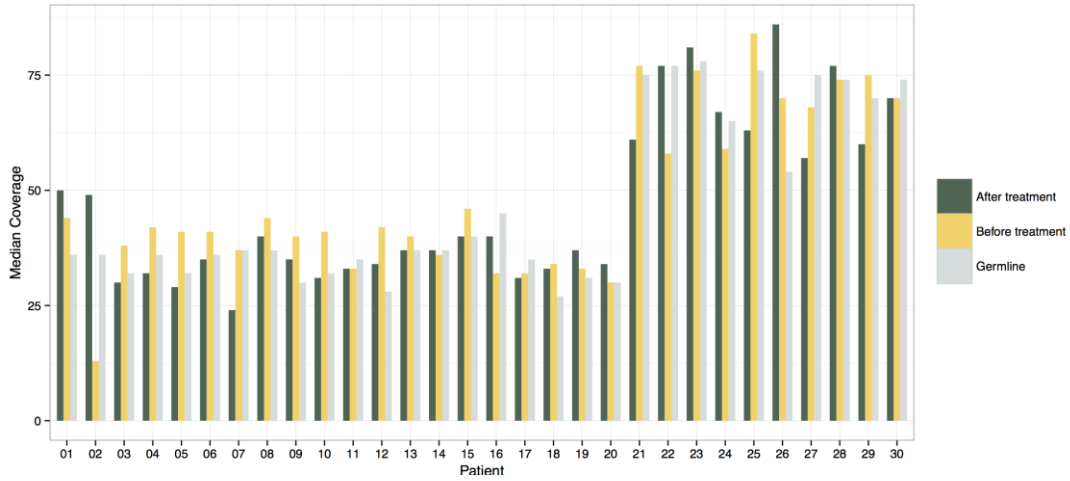
Table 5.1: Clinical and pathological patient characteristics

Patient details			Pathological details			pTR		PET characteristics		Overall survival		Recurrence	
No.	Age	Gender	Grade	pT	pN	Y/N	TRG	mTR	Nodal avidity	2 yr	5 yr	2 yr	5 yr
1	69	Male	Mod	3	1	Yes	3	Yes	0	0	0	1	1
2	78	Male	Poor	2	1	Yes	3	Yes	1	0	0	1	1
3	49	Male	Poor	3	1	Yes	3	Yes	0	0	0	1	1
4	53	Male	Poor	3	3	Yes	3	No	0	1	0	1	1
5	73	Male	Poor	3	0	Yes	2	Yes	0	1	0	0	1
6	63	Male	Mod	3	0	Yes	2	No	0	1	1	0	0
7	68	Male	Poor	2	1	Yes	3	Yes	1	1	1	0	0
8	54	Male	Mod	3	0	Yes	2	Yes	0	1	1	0	0
9	58	Female	Mod	1	0	Yes	3	Yes	0	1	1	0	0
10	61	Male	Poor	2	1	Yes	3	Yes	0	1	1	0	0
11	71	Male	Mod	2	2	No	4	Yes	0	0	0	1	1
12	56	Female	Mod	3	3	No	5	No	0	1	0	1	1
13	67	Male	Mod	2	3	No	4	Yes	0	0	0	1	1
14	62	Male	Poor	3	2	No	4	Yes	0	1	0	1	1
15	67	Male	Poor	3	3	No	4	No	0	0	0	1	1
16	60	Male	Mod	3	2	No	4	Yes	1	1	1	0	0
17	77	Male	Poor	2	0	No	5	Yes	0	1	0	0	1
18	68	Female	Mod	2	0	No	5	No	0	1	1	0	0
19	65	Male	Poor	3	0	No	4	No	0	1	1	0	0
20	65	Male	Poor	3	2	Yes	3	Yes	1	1	1	0	0
21	70	Male	Mod	2	3	No	4	No	0	1	0	0	1
22	60	Male	Mod	3	0	Yes	3	Yes	0	1	1	0	1
23	73	Male	Poor	2	0	Yes	3	Yes	0	1	1	0	0
24	57	Male	Mod	1	1	No	5	No	1	0	0	1	1
25	65	Female	Poor	4	3	No	4	Yes	0	0	0	1	1
26	76	Male	Poor	2	0	No	4	No	0	1	1	0	0
27	78	Female	Well	1	0	Yes	3	No	0	1	1	0	0
28	74	Male	Poor	2	2	Yes	3	Yes	0	1	0	0	1
29	29	Male	Mod	3	0	No	5	Yes	0	0	0	1	1
30	69	Female	Mod	2	0	No	4	Yes	1	1	1	0	0

5.3.3 Sequencing, coverage and contamination

Sequencing was performed in two rounds of 20 and 10 patients (patients 1-20; 21-30) by the High-Throughput Genomics Group at the WTCHG. Libraries were constructed using Illumina TruSeq and Nextera preparation kits respectively. Median depth was: 40X (IQR 33.8-65.5) for normal samples, 40X (33.8-63.5) before chemotherapy, and 40X (33.8-61.5) after chemotherapy. Median percentage of bases with 10X coverage was 88.0% (87.0-90.0%) for all three (figure 5.1). Median contamination fraction was estimated by PurBayes to be 0.141% ($5.00x10^{-5}$-0.749%) before chemotherapy, and 0.137% ($4.86x10^{-5}$-0.746) after. As this was an extremely low estimate, analyses were therefore not corrected for this.

Figure 5.1: Median sequencing coverage of normal tissue and tumour before and after NAC



5.3.4 Mutational burden before and after chemotherapy

5.3.4.1. *Single nucleotide variants and short insertions / deletions*

Two tumours (patients 18 and 30; table 5.2) had a very high number of mutations, suggestive of MSI. The exomes of both were assessed for potentially functional mutations (non-synonymous, truncating, or splice site) in DNA MMR pathway genes. Missense mutations were identified in two (*RFC4* and *RPA4*) in patient 18; none were identified in 30. Both were included with caution in primary analyses; secondary analyses were repeated excluding both tumours.

Before NAC, a median 278.5 mutations (IQR 198.2-443.5) were identified per tumour; 200.5 mutations (66.8-389.5) were detected after (p=0.074, Mann Witney U test). These involved a median 242 (193.2-410.2) and 190 (63.3-352.2) genes respectively (p=0.095).

In total, 13,127 genes were mutated (3,321 potentially functional as determined by SnpEff annotation) before NAC, and 12,899 (3,168) after (supplementary tables 5.1 and 5.2). 4,300 (2,336) mutated genes were shared by at least 2 patients before NAC; 4,001 (441) after; 536 (222) were shared. 129 (6 potentially functional) mutated genes were shared by 6 (20%) of tumours before chemotherapy, 53 (4) afterwards, with 17 (2) persisting.

5.3.4.2. Copy number variants before and after chemotherapy

48 CNV segments were reliably identified (p<0.01) before NAC, affecting 18 tumours. Median length was 9.33Mb (IQR 3.06-19.9Mb). 49 segments were identified after NAC, affecting 15 tumours. Median length was 7.14 MB (2.71-19.7MB; p=0.289). None involved any driver gene loci.

5.3.5 Driver mutations and genes

5.3.5.1. Drivers before chemotherapy

Candidate drivers/significantly mutated genes (SMGs) were identified using IntOGen Mutations v2.3.0 functional impact bias (OncodriveFM, generating an FM p value of bias towards accumulation of functional mutations) and regional clustering (OncodriveCLUST, generating q value of mutation clustering within functional protein regions). Parallel analysis was performed using MutSigCV v1.3.01, using abundance of mutations relative to background mutation rate, functional clustering within genes, and conservation of mutated positions to generate p and FDR-corrected q values. Both identify genes as either significantly (q<0.05) or possibly significantly mutated (q<0.250).

Three genes were identified by FMbias (q<0.05; table 5.2): *TP53* (n=22/30 tumours with non-silent mutations; $q<1x10^{-16}$), *ARID1A* (n=6; $q=1.47x10^{-4}$) and *NRP2* (n=2; p=0.029). A further 22 had possible evidence of association (q<0.250). *TP53* was the sole variant identified by OncoClust (q=0.011). The frequency of mutations, the function of the relevant genes,

whether they have previously been implicated in carcinogenesis, and data from the ICGC and C-bio portal OAC samples are presented in table 5.4.

Only 5 genes were mutated in more than 2 tumours: *TP53* (22), *ARID1A* (6), *SMAD4* (3), *MYH10* (5) and *SEC31A* (3). Most (20; 80.0%) genes identified had plausible functions; most notably chromatin remodelling (n=5: *ARID1A, ARID1B, SETD2, MLL4,* and *PHF2*), and apoptosis (n=4; *TP53, BAX, UACA* and *SMEK1*). However, a significant minority (5; 20%) identified were either protein products of unclear function or without an obvious role in carcinogenesis (for example, myosin or protein transfer), potentially representing false positive results.

MutSigCV also identified *TP53* as significantly mutated (n=21; $p<1.68x10^{-11}$). A further 29 genes were possibly significant ($p<0.02$); supplementary table 5.3. Fifteen (50.0%) genes had potentially plausible functions, including the TGF-ß pathway (n=3: *SMAD4, GDF3* and *BMPR1A*) pro-apoptotic factors (n=4: *TP53, MAGEA4, FAM162A* and *PIH1D1*), and chromatin remodelling (n=1; *SMARCA4*). Just 3 such genes were mutated in more than 2 tumours: *TP53* (n=21), *SMARCA4* (n=4) and *ADAM32*. However, a number of likely false positive associations were identified, typically large genes prone to accumulating passenger mutations, and suggesting the algorithm to compensate inadequately for background mutation rate. Overall, there was overlap between platforms for one gene: *TP53*.

Of these genes, *TP53* (genomic stability and apoptosis), *ARID1A* and *SMARCA4* (chromatin remodelling), *SMAD4* (TGF-β pathway) have previously been identified as candidate OAC drivers. In addition *ARID1B* (a mutually-exclusive homolog of *ARID1A*, contributing to the SWI/SNF nucleosome remodelling complex (Helming et al., 2014)) has been suggested to play a role in oesophageal carcinogenesis. By contrast, whilst *NRP2* (a VEGF co-factor) has been reported as a candidate oncogene in a number of gastrointestinal ACs, it has only been reported in oesophageal cancer in SCC at the RNA level. It therefore may represent a novel association. Similar such associations with SCC at the mRNA level include: *DPYD* (pyrimidine metabolism) and *UACA* (apoptosis). A number of other genes have previously been identified in other malignancies, such as *MLL4* (chromatin remodelling) and *ADD3* (cell adhesion) in breast cancer.

Overall, 12 pre-treatment tumours (40.0%) were mutated for the above chromatin remodelling genes (*ARID1A, ARID1B, SETD2, MLL4, PHF2* and *SMARCA4*); in 7 (23.3%) tumours more than 2 genes were mutated. Eight (26.7%) tumours demonstrated mutations

in the three TGF-β genes above (*SMAD4, GDF3* and *BMPR1A*); 23 (76.7%) had mutations in the apoptotic regulators identified above (*TP53, BAX, SMEK1* and *UACA*).

To determine what bias the two MSI tumours might exert, analyses were repeated excluding both (Supplementary tables 5.4-5). Overall, as expected fewer genes were identified; two additional candidates were identified: *NOTCH1* (n=7; 23.3%) and *RPS6KA2* (n=2; 6.67%). *NOTCH1* (a transmembrane receptor, involved in cell fate decisions) has been reported as a driver in oesophageal SCC; *RPS6KA2* (a serine/threonine kinase) has been identified in ovarian cancer.

Table 5.2: Significantly mutated (non-silent) genes identified by IntOGen before NAC

Gene Function	Before NAC						After NAC		p	Previously identified? CBP frequency ICGC frequency
	N	%	FM		OncoClust		N	%		
			p	q	p	q				
TP53 Apoptosis, genomic stability	22	73.3	$<1x\ 10^{-16}$	$<1x\ 10^{-16}$	0.020	0.020	14	46.7	0.064	AC, SCC (Weaver et al., 2014, Dulak et al., 2013) CBP 71.9% ICGC 66.3%
ARID1A Chromatin remodelling	6	20.0	$6.6\ x10^{-7}$	$1.47\ x10^{-4}$	NA	NA	4	13.3	0.731	AC (Streppel et al., 2014) CBP 8.90% ICGC 7.4%
NRP2 VEGF co-factor	2	6.67	$1.96\ x10^{-4}$	0.029	NA	NA	3	10.0	0.472	SCC (expression) (Fung, 2013) CBP 0.00% ICGC 0.00%
Possibly significant										
SMAD4 TGF-β pathway	3	10.0	$1.04\ x10^{-3}$	0.116	NA	NA	3	10.0	0.707	AC (Weaver et al., 2014) CBP 8.40 % ICGC 8.40%
DPYD Pyrimidine metabolism	2	6.67	$2.05\ x10^{-3}$	0.131	NA	NA	4	13.3	1	SCC (mRNA) (Kimura et al., 2011) CBP 4.80% ICGC 1.14%
HECTD4 Ubiquitin ligase	2	6.67	$2.05\ x10^{-3}$	0.131	NA	NA	3	10.0	1	No CBP 2.1% ICGC 1.05%
MYH10 Myosin	5	16.7	$1.89\ x10^{-3}$	0.131	NA	NA	4	13.3	1	NA CBP 3.40% ICGC 2.10%
UACA Pro-apoptotic	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	1	3.33	1	SCC (mRNA) (Su et al., 2011) CBP 2.10% ICGC 0.00%
SEC31A JAK/STAT signalling	3	10.0	$1.39\ x10^{-2}$	0.248	NA	NA	4	13.3	1	No (NHL) (Van Roosbroeck et al., 2011) CBP 1.40% ICGC 1.05%
SMEK1 Pro-apoptotic	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	1	13.3	1	No (Ovarian) (Byun et al., 2012) CBP 0.70% ICGC 0.00%
TRIM26 Unknown	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	2	6.67	1	NA CBP 1.40% ICGC 0.00%
SETD2 Chromatin remodelling	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	4	13.3	1	No (renal) (Dalgliesh et al., 2010) CBP 2.10% ICGC 0.00%
ADD3 Cell adhesion	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	3	10.0	1	No (breast, mRNA) (Eswaran et al., 2013) CBP 0.7% ICGC 0.00%
PHF2 Chromatin remodelling	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	2	6.67	1	No CBP 0.70% ICGC 0.00%
ARID1B Chromatin remodelling	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	2	6.67	1	ESCC (protein) (Kano et al., 2013, Helming et al., 2014) CBP 0.70% ICGC 1.05%
DNM2 Endocytosis, cytoskeleton	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	2	6.67	1	NA CBP 2.70% ICGC 0.00%
RPL22 Ribosomal protein	2	6.67	$1.39\ x10^{-2}$	0.248	NA	NA	3	10.0	1	NA CBP 0.00% ICGC 0.00%

KMT2D Chromatin remodelling	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	3	10.0	1.000	No (breast) (Kim et al., 2014) CBP 2.10% ICGC 2.11%
VPS13B Protein transfer	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	3	10.0	1	NA CBP 4.80% ICGC 1.05%
BAX Pro-apoptotic	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	2	6.67	1	SCC (mRNA) (Joubert et al., 2005) CBP 0.00% ICGC 0.00%
UNK Zinc finger	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	2	6.67	1	NA CBP 0.00% ICGC 0.00%
ZMPSTE24 Metallopeptidase	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	2	6.67	1	No CBP 0.00% ICGC 0.00%
MDN1 Nuclear chaperone	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	3	10.0	1	No CBP 6.20% ICGC 0.00%
TTF1 Replication fork arrest	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	2	6.67	1	No (lung)(Bai and Shen, 2008) CBP 1.40% ICGC 0.00%
SENP6 *Ubiquitin*-like	2	6.67	1.39 $\times10^{-2}$	0.248	NA	NA	2	6.67	1	No CBP 0.70% ICGC 0.00%

CBP=C-bioportal; ICGC=International Cancer Genome Consortium

5.3.5.2. Driver pathways before chemotherapy

Forty-one pathways were identified as significantly enriched (disproportionately mutated) before NAC ($q<0.05$), mutated in 16 (53.3%)-29 (96.7%) tumours (table 5.3). Notably, these included the HSA00230 Purine metabolism pathway was also identified via GSEA in patients with BO (Chapter 3). This analysis was repeated following the exclusion of both MSI tumours, without significantly altering the findings.

Table 5.3 Significantly enriched pathways before NAC

KEGG ID	Pathway	Function	Before NAC				After NAC		p
			N	%	IntOGen		N	%	
					p	q			
hsa05210	Colorectal cancer	Genomic instability	25	83.3	6.28×10^{-12}	8.85×10^{-10}	19	63.3	0.143
hsa04210	Apoptosis	Apoptosis	24	80.0	1.36×10^{-11}	9.62×10^{-10}	20	66.7	0.382
hsa04310	Wnt signalling pathway	Cell growth, angiogenesis, differentiation	26	86.7	5.43×10^{-11}	2.55×10^{-09}	17	56.7	0.02
hsa05016	Huntington's disease	Mitochondrial and nuclear toxicity	27	90.0	9.60×10^{-11}	3.38×10^{-9}	19	63.3	0.03
hsa05014	Amyotrophic lateral sclerosis	Oxidative stress, mitochondrial toxicity	22	73.3	3.14×10^{-10}	8.85×10^{-9}	15	50.0	0.11
hsa05202	Transcriptional misregulation in cancer	Transcriptional misregulation	29	96.7	4.64×10^{-10}	1.09×10^{-8}	23	76.7	0.052
hsa05213	Endometrial cancer	DNA mismatch repair	27	90.0	4.98×10^{9}	1.00×10^{-7}	18	60.0	0.015
hsa05222	Small cell lung cancer	Apoptosis	27	90.0	6.73×10^{-9}	1.19×10^{-7}	21	70.0	0.104
hsa05217	Basal cell carcinoma	Sonic hedgehog signalling	25	83.3	1.09×10^{-8}	1.71×10^{-7}	16	53.3	0.025
hsa05216	Thyroid cancer	Chromosomal instability	24	80.0	4.05×10^{-8}	5.71×10^{-7}	17	56.7	0.095
hsa05162	Measles	Apoptosis, JAK-STAT pathway	26	86.7	7.65×10^{-8}	9.81×10^{-7}	21	70.0	0.209
hsa04115	p53 signalling pathway	TP53-mediated functions	23	76.7	1.17×10^{-7}	1.38×10^{-6}	16	53.3	0.103
hsa04722	Neurotrophin signalling pathway	Receptor tyrosine kinase signalling	26	86.7	1.35×10^{-7}	1.47×10^{-6}	20	66.7	0.125
hsa05223	Non-small cell lung cancer	Proliferation, apoptosis	26	86.7	4.46×10^{-7}	4.50×10^{-6}	19	63.3	0.072
hsa05160	Hepatitis C	Proliferation, apoptosis	26	86.7	6.22×10^{-7}	5.48×10^{-6}	20	66.7	0.125
hsa05161	Hepatitis B	Proliferation, apoptosis	27	90.0	6.14×10^{-7}	5.48×10^{-6}	21	70.0	0.104
hsa05212	Pancreatic cancer	Proliferation, genomic instability	27	90.0	8.47×10^{-7}	7.02×10^{-6}	20	66.7	0.058
hsa04110	Cell cycle	Cell cycle regulation	27	90.0	1.24×10^{-6}	9.70×10^{-6}	19	63.3	0.03
hsa05220	Chronic myeloid leukemia	Chromosomal instability	25	83.3	1.37×10^{-6}	1.02×10^{-5}	20	66.7	0.233
hsa05219	Bladder cancer	Invasion and metastasis	24	80.0	1.67×10^{-6}	1.18×10^{-5}	18	60.0	0.158
hsa05218	Melanoma	Tumour suppression signalling pathways	26	86.7	3.76×10^{-6}	2.53×10^{-5}	20	66.7	0.125
hsa05203	Viral carcinogenesis	Genomic instability; apoptosis	26	86.7	6.21×10^{-6}	3.98×10^{-5}	21	70.0	0.209
hsa05166	HTLV-I	Proliferation; apoptosis	28	93.3	9.10×10^{-6}	5.58×10^{-5}	24	80.0	0.252
hsa05168	Herpes simplex infection	Immune response	29	96.7	9.81×10^{-6}	5.76×10^{-5}	26	86.7	0.353
hsa05214	Glioma	Multiple cancer processes	26	86.7	1.07×10^{-6}	6.04×10^{-5}	19	63.3	0.072
hsa04972	Pancreatic secretion	cAMP functions	20	66.7	1.10×10^{-4}	5.98×10^{-5}	20	66.7	1
hsa05169	Epstein-Barr virus	Viral carcinogenesis	28	93.3	1.29×10^{-4}	6.75×10^{-4}	27	90.0	1
hsa04610	Complement and coagulation cascades	Complement, inflammation	16	53.3	2.58×10^{-4}	1.30×10^{-3}	12	40.0	0.44
hsa04010	MAPK signaling pathway	Proliferation, differentiation	27	90.0	3.84×10^{-4}	1.87×10^{-3}	26	86.7	1
hsa05215	Prostate cancer	Proliferation; apoptosis	27	90.0	4.79×10^{-4}	2.25×10^{-3}	21	70.0	0.104
hsa04970	Salivary secretion	cAMP, calcium signalling	22	73.3	1.12×10^{-3}	5.08×10^{-3}	20	66.7	0.779
hsa04971	Gastric acid secretion	cAMP, calcium signalling	23	76.7	1.38×10^{-3}	5.74×10^{-3}	19	63.3	0.399
hsa00230	Purine metabolism	Nucleotide metabolism	20	66.7	1.31×10^{-3}	5.74×10^{-3}	21	70.0	1
hsa04540	Gap junction	Cellular adhesion	21	70.0	1.35×10^{-3}	5.74×10^{-3}	19	63.3	0.785
hsa04976	Bile secretion	Cellular transport	21	70.0	5.00×10^{-3}	0.020	19	63.3	0.785
hsa04961	Endocrine and other factor-regulated calcium reabsorption	Cellular homeostasis	18	60.0	6.08×10^{-3}	0.024	15	50.0	0.604
hsa05200	Pathways in cancer	Multiple cancer pathways	29	96.7	6.86×10^{-3}	0.026	26	86.7	0.353
hsa04320	Dorso-ventral	Embryogenesis	14	46.7	7.05×10^{-3}	0.026	6	20.0	0.054

	axis formation								
hsa04725	Cholinergic synapse	cAMP signalling	25	83.3	$8.03x10^{-3}$	0.029	22	73.3	0.532
hsa04721	Synaptic vesicle cycle	Cellular homeostasis	19	63.3	0.012	0.041	18	60.0	0.353
hsa04151	PI3K-Akt signaling pathway	Proliferation ,apoptosis	29	96.7	0.012	0.041	26	0.867	1

5.3.5.3. Driver mutations after chemotherapy

After NAC, *TP53* mutations were again identified by all algorithms (n=14/30; FMbias $q=5.43x10^{-10}$; OncoClust $q=5.13x10^{-3}$; MutSigCV $q=4.66x10^{-5}$; table 5.4; supplementary table 5.6). Intriguingly, there was evidence of considerable temporal heterogeneity: *TP53* mutations seemed to be less prevalent after NAC (n=14) than before (n=22).

FMbias again identified *ARID1A* (n=4 13.3%; q=0.011; chromatin remodelling), in addition to 37 genes with FDR<0.25. 24 genes with p<0.02 were identified by MutSigCV. Overall, 24 (64.9%) genes identified by FMbias were plausible; again, chromatin remodelling genes were frequently identified (n=5 genes: *ARID1A*, *ARID1B, SETD2, ASXL1* and *PHF2*), mutated in 12 (40.0%) tumours.

Again, a number of seemingly spurious associations were identified by MutSigCV: just 7 (29.1%) of genes were plausible. These analyses were repeated excluding the 2 MSI tumours; as for the pre-NAC analyses, fewer genes were identified, although no new candidates were apparent (data not shown due to reasons of space).

Table 5.4: Significantly mutated genes identified by IntOGen after chemotherapy

Gene Function	After NAC						Before NAC		p	Previously implicated?
	N	%	FM		OncoClust		N	%		
			p	q	p	q				
Significant										
TP53 Apoptosis, genome stability	14	46.7	1.44×10^{-12}	5.34×10^{-10}	5.12×10^{-4}	5.12×10^{-4}	22	73.3	0.064	AC, SCC(Dulak et al., 2013)
ARID1A Chromatin remodelling	4	13.3	5.76×10^{-5}	0.011	NA	NA	6	20.0	0.731	AC(Streppel et al., 2014)
Possibly significant										
SETD2 Chromatin remodelling	3	10.0	1.05×10^{-3}	0.130	NA	NA	2	6.67	1.000	Renal (Dalgliesh et al., 2010)
SYNE2 Nuclear organisation	2	6.67	0.022	0.216	NA	NA	4	13.3	0.424	AC (CNV)(Frankel et al., 2014)
SEC31A JAK/STAT signalling	3	10.0	0.022	0.216	NA	NA	3	10.0	1.000	NHL (Van Roosbroeck et al., 2011)
COL24A1 ECM component	2	6.67	0.022	0.216	NA	NA	1	3.33	1.000	No
SLC4A2 Solute transport	2	6.67	0.004	0.216	NA	NA	1	3.33	1.000	No
TRIM26 DNA binding?	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
HECTD4 Ubiquitin ligase	2	6.67	0.003	0.216	NA	NA	2	6.67	1.000	No
ADD3 Cell adhesion	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	Breast RNA(Eswaran et al., 2013))
SYNE1 Nuclear organisation	5	16.7	0.018	0.216	NA	NA	6	20.0	0.399	AC(Chong et al., 2013)
LRP1 Lipoprotein receptor	3	10.0	0.004	0.216	NA	NA	0	0.00	0.237	No
PHF2 Chromatin remodelling	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
ASXL1 Chromatin remodelling	2	6.67	0.022	0.216	NA	NA	1	3.33	1.000	Myelodysplasia (Chen et al., 2014))
ARID1B Chromatin remodelling	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	SCC (protein)(Kano et al., 2013, Helming et al., 2014)
FBXW7 Ubiquitin ligase	3	10.0	0.008	0.216	NA	NA	2	6.67	1.000	SCC(Lin et al., 2014)
SDC1 ECM component	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
RPL22 Ribosomal protein	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
TSHZ3 Transcriptional regulator	2	6.67	0.022	0.216	NA	NA	3	10.0	0.707	Breast/prostate(Yamamoto et al., 2011)
ATG16L2 Autophagy	2	6.67	0.022	0.216	NA	NA	3	10.0	1.000	No
EPPK1 Epithelial migration	2	6.67	0.022	0.216	NA	NA	3	10.0	1.000	No
CPD Metallocarboxypeptidates	3	10.0	0.004	0.216	NA	NA	2	6.67	1.000	No
POP1 Cell cycle regulation	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
ITGA3 Cell adhesion	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
VPS13B Protein transfer	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
CDK14 Cyclin dependent kinase	2	6.67	0.022	0.216	NA	NA	1	3.33	0.612	No
BAX Pro-apoptotic	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	SCC (mRNA)(Joubert et al., 2005)
UNK	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No

Zinc finger										
COL5A1 Collagen	2	6.67	0.022	0.216	NA	NA	3	10.0	0.707	No
MACF1 Cytoskeleton	4	13.3	0.016	0.216	NA	NA	3	10.0	0.707	No
NIPBL Developmental regulation	2	6.67	0.022	0.216	NA	NA	3	10.0	1.000	No
ATP8A1 ATP transport	3	3.33	0.019	0.216	NA	NA	1	3.33	1.000	No
ZMPSTE24 Metallopeptidase	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
MDN1 Nuclear chaperone	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No
TTF1 Replication fork arrest	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	Lung (Bai and Shen, 2008)
MYH10 Myosin	3	10.0	0.005	0.216	NA	NA	5	16.7	1.000	No
OPRL1 Nociception	2	6.67	0.011	0.216	NA	NA	1	3.33	1.000	No
SENP6 Ubiquitin-like	2	6.67	0.022	0.216	NA	NA	2	6.67	1.000	No

5.3.5.4. Driver pathways identified after chemotherapy

Forty-two significantly enriched pathways were identified after NAC (table 5.5). Intriguingly, whilst the same pathways were typically enriched as before NAC, they were without exception mutated in a lower proportion of tumours then before: 14 (46.7%) to 26 (86.7%).

Table 5.5: Significantly over-mutated pathways after NAC

KEGG ID	Pathway	After NAC				Before NAC		p
		N	%	IntOGen		N	%	
				p	q			
hsa05222	Small cell lung cancer	21	70.0	5.03×10^{-9}	7.04×10^{-7}	27	90.0	0.104
hsa05218	Melanoma	20	66.7	4.87×10^{-8}	3.41×10^{-6}	26	86.7	0.125
hsa05223	Non-small cell lung cancer	19	63.3	3.39×10^{-7}	9.59×10^{-6}	26	86.7	0.072
hsa05203	Viral carcinogenesis	21	70.0	2.19×10^{-7}	9.59×10^{-6}	26	86.7	0.209
hsa05210	Colorectal cancer	19	63.3	3.43×10^{-7}	9.59×10^{-6}	25	83.3	0.143
hsa04210	Apoptosis	20	66.7	6.76×10^{-7}	1.58×10^{-5}	24	80.0	0.382
hsa05216	Thyroid cancer	17	56.7	1.15×10^{-6}	2.31×10^{-5}	24	80.0	0.095
hsa05016	Huntington's disease	19	63.3	2.70×10^{-6}	4.73×10^{-5}	27	90.0	0.030
hsa05212	Pancreatic cancer	20	66.7	7.14×10^{-6}	1.11×10^{-4}	27	90.0	0.058
hsa05213	Endometrial cancer	18	60.0	8.43×10^{-6}	1.18×10^{-4}	27	90.0	0.015
hsa05214	Glioma	19	63.3	1.00×10^{-6}	1.28×10^{-4}	26	86.7	0.072
hsa05161	Hepatitis B	21	70.0	1.73×10^{-6}	1.93×10^{-4}	27	90.0	0.104
hsa05162	Measles	21	70.0	1.93×10^{-6}	1.93×10^{-4}	26	86.7	0.209
hsa04110	Cell cycle	19	63.3	1.80×10^{-5}	1.93×10^{-4}	27	90.0	0.030
hsa05166	HTLV-I	24	80.0	7.46×10^{-5}	6.96×10^{-4}	28	93.3	0.254
hsa04540	Gap junction	19	63.3	8.87×10^{-5}	7.76×10^{-4}	21	70.0	0.785
hsa05219	Bladder cancer	18	60.0	1.62×10^{-4}	1.33×10^{-3}	24	80.0	0.158
hsa04115	p53 signaling pathway	16	53.3	1.93×10^{-4}	1.50×10^{-3}	23	76.7	0.103
hsa04060	Cytokine-cytokine receptor interaction	20	66.7	2.58×10^{-4}	1.90×10^{-3}	25	83.3	0.233
hsa04972	Pancreatic secretion	20	66.7	3.02×10^{-4}	2.11×10^{-3}	20	66.7	1.000
hsa05014	Amyotrophic lateral sclerosis	15	50.0	3.58×10^{-4}	2.39×10^{-3}	22	73.3	0.110
hsa05220	Chronic myeloid leukemia	20	66.7	4.20×10^{-4}	2.67×10^{-3}	25	83.3	0.233
hsa04971	Gastric acid secretion	19	63.3	4.79×10^{-4}	2.80×10^{-3}	23	76.7	0.399
hsa05202	Transcriptional misregulation in cancer	23	76.7	4.80×10^{-4}	2.80×10^{-3}	29	96.7	0.052
hsa04970	Salivary secretion	20	66.7	6.37×10^{-4}	3.57×10^{-3}	22	73.3	0.779
hsa05160	Hepatitis C	20	66.7	6.71×10^{-4}	3.61×10^{-3}	26	86.7	0.125
hsa04976	Bile secretion	19	63.3	1.04×10^{-4}	5.40×10^{-3}	21	70.0	0.785
hsa05168	Herpes simplex infection	26	86.7	1.42×10^{-3}	6.87×10^{-3}	29	96.7	0.353
hsa05217	Basal cell carcinoma	16	53.3	1.42×10^{-3}	6.87×10^{-3}	25	83.3	0.025
hsa04722	Neurotrophin signaling pathway	20	66.7	1.54×10^{-3}	7.21×10^{-3}	26	86.7	0.125
hsa04725	Cholinergic synapse	22	73.3	2.64×10^{-3}	0.012	25	83.3	0.532
hsa04730	Long-term depression	14	46.7	3.59×10^{-3}	0.016	17	56.7	0.606
hsa00230	Purine metabolism	21	70.0	4.05×10^{-3}	0.017	20	66.7	1.000
hsa04512	ECM-receptor interaction	23	76.7	4.87×10^{-3}	0.020	26	86.7	0.506
hsa05200	Pathways in cancer	26	86.7	6.23×10^{-3}	0.024	29	96.7	0.353
hsa04151	PI3K-Akt signaling pathway	26	86.7	6.23×10^{-3}	0.024	29	96.7	0.353
hsa05146	Amoebiasis	23	76.7	6.80×10^{-3}	0.025	24	80.0	1.000
hsa05215	Prostate cancer	21	70.0	6.77×10^{-3}	0.025	27	90.0	0.104
hsa00310	Lysine degradation	14	46.7	7.90×10^{-3}	0.028	17	56.7	0.606
hsa04721	Synaptic vesicle cycle	18	60.0	9.22×10^{-3}	0.032	19	63.3	1.000
hsa04713	Circadian entrainment	21	70.0	0.011	0.036	27	90.0	0.104
hsa04630	Jak-STAT signaling pathway	17	56.7	0.011	0.037	20	66.7	0.596

5.3.6 Drivers of chemosensitivity and chemoresistance

To identify genes and pathways associated with chemosensitivity, as well as differences in mutation burden, analyses were performed for the 15 tumours demonstrating a pTR, versus those 15 not.

5.3.6.1. Responding versus non-responding tumours before chemotherapy: all and driver mutations

IntOGen identified *TP53* as significantly mutated in both non-responding (n=10; FM $p=3.91x10^{-7}$; FM $q=9.57x10^{-3}$; OncoClust p&q=0.012) and responding tumours (n=12; FM $p=2.88x10^{-12}$; FM $q=1.47x10^{-10}$). IntOGen also identified *SMARCA4* (n=2; FM $q=2.20x10^{-3}$), *NRP2* (n=4; q=0.011), *SMAD4* (n=2; q=0.018) and *ARID1A* (n=3; q=0.018) as significantly mutated in responding tumours. In addition, *RPS6KA2* (ribosomal protein kinase) was identified as possibly significant (n=2; q=0.120; table 5.6).

Overall, non-responding and responding tumours had a comparable number of potentially functional mutations before NAC: median 51.0 (37.0-101.0) versus 67.0 (44.0-89.0; p=0.521 Mann-Witney test) respectively. However, this was heavily influenced by the two MSI tumours, distorting this distribution (mean 203.9 versus 78.9 non-silent mutations), although no significant difference was demonstrated when they were excluded (p=0.160).

When the distribution of individual significantly mutated genes was considered, no differences were identified (table 5.10). Consequently, whilst there might be fundamental genetic difference between these populations before NAC, the mutational status of individual genes in isolation was not associated with chemosensitivity.

Table 5.6: Mutations before chemotherapy and pathological response—IntOGen

Gene Function	No response						Response						p
	N	%	FM		OncoClust		N	%	FM		OncoClust		
			p	q	p	q			p	q	p	q	
Significant													
TP53	10	66.7	3.91×10^{-7}	9.57×10^{-5}	0.012	0.012	12	80.0	2.88×10^{-12}	1.47×10^{-10}	0.461	0.461	0.681
SMARCA4	4	26.7	0.890	1.000	NA	NA	2	13.3	8.62×10^{-5}	2.20×10^{-3}	1.000	1.000	0.651
NRP2 VEGF cofactor	2	13.3	NA	NA	NA	NA	4	26.7	6.51×10^{-4}	0.011	1.000	1.000	0.651
SMAD4 TGFβ pathway	3	20.0	NA	NA	NA	NA	2	13.3	1.78×10^{-3}	0.018	1.000	1.000	1.000
ARID1A Chromatin remodelling	3	20.0	1.34×10^{-3}	0.164	NA	NA	3	20.0	1.76×10^{-3}	0.018	1.000	1.000	1.000
Possibly significant													
RPS6KA2 Ribosomal protein kinase	1	6.67	NA	NA	NA	NA	2	13.3	0.014	0.117	1.000	1.000	1.000
Other candidates													
DPYD Pyrimidine metabolism	3	20.0	NA	NA	NA	NA	2	13.3	NA	NA	NA	NA	1.000
HECTD4 Ubiquitin ligase	3	20.0	3.64×10^{-3}	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.224
MYH10 Myosin	4	26.7	0.015	0.028	NA	NA	1	6.67	NA	NA	NA	NA	0.330
UACA Pro-apoptotic	1	6.67	NA	NA	NA	NA	1	6.67	NA	NA	NA	NA	1.000
SEC31A JAK/STAT	3	20.0	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.224
SMEK1 Pro-apoptotic	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
TRIM26 DNA binding	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
SETD2 Chromatin remodelling	3	20.0	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.224
ADD3 Cell adhesion	3	20.0	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.224
PHF2 Chromatin remodelling	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ARID1B Chromatin remodelling	1	6.67	NA	NA	NA	NA	1	6.67	NA	NA	NA	NA	1.000
DNM2 Cytoskeleton	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
RPL22 Ribosomal protein	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
KMT2D Chromatin remodelling	2	13.3	NA	NA	NA	NA	1	6.67	NA	NA	NA	NA	1.000
VPS13B Protein transfer	3	20.0	0.025	0.280	NA	NA	1	6.67	NA	NA	NA	NA	0.598
BAX Pro-apoptotic	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
UNK Zinc finger	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ZMPSTE24 Metallopeptidase	1	6.67	NA	NA	NA	NA	1	6.67	NA	NA	NA	NA	1.000
MDN1 Nuclear chaperone	4	26.7	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.100
TTF1 Replication fork arrest	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483

SENP6 Ubiquitin-like	2	13.3	0.025	0.280	NA	NA	0	0.00	NA	NA	NA	NA	0.483

5.3.6.2. Responding versus non-responding tumours before chemotherapy: enriched pathways

Before NAC, 31 pathways were identified in responding tumours, and 30 in non-responsive tumours. Similar to individual genes, responsive tumours were more likely to have a mutation in these pathways (OR=1.16 [1.06-1.27]; $p=2.00x10^{-3}$). Again, there were no significant differences at the level of individual pathways (supplementary tables 5.7-8).

5.3.6.3. Responding versus non-responding tumours after chemotherapy: all and driver mutations

Two significant drivers were identified after chemotherapy in responsive tumours: *TP53* (n=4; 26.7%; q=0.013) and *CPD* (n=2; 13.3%; q=0.018). In non-responsive tumours, one significant gene was identified: *TP53* (n=10; 66.7%; $q=1.66x10^{-4}$), in addition to 32 possibly significant genes (table 5.7).

All but one of these had been previously identified in earlier analyses: *STAG2*, a cohesion complex subunit, involved in chromatid cohesion (and therefore the avoidance of aneuploidy). This has not previously been reported in oesophageal cancer, although is a reported tumour suppressor in bladder (Solomon et al., 2013) and pancreatic cancer (Evers et al., 2014). This therefore represents a potentially novel association.

There were no statistically significant differences (Fisher's exact test) between the mutational frequency of any genes and pTR. However, there was a trend towards lower frequency of *TP53* mutations in tumours that had responded: 4 (26.7%) versus 10 (66.7%; p=0.066). This intriguing observation suggested that, whilst *TP53* mutations before NAC did not predict subsequent pTR, lack of a mutation after NAC might be associated with prior pTR. This would be in keeping in part with the evidence from Chapter 4, but suggests that that temporal heterogeneity before and after NAC (i.e. a genetic response, gTR), might be more pronounced in tumours demonstrating a pTR.

Further supportive evidence was found on the basis of mutational burden. Although responsive tumours tended to have more non-silent mutant genes than non-responsive tumours before NAC, they had fewer after: median 14.0 (10.5-45.5), versus 85.0 (29.0-122.5; p=0.044; Mann Witney test).

Table: 5.7 Driver mutations after chemotherapy, in chemoresponsive tumours versus nonresponsive (IntOGen)

Gene	No response						Response						p
	N	%	FM		OncoClust		N	%	FM		OncoClust		
Function			p	q	p	q			p	q	p	q	
Significant													
TP53 Apoptosis, genome stability	10	66.7	5.71×10^{-7}	1.66×10^{-4}	3.89×10^{-3}	3.89×10^{-3}	4	26.7	1.22×10^{-3}	0.013	0.461	0.461	0.066
CPD Metallocarboxypeptidase	1	6.67	NA	NA	NA	NA	2	13.3	3.29×10^{-3}	0.018	NA	NA	1.000
Possibly significant													
SETD2 Chromatin remodelling	3	20.0	1.64×10^{-3}	0.159	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ARID1A Chromatin remodelling	3	20.0	1.64×10^{-3}	0.159	NA	NA	1	6.67	NA	NA	NA	NA	0.598
SYNE2 Nuclear organisation	4	26.7	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.100
SEC31A KAK/STAT signalling	3	20.0	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.224
COL24A1 ECM component	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
SLC4A2 Solute transporter	2	13.3	5.76×10^{-3}	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
TRIM26 DNA binding	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
HECTD4 Ubiquitin ligase	2	13.3	4.58×10^{-3}	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ADD3 Cell adhesion	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
LRP1 Lipoprotein receptor	3	20.0	4.77×10^{-3}	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.224
PHF2 Chromatin remodelling	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ASXL1 Chromatin remodelling	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
FBXW7 Ubiquitin ligase	4	20.0	0.011	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.100
SDC1 ECM component	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
RPL22 Ribosomal protein	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ATG16L2 Autophagy	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
EPPK1 Epithelial migration	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
KMT2D Chromatin remodelling	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
ITGA3 Cell adhesion	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483

VPS13B Protein transfer	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
CDK14 Cell cycle	1	6.67	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	1.000
BAX Apoptosis	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
UNK Zinc finger	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
COL5A1 Collagen	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
STAG2 Chromatin cohesion	2	13.3	0.022	0.249	NA	NA	1	6.67	NA	NA	NA	NA	0.483
MACF1 Cytoskeleton	4	26.7	0.019	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.100
NIPBL Developmental regulation	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
MDN1 Nuclear chaperone	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
TTF1 Replication fork arrest	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
MYH10 Myosin	3	20.0	0.007	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.224
OPRL1 Nociception	2	13.3	0.013	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483
SENP6 Ubiquitin-like	2	13.3	0.028	0.249	NA	NA	0	0.00	NA	NA	NA	NA	0.483

5.3.6.4. Comparison of responding and non-responding tumours after chemotherapy: pathways

Just 4 significantly enriched pathways were identified in responding tumours, indicative of a reduction in mutational burden, compared with 38 in non-responding tumours (supplementary tables 5.9-10). No new pathways were identified. As with individual genes, there was a highly significant difference in the likelihood of individual pathways being mutated: OR pTR 0.32 (0.29-0.35; $p=1.19 \times 10^{-67}$), indicative of significant underlying genetic differences.

A number of pathways were disproportionally mutated in non-responsive tumours with nominal significance ($p<0.05$). None had an FDR<0.05, although 6 demonstrated an FDR of 0.171: HSA05216 Thyroid cancer involved in TP53-dependent regulation, HSA05161 Hepatitis B, involved in cell signalling, differentiation, proliferation and apoptosis, HSA04110 Cell cycle HAS04520 Cell Adherens Junction, HSA05014 Amyotrophic lateral sclerosis, involved in cellular homeostasis and cell death, and HSA05202 Transcriptional misregulation in cancer.

5.3.6.5. Lack of associations with survival, recurrence and stage

Similar analyses were performed on the basis of 5-year survival and recurrence, pT and pN and the presence of FDG-avid nodes, for variants before and after chemotherapy. However, no novel drivers or statistically significant differences were identified (data not shown, due to reasons of space). Similar pathway analyses were performed.

5.3.7 Temporal heterogeneity

5.3.7.1. Genes, mutations and pathways

Temporal heterogeneity was evidenced at a number of levels: the differential number of non-silent mutations before and after NAC, the proportion of mutations persisting during NAC (i.e. present both before and after), and apparent alterations in mutational frequency of individual genes and pathways as discussed above. This was supported by comparison of individual gene mutation frequency, and pathway enrichment comparing tumours before and after chemotherapy.

There were a median 54.0 (42.0-89.5) non-silent mutant genes before NAC overall, compared with 39.0 (11.30-96.3) after ($p=0.080$, Mann Witney test). However, when considering this within the context of pTR, responsive tumours had fewer mutant genes after NAC than before, whilst non-responsive tumours tended to have more: median percentage change -62.6% (-85.8- -45.3), versus 15.0% (-24.7-30.2; $p=9.97\times10^{-4}$).

At the level of individual non-silent mutations and individual cancers, dramatic differences were seen after NAC. Overall, only 21.7% (8.47-50.9%) of mutant genes were mutated both before and after chemotherapy. Again, this was heavily influenced by pTR: responsive tumours 9.09% (6.54-23.2) versus 41.0% (15.6-54.4; $p=0.026$).

5.3.7.2. *Variant allele frequency*

There was further evidence as regards alterations in VAF: the proportion of mutant reads of the total reads for a particular site/mutation, and a surrogate measure of clonality.

Compared with binary mutational status, comparison of VAF (both overall, and for individual genes) allows for a greater sensitivity.

Before NAC, overall each mutation had a median VAF of 14.5 (4.44-25.0%); after NAC, this increased to 21.9 (0.00-33.3%; $p<2.2x10^{-16}$), representing a tendency for individual mutations to be present in a greater proportion of cells (and therefore clones) after NAC. However, again this was dramatically different between responding and non-responding tumours: overall VAF reduced in responding tumours from 16.7 (8.10-27.3%) to 0.00 (0.00-7.09%; $p<2.2x10^{-16}$), whilst it increased in non-responsive tumours, from 14.0 (1.82-24.1%) to 27.6 (15.1-36.3%; $p<2.2x10^{-16}$).
As considering overall alterations in VAF might be complicated by reductions and increases in different mutations or cancers cancelling each other out, an absolute ΔVAF for each mutation was also considered, as a measure of overall heterogeneity. For responsive tumours this was greater: 18.9 (11.1-29.5%), versus 15.8 (8.70-25.0%) for non-responsive tumours ($p<2.2x10^{-16}$).

These results suggest that tumours undergo considerable mutational change during chemotherapy; this is the case even for pathologically non-responsive tumours, but is most marked in tumours demonstrating a response. Overall, responsive tumours demonstrated a dramatic loss in non-silent mutations, both in absolute terms and as regards VAF, whilst non-responsive tumours demonstrated a much smaller loss of mutations, whilst VAF increased overall.

5.3.7.3. *Implications for driver genes and response*

There was evidence of considerable heterogeneity, both of mutation frequency and VAF, of driver genes (previously reported and candidates identified in this study; supplementary table 5.11)

The relative frequency of tumours with *TP53* mutations reduced from 22 (73.3%) to 14 (46.7%) following NAC (p=0.064, Fisher's exact test). Comparable reductions (although from lower baseline proportions) were seen for *NOTCH1*, 7 (23.3%) to 1 (3.33 %; p=0.052), *ARID1A* 6 (20.0%) to 4 (13.3%) p=0.731), *RPS6KA2* and *NRP2*, 2 (6.67%) to 0 (0.00%; p=0.492). More minor alterations were evident in a number of other drivers, from this study and the literature.

For the case of *TP53*, 9 tumours mutant before NAC (each with 1 mutation) demonstrated regression (to wild-type). One initially mutant tumour had 2 mutations before chemotherapy: 1 regressed, whilst one persisted. In a further 12 tumours, mutations persisted. One initially wild-type tumour developed a *de novo* mutation during NAC. Furthermore, in addition a reduction in median VAF was seen from 22.1% (11.6-39.2) to 15.3% (0.00-41.7; p=0.219).

Notably, all 9 initially mutant tumours regressing to wild-type demonstrated a pTR (supplementary table 5.12). Indeed, just 3 persistently *TP53* mutant tumours responded, whilst one tumour developed a *de novo* mutation despite response. By contrast, the remaining 9 persistently mutant tumours failed to respond. The persistence of a *TP53* mutation was strongly associated with chemoresistance: OR 0.03 (2.47×10^{-3}-0.31; $p=3.74 \times 10^{-3}$, univariate binary logistic regression). This was in contrast to the presence of a *TP53* mutation before chemotherapy: OR 2.00 (0.38-10.5; p=0.419). This therefore suggests that, in contrast to previous hypotheses that the association of TP53 mutation after chemotherapy and pathological response was a consequence of lack of response, rather than a contributory factor. Similar evidence was apparent from ΔVAF: responsive tumours demonstrated a dramatic loss of VAF: -30.8% (-45.2-9.02%), whilst non-responding tumours demonstrated gain: +14.2% (0.63-31.9%). These changes were not obviously accounted for by copy number loss or gain.

A similar pattern was evident for the 46 candidate or reported driver genes (supplementary table 5.12) Non-silent driver mutations in responding tumours were typically lost during chemotherapy (75.6%), with only a small minority persisting (14.0%) or developing de novo (10.5%); by contrast non-responding tumours lost only a minority (21.9%), with the majority (61.9%) persisting or developing de novo (20.8%; $p<2.2 \times 10^{-16}$). In tandem with this, there was typically a reduction in VAF for responding tumours, and a gain for non-responding tumours. Relative to loss, the persistence of driver mutations despite chemotherapy was strongly associated with non-response: OR 0.05 (0.03-0.11; $p=8.88 \times 10^{-15}$), as was the development of driver mutations de novo: 0.11 (0.05-0.28; $p=9.82 \times 10^{-7}$). Overall, functional VAF reduced from a median 0.138 (0.017-0.251) to 0.051 (0.003-0.200; $p<2.2 \times 10^{-16}$; Mann Witney U test). Median ΔVAF was 0.171 (0.098-0.269; $p<2.2 \times 10^{-16}$).

5.3.7.4. *Implications for biomarkers and therapeutic targets*

Comparable heterogeneity was seen for: candidate tumour drivers (0.168 [0.099-0.273; $p=<2.2x10^{-16}$), therapeutic targets (0.157 [0.091-0.270]; $p=2.2x10^{-16}$), and reported DNA biomarkers of outcome and response identified in chapter 4 (0.150 [0.097-0.301]; $p<2.2x10^{-16}$; supplementary tables 5.3 and 5.14)

5.3.8 A theory of genomic tumour response

Assuming driver gene mutation status to be representative of the major clone, this might be explained by a clonal response to NAC: a genomic tumour response (gTR). In non-responding tumours there was typically persistence/development of driver mutations, indicative of persistence and evolution of the major clone(s) despite NAC (lack of gTR). The increase seen in VAF would suggest that this clone consolidates its position, becoming yet more dominant (at the expense of minor clones, evidenced by the overall reduction in (non-driver) mutations and VAF).

By contrast, responding tumours typically went through a qualitative 'genetic bottleneck', losing their driver mutations and therefore cells, suggesting that the major clone was inherently chemosensitive. The residual cancer clones were typically characterised by a low overall somatic mutation burden, and being driver gene wild type. As loss of driver tumour suppressor functions would confer a growth advantage for the major clone, the fact that the residual clones were wild type might suggest they had been out-competed by the major mutant clone before chemotherapy, and therefore explaining why they had not been observed before treatment.

5.3.8.1. Discordant genomic and pathological response

Notably, 3 tumours (patients 10, 23 and 27) demonstrated a pTR (Mandard 3) without a gTR: a stable or increasing non-silent mutational burden (median 48.0 [45.0-68.0] non-silent mutant genes before chemotherapy, and 60.0 [48.0-67.0] after), an increase in VAF (median 3.70% [0.00-19.0] before, 14.3% [5.16-31.8] after), and a large proportion of persistent mutations (median 51.2% [29.4-58.6]). In these cancers, it may be that the responsive elements were minor clones, or only part of the major clone.

By contrast, 5 tumours (patients 12, 14, 25, 26 and 29) did not demonstrate a pTR, yet their genomic pattern suggested gTR: although the number of non-silent mutant genes remained stable (median 15.0 [7.00-35.0] before, median 15.0 [16.0-35.0] after), just 10.8% (8.30-15.8) of such mutations persisted during chemotherapy, and they demonstrated a dramatic reduction in VAF: median 13.6 (6.18-24.5) before, reducing to 0.00 (0.00-6.60). This would be consistent with a major clone gTR, with elimination of the majority of mutations and a genetic bottleneck, but rapid regrowth of an aggressive subclone by the time of sampling to give the appearance of a lack of response. Interestingly, in addition to their low starting mutational burden, all were *TP53* wild-type, and demonstrated an mTR (>30% reduction in SUVmax).

5.3.9 Survival associations

5.3.9.1. Traditional markers

Multivariate cox proportional hazards regression models were generated, for both OS DFS, comprising a number of variables: patient (age, gender and ASA grade), tumour (grade and the presence of FDG-avid nodes on PET-CT after chemotherapy), pTR and stage (pT, pN, R, pL and pV). Following this, male gender, lack of pTR, and pN demonstrated negative survival associations, although the wide confidence intervals for some variables suggested that the analysis was inevitably underpowered (supplementary table 5.15)

5.3.9.2. Mutational burden

The survival associations of global metrics of mutational burden and clonality were tested, adjusted for all factors above other than pTR (due to their interdependence; table 5.8). Overall, a greater mutational burden (non-silent mutant genes) before NAC was associated with improved OS (HR 0.96 [0.93-0.99]; p=0.021) and DFS (0.97 [0.94-1.00]; 0.034). After NAC, however, the opposite was true: whilst there was no association with the number of mutant genes, or the number persisting during chemotherapy, there was a negative association with greater VAF (%): OS 1.13 (1.01-1.26; p=0.031); DFS 1.14 (1.01-1.28; 0.021). Similarly, there was an association between VAF progression (ΔVAF) and worse prognosis: OS 1.07 (1.00-1.13; p=0.048); DFS 1.07 (1.00-1.14; 0.042).

This suggests that the more non-silent mutations accumulated by a cancer before chemotherapy (or rather, the detectable major clone), the more likely it is to be

chemosensitive, and therefore have a better prognosis. By contrast, after chemotherapy the strong association between residual VAF and outcome, and a lower/absent reduction in ΔVAF suggests that progressive consolidation and domination by a chemoresistant clone despite chemotherapy is associated with a dramatically worse prognosis.

Table 5.8: Tumour mutational metrics and survival (full model, excluding pTR)

Factor	OS HR (95% CI)	p	DFS HR (95% CI)	p
Adjusted for age, gender, ASA grade, tumour grade, avid nodal stage and pathological stage Not adjusted for pTR				
Before NAC				
Non-silent mutant / genes	0.96 (0.93-0.99)	0.021	0.97 (0.94-1.00)	0.034
Non-silent VAF (%)	0.92 (0.82-1.04)	0.190	0.92 (0.82-1.05)	0.220
After NAC				
Percentage non-silent mutations persisting	0.08 (0.00-42.2)	0.430	0.10 (0.00-37.1)	0.450
Non-silent mutant / genes	1.00 (0.99-1.02)	0.800	1.00 (0.99-1.01)	0.690
Non-silent VAF (%)	1.13 (1.01-1.26)	0.031	1.14 (1.01-1.28)	0.021
Non-silent ΔVAF (%)	1.07 (1.00-1.13)	0.048	1.07 (1.00-1.14)	0.042

5.3.9.3. Driver gene status

A similar pattern was evident for driver mutations, both all candidates analysed above and *TP53* in isolation (table 5.9). *TP53* mutant status before and after NAC was not associated with prognosis, although in keeping with the above findings the direction of effect reversed, suggestive of a non-significant trend for worse prognosis for *TP53* mutant tumours after NAC. Similar findings were evident for all drivers together. However, there were strong negative associations for *TP53* / driver gene VAF after chemotherapy, and ΔVAF.

Again this provides further evidence of the gTR proposed above, and from a translatable perspective again suggests that the presence of a *TP53* mutation despite NAC, may be a marker of worse prognosis as a surrogate marker of lack of pTR. As by far the most prevalent driver gene in oesophageal cancer, *TP53* VAF and ΔVAF after chemotherapy therefore may represent a more efficient way of monitoring response, via targeted resequencing rather than WES.

Table 5.9: Non-silent driver mutational metrics and survival (full model, excluding pTR)

Factor	Overall survival HR (95% CI)	p	Disease-free survival HR (95% CI)	p
Adjusted for age, gender, ASA grade, tumour grade, avid nodal stage and pathological stage Not adjusted for pathological response				
Before NAC				
TP53 mutation	0.63 (0.04-10.2)	0.750	0.62 (0.04-9,94)	0.730
TP53 VAF (%)	0.98 (0.93-1.03)	0.380	0.98 (0.93-1.03)	0.420
Driver mutation	0.86 (0.66-1.11)	0.250	0.87 (0.68-1.12)	0.300
Driver VAF (%)	0.97 (0.92-1.04)	0.420	0.98 (0.92-1.04)	0.480
After NAC				
TP53 mutation	1.37 (0.27-6.92)	0.700	1.34 (0.26-6.96)	0.730
TP53 VAF (%)	1.07 (1.03-1.13)	6.30×10^{-3}	1.07 (1.02-1.13)	7.60×10^{-3}
TP53 ΔVAF (%)	1.07 (1.02-1.12)	4.30×10^{-3}	1.06 (1.02-1.11)	6.20×10^{-3}
Driver mutation	0.99 (0.88-1.12)	0.890	0.99 (0.88-1.12)	0.930
Persistent driver mutation	0.97 (0.82-1.15)	0.710	0.97 (0.82-1.15)	0.750
Driver VAF (%)	1.10 (1.02-1.86)	0.021	1.10 (1.02-1.94)	0.020
Driver ΔVAF (%)	1.10 (1.01-1.87)	0.021	1.03 (0.99-1.08)	0.160

5.4 DISCUSSION

5.4.1 Summary of findings and implications

This is first study of which the author is aware, firstly to explore potential associations between the OAC exome, chemotherapy response and clinical outcome, and secondly to assess temporal heterogeneity and genomic evolution within the context of NAC.

5.4.1.1. Driver genes and pathways

A number of previously described driver genes were identified both before and after chemotherapy, in addition to the novel identification of a number of candidate driver pathways. Two possible biologically plausible genes were identified: *STAG2* and *NRP2*. The former is a cohesion complex subunit, involved in chromatid cohesion (and therefore the avoidance of aneuploidy), and is a reported tumour suppressor in bladder (Solomon et al., 2013) and pancreatic cancer (Evers et al., 2014). The latter is a VEGF co-factor, reported as a candidate oncogene in a number of cancers including pancreatic (Dallas et al., 2008) and colorectal AC, but reported in oesophageal SCC at the RNA level (Fung, 2013).

Importantly, whilst there were apparent differences between responding and non-responding tumours, there were no statistically significant differences between individual genes or pathways. Whilst this may relate to under-powering, one conclusion is that whilst chemosensitivity has a strong underlying somatic genomic basis, at present this is too complicated to be predicted on the basis of individual genes or pathways.

5.4.1.2. Temporal heterogeneity

The most interesting findings were, however, that of temporal heterogeneity seen before and after NAC. In the first instance, there was strong evidence that tumours after NAC were dramatically different compared to the 'same' tumour before: this was suggestive both at the level of individual genes, SNVs and pathways, and profoundly so when considering all SNVs. Overall, responsive tumours tended to lose mutations following NAC, whilst non-responsive tumours tended to accrue additional mutations. Overall just 21.7% of mutant genes were present both before and after chemotherapy, suggesting post-NAC tumours to be dramatically different to those pre-NAC. Again, this was heavily influenced by pTR; in

responsive tumours just 9.09% persisted. Further evidence was provided by alterations in VAF: this reduced dramatically in responding tumours, whilst increasing in non-responsive tumours.

Overall, responsive tumours demonstrated a dramatic loss in non-silent mutations (primarily drivers), both in absolute terms and as regards VAF, whilst non-responsive tumours demonstrated a much smaller loss of mutations, with an overall increase in VAF. Similar heterogeneity was evident for therapeutic target genes and biomarkers, highlighting the inherent problems with using biopsies at one time point to guide therapy. Indeed, the findings from Chapter 4 that *TP53* mutation was associated with worse prognosis and chemosensitivity were illuminated: specifically, that these associations may rather be a consequence of lack of pTR, rather than a contributory factor.

In addition, a number of new survival associations were identified. The favourable association seen between number of SMGs and survival before NAC, might be explained by greater dysregulation resulting in greater chemosensitivity. Conversely, the negative associations seen between both VAF overall (and that of *TP53*) and survival suggest that these are markers of a lack of gTR.

5.4.1.3. A theory of genomic response

Finally, these findings suggested strongly that in non-responding tumours there was typically persistence/development of driver mutations, indicative of persistence, consolidation and evolution of the major clone(s) despite NAC. By contrast, responding tumours pass through a 'genetic bottleneck', losing their driver mutations and therefore (inherently chemosensitive) cells. The residual chemoresistant clones were had a low overall somatic mutation burden, typically with wild-type driver genes, most likely representing relatively indolent clones.

The discordance seen between genomic and pathological response in a minority of tumours highlights an important possibility: that in some tumours the major clone responds, passes through a genetic bottleneck, but then undergoes rapid regrowth of an aggressive subclone by the time of sampling to give the appearance of a lack of response. From a translatable perspective it may be possible to identify these patients by serial genomic monitoring (similarly to interval PET-CT to assess metabolic response), perhaps using targeted resequencing of *TP53* or driver gene panels. Clearly, at present gTR represents a qualitative

concept, rather than a quantitative classification. However, future research may allow stratification of gTR, analogous to that of pTR using the Mandard TRG.

5.4.2 Limitations of this study

The primary limitation of this study is that of spatial heterogeneity and contamination; pre-NAC biopsies had to be obtained endoscopically, and no reliable means exist to map such biopsies to areas of the resection specimen. Consequently, it could be argued that these findings in fact represent a degree of artefact; either the sampling of unrelated areas of tumour or even normal tissue. In particular, the reduction in VAF seen in responsive tumours might be explained by 'dilution' by residual areas of fibrosis. However, subsequent histological review of immunohistochemistry sections of adjacent biopsies (i.e. those next to those sequenced) by Dr Lai Mun Wang indicated typically at least 50% tumour cells, to be present with no apparent differences between responding and non-responding tumours, and a low VAF (5-10%) is typically found in cancers (Kandoth et al., 2013). Despite the low overall VAFs and number of mutations seen, these tumours still tended to possess some driver mutations in high VAF, suggestive of tumour.

An ideal study to resolve the issue of spatial heterogeneity would involve multi-region sequencing of pre-determined areas which could be reliably mapped in the resection specimen. This might be possible endoscopically using small areas of tattooing, although in reality might prove prohibitively difficult. However, pre-NAC biopsies typically contained mutations with VAFs at approximately 20% and therefore unlikely to represent minority evolutionary branches of tumour. Furthermore, it is unlikely that the consistent reductions in driver VAF seen could be explained by random sampling bias.

Finally, there remain issues regarding pTR. In the first instance, the necessary classification of response to a binary state does not completely reflect the inherent complexity of pathological response to NAC. In the second, as discussed in Chapter 1, classification remains subjective although this was minimised by double pathological review being undertaken prior to molecular work being carried out.

5.4.3 Future work

This study suggests a large number of potentially fascinating avenues of future research. Firstly, it remains to be determined whether there are similar differences evident in tumours before and after NAC at the level of the epigenome, transcriptome and proteome. A particularly fascinating question would be whether differential expression of, for example cell cycle regulators, might corroborate the theory that the subclone seen after response represents a 'primordial' and indolent tumour, and conversely that the clone seen after rapid regrowth of tumours demonstrating a genomic but not pathological response indeed does demonstrate an aggressive growth phenotype.

At a translatable level, these data suggest that pre-NAC biopsies are very poorly predictive of subsequent response. Rather, genomic monitoring (either by targeted/exome/genome sequencing of the primary tumour or circulating tumour DNA) during therapy might provide valuable information, perhaps using *TP53* as an exemplar. In the presence of response it may be possible to observe the emergence of a resistant clone; this may then be amenable to either additional therapies or rapid resection should it behave aggressively. Conversely, the lack of a genomic bottleneck might suggest failure of therapy, mandating resection.

5.4.4 Conclusions

In conclusion, this study found evidence that OAC responding to neoadjuvant oxaliplatin and 5-fluorouracil chemotherapy typically go through a major genetic bottleneck, consistent with prior reports from smaller sets of haematological malignancies. However, this loss of cells primarily affects major clones and driver mutations (as exemplified by *TP53*), which are typically lost or greatly reduced in frequency. They are generally replaced by a perhaps previously unobserved clone; this tends to have few mutations, and suggests a model in which the primordial clone is out-competed by the major clone before NAC, but has greater intrinsic resistance to chemotherapy. Conversely, in most non-responding cancers, the major clone seems intrinsically resistant, and continues to consolidate its position evolve (often dramatically) during NAC. However, chemoresistance appears extremely complicated and at present unpredictable on the basis of pre-NAC biopsies. This, and the findings that mutations in biomarker and therapeutic target demonstrate considerable temporal heterogeneity, strongly suggest that serial sequencing may have a role to play in adapting therapy.

CHAPTER 6

PRECISION STAGING OF OESOPHAGEAL CANCER USING DECISION THEORY: SELECTIVE ENDOSCOPIC ULTRASOUND, POSITRON EMISSION TOMOGRAPHY AND LAPAROSCOPY

6. CHAPTER 6: PRECISION STAGING OF OESOPHAGEAL CANCER USING DECISION THEORY: SELECTIVE ENDOSCOPIC ULTRASOUND, POSITRON EMISSION TOMOGRAPHY AND LAPAROSCOPY

6.1 SYNOPSIS

6.1.1 Introduction

Following CT, international guidelines for the staging of oesophageal and GOJ cancer recommend EUS, PET-CT, and laparoscopy for T3/T4 GOJ tumours. However, these recommendations are based on the generic utilities of each test; it is unknown whether for some patients test risk in fact outweighs potential benefit. The aim of this chapter was to quantify investigation risks, benefits and utilities for the first time, and develop pragmatic, personalised staging recommendations.

6.1.2 Methods

All patients with oesophageal/GOJ cancer staged in OUH beyond CT from May 2006-July 2013 comprised a development set; those from July 2013-July 2014 a prospective validation set. Probability thresholds of altering management were calculated and predictive factors identified for each modality and endpoint. A threshold of >T1N0 disease was used for selecting patients for NAC. Algorithms and models (decision tree analysis, logistic regression, and artificial neural networks) were validated internally and independently.

6.1.3 Results

953 patients were staged following CT, by ^{18}F-FDG PET-CT (n=918), EUS (799) and laparoscopy (458). 829 comprised the development set (n=800, 698 and 397); 124 validation (n=118, 101 and 61). EUS utility in the 71.8% patients with T2-T4a disease on CT was minimal (0.40%), its risk exceeding benefit. PET-CT could further refine selection on the basis of avidity. EUS was moderately accurate for pT1N0 stage. Predictive models were developed for patient selection. PET-CT altered management in 23.0%; laparoscopy in 7.29%,

including T2 and distal oesophageal tumours. A number of factors predicted metastases on PET-CT and laparoscopy, although none could inform an algorithm.

6.1.4 Conclusions

Whilst EUS provides additional information on T and N stage, this study found its risk to outweigh potential benefit in most patients (those with T2-T4a disease on CT) when a threshold of >T1N0 disease was used to select patients for NAC. Laparoscopy is justified for ≥T2 distal oesophageal tumours, supporting current UK guidelines.

6.2 INTRODUCTION

As discussed in Chapter 1.3.5, there is largely consensus internationally as to initial staging algorithms for oesophageal cancer: AUGIS, (in conjunction with the British Society of Gastroenterology and British Association of Surgical Oncology), the USA STS and NCCN, and in Europe ESMO, recommend CT and EUS for all patients, followed by PET-CT for T1b-T4 disease, in the absence of definitive metastatic disease (Varghese et al., 2013, National Comprehensive Cancer Guidelines, 2014, Stahl et al., 2010, Allum et al., 2011). In the USA and Europe, laparoscopy is reserved for T3/4 disease involving the GOJ, although in the UK it is also recommended for distal oesophageal tumours. These recommendations reflect the complementary roles of each modality: EUS is most accurate for T and N stage (Puli et al., 2008a), PET-CT for distant metastases (Gillies et al., 2012, Richardson and Khan, 2012) and laparoscopy for peritoneal disease[12].

However, using all modalities by default consumes resources, delays treatment, confers small but clinically significant risks, and yet may in practice not alter management decisions. The primary utility of staging modalities is to partition patients into optimal management groups: identifying patients with early disease (T1aN0 intramucosal cancer for ER; T1bN0 for resection of submucosal cancer without neoadjuvant therapy), locally advanced (T1bN1-T4a; typically resection with neoadjuvant therapy), and unresectable T4b and metastatic disease.

By contrast, using all modalities will provide optimal staging precision and useful prognostic information (for example, distinguishing between T2N1 and T3N3 disease), but without typically altering management. It is not known whether patient subgroups exist for whom

these primary test utilities differ. Similarly, decision analytic metrics are not available to guide staging (i.e. whether this utility justifies test risk).

The primary aim of this study was to (for the first time) calculate the contemporary net benefits and risks of EUS, PET-CT and laparoscopy, their primary utilities (probability of altering management), and probability thresholds (at which test benefit equals risk). Secondary aims were to identify clinical, radiological and histopathological factors associated with these endpoints, and generate predictive models to identify patient subgroups for selective staging. And finally, to use these findings to refine existing staging algorithms (which achieve optimal precision) on the basis of optimal pragmatism, maximal efficiency and minimal patient risk.

6.3 RESULTS

6.3.1 Patients

All patients with oesophageal/GOJ cancer staged in OUH beyond CT from May 2006-July 2013 comprised a development set; those from July 2013-July 2014 a prospective validation set (table 6.1)**.** 953 consecutive patients were staged following CT by PET-CT (n=918, using one of two scanners, before and after 3rd November 2009), EUS (799) and laparoscopy (458). 829 comprised the development dataset (n=800, 698 and 397 respectively; table 6.1). 124 the validation set (n=118, 100 and 61; table 6.1). Potential operator bias was adjusted for using referring centre and PET scanner within multivariate models, but not EUS operator as possible T1N0 tumours were selectively referred for EUS+/-ER. As discussed in Chapter 2.4.3, a hybrid of the TNM 6th and 7th editions was used: all T stages were converted to the 7th edition, and all N stages to the 6th (as insufficient data were available to convert to the 7th).

Table 6.1—Patient and tumour characteristics

Characteristic	Development set (n = 829)	Validation set (n = 124)
Age (years; median; IQR)	66 (13, 29–88)	68 (13, 40–80)
Sex		
Female	204 (24.6%)	35 (28.2%)
Male	625 (75.4%)	89 (71.8%)
Cell type		
AC	639 (77.1%)	104 (83.9%)
SCC	165 (19.9%)	18 (14.5%)
Adenosquamous	7 (0.84%)	2 (1.61%)
Neuroendocrine	3 (0.4%)	0 (0%)
Small cell	3 (0.4%)	0 (0%)
Undifferentiated	12 (1.4%)	0 (0%)
Grade		
Well	71 (8.6%)	5 (4.00%)
Moderate	347 (41.9%)	58 (46.8%)
Poor	389 (46.9%)	60 (48.4%)
Undifferentiated	22 (2.7%)	1 (0.80%)
Tumour site		
Proximal	23 (2.8%)	1 (0.80%)
Mid	67 (8.1%)	12 (9.70%)
Distal	237 (28.6%)	42 (33.9%)
Siewert 1	197 (23.8%)	38 (30.6%)
Siewert 2	195 (23.5%)	16 (12.9%)
Siewert 3	105 (12.7%)	14 (11.3%)
Multifocal	5 (0.6%)	1 (0.80%)
Pre-treatment stage (TNM 6th edition)		
I	52 (6.3%)	11 (8.90%)
IIA	157 (18.9%)	21 (16.9%)
IIB	57 (6.9%)	15 (12.1%)
III	373 (45.0%)	55 (44.4%)
IV	190 (22.9%)	22 (17.7%)
Impassable at OGD		
No	699 (84.3%)	107 (86.3%)
Yes	130 (15.7%)	17 (13.7%)
Management decisions		
Palliative (performance status)	143 (17.2%)	12 (9.7%)
Palliative (incurable)	185 (22.3%)	22 (17.7%)
Endoscopic therapy	26 (3.2%)	11 (8.90%)
Surgery	58 (7.0%)	5 (4.03%)
Neoadjuvant therapy + surgery	378 (45.6%)	67 (54.0%)
DCRT	37 (4.5%)	7 (5.65%)
Died during staging	2 (0.2%)	0 (0%)
Endoscopic ultrasonography		
Not requested	127** (15.3%)	24*** (19.4%)
Performed (passable)	543 (65.5%)	75 (60.5%)
Performed (impassable+miniprobe)	49 (5.91%)	1 (0.81%)
Performed (impassable, no miniprobe)	106 (12.8%)	24 (19.4%)
Abandoned	4 (0.48%)	0 (0.00%)

EUS not requested due to: metastases on PET-CT (79) laparoscopy (5), patient choice (22), prohibitive anatomy (10), DCR (7), and death during staging (2), and unclear reasons (2). * metastases on PET-CT (10) or laparoscopy (1), patient choice/performance status (9), prohibitive anatomy (1) decision to proceed to DCR (3)

6.3.2 Endoscopic ultrasound

The primary utility of EUS was considered to be that altering management by identifying/refuting T1N0 or T4b disease, or unsuspected metastases (distant organ, or nodal outside a standard resection field); PET-CT by identifying/confirming metastases, or unsuspected lesions altering management following investigation (e.g. treatment of a synchronous cancer, detection of high-risk lesions such as polyps requiring colonoscopic surveillance (Cairns et al., 2010)).

6.3.2.1. EUS (development dataset): T1N0

Tx/T1N0 disease was reported in 51 patients (7.31%). In 4 tumours impassable to the echoscope, possible T1N0 disease was demonstrated in the visualized tumour. However, in none was T1N0 confirmed by mini-probe. T1N0 disease was associated with earlier CT T group, CT N0 group and a passable tumour at OGD (table 6.2).

49 patients were identified in the 128 with possible T1 disease on CT (38.3%; 95% CI 30.0-46.9%), 2 in the 501 with CT T2-T4a disease (0.40%; 95% CI $1x10^{-4}$-1.54%). There was zero incidence in the 69 patients with possible T4b disease on CT (95% CI 0.00-4.35%) and 81 with impassable tumours at OGD (95% CI 0.00-3.70%). Multivariate regression was therefore not possible for impassable tumours, it being a perfect separator; earlier CT T group independently predicted EUS T1N0 disease (table 6.2).

675 patients subsequently underwent PET-CT (n=38 EUS T1N0). There was zero incidence of EUS T1N0 disease in the 259 with FDG-avid nodes (95% CI 0.00-10.9%). Multivariate regression (excluding these) demonstrated shorter avid length and earlier CT T group to predict T1N0 disease.

Table 6.2: Factors associated with EUS T1N0 disease: overall and multivariate logistic regression

Characteristic	EUS T1N0 (n=51)	EUS T1N1 and above (n=647)	p (corrected α=0.0042)	Multivariate OR* (95% CI)	p
Gender					
Female	12 (23.5%)	158 (24.4%)	1.00[a]	Ref	Ref
Male	39 (76.5%)	489 (75.6%)		0.702 (0.176-2.80)	0.617
Age (median) (IQR, range)**	69 (59-72; 32-84)	66 (64-75; 29-88)	0.0187[b]	1.00 (1.00-1.00)	0.649
Cell type					
AC	46 (90.2%)	494 (76.4%)	0.209[c]	Ref	Ref
SCC	5 (9.80%)	133 (20.6%)		0.207 (0.02-2.20)	0.192
AS	0 (0.00%)	7 (1.08%)		NA	NA
NEC	0 (0.00%)	3 (0.46%)		NA	NA
Small cell	0 (0.00%)	2 (0.31%)		NA	NA
Undifferentiated	0 (0.00%)	8 (1.24%)		NA	NA
Grade					
Well	6 (11.8%)	59 (9.12%)	0.525[c]	Ref	Ref
Moderate	25 (49.0%)	269 (41.6%)		2.54 (0.321-20.1)	0.367
Poor	19 (37.3%)	302 (46.7%)		3.03 (0.370-24.8)	0.302
Undifferentiated	1 (1.96%)	17 (2.63%)		5.50×10^{-4} (0-∞)	0.996
Tumour site					
Proximal	0 (0.00%)	14 (2.16%)	0.0497[c]	NA	NA
Mid	4 (7.84%)	56 (8.66%)		Ref	Ref
Distal	21 (41.2%)	173 (26.7%)		0.705 (0.034-14.7)	0.822
Siewert 1	17 (33.3%)	160 (24.7%)		1.67 (0.071-39.8)	0.750
Siewert 2	8 (15.7%)	160 (24.7%)		0.421 (0.018-9.88)	0.591
Siewert 3	1 (1.96%)	80 (12.4%)		2.16 (0.503-9.25)	0.534
Multifocal	0 (0.00%)	4 (0.62%)		NA	NA
Impassable (OGD)					
No	51 (100%)	566 (87.5%)	0.0024[c]	NA	NA
Yes	0 (0.00%)	81 (12.5%)			
CT T stage					
Possible T1	49 (96.1%)	79 (12.2%)	$<2.2\times10^{-16}$[c]	Ref	Ref
T2-T4a	2 (3.90%)	499 (77.1%)		0.025 (0.004-0.160)	1.06×10^{-4}
Possible T4b	0 (0.00%)	69 (10.7%)		NA	NA
CT N stage					
N0	49 (96.1%)	274 (42.2%)	2.1×10^{-15} [c]	Ref	Ref
N1	2 (3.90%)	373 (57.7%)		0.747 (0.076-7.39)	0.803
CT centre					
OUH	17 (33.3%)	226 (34.9%)	0.954[c]	2.61 (0.624-11.0)	0.188
Centre 1	12 (23.5%)	157 (24.3%)		Ref	Ref
Centre 2	13 (25.5%)	150 (23.2%)		2.16 (0.503-9.25)	0.301
Centre 3	8 (15.7%)	92 (14.2%)		0.437 (0.031-6.24)	0.542
Other	1 (1.96%)	22 (3.40%)		29.4 (0.576-1504)	0.100
SUVmax (median)	4.15	10.9	$<2.2\times10^{-16}$ [b]	0.056 (0.009-3.37)	0.168
(IQR)	(2.50-5.130	(7.50-16.2)			
(Range)	(2.5-11.8)	(2.50-52.6)			
Not performed	13 (25.5%)	10 (1.55%)			
PET length (median)	1.00	5.40			
(IQR)	(0.00-1.78;	(3.70-7.00)	$<2.2\times10^{-16}$ [b]	0.53 (0.29-0.95)	0.034
(Range)	(0.00-6.90)	(0.00-20.0)			
Not performed	13 (25.5%)	10 (1.55%)			
PET Avid nodes					
No	38 (74.5%)	378 (58.4%)	8.29×10^{-9} C	NA	NA
Yes	0 (0.00%)	259 (40.0%)			
Not performed	13 (25.5%)	10 (1.55%)			
PET scanner					
Before Nov 2009	14 (27.5%)	304 (47.0%)	NA	2.11 (0.668-6.66)	0.203
After Nov 2009	24 (47.1%)	331 (51.2%)			
Not performed	13 (25.5%)	12 (1.85%)			

*following exclusion of perfect separators; **Shapiro-Wilk's $p=7.54\times10^{-08}$; Ref=reference category;

Table 3: Factors associated with EUS T4b disease: overall and binary logistic regression

Characteristic	EUS T4b (n=26)	EUS <T4b (n=672)	p (α=4.17 x10^{-3})	Multivariate OR (95% CI)*	p
Gender					
Female	2 (7.69%)	168 (25.0%)	0.059[a]	Ref	Ref
Male	24 (92.3%)	504 (75.0%)		0.490 (1.72-1.40)	0.182
Age (median)	67.5	66	0.475[b]	1.00 (1.00-1.00)	0.790
(IQR)	(58.3-69.8)	(59.8-72.3)			
(Range)*	(48.0-80.0)	(44.0-83.0)			
Cell type					
AC	7 (26.9%)	533 (79.3%)	2.05x10^{-7} [c]	Ref	Ref
SCC	17 (65.4%)	121 (18.0%)		4.98 (1.27-19.5)	0.021
AS	1 (3.85%)	6 (0.89%)		54.1 (2.68-1092)	9.24x10^{-3}
NEC	0 (0.00%)	3 (0.45%)		NA	NA
Small cell	1 (3.85%)	1 (0.15%)		NA	NA
Undifferentiated	1 (3.85%)	8 (1.19%)		NA	NA
Grade					
Well	4 (15.4%)	61 (9.08%)	0.102[c]	Ref	Ref
Moderate	12 (46.2%)	282 (42.0%)		0.250 (0.053-1.18)	0.081
Poor	8 (30.8%)	313 (46.6%)		0.200 (0.037-1.10)	0.065
Undifferentiated	2 (7.69%)	16 (2.38%)		2.84x10^{-6} (0-∞)	0.991
Tumour site					
Proximal	2 (7.69%)	12 (1.79%)	1.05x10-8 [c]	Ref	Ref
Mid	12 (46.2%)	48 (7.14%)		1.24 (0.200-7.89)	0.865
Distal	7 (26.9%)	187 (27.8%)		0.312 (0.047-2.07)	0.228
Siewert 1	3 (11.5%)	174 (25.9%)		0.049 (3.51x10^{-3}-0.682)	0.025
Siewert 2	0 (0.00%)	168 (25.0%)		NA	NA
Siewert 3	1 (3.85%)	80 (11.9%)		0.130 (7.36x10^{-3}-2.30)	0.164
Multifocal	1 (3.85%)	3 (0.45%)		0.516 (0.018-14.7)	0.699
Impassable					
No	17 (65.4%)	600 (89.3%)	1.58x10^{-3}[c]	Ref	Ref
Yes	9 (34.6%)	72 (10.7%)		4.15 (1.31-13.2)	0.015
CT T stage					
Possible T1	0 (0.00%)	128 (19.0%)	<2.2x10^{-16}[c]	NA	NA
T2-T4a	0 (0.00%)	501 (74.6%)		NA	NA
Possible T4b	26 (100.0%)	43 (6.40%)		NA	NA
CT N stage					
N0	12 (46.2%)	311 (46.3%)	1.00[a]	Ref	Ref
N1	14 (53.8%)	361 (53.7%)		0.897 (0.298-2.70)	0.846
CT centre					
OUH	9 (34.6%)	243 (36.2%)	0.664[c]	0.435 (0.120-1.57)	0.205
Centre 1	9 (34.6%)	156 (23.2%)		Ref	Ref
Centre 2	4 (15.4%)	159 (23.7%)		0.215 (0.044-1.06)	0.059
Centre 3	3 (11.5%)	90 (13.4%)		0.443 (0.078-2.51)	0.358
Other	1 (3.85%)	34 (5.06%)		1.42 (0.115-17.6)	0.785
SUVmax (median)	13.80	10.30	0.062[b]	0.360 (0.117-1.11)	0.074
(IQR)	(9.70-16.80)	(7.00-15.90)			
(Range)	(2.90-32.50)	(2.50-52.60)			
PET length (median)	7.00	5.00	8.42x10^{-4} [b]	1.63 (1.29-2.06)	4.99x10^{-5}
(IQR)	(5.93-8.28)	(3.50-7.00)			
(Range)	(2.00-14.40)	(0.00-20.00)			
Avid nodes					
No	12 (46.2%)	244 (61.6%)	0.337 [a]	1.03 (0.316-3.34)	0.965
Yes	12 (46.2%)	396 58.9%)			
Not performed	2 (7.69%)	32 (4.76%)			
PET scanner					
Before Nov 2009	7 (26.9%)	303 (46.5%)	NA	0.360 (0.117-1.10)	0.074
After Nov 2009	17 (65.4%)	348 (53.8%)			
Not performed	2 (7.69%)	23 (4.55%)			

*following exclusion of perfect separators; A=Fisher's exact test; b=Mann-Witney U test; c=Pearson's Chi square

6.3.2.2. EUS (development dataset): T4b

T4b disease was confirmed in 26 of 69 (37.7%) patients suspected on CT (n=4 mini-probe), and refuted in 31 (n=3 mini-probe). A further 12 with impassable tumours were managed as T3. No additional cases were identified. On multivariate analysis (unadjusted for CT T group as a perfect separator, table 6.3) cell type (SCC and adenosquamous), site (non-GOJ tumour), impassability and longer PET length independently predicted T4b disease.

6.3.2.3. EUS (development dataset): metastases

Possible M1 nodal metastases were identified by EUS in 5 cases; 3 were excluded by FNA; 2 were confirmed, but also evident on PET-CT.

6.3.2.4. EUS: utility

EUS therefore altered management decisions in 77 patients (11.0%). Metastases were subsequently demonstrated in 96 (14.2%) patients by further staging with PET-CT +/- laparoscopy. Notably, for patients down-staged by EUS from possible T4b disease, metastases on PET-CT were subsequently demonstrated in 10 of 43 patients (23.3%).

Stratification by CT T group demonstrated distinct utilities: in patients with Tx/possible T1 (early) disease (n=128; 18.3%), EUS confirmed T1N0 in 49 (38.3%). In patients with possible T4b disease (n=69; 9.89%) EUS was confirmatory in 26 (37.7%). However, in 501 patients (71.8%) without possible T1 or T4b disease on CT, EUS altered management in just 2 (0.40%). In the 81 patients with impassable tumours, EUS altered management in 3 (1.94%, confirming T4b with mini-probe.

6.3.2.5. EUS: comparison with pathological staging

367 patients underwent resection; 81 (21.3%) without neoadjuvant therapy: ER (19), ER plus surgery (7), and surgery (55). Of the 51 EUS T1N0 tumours, 46 were resected: (17, 7 and 22 respectively). Of the 17 patients undergoing ER only, pT1 was confirmed in 11. Of the 29 treated with surgery+/-ER, pT1N0 was confirmed in 24. 5 tumours were upstaged by T stage, 2 also to N1 disease. 5 other tumours were down-staged to pT1N0. Excluding the 17 ER-only patients, EUS was 82.8% sensitive and 84.4% specific for pT1N0 (PPV 82.8%; NPV

84.4%). On multivariate regression, no variables predicted pT1N0 disease (supplementary table 6.1).

6.3.2.6. EUS: decision theory

Probability thresholds (Pt) were calculated to inform decision theory and models (Pauker and Kassirer, 1980) using sensitivity, FPR, treatment risk, net benefit and test risk for each investigation. These were taken from this study or the highest level of evidence in the literature if not available. P_t is the probability of an outcome (e.g. identifying T1N0 disease) at which the risk of a test is equal to its potential benefit; i.e. if the probability is $\geq P_t$, the test is justified; if $<P_t$, the risk exceeds potential benefit.

There was one instance of perforation (0.14%). The P_t for EUS T1N0 disease was 0.95% (the probability of identifying T1N0 at which the benefit of EUS equals its risk). As the probability in patients with impassable tumours (0.00%) or T2-T4a disease on CT (0.4%) was lower, EUS risk to these patients outweighed its potential benefit of altering management. The P_t for EUS T4b disease was 2.02 % (based on T4 disease overall).

6.3.3 PET-CT

6.3.3.1. PET-CT: utility

In 100 of 800 patients, prior CT suggested metastatic disease. This was confirmed in 57 (57.0%; in 28 [49.1%] of whom additional unsuspected metastases were demonstrated), and refuted in 43 (43.0%). In the 700 without prior suggestion, PET-CT identified metastases in 104 (14.9%). In total, metastatic disease was demonstrated in 161 patients (n=144 unequivocal, n=17 requiring confirmatory biopsy). 5 further lesions were biopsied demonstrating: benign disease (n=4) and chronic lymphocytic leukaemia (n=1).

In 103 patients, 119 non-metastatic lesions were identified. 78 were investigated, confirming significant pathology in 17 (2.12%): 8 synchronous cancers (thyroid, colon, lung, prostate [n=2 each], bladder [n=1]), high-risk colonic polyps in 9. In 6 patients (0.75%), PET-CT also staged known synchronous cancers. Consequently, PET-CT altered management in 23.0%:

confirming metastases (7.12%), identifying unsuspected metastases (13.0%), additional pathology (2.12%), and staging synchronous cancers (0.75%).
Higher SUVmax was associated with SCC, advanced stage, female sex and younger age; longer length with advanced stage; FDG-avid nodes with SCC, advanced stage and age. The later PET-CT scanner was associated with higher SUVmax and avid nodes; all associations were independent of this (supplementary tables 6.2-6.4).

6.3.3.2. PET-CT: predicting unsuspected metastases

Analysis was restricted to the 700 patients with CT M0 examinations (supplementary table 6.6). Although EUS T and CT N stage independently predicted metastases, no factors could identify patients with a probability <Pt (0.083%; supplementary table 6.5. Although there was zero incidence in EUS T1 disease, the 95% CI was 0.00-6.12%.

6.3.4 Laparoscopy

397 patients underwent laparoscopy (56 with jejunostomy insertion); metastases were demonstrated in 28 (7.05%). In patients undergoing laparoscopy alone (jejunostomy cases being excluded due to risk from the additional procedure) there was one major (pneumonia; 0.29%) and 4 minor complications (1.17%; urinary retention). Notably, metastases were demonstrated in 2 (3.70%) distal oesophageal tumours not involving the GOJ endoscopically. An impassable tumour, undifferentiated grade, CT possible T4b disease, and lower SUVmax predicted unsuspected peritoneal metastases (supplementary table 6.6). No factors could identify patients below the Pt (0.38%).

6.3.5 Refinement of existing algorithm

These findings suggest that EUS should be reserved for patients with possible T1 or T4b disease on CT. Retrospectively, this would have been 96.1% sensitive and 87.8% specific for T1N0, and 100% sensitive and 93.6% specific for T4b disease at EUS, with very high NPV (99.7% and 100% respectively; table 6.4; figure 6.1). Compared with existing practice there was no significant change in sensitivity, but specificity improved ($p<2.2x10^{-16}$; paired McNemar's test) and net benefits increased. This would be accompanied by a 71.8% reduction in examinations, procedural risk and expenditure. DCA demonstrated minimal

change in net algorithm benefits with extreme variations in perforation risk; i.e. although perforation risk was based on just one event, even dramatic fluctuations up to 1% had little effect on P_t (figure 6.2).

Figure 6.1: CT-guided algorithm (and DTA 1) for performing EUS for T1N0 disease before PET-CT

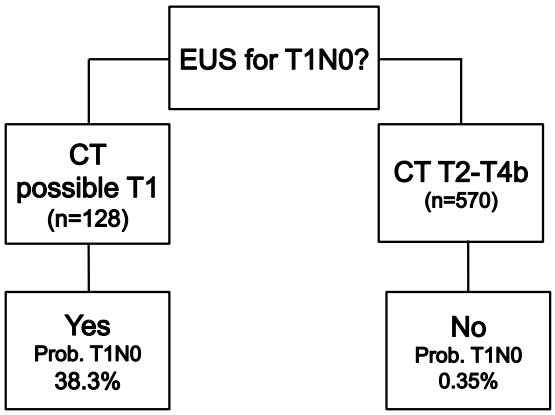
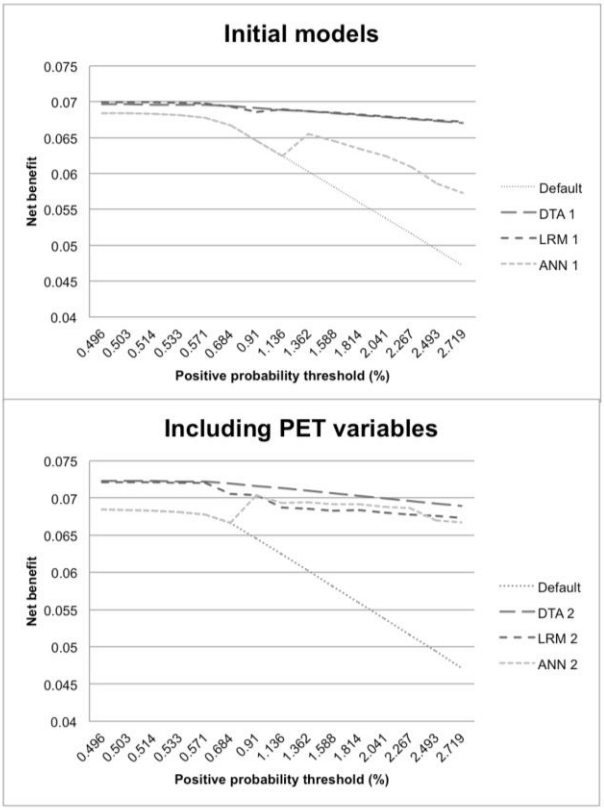


Figure 6.2: Decision curve analysis for EUS staging algorithms



The zero incidence of T1N0 disease in impassable tumours also suggests EUS could be omitted in these patients. However, this would have only applied to 2 of 128 patients with possible T1 disease on CT. Similarly, the zero incidence in patients with FDG-avid nodes applied only to 17 of 113 patients, for whom confirmatory assessment by EUS +/- FNA may be warranted.

This CT-guided algorithm was validated internally (using bootstrapping) and independently. Internal validation demonstrated minimal over-fitting and sampling bias: adjusted T1N0 sensitivity was 94.1% and 88.1%; T4b: 96.2% and 92.6% (table 6.4). Independent validation demonstrated 100.0% sensitivity and 85.5% specificity for T1N0 (n=11 EUS T1N0).

91 validation patients underwent PET-CT; there was zero incidence of T1N0 disease in the 60 with avid nodes. 12 had possible T4b disease on CT; 7 underwent EUS refuting T4b; in 5 EUS was omitted (table 6.1). EUS was performed in 8 of 17 patients with impassable tumours; 2 were down-staged from possible T4b disease.

6.3.6 Predictive modelling

Modelling was performed to corroborate the CT-guided pragmatic EUS algorithm, and determine whether unidentified interactions could refine this (identifying the remaining 6.4% patients with EUS T1N0 disease), or guide novel PET-CT and laparoscopy algorithms.

The optimal model for identifying T1N0 disease by EUS before PET-CT was a decision tree (DTA 1): this reserved EUS for those with possible T1 disease on CT, and was identical to the pragmatic CT-guided algorithm (figure 6.1; table 6.4; supplementary table 6.7). After PET-CT, the optimal model was a modified decision tree (DTA 2): this reserved EUS for patients with possible T1 disease on CT without FDG-avid nodes, or CT T2-4a disease with SUVmax<6.38 and length <3.4cm on PET-CT (figure 6.3). This was 100% sensitive and 84.0% specific with minimal over-fitting on internal validation (table 6.4). This could not be independently validated, as no validation patients with CT T2-T4a disease had EUS T1N0 disease. DCA demonstrated variations in EUS perforation rate to have minimal effects on model net benefits (figure 6.2).

Again, the optimal model for identifying T4b disease by EUS was a decision tree identical to the proposed algorithm: this reserved EUS for patients with possible T4b disease on CT (100% sensitivity; table 6.4). ANN could be generated. No models had clinical utility in

predicting pT1N0 disease (supplementary table 6.7, and unsuspected metastases on PET-CT and at laparoscopy.

Figure 6.3: Decision tree analysis (DTA 2) for performing EUS for T1N0 disease, after PET-CT

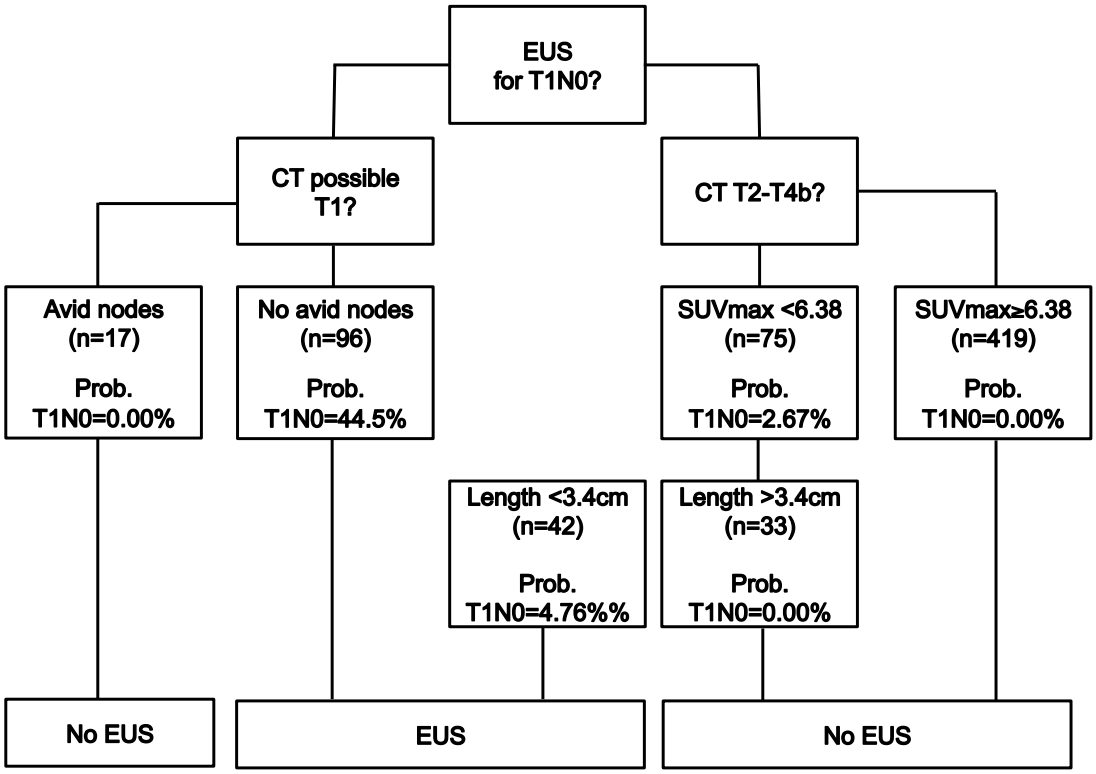


Table 6.4: Apparent, internally validated and temporally validated performance metrics of existing and novel algorithms, plus DTA, LRM and ANN in predicting EUS T1N0, T4b and pT1N0 disease

Algorithm / model	Sens	Spec	PPV	NPV	Brier	Kappa	O:E	AUC	Net benefit	Total cost (GBP)
EUS T1N0 (n=51 development; n=11 temporal validation)										
Default	1.000	0.00	0.738	0.000	NA	0.000	0.731	0.500	0.050	£380,410
Novel (apparent)	0.961	0.878	0.383	0.997	0.046	0.495	0.398	0.919	0.071	£84,412
Novel (internal)	0.941	0.881	0.384	0.995	0.137	0.493	0.349	0.928	0.067	£91738
Novel (independent)	1.000	0.855	0.478	1.000	0.048	0.581	0.478	0.928	0.107	NA
Models before PET-CT										
DTA 1 (apparent)	0.961	0.878	0.383	0.997	0.046	0.495	0.398	0.919	0.071	£84,412
DTA 1 (internal)	0.941	0.881	0.384	0.995	0.137	0.493	0.349	0.928	0.070	£91738
DTA 1 (independent)	1.000	0.855	0.478	1.000	0.048	0.581	0.478	0.928	0.107	NA
LRM1 (apparent)	0.980	0.535	0.157	0.997	0.0549	0.152	0.160	0.758	0.068	£230,637
LRM 1 (internal)	0.980	0.535	0.157	0.997	0.0549	0.152	0.160	0.758	0.068	£230,637
LRM 1 (independent)	1.000	0.723	0.324	1.000	0.052	0.379	0.324	0.861	0.107	NA
ANN 1 (apparent)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ANN 1 (internal)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ANN 1 (independent)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Models after PET-CT										
DTA 2 (apparent)	1.000	0.840	0.331	1.000	0.031	0.436	0.331	0.920	0.072	£113,360
DTA 2 (internal)	0.944	0.866	0.225	0.996	0.038	0.401	0.298	0.907	0.051	£128,012
LRM 2 (apparent)	0.947	0.734	0.310	0.991	0.049	0.359	0.301	0.953	0.100* (0.069)	£128,012
LRM 2 (internal)	0.947	0.734	0.310	0.991	0.052	0.0359	0.301	0.946	0.100* (0.069)	£128,012
ANN 2 (apparent)	0.974	0.835	0.261	0.998	0.022	0.354	0.261	0.904	0.069	£120,686
ANN 2 (internal)	0.974	0.835	0.261	0.998	0.018	0.354	0.261	0.904	0.068	£120,686
EUS T4b (n=26 development; n=0 temporal validation)										
Default	1.000	0.000	0.038	0.000	NA	NA	NA	0.500	0.009	£380,410
Novel (apparent)	1.000	0.936	0.377	1.000	0.038	0.522	0.377	0.968	0.035	£37,605
Novel (internal)	0.962	0.926	0.333	0.998	0.029	0.466	0.333	0.944	0.034	£65,437
DTA (apparent)	1.000	0.936	0.377	1.000	0.038	0.522	0.377	0.968	0.035	£37,605
DTA (internal)	0.962	0.926	0.333	0.998	0.029	0.466	0.333	0.944	0.034	£65,437
ANN (apparent)	1.000	0.700	0.114	1.000	0.019	0.147	0.114	0.849	0.028	£37,605
ANN (internal)	1.000	0.702	0.115	1.000	0.0851	0.147	0.115	0.851	0.028	£37,605
pT1N0										
Default	1.000	0.000	0.564	0.000	NA	NA	NA	0.500	0.466	NA
DTA (apparent)	1.000	0.348	0.580	1.000	0.200	0.333	0.580	0.790	0.470	NA
DTA (internal	0.667	0.708	0.741	0.630	0.421	0.370	0.889	0.685	0.308	NA
LRM (apparent)	1.000	0.111	0.590	1.000	0.102	0.139	0.583	0.556	0.440	NA
LRM (internal)	1.000	0.111	0.590	1.000	0.102	0.139	0.583	0.556	0.440	NA
ANN (apparent)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ANN (internal)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*LRM net benefit derived on smaller subset, adjusted benefit for whole cohort in parentheses; Sens=sensitivity; Spec=specificity; PPV=positive predictive value; NPV=negative predictive value; internal=internal validation; independent=independent validation

6.4 DISCUSSION

6.4.1 Summary of findings

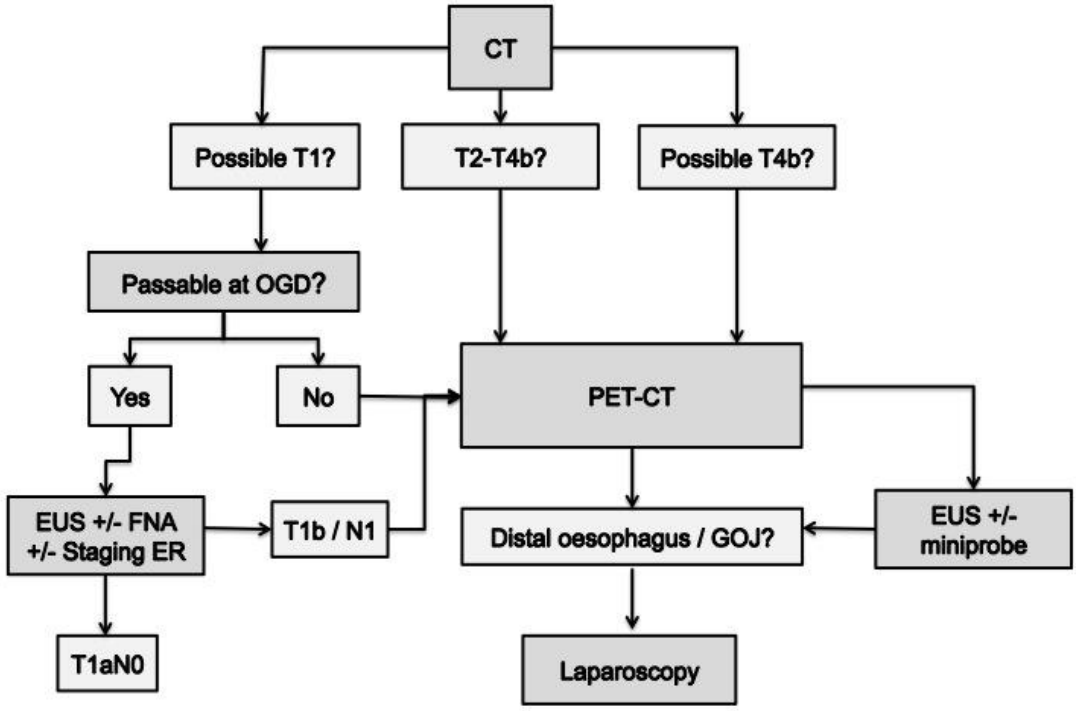
This is the first study of which the author is aware to quantify the staging utilities of EUS, PET-CT and laparoscopy for oesophageal/GOJ cancer, within the context of risk, benefit and probability thresholds. A number of factors predicted EUS T1N0 and T4b disease, identifying patients above and below these thresholds. Whilst EUS undoubtedly provides additional precision and information as regards T and N stage over CT and PET-CT, this had no added utility in the majority of patients: those without possible T1 or T4b disease on CT. For these patients, EUS risk (whilst small) exceeded potential benefit (as defined by its primary utility). This pragmatic algorithm was internally and independently validated. Furthermore, there was evidence that PET-CT could further sub-select patients on the basis of SUVmax, length and FDG-avid nodes.

The primary findings regarding utilities of EUS, PET-CT and laparoscopy, and CT T group and impassable tumours are likely to be robust, due to the size of the cohorts involved. Whilst the lower resolution of CT confers lower sensitivity and specificity for T and N stage than EUS (Lowe et al., 2005, van Vliet et al., 2008), it typically demonstrates early Tx/possible T1, possible T4b, or locally advanced T2-T4a disease; this appears sufficient to guide use of EUS in our centre. The secondary findings, regarding sub-selection using SUVmax, length and FDG-avid nodes are based on a cohort biased towards >EUS T1N0 disease, and confounded by the use of two PET-CT scanners (although internal validation demonstrated little over-fitting and analyses were adjusted accordingly).

6.4.2 Suggested staging algorithm

Based on these findings, the following algorithm after initial CT is suggested when considering patients for resection (figure 6.4): EUS (+/-FNA+/-staging ER) should be reserved for patients with either: Tx/possible T1 disease on CT passable at EGD; or patients with possible T4b disease without metastases on PET-CT. For all other patients EUS can be omitted, thereby reducing risk, delay and expenditure.

Figure 6.4: A suggested pragmatic algorithm for staging oesophageal cancer



6.4.3 Generalizability and other implications

However, as the relative merits and results of investigations vary with local resources, imaging and software platforms, external evaluation of these algorithms is required to assess generalizability to other centres. In addition, these recommendations are based on pragmatic definitions of the primary utilities of EUS: the identification of patients for endoscopic resection and neoadjuvant therapy (using a threshold of T1N0 disease). The benefit of the latter in patients with T2N0 disease has not been established unequivocally (as reflected by variances in study protocols), and so whilst OUH (in common with many others centres) uses a threshold stage of T1N1/T2N0 disease to guide therapy, these findings will require further exploration in centres using different thresholds. In selected patients, EUS may also have secondary utilities, such as providing additional prognostic or anatomical information (for example, distinguishing between T2N0 and T3N3 disease). Due to the limitations of predicting outcome on the basis of EUS T and N stage before potentially downstaging neoadjuvant therapy, such information is likely to alter management infrequently. However, in such instances EUS may have a role to play in patients with T2-T4a disease on CT. As discussed, the secondary findings of this study also require validation, but suggest considering EUS in patients CT T2-T4a disease on CT, with SUVmax<6.38, length <3.4cm and no avid nodes.

Whilst variables were associated with metastases on PET-CT and at laparoscopy, comparable groups could not be identified. No benefit was seen for PET-CT in the few tumours staged as T1N0, but due to the significant EUS false positive rate (8.90%) the possibility of metastases in this group exists, reinforcing the need for staging ER (Allum et al., 2011). Indeed, PET variables could predict false positive T1N0s. Notably, there was benefit of laparoscopy for T2 and distal oesophageal tumours, which whilst conflicting with American and European guidelines (Varghese et al., 2013, Stahl et al., 2010, National Comprehensive Cancer Guidelines, 2014) supports UK practice.

This study also corroborated prior findings that AC and SCC differ in their FDG avidity (Gillies et al., 2011a), and identified novel associations with FDG-avid nodes, SUVmax, length. The role of mini-probe EUS in impassable tumours is unclear; whilst there was benefit in patients with possible T4b disease on CT, there was none for those without, although the 95% confidence interval for the latter (0.00-3.70%) exceeds the Pt. Whilst this is a single centre retrospective study, data were recorded prospectively. Although CT was performed in multiple centres, this was not a significant confounder and was adjusted for. The advantages and disadvantages of DTA, LRM and ANN were mitigated by comparing all three.

6.4.4 Conclusions

In conclusion, on the basis of pragmatic primary staging utilities this chapter found the risk of EUS to typically exceed its benefit in patients with either T2-T4a disease on CT, impassable tumours at OGD or FDG-avid nodes on PET-CT. There may be further roles for decision tree and logistic regression models in guiding further selection. Laparoscopy was beneficial in patients with distal oesophageal tumours and T2 disease, supportive of current UK practice but in contrast to USA and European guidelines.

CHAPTER 7

RESTAGING OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY WITH POSITRON EMISSION TOMOGRAPHY:

NEW CLASSIFICATIONS OF METABOLIC NODAL STAGE AND RESPONSE, AND THEIR UTILITY IN PREDICTING PROGRESSION TO INCURABLE DISEASE

7. CHAPTER 7: RESTAGING OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY WITH POSITRON EMISSION TOMOGRAPHY

7.1 SYNOPSIS

7.1.1 Introduction

In contrast to staging, it is unknown whether restaging PET-CT is more useful than contrast-enhanced CT in detecting interval progression after neoadjuvant therapy. There are also no biomarkers/models to identify high-risk patients. This chapter aimed firstly to compare the performance of CT and PET-CT in detecting interval progression. Secondly, it aimed to use decision theory to calculate the Pt for restaging PET-CT, and determine whether clinical/pathological/imaging factors (including generation of the first classification of mN stage and response) could identify patients with a probability of interval progression $<P_t$ (i.e. those in whom re-staging PET-CT was not required). Thirdly, it aimed to generate predictive models for use in stratifying/predicting risk of progression to incurable disease (metastases on restaging examination, and the finding of unsuspected unresectable disease at surgery)

7.1.2 Methods

All patients with oesophageal cancer staged by PET-CT, and re-staged with either CT or PET-CT after NAC within OUH were identified from the cohort of patients analysed in Chapter 6 (from May 2006-November 2014). Pts were calculated. LR, DRA and ANN models were generated and validated. A new classification of mN stage and response (mNR) were generated.

7.1.3 Results

383 patients were restaged (103 CT, 280 PET-CT). Incurable disease was detected by CT in 3 (2.91%) and PET-CT in 17 (6.07%). Despite restaging unsuspected incurable disease was encountered at surgery in 34/336 patients (10.1%). PET-CT was more sensitive than CT ($p=4.58x10^{-3}$; McNemar's test). A new classification of FDG-avid nodal stage (mN) before

NAC (plus tumour FDG-avid length) predicted subsequent progression, independent of EUS nodal stage. The presence of FDG-avid nodes after NAC and an impassable tumour stratified risk of incurable disease at surgery into high (75.0%; both risk factors), medium (22.4%; either) and low risk (3.87%; neither) groups ($p=7.48x10^{-9}$, Fisher's exact test). Decision theory supported restaging PET-CT.

7.1.4 Conclusions

PET-CT is more sensitive than CT for detecting interval progression, however, is insufficient in at least higher risk (and probably all) patients. Two novel parameters were generated (mN stage and mNR), which in combination with primary tumour characteristics (primarily impassability) can stratify this risk.

7.2 INTRODUCTION

The majority (85%) of patients with oesophageal cancer receive neoadjuvant therapy (NAC or NACR) (Royal College of Surgeons Clinical Effectiveness Unit, 2012, Pennathur et al., 2013). However, 50-60% of tumours do not demonstrate a significant pTR (Urschel et al., 2002, Campbell and Villaflor, 2010), and without markers to predict this some patients inevitably progress to metastatic disease during therapy. This mandates restaging before surgery, but despite this unresectable disease is often encountered, with consequent psychological and physiological trauma from an ultimately futile trial resection.

However, no restaging guidelines exist; whilst intuitively PET-CT might be expected to be superior to CT, this has not been demonstrated. Baseline PET variables (primarily SUVmax and avid length) are associated with metastatic disease at presentation, and dynamic variables with pathological response, prognosis, recurrence and nodal/distant metastases following treatment (Gillies et al., 2012, Moon et al., 2014, Kwee, 2010). However, no studies have assessed whether factors can predict progression during therapy, unsuspected unresectable disease or early recurrence. One candidate is FDG-avid nodal burden; in 121 unselected patients in OUH their presence before NAC was associated with worse prognosis. However, neither a classification for avid nodal stage or response has been described.

This chapter aimed firstly to compare the performance of CT and PET-CT in detecting interval progression. Secondly, it aimed to use decision theory to calculate the Pt for

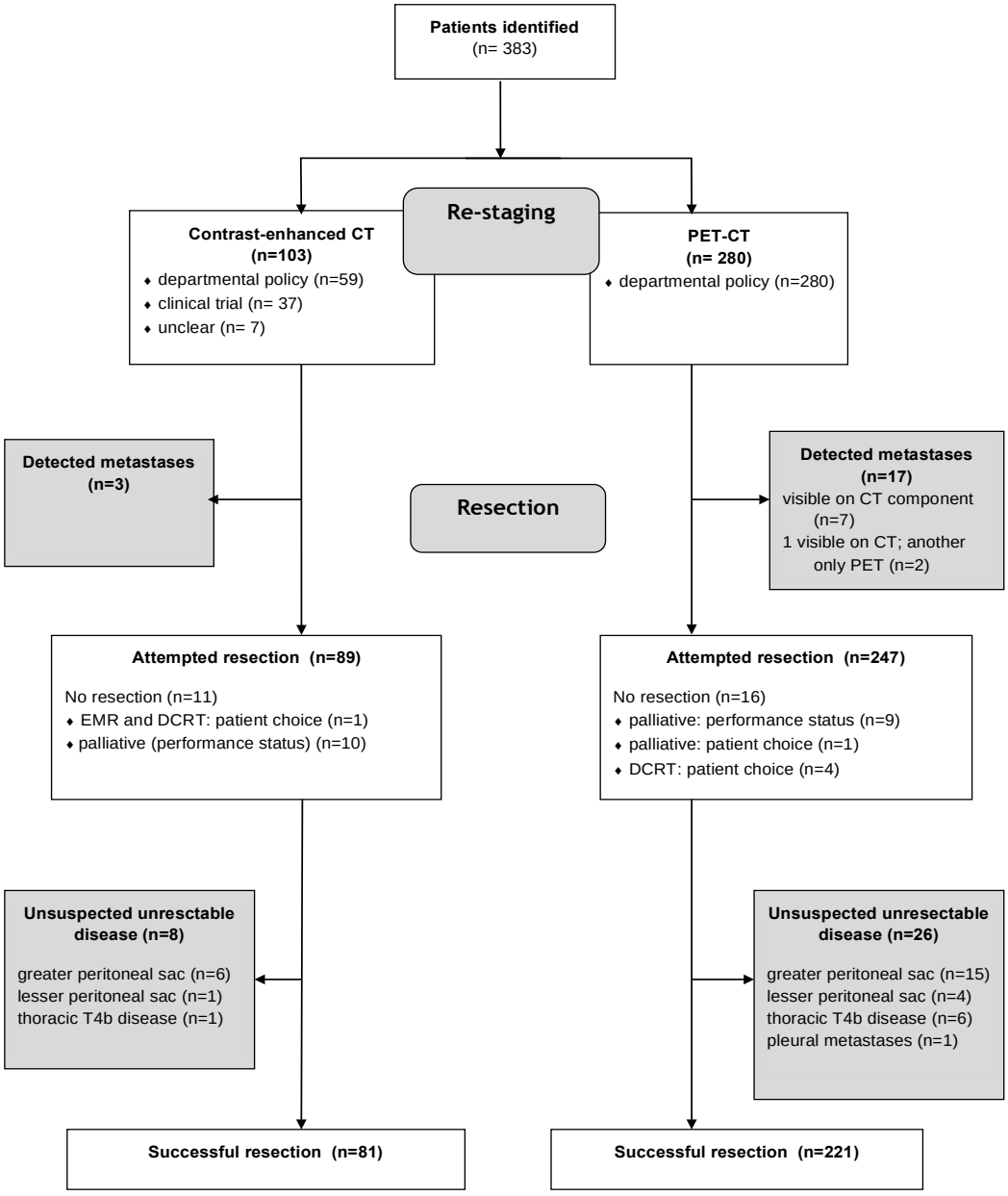
restaging PET-CT, and determine whether clinical/pathological/imaging factors (including generation of the first classification of mN stage and response) could identify patients with a probability of interval progression <Pt (i.e. those in whom re-staging PET-CT was not required). Thirdly, it aimed to generate predictive models for progression to incurable disease (metastases on restaging examination, and the finding of unsuspected unresectable disease at surgery)

7.3 RESULTS

7.3.1 Patients

383 patients were re-staged with PET-CT (n=280; 73.1%) or CT (n=103; 36.9%; figure 7.1). There were nominally significant differences as regards patient age and tumour site but after correction for multiple comparisons only that of initial PET-CT scanner remained (table 7.1).

Figure 7.1: Study structure



7.3.2 Interval progression to incurable disease

7.3.2.1. Progression to incurable disease on restaging examination

Overall, metastases were identified in 20 (5.22%) patients: nodal (n=9), liver (n=5), liver and nodal (n=1), liver and bone (n=1), bone (n=2), lung (n=1), or disseminated (n=1). Indirect comparison demonstrated detection by CT in 3 cases (2.91% examinations) and PET-CT in 17 (6.07%; p=0.303 Fisher's exact test). Twelve (3.13%) tumours were initially non-avid;

none demonstrated metastatic progression. Before considering false negatives (unsuspected incurable disease found at surgery), this suggested PET-CT to be more sensitive than CT: relative sensitivities 100-v-47.1%).

All 17 PET-CT examinations were reviewed for direct comparison between PET and CT components. In all cases metastases were visible on the PET component. In 7 (41.2%) metastases were also visible on the CT component; in 2 (11.8%) one metastasis was visible on CT, but the other only with PET; in 8 (47.1%) metastases were only visible with PET (p=0.006, Fisher's exact test).

7.3.2.2. Progression to unsuspected incurable disease at surgery

336 patients underwent surgery after re-staging scan (n=247 PET-CT, n=89 CT). In 34 (10.1%) unsuspected unresectable disease was found (n=26 PET-CT, n=8 CT): n=21 greater sac (61.8%; peritoneal metastases [+/-liver] n=15, liver metastases n=6), n=5 lesser sac (14.7%; peritoneal metastases+/-T4b disease n=3, T4b disease n=2), n=7 thoracic T4b disease (20.6%), n=1 pleural metastases (2.94%). Twenty- seven patients without metastases did not undergo surgery (n=16 PET-CT; n=11 CT). However, as these patients typically received additional palliative therapies, were not routinely re-imaged, and the temporal significance of subsequent additional metastases would be unclear, they were not considered reliable indicators of additional false negatives.

Excluding these 27 patients, PET-CT identified 17 true positives, 26 false negatives, and 221 true negatives. Resultant sensitivity was 39.5% (95% CI 25.0-55.6), specificity 100.0% (97.8-100.0), and negative predictive value 89.4% (84.8-92.9%). CT identified 3 true positives, 8 false negatives and 81 true negatives. Resultant sensitivity was 27.3% (7.33-60.7), specificity 100.0% (94.4-100.0) and negative predictive value 91.0 (82.6 -95.8%). In a subsidiary analysis considering the PET and CT components separately for patients restaged by PET-CT, PET identified 17 true positives, 26 false negatives, and 221 true negatives, and CT 9, 34 and 221 respectively. PET-CT was significantly more sensitive than CT alone (39.5% versus 20.9%; ($p=4.68x10^{-3}$; McNemar's test for paired sensitivities).

All patients with peritoneal disease had undergone staging laparoscopy. Two patients with liver metastases had tiny equivocal lesions on re-staging PET-CT. For the first, this was reported as benign by MRI and percutaneous biopsy; the metastasis at surgery was not retrospectively apparent. For the second, the lesion was not amenable to biopsy; MRI was

reported as benign. Both patients were re-staged using the first PET-CT scanner, so were therefore excluded from model development anyway. Of the 7 patients with thoracic T4b disease all had undergone EUS, demonstrating T2 (n=1) and T3-T4a disease (n=6) prior to NAC, although for 2 impassable tumours mini-probe was not available.

7.3.2.3. Decision theory

Pt for PET-CT was calculated at 0.084%; as this is considerably less than the probability of progression, routine re-staging with PET-CT is justified clinically. The number of PET-CT examinations (rather than CT) required to identify one additional patient with progression and prevent inappropriate oesophagectomy was 23.3, at a net cost of £6,631.5 / US$10,669.90, and net 0.24% reduction in lifetime cancer risk.

7.3.3 Predictive factors for interval progression

7.3.3.1. Pre-chemotherapy factors predicting progression to metastases

Multivariate analysis (table 7.2; supplementary table 7.1) demonstrated 2 pre-NAC PET-CT characteristics to predict progression: FDG-avid length (OR 1.45 [95%CI 1.09-1.92]; p=0.010) and the presence/number of FDG-avid nodes.

7.3.3.2. Development of metabolic nodal stage

There was prohibitive multicollinearity between nodal number and compartments; mN stage was therefore classified as mN0 (0 nodes), mN1 (1-2 nodes) and mN2 (>2 nodes). mN before NAC predicted progression: mN1: OR=17.94 (2.62-122.97), p=3.29x10-3; mN2: OR 33.85 (4.58-250.43), p=5.64x10-4.

Interestingly, this association was independent of traditional N staging by EUS: overall, 92 patients were staged as N0/mN0, 150 N1/mN0, 105 N1/mN1, and 17 as N0/mN1. Progression occurred in 1 (1.10%), 1 (0.67%), 14 (11.8%) and 4 (19.0%) respectively (p=2.57x10-6, Fisher's exact test), suggesting risk in fact to be minimal for patients with non-FDG-avid N1 disease.

There were no associations between additional PET variables and progression (including MTV, TGV and nodal SUVmax, although potentially biased by the limited number of events; (n=8) Supplementary table 7.1).

Table 7.2 – Pre-NAC variables and subsequent progression to metastases

n=354 patients	Univariate OR (95% CI)	p value	Multivariate OR (95% CI)	p value
Age	1.00 (1.00-1.00)	0.972	1.00 (1.00-1.00)	0.010
Sex				
Female	Ref	Ref	Ref	Ref
Male	0.50 (0.20-1.27)	0.144	0.39 (0.10-1.53)	0.178
Cell type*				
AC	Ref	Ref	Ref	Ref
SCC	1.20 (0.39-3.72)	0.751	0.62 (0.10-4.05)	0.621
Pre-treatment Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.33 (0.15-11.4)	0.796	1.41 (0.10-20.37)	0.802
Poor	2.44 (0.31-19.5)	0.398	2.39 (0.17-32.97)	0.517
Undifferentiated	5.50 (0.30-100)	0.250	169.02 (0.27-105x10^3)	0.118
Pre-treatment T stage				
1	NA	NA	NA	NA
2	Ref	Ref	Ref	Ref
3	1.37 (0.30-6.20)	0.683	0.73 (0.11-4.82)	0.747
4a	4.32 (0.74-25.2)	0.104	1.76 (0.15-21.17)	0.656
Pre-treatment N stage				
0	Ref	Ref	Ref	Ref
1	1.28 (0.45-3.60)	0.644	0.63 (0.13-3.06)	0.569
Impassable				
No	Ref	Ref	Ref	Ref
Yes	0.81 (0.18-3.60)	0.778	0.43 (0.06-3.24)	0.421
Site				
Mid	Ref	Ref	Ref	Ref
Distal	0.98 (0.24-3.99)	0.978	2.65 (0.26-28.87)	0.410
Siewert 1	0.40 (0.08-1.92)	0.252	0.50 (0.04-6.40)	0.596
Siewert 2	0.22 (0.04-1.13)	0.070	0.24 (0.02-3.28)	0.284
Siewert 3	0.30 (0.05-1.89)	0.199	0.20 (8.61x10^{-3}-4.49)	0.308
Restaging scan				
CT	Ref	Ref	Ref	Ref
PET-CT	2.20 (0.63-7.67)	0.251	5.54 (0.76-40.19)	0.091
Days to scan (log)	5.47 (0.07-422)	0.444	2.11 (2.53x10^{-3}-1765)	0.828
Initial SUVmax (log)	1.36 (1.16-1.60)	0.114	0.33 (0.01-8.98)	0.511
Initial PET length (cm)	3.27 (0.75-14.4)	2.06x10^{-4}	1.45 (1.09-1.92)	0.010
Initial PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	0.61 (0.24-1.52)	0.289	0.13 (0.03-0.61)	0.010
mN stage				
mN0 (0 avid nodes)	Ref	Ref	Ref	Ref
mN1 (1-2)	11.6 (2.29-58.9)	3.10x10^{-3}	17.94 (2.62-122.97)	3.29x10^{-3}
mN2 (>2)	26.7 (5.81-123)	2.46x10^{-5}	33.85 (4.58-250-43)	5.64x10^{-4}
NAC regimen				
Dual	Ref	Ref	Ref	Ref
Triple	2.24 (0.90-5.59)	0.084	3.50 (0.75-16.29)	0.110

*Other cell types excluded as perfect separators

7.3.3.3. Pre-chemotherapy factors predicting progression to unresectable disease

Three pre-NAC variables predicted unresectable disease: an impassable tumour (OR 57.00 [14.65-221.78]; p=5.50x10^{-9}), FDG-avid length (OR 1.55 [1.21-1.98]; p=4.71x10^{-4}) and SUVmax (logSUVmax OR 0.04 [2.48x10^{-3}-0.49]; p=0.012]; table 7.3; supplementary table 7.2).

7.3.3.4. Post- chemotherapy factors predicting progression to unsuspected incurable disease at surgery

On multivariate analysis, 3 post-NAC variables predicted incurable disease (table 7.4; Supplementary Table 7.3): FDG-avid length (OR 2.07 [1.41-3.05], p=2.10x10^{-4}), logSUVmax (OR 4.68x10-4 [3.40x10^{-6}-0.06] p=2.29x10-3), and mN stage (again independent of N stage: mN1 OR 12.52 [0.91-172.00], p=0.059; mN2, OR 549.50 [22.43-13463.26], p=1.11x10-4), in addition to an impassable tumour. These associations remained significant for patients staged with either scanner. There were no associations with either nodal SUVmax or additional primary tumour PET variables.

Table 7.3. Pre-NAC factors associated with progression to unsuspected unresectable disease

n=306 patients	Univariate OR (95% CI)	p value	Multivariate OR (95% CI)	p
Age	1.00 (1.00-1.00)	0.417	1.00 (1.00-1.00)	0.926
Sex				
Female	Ref	Ref	Ref	Ref
Male	0.59 (0.28-1.25)	0.168	0.38 (0.11-1.23)	0.104
Cell type*				
AC	Ref	Ref	Ref	Ref
SCC	1.21 (0.47-3.09)	0.693	0.72 (0.10-5.32)	0.751
Pre-treatment grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.65 (0.36-7.65)	0.520	7.36 (0.50-108.53)	0.146
Poor	1.74 (0.38-7.95)	0.477	8.31 (0.54-126.64)	0.128
Undifferentiated	NA	NA	NA	NA
Pre-treatment T stage				
1	NA	NA	Ref	Ref
2	0.61 (0.06-6.26)	0.676	0.36 (6.25×10^{-3}-20.50)	0.699
3	0.757 (0.09-6.41)	0.798	0.50 (0.01-23.48)	0.724
4a	2.06 (0.20-21.0)	0.542	2.65 (0.04-159.52)	0.641
Pre-treatment N Stage				
0	Ref	Ref	Ref	Ref
1	1.18 (0.53-2.63)	0.688	1.89 (0.51-6.95)	0.340
Passable	Ref	Ref	Ref	Ref
Impassable	17.2 (7.62-38.9)	7.69×10^{-12}	57.00 (14.65-221.78)	5.50×10^{-9}
Site				
Proximal	Ref	Ref	Ref	Ref
Mid	0.80 (0.06-9.92)	0.862	1.04 (0.0.03-40.25)	0.751
Distal	0.46 (0.04-5.00)	0.525	0.44 (0.01-18.41)	0.983
Siewert 1	0.34 (0.03-3.70)	0.378	0.24 (0.01-10.90)	0.663
Siewert 2	0.24 (0.02-2.55)	0.236	0.08 (1.54×10^{-3}-3.60)	0.462
Siewert 3	0.024 (0.02-2.87)	0.260	0.05 (9.36×10^{-5}-2.95)	0.189
Multifocal	3.00 (0.08-107)	0.547	1.21 (3.22×10^{-3}-454.96)	0.152
Restaging scan				
CT	Ref	Ref	Ref	Ref
PET-CT	1.01 (0.45-2.26)	0.976	6.61 (1.30-33.65)	0.023
Days to scan (log)	13.7 (0.36-515)	0.158	0.73 (1.56×10^{-3}-342.50)	0.920
Days to surgery (log)	0.55 (0.13-2.23)	0.398	0.76 (0.08-7.03)	0.0.806
Initial SUVmax (log)	0.92 (0.26-3.26)	0.891	0.04 (2.48×10^{-3}-0.49)	0.012
Initial PET length (cm)	1.21 (1.05-1.39)	9.83×10^{-3}	1.55 (1.21-1.98)	4.71×10^{-4}
Initial PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	0.54 (0.26-1.12)	0.097	0.09 (0.02-0.44)	2.65×10^{-3}
mN stage				
0	Ref	Ref	Ref	Ref
1	1.51 (0.60-3.81)	0.382	1.87 (0.41-8.52)	0.421
2	2.66 (1.11-6.36)	0.028	2.86 (0.52-15.60)	0.225
Dual agent NAC	Ref	Ref	Ref	Ref
Triple agent NAC	1.39 (0.63-3.06)	0.416	6.12 (1.35-27.59)	0.018

*Other cell types excluded as perfect separators

Table 7.4 – Post-chemotherapy variables associated with incurable disease at surgery, adjusted for relevant baseline variables

n=226 patients	Univariate OR (95% CI)	p value	Multivariate OR (95% CI)	p
Age	1.00 (1.00-1.00)	0.434	1.00 (1.00-1.00)	0.894
Sex				
Female	Ref	Ref	Ref	Ref
Male	0.49 (0.21-1.15)	0.101	0.32 (0.07-1.54)	0.154
Cell type*				
AC	Ref	Ref	Ref	Ref
SCC	1.07 (0.34-3.31)	0.911	0.09 (4.80×10^{-3}-1.84)	0.119
Pre-treatment Grade				
Well	NA	NA	NA	N
Moderate	Ref	Ref	Ref	Ref
Poor	1.20 (0.53-2.75)	0.661	5.10 (0.84-30.91)	0.076
Undifferentiated	NA	NA	NA	NA
Pre-treatment T stage				
1	Ref	Ref	Ref	Ref
2	0.53 (0.05-5.69)	0.597	2.78 (3.04×10^{-4}-254×10^{3})	0.826
3	0.60 (0.07-5.39)	0.645	2.81 (3.71×10^{-4}-225×10^{3})	0.816
4a	1.25 (0.10-15.1)	0.861	5.25 (6.21×10^{-4}-444×10^{3})	0.719
Pre-treatment N Stage				
0	Ref	Ref	Ref	Ref
1	1.21 (0.46-3.18)	0.693	5.91 (0.76-46.02)	0.090
Passable	Ref	Ref	Ref	Ref
Impassable	16.0 (6.23-41.1)	8.23×10^{-9}	118.75 (13.40-1052)	1.78×10^{-5}
Site				
Proximal	Ref	Ref	NA	Ref
Mid	0.40 (0.02-6.85)	0.862	0.05 (5.56×10^{-4}-4.84)	0.201
Distal	0.44 (0.04-5.52)	0.525	0.06 (4.41×10^{-4}-7.30)	0.467
Siewert 1	0.22 (0.02-2.78)	0.378	0.01 (6.56×10^{-5}-1.50)	0.072
Siewert 2	0.16 (0.01-2.03)	0.236	3×10^{-3} (1.30×10^{-5}-0.63)	0.034
Siewert 3	0.21 (0.01-3.12)	0.260	8×10^{-3} (3.25×10^{-5}-1.76)	0.079
Multifocal	NA	NA	NA	NA
Days to scan (log)	8.04 (0.12-555)	0.335	4.08 (1.69×10^{-3}-9839	0.723
Days to surgery (log)	0.74 (0.13-4.14)	0.733	2.60 (0.14-46.67)	0.517
SUVmax (log)	1.68 (0.38-7.43)	0.493	4.68×10^{-4} (3.40×10^{-6}-0.06)	2.29×10^{-3}
PET length (cm)	1.15 (0.99-1.34)	0.075	2.07 (1.41-3.05)	2.10×10^{-4}
Initial PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	0.47 (0.21-1.07)	0.073	0.18 (6.09×10^{-3}-5.56)	0.331
Restaging PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	0.38 (0.17-0.87)	0.023	0.11 (4.03×10^{-3}-3.25)	0.204
mN stage				
0	Ref	Ref	Ref	Ref
1	1.70 (0.35-8.17)	0.511	12.52 (0.91-172)	0.059
2	7.63 (2.84-20.5)	5.95×10^{-5}	549.50 (22.43-13463)	1.11×10^{-4}
NAC regimen				
Dual agent	Ref	Ref	Ref	Ref
Triple agent	1.78 (0.74-4.27)	0.196	9.57 (1.62-56.41)	0.013

*Other cell types excluded as perfect separators

7.3.3.5. Metabolic tumour response and unsuspected incurable disease at surgery

No classification of mTR (staged and re-staged using the same scanner; table 7.5; supplementary table 7.4) predicted unresectable disease. Notably, 5 patients (9.09%) with CMR had unresectable disease.

7.3.3.6. Generation of metabolic nodal response

In contrast to mTR, mNR did predict incurable disease at surgery. This was true for a lesser reduction in mN stage (supplementary table 7.5), in addition to nodal SUVmax (p=0.025) and nodal PERCIST response (p=0.022). The optimal ROC nodal SUVmax threshold for predicting unresectable disease was ≥31.7% reduction, but with no difference in predictive utility compared with the 30.0% PERCIST threshold.

mNR was therefore classified as: no avid nodal disease (no avid nodes), CMR, PMR (reduction in mN stage, or ≥30% reduction of nodal SUVmax), SMD or PMD (increase in stage, or ≥30% increase in nodal SUVmax). This strongly predicted the finding of incurable disease at surgery on multivariate analysis: nodal SMD, OR 17.26 (1.85-160.76; p=0.012); nodal PMD, OR 126.45 (8.19-1951.99; $p=5.28\times10^{-4}$; table 7.5). This remained significant for patients staged using the most recent PET-CT scanner, and was borderline for the first scanner (SMD p=0.089). Overall, of 43 patients with FDG-avid nodes (but no apparent metastases) despite NAC, incurable progression was encountered in 11 (25.6%), compared with 15/197 (7.60%) without ($p=1.80\times10^{-3}$; Fisher's exact test).

Table 7.5: Metabolic response to NAC and incurable disease at surgery

n=209 patients	Univariate OR (95% CI)	p value	Multivariate OR (95% CI)	p
Age	1.00 (1.00-1.00)	0.434	1.00 (1.00-1.00)	0.377
Sex				
Female	Ref	Ref	Ref	Ref
Male	0.49 (0.21-1.15)	0.101	0.46 (0.11-2.01)	0.304
Cell type*				
AC	Ref	Ref	Ref	Ref
SCC	1.07 (0.34-3.31)	0.911	0.26 (0.02-3.19)	0.292
Pre-treatment Grade				
Well	NA	NA	NA	NA
Moderate	Ref	Ref	Ref	Ref
Poor	1.20 (0.53-2.75)	0.661	1.65 (0.44-6.25)	0.460
Undifferentiated	NA	NA	NA	NA
Pre-treatment T stage				
1	Ref	Ref	Ref	Ref
2	0.53 (0.05-5.69)	0.597	0.46 (0.01-33.31)	0.720
3	0.60 (0.07-5.39)	0.645	0.74 (0.01-40.13)	0.882
4a	1.25 (0.10-15.1)	0.861	2.45 (0.03-171.97)	0.679
Pre-treatment N Stage				
0	Ref	Ref	Ref	Ref
1	1.21 (0.46-3.18)	0.693	2.91 (0.48-17.79)	0.247
Impassable				
No	Ref	Ref	Ref	Ref
Yes	16.0 (6.23-41.1)	8.23×10^{-9}	37.63 (7.73-183.24)	7.07×10^{-6}
Site				
Proximal	Ref	Ref	NA	Ref
Mid	0.40 (0.02-6.85)	0.862	0.40 (3.92×10^{-3}-40.57)	0.697
Distal	0.44 (0.04-5.52)	0.525	0.41 (4.33×10^{-3}-38.14)	0.698
Siewert 1	0.22 (0.02-2.78)	0.378	0.11 (1.10×10^{-3}-11.69)	0.357
Siewert 2	0.16 (0.01-2.03)	0.236	0.06 (5.62×10^{-4}-7.13)	0.252
Siewert 3	0.21 (0.01-3.12)	0.260	0.08 (6.39×10^{-4}-9.94)	0.304
Multifocal	NA	NA	NA	NA
Days to scan (log)	8.04 (0.12-555)	0.335	4.25 (0.01-1599.77)	0.633
Days to surgery (log)	17.6 (0.02-16230)	0.411	1.25 (0.09-17.48)	0.870
%SUVmax response	1.68 (0.38-7.43)	0.493	6.66 (0.66-66.93)	0.107
%Avid length response	1.15 (0.99-1.34)	0.075	0.26 (0.05-1.28)	0.098
mNR				
mN0	Ref	Ref	Ref	Ref
CMR	1.32 (0.43-4.06)	0.633	1.49 (0.30-7.52)	0.628
PMR	2.42 (0.46-12.60)	0.294	2.92 (0.22-39.31)	0.419
SND	5.19 (1.16-23.20)	0.031	17.27 (1.85-160.76)	0.012
PMD	9.08 (2.63-31.34)	4.87×10^{-4}	126.45 (8.19-1951.99)	5.28×10^{-4}
NAC regimen				
Dual agent	Ref	Ref	Ref	Ref
Triple agent	1.78 (0.74-4.27)	0.196	7.78 (1.74-34.88)	7.36×10^{-3}
PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	0.47 (0.21-1.07)	0.073	0.11 (0.03-0.45)	2.16×10^{-3}

*Other cell types excluded as perfect separators

Table 7.6: mNR and incurable disease at surgery

	Resectable (n=84)	Unresectable (n=16)	p (α=0.013)
mN stage response			
No avid nodal disease	138 (NA)	11 (NA)	0.009[a]
CMR	51(60.7%)	5 (31.3%)	
PMR	5 (5.95%)	1 (6.25%)	
SND	19 (22.6%)	8 (50.0%)	
PMD	9 (10.7%)	2 (12.5%)	
% Nodal SUVmax reduction	100%	24.8%	0.025 [b]
(median; IQR; range)	46.8-100; -33.4-100%	6.44-100; -77.7-100%	
Nodal PERCIST response			
CMR	51 (60.7%)	5 (31.3%)	0.020 [a]
PMR	13 (15/5%)	1 (6.25%)	
SND	15 (17.9%)	8 (50.0%)	
PMD	5 (5.95%)	2 (12.5%)	
Overall mNR			
No avid nodal disease	138 (NA)	10 (NA)	3.88×10^{-3} [a]
CMR	51 (60.7%)	5 (31.3%)	
PMR	13 (15.5%)	2 (12.5%)	
SND	10 (11.9%)	6 (37.5%)	
PMD	10 (11.9%)	3 (18.8%)	

7.3.4 Predictive modelling

7.3.4.1. Predicting progression to metastases on restaging examination before chemotherapy

Modelling was performed to determine whether sufficiently low-risk patients (probability of progression <0.084%) could be identified using these associations identified, in whom the risk of progression was so low that re-staging PET-CT was not justified. LR comprised FDG-avid length and mN stage (supplementary table 7.5). DTA partitioned using FDG-avid length: predicting avid tumours<3.15cm (n=27) had zero probability of progression. A useful ANN could not be generated. No models, however demonstrated utility using this P_t (table 7.7). However, using the optimal threshold (0.06, determined by ROC), LR predicted progression with 87.5% sensitivity (44.4% independently validated) and 72.6% specificity (87.7%).

Table 7.7: Apparent, internal and independently validated performances of models predicting progression to metastatic disease

Model	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	O:E	AUC	Net benefit
Default							
Default (PET-CT)	100.0% (62.9-100)	0.00% 0.00-2.44)	4.97% (2.18-9.56)	NA	0.050	0.500	$3.70x10^{-4}$
Logistic regression – using Pt							
Apparent performance	100.0 (69.2-100.0)	0.61 (0.00-3.36)	4.65% (2.04-8.96)	100.0% (16.7-100)	0.047	0.523	$3.68x10^{-4}$
Independent validation	100.0% (66.2-100)	0.00% (0.00-4.98)	11.0% (5.15-19.8)	NA	0.098	0.500	$3.70x10^{-4}$
Logistic regression – using p>0.06							
Apparent performance	87.5% (47.4-97.9)	72.6% (65.1-79.2)	13.5% (2.04-8.96)	99.2% (95.4-99.9)	0.154	0.800	NA
Independent validation	44.4% (14.0-78.6)	87.7% (77.9-94.2)	30.8% (9.28-61.4)	92.3% (84.8-97.6%)	0.629	0.661	NA
Internal validation	87.5% (44.7-97.9)	72.4% (64.9-79.1)	13.5% (5.61-25.9)	99.2% (95.4-99.9)	0.148	0.800	NA
Decision tree analysis – using Pt							
Apparent performance	100% (62.9-100)	12.1% (7.45-18.3)	5.48% (2.40-10.5)	100% (82.9-100)	0.055	0.578	0.041
Independent validation	100% (66.2-100)	16.4% (8.80-27.0)	12.9% (6.06-23.0)	100% (73.4-100)	0.129	0.564	0.115
Internal validation	0.00% (0.00-37.1)	97.6% (93.9-99.3)	0.00% (0.00-59.8)	95.2% (90.8-97.9)	2.000	0.476	0.000
Artificial neural network – using Pt							
Apparent performance	100% (62.9-100)	0.00% 0.00-2.44%)	4.97% (2.18-9.56)	NA	0.050	0.500	0.040
Independent validation	100% (0.00-6.07)	0.00% (0.00-5.04)	0.00% (0.00-5.04)	NA	0.181	0.500	0.040
Validated	100% (62.9-100)	0.00% (0.00-2.44)	4.97% (2.18-9.56)	NA	0.050	0.500	0.040

O:E=observed:expected ratio

7.3.4.2. Predicting unsuspected incurable disease at surgery after restaging PET-CT

An LR model comprised tumour impassability, and FDG-avid length, SUVmax and mN stage after NAC. This was highly discriminant (AUC 0.903, r^2=0.471). Using a threshold of 0.122, sensitivity was 92.3% and specificity 86.3% (61.5% and 93.1%, independent validation) with minimal over-fitting (table 7.8). DTA identified patients with either an impassable tumour or a passable tumour but mN stage≥1. This was 76.9% sensitive and 76.6% specific (75.0 %and 92.5%). The optimal ANN had no utility.

7.3.4.3. Predicting unresectable disease using metabolic tumour and nodal response

An LR model comprised tumour impassability and mNR (optimal threshold 0.055; table 7.8). This performed worse than LR using absolute re-staging values (r^2=0.314; AUC=0.814; 76.9% sensitive and 76.6% specific [69.2% and 83.1%] with minimal over-fitting). DTA identified patients with an impassable tumour, or without nodal CMR/no avid nodes: 76.9% sensitive, 76.6% specific (75.0% and 92.5%), effectively identical to the DTA above. A useful ANN could not be validated. Composite models of post-NAC and metabolic response variables had no additional benefit (data not shown due to reasons of space).

7.3.5 Simple risk stratification for incurable disease at surgery

Simple risk stratification was performed using mN stage after NAC/ mNR, and an impassable tumour. As the presence of avid nodes after NAC and/or an impassable tumour appeared the most reliable risk factors for incurable disease at surgery, three risk groups were derived: low (neither factor; n=181), medium (one; n=58) and high (both; n=8). The risk of incurable disease increased dramatically: low (7/181; 3.87%), medium (13/58; 22.4%) and high (6/8; 75.0%; $p=7.48\times10^{-9}$, Fisher's exact test)

Table 7.8: Model performance and validation in predicting unresectable disease

Model	Sensitivity	Specificity	PPV	NPV	O:E	AUC
Re-staging PET-CT						
LR (using 12.2% threshold)						
Apparent	92.3% (63.9-98.7)	86.3% (79.5-91.6)	38.7% (21.9-57.8)	99.2% (95.3-99.9)	0.419	0.893
Independent validation	61.5% (31.6-86.0)	93.1% (83.3-98.1)	66.7% (35.0-89.9)	91.5% (81.3-97.2)	1.080	0.791
Internal validation	92.3 (63.9-98.7)	86.3 (79.5-91.6)	38.7% (21.9-57.8)	99.2% (95.3-99.9)	0.419	0.893
Decision tree analysis						
Apparent	76.9% (46.2-94.7)	77.3% (69.5-83.9)	23.8% (12.1-39.5)	97.3% (92.4-99.4)	0.310	0.606
Independent validation	75.0% (42.8-94.2)	92.5% (81.8-97.9)	69.2% (38.6-90.7)	94.2% (84.0-98.7)	0.474	0.699
Internal validation	0.00% (0.00-24.8)	99.3% (96.1-99.8)	0.00% (0.00-83.5)	91.5% (85.9-95.4)	13.00	0.458
Artificial neural networks						
Apparent	53.9% (25.2-80.7)	100% (97.4-100)	100% (58.9-100)	95.9% (91.2-98.6)	1.86	0.979
Independent validation	15.4% (2.37-45.5)	98.2% (90.2-99.7)	66.7% (11.6-94.5)	83.1% (71.7-91.2)	0.231	0.749
Internal validation	0.00% (0.00-24.9)	100% (97.4-100)	NA	91.5% (85.8-95.4)	NA	0.500
mTR / mNR						
Logistic regression (threshold=5.48%)						
Apparent	76.9% (46.2-94.7)	76.6% (68.7-83.3)	23.3% (11.8-38.6)	97.3% (92.3-99.4)	0.302	0.768
Independent validation	69.2% (38.6-90.7)	83.1% (71.0-91.6)	47.4% (24.5-71.1)	92.5% (81.8-97.9%)	0.684	0.699
Internal validation	76.9% (46.2-94.7)	76.6% (68.7-83.3)	23.3% (11.8-38.6)	97.3% (92.3-99.4)	0.302	0.768
DTA						
Apparent	76.9% (46.2-94.7)	76.6% (68.7-83.3)	23.3% (11.8-38.6)	97.3% (92.2-99.4)	0.302	0.768
Independent validation	75.0% (42.8-94.2)	92.5% (81.8-97.9)	69.2% (38.6-90.7)	94.2% (84.0-98.7)	1.080	0.699
Internal validation	15.4% (2.37-45.5)	98.6% (94.9-99.8)	50.0% (8.30-91.7)	92.6% (87.1-96.3)	3.250	0.713
ANN						
Apparent	76.9% (46.2-94.7)	100% (97.4-100)	100% (69.0-100)	97.9% (93.9-99.5)	1.300	0.917
Independent validation	15.4% (2.37-45.5)	100% (93.8-100)	100% (19.3-100)	84.1% (73.3-91.8)	0.154	0.920
Validated	0.00% (0.00-24.9)	100% (97.4-100)	NA	91.5% (85.8-95.4)	NA	0.500

7.4 DISCUSSION

7.4.1 Summary of findings

This chapter found—by both indirect and direct comparison—PET-CT to be significantly more sensitive for progression than CT, associated with a reduced radiation dose and minimal additional cost. A new classification of FDG-avid nodal stage (mN) before NAC predicted disease progression to metastases on restaging scan. mN stage after NAC, and a new classification of mNR, plus primary tumour impassability and FDG-avidity predicted unsuspected incurable disease at surgery (in notable contrast to mTR). These variables and derivative LR and DTA models identified patients (before and after NAC) at high risk of progression and abandoned resections, demonstrating that avid nodes after NAC and an impassable tumour can stratify patient risk easily.

7.4.2 Importance of nodal FDG-avidity

Whilst avid nodes predict worse prognosis in oesophageal cancer (Gillies et al., 2012), cholangiocarcinoma (Park et al., 2014) and uterine carcinoma (Im et al., 2014), the author believes this to be the first association with disease progression. Intriguingly, this was independent of traditional N staging: patients with ≥N1 but mN0 disease seem to have the same low risk as N0 mN0 disease. This suggests that FDG-avidity is an important surrogate marker of more aggressive metastatic clones, although possibly explained in part by over-estimation of nodal stage by EUS (Puli et al., 2008a). Risk increased with nodal burden; this was classified analogous to the TNM 7th edition N stage (mN0, mN1 and mN2), although a reliable mN3 stage could not be generated. The author also believes this to be the first description of mNR, and similarly its important association with progression. Future questions include whether the proportion of EUS-identified nodes that are avid has predictive ability. Whether mN stage and mNR predict pTR, recurrence and survival is assessed in Chapters 8 and 9.

7.4.3 Other factors

An impassable tumour (at baseline) strongly predicted unresectable disease, independent of T stage and FDG-avid length. This might represent clonal evolution within a larger tumour, or nutritional compromise (although these patients are routinely supported in OUH via jejunostomy tube). Progression was more likely with a longer FDG-avid tumour (perhaps again representing volume), but unresectable disease was associated with a lower SUVmax (presumably representing less FDG-avid metastases from less FDG-avid primaries).

7.4.4 High rate of unsuspected incurable disease at surgery

The high rate of incurable disease encountered at surgery suggests that whilst re-staging PET-CT is preferable to CT it is insufficient in isolation (perhaps due to limitations in primary tumour avidity as discussed), although a lack of evidence and individual case variation precludes making general recommendations. However, it seems logical that patients with thoracic disease either impassable at OGD or ≥T3 may require additional re-staging cross-sectional imaging. The morbidity of laparoscopy is sufficiently low (Richardson and Khan, 2012) that re-staging laparoscopy (including the lesser sac) should be considered in all patients with distal oesophageal/GOJ disease, perhaps even before PET-CT. In this group, even the lowest risk group of patients (those with passable tumours and mN0 disease) had a 3.87% risk of unresectable disease. In the National Health Service, this would be cost-neutral: 9.52 procedures (£14,613) required to prevent an abandoned oesophagectomy (£12,274).

The rate of such incurable disease found at surgery in this study is relatively high compared with 3 recent studies: 0/89 (0.00%) (Elliott et al., 2014), 1/46 (2.17%) (Blom et al., 2011) and 2/57 patients (3.57%) (Bruzzi et al., 2007b), potentially related to differences in patient selection and treatment. Firstly, all 3 studies used neoadjuvant chemoradiotherapy rather NAC, potentially resulting in improved local control. Secondly, there were differences in stage, particularly N1 disease before therapy. This could not be ascertained from two studies (Blom et al., 2011, Elliott et al., 2014), and there were considerably fewer node positive patients in the other (Bruzzi et al., 2007b). Thirdly, nodal avidity after therapy is important; in one study no patients had residual FDG-avid nodes (Bruzzi et al., 2007b), whilst the other two papers did not report this.

7.4.5 Study limitations

This study has a number of limitations, including its opportunistic single centre retrospective design, an indirect comparison of two modalities, and use of two PET-CT scanners (although control was performed for confounding variables including scanner, replicated findings for individual scanners, and generated models from the later group with validation in the former). Additionally, the relative infrequency of events resulted in wide regression confidence intervals, and the utility of re-staging PET-CT in (initially) non-avid tumours would not be precisely ascertained. However, whilst these broad intervals might limit precision, the author believes their associations to be genuine, on the basis of consistently strong associations on direct comparison (for example, impassable tumours and unresectable disease: $p=6.47x10^{-15}$, Fisher's exact test). Consequently, LR models may have less generalizability than DTA which do not use precise effect sizes. Whilst PET-CT detected twice as many instances of progression, this was not significant on direct comparison using Fisher's exact test, most likely indicative of the lower CT sample size. However, the author again believe these rates to be representative; when considering those metastases evident on PET-CT, a similar proportion were evident on the CT component (plus PET) and on the PET component alone. Inevitably, further potential sources of bias are technological (in particular slice thickness) and clinical evolution towards triple agent NAC during this period, although again these were adjusted.

Three modelling techniques were used to mitigate advantages and disadvantages. Whilst the re-staging PET-CT LR model outperformed DTA for unresectable disease, its lower independent validation performance highlights difficulties in partitioning data with continuous variables, also limited by wide confidence intervals. By contrast DTA partitioned on pragmatic and more reliable categories—an impassable tumour and the presence of avid nodes despite NAC—with better validation performance. All models and markers require further external validation before being used to guide decision-making. However, the author believes patients with either an impassable tumour or avid nodes after NAC can be easily identified to stratify risk.

7.4.6 Conclusions

In conclusion, re-staging with PET-CT rather than CT appears significantly more sensitive for disease progression after NAC, with the caveats of comparison across a time period. New classifications of mN stage and response, in addition to impassability and perhaps primary tumour FDG-avidity, can identify patients at risk. There is therefore a strong argument for evaluating nodal avidity in addition to traditional N stage within the TNM staging manual, and also considering interval metabolic response of both the primary tumour and nodal metastases. However, there remains a need for additional staging in high and medium risk patients as a minimum; the most likely modalities being re-staging laparoscopy (including the lesser sac) and cross-sectional imaging beyond CT.

CHAPTER 8

PREDICTING PATHOLOGICAL RESPONSE OF OESOPHAGEAL CANCER TO NEOADJUVANT CHEMOTHERAPY:

METABOLIC TUMOUR AND NODAL RESPONSE AND THEIR IMPLICATIONS FOR PERSONALISED THERAPY

8. CHAPTER 8: PREDICTING PATHOLOGICAL RESPONSE OF OESOPHAGEAL CANCER TO NEOADJUVANT CHEMOTHERAPY

8.1 SYNOPSIS

8.1.1 Introduction

Only a minority of oesophageal tumours demonstrate a pTR to NAC. ^{18}F-FDG PET-CT is increasingly used after 1 cycle to identify unresponsive tumours. However, the reported ΔSUVmax thresholds for classifying mTR vary. The aim of this chapter was to re-evaluate these, compare and assess novel response parameters of both tumour and nodes, generate predictive models, and determine their implications for precision oncology.

8.1.2 Methods

The study population comprised those patients analysed in Chapter 7 (staged before NAC with PET-CT), and restaged after NAC by CT or PET-CT in OUH, proceeding to successful resection. pTR was defined as Mandard TRG 1-3. Variables included routine clinical and pathological data; imaging parameters included SUVmax/mean/peak, MTV, TGV mean/peak, nodal SUVmax, mN stage and mNR (developed in Chapter 7). Predictive factors were identified using direct group comparison, and univariate and multivariate binary logistic regression was performed. Predictive modelling was performed using LR, DTA and ANNs. Optimal thresholds for each metric for discrimination, sensitivity and specificity were determined using ROC and compared with existing 30% (PERCIST) and 35% (MUNICON) thresholds.

8.1.3 Results

Eighty-two (27.2%; 95% CI 22.3-32.7) of 301 patients demonstrated pTR. No pre-NAC imaging parameters predicted response. In 220 patients re-staged by PET-CT, ΔSUVmax and Δlength independently predicted pTR. The optimal tumour ΔSUVmax threshold was -77.8%. This was as sensitive as the standard -30% threshold, but more specific with a better negative predictive value ($p<9.38x10^{-5}$). Composite avidity/spatial metrics (e.g. ΔMTV)

outperformed ΔSUVmax. Whilst mNR was associated with pTR, discrepancy was observed in 42/219 (19.2%), usually mNR without pTR/mTR.

8.1.4 Conclusions

Current ΔSUVmax thresholds imperfectly predict pTR and should be revised. Metrics using metabolic tumour burden perform better, and should be evaluated urgently. mNR is a novel independent factor. Studies using mTR (ΔSUVmax) in isolation omit both spatial and nodal response, which is often independent, and so may abort/continue NAC inappropriately.

8.2 INTRODUCTION

As discussed in Chapters 1.3.8 and 7, the ability to predict pTR to neoadjuvant therapy for patients with oesophageal cancer at the outset would be invaluable. As demonstrated in Chapters 4 and 5, there is limited evidence that molecular markers, and perhaps PET markers, may have predictive ability (Hatt et al., 2011). However, clinical utility has not been demonstrated robustly. Interval assessment of response during therapy is therefore the next best option, and indeed mTR on PET has been shown to associate with pTR, albeit imperfectly. A 35% reduction of SUVmax is most commonly used (Weber et al., 2001, Ott et al., 2006) , and formed the basis of the landmark MUNICON trial: NAC was aborted after a single cycle in patients not demonstrating mTR, defined as a reduction in SUVmax ≥35% (Lordick et al., 2007).

After NAC, the PERCIST criteria use a 30% reduction to classify mTR (Wahl et al., 2009). However, these may not be optimal; the PERCIST threshold is neither tumour nor context-specific, whilst the MUNICON threshold derives from just 40 patients 14 years ago. Furthermore, SUVmax provides no spatial information, and assesses only the primary tumour; as identified in Chapter 7 nodal FDG-avidity (mN stage and mNR) may have major and clinical implications independent of primary tumour stage and response.

The aims of this chapter were firstly to comprehensively re-examine the utility of PET-CT in predicting pTR to NAC, as well as routine clinical and pathological variables. Firstly, to assess the predictive ability of clinical, pathological and imaging factors available before NAC. Secondly, to refine associations and thresholds of mTR after NAC, and assess for the first time the novel concept of mNR developed in Chapter 7. Thirdly, to generate and validate

predictive models for clinical use. And finally, to draw lessons for studies using interval mTR to personalise therapy.

8.3 RESULTS

8.3.1 Patients and pathological response

302 patients underwent successful resection following NAC (table 8.1). Mandard TRG was available for 301. pTR was evident in 82 (27.2%): TRG 1 n=14 (4.65%), TRG 2 n=13 (4.32%), TRG 3 n=55 (18.3%), TRG 4 n=162 (53.8%) TRG 5 n=67 (22.2%).

Table 8.1: Patient characteristics and management and tumour response

Baseline factor	Overall (n=301)	pTR (n=82)	No pTR (n=219)	p $\alpha=4.55\times10^{-3}$ pre-NAC $\alpha=2.63\times10^{-3}$ post-NAC
Age *Median; IQR; range*	64.0 (58.0-70.0; 36.0-80.0)	62.5 (57.3-69.0; 36.0-79.0)	64.0 (58.0-70.0; 38.0-80.0)	0.369[a]
Gender *Male* *Female*	 228 (75.7%) 73 (24.3%)	 61 (74.4%) 21 (25.6%)	 167 (76.3%) 52 (23.7%)	 0.764[b]
Tumour				
Cell type *AC* *SCC* *AS* *NEC* *SC* *Anaplastic*	 249 (82.7%) 44 (14.6%) 5 (1.66%) 0 (0.00%) 1 (0.33%) 2 (0.66%)	 68 (82.9%) 13 (15.9%) 1 (1.22%) 0 (0.00%) 0 (0.00%) 0 (0.00%)	 181 (82.6%) 31 (14.2%) 4 (1.83%) 0 (0.00%) 1 (0.46%) 2 (0.91%)	 0.979[b]
Grade *Well* *Moderate* *Poor* *Undifferentiated*	 28 (9.30%) 128 (42.5%) 140 (46.5%) 5 (1.66%)	 5 (6.10%) 35 (42.7%) 42 (51.2%) 0 (0.00%)	 23 (10.5%) 93 (42.0%) 98 (44.7%) 5 (2.28%)	 0.338[b]
Tumour site *Proximal 1/3* *Mid 1/3* *Distal 1/3* *GOJ 1* *GOJ 2* *GOJ 3* *Multifocal*	 0 (0.00%) 18 (5.98%) 52 (17.3%) 72 (23.9%) 107 (35.5%) 51 (16.9%) 1 (0.33%)	 0 (0.00%) 9 (11.0%) 11 (13.4%) 23 (28.0%) 20 (24.4%) 19 (23.2%) 0 (0.00%)	 0 (0.00%) 11 (5.02%) 41 (18.7%) 49 (22.4%) 85 (38.8%) 32 (14.6%) 1 (0.46%)	 0.033[b]
Pre-NAC staging				
T stage *1* *2* *3* *4a* *4b*	 7 (2.33%) 46 (15.3%) 231 (76.7%) 17 (7.76%) 0 (0.00%)	 2 (2.43%) 19 (23.2%) 56 (68.3%) 5 (6.10%) 0 (0.00%)	 5 (2.28%) 27 (12.3%) 175 (79.9%) 12 (35.48%) 0 (0.00%)	 0.114[b]
N stage				

0	88 (29.3%)	58 (26.5%)	30 (36.6%)	0.090[b]
1	213 (70.7%)	161 (73.5%)	52 (63.4%)	
Initial PET CT				
FDG-avid	290 (96.7%)	75 (91.5%)	215 (98.2%)	0.011[b]
FDG-negative	11 (3.65%)	7 (8.54%)	4 (1.83%)	
Initial PET-CT scanner				
Before Nov 2009	142 (47.7%)	38 (46.3%)	104 (47.5%)	0.897[b]
After Nov 2009	159 (52.3%)	44 (55.7%)	115 (52.5%)	
NA	0 (0.00%)			
Restaging PET-CT scanner				
Before Nov 2009	62 (20.6%)	16 (19.5%)	46 (21.0%)	0.739[b]
After Nov 2009	158 (52.5%)	46 (56.1%)	112 (51.19%)	
CT	81 (26.9%)	20 (24.4%)	61 (27.9%)	
mN stage				
0	209 (69.4%)	54 (65.9%)	155 (70.8%)	0.371[b]
1	54 (17.9%)	14 (17.1%)	40 (18.3%)	
2	38 (12.6%)	14 (17.1%)	24 (11.0%)	
NA	0 (0.00%)			
Impassable at OGD?	278 (92.4%)	77 (93.9%)	201 (92.8%)	0.633[b]
No	23 (7.60%)	5 (6.10%)	18 (8.20%)	
Yes				
Surgical approach				
Resection				
LTO	200 (66.4%)	12 (14.6%)	156 (71.3%)	0.003[b]
ILO	46 (15.3%)	44 (53.7%)	34 (15.5%)	
3 stage	10 (3.32%)	5 (6.10%)	5 (2.28%)	
THO	1 (0.33%)	1 (1.22%)	0 (0.00%)	
ETG	44 (14.6%)	20 (24.4%)	24 (11.0%)	
Response to chemotherapy				
Chemotherapy				
Dual	230 (76.4%)	48 (58.5%)	182 (83.1%)	2.69×10^{-5} [b]
Triple	71 (23.6%)	34 (41.5%)	37 (16.9%)	
Days to re-staging	82.0 (71.0-93.0)	88.5 (71.3-106.8;	82.0 (71.0-91.0;	0.036[b]
Median; IQR; range		43.0-167)	40.0-165)	
Days scan to surgery	24.0 (17.0-33.0)	23.0 (18.3-31.8; 5.0-52.0)	23.0 (15.0-33; 4.0-72.0)	0.283[b]
Median; IQR; range				
pTR				
No	82 (27.2%)	NA	NA	NA
Yes	219 (72.8%)			
mTR				
Non-avid	7 (2.33%)	5 (8.06%)	2 (1.27%)	5.38×10^{-13} [b]
CMR	48 (15.9%)	33 (53.3%)	15 (9.49%)	
PMR	108 (35.9%)	20 (32.4%)	88 (55.7%)	
SMD	43 (14.3%)	4 (1.33%)	39 (24.7%)	
PMD	14 (4.65%)	0 (0.00%)	14 (8.86%)	
NA	81 (26.9%)	20 (NA)	61 (NA)	
mNR				
No avid nodes	138 (45.8%)	39 (62.9%)	99 (62.6%)	1.23×10^{-4} [b]
CMR	50 (16.6%)	21 (33.9%)	29 (18.4%)	
PMR/SMD/PMD	32 (10.6%)	2 (3.22%)	30 (19.0%)	
NA	81 (26.9%)	20 (NA)	61 (NA)	

a=Mann-Witney test; b=Fisher's exact test; LTO=left thoracoabdominal oesophagectomy; ILO=Ivor-Lewis oesophagectomy; THO=transhiatal oesophagectomy; ETG=extended total gastrectomy;

8.3.2 Predicting pathological response

8.3.2.1. Predicting pathological response before chemotherapy

On multivariate analysis of all baseline variables, the use of triple agent NAC was associated with pTR: OR 5.98 (95% confidence interval [CI] 2.44-14.7; $p=8.94x10^{-5}$). No tumour variables before NAC predicted pTR (table 8.2; supplementary table 8.1). However, for stage mN1 and 2 tumours (n=79), progressive FDG-avidity of the most avid node was strongly associated with lack of response: adjusted OR 462 (4.25-50228; p=0.010).

8.3.2.2. Predicting pathological response after chemotherapy

For the 220 cases re-staged by PET-CT, a greater SUVmax of the primary tumour after NAC was strongly and negatively associated with pTR: logSUVmax OR $3.84x10^{-4}$ ($1.17x10^{-5}$-.002; $p=9.89x10^{-6}$). On subgroup analysis of the 158 patients (re-staged with the most recent scanner) with additional PET variables, all metrics (were strongly and negatively associated with response ($p<3.90x10^{-3}$; table 8.3; supplementary table 8.2).

Table 8.2: Pre-NAC factors associated with pathological response

Factor (n=291)	pTR			
	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Age (Median; IQR)	1.00 (1.00-1.00)	0.536	1.00 (1.00-1.00)	0.949
Sex				
Female	Ref	Ref	Ref	Ref
Male	0.90 (0.50-1.62)	0.722	0.94 (0.45-1.95)	0.859
Cell*				
AC	Ref	Ref	Ref	Ref
SCC	1.14 (0.56-2.32)	0.716	0.87 (0.31-2.45)	0.792
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.77 (0.62-5.02)	0.284	1.07 (0.33-3.49)	0.906
Poor	1.99 (0.71-5.58)	0.194	1.53 (0.47-4.97)	0.477
Undifferentiated	NA	NA	NA	NA
Site*				
Mid 1/3	Ref	Ref	Ref	Ref
Distal 1/3	0.30 (0.10-0.91)	0.034	0.21 (0.05-0.79)	0.021
GOJ 1	0.51 (0.18-1.54)	0.200	0.34 (0.09-1.26)	0.106
GOJ 2	0.28 (0.10-0.78)	0.015	0.17 (0.04-0.69)	0.013
GOJ 3	0.68 (0.23-1.98)	0.480	0.15 (0.03-0.64)	0.020
T stage				
1	Ref	Ref	Ref	Ref
2	1.76 (0.31-10.0)	0.525	2.33 (0.34-16.0)	0.390
3	0.83 (0.16-4.42)	0.830	0.98 (0.15-6.27)	0.986
4a	1.05 (0.15-7.27)	0.967	1.13 (0.13-10.0)	0.916
N stage				
0	Ref	Ref	Ref	Ref
1	0.64 (0.37-1.10)	0.105	0.60 (0.13-1.16)	0.129
Passable	Ref	Ref	Ref	Ref
Impassable	0.63 (0.20-1.93)	0.416	0.50 (0.13-1.95)	0.317
Chemotherapy				
NAC regimen				
Dual	Ref	Ref	Ref	Ref
Triple	3.48 (1.97-6.14)	1.76×10^{-5}	5.98 (2.44-14.7)	8.94×10^{-5}
Log days to restaging	63.9 (4.24-964)	2.66×10^{-3}	10.8 (0.42-280)	0.152
Log days to surgery	0.93 (0.31-2.79)	0.896	1.12 (0.29-4.33)	0.873
PET-CT variables				
PET scanner				
1	Ref	Ref	Ref	Ref
2	1.07 (0.64-1.79)	(0.796)	0.69 (0.36-1.32)	0.267
mN stage				
0	Ref	Ref	Ref	Ref
1	0.94 (0.46-1.89)	0.857	1.42 (0.60-3.34)	0.426
2	1.67 (0.80-3.48)	0.720	1.72 (0.67-4.45)	0.261
Log SUVmax	0.43 (0.16-1.11)	0.081	0.54 (0.15-1.92)	0.343
Log -avid length	0.90 (0.81-1.01)	0.070	0.89 (0.77-1.04)	0.145
Subset of patients with FDG-avid nodes (n=79)**				
Log nodal SUVmax	7.00 (0.94-51.8)	0.057	462 (4.25-50228)	0.010
Subset of patients staged using second PET-CT scanner (n=155)				
SUVmean	1.47 (0.09-23.4)	0.784	1.56 (0.04-65.8)	0.814
SUVpeak	2.53 (0.41-15.8)	0.320	1.85 (0.50-6.77)	0.356
MTV	1.55 (0.79-3.04)	0.203	1.70 (0.66-4.39)	0.276
TGVmax	1.53 (0.84-2.76)	0.163	1.72 (0.74-3.99)	0.230
TGVmean	1.45 (0.86-2.44)	0.164	1.64 (0.78-3.42)	0.189

*other cell types and sites excluded as perfect separators **effect sizes for subgroup with FDG-avid nodes only

Table 8.3: Post-neoadjuvant chemotherapy factors associated with pathological response

Factor (n=213)	pTR			
	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Chemotherapy				
Dual agent	Ref	Ref	Ref	Ref
Triple agent	4.30 (2.16-8.55)	3.23×10^{-5}	17.6 (4.39-70.1)	5.00×10^{-5}
Log time to restaging	25.1 (1.10-574)	0.044	0.32 (0.00-69.2)	0.678
Log time to surgery	2.28 (0.57-9.07)	0.241	0.52 (0.06-4.82)	0.567
PET-CT variables				
Restaging PET scanner				
Before Nov 2009	Ref	Ref	Ref	Ref
After Nov 2009	1.30 (0.66-2.57)	0.446	5.24 (0.95-28.9)	0.057
Restaging mN stage				
0	Ref	Ref	Ref	Ref
1	0.16 (0.02-1.28)	0.084	1.07 (0.07-16.8)	0.959
2	0.16 (0.02-1.28)	0.084	2.39 (0.18-31.6)	0.509
Restaging log SUVmax	2.37×10^{-3} (4.21×10^{-4}-0.01)	6.93×10^{-12}	3.84×10^{-4} (1.17×10-5-0.02)	9.89×10^{-6}
Restaging log length	0.61 (0.51-0.73)	3.80×10^{-8}	1.01 (0.76-1.34)	0.951
Restaging log MTL	0.03 (0.01-0.10)	3.88×10^{-10}	0.02 (4.03×10^{-3}-0.06)	6.19×10^{-9}
Subset of patients with FDG-avid nodes (n=30)				
Log nodal SUVmax*	8.71 (0.01-5787)	0.514	NA	NA
Subset of patients staged using second PET-CT scanner (n=155)				
Log SUVmean	1.58×10^{-4}(7.51×10^{-6}-0.003)	1.78×10^{-4}	1.13×10^{-7} (8.55×10^{-12}-0.001)	9.32×10^{-5}
SUVpeak	5.05×10^{-3} (1.81×10^{-4}-0.14)	1.85×10^{-3}	0.57 (0.39-0.84)	3.90×10^{-3}
Log MTV	0.28 (0.18-0.44)	0.203	0.09 (0.03-0.28)	2.03×10^{-5}
Log TGVmax	0.32 (0.21-0.48)	3.91×10^{-8}	0.11 (0.04-0.31)	2.72×10^{-5}
Log TGVmean	0.30 (0.19-0.46)	3.27×10^{-8}	0.10 (0.03-0.29)	2.29×10^{-5}
Pre-NAC variables				
Age	1.00 (1.00-1.00)	0.590	1.00 (1.00-1.00)	0.526
Female	Ref	Ref	Ref	Ref
Male	0.82 (0.41-1.62)	0.565	1.03 (0.30-3.56)	0.969
Cell*				
AC	Ref	Ref	Ref	Ref
SCC	1.01 (0.44-2.35)	0.973	0.49 (0.10-2.35)	0.372
Grade*				
Well	Ref	Ref	Ref	Ref
Moderate	1.13 (0.33-3.84)	0.851	0.60 (0.07-5.04)	0.637
Poor	1.11 (0.33-3.78)	0.861	0.40 (0.05-3.24)	0.391
Site*				
Mid 1/3	Ref	Ref	Ref	Ref
Distal 1/3	0.32 (0.08-1.25)	0.101	0.13 (0.02-0.95)	0.044
GOJ 1	0.48 (0.14-1.67)	0.246	0.24 (0.03-1.64)	0.145
GOJ 2	0.27 (0.08-0.96)	0.042	0.08 (0.01-0.97)	0.019
GOJ 3	0.61 (0.16-2.38)	0.477	0.08 (0.01-0.97)	0.048
T stage				
1	Ref	Ref	Ref	Ref
2	1.25 (0.20-7.68)	0.810	1.53 (0.14-16.9)	0.728
3	0.68 (0.12-3.85)	0.661	1.61 (0.18-14.7)	0.674
4a	1.25 (0.16-9.54)	0.830	4.64 (0.31-70.4)	0.269
N stage				
0	Ref	Ref	Ref	Ref
1	0.52 (0.27-0.98)	0.043	0.43 (0.16-1.15)	0.093
Passable	Ref	Ref	Ref	Ref
Impassable	0.66 (0.18-2.45)	0.532	0.24 (0.03-1.80)	0.165

Staging PET scanner *Before Nov 2009* *After Nov 2009*	 Ref 1.09 (0.58-2.07)	 Ref 0.782	 Ref 0.10 (0.02-0.55)	 Ref 0.008

8.3.2.3. Predicting pathological tumour response using metabolic tumour and nodal response

As expected, mTR predicted pTR (tables 8.1 and 8.4). However, this was true both for ΔSUVmax and Δlength independently on regression: ΔSUVmax OR=1.03 (1.01-1.06), $p=3.24x10^{-3}$; Δlength OR=1.02 (1.00-1.03); p=0.019. Thereafter, a composite of the two (MTL; metabolic tumour length) was generated for comparison. Interestingly, whilst a PERCIST PMR (reduction >30%) was associated with pTR, the 35% (MUNICON) threshold was not, once Δlength was adjusted for. All additional metrics of mTR were strongly associated with pTR.

mNR was associated with pTR using Fisher's exact test (table 8.1), but not multivariate regression (table 8.4). Notably, mNR and pTR were discordant in 42/220 (19.1%) patients; in 41 cases this was either a nodal CMR or PMR despite lack of pTR (table 8.5). However, this represents 51.5% of those 82 patients with FDG-avid nodes before NAC, mTR and mNR were also compared (table 8.5): in 13 (5.90%) of all cases these were discordant (15.8% of those with FDG-avid nodes before NAC). Again, typically discordance was a mNR in the absence of mTR (10; 4.6% / 12.2% respectively).

Table 8.4: Metabolic response and other factors associated with pathological response to neoadjuvant chemotherapy (patients staged and restaged using same PET scanner)

Factor (n=196)	pTR			
	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Chemotherapy				
NAC regimen				
Dual	*Ref*	*Ref*	*Ref*	*Ref*
Triple	4.30 (2.16-8.55)	$3.23x10^{-5}$	20.3 (4.50-91.4)	$8.84x10^{-5}$
Log time to restaging	69.1 (1.86-2571)	0.022	0.22 (0.00-172)	0.658
Log time to surgery	1.75 (0.41-7.44)	0.452	0.70 (0.06-8.36)	0.781
PET-CT variables				
PET scanner				
1	*Ref*	*Ref*	*Ref*	*Ref*
2	0.87 (0.40-1.88)	0.718	0.71 (0.21-2.38)	0.580
Avid nodal response				
NA	*Ref*	*Ref*	*Ref*	*Ref*
CMR	1.93 (0.93-4.01)	0.076	2.01 (0.54-7.51)	0.300
PMR	0.45 (0.05-3.87)	0.465	11.2 (0.64-197.3)	0.098
SMD	0.27 (0.03-2.18)	0.219	1.15 (0.09-14.4)	0.911
PMD	NA (NA)	NA	NA (NA)	NA
Reduction logSUVmax %	1.04 (1.02-1.05)	$6.65x10^{-8}$	1.03 (1.01-1.06)	$3.24x10^{-3}$
Reduction avid length %	1.03 (1.02-1.04)	$9.37x10^{-8}$	1.02 (1.00-1.03)	0.019
Additional metrics in all patients (n=202)				
Reduction MTL (%)	1.05 (1.03-1.07)	$2.86x10^{-6}$	1.11 (1.05-1.16)	$1.16x10^{-5}$
PERCIST				
CMR	*Ref*	*Ref*	*Ref*	*Ref*
PMR	0.10 (0.04-0.22)	2.24x10-8	0.08 (0.02-0.32)	$3.53x10^{-5}$
S/PMD	0.04 (0.01-0.14)	2.18x10-7	0.06 (0.01-0.49)	$8.46x10^{-4}$
MUNICON	*Ref*	*Ref*	*Ref*	*Ref*
No response	5.21 (2.08-13.0)	4.22x10-5	1.63 (0.41-6.45)	0.484
Response				
Subset of patients staged using second PET-CT scanner (n=155)				
Reduction SUVmean (%)	1.03 (1.02-1.04)	$2.25x10^{-8}$	1.05 (1.02-1.09)	$1.90x10^{-3}$
Reduction SUVpeak (%)	1.09 (1.03-1.15)	$1.91x10^{-5}$	1.04 (1.02-1.05)	$2.20x10^{-3}$
Reduction MTV (%)	1.44 (1.09-1.92)	$2.70x10^{-5}$	1.16 (1.07-1.25)	0.011
Reduction TGVmax (%)	1.30 (1.12-1.52)	$5.82 x10^{-3}$	2.31 (1.27-4.20)	$2.72x10^{-5}$
Reduction TGVmean (%)	1.23 (1.10-1.37)	$3.91x10^{-8}$	1.87 (1.20-2.90)	$2.29x10^{-5}$
Baseline variables				
Age	1.00 (1.00-1.00)	0.380	1.00 (1.00-1.00)	0.866
Sex				
Female	*Ref*	*Ref*	*Ref*	*Ref*
Male	0.74 (0.35-1.54)	0.415	0.73 (0.18-2.95)	0.658
Cell				
AC	*Ref*	Ref	Ref	Ref
SCC	1.47 (0.61-3.53)	0.395	0.45 (0.07-2.97)	0.408
Grade				
Well	*Ref*	*Ref*	*Ref*	*Ref*
Moderate	0.87 (0.21-3.67)	0.850	0.20 (0.02-1.89)	0.162
Poor	0.90 (0.22-3.74)	0.885	0.13 (0.01-1.21)	0.073
Undifferentiated	NA	NA	NA	NA
Site				
Proximal 1/3	NA	NA	NA	NA
Mid 1/3	Ref	Ref	Ref	Ref
Distal 1/3	0.35 (0.09-1.39)	0.136	0.15 (0.02-1.37)	0.093

GOJ 1	0.39 (0.11-1.42)	0.155	0.17 (0.02-1.63)	0.125
GOJ 2	0.25 (0.07-0.90)	0.034	0.08 (0.01-0.89)	0.040
GOJ 3	0.59 (0.15-2.33)	0.449	0.06 (0.00-1.01)	0.051
Multifocal	NA	NA	NA	NA
T stage				
1	*Ref*	*Ref*	*Ref*	*Ref*
2	0.78 (0.11-5.34)	0.802	0.63 (0.04-9.28)	0.739
3	0.50 (0.08-3.09)	0.452	0.33 (0.03-4.08)	0.390
4a	0.86 (0.10-7.51)	0.889	2.81 (0.13-59.8)	0.508
N stage				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	0.56 (0.28-1.12)	0.101	0.21 (0.06-0.70)	0.011
Passable at OGD?				
Yes	*Ref*	*Ref*	*Ref*	*Ref*
No	0.45 (0.10-2.12)	0.316	0.04 (0.00-0.65)	0.024

Table 8.5: Comparison of tumour and nodal metabolic response

Tumour response	mNR				
	NA	CMR	PMR	SMD	PMD
Pathological response					
pTR	39 (17.7%)	21 (9.55%)	1 (0.45%)	1 (0.45%)	0 (0.00%
No pTR	99 (45.0%)	29 (13.2%)	12 (5.45%)	13 (5.91%)	5 (22.7%)
Metabolic response					
NA	6 (2.73%)	1 (0.45%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
CMR	32 (14.5%)	14 (1.82%)	1 (0.45%)	1 (0.45%)	0 (0.00%)
PMR	68 (30.9%)	29 (13.2%)	8 (3.64%)	2 (0.91%)	0 (0.00%)
SMD	22 (9.09%)	5 (2.27%)	4 (1.82%)	10 (4.55%)	3 (1.36%)
PMD	10 (4.55%)	1 (0.45%)	0 (0.00%)	1 (0.45%)	2 (0.91%)

8.3.3 Refining and comparing metabolic response thresholds

8.3.3.1. Comparing metrics overall

The ability of each metric to predict pTR was compared, using sensitivity, specificity and ROC AUC (tables 8.6; supplementary tables 8.4-8.6). Comparisons were performed for patients staged and re-staged using the most recent PET-CT scanner using ROC (n=144). All were moderately and similarly discriminant: AUC ranged from 0.802 (FDG-avid length) to 0.862 (TGVmax and TGVmean response); that of SUVmax was 0.844. There were no statistically significant differences (table 8.6). However, both the PERCIST and MUNICON thresholds (whilst 100% sensitive) appeared poorly specific.

Table 8.6: Comparison of receiver operator curve statistics for metrics of metabolic response (patients staged and re-staged using most recent PET-CT scanner (n=144)

Metric	AUC	p value (α=1.79x10^{-3})							
		SUVmax	Length	MTL	SUVmean	SUVpeak	MTV	TGVmax	TGV mean
SUVmax	0.841	NA	0.318	0.768	0.050	0.194	0.445	0.244	0.271
Length	0.802	0.138	NA	0.186	0.541	0.417	0.123	0.111	0.116
MTL	0.835	0.768	0.186	NA	0.791	0.884	0.446	0.352	0.376
SUVmean	0.825	0.050	0.541	0.791	NA	0.710	0.145	0.074	0.085
SUVpeak	0.830	0.194	0.417	0.884	0.710	NA	0.178	0.088	0.101
MTV	0.859	0.445	0.123	0.446	0.145	0.178	NA	0.393	0.233
TGVmax	0.862	0.244	0.111	0.352	0.074	0.088	0.393	NA	1.000
TGVmean	0.862	0.271	0.116	0.376	0.085	0.101	0.233	1.000	NA

MTL=metabolic tumour length (SUVmax x length)

8.3.3.2. Determining optimal thresholds

The optimal thresholds were then determined using ROC (table 8.7) and validated (supplementary table 8.7): overall (the optimal balance of sensitivity and specificity), for sensitivity, and for specificity. Notably, the optimal percentage reduction in SUVmax for sensitivity (100% [89.9-100]) was 27.4-30.6%. This was identical to that of the PERCIST criteria (threshold 30%), and very similar to the MUNICON threshold (35%: sensitivity 97.1% [85.0-100]). These, however, had poor specificity: 33.0% (23.8-42.6); 41.8% (32.0-52.2). By contrast, the optimal ΔSUVmax threshold for balancing sensitivity (73.6% [58.6-82.7]) and specificity (84.5% [78.7-89.1]) was dramatically different. Rounded down to a more pragmatic 75.0%, sensitivity was identical, whilst specificity reduced non-significantly to 84.0

Table 8.7: Receiver operator curve statistics and thresholds for metrics of mTR

		Optimal			Sensitivity			Specificity		
Metric	AUC (95% CI)	Thresh.	Sensitivity (95%CI)	Specificity (95% CI)	Thresh	Sensitivity (95%CI)	Specificity (95% CI)	Thresh	Sensitivity (95%CI)	Specificity (95% CI)
SUVmax	0.811 (0.729-0.861)	-77.8%	0.736 (0.586-0.827)	0.845 (0.787-0.891)	-6.90%	1.00 (1.00-1.00)	0.127 (0.087-0.180)	-97.9%	0.693 (0.448-0.690)	0.894 (0.842-0.934)
Avid length	0.779 (0.695-0.856)	-57.3%	0.692 (0.558-0.808)	0.894 (0.750-0.872)	-61.1%	0.981 (0.942-1.00)	0.007 (0.00-0.021)	-87.6%	0.509 (0.385-0.84)	0.917 (0.857-0.950)
MTL	0.797 (0.719-0.870)	-76.1%	0.769 (0.654-0.885)	0.778 (0.707-0.850)	-29.7%	1.000 (1.00-1.000)	0.057 (0.021-0.100)	-97.5%	0.538 (0.404-0.673)	0.914 (0.864-0.957)
PERCIST (30%)	NA	-30%	0.906 (0.862-0.983)	0.331 (0.268-0.480)	NA	NA	NA	NA	NA	NA
MUNICON (35%)	NA	-35%	0.807 (0.793-0.962)	0.408 (0.331-0.493)	NA	NA	NA	NA	NA	NA
Based on subset staged and re-staged with most recent PET-CT scanner (n=144)										
SUVmax	0.845 (0.777-0.905)	-77.8%	0.795 (0.667-0.887)	0.812 (0.742-0.881)	27.4%	1.00 (1.00-1.00)	0.327 (0.238-0.426)	-96.5%	0.615 (0.462-0.744)	0.861 (0.792-0.921)
Avid length	0.802 (0.712-0.883)	-57.1%	0.737 (0.605-0.868)	0.820 (0.740-0.890)	-2.66%	0.974 (0.921-1.00)	0.070 (0.020-0.120)	-85.3%	0.526 (0.368-0.684)	0.890 (0.830-0.950)
MTL	0.835 (0.758-0.906)	-76.0%	0.816 (0.684-0.921)	0.800 (0.720-0.800)	8.30%	1.00 (1.00-1.00)	0.180 (0.110-0.250)	-97.5%	0.526 (0.368-0.684)	0.900 (0.84-0.950)
PERCIST (30%)	NA	-30%	1.00 (1.00-1.00)	0.330 (0.238-0.426)	NA	NA	NA	NA	NA	NA
MUNICON (35%)	NA	-35%	0.974 (0.923-1.000)	0.420 (0.327-0.515)	NA	NA	NA	NA	NA	NA
SUVmean	0.825 (0.750-0.894)	-36.8%	0.789 (0.658-0.921)	0.800 (0.710-0.870)	-7.64%	1.00 (1.00-1.00)	0.320 (0.230-0.410)	-81.8%	0.632 (0.474-0.890)	0.860 (0.780-0.930)
SUVpeak	0.830 (0.746-0.896)	-69.8%	0.889 (0.778-0.972)	0.667 (0.566-0.798)	-2.21%	1.00 (1.00-1.00)	0.081 (0.03-0.141)	-95.8%	0.611 (0.444-0.778)	0.869 (0.799-0.929)
MTV	0.859 (0.797-0.914)	-89.6%	0.972 (0.917-1.00)	0.687 (0.597-0.778)	-80.0%	1.00 (1.00-1.00)	0.576 (0.485-0.667)	-99.9%	0.611 (0.444-0.778)	0.869 (0.799-0.929)
TGVmax	0.862 (0.804-0.913)	-93.8%	0.974 (0.921-1.00)	0.680 (0.590-0.770)	-91.9%	1.00 (1.00-1.00)	0.640 (0.550-0.730)	-100%	0.612 (0.474-0.790)	0.860 (0.790-0.930)
TGVmean	0.862 (0.903-0.915)	-89.2%	1.00 (1.00-1.00)	0.640 (0.550-0.730)	-89.2%	1.00 (1.00-1.00)	0.640 (0.550-0.730)	-99.9%	0.612 (0.474-0.790)	0.860 (0.790-0.930)

Thresh=threshold

8.3.3.3. Comparing and validating predictive ability of optimal thresholds

The predictive ability of these thresholds were then compared (supplementary tables 8.4-8.8) and validated internally (bootstrapping) and independently (first PET-CT scanner group).

Overall, ΔMTV, ΔTGVmax and ΔTGVmean thresholds outperformed PERCIST and MUNICON: they were comparably sensitive, but significantly more specific ($p<4.11\times10^{-4}$) and discriminant ($p<9.38\times10^{-5}$), and were supported by internal (bootstrapping) validation. Although they did not significantly outperform ΔSUVmax (77.8%), this suggests that composite metrics of avidity and volume perform better than avidity alone.

The most sensitive were: PERCIST (100%), MUNICON (97.1%), ΔMTV (97.1%), ΔTGVmax (97.1%) and ΔTGVmean (94.3%). These were significantly more sensitive than ΔL ($<4.68\times10^{-3}$; FDR=0.046). Whilst these were more sensitive than thresholds such as ΔSUVmax, this difference was not statistically significant.

The most specific thresholds were ΔSUVmax (77.8%; 81.7% specific) and ΔL (53.1%; 82.7% specific); these were significantly more specific than the most sensitive thresholds above ($p<4.11\times10^{-4}$). ΔSUVmax (77.8%), ΔMTV, ΔTGV and ΔTGVmean thresholds were significantly more discriminant than PERCIST and MUNICON ($p<9.38\times10^{-5}$).

8.3.4 Predictive models for pathological response

Predictive models were generated using pre-NAC, post-NAC and mTR variables. The most successful was a LR model comprising Δlength + ΔSUVmax; this was highly sensitive (91.4%), moderately specific (71.4%) and discriminant (0.814); this sensitivity persisted during internal and independent validation (although with relatively poor specificity and discrimination). However, ultimately none of the composite models outperformed individual mTR thresholds (supplementary tables 8.4-8.8). Typically, ANN had little utility.

8.4 DISCUSSION

8.4.1 Main findings and clinical implications

In this study of 301 patients treated with NAC and surgery—the largest conducted to date in oesophageal cancer, and the first of which the author is aware to compare standard and experimental PET-CT metrics—no variables were found to predict pTR at baseline. However, both absolute tumour restaging metrics of avidity and mTR were strongly associated with pTR on multivariate analysis, and whilst not derived from interval assessment, a number of clinically relevant implications were identified.

Firstly, the significantly better performance of a new optimal ΔSUVmax threshold (77.8%) than the generic PERCIST threshold (30%) suggests that the latter should be raised considerably for oesophageal cancer to improve stratification of mTR. This threshold was nominally significantly better than the 35% MUNICON threshold, but as the latter is based on interval assessment the significance of this is unclear.

Secondly, assessing mTR using ΔSUVmax in isolation is suboptimal. At a most basic level, Δlength is an independent factor, and improves prediction. However, composite spatial and avidity thresholds (ΔMTV, ΔTGVmax and ΔTGVmean) outperformed the existing recommended PERCIST SUVmax threshold of a 30% reduction. They were comparably sensitive, but more specific ($p<4.11\times10^{-4}$) and discriminant ($p<9.38\times10^{-5}$), and were supported by internal (bootstrapping) validation. They may therefore have improved predictive ability in clinical trials using ΔSUVmax alone (such as those based on the MUNICON trial 35% threshold). In particular, their superior specificity and high NPV (98.5-100%) would identify more non-responders for cessation of therapy.

Thirdly, primary and nodal tumour often demonstrated a differential response potentially very important as the latter is not used in clinical practice. Consequently, mNR may represent a new and important method of assessing response to NAC of metastatic sub-clones: i.e. those most likely responsible for local and distant disease relapse.

8.4.2 Predicting response at baseline and during therapy

The lack of associations between baseline factors and chemosensitivity is not surprising, and reflects the daunting complexity involved. Rather than being a binary state with a single cause, 'chemoresistance' is multifactorial and constitutes a spectrum of sensitivity, which depends upon numerous macroscopic, microscopic and molecular factors modulating chemotoxicity (Siddik, 2003, Reardon et al., 1999). Intratumoural heterogeneity further complicates this, with a number of sub-clones which may demonstrate differential response and baseline characteristics (in addition to heterogeneity between tumour and nodal metastases).

This notwithstanding, the landmark MUNICON trial demonstrated that interval mTR (ΔSUVmax >35%) after one cycle of NAC could be used feasibly to identify non-responsive tumours, in whom NAC could then be aborted (Lordick et al., 2007). However, these associations were imperfect; whilst no major pTR was identified in metabolic non-responders these patients by definition had just one cycle of chemotherapy; metabolic responders by contrast were much more likely (48.0%) to demonstrate an ultimate (major) pathological response after complete therapy. Although interval ΔSUVmax and other thresholds could not be refined, the findings of this study after NAC suggest that the predictive ability of this approach may be dramatically improved by considering metabolic spatial response in conjunction: Δlength at a most basic level, or ideally a composite metric such as ΔMTV or ΔTGVmax/mean.

In addition, the interval ΔSUVmax MUNICON threshold of 35% is based on a small pilot study population in 2001; whilst clearly useful it likely represents an approximation. It is highly probable that a limited number of interval metabolic non-responders as assessed by ΔSUVmax might have ultimately derived benefit. With negative predictive values of 98.5-100% the author would recommend strongly that future studies consider composite metrics to this end.

8.4.3 Tumour and nodal response discordance

An additional parameter unassessed until now is mNR. Nodal metastases by definition contain an aggressive subpopulation of cancer clones originating from the primary tumour, which then evolve differently at a genetic and phenotypic level (Jones et al., 2008). A crucial phenotype is chemosensitivity, and whilst clearly mNR (as with the primary tumour) is likely

an imperfect surrogate of response, no pathological grading systems for nodal response are in use. The prognostic significance of this is unclear, but is assessed in Chapter 9: the presence of persistent nodal metastases has been shown to be an independent marker of survival by some (Verlato et al., 2010), but not all studies(Mandard et al., 1994, Hermann et al., 2006). However, these findings of disparity between pTR and mNR add to those of Chapter 7, in which mN stage after NAC / lack of a nodal CMR was strongly associated with the risk of disease progression from curable to incurable disease, independently of mTR.

8.4.4 Limitations

This study has a number of limitations, including that of pTR itself. Whilst the gold standard technique for assessing primary tumour response is direct histopathological assessment, this remains imperfect. Indeed all classifications remain relatively subjective, and are tempered by inter-observer variability, due to the lack of a standardized approach in determining TRG. In particular, response may vary with a 'single' tumour with resultant sampling bias (Chetty et al., 2012). A further caveat is the lack of knowledge of the processes occurring during and after therapy, considerable opportunities for cancer evolution, as evidenced in Chapter 5.

Other limitations of this study include its single centre retrospective design over a time period, necessitating the use of two PET-CT scanners as for Chapter 6 and 7. However, this was mitigated by adjusting analyses for the scanner used and by restricting model development to the more recent representative scanner (and validating in the group of patients scanned using the earlier). Despite this, this represents the most appropriately powered study performed for oesophageal cancer of which the author is aware, and therefore the results and conclusions in general are likely to be robust. Whether they can be extrapolated to NACR is not clear, but as the clinical stakes are high, this should be assessed urgently.

8.4.5 Conclusions

In conclusion, this chapter found evidence that the traditional thresholds used for ΔSUVmax after NAC as a surrogate of pTR for oesophageal cancer are too low, and should be raised. Whether this is true for assessing interval response cannot be inferred, however, there was strong evidence that assessing spatial response, either in isolation or as a combination metric improves predictive ability. This, and an assessment of nodal response (which is often

discordant with the primary tumour and allows assessment of metastatic sub-clones, most likely responsible for local and distant disease relapse) should therefore be able to improve interval assessment and personalisation of both NAC and NACR).

CHAPTER 9

PREDICTING SURVIVAL AND RECURRENCE OF OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY AND SURGERY:

PATHOLOGICAL AND NOVEL ^{18}F-FDG POSITRON EMISSION TOMOGRAPHY MARKERS AND MODELS

9. CHAPTER 9: PREDICTING SURVIVAL AND RECURRENCE OF OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY AND SURGERY

9.1 SYNOPSIS

9.1.1. Introduction

There remains an urgent need to stratify prognosis after neoadjuvant therapy and surgical resection of oesophageal cancer. The aim of this chapter was firstly to determine the added utility of ^{18}F-FDG PET-CT to do so before and after surgery. Secondly, to explore the significance of discordant tumour and nodal response to NAC identified in Chapter 8. Thirdly, to validate and refine tumour 'down-staging' as a marker of prognosis. And finally, to generate prognostic models.

9.1.2. Methods

The study population comprised patients from Chapter 8. Following exclusion of 8 deaths within 30 days or in-hospital (2.65%), 294 patients with oesophageal cancer staged with PET-CT before NAC, re-staged by CT/PET-CT after NAC and successfully resected were included. Tumour pTR was defined and validated as Mandard TRG 1-3 using Cox regression analysis. mTR and mNR were defined using absolute and threshold reductions in PET parameters explored in Chapters 7 and 8. Multivariate Cox regression analysis was performed for OS, DFS and recurrence. Predictive models (LR, DTA and ANN) were generated as for Chapters 6-8.

9.1.3. Results

Before surgery, both mTR and mNR independently predicted prognosis. After surgery, pTR (but not mTR), R, pN stage and mNR predicted prognosis. The 14% of patients with FDG-avid nodal disease after NAC had a worse prognosis than non-avid and node-negative disease. They were at higher risk recurrence or death at 1 and 2 years (44.8% and 71.4%; p=0.019, p=0.024, Fisher's exact test). Down-staging was validated, but prediction was improved by considering T and N separately. A simple risk score (0-5) was generated using pTR, R status, pN stage and mNR. This was strongly associated with prognosis.

9.1.4. Conclusion

PET-CT after NAC helps stratify prognosis after NAC and surgery. mNR is a novel and important prognostic factor, independent of conventional N stage. Primary and nodal tumours often respond discordantly, are statistically independent and must be considered separately. These factors can identify high-risk patients who can be counselled and might opt for non-operative treatment strategies, and tailored follow-up.

9.2 INTRODUCTION

Despite improvements in perioperative technique and outcomes (McCulloch et al., 2003, Findlay et al., 2014a), oesophagectomy and extended total gastrectomy entail considerable risk, compounded by often profound and long term reductions in quality of life (Findlay et al., 2014d, Carrott et al., 2012, Markar et al., 2012). This is further compounded by a disappointing prognosis following seemingly successful resections (Rouvelas et al., 2005, Jamieson et al., 2004). The ability to stratify prognosis both before and after surgery would be invaluable, allowing for more individualised decisions by both patients and clinicians as regards whether to proceed to surgery, and more tailored adjuvant treatment and follow up.

As discussed in section 1.3.8, a number of markers have been investigated, mainly involving pathological stage after surgery without neoadjuvant therapy. After NAC, a major determinant of survival is pTR, but high recurrence rates indicate that other factors are important, such pN stage (albeit variably demonstrated) (Mandard et al., 1994, Schneider et al., 2005). Indeed, a recent large study reported tumour down-staging to NAC (a reduction from clinical pre-treatment to pathological post-treatment TNM 6th edition stage) and R status to be the primary determinants of survival (although notably, pTR was not assessed) (Davies et al., 2014a). In a separate analysis, the same group reported lack of pTR, progressive TNM stage, grade and a positive resection margin to predict early recurrence/death (Davies et al., 2014b).

However, PET-CT may provide complementary information. Whilst tumour SUVmax after neoadjuvant therapy negatively associates with survival, no studies have assessed nodal avidity (Swisher et al., 2004). And notably, whilst mTR associates positively with survival, it is unclear whether this represents a surrogate of pTR or has independent utility (Ott et al., 2006).

The aims of this chapter were: firstly, to identify factors before and after NAC and surgery that predict OS, DFS and recurrence. These included metrics of mTR, both routine (e.g. SUVmax in isolation) and novel metrics (e.g. composite spatial and avidity) plus the new thresholds explored in Chapter 8, and the new concepts of mN stage and mNR developed in Chapter 7. Secondly, to evaluate the prognostic significance of disparity between tumour and nodal response. Thirdly, to validate previous reports of factors predicting early recurrence and death (Davies et al., 2014a, Davies et al., 2014b). And finally, to generate and validate clinically useful prognostic models that might inform individualised treatment decisions before surgery, and allow a tailored post-operative surveillance programme.

9.3 RESULTS

9.3.1 Patients, survival, recurrence and follow-up

302 patients underwent resection after NAC (table 9.1). Eight (2.65%) deaths within 30 days or in hospital were excluded.

Median follow-up duration (using the reverse Kaplain-Meier estimator) was 1353 days (95% CI 1198-1619). There were 149 deaths and 116 instances of recurrence. One-hundred-and-five (90.5 %) of the latter were detected within 2 years. Median OS was 1008 days (791-1511). Median DFS was 918 days (647-1513; supplementary table 9.1.

Table 9.1: Patients

Factor	n=294
Patient	
Age (*Median; IQR; range)*	64.0 (58.0-70.0; 36.0-80.0)
Gender	
Male	221 (75.2%)
Female	73 (24.68)
ASA	
I	18 (6.12%)
II	216 (73.5%)
III	58 (19.7%))
IV	2 (0.68%)
Tumour	
Cell type	
AC	244 (83.0%)
SCC	42 (14.3%)
AS	5 (1.70%)
SC	1 (0.34%)
Anaplastic	2 (0.68%)
Grade of differentiation	
Well	28 (9.52%)
Moderate	124 (42.2%)
Poor	137 (46.6%)
Undifferentiated	5 (1.71%)
Tumour site	
Mid 1/3	20 (6.80%)
Distal 1/3	49 (16.7%)
GOJ 1	68 (23.2%)
GOJ 2	106 (36.1%)
GOJ 3	50 (17.0%)
Multifocal	1 (0.34%)
Pre-NAC staging	
T stage (7th edition)	
1	5 (1.70%)
2	44 (15.0%)
3	228 (77.6%)
4a	17 (5.78%)
4b	0 (0.00%)
N stage (6th edition)	
0	87 (29.6%)
1	207 (70.4%)
FDG-avid	283 (96.3%)
FDG-negative	11 (3.74%)
Initial PET-CT scanner	
Before Nov 2009	139 (47.3%)
After Nov 2009	155 (52.7%)
mN stage	
0	204 (69.4%)
1	52 (17.7%)
2	38 (12.9%)
Impassable	
No	271 (92.2%)
Yes	23 (7.82%)
Surgical approach	
Resection	
LTO	197 (67.0%)

ILO	43 (14.6%)
3 stage	10 (3.40%)
THO	1 (0.34%)
ETG	43 (14.6%)
Response to chemotherapy	
Chemotherapy	
Dual	224 (76.2%)
Triple	70 (23.9%)
pTR	
No	79 (26.9%)
Yes	214 (72.8%)
Unknown	1 (0.34%)
Overall downstaging response	
No	186 (63.3%)
Yes	105 (35.7%)
Unknown	3 (1.02%)
Restaging PET-CT scanner	
Before Nov 2009	59 (20.1%)
After Nov 2009	154 (52.3%)
CT	81 (27.6%)
mNR	
No avid nodes	133 (62.4%)
CMR	49 (23.0%)
PMR	13 (6.10 %)
SMD	14 (6.57%)
PMD	4 (1.88%)
NA	81 (NA)
Pathological stage	
pT	
0	12 (4.08%)
1	23 (7.82%)
2	79 (26.9%)
3	163 (55.4%)
4	14 (4.76%)
Unknown	3 (1.02%)
pN	
0	96 (32.7%)
1	73 (24.5%)
2	61 (20.7%)
3	61 (20.7%)
Unknown	3 (1.02%)
R status	
0	224 (76.2%)
CRM alone	57 (19.4%)
LRM alone	6 (2.04%)
CRM and LRM	4 (1.36%)
Unknown	3 (1.02%)
L	
0	131 (44.6%)
1	160 (54.4%)
NA	3 (1.02%)
V	
0	173 (58.8%)
1	118 (40.1%)
NA	3 (1.02%)

LTO=left thoracoabdominal oesophagectomy; ILO=Ivor-Lewis oesophagectomy; THO=transhiatal oesophagectomy; ETG=extended total gastrectomy

9.3.2 Markers of prognosis

Three full multivariate models were generated after exclusion of perfect separators; (1) pre-NAC adjusting for age, gender, T and N stage, ASA grade, grade, cell type, tumour site, PET-CT metrics and scanner used; (2) post-NAC this also included re-staging PET-CT and NAC data; (3) post-operatively, this included operative and pathological data. Final (restricted) models were then generated using independently predictive factors identified; for (2) this included scanner and cell type (both influencing tumour avidity, as demonstrated in Chapter 6); for (3) also pathological variables. For additional PET-CT metrics, overlapping metrics were excluded.

9.3.2.1 Pre-chemotherapy markers of prognosis

The only pre-NAC factor predicting survival was radiological nodal disease (≥N1 TNM 6th edition; supplementary table 9.2; OS HR 1.55 [1.03-2.34]; p=0.037; DFS HR 1.54 [1.03-2.31]; p=0.035).

9.3.2.2 Conventional pathological post-chemotherapy markers of prognosis

After surgery, in multivariate analysis of pathological variables pTR (TRG 1-3) was associated with better survival: HR OS 0.55 (0.32-0.97; p=0.037); DFS 0.55 (0.32-0.96; p=0.035). There were no differences between TRG 1-3, with a significant difference being demonstrated for TRG 4-5, validating this definition of pTR (supplementary table 9.3). In addition, pN (TNM 7th edition) and R status also predicted OS and DFS (supplementary table 9.4; supplementary figures 9.1-9.3).

9.3.2.3 Metabolic tumour response and prognosis

In multivariate analysis adjusting only for variables available prior to surgery (model 2), mTR independently predicted survival (table 9.2; supplementary tables 9.5-6). Assessed as continuous variables, a greater reduction in tumour avidity (logSUVmax) but not length was associated with both improved OS and DFS: HR for each percentage reduction 0.99 (0.99-1.00; p=0.017) and 0.99 (0.99-1.00; p=0.015) respectively. Of the composite metrics of avidity and tumour volume, only ΔMTV was significant in the final model: OS (HR for each percentage reduction 0.99 [0.99-1.00]; p=0.030) and recurrence (HR 0.99 [0.99-1.00];

p=0.041; supplementary table 9.3. Using the threshold values described in Chapter 8, ΔSUVmax (-77.8%) demonstrated utility: HR OS 0.46 (0.24-0.88; p=0.019; DFS 0.44 (0.23-0.83; p=0.011), as did ΔSUVpeak [-69.8%]; supplementary table 9.5).

Table 9.2: Associations of mTR and mNR with prognosis: final model

Factor	Overall Survival (n=207)		Disease-free survival		Time to recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Post-chemotherapy PET-CT						
ΔSUVmax	0.99 (0.99-1.00)	0.017	0.99 (0.99-1.00)	0.015	0.99 (0.99-1.00)	0.100
ΔLength	1.00 (1.00-1.01)	0.360	1.00 (1.00-1.01)	0.340	1.00 (0.99-1.01)	0.990
mNR						
No avid nodes	Ref	Ref	Ref	Ref	Ref	Ref
CMR	0.92 (0.51-1.66)	0.790	0.96 (0.54-1.69)	0.880	0.87 (0.44-1.72)	0.690
PMR/SMD/PMD	1.75 (0.99-3.07)	0.053	2.03 (1.16-3.55)	0.013	2.06 (1.10-3.83)	0.023
Conventional confounders						
N stage						
0	Ref	Ref	Ref	Ref	Ref	Ref
1	1.81 (1.07-3.07)	0.028	1.73 (1.03-2.90)	0.039	1.84 (1.00-3.38)	0.050
PET-CT confounders						
Scanner						
1	Ref	Ref	Ref	Ref	Ref	Ref
2	0.73 (0.46-1.16)	0.180	0.71 (0.45-1.11)	0.120	0.75 (0.44-1.28)	0.290
Cell type						
AC	Ref	Ref	Ref	Ref	Ref	Ref
SCC	1.18 (0.67-2.11)	0.570	1.09 (0.63-1.91)	0.750	0.59 (0.26-1.3)	0.190

However, adjusting for the post-operative variables identified above (model 3; pTR, pN stage and R status) these associations did not persist (table 9.3). pTR, R and pN again predicted survival, although the association between pTR and OS was borderline (p=0.071). This indicated multicollinearity between pTR and mTR. To determine whether this obscured a subtle association, data were partitioned into patients demonstrating pTR, and those not and the analysis repeated. However, again no associations were demonstrated, suggesting no additive prognostic utility of mTR beyond its use as an imperfect surrogate of pTR.

Table 9.3: Tumour metabolic response, overall survival and time to recurrence: final model

Factor	Overall Survival (n=205)		Disease-free survival		Recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
PET-CT						
ΔSUVmax (%)	1.00 (0.99-1.00)	0.540	1.00 (0.99-1.00)	0.330	1.00 (0.99-1.00)	0.440
ΔAvid length(%)	1.01 (1.00-1.01)	0.170	1.01 (1.00-1.01)	0.160	1.00 (0.99-1.01)	0.640
mNR mN0 CMR PMR/SMD/PMD	 Ref 1.16 (0.64-2.13) 2.36 (1.29-4.33)	 Ref 0.620 $5.20x10^{-3}$	 Ref 1.08 (0.60-1.93) 2.28 (1.26-4.12)	 Ref 0.810 $6.40X10^{-3}$	 Ref 0.91 (0.46-1.83) 1.96 (1.01-3.81)	 Ref 0.800 0.046
Pathological						
pTR No Yes	 Ref 0.48 (0.23-0.99)	 Ref 0.046	 Ref 0.49 (0.24-1.00)	 Ref 0.049	 Ref 0.58 (0.26-1.30)	 Ref 0.190
pN 0 1 2 3	 Ref 2.11 (1.07-4.19) 1.84 (0.88-3.83) 3.89 (1.89-7.99)	 Ref 0.032 0.110 $2.20x10^{-4}$	 Ref 2.10 (1.06-4.15) 2.33 (1.13-4.79) 3.86 (1.88-7.89)	 Ref 0.033 0.021 $2.20x10^{-4}$	 Ref 1.91 (0.87-4.20) 3.03 (1.36-6.76) 3.83 (1.71-8.60)	 Ref 0.110 $6.60x10^{-4}$ $1.10x10^{-4}$
R 0 1	 Ref 2.54 (1.53-4.20)	 Ref $2.00x10^{-4}$	 Ref 1.82 (1.11-2.98)	 Ref 0.017	 Ref 1.07 (0.59-1.96)	 Ref 0.110
PET-CT confounders						
PET-CT scanner 1 2	 Ref 0.74 (0.46-1.18)	 Ref 0.210	 Ref 0.74 (0.46-1.18)	 Ref 0.210	 Ref 0.77 (0.45-1.32)	 Ref 0.340
Cell type AC SCC	 Ref 1.01 (0.55-1.88)	 Ref 0.970	 Ref 0.98 (0.54-1.76)	 Ref 0.950	 Ref 0.60 (0.26-1.31)	 Ref 0.210

9.3.2.4 Metabolic nodal response and prognosis

Before surgery (in model 2), mNR was associated with both survival and time to recurrence. This was independent of mTR and other pre-operative variables (table 9.3; supplementary table 9.5). Patients with FDG-avid nodes before NAC undergoing a CMR had a similarly good prognosis as patients without avid nodes before NAC (figure 9.2). However, patients without a CMR (either PMR, SMD or PMD) had a worse prognosis: OS HR 1.75 (0.99-3.07; p=0.053); DFS HR 2.03 (1.16-3.55; p=0.013); recurrence HR 2.06 (1.10-3.38; p=0.023). Importantly, this was independent of pre-NAC conventional nodal stage.

Thirty-one patients did not demonstrate a nodal CMR, and had persistent FDG-avid nodes after NAC. Thirty had complete 1 year follow up. Only 16 (53.3%) were alive and disease-free at 1 year, compared with 135 (74.2%) of the 182 patients without (p=0.050; Fisher's

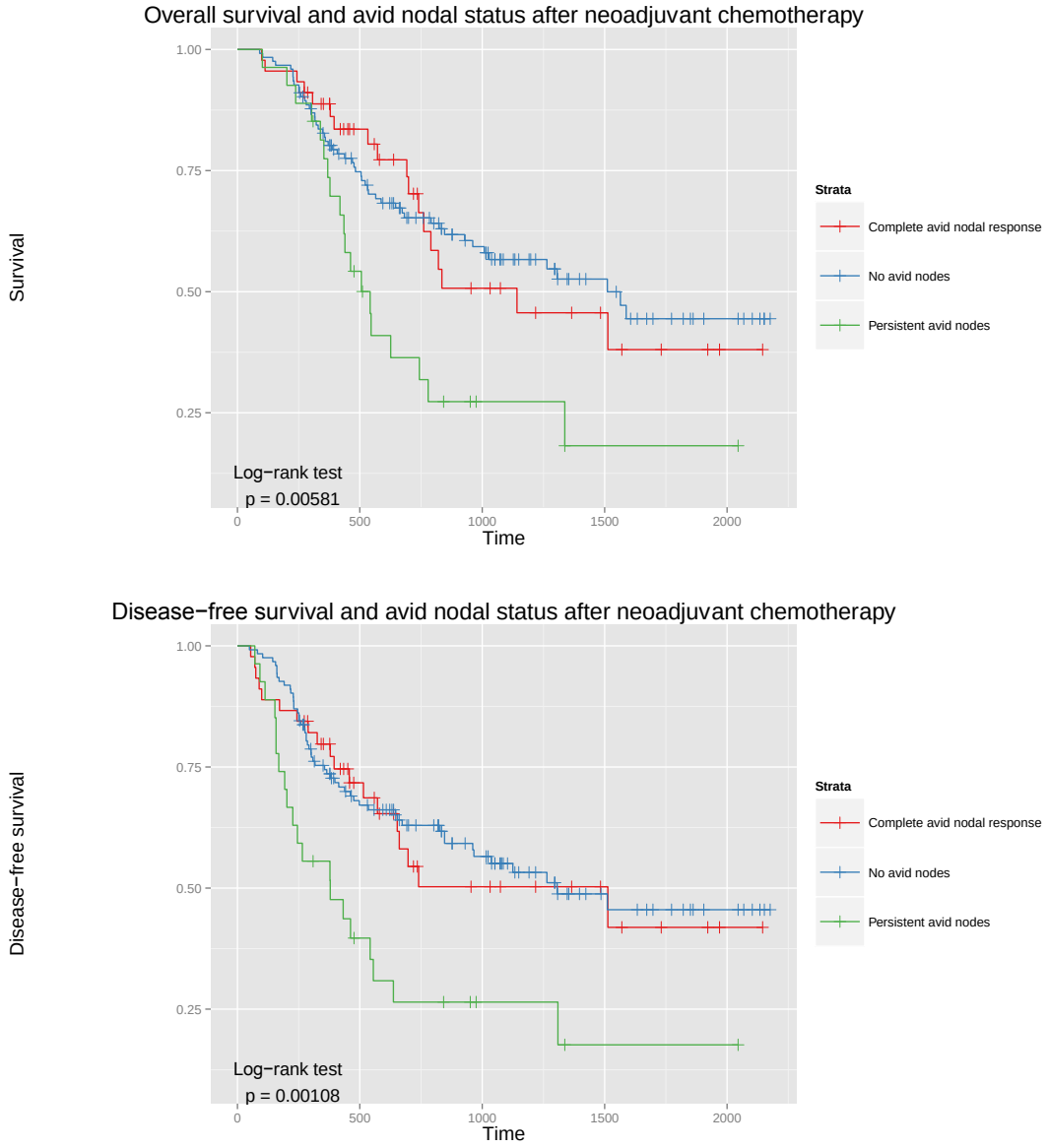
exact test). Median DFS was just 379 days (95% CI 226-1309), compared with 1511 (846-not reached); p=2.84x10^{-3}, likelihood ratio test; supplementary table 9.2).

In contrast to mTR, this association with mN stage/mNR after NAC remained associated with both OS and DFS in model 3, adjusted for post-operative variables (tables 9.2 and 9.3; supplementary table 9.6). Again patients with a nodal CMR had a similarly favourable prognosis as those without pre-NAC avid nodes. However, patients without CMR had a worse prognosis: OS HR 2.36 (1.29-4.33; p=5.20x10^{-3}; DFS 2.28 (1.29-4.12; p=6.40x10^{-3}; time to recurrence 1.96 (1.01-3.81; p=0.046). There were no significant differences when nodal PMR, SMD and PMD were considered in isolation.

As this was independent of the strong associations seen with progressive pN stage, a *post hoc* analysis was performed to explore a composite of pN and mN stage using the following groups: pN0 mN0, ≥pN1 mN0 and ≥pN1 ≥mN1 (supplementary table 9.7). Whilst patients with ≥pN1 mN0 disease had a worse prognosis (OS HR 2.07 [1.10-3.91], p=0.025; DFS 2.11 [1.120-3.97], p=0.021), for patients with ≥pN1 ≥mN0 disease this was even worse: OS 4.14 (1.83-9.30, p=6.30x10^{-4}); DFS 4.19 (1.87-9.40, p=5.10x10^{-4}),

In Chapter 7, this cohort of patients plus the 34 in whom resections were abandoned, the presence of FDG-avid nodes after NAC conferred a 25.6% probability of unsuspected unresectable disease. These data demonstrate that the remaining 73.5% of these patients have a poor prognosis, despite seemingly curative resection (table 9.2) Thirty-one patients had persistent FDG-avid nodes after NAC, 30 with full 1year follow up. Only 16 (53.3%)) were alive and disease-free at 1 year, compared with 135 (74.2%) of the 182 patients without (p=0.050; Fisher's exact test; table 2). Median DFS was just 379 days (95% CI 226-1309), compared with 1511 (846-not reached); p=2.84x10^{-3}, likelihood ratio test). Combining all patients, before surgery patients with FDG-avid nodes after NAC had only a 38.2% probability of being alive and disease-free at 1 year (11 of 42 patients, with one censored) dropping to 20.0% (8/40) at 2 years.

Figure 9.3: Overall and disease-free survival and avid nodal response to chemotherapy



9.3.2.5 Development of a simple risk score

Ultimately, 4 factors were independently associated with prognosis after NAC and surgery: pTR and R status of the primary tumour, and avidity and pN stage (TNM 7th edition) of nodal tumour (table 9.4). The HR for the first three were very similar: approximately 2, as was that of both pN1 and pN2 disease. The HR of pN3 disease was approximately twice that, at 4.

A simple risk score was then generated from 0-5, with patients scoring 1 point each for lack of pTR, R1 status and lack of a nodal CMR, plus 1 point for pN1 or pN2 disease, or 2 points for pN3). This was strongly associated with survival: OS $p=1.70 \times 10^{-10}$; DFS $p=4.74 \times 10^{-11}$;

likelihood ratio test) and recurrence ($p=2.06x10^{-8}$): patients with a score of 0-1 had an extremely good prognosis, whilst, prognosis worsened significantly and progressively with scores 2-4 (table 9.4; supplementary table 9.1; figures 9.3-4).

Figure 9.3: Risk score

Pathological response	
Mandard TRG 1-3	0
Mandard TRG 4-5	1

Resection margin	
R0	0
R1 (CRM or LRM)	1

pN stage	
pN0	0
pN1	1
pN2	1
pN3	2

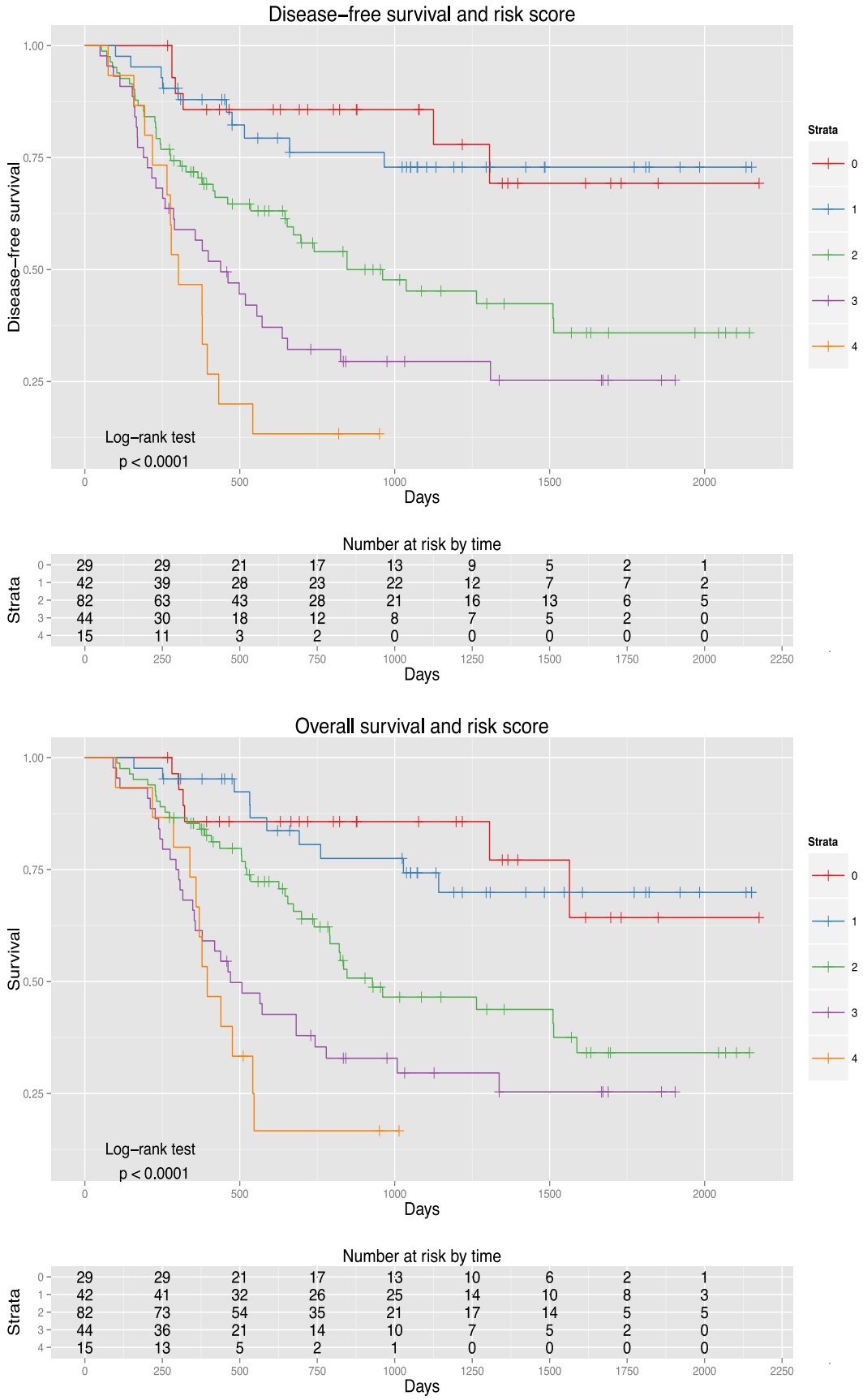
Restaging mN stage	
mN0	0
mN1-2	1

Score	Disease free survival		
	1 year	2 years	5 years
0	85.7%	81.0%	68.8%
1	87.2%	70.0%	59.3%
2	70.0%	47.6%	32.1%
3	55.9%	28.6%	18.4%
4	46.7%	14.3%	0.00%
5	NA	NA	NA

Table 9.4: Risk score and prognosis

Score (n=212)	Overall Survival		Disease-free survival		Time to recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
0 (n=29)	Ref	Ref	Ref	Ref	Ref	Ref
1 (n=42)	1.11 (0.40-3.04)	0.840	1.20 (0.44-3.30)	0.720	2.16 (0.58-7.98)	0.250
2 (n=82)	2.81 (1.19-6.65)	0.019	3.17 (1.35-7.47)	$8.30x10^{-3}$	4.57 (1.39-15.0)	0.012
3 (n=44)	5.00 (2.08-12.0)	$3.10x10^{-4}$	5.23 (2.18-12.6)	$2.10x10^{-4}$	7.61 (2.28-25.4)	$9.60x10^{-4}$
4 (n=15)	7.93 (2.95-21.3)	$4.20x10^{-5}$	8.52 (3.21-22.6)	$1.70x10^{-5}$	10.8 (2.90-40.3)	$3.80x10^{-4}$
5 (n=0)	NA	NA	NA	NA	NA	NA

Figure 9.4: Risk score and prognosis



9.3.3 Validating downstaging and comparison with pathological response

On univariate analysis, tumour downstaging was associated with OS (HR 0.60 [0.43-0.85]; $p=3.37\times10^{-3}$) and DFS (0.62 [0.44-0.87]; $p=5.55\times10^{-3}$). Adjusting for the same confounders as the original study, significance persisted for OS (HR 0.66 [0.44-1.00], p=0.049) but not DFS (HR 0.72 [0.48-1.10], p=0.130). The reasons for this were explored. Tumour downstaging was imperfectly associated with pTR (supplementary table 9.10), the latter being the stronger predictor of both OS (r^2=0.029 versus 0.065) and DFS (0.026 versus 0.074). However, when both were used in the final model, multicollinearity indicated significant overlap.

9.3.4 The value of considering tumour and nodal response independently

A number of *post hoc* analyses were then performed to explore whether considering both tumour and nodal response as independent variables improved prediction of prognosis.

9.3.4.1 Modified tumour down-staging

As conventional downstaging as previously described does not distinguish between T and N, these were considered separately in a *post hoc* analysis for both the 6th (n=291) and 7th TNM editions (n=141). For the 6th edition, response was concordant (i.e. both T & N demonstrating response, stable or progressive disease) for only 206 (69.9%) of patients; 76 (26.0%) demonstrated partial discordance (i.e. a reduction in T stage with stable N stage, or *vice versa*) whilst 12 (4.11%) demonstrated complete discordance (i.e. a progression in N stage and a reduction in T stage, *or vice versa*). Using the 7th edition, this was more pronounced: 67 (47.5%), 60 (42.6%) and 14 (9.93%) respectively.

T and N down-staging (7th edition) individually were then evaluated using multivariate analysis. Initially, response, stable disease and progressive disease were considered; whilst response of either was associated with survival, T progression was not. Therefore a binary state of response or not was used. Both T and N response independently predicted survival: T OS HR 0.39 (0.19-0.77; p=0.011) and N OS 0.36 (0.16-0.79; $p=7.20\times10^{-3}$); DFS 0.48 (0.24-0.95; p=0.035) and 0.38 (0.17-0.81; p=0.013) respectively.

9.3.4.2 Predictive utility of each classification

This was evaluated further, by using various permutations of conventional down-staging, or T/N down-staging individually, to see whether this improved prediction of 2 year survival (using sensitivity, specificity and discrimination; supplementary tables 9.11-9.12). This demonstrated that splitting down-staging into both T and N stage, significantly improved discrimination and sensitivity.

Conventional down-staging and pTR in isolation were similarly predictive. However, considering pTR in combination with nodal response (either pN or N down-staging) significantly improved AUC, sensitivity and specificity.

9.3.4.3 Discordance of pathological tumour and nodal response

A final assessment was performed, to assess discordance of tumour and nodal response (TNM 7th edition; n=141). Of patients demonstrating pTR, 24/40 (62.5%) either remained or were down-staged to pN0 disease (indicating a nodal response), 6 (15.0%) remained node positive, whilst 9 (22.5%) developed progressive nodal metastases. Conversely, for tumours not demonstrating pTR, these figures were 24/101 (23.8%), 28 (27.7%) and 49 (48.5%) respectively.

9.3.5 Validation of predictors of early recurrence and/or death

Thirty-four of 116 patients with recurrence died within 1 year and were available for validation of previously identified factors: pTNM stage, R, grade and pTR, adjusted for age and lymphovascular invasion (Davies et al., 2014b). All, except grade, demonstrated significant univariate associations, but none were significant on multivariate analysis. As this might be due to the number of events compared with the original study (n=34 versus 100), and that patients dying within a year may have undiagnosed recurrence, death *or* recurrence within 1 year (n=82) was used as a surrogate (i.e. DFS<1 year). pTNM stage demonstrated significance (for disease beyond T0-2N1). The four factors identified previously were again considered for this endpoint; again lack of pTR, R status, pN and lack of nodal CMR were strongly associated on univariate analysis. On multivariate analysis, associations were

borderline (p=0.051-0.10), but remained significant for avid nodes (OR 2.57 [1.02-6.48]; p=0.045; supplementary table 9.13).

9.3.6 Prognostic models and comparison with risk score

Models were then generated for predicting OS and DFS at 1,2 and 5 years, using the binary prediction of alive or dead. LR and DTA models performed better than ANN. Performance was best at 2 years, typically predicting OS and DFS with approximately 70-75% sensitivity, specificity, AUC and NPV (supplementary tables 9.14-9.15). As these models used a binary prediction, they could not be directly compared with the risk score above, which stratified patients from 0-5. To do so, the optimal threshold scores or the risk score were determined for each endpoint (typically a risk score of 2 or 3). This demonstrated that the risk score performed at least as well as these models, and was better supported by internal (bootstrapping) validation.

Furthermore, the risk score provided greater precision for risk stratification as described in table 9.4, supplementary table 9.1 and figures 9.3-4. Of patients with a risk score of 0/5 (i.e. a pTR, R0 resection, without nodal metastases or avidity after NAC) 85.7% were alive and disease-free at 1 year, 81.0% at years, and 68.8% at 5 years. These percentages were similar for patients with a risk score of 1. Survival reduced substantially thereafter; for patients with a risk score of 3 DFS was 55.9% at 1 year, and just 28.6% at 2 years and 18.4% at 5 years. For patients with a risk score of 4, respective DFS were just 46.7% and 14.3%, with no patients alive and disease-free at 5 years.

9.4 DISCUSSION

9.4.1 Rationale for this study

Despite refinements of neoadjuvant therapy and perioperative care, the prognosis for patients with oesophageal cancer following seemingly successful neoadjuvant therapy and surgery remains disappointing. Of particular concern is the significant risk of recurrence in patients with tumours demonstrating a pathological response (pTR). This incongruity suggests three points: that additional (perhaps unidentified) factors are important; considering the primary tumour in isolation is insufficient; and that traditional pathological characteristics (tumour stage, grade and response) provide an incomplete snapshot of tumour phenotype. By providing a biological perspective, PET is a strong candidate to provide complementary information and one that is widely used before, but less so after, NAC.

9.4.2 Summary of main findings

This chapter identifies a number of findings of potential clinical relevance. Firstly, that whilst radiological nodal stage at baseline is associated with prognosis, staging information before NAC is insufficient to personalise therapy, in agreement with the findings of Chapter 8.

Secondly, before surgery, mTR is associated with improved prognosis, in agreement with the literature. However, a thus far un-assessed parameter, mNR, is independent this. This suggests (again in agreement with Chapter 8) that for centres and trials using interval mTR in isolation to guide therapy, mNR should also be evaluated urgently as it may identify patients in whom neoadjuvant therapy should be aborted or continued with greater precision. In Chapter 7, it was found that patients with FDG-avid nodes despite NAC had a 26.5% risk of occult unresectable disease and an abandoned resection. It seems that those three quarters in whom resection can be performed, have a very poor prognosis. Combining both studies, patients with FDG-avid nodes after NAC had only a 21.5% probability of 2 year DFS. These high-risk patients can now be counselled, to allow them to balance the morbidity, mortality and quality of life implications of surgery with their prognosis.

After surgery, pTR and mTR were often discordant, and the former was the stronger and independent predictor of prognosis, in conjunction with R, pN and mNR. The independence

of the last two suggests that nodal avidity provides additional biological information. We found further considerable evidence that prognostication is dramatically improved when the primary and nodal tumours are considered independently (pTR and R, and pN and mNR respectively). These could be combined into an intuitive risk score to stratify patient risk after surgery. This performed better than more complicated models, and was reinforced by internal (bootstrapping) validation.

9.4.3 Biological consistency

These findings are consistent with theories regarding clonal evolution of cancer, in which branched evolution within a tumour gives rise to sub-clonal populations with different phenotypes (Greaves and Maley, 2012). It is therefore highly likely that the primary tumour and its nodal metastases contain different populations, with PET-CT potentially providing information as regards their relative biology (Vander Heiden et al., 2009). We found that whilst primary tumour avidity and MR was strongly associated with survival before surgery, this appeared to be due to imperfect surrogacy of pTR. Consequently, it is likely that nodal avidity and MR represents a similarly imperfect surrogacy of nodal PR, although no validated mechanisms for assessing this are in clinical use

9.4.4 Limitations

Despite efforts to ensure data reliability and adjust for confounding variables, there remain a number of limitations of this study, including its retrospective single centre design and use of pTR, which whilst a gold standard technique remains somewhat subjective (Chetty et al., 2012). Mandard *et al*'s original classification of response as TRG 1-3 (Mandard et al., 1994) was validated, however. In addition, by necessity there remains a delay between completing NAC and imaging, and so as evidenced in Chapter 5, how tumours evolve during this window is not clear. Other limitations include evolving clinical practice, with multiple NAC regimens and the use of two PET-CT scanners, although both were adjusted for.

Consequently, external validation will be required to assess generalizability to centres using different imaging platforms and software. Similarly, it is at present unclear whether these findings apply to patients treated with neoadjuvant chemoradiotherapy, although the clinical stakes involved mean that this merits evaluation.

The risk score described is intriguing, as it is easily calculated and can be used to stratify risk intuitively. Whether risk does precisely follow this linear and additive model is unlikely, but the consistency of HR for each variable (~2; ~4 for pN3) lend themselves to it. They were also very similar to those HR in individual LR models. The score was validated partly, by bootstrapping of the optimal threshold (e.g. score <3 or <2) for predicting survival, and seemed reliable. However, ultimately this will require temporal and external validation before being used to inform practice. However, at the very least it may be that follow-up resources can be focussed on (the majority) of patients with risk factors. Conversely, patients with 0 or 1 point can be cautiously reassured as to their prognosis (although perhaps less so for patients with FDG-avid nodes after chemotherapy, or a positive resection margin).

9.4.5 Conclusions

In conclusion, this chapter found that incorporating FDG-PET/CT response parameters can improve prognostic modelling in patients with oesophageal cancer undergoing NAC followed by surgery. Primary and nodal tumours often respond discordantly and must be considered separately; mNR is a new and important marker of poor prognosis, is independent of established risk factors. In particular, the 15% of patients who have FDG-avid nodes after NAC are not only at high risk of interval progression, but also medium and long term recurrence and death. Survival and recurrence risk can be stratified using a simple risk score, to identify high-risk patients. These patients could then be counselled to allow them to make a more informed decision whether to proceed with surgery or not in light of their response to NAC. After surgery, patients at higher risk for recurrence/death can be better counselled as to their prognosis, and follow-up resources deployed appropriately.

CHAPTER 10

GENERAL DISCUSSION

10 CHAPTER 10: GENERAL DISCUSSION

This thesis aimed to explore the potential of genomic, imaging and pathological markers to guide precision staging and management of BO and oesophageal cancer.

From a genetic perspective, the roles of germline and somatic markers of susceptibility to BO, dysplasia and OAC were evaluated, as were those of oesophageal cancer outcome and stage. Overall, whilst markers have frequently been associated statistically with these endpoints, that none have yet filtered into clinical practice is illuminating. The biology of these conditions is complex, with innumerable interactions between molecular processes and macroscopic and environmental modulators. This places fundamental limits on both candidate and GWAS-identified biomarkers, including those identified in Chapters 3 and 4. Beyond this, the more reliable markers (those from large-scale GWAS) appear to have modest effect sizes. Therefore the immediate translatable utility of germline SNPs is minimal, and the associations demonstrated in Chapter 3 may actually be more useful as indicators of novel biological processes rather than robust markers of individual risk. For them to constitute the latter would require external validation in an additional large robust dataset, with adjustment for established clinical risk factors.

However, relatively high estimates of heritability (Ek et al., 2013) suggest that in the future multi-SNP arrays may yet have a role in risk stratification. This might be used in combination with targeted re-sequencing of DNA and mRNA, and perhaps proteomics and metabolomics of BO epithelium (or even surveillance of 'normal' squamous epithelium), to predict risk and guide tailored intervention. A simple prototype of such an approach is currently being assessed by the BEST2 trial, using the Cytosponge to assay protein expression and CIN (Benaglia et al., 2013). This reflects the seemingly more reliable and plausible associations demonstrated for a limited number of markers of prognosis: namely *TP53* mutations, *ERBB2*/*HER2* CNV and CIN. However, future studies must move from narrow assessments, to large, methodologically robust and generalizable studies

Beyond this, these associations may be heavily influenced (and perhaps dependent) upon clonal evolution. The initial goal of Chapter 5 was to assess for novel driver genes and markers of clinical endpoints. This ultimately proved disappointing, however, by far the more interesting (and unexpected) finding was that of profound genetic changes occurring during NAC. This suggested a new model of gTR, which further complicates and limits the usefulness of markers due to clonal evolution/temporal heterogeneity during therapy.

Although this model requires refinement and validation in a larger dataset, ideally using whole genome and multi-region sequencing, it provides further evidence that precision oncology is not yet possible on the basis of biopsies alone. However, it does suggest that serial monitoring of gTR during therapy (analogous to interval assessment of mTR, which could be performed in parallel) may have a role. Perhaps even changes in *TP53* or cancer driver panels assessed with targeted resequencing might be used as a surrogate, possibly even via circulating DNA.

Ultimately, the comparison of gTR, mTR and pTR is intriguing. Is it that mTR is a surrogate of pTR, itself a surrogate of gTR, or do all three represent related but distinct concepts? As recurrence occurs in a significant minority of patients despite a pTR, resection and lymphadenectomy, perhaps additional unidentified factors are involved, or pTR as a concept is too simplistic. A pTR of sub-clones without immediate metastatic potential is arguably irrelevant, as these cells will be resected. However, if in parallel clones with metastatic potential (i.e. those ultimately causing death) do not exhibit a pTR, this resection may ultimately be futile. This is illustrated by the 'emergence' of previously unidentified clones from a genetic bottleneck. Interval molecular assessment as discussed above may be able to map and profile individual clones in this regard.

In the second half of this thesis, a comprehensive analysis of all patients with oesophageal cancer staged beyond CT in OUH from 2006-2014 was performed. This cohort was then followed through initial staging, restaging, neoadjuvant chemotherapy and resection, and beyond. These analyses were limited by a number of factors which mandate external validation, most significantly their retrospective and single-centre design, as well as spanning changes in technology and practice. However, the application of decision theory indicated in principle that routine clinical data can be used to stratify patients for personalised staging. The relative imprecision of EUS for pT1N0 disease (83% sensitivity, 84% specificity) highlights the significant chance of both under and over-staging patients. This then suggests that additional data (most notably that from PET-CT) can be used to flag up patients for repeat evaluation: for example the patient under-staged as T1N0, but with an extremely avid tumour suggestive actually of more advanced disease; or sub-centimetre avid nodes suggestive of small nodal metastases.

This potential importance and marker role of avid nodes was then refined, with the development of new concepts of mN stage and mNR. These were found to be strongly and progressively associated with disease progression during NAC. This suggests that avid nodes

despite NAC represent a metastatic, aggressive and chemoresistant clonal population, and that PET-CT can monitor their response. When considering mTR as a surrogate of pTR, SUVmax in isolation was found to be suboptimal, with prediction improved by composite avidity and volume metrics. However, again mNR appeared important, with differential response of nodes and primary tumours found to be frequent.

Beyond the immediate clinical relevance of this for precision oncology, mNR was found to be an independent marker of prognosis, above and beyond pN stage, and pTR and resection margin status of the primary tumour. By contrast, mTR appeared useful only before surgery, as an imperfect surrogate of pTR. This presence of avid nodes before NAC was previously suggested to be an adverse prognostic factor in unselected patients (Gillies et al., 2012). Whilst this does associate with a greater likelihood of disease progression, it appears rather that is the response of these nodes to NAC that is important. Patients with avid nodes before NAC that demonstrated a CMR had a similarly favourable prognosis as those without avid nodes. It is likely that this mNR represents a surrogate of pathological nodal response, although this has yet to be described. Ultimately, mNR as a prognostic factor provides a mechanism for identifying high-risk patients after NAC: those with nodal metastases that are chemoresistant, and therefore very possibly micrometastases outside a resection field. Although requiring external validation and refinement first, this may be of use before surgery, as an opportunity to counsel patients that their lack of mNR indicates just a 38.2% chance of being alive and disease-free at 1 year, reducing to 20.0% at 2 years. Ultimately, as proceeding to surgery carries the psychological, physical and quality of life costs of either a 25% chance of an abandoned laparotomy or thoracotomy, or a 75% chance of a major resection, for certain patients this may not be appropriate. After surgery, mNR in combination with the additional factors identified (perhaps in the form of a simple risk score such as that developed in Chapter 9), might be used to both inform patients as to their likely prognosis, and to allow centres to target higher risk patents for radiological follow-up. Indeed, there is limited evidence that such earlier detection and treatment of post-operative recurrence may improve survival (Jingu et al., 2012b, Jingu et al., 2012a).

An attempt to link these imaging and pathological findings with their genetic basis was made in Chapter 5, although was unsuccessful in part undoubtedly due to lack of power. However, a fascinating future study would be one performing multi-region sequencing of multiple areas of primary and nodal tumour, including nodes that were persistently FDG-avid despite NAC, those exhibiting a mNR, and those persistently non-avid. However, until the molecular

explanations for mN stage and mNR are elucidated, serial monitoring of both gTR and mNR during NAC may have the potential to fundamentally personalise patient management.

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12. SUPPLEMENTARY DATA

.

CHAPTER 3: GENETIC MARKERS OF SUSCEPTIBILITY TO BARRETT'S OESOPHAGUS, DYSPLASIA AND ADENOCARCINOMA

Section 3.3.2: Present study: GWAS replication phase 2

Supplementary table 3.1: 83 SNPs prioritised for genotyping in replication stage 3b

SNP	Chr	Position (Build 36)	iPLEX design?	iPLEX call rate	Meta-analysed OR (95%CI) for replication stage 3b	p value	Number cohorts genotyped	Meta-analysed OR (95% CI) for all phases (discovery-replication stage 3b)	p value
Discovery $P<1x10^{-4,}$ but not replicated on Immunochip									
rs6480314	10	69637453	YES	0.989	1.03 (0.90-1.16)	0.659	2	1.16 (1.08-1.24)	$2.02x10^{-4}$
rs9404438	6	104055272	YES	0.989	0.99 (0.88-1.09)	0.832	2	0.89 (0.82-0.96)	$5.08x10^{-4}$
rs4674923	2	225426527	YES	0.999	1.08 (0.98-1.17)	0.141	2	1.08 (1.02-1.14)	$1.24x10^{-2}$
rs2319335	3	147364330	YES	0.998	0.89 (0.78-1)	0.036	2	1.07 (1.01-1.14)	$4.02x10^{-2}$
rs1158793	4	96223364	YES	0.962	1.08 (0.97-1.19)	0.171	2	0.87 (0.8-0.94)	$3.43x10^{-5}$
rs1357111	7	45651069	YES	1.000	0.98 (0.82-1.14)	0.796	2	0.86 (0.76-0.95)	$1.65x10^{-3}$
rs2854925	9	135108551	YES	0.988	1.02 (0.92-1.11)	0.759	2	1.10 (1.04-1.17)	$1.65x10^{-3}$
rs12156009	8	11322629	YES	0.996	0.97 (0.88-1.06)	0.444	5	0.91 (0.87-0.96)	$1.18x10^{-4}$
rs6926118	6	3320615	NO	NA	0.98 (0.82-1.14)	0.816	3	0.86 (0.76-0.95)	$1.30x10^{-3}$
rs7042370	9	12775073	YES	0.999	1.03 (0.93-1.12)	0.619	2	0.90 (0.84-0.96)	$4.84x10^{-4}$
rs4858738	3	19826462	YES	0.998	1.04 (0.93-1.15)	0.501	2	0.88 (0.81-0.95)	$2.55x10^{-4}$
rs10903038	1	24363645	YES	0.999	0.98 (0.87-1.08)	0.656	2	1.12 (1.05-1.18)	$7.36x10^{-4}$
Meta-analysis $p<1 x10^{-4}$									
rs3072	2	20741887	YES	1.000	1.13 (1.04-1.23)	0.007	5	1.15 (1.11-1.2)	$4.04x10^{-9}$
rs6751791	2	35435501	YES	0.998	0.94 (0.85-1.03)	0.164	5	0.88 (0.84-0.93)	$1.82x10^{-7}$
rs10083033	12	105899685	YES	1.000	1.00 (0.91-1.09)	0.943	5	0.90 (0.85-0.94)	$5.82x10^{-6}$
rs189247	15	95387634	YES	0.999	0.91 (0.82-1.00)	0.049	5	0.88 (0.83-0.92)	$5.00x10^{-8}$
rs2043633	16	5759275	YES	0.998	0.88 (0.79-0.97)	0.005	5	0.87 (0.82-0.92)	$6.02x10^{-9}$

rs3923500	13	85657509	YES	1.000	0.94 (0.8-1.07)	0.336	5	1.13 (1.07-1.19)	$5.96x10^{-5}$
rs2731672	5	176842474	YES	0.999	0.91 (0.81-1.02)	0.082	5	0.88 (0.82-0.93)	$1.07x10^{-6}$
rs2701108	12	114674261	YES	0.993	0.89(0.8-0.98)	0.011	5	0.88 (0.83-0.93)	$1.39x10^{-7}$
rs3734960	7	154684153	YES	0.999	0.99(0.89-1.09)	0.814	5	1.11 (1.05-1.16)	$1.35x10^{-4}$
rs6921589	6	25530348	YES	1.000	1.01(0.88-1.14)	0.880	5	1.15 (1.08-1.21)	$6.48x10^{-5}$
rs4792891	17	41329294	YES	0.995	1.00 (0.91-1.09)	0.971	5	0.91 (0.86-0.96)	$1.44x10^{-4}$
rs7825744	8	72180729	YES	1.000	1.08(0.93-1.23)	0.316	5	1.18 (1.1-1.26)	$2.43x10^{-5}$
rs1473857	3	103118335	YES	1.000	0.98(0.87-1.1)	0.788	5	1.11 (1.05-1.17)	$4.43x10^{-4}$
rs9074	20	44122072	YES	1.000	1.02(0.92-1.12)	0.676	5	0.91 (0.86-0.97)	$6.90x10^{-4}$
rs6032951	20	10717074	NO	NA	1.06 (0.95-1.18)	0.295	5	1.13 (1.07-1.19)	$3.16x10^{-5}$
rs4742902	9	105896064	YES	1.000	1.01 (0.91-1.11)	0.815	5	0.91 (0.86-0.96)	$5.12x10^{-4}$
rs1333916	9	121635857	YES	1.000	1.03 (0.93-1.13)	0.582	5	0.92 (0.87-0.97)	$1.03x10^{-3}$
rs13087427	3	117878806	YES	0.995	1.02 (0.88-1.16)	0.776	5	0.88 (0.81-0.96)	$7.29x10^{-4}$
rs958889	11	127772438	YES	1.000	0.99 (0.88-1.09)	0.778	5	0.91 (0.85-0.96)	$2.84x10^{-4}$
rs12985909	19	18300383	YES	0.999	1.11 (1.02-1.2)	0.027	5	1.11 (1.07-1.16)	$4.20x10^{-6}$
rs4775330	15	59022174	YES	0.998	0.98 (0.88-1.07)	0.641	5	1.08 (1.03-1.13)	$1.24x10^{-3}$
rs563198	9	77521795	YES	0.999	0.94 (0.85-1.04)	0.245	5	0.90 (0.85-0.95)	$5.38x10^{-5}$
rs2902637	10	105568720	NO	NA	1.01 (0.908-1.11)	0.905	5	1.10 (1.05-1.16)	$5.71x10^{-4}$
rs851727	7	146997887	YES	1.000	1.09 (0.87-1.3)	0.444	5	0.85 (0.74-0.96)	$2.76x10^{-3}$
rs9379897	6	26709505	YES	1.000	0.86 (0.73-0.99)	0.022	5	0.86 (0.79-0.92)	$7.69x10^{-6}$
rs9524596	13	94166840	YES	0.999	0.94 (0.85-1.03)	0.156	5	0.91 (0.86-0.96)	$5.69x10^{-5}$
rs6903535	6	28525201	YES	0.999	0.98 (0.89-1.08)	0.731	5	0.92 (0.87-0.97)	$5.42x10^{-4}$
rs1325190	1	197509851	YES	1.000	1.05 (0.95-1.15)	0.369	5	0.92 (0.87-0.97)	$1.82x10^{-3}$
rs13385191	2	20751746	YES	0.999	1.05 (0.94-1.15)	0.375	5	1.11 (1.06-1.17)	$1.06x10^{-4}$
rs340620	2	20787591	YES	0.982	0.94 (0.84-1.03)	0.175	5	0.9 (0.85-0.95)	$1.52x10^{-5}$
rs12993283	2	35445909	YES	0.999	0.93 (0.84-1.02)	0.121	5	0.89 (0.84-0.93)	$2.15x10^{-7}$
rs819848	3	157218708	YES	0.994	1.02 (0.92-1.11)	0.755	5	1.10 (1.05-1.15)	$3.75x10^{-4}$
rs9824398	3	187864272	YES	1.000	1.06 (0.97-1.15)	0.241	5	1.10 (1.05-1.15)	$5.65x10^{-5}$

rs9879899	3	187864410	YES	0.993	0.95 (0.86-1.05)	0.321	5	0.91 (0.86-0.96)	$6.59x10^{-5}$
rs13157599	5	122668972	YES	1.000	0.92 (0.72-1.13)	0.461	5	1.19 (1.08-1.29)	$1.43x10^{-3}$
rs35936561	7	155880035	YES	0.998	0.98 (0.86-1.09)	0.682	5	0.91 (0.86-0.96)	$1.01x10^{-4}$
rs2898290	8	11471318	YES	0.989	0.92 (0.83-1.01)	0.083	5	0.90 (0.86-0.95)	$1.42x10^{-5}$
rs13267835	8	11542328	YES	0.998	0.97 (0.83-1.10)	0.615	5	0.87 (0.80-0.94)	$9.11x10^{-5}$
rs13273672	8	11649790	YES	0.999	0.98 (0.89-1.08)	0.739	5	1.09 (1.04-1.14)	$6.90x10^{-4}$
rs8180912	8	11677400	YES	0.999	1.00 (0.89-1.12)	0.988	5	0.90 (0.84-0.96)	$3.73x10^{-4}$
rs7895043	10	71016742	YES	0.999	1.04 (0.95-1.13)	0.428	5	1.09 (1.05-1.14)	$1.52x10^{-4}$
rs7168393	15	95353461	YES	0.999	1.01 (0.85-1.17)	0.866	5	1.14 (1.07-1.21)	$1.96x10^{-4}$
rs9941024	16	5734313	YES	1.000	0.90 (0.81-0.99)	0.030	5	0.89 (0.84-0.94)	$6.72x10^{-7}$
rs11866983	16	5743925	YES	0.997	1.09 (0.99-1.19)	0.088	5	1.12 (1.07-1.17)	$4.93x10^{-6}$
rs9936833	16	84960619	YES	0.997	1.02 (0.91-1.14)	0.669	5	1.15 (1.10-1.20)	$4.68x10^{-8}$
rs889592	16	85076757	YES	1.000	0.99 (0.87-1.11)	0.921	5	0.90 (0.83-0.96)	$3.00x10^{-4}$
Excluded									
rs2218260	15	56001502	YES	0.935	1.05 (0.96-1.14)	0.313	5	1.11 (1.06-1.16)	$1.11x10^{-5}$
rs2425752	20	44135527	YES	0.936	0.97(0.86-1.07)	0.528	5	0.90 (0.85-0.95)	$7.94x10^{-5}$
rs1935671	20	8331096	YES	0.863	1.00 (0.89-1.1)	0.935	4	1.10 (1.04-1.16)	$8.66x10^{-4}$
rs7255	2	20742301	YES	0.901	0.87 (0.78-0.96)	0.003	5	0.87 (0.83-0.92)	$1.09x10^{-8}$
rs6903130	6	32840188	NO	NA					
rs6727683	2	35429477	NO	NA					
rs7836059	8	11309574	NO	NA					
rs12677326	8	11476634	NO	NA					
rs1265496	12	1.13E+08	NO	NA					
rs12903220	15	59040471	NO	NA					
rs7173314	15	95353711	NO	NA					
rs2535483	15	95357916	NO	NA					
rs7200175	16	5742063	NO	NA					
rs1532167	16	84961705	NO	NA					

rs7187365	16	85069416	NO	NA					
rs4792827	17	41487141	NO	NA					
rs6040146	20	10701237	NO	NA					
Evidence of gender specific effect: $p<1 \times 10^{-4}$									
rs10210285	2	142746158	YES	0.998	1.06 (0.87-1.25)	0.538	2	1.14 (1.02-1.26)	3.08×10^{-2}
rs7609738	3	182733412	YES	0.999	1.06 (0.96-1.16)	0.251	2	1.05 (0.99-1.11)	9.39×10^{-2}
rs7816766	8	4523552	YES	0.991	0.88 (0.78-0.97)	0.008	2	1.12 (1.06-1.18)	2.48×10^{-4}
rs719527	11	102945919	YES	0.998	0.99 (0.88-1.1)	0.872	2	1.08 (1.01-1.15)	2.33×10^{-2}
Excluded									
rs292808	1	33819233	NO	NA					
Previously described candidate variant (MSR1)									
rs41341748	8	16056965	YES	1	1.09 (0.64-1.54)	0.701	1	1.09 (0.64-1.54)	0.701
rs2715425	15	97278619	YES	0.998	0.97 (0.86-1.09)	0.647	1	0.97 (0.86-1.09)	0.647
rs6898743	5	42638249	YES	0.999	1.03 (0.91-1.15)	0.611	1	1.03 (0.91-1.15)	0.611

Section 3.4: Fine mapping and functional annotation

Supplementary table 3.2: Annotation of rs3072, and non-coding variants in moderate/strong linkage disequilibrium

Variant			LD metrics (ref rs3072)		Variant		Sequence conserved?		Chromatin effects?		DNAse hypersensitivity?	Binding site?	eQTL in specific tissues?	Regulatory motifs altered?	Regulome DB
SNP	Chr	Pos	r^2	D'	SNP (MAF*)	Flanking genes (distance)	SiPhy	GERP	Promoter histone marks	Enhancer histone marks (cell line)		Proteins bound?			
rs3072	2	20878406	NA	NA	T/C 0.36	C2orf43 (6410b) GDF7 (7156b)	No	No	No	Yes (GM12878)	No	No	No	GATA Gfi1	5
rs10193919	2	20880833	0.92	1.00	C/T 0.36	C2orf43 (3983b) GDF7 (9583b)	Yes	Yes	No	No	No	No	No	BCL, EBF, HEY1, Rad21, YY1, Zbtb3	NA
rs9306894	2	20878105	0.97	0.99	A/G 0.36	GDF7 (6855) C2orf43 (6713)	No	Yes	No	Yes (GM12878)	Yes (NDHF-neo)	No	No	AP-1, BATF, Irf, KAP1, RXRA	1f
rs9306895	2	20878153	0.97	1.00	T/C 0.36	GDF7 (6903) C2orf43 (6665)	No	Yes	No	Yes (GM12878)	Yes (NDHF-neo)	No	No	HMG-IY, Lhx3, Pou3f3	5
rs2289081	2	20881840	0.87	0.95	G/C 0.36	C2orf43 (2978) GDF7 (10590)	No	No	No	No	No	No	No	Egr-1, NRSF, Pax-5, TCF12	NA
rs7255	2	20878820	0.60	0.95	T/C 0.53	C2orf43 (5998) GDF7 (7570)	Yes	Yes	No	Yes (GM12878)	No	No	No	ZF1, Gm397, PLZF	6
rs10170771	2	20883216	0.47	0.89	T/C 0.25	C2orf43 (1602) GDF7 (11966)	No	No	No	No	No	No	Gibbs frontal cortex, pons, temporal cortex	EWSR1-FLI1, Elf5, Ets, HDAC2	6
rs13394027	2	20882056	0.5	0.97	G/A 0.23	C2orf43 (2762) GDF7 (10806),	No	No	No	No	Yes (30 cell types)	CTCF, RAD21, SMC3	Gibbs frontal cortex, pons, temporal cortex, Schadt_Liver	Pbx3	1f
rs12622106	2	20883561	0.47	0.89	C/T 0.25	C2orf43 (1257) GDF7 (12311)	No	No	No	No	No	CJUN, JUND	Gibbs frontal cortex, pons, temporal cortex	No	1f
rs10171934	2	20884546	0.48	0.92	A/C 0.25	C2orf43 (270)	No	No	No	Yes (HepG2)	Yes (HepG2)	FOXA1, P300	CEBPA	No	4
rs10182643	2	20884586	0.48	0.92	G/C 0.25	C2orf43 (230)	No	No	No	Yes (HepG2)	Yes (HepG2)	FOXA1, P300, SP1	Gibbs frontal cortex, pons, temporal cortex	NRSF,STAT	1f
rs13385191	2	20888265	0.46	0.88	A/G 0.25	C2orf43 (intronic)	No	No	No	No	No	No	Gibbs frontal cortex, pons, temporal cortex	Esr2	6

*European frequency for for 1000 genomes phase 1; RDB=RegulomeDB score;

Supplementary table 3.3: Annotation of rs2701108, and non-coding variants in moderate/strong linkage disequilibrium

SNP	Chr	Pos	LD metrics (ref rs3072)		Variant		Sequence conserved?		Chromatin effects?		DNAse hypersensitivity?	Binding site?	eQTL in specific tissues?	Regulatory motifs altered?	Regulome DB
			R2	D'	SNP (MAF*)	Flanking genes (distance)	SiPhy	GERP	Promoter histone marks	Enhancer histone marks		Proteins bound?			
rs2701108	12	114674261	NA	NA	T/C 0.36	RBM19 (270085) TBX5 (117474)	No	No	No	No	No	No	No	BDP1, Evi-1,TBX5, ZEB1	NA
rs1247942	12	114673723	0.93	0.97	G/C 0.37	RBM19 (269547) TBX5 (118012)	No	No	No	No	No	No	No	BCL,Bcl6b, Irf, Mef2, PU.1.5, RXRA, STAT	6
rs11067002	12	114667046	0.83	0.96	T/A 0.34	RBM19 (262870) TBX5 (124689)	No	No	No	No	No	No	No	CIZ,Evi-1	NA
rs2555015	12	114673774	0.65	1.00	T/C 0.47	RBM19 (269598) TBX5 (117961)	No	No	No	No	No	No	No	Nkx2,Pbx3	NA
rs2555009	12	114666099	0.51	0.98	A/G 0.52	RBM19 (261923) TBX5 (125636)	No	No	No	Yes (HepG2)	No	No	No	No	NA
rs2555019	12	114668618	0.52	0.99	0.52	RBM19 (264442) TBX5 (123117)	No	No	No	No	No	No	No	BCL, GATA, HDAC2, HMGN3	6
rs2555016	12	114670663	0.52	0.99	T/G0.52	RBM19 (266487), TBX5 (121072)	No	No	No	No	No	No	No	BRCA1, Evi-1, PEBP	NA
rs2701109	12	114671825	0.52	0.99	T/A 0.52	RBM19 (267649), TBX5 (119910)	No	No	No	No	No	No	No	AP-3, Foxa, Pou1f1, Pou2f2, STAT	NA
rs1247943	12	114673421	0.53	0.99	G/A 0.52	RBM19 (269245), TBX5 (118314)	No	No	No	No	No	No	No	RFX5	NA
rs201604604	12	114676112	0.53	0.84	T/TAC 0.43	RBM19 (271936), TBX5 (115623)	No	No	No	Yes (K562)	No	No	No	AP-4, Myc, SREBP, Sin3Ak-20	NA
rs11382177	12	114676113	0.52	0.84	A/AC 0.44	RBM19 (271937), TBX5 (115622)	No	No	No	Yes (K562)	No	No	No	AP-4, LBP-1, LBP-9, Myc, SREBP	5
rs1270886	12	114676470	0.58	0.87	C/T 0.82	RBM19 (272294) TBX5 (115265)	No	No	No	Yes (K562)	No	No	No	NRSF	NA
rs1265496	12	114676983	0.58	0.87	C/T 0.43	RBM19 (272807) TBX5 (114752)	No	No	No	No	No	No	No	Pax-4	5
rs2555014	12	114677491	0.56	0.87	G/T 0.43	RBM19 (273315) TBX5 (114244)	No	No	No	No	No	No	No	Hoxb6, NRSF, Pdx1	6
rs2555013	12	114678318	0.60	1.00	T/C 0.50	RBM19 (274142) TBX5 (113417)	No	No	No	No	No	No	No	AP-1, Foxc1, PPAR, ZEB1	6
rs2555012	12	114678725	0.56	0.87	C/T 0.44	RBM19 (274549) TBX5 (113010)	No	No	No	No	Osteoblasts	No	No	GR, RXRA	NA

rs2252414	12	114679137	0.56	0.87	G/A 0.44	RBM19 (274961) TBX5 (112598)	No	No	No	No	No	No	No	Fox, Foxa, Foxd1, foxi1, foxj1, foxj2, foxk1, foxo, foxp1, HDAC2, TCF12, p300	6
rs1270885	12	114681552	0.49	0.79	A/G 0.44	RBM19 (277376) TBX5 (110183)	No	No	No	No	No	No	No	CDP, Irx, RXRA	NA
rs1247940	12	114682651	0.56	0.87	T/C 0.44	RBM19 (278475) TBX5 (109084)	No	No	No	No	No	No	No	Pax-5	NA
rs1247938	12	114683568	0.56	0.87	G.A 0.44	RBM19 (279392) TBX5 (108167)	No	No	No	No	12 cell types	CTCF, RAD21	No	NRSF, Spz1	2b
rs1269789	12	114684542	0.56	0.87	T/C 0.44	RBM19 (280366) TBX5 (107193)	No	No	No	No	No	No	No	Irx	NA
rs2253207	12	114685437	0.56	0.87	T/C 0.44	RBM19 (281261) TBX5 (106298	No	No	No	No	No	No	No	Nkx2	NA
rs1247928	12	114686840	0.56	0.87	T/C 0.44	RBM19 (282664) TBX5 (104895)	No	No	No	No	No	No	No	YY1,Zfp691	NA
rs1247927	12	114687056	0.56	0.87	T/C 0.44	RBM19 (282880) TBX5 (104679)	No	No	No	No	No	No	No	Foxq1, Mef2, Pou1f1, SRF, TATA	6
rs1247926	12	114687311	0.56	0.87	T/C 0.44	RBM19 (283135), TBX5 (104424)	No	No	No	No	No	No	No	Foxj1, GR, STAT	6
rs1920562	12	114660658	0.61	0.84	T/C 0.35	RBM19 (256482), TBX5 (131077)	No	No	No	Yes (H1, NHLF)	No	No	No	Evi-1, Myc, Osf2, PEBP, Six5	3a
rs12828548	12	114661066	0.61	0.84	C/G 0.35	RBM19 (256890) TBX5 (130669)	No	Yes	No	Yes (H1)	No	No	No	Foxj1, Rad21, Zfp105	6
rs11066998	12	114661789	0.64	0.88	A/T 0.35	RBM19 (257613), TBX5 (129946)	No	No	No	No	No	No	No	Bach2	NA
rs1920568	12	114669732	0.55	1.00	G/C 0.49	RBM19 (265556, TBX5 (122003)	No	Yes	No	No	No	No	No	BRCA1, Evi-1, PEBP	NA
rs1270884	12	114685571	0.60	1.00	A/G 0.50	RBM19 (281395) TBX5 (106164)	No	No	No	No	No	No	No	SRF, TFIIA	NA
rs2555004	12	114686645	0.60	1.00	G/A 0.49	RBM19 (273315) TBX5 (114244)	No	No	No	No	No	NRSF	No	Foxa, Foxc1, Six5, Znf143	5
rs201882298	12	114685665	0.51	0.83	T/TG 0.43	RBM19 (279143) TBX5 (108416)	No	No	No	Yes (H1)	No	No	No	HNF4, Pax-2	NA
rs201288186	12	114685665	0.42	0.71	TTA/T 0.41	RBM19 (281489) TBX5 (106070)	No	No	No	No	No	No	No	Dbx1, Dbx2, Evi-1, Foxk1, Foxo, Foxp1, HDAC2, HNF1, Hoxa9, Hoxd8, Irf, Mef2, Ncx, Nkx6, Pax-4, Pou2f2, Sox, TATA, Zfp105, p300	NA
rs2252923	12	114683320	0.56	0.87	A/G 0.42	RBM19 (279144) TBX5 (108415)	No	No	No	Yes (H1)	No	No	No	HNF4, Pax-2, RXRA	NA

rs200186585	12	114683322	0.49	0.79	AC/A 0.42	RBM19 (279146), TBX5 (108413)	No	No	No	Yes (H1)	No	No	No	Pax-2	NA
rs2252924	12	114683323	0.56	0.87	C/A 0.43	RBM19 (279147 TBX5 (108412)	No	No	No	Yes (H1)	No	No	No	Pax-2	NA
rs10706438	12	114685657	0.44	0.73	AT/A 0.41	RBM19 (281481) TBX5 (106078)	No	No	No	No	No	No	No	Cdx, Evi-1, Foxo, Foxp1, HDAC2, HNF1, Hoxd8, Irf, Mef2, PLZF, Zfp105, p300	6
rs35484355	12	114686244	0.51	0.83	GC/G 0.43	RBM19 (282068) TBX5 (105491)	No	No	No	No	No	No	No	LBP-1	NA

Supplementary table 3.4: Annotation of rs1920562, and non-coding variants in moderate/strong linkage disequilibrium

SNP	Chr	Pos	LD metrics (ref rs3072)		Variant		Sequence conserved?		Chromatin effects?		DNAse hypersensitivity?	Binding site?	eQTL tissues?	Regulatory motifs altered?	Regulome DB
			R^2	D'	SNP MAF	Flanking genes (distance)	SiPhy	GERP	Promoter histone marks	Enhancer histone marks (cell line)		Proteins bound?			
rs1920562	12	114660658	NA	NA	T/C 0.35	RBM19 (256482), TBX5 (131077)	No	Yes	No	Yes (H1, NHLF)	No	No	No	Evi-1, Myc, Osf2, PEBP, Six5	3a
rs12828548**	12	114661066	0.99	1.00	C/G 0.35	RBM19 (256890) TBX5 (130669)	No	Yes	No	Yes (H1)	No	No	No	Foxj1, Rad21, Zfp105	6
rs11066998**	12	114661789	0.97	1.00	A/T 0.35	RBM19 (257613), TBX5 (129946)	No	No	No	No	No	No	No	Bach2	NA
rs11067002**	12	114667046	0.83	0.96	T/A 0.34	RBM19 (262870) TBX5(124689)	No	No	No	No	No	No	No	CIZ,Evi-1	NA
rs1247942**	12	114673723	0.67	0.89	G/C 0.37	RBM19 (269547) TBX5 (118012)	No	No	No	No	No	No	No	BCL, Bcl6b, Irf, Mef2, PU.1,5, RXRA, STAT	6
rs2701108**	12	114674261	0.61	0.84	T/C 0.36	RBM19 (270085) TBX5 (117474)	No	No	No	No	No	No	No	BDP1, Evi-1, TBX5, ZEB1	NA
rs1465548	12	114656829	0.48	1.00	T/C 0.19	RBM19 (19945) TBX5 (134904)	No	No	No	No	No	No	No	Bcl, Irf, MAZR, SP1, Sin3Ak-20, TATA	NA
rs1247938**	12	114683568	0.41	0.80	G/A 0.44	RBM19 (279392) TBX5 (108167)	No	No	No	No	12 cell types	CTCF, RAD21	No	NRSF, Spz1	2b
rs1465550	12	114658037	0.50	1.00	C/T 0.20	RBM19 (18737) TBX5 (133696)	No	No	No	No	No	No	No	ERalpha-a, RXRA, Sin3Ak-20	6
rs11066995	12	114659981	0.50	1.00	C/T 0.20	RBM19 (16783) TBX5 (131752)	No	No	No	Yes (NHLF, H1)	Yes (6 cells)	CTCF	No	CTCF, PLAG1, VDR	3a
rs11066997	12	114661524	0.50	1.00	C/T 0.20	RBM19 (15250) TBX5 (130209)	No	No	No	No	No	No	No	Pou2f2, Pou3f2, Pou3f3	NA
rs11066999	12	114663333	0.50	1.00	G/T 0.20	RBM19 (13441) TBX5 (128400)	No	No	No	No	Yes (osteoblasts)	No	No	AP-1, HNF1, p300	NA
rs4766726	12	114667739	0.46	0.93	A/G 0.20	RBM19 (9035) TBX5 (123994)	No	No	No	No	No	No	No	Pou2f2	NA
rs4766727	12	114667772	0.46	0.93	G/C 0.20	RBM19 (9002) TBX5 (123961)	No	No	No	No	No	No	No	No	6
rs7980132	12	114678673	0.41	0.93	A/G 0.19	RBM19 (within) TBX5 (113060)	No	No	No	No	No	No	No	No	NA

rs11067013	12	114681027	0.41	0.93	C/T 0.19	RBM19 (1451) TBX5 (110706)	No	No	No	No	No	No	No	DMRT1, GATA, MIF-1, RFX5	NA
rs1543909	12	114632506	0.46	0.78	T/C 0.41	RBM19 (44262) TBX5 (159227)	No	No	No	No	No	No	No	Pouf51	NA
rs4767234	12	114637848	0.48	0.78	A/C 0.41	RBM19 (38926) TBX5 (153885)	No	No	No	No	No	No	No	GATA, Rad21, SRF, THAP1, YY1	6
rs1920557	12	114641793	0.48	0.78	A/G 0.41	RBM19 (34981) TBX5 (149940)	No	No	No	No	No	No	No	ERalpha-a, EGR-1, SRF	NA
rs7967272	12	114644733	0.48	0.78	T/G 0.41	RBM19 (32041) TBX5 (14700)	No	No	No	No	No	No	No	Pou2f2	6
rs978498	12	114645356	0.48	0.78	G/T 0.41	RBM19 (31418) TBX5 (146377)	No	No	No	No	No	No	No	Nkx2	5
rs978500	12	114645639	0.48	0.78	G/A 0.41	RBM19 (31135) TBX5 (146094)	No	No	No	No	No	No	No	No	6
rs2003611	12	114652636	0.49	0.82	T/C 0.42	RBM19 (24138) TBX5 (139097)	No	No	No	No	No	No	No	RXA, SMAD	6
rs714253	12	114641284	0.37	0.92	A/G 0.19	RBM19 (35490) TBX5 (150449)	No	No	No	No	No	ZNF253	No	Irf	5
rs12367740	12	114641800	0.44	0.93	C/T 0.19	RBM19 (34973) TBX5 (149933)	No	No	No	No	No	No	No	ERalpha, SP1, SRF, p53	NA
rs2081805	12	114654496	0.41	1.00	G/A 0.19	RBM19 (22278) TBX5 (137237)	No	No	No	No	No	No	No	EWSR1, GATA, HDAC2, Irf, Nanog, TATA, Zfp105, p300	6
rs1465548	12	114656829	0.48	1.00	T/C 0.19	RBM19 (19945) TBX5 (134904)	No	No	No	No	No	No	No	Bcl, Irf, MAZR, SP1, Sin3Ak-20, TATA	NA
rs1465550	12	114658037	0.50	1.00	C/T 0.19	RBM19 (18737) TBX5 (133696)	No	No	No	No	No	No	No	ERalpha-a, RXRA, Sin3Ak-20	6
rs11067004	12	114671418	0.47	0.88	A/G 0.23	RBM19 (5356) TBX5 (120315)	No	No	No	No	No	No	No	Hoxa10, Hoxc10, Hoxd10, ZBTB7A	6
rs10850292	12	114672508	0.44	0.88	C/A 0.23	RBM19 (4266) TBX5 (119225)	No	No	No	No	No	No	No	BRCA1, FAC1, Foxa, Foxj2, Foxl1, Foxp1, RREB-1	6
rs34388546	12	114683214	0.41	0.93	C/T 0.19	RBM19 (3638) TBX5 (108519)	No	No	No	H1	No	No	No	Irx	NA
rs759928	12	114610511	0.44	0.93	G/A 0.19	RBM19 (59216) TBX5 (181222)	No	No	No	No	No	No	No	Nkx2, PPAR	6
rs71467958	12	114610805	0.38	0.81	T/A 0.19	RBM19 (59510) TBX5 (180928)	No	No	No	No	No	No	No	KAP1, PU.1, SBIP, TATA	NA
rs4767232	12	114612986	0.44	0.93	G/T 0.19	RBM19 (6961) TBX5 (178747)	No	No	No	No	No	No	No	No	NA

rs12832663	12	114615472	0.42	0.87	G/A 0.19	RBM19 (31135) TBX5 (146094)	No	No	No	No	No	No	No	Foxc1, Mef2, NF-AT, Sox	6
rs11066980	12	114632071	0.44	0.93	G/A 0.19	RBM19 (44703) TBX5 (159662)	No	No	No	H1	iPS	No	No	BDP1, Zic	5
rs11066982	12	114635443	0.44	0.93	C/T 0.18	RBM19 (41331) TBX5 (156290)	No	No	No	No	No	No	No	No	NA
rs4767235	12	114637909	0.44	0.93	A/G 0.19	RBM19 (38865) TBX5 (153824)	No	No	No	No	No	No	No	ERalpha-a, RXRA, TAL1	6
rs34536114	12	114638515	0.44	0.93	T/C 0.19	RBM19 (38259) TBX5 (153218)	No	No	No	No	No	No	No	E2A, GA, RREB-1, SP1, UF1H3BETA, VDR, ZEB1, Zfp281	6
rs11066991	12	114650495	0.44	0.93	C/T 0.19	RBM19 (26279) TBX5 (141238)	No	No	No	No	No	No	No	RORalpha1	NA
rs11066975	12	114615910	0.44	0.93	G/A 0.19	RBM19 (60864) TBX5 (175823)	No	No	No	No	No	No	No	AIRE, Foxc1, Foxo, Irx, p53	NA
rs4766725	12	114617734	0.38	0.81	C/A 0.20	RBM19 (59040) TBX5 (173999)	No	No	No	No	No	No	No	NMyf	NA
rs7965491	12	114618506	0.38	0.81	C/T 0.21	RBM19 (58268) TBX5 (173227)	No	No	No	No	No	No	No	Foxo, GATA, Rad21, TAL1	NA
rs7304416	12	114620070	0.38	0.81	G/C 0.21	RBM19 (56704) TBX5 (171663)	No	No	No	No	No	No	No	Pou3f3	NA
rs16943778	12	114620336	0.36	0.80	C/T 0.21	RBM19 (56438) TBX5 (171397)	No	No	No	No	No	No	No	No	NA
rs12424280	12	114626694	0.38	0.81	G/A 0.21	RBM19 (50080) TBX5 (165039)	No	No	No	No	No	No	No	CHX10, Evi-1, GR, Hoxa4. Hoxa5, Hoxb6, Pou2f2, Pou6f1, Prrx2	6
rs10850286	12	114627326	0.38	0.81	C/G 0.21	RBM19 (49448) TBX5 (164407)	No	No	No	No	No	No	No	PEBP	NA
rs10774736	12	114628140	0.38	0.81	A/G 0.21	RBM19 (48634) TBX5 (163593)	No	No	No	No	No	No	No	HIF1::Arnt, Zbtb12	NA
rs16943814	12	114640718	0.44	0.93	C/A 0.19	RBM19 (36056) TBX5 (151015)	No	No	No	No	No	No	No	STAT	NA
rs4007267	12	114685668	0.41	0.76	A/T ?	RBM19 (6092) TBX5 (106065)	No	No	No	No	No	No	No	Cdx, Dbx1, Dbx2, Evi-1, Foxa, Foxd3, Foxp1, HDAC2, HMG-IY, HNF1, Hoxd8, Ncx, Nkx6-1, PLZF, Pax-4, Pou2f2, Sox, TATA, Zfp105, p300	6
rs2555015**	12	114673774	0.65	1.00	T/C 0.47	RBM19 (269598) TBX5 (117961)	No	No	No	No	No	No	No	Nkx2,Pbx3	6

rs201604604**	12	114676112	0.53	0.84	T/TAC 0.43	RBM19 (271936), TBX5 (115623)	No	No	No	Yes (K562)	No	No	No	AP-4, Myc, SREBP, Sin3Ak-20	NA
rs1270886**	12	114676470	0.58	0.87	C/T 0.82	RBM19 (272294) TBX5 (115265)	No	No	No	Yes (K562)	No	No	No	NRSF	NA
rs1265496**	12	114676983	0.58	0.87	C/T 0.43	RBM19 (272807) TBX5 (114752)	No	No	No	No	No	No	No	Pax-4	5
rs2555014**	12	114677491	0.56	0.87	G/T 0.43	RBM19 (273315) TBX5 (114244)	No	No	No	No	No	No	No	Hoxb6, NRSF, Pdx1	6
rs2555012**	12	114678725	0.56	0.87	C/T 0.44	RBM19 (274549) TBX5 (113010)	No	No	No	No	Osteoblasts	No	No	GR, RXRA	NA
rs2252414	12	114679137	0.41	0.80	G.A 0.44	RBM19 (within) TBX5 (112596)	No	No	No	No	No	No	No	Fox, Foxa, Foxd1, Foxi1, Foxj1, Foxj2, Foxk1, Foxo, Foxp1, HDAC2, TCF12, p300	6
rs1270885**	12	114681552	0.49	0.79	A/G 0.44	RBM19 (277376) TBX5 (110183)	No	No	No	No	No	No	No	CDP, Irx, RXRA	6
rs1247940**	12	114682651	0.56	0.87	T/C 0.44	RBM19 (278475) TBX5 (109084)	No	No	No	No	No	No	No	Pax-5	NA
rs201882298**	12	114685665	0.51	0.83	T/TG 0.43	RBM19 (279143) TBX5 (108416)	No	No	No	Yes (H1)	No	No	No	HNF4, Pax-2	NA
rs201288186**	12	114685665	0.42	0.71	TTA/T 0.41	RBM19 (281489) TBX5 (106070)	No	No	No	No	No	No	No	Dbx1, Dbx2, Evi-1, Foxk1, Foxo, Foxp1, HDAC2, HNF1, Hoxa9, Hoxd8, Irf, Mef2, Ncx, Nkx6, Pax-4, Pou2f2, Sox, TATA, Zfp105, p300	NA
rs2252923**	12	114683320	0.56	0.87	A/G 0.42	RBM19 (279144) TBX5 (108415)	No	No	No	Yes (H1)	No	No	No	HNF4, Pax-2, RXRA	NA
rs2252924**	12	114683323	0.56	0.87	C/A 0.43	RBM19 (279147 TBX5 (108412)	No	No	No	Yes (H1)	No	No	No	Pax-2	NA
rs2253207**	12	114685437	0.56	0.87	T/C 0.44	RBM19 (281261) TBX5 (106298	No	No	No	No	No	No	No	Nkx2	NA
rs10706438**	12	114685657	0.44	0.73	AT/A 0.41	RBM19 (281481) TBX5 (106078)	No	No	No	No	No	No	No	Cdx, Evi-1, Foxo, Foxp1, HDAC2, HNF1, Hoxd8, Irf, Mef2, PLZF, Zfp105, p300	6
rs35484355**	12	114686244	0.51	0.83	GC/G 0.43	RBM19 (282068) TBX5 (105491)	No	No	No	No	No	No	No	LBP-1	NA
rs1247928**	12	114686840	0.56	0.87	T/C 0.44	RBM19 (282664) TBX5 (104895)	No	No	No	No	No	No	No	YY1,Zfp691	NA

rs1247927**	12	114687056	0.56	0.87	T/C 0.44	RBM19 (282880) TBX5 (104679)	No	No	No	No	No	No	No	Foxq1, Mef2, Pou1f1, SRF, TATA	6
rs1247926**	12	114687311	0.56	0.87	T/C 0.44	RBM19 (283135), TBX5 (104424)	No	No	No	No	No	No	No	Foxj1, GR, STAT	6
rs10850289	12	114650143	0.32	0.92	C/A 0.59	RBM19 (26631), TBX5 (141590)	No	No	No	No	No	No	No	AP-3, Hoxa4, Mrg1::Hoxa9, Nkx2, Pou2f2, RORalpha1, SRF, Zfp410	NA
rs10850291	12	114655185	0.40	1.00	C/T 0.60	RBM19 (21589), TBX5 (136548)	No	No	No	No	No	No	No	Foxa, Nanog, RREB-1, Sox	6
rs1920564	12	114664513	0.43	1.00	A/T 0.60	RBM19 (12261), TBX5 (127220)	No	No	No	No	6 cell types	CTF, RAD21	No	BDP1, Irf, Klf7, MAZR, Pax-4, RXRA, SP1, STAT, Zfp281	2b

**Also presented in table 3.7 as in linkage disequilibrium with rs2701108

Section 3.4.3: Systematic review and meta-analysis of germline and somatic genetic biomarkers of susceptibility to Barrett's Oesophagus and progression

Supplementary tables 4-10 for this section are available online at http://link.springer.com/article/10.1007%2Fs10620-015-3884-5, accompanying the published version of this section.

Section 3.4.4: Identification of novel germline variants associated with progression to dysplasia, and adenocarcinoma

Supplementary table 3.5: Annotation of variants associated with progression in Discovery and UK rep2 cohorts

Variant					Sequence conserved?		Chromatin effects?		DNAse hypersensitivity?	Binding site?	Regulatory motifs altered?	RDB	eQTL	
SNP	Chr	Pos	Flanking genes (distance)	Gene function	SiPhy	GERP	Promoter histone marks	Enhancer histone marks (cell line)		Proteins bound?			HaploReg	GeneVar
rs587139	2	20812160	C2orf43 (intronic)	Unknown	No	No	No	Yes (K562)	No	No	GATA,RREB-1,THAP1	5	No	No
rs9880789	3	72069948	LINC0877 (97411), PROK2 (152900)	Unknown Circadian rhythm-related	No	No	No	No	No	No	cdx, Evi-1, Pou3f2, Pou3f3, Pou6f1	6	No	Yes- SHQ1 (ribosomal RNA function) $p=7x10^{-4}$ (Stranger 2012)
rs16964327	15	35003879	MEIS2 (intronic)	Homeotic transcriptional regulator	No	No	No	No	Yes	No	TR4, p300	5	No	No
rs7367706	1	66480444	PDE4B (intronic)	Phosphodiesterase (signal transduction)	No	No	No	No	No	No	AFP1, Arid_3a, Dlx2, ERalpha-a, Hlx1, Hoxa10, Hoxb6, Hoxb8, Hoxc9, Hoxd10, Nkx6 , RAR, Roralpha1, RXRA, Rhox11	NA	No	No
rs283741	8	128322175	CCAT1 (21659) POU5F1B (174862)	Unknown Transcriptional activator	No	No	No	Yes (GM12878, Huvec, NHEK)	No	No	AIRE,Ik-1,Ik-2	NA	No	No
rs1631576	18	43657481	SMAD2 (intronic)	TGFβ-family transcriptional regulator	No	No	No	No	No	CTCF	AP-2rep, Nanog, Pou5f1, Sox	3a (CTCF)	No	Yes (SMAD2) $p=6.8x10^{-5}$ (Stranger 2012); p=6.7x10-7 (Dimas 2009); $p=4.0x10^{-4}$ (Nica 2011); $p=1.0x10^{-6}$ (Grundberg 2012)

rs6716108	2	85537721	Y_RNA (3056) SH2D6 (20057)	Unknown Unkown	No	No	No	Yes (HMEC, NHEK)	No	No	NRSF,PPAR	NA	No	No
rs7869944	9	89422222	DAPK1 (Intronic)	Pro-apoptotic tumour suppressor	No	No	No	No	No	No	No	NA	No	No
rs997185	4	67351175	U6 (94333) CENPC1 (669407)	Splicosomal RNA Anaphase regulation	No	No	No	No	No	No	HMGN3	NA	No	Yes (STAP1: B cell antigen signalling) $p<1x10^{-3}$ (Grundberg 2012)
rs12351988	9	129053122	GARNL3 (intronic)	GTP-ase regulation	Yes	Yes	No	No	No	No	EBF, Irf, Pou2f2, STAT	6	No	No
rs10753834	1	196945716	PTPRC (intronic)	Cytokine tyrosine kinase (growth, mitosis)	No	No	No	No	No	No	No	6	No	NA
rs7722480	5	149762613	CD74 (intronic)	MHC, antigen presentation	No	No	No	No	CD4, CD20 cells	POL2	CTCF	4	No	Yes – GM2A (glycolipid transport) $p=3x10^{-4}$ (Stranger, 2012)
rs220137	21	42406163	UMODL1 (intronic)	Peptidase inhibitor, calcium binding	No	No	No	No	33 cell types	P300	Pax5	4	No	No
rs1203578	7	40864663	C7orf10 (intronic)	Co-acetyle transferase	No	No	No	No	No	No	AP-2,CEBPB,GR	NA	No	No
rs9525688	13	43393917	FAM216B (304000) C13orf30 (23028)	Unknown Unknown	No	No	No	No	No	No	No	NA	No	No
rs2937952	15	84141529	KLHL25 (2263)	Unknown	No	No	No	No	No	No	No	NA	No	Yes (AKAP13); $p=8x10^{-4}$ (Stranger 2012, Nica 2011, Grundberg 2012)
rs2763321	10	28498908	MPP7 (intronic)	Cell adhesion	No	No	No	No	No	No	NERF1a, Nanog, Nkx2, Nkx3	6	No	No
rs6718783	2	11686323	GREB1 (intronic)	Oesotrogen-mediated cell proliferation	No	No	No	No	Fibroblasts	No	No	NA	No	No

rs9544021	13	75151355	LMO7 (intronic)	Protein-protein interactions	No	No	No	HepG2	No	No	Hsf,RP58,TATA	NA	No	No
rs7679085	4	83883231	SCD5 (intronic)	Fat metabolism	No	No	No	HMEC, NHEK	Osteoblasts	No	AP-2, Bdp1, PLAG1, Rbp-J, STAT	NA	No	No
rs12406216	1	34420965	C1orf94 (intronic)	Unknown	No	No	No	No	Fibroblasts	No	MZF1	NA	No	No
rs284541	2	217077084	RPL37A (intronic)	Ribosomal subunit	No	No	No	No	BE2_C, HCT-116, HFF-Myc, Jurkat	POL2	CCNT2, GATA, Rad21, TAL1, Zec	4	No	Yes - SMARKAL1 (transcritption factor) & IGFBP2 (IGF-binding) $p=1x10^{-6}$ (Grundberg 2012), MARCH4 8(ubiquitin protein ligase) $p=7x10^{-4}$ (Nica 2011)
rs17595975	14	34610515	FAM177A1 (intronic)	Unknown	No	No	No	No	No	No	Bbx, Cphx, Duxl, Evi-1, HDAC2, HMG-IY, Pou1f1, Sox, Zfp105	6	No	No
rs1346139	15	67824893	AC009677 (14677) C15orf50 (89732)	miRNA RNA	No	No	No	No	No	No	AP-1, Hoxa7, NF-Y, ZBRK1	5	No	No
rs1936688	1	190681611	AL136987 (45269) RGS21 (78573)	miRNA G-protein coupling	No	No	No	Huvec	No	CTCF	E2A, GR, Maf, TCF-12, ZEB1	3a	No	No
rs4638779	2	65008776	LOC400958 (intronic)	RNA, unknown	No	No	No	GM12878	No	No	DMRT3, DMRT4, ELF1, PLZF	6	Yes	Yes – SLC1A4 ( ) $p=9.5x10^{-3}$ (Stranger 2012)

Supplementary table 3.4: Annotation of variants association with OAC within ChOPIN and Dutch cohorts (Immunochip analysis)

Variant					Sequence conserved?		Chromatin effects?		DNAse hypersensitivity?	Binding site?	Regulatory motifs altered?	RDB	eQTL	
SNP	Chr	Pos	Flanking genes (distance)	Gene function	SiPhy	GERP	Promoter histone marks	Enhancer histone marks (cell line)		Proteins bound?			Haplo Reg	GeneVar
rs35272229	2	197654637	ANKRDD (synonymous, within gene)	Recognition phosphoproteins	Yes	Yes	No	No	No	No	BCL, Irf, NRSF, PU.1, RAD21, SETDB1	NA	No	NA
rs13070425	3	144329972	RP11-80H8.4 (intronic) CHST2 (5469)	LincRNA Sulphite-dependent lymphocyte homing	No	No	No	Yes (GM12878, NHLF)	Yes (Ha-sp, HCPEpiC, HMVEC-LBl, HMVEC-dBL-Ad, HVMF, WI-38)	No	ETS, GATA, Mef2, Nanog, PRDM1, Pax-5, p300)	5	No	No
rs12658568	5	31139623	RP11-152K4.2 (intronic) CDH6 (89894)	Pseudogene Cell adhesion	No	No	No	No	No	No	Arid3a, Cdx2, MHG-IY, Hoxa10, Hoxb13, Hoxb9, Hoxd10, Pou3f2, Pou3f3, Sox2, Sox-19, Zfp105	6	No	Yes (CDH6) p<1x10-5 (Grundberg 2012, A)
rs12515934	5	40412687	AC108105.1 (56412) PTGER4 (303100)	miRNA Prostaglandin 4 receptor	No	No	No	Yes (GM12878)	CD20+,GM12864, GM12865	SPI1	Foxo, Foxp1, Irf, Pou2f2, Sox3, Sox13, Sox6, Sox7, TATA, Zfp105, p300	2a (SPI1)	No	No
rs6601062	5	178998248	C5orf60 (3902)	Unknown	No	No	No	No	Chorion,Hepatocytes,ProgFib	No	Foxa, p300	6	No	Yes – SqSTM1 (differentiation, apoptosis) p=8.00x10^{-4} (Stranger 2012, L)
rs3734262	6	34946555	UHRF1BP1 (intronic)	Cell growth regulator	No	No	No	No	No	No	Foxa, p300	NA	No	Yes – CLPS (lipid hydrolysis) p=9x10^{-4} (Stranger 2012)
rs1737727	6	35012466	ANKS1A (intronic)	Cell growth	No	No	No	No	HSMMtube	No	Irf7	NA	No	No

rs1996913	6	35108431	ANKS1A (intronic)	Cell growth	No	No	No	Yes (HepG2)	No	No	Nkx2, p300	NA	No	No
rs73411720	6	35295138	DEF6 (5607)	Th2 activation	No	No	No	Yes (HepG2)	No	No	Ap-1, CPhx	4	No	NA
rs4750290	10	13045926	CCDC3 (intronic)	Fat (abdominal) metabolism	No	No	No	No	No	No	Myc,Sin3Ak-20,XBP-1	NA	No	No
rs7923097	10	61634552	ANK3 (intronic)	Cell adhesion and motility	No	No	No	No	No	No	AIRE,AP-1,GATA	NA	No	NA
rs2801539	10	90830632	MIR4679 (17483)	microRNA	No	No	No	Yes (NHLF, Huvec)	Yes (ADI.MSC, MUS.SC, CD34, MSC.ADIPC, CD4.MP, PFF.1)	GATA1, GATA2	No	4	No	No
rs1048932	11	114550060	CADM1 (intronic)	Cell adhesion	Yes	Yes	No	No	No	No	AP-1, Irf1, RFX5, RREB-1, TATA	6	Yes – Gibbs Cerebellum, Frontal Cortex, Pons, Temporal cortex	Yes – CADM1 p=1.1x10-11 (Nica, 2011, L, A, S) & (Grundberg, 2012 A, L, S) ZBTB16 (tissue differentiation) $p=1x10^{-3}$ (Stranger 2012; L)
rs7132203	12	38787992	SLC2A13 (2063)	Glucose transporter	No	No	Yes (NHLF, HepG2, GM12878, K562)	Yes (HSMM, HMEC)	No	Yes (CTCF)	SRF,Sp100, p300	4	No	NA

A=adipocytes; L=lymphoblastoid; S=skin

Section 3.4.5: Gene set enrichment (pathway) analysis

Supplementary table 3.7: Significantly enriched iGSEA canonical pathways for Barrett's Oesophagus

Pathway/Gene set name	All genes	P-value	FDR	Significant genes	Selected genes	n SNPs
HSA04612 ANTIGEN PROCESSING AND PRESENTATION	83	$1.00x10^{-3}$	$5.50x10^{-3}$	26	63	734
HSA04940 TYPE I DIABETES MELLITUS	45	$<1.00x10^{-3}$	$7.00x10^{-3}$	28	42	1223
CARM	30	$< 1.00x10^{-3}$	$1.22x10^{-2}$	14	22	1106
HSA04514 CELL ADHESION MOLECULES	134	$<1.00x10^{-3}$	$1.22x10^{-2}$	70	121	7817
HSA04130 SNARE INTERACTIONS IN VESICULAR TRANSPORT	36	$7.00x10^{-3}$	0.1934	15	31	NA
STRESSPATHWAY	25	$6.00x10^{-3}$	0.215	12	24	NA
BREAST CANCER ESTROGEN SIGNALING	101	$2.00x10^{-3}$	0.217	44	87	NA
HDACPATHWAY	30	$6.00x10^{-3}$	0.227	18	29	NA
HSA00230 PURINE METABOLISM	145	$2.00x10^{-3}$	0.229	57	129	NA

ES=enrichment score; NES=normalised enrichment score

Supplementary table 3.8: Significantly enriched GenGen canonical pathways for Barrett's Oesophagus

Pathway/Gene set name	n genes	ES	NES	p	FDR	FWER
STARCH AND SUCROSE METABOLISM	31	0.904	8.273	$<1.00x10^{-3}$	$<1.00x10^{-3}$	$<1.00x10^{-3}$
HSA00052 GALACTOSE METABOLISM	31	0.913	8.146	$<1.00x10^{-3}$	$<1.00x10^{-3}$	$<1.00x10^{-3}$
TRYPTOPHAN METABOLISM	52	0.651	6.077	$<1.00x10^{-3}$	$<1.00x10^{-3}$	$<1.00x10^{-3}$
HSA03320 PPAR SIGNALING	66	0.503	4.788	$<1.00x10^{-3}$	$<1.00x10^{-3}$	0.001
HSA00230 PURINE METABOLISM	141	0.454	4.138	$<1.00x10^{-3}$	0.001	0.007
TOLLPATHWAY	31	0.618	3.839	$<1.00x10^{-3}$	0.002	0.022
PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	82	0.518	3.656	$<1.00x10^{-3}$	0.005	0.048
HSA00564 GLYCEROPHOSPHOLIPID METABOLISM	66	0.496	3.647	$<1.00x10^{-3}$	0.004	0.049
HSA04920 ADIPOCYTOKINE SIGNALING	70	0.495	3.627	0.001	0.005	0.054
GAMMA HEXACHLOROCYCLOHEXANE DEGRADATION	29	0.652	3.211	$<1.00x10^{-3}$	0.014	0.184
HSA04742 TASTE TRANSDUCTION	49	0.534	3.081	0.002	0.020	0.260
GLYCEROLIPID METABOLISM	43	0.548	2.935	0.003	0.029	0.362
HSA00591 LINOLEIC ACID METABOLISM	31	0.561	2.891	0.002	0.031	0.392
WNT SIGNALING	56	0.534	2.858	0.002	0.032	0.414
HSA00510 N GLYCAN BIOSYNTHESIS	42	0.509	2.843	0.001	0.032	0.420
PPARAPATHWAY	50	0.511	2.640	0.003	0.055	0.611
HSA05120 EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	66	0.426	2.597	0.001	0.061	0.651
N GLYCAN BIOSYNTHESIS	22	0.564	2.577	0.001	0.062	0.669
OVARIAN INFERTILITY GENES	24	0.591	2.574	0.005	0.060	0.671
ALKPATHWAY	31	0.590	2.558	0.003	0.060	0.683
HSA04940 TYPE I DIABETES MELLITUS	42	0.544	2.493	0.013	0.070	0.746
NFKBPATHWAY	22	0.560	2.403	0.008	0.086	0.813
HSA04910 INSULIN SIGNALING PATHWAY	130	0.392	2.288	0.011	0.112	0.878

HSA04110 CELL CYCLE	108	0.370	2.233	0.023	0.124	0.896
HSA00790 FOLATE BIOSYNTHESIS	42	0.422	2.232	0.020	0.120	0.897
INFLAMPATHWAY	28	0.501	2.104	0.021	0.158	0.957
HSA04530 TIGHT JUNCTION	131	0.425	2.096	0.020	0.157	0.962
ANDROGEN AND ESTROGEN METABOLISM	21	0.549	2.038	0.016	0.174	0.975
HSA00251 GLUTAMATE METABOLISM	31	0.469	1.993	0.012	0.188	0.981
HSA00980 METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	69	0.447	1.976	0.024	0.190	0.984
ST TUMOR NECROSIS FACTOR PATHWAY	29	0.467	1.958	0.025	0.193	0.986
STRESSPATHWAY	25	0.509	1.944	0.031	0.194	0.988
HSA00602 GLYCOSPHINGOLIPID BIOSYNTHESIS NEO LACTOSERIES	21	0.520	1.943	0.017	0.190	0.988
ST FAS SIGNALING PATHWAY	60	0.383	1.939	0.028	0.186	0.988
HSA04612 ANTIGEN PROCESSING AND PRESENTATION	74	0.415	1.936	0.015	0.183	0.988
HSA05010 ALZHEIMERS DISEASE	27	0.495	1.933	0.027	0.180	0.988
NUCLEAR RECEPTORS	39	0.550	1.926	0.029	0.179	0.988
HSA04350 TGF BETA SIGNALING PATHWAY	89	0.460	1.897	0.024	0.186	0.988
HSA00561 GLYCEROLIPID METABOLISM	55	0.475	1.878	0.032	0.191	0.992
GLUTAMATE METABOLISM	24	0.491	1.855	0.025	0.197	0.994
GPCRDB CLASS A RHODOPSIN LIKE	156	0.410	1.797	0.039	0.219	0.998
NTHIPATHWAY	22	0.508	1.778	0.048	0.223	0.998
HSA00252 ALANINE AND ASPARTATE METABOLISM	32	0.453	1.776	0.029	0.220	0.998
STRIATED MUSCLE CONTRACTION	34	0.429	1.712	0.036	0.248	1.000
HSA05220 CHRONIC MYELOID LEUKEMIA	76	0.404	1.699	0.047	0.250	1.000

FDR=false discovery rate; FWER=family-wise error rate

Supplementary table 3.9: Genes shared by the HSA 04940 Type I Diabetes Mellitus and HSA04612 Antigen Processing and Presentation canonical pathways

Shared genes	Function
HLA-B	MHC I antigen
HLA-DQA2	MHC 2 antigen
HLA-DQB1	MHC 2 antigen
HLA-DQA1	MHC 2 antigen
HLA-DRA	MHC 2 antigen
HLA-DOB	MHC 2 antigen
HLA-C	MHC I antigen
HLA-DQB2	MHC 2 antigen
LTA (TNFA)	Inflammatory cytokine
HLA-DRB5	MHC 2 antigen
HLA-DRB1	MHC 2 antigen
HLA-F	MHC I antigen
HLA-G	MHC I antigen

MHC=major histocompatibility complex

Supplementary table 3.10: Significantly enriched iGSEA GO biological processes for Barrett's Oesophagus

Pathway/Gene set name	All genes	P value	FDR	Significant genes	Selected genes
VESICLE MEDIATED TRANSPORT	194	< 0.001	$6.00x10^{-3}$	82	175
GOLGI VESICLE TRANSPORT	48	< 0.001	0.0375	22	45
POSITIVE REGULATION OF CELL DIFFERENTIATION	25	< 0.001	0.099	16	22
SECRETORY PATHWAY	84	0.0020	0.100	36	78
REGULATION OF GENE EXPRESSION EPIGENETIC	30	0.0020	0.1075	13	27
CALCIUM ION TRANSPORT	27	0.0010	0.166	17	26
SECRETION BY CELL	117	0.0040	0.167	48	108
POSITIVE REGULATION OF DEVELOPMENTAL PROCESS	218	0.0020	0.188	89	195
PROTEIN MODIFICATION BY SMALL PROTEIN CONJUGATION	43	0.0080	0.195	18	41
NEURON DIFFERENTIATION	76	0.0010	0.200	43	71
CELLULAR MORPHOGENESIS DURING DIFFERENTIATION	49	0.0030	0.217	29	46
DI TRI VALENT INORGANIC CATION TRANSPORT	32	0.0050	0.230	19	31
NEURITE DEVELOPMENT	52	0.0050	0.231	30	50
EMBRYONIC DEVELOPMENT	58	0.0030	0.248	32	53

FDR=false discovery rate

Supplementary table 3.11: Significantly enriched GenGen GO biological processes for Barrett's Oesophagus

Pathway/Gene set	n genes	ES	NES	P	FDR	FWER
CELLULAR MACROMOLECULE CATABOLIC PROCESS	100	0.773	13.703	<0.00001	0.000	<0.00001
MACROMOLECULE CATABOLIC PROCESS	132	0.711	13.589	<0.00001	0.000	<0.00001
BIOPOLYMER CATABOLIC PROCESS	114	0.735	13.420	<0.00001	0.000	<0.00001
CARBOHYDRATE METABOLIC PROCESS	170	0.672	11.684	<0.00001	0.000	<0.00001
CELLULAR CARBOHYDRATE METABOLIC PROCESS	119	0.724	11.093	<0.00001	0.000	<0.00001
CARBOHYDRATE CATABOLIC PROCESS	22	0.938	6.107	<0.00001	0.000	<0.00001
CELLULAR CARBOHYDRATE CATABOLIC PROCESS	21	0.942	5.741	<0.00001	0.000	<0.00001
SEXUAL REPRODUCTION	124	0.432	4.514	<0.00001	0.000	0.00100
GAMETE GENERATION	103	0.452	4.491	<0.00001	0.000	0.00100
CELLULAR LIPID CATABOLIC PROCESS	34	0.615	4.400	<0.00001	0.000	0.00300
LIPID CATABOLIC PROCESS	37	0.593	4.225	<0.00001	0.001	0.00900
CYTOKINE BIOSYNTHETIC PROCESS	38	0.561	3.942	<0.00001	0.003	0.02100
FATTY ACID METABOLIC PROCESS	62	0.486	3.900	<0.00001	0.003	0.02300
TISSUE DEVELOPMENT	133	0.468	3.861	<0.00001	0.003	0.02700
CYTOKINE METABOLIC PROCESS	39	0.551	3.836	<0.00001	0.003	0.03100
STEROID METABOLIC PROCESS	68	0.481	3.746	0.00100	0.004	0.04300
ION TRANSPORT	176	0.465	3.467	<0.00001	0.009	0.10900
MEIOTIC CELL CYCLE	33	0.558	3.410	<0.00001	0.011	0.12700
ORGANIC ACID METABOLIC PROCESS	172	0.406	3.269	<0.00001	0.015	0.18800
CENTRAL NERVOUS SYSTEM DEVELOPMENT	118	0.541	3.256	0.00200	0.015	0.19100
MESODERM DEVELOPMENT	20	0.689	3.202	0.00100	0.017	0.21400
CARBOXYLIC ACID METABOLIC PROCESS	170	0.404	3.156	<0.00001	0.019	0.23900
DNA REPLICATION	96	0.380	3.146	0.00200	0.019	0.24100

MONOCARBOXYLIC ACID METABOLIC PROCESS	85	0.438	3.082	0.00200	0.021	0.28300
DNA RECOMBINATION	45	0.478	3.064	0.00100	0.022	0.30400
MEIOSIS I	20	0.691	3.055	<0.00001	0.021	0.30700
RESPONSE TO BACTERIUM	28	0.549	3.021	<0.00001	0.022	0.32800
POSITIVE REGULATION OF I KAPPAB KINASE NF KAPPAB CASCADE	81	0.411	3.019	0.00100	0.022	0.33000
GLYCOPROTEIN METABOLIC PROCESS	88	0.468	2.988	0.00400	0.023	0.35400
DETECTION OF EXTERNAL STIMULUS	23	0.612	2.947	<0.00001	0.025	0.39100
REPRODUCTIVE PROCESS	152	0.397	2.856	0.00200	0.031	0.46800
DI TRI VALENT INORGANIC CATION TRANSPORT	32	0.609	2.726	0.00400	0.045	0.58000
REGULATION OF PROTEIN SECRETION	21	0.571	2.596	0.00800	0.064	0.71400
RECEPTOR MEDIATED ENDOCYTOSIS	31	0.535	2.443	0.00800	0.093	0.83700
I KAPPAB KINASE NF KAPPAB CASCADE	106	0.382	2.436	0.00800	0.092	0.84500
CATION TRANSPORT	140	0.459	2.428	0.00700	0.091	0.85000
EMBRYONIC DEVELOPMENT	54	0.573	2.382	0.01000	0.100	0.88200
NUCLEOBASE NUCLEOSIDE AND NUCLEOTIDE METABOLIC PROCESS	49	0.453	2.354	0.01100	0.105	0.89700
DEFENSE RESPONSE TO BACTERIUM	23	0.536	2.315	0.00200	0.113	0.92200
DEVELOPMENT OF PRIMARY SEXUAL CHARACTERISTICS	26	0.580	2.310	0.00600	0.112	0.92400
PROTEIN AMINO ACID N LINKED GLYCOSYLATION	29	0.564	2.309	0.01200	0.109	0.92400
CELLULAR HOMEOSTASIS	131	0.436	2.293	0.01200	0.110	0.93700
REGULATION OF I KAPPAB KINASE NF KAPPAB CASCADE	87	0.381	2.273	0.01000	0.113	0.94800
DEPHOSPHORYLATION	67	0.489	2.225	0.01000	0.125	0.96500
DETECTION OF STIMULUS	45	0.482	2.217	0.01800	0.125	0.96700
LIPID BIOSYNTHETIC PROCESS	92	0.394	2.174	0.02000	0.136	0.97900
REGULATION OF CYTOSKELETON ORGANIZATION AND BIOGENESIS	28	0.505	2.159	0.01300	0.138	0.98300
CELL CELL ADHESION	85	0.499	2.155	0.01700	0.137	0.98400
CALCIUM ION TRANSPORT	27	0.603	2.131	0.01800	0.141	0.98600
POSITIVE REGULATION OF CYTOKINE BIOSYNTHETIC PROCESS	23	0.533	2.122	0.00600	0.141	0.98900
ANION TRANSPORT	29	0.486	2.116	0.01700	0.140	0.99100
PROTEIN AMINO ACID DEPHOSPHORYLATION	60	0.504	2.084	0.01600	0.148	0.99400
PHOSPHOINOSITIDE MEDIATED SIGNALING	44	0.527	2.055	0.02600	0.155	0.99400
EPIDERMIS DEVELOPMENT	69	0.401	2.050	0.02500	0.154	0.99500
MULTI ORGANISM PROCESS	156	0.355	2.043	0.02100	0.154	0.99500
CALCIUM INDEPENDENT CELL CELL ADHESION	22	0.546	2.038	0.01000	0.153	0.99600
VIRAL REPRODUCTIVE PROCESS	36	0.461	2.034	0.02600	0.151	0.99600
NEURON DEVELOPMENT	60	0.541	2.034	0.02700	0.149	0.99600
SECRETION	174	0.388	2.026	0.01800	0.149	0.99600
REGULATION OF HEART CONTRACTION	24	0.577	2.018	0.02500	0.149	0.99600
MEMBRANE LIPID METABOLIC PROCESS	94	0.376	2.001	0.02500	0.153	0.99600
MUSCLE DEVELOPMENT	87	0.469	1.993	0.02400	0.154	0.99600
POSITIVE REGULATION OF SECRETION	20	0.581	1.974	0.02100	0.157	0.99800
POSITIVE REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	35	0.511	1.966	0.02700	0.158	0.99800
VIRAL REPRODUCTION	41	0.443	1.963	0.02500	0.157	0.99800
REGULATION OF SECRETION	39	0.486	1.961	0.02500	0.155	0.99800
BONE REMODELING	28	0.561	1.933	0.02900	0.164	0.99900

G PROTEIN SIGNALING COUPLED TO IP3 SECOND MESSENGER PHOSPHOLIPASE C ACTIVATING	41	0.526	1.917	0.03600	0.167	0.99900
PROTEIN SECRETION	31	0.496	1.906	0.03600	0.169	0.99900
CYTOKINE PRODUCTION	69	0.438	1.901	0.03200	0.168	0.99900
SECRETION BY CELL	114	0.402	1.894	0.02700	0.169	0.99900
HEART DEVELOPMENT	34	0.544	1.892	0.02900	0.167	0.99900
TISSUE REMODELING	29	0.551	1.857	0.03700	0.179	1.00000
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	25	0.582	1.853	0.03300	0.178	1.00000
ION HOMEOSTASIS	114	0.407	1.837	0.03700	0.182	1.00000
CARBOHYDRATE BIOSYNTHETIC PROCESS	44	0.539	1.825	0.03400	0.184	1.00000
M PHASE	107	0.312	1.779	0.04500	0.201	1.00000
LEUKOCYTE ACTIVATION	64	0.432	1.766	0.03700	0.204	1.00000
REGULATION OF ORGANELLE ORGANIZATION AND BIOGENESIS	37	0.465	1.760	0.03500	0.204	1.00000
NEURITE DEVELOPMENT	52	0.548	1.757	0.04500	0.203	1.00000
CELLULAR CATION HOMEOSTASIS	92	0.424	1.737	0.04800	0.209	1.00000
ACTIN FILAMENT BASED PROCESS	112	0.390	1.735	0.04600	0.207	1.00000
STEROID BIOSYNTHETIC PROCESS	22	0.507	1.725	0.04400	0.210	1.00000
NUCLEOTIDE METABOLIC PROCESS	39	0.461	1.720	0.04000	0.209	1.00000
HOMEOSTATIC PROCESS	190	0.396	1.714	0.04500	0.209	1.00000
CATION HOMEOSTASIS	95	0.419	1.697	0.04100	0.214	1.00000
ACTIN FILAMENT ORGANIZATION	24	0.463	1.681	0.02300	0.219	1.00000
VIRAL INFECTIOUS CYCLE	32	0.441	1.677	0.04800	0.218	1.00000
NITROGEN COMPOUND CATABOLIC PROCESS	28	0.467	1.671	0.05300	0.218	1.00000
METAL ION TRANSPORT	114	0.459	1.642	0.05400	0.228	1.00000
GLYCOPROTEIN BIOSYNTHETIC PROCESS	73	0.432	1.609	0.05300	0.242	1.00000
REGULATION OF GENE EXPRESSION EPIGENETIC	29	0.459	1.608	0.04000	0.239	1.00000
RESPONSE TO WOUNDING	180	0.349	1.608	0.05500	0.237	1.00000
ENERGY DERIVATION BY OXIDATION OF ORGANIC COMPOUNDS	35	0.437	1.588	0.06800	0.245	1.00000
ANATOMICAL STRUCTURE FORMATION	54	0.502	1.576	0.05900	0.248	1.00000
FEMALE PREGNANCY	51	0.410	1.575	0.07200	0.246	1.00000
NITROGEN COMPOUND METABOLIC PROCESS	144	0.380	1.571	0.06100	0.245	1.00000
EXTRACELLULAR STRUCTURE ORGANIZATION AND BIOGENESIS	32	0.491	1.568	0.05800	0.244	1.00000

FDR=false discovery rate; FWER=family-wise error rate

Supplementary table 3.12: Significantly enriched iGSEA canonical pathways for progression to dysplasia/carcinoma

Pathway/Gene set name	P-value	FDR	Significant genes	Selected genes	All genes
DEATHPATHWAY	$<1.00\times10^{-3}$	$<1.00\times10^{-3}$	17	32	33
APOPTOSIS	$<1.00\times10^{-3}$	2.00×10^{-3}	30	63	71
HSA04210 APOPTOSIS	$<1.00\times10^{-3}$	0.011	33	75	84
SIG PIP3 SIGNALING IN B LYMPHOCYTES	$<1.00\times10^{-3}$	0.017	19	29	36
HSA04520 ADHERENS JUNCTION	$<1.00\times10^{-3}$	0.019	45	71	75
HSA00410 BETA ALANINE METABOLISM	$<1.00\times10^{-3}$	0.020	15	22	25
P38MAPKPATHWAY	$<1.00\times10^{-3}$	0.020	21	34	40
KERATINOCYTEPATHWAY	$<1.00\times10^{-3}$	0.020	25	41	46
HIVNEFPATHWAY	$<1.00\times10^{-3}$	0.021	23	53	58
HSA04650 NATURAL KILLER CELL MEDIATED CYTOTOXICITY	$<1.00\times10^{-3}$	0.021	50	112	132
CASPASEPATHWAY	2.00×10^{-3}	0.023	10	21	23
HSA04670 LEUKOCYTE TRANSENDOTHELIAL MIGRATION	1.00×10^{-3}	0.027	48	96	115
ST ADRENERGIC	1.00×10^{-3}	0.029	19	32	37
HSA04360 AXON GUIDANCE	$<1.00\times10^{-3}$	0.034	66	114	128
ST MYOCYTE AD PATHWAY	$<1.00\times10^{-3}$	0.034	14	22	28
CHEMICALPATHWAY	7.00×10^{-3}	0.047	10	20	22
CIRCADIAN EXERCISE	4.00×10^{-3}	0.053	17	40	47
HSA05216 THYROID CANCER	4.00×10^{-3}	0.057	16	28	29
NFKBPATHWAY	4.00×10^{-3}	0.060	12	21	23
MAPKPATHWAY	4.00×10^{-3}	0.066	37	79	87
HSA04930 TYPE II DIABETES MELLITUS	5.00×10^{-3}	0.073	23	42	44
CREBPATHWAY	6.00×10^{-3}	0.075	14	24	27
ST TUMOR NECROSIS FACTOR PATHWAY	9.00×10^{-3}	0.075	11	27	29
CALCIUM REGULATION IN CARDIAC CELLS	2.00×10^{-3}	0.078	69	124	143
APOPTOSIS GENMAPP	6.00×10^{-3}	0.081	19	41	44
HSA05222 SMALL CELL LUNG CANCER	8.00×10^{-3}	0.087	38	79	87
HSA04664 FC EPSILON RI SIGNALING PATHWAY	9.00×10^{-3}	0.094	31	70	75
HSA04020 CALCIUM SIGNALING PATHWAY	8.00×10^{-3}	0.108	78	150	175
ST DIFFERENTIATION PATHWAY IN PC12 CELLS	0.01	0.112	19	37	47
CXCR4PATHWAY	0.017	0.133	13	22	24
BETA ALANINE METABOLISM	0.015	0.137	14	25	27
SIG PIP3 SIGNALING IN CARDIAC MYOCTES	0.014	0.149	28	55	67
HSA00650 BUTANOATE METABOLISM	0.023	0.161	18	41	45
ST JNK MAPK PATHWAY	0.018	0.164	20	36	40
ST INTEGRIN SIGNALING PATHWAY	0.015	0.166	35	72	82
HSA05212 PANCREATIC CANCER	0.021	0.172	30	68	73
GSK3PATHWAY	0.024	0.186	14	23	27
HSA04662 B CELL RECEPTOR SIGNALING PATHWAY	0.026	0.190	26	53	64
HSA00903 LIMONENE AND PINENE DEGRADATION	0.039	0.191	12	25	29
HSA00280 VALINE LEUCINE AND ISOLEUCINE DEGRADATION	0.03	0.194	19	41	44
HSA05010 ALZHEIMERS DISEASE	0.034	0.195	11	25	28
FCER1PATHWAY	0.036	0.203	18	33	38
SMOOTH MUSCLE CONTRACTION	0.03	0.205	60	127	150
OXIDATIVE PHOSPHORYLATION	0.049	0.250	19	51	63

FDR=false discovery rate

Supplementary table 3.13: Significantly enriched iGSEA GO pathways for progression to dysplasia/carcinoma

Pathway/Gene set name	P-value	FDR	Significant genes	Selected genes	All genes
RESPONSE TO OTHER ORGANISM	$<1.00\times10^{-3}$	0.006	30	71	83
EXOCYTOSIS	$<1.00\times10^{-3}$	0.039	12	23	25
POSITIVE REGULATION OF DEVELOPMENTAL PROCESS	$<1.00\times10^{-3}$	0.040	88	195	210
RESPONSE TO BIOTIC STIMULUS	$<1.00\times10^{-3}$	0.040	38	105	210
RESPONSE TO BACTERIUM	3.00×10^{-3}	0.044	12	27	31
DEFENSE RESPONSE TO BACTERIUM	9.00×10^{-3}	0.077	9	22	25
SKELETAL DEVELOPMENT	$<1.00\times10^{-3}$	0.078	53	96	103
POSITIVE REGULATION OF NUCLEOBASE NUCLEOSIDE NUCLEOTIDE AND NUCLEIC ACID METABOLIC PROCESS	1.00×10^{-3}	0.079	68	139	154
RESPONSE TO VIRUS	4.00×10^{-3}	0.080	18	42	50
SECRETORY PATHWAY	$<1.00\times10^{-3}$	0.081	35	77	84
CAMP MEDIATED SIGNALING	1.00×10^{-3}	0.082	34	56	65
PATTERN SPECIFICATION PROCESS	2.00×10^{-3}	0.083	18	26	31
BRAIN DEVELOPMENT	$<1.00\times10^{-3}$	0.087	27	42	51
AMINE CATABOLIC PROCESS	3.00×10^{-3}	0.089	13	21	27
DETECTION OF STIMULUS	2.00×10^{-3}	0.092	21	43	47
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	1.00×10^{-3}	0.092	12	23	26
POSITIVE REGULATION OF TRANSCRIPTION	2.00×10^{-3}	0.094	64	129	144
POSITIVE REGULATION OF TRANSCRIPTION DNA DEPENDENT	2.00×10^{-3}	0.095	54	107	118
MULTI ORGANISM PROCESS	1.00×10^{-3}	0.096	54	138	166
POSITIVE REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	3.00×10^{-3}	0.098	16	34	36
G PROTEIN SIGNALING COUPLED TO CAMP NUCLEOTIDE SECOND MESSENGER	1.00×10^{-3}	0.102	33	55	64
MUSCLE CELL DIFFERENTIATION	3.00×10^{-3}	0.103	13	20	22
CARBOHYDRATE METABOLIC PROCESS	1.00×10^{-3}	0.115	72	159	180
CHROMOSOME ORGANIZATION AND BIOGENESIS	6.00×10^{-3}	0.115	40	102	124
CELL PROJECTION BIOGENESIS	7.00×10^{-3}	0.117	12	22	26
NEGATIVE REGULATION OF NUCLEOBASE NUCLEOSIDE NUCLEOTIDE AND NUCLEIC ACID METABOLIC PROCESS	8.00×10^{-3}	0.119	78	183	211
POSITIVE REGULATION OF RNA METABOLIC PROCESS	5.00×10^{-3}	0.123	54	109	120
NEGATIVE REGULATION OF TRANSFERASE ACTIVITY	8.00×10^{-3}	0.123	14	29	35
CARBOHYDRATE CATABOLIC PROCESS	0.017	0.124	8	21	24
GROWTH	9.00×10^{-3}	0.125	31	68	77
AMINO ACID CATABOLIC PROCESS	6.00×10^{-3}	0.125	12	20	25
ESTABLISHMENT AND OR MAINTENANCE OF CHROMATIN ARCHITECTURE	0.010	0.127	23	59	77
POSITIVE REGULATION OF SIGNAL TRANSDUCTION	6.00×10^{-3}	0.129	44	114	126
REGULATION OF ORGANELLE ORGANIZATION AND BIOGENESIS	0.013	0.129	15	36	40
CHROMATIN MODIFICATION	0.020	0.152	16	43	55
DETECTION OF EXTERNAL STIMULUS	0.020	0.154	8	20	23
G PROTEIN SIGNALING ADENYLATE CYCLASE ACTIVATING PATHWAY	0.011	0.155	13	21	25
NITROGEN COMPOUND CATABOLIC PROCESS	0.011	0.155	13	23	29
CELLULAR CARBOHYDRATE METABOLIC PROCESS	0.017	0.156	47	110	126
NEGATIVE REGULATION OF CATALYTIC ACTIVITY	0.012	0.156	28	58	69
REGULATION OF CYTOSKELETON ORGANIZATION AND BIOGENESIS	0.015	0.158	11	27	30
NEGATIVE REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	0.022	0.161	10	22	29
CELLULAR CARBOHYDRATE CATABOLIC PROCESS	0.032	0.164	7	20	23
STRIATED MUSCLE DEVELOPMENT	0.022	0.165	21	36	40
PROTEOLYSIS	0.023	0.165	64	166	191
NEGATIVE REGULATION OF RNA METABOLIC PROCESS	0.017	0.165	50	111	132
NEGATIVE REGULATION OF TRANSCRIPTION DNA	0.017	0.165	50	111	130

DEPENDENT					
MRNA METABOLIC PROCESS	0.027	0.169	21	74	85
AMINE METABOLIC PROCESS	0.017	0.170	54	116	141
TISSUE REMODELING	0.011	0.170	19	29	30
SECRETION BY CELL	0.02	0.171	43	107	117
NEGATIVE REGULATION OF TRANSCRIPTION	0.011	0.172	71	166	188
AXONOGENESIS	0.022	0.172	25	40	43
POSITIVE REGULATION OF CELLULAR PROTEIN METABOLIC PROCESS	0.021	0.173	26	66	73
NUCLEOBASE NUCLEOSIDE NUCLEOTIDE AND NUCLEIC ACID TRANSPORT	0.03	0.176	9	29	32
BONE REMODELING	0.021	0.181	18	28	29
REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	0.029	0.185	45	114	126
POSITIVE REGULATION OF PHOSPHATE METABOLIC PROCESS	0.032	0.185	12	28	28
POSITIVE REGULATION OF CATALYTIC ACTIVITY	0.026	0.187	63	143	165
REGULATION OF MAP KINASE ACTIVITY	0.027	0.201	30	58	67
POSITIVE REGULATION OF TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER	0.027	0.210	30	58	65
REGULATION OF GROWTH	0.034	0.226	22	51	58
NITROGEN COMPOUND METABOLIC PROCESS	0.034	0.231	56	126	155

Supplementary table 3.14: Genes shared by GO processes associated with progression

Response to other organisms, biotic stimulus and bacterium GO biological processes	
AZU1	Monocyte and fibroblast chemotactant
BCL10	Pro-apoptotic mediator
CAMP	Intracellular signalling
CD1D	Antigen presentation (to T helper and NK cells)
CD24	B cell activation
CFP	Alternative complement pathway
CHIT1	Pathogen degredation enzyme
DEFB103A	Antimicrobial
DEFB127	Antimicrobial
IL10	Cytokine
IL12A	Cytokine
LALBA	Lactose synthetase
NLRC4	Apopotic activator
NOD1	Apopotic activator
NOS2	Nitric oxide synthesis
PGLYRP1	Bactericidal protein
DEFB118	Antimicrobial
DMBT1	Mucosal defence, cellular differentiaton
NOD2	Apopotic activator
PGLYRP2	Bactericidal protein
PGLYRP3	Bactericidal protein
PGLYRP4	Bactericidal protein
S100A7	Cell cycle progression and differentiation
SLC11A1	Antimicrobial
SPACA3	Spermatogenesis
SPN	T lymphocyte glycoprotein
STAB1	Angiogenesis, cell adhesion, lymphocyte homing
STAB2	Angiogenesis, cell adhesion, lymphocyte homing
TLR3	Pathogen recognition and pro-inflammatory response
TLR6	Pathogen recognition and pro-inflammatory response
WFDC12	Antibacterial

CHAPTER 4: A SYSTEMATIC REVIEW AND META-ANALYSIS OF SOMATIC AND GERMLINE DNA SEQUENCE BIOMARKERS OF OESOPHAGEAL CANCER SURVIVAL, THERAPY RESPONSE AND STAGE

Supplementary tables 1-23 are available online, accompanying the published version of this chapter (http://annonc.oxfordjournals.org/content/26/4/624)

CHAPTER 5: EXOME SEQUENCING OF OESOPHAGEAL ADENOCARCINOMA BEFORE AND AFTER NEOADJUVANT CHEMOTHERAPY: A NEW MODEL OF GENOMIC RESPONSE AND IMPLICATIONS FOR PRECISION ONCOLOGY

Section 5.3.4: Mutational burden before and after chemotherapy

Supplementary table 5.1: Total number of mutations and mutant genes per patient

Patient	Relationship to chemotherapy									
	Before		After		Before only		Before and after		After only	
	Mut	Genes	Mut	Genes	Mut	Genes	Mut	Genes	Mut	Genes
1	281	256	149	133	179	166	102	92	47	44
2	221	214	28	26	216	211	5	5	23	22
3	374	351	39	40	341	323	33	34	6	7
4	186	176	45	40	148	143	38	35	7	7
5	325	302	70	65	287	276	38	33	32	33
6	442	411	219	213	308	284	134	129	85	85
7	440	400	336	324	264	250	176	172	160	154
8	991	880	83	82	967	861	24	24	59	59
9	546	507	216	205	511	477	35	32	181	174
10	211	196	189	180	64	60	147	139	42	42
11	234	212	604	550	72	70	162	145	442	412
12	71	63	212	203	13	13	58	52	154	154
13	318	295	454	416	65	63	253	234	201	189
14	221	211	94	89	137	136	84	79	10	11
15	288	228	224	200	125	86	163	157	61	61
16	638	546	678	623	284	237	354	328	324	307
17	217	203	267	250	33	34	184	171	83	82
18	6018	4900	6086	4944	516	495	5502	4531	584	555
19	196	188	482	453	71	72	125	118	357	338
20	86	85	31	32	68	68	18	19	13	14
21	543	498	536	499	181	173	362	342	174	162
22	1009	883	115	115	998	873	11	12	104	104
23	448	410	368	331	147	142	301	274	67	63
24	906	814	1007	895	290	270	616	568	391	354
25	115	112	57	58	75	73	40	41	17	18
26	276	261	49	44	254	243	22	20	27	25
27	199	195	648	512	138	135	61	62	587	463
28	109	104	70	67	86	85	23	22	47	47
29	41	37	50	51	29	26	12	13	38	39
30	7523	5912	9024	6908	1167	1077	6356	5173	2668	2382
Median	284.5	258.5	214	201.5	163.5	154.5	93	85.5	75	72.5
IQR (1st)	202.0	195.25	70.0	65.5	72.75	72.25	33.5	32.3	33.5	34.5
IQR (3rd)	519.25	476.25	475.0	443.75	289.25	274.5	182.0	171.8	196.0	185.25

Mut=mutations

Supplementary table 5.2: Total number of potentially functional mutations and affected genes per patient

Patient	Relationship to chemotherapy									
	Before		After		Before only		Before and after		After only	
	Mut	Genes	Mut	Genes	Mut	Genes	Mut	Genes	Mut	Genes
1	50	50	19	20	37	37	13	14	6	7
2	43	44	5	6	40	41	3	4	2	3
3	88	86	10	11	80	79	8	9	2	3
4	42	40	11	10	32	32	10	9	1	2
5	71	71	12	13	66	66	5	6	7	8
6	76	75	41	42	47	46	29	30	12	13
7	90	90	50	49	64	64	26	27	24	24
8	207	194	13	13	202	189	5	6	8	8
9	123	123	46	46	116	116	7	8	39	39
10	48	47	45	44	11	12	37	36	8	9
11	51	48	107	100	24	25	27	25	80	77
12	6	7	35	36	2	3	4	5	31	32
13	62	61	85	84	10	11	52	51	33	34
14	57	58	23	24	35	36	22	23	1	2
15	42	41	37	38	15	14	27	28	10	11
16	114	108	137	133	41	38	73	71	64	64
17	43	43	53	53	5	6	38	38	15	16
18	939	888	944	891	106	107	833	786	111	111
19	34	35	108	106	15	16	19	20	89	87
20	20	21	4	5	18	19	2	3	2	3
21	88	85	100	97	22	23	66	63	34	35
22	190	180	14	15	188	178	2	3	12	13
23	67	65	60	56	24	25	43	41	17	16
24	173	166	199	192	49	49	124	122	75	73
25	15	16	7	8	12	13	3	4	4	5
26	40	41	6	7	39	40	1	2	5	6
27	45	46	112	106	34	35	11	12	101	95
28	23	23	10	11	19	20	4	5	6	7
29	8	9	5	6	7	8	1	2	4	5
30	1387	1301	1670	1529	230	225	1157	1093	513	493
Median	54	54	39	40	34.5	35.5	16	17	12	13
IQR (1st)	42	41	11.25	11.5	15.75	16.75	4.25	5.25	5.25	6.25
IQR (3rd)	89.5	89	96.25	93.75	60.25	60.25	37.75	37.5	37.75	38

Mut=mutations

Section 5.3.5: Driver mutations and genes

Supplementary table 5.3: Significantly mutated genes identified by MutSigCV before NAC

Gene Function	Before NAC				After NAC		p	Previously implicated? CBP frequency ICGC frequency
	N	%	MutSigCV p	MutSigCV q	N	%		
TP53 Apoptosis, genome stability	21	0.700	8.88×10^{-18}	1.68×10^{-11}	12	0.400	0.037	AC, SCC CBP 71.9% ICGC 66.3%
Possibly significant								
DEFB127 Defensin	2	0.067	2.47×10^{-3}	1	2	0.067	1.000	No CBP 0.00% ICGC 0.00%
FAM162A Hypoxia, apoptosis	2	0.067	6.77×10^{-3}	1	1	0.033	1.000	No CBP 0.70% ICGC 0.00%
KLK15 Serine peptidase	2	0.067	8.66×10^{-3}	1	3	0.100	1.000	No (prostate, germline (Batra et al., 2011)) CBP 1.40% ICGC 1.05%
C1orf68 Unknown	2	0.067	8.70×10^{-3}	1	1	0.033	1.000	CBP 0.00% ICGC 0.00%
C1QTNF2 C1 and TNF-binging	2	0.067	9.11×10^{-3}	1	2	0.067	1.000	No CBP 0.70% ICGC 1.05%
MAGEA4 Pro-apoptotic	2	0.067	9.49×10^{-3}	1	0	0.000	0.492	No CBP 1.00% ICGC 0.00%
LIPC Lipid metabolism	3	0.100	9.57×10^{-3}	1	0	0.000	0.273	No CBP 0.00% ICGC 1.05%
KLK3 Serine peptidates	2	0.067	1.01×10^{-3}	1	1	0.033	1.000	No (prostate, germline)(Kote-Jarai et al., 2011) CBP 0.00% ICGC 0.00%
SP7 Osteoblast transcription factor	3	0.100	1.08×10^{-3}	1	2	0.067	1.000	No CBP 0.00% ICGC 0.00%
OR6Y1 Olfactor receptor	2	0.067	1.08×10^{-2}	1	1	0.033	1.000	No CBP 2.10 % ICGC 0.00%
ODF3L2 Unknown	2	0.067	1.10×10^{-2}	1	1	0.033	1.000	No CBP 0.00% ICGC 0.00%
DUPD1 Tyrosine phosphatase	2	0.067	1.11×10^{-2}	1	1	0.033	1.000	No CBP 0.00% ICGC 0.00%
LPAR4 Signal transduction	2	0.067	1.13×10^{-2}	1	1	0.033	1.000	No CBP 2.10% ICGC 0.00%
KCNF1 Potassium channel	3	0.100	1.21×10^{-2}	1	3	0.100	1.000	No CBP 1.10% ICGC 0.00%
ZNF670 Transcriptional regulation?	2	0.067	1.36×10^{-2}	1	0	0.000	0.492	No CBP 0.00% ICGC 0.00%
BMPR1A TGF-β pathway	2	0.067	1.39×10^{-2}	1	1	0.033	1.000	No CBP 1.40% ICGC 0.00%
SPERT Unknown	3	0.100	1.42×10^{-2}	1	3	0.100	1.000	No CBP 1.40 % ICGC 0.00%
MAPK3 Cell signalling	2	0.067	1.43×10^{-2}	1	1	0.033	1.000	No (colorectal)(Baba et al., 2010) CBP 0.70% ICGC 0.00%
PIH1D1 Pro-apoptotic	2	0.067	1.43×10^{-2}	1	2	0.067	1.000	No CBP 0.00% ICGC 0.00%
SMAD4 TGF-β pathway	2	0.067	1.46×10^{-2}	1	1	0.033	1.000	SCC (mRNA)(Natsugoe et al., 2002) CBP 8.40 % ICGC 8.40%
TRIM63 Creatine kinase regulation	2	0.067	1.49×10^{-2}	1	0	0.000	0.492	No CBP 1.40% ICGC 0.00%
SAMD8 Protein secretion	2	0.067	1.49×10^{-2}	1	1	0.033	1.000	No CBP 0.70% ICGC 0.00%
SLC22A12 Urate transport	2	0.067	1.75×10^{-2}	1	1	0.033	1.000	No CBP 1.40% ICGC 0.00%
GDF3 TGF-β pathway	2	0.067	1.80×10^{-2}	1	1	0.033	1.000	no (breast – mRNA)(Li et al., 2012) CBP 1.30% ICGC 0.00%
LSM7 mRNA splicing	1	0.033	1.80×10^{-2}	1	1	0.033	1.000	No CBP 0.00% ICGC 0.00%
ADAM32 Metalloendopeptidase	3	0.100	1.82×10^{-2}	1	2	0.067	1.000	No CBP 0.00% ICGC 0.00%
SMARCA4	4	0.133	1.89×10^{-2}	1	3	0.100	1.000	AC(Dulak et al., 2013)

Chromatin remodeling								CBP 6.80% ICGC 8.40%
MFAP5 ECM	1	0.033	$1.89x10^{-2}$	1	0	0.000	1.000	No CBP 0.00% ICGC 0.00%
LSM14A DNA binding	2	0.067	$1.96x10^{-2}$	1	2	0.067	1.000	No CBP 0.00% ICGC 0.00%

ECM=extracellular matrix

Supplementary table 5.4: Significantly mutated genes identified by IntOGen before NAC (microsatellite stable tumours only; n=28)

Gene	Before NAC						After NAC		p	Previously implicated?
	N	%	FM		OncoClust		N	%		CBP %
Function			p	q	p	q				ICGC %
TP53 Apoptosis, genomic stability	20	0.714	$<1x\ 10^{-17}$	$<1x\ 10^{-17}$	0.01 1	0.01 1	12	0.429	0.05 8	AC, SCC CBP 0.719 ICGC 0.663
SMARCA4 Chromatin remodelling	4	0.143	$2.44x\ 10^{-7}$	$1.39x\ 10^{-5}$	NA	NA	4	0.143	1	AC(Dulak et al., 2013) CBP 0.068 ICGC 0.084
ARID1A Chromatin remodelling	4	0.143	$2.74x\ 10^{-5}$	$1.04x\ 10^{-3}$	NA	NA	2	0.071	0.7	AC(Streppel et al., 2014) CBP 0.089 ICGC 0.074
SMAD4 TGF-β pathway	3	0.107	$7.53x\ 10^{-5}$	$2.10\ x10^{-3}$	NA	NA	1	0.036	0.61 1	AC (Weaver et al., 2014) CBP 0.084 ICGC 0.084
MYH10 Myosin	3	0.107	$9.20x\ 10^{-5}$	$2.10x\ 10^{-3}$	NA	NA	2	0.071	1	No CBP 0.034 ICGC 0.021
NRP2 VEGF co-factor	4	0.143	$5.52x\ 10^{-4}$	0.010	NA	NA	1	0.036	0.35 2	SCC (expression)(Fung, 2013) CBP 0.000 ICGC 0.000
ARID1B Chromatin remodelling	4	0.071	$2.08x\ 10^{-3}$	0.034	NA	NA	2	0.071	1	OSCC (protein)(Kano et al., 2013, Helming et al., 2014) CBP 0.007 ICGC 0.053
Possibly significant										
C3 Complement component	3	0.107	$8.97x\ 10^{-3}$	0.128	NA	NA	2	0.071	1	No CBP 0.014 ICGC 0.011
RPS6KA2 Serine/threonine protein kinase	2	0.071	0.017	0.195	NA	NA	0	0.000	0.49 1	No (ovarian)(Bignone et al., 2007) CBP 0.014 ICGC 0.000
NOTCH1 NOTCH signalling	7	0.286	0.017	0.195	NA	NA	0	0.0	0.01 0	SCC (Agrawal et al., 2012) CBP 0.027 ICGC 0.042

Supplementary table 5.5: Significantly mutated genes identified by MutSigCV before NAC (microsatellite stable tumours only; n=28)

Gene Function	Before NAC				After NAC		p	Previously implicated? CBP frequency ICGC frequency
	N	%	MutSigCV		N	%		
			p	q				
TP53 Apoptosis, genome stability	21	0.700	$<1x10^{-16}$	$<1x10^{-16}$	11	0.393	0.031	AC, SCC CBP 0.719 ICGC 0.663
Possibly significant								
SUB1 Transcription co-activator	1	0.036	$7.93x10^{-3}$	1.000	0	0.000	1.000	No CBP 0.000 ICGC 0.000
MAGEA4 Pro-apoptotic	2	0.071	$9.02x10^{-3}$	1.000	0	0.000	0.491	No (melanoma)(Caballero et al., 2010) CBP 0.010 ICGC 0.000
SP7 Osteoblast transcription factor	2	0.071	$1.06x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.000 ICGC 0.000
ODF3L2 Spermatid protein	2	0.071	$1.11x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.000 ICGC 0.011
MFAP5 ECM component	1	0.036	$1.12x10^{-2}$	1.000	0	0.000	1.000	No CBP 0.000 ICGC 0.375
LPAR4 Lipid binding	2	0.071	$1.16x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.002 ICGC 0.000
SMARCA4 Chromatin remodelling	4	0.143	$1.16x10^{-2}$	1.000	3	0.107	1.000	AC(Dulak et al., 2013) CBP 0.068 ICGC 0.084
SMAD4 TGF-β pathway	2	0.071	$1.24x10^{-2}$	1.000	1	0.036	1.000	AC(Weaver et al., 2014) CBP 0.084 ICGC 0.084
KCNF1 Potassium channel	2	0.071	$1.26x10^{-2}$	1.000	2	0.071	1.000	No CBP 0.010 ICGC 0.000
LIPC Lipid metabolism	2	0.071	$1.27x10^{-2}$	1.000	0	0.000	0.491	No CBP 0.010 ICGC 0.011
MAP2K3 MAP kinase	2	0.071	$1.29x10^{-2}$	1.000	0	0.000	0.491	No CBP 0.000 ICGC 0.000
LSM7 mRNA splicing	1	0.036	$1.30x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.000 ICGC 0.000
DCX Neuronal migration	2	0.071	$1.32x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.000 ICGC 0.000
DEFB127 Defensin	1	0.036	$1.33x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.000 ICGC 0.000
ZNF670 Transcriptional regulation?	2	0.071	$1.40x10^{-2}$	1.000	0	0.000	0.491	No CBP 0.000 ICGC 0.000
TLK1 Chromatin reassembly	2	0.071	$1.51x10^{-2}$	1.000	2	0.071	1.000	No (breast)(Sunavala-Dossabhoy et al., 2003) CBP 0.007 ICGC 0.000
MARVELD2 Epithelial barrier	2	0.071	$1.67x10^{-2}$	1.000	1	0.036	1.000	No CBP 0.027 ICGC 0.000
DFNB31 Inner ear cilial protein	2	0.071	$1.92x10^{-2}$	1.000	0	0.000	0.491	No CBP 0.021 ICGC 0.000

Supplementary table 5.6: Possible driver genes identified after NAC by MutSigCV

Gene Function	After NAC				Before NAC		p	Previously implicated?
	N	%	MutSigCV		N	%		
			p	q				
TP53 Apoptosis, genome stability	12	40.0	2.47x10-9	4.66 x10^{-5}	21	0.700	0.037	AC, SCC(Dulak et al., 2013)
DEFB127 Defensin	2	6.67	2.38x10^{-3}	1.000	2	0.067	1.000	No
KLK15 Serine peptidase	2	6.67	6.08x10^{-3}	1.000	2	0.067	1.000	No (prostate, germline (Batra et al., 2011))
OR6P1 Olfactory receptor	2	6.67	6.93x10^{-3}	1.000	0	0.000	0.492	No
KCNMB4 Potassium chanel	2	0.067	7.81x10^{-3}	1.000	0	0.000	0.492	No
C1QTNF2 C1 & TNF-binding protein	2	0.067	8.00x10^{-3}	1.000	2	0.067	1.000	No
HTR1E Serotonin receptor	3	10.0	9.15x10^{-3}	1.000	1	0.033	0.612	No
OR5V1 Olfactory receptor	2	0.067	9.76x10^{-3}	1.000	0	0.000	0.492	No
MRGPRX2 G protein-coupled receptor	2	0.067	0.010	1.000	1	0.033	1.000	No
PPP3R1 Protein phosphatase	1	0.033	0.011	1.000	0	0.000	1.000	No
KCNF1 Potassium channel	3	0.100	0.012	1.000	3	0.100	1.000	No
MSRA Oxidative damage repair	2	0.067	0.013	1.000	0	0.000	0.492	No (HCC)(Lei et al., 2007)
OR4X1 Olfactory receptor	2	0.067	0.014	1.000	1	0.033	1.000	No
SPERT Spermatid protein	3	0.100	0.014	1.000	3	0.100	1.000	No
MPP2 Cell adhesion	2	6.67	0.015	1.000	1	0.033	0.612	No
MRP63 Mitochondrial ribosomal protein	1	0.033	0.016	1.000	1	0.033	1.000	No
EDNRB Endothelin receptor	2	0.067	0.016	1.000	0	0.000	0.492	No
WSCD1 Sulphotransferase	2	0.067	0.016	1.000	0	0.000	0.492	No
SLC22A17 Solute transport	2	0.067	0.016	1.000	2	0.067	1.000	No
HINFP DNA methylation	2	0.067	0.017	1.000	2	0.067	1.000	No
GABRA3 GABA receptor	2	0.067	0.017	1.000	0	0.000	0.491	No
OPRL1 Opiod receptor	2	0.067	0.017	1.000	1	0.033	1.000	No
PIH1D1 Pro-apoptotic	2	0.067	0.018	1.000	2	0.067	1.000	No
COX6C Mitochondrial respiratory chain	1	0.033	0.018	1.000	1	0.033	1.000	No
HIST1H3E Histone	1	0.033	0.019	1.000	0	0.000	1.000	No

Section 5.3.6: Drivers of chemosensitivity and chemoresistance

Supplementary table 5.7: Significantly enriched pathways before chemotherapy in non-responding tumours

KEGG ID	Pathway	Non-responding				Responding		p*
		N	%	IntOGen		N	%	
				p	q			
hsa04210	Apoptosis	11	0.733	$6.76x10^{-6}$	$2.90 x10^{-4}$	13	0.867	0.651
hsa05016	Huntington's disease	12	0.800	$5.10x10^{-6}$	$2.90 x10^{-4}$	15	1.000	1.000
hsa05210	Colorectal cancer	12	0.800	$7.02x10^{-6}$	$2.90 x10^{-4}$	13	0.867	1.000
hsa04110	Cell cycle	13	0.867	$9.37x10^{-6}$	$2.91 x10^{-4}$	14	0.933	1.000
hsa05222	Small cell lung cancer	13	0.867	$1.35x10^{-5}$	$3.35 x10^{-4}$	14	0.933	1.000
hsa05202	Transcriptional misregulation in cancer	14	0.933	$4.74x10^{-5}$	$9.79 x10^{-4}$	15	1.000	1.000
hsa05161	Hepatitis B	13	0.867	$8.64x10^{-5}$	$1.53 x10^{-3}$	14	0.933	1.000
hsa05162	Measles	13	0.867	$3.04x10^{-4}$	$4.19 x10^{-3}$	13	0.867	1.000
hsa05216	Thyroid cancer	12	0.800	$2.80x10^{-4}$	$4.1 x10^{-3}$	12	0.800	1.000
hsa04115	p53 signaling pathway	11	0.733	$3.82x10^{-4}$	$4.30 x10^{-3}$	12	0.800	1.000
hsa05213	Endometrial cancer	13	0.867	$3.62x10^{-4}$	$4.30 x10^{-3}$	14	0.933	1.000
hsa04610	Complement and coagulation cascades	9	0.600	$5.26x10^{-4}$	$4.66 x10^{-3}$	7	0.467	0.715
hsa05203	Viral carcinogenesis	13	0.867	$4.96x10^{-4}$	$4.66 x10^{-3}$	13	0.867	1.000
hsa05218	Melanoma	12	0.800	$4.85x10^{-4}$	$4.66 x10^{-3}$	14	0.933	0.598
hsa05223	Non-small cell lung cancer	13	0.867	$6.80x10^{-4}$	$5.27 x10^{-3}$	13	0.867	1.000
hsa05014	Amyotrophic lateral sclerosis	10	0.667	$6.71x10^{-4}$	$5.27 x10^{-3}$	12	0.800	0.682
hsa05212	Pancreatic cancer	13	0.867	$8.24x10^{-4}$	$6.01 x10^{-3}$	14	0.933	1.000
hsa04310	Wnt signaling pathway	13	0.867	$1.01x10^{-3}$	$6.94 x10^{-3}$	13	0.867	1.000
hsa05217	Basal cell carcinoma	13	0.867	$2.73x10^{-3}$	0.018	12	0.800	1.000
hsa05160	Hepatitis C	12	0.800	$3.68x10^{-3}$	0.023	14	0.933	0.598
hsa00230	Purine metabolism	9	0.600	$4.10x10^{-3}$	0.024	11	0.733	0.700
hsa04722	Neurotrophin signaling pathway	11	0.733	$5.94x10^{-3}$	0.031	15	1.000	0.100
hsa04972	Pancreatic secretion	9	0.600	$5.58x10^{-3}$	0.031	11	0.733	0.700
hsa04320	Dorso-ventral axis formation	8	0.533	$5.89x10^{-3}$	0.031	6	0.400	0.715
hsa00310	Lysine degradation	8	0.533	$6.97x10^{-3}$	0.035	9	0.600	1.000
hsa04540	Gap junction	8	0.533	$7.52x10^{-3}$	0.036	13	0.867	0.109
hsa05214	Glioma	12	0.800	$9.10x10^{-3}$	0.042	14	0.933	1.000
hsa05168	Herpes simplex infection	14	0.933	$1.01x10^{-2}$	0.042	15	1.000	1.000
hsa05220	Chronic myeloid leukemia	12	0.800	$9.81x10^{-3}$	0.042	13	0.867	1.000
hsa05219	Bladder cancer	12	0.800	$9.47x10^{-3}$	0.042	12	0.800	1.000

*Fisher's exact test

Supplementary table 5.8: Significantly enriched pathways before chemotherapy in responding tumours

KEGG ID	Pathway	Non-responding				Responding		p*
		N	%	IntOGen		N	%	
				p	q			
hsa04310	Wnt signaling pathway	13	0.867	$9.14x10^{-13}$	$4.11x10^{-11}$	13	0.867	1.000
hsa05014	Amyotrophic lateral sclerosis	12	0.800	$9.00x10^{-10}$	$2.03x10^{-8}$	10	0.667	0.682
hsa05210	Colorectal cancer	13	0.867	$2.42x10^{-9}$	$3.63x10^{-8}$	12	0.800	1.000
hsa05217	Basal cell carcinoma	12	0.800	$4.58x10^{-9}$	$5.15x10^{-8}$	13	0.867	1.000
hsa04210	Apoptosis	13	0.867	$1.27x10^{-8}$	$1.14x10^{-7}$	11	0.733	0.651
hsa04722	Neurotrophin signaling pathway	15	1.000	$1.58x10^{-8}$	$1.19x10^{-7}$	11	0.733	0.100
hsa05213	Endometrial cancer	14	0.933	$2.88x10^{-8}$	$1.85x10^{-7}$	13	0.867	1.000
hsa05202	Transcriptional misregulation in cancer	15	1.000	$3.99x10^{-8}$	$2.24x10^{-6}$	14	0.933	1.000
hsa05166	HTLV-I	15	1.000	$2.71x10^{-7}$	$1.22x10^{-6}$	13	0.867	0.483
hsa05016	Huntington's disease	15	1.000	$2.70x10^{-7}$	$1.22x10^{-6}$	12	0.800	0.224
hsa05220	Chronic myeloid leukemia	13	0.867	$4.51x10^{-7}$	$1.85x10^{-6}$	12	0.800	1.000
hsa05160	Hepatitis C	14	0.933	$6.29x10^{-7}$	$2.36x10^{-6}$	12	0.800	0.598
hsa05169	Epstein-Barr virus	14	0.933	$8.63x10^{-7}$	$2.99x10^{-6}$	14	0.933	1.000
hsa05219	Bladder cancer	12	0.800	$1.54x10^{-6}$	$4.94x10^{-6}$	12	0.800	1.000
hsa04010	MAPK signaling pathway	15	1.000	$2.58x10^{-6}$	$7.74x10^{-6}$	12	0.800	0.224
hsa05216	Thyroid cancer	12	0.800	$2.89x10^{-6}$	$8.12x10^{-6}$	12	0.800	1.000
hsa05162	Measles	13	0.867	$4.05x10^{-6}$	$1.07x10^{-5}$	13	0.867	1.000
hsa05168	Herpes simplex infection	15	1.000	$4.97x10^{-6}$	$1.24x10^{-5}$	14	0.933	1.000
hsa04115	p53 signaling pathway	12	0.800	$8.43x10^{-6}$	$2.00x10^{-5}$	11	0.733	1.000
hsa05223	Non-small cell lung cancer	13	0.867	$1.55x10^{-5}$	$3.31x10^{-5}$	13	0.867	1.000
hsa05214	Glioma	14	0.933	$1.55x10^{-5}$	$3.31x10^{-5}$	12	0.800	0.598
hsa05222	Small cell lung cancer	14	0.933	$2.81x10^{-5}$	$5.75x10^{-5}$	13	0.867	1.000
hsa05212	Pancreatic cancer	14	0.933	$3.02x10^{-5}$	$5.91x10^{-5}$	13	0.867	1.000
hsa05218	Melanoma	14	0.933	$3.16x10^{-4}$	$5.90x10^{-4}$	12	0.800	0.598
hsa05161	Hepatitis B	14	0.933	$3.53x10^{-4}$	$6.35x10^{-5}$	13	0.867	1.000
hsa05203	Viral carcinogenesis	13	0.867	$1.25x10^{-3}$	$2.16x10^{-3}$	13	0.867	1.000
hsa04270	Vascular smooth muscle contraction	12	0.800	$2.18x10^{-3}$	$3.64x10^{-3}$	10	0.667	0.682
hsa05215	Prostate cancer	13	0.867	$3.22x10^{-3}$	$5.17x10^{-3}$	14	0.933	1.000
hsa04110	Cell cycle	14	0.933	$4.16x10^{-3}$	$6.46x10^{-3}$	13	0.867	1.000
hsa04020	Calcium signaling pathway	15	1.000	$4.35x10^{-3}$	$6.53x10^{-3}$	13	0.867	1.000
hsa05200	Pathways in cancer	14	0.933	$4.95x10^{-3}$	$7.18x10^{-3}$	15	1.000	1.000

*=Fisher's exact test

Supplementary table 5.9: Significantly enriched pathways after chemotherapy in responding tumours

KEGG ID	Pathway	Responding				Non-responding		p*
		N	%	IntOGen		N	%	
				p	q			
hsa05166	HTLV-I infection Proliferation and apoptosis	10	66.7	$4.66x10^{-5}$	$2.33x10^{-4}$	14	93.3	0.167
hsa04010	MAP Kinase signalling pathway Cellular proliferation, differentiation	12	80.0	$8.16x10^{-3}$	0.014	14	93.3	0.598
hsa05200	Pathways in cancer Multiple cancer pathways	12	80.0	$5.97x10^{-3}$	0.014	14	93.3	0.598
hsa05169	Epstein Barr Virus Cellular proliferation	13	86.0	0.018	0.225	14	93.3	1.000

*=Fisher's exact test

Supplementary table 5.10: Significantly enriched pathways before chemotherapy in non-responding tumours

KEGG ID	Pathway	Non-responding				Responding		p*	FDR
		N	%	IntOGen		N	%		
				p	q				
hsa05222	Small cell lung cancer	12	80.0	4.80×10^{-7}	6.44×10^{-5}	9	60.0	0.427	0.518
hsa05016	Huntington's disease	11	73.3	1.02×10^{-6}	6.86×10^{-5}	8	53.3	0.450	0.518
hsa05203	Viral carcinogenesis	13	86.7	2.95×10^{-6}	1.32×10^{-4}	8	53.3	0.109	0.232
hsa05218	Melanoma	12	80.0	7.45×10^{-6}	2.30×10^{-4}	8	53.3	0.245	0.321
hsa05210	Colorectal cancer	12	80.0	8.59×10^{-6}	2.30×10^{-4}	7	46.7	0.128	0.232
hsa05223	Non-small cell lung cancer	12	80.0	2.17×10^{-5}	4.85×10^{-4}	7	46.7	0.128	0.232
hsa04210	Apoptosis	13	86.7	3.31×10^{-5}	5.55×10^{-4}	7	46.7	0.050	0.209
hsa05216	Thyroid cancer	12	80.0	3.12×10^{-5}	5.55×10^{-4}	5	33.3	0.025	0.171
hsa05213	Endometrial cancer	12	80.0	1.46×10^{-4}	2.17×10^{-3}	6	40.0	0.060	0.209
hsa05212	Pancreatic cancer	12	80.0	2.55×10^{-4}	3.41×10^{-3}	8	53.3	0.245	0.321
hsa05161	Hepatitis B	14	93.3	5.98×10^{-4}	6.67×10^{-3}	7	46.7	0.014	0.171
hsa04110	Cell cycle	13	86.7	5.91×10^{-4}	6.67×10^{-3}	6	40.0	0.021	0.171
hsa05214	Glioma	12	80.0	6.69×10^{-4}	6.90×10^{-3}	7	46.7	0.128	0.232
hsa00310	Lysine degradation	9	60.0	9.86×10^{-4}	8.81×10^{-3}	5	33.3	1.000	1.000
hsa04540	Gap junction	12	80.0	9.64×10^{-4}	8.81×10^{-3}	7	46.7	0.128	0.232
hsa05162	Measles	13	86.7	1.47×10^{-3}	0.012	8	53.3	0.109	0.232
hsa04972	Pancreatic secretion	12	80.0	1.48×10^{-3}	0.012	8	53.3	0.245	0.321
hsa05146	Extracellular matrix	13	86.7	2.01×10^{-3}	0.015	10	66.7	1.000	1.000
hsa04971	Gastric acid secretio	11	73.3	2.32×10^{-3}	0.016	8	53.3	0.450	0.518
hsa04970	Salivary secretion	12	80.0	3.03×10^{-3}	0.018	8	53.3	0.245	0.321
hsa04520	Cell adherens junction	13	86.7	3.03×10^{-3}	0.018	6	40.0	0.021	0.171
hsa05014	Amyotrophic lateral sclerosis	11	73.3	3.15×10^{-3}	0.018	4	26.7	0.027	0.171
hsa04730	Cerebellar long term depression	10	66.7	3.24×10^{-3}	0.018	4	26.7	0.066	0.209
hsa04060	Cytokine cytokine receptor interaction	12	80.0	3.03×10^{-3}	0.018	8	53.3	0.245	0.321
hsa04512	Extracellular matrix-recetor interaction	14	93.3	3.38×10^{-3}	0.018	9	60.0	0.080	0.232
hsa05168	Herpes simplex infection	14	93.3	4.71×10^{-3}	0.023	12	80.0	0.598	0.668
hsa04976	Bile secretion	12	80.0	4.68×10^{-3}	0.023	7	46.7	0.128	0.232
hsa05166	HTLV-I	14	93.3	5.36×10^{-3}	0.026	10	66.7	0.187	0.321
hsa05219	Bladder cancer	11	73.3	5.52×10^{-3}	0.026	7	46.7	0.264	0.334
hsa04510	Focal adhesion	15	100	6.99×10^{-3}	0.031	10	66.7	0.042	0.209
hsa00230	Purine metabolism	13	86.7	7.21×10^{-3}	0.031	8	53.3	0.109	0.232
hsa04722	Neurotrophin signaling pathway	13	86.7	8.00×10^{-3}	0.033	7	46.7	0.050	0.209
hsa04721	Synaptic vesicle cycle	9	60.0	8.21×10^{-3}	0.033	9	60.0	1.000	1.000
hsa04115	p53 signaling pathway	11	73.3	8.47×10^{-3}	0.033	5	33.3	0.066	0.209
hsa05160	Hepatitis C	12	80.0	8.84×10^{-3}	0.034	8	53.3	0.245	0.321
hsa05220	Chronic myeloid leukemia	12	80.0	9.43×10^{-3}	0.035	8	53.3	0.245	0.321
hsa04725	Cholinergic synapse	12	80.0	9.71×10^{-3}	0.035	10	66.7	0.682	0.740
hsa05202	Transcriptional misregulation in cancer	15	100	0.013	0.047	8	53.3	0.006	0.171

*=Fisher's exact test

Section 5.3.8: Temporal heterogeneity

Supplementary table 5.11: Temporal heterogeneity of functional mutations before and after chemotherapy: candidate drivers this study and the literature

Gene	Relationship to NAC			VAF (%) (median, IQR)		
	Before only	Before and after	After only	Before	After	p
Candidates from this study						
TP53	9	12	1	22.1 (11.6-39.2)	15.3 (0.00-41.7)	0.219
SYNE1	3	3	2	14.2 (7.54-26.4)	26.7 (3.23-31.9)	0.568
NOTCH1	6	1	0	18.8 (14.9-23.4)	0.00 (0.00-0.00)	3.44×10^{-3}
SMARCA4	2	2	1	27.6 (4.30-71.4))	14.1 (0.00-17.2)	0.398
ARID1A	3	3	1	25.0 (11.3-33.9)	26.1 (0.00-35.0)	0.965
SMAD4	2	1	0	16.7 (15.7-22.6)	0.00 (0.00-12.5)	0.376
NRP2	2	0	0	50.7 (49,7-51.7)	0.00 (0.00-0.00)	0.221
ARID1B	0	2	0	23.9 (22.8-25.0)	38.9 (32.3-45.5)	0.667
RPS6KA2	2	0	0	31.9 (22.9-41.0)	0.00 (0.00-0.00)	0.221
CPD	0	1	2	1.40 (0.7-4.00)	14.7 (10.1-19.7)	0.200
PHF2	0	2	0	24.8 (21.8-27.8)	25.3 (23.0-27.7)	1.000
FBXW7	0	2	1	7.41 (3.70-24.8)	14.0 (9.36-50.2)	0.700
DPYD	2	0	1	13.0 (6.48-20.8)	0.00 (0.00-7.64)	0.643
BAX	0	2	0	20.2 (16.7-23.8)	41.8 (31.3-45.4)	0.333
KMTD2	1	2	2	6.90 (0.00-34.4)	25.9 (24.3-30.8)	0.672
SMEK1	1	1	0	30.4 (22.1-387.7)	17.1 (9,92-24.4)	0.667
SEC31A	0	3	0	33.9 (24.4-40.6)	31.9 (18.5-47.0)	1.000
SETD2	0	2	1	15.4 (9.68-18.3)	20.8 (15.6-23.9)	0.343
ASXL1	0	1	1	16.4 (8.20-24.6)	17.9 (17.4-18.3)	1.000
STAG2	0	2	1	14.3 (7.14-35.4)	70.0 (54.3-70.7)	0.200
Candidates from the literature						
ELMO1 (Dulak et al., 2013)	1	0	0	20.8 (NA)	0.00 (NA)	1.000
DOCK2 (Dulak et al., 2013)	2	1	0	20.3 (15.0-21.1)	0.00 (0.00-4.29)	0.124
CDKN2A (Dulak et al., 2013)	0	1	0	0.334 (NA)	0.429 (NA)	1.000
EYS (Dulak et al., 2013)	2	0	1	19.2 (9.57-33.5)	0.61 (0.30-12.8)	0.825
SLC39A12 (Dulak et al., 2013)	0	0	0	NA	NA	NA
SPG020 (Dulak et al., 2013)	0	0	0	NA	NA	NA
AKAP6 (Dulak et al., 2013)	0	1	0	18.8 (NA)	30.1 (NA)	1.000
TLL1 (Dulak et al., 2013)	0	0	0	NA	NA	NA
ARID2 (Dulak et al., 2013)	0	1 (2 mutations)	0	11.7 (9.17-14.2)	31.3 (29.3-33.3)	1.000
HECW1 (Dulak et al., 2013)	1	1	0	16.3 (11.7-20.9)	19.6 (9.82-29.5)	1.000
AJAP1 (Dulak et al., 2013)	1	0	0	55.6 (NA)	0.00 (NA)	1.000
C6orf118 (Dulak et al., 2013)	0	0	0	NA	NA	NA
ACTL7B (Dulak et al., 2013)	1	0	0	11.5 (NA)	2.50 (NA)	1.000
F5 (Dulak et al., 2013)	0	0	1	0.00 (NA)	20.0 (NA)	1.000
KCNU1 (Dulak et al., 2013)	0	0	0	NA	NA	NA
TLF4 (Dulak et al., 2013)	0	0	0	NA	NA	NA
KATA6 (Dulak et al., 2013)	0	0	0	NA	NA	NA
SCN10A (Dulak et al., 2013)	1	0	2	0.00 (0.00-12.5)	33.3 (16.7-35.8)	0.354
CNTNAP5 (Dulak et al., 2013)	4 (5 mutations)	1	1	16.5 (14.4-17.8)	0.00 (0.00-5.90)	0.115
MYO18B (Weaver et al., 2014)	2	0	1	17.1 (8.57-22.1)	0.00 (0.00-10.8)	0.643
SEMA5A (Weaver et al., 2014)	0	1	0	6.25 (NA)	10.4 (NA)	1.000
ABCB1 (Weaver et al., 2014)	1	0	0	20.0 (NA)	0.00 (NA)	1.000
PIK3CA (Chong et al., 2013)	0	2	0	29.0 (22.8-30.2)	25.4 (17.8-33.0)	1.000
USH2A (Chong et al., 2013)	3	2	1 (2 mutations)	14.7 (5.45-19.8)	9.90 (0.00-16.4)	0.514
NOTCH2 (Agrawal et al., 2012)	0	2 (3 mutations)	0	18.0 (15.2-25.2)	47.2 (41.7-48.6)	0.100
NOTCH3 (Agrawal et al., 2012)	0	0	0	NA	NA	NA

Supplementary table 5.12: Differential loss and retention of driver genes by responsive and non-responsive tumours

Gene	pTR			No pTR			p*	Response	Non-response	p**
	BTo	BT&AT	ATo	BTo	BT&AT	ATo		ΔVAF	ΔVAF	
OVERALL	65	12	9	29	104	35	$<2.2 \times 10^{-16}$	-1.42 (-19.2-15.3)	+0.32 (-18.0-14.8)	7.35×10^{-16}
TP53	9	3	1	0	9	0	9.83×10^{-4}	-30.8 (-45.2-9.02)	+14.2 (0.63-31.9)	1.91×10^{-3}
SYNE1	2	0	1	1	2	1	0.125	-9.83 (-20.5-24.3)	+8.15 (2.17-11.9)	0.667
NOTCH1	4	0	0	2	1	0	0.429	-17.9 (-20.0- -15.2)	-12.8 (-21.5- -9.90)	0.857
SMARCA4	1	1	0	1	1	1	1.00	-39.0 (-58.7 - -19.3)	+12.3 (-29.6-13.2)	0.400
ARID1A	3	0	1	0	3	0	7.94×10^{-3}	-20.7 (-31.5- -7.02)	+12.9 (10.0-16.6)	0.032
SMAD4	2	0	0	0	1	0	0.333	-15.8 (-16.2- -15.3)	-3.64 (NA)	0.667
NRP2	2	0	0	0	0	0	0.483	-50.7 (-51.7- - 49.7)	NA	NA
ARID1B	0	1	0	0	1	0	1.000	-0.47 (NA)	30.4 (NA)	1.000
RPS6KA2	2	0	0	0	0	0	0.483	-32.0 (-41.0 - -22.9)	NA	NA
CPD	0	0	2	0	1	0	0.333	+9.34 (6.62-12.0)	18.0 (NA)	0.667
PHF2	0	0	0	0	2	0	0.483	NA	+0.58 (-4.75-5.90)	NA
FBXW7	0	0	0	0	2	1	0.224	NA	+13.9 (-11.7-46.4)	NA
DPYD	1	0	0	1	0	1	1.000	-20.6 (NA)	+1.16 (-5.90-8.21)	0.667
BAX	0	0	0	0	2	0	0.483	NA	+21.6 (14.5-20.7)	NA
KMTD2	0	0	1	1	1	1	1.000	+25.9 (NA)	+3.86 (-7.74-8.95)	0.800
SMEK1	0	0	0	1	1	0	0.483	NA	-13.3 (-14.4- -12.2)	NA
SEC31A	0	0	0	0	3	0	0.224	NA	+3.65 (-7.22-13.4)	NA
SETD2	0	0	0	0	2	1	0.224	NA	+6.16 (-3.73-15.9)	NA
ASXL1	0	0	0	0	1	1	0.483	NA	1.46 (-6.29-9.23)	NA
STAG2	0	0	1	0	2	0	0.333	+71.4 (NA)	+18.9 (0.53-37.3)	0.667
ELMO1 (Dulak et al., 2013)	1	0	0	0	0	0	1.000	-20.8 (NA)	NA	NA
DOCK2 (Dulak et al., 2013)	1	0	0	1	1	0	1.000	-22.6 (NA)	-20.1 (-20.3- -1.43)	0.500
CDKN2A (Dulak et al., 2013)	0	1	0	0	0	0	1.000	+9.50 (NA)	NA	NA
EYS (Dulak et al., 2013)	2	0	0	0	0	1	0.333	-33.3 (-40.3- -26.2)	+25.0 (NA)	0.667
SLC39A12 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
SPG020 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
AKAP6 (Dulak et al., 2013)	0	0	0	0	1	0	1.000	NA	+11.3 (NA)	NA
TLL1 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
ARID2 (Dulak et al., 2013)	0	0	0	0	1	0	1.000	NA	19.6 (NA)	NA
HECW1 (Dulak et al., 2013)	1	0	0	0	1	0	1.000	-7.11 (NA)	+13.8 (NA)	1.000
AJAP1 (Dulak et al., 2013)	1	0	0	0	0	0	1.000	-55.6 (NA)	NA	NA
C6orf118 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
ACTL7B (Dulak et al., 2013)	0	0	0	1	0	0	1.000	NA	-9.29 (NA)	NA
F5 (Dulak et al., 2013)	0	0	1	0	0	0	1.000	+20.0 (NA)	NA	NA
KCNU1 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
TLF4 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
KATA6 (Dulak et al., 2013)	0	0	0	0	0	0	NA	NA	NA	NA
SCN10A (Dulak et al., 2013)	1	0	0	0	0	1	0.333	-25.0 (NA)	+35.8 (34.6-37.0)	0.667
CNTNAP5 (Dulak et al., 2013)	2	0	1	1	1	0	0.600	-15.1 (-16.8- -4.19)	-18.8 (-19.8 - -17.7)	0.267
MYO18B (Weaver et al., 2014)	2	0	0	0	0	1	0.333	-22.2 (-24.6- -19.7)	21.5 (NA)	0.667
SEMA5A (Weaver et al., 2014)	0	0	0	0	1	0	1.000	NA	4.04 (NA)	NA

ABCB1 (Weaver et al., 2014)	1	0	0	0	0	0	1.000	-20.0 (NA)	NA	NA
PIK3CA (Chong et al., 2013)	0	0	0	0	2	0	0.483	NA	-3.60 (-9.95-2.76)	NA
USH2A (Chong et al., 2013)	2	0	0	0	3	0	0.238	-17.3 (-20.4- -14.1)	+8.40 (-2.39-9.90)	0.191
NOTCH2 (Agrawal et al., 2012)	0	0	0	0	2	0	0.483	NA	+29.3 (16.5-33.4)	NA
NOTCH3 (Agrawal et al., 2012)	0	0	0	0	0	0	NA	NA	NA	NA

*Fisher's exact test; **Mann Witney test; BTo=before treatment only; ATo=after treatment only; BTAT=both before and after treatment

Supplementary table 5.13: Temporal heterogeneity of therapeutic target genes

Gene	Functional mutations			VAF (median, IQR)		
	BTo	BT&ATr	ATo	Before	After	p
Therapeutic targets(Garnett et al., 2012)						
TP53	9	13	1	22.1 (11.6-39.2)	15.3 (0.00-41.7)	0.219
EGFR (Erlotinib / Lapatinib)	0	2	0	18.2 (15.3-21.0)	32.1 (27.2-37.1)	0.667
ERBB2 (Lapatinib)	0	1	0	6.70 (NA)	10.3 (NA)	1.000
MTOR (Rapamycin / Torin 1)	0	0	0	NA	NA	NA
PDGFRA (Sunitinib / Sorafenib)	0	0	1	2.90 (NA)	34.1 (NA)	1.000
PDGFRB (Sunitinib / Sorafenib)	0	0	0	NA	NA	NA
VEGFR (Sunitinib / Sorafenib)	0	0	0	NA	NA	NA
VEGFA (Bevacuzimab)	0	0	0	NA	NA	NA
FLT3 (Sunitinib / Sorafenib)	0	0	0	NA	NA	NA
KIT (Sunitinib / Sorafenib / Imatinib)	0	1	0	12.2 (NA)	10.4 (NA)	1.000
ABL1 (Imatinib / Dasatinib / GNF-2)	0	0	0	NA	NA	NA
ABL2 (Imatinib / Dasatinib / GNF-2)	0	1	0	11.8 (NA)	33.3 (NA)	1.000
BRAF (AZ628)	0	0	1	0.00 (NA)	18.6 (NA)	1.000
AURKA (VX680)	0	0	0	NA	NA	NA
AURKB (VX680)	0	0	0	NA	NA	NA
AURKC (VX680)	0	0	0	NA	NA	NA
ALK (Crizotinib / NVP-TAE684)	2	1	1	16.2 (9.93-22.20)	3.40 (0.00-12.7)	0.460
MET (Crizotonib)	1	0	0	25.1 (NA)	0.40 (NA)	1.000
SRC (Saracatanib / Dasatanib)	0	0	0	NA	NA	NA
KIF11 (NSC83265)	0	0	0	NA	NA	NA
APH1A (Z-LLNle-CHO)	0	0	0	NA	NA	NA
APH1B (Z-LLNle-CHO)	0	0	0	NA	NA	NA
BCR (GNF-2)	0	0	0	NA	NA	NA
CDK1 (CGP-60474 / Seliciclib)	0	0	0	NA	NA	NA
CDK2 (CGP-60474 / Seliciclib)	0	0	0	NA	NA	NA
CDK4 (CGP-082996 / Seliciclib)	1	0	0	68.8 (NA)	2.40 (NA)	1.000
CDK5 (CGP-60474 / Seliciclib)	0	1	0	31.3 (NA)	41.2 (NA)	1.000
CDK7 (CGP-60474 / Seliciclib)	0	0	0	NA	NA	NA
CDK9 (CGP-60474 / Seliciclib)	0	0	0	NA	NA	NA
BMX (WZ-1-84)	0	0	0	NA	NA	NA
TEC (WZ-1-84)	0	0	0	NA	NA	NA
PLK1 (BI-2536 / GW843682X)	0	1	0	31.1 (NA)	18.4 (NA)	1.000
PLK2 (BI-2536)	0	0	0	NA	NA	NA
PLK3 (BI-2536)	0	0	0	NA	NA	NA
IGF1R (BMS-536924)	0	0	0	NA	NA	NA
ITK (BMS-509744)	0	0	0	NA	NA	NA
RPS6KA2	2	0	0	31.9 (22.9-41.0)	0.00 (0.00-0.00)	0.221
DHFR (Daraprim)	0	0	0	NA	NA	NA
AKT1 (A-443654)	1	0	0	10.9 (NA)	1.10 (NA)	1.000
AKT2 (A-443654)	0	0	0	NA	NA	NA
AKT3 (A-443654)	0	0	0	NA	NA	NA
NFKB (Parthenolide)	0	0	0	NA	NA	NA
IKKE (KIN001-135)	0	0	0	NA	NA	NA
ERK5 (XMD8-85)	0	0	0	NA	NA	NA
PPP1R15A (Salubrinal)	0	0	0	NA	NA	NA
ROCK1 (GSK269962A)	0	0	0	NA	NA	NA
ROCK2 (GSK269962A)	0	0	0	NA	NA	NA
PRKAA1 (A-769662)	0	0	0	NA	NA	NA
PRKAA2 (A-769662)	0	0	0	NA	NA	NA
PRKAB1 (A-769662)	0	0	0	NA	NA	NA
PRKAB2 (A-769662)	0	0	0	NA	NA	NA
PRKAG1 (A-769662)	0	0	0	NA	NA	NA

PRKAG2 (A-769662)	0	1	0	17.4 (NA)	4.85 (NA)	1.000
PRKAG3 (A-769662)	0	0	0	NA	NA	NA
SHP1 (NSC-87877)	0	0	0	NA	NA	NA
SHP2 (NSC-87877)	0	0	0	NA	NA	NA
GSK3B (CHIR-99021)	0	0	0	NA	NA	NA
PI3KB (AZD6482)	0	0	0	NA	NA	NA
JNK (JNK-9L)	0	0	0	NA	NA	NA
FAK (PF-562271)	0	0	0	NA	NA	NA
P4HA1 (DMOG)	0	0	0	NA	NA	NA
P4HA3 (DMOG)	0	0	0	NA	NA	NA
FNTA (FTI-277)	0	0	0	NA	NA	NA
XIAP (Embelin)	0	0	0	NA	NA	NA
CASP3 (PAC-1)	0	0	0	NA	NA	NA
PAK (IPA-3)	0	0	0	NA	NA	NA
SGK3 (GSK-650394)	0	0	0	NA	NA	NA
SYK (BAY 61-3606)	0	0	0	NA	NA	NA
BCL2 (Obatoclax Mesylate)	0	0	0	NA	NA	NA
BCLXL (Obatoclax Mesylate)	0	0	0	NA	NA	NA
MCL2 (Obatoclax Mesylate)	0	0	0	NA	NA	NA
BTK (LFM-A13)	0	0	0	NA	NA	NA
HSP90 (AUY922)	0	0	0	NA	NA	NA
PRKC (Bryostatin 1)	0	0	0	NA	NA	NA
MEK1 (RDEA119)	0	0	0	NA	NA	NA
MEK2 (RDEA119)	0	0	0	NA	NA	NA
PARP1 (AZD-2281)	0	0	0	NA	NA	NA
PARP2 (AZD-2281)	0	0	0	NA	NA	NA
TNFA (Lenalidomide)	0	0	0	NA	NA	NA
CHK1 (AZD7762)	0	0	0	NA	NA	NA
CHK2 (AZD7762)	0	0	0	NA	NA	NA
NTRK1 (GW 441756)	0	0	0	NA	NA	NA
JAK (Lestaurtinib)	0	0	0	NA	NA	NA
RET (Lestaurtinib)	1	0	0	9.80 (NA)	1.10 (NA)	1.000
MAPK11 (VX-702)	0	0	0	NA	NA	NA
MAPK12 (VX-702)	0	0	0	NA	NA	NA
MAPK13 (VX-702)	0	0	0	NA	NA	NA
MAPK14 (VX-702)	0	0	0	NA	NA	NA
ATM (KU-55933)	0	0	0	NA	NA	NA
HSP70 (Elesclomol)	0	0	0	NA	NA	NA
SMO (Vismodegib)	0	0	0	NA	NA	NA
TBK1 (BX-795)	0	0	0	NA	NA	NA
PDK1 (BX-795)	0	0	0	NA	NA	NA
IKK (BX-795)	0	0	0	NA	NA	NA
DNAPK (NU-7441)	0	0	0	NA	NA	NA
PIM3 (SL 0101-1)	0	0	0	NA	NA	NA
WEE1 (681640)	0	0	1	0.00 (NA)	40.0 (NA)	1.000
MDM2 (Nutlin-3a)	0	0	0	NA	NA	NA
FGFR1 (PD-173074)	0	0	0	NA	NA	NA
FGFR2 (PD-173074)	1	0	0	10.4 (NA)	0.30 (NA)	1.000
FGFR3 (PD-173074)						

Supplementary table 5.14: Temporal heterogeneity of biomarker genes from Chapter 4)

Gene	Non-silent mutations			VAF (median, IQR)		
	Before only	Before and after	After only	Before	After	p
Survival biomarkers: mutations and copy number variants)						
PIK3CA	0	2	0	29.0 (22.8-30.2)	25.4 (17.8-33.0)	1.000
TP53	9	13	1	22.1 (11.6-39.2)	15.3 (0.00-41.7)	0.219
EGFR	0	2	0	18.2 (15.3-21.0)	32.1 (27.2-37.1)	0.667
ERBB2	0	1	0	6.70 (NA)	10.3 (NA)	1.000
MET	1	0	0	25.1 (NA)	0.40 (NA)	1.000
FGF3	0	0	0	NA	NA	NA
ERCC1	0	0	0	NA	NA	NA
ERCC2	0	0	0	NA	NA	NA
IL6	1	0	0	5.40 (NA)	0.00 (NA)	1.000
MDM2	0	0	0	NA	NA	NA
AURKA	0	0	0	NA	NA	NA
VEGFA	0	0	0	NA	NA	NA
GNAS1	0	0	0	NA	NA	NA
ECRG2	0	0	0	NA	NA	NA
MTHFR	0	0	0	NA	NA	NA
ABCB1	1	0	0	20.0 (NA)	0.00 (NA)	1.000
F2R	0	0	0	NA	NA	NA
Chemo/chemoradiotherapy sensitivity(Findlay et al., 2014b)						
TP53	9	13	1	22.1 (11.6-39.2)	15.3 (0.00-41.7)	0.219
GNAS1	0	0	0	NA	NA	NA
ERCC1	0	0	0	NA	NA	NA
XRCC1	0	1	0	14.5 (NA)	25.0 (NA)	1.000

Section 5.3.10: Survival associations

Supplementary table 5.15: Multivariate cox proportional hazards models for overall and disease-free survival

Factor	OS HR (95% CI)	p	DFS HR (95% CI)	p
Patient				
Age	1.03 (0.94-1.13)	0.520	1.04 (0.95-1.13)	0.440
Gender				
Female	Ref	Ref	Ref	Ref
Male	75.4 (1.85-3069.8)	0.022	23.7 (0.86-652.5)	0.061
ASA				
2	Ref	Ref	Ref	Ref
3	0.07 (0.00-2.39)	0.140	0.12 (0.00-3.66)	0.220
Tumour				
Grade				
Moderate	Ref	Ref	Ref	Ref
Poor	0.39 (0.07-2.09)	0.270	0.32 (0.06-1.73)	0.190
Avid nodes after NAC				
No	Ref	Ref	Ref	Ref
Yes	0.09 (0.00-1.61)	0.100	0.06 (0.00-1.18)	0.064
Pathological response				
pTR	Ref	Ref	Ref	Ref
No pTR	0.10 (0.02-0.61)	0.013	0.08 (0.01-0.54)	9.60×10^{-3}
Pathological stage				
pT				
1	Ref	Ref	Ref	Ref
2	0.00 (0.00-1.77)	0.076	0.01 (0.00-2.16)	0.094
3	0.05 (0.00-16.6)	0.320	0.12 (0.00-19.9)	0.420
4a	26.2 (0.01-58404.5)	0.410	13.1 (0.01-17285.9)	0.480
pN				
0	Ref	Ref	Ref	Ref
1	113.2 (4.45-2881.4)	4.20×10^{-3}	133.2 (4.95-3588.1)	3.60×10^{-3}
2	4.81 (0.25-92.7)	0.300	5.40 (0.26-111.7)	0.270
3	49.4 (1.33-1831.8)	0.034	28.30 (0.86-929.8)	0.061
pR				
0	Ref	Ref	Ref	Ref
1	3.13 (0.28-34.9)	0.350	2.70 (0.21-20.7)	0.540
pL				
0	Ref	Ref	Ref	Ref
1	3.68 (0.18-74.3)	0.400	5.27 (0.24-116.1)	0.290
pV				
0	Ref	Ref	Ref	Ref
1	0.10 (0.00-2.84)	0.180	0.10 (0.00-2.60)	0.170

CHAPTER 6: PRECISION STAGING OF OESOPHAGEAL CANCER USING DECISION THEORY: SELECTIVE ENDOSCOPIC ULTRASOUND, POSITRON EMISSION TOMOGRAPHY AND LAPAROSCOPY

Section 6.3.2: Endoscopic ultrasound

Supplementary table 6.1 Factors association with pT1N0 disease: binary logistic regression

Characteristic	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Gender				
Female	Ref	Ref	Ref	Ref
Male	1.41 (0.27-7.26)	0.680	1.13 (0.04-34.5)	0.943
Age (squared)	1.00 (1.00 -1.00)	0.765	1.00 (1.00 -1.00)	0.347
Cell type* NA				
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	0.67 (0.09-4.80)	0.687	0.03 ($1.12x10^{-4}$ -6.08)	0.190
Poor	0.52 (0.07-4.00)	0.529	0.123 (0.001-13.9)	0.114
Undifferentiated	NA	NA	NA	NA
Tumor location				
Distal	Ref	Ref	Ref	Ref
Siewert 1	1.11 (0.28-4.28)	0.881	1.83 (0.03-1.22)	0.778
Siewert 2	3.33 (0.632-17.6)	0.156	135 (0.09-$2.05x10^{5}$)	0.190
Siewert 3	NA	NA	NA	NA
Multifocal	NA	NA	NA	NA
Impassable tumor	NA	NA	NA	NA
CT T stage				
T1	Ref	Ref	Ref	Ref
T2-T4a	0.05 (8.88-$x10^{-3}$-0.272)	5.57 $x10^{-5}$	0.09 ($3.05x10^{-4}$-29.4)	0.420
T4b	NA	NA	NA	NA
CT N stage				
N0	Ref	Ref	Ref	Ref
N1	0.23 (0.02-2.20)	0.200	6.94 ($7.43x10^{-3}$-6486)	0.579
CT center				
OUH	1.33 (0.24-7.56)	0.745	7.89 (0.14-466)	0.321
Center 1	Ref	Ref	Ref	Ref
Center 2	1.60 (0.24-10.8)	0.630	8.20 (0.03-2125)	0.458
Center 3	0.89 (0.09-9.16)	0.921	4.30 ($7.80x10^{-3}$-2943)	0.632
Other	NA	NA	NA	NA
EUS T stage				
T1	Ref	NA	Ref	Ref
T2	NA	NA	NA	NA
T3	0.10 (0.02-0.43)	$2.12x10^{-3}$	0.14 ($4.08x10^{-4}$-50.6)	0.517
T4a	NA	NA	NA	NA
EUS N stage				
0	Ref	Ref	Ref	Ref
1	0.15 (0.03-0.82)	0.03	0.12 ($9.55x10^{-4}$-18.9)	0.376
PET SUV (Log)	$1.98x10^{-3}$ ($2.96x10^{-5}$-0.13)	$3.68x10^{-3}$	$8.28x10^{-3}$ ($8.46x10^{-8}$-810)	0.414
PET length	0.40 (0.22-0.73)	$2.32x10^{-3}$	0.38 (0.08-1.94)	0.245
PET scanner				
1	Ref	Ref	Ref	Ref
2	2.89 (0.83-10.0)	0.09	18.2 (0.54-616	0.106
PET avid nodes	NA	NA	NA	NA

*NA as perfect separators

Section 6.3.3: PET-CT

Supplementary table 6.2: Linear regression – factors associated with LogSUVmax

Characteristic	Univariate OR (95% CI)	p value	Multivariate OR (95% CI)	p value
Gender				
Female	Ref	Ref	Ref	
Male	0.924 (0.884-0.965)	4.41×10^{-5}	0.956 (0.91-0.997)	0.037
Cell type				
AC	Ref	Ref	Ref	Ref
SCC	1.15 (1.10-1.20)	1.07×10^{-8}	1.10 (1.04-1.16)	7.38×10^{5}
AS	1.13 (0.927-1.38)	0.226	1.10 (0.915-1.32)	0.315
NEC	1.06 (0.781-1.43)	0.716	0.982 (0.740-1.31)	0.901
Small cell	1.02 (0.704-1.48)	0.910	0.816 (0.555-1.20)	0.303
Undifferentiated	1.13-0.966-1.33)	0.124	0.928 (0.731-1.18)	0.541
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.07 (0.995-1.15)	0.072	1.05 (0.988-1.12)	0.244
Poor	1.07 (1.00-1.15)	0.043	1.06 (0.996-1.13)	0.069
Undifferentiated	1.18 (1.04-1.36)	0.013	1.18 (0.968-1.43)	0.104
Tumor site				
Proximal	Ref	Ref	Ref	
Middle	0.925 (0.813-1.05)	0.239	0.971 (0.862-1.09)	0.623
Distal	0.874 (0.778-0.983)	0.025	0.967 (0.866-1.08)	0.553
Siewert 1	0.862 (0.767-0.969)	0.013	0.969 (0.865-1.09)	0.594
Siewert 2	0.852 (0.757-0.958)	7.68×10^{-3}	0.959 (0.854-1.08)	0.481
Siewert 3	0.86 (0.789-0.957)	7.83×10^{-3}	0.914 (0.810-1.03)	0.142
Multifocal	1.06 (0.816-1.38)	0.658	1.03 (8.10-1.031)	0.825
Impassable tumor	1.08 (1.02-1.14)	4.49×10^{-3}	1.02 (0.971-1.07)	0.142
Age (squared)	1.000 (1.000)	5.85×10^{-3}	1.00 (1.00-1.00)	0.038
Stage				
I	Ref	Ref	Ref	Ref
IIA	1.41 (1.30-1.54)	2.31×10^{-14}	1.41 (1.29-1.53)	3.05×10^{-14}
IIB	1.44 (1.30-1.60)	3.03×10^{-12}	1.42 (1.29-1.57)	1.00×10^{-11}
III	1.58 (1.46-1.72)	$<2.2\times10^{-16}$	1.55 (1.43-1.68)	$<2.2\times10^{-16}$
IV	1.69 (1.56-1.85)	$<2.2\times10^{-16}$	1.66 (1.52-1.81)	$<2.2\times10^{-16}$
PET scanner				
1	Ref	Ref	Ref	Ref
2	1.362 (1.131)	0.0122	1.05 (1.01-1.09)	9.80×10^{-3}

Supplementary table 6.3: Linear regression – factors associated with PET length

Characteristic	Univariate OR (SE)	p value	Multivariate OR (95% CI)	p value
Gender				
Female	Ref	Ref	Ref	Ref
Male	0.798 (0.502-1.27)	0.341	0.836 (0.537-1.30)	0.429
Cell type				
AC	Ref	Ref	Ref	Ref
SCC	1.13 (0.686-1.86)	0.635	1.07 (0.604-1.90)	0.815
ASS	0.500 (0.059-4.20))	0.523	0.324 (0.047-2.21)	0.251
NEC	4.04 (0.158-103.4)	0.399	2.45 (0.124-48.6)	0.557
Small cell	28.9 (0.156-1526)	0.097	4.43 (0.078-254.5)	0.472
Undifferentiated	12.9 (2.34-70.7)	3.40×10^{-3}	2.21 (0.179-27.1)	0.537
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.37 (0.655-2.86)	0.404	1.30 (0.664-2.54)	0.446
Poor	2.29 (1.10-4.74)	0.026	1.80 (0.920-3.52)	0.086
Undifferentiated	17.8 (4.33-73.1)	7.13×10^{-5}	3.38 (0.435-26.2)	0.245
Tumor site				
Proximal	Ref	Ref	Ref	Ref
Middle	0.591 (0.152-2.30)	0.448	0.828 (0.239-0.287)	0.767
Distal	0.452 (0.132-1.55)	0.206	0.723 (0.226-2.32)	0.585
Siewert 1	0.587 (0.171-2.02)	0.399	1.05 (0.317-3.47)	0.936
Siewert 2	0.761 (0.221-2.62)	0.665	1.14 (0.335-0.385)	0.838
Siewert3	0.964 (0.265-3.50)	0.955	0.908 (0.257-3.21)	0.881
Multifocal	15.128 (0.952-240.3)	0.055	7.31 (0.599-89.1)	0.119
Impassable tumor				
Yes	2.11 (1.20-3.70)	9.98×10^{-3}	1.29 (0.771-2.17)	0.331
Age (squared)	1.00 (1.00-1.00)	1.95×10^{-3}	1.00 (1.00-1.00)	0.059
Stage				
I	Ref	Ref	Ref	Ref
IIA	23.6 (9.59-58.1)	1.22×10^{-11}	21.4 (8.65-52.9)	6.26×10^{-11}
IIB	20.6 (7.22-58.6)	2.10×10^{-8}	19.6 (6.89-56.0)	3.49×10^{-8}
III	108.8 (46.7-253.6)	$<2.2 \times 10^{-16}$	89.0 (37.7-210)	$<2.2 \times 10^{-16}$
IV	2643.3 (109.3-639.4)	$<2.2 \times 10^{-16}$	183.2 (73.9-454.6)	$<2.2 \times 10^{-16}$
PET scanner				
1	Ref	Ref	Ref	Ref
2	0.919 (0.616-1.37)	0.679	0.891 (0.615-1.29)	0.540

Supplementary table 6.4: Binary logistic regression–factors associated with PET avid nodes

Characteristic	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Gender				
Female	Ref	Ref	Ref	Ref
Male	0.791 (0.567-1.10)	0.167	0.796 (0.528-1.20)	0.274
Cell type				
AC	Ref	Ref	Ref	Ref
SCC	1.71 (1.19-2.45)	3.48×10^{-3}	1.74 (1.03-2.94)	0.037
AS	1.07 (0.236-4.80)	0.934	1.45 (0.275-7.67)	0.660
NEC	2.84 (0.256-31.5)	0.394	3.87 (0.290-51.7)	0.306
Small cell	1.42 (0.088-22.8)	0.804	0.661 (0.025-17.2)	0.804
Undifferentiated	3.32 (0.849-13.0)	0.088	2.92 (0.294-29.0)	0.360
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	0.992 (0.572-1.72)	0.976	0.835 (0.441-1.58)	0.578
Poor	1.60 (0.923-2.75)	0.088	1.31 (0.693-2.46)	0.410
Undifferentiate	2.74 (0.953-7.90)	0.062	0.786 (0.141-4.39)	0.783
Tumor site				
Proximal	Ref	Ref	Ref	Ref
Middle	0.350 (0.126-0.969)	0.043	0.446 (0.142-1.39)	0.165
Distal	0.362 (1.43-0.918)	0.032	0.407 (0.139-1.19)	0.101
Siewert 1	0.371 (1.46-0.945)	0.038	0.475 (0.158-1.43)	0.185
Siewert 2	0.270 (0.106-0.688)	6.12×10^{-3}	0.383 (0.125-1.18)	0.095
Siewert 3	0.375 (0.142-0.988)	0.047	0.357 (0.112-1.40)	0.082
Multifocal	NA	NA	NA	NA
Impassable tumor	1.24 (0.829-1.86)	0.292	0.935 (0.583-1.50)	0.782
Age (squared)	1.00 (1.00-1.00)	1.74×10^{-4}	1.00 (1.00-1.00)	1.54×10^{-3}
Stage				
I	NA	NA	NA	NA
IIA	Ref	Ref	Ref	Ref
IIB	5.19 (2.51-10.7)	8.53×10^{-6}	4.73 (2.23-10.0)	5.03×10^{-5}
III	5.93 (3.47-10.1)	6.56×10^{-11}	5.49 (3.16-9.53)	1.39×10^{-9}
IV	19.7 (10.9-35.5)	$<2.2 \times 10^{-16}$	19.4 (10.4-36.1)	$<2.2 \times 10^{-16}$
PET scanner				
1	Ref	Ref	Ref	Ref
2	1.96 (1.46-2.62)	6.97×10^{-6}	2.09 (1.48-2.95)	2.58×10^{-5}

Supplementary table 6.5: Factors associated with demonstration of unsuspected PET-CT metastases (binary logistic regression; excluding perfect separators)

Characteristic	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Gender				
Female	Ref	Ref	Ref	Ref
Male	1.90 (0.942-3.84)	0.073	2.10 (0.95-0.463)	0.066
Tumor site				
Proximal	Ref	Ref	Ref	Ref
Mid	0.652 (0.112-3.79)	0.634	0.518 (0.069-3.87)	0.522
Distal	1.22 (0.257-5.78)	0.803	1.26 (0.213-7.38)	0.802
GOJ (Type I)	0.732 (0.149-3.59)	0.700	0.713 (0.114-4.47)	0.718
GOJ (Type II)	0.451 (0.088-2.30)	0.338	0.383 (0.058-2.52)	0.318
GOJ (Type III)	0.844 (0.162-4.40)	0.840	0.695 (0.100-4.84)	0.714
Multifocal	NA	NA	NA	NA
EUS T stage				
1	NA	NA	NA	NA
2	Ref	Ref	Ref	Ref
3	3.92 (1.19-12.8)	0.024	3.88 (1.03-14.6)	0.045
4A	4.14 (0.946-18.3)	0.061	3.07 (0.567-16.6)	0.193
4B	8.54 (1.85-39.4)	5.95×10^{-3}	6.57 (0.918-47.1)	0.061
EUS N stage				
0	Ref	Ref	Ref	Ref
1	1.71 (0.921-3.18)	0.089	0.869 (0.426-1.77	0.700
CT T stage				
Possible T1	Ref	Ref	Ref	Ref
T2-T4a	1.37 (0.522-3.58)	0.525	0.430 (0.137-1.35))	0.149
Possible T4b	3.69 (1.24-11.0)	0.019	0.909 (0.209-3.95)	0.899
CT N stage				
N0	Ref	Ref	Ref	Ref
N1	5.69 (2.75-11.7)	2.59×10^{-6}	6.27 (2.69-14.6)	2.17×10^{-5}
Cell type				
AC	Ref	Ref	Ref	Ref
SCC	0.914 (0.469-1.78)	0.791	0.775 (0,315-1.90)	0.579
AS	1.58 (0.181-13.8)	0.678	1.67 (1.30-21.5)	0.692
NEC	NA	NA	NA	NA
Small cell	NA	NA	NA	NA
Undifferentiated	7.92 (1.92-32.7)	4.22×10^{-3}	3.25 (0.179-59.3)	0.426
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	0.795 (0.282-2.24)	0.663	0.829 (0.272-2.53)	0.742
Poor	1.51 (0.562-4.04)	0.416	1.19 (0.403-3.50)	0.755
Undifferentiated	5.11 (1.22-21.7)	0.025	1.73 (0.128-23.6)	0.679
Impassable tumor	1.39 (0.691-2.80	0.355	1.33 (0.600-2.97)	0.479
Age	1.00 (1.00-1.00)	0.075	1.00 (1.00-1.00)	0.132
PET scanner				
1	Ref	Ref	Ref	Ref
2	0.969 (0.580-1.62)	0.905	0.998 (0.557-1.79)	0.993

Section 6.3.4: Laparoscopy

Supplementary table 6.6: Factors associated with metastases at staging laparoscopy: binary logistic regression*

Characteristic	Univariate OR (95%CI)	p	Multivariate OR (95% CI)	p
Age (squared)	1.00 (0.999-1.00)	0.306	1.000 (1.000)	0.638
Gender				
Female	Ref	Ref	Ref	Ref
Male	2.20 (0.499-9.75)	0.297	4.09 (0.623-26.8)	0.143
Tumor location				
Distal	Ref	Ref	Ref	Ref
Siewert 1	0.242 (0.025-2.39)	0.225	0.207 (0.017-2.47)	0.213
Siewert 2	1.73 (0.457-6.54)	0.420	1.28 (0.287-5.69)	0.747
Siewert 3	2.56 (0.629-10.4)	0.189	1.30 (0.238-7.15)	0.761
EUS T stage				
T1	NA	NA	NA	NA
T2	Ref	Ref	Ref	Ref
T3	1.22 (0.266-5.60)	0.798	0.693 (0.121-3.98)	0.681
T4a	6.45 (1.2034.8)	0.030	3.66 (0.473-28.3)	0.214
EUS N stage				
N0	Ref	Ref	Ref	Ref
N1	1.43 (0.507-3.52)	0.502	1.06 (0.302-3.73)	0.927
CT T stage				
Possible T1	Ref	Ref	Ref	Ref
T2-T4a	2.53 (0.336-19.5)	0.375	1.52 (0.155-14.9)	0.718
Possible T4b	18.5 (1.64-208.7)	0.018	20.9 (1.01-431.5)	0.049
CT N stage				
N0	Ref	Ref	Ref	Ref
N1	1.42 (0.570-3.52)	0.453	1.02 (0.305-3.41)	0.974
Cell type	NA	NA	NA	NA
Grade				
Well	NA	NA	NA	NA
Moderate	Ref	Ref	Ref	Ref
Poor	0.682 (0.269-1.73)	0.420	0.815 (0.262-2.53)	0.723
Undifferentiated	12.5 (1.59-98.4)	0.016	37.1 (3.00-458.8)	4.86×10^{-3}
Impassable	3.20 (1.08-9.43)	0.035	4.95 (1.34-18.1)	0.016
Age	1.00 (0.999-1.00)	0.355	1.000 (0.999-1.00)	0.651
SUVmax (log)	0.202 (0.034-1.20)	0.078	0.014 (7.75×10^{-4}-0.246)	3.54×10^{-3}
PET length	1.03 (0.877-1.21)	0.723	1.09 (0.891-1.33)	0.47
Avid nodes	1.37 (0.548-3.41)	0.503	2.58 (0.696-9.58)	0.156
PET scanner				
1	Ref	Ref	Ref	Ref
2	0.979 (0.403-2.38)	0.962	0.574 (0.170-1.94)	0.372

Section 6.3.6: Predictive modelling

Supplementary table 6.7: Statistical comparison of model performance (McNemar's test)

Algorithm / model	Sens	Spec	p –v- default		p –v- DTA/internal validation		p –v- LRM/internal validation	
			Sens	Spec	Sens	Spec	Sens	Spec
EUS T1N0								
Models before PET-CT								
DTA 1 (apparent)	0.961	0.878	0.157	$<2.2x10^{-16}$	NA	NA	0.317	$<2.2x10^{-16}$
DTA 1 (internal)	0.941	0.881	0.083	$<2.2x10^{-16}$	NA	NA	0.083	$<2.2x10^{-16}$
LRM 1 (apparent)	0.980	0.535	0.157	$<2.2x10^{-16}$	1.000	$<2.2x10^{-16}$	NA	NA
LRM 1 (internal)	0.980	0.535	0.157	$<2.2x10^{-16}$	1.000	$<2.2x10^{-16}$	NA	NA
Models after PET-CT								
DTA 2 (apparent)	1.00	0.840	1.000	$<2.2x10^{-16}$	NA	NA	1.00	$7.10x10^{-6}$
DTA 2 (internal)	0.944	0.866	0.083	$<2.2x10^{-16}$	NA	NA	1.00	$7.10x10^{-6}$
LRM 2 (apparent)	0.947	0.734	0.083	$<2.2x10^{-16}$	1.000	$7.10x10^{-6}$	NA	NA
LRM 2 (internal)	0.947	0.734	0.083	$<2.2x10^{-16}$	1.000	$7.10x10^{-6}$	0.228	0.996
EUS T4b								
DTA (apparent)	1.000	0.936	1.000	$<2.2x10^{-16}$	NA	NA	NA	NA
DTA (internal)	0.962	0.926	0.317	$<2.2x10^{-16}$	NA	NA	NA	NA
pT1N0								
DTA (apparent)	1.000	0.348	1.000	$<2.2x10^{-16}$	NA	NA	1.000	$4.68x10^{-3}$
DTA (internal)	0.958	0.300	0.317	$<2.2x10^{-16}$	NA	NA	1.000	$<2.2x10^{-16}$
LRM (apparent)	1.000	0.100	1.000	$<2.2x10^{-16}$	1.000	$4.68x10^{-3}$	NA	NA
LRM (internal)	1.000	0.00	1.000	1.00	0.317	0.014	NA	NA

*For LRM, comparative DTA and ANN metrics were obtained from the LRM subset only

EUS T1N0 LRM1: equation=-0.277-4.329[CT T2-T4a]-1.797[CT N1])

EUS T1N0 LRM 2: equation=1.279-3.485[CT T2-T4a]-0.863[PET length])

pT1N0 LRM: LRM: 5.932-2.467[CT T2-T4a]-6.746[LogSUV]-0.031[length])

CHAPTER 7: RESTAGING OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY WITH POSITRON EMISSION TOMOGRAPHY

Section 7.3.3: Predictive factors for interval progression

Supplementary table 7.1: Pre-NAC factors associated with progression to overt metastases

	No metastases (n=363)	Metastases (n=20)	p ($\propto$=2.17x10^{-3})
All patients			
Age (median) IQR, range	64.0 (57.0-70.0; 34.0-80.0)	63.0 (55.8-70.3;47.0-83.0)	0.821[a]
Gender *Female* *Male*	 91 (25.1%) 272 (74.9%)	 8 (40.0%) 12 (60.0%)	 0.222[b]
Cell type *AC* *SCC* *AS* *Small cell* *Anaplastic*	 293 (80.7%) 61 (16.8%) 5 (1.38%) 1 (0.28%) 3 (0.83%)	 16 (80.0%) 4 (20.0%) 0 (0.00%) 0 (0.00%) 0 (0.00%)	 0.315[b]
Grade *Well* *Moderate* *Poor* *Unidifferentiated*	 33 (9.09%) 155 (42.7%) 169 (46.6%) 6 (1.65%)	 1 (5.00%) 6 (30.0%) 12 (60.0%) 1 (5.00%)	 0.298[b]
Pre-treatment T stage *1* *2* *3* *4a*	 8 (2.20%) 54 (14.9%) 276 (76.0%) 25 (6.89%)	 0 (0.00%) 2 (10.0%) 14 (70.0%) 4 (20.0%)	 0.229[b]
Pre-treatment N Stage *0* *1*	 108 (29.8%) 255 (70.2%)	 5 (25.0%) 15 (75.0%)	 0.803[b]
Impassable *No* *Yes*	 319 (87.9%) 44 (12.1%)	 18 (90.0%) 2 (10.0%)	 1.000[b]
Site *Proximal* *Mid* *Distal* *Siewert 1* *Siewert 2* *Siewert 3* *Multifocal*	 5 (1.38%) 25 (6.89%) 70 (19.3%) 86 (23.7%) 118 (32.5%) 57 (15.7%) 2 (0.55%)	 0 (0.00%) 3 (15.0%) 8 (4010%) 4 (200%) 3 (15.0%) 2 (10.0%) 0 (0.00%)	 0.190[b]
Restaging scan *CT* *PET-CT*	 100 (27.5%) 263 (72.5%)	 3 (15.0%) 17 (85.0%)	 0.303[b]
Days to scan (median) (IQR; range)	82.5 (73.0-97.3; 40.0-178.0)	86.0 (79.5-95.0; 50.0-157.0)	0.357[a]
Initial PET scanner *Before Nov 2009* *After Nov 2009*	 173 (47.7%) 190 52.3%)	 12 (60.0%) 8 (40.0%)	 0.360[b]

Initial SUVmax (median) (IQR; range)	10.25 (7.18-15.9; 2.5-52.6)	13.7 (9.45-16.75; 6.50-46.1)	0.354[a]
Initial PET length (median) (IQR; range)	5.00 (3.50-6.53; 0.00-14.6)	7.40 (5.20-9.05; 3.20-15.5)	1.26×10^{-3} [a]
Initial avid nodes *0* *1* *2* *3+*	 241 (66.4%) 40 (24.2%) 27 (7.44%) 55 (15.2%)	 2 (10.0%) 5 (25.0%) 2 (10.0%) 11 (55.0%)	8.93×10^{-7} [b]
Initial avid nodes compartments *0* *1* *2* *3*	 241 (66.4%) 88 (24.2%) 31 (8.54%) 3 (0.83%)	 2 (10.0%) 10 (50.0%) 7 (35.0%) 1 (5.00%)	6.09×10^{-7} [b]
Avid tumour *Avid* *Non avid*	 351 (96.7%) 12 (3.31%)	 20 (100%) 0 (0.00%)	1.000[b]
Neoadjuvant regimen *Dual* *Triple*	 271 (74.7%) 91 (25.3%)	 11 (55.0%) 9 (45.0%)	0.067[b]
Patients with avid nodes			
Avid nodal SUVmax (median); (IQR, range)	6.59 (4.26-9.44; 21.6-27.5)	7.46 (5.82-8.79; 2.70-20.3)	0.485
Additional PET parameters – patients staged using most recent scanner			
	(n=185)	(n=8)	
SUVmean (median) (IQR; range)	6.31 (5.28-7.88; 2.50-13.4)	6.45 (5.75-8.43; 5.34-15.4)	0.547[a]
SUVpeak (median) (IQR; range)	9.78 (6.93-13.7; 2.50-32.2)	10.1 (7.98-14.9; 6.77-33.8)	0.512[a]
MTV (median) (IQR; range)	16.6 (7.07-37.0; 0.00-217)	24.2 (13.4-92.6; 5.26-124.2)	0.197[a]
TGVmean (median) (IQR; range)	113.7 (40.2-277.5; 0.00-2561)	169.9 95.5-560.6; 28.1-1907)	0.211[a]
TGVmax (median) (IQR; range)	170.8 (44.5563.6; 0.00-7247)	236.8 (101.8-490.1; 0.00-127.4)	0.709[a]

a=Mann-Witney test; b=Fisher's exact test' c=Pearson's Chi square test

Supplementary table 7.2: Pre-neoadjuvant NAC factors associated with progression to unresectable disease at surgery

	Resectable (n=302)	Unresectable (n=34)	p ($\propto$=2.17x10^{-3})
Age (median) IQR, range	64.0 (57.3-70.0; 36.0-80.0)	63.5 (54.0-69.0; 34.0-78.0)	0.599a
Gender *Female* *Male*	 73 (24.2%) 229 (75.8%)	 12 (35.3%) 22 (64.7%)	 0.228[b]
Cell type *AC* *SCC* *AS* *Small cell* *Anaplastic*	 250 (82.8%) 44 (14.6%) 5 (1.66%) 1 (0.33%) 2 (0.66%)	 28 (82.4%) 6 (17.6%) 0 (0.00%) 0 (0.00%) 0 (0.00%)	 0.840[c]
Pre-treatment Grade *Well* *Moderate* *Poor* *Undifferentiated*	 28 (9.27%) 129 (42.7%) 140 (46.4%) 5 (1.66%)	 2 (5.88%) 15 (44.1%) 17 (50.0%) 0 (0.00%)	 0.938
Pre-treatment T stage *1* *2* *3* *4a*	 7 (2.32%) 46 (15.2%) 232 (76.8%) 17 (5.63%)	 1 (2.94%) 4 (11.8%) 24 (70.6%) 5 (14.7%)	 0.203[c]
Pre-treatment N Stage *0* *1*	 88 (29.1%) 214 (70.9%)	 9 (26.5%) 25 (73.5%)	 0.843
Impassable *No* *Yes*	 279 (92.4%) 23 (7.62%)	 15 (44.1%) 19 (55.9%)	 6.47x10^{-15} [c]
Site *Proximal* *Mid* *Distal* *Siewert 1* *Siewert 2* *Siewert 3* *Multifocal*	 3 (0.99%) 16 (5.30%) 52 (17.2%) 72 (23.8%) 107 (35.4%) 51 (16.9%) 1 (0.33%)	 1 (2.94%) 3 (8.82%) 9 (26.5%) 8 (23.5%) 8 (23.5%) 4 (11.8%) 1 (2.94%)	 0.146[c]
Restaging scan *CT* *PET-CT*	 81 (26.8%) 221 (73.2%)	 8 (23.5%) 26 (76.5%)	 0.838[c]
Days to scan (median) (IQR; range)	82.0 (71.0-93.8; 40.0-167.0)	84.0 (75.0-102.8; 56.0-135.0)	0.136
Days to surgery (median) (IQR; range)	24.0 17.0-33.0; 1.00-72.0	22.0 (13.3-38.8; 4.00-57.0)	0.920
Avid tumour *Avid* *Non avid*	 291 (96.4%) 11 (3.64%)	 33 (97.1%) 1 (2.94%)	 1.000[b]
Initial SUVmax (median) (IQR; range)	10.2 (7.10-15.6; 2.50-38.7)	10.2 (7.10-15.72; 2.50-52.6)	0.799[a]
Initial PET length (median) (IQR; range)	5.00 (3.50-6.50; 0.00-14.6)	6.00 (5.00-7.30; 0.00-11.5)	0.013[a]
Initial mN stage *0* *1* *2*	 210 (69.5%) 54 (17.9%) 38 (12.6%)	 18 (52.9%) 7 (20.6%) 9 (26.5%)	 0.063

Nodal SUVmax (median) (IQR, range) Unknown	6.49 (4.21-8.52; 2.16-27.5) 8	6.48 (3.92-8.97; 2.87-15.6) 0	0.921
Initial PET scanner *Before Nov 2009* *After Nov 2009*	 142 (47.0%) 160 (53.0%)	 20 (58.8%) 14 (41.2%)	0.259
Neoadjuvant regimen *Dual* *Triple*	 231 (76.7%) 71 (23.3%)	 24 (70.6%) 10 (29.4%)	0.525[b]
Additional PET-CT variables			
	Resectable (n=160)	Unresectable (n=14)	
SUVmean (median) (IQR, range) Unknown	6.130 (5.35-7.93; 4.32-13.4) 6	6.42 (5.28-7.76; 4.72-10.1) 0	0.709[a]
SUVpeak (median) (IQR, range) Unknown Incalculable (therefore=2.5)	9.22 (6.70-13.7; 2.5-32.2) 7 19	11.3 (7.45-13.9; 2.5-20.7) 0 1	0.551[a]
MTV (median) (IQR, range) Unknown	13.5 (6.65-37.0; 0.1-191) 6	26.8 23.5-62.5; 2.86-170.5) 0	0.050[a]
TGVmean (median) (IQR, range) Unknown	97.3 39.6-299.6; 0.44-2561) 6	192.9 123.1-368.6; 13.5-1098)	0.074[a]
TGVmax (median) (IQR, range) Unknown	139 (39.6-618.6; 0.00-7247) 6	293.0 (96.6-620.9; 18.0-2346)	0.196[a]

A=Mann Witney test; b=Pearson's Chi square; c=Fisher's exact test; IQR=interquartile range; MTV=metabolic tumour volume; TGV=tumour glycolytic volume; SUV=standardized uptake value

Supplementary table 7.3: Factors associated with unresectable disease at surgery: re-staging PET-CT

	Resectable (n=221)	Unresectable (n=26)	p value ($\propto$=2.38x10^{-3}
Age (median) IQR, range	65.0 (59.0-71.0;	66.0 (51.0-71.3; 34.0-78.0)	0.725a
Sex *Female* *Male*	 51 (23.1%) 170 (26.9%)	 10 (38.5%) 16 (61.5%)	0.139[b]
Cell type *AC* *SCC* *AS* *Small cell* *Anaplastic*	 183 (82.8%) 32 (14.5%) 3 (1.36%) 1 (0.45%) 2 (0.90%)	 22 (84.6%) 4 (15.4%) 0 (0.00%) 0 (0.00%) 0 (0.00%)	1.000[c]
Pre-treatment Grade *Well* *Moderate* *Poor* *Undifferentiated*	 15 (6.79%) 95 (43.0%) 107 (48.4%) 4 (1.81%)	 1 (3.85%) 10 38.5%) 15 (57.7%) 0 (0.00%)	0.938
Pre-treatment T stage *1* *2* *3* *4a*	 6 (2.71%) 39 (17.6%) 163 (73.8%) 13 (5.88%)	 1 (3.85%) 4 (15.4%) 18 (69.2%) 3 (11.5%)	0.567[c]
Pre-treatment N Stage *0* *1*	 59 26.7%) 162 (73.3%)	 6 (23.1%) 20 (76.9%)	0.816
Impassable *No* *Yes*	 204 (92.3%) 17 (7.69%)	 12 (46.2%) 14 (53.8%)	1.49x10^{-10} [b]
Site *Proximal* *Mid* *Distal* *Siewert 1* *Siewert 2* *Siewert 3* *Multifocal*	 2 (0.90%) 11 (4.98%) 37 (16.7%) 61 (27.6%) 80 (36.2%) 29 (13.1%) 1 (0.45%)	 1 (3.85%) 1 (3.85%) 9 (34.6%) 6 (23.1%) 6 (23.1%) 3 (11.5%) 0 (0.00%)	0.239[c]
Restaging scanner *Pre Nov 2009* *Post Nov 2009*	 62 (28.1%) 159 (71.9%)	 13 (50.0%) 13 (50.0%)	0.038[b]
Days to surgery** (median) (IQR; range)	23.0 16.0-32.0; 4.00-72.0	22.0 (13.0-37.0; 4.00-57.0)	0.746[a]
Restaging SUVmax*** (median) (IQR; range)	5.90 (neg-8.90; neg-29.7)	6.75 (4.40-11.3; neg-18.3)	0.799[a]
Restaging PET negative *No* *Yes*	 165 (74.7%) 56 (25.3%)	 21 (80.8%) 5 (19.2%)	0.633[c]
Restaging PET length****(median) (IQR; range)	3.00 (1.00-5.00 0.00-12.5)	4.35 (2.23-5.88; 0.00-10.6)	0.008[a]
Restaging mN stage *0* *1* *2*	 191 (86.4%) 16 (7.24%) 14 (6.33%)	 15 (57.7%) 2 (7.69%) 9 (34.6%)	1.94x10-4
Nodal SUVmax (median) (IQR, range) Unknown	4.38 (3.66-6.33; 2.15-14.4) 0	4.70 (2.90-6.71; 2.50-7.78) 2	0.900
Neoadjuvant regimen Double Triple	 174 (78.7%) 47 (21.3%)	 17 (65.4%) 9 (34.6%)	0.139[c]

Additional PET-CT variables			
	Resectable disease (n=149)	Unresectable disease (n=13)	
SUVmean Unknown	5.06 (4.46-5.85; 4.01-10.8) 13	5.73 (4.75-7.11; 4.09-10.1) 1	0.286
SUVpeak	Negative (Neg-8.19; Neg-20.5)	7.81 (5.63-10.8; neg-17.4)	0.040
MTV	3.73 (0.78-10.0; 0.02-103.9)	15.3 (9.26-26.3; 0.05-49.9)	0.024
TGVmean	19.2 (3.43-58.1; 0.08-751.1)	109 (49.2-140.9; 0.20-501.5)	0.026
TGVmax	28.2 (4.36-108.8; 0.08-1823)	213.5 (84.6-236.5; 0.21-1066)	0.031

A=Mann Witney test; b=Pearson's Chi square; c=Fisher's exact test; IQR=interquartile range; MTV=metabolic tumour volume; TGV=tumour glycolytic volume; SUV=standardized uptake value

Supplementary table 7.4: Metabolic response of avid tumors and unresectable disease

	Resectable (n=215)	Unresectable (n=26)	p (α=6.25x10^{-3})
% SUVmax response (median) (IQR, range) Unknown	57.9% (25.2-86.9; -284.6-100%) 0	51.7% (17.6-78.6 -31.8-100%)	0.673a
SUVmax PERCIST *Non avid* *CMR* *PMR* *SMD* *SMD*	 7 49 108 43 14	 0 5 13 7 1	0.903[b]
% Length response (median) (IQR; range) Unknown	29.8% (0.00-74.2; -132.1-100.%) 3	27.3% (10.4-57.8; -97.0-100) 0	0.887[a]
Second PET scanner initially avid tumors (n=186)			
SUVmean response	27.1% 9.23-100; -26.2-100%)	13.4% 8.24-23.8; -11.7-100%	0.114 [a]
SUVpeak response	66.8% 25.4-95.8; -164-100%	17.6% 8.20-51.0; -10.6-100%	0.038 [a]
% MTV response (median) (IQR, range) Unknown	87.6% (57.2-100; -353.3-100) NA	55.0% (47.8-97.1; -157.1-100) 1	0.216[a]
% TGVmean response (median) Unknown	91.8% 64.5-1.00; -367.8-100 5	62.8% 50.3-97.5; -135.9-100 1	0.144 [a]
% TGVmax response (median) Unknown	93.5% 70.0-100; -427.8-100) 5	74.6% (48.9-98.2; -123.3-100) 1	0.155 [a]

A=Mann Witney test; b=Fisher's exact test; IQR=interquartile range;

Section 7.3.4: Predictive modelling

Supplementary table 7.5: Logistic regression formulae

Outcome	Inputs	Formula
Metastases	Baseline	-5.87 + 0.26*length [cm] + 1.46*mN1 + 1.93*mN2
Unresectable	Baseline	-3.39 + 4.14*Impassable tumour + 2.71*mN1 + 4.11*mN2 + 0.67*avid length -5.27*logSUVmax.
Unresectable	Re-staging	-3.64 + 2.582*Impassable + 0.046*nodal CMR + 1.237*nodal PMR + 2.300*nodal SND+ 1.542*nodal PND

CHAPTER 8: PREDICTING PATHOLOGICAL RESPONSE OF OESOPHAGEAL CANCER TO NEOADJUVANT CHEMOTHERAPY

Section 8.3.2: Predicting pathological response

Supplementary table 8.1: Pre-NAC factors associated with pTR

Factor	Response		p $\alpha=2.94x10^{-3}$
	Response (n=82)	No response (n=219)	
Age	62.5 (57.3-69.0; 36.0-79.0)	64.0 (58.0-70.0; 38.0-80.0)	0.369[a]
Sex			
Male	61 (74.4%)	167 (76.3%)	0.764[b]
Female	21 (25.6%)	52 (23.7%)	
Cell			
AC	68 (82.9%)	181 (82.6%)	0.979[b]
SCC	13 (15.9%)	31 (14.2%)	
AS	1 (1.22%)	4 (1.83%)	
NEC	0 (0.00%)	0 (0.00%)	
SC	0 (0.00%)	1 (0.46%)	
Anaplastic	0 (0.00%)	2 (0.91%)	
Chemo			
Dual	48 (39.0%)	37 (16.9%)	$2.69x10^{-5}$ [b]
Triple	32 (61.0%)	182 (83.2%)	
Site			
Proximal 1/3	0 (0.00%)	0 (0.00%)	0.033[b]
Mid 1/3	9 (11.0%)	11 (5.02%)	
Distal 1/3	11 (13.4%)	41 (18.7%)	
GOJ 1	23 (28.0%)	49 (22.4%)	
GOJ 2	20 (24.4%)	85 (38.8%)	
GOJ 3	19 (23.2%)	32 (14.6%)	
Multifocal	0 (0.00%)	1 (0.46%)	
T stage			
1	2 (2.43%)	5 (2.28%)	0.114 [b]
2	19 (23.2%)	27 (12.3%)	
3	56 (68.3%)	175 (79.9%)	
4a	5 (6.10%)	7 (3.20%)	
N stage			
0	30 (36.6%)	58 (26.5%)	0.090[b]
1	52 (63.4%)	161 (73.5%)	
PET scanner			
1	38 (46.3%)	104 (47.5%)	0.897[b]
2	44 (55.7%)	115 (52.5%)	
mN stage			
0	54 (65.9%)	155 (70.8%)	0.371[b]
1	14 (17.1%)	40 (18.3%)	
2	14 (17.1%)	24 (11.0%)	
Nodal SUVmax	7.05 (4.84-10.8; 2.53-27.5)	5.68 (4.18-8.49; 2.16-18.2)	0.253[a]
NA	54	155	
FDG-avid tumours			
SUVmax	9.05 (5.63-15.7; 2.50-38.7)	10.5 (7.50-15.6; 2.50-38.6)	0.073[a]
Avid length	4.40 (3.00-6.00; 1.00-14.0)	5.10 (3.70-6.55; 1.00-14.6)	0.013[a]
Patients staged using most recent PET scanner			
SUVmean	6.58 (5.10-8.41; 2.50-13.4)	6.08 (5.24-7.62; 2.50-11.6)	0.579[a]
NA	42		
SUVpeak	9.91 (5.97-14.9; 2.50-32.2)	9.15 (6.55-13.1; 2.50-29.6)	0.457[a]
NA	42		
MTV	16.4 (7.21-58.9; 0.51-191)	13.5 (6.55-36.2; 0.00-217)	0.766[a]
TGVmax	170.8 (63.3-851.2; 0.00-7247)	181.4 (50.0-535.9; 0.00-5426)	0.901[a]
TGVmean	90.0 (39.1-389; 0.00-2561)	93.8 (34.4-240.4; 0.00-2511)	0.655[a]

a=Mann-Witney U Test; b=Fisher's exact test;

Supplementary table 8.2: Re-staging PET-CT factors associated with pathological response to neoadjuvant chemotherapy

Factor	Response		p $\alpha=2.94\times10^{-3}$
	Response(n=62)	No response (n=158)	
Age	63.0 (56.4-70.8; 36.0-79.0)	65.0 (59.3-71.0; 39.0-80.0)	0.468[a]
Sex			
Male	46 (74.2%)	123 (77.8%)	0.764[b]
Female	16 (25.8%)	35 (22.2%)	
Cell			
AC	52 (83.9%)	130 (82.3%)	1.000[b]
SCC	9 (14.5%)	23 (14.6%)	
AS	1 (1.61%)	2 (1.27%)	
NEC	0 (0.00%)	0 (0.00%)	
SC	0 (0.00%)	1 (0.63%)	
Anaplastic	0 (0.00%)	2 (1.27%)	
Site			
Proximal 1/3	0 (0.00%)	0 (0.00%)	0.178[b]
Mid 1/3	6 (9.68%)	7 (4.43%)	
Distal 1/3	9 (14.5%)	28 (17.7%)	
GOJ 1	20 (32.3%)	41 (25.9%)	
GOJ 2	16 (25.8%)	63 (39.9%)	
GOJ 3	11 (17.7%)	18 (11.4%)	
Multifocal	0 (0.00%)	1 (0.46%)	
T stage			
1	2 (3.23%)	4 (2.53%)	0.247[b]
2	15 (24.2%)	24 (15.2%)	
3	40 (64.5%)	122 (7272%)	
4a	5 (8.06%)	8 (5.06%)	
N stage			
0	23 (37.1%)	36 (22.8%)	0.042[b]
1	39 (62.9%)	122 (71.2%)	
Impassable at OGD?			
No	58 (93.5%)	145 (91.8%)	0.784[b]
Yes	4 (6.45%)1	13 (8.20%)	
Neoadjuvant chemotherapy			
Chemo			
Dual	37 (59.7%)	136 (86.1%)	4.16×10^{-5} [b]
Triple	25 (40.3%)	22 (13.9%)	
Days to restaging	88.5 (71.3-106.8; 43.0-167)	82.0 (71.0-91.0; 40.0-165)	0.036[b]
Days to surgery	23.0 (18.3-31.8; 5.0-52.0)	23.0 (15.0-33; 4.0-72.0)	0.283[b]
PET-CT variables			
Initial PET scanner			
1	20 (32.3%)	52 (32.9%)	1.000[b]
2	42 (67.2%)	106 (61.1%)	
Restaging scanner			
1	16 (25.8%)	46 (29.1%)	0.739[b]
2	46 (74.2%)	112 (70.9%)	
Restaging mN stage			
0	60 (96.8%)	130 (82.3%)	0.018[b]
1	1 (1.61%)	15 (9.49%)	
2	1 (1.61%)	13 (8.23%)	
Restaging avid nodal SUVmax	2.50 (2.50-2.50; 2.50-8.04)	2.50 (2.50-2.70; 2.15-14.4)	7.44×10^{-3}[a]
SUVmax	2.50 (2.50-4.95; 2.50-8.30)	7.20 (5.03-10.9; 2.50-29.7)	9.25×10^{-16} [a]
Avid length	0.50 (0.00-2.50; 0.00-12.5)	3.80 (2.30-5.13; 0.00-10.0)	1.51×10^{-11} [a]
Patients staged using most recent PET scanner (n=158)			
SUVmean	2.50 (2.50-4.30; 2.50-4.97)	5.05 (4.29-5.86; 2.50-10.8)	7.30×10^{-11} [a]
SUVpeak	2.50 (2.50-2.50; 2.50-6.66)	2.50 (2.50-8.31; 2.50-20.5)	2.26×10^{-5} [a]
MTV	0.00 (0.00-0.24; 0.00-31.6)	3.36 (0.49-9.37; 0.00-103.9)	2.46×10^{-10} [a]
TGVmax	0.00 (0.00-1.14; 0.00-195.5)	26.0 (2.58-107.7; 0.00-1823)	1.47×10^{-10} [a]
TGVmean	0.00 (0.00-1.03; 0.00-145)	17.5 (2.20-57.7; 0.00-751.1)	1.87×10^{-10} [a]

a=Mann-Witney U Test; b=Fisher's exact test;

Supplementary table 8.3: Metabolic response and pathological response to neoadjuvant chemotherapy (patients staged and re-staged using the same PET-CT scanner)

Factor	Response		p $\alpha=2.94\times10^{-3}$
	Response (n=58)	No response (n=144)	
Age	63.0 (55.3-70.8; 36.0-79.0)	65.0 (59.0-70.0; 39.0-80.0)	0.485[a]
Sex			
Male	43 (74.1%)	114 (79.2%)	0.458[b]
Female	15 (25.9%)	30 (20.8%)	
Cell			
AC	48 (82.8%)	121 (84.0%)	0.924 [b]
SCC	9 (15.5%)	19 (13.2%)	
AS	1 (1.72%)	2 (1.39%)	
NEC	0 (0.00%)	0 (0.00%)	
Small cell	0 (0.00%)	1 (0.69%)	
Anaplastic	0 (0.00%)	1 (0.69%)	
Grade			
Well	4 (6.90%)	7 (4.86%)	0.784[b]
Moderate	25 (43.1%)	61 (42.4%)	
Poor	29 (50.0%)	73 (50.7%)	
Undifferentiated	0 (0.00%)	3 (2.08%)	
Site			
Proximal 1/3	0 (0.00%)	0 (0.00%)	0.105 [b]
Mid 1/3	6 (10.3%)	6 (4.17%)	
Distal 1/3	9 (15.5%)	23 (16.0%)	
GOJ 1	19 (32.8%)	40 (27.8%)	
GOJ 2	13 (22.4%)	57 (39.6%)	
GOJ 3	11 (19.0%)	17 (11.8%)	
Multifocal	0 (0.00%)	1 (0.69%)	
T stage			
1	2 (3.45%)	4 (2.78%)	0.385 [b]
2	13 (22.4%)	23 (16.0%)	
3	38 (65.5%)	110 (76.4%)	
4a	5 (8.62%)	7 (4.86%)	
N stage			
0	21 (36.2%)	32 (22.2%)	0.052 [b]
1	37 (63.8%)	112 (77.8%)	
Impassable			
No	55 (94.8%)	131 (91.0)	0.565 [b]
Yes	3 (5.2%)	13 (9.03%)	
Chemotherapy			
Chemo			
Dual	33 (56.9%)	124 (86.1%)	2.21×10^{-5} [b]
Triple	25 (53.1%)	20 (13.9%)	
Days to restaging	88.5 (70.5-105.5; 43.0-167.0)	81.5 (70.0-90.3; 54.0-165.0)	0.030 a
Days to surgery	23.0 (18.3-30.8; 5.00-52.0)	23.0 (15.0-33.0; 4.00-72.0)	0.415 a
PET-CT variables			
PET scanner			
1	16 (27.6%)	42 (29.2%)	0.865 [b]
2	42 (72.4%)	102 (70.8%)	
mNR			
NA	37 (63.8%)	91 (63.0%)	
CMR	19 (32.8%)	27 (18.8%)	0.032 [b]
PMR	1 (1.72%)	11 (7.64%)	
SMD	0 (0.00%)	9 (6.25%)	
PMD	1 (1.72%)	6 (4.17%)	
Reduction SUVmax (%)	100% (74.8-100; -6.67-100)	46.1% (14.2-69.0; -284.6-100)	1.58×10^{-11} [a]
Reduction avid length (%)	89.6% (49.7-100; -132.1-100)	23.1% (0.00-50.0; -97.1-100)	3.78×10^{-9} [a]
PERCIST (30%)			
Negative	5 (8.62%)	2 (1.39%)	4.68x10-12 b
CMR	32 (55.2%)	15 (10.4%)	
PMR	17 (23.9%	81 (56.3%)	
SMD	4 (6.90%)	35 (24.3%)	
PMD	0 (0.00%	11 (7.64%)	
MUNICON (35%)			
Response	47 (81.0%)	84 (58.3%)	8.24×10^{-6} b

No response *Non avid*	6 (10.3%) 5 (8.62%)	58 (40.3%) 2 (1.39%)	
Patients staged using most recent PET scanner (n=158)			
Reduction SUVmean (%)	100% (45.7-100; 8.45-100)	19.8% (4.58-34.6; -26.2-100)	$2.74x10^{-9}$ a
Reduction SUVpeak (%)	100% (74.3-100; 0.00-100)	54.0% (7.70-75.1; -163.6-100)	$7.43x10^{-8}$ a
Reduction MTV (%)	100% (96.0-100; 80.4-100)	71.7% (34.6-94.9; -353.3-100)	$1.34x10^{-10}$ a
Reduction TGVmax (%)	100% (99.0-100; 92.0-100)	83.5% (50.1-98.1; -427.8-100)	$3.69x10^{-11}$ a
Reduction TGVmean (%)	100% (98.2-100; 89.2-100)	80.3% (40.5-96.9; -367.8-100)	$3.67x10^{-11}$a

a=Mann-Witney U Test; b=Fisher's exact test;

Section 8.3.3: Refining and comparing metabolic response thresholds

Supplementary table 8.4: Comparison of receiver operator curve statistics for thresholds and models of metabolic response (patients staged and re-staged using recent PET-CT scanner; n=144)

Model / Metric	AUC	p value (α=5.49x10^{-4}) (P<4.68x10-3 =FDR<0.046)													
		LR NAC+ ΔL+ΔSUVmax	LR ΔL+ ΔSUVmax	LR ΔSUVmean +ΔL	LR ΔSUVpeak +ΔL	ΔSUV max 77.8%	ΔSUVmax 30.0%	ΔSUV max 35%	ΔSUV mean 36.1%	ΔSUV peak 69.8%	ΔL 57.3%	ΔMTL 76.1%	ΔMTV 89.6%	ΔTGV max 93.8%	ΔTGV mean 89.2%
LR NAC+ ΔL+ΔSUVmax	0.855	NA	0.196	0.283	0.067	0.264	1.17 x10^{-11}	4.72x10^{-6}	0.070	7.42x10^{-3}	0.112	0.314	0.264	0.275	0.138
LR ΔL+ΔSUVmax	0.814		NA	0.816	0.469	0.815	8.68x10^{-6}	1.57x10^{-4}	0.575	0.207	0.425	0.914	0.698	0.631	0.827
LR ΔSUVmean +ΔL	0.823			NA	0.143	1.000	7.00x10^{-5}	1.24x10^{-3}	0.076	0.088	0.353	0.889	0.899	0.885	0.979
LR ΔSUVpeak+ΔL	0.761				NA	0.275	5.61x10^{-3}	0.020	0.883	0.653	0.907	0.371	0.310	0.313	0.424
ΔSUVmax (77.8%)	0.822					NA	9.38x10^{-5}	9.07x10^{-4}	0.064	0.075	0.413	0.890	0.897	0.881	0.978
ΔSUVmax (30%)	0.663						NA	0.110	1.56x10^{-3}	2.44x10^{-3}	7.12x10^{-3}	1.42x10^{-4}	6.23 x10^{-9}	3.09 x10^{-9}	2.97 x10^{-11}
ΔSUVmax (35%)	0.695							NA	0.017	0.017	0.046	1.79x10^{-3}	2.53 x10^{-5}	2.53 x10^{-9}	4.35 x10^{-7}
ΔSUVmean (36.1.%)	0.793								NA	0.547	0.856	0.554	0.406	0.352	0.472
ΔSUVpeak (69.8%)	0.775									NA	0.810	0.227	0.156	0.092	0.191
ΔL (57.3%)	0.743										NA	0.334	0.296	0.299	0.405
ΔMTL (76.1%)	0.817											NA	0.761	0.771	0.912
ΔMTV (89.6%)	0.828												NA	1.000	0.725
ΔTGVmax (93.8%)	0.828													NA	0.727
ΔTGVmean (89.2%)	0.821														NA

Supplementary table 8.5: Comparison of sensitivities for thresholds and models of metabolic response (patients staged and re-staged using recent PET-CT scanner (n=144)

Model / Metric	AUC	p value ($\alpha=5.49\times10^{-4}$) (P<4.68x10-3 =FDR<0.046)													
		LR (NAC+ ΔL+ΔSUVmax	LR ΔL+ΔSUVmax	LR ΔSUVmean +ΔL	LR ΔSUVpeak +ΔL	ΔSUVmax 77.8%	ΔSUVmax 30.0%	ΔSUVmax 35%	ΔSUVmean 36.1%	ΔSUVpeak 69.8%	ΔL 57.3%	ΔMTL 76.1%	ΔMTV 89.6%	ΔTGVmax 93.8%	ΔTGVmean 89.2%
LR NAC+ ΔL+ΔSUVmax	0.855	NA	0.317	0.025	8.15×10^{-3}	0.025	0.083	0.157	0.102	0.534	0.011	0.058	1.000	1.000	1.000
LR ΔL+ΔSUVmax	0.814		NA	0.180	0.025	0.180	8.68×10^{-6}	1.57×10^{-4}	0.575	0.207	0.014	0.083	0.317	0.157	0.083
LR ΔSUVmean +ΔL	0.823			NA	0.157	1.000	0.014	0.025	0.317	0.157	0.257	1.000	0.059	0.025	0.083
LR ΔSUVpeak+ΔL	0.761				NA	0.317	4.67×10^{-3}	8.15×10^{-3}	0.564	0.046	0.655	0.317	0.082	8.15×10^{-3}	4.68×10^{-3}
ΔSUVmax (77.8%)	0.822					NA	0.014	0.025	0.317	0.157	0.317	1.000	0.059	0.025	0.014
ΔSUVmax (30%)	0.663						NA	0.317	8.15×10^{-3}	0.046	2.70×10^{-3}	0.014	0.317	0.317	1.000
ΔSUVmax (35%)	0.695							NA	0.014	0.083	4.68×10^{-3}	0.025	1.000	1.000	0.317
ΔSUVmean (36.1.%)	0.793								NA	0.083	0.480	0.705	0.034	0.014	8.15×10^{-3}
ΔSUVpeak (69.8%)	0.775									NA	0.059	0.317	0.180	0.083	0.046
ΔL (57.3%)	0.743										NA	0.180	4.68×10^{-3}	4.68×10^{-3}	2.67×10^{-3}
ΔMTL (76.1%)	0.817											NA	0.025	0.025	0.014
ΔMTV (89.6%)	0.828												NA	1.000	0.317
ΔTGVmax (93.8%)	0.828													NA	0.317
ΔTGVmean (89.2%)	0.821														NA

Supplementary table 8.6: Comparison of specificities for thresholds and models of metabolic response (patients staged and re-staged using recent PET-CT scanner (n=144)

Model / Metric	AUC	p value (α=5.49x10^{-4}) (P<4.68x10-3 =FDR<0.046)													
		LR (NAC+ ΔL+ΔSUVmax	LR ΔL+ ΔSUV max	LR ΔSUV mean+ΔL	LR ΔSUV peak +ΔL	ΔSUV max 77.8%	ΔSUV max 30.0%	ΔSUVmax 35%	ΔSUVmean 36.1%	ΔSUVpeak 69.8%	ΔL 57.3%	ΔMTL 76.1%	ΔMTV 89.6%	ΔTGVmax 93.8%	ΔTGVmean 89.2%
LR NAC+ ΔL+ΔSUVmax	0.855	NA	0.419	0.019	0.083	8.15x10^{-3}	5.52x10-^{10}	4.20x10^{-7}	0.206	0.059	0.059	0.083	0.109	0.109	0.109
LR ΔL+ΔSUVmax	0.814		NA	7.52x10^{-3}	0.013	3.89x10^{-3}	1.87x10^{-9}	4.58x10^{-7}	0.071	0.197	2.28x10^{-3}	2.70x10^{-3}	0.439	0.439	0.108
LR ΔSUVmean +ΔL	0.823			NA	0.655	1.000	4.26x10^{-12}	1.24x10^{-9}	0.083	2.75x10^{-4}	0.763	0.657	7.89x10^{-4}	7.89x10^{-4}	0.108
LR ΔSUVpeak+ΔL	0.761				NA	0.655	7.10x10-^{12}	7.07x10^{-10}	0.414	1.83x10^{-3}	0.572	1.000	2.70x10^{-3}	2.70x10^{-3}	3.47x10^{-3}
ΔSUVmax (77.8%)	0.822					NA	5.26x10^{-12}	4.24x10^{-12}	0.083	1.08x10^{-5}	0.763	0.655	3.11x10^{-4}	3.11x10^{-4}	3.74x10^{-5}
ΔSUVmax (30%)	0.663						NA	2.70x10^{-3}	1.97x10^{-11}	2.43x10^{-8}	6.82x10^{-12}	7.10x10^{-12}	3.30x10^{-9}	3.30x10^{-9}	2.58x10^{-8}
ΔSUVmax (35%)	0.695							NA	5.22x10^{-9}	2.52x10^{-6}	1.64x10^{-9}	1.87x10^{-9}	3.41x10^{-7}	3.41x10^{-7}	2.73x10^{-6}
ΔSUVmean (36.1.%)	0.793								NA	2.70x10^{-3}	0.285	0.480	0.012	0.012	1.75x10^{-3}
ΔSUVpeak (69.8%)	0.775									NA	6.47x10^{-4}	9.67x10^{-5}	0.617	0.617	0.655
ΔL (57.3%)	0.743										NA	0.480	1.75x10^{-3}	1.75x10^{-3}	2.39x10^{-4}
ΔMTL (76.1%)	0.817											NA	1.34x10^{-3}	1.34x10^{-3}	1.62x10^{-4}
ΔMTV (89.6%)	0.828												NA	1.000	0.046
ΔTGVmax (93.8%)	0.828													NA	0.046
ΔTGVmean (89.2%)	0.821														NA

Supplementary table 8.7: Validation of thresholds of mTR and pTR

Threshold	Sensitivity	Specificity	PPV	NPV	O:E	AUC
ΔSUVmax=77.8%						
Apparent	0.829 (0.663-0.934)	0.816 (0.725-0.887)	0.617 (0.464-0.755)	0.930 (0.854-0.974)	0.745	0.822 (0.741-0.891)
Internal	0.829 (0.663-0.934)	0.806 (0.714-0.879)	0.604 (0.453-0.742)	0.929 (0.853-0.974)	0.814	0.817 (0.774-0.885)
Independent	0.714 (0.419-0.914)	0.585 (0.421-0.737)	0.370 (0.194-0.576)	0.857 (0.673-0.959)	0.519	0.650 (0.607-0.782)
ΔSUVmax=30%						
Apparent	1.00 (89.9-1.00)	0.330 (0.242-0.435)	0.347 (0.255-0.448)	1.00 (0.893-1.00)	0.347	0.663 (0.617-0.709)
Internal validation	1.00 (89.9-1.00)	0.296 (0.208-0.397)	0.337 (0.247-0.458)	1.00 (0.879-1.00)	0.337	0.648 (0.602-0.694)
Independent validation	0.714 (0.419-0.914)	0.342 (0.201-0.506)	0.270 (0.138-0.441)	0.778 (0.524-0.935)	0.378	0.528 (0.384-0.659)
ΔSUVmax=35%						
Apparent	0.971 (0.850-1.00)	0.418 (0.320-0.522)	0.374 (0.274-0.481)	0.976 (0.274-0.481)	0.374	0.695 (0.639-0.755)
Internal validation	1.00 (89.9-1.00)	0.327 (0.235-0.429)	0.347 (0.255-0.448)	1.00 (0.890-1.00)	0.347	0.648 (0.607-0.694)
Independent validation	0.714 (0.419-0.914)	0.390 (0.242-0.555)	0.286 (0.147-0.463)	0.800 (0.563-0.941)	0.400	0.552 (0.409-0.645)
ΔL=57.3%						
Apparent	0.743 (0.567-0.875)	0.827 (0.737-0.896)	0.605 (0.441-0.750)	0.900 (0.819-0.0.953)	0.814	0.785 (0.532-0.820)
Internal validation	0.829 (0.663-0.934)	0.786 (0.691-0.862)	0.580 (0.432-0.781)	0.928 (0.849-0.973)	0.720	0.807 (0.728-0.880)
Independent validation	0.600 (0.323-0.836)	0.854 (0.780-0.944)	0.600 (0.323-0.836)	0.854 (0.708-0.944)	0.933	0.748 (0.605-0.880)
ΔSUVmean (36.1%)						
Apparent	0.800 (0.631-0.915)	0.786 (0.691-0.862)	0.571 (0.422-0.712)	0.917 (0.836-0.957)	0.714	0.793 (0.713-0.865)
Internal validation	0.875 (0.710-0.964)	0.733 (0.635-0.816)	0.509 (0.371-0.646)	0.949 (0.874-0.986)	0.673	0.778 (0.699-0.854)
ΔSUVpeak (69.8%)						
Apparent	0.886 (0.732-0.967)	0.663 (0.561-0.756)	0.484 (0.358-0.613)	0.942 (0.858-0.984)	0.547	0.775 (0.696-0.839)
Internal validation	0.886 (0.732-0.967)	0.663 (0.561-0.756)	0.484 (0.358-0.613)	0.942 (0.858-0.984)	0.547	0.775 (0.696-0.839)
ΔMTL=76.1%						
Apparent	0.892 (0.746-0.969)	0.717 (0.618-.803)	0.541 (0.409-0.669)	0.947 (0.869-0.985)	0.607	0.817 (0.735-0.867)
Internal validation	0.886 (0.732-0.967)	0.725 (0.625-0.810)	0.535 (0.399-0.667)	0.947 (0.869-0.985)	0.574	0.805 (0.735-0.874)
Independent validation	0.714 (0.419-0.914)	0.463 (0.306-0.626)	0.313 (0.161-0.500)	0.826 (.612-0.949)	0.438	0.589 (0.435-0.723)
ΔMTV=89.6%						
Apparent	0.971 (0.850-0.995)	0.684 (0.582-0.774)	0.523 (0.396-0.649)	0.985 (0.921-0.998)	0.538	0.828 (0.772-0.878)
Internal validation	0.971 (0.850-0.995)	0.653 (0.550-0.746)	0.500 (0.376-0.624)	0.985 (0.917-0.997)	0.515	0.812 (0.754-0.862)
ΔTGVmax=93.8%						
Apparent	0.943 (0.808-0.991)	0.684 (0.582-0.774)	0.516 (0.387-0.643)	0.971 (0.899-0.996)	0.547	0.813 (0.750-0.868)
Internal validation	0.971 (0.850-0.995)	0.653 (0.550-0.746)	0.500 (0.376-0.624)	0.985 (0.917-0.997)	0.515	0.812 (0.754-0.862)
ΔTGVmean=89.2%						
Apparent	1.00 (0.899-1.00)	0.643 (0.540-0.737)	0.500 (0.378-0.622)	1.00 (0.943-1.00)	0.547	0.821 (0.750-0.868)
Internal validation	1.00 (0.899-1.00)	0.592 (0.488-0.690)	0.467 (0.351-0.586)	1.00 (0.938-1.00)	0.467	0.796 (0.745-0.847)

Supplementary table 8.8: Models predicting pathological response using metabolic response

Model	Optimal		p value (α=1.11x10^{-3})			
	Sensitivity	Specificity	PPV	NPV	O:E	AUC
Logistic regression: equation=-6.421+3.211*(Triple agent NAC) + 0.056*(ΔSUVmax) + 0.07*(ΔL); Pt=0.150						
Apparent (n=144)*	0.973 (0.858-0.996)	0.737 (0.639-0.921)	0.581 (0.449-0.705)	0.987 (0.927-0.998)	0.712	0.855 (0.801-0.901)
Internal validation	0.919 (0.781-0.982)	0.758 (0.661-0.838)	0.586 (0.449-0.714)	0.962 (0.892-0.992)	0.638	0.838 (0.773-0.894)
Independent (n=68)**	0.571 (0.289-0.822)	0.875 (0.732-0.958)	0.615 (0.316-0.860)	0.854 (0.708-0.944)	1.08	0.723 (0.591-0.866)
Logistic regression= -4.491 +0.046*(ΔSUVmax) + 0.006*(ΔL) Pt=0.213						
Apparent (n=144)*	0.914 (0.769-0.981)	0.714 (0.614-0.801)	0.533 (0.400-0.663)	0.959 (0.884-0.991)	0.583	0.814 (0.745-0.876)
Internal validation	0.865 (0.712-0.954)	0.571 (0.432-0.703)	0.571 (0.432-0.703)	0.865 (.712-0.954)	0.723	0.816 (0.745-0.881)
Independent (n=68)**	0.846 (0.651-0.956)	0.463 (0.307-0.626)	0.500 (0.346-0.654)	0.826 (0.612-0.949)	0.438	0.589 (0.444-0.721)
Restaging SUVmean: -2.746 + 0.029*(ΔSUVmean) + 0.006*(ΔL); Pt=0.200						
Apparent (n=144)*	0.838 (0.680-0.938)	0.808 (0.717-0.880)	0.620 (0.472-0.753)	0.930 (0.854-0.974)	0.740	0.823 (0.752-0.892)
Internal validation	0.838 (0.680-0.938)	0.778 (0.683-0.938)	0.585 (0.441-0.719)	0.928 (0.849-0.973)	0.698	0.808 (0.732-0.877)
Restaging SUVpeak: -4.404 + 0.042*(ΔSUVpeak) + 0.008*(ΔL); Pt=0.307						
Apparent (n=144)*	0.829 (0.663-0.934)	0.731 (0.629-0.818)	0.537 (0.391-0.637)	0.919 (0.832-0.970)	0.761	0.761 (0.686-0.734)
Internal validation	0.657 (0.448-0.809)	0.827 (0.637-0.896)	0.575 (0.409-0.730)	0.871 (0.785-0.931)	0.875	0.742 (0.652-0.828)
Decision tree analysis: selecting patients with ΔSUVmax>77.8%						
Apparent (n=144)*	0.829 (0.663-0.934)	0.816 (0.725-0.887)	0.617 (0.464-0.755)	0.930 (0.854-0.974)	0.745	0.822 (0.741-0.891)
Internal validation	0.571 (0.394-0.737)	0.918 (0.845-0.964)	0.714 (0.513-0.867)	0.857 (0.775-0.918)	1.25	0.754 (0.664-0.836)
Independent (n=68)**	0.714 (0.419-0.914)	0.585 (0.421-0.737)	0.370 (0.194-0.576)	0.857 (0.673-0.959)	0.519	0.650 (0.607-0.782)
Artificial neural network: NAC NAC regimen, ΔSUVmax, ΔL						
Apparent (n=144)*	0.657 (0.478-0.809)	0.969 (0.913-0.993)	0.885 (0.698-0.974)	0.888 (0.812-0.941)	1.36	0.813 (0.728-0.890)
Internal validation	0.00 (0.00-0.101)	1.00 (0.963-1.00)	0.00 (NA)	0.737 (0.654-0.809)	NA	0.500 (0.500-0.500)
Independent (n=68)**	0.125 (0.02-0.384)	1.00 (0.951-1.000)	1.00 (0.193-1.00)	0.750 (0.616-0.856)	0.125	0.507 (0.410-0.604)

CHAPTER 9: PREDICTING SURVIVAL AND RECURRENCE OF OESOPHAGEAL CANCER AFTER NEOADJUVANT CHEMOTHERAPY AND SURGERY

Section 9.3.1: Patients, survival, recurrence, and follow up

Supplementary table 9.1: Overall survival, disease-free survival and recurrence:

	Overall (n=294)	Risk Score (n=212)						Avid nodes after NAC (n=31)
		0	1	2	3	4	5	Yes
Median OS (95% CI) days	1008 (791-1511)	NR (1564-NR)	NR (NR-NR)	938 (790-NR)	470 (356-1008)	395 (359-431)	NA	518 (435-1337)
Median DFS (95% CI) days	918 (647-1514)	NR (1306-NR)	NR (NR-NR)	961 (653-NR)	438 (286-654)	302 (265-542)	NA	379 (226-1309)
Median days to Rec (95% CI)	1077 (975-1306)	NR (NR-NR)	NR (NR-NR)	709 (651-NR)	654 (289-NR)	379 (276-NR)	NA	432 (245-NR)
1 year OS								
Alive	236 (82.8%)	25 (89.3%)	37 (94.9%)	66 (85.7%)	27 (61.4%)	10 (66.7%)	NA	23 (76.7%)
Dead	49 (17.2%)	3 (10.7%)	2 (5.13%)	11 (14.3%)	17 (38.6%)	5 (33.3%)	NA	7 (23.3%)
Censored	9	1	3	5	0	0	NA	1
2 year OS								
Alive	141 (56.4%)	17 (81.0%)	26 (48.9%)	37 (35.5%)	15 (57.8%)	2 (44.2%)	NA	10 (35.7%)
Dead	109 (43.6%)	4 (19.0%)	7 (51.1%)	26 (64.5%)	27 (42.2%)	12 (55.8%)	NA	18 (64.3%)
Censored	44 (NA)	8	9	19	2	1	NA	3
5 year OS								
Alive	88 (39.5%)	12 (75.0%)	18 (66.7%)	18 (44.0%)	8 (21.1%)	0 (0.00%)	NA	5 (20.0%)
Dead	135 (60.%)	4 (25.0%)	9 (33.3%)	35 (66.0 %)	30 (78.9%)	12 (100 %)	NA	20 (80.0%)
Censored	71 (NA)	13	15	29	6	3	NA	6
1 year DFS								
Yes	200 (70.2%)	24 (85.7%)	34 (87.2%)	53 (70.7%)	24 (55.9%)	7 (46.7%)	NA	16 (53.3%)
No	85 (29.8)	4 (14.3%)	5 (12.8%)	22 (29.3%)	19 (44.1%)	8 (53.3%)	NA	14 (48.7%)
Censored	9 (NA)	1	3	7	1	0	NA	1
2 year DFS								
Yes	118 (47.0%)	17 (81.0%)	23 (70.0%)	30 (47.6%)	12 (28.6%)	2 (14.3%)	NA	8 (28.6%)
No	132 (53.0%)	4 (19.0%)	10 (30.0%)	33 (52.4%)	30 (71.4%)	12 (85.7%)	NA	20 (71.4%)
Censored	44 (NA)	8	9	19	2	1	NA	3
5 year DFS								
Yes	81 (36.3%)	11 (68.8%)	16 (59.3%)	17 (32.1%)	7 (18.4%)	0 (0.00%)	NA	5 (20.0%)
No	142 (63.7%)	5 (31.2%)	11 (40.7%)	36 (67.9%)	31 (81.6%)	12 (100%)	NA	20 (80.0%)
Censored	71 (NA)	13	15	29	6	3	NA	6
1 year REC								
Recurrence	68 (25.4%)	2 (7.69%)	4 (10.5%)	18 (25.4%)	15 (38.5%)	7 (50.0%)	NA	13 (44.8%)
No	200 (74.6%)	24 (92.3%)	34 (89.5%)	53 (74.6%)	24 (61.5%)	7 (50.0%)	NA	16 (55.2%)
Censored	26 (NA)	3	4	10	4	1	NA	2
2 year REC								
Recurrence	105 (47.1%)	2 (10.5%)	8 (74.2%)	28 (48.3%)	21 (63.6%)	9 (81.8%)	NA	16 (66.7%)
None	118 (52.9%)	17 (89.5%)	23 (25.8%)	30 (51.7%)	12 (36.4%)	2 (18.2%)	NA	8 (32.3%)
Censored	71 (NA)	10	11	24	11	4	NA	7
5 year REC								
Recurrence	111 (50.2%)	2 (10.5%)	9 (29.0%)	30 (53.6%)	22 (66.7%)	9 (81.8%)	NA	16 (66.7%)
None	110 (49.8%)	17 (89.5%)	22 (71.0%)	26 (46.4%)	11 (33.3%)	2 (18.2%)	NA	8 (32.3%)
Censored	73 (NA)	10	11	26	11	4	NA	7

NR=not reached

Section 9.3.2: Markers of prognosis

Supplementary table 9.2: Pre-NAC factors and prognosis

Factor	Overall Survival		Disease-free survival		Recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Patient						
Age[2]	1.00 (1.00-1.00)	0.860	1.00 (1.00-1.00)	0.980	1.00 (1.00-1.00)	0.260
Sex						
Female	Ref	Ref	Ref	Ref	Ref	Ref
Male	1.18 (0.76-1.84)	0.460	1.07 (0.69-1.67)	0.760	0.91 (0.55-1.50)	0.710
ASA						
I	Ref	Ref	Ref	Ref	Ref	Ref
II	0.97 (0.45-2.10)	0.950	1.02 (0.47-2.21)	0.950	0.93 (0.40-2.14)	0.870
III	1.25 (0.55-2.86)	0.600	1.25 (0.55-2.87)	0.590	0.96 (0.38-2.41)	0.930
IV	NA	NA	NA	NA	NA	NA
Tumour						
Cell						
AC	Ref	Ref	Ref	Ref	Ref	Ref
SCC	0.89 (0.48-1.62)	0.690	0.89 (0.49-1.63)	0.710	0.56 (0.25-1.25)	0.160
Grade						
Well	Ref	Ref	Ref	Ref	Ref	Ref
Moderate	1.22 (0.65-2.31)	0.540	1.23 (0.65-2.33)	0.520	1.14 (0.55-2.35)	0.720
Poor	1.60 (0.84-3.05)	0.150	1.58 (0.83-2.99)	0.160	1.35 (0.65-2.80)	0.420
Undifferentiated	NA	NA	NA	NA	NA	NA
Site						
Proximal 1/3	Ref	Ref	Ref	Ref	Ref	Ref
Mid 1/3	1.13 (0.49-2.62)	0.770	1.17 (0.50-2.73)	0.720	1.43 (0.43-4.74)	0.560
Distal 1/3	0.93 (0.40-2.15)	0.860	0.96 (0.41-2.27)	0.930	1.03 (0.31-3.47)	0.960
GOJ 1	0.83 (0.35-2.00)	0.680	0.93 (0.39-2.26)	0.880	1.44 (0.43-4.89)	0.550
GOJ 2	0.71 (0.28-1.81)	0.470	0.74 (0.29-1.93)	0.540	1.02 (0.28-3.68)	0.970
GOJ 3	NA	NA	NA	NA	NA	NA
Impassable at OGD?	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
No	1.11 (0.52-2.36)	0.790	1.11 (0.55-2.27)	0.770	1.04 (0.44-2.49)	0.930
Yes						
Baseline T stage						
1	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
2	0.59 (0.16-2.15)	0.430	0.53 (0.15-1.93)	0.340	0.39 (0.10-1.48)	0.170
3	1.06 (0.31-3.55)	0.930	0.93 (0.27-3.12)	0.900	0.72 (0.22-2.43)	0.600
4a	0.60 (0.13-2.67)	0.500	0.46 (0.10-2.07)	0.310	0.33 (0.07-1.56)	0.16
Baseline N stage						
0	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
1	1.55 (1.03-2.34)	0.037	1.54 (1.03-2.31)	0.035	1.47 (0.93-2.31)	0.100
PET-CT						
PET scanner						
1	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
2	0.84 (0.57-1.24)	0.380	0.74 (0.51-1.08)	0.120	0.77 (0.51-1.18)	0.230
logSUVmax	0.55 (0.24-1.27)	0.160	0.50 (0.22-1.16)	0.110	0.46 (0.17-1.20)	0.110
Length	1.04 (0.96-1.14)	0.330	1.05 (0.96-1.14)	0.270	1.03 (0.94-1.39)	0.510
Avid nodal stage						
mN0	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
mN1	0.93 (0.57-1.52)	0.760	0.96 (0.59-1.57)	0.880	1.14 (0.66-1.97)	0.690
mN2	1.44 (0.79-2.62)	0.230	1.53 (0.85-2.75)	0.150	1.28 (0.63-2.60)	0.490
Nodal logSUVmax	1.29 (0.25-6.74)	0.760	1.21 (0.24-5.98)	0.820	1.47 (0.23-9.47)	0.690
Additional PET-CT metrics (n=151)						
logSUVpeak	0.58 (0.17-1.91)	0.370	0.63 (0.20-1.97)	0.430	1.18 (0.33-4.17)	0.800
logSUVmean	0.45 (0.04-5.60)	0.530	0.52 (0.04-6.05)	0.600	3.22 (0.22-47.4)	0.390
MTV	1.00 (0.99-1.01)	0.560	1.00 (0.99-1.01)	0.530	1.00 (0.99-1.00)	0.820
TGVmax	1.00 (1.00-1.00)	0.330	1.00 (0.99-1.00)	0.290	1.00 (1.00-1.00)	0.610
TGVmean	1.00 (1.00-1.00)	0.430	1.00 (0.99-1.00)	0.410	1.00 (1.00-1.00)	0.910

Supplementary table 9.3: Mandard tumour regression grades and univariate survival; n=294)

TRG	Overall survival HR (95% CI)	p	Disease-free survival HR (95% CI)	p
TRG1	*Ref*	*Ref*	*Ref*	*Ref*
TRG2	1.08 (0.15-7.65)	0.940	1.11 (0.16-7.89)	0.920
TRG3	1.83 (0.05-66.2)	0.420	1.79 (0.42-7.73)	0.430
TRG4	4.43 (1.08-18.0)	0.037	4.48 (1.10-18.2)	0.036
TRG5	4.85 (1.17-20.1)	0.030	4.25 (1.02-17.6)	0.046

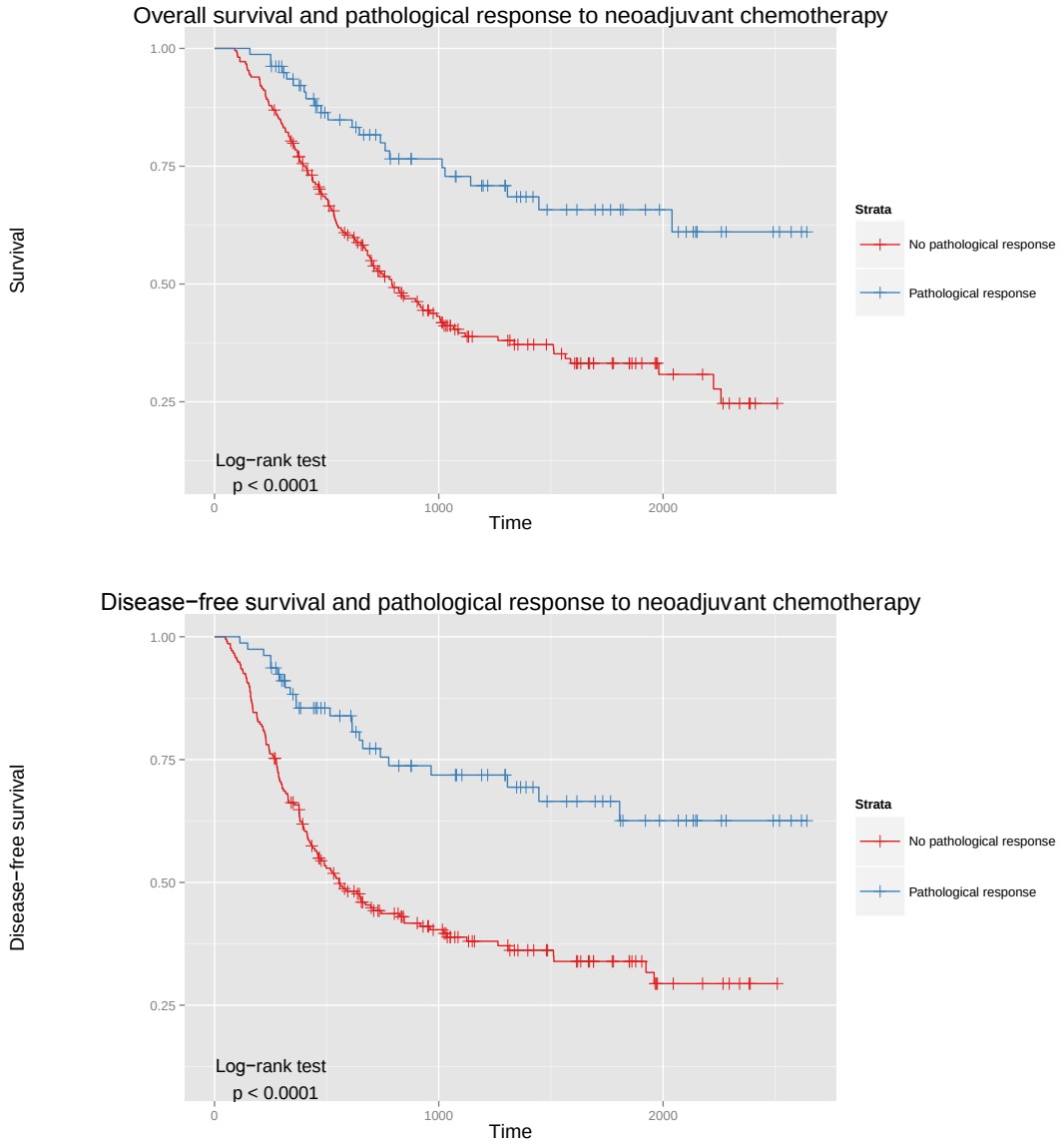
Supplementary table 9.4: Pathological factors associated with overall survival and disease-free survival after neoadjuvant chemotherapy and successful resection

Factor	Overall Survival		Disease-free survival	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Patient				
Age[2]	1.00 (1.00-1.00)	0.350	1.00 (1.00-1.00)	0.410
Sex				
Female	Ref	Ref	Ref	Ref
Male	1.21 (0.77-1.91)	0.410	1.05 (0.67-2.49)	0.830
ASA				
I	Ref	Ref	Ref	Ref
II	0.98 (0.45-2.13)	0.960	0.94 (0.43-2.02)	0.860
III	1.28 (0.54-3.04)	0.570	1.01 (0.43-2.39)	0.970
IV	NA	NA	NA	NA
Tumour				
Cell				
AC	Ref	Ref	Ref	Ref
SCC	1.04 (0.55-1.98)	0.910	1.89 (0.55-6.45)	0.790
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.11 (0.57-2.17)	0.750	1.22 (0.62-2.39)	0.560
Poor	1.42 (0.74-2.74)	0.290	1.29 (0.67-2.49)	0.450
Undifferentiated	NA	NA	NA	NA
Site				
Proximal 1/3	NA	NA	NA	NA
Mid 1/3	Ref	Ref	Ref	Ref
Distal 1/3	0.73 (0.30-1.78)	0.490	1.89 (0.55-6.45)	0.560
GOJ 1	0.70 (0.28-1.75)	0.440	0.76 (0.30-1.91)	0.560
GOJ 2	0.45 (0.17-1.15)	0.940	0.54 (0.21-1.40)	0.210
GOJ 3	0.52 (0.15-1.77)	0.300	0.71 (0.21-2.40)	0.580
Multifocal	NA	NA	NA	NA
Pathological response and stage				
pTR				
No (MTRG4-5)	Ref	Ref	Ref	Ref
Yes (MTRG1-3)	0.55 (0.32-0.97)	0.039	0.55 (0.32-0.96)	0.035
pT				
0	Ref	Ref	Ref	Ref
1	0.92 (0.10-8.76)	0.940	0.98 (0.10-9.61)	0.990
2	0.91 (0.11-7.44)	0.930	1.06 (0.13-8.49)	0.950
3	1.17 (0.14-9.74)	0.880	1.25 (0.15-10.2)	0.830
4	1.28 (0.13-12.2)	0.830	1.20 (0.13-11.5)	0.870
pN				
0	Ref	Ref	Ref	Ref
1	1.89 (1.00-3.56)	0.049	2.25 (1.22-4.16)	9.90×10^{-3}
2	2.40 (1.26-4.59)	7.90×10^{-3}	2.57 (1.35-4.89)	4.00×10^{-3}
3	4.18 (2.19-7.98)	1.40×10^{-5}	4.18 (2.20-7.93)	1.20×10^{-5}
R				
0	Ref	Ref	Ref	Ref
1	1.70 (1.10-2.61)	0.016	1.53 (0.99-2.35)	0.054

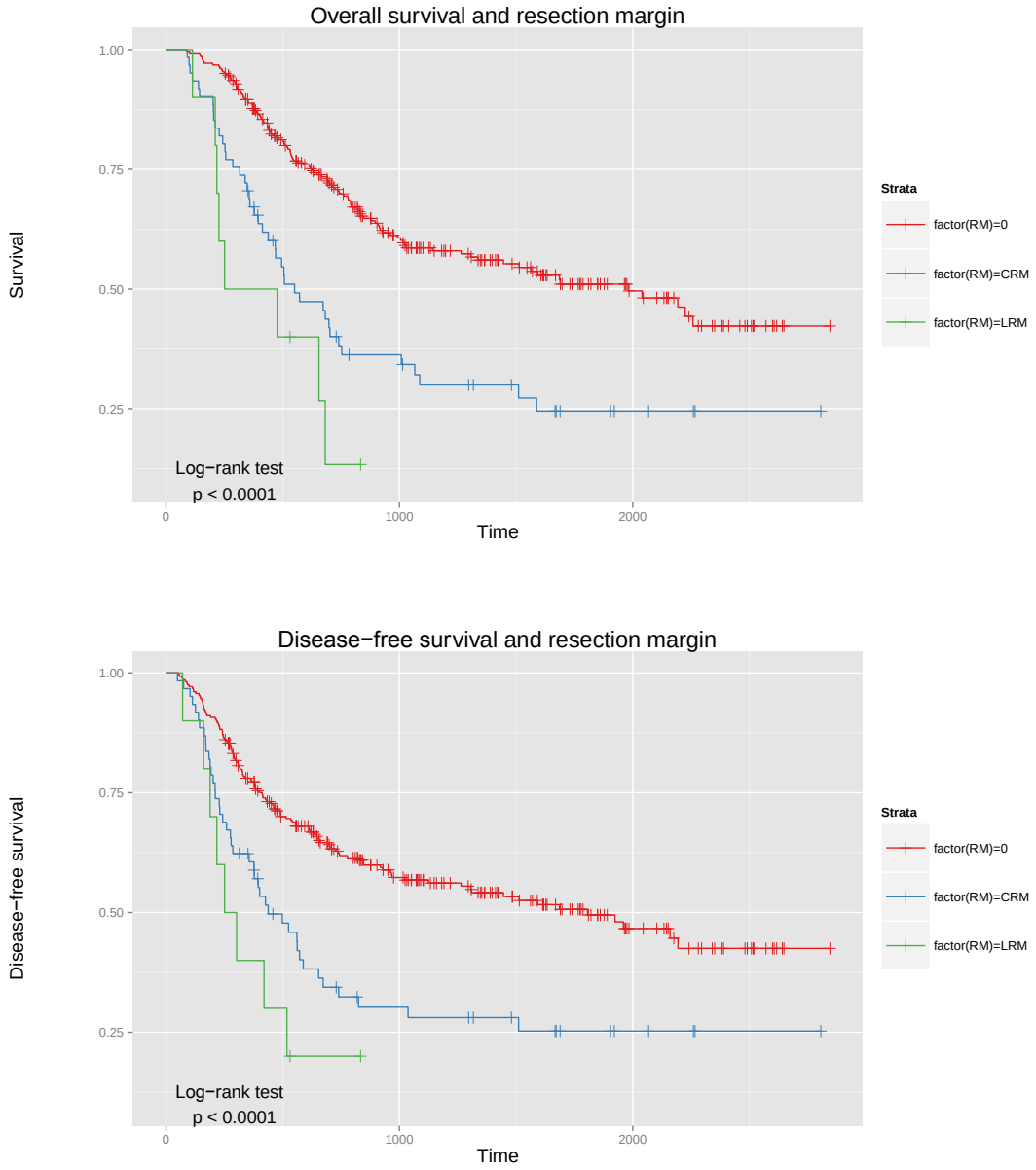
L *0* *1*	 Ref 1.13 (0.69-1.72)	 Ref 0.710	 Ref 1.06 (0.64-1.73)	 Ref 0.830
V *0* *1*	 Ref 1.09 (0.69-1.72)	 Ref 0.710	 Ref 1.14 (0.81-1.82)	 Ref 0.590
Neoadjuvant chemotherapy and operation				
Chemotherapy regimen *Double agent* *Triple agent*	 Ref 0.78 (0.43-1.40)	 Ref 0.400	 Ref 0.78 (0.43-1.41)	 Ref 0.420
Operation *LTO* *ILO* *3 stage* *ETG*	 Ref 0.82 (0.46-1.45) 0.30 (0.08-1.12) 1.25 (0.54-2.93)	 Ref 0.490 0.070 0.600	 Ref 0.95 (0.55-1.67) 0.37 (0.10-1.36) 1.15 (0.50-1.67)	 Ref 0.870 0.740 0.130
Log days to restaging	0.27 (0.03-2.81)	0.270	0.21 (0.02-2.12)	0.180
Log days to surgery	0.83 (0.39-1.75)	0.620	0.92 (0.43-1.96)	0.820

*denotes for subset of patients staged and re-staged using most recent PET-CT scanner (n=145), adjusted for all other variables above; **not included in full model

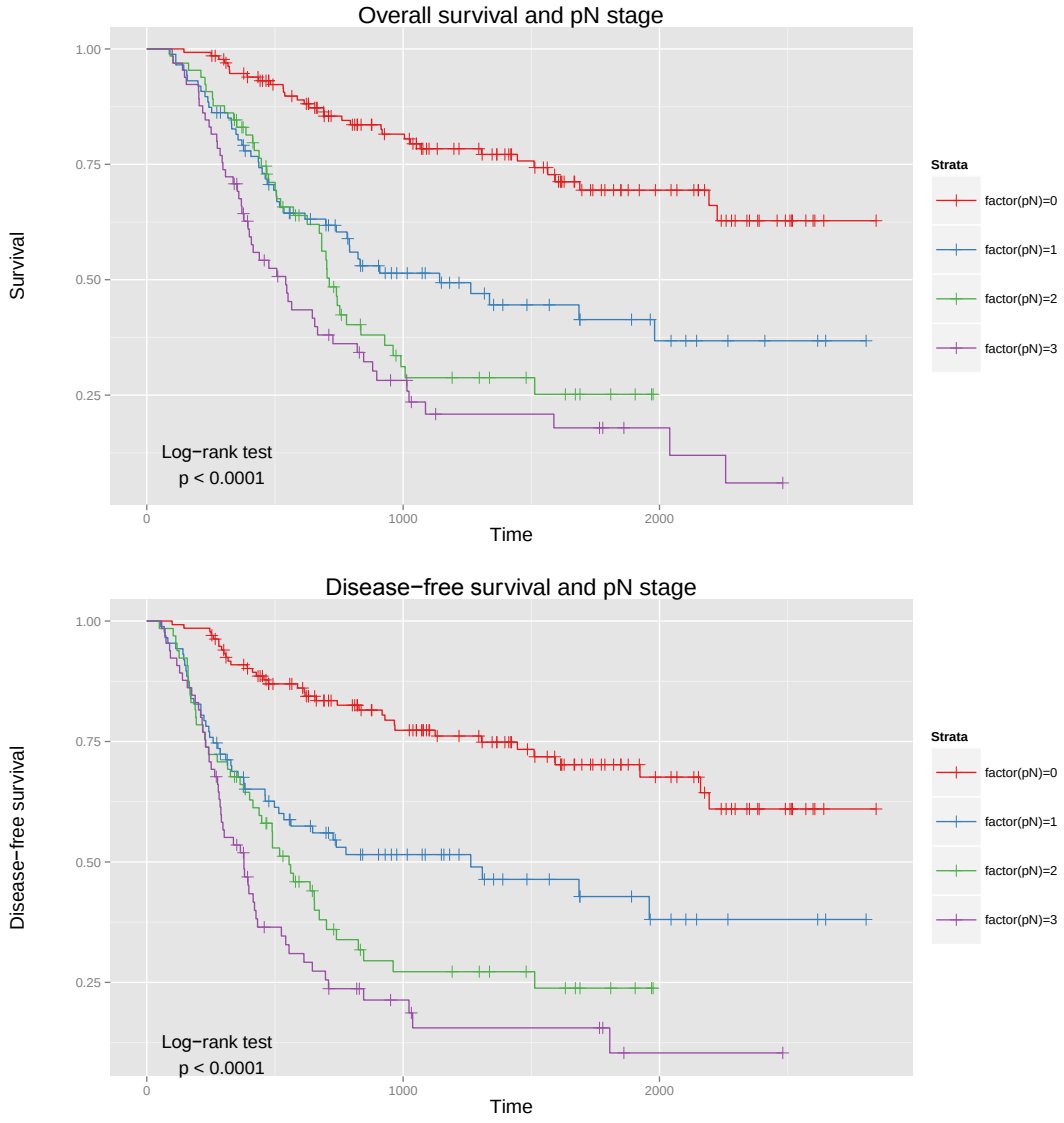
Supplementary figure 9.1



Supplementary figure 9.2



Supplementary figure 9.3



Supplementary table 9.5: Post-NAC pre-operative factors associated with prognosis (patients staged and restaged using same PET-CT scanner; n=195)

Factor	OS		DFS		Recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Patient						
Age[2]	1.00 (1.00-1.00)	0.570	1.00 (1.00-1.00)	0.610	1.00 (1.00-1.00)	0.440
Sex						
Female	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
Male	1.18 (0.65-2.15)	0.580	1.07 (0.60-1.94)	0.810	0.90 (0.45-1.82)	0.780
ASA grade						
1	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
2	0.91 (0.40-2.05)	0.820	0.96 (0.43-2.15)	0.920	0.76 (0.31-1.86)	0.550
3	0.99 (0.39-2.53)	0.980	0.99 (0.38-2.55)	0.990	0.55 (0.18-1.69)	0.300
4	NA	NA	NA	NA	NA	NA
Cell						
AC	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
SCC	1.09 (0.49-2.42)	0.840	1.04 (0.47-2.28)	0.920	0.54 (0.18-1.59)	0.270
Grade						
Well	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
Moderate	0.96 (0.35-2.62)	0.940	1.03 (0.39-2.70	0.950	0.78 (0.27-2.24)	0.650
Poor	1.52 (0.56-4.08)	0.210	1.51 (0.58-3.96)	0.400	0.97 (0.34-2.76)	0.950
Undifferentiated	NA	NA	NA	NA	NA	NA
Site						
Mid 1/3	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
Distal 1/3	1.00 (0.35-2.87)	1.00	1.24 (0.43-3.58)	0.690	2.19 (0.43-11.2)	0.340
GOJ 1	0.75 (0.25-2.20)	0.590	0.87 (0.30-2.56)	0.800	1.04 (0.20-5.39)	0.970
GOJ 2	0.64 (0.22-2.90)	0.420	0.84 (0.28-2.46)	0.760	1.64 (0.32-8.31)	0.550
GOJ 3	0.72 (0.19-2.76)	0.630	0.97 (0.25-3.73)	0.960	2.03 (0.32-13.1)	0.460
Multifocal	NA	NA	NA	NA	NA	NA
Impassable						
No	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
Yes	0.69 (0.20-2.36)	0.550	0.80 (0.27-2.33)	0.680	0.58 (0.13-2.58)	0.470
Baseline T stage						
1	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
2	0.90 (0.10-7.79)	0.920	0.96 (0.11-8.24)	0.970	0.73 (0.08-7.00)	0.790
3	1.76 (0.22-14.0)	0.590	1.72 (0.22-13.7)	0.610	1.22 (0.14-10.5)	0.860
4a	0.49 (0.02-6.02)	0.570	0.55 (0.04-6.65)	0.640	0.66 (0.06-7.64)	0.740
Baseline N stage						
0	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
1	1.85 (1.05-3.27)	0.033	1.74 (1.01-3.00)	0.048	1.71 (0.90-3.24)	0.100
PET-CT						
PET scanner						
1	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
2	0.66 (0.40-1.12)	0.120	0.69 (0.42-1.15)	0.160	0.77 (0.42-1.41)	0.390
ΔSUVmax	0.99 (0.99-1.00)	0.035	0.99 (0.99-1.00)	0.026	0.99 (0.99-1.00)	0.096
ΔLength	1.00 (0.99-1.01)	0.790	1.00 (0.99-1.01)	0.680	1.00 (0.99-1.01)	0.800
nMR						
No avid nodes	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
CMR	0.76 (0.41-1.12)	0.390	0.80 (0.44-1.46)	0.470	0.79 (0.38-1.63)	0.520
PMR/SMD/PMD	1.34 (0.71-2.51)	0.360	1.55 (0.84-2.85)	0.160	1.69 (0.84-3.39)	0.140
Additional PET-CT variables (patients stated and re-staged using second PET-CT scanner)						
ΔSUVpeak	0.99 (0.98-1.00)	0.063	0.99 (0.97-1.00)	0.027	0.98 (0.97-1.00)	0.020
ΔSUVmean	1.00 (0.98-1.01)	0.530	1.00 (0.99-1.01)	0.720	1.00 (0.98-1.01)	0.610
ΔMTV	0.99 (0.99-1.00)	0.036	1.00 (0.99-1.00)	0.100	0.99 (0.99-1.00)	0.090
ΔTGVmax	1.00 (0.99-1.00)	0.065	1.00 (0.99-1.00)	0.150	1.00 (0.99-1.00)	0.120
ΔTGVmean	0.99 (0.99-1.00)	0.045	1.00 (0.99-1.00)	0.130	1.00 (0.99-1.00)	0.120
Chemotherapy						
Double agent	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*	*Ref*
Triple agent	0.70 (0.35-1.40)	0.310	0.84 (0.43-1.62)	0.600	0.87 (0.40-1.86)	0.720
Delay to re-staging	0.30 (0.02-4.76)	0.400	0.12 (0.01-1.72)	0.120	0.03 (0.00-0.46)	0.013

Supplementary table 9.6: Post-NAC factors associated with survival (including pathological stage and response)

Factor	Overall Survival		Disease-free survival	
	Multivariate HR (95%)	p	Multivariate HR (95%)	p
Patient				
Age[2]	1.00 (1.00-1.00)	0.260	1.00 (1.00-1.00)	0.300
Sex				
Female	*Ref*	*Ref*	*Ref*	*Ref*
Male	1.19 (0.67-2.12)	0.560	1.12 (0.63-1.98)	0.790
ASA				
I	*Ref*	*Ref*	Ref	Ref
II	1.23 (0.51-2.99)	0.640	1.02 (0.44-2.38)	0.970
III	1.34 (0.50-3.61)	0.560	1.10 (0.42-2.89)	0.840
IV	NA	NA	NA	NA
Tumour				
Cell				
AC	*Ref*	*Ref*	*Ref*	*Ref*
SCC	1.06 (0.47-2.38)	0.890	1.14 (0.52-2.53)	0.730
Grade				
Well	*Ref*	*Ref*	*Ref*	*Ref*
Moderate	11.32 (0.48-3.67)	0.590	1.38 (0.49-3.91)	0.540
Poor	1.89 (0.66-5.39)	0.230	1.69 (0.59-4.86)	0.330
Undifferentiated	NA	NA	NA	NA
Site				
Proximal 1/3	NA	NA	NA	NA
Mid 1/3	*Ref*	*Ref*	*Ref*	*Ref*
Distal 1/3	0.90 (0.29-2.76)	0.850	0.83 (0.27-2.57)	0.740
GOJ 1	0.68 (0.22-2.17)	0.520	0.63 (0.20-2.03)	0.440
GOJ 2	0.45 (0.14-1.46)	0.180	0.46 (0.14-1.53)	0.210
GOJ 3	0.46 (0.09-2.41)	0.360	0.48 (0.09-2.46)	0.380
Multifocal	NA	NA	NA	NA
Impassable				
No	*Ref*	*Ref*	*Ref*	*Ref*
Yes	0.28 (0.08-1.01)	0.053	0.33 (0.11-1.03)	0.056
PET-CT				
PET scanner				
1	*Ref*	*Ref*	*Ref*	*Ref*
2	0.57 (0.26-1.27)	0.170	0.98 (0.45-2.14)	0.960
Restaging mN stage				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	2.40 (1.01-5.72)	0.048	2.87 (1.21-6.82)	0.017
2	0.93 (0.34-2.55)	0.890	0.93 (0.36-2.37)	0.870
Restaging logSUVmax	2.75 (0.74-10.1)	0.130	2.54 (0.71-9.08)	0.150
Restaging avid length	0.89 (0.77-1.03)	0.130	0.890 (0.77-1.02)	0.110
pN/AvidN stage**				
N0	*Ref*	*Ref*	*Ref*	*Ref*
≥N1 non avid	1.67 (0.79-3.55)	0.180	1.68 (0.80-3.51)	0.170
≥N1 avid	2.87 (1.00-8.33)	0.050	2.90 (1.05-8.03)	0.041
Log SUVmean*	22.8 (0.56-920.2)	0.098	8.00 (0.35-180.9)	0.190
Log SUVpeak*	14.9 (2.81-79.3)	1.50×10^{-3}	9.61 (2.32-39.9)	1.80×10^{-3}
Log MTV*	1.01 (0.99-1.04)	0.290	1.01 (0.99-1.03)	0.370
Log TGVmax*	1.10 (0.77-1.58)	0.600	0.96 (0.72-1.29)	0.800
Log TGVmean*	1.15 (0.79-1.68)	0.460	0.99 (0.72-1.35)	0.940
Log avid nodal SUVmax*	1.70×10^{5} (0.06-4.73×10^{11})	0.110	8266.8 (1.30-5.28×10^{-7})	0.044
Pathological response and stage				
ypTR				
No	*Ref*	*Ref*	*Ref*	*Ref*
Yes	0.57 (0.27-1.21)	0.150	0.54 (0.26-1.13)	0.100
pT				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	1.09 (0.20-5.95)	0.920	1.22 (0.22-6.61)	0.820

2	0.61 (0.12-3.09)	0.550	0.74 (0.15-3.76)	0.720
3	0.85 (0.17-4.25)	0.840	1.08 (0.22-5.42)	0.930
4	1.19 (0.19-7.50)	0.860	1.49 (0.23-9.53)	0.670
pN				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	1.85 (0.86-3.98)	0.110	1.76 (0.83-3.72)	0.140
2	1.81 (0.79-4.13)	0.160	2.09 (0.93-4.68)	0.073
3	3.67 (1.58-8.54)	2.50×10^{-3}	3.40 (1.60-7.69)	3.40×10^{-3}
R				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	2.28 (1.25-4.16)	7.40×10^{-3}	11.43 (0.79-2.57)	0.230
L				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	1.27 (0.67-2.44)	0.460	1.19 (0.61-2.31)	0.610
V				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	0.88 (0.46-1.66)	0.690	1.02 (0.54-1.91)	0.950
Neoadjuvant chemotherapy and operation				
Double agent	*Ref*	*Ref*	*Ref*	*Ref*
Triple agent	0.79 (0.36-1.73)	0.560	0.99 (0.49-2.00)	0.980
Operation				
LTO	*Ref*	*Ref*	*Ref*	*Ref*
ILO	0.84 (0.44-1.60)	0.600	0.89 (0.77-1.02)	0.740
3 stage	1.00 (0.21-4.86)	0.210	0.98 (0.20-4.82)	0.980
ETG	2.03 (0.67-6.19)	1.000	1.72 (0.57-5.14)	0.330
Log days to restaging	0.08 (0.00-2.47)	0.590	0.14 (0.01-3.67)	0.240
Log days to surgery	0.70 (0.19-2.55)	0.150	0.78 (0.22-2.78)	0.700

*denotes for subset of patients staged and re-staged using most recent PET-CT scanner (n=145), adjusted for all other variables above; **not included in full model

Supplementary table 9.7: Post-chemotherapy PET-CT factors associated with overall survival and disease-free survival: final model

Factor	Overall Survival (n=205)		Disease-free survival			
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Post-chemotherapy PET-CT						
LogSUVmax	1.30 (0.34-4.47)	0.680	1.30 (0.39-4.28)	0.670	1.51 (0.41-6.19)	0.510
Avid length	0.92 (0.81-1.04)	0.190	0.94 (0.83-1.06)	0.280	0.94 (0.82-1.08)	0.410
Avid nodes *No* *Yes*	 Ref 2.17 (1.15-4.09)	 Ref 0.016	 Ref 2.15 (1.14-4.05)	 Ref 0.017	 Ref 1.91 (0.94-3.89)	 Ref 0.076
Pathological						
pTR No Yes	 Ref 0.52 (0.25-1.06)	 Ref 0.071	 Ref 0.50 (0.26-0.98)	 Ref 0.045	 Ref 0.57 (0.26-3.89)	 Ref 0.170
pN *0* *1* *2* *3*	 *Ref* 2.18 (1.10-4.32) 1.97 (0.96-4.05) 4.25 (2.09-8.67)	 *Ref* 0.025 0.065 6.50×10^{-5}	 *Ref* 2.05 (1.05-3.99) 2.40 (1.21-4.77) 4.11 (2.07-8.15)	 *Ref* 0.035 0.012 5.30×10^{-5}	 *Ref* 1.92 (0.88-4.19) 2.94 (1.33-6.49) 3.91 (1.77-8.61)	 *Ref* 0.100 7.70×10^{-3} 7.30×10^{-5}
R *0* *1*	 Ref 2.40 (1.48-3.90)	 Ref 4.20×10^{-4}	 *Ref* 1.68 (1.05-2.69)	 *Ref* 0.032	 Ref 1.12 (0.62-2.01)	 Ref 0.710
PET-CT confounders						
PET-CT scanner *1* *2*	 Ref 0.75 (0.47-1.19)	 Ref 0.220	 *Ref* 0.69 (0.44-1.10)	 *Ref* 0.120	 *Ref* 0.76 (0.44-1.30)	 *Ref* 0.310
Cell type *AC* *SCC*	 Ref 1.05 (0.58-1.90)	 *Ref* 0.870	 *Ref* 1.02 (0.59-1.77)	 *Ref* 0.950	 *Ref* 0.57 (0.26-1.27)	 *Ref* 0.170

Supplementary table 9.8: Metabolic response and survival (including pathological stage and response; patients staged and restaged using same PET-CT scanner n=195)

Factor	Overall Survival		Disease-free survival	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Patient				
Age[2]	1.00 (1.00-1.00)	0.190	1.00 (1.00-1.00)	0.350
Sex				
Female	Ref	Ref	Ref	Ref
Male	1.25 (0.66-2.38)	0.490	1.20 (0.62-2.33)	0.580
ASA				
I	*Ref*	*Ref*	Ref	Ref
II	1.23 (0.51-2.99)	0.640	1.02 (0.44-2.38)	0.970
III	1.34 (0.50-3.61)	0.560	1.10 (0.42-2.89)	0.840
IV	NA	NA	NA	NA
Tumour				
Cell				
AC	Ref	Ref	Ref	Ref
SCC	2.03 (0.22-18.6)	0.530	1.73 (0.19-15.5)	0.620
Grade				
Well	Ref	Ref	Ref	Ref
Moderate	1.01 (0.31-3.34)	0.980	0.97 (0.29-3.18)	0.950
Poor	1.12 (0.34-3.67)	0.530	0.93 (0.29-3.05)	0.910
Undifferentiated	NA	NA	NA	NA
Site				
Mid 1/3	Ref	Ref	Ref	Ref
Distal 1/3	1.00 (0.29-3.40)	1.000	0.83 (0.25-2.78)	0.770
GOJ 1	0.76 (0.22-2.59)	0.660	0.68 (0.20-2.29)	0.530
GOJ 2	0.41 (0.11-1.50)	0.180	0.42 (0.12-1.50)	0.180
GOJ 3	0.33 (0.10-1.11)	0.440	0.52 (0.10-2.81)	0.450
Multifocal	NA	NA	NA	NA
Impassable				
No	Ref	Ref	Ref	Ref
Yes	0.33 (0.11-1.11)	0.074	0.39 (0.13-1.14)	0.085
PET-CT				
PET scanner				
1	Ref	Ref	Ref	Ref
2	0.61 (0.33-1.11)	0.100	00.68 (0.38-1.22)	0.200
mNR				
No avid nodes	Ref	Ref	Ref	Ref
CMR	1.15 (0.59-2.25)	0.670	1.15 (0.61-2.17)	0.170
PMR	1.89 (0.76-4.74)	0.170	2.02 (0.80-5.11)	0.870
SMD	1.48 (0.45-4.82)	0.520	1.86 (0.62-5.60)	0.140
PMD	4.57 (1.45-14.4)	9.60×10^{-3}	2.92 (0.96-8.95)	0.060
ΔSUVmax (%)	1.00 (0.99-1.02)	0.580	0.99 (0.99-1.00)	0.094
ΔLength (%)	0.99 (0.98-1.00)	0.220	1.01 (1.00-1.02)	0.077
ΔSUVmean*	1.00 (0.99-1.00)	0.470	1.00 (0.99-1.00)	0.970
ΔSUVpeak*	1.00 (0.99-1.01)	0.630	0.99 (0.98-1.00)	0.087
ΔMTV*	1.00 (1.00-1.01)	0.740	1.00 (0.99-1.00)	0.550
ΔTGVmax*	0.70 (0.15-3.22)	0.650	1.00 (0.99-1.00)	0.410
ΔTGVmean*	1.00 (1.00-1.01)	0.740	1.00 (0.99-1.00)	0.510
Thresholds				
Δ SUVmax (77.8%)	1.45 (0.51-4.16)	0.490	0.56 (0.23-1.32)	0.180
Δ Length (57.3%)	0.92 (0.30-2.81)	0.880	1.27 (0.59-2.73)	0.540
Δ SUVmean (36.3%)*	1.30 (0.55-3.09)	0.550	0.88 (0.41-1.89)	0.740
Δ SUVpeak (69.8%)*	0.42 (0.18-1.00)	0.050	0.41 (0.19-0.87)	0.020
Δ MTL (76.1%)*	1.63 (0.72-3.70)	0.550	1.42 (0.62-3.25)	0.410
Δ MTV (89.6%)*	1.38 (0.64-2.96)	0.240	1.33 (0.70-2.52)	0.380
Δ TGVmax (93.8%)*	1.40 (0.66-2.98)	0.380	1.29 (0.68-2.43)	0.440
Δ TGVmean (89.2%)*	1.37 (0.65-2.89)	0.410	1.28 (0.72-2.29)	0.400
Pathological response and stage				
pTR				
No	*Ref*	*Ref*	Ref	Ref
Yes	0.57 (0.27-1.21)	0.150	0.54 (0.26-1.13)	0.100

pT				
0	*Ref*	*Ref*	Ref	Ref
1	1.09 (0.20-5.95)	0.920	1.22 (0.22-6.61)	0.820
2	0.61 (0.12-3.09)	0.550	0.74 (0.15-3.76)	0.720
3	0.85 (0.17-4.25)	0.840	1.08 (0.22-5.42)	0.930
4	1.19 (0.19-7.50)	0.860	1.49 (0.23-9.53)	0.670
pN				
0	*Ref*	*Ref*	Ref	Ref
1	1.85 (0.86-3.98)	0.110	1.76 (0.83-3.72)	0.140
2	1.81 (0.79-4.13)	0.160	2.09 (0.93-4.68)	0.073
3	3.67 (1.58-8.54)	2.50×10^{-3}	3.40 (1.60-7.69)	3.40×10^{-3}
R				
0	*Ref*	*Ref*	Ref	Ref
1	2.28 (1.25-4.16)	7.40×10^{-3}	11.43 (0.79-2.57)	0.230
L				
0	*Ref*	*Ref*	Ref	Ref
1	1.27 (0.67-2.44)	0.460	1.19 (0.61-2.31)	0.610
V				
0	*Ref*	*Ref*	Ref	Ref
1	0.88 (0.46-1.66)	0.690	1.02 (0.54-1.91)	0.950
Neoadjuvant chemotherapy and operation				
Double agent	*Ref*	*Ref*	R*ef*	*Ref*
Triple agent	0.79 (0.36-1.73)	0.560	0.99 (0.49-2.00)	0.980
Operation				
LTO	*Ref*	*Ref*	Ref	Ref
ILO	0.84 (0.44-1.60)	0.600	0.89 (0.77-1.02)	0.740
3 stage	1.00 (0.21-4.86)	0.210	0.98 (0.20-4.82)	0.980
ETG	2.03 (0.67-6.19)	1.000	1.72 (0.57-5.14)	0.330
Log days to restaging	0.08 (0.00-2.47)	0.590	0.14 (0.01-3.67)	0.240
Log days to surgery	0.70 (0.19-2.55)	0.150	0.78 (0.22-2.78)	0.700

*denotes for subset of patients staged and re-staged using most recent PET-CT scanner (n=145), adjusted for all other variables above; **not included in full model

Supplementary table 9.9: Pre-operative associations of metabolic response metrics of primary and nodal tumour with survival and recurrence (final model)

Factor	Overall Survival		Disease-free survival		Time to recurrence	
	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate HR (95% CI)	p
Primary tumour (SUV metrics adjusted for length; all adjusted for pre-chemo N stage and cell type)						
LogΔSUVmax	0.99 (0.99-1.00)	0.017	0.99 (0.99-1.00)	0.015	0.99 (0.99-1.00)	0.100
ΔLength	1.00 (1.00-1.01)	0.360	1.00 (1.00-1.01)	0.340	1.00 (0.99-1.01)	0.990
ΔSUVpeak	0.99 (0.98-1.00)	0.120	0.99 (0.98-1.00)	0.150	0.99 (0.98-1.00)	0.073
ΔSUVmean	1.00 (0.99-1.01)	0.640	1.00 (0.99-1.00)	0.350	0.99 (0.98-1.00)	0.270
ΔMTV	0.99 (0.99-1.00)	0.030	1.00 (0.99-1.00)	0.100	0.99 (0.99-1.00)	0.041
ΔTGVmax	1.00 (0.99-1.00)	0.093	1.00 (0.99-1.00)	0.160	1.00 (0.99-1.00)	0.170
ΔTGVmean	1.00 (0.99-1.00)	0.100	1.00 (0.99-1.00)	0.150	1.00 (0.99-1.00)	0.100
Primary tumour thresholds						
ΔSUVmax -77.8%	0.46 (0.24-0.88)	0.019	0.44 (0.23-0.83)	0.011	0.44 (0.23-0.83)	0.011
ΔLength -55.3%	0.83 (0.47-1.43)	0.510	0.79 (0.45-1.37)	0.390	0.79 (0.45-1.37)	0.390
ΔSUVpeak -69.8%	0.65 (0.41-1.03)	0.068	0.61 (0.38-0.96)	0.033	0.61 (0.38-0.98)	0.033
ΔSUVmean -36.1%	0.91 (0.57-1.43)	0.670	0.89 (0.57-1.39)	0.600	0.89 (0.57-1.39)	0.600
ΔMTV -89.6%	0.71 (0.45-1.10)	0.120	0.71 (0.46-1.09)	0.120	0.71 (0.46-1.09)	0.120
ΔTGVmax -93.8%	0.75 (0.48-1.18)	0.210	0.74 (0.48-1.14)	0.170	0.74 (0.48-1.14)	0.170
ΔTGVmean -89.2%	0.71 (0.46.-1.10)	0.130	0.72 (0.47-1.10)	0.130	0.72 (0.47-1.10)	0.130

Section 9.3.3: Validation of tumour downstaging and comparison with pathological response

Supplementary table 9.10: Association of pathological response with downstaging, resection margin and pathological stage

Factor	Pathological response		
	Yes	No	p value
Resection margin			
R0	73 (91.0%)	158 (72.5%)	1.00×10^{-3} [a]
R1	8 (9.01%)	60 (27.5%)	
pT			1.38×10^{-11}
0	14 (17.3%)	0 (0.00%)	
1	10 (12.3%)	16 (7.34%)	
2	30 (37.0%)	49 (22.5%)	
3	25 (30.9%)	140 (64.2%)	
4	2 (2.50%)	13 (5.9%)	
pN			
0	48 (59.3%)	52 (23.9%)	2.76×10^{-7}
1	16 (19.8%)	60 (27.5%)	
2	9 (11.1%)	53 (24.3%)	
3	8 (9.88%)	53 (24.3%)	
L			
0	57 (70.4%)	81 (37.2%)	4.81×10^{-7}
1	24 (29.6%)	137 (62.8%)	
V			
0	66 (81.5%)	115 (52.8%)	4.70×10^{-6}
1	15 (18.5%)	103 (47.2%)	
Downstaged			
No	38 (46.9%)	152 (67.9%)	4.06×10^{-4}
Yes	43 (53.1%)	66 (72.1%)	
Nodal alteration			
Negative	22 (27.2%)	17 (7.80%)	1.74×10^{-7}
Loss	26 (32.1%)	35 (16.1%)	
Stable/progression	25 (30.9%)	126 (57.8%)	
New	8 (9.88%)	40 (18.3%)	

Section 9.3.4: The added value of considering tumour and nodal response separately

Supplementary table 9.11: Comparison of tumour and nodal response metrics for predicting overall survival at 2 years

Model	p value					
	AUC	Downstaging	T & N downstaging	pTR	pTR + pN	pTR + N downstaging
AUC						
AUC	NA	0.598	0.692	0.633	0.691	0.696
Downstaging	0.598	NA	$2.92x10^{-3}$	0.519	0.088	0.042
T & N downstaging	0.692	$2.92x10^{-3}$	NA	0.255	0.980	0.914
pTR	0.633	0.519	0.255	NA	0.242	0.076
pTR + pN	0.691	0.088	0.980	0.242	NA	0.895
pTR + N downstaging	0.696	0.042	0.914	0.076	0.895	NA
Sensitivity						
Sensitivity	NA	0.421	0.649	0.386	0.789	0.596
Downstaging	0.421	NA	$3.11x10^{-4}$	0.549	$5.31x10^{-5}$	0.033
T & N downstaging	0.649	$3.11x10^{-4}$	NA	$1.09x10^{-3}$	0.033	0.366
pTR	0.386	0.549	$1.09x10^{-3}$	NA	$9.63x10^{-7}$	$3.11x10^{-4}$
pTR + pN	0.789	$5.31x10^{-5}$	0.033	$9.63x10^{-7}$	NA	$9.11x10^{-4}$
pTR + N downstaging	0.596	0.033	0.366	$3.11x10^{-4}$	$9.11x10^{-4}$	NA
Specificity						
Specificity		0.776	0.735	0.898	0.592	0.796
Downstaging	0.776	NA	0.157	0.058	0.022	0.705
T & N downstaging	0.735	0.157		0.021	0.071	0.257
pTR	0.898	0.058	0.021	NA	$2.75x10^{-4}$	0.025
pTR + pN	0.592	0.022	0.071	$2.75x10^{-4}$	NA	$3.89x10^{-3}$
pTR + N downstaging	0.796	0.705	0.257	0.025	$3.89x10^{-3}$	NA

Supplementary table 9.12: Comparison of tumour and nodal response metrics for predicting disease-free survival at 2 years

Model	p value					
	AUC / sensitivity / specificity	Downstaging	T & N downstaging	pTR	pTR + pN	pTR + N downstaging
AUC						
AUC	NA	0.579	0.678	0.619	0.664	0.692
Downstaging	0.579	NA	4.41×10^{-3}	0.495	0.068	0.029
T & N downstaging	0.678	4.41×10^{-3}	NA	0.269	0.729	0.739
pTR	0.619	0.496	0.269	NA	0.292	0.051
pTR + pN	0.664	0.068	0.729	0.292	NA	0.340
pTR + N downstaging	0.692	0.029	0.739	0.051	0.340	NA
Sensitivity						
Sensitivity	NA	0.417	0.667	0.375	0.500	0.625
Downstaging	0.417	NA	5.32×10^{-4}	0.670	0.285	0.025
T & N downstaging	0.667	5.32×10^{-4}	NA	2.83×10^{-3}	0.021	0.527
pTR	0.375	0.670	2.83×10^{-3}	NA	0.083	5.32×10^{-4}
pTR + pN	0.500	0.285	0.021	0.083	NA	0.014
pTR + N downstaging	0.625	0.025	0.527	5.32×10^{-4}	0.014	NA
Specificity						
Specificity	NA	0.741	0.690	0.862	0.828	
Downstaging	0.741	NA	0.083	0.052	0.096	0.739
T & N downstaging	0.690	0.083	NA	7.53×10^{-3}	4.68×10^{-3}	0.157
pTR	0.862	0.052	7.53×10^{-3}	NA	0.480	0.014
pTR + pN	0.828	0.096	4.68×10^{-3}	0.480	NA	0.046
pTR + N downstaging	0.759	0.739	0.157	0.014	0.046	NA

Section 9.3.5: Validation of predictors of early recurrence and/or death

Supplementary table 9.13: Final model factors predicting death or recurrence within 1 year: (patients staged and restaged using same PET-CT scanner; n=195)

Factor	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Post-chemotherapy PET-CT				
Avid nodes				
No	Ref	Ref	Ref	Ref
Yes	2.76 (1.23-6.18)	0.014	2.57 (1.02-6.48)	0.045
Pathological				
pTR				
No	Ref	Ref	Ref	Ref
Yes	2.12 (1.17-3.83)	0.013	2.09 (0.95-4.64)	0.069
pN				
0	*Ref*	*Ref*	*Ref*	*Ref*
1	3.78 (1.64-8.68)	1.72×10^{-3}	1.61 (0.53-4.87)	0.396
2	4.16 (1.78-9.75)	1.05×10^{-3}	2.93 (0.97-8.89)	0.058
3	2.12 (1.17-3.83)	1.01×10^{-5}	0.43 (0.19-1.05)	1.04×10^{-4}
Resection margin				
R0	Ref	Ref	*Ref*	*Ref*
R1	2.40 (1.48-3.90)	4.20×10^{-4}	1.96 (1.20-3.18)	6.70×10^{-3}
PET-CT confounders				
PET-CT scanner				
1	Ref	Ref	*Ref*	*Ref*
2	0.58 (0.30-1.13)	0.109	0.53 (0.25-1.09)	0.083
Cell type				
AC	*Ref*	*Ref*	*Ref*	*Ref*
SCC	1.01 (0.49-2.08)	0.987	1.07 (0.42-2.70)	0.887

Section 9.3.6: Prognostic models and comparison with risk score

Supplementary table 9.14: Prognostic models: overall survival after neoadjuvant chemotherapy and surgery

Model	Optimal					
	Sensitivity	Specificity	PPV	NPV	O:E	AUC
Risk score						
1 yr OS: Risk score <3						
Apparent (n=203)	0.776 (0.704-0.837)	0.579 (0.408-0.737)	0.889 (0.826-0.936)	0.373 (0.250-0.509)	1.15	0.677 (0.588-0.758
Internal	0.776 (0.704-0.837)	0.579 (0.408-0.737)	0.889 (0.826-0.936)	0.373 (0.250-0.509)	1.15	0.677 (0.588-0.758
2 yr OS: Risk score <3						
Apparent (n=203)	0.825 (0.734-0.895)	0.513 (0.369-0.630)	0.684 (0.592-0.767)	0.696 (0.558-0.812)	0.829	0.669 (0.603-0.737)
Internal	0.825 (0.734-0.895)	0.513 (0.369-0.630)	0.684 (0.592-0.767)	0.696 (0.558-0.812)	0.829	0.669 (0.603-0.737)
5 yr OS: Risk score <2						
Apparent (n=203)	0.857 (0.738-0.936)	0.467-0.361-0.575)	0.500 (0.396-0.604)	0.840 (0.789-0.928)	0.583	0.662 (0.594-0.730)
Internal	0.857 (0.738-0.936)	0.467-0.361-0.575)	0.500 (0.396-0.604)	0.840 (0.789-0.928)	0.583	0.662 (0.594-0.730)
Logistic regression						
1 year OS: 2.888 -1.096 *(pN1) -0.905*(pN2) -1.790*(pN3) -0.831*(pR1); Pt=0.808						
Apparent (n=293)	0.696 (0.632-0.754)	0.622 (0.465-0.726)	0.904 (0.851-0.943)	0.286 (0.199-0.386)	1.30	0.659 (0.577-0.736)
Internal validation	0.696 (0.632-0.754)	0.622 (0.465-0.726)	0.904 (0.851-0.943)	0.286 (0.199-0.386)	1.30	0.659 (0.577-0.736)
2 year OS: 1.273 -15.457*(pT1) -0.6032(pN1)-0.6538*(pN2)-1.953 *(pN3) -1.3066*(pR1) + 0.8983 *(pTR) -0.8709(Avid nodes after NAC); Pt-0.471						
Apparent (n=173)	0.784 (0.688-0.871)	0.692 (0.578-0.792)	0.760 (0.664-0.840)	0.720 (0.604-0.818)	0.97	0.734 (0.666-0.799)
Internal validation	0.732 (0.638-0.812)	0.723 (0.598-0.827)	0.814 (0.723-0.886)	0.618 (0.500-0.728)	1.11	0.716 (0.650-0.785)
5 year OS : 0.418 -1.134 *(pN1)-1.810 *(pN2)-2.509*p(N3)+1.213*(pTR) Pt=0.529						
Apparent (n=222)	0.718 (0.615-0.804)	0.785 (0.709-0.849)	0.644 (0.534-0.744)	0.837 (0.764-0.895)	0.897	0.740 (0.678-0.795)
Internal validation	0.718 (0.615-0.804)	0.785 (0.709-0.849)	0.644 (0.534-0.744)	0.837 (0.764-0.895)	0.897	0.740 (0.678-0.795)
Recursive partitioning						
1 year OS: (NA)						
NA	NA	NA	NA	NA	NA	NA
2 year OS: Partitioning 1st on pN stage (0–v 1-3); then pR the >pN0 (0-v-1), then presence of avid nodes after NAC						
Apparent (n=173)	0.763 (0.666-0.843)	0.724 (0.609-0.820)	0.779 (0.682-0.858)	0.705 (0.691-0.803)	1.02	0.743 (0.679-0.801)
Internal validation	0.794 (0.700-0.869)	0.579 (0.460-0.691)	0.706 (0.612-0.790)	0.688 (0.559-0.798)	0.890	0.686 (0.618-0.754)
5 year OS: selecting patients with >pN0 disease, then those with pTR						
Apparent (n=222)	0.644 (0.644-0.734)	0.837 (0.764-0.895)	0.718 (0.605-0.814)	0.785 (0.709-0.849)	1.10	0.740 (0.678-0.801)
Internal validation	0.621 (0.510-0.723)	0.822 (0.747-0.883)	0.692 (0.578-0.792)	0.771 (0.694-0.837)	1.12	0.722 (0.661-0.780)

Supplementary table 9.15: Prognostic models: disease-free survival after neoadjuvant chemotherapy and surgery

Model	Optimal					
	Sensitivity	Specificity	PPV	NPV	O:E	AUC
Risk score						
1 year DFS: Risk score <3						
Apparent (n=200)	0.833 (0.736-0.906)	0.472 (0.365-0.581)	0.598 (0.504-0.688)	0.750 (0.616-0.856)	0.718	0.653 (0.588-0.719)
Internal validation	0.833 (0.736-0.906)	0.472 (0.365-0.581)	0.598 (0.504-0.688)	0.750 (0.616-0.856)	0.718	0.653 (0.588-0.719)
2 year DFS: risk score <3						
Apparent (n=173)	0.782 (0.705-0.847)	0.466 (0.333-0.601)	0.782 (0.705-0.847)	0.466 (0.333-0.601)	1.000	0.624 (0.549-0.700)
Internal validation	0.782 (0.705-0.847)	0.466 (0.333-0.601)	0.782 (0.705-0.847)	0.466 (0.333-0.601)	1.000	0.624 (0.549-0.700)
5 year DFS: risk score <2						
Apparent (n=200)	0.529 (0.385-0.671)	0.832 (0.741-0.901)	0.394 (0.272-0.423)	0.628 (0.467-0.770)	1.19	0.681 (0.605-0.758)
Apparent (n=146)	0.529 (0.385-0.671)	0.832 (0.741-0.901)	0.394 (0.272-0.423)	0.628 (0.467-0.770)	1.19	0.681 (0.605-0.758)
Logistic regression						
1 year DFS: 1.512 -0.757 (pN1) -0.759 (pN2) – 1.360(pN3) +0.931(pTR)-0.579 (Avid nodes after NAC); Pt=0.705						
Apparent (n=200)	0.869 (0.778-0.933)	0.405 (0.315-0.500)	0.514 (0.429-0.599)	0.810 (0.686-0.901)	0.519	0.663 (0.594-0.727)
Internal validation	0.500 (0.428-0.572)	0.817 (0.716-0.894)	0.868 (0.793-0.924)	0.404 (0.328-0.482)	1.736	0.659 (0.602-0.715)
2 year DFS: 1.007 -0.924(pN1) -1.055(pN2) -2.209(pN3) + 0.767(pTR)-0.717(pR)-0.838(Avid nodes after NAC); Pt=0.519						
Apparent (n=173)	0.728 (0.618-0.821)	0.728 (0.626-0.816)	0.702 (0.593-0.797)	0.753 (0.650-0.838)	0.964	0.728 (0.659-0.796)
Internal validation	0.810 (0.686-0.900)	0.670 (0.574-0.756)	0.556 (0.447-0.668)	0.872 (0.783-0.934)	0.726	0.701 (0.636-0.764)
5 year DFS: 0.286 -1.079(pN1) -1.668(pN2) -3.006 (pN3) + 1.164 (pTR)-0.717(pR)-0.838*(Avid nodes after NAC); Pt=0.379						
Apparent (n=146)	0.651 (0.541-0.751)	0.816 (0.741-0.877)	0.691 (0.579-0.789)	0.787 (0.710-0.852)	1.06	0.739 (0.679-0.797)
Internal validation	0.651 (0.541-0.751)	0.816 (0.741-0.877)	0.691 (0.579-0.789)	0.787 (0.710-0.852)	1.06	0.739 (0.679-0.797)
Recursive partitioning						
1 year DFS: selecting patients with pN0; for patients with pN1-3, partitioning on R; for patients with pR0, those with avid nodes after NAC						
Apparent	0.921 (0.864-0.960)	0.379 (0.255-0.516)	0.782 (0.711-0.842)	0.667 (0.482-0.820)	0.861	0.644 (0.577-0.711)
Internal validation	0.909 (0.849-0.950)	0.293 (0.181-0.427)	0.759 (0.687-0.821)	0.567 (0.374-0.745)	0.835	0.601 (0.539-0.661)
2 year DFS: selecting patients with pN0; for patients with pN1-3, partitioning on R; for patients with R0, those with avid nodes after NAC						
Apparent	0.721 (0.614-0.812)	0.747 (0.643-0.834)	0.738 (0.631-0.828)	0.730 (0.626-0.819)	0.977	0.734 (0.666-0.798)
Internal validation	0.721 (0.614-0.812)	0.719 (0.614-0.812)	0.713 (0.606-0.805)	0.727 (0.622-0.817)	0.966	0.729 (0.659-0.792)
5 year DFS: partitioning 1st on pN (pN0-v-pN1-3); and 2nd on pTR (0-v-1)						
Apparent	0.678 (0.564-0.781)	0.806 (0.731-0.867)	0.654 (0.540-0.757)	0.823 (0.750-0.882)	1.167	0.739 (0.678-0.798)
Internal validation	0.519 (0.404-0.631)	0.844 (0.773-0.900)	0.656 (0.527-0.771)	0.753 (0.678-0.818)	1.523	0.681 (0.622-0.741)