Utilising novel therapies in the treatment of gastrointestinal cancers

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Declaration

I declare that the work presented in this thesis is my own. I have stated the contribution from other parties in the acknowledgements section and at points within the text.

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Abstract

Background: Gastrointestinal (GI) malignancies are common and lethal diseases. Novel and better tolerated treatments which improve survival are urgently required.

Methods: Clinical and translational data were analysed from 3 early phase clinical trials evaluating whether targeting MYC 'the master regulator', and the immune modulating effects of Wnt signalling and epigenetic modulation, could be therapeutically exploited for patient benefit in oesophagogastric (OG) and colorectal cancer (CRC).

Results: 1. Eight patients with advanced OG cancer were treated with the BTK inhibitor, ibrutinib, in the iMYC study. No responses were observed however 1 patient with a dual c-MYC and HER2 co-amplified tumour achieved disease control for 32 weeks. Grade ≥ 3 GI haemorrhage occurred in 3 patients which was considered a new safety finding for ibrutinib in this population. **2.** Domatinostat, an HDAC inhibitor, plus avelumab, anti-PD-L1 antibody, was found to be safe and tolerable in 13 patients with advanced pMMR/MSS OGA or CRC treated in the phase IIa EMERGE study. 5 CRC patients experienced disease control at 6 months and 1 OGA patient had a PR. Domatinostat 200mg BD combined with avelumab 10mg/kg was determined as the RP2D. **3.** DKN-01, anti-DKK1 antibody, plus atezolizumab, anti-PD-L1 antibody, was found to be safe and tolerable in 11 patients with advanced pMMR/MSS OGA treated in the phase IIa WAKING study. One patient with a DKK1-high tumour achieved a PR. DKN-01 600mg with atezolizumab 840mg was the RP2D for the ongoing expansion phase.

Conclusion: Some signals of efficacy have been seen in these early trials of novel therapies in GI cancer patients. However, future trials of novel therapies need to consider how to deal with intratumoural heterogeneity, and incorporate novel trial design, to maximise the chance of therapeutic success.

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Abbreviations

4SC-202:	Domatinostat
5-FU:	5-Fluorouracil
ADA:	Anti-drug antibody
AE:	Adverse event
AF:	Atrial fibrillation
ALK:	Anaplastic lymphoma kinase
ALT:	Alanine transaminase
APC:	Antigen presenting cell
Arg1:	Arginase production gene
AST:	Aspartate transaminase
AUC:	Area under the curve
AZA:	5-Azacitadine
β-CTX:	β-C-terminal telopeptide
B2M:	Beta-2 microglobulin
B7-1:	Cluster of differentiation 80 (CD80)
Bax:	Bcl-2-associated X protein
BCL9:	B-cell CLL/lymphoma 9 protein
BD:	Twice daily
BET:	Bromodomain and extra-terminal domain
BRAF:	v-raf murine sarcoma viral oncogene homolog B1
BRCA:	Breast Cancer gene
BRD4:	Bromodomain-containing 4
BSC:	Best supportive care
BTK:	Bruton’s tyrosine kinase
C1D1:	Cycle 1, day 1
C1D8:	Cycle 1, day 8
C1D10:	Cycle 1, day 10

C4D1:	Cycle 4, day 1
Ca 19-9:	Cancer Antigen 19-9
CAF:	Cancer-associated fibroblast
CAPA:	Corrective and Preventative Action
CAPOX:	Capecitabine and oxaliplatin
CCL4:	Chemokine (C-C motif) ligand 4
CCNE1:	Cell cycle regulator cyclin E1
CD3:	Cluster of differentiation 3
CD4:	Cluster of differentiation 4
CD8:	Cluster of differentiation 8
CD11B:	Cluster of differentiation 11B
CD15 Fitc:	3-fucosyl-N-acetyl-lactosamine fluorescein isothiocyanate
CD33:	Sialic acid binding Ig-like lectin 3
CD45:	Leucocyte common antigen
CD47:	Cluster of differentiation 47
CD62L:	L-Selectin
CD66B PC5.5:	Anti-human CD66b antibody
CD68:	Cluster of differentiation 68
CD80:	Cluster of differentiation 80
CD206:	Cluster of differentiation 206 (mannose receptor)
CDH1:	Cadherin-1
CDK1:	Cyclin-dependent kinase 1
CDKN2A:	Cyclin-dependent kinase inhibitor 2A
CEP8:	Centromeric probe for chromosome 8
cfDNA:	Cell-free DNA
CHK1:	Cell cycle checkpoint
CI:	Confidence interval
CI:	Chief Investigator
CIMP:	CpG island methylator phenotype
CIN:	Chromosomal instability

CISH:	Chromogenic in-situ hybridization
CK1alpha:	Casein kinase 1alpa
CKAP4:	Cytoskeleton associated protein 4
CKD7:	Cyclin-dependent kinase 7
CKD9:	Cyclin-dependent kinase 9
CLK1:	CDC like kinase 1
CLL:	Chronic lymphocytic leukaemia
CLN18:	Claudin18
Cmax:	Maximum concentration
CMS:	Consensus molecular subtype
CNS:	Central Nervous System
CpG:	CG dinucleotide repeat
CPS:	Combined positive score
CR:	Complete response
CRC:	Colorectal cancer
CRF:	Case report form
CRISPR:	Clustered regularly interspaced short palindromic repeats
CT26:	Murine colorectal carcinoma cell line
CTCAE:	Common Terminology Criteria for Adverse Events
ctDNA:	Circulating tumour DNA
CTL:	Cytotoxic T lymphocytes
CTLA-4:	Cytotoxic T-lymphocyte-associated protein 4
CTNNB1:	Catenin beta 1
CXCL1:	Chemokine (C-X-C motif) ligand 1
CXCL9:	Chemokine (C-X-C motif) ligand 9
CXCL10:	Chemokine (C-X-C motif) ligand 10
CXCL12:	Chemokine (C-X-C motif) ligand 12
CYP 3A4:	Cytochrome P450 enzyme 3A4
DAAM1:	Disheveled-associated activator morphogenesis 1
DATIX:	Risk Management Information System

DC:	Dendritic cell
DCR:	Disease Control Rate
ddPCR:	Digital droplet PCR
DEXA:	Dual energy X-ray absorptiometry
DKK:	Dickkopf
DKK1:	Dickkopf 1
DKK1/2:	Dickkopf 1/2
DLT:	Dose Limiting Toxicity
dMMR:	Deficient mismatch repair
DNA:	Deoxyribonucleic acid
DNMT:	DNA methyltransferase
DNMT1:	DNA methyl transferase type 1
DNMT3B:	DNA methyl transferase type 3B
DNMTi:	DNA methyltransferase inhibitor
Dvl:	Disheveled
E-box:	Enhancer box
EBV:	Epstein-Barr virus
ECHO:	Echocardiogram
ECM:	Extracellular matrix
ECOG:	Eastern Cooperative Oncology Group
EGFR:	Epidermal growth factor
EMA:	European Medicines Agency
EMT:	Epithelial-to-mesenchymal transition
EORTC:	European Organisation for Research and Treatment of Cancer
ERBB2:	Human epidermal growth factor receptor 2
ERBB3:	Human epidermal growth factor receptor 3
ERK:	Extracellular signal regulated kinase
ESMO:	European Society for Medical Oncology
FDA:	Food and Drug Administration
FDG:	18F-fluorodeoxyglucose

FFPE:	Formalin-fixed paraffin-embedded
FGFR:	Fibroblast growth factor receptor
FISH:	Fluorescent *in situ* hybridisation
FOLFIRI:	Folinic acid, fluorouracil and irinotecan
FOLFOX:	Folinic acid, fluorouracil and oxaliplatin
FOLFOXIRI:	Folinic acid, fluorouracil, oxalipatin and irinotecan
FOXP3:	Forkhead box P3
FZD/Fzd:	Frizzled
GATA6:	GATA binding factor 6
GC:	Gastric cancer
GCP:	Good Clinical Practice
GGT:	Gamma-glutamyltransferase
GI:	Gastrointestinal
GLS:	Glutaminase
GSK2beta:	Glycogen synthase kinase 3 beta
GTPase:	Guanosine triphosphatase
H. pylori:	Helicobacter pylori
HAT:	Histone acetyltransferase
HDAC:	Histone deacetylase
HDACi:	Histone deacetylase inhibitor
HDM:	Histone demethylase
HER2:	Human epidermal growth factor receptor 2
HIV:	Human immunodeficiency virus
HLA-DR:	Human Leukocyte Antigen – DR type
HMT:	Histone methyltransferase
HPLC-MS/MS:	High Performance Liquid Chromatography - Mass Spectrometry
HR:	Hazard ratio
HRA:	Health and Care Research Approval
HRQoL:	Health-related quality of life
ICI:	Immune checkpoint inhibitor

ICR:	Institute of Cancer Research
IDMC:	Independent Data Monitoring Committee
IDO-1:	Indoleamine 2,3-dioxygenease 1
IFN-γ:	Interferon-gamma
IHC:	Immunohistochemistry
IL-4:	Interleukin 4
IL-10:	Interleukin 10
IO:	Immuno-oncology
iPR:	Immune partial response
IQR:	Interquartile range
IRAE:	Immune-related adverse event
iRECIST:	Immune Response Evaluation Criteria in Solid Tumours
iSD:	Immune stable disease
ITSDMC:	Independent Trial Steering and Data Monitoring Committee
IV:	Intravenous
JNK:	Jun N-terminal kinase
KRAS:	Kirsten rat sarcoma viral oncogene homolog
LAG3:	Lymphocyte activation gene 3
LAMB3:	Laminin subunit beta 3
LEF:	Lymphoid enhancer-binding factor
LGR5:	Leucine-rich repeat containing G protein coupled receptor
LRP:	Low-density lipoprotein
LRP5/6:	Low-density lipoprotein receptor-related proteins 5/6
M1 macrophage:	Pro-inflammatory macrophage
M2 macrophage:	Alternatively activated macrophage
MACRO:	Electronic Data Capture System
MAGE-3:	Melanoma associated antigen-A3
MAIT:	Mucosal associated invariant T
MAPK:	Mitogen-activated protein kinase
MAP2K2:	Mitogen-activated protein kinase kinase 2

MAP2K3:	Mitogen-activated protein kinase kinase 3
Max:	Maximum
MCP-1:	Monocyte chemoattractant protein -1
mCRC:	Metastatic colorectal cancer
MDSC:	Myeloid-derived suppressor cell
MEK:	Mitogen-activated protein kinase kinase
mFOLFOX6:	Modified oxaliplatin, leucovorin and 5-flouorouracil
MHC:	Major histocompatibility complex
MHLW:	Ministry of Health, Labour, and Welfare (Japan)
MLH1:	DNA mismatch repair protein MIh1
MLL2:	Tumour suppressor gene encoding histone H3 lysine 4 mono-methyltransferase
MMP-2:	Matrix metallopeptidase 2
MMP-9:	Matrix metallopeptidase 9
MMR:	Mismatch repair
mRNA:	Messenger ribonucleic acid
MSI:	Microsatellite instability
MSI-H:	Microsatellite instability-high
MSS:	Microsatellite stable
MTD:	Maximum tolerated dose
mTOR:	Mammalian target of rapamycin
Mut/Mb:	Mutations/megabase
MYC:	Proto-oncogene encoding c-myc, l-myc and n-myc
NCI-CTCAE:	National Cancer Institute - Common Terminology Criteria for Adverse Events
NCT:	National clinical trial
NF1:	Neurofibromatosis type 1
NFE2L2:	Nuclear factor erythroid-derived 2-like 2
NGS:	Next generation sequencing
NICE:	National Institute for Health and Care Excellence

NK:	Natural killer
NOTCH1:	Neurogenic locus notch homolog protein 1
Nrf2:	Nuclear factor erythroid 2 -related factor 2
NSCLC:	Non-small cell lung cancer
NTRK:	Neurotrophic tyrosine receptor kinase
NY-ESO-1:	New York esophageal squamous cell carcinoma 1
OAC:	Oesophageal adenocarcinoma
OBD:	Optimal biologically active dose
OD:	Once daily
OG:	Oesophagogastric
OGA:	Oesophagogastric adenocarcinoma
OGJ:	Oesophagogastric junction
ORR:	Overall response rate
OS:	Overall survival
OSCC:	Oesophageal squamous cell carcinoma
P:	Probability
PARP:	Poly (ADP-ribose) polymerase
PBS:	Phosphate Buffered Saline
PCR:	Polymerase chain reaction
PD:	Progressive disease
PD-1:	Programmed cell death-1
PD-L1:	Programmed cell death 1 ligand 1
PD-L2:	Programmed cell death 1 ligand 2
PDX:	Patient derived xenograft
PET:	Positron emission tomography
PET-CT:	Positron emission tomography-computed tomography
PFS:	Progression free survival
PI3K:	Phosphatidylinositol-3 kinase
PIK3CA:	Phosphatidylinositol-4,5-biphospate 3-kinase catalytic subunit alpha
PIM1:	Serine/threonine-protein kinase 1

PIN1:	Peptidyl-prolyl cis/trans isomerase
PK:	Pharmacokinetic
PK/PD:	Pharmacokinetic/pharmacodynamic
PLC:	Phospholipase C
PLK1:	Polo-like kinase 1
PMD:	Progressive metabolic disease
pMMR:	Proficient mismatch repair
PMR:	Progressive metabolic response
POLE:	DNA polymerase epsilon
PORCNi:	Porcupine inhibitor
PPA2:	Inorganic pyrophosphatase
PPIB:	Peptidyl-prolyl cis-trans isomerase B
PR:	Partial response
PRKCA:	Protein kinase C alpha
PROM:	Patient reported outcome measure
PS:	Performance status
PTEN:	Phosphatase and tensin homolog
q2w:	Once every 2 weeks
q3W:	Once every 3 weeks
QLQ-C30:	EORTC core quality of life questionnaire
QLQ-STO22:	EORTC Quality of Life Questionnaire-Stomach
QTc:	Corrected QT interval
RAF:	Rapidly accelerated fibrosarcoma
RAS:	Rat sarcoma virus
REC:	Research Ethics Committee
RECIST:	Response Evaluation Criteria in Solid Tumours
RhoA:	Ras homolog family member A
RNA:	Ribonucleic acid
RM-ICR:	Royal Marsden – Institute of Cancer Research
RMH:	Royal Marsden Hospital

RNAi:	RNA interference
RNF43:	Ring finger protein 43
ROCK:	Rho-associated protein kinase
ROR:	Regulator of reprogramming
RP2D:	Recommended Phase II dose
RSPO:	R-spondin
RTK:	Receptor tyrosine kinase
SCNA:	Somatic chromosomal copy number aberration
SD:	Stable disease
sFRP2:	Secreted Frizzled-related protein 2
SMAD4:	Mothers against decapentaplegic homolog 4
SMD:	Stable metabolic disease
SOX2:	Sex determining region Y-box 2
SPK2:	Sphingosine kinase 2
SIRT:	Sir2-like protein
siRNA:	Small interfering RNA
SRC:	Safety Review Committee
STAT1:	Signal transducer and activator of transcription 1
STAT3:	Signal transducer and activator of transcription 3
SUSAR:	Suspected Unexpected Serious Adverse Reaction
SUVmax:	Maximum standardised uptake value
TAM:	Tumour associated macrophage
TAS-102:	Trifluridine-tipiracil
tCA:	Transcinnamic acid
TCF:	Transcription factor
TCF7:	Transcription factor 7
TCGA:	The Cancer Genome Atlas
TDD:	Total daily dosing
TEC:	Ternary elongation complex
TERT:	Telomerase reverse transcriptase

TGCA:	The Global Cancer Genome Atlas
TGFβ:	Transforming growth factor beta
TGFBR2:	Transforming growth factor-beta receptor II
Th1:	T helper type 1
TLE:	Transducin-like enhancer of split
TNFα:	Tumour necrosis factor alpha
TNF:	Tumour necrosis factor
TMB:	Tumour mutational burden
TME:	Tumour microenvironment
TMG:	Trial Management Group
TP53:	Tumour Protein 53
TPS:	Tumour percentage score
TRAE:	Treatment related adverse event
TrCP:	Transducin repeat-containing protein
Tregs:	Regulatory T cells
TRK:	Tropomyosin receptor kinase
TSA:	Trichosstatin
TSG:	Tumour suppressor gene
U.K.:	United Kingdom
UPD:	Unconfirmed PD
US:	United States of America
USP7:	ubiquitin specific protease 7
VEGF-A:	Vascular endothelial growth factor-A
VEGF-C:	Vascular endothelial growth factor-C
VEGFR-1:	Vascular endothelial growth factor receptor type 1
VEGFR-2:	Vascular endothelial growth factor receptor type 2
WHO:	World Health Organisation
Wnt:	“Wingless-related integration site”
Wnt3:	Proto-oncogene Wnt-3
WNT1:	Wnt family member 1

WNT2b:	Wnt family member 2 (formerly WNT13)
WNT5:	Wnt family member 5
WNT5a:	Wnt family member 5a
WNT6:	Wnt family member 6
WNT10a:	Wnt family member 10a
ZNF750:	Zinc Finger Protein 750
ZNRF3:	Zinc and Ring Finger 3

Introduction

1.1 Advanced oesophagogastric cancer as an unmet need

1.1.1 Epidemiology and risk factors

Oesophagogastric (OG) cancers are common and lethal malignancies. In 2020, more than 1.7 million new cases were diagnosed worldwide, and in the U.K, over 70% of these are at an advanced stage.(1–3) Fewer than 20% of patients are alive at 5 years from diagnosis, ranking gastric and oesophageal cancers as the 4th and 6th leading causes of global cancer-related death respectively.(1,4,5) The incidence of gastric cancer (GC) is twice as common in males, and oesophageal cancer is 3 times more common in males than females.(1) There is considerable variation in prevalence of OG cancers globally, largely reflected by the differing aetiologies of this group of diseases.

Broadly, oesophageal cancers are divided histologically into adenocarcinomas and squamous cell carcinomas. Adenocarcinomas typically arise from Barrett's oesophagus in the lower oesophagus because of gastroesophageal reflux disease, and account for two thirds of oesophageal cancer cases in the West.(6,7) The rising prevalence of obesity and gastroesophageal reflux disease is thought to contribute to the increasing incidence of oesophageal adenocarcinoma (OAC) in Northern Europe, North America, and Oceania.(8) Decreasing incidence of Helicobacter pylori (H. pylori) infection, inversely linked to development of OAC, in these regions may also be contributory.(8) On the other hand, oesophageal squamous cell carcinoma (OSCC) develops in the stratified squamous epithelium which lines the upper two-thirds of the oesophagus and is most prevalent in Eastern Asia, particularly China, but also Southern and Eastern Africa and South-Central Asia.(1) Squamous cell cancers occur due to chronic irritation and inflammation through carcinogen exposure or recurrent physical injury to the oesophageal mucosa. In the West, smoking and heavy alcohol consumption are established risk factors, particularly in combination.(9) Other likely causative agents, more relevant globally, include dietary factors such as nitrosamines, nutritional deficiencies, pickled vegetables,

betel quid chewing, and ingesting very hot food and drinks.(6,10–13) Worldwide OSCC is the dominant histological subtype, accounting for around 90% of oesophageal cancer cases, however the incidence of this subtype is on the decline both in Asia, due to dietary improvements, and in Western countries due to less cigarette smoking.(6,14)

Gastric cancer is most prevalent in South Central Asia, Eastern Asia, Eastern Europe, and South America.(15) It is generally divided into two anatomical subsites, the cardia (upper stomach, close to the oesophagogastric junction (OGJ)) and non-cardia (lower stomach). Non-cardia tumours are by far the most prevalent tumours, making up more than 80% of cases globally, almost all of which are caused by chronic H. pylori infection.(14,16) Other risk factors include alcohol consumption, eating processed meats, and low intake of fruits and vegetables.(14,17) The incidence of non-cardia tumours has been slowly declining over the past 50 years due to the decreasing prevalence of H. pylori infections, and reduced reliance of salt and pickling to preserve food.(1,18) Similarly, to OAC, gastric cardia tumours are more frequent in North America and Western Europe and are associated with obesity and gastroesophageal reflux disease.(8,17) Importantly, cases of both cardia and non-cardia GCs are rising in incidence in the young adult population (<50 years), particularly in more affluent countries such as the U.K. and U.S.(19) Disruption of the gut microbiome, for example, with antibiotics and gastric acid suppressants and higher rates of autoimmune gastritis, are suggested reasons for this increase.(20,21)

1.1.2 Clinical, biological, and molecular characteristics

Dysphagia or odynophagia and unintentional weight loss are the main presenting symptoms of oesophageal and proximal gastric cancers. Other symptoms of OG cancer include poor appetite, indigestion, early satiety, bleeding, and abdominal pain. Unfortunately, by the time a patient becomes symptomatic and seeks medical attention, these cancers have often spread and are diagnosed at an advanced or metastatic stage which cannot be cured. For the purposes of

determining treatment options, OG cancers are divided by histopathological subtype. Typically, squamous cell carcinomas respond very well to radiotherapy, and thus, the preferred treatment paradigm for locally advanced OSCC is neoadjuvant or definitive chemoradiotherapy.(22) By contrast, many patients with locally advanced OAC will receive perioperative chemotherapy alone, although neoadjuvant chemoradiotherapy is also offered in some parts of the world for oesophageal and OGJ adenocarcinoma.(22) Perioperative chemotherapy is also considered the standard of care management of operable GC, and, in the advanced disease setting, oesophageal, OGJ and gastric adenocarcinoma (collectively OGA) are currently managed in the same way.(22,23) However, there are important molecular and clinical differences between these anatomical and histological subtypes, and clinical trials are increasingly distinguishing between OSCC and adenocarcinoma within inclusion criteria and subgroup analyses. Patients with OSCC also derive relatively greater benefit from immune checkpoint inhibitors, particularly in the second line, which has been a major turning point in their management and discussed further in section 1.1.4.

The Global Cancer Genome Atlas (TCGA) project has reported distinct differences between the molecular profiles of OSCC, OAC, and GC.(24,25) In OACs and OSCCs, the only shared frequently mutated gene was TP53.(24) Otherwise in OSCCs; NFE2L2, MLL2, ZNF750, NOTCH1 and TGFBR2 were often mutated.(24) Whereas OACs displayed increased mutations in CDKN2A, ARID1A, SMAD4 and ERBB2.(24) There were also significant variations in somatic copy number alterations (SCNAs), which are considered the predominant type of genetic driver alterations, rather than mutations in driver genes, between OAC and OSCCs.(24) For example, amplifications in VEGFA, ERBB2, GATA6, CCNE1, and deletion of SMAD4, were unique to OAC. Common SCNAs in OSCC included amplifications of SOX2, TERT, and FGFR1.(24) Alterations to cell cycle regulators were common features of both OSCCs and OACs, although different genes were altered in each case.(24) Alterations in receptor tyrosine kinases (RTKs) and components of their downstream signalling pathways e.g., EGFR and PIK3CA were more common in OACs, and beta-catenin was more frequently activated in OAC than OSCC.(24) Further comprehensive molecular characterisation, including analysis of mRNA

expression and DNA methylation data, showed that OSCC was much more akin to squamous cell carcinoma of the head and neck than to OAC.(24) This corroborates the notion that OSCC and OAC are two discrete diseases which simply share an anatomical site, and thus, would require divergent treatment strategies. The TGCA has further defined 3 subtypes of OSCCs. OSCC1, which have high levels of NRF2 pathway alterations, OSCC2, which have high rates of NOTCH1 or ZNF750 mutation, and OSCC3, which display features expected to cause activation of the PI3K pathway.(24) Interestingly these 3 subtypes also displayed geographic clustering with OSCC1 more common in Asian patients, OSCC2, more common in Eastern European and South American patients, and OSCC3, exclusively confined to the U.S. and Canada.(24)

The subtypes of GCs are classed as having, (1) Epstein-Barr virus (EBV) infection, (2) microsatellite instability (MSI), (3) chromosomal instability (CIN) and (4) genomic stability (GS).(24,25) By contrast to OSCC, the molecular profiles of OACs distinctly resemble CIN GCs.(24) This convergent underlying biology supports the current therapeutic approach where OACs and GCs are managed in the same way. Interestingly the CIN phenotype appears to increase more proximally from the stomach towards the lower oesophagus, with OACs, junctional tumours and proximal GCs displaying the highest rates of hypermethylation.(25) Of the four molecular subtypes, CIN GCs are the most common, making up 50% of cases, and have also been associated with the worst prognosis.(25) CIN tumours are characterised by aneuploidy via SCNAs, and amplification of RTK genes such as HER2, FGFR2, and MET.(25,26) By contrast, EBV positive tumours are more commonly located in the gastric fundus or body.(25) The EBV positive subgroup exhibit profound levels of hypermethylation (CIMP), high levels of PIK3CA mutations, and overexpression of PD-L1 and PD-L2.(25,27,28) Microsatellite unstable tumours show high levels of hypermethylation, including characteristic MLH1 hypermethylation, and high levels of mutations in oncogenic genes such as TP53, KRAS, ARID1A, PIK3CA, ERBB3 and PTEN, and major histocompatibility complex (MHC) class I genes.(25) Finally, the GS subtype, associated with diffuse histology and an earlier age of diagnosis, are enriched with mutations in RHOA, which plays a part

in cell motility, and fusions of CLN18, which is involved in cell adhesion.(25) Table 1 outlines the differences between the TCGA subtypes of OSCC, OAC and GC.

The definition of these subgroups has dramatically deepened our understanding of the biology of GC and have become increasingly therapeutically relevant. For example, EBV positive and MSI-high gastric cancers have shown the greatest benefit from immune checkpoint inhibitors (ICIs) as single agents.(29,30) However, in stage IV disease, EBV positive and MSI-high tumours represent only 6% of the population in each case.(31) Currently the only routinely tested biomarkers to determine treatment of OG cancer are mismatch repair deficiency, HER2 and PD-L1. In future, knowledge of these molecular differences between subtypes, will no doubt enhance the development and clinical application of novel therapies going forward.

	OSCCs			OAC	GC			
Histology	Squamous cell carcinoma			Adenocarcinoma	Adenocarcinoma			
Subtypes	OSCC1	OSCC2	OSCC3		EBV	MSI	CIN	GS
Geographical hotspots	Asia	Eastern Europe and South America	U.S. and Canada	Northern Europe, U.S., and Oceania				
Lauren histologic type (gastric cancer)	-			-	-	-	Intestinal	Diffuse
Anatomical location	Upper two-thirds oesophagus			Distal oesophagus	Gastric fundus/ body	Antrum/pylorus	Lower oesophagus/proximal stomach	Antrum/pylorus
DNA methylation	-			-	EBV-CIMP	Gastric-CIMP	-	-
Mutated genes	TP53 NFE2L2 MLL2 ZNF750 NOTCH1 TGFBR2			TP53 CDKN2A ARID1A SMAD4 ERBB2	PIK3CA ARID1A BCOR	TP53 KRAS ARID1A PIK3CA ERBB2/3 PTEN EGFR MHC class I genes	TP53	RHOA CDH1 CLN18-ARHGAP fusions
Somatic copy number alterations	Amplifications: SOX2, TER, FGFR1, MDM2, NFX2-1, CDK6 Deletions: RB1			Amplifications: VEGFA, ERBB2, GATA6, CCNE1 Deletions: SMAD4	Amplifications: JAK2, ERBB2		Amplifications: ERBB2, ERBB3, VEGFA, EGFR, FGFR2, MET	
Alterations in receptor tyrosine kinases/downstream signalling components	EGFR (19%), PIK3CA, PTEN PIK3R1, ERBB2 (3%)			EGFR, PIK3CA, ERBB2 (32%), KRAS, IGF1R, VEGFA			EGFR, ERBB2, FGFR2, MET	

Table 1 TCGA molecular subtypes of oesophageal and gastric cancers

1.1.3 Pre-requisites for effective anti-cancer immunity and the immune landscape in OSCC and OGA

Immune checkpoint inhibitors have transformed the management of several malignancies such as melanoma, non-small cell lung cancer (NSCLC), and renal cancers.(32–34) However, responses to ICIs in OG cancer have, generally, been far less dramatic, particularly as single agents in chemo-refractory patients, where they were initially evaluated. In the past couple of years, the positive results of large phase III trials assessing chemotherapy plus ICI, or dual ICI, combinations (section 1.1.4), have broadened the applicability of ICI-use in OG cancer. However, there is still further progress to be made, particularly in the identification of better predictors of response to these immune therapies. Thus far, PD-L1 status (measured by either combined positive score (CPS); the number of PD-L1 positive cells, including tumour cells, macrophages, and lymphocytes, divided by the total number or tumour cells, and multiplied by 100, or tumour proportion score (TPS); the number of positive tumour cells divided by the total number of viable tumour cells multiplied by 100), has been used to select and stratify patients with OG cancer onto ICI trials.(35,36) However, it is an imperfect biomarker, as responses to ICIs have been observed in both PD-L1 positive and negative patients,(37) and survival has not been vastly improved, by more than a month or so, in PD-L1 positive compared to PD-L1 negative patients who received ICI monotherapy beyond the first line.(38,39) It also appears to be a dynamic biomarker in OG cancer, as sequential biopsies from patients during ICI treatment have revealed significant discordance in PD-L1 status.(40) Moreover, there is the challenge of substantial spatial and temporal heterogeneity with which to contend, as PD-L1 expression between the primary and metastasis from the same patient is only 60% concordant and, disparity of PD-L1 positivity has been observed in 40% of pre-treatment diagnostic biopsies compared to fresh biopsy.(41) This makes it challenging to use reliably when determining treatment options, particularly in later line settings and in patients with a large volume of metastatic disease. Finally, PD-L2, which is expressed in up to two thirds of OG cancers, can also interact with PD-L1 and potentially hamper ICI responses.(42–44) Whereas, PD-L1 expression is driven by interferon-gamma and associated with a pro-inflammatory environment, PD-L2 expression is driven

by IL-4 and associated with immune suppression, potentially due to infiltration of myeloid-derived suppressor cells (MDSCs).(43–45) PD-L2 expression has also been linked to lower levels of CD8+ T cell infiltration in OSCC.(46) Therefore, just targeting the PD-1/PD-L1 interaction with ICIs may not be enough to activate the immune infiltrate within the tumour. A deeper understanding of the immune landscape across OG cancer subtypes, will help to develop better predictive biomarkers and more personalised strategies in immune-oncology drug development.

The Cancer-Immunity Cycle, a term first coined by Chen and Mellman in 2013, describes the seven main steps required for the immune system to carry out its final effector function of killing of cancer cells (Figure 1).(47) PD-L1 and PD-L2 are expressed by tumour cells and antigen presenting cells (APCs) and bind to PD-1 on effector T cells.(48) This creates an inhibitory signal which impairs effector T cell function. Most currently available ICIs work at the seventh step, by blocking the PD-1/PD-L1 pathway, which usually acts as an ‘immunostat’ to prevent autoimmunity, but can also be up-regulated in patients with cancer and results in immune evasion.(49) Application of anti-PD-1/PD-L1-based therapy, releases the inhibition on effector T cells and reinstates the host anti-cancer response.(50) However, if the other key events/steps in the cycle have not occurred beforehand, the anti-cancer response will not be fully potentiated by PD-1/PD-L1 blockade alone.(47)

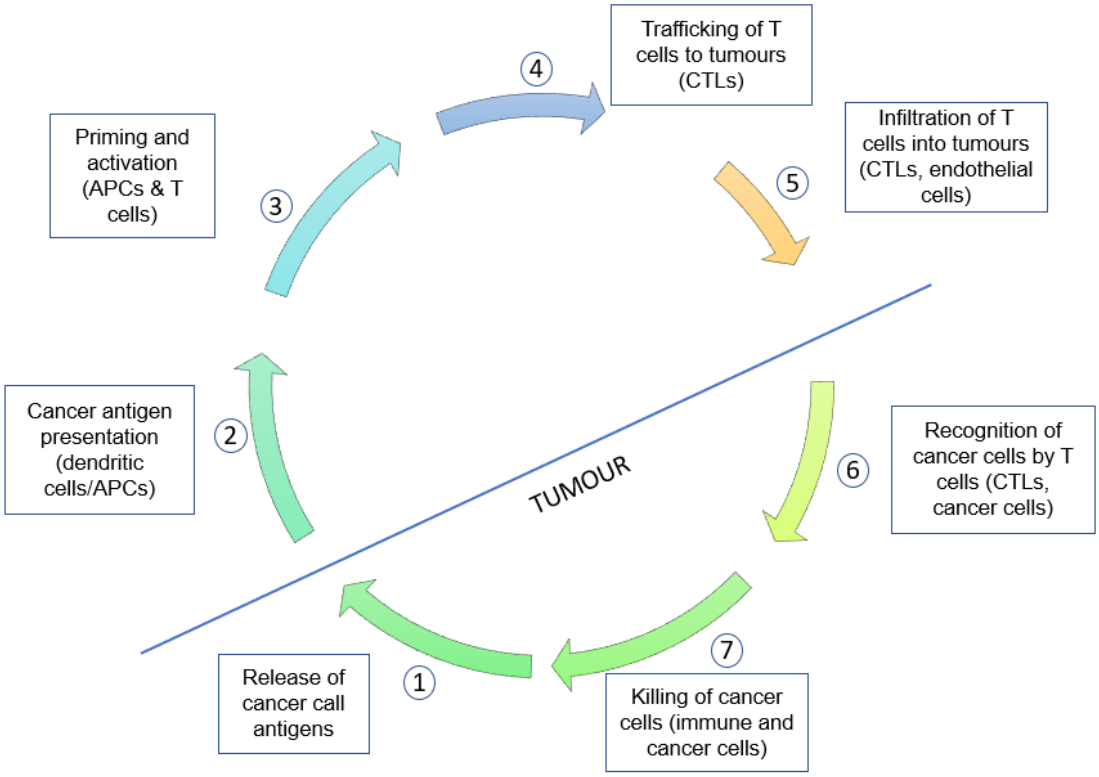


Figure 1 The Cancer-Immunity Cycle.(47) Firstly, neoantigens are released by dying cancer cells. These are subsequently picked up by antigen presenting cells (APCs), which travel to lymph nodes to prime and activate T cells. This is a key step which requires the correct balance between co-stimulatory and inhibitory factors in the TME to promote stimulation of cytotoxic T cells, rather than pro-tumoral Tregs. Effector T cells then must travel to the site of the tumour, creep into the core of tumour, recognize the cancer cells on site and finally effectively kill them. In tumours which respond to single agent PD-1/PD-L1 blockade, only this final step in the sequence is flawed, however, several tumour types, including most GI cancers, have other malfunctioning steps in the pathway which would also require attention before effective PD-1/PD-L1 blockade can be realised

The first step in the Cancer-Immunity Cycle involves release of cancer antigens. Tumours with higher numbers of mutations or 'neoantigens' are more likely to be recognized by the host immune system as foreign and, therefore, be picked up by MHC class I molecules and stimulate a strong effector T cell response.(47) Thus, tumour mutational burden (TMB), can predict sensitivity to ICIs across tumour types. Melanoma, where use of ICIs has been most efficacious, has a median mutational burden of around 14 mutations/megabase (mut/Mb), which is high compared to other malignancies.(51) On the other hand, the median TMB has been reported as 3.9 mut/Mb in OSCC,(52) 5 mut/Mb in GC,(51) and 9.9

mut/Mb in OAC.(53,54) Virus-associated cancers and cancers with microsatellite instability, or loss of mismatch repair (MMR) proteins, also display increased mutational loads and immune exhaustion,(55–57) which is likely why the EBV-positive and MSI TCGA subtypes respond best to ICIs as discussed.(29,30) However, based on ICI monotherapy studies in patients with OSCC and OGA, efficacy of ICIs is slightly better in OSCC.(38,58–60) Therefore, there are clearly other factors which influence ICI efficacy above TMB.

In 2017, Chen and Mellman went on to describe three distinct immune phenotypes which could explain the mechanism of a tumour's resistance to anti-cancer immunity.(61) So called 'inflamed' tumours are most likely to respond to PD-1/PD-L1 blockade as they are already permeated by plenty of different immune cells, including cytotoxic T cells.(61) However, they may have just been driven to an exhausted state. The 'inflamed' tumours might also harbor Tregs, MDSCs, B cells and cancer-associated fibroblasts (CAFs) which are generally inhibitory, but the balance is tipped towards a more immune permissive TME, where PD-1/PDL-1 inhibitor administration is more likely to boost the effector T cell activity of this type of tumour than the other two 'non-inflamed' phenotypes.(61) In 'immune-desert' tumours, cytotoxic T cells are either a rarity or completely absent, possibly from a lack of appropriate T cell priming or activation in the lymph nodes.(61) In 'immune-excluded' tumours, the T cells are nearby but they cannot get into the tumour due to stromal or vascular barriers.(61) The Cancer Immunogram nicely plots the various factors in the immune milieu which may either be present or lacking in a patient's tumour, and thus outlines an agenda for successful individualized immunotherapy treatment.(62) Ultimately, the success or failure of immunotherapy in cancer relies upon the tumour being able to initiate a host anti-cancer response, an immune-permissive TME, and a sensitivity to immune effector mechanisms, which may also be impacted external factors such as obesity, genetics, or the gut microbiome.(61) As described in detail in chapter 2, epigenetic mechanisms also play a key role in influencing immunogenicity at almost every step of the Cancer-Immunity Cycle.

Recent characterisation of tumour and immune cell subsets in primary OGAs has revealed distinct differences in the tumour microenvironment (TME) amongst

OGA subtypes, which may explain the differential responses to ICIs seen in the clinic. The EBV subtype appears to have the highest CD8+ T cell infiltrate at the centre of the tumour and was thus the most 'inflamed', or immunogenically 'hot', of all the subtypes, consistent over multiple samples.(26) By contrast, in the CIN subtype, the CD8+ T cells appeared stuck at the invasive margin and thus 'immune-excluded'.(26) Greater PD-L1 expression was found in the MSI and EBV subtypes compared to CIN or GS tumours, as expected.(26) However, not all MSI OGAs had a high T cell infiltrate, in fact, roughly half had a TME more akin to CIN tumours,(26) which may explain why not all MSI cancers respond to ICIs. The CIN tumours were also characterised by high levels of CD68+ myeloid cells and neutrophils.(26) The sub-populations of CD68+ myeloid cells were not established,(26) but MDSCs, M2 macrophages, derived from the myeloid lineage, and neutrophils, are generally immune inhibitory by nature.(63) Based on clustering of CD4+ and CD8+ T cells, around 50% of the CIN tumours were classed as CIN 'hot' and the other half as CIN 'cold'.(26) The CIN 'cold' tumours were located more distally than the 'hot' ones, and had more activated signalling pathways related to MYC, which drives cellular proliferation,(64) and more abundant amplifications in cell cycle regulator cyclin E1 (CCNE1).(26) Interestingly, around half of the GS subtype tumours had evidence of tertiary lymphoid structures,(26) which are ectopic lymphoid propagations in non-lymphoid tissue which can form at sites of chronic inflammation and within tumours.(65) They are areas usually replete with B-cells, T-cells, and dendritic cells, and have been associated with improved responses to ICIs.(65) Therefore, there may be potential to improve the applicability of ICIs to GS OGA tumours in future. In OSCC, CD8+ and CD4+ T cells, which boost the antigen detecting capabilities of CD8+ T cells,(66) appear to be in plentiful supply in the TME however they largely confined to the stroma.(67,68) This may inhibit their effector function.

Wnt pathway activation, which usually triggers immune exclusion, and inactivation of genes involved in immune signalling, such as B2M,(69,70) has also been associated with hyper-mutated cases of OGA.(71) This could be the underlying mechanism to explain why some hypermutated, or 'hot', OGA tumours do not respond as expected to ICIs. Furthermore, specific driver mutations can

also influence overall mutational burden and, thus, the immune response. For example, KRAS is an oncogene common to OGA and frequently mutated in many solid tumours, particularly pancreatic and colorectal cancer.(72) It causes a high number of mutations and is associated with a poor prognosis and poor response to standard therapies.(72) While KRAS mutations have been shown to induce an inflammatory TME in pancreatic cancer, by recruitment of M1 macrophages and release of pro-inflammatory cytokines such as tumour necrosis factor (TNF), it has also been associated with upregulation of PD-L1 expression and enhanced infiltration of MDSCs, leading to immune escape.(73) TP53 has also been associated with immune evasion in pancreatic cancer.(73) On the other hand, tumours with PIK3CA aberrations, which are enriched in the EBV and MSI OGA subtypes which respond best to ICIs,(26,29) have been associated with a high T cell infiltrate.(74) While overall TMB might be lower in OSCC compared to OGA, PD-L1 expression is observed to be 4-fold higher in OSCC tumours, compared to OGA.(75) This may, of course, contribute to the improved responses seen with ICI monotherapy in OSCC compared to OGA thus far.

In addition to the relative populations of innate and adaptive immune cells within the TME, other factors including stromal cells with immunosuppressive features, such as cancer-associated fibroblasts (CAFs), endothelial cells, extracellular matrix (ECM), and secreted factors, contribute its complex composition.(76) The most immunosuppressive immune cells within the TME, largely already mentioned, include MDSCs, regulatory T cells (Tregs) and tumour associated macrophages (TAMs).(76) By contrast, immune cells including cytotoxic T lymphocytes, T helper type 1 (Th1) cells, natural killer NK cells and M1 macrophages, and their cytokines, promote the host anti-cancer immune response.(76) The correct balance between immune permissive versus immune suppressive features within the TME is of critical importance to successful progression through the Cancer-Immunity Cycle, and thus, the success or failure of ICI therapy. Typically, in MMR proficient (pMMR)/microsatellite stable (MSS) GI cancers, the balance of factors is shifted more towards immunosuppression and thus ICIs as single agents are not efficacious (Figure 2). Mucosal associated invariant T (MAIT) cells, are a type of innate T cells which have cytotoxic effects like NK cells in tumours.(77) They make up around a third of tumour infiltrating

CD8+ T cells in OACs and have been correlated to favourable prognosis, but demonstrate markers of exhaustion which could correlate with ICI efficacy.(78) CAFs generate the ECM, recruit TAMs, and promote immunosuppression.(79) They are present in 93% of OAC tumours, and secrete the chemokine CXCL12, which generates tumour cell invasion, angiogenesis, metastasis, and immune evasion in both OAC and OSCC.(80) The chemokine MCP-1 appears to encourage infiltration of TAMs into the TME, which in turn promotes angiogenesis.(81) Clinicopathologically, MCP-1 is expressed in 55% of OSCC and corelated with advanced stage disease and worse survival rates.(81) Similarly, MDSCs, which are a type of alternatively activated macrophage (M2), rather than the classically activated M1 phenotype, which have antitumour properties, potently dampen the host immune response through T cell apoptosis and suppression of T-cell activation.(45,63,82) MDSCs are abundant in the CIN subtype of OGA, as described,(26) but also present in significantly increased levels in OSCC,(82) and are associated with poor prognosis.(82,83) It is likely that a TME enriched with MDSCs would confer resistance to ICIs. A better understanding of the complex interplay of cells and factors that make up the TME in OG cancer, and identification of new, rational targets, will be required to enhance ICI efficacy and improve patient outcomes.

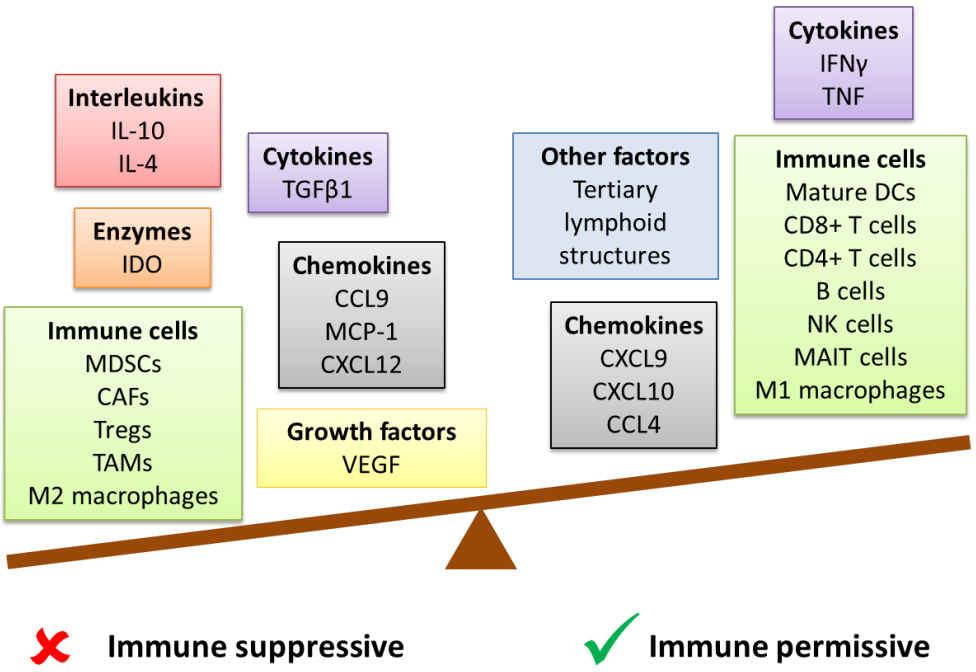


Figure 2 The balance of immune suppressive and immune permissive factors within the TME of OG cancer and CRC (adapted from Turkes et al).(84) Generally, presence of immune suppressive factors outweighs the immune permissive factors in pMMR/MSS OG cancer and pMMR/MSS CRC leading to an 'immune-excluded' or 'immune-desert' phenotype.

1.1.4 Systemic therapy options for advanced oesophagogastric cancer

Since the inception of this thesis there has been a therapeutic revolution in the management of OSCC, with ICIs at the helm. Historically, OSCC was managed with the same chemotherapy options as OACs, namely, platinum-fluoropyrimidine doublet in the first line, followed by taxanes or irinotecan in the second line. This was the case for decades, even though randomised trials of these agents were largely conducted in patients with OAC. The first trial to change the first line treatment paradigm for advanced OSCC was the phase III KEYNOTE-590 trial, which evaluated the combination of cisplatin-5-FU with the ICI, pembrolizumab, in patients with advanced, untreated oesophageal or OGJ cancer.(85) In this trial, 749 patients were allocated to receive pembrolizumab

plus chemotherapy, or placebo plus chemotherapy. Of patients included, 73% had advanced OSCC, and around half of the patients with OAC had Siewert type 1 tumours, defined as tumours located 1-5cm above the OG junction.(85) The trial did not require known PD-L1 status upfront, but OSCC and PD-L1 combined positive score (CPS) ≥10, OSCC, PD-L1 CPS ≥10 and all randomised patients, were pre-specified subgroups for analysis.(85) The trial demonstrated a survival benefit of almost 2 months for the chemotherapy and pembrolizumab arm across all subgroups, but there was a particularly strong effect in both OSCC subgroups.(85) The median overall survival for patients with OSCC and PD-L1 CPS ≥10 was 13.9 months vs 8.8 months; HR 0.57. In all patients with OSCC, overall survival was 12.6 months in the pembrolizumab arm, versus 9.8 months in the placebo arm; HR 0.72.(85) In a post-hoc analysis, clinical benefit for patients with PD-L1 CPS <10 tumours was negligible.(85) Following the presentation of these results in 2021, chemotherapy and pembrolizumab was licensed by the FDA for treatment of all patients with advanced OSCC or OG tumours 1-5cm above the OG junction. The EMA and NICE followed with similar approvals, however only for patients with tumours expressing PD-L1 CPS ≥10 (Table 2). Notably the EMA and NICE included any junctional tumours, possibly because Siewert Type 1 tumours were considered too difficult to define, particularly in the advanced disease setting.

The phase III Checkmate-648 trial also evaluated a first line chemotherapy and ICI combination, and an additional dual ICI combination, in patients with untreated advanced OSCC. A total of 970 patients were randomised to receive either chemotherapy alone, nivolumab, anti-PD-1 antibody, plus chemotherapy, or nivolumab plus ipilimumab, anti-CTLA-4 antibody.(36) The trial was for all-comers but PD-L1 status, assessed centrally, was used as a stratification factor in randomisation. Conversely to KEYNOTE-590, tumour cell PD-L1 expression (TPS), rather than PD-L1 CPS, was used to divide patients with a cut-off of 1%.(36) Overall survival was significantly improved in patients treated with chemotherapy and nivolumab, with the greatest benefit in patients with PD-L1 TPS ≥ 1%; overall survival was 15.4 months compared to 9.1 months with chemotherapy alone.(36) Survival was also significantly longer in patients with PD-L1 TPS ≥ 1% treated with nivolumab plus ipilimumab compared to

chemotherapy alone; median overall survival of 13.7 months versus 9.1 months respectively.(36) In addition, the immunotherapy containing arms led to higher numbers of complete and durable responses compared to chemotherapy alone. In the dual immunotherapy arm, there was an increase in early deaths and lower initial response rates compared to chemotherapy until around 6 months.(36) Delayed responses and early progressions are a well-known phenomenon of ICIs, and thus this chemotherapy-free option is likely to be reserved for patients with low volume disease, or where chemotherapy side effects are to be avoided. The FDA have approved nivolumab plus chemotherapy, and nivolumab plus ipilimumab, as first line therapy for all patients with advanced OSCC regardless of PD-L1 expression. On the other hand, the EMA have approved both nivolumab-containing regimens for patients with tumour cell PD-L1 expression ≥ 1%. Currently the NICE draft guidance from October 2022 advises approval of chemotherapy and nivolumab in patients with tumour cell PD-L1 expression ≥ 1% only. If the NICE guidance comes into effect this will give patients with OSCC or junctional OAC more options for accessing immunotherapy in the U.K, with tumours with CPS ≥ 10 likely treated with chemotherapy and pembrolizumab, and for those with TPS ≥ 1% and CPS < 10, with chemotherapy and nivolumab. However, it is anticipated that, both PD-L1 CPS and TPS testing will, therefore, be required which may be challenging in practice.

In KEYNOTE-590, the PD-L1 IHC 22C3 assay was used to determine CPS, with a cut-off for 'positivity' of ≥ 10. Whereas, in CheckMate 648, the PD-L1 IHC 28-8 pharmDx assay was used to determine TPS, with a cut-off of 1%. The concordance between both assays is high in other tumour types, such as lung cancer, but currently they are not considered interchangeable in OSCC.(86) In KEYNOTE-590, 51% of patients had tumours with PD-L1 CPS ≥10, and in CheckMate 648, 49% of patients had tumours with PD-L1 TPS 1% or more. Notably, PD-L1 CPS was also evaluated as an exploratory analysis in CheckMate 648, and 91% of all included patients had PD-L1 CPS of 1 or higher. Furthermore, the clinical benefit seen across the PD-L1 TPS "positive" and CPS "positive" subgroups corresponded.(36) This suggests that there is clinical use for both methods but has introduced complexity. In gastric cancer, there appears to be some discrepancy between use of the 28-8 assay and 22C3 assay for CPS

determination, as the 28-8 assay appears to assign a higher number of cases with higher PD-L1 scores.(87) Therefore, assays should not be considered interchangeable at this stage.

In the second line setting, single agent nivolumab is an option for patients with OSCC treated with chemotherapy, based on the results of the ATTRACTION-3 study.(59) Compared to chemotherapy, nivolumab was associated with a 1.5-month overall survival benefit, regardless of PD-L1 expression, and a lower incidence of grade 3 or 4 toxicities.(59) However, approvals for nivolumab monotherapy existed before the positive results of the more recent chemotherapy and checkpoint inhibitor combination studies. Therefore, if patients progress on a prior chemotherapy and ICI combination regimen, it is not clear whether ICI monotherapy would be as clinically beneficial. Furthermore, a largely Asian population were recruited to ATTRACTION-3, and so, it is not certain whether the results would be as applicable to Western patients. Nevertheless, nivolumab is approved within the NHS setting for patients with OSCC in the second line (Table 2). A phase III study evaluating tislelizumab versus standard of care chemotherapy produced similar results to ATTRACTION-3 however, again, the study population was predominantly Asian.(88) An approval for tislelizumab for this indication is still pending. KEYNOTE-181 had a lower relative proportion of Asian patients included, compared to the other two studies. Prolonged survival with pembrolizumab over standard of care second line chemotherapy was also demonstrated, but only in patients with PD-L1 CPS ≥10%.(60) Table 2 summarises the current indications and approvals of ICIs in the management of advanced OG cancer. Beyond the second line, irinotecan or taxane-based chemotherapy are possibilities.(22) Molecularly targeted agents, other than ICIs, currently play no role in the management of OSCC.

Regimen/agent	Subtype	Biomarker	Positive trial	Approving body (year)
First line				
Fluoropyrimidine- and platinum-based chemotherapy + pembrolizumab	OSCC/OGJ adenocarcinoma	-	KEYNOTE-590	FDA (2021)
Fluoropyrimidine- and platinum-based chemotherapy + pembrolizumab	OSCC/OGJ adenocarcinoma	PD-L1 CPS ≥10	KEYNOTE-590	EMA (2021), NICE (2021)
Nivolumab + ipilimumab	OSCC	-	Checkmate-648	FDA (2022)
Nivolumab + ipilimumab	OSCC	PD-L1 TPS ≥1%	Checkmate-648	EMA (2022)
Nivolumab + fluoropyrimidine- and platinum-based chemotherapy	OSCC	-	Checkmate-648	FDA (2022)
Nivolumab + fluoropyrimidine- and platinum-based chemotherapy	OSCC	PD-L1 TPS ≥1%	Checkmate-648	EMA (2022), anticipated approval NICE 2023
Nivolumab + fluoropyrimidine- and platinum-based chemotherapy	OGA	-	Checkmate-649	FDA (2021)
Nivolumab + fluoropyrimidine- and platinum-based chemotherapy	OGA	PD-L1 CPS ≥5	Checkmate-649	EMA (2021), NICE (2023)
Second line				
Pembrolizumab	OSCC	PD-L1 CPS ≥10	KEYNOTE-181	FDA (2019)
Nivolumab	OSCC	-	ATTRACTION-3	FDA (2020), EMA (2020), MHLW (2020), NICE (2021)
Third line and beyond				
Nivolumab	GC	-	ATTRACTION-2	MHLW (2020)
Pembrolizumab	GC/OGJ adenocarcinoma	PD-L1 CPS ≥1	KEYNOTE-059	FDA (2017, withdrawn 2021)

Table 2 Currently approved immune checkpoint inhibitors for management of advanced oesophagogastric cancer

In advanced disease, OAC, OGJ and GCs are currently managed in the same way.(23) In the first line setting, combination chemotherapy, usually a platinum-fluoropyrimidine doublet, is standard of care. These agents improve overall survival to around 1 year, from 3-4 months with best supportive care alone.(89,90) HER2 amplification or HER2 protein overexpression should be

tested at baseline in advanced disease, as patients with HER2 positive cancers benefit from the addition of the anti-HER2 antibody, trastuzumab, to chemotherapy.(91) This was demonstrated by the phase III TOGA trial where patients with HER2 positive tumours who were randomised to cisplatin and plus capecitabine/fluorouracil and trastuzumab, had a prolonged median survival of 13.8 months compared to 11.1 months in those treated with chemotherapy alone.(91) In patients with HER2 immunohistochemistry (IHC) score 3+ or HER2 IHC 2+, and fluorescent in situ hybridisation (FISH)-positive, the overall survival was even greater at 16 months for those treated with chemotherapy plus trastuzumab.(91) HER2 positivity is present in around 20% of OGAs, most commonly in the CIN subgroup and in the proximal stomach or OGJ.(24,25,91) Since the positive results of the TOGA trial were reported in 2010, several other large phase III trials evaluating the efficacy of other molecularly targeted agents and antibodies combined with chemotherapy were published in succession. Sadly, these were all negative trials (Table 3), and were criticised for suboptimal patient selection, inadequately enriched for relevant genomic alterations or vulnerabilities, and failure to address intra-tumoural heterogeneity which is a defining hallmark of OG cancer.(92) Therefore, well-designed smaller studies with a comprehensive translational component may be the key to gaining a deeper understanding of the potential mechanisms of response and resistance to novel therapies in OGA - essential prerequisites for a successful phase III study.

The first line chemotherapy paradigm for advanced OGA has, however, been recently enriched by the addition of nivolumab in a landmark study. In CheckMate-649, the overall survival of patients treated with platinum-fluoropyrimidine doublet chemotherapy plus nivolumab was 13.1 months, compared to 11.1 months with chemotherapy alone in all patients.(93) In patients with PD-L1 CPS ≥ 5, measured with the 28-8 pharmDx assay, the median OS was improved by a further 3.3 months, to 14.4 months.(93) Based on this data, the FDA granted approval of nivolumab plus chemotherapy in the first line treatment of advanced OGA for all patients, whereas the EMA and NICE have approved the combination only for patients whose tumours have PD-L1 CPS ≥ 5 (Table 2). First line pembrolizumab as a single agent was previously found to

result in non-inferior survival compared to platinum-fluoropyrimidine doublet chemotherapy in patients with PD-L1 CPS ≥ 1.(94) However, response rates were lower and the PFS was less favourable to chemotherapy, and therefore, it is not advocated in current guidelines.(23) As described previously, pembrolizumab plus cisplatin-5-FU was found to be superior to cisplatin-5-FU alone in patients with PDL1 CPS ≥ 5 OGJ tumours and so is another available option for treatment-naïve patients with junctional adenocarcinoma.(85)

Following failure of first line therapy, standard-of-care chemotherapies have modest success, with an improvement in median overall survival of approximately six weeks.(95,96) Options include docetaxel, paclitaxel, and irinotecan, and the choice will largely depend on the most suitable toxicity profile.(95–97) The phase III RAINBOW study demonstrated a PFS and OS benefit, and improved response rates, with ramucirumab (VEGFR2 antibody) plus paclitaxel compared to paclitaxel alone in patients with GC/OGJ adenocarcinoma.(98) However, widespread use of ramucirumab is limited in the U.K due to lack of funding. Ramucirumab is also approved as a monotherapy in the second line, but response rates are limited.(99) The antibody-drug conjugate, trastuzumab deruxtecan, is only approved in the U.S and Japan for patients with HER2 positive disease after first line treatment with a trastuzumab-containing regimen.(100) In the phase III KEYNOTE-061 trial, pembrolizumab was not superior to paclitaxel in PD-L1 positive patients (CPS ≥ 1) in the second line,(39) however in a post-hoc analysis of the patients with MSI-H tumours the response rates surpassed 50%,(101) and in patients with PD-L1 CPS ≥ 10 tumours, response rates were around 25%.(102) Response rates to pembrolizumab monotherapy in the dMMR/MSI-H gastric cancer cohort of the phase II KEYNOTE-158 study, who had progressed on all standard therapies, were equally as impressive.(103) Results of KEYNOTE-158 led to two tumour-agnostic approvals for use of pembrolizumab in any solid tumour with evidence of dMMR/MSI-H, or with a TMB of ≥ 10 mut/Mb.(104,105) However, a limited number of patients with OGA are well enough for further treatment after failure of second line therapies. Only 14% of patients with OGA went on to receive third line treatment in a U.K. tertiary cancer centre and, after first line treatment, response rates and survival are significantly reduced.(106) Thus, there is a real need for more effective strategies

to improve response rates and survival for patients with advanced OGA cancer, particularly for HER2 negative, PD-L1 negative patients, and after progression on first line regimens. Based on the evolving research into the genomic and immune landscape of these tumours, there is a potential to make future therapies more personalised, and scope to enhance the role of ICIs in this disease.

Target	Agent	Trial	Trial design	Biomarker	Overall survival experimental vs control (months)
First line					
Dual-HER-2	Trastuzumab/ pertuzumab	JACOB(107)	Trastuzumab plus chemotherapy with pertuzumab or placebo	HER2	17.5 vs 14.2 (HR 0.84 [95% CI 0·71-1·00]); p=0·057)
HER2	Lapatinib	LOGiC(108)	CAPOX plus lapatinib or placebo	HER2	12.2 vs 10.5 (HR 0.91 [95% CI, 0.73 – 1.12]); p=0.3492
EGFR	Cetuximab	EXPAND(109)	Chemotherapy with or without cetuximab	None	9.4 vs 10.7 (HR 1.00 [95% CI 0·87–1·17]); p=0·95
EGFR	Panitumumab	REAL-3(110)	Chemotherapy with or without panitumumab	None	8.8 vs 11.8 (HR 1.37 [95% CI 1·07-1·76]); p=0·013
MET	Onartuzumab	METGastric(111)	mFOLFOX6 with onartuzumab or placebo	MET and HER2 -ve	11.0 vs 11.3 (HR 0.82 [95% CI, 0.59-1.15]); p=0.24
MET	Rilotumumab	RILOMET(112)	Chemotherapy with rilotumumab or placebo	MET and HER2 -ve	8.8 vs 10.7 (HR 1.34 [95% CI 1·10-1·63]); p=0·003
Second line					
HER2	Lapatinib	TyTAN(113)	Lapatinib plus paclitaxel vs paclitaxel alone	HER2	11.0 vs 8.9 (HR 0.84 [95% CI, 0.64-1.11]); p=0.1044
HER2	Trastuzumab emtansine	GATSBY(114)	Trastuzumab emtansine vs paclitaxel	HER2	7.9 vs 8.6 (HR 1.15 [95% CI 0.87-1.51]); p=0.86
STAT3	Napabucasin	BRIGHTER(115)	Paclitaxel with napabucasin or placebo	None	6.93 vs 7.36 (HR 1.01 [95% CI 0.86-1.20]); p=0.8596
PARP	Olaparib	GOLD(116)	Paclitaxel with olaparib or placebo	None	8.8 vs 6.9 (HR 0.79 [97.5% CI 0.63-1.00]); p=0·026
FGFR2	AZD4547	SHINE(117)	AZD4547 vs paclitaxel	FGFR2	PFS 1.8 vs 3.5 (HR 1.57 [80% CI, 1.12-2.21]); p=0.96
Third line					
mTOR	Everolimus	GRANITE(118)	Everolimus vs placebo	None	5.4 versus 4.3 (HR 0.90 [95% CI, 0.75 to 1.08]); p= 0.0124

Table 3 Selected negative randomised phase II/III studies of molecularly targeted agents in patients with oesophagogastric cancer

1.2 Advanced colorectal cancer as an unmet need

1.2.1 Epidemiology and risk factors

Colorectal cancer (CRC) is the third most diagnosed cancer in the world, and the second most common cause of cancer related death.(1) Incidence rates of CRC appear to be increasing in all global regions, but remain highest in the more socioeconomically developed countries such as Europe, Australia, New Zealand, and North America.(1) The rates of CRC among women in these countries are around 10% lower than men, as is the mortality.(1) Recognised risk factors for CRC development include obesity,(119) consumption of red or processed meat,(120) cigarette smoking,(121) and alcohol drinking.(122) Hereditary bowel cancer syndromes are responsible for around 5-7% of cases,(123) whereas a positive family history, without a known underlying genetic syndrome, is attributed to up to 20% of cases.(124) Protective factors include a diet rich in whole grains, fibre, tree nuts, and dairy products.(125,126) The institution of bowel cancer screening programmes has resulted in a decline in cases in certain populations.(127) In the U.K., bowel cancer screening is offered every 2 years to men and women between the ages of 50 and 74.(128) However, as with OG cancer, there appears to be an increasing number of CRC diagnoses in younger patients under the age of 50, more so in the West, and particularly rectal cancer and left-sided colon cancer.(129,130) The reasons for this rising trend amongst younger patients are unclear but modern lifestyle influences, obesity and diet are all thought to play a role. The United States have responded to this by lowering the age of bowel cancer screening to 45 years.(131)

1.2.2 Clinical, biological, and molecular characteristics

Colorectal cancer (CRC) is frequently entirely asymptomatic in the early stages of the disease. Presenting features often include rectal bleeding, change in bowel habit, anaemia, or abdominal pain. In right-sided tumours, bleeding is often occult, and patients frequently present later, and with more advanced stage

disease. Around 20% of patients with CRC have metastatic disease at the time of diagnosis.(132) The frequent sites of metastases are the liver, most commonly, followed by the peritoneum, lung, and lymph nodes.(133) Locoregional therapy can improve survival rates in select cases of oligo-metastatic disease, otherwise the 5-year survival rate for patients diagnosed with stage IV disease is around 10%.(134)

Up to 90% of CRCs develop from the adenoma-carcinoma pathway, through a sequential accumulation of genetic and epigenetic events.(135) Classically, the pathway is triggered by an APC mutation, which occurs in the germline in cases of familial adenomatous polyposis, or in a sporadic fashion, which is most often the case.(135) APC mutations activate the Wnt-beta-catenin pathway in >90% of cases. APC mutations are followed by an aberration in KRAS, followed by TP53, resulting in an MSS phenotype, which make up 95% of advanced CRCs.(135) On the other hand, the sessile serrated neoplasia pathway, which makes up 10-20% of CRCs, often begins with a BRAF or KRAS mutation, followed by methylation of tumour suppressor genes.(136) This results in the CpG island methylator phenotype (CIMP) which can become a MSI or MSS tumour, depending on which genes have been epigenetically silenced.(135) Approximately, 5% of advanced colorectal cancers will demonstrate microsatellite instability, through loss of germline MMR proteins which gives rise to hypermutation.(137,138) This is often a sporadic event e.g., due to epigenetic hypermethylation of genes such as MLH1, but can be associated with Lynch syndrome.(137)

As with OG cancer, distinct consensus molecular subtypes (CMSs) have been defined for CRCs.(139) CMS1 (MSI immune) tumours were characterised by hypermethylation and a low number of SCNAs. The majority of MSI tumours fell into the CMS1 category, which demonstrated overexpression of proteins linked to DNA damage repair. Mutations in BRAF were frequently mutated in CMS1 and the MAPK pathway, which regulates several cellular processes such as proliferation, differentiation, and apoptosis, was significantly activated.(139) CMS1 tumours were more common in females, right-sided lesions and associated with poor survival after relapse.(139) Additionally, CMS1 tumours

showed upregulation of genes involved in the immune response, and genes linked to an immune infiltrate rich in cytotoxic T cells with evidence upregulated immune checkpoints.(139,140) This underlying biology likely explains why ICIs are so effective when used in patients with dMMR/MSI-H CRC in the clinic and, it is expected that the poor prognosis correlated to this subtype in 2015, would now be much improved since ICIs became standard of care for dMMR/MSI-H tumours. The other CMS tumours had much higher levels of CIN, which was most prevalent in CMS2 (canonical) samples. CMS2 tumours were the most common subtype amongst the samples analysed making up 37%.(139) They were characterised by upregulation of proteins involved in the MYC and WNT pathways, typically left-sided location, and associated with the best prognosis. On the other hand, CMS3 (metabolic) tumours were characterised by metabolic dysregulation, low levels of CIMP, and low numbers of SCNAs, although a higher proportion of KRAS mutations than the other subtypes.(139) Molecularly, CMS3 was like the GS OGA subtype already described. CMS4 (mesenchymal) tumours, were enriched with genes which promote epithelial-to-mesenchymal transition (EMT), stromal invasion and angiogenesis.(139) Of all subtypes, CMS4 was associated with the poorest survival and the more advanced stage of disease.(139)

Further to these subtypes, a greater differentiation and clarification between the biology of left (defined as splenic flexure, descending colon, sigmoid and rectosigmoid) and right tumours (caecum, ascending colon, and hepatic flexure), which includes other host factors, has emerged. The basis for the differences is thought to start with the discrete embryological origins of the right and left colon, which develop from the midgut and hindgut, respectively, under the influence of different genes.(141) Other factors attributed to these differences include the gut microbiome, which is markedly dissimilar in patients who develop right and left CRC. In particular, bacterial biofilms were found in 89% of right sided CRCs compared to only 12% of left sided CRCs, and are thought to be related to the pathogenesis of right sided tumours.(142) Higher levels of bile acid concentrations in right sided tumours may also play a role in the development in right sided tumours.(143) The impact of these differences on the biology of left and right sided lesions has also translated into the clinic. RAS wild-type status,

which does not appear to be unique to one subtype, is a known predictor of sensitivity to anti-EGFR therapy. However, patients with right sided tumours who were treated with first line chemotherapy and cetuximab have shown limited survival benefit, compared to those who did not receive cetuximab.(144,145) Whereas overall survival was significantly improved for patients with left sided RAS wild-type tumours.(144,145)

1.2.3 Immune landscape of CRC

The landscape of the immune cell infiltrate was initially found to be prognostic in CRCs, as tumours with a high number of effector and memory T cells were correlated with more localised disease.(146,147) Tumours with higher T cell densities (CD3+ and CD8+) at the tumour centre and invasive margin, were also less likely to recur.(148) Whereas tumours with low levels of CD8+ and CD3+ and memory T cells (CD45RO+) in these regions, were correlated with distant metastases and poorer overall survival.(148,149) Based on these observations, and the numbers of CD8+ and CD45RO+ T cells at the tumour centre and invasive margin, the Immunoscore was developed and validated as a prognostic biomarker for early-stage CRCs.(148) Patients with high Immunoscores were found to have a 50% lower chance of recurrent disease than those with low immunoscores.(149) Subsequently, tumours with a high level of FOXP3+CD4+ T cells (a type of Treg cell) and MDSCs, were also correlated to poorer survival.(150)

As well as demonstrating high levels of hypermutation and an immune infiltrate rich with effector T cells, the CMS1 tumours also show upregulation of genes involved in IFN-γ signalling, which promote expansion of Th1 cells, formation of tertiary lymphoid structures, and expression of immune permissive chemokines which mobilise T cells toward the tumour, such as CXC9 and CXCL10.(139,151–153) Conversely, the remaining MSS subtypes, with lower mutational loads, were enriched with Tregs and MDSCs, immune inhibitory cytokines and a lack of immune checkpoints compared to MSI-H CRCs, and other tumour types with

higher overall TMB, such as melanoma or NSCLC.(151,154) As mentioned previously, activation of Wnt- beta-catenin signalling, which is almost ubiquitous to MSS CRCs, is also potently associated with immune exclusion.(155) TGFβ expression is another immunosuppressive mechanism, which hampers the activity of T cells and dendritic cells, inherent to MSS CRCs.(139,156) As MSS CRCs have a low mutation rate and are poorly immunogenic,(151,154) these immunosuppressive mechanisms have a greater influence on shaping the TME. Additionally, PD-1 and PD-L1 expression appears to be negatively correlated to tumour stage, with the lowest levels in stage IV disease.(154) This has likely contributed to ineffective targeting of the PD-1/PD-L1 axis with anti-PD-1 and anti-PD-L1 therapy in advanced disease. Furthermore, while the genomic profile between CRC primary and metastatic lesions appears to be preserved for most genes,(157,158) there is a disparity of immune cell infiltration patterns between the primary and metastasis, with a lower proportion of CD8+ T cells in the latter.(74) Conversion from an immunologically 'cold' TME to an immunologically 'hot' TME in CRC, will likely require ways to reduce levels of immune cells which cause immunosuppression like MDSCs and Tregs, increase levels of effector T cells, particularly at sites of metastases, and potentially dampen down Wnt-beta-catenin signalling. Addressing these barriers to immunogenicity by reprogramming the TME may improve chances of response to ICIs.

As with OG cancer, specific gene mutations can also influence the immune milieu in CRC. For example, KRAS mutations appear to drive an immunosuppressive phenotype via reduced IFN-γ signalling, reduced levels of STAT1, which drives transcription of MHC class II molecules, and reduced levels of CXCL10, which favours the Th1 phenotype.(159) These features were displayed across all CMS clusters, but particularly predominant in CMS2, which is the most common subtype of MSS CRC,(139) and may explain why MSS CRC is so resistant to ICI monotherapy. Interestingly, MSS tumours with POLE exonuclease domain mutations, which are associated with an ultra-mutated phenotype and very high neoantigen loads, have abundant effector T cells in the immune infiltrate.(160) Therefore, high neoantigen burdens driven by POLE can result in an immunologically 'hot' TME, independent of MMR status. Patients with POLE mutated CRCs treated with chemotherapy for stage III or IV disease, have also

been reported to have inferior survival compared to POLE wild type equivalent patients.(161) Therefore, an immunotherapy-based approach, possibly just with ICI monotherapy, would likely be a superior choice for these patients. However, POLE mutations are an uncommon phenomenon in CRC, occurring in around 1% of patients.(162) On the other hand, tumours harbouring PIK3CA mutations were also found to have high a higher TMB and, as these occur in 25% of CRCs,(163) may be more relevant to consider targeting with ICIs in future treatment strategies. Interestingly, treatment of RAS wild type mCRC with cetuximab, appears to induce immunogenic cell death and promote cytotoxic T cell infiltration into the TME, as well as upregulating immune checkpoints such as PD-L1 and LAG3.(164,165) Therefore, patients with RAS wild type disease treated with prior cetuximab, may derive benefit from subsequent ICI-based approaches.

1.2.4 Systemic therapy options for advanced colorectal cancer

Current guidelines recommend testing for MMR status and RAS and RAF mutations at baseline to select the most appropriate therapy.(133) Testing HER2 status is also recommended in the second line setting and, where possible, NTRK fusions,(133) however these are extremely rare in advanced CRC.(139) There has been a seismic shift in the management of patients with advanced dMMR/MSI-H colorectal tumours, enriched with neoantigens which beckon to the host immune system, resulting in deep and durable responses to ICIs.(166,167) The profound benefit shown in earlier phase II studies of ICI monotherapy were confirmed with a landmark phase III trial of first line pembrolizumab monotherapy in patients with dMMR/MSI-H metastatic CRC.(168) The overall survival was not significantly different, likely due to crossover within the trial, but the PFS benefit with pembrolizumab over standard chemotherapy was more than 8 months.(168) Pembrolizumab monotherapy is therefore the recommended upfront treatment for dMMR/MSI-H disease. However, most patients with advanced CRC (>95%) have pMMR/MSS tumours and ICIs currently play no role in their management.(138)

In the U.K., patients with RAS mutant mCRC (around 50%) are treated with doublet chemotherapy, containing a fluoropyrimidine (either intravenous 5-FU or capecitabine) and either oxaliplatin (FOLFOX/CAPOX), or irinotecan (FOLFIRI).(169–171) The choice will depend on the most suitable toxicity profile, as both agents are equally as active. In certain cases, a triplet regimen, FOLFOXIRI, may be considered as it has been associated with improved response rates, but the toxicity is high.(172) In view of the aggressive nature and poor survival rates associated with BRAF mutated CRCs, however, triplet chemotherapy is more often considered for these patients.(173) Bevacizumab, a selective VEGF-A inhibitor, has shown some added survival benefit when combined with doublet chemotherapy,(174,175) but it is not funded in the U.K. Patients with RAS wild type tumours (50%), are treated with doublet chemotherapy plus an anti-EGFR inhibitor (either cetuximab or panitumumab) in the first line setting.(176–179) Panitumumab may be preferred in certain centres as the administration time is slightly less than cetuximab. Initial trials of chemotherapy and cetuximab, or panitumumab, were carried out in unselected populations where improved response rates and survival were revealed in the RAS wild type population in later subgroup analyses.(177) One exception is in the COIN trial, which evaluated CAPOX plus cetuximab, where no survival benefit was found, likely due to delays and dose reductions required in view of increased toxicity with the combination.(180) As outlined previously, anti-EGR antibodies are not recommended for right-sided tumours. Therefore, where available, bevacizumab plus a fluoropyrimidine doublet is given for right-sided tumours, irrespective of RAS/RAF status. Whereas, for a RAS wild type left sided tumour, cetuximab plus a fluoropyrimidine doublet, would be preferred.(145,181)

In the second line setting, choice of agents will depend upon which combination was given upfront. If FOLFOX was given first, then FOLFIRI would usually be given next, and vice versa.(182) If available, and not given initially, bevacizumab and anti-EGFR antibodies can be given with chemotherapy in the second line. Ramucirumab, a human monoclonal antibody that binds VEGFR-2, has also demonstrated a survival benefit combined with chemotherapy in the second line,(183) however it is not funded in the U.K. The positive results of the BEACON trial were an important milestone in treatment of BRAF mutant disease. This

phase III trial evaluated encorafenib, a BRAF inhibitor, and cetuximab, with or without binimetinib, a MEK inhibitor, versus chemotherapy in patients with BRAF-V600E mutant disease in the second or third line.(184) Median overall survival for both the triplet and doublet was 9.3 months, compared to just 5.9 months for the patients on standard chemotherapy regimens.(184) Response rates were also improved in the experimental arms and toxicity was better than with chemotherapy.(184) This resulted in the approval of encorafenib and cetuximab for this indication, and set a new standard of care for patients with BRAF-mutant disease. Trials evaluating this combination in the first line setting for these patients are currently in progress. Based on the results of CheckMate-142, nivolumab and ipilimumab, anti-CTLA4 antibody, is good option for patients with dMMR/MSI-H disease in the second line.(185) Although only a single arm study with no comparator, for patients treated with this ICI combination ORR was 55%, 3-month disease control rate was 80%, and median duration of response was not reached.(185)

Beyond second line, TAS-102 (trifluridine/tipiracil) is available in the U.K., however treatment is associated with marginal response rates (< 2%), and bone marrow suppression is a significant toxicity.(186) Globally, other options include anti-EGFR antibodies as monotherapies, however survival compared to best supportive care (BSC) is limited,(187,188) and regorafenib, an oral multi-kinase inhibitor which inhibits VEGFR1-3 and stromal and oncogenic RTKs, which has a similar margin of clinical benefit as trifluridine-tipiracil, but a different toxicity profile.(189) With sequential chemotherapy, with or without anti-EGFR agents and anti-angiogenic agents (not funded in the NHS setting), median survival for patients with mCRC is around 30 months.(179,190) However, given that NHS patients cannot access anti-angiogenic agents, this may be an overestimate in the U.K population. More personalised, efficacious, and better tolerated treatments are warranted.

To date, definitive clinical benefit from ICI monotherapy in advanced pMMR/MSS CRC has not been established. Since the initial results of the pembrolizumab monotherapy study in MSS CRC were published in 2015,(57) attempts to augment ICI efficacy in CRC with rational agents to convert these

immunologically ignorant tumours, to inflamed tumours, have been widely explored. Given that 95% of patients with advanced CRC have pMMR/MSS tumours, and that CRC is the second most deadly cancer in the world, such efforts to identify novel strategies have been much welcomed. However, many of the combinatorial strategies trialled thus far have had low success rates (Table 4). More recent studies such as the phase Ib REGONIVO trial, evaluating regorafenib combined with nivolumab in patients with MSS mCRC and GC, have demonstrated response rates of 33% in the mCRC cohort,(191) and thus restored interest in evaluation of immunotherapy combinations in MSS mCRC patients. Therefore, development of novel, better tolerated treatments, which improve survival for patients with pMMR/MSS mCRC, may include rational combinations with immunotherapy.

Target	Drug	Combination partner	Phase and setting	Result	Ref
Checkpoint inhibition	Tremelimumab	Durvalumab	Randomised phase II (Exhausted all therapies)	OS 6.6 (experimental arm vs 4.1 months with BSC (HR 0.72, p=.0.07)	(192)
	Ipilimumab	Nivolumab	Phase II basket (Exhausted all therapies)	ORR 10% (close due to futility)	(193)
	Favezelimab	Pembrolizumab	Phase I (Exhausted all therapies)	In patients with PD-L1 CPS ≥ 1 tumours ORR 11.1%, OS 12.7months	(194)
Chemotherapy	FOLFOXIRI + bevacizumab	Atezolizumab	Randomised phase II (1st line)	PFS 12.9 vs 11.4 months (pMMR/MSS patients)	(195)
	mFOLFOX6 x6	Durvalumab + tremelimumab x6 followed by maintenance durvalumab	Phase II (First-line, RAS mutated)	-5% patients were MSI-H -ORR 61%, median PFS 8.4 months -High PDL1+ MDSC associated with poor PFS	(196)
	mFOLFOX6 + cetuximab	Avelumab	Phase II (First-line, RAS/RAF wild-type)	-2 patients MSI-H -ORR 79.5% -Early ctDNA decrease associated with response	(197)
VEGFR	Regorafenib	Nivolumab	Phase I (≥ 2 prior lines)	-ORR 33% -PFS 7.9 months -Absence of liver metastases correlated with response	(191)
	Regorafenib	Nivolumab	Phase I/II (≥ 3 prior lines)	-ORR 7.1% (all patients) - ORR 21.7 % (no liver metastases)	(198)
	Lenvatinib	Pembrolizumab	Phase II (third line)	-ORR 22% -OS 7.5 months	(199)
Small molecules	Cobimetinib	Atezolizumab	Phase III (Third line)	-ORR 3% - OS 8.87 months vs 8.51 months (control)	(200)
	Azacitadine	Pembrolizumab	Phase II (Exhausted all therapies)	-ORR 3% - OS 6.2 months	(201)
	Zabadinostat	Nivolumab	Phase I/2 (≥ 2 prior lines)	-OS 7 months - 7.3% still alive at 3 years	(202)
	Entinostat	Pembrolizumab	Phase Ib/2 (≥ 2nd line)	-ORR 6%	(203)

Table 4 Selected trials of different combination approaches in largely pMMR/MSS advanced CRC

Hypothesis

Recently elucidated oncogenic pathways in oesophagogastric and colorectal cancer, encompassing MYC ‘the master regulator’, and the immune modulating effects of Wnt and epigenetic modulation, can be therapeutically exploited for patient benefit

Aims

1. To determine whether BTK inhibition with ibrutinib can lead to clinical benefit in patients with c-MYC and/or HER-2 amplified advanced/ metastatic OG cancer

2. To evaluate whether the combination of a class I HDAC inhibitor and epigenetic modifier, domatinostat, and anti-PD-L1 antibody, avelumab, is safe and tolerable and/or demonstrates any signal of activity in the treatment of pMMR/MSS OGA and CRC

3. To evaluate whether the combination of a Wnt signalling modulator, DKN-01, and anti-PD-L1 antibody, atezolizumab, is safe and tolerable and/or demonstrates any signal of activity in the treatment of pMMR/MSS OGA

Chapter 1 Evaluating use of ibrutinib in c-MYC and HER2 amplified oesophagogastric carcinoma: results of the proof-of-concept iMYC study

1.1 Introduction

1.1.1 MYC dysregulation in oesophagogastric cancer

The MYC family of proto-oncogenes, made up of C-MYC, MYCN, and MYCL, which code for transcription factors c-Myc, N-Myc and L-Myc, respectively, are frequently dysregulated in over 70% of human cancers.(64,204) They are considered ‘master regulators’, controlling the transcription of genes responsible for a range of cellular processes such as cell growth, differentiation, metabolism, DNA damage repair and apoptosis, in both health and disease.(64) MYC is frequently activated and/or overexpressed by a number of mechanisms including chromosomal translocation or genomic amplification, which cause increased MYC mRNA expression, or through activation of upstream regulatory pathways, which can lead to increased or decreased transcription of the MYC oncogene, or by post-translation modifications of the MYC protein, which cause stabilisation of MYC and thus its increased activation.(205,206) MYC-driven tumours are considered ‘oncogene addicted’ tumours, where the tumour becomes dependent on MYC signalling for maintenance and survival.(207) This has been demonstrated through in vivo experiments using mouse models of MYC-driven solid tumours including hepatocellular carcinoma, renal cell carcinoma, lung adenocarcinoma, and pancreatic cancer, where inactivation of MYC signalling has caused tumour regression through different mechanisms, culminating in cell cycle arrest and apoptosis.(208–212) c-MYC (commonly referred to as just MYC) is the paralogue most commonly implicated in both the initiation and maintenance of cancer.(213) While elevated MYC levels have shown to correspond with susceptibility to apoptosis in normal cells,(208,214) in malignancy, MYC

activation can circumvent apoptosis by upregulating other mechanisms to promote cancer cell proliferation, such as facilitating more rapid progression through the cell cycle.(215,216) MYC can also affect cell growth and metabolism by increased generation of ribosomes and proteins, and by upregulating genes involved in angiogenesis.(217,218) Additionally, MYC can control cellular invasion, migration, and metastasis.(219) More recently, MYC has been shown to impact host anti-tumour responses and the TME. For example, MYC-driven tumours can evade immune surveillance by upregulating the expression of immune checkpoints such as PD-L1 (which suppresses the immune response) and CD47 (which suppresses macrophages and T cells), (220–222) and MHC class I.(223–225) MYC overexpression also favours a T cell excluded TME deplete of T, B and NK cells, mainly by upregulation of chemokine CCL9.(226) Therefore, MYC expression may predict response to immunotherapy.

c-MYC amplification has been reported in 25-30% of GCs, (227,228) 23% of OSCCs, and 32% of OACs.(24) In OSCC, c-MYC expression is associated with more advanced stage of disease, presence of lymph node metastasis, poorly differentiated disease, and greater depth of tumour invasion, which are features predictive of poorer survival.(229–231) c-MYC expression appears to be positively correlated with PD-L1 status in OSCC, and associated with worse survival when both c-MYC and PD-L1 positivity co-exist, compared to a c-MYC negative or positive, or PD-L1 negative or positive tumour alone.(232) In vitro experiments have confirmed that c-MYC expression appears to regulate PD-L1 expression in OSCC, and thus, may contribute to immune escape.(232) In OAC and GC, increased MYC expression has also been associated with deeper tumour invasion, older age, and presence of metastases.(218,233) Increased MYC protein expression was observed more frequently in intestinal-type histology, whereas MYC hypomethylation or MYC mutations, were more common in diffuse-type disease.(233) MYC amplified OACs also appear to have higher levels of proangiogenic factors, VEGF-A and VEGF-C, compared to non MYC amplified OAC samples.(218) In breast and ovarian cancer, c-MYC expression is associated with better responses to neoadjuvant chemotherapy.(234,235) Similar therapies i.e. platinum agents, taxanes and fluoropyrimidines, are used in the management of OSCC and OGA, however, studies evaluating molecular features

of GC and OAC and response to neoadjuvant chemotherapy, have produced conflicting results.(236,237) In OAC, MYC overexpression was a predominant feature of non-responders, whereas, in GC, MYC amplification was linked to chemotherapy response. In advanced OG cancer, MYC amplification status does not appear to significantly influence responses to first-line chemotherapy.(238) Given that MYC amplifications occur in a third of OG cancers, are associated with poor prognostic features, and limited responses to conventional chemotherapeutic agents, novel approaches to target MYC expression in OG cancer would be desirable.

1.1.2 Targeting MYC in the clinic

As MYC alterations are so commonplace and pervasive in human cancer, potential strategies to therapeutically target MYC have been highly sought after for years. However, MYC has been historically challenging to target for number of reasons. Firstly, because MYC acts as a transcription factor within the cell nucleus, it is not easily accessed by antibody-based agents which usually work on the cell surface. MYC also has a disordered structure with no clear binding site for small molecules,(239) and its half-life, as with most transcription factors, is very short. Finally, the mechanisms by which MYC is activated in cancer, in additional to the broad range of cellular processes that it controls in normal tissues, present further obstacles to successful drug development, particularly due to potentially serious side effects in normal tissues.

To date, the most promising strategy to directly inhibit MYC has been with an agent called Omomyc, a c-MYC dominant negative miniprotein, which is able to dimerize with c-Myc and its partner Max (the c-Myc-Max dimer binds to a specific DNA sequence called the Enhancer-box (E-box), which then activates gene transcription).(240) The first in vitro study, over 20 years ago, showed that by interfering with Myc-Max dimerization, Omomyc inhibited transcriptional activity of Myc by 50%, and caused a tenfold reduction in proliferation of fibroblast cell colonies.(240) Several successive pre-clinical studies have since shown that

Omomyc caused tumour regression and apoptosis in a number of cancer mouse models such as pancreatic cancer, lung cancer, gliomas and triple negative breast cancer,(212,241–245) with mild, reversible effects on normal proliferating tissues.(242) Following on from these encouraging results, the first phase I/II clinical trial investigating the safety and tolerability of 5 dose levels of OMO-103 (Omomyc), and its anti-tumour activity, in patients with solid tumours opened to recruitment in 2021 (NCT04808362).

While the bench to bedside journey of Omomyc has been encouraging, pharmacological endeavours to target long-pursued MYC have also focussed their attention on reducing MYC expression via indirect routes at all levels of its regulation. These strategies include, decreasing MYC gene transcription or signal transduction from MYC to RNA polymerase, preventing MYC mRNA translation, targeting MYC post-translational modifiers/MYC stability, and using the concept of 'synthetic lethality' to quell MYC overexpression. Figure 3 summarises some of the strategies to target MYC currently under evaluation in the laboratory and/or the clinic. Of these, BET proteins, such as BRD4, which activate MYC transcription by modifying chromatin and enabling the recruitment of transcription factors,(246) have probably been the most intensely investigated to date. Preclinically, BET inhibitors have demonstrated potent suppression of MYC expression and anti-cancer effects in breast, pancreatic, leukaemia, and lymphoma cancer models.(247,248) There are now several compounds being tested in the clinic, however, some previous early phase trials were stopped prematurely due to toxicity concerns such as grade 3 headache, vomiting, and back pain,(249) possibly due to off target effects and suppression of genes other than MYC.

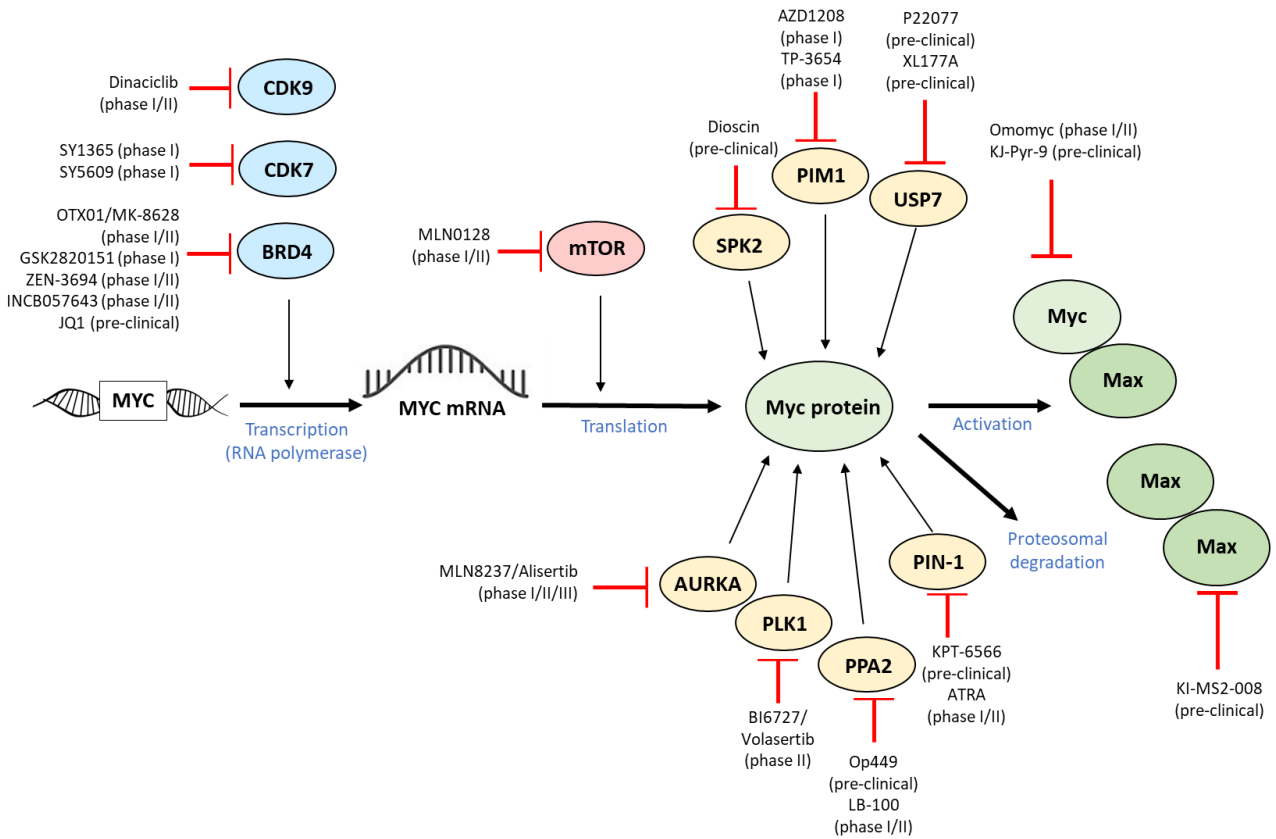


Figure 3 Potential strategies to target MYC and the phase of pre-clinical study or clinical trial that is currently being evaluated (updated from Chen et al)(250). These include proteins regulating MYC expression/transcription (blue), MYC mRNA translation (red), MYC function and stability at the posttranslational level (yellow), targeting Myc-Max or Max-Max interaction (green). BRD4 bromodomain-containing 4, CDK7 cyclin-dependent kinase 7, CDK9 cyclin-dependent kinase 9, mTOR mammalian target of rapamycin, AURKA aurora kinase A, PLK1 polo-like kinases 1, PPA2 inorganic pyrophosphase 2, PIN-1 peptidyl-prolyl cis/trans isomerase, SPK2 sphingosine kinase 2, PIM1 serine/threonine-protein kinase pim-1, USP7 ubiquitin specific protease 7

1.1.3 Synthetic lethality as an alternative MYC targeting strategy in oesophagogastric cancer: background to the development of the iMYC trial

Synthetic lethality, a term first coined in the 1940s, occurs when inhibition or mutation of two genes simultaneously causes cell death, but inhibition of either gene alone is still viable for the cell.(251) This concept has since been applied to cancer therapeutics and is particularly pertinent to tumour suppressor genes (TSGs), which result in a loss of function within the cell, and therefore do not otherwise have a pharmacological target to inhibit, but also to oncogene-addicted tumours, which can be challenging to target due to their multiple downstream effects.(251) Synthetic lethality can be grouped into 3 categories – oncogene addicted, non-oncogene based, and drug-based.(252) An example of oncogene addicted synthetic lethality would be to disrupt a gene associated with maintenance and progression of cancer cells that is dependent upon/addicted to a mutated oncogene, e.g., KRAS, which will consequently prove lethal to the KRAS-driven cells.(252) Non-oncogene based synthetic lethality refers to the loss or mutation of a gene which consequently makes tumours dependent upon an interrelated pathway, and thus, more sensitive to inhibition of this pathway.(252) The most well-known example of a synthetic lethal pair is a BRCA gene mutation and poly (ADP-ribose) polymerase (PARP) inhibitor, which result in cell death due to failure of DNA damage repair mechanisms.(253,254) Following on from the results in these pivotal experiments, PARP inhibitors are now widely used in the clinic to treat BRCA-mutated ovarian, breast, and pancreatic cancer. Finally, drug-based synthetic lethality refers to the requirement of more than one drug to overcome intrinsic resistance mechanisms due to the role of compensatory pathways, which can maintain tumour growth in the case of monotherapy.(252) An example is simultaneous inhibition of the EGFR and BRAF pathway in cancers with BRAF V600E mutations, where a BRAF inhibitor alone is insufficient.(255)

Synthetic lethality can be harnessed in MYC overexpression because MYC-driven tumours are susceptible to apoptosis.(256) Thus, targeting a gene that is synthetically lethal with MYC overexpression, should only kill cancer cells and

spare normal cells. MYC overexpression was first found to be synthetically lethal with inhibition of cyclin-dependent kinase 1 (CDK1), which regulates progression through the cell cycle,(257) in MYC-driven models of liver cancer and lymphoma.(258) Additionally, CDK1 inhibition resulted in apoptosis and tumour regression in models of MYC-driven triple-negative breast cancer.(259) However, in the clinic, there have been concerns regarding CDK1 inhibition and toxicities in patients with chronic lymphocytic leukaemia (CLL), such as tumour lysis syndrome.(260) Other synthetic lethal partners to MYC overexpression include inhibitors of CHK1, which coordinates cell cycle checkpoint control. Application of CHK1 inhibitors caused cell death in MYC-amplified neuroblastoma and lymphoma cell models.(261,262) Additionally, inhibitors of GLS, an enzyme involved in glutamine metabolism that is highly expressed in cancer cells, resulted in apoptosis in MYC-driven neuroblastoma models.(263)

There are number of different methods to identify new synthetic lethal interactions, which facilitate the potential for novel targeting of genetic aberrations in cancer where treatment options are scarce. These include direct screenings using yeast, drugs, RNA interference (RNAi) and CRISPR technology; genomic based screens developed from databases of genetic interactions in tumours, and cell line and bioinformatics screens.(264) The foundation for the iMYC trial came from research led by our group at the Institute of Cancer Research, who demonstrated that MYC-amplified oesophageal cancer models were profoundly addicted to BTK.(265) They put together genomic mutational profiles of 17 oesophageal tumour cell lines (9 SCC and 8 EAC), from array comparative genomic hybridisation and exome sequencing data. They then used his data to classify the cells lines in accordance with the presence or absence of cancer driver alterations. Based on RNAi screening data (266) for the same cell lines, they found RNAi reagents that could specifically target these genetic drivers. Finally, they integrated the RNAi screening data and drug sensitivity profiles of the cell lines with the knowledge of their driver alterations, and performed drug sensitivity screens with 80 molecular inhibitors, assessing cell viability after 5 days.(265)

In accordance with the previously characterised molecular profiles of oesophageal cancer samples from patients, the mutational landscape of the 17 oesophageal cell lines was comparable. For example, TP53 mutations were detected across all cell lines,(53,267) HER2 was amplified in around one third (consistent with the prevalence in primary OAC), and 9 out of 17 oesophageal tumour cell lines had deletions in CDKN2A (CDKN2A deletions have been described in 76% of clinical OSCC and OAC cases).(24) MYC amplifications were observed in 5 of these cell lines (both OSCC and OAC), again consistent with expected frequencies in both OSCC and OAC in clinical cases, 32% and 23%, respectively.(24) They treated each cell line to 80 different drugs, which were either established anti-cancer drugs or drugs in a mature phase of development, at four different concentrations.(265) They found that, for example, almost all cell lines were susceptible to standard chemotherapeutic agents used to treat the clinical disease e.g., taxanes. When they integrated the small molecule inhibitor screen with the genomic profiles of the cell lines to determine the cancer driver alterations, they found that, for example, cell lines with HER2 or EGFR amplifications were susceptible to lapatinib, the dual tyrosine kinase inhibitor which interrupts the HER2 and EGFR pathways.(265)

When the genomic profiles of the oesophageal tumour cells were then integrated with RNAi screening data, they found that, for example, an RNAi reagent designed to target HER2, favourably selected the HER2 amplified cell lines.(265) This suggested that HER2 amplified tumour models were addicted to HER2. Additionally, the HER amplified tumour cell lines were also found to have genetic dependencies on genes in the mitogen-activated protein kinase signalling pathway, such as MAPK2K2 and MAP2K3, downstream of HER2.(265) The MYC oesophageal tumour cell lines were found to have dependencies on several genes including, CLK1, BTK, ALK and PRKCA.(265) The greatest synthetic lethal interaction was the BTK/MYC association which was recapitulated using several different BTK-targeting siRNAs.(265) This synthetic lethal interaction also resulted in reduced cell viability and reduced BTKC mRNA expression, when a specific BTK-C siRNA was used, suggesting that BTKC was the isoform expressed in these oesophageal cancer models, which the group also confirmed.(265) BTK is a cytoplasmic protein tyrosine kinase typically involved in

B cell receptor signalling, however BTK proteins do also exist in solid tumours such as gastric,(268) and colon cancers.(269) Next, in place of the BTK targeting siRNA, the clinical BTK inhibitor, ibrutinib, was applied to the oesophageal cell lines at concentrations 0.1-10μM.(265) Ibrutinib is a specific, irreversible, and potent inhibitor of BTK, and approved for treatment of various haematological malignancies. In addition, ibrutinib has been shown to target HER2 in HER2 positive breast cancer cells at nanomolar concentrations, and hamper downstream AKT and MAPK signalling.(270) The group found that ibrutinib reduced levels of MYC protein and induced G1 cell cycle arrest in both the MYC-amplified and MYC and HER2 co-amplified oesophageal tumour cell lines.(265) Notably, increased levels of markers of apoptosis, cleaved PARP and annexin V, were only observed in the MYC and HER2 co-amplified oesophageal tumour cell lines.(265) BTK is known to signal via the canonical RAS-RAF-MEK-ERK pathway, and ERK is a known mediator of MYC phosphorylation.(269) Finally, the group was able to show that the mechanism of action of ibrutinib in the MYC amplified and MYC and HER2 co-amplified oesophageal cancer cell lines, was via reduced phosphorylation of BTK, ERK and MYC phosphorylation (required to stabilise MYC), which ultimately led to reduced MYC protein levels.(265)

These preclinical observations led to the development of the proof-of-concept iMYC study, to evaluate the efficacy of ibrutinib in patients with MYC and/or HER2 amplified advanced OG cancer. This chapter outlines the analysis and results of the iMYC study. I became the Trial Physician for the iMYC study in August 2019, having been handed over the trial from Michael Davidson, Clinical Fellow, who set-up the trial with the Chief Investigator (CI), Ian Chau. Prior to this, I was involved in patient recruitment and clinical review of patients on study in weekly NHS follow-up clinics, and research clinics, respectively. During my time as the Trial Physician and as a member of the Trial Management Group (TMG), I was responsible for the day to day running of the trial e.g., assessing safety events as delegate of the CI, preparing TMG reports with the study statistician, Maria Aresu, and responding to queries from site. I cleaned all patient data in preparation for the interim analysis and, with Maria, co-authored the statistical analysis plan for the final analysis. Having reviewed the results, I requested re-analysis or addition of information to generate the final data set. Having analysed and interpreted the

data, I also prepared and/or revised the figures and tables of results and drafted the full manuscript as first author, which was published in *Current Oncology* in March 2022. Janssen provided funding, educational support and supply of ibrutinib for iMYC, but they had no role in the design or management of the study.

1.2 Materials and methods

1.2.1 Patient eligibility

The iMYC trial recruited adult patients with advanced, metastatic, or locally advanced inoperable OG cancer (both squamous cell carcinoma and adenocarcinoma histological subtypes). Patients with disease progression after at least one prior line of chemotherapy for advanced disease were eligible. Patients who had disease progression at any point during neoadjuvant/adjuvant chemotherapy or definitive chemoradiotherapy, or within 6 months after the last dose of neoadjuvant/adjuvant chemotherapy or definitive chemoradiotherapy, were also permitted to be enrolled. As it was a second or later line study and HER2 targeted treatments are only reimbursed in the first line setting in the U.K., it was expected that patients with HER2 positive tumours would have already received a HER2 targeted agent. If they had not, then, as there was no standard HER2 targeted agent available in the second line, patients who had had chemotherapy, with or without a HER2 targeted agent, were permitted entry into the trial. Patients were required to have at least one measurable target lesion as per Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1,(271) and a World Health Organisation (WHO) performance status 0-2. All patients were required to provide archival tumour tissue to determine c-MYC or HER2 gene amplification.

In the case that an archival tumour sample was not available, consent to a fresh biopsy to provide tissue for c-MYC and or HER2 analysis was mandatory. Patients were required to have tumours with c-MYC or HER2 gene amplification as defined in section 1.2.3. Exclusion criteria included clinically significant cardiovascular disease or known brain metastases. Use of strong P450 (CYP) 3A4 inhibitors also precluded study entry. As Ibrutinib is primarily metabolised by cytochrome P450 enzyme 3A4/5, concurrent use of ibrutinib with CYP 3A4 could increase overall exposure to ibrutinib. Exclusion criteria were updated during recruitment to exclude any actively bleeding tumour, any prior or current therapeutic anticoagulant treatment, or any oesophageal stent in situ.

1.2.2 Clinical trial design

The iMYC trial was an open-label, non-randomised, single arm biomarker-selected phase II trial, in patients with previously treated advanced c-MYC/HER2 amplified OG cancer. Written informed consent was obtained from all patients. The study was carried out in accordance with the Declaration of Helsinki and approved by local institutional review boards. c-MYC testing was performed centrally by Fluorescence in situ Hybridisation (FISH), and a c-MYC ratio of ≥ 2.5 was required for study entry. HER2 testing was only performed if not available from previous local testing results. A tumour biopsy (either an archival diagnostic formalin-fixed paraffin-embedded (FFPE) sample or, if not available, a fresh sample), was provided for FISH testing. C-MYC/HER2 testing was performed for patients treated with any line of therapy, who the investigator considered might have become eligible on disease progression during the recruitment period (Stage 1: pre-screening). Eligible patients, as per their c-MYC or HER2 status, later provided consent to the main study (Stage 2: main study). Treatment consisted of 560mg ibrutinib (four 140mg capsules) orally once daily on a 28-day cycle, and patients could continue until disease progression, unacceptable toxicity, or study withdrawal. The 560mg ibrutinib dosage was chosen in line with other studies using ibrutinib in solid tumours at the time.(272,273) Patients could receive up to two dose reductions for adverse events (AEs).

1.2.3 Definition of c-MYC or HER2 gene amplification

FISH is a sensitive and specific technique to identify c-MYC and/or HER2 amplification in tumour specimens.(274,275) A dual probe FISH assay was specifically designed and validated to determine tumour MYC amplification in OG cancer in house. The most common MYC aberrations are translocations in haematological cancers, and, at the time, there was only one probe from Vysis/Abbott Molecular available for this purpose (https://www.molecular.abbott/int/en/chromosome/8). In addition to the MYC probe, mapping to chromosome 8q24, covering the entire coding region of the

MYC gene from exons 1-3, the centromeric probe (CEP8), also for chromosome 8, was used as a control. The testing was performed at the Clinical Cytogenetics lab at The Royal Marsden Hospital, which is a diagnostic lab. Testing was first performed on oesophageal cancer cell lines whose MYC amplification status was known. Once the protocol was optimised, patient samples were then used for further testing and refining of scoring criteria for positive and negative samples, which included optimisation of probe concentration, incubation time, and temperature. The dual probe FISH assay was performed on FFPE tissue sections using a standard protocol and reported in a standardised manner. The range and modal ratio of CEP8 and MYC signals were recorded. This was used to distinguish between increased copies of chromosome 8 (polysomy), and true MYC amplification. A tumour was considered as having c-MYC amplification based on c-MYC: CEP8 FISH ratio ≥ 2.5, in line with the established HER2 positive threshold by FISH testing in breast cancer.(276) If MYC amplification was detected, the proportion of cells displaying the amplified signal was recorded. Patients whose tumours demonstrated c-MYC amplification and a c-MYC ratio of ≥ 2.5 were considered eligible. Figure 4 displays examples of MYC pre-screening results by FISH. If local HER2 results were not available, HER2 testing was also performed centrally. Tumours were considered HER2 positive in accordance with established guidelines.(277)

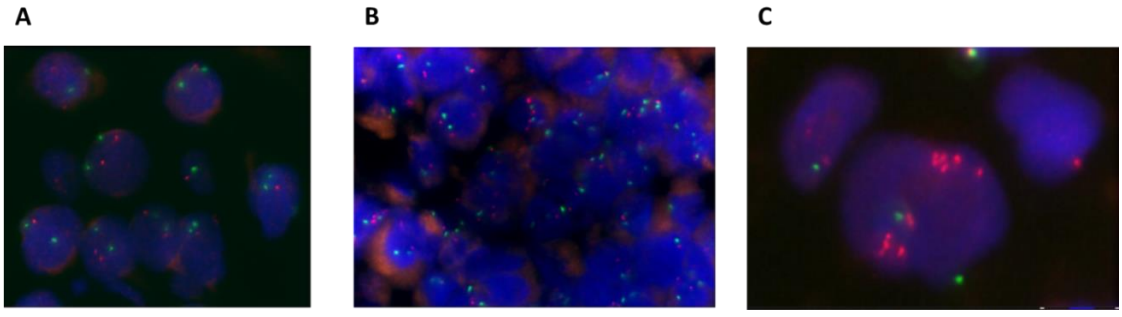


Figure 4 Examples of MYC testing results on oesophagogastric tumour samples detected by FISH for the iMYC trial (red = MYC; green = CEP8). (A) Non- MYC-amplified (normal diploid pattern) results (2 MYC and 2 CEP8 signals per cell). (B) Polysomy of chromosome 8 (additional signals of MYC and CEP8 without MYC amplification). (C) MYC-amplified result (increased ratio of MYC to CEP8 signals). Images for figure kindly provided by Lauren Aronson.

The MYC amplification status of 162 patient samples from the iMYC trial has previously been reported.(238) Out of 135 of the 162 samples which were successfully analysed, polysomy without MYC amplification was most frequently observed in 80 (59%) patient samples (Figure 4B). A normal diploid pattern was detected in 24 (18%) of samples (Figure 4A). MYC amplification without polysomy was observed in 16 cases (12%) (Figure 4C), and MYC amplification with polysomy was seen in 15 cases (11%). No significant correlation was found between MYC amplification status and histological subtype, nor was there an association between HER2 positivity and MYC amplification status.(238)

1.2.4 Study assessments and toxicity

Imaging was performed during screening (baseline), at week 8 after starting treatment and every 8 weeks thereafter until disease progression. 18F-fludeoxyglucose positron emission tomography (FDG-PET) scans were performed prior to starting ibrutinib (baseline), at day 14, and at 8 weeks during treatment. Up to 3 target lesions: >1cm, the largest or most FDG avid lesions, were recorded for data analysis. Optional tumour biopsies were planned on day 10-14, week 8-9 (in responders only), and on progression. A blood sample to assess plasma c-MYC copy number variation (by digital droplet (dd) polymerase chain reaction (PCR) was taken at pre-screening, screening, cycle 1 day 15, cycle 3 day 1, and every 8 weeks thereafter until ibrutinib discontinuation. Health-related quality of life (HRQoL) assessments were completed prior to each cycle before any study-related assessments were performed at the study visit. Toxicity was assessed according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Safety data were summarised according to grade and time point.

1.2.5 Outcomes

The primary endpoint was overall response rate (ORR), defined as complete response (CR) and partial response (PR), assessed according to RECIST 1.1, at 8 weeks. Secondary endpoints were disease control rate (DC), defined as CR, PR, or SD at 8 weeks, PFS (defined as time from start of study treatment to progression or death from any cause), and OS. Additional secondary endpoints included safety and tolerability of ibrutinib treatment in advanced OG cancer, and patient reported outcomes as assessed by EORTC QLQ-C30 and STO22. Assessment of the pharmacodynamic activity of ibrutinib on FDG PET-CT was also a secondary objective. PET-CT is an established non-invasive, functional imaging modality to assess the effects of a drug on a tumour, and, thus, enhance the understanding of the mechanism of action of a novel therapy.(278) For example, where conventional criteria such as determining tumour shrinkage by RECIST 1.1 may not be suitable, PET-CT may still demonstrate drug activity at the site of the tumour, in the absence of a radiological response. This can provide valuable information as to the potential therapeutic benefit of a novel therapy in development, particularly where inhibition of cellular proliferation is expected rather than tumour shrinkage.(279) In OG cancer, early metabolic responses on PET have also been shown to correspond to objective responses to neoadjuvant chemotherapy and improved overall survival.(280) Thus, FDG response data can be a helpful adjunct in early phase trials, particularly to identify potential non-responders who may benefit from an alternative treatment, if other options were available.

1.2.6 Statistical plan

The planned sample size was between 9-17 evaluable patients. To assess the primary endpoint assuming that ibrutinib is active with a true underlying response rate of > 20%, the chance of seeing no responses in a cohort of 17 patients would be low (approximately 3%, calculated on binomial probabilities). A Simon two stage design was planned to be incorporated for primary endpoint analysis as

follows: if 1 or more patients in the first 9 demonstrate a response then the study may expand to 17 patients. If no patient in the first 9 demonstrates a response, then study closure would be considered. If 3 or more patients out of 17 demonstrate a response, then further research would be indicated. This gives 80% power with an alpha of 5%, to detect a 25% response rate while ruling out a 5% activity rate. Within the first projected recruitment of 9 patients, at least 4 were to demonstrate c-MYC amplification. The remaining 5 would show either c-MYC or HER2 amplification, or co-amplification of both.

However due to slow recruitment, the Covid-19 pandemic, and no further pre-screening capacity in the cytogenetics lab, the TMG and Independent Data Monitoring Committee (IDMC) made the decision to close the trial in June 2020. Prior to study closure, I checked through all patients who were pre-screened during their previous line of treatment, and potentially eligible, to ensure that there was no patient who could imminently be recruited onto the main study. I found that no patient who had a MYC/HER2 amplified tumour identified during pre-screening, could be considered for the main study as they were either deceased, receiving other treatment, or had commenced anticoagulation due to thrombosis which had become an exclusion criterion. The final analysis was therefore performed after 8 patients had received treatment.

1.3 Results

1.3.1 Patient characteristics

Between July 2016 and December 2019, 190 patients with advanced OG cancer were screened by FISH for the presence of c-MYC amplification. Pre-screening identified c-MYC amplification in 43 patients (23%), and 36 (19%) of pre-screened patients had HER2 positive tumours. Of these, 11 patients were registered on the main study and 8 patients received treatment with ibrutinib. One patient died before having the first response assessment and, therefore, was not evaluable for the primary endpoint (Figure 5). Patients' characteristics are provided in Table 5. The median age was 63 years (range 58-69 years), and most patients were male (82%). Most patients' tumours were adenocarcinoma (73%), rather than squamous carcinoma (27%), and half were HER2 positive (55%). Of the 11 registered patients, 5 were pre-screened during their first-line treatment, and 3 were pre-screened shortly after completion of their first line. Eight patients had liver metastases, 3 patients had lymph node metastases, 2 patients had lung metastases, and 1 patient had bone metastases (Table 6). Eight of the 11 registered patients had tumours which were c-MYC amplified, 6 were HER2 positive, and 3 were c-MYC and HER2 co-amplified**.**

Of the 8 patients who received study treatment, no dose reductions were required. However, 2 patients had a dose interruption at cycle 4. For one patient this was due to toxicity and the other was not treatment related.

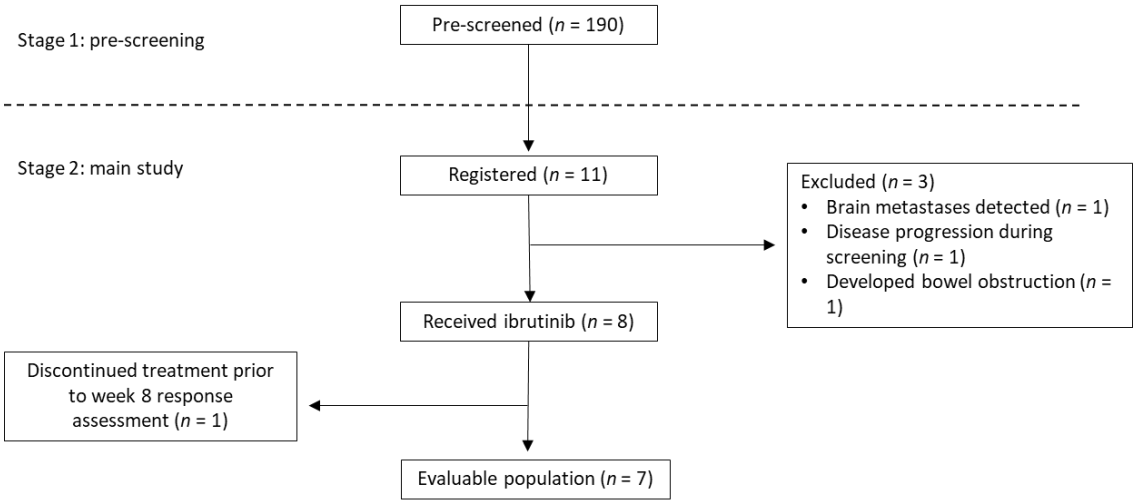


Figure 5 Flow diagram for the iMYC trial

	Pre-screened (n=190)	Registered (n=11)	Patients starting Ibrutinib (n=8)
	N(%)	N(%)	N(%)
Gender			
Female	40(21)	2(18)	1(13)
Male	150(79)	9(82)	7(87)
Histology			
Adenocarcinoma	155(82)	8(73)	5(62)
Squamous carcinoma	33(17)	3(27)	3(38)
Mixed	2(1)	0(0)	0(0)
HER2 status			
Negative	154(81)	5(45)	3(38)
Positive	36(19)	6(55)	5(62)
C-myc status			
Amplified	43(23)	8(73)	6(75)
Not amplified	120(63)	2(18)	1(12)
Failed Testing	25(13)	1(9)	1(12)
Other		-	-
No tumour	1(1)	-	-
Not tested	1(1)	-	-
Coamplified	8(2)	3(27)	3(38)
Tumour location			
GOJ	60(31)	4(36)	2(25)
Gastric	40(21)	1(9)	1(13)
Oesophagus	90(48)	6(55)	5(63)
Disease status at time of pre-screening			
1st line on treatment	85(45)	5(45)	4(50)
1st line completed treatment	40(21)	3(27)	2(25)
2nd line on treatment	27(14)	1(9)	1(12)
2nd line completed treatment	21(11)	1(9)	1(12)
3rd line on treatment	8(4)	0(0)	0(0)
3rd line completed treatment	9(5)	1(9)	0(0)

Table 5 Baseline characteristics

	Registered (n=11)
	N
Metastatic	10
Locally advanced inoperable	1
Site of metastatic disease (n=10)	
Brain/CNS	0
Other CNS	0
Lung	2
Liver	8
Lymph nodes	3
Adrenal	0
Peritoneal	0
Bone	1
Sigmoid	1

Table 6 Sites of metastatic disease patients registered to the main study

1.3.2 Therapeutic response

After 8 weeks of treatment, the best ORR rate (CR/PR) was 0% in the evaluable population. Four patients had progressive disease, and 3 patients had stable disease by RECIST 1.1 criteria (Table 7). After 16 weeks of treatment, a further patient came off study due to progressive disease, and by the third response assessment, after 6 cycles of treatment, only one patient remained on study. This patient with ongoing stable disease was one of the 3 evaluable patients whose tumour was co-amplified for c-MYC and HER2. She was a 70-year-old female patient who had poorly differentiated OGJ adenocarcinoma with metastatic disease from the outset, by virtue of para-aortic lymph node involvement. Her tumour was pMMR and HER2 3+ positive, as tested on tissue from her diagnostic biopsy. Prior to entry into iMYC, she had progressed through platinum/fluoropyrimidine doublet plus trastuzumab and second line paclitaxel, however, these progressions were always in the primary tumour, and she did not develop any new sites of metastatic disease. She did not have many disease-related symptoms at trial entry, so it was difficult to assess initial clinical response. However, after the first 2 cycles of ibrutinib, her Ca 19-9 dropped to almost half of pre-treatment levels, consistent with an encouraging biochemical response. In addition to her tumour being co-amplified for MYC and HER2, it was also strongly MYC positive – CEP8: MYC ratio 1:10 (Table 8). In total, she completed 9 cycles of treatment, and achieved disease control for 32 weeks. While this patient did not have a confirmed response by RECIST 1.1 criteria (only 20% total reduction of target lesion sum from baseline as best response), comparison of FDG-PET scans in this patient between baseline and day 14, showed a marked partial metabolic response in the primary tumour according to PET/CT sub-study EORTC1999 Response Criteria, which was maintained for 8 weeks (Figure 6).(166) Of the other 2 patients with a CEP8:MYC ratio 1:10, the best FDG response by PET-CT criteria was stable metabolic disease (SMD) (Table 8).

8 Weeks (Best Response)				16 Weeks			24 Weeks			32 Weeks	
RECIST Response	**N**	**%**		**N**	**%**		**N**	**%**		**N**	**%**
CR	0	0		0	0		0	0		0	0
PR	0	0		0	0		0	0		0	0
SD	3	43%		2	29%		1	14%		0	0
PD	4	57%		5	71%		6	86%		7	100%

Table 7 Best overall response rate in the evaluable population n=7 at 8 weeks and ORR at 16,24, and 32 weeks

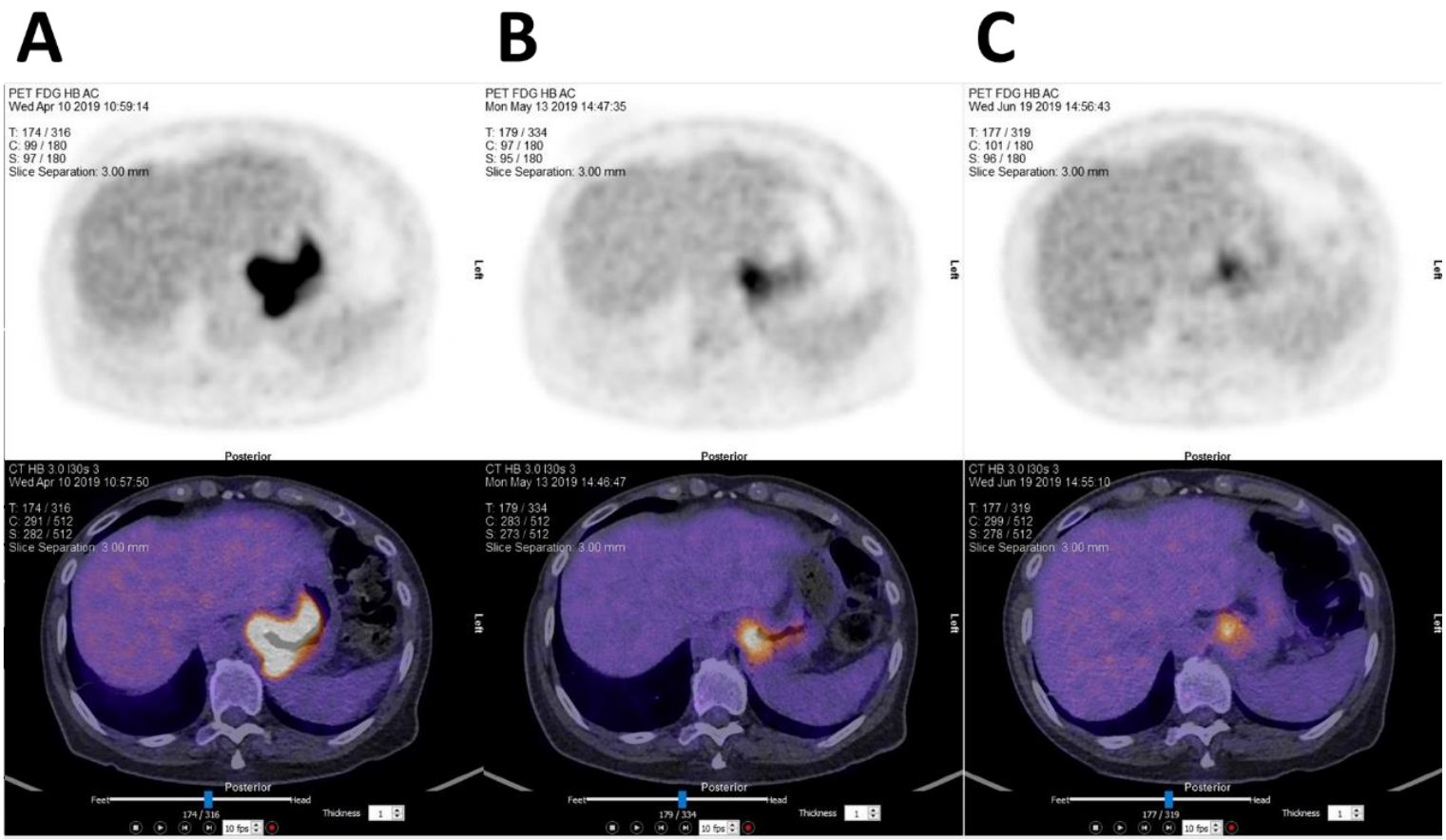


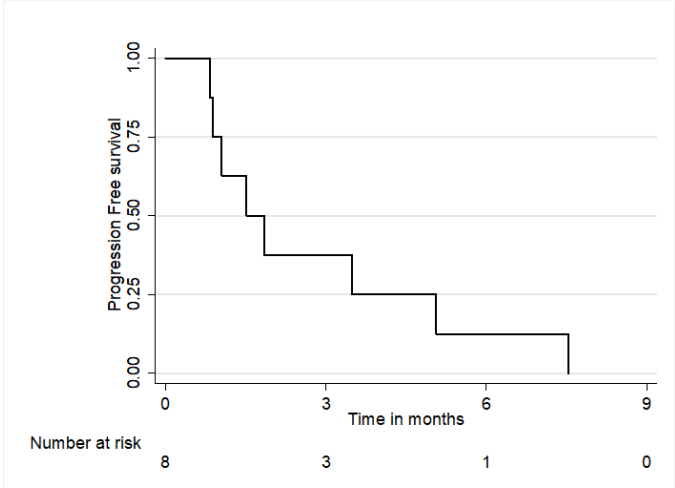
Figure 6 Sequential PET-CT scan images from a study patient showing reducing FDG-avidity (SUVmax) in the primary OGJ tumour during ibrutinib treatment. (A) shows the primary tumour in the oesophagogastric junction/cardia at baseline (SUVmax was 14.77), (B) at day 14 (SUVmax 7.88) and (C) at week 8 (SUVmax 6.47). SUVmax reduced by 56% between baseline and week 8 consistent with a partial metabolic response.

Patient	Age (yrs)	Sex	Adeno- (A)/ Squamous cell carcinoma (S)	HER2	% cells with amplification signals	Modal CEP8: MYC ratio	No of cycles	Best response by RECIST 1.1	Best FDG response
1	84	M	A	neg	87	1:3	2	PD	PMD
2	69	M	A	3+	(failed)	(failed)	2	PD	SMD
3	62	M	A	neg	71	1:10	4	SD	SMD
4	70	F	A	3+	47	1:10	9	SD	PMR
5	60	M	S	neg	30	1:2.5	5	SD	SMD
6	60	M	A	3+	Not amplified	Not amplified	1	-	PMD
7	52	M	S	3+	10	1:2.5	1	PD	PMD
8	58	M	A	3+	77	1:10	1	PD	SMD

Table 8 MYC amplification testing results for all treated patients and corresponding best response by RECIST 1.1 and best FDG response by EORTC recommendations

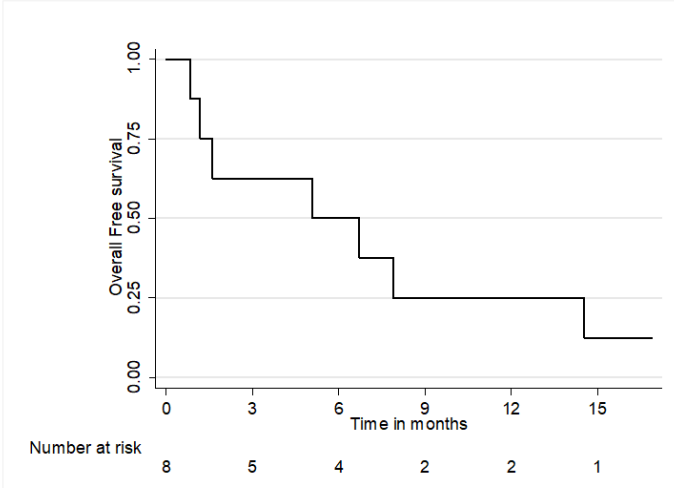
All patients were off treatment at the time of analysis, and all but one patient was deceased. The median number of cycles received was 2 (IQR 1-4). Six patients stopped ibrutinib due to disease progression, 1 patient stopped due to toxicity, and 1 patient died while on treatment. Median PFS was 1.5 months (95% CI; 0.8 - 5.1) and the median OS was 5.1 months (95% CI; 0.8 – 14.5) (Figure 7).

A



Number of observations	Median PFS & IQR (months)	95%CI for the median
8	1.5[0.9 – 3.5]	0.8 – 5.1
Time point(months)	**Proportion Progression Free (%)**	**95% CI**
3	37.5	8.7 – 67.4
6	12.5	0.6 – 42.3
9	-	-

B



Number of observations	Median OS & IQR (months)	95%CI for the median
8	5.1[1.2 – 7.9]	0.8 – 14.5
Time point(months)	**Proportion still alive (%)**	**95% CI**
3	63.5	22.9 – 86.1
6	50.0	15.2 – 77.5
9	25.0	3.7 – 55.8
12	25.0	3.7 – 55.8
15	12.5	0.66 – 42.3

Figure 7 (A) Progression free survival and (B) overall survival in the treated population

1.3.3 Treatment tolerance and toxicity

The most common adverse events were anaemia, constipation, diarrhoea, fever, vomiting, and fatigue, in line with toxicities known to be associated with ibrutinib (Table 9). Grade ≥ 3 AES included fever, infection, neutropenia, and vomiting. Eleven serious adverse events were reported in patients who received ibrutinib during the study including gastrointestinal haemorrhage (n=3), which resulted in death in two cases and were reported as SUSARs. All 3 of these patients had their primary tumours in situ and 2 were on therapeutic anticoagulation prior to starting ibrutinib. Of these, the patient who died had a pre-existing oesophageal stent in situ. The third patient had an oesophageal stent inserted after an episode of haematemesis during cycle 5 of ibrutinib. The patient died shortly after receiving treatment for a chest infection with evidence of recent upper gastrointestinal bleeding. The last dose of ibrutinib had been 5 weeks previously.

Recruitment to the main study was paused from January 2018, until the IDMC could convene to review this emergent safety concern. The study reopened in May 2018 after a substantial amendment was approved which excluded patients with prior or current anticoagulation treatment and recommended that ibrutinib should be discontinued for a minimum of 7 days if an oesophageal stenting procedure was required while on study. Patients with their primary tumour in-situ who had previously undergone an oesophageal stenting procedure, were also excluded going forward.

Toxicity	Grade				
	1	2	3	4	5
	N	N	N	N	N
Anaemia	1	5	1		
Arrhythmia	1				
Constipation	2	1			
Diarrhoea	3				
Dry skin	2				
Fatigue	5	1			
Fever	2		1		
Gastrointestinal haemorrhage			1		2
Hypotension			1		
Infection			1		
Mucositis	1				
Nausea	1	1			
Neutropenia			1		
Pruritis	1				
Skin rash/desquamation	1				
Thrombocytopenia	1				
Vomiting	1	1	1		

Table 9 Frequency of maximum grade of each toxicity in the treated population (n=8)

1.4 Discussion

To our knowledge, this is the first clinical trial to assess the role of ibrutinib monotherapy in patients with c-MYC and/or HER2 amplified OG cancer. Following pre-screening of tissue from 190 OG cancer patients, we detected a c-MYC amplification rate of 25%, in line with previously reported frequencies.(24,227,228) The number of pre-screened patients with HER2 positive tumours was also consistent with the expected rate.(282,283) The iMYC trial was developed based on pre-clinical observations which identified a profound synthetic lethal interaction with BTK inhibition and c-MYC amplification in OG cancer models.(265) Subsequent application of ibrutinib, a potent inhibitor of BTK which also inhibits HER2, resulted in cell cycle arrest and apoptosis and reduced ERK-mediated MYC phosphorylation, in MYC-amplified, and MYC and HER2 co-amplified, oesophageal tumour cell lines. However, among the 8 patients with c-MYC and/or HER2 amplified advanced OG cancer treated with ibrutinib in the trial, clinical benefit was limited, and no radiological tumour shrinkage was demonstrated.

Notably, one patient with a c-MYC and HER2 co-amplified tumour, continued ibrutinib for 32 weeks and, over 8 weeks, had a marked metabolic response in her primary tumour on sequential FDG-PET imaging. There are a few observations to highlight from this patient's case. Firstly, she was one of 3 patients treated within the trial who had a c-MYC and HER2 co-amplified tumour and, in the pre-clinical work, the greatest effect with ibrutinib, including induction of apoptosis, occurred in the MYC and HER2 co-amplified cell lines. Therefore, based on these observations, it was anticipated that the MYC and HER2 co-amplified group would be most likely to respond clinically. However, MYC and HER2 co-amplification is rare in OG cancer, occurring in 2% of patients from previous reports,(284,285) and 2% of our pre-screened population. Therefore, restricting the inclusion to patients with co-amplified tumours in a phase II trial would not have been feasible, as it would have required multiple participating centres, more funding, and more capacity to perform the pre-screening to identify these rare patients, which was not available at the time. Given that ibrutinib also caused cytostatic effects and reduced MYC protein levels in the MYC amplified

OG cell lines, it was rational to also include MYC-amplified OG cancer patients in the trial. However, it may have been ambitious to expect clinically meaningful tumour shrinkage in the patients who were not co-amplified. Secondly, this patient was one of 3 patients who had a high level MYC amplification (CEP8: MYC ratio 1:10). Of the 3 patients who stayed on study the longest, 2 of them had tumours with a CEP8: MYC ratio 1:10, receiving 4 and 9 cycles of treatment respectively. The MYC: CEP8 FISH ratio of ≥ 2.5, used in the trial to assign MYC amplified cases, was determined based on thresholds derived from calling HER2 'positivity' in breast cancer, where, in fact, clinical benefit with HER2 targeted therapy in breast cancer patients has also been seen at even lower HER2 FISH ratios.(276,286) Therefore, this threshold may not be correct benchmark when looking to target other RTKs in other tumour types. For example, much higher levels of HER2 or FGFR2 amplification are required before effective therapeutic targeting is achieved with HER2 or FGFR2 inhibition in gastric cancer.(172,173) Therefore, in future efforts to target MYC in OG cancer, higher thresholds of MYC amplification may be required.

Digital polymerase chain reaction can be a sensitive method to detect gene expression if DNA yield from tissue is low. A digital droplet PCR (ddPCR) assay was developed to detect MYC amplifications in the tissue samples collected during pre-screening for the iMYC trial.(238) ddPCR analysis was performed on 127 (78%) of patient samples and was successful in 98 (93%) of cases. There was no observed difference in the ability of the ddPCR assay to identify MYC amplification in cases where less than 35% of cells were MYC amplified, but the ddPCR assay appeared more sensitive at picking up cases where MYC was amplified in >70% of cells. Thus, going forward, ddPCR could be an alternative method to determine clinically meaningful MYC amplification in the future. Finally, it is notable that this patient who had the partial metabolic response in her primary tumour, did not have a large volume of other metastatic disease. Therefore, the diagnostic biopsy from her primary tumour which was used to determine c-MYC/HER2 status, may have been more reflective of the biology of her overall disease burden, compared to other patients who had a greater burden of metastatic disease at trial entry.

An increasingly recognised limitation of delivering personalised medicine in metastatic OG cancer, is intra-tumoral heterogeneity and clonal evolution.(289) This presents two major challenges, firstly, spatially – how do we know that the genetic and epigenetic landscape of the area of tumour biopsied is representative of the entire disease? And, secondly, temporally, how to we know that the clonal composition of the tumour sample is still representative of the current disease over time e.g., after one or two lines of therapy? In the case of HER2 positivity, the latter has already been demonstrated by multiple groups, as re-biopsy of OG tumours following treatment with first-line trastuzumab, has shown a loss of HER2 positive status in 1/3 of patients, compared to before treatment.(290,291) This loss of highly amplified HER2 cancer clones due to selective pressure of first line treatment, has been proposed as a key contributory factor to the lack of efficacy seen with second-line line HER2 targeting agents.(113,292)

In the iMYC trial, the tissue used to determine c-MYC/HER2 status in 7/8 treated patients, was from archival diagnostic specimens of the primary tumour. Therefore, by the time of trial entry, after one or two lines of treatment, multiple different subclones could have developed which could have either evolved into new ‘drivers’ of the disease, other than MYC or HER2, or developed acquired resistance to MYC/HER2-targeting agents. Therefore, the single archival biopsy used to determine MYC or HER2 status, may not have accurately distinguished the dominant clones in the patient’s tumour at the time of study enrolment. This could have contributed to the varied responses/lack of efficacy seen. One potential way to mitigate this would have been to re-biopsy the patient at the time of iMYC trial entry to re-assess c-MYC and HER2 status. If acceptable to the patient, re-biopsy of both the primary and metastatic disease, could have provided even more valuable information to use to assign treatment. At a single timepoint, marked discordance of genomic alterations, particularly gene amplifications, between samples of OG cancer primary tumours and metastatic disease have been recorded.(92,228,293) Subsequent targeting of actionable aberrations detected in the metastatic disease could therefore prove more fruitful, and indeed more ‘personalised’ and context specific. This is the stance in breast cancer, when discordance is detected between a primary tumour and metastasis at re-biopsy, the recommendation is to assign treatment based on the

metastasis.(294) c-MYC amplification appears to occur more frequently in distant metastatic disease compared to the primary tumour and, therefore, likely represents an acquired event through tumour evolution rather than a functional driver event.(295,296) Therefore, it is also possible that a more contemporary biopsy of patients metastatic disease at the point of trial entry, after one or two lines of treatment, may have identified a higher number of c-MYC amplified patients through pre-screening, who could have benefited from the trial.

An alternative to repeated invasive tissue sampling to address the challenges of spatial and temporal heterogeneity, could be to harness 'liquid biopsies' using cell-free circulating tumour DNA (ctDNA) profiling. In the previously published iMYC pre-screening results, 31 patient specimens were evaluated for intra-tumoral heterogeneity of MYC amplification status, and wide ranges of MYC: CEP8 ratios were observed within the same sample in 22 (71%) cases.(238) In contrast to the potential for needle sampling errors with tissue biopsy, ctDNA next generation sequencing (NGS), allows for the detection of ctDNA shed from all tumour sources from within the body, and thus, may produce a more representative landscape of potentially targetable somatic mutations within the patient.(297) In particular, detection of genomic aberrations from ctDNA is most successful in patients with liver metastases,(298) which the majority of the treated iMYC population had. As discussed previously, high gene copy numbers are required for targeted therapies to be efficacious in OG cancer. One potential limitation of liquid biopsy is that very high gene amplification in tissue is required for it to be identified in plasma.(298) However, given that the greatest clinical benefit in the iMYC population was observed in those with c-MYC amplifications of ≥ 10, then perhaps only patients with ctDNA detectable MYC amplifications should be evaluated in future efforts to target MYC in OG cancer. Successful ddPCR analysis of ctDNA from plasma samples of the iMYC pre-screening cohort was only feasible in 75/127 (59%) of cases.(238) There was a high rate of instances where the yield of DNA was too low for extraction, which led to failure of ctDNA analysis in almost half of the patients. This was probably in part a problem with the timing of the plasma sample collection as, per protocol, pre-screening for iMYC could take place at any time before, during, or after a treatment line, and we know that patients who are either on treatment, or who

have had effective treatment, generally have a lower ctDNA yield.(298) Therefore, in future, MYC amplification should probably be assessed in plasma at the time of prior treatment failure, in order to yield enough DNA for analysis, and/or, at the time of initiation of a new biomarker-directed therapy, in order to identify patients who have appropriate evidence of the biomarker at the correct timepoint.

Four out of 7 evaluable patients developed progressive disease after 2 cycles of ibrutinib treatment. BTK usually signals via the RAS/RAF/MEK/ERK protein kinase pathway,(299) and exposure to ibrutinib resulted in reduced ERK-mediated MYC phosphorylation in the MYC amplified preclinical oesophageal cancer models.(265) However, in the clinic, primary resistance to BTK inhibition has been observed with ibrutinib use in haematological malignancies (around 30%),(300) due to simultaneous activation of other pathways, such as PI3K-AKT signalling, which is also a key pathway involved in cell growth and survival in OG cancer.(301,302) Therefore, targeting a single genetic vulnerability such as c-MYC amplification may not have been sufficient to overcome intrinsic resistance mechanisms, and highlights the potential need for combination therapy, e.g. with PI3K inhibitors, to target activated interrelated pathways. A limitation of the preclinical work is that no correlative tissue experiments were performed, as cell lines do not account for the molecular heterogeneity so frequently observed in OG cancer in the clinic. Future work might, therefore, seek to evaluate potential mechanisms of resistance to BTK inhibition in humanised mouse models, patient derived tumour organoids, or patient-derived xenografts (PDXs).

Addressing acquired resistance mechanisms will also be important in future efforts to target MYC or and HER2 in the clinic. Firstly, identification of resistance mutations developed from prior targeted therapies with be required to determine the best next treatment strategy. For example, acquired mutations in KRAS, NF1, and PIK3CA, detected by ctDNA profiling, have all been observed following treatment with HER2 directed therapy,(298) and, therefore, may also require targeting as part of a combination strategy, along with MYC and HER2, in the next treatment line. Secondly, it will be important to track mechanisms of resistance during treatment with MYC and/or HER2 targeted therapy, to

understand why treatment failed, and to develop better treatments in future. Liquid biopsies may be the most convenient, least invasive way to do this, and are increasingly being used for this purpose.(303) In the iMYC trial, optional sequential biopsies were part of the protocol however none were eventually collected during the study. The reasons for this are unclear however could have been because biopsies were not encouraged by the study team, or because patients did not wish to undergo repeated biopsy. As a Clinical Research Fellow, I was fortunate enough to have been involved with the writing and set-up of two early phase clinical trials. After careful consideration, we opted to make sequential trial biopsies mandatory in these two studies, particularly as many patients with GI cancers have expressed a wish to contribute to scientific research which may benefit others.(304) Given that so many phase III trials of targeted agents in OG cancer failed in past (Table 3), translational work is extremely important to add insight and justification to clinical observations. However, given the limitations of single site tissue biopsies in such a highly heterogenous disease, as already discussed, ctDNA profiling for biomarker detection, tracking clinical response, and monitoring for resistance mechanisms, will likely supersede repeated tissue biopsy in the future personalised medicine strategies for OG cancer.

Disappointingly, the iMYC trial closed early because of the Covid-19 pandemic and loss of pre-screening capacity in the cytogenetics lab. However, there were other challenges to recruitment reflected in the fact that pre-screening identified 79 patients with HER2 positive/c-MYC amplified tumours, but only 8 were treated over a period of 4 years. Several factors likely contributed to this high attrition rate. Firstly, patients were often pre-screened during their previous line of therapy, as per protocol, however by the time a change in treatment was required i.e., when entry to the iMYC trial became an option, they may not have met eligibility criteria at this stage, for example, due to deterioration of baseline organ function or fitness. The thrombosis rate among OG cancer patients undergoing first line chemotherapy is reported as 10%,(305) and anticoagulation use became an exclusion criterion, which further precluded patients. We know that, sadly, only around 40% of advanced OG cancer patients get to second line treatment,(106) and, of these, perhaps 20-30% might be fit for a clinical trial, so there is often a

narrow window in which therapy needs to start. The FISH results usually took a couple of weeks to be reported, however often patients' archival tissue had to be retrieved from other centres which may have added on a further 2-4 weeks. If patients' pre-screening results were not yet back and a change in therapy was required, they likely would have had to start on an alternative therapy without being able to wait for the c-MYC result. Furthermore, during the 5 month period that recruitment was paused for the IDMC to assess and advise upon the bleeding events, many patients that were previously pre-screened and found to have a c-MYC/HER2 amplification, had already started other treatments during this time and were not fit enough for the trial by the time third line options were being considered.

Although no CR or PR was achieved, 3 patients experienced disease stabilisation after 8 weeks. Given that standard second line chemotherapy improves survival by approximately 6 weeks,(95,96) and only 12 - 20% of tumours reach a best response of PR after treatment with established second- or third-line regimens,(106) it may have been ambitious to aim for response rates (CR/PR) of > 20% in our small sample size. Patient reported outcome measures (PROMs) can enhance clinical endpoints in determining tolerability of treatment.(306) However, we did not collect enough health questionnaires to meaningfully confer quality of life benefit from ibrutinib, as only 4 patients completed them at the start of treatment and most patients progressed after only 2 cycles. It is noteworthy that baseline tumour assessment scans took place up to 28 days before patients started ibrutinib as this is a relatively long time in such an aggressive disease. Therefore, the progressions seen at the first response assessment may have represented the fast pace of the disease process, rather than ineffective treatment. Almost all treated patients had liver metastases at trial entry and the protocol allowed up to performance status (PS) of 2, both of which are poor prognostic features of this disease.(307,308) In future design of second line trials in this patient population, we should consider whether a PS of up to 2 should be reduced to 0-1, and whether other better prognostic indicators, such as response to first line therapy, should be considered. This may result in a more stable patient population, and thus, a fairer starting point from which to evaluate a new therapy.

Achieving disease stabilisation in this population of pre-treated patients might also be a clinically meaningful goal in future trial design.

The adverse event profile in this study was consistent with commonly reported side effects of ibrutinib apart from the 3 out of 8 patients who suffered unexpected major gastrointestinal bleeding, which is a much higher rate of major bleeding than would be expected with this drug. This was therefore considered a new safety finding for ibrutinib in this patient population, and later included in the Reference Safety Information (risk of any grade haemorrhage with ibrutinib is 32% and ≥ grade 3 is 1%).(309) A phase III study of ibrutinib combined with chemotherapy in patients with advanced pancreatic cancer, has also reported major haemorrhage in 6% of patients.(273) Thus the rate of significant bleeding with ibrutinib appears to be particularly high in patients with gastrointestinal tumours. Ibrutinib is the first human BTK inhibitor which was developed in 2009 and is approved for the treatment of several haematological malignancies. The mechanism for ibrutinib-related bleeding occurs largely via its effects on platelets, through inhibition of both BTK and TEC, which is another tyrosine kinase irreversibly inhibited by ibrutinib at clinically meaningful concentrations.(310) It is thought to be the combination of BTK and TEC inhibition which results in bleeding, rather than inhibition BTK or TEC alone.(310) Given the inherently high incidence of gastrointestinal bleeding and associated mortality in OG cancer patients,(311,312) it will be of critical importance to account for the bleeding risks associated with BTK inhibition in any future studies. For example, exclusion of patients on therapeutic anticoagulation, guidance on stopping of ibrutinib for invasive procedures, and concurrent use of anti-platelet therapy for cardiovascular disease, or non-steroidal anti-inflammatory drugs which inhibit platelet function, should all be considered. Newer generation BTK inhibitors which have more selectivity for BTK, such as alacabrutinib or zanubrutinib, are available and are associated with lower frequency of bleeding events in patients with haematological malignancies, however these drugs also have lower selectivity for HER2.(310,313,314) Therefore, future targeting of MYC and HER2 co-amplification in the clinic, may instead benefit from a combination of a second generation BTK inhibitor, plus a dedicated selective anti-HER2 agent in attempt to mitigate unwanted off-target effects such as bleeding.

Several exploratory analyses were intended as part of the iMYC study but, unfortunately, these were not pursued due to lack of funding. The intention was to investigate the possible PK/PD relationships between plasma ibrutinib exposure and plasma concentrations of phospho-BTK and phospho-HER2, to establish whether the intended mechanism of action of ibrutinib, and the in vitro findings, were in fact recapitulated in patients with clear target engagement. Another aim was to explore the association between c-MYC copy number, c-MYC FISH ratio, c-MYC copy number in serum, and the efficacy of ibrutinib. While we have seen a trend towards greater clinical benefit with MYC ratios ≥ 10 in tissue, the numbers of these patients were small. Future work in this area would benefit from exploring whether higher ratios and copy numbers of MYC amplification led to more clinical efficacy, and this could potentially be done with use of a ddPCR assay on plasma samples taken prior to study entry. If sequential tumour biopsies had been collected, the goal would have been to analyse downstream HER2 signalling pathways and MYC target genes at baseline and on-treatment and correlate with tumour size. In addition, the plan would have been to prepare tissue arrays to develop molecular signatures of response and resistance to treatment. As discussed, this could also be done in vitro first with use of PDX models, as optional sequential biopsies were challenging to collect on this study. Alternatively, as discussed, ctDNA tracking with genomic profiling of sequential 'on-treatment' plasma samples, in future clinical studies, could also identify development of new gene mutations and amplifications which could drive therapy resistance.

The iMYC trial was negative for the primary endpoint however the findings, together with the earlier pre-clinical findings from our group, suggest that it may be worthwhile focussing on the synthetic lethal interaction between BTK inhibition and co-amplification of MYC and HER2 in future. Given that this phenomenon occurs at an extremely low frequency in the OG patient population,(284,285) a "basket"-trial approach, where novel therapies with rare driver mutations can be evaluated in patients with any tumour type, could be pursued. This type of trial design was very successful in evaluating the efficacy of larotrectinib, an oral tropomyosin receptor kinase (TRK) inhibitor, in patients with any TRK fusion-positive cancer, which occurs in approximately 1% of all solid tumours,(315) and

led to a tumour agnostic approval. This may be more valuable for tumour types where prevalence of MYC and HER2 co-amplification is more common such as breast cancer,(316) but still worth evaluating when there is such a great unmet need for better, more personalised treatments in OG cancer and others. Furthermore, MYC and HER2 amplification in ctDNA could be a simple and convenient way to screen for these rare co-amplifications. Given that MYC amplification results in an oncogene addiction phenotype, partly by activation of other RTKs, concurrent inhibition of MYC and other pathways could synergise. For example, in the pre-clinical work, ALK was also selectively lethal in MYC amplified oesophageal cell lines,(265) and in neuroblastoma cells, BTK overexpression appears to enhance the stability and expression levels of ALK.(317) Subsequently, combination of ibrutinib with the ALK inhibitor, crizotinib, led to a greater decrease in cell proliferation and apoptosis than ibrutinib alone.(317) This combination may therefore also be relevant to MYC amplified, ALK positive GI cancers. ALK positivity has been reported in 8.4% of resected gastric cancers and is associated with signet ring cell histology, worse survival, and younger age.(318) The prevalence of both MYC amplification and ALK positivity is likely to much lower, but could also be evaluated in a “basket” trial if initial preclinical studies looked promising.

MYC inactivation can trigger an adaptive immune response, resulting in activation of macrophages and CD4+ T cells,(319) in addition to downregulation of immune checkpoints CD47 and PD-L1,(220) thus potentially restoring the host immune response against cancers. Furthermore, BTK inhibition with ibrutinib can reduce formation of MDSCs and promote MDSC differentiation into mature DCs, thus reducing overall levels of MDSCs in the TME, and increasing proliferation and effector function of CD8 cytotoxic T cells.(320–322) Subsequent application of ibrutinib in combination with anti-PD-L1 therapy to in vitro models of solid tumours, have demonstrated synergistic effects with greater tumour shrinkage with both agents, compared to anti-PD-L1 alone.(320) Thus far, trials of BTK inhibition plus checkpoint blockade in solid tumours, other than OG cancer, have had limited clinical benefit.(323–325) However, in some correlative translational studies, reduced levels of peripheral MDSCs and increased CD8 T cell numbers were observed.(324,325) Some of the lack of efficacy seen is presumed due to

rapid elimination of drug in patients,(326) and different drug delivery systems for BTK inhibitors, such as encapsulation in a nanocomplex, have been explored.(327) Therefore, there may still be milage in evaluating the combination of BTK inhibition with checkpoint blockade in OG cancer, particularly in the more immunologically ‘cold’ subtypes, in future efforts to target MYC.

1.5 Conclusion

Single agent ibrutinib had limited clinical efficacy in patients with c-MYC and/or HER2 amplified OG cancer in this proof-of-concept study. These data highlight the need for novel drug development as well as the assessment of drug combinations to overcome potential resistance mechanisms to monotherapy. Novel approaches to address the multiple clonal subpopulations within metastatic OG tumours, and the dynamic expression status of potential biomarkers during therapy will also be required and liquid biopsy may be the best way to do this. Given that MYC overexpression is correlated to an immunosuppressive TME, future combinations involving MYC targeting agents with checkpoint inhibition may be a promising strategy in OG cancer, where responses to ICI monotherapy are limited.

Chapter 2 Epigenetic modulation of the immune response in gastrointestinal cancers: phase IIa results of the EMERGE trial

2.1 Introduction

2.1.1 The role of epigenetics in colorectal and oesophagogastric cancer

Epigenetics refers to non-heritable alterations of gene expression without changing the fundamental DNA sequence.(328) These alterations include disruption of DNA methylation homeostasis and changes (post-translational modification) to histone proteins, chromatin remodelers, and non-coding RNAs (which regulate transcription at a post-transcriptional level). The resulting abnormally deregulated genes have a key role in the pathogenesis and progression of cancer.(328,329) Importantly, these changes to the epigenome appear to occur very early on in CRC and OGA development and involve virtually every signalling pathway associated with cancer growth and progression.(330,331) Additionally, they have been associated with chemoresistance, higher tumour grade, and poor survival. Therefore, the potential targeting of epigenetic alterations in treatment strategies for CRC and OGA, has become an important area of cancer research.

DNA methylation, carried out by enzymes known as DNA methyltransferases (DNMTs), which add a methyl group to cytosine residues at CpG dinucleotide sites (*"writers"*), is the most extensively investigated epigenetic modification thus far.(332) CpG islands, which contain many CG dinucleotide repeats (CpGs), and are located close to gene promoters, are usually unmethylated.(333) Hypermethylation at CpG promoter regions is typically associated with gene repression, and can initiate cancer by suppressing transcription of TSGs. For example, epigenetic silencing of MLH1,(334) and APC,(335) has been strongly linked to development of sporadic MSI-H CRC, and MSS CRC, respectively.

Aberrant DNA methylation in specific CpG islands is known CIMP, as mentioned in the introduction. CIMP-high CRCs are enriched in the CMS1 cluster, and associated with MSI-high status, right sided tumour location, BRAF mutations, and are more common in women.(139) By contrast, CIMP-low CRCs are associated with KRAS mutations, and are more common in men.(139) CIMP-negative CRCs, are also more strongly linked to TP53 mutations.(139) In GC, CIMP tumours are enriched in the MSI and EBV subtypes.(25) Hypermethylation of the CDH1 promoter, causing loss of the TSG CDH1 through epigenetic silencing, has also been found in almost half of all hereditary diffuse GCs, and associated with poor survival.(336–338) Loss of CDH1 appears to upregulate other oncogenic pathways such as Wnt beta-catenin and mTOR. On the other hand, global DNA hypomethylation has been found to directly enhance expression of proto-oncogenes, such as MYC, or regulation of beta-catenin, and is predominantly an early event in oesophageal and colorectal carcinogenesis.(339–341) However, hypomethylated genes do appear increased in more advanced colonic tumours and, therefore, may also contribute to CRC progression.(342,343) Interestingly, DNMT protein expression appears to be higher in OSCC compared to OAC.(331) Figure 8 displays selected epigenetic machinery relevant to OG cancer and CRC.

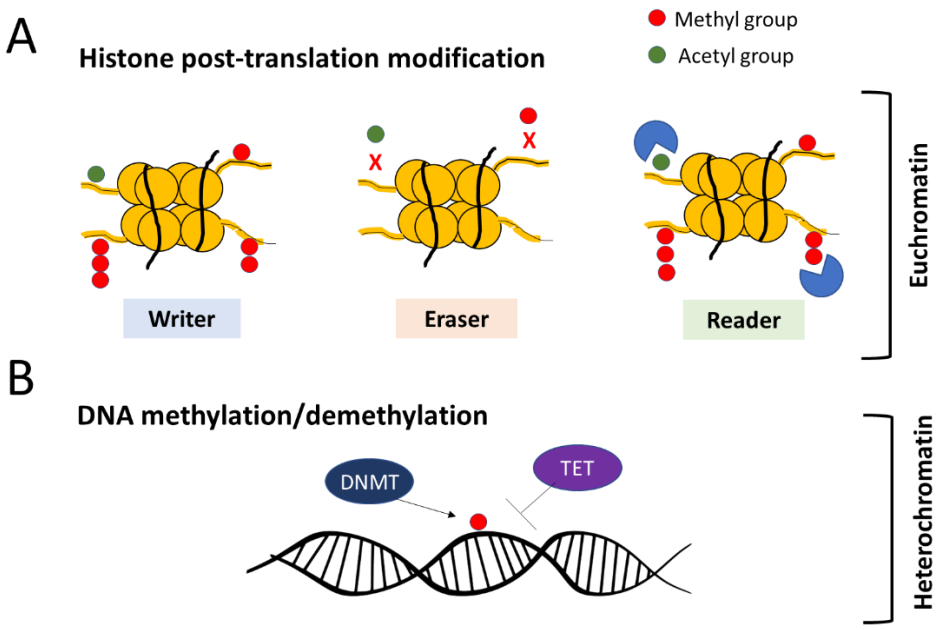


Figure 8 Epigenetic machinery in OG cancer and CRC (Adapted from Lopes et al).(331) (A) Histone writers, erasers, and readers; (B) DNA methylation regulators. DNMT – DNA methyltransferases; TET – DNA demethylases.

Post-translational modification of histone proteins commonly involves enzymes which either add acetyl groups (histone acetyltransferases, HATs; *"writers"*), or remove them (histone deacetylases, HDACs; *"erasers"*). Acetylation reduces the affinity between DNA and the histone protein, thus, the DNA is less tightly wound and so more accessible to transcription factors.(344) Histone deacetylation at sites of promotor regions of TSGs, is associated with the initiation and progression of cancer,(329,345) and overexpression of HDACs has been linked with inferior survival in several gastrointestinal cancers including CRC and OGA.(329) Additionally, molecular characterization of oesophageal carcinomas has revealed that elevated levels of HDAC1 and HDAC2 (class I HDACs), are associated with more aggressive and advanced stage disease.(346,347)

Histone methylation is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). These add or remove methyl groups to histone

tails, which create docking sites for proteins which promote expression of genes involved in Wnt beta-catenin signalling, commonly deregulated in both CRC and OGA, as previously described.(348–350) BET proteins, e.g. BRD4, mentioned in the last chapter, are epigenetic *"readers",* capable of recognising histone modifications and regulating gene expression, e.g. MYC transcription.(246) BRD4 has been shown to recognise transcription factors which activate EMT and promote metastatic growth in GC and CRC,(351,352) and induce the overexpression of MYC in GC.(353) BRD2 can also activate transcription of LAMB3, a pro-metastatic gene, in CRC, causing tumour proliferation and metastasis.(354) Knowledge of the epigenetic landscape of CRC and OGA is therefore increasingly important in understanding the formation and progression these tumours, and to identify potential novel therapeutic targets. Furthermore, because these changes occur in the epigenome, rather than the underlying genetic material of cells, they are essentially reversible and, thus, an attractive target for therapy.(355)

2.1.2 Targeting the epigenetic machinery in CRC: pre-clinical and clinical data

Various epigenetic modifiers are approved and clinically beneficial in several diseases including haematological malignancies as single agents. However, when evaluated in patients with myelodysplastic syndromes, median time to initial response was around 3 months, which is a long time for aggressive, metastatic solid tumours.(356) This led to use of epigenetic regimens being explored in combination strategies in solid tumours, such as CRC and OGA, with other established anti-cancer agents like chemotherapies. To date, inhibitors of histone deacetylases (HDACi) and DNA methyltransferases (DNMTi), are the most extensively evaluated epigenetic modulators. Preclinical CRC xenograft models treated with transcinnamic acid (tCA), which inhibits HDAC I and II, resulted in decreased expression of PARP, which helps to repair DNA damage, and increased expression of Bax, which mediates apoptosis, leading to reduced cell viability.(357) However, when entinostat, a class II HDACi, was applied to 5 different CRC models, there were differential responses, as tumour attenuation

was only observed in the HT29 and HCT116 models.(358) As the HT29 and HCT116 models also upregulate expression of genes involved in cell adhesion, the authors postulated that entinostat may, therefore, help to limit metastatic spread of the clinical disease.(358) Preclinically, HDAC inhibitors have also demonstrated synergy in combination with 5-FU, through downregulation of thymidylate synthase.(359) This was observed through combination of trichosstatin (TSA) and 5-FU, which decreased CRC cell viability and reduced expression of pro-tumourigenic proteins, such as KRAS, c-MYC, and AKT/mTOR.(360) The HDACi, romidepsin, has also demonstrated synergy with 5-FU in HCT116, HT29, and SW48 CRC cells, resulting much greater levels of cell cycle arrest, proteolytic cleavage, and upregulation of MHC class II genes, than with 5-FU alone.(361) The use of HDAC inhibitors, in combination with chemotherapy, was therefore considered promising in CRC, and, potentially, particularly useful in earlier stages of disease.

In CRC cell line in vitro studies, inhibition of DNMT1 and DNMT3B, appeared to reverse hypermethylation of TSGs, resulting in apoptosis and decreased cellular proliferation.(362,363) When combined with irinotecan and 5-FU, the DNMT inhibitor, 5-azacitadine (AZA), caused greater tumour regression in in vitro and in vivo CRC models, than with either agent alone.(364) Responses in the CRC models were not ubiquitous, with some responding straight away, and others taking several weeks to respond, such as HCT116, with some not responding at all. Interestingly, treatment with AZA also appeared to downregulate the efflux transporter ABCC1, which is associated with irinotecan resistance.(364) This enhanced sensitivity to irinotecan with AZA was also reported by another group, but was only observed in MSS CRC cell lines and not MSI CRC cell lines.(365) As CMS1 CRCs are linked to CIMP and have a poor prognosis, demethylating agents could be especially valuable in these tumours. Furthermore, BRAF mutated tumours, which have greater activation of the PI3K/AKT pathway in CRC, where many of the pathway proteins are susceptible to epigenetic influences, may also be good candidates for epigenetic therapy. Indeed, treatment of BRAF mutated cell lines and mouse models, with PLX4720, a DNMTi, combined with a PI3K inhibitor, resulted in synergistic anti-proliferative effects and decreased AKT activation.(366) Therefore, epigenetic modulators

may also be relevant for overcoming resistance to traditional BRAF targeted therapies.

The clinical utility of “epi-drugs” as single agents in mCRC has been limited. For example, in pre-treated chemo-refractory patients with mCRC, no tumour shrinkage was observed with romidepsin, an HDACi, as a single agent.(367) A similar lack of response was seen with vorinostat, another HDACi, in heavily pre-treated patients with CRC, and toxicity rates were high.(368) Interestingly, the combination of the HDACi, entinostat, with the DNMTi, AZA, resulted in some excellent responses in some patients with NSCLC. The study included 45 patients with heavily pre-treated NSCLC and used much lower doses of either drug that had previously been evaluated as a monotherapy.(369) The treatment was very well tolerated and resulted in 1 CR and 2 PRs, and 10 patients experienced stable disease lasting at least 3 months.(369) However, in a similar study carried out in patients with CRC, no responses were observed at all.(370) A few patients did experience stable disease for up to 10 months and, interestingly, the patients with disease stabilisation, showed greater levels DNA demethylation from analysis of paired biopsies at baseline and at cycle 2.(370)

Efforts to combine HDACi and chemotherapy have also been largely disappointing in the clinic. For example, a dose finding trial of continuous dosing of oral vorinostat, with 5-FU, was terminated early due to high toxicity.(371) A subsequent trial of two different vorinostat dosing regimens were evaluated with 5-FU (800 or 1400mg/day taken for 3 days, every two weeks, with 5-FU was given on days 2 and 3 of vorinostat), in CRC patients who had exhausted all standard therapy, including 5-FU. In the lose dose vorinostat arm, one PR was observed, and overall survival was over 6 months.(372) This regimen was also better tolerated, however the trial did not reach its primary endpoint.(372) Table 10 displays findings of some previous clinical trials which evaluated epigenetic agents in CRC and OGA.

Epigenetic target	Drug	Combination partner	Clinical trial phase	Result	Ref
DNMTi	Azacitadine	Capecitabine and oxaliplatin	I/II in mCRC	SD in 17 patients 4 patients with SD >4 months	(373)
	Decitabine	Carboplatin	Phase I in solid tumours	PD was best response in 7 patients with mCRC	(374)
	Azacitadine	Epirubicin, capecitabine and oxaliplatin neoadjuvant chemotherapy (Azacitadine given before each cycle)	Phase I in patients with resectable OG cancer	67% overall response rate, 25% complete response	(375)
HDACi	Vorinostat	FOLFOX	Phase I mCRC	21 patients treated, 2% had SD	(376)
	Vorinostat	5-FU and leucovorin	Phase I refractory solid tumours	- 43 patients treated (38 had CRC, 2 had oesophageal cancer and 1 had GC - 1 had PR, 23 had SD	(377)
	Vorinostat	5-FU and leucovorin	Phase I/II in mCRC	- 10 patients treated; 2 patients had SD lasting >4 months - 2 DLTs occurred at starting dose level so study terminated	(371)
	Vorinostat	Capecitabine and cisplatin	Phase II in patients with advanced OG cancer	42% overall response rate, toxicities increased with the combination	(378)

Table 10 Selected trials of epigenetic drugs in combination with chemotherapy in CRC and OGA

2.1.3 Targeting the epigenetic machinery in OG cancer: pre-clinical and clinical data

Use of the DNMTi, decitabine, in GC cell lines has been shown to reduce levels of hypermethylation and significantly reduced proliferation of CIMP positive GC cell lines, compared to CIMP negative GC cell lines, as expected.(379) Decitabine has also been observed to inhibit the activity of MMP-2 and MMP-9, thus reducing cellular invasion.(380) Additionally, DNMT silencing in mouse models of OSCC resulted in reduced invasion and metastases, and led to apoptosis.(381,382) Therefore, DNMT appears to be related to cellular proliferation, invasion, and metastasis, in OG cancer. Preclinical models evaluating HDAC expression in OG cancer models, demonstrate that HDACs have similar oncogenic effects. For example, different studies have shown that silencing of HDAC1, HDAC2, HDAC4, and HDAC6, resulted in decreased proliferation, migration, and invasion of OSCC cells, and promoted cell cycle arrest.(383–386) Like in models of CRC, HDACi and DNMTi, can also reverse resistance mechanisms acquired by epigenetic alterations, and re-sensitise OG tumours to chemotherapy. For example, in GC, inhibition of HDAC9 potentiated the effects of cisplatin and resulted in cell cycle arrest and apoptosis.(387) Combination of cisplatin and decitabine also caused significant tumour attenuation in CIMP-positive GC cell lines, indicating a synergising effect from the combination.(379) Combination of SAHA, an HDACi, also had a synergistic effect when combined with taxanes, in GC cell lines which were taxane resistant, resulting in attenuated tumour growth.(388)

In the clinic, combinations of “epi-drugs” with chemotherapy have been less frequently explored in OG cancer, than in CRC. However, as in CRC, clinical results thus far have not been very fruitful. For example, in a phase I dose finding study of escalating doses of vorinostat and 5-FU, the 2 included patients with advanced oesophageal cancer, and one patient with GC, did not respond.(377)

Therefore, despite the positive pre-clinical data demonstrating that HDACi and DNMTi enhance the activity of chemotherapy in OG and CRC, there must be other underlying reasons for their lack of effect in humans. High systemic

toxicities, such as GI toxicity, cytopaenias, and severe fatigue with HDAC inhibitors, are at least partly attributable for the failure of these combinations in patients with solid tumours, which have led to several trials of chemotherapy combined with HDACi or DNMTi being stopped prematurely.(328) Other limitations include the pharmacokinetic properties and bioavailability of these drugs as, for example, the half-lives of decitabine and AZA range from only 15-25 mins, as they are degraded rapidly.(389,390) Furthermore, the dosing regimens and route of administration used in trials have not been consistent in clinical trials, so it is unclear as to whether the lack of effect could be due to subtherapeutic dosing. Therefore, it is likely that future endeavours planning to evaluate “epi-drugs” in combination studies, should include PK samples and PD studies, to ensure adequate drug levels and target effects. Moreover, predictive biomarkers, for example CIMP status, analysis of target gene de-repression, or HDAC overexpression, may also be valuable to select appropriate patients who may derive more benefit from the combinations.

2.1.4 Epigenetics and immune function

Epigenetic regulation of chromatin is a key process which determines the fate of stem cell differentiation into all cells of the body, including immune cells. Epigenetic mechanisms can regulate accessibility of transcription factors, such as TCF7, which is involved in differentiation of naïve CD8+ T cells to effector CD8+ T cells.(391) This appears to be a dynamic process which can be reversed by demethylation of select genes, resulting in dedifferentiation of effector CD8+ T cells to memory T cells.(392,393) Blocking DNA methylation also appears to stave off CD8+ T cell exhaustion due to persistent antigen stimulation,(393,394) and thus may sensitize tumours to reinvigoration with checkpoint inhibition. Epigenetic mechanisms also control the differentiation and activation of myeloid cells, e.g., MDSCs,(395–397) which have potent immunosuppressive features, such as inhibiting T cell activation and proliferation. Additionally, DNA methylation status controlled by histone regulators, appears to play a key role in the activation of dendritic cells which are required for an effective T cell response.(398,399)

In tumour cells, epigenetics can disrupt the antigen presenting machinery such as suppression of MHC class I,(400,401) which means that the tumour is less recognisable to the host immune system and T cells – one of the defining features of an immunologically ‘hot’ or ‘cold’ tumour, leading to lower levels of immune activation. Epigenetic enzymes can also regulate expression of Th1-type chemokines, e.g., CXCL9 and CXCL10, required by T cells for T cell recruitment and infiltration,(402) in addition to regulating the expression of STAT1,(403) a transcription factor which mediates the interferon pathway, which plays a key role in cellular immunity. Importantly, epigenetic mechanisms also contribute to upregulation of PD-L1 expression on tumour cells, which leads to immune evasion and T cell exhaustion. (404,405)

Thus, epigenetic mechanisms play a crucial role in both immune cells and tumour cells at almost every step of the Cancer-Immunity Cycle. Therefore, if these effects could be reversed with epigenetic modifying agents such as HDAC inhibitors, this may, in simplest terms, covert an immunologically ‘cold’ tumour to ‘hot’ one and reinstate the anti-cancer immune response for collaboration with ICIs. This has already been demonstrated both in vivo and in the clinic. For example, HDAC inhibition was shown to increase levels of tumour associated antigens and upregulate MHC and co-stimulatory molecules in melanoma mouse models.(406) In addition, PD-L1 expression was upregulated, suggesting that targeting the anti-PD1/anti-PD-L1 axis might be efficacious.(406) In mouse models of NSCLC, HDAC inhibition led to increased expression of T cell chemokines and, in mice with intact immune systems, tumour regression was also observed.(392) Furthermore, combination of an HDACi with an anti-PD-1 antibody in mouse models of NSCLC, led to long-term tumour eradication in the majority.(407) The following section will describe the pre-clinical and clinical data demonstrating synergistic effects from combining ICIs with HDAC and DNMT inhibitors.

2.1.5 Epigenetic modulators and synergy with checkpoint inhibition in CRC and OGA

Pre-clinical data in OGA and CRC suggest that combinations of DNMT inhibition or HDAC inhibition with ICIs may also be efficacious. CRC cell lines subjected to 5-AZA, were found to upregulate 15 gene sets (termed immune AIM genes) involved in antigen presentation, interferon signalling, and chemokine and cytokine signalling, after 8 weeks.(408) Higher basal levels of AIM gene expression was also correlated to CIMP-high status in CRCs.(408) This data suggests that AZA could favourably reverse the cancer immune evasion phenotype in patients with CIMP-low MSS CRC (most CRC patients), and thus increase sensitivity to ICIs. CT26 CRC cell lines and mouse models are rapidly growing, aggressive tumours, which quickly metastasise and are modestly immunogenic. Similarly, 4T1 CRCs are poorly immunogenic, metastatic tumours. Mouse model work showed that CT26 and 4T1 CRCs, which were in no way sensitive to combined PD-L1 and CTLA-4 antibodies alone, were subsequently completely eradicated following co-treatment with 5-AZA and entinostat (a class I HDACi) with the ICIs.(409) The dual HDACi and DNMTi approach, significantly reduced MDSC populations, which allowed for expansion of CD8+ T cell cohorts, thus suggesting that MDSC depletion, is the key mechanism by which these drugs favourably alter the TME.(409) These effects were much more profound with the drugs in combination, than with either the 5-AZA, or entinostat, on their own.(409) This suggests that the combination of a HDACi and a DNMTi might demonstrate greater synergy together, by shifting the immune cells in the TME toward a more immune permissive state and, thus, predispose to greater benefit from ICI therapy. Decitabine has also been shown to increase expression of antigen processing and presenting genes, and cytokine and chemokine-related genes, such as CXCL1, in CT26 CRC cells and PDX models.(410) On the other hand, genes involved in cell proliferation were downregulated, and there was influx of CD4+ and CD8+ T cells into the tumours.(410) When decitabine and PD-1 blockade were administered concurrently, tumour growth was significantly attenuated, and tumour bearing mice had survived longer with the combination, than with either drug alone.(410)

Exposure of oesophageal cancer cell lines to an HDACi led to increased expression of MAGE-3, which is a tumour associated antigen corelated to tumour immunogenicity, and often aberrantly expressed in oesophageal carcinoma.(411) Furthermore, in a phase I study of decitabine in patients with solid tumours including oesophageal cancer, the tumour associated antigens, NY-ESO-1, MAGE-3, and p16, were found to be significantly induced in the on-treatment biopsies compared to baseline.(411,412) This study provided proof of concept that epigenetic modifying therapy could enhance neoantigen expression in upper GI cancer. One recent study evaluating three different HDAC inhibitors (Trichostatin A, SAHA, and sodium butyrate), showed that these agents reduce IFN-γ-induced B7-H1 expression (which protects tumour cells from cytotoxic killing by T cell apoptosis, T cell exhaustion, or IL-10 production) in mouse models of gastric cancer,(413) resulting in decreased tumour growth.(414) Those treated with SAHA also demonstrated higher levels of infiltrating CD8+ T cells within the tumours.(414) Thus, HDAC inhibition could overcome immune exhaustion and improve responses to ICs. In another study, mice with OAC exposed to one week of DNMT treatment or placebo, followed one week of HDACi treatment or placebo, followed by a dose of PD-1 inhibitor or placebo on day 12 of a 14-day cycle, displayed a decrease in tumour volume and an increase in CD3+ and CD8+ T cells compared to placebo, however there was no difference in survival.(415) Expression of CD206, TNFα, and Arg1, which are involved in activation of TAMs, were also significantly upregulated in mice who received the active drugs compared to placebo.(415) Thus, sequential DNMT inhibition followed by HDAC inhibition, appears to favourably reprogramme the TME by increasing levels of activated immune cells in the vicinity which may, therefore, be able to more effectively collaborate with ICIs.

In the clinic, several trials of ICIs combined with “epi-drugs” are currently underway in patients with mCRC, and some have reported early results. A pilot study in 24 patients with chemotherapy-refractory MSS mCRC who received either 5-AZA, romidepsin (HDACi), or combination of both, followed by administration of pembrolizumab, established that the combination of these agents with checkpoint blockade, was safe and tolerable.(416) A similar study of low-dose AZA combined with pembrolizumab in 30 patients with pre-treated

mCRC also established safety of the combination, with the majority of TRAEs experienced being grade 1 or 2 only.(417) The trial was stopped early due to lack of clinical efficacy (ORR 3%; median PFS 1.9 months; median OS 6.3 months) however, correlative translational work demonstrated increased CD8+ TIL infiltration in the 'on-treatment' biopsies compared to baseline biopsies,(417) suggesting that AZA and pembrolizumab results in a more T cell 'inflamed' TME. Combination of the class-I selective HDACi, entinostat, with pembrolizumab in patients with heavily pre-treated pMMR/MSS mCRC, has also established safety and encouraging efficacy with 6 patients (1 PR, 5 SD) out of 16 still on treatment at the time of data cut-off.(203) Results of the phase II Carosell trial, evaluating zabadinostat, HDACi, with nivolumab in patients with pre-treated MSS CRC also appear promising. Almost half of the 46 evaluable patients had prolonged disease control, and 4 patients are still alive after 3 years.(202) The combination was well-tolerated, and no patient came off study due to toxicity.(202)

In advanced OGA, a phase II study assessing the safety and efficacy of the combination of tucidinostat, a class I HDACi, with toripalimab, PD-1 inhibitor, is currently recruiting (NCT05163483). Additionally, results from a phase II study evaluating the benefit of epacadostat, an inhibitor of IDO-1, which impedes host anti-tumour immune responses through catabolism of tryptophan, which is required for T cell activity,(418) plus pembrolizumab in advanced OGA, are awaited (NCT03196232).

2.1.6 The histone deacetylase inhibitor (HDACi) domatinostat

The HDAC family consists of 18 enzymes split into four classes. The "classical" HDACs include class I (HDAC1-3 and HDAC8), class II (HDAC4-7, HDAC9, and HDAC10), and class IV (HDAC11) proteins.(419) Class III include the Sir2-like proteins (SIRT1-7). Domatinostat (4SC-202) is an oral selective inhibitor of class I HDAC with an IC50 of 1.20µM, 1.12µM, and 0.57µM for HDAC1, HDAC2, and HDAC3, respectively. Preclinically, it has demonstrated effective inhibition of Wnt

and Hedgehog signalling pathways, as well as inhibition of tumour growth and angiogenesis in multiple cell lines including CRC.(420–425)

In syngeneic mouse models, domatinostat has been shown to favourably influence the immune TME by upregulating tumour associated antigen expression, MHC class I and II expression, as well as increased cytotoxic T cell infiltration, which are all features known to correlate with clinical responses to anti-PD-1/PD-L1 antibodies.(426) Furthermore, the combination of anti-PD-L1 therapy and domatinostat reduced tumour growth in CT26 mouse models of CRC, which are known to have low intrinsic responses to ICI therapy, more so than with domatinostat alone.(426) Response rates were higher in C38 CRCs, which have higher levels of pre-existing cytotoxic CD8+ cells, as would be expected. Intratumoral cytotoxic CD8+ cell infiltration was increased in both mouse models of CRC, but more so in the model which had lower CD8+ cells to begin with, in addition to upregulation of genes involved in IFN-γ signalling, which promotes anti-tumour immune responses in several ways, for example, by upregulating the expression of MHC molecules on tumour and immune cells, and promoting T cell trafficking through upregulating chemokines CXCL10 and CXCL11.(426) Interestingly, in the C38 models, which have higher baseline cytotoxic T cell levels, domatinostat appeared to induce a larger population of T cells which co-expressed PD-L1+/LAG3+.(426) Successively, when a triple combination of domatinostat, anti-PD-1, and anti-LAG3 were applied to the C38 tumour model, even greater responses were seen than with the domatinostat and anti-PD-1 combination.(426) Importantly, domatinostat alone was only able to reduce tumour growth in immunocompetent mice and not in immunodeficient mice, suggesting that an intact immune system is required for domatinostat antitumoral efficacy.(426) The combination of domatinostat and anti-PD-L1 therapy reduced MDSC infiltration in the CT26 tumour model, but not peripheral MDSC levels.(426) This suggests that the tumour specific changes were due to modification of the TME rather than general immune cell numbers.

Domatinostat was first evaluated in humans in the phase I TOPAS study.(427) In this study, 24 patients with advanced haematological malignancies were treated with 7 different doses of domatinostat either once or twice daily, day 1-14 with 7

days off, or with continuous daily dosing (up to 400mg total daily dosing (TDD), in a “3+3” design.(427) Domatinostat was well tolerated, and only 1 DLT (grade 4 hypercalcaemia was observed). The MTD was not determined and the recommended phase II dose for further domatinostat monotherapy studies was 400mg TDD in a 200mg twice daily schedule, for 14 days on and 7 days off. The most common adverse events were nausea (29%), diarrhoea (38%), and fatigue (46%). Out of 24 patients, 18 patients had stable disease as best response, 1 patient had a CR and 1 patient had a PR.

In phase Ib of the SENSITIZE study, 40 patients with progressive, unresectable, or metastatic cutaneous melanoma with a best response of SD or PD to prior anti-PD-1 therapy, were treated with domatinostat in combination with pembrolizumab.(428) Similarly, to the TOPAS study, the most frequent TRAEs were diarrhoea (23%), nausea (20%), and fatigue (20%), 8 patients (20%) had ≥ grade 3 AEs. There was also a signal of activity as 30% of patients achieved disease control across all dose levels examined.(428) Relatively few baseline and on-treatment biopsies were collected, however after 14 days of “priming” with 100mg of domatinostat, analysis of gene expression profiles correlated with the findings seen with domatinostat in mouse models.(428) Domatinostat appeared to increase IFN-γ expression and gene expression signatures, which have been associated with response to pembrolizumab. Additionally, domatinostat treatment resulted in higher expression of MHC genes and greater T cell infiltration.(428) Interestingly the patient who that the highest upregulation of gene expression was one who had the lowest number of immune cells present in the pre-treatment biopsy.(428) These data suggest that ‘priming’ with domatinostat can indeed reset a TME both lacking in T cells, and which has developed intrinsic resistance to prior ICI therapy, into an ‘inflamed’, cooperative environment again, for ICIs to work effectively. The immune priming effects of domatinostat, determined from these preclinical and clinical studies, therefore hold promise for patients with pMMR/MSS OGAs and CRCs, which are typically immune-desert or immune-excluded tumours, poorly infiltrated by lymphocytes.

2.1.7 The PD-L1 inhibitor avelumab

Avelumab is a fully human antibody which binds to PD-L1 on tumour cells and blocks its interaction with PD-1 on T cells. By removing the suppressive effect of PD-L1 on anti-tumour CD8+ T cells, cytotoxic T cell responses are restored. Avelumab is currently approved for the treatment of metastatic Merkel-cell carcinoma, in combination with axitinib for first line treatment of advanced renal cell carcinoma, and for locally advanced/metastatic urothelial cell carcinoma after progression during or following platinum-containing chemotherapy and/or as a maintenance for patients who have not progressed during first line platinum-containing chemotherapy. It has a well described safety profile which was superior to chemotherapy in patients with advanced gastric cancer when used as a maintenance therapy as a single agent compared to continuing chemotherapy, however overall survival was not improved in this population with maintenance avelumab.(429) Based on the efficacy data across multiple tumour types and the excellent safety profile of the drug, avelumab was considered a promising agent for combination with domatinostat in the EMERGE trial.

This chapter outlines the analysis and results of phase IIa of the EMERGE trial. I became the Trial Physician for the EMERGE trial in May 2019. During my time as Trial Physician, I ran all aspects of the day-to-day conduct of the phase IIa component of the study, which included responding to queries from sites, patient recruitment, assessing safety events as delegate of the CI, writing protocol amendments, and data cleaning prior to each Safety Review Committee (SRC) meeting. As a member of the TMG, I also prepared TMG reports and was involved in data analysis. Together with the Trial Manager, Claire Saffrey, Statistician, Ria Kalaizaki, and database programmer, I designed the electronic case report forms (CRFs) and trial database, as the trial initially opened on paper CRFs. I took the study through 3 SRC meetings where the safety data of each dose cohort were reviewed once 3 patients had completed the Dose Limiting Toxicity (DLT) period at each dose level. Prior to each SRC meeting, I thoroughly cleaned all patient data, prepared the SRC report alongside the study statistician, and prepared additional data as requested by the SRC chair. The results of the phase IIa component of the trial were presented as a poster at the ESMO 2020 annual

congress (see appendix for copy of poster). The following chapter includes updated safety and efficacy results, and survival data.

2.2 Methods

2.2.1 Study design and treatment

This was an open-label, single-arm phase IIa/b trial to assess the safety, tolerability, and efficacy of domatinostat plus avelumab in patients with previously treated, immunotherapy-naïve, advanced, unresectable pMMR/MSS OGA and CRC. Figure 9 displays the EMERGE study schema. The primary endpoint of phase IIa (safety run-in phase) was to establish a safe and tolerable dose of domatinostat combined with avelumab for use in the main efficacy phase (phase IIb); the evaluation of anti-tumour activity in phase IIa was an exploratory objective. The primary endpoint for the phase IIb (efficacy phase) was the best ORR according to RECIST 1.1 (either CR or PR) by 6 months from the initiation of combination treatment in each cohort (OGA and CRC). Secondary endpoints included safety and tolerability of the study treatment in the safety population according to NCI-CTCAE version 4.03, PFS, and OS.

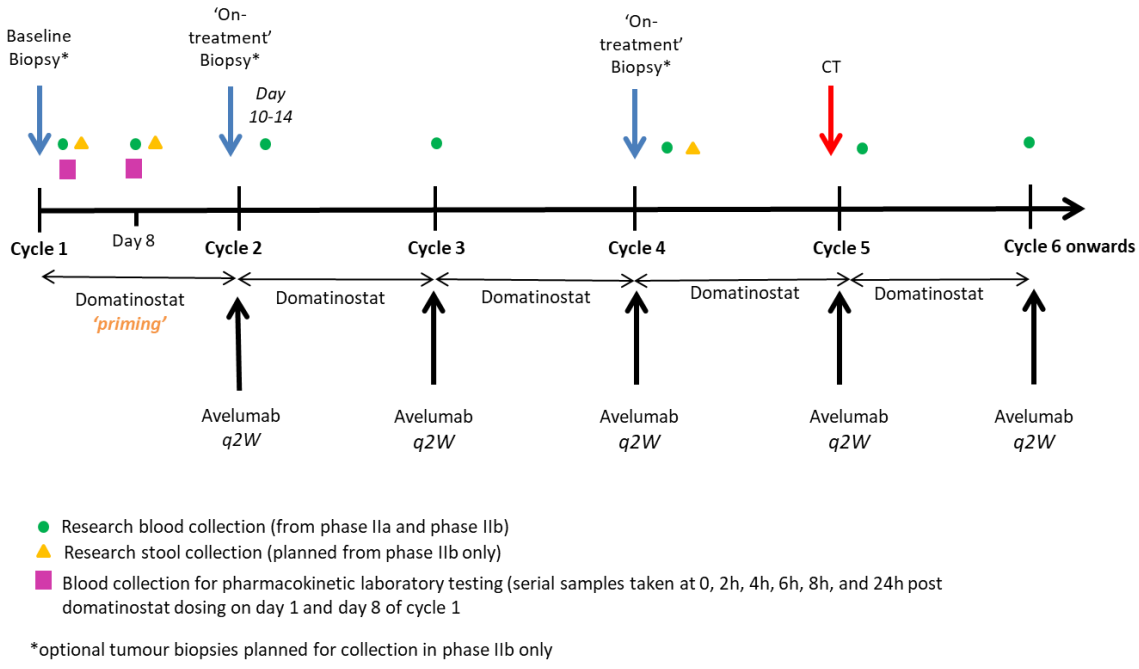


Figure 9 EMERGE study schema

Phase IIa was a dose-defining phase that followed a “3+3” design. Three dose levels of oral domatinostat (100mg OD, 200mg OD, and 400mg total daily dose (TDD)) taken continuously, combined with a fixed dose of avelumab (10mg/kg) ever 2 weeks (Table 11), were assessed for DLT. Based on the first in human study with domatinostat monotherapy in patients with haematological malignancies, the recommended phase II dose (RP2D) was 200mg twice daily on day 1-14, every 3 weeks. The rationale for dosing below this RP2D for domatinostat monotherapy in the EMERGE study, was because domatinostat was being used to prime the TME rather than for its direct anti-tumour effects, therefore lower doses may have been sufficient (particularly to mitigate any potentially additive toxicity from the ICI). Furthermore, in the mouse CRC studies where domatinostat and anti-PD1/anti-PD-L1 were evaluated, the mice were given a daily dose of either 40mg/kg or 60mg/kg domatinostat continuously for 12-14 days (CT26) or up to 24 days (C38), anti-PD-1 was given twice weekly for two weeks, and anti-PD-L1 every 3 days for eight injections. Therefore, from this preclinical data, it was presumed that continuous dosing of domatinostat would be needed for optimal effects, rather than the two weeks on, one week of regimen. Oral domatinostat monotherapy was administered for 2 weeks prior to adding in avelumab. A DLT was defined as an AE occurring during the first two cycles of combination domatinostat and avelumab, that met predefined criteria. The SRC reviewed all data before escalating to a higher dose level. Per protocol, no escalation beyond domatinostat 400mg TDD was permitted. If a maximum tolerated dose (MTD) could not be established after evaluation of all dose levels, the dose for expansion would be determined based on the safety data.

Originally, the final dose level of domatinostat in the EMERGE protocol was set at 300mg TTD, however, in September 2019, results of the SENSITIZE study, which evaluated the efficacy of domatinostat and pembrolizumab in patients with checkpoint-inhibitor refractory advanced melanoma, were presented at the ESMO Annual Congress. The data showed a trend toward dose-dependent clinical activity with domatinostat 400mg TDD (split 200mg BD) in combination pembrolizumab, as patients stayed on study treatment for longer, and without additional toxicity.(430) Furthermore, in the TOPAS study, both AUC and Cmax values for BD dosing were higher at single, and during steady state timepoints.

Therefore, split dosing of 200mg BD may increase drug plasma levels, potentially leading to more beneficial modulation of the immune microenvironment. I therefore discussed changing the final dose level in the EMERGE study from domatinostat 300mg TDD, to 400mg TDD (split 200mg BD) firstly with 4SC, the Pharma company who funded the trial and supplied domatinostat, and subsequently with the EMERGE Chief Investigator and TMG. With TMG agreement, I amended the protocol and study documents to change the final dose level to domatinostat 200mg BD.

	Dose level 1 (1st cohort)	**Dose level 2** (2nd cohort)	**Dose level 3** (3rd cohort)
Domatinostat (Continuous dosing)	100mg OD	200mg OD	400mg TDD Split dose 200mg mane/200mg nocte
Avelumab	10mg/kg	10mg/kg	10mg/kg

Table 11 Dose levels during the EMERGE safety run-in phase

Patients could receive domatinostat plus avelumab until confirmed disease progression, intolerability, death, or patient withdrawal. Grade 3/4 toxicities were managed by dose delays (up to 4 weeks i.e., 6 weeks from day 1 of the previous cycle) and/or reductions. Only two dose reductions were permitted for domatinostat. Dose reductions of avelumab were not permitted.

2.2.2 Patients

Adult patients with histologically confirmed MSS, or pMMR, advanced and/or inoperable, or metastatic OGA or CRC, who had disease progression after at least one prior line of chemotherapy treatment were eligible. Patients with no established treatment option, or patients who decided against an established treatment option were also eligible. Patients were required to have adequate bone marrow, renal, and liver function, and an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1. I updated inclusion criteria during recruitment as a protocol amendment, to only permit patients with measurable disease (at least one target lesion) by RECIST 1.1,(271) as originally patients with radiologically non-evaluable disease were also allowed. This was necessary because the primary endpoint of the phase IIb was ORR and, thus, measurable disease at baseline is a prerequisite for this assessment.(271) Patients on oral anticoagulation needed to change to low molecular weight heparin to be eligible, and it was necessary for all sexually active patients to use highly effective contraception if there was a possibility of conception.

Patients with any prior immunotherapy treatment or other immunomodulatory drugs, other active malignancy, immunodeficiency disorder, or active autoimmune disease requiring systemic treatment in the past 2 years (excluding replacement therapy such as thyroxine, insulin, or physiologic corticosteroid replacement therapy), were excluded. Other exclusion criteria included any patients with brain metastases which were unstable, symptomatic, or required steroids, cerebrovascular disease within the previous 6 months, active infection including HIV or known acquired immunodeficiency syndrome, hepatitis A or C or active hepatitis B, patients unable to swallow orally administered medication, or any malabsorption disorder, and any significant cardiovascular disease. Although not reported when domatinostat was evaluated in patients with haematological malignancies in the TOPAS study,(427) as QTc-prolongation is a known class effect of HDAC inhibitors,(431) patients with evidence of baseline prolongation of QT/QTc interval, or taking concomitant agents known to prolong the QT interval, were also excluded.

2.2.3 Safety assessment

Safety evaluations were performed throughout the study, and all adverse events (AEs) were graded for severity according to the CTCAE v 4.03, and relationship with domatinostat or avelumab. DLT criteria included any ≥ grade 3 toxicity that was possibly, probably, or definitely related to avelumab or domatinostat, occurring during the first two cycles of combination treatment, except any of the following:

- Grade 3 infusion-related reaction resolving within 6 hours and controlled with medical management.
- Transient (≤ 6 hours) grade 3 flu-like symptoms or fever, which is controlled with medical management.
- Transient (≤ 24 hours) grade 3 fatigue, local reactions, headache, nausea, emesis that resolves to ≤ grade 1.
- Grade 3 diarrhoea, grade 3 skin toxicity, or grade 3 liver function test (ALT, AST, or GGT) increase that resolves to ≤ grade 1 in less than 7 days after medical management (e.g., immunosuppressant treatment) has been initiated.
- Single laboratory values out of normal range that are unlikely related to trial treatment according to the investigator, do not have any clinical correlate, and resolve to ≤ grade 1 within 7 days with adequate medical management.
- Tumour flare phenomenon defined as local pain, irritation, or rash localized at sites of known or suspected tumour.

Patients were reviewed at 30 days +/- 7 days after completion of study treatment for an end of treatment review and adverse event reporting. An additional extended safety follow-up was performed 90 days after the last dose of avelumab administration.

2.2.4 Pharmacokinetics

Serial blood samples were taken at 0 (30 minutes pre domatinostat dosing) and 2h, 4h, 6h, 8h, and 24h after domatinostat dosing, on day 1, and day 8, of cycle 1 (domatinostat monotherapy). Samples were centrifuged within 30 minutes of collection and plasma was frozen at - 80°C before shipping to SwissBioQuant AG, Switzerland on dry ice. Samples were analysed for serum concentrations of domatinostat using a validated HPLC-MS/MS method.

2.2.5 Antitumour activity

Imaging assessments were performed at screening (within 20 days prior to starting treatment), at 8 weeks from treatment initiation, and every 6 weeks thereafter. All responses to treatment (CR, PR, and SD) were confirmed with repeat imaging within 4-6 weeks. Response was evaluated by RECIST 1.1 and iRECIST.(271,432)

2.2.6 Statistical plan and data analysis

In the phase IIa safety run-in a standard “3+3” design was utilised. Therefore, with 3 potential dose levels, the phase IIa was expected to recruit between 3-18 patients (both OGA and CRC patients). In the phase IIb efficacy phase, a Simon two stage optimal design was used with different *a priori* expectations of response in each cohort. In the CRC cohort, to rule out a best ORR of 5% while aiming for a rate of 20%, with significance level of 5% and 80% power, requires 1/10 responses for the first stage and 4/29 for the second stage. In the OGA cohort, to rule out a best ORR of 15% while aiming for a rate of 35%, with a significance level of 5% and 80% power, requires 2/9 responses for the first stage and 9/34 for the second stage. A planned interim analysis of the accumulating efficacy and toxicity data was to be performed according to the Simon two stage design after

10 and 9 patients, respectively, in CRC and OGA cohorts, have reached the 6-month mark from combination treatment initiation. The phase IIa was only to recruit from The Royal Marsden Hospital. Allowing for up to 18 patients to be recruited, it was anticipated that this could take up to 9 months to complete.

Safety data was reviewed from all patients who received at least one dose of either trial drug. Dose limiting toxicities (DLTs) were recorded during the patient's DLT period which was defined as 28 days from the initiation of combination treatment (equivalent to two cycles of combination treatment). If a patient experienced a break in treatment during the DLT period, an extension to the DLT period was applied which was equivalent to the length of break in treatment. Patients were to be replaced for DLT assessment if they had either (i) discontinued treatment during their DLT period and did not experience a DLT, (ii) experienced a treatment break longer than 4 weeks during the DLT period due to treatment-related toxicity, or (iii) experienced a treatment break longer than 3 weeks during the DLT period for reasons other than treatment-related toxicity.

Adverse events were summarised descriptively, and no statistical inference was performed. Anti-tumour activity was assessed in patients who received at least one cycle of domatinostat plus avelumab combination, and were either evaluable for tumour assessment, or experienced clinical progression. The two-sided 95% confidence intervals (CIs) of ORR and PR rates were calculated using the Clopper-Pearson method. Median PFS, OS, and disease control rates at 6 and 12 months from the start of combination treatment were summarised by Kaplan-Meier estimates and presented alongside two-sided 95% CIs. Median follow-up was calculated using the reverse Kaplan-Meier method.

2.3 Results

2.3.1 Patient characteristics

Between 5th February 2019 and 10th December 2020, 13 patients with advanced OGA and CRC were enrolled and included in the safety and efficacy analysis. Domatinostat was taken by 4 patients at 100mg/day, 3 patients at 200mg, and 6 patients at 400mg/day, in combination with avelumab from cycle 2 onwards. At the data cut-off date (12th August 2022), the median duration of follow-up was 14.6 months (95% CI lower bound 9.9 months – upper bound not quantified) in the CRC cohort, and 14 months in the OGA cohort (95% CI could not be quantified). The patients' characteristics are presented in Table 12. The median age was 55 years, with 54% being male. Most patients had CRC (85%) rather than OGA (15%), and most had received at least 2 prior lines of anti-cancer therapy (77%). Most patients had liver (46%), and/or lung metastases (46%), at trial entry. The median time from initial diagnosis to trial entry was 2.8 years, and the median duration of patients' last line of anti-cancer therapy prior to trial entry was 2.5 months.

Characteristic (N=13)	
Gender, n (%)	
Female	6 (46)
Male	7 (54)
Age, year	
Median (IQR)	55 (50 – 66)
Min - Max	36 - 81
Ethnicity, n (%)	
Caucasian	10 (77)
Other	3 (23)
Cancer cohort, n (%)	
Colorectal (CRC)	11 (85)
Oesophagogastric (OGA)	2 (15)
ECOG, n (%)	
0	2 (15)
1	11 (85)
Number of prior lines for advanced disease, n (%)	
1	3 (23)
≥ 2	10 (77)
Sites of metastases at trial entry, n (%)	
Liver	6 (46)
Lung	6 (46)
Distant lymph nodes	3 (23)
Regional lymph nodes	2 (15)
Peritoneum	3 (23)
Bone	1 (8)
Other	2 (15)
Disease stage at diagnosis, n (%)	
I	0 (0)
II	1 (8)
III	6 (46)
IV	6 (46)
Any history of prior radiotherapy, n (%)	
Yes	5 (38)
No	8 (62)
Time from initial diagnosis to trial entry, year	
Median (IQR)	2.8 (2.2 – 3.4)
Min - Max	0.9 – 9.8
Duration of last line of prior anti-cancer therapy, month	
Median (IQR)	2.5 (1.4 – 3.0)
Min - Max	1.0 – 6.5

Table 12 Baseline patient demographic and clinical characteristics

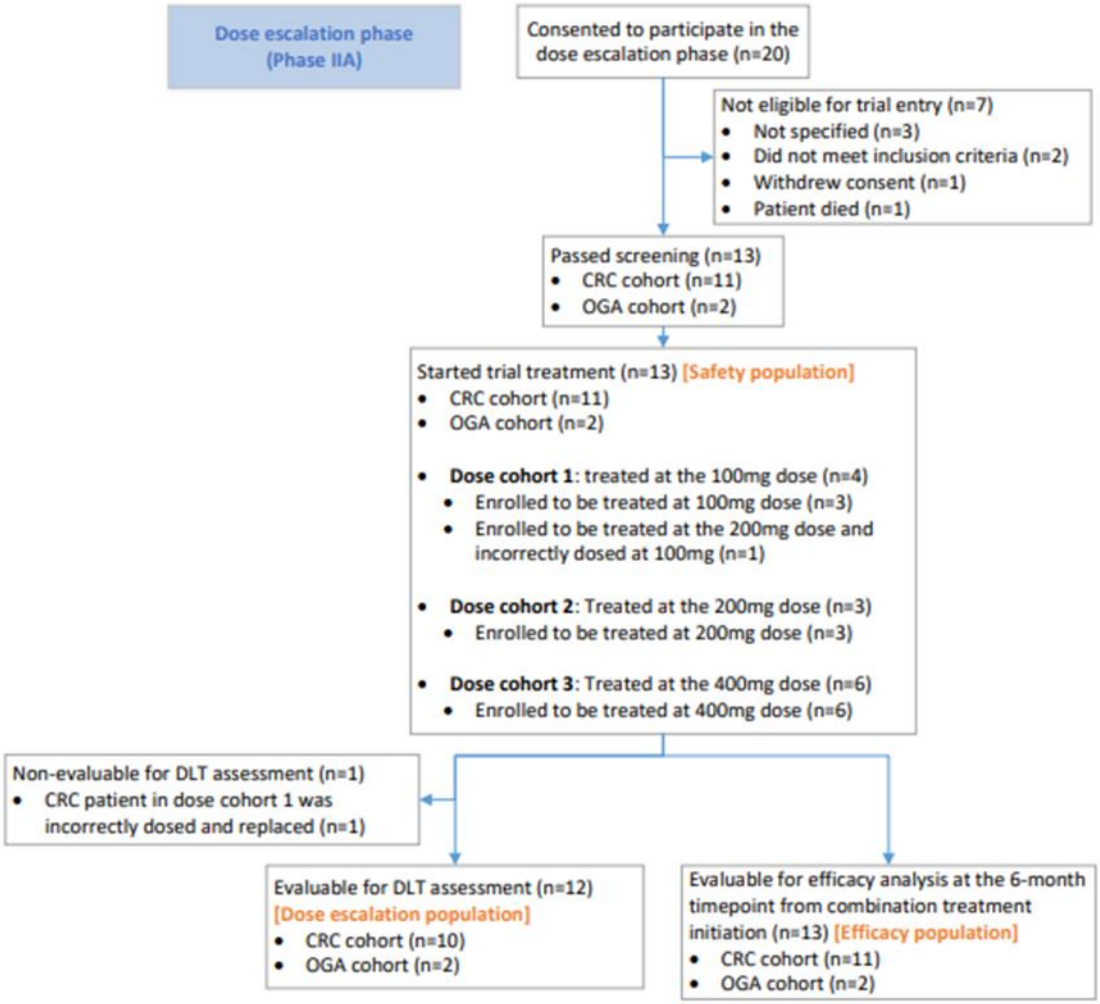


Figure 10 EMERGE Phase IIa flow diagram

The fourth patient enrolled onto the trial was meant to be treated as the first patient in the second dose cohort but was erroneously treated with domatinostat 100mg OD rather than 200mg OD. As trial physician, I immediately brought this to the attention of the CI and reported the incident to our internal Good Clinical Practice (GCP) team. The research site team reported the event via our local incident reporting pathway (DATIX), and the patient was informed as a priority. Upon investigation into the potential cause of this error, I uncovered that the electronic clinical trial prescription was set-up incorrectly as the domatinostat dose was fixed at 100mg, rather than in a variable dose format which could be altered dependent upon on cohort allocation (i.e., 100mg OD, 200mg OD, or 400mg TDD). Consequently, the electronic prescription proforma was swiftly corrected. Together with the Trial Manager, Claire Saffrey, I also instructed a better system of communication to the pharmacy team to indicate when we were moving onto the next dose cohort, as one of the corrective and preventative actions (CAPAs) put in place.

Following discussion with the CI and GCP team, we concluded that the event did not affect patient safety, or the scientific value of the trial. As the 100mg dose had already been determined as safe and tolerable following the SRC meeting on 19th June 2019, there were no safety concerns regarding the patient having received this dose. As this patient would not count towards the second dose cohort, we replaced this patient with another patient who was treated with the 200mg dose (Figure 10), thus maintaining the scientific value of the trial. After learning of the dosing error, and following full reassurance of lack of safety concerns at this dose level thus far, the patient still wanted to continue on trial at the 100mg dose. All scheduled assessments took place as per the study protocol except collection of PK bloods samples, as these had already been collected from 3 patients at this dose level. I informed the SRC chair of the event, and the Trial Manager informed the Research Ethics Committee (REC) chair.

2.3.2 Treatment exposure and compliance

No patient remained on treatment at the time of data cut-off; the final patient received their last cycle on 8th March 2022. Of the 13 patients treated during the dose escalation phase, 12 (92%) patients discontinued treatment due to radiological progression, and 1 (8%) patient for clinical progression.

All patients took at least 80% of all doses of domatinostat during their time on treatment. Six patients (46%) missed at least 1 domatinostat tablet across their cycles, and 1 patient required a dose reduction in 1 cycle from 400mg to 200mg domatinostat due to AEs (grade 2 fatigue, anorexia, constipation, and nausea). Six patients (46%) required a treatment delay mostly due to AEs.

The median time on treatment (measured from combination treatment initiation to treatment discontinuation or death) for the 11 patients in the CRC cohort was 3.2 months (min: 1.3, max: 11.7), and 10.5 months for the 2 patients in the OGA cohort (min: 7.9, max: 13.1). The median number of treatment cycles received by all 13 patients was 8 cycles (min: 4, max: 29); 6 cycles (min: 4, max: 25) in the CRC cohort, and 20 cycles (min: 11, max: 29) in the OGA cohort.

2.3.3 Safety and tolerability

The most common TRAEs reported during the study are displayed in Tables 13, 14, and 15 (any grade in ≥ 2 patients; grade ≥ 3 in at least 1 patient). During the trial 75 TRAEs were reported – of these, 71 were reported as related to domatinostat, 66 were reported as related to avelumab, and 61 were reported as either probably or possibly related to both avelumab and domatinostat across the trial. The most common TRAEs of any grade related to either domatinostat or avelumab were fatigue (77%), anorexia (38%), nausea (38%), and anaemia (31%). There was only 1 (8%) grade ≥ 3 TRAE of increased alkaline phosphatase. Immune-related AEs (IRAEs) of any grade reported in more than one patient included maculo-papular rash (15%), hypothyroidism (8%),

hyperthyroidism (8%), aspartate aminotransferase increased (8%), and pruritus (8%); no events were grade ≥ 3 (Table 15). There were no serious adverse events related to domatinostat and/or avelumab, and no treatment-related deaths occurred.

Adverse event	All grades n (%)	Grade ≥ 3 n (%)
Fatigue	10 (77)	0 (0)
Anorexia	5 (38)	0 (0)
Nausea	5 (38)	0 (0)
Anaemia	4 (31)	0 (0)
Alkaline Phosphatase Increased	3 (23)	1 (8)
Diarrhoea	3 (23)	0 (0)
Dry Skin	3 (23)	0 (0)
Insomnia	3 (23)	0 (0)
Constipation	3 (15)	0 (0)
Electrocardiogram Qt Corrected Interval	2 (15)	0 (0)
Mucositis Oral	2 (15)	0 (0)
Rash Maculo-Papular	2 (15)	0 (0)
Serum Amylase Increased	2 (15)	0 (0)

Table 13 TRAEs related to domatinostat experienced by ≥ 2 patients (any grade) and any grade ≥ 3 TRAE experienced by one or more patients

Adverse event	All grades n (%)	Grade ≥ 3 n (%)
Fatigue	9 (69)	0 (0)
Anorexia	5 (38)	0 (0)
Anaemia	3 (23)	0 (0)
Diarrhoea	3 (23)	0 (0)
Dry Skin	3 (23)	0 (0)
Serum Amylase Increased	3 (23)	0 (0)
Alkaline Phosphatase Increased	2 (15)	1 (8)
Constipation	2 (15)	0 (0)
Fever	2 (15)	0 (0)
Hypothyroidism	2 (15)	0 (0)
Insomnia	2 (15)	0 (0)
Nausea	2 (15)	0 (0)
Rash Maculo-Papular	2 (15)	0 (0)
Vomiting	2 (15)	0 (0)

Table 14 TRAEs related to avelumab experienced by ≥ 2 patients (any grade) and any grade ≥ 3 TRAE experienced by one or more patients

Adverse event	All grades n (%)	Grade ≥ 3 n (%)
Aspartate Aminotransferase Increased	1 (8)	0 (0)
Hyperthyroidism	1 (8)	0 (0)
Hypothyroidism	2 (15)	0 (0)
Pruritus	1 (8)	0 (0)
Maculo-Papular rash	2 (15)	0 (0)

Table 15 Immune-related adverse events experienced by one or more patients

No DLT was observed and no formal MTD was reached. Three patients had an extension to their DLT period due to adverse events: one patient in the 100mg domatinostat dose cohort by 17 days due to grade 3 infection, and 2 patients in the 400mg domatinostat dose cohort by 7 days each due to grade 2 fatigue, and grade 3 atrial fibrillation (AF), respectively. Grade 2 fatigue was considered possibly related to study treatment. Both the grade 3 TRAEs were initially reported as SUSARs but then downgraded to SAEs once further information was available, and therefore, were not considered DLTs. The patient with grade 3 infection was admitted with hypotensive sepsis based on clinical and biochemical parameters when they attended for their third cycle of treatment. No infective source was found, however, imaging suggested direct tumour invasion into the hepatobiliary system and, therefore, a potential cause. Following discussion and agreement with the CI, I therefore downgraded the event to an SAE. The patient with grade 3 AF experienced two episodes of fast AF during the DLT period. Initially this was presumed to be secondary to an infection, but after the second occurrence, possible cardiotoxicity of either drug could not initially be excluded, and the event was reported as a SUSAR. After a series of cardiac investigations and assessment by a cardiologist, it was felt that due to the patients age, dilated left atrium on ECHO, and a series of tachy-brady arrhythmias on a 24h Holter, that the patient was at high risk of developing AF independent of trial treatment. The patient therefore continued on trial, and the event was considered an SAE.

2.3.4 Pharmacokinetics

PK samples were analysed in 7 patients (4 patients in the 100mg dose cohort, and 3 patients in the 200mg dose cohort). Preliminary data indicate that domatinostat concentrations peaked at 2 hours post dosing. The geometric mean (%CV) Cmax and area under the concentration-time curve of domatinostat ranged from approximately 67-156 ng/mL and 123-220 ng/mL, and 598-1740ng/mL x hour and 1860-2460 ng/mL x hour, respectively, across the 100-200mg domatinostat once daily dosing (D1-14 q2w) at cycle 1 day 1 (single dose), and cycle 1 day 8 (steady state). Accumulation of domatinostat exposure was thus greater with the 200mg once daily dosing after 7 days, compared to 100mg once daily dosing. These PK profiles were consistent with those previously reported for domatinostat monotherapy. Unfortunately, 4SC, the drug company who provided the funding and supply of domatinostat for the EMERGE study, chose not to fund any further PK analysis after these results. Therefore, no further data is available for these 7 patients, and the samples taken from the 6 patients treated with 400mg TDD were never analysed.

2.3.5 Anti-tumour activity

The efficacy population consisted of all 13 treated patients (11 CRC, 2 OGA). Of the 11 evaluable patients in the CRC cohort, 5 patients experienced disease control during the 6 months from combination treatment initiation. The best DCR at 6 months from combination treatment initiation in the CRC cohort was, therefore, 45.5% (95% CI: 16.7, 76.6), and median duration of disease-control was 5.7 months (IQR: 3.5, 7.0). No CR or PR was achieved in the CRC cohort. One patient in the CRC cohort stayed on study treatment for almost 1 year, receiving 25 cycles. At the time of study entry, he was 51 years old and had low volume metastatic disease in his lungs (sub-centimetre lesions), which had recently progressed following first line FOLFIRI chemotherapy. His disease was evaluable but non-measurable by RECIST 1.1 criteria, and stayed stable for almost 12 months on treatment. Six patients (55%) in the CRC cohort had liver

metastases at trial entry, and all but 1 of these patients had progressed by the time of the first on-treatment scan, or before.

Both patients in the OGA cohort experienced disease control during the 6 months from combination treatment initiation. The best DCR at 6 months from combination treatment initiation in the OGA cohort was, therefore, 100% (1-sided 97.5% CI: 15.8, 100), and median duration of disease control was 5.4 months (IQR: 4.5, 6.2). One patient in the OGA cohort experienced a PR (Figure 12), therefore best ORR during the 6 months from combination treatment initiation in the OGA cohort was 50% (95% CI: 1.3, 98.7). The duration of this one response was 6.2 months and this patient stayed on study treatment for over 12 months (Figure 11), receiving 28 cycles of treatment without significant toxicity. This patient was 54 years old at the time of trial entry and had MSS, HER2 positive OGJ adenocarcinoma with nodal metastases. He entered EMERGE after progressing on maintenance trastuzumab, following initial disease control from 6 cycles of cisplatin, capecitabine, and trastuzumab. The PD-L1 CPS from his tumour, assessed with the DAKO 223C assay, was 9. PD-L1 status was not assessed on tumour tissue from the other OGA patient. None of the patients in the OGA cohort had liver metastases at trial entry.

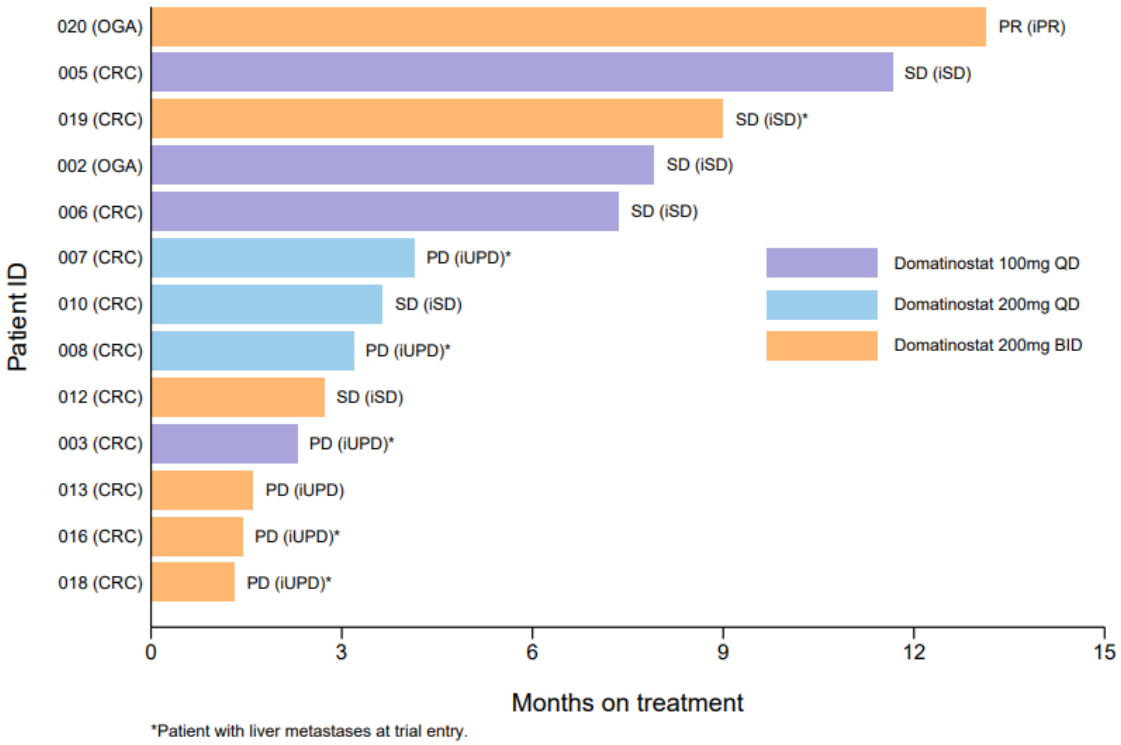


Figure 11 Duration of treatment and best response by RECIST 1.1 and iRECIST (n=13). Patients with liver metastases at trial entry are marked with an asterisk. Duration of treatment is measured from treatment initiation (day 1 of cycle1).

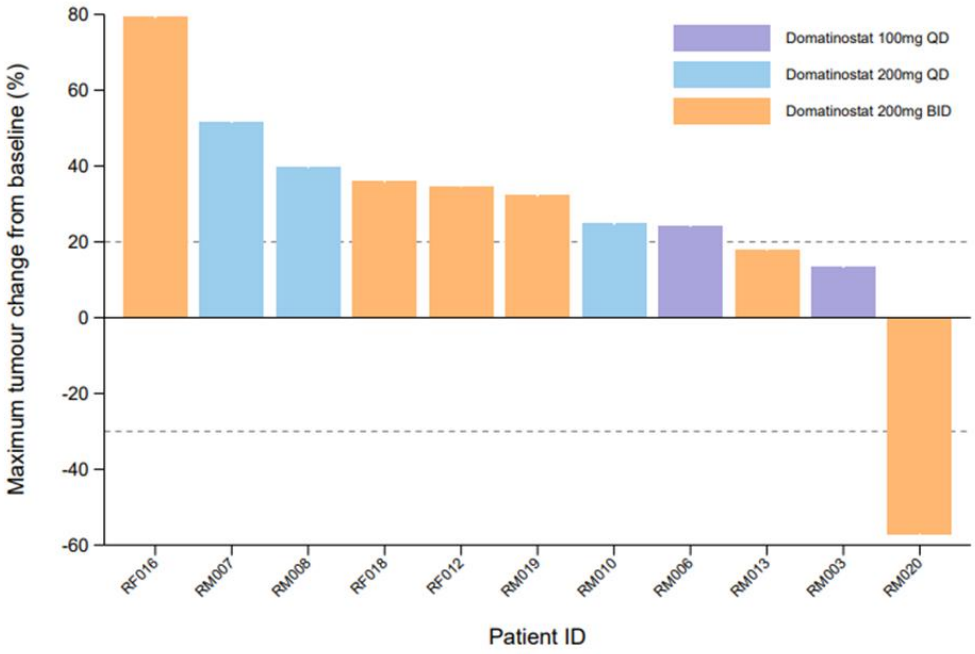


Figure 12 Waterfall plot (n=11). Maximum tumour change from baseline by patient across all follow-up scans (RM002 and RM005 are excluded from the waterfall plots because they did not have measurable disease, i.e., they had no target lesions measured at baseline and only had non-target lesions)

All 13 treated patients were evaluable for PFS and OS analysis. Of the 11 CRC patients, 5 patients progressed, and 3 patients died within 6 months from the start of combination treatment; 6-month PFS rate in the CRC population was, therefore, 27.3% (95% CI: 6.5, 53.9), and 6-month OS rate was 72.7% (95% CI: 37.1, 90.3) (Table 16). None of the 2 OGA patients progressed or died during this 6-month period. Across all patients, the 6-month PFS rate was 38.5% (95% CI: 14.1, 62.8), and the 6-month OS rate was 76.9% (95% CI: 44.2, 91.9) (Table 17).

Tumour cohort	No. of patients at risk	No. of patients with disease progression/death during the 6m period	6-month PFS (95% CI)
CRC	11	8	27.3 (6.5, 53.9)
OGA	2	0	100 (*)
All patients	13	8	38.5 (14.1, 62.8)

*95% CI could not be quantified.

Table 16 Progression-free survival rates at 6-months from combination treatment initiation

Tumour cohort	No. of patients at risk	No. of deaths during the 6m period	6-month OS (95% CI)
CRC	11	3	72.7 (37.1, 90.3)
OGA	2	0	100 (*)
All patients	13	3	76.9 (44.2, 91.9)

*95% CI could not be quantified.

Table 17 Overall survival rates at 6-months from combination treatment initiation

2.4 Discussion

In this phase IIa trial, domatinostat in combination with avelumab was well-tolerated across a heterogenous population of patients with pre-treated, advanced CRC and OGA. No DLT was observed and no MTD was reached, consistent with findings from combining domatinostat and pembrolizumab in checkpoint-inhibitor refractory melanoma where, similarly, no MTD was reached.(428) Most drug-related AEs were grade 1-2. Only 1 grade 3 TRAE was observed, and no patient dropped out or died due to treatment. The 14-day cycle of avelumab 10mg/kg on day 1, with daily domatinostat 200mg BD continuously, was established as the RP2D.

In the PK analysis, after a single dose of domatinostat, the mean Cmax and AUC increased by dose escalation (100mg OD to 200mg OD). After 2 doses, the exposure degree of domatinostat was only increased in the 200mg OD dose group. However, as domatinostat was being used to 'prime' the TME in this study, rather than for its direct anti-tumour effects, it is unclear how the plasma concentration of domatinostat and rate of clearance over time, would affect the immune cell populations in our study without corresponding translational work. In the SENSITIZE study, domatinostat (up to 400mg TDD) was taken D1-14, q3W or day 1-21, q3w (continuous dosing), and PK sampling was performed on a slightly different schedule to EMERGE at C1D1, C1D14, and C2D1.(428) In this study, the drug exposure AUC increased in a dose dependent manner until steady state across all dose cohorts up until day 14. In pre-clinical work, domatinostat dose-dependently increased the expression of tumour associated antigens and MHC class I and II molecules in melanoma cells.(406) Therefore, ideally, in EMERGE, a baseline and on-treatment biopsy during the domatinostat 'priming' period, either cycle 1 day 8, or cycle 1 day 14, should have been collected. This could then have been correlated with the plasma concentration of domatinostat at the time of the on-treatment biopsy to determine whether increasing doses of domatinostat, correlated with increased expression of tumour antigens and MHC class I and II molecules in the TME, in a dose dependent manner compared to baseline. Based on the preclinical studies using domatinostat in poorly immunogenic CRC mouse models, which demonstrated a

shift in immune cell populations in the TME, favouring influx of CD8+ cells, it would also have been valuable to assess CD8+/Treg ratio on these biopsies after domatinostat priming, to confirm target effect. Importantly, in the poorly immunogenic mouse CRCs, the percentages of immune cell subpopulations in blood were unaffected after domatinostat treatment and, therefore, tissue biopsies would be required to assess the shift in immune cell populations directly within the TME.

The most common TRAEs to either domatinostat or avelumab were fatigue (77%), anorexia (38%), nausea (38%), and anaemia (31%), which are consistent with previous clinical experience of domatinostat monotherapy, or in combination studies with domatinostat and checkpoint inhibitors.(427,428,430) In addition, we report two instances of QTc interval prolongation, a known class-specific effect for HDACi, but not previously observed with domatinostat use in patients with normal baseline ECGs. Importantly, there was no evidence of synergistic immune toxicity or infusion reactions.

Assessment of anti-tumour activity was an exploratory objective of this phase IIa dose-finding study. The ORR (PR/CR) in the CRC patients was 0%, which would be the expected response to single-agent checkpoint inhibitor therapy in MSS mCRC.(57) However, half of the CRC patients in our study still had stable disease at 6 months. Given that standard of care third line therapies for mCRC improve overall survival by less than 2 months, with response rates of less than 2% in randomised trials,(186,189) our preliminary results in MSS CRC may be promising. Limited responses but prolonged stable disease has also been observed in some patients with chemorefractory pMMR/MSS mCRC treated with the HDAC inhibitors, entinostat, and zabadinostat, with pembrolizumab, and nivolumab, respectively, in comparable preliminary studies.(202,203) Our results therefore echo these findings.

Six out of 11 patients in our CRC cohort had liver metastases, and in all but one of these patients, the best response was PD. This is consistent with data from other trials evaluating anti-angiogenic drug combinations with PD-1 or PD-L1 targeting in patients with MSS mCRC, where improved responses were observed

in the absence of liver metastases.(433,434) This phenomenon appears to be due to a unique TME within the liver metastases, which compared to tumours without liver metastases, have lower populations of cytotoxic T cells and higher numbers of immunosuppressive cells.(435) Furthermore, the TME within liver metastases appears to recruit monocyte-derived macrophages, which drive cytotoxic CD8+ T cell apoptosis. In turn, this loss of cytotoxic T cells appears to induce a systemic immunosuppressive effect, which may explain the poorer responses to ICIs in patients with liver metastases.(435) Notably, the one patient with liver metastases who had stable disease for 8 weeks in our study, was the only patient with liver metastases treated at the RP2D. However more patients would need to be enrolled to determine whether the 200mg BD domatinostat dose is associated with a higher chance of response in CRC with hepatic metastases. Interestingly, the patient in the CRC cohort with lung metastases and no liver metastases, had the best outcome to treatment, experiencing prolonged disease stabilisation for almost 1 year. This correlates with observations in the REGONIVO study, where improved response rates were seen in patients with MSS CRC and GC who had lung metastases.(191) Therefore, it may be worthwhile to explore these clinical factors as predictors of response to ICIs in future clinical trials combining ICIs and HDAC inhibitors.

Only 2 patients were enrolled into the OGA cohort of the phase IIa dose-finding component of this trial. Nevertheless, one of these patients achieved a PR which was maintained for over 1 year, and the other had disease control for over 6 months. Given that standard of care second line therapies improve survival by only 6 weeks in OGA patients, these results may also be encouraging. However, it is noteworthy that the responder had a baseline PD-L1 CPS score of 9. This response may, therefore, have been independent to any immune modulating effects of domatinostat. It would be important to assess baseline PD-L1 status on all patients in future studies of ICI combinations in OG cancer as it is a validated predictive biomarker of ICI efficacy and, therefore, should be considered when interpreting outcomes to treatment. It is also notable that the patient in the OGA cohort who responded did not have liver metastases, which may have contributed to this positive outcome.

The phase IIb dose expansion component of the EMERGE study, to confirm efficacy for the domatinostat 200mg BD dose, was enrolling patients throughout 2021. At the time of the interim analysis, 21 patients had been enrolled and treated at the RP2D across both phase IIa and IIb. Of the 10 patients in the CRC cohort evaluable for efficacy, no responses were observed, and the median duration of therapy was 2 months.(436) Of the 9 patients treated in the OGA cohort, the best ORR was 22.2% [95%: 2.8, 60.0], and 1 CR and 1 PR were observed. PD-L1 CPS testing was performed on all archival tumour tissue from patients in the expansion phase of the study, where available.(436) In parallel to the PR seen in the OGA patient with a baseline PD-L1 CPS of 9 in the phase IIa, the OGA patient who had the PR in the expansion phase, also had 'positive' PD-L1 expression (CPS ≥ 5). The tumour tissue of the patient who had the CR was exhausted and he declined a new baseline biopsy at study entry, so the PD-L1 status for this patient remains unknown. The effect of higher PD-L1 scores as the potential key cause of these responses to avelumab therefore cannot be excluded, and the contribution of the domatinostat to these responses is therefore unclear. Of note, data from the EMERGE interim analysis did not uncover any additional safety signals. Unfortunately, in February 2022, a commercial decision was taken to discontinue the domatinostat development programme, and consequently recruitment to the EMERGE trial has closed.

Limitations of our study include a lack of early translational data to assess the immune modulatory effects of domatinostat on the TME in OGA and CRC patients in the dose finding phase. This may have provided some insight into mechanisms of response and resistance to combination HDACi and checkpoint inhibitor therapy in our patients and allowed for better patient selection and stratification in the expansion phase. In addition to collecting baseline and on-treatment biopsies to establish the pharmacodynamic effects of increasing doses domatinostat on the CD8+/Treg ratios in the TME, and expression of TAMs and MHC class I and II expression, other translational endpoints would also have been valuable. For example, evaluation of whether elevated levels of HDACs at baseline correlated with response and, pharmacodynamically, whether domatinostat induced upregulation of the IFN-γ signature, as in the preclinical work, which is correlated to ICI response. In preclinical models of poorly

immunogenic CRCs, domatinostat appeared to decrease MDSC populations in the TME, which we know are a potently immunosuppressive cell population. Any shifts of immune cell populations could have been analysed in the sequential biopsies and correlated with response. While changes in peripheral immune cell populations were not observed with domatinostat in the in vivo preliminary work, a study of entinostat and pembrolizumab in patients with NSCLC, found a correlation between high levels of baseline HLA-DR high classical monocytes and clinical benefit. HLA-DR is an MHC class II antigen-presenting molecule which is upregulated by IFN-γ and correlated with response to ICIs.(437) As blood samples are likely less costly, easier to perform, and potentially more acceptable to the patient, levels of HLA-DR high classical monocytes at baseline and at treatment response, could also be explored in future studies of HDACi and ICIs.

We also did not routinely collect or consider the molecular landscape of tumours, beyond MMR status (and PD-L1 status in the expansion phase), and, as discussed in the introduction, this is likely to be increasing important in evaluating ICI combinations in CRC and OGA in future. For example, KRAS mutations drive an immunosuppressive phenotype by reduced IFN-γ signalling and reduced transcription of MHC class II molecules, and have been shown to confer resistance to ICIs.(159,434) Interestingly, KRAS mutant cells also appear to upregulate HDAC6 and MYC, and are less sensitive to HDAC inhibition than KRAS wild type cells.(438) Therefore, the combination of a KRAS inhibitor and HDAC inhibitor may be required to overcome the immunosuppressive phenotype induced by MYC, before ICI would be effective. By contrast, CRCs with BRAF V600E mutations are typically associated with a more ‘inflamed’ TME, which may enhance the possibility of response to these agents.(434,439) In OGA, another predictor of ICI sensitivity, distinct from dMMR/MSI-H status, is EBV positivity. As the ORR to ICI monotherapy in EBV positive OGA is 100%,(30) although relatively uncommon, it would be important to know EBV status in all OGA patients enrolled into combination ICI trials. Future trials evaluating immunotherapy combinations in CRC and OGA patients should therefore include more comprehensive data capturing, to ensure that relevant molecular and genomic features of the tumour are included for correlation with treatment response and resistance.

4SC have discontinued the development programme of domatinostat and have instead focused on the clinical development of resminostat which inhibits class I, IIB, and IV HDAC enzymes, particularly HDAC6. HDAC6 is often active in metastases and, as described, is upregulated by KRAS mutant cancers which make up 50% of CRCs. This may therefore be an attractive future combination partner for ICIs in MSS CRCs. However, the most compelling preclinical data of epigenetic modifiers in TME reprogramming in CRCs, thus far, has been with use of both an HDAC inhibitor, together with a DNMT inhibitor. The combination of these two epigenetic agents caused much greater influx of CD8+ T cells, and efflux of MDSCs, within the TME, than either drug alone. Furthermore, in humans, combined HDAC and DNMT inhibition with pembrolizumab, was found to be safe and tolerable in patients with pMMR/MSS CRC. Therefore, this combination may be the most rational to take forward in future efforts to combine epigenetic modifiers with ICIs. Another preclinical finding that should be brought to the fore is the domatinostat -induced expression of the immune checkpoint LAG3 on the CD8+ T cells in the modestly immunogenic CRC mouse models, which subsequently dramatically responded to a triple combination of domatinostat, anti-PD-1, and anti-LAG3. Recently, preliminary data combining the LAG-3 inhibitor, favezelimab, with pembrolizumab has been reported. The combination was tolerable, and ORR was 11.1% in patients with mCRC with baseline PD-L1 CPS ≥ 1 tumours.(194) Interestingly overall survival in patients with PD-L1 CPS ≥ 1 tumours was over a year, and therefore this combination looks promising.(194) It is possible that, based on the compelling preclinical data, the addition of an HDAC inhibito,r may further augment the clinical benefit from dual LAG3 and PD-(L)1 blockade.

2.5 Conclusion

In summary, the HDACi, domatinostat, was safe and tolerable in combination with avelumab in patients with advanced pMMR/MSS CRC and OGA. Common AEs were manageable with no evidence of enhanced immune-related toxicity. The ORR in the OGA subgroup would have warranted further evaluation, however the trial was closed early due to termination of the clinical development programme for domatinostat. Future dose-finding studies combining “epi-drugs” with checkpoint inhibitors in GI cancers, should consider inclusion of pharmacodynamic biomarker sampling and translational endpoints to demonstrate sufficient proof-of-concept and biological activity.

Chapter 3 Wnt inhibition in oesophagogastric cancer: phase IIa results of the WAKING trial

3.1 Introduction

3.1.1 Wnt signalling in oesophagogastric adenocarcinoma

The Wnt signalling pathway is involved in several key processes in humans including embryonic development, cell proliferation, apoptosis, and "stemness".(440) It is broadly divided into beta-catenin dependent (canonical) and independent (non-canonical) pathways. Canonical Wnt signalling essentially controls the availability of the transcriptional co-activator beta-catenin, through its phosphorylation by the destruction complex (made of Axin, APC, and GSK2beta and CK1alpha) and ubiquitination by beta-TrCP, which encourages proteasomal degradation.(441) Beta-catenin is thus unable to reach the cell nucleus and, in its place, its binding partner TCF/LEF forms a repressive complex with Groucho/TLE which recruits HDACs to repress target genes.(440) Wnt signalling is activated when Wnt ligand binds to Frizzled (FZD) and low-density lipoprotein (LRP) co-receptors. This initiates a phosphorylation cascade whereby the destruction complex is inactivated, allowing beta-catenin to accumulate and translocate to the nucleus, resulting in transcription of Wnt-associated genes.(442) Figure 13 gives an overview of the beta-catenin dependent Wnt signalling, and some of the therapeutic targeting opportunities in cancers with Wnt signalling aberrations. In non-canonical Wnt signalling, beta-catenin is not required for signal transduction. Wnt activation leads to binding of ROR and FZD, which forms a complex allowing activation of Dishevelled (Dvl).(440) Dvl then activates DAAM1 and RHOA GTPase, which triggers ROCK and JNK. A simplified schema of beta-catenin independent Wnt signalling is shown in Figure 14. This leads to cytoskeleton remodelling or transcription. Wnt/Ca2+ signalling is activated by phosphorylation of PLC, after activation of Dvl, this then initiates a downstream cascade resulting in activation of calcium-dependent transcription and cytoskeletal changes.(440)

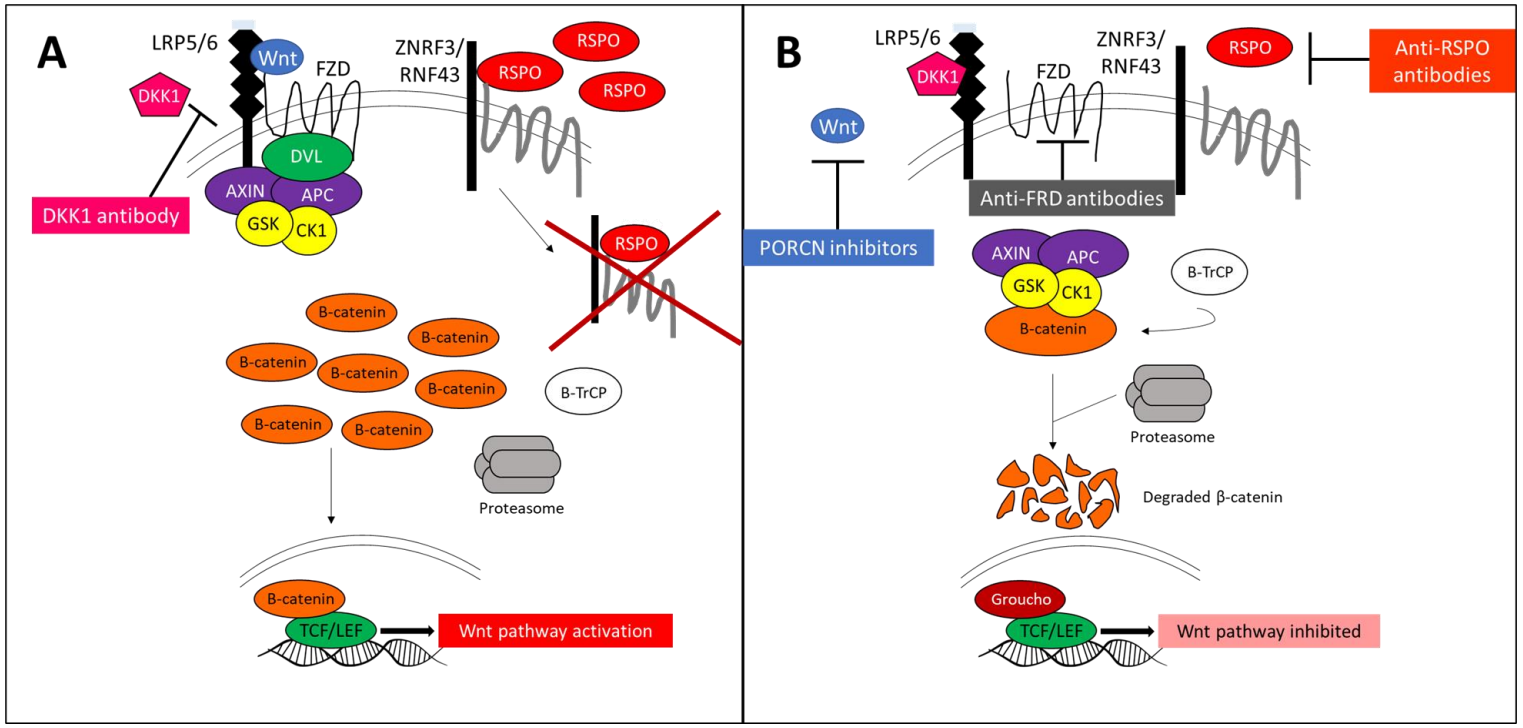


*Figure 13 Overview of beta-catenin-dependent Wnt signalling and some of the therapeutic targeting opportunities in cancers with Wnt signalling aberrations. (Adapted from ter Steege et al)(443) (**A**) Binding of Wnt to a FZD receptor and the LRP5/6 co-receptor causes disruption of the destruction complex and stabilises beta-catenin which then travels to the nucleus and promotes transcription of Wnt target genes. Overexpression of RSPOs causes clearance of negative regulators ZNR53/RNF43 from the membrane and enhances Wnt-receptor availability and Wnt pathway activation. DKK1 usually inhibits Wnt pathway activation by binding LRP5/6 thus blocking Wnt interaction. DKK1 antibody blocks this action thus also potentiates Wnt signalling. **(B)** In the absence of Wnt, beta-catenin is bound by the destruction complex and beta-catenin degradation is induced, preventing the transcription of Wnt target genes. When DKK1 is bound to LRP5/6 Wnt signalling is inhibited. PORCN inhibitors block the interaction of Wnt ligands and membrane receptors, thus inhibiting Wnt signalling. Anti-FZD antibodies prevent FZD from binding to Wnt ligand and LRP5/6 co-receptors and inhibit Wnt signalling. Anti-RSPO antibodies stop RSPOs from clearing ZNRF3/RNF43 and thus also inhibits the pathway.*

Wnt pathway dysregulation occurs frequently in cancer and is associated with tumour growth.(440) Wnt signalling is relatively complex, and it can also crosstalk with other signalling pathways, such as Hedgehog and Notch, which co-operate to drive tumour progression. Hyperactivation of Wnt signalling as a driver of CRC development and progression, most commonly via loss of function APC mutations and CTNNB1 (beta-catenin) stabilising mutations, is particularly well-described, however aberrations in Wnt signalling have also been observed in up to 50% of gastric tumours,(444) proposing a potential target for therapy. Components of Wnt signalling appear to be dysregulated at all levels of both the canonical and non-canonical pathway in OGA. For example, MYC is a Wnt target gene and, as described in chapter 1, is amplified in around a third of OGAs.(227) In GC models, MYC upregulation was shown to be dependent on Frd7, highlighting the dependency of MYC overexpression on Wnt signalling.(445)

LGR5 is another Wnt target gene, expressed through activation of Wnt beta-catenin, which is associated with proliferation, migration, and invasion in GC. This was corroborated by experiments causing knockout of LGR5, which was observed to stop GC proliferation and invasion.(446) ZNRF/RNF43 also appears to play an important role as a TSG in the stomach. ZNRF/RNF43 negatively regulates Wnt signalling, by causing ubiquitin-mediated internalisation and degradation of Frizzled receptors, and thus decreased Wnt signalling, as one of its mechanisms.(447) RNF43 mutations are more frequent in MSI-H tumours, and lower levels of RNF43 expression are associated with poorly differentiated, and more advanced disease.(448) By contrast, RNF43 overexpression results in apoptosis of GC cell lines.(448) RNF43 knockout mice developed neoplasia in the intestine through secretion of Wnt3.(449) Subsequently, when a porcupine inhibitor (PORCNi), which prevents Wnt from being transported to the extracellular membrane, in turn, preventing the production of beta-catenin, was applied to the RNF43 knockout mice, the tumours were attenuated.(449) While porcupine inhibitors were not specifically demonstrated to have anti-tumour effects in RNF43 mutated GC, this may warrant further investigation. R-spondin (RSPO) proteins are also important promoters of Wnt pathway activation.(443) They hijack the negative feedback mechanisms instilled by ZNRF3/RNF43 by

binding to LGR4/5/6 and ZNRF3/RNF43, which causes clearance of ZNRF3/RNF43 from the cell membrane.(443) This, in turn, increases Wnt receptor availability and greater pathway potentiation (Figure 13). RSPO gene fusions and overexpression have more frequently been described in CRC, but have also been implicated in GC and multiple other cancer types, thus also presenting a potentially promising target for novel therapies.(443) RSPO activity can either be pharmacologically targeted directly by targeting the RSPO ligands, or indirectly with PORCNi, which impair RSPO activity by blocking the production and activity of Wnt ligands. Some currently recruiting trials evaluating the safety and efficacy of PORCNi are specifically recruiting patients with evidence of RNF43/RSPO as a potential predictive biomarker of enhanced response (Table 18).

Mutations in AXIN/12, CTNNB1, and APC, appear commonly in gastric cancer,(450) and are associated with aberrant Wnt signalling. Several Wnt ligands including WNT1, WNT2b, WNT5a, WNT6, and WNT10a, are upregulated in OGA.(451) WNT5 and WNT6 appear to be associated with more advanced stage disease and metastases,(452) and an anti-Wnt5a antibody was able to supress development of liver metastases in preclinical models of GC compared to controls.(453)

In gastric cancer, Wnt components appear to be most commonly deregulated at the level of the ligand/receptor e.g. by hypermethylation and epigenetic silencing of Dickkopf 1/2 (DKK1/2), which usually binds to LRP5/6, blocking Wnt interaction, and secreted Frizzled-related protein 2 (sFRP2), which typically binds to Fzd and downregulates Wnt signalling.(454) Increased expression of the RYK co-receptor, which triggers non-canonical Wnt signalling, is also common in GC and linked to advanced stage disease and metastasis.(455) Upregulation of all 10 Fzd receptors have been implicated in GC,(456) with Fzd7 having been identified and the key mediator of Wnt signalling in GC.(445) Use of the anti-frizzled antibody, OMP-18R5 (vantictumab), which targets Fzd1,2,5,7, and 8, or the PORCNi, IWP-2, were able to inhibit tumour growth in in vivo models of GCs.(445)

It may, therefore, be theoretically possible to target Wnt signalling anywhere along the course of the pathway. Furthermore, as activation of Wnt is strongly correlated to promotion of EMT and metastases in GC,(457) Wnt inhibition may be a particularly attractive target for advanced disease. Preliminary clinical studies of Wnt signalling modulators as monotherapies including PORCNi, Frizzled receptor targeting agents, a Wnt5a-mimetic, and agents that block beta-catenin binding the transcriptome, have so far demonstrated promising safety profiles in first-in-human studies, where side effects appear to be manageable.(458–463) However, an important toxicity associated with Wnt inhibitors are their unfavourable effects on bone, including iatrogenic osteopenia and pathological fractures. One patient who had a pathological fracture on a dose escalation study with vantictumab, had a 4-fold rise in levels of β-C-terminal telopeptide (β-CTX), which is a marker for bone degradation.(461) The trial then mitigated for this by measuring levels of β-CTX and, if it rose by 2-fold, zolendronic acid was administered.(461) Two patients were eventually given zolendronic acid, and their β-CTX level returned to baseline.(461) No further fractures were observed.(461) In another study of the Wnt inhibitor, ipafricept, combined with chemotherapy in patients with advanced pancreatic cancer, bone markers were monitored from the outset and bisphosphonates given as required.(464) No fractures were observed on this study.(464) Signals of single agent efficacy with Wnt pathway modulating agents have yet to be confirmed. Table 18 displays selected Wnt modulating agents currently in development.

Mechanism of action	Drug	Combination partner drug	Biomarker	Phase	Study population	Trial identifier
PORCN inhibitors	IWP-2	-	-	Pre-clinical	In vivo models of GC	-
	CGX1321	pembrolizumab	-	Ia/Ib	All GI tumours	NCT02675946
	ETC-1922159	pembrolizumab	RSPO fusions (in 2 groups of MSS CRC patients)	Ia/Ib	Advanced MSS solid tumours	NCT02521844
	LGKK974	+/- PDR001 (anti-PD-1 antibody)	RNF43/ RSPO aberration	Ia/b	Malignancies dependent on Wnt ligands inc BRAF mutant CRC and OSCC	NCT01351103
	RXC004	+/- nivolumab	RNF43/ RSPO aberration	II	MSS mCRC	NCT04907539
Anti-RSPO monoclonal antibody	OMP-131R10	+/- FOLFIRI	-	Ia/b	Advanced solid tumours and pre-treated mCRC	NCT02482441
Anti-Frizzled antibody	OMP-18R5 (vantictumab)	-	-	Pre-clinical	In vivo models of GC	-
	OMP-18R5 (vantictumab)	-	-	Ia	Advanced solid tumours	NCT01345201
Anti-Wnt5a antibody	Foxy-5	-	-	Pre-clinical	In vivo models of GC	-
DKK1 antibody	DKN-01	Atezolizumab	-	II	Advanced OGA (2^{nd}/3rd line)	NCT04166721
	DKN-01	Tislelizumab + chemotherapy	-	II	Advanced OGA (1^{st} line)	NCT04363801
	DKN-01	Tislelizumab	DKK1-high	II	Advanced OGA (2^{nd} line)	NCT04363801
	DKN-01	FOLFIRI/FOLFOX and bevacizumab	-	II	Advanced CRC (2^{nd} line)	NCT05480306

Table 18 Selected Wnt pathway modulating agents currently in development

3.1.2 Wnt signalling and immune modulation

In parallel, mounting evidence indicates a critical role for Wnt beta-catenin signalling in immunomodulation of the TME at multiple steps of the Cancer-Immunity Cycle, ultimately rendering tumours immunologically 'cold'.(465) Immune 'desert' or T-cell excluded tumours show enrichment of activating mutations in Wnt beta-catenin signaling components including CTNNB1, APC, and AXIN.(155,466) Thus, restoring immune cell infiltration into the TME by modulating Wnt beta-catenin signaling, may enhance the magnitude of benefit from treatment with ICIs.

Activated Wnt beta-catenin signaling also appears to accentuate tumour immune exclusion by suppressing DC recruitment into the TME, via down-regulation of the chemokine CCL4.(467) Consequently, levels of CXCL9/10 and IL-10, secreted by DCs, are exhausted, resulting in impaired priming of effector T cells in melanoma mouse models, which were subsequently resistant to immune checkpoint blockade.(467,468) When Wnt beta-catenin signaling is inactivated, by blockage of binding of Wnt ligand to co-receptors LRP5/6 and Fzd, presentation of cancer associated antigens and T cell priming appear to be reinstated.(469)

In addition to negative effects on cancer antigen presentation and T cell priming, aberrant Wnt beta-catenin signaling also regulates presence of effector T cells in the TME. Namely, Wnt signaling seems to favour survival and influx of inhibitory Tregs, while inactivating effector T cells.(470,471) As alluded in chapter 1, MYC overexpression is linked to an unfavourable immune milieu. Driven by Wnt, MYC overexpression also triggers apoptosis of naïve T cells,(471) contributing to a reduced anti-cancer immune response. Blocking Wnt3a beta-catenin signaling seems to reestablish levels of CD8+ effector T cells, and reduce tumour growth.(472) Similarly, blocking the interaction between beta-catenin and its coactivators, BCL9 and B9L, appears to suppress tumour growth in animal models, reduce levels of regulatory T cells, and increase levels of DCs.(473) This preclinical work suggests that the host anti-cancer immune response can be restored by inhibiting Wnt signaling. In tumour cells, activated Wnt beta-catenin

signaling can in turn cause MYC to upregulate immune checkpoints PD-L1 and CD47,(220,471) thus hampering immune surveillance. Furthermore, MYC inhibitors, such as MYCi361, have been shown to cause increased tumour immune cell infiltration, and effective tumour reduction, when combined with anti-PD-1 agents.(474) Therefore, agents targeting components of the Wnt pathway, could improve responses to ICIs.

3.1.3 The rationale for DKK1 inhibition in advanced OGA and combination with checkpoint blockade

The Dickkopf (DKK) family is made up of 4 extracellular glycoproteins (DKK1-4), with DKK1, the most well-characterized.(475) DKK1 has a critical role in embryonic head development.(476) In adults it is less widely expressed, but appears to play a role in mediating bone health and disease.(477) As described previously, in beta-catenin Wnt signaling, DKK1 acts as an antagonist, by binding to the LRP6 co-receptor with high affinity and, thus, inhibits Wnt interaction, and subsequent beta-catenin Wnt signaling (Figure 13).(478) Activated beta-catenin Wnt signaling can, in turn, cause upregulation of DKK1 by negative feedback mechanisms.(479) However, through mechanisms yet to be fully elucidated, DKK1 has also been shown to activate Wnt signaling independent of beta-catenin in both oncology models, and in the pathogenesis of Alzheimer's disease.(478) Therefore the effects of DKK1 on cells are complex, and it is thought that the inhibitory effect it exerts on the beta-catenin Wnt pathway is counterbalanced by greater activation of beta-catenin independent Wnt signaling.(478) For example, knockdown of DKK in models of liver cancer resulted in a decrease in phosphorylation of JNK, which is a downstream target of the Wnt/PCP pathway, and decreased metastases.(480) Furthermore, in models of osteosarcoma, elevated levels of DKK1 expression resulted in increased tumour growth, and higher levels of RHOA expression and JNK phosphorylation.(481) Given that DKK1 inhibits Wnt signaling, the initial presumption was that DKK1 acted as a TSG.(478) This was supported by preclinical studies indicating that DKK1 could limit tumour growth and proliferation, induce apoptosis, and inhibit

angiogenesis.(482–485) However accumulating evidence has linked elevated levels of DKK1 with promotion of cancer pathogenesis.(478) The oncogenic action of DKK1 may also be, in part, due to communication with the CKAP4-AKT signaling pathway (Figure 14).(486) This has been demonstrated by preclinical models which have shown that DKK1 binds to CKAP4, which caused internalization of CKAP4 and activated AKT by forming a complex with PI3K, thereby resulting in tumour growth.(486) Furthermore, addition of an anti-CKAP4 antibody, stopped DKK1 binding to CKAP4, and reduced activity of AKT which resulted in decreased tumour growth.(486) Therefore, the role of DKK1 in driving tumour growth, or its suppression, is presumed to depend on the interplay of several factors such as the TME, type of cancer, degree of heterogeneity, and the influence of other components of Wnt signaling network.(478)

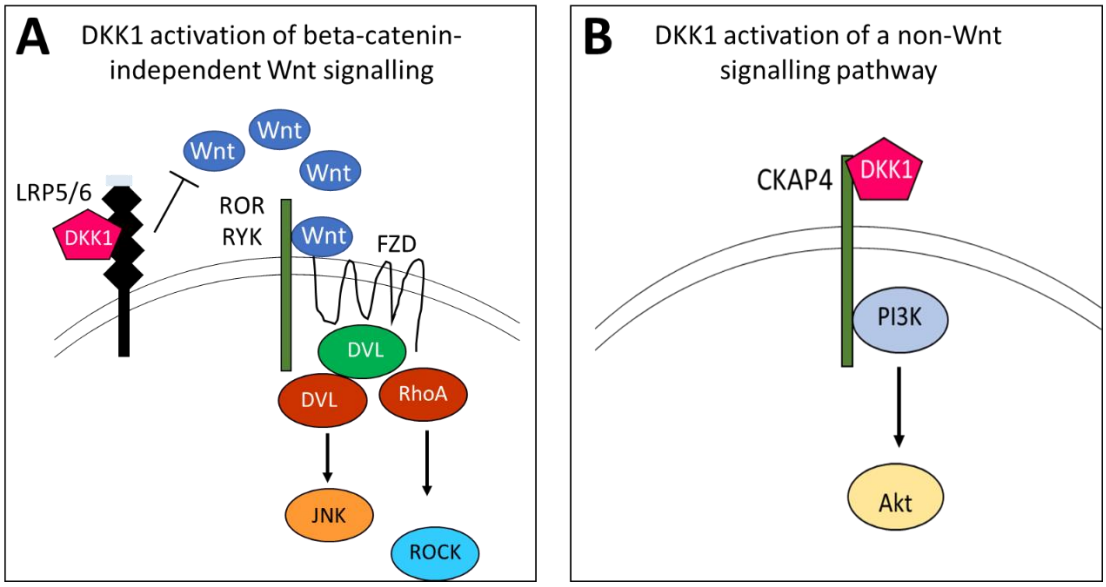


Figure 14 DKK1 regulation of signalling pathways other than beta-catenin dependent Wnt signalling (from Kagey et al). ***(A)*** *DKK1 binds to the LRP5/6 co-receptor (inhibiting beta-catenin Wnt signalling) which steer the unbound Wnt ligand and FZD receptors towards beta-catenin independent signalling pathways such as the Wnt/PCP pathway. (B) DKK1 binds to the CKAP4 receptor and promotes PI3K/Akt signalling.*

Multiple studies have observed that elevated levels of tumoural DKK1 correlate with poor overall, and disease-free survival in OG cancer.(487–490) DKK1 can also be detected in serum, and serum DKK1 levels were significantly greater in OG cancer patients (63%), compared to controls (5%).(491) In addition to poorer

survival outcomes, DKK1 overexpression is associated with increased vascular and lymphatic invasion, and distant metastases in OG cancer.(484) This is likely related to the effects of DKK1 on expression of MMP, which has a role in cell migration, and increased expression of proteins involved in angiogenesis.(478) Interestingly, while DKK1 inhibits beta-catenin Wnt signaling, as described, the relationship between DKK1 mRNA expression and Wnt signaling in patients with OGA has been recently explored. This has revealed that patients with high levels of DKK1 expression were more likely to harbor Wnt activating mutations such as stabilizing mutations in CTNNB1 (beta-catenin), loss of function mutations in APC, and loss of function mutations in RNF43.(Data from DKN-01 Investigator's Brochure, Version 8.0) This further emphasizes the complexities of the interplay of components in Wnt signaling, and the number of positive and negative effects that DKK1 has on the modulation of Wnt pathways.

In addition to its effects on cancer cells, DKK1 also has immunomodulatory abilities. Specifically, DKK1 has been shown to reduce beta-catenin levels in mice and human MDSCs, and higher DKK1 expression is positively correlated with MSDC accumulation in tumour tissues.(492) DKK1 has also been shown to stimulate polarization of immune inhibitory Th2 macrophages, as well as decrease Th1 macrophage populations and, therefore, the secretion of pro-inflammatory cytokines such as IFN-γ.(493) Subsequently, administration of a DKK1 neutralizing antibody, appeared to significantly reduce MDSC levels in tumour-bearing mice, which resulted in an influx of CD4+ and CD8+ T cells, and reduction of tumour growth.(492) DKK1 also appears to reduce expression of NK cell activating ligands and numbers of NK cells in the TME, in lung and breast cancer models.(494) More recent work has shown that the murine anti-DKK1 antibody, mDKN-01, can instigate favorable changes in the TME, by decreasing levels of MDSCs, upregulating PD-L1 on MDSCs, and activating NK cells, resulting in decreased tumour growth of melanoma and breast cancer models.(495) Additionally, lung metastases in a breast cancer model were significantly reduced after application of mDKN-01.(495) Furthermore, mDKN-01 combined with anti-PD-1 resulted in greater tumour reduction in melanoma models, than mDKN-01 alone.(495) DKK2, which can be upregulated by APC loss, has similar immune suppressive effects on the TME, by reducing activity of

NK cells and CD8+ T cells in models of CRC.(496) When an anti-DKK2 antibody is applied, NK and CD8+ T cells are reactivated, and tumour growth is attenuated.(496) CRC tumour growth is subsequently more greatly impeded by the addition PD-1 blockade.(496) These results suggest that DKK targeting therapies may favourably reprogram the TME for collaboration with checkpoint inhibitors, which could be an attractive therapeutic strategy. Furthermore, DKN-01 has demonstrated promising clinical activity in OG cancers when combined with paclitaxel or pembrolizumab, without additional toxicity. Taken together, these studies provide rationale for inhibiting DKK1 in OG cancer.

3.1.4 DKN-01

DKN-01 is a humanized monoclonal IgG4 neutralising antibody against DKK1. It binds with high affinity and specificity to DKK1 in humans and neutralizes DKK1 in cell-based assays. As a monotherapy and in combination with paclitaxel, and gemcitabine and cisplatin, DKN-01 showed a dose-dependent increase in Cmax.(497–499) PK/PD modeling also showed a dose-dependent decrease in free DKK1 concentrations.

In the clinic, DKN-01 has been evaluated in both the phase I and phase II setting. In a phase I study of DKN-01 monotherapy in patients with multiple myeloma or advanced solid tumours, no DLT was observed and no MTD was reached.(500) The dose taken forward into Part B was 300mg DKN-01 Q2W. In total, 32 patients were enrolled across Part A and B of the study, including 24 patients with relapsed/refractory NSCLC. Generally, the TRAEs, most commonly nausea, fatigue, and decreased appetite, were manageable and there was no grade 3 TRAE or discontinuation due to a TRAE.(500) One patient had a CR on study, 4.2% had a PR, and 42% had stable disease. The median PFS of the 24 patients with NSCLC was 2.2 months [95% CI 1.8-2.9].(500) Successively, the safety profile of DKN-01 in combination with standard chemotherapy in patients with

advanced cholangiocarcinoma,(497) OG cancer,(499) and as monotherapy, and in combination with paclitaxel in advanced gynaecological malignancies,(501) has not uncovered further untoward safety data. In the combination studies, grade 3/4 TRAEs included anaemia, neutropenia, thrombocytopenia, and fatigue, however this was not significantly increased from the expected toxicity of the chemotherapy alone.

The combination of DKN-01 and the anti-PD1 antibody, pembrolizumab, has also been evaluated in patients with advanced OG cancer. In this phase Ib study, DKN-01 (150mg or 300mg) on Days 1 and 15, plus pembrolizumab on Day 1 of a 3-weekly cycle, were examined.(502) Among 45 treated patients, no DLTs were identified, and no new safety signals indicative of additive toxicity were found. The main ≥ grade 3 TRAEs were hyponatraemia and anorexia. Of the evaluable patients who were checkpoint inhibitor naïve, the ORR was 17.4% and all patients with PRs had MSS tumours.(503) Subsequent retrospective translational work, revealed that patients with high tumoral DKK1 expression at baseline, measured by RNAscope, with the upper tertile defined as high, had improved clinical outcomes; DKK1-high patients had an ORR of 50% versus 0% for DKK1 low patients, and a median PFS of 22.1 weeks versus 5.9 weeks, respectively (HR 0.24, 95% CI 0.08-0.67).(502) Furthermore, this clinical benefit appeared to be independent of PD-L1 status.(502) DKN-01 has most recently been evaluated as a first line therapy in OG cancer in combination with the anti-PD-1 antibody, tislelizumab plus chemotherapy in an ongoing phase II study. Recently presented results showed an ORR of 90% in the DKK1-high population, and in the 6 patients who had PD-L1 low (CPS <5) and DKK-high expression, on their baseline tumours, ORR was 100%.(504) Overall median PFS was 11.3 months (11.3 months in DKK-1 high and 12.0 months in DKK1-low groups).(504) However, so far, DKK1 has only been correlated with response to DKK1-based therapies retrospectively in a modest number of patients with OG cancer. A randomized controlled phase II study of DKN-01 +/- tislelizumab and chemotherapy in first line OGA has recently started recruiting (NCT04363801). In this randomized component of the study, part C, patients will be stratified at randomization for 1. DKK1 RNAscope tumour percentage score (TPS) (≥20% versus <20%), and 2. PD-L1 CPS (≥5 versus <5). The data derived from the randomized portion of the

study will help to validate the clinical utility of DKK1 as a predictive biomarker for DKK1-based therapy, through secondary analyses using an interaction test between the treatment and the biomarker in a statistical model.

3.1.5 Atezolizumab

Atezolizumab is a humanized IgG1 monoclonal antibody that targets PD-L1 and inhibits the interactions between PD-L1 and its receptors, PD-1, and B7-1 (also known as CD80), both of which function as inhibitory receptors expressed on T cells. Therapeutic blockage of PD-L1 binding by atezolizumab has been shown to enhance the magnitude and quality of tumour-specific T-cell responses, resulting in improved anti-tumour activity.(505,506) Atezolizumab is currently approved as monotherapy, or in combination with other agents, for the treatment of locally advanced or metastatic urothelial carcinoma, advanced NSCLC, extensive stage small cell lung cancer, triple negative breast cancer, hepatocellular carcinoma and BRAF V600 mutated melanoma. It has a well described safety profile and has been used in several ongoing studies in patients with OGA.

Given that anti-PD-1 and anti-PD-L1 therapies have purported only modest success as single agents in MSS OGA, approaches to improve responses to these therapies are warranted. Preclinical data implicates DKK1 in contributing to an immunosuppressive TME, and inhibiting host anti-tumour immune responses by activating MDSCs, and downregulating NK activating ligands on cancer cells. DKN-01 in combination with checkpoint blockade, in preclinical models, has additive effects on hampering tumour growth with corresponding favorable effects on the TME and, in the clinic, DKN-01 combined with checkpoint blockade is well-tolerated in patients with OGA. The WAKING study was conceived with the hypothesis that DKN-01 would have additive activity with atezolizumab, in part by targeting innate immunity. Since WAKING opened, DKN-01 has made its way into first line trials in combination with chemotherapy, and corresponding translational work has shown a convincing trend towards improved

clinical benefit from DKN-01 in DKK1-high tumours. Exploratory translational work performed on samples from patients enrolled into WAKING presented in this chapter will include tumoural DKK1 analysis.

This chapter outlines the analysis and results of phase IIa of the WAKING study. I have been the Trial Physician for WAKING since July 2018. In this role, I co-authored the IRAS form, along with the Trial Manager, Richard Crux, and study documents including trial protocol, patient information sheets, GP letter and lay summary, incorporating comments from Patient and Public Involvement. Additionally, I co-drafted the trial pharmacy manual alongside the GI trials pharmacist. In July 2019 I attended the REC meeting on behalf of the CI to discuss the trial and resolve any questions or concerns from the REC panel. Shortly afterwards, REC and Health and Care Research Approval (HRA) approval was granted. When the study opened in February 2020, I was involved in patient selection and recruitment. Unfortunately, recruitment to all trials at our institution was held for 3 months at the start of the Covid-19 pandemic which meant that recruitment was slower than planned. In addition to authoring the study documents, together with Ria Kalaitzaki, study Statistician, and the database programming team, I designed and developed the trial database on MACRO to capture all patient data. As in my role as Trial Physician for the EMERGE study, I ran all aspects of the day-to-day conduct of the phase IIa component of WAKING, which included responding to queries from sites, assessing safety events as delegate of the CI, protocol amendments and data cleaning. Alongside the study Statistician, Amina Tran, I analysed patient data at each dose level and co-authored the SRC report prior to each SRC meeting.

As described, this chapter will also report some translational work from WAKING. My role in the translational plan for the study included drafting the biospecimen collection and analysis plan together with the translational team at Roche, who were kindly funding the work and coordinating central analysis of samples with their vendors. This also involved contributing to the set-up and implementation of the imCORE master contract between our institution, the ICR, and Roche,

delineating terms which included biosample management. The imCORE network is a strategic collaboration between cancer immunotherapy research institutions and Roche's immunotherapy division, to accelerate progress in cancer immunotherapy research. RM-ICR is one of 2 imCORE sites in the UK, and this master contract has since been used across the Trust and ICR for other academic collaborations with Roche. I also worked with Alan Dunlop, head of immunophenotyping at the ICR, and put together a plan to analyse immune cell populations by flow cytometry at various timepoints. Based on the promising translational data which supported previous clinical studies evaluating DKN-01 and its effects on the TME, I sought approval from the study CI to commence collection of translational samples from phase IIa, as I anticipated that this data may be valuable. Concurrently, I drafted the laboratory manual with Isma Rana, Translational Research Manager. When tumoural DKK1 expression emerged as a potentially important predictor of sensitivity to DKN-01 and checkpoint inhibitor efficacy, I worked closely with Leap Therapeutics (supplier of DKN-01 for WAKING) and Isma to expedite sample shipment to their partner lab to run the DKK1 analysis with a Material Transfer Agreement. All tissue samples needed to be cut at RMH before shipment, and based on tumour content in each sample, I helped to coordinate this with Isma. I was not involved in any of the translational analyses at the bench, but instead led on the interpretation of results. For all the translational work, I was involved in drawing up costings together with the Trial Manager, Richard Crux.

I presented the phase IIa results of WAKING as a poster at the ESMO 2022 annual congress (see appendix for copy of poster). The following chapter outlines those results.

3.2 Materials and methods

3.2.1 Study design and treatment

This was an open-label, single-arm phase IIa/b trial to assess the safety, tolerability, and efficacy, of DKN-01 plus atezolizumab, in patients with previously treated, immunotherapy-naïve, advanced, unresectable pMMR OGA. Figure 15 displays the WAKING study schema. The primary endpoint of the phase IIa (safety run-in phase) was to establish a safe and tolerable dose of DKN-01 combined with atezolizumab, for use in the main efficacy phase (phase IIb); evaluation of anti-tumour activity in the phase IIa, assessment of the dynamic status of tumoral DKK1 expression, PD-L1 status, and peripheral MDSC levels during treatment, were exploratory objectives. The primary endpoint for the phase IIb (efficacy phase) was best ORR during treatment, according to RECIST 1.1 (either CR or PR). iRECIST was to also be used in a sensitivity analysis. Secondary endpoints included safety and tolerability of the study treatment in the safety population according to NCI-CTCAE version 5.0, PFS, and OS.

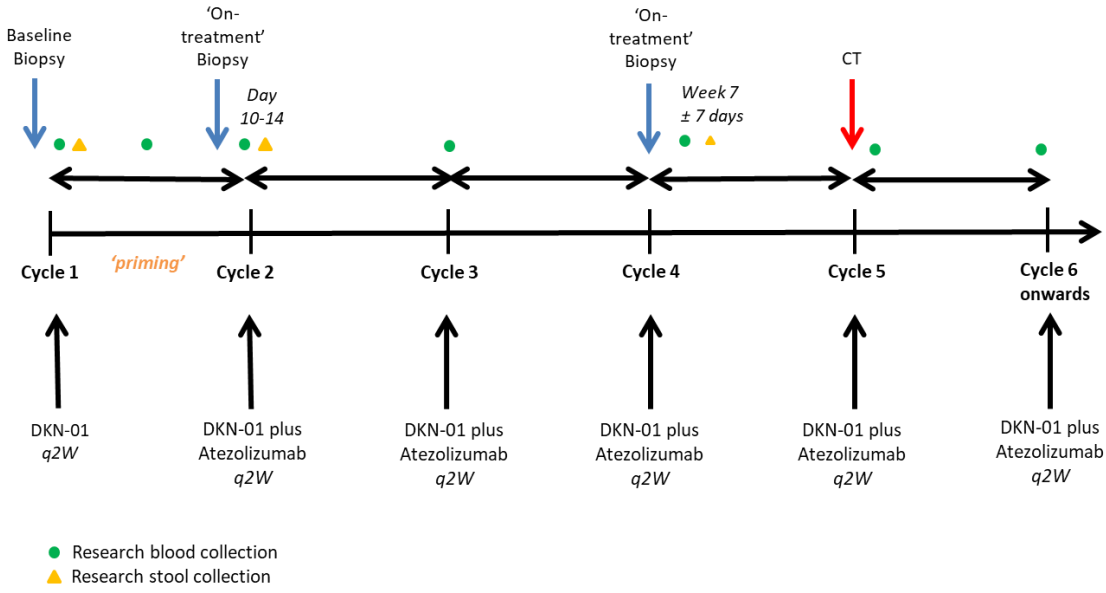


Figure 15 WAKING study schema

Phase IIa was a dose-defining phase that followed a “3+3” design. Two dose levels of intravenous DKN-01 (300mg and 600mg) Q2W, combined with a fixed dose of atezolizumab 840mg Q2W, were assessed for DLT (Table 19). DKN-01 monotherapy was administered two weeks before starting combination DKN-01 plus atezolizumab.(499)

The dose levels of DKN-01 were selected based on previous PK studies in OGA patients treated with DKN-01 on day 1, and day 15 of a 28-day cycle, as a monotherapy, or in combination with paclitaxel. Seventy-one patients were dosed with 300mg DKN-01, 37 as monotherapy, and 34 in combination with paclitaxel, and 19 patients were dosed with 600mg DKN-01, 8 as monotherapy, and 11 in combination with paclitaxel. After 14 days, before the second dose of DKN-01, DKN-01 AUC_{0-7} were not detectable for most patients treated with 300mg. After the first dose in cycle 1, the exposure degree at least doubled for the 2-fold change in DKN-01 concentration. Steady state was reached by the 6^{th} to 8^{th} cycle with both dose levels. No ADA were detected from patients in this study and so this did not impact PK exposure. Previous clinical studies of DKN-01 in patients with cancer have assessed the pharmacodynamic relationship of DKN-01 and total DKK1 concentrations, the DKN-01 target, in serum. Data have shown that higher drug exposure is associated with lower free DKK1 in plasma. In preclinical studies, the lower the DKK1 levels became, the greater tumour growth was attenuated. Furthermore, in patients treated with 300mg DKN-01, those who derived the most clinical benefit (either SD or PR), had lower DKK1 trough levels than patients who progressed on treatment. DKN-01 300mg and 600mg are expected to reduce DKK1 levels to a trough of 0.8 and 0.4ng/mL, respectively. Therefore, higher dose levels appear to lead to greater DKK1 neutralisation and greater clinical efficacy. This information combined with the data from clinical studies, which have shown that 600mg DKN-01 appears to be safe and tolerable, led to the rationale to explore DKN-01 doses up to 600mg in the WAKING study.

A DLT was defined as an AE occurring during the first two cycles of combination DKN-01 and atezolizumab, that met predefined criteria. The SRC reviewed all data before escalating to a higher dose level. Per protocol, no escalation beyond 600mg DKN-01 was permitted. If an MTD could not be established after

evaluation of all dose levels, the dose for expansion would be determined based on safety data.

Drug	Dose level 0	Dose level +1	Dose level -1
DKN-01	300mg IV Q2W	600mg IV Q2W	150mg IV Q2W
Atezolizumab	840mg IV Q2W	840mg IV Q2W	840mg IV Q2W

Table 19 Dose levels during the WAKING safety run-in phase

Patients could receive DKN-01 plus atezolizumab until confirmed disease progression, intolerability, death, or patient withdrawal. Grade 3/4 toxicities were managed by dose delays (up to 4 weeks i.e., 6 weeks from day 1 of the previous cycle), and/or reductions of DKN-01. Dose reductions of atezolizumab were not permitted.

3.2.2 Patients

Adult patients with histologically confirmed MSS or pMMR, advanced and inoperable, or metastatic OGA, who had disease progression after one or two prior lines of treatment for advanced disease, one of which must have been a platinum and fluoropyrimidine combination, were eligible. Patients were required to have adequate bone marrow, renal and liver function, and an ECOG performance status of 0-1. Measurable, or non-measurable but evaluable disease, was required. Patients had to agree to undergo biopsies for translational endpoints and tumours had to be amenable to safe repeated biopsies. Patients on oral anticoagulation needed to change to low molecular weight heparin to be eligible, and it was necessary for all sexually active patients to use highly effective contraception if there was a possibility of conception.

Patients with any prior immunotherapy treatment or other immunomodulatory drugs, other active malignancy, immunodeficiency disorder, or active autoimmune disease requiring systemic treatment in the past 2 years (excluding replacement therapy such as thyroxine, insulin, or physiologic corticosteroid replacement therapy), were excluded. Other exclusion criteria included any patients with brain metastases which were unstable, symptomatic, or required steroids, cerebrovascular disease within the previous 6 months, active infection including HIV or known acquired immunodeficiency syndrome, hepatitis A or C or active hepatitis B, patients unable to swallow orally administered medication or any malabsorption disorder, and any significant cardiovascular disease.

Any antibiotics, opioid analgesics, proton pump inhibitors and corticosteroid use up to two months prior to study enrolment, were recorded where possible, due to their potential impact on the patient's luminal gut microbiome. Stool was collected at the timepoints indicated in Figure 15 to assess the role of the microbiome in response to DKN-01 + atezolizumab treatment. There is ever-increasing data to show that the gut microbiome composition and diversity can modulate antitumour immunity and predict responses to ICIs,(507) therefore, this was an important exploratory objective of the trial.

3.2.3 Safety assessment

Safety evaluations were performed throughout the study, and all adverse events (AEs) were graded for severity according to the CTCAE v 5.0, and relationship with DKN-01 or atezolizumab. A DLT was defined as an AE during the 28-day DLT period that was at least possibly related to the study drugs, and fulfilled at least 1 of the following:

- Grade 4 neutropenia lasting ≥ 5 days or grade 3 or 4 neutropenia with fever and/or infection
- Grade 4 thrombocytopenia (or grade 3 with bleeding)
- Grade 4 anaemia
- Grade 3 or 4 non-haematological toxicity (excluding: grade 3 vomiting and Grade 3 diarrhoea including the clinical sequelae (e.g., electrolyte abnormalities) despite optimal supportive care and excluding alopecia)
- Dosing delay greater than 14 days due to treatment-related AEs or related severe laboratory abnormalities
- Grade 3 hypersensitivity reaction to DKN-01 with premedication (grade 3 hypersensitivity reaction to DKN-01 without premedication is not considered a DLT)
- Grade 4 hypersensitivity reaction to DKN-01 with or without premedication
- Any Grade 5 AE
- Any treatment-related AE that causes the patient to discontinue treatment during the DLT period

Any of the following were not to be considered as DLTs:

- Grade 3 infusion-related reaction resolving within 6 hours and controlled with medical management
- Transient (≤ 6 hours) grade 3 flu-like symptoms or fever, which is controlled with medical management
- Transient (≤ 24 hours) grade 3 fatigue, local reactions, headache, nausea, emesis that resolves to ≤ grade 1

- Grade 3 diarrhoea, grade 3 skin toxicity, or grade 3 liver function test (ALT, AST, or GGT) increase that resolves to ≤ grade 1 in less than 7 days after medical management (e.g., immunosuppressant treatment) has been initiated
- Single laboratory values out of normal range that are unlikely related to trial treatment according to the investigator, do not have any clinical correlate, and resolve to ≤ grade 1 within 7 days with adequate medical management
- Tumour flare phenomenon defined as local pain, irritation, or rash localized at sites of known or suspected tumour

Patients were reviewed at 30 days +/- 3 days after completion of study treatment for an end of treatment review and adverse event reporting. An additional extended safety follow-up was performed 135 days after the last dose of atezolizumab administration.

3.2.4 Laboratory assessments

Blood tests for analysis of peripheral MDSC levels were taken at baseline, C1D8, C1D10, and C4D1. A whole blood assay was used to measure the peripheral MDSCs by flow cytometric analysis. Samples were analysed within 24 hours of collection at the Immunophenotyping Laboratory, The Royal Marsden Hospital. 200ul of fresh whole blood was transferred to a 4ml falcon tube, with the following antibodies added CD15 Fitc, CD33PE, CD66B PC5.5, CD14PC7, CD11B APC, CD62L APC H7, HLADR Pacific blue and CD45 Pacific Orange. After mixing well by vortexing, samples were incubated for 15 minutes at room temperature. After 15 minutes, red blood cells were lysed by the addition of 2.5ml of ammonium chloride lysis buffer. Samples were incubated for 10mins and then spun for 3mins at 300G. The supernatant was discarded, and a vortex tube used to resuspend the cell pellet. 2ml of Phosphate Buffered Saline was added and the sample spun again for 3 mins at 300G. The supernatant was discarded and resuspended in 300ul of PBS. Samples were then run on a cytoflex flow cytometer for 3 minutes.

Tumour biopsies of the same lesion were collected at baseline before starting treatment, at C1D10 during DKN-01 monotherapy, and at C4D1 +/- 7 days after 4 weeks of DKN-01 plus atezolizumab had been administered. FFPE tumour tissue was evaluated for DKK1 expression by Advanced Cell Diagnostics at Flagship Biosciences (Broomfield, CO, USA).(508) A 5µm slide section was stained for DKK1 mRNA by a RNAscope chromogenic in-situ hybridization (CISH) assay on the Leica Biosystems BOND RX platform. FFPE slide sections were quality controlled for background and RNA degradation using dapB and PPIB probes respectively. Tumour percentage score (TPS) of DKK1 staining (≥ 1 dot / cell) was determined on scanned slides using an image analysis algorithm developed by Flagship Biosciences,(509) or manually on glass slides by a pathologist. Initially an H-score was calculated by determining the percentage of low (1-3 dots/cell), medium (4-9 dots/cell), and high (10+ dots/cell). An H-score cut-off of 35 was set for high versus low DKK1 expression. For the WAKING study a switch was made from H-score to TPS, as TPS was easier and faster for pathologists to determine. An H-score of 35 corresponds to TPS of 20%, therefore a tumour with TPS ≥ 20% was considered as having high DKK-1 expression.

3.2.5 Antitumour activity

Imaging assessments were performed at screening (within 28 days prior to commencing study treatment), at 8 weeks from treatment initiation (+/- 1 week), and every 6 weeks (+/- 1 week) thereafter. All responses to treatment (CR, PR, and SD) were confirmed with repeat imaging within 6 weeks. Objective response was evaluated using RECIST v1.1 and iRECIST.(271,432)

3.2.6 Statistical analysis

In the phase IIa safety run-in, a standard “3+3” design was utilised. Therefore, with 3 potential dose levels, the phase IIa was expected to recruit between 3-18

patients. Phase IIa patients treated at the recommended dose were to count towards the phase IIb sample size. In the phase IIb efficacy phase, a single stage A'hern design was used. We were aiming for a meaningful best ORR during treatment of 25% and wished to exclude an ORR of 10%. With one-sided α of 5% and 80% power, we would need to observe at least 8 responses out of a total of 40 patients. The total sample size would be between 3 and 52 patients (6 patients from phase IIa would contribute towards the phase IIb sample size), dependent on the success of the phase IIb.

Safety data was reviewed from all patients who received at least one dose of either trial drug. Dose limiting toxicities (DLTs) were recorded during the patient's DLT period, which was defined as 28 days from the initiation of combination treatment (equivalent to two cycles of combination treatment). If a patient experienced a break in treatment during the DLT period, an extension to the DLT period was applied, which was equivalent to the length of break in treatment. Patients were to be replaced for DLT assessment if they had either (i) discontinued treatment during their DLT period and did not experience a DLT, (ii) experienced a treatment break longer than 4 weeks during the DLT period due to treatment-related toxicity, or (iii) experienced a treatment break longer than 3 weeks during the DLT period, for reasons other than treatment-related toxicity. Adverse events were summarised descriptively, and no statistical inference was performed. Anti-tumour activity was assessed in patients who received at least one cycle of DKN-01 plus atezolizumab combination and were either evaluable for tumour assessment or experienced clinical progression. Disease control rate (DCR) included the number of patients exhibiting CR, PR, or SD within 6 months from treatment initiation. The two-sided 95% confidence intervals (CIs) were calculated using the Clopper-Pearson method. Median PFS, OS, and disease control rates at 6 and 12 months from the start of combination treatment, were summarised by Kaplan-Meier estimates and presented alongside two-sided 95% CIs. Median follow-up was calculated using the reverse Kaplan-Meier method.

3.3 Results

3.3.1 Patient characteristics

Between 19th March 2020 and 17th June 2022, 12 patients with advanced OGA were enrolled and included in the safety analysis. Nine patients were evaluable for DLT assessment, and 10 patients were included in the efficacy analysis (Figure 16). Five patients were treated with 300mg, and 7 patients were treated with 600mg DKN-01, in combination with atezolizumab 840mg from cycle 2 onwards. At the data cut-off date (16th August 2022), the median duration of follow-up was 10.1 months (95% CI lower bound 2.3 months – upper bound not quantified). The patients' characteristics are presented in Table 20. The median age was 61 and most patients (75%) had received only 1 prior line of therapy for advanced disease. Most patients had HER2 negative tumours, 1 patient had a HER2 positive tumour. Six patients (50%) had DKK1-low tumours, and a third of patients (33%) had DKK1-high tumours. Half of patients had lymph node metastases (50%), a third had liver metastases (33%), and a third had peritoneal disease (33%). The median time from initial diagnosis to trial entry was 13 months, and the median duration of last line of anti-cancer therapy prior to trial entry was 2.1 months.

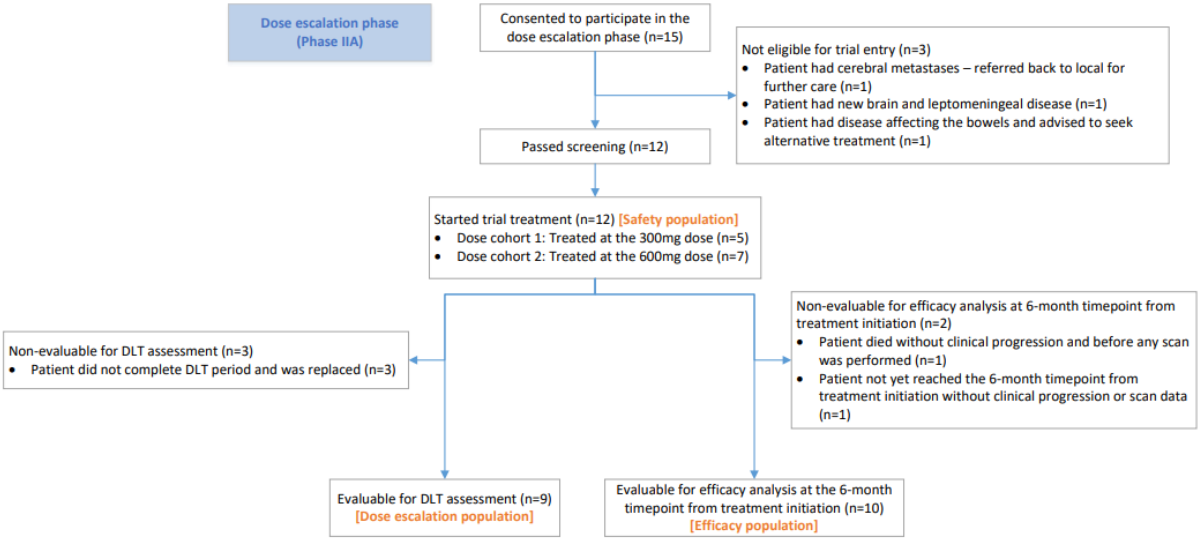


Figure 16 WAKING phase IIa flow diagram

Characteristic (N=12)		
Gender, n (%)		
Female	5	(42)
Male	7	(58)
Age, year		
Median (IQR)	61 (54 – 69)	
Min - Max	36 - 72	
Ethnicity, n (%)		
Caucasian	9	(75)
Mixed race	1	(8)
Asian	1	(8)
Other	1	(8)
HER2 status, n (%)		
Positive	1	(8)
Negative	11	(92)
Baseline DKK1 (TPS) expression, n (%)		
Low (<20%)	6	(50)
High (≥20%)	4	(33)
N/A	2	(17)
ECOG, n (%)		
0	5	(42)
1	7	(58)
Number of prior lines for advanced disease, n (%)		
1	9	(75)
2	3	(25)
Sites of metastases at trial entry, n (%)		
Liver	4	(33)
Lung	3	(25)
Lymph nodes	6	(50)
Peritoneum	4	(33)
Other	9	(75)
Any history of prior radiotherapy, n (%)		
Yes	1	(8)
No	11	(92)
Time from initial diagnosis to trial entry, month		
Median (IQR)	13 (7.4 – 31.6)	
Min - Max	3.2 – 61.8	
Duration of last line of prior anti-cancer therapy, month		
Median (IQR)	2.1 (2.0 – 4.5)	
Min - Max	1.0 – 9.0	

Table 20 Baseline patient demographic and clinical characteristics

3.3.2 Treatment exposure and compliance

Of the 12 patients treated during the dose escalation phase, 1 patient was still on treatment at the time of data cut-off; their last recorded treatment date was 20th July 2022. Of the 11 patients who have since come off treatment, 7 (58%) patients discontinued due to radiological progression, 3 (25%) for clinical progression and 1 (8%) for being unwell and not suitable for further treatment.

The median time on treatment (measured from treatment initiation (C1D1 DKN-01) to treatment discontinuation or death) for the 12 treated patients at the time of data-cut off was 1.9 months (min 0.5, max: 6.7).

Three patients did not complete the DLT period due to worsening of disease-related symptoms and were replaced for DLT assessment. No patient required a dose reduction, and no patient experienced an incident during intravenous administration of treatment. Five patients experienced a delay in receiving atezolizumab or DKN-01. Three of these patients were impacted by DKN-01 drug supply issues in at least one cycle as below:

- RM4976008 received atezolizumab alone (without DKN-01) in cycle 7; cycle 6 was also delayed by 1 week due to drug supply issues.
- RM4976010 received atezolizumab alone (without DKN-01) in cycle 6; cycle 5 was also delayed by 1 week due to drug supply issues.
- RM4976011 received atezolizumab alone (without DKN-01) in cycles 4 and 5.

The lack of DKN-01, supplied by Leap Therapeutics, was due to the drug not being available in the UK depot. This affected 3 patients on the trial in the second dose cohort. An order was placed for DKN-01 in line with the pharmacy guidelines provided by Leap Therapeutics in mid-July 2021, however we were informed by Leap Therapeutics that we would not receive the stock until end of September 2021. All 3 patients were informed of this issue, and all expressed that they would like to continue participating in the trial despite lack of DKN-01. All 3 patients were outside the DLT window before this drug supply issue occurred. Due to the potential impact on the scientific value of the trial, we discussed this with our

internal GCP team and, together with the Trial Manager and GCP team, I prepared paperwork to notify the MHRA of this serious breach. Subsequently an ITSDMC meeting was held to determine the impact of this event on the data integrity and scientific value of the trial. It was felt that as the patients had already passed the DLT period, they could be included in the safety analysis. It was also felt that because these patients only missed 1 or 2 doses of DKN-01, relative to the number of cycles they had received in total, cycles without DKN-01 were a small proportion, and so these patients could also be included in the efficacy analysis. We made the decision to halt further recruitment to the trial until we could secure a 3-month supply of DKN-01 for these 3 patients who were currently still on the trial and had assurances from Leap Therapeutics of continued drug supply going forward. To assist with this, I had weekly meetings with Leap Therapeutics and have since provided an updated IMP forecast every 4 weeks at their request, in attempt to mitigate any further supply issues. Recruitment recommenced in November 2021.

3.3.3 Treatment tolerance and toxicity

The most common TRAEs reported during the study, related to DKN-01 and atezolizumab respectively, are displayed in Tables 21 and 22. During the trial 26 TRAEs were reported. Of these, 18 were reported as related to DKN-01, and 16 were reported as related to atezolizumab. Eight TRAEs were reported as either probably or possibly related to both DKN-01 and atezolizumab across the trial. The most common TRAEs of any grade related to either DKN-01 or atezolizumab were fatigue, anaemia, hypothyroidism, and pain. There was only 1 grade ≥ 3 TRAE of urticaria related to atezolizumab. Immune-related AEs of any grade, or experienced in any patient, were hyperthyroidism, hypothyroidism, infusion-related reaction, and pneumonitis; no events were grade ≥ 3 (Table 23). Grade 2 pneumonitis related to atezolizumab was experienced by 1 patient and reported as an SAE, as it was an AE of special interest. No treatment-related deaths occurred.

CTCAE term	All grades n (%)	Grade ≥ 3 n (%)
Fatigue	4 (33)	0 (0)
Anaemia	2 (17)	0 (0)
Pain	2 (17)	0 (0)
Vomiting	2 (17)	0 (0)

Table 21 TRAEs related to DKN-01 experienced by ≥ 2 patients (any grade) and any TRAEs if they were grade ≥ 3

CTCAE term	All grades n (%)	Grade ≥ 3 n (%)
Fatigue	2 (17)	0 (0)
Hypothyroidism	2 (17)	0 (0)
Pain	2 (17)	0 (0)
Urticaria	1 (8)	1 (8)

Table 22 TRAEs related to atezolizumab experienced by ≥ 2 patients (any grade) and any TRAEs if they were grade ≥ 3

CTCAE term	All grades n (%)	Grade ≥ 3 n (%)
Hyperthyroidism	1 (8)	0 (0)
Hypothyroidism	2 (17)	0 (0)
Infusion-related reaction	1 (8)	0 (0)
Pneumonitis	1 (8)	0 (0)

Table 23 Immune-related adverse events experienced by one or more patients

No DLT was observed and no MTD was reached. Two patients had an extension to their DLT period due to adverse events. For 1 patient, the DLT period was extended by 14 days due to grade 3 urticaria which required steroid treatment. Trial treatment resumed once prednisolone had been weaned to 10mg. The other patient's DLT period was extended by 8 days due to grade 2 ascites, which required elective drainage. Both patients who had a DLT period extension were in the 600mg DKN-01 cohort.

3.3.4 Anti-tumour activity

At the time of data cut-off, 10 of the 12 patients were evaluable for response and included in the efficacy population. Of the 2 non-evaluable patients, 1 patient did not yet have scan data available, whereas the other patient died without scans being performed, and without clinical progression.

Of the 10 patients evaluable for response, only one patient experienced a PR during treatment, and during the 6 months from treatment initiation (Figure 21). Best ORR during treatment, and during the 6 months from treatment initiation, was 10% (95% CI 0.3, 44.5), and the duration of the one response was 2.7 months. This patient stayed on study treatment for over 6 months (Figure 20), receiving 14 cycles. She was a 73-year-old patient with HER2 negative OGJ adenocarcinoma with low volume lymph node metastases in the pelvis and intramuscular metastases, which had recently progressed on first line CAPOX chemotherapy. During cycle 3 of treatment on WAKING, she received palliative radiotherapy to her left inner thigh for symptom relief. Three patients with the longest time on treatment, received 600mg DKN-01 (Figure 20).

Of the 10 patients evaluable for response, 5 (50%) patients experienced disease control during 6 months from treatment initiation. The median duration of disease control was 1.9 months (IQR: 1.6, 2.7).

At the time of data cut-off, 1 patient in the safety population had not yet been followed up for 6 months from treatment initiation, so was excluded from the PFS and OS analysis.; therefore 11 out of the 12 treated patients were analysed for PFS and OS. Of the 11 treated patients, 10 (91%) patients either progressed or died during the 6-month period. The 6-month PFS rate in the safety population was therefore 9.1% (95% CI: 0.5, 33.3) (Table 24), and the 6-month OS rate in the safety population was 45.5% (95% CI: 14.1, 72.8) (Table 25).

Population	No. of patients at risk	No. of pts with disease progression/death during the 6m period	6-month PFS (95% CI)
Safety	11	10	9.1 (0.5, 33.3)
Efficacy	10	9	10.0 (0.6, 35.8)

Table 24 Progression-free survival rates at 6-months from treatment initiation

Population	No. of patients at risk	No. of pts with disease progression/death during the 6m period	6-month PFS (95% CI)
Safety	11	10	9.1 (0.5, 33.3)
Efficacy	10	9	10.0 (0.6, 35.8)

Table 25 Overall survival rates at 6-months from treatment initiation

3.3.5 DKK1 expression and clinical outcomes

DKK expression by RNAscope was reportable in 21 samples from 11 patients treated with DKN-01 and atezolizumab. TPS scores for these samples ranged from 3-81% (Figure 19). DKK1 expression was assessable in baseline biopsies of 10 patients and, of these, 4 (33%) had TPS ≥ 20%. Six patients had a screening biopsy and at least one on-treatment biopsy. Of these, 3 patients had two on-treatment biopsies (C1D10 and C4D1). There were no noticeable trends in DKK1 staining levels for the on-treatment biopsies relative to the screening biopsies (Figure 18). Figure 17 displays examples of DKK-high staining and DKK-low staining from patient samples in the WAKING study.

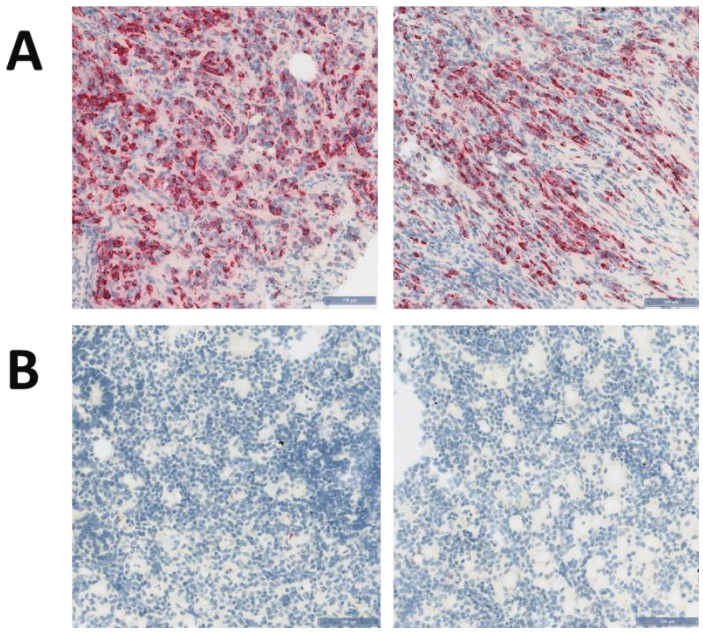


Figure 17 (A) DKK1 staining in the baseline biopsy from patient 008 with DKK1-high expression (TPS 81%), (B) DKK1 staining in the baseline biopsy from patient 013 with DKK1-low expression (TPS 6%). Images kindly provided by Mike Kagey from Leap Therapeutics.

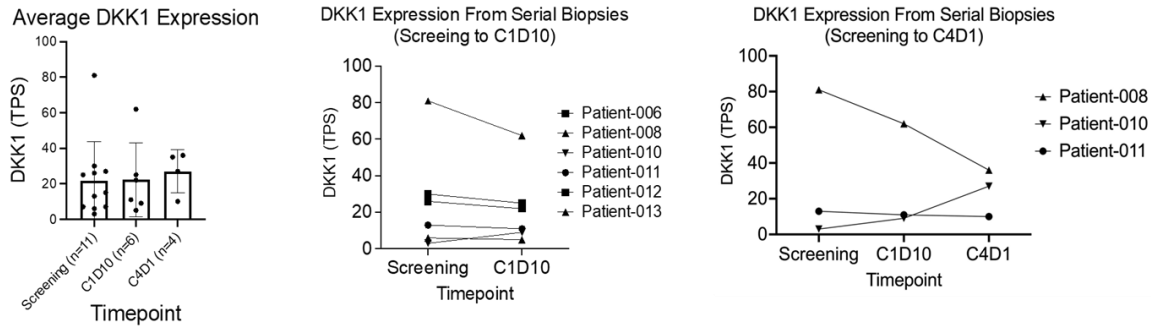


Figure 18 Average DKK1 expression by timepoint. Figure kindly provided by Mike Kagey from Leap Therapeutics.

The 1 patient who achieved a PR had a baseline DKK1 TPS of 81% (Figure 19). The best ORR for the 4 DKK1-high patients was 25% (1 PR, 1 SD, 1 PD, and 1 NE) versus 0% in the DKK-low patients (Table 26).

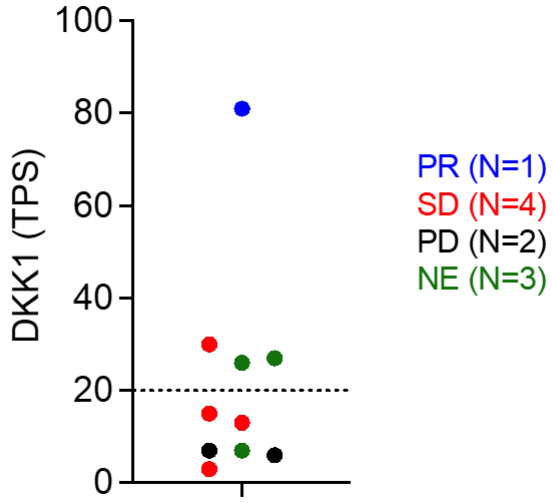


Figure 19 Association of screening biopsy DKK1 expression and best response by RECIST v1.1. Figure kindly provided by Mike Kagey from Leap Therapeutics.

	Partial response	Stable disease	Progressive disease*	Non-evaluable
DKK1-high (N=4)	1 (25)	1 (25)	1 (25)	1 (25)
DKK1-low (N=6)	0	3 (50)	3 (50)	0
DKK1-unknown (N=2)	0	0	1 (50)	1 (50)

*Includes radiological or clinical disease progression; percentages calculated across rows.

Table 26 Baseline DKK1 expression and best response by RECIST v1.1, n (%) (N=12)

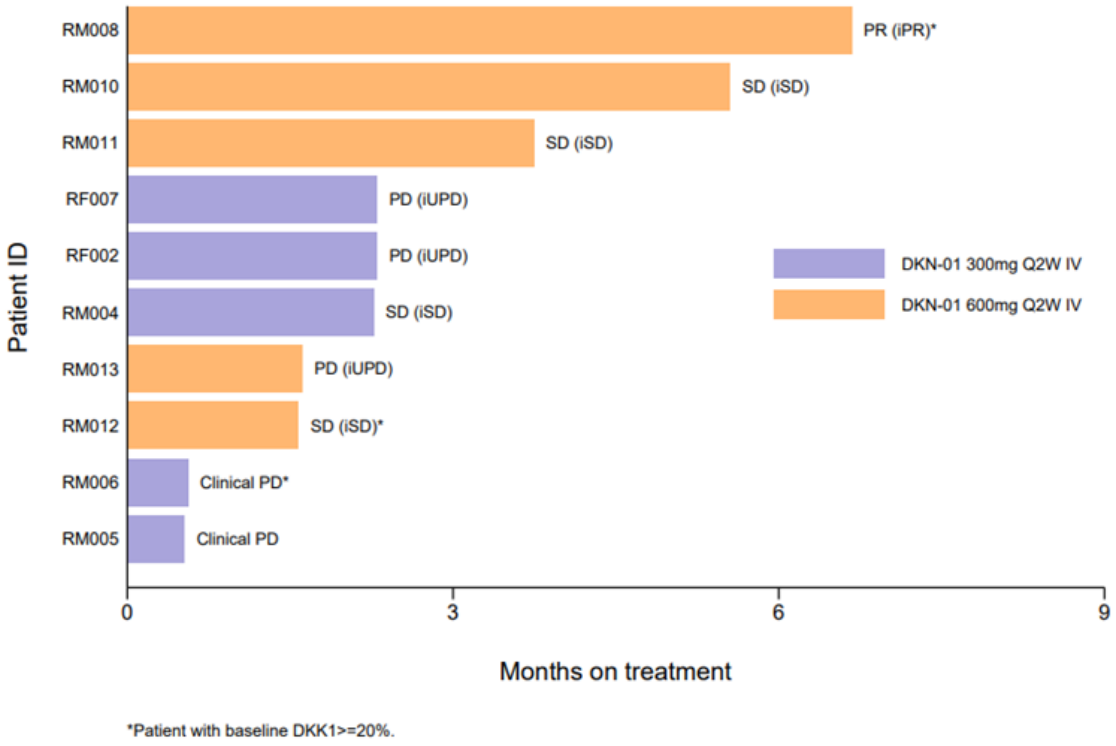


Figure 20 Duration of treatment and best response by RECIST v1.1 (iRECIST) (n=10). Of the 12 treated patients, 2 were non-evaluable (1 patient had no scan data available yet, and 1 patient died without a scan/PD)

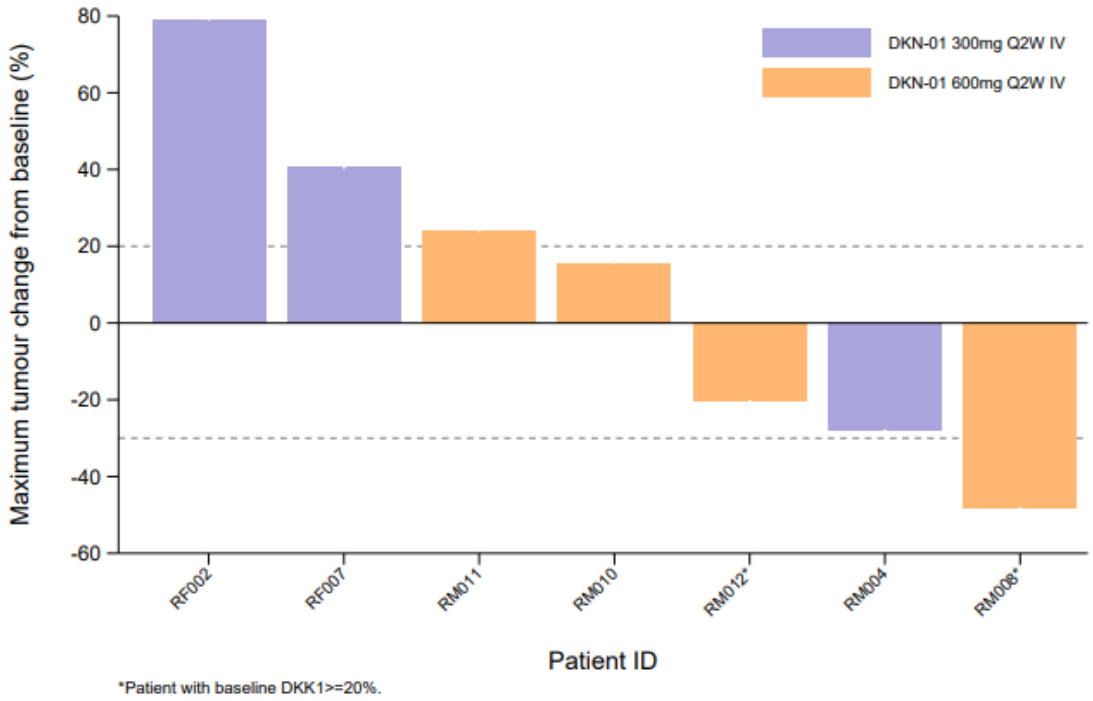


Figure 21 Maximum tumour change from baseline (n=7). Of the 10 treated patients, 1 patient did not have measurable disease and 2 patients clinically progressed with no scans performed

3.3.6 Peripheral MDSC levels and clinical outcomes

Peripheral MDSC levels were reportable in 25 samples from 9 patients treated with DKN-01 and atezolizumab. MDSC levels for these samples ranged from 2.5-81. There were no obvious trends in MSDC levels over the course of trial treatment, or associations with response (Figure 22). However, patient 002, who had the greatest increase in tumour volume during treatment (Figure 21) also had an MSDC level taken at progression, and it had doubled in between C4D1 and PD timepoints; he came off treatment after 6 cycles (data not included on Figure 22). In his case, there is a clear stepwise increase in peripheral MDSC levels over time until progressive disease.

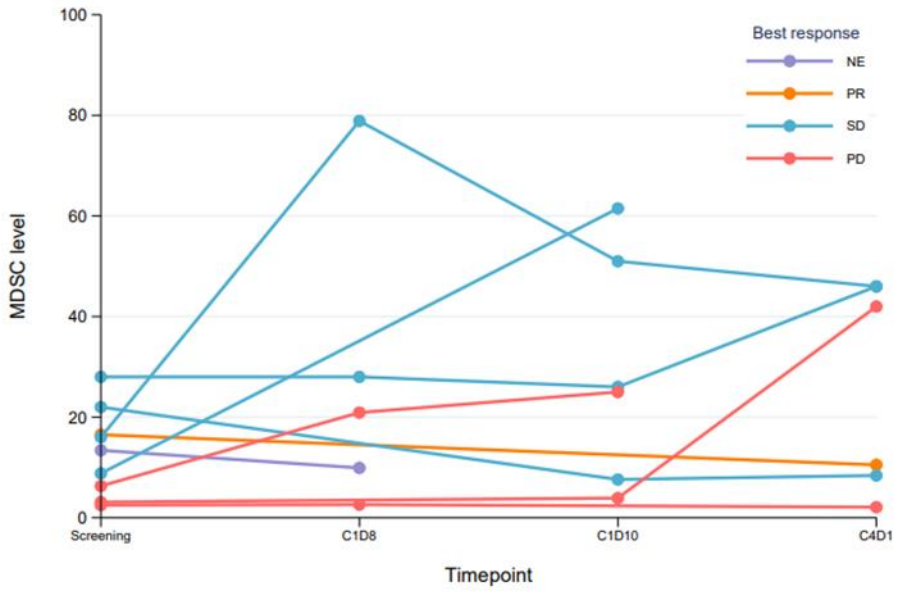


Figure 22 MDSC level by timepoint and best response during treatment

3.4 Discussion

In this phase IIa trial, the combination of DKN-01 and atezolizumab was well-tolerated across a heterogeneous population of patients with pre-treated pMMR OGA. No DLT was observed and no MTD was reached, consistent with findings from a similar phase Ib trial evaluating DKN-01 plus pembrolizumab in patients with advanced OGA.(503) Most drug-related AEs were grade 1-2. Only 1 grade 3 TRAE was observed, and no patient dropped out or died due to treatment. The 14-day cycle of atezolizumab 840mg and 600mg DKN-01 on day 1 was established as the RP2D.

The most common TRAEs were fatigue, anaemia, hypothyroidism, pain, and vomiting. Fatigue and anaemia were commonly reported with DKN-01 in combination with pembrolizumab in a comparable patient population.(502) We report one instance of grade 2 pneumonitis due to atezolizumab, which is not a common occurrence with anti-PD-L1 antibody use in OG patients, but has been observed in 2.5% of patients with OG cancer treated with maintenance avelumab, another anti-PD-L1 antibody.(429,510) One episode of immune-related grade 3 urticaria was well-managed with a treatment interruption and short course of oral prednisolone. There was no evidence of synergistic immune toxicity or infusion reactions. Importantly, the pathological fracture rate with Wnt inhibitor therapy is 4.3%.(461) Unlike in other early clinical studies evaluating Wnt modulating therapies, where markers of bone loss were monitored, and bisphosphonates administered as required, no such monitoring took place on this study. Even though there have been no reports of pathological fracture related to DKN-01 from its use in previous clinical studies, this is a significant adverse effect. DKK1 itself has been implicated in impaired bone healing and promotion of osteolytic metastasis.(477,511,512) Furthermore, mouse models with decreased DKK1 expression have resulted in an increase in bone mass.(477) Therefore, neutralising DKK1 may, in fact, promote bone healing and osteoblastic activity however, this has, so far, not be assessed in the clinic. Future studies with DKN-01 based therapies should, therefore, consider inclusion of bone density monitoring, for example, by measuring β-CTX levels, or with use of DEXA scans.

Assessment of anti-tumour activity and analysis and correlation of DKK1 expression and MDSC levels with clinical outcomes, were exploratory in this small dose-finding study. While clinical activity of DKN-01 and atezolizumab in this unselected heterogenous population was modest at 10% ORR, there was a trend towards dose-dependent efficacy, as 3 patients who stayed on study the longest were treated with the 600mg DKN-01 dose. In line with other data evaluating DKK1 expression in patients with advanced OGA, approximately one third of our population had DKK1-high tumours (TPS ≥ 20%), which is also considered a poor prognostic group.(487–490) Within our DKK1-high group, the ORR was 25% suggesting that elevated baseline DKK1 expression may predict sensitivity to DKK1-targeting therapy. To the best of our knowledge, we are the first group to assess DKK1 expression status directly prior to commencement of DKK1-inhibiting treatment with a 'fresh' tumour biopsy, and to assess the dynamic status of tumoral DKK1 expression over the course of therapy with two sequential on-treatment biopsies. While no obvious trend was observed, the number of patients with both a screening, and at least one on-treatment biopsy, was small and thus interpretation of this data is limited at this stage. Ongoing assessment of dynamic DKK1 expression status is continuing in phase IIb and may provide further insight.

As discussed previously, spatial and temporal genomic heterogeneity is a major stumbling block for personalised medicine efforts in OG cancer. While DKK1 expression analysis was retrospective, by being able to analyse this from a fresh tumour sample just prior to trial entry, we hope to have captured the most accurate molecular landscape of a patient's individual tumour from which to identify a potential predictive biomarker. By choosing to collect all intra-patient biopsies from the same lesion, the focus was an accurate representation of DKK1 expression status over the course of therapy. Therefore, the matter of intra-patient tumoural heterogeneity, and whether the DKK1 expression status of the biopsied lesion was reflective of the individual's entire disease burden (from primary tumour to various metastases) at the time of study entry, still exists. Given that elevated DKK1 levels can also be detected in the serum of OG cancer patients,(491) it may be possible to develop an assay to determine DKK1-high status as a potential blood-based biomarker. This would be a more convenient

method of sampling during treatment, and may also overcome the intra-patient heterogeneity hurdle, and potential for needle sampling errors with tissue biopsies.

Importantly, assessment of PD-L1 status on trial biopsies is ongoing. While two ongoing studies have demonstrated that DKK1 and PD-L1 expression are not correlated,(502,504) as DKK1-high patients respond to DKN-01 and IO combinations despite PD-L1 status,(502,504) PD-L1 positive tumours (particularly CPS ≥ 5) can predict benefit from anti-PD-1/PD-L1 therapy.(93) Therefore, this data is necessary to help determine whether potential clinical benefit from DKN-01 and atezolizumab in the DKK1-high patients is independent to a purely increased sensitivity to atezolizumab alone due to high PD-L1 CPS.

MDSC expansion and activation by the Wnt beta-catenin pathway is a proposed mechanism for immune escape.(492,513) Preclinically a DKK1 neutralising antibody demonstrated reduction in MDSC numbers and increased levels of CD4+ and CD8+ T cells within tumours of mouse models.(492) In an exploratory analysis, we evaluated the dynamic status of circulating MDSC levels before and during treatment with DKN-01 and atezolizumab. No clear trend in peripheral MDSC levels during treatment, or associated with response, have been observed thus far. Peripheral MDSC level evaluation is continuing in phase IIb.

It took over two years to recruit 12 patients and evaluate two different doses of DKN-01 in combination with atezolizumab. This was in part related to pauses in recruitment due, firstly, to the Covid-19 pandemic, and later, in view of the DKN-01 supply issue, which together added 6 months to the enrolment period. Unfortunately, the original 6th patient in the final dose cohort developed obstructive jaundice due to biliary obstruction, caused by periportal lymphadenopathy, during cycle 1 of DKN-01 monotherapy. His treatment was therefore held for 5 weeks for biliary stent insertion but, eventually, he had to come off trial. Given that the protocol indicated that 2 DLTs were required before dose de-escalation, and because no concerning safety signals or DLTs had been seen thus far, I proposed opening the dose expansion phase at this juncture, after 5 patients had been treated at the 600mg DKN-01 dose, to the CI. The aim was

to speed-up recruitment to the trial as a first line trial of chemotherapy and IO and DKN-01 was already in progress, and European approvals for first line chemotherapy and nivolumab in PD-L1 CPS ≥ 5 were anticipated, based on the Checkmate 649 data. This might have made further recruitment to WAKING more challenging if patients were able to access ICIs in the first line, as a requirement for entry was that patients were IO naïve. The CI agreed with this strategy and the SRC chair also agreed. Therefore the 6th patient treated at the 600mg DKN-01 dose was also the first patient in the expansion phase.

At the time of data cut off, 18 patients were enrolled in the WAKING study across both phase IIa and IIb. In addition to tumour PD-L1 status, other translational analyses including T cell receptor analysis, analysis of tumour genomics, including expression of immune and Wnt related genes, and evaluation of stool microbiome are ongoing. As described previously, patients with high levels of DKK1 expression also appear to have Wnt activating mutations such as stabilizing mutations in CTNNB1), loss of function mutations in APC, and loss of function mutations in RNF43. Given that RNF43 mutations are currently being evaluated as a potential biomarker for sensitivity to PORCNi, another class of Wnt modulating drug, it would be of interest to correlate the expression of Wnt related genes and potential response or resistance to DKN-01. Furthermore, as the preclinical data evaluating the effects of DKK1 on immune cells demonstrate a positive correlation with MSDCs within the tissue as one of its most significant immune inhibitory effects, it would be important to also assess MDSC number and function within the tumour tissue itself. As described in chapter 2, shifts in immune cell populations in response to HDACi therapy were only captured within the tumour tissue rather than blood, and, therefore, this may also be the case with DKN-01. Patients with higher MDSCs in their baseline tumour sample, may also benefit the most from a DKN-01 and ICI combination. Based on the preclinical data, levels of expression of NK cell activating ligands and numbers of NK cells in the TME in response to DKN-01, would likely be the other most valuable TME modulating effects to explore. This would also validate the rationale to combine DKN-01 with ICIs, particularly in patients previously unresponsive to ICI therapy.

The gut microbiome has been shown to influence both tumorigenesis and the immune response. In gastric cancer, H. pylori status appears to be significantly correlated with PD-L1 expression, with a greater proportion of H pylori positive tumours having PD-L1 CPS ≥1.(514) Therefore, responses to anti-PD(L)1 therapies may be greater in H. pylori positive patients with OG cancer. Furthermore, responses to anti-CTLA4 therapy appears to depend upon Bacteroides species – presence of B. fragilis appears to enhance anti-CTLA4 efficacy.(515) Abundance of Bifidobacteria has also been linked to a more immune permissive TME,(516) and therefore may also enhance responses to ICIs in OG cancer. The luminal microbiome and correlation with treatment efficacy will be assessed as another exploratory objective of the WAKING study.

Given that addition of checkpoint blockade to chemotherapy has now become standard of care for patients with OGA and PD-L1 CPS ≥5 in the U.K., it is likely that ongoing recruitment to WAKING will, by default, become enriched with either a PD-L1 negative population, or a population with PD-L1 CPS <5. Therefore, if the results of WAKING phase IIB were to be positive, the role of DKN-01 in the current OGA treatment schema would likely be in combination with ICI in the second line, in patients with PD-L1 CPS <5 tumours. However, depending on the results of the randomised first line study evaluating chemotherapy plus tislelizumab +/- DKN-01, it may be that the greatest patient population who benefit from the addition of DKN-01, are those with PD-L1 CPS < 5 tumours. In which case, it would be unlikely that there would be a further role for more DKN-01 in the second line with the current level of data. The results of these trials are eagerly anticipated.

3.5 Conclusion

In summary, the DKK1 neutralising antibody, DKN-01, was safe and tolerable in combination with atezolizumab in patients with advanced pMMR/MSS OGA. TRAEs were manageable with no evidence of enhanced immune-related toxicity. Elevated baseline DKK1 expression may be associated with clinical response, and this will be further explored in the ongoing expansion phase. Future dose-finding studies combining novel agents with checkpoint inhibitors in OG cancer, should consider innovative trial design to accelerate determination of RP2D and maximise the potential benefit to patients with limited treatment options.

Conclusion

This thesis describes the results of three clinical trials of novel therapies for advanced OG cancer and CRC. The iMYC trial raised a particularly important issue regarding robust and reliable biomarker determination in advanced OG cancer. Spatial heterogeneity is an important barrier to personalised medicine efforts in OG cancer, and the challenges faced by the iMYC trial have been echoed by many other trials evaluating biomarker-directed therapies in this disease to date. To address this, newer, larger trials, have started utilising 'liquid biopsies' prospectively, to assign personalised therapies to patients with advanced gastrointestinal cancers progressing on first line treatments, based on their ctDNA genotyping results.(92,517,518) This 'umbrella' trial approach has so far screened over 700 patients with advanced gastric cancer and assigned 14.7% of patients with a biomarker targeted drug, with encouraging response rates compared to standard second line chemotherapy.(519) Given that the genomic profiles of ctDNA are > 85% concordant with metastatic lesions,(92) 'liquid biopsies' as predictive biomarkers should be incorporated into future personalised medicine efforts for gastrointestinal cancers, in attempt to avoid sub-optimal treatments.

Another benefit of ctDNA profiling for guiding therapy selection is the significantly shorter turnaround time compared to tissue-based assays, meaning patients may be enrolled onto trials, and started on therapies more quickly.(517) This is particularly important in aggressive diseases such as OG cancer, where patients can quickly become symptomatic and unwell while off treatment. In the EMERGE trial, we biasedly recruited more patients with CRC to the safety run-in phase, as these patients are often more 'stable' than OG cancer patients. Of course, in WAKING, only OG cancer patients were included, and 3 of the 12 patients who started trial treatment had to be replaced as they did not complete the DLT period due symptomatic, progressive disease. Together with the nature of the "3+3" design, this led to recurrent delays with further recruitment, which then hampered the progression of the safety run-in within the anticipated time frame. Since WAKING opened in 2019, the standard of care for first line treatment of OG

cancer has changed. This not only narrows the potential future pool of patients who may be eligible for WAKING, but also makes the future place for DKN-01 and checkpoint blockade in the treatment paradigm less clear. As a clinical trials unit, we run several phase I-III studies, however our experience with dose-finding studies is proportionally less. In the current era, it would have been quicker, and potentially more cost-effective, to use an adaptive trial design such as the continual reassessment method (CRM) rather than the “3+3”. The CRM includes a Bayesian estimation of the MTD, and integrates accumulated observed data throughout the trial to estimate DLT risk, thus, it can then recommend the best MTD estimate for the next patient.(520) Furthermore, the time-to-event continual reassessment method (TITE-CRM) is particularly useful for determining late onset toxicities, which are common in immunotherapy combination trials, and which may not be reliably captured in a “3+3” design with a DLT period confined to 1 or 2 cycles at treatment initiation.(520) This method does not stagger recruitment, and considers the length of time each patient has been on study in the absence of a DLT.(520) If no DLTs are observed, then the method reverts to the original CRM.

Another important point to consider in future early phase studies of immunotherapy combinations is the concept of MTD, which is a term derived from early studies with chemotherapeutic agents, where it was presumed that the higher the dose delivered, the more cytotoxic effects would be realised. However, in the case of utilising novel agents to ‘prime’ the immune system for collaboration with ICIs, the goal should be to identify the optimal biologically active dose (OBD), as more is not necessarily better. This can only be done by measuring target effect with biological endpoints such as drug concentration in plasma, or immunological parameters such as flux of CD8+ cells or MDSCs in the TME. However, as discussed in chapter 1, in heavily pre-treated patients, reliable PD assessment may be influenced by prior therapies and development of resistant subclones. Therefore, use of window of opportunity trials, where novel treatments are given neoadjuvantly for a few weeks prior to planned surgery, could be utilised. Over this short period, biopsies and blood samples for biomarker assessment could be taken before, during, and after treatment, and functional imaging such as FDG-PET-CT could be used to assess target engagement and

immunomodulatory effects, in a more controlled situation. The added benefit is that the surgical resection specimen could also be examined after the window, which could provide valuable information on the architecture and spatial relationships of immune cell populations in response to therapy as a 'proof-of-concept' of target effect. Importantly, cancer is always evolving, so new treatments/combinations should be tailored according to the phase of evolution of cancer, for example, as discussed, epigenetic modifications tend to occur early on in cancer development and, therefore, targeting with "epi-drugs" may be more effective in the adjuvant/neoadjuvant setting.

Unfortunately, all 3 of the trials presented in this thesis were affected by the Covid-19 pandemic. In addition to the delays to recruitment due to trial closure for 3-4 months, there were other difficulties presented by the restrictions imposed. For example, trial biopsies were put on hold at the start which meant that some patients were not biopsied on the WAKING study, so we have fewer patients with known DKK1 status from which to correlate responses. There were also difficulties with some of the data collection as we tried to reduce hospital attendances as much as possible. This meant that some scheduled appointments were carried out over the phone and, for patients who lived far away, some routine blood tests were missed. Nevertheless, despite these added challenges, as a team, I believe we navigated them well. Ultimately, I was glad to have been able to conduct research and complete the first part of 3 clinical studies which, despite only hinting at a tiny subgroup of patients who might benefit from these therapies, no doubt still contributes to the field.

It is also a field which is ever evolving, and since these trials opened much has changed in the treatment landscape, particularly in OG cancer, with the addition of ICIs. The current unmet need in Europe for patients with OG cancer is now how best to manage patients who have PD-L1 low tumours, or what to offer after ICIs when resistance develops. Perhaps some of these questions will be answered by looking to other tumour types to understand potential resistance mechanisms, and potentially epigenetic modulation and/or Wnt signalling will be important. Expanding the applicability of immune therapies to pMMR/MSS CRC, the third most common cancer in the world, remains a great challenge.

Researchers will need better understanding of tumour biology, primary resistance mechanisms, such as presence of liver metastases, and improved biomarker selection, to take on this exciting but enormous task.

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Publications and presentations associated with this area of research

Publications

2022 **Turkes F,** Bryant A, Begum, R, Davidson M, Kalaitzaki E et al. Ibrutinib in c-MYC and HER2 amplified oesophagogastric carcinoma: results of the proof-of-concept iMYC study. *Current Oncology.* 2022, 29, 2174-2184 PMID:

2020 **Turkes F,** Mencel J, Starling N. Targeting the immune milieu in gastrointestinal cancer. *Journal of Gastroenterology: The Japanese Society of Gastroenterology.* 2020 Oct;55(10_:909-926. PMID: 32748171

2019 **Turkes F**, Carmichael J, Cunningham D, Starling N. Contemporary tailored oncology treatment of biliary tract cancers *Gastroenterology Research and Practice*. 2019 Dec; vol. 2019. PMID: 31929787

2019 **Turkes F**, Chau I. Ramucirumab and its use in the treatment of hepatocellular carcinoma*. Future Oncol.* 2019 Mar;15(9):979-988. PMID: 30644314

Presentations and invited lectures

2022 **Fiona Turkes**, Richard Crux, Amina Tran, Elizabeth Cartwright, Isma Rana, Ed Johnston, Alan Dunlop, Jan Thomas, Alice Smith, Elizabeth Smyth, Charlotte Fribbens, Sheela Rao, David Watkins, Ian Chau, Naureen Starling, David Cunningham, Safety, and efficacy of Wnt inhibition with a DKK1 inhibitor, DKN-01, in combination with atezolizumab in patients with advanced oesophagogastric adenocarcinoma: Phase IIa results of the WAKING trial. Poster presentation, ESMO Annual Congress, September 2022

2022 S. Slater*, E. Cartwright*, C. Saffery, A. Tran, G. Smith, M. Bacason, O. Zhitkov, I. Rana, E. Johnston, M. Aresu, D. Kohoutova, M. Terlizzo, **F. Turkes**, E. Smyth, W. Mansoor, C. Fribbens, S. Rao, D. Watkins, N. Starling, I. Chau, D. Cunningham, EMERGE: a multicentre, non-randomised, single-arm phase II study investigating domatinostat (selective class I HDAC inhibitor) plus avelumab in patients with previously treated advanced mismatch repair proficient oesophagogastric and colorectal adenocarcinoma. Poster presentation, ESMO World GI, June 2022

2021 E. Cartwright, **F. Turkes**, C. Saffery, A. Tran, G. Smith, S. Esteban Moreno, S.Hatt, A. Renn, E. Johnston, D. Kohoutova, R. Begum, E. Smyth, C. Peckitt, C. Fribbens, S. Rao, D. Watkins, I. Chau, N. Starling, D. Cunningham, EMERGE: A multicenter phase II non-randomised trial assessing the efficacy of domatinostat plus avelumab in patients with previously treated advanced mismatch repair proficient oesophagogastric and colorectal cancers – phase IIA dose finding. Poster presentation, ESMO Annual Congress, September 2021

2021 **Fiona Turkes**, Richard Crux, Elizabeth Cartwright, Isma Rana, Ruwaida Begum, Jan Thomas, Alan Dunlop, Amina Tran, Sheela Rao, David Watkins, Ian Chau, Naureen Starling, David Cunningham, WAKING: Wnt and checkpoint Inhibition in gastric cancer. Mini oral live interaction session, 4th IMCORE Scientific summit, March 2021

2020 **F Turkes.** Utilising novel therapies in the treatment of gastrointestinal cancer. Oral presentation, ICR virtual conference, 25th June 2020

2019 **F S Turkes**, R Crux, D Cunningham, A Athauda, E Kalaitzaki, A Musallam, R Begum, K De Paepe, N Fotiadis, A Riddell, K Von Loga, J Kinross, J R Marchesi, J Teare, D Morganstein, S Rao, D Watkins, I Chau, M Gerlinger, N Starling, 661TiP iSCORE: Immunotherapy sequencing in colon and rectal cancer, *Annals of Oncology*, Volume 30, Issue Supplement_5. Poster presentation, ESMO Annual Congress, October 2019

2019 E Cartwright, **F Turkes**, C Saffery, E Kalaitzaki, R Powell, A Wotherspoon, K De Paepe, K von Loga, M Hubank, S Rao, D Watkins, I Chau, N Starling, D Cunningham, 670TiP EMERGE: Epigenetic modulation of the immune response in gastrointestinal cancers, *Annals of Oncology*, Volume 30, Issue Supplement_5. Poster presentation, ESMO Annual Congress, October 2019

2019 M Davidson, C Butler, O Butters, C Fong, **F Turkes**, C Cafferkey, K Kouvelakis, D Cunningham, D Watkins, S Rao, N Starling, I Chau, P-201 Treatment of relapsed oesophagogastric adenocarcinoma after perioperative chemotherapy and surgery, *Annals of Oncology*, Volume 30, Issue Supplement_4, July 2019, mdz155.200. Poster presentation, ESMO World GI, Barcelona, June 2019

2019 **Fiona Turkes**. ‘Treatment of advanced colorectal cancer’, Invited lecture, Gastrointestinal Interdisciplinary Education (GuIDE) Teaching Programme, RMH, 10th April 2019

2017 **Fiona Turkes**. ‘Immuno-oncology in gastrointestinal Cancer’, Invited lecture, Gastrointestinal Interdisciplinary Education (GuIDE) Teaching Programme, RMH, 1st November 2017

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APPENDICES

EMERGE: A multicenter phase II non-randomised trial assessing the efficacy of domatinostat plus avelumab in patients with previously treated advanced mismatch repair proficient oesophagogastric and colorectal cancers – phase IIA dose finding

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Background

- Domatinostat is a histone deacetylase (HDAC) 1,2 and 3 inhibitor, which has demonstrated single agent activity in haematological malignancies[1]
- Preclinical studies with domatinostat have shown beneficial effects on tumour microenvironment by
  - Upregulating the expression of tumour associated antigens
  - Increasing CD8 T cell infiltrate[2]
- Domatinostat has been shown to act synergistically with anti-PD-L1 therapy to decrease tumour volume in mouse models[3]
- Avelumab is an anti-PDL1 monoclonal antibody demonstrating activity in multiple tumour types

Methods

Key eligibility criteria

- Adults aged ≥ 18 years with advanced inoperable or metastatic oesophagogastric or colorectal adenocarcinoma who have received at least one prior chemotherapy treatment
- MMRp
- PS 0-1

Study Design

- EMERGE is a multicentre, phase II, non-randomised trial
- Phase IIA comprises the dose escalation part of the trial
- Domatinostat 100mg once daily (QD), 200mg (QD) and 200mg twice daily (BID) were administered orally, continuous daily dosing in combination with avelumab 10mg/kg q2w from cycle 2 onwards (1 cycle = 2 weeks; **Figure 1**)
- Sequential cohorts of patients were enrolled using a standard 3+3 dose escalation design (**Table 1**)
- Treatment was continued until disease progression, unacceptable toxicity or patient withdrawal of consent

Figure 1. Treatment schema

Table 1. Dosing cohorts

Cohort	Domatinostat	Avelumab	No. of patients
1	100mg/day	10mg/kg	4*
2	200mg/day	10mg/kg	3
3	400mg/day	10mg/kg	3
MTD expansion	400mg/day	10mg/kg	3

MTD, maximum tolerated dose
*Additional patient treated in cohort 1

Study endpoints

- Phase IIA: To recommend a safe and tolerable dose for use in the main efficacy phase of the study
- Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), version 4.0
- Anti-tumour activity was evaluated using Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 and immune RECIST (iRECIST)

Results

Patients

- 13 patients (OGA 2; CRC 11) were enrolled in the dose finding phase of the study, IIA (**Table 2**)
- 200mg BID was found to be the recommended phase 2 dose (RP2D)

Table 2. Baseline patient characteristics

Characteristic	
Gender, n (%)	
Female	6 (46)
Male	7 (54)
Age, year	
Median (range)	55 (50 – 61)
Ethnicity, n (%)	
Caucasian	10 (77)
Other	3 (23)
Cancer cohort	
Colorectal (CRC)	11 (85)
Oesophagogastric (OGA)	2 (15)
ECOG, n (%)	
0	2 (15)
1	11 (85)
Number of prior chemotherapy lines, n (%)	
1	2 (15.4)
2	2 (15.4)
3	6 (46.2)
4	3 (23.1)

ECOG,Eastern Cooperative Oncology Group

Safety

- No dose limiting toxicities were reported
- 12 patients (92.3%) experienced at least 1 treatment related adverse event (TRAE)
- The most common TRAEs of all grades are shown in **Table 3**
- Immune related adverse events (irAE) are shown in **Table 4**
- 1 patient (7.7%) experienced a grade 3 TRAE (rise in alkaline phosphatase). 0 patients experienced a grade 4 or 5 TRAE
- 3 patients (23.1%) experienced ≥ 1 serious adverse event (SAE), none were treatment related
- 6 patients (46.2%) experienced a treatment delay
- 0 patients discontinued treatment due to adverse events

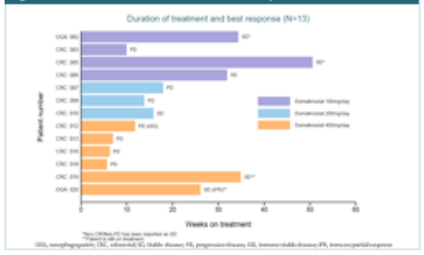
Table 3. TRAEs experienced by ≥ 2 patients

Adverse event, n (%)	All grades (N=13)	Grade 3/4 (N=13)
Fatigue	10 (77)	0 (0)
Anorexia	5 (38)	0 (0)
Nausea	5 (38)	0 (0)
Anaemia	4 (31)	0 (0)
Alkaline Phosphatase Increased	3 (23)	1 (8)
Dry Skin	3 (23)	0 (0)
Insomnia	3 (23)	0 (0)
Serum Amylase Increased	3 (23)	0 (0)
Constipation	2 (15)	0 (0)
Diarrhoea	2 (15)	0 (0)
Electrocardiogram QT Corrected Interval Prolonged	2 (15)	0 (0)
Fever	2 (15)	0 (0)
Hypothyroidism	2 (15)	0 (0)
Mucositis Oral	2 (15)	0 (0)

Table 4. irAEs

Adverse event, n (%)	All grades (N= 13)	Grade 3/4 (N=13)
Hypothyroidism	2 (15)	0 (0)
Aspartate aminotransferase increased	1 (8)	0 (0)
Maculopapular rash	1 (8)	0 (0)

Figure 3. Duration of treatment and best response



Efficacy

- The median duration of treatment is 3.6 months. 2 of the 13 patients are still on treatment (**Figure 3**)
- At the time of data cut of 7 patients have so far achieved stable disease (SD) with 1 of these patients experiencing partial response by iRECIST in the OGA cohort (**Figure 4**)

Figure 4. Maximum tumour change

Conclusions

- Domatinostat up to 200mg BID in combination with avelumab ≥ 10mg/kg was considered safe and 200mg BID was determined as the recommended phase 2 dose (RP2D)
- Cohort expansion in the phase IIB is now recruiting. At time of data cut off on 19th August 23 patients (OGA 16; CRC 7) were enrolled in the study across both Phase IIA and IIB

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Safety and efficacy of Wnt inhibition with a DKK1 inhibitor, DKN-01, in combination with atezolizumab in patients with advanced oesophagogastric adenocarcinoma (OGA): Phase IIa results of the WAKING trial

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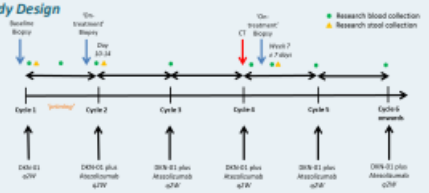
BACKGROUND

- Dickkopf-1 (DKK1) modulates Wnt/β-catenin signalling and promotes an immunosuppressive tumour microenvironment (TME) by activating MDSCs and downregulating NK cell activity [1,2,3]
- DKK1 is frequently overexpressed in OGA and associated with poor prognosis [1,4]
- DKN-01 is a DKK1 neutralising antibody
- Preclinical studies show that DKN-01 can favourably reprogram the TME by:
  - Increasing infiltration of NK cells[5]
  - Reducing function of MDSCs[5]
  - Upregulating PD-L1 expression[5]
- Clinical studies have shown promising response rates with DKN-01 combined with anti-PD-1 therapy ± chemotherapy in patients with microsatellite stable OGA [6,7]
- Patients with DKK1-high tumours appear to benefit most from these combinations [6,7]
- We report the safety and anti-tumour activity of DKN-01 and atezolizumab, anti-PD-L1 antibody, as the second or third-line therapy in patients with advanced OGA enrolled onto the dose escalation phase of the WAKING study

METHODS

Study Design



- WAKING is an open-label, single-arm phase IIa/b non-randomised trial
- Phase IIa is the dose-finding phase that followed a "3+3" design
- Primary objective: to recommend a safe and tolerable dose of DKN-01 combined with atezolizumab for use in the main efficacy phase of the study
- Tumoural DKK1 mRNA expression: assessed by RNAscope on tissue samples (Advanced Cell Diagnostics at Flagship biosciences) and assigned a tumour percentage score (TPS). TPS ≥ 20% was considered DKK1-high
- Tumoural MDSC level: assessed by flow cytometric analysis on peripheral blood

Key eligibility criteria

- Aged ≥18 years
- PD-L1 unselected
- PS 0-1
- ≤ 2 prior lines
- Histologically/cytologically confirmed advanced OGA
- Immunotherapy naïve
- MMRp/MSS

Dosing cohorts

Drug	Dose level 0	Dose level +1	Dose level -1
DKN-01	300mg	600mg	150mg
Atezolizumab	840mg	840mg	840mg

RESULTS

Treatment

- 12 patients were treated in phase IIa
- One patient was still on treatment at data cut-off date (16th August 2022)
- 7 patients discontinued due to radiological progression
- 4 patients discontinued due to clinical progression
- Median time on treatment was 1.9 months
- Longest time on treatment was 6.7 months

Safety

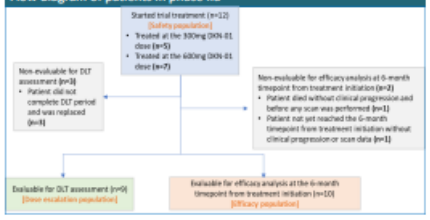
- No DLT was observed and no formal MTD was reached
- 23 TRAEs were reported during phase IIa; 17 related to DKN-01, 14 related to atezolizumab
- 1 patient experienced a grade 3 TRAE (urticaria)
- No treatment-related deaths occurred
- No dose reductions were required
- 7 patients experienced AEs which led to treatment being delayed/discontinued

Patient characteristics (n=12)

Gender, n (%)	
Female	5 (42)
Male	7 (58)
Age, year	
Median (IQR)	61 (54 – 69)
Min - Max	36 - 72
Ethnicity, n (%)	
Caucasian	9 (75)
Mixed race	1 (8)
Asian	1 (8)
Other	1 (8)
HER2 status, n (%)	
Positive	1 (8)
Negative	11 (92)
Baseline DKK1 (TPS) expression, n (%)	
Low (<20%)	6 (50)
High (≥20%)	4 (33)
N/A	2 (17)
ECOG, n (%)	
0	5 (42)
1	7 (58)
Number of prior lines for advanced disease, n (%)	
1	9 (75)
2	3 (25)
Sites of metastases at trial entry, n (%)	
Liver	4 (33)
Lung	3 (25)
Lymph nodes	6 (50)
Peritoneum	4 (33)
Other	9 (75)
Any history of prior radiotherapy, n (%)	
Yes	1 (8)
No	11 (92)
Time from initial diagnosis to trial entry, month	
Median (IQR)	13 (7.4 – 31.6)
Min - Max	3.2 – 61.8
Duration of last line of prior anti-cancer therapy, month	
Median (IQR)	2.1 (2.0 – 4.5)
Min - Max	1.0 – 9.0

ECOG, Eastern Cooperative Oncology Group

Flow diagram of patients in phase IIa



TRAEs related to DKN-01 experienced by ≥ 2 patients or any grade ≥ 3 (n=12)

Adverse event	All grades, n (%)	Grade ≥ 3, n (%)
Fatigue	4 (33)	0 (0)
Anaemia	2 (17)	0 (0)
Pain	2 (17)	0 (0)
Vomiting	2 (17)	0 (0)

n(%), number [proportion] of patients experiencing each TRAE

TRAEs related to atezolizumab experienced by ≥ 2 patients or any grade ≥ 3 (n=12)

Adverse event	All grades, n (%)	Grade ≥ 3, n (%)
Fatigue	2 (17)	0 (0)
Hypothyroidism	2 (17)	0 (0)
Pain	2 (17)	0 (0)
Urticaria	1 (8)	1 (8)

n(%), number [proportion] of patients experiencing each TRAE

Immune-related AEs (n=12)

Adverse event	All grades, n (%)	Grade ≥ 3, n (%)
Hyperthyroidism	1 (8)	0 (0)
Hypothyroidism	2 (17)	0 (0)
Infusion related reaction	1 (8)	0 (0)
Pneumonitis	1 (8)	0 (0)

n(%), number [proportion] of patients experiencing each irAE

Efficacy

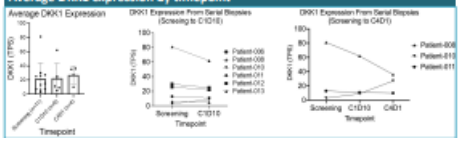
- 10 patients were evaluable for response at the time of data cut-off
- 1 patient with baseline DKK1 expression of 81% TPS had a PR during treatment (best ORR 10%); duration of this response was 2.7 months
- Disease control rate during treatment was 50%; median duration of disease control was 1.9 months (IQR 1.6, 2.7)]
- 3 patients with the longest time on treatment received 600mg DKN-01
- Elevated baseline DKK1 expression (TPS ≥ 20%) may be associated with clinical response (of the 4 DKK1-high patients: Best ORR 25% (1 PR, 1 SD, 1 PD 1 NE))
- DKN-01 and atezolizumab do not appear to effect DKK1 expression or peripheral MDSC levels over time

Best response on treatment by baseline DKK1 expression (RECIST 1.1), n(%) (n=12)

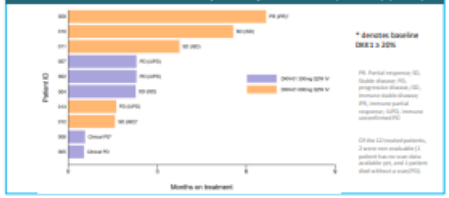
	Partial response	Stable disease	Progressive disease*	Non-evaluable
DKK1-high (N=4)	1 (25)	1 (25)	1 (25)	1 (25)
DKK1-low (N=6)	0	3 (50)	3 (50)	0
DKK1-unknown (N=2)	0	0	1 (50)	1 (50)

*Includes radiological or clinical disease progression; percentages calculated across rows.

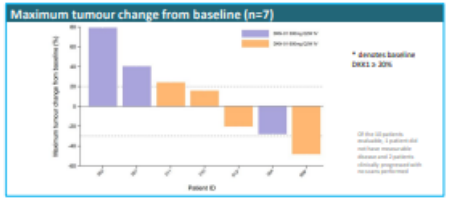
Average DKK1 expression by timepoint



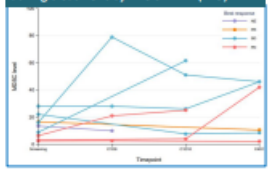
Duration of treatment and best response by RECIST v1.1 (iRECIST) (n=10)



Maximum tumour change from baseline (n=7)



MDSC level by timepoint and best response during treatment by RECIST v1.1 (n=9)



CONCLUSIONS

- DKN-01 up to 600mg in combination with atezolizumab 840mg was considered safe
- DKN-01 600mg was determined as the recommended phase 2 dose
- Phase IIB is now recruiting
- At time of data cut off (16th August 2022), 18 patients were enrolled in the study across both Phase IIA and IIB
- Translational analyses and assessment of PD-L1 status are ongoing

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Disclosures: F Turkes has no conflicts of interest to declare

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