Tumour immune microenvironment biomarkers predicting cytotoxic chemotherapy efficacy in colorectal cancer

Kate Wilkinson, Weng Ng, Tara Laurine Roberts, Therese M Becker, Stephanie Hui-Su Lim, Wei Chua, Cheok Soon Lee

ABSTRACT
The role of the local tumour and stromal immune landscape is increasingly recognised to be important in cancer development, progression and response to therapy. The composition, function, spatial orientation and gene expression profile of the infiltrate of the innate and adaptive immune system at the tumour and surrounding tissue has an established prognostic role in colorectal cancer (CRC). Multiple studies have confirmed that a tumour immune microenvironment (TIME) reflects of a type 1 adaptive immune response is associated with improved prognosis. There have been significant efforts to evolve these observations into validated, histopathology-based prognostic biomarkers, such as the Immunoscore. However, the clinical need lies much more in the development of predictive, not prognostic, biomarkers which have the potential to improve patient outcomes. This is particularly pertinent to help guide cytotoxic chemotherapy use in CRC, which remains the standard of care. Cytotoxic chemotherapy has recognised immunomodulatory activity distinct from its antimitotic effects, including mechanisms such as immunogenic cell death (ICD) and induction/inhibition of key immune players. Response to chemotherapy may differ with regard to molecular subtype of CRC, which are strongly associated with immune phenotypes. Thus, immune markers are potentially useful, though under-reported, predictive biomarkers. In this review, we discuss the impact of the TIME on response to cytotoxic chemotherapy in CRC, with a focus on baseline immune markers, and associated genomic and transcriptomic signatures.

INTRODUCTION
The tumour immune microenvironment (TIME) has an important role in mediating cytotoxic drug response and resistance, as illustrated by the differences in efficacy between in vitro, ectopic tumour mouse models and humans. The TIME is extremely complex in colorectal cancer (CRC), reflecting genomic, host immunity and environmental (including microbiome) diversity. The immune visibility and susceptibility of CRCs can vary widely, and explain differential prognosis. The baseline TIME may facilitate immune evasion through low antigenicity, paucity of immune effectors or immunosuppressive mechanisms, which may contribute to primary resistance to chemotherapy. However, it is hypothesised that immunostimulatory chemotherapy may overcome these deficits specifically to improve prognosis, or conversely be redundant in an optimally infiltrated tumour. There is a significant clinical need to identify biomarkers of response to the standard cytotoxics used in CRC—the antimetabolites (5-fluorouracil (5-FU) and capecitabine), platinum derivatives (oxaliplatin) and topoisomerase inhibitors (irinotecan). This review will summarise the key literature and studies that focus on baseline, pretreatment TIME histopathological markers as potential predictive and prognostic biomarkers in patients with CRC receiving cytotoxic chemotherapy. Biomarkers relevant to radiotherapy and novel immunotherapies are outside the scope of this review.

TIME ASSESSMENT IN CRC
The TIME is composed of various infiltrating cells of the innate and adaptive immune system and their associated mediators. Immune cells can be identified in the core of the tumour (tc), both in intraepithelial cancer cell nests, or the tumour stroma (ts); at the invasive margin (tm), and in organised tertiary lymphoid structures distant from the tumour (tls) (figure 1). This nomenclature will be used in the review to identify biomarker location where identified in respective papers. The cell type, location, density and functional orientation are all relevant for prognostication. Peritumoural infiltrates can be assessed on H&E-stained slides, using semiquantitative validated scoring systems including the Klintrup-Mäkinen (KM) grade and the Jass score. Multiplex immunohistochemical (IHC) techniques in clinically annotated tumour slides, to identify specific immune cells based on surface markers, is currently one of the key assessments of the TIME. Whole slides can be assessed, or tissue microarray techniques used to allow high throughput of samples. Cell density estimation can be performed manually, or assessed through digital image analysis and machine learning algorithms to allow objective quantification, although scoring methodology varies widely. Advances in RNA sequencing, proteomics and single-cell technologies are also increasingly used to assess the TIME. Techniques such as CIBERSORT and MCP-counter can estimate the abundance of immune infiltrate in the tumour using the gene expression data from bulk tissues. Mass cytometry provides data at the individual cell level, and single-cell RNA sequencing allows profiling and classification of individual immune cells. Tumour heterogeneity

Correspondence to
Dr Kate Wilkinson, Liverpool Cancer Therapy Centre, Liverpool Hospital, Liverpool, NSW 2170, Australia; kate.wilkinson1@health.nsw.gov.au

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and sampling issues add complexity to the use of biopsy-driven TIME biomarkers. Key cell types analysed using IHC techniques are listed in table 1, in addition to a summary of their known prognostic and predictive associations.

IMMUNOMODULATORY MECHANISM OF ACTION OF CYTOTOXIC CHEMOTHERAPY

Many chemotherapeutic agents, including oxaliplatin, fluoropyrimidines and irinotecan, have local and systemic immunomodulatory effects beyond their cytostatic mechanisms.10–12 Preclinical models demonstrate that chemotherapy can augment immune responses directly by activation of immune effector cells (eg, production of interferon (IFN)γ) or inhibition of immunosuppressive factors (such as circulating regulatory T cells (Tregs)), or act on tumours directly to increase antigenicity or immunogenicity or susceptibility to immune attack through other mechanisms.13–16 A small repertoire of chemotherapeutics, including oxaliplatin, can generate a specific mechanism of cell death, termed ‘immunogenic cell death’ (ICD), whereby release of specific danger signals from dying tumour cells stimulates a dendritic cell (DC)-mediated, cytotoxic T-helper 1 (Th1) response to eradicate residual tumour cells.17–19 Platinum cytotoxics can cause DC maturation,14 downregulate immune checkpoints and thus increase CD8+ T cell activation.20–21 In vivo, fluoropyrimidines selectively deplete immunosuppressive myeloid-derived suppressor cells (MDSCs),22 although have also been associated with a pro-tumour Th17 response.23–24 The immunogenicity of irinotecan is less certain, although in vivo work has reported influence on Treg and MDSC infiltration,25 and upregulation of tumour PD-L1.26 For clinical correlation, patients receiving neoadjuvant (preoperative) 5-FU/oxaliplatin show increased infiltration of CD3+ and CD8+ natural killer (NK) and CD8+ cells27–28 in resected liver metastases compared with patients undergoing upfront surgery. Neoadjuvant fluoropyrimidines increase the density of CD3+ and CD8+ cells in patients with resected rectal cancer compared with pretreatment biopsies.29–31

TIME BIOMARKERS

Inflammatory infiltrate

Increased tumour inflammatory infiltrate is strongly associated with improved survival,32 although most studies do not specify survival by subgroups based on chemotherapy utilisation. For those studies that do, an increased infiltrate seems to confer a positive prognostic advantage in patients receiving chemotherapy, mirroring the trend in the untreated population. A higher KM grade (more florid infiltrate at invasive margin) is associated with improved overall survival (OS) in patients receiving adjuvant chemotherapy (unspecified regimes)33 34 and FOLFOX (infusional 5-FU and oxaliplatin) chemotherapy.35 36 Tumour-infiltrating lymphocyte (TIL) density CT and IM was not prognostic in stage II/III patients receiving adjuvant 5-FU plus oxaliplatin regimes; however, increased primary TIL density was associated with improved response rates (79% vs 48%, p=0.025) to doublet chemotherapy (oxaliplatin or irinotecan based) in patients with metastatic disease.38 This is notable as the primary tumour TIME appeared to impact on response rates at distant metastatic sites. Morris et al39 reported a significant survival benefit with adjuvant 5-FU chemotherapy versus observation in stage III patients (n=1156) with peritumoural TILs present (HR 0.22, p<0.001) which was not evident in patients with absent TILs (HR 0.84, p=0.29). This suggests a possible predictive role, with 5-FU being more efficacious in patients with pre-existing immune recognition; however, non-standardised methods were used to identify TILs in this study which may impact validity.

CD3+/CD8+ T cells

The predominant infiltrating immune cells in CRC are T lymphocytes, identified by the generic CD3+ surface marker. Cytotoxic CD8+ T lymphocytes recognise tumour antigen presented by MHC class I molecules, thus providing the key antitumour immune response. High density of CD3+ and CD8+ T cells in the core tumour and invasive margin are well established as a positive prognostic marker in the majority of CRC studies.32

Figure 1  Key cells and locations in the tumour immune microenvironment.
### Table 1: Primary tumour prognostic and predictive IHC-based TIME biomarkers in patients receiving chemotherapy

<table>
<thead>
<tr>
<th>Immune biomarker</th>
<th>Location</th>
<th>Prognostic role in early stage patients receiving adjuvant chemotherapy (regime)</th>
<th>Prognostic role in stage IV patients receiving palliative chemotherapy (regime)</th>
<th>Predictive role or differential biomarker prognostic role by treatment group</th>
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<tbody>
<tr>
<td><strong>Specific immune cell</strong></td>
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<tr>
<td>CD3+</td>
<td>CT</td>
<td>Most studies - î density=positive prognostic assoc</td>
<td>î density=positive prognostic assoc</td>
<td>Possible negative predictive role (adjuvant chemotherapy unspecified)</td>
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<tr>
<td></td>
<td></td>
<td>► Improved OS (5-FU), DFS (5-FU)</td>
<td>► Improved OS (unspecified regime)</td>
<td>►î density (vs low density)=improved OS in observation group (not chemotherapy group)</td>
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<td>► Improved DFS (FOLFOX)</td>
<td>► Improved OS (unspecified regime)</td>
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<td></td>
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<td>Few studies – no association</td>
<td>No association (DFS (5-FU), OS (unspecified regimes))</td>
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<td>► OS (unspecified regimes)</td>
<td>►DFS (5-FU), OS (unspecified regimes)</td>
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<td></td>
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<td>► CD4+ CT î density=mixed findings</td>
<td>No association (DFS (5-FU),OS (unspecified regimes))</td>
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<td></td>
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<td>►CD4+ CT î density=positive prognostic association</td>
<td>No association (OS (unspecified regimes))</td>
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<td>► Improved OS (FOLFOX)</td>
<td>►OS (unspecified regime)</td>
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<td>► Improved DFS (FOLFOX), CAPOX</td>
<td>► No association OS (FOLFOX)</td>
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<td>Few studies – no association</td>
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<td>► OS (5-FU), OS (unspecified regimes)</td>
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<td><strong>Immunoscore (CD3+ and CD8+)</strong></td>
<td>0–4</td>
<td>High score=positive prognostic assoc</td>
<td>High score=positive prognostic assoc</td>
<td>Positive predictive role stage III (various adjuvant regimes)</td>
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<td></td>
<td></td>
<td>► Improved DFS high-risk stage II (5-FU)</td>
<td>► High IS (2–4)=DFS benefit with adjuvant chemotherapy (vs low IS 0–1=no benefit)</td>
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<td></td>
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<td>► Improved DFS stage III (FOLFOX)</td>
<td>► Not predictive stage II (5-FU)</td>
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<td></td>
<td></td>
<td>► Improved OS stage III (5-FU), variable regimes</td>
<td>► Mixed findings</td>
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<tr>
<td><strong>Foxp3</strong> (Treg)</td>
<td>CT</td>
<td>î density=mixed findings</td>
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<td>► Improved OS (5-FU)</td>
<td>► Improved OS (FOXFOX), 5-FU oxaliplatin or irinotecan</td>
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<td>► No association DFS (5-FU)</td>
<td>► No association OS (unspecified regime)</td>
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<td>► Worse DFS/OS (unspecified regime)</td>
<td>► Mixed findings</td>
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<td>►FS/OS on multivariate analysis (unspecified regime)</td>
<td>► Mixed findings</td>
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<td><strong>CD66b</strong> (TAN)</td>
<td>CT</td>
<td>î density=positive prognostic association</td>
<td>Mixed findings</td>
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<td>► Improved DFS/OS (5-FU), unspecified regime</td>
<td>► Possible positive predictive role (adjuvant chemotherapy unspecified)</td>
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<td></td>
<td>► Improved DFS (5-FU), OS (unspecified regimes)</td>
<td>► i density (vs low density)=improved OS in adjuvant chemotherapy group</td>
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<tr>
<td><strong>IM</strong></td>
<td></td>
<td>No prognostic association</td>
<td>No prognostic association</td>
<td>Possible negative predictive role (adjuvant chemotherapy unspecified)</td>
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<td>► DFS/OS on multivariate analysis (unspecified regime)</td>
<td>► i density (vs low density)=improved OS in observation group (not chemotherapy group)</td>
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<tr>
<td><strong>CD68+</strong> (general TAM marker)</td>
<td>CT</td>
<td>No prognostic assoc stage II</td>
<td>i density=negative prognostic association</td>
<td>Possible positive predictive role (adjuvant chemotherapy)</td>
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<td></td>
<td></td>
<td>► DFS/OS (5-FU)</td>
<td>► Worse OS (unspecified regimes)</td>
<td>► i density (vs low density)=improved DFS in chemotherapy group only (not observation group)</td>
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<td></td>
<td>► Improved DFS (5-FU)</td>
<td>► OS (unspecified regime)</td>
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<td><strong>CD163+ (M2 polarised TAM)</strong></td>
<td>CT</td>
<td>î density=negative prognostic association</td>
<td>Mixed findings</td>
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<td>► Worse DFS/OS (unspecified regime)</td>
<td>► Possible negative predictive role (adjuvant chemotherapy unspecified)</td>
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<td>► Improved DFS (5-FU)</td>
<td>► i density=no OS/DFS benefit from adjuvant chemotherapy (vs observation); vs low density=positive OS/DFS detriment with adjuvant chemotherapy</td>
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<tr>
<td><strong>IM</strong></td>
<td></td>
<td>No prognostic association</td>
<td>► Positive predictive role (adjuvant chemotherapy)</td>
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<td>► DFS/OS (5-FU)</td>
<td>► i density (vs low density)=improved DFS in adjuvant chemotherapy group</td>
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<td>► Improved DFS (5-FU)</td>
<td>► worse DFS in chemotherapy group (not observation group)</td>
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<tr>
<td><strong>CD206+ (M2 polarised TAM)</strong></td>
<td>CT</td>
<td>î density=negative prognostic association</td>
<td>Mixed findings</td>
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<tr>
<td></td>
<td></td>
<td>► Worse DFS/OS (5-FU)</td>
<td>► Positive negative predictive role (adjuvant chemotherapy unspecified)</td>
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<tr>
<td><strong>IM</strong></td>
<td></td>
<td>No prognostic association</td>
<td>► i density (vs low density)=improved DFS in chemotherapy group (not observation group)</td>
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<td></td>
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<td>► OS (unspecified regime)</td>
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<tr>
<td><strong>CD45RO</strong> (memory T cell)</td>
<td>CT</td>
<td>î density=positive prognostic association</td>
<td>Mixed findings</td>
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<td>► Improved OS (5-FU),</td>
<td>Possible positive predictive role (adjuvant chemotherapy)</td>
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<td></td>
<td></td>
<td>► Improved OS (5-FU), OS (unspecified regimes)</td>
<td>► i density (vs low density)=improved DFS with adjuvant chemotherapy (vs low ratio—no benefit)</td>
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</table>

Continued
However, location is relevant—tumours demonstrating a paucity of CD8+ cells in the tumour core, and lacking the activation markers granzyme-B and IFNγ, have been termed ‘infiltrated excluded’ with worse survival outcomes. The prognostic associations in chemotherapy-treated patients are less well reported. Retrospective studies have confirmed a positive survival association of increased density CD3+ in patients receiving adjuvant FOLF旭 cetuximab (an epidermal growth factor receptor inhibitor) in a large prospective phase III trials of patients receiving adjuvant 5-FU chemotherapy, although some groups have found no relationship. CD3+ was not prognostic for single agent 5-FU chemotherapy, and this may reflect the phenomenon of the ‘infiltrated excluded’ tumour discussed above, which could impact on 5-FU efficacy. In contrast, increased density of CD3+ in patients receiving single agent 5-FU chemotherapy (an epidermal growth factor receptor monoclonal antibody), it is possible that the addition of oxaliplatin to 5-FU may influence the prognostic impact of invasive margin T cells. Increased density of CD8+ in patients receiving adjuvant FOLF旭 cetuximab (an epidermal growth factor receptor monoclonal antibody), and high CD8+ was associated with improved DFS in patients receiving oxaliplatin doublet adjuvant chemotherapy. Some studies have reported that the relative survival benefit of adjuvant 5-FU chemotherapy is much greater for patients with increased density of CD8+ compared with patients with low density, supporting Morris et al’s findings, and suggesting fluoropyrimidines may be more efficacious when a pre-existing Th1 response is present. However, a treatment interaction has not been confirmed by other groups. CD8+ as a prognostic marker in stage IV patients has shown contradictory results (see table 1). Multiple studies have correlated high pretreatment CD3+ and CD8+ cell density on rectal biopsy with increased response rates to neoadjuvant therapy and improved survival, although this has not been replicated in all reports, and outcomes are mediated by the effects of radiotherapy and are thus outside the scope of this review.

### Immunoscore

The Immunoscore (IS) was designed as a digitally quantified IHC assessment of CD8+ and memory T cell (CD45RO+) densities added to produce a cumulative score. It has been validated to show prognostic ability superior to the traditional tumour/node/metastasis (TNM) staging system, with high scores conferred superior survival. CD3 later replaced CD45RO due to superior antibody performance (figure 2). Its validity as a prognostic marker in patients receiving adjuvant 5-FU alone and FOLFOX chemotherapy has been reported, but its role as a predictive marker is less clear. In a recent multinational trial of stage III patients, those with a low IS (0–1) did not benefit from adjuvant chemotherapy (various regimes), whereas those with IS 2–4 did, and the magnitude of the survival benefit was greater the higher the IS. In high-risk stage II disease, high IS was prognostic, but not a predictive discriminator of 5-FU benefit. Interestingly, in an analysis of stage III patients in the IDEA collaboration (3 months vs 6 months of adjuvant FOLFOX), patients with IS 2–4 had a significantly improved DFS with 6 months vs 3 months of FOLFOX (HR 0.53, p=0.0003), whereas patients with IS 0–1 did not derive a significant DFS benefit with extended treatment (HR 0.84, p=0.27). This suggests again possible futility of doublet regimes, irrespective of cumulative dose, in immune-excluded disease and a dose-dependent benefit of oxaliplatin regimes in tumours with a baseline cytotoxic T lymphocyte response.

### CD4+/Foxp3+ T cells

CD4+ helper T cells, which aid tumour immune responses by activation of signalling to facilitate CD8+ T cell-mediated cell death, can exert both Th1 responses which promote antitumour effects with good prognostic association, and Th2 responses which are tumourigenic. CD4+ CT as a prognostic marker has shown positive association in only half of the studies it has been assessed, and in no studies of CD4+ IM. Adjuvant studies referring to chemotherapy are lacking. Increased primary tumour CD4+ CT and IM was prognostic in some studies of stage IV patients receiving mixed palliative regimes but not in other cohorts.

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**Table 1**

<table>
<thead>
<tr>
<th>Immune biomarker</th>
<th>Location</th>
<th>Prognostic role in early stage patients receiving adjuvant chemotherapy (regime)</th>
<th>Prognostic role in stage IV patients receiving palliative chemotherapy (regime)</th>
<th>Predictive role or differential biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM + density=positive prognostic association</td>
<td>Improved OS (unspecified regime)</td>
<td>No predictive role (adjuvant chemotherapy unspecified)</td>
<td>I density=improved OS in patients treated with and without adjuvant chemotherapy</td>
<td></td>
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</tbody>
</table>

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CS, core tumour stroma; CT, core tumour; DFS, disease-free survival; FOLFOX, F-FU, 5-fluorouracil; IHC, immunohistochemical; IM, invasive margin; OS, overall survival; TAM, tumour-associated macrophage; TAN, tumour-associated neutrophil; TME, tumour immune microenvironment; TLS, tertiary lymphoid structures.
receiving oxaliplatin regimes.\textsuperscript{56} T\textsubscript{em} constitute a specific subtype of CD4\textsuperscript+ T cell, identified by immunoprofile CD25\textsuperscript+ Foxp3\textsuperscript+ and have high tumour-infiltrating CD8\textsuperscript+ infiltration and better therapeutic efficacy.\textsuperscript{80} Future exploration in patient cohorts using differential chemotherapy regimes is required.

**CD45RO\textsuperscript+ T cells**

Central and effector memory T cells (characterised by CD45RO\textsuperscript+ marker) drive secondary immune responses post exposure to primary antigens. Meta-analyses suggest a positive prognostic association for increased density of these cells both in the core tumour and invasive margin.\textsuperscript{74} High density of primary tumour CD45RO\textsuperscript+\textsuperscript{72} and CD45RO\textsuperscript+\textsuperscript{62} is an independent prognostic factor for improved OS in early stage disease patients receiving 5-FU. High density was associated with better survival in patients with stage IV CRC undergoing adjuvant oxaliplatin or irinotecan chemotherapy post-curative intent resection (estimated 3-year survival 62\% vs 27\%, p=0.007).\textsuperscript{54} High CCR7\textsuperscript+ (used to identify CD8\textsuperscript+ naïve and central memory T cells) was associated with improved OS in patients receiving palliative oxaliplatin-based regimes.\textsuperscript{31}

**Gamma delta (\(\gamma\delta\)) T cells**

Gamma delta T cells are a rare subset of predominantly mucosal CD8\textsuperscript+ CD4\textsuperscript- T cells with a broad functional role in cytokine (IFN-\(\gamma\), tumour necrosis factor (TNF)-\(\alpha\), interleukin (IL)-17) and chemokine (RANTES, IP-10, lymphoactin) production, cytosis and coordination of antigen presentation.\textsuperscript{82} In vivo studies show that ICD-inducing chemotherapy causes a rapid invasion of \(\gamma\delta\) T lymphocytes prior to the invasion of CD8\textsuperscript+ T cells, and that in TCR \(\delta\) mouse, the therapeutic efficacy of chemotherapy was reduced.\textsuperscript{83} Increased expression of \(\gamma\delta\) T cells has been associated with improved DFS in patients with CRC.\textsuperscript{75} While results from CRC cohorts receiving chemotherapy are under-reported, a series (n=463) of patients with gastric cancer receiving adjuvant 5-FU chemotherapy suggest a significant survival advantage of chemotherapy versus observation if infiltrating \(\gamma\delta\) T cells were increased.\textsuperscript{85}

**B cells**

B cells also recognise tumour antigens, produce tumour-specific antibodies and are identified through CD19\textsuperscript+, CD20\textsuperscript+ and CD78\textsuperscript+ markers. High CD20\textsuperscript+ has been associated with better prognosis in CRC.\textsuperscript{86} as has the presence of TLJs, which contain concentrated B cells.\textsuperscript{87} However, CD20\textsuperscript+\textsuperscript{88} was not prognostic in patients receiving adjuvant FOLFOX.\textsuperscript{85}

**Immune checkpoints**

Multiple stimulatory and inhibitory immune checkpoints, crucial for self-tolerance, and co-opted by tumours to evade immunosurveillance, have been identified in the TIME. One such checkpoint, programmed death-ligand 1 (PD-L1), is predominantly derived from the immune infiltrate,\textsuperscript{88} not tumour cells, in CRC. Immunodeficient murine xenograft models of PD-L1 knockout tumours display resistance to oxaliplatin,\textsuperscript{89} which contrasts with models in other tumour types. In early stage patients receiving 5-FU chemotherapy, high tumour PD-L1 was not prognostic in some studies,\textsuperscript{40} although negatively impacted on DFS in another stage III cohort receiving adjuvant chemotherapy.\textsuperscript{90} In contrast, PD-L1 expression on immune infiltrating mononuclear cells was associated with longer DFS. Dunne et al\textsuperscript{40} reported that in stage III CRC (n=201), PD-L1\textsuperscript+ tumours conferred a significant DFS benefit from adjuvant chemotherapy versus observation (adjusted HR 0.44, p=0.0062), and the use of adjuvant chemotherapy was able to overcome the negative prognostic impact of...
low PD-L1. However, in contrast, PD-L1\textsubscript{high} expression resulted in inferior DFS post adjuvant chemotherapy versus observation (unadjusted HR 4.95, 95% CI 1.10 to 22.35, \(p=0.02\)), although the significance was lost on multivariate analysis. This is one of the first series to suggest a possible detrimental effect of chemotherapy in tumours which overexpress PD-L1.

**Immune markers associated with microsatellite instability**

Tumours harbouring microsatellite instability (MSI) have defects in the DNA mismatch repair system (dMMR), and thus display a hypermutable phenotype. The differential improved survival in patients with early stage dMMR tumours has been extensively reported and is partly attributable to increased immune stimulation in these tumours due to the increased neoantigen load. MSI tumours have a dense infiltration of CD8\textsuperscript{+} cells, a T\textsubscript{H}1 cytokine response, and also overexpress many inhibitory immune checkpoints (including PD-1, PD-L1, CTLA-4, LAG-3 and IDO). However, MSI tumours are more chemoresistant to 5-FU than microsatellite stable (MS\textsubscript{S}) lines in preclinical models. The relative survival benefit from FOLFOX compared with 5-FU is much greater in stage II-III dMMR patients compared with pMMR, suggesting possible resistance to 5-FU alone. However, in a recent report from the FoXTROT trial, dMMR colon cancers showed significantly reduced pathological response rates to neoadjuvant oxaliplatin doublet chemotherapy than pMMR, and clinical progression through this chemotherapy regime was more common in dMMR than pMMR rectal cancers (29% vs 0%, \(p=0.001\)).

In contrast, in the metastatic setting, MSI status did not affect response rates to palliative FOLFOX chemotherapy. Regarding irinotecan therapy, in both cell lines and tumour xenografts, dMMR tumours are more sensitive to irinotecan than MMR proficient (pMMR) lines. In a small retrospective cohort, response rates to palliative 5-FU plus irinotecan were much higher in MSI than MS\textsubscript{S} disease (57% vs 10%, \(p=0.0009\)) and DFS was longer in MSI tumours receiving an irinotecan containing regime in a separate cohort. In a large adjuvant phase III trial, only patients with dMMR tumours received a DFS benefit from adding irinotecan to 5-FU, but this MSI/treatment interaction was not confirmed in another similar trial. The relevance of immunological variation on chemotherapeutic response in the context of genetic alterations is largely unknown.

**Immunogenic Cell Death markers**

ICD is the cornerstone of the immunomodulatory action of oxaliplatin and associated markers are potential predictive biomarkers (figure 3). DC activation is a key step in ICD. However, identification of DCs, which show functional diversity and heterogeneous activation states, can be challenging and markers are variably reported between studies and may account for conflicting results reporting both good and bad prognostic association. In vivo studies have demonstrated that blockade of surface calreticulin exposure and HMGB1-dependent TLR\textsubscript{4} signalling, both key steps in ICD, severely compromised the cytotoxicity of oxaliplatin chemotherapy.

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**Figure 3** Key cells and pathways in immunogenic cell death as potential predictive biomarkers. IFN\(\gamma\), interferon; IL, interleukin.
Stromal calreticulin expression is associated with infiltration of CD45RO+ cells and improved OS in univariate analysis in patients receiving adjuvant 5-FU.113

Stromal markers
The tumour stroma plays a direct and indirect role in modulating response to immunomodulatory chemotherapy. De novo drug resistance may occur from environment-mediated phenomena, where cancer cells are protected from treatment-induced apoptosis by ‘barriers’, including either soluble secreted factors or cell-adhesion-mediated mechanisms.114 The tumour:stroma percentage is a validated prognostic marker, with increased stromal percentage associated with poorer prognosis, including in chemotherapy-treated patients.115 Cancer-associated fibroblasts (CAF) are a heterogeneous group of fibroblast-like cells that release certain cytokines, growth factors and proinflammatory factors. In vitro cell line studies suggest CAFs trigger a JAK/STAT pathway signalling cascade that leads to reduced response rates to oxaliplatin and 5-FU.116 and stromal CAF-derived conditioned medium primed the growth of cancer stem cells after treatment with 5-FU and oxaliplatin, thus increasing their inherent chemoresistance.117 High CAF infiltration is associated with worse DFS in adjuvant-treated patients118 and associated-induced expression of their surrogate markers smooth muscle actin and survivin have been related to worse survival in 5-FU119 and oxaliplatin-treated advanced patients.120

Genomic markers and transcriptomic profiles
Recent advances in high-throughput gene testing technology have led to the development of some molecular signatures for chemotherapy prediction. Increased expression of infiltrating immune cells, as identified by CIBERSORT, showed a trend to improved overall survival in patients receiving chemotherapy.121 Multiple classifications of CRC, based on molecular transcriptomic data, have been proposed in recent years, and unified into the Consensus Molecular Subtypes (CMS). This incorporates gene expression profiles from the tumour, stroma and immune cells to differentiate four groups (CMS1-4) and are highly correlated with immune cell infiltration patterns.122 The CMS1 subgroup (MSI-like) is enriched for genes coding for CD8+ and CD68+ cells, T-cell attracting chemokines, TLS and Th1 cytokines. The CMS4 subgroup (mesenchymal) is enriched for expression of genes encoding CD8+ cells, MDSCs, Treg, T17+ cells, angiogenic factors and immunosuppressive molecules (eg, TGFβ1). Both CMS2 (canonical) and CMS3 (metabolic) subgroups exhibit low-immune and low-inflammatory signatures. In a retrospective taxonomy study, only CMS2 and 3 subgroups derived a benefit from adjuvant chemotherapy (unspecified) in stage III disease, with CMS4 showing a trend to benefit.123 Song et al124 used an alternative transcriptomic classifier (CRCA) to examine patients in the NSABP-07 trial (adjuvant FOLFOX vs 5-FU), and reported only patients with an ‘enterocyte’ subtype (with immune features similar to the ‘cold’ CMS2) derived a benefit from the addition of oxaliplatin, with a significant interaction test. The same group repeated the analysis using patients enrolled on the MOSAIC trial (adjuvant CAPOX vs capcitabine) but did not find any association,125 which may be due to different fluoropyrimidine use or oxaliplatin schedule, which have shown different interactions in other immune biomarker studies.126 CMS1 patients have worse OS with FOLFIRI-based regimes compared with the other CMS subtypes in the FIRE-3 trial127; however, they also show improved OS with the addition of bevacizumab in the metastatic setting.127 Published studies suggest a trend to 5FU/oxaliplatin resistance in CMS4 (or similar classifier) patients, both in the adjuvant128 and metastatic setting,129 where first-line irinotecan regimes showed better response rates and survival.130 131

IMMUNE INFILTRATE IN RESECTED METASTASES
Several reports have assessed the prognostic and predictive impact of the TIME from resected metastases, predominantly liver metastases,132 which appears to correlate with the primary tumour. However, many of the studies include patients receiving neoadjuvant therapy, which can alter the immune infiltrate substantially. Metastatic disease has a different immunological milieu which is defined by tumour immune evasion. Liver metastases with pretreatment high Immunoscore (and high CD3+, CD8+ and CD20+ cells133) are associated with increased response to chemotherapy (p=0.009) and improved DFS and OS.134 The type of postoperative chemotherapy/adjuvant did not impact survival. However, a high IS is not tantamount to excellent prognosis in this setting (as opposed to with early stage disease) as most patients relapsed after surgery. The authors showed the density of CD8+/CD20+/CT+IM to be an additional strong prognostic discriminator. A high ‘density score’ (based on a cumulative density of CD3+, CD8+ and granzyme B in liver metastases) was also reported by Halama et al134 to have significant prognostic ability in stage IV patients receiving any regime of chemotherapy (HR OS 0.06, p<0.01).

CONCLUSIONS
Here, we have reviewed CRC studies focusing on the TIME and found that the prognostic ability of these markers in CRC is mediated in the context of chemotherapy, and true predictive studies are under-reported. While prognostic biomarkers have been used as a surrogate for predictive markers, with an assumption that patients with ‘poor’ prognosis will gain a greater absolute benefit from chemotherapy, this may be untrue, especially if the biomarker is also a marker of therapy resistance. Nevertheless, current reports indicate that the relative benefit of 5-FU chemotherapy may be enhanced in the context of some pre-existing CD8+/CD3+ infiltration in core tumour, but may be unnecessary or importantly even detrimental in the milieu of a highly inflamed TIME. CRGs with high immunosuppressive pathways may also be more resistant to oxaliplatin doublets. Furthermore, chemotherapy may improve prognosis in cancers driven by specific immune cell populations, such as TAMs and TANS. The emerging move

Take home messages
► The tumour immune microenvironment has an important role to play in mediating cytotoxic chemotherapy response and primary resistance.
► Many chemotherapy agents used in colorectal cancer have local and systemic immunomodulatory effects.
►Baseline tumour immune cells, including T cell subsets, tumour-associated neutrophils and macrophages, may represent potential predictive biomarker predicting response and resistance to cytotoxic chemotherapy.
►Prospective trials using standardised validated markers, such as the Immunoscore, are required to rationalise the use of adjuvant chemotherapy and target different palliative chemotherapy regimes and adjuncts to patients more likely to respond.
to standardise assessment of TILs/IHC-based markers in CRC reporting has the potential for more robust prospective trials. Such trials are needed to develop better clinical biomarkers for therapy benefit and cytotoxic effects of chemotherapy. Patients likely having adverse effects may require de-escalated or even no therapy, and some may require alternative or combination agents, which have shown early promise.153 Significant research and development is in progress with regard to such adjuncts, which include various combination approaches with synergistic benefits154 and novel immunotherapies, to improve precision medicine in the future.

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ORCID iD Kate Wilkinson http://orcid.org/0000-0001-8318-4615

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