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Original Research

The correlation between immune subtypes and consensus molecular subtypes in colorectal cancer identifies novel tumour microenvironment profiles, with prognostic and therapeutic implications

B. Soldevilla [a,b,1], C. Carretero-Puche [a,b,1], G. Gomez-Lopez [c], F. Al-Shahrour [c], M.C. Riesco [a,d], B. Gil-Calderon [a,b], L. Alvarez-Vallina [e,f,g], P. Espinosa-Olarte [a,d], G. Gomez-Esteves [a,b], B. Rubio-Cuesta [a,b], J. Sarmentero [a,b], A. La Salvia [a,d], R. Garcia-Carbonero [a,d,h,*]

[a] *Laboratorio de Oncología Clínico-Traslacional, Unidad de Investigación en Tumores Digestivos, Instituto de Investigación I+12, Hospital 12 de Octubre, 28041, Madrid, Spain*
[b] *Centro Nacional de Investigación Oncológica (CNIO), 28029, Madrid, Spain*
[c] *Bioinformatics Unit, Centro Nacional de Investigación Oncológica (CNIO), 28029, Madrid, Spain*
[d] *Oncology Department, Hospital Universitario Doce de Octubre, Instituto de Investigación Sanitaria Hospital 12 de Octubre (imas12), UCM, CNIO, CIBERONC, Madrid, Spain*
[e] *Cancer Immunotherapy Unit (UNICA), Department of Immunology, Hospital Universitario 12 de Octubre, 28041, Madrid, Spain*
[f] *Immuno-Oncology and Immunotherapy Group, Instituto de Investigación Sanitaria 12 de Octubre (i+12), 28041, Madrid, Spain*
[g] *Immunotherapy and Cell Engineering Laboratory, Department of Engineering, Aarhus University, 8000, C Aarhus, Denmark*
[h] *Complutense University of Madrid, Madrid, Spain*

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Abstract ***Background:*** Solid tumour growth is the consequence of a complex interplay between cancer cells and their microenvironment. Recently, a new global transcriptomic immune classification of solid tumours has identified six immune subtypes (ISs) (C1–C6). Our aim was to specifically characterise ISs in colorectal cancer (CRC) and assess their interplay with the consensus molecular subtypes (CMSs).

* *Corresponding author*: Medical Oncology Department, Hospital Universitario Doce de Octubre, Avenida de Cordoba s/n, 28041, Madrid, Spain.Fax: +34 91 4603310
E-mail address: rgcarbonero@gmail.com (R. Garcia-Carbonero).
[1] These authors contributed equally to this study.

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Tumour microenviroment; Immunotherapy

Methods: Clinical and molecular information, including CMSs and ISs, were obtained from The Cancer Genome Atlas (TCGA) (N = 625). Immune cell populations, differential gene expression and gene set enrichment analysis were performed to characterise ISs in the global CRC population by using CMSs.

Results: Only 5 ISs were identified in CRC, predominantly C1 *wound healing* (77%) and C2 *IFN-γ dominant* (17%). CMS1 showed the highest proportion of C2 (53%), whereas C1 was particularly dominant in CMS2 (91%). CMS3 had the highest representation of C3 *inflammatory* (7%) and C4 *lymphocyte depleted* ISs (4%), whereas all C6 *TGF-β dominant* cases belonged to CMS4 (2.3%). Prognostic relevance of ISs in CRC substantially differed from that reported for the global TCGA, and ISs had a greater ability to stratify the prognosis of CRC patients than CMS classification. C2 had higher densities of CD8, CD4 activated, follicular helper T cells, regulatory T cells and neutrophils and the highest M1/M2 polarisation. C2 had a heightened activation of pathways related to the immune system, apoptosis and DNA repair, mTOR signalling and oxidative phosphorylation, whereas C1 was more dependent of metabolic pathways.

Conclusions: The correlation of IS and CMS allows a more precise categorisation of patients with relevant clinical and biological implications, which may be valuable tools to improve tailored therapeutic interventions in CRC patients.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death. CRC management is primarily based on tumour location and extent of disease, with a very limited repertoire of molecular biomarkers to guide personalised patient care. CRC is however a heterogeneous disease with widely variable clinical outcomes, both in terms of prognosis and drug response. Tumour diversity is also reflected at the molecular level, and molecular classifications of CRC have substantially evolved in recent years.

At the genomic level, two major groups can be identified as follows: i) hypermutated (~15% of CRCs), often with microsatellite instability (MSI) and are associated with a rich immune cell infiltration, a favourable prognosis in early stages and a particular susceptibility to immune checkpoint inhibition in advanced disease and ii) non-hypermutated (~85%), that generally present chromosomal instability (CIN), and are associated with a worse prognosis and a poor response to immunotherapy. However, the only genomic events well established to guide standard therapy in the clinic are RAS mutations, to exclude epidermal growth factor receptor (EGFR)-targeted therapy, and MSI, to select patients for treatment with immune checkpoint inhibitors.

But cancer initiation and progression are the consequence of a complex interplay between tumour cells and their microenvironment. In this regard, transcriptomic classifications have improved the biological characterisation of CRC, as they encompass not only the tumour cells but also the immune and stromal components of cancer [1–6]. In 2015, the international CRC Subtyping Consortium proposed a unified transcriptomic classification that identified four biologically distinct consensus molecular subtypes (CMSs) [7]: CMS1 (MSI immune subtype [IS], 14%), characterised by BRAF mutation–enriched, hypermutated and hypermethylated tumours, with a strong immune activation; CMS2 (canonical subtype, 37%), commonly CIN tumours with upregulation of WNT and MYC signalling; CMS3 (metabolic subtype, 13%), encompasses epithelial tumours with metabolic deregulation, enriched in KRAS mutations; and CMS4 (mesenchymal subtype, 23%), defined by a strong activation of epithelial–mesenchymal transition, angiogenesis and stemness pathways; this is the CMS with the worst outcome [7]. The CMS is the most robust classification currently available to stratify CRC. However, trained on disease biology rather than on clinical outcome, it has been associated with prognosis but its predictive value remains to be properly addressed [8–10].

In addition to focussing on tumour microenvironment, Becht *et al.*, [11] analysed the composition and functional orientation of the immune and stromal components of CRC by CMS. In this study, CMS2 and CMS3 were found to be immunologically 'cold', as they were devoid of lymphoid and myeloid infiltration, and had poor expression of major histocompatibility complex(MHC1) genes. On the contrary, CMS1, associated with a good prognosis, presented overexpression of genes specific to cytotoxic lymphocytes, and CMS4, associated with a poor prognosis, overexpressed markers of lymphocytes and monocytes and signatures characteristic of angiogenesis, inflammation and

immunosuppression [11], as well as the highest fibroblast density.

Recently, Thorsson *et al* [12]. have developed a new global immune classification of solid tumours based on the transcriptomic profiles of over 10,000 patients from all 33 non-haematological The Cancer Genome Atlas (TCGA) cancer types. They identified 6 distinct immune subtypes (ISs). The *wound healing* (C1) showed an elevated expression of angiogenic genes, a high proliferation rate and a low Th1/Th2 ratio related to the adaptive immune infiltrate. The *IFN-γ dominant* (C2) presented a high proliferation rate, the highest intratumoral heterogeneity, macrophages M1/M2 polarisation and CD8 T cell population and the greatest T-cell receptor (TCR) diversity. The *inflammatory* (C3) was defined by elevated Th17 and Th1 genes, low to moderate proliferation, lower levels of aneuploidy, higher somatic copy-number alterations and the most favourable prognosis. The *lymphocyte depleted* (C4) presented moderate cell proliferation and intratumoral heterogeneity, and a prominent macrophage signature with Th1 suppressed and a high M2 response; consistent with these features, it was associated with a poor outcome. The *immunologically quiet* (C5) displayed the lowest lymphocyte and highest macrophage responses, dominated by M2, and had low rates of proliferation and heterogeneity. Finally, the *TGF-β dominant* (C6) was a small group of mixed tumours with the highest TGF-β signature and a high lymphocytic infiltrate with a balanced Th1:Th2 ratio. Together with C4, C6 was associated with the worst prognosis [12].

This recent immune classification, thus, spans across traditional cancer classifications based on anatomical site of origin and suggests that certain therapeutic approaches may be considered regardless of tumour location or histology. However, individual tumour types varied substantially in their proportion of ISs and in their prognostic impact. In this context, the purpose of our study was to specifically characterise, from a clinical and molecular perspective, the ISs in a large cohort of CRC patients. We aimed to explore the distribution by IS of relevant clinical and pathological features, genomic and transcriptomic profiles and assess their interplay with the CMS classification of CRC. We also analysed the composition and functional orientation of immune and stromal populations of the tumour microenvironment, as well as specific

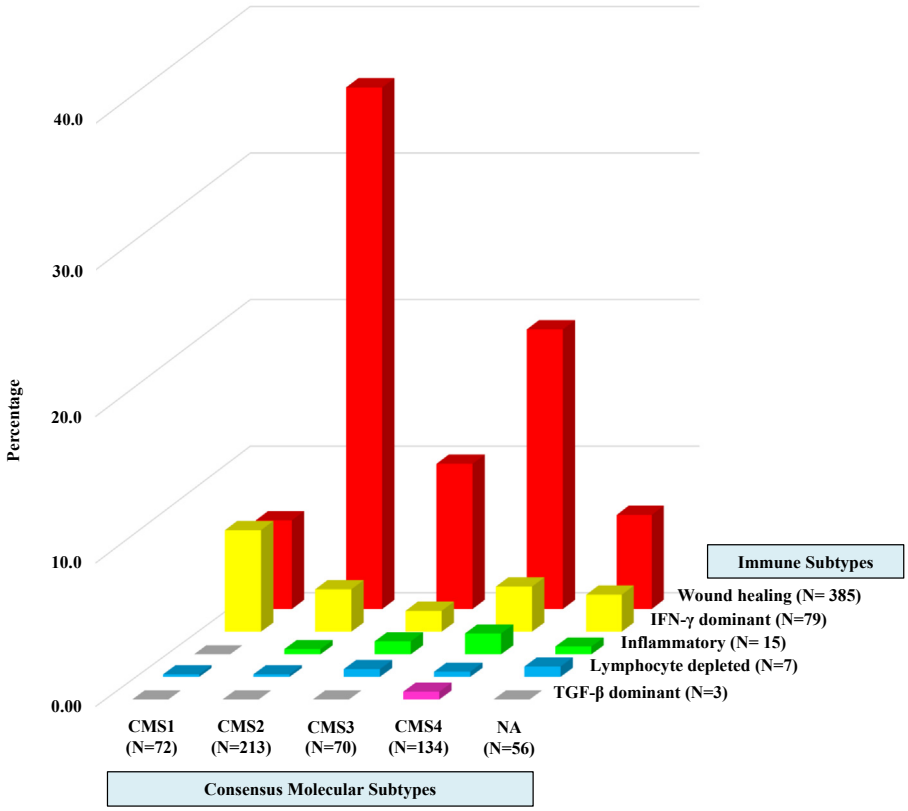

Fig. 1. **Distribution of colorectal cancer patients according to consensus molecular subtypes and immune subtypes**. 3D bar plot showing the interconnectivity between CMSs (N = 545) and ISs (N = 489). The X-axis represents the CMS (CMS1 (N = 72), CMS2 (N = 213), CMS3 (N = 70) and CMS4 (N = 134)), the Y-axis shows the ISs (*wound healing* (N = 384), *IFN-γ dominant* (N = 79), *inflammatory* (N = 15), *lymphocyte depleted* (N = 7) and *TGF-β dominant* (N = 3)) and the Z-axis represents the percentage of patients who belong to a specific group.

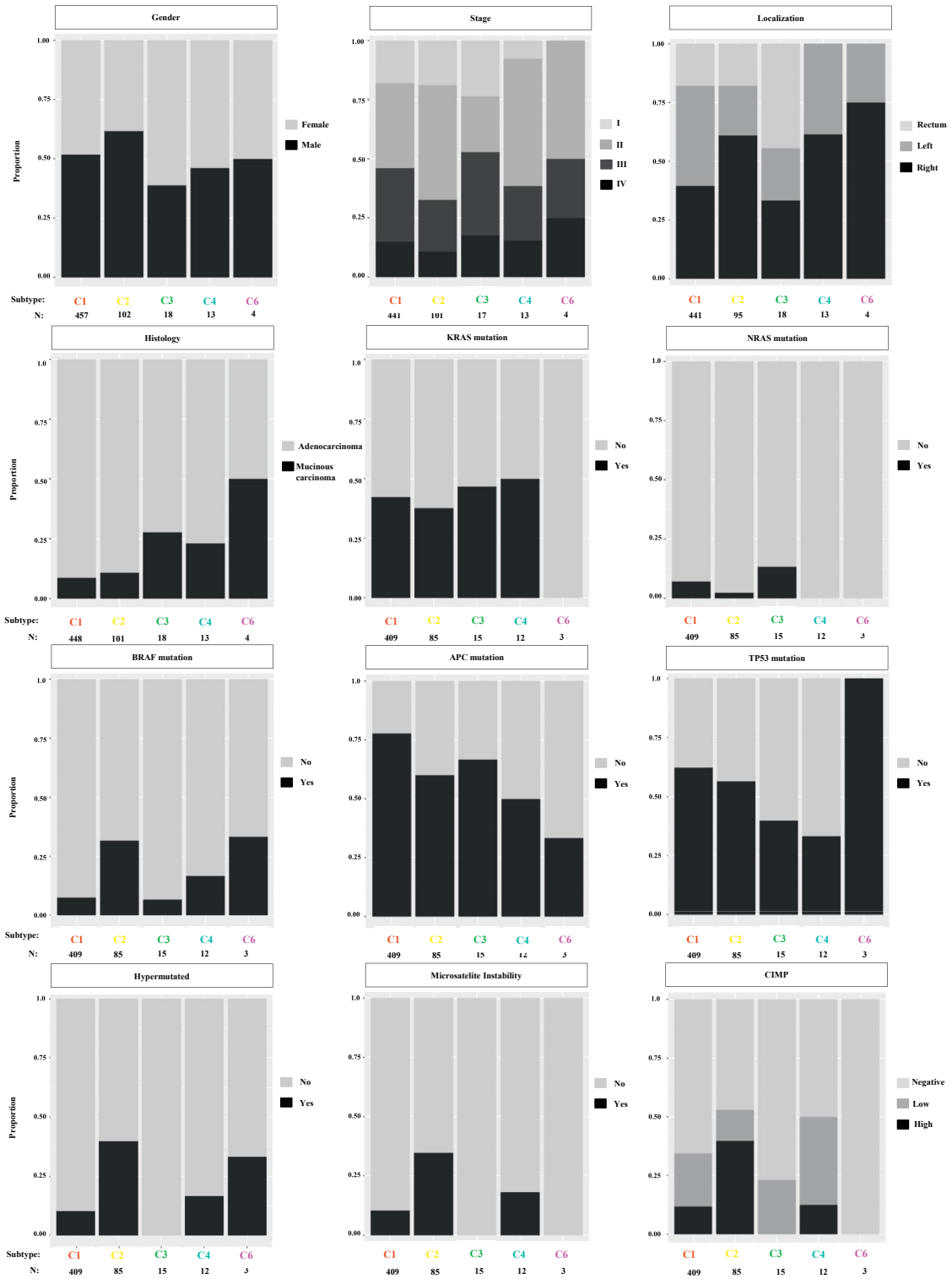

Fig. 2. **Clinical and molecular characteristics of colorectal cancer (CRC) patients according to immune subtypes**. Bar plots showing the proportion of gender, stage, primary tumour localisation, histology, KRAS, NRAS, BRAF, APC, and TP53 mutations, hypermutated phenotype, microsatellite instability and CpG island methylator phenotype (CIMP) in immune subtypes C1 (*wound healing*), C2 (*IFN-γ dominant*), C3 (*inflammatory*), C4 (*lymphocyte depleted*) and C6 (*TGF-β dominant*). The number of patients with specific clinical information for each immune subtype is detailed below the bar plot.

genes and pathways by using IS in this patient population.

2. Material and methods

2.1. Study population and datasets

The study population included all CRC patients from TCGA [13] (N = 625) with available information on ISs [12](N = 597). Clinical and molecular information, CMS classification and transcriptomic profiles were obtained from different public data platforms (see Supplementary Material and Methods). Main characteristics of the study population are summarised in Supplementary Tables 1 and 2).

2.2. Clinical and molecular data analyses

Associations of categorical variables and CRC subtypes were assessed by using the Fisher's exact test. Overall Survival (OS) was estimated according to the *Kaplan-Meier* method [14]. Cox proportional-hazard univariate and multivariate analyses were also conducted, including relevant known clinical and pathological prognostic factors as well as the IS and CMS classifications (see Supplementary Material and Methods).

2.3. Immune cell population analysis

Differences in the fraction of immune cell types, M1/M2 ratio, B-cell receptor and TCR diversity among ISs were performed by using the Wilcoxon signed-rank test. Immune and stromal cell populations were estimated by using microenviroment cell populations (MCP)-counter [11]. Statistical differences between ISs were evaluated using the Student's *t-test.* ComplexHeatmap R package [15] and Z-score values obtained from all samples were used to generate immune population and IS heatmaps (see Supplementary Material and Methods).

2.4. Differential expression and gene set enrichment analysis

Differential expression analysis (DEG) between *wound healing* and *IFN-γ dominant* ISs was performed using Bioconductor's DESeq2 package [16]. Genes with FDR <0.05 were selected as differentially expressed between both subtypes. Gene set enrichment analysis (GSEA) was computed with ssGSEA algorithm implemented in Bioconductor's GSVA R package [17], Hallmarks and KEGG gene sets (details of differentially expressed genes (DEG) and GSEA included in Supplementary Material and Methods).

2.5. Analysis of immune modulator genes

Only samples with RNA-seq data available were used for immune modulator gene expression analysis (N = 590). Kruskal-Wallis or Wilcoxon signed-rank test was performed depending on the number of ISs considered in each analysis (see Supplementary Material and Methods).

3. Results

3.1. Immune subtype distribution in the global colorectal cancer population by consensus molecular subtype

First, we assessed the distribution of ISs in the global CRC population (N = 573), and we found that the 2 predominant ISs were the C1 *wound healing* subtype, identified in 459 samples (77%) and the C2 *IFN-γ dominant* subtype, present in 103 samples (17%). Other ISs were far less commonly encountered, such as the C3 *inflammatory* (18 samples, 3%), C4 *lymphocyte depleted* (13 samples, 2%) and C6 *TGF-β dominant* (4 samples, 0.7%) subtypes. Of note, no CRC sample belonged to the C5 *immunologically quiet* IS (Supplementary Table 1).

Next, we explored the distribution of ISs by CMS to assess the interplay of both molecular classifications (Fig. 1, Supplementary Tables 3 and 4). The 2 main CRC ISs, C1 and C2, were present in all CMS groups but their relative distribution differed by CMS. Indeed, the C1 *wound healing* IS was particularly dominant in CMS2 (91%) but far less common in CMS1 (46%) tumours, whereas the proportion of C1 in CMS3 and CMS4 tumours was intermediate (77–78%). On the contrary, the C2 *IFN-γ dominant* was the most common IS observed in CMS1 (53%) and was under-represented in CMS2 (8%), whereas the proportion of C2 in CMS3 and CMS4 was slightly higher (11–13%). Other ISs were barely present in CMS1 and CMS2. The immunological landscape of CMS3 and CMS4 was more diverse: they were enriched in the C3 *inflammatory* subtype (7% and 6%, respectively), as compared with CMS1/2, CMS3 had the highest representation of the C4 *lymphocyte depleted* subtype (4%), and all 3 cases of *TGF-β dominant* phenotype belonged to the CMS4 subgroup (2.3%). Distribution of CMS by ISs is detailed in Fig. 1 and Supplementary Table 4.

3.2. Clinical and molecular features of colorectal cancer by IS

The main clinical and molecular characteristics by IS in the global CRC population are depicted in Fig. 2 and detailed in Supplementary Table 4, and analysed by CMS in Supplementary Figs. S2–5 and Supplementary Tables 5–8. C2 *IFN-γ dominant*, C4 *lymphocyte depleted* and C6 *TGF-β dominant* ISs were enriched in tumours located in the right colon, whereas C1 *wound healing* and C3 *inflammatory* ISs were frequently encountered in left-

sided tumours. No significant associations were observed with gender or tumour stage.

All ISs presented a similar proportion of KRAS mutations, except for the *TGF-β dominant* IS, where no KRAS mutations were identified. BRAF mutations were more commonly observed in *IFN-γ dominant* (25%), *lymphocyte depleted* (17%) and *TGF-β dominant* (33%) subtypes, although these figures are not particularly accurate in these last subgroups because of the small sample size. MSI, CpG island methylator phenotype, and hypermutated phenotypes were consistently more common in the *IFN-γ dominant* subtype. Most frequent genetic alterations encountered in CRC are provided by IS in Supplementary Table 9. Distribution of clinical and molecular characteristics by CMS is consistent with the previous data [7] (Supplementary Fig. S1 and Supplementary Table 3).

3.3. Prognostic impact of immune subtypes in colorectal cancer

The immune phenotype had a major influence on patient's prognosis, although its impact on CRC patients significantly differed from the overall TCGA, which included over 30 different solid tumour types [12] (Fig. 3A). Indeed, in our CRC study cohort, patients with *TGF-β dominant* and *wound healing* ISs showed better prognosis (5-year OS of 100% and 65%, respectively), whereas *IFN-γ dominant* and *inflammatory* ISs were associated with the worst outcome (5-year OS of 49% and 23%, respectively) (C2 vs C1, *HR 1.59, P = 0.004*; C3 vs C1, *HR 2.77, P = 0.02*) (Fig. 3B, Supplementary Table 10). Similar trends were observed when analysed within each CMS (Supplementary Fig. S6 and Supplementary Table 10).

Cox multivariate analysis showed that ISs were significantly associated with survival, independent of other well-established prognostic factors in CRC such as age, stage at diagnosis or primary tumour site (Supplementary Table 11), and showed that the immune phenotype had a significantly greater influence on OS than CMS.

3.4. Immune subtypes of colorectal cancer show distinct immune and stromal cell population patterns

Different patterns for immune and stromal cell population abundance were found among ISs in CRC patients. The most relevant differences were identified between the two major subtypes (C1 and C2). *IFN-γ dominant* tumours presented higher levels of CD8, CD4, follicular helper T cells and M1 macrophages. High levels of NK cells, M2 macrophages, M1/M2 ratio and neutrophils were also found in this subtype (Fig. 4). No differences were found in endothelial cells and fibroblasts abundance between these two groups, although the proportion of these populations was strikingly high in the TGF-β subtype (Fig. 4B). When analysed by CMS group, similar differences were observed in the CMS1 group (Supplementary Fig. S7), but these were diluted in the CMS2-4 groups (Supplementary Figs. 8–10).

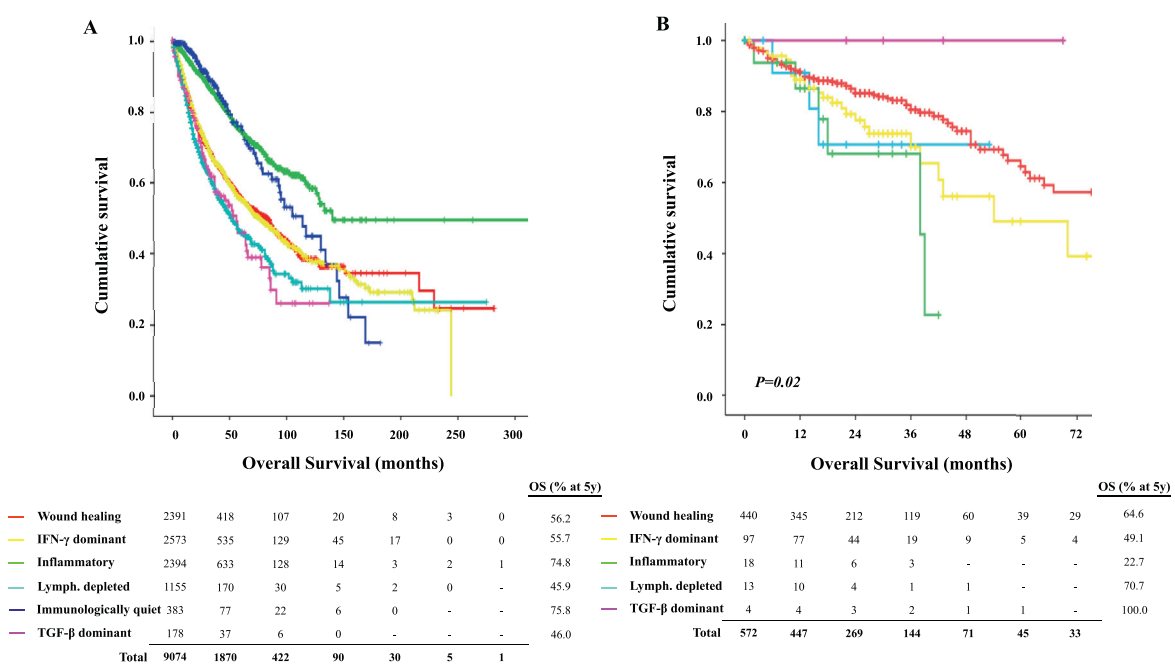

Fig. 3. **Impact of immune subtypes on survival of colorectal cancer patients**. **A)** Overall survival (OS) by immune subtypes in the overall TCGA solid tumour population (N = 9074). **B)** Overall survival by immune subtypes in the TCGA CRC cohort (N = 572). Patients at risk at the corresponding time point and survival rates at five years in each subtype are provided. *P-value* was calculated by log-rank test.

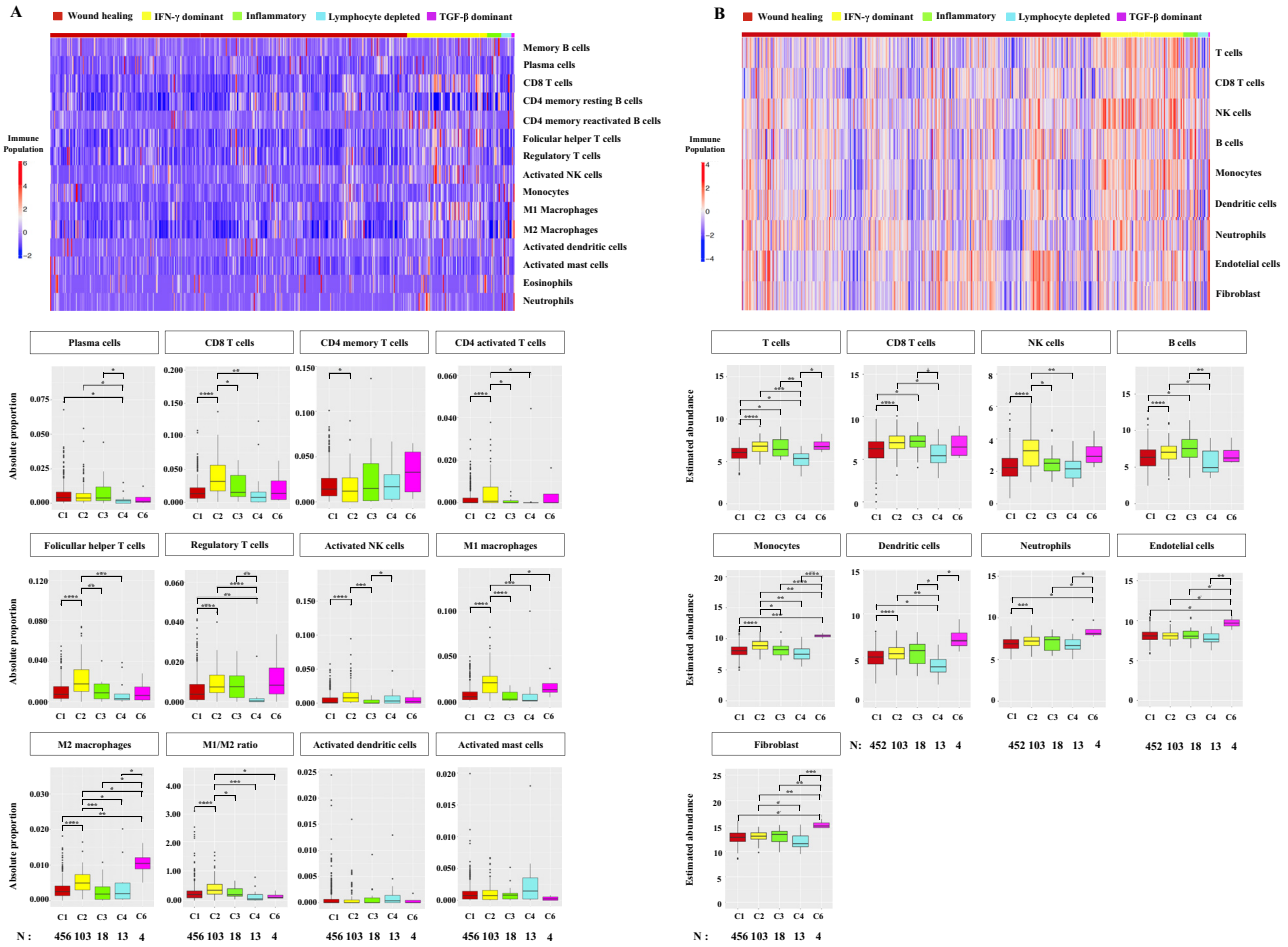

Fig. 4. **Immune and stromal signatures of the immune subtypes in colorectal cancer patients**. **A)** CIBERSORT heatmap showing the distribution of lymphoid and myeloid lineages in the colorectal cancer cohort by immune subtype. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using Wilcoxon signed-rank test. **B)** MCP counter heatmap showing the estimated abundance of several immune and stromal cell populations in colorectal cancer patients by immune subtypes. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using *Student's t-test*.

3.5. Immune subtypes show differential expression of immunomodulatory genes

Next, we examined gene expression, amplification or deletion of a large set of immunomodulators (IMs) genes across ISs. Upregulation of several IMs was found in the *IFN-γ dominant* subtype, such as CD80 (*FDR = 1.69E-12*), CD28 (*FDR = 5.20-9*), CD274 (PDL-1) (*FDR = 6.82E-23*), CXCL10 (*FDR = 1.83E-24*), LAG3 (*FDR = 6.94E-21*), ICAM1 (*FDR = 1.41E-11*), HLA-DQA1 (*FDR = 3.84E-16*), HLA-DRA (*FDR = 3.38E-17*), IDO1 (*FDR = 8.44-20*) and GZMA (*FDR = 3.34E-18*) (Fig. 5A and Supplementary Fig. 12). Immune inhibitors with the greatest differences between subtypes included PDL-1, PD-1, CTLA-4, IDO1 or LAG3 and were most highly expressed in the *IFN-γ dominant* subtype (Fig. 5B). Significant differences observed between the 2 major immune phenotypes (C1 and C2) were maintained within each CMS (Supplementary Fig. 11). Copy-number variations affected multiple IMs and both amplifications and deletions were frequently found in the *wound healing IS*, whereas other ISs showed fewer alterations (Fig. 5A). Thus, SLAMF4, TNFSF4, CX3CL1, IL10, IL2, ENDRB, CD40, IDO1 and HMGβ1 genes were most frequently amplified, whereas VTCN1, CD276 (PDL-1), IL4, IL13, TNGRS-F18, TNGRS-F4, TNGRS-F9, TNGRS-14, and ADORA2A were most frequently deleted in C1 IS.

We also found that both tumour mutational burden and antigen-specific TCR repertoires, which determine the robustness of the antitumour response, were enriched in *IFN-γ dominant* (N = 56) compared with *wound healing* (N = 258) IS. A trend was observed in BCR diversity between these main ISs (Fig. 5 and Supplementary Fig. S11).

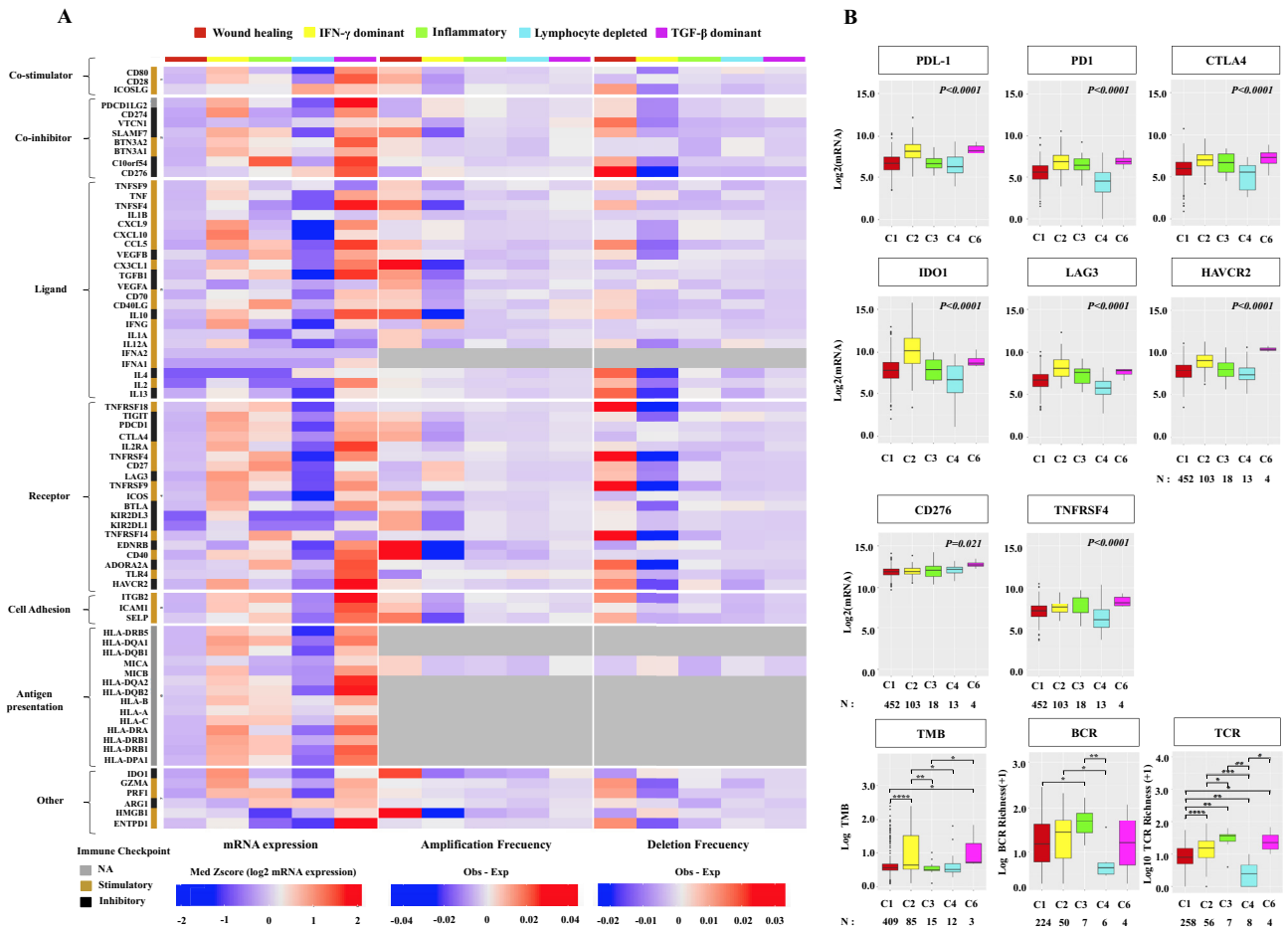

Fig. 5. **Regulation of immunomodulators**. **A)** mRNA expression (median z-score), amplification frequency (difference between the observed versus expected fraction of samples in which an IM is amplified) and deletion frequency (difference between the observed versus expected fraction of samples in which an IM is deleted) for 75 immunomodulator genes by immune subtype. **B)** Distribution of expression levels for immune checkpoints (log2-transformed), tumour mutational burden (TMB), BCR and TCR diversity (log+1 transformed) by immune subtypes: C1 (*wound healing*), C2 (*IFN-γ dominant*), C3 (*inflammatory*), C4 (*lymphocyte depleted*) and C6 (*TGF-β dominant*). The number of patients with gene expression data for each immune subtype is detailed below the bar plot. *P-value* was calculated by Kruskal-Wallis test for immune checkpoints and by Wilcoxon singed-rank test for TMB, BCR and TCR analysis.

3.6. Differential gene expression signatures between wound healing and IFN-γ dominant subtypes identify key genes and pathways of potential diagnostic or therapeutic relevance in CRC

A total of 3150 and 1362 differentially expressed genes achieved statistical significance ($FDR < 0.05$) between *wound healing* and *IFN-γ dominant* subtypes when we analysed all and CMS1 CRC patients, respectively. The expression profile of an important number of genes was specific to each CMS, but 21 genes were consistently found to be differentially expressed in all analysis performed (Fig. 6A and Supplementary Table 13).

GSEA pathway analysis showed that the *wound healing* subtype was enriched in metabolic pathways, and had greater activation of WNT and hedgehog signalling (Fig. 6B). In contrast, the *IFN-γ dominant* subtype presented greater activation of pathways related to the immune system, apoptosis and DNA repair, as well as mTOR signalling and oxidative phosphorylation (Fig. 6B and Supplementary Table 14). Pathway analyses by IS within each CMS are provided in Supplementary Fig. 12 and Supplementary Tables 15–18.

4. Discussion

An increasing body of evidence supports the major role that tumour microenvironment and, in particular, the immune system play in cancer fate. An improved understanding of the immune landscape of cancer is therefore critical to refine current immunotherapeutic strategies that have revolutionised cancer care, but are still ineffective in a great proportion of CRC

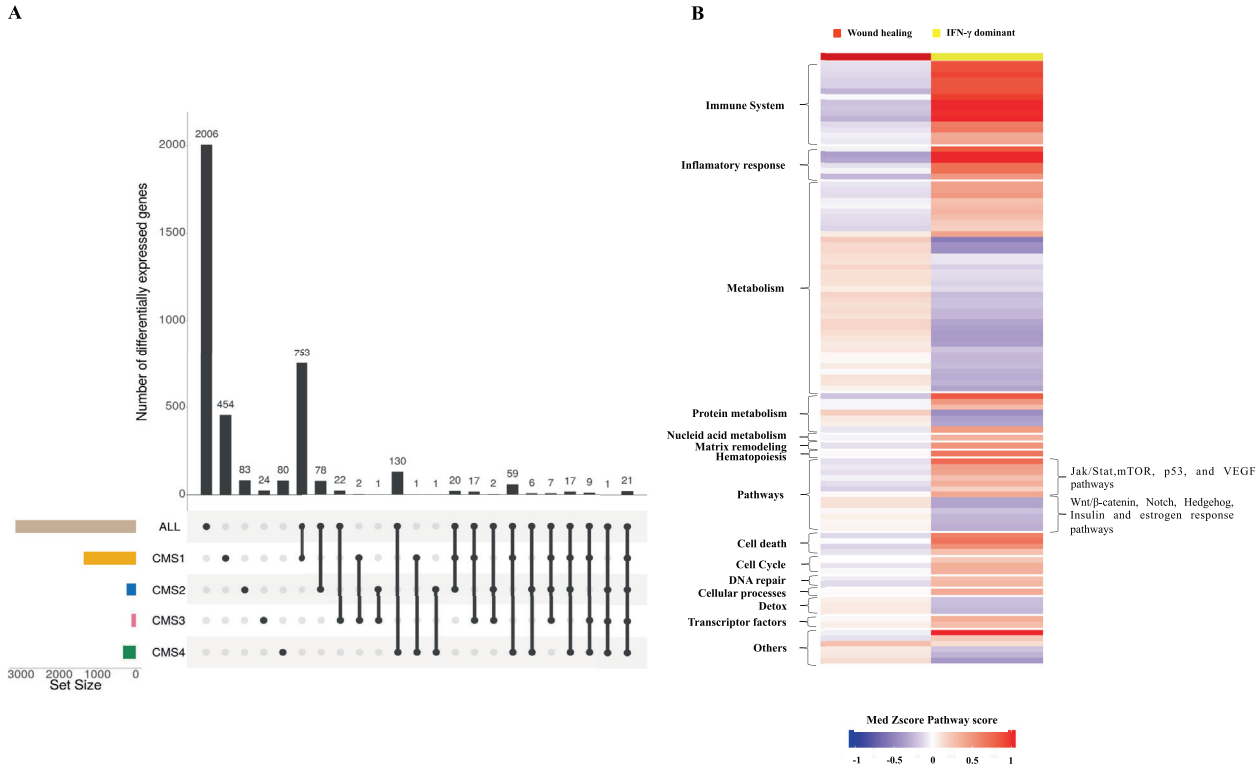

Fig. 6. Differential expression of genes and pathways between wound healing and IFN-γ dominant immune subtypes in CRCcolorectal cancer patients. **A)** An histogram displaying the differential gene expression profile of wound healing versus IFN-γ dominant immune subtypes by CMS. The number of genes with significantly different expression levels (FDR<0.05) were provided for all (N = 507), CMS1 (N = 71), CMS2 (N = 206), CMS3 (N = 62) and CMS4 (N = 120) colorectal patients. **B)** Heatmap showing the gene set mRNA enrichment analysis (ssGSEA) of signatures (median z-scores) of special interest in CRC in the 2 predominant immune subtypes, *wound healing* and *IFN-γ dominant*.

patients. Recently, Thorsson *et al.* developed a pan-cancer immune classification that encompasses nearly all human malignancies, and consists of 6 (ISs with distinct immunogenomic features and clinical outcomes [12]. However, distribution of these ISs substantially differed across the 33 solid tumours included, and their association with patients' prognosis was also dependent on tumour type. In the present study, we specifically characterised the ISs in the TCGA cohort of CRC patients, and assessed their interplay with the most robust molecular classification reported to date in CRC, the CMS classification [7]. In our study, we show that only 5 of the 6 ISs are present in CRC patients, with 2 predominant ones (the C1 *wound healing* subtype [77%] and the C2 *IFN-γ dominant* subtype [17%]), and demonstrate significant heterogeneity of immune phenotype distribution among CMS subgroups. Importantly, we observed that the immune phenotype has greater influence in survival than the CMS classification. These findings may have relevant implications in terms of prognosis stratification and prediction of response to therapy, and suggest the immune phenotype may allow a more accurate classification of patients to assist clinicians in personalised patient care.

Indeed, the C1 *wound healing* IS was particularly dominant in CMS2 (91%) but far less common in CMS1 (46%) tumours, and the C2 *IFN-γ dominant* was the most common IS observed in CMS1 tumours (53%) and was under-represented in CMS2 tumours (8%). Other ISs were barely present in CMS1 and CMS2 tumours, whereas the immunological landscape of CMS3 and CMS4 was more diverse: they had some representation of the C3 *inflammatory* (7% and 6%, respectively) and C4 *lymphocyte depleted* (4.3% and 1.5%, respectively) subtypes, and all 3 cases with *TGF-β dominant* phenotype belonged to the CMS4 subgroup (2.3%). Of note, ISs were significantly associated with survival in CRC patients, independent of other well established prognostic factors (age, stage at diagnosis or primary tumour site), whereas the CMS lost statistical significance in the Cox multivariate analysis.

These observations are consistent with the fact that clinical and molecular features enriched in CMS1 were also over-represented in *IFN-γ dominant* patients, and the strong immune activation observed in CMS1 [7,8] could be explained by the enrichment of *IFN-γ dominant* tumours in this CMS. To date, CMS1 (MSI-like immune) patients have been postulated to be the most likely to benefit from immunotherapy, as this

subtype includes most MSI/highly mutated tumours [18,19]. Nevertheless, not all CMS1 are MSI and other CMS subgroups (i.e CMS3) also include a significant proportion of MSI tumours. This is relevant as MSI is the biomarker used in pivotal studies that demonstrated the clinical efficacy of checkpoint inhibitors in CRC, and the only available biomarker in standard clinical practice to identify immunogenic CRC [11]. Moreover, our results reported that the immune landscape of CMS1 is diverse and this may potentially impact response to therapy. Given the fact that *IFN-γ dominant* tumours presented a strong immune activation (high proportion of CD8, follicular helper T cells and M1 macrophages, as well as Tregs, M2 macrophages, dendritic cells and neutrophils) and upregulation of several immune regulatory genes (PD-1/PD-L1, CTLA-4, IDO1 and LAG3) potentially involved in immune escape mechanisms, it is reasonable to assume that the *IFN-γ dominant* IS may be accountable for the susceptibility of CMS1 tumours to immune checkpoint inhibition, whereas CMS1 tumours of the *wound healing* subtype may likely be more resistant to these agents and would require other therapeutic approaches. In addition, *IFN-γ dominant* tumours belonging to CMS2-4 subgroups could also potentially benefit of immune checkpoint–targeted therapy. This is particularly relevant as MSI tumours only represent a small proportion of patients with advanced CRC (~5%), and despite the unquestionable success of immunotherapy in this subgroup of patients, still a significant proportion of MSI tumours (40–60%) do not respond to immune checkpoint inhibitors for yet unexplored biological grounds. On the contrary, a subset of MSS tumours also show increased expression of immune genes, indicating that other factors also determine immune contexture and clinical outcomes [20,21]. The IS classification could be therefore a new valuable tool to aid in the selection of patients for immune therapy. Like CMS1, CMS4 tumours are also characterised by a prominent immune activation [7,8], and it has been postulated that this CMS subgroup could also benefit from immune checkpoint inhibitors but most likely in combination with other treatment strategies [8].The most common ISs in CMS4 tumours were the *wound healing* (78%) followed by the *IFN-γ dominant* (13%) subtypes, but, together with CMS3, these tumours were also relatively enriched in the *inflammatory* subtype (6%). The *inflammatory* phenotype has been associated with cancer progression by multiple mechanisms and, consistently, these samples were part of the CMS4 group, the CMS with the poorest prognosis. It is also important to highlight that all *TGF-β dominant* tumours belonged to the CMS4 subtype, although the number of patients was very low. TGF-β phenotype in CRC has recently been associated with a reduced cytotoxic T-cell response against tumour cells and resistance to PD-1 blockade, being a primary mechanism of immune evasion [22].

Another relevant finding of our study was that the prognostic impact of ISs in CRC was substantially different from that reported for the global TCGA [12]. Indeed, CRC patients with the *TGF-β dominant* and *wound healing* ISs showed better prognosis (5-year OS: 100% and 65%, respectively), whereas *IFN-γ dominant* and *inflammatory* subtypes were associated with the worst outcome (5-year OS: 49% and 23%, respectively). This in sharp contrast with survival rates by IS reported for the overall solid tumour population, where the *inflammatory* subtype had the best prognosis, and the *lymphocyte depleted* and *TGF-β dominant* were the ISs associated with the worst survival [12]. On the other hand, the excellent prognosis of *TGF-β dominant* tumours was certainly unexpected, despite they all belonged to the CMS4 group, and particularly considering TGF-β signalling in stromal cells is a defining feature of poor prognosis CRC subtypes [23]. However, only 4 CRC samples (0.7%) were *TGF-β dominant*, which preclude firm conclusions to be drawn.

Our observations may be partially conditioned by some caveats inherent to the use of TCGA data. First is the fact that for most tumour types, a tumour cell component >50% was required for study entry; this introduces a significant bias as the epithelial cell component is likely over-represented. In addition, survival rates and patient follow-up substantially differed across tumour types, antitumour therapy is presumably very heterogeneous, and its impact on clinical outcomes is disregarded. Finally, it should be pointed out that the IS classification was developed in a large but very heterogeneous cancer population, which may dilute distinct, potentially relevant tumour-specific transcriptomic profiles. Thus, further refinement of this classification specifically adjusted for CRC is certainly warranted.

In summary, in line with the changing treatment paradigm, that is shifting from the traditional one predominantly focused on targeting the epithelial compartment, to the development of more integrated approaches targeting tumour microenvironment, in-depth study of the immune landscape of tumours provides very valuable information for cancer management. In the present study, we have characterised the recently described pan-cancer IS classification in a large cohort of CRC patients, demonstrating distinct clinical and biological implications of ISs in this cancer type. We have also identified substantial heterogeneity in the distribution of ISs by CMS subgroups, and demonstrate that ISs have greater ability to stratify prognosis of CRC patients than the CMS classification. Profound biological differences observed among ISs are expected to translate into heterogeneous drug responses, both to conventional cytotoxic drugs and to alternative treatment strategies targeting the tumour ecosystem,

including immunotherapy. We believe these results are highly relevant and should be taken into account for the design of future therapeutic strategies that may eventually improve the fate of CRC patients.

5. Conclusions

Cancer is the consequence of a complex interplay between tumour cells and their microenvironment. Recently, a new global transcriptomic immune classification of solid tumours has identified six ISs (C1–C6) which spans across traditional cancer classifications based on anatomical site of origin and suggests that certain therapeutic approaches may be considered regardless of tumour location or histology. However, individual tumour types varied substantially in their proportion of ISs and in their prognostic impact. Thus, we have characterised, from a clinical and molecular perspective, the ISs in a large cohort of CRC patients, and we have assessed their interplay with the most accurate classification system in CRC, CMSs. CMSs show substantial heterogeneity in the distribution of ISs with relevant clinical and biological implications and a significant impact on patients' survival. Profound biological differences observed among ISs could be translated into heterogeneous drug responses to conventional cytotoxic drugs and to novel strategies such as immunotherapies. These results are highly relevant and should be taken into account for the design of future therapeutic strategies that may eventually improve the fate of CRC patients.

Conflict of interest statement

The authors declare no competing interests related to the published work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.09.008.

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