USCAP

ARTICLE

CINSARC signature outperforms gold-standard TNM staging and consensus molecular subtypes for clinical outcome in stage II–III colorectal carcinoma

Anne-Cécile Brunac[1], Joanna Fourquet[2], Gaëlle Perot[2], Marion Jaffrelot[3], Julie Meilleroux[1], Marie Danjoux[1], Thomas Filleron[4], Vincent Nicolaï[5], Rosine Guimbaud[3], Samira Icher[1], Nadim Farés[3], Janick Selves[1,6] and Frédéric Chibon[2,6]✉

The outcome of stage II–III colorectal cancer (CRC) is highly variable and therapeutic choice is currently based on TNM staging with a few additional biomarkers. However, studies show that some stage III patients have a better prognosis than some stage II patients. A promising consensus molecular (CMS) classification with prognostic relevance has been developed, but it is not used in daily practice. Our team developed CINSARC, a 67-gene expression prognostic signature, whose prognostic value has been demonstrated in many cancer types. It is applicable to formalin-fixed, paraffin-embedded (FFPE) blocks using NanoString® technology. We investigated whether it could predict outcome in stage II–III CRC. We established the CINSARC classification on the TCGA retrospective cohort comprising 297 stage II–III CRC patients using RNA sequencing and on a second independent cohort comprising 169 cases using NanoString® technology. We compared its recurrence-free and overall survival prognostic value with TNM staging and CMS classification. In the TCGA cohort, we showed that CINSARC significantly splits the population of stage II–III CRC into two groups with different progression-free interval ($P = 1.68 \times 10^{-2}$; HR = 1.87 [1.11–3.16]) and overall survival ($P = 3.73 \times 10^{-3}$; HR = 2.45 [1.31–4.59]) and is a strong prognostic factor in multivariate analysis, outperforming TNM staging and CMS classification. We validated these results in the second cohort by applying CINSARC on FFPE samples with Nanostring® technology. CINSARC is a ready-to-use tool with a robust independent prognostic value in stage II–III CRC.

Modern Pathology (2022) 35:2002–2010; https://doi.org/10.1038/s41379-022-01166-9

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer with over 1.5 million new cases diagnosed every year and accounts for 10% of cancer-related death worldwide[1], with a 5-year overall survival (OS) rate of 64%[2]. CRC is characterized by etiological, genetic and clinical heterogeneity, thus rendering prognosis estimation and therapeutic management difficult.

The most widely used staging system for prognosis evaluation and treatment guidance is the AJCC/IUCC/TNM (American Joint Committee on Cancer/Union for International Cancer Control/Tumor Node Metastasis) classification, currently in its 8th edition[3]. It predicts the prognosis of CRC patients, with lower-stage cancers having a better prognosis than higher-stage ones. However, TNM staging is not sufficiently robust to predict the survival of stage II–III patients, who have reported 5-year survival rates of 73% an 53% respectively[3]. This remains a challenge despite incorporating additional prognostic factors (vascular/lymphatic invasion—perineural invasion (VELIPI), grade, tumor budding, perforation, *KRAS* status, MisMatch Repair (MMR) status and *BRAF* status)[3]. This is especially illustrated by the "stage paradox", i.e., prognosis is better for stage IIIA patients than for stage IIB/IIC ones[4].

Surgery is the cornerstone of curative intent treatment. Adjuvant therapy with fluoropyrimidine + oxaliplatin-based chemotherapy is recommended and improves survival in stage III and high-risk stage II CRC studies[5], but the lack of sufficiently robust biomarkers may result in the under- or over-treatment of some patients. Therefore, there is an urgent need to identify new robust biomarkers that are easily applicable in routine clinical practice to better stratify individual risk.

In 2015, an international consortium established the consensus molecular classification which distinguishes four consensus molecular subtypes (CMS): CMS1 Immune, hypermutated, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), *BRAF* mutation and immune activation; CMS2 Canonical, epithelial, chromosomal instability (CIN), marked WNT and MYC signaling activation; CMS3 Metabolic, epithelial, *KRAS* mutation and metabolic dysregulation; and CMS4 Mesenchymal, epithelial-mesenchymal transition, transforming growth factor-β activation, stromal invasion

[1]Department of Pathology, Institut Universitaire du Cancer-Oncopole de Toulouse, Centre Hospitalier Universitaire (CHU), Toulouse, France. [2]Oncogenesis of Sarcomas, INSERM UMR1037, Cancer Research Centre of Toulouse, Toulouse, France. [3]Department of Digestive Oncology, Centre Hospitalier Universitaire (CHU), Toulouse, France. [4]Department of Biostatistics, Institut Claudius-Regaud, Institut Universitaire du Cancer-Oncopole de Toulouse, Toulouse, France. [5]Department of Medical Oncology, Institut Universitaire du Cancer-Oncopole de Toulouse, Centre Hospitalier Universitaire (CHU), Toulouse, France. [6]These authors contributed equally: Janick Selves, Frédéric Chibon.
✉email: frederic.chibon@inserm.fr

Received: 21 June 2022 Revised: 9 August 2022 Accepted: 10 August 2022
Published online: 6 October 2022

Table 1. Summary of patient information from two cohorts in this study.

Characteristics	Cohort 1 ($n = 297$)	Cohort 2 ($n = 169$)	*P* value
Mean age (min–max) (years)	67.3 (31–90)	68.8 (25–90)	0.68
Gender			0.93
Male	152 (51.2%)	88 (52.1%)	
Female	145 (48.8%)	81 (47.9%)	
Neoadjuvant treatment			0.54
Yes	2 (0.7%)	0	
No	295 (99.3%)	169 (100%)	
Perforation status			NA
No perforation	NA	160 (94.7%)	
Perforation	NA	9 (5.3%)	
ND	297 (100%)	0	
Tumor location			2.92×10^{-17}
Right colon	144 (48.5%)	73 (43.2%)	
Left colon	87 (29.3%)	62 (36.7%)	
Recto-sigmoid junction/rectum	4 (1.3%)	33 (19.5%)	
Colon NOS	62 (20.9%)	1 (0.6%)	
Tumor grade			*NA*
Low-grade	NA	158 (93.5%)	
High-grade	NA	11 (6.5%)	
ND	297 (100%)	NA	
Lymphovascular and/or perineural invasion (VELIPI)			9.53×10^{-9}
Yes	131 (44.1%)	62 (36.7%)	
No	61 (20.5%)	104 (61.5%)	
ND	105 (35.4%)	3 (1.8%)	
TNM stage			0.75
II	171 (57.6%)	94 (55.6%)	
III	126 (42.4%)	75 (44.4%)	
Lymph nodes examined			0.13
<12	27 (9.1%)	25 (14.8%)	
≥12	254 (85.5%)	144 (85.2%)	
ND	16 (5.4%)	NA	
MMR status (IHC)			0.99
MSS	184 (62%)	141 (83.4%)	
MSI	38 (12.8%)	28 (16.6%)	
ND	75 (25.2%)	NA	
CDX2 status (IHC)			NA
CDX2-negative	NA	16 (9.5%)	
CDX2-positive	NA	153 (90.5%)	
ND	297 (100%)	NA	
Adjuvant chemotherapy			NA
Yes	96 (32.3%)	NA	
No	151 (50.9%)	NA	
ND	50 (16.8%)	169 (100%)	
Mean follow-up duration (minimum–maximum) (years)	2.6 (0–12.3)	5.6 (0–12)	0.76
Progression-free interval (PFI)/Disease-free survival (DFS)			0.73
Progression	67 (22.6%)	41 (24.3%)	
No progression	230 (77.4%)	127 (75.1%)	
ND	NA	1 (0.6%)	
Overall survival			0.26
Dead	53 (17.8%)	38 (22.5%)	
Alive	244 (82.2%)	130 (76.9%)	
ND	NA	1 (0.6%)	

and angiogenesis[6]. Its stage-independent prognostic value was demonstrated with a worse outcome for both relapse-free survival and OS in CMS4 tumors[6]. However, the requirement for sufficient tumor material and the cost and technology required for genome-wide expression analysis restrict its use. Trinh et al. developed an immunohistochemistry-based method to stratify patients into three CMS groups: 1 immune, 2/3 epithelial and 4 mesenchymal, with a prognostic value in stage II CRC[7], but this remains to be validated in

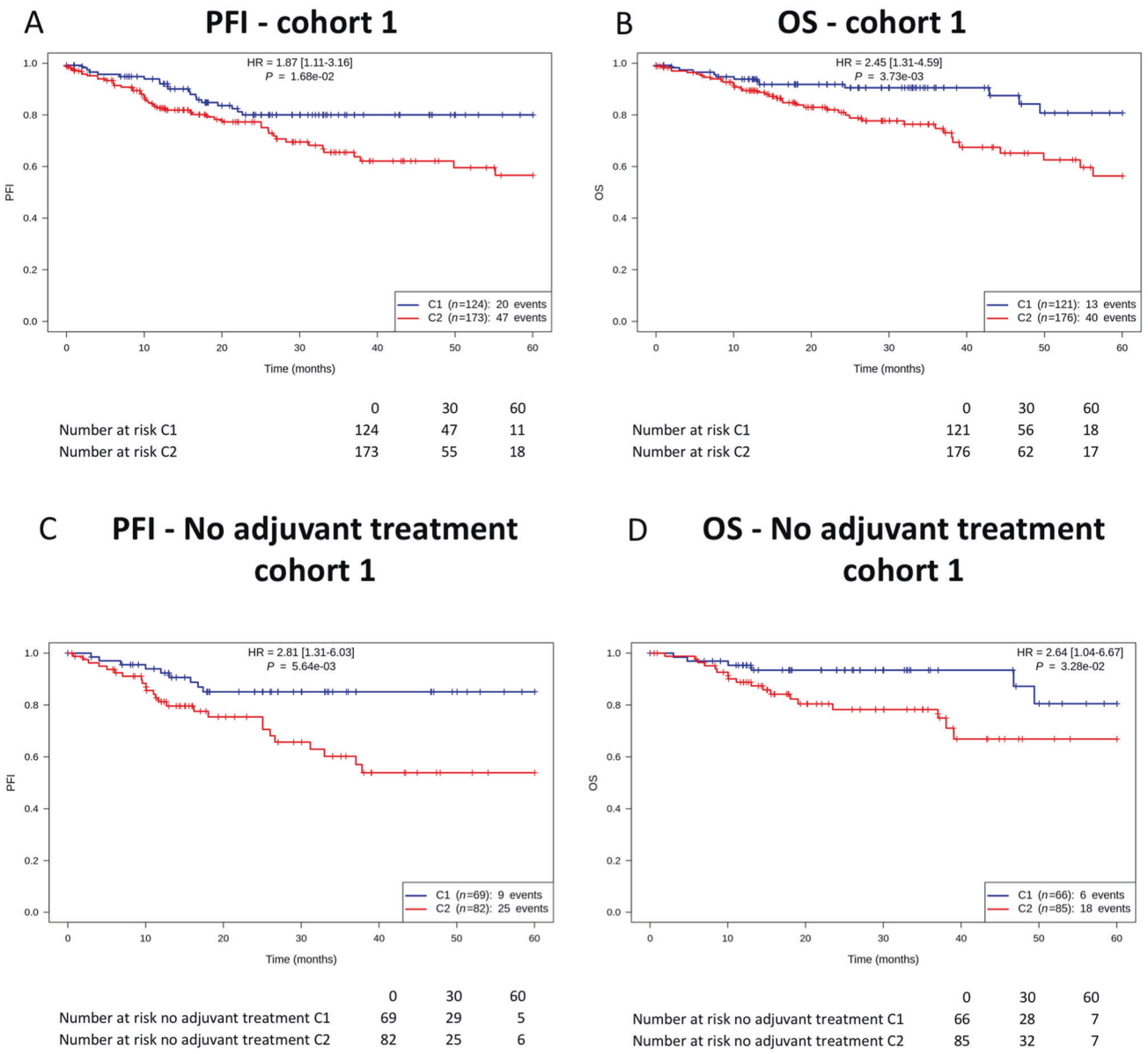

Fig. 1 CINSARC in cohort 1. PFI (**A**) and OS (**B**) analyses according to CINSARC classification for stage II–III CRC patients; PFI (**C**) and OS (**D**) analyses according to CINSARC classification for stage II–III CRC patients without adjuvant treatment; PFI (**E**) and OS (**F**) analyses according to CINSARC classification for stage II–III CRC patients with adjuvant treatment; PFI (**G**) and OS (**H**) analyses according to CINSARC classification for stage II–III CRC patients with and without adjuvant treatment in cohort 1.

larger cohorts, as the distribution of patients is heterogeneous between studies[7,8]. Later, Dalerba et al. proposed the use of transcription factor CDX2 expression as sole prognostic factor, with its loss of expression associated with a worse prognosis[9]. To date, there is no recommendation in international guidelines to use these classifications for adjuvant treatment[10].

In 2010, our team developed a prognostic transcriptomic signature in sarcomas that predicts metastatic outcome and outperforms the gold-standard Fédération Française des Centres de Lutte Contre le Cancer grading system. The Complexity INdex in SARComas (CINSARC) signature comprises 67 genes related to chromosome biogenesis, mitosis control, and chromosome segregation and is correlated with CIN[11]. It has also demonstrated its prognostic value in 21 out of 39 cancer types, outperforming more than 15000 pre-existing signatures[12]. Initially established on frozen material with DNA microarrays and then applied with RNA sequencing, it was recently validated on formalin-fixed paraffin-embedded (FFPE) tissue thanks to the NanoString® technology with the code set NanoCind® developed by our team[13].

In the present report, we investigated whether CINSARC better predicted tumor recurrence and survival in stage II–III CRC in two independent retrospective cohorts.

MATERIALS AND METHODS

Patient selection

Two independent retrospective patient cohorts were analyzed in this study. Cohort 1 was retrieved from The Cancer Genome Atlas-Colon Adenocarcinoma (TCGA-COAD) data and included 297 stage II–III CRC patients. RNA sequencing, clinicopathological data including TNM stage and follow-up data were obtained for each case[14]. Cohort 2 consisted of 169 stage II–III CRC patients who were surgically resected at the Centre Hospitalo-Universitaire in Toulouse, France, between 2008 and 2013, for

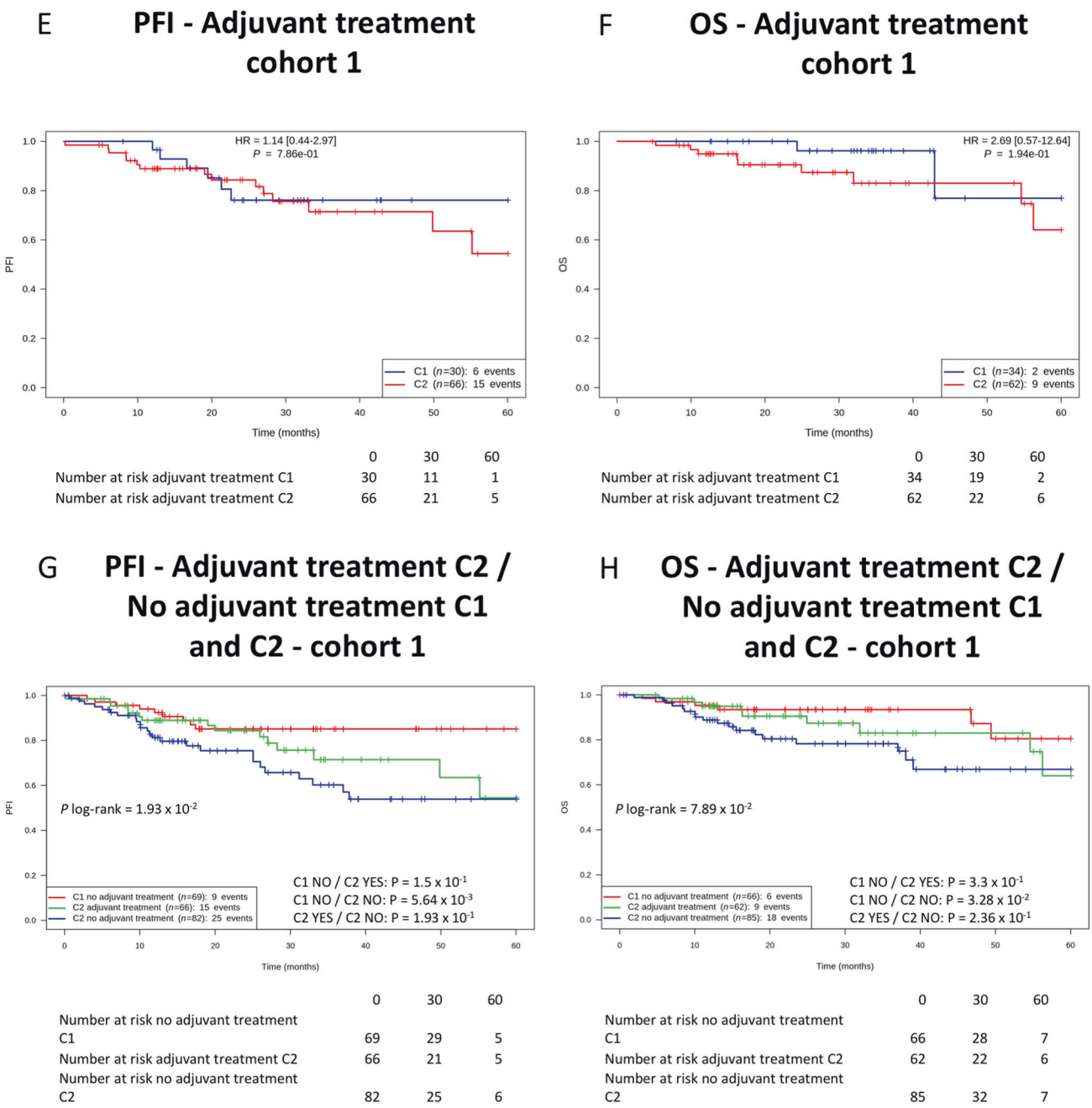

Fig. 1 (Continued)

whom we obtained FFPE blocks, clinicopathological data including TNM stage (defined for each patient in Digestive Oncology Multidisciplinary Consultation Meetings) and follow-up data. For all cases, FFPE blocks of surgical resections were used to build tissue microarrays (TMAs). Three pathologists performed a review of hematoxylin- and eosin-stained (H&E-stained) tumor sections to define diagnostic areas (JM, ACB, JS). Two representative cores (2 mm-punch size) were taken so each case was included in duplicate in the array.

Long-term oncologic outcomes were analyzed based on progression-free interval (PFI) (cohort 1) or disease-free survival (DFS) (cohort 2) and OS (cohorts 1 and 2). PFI was defined as the time from diagnosis to a new tumor event (progression of disease, local recurrence, distant metastasis, new primary tumors, death), DFS was defined as the time from surgery to the first event of relapse of CRC (local recurrence or distant metastasis), and OS was defined as the time from surgery to death from any cause. Cases were censored after 5 years of follow-up.

Clinicopathological features and outcomes are described in Table 1.

Immunohistochemistry (IHC) staining, MMR and CDX2 status

MMR status data identified by IHC was available for 222/297 patients in cohort 1. MMR status was identified by IHC for all cases in cohort 2. TMA slides were stained with anti-MLH1 (ES05, Agilent (Santa Clara, California, United-States), ready-to-use (RTU)), anti-PMS2 (EP51, Agilent, RTU), anti-MSH2 (FE11, Agilent, RTU), anti-MSH6 (EP49, Agilent, RTU) and anti-CDX2 (EP25, Leica (Wetzlar, Germany), RTU). Tissue slides were stained on a Bond III automatic stainer (Leica) and revelation was performed with the Bond Polymer Refine Detection kit (DS9800, Leica). For interpretation, the slides were evaluated by light microscopy by pathologists experienced with interpreting IHC and MMR studies (JM, ACB, JS)[15], and CDX2 status was identified as previously described (ACB, JS)[9].

RNA extraction, NanoCind®, and CINSARC classifications

To establish CINSARC classification in cohort 1, we used the RNA-sequencing data downloaded from the TCGA-COAD database on the

Table 2. Univariate and multivariate analyses for PFI and OS in patients with stage II–III CCR in cohort 1.

Variable	Progression-free interval (PFI)				Overall survival (OS)			
	Univariate analysis		Multivariate analysis ($n = 122$)		Univariate analysis		Multivariate analysis ($n = 222$)	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
CINSARC classification (C1/C2)	1.87 (1.11–3.16)	1.68×10^{-2}	4.55 (1.74–13.87)	1.54×10^{-3}	2.45 (1.31–4.59)	3.73×10^{-3}	3.15 (1.33–8.93)	7.82×10^{-3}
TNM (II/III)	1.68 (1.04–2.71)	3.33×10^{-2}	1.01 (0.45–2.33)	9.89×10^{-1}	2.38 (1.37–4.15)	1.54×10^{-3}	2.14 (1.02–4.65)	4.33×10^{-2}
CMS (1/2/3/4)	NA	3.32×10^{-2}	0.60 (0.40–0.88)	9.47×10^{-3}	NA	1.34×10^{-1}	NA	NA
MMR status (MSI/MSS)	3.31 (1.03–10.66)	3.34×10^{-2}	2.39 (0.69–12.53)	1.85×10^{-1}	91138956.25 (0–INF)	1.09×10^{-2}	16.49 (2.30–2093.39)	1.07×10^{-3}
VELIPI (absence/presence)	3.75 (1.58–8.89)	1.34×10^{-3}	1.59 (0.58–4.99)	3.81×10^{-1}	2.11 (0.9–4.97)	8×10^{-2}	NA	NA
Lymph nodes examined (<12≥)	0.94 (0.4–2.18)	8.85×10^{-1}	NA	NA	1.76 (0.86–3.63)	1.19×10^{-1}	NA	NA

Genomic Data Commons (GDC) Data Portal. For cohort 2, RNA was extracted from all FFPE blocks ($n = 169$) using the High Pure FFPET RNA Isolation Kit (Roche, Bâle, Switzerland) according to the manufacturer's instructions. RNA extraction was performed from full sections of tumors on a representative FFPE block from the initial surgical resection material and the whole tumor area was selected. One to ten 10 µm-slides were prepared depending on the tumor area which was evaluated on H&E stained-slides by a pathologist, with a minimum area required of 8 mm2 up to more than 100 mm2 (ACB). To establish CINSARC classification, we used the NanoString® technology with the nCounter code set (NanoCind®) developed by the team, comprising a panel of 75 probes, including 67 distinct test probes derived from the 67 CINSARC genes (Supplementary Table 1) and 8 from housekeeping genes for normalization purposes[13].

Subjects from the two cohorts were assigned to two groups (C1: good prognosis and C2: poor prognosis) using the nearest centroid method as previously described[11]. Centroids C1 and C2 were defined in each cohort from selected cases as follows: for PFI/DFS, patients without disease after 3 years of follow-up and patients with a progression/relapse; for OS, patients alive after 3 years of follow-up and patients deceased within 5 years after surgery.

CMS classification

To compare its prognostic value with the CINSARC classification, we established the CMS classification for cohort 1. The RNA-sequencing-based CMS classifier developed by Guinney et al. was applied to RNA-sequencing data from cohort 1 as previously described[6].

Functional enrichment analysis

For cohort 1, RNA-sequencing raw data was obtained from the TCGA-COAD database on the GDC data portal with the R GenomicDataCommons package. Differential gene expression analysis between C1 and C2 groups was performed with the R DESeq2 package. Gene Set Enrichment Analysis with the (GSEA) was performed with https://www.gsea-msigdb.org/gsea/index.jsp using the database MSigDB c5.go.bp.v7.4 available on http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp with 4010 gene sets and with the CINSARC gene set.

Statistics

All bioinformatic and statistical analyses were conducted with R statistical software version ≥4.0.4. Cohorts were compared with χ^2 test for categorical predictors (Fisher's exact test for theoretical numbers less than 5) and Student's test for continuous predictors. Patient subgroups were compared with respect to DFS, PFI and OS by using Kaplan–Meier survival curves, log-rank tests, and multivariate analyses based on the Cox proportional hazards method.

RESULTS

CINSARC is a significant prognostic factor and outperforms TNM staging system and CMS classification

The prognostic value of CINSARC was tested on cohort 1 comprising 297 stage II–III CRC. We established the classification from the available RNA-sequencing data and all cases were interpretable. For PFI, 124 cases were classified C1 and 173 were classified C2, whereas for OS, 121 cases were classified C1 and 176 were classified C2. Clinicopathological features and outcomes of each group are described in Supplementary Table 2. CINSARC split the population into two groups with different prognoses in terms of survival: the C2 group had a higher risk of progression (PFI: $P = 1.68 \times 10^{-2}$; HR = 1.87 [1.11–3.16]) and a higher risk of death ($P = 3.73 \times 10^{-3}$; HR = 2.45 [1.31–4.59]) than the C1 group (Fig. 1).

In univariate analysis, CINSARC classification, TNM stage, CMS classification, MMR status and VELIPI were significant prognostic factors for PFI (Supplementary Fig. 1). Among these, CINSARC and CMS classifications were independent prognostic factors, CINSARC classification being the most significant (Table 2). Regarding OS, CINSARC classification, TNM stage and MMR status were prognostic factors (Supplementary Fig. 1). All were independent prognostic factors, MMR status being the most significant (Table 2). Therefore, in that cohort, CINSARC proved to be an independent prognostic factor outperforming the gold-standard TNM classification.

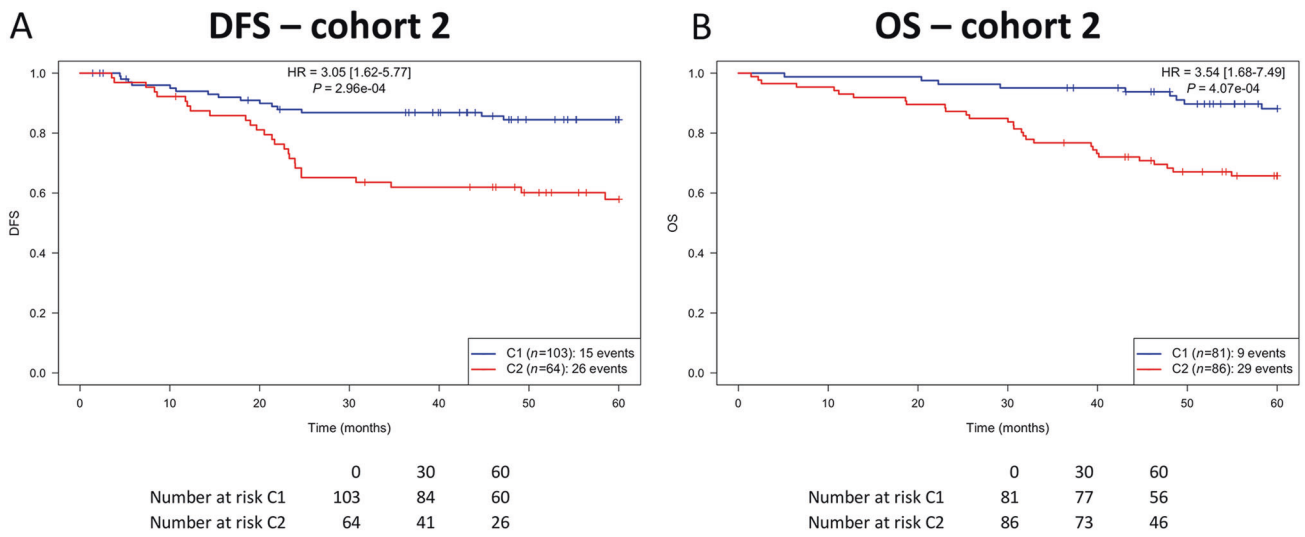

Fig. 2 CINSARC in cohort 2. DFS (**A**) and OS (**B**) analyses according to CINSARC classification for stage II–III CRC in cohort 2.

To evaluate the impact of adjuvant treatment on CINSARC classification, we next analyzed CINSARC classification in sub-groups of patients having received an adjuvant treatment or not. This showed that for patients who did not receive an adjuvant treatment, CINSARC classification split the population into two groups with significantly different survival, regardless of the outcome (PFI: $P = 5.64 \times 10^{-3}$; HR = 2.81 [1.31–6.03]; OS: $P = 3.28 \times 10^{-2}$; HR = 2.64 [1.04–6.67]). On the contrary, there was no significant difference between C1 and C2 groups in the population of patients who received an adjuvant treatment (PFI: $P = 7.86 \times 10^{-1}$; HR = 1.14 [0.44–2.97]; OS: $P = 1.94 \times 10^{-1}$; HR = 2.69 [0.57–12.64]) (Fig. 1). Furthermore, there was no significant survival difference between the C1 non-treated group and the C2 treated one (PFI: $P = 1.5 \times 10^{-1}$; HR = 1.82 [0.8–4.16]; OS: $P = 3.3 \times 10^{-1}$; HR = 1.66 [0.59–4.68]) (Fig. 1).

Validation of CINSARC's prognostic value on an independent cohort

To validate these results, CINSARC was applied to a second cohort comprising 169 tumors. The CINSARC classification was applied to RNA extracted from FFPE blocks (see M&M) and 168/169 (99.4%) cases were interpretable. DFS was available for 167 patients: 103 cases were classified C1 and 64 were classified C2. For OS, information was available for 167 patients: 81 were classified C1 and 86 were classified C2. Clinicopathological features and outcomes of each group are described in Supplementary Table 3. In this cohort, CINSARC significantly split the population of CRC into two groups, regardless of the outcome (DFS: $P = 2.96 \times 10^{-4}$; HR = 3.05 [1.62–5.77] and OS: $P = 4.07 \times 10^{-4}$; HR = 3.54 [1.68–7.49]) (Fig. 2).

In univariate analysis, CINSARC classification, TNM stage and CDX2 status were significant prognostic factors for DFS. All three were independent risk factors in the multivariate analysis, CINSARC classification being the most significant (Table 3). With regard to OS, CINSARC classification and CDX2 status were prognostic factors and CINSARC classification was independent in multivariate analysis (Table 3). Thus, these results validate CINSARC classification as a strong prognostic factor for stage II–III CRC, outperforming the TNM classification and other factors used in clinical practice such as perforation status, tumor grade, VELIPI, number of examined lymph nodes and MMR status.

In TNM sub-group analysis, CINSARC predicted DFS and OS in stage II CRC with a worse outcome in the C2 group (DFS: $P = 1.38 \times 10^{-3}$; HR = 5.4 [1.69–17.23]; OS: $P = 1.99 \times 10^{-2}$; HR = 3.19 [1.14–8.96]) but significantly predicted only OS in stage III CRC (DFS: $P = 9.4 \times 10^{-2}$; HR = 1.91 [0.89–4.12]; OS: $P = 1.11 \times 10^{-2}$; HR = 3.75 [1.25–11.23]) (Fig. 3). We also investigated its performance among microsatellite stable (MSS) patients which split this sub-population in two groups with significantly distinct DFS and OS (DFS: $P = 5.91 \times 10^{-4}$; HR = 3.24 [1.59–6.6]; OS: $P = 1.03 \times 10^{-3}$; HR = 3.42 [1.57–7.48]) (Supplementary Fig. 2).

CINSARC genes are overexpressed in C1 tumors

To decipher the biological mechanisms involved in the different outcome between groups C1 and C2, we performed GSEA for the 297 cases included in cohort 1. No gene set was significantly enriched in C2 tumors (FDR < 0.05). Interestingly, the CINSARC gene set was significantly enriched in group C1 (FDR = 0; NES = 2.08) (Supplementary Fig. 3). The five most enriched gene sets in group C1 were: negative regulation of cell cycle phase transition (FDR = 1.12×10^{-2}; NES = −2.26), metaphase anaphase transition of cell cycle (FDR = 8.49×10^{-3}; NES = −2.23), negative regulation of mitotic cell cycle (FDR = 6.44×10^{-3}; NES = −2.23), chromosome segregation (FDR = 5.82×10^{-3}; NES = −2.22) and regulation of chromosome separation (FDR = 5.04×10^{-3}; NES = −2.22) (Supplementary Fig. 3). The overexpression of CINSARC genes in group C1 was confirmed in cohort 2 (Supplementary Fig. 4).

DISCUSSION

CRC is the second most common cause of cancer death worldwide[1] and patient management depends mostly on the stage of the disease determined by TNM staging[3]. However, predicting clinical outcome in stage II–III CRC remains a challenge, despite the variety of available biomarkers[3,6,9,16–18] which are neither precise enough nor immediately applicable in routine practice. In this study, we show that CINSARC improves the ability to discriminate the risks of recurrence and death specifically in stage II–III CRC. In two independent stage II–III CRC cohorts, it identified a group of tumors with a poor outcome, whereas TNM staging either did not significantly differentiate tumors or was less discriminating than CINSARC. Furthermore, it outperformed other prognostic factors used in clinical practice and was more significantly discriminating than recently proposed ones such as CMS classification and CDX2 expression[6,9].

Our results show that the CMS classification cannot be used as a prognostic tool for CRC[6]. A high percentage of patients were not classified in any of the CMS groups (62 patients, 20.9 %), a feature not consistent with its use in clinical practice. Despite being higher than in previous studies (0–14.5%)[6,7,16,19–21], this high percentage

Table 3. Univariate and multivariate analyses for DFS and OS in patients with stage II–III CCR in cohort 2.

Variable	Disease-free survival (DFS) ($n = 167$)				Overall survival (OS) ($n = 167$)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR [95% CI]	*P* value	HR [95% CI]	*P* value	HR [95% CI]	*P* value	HR [95% CI]	*P* value
CINSARC classification (C1/C2)	3.05 [1.62–5.77]	2.96×10^{-4}	3.32 [1.79–6.39]	1.18×10^{-4}	3.54 [1.68–7.49]	4.07×10^{-4}	2.46 [1.40–4.53]	1.56×10^{-3}
TNM stage (II/III)	2.81 [1.47–5.36]	1.07×10^{-3}	2.35 [1.28–4.48]	5.46×10^{-3}	1.5 [0.79–2.83]	2.12×10^{-1}	NA	NA
MMR status (MSI/MSS)	1.15 [0.45–2.93]	7.7×10^{-1}	NA	NA	0.64 [0.29–1.39]	2.56×10^{-1}	NA	NA
Perforation Status	1.83 [0.65–5.13]	2.45×10^{-1}	NA	NA	1.49 [0.46–4.85]	5.03×10^{-1}	NA	NA
Tumor Grade	0.87 [0.21–3.6]	8.48×10^{-1}	NA	NA	1.34 [0.41–4.35]	6.27×10^{-1}	NA	NA
VELIPI (absence/presence)	1.62 [0.86–3.07]	1.33×10^{-1}	NA	NA	1.7 [0.9–3.32]	9.78×10^{-2}	NA	NA
Lymph nodes examined (<12≥)	0.86 [0.34–2.20]	7.54×10^{-1}	NA	NA	1.79 [0.82–3.9]	1.39×10^{-1}	NA	NA
CDX2 status (positive/negative)	3.37 [1.49–7.63]	1.93×10^{-3}	3.86 [1.60–8.16]	4.21×10^{-3}	3.73 [1.76–7.88]	2.19×10^{-4}	2.03 [0.94–3.95]	6.93×10^{-2}

represents a major limitation. The CMS4 group was initially described as having a poor progression and OS in localized stages[6,7,21]. However, we did not observe this in our stage II–III cohort 1 (Supplementary Fig. 1), suggesting that the CMS lacks robustness. Recently, Marisa et al. studied intratumor heterogeneity in the PETACC8 trial cohort of 1779 patients by using their deconvoluted transcriptomic profiles. They discovered that up to 55% of tumors corresponded to a mixture of at least two different CMS groups and that this heterogeneity was more significantly associated with a poor DFS and OS than each CMS group separately[22]. Furthermore, the current lack of standardization of the tools used to establish the CMS classification (different classifiers[6], IHC panels[7,16] and NanoString® panels[19,20,23]) further limits its use in routine. Therefore, while the CMS is useful for unraveling the biology of these tumors and could be used to develop new precision therapeutics, it is not suitable for use as a prognostic biomarker in clinical practice.

As for guiding treatment decision-making, CINSARC is currently being tested prospectively in sarcomas in the multicenter clinical trial CHIC-STS led by our team (NCT04307277)[24]. We hypothesize that in addition to being a prognostic biomarker of CRC, CINSARC could serve to better select patients for adjuvant chemotherapy. For example, there was no significant survival difference in cohort 1 between the C1 non-treated group and the C2 treated one, likely meaning that patients with stage II–III and CINSARC C2 CRC benefit from adjuvant treatment and that the latter might be an option for patients with stage II C2 CRC, who are commonly treated with surgery alone. On the contrary, patients with stage III C1 CRC, who are commonly treated with surgery and adjuvant treatment and are thus potentially exposed to chemotherapy-induced side-effects, might be protected from them. Dedicated prospective trials are needed to validate this hypothesis.

To better understand the biological mechanisms involved in the different outcome between C1 and C2 CRC, we performed functional enrichment analysis. As previously observed in different types of sarcomas and breast carcinomas, we expected an enrichment of the biological mechanisms implicated in cell cycle and mitosis due to aggressive cell proliferation, as well as an enrichment of the CINSARC gene set in the C2 group[11,25], thus explaining the worse prognosis of these tumors. Strikingly, we observed the opposite, with a significant enrichment of these biological pathways as well as an overexpression of CINSARC genes in the C1 group. These biological pathways and the CINSARC signature indirectly reflect CIN[11]. The acquisition of genomic instability is a crucial feature of CRC oncogenesis with three major pathways: CIN, MSI/hypermutated and CIMP pathways[14,26]. CIN plays a major role in CRC oncogenesis, as it is implicated in 65–70% of sporadic CRC[27]. It is characterized by aneuploidy and loss of heterozygosity, it may arise from defects in chromosomal segregation, telomere stability and the DNA damage response[27], and it is reflected by the Global Genomic Index (GGI)[28], which corresponds to the fraction of rearranged genome. However, its role in CRC prognosis is still debated[28,29]. Orsetti et al. observed a non-linear association between GGI level and prognosis: the best prognosis was found for tumors with a low GGI and the worst for tumors with a median GGI, whereas tumors with a high GGI had an intermediate outcome[28]. Similarly, Andor et al. studied the association between copy number variants (CNVs) and clinical outcome in a variety of tumor types. They found that tumors with either the lowest or highest rate of CNVs had the most favorable outcomes, suggesting that either too little or too much CIN can be detrimental for tumor cells[29]. Thus, we hypothesize that the low expression of CINSARC genes in CRC is correlated with an intermediate level of CIN. No significantly enriched gene sets were highlighted in the C2 group, which suggests that it comprises more heterogeneous molecular intrinsic subtypes than the C1 group. As previously observed in sarcomas[30], there might not be just one group of C2 tumors but several, explaining the lack of identified biological processes implicated in their poor prognosis. Other parameters involved in

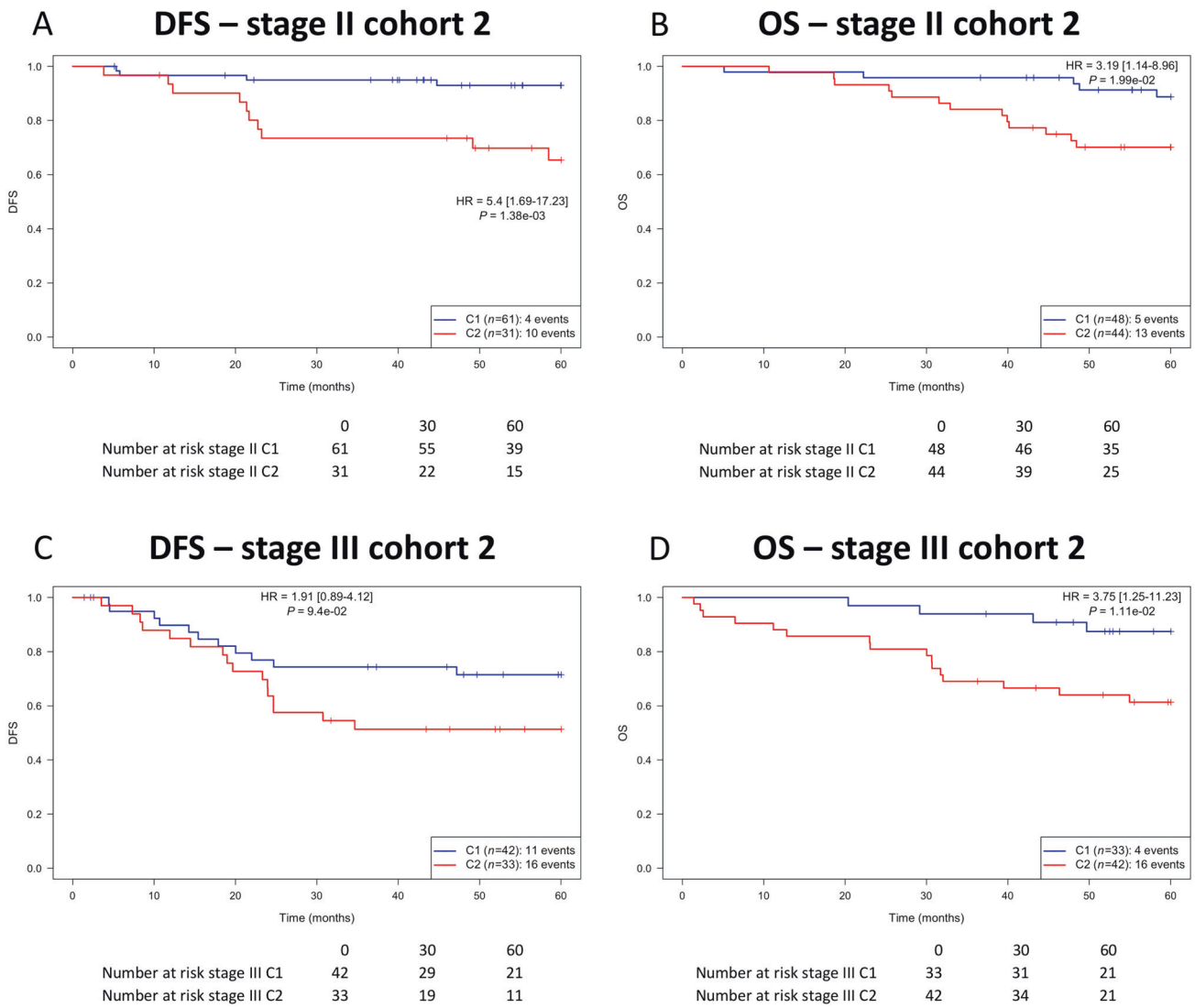

Fig. 3 CINSARC in cohort 2 by stage. DFS (**A**) and OS (**B**) analyses according to CINSARC classification for stage II CRC; DFS (**C**) and OS (**D**) analyses according to CINSARC classification for stage III CRC in cohort 2.

the prognosis such as the immune contexture[17,18], which was not taken into account in our study, could also explain this observation. Thus, it would be interesting to further explore these tumors with microenvironment data as well as to compare the CINSARC classification with the prognostic performance of an immune classification such as the Immunoscore[18].

Our study has some limitations. First, it was retrospective. Second, there may be a technological bias since we used different technologies to quantify RNA expression in both cohorts (RNA-sequencing data in cohort 1 and NanoCind® data in cohort 2) and with different types of samples (frozen samples in cohort 1 and FFPE samples in cohort 2). However, we believe it only reinforces the robustness of the CINSARC classification by showing that regardless of the technology used, it remains a significant prognostic biomarker. In cohort 2, we did not evidence any prognostic value of MMR status, a biomarker currently used in routine clinical practice. However, to detect a benefit associated with MSI with an HR of 0.65 with 80% power and 5% type I error, 300 events would be required, demonstrating the lack of power of our cohort and explaining this result[31].

In conclusion, CINSARC reliably estimated prognostic risk in stage II–III CRC patients in two independent cohorts, representing a total of 466 stage II–III CRC patients. It is immediately applicable in clinical practice when used with NanoString® technology and the NanoCind® code set. There was a correlation between low expression of the CINSARC genes and a poor prognosis in the high-risk group, possibly due to an intermediate level of CIN. Further studies are needed to validate these results in prospective cohorts including other new prognostic factors (immune contexture and tumor budding) and to clarify all the determinants of the poor prognosis in the heterogenous group of high-risk C2 tumors.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A. & Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394–424 (2018).
2. Siegel, R. L., Miller, K. D., Goding Sauer, A., Fedewa, S. A., Butterly, L. F., Anderson, J. C. et al. Colorectal cancer statistics, 2020. *CA A Cancer J Clin* **70**, 145–164 (2020).
3. *TNM classification of malignant tumours*. (John Wiley & Sons, Inc, 2017).

4. Kim, H. S., Kim, K. M., Lee, S. B., Kim, G. R., Han, Y. D., Cho, M. S. et al. Clinicopathological and biomolecular characteristics of stage IIB/IIC and stage IIIA colon cancer: Insight into the survival paradox. *J Surg Oncol* **120**, 423–430 (2019).
5. André, T., Boni, C., Navarro, M., Tabernero, J., Hickish, T., Topham, C. et al. Improved Overall Survival With Oxaliplatin, Fluorouracil, and Leucovorin As Adjuvant Treatment in Stage II or III Colon Cancer in the MOSAIC Trial. *J Clin Oncol* **27**, 3109–3116 (2009).
6. Guinney, J., Dienstmann, R., Wang, X., de Reyniès, A., Schlicker, A., Soneson, C. et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* **21**, 1350–1356 (2015).
7. Trinh, A., Trumpi, K., De Sousa, E Melo, F., Wang, X., de Jong, J. H., Fessler, E. et al. Practical and Robust Identification of Molecular Subtypes in Colorectal Cancer by Immunohistochemistry. *Clin Cancer Res* **23**, 387–398 (2017).
8. Li, Y., Yao, Q., Zhang, L., Mo, S., Cai, S., Huang, D. et al. Immunohistochemistry-Based Consensus Molecular Subtypes as a Prognostic and Predictive Biomarker for Adjuvant Chemotherapy in Patients with Stage II Colorectal Cancer. *Oncol* **25**, 1968-e1979 (2020).
9. Dalerba, P., Sahoo, D., Paik, S., Guo, X., Yothers, G., Song, N. et al. CDX2 as a Prognostic Biomarker in Stage II and Stage III Colon Cancer. *N. Engl J Med* **374**, 211–222 (2016).
10. Argilés, G., Tabernero, J., Labianca, R., Hochhauser, D., Salazar, R., Iveson, T. et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **31**, 1291–1305 (2020).
11. Chibon, F., Lagarde, P., Salas, S., Pérot, G., Brouste, V., Tirode, F. et al. Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. *Nat Med* **16**, 781–787 (2010).
12. Lesluyes, T., Delespaul, L., Coindre, J.-M. & Chibon, F. The CINSARC signature as a prognostic marker for clinical outcome in multiple neoplasms. *Sci Rep.* **7**, 5480 (2017).
13. Le Guellec, S., Lesluyes, T., Sarot, E., Valle, C., Filleron, T., Rochaix, P. et al. Validation of the Complexity INdex in SARComas prognostic signature on formalin-fixed, paraffin-embedded, soft-tissue sarcomas. *Ann Oncol* **29**, 1828–1835 (2018).
14. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330–337 (2012).
15. Jaffrelot, M., Farés, N., Brunac, A. C., Laurenty, A. P., Danjoux, M., Grand, D. et al. An unusual phenotype occurs in 15% of mismatch repair-deficient tumors and is associated with non-colorectal cancers and genetic syndromes. *Mod Pathol* **35**, 427–437 (2022).
16. Li, X., Larsson, P., Ljuslinder, I., Ling, A., Löfgren-Burström, A., Zingmark, C. et al. A modified protein marker panel to identify four consensus molecular subtypes in colorectal cancer using immunohistochemistry. *Pathol Res Pr* **220**, 153379 (2021).
17. Allard, M.-A., Bachet, J. B., Beauchet, A., Julie, C., Malafosse, R., Penna, C. et al. Linear quantification of lymphoid infiltration of the tumor margin: a reproducible method, developed with colorectal cancer tissues, for assessing a highly variable prognostic factor. *Diagn Pathol* **7**, 156 (2012).
18. Pagès, F., Mlecnik, B., Marliot, F., Bindea, G., Ou, F.-S., Bifulco, C. et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **391**, 2128–2139 (2018).
19. Lenz, H.-J., Ou, F.-S., Venook, A. P., Hochster, H. S., Niedzwiecki, D., Goldberg, R. M. et al. Impact of Consensus Molecular Subtype on Survival in Patients With Metastatic Colorectal Cancer: Results From CALGB/SWOG 80405 (Alliance). *J Clin Oncol* **37**, 1876–1885 (2019).
20. Piskol, R., Huw, L., Sergin, I., Kljin, C., Modrusan, Z., Kim, D. et al. A Clinically Applicable Gene-Expression Classifier Reveals Intrinsic and Extrinsic Contributions to Consensus Molecular Subtypes in Primary and Metastatic Colon Cancer. *Clin Cancer Res* **25**, 4431–4442 (2019).
21. Haasnoot, K. J. C., Backes, Y., Moons, L. M. G., Kranenburg, O., Trinh, A., Vermeulen, L. et al. Associations of non-pedunculated T1 colorectal adenocarcinoma outcome with consensus molecular subtypes, immunoscore, and microsatellite status: a multicenter case-cohort study. *Mod Pathol* **33**, 2626–2636 (2020).
22. Marisa, L., Blum, Y., Taieb, J., Ayadi, M., Pilati, C., Le Malicot, K. et al. Intratumor CMS Heterogeneity Impacts Patient Prognosis in Localized Colon Cancer. *Clin Cancer Res* **27**, 4768–4780 (2021).
23. Morris, J. S., Luthra, R., Liu, Y., Duose, D. Y., Lee, W., Reddy, N. G. et al. Development and Validation of a Gene Signature Classifier for Consensus Molecular Subtyping of Colorectal Carcinoma in a CLIA-Certified Setting. *Clin Cancer Res* **27**, 120–130 (2021).
24. Filleron, T., Le Guellec, S., Chevreau, C., Cabarrou, B., Lesluyes, T., Lodin, S. et al. Value of peri-operative chemotherapy in patients with CINSARC high-risk localized grade 1 or 2 soft tissue sarcoma: study protocol of the target selection phase III CHIC-STS trial. *BMC Cancer* **20**, 716 (2020).
25. Ferrari, A., Iannó, M. F., Carenzo, A., Fortunato, O., Casanova, M., Chiaravalli, S. et al. Complexity index in sarcoma and genomic grade index gene signatures in rhabdomyosarcoma of pediatric and adult ages. *Pediatr Blood Cancer* **68**, e28987 (2021).
26. Fearon, E. R. Molecular Genetics of Colorectal Cancer. *Annu Rev Pathol Mech Dis* **6**, 479–507 (2011).
27. Pino, M. S. & Chung, D. C. The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology* **138**, 2059–2072 (2010).
28. Orsetti, B., Selves, J., Bascoul-Mollevi, C., Lasorsa, L., Gordien, K., Bibeau, F. et al. Impact of chromosomal instability on colorectal cancer progression and outcome. *BMC Cancer* **14**, 121 (2014).
29. Andor, N., Graham, T. A., Jansen, M., Xia, L. C., Aktipis, C. A., Petritsch, C. et al. Pan-cancer analysis of the extent and consequences of intratumor heterogeneity. *Nat Med* **22**, 105–113 (2016).
30. Lesluyes, T. & Chibon, F. A global and integrated analysis of CINSARC-associated genetic defects. *Cancer Res* **80**, 5282–5290 (2020).
31. Popat, S., Hubner, R. & Houlston, R. S. Systematic Review of Microsatellite Instability and Colorectal Cancer Prognosis. *J Clin Oncol* **23**, 609–618 (2005).

ACKNOWLEDGEMENTS

We would like to thank the Biological Resources Center of the University Hospital of Toulouse for providing study material and patients. We thank the Department of Pathology at the Institut Universitaire du Cancer de Toulouse—Oncopole of Toulouse for performing immunohistochemistry and the technological pole of the Cancer Research Center in Toulouse.

AUTHOR CONTRIBUTIONS

A.-C.B., N.F., R.G., J.S., and F.C. performed study concept and design; A.-C.B., J.M., M.D., N.F., R.G., and J.S. provided study material and patients; A.-C.B., J.M., M.J., N.F., R.G., and J.S. performed collection and assembly of data; J.F., G.P., A.-C.B., V.N., T.F., J.S., and F.C. performed data analysis and interpretation; S.I. provided administrative Support; A.-C.B., J.F., N.F., J.S. and F.C. wrote the paper; all authors performed final approval of the paper, are accountable for all aspects of the work, confirm that they had full access to all the data in the study and accept responsibility to submit for publication.

FUNDING

Financial support was provided by ITMO Cancer Aviesan and the "Fonds d'Aide à la Recherche et à l'Évaluation" from the Société Nationale Française de Gastro-Entérologie.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Patients' samples were obtained after informed consent in accordance with the Declaration of Helsinki and stored at the CRB Cancer des Hôpitaux de Toulouse (BB-0033-00014) collection. According to French law, CRB Cancer collection has been declared to the Ministry of Higher Education and Research (DC-2008-463) and obtained a transfer agreement (AC-2013-1955) after approbation by ethical committees. Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41379-022-01166-9.

Correspondence and requests for materials should be addressed to Frédéric Chibon.