CK7 and consensus molecular subtypes as major prognosticators in V600E BRAF mutated metastatic colorectal cancer

CK7 and Consensus Molecular Subtypes as major prognosticators in ^{V600E}*BRAF* mutated metastatic colorectal cancer

Authors and affiliations

Fotios Loupakis[1], Paola Biason[1], Alessandra Anna Prete[1], Chiara Cremolini[2], Filippo Pietrantonio[3,4], Nicoletta Pella[5], Emanuela Dell'Aquila[6], Elisa Sperti[7], Clizia Zichi[7], Rossana Intini[1], Vincenzo Dadduzio[1], Marta Schirripa[1], Francesca Bergamo[1], Carlotta Antoniotti[2], Federica Morano[3], Francesco Cortiula[5,8], Giovanna De Maglio[9], Lorenza Rimassa[10], Valeria Smiroldo[10], Lorenzo Calvetti[11], Giuseppe Aprile[11], Lisa Salvatore[12*], Daniele Santini[6], Giada Munari[1,13], Roberta Salmaso[13], Vincenza Guzzardo[13], Claudia Mescoli[13], Sara Lonardi[1], Massimo Rugge[13], Vittorina Zagonel[1], Massimo Di Maio[7], Matteo Fassan[13].

[1]Department of Oncology, Veneto Institute of Oncology IOV – IRCCS, Padua, Italy
[2]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy
[3]Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[4]Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy
[5]Department of Oncology, University and General Hospital, Udine, Italy.
[6]Department of Medical Oncology, Campus Bio-Medico University of Rome, Rome, Italy.
[7]Department of Oncology, University of Turin at Umberto I "Ordine Mauriziano" Hospital, Turin, Italy.
[8]Department of Medicine (DAME), University of Udine, Italy.
[9]Department of Pathology, University Hospital of Udine, Udine, Italy.
[10]Medical Oncology and Hematology Unit, Humanitas Cancer Center, Humanitas Clinical and Research Center - IRCCS Rozzano (Milan), Italy
[11]Department of Oncology, General Hospital San Bortolo, Unità Locale Socio-Sanitaria 8 Berica, Vicenza, Italy
[12]Medical Oncology, Polyclinic University Foundation A. Gemelli IRCCS, Roma, Italy
[13]Surgical Pathology & Cytopathology Unit, Department of Medicine (DIMED), University of Padua, Italy

*_Former Affiliation_: Unit of Oncology, Polyclinic GB Rossi, AOUI Verona, Italy

Running title

Extensive profiling of ^{V600E}*BRAF* mutated metastatic CRC

Keywords

Colorectal cancer, metastases, ^{V600E}*BRAF*, immunohistochemistry, prognostic markers

Additional information

Financial support

Regione Veneto – RP-2014-00000395., DOR Funds University of Padua

Corresponding authors

Fotios Loupakis, MD, PhD. Veneto Institute of Oncology IOV – IRCCS, via Gattamelata 64, Padua, Italy.
Phone: + 39 049/8215919. Fax: + 39 049/8217929.
E mail address: fotios.loupakis@iov.veneto.it

Matteo Fassan, MD, PhD. Surgical Pathology & Cytopathology Unit, Department of Medicine (DIMED), University of Padua, via Gabelli 61, 35121 Padua, Italy. Fax: +39 049 827 2277 Phone: +39 049 821 1312. E mail address: matteo.fassan@unipd.it

Conflict of interest

The authors declare no conflict of interest regarding the publication of this article

Word count

Running Title: 55

Keywords: 5

Translational relevance: 148

Abstract: 225

Main text: 3239

References: 28

Total number of tables: 4 ordinary plus 4 supplementary

Total number of figures: 1 ordinary plus 1 supplementary

Translational Relevance

Based on a large cohort of ^{V600E}*BRAF* mutated metastatic colorectal cancer (mCRC), the present study provides new data on this rare and aggressive disease.

We selected a panel of the most intriguing and promising markers for which prognostic significance has been hypothesized several times, based on a robust rationale, but up today never properly explored. Analyses focused on cytokeratins' profile, consensus molecular subtypes, ^{V600E}*BRAF* mutated-specific subtypes as defined by Barras and colleagues, and tumor infiltrating lymphocytes. Molecular data were challenged in a robust multivariate model including an innovative and validated clinical score.

A comparison between primary tumor and metastases for a large number of coupled samples was conducted, finding a high concordance level and therefore answering to a question that is always left open in several exploratory analyses. Taken altogether, data presented here will provide clinicians and researchers with new tools for predicting prognosis in ^{V600E}*BRAF* mutated mCRC.

Abstract

Introduction ^{V600E}*BRAF* mutated metastatic colorectal cancer (mCRC) is a subtype(10%) with overall poor prognosis, but the clinical experience suggests a great heterogeneity in survival at the single patient level. In a previous work we built a clinical score to stratify prognosis of ^{V600E}*BRAF* mCRC patients. It is still unexplored which is the real distribution of traditional and innovative biomarkers among ^{V600E}*BRAF* mutated mCRC and to which extent those determinants may further improve the clinical prediction of survival outcomes.

Materials and methods Data and tissue specimens from 155 ^{V600E}*BRAF* mutated mCRC patients treated at 8 Italian Units of Oncology were collected. Specimens were analysed by means of immunohistochemistry profiling performed on tissue microarrays. For each biomarker, primary endpoint was overall survival (OS).

Results CDX2 loss conferred worse OS (HR=1.72, 95%CI1.03-2.86, p=0.036), as well as high CK7 expression (HR=2.17, 95%CI1.10-4.29, p=0.026). According to Consensus Molecular Subtypes (CMS), CMS1 patients had better OS compared to CMS2-3/CMS4 (HR=0.37, 95%CI 0.19-0.71, p=0.003). Samples showing less TILs had worse OS (HR=1.72, 95%CI 1.16-2.56, p=0.007). Progression-free survival analyses led to similar results. At multivariate analysis for OS challenging molecular results with robust clinical score data, CK7 and CMS subgrouping emerged as the strongest prognosticators.

Conclusion The present study provides new data on how several well-established biomarkers perform in a homogenous ^{V600E}*BRAF* mutated mCRC population, with important and independent information added to standard clinical prognosticators.

Introduction

^{V600E}*BRAF* mutation is detected in 8-12% of colorectal cancer (CRC) patients, accounting for more than 90% of CRC *BRAF* mutations (1). It is an independent negative prognostic factor in CRC across all stages (2,3).Furthermore, a recent consensus work identified *BRAF* mutational status as one of the top five fundamental stratification characteristics in the initial evaluation of metastatic CRC (mCRC) patients, together with *RAS* mutations, patients' performance status, primary tumor sidedness and presence of liver-limited disease (4).

Despite the evidence of its prognostic significance, great heterogeneity in survival outcome is evident among ^{V600E}*BRAF* mutated mCRC (5). Indeed, daily clinical practice as well as results from recent trials show that some patients with ^{V600E}*BRAF* mutated mCRC may experience prolonged survival and durable response to therapies, while other patients develop rapid resistance, disease progression and or clinical worsening (6). These observations led to the hypothesis that a better stratification based on clinical and molecular features should be explored when considering ^{V600E}*BRAF*mCRC as a separate disease. To achieve this goal, correct methodology, homogeneous patients' cohorts and adequate sample size are of crucial importance. We recently proposed a clinical risk score prognostic calculator based on ECOG PS, tumor grading, presence of liver metastases, presence of lung metastases and presence of nodal involvement, CA19.9, CEA, LDH levels and neutrophils/lymphocytes ratio (7). Moreover, a "simplified" version based only on the first five covariates was subsequently developed for a more practical clinical application and as functional and reliable tool for multivariate modelling of translational analyses.

Caudal type homeobox 2 (*CDX2*) is a gene encoding a protein involved in cell differentiation, adhesion and polarity. It has been hypothesized that ^{V600E}*BRAF* mutation and loss of CDX2, which are significantly associated, might cooperate in promoting CRC tumorigenesis (8,9). Dalerba et al. demonstrated that loss of CDX2 expression may confer poor prognosis to stage II-III CRC patients (10). Notwithstanding, no or limited information regarding the relative impact of *BRAF* mutations and other prognostic features were available(11).

CRC has been classically associated to a CK20-positive and CK7-negative profile (12,13). Literature data suggest that among ^{V600E}*BRAF* mutated CRC, a higher prevalence of CK20-negative tumors may be found (14,15).

Most importantly, up to 30% of ^{V600E}*BRAF* mutated cases show microsatellite instability (MSI), a condition caused by either germline or somatic mutations in *MLH1*, *PMS2*, *MSH2* and *MSH6* genes or *MLH1* hyper-methylation. In early stages, ^{V600E}*BRAF* mutated microsatellite stable (MSS) CRC are characterized by augmented clinical aggressiveness of the disease with poor prognosis (16-18); conversely, the prognostic impact of microsatellite instability-high (MSI-H) status in ^{V600E}*BRAF* mutated patients is still debated. Venderbosch et al. (2) retrospectively analysed a large cohort of 3,063 patients from 4 different studies aiming to describe mutual influence on prognosis of microsatellite instability in *BRAF* mutated stage IV CRC and vice-versa. The prognostic influence of ^{V600E}*BRAF* mutation in MSS was confirmed, but other definitive conclusions were limited by excessive subgrouping. Another retrospective study including only 14 ^{V600E}*BRAF* mutated patients out of 55 MSI-H cases suggested a negative impact of ^{V600E}*BRAF* mutation in MSI-H patients, but again small sample size limited any reliable consideration on the prognostic impact of microsatellite instability among^{V600E}*BRAF* mutated patients (19).

A remarkable step forward in the description of CRC heterogeneity has been made by the Consensus Molecular Subtypes (CMS) (20). The majority of ^{V600E}*BRAF* (up to 70%) are classified into CMS1 subgroup, while 7% and 17% are grouped in CMS2-3 and CMS4, respectively. This heterogeneous distribution supports the rationale for exploring the prognostic relevance of CMS subgrouping among ^{V600E}*BRAF* mCRC.

Another active field of interest in the definition of mCRC prognosis is the presence of tumor infiltrating lymphocytes (TILs). So far, no specific studies are available in literature concerning their role in ^{V600E}*BRAF* mutated tumors.

Finally, Barras et al. categorized ^{V600E}*BRAF* mutated CRC into two groups based on gene expression signatures: BM1 patients, accounting for approximately one third of cases, show activation of KRAS/mTOR/AKT/4EBP1 pathway, while BM2 group is characterized by dysregulation in the cell-cycle (5). This study tried to elucidate possible differences in

proliferation mechanisms among ^{V600E}*BRAF* mutated CRC, thus giving molecular bases to the observation of heterogeneity in this disease.

Given the above reported assumptions, the aim of our work is to investigate the prognostic role of the most important and biologically sound prognostic markers in a large set of ^{V600E}*BRAF* mutated mCRC patients, in order to better explain the wide inter-patient heterogeneity observed in routine clinical practice.

Materials and Methods

Clinical and molecular data of ^{V600E}*BRAF* mutated mCRC patients referred to 8 Italian Oncology Units between January 2005 and December 2016 were collected. In particular, for each patient data on demographic, tumor characteristics, 1st line systemic treatment, RECIST1.1 response and survival were retrieved.

Patients were deemed eligible if clinical data and archival tissue either of primary tumor and/or metastases were available.

Available primary and/or metastatic formalin-fixed paraffin-embedded (FFPE) surgical samples were processed using the Galileo CK3500 Arrayer (www.isenet.it), a semiautomatic and computer-assisted Tissue microarray (TMA) platform. Tissue cores (3 cores per sample; 1 mm in diameter) were obtained from each primary and metastatic lesion, respectively. Small biopsy samples were processed separately. Immunohistochemical stainings were automatically performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) on 4 µm-thick sections. Primary antibodies, dilutions and scoring evaluation are available upon request.

CDX2 expressions values were defined according to H-score, defined as the aggregate of total percentage of tumor cells expressing CDX2 at each particular intensity level from 0, +1 (weak intensity), +2 (moderate intensity) or +3 (strong intensity). In brief, the H-score was defined as: (Percent of CDX2 1+ tumor cells multiplied by intensity of 1) + (Percent of CDX2 2+ tumor cells multiplied by intensity of 2) + (Percent of CDX2 3+ tumor cells multiplied by intensity of 3). Thus, this composite score can range from 0 (a tumor which is completely negative) to a maximum of 300 (a tumor in which all the cells feature a 3+ staining). CDX2 results were split in tertiles as follows: 0-24 (low expression), 25-120 (intermediate expression), 121-300 (high expression).

Cytokeratin expression pattern was evaluated by CK7 and CK20 expression. CK7 expression was categorized in low (values 0-1) and high (values 2-3), whereas CK20 expression was indicated as negative (no expression) or positive (values 1,2 or 3), according to staining intensity in more than 10% of cancer cells.

MSI-H status was defined in the absence of nuclear immunostaining for one of the couples MLH1/PMS2 or MSH2/MSH6 in tumor cell. The diagnostic performance of immunohistochemistry in identifying MSI-H cases was tested by microsatellite analysis (Titano kit, Diatech Pharmacogenetics) in a series of 20 MMRd and 20 MMRp tumors (21).

CMS were assigned by assessing 4 IHC markers (FRMD6, ZEB1, HTR2B, CDX2) in combination with pan-cytokeratin (KER) to normalize results as reported in literature (22). Primary tumors and/or metastasis were then categorized into the 3 CMS classes (CMS1, CMS2/3 or CMS4) using the online classification tool (https://play.google.com/store/apps/details?id=com.cordova.braf).

Presence of TILs was evaluated on haematoxylin and eosin (H&E) stained slides and dichotomized by using a cut-off of 2.0: low number of TILs for tumors showing an average number of TILs<2.0, high number of TILs for tumors with ≥2.0 TILs (23).

To stratify tumors according to Barras et al. (5) in BM1 and BM2 groups, we exploratively categorized each tumor based on the presence/absence of these 5 markers: CDK1, ATM, Phospho-Akt (Ser473), Cyclin D1, and Phospho-4E-BP1 (Thr70). Since BM1 is characterized by activation of PI3K/mTOR/AKT pathway, while BM2 of cell cycle pathway we assigned samples to BM1 or BM2 based on the coherence of the following parameters. Overexpression of Phospho-Akt, Phospho-4E-BP1, ATM and Cyclin D1 and downregulation of CDK1 were consistent with a BM1 profile. On the other hand, BM2 cases were characterized by overexpression of CDK1 and downregulation of the remaining markers. A tumor was considered positive in ATM if >10% of tumor cells were positive for nuclear ATM staining. The activation of the AKT/4E-BP1 cascade was defined in the presence of high expression levels of the phosphorylated forms of AKT and/or 4E-BP1. High levels of Cyclin D1 and CDK1 expression were defined in the presence of at least 50% of cancer cells positive (Cyclin D1 in the nucleus, CDK1 both in the nucleus and cytoplasm). Samples with 4 or 5 coherent parameters were defined as BM1 or BM2, whereas tumors in which 3 out of 5 parameters were coherent with the hypothesis were

defined as borderline BM1 or BM2. Tumors with only 1 or 2 parameters coherent with the original classification were defined as not evaluable.

Simplified score for estimating the prognostic impact of major clinical and pathological was calculated considering 5 parameters as previously described (7): grading, ECOG PS at diagnosis of metastatic disease and sites of metastases at diagnosis (liver, lung, nodes). To calculate the score the following criteria were applied: ECOG PS 0 = 0 points; ECOG PS 1 = 2 points; ECOG PS 2-3 = 3 points. Tumor grading 1 or 2 = 0 points, tumor grading 3 or 4 = 1 point. Presence of liver metastases = 1 point; presence of lung metastases = 2 points, presence of distant nodes metastases = 2 points. The score is calculated as the total sum of points. Patients were classified as “low-risk” if they had a score ranging from 0 to 2; they were classified as “intermediate-risk” if the score was 3 or 4; they were classified as “high-risk” if their score ranged from 5 to 9.

Statistical analysis

The primary endpoint of the present analysis was Overall Survival (OS) for each variable analyzed. OS was defined as time from metastatic disease diagnosis to death due to any cause. Secondary endpoints included: Progression Free Survival (PFS) for each variable (PFS was defined as the time from 1st line treatment start date to 1st progression); the reproducibility of BM1/BM2 subgrouping and CMS distribution as assigned by means of IHC/TMA. For each determinant, comparison between samples from primary tumor and metastatic lesions was performed in order to explore their concordance.

Both OS and PFS and 95%CI were calculated using Kaplan-Meier method. Cox proportional Hazard model was adopted in the multivariate analysis including all covariates significantly correlated with survival in the univariate analysis.

PFS and OS were calculated in univariate analysis for the following molecular factors: CDX2,CK7, CK20 expression, CMS groups, BM1/BM2 groups, and presence of TILs. Factors found significant at univariate analysis were included into the multivariate analysis for both OS and PFS including clinical score data as covariate.

Results

A total of 155 patients were included. Males and females were equally represented (50.3%/49.7%, respectively). As expected, frequent features were: right-sidedness of primary tumor (74.2%), presence of synchronous metastases (65.8%), hepatic or nodal involvement (53% and 38% respectively), with 63% of patients having a single metastatic site at the time of stage IV disease diagnosis. A large proportion of patients had previous primary tumor resection (87.1%). Baseline characteristics and major clinical parameters are summarized in **Table 1**.

The vast majority of patients (89%) received at least 1 treatment for metastatic disease: first-line treatment was monochemotherapy +/- a biologic agent (anti-EGFR monoclonal antibody or bevacizumab) in 9.4% of treated patients, doublet +/- a biologic agent in 55.8%, triplet +/- a biologic agent in 24.6%, immunotherapy in 7.3% and anti-BRAF treatment in 2.2%.

Distribution of molecular variables analyzed in whole population is shown in **Table 2,** correlation data between paired single parameters are reported in **Supplementary Table 1**. For 46 patients, paired primary and metastasis samples were available: data obtained from IHC analyses were concordant in most cases, as shown in **Supplementary Table 2**.

After a median follow-up of 27.9 months (95%CI 20.3-35.5), 104 patients (67.1%) died.Median OS of the whole population was 18.5 months (95%CI 13.3-23.7), median PFS from the beginning of the first line treatment was 7.6 months (95%CI 5.2-10.0).

Univariate analyses

Results on OS and PFS are reported in **Table 3** and **Supplementary Table 3** and graphically represented in **Figure 1** and **Supplementary Figure 1**, respectively and described below for each single variable.

CDX2. Patients with low or intermediate expression had a shorter OS compared to patients with high expression (HR=1.72, 95%CI 1.03–2.86, p=0.036). Similar trend, but no significant differences were detected in terms of PFS (HR=1.41, 95%CI 0.86 – 2.30, p=0.169).

CK7 – CK20. Patients with higher CK7 expression had a shorter OS compared to patients with lower CK7 expression (HR=2.17, 95%CI 1.10-4.29, p=0.026). No significant differences were detected in terms of PFS (HR=1.13, 95%CI 0.56-2.29, p=0.74). Patients with negative CK20 had a shorter OS compared to patients with positive CK20 (HR=1.75, 95%CI 0.83-3.69, p=0.14). Similar trend, but no significant differences were detected in terms of PFS (HR=1.72, 95%CI 0.73-4.05, p=0.21).

CMS. CMS2-3 or CMS4 patients had a shorter OS compared to CMS1 (HR=2.70, 95%CI 1.41-5.26, p=0.003), similar results were reported for PFS (HR=2.22, 95%CI 1.14-4.35, p=0.02).

TILs. Patients with low TILs levels had a shorter OS compared to patients with high levels (HR=1.72, 95%CI 1.16-2.56, p=0.007), results confirmed also in PFS (HR=1.72, 95%CI 1.18-2.56, p=0.005).

BM1/BM2. No significant differences between BM1 and BM2 patients were detected in terms of OS (HR=1.37, 95%CI 0.87-2.17, p=0.18), or PFS (HR=1.27, 95%CI 0.80-2.03, p=0.31).

Clinical Score. As expected, patients with high score had a shorter OS compared to patients with intermediate or low score (HR=2.61, 95%CI 1.53-4.48, p<0.001), similar results were obtained in terms of PFS (HR=2.13, 95%CI 1.21-3.75, p=0.009).

Multivariate analysis

Since the clinical score was built on OS data, it was included to adjust for integrating clinical and molecular prognostication. At multivariable analysis, CK7 overexpression was independently associated with worse OS with a HR of 2.11 (95%CI 1.03-4.34, p=0.041), as was CMS2-3 and 4 over CMS1, with a HR of 2.22 (95%CI 1.03-050, p=0.049). Complete data are reported in **Table 4**. The poor prognostic score that was determined clinically retained an independent prognostic impact (HR=2.42, 95%CI 1.16-5.05, p=0.019).

Similarly, in multivariate analysis for PFS, CMS2-3 and 4 were significant determinants of worse outcome over CMS1 (HR 2.17, 95%CI1.01-4.76, p=0.049). Complete data are reported in **Supplementary Table 4**

Discussion

In the present study, we explored and clarified the prognostic role of CDX2, CK7 and 20, TILs, CMS and BM1/BM2 subtypes in ^{V600E}*BRAF* mutated mCRC with a modern multivariate model including a validated clinical prognostic score as covariate. For each variable, we verified the impact on OS and secondarily on PFS. Moreover, level of concordance between primary tumors and metastatic sites was studied for each parameter.

CRC is usually associated to a CK7 negative and CK20/CDX2 positive profile (12,13). Notwithstanding, some evidence in literature suggests that in ^{V600E}*BRAF* mutated mCRC this profile may be different, with a higher prevalence of CK20/CDX2 negative tumors(14,15). On the other hand, it has never been properly explored how the cytokeratins' profile affects prognosis among *BRAF* mutated patients, moreover none of the survival analyses conducted so far included important covariates such as MSI status (11). In 2014 Landau et al. documented lower expression of CDX2 in ^{V600E}*BRAF* mutated mCRC compared to ^{V600E}*BRAF* wild type mCRC, irrespective of MSI status: these results suggest that loss of expression of CDX2 could depend on *BRAF* mutations more than on microsatellite status. Of note, in the same study a higher prevalence of CK7 expression was found in *BRAF* mutated MSS CRC. Unfortunately, no survival analyses were planned in that study (24).Recently, another work described the relationship between CDX2 and prognosis in CRC: in this study, loss of CDX2 expression was associated with significantly worse OS, but specific analyses for stage IV ^{V600E}*BRAF* patients were not possible due to small sample size (25). According to our data, low CDX2 expression was associated to worse OS (HR of intermediate/low vs high expression = 1.72, 95%CI: 1.03–2.86, p=0.036).

We were also able to report on the prognostic role of CK7 expression, emerging as a strong determinant of outcome even in the multivariate model (HR of CK7 positive vs negative = 2.11, 95%CI 1.03–4-34, p=0.041). From a mechanistic perspective, this finding is in line with what Harbaum et al. described earlier: a high prevalence of CK7 positive cells at the invasive front of tumor buds in samples of CRC. The same authors recorded a trend toward a higher risk of disease progression or mortality in patients with high CK7 expression (26). The authors argued that CK7 could be associated with epithelial-to-mesenchymal transition. Again, no specific information was available regarding ^{V600E}*BRAF* mutated patients in this study.

In our study, CMS2-3 and 4 were associated with significantly worse OS and PFS (HR=2.70, 95%CI 1.41-5.26, p=0.003 and HR=0.22, 95%CI 1.14 – 4.35, p=0.02, respectively) when compared to CMS1 also in multivariate analyses. Given that we arbitrarily assigned MSI-H tumors to the CMS1 subgroup as previously described (22,27), our data clarify the effect of MSI-H phenotype on ^{V600E}*BRAF* mutated mCRC. This finding provides an answer to an open issue still unsolved from studies conducted so far (2,19). It is unlikely that treatment with anti-PD1 could have influenced these results since the percentage of patients treated with those agents is below 10% in our series. Given the rapid development of anti-BRAF and immunotherapy with checkpoint inhibitor-based treatments in mCRC, our data would be quite useful to inform the design of future clinical trials.

Another interesting observation is that presence of TILs was related to better OS and PFS outcome (respectively, HR=0.58, 95%CI 0.39–0.86 for OS and HR=0.58, 95%CI 0.39–0.85 for PFS). Recently, Shibutani et al. described a correlation between high immune infiltrate in primary tumor and response to chemotherapy in a cohort of 57 mCRC patients, with higher response rate to chemotherapy (79.3% vs. 48.1%, p=0.025) and better PFS (10.1 months vs. 7.3 months, p=0.013) in the high-TILs group. Of note, in the high-TILs group a significantly better OS was observed compared to low-TILs group (35.5 months vs. 22.4 months, p=0.022) (28). Taken together, data on TILs and CMS suggest a role for tumor-immune system interaction in affecting prognosis of ^{V600E}*BRAF* mutated mCRC.

We also explored the reproducibility of BM1/BM2 categorization with IHC based surrogate markers and correlated those results with outcome. Similarly to what Barras et al. previously reported (5), no differences were found in PFS and OS between the two groups.

Major points of strength of our data rely on: a) clinical homogeneity (i.e. all stage IV patients), b) large numbers (^{V600E}*BRAF* mutated mCRC constitutes around 8% and 155 patients with detailed clinical data and biologic material constitute one the biggest cohort ever studied), c) adjustment with a modern, robust and validated clinical prognostic

score, d) real world data. The latter is a two-faced point, with its intrinsic pros and cons. From one side, being *BRAF* a determinant of extremely bad prognosis, it has been reported how patients with available biologic material or enrolled in trials do not resemble exactly in terms of incidence and specific characteristics. From this may obviously originate a dangerous selection bias when analyzing patients and samples from trials (1). From the other side, we should admit that quality and detail of real-world data are certainly limited compared to prospective controlled trials. In fact, the most relevant limitation of our study is its retrospective nature. Patients have been selected according to stage, but not for type or number of previous lines of treatment received: in this way, a slight selection bias could not be excluded.

In conclusion, present data provide new, original and informative observations. These findings would deserve external confirmatory studies, but give already intriguing answers on how to interpret and prognosticate clinical heterogeneity within the subgroup of ^{V600E}*BRAF* mCRC.

References

1. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S*, et al.* Mutations of the BRAF gene in human cancer. *Nature* **2002**;417(6892):949-54 doi 10.1038/nature00766.
2. Venderbosch S, Nagtegaal ID, Maughan TS, Smith CG, Cheadle JP, Fisher D*, et al.* Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. *Clin cancer res* **2014**;20(20):5322-30 doi 10.1158/1078-0432.ccr-14-0332.
3. Sinicrope FA, Shi Q, Smyrk TC, Thibodeau SN, Dienstmann R, Guinney J*, et al.* Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. *Gastroenterology* **2015**;148(1):88-99 doi 10.1053/j.gastro.2014.09.041.
4. Goey KKH, Sørbye H, Glimelius B, Adams RA, André T, Arnold D*, et al.* Consensus statement on essential patient characteristics in systemic treatment trials for metastatic colorectal cancer: Supported by the ARCAD Group. *Eur J Cancer* **2018**;100:35-45 doi 10.1016/j.ejca.2018.05.010.
5. Barras D, Missiaglia E, Wirapati P, Sieber OM, Jorissen RN, Love C*, et al.*BRAF V600E Mutant Colorectal Cancer Subtypes Based on Gene Expression. *Clin cancer res* **2017**;23(1):104-15 doi 10.1158/1078-0432.ccr-16-0140.
6. Corcoran RB, André T, Atreya CE, Schellens JHM, Yoshino T, Bendell JC*, et al.*Combined BRAF, EGFR, and MEK Inhibition in Patients with. *Cancer Discov* **2018**;8(4):428-43 doi 10.1158/2159-8290.CD-17-1226.
7. Intini R, Loupakis F, Cremolini C, Sartore-Bianchi A, Pietrantonio F, Pella N*, et al.*Clinical prognostic score of BRAF V600E mutated (BM) metastatic colorectal cancer (mCRC): Results from the "BRAF, BeCool" platform. *Journal of Clinical Oncology* **2018**;36(4_suppl):639- doi 10.1200/JCO.2018.36.4_suppl.639.
8. Sakamoto N, Feng Y, Stolfi C, Kurosu Y, Green M, Lin J*, et al.* BRAF V600E cooperates with CDX2 inactivation to promote serrated colorectal tumorigenesis. *Elife* **2017**;6 doi 10.7554/eLife.20331.
9. Tong K, Pellon-Cardenas O, Sirihorachai VR, Warder BN, Kothari OA, Perekatt AO*, et al.* Degree of Tissue Differentiation Dictates Susceptibility to BRAF-Driven Colorectal Cancer. *Cell reports* **2017**;21(13):3833-45 doi 10.1016/j.celrep.2017.11.104.
10. 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* **2016**;374(3):211-22 doi 10.1056/NEJMoa1506597.
11. Schirripa M, Loupakis F, Lenz HJ. CDX2 as a Prognostic Biomarker in Colon Cancer. *N Engl J Med* **2016**;374(22):2183 doi 10.1056/NEJMc1602584.
12. Moll R, Zimbelmann R, Goldschmidt MD, Keith M, Laufer J, Kasper M*, et al.* The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation* **1993**;53(2):75-93.
13. Werling RW, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* **2003**;27(3):303-10.
14. Kim JH, Rhee YY, Bae JM, Cho NY, Kang GH. Loss of CDX2/CK20 expression is associated with poorly differentiated carcinoma, the CpG island methylator phenotype, and adverse prognosis in microsatellite-unstable colorectal cancer. *Am J Surg Pathol* **2013**;37(10):1532-41 doi 10.1097/PAS.0b013e31829ab1c1.
15. Zlobec I, Bihl M, Foerster A, Rufle A, Lugli A. Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low, and -negative colorectal cancers based on protein marker expression and molecular features. *J pathol* **2011**;225(3):336-43 doi 10.1002/path.2879.
16. Popovici V, Budinska E, Tejpar S, Weinrich S, Estrella H, Hodgson G*, et al.* Identification of a poor-prognosis BRAF-mutant-like population of patients with colon cancer. *J clin oncol* **2012**;30(12):1288-95 doi 10.1200/jco.2011.39.5814.
17. Tie J, Gibbs P, Lipton L, Christie M, Jorissen RN, Burgess AW*, et al.* Optimizing targeted therapeutic development: analysis of a colorectal cancer patient population with the BRAF(V600E) mutation. *Int j cancer* **2011**;128(9):2075-84 doi 10.1002/ijc.25555.
18. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH*, et al.* Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer* **2011**;117(20):4623-32 doi 10.1002/cncr.26086.
19. Goldstein J, Tran B, Ensor J, Gibbs P, Wong HL, Wong SF*, et al.* Multicenter retrospective analysis of metastatic colorectal cancer (CRC) with high-level microsatellite instability (MSI-H). *Ann Oncol* **2014**;25(5):1032-8 doi 10.1093/annonc/mdu100.
20. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C*, et al.* The consensus molecular subtypes of colorectal cancer. *Nat Med* **2015**;21(11):1350-6 doi 10.1038/nm.3967.
21. Remo A, Fassan M, Lanza G. Immunohistochemical evaluation of mismatch repair proteins in colorectal carcinoma: the AIFEG/GIPAD proposal. *Pathologica* **2016**;108(3):104-9.
22. Ten Hoorn S, Trinh A, de Jong J, Koens L, Vermeulen L. Classification of Colorectal Cancer in Molecular Subtypes by Immunohistochemistry. *Methods Mol Biol* **2018**;1765:179-91 doi 10.1007/978-1-4939-7765-9_11.
23. Williams DS, Mouradov D, Jorissen RN, Newman MR, Amini E, Nickless DK*, et al.* Lymphocytic response to tumour and deficient DNA mismatch repair identify subtypes of stage II/III colorectal cancer associated with patient outcomes. *Gut* **2018** doi 10.1136/gutjnl-2017-315664.
24. Landau MS, Kuan SF, Chiosea S, Pai RK. BRAF-mutated microsatellite stable colorectal carcinoma: an aggressive adenocarcinoma with reduced CDX2 and increased cytokeratin 7 immunohistochemical expression. *Hum Pathol* **2014**;45(8):1704-12 doi 10.1016/j.humpath.2014.04.008.

25. Bruun J, Sveen A, Barros R, Eide PW, Eilertsen I, Kolberg M*, et al.* Prognostic, predictive, and pharmacogenomic assessments of CDX2 refine stratification of colorectal cancer. *Mol Oncol* **2018** doi 10.1002/1878-0261.12347.
26. Harbaum L, Pollheimer MJ, Kornprat P, Lindtner RA, Schlemmer A, Rehak P*, et al.* Keratin 7 expression in colorectal cancer--freak of nature or significant finding? *Histopathology* **2011**;59(2):225-34 doi 10.1111/j.1365-2559.2011.03694.x.
27. Trinh A, Trumpi K, De Sousa EMF, Wang X, de Jong JH, Fessler E*, et al.* Practical and Robust Identification of Molecular Subtypes in Colorectal Cancer by Immunohistochemistry. *Clin cancer res* **2017**;23(2):387-98 doi 10.1158/1078-0432.ccr-16-0680.
28. Shibutani M, Maeda K, Nagahara H, Fukuoka T, Iseki Y, Matsutani S*, et al.* Tumor-infiltrating Lymphocytes Predict the Chemotherapeutic Outcomes in Patients with Stage IV Colorectal Cancer. *In vivo* **2018**;32(1):151-8 doi 10.21873/invivo.11218.

Tables

Table 1.*Baseline* characteristics and major clinical parameters*

**i.e. at the time of first-line treatment start or, for candidates to BSC only, at the first visit for metastatic disease*

Characteristic		TOT= 155 N (%)
Sex	Female	77 (49.7%)
	Male	78 (50.3%)
Age	Median (range)	66 (28-85)
Age	>70	55 (35.5%)
	≤70	100 (64.5%)
Baseline ECOG PS	0	112 (72.2%)
	1	37 (23.9%)
	≥2	6 (3.9%)
Primary tumor resected	Yes	135 (87.1%)
	No	20 (12.9%)
Primary tumor location	Right	115 (74.2%)
	Left	31 (20.0%)
	Rectal	9 (6.8%)
Presentation of metastases	Synchronous	102 (65.8%)
	Metachronous	53 (34.2%)
Number of metastatic sites	Single	97 (63%)
	Multiple	57 (37%)
	Missing	*1*
Sites of metastases at diagnosis	Liver	82 (53%)
	Lung	27 (17.4%)
	Distant Nodes	59 (38%)
	Other	28 (18.1%)
	Missing	*1*

Table 2. Distribution of molecular variables analyzed in whole population

Characteristic		TOT= 155 N (%)
CDX2	Low	47 (32.6%)
	Intermediate	50 (34.8%)
	High	47 (32.6%)
	NE[#]	*11*
CK7	Low	81 (87.1%)
	High	12 (12.9%)
	NE	*9*
	Not tested	*53*
CK20	Low	11 (11.8%)
	High	82 (88.2%)
	NE[#]	*9*
	Not tested	*53*
CMS	1	44 (39.7%)
	2-3	47 (42.3%)
	4	20 (18.0%)
	NE[#]	*44*
TILs	Low	61 (39.6%)
	High	93 (60.4%)
	NE[#]	*1*
Barras et al subtypes	BM1	51 (49%)
	BM2	53 (51%)
	NE[#]	*51*
*Clinical prognostic score**	Low	69 (44.8%)
	Intermediate	59 (38.3%)
	High	26 (16.9%)
	NE[#]	*1*

[#] Not Evaluable
* Simplified version

Table 3. Univariate analysis for Overall Survival

Characteristics		Median OS (months)	Overall Survival		
			HR	95% CI	p
CDX2	High	22.3	1	-	-
	Intermediate	16.0	1.72	1.03–2.86	**0.036**
	Low	12.7			
CK7	Low	22.3	1	-	-
	High	7.2	2.17	1.10 – 4.29	**0.026**
CK20	Pos	22.0	1	-	
	Neg	9.7	1.75	0.83 – 3.69	0.14
CMS	1	26.3	1	-	-
	2-3	19.2	2.70	1.41 – 5.26	**0.003**
	4	12.7			
TILs	High	22.0	1	-	-
	Low	13.9	1.72	1.16– 2.56	**0.007**
BM	2	22.0	1	-	-
	1	15.6	1.37	0.87 – 2.17	0.177
Simplified score	Low	23.3	1	-	-
	Intermediate	19.5			
	High	6.6	2.61	1.53 – 4.48	**<0.001**

Table 4. Multivariate analysis for Overall Survival

Characteristics		Overall Survival		
		HR	**95% CI**	**p**
CDX2	High	1	-	-
	Low + Intermediate	1.92	0.94– 4.00	0.070
CK7	Low	1	-	-
	High	2.11	1.03 – 4-34	**0.041**
CMS	CMS1	1	-	-
	CMS2-3 +CMS4	2.22	1.03 – 0.50	**0.049**
TILs	High	1	-	-
	Low	1.19	0.66–2.17	0.55
Simplified score	Intermediate +Low	1	-	-
	High	2.42	1.16 – 5.05	**0.019**

Supplementary Table 1. Correlation between paired single parameters in terms of percentage of positive cases out of total evaluable for both analyses.

	CK7+	CK20+	CMS1	CMS2-3	CMS4	TILs+	BM1	BM2
CDX-2 intermediate	2.1%	27.9%	10.1%	14.7%	7.3%	18.9%	17.3%	13.5%
CDX-2 high	5.4%	34.4%	11.0%	21.1%	0.9%	21.0%	17.3%	17.3%
CK7+		10.7%	4.3%	5.4%	3.2%	6.4%	6.5%	5.4%
CK20+			32.2%	39.8%	16.1%	58.1%	36.9%	51.1%
CMS1						37.8%	9.7%	25.2%
CMS2-3						21.6%	27.2%	18.4%
CMS4						6.3%	11.6%	7.7%
TILs+							26.0%	37.5%

Supplementary Table 2. Concordancy for primary/mets paired

		TOT= 46 paired N (%)	***Prim→Mets N (%)***	***N (%)***
CDX2	Concordant	41 (89.1%)		
	Discordant	5 (10.9%)	Low→High	1 (20%)
			Int→Low	3 (60%)
			High→Low	1 (20%)
CK7	Concordant	46 (100%)		
	Discordant	0		
CK20	Concordant	46 (100%)		
	Discordant	0		
CMS	Concordant	35 (76.1%)		
	Discordant	11 (23.9%)	CMS2-3→CMS4	5 (45.5%)
			CMS4→CMS2-3	6 (54.5%)
TILs	Concordant	45 (97.8%)		
	Discordant	1 (2.2%)	Low→High	1 (100%)
BM	Concordant	20 (50%)		
	Discordant/NAs	20 (50%)	BM1 border	8 (40%)
			BM2 border	12 (60%)
	*NE**	*6*		

** Not Evaluable*

Supplementary Table 3. Univariate analysis for Progression-Free Survival

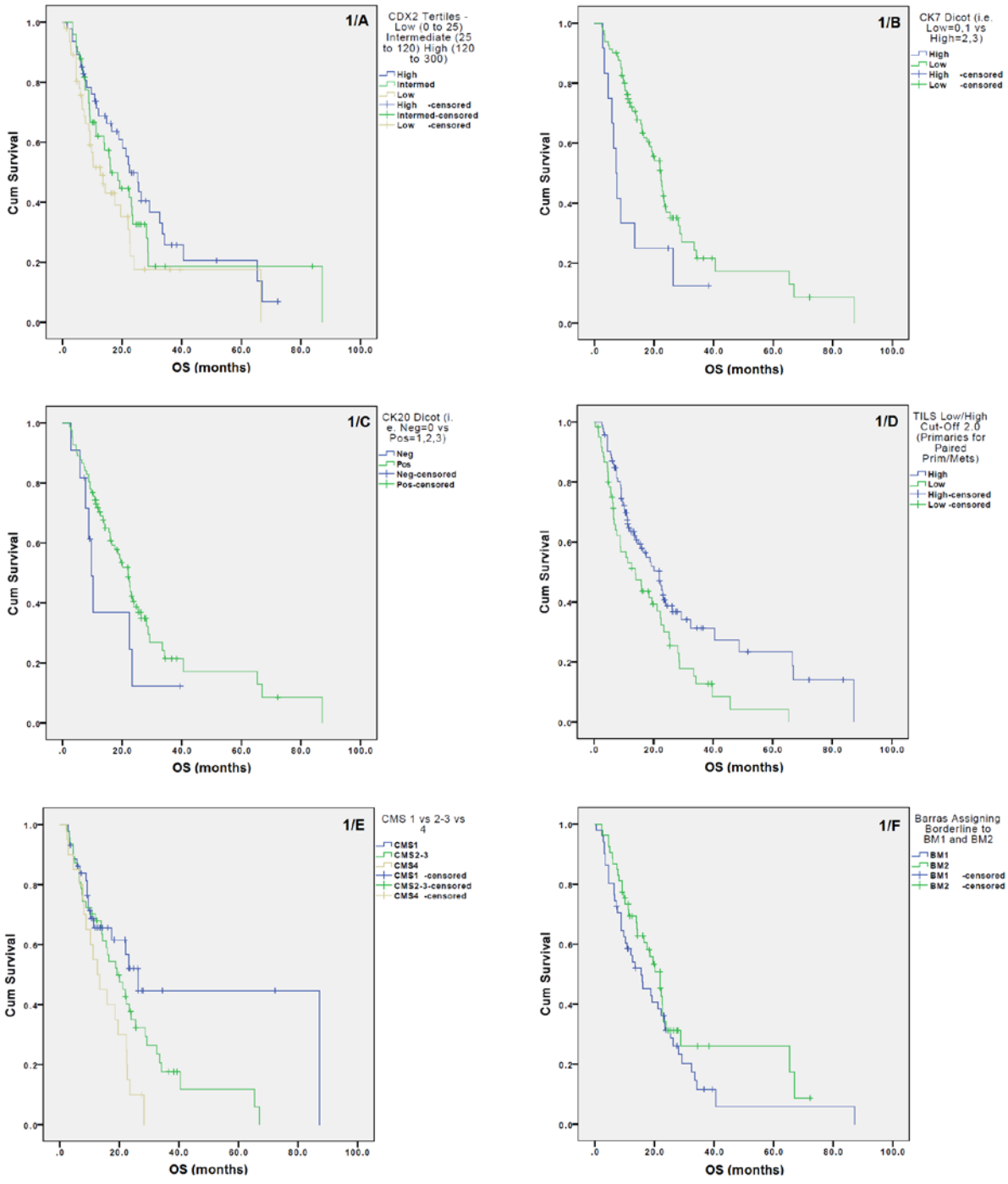
Characteristics		Median PFS (months)	Progression Free Survival		
			HR	95% CI	p
CDX2	High	9.6	1	-	-
	Intermediate	5.8	1.41	0.86 – 2.30	0.169
	Low	5.6			
CK7	Low	8.5	1	-	-
	High	4.3	1.13	0.56 – 2.29	0.740
CK20	Pos	8.0	1	-	
	Neg	3.5	1.72	0.73 – 4.05	0.213
CMS	1	11.0	1	-	-
	2-3	7.0	2.22	1.14 – 4.35	**0.020**
	4	8.0			
TILs	High	8.9	1	-	-
	Low	5.8	1.72	1.18– 2.56	**0.005**
BM	2	9.1	1	-	-
	1	5.5	1.27	0.80 – 2.03	0.309
Simplified score	Low	9.0	1	-	-
	Intermediate	8.0			
	High	3.4	2.13	1.21 – 3.75	**0.009**

Supplementary Table 4. Multivariate analysis for Progression Free Survival

Characteristics		Progression Free Survival		
		HR	**95% CI**	**p**
CMS	CMS1	1	-	-
	CMS2-3 +CMS4	2.17	1.01–4.76	**0.049**
TILs	High	1	-	-
	Low	1.16	0.67– 1.96	0.593
Simplified score	Intermediate +Low	1	-	-
	High	2.52	1.25–5.11	**0.010**

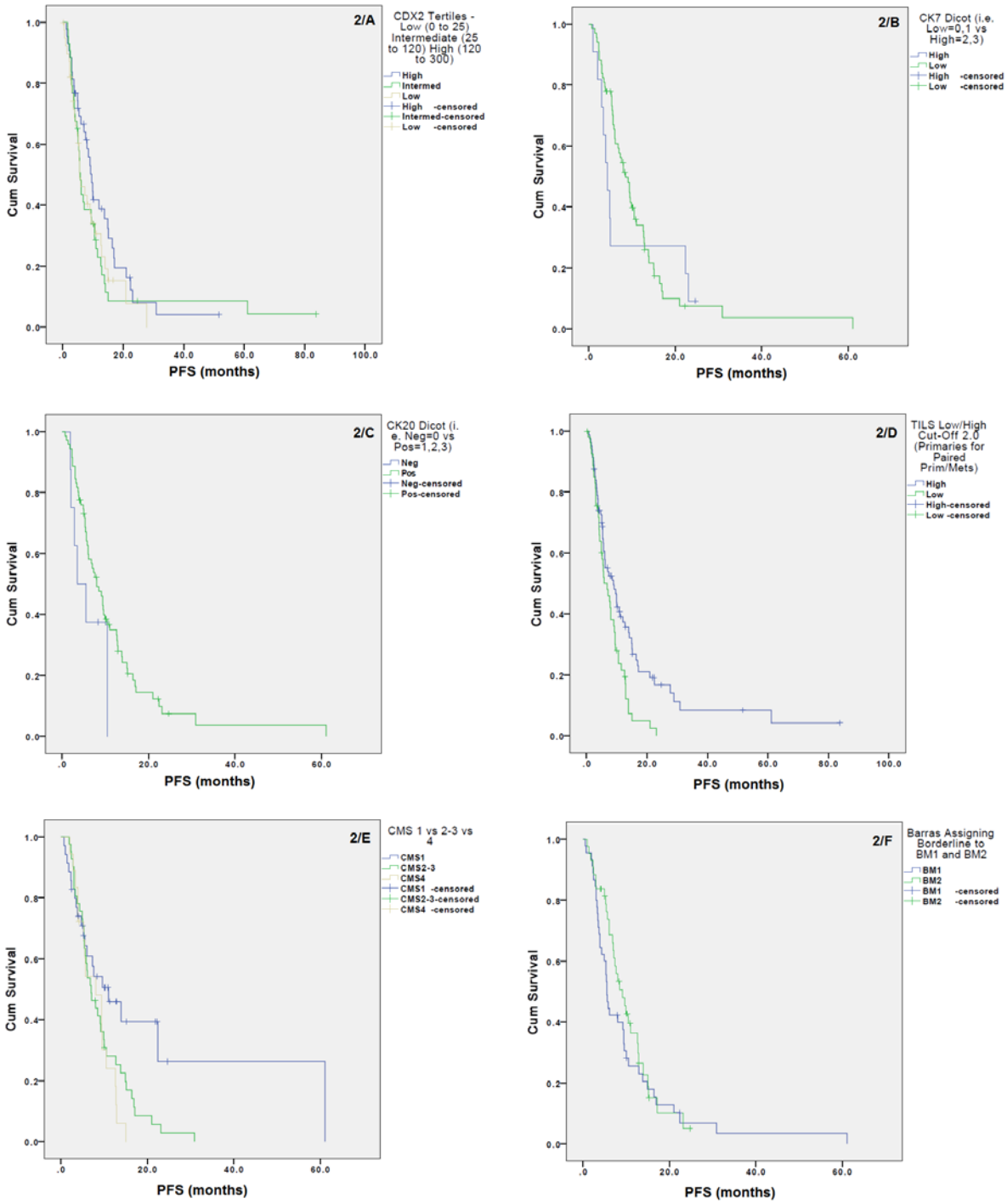
FIGURES LEGEND

Figure 1 –Kaplan-Meier curves for Overall survival (OS).



1/A - CDX2 tertiles expression (low vs intermediate and high)
1/B - CK7 expression (high vs low)
1/C - CK20 expression (negative vs positive)
1/D - TILs expression (low vs high)
1/E - CMS classification (CMS2/3 and CMS4 vs CMS1)
1/F - Barras classification (BM1 vs BM2)

Supplementary Figure 1 – Kaplan-Meier curves for Progression-free survival (PFS).



2/A - CDX2 tertiles expression (low vs intermediate and high)
2/B - CK7 expression (high vs low)
2/C - CK20 expression (negative vs positive)
2/D - TILs expression (low vs high)
2/E - CMS classification (CMS2/3 and CMS4 vs CMS1)
2/F - Barras classification (BM1 vs BM2)