Title:

An Integrated Clinical-Molecular Classification of Colorectal Liver Metastases: A Biomarker Analysis of the Randomized Phase III New EPOC Trial

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Key Points

Question: Are biologically-derived molecular subtypes and integrated clinical-molecular risk stratification of colorectal liver metastases prognostic in an independent cohort from the randomized, controlled phase III New EPOC trial?

Findings: The biological phenotype of each molecular subtype in the validation cohort was concordant with the discovery cohort. The immune subtype (best prognosis) demonstrated an improved 5-year PFS and OS, compared to the canonical subtype (worst prognosis). The low-risk integrated group demonstrated 5-year PFS of 44% and OS of 78%, superior to the high-risk group at 16% and 43%, respectively.

Meaning: Molecular subtypes of oligometastatic colorectal liver metastases and integrated risk stratification are prognostic and warrant further study as a possible predictive biomarker to personalize therapies.

Abstract

Importance: Personalized treatment approaches for patients with oligometastatic colorectal liver metastases are critically needed. We previously defined three biologically distinct molecular subtypes of colorectal liver metastases: (1) canonical, (2) immune, and (3) stromal.

Objective: We independently validate these molecular subtypes in the randomized, controlled phase III New EPOC trial

Design: Secondary analysis of a randomized, phase III trial

Setting: Retrospective bi-institutional discovery cohort and multi-institutional validation cohort from New EPOC, a randomized phase III trial

Participants and Interventions: Discovery cohort comprised 93 patients who underwent hepatic resection for limited colorectal liver metastases (98% received peri-operative chemotherapy) between 1994 and 2012. Resected metastases underwent RNA sequencing and miRNA profiling. The validation cohort comprised 147 patients who underwent hepatic resection for liver metastases with peri-operative chemotherapy (fluorouracil, oxaliplatin, and irinotecan-based) with or without cetuximab between 2007 and 2012. Resected metastases underwent mRNA and miRNA profiling with microarray.

Main Outcomes and Measures: A 31-feature (24 mRNAs and 7 miRNAs) neural network classifier was trained to predict molecular subtypes in the discovery cohort and applied to the validation cohort. Integrated clinical-molecular risk groups were designated based on molecular subtypes and the Clinical Risk Score. The unique biological phenotype of each molecular subtype was validated using gene set enrichment analyses and immune deconvolution. The primary clinical endpoints were progression-free survival and overall survival.

Results: In the validation cohort, 73 (50%), 28 (19%), and 46 (31%) patients were classified as having canonical, immune, and stromal metastases, respectively. The biological phenotype of each subtype was concordant with the discovery cohort. The immune subtype (best prognosis) demonstrated 5-year PFS of 43% (95% CI, 25%-60%; Cox HR 0.37, 95% CI, 0.20-0.68) and OS of 63% (95% CI, 40%-79%; HR 0.38, 95% CI, 0.17-0.86), significantly higher than the canonical subtype (worst prognosis) at 14% (95% CI, 7%-23%) and 43% (95% CI, 32%-55%), respectively. Adding molecular subtypes to the Clinical Risk Score improved prediction (Gönen and Heller's *K* for discrimination) from 0.55 (95% CI, 0.49-0.61) to 0.62 (95% CI, 0.57-0.67) for PFS and 0.59 (95% CI, 0.52-0.66) to 0.63 (95% CI, 0.56-0.70) for OS. The low-risk integrated group demonstrated 5-year PFS of 44% (95% CI, 20%-66%; HR 0.38, 95% CI, 0.19-0.76) and OS of 78% (95% CI, 44%-93%; HR 0.26, 95% CI, 0.08-0.84), superior to the high-risk group at 16% (95% CI, 10%-24%) and 43% (95% CI, 32%-52%), respectively.

Conclusions and Relevance: Biologically-derived colorectal liver metastasis molecular subtypes and integrated clinical-molecular risk groups were highly prognostic in the phase III New EPOC trial. This novel molecular classification warrants further study as a predictive biomarker to potentially personalize systemic treatment approaches for colorectal liver metastases.

Trial Registration: ISRCTN22944367

Introduction

Approximately 25% of patients with colorectal cancer eventually develop liver metastases, which is typically associated with poor survival.[1] However, patients undergoing surgical resection of limited liver metastases (i.e. oligometastases) demonstrate 5-year disease-free survival of 20-25% and overall survival (OS) of 30-40%.[2–5] Oligometastatic colorectal cancer exhibits a wide spectrum of clinical behavior, and multiple randomized trials of adjuvant chemotherapy have failed to improve OS.[6–9] Prognostic biomarkers are critically needed to improve risk stratification and facilitate personalized selection of peri-operative systemic therapies.

In this context, various prognostic models encompassing clinico-pathologic features have been developed.[4,5,10,11] A limitation of clinical risk stratification is a failure to account for the underlying biological features that impact metastatic virulence and ultimately survival after surgical resection. We previously defined three robust, biologically distinct molecular subtypes of colorectal cancer liver metastasis based on messenger RNA (mRNA) and microRNA (miRNA) expression patterns.[12] Metastases were classified as: (1) canonical (associated with altered cell cycle signaling, increased cellular proliferation, and an immune-depleted microenvironment), (2) immune (exhibiting robust innate and adaptive immune infiltration), and (3) stromal (demonstrating increased epithelial-mesenchymal transition [EMT], angiogenesis, KRAS signaling, and inflammatory immune infiltration).

Although these subtypes were derived from their biological properties alone and not their association with clinical outcomes, molecular subtypes were independently prognostic, even when accounting for clinical risk stratification. Furthermore, integrated clinical-molecular risk groups predicted distinct clinical outcomes, with low-, intermediate-, and high-risk patients

exhibiting 10-year OS of 94%, 45%, and 19%, respectively. Importantly, low-risk patients exhibited an oligometastatic pattern of failure and pace of progression with all instances of tumor recurrence being confined to only 1-3 additional liver metastases, in stark contrast to intermediate- and high-risk groups where recurrences were commonly more widespread and involving multiple organ sites. Validation of these molecular subtypes as prognostic biomarkers was required before advocating for their widespread use.

Here, we present the first clinical validation of a novel 31-gene classifier that accurately predicts the colorectal liver metastasis molecular subtypes as a secondary analysis of the large multicenter, randomized, controlled phase III New EPOC trial.[13,14] Importantly, we confirm our hypothesis that integrated clinical-molecular risk groups are highly prognostic for survival and confirm that a low-risk integrated subgroup achieves excellent OS after surgical resection.

Methods

Study Design and Participants

Study results were reported following REMARK guidelines.[15] We trained a neural network molecular classifier in a retrospective discovery cohort consisting of 93 patients (**Figure 1**) treated at The University of Chicago Medical Center (Chicago, IL) and NorthShore University Hospital (Evanston, IL) between 05/31/94 and 08/14/12. Patients with colorectal adenocarcinoma underwent hepatic resection for limited liver metastases that presented either synchronously or metachronously (typically 1-5 lesions involving one or both lobes). 98% of patients received peri-operative fluorouracil and platinum-based chemotherapy. Data collection was approved by Institutional Review Boards at each respective cancer center.

An independent validation cohort consisted of 147 patients (**Figure 1**) enrolled in the multicenter, randomized, controlled phase III New EPOC trial (registration ID: ISRCTN22944367) that underwent molecular profiling of colorectal liver metastases (study protocol previously published).[13,14] The study was approved by the South West Research Ethics Committee. Patients with operable colorectal cancer liver metastases (including those deemed suboptimally resectable or at high risk of positive resection margins) underwent hepatic resection with peri-operative chemotherapy (fluorouracil, oxaliplatin, irinotecan-based) with or without cetuximab between 02/26/07 and 11/01/12 (median follow-up was 53.4 months). Patients were excluded if they were ineligible for chemotherapy or had extrahepatic distant metastases. Thus, both cohorts were similar, representing patients undergoing surgery with peri-operative systemic therapy for limited colorectal cancer liver metastases.

Specimen Processing and Development of Molecular Subtype Classifier

Specimen processing, training, and application of the neural network classifier for colorectal liver metastasis molecular subtypes are outlined in detail in the **Supplement 2**. For the discovery cohort, formalin-fixed paraffin-embedded (FFPE) specimens from hepatic resections underwent whole transcriptome RNA sequencing and miRNA profiling.[12] For the validation cohort, archival liver metastasis and primary tumor FFPE blocks at the time of resection from the New EPOC trial underwent mRNA and miRNA profiling with microarray.[13,14]

In the discovery cohort, a machine learning neural network classifier was trained to classify colorectal liver metastases into one of three molecular subtypes (canonical, immune, and stromal) using mRNA and miRNA expression data (**eFigure 1; Supplement 2).** In this cohort, we previously defined molecular subtypes using the similarity network fusion (SNF) clustering algorithm, and these served as the reference standard for training the neural network classifier.[12]

Importantly, although molecular subtypes were ultimately associated with survival in our discovery set, the original SNF algorithm clustered tumors based only on molecular features and not survival outcomes. The final classifier contained 31 features (24 mRNAs and 7 miRNAs). For each patient in the validation cohort, the neural network classifier was applied to predict the molecular subtype of the corresponding liver metastasis.

Molecular subtypes of the liver metastases were utilized for the primary statistical analyses. To investigate if the signature's prognostic performance was specific to application to liver metastases only, the subtypes were also predicted for matched primary tumors. Consensus molecular subtypes (CMSs) of both metastases and primary tumors were also determined to compare their prognostic performance with our study's liver metastasis subtypes.[16]

Outcomes

Unlike the discovery cohort, no gold standard reference existed against which to compare the computed subtypes in the validation cohort. To confirm that the neural network classifier accurately captured the expected biological phenotype of the computed molecular subtypes within the validation cohort, single sample gene-set enrichment analysis (ssGSEA) and immune deconvolution were performed utilizing gene expression data for each liver metastasis (detailed methodology in **Supplement 2**).[17,18]

All patients were annotated with baseline demographic, clinical, and pathologic information from which the Clinical Risk Score (CRS) was computed (**Supplement 2)**.[4] As previously defined, an integrated clinical-molecular risk group was designated for each patient, combining the computed molecular subtype with high (≥2) or low (<2) CRS.[12] Low-risk patients were defined as exhibiting an immune or canonical subtype with low CRS. Intermediate-risk patients

were defined as demonstrating an immune subtype with high CRS or stromal subtype with low CRS. High-risk patients were defined as having a canonical or stromal subtype with high CRS.

The primary clinical endpoints of this study were PFS and OS in the validation cohort. PFS was defined as time to recurrence, progression, or death (whichever occurred first), and OS was defined as time to death. Time-to-event outcomes were measured from date of surgery in the discovery cohort and date of randomization on trial in the validation cohort.

Statistical Analysis

We hypothesized that the immune subtype would exhibit the best PFS and OS (compared to the canonical and stromal subtypes) and that integrated clinical-molecular risk stratification would be strongly associated with both PFS and OS in the validation cohort, based on previously published analysis of the discovery cohort.[12] Patients were excluded if they did not undergo surgery or did not undergo molecular profiling of their liver metastases. Patients were excluded in the integrated risk group analysis if CRS could not be computed due to unavailable data (N=3). PFS and OS were analyzed using the Kaplan-Meier method and log-rank tests. Multivariable Cox proportional hazards models for PFS and OS were generated in the validation cohort. In multivariable models of the molecular subtypes, CRS and randomization to cetuximab were included as covariates. In multivariable models of the integrated risk groups, randomization to cetuximab was included as a covariate. In sensitivity analyses, the multivariable models were extended to also include age, tumor differentiation, resection margin status, WHO performance status, *KRAS* and *BRAF* mutation status, and primary tumor location. CRS was analyzed as a categorical variable when included as a covariate. Model discrimination was evaluated by Gönen and Heller's *K* concordance statistic. Statistical analyses were performed using StataIC 16.1.

Statistical analysis for ssGSEA enrichment scores and immune deconvolution features consisted of t-tests for pairwise comparison between subtypes. To correct for multiple comparisons, *P* values were adjusted by controlling the false discovery rate (FDR < 0.05).

Results

Cohort Characteristics

Patient characteristics are summarized in **Table 1**. Overall, both the discovery (N=93) and validation (N=147) cohorts were representative of patients who underwent hepatic resection for limited colorectal liver metastases in the setting of peri-operative chemotherapy. The prevalence of *KRAS* and *BRAF* alterations and microsatellite instability are reported in **Supplement 2**.

Classification of Molecular Subtypes

Training of the neural network classifier for liver metastasis molecular subtypes is detailed in **Supplement 2**. A 31-feature signature consisting of 24 mRNAs and 7 miRNAs resulted in optimal model performance with an average accuracy of 96% across cross-validation testing sets (**eFigure 2, eTable 1, eFigure 3; Supplement 2** and neural network coefficient matrices in **Supplement 3**). Molecular subtypes were predicted, and integrated clinical-molecular risk groups were determined in the validation cohort (**eFigure 4; Supplement 2**). Across molecular subtypes, there were no differences in several clinico-pathologic features, including the risk factors comprising the CRS, tumor and nodal staging, tumor differentiation, age, or sex (**eFigure 5; Supplement 2**). PFS and OS were highly concordant between the discovery and validation cohorts (**eFigure 6; Supplement 2**) by total cohort, molecular subtype, or integrated clinical-molecular risk group.

Biological Phenotypes in the Validation Cohort

To corroborate the phenotype of each molecular subtype in the validation cohort, we performed an ssGSEA analysis (**Figure 2A** and **eFigure 7A; Supplement 2**). Consistent with previous findings, the canonical subtype exhibited increased enrichment scores corresponding to DNA repair pathways, cell cycle regulation/proliferation (including E2F, G2M, mitotic spindle pathways), and MYC signaling. The stromal subtype demonstrated enrichment for EMT, angiogenesis, inflammatory response, and KRAS signaling. In addition, the immune subtype exhibited lower enrichment scores for KRAS signaling, angiogenesis, cell proliferation, and TGFβ signaling pathways.

Immune deconvolution analysis was performed in the validation cohort to evaluate the abundance of specific immune cells by molecular subtype (**Figure 2B** and **eFigure 7B; Supplement 2**). The majority of immune cells were decreased in the canonical subtype, whereas the immune subtype demonstrated enrichment for B cells, NK cells, CD8 T cells, and cytotoxic lymphocytes. By contrast, the stromal subtype exhibited depletion of B lymphocytes and NK cells and enrichment for fibroblast, monocytes, and myeloid dendritic cells in the context of CD8 T cells and cytotoxic lymphocytes. Though the presence of CD8 T and cytotoxic lymphocytes were similar between the immune and stromal subtypes, histological evaluation of the discovery cohort previously demonstrated that the spatial distribution of T cells in the tumor microenvironment was distinct.[12] Immune metastases displayed dense band-like peritumoral and intratumoral infiltration of CD8 T lymphocytes, whereas stromal metastases exhibited significant fibrosis resulting in peritumorally restricted T lymphocytic infiltrate, which is consistent with increased fibroblasts in the stromal subtype. Collectively, these findings corroborated the distinct underlying biological phenotypes associated with each subtype.

Clinical Outcomes in the Validation Cohort

PFS and OS were analyzed in the validation cohort by molecular subtype of the liver metastasis and integrated clinical-molecular risk group to validate both as prognostic biomarkers. The immune subtype demonstrated superior PFS and OS to canonical and stromal subtypes, consistent with our previous findings (**Figure 3A**). The 5-year PFS was 43% (95% CI, 25%-60%), 14% (95% CI, 7.0%-23%), and 26% (95% CI, 14%-39%) for immune, canonical, and stromal subtypes, respectively. Differences in PFS were statistically significant across subtypes (log-rank *P*=0.004). Similarly, the 5-year OS was 63% (95% CI, 40%-79%), 43% (95% CI, 32%-55%), and 49% (95% CI, 34%-63%) for immune, canonical, and stromal subtypes, respectively (log-rank *P*=0.083, **Figure 3B**). By pairwise comparison, this resulted in a statistically significant difference in OS between immune versus canonical/stromal subtypes (log-rank *P*=0.045).

When applied to primary tumor expression data (N=124), there was no association between predicted molecular subtypes in primary tumors and PFS or OS (**eFigure 8; Supplement 2**). Similarly, neither the CMS subtype of the primary tumor nor CMS subtype of the matched liver metastasis were associated with PFS and OS (**eTable 2, eFigure 9; Supplement 2**). Thus, liver metastasis molecular subtypes were only prognostic when applied to the metastatic tumor.

By integrated clinical-molecular risk group, 5-year PFS was 44% (95% CI, 20%-66%), 40% (95% CI, 21%-58%), and 16% (95% CI, 10%-24%) for the low-, intermediate-, and high-risk groups, respectively (log-rank *P*=0.0023, **Figure 3C**). The superior PFS of patients in the low-risk group also translated to improved OS. 5-year OS was 78% (95% CI, 44%-93%), 56% (95% CI, 34%-74%), and 43% (95% CI, 32%-52%) for the low-, intermediate-, and high-risk groups, respectively (**Figure 3D**).

Multivariable Cox models were computed in the validation cohort (**Table 2**), which also includes randomization to cetuximab since cetuximab was associated with decreased survival in the New EPOC trial. The addition of molecular subtypes to the Clinical Risk Score provided further prognostic value for PFS, increasing Gönen and Heller's *K* concordance statistic from 0.55 (95% CI, 0.49-0.61) to 0.62 (95% CI, 0.57-0.67). For PFS, the immune subtype demonstrated a HR of 0.37 (95% CI, 0.20-0.68; P=0.0014) and the stromal subtype demonstrated a HR of 0.56 (95% CI, 0.36-0.89; P=0.014) compared to canonical when controlling for the CRS. For OS, the addition of molecular subtypes to the Clinical Risk Score similarly improved model performance, increasing Gönen and Heller's *K* from 0.59 (95% CI, 0.52-0.66) to 0.63 (95% CI, 0.56-0.70). The immune subtype exhibited a HR of 0.38 (95% CI, 0.17-0.86; P=0.020). Thus, the immune subtype demonstrated greater PFS and OS.

Furthermore, the integrated clinical-molecular risk score remained strongly associated with both PFS and OS. Relative to high-risk, the HR for the low-risk integrated group was 0.38 (95% CI, 0.19-0.76, P=0.0062) for PFS and 0.26 (95% CI, 0.08-0.84; P=0.024) for OS. Randomization to cetuximab did not notably impact the prognostic effect size of the molecular subtypes or integrated risk groups. There were no significant interaction effects between molecular subtype and CRS, molecular subtype and cetuximab, and integrated risk group and cetuximab ($P > 0.05$). Finally, the prognostic effect of the integrated clinical-molecular risk grouping and molecular subtypes persisted in sensitivity analyses that included randomization to cetuximab, age, tumor differentiation, margin status, WHO performance status, *KRAS* and *BRAF* mutation statuses, and primary tumor location in the model (**eTable 3; Supplement 2**). For PFS, the addition of integrated risk grouping to the aforementioned variables increased Gönen and Heller's *K* from 0.61 (95% CI, 0.56-0.66) to 0.65 (95% CI, 0.60-0.70). For OS, the addition of integrated risk

grouping increased Gönen and Heller's *K* from 0.66 (95% CI, 0.61-0.71) to 0.69 (95% CI, 0.63-0.75). In summary, integrated clinical-molecular risk stratification was highly prognostic in this independent validation cohort, defining a low-risk subgroup with an OS of 78% (95% CI, 44%-93%) at 5 years.

Discussion

We developed a novel classification of colorectal cancer liver metastases that was biologically derived and not empirically developed based on association with clinical outcome. We validated its prognostic significance in the multicenter randomized phase III trial, New EPOC. As previously published for the discovery cohort, molecular subtypes independently add to clinical risk stratification for oncologic outcomes after hepatic resection and an integrated clinical-molecular risk grouping remains highly prognostic for survival.

The above findings may improve the management of oligometastatic colorectal liver metastases in several aspects. First, this study presents a novel molecular classification system in a context where prognostic biomarkers are generally lacking and rarely integrated with well-established clinical risk stratification. Although CMS exists for primary tumors, their prognostic utility is limited in liver metastases, where one third of tumors are unclassifiable.[12] Thus, it is crucial to molecularly stage the metastasis separately from the primary tumor. While other prognostic features include histopathological growth patterns and the Immunoscore, risk stratification based on gene expression is sparse[19–22]. Balachandran et al reported a multigene molecular risk score for colorectal liver metastases that was prognostic and validated in a retrospective European cohort.[23] By contrast, the molecular subtypes in this study were not derived empirically based on their association with survival, but instead defined by their underlying biological phenotype. For PFS and OS, the improvement in model discrimination with the addition of molecular subtyping

to the Clinical Risk Score is comparable to other prognostic molecular biomarkers[24–26]. The validation of an integrated clinical-molecular risk stratification of liver metastases potentially defines a novel framework to stage metastatic disease using both clinical and molecular features.

Second, we propose that these molecular subtypes and integrated risk stratification warrant further study as possible predictive biomarkers. While adjuvant chemotherapy is commonly administered after surgery for liver metastases, multiple randomized trials have failed to demonstrate improvements in OS, and predictive biomarkers are needed to optimally personalize systemic therapies after aggressive local therapies.[2,6–9,27,28] Our novel classification identifies patients with the greatest risk of relapse and thus, may help select patients for peri-operative systemic therapy. Moreover, because these molecular subtypes are characterized by distinct biological phenotypes, they uniquely present a potential opportunity to personalize the classes of therapies utilized. In this context, we demonstrated that the CIN70 gene expression signature predicts response to DNA-damaging chemotherapy in colorectal liver oligometastases.[29] Thus, particular classes of adjuvant systemic therapies (including cytotoxic chemotherapy, immunotherapy, anti-angiogenesis agents, or other targeted therapies) may demonstrate differential benefit in specific molecular subtypes or integrated risk groups, justifying further investigation in future biomarker-driven trials or inclusion as stratification factors. Similarly, the molecular subtypes in this study may complement liquid biomarkers. While circulating tumor DNA (ctDNA) helps identify patients with minimal residual disease, colorectal liver metastases exhibit a wide spectrum of metastatic behavior that are partially elucidated by molecular classification.[30]

Limitations of this study include that this classifier was developed in patients undergoing surgery only. Future study would extend investigation to other local therapies, including radiation

therapy and ablation.[31,32] Finally, the magnitude of the concordance probability estimates in this study highlight the significant clinical heterogeneity of colorectal liver oligometastases. Subsequent studies in larger patient cohorts may further clarify the role of molecular subtyping in this context.

In conclusion, colorectal liver metastasis molecular subtypes are associated with differential PFS and OS in an independent cohort from the New EPOC phase III randomized trial. When combined with the CRS, integrated risk stratification is strongly associated with long-term survival after resection for limited colorectal liver metastases. This study illustrates how integrated clinical and molecular risk stratification characterizes the diverse phenotypic spectrum of clinical metastases. It may serve as a framework that is broadly applicable to many human cancers for the development of biomarkers that influence the utilization of local and systemic therapies in metastatic disease.

Declaration of Competing Interests

SAP reports payment or honoraria from Merck. TSM reports from funding support for the S:CORT consortium from the MRC and Cancer Research UK, provision of cetuximab for the New EPOC trial by Merck KGaA, consulting fees from AstraZeneca, participation on a Data Safety Monitoring Board or Advisory Board for Pierre Fabre, appointments as trustee for the Institute for Cancer Research and chair of the strategy group for the National Cancer Research Institute. RRW reports research grants from Varian and Regeneron, payment or honoraria from AstraZeneca, Boehringer Ingelheim, and Merck Serono SA, participation on a Data Safety Monitoring Board or Advisory Board for NKMax America Inc and Highlight Therapeutics SL, and stock or stock options in Boost Therapeutics, Immvira LLC, Reflexion Pharmaceuticals Inc, Coordination Pharmaceuticals Inc, Magi Therapeutics, Oncosenescence, and Aqualung Therapeutics Corporation. SPP reports funding supporting the present study from the Ludwig Cancer Research Foundation. Both SPP and RRW report issued patents for "Methods and Kits for Diagnosis and Triage of Patients with Colorectal Liver Metastases" and provisional patents for "Molecular Subtyping of Colorectal Liver Metastases to Personalize Treatment Approaches". The other authors declare no conflicts of interest.

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Role of Funder / Sponsor Statement

Organizations providing funding support for this study had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Access to Data and Data Analysis

RRK and SPP had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data Sharing Statement

De-identified gene expression data are available through European Genome-Phenome Archive (EGAC00001000904) for the discovery cohort. Additional data, including de-identified individual participant data, for the discovery cohort can be made available upon reasonable request with publication. De-identified individual participant data, including gene expression data and corresponding clinical annotation, for the validation cohort (as part of the New EPOC trial) can be made available by application to the S:CORT consortium.

Contributors

SPP conceived the study concept and design. RRK, CAM, and SPP performed data curation, data analysis, and visualization. CAM and ED were involved in development of methodology, especially pertaining to bioinformatics analyses of gene expression data. RRK and SPP prepared the original manuscript draft and were involved in review and editing. SAP, JAB, JNP, ED, TSM, and the S:CORT consortium were responsible for data collection and data curation of the validation cohort (New EPOC trial participants) and were involved in preparation of the

manuscript, including revisions. MT, MCP, RRW, and SPP were responsible for data collection and data curation of the discovery cohort (UChicago and NorthShore participants) and were involved in the preparation of the manuscript, including revisions.

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Figure Legends:

Figure 1: Discovery and Validation Cohorts

Flowchart demonstrating patients included in analyses, including the retrospective discovery cohort and New EPOC trial validation cohort

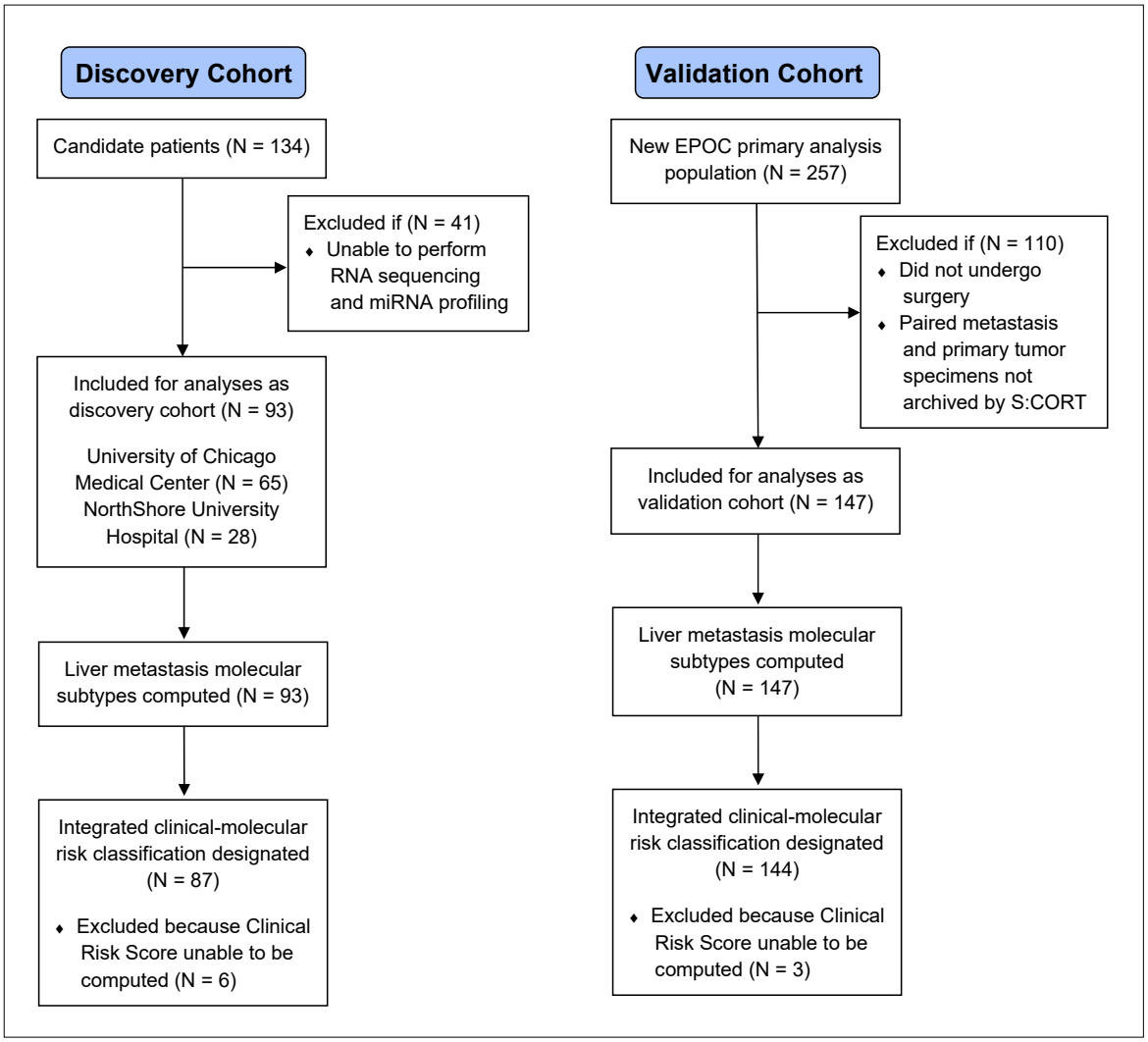

Figure 2: Gene Set Enrichment Analysis and Immune Deconvolution

Heat map representing enrichment and depletion of multiple gene sets and immune compartments. (A) Single-sample gene set enrichment analysis across molecular subtypes in the validation cohort; (B) Immune deconvolution across molecular subtypes in the validation cohort.

Statistical analysis of enrichment scores and immune deconvolution consisted of t-tests for pairwise comparison between subtypes, with *P* values adjusted by controlling the false discovery rate (FDR < 0.05) to account for multiple comparisons.

If a row is annotated with a single subtype label (C: Canonical, I: Immune, or S: Stromal), that molecular subtype was enriched for the corresponding pathway with adjusted $P < 0.05$ (compared to each of the other subtypes). If a row is annotated with two subtype labels, both subtypes were enriched with adjusted $P < 0.05$, compared to the other subtype. Additional detailed view is presented in eFigure 7 in Supplement 2.

Figure 2

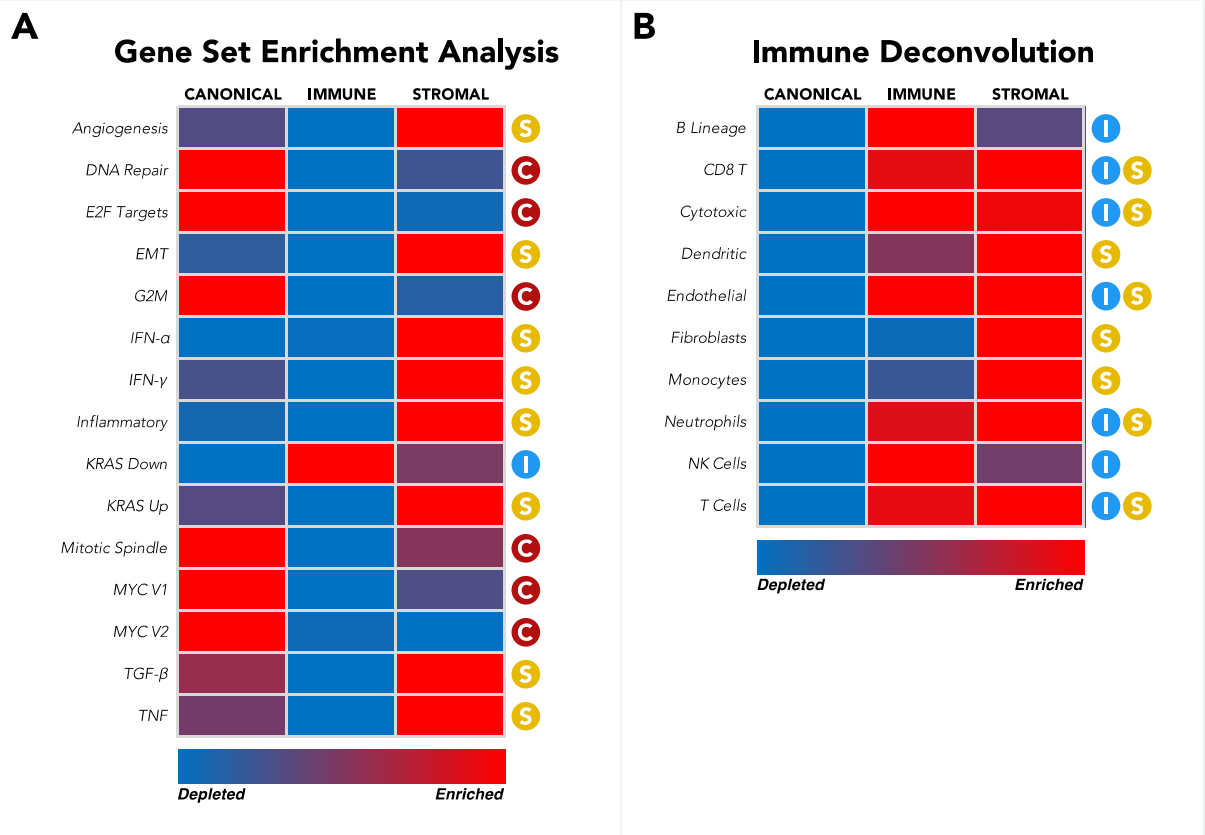

Figure 3: **PFS and OS by Molecular Subtype and Integrated Risk Stratification**

Survival outcomes in validation cohort; X-axis represents time after randomization on the New EPOC trial in months. Of 147 total patients, PFS events occurred in 113, 16, 63, and 34 patients in the overall cohort, immune, canonical, and stromal subtypes, respectively, and OS events occurred in 75, 9, 43, and 23 patients, respectively. By integrated clinical-molecular risk group (N=144), PFS events occurred in 9, 15, and 86 patients in the low-, intermediate-, and high-risk groups, respectively, and OS events occurred in 3, 10, and 61 patients, respectively. (A) PFS by molecular subtype (B) OS by molecular subtype (C) PFS by integrated risk group (D) OS by integrated risk group

Figure 3

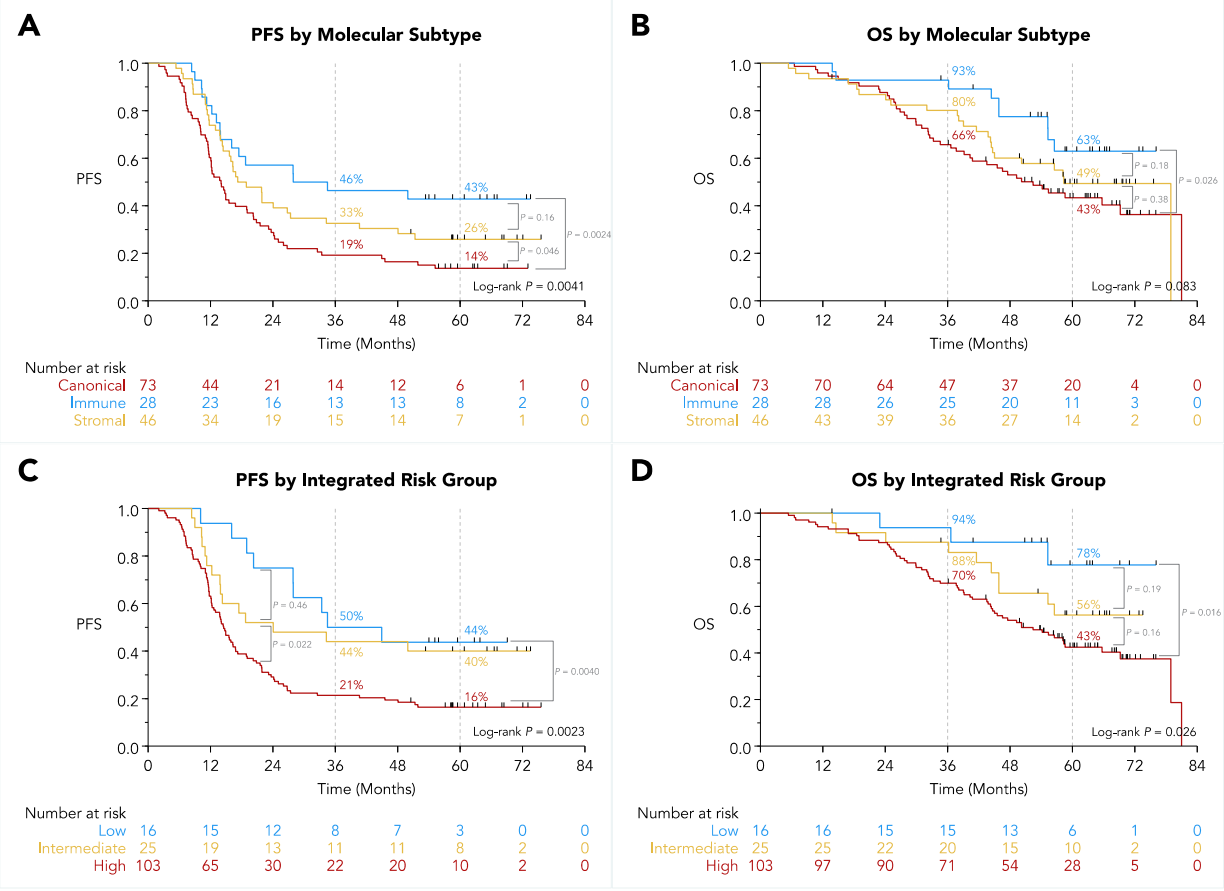

Table 1: Baseline Patient Characteristics

	Total	**Discovery Cohort / Chicago**	**Validation Cohort / UK**
	N=240	N=93	N=147
Age, years, mean (range)	63.0 (56.3-68.0)	60.8 (52.3-65.6)	64.0 (59.0-69.0)
Sex			
Female	89 (37%)	39 (42%)	50 (34%)
Male	151 (63%)	54 (58%)	97 (66%)
Clinical Risk Score			
CRS < 2	53 (23%)	32 (37%)	21 (15%)
CRS ≥ 2	178 (77%)	55 (63%)	123 (85%)
Incomplete	9	6	3
Number of Liver Metastases > 1	151 (64%)	39 (42%)	112 (78%)
Node-Positive Primary	151 (67%)	55 (64%)	96 (69%)
Pre-operative CEA > 200 ng/mL	12 (5.5%)	3 (3.9%)	9 (6.3%)
Disease-Free Interval < 12 months	157 (65%)	51 (55%)	106 (72%)
Metastasis Size > 5cm	61 (25%)	23 (25%)	38 (26%)
Shortest Margin Between Cancer and Cut Surface			
Margin ≥ 1cm	76 (34%)	24 (28%)	52 (38%)
Margin < 1cm	120 (54%)	48 (56%)	72 (53%)
No Margin (Cancer Visible on Cut Surface)	27 (12%)	14 (16%)	13 (9%)
Not Available*	17	7	10

*Margin distance between cancer and cut surface was designated “Not Available” if considered not evaluable (e.g. due to ablation being performed as part of hepatic resection)

588 **Table 2: Multivariable Cox Models for PFS and OS in Validation Cohort**

589 Cox proportional hazards model for PFS and OS in validation cohort.

Primary Cox Models			***Cox Models Including Cetuximab Randomization***		
PFS by Molecular Subtype			**PFS by Molecular Subtype**		
Variable	**Hazard Ratio (95% CI)**	***P***	**Variable**	**Hazard Ratio (95% CI)**	***P***
Molecular Subtype			Molecular Subtype		
Canonical	Reference		Canonical	Reference	
Immune	0.37 (0.20 to 0.68)	0.0014	Immune	0.37 (0.20 to 0.68)	0.0013
Stromal	0.56 (0.36 to 0.89)	0.014	Stromal	0.56 (0.35 to 0.88)	0.013
Clinical Risk Score			Clinical Risk Score		
1	Reference		1	Reference	
2	1.7 (0.92 to 3.3)	0.09	2	1.8 (0.92 to 3.4)	0.088
3	2.0 (1.0 to 3.7)	0.037	3	2.0 (1.1 to 3.7)	0.035
4	2.0 (0.87 to 4.6)	0.10	4	2.0 (0.84 to 4.6)	0.12
5	1.2 (0.15 to 9.4)	0.86	5	1.1 (0.15 to 9.2)	0.88
			Cetuximab		
			No	Reference	
			Yes	1.1 (0.71 to 1.6)	0.72
OS by Molecular Subtype			**OS by Molecular Subtype**		
Variable	**Hazard Ratio (95% CI)**	***P***	**Variable**	**Hazard Ratio (95% CI)**	***P***
Molecular Subtype			Molecular Subtype		
Canonical	Reference		Canonical	Reference	
Immune	0.38 (0.17 to 0.86)	0.020	Immune	0.36 (0.16 to 0.81)	0.014
Stromal	0.66 (0.38 to 1.2)	0.14	Stromal	0.58 (0.33 to 1.01)	0.056
Clinical Risk Score			Clinical Risk Score		
1	Reference		1	Reference	
2	1.7 (0.61 to 4.5)	0.32	2	1.8 (0.68 to 5.0)	0.23
3	2.7 (1.0 to 6.9)	0.043	3	3.3 (1.2 to 8.5)	0.016
4	2.7 (0.87 to 8.2)	0.087	4	2.6 (0.83 to 7.9)	0.10
5	2.2 (0.25 to 18.8)	0.49	5	1.8 (0.20 to 15.5)	0.61
			Cetuximab		

			No	Reference	
			Yes	2.0 (1.2 to 3.4)	0.0083
PFS by Integrated Risk			**PFS by Integrated Risk**		
Variable	**Hazard Ratio (95% CI)**	***P***	**Variable**	**Hazard Ratio (95% CI)**	***P***
Integrated Risk			Integrated Risk		
Low	0.38 (0.19 to 0.76)	0.0062	Low	0.38 (0.19 to 0.76)	0.0062
Intermediate	0.52 (0.30 to 0.91)	0.021	Intermediate	0.52 (0.30 to 0.91)	0.021
High	Reference		High	Reference	
			Cetuximab		
			No	Reference	
			Yes	1.01 (0.70 to 1.48)	0.94
OS by Integrated Risk			**OS by Integrated Risk**		
Variable	**Hazard Ratio (95% CI)**	***P***	**Variable**	**Hazard Ratio (95% CI)**	***P***
Integrated Risk			Integrated Risk		
Low	0.26 (0.08 to 0.84)	0.024	Low	0.25 (0.08 to 0.79)	0.019
Intermediate	0.62 (0.32 to 1.21)	0.16	Intermediate	0.64 (0.33 to 1.26)	0.20
High	Reference		High	Reference	
			Cetuximab		
			No	Reference	
			Yes	1.57 (0.99 to 2.51)	0.057

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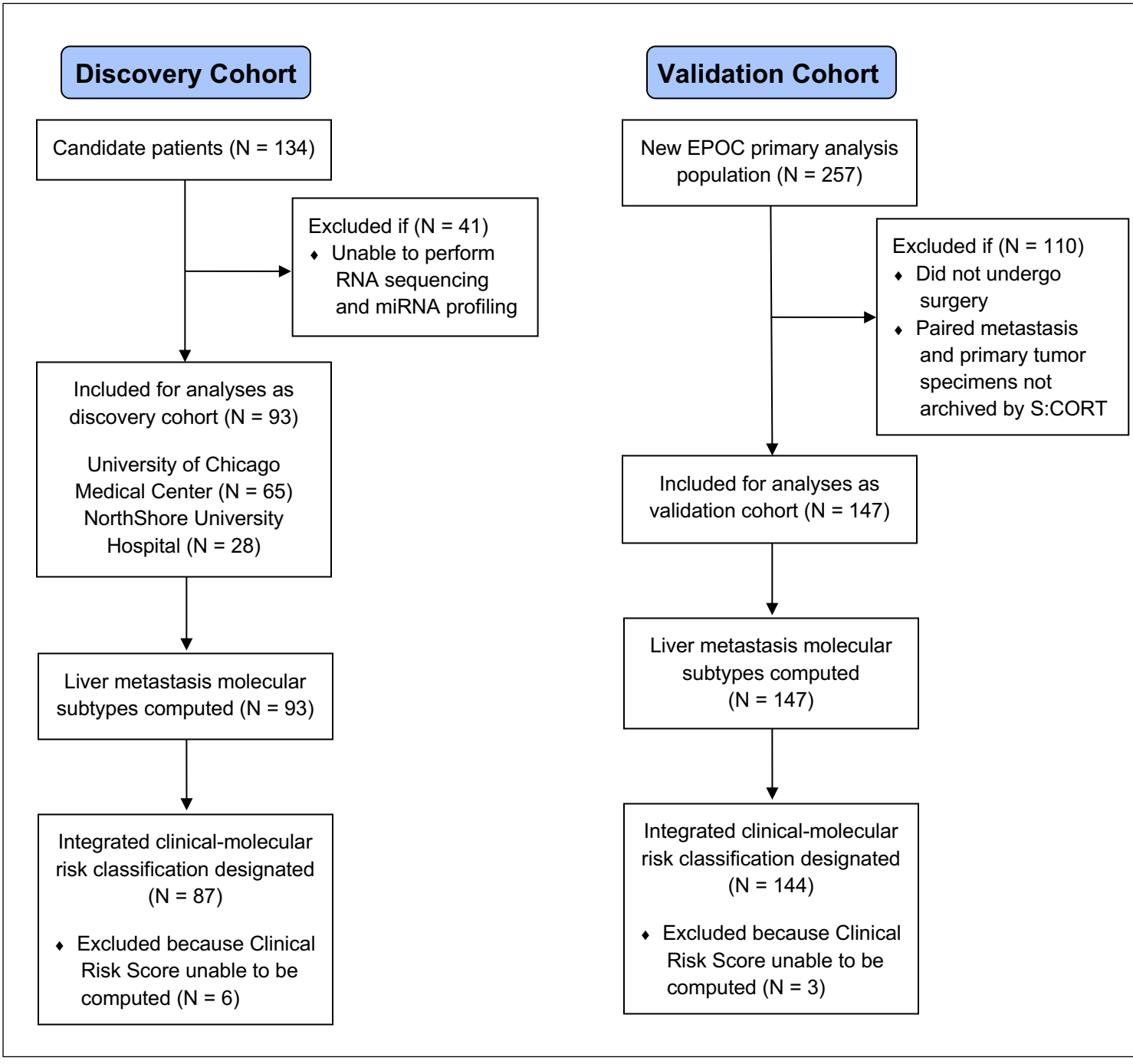

Discovery Cohort
Candidate patients (N = 134)
Excluded if (N = 41)
♦ Unable to perform RNA sequencing and miRNA profiling
Included for analyses as discovery cohort (N = 93)
University of Chicago Medical Center (N = 65)
NorthShore University Hospital (N = 28)
Liver metastasis molecular subtypes computed (N = 93)
Integrated clinical-molecular risk classification designated (N = 87)
♦ Excluded because Clinical Risk Score unable to be computed (N = 6)
Validation Cohort
New EPOC primary analysis population (N = 257)
Excluded if (N = 110)
♦ Did not undergo surgery
♦ Paired metastasis and primary tumor specimens not archived by S:CORT
Included for analyses as validation cohort (N = 147)
Liver metastasis molecular subtypes computed (N = 147)
Integrated clinical-molecular risk classification designated (N = 144)
♦ Excluded because Clinical Risk Score unable to be computed (N = 3)

Figure 2

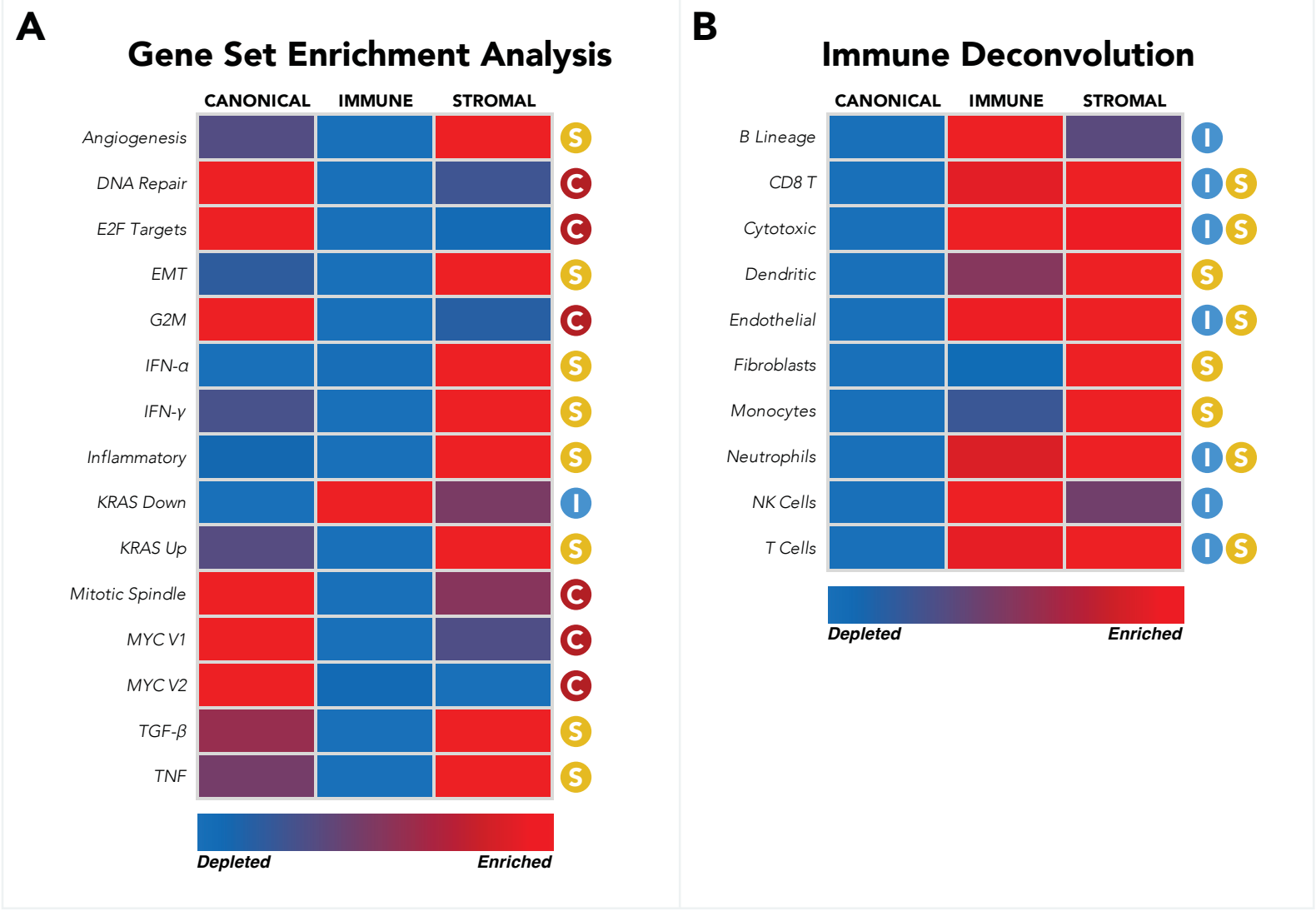
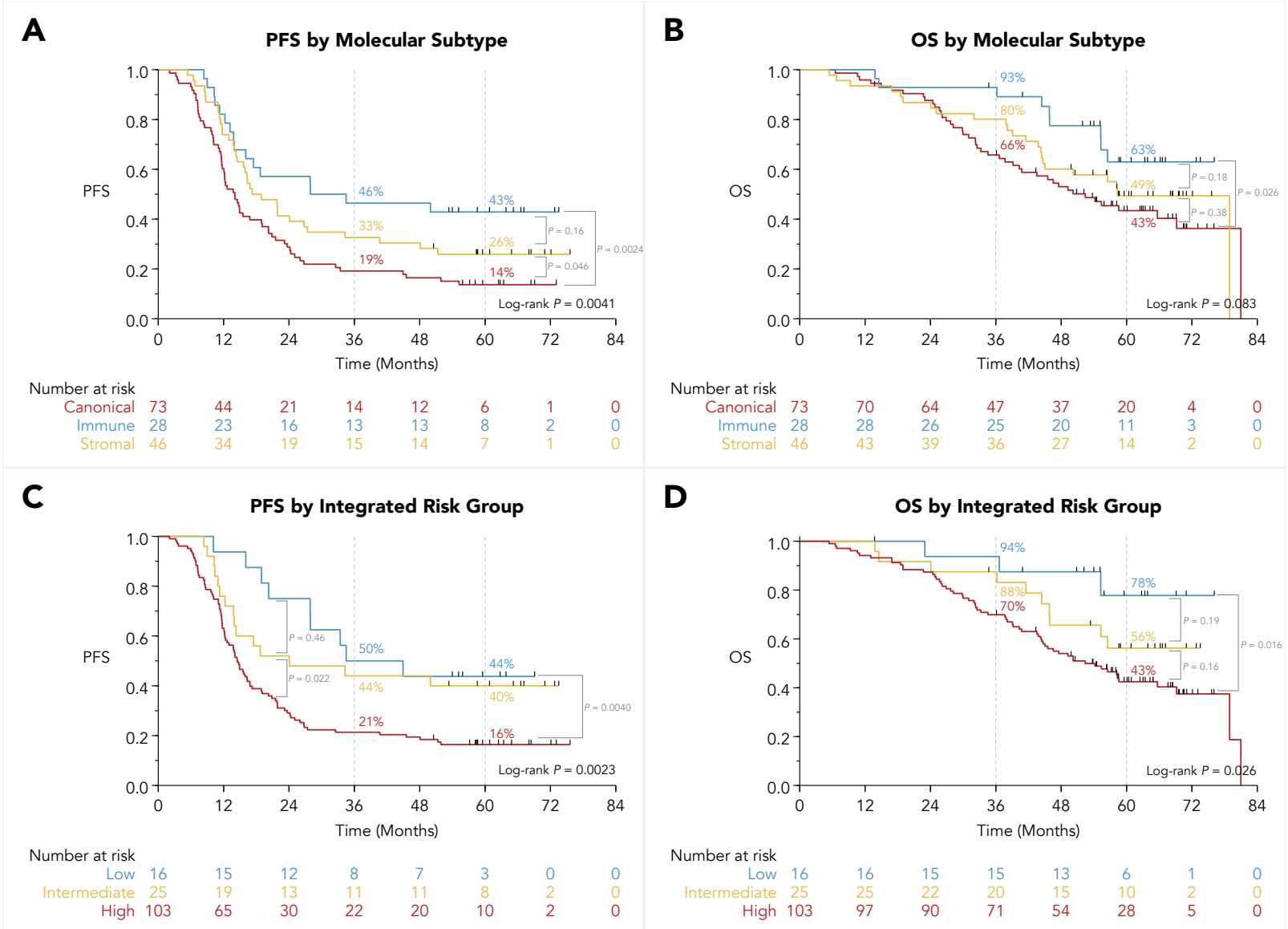

A
Gene Set Enrichment Analysis
CANONICAL
IMMUNE
STROMAL
Angiogenesis
DNA Repair
E2F Targets
EMT
G2M
IFN-α
IFN-γ
Inflammatory
KRAS Down
KRAS Up
Mitotic Spindle
MYC V1
MYC V2
TGF-β
TNF
S
C
C
S
C
S
S
S
I
S
C
C
C
S
S
Depleted
Enriched
B
Immune Deconvolution
CANONICAL
IMMUNE
STROMAL
B Lineage
CD8 T
Cytotoxic
Dendritic
Endothelial
Fibroblasts
Monocytes
Neutrophils
NK Cells
T Cells
I
I S
I S
S
I S
S
S
I S
I
I S
Depleted
Enriched

Figure 3


A
PFS by Molecular Subtype
PFS
Time (Months)
46%
33%
19%
43%
26%
14%
P = 0.16
P = 0.046
P = 0.0024
Log-rank P = 0.0041
Number at risk
Canonical 73 44 21 14 12 6 1 0
Immune 28 23 16 13 13 8 2 0
Stromal 46 34 19 15 14 7 1 0
B
OS by Molecular Subtype
OS
Time (Months)
93%
80%
66%
63%
49%
43%
P = 0.18
P = 0.38
P = 0.026
Log-rank P = 0.083
Number at risk
Canonical 73 70 64 47 37 20 4 0
Immune 28 28 26 25 20 11 3 0
Stromal 46 43 39 36 27 14 2 0
C
PFS by Integrated Risk Group
PFS
Time (Months)
50%
44%
21%
44%
40%
16%
P = 0.46
P = 0.022
P = 0.0040
Log-rank P = 0.0023
Number at risk
Low 16 15 12 8 7 3 0 0
Intermediate 25 19 13 11 11 8 2 0
High 103 65 30 22 20 10 2 0
D
OS by Integrated Risk Group
OS
Time (Months)
94%
88%
70%
78%
56%
43%
P = 0.19
P = 0.16
P = 0.016
Log-rank P = 0.026
Number at risk
Low 16 16 15 15 13 6 1 0
Intermediate 25 25 22 20 15 10 2 0
High 103 97 90 71 54 28 5 0