Association of Consensus Molecular Subtypes and Molecular Markers With Clinical Outcomes in Patients With Metastatic Colorectal Cancer: Biomarker Analyses From LUME-Colon 1

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Abstract

LUME-Colon 1 (NCT02149108) was a randomized, controlled, phase III study of nintedanib in 765 patients with advanced colorectal cancer. Pre-specified biomarker analyses investigated the association of colorectal cancer consensus molecular subtypes and tumor genomic/circulating biomarkers with clinical outcomes. **Unclassified/mixed consensus molecular subtype was potentially predictive for nintedanib response; somatic mutations were highly concordant between tumor tissue and cell-free DNA.**

Introduction: LUME-Colon 1 (NCT02149108) was a global, placebo-controlled phase III study of nintedanib in advanced colorectal cancer (CRC). Pre-specified biomarker analyses investigated the association of CRC consensus molecular subtypes (CMS) and tumor genomic and circulating biomarkers with clinical outcomes.

Materials and Methods: Archival tumor tissue, cell-free DNA (cfDNA), and plasma samples were collected for genomic, transcriptomic, and proteomic analyses to investigate potential associations between CRC CMS and other biomarkers with nintedanib response and clinical outcomes. **Results:** Of the 765 treated patients, 735, 245, and 192 patient samples were analyzed in the circulating protein, tumor tissue, and cfDNA datasets, respectively. Patients were classified as CMS1 (1.7%), CMS2 (27.7%), CMS3 (0.9%), CMS4 (51.5%), or unclassified (18.2%). Unclassified/mixed CMS was associated with longer overall survival (OS) with nintedanib vs. CMS2 or CMS4 (interaction P -value = .0086); no association was observed for CMS4. Gene expression-based pathway analysis revealed an association between vascular endothelial growth factor-related signaling and OS for nintedanib (P = .0498). The most frequently detected somatic mutations were *APC* (72.0% [tumor tissue] vs. 56.8% [cfDNA]), *TP53* (47.1% vs. 34.9%), *KRAS* (40.8% vs. 28.6%), and *PIK3CA* (16.6% vs. 11.5%); concordance rates were > 80%. Median OS differences were observed for *APC* and *TP53* mutations vs. wild-type in cfDNA, indicating a potential prognostic value. Circulating ANG-2, CA-9, CEACAM1, collagen-IV, IGFBP-1, ICAM-1, IL-8, and uPAR were potentially prognostic for both OS and progression-free survival. **Conclusion:** We demonstrated the feasibility of large-scale biomarker analyses and CMS classification within a global clinical trial, and identified signals suggesting a potential for greater nintedanib treatment response in the unclassified/mixed CMS subgroup, despite these tumors showing heterogeneous patterns of CMS mixtures. Our results revealed a high degree of

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concordance in somatic mutations between tumor tissue and cfDNA. Associations with prognosis for cfDNA somatic mutations, as well as several protein-based biomarkers, may warrant further investigation in future trials.

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Introduction

Biomarkers in Colorectal Cancer

Colorectal cancer (CRC) is one of the most common types of cancer; it is the third most common in men and the second most common in women.¹ There are multiple genes and pathways known to be involved in its initiation, progression, and metastasis, resulting in a high degree of genetic heterogeneity.^{2,3} Transcriptomic characterization of CRC offers the potential to overcome some of the therapeutic challenges of genetic heterogeneity by identifying activated genes or pathways (ie, personalized therapeutic targets).^{3,4}

Potential targets implicated in the initiation and progression of CRC include the WNT, RAS-MAPK, phosphoinositide 3-kinase (PI3K), transforming growth factor beta (TGF- β), p53, and DNA mismatch-repair pathways.² Their key role in the development and proliferation of CRC identifies these pathways (and related mutations) as being of potential utility as clinical biomarkers of treatment response (predictive biomarkers) and/or patient outcomes (prognostic biomarkers).

Adenomatous polyposis coli (APC) protein is a component of the Wnt/ β -catenin signaling pathway, which negatively regulates cell growth. Inactivation of APC appears to be an early (perhaps initiating) event in ~70% to 80% of colorectal adenomas and carcinomas. However, APC has thus far shown limited potential as a clinical biomarker.^{2,5}

RAS and *BRAF* mutations are present in an estimated 40% and 5% to 10% of patients with CRC, respectively, and are of interest as potential predictors of response to epidermal growth factor receptor (EGFR) therapy.^{2,5}

Mutations in the tumor protein p53 (*TP53*) gene are observed in 50% to 75% of CRCs. The TP53 protein has a regulatory role in cell growth arrest, DNA repair and apoptosis, oxidative stress, DNA damage, and cell aberrant proliferation; thus maintaining cell cycle homeostasis. Dysfunctions in TP53 have a critical role in oncogenesis in a wide range of tumor types, including CRC.^{2,3,6}

EGFR signaling has also been shown to play a critical role in tumor survival (including CRC), resulting in the development and approval of anti-EGFR targeted therapies. Human epidermal growth factor receptor 2 (HER2) signaling represents one potential mechanism of acquired resistance to anti-EGFR therapies. HER2 amplification has been detected in approximately 5% of *KRAS* wild-type cancers, and there is evidence to support HER2 expression as a predictive biomarker for anti-HER2 treatment response.^{2,3,6}

A leading cause of mutations found in CRC is dysfunction in the mismatch repair system, resulting in the well-established microsatellite instability (MSI) phenotype. MSI is reported in approximately 15% of all CRCs. Hypermutated CRC is highly correlated with

MSI, and is frequently observed together with *BRAF* V600E mutation. The evidence to support MSI status as a biomarker for potential treatment response remains equivocal at this time. However, recent reports describe significantly better clinical outcomes in patients with MSI-high CRC compared with non-MSI CRC, suggesting potential utility for MSI status and hypermutated phenotypes as predictive markers for response to immunomodulating therapy.⁷

The role of dysregulated angiogenesis is well-established in CRC, and is underpinned by the approval of several anti-angiogenic drugs that target vascular endothelial growth factor (VEGF) ligands (eg, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor [PlGF]), their receptors (VEGFR1-3, Tie2 receptor tyrosine kinase), and other related proliferative pathways (platelet-derived growth factor receptor [PDGFR] and fibroblast growth factor receptor [FGFR]). However, cross-talk between VEGF and other crucial pathways is complex, and many patients demonstrate pre-existing or acquired resistance to anti-angiogenic agents.^{8,9} The lack of a definitive predictive biomarker for anti-angiogenic treatment highlights the need for further biomarker analyses in large-scale clinical trials.⁸

Consensus Molecular Subtype (CMS) Classification

The CMS classification system is defined by the pathways implicated in CRC.¹⁰ It comprises 4 distinct subgroups defined by gene expression levels: (1) CMS1 (MSI immune), hypermutated, microsatellite unstable, and strong immune activation; (2) CMS2 (canonical), epithelial, marked WNT and MYC signaling activation; (3) CMS3 (metabolic), epithelial and evident metabolic dysregulation; and (4) CMS4 (mesenchymal), prominent TGF β activation, stromal invasion, and angiogenesis.

CMS has been shown to be a robust approach to CRC subtyping, and is of substantial interest for its potential as a predictive biomarker.¹¹⁻¹⁵ Nevertheless, the ability of CMS to predict treatment response remains unclear owing to conflicting results from phase III trials, for example, in the CALGB/SWOG 80405 trial and FIRE3 trials.^{12,13} The predictive and prognostic values of CMS were evaluated in a heterogeneous population of patients with advanced CRC (n = 581) in the CALGB/SWOG 80405 trial.¹² CMS subgroup was found to be associated with survival outcomes ($P < .001$ for both overall survival [OS] and progression-free survival [PFS]). Patients in the CMS1 cohort who received bevacizumab had significantly longer OS than those treated with cetuximab ($P < .001$) and, conversely, patients in the CMS2 cohort treated with cetuximab had significantly longer OS than patients treated with bevacizumab ($P = .0046$).¹² In the FIRE3 trial in patients with metastatic CRC (n = 592), CMS was also found to be

a predictor of OS ($P < .001$) and PFS ($P < .001$).¹³ However, there was no difference in OS among patients who received bevacizumab compared with those who received cetuximab in either CMS1 ($P = .28$) or CMS2 ($P = .40$).¹³ These findings suggest that further research is needed in additional clinical trial populations to determine the role of CMS classification as a biomarker in identifying patients with metastatic CRC who are most likely to benefit from specific targeted therapies.

LUME-Colon 1 Biomarker Analyses

LUME-Colon 1 (NCT02149108) was a large-scale, global, randomized, double-blind, placebo-controlled, phase III trial investigating the efficacy and safety of nintedanib (vs. placebo) plus best supportive care in patients with metastatic CRC refractory to standard therapies. Results from the primary analysis have been published elsewhere.¹⁶

Here, we present the results from the biomarker analyses from LUME-Colon 1. At the time of study design, a comprehensive exploratory biomarker analysis was planned to provide molecular profiling data, based on targeted next-generation sequencing (NGS) of tumor tissue and cell-free DNA (cfDNA) samples, including a concordance analysis of mutations between tumor tissue and cfDNA samples. The analysis also included transcriptomic characterization of tumor tissue and an evaluation of protein biomarkers in plasma samples.

The approach taken with this global, phase III study provides an opportunity to explore whether a differential treatment effect was evident in any biomarker-defined subpopulations treated with nintedanib. The objective was to molecularly characterize the patients' tumors (ie, identify the frequency of potential clinical biomarkers) as well as to assess the utility of the CRC CMS classification system (and of other potential biomarkers proposed within the CRC literature) for their association with treatment response and/or clinical outcomes. The CMS4 subgroup was of particular interest as it is described as being associated with angiogenic pathway activation, and might, therefore, be more strongly associated with response to nintedanib (an anti-angiogenic agent) vs. other CMS subgroups.

Materials and Methods

Study Design and Biomarker Sample Collection

LUME-Colon 1 was a global, randomized, phase III study investigating the efficacy and safety of nintedanib (200 mg twice daily) vs. placebo in patients with advanced CRC. Detailed information about the trial design has been previously described.¹⁶

Plasma samples for circulating protein biomarker analysis were collected at randomization (before intake of study drug at cycle 1, visit 1), before intake of study drug at the start of treatment course 2, and at the end of the trial.

Archival formalin-fixed, paraffin-embedded (FFPE) tumor tissue for genomic and transcriptomic profiling was collected and analyzed from all patients with sufficient and evaluable tumor tissue.

The final biomarker analysis in tumor tissue and cfDNA samples was performed on a subset of samples that were selected according to the following quality-based criteria: sample availability; tumor content $> 40\%$ (before macrodissection); RNA mean concentration > 20 ng/ μ L; RNA DV₂₀₀ (percentage of RNA fragments > 200 nt

in size) $> 28\%$; availability of baseline ethylene diamine tetraacetic acid blood sample as germline reference for mutation profiling; and availability of cycle 1, visit 1 plasma cfDNA sample.

Outcome Measures in Biomarker Cohorts

To assess the similarity of the biomarker cohorts and identify any sources of potential selection bias, the co-primary endpoints (OS and PFS) from the LUME-Colon 1 trial were evaluated for each biomarker cohort, and compared between cohorts and against the results for the overall trial population. Outcomes were evaluated as hazard ratios (HRs) with 95% confidence intervals (CIs) for both PFS and OS.

Evaluation of Prognostic and Predictive Utility

All investigational biomarkers were investigated for their association with patient outcomes (prognostic potential) and response to treatment (predictive potential).

Prognostic Analysis. To eliminate any potential confounding by treatment effect, evaluation of the prognostic potential of investigational biomarkers was conducted only in the placebo arm of the study. The analysis evaluated whether the presence or elevation of a particular biomarker at baseline, or classification within a specific CMS category, was associated with reduced or prolonged survival outcomes (OS and PFS).

Predictive Analysis. The predictive evaluation of investigational biomarkers included the nintedanib and placebo treatment arms, and assessed whether allocation to a particular biomarker subgroup (or to a specific CMS category) resulted in a relative treatment benefit in terms of OS and PFS in favor of nintedanib compared with placebo.

Genomic Analysis

Evaluation of the frequency of somatic cancer-associated mutations and hypermutations of DNA repair genes was performed in DNA samples isolated from FFPE tumor tissue, and from cfDNA isolated from plasma. The NGS panel was a self-designed Roche SeqCap EZ Choice panel comprising a total of 44 genes, including nintedanib target genes, commonly altered CRC genes, and somatic cancer-associated mutations (eg, *APC*, *PIK3CA*, and *TP53*), as well as hypermutations of DNA repair genes (eg, *MLH1*, *MSH2*, *MSH3*, *MSH6*, and *POLE*). See Supplemental Table 1 (in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>) for a complete list. Targeted DNaseq libraries were prepared from DNA isolated from FFPE tumor tissue as well as from cfDNA isolated from plasma samples using the SeqCap EZ together with the KAPA HyperPlus kit; sequencing was performed on an Illumina NextSeq 500 instrument using the NextSeq 500/550 Mid-Output v2 kit according to the manufacturer's instructions. Concordance between mutations detected in matching cfDNA and tumor tissue samples was also evaluated.

The tumor tissue dataset was used to further profile the LUME-Colon 1 population by the CRC CMS classification based on transcriptome (RNAseq) data. RNAseq libraries were generated from RNA isolated from FFPE tumor tissue using the Illumina

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TruSeq Stranded Total RNA LT (with Ribo-Zero Gold) kit and sequenced on an Illumina HiSeq 3000/4000 instrument using the HiSeq 3000/4000 SBS kit according to the manufacturer's instructions. Associations between the CRC CMS with PFS and OS were investigated, considering in particular the predictive potential of the angiogenesis-related CMS4 for response to nintedanib treatment.

Plasma Protein Analysis

Baseline plasma samples were used to identify potential plasma-derived protein biomarkers. In total, 55 soluble angiogenic factors were evaluated and analyzed using the Human AngiogenesisMAP (Myriad RBM), including: angiopoietin-2 (ANG-2), carbonic anhydrase 9 (CA-9), tenascin-C (TN-C), basic fibroblast growth factor (bFGF), VEGF A and D, VEGFR1-3, and PlGF. See [Supplemental Table 2](https://doi.org/10.1016/j.clcc.2020.09.003) (in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>) for a complete list.

Statistical Analysis

Descriptive statistics were used to summarize data, including CMS distribution and mutational and pathway activation status. Kaplan-Meier methods were used in time-to-event analyses, and Cox regression was used to assess the predictive and prognostic potential of biomarkers. All analyses were exploratory; no additional confirmatory statistical analyses were conducted.

The association between plasma protein markers or molecular characteristics and response to nintedanib treatment was evaluated using the co-primary endpoints from the LUME-Colon 1 trial (centrally-assessed PFS and OS), and a treatment-by-biomarker interaction test based on 2 independent tests, both with a 2-sided significance level of .05. The prognostic potential of plasma protein and molecular characteristics was assessed in the placebo group via pairwise comparisons of individuals with/without the characteristic of interest. Subgroup analyses were only performed if ≥ 10 patients were available per subgroup and per treatment.

Mutation profiling in cfDNA samples in the placebo-treated group were significantly associated with treatment benefit at a level of $P < .1$ (false discovery rate [FDR]-adjusted P -value). Protein biomarkers were significantly associated with lack of treatment benefit at a level of $P < .01$ (FDR-adjusted P -value).

CMS Analysis

CMS classification analysis was conducted using the R package *CMSclassifier*, in line with published methodology (ie, centroid-based and default settings).¹⁰ Any dataset-specific refinements to the published methodology were agreed in consultation with the Swiss Institute of Bioinformatics, Lausanne, Switzerland. NGS gene expression data (expressed in transcripts per million reads) were $\text{Log}_2(x+1)$ transformed for use with the CMS classifier. Direct mapping of Ensemble IDs to Entrez IDs was not possible in approximately 10 cases.

Results

Data Availability

In total, 765 patients were treated in LUME-Colon 1 ($n = 384$ nintedanib; $n = 381$ placebo).¹⁶ In this biomarker analysis, 735 patients contributed to the plasma protein dataset ($n = 368$

[95.8%] nintedanib; $n = 367$ [96.3%] placebo); 245 patients contributed to the tumor tissue dataset ($n = 125$ [32.6%] nintedanib; $n = 120$ [31.5%] placebo); and 192 patients contributed to the cfDNA dataset ($n = 104$ [27.1%] nintedanib; $n = 88$ [23.1%] placebo) ([Figure 1](#)). Matched tumor tissue and cfDNA data were available for concordance analysis for 118 patients ($n = 57$ [14.8%] nintedanib; $n = 61$ [16.0%] placebo).

Baseline Characteristics

There were no notable differences in patient demographics or clinical characteristics in the overall LUME-Colon 1 population ([Table 1](#)) or between different biomarker cohorts (see [Supplemental Table 3](#) in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>). There were no clinically meaningful differences in outcomes (PFS or OS) between any of the biomarker cohorts, or between any of the cohorts and the overall LUME-Colon 1 trial population (see [Supplemental Table 4](#) in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>).

CMS Categorization

Of the 245 patient samples in the tumor tissue dataset, 231 (94.3%) had evaluable RNA-Seq gene expression data and met the necessary quality criteria for CMS classification. Four (1.7%) patients were CMS1; 64 (27.7%) were CMS2; 2 (0.9%) were CMS3, and 119 (51.5%) were CMS4. The remaining 42 (18.2%) patients were unclassifiable or of mixed phenotype by CMS categorization (hereafter referred to as 'unclassified') (see [Supplemental Table 5](#) in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>).

CMS distribution was similar in the nintedanib and placebo groups. Demographic characteristics and oncological history were similar for the CMS subgroups and the overall tumor tissue cohort, with the exception of prior surgical tumor removal, which was more common in CMS4 patients than other groups (nintedanib: 95.0% vs. 82.5%, respectively; placebo: 91.5% vs. 76.4%, respectively).

Across all CMS subgroups, right-sided tumors were less common than left-sided tumors, with no right-sided tumors detected in the CMS1 and CMS3 subgroups and an incidence of 12.5%, 9.2%, and 9.5% for right-sided tumors in the CMS2, CMS4, and unclassified/mixed phenotype subgroups, respectively. The frequency of common CRC mutations varied across CMS subgroups, but *APC* mutations were consistently the most common, with an incidence ranging from 62.7% (47/75 samples) in CMS4 to 100% (3/3 and 1/1 samples, respectively) in CMS1/CMS3. Only 1 class III *BRAF* mutation was detected (in a patient with CMS2 CRC).

Owing to the low number of patients categorized as CMS1 or CMS3, the predictive/prognostic biomarker analyses omitted these groups and only considered the CMS2, CMS4, and unclassified subgroups.

The predictive biomarker analysis revealed an association between the unclassified CMS category and longer OS (interaction P -value = .0086); patients in this subgroup derived greater benefit from nintedanib treatment than those in either CMS2 or CMS4. The median OS in the unclassified/mixed subgroup was 11.1 months in the nintedanib arm vs. 4.7 months in the placebo arm ([Figure 2](#)). In contrast, the CMS4 subgroup (characterized by angiogenic pathway activation, among other features) was not associated with nintedanib treatment effect vs. non-CMS4

Figure 1 Availability of Biomarker Samples (CONSORT Diagram)

Abbreviations: cfDNA = Cell-free DNA; FFPE = formalin-fixed, paraffin-embedded.

subgroups, in terms of either OS or PFS (interaction P -values .6634 and .8278, respectively) (see [Supplemental Table 6](#) in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>).

Signaling Pathways

Gene expression data were used to evaluate pre-defined signaling pathway activation. The VEGF-related signaling pathway showed a predictive potential for nintedanib (for OS only; unadjusted P -value = .0498); based on an analysis of the placebo group, there was no prognostic potential for either OS or PFS. No prognostic/predictive associations were found for other signaling pathways, including activated vs. non-activated angiogenic pathways.

Mutation Profiling

Somatic mutations were assessed in 157 tumor tissue samples ($n = 74$ nintedanib; $n = 83$ placebo) and 192 cfDNA samples ($n = 104$ nintedanib; $n = 88$ placebo). The most frequently observed somatic mutations were in: *APC* ($n = 113$ [72.0%] tumor tissue vs. $n = 109$ [56.8%] cfDNA); *TP53* ($n = 74$ [47.1%] vs. $n = 67$ [34.9%]); *KRAS* ($n = 64$ [40.8%] vs. $n = 55$ [28.6%]), and *PIK3CA* ($n = 26$ [16.6%] vs. $n = 22$ [11.5%]) ([Figure 3](#)). Detection rates for these genes were similar in both the nintedanib and placebo treatment groups. Of note, the frequency of *BRAF* mutations in the analyzed patient cohort was very low; only 1 (0.6%) *BRAF* D594G mutation was observed, which was detected in the tumor tissue as well as the plasma cfDNA samples of the patient.

Concordance was assessed in 118 patients with matched tumor and cfDNA samples, with a significant association ($R^2 = 0.9912$;

$P < .0001$) between the tumor tissue cohort and the cfDNA cohort for the 20 most commonly mutated genes ([Figure 4](#)). For the most frequently mutated genes (*APC*, *TP53*, *KRAS*, and *PIK3CA*), concordance was 82.2%, 81.4%, 87.3%, and 89.8%, respectively.

Differences in median OS were observed in cfDNA samples of patients with *APC* and *TP53* mutations compared with patients with no mutations (in the placebo-treated group), at a significance level of $P < .1$ (FDR-adjusted P -value); suggestive of a potential prognostic value ([Figure 5](#)). The median OS for *APC* was 5.7 vs. 7.8 months (mutant vs. wild-type; HR, 1.74; 95% CI, 1.05-2.89; $P = .0656$). The median OS for *TP53* was 5.7 vs. 7.3 months (HR, 1.93; 95% CI, 1.14-3.25; $P = .0543$). The survival difference was not significant for *PIK3CA*: the median OS was 5.9 vs. 6.2 months (HR, 1.75; 95% CI, 0.79-3.86; $P = .1683$). For *KRAS*, an association was observed for both OS and PFS at a significance level of $P < .1$; however, the median OS and PFS values were similar for mutated and wild-type patients: the median OS was 6.2 vs. 6.1 months (HR, 1.75; 95% CI, 1.00-3.07; $P = .0658$), and the median PFS was 1.3 vs. 1.4 months (HR, 1.97; 95% CI, 1.15-3.38; $P = .0546$). No potentially prognostic signals were detected in the tumor tissue (FFPE) samples.

Hypermutation status (defined by the presence of any alteration in any of the DNA repair genes investigated) was not associated with either OS or PFS (see [Supplemental Figure 1](#) in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>).

Protein Biomarkers

A panel of 55 circulating protein biomarkers was analyzed, which included regulators of growth, survival, angiogenesis, adhesion, and

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Table 1 Baseline Characteristics in LUME-Colon 1 (Full Population and Biomarker Sub-population)

	LUME-Colon 1, n (%)			LUME-Colon 1 Biomarker Sub-population, ^a n (%)		
	Placebo	Nintedanib	Total	Placebo	Nintedanib	Total
No. patients	382 (100)	386 (100)	768 (100)	120 (100)	125 (100)	245 (100)
Age, y						
Mean	61.1	61.0	61.1	59.9	59.8	59.9
Range	23-83	22-85	22-85	28-82	30-85	28-85
Gender						
Female	164 (42.9)	150 (38.9)	314 (40.9)	59 (49.2)	51 (40.8)	110 (44.9)
Male	218 (57.1)	236 (61.1)	454 (59.1)	61 (50.8)	74 (59.2)	135 (55.1)
Geographical region						
Asia	98 (25.7)	95 (24.6)	193 (25.1)	32 (26.7)	31 (24.8)	63 (25.7)
Australia	12 (3.1)	14 (3.6)	26 (3.4)	6 (5.0)	6 (4.8)	12 (4.9)
North America	29 (7.6)	34 (8.8)	63 (8.2)	6 (5.0)	8 (6.4)	14 (5.7)
Western Europe	186 (48.7)	183 (47.4)	369 (48.0)	54 (45.0)	60 (48.0)	114 (46.5)
Rest of world	57 (14.9)	60 (15.5)	117 (15.2)	22 (18.3)	20 (16.0)	42 (17.1)
Race						
Asian	104 (27.2)	97 (25.1)	201 (26.2)	34 (28.3)	31 (24.8)	65 (26.5)
White	268 (70.2)	279 (72.3)	547 (71.2)	82 (68.3)	91 (72.8)	173 (70.6)
Other	3 (0.8)	5 (1.3)	8 (1.0)	0 (0.0)	1 (0.8)	1 (0.4)
Missing ^b	9 (2.4)	5 (1.3)	14 (1.8)	4 (3.3)	2 (1.6)	6 (2.4)
ECOG PS at baseline						
0	142 (37.2)	162 (42.0)	304 (39.6)	42 (35.0)	48 (38.4)	90 (36.7)
1	240 (62.8)	223 (57.8)	463 (60.3)	78 (65.0)	76 (60.8)	154 (62.9)
≥ 2	0 (0.0)	1 (0.3)	1 (0.1)	0 (0.0)	1 (0.8)	1 (0.4)
Primary tumor location ^c						
Left	295 (77.2)	298 (77.2)	593 (77.2)	91 (75.8)	95 (76.0)	186 (75.9)
Right	86 (22.5)	88 (22.8)	174 (22.7)	29 (24.2)	30 (24.0)	59 (24.1)
Missing	1 (0.3)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)
Time since first histologic diagnosis, mos						
Mean (SD)	47.4 (29.9)	46.5 (29.5)	46.9 (29.7)	43.2 (27.4)	43.1 (27.8)	43.1 (27.6)
Range	2-155	6-245	2-245	8-138	6-208	6-208
Number of lines of previous anticancer therapies						
1	11 (2.9)	7 (1.8)	18 (2.3)	4 (3.3)	4 (3.2)	8 (3.3)
2	75 (19.6)	82 (21.2)	157 (20.4)	23 (19.2)	30 (24.0)	53 (21.6)
3	109 (28.5)	90 (23.3)	199 (25.9)	39 (32.5)	26 (20.8)	65 (26.5)
4	70 (18.3)	87 (22.5)	157 (20.4)	19 (15.8)	33 (26.4)	52 (21.2)
5	54 (14.1)	59 (15.3)	113 (14.7)	10 (8.3)	19 (15.2)	29 (11.8)
≥ 6	63 (16.5)	61 (15.8)	124 (16.1)	25 (20.8)	13 (10.4)	38 (15.5)
Location of metastatic sites at screening						
Lung	305 (79.8)	316 (81.9)	621 (80.9)	95 (79.2)	106 (84.8)	201 (82.0)
Liver	266 (69.6)	277 (71.8)	543 (70.7)	79 (65.8)	86 (68.8)	165 (67.3)
Lymph nodes	191 (50.0)	184 (47.7)	375 (48.8)	63 (52.5)	54 (43.2)	117 (47.8)
Peritoneum/omentum/malignant ascites	84 (22.0)	93 (24.1)	177 (23.0)	23 (19.2)	28 (22.4)	51 (20.8)
Bone	46 (12.0)	35 (9.1)	81 (10.5)	16 (13.3)	6 (4.8)	22 (9.0)
Other	120 (31.4)	143 (37.0)	263 (34.2)	37 (30.8)	38 (30.4)	75 (30.6)

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; FFPE = formalin-fixed, paraffin-embedded; SD = standard deviation.

^aBased on patient subset with archival FFPE tumor tissue.^bIncludes data from countries where collection of patient race data is not permitted.^cRight-sided defined as cecum, ascending colon, and transverse colon. Left-sided defined as descending colon, sigmoid colon, and rectum.

Figure 2 OS (A) and PFS (B) Analyses for Individual CMS Subgroups

Only subgroups comprising ≥ 10 patients were included in the analysis.

Abbreviations: CI = confidence interval; CMS = consensus molecular subtype; HR = hazard ratio; OS = overall survival; PFS = progression-free survival.

other oncogenic pathways; most of which were present at detectable levels. At a significance level of $P < .01$ (FDR-adjusted P -value), 8 of the 55 protein biomarkers were found to be prognostic for both OS and PFS: ANG-2; CA-9; carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1); collagen IV; insulin-like growth factor-binding protein (IGFBP)-1; intercellular adhesion molecule (ICAM)-1, IL-8, and urokinase receptor (uPAR). For these 8 proteins, high plasma concentrations at baseline were correlated with shorter OS and PFS. In addition to those 8 proteins, 21 protein biomarkers were prognostic for OS only. No protein biomarkers were predictive for PFS or OS at a significance level $< .01$ (FDR-adjusted interaction P -value).

Discussion

Distribution of CMS Subgroups in LUME-Colon 1

There were notable differences between the CMS distribution in the LUME-Colon 1 biomarker population and that reported in the

literature (Figure 6): CMS1 (1.7% observed vs. 9%–17% reported); CMS2 (27.7% vs. 37%–50%); CMS3 (0.9% vs. 2%–13%); CMS4 (51.5% vs. 23%–45%); and unclassified/mixed (18.2% vs. 0%–13%). Importantly, the observed proportion of unclassified/mixed CMS patients is higher in our study than previously reported; although recent data from the ColoType classifier report approximately 16% unclassified samples, and data from PETACC-3 show $> 20\%$ unclassified samples.¹⁷ As the CMS classification was conducted in accordance with published methods,¹⁰ it is important to consider other potential factors for these differences, which might include the range of tumor sample sites that contributed to the analysis, the range of disease stages, and the number of previous lines of treatment within the analyzed population at baseline.

The strict patient eligibility criteria of LUME-Colon 1 (advanced, refractory CRC) compared with those of the CMS consensus paper (which analyzed a broad range of patients with CRC across 18 datasets) might have resulted in a different distribution of patient

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Figure 3 Frequency of Somatic Mutations in LUME-Colon 1 (FFPE vs. cfDNA Samples) and Other Publically Available Datasets

Abbreviations: cfDNA = cell-free DNA; CRC = colorectal cancer; FFPE = formalin-fixed, paraffin-embedded; TCGA = The Cancer Genome Atlas Network.

characteristics.^{10,16} Within the tumor tissue cohort, the majority of samples were taken from primary tumors (n = 182; 79%), but a proportion were also taken from metastases (n = 37; 16%) or from an unknown source (n = 12; 5%). All patients had stage IV disease; approximately one-half of the patients in LUME-Colon 1 were randomized more than 36 months after their first histologic diagnosis. At the time of randomization, 25% of the patients had received 1 to 2 previous lines of treatment; 48% of the patients had received 3 to 4 previous lines of treatment; and 27% of patients had received ≥ 5 previous lines of treatment. Both factors — chemo-refractory setting and analysis of metastatic tissue — have been reported to impact on the distribution of CMS as well as the number of unclassified/mixed samples.^{18,19} Finally, an unusually low frequency of *BRAF* mutations was observed in this trial (0.6% observed vs. 5%-10% typically reported).² No instances of *BRAF*V600 mutations were observed in the LUME-Colon 1 population.

In turn, this altered distribution of CMS subgroups could have affected the biomarker analysis and limited its accuracy. It is also important to note that, although the biomarker analysis was performed on archival FFPE tumor tissue samples, the observed distribution of the CMS from the LUME-Colon 1 analysis may reflect the prognostic effect of the individual subtypes, and thus be more similar to the chemo-refractory setting and analysis of metastases. Indeed, this may also explain the observed low frequency of CMS1 (and *BRAF* mutation frequency) in the dataset, as the prognostic effect of *BRAF* mutation may have affected enrollment into the study.

Predictive Potential of CMS Subgroups

The observed association between the unclassified/mixed CMS subgroup and longer OS highlights a gap in our knowledge of CRC, though should be interpreted with caution owing to the low

number of patients (n = 42 unclassified/mixed patients). The literature describes as many as 13% of patients being of unclassified or mixed CMS category.¹⁰ In this study, the proportion of samples in the unclassified/mixed subgroup may have been higher owing to the factors described above. Forcing the samples into 1 of the 4 CMS groups by applying ‘nearest CMS’ classification resulted in distribution of the ‘unclassified’ samples across CMS1 (n = 6), CMS2 (n = 12), CMS3 (n = 3), and CMS4 (n = 21); there was no association for any of the adjusted CMS groups with clinical parameters (PFS or OS).

Somewhat surprisingly, there was no association between CMS4 (which is characterized by angiogenic pathway activation) and either OS or PFS, as compared with other CMS subgroups. However, a separate analysis of VEGF pathway activation, based on gene expression profile data, did suggest that nintedanib treatment was associated with OS.

Mutational Analyses in Tumor Tissue and cfDNA

Results from the mutational analysis are consistent with those reported in the literature. Mutations in *KRAS* were detected in 40.8% (tumor tissue) and 28.6% (cfDNA) of patients with CRC in LUME-Colon 1 vs. an estimated 40% of patients with CRC in published studies.^{2,5} Meanwhile, *TP53* mutations were observed in 47.1% (tumor tissue) and 34.9% (cfDNA) of patients with CRC, as compared with 50% to 75% of patients with CRC in published studies.^{2,3,6}

The LUME-Colon 1 biomarker analysis revealed a high concordance of mutations detected in tumor tissue and cfDNA samples. This supports the use of plasma samples in routine clinical practice as a means to classify CRC by mutation status to monitor response to treatment in real-time, and detect both minimal residual

Figure 4 Correlation Between Missense Mutation Frequencies in Tumor Tissue and cfDNA Samples

Graph shows missense mutation frequencies in tumor tissue and cfDNA for the 20 most frequently mutated genes observed in tumor tissue samples. Abbreviations: cfDNA = cell-free DNA; CI = confidence interval.

disease and the emergence of targeted treatment-resistant clones.²⁰ As the routine collection of plasma isolated cfDNA during serial assessments becomes more established in clinical practice, patients

with CRC undergoing genomic profiling may increasingly benefit from this minimally invasive procedure.²⁰ Nevertheless, concordance between cfDNA and tumor samples fell short of 100%,

Figure 5 Prognostic Potential of Selected Mutations Measured in cfDNA on OS (Placebo Group). *TP53* (A), *APC* (B), *KRAS* (C) and *PIK3CA* (D)

Abbreviations: CI = confidence interval; FDR = false discovery rate; OS = overall survival.

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Figure 6 CMS Distribution in LUME-Colon 1 and Other Published CRC Clinical Trials

Adapted from results in published references.¹⁰⁻¹⁴

Abbreviations: CMS = consensus molecular subtype; FOLFIRI = folinic acid, fluorouracil, and irinotecan chemotherapy; FOLFOX = folinic acid, fluorouracil, and oxaliplatin chemotherapy; MSI = microsatellite instability.

*Stages I-IV; †Stage IV; nintedanib vs. placebo; CMS analysis by RNAseq; ‡Stage IV; regorafenib vs. placebo; CMS analysis by Affymetrix; §Stage IV; bevacizumab + chemotherapy vs. cetuximab + chemotherapy; CMS analysis by nanoString (custom designed); ¶Stage IV; bevacizumab + FOLFIRI vs. cetuximab + FOLFIRI; CMS analysis by Affymetrix (ALMAC Xcel); **Stage III; FOLFOX vs. FOLFOX + cetuximab; CMS analysis by nanoString (196 genes).

which is reflected in the observation that only *APC* and *TP53* were identified as having potential prognostic value via the cfDNA cohort analysis, and none of the most frequently mutated genes (*APC*, *PIK3CA*, *KRAS*, or *TP53*) were found to have either predictive or prognostic utility in the tumor tissue cohort analysis. *KRAS* mutations in cfDNA were found to be potentially prognostic for both PFS and OS, although the median PFS and OS values were similar for mutated and non-mutated patients. A recent publication reports a similar concordance rate of 85% between blood and tumor tissue, as well as a prognostic effect of *KRAS* mutation for OS, which is strongest in patients with concordant *KRAS* mutation status in both blood and tumor tissue samples.²¹

These results should be interpreted with caution, owing to the limited number of samples involved in our analysis; it should also be noted that patients with higher levels of cfDNA typically have a greater tumor burden and worse outcomes.^{22,23} Differences such as this may be the result of incomplete overlap between the cfDNA and tumor tissue cohorts (only ~61% of patients from the tumor tissue set were included in the cfDNA sample set) and also the fact that, although plasma samples were collected from patients on entry to the LUME-Colon 1 trial, the tumor tissue samples were archival and (in most cases) collected several years before start of the trial, giving rise to the potential for divergence.

Analysis of Protein Biomarkers

Several protein biomarkers were found to be associated with both OS and PFS (ANG-2, CA-9, CEACAM1, collagen IV, IGFBP-1, ICAM-1, IL-8, and uPAR), including members of angiogenesis, adhesion, and proliferation pathways. Other exploratory biomarker studies in patients with metastatic CRC have identified similar

protein markers or signatures.^{8,24-26} For example: circulating angiogenic profiles (which signify tumor hypoxia) seem to be associated with worse outcomes²⁵; in patients treated with bevacizumab, plasma cytokine and angiogenic factors are associated with PFS and OS²⁴; in the CORRECT trial of regorafenib, a greater effect on OS was observed in patients with high TIE-1 expression levels (a factor that supports tumor-associated endothelial survival) than those with low TIE-1 levels²⁶; and in a study of sunitinib and sorafenib, levels of angiogenic factors can change in response to treatment, perhaps reflecting a susceptibility of intratumoral blood vessels to anti-angiogenic treatment.²⁵

Given the poor treatment outcomes associated with metastatic CRC and the complexity of angiogenesis pathways, as well as the dearth of validated response markers, more biomarker analyses from large-scale clinical trials will be essential to conclusively identify circulating protein biomarkers that can robustly predict for response to anti-angiogenic agents such as nintedanib.⁸

Limitations

This was an exploratory biomarker analysis and not powered to detect statistical significant or clinically significant associations between investigational biomarkers and clinical endpoints. The sample cohorts used in the mutational and genomic analyses involved a subset ($n = 245$; 32%) of the total patients randomized in the LUME-Colon 1 trial ($N = 768$), with sample selection limited by parameters, such as availability of good quality archival tumor tissue and paired pre-treatment plasma cfDNA samples, and other quality control measures. Nevertheless, the consistency of OS and PFS outcomes observed for the different biomarker cohorts, and for the overall LUME-Colon 1 population, provides confidence that biomarker sample selection

did not result in any clinically meaningful selection bias. Finally, as LUME-Colon 1 did not meet its primary objective, it is challenging to definitively identify biomarkers associated with treatment response.

Conclusion

In conclusion, analysis of the CRC CMS using RNAseq was feasible in this large-scale, global, phase III clinical trial — although there were notable differences between the carefully defined LUME-Colon 1 trial population and the more varied patients included in the original CRC CMS sub-group analysis. Of note, the predictive signal observed with the unclassified/mixed CMS group may warrant further research once future refinements of the disease classification are available; for example, with potential intra-CMS subgroups and with better characterization of samples with mixed phenotypes. Predictive and/or prognostic potential was observed for *APC* and *TP53* mutations and for a range of protein biomarkers, suggesting avenues for future research in patients with advanced CRC. Finally, the high degree of concordance between tumor tissue and cfDNA in the mutational analysis strengthens the rationale for the use of liquid biopsies to help guide treatment decisions in the clinic.

Clinical Practice Points

- Several anti-angiogenic drugs are approved for the treatment of CRC. However, cross-talk between proliferative pathways is complex; many patients display resistance, revealing a need for biomarker analyses in large-scale clinical trials.
- LUME-Colon 1 (NCT02149108) was a placebo-controlled, phase III study of nintedanib in 765 patients with advanced CRC. Pre-planned biomarker analyses investigated the association of CRC CMS and tumor genomic and circulating biomarkers with clinical outcomes. Evaluation of CMS4 was of particular interest owing to its association with angiogenic pathway activation and nintedanib's anti-angiogenic mechanism of action. Overall, 735, 245, and 192 patient samples contributed to the plasma protein, tumor tissue, and cfDNA datasets, respectively.
- CMS distribution in the LUME-Colon 1 biomarker population differed from the published literature: CMS1 (1.7%); CMS2 (27.7%); CMS3 (0.9%); CMS4 (51.5%); and unclassified/mixed (18.2%). An association between unclassified CMS and OS in the nintedanib-treated group highlights an important gap in our knowledge of CRC. Although there was no association with CMS4, VEGF pathway activation was identified as a marker for nintedanib treatment effect. Mutations in *APC*, *TP53*, *KRAS*, and *PIK3CA* (in cfDNA) were associated with OS. Circulating ANG-2, CA-9, CEACAM1, collagen-IV, IGFBP-1, ICAM-1, IL-8, and uPAR were potentially prognostic for both OS and PFS.
- In summary, we conducted a large-scale biomarker analysis and CMS classification within a global clinical trial setting. Unclassified/mixed CMS was potentially predictive for nintedanib response; somatic mutations were highly concordant between

tumor tissue and cfDNA; several biomarkers may warrant further investigation in future trials.

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Supplemental Data

Supplemental figure and tables accompanying this article can be found in the online version at <https://doi.org/10.1016/j.clcc.2020.09.003>.

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Supplemental Data

Supplemental Figure 1 Mutation Profile (A) and prognostic Analysis (B) of OS and PFS in Putative Hypermutated[§] vs. Non-hypermutated Tumors

[†]HR < 1 favored hypermutated samples. [‡]HR, CI, and P-value were obtained from a model adjusted for treatment, regorafenib pre-treatment, time from onset of metastatic disease until randomization, and region. [§]Hypermutation status was defined by the presence of a mutation in at least one of the DNA repair genes included in the gene panel (ie, MLH1, MSH2, MSH3, MSH6, or POLE). Abbreviations: CI = confidence interval; HR = hazard ratio; OS = overall survival; PFS = progression-free survival.

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Supplemental Table 1 Genes Included in the LUME-Colon 1 Targeted NGS Panel			
<i>ALK</i>	<i>FBXW7</i>	<i>MET</i>	<i>PIK3CA</i>
<i>AMER1 (FAM123B)</i>	<i>FGFR1^a</i>	<i>MLH1</i>	<i>POLE</i>
<i>APC</i>	<i>FGFR2^a</i>	<i>MSH2</i>	<i>PTEN</i>
<i>ARID1A</i>	<i>FLT1^a</i>	<i>MSH3</i>	<i>RNF43</i>
<i>ATM</i>	<i>FLT3^a</i>	<i>MSH6</i>	<i>ROS1</i>
<i>BRAF</i>	<i>FLT4^a</i>	<i>MTOR</i>	<i>SMAD2</i>
<i>CTNNB1</i>	<i>KDR^a</i>	<i>MYC</i>	<i>SMAD4</i>
<i>EGFR</i>	<i>KMT2C</i>	<i>NRAS</i>	<i>SRC^a</i>
<i>ERBB2</i>	<i>KRAS</i>	<i>NTRK3</i>	<i>TCF7L2</i>
<i>ERBB3</i>	<i>LCK^a</i>	<i>PDGFRA^a</i>	<i>TET1</i>
<i>ERBB4</i>	<i>MAP2K4</i>	<i>PDGFRB^a</i>	<i>TP53</i>

In total, 44 genes were evaluated, consisting of nintedanib target genes, commonly altered CRC genes, and somatic cancer-associated mutations. This was a custom-designed gene panel covering all exons of each individual gene and utilizing a Roche SeqCap EZ hybridization capture-based target enrichment method. Sequencing was performed on DNA samples extracted from archival FFPE tumor tissue, plasma-isolated cfDNA, and gDNA isolated from whole blood, which permitted the identification of somatic alterations and enabled comparison between tumor tissue and cfDNA. Abbreviations: cfDNA = cell-free DNA; CRC = colorectal cancer; gDNA = genomic DNA; FFPE = formalin-fixed, paraffin-embedded; NGS = next-generation sequencing.

^aNintedanib target genes.

Supplemental Table 2 Proteins Included in the Plasma Biomarker Analysis

Analyte Name	Abbreviated Name	UniProtKB/ SwissProt ID	HGNC	Type
6Ckine		000585	CCL21	Cytokine
Angiopoietin-1	ANG-1	Q15389	ANGPT1	Growth factor
Angiopoietin-2	ANG-2	O15123	ANGPT2	Growth factor
Cancer antigen 15-3	CA-15-3	NA	NA	NA
Carbonic anhydrase 9	CA-9	Q16790	CA9	Enzyme
Carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	P13688	CEACAM1	Other
Collagen IV		P02462/Q01955	COL4A1/A3	Other
Decorin		P07585	DCN	Other
Endoglin		P17813	ENG	TM receptor
Epidermal growth factor	EGF	P01133	EGF	Growth factor
Epidermal growth factor receptor	EGFR	P00533	EGFR	Kinase
Fatty acid-binding protein, adipocyte	FABP, adipocyte	P15090	FABP4	Transporter
Fibroblast growth factor, basic	bFGF	P09038	FGF2	Growth factor
Granulocyte-macrophage colony-stimulating factor	GM-CSF	P04141	CSF2	Cytokine
Heparin-binding EGF-like growth factor	HB-EGF	Q99075	HBEGF	Growth factor
Hepatocyte growth factor receptor	HGF receptor	P08581	MET	Kinase
Hepsin		P05981	HPN	Peptidase
Human epidermal growth factor receptor 2	HER-2	P04626	ERBB2	Linase
Insulin-like growth factor-binding protein 1	IGFBP-1	P08833	IGFBP1	Other
Insulin-like growth factor-binding protein 2	IGFBP-2	P18065	IGFBP2	Other
Intercellular adhesion molecule 1	ICAM-1	P05362	ICAM1	TM receptor
Interferon gamma	IFN-gamma	P01579	IFNG	Cytokine
Interferon-inducible T-cell alpha chemoattractant	ITAC	O14625	CXCL11	Cytokine
Interleukin-1 alpha	IL-1 alpha	P01583	IL1A	Cytokine
Interleukin-1 beta	IL-1 beta	P01584	IL1B	Cytokine
Interleukin-10	IL-10	P22301	IL10	Cytokine
Interleukin-2	IL-2	P60568	IL2	Cytokine
Interleukin-6	IL-6	P05231	IL6	Cytokine
Interleukin-8	IL-8	P10145	CXCL8	Cytokine
Kallikrein 5	KLK5	Q9Y337	KLK5	Peptidase
Kallikrein-7	KLK7	P49862	KLK7	Peptidase
Macrophage inflammatory protein-3 beta	MIP-3 beta	Q99731	CCL19	Cytokine
Macrophage inflammatory protein-1 alpha	MIP-1 alpha	P10147	CCL3	Cytokine
Macrophage inflammatory protein-1 beta	MIP-1 beta	P13236	CCL4	Cytokine
Macrophage migration inhibitory factor	MIF	P14174	MIF	Cytokine
Maspin		P36952	SERPINB5	Other
Matrix metalloproteinase-3	MMP-3	P08254	MMP3	Peptidase
Matrix metalloproteinase-9	MMP-9	P14780	MMP9	Peptidase
Mesothelin	MSLN	Q13421	MSLN	Other
Monocyte chemotactic protein 1	MCP-1	P13500	CCL2	Cytokine
Placenta growth factor	PIGF	P49763	PGF	Growth factor
Platelet endothelial cell adhesion molecule	PECAM-1	P16284	PECAM1	Other
Platelet-derived growth factor BB	PDGF-BB	P01127	PDGFB	Growth factor
Stem cell factor	SCF	P21583	KITLG	Growth factor
Stromal cell-derived factor-1	SDF-1	P48061	CXCL12	Cytokine
Tenascin-C	TN-C	P24821	TNC	Other
Transforming growth factor alpha	TGF-alpha	P01135	TGFA	Growth factor
Tumor necrosis factor alpha	TNF-alpha	P01375	TNF	Cytokine
Tumor necrosis factor beta	TNF-beta	P01374	LTA	Cytokine
Urokinase-type plasminogen activator receptor	uPAR	Q03405	PLAUR	TM receptor
Vascular endothelial growth factor	VEGF	P15692	VEGFA	Growth factor
Vascular endothelial growth factor B	VEGF-B	P49765	VEGFB	Growth factor

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Supplemental Table 2 Continued

Analyte Name	Abbreviated Name	UniProtKB/ SwissProt ID	HGNC	Type
Vascular endothelial growth factor D	VEGF-D	O43915	FIGF	Growth factor
Vascular endothelial growth factor receptor 1	VEGFR-1	P17948	FLT1	Kinase
Vascular endothelial growth factor receptor 2	VEGFR-2	P35968	KDR	Kinase
Vascular endothelial growth factor receptor 3	VEGFR-3	P35916	FLT4	TM receptor

Abbreviations: NA = no annotation provided by Myriad RBM; TM = transmembrane.

Supplemental Table 3 Baseline Characteristics (Overall Population, and Plasma Protein, Tumor Tissue, cfDNA, and Tumor Tissue/cfDNA Concordance Sub-Populations)

	Total Population, n (%)		Plasma Protein Sub-population, n (%)		Tumor Tissue Sub-population, n (%)		cfDNA Sub-population, n (%)		Concordance Population, ^a n (%)	
	Nintedanib (n = 386)	Placebo (n = 382)	Nintedanib (n = 368)	Placebo (n = 367)	Nintedanib (n = 125)	Placebo (n = 120)	Nintedanib (n = 104)	Placebo (n = 88)	Nintedanib (n = 88)	Placebo (n = 82)
Mean age, y (range)	61.0 (22-85)	61.1 (23-83)	61.0 (28-85)	61.4 (28-82)	59.8 (30.0-85)	59.9 (28-82)	60.8 (28-85)	60.5 (28-82)	60.9 (34-85)	61 (28-82)
Gender (male)	236 (61.1)	218 (57.1)	221 (60.1)	211 (57.5)	74 (59.2)	61 (50.8)	59 (56.7)	47 (53.4)	49 (55.7)	43 (52.4)
Race										
Caucasian	279 (72.3)	268 (70.2)	261 (70.9)	253 (68.9)	91 (72.8)	82 (68.3)	84 (80.8)	62 (70.5)	69 (78.4)	57 (69.5)
Asian	97 (25.1)	104 (27.2)	97 (26.4)	104 (28.3)	31 (24.8)	34 (28.3)	19 (18.3)	25 (28.4)	18 (20.5)	24 (29.3)
Other	5 (1.3)	3 (0.8)	5 (1.4)	3 (0.8)	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing ^b	5 (1.3)	9 (2.4)	5 (1.4)	9 (2.5)	2 (1.6)	4 (3.3)	1 (1.0)	1 (1.1)	1 (1.1)	1 (1.2)
ECOG PS at baseline										
0	162 (42.0)	142 (37.2)	152 (41.3)	135 (36.8)	48 (38.4)	42 (35.0)	41 (39.4)	32 (36.4)	34 (38.6)	30 (36.6)
1	223 (57.8)	240 (62.8)	215 (58.4)	232 (63.2)	76 (60.8)	78 (65.0)	62 (59.6)	56 (63.6)	53 (60.2)	52 (63.4)
> 1	1 (0.3)	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.8)	0 (0.0)	1 (1.0)	0 (0.0)	1 (1.1)	0 (0.0)
Time from onset of metastatic disease until randomization, mos										
< 24	108 (28.0)	110 (28.8)	102 (27.7)	109 (29.7)	45 (36.0)	36 (30.0)	34 (32.7)	29 (33.0)	32 (36.4)	27 (32.9)
≥ 24	278 (72.0)	272 (71.2)	266 (72.3)	258 (70.3)	80 (64.0)	84 (70.0)	70 (67.3)	59 (67.0)	56 (63.6)	55 (67.1)
Primary site of disease										
Colon	227 (58.8)	206 (53.9)	217 (59.0)	198 (54.0)	77 (61.6)	68 (56.7)	62 (59.6)	51 (60.0)	55 (62.5)	47 (57.3)
Rectum	130 (33.7)	154 (40.3)	124 (33.7)	148 (40.3)	39 (31.2)	46 (38.3)	33 (31.7)	31 (35.2)	28 (31.8)	30 (36.6)
Cecum	29 (7.5)	21 (5.5)	27 (7.3)	20 (5.4)	9 (7.2)	6 (5.0)	9 (8.7)	6 (6.8)	5 (5.7)	5 (6.1)
Other/unknown	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Previous treatments										
Prior lines of systematic anticancer therapies, mean (SD)	3.9 (1.8)	3.9 (1.8)	3.9 (1.8)	3.8 (1.8)	3.7 (1.79)	3.8 (1.76)	3.8 (1.95)	3.8 (1.78)	3.8 (1.88)	3.8 (1.74)
≥ 3	297 (76.9)	296 (77.5)	285 (77.4)	285 (77.7)	91 (72.8)	93 (77.5)	77 (74.0)	66 (75.0)	65 (73.9)	62.9 (75.6)
Oxaliplatin	386 (100.0)	382 (100.0)	368 (100.0)	367 (100.0)	125 (100.0)	120 (100.0)	104 (100.0)	88 (100.0)	88 (100.0)	82 (100.0)
Fluoropyrimidine	386 (100.0)	382 (100.0)	368 (100.0)	367 (100.0)	125 (100.0)	120 (100.0)	104 (100.0)	88 (100.0)	88 (100.0)	82 (100.0)
Irinotecan	386 (100.0)	382 (100.0)	368 (100.0)	367 (100.0)	125 (100.0)	120 (100.0)	104 (100.0)	88 (100.0)	88 (100.0)	82 (100.0)
Bevacizumab	368 (95.3)	370 (96.9)	352 (95.7)	355 (96.7)	119 (95.2)	117 (97.5)	99 (95.2)	86 (97.7)	84 (95.5)	81 (98.8)
Aflibercept	48 (12.4)	47 (12.3)	45 (12.2)	44 (12.0)	18 (14.4)	15 (12.5)	12 (11.5)	10 (11.4)	10 (11.4)	8 (9.8)
Regorafenib	141 (36.5)	144 (37.7)	135 (36.7)	140 (38.1)	41 (32.8)	41 (34.2)	39 (37.5)	28 (31.8)	33 (37.5)	26 (31.7)
Trifluridine/tipiracil	55 (14.2)	53 (13.9)	55 (14.9)	53 (14.4)	11 (8.8)	11 (9.2)	9 (8.7)	9 (10.2)	8 (9.1)	8 (9.8)

Supplemental Table 3 Continued

	Total Population, n (%)		Plasma Protein Sub-population, n (%)		Tumor Tissue Sub-population, n (%)		cfDNA Sub-population, n (%)		Concordance Population, ^a n (%)	
	Nintedanib (n = 386)	Placebo (n = 382)	Nintedanib (n = 368)	Placebo (n = 367)	Nintedanib (n = 125)	Placebo (n = 120)	Nintedanib (n = 104)	Placebo (n = 88)	Nintedanib (n = 88)	Placebo (n = 82)
Other	331 (85.8)	331 (86.6)	314 (85.3)	317 (86.4)	101 (80.8)	103 (85.8)	76 (86.4)	84 (80.8)	70 (79.5)	70 (85.4)
Prior radiotherapy	104 (26.9)	132 (34.6)	97 (26.4)	123 (33.5)	31 (24.8)	44 (36.7)	27 (26.0)	28 (31.8)	22 (25.0)	27 (32.9)
KRAS wild-type and other RAS wild-type patients only	Nintedanib (n = 158)	Placebo (n = 176)	Nintedanib (n = 150)	Placebo (n = 171)	Nintedanib (n = 40)	Placebo (n = 55)	Nintedanib (n = 39)	Placebo (n = 40)	Nintedanib (n = 32)	Placebo (n = 38)
Prior cetuximab or panitumumab	158 (100.0)	175 (99.4)	150 (100.0)	170 (99.4)	40 (100.0)	55 (100.0)	39 (100.0)	40 (100.0)	32 (100.0)	38 (100.0)

Abbreviations: cfDNA = cell-free DNA; ECOG PS = Eastern Cooperative Oncology Group performance status; NS = not specified; SD = standard deviation.

^aSubpopulation with matched tumor tissue and cfDNA samples.

^bCan include patients living in a country where race is not allowed to be collected.

Supplemental Table 4 Co-primary Endpoints (Overall Population and Biomarker Subgroups)

Cohort	Patients Placebo Nintedanib	Events Placebo Nintedanib	HR (95% CI)
Overall survival			
Overall LUME-Colon 1 population (N = 735)	367 368	286 306	1.04 (0.88-1.22)
Tumor tissue			
CMS cohort (n = 231)	114 117	86 90	0.87 (0.65-1.17)
Mutations CNV cohort (n = 157)	84 74	61 60	0.97 (0.67-1.39)
cfDNA (n = 192)	88 104	66 85	0.96 (0.69-1.33)
Protein biomarker (n = 735)	367 368	286 306	1.04 (0.88-1.22)
Progression-free survival			
Overall LUME-Colon 1 population (N = 735)	367 368	325 334	0.66 (0.57-0.78)
Tumor tissue			
CMS cohort (n = 231)	114 117	98 110	0.60 (0.45-0.79)
Mutations CNV cohort (n = 157)	83 74	72 68	0.60 (0.43-0.85)
cfDNA (n = 192)	88 104	74 102	0.46 (0.33-0.65)
Protein biomarker (n = 735)	367 368	325 334	0.66 (0.57-0.78)

Abbreviations: cfDNA = Cell-free DNA; CI = confidence interval; CMS = consensus molecular subtype; CNV = copy number variation; HR = hazard ratio.

Supplemental Table 5 Distribution and Clinical Characteristics of CMS Subgroups (Pooled Nintedanib and Placebo Arms)

CMS Category	Sample Site, n (%)		Sidedness, n (%)		Incidence of Mutations, n (%)				
						KRAS	BRAF	APC	TP53
CMS1 (N = 4)	Primary	2 (50.0)	Right	0 (0.0)	Samples	3			
	Metastasis	2 (50.0)	Left	0 (0.0)	Mutations	0 (0.0)	0 (0.0)	3 (100.0)	1 (33.3)
	NA	0 (0.0)	NA	4 (100.0)					
CMS2 (N = 64)	Primary	50 (78.1)	Right	8 (12.5)	Samples	39			
	Metastasis	11 (17.2)	Left	31 (48.4)	Mutations	14 (35.9)	1 (2.6)	33 (84.6)	15 (38.5)
	NA	3 (4.7)	NA	25 (39.1)					
CMS3 (N = 2)	Primary	2 (100.0)	Right	0 (0)	Samples	1			
	Metastasis	0 (0.0)	Left	1 (50.0)	Mutations	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)
	NA	0 (0.0)	NA	1 (50.0)					
CMS4 (N = 119)	Primary	96 (80.7)	Right	11 (9.2)	Samples	75			
	Metastasis	17 (14.3)	Left	54 (45.4)	Mutations	33 (44.0)	0 (0.0)	47 (62.7)	39 (52.0)
	NA	6 (5.0)	NA	54 (45.4)					
Unclassified/mixed phenotype (N = 42)	Primary	32 (76.2)	Right	4 (9.5)	Samples	25			
	Metastasis	7 (16.7)	Left	18 (42.9)	Mutations	9 (36.0)	0 (0.0)	18 (72.0)	12 (48.0)
	NA	3 (7.1)	NA	20 (47.6)					

Abbreviations: CMS = Consensus molecular subtype; NA = not available.

Biomarker Analysis: Nintedanib in LUME-Colon 1

Supplemental Table 6 Predictive Analysis of CMS Subgroups

	CMS4		Other ^a (Non-CMS4)	
	Placebo	Nintedanib	Placebo	Nintedanib
Overall survival				
Number of patients, N (%)	59 (100.0)	60 (100.0)	55 (100.0)	57 (100.0)
OS events, n (%)	41 (69.5)	48 (80.0)	45 (81.8)	42 (73.7)
Median time to event, months (95% CI) ^b	6.4 (4.5-8.4)	7.5 (5.3-9.5)	6.6 (4.4-8.0)	6.9 (6.2-8.9)
Nintedanib vs. placebo, HR (95% CI) ^c	0.92 (0.60-1.41)		0.81 (0.53-1.25)	
Interaction <i>P</i> -value ^d	.6634			
Progression-free survival (central assessment)				
Number of patients, N (%)	59 (100.0)	60 (100.0)	55 (100.0)	57 (100.0)
PFS events, n (%)	50 (84.7)	55 (91.7)	48 (87.3)	55 (96.5)
Median time to event, months (95% CI) ^b	1.3 (1.3-1.4)	2.2 (1.4-2.7)	1.4 (1.3-1.4)	2.6 (1.4-2.7)
Nintedanib vs. placebo, HR (95% CI) ^c	0.60 (0.40-0.91)		0.56 (0.37-0.85)	
Interaction <i>P</i> -value ^d	.8278			

Abbreviations: CI = Confidence interval; CMS = consensus molecular subtype; HR = hazard ratio; OS = overall survival; PFS = progression-free survival.

^aOther includes all evaluable patients not classified as CMS4.

^bEstimated from an unadjusted Kaplan-Meier curve for each treatment arm.

^cHR < 1 favors nintedanib; model adjusted for treatment, regorafenib pre-treatment, time from onset of metastatic disease until randomization, and region.

^dModel adjusted for the 3 stratification factors: treatment, subgroup, and treatment by subgroup interaction.