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IMPACT OF KRAS/NRAS MUTATIONAL HETEROGENEITY ON CLINICAL OUTCOMES IN COLORECTAL CANCER

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A

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Jonathan M. Loree, MD

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Dedication

To Allison, my amazing wife. Thank you for all of your support on this adventure. You give me the strength to take on new challenges and are my best friend.

To my parents, thank you for being a continual source of love and support in everything I do.

To my patients, the desire to make things better is my motivation for research and your strength inspires me to do more.

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Abstract

IMPACT OF *KRAS/NRAS* MUTATIONAL HETEROGENEITY ON CLINICAL OUTCOMES IN COLORECTAL CANCER

Jonathan Michael Loree, MD

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Introduction: Mutations in *KRAS/NRAS* (*RAS*) predict a lack of benefit from anti-EGFR agents in metastatic colorectal cancer (mCRC). As next generation sequencing (NGS) has advanced, we are discovering atypical and low allele frequency mutations. We aimed to evaluate how NGS can optimally define *RAS* mutant CRC and the role of relative mutant allele frequency (rMAF) as a biomarker.

Methods: Using institutional and public cohorts of mCRC patients with NGS results, we described the prevalence and clinical impact of atypical (not in current guidelines) and low rMAF *RAS* mutations (<50%). rMAF was defined by dividing *RAS* MAF by the MAF of the mutated gene with the highest allele frequency to normalize for tumor content. Functional annotation of 113 *RAS* mutations was performed and functionality of mutations was compared to rMAF.

Results: *RAS* mutations were noted in 4244/8609 patients (49.3%), with atypical mutations in 1.3% of patients. The most prevalent atypical mutations were *KRAS* Q22K (0.2%), *KRAS* D33E (0.1%) and *KRAS* T50I (0.1%). Of 113 functionally characterized *RAS* mutations, all 23 non-activating mutations were atypical, while every guideline cited mutation was activating. Atypical variants (HR 2.45, P=0.0092) and those that resulted in MAPK activity greater than *KRAS* exon 2 (HR 1.40, P=0.028) had a worse OS. A *RAS* rMAF >50% was associated with worse OS than rMAF <50% in one of two cohorts (P= 0.075 & P=0.0058) and having any *RAS* mutation was

associated with a worse OS than wild-type patients. The rMAF of any mutated gene was also associated with functional significance in a clinically annotated database, yet the magnitude of difference in rMAF was not sufficient to warrant clinical utility in tissue cohorts. However, a cfDNA cohort did show striking results demonstrating rMAF was associated with a variants functional characterization.

Conclusions: Through a comprehensive atlas of *RAS* functional characterization, we show that several atypical variants appear clinically relevant. Although rMAF was not useful in characterizing variants as damaging, our findings that *RAS* rMAF is associated with prognosis suggests allele frequency may be useful information in standard clinical reports.

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Chapter 1: Introduction

Colorectal Cancer

Colorectal Cancer (CRC) is the third leading cause of cancer for both men and women in the United States, resulting in an estimated 135,430 new cases and 50,260 deaths in 2017.[1] Although surgical resection is curative for many, 21% of patients present with synchronous metastases at the time of initial diagnosis and 10-25% of stage II/III patients will recur.[2] Curative intent surgical or ablative maneuvers are options in select cases of oligometastatic CRC and result in improved overall survival (OS).[3] However, most patients with metastatic CRC (mCRC) are not curable and will require systemic therapy. In the first and second line setting, this typically consists of a fluoropyrimidine doublet (FOLFOX/CAPOX or FOLFIRI/CAPIRI) combined with a biologic agent that either targets angiogenesis (bevacizumab, ramicurumab, or ziv-aflibercept), or the epidermal growth factor receptor (EGFR) (panitumumab or cetuximab) in patients without *KRAS/NRAS* (*RAS*) mutations [Figure 1].[4–6] First line FOLFOXIRI +/- bevacizumab can also be considered, particularly in patients requiring maximal response or those with aggressive biology.[7–9] Subsequent therapeutic options in the ≥3rd line setting include anti-EGFR +/- cytotoxics (in *RAS* wild type patients who have not previously progressed on cetuximab or panitumumab), regorafenib, TAS-102 and investigational agents.[10–13]

Though cytotoxic agents represent the backbone of systemic therapy, advances in the molecular characterization of mCRC and the advent of targeted therapies are expanding treatment options for patients beyond conventional chemotherapy. The recent approvals of pembrolizumab and nivolumab in mCRC with microsatellite instability (MSI) or mismatch repair deficiency (MMRd) are representative examples of both the successes and challenges of

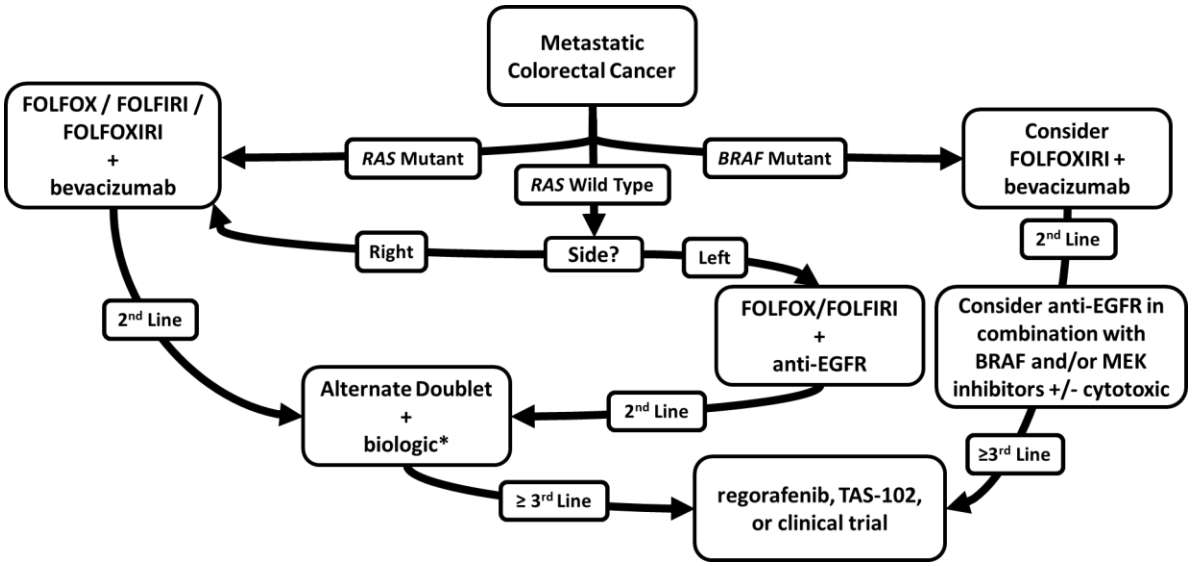


Figure 1. Treatment algorithm for microsatellite stable metastatic colorectal cancer.

Reproduced with permission from: Loree JM, Kopetz S. Recent developments in the treatment of metastatic colorectal cancer. *Ther Adv Med Oncol*. 2017;9(8):551-564.

targeted agents in mCRC. Though response rates were 26-40% with anti-PD1 therapy, they were largely limited to the ~5% of mCRC patients who have MSI-H tumors.[14, 15] Successes have been noted in several other small subgroups with molecularly matched therapies. In the 8-10% of patients with *BRAF* mutated mCRC, the addition of vemurafenib to irinotecan and cetuximab resulted in improved progression free survival (PFS) (HR 0.42, 95% CI 0.26-0.66, P<0.001) in SWOG 1406.[16] For the 3-4% of *ERBB2* amplified mCRC, the HERACLES trial with trastuzumab and lapatinib had a 30% response rate (RR) in heavily pre-treated patients, while trastuzumab and pertuzumab had a 23% RR in the MyPathway basket study.[17, 18] Targeted agents represent promising new strategies in treating mCRC, however defining the ideal population for each of these treatments is essential to their appropriate inclusion into standard of care treatment algorithms.

KRAS/NRAS (*RAS*) Mutations in Colorectal Cancer

Alterations in the canonical mitogen-activated protein kinase (MAPK) pathway are key determinants of clinical course in mCRC and are present in an estimated 59% of non-hypermutated CRC and 80% of hypermutated tumors.[19] While there is significant cross talk between the MAPK-ERK pathway and other signaling cascades, such as the PIK3CA/AKT and Wnt pathways, we will focus on the implications of alterations in the MAPK-ERK pathway downstream of the epidermal growth factor receptor (EGFR). Alterations in these genes have been shown to be associated with resistance to the anti-EGFR agents cetuximab and panitumumab. Key proteins involved in this pathway include EGFR, *KRAS*/N*RAS*, BRAF, MEK 1/2, and ERK 1/2.

In a simplified version of this signaling cascade [Figure 2], extracellular ligand binding to growth factor receptors, such as the EGFR transmembrane receptor, results in activation of the

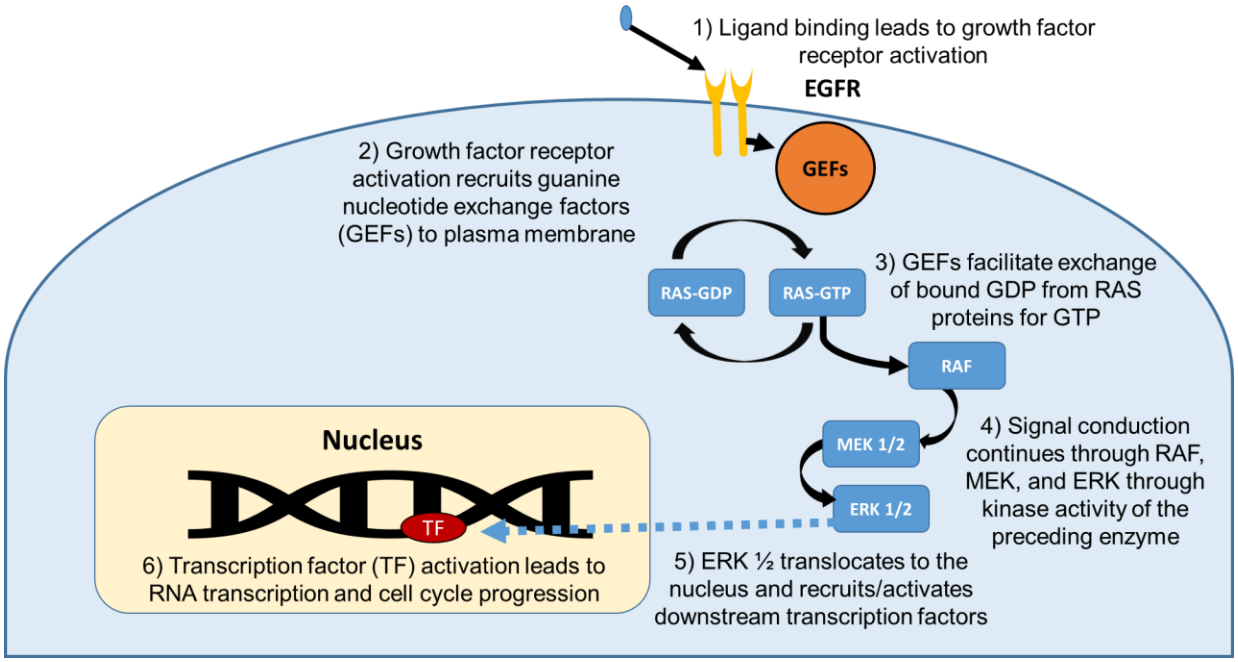


Figure 2. Simplified schematic of MAPK signaling in colorectal cancer.

cascade. Following activation, these receptors recruit guanine nucleotide exchange factors (GEFs) to the plasma membrane where RAS proteins are anchored. The geographic coupling of GEFs near RAS results in GEFs catalyzing the exchange of bound GDP for GTP.[20] The RAS family of proteins are GTPases whose functional status is determined by the phosphorylation state of bound guanine. RAS assumes an active state when bound to GTP and is subsequently able to activate downstream effectors, such as the RAF family of proteins. BRAF is the stereotypical RAF family protein with clinical significance in mCRC. Activated BRAF phosphorylates and activates the kinase enzymes MEK1/2 which then phosphorylate ERK1/2. Activated ERK1/2 subsequently translocates into the nucleus and causes downstream activation of transcription factors and cell cycle progression.

Though alterations are possible at each step in the MAPK-ERK pathway, *RAS* mutations are the most common.[21, 22] In early stage disease, the prognostic impact of *RAS* alterations is unclear. While the Alliance NO147 trail showed the strongest evidence that these mutations may have prognostic impact, numerous other trials and retrospective studies have not shown consistent results.[23–25] Following curative intent resection of liver metastases, we have shown that *RAS* mutations are associated with a worse median overall survival (OS) when *RAS* is considered alone (48 vs 71 months, P<0.001) and a worse relapse free survival (RFS) following hepatectomy when co-mutated with *TP53* (9 vs 11 months, P<0.001).[26] In the metastatic setting, *RAS* mutations appear to have a prognostic impact on OS in the era of anti-EGFR based therapies, however may not have clinical implications independent of these therapies.[27] In trials assessing the impact of anti-EGFR based therapies with a control arm (NCIC CO.17 and 20020408), the OS and PFS point estimates for *RAS* mutant and *RAS* wild type patients receiving best supportive care appear to overlap, suggesting that these molecular alterations are only responsible for altering survival in the setting of targeted therapies.[10, 11, 28]

The major clinical relevance of these variants stems from the fact that *RAS* mutations are a predictive biomarker of a lack of response to anti-EGFR agents. Testing for these mutations has become a mandatory companion test prior to treatment with cetuximab or panitumumab due to this predictive capacity.[10, 29] Identification of patients with these alterations is important not only to avoid the potential toxicity and financial implications of ineffective therapy, but *RAS* mutations may also predict harm in the setting of anti-EGFR treatment. In the PRIME study, *RAS* mutant mCRC patients treated with panitumumab + FOLFOX4 had a worse mPFS than patients who received FOLFOX4 alone (HR 1.31, 95% CI 1.07-1.60, P=0.008).[28, 30]

Expanding the Definition of *RAS* Mutant Metastatic Colorectal Cancer

Although mutations in *KRAS* exon 2 were the first noted predictive alterations, extended *RAS* testing to include *KRAS/NRAS* exon 2 (codon 12 & 13), exon 3 (codon 59 & 61), and exon 4 (codon 117 and 146) is now considered the standard of care.[28, 29] Including extended *RAS* mutations, 55.9% of mCRC patients are predicted to have an alteration that would result in a lack of benefit from anti-EGFR agents.[31] While the evidence to support *KRAS* exon 2 mutations as a predictive biomarker is quite firm, with a positive interaction test in a placebo controlled trial, the predictive nature of many of the less common variants remains unclear.[10] For example, only 7 patients with codon 59 mutations were identified in the PRIME trial that demonstrated extended *RAS* variants had clinical relevance.[28] These mutations were not part of the extended *RAS* mutation analysis, but rather were assessed in a post-hoc analysis that showed that removing them from the wild type population resulted in a smaller hazard ratio favoring FOLFOX + panitumumab.[30] Case reports demonstrating activity of anti-EGFR agents for patients with *RAS* mutations are numerous and even among exon 2 mutations, there has been much exploration about whether all variants are equally relevant.[32, 33] Prior retrospective studies suggested that patients with *KRAS* G13D mutations had improved OS and PFS following treatment with anti-EGFR agents compared to other *KRAS* mutations.[34, 35] This

resulted in the prospective ICECREAM trial which attempted to validate the hypothesis that *KRAS* G13D mutated mCRC may still benefit from anti-EGFR treatment. Unfortunately, there were no responses in G13D mutated mCRC patients in the trial.[36]

Beyond extended *RAS* mutations, other "atypical" variants in *KRAS* and *NRAS* have been noted and it remains unclear whether these variants are functionally activating or have clinical relevance.[37] As sequencing capacity improves and we move beyond single gene and hot-spot annotation, these mutations are likely to become of increasing importance. Despite G13D being one of the most common *RAS* mutations, the ICECREAM study took over 2 years to recruit 53 patients and demonstrated the difficulty in studying rare variants prospectively. If a similar prospective strategy is utilized to validate all of the rare atypical variants that are being discovered, it is unlikely that we will be successful in answering our question as to whether these variants have clinical relevance and alternative strategies are required.

Beyond defining the individual mutations of relevance within *RAS*, understanding the impact of tumor heterogeneity and low frequency mutations in these genes raises further questions about defining the optimal population for treatment with anti-EGFR agents. While traditional PCR or Sanger sequencing identified mutations if >10% of cells had a mutation, newer techniques have sensitivities to detect variants in as few as 0.01% of cells.[38, 39] These low frequency mutations may be responsible for treatment failure of anti-EGFR therapies in patients previously defined as wild type. In the CAPRI-GOIM trial, the use of a more sensitive next generation sequencing (NGS) assay than standard of care testing resulted in a further 15.9% of patients being identified as *RAS* mutant despite similar assay coverage.[40] These patients had inferior outcomes to *RAS* wild type patients and appeared to have a similar prognosis to high allele frequency *RAS* mutant patients. These findings have been demonstrated by numerous other groups, however it remains unclear if there is an allele frequency threshold at which anti-EGFR agents may still be active.[41–43] In CRYSTAL, the use of high sensitivity BEAMing to

determine *RAS* status was able to evaluate allele frequencies down to 0.1%.[44] This demonstrated that at low but detectable allele frequencies, the addition of anti-EGFR agents may still benefit patients (HR 0.57, 95% CI 0.33-1.01) and there was a gradual increase in the HR favoring FOLFIRI alone as the allele frequency of *RAS* variants increased. Taken together, it seems reasonable that both the specific *KRAS/NRAS* variant and the allele frequency of a mutation may have clinical impact, however these concepts remain relatively unexplored.

Summary of Introduction and Specific Aims of Thesis

Over the past two decades there have been considerable advances in the treatment of CRC, with the introduction of new cytotoxic and targeted agents. Molecularly targeted agents are an important step forward in oncology, however are heavily reliant on predictive biomarkers to select the right patient for the right drug. Mutations in *RAS* have been shown to predict a lack of benefit from anti-EGFR agents and are present in a large proportion of mCRC patients. However, there is significant heterogeneity in the clinical course of *RAS* mutant patients and many *RAS* wild type patients still fail to respond to anti-EGFR therapy. As sequencing technology has advanced, we are now becoming aware of novel variants that are either at locations that were previously not sequenced or at allele frequencies that were not detected. Further characterization of these mutations and a greater understanding of how tumor heterogeneity impacts outcomes is required and we are left with the most important scientific question remaining unanswered. How do we apply these findings to our patients? The specific aims of this thesis are:

Aim 1: To describe the functional and clinical significance of atypical *RAS* mutations.

Aim 2: To assess the impact of *RAS* mutant allele frequency on clinical outcomes in mCRC.

Aim 3: To evaluate whether allele frequency of mutations can be used as a predictor of functional impact.

Chapter 2: Materials and Methods

Description of Study Population and Development of Cohorts

Two separate clinical cohorts were built to facilitate the analyses performed in this thesis at MD Anderson (MDA) with data extracted from a mixture of sources including the MDA tumor registry, the MDA Pathology Department, and from clinical chart review.

1. MDA T200 Cohort – This cohort consists of 207 patients with mCRC who had a 201 gene NGS panel performed on their tumor. These patients were seen at MDA between January 1, 2012 and September 1, 2016 and the database has an OS event rate of 87.4%. Available variables include age, gender, date of diagnosis, stage at diagnosis, date of stage IV diagnosis, site of biopsy, date of death or last follow up, vital status, histology, MSI status, tumor location, tumor content in biopsy, and mutation status including single nucleotide variants (SNV), insertions/deletions (indels), allele frequency and copy number alterations.
2. MDA CMS 46 Cohort – This cohort consists of 1877 patients with mCRC who had a 46 gene NGS panel performed on their tumor. These patients were seen at MDA between January 1, 2012 and September 1, 2016 and the database has an OS event rate of 58.7%. Available variables include age, gender, date of diagnosis, stage at diagnosis, date of stage IV diagnosis, site of biopsy, date of death or last follow up, vital status, histology, MSI status, tumor location, tumor content in biopsy and mutation status including single nucleotide variants and allele frequency.

In addition to the 2 cohorts described above, 5 separate cohorts with NGS results but minimal clinical annotation were utilized to facilitate assessment of atypical *RAS* mutation prevalence. These cohorts included:

1. cfDNA Cohort – This cohort consists of 1397 CRC patients who underwent cfDNA testing between June 1, 2014 and May 18, 2016 with a Guardant360^{TM} NGS assay globally. Patients who had an assay without a variant detected were not included in the

analysis (272 patients excluded). The Guardant360™ assay has been previously described and was performed centrally by Guardant Health.[45] The assay has a predicted sensitivity of 0.1% mutant allele frequency.

2. Project Genie Cohort – Publicly available data from the AACR Project Genie was used and consists of 2081 patients with CRC who had an NGS panel performed at 1 of 8 international centers. Each center that is part of Project Genie utilizes its own sequencing assay and bioinformatic pipeline, each of which are described in Project Genie's data guide.[46]
3. Caris Life Sciences Molecular Diagnostics Cohort – In collaboration with Caris Life Sciences, we have assessed their sequencing database for the prevalence of all *RAS* mutations detected using their NGS assay in CRC patients. No clinical annotation or mutational data outside of the *KRAS/NRAS* genes is available for this data set.
4. The Cancer Genome Atlas (TCGA) CRC Cohort – Publicly available data from 228 patients in the TCGA colorectal characterization was downloaded to assess atypical *RAS* mutation prevalence in this cohort which includes whole exome sequencing results.[19] Clinical annotation was not downloaded.
5. Nurses' Health Study/ Health Professionals Study (NHS/HPFS) Colorectal Cohort – Publicly available data from 619 patients in the NHS/HPFS with colorectal cancer and whole exome sequencing was downloaded to assess atypical *RAS* mutation prevalence in this cohort which includes whole exome sequencing results.[47]

Molecular Techniques

Next Generation Sequencing for MDA CMS 46 Cohort

Tumor sequencing was performed in the CLIA environment of MDA's Molecular Diagnostics Laboratory. Archival formalin-fixed paraffin embedded (FFPE) samples from either surgical resection specimens or tissue biopsies with >20% tumor content were used for sequencing after macro-dissection. Sequencing results from primary tumors or metastatic tumor biopsies

were considered together as prior evidence suggests a high level of concordance between primary and metastatic lesions.[29] DNA extraction used a PicoPure DNA extraction kit (Arcturus, Mountain View, CA) and was purified using an Agencourt AMPure XP kit (Agencourt Biosciences, Beverly, MA). DNA quantification was performed with a Qubit DNA assay kit (ThermoFisher Scientific, Waltham, MA). Library preparation was performed using the Ion Torrent AmpliSeq 2.0 Beta kit and Ion Torrent Ampliseq Cancer Panel Primers (Life Technologies, Grand Island, NY). Until September 10, 2013, version 1.0 of the panel which included 46 cancer related genes was utilized. Subsequently, version 2.0 of the panel was used which included an additional 4 genes. For patients who had version 2.0, only the 46 genes used for the entire cohort were assessed. Sequencing was performed using an Ion Torrent Personal Genome Machine Sequencer and adequately covered amplicons were defined as those with a depth of ≥250X. Sequence alignment and base calling were performed using the Torrent Suite software version 2.01 and variant calling was performed with Torrent Variant Caller software version 1.0 with Human Genome Build 19 as the reference. Routine germ line testing was not performed. Tested codons and genes have been previously described[48].

Next Generation Sequencing for MDA T200 Cohort

DNA extraction and purification was performed using the same techniques as the CMS46 cohort, however matched germline DNA was also extracted and sequenced. The T200 panel is a capture based targeted exome panel that provides high depth coverage for all exons in 201 cancer-related genes and provides information regarding mutations, indels, and copy number alterations. The panel has previously been described and contains 4874 exons encoding 938,607 bases.[49] DNA from each sample is sheared by sonication and prepared for library creation using the KAPA library prep kit (Woburn, MA). Targeted capture is performed with biotin labeled Roche Nimblegen DNA probes (Indianapolis, IN). The capture process occurred via the manufacturer's protocol. Captured libraries were sequenced on an Ilumina HiSeq 2000

(San Diego, CA) on a version 3 TruSeq paired end flow cell. FASTQ files were demultiplexed using CASAVA 1.8.2 and regions required >20 reads to be considered adequately covered. Sequence alignment was performed with BWA using human reference genome hg19 and duplicated reads were removed using Picard.[50, 51] VarScan2 was used to call single nucleotide variants (SNVs), and copy number was called using Lonigro et al's previously published pipeline.[52, 53] Sequencing results from tumor and germline were compared and germline variants were filtered from tumor sequencing results.

Microsatellite Instability (MSI) Testing

MSI status was retrospectively reviewed from patient's charts for both MDA cohorts and was only evaluated in patients who had testing performed as part of their standard care. Testing consisted of a mixture of immunohistochemical (IHC) staining for mismatch repair protein deficiency (MLH1, MSH2, PM2, MSH6) and PCR based assessment of microsatellite status. For the PCR based assessment, a total of 7 markers (BAT25, BAT26, D2S123, D5S346, D17S250, TGFβRII, and BAT40) were tested in a multiplex PCR assay that incorporates fluorescently labeled primers to detect instability of nucleotide repeats in microsatellites. Tumors were defined as MSI-H if either method (IHC or PCR) of detection was abnormal.

Relative Impact of Atypical *RAS* Mutations on Outcomes

Prevalence of *RAS* Mutations

In order to determine the prevalence of specific *RAS* variants and their corresponding categorization we utilized internal, external, and publicly available databases that included NGS results from patients with CRC. A total of 8609 patients were included in the analysis from 7 cohorts [Table 1]. For patients with more than one mutation in *KRAS* or *NRAS*, variants were considered independently for calculating the specific mutations prevalence. However, these patients were categorized into typical (*KRAS* codon 12 & 13), extended (*KRAS* codon 59, 61, 117, 146 or *NRAS* codon 12, 13, 59, 61, 117, 146), or atypical (all other *KRAS*/*NRAS* variants) with preference given in descending order from typical to extended to atypical mutations for all

Table 1. Cohorts utilized to characterize relative prevalence of *RAS* mutations in colorectal cancer and their characteristics.

Cohort	**MDA CMS 46**[69]	**MDA T200**[49]	**CARIS**	**Project Genie**[46]	**TCGA**[19]	**NHS & HPFS**[47]	**Guardant 360**[45]
Number of Patients	1877	207	2200	2081	228	619	1397
RAS Coverage	Hot spot	All exons	All exons	Mixed	All exons	All exons	All exons
Assay Type	Multiplex	Capture Based	Multiplex	Mixed	Exome	Exome	cfDNA
Assay Depth	≥250X	Median 906X (tumor)	>750X	Varied by Platform	>20X for 80% of exons	Median 88X (tumor)	8000X
Tumor Cellularity	>20%	>20%	>20%	>10%	≥60%	Average 45%	n/a
Stage of Patients	Stage IV	Stage IV	Stage IV	Mostly Stage IV	Stage I-IV	Stage I-IV	Mostly Stage IV
Publicly Available	No	No	No	Yes	Yes	Yes	No

further analysis. *RAS* mutations were categorized into typical, extended and atypical variants based on prior literature.[29]

Functional Characterization of *RAS* Mutations

NovellusDx Functional Annotation for Cancer Treatment (FACT) Assay

Functional significance was assessed for all *RAS* variants (a) detected at MDA among patients who received a CMS 46 NGS assay for any malignancy, (b) present in a CRC patient in the CARIS Life Sciences Molecular Diagnostics database, or (C) noted to be of clinical significance or with prior functional annotation in PubMed or COSMIC.[62] For example, *KRAS* P34R has been associated with cardiofaciocutaneous syndrome (CFC) and *KRAS* T58I is associated with Noonan syndrome. As these have both been well characterized to increase cellular proliferation, decrease *KRAS* GTPase activity, and stimulate down-stream phosphorylation of MEK, they served as a reasonable control for activating atypical alterations.[63, 64]

Characterization occurred using the NovellusDx FACT Assay in a CLIA-certified laboratory. Mutations were generated over a wild-type transfection vector and co-transfected into a HeLa cell line based assay with a GFP-ERK reporter construct. Upon phosphorylation and activation, ERK moves from the cytoplasm to the nucleus. Using this assay, MAPK pathway activation was characterized as the relative localization of GFP-ERK to the nucleus using fluorescent microscopy for detection. Each mutation was then normalized to a wild-type transfection by establishing the assay output of wild type transfection as a value of 1.0, and converting all other measurements to fold change from wild-type. Within each run of the assay, thousands of cells undergo transfection within each well as biologic repeats and each mutation had 8 technical repeats of the assay performed. Changes from wild-type transfection were compared using Student's t-test and a value of $P<0.05$ was deemed significant. Correction for multiple testing was not performed. Graphical presentation represents mean +/- standard deviation.

In the analysis comparing OS based on functional activity as defined by the NovellusDx assay, a cut point of the median functional activity of all variants characterized (1.46) was utilized rather than a patient weighted median (1.35), as the patient weighted median would have split the *KRAS* exon 2 mutations evenly between "high" and "low" activity groups, and these variants are known to be predictive biomarkers for resistance to anti-EGFR agents. *BRAF V600* mutated patients were excluded from all survival analysis to ensure differences between groups were not dependent on other MAPK alterations.

Ba/F3 Transformation Assay

In order to validate the findings of the NovellusDx FACT assay, we utilized the Ba/F3 Transformation Assay. A selection of 13 *RAS* mutations representing typical, extended, atypical, activating, and non-activating (per the NovellusDx assay) mutations were assessed using the well described Ba/F3 transformation assay in collaboration with Dr. Gordon Mills and Dr. Patrick Kwok-Shing Ng.[65, 66] Ba/F3 cells were cultured using RPMI medium with 5% FBS and 1 ng/mL of IL-3. The 13 mutations and a wild type construct were cloned into pHAGE vector by HiTMMoB technique.[67] Lentivirus was generated in LentiX-293T cells by transfecting the pHAGE and two packaging plasmids (psPAX2 and pMD2.G). The virus was harvested 3 days after transfection by filtering with 0.45 µM filter paper. Ba/F3 cells (0.6 million cells) were transduced with 900 µL of virus containing medium by spinoculation at 1000X gravity for 3 hours in the presence of polybrene (8µg/mL). After spinoculation, cells were suspended in medium without IL-3. Transduced cells were incubated at 37ºC for 3 days and cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Cell viability was compared to wild type using Student's t-test. Ba/F3 cells are normally dependent on IL-3 for survival and require its addition to media. After successful transfection of a functionally active oncogene these cells are able to survive the withdrawal of IL-3, while mutations that are not transforming will result in cells death. Wild type cell viability was assessed with 4 technical repeats and each mutation was assessed with 2 technical repeats.

Comparison of *RAS* Mutations Based on Allele Frequency

RAS mutation allele frequency was assessed for clinical significance in patients from the MDA T200 cohort and the MDA CMS 46 cohort. In order to normalize the allele frequency of mutations to tumor content, we performed a calculation that directly compared *RAS* mutant allele frequency to the allele frequency of whichever mutation was detected in that patient at the highest allele frequency. This high allele frequency mutation was considered the most "truncal" mutation and chosen to represent tumor content.

$$\textbf{rMAF}^{RAS} = \frac{\boldsymbol{RAS\ Mutation\ Allele\ Frequency}}{\boldsymbol{Truncal\ Mutation\ Allele\ Frequency}}$$

Sensitivity analyses were performed using the pathologist assessed tumor content in place of the truncal mutation. For patients with a T200 panel, copy number information was available. Correction for copy number alterations was applied to take into account amplifications of *RAS* or copy number losses in key truncal tumor suppressors such as *TP53*. The modified formula was as follows:

$$\textbf{rMAF}^{RAS}\ \textbf{corrected for CNA} = \frac{\boldsymbol{RAS\ Mutation\ Allele\ Frequency} * \left(\frac{\mathbf{2}}{\boldsymbol{RAS\ Copy\ Number}}\right)}{\boldsymbol{Truncal\ Mutation\ Allele\ Frequency} * \left(\frac{\mathbf{2}}{\boldsymbol{Truncal\ Copy\ Number}}\right)}$$

For all comparisons between groups that utilized rMAF *RAS* to stratify patients, we excluded *BRAF V600* mutant patients. A cut point to dichotomize patients into high and low allele *RAS* mutant groups was chosen at an rMAF of 50% with the biologic rationale that this would be comparing patients with greater than half of their tumor comprised of *RAS* mutant clones to those with less than half. The median was not chosen as it was a *RAS* rMAF of >75%, which from a biologic perspective was not deemed a reasonable cut point. This would have resulted in many of the patients in the "low" allele frequency group having a nearly clonal mutation. Given the debate regarding the discrepancy between pathologist estimates of tumor content

and true tumor content, we a-priori planned to primarily use the molecularly defined rMAF for all analyses.[54]

Relative Mutant Allele Frequency as a Predictor of Functional Significance

In addition to assessing the impact of *RAS* rMAF, we aimed to determine whether rMAF of a variant could be used as a predictor of a mutations functional significance. This work utilized the MDA T200, MDA CMS46, cfDNA, and Project Genie cohorts described in the methods. All variants detected within each of the cohorts had their functional significance categorized as described below.

Assignment of Functional Significance to NGS Variants in Aim 3

Functional significance of variants was performed by cross referencing individual variants with 4 separate measures of functional significance. These methods included:

1. "PODS" Functional Annotation - The Precision Oncology Decision Support Core at MDA has a large database of functionally annotated variants that categorizes variants as activating, inactivating, likely benign, or unknown based on a review of all available literature as well as *in-vitro* assessment of functional significance for many variants and has been previously described.[55]
2. SIFT scores – A SIFT score predicts whether the amino acid substitution that results from a mutation will be functionally relevant and is computationally defined.[56] A score of 0-0.05 is considered "deleterious" and a score of 0.05 to 1.0 is considered "tolerated." SIFT scores were assigned to the MDA T200 cohort and Project Genie cohort during their primary bioinformatic work and these values were used for analysis. For the MDA CMS 46 and cfDNA cohorts, all variants were cross referenced to assigned SIFT scores in the Project Genie cohort to ensure uniformity.
3. Polymorphism Phenotyping (Polyphen) scores - A Polyphen score predicts whether the amino acid substitution that results from a mutation will be functionally relevant and is computationally defined.[57] Polyphen assigns a score between 0.0 (benign) to 1.0

(damaging) and patients are grouped into benign, possibly damaging, and probably damaging based on its score and a qualitative assessment of the algorithm. Polyphen scores were assigned to the Project Genie cohort during its bioinformatic pipeline. The MDA CMS 46, MDA T200 and cfDNA cohorts had all variants cross referenced to assigned Polyphen scores in the Project Genie cohort to insure uniformity.

4. SIFT/Polyphen Merged Predicted Functional Significance – This measure was composed of a score between 0 and 4, with points assigned based on the SIFT and Polyphen scores considered together for each variant. In SIFT, a point was given for variants deemed to be possibly deleterious (low confidence) and 2 points were given for deleterious variants. In Polyphen, 1 point was given for possible damaging and 2 points for probably damaging. Variants were only considered if at least 1 of the scores made a functional prediction for that mutation.

In addition to studying predictive scores for defining the functional significance of a variant, we also performed two case-studies to evaluate whether a model of increasing rMAF was compatible with a determination of functional significance. The first case-study reviewed rMAF of *BRAF* V600 and non-V600 mutations across all 4 previously described cohorts. There has been growing evidence that *BRAF* V600 mutations are responsible for the negative prognostic significance of *BRAF* mutations and that non-V600 mutations have less impact. [58, 59] As such, we predicted that *BRAF* V600 mutations would occur at higher rMAFs.

Using the cfDNA cohort, we also aimed to determine whether there was a correlation between the number of mechanisms of resistance detected within a patient to anti-EGFR therapy to the rMAF of those mechanisms of resistance. From an evolutionary perspective, the development of resistance often comes at a cost for a microorganism or tumor cell. These costs can be either a reduced fitness or the uncovering of a collateral sensitivity.[60, 61] Based on this theory, if a malignant cell has developed a mechanism of resistance to a targeted agent, there would be little benefit to developing multiple other mechanisms within the same cell. There may however

be other clonal populations that exist within the patient that develop alternate mechanisms of resistance. cfDNA allows the sampling of all clones secreting DNA into a patient's blood stream and provides a contemporary view of the genomic landscape within a patient at the time that blood is drawn. We predicted that patients with a single mechanism of resistance detected may have this mutation at a high allele frequency. In patients with multiple concurrent alterations, the average rMAF of those alterations should be lower, as these patients likely have a more diverse clonal structure and we would expect that each of those mechanisms would only be present in a portion of the total tumor cell population. There was no clinical annotation available for this cohort to confirm prior receipt of an anti-EGFR agent in patients. Mechanisms of resistance included alterations to *EGFR, KRAS, NRAS, BRAF, MAP2K1, ERBB2, MET*, and *KIT*. A mechanism of resistance could include either an amplification or a single nucleotide variant, however only single nucleotide variants were assessed for average rMAF.

Statistical Analysis

Categorical characteristics were compared using the χ^2 test or Fisher's exact test as appropriate, while continuous variables were compared with the Mann-Whitney or Kruskal-Wallis tests when a median is reported and the Student's t-test or ANOVA when averages are shown. Correction for multiple testing was not performed. $P<0.05$ is considered significant for all analyses. Right sided tumors were defined based on pathology and surgical reports as those occurring from the cecum up to but not including the splenic flexure. Left sided tumors were defined as those occurring from the splenic flexure to the rectum. OS was defined as the time from diagnosis with stage IV CRC until death or last follow up. Patients alive at the time of last follow up were censored. OS was summarized using Kaplan-Meier curves and compared using the log-rank test and Cox-regression analysis. Where multivariate models were performed, a forward likelihood ratio selection was used. Variables with $p<0.1$ were included. All variables met the proportional hazards assumption and were chosen based on differences in baseline characteristics between groups or known prognostic features in CRC.

Analysis was performed using Graph Pad Prism software version 5.0 (La Jolla, California), SPSS version 22.0 (Armonk, New York) and R studio version 3.30 (Boston, MA). Data visualization also utilized the R package ggplot2.[68]

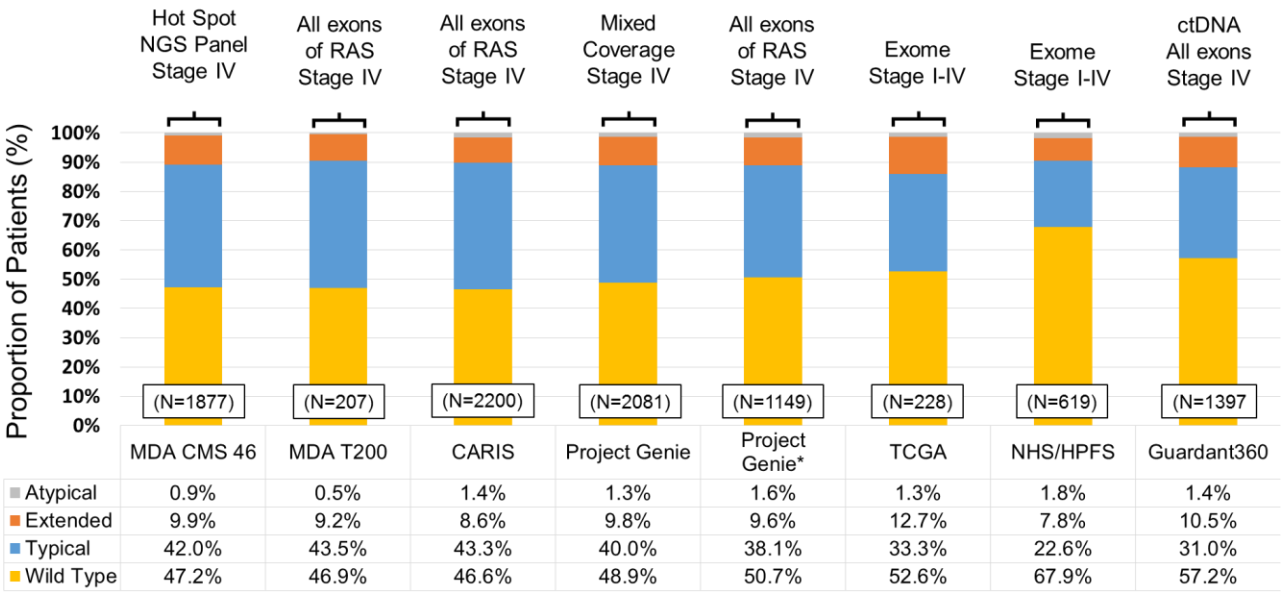
Chapter 3: Results

Aim 1: To describe the functional and clinical significance of atypical *RAS* mutations.

Prevalence of Atypical *RAS* Mutations

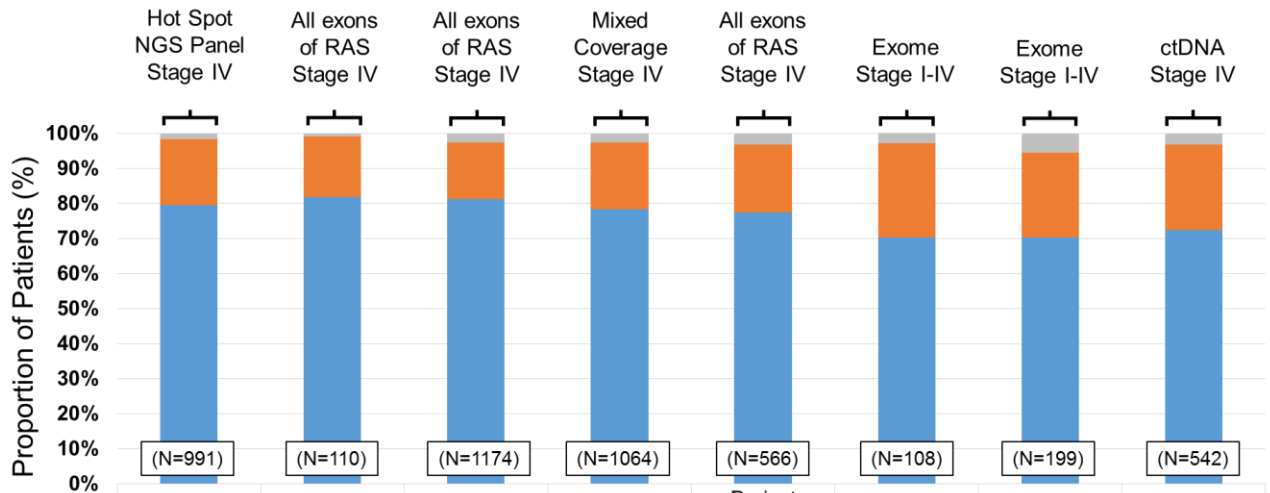
The prevalence of missense, nonsense, and indel mutations in *RAS* was assessed across 7 cohorts [Figure 3 & 4]. Mutations in *RAS* were noted in 4244/8609 patients (49.3%), and varied significantly between cohorts (P<0.0001) with a range in prevalence from 32.1% to 53.3%. Data from the Project Genie collaboration includes patients with a variety of NGS platforms. Given the heterogeneity in techniques used, data is presented for the entire available cohort (N=2081) and for only those patients who utilized assays that would cover all exons of *KRAS/NRAS* (depicted with a *). There was no statistically significant difference in *RAS* mutation frequency (P=0.31) or distribution of *RAS* mutation category (ie. typical, extended, atypical, P=0.65) noted between the two versions of the cohort.

A total of 3314/4244 (78.1%) *RAS* mutant patients had typical mutations, 822/4244 (19.4%) had extended *RAS* mutations and 108/4244 (2.5%) had atypical *RAS* mutations.[29] One hundred and twenty three atypical variants were detected, with 84 occurring in *KRAS* and 39 in *NRAS*. Fifteen of these variants occurred in patients who had a co-occurring typical or extended mutation and they were categorized according to their more common variant. Prevalence of individual variants are summarized in Table 2, and although most atypical variants occur at very low frequencies, certain variants are present in a larger proportion of patients than guideline cited variants. For example, *KRAS* Q22K was noted in 13/8609 (0.2%) patients, and yet not a single *NRAS* codon 117 or 146 variant was detected in the entire cohort and only 1 *NRAS* codon 59 variant was detected. Other atypical variants occurring at frequencies of ≥0.1% include *KRAS* L19F (7/8609, 0.1%), *KRAS* D33E (7/8609, 0.1%), and *KRAS* T50I (5/8609, 0.1%).



	MDA CMS 46	MDA T200	CARIS	Project Genie	Project Genie*	TCGA	NHS/HPFS	Guardant360
Atypical	0.9%	0.5%	1.4%	1.3%	1.6%	1.3%	1.8%	1.4%
Extended	9.9%	9.2%	8.6%	9.8%	9.6%	12.7%	7.8%	10.5%
Typical	42.0%	43.5%	43.3%	40.0%	38.1%	33.3%	22.6%	31.0%
Wild Type	47.2%	46.9%	46.6%	48.9%	50.7%	52.6%	67.9%	57.2%

Figure 3. Prevalence of *RAS* mutations based on category of mutation in colorectal cancer across 7 cohorts. Project Genie* = Project Genie cohort restricted to only the 1149 patients with all exons of *KRAS/NRAS* sequenced.



RAS MT	MDA CMS 46	MDA T200	CARIS	Project Genie	Project Genie*	TCGA	NHS/HPFS	Guardant360
Atypical	1.6%	0.9%	2.6%	2.5%	3.2%	2.8%	5.5%	3.2%
Extended	18.8%	17.3%	16.2%	19.2%	19.4%	26.9%	24.1%	24.4%
Typical	79.6%	81.8%	81.2%	78.3%	77.4%	70.4%	70.4%	72.4%

Figure 4. Proportion of *RAS* mutations that are defined as typical, extended, or atypical in colorectal cancer across 7 cohorts. Project Genie* = Project Genie cohort restricted to only the 1149 patients with all exons of *KRAS/NRAS* sequenced.

Table 2. Prevalence of specific *RAS* mutations detected across 7 cohorts.

Gene	Alteration	N	Prevalence (%)	**Gene**	Alteration	N	Prevalence (%)
KRAS	L6P	1	0.0%	*NRAS*	A11S	1	0.0%
	V7E	1	0.0%		A11T	1	0.0%
	V9_G10dup	1	0.0%		G12A	6	0.1%
	G10dup	1	0.0%		G12C	17	0.2%
	A11_G12dup	1	0.0%		G12D	55	0.6%
	G12A	172	2.0%		G12R	2	0.0%
	G12C	268	3.1%		G12S	7	0.1%
	G12D	1164	13.5%		G12V	11	0.1%
	G12E	1	0.0%		G13C	2	0.0%
	G12F	5	0.1%		G13D	13	0.2%
	G12L	1	0.0%		G13R	15	0.2%
	G12R	51	0.6%		G13V	4	0.0%
	G12S	166	1.9%		Q22K	1	0.0%
	G12V	769	8.9%		Q25*	1	0.0%
	GC12-13ES	1	0.0%		Q25H	1	0.0%
	G13C	16	0.2%		V29L	1	0.0%
	G13D	693	8.0%		E31K	1	0.0%
	G13dup	1	0.0%		E49*	1	0.0%
	G13H	1	0.0%		L53F	1	0.0%
	G13R	6	0.1%		D54V	1	0.0%
	G13V	2	0.0%		D57Y	2	0.0%
	V14I	4	0.0%		A59T	1	0.0%
	G15S	1	0.0%		G60E	1	0.0%
	A18D	1	0.0%		G60R	1	0.0%
	L19F	7	0.1%		Q61H	20	0.2%
	T20M	1	0.0%		Q61K	104	1.2%
	I21T	1	0.0%		Q61L	43	0.5%
	Q22K	13	0.2%		Q61R	53	0.6%
	E31E	1	0.0%		E62K	1	0.0%
	E31K	1	0.0%		E62L	1	0.0%
	D33E	7	0.1%		A66T	1	0.0%
	S39Pfs*6	1	0.0%		E98V	1	0.0%
	E49X	1	0.0%		D105Tfs*9	1	0.0%
	T50I	5	0.1%		S106L	1	0.0%
	L56V	1	0.0%		D108G	1	0.0%
	D57N	1	0.0%		V114A	1	0.0%
	A59E	3	0.0%		T122R	1	0.0%
	A59G	7	0.1%		D126V	1	0.0%
	A59T	14	0.2%		Q129H	1	0.0%
	G60D	1	0.0%		E132K	3	0.0%
	G60V	1	0.0%		K147R	1	0.0%
	Q61K	20	0.2%		E162*	1	0.0%
	Q61L	40	0.5%		R164C	2	0.0%

Gene	Alteration	N	Prevalence (%)
KRAS	Q61H	131	1.5%
	Q61P	1	0.0%
	Q61R	17	0.2%
	E62K	1	0.0%
	A66_M67ins*	1	0.0%
	R68S	4	0.0%
	Y71D	1	0.0%
	M72L	1	0.0%
	E76G	1	0.0%
	E98*	1	0.0%
	E98X	1	0.0%
	V109A	1	0.0%
	N116H	1	0.0%
	K117N	30	0.3%
	K117R	1	0.0%
	Q131H	1	0.0%
	T144I	1	0.0%
	A146P	11	0.1%
	A146T	173	2.0%
	A146V	49	0.6%
	K147E	1	0.0%
	K147N	2	0.0%
	K147T	1	0.0%
	F156V	1	0.0%
	L159Wfs*2	1	0.0%
	R164*	1	0.0%
	R164Q	1	0.0%
	E168fs	1	0.0%
	K170Q	1	0.0%
	K176N	1	0.0%
	K177del	1	0.0%
	K178del	1	0.0%
	P178L	1	0.0%
	K185fs	1	0.0%
	c.291-10delT	1	0.0%
	Wild Type	4708	54.7%
	Total	8609	100.0%

Gene	Alteration	N	Prevalence (%)
NRAS	G138R	1	0.0%
	R167Q	1	0.0%
	M168I	1	0.0%
	K170N	1	0.0%
	P185A	1	0.0%
	V188M	1	0.0%
	c.451-11dupT	1	0.0%
	Wild Type	8217	95.4%
	Total	8609	100.0%

Functional Impact of Specific *RAS* Mutations

Using the NovellusDx FACT assay we transfected 113 different *RAS* mutations (61 *KRAS* and 52 *NRAS*) in a live cell reporter assay. Mutations that resulted in nuclear localization of GFP-ERK that was significantly greater than wild type transfection are considered “activating,” while those that did not significantly increase nuclear localization of GFP-ERK were deemed “non-activating” [Figure 5 & 6]. Values represent the average of 8 repeats for each mutation and have been normalized to the wild type transfection of *KRAS* or *NRAS* (mutation dependent) to represent a fold change from wild type transfection. Of 61 *KRAS* mutations characterized, 6 were not activating and included I21L, E49*, D57N, E76G, E98* and K176N. Of 52 *NRAS* mutations characterized, 17 were not activating and included G10E, A11T, A18T, I21V, V29L, E31K, D54V, D57Y, A66T, M67I, D126V, E132K, K135N, G138R, R164C, R167Q and P185A.

All non-activating *RAS* variants were atypical mutations and all typical or extended mutations were shown to be activating. Relative MAPK activity summarized by atypical/typical/extended categorization is summarized in Figure 7 and demonstrates that extended mutations resulted in the highest MAPK activity, which was greater than both typical (P=0.0018) or atypical (P<0.0001) mutations. After weighting each variant based on their prevalence in the MDA CMS 46 cohort of 1877 mCRC patients [Figure 7- C], we noted that the recurrent atypical variants present in patients were those of higher activity, rather than the non-activating mutations. In fact, all atypical variants in this cohort were those that resulted in a functional activity in the top 10%ile of all patients.

More atypical mutations were shown to be non-activating for *NRAS* (17/26 tested atypical mutations) than for *KRAS* (6/31 tested atypical mutations) (P=0.0004). In addition, though the median MAPK activity of all assessed *KRAS* (Median 1.46, IQR 1.27-1.60) and *NRAS* (Median 1.53, IQR 1.15-1.90) mutations did not differ (P=0.52), when we weighed the

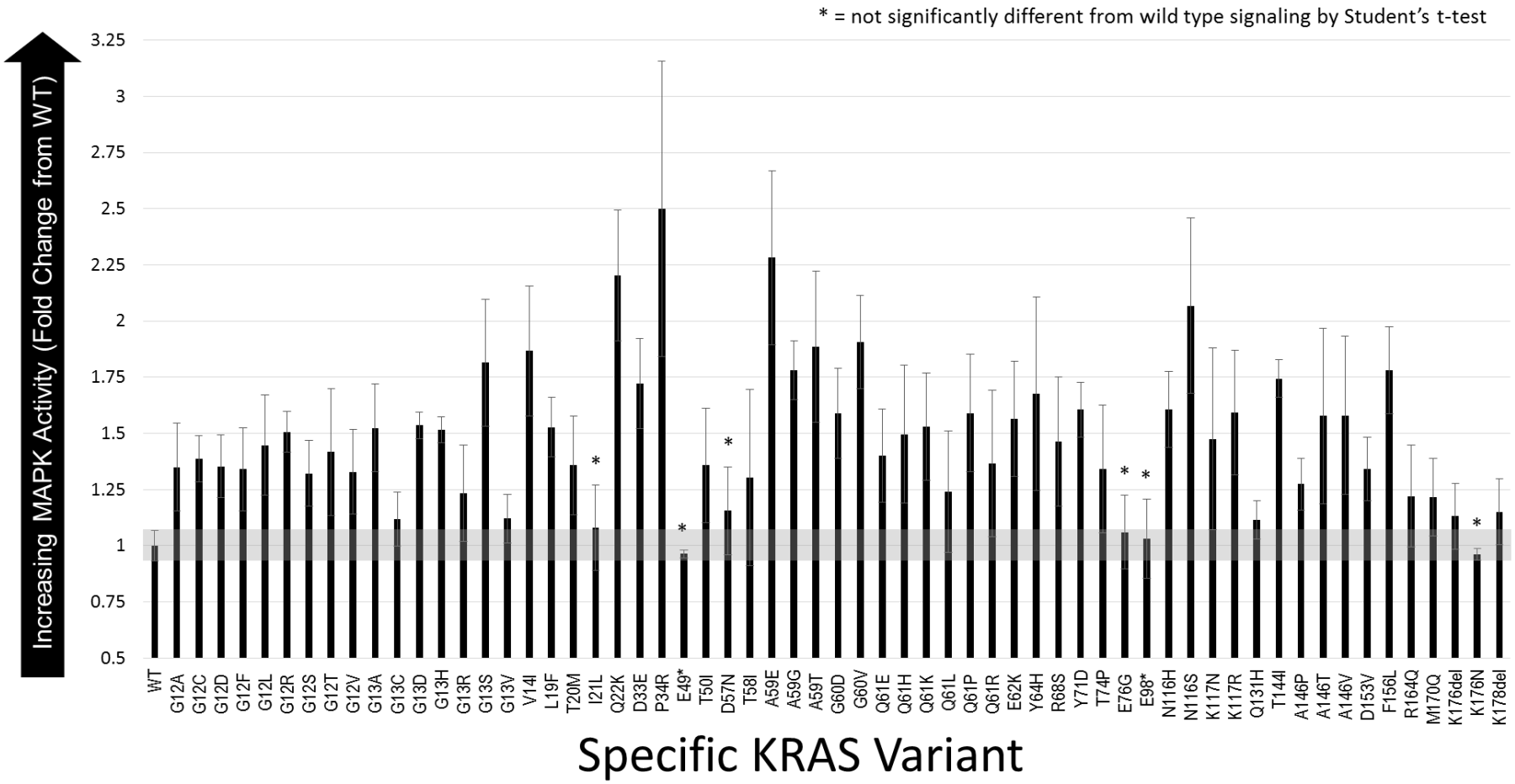


Figure 5. Functional impact of *KRAS* variants on MAPK signaling and nuclear GFP-ERK localization following transfection in the Novellus Dx FACT assay. Values have been normalized to a wild type transfection and represent fold change from wild type. Each mutation was assessed with 8 technical repeats. Values represent mean +/- standard deviation.

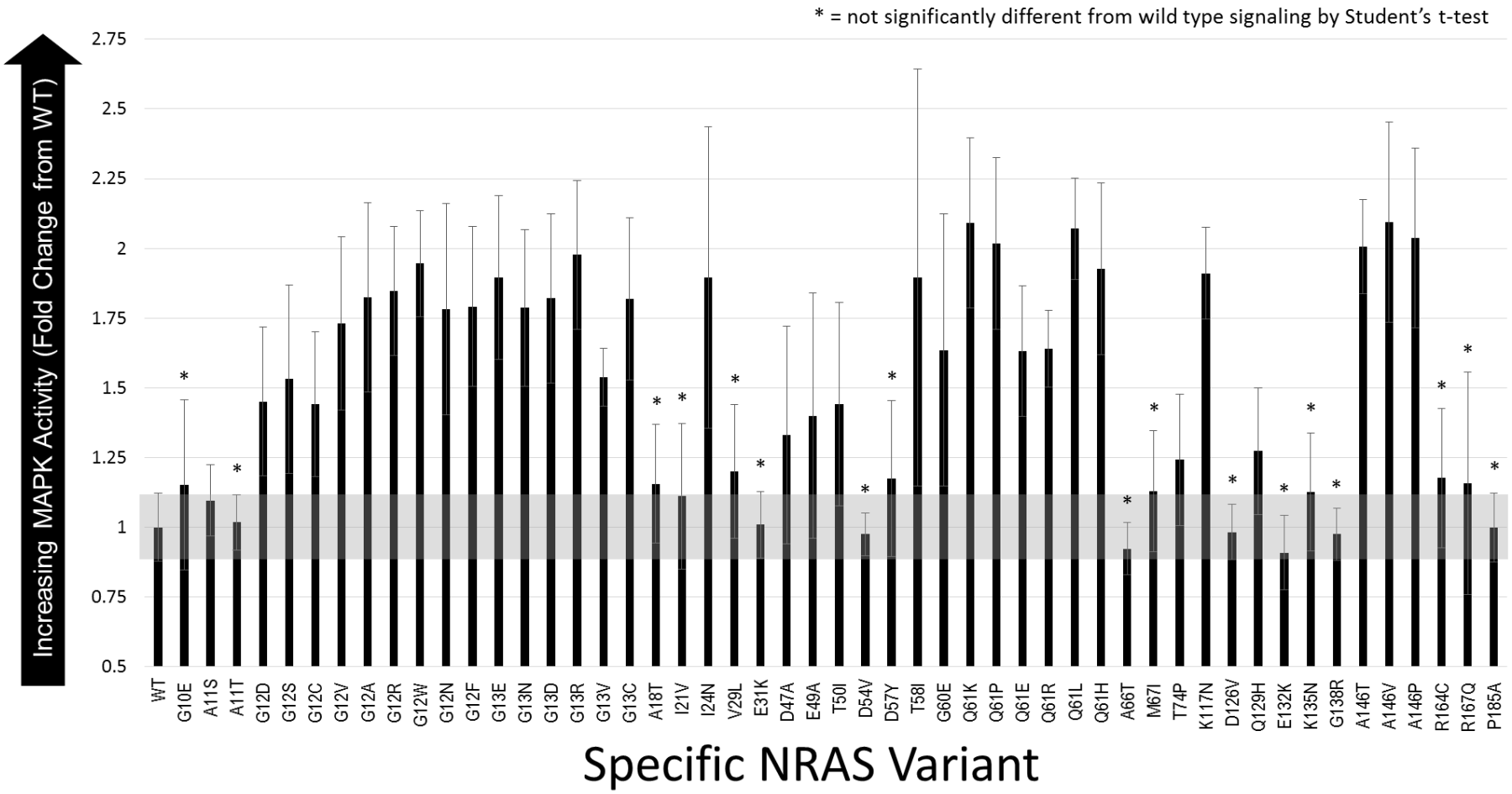


Figure 6. Functional impact of *NRAS* variants on MAPK signaling and nuclear GFP-ERK localization following transfection in the Novellus Dx FACT assay. Values have been normalized to a wild type transfection and represent fold change from wild type. Each mutation was assessed with 8 technical repeats. Values represent mean +/- standard deviation.

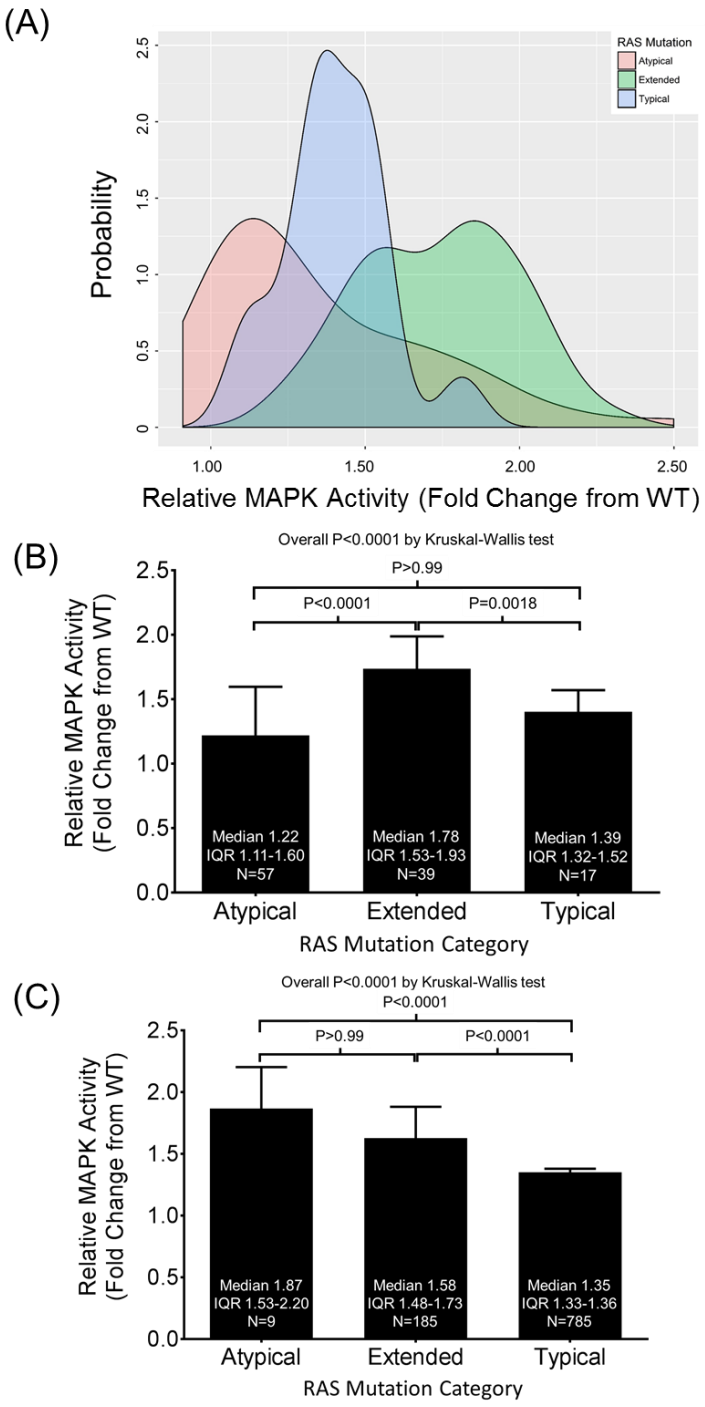


Figure 7. Functional impact of all *KRAS* and *NRAS* mutations on MAPK pathway signaling categorized by mutation category. (A) Values represent Kernel Density plot of probability at each functional activity fold change compared to wild type which was considered an activity of 1.0. (B) Median activity of each assessed variant and (C) weighted median activity based on prevalence of each assessed mutation.

activity scores by the prevalence of each variant in our MDA CMS 46 cohort, patients with *NRAS* mutations (Median 1.93, IQR 1.45-2.09) had mutations resulting in higher activity than patients with *KRAS* mutations (Median 1.35, IQR 1.35-1.38) (P<0.0001) [Figure 8 - C]. Of all 23 mutations that did not increase MAPK signaling, only *KRAS* E98*, *NRAS* D57Y, *NRAS* E132K, and *NRAS* R164C were recurrently identified in >1 CRC in our pooled cohort of 8609 mCRC patients. As well, *KRAS* I21L, *NRAS* G10E, *NRAS* A18T, *NRAS* I21V, *NRAS* M67I and *NRAS* K135N have only been identified in non-CRC malignancies. In total, only 22/8609 patients (0.3%) had a *RAS* mutation that was non-activating in the pooled cohort.

In the hopes of identifying patients with mutations that may have signaling activity close to wild type, we reviewed the specific variants that occurred at the lowest functional activities. No atypical variants fell in this category. Variants detected commonly in the MDA CMS 46 cohort in the bottom 10% of signaling activity included *KRAS* G13C (3/1877), *KRAS* G13R (2/1877), *KRAS* Q61L (12/1877), *KRAS* A146P (4/1877), *KRAS* G12S (34/1877). Any variants with signaling higher than these mutations were well characterized activating *KRAS* exon 2 mutations.

In addition to the NovellusDx FACT assay, a select number of *RAS* mutations representing typical, extended, atypical, activating, and non-activating mutations were assessed using the well described Ba/F3 transformation assay in collaboration with Dr. Gordon Mills and Dr. Patrick Kwok-Shing Ng.[65, 66] All 13 mutations assessed using the Ba/F3 assay were concordant in their categorization of mutations as activating/non-activating with the NovellusDx FACT assay [Figure 9]. This included 2 non-activating atypical mutations (*KRAS* D57N and *NRAS* K135N) that were concordant between the two assays and a third atypical mutation (*KRAS* R68S) that was activating in both assays.

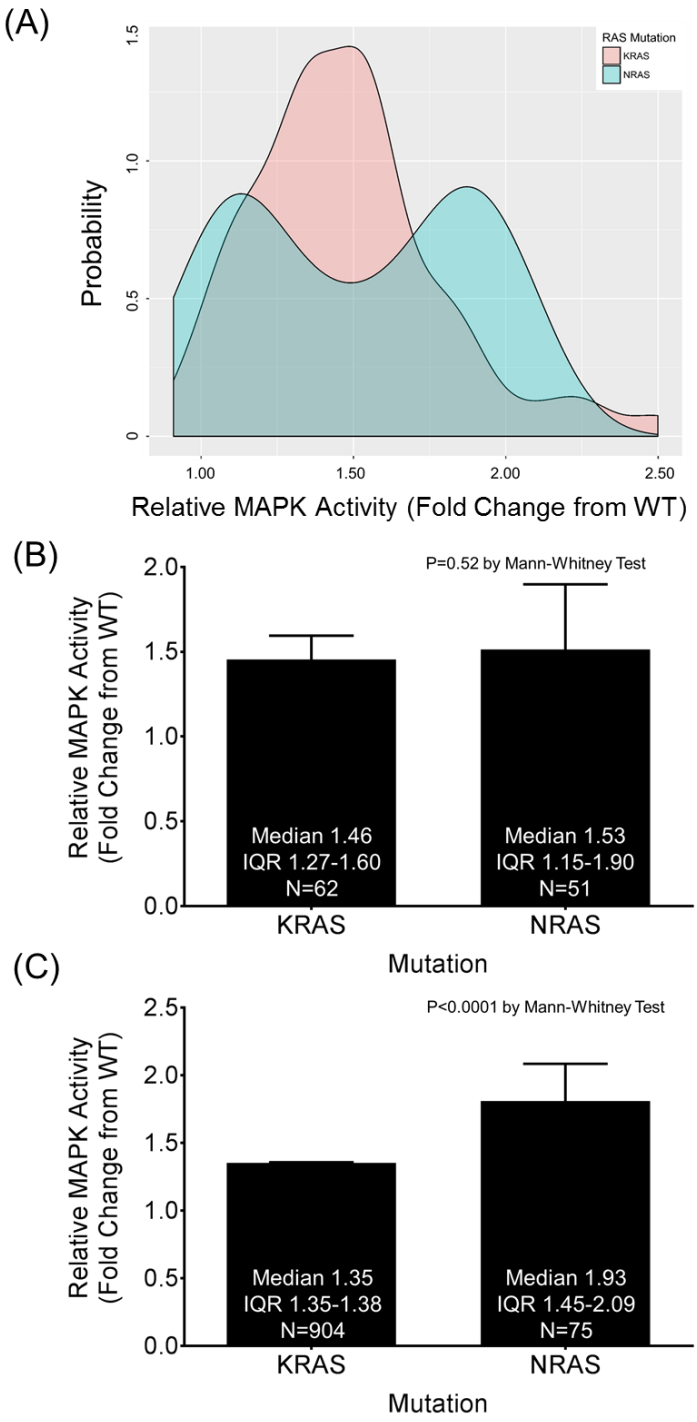


Figure 8. Functional impact of all *KRAS* and *NRAS* mutations on MAPK pathway signaling categorized by gene. (A) Values represent Kernel Density plot of probability at each functional activity fold change compared to wild type which was considered an activity of 1.0. (B) Median activity of each assessed variant and (C) weighted median activity based on prevalence of each assessed mutation.

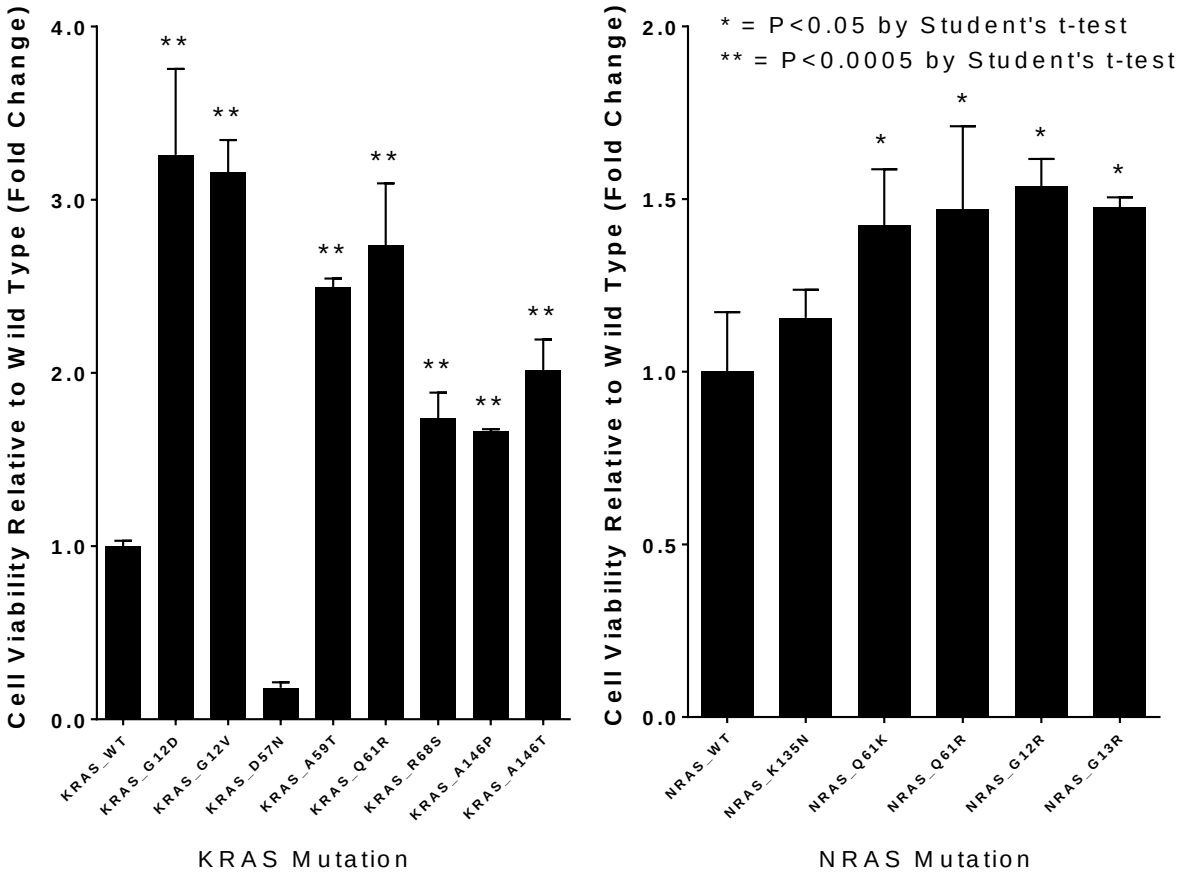


Figure 9. Functional impact of *KRAS*/*NRAS* mutations introduced into the Ba/F3 cell transformation assay. Values represent mean +/- standard deviation.

Impact of *RAS* Mutation Category and Functionality on Clinical Outcomes

Baseline characteristics stratified by *RAS* mutation category and functional annotation for the MDA CMS 46 cohort are shown in Table 3 and 4. Atypical mutations were numerically less common with mucinous or signet ring histology (P=0.065) and were more commonly associated with *BRAF* V600 mutations (P<0.0001). *RAS* mutations with activity above the median fold change for all characterized mutations were numerically less common with mucinous or signet ring histology (P=0.070). None of the atypical mutant patients from MDA with mCRC ever received an anti-EGFR agent.

Prognostic impact of variants was assessed in only the MDA CMS 46 cohort. Patients with typical (HR 1.43, 95% CI 1.26-1.65, P<0.0001), extended (HR 1.61, 95% CI 1.39-2.20, P<0.0001), and atypical (HR 3.45, 95% CI 3.15-40.83, P=0.0002) *RAS* mutations had a worse OS than *RAS*/*BRAF* wild type mCRC patients [Figure 10]. There were no statistically significant differences between extended and typical *RAS* mutations (P=0.20), however atypical *RAS* mutations were associated with a worse OS than typical (HR 2.45, 95% CI 1.43-12.5, P=0.0092) but not extended (HR 1.97, 95% CI 0.99-6.92, P=0.055) *RAS* mutations. In multivariate models controlling for primary tumor location, synchronous metastases at diagnosis, histology, age, gender and MSI, typical (P<0.0001) and extended (P=0.0010) mutations remained significantly associated with a worse OS, while atypical variants showed strong trends to worse OS (HR 2.62, 95% CI 0.97-7.06, P=0.055).

Similar results were seen when considering *KRAS* mutations alone by category, where typical (HR 1.43, 95% CI 1.26-1.65, P<0.0001), extended (HR 1.53, 95% CI 1.24-2.20, P=0.0012), and atypical (HR 3.26, 95% CI 2.49-35.77, P=0.0064) *KRAS* mutations were associated with a worse OS. Among *NRAS* mutated patients, only 1 atypical variant was noted. Extended *NRAS*

Table 3. Baseline characteristics of metastatic colorectal cancer patients with *RAS* mutations according to category of mutation.

RAS Mutation Status	Typical (N=787)	Extended (N=186)	Atypical (N=11)	Wild Type (N=893)	P (RAS Mutant Groups)	P (Overall)
Median Age (Interquartile Range)	55 (45-63)	56 (46-63)	57 (46-69)	55 (46-62)	0.99	0.91
Male (%)	400 (50.8%)	101 (54.3%)	5 (45.5%)	552 (61.8%)	0.64	<0.0001
Female (%)	387 (49.2%)	85 (45.7%)	6 (54.5%)	341 (38.2%)		
MSI-H (% of known)	18 (2.8%)	7 (4.5%)	0 (0%)	32 (4.4%)	0.47	0.39
Mucinous or Signet Histology	147 (18.7%)	22 (11.8%)	1 (9.1%)	107 (12.0%)	0.065	0.0010
Right Sided	298 (37.9%)	62 (33.3%)	6 (54.5%)	214 (24.0%)	0.25	<0.0001
Metastatic at Diagnosis	506 (64.3%)	123 (66.1%)	5 (45.5%)	581 (65.1%)	0.37	0.56
BRAF V600	0 (0%)	1 (0.5%)	1 (9.1%)	113 (12.7%)	<0.0001	<0.0001

Table 4. Baseline characteristics of metastatic colorectal cancer patients with *RAS* mutations according to activity of mutation.

RAS Mutation Activity	RAS MT Activity < Median (N=821)	RAS MT Activity > Median (N=156)	Wild Type* (N=780)	P (RAS Mutant Groups)	P (Overall)
Median Age (Interquartile Range)	55 (45-63)	55 (46-63)	54 (45-62)	0.59	0.30
Male (%)	417 (50.8%)	86 (55.1%)	500 (64.1%)	0.32	<0.0001
Female (%)	404 (49.2%)	70 (44.9%)	280 (35.9%)		
MSI-H (% of known)	20 (3.0%)	4 (3.1%)	19 (3.0%)	0.99	0.99
Mucinous or Signet Histology	149 (18.1%)	19 (12.2%)	68 (8.7%)	0.070	<0.0001
Right Sided	312 (38.0%)	52 (33.3%)	143 (18.4%)	0.27	<0.0001
Metastatic at Diagnosis	522 (63.6%)	109 (69.9%)	498 (63.8%)	0.13	0.31

*BRAF V600 mutant patients excluded

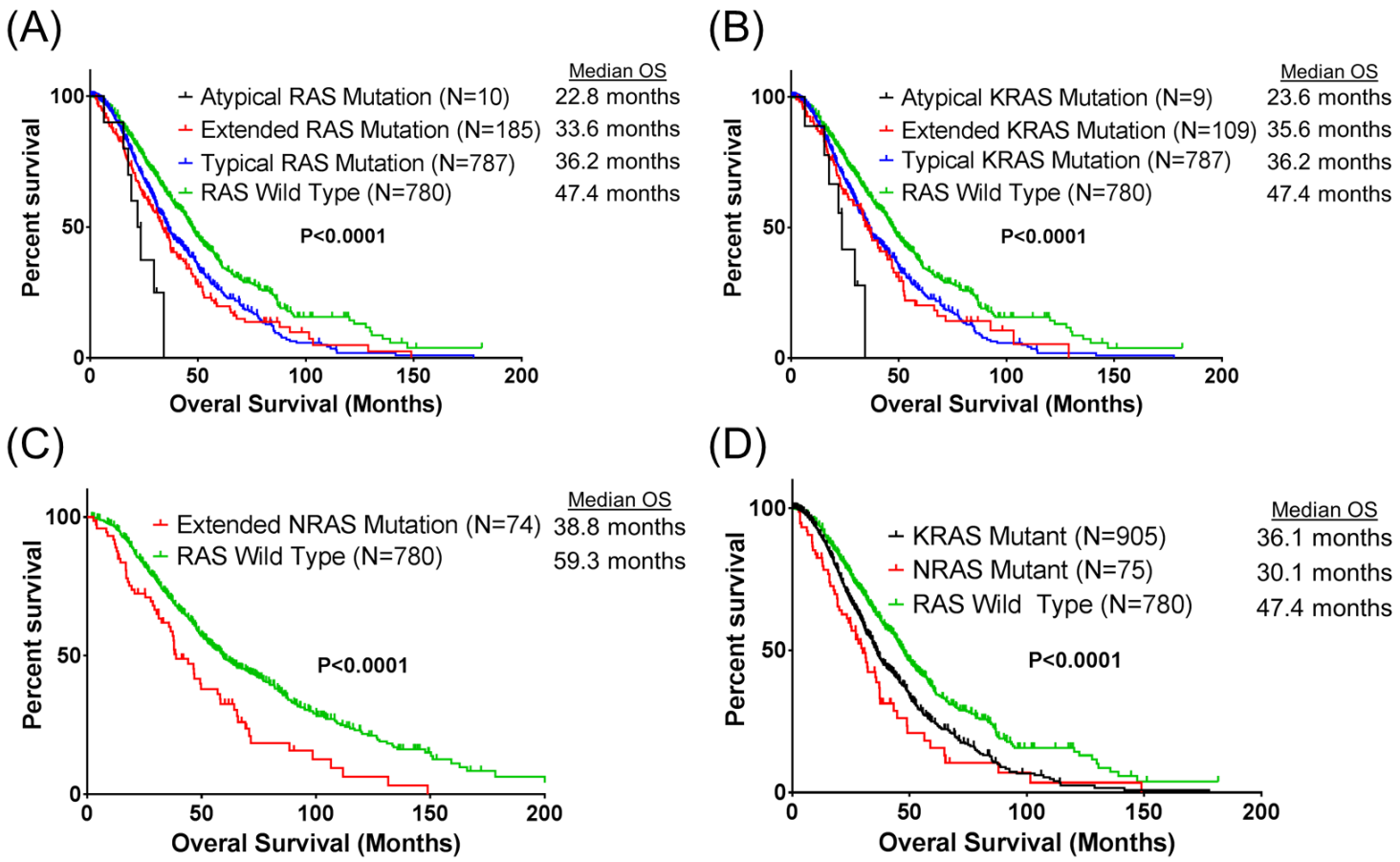


Figure 10. (A) Overall survival of patients with metastatic colorectal cancer stratified by category of *RAS* mutation, (B) *KRAS* mutation category and (C) *NRAS* mutation category. Panel (D) demonstrates the prognostic relevance of *KRAS*/*NRAS* without categorization. Patients with *BRAF* V600 mutations were excluded from analysis.

mutations were associated with a worse overall survival (HR 1.74, 95% CI 1.43-2.88, P<0.0001) compared to wild type patients. When comparing all mutation categories together, *NRAS* mutations were associated with a worse OS than *KRAS* mutations (HR 1.34, 95% CI 1.02-1.89, P=0.036). This appeared independent of the particular exon mutated in *NRAS* [Figure 11].

Due to the wide dynamic range of activity noted in the NovellusDx FACT assay, we also compared *RAS* variants based on their functional activity. Of the 113 characterized mutations, the median functional activity was 1.46. Patients with *RAS* variants either below (HR 1.43, 95% CI 1.27-1.65, P<0.0001) or above (HR 1.66, 95% CI 1.42-2.37, P<0.0001) the median had a worse OS than patients with *RAS*/*BRAF* V600 wild type tumors [Figure 12 - A] but did not differ from each other in univariate (HR 1.16, 95% CI 0.94-1.46, P=0.17) or multivariate models (P>0.1).

Because *KRAS* exon 2 mutations have been well characterized as a predictive biomarker, we next compared variants that were statistically higher or lower than the pooled average of *KRAS* exon 2 mutations. No patient had a variant that was significantly lower than *KRAS* exon 2, however 66 patients had variants that resulted in significantly higher signaling. These high signaling patients had variants that included *KRAS* V14I, *KRAS* Q22K, *KRAS* D33E, *KRAS* A146V, *NRAS* G12V, *NRAS* G13R, *KRAS* A59T and *NRAS* Q61R/L/K/H. Having one of these high signaling variants resulted in a worse OS (HR 1.40, 95% CI 1.04-2.08, P=0.028) than having a *KRAS* exon 2 mutation [Figure 12 - B]. In a multivariate model controlling for co-variates, mutations with signaling activity significantly higher than exon 2 mutations (HR 1.81, 95% CI 1.28-2.57, P=0.0010) were associated with a larger prognostic difference from *RAS* wild type patients than exon 2 mutations (HR 1.37, 95% CI 1.17-1.61, P<0.0001), however they were not significantly different when directly compared to each other [Table 5]. If a patient's

MAPK activity change was considered as a continuous variable in Cox-regression models, it was associated with worse OS (HR 1.94, 95% CI 1.46-2.59, P<0.0001) [Table 3.5 – Model 3].

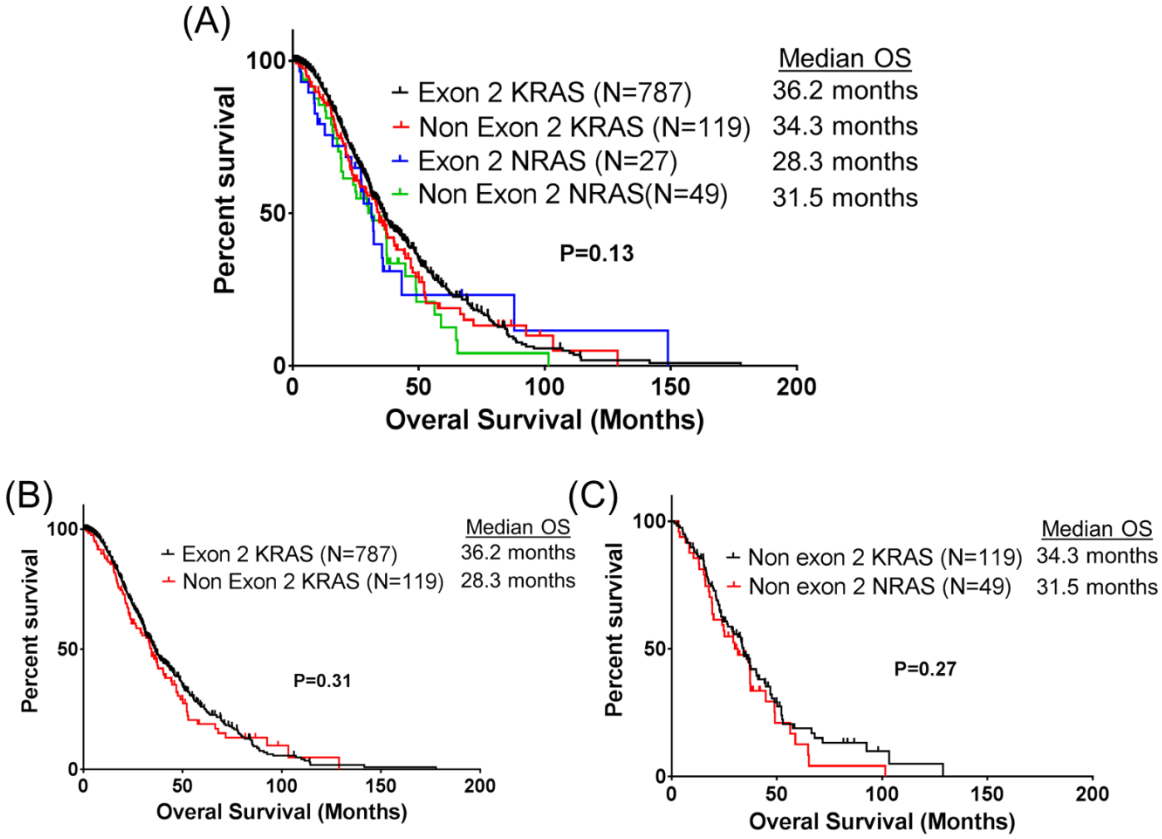


Figure 11. (A) Overall survival of patients with metastatic colorectal cancer stratified by exon of *KRAS* or *NRAS* mutations showing no significant difference between (B) *KRAS* and *NRAS* exon 2 mutations or (C) *KRAS* and *NRAS* non-exon 2 mutations. Patients with BRAFV600 mutations were excluded from analysis.

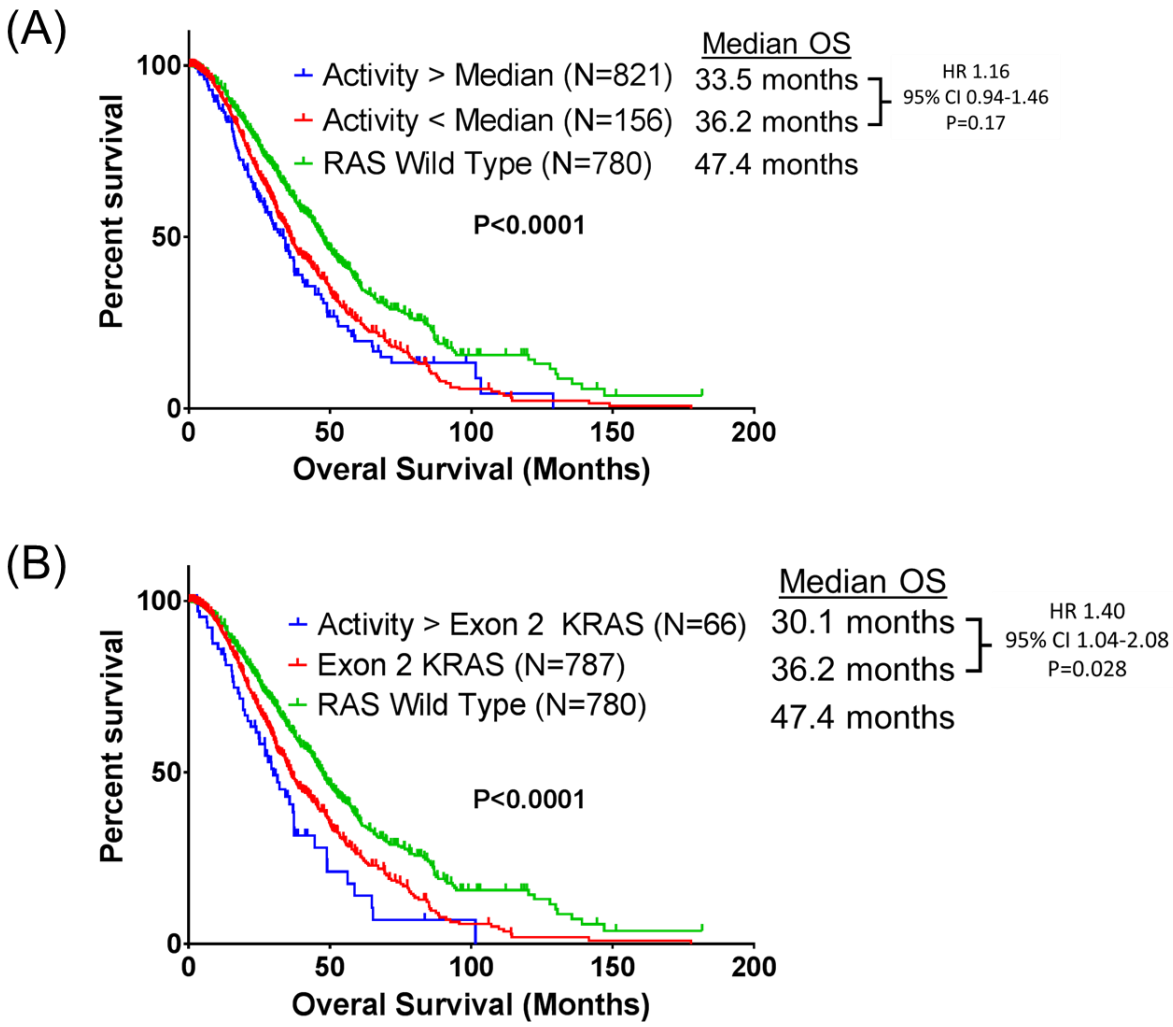


Figure 12. Overall survival of patients with metastatic colorectal cancer stratified by functional activation of a patients *RAS* variant stratified at the (A) median activity of 113 classified mutations and (B) by comparing patients with *KRAS* exon 2 mutations to any patients with a mutation resulting in MAPK activity significantly higher than *KRAS* exon 2.

Table 5. Multivariate models comparing the impact of *RAS* mutation category and functional characterization on overall survival in metastatic colorectal cancer. Model 1 considers *RAS* mutations based on their category, model 2 considers *RAS* mutations based on their functional activity compared to *KRAS* exon 2 mutations, and model 3 considers the activity of *RAS* mutations as a continuous variable.

Co-Variate	**HR (95% CI)**	**P**
Model 1		
RAS Wild Type	Reference	
Typical RAS Variant	1.38 (1.18-1.61)	<0.0001
Extended RAS Variant	1.48 (1.18-1.86)	0.001
Atypical RAS Variant	2.62 (0.97-7.06)	0.055
Right Sided Primary	1.33 (1.14-1.56)	<0.0001
Metastatic at Diagnosis	1.47 (1.26-1.71)	<0.0001
Mucinous or Signet Histology	1.33 (1.08-1.64)	0.0070
Model 2		
RAS Wild Type	Reference	
Exon 2 Mutation	1.37 (1.17-1.61)	<0.0001
Activity > Exon 2 Mutations	1.81 (1.28-2.57)	0.0010
Right Sided Primary	1.42 (1.21-1.68)	<0.0001
Metastatic at Diagnosis	1.52 (1.30-1.79)	<0.0001
Mucinous or Signet Histology	1.30 (1.05-1.62)	0.017
Model 3		
RAS Activity (continuous)	1.94 (1.46-2.59)	<0.0001
Right Sided Primary	1.37 (1.17-1.59)	<0.0001
Metastatic at Diagnosis	1.45 (1.24-1.68)	<0.0001
Mucinous/Signet Ring	1.35 (1.10-1.65)	0.0050

Aim 2: To assess the impact of *RAS* mutant allele frequency on clinical outcomes in mCRC.

Aim 2 utilized the MDA T200 cohort as a discovery cohort to evaluate cut points and perform sensitivity analyses, while the MDA CMS 46 cohort served as the validation cohort for prognostic differences between groups.

Relative Mutant Allele Frequency as a Prognostic Marker in MDA T200 Cohort

Of 207 sequenced mCRC cases, low allele frequency *RAS* mutations (rMAF <50%) occurred in 21 patients (10.1%) and high allele frequency *RAS* mutations (rMAF >50%) occurred in 89 patients (43.0%). The median *RAS* rMAF was 76.1%. After correcting for copy number alterations, 29 patients (14.0%) had low allele frequency *RAS* mutations and 81 patients (39.1%) had high allele frequency mutations. Median copy number corrected *RAS* rMAF was 76.8%. Distribution of uncorrected and corrected rMAF is presented in Figure 13 and Figure 14 and demonstrates the relative change in rMAF with correction for copy number alterations. Specific alterations used as the denominator in calculating rMAF are shown in Figure 14 - B. Patients were dichotomized into two groups at a 50% rMAF cut point for all further analysis.

As seen in Table 6, there were no significant differences in baseline characteristics or mutational status based on allele frequency except low frequency variants were less common among patients with synchronous metastatic disease at diagnosis (38.1% vs 65.2%, P=0.023). Despite this, there was no difference in the distribution of site of biopsy (primary tumor vs metastases) between the two groups (P=0.65). Both *RAS* mutant groups were more likely to have right sided tumors (P=0.0062) or PIK3CA mutations (P=0.0022) than wild type patients and there were no BRAF V600 mutations among the *RAS* mutant groups but 8 among wild type patients (P=0.0062). Average sequencing coverage was lower in patients with *RAS* mutations at rMAF <50% compared to those with mutations at rMAF >50% (Average depth

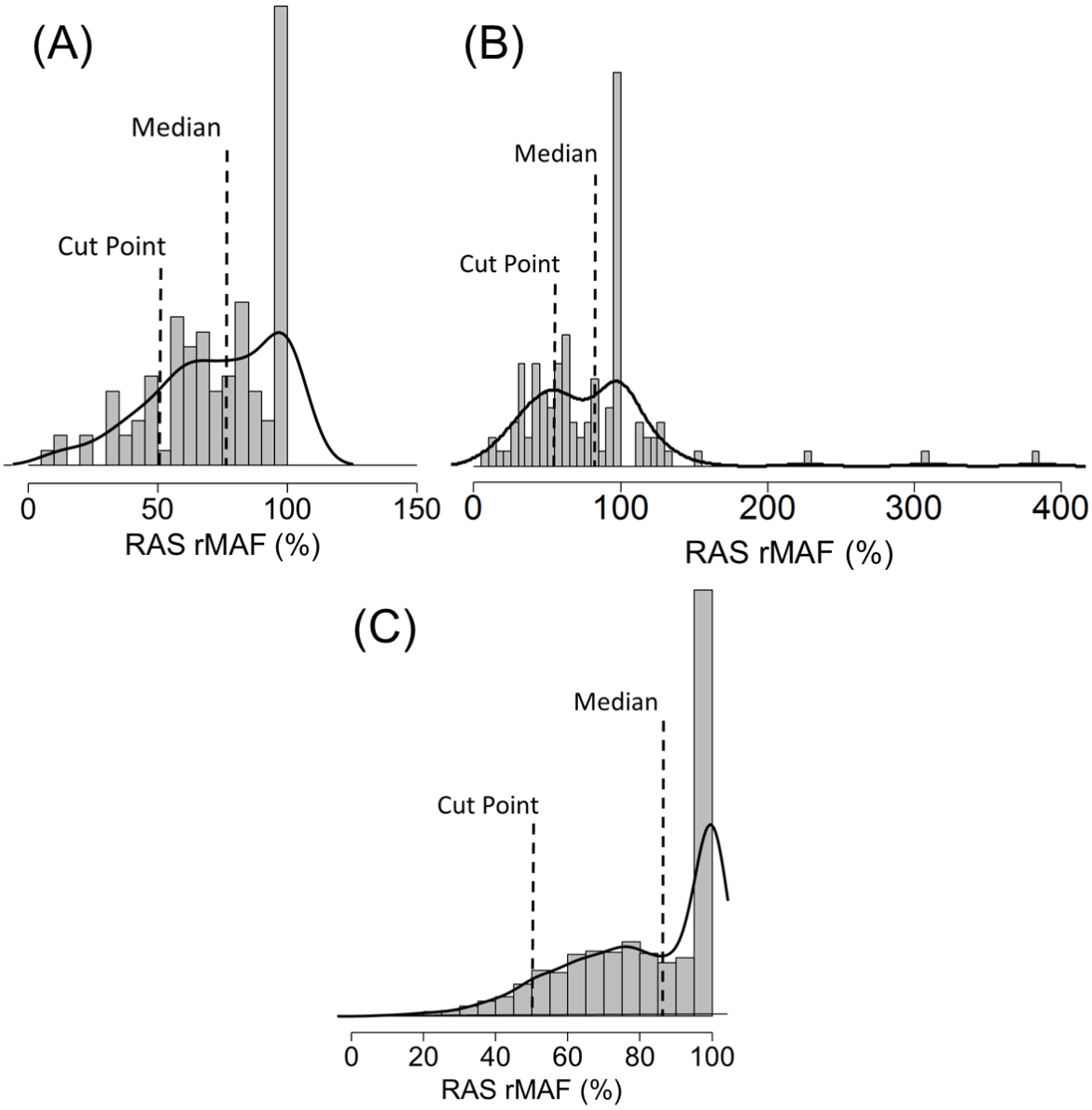
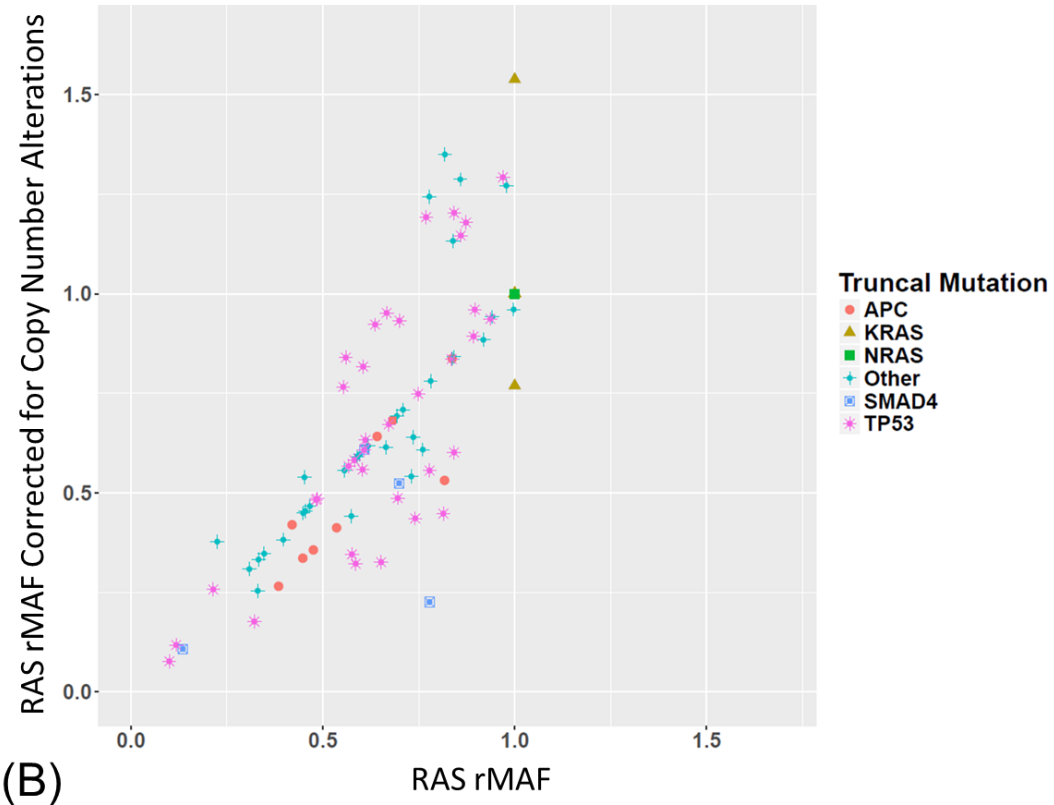


Figure 13. Histogram of (A) *RAS* mutation relative mutant allele frequency (rMAF) and (B) *RAS* mutation rMAF corrected for copy number alterations among patients with metastatic colorectal cancer who received NGS with a T200 panel or (C) a CMS 46 panel.

(A)



(B)

Truncal Mutation	Frequency	% of Total
TP53	78	37.7%
KRAS	24	11.6%
APC	21	10.1%
SMAD4	6	2.9%
ARID1A	4	1.9%
NRAS	4	1.9%
CTNNB1	3	1.4%
EGFR	3	1.4%
Mutation truncal in only one/two patients	64	30.9%

Figure 14. (A) Impact of copy number alterations on the relative mutant allele frequency (rMAF) of *RAS* mutations and (B) mutations selected as the most truncal mutation for calculation of rMAF.

Table 6. Baseline characteristics of patients with metastatic colorectal cancer who underwent sequencing with a T200 panel stratified by *RAS* mutation relative mutant allele frequency (rMAF).

Group	RAS Mutation <50% rMAF (N=21)	RAS Mutation >50% rMAF (N=89)	RAS WT (N=97)	P (RAS Mutant)	P (All)
Median Age (Interquartile Range)	46 (41-57)	51 (42-58)	49 (44-57)	0.53	0.77
Male (%)	12 (57.1%)	43 (48.3%)	56 (57.7%)	0.47	0.41
Female (%)	9 (42.9%)	46 (51.7%)	41 (42.3%)		
MSI-H (% of known)	1 (4.8%)	2 (2.3%)	1 (1.0%)	0.52	0.51
Mucinous or Signet Histology	4 (19.0%)	12 (13.5%)	11 (11.3%)	0.52	0.63
Metastatic at Diagnosis	8 (38.1%)	58 (65.2%)	62 (63.9%)	0.023	0.060
Biopsy Site (Metastasis:Primary)	7:13*	36:53	43:53*	0.65	0.67
Side					
Left	12 (57.1%)	52 (58.4%)	77 (79.4%)		
Right	9 (42.9%)	36 (40.4%)	20 (20.6%)	0.87	0.0062
Unknown	0 (0%)	1 (1.1%)	0 (0%)		
Differentiation					
Well/Moderately Differentiated	18 (85.7%)	81 (91.0%)	71 (74.0%)	0.47	0.0087
Poorly Differentiated	3 (14.3%)	8 (9.0%)	25 (26.0%)		
Average Coverage	485X	671X	645X	0.027	0.073
Average RAS Coverage	630X	852X	n/a	0.076	
BRAF V600 Mutated	0 (0%)	0 (0%)	8 (8.2%)	1	0.009
KRAS Mutant	20 (95.2%)	82 (92.1%)	0 (0%)	0.62	
NRAS Mutant	1 (4.8%)	8* (9.0%)	0 (0%)	1	
TP53 Mutant	14 (66.7%)	60 (67.4%)	68 (70.1%)	0.95	0.20
APC Mutant	16 (76.2%)	50 (56.2%)	52 (53.6%)	0.092	0.14
PIK3CA Mutant	7 (33.3%)	24 (27.0%)	9 (9.3%)	0.56	0.0022
SMAD4 Mutant	6 (28.6%)	16 (18.0%)	13 (13.4%)	0.28	0.23
FBXW7 Mutant	2 (9.5%)	11 (12.4%)	10 (10.3%)	1	0.88

*One patient with rMAF <50% and one *RAS* wild type patient had missing information regarding the site of biopsy.

485X vs 671X, P=0.027) and there were trends towards lower *RAS* coverage among the low allele frequency group (Average coverage 630X vs 852X, P=0.076).

When comparing OS based on rMAF, patients with *RAS* rMAF >50% showed strong trends towards an association with worse OS than patients with *RAS* rMAF <50% (HR 1.59, 95% CI 0.97-2.40, P=0.075) [Figure 15 - A]. Median OS was estimated at 55.4 months, 32.2 months, and 52.7 months for patients with *RAS* rMAF <50%, >50%, and wild type patients respectively. In multivariate models that controlled for age, MSI status, stage at diagnosis, age, histology, and primary tumor location, having a *RAS* mutation with an rMAF >50% was associated with a worse OS than wild type patients (HR 1.72, 95% CI 1.23-2.40, P=0.0010), while *RAS* mutant patients with rMAF <50% did not have a worse prognosis (HR 1.08, 95% CI 0.61-1.90, P=0.79). Other variables significant in the model included having stage IV disease at diagnosis and having a right sided primary [Table 7]. When high and low rMAF mutations were directly compared, they did not significantly differ.

After correcting rMAF for copy number alterations, similar trends suggesting that higher allele frequency may be associated with a worse outcome were noted (HR 1.41, 95% CI 0.91-2.12, P=0.13), but again were not significant [Figure 15 – B]. In multivariate models using the copy number corrected rMAF, patients with rMAF >50% had a worse prognosis than wild type patients (HR 1.87, 95% CI 1.34-2.61, P<0.0001) but low allele frequency *RAS* mutations did not differ from wild type patients (HR 1.36, 95% CI 0.85-2.17, P=0.21). Of the other variables tested for inclusion in the multivariate model, only the presence of stage IV disease at diagnosis met criteria to remain in the model (HR 1.43, 95% CI 1.03-1.98, P=0.033) [Table 7]. When high and low rMAF mutations were directly compared, they did not significantly differ.

As *RAS* mutations were occasionally truncal and may be impacted by amplifications, we performed a sensitivity analysis to ensure that patients with *RAS* mutations occurring in the

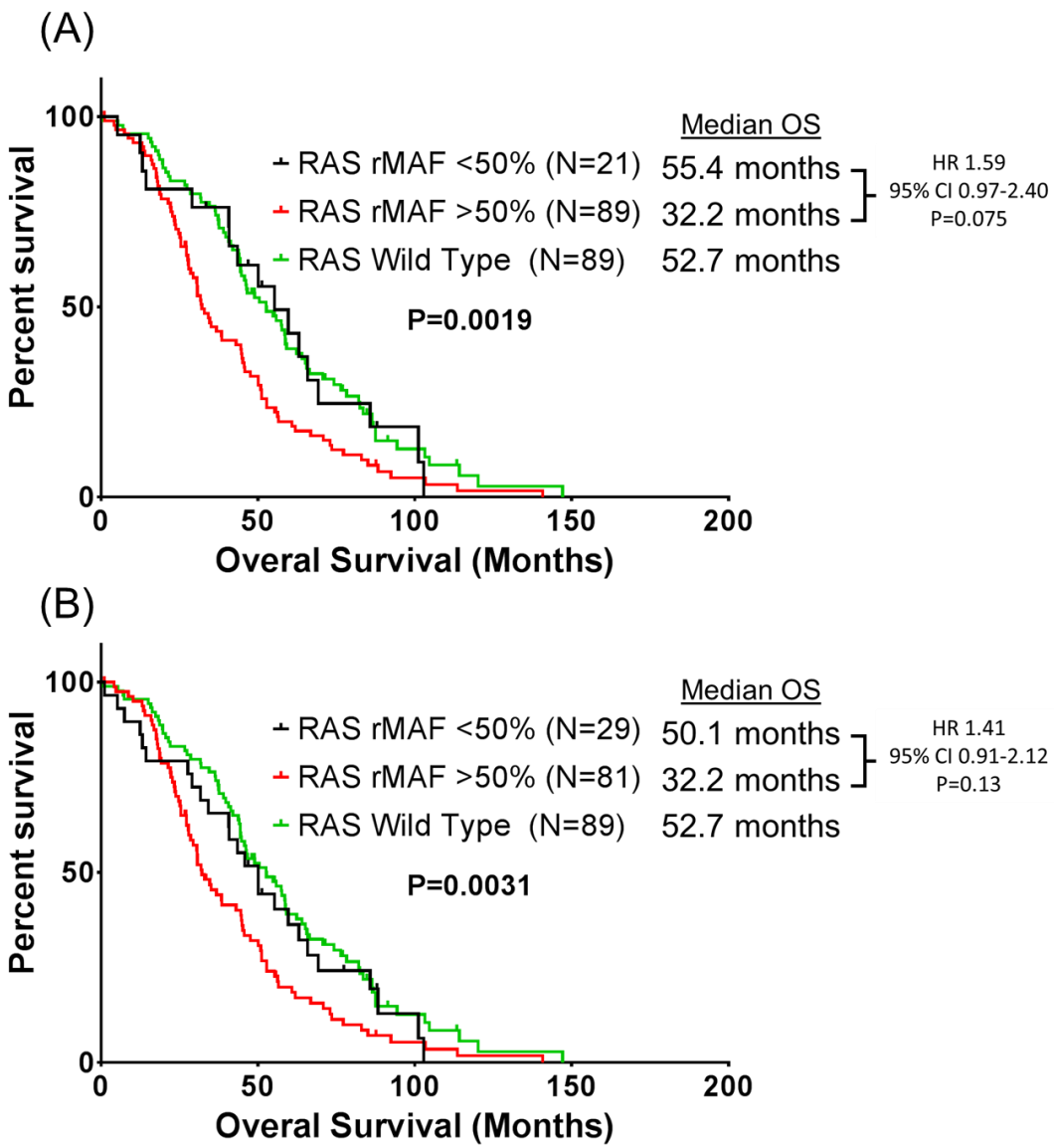


Figure 15. Impact of *RAS* mutation relative mutant allele frequency (rMAF) on overall survival in metastatic colorectal cancer. rMAF was calculated (A) as a direct comparison between *RAS* and the mutation with the highest allele frequency for a patient and (B) with consideration of copy number alterations.

Table 7. Multivariate models assessing the impact of *RAS* mutation relative mutant allele frequency (rMAF) on overall survival in metastatic colorectal cancer. Model 1 utilizes an uncorrected rMAF, while model 2 uses rMAF adjusted for copy number alterations.

Model 1. Comparison of RAS mutant rMAF		
Co-Variate	HR (95% CI)	P
RAS Wild Type	Reference	
RAS rMAF <50%	1.08 (0.61-1.90)	0.79
RAS rMAF >50%	1.72 (1.23-2.40)	0.0010
Metastatic at Diagnosis	1.43 (1.03-1.99)	0.033
Right Sided Primary	1.41 (1.01-1.97)	0.044
Model 2. Comparison or RAS mutant rMAF with correction for copy number alterations		
RAS Wild Type	Reference	
RAS rMAF <50%	1.36 (0.85-2.17)	0.21
RAS rMAF >50%	1.87 (1.34-2.61)	<0.0001
Metastatic at Diagnosis	1.43 (1.03-1.98)	0.033

numerator and denominator of their rMAF calculation were not skewing results. The second highest allele frequency mutation was chosen as the truncal mutation and used in the copy number corrected rMAF calculation for this analysis [Figure 16 – A]. Prognostic differences between rMAF >50% and rMAF <50% groups became statistically significant (HR 1.55, 95% CI 1.01-2.30, P=0.047). A second sensitivity analysis was performed with a replacement of TP53 whenever it was chosen as the truncal mutation for calculating rMAF, as TP53 is prone to copy number losses. In this second sensitivity analysis, prognostic differences between rMAF >50% and rMAF <50% groups showed similar trends to all other analyses but were not significant (HR 1.48, 95% CI 0.94-2.22, P=0.098) [Figure 16 – B].

Our analysis was also repeated using the pathologist estimate for tumor content in sequenced samples. Seven patients with *RAS* mutations did not have reported tumor content and were excluded. Using pathologist estimates of tumor content resulted in 50 patients being classified to the rMAF <50% group and 52 patients being classified as rMAF >50%. This redistribution of groups resulted in non-significant differences between *RAS* mutant patients based on allele frequency (HR 1.01, 95% CI 0.67-1.52, P=0.95) [Figure 16 –C]. However, if the groups were re-divided so a similar proportion of patients were in each of the high vs low allele frequency groups as were noted in the main rMAF analysis, similar finding to our main analysis were noted, with patients having a higher *RAS* rMAF showing trends towards worse OS (HR 1.54, 95% CI 0.94-2.38, P=0.098) [Figure 16 – D].

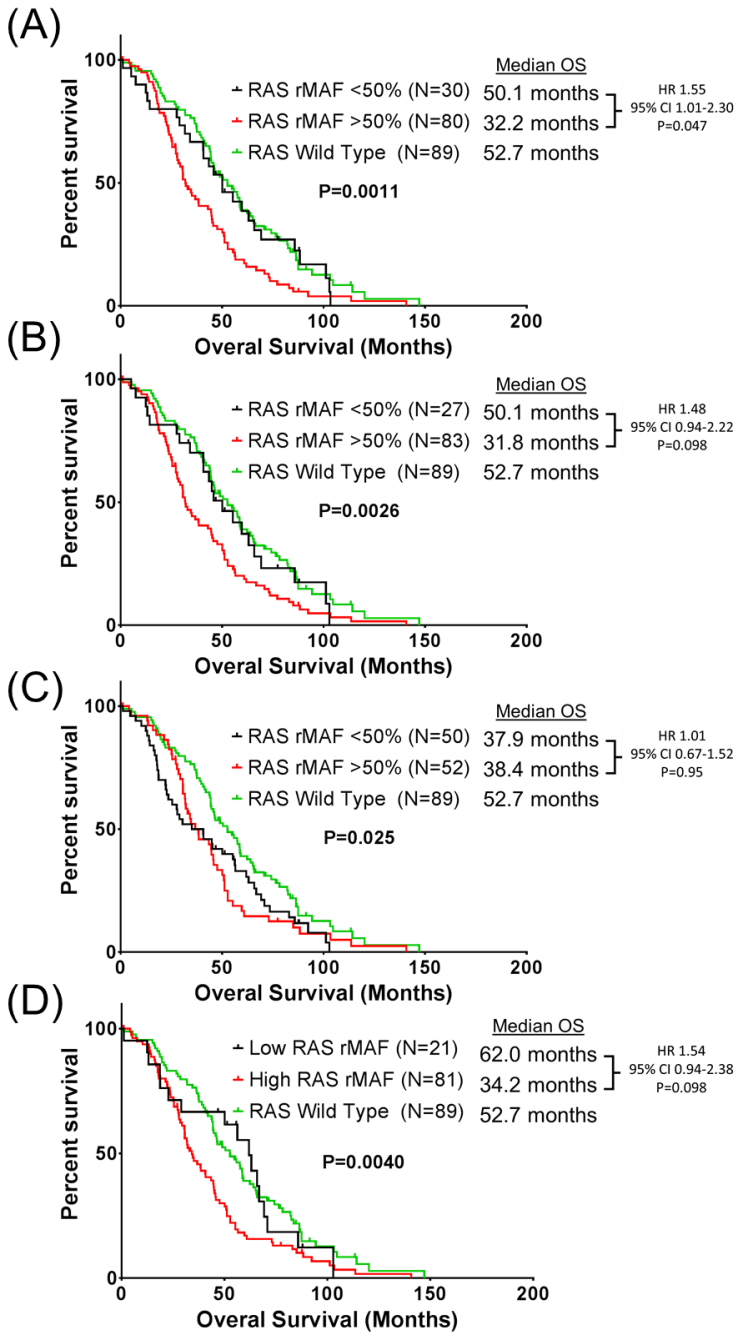


Figure 16. Sensitivity analysis of the impact of *RAS* mutation relative mutant allele frequency (rMAF) on overall survival in metastatic colorectal cancer with (A) replacement of *RAS* as truncal mutation whenever found to be truncal, (B) replacement of TP53 as truncal mutation whenever found to be truncal, (C) use of pathology assessed tumor content to determine rMAF and (D) use of pathology assessed tumor content with an altered rMAF cut point.

Relative Mutant Allele Frequency as a Prognostic Marker in MDA CMS46 Cohort

Of 1877 mCRC patients who had a CMS 46 NGS panel performed, 892 (47.5%) were *RAS* wild type, 85 (4.5%) had low allele frequency *RAS* mutations (rMAF <50%), and 900 (48.0%) had high allele frequency *RAS* mutations (rMAF >50%). rMAF distribution is displayed in Figure 13 – C. As seen in Table 8, baseline characteristics were very similar between patients classified as low or high *RAS* rMAF. The only difference noted was that rMAF <50% patients were more likely to have left sided tumors than rMAF >50% patients (P=0.0065). Patients with any *RAS* mutation were more likely to be female (P<0.0001), or have a SMAD4 (P<0.0001) or PIK3CA mutation (P<0.0001) and less likely to have mucinous/signet ring cell histology (P=0.0060) or have a BRAF V600 mutation (P<0.0001) than wild type patients. Unlike the T200 cohort, there was no difference in stage at diagnosis between low and high allele frequency *RAS* mutant patients (P=0.68). Copy number information was not available from the CMS 46 panel.

Patients with *RAS* rMAF >50% had a worse prognosis compared to *RAS* wild type patients (HR 1.52, 95% CI 1.35-1.74, P<0.0001) while patients with *RAS* rMAF <50% did not (HR 1.01, 95% CI 0.74-1.39, P=0.95) [Figure 17]. These findings remained constant in a multivariate model that controlled for age, gender, stage at diagnosis, histology, primary tumor location, and MSI status [Table 9 – Model 1]. Other variables that were significant in the model included having stage IV disease at the time of diagnosis (HR 1.45, 95% CI 1.25-1.69, P<0.0001), mucinous or signet ring cell histology (HR 1.31, 95% CI 1.06-1.60, P=0.012), and a right sided primary tumor location (HR 1.32, 95% CI 1.13-1.54, P=0.0010). When directly comparing *RAS* mutant patients based on allele frequency, *RAS* rMAF >50% was associated with a worse OS than *RAS* rMAF <50% in both univariate (HR 1.55, 95% CI 1.12-1.88, P=0.0058) and multivariate models (HR 1.49, 95% CI 1.04-2.13, P=0.031). The multivariate model included age, gender, stage at diagnosis, histology, primary tumor location, and MSI status [Table 9 – Model 2]. However, the only co-variate besides allele frequency that remained significant was having stage IV disease at the time of diagnosis (HR 1.28, 95% CI 1.06-1.55, P=0.011).

In addition to comparing *RAS* rMAF as defined by a truncal mutation detected on the CMS 46 panel, we also used the pathologist determined tumor content [Figure 17 – B]. This analysis shifted the distribution of groups so that more patients were considered to have low allele frequency mutations. Though all patients with *RAS* mutations had a worse prognosis than wild type patients (P<0.0001), there were no differences based on *RAS* rMAF in this pathologist defined groups (HR 1.10, 95% CI 0.91-1.33, P=0.31). As this may have been due to a shifting of the group proportions, we repeated the analysis and split the *RAS* mutant patients so a similar proportion of patients to the molecularly defined rMAF were included in the low frequency group. Once again, no differences were demonstrated between pathologically defined *RAS* rMAF groups (HR 1.09, 95% CI 0.82-1.44, P=0.58) [Figure 17 – C].

Table 8. Baseline characteristics of patients with metastatic colorectal cancer who underwent sequencing with a CMS 46 NGS panel stratified by relative mutant allele frequency (rMAF) of *RAS* mutations.

Group	RAS Mutation <50% rMAF (N=85)	RAS Mutation >50% rMAF (N=900)	RAS WT (N=892)	P (RAS Mutant)	P (All)
Median Age (Interquartile Range)	55 (46-63)	55 (45-63)	55 (46-62)	0.48	0.77
Male (%)	37 (43.5%)	469 (52.1%)	552 (61.9%)	0.13	<0.0001
Female (%)	48 (56.5%)	431 (47.9%)	340 (38.1%)		
MSI-H (% of known)	3 (4.4%)	23 (3.1%)	31 (4.3%)	0.49	0.49
Mucinous or Signet Histology	15 (17.6%)	155 (17.2%)	107 (12.0%)	0.95	0.0060
Metastatic at Diagnosis	57 (67.1%)	578 (64.2%)	580 (65.0%)	0.68	0.85
Biopsy Site (Metastasis:Primary)	29:56	339:555	358:529	0.49	0.37
Side					
Left	65 (76.5%)	554 (61.6%)	677 (76.0%)	0.0065	<0.0001
Right	20 (23.5%)	346 (38.4%)	214 (24.0%)		
BRAF V600 Mutated	1 (1.2%)	1 (0.1%)	113 (12.7%)	0.17	<0.0001
KRAS Mutant	76 (89.4%)	833 (92.6%)	0 (0%)	0.17	
NRAS Mutant	9 (10.6%)	70 (7.8%)	0 (0%)	0.38	
TP53 Mutant	59 (69.4%)	534 (59.3%)	621 (69.6%)	0.085	<0.0001
APC Mutant	49 (57.6%)	452 (50.2%)	346 (38.8%)	0.22	<0.0001
PIK3CA Mutant	17 (20.0%)	181 (20.1%)	89 (10.0%)	0.95	<0.0001
SMAD4 Mutant	19 (22.4%)	139 (15.4%)	75 (8.4%)	0.11	<0.0001
FBXW7 Mutant	9 (10.6%)	82 (9.1%)	51 (5.7%)	0.67	0.014

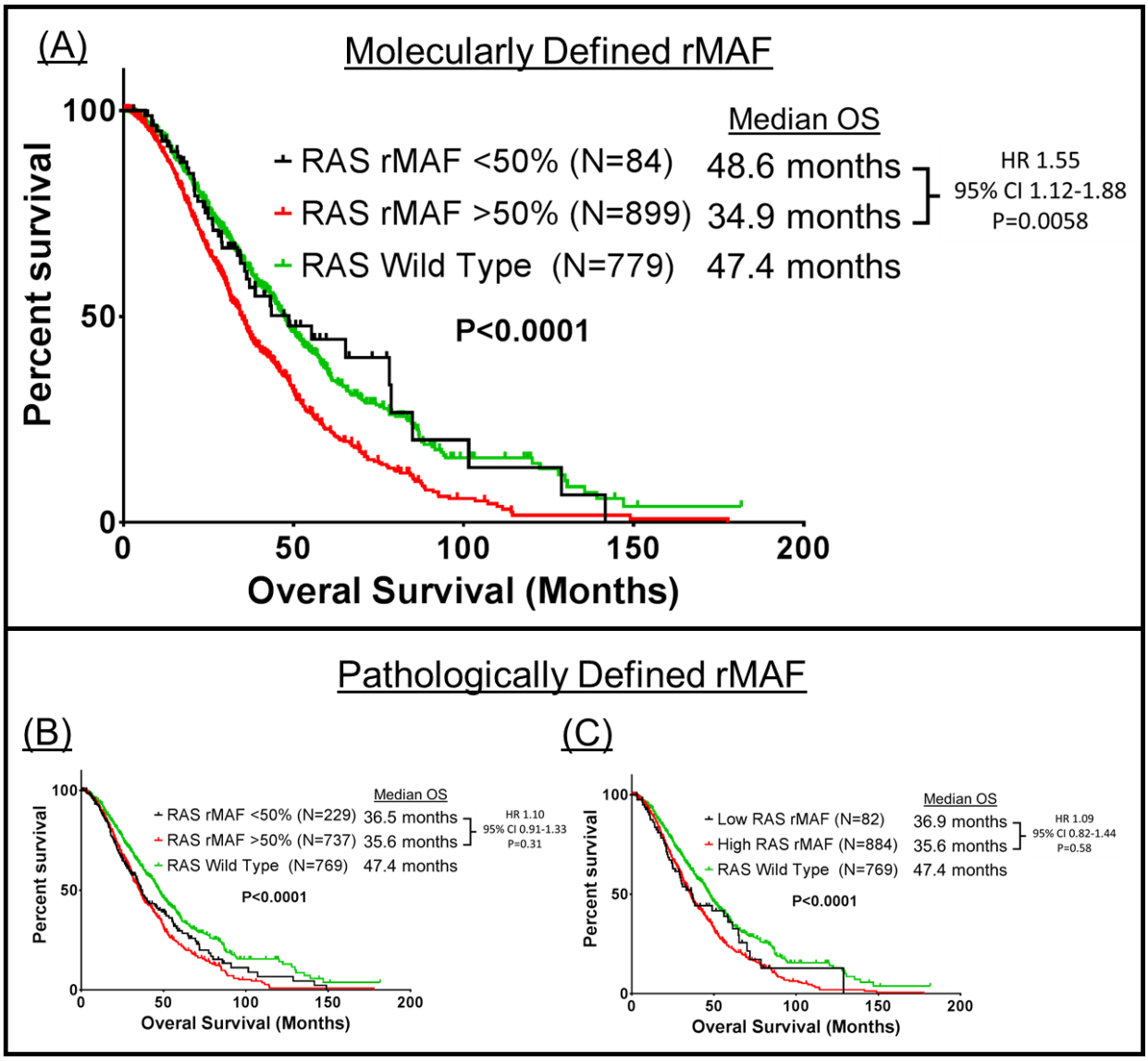


Figure 17. Impact of *RAS* mutation relative mutant allele frequency (rMAF) on overall survival in metastatic colorectal cancer patients undergoing NGS with a CMS 46 panel. rMAF was calculated (A) using a truncal mutation to define tumor content and (B) using pathologist defined tumor content. Even after adjusting the cut point between groups in the pathologically defined rMAF analysis (C), prognostic differences were not noted based on rMAF.

Table 9. Multivariate models assessing the impact of *RAS* mutation relative mutant allele frequency (rMAF) on overall survival in metastatic colorectal cancer. Model 1 compares *RAS* mutant patients to wild type patients as the reference, while model 2 directly compares *RAS* rMAF groups.

Model 1. Comparison of RAS mutant rMAF with wild type patients as reference		
Co-Variate		
RAS Wild Type	Reference	
RAS rMAF <50%	1.01 (0.70-1.46)	0.96
RAS rMAF >50%	1.46 (1.25-1.70)	<0.0001
Metastatic at Diagnosis	1.45 (1.25-1.69)	<0.0001
Mucinous or Signet Histology	1.31 (1.06-1.60)	0.012
Right Sided Primary	1.32 (1.13-1.54)	0.0010
Model 2. Comparison or RAS mutant rMAF with rMAF <50% as reference		
RAS rMAF <50%	Reference	
RAS rMAF >50%	1.49 (1.04-2.13)	0.031
Metastatic at Diagnosis	1.28 (1.06-1.55)	0.011

Aim 3: To evaluate whether allele frequency can be used as a predictor of functional impact.

Given our findings that *RAS* rMAF was associated with prognostic differences, we next evaluated whether rMAF could be used as a predictive marker of functional significance of any gene. This analysis used the MDA T200, MDA CMS 46, cfDNA, and Project Genie cohorts. Coding variants from any gene were considered individually in this analysis.

PODS Functional Annotation

Based on the MDA PODS functional annotation, inactivating mutations occurred at the highest rMAF in all 4 cohorts, while activating mutations were the next highest rMAF category in 3 of 4 cohorts [Figure 18]. In the MDA CMS 46 cohort, likely benign variants had the second highest rMAF. In both the T200 and the cfDNA cohorts, there were statistically significant differences between functionally significant (activating/inactivating) variants and likely benign variants that were also numerically large (25.2% and 27.1%, respectively), however in the MDA CMS 46 these differences were not significant and in the Project Genie cohort the difference in rMAF was only significant when comparing inactivating to likely benign variants. The range of difference between inactivating variants and likely benign variants was 2.6%-27.1%, with the largest range occurring in the cfDNA cohort. Variants that had no functional annotation occurred at the lowest rMAF in the MDA CMS 46 and MDA T200 cohorts, while they had the second lowest rMAF in the Project Genie cohort. The cfDNA cohort differed from the other 3 panels as it also had information about synonymous mutations which should have no functional impact on a protein. These alterations occurred at the lowest rMAF.

SIFT/Polyphen Functional Annotation

In using bioinformatic algorithms to predict functional significance, we saw significantly different results based on whether mutations were categorized using SIFT or Polyphen [Figure 19 & 20]. The SIFT score consistently showed an association between deleterious variants and a high rMAF across all 4 cohorts. Deleterious variants had an rMAF that was statistically higher than

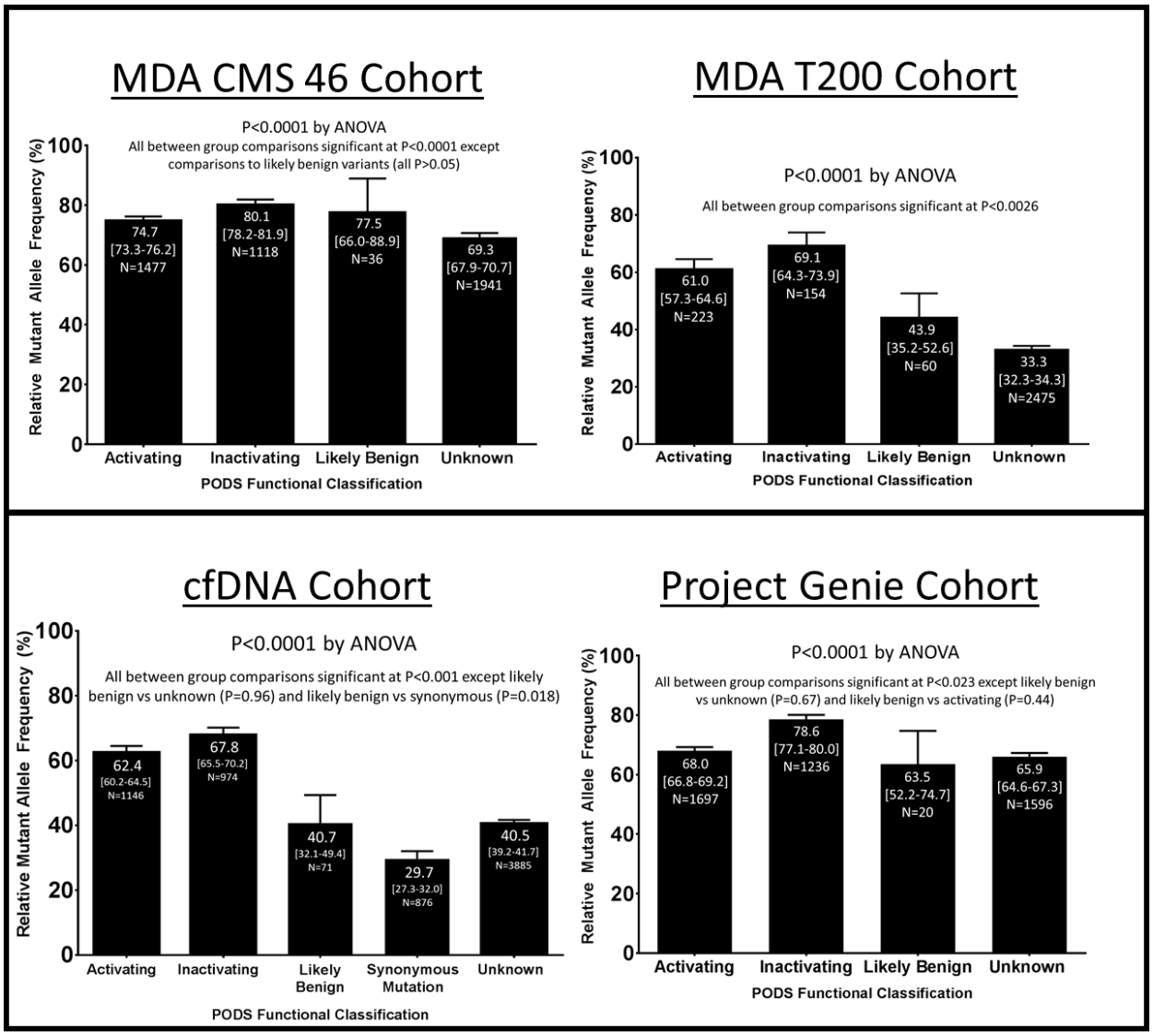


Figure 18. Impact of the Precision Oncology Decision Support (PODS) Core defined functional significance of a mutation on the relative mutant allele frequency of that mutation. Values represent mean +/- 95% confidence interval.

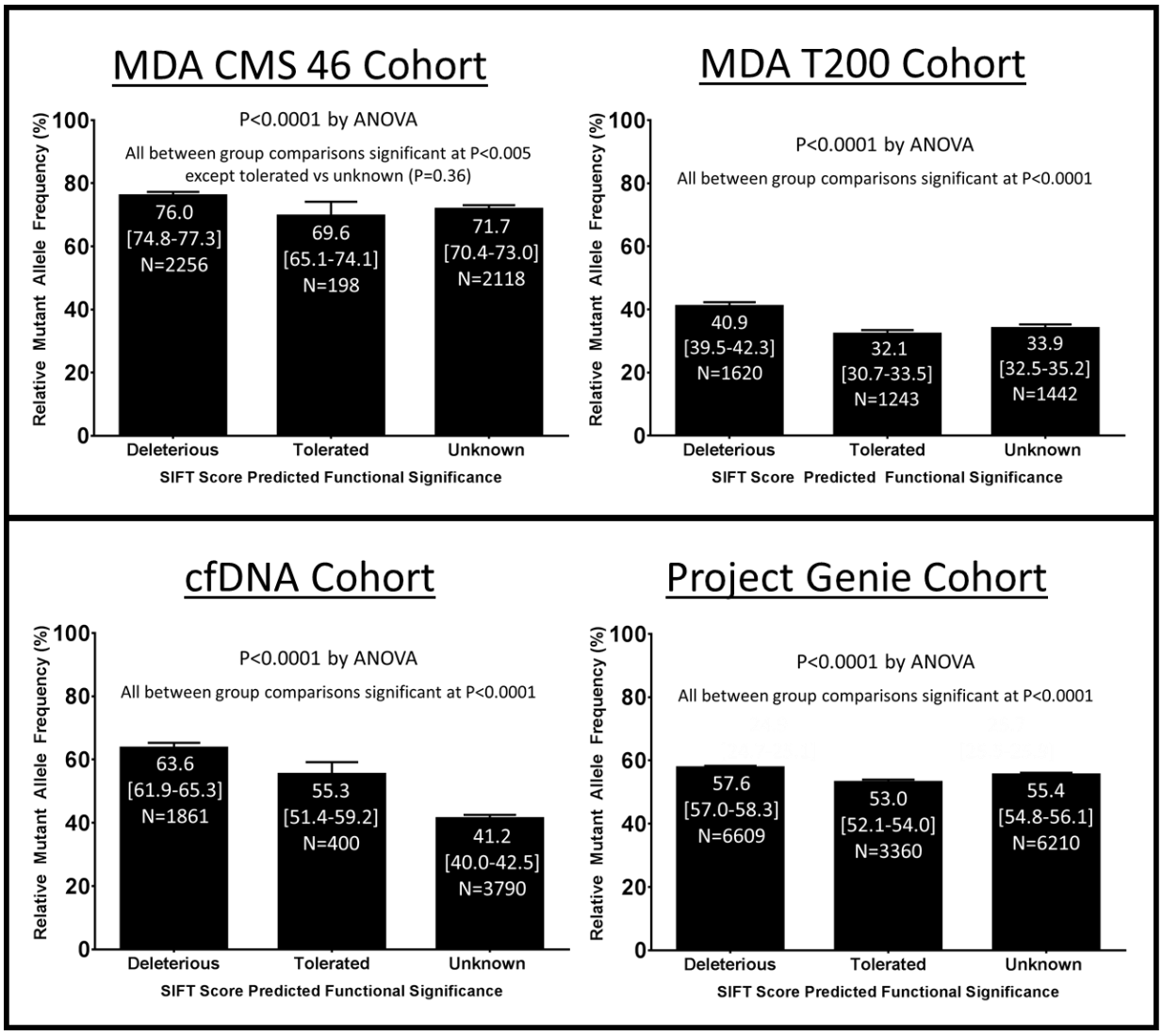


Figure 19. Impact of the SIFT score defined functional significance of a mutation on the relative mutant allele frequency of that mutation. Values represent mean +/- 95% confidence interval.

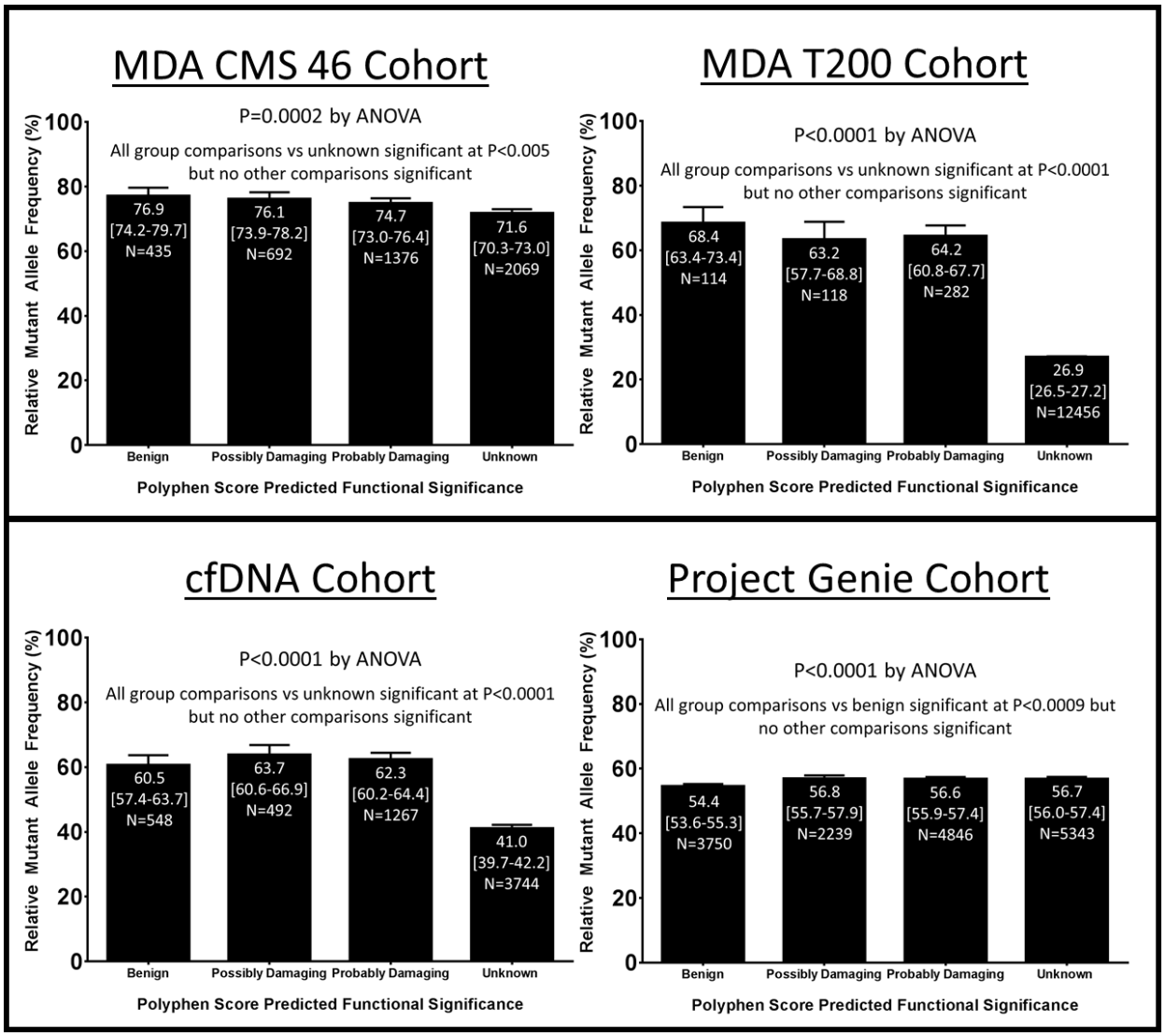


Figure 20. Impact of the Polyphen score defined functional significance of a mutation on the relative mutant allele frequency of that mutation. Values represent mean +/- 95% confidence interval.

both tolerated and unknown variants. The numerical magnitudes of differences in rMAF were however very small (range 4.6%-8.8%), suggesting much of the statistical significance was driven by large sample sizes. rMAF differences were largest in the cfDNA group (8.3%). When Polyphen was used to categorize variants, there were significant differences across all groups, however no individual between group comparisons between benign and pathologic mutations were significant except in the Project Genie cohort where all groups had a higher rMAF than benign variants. Benign variants had a higher rMAF than probably damaging variants in 2 of the 4 cohorts and the range in difference between probably damaging and benign variants was -4.2% to 2.2%, with the largest difference occurring in the MDA T200 cohort, however the relationship was inverse.

When SIFT/Polyphen were considered together with a merged score, there appeared to be a gradual trend of increasing rMAF with higher scores, however variants that received a score of 0 had the highest rMAF in the MDA CMS 46 and cfDNA cohorts [Figure 21]. Although variants with a score of 1, 3, or 4 had higher rMAF than those with a score of 0, these groups had very small numbers compared to other comparisons. Overall, the magnitude of difference between variants with a score of 1 and a score of 4 ranged from 3.5% to 9.1%, with the largest magnitude of difference occurring in the cfDNA cohort.

Case Study #1 - BRAF Mutations

Given that our findings suggested that rMAF differed based on functional classification, we decided to choose a well characterized oncogene in CRC to apply this model towards. BRAF V600 mutations confer a negative prognosis in patients with mCRC and recent evidence has shown that non-V600 variants appear to be of less clinical significance.[58, 59] BRAF V600 mutations occurred at higher rMAFs than non-V600 mutations in the MDA CMS 46 cohort (P=0.0038) and cfDNA cohort (P<0.0001), however did not differ among the Project Genie (P=0.25) or MDA T200 cohorts (P=0.22) [Figure 22]. Only 23 BRAF mutations were available

for assessment in the MDA T200 cohort, however all other cohorts had 145 or more BRAF mutations to consider.

Case Study #2 – rMAF Stratified by Number of Mechanisms of Resistance

From an evolutionary perspective, the development of resistance often comes at a cost for a microorganism or tumor cell and there is little benefit to having multiple mechanisms of resistance to the same drug. Using the cfDNA cohort, we aimed to determine whether there was a correlation between the number of mechanisms of resistance to anti-EGFR therapy with the rMAF of those mechanisms of resistance. As seen in Figure 2.6, a continuous decrease in average rMAF of a resistance mechanism was seen as patients developed an increasing number of mechanisms of resistance ($P<0.0001$). If a patient had a single mechanism of resistance, the average rMAF of that alteration was 74%, compared to 16% when a patient had 6+ potential mechanisms of resistance.

Case Study #3 – rMAF Stratified by RAS Categorization and Functional Significance

We next sought to evaluate whether the allele frequency of RAS variants differed based on RAS categorization (typical, extended, or atypical) or functional significance based on the NovellusDx FACT assay. As seen in Figure 24, atypical variants occurred at a lower rMAF than typical variants in 3 of the 4 cohorts and the 4th cohort (MDA T200) only had 2 atypical variants to evaluate. Extended RAS mutations did not show consistent trends in their relationship to typical variants across the 4 cohorts. Figure 25 shows that variants which resulted in signaling above that of KRAS exon 2 mutations were of lower rMAF than KRAS exon 2 mutations in 3 out of 4 cohorts.

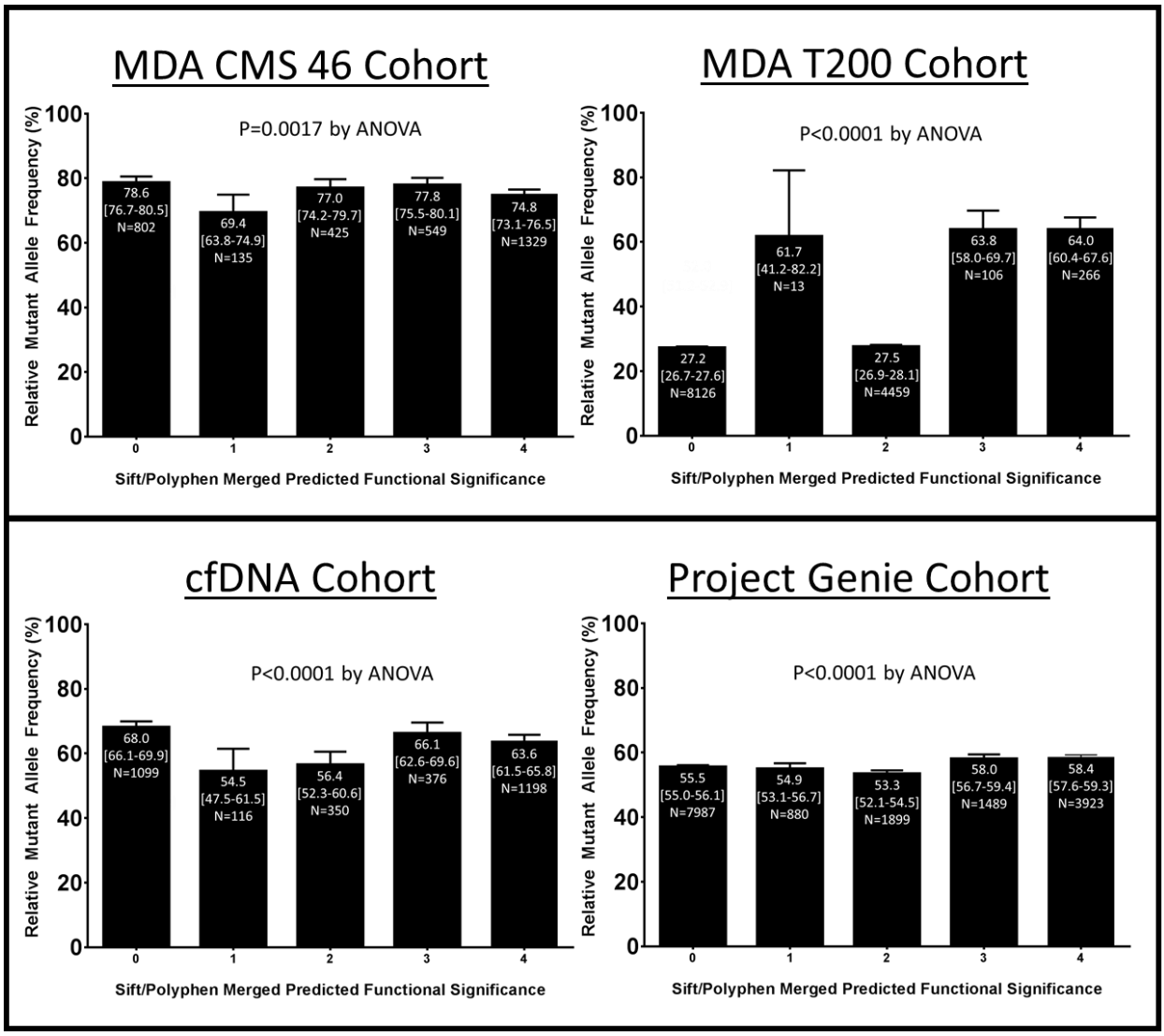


Figure 21. Impact of the SIFT/Polyphen Merged predicted functional significance of a mutation on the relative mutant allele frequency of that mutation. Values represent mean +/- 95% confidence interval.

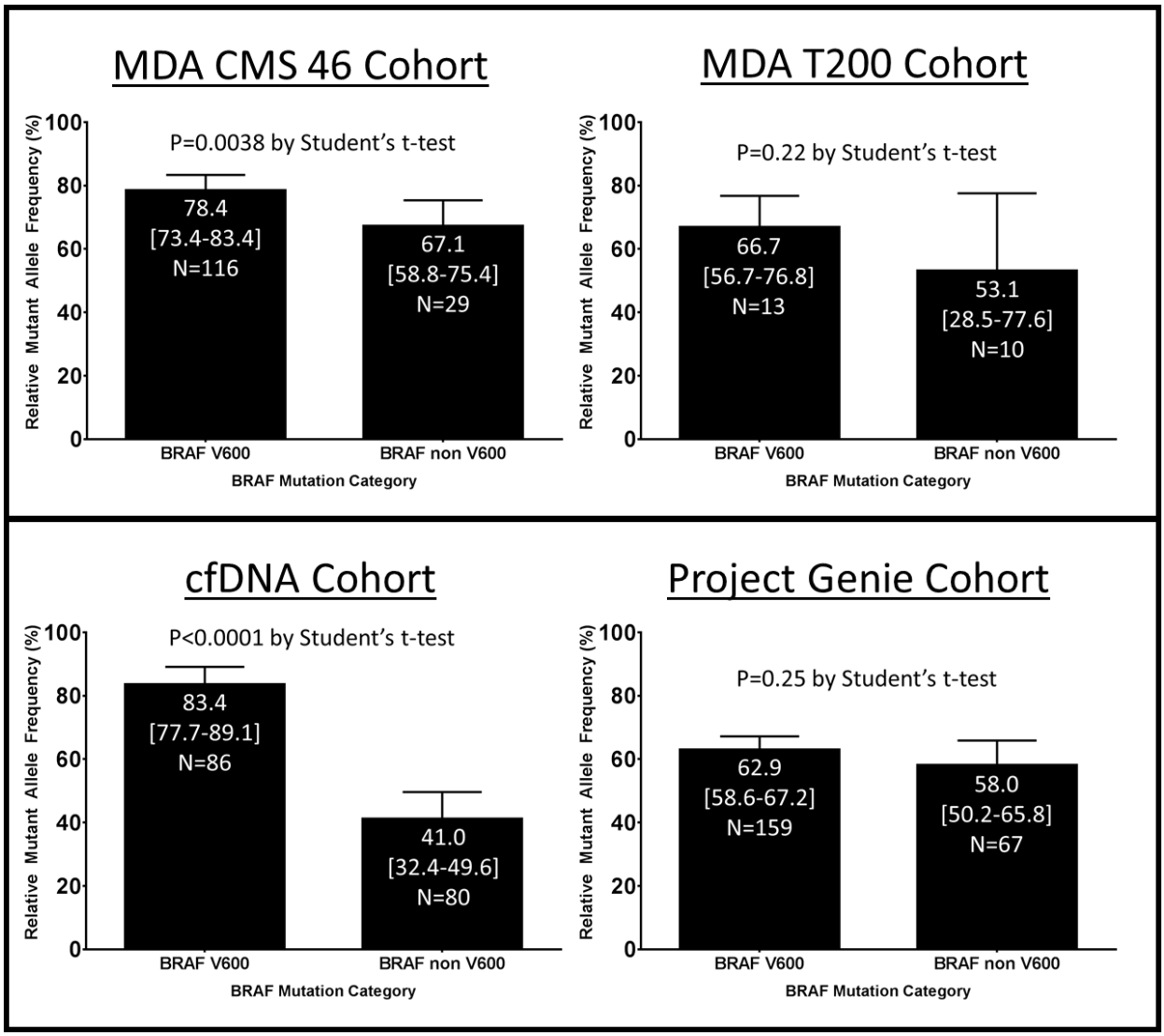


Figure 22. Comparison of the relative mutant allele frequency (rMAF) of BRAF V600 and non-V600 mutations in colorectal cancer. Values represent mean +/- 95% confidence interval.

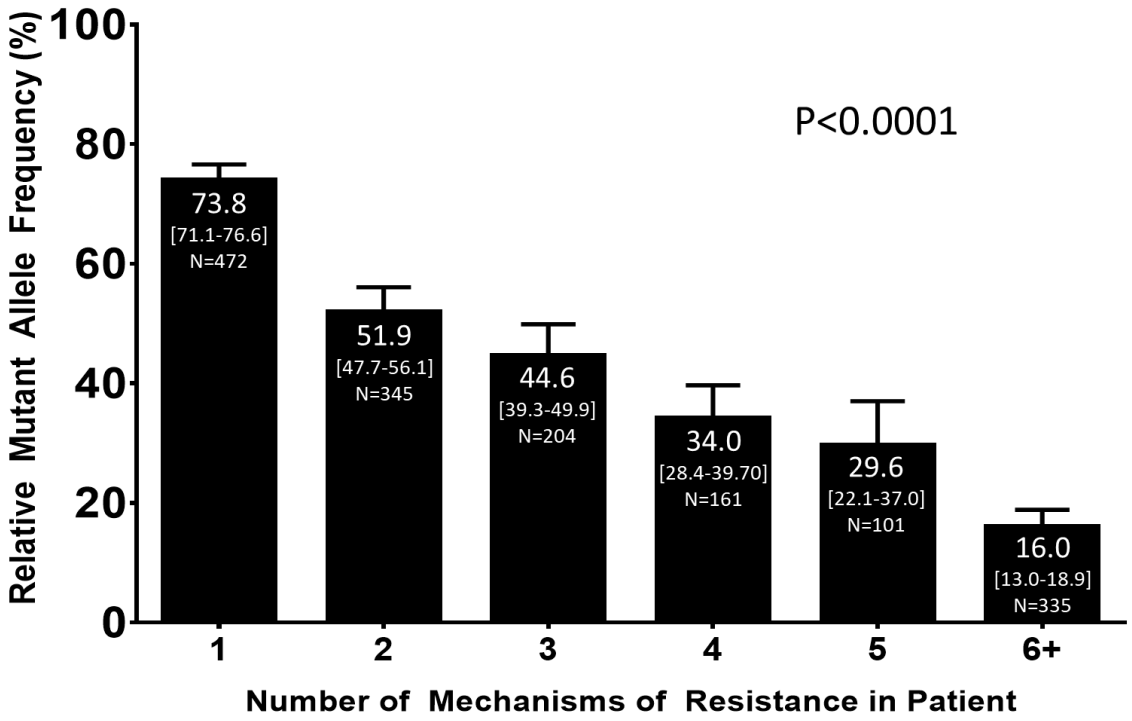


Figure 23. Average relative mutant allele frequency of single nucleotide variants that result in resistance to an anti-EGFR agent in colorectal cancer stratified by the number of mechanisms of resistance that are present in that patient. Values represent mean +/- 95% confidence interval.

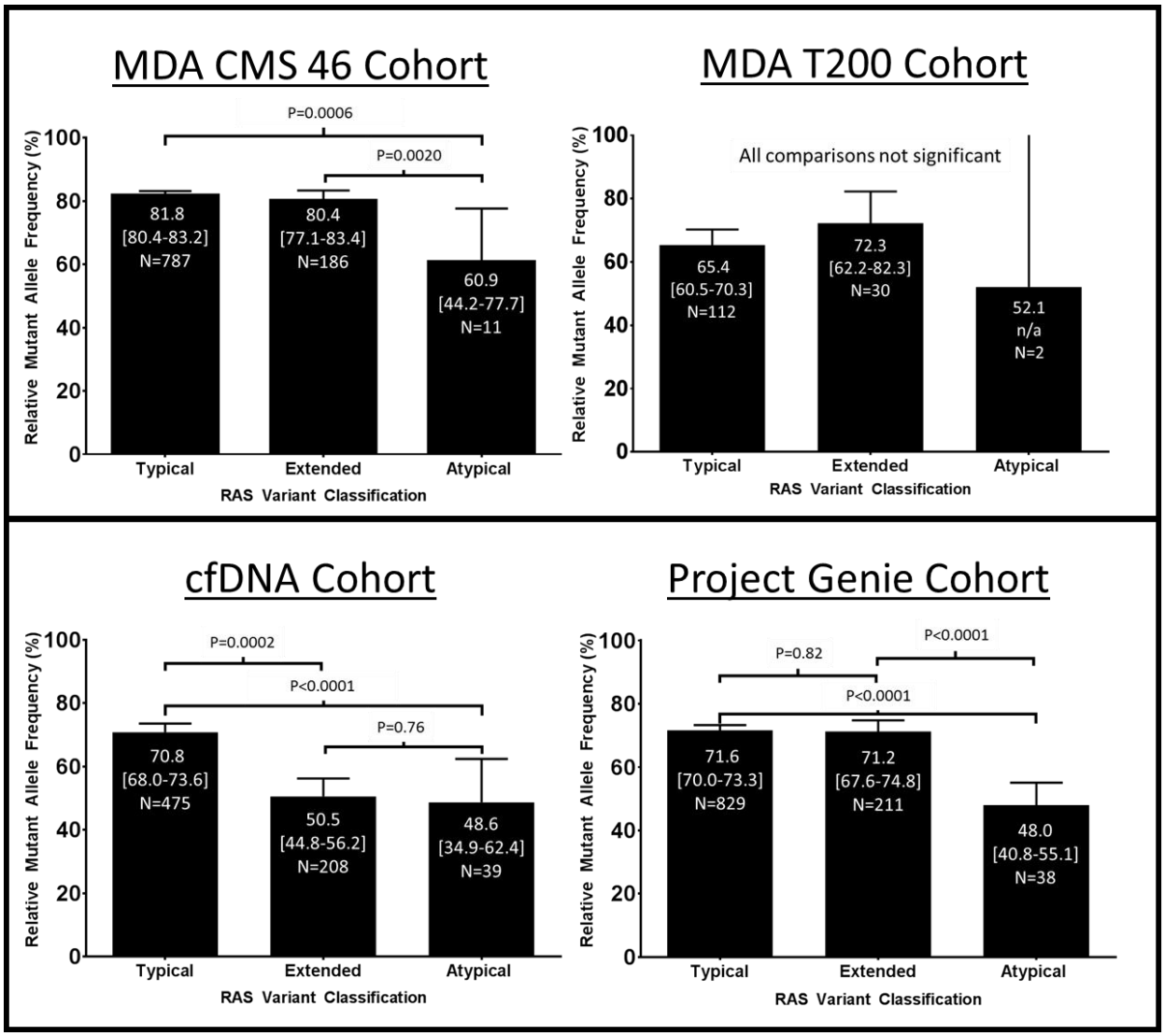


Figure 24. Average relative mutant allele frequency of RAS variants based on the category of a RAS variant (typical vs extended vs atypical). Values represent mean +/- 95% confidence interval.

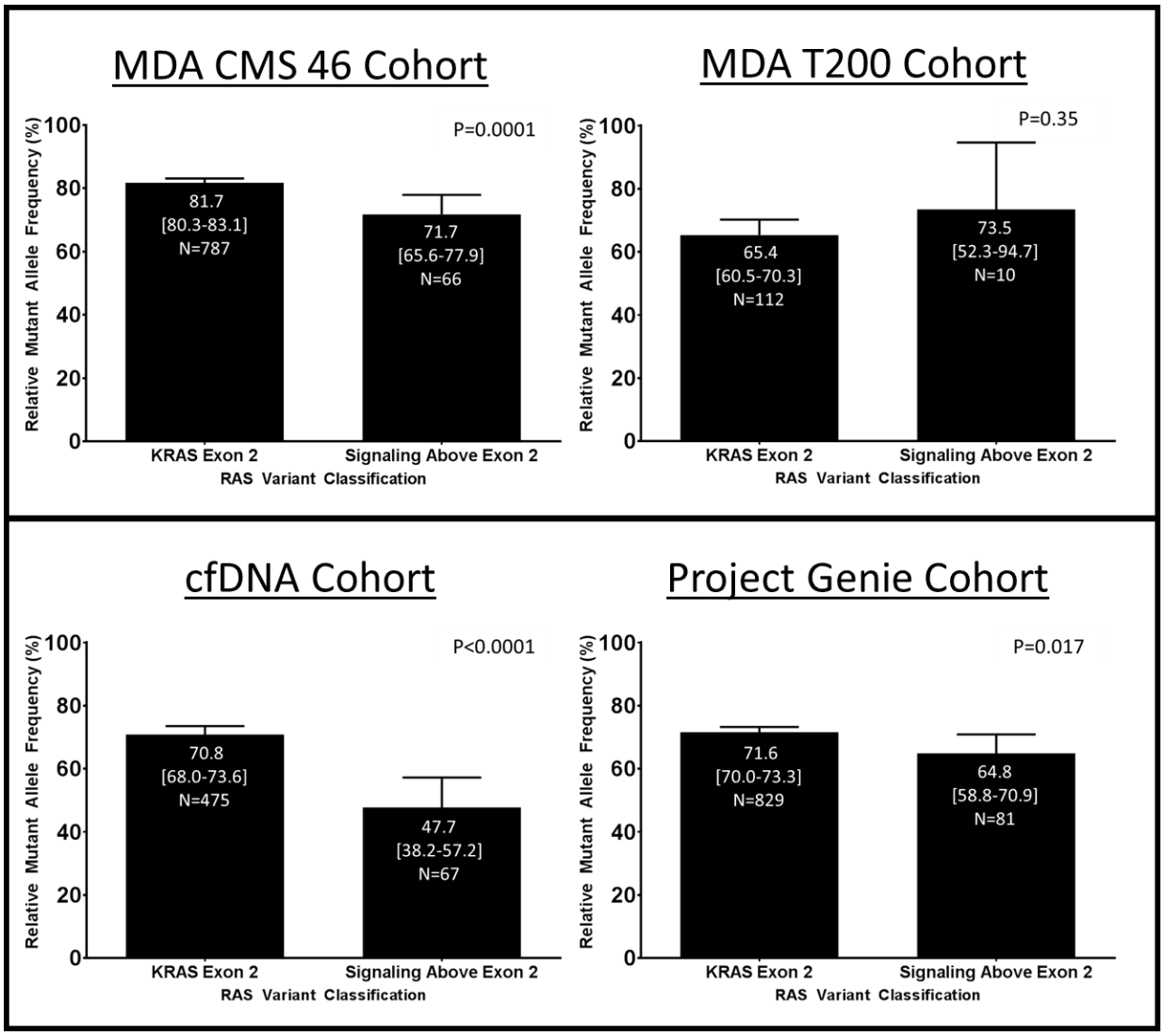


Figure 25. Average relative mutant allele frequency of RAS variants based on functional activity of the variant. Patients were divided into groups based on whether they had a KRAS exon 2 mutation or a mutation that resulted in significantly more downstream signaling based on the NovellusDx FACT assay. Values represent mean +/- 95% confidence interval.

Chapter 4: Discussion

With recent advancements in genome sequencing capabilities and the exponential decline in sequencing costs over the past decade, it is now possible to move precision oncology from the bench to the clinic.[38] However, the abundance of data now available has expanded our awareness of the unanswered questions that arise from these new capabilities. Two particular sequencing capacities that have unearthed these questions include the ability to sequence at higher sensitivities and the ability to increase coverage over larger regions of the genome. Despite significant developments in the molecular characterization of CRC over the past decade, *RAS* mutations still represent one of the most defining molecular characteristics of CRC. This body of work aims to determine how NGS can be leveraged to optimally define *RAS* mutant CRC and how to integrate functional characterization into the assessment of unknown variants. Given the vast number of novel variants continually discovered, annotation of which mutations have clinical significance is of utmost importance.

Aim 1: Describe the functional and clinical significance of atypical *RAS* mutations

Given the increasing use of more comprehensive sequencing platforms as part of standard care, we need robust mechanisms to annotate variants that are not well described. In collaboration with NovellusDx, we identified a comprehensive list of *RAS* variants that were either seen at our institution, Caris Life Sciences, or reported in the literature and performed functional annotation while also providing a real-world description of the prevalence of these alterations. Although pooled estimates of *RAS* mutation prevalence from clinical trials are available, these cohorts may not be representative of the entire CRC population. Clinical trial patients are frequently younger, have better performance status and may have other clinical characteristics that make them more likely to seek enrollment on a clinical trial. After pooling 8609 patients with mCRC from institutional and publicly available cohorts, we identified *RAS*

mutations in 49.3% (95% CI 48.2%-50.4%) of patients. This was statistically lower than the 55.9% reported by Peeters et al in a pooled analysis from 5 randomized controlled trials and highlights the importance of considering real-world data in addition to prospective clinical trials (P<0.0001).[31] Although there are always inherent biases in retrospective studies, the sheer size of our population and the fact that all included panels covered the guideline mandated codons strongly supports our point estimate as being one of the most robust available.[29]

Another important finding from our survey of these 7 databases was that mutations in certain codons described in current clinical guidelines are extremely uncommon. For example, out of 8609 patients, we did not note a single *NRAS* codon 117 or 146 mutation and only 1 *NRAS* codon 59 mutation was detected. Given that these variants were also not detected in the PRIME study which first evaluated extended *RAS* mutations, there is essentially no evidence to support their role as a predictive biomarker and their inclusion in standard of care panels is likely not warranted.[30] On the contrary, we noted several variants that although uncommon, still occurred at a frequency that rivaled many guideline cited variants and were shown to be functionally activating. These included *KRAS* Q22K, *KRAS* L19F, *KRAS* D33E, and *KRAS* T50I. Though these variants have not been evaluated as markers of resistance to anti-EGFR agents, their functional annotation and recurrent nature would suggest that they should be considered pathologic.

Although the NovellusDx FACT assay is a relatively new method for assessing functional significance, we have several sources of information that confirm the results obtained. A number of the variants included for functional characterization do not have clinical relevance in CRC. They were chosen specifically because of their pathogenesis in other diseases. *KRAS* V14I, P34R, T58I, and F156L have all been associated with Noonan syndrome or cardio-facio-cutaneous syndrome and are well characterized as activating variants that result in decreased GTP hydrolysis and varying degrees of increased activity of downstream signaling.[63, 64] The

NovellusDx assay correctly identified all of these variants as activating. The *NRAS* A18T variant was also included and in melanoma this variant is associated with a good prognosis with preliminary *in-vitro* work suggesting that it is not activating.[76] In the Novellus Dx FACT assay, this variant was also not deemed activating. We also performed our own *in-vitro* confirmation of 13 variants that represented a mixture of typical, extended, atypical, activating, and non-activating variants using a Ba/F3 transformation assay. The NovellusDx FACT and Ba/F3 transformation assays were concordant on 13/13 variants. Using a mixture of primary literature resources and this *in-vitro* work, we believe the NovellusDx FACT assay is a robust mechanism for assessing the functional relevance of *RAS* alterations.

One of the hopes in functionally characterizing such a large number of *RAS* variants was that we may be able to find patients with signaling that resembled wild type *RAS*. This pre-clinical information would help identify patients who may benefit from anti-EGFR therapy. Unfortunately, while we identified a number of non-activating variants, all of the atypical variants in the MDA CMS 46 or T200 cohorts were those that were activating. Based on the prevalence of individual atypical variants, it appears the functionally activating atypical variants are the ones that are recurrent in CRC. Importantly, we also noted that a comprehensive assessment of the guideline cited mutations did not reveal any lacking non-activating variants. This is crucial information as there are repeatedly attempts to identify whether rare mutations in that group may still respond to cetuximab/panitumumab. For example, retrospective studies suggested that *KRAS* G13D patients may still respond to anti-EGFR therapy. This lead to the ICECREAM trial which was a tremendous effort to prospectively validate whether G13D mutants respond to cetuximab, however the trial was negative.[36]

On the opposite end of the spectrum, we also identified a number of variants that were functionally more activating than the well-defined *KRAS* exon 2 variants. Having one of these highly activating mutations was associated with a worse OS than an exon 2 mutation (HR 1.40,

P=0.028). Many of these mutations were atypical mutations (*KRAS* V14I, Q22K, D33E) or were mutations in *NRAS*. Prognostic differences did not remain in multivariate models, however highly activating mutations resulted in a larger HR compared to wild type patients than exon 2 mutations. Though this information is intriguing, it has little clinical utility outside of prognostic stratification as all of these patients would be deemed ineligible for anti-EGFR agents.

More interestingly, this analysis led to a direct comparison of *KRAS* and *NRAS* mutant patients. These two groups have never been shown to have prognostic differences, however we demonstrated that *NRAS* mutations were associated with a worse OS than *KRAS* mutations. As seen in the functional characterization of *RAS* variants, *NRAS* alterations were generally associated with a larger fold change compared to *KRAS* alterations. Taken together, these two findings suggest that different signaling mechanisms may be responsible for differing behavior. *NRAS* mutations activate the stereotypical MAPK pathway, but are also responsible for activating the Rho GTPase Rac1 and Rho A.[77, 78] Downstream of these two proteins, the PAK-1/PDK signaling complex is responsible for activating actin and microtubule cytoskeletons leading to increased motility and invasion.[79] Other differences between *KRAS* and *NRAS* include the fact that *NRAS* mutations are more common at codon 61, while *KRAS* is predominantly codons 12 and 13. Codon 12 and 13 mutations result in *RAS* being insensitive to inactivation by GTPase activating proteins, while codon 61 mutations inhibit intrinsic *RAS* activity. We noted that the poor prognosis of NRAS variants appeared independent of the exon mutated. These findings suggest a need to stratify *KRAS*/*NRAS* mutant patients as targeted therapy combination trials are launched.

The functional characterization of the large number of *RAS* variants evaluated in our study will hopefully serve as an atlas for clinical relevance of atypical variants should they be seen in clinical sequencing results. Although we cannot prove that activating variants predict a lack of response to therapy, this study presents some of the best available evidence to help stratify

atypical variants. By pooling atypical variants together and showing that they have a similar or worse prognosis than other *RAS* mutant CRC and showing that most of the recurrent atypical variants result in increased MAPK activity, we provide compelling evidence to consider these variants as pathologic.

Future Directions

Given the rarity of each of the individual extended and atypical variant characterized, large scale collaborations are required to provide clinical annotation and further refine our understanding of whether these variants are pathologic. One of the major limitations of our study was the small number of atypical and extended mutations with clinical outcomes and treatment information. In collaboration with the Mayo Clinic, Caris Life Sciences, and the BC Cancer Agency, we will obtain treatment information on patients with atypical variants to try and provide further evidence of their clinical impact. Given the fact that some of these patients will have had variants that may have been undetected in older versions of *RAS* testing, we predict that there will be some patients who received anti-EGFR therapy that can be evaluated for response rates to help establish whether these mutations are predictive.

Aim 2: Impact of *RAS* mutant allele frequency on clinical outcomes in mCRC

Though low allele frequency *RAS* mutations are being investigated, it is unclear how they impact clinical outcomes and assays that are able to detect these mutations have not made their way into the clinic.[10, 30, 44] In our analysis of *RAS* mutation rMAF, we noted an association of high *RAS* rMAF with OS in univariate and multivariate models for the CMS 46 cohort (HR 1.55, P=0.0058), with strong trends also seen in the considerably smaller T200 cohort (HR 1.59, P=0.075). These results were consistent regardless of whether copy number alterations were considered, however *RAS* mutation rMAF was less able to discriminate a high from low risk group when a pathologist's estimate of tumor content was used in place of a truncal mutation. The fact that results were consistent in the T200 cohort with and without copy number correction supports the feasibility of using uncorrected rMAF calculations in the CMS46

cohort where copy number data was not available and demonstrates that our findings are not just highlighting a poor prognosis for patients with *RAS* amplification or loss of heterozygosity for *APC* or *TP53*. We also noted that the molecularly defined rMAF was better able to divide patients into a low and high risk group than when we used the pathologist defined tumor content.[54]

Our findings suggest that *RAS* mutation allele frequency may be an important determinant of outcome. While this conclusion seems intuitive, *RAS* mutations have not been shown to be prognostic outside of the receipt of anti-EGFR agents. Although we do not have the full treatment history for all patients evaluated, the fact that they had a *RAS* mutation identified on their clinically requisitioned assay means they likely did not receive anti-EGFR therapy. Our finding that these low rMAF patients have a better prognosis than high rMAF patients despite presumed similar treatment is thus novel and requires further exploration. Given that we assessed the OS of these patients from the time of their stage IV diagnosis and had over 900 *RAS* mutant patients to consider, our findings may be novel due to the power of our study. Most prior retrospective studies evaluating *RAS* as a prognostic marker include patients that were on a clinical trial for a single line of therapy.[10, 11] These patients were followed for a limited time rather than their entire disease course. Many of these studies had only a few hundred *RAS* mutant patients to follow and based on the timing of these studies, they do not include high sensitivity assays that provide full coverage of all extended *RAS* mutations. Given that both of our cohorts had excellent long term follow up with a high event rate, had full *RAS* coverage with highly sensitive NGS assays and such large numbers, our results may be uncovering a true prognostic impact of *RAS* mutations that might not be noted in other studies.

The finding that *RAS* rMAF is prognostic differs from a similar study recently performed by Dienstmann et al which suggested that allele frequency of *KRAS* mutations did not impact OS and that allele frequency was not predictive of time to progression in patients with *RAS*

mutations receiving anti-MEK therapy.[70] This difference may be explained by the fact that their study was smaller (N=322) than our CMS 46 cohort and that they only evaluated *KRAS* allele frequencies as a continuous variable rather than including all *RAS* mutant patients and using a cut-point to create two groups. This difference would suggest that the characteristics of the low allele frequency group we defined may be important to consider. As is seen in Table 6 and 8, we did not note any consistent difference between *RAS* mutant patients based on allele frequency. However, the T200 cohort demonstrated that *RAS* mutations with rMAF <50% were associated with an earlier stage at diagnosis. While this may suggest patients with a low rMAF *RAS* variant gained it at a different time during their disease course, the fact that there was no difference in allele frequency based on whether a primary or metastatic lesion was sampled does not support this argument.

The evolutionary context of *RAS* mutations has been investigated by others and though *RAS* mutations typically occur as early clonal events, others have shown that acquired *RAS* alterations occur at lower allele frequency than the more common early alterations.[22, 71, 72] This likely explains why such a small proportion of our *RAS* mutant patients were classified as having an rMAF <50%. Most patients will have early truncal *RAS* mutations, while the low rMAF group may be acquired later and behave differently. Siravegna et al have previously shown that *KRAS* clones which develop during anti-EGFR therapy can subsequently disappear following withdrawal of treatment and re-challenge can result in responses.[73] These acquired mutations may not have the clonal stamina to persist as the dominant clone following removal of selective pressures. In this setting, the patient's tumor bulk may behave biologically like a wild-type CRC as this is the genotype of the dominant clone. Alternatively, given that we do not have complete treatment information for all patients, the reason we are seeing this low allele frequency group having a better prognosis may be that these patients were initially thought to be wild type, received the benefit from anti-EGFR therapy and subsequently acquired low rMAF *RAS* mutations.

In order to help evaluate this possible explanation, we reviewed the cfDNA cohort, which though lacking treatment information for most patients, does include at least some patients with known prior anti-EGFR therapy. Given current practice patterns regarding cfDNA use, patients in this cohort are more likely to be heavily pre-treated and who may have already received anti-EGFR therapy. In the cfDNA cohort, 236/722 detected *RAS* variants (32.7%) occurred at an rMAF of <50%, significantly more than the patients with tissue based sequencing (P<0.0001). This difference may just show the enhanced ability of "liquid biopsies" to sample all parts of a tumor concurrently. Alternatively, if these low allele frequency *RAS* mutations are all acquired, this finding may support the notion that most mutations within a tumor mass occur early and later acquired mutations are less stable and remain subclonal. This notion is supported by a proposed "big bang" model of CRC carcinogenesis in which most of the mutations in a cancer are derived early during development and later clonal expansions are less responsible for tumor heterogeneity than pre-existing subclonal mixing.[74]

An important limitation of our study was that patients identified as "low allele frequency" mutants represent a range of rMAF values up to 50% and patients with a *RAS* mutation occurring at 0.1% may be very different from a patient with a mutation occurring at 49%. This is a major limitation of the two assays used in our MDA cohorts. Both assays used a lower threshold for confidently calling mutations in the range of 3-5%. This lower limit of detection is important. In the CRYSTAL clinical trial comparing FOLFIRI + cetuximab to FOLFIRI in the first line setting, the hazard ratio for PFS was shown to move further in favor of FOLFIRI + cetuximab as *RAS* mutation allele frequency decreased. At a *RAS* mutant allele frequency of 0.1%, there was a strong trend towards continued benefit of an anti-EGFR containing regimen (HR 0.57, 95% CI 0.33-1.01).[75] Another major limitation of our study is the lack of treatment annotation and the inability of these cohorts to answer questions surrounding the predictive nature of *RAS* allele frequency. Given that the major clinical implication of *RAS* mutations is

defining whether a certain allele threshold predicts resistance to anti-EGFR agents, this can only be defined in a population of patients who has received cetuximab or panitumumab. Our work does however provide valuable insights into the biological relevance of allele frequency and the importance of considering clonal architecture when interpreting genomic information.

Future Directions

Given the importance of assessing ultra-low allele frequency mutations and the need for prospective validation of the findings in our retrospective study, we are currently evaluating the role of low frequency *KRAS*/*NRAS* mutations in the CO.17 clinical trial comparing cetuximab to best supportive care in the third line setting.[10] This analysis will use a BEAMing digital PCR assay. Since the trial includes a best supportive care arm, this analysis will be positioned to evaluate the predictive capacity of low allele frequency *RAS* mutations.

Aim 3: Utility of allele frequency as a predictor of functional impact.

With the expansion of sequencing panels to provide broader coverage of the genome, variants of unknown significance are becoming an increasing challenge. Even though a gene may be actionable, particular mutations may not be. From an evolutionary perspective, alterations that result in a survival advantage are likely to be those that drive clonal expansion and tumor progression. We hypothesized that more damaging variants may be present at higher allele frequencies. Using the clinically annotated PODS database and the SIFT score, we were able to demonstrate that pathologic variants were associated with a higher allele frequency. Unlike the SIFT score, Polyphen did not appear to differentiate variants of different categories based on allele frequency. Both SIFT and Polyphen are useful tools, however bioinformatic approaches to functional characterization are often challenged by issues of low specificity and multiple concurrent characterizations are ideally required.[80, 81] We attempted to use this approach by creating a score that merged SIFT and Polyphen scores but this method was also hindered by little discrimination between functional groupings.

Though we did note statistically significant differences between groups based on functional classification, unfortunately outside of the cfDNA cohort most differences were of such small magnitude that they would not be clinically useful and statistical significance was likely achieved due to the large number of variants utilized in between group comparisons. Even when we chose to compare BRAF V600 to non V600 mutations, we had inconsistent findings between cohorts that would support the use of rMAF as a determinant of clinical relevance. Also arguing against the use of rMAF as a marker in the context of BRAF, was the fact that Dienstmann et al demonstrated that BRAF V600E mutations occurred at lower rMAFs than non V600 variants in their cohort, showing inconsistent findings and suggesting this metric is not readily transferable between platforms.[70] Dienstmann et al also did not observe an association between allele frequency and PFS in patients on targeted therapies matched to *KRAS*, *BRAF*, or *PIK3CA* mutations. Given that Dientsmann and others have shown that driver mutations in different genes occur at different allele frequencies, a simple application of rMAF to define functional significance may not take into account the evolutionary context of a patient's cancer and the current biologic relevance of a particular clone to the proliferation of a tumor.[70, 72]

The cfDNA cohort does show some interesting findings however regarding the utility of rMAF. The fact that synonymous mutations were shown to occur at the lowest rMAF supports our theory of the utility in using rMAF as a marker of functional significance. This cohort also showed the largest delta between benign and pathologic variants. Given that the other cohorts using the PODS annotation did not show as large of a difference in rMAF, we may be seeing evidence that rMAF is associated with the relevance of a mutation to the biologic process currently driving proliferation rather than whether a mutation is present anywhere in the total tumor mass. cfDNA has a very short half-life of 30 minutes to 2 hours.[82] As such, evolutionarily quiescent populations are likely to be under represented but may still be present in the total tumor mass. These cells may stay in "reserve" until called upon by some evolutionary stressor, however the bulk of the cfDNA will be driven by whichever population is rapidly expanding at

that moment. This idea that cfDNA may represent a snap shot of the active ecosystem within a patient is supported by our second case study that shows the rMAF of a mechanism of resistance falls as the patient accumulates more mechanisms of resistance. Figure 23 shows that evolutionarily there is no reason for redundancy in mechanisms of resistance and those patients who have multiple mechanisms detected, likely have numerous different populations competing to become the dominant clone and each of these mechanisms of resistance is likely in a private population. However, in the patients that have only a single mechanism of resistance, we are seeing that this clone has become dominant as no other independent resistance mechanism has developed.

A major limitation in assessing the utility of rMAF in functional characterization is the design of the NGS panels used for the cohorts we analyzed. These panels are designed for clinical annotation and have an enrichment for pathologic variants that are deemed actionable. As such, there are few benign variants detected. Repeating this experiment in patients with whole genome or whole exome sequencing may yield different results, however we focused on NGS panels as the aim was determine whether rMAF could be used as a clinical tool and exome/genome sequencing is not a clinical reality for most patients. Panel design may also explain why the cfDNA showed the largest variance in rMAF based on functional characteristics. The cfDNA assay used in our study has a lower limit of detection of 0.1%.[45] Given that the NGS panels used in the other cohorts have thresholds of ~5%, cfDNA may be picking up more truly low allele frequency mutations that are missed by the assays in the tissue cohorts.

Future Directions

Though we failed to demonstrate the utility of using rMAF as a tool for defining the functional relevance of a mutation, the findings in our cfDNA cohort are striking and demonstrate potential clinical utility. There have already been a number of studies demonstrating the utility in tracking

clonal evolution with cfDNA to integrate this information into therapeutic decisions such as with anti-EGFR re-challenge or tracking for tumor recurrence. Current work by our group is assessing how to incorporate cfDNA assessments into clinical care and I am analyzing a cohort of patients with serial blood draws to demonstrate how useful following clonal dynamics is on a larger scale. Most current reports tracking serial cfDNA outside of surveillance studies have not yet demonstrated at what frequency clonal architecture changes of clinical significance occur on serial samplings. Of particular interest, I will be evaluating whether mechanisms of resistance detected on cfDNA are stable over time and the kinetics of how these mechanisms may be lost.

Conclusions

We present a comprehensive functional annotation of *RAS* mutations that will hopefully help guide clinicians when these variants are found during clinical sequencing. Our results show that many recurrent atypical variants are functionally activating and associated with a poor prognosis. These findings support the inclusion of all *RAS* exons in clinical sequencing panels and demonstrate the need for a personalized approach to handling variants of unknown significance. Though rMAF was not useful in characterizing variants as functional vs benign, our findings that *RAS* rMAF is associated with prognosis suggests allele frequency may be useful information to include in standard clinical reports. The ability of this work to study rare variants using pre-existing databases and many publicly available data sets supports open access to sequencing results and de-identified clinical data to optimize patient outcomes in rare subgroups. As we move into evaluating therapies in smaller subgroups of patients, these collaborative large scale projects will be key to ensuring our decisions are based on best available evidence.

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Vita

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