nature portfolio

# Peer Review File

# Ligand-activated EGFR/MAPK signaling but not PI3K, are key resistance mechanisms to EGFR-therapy in colorectal cancer

Corresponding Author: Dr Carlos Bais

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript submitted by Qu et al., shows mainly the genomic alterations acquired after anti-cancer treatment (anti-EGFR and anti-VEGF) in a large cohort of metastatic colorectal tumours (n=243). The study analyses data from a phase 3 clinical trial called IMblaze370 trial 85 (NCT02788). The outcome is that anti-EGFR treatment increases mutations in EGFR/MAPK signalling (high frequency in EGFR extracellular domain and increase in EGFR ligands).
This work is of high significance because the acquired resistance after treatment is the reason of most of the deaths in cancer. The team counts with the access to a large cohort of mCRC patients which is one of the biggest collection known at present to study resistance in cancer.

Major points:
1. The heterogeneity of tumours is well known, and has been well described that advanced tumours are composed of many different clusters. The analysis of primary tumour (archival tissue) should be done in more than one region of each tumour to confirm that the analysis of plasma vs tissue is correct.
2. The experiments with the inhibitors using three cell lines should be done in CRC organoids.
3. The abstract mentions that mechanisms are described in the manuscript however more mechanistically data should be done to demonstrate the impact of treatments on oncogenic signalings.
4. FACT is sequenced to a depth of ~5000X and FoundationOne more than 500X, due to sample origin differences. Can they proof that these methodology differences does not affect the outcome?

Minor points:
1. Title does not reflect the resistance data which is shown in the manuscript or is not clear enough in my opinion.
2. How CMS classification was done should be better explained in methods. Were authors able to classify all the tumours?
3. What type of metastatic CRC were in the cohort should be shown and survival data of patients.
4. Western blots should be n=3 and authors should show quantification.
5. p values in figure 1c should be clear to understand what exactly is compared.
6. Is confusing when in suppl fig 1 the table has been written: anti VEGF but not anti-EGFR treatment but in the left box only it is written anti-EGFR treatment. If written the same way should be: anti-EGFR but not anti-VEGF treatment.
7. Figure 5b can be improved, specially the matrix data.
8. Figure 2, there are no p-values.
9. Drug sensitivity assays methodology text don't mention the number of replicates that are shown in the graphs.

Is there enough detail provided in the methods for the work to be reproduced?

Reviewer #2

(Remarks to the Author)
This manuscript investigates differences in newly emerging mutations (and the associated affected pathways) in mCRC patients treated with either chemo+anti-EGFR or chemo+anti-VEGF to try and elucidate resistance mechanisms observed in patients for both these combination treatments. The authors have gathered an impressive cohort of paired samples from the Imblaze 370 study (tested with Foundation F1 or FACT assay) as well as a validation cohort tested with Guardant360. Overall, the paper provides interesting insights particularly for anti-EGFR resistance. However I have some specific concerns as specified below:

Major comments:
1) Although the presence of paired samples represents a strong point of the study, I am worried about the use of cfDNA for the measurement after treatment. More particularly, I am worried about varying contributions of ctDNA to the overall cfDNA pool, which will influence the ability of actually detecting emerging mutations after treatment. I would urge the authors to investigate the potential influence of this by comparing the estimated ctDNA fraction between the EGFR and VEGF cohorts and correct for this if necessary. From the Figures it seems that more mutations are detected in cfDNA samples from the EGFR cohort compared to VEGF, which could indicate a difference in ctDNA fraction between these cohorts. If true this hampers the comparison as it is presented now.

2) Similarly, when grouping samples based on the presence of emerging mutations in EGFR/MAPK genes, this may be confounded by the amount of ctDNA. The fact that they find more highly proliferative CMS2 tumors in the group with emerging mutations suggests to me that in this group ctDNA fraction might be higher which also increases the chances of finding an emerging mutation.

3) For CNV analyses the ctDNA fraction may represent an even bigger bias, since detection of these CNVs is known to be less sensitive than SNVs or indels. The recurrent alterations mentioned by the authors are well-known from primary CRC, so are these really newly arising alterations or are these already observed in the baseline tissue data? And if so is the difference for 8q between the treatments also seen at baseline or only after treatment, where this difference between treatments could also be due to different ctDNA contributions?

4) The authors suggest that tumors with high MYC, EGFR ligands, WNT activation and Tgd cells are more susceptible to EGFR-inhibition and subsequently develop resistance mutations, whereas tumors with a high stromal and immune gene signature at baseline are more likely to display primary resistance. However, no data is shown to prove that one group responds better to EGFR-inhibition than the other group. To support this statement, authors need to provide for example PFS data for patients in both groups to demonstrate better response in 1 group and primary resistance in the other.

5) I wonder whether the authors have also investigated mutations that arise at similar frequencies in both treatment groups? Could these represent common resistance mechanisms or perhaps resistance mechanisms to the chemotherapy backbone, which is neglected in the current presentation of the data?

6) With respect to the cell line experiments I feel interpretation is difficult since, if I understand correctly, 3 different cell lines were used. Since the genetic backbone of these cells is very different, it is impossible to conclude that differences in sensitivity are due to the one mutation of interest to the authors. In fact the differences in sensitivity could also be due to other differences between the cell lines.

Minor comments:
1) With respect to the organoid experiments it is currently unclear from the results section that these organoids are derived from mice instead of patients.

2) For the CNVs it would be easier to also include cytoband locations instead of only bp positions.

3) In the discussion section it states that Tgd cells contribute to the response to ICI treatment and that this suggests a link between Tdg cell driven immune activity and resistance to anti-EGFR. This statement is unclear to me?

4) It states multiple times that patients were selected that received anti-EGFR or anti-VEGF but not anti-EGFR. I assume this also means that patients on anti-EGFR did not receive anti-VEGF, but strictly speaking this is not specified in the text?

5) Please explain why CMSCaller was used (which was developed for cell lines) instead of the original CMS classifier package (https://github.com/Sage-Bionetworks/CMSclassifier)?

Reviewer #3

(Remarks to the Author)
A relevant paper published in Nature 10 years ago in 2014 by Bertotti et al. entitled “The genomic landscape of response to EGFR blockade in colorectal cancer” was not cited or discussed. This oversight or omission diminishes the novelty of the current manuscript. One would expect a thorough discussion of similarities and differences as well as clear indication of novel findings versus the previous landmark paper.

While the paper makes a point about anti-VEGF therapy there is little if any novel insight into the resistance mechanisms

other than they don’t see what is seen with anti-EGFR therapies.

Overall the findings are incremental and of interest to a more specialized audience due to limited novelty or clinical actionability.

Reviewer #4

(Remarks to the Author)
The study comparing intrinsic and acquired mechanisms of resistance to anti-EGFR versus anti-VEGF plus chemotherapy therapies in metastatic colorectal cancer (mCRC) patients is comprehensive and sheds light on several key aspects of treatment resistance. However, there are several areas that require attention and potential improvement:

1) Data integration is a missed opportunity for this paper. It must be done more thoroughly to support the claims. I would integrate both RNA-seq and pre-treatment mutation status from the patients and use a multi-omics approach for clustering and supervised analysis. The obtained NMF clusters are not representative of acquired mutation status. There are many multi-omics data integration tools, one of which should be used for this purpose in both supervised and unsupervised setting. One can also use results from xCell and pathway analysis as input to multi-omics data integration tools as input to get a more unified picture. The figures do not show a clear separation of patients dependent on their acquired mutation status, multi-omics integration analysis can show such separation if there is signal in the data.

2) If EGFR ligands or MYC activation have predictive importance beyond this study as claimed at the end of the abstract, one can easily explore this by integrating TCGA or even Novartis PDX ( 10.1038/nm.3954) data to see if these ideas have any ground. For TCGA, I would check what percentage of CRC tumors have ligands highly expressed or any other subsetting of patient population based on predicted markers in this study and see if there is any survival difference for that subsetting. For Novartis PDX data, I would check if there is any evidence of treatment response alteration depending on the ligand status.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In general, my concerns have been adequately addressed. The authors have acknowledged some limitations of the project, which I fully agree with. Despite these caveats, I believe the study is of significant relevance and value to the field. In my opinion, the quality of the work and its potential contribution for current and future cancer patients treatments justify its publication.

Reviewer #2

(Remarks to the Author)
The authors have sufficiently addressed my comments.

Reviewer #4

(Remarks to the Author)
The authors validated their approach with new datasets and analysis. The new analysis strengthened the claims and satisfactorily answered my previous comments.

**RESPONSE TO REVIEWERS' COMMENTS**

**REVIEWER COMMENTS**

Reviewer #1, expertise in colorectal cancer organoids and functional genomics (Remarks to the Author):

The manuscript submitted by Qu et al., shows mainly the genomic alterations acquired after anti-cancer treatment (anti-EGFR and anti-VEGF) in a large cohort of metastatic colorectal tumours (n=243). The study analyses data from a phase 3 clinical trial called IMblaze370 trial 85 (NCT02788). The outcome is that anti-EGFR treatment increases mutations in EGFR/MAPK signalling (high frequency in EGFR extracellular domain and increase in EGFR ligands).
This work is of high significance because the acquired resistance after treatment is the reason of most of the deaths in cancer. The team counts with the access to a large cohort of mCRC patients which is one of the biggest collection known at present to study resistance in cancer.

We greatly appreciate the reviewer's recognition of the significance of our work and the value of using a large cohort of mCRC patients to study acquired resistance to anti-EGFR and anti-VEGF treatments. We agree that investigating the molecular mechanisms driving acquired resistance is crucial to further improve therapeutic outcomes in cancer treatment, and we are encouraged that the reviewer found this aspect of our study to be of high significance.

Major points:
1. The heterogeneity of tumours is well known and has been well described that advanced tumours are composed of many different clusters. The analysis of primary tumour (archival tissue) should be done in more than one region of each tumour to confirm that the analysis of plasma vs tissue is correct.
We appreciate the reviewer's insightful comment regarding tumor heterogeneity and the suggestion to analyze multiple regions of each tumor. We fully agree that advanced tumors exhibit significant intertumoral heterogeneity, which is an important factor to consider in investigating resistance mechanisms. However, due to the constraints associated with using archival tumor tissue from a large cohort of mCRC patients in the IMblaze370 trial, it was not feasible obtaining multiple regions from each tumor for analysis.

Despite this limitation, the striking difference in the genomic alterations acquired in anti-EGFR treated patients but not observed in anti-VEGF treated patients, and the historical data on the genomic landscape of baseline (pre-treatment) CRC support the robustness and interpretation of our plasma vs. tissue analysis. Furthermore, these findings were independently validated in a separate dataset of 274 mCRC patients who underwent the Guardant 360 cDNA testing both before and after treatment (longitudinal) with anti-EGFR (N=131) or anti-VEGF(N=142) therapies. This consistency across two

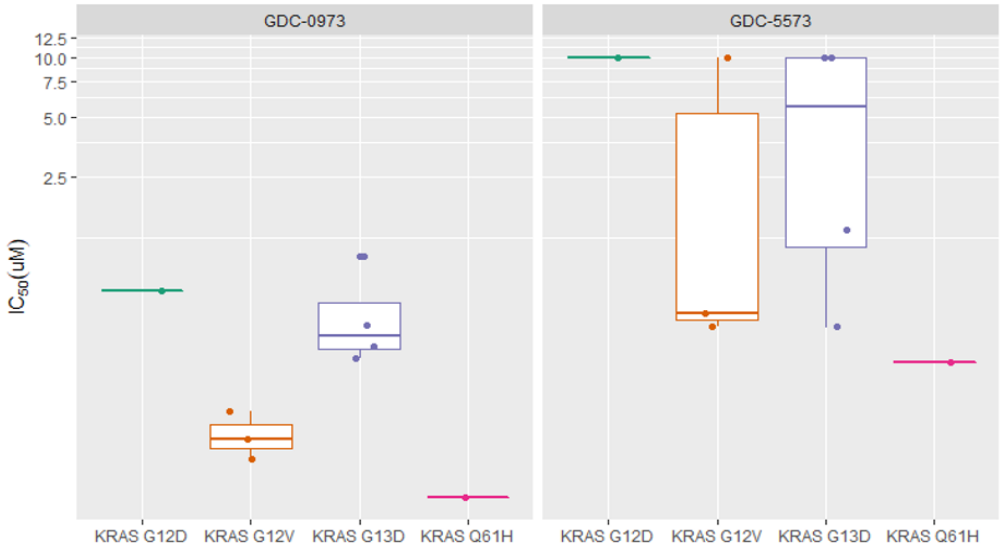
independent cohorts underscores the reliability of our conclusions and reinforces the validity of our plasma vs. tissue analysis.

2. The experiments with the inhibitors using three cell lines should be done in CRC organoids.

We appreciate the reviewer's suggestion to conduct experiments using CRC organoids, as these models would indeed provide a more physiologically relevant system for evaluating treatment responses in patients. While we recognize the value that organoid-based studies would add to the translational relevance of our findings, we currently face resource limitations that preclude us from performing these experiments. Specifically, the team members who conducted the original organoid experiments have left the company, and the current team lacks the required infrastructure and expertise to establish and conduct organoid experiments.

To address this limitation, we have expanded our analysis to include the data from 9 CRC cell lines. As shown in Supplementary Fig. 7, the results from these additional cell lines reinforce the robustness of our findings and validate the unique resistance mechanism of KRAS Q61H to anti-EGFR therapy.

We acknowledge that incorporating organoid models would further validate and enhance the translational impact of this work, but this is beyond the scope of the present manuscript. We hope that future studies will build upon our initial findings in this direction.




**Suppl. Fig. 7** Comparison of relative viability of KRAS $^{Q61H}$ CRC cell line with KRAS$^{G12D}$, KRAS $^{G12V}$, and KRAS $^{G13D}$ CRC cell lines in response to MEK inhibitor GDC-0973 and pan-RAF inhibitor GDC-5573 treatment. The reported $IC_{50}$ is the dose at which the estimated inhibition is 50% relative to untreated wells. The bar indicated the median.

3. The abstract mentions that mechanisms are described in the manuscript however more mechanistically data should be done to demonstrate the impact of treatments on oncogenic signaling.

We thank the reviewer for highlighting the importance of providing additional mechanistic insights into the impact of treatments on oncogenic signaling. Although the retrospective nature of this study limited our ability to conduct further experimental validation, we believe our findings provide robust evidence of treatment-specific resistance mechanisms, emphasizing specific biochemical alterations emerging at distinct frequencies that lay a foundation and rationale for future mechanistic studies.

This study identifies key distinctions in resistance pathways between anti-VEGF and anti-EGFR therapies using multi-omics data from 254 mCRC patients in the IMblaze370 trial. We demonstrated that anti-VEGF therapy does not select for secondary oncogenic mutations within the panel of common alterations that we evaluated, whereas resistance to anti-EGFR therapy predominantly converges on the EGFR/MAPK pathway. Notably, the observation that acquired KRAS Q61H mutation, an extremely rare mutation at diagnosis, was the most prevalent downstream alteration following anti-EGFR treatment, suggests its unique role in preferentially activating MAPK signaling. Consistent with this hypothesis, KRAS Q61H is known to disrupt intrinsic nucleotide exchange and lacks intrinsic GTP hydrolysis (Hunter et al., Mol Cancer Research, 2015, PMID:26037647), decouples KRAS from upstream regulation (Gebregiworgis et al., Nature, 2021, PMID: 34725361), leading to constitutive activation of the MAPK pathway. It also preferentially binds to RAF over PI3K (Zhou et al., Cancer Res, 2020, PMID: 32605999), promoting selective MAPK activation and contributing to treatment resistance. These findings are consistent with our observation that organoids harboring KRAS Q61H preferentially activate MAPK over PI3K signaling, and CRC cell line with KRAS Q61H mutations is more sensitive to MAPK pathway inhibitors compared to those with other KRAS mutations (Figure 3, and Supplementary Fig. 7)

We also observed frequent mutations in its extracellular domain of EGFR, but very rare mutations in its tyrosine kinase intracellular domain in anti-EGFR-treated patients, which correlated to prior higher baseline expression of EGFR ligands. This finding underscores an oncogenic ligand-activated EGFR signaling dependency mechanism in CRC, aligning with prior studies that identified EGFR ligands as predictive biomarkers for anti-EGFR therapy (Seligmann et al., JAMA Oncol, 2016, PMID: 26867820; Smyth et al., JAMA Oncol, 2016, PMID: 26869404), and the acquired genomic alterations in EGFR primarily located in EGFR extracellular domain but not in intracellular RTK domain (Strickler et al, Cancer Discov.,2018, PMID: 2919463).

While this study primarily focuses on identifying resistance mechanisms and generating testable clinical hypotheses, we agree that additional mechanistic experiments would further enrich our understanding. Future studies, beyond the scope of the present manuscript, could build on these insights by evaluating EGFR and pan-KRAS inhibitor combinations, as well as ligand blockers, to improve future targeted drug development strategies in CRC.

4. FACT is sequenced to a depth of ~5000X and FoundationOne more than 500X, due to sample origin differences. Can they proof that these methodology differences does not affect the outcome?

We thank the review for raising this important point regarding differences in sequencing depth between FACT and FoundationOne and their potential impact on the findings.

The difference in depth is primarily to account for tumor purity differences. Since tissue samples have tumor purity requirements of at least 20% tumor purity, a depth of 500x enables detection of nearly all alteration, including sub clonal ones with allele fraction as low as 1%. In contrast, liquid biopsies typically have tumor shed levels well below 20%, necessitating a sequencing depth of 5,000x to achieve comparable sensitivity. As both FACT and FoundationOne are FDA- approved tests, extensive validations have been performed to ensure their sensitivity and specificity for detecting genomic alterations in their respective sample types (FDA validation references: https://www.accessdata.fda.gov/cdrh_docs/pdf19/P190032S010B.pdf; https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019S006B.pdf).

Furthermore, the strength of our analysis is the inclusion of both anti-EGFR and anti-VEGF treated patient groups. The observation that the acquired genomic alterations converge to EGFR/MAPK pathway specifically in anti-EGFR treated patients but not in anti-VEGF treated patients suggests that the methodological differences are unlikely to account for our findings. Furthermore, many of the mutations reported here are consistent with prior publications in this topic and with the Guardant longitudinal collection dataset presented here.

Minor points:

1. Title does not reflect the resistance data which is shown in the manuscript or is not clear enough in my opinion.

We thank the reviewer for their opportune comment regarding the previous title of our manuscript. In response to this valuable suggestion, we have revised the title to better emphasize our findings about mechanism of resistance. The update title now reads: Genomic and Expression Analysis Reveals Ligand-Activated Signaling, and Downstream MAPK and MYC, but not PI3K, as Critical Mechanisms of Acquired Resistance to Anti-EGFR Therapy in Colorectal Cancer.

2. How CMS classification was done should be better explained in methods. Were authors able to classify all the tumours?

We thank the reviewer for highlighting this point. CMS classification was performed using the CMScaller R package (https://www.nature.com/articles/s41598-017-16747-x), which implements the Nearest Template Prediction (NTP) algorithm proposed by Yujin Hoshidato. This algorithm assigns CMS subtypes based on gene expression profiles and includes a small ensemble of functions for evaluating and visualizing classification results. We have added these details to the Methods Section. Additional details about the CMScaller package can be found on Github(https://github.com/peterawe/CMScaller) and in its original publications (Guinney et al., Nature Medicine, 2015, PMID: 26457759; Eide et al., Sci Rep, 2017, PMID: 29192179). Using this method, we successfully

classified most tumors. However, consistent with the original study (Guinney et al., Nature Medicine, 2015, PMID: 26457759), 7% of tumors could not be classified due to mixed or indeterminate phenotypes.

3. What type of metastatic CRC were in the cohort should be shown and survival data of patients.
We thank the reviewer for their thoughtful suggestion. The tumor tissue in our cohort from the IMblaze 370 study includes both primary and metastatic tumors. We have now added details regarding the tumor sample origin and tumor types to Supplemental table S1 for clarity.

Regarding the survival data, we would like to clarify that tumor samples were collected before any treatment, and their matching plasma samples were collected after two or three lines of prior therapies but before receiving any IMblaze370 treatment regimens. Therefore, the survival data available from Imblaze370 study is not relevant to this analysis, and we did not include it in this manuscript.

4. Western blots should be n=3 and authors should show quantification.
We thank the reviewer for their helpful comment. We would like to clarify that the Western blot experiments were performed with 3 biological replicates, but the images shown in the manuscripts are representative of these replicates.

Regarding the request for quantification, although we agree that, ideally the data should be presented in a quantitative format, we regret to inform the reviewer that many of us have left the company since then, and we are unable to perform the quantification after our departure. While we acknowledge that quantification would strengthen the point we are making with this result, we are convinced that the data is reproducible and hope that the representative blots presented in the manuscript are sufficient for convincingly conveying our results. However, if the reviewer does not agree with this approach, and since this western blot is not a major point of this paper, we are open to remove this data from the manuscript.

We appreciate the reviewer's understanding and flexibility.

5. p values in figure 1c should be clear to understand what exactly is compared.
We appreciate the reviewer's comment. In Figure 1C, the p values were calculated using a two-tailed Fisher's exact test to compare the percentage of patients with emerging alterations between those who received prior anti-EGFR therapies and those who received prior anti-VEGF therapies.

To make this clearer, we have updated the figure legend to explicitly indicate the comparison between these two patient groups. We hope this clarification helps improve the interpretation of the data.

6. Is confusing when in suppl fig 1 the table has been written: anti VEGF but not anti-EGFR treatment but in the left box only it is written anti-EGFR treatment. If written the same way should be: anti-EGFR but not anti-VEGF treatment.

We thank the reviewer for pointing this out and provide us with an opportunity to further clarify this text that may be confusing. The label in Supplemental Figure 1 is indeed correct, as most of the patients who received anti-EGFR treatment also received anti-VEGF/R treatment. Specifically, out of the 113 patients who received anti-EGFR treatment, only 24 patients received anti-EGFR treatment without anti-VEGF/R treatment.

To address this potential confusion, we have added a sample summary table from the IMblaze370 study (Supplementary table 1), and an additional column to the Supplemental Table 10 that details the treatment regimens of patients who received anti-EGFR treatment. As shown in the revised Supplementary table S10, the percentage of KRAS/NRAS/BRAF wild-type patients with acquired genomic alteration in EGFR/MAPK pathways was comparable between those who received anti-EGFR treatment alone (10 out of 15 patients: 66.67%) and those who received both anti-EGFR and anti-VEGF/R treatment (31 out of 65 patients: 53%). These findings support our observations that the resistance to anti-VEGF therapy is likely driven by non-genomic adaptive mechanisms, while the observed acquired genomic alterations in patients receiving both anti-EGFR and anti-VEGF/VEGFR treatment were predominantly attributed to the anti-EGFR treatment.

We hope these clarifications and revisions resolve the confusion and provide additional context to our findings.

7. Figure 5b can be improved, specially the matrix data.

We thank the reviewer for their comment. The figure has been revised to include the annotation for the acquired mutations and the tumor sample collection location. The matrix presented is a consensus matrix derived from 100 runs of Non-negative Matrix Factorization (NMF), illustrating the probability of one sample clustering with another. Such plots are typical output for NMF clustering. The cophenetic correlation coefficient, a numeric manifestation of the consensus matrix (i.e. the average of connectivity matrices), was introduced by Brunet et al. (PNAS, 2004, PMID: 15016911) to assess the stability of the clusters obtained through NMF. This matric provides a robust measure of clustering consistency.

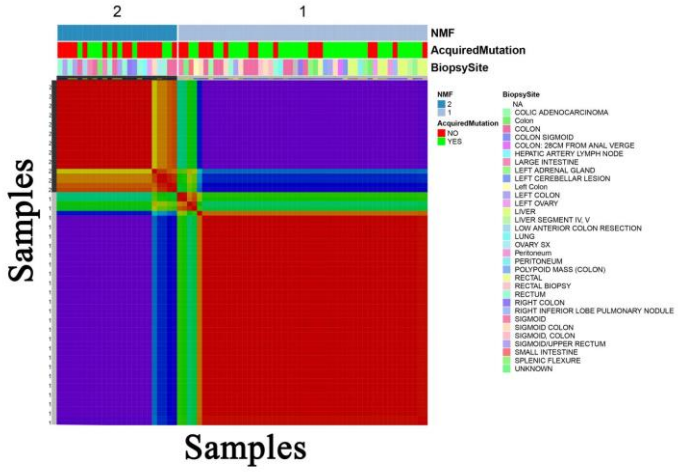



Figure 5b Consensus patient similarity matrix depicting clusters (k=2). The pie chart depicts the fraction of patients in each subtype with acquired mutations.

8. Figure 2, there are no p-values.
We thank the reviewer for this comment. We agree that including P-value is important and have included p-values in the revised figure for clarity.

9. Drug sensitivity assays methodology text don't mention the number of replicates that are shown in the graphs.
We thank the reviewer for this comment. For all the drug sensitivity assay, experiments were conducted with 3 biological replicates, meaning each screen was set up on different days. We have added this information in the Method section for clarity.

Is there enough detail provided in the methods for the work to be reproduced?
We appreciate the reviewer's question. While we have provided sufficient detail in the Methods section for producibility of the analysis, we must note that there are certain datasets, such as the FMI data, for which privacy concerns and compliance restrictions prevent us from releasing the raw data. However, we believe that all other experimental procedures and analysis are clearly described to allow for reproducibility.

If there are specific areas that the reviewer feels require further clarification, we would be happy to address those concerns and provide additional information where possible. We are committed to ensuring the methods are clearly outlined for reproducibility.

Reviewer #2, expertise in colorectal cancer genomics (Remarks to the Author):

This manuscript investigates differences in newly emerging mutations (and the associated affected pathways) in mCRC patients treated with either chemo+anti-EGFR or chemo+anti-VEGF to try and elucidate resistance mechanisms observed in patients for both these combination treatments. The authors have gathered an impressive cohort of paired samples from the Imblaze 370 study (tested with Foundation F1 or FACT

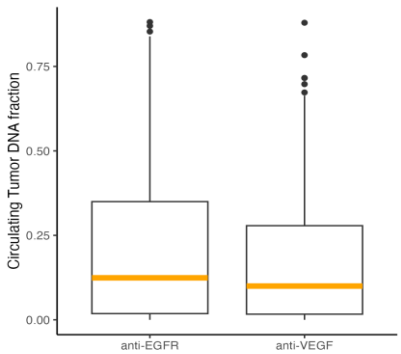
assay) as well as a validation cohort tested with Guardant360. Overall, the paper provides interesting insights particularly for anti-EGFR resistance. However, I have some specific concerns as specified below:

We greatly appreciate the reviewer's positive feedback and thoughtful evaluation of our manuscript. We are pleased that the reviewer found our investigation into the resistance mechanisms to chemo + anti-EGFR or chemo + anti-VEGF treatment in mCRC patients was of interest, particularly with respect to anti-EGFR resistance. We also appreciate the reviewer's recognition of the robust cohorts gathered from the IMblaze370 study, and the validation cohort tested with Guardant 360.

Major comments:
1) Although the presence of paired samples represents a strong point of the study, I am worried about the use of cfDNA for the measurement after treatment. More particularly, I am worried about varying contributions of ctDNA to the overall cfDNA pool, which will influence the ability of actually detecting emerging mutations after treatment. I would urge the authors to investigate the potential influence of this by comparing the estimated ctDNA fraction between the EGFR and VEGF cohorts and correct for this if necessary. From the Figures it seems that more mutations are detected in cfDNA samples from the EGFR cohort compared to VEGF, which could indicate a difference in ctDNA fraction between these cohorts. If true this hampers the comparison as it is presented now.

We appreciate the reviewer's thoughtful concern regarding the potential influence of varying contributions of ctDNA to the overall cfDNA pool and its effect on detecting emerging mutations. While we acknowledge this possibility, the observed differences in acquired genomic alterations were specific to genes in EGFR/MAPK pathway genes but were not observed in other genes. This suggests that the findings are unlikely to be primarily attributed to differences in ctDNA fractions between the two treatment groups.

To further address this concern, we conducted an analysis comparing the tumor fraction estimates in the cfDNA samples from the anti-EGFR and anti-VEGF treated groups. Our analysis revealed similar levels of ctDNA fraction between the two groups, with medians of 12% and 10% for anti-EGFR and anti-VEGF treatment, respectively (p=0.25). These findings indicate that the ctDNA fraction is comparable between the two groups and that it does not introduce any significant bias into our comparisons.

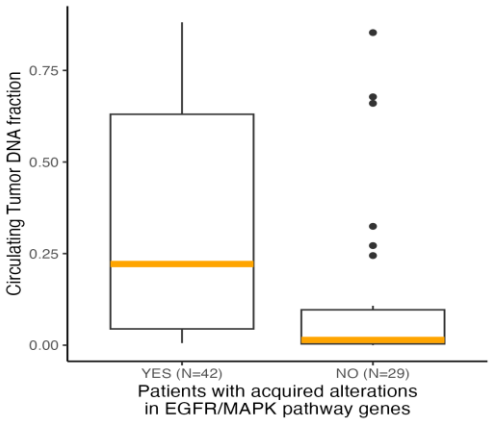


**Reviewer Figure 1:** Comparison of estimated circulating tumor DNA fraction in the baseline plasma from IMblaze370 patients following anti-EGFR therapies (N=110) and anti-VEGF therapies (N=139). Orange bars represent the median circulating tumor DNA fraction. P value was generated using a Mann-Whitney U test.

Lastly, our data is highly consistent with vast previous published literature. Several of the specific acquired mutations reported here in ctDNA after anti-EGFR treatment were previously detected by others in paired tissue biopsies and in pre vs. post genomic surveys of multiple metastatic lesions or plasma samples in individual patients (Bertotti et al., Nature, 2015, PMID: 26416732; Russo et al., Cancer Discov. 2016, PMID: 26644315). No consistent reported mutations in response to anti-VEGF therapy has been reported to date.

2) Similarly, when grouping samples based on the presence of emerging mutations in EGFR/MAPK genes, this may be confounded by the amount of ctDNA. The fact that they find more highly proliferative CMS2 tumors in the group with emerging mutations suggests to me that in this group ctDNA fraction might be higher which also increases the chances of finding an emerging mutation.

We thank the reviewer's insightful comments. To address the concern regarding potential confounding by the amount of ctDNA, we compared the estimated circulating tumor DNA fractions between the anti-EGFR treated patients with acquired mutations in EGFR/MAPK genes and those without. We found that the circulating tumor DNA fraction was significantly higher in the anti-EGFR treated patients with acquired mutations in EGFR/MAPK genes compared to those without (p = 5.7E-05). Based on this finding, we have included this data in the main text and as a supplemental figure (Supplementary Fig.9).




**Supplementary Figure 9:** Comparison of estimated circulating tumor DNA fraction between anti-EGFR treated patients with acquired mutations in EGFR/MAPK genes and those without from IMblaze 370 study. P value was generated using a Mann-Whitney U test.

3) For CNV analyses the ctDNA fraction may represent an even bigger bias, since detection of these CNVs is known to be less sensitive than SNVs or indels. The recurrent alterations mentioned by the authors are well-known from primary CRC, so are these really newly arising alterations or are these already observed in the baseline tissue data? And if so is the difference for 8q between the treatments also seen at baseline or only after treatment, where this difference between treatments could also be due to different ctDNA contributions?

We appreciate the reviewer's valuable comment regarding the potential bias introduced by the ctDNA fraction in CNV analysis, particularly given the lower sensitivity of CNV detection compared to SNVs or indels. As shown in Reviewer Fig. 1 and mentioned in the response to the major comment 1 of the reviewer, no significant differences in the tumor shed was observed between the two treatment groups, suggesting that the differences between the two groups are very unlikely influenced by ctDNA contribution.

Regarding the calling of gains, such as 8q gain, we utilized a method that allows for the detection of low-level gains rather than just high-level amplifications. When there is sufficient tumor DNA (as is the case with baseline tumor samples), the sensitivity to detect gains is high. Based on our analysis, it appears that the 8q gains are de novo in a high fraction of the EGFR -treated samples, rather than being pre-existing alterations at the baseline.

4) The authors suggest that tumors with high MYC, EGFR ligands, WNT activation and Tgd cells are more susceptible to EGFR-inhibition and subsequently develop resistance mutations, whereas tumors with a high stromal and immune gene signature at baseline are more likely to display primary resistance. However, no data is shown to prove that one group responds better to EGFR-inhibition than the other group. To support this statement, authors need to provide for example PFS data for patients in both groups to demonstrate better response in 1 group and primary resistance in the other.

We thank the reviewer for their insightful comments. We agree that PFS and/or survival analysis could provide valuable insights into the predictive value of those two groups. However, as mentioned in the response to Reviewer 1's minor comment 3, the tumor samples in the IMblaze370 study were collected prior to any treatment, while its matching plasma samples were collected after two or three lines of prior therapies but before receiving any IMblaze370 treatment regimens. Thus, available survival data from the IMblaze370 study is not relevant for evaluating the response to anti-EGFR treatment. Additionally, while the TCGA dataset has survival and gene expression data available, it can't be used for this analysis due to the lack of detailed prior treatment history for most patients.

To address the reviewer's concern, we utilized the data from Novartis's patient-derived xenografts (PDX) datasets (Gao et al. Nat Medicine, 2015, PMID: 26479923). This dataset included a cohort of 244 cetuximab-treated PDX samples with available response and gene expression data. To classify each PDX sample into NMF subtypes, we trained a random forest machine learning algorithm (R package random Forest) using the IMblaze 370 RNAseq data as the training set. The classifier, based on an ensemble of binary decision trees, predicts the class of a given sample by evaluating its

similarity to patterns observed in the training set. Using this approach, we assigned NMF subtypes to each PDX sample. As hypothesized, PDX samples classified as NMF2.1 demonstrated significantly greater responsiveness to cetuximab treatment compared to those classified as NMF 2.2, as evidenced by a smaller increase in tumor volume at 3 weeks post treatment (Wilcoxon p=9e-04, Figure 5d).

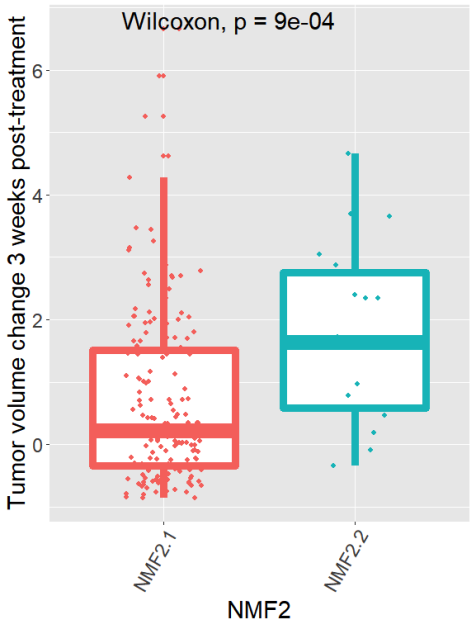



**Figure 5d:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models shown by NMF subtypes. P-value determined using a two-sided Wilcoxon rank sum test.

Consistently, PDX samples with high EREG expression (above median) exhibited significantly better response to anti-EGFR treatment compared to those with lower EREG expression (below median) (Wilcoxon p=1.2e-07, Figure 6d).

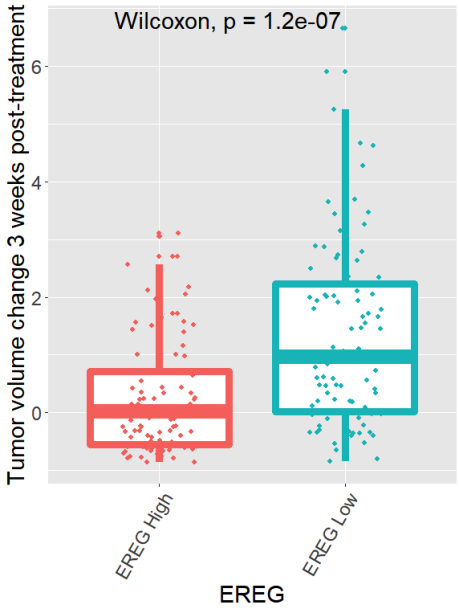
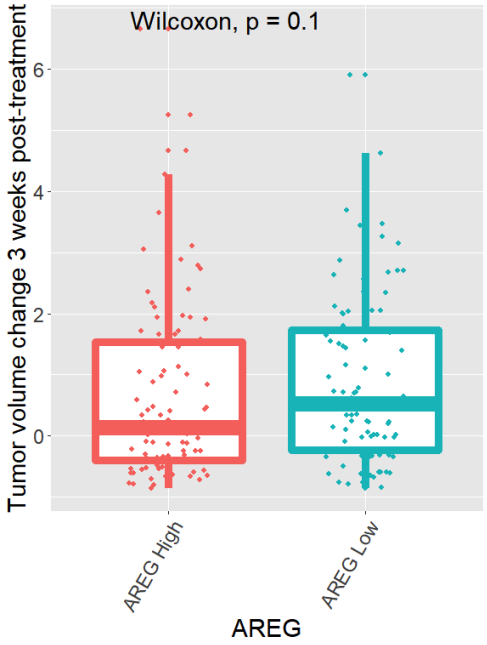
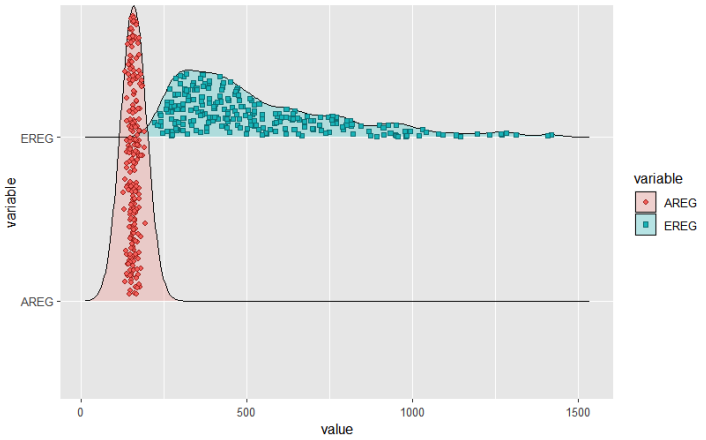



**Figure 6d:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models with EREG high (above median n=122) and EREG low (below median n=122) tumors. P-value determined using a two - sided Wilcoxon rank sum test.

Unexpectedly, no significant difference in response was observed between PDX samples with high versus low AREG expression (Wilcoxon p=0.1, Supplementary Figure10a). However, as shown in the ridge plot comparing the expression of these two ligands, AREG expression was much lower than EREG expression in this dataset Supplementary Figure10b), which may account for the lack of significant association.



**Supplementary Figure 10a:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models with AREG high (above median n=122) and AREG low (below median n=122) tumors. P-value determined using a two-sided Wilcoxon rank sum test.




**Supplementary Figure 10b:** The ridge plot shows the distribution of the expression levels of AREG and EREG in 244 PDX models.

Collectively, these findings provide preliminary evidence supporting our hypothesis that NMF2.1 tumors characterized by high MYC, WNT activation and Tgd cells are more susceptible to EGFR inhibition. In contrast, NFM2.2 tumors with high stromal and immune gene signature exhibit intrinsic resistance to EGFR inhibition. Future validation with larger datasets and clinical samples will be essential to confirm and expand upon these observations.

5) I wonder whether the authors have also investigated mutations that arise at similar frequencies in both treatment groups? Could these represent common resistance mechanisms or perhaps resistance mechanisms to the chemotherapy backbone, which is neglected in the current presentation of the data?

We thank the reviewer for their insightful comments. As described in the manuscript and shown in Figure 1c, the gene-level prevalence analysis demonstrates that the prevalence of genomic alterations detected in both anti-EGFR and anti-VEGF treated patients are highly concordant between ctDNA and archival tissue for all genes, except for the key genes in EGFR/MAPK pathways in anti-EGFR treated patients. However, we did identify four genes, FLT3, PIK3Ca, BRCA2, FGFR2, with emerging mutations detected in more than 2% of patients in both treatment groups.

These mutations are of interest due to their potential role in chemotherapy resistance. FLT3 mutations have been associated with resistance to chemotherapy in hematological malignancies, where FLT3 inhibitors are used to overcome resistance

mechanisms (Kiyoi et al, Cancer Sci., 2020, PMID: 31821677). PIK3CA mutations are known to contribute to resistance to both chemotherapies and targeted therapies (Hu et al, Ann Transi Med, 2021, PMID: 33842631, Strickler et al, Cancer Disc. 2018, PMID: 29196463,). BRCA2 mutations, which impair DNA repair, are linked to resistance to platinum-based therapies (Mylavarapu et al., Front Oncol, 2018, PMID: 29459887). FGFR2 mutations have been implicated in resistance to several therapies, including chemotherapy, by promoting tumor growth and survival (Zhou et al, J Cancer, 2020, PMID: 32127928).

Given the similar prevalence of these mutations in both treatment groups, it is plausible that they may represent common resistance mechanisms that affect not only the targeted therapies but also the chemotherapy backbone. While the retrospective nature of this study and the scope of this manuscript limited our ability to further explore this in the current manuscript, we acknowledge that investigating the role of these mutations in chemotherapy resistance could provide valuable insights and warrants further investigation.

6) With respect to the cell line experiments I feel interpretation is difficult since, if I understand correctly, 3 different cell lines were used. Since the genetic backbone of these cells is very different, it is impossible to conclude that differences in sensitivity are due to the one mutation of interest to the authors. In fact, the differences in sensitivity could also be due to other differences between the cell lines.
We thank the reviewer for their thoughtful comment. We agree that the different genetic background of the cell lines could introduce variability in sensitivity, and that differences in sensitivity could be influenced by other factors inherent to different cell lines.

To address this concern, we included 6 additional cell lines with other KRAS mutations (G12V and G13D) to broaden the scope of our analysis. Unfortunately, CL-11 is the only available cell line with the KRAS 61H mutation specifically. Despite the potential genetic differences between these cell lines, as shown in Suppl Fig. 7, the results are consistent with our previous observations, confirming that the CRC cell line with the KRAS 61H mutation are more sensitive to MEK or pan-RAF inhibitors compared to CRC cell lines with other KRAS mutations. While we acknowledge that the potential differences in the genetic backbone of the cell lines limits our ability to conclusively attribute differences in sensitivity solely to this mutation, the consistency with prior findings strengthens the relevance of our conclusions.

We have added this limitation to the discussion section of the manuscript and suggest that future studies using isogenic cell lines or more controlled models would be valuable to better isolate the effects of the KRAS 61H mutation on treatment sensitivity.

Minor comments:
1) With respect to the organoid experiments it is currently unclear from the results section that these organoids are derived from mice instead of patients.

We thank the reviewer for bringing this to our attention. Apologies for the confusion. To clarify, the organoids were derived from mice, not patients. We have updated the manuscript to accurately state this.

2) For the CNVs it would be easier to also include cytoband locations instead of only bp positions.
We appreciate the reviewer's suggestion. Analyses were performed using base pairs rather than cytoband locations to maintain higher resolution, as breakpoints can occur within a cytoband. Using base pairs ensues more precise identification of these breakpoints. However, if necessary, we will be happy to call out cytoband ranges that are included in the gained/lost regions, though these can already be directly inferred from the reported base pairs in the text and figures.

3) In the discussion section it states that Tgd cells contribute to the response to ICI treatment and that this suggests a link between Tdg cell driven immune activity and resistance to anti-EGFR. This statement is unclear to me?
We appreciate the reviewer's comment. We agree that the link between Tgd cells and resistance to anti-EGFR therapy was not clearly articulated. After further consideration, we have decided to remove this statement from the discussion, as the connection between Tgd cell-driven immune activity and anti-EGFR resistance requires further investigation. We have revised the manuscript to better reflect this.

4) It states multiple times that patients were selected that received anti-EGFR or anti-VEGF but not anti-EGFR. I assume this also means that patients on anti-EGFR did not receive anti-VEGF, but strictly speaking this is not specified in the text?
We thank the reviewer for pointing this out. As addressed in our response to Reviewer 1's minor comment 6, the statement regarding the treatment regimen is correct, as most patients who received anti-EGFR treatment also received anti-VEGF treatment. We have clarified this in the manuscript and provided additional treatment details in Supplemental table S1 and S10. Specifically, we observed that the percentage of KRAS/NRAS/BRAF wild-type patients with acquired genomic alteration in EGFR/MAPK pathways was comparable between patients who received anti-EGFR treatment alone and those who received both anti-EGFR and anti-VEGF/R treatment (Supplemental table S10). We hope this clarification resolves any confusion and provides additional context to our findings.

5) Please explain why CMSCaller was used (which was developed for cell lines) instead of the original CMS classifier package (https://github.com/Sage-Bionetworks/CMSclassifier)?
We appreciate the reviewer's question. Internal benchmarking demonstrated consistent results between CMScaller and the original CMSclassifier. We chose to use CMScaller to get consistent calls between our patient samples and preclinical models as described in the study by Radia et al. ( https://www.nature.com/articles/s41598-017-16747-x).

Additionally, at the time the work was conducted, the CMSclassifier algorithm was not available for commercial use, which further influenced our decision to use CMSCaller.

Reviewer #3, expertise in colorectal cancer clinical trials and precision medicine (Remarks to the Author):

A relevant paper published in Nature 10 years ago in 2014 by Bertotti et al. entitled “The genomic landscape of response to EGFR blockade in colorectal cancer” was not cited or discussed. This oversight or omission diminishes the novelty of the current manuscript. One would expect a thorough discussion of similarities and differences as well as clear indication of novel findings versus the previous landmark paper.

While the paper makes a point about anti-VEGF therapy there is little if any novel insight into the resistance mechanisms other than they don’t see what is seen with anti-EGFR therapies.

Overall the findings are incremental and of interest to a more specialized audience due to limited novelty or clinical actionability.
We sincerely thank the reviewer for their insightful comments and apologize for the inadvertent omission of this important publication by Bertotti et al (Nature, 2015, PMID: 26416732) in our original submission. We fully acknowledge this landmark study and its critical contribution, along with other key publications (Ref.13-20), to advancing our understanding of resistance mechanisms to anti-EGFR therapies in CRC.

To address this oversight, we have added Bertotti et al in the revised manuscript. Additionally, we have elaborated on how our study builds upon these prior insights and advances the field in several meaningful ways. The similarities between our findings and those of Bertotti et al and others confirm the reproducibility and robustness of resistance mechanisms to anti-EGFR therapies. However, our study extends this knowledge and takes this further by analyzing a larger, well-controlled cohort.

Unlike Bertotti et al., and other prior studies, our inclusion of anti-VEGF treated group as a control ensures that the observed acquired mutations are not ctDNA artifacts but represent genuine resistance mechanisms specific to anti-EGFR therapies. Furthermore, the larger patient cohort analyzed in our study provides insights into frequencies and biochemical dependency of certain alterations over others. These provide critical insights that could define priority combinations or mechanisms of action to evaluate in patients.

In addition, our data reveal novel insights into resistance mechanisms, such as the role of 8q gain and PI3K, which were not described in the prior work published by Bertotti et al and others. The baseline gene expression and subtype analysis in the context of likelihood of resistance mutation acquisition describes unique biological insights that, to our knowledge, have not been previously reported.

In summary, we believe that the totality of the data presented here not only validates and adds on the legacy of published data by Bertotti et al. and others, but also uncovers new critical insights that may contribute to a comprehensive molecular roadmap for future drug development in CRC. We greatly appreciate the reviewer bringing attention to this critical reference and providing us with the opportunity to enhance our manuscript.

Reviewer #4, expertise in machine learning and subtype classification for colorectal cancer (Remarks to the Author):

The study comparing intrinsic and acquired mechanisms of resistance to anti-EGFR versus anti-VEGF plus chemotherapy therapies in metastatic colorectal cancer (mCRC) patients is comprehensive and sheds light on several key aspects of treatment resistance. However, there are several areas that require attention and potential improvement:
We thank the reviewer for their insightful and constructive feedback on our study. We appreciate the reviewer's expertise and input, which is invaluable in helping us refine our work. Below, we outlined how we addressed these suggestions:

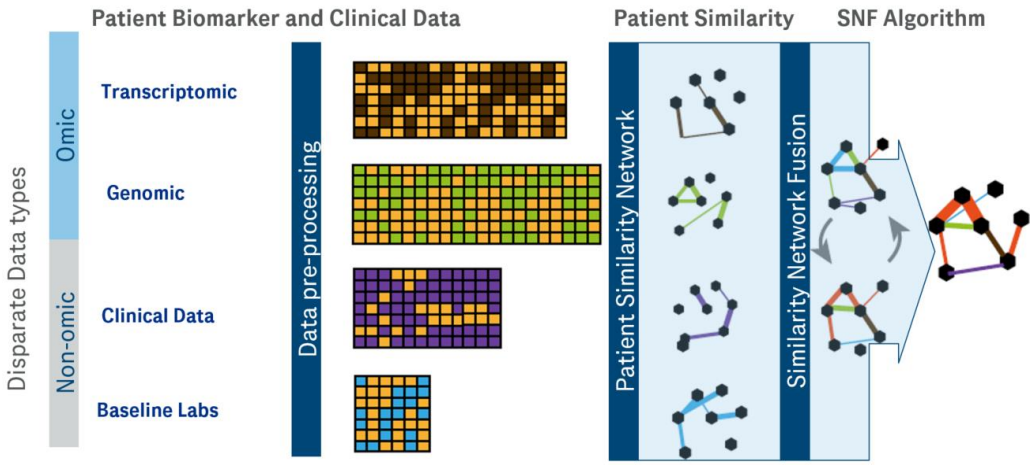
1) Data integration is a missed opportunity for this paper. It must be done more thoroughly to support the claims. I would integrate both RNA-seq and pre-treatment mutation status from the patients and use a multi-omics approach for clustering and supervised analysis. The obtained NMF clusters are not representative of acquired mutation status. There are many multi-omics data integration tools, one of which should be used for this purpose in both supervised and unsupervised setting. One can also use results from xCell and pathway analysis as input to multi-omics data integration tools as input to get a more unified picture. The figures do not show a clear separation of patients dependent on their acquired mutation status, multi-omics integration analysis can show such separation if there is signal in the data.
We thank the reviewer for their insightful and constructive comments regarding the value of multi-omics data integration. We fully agree that such approaches can provide a more comprehensive understanding of the data by multiple sample types.

However, as noted in the response to the Reviewer 1's minor comment #3, there are inherent challenges in interpreting these different data due to the differences in their collection time. Specifically, tumor samples were collected prior to any treatment, whereas baseline lab measurements and clinical data were collected after two or three lines of prior therapies but before any treatment with IMblaze370 treatment regimen. Consequently, integrating these different layers may be hard to interpret.

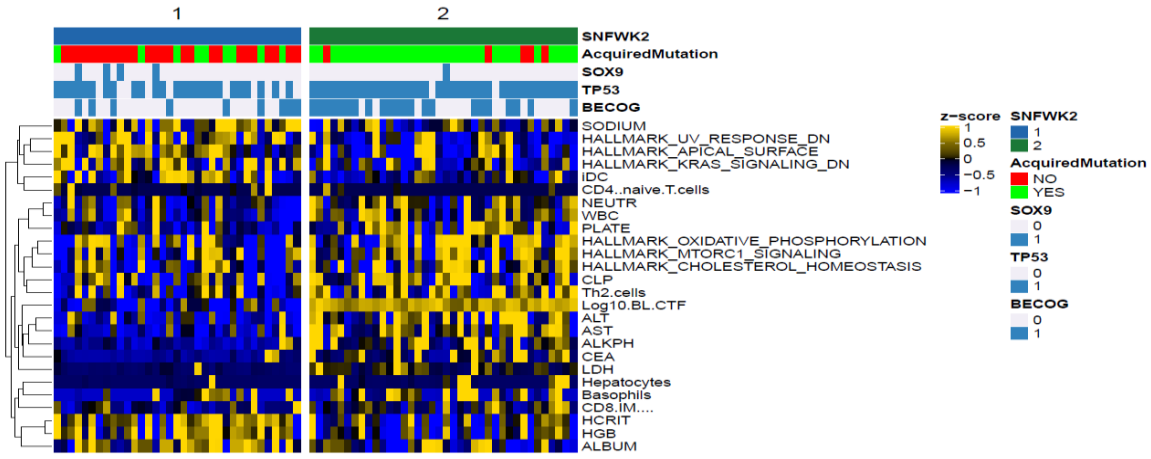
For the unbiased integrative analysis, a similarity network fusion (SNF) algorithm (Wang et al Nature Methods,2014, PMID: 24464287) was applied to pre-processed RNAseq data (layer 1), mutational data (layer 2), baseline lab measurements (layer 3) and baseline clinical data (layer 4) whereby patient similarity networks were constructed for each of the data types using pairwise correlation (SNFTool R package; cran.r-project.org/web/packages/SNFtool). Each patient similarity network was iteratively integrated, making them more similar with each step, using the SNF novel fusion algorithm (with default settings) to generate the integrated network. Data were imputed in samples with less than 25% missingness using the K nearest neighbor algorithm (Reviewer Figure. 2)

While this preliminary analysis showed a more significant association with acquired resistance mutational (chi-squared $p$=4.62x10-7) than NMF subtypes derived from RNA-seq data alone (chi-squared $p$=0.03) (Reviewer Figure 3), the interpretation of these findings remains limited due to the different data collection time.

Given these caveats, we have decided not to include this analysis in the revised manuscript. Again, we appreciate the reviewer's suggestion and agree that a more thorough multi-omics integration would be an important area for future work, ideally in a dataset where all data layers are collected under more consistent conditions.



**Reviewer Figure 2:** Schematic of the SNF algorithm. Patient similarity matrices are constructed for each data type using pairwise correlation. Patient similarity matrices are equivalent to patient similarity networks, whereby the nodes represent patients and lines represent patients' pairwise similarities. Each patient similarity network is updated iteratively with the information from the other networks, making them more similar with each step. The final fused network of patients to which the SNF process has converged.




Reviewer Figure 3: Heatmap show patient samples sorted by SNF cluster assignment in the column and the most significantly (Krueskal Wallis (numeric) or Chi-squared (Categorical) p-value<0.05) SNF associated variables associated.

2) If EGFR ligands or MYC activation have predictive importance beyond this study as claimed at the end of the abstract, one can easily explore this by integrating TCGA or even Novartis PDX ( 10.1038/nm.3954) data to see if these ideas have any ground. For TCGA, I would check what percentage of CRC tumors have ligands highly expressed or any other subsetting of patient population based on predicted markers in this study and see if there is any survival difference for that subsetting. For Novartis PDX data, I would check if there is any evidence of treatment response alteration depending on the ligand status.

We thank the reviewer for their insightful comments and great suggestions to use The TCGA or Novartis PDX model to validate our findings.

To address the reviewer's concern, we utilized the data from Novartis's patient-derived xenografts (PDX) datasets (Gao et al. Nat Medicine, 2015, PMID: 26479923). This dataset included a cohort of 244 cetuximab-treated PDX samples with available response and gene expression data. To classify each PDX sample into NMF subtypes, we trained a random forest machine learning algorithm (R package random Forest)

using the IMblaze 370 RNAseq data as the training set. The classifier, based on an ensemble of binary decision trees, predicts the class of a given sample by evaluating its similarity to patterns observed in the training set. Using this approach, we assigned NMF subtypes to each PDX sample. As hypothesized, PDX samples classified as NMF2.1 demonstrated significantly greater responsiveness to anti-EGFR treatment compared to those classified as NMF 2.2, as evidenced by a smaller increase in tumor volume at 3 weeks post cetuximab treatment (Wilcoxon p=9e-04, Fig. 5d)

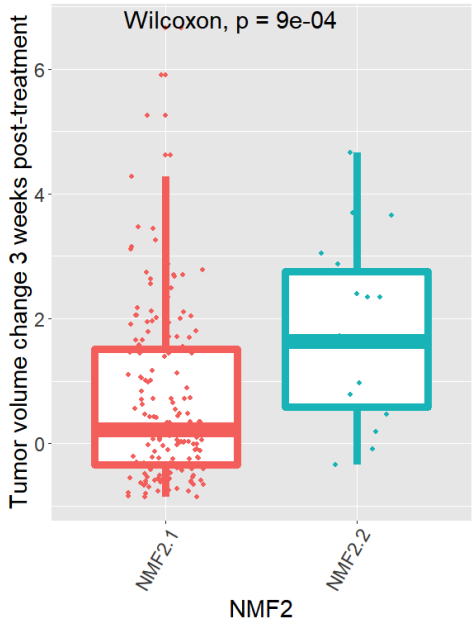
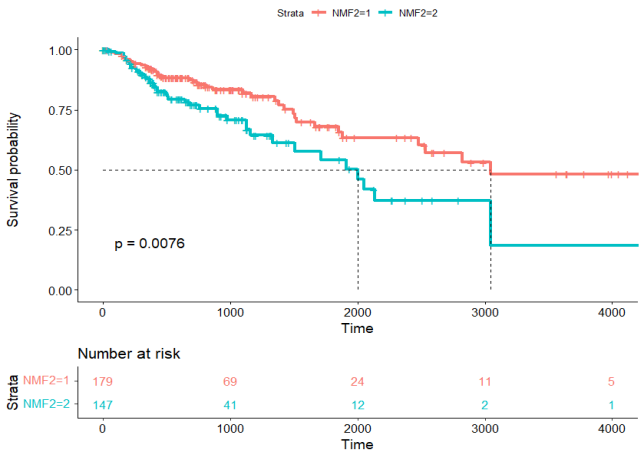



**Figure 5d:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models shown by NMF subtypes. P-value determined using a two-sided Wilcoxon rank sum test.

We also applied our classifier to TCGA data to predict NMF subtypes. Preliminary analysis showed that NMF2.1 tumors were associated with significantly longer OS compared to NMF2.2 tumors (p=0.0076, Reviewer Figure. 4). However, we chose not to include this data in the manuscript due to limited information on prior treatment history in the TCGA dataset, which prevented us from distinguishing prognostic effects from predictive value.




**Reviewer Figure 4:** Overall survival of NMF2.1 and NMF2.2 tumors in TCGA dataset

Consistently, PDX samples with high EREG expression (above median) exhibited significantly better response to cetuximab treatment compared to those with lower EREG expression (below median) (Wilcoxon p=1.2e-07, Fig. 6d). Unexpectedly, no significant difference in response was observed between PDX samples with high versus low AREG expression (Wilcoxon p=0.1, Supplementary Figure10a). However, as shown in the ridge plot comparing the expression of these two ligands, AREG expression was much lower than EREG expression in this dataset Supplementary Figure10b), which may explain the lack of significant association.

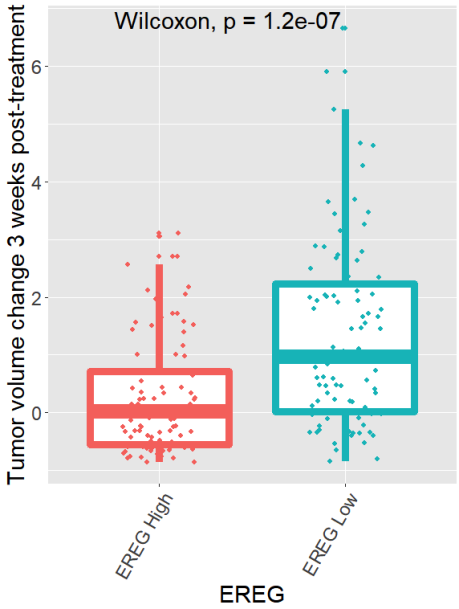
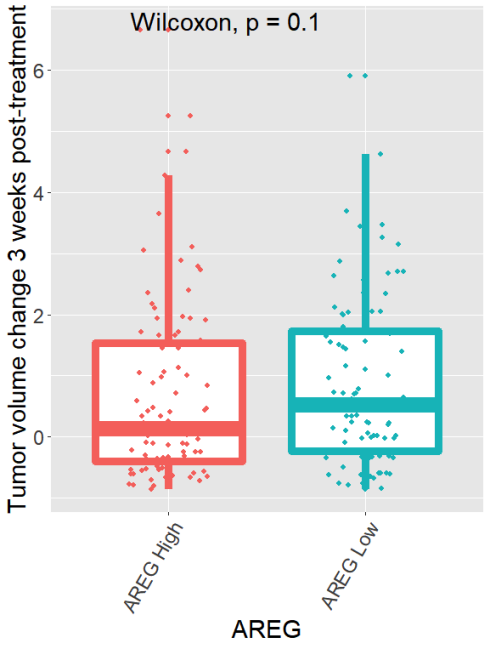
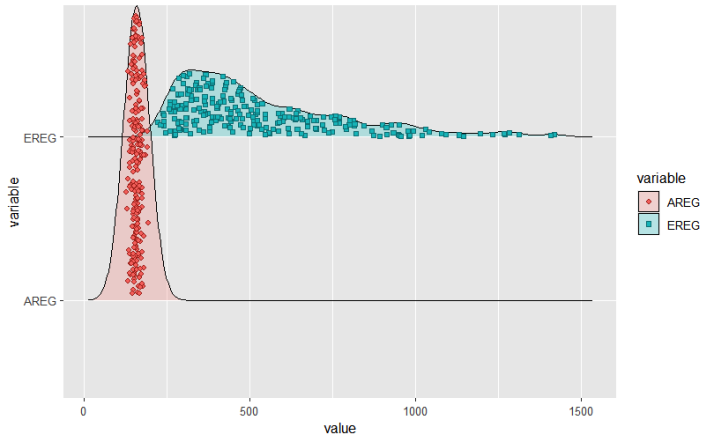



**Figure 6d:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models with EREG high (above median n=122) and EREG low

(below median n=122) tumors. P-value determined using a two - sided Wilcoxon rank sum test.




**Supplementary Figure 10a:** Changes in tumor volume from baseline to 3 weeks post Cetuximab treatment in 244 PDX models with AREG high (above median n=122) and AREG low (below median n=122) tumors. P-value was calculated using two-sided Wilcoxon rank sum test.




**Supplementary Figure 10b:** The ridge plot shows the distribution of the expression levels of AREG and EREG in 244 PDX models.

Consistent with our findings, previous studies have reported that high EGFR ligand expression is a predictive biomarker for anti-EGFR therapy in RAS WT patients. (Seligmann et al., JAMA Oncol, 2016, PMID: 26867820; Smyth et al., JAMA Oncol, 2016, PMID: 26869404).

Collectively, these findings further support the predictive value of EGFR ligand, particularly EREG, for anti-EGFR treatment in mCRC. These results provide a strong rationale for incorporating high EREG expression as biomarker for patient selection in future clinical studies of anti-EGFR therapies in mCRC.