PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Development of a hoRizontal data intEgration classifier for NOninvasiVe early diAgnosis of breasT cancEr: the RENOVATE study protocol
AUTHORS	Ravera, Francesco; Cirmena, Gabriella; Dameri, Martina; Gallo, Maurizio; Vellone, Valerio; Fregatti, Piero; Friedman, Daniele; Calabrese, Massimo; Ballestrero, Alberto; Tagliafico, Alberto; Ferrando, Lorenzo; Zoppoli, Gabriele

VERSION 1 – REVIEW

REVIEWER	Guttery, David University of Leicester
REVIEW RETURNED	21-Jul-2021

GENERAL COMMENTS	Ravera et al. propose a study protocol towards multi-analyte analysis combined with radiomics towards developing a HDI classifier for improved diagnosis of screen-detected breast cancers. This is a very interesting proposal that could answer a very important questions; however, there are significantly concerns that require attention prior to publication. Major concerns: 1) More definitive details are required regarding the amount of plasma that will be used for extraction of cfDNA. In order to obtain enough for appropriate analysis, in my experience at least 4ml is required, maybe more in these early stage BCs. 2) The authors discuss obtaining samples for RNA analysis but then don't really explain how this will into the model. Is this a part of it or will they be analysed in future and integrated into the workflow? If they will be analysed, how much RNA will be analysed? What endogenous genes will be analysed? How will the anlysis be performed? 3) The authors have developed a ctDNA sequencing panel based on the GENIE dataset. Why was only this dataset chosen? In my opinion mutations in the TCGA and METABRIC cohorts should also be considered. 4) Why are only selected regions of TP53 and PIK3CA being analysed? Would a whole exon approach for each gene be more appropriate? 5) The authors state that sequencing of 20 ng of cfDNA at 20,000x is sufficient for detection at 0.1% VAF. I would urge caution here since a lot of things need to be taken into account. Given that

	Page et al (2021, Annals of Oncology) only detected a small number of ctDNA positive screen-detected BCs, sequencing up to 50 ng of cfDNA at >100,000x looking for up to 16 mutations, i am sceptical that the authors sequencing approach looking at over 100 regions is going to detect any ctDNA positive patients. Not only this, but how are the authors going to define ctDNA positivity? Will be it be 2 molecular families? 3 families? This needs to be taken into account. 6) Will the authors be determine sensitivity/specificity of their tests? If so this should be stated. 7) How will the authors define positivity in the methylation analysis? Will it be changes in hyper/hypo methylation? If so in which regions? Promoter regions? EREs? 8) Why is validation only being performed on protein markers?

REVIEWER	Moscetti , Luca Universitaria di Modena, Oncology
REVIEW RETURNED	22-Sep-2021

GENERAL COMMENTS	The study protocol is innovative and very well designed. In the study design was reported that the patients with a stage IIIA breast cancer has been included but no mention of the different subtypes are indicated. Can the lack of differentiation of the different subtypes have an impact on the final statistical analysis? Please comment

VERSION 1 – AUTHOR RESPONSE

Reviewer #1, Dr. David Guttery, University of Leicester.

Ravera et al. propose a study protocol towards multi-analyte analysis combined with radiomics towards developing a HDI classifier for improved diagnosis of screen-detected breast cancers. This is a very interesting proposal that could answer a very important questions; however, there are significant concerns that require attention prior to publication.

We wish to thank Dr. Guttery for his appreciation of our effort.

More definitive details are required regarding the amount of plasma that will be used for extraction of cfDNA. In order to obtain enough for appropriate analysis, in my experience at least 4ml is required, maybe more in these early stage BCs.

This has now been amended in the text. We thank the Dr. Guttery for his remark.
See page 9 of the clean manuscript.

The authors discuss obtaining samples for RNA analysis but then don't really explain how this will into the model. Is this a part of it or will they be analysed in future and integrated into the workflow? If they will be analysed, how much RNA will be analysed? What endogenous genes will be analysed? How will the anlysis be performed?

On average we manage to obtain 100 micrograms of RNA from PBMCs in Tempus tubes. We deliberately did not specify which kit we are going to use, since the technology has been progressing over the last few years. Ideally, we would now favor methods such as 3'-directed RNA sequencing due to their relative ease of analysis and economic advantages compared to conventional transcriptome sequencing. We do not envisage these analyses to be initially included in our multianalyte model, but may be added as a second step.
See page 11 of the clean manuscript.

The authors state that sequencing of 20 ng of cfDNA at 20,000x is sufficient for detection at 0.1% VAF. I would urge caution here since a lot of things need to be taken into account. Given that Page et al (2021, Annals of Oncology) only detected a small number of ctDNA positive screen-detected BCs, sequencing up to 50 ng of cfDNA at >100,000x looking for up to 16 mutations, I am sceptical that the authors sequencing approach looking at over 100 regions is going to detect any ctDNA positive patients. Not only this, but how are the authors going to define ctDNA positivity? Will be it be 2 molecular families? 3 families? This needs to be taken into account.

We appreciate the remark, and have now corrected our expectations. Indeed, we have followed the approach delineated by Cohen et al (Science 2018), which made use of tagged-amplicon based panel to detect approximately the same number of regions we have designed. We presume the reviewer refers to the 2021 Annals of Oncology paper by Magbanua et al., which adopts a different approach, i.e. that of generating patient-specific ctDNA panels and assessing the presence and clearance of patient-specific mutations after neoadjuvant chemotherapy. In our panel, the design is fixed and aimed at common mutations in breast cancer. As a key difference compared with the fixed panel of CancerSeek, it is designed for BC only and not for different tumor histologies, and as such maintains a limited number of amplicons while showing greater in silico coverage for BC recurrent mutations. Three families are considered as positive by ThermoFisher, but this threshold is arbitrary. We may have to adapt the positivity threshold based on the actual class the sample falls in, in order to optimize the accuracy of our multiomic test. This has been specified in the text.
See page 9 of the clean manuscript.

The authors have developed a ctDNA sequencing panel based on the GENIE dataset. Why was only this dataset chosen? In my opinion mutations in the TCGA and METABRIC cohorts should also be considered.

We indeed took into consideration those large datasets in the beginning, but since all common BC mutations are covered by all the three datasets, and since GENIE had, at the time we designed our panel, a greater coverage and a larger number of patients than the other two datasets, in the end we chose to use only GENIE to increase the homogeneity of the dataset used to design our panel. Furthermore, looking at the most frequent, non-overlapping (i.e. mutually exclusive) mutations, the sample size of GENIE was deemed as adequate for the panel design.
See page 8 of the clean manuscript.

Why are only selected regions of TP53 and PIK3CA being analysed? Would a whole exon approach for each gene be more appropriate?

We are sorry for the misunderstanding: by regions, we mean the exonic ones to be targeted. TP53 is planned to be analyzed, together with CDH1, for all its exons. On the other hand, PIK3CA presents mostly hotspot mutations in exons 9 and 20, hence it would be a waste of amplicons for a targeted panel. Hence, only those two exons are going to be covered by our design. We have clarified that in the text.
See page 8 of the clean manuscript.

Will the authors be determine sensitivity/specificity of their tests? If so this should be stated.

Yes. This has now been amended in the text. We thank the Reviewer for his remark.
See page 11 of the clean manuscript.

How will the authors define positivity in the methylation analysis? Will it be changes in hyper/hypo methylation? If so in which regions? Promoter regions? EREs?

cfMeDIP-Seq as a method leverages methyl-cytosine pull-down by a specific antibody. As such, it is agnostic in regard to the regions to be analysed. Both types of regions will therefore be assessed, provided they are captured and sequenced and found to be differentially expressed by the sample classes.

Why is validation only being performed on protein markers?

We believe this was a mistake. Validation and dummy-down, i.e. resorting to cheaper, production-scale technologies assessing fewer markers, faster and at lower costs, are integral parts of the project. This has now been amended in the text.
See page 11 of the clean manuscript.

Reviewer #2, Dr. Luca Moscetti, Università di Modena

The study protocol is innovative and very well designed.

We wish to thank Dr. Moscetti for his appreciation of our effort.

In the study design was reported that the patients with a stage I-IIA breast cancer has been included but no mention of the different subtypes are indicated. Can the lack of differentiation of the different subtypes have an impact on the final statistical analysis?
Please comment

VERSION 2 – REVIEW

REVIEWER	Guttery, David University of Leicester
REVIEW RETURNED	02-Nov-2021

GENERAL COMMENTS	The authors have addressed my concerns and i am happy to recommend publication.