Peer Review File

Article information: https://dx.doi.org/10.21037/tp-22-105

Reviewer A

The authors wrote a nice narrative review on untargeted metabolomics in diagnostics of IEM.

My main concern is the discussion on the use of untargeted metabolomics for therapeutic monitoring. Given the non or semi-quantitative nature of metabolomics I feel that untargeted metabolomics may reveal biomarkers that reflect response to therapy that have previously not been measured, but proper therapeutic follow up will require quantitative data.

We thank the reviewer for these comments, and agree that if non-traditional, not validated or rather "novel candidate" biomarkers are encountered by untargeted methods, these novel markers need to be further validated to be used a proper biomarkers for monitoring purposes. We also agree that targeted assays are more likely to provide traditional, quantitative data. As such, we have adjusted section 4.2 accordingly including its title, page 11, line 259.

Discussion on integration with genetics: this shows how metabolomics can validate a VUS, there are multiple such examples. It may also be worthwile to discuss broad integration of genome and metabolomics (PMID: 32443577, PMID: 29721916).

As recommended, we have added further discussion and the references to section 4.4 (Untargeted metabolomics as a diagnostic companion to genomics), page 14, lines 313.

Line 81 onwards: the statement on the pubmed hits: This suggests that untargeted metabolomic technology is increasingly being utilized in the investigation of patients with IEMs." Please validate statement, since individual search terms (metabolomics, diagnosis) show the same trend?

We thank the reviewer for the comment and upon re-evaluation we decided to remove this figure from the manuscript and modified text accordingly and including a reference, page 4, line 84.

Line 59: "techniques, to detect around 30 disorders in the pre-symptomatic phase"the number of diseases screened for is highly dependent on country and region

As advised, we have added further clarification to Introduction "…Depending on the country and region, these programs can detect around 30 disorders…" page 3, line 60

Textual:

Line 27 and 31 remove brackets "targeted metabolomic"

"untargeted metabolomics":

Removed brackets.

Line 35: revolutionize the diagnosis -> rather it revoutionizes that diagnostics, not the diagnosis

Diagnosis is changed to diagnostics.

Line 120 space is missing

Space is added.

Reviewer B

This mini review addresses the application of untargeted metabolomics in the field of IEM. The review is structured well, in particular section 4 is very useful.
We thank the reviewer for these comments.

Major points:
The review lacks discussion on the current application of untargeted metabolomics as a screening tool to diagnose IEM. The review title - integration of metabolomics into clinical practice for IEM - implies inclusion of clinical IEM screening by untargeted metabolomics, but this is only just mentioned in section 1, lines 71-75. The successful application of this approach by a number of labs merits attention. The authors should elaborate on this in addition to the applications related to discovery described in section 4. Please also add literature references (original research papers such as PMID 32828637, 32445384, 31779119, 30641898) that report the first applications of untargeted metabolomics in clinical screening for IEM.
We agree with this comment and thank the reviewer for bringing this to our attention. To emphasize this important point more, we added content and appropriate references addressing the use of metabolomics in screening/diagnosis of IEMs, page 8, line 181 onward.

In section 5 limitations of untargeted metabolomics are discussed. In my opinion a considerable limitation is the fact that 'the metabolome' is far from established. Where genomics can use the reference genome and established variants in eg ClinVar, clinical scientists using untargeted metabolomics are only able to identify a small fraction of the metabolites measured in a sample (typically <500), i.e. with m/z, fragmentation data, isotope patterns and retention time, the latter only when LC is used. The remainder of the features is annotated with limited certainty, usually with just m/z numbers and databases such as HMDB. Better metabolite identification is in my view the major challenge in the use of metabolomics too perform reliable and fast clinical screening. The authors should elaborate on this.
We thank the reviewer for these comments and have incorporated these comments into the limitations section (5. Limitations of untargeted metabolomics) to bring it to the reader's attention. Page 15, lines 350-355. Whilst identifying this as a limitation, we have also included information from the Human Metabolome Database for 2022, to demonstrate the progress is understanding and characterizing the metabolome, page 15, lines 363-370.

Minor points:
Line 52: rare > individually rare.
Added "individually" page 3, line 52.

Lines 71-72: Advances in HRAM-MS have been pivotal in development of untargeted metabolomics.
Included this important comment on "high resolution accurate mass spectrometers" in the rephrased sentence, please see page 3, line 74.

Line 124: Do the authors mean by LC-MS/MS a triple quad (tandem MS) with mass resolution

up to 0.1 Da? Untargeted metabolomics can not be performed using such equipment. High resolution MS is required, although these may be used to generate fragment spectra in addition to full scan data. This point needs to be clarified in the text.
The sentence has been modified to clarify this point "Untargeted metabolomics can be performed by ultra-high performance mass spectrometry-based approaches in tandem with liquid chromatography…" and Evans *et al.,* 2009 added as reference, page 6, line 124.

Lines 126-130 LC coupled to TOF or Orbitrap are worth mentioning here, since these techniques are most promising in untargeted metabolomics for diagnostic purposes.
We added this information to section 2 (Evaluating the Metabolome), page 6, line 127.

Lines 134-135 This is a puzzling sentence as one would expect bioinformatics to enable analysis of more compounds (compared to manual curation of data).
Thank you for bringing this to our attention we modified the sentence to make it clear: "Automated bioinformatic curation, based on population z-scores, reduces the number of compounds requiring manual review downstream (32, 33)." page 6, line 137.

Additional changes by authors:

Modified affiliation 4 to read "Specialty of Genomic Medicine, Faculty of Medicine and Health, …" page 1, line 15.

Deleted additional "Tel" (page 1, line 19)

Deleted: "The authors declare no conflict of interest." Page 17, line 398, instead added "All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare"per checklist.

Changed Figure 2 to Figure 1 (due to deletion of Figure 1 per Reviewer A's comment above)

Added a statement per checklist **"Copyright**: The figure is original and has not been published previously." Page 18, line 429.

Added Figure Leged separately also as requested in checklist (page 24, line 658)