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Peer Review File

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Reviewer A

The present study by Wang, et al. aims to investigate the etiological diagnosis for newborns with cholestasis after emerging molecular diagnostics. The study follows the premise that most cholestatic patients show symptoms of cholestasis at 0-3 months of age and usually show specific genetic defects after failing to obtain a definite diagnosis by routine evaluation. The authors performed a retrospective study to evaluate the clinical characteristics, etiologies and outcomes in infants with neonatal cholestasis. Of the 160 cases reviewed, ~49% were diagnosed with PNAC, 18% with cardiovascular and circulatory disorders, 13% with biliary anatomic obstruction, 9% with infection and about 6% with genetic disorders. While the study is well designed and demonstrates the variable causes of neonatal cholestasis in newborns, several concerns are noted:

Reply A:

a) I am unsure if the title, "Etiology of neonatal cholestasis after emerging molecular diagnostics". Do the authors imply that certain diagnostic measures routinely used in the laboratories identify a diverse range of causes of neonatal cholestasis? Aren't these diagnostic measures and clinical indices common in diagnosing a newborn infant referred to the clinic for cholestasis evaluation?

Reply: Thanks for getting our message. So far genetic test hasn't been a routine test for identifying the causes of neonatal cholestasis from literature. Genetic molecular diagnosis did help increasing the diagnostic rate in neonatal population, and improved the etiology understanding in some of the uncertain cases from our work. We would like to refer it as a common measurement in neonatal cholestasis evaluation, especially in the cases without clear etiology.

b) The study is observational and does not identify a "remedy" to the solution. Although the title authors note in the Conclusion that molecular diagnostics can improve the etiological diagnosis for newborns with cholestasis, no novel techniques are employed to diagnose these patients. Can the authors comment on this?

Reply: Thanks. It's true, this was an observational study, the improving of the etiological diagnosis may not change our clinical management to the individual cholestasis case, because it may require about one month to achieve these genetic reports in clinical practice. But compare to our previous study published in 2012, the diagnostic rates were improved. And we do believe this would help neonatologists to figure out the cases who need long-term follow up, and some treatments pointing to the mechanism of the cholestasis may be remedy in the future.

c) The authors also suggest that "quite amount of causes are remediable" and "gene test may

help to eliminate genetic causes". How do the authors intend to remedy these causes? No specific information is readily evident to this effect from the manuscript. Second, by using standard genetic analysis tools, how do the authors envisage to eliminate the genetic causes. Can the authors please comment on these two important conclusions drawn in the current study?

Reply: Thanks for the hint. We may use inappropriate words here, the first half of the sentence was pointing at the other causes of cholestasis such as infection and PNAC, not for the genetic causes. And the word of "eliminate" should change to "rule out", which means the genetic etiology could be ruled out. Such as PNAC, the diagnosis of PNAC absent diagnostic gold standard in clinical, for some preterm cases having TPN course with potential genetic etiological diagnosis could be misdiagnosed.

But still, our study showed quite amount of causes are remediable and transient during the neonatal period, genetic test may help to rule out genetic causes and enhance confidence in judging prognosis.

d) Manuscript and presentation language needs improvement.

Reply: Thanks. We have sent this manuscript to English native language specialists to revise. We would like to have the professional staff from TP for polish if necessary.

Changes in the text:

We change the word of "eliminate" to "rule out" (see page 3, line 52) and (see page 18, line 351).

Reviewer B

The study is interesting and well written, however, has several caveats. Comments are as follows:

Introduction:

Reply B

- According with the authors, "neonatal cholestasis is never physiological" I suppose it means that is not pathognomonic for hepatobiliary or metabolic disorders.

Reply: Thanks. This was relative to unconjugated bilirubinemia, and neonatal cholestasis equivalent to neonatal conjugated bilirubinemia. Neonatal unconjugated bilirubinemia could be described as physiological and pathological.

Methods:

- It would be appreciated if the analysed genes were included in the manuscript, perhaps as supplementary material.

Reply: We will add these genetic results as supplementary material at the end of our manuscript.

- "Except for the genetic tests…" Maybe means "Besides"?

Reply: Do you point at this sentence "Except for the genetic tests, investigations for the etiology of neonatal cholestasis included infections, anatomic obstruction of the biliary system,

endocrinopathies, metabolic disorders, cardiovascular and circulatory disorders, toxin and drug exposures, et al." We are sorry for making the confusion, "besides" is more felicitous.

Results:

- The percentage in the line 135 was wrong, it is 3.14%

Reply: Thanks very much for the correction, it is 3.14%. This was a mistake and never noticed.

- The abbreviations are not well explained, GA? BW? They not are defined in its first appearance.

Reply: Thanks for the reminding. We will add the annotations of GA and BW. GA is gestational age, and BW is birth weight.

- The percentage in the line 142 (71.2%) is 71.3%

Reply: Thanks for your meticulous review, we are very sorry for making this mistake. We will correct it.

- In the first paragraph in the "Causes of neonatal cholestasis" Percentages not correctly rounded, and the sum of all of them is greater than 100

Reply: Thank you very much. We did mistake of "post Rh or ABO incompatibility 5.0%", it should be 2.5%. We are very sorry for the silly mistakes. And meanwhile the rate of infection is also not accurate, should be 8.8%, and as well as neonatal hemochromatosis, the rate should be 1.2%. This paragraph should be as below:

The most common etiology of cholestasis was PNAC 48.8 % (n=78), followed by cardiovascular and circulatory disorders 18.1% (n=29), biliary anatomic obstruction 12.5% (n=20) (including surgery confirmed BA, n=17), infection 8.8% (n=14, including cytomegalovirus, n=7; enterovirus, n= 4; fungus, n=1; mycoplasma, n= 1 and syphilis, n=1), genetic and metabolic disorders 5.6% (n=9), post Rh or ABO incompatibility 2.5% (n=4, including Rh incompatibility, n=3 and ABO, n=1), neonatal hemochromatosis 1.2% (n=2), and unknown 2.5% (n=4).

- I do not understand the second paragraph in the "Causes of neonatal cholestasis": "PNAC and biliary anatomic obstruction were the most common etiology…" but in the figure, looks like Cardiovascular and circulatory disorders are more common than Biliary anatomic obstruction (29 vs 20 patients)

Reply: Thanks. "PNAC and biliary anatomic obstruction were the most common etiology of cholestasis for preterm and term infants, respectively" this means "PNAC" was the most common etiology of cholestasis for "preterm", and "biliary anatomic obstruction" was the most common etiology of cholestasis for "term". In "Cardiovascular and circulatory disorders" group, there were 20 preterm and 9 term, so the total number was 29.

- A lot of information is missing in the section on genetic study so, maybe, involve the geneticist in the manuscript preparation, can be a good contribution to improve this part.

Reply: Thanks for the advice. One of the authors, the second is geneticist. We would add the information of this part.

o Only 36 variants on 140 cases in hard to believe: Are you only reporting damaging variants? Which filters have been used for variant selection?
Reply: The 36 variants were reported according to the original diagnosis of cholestasis, or associating with bilirubinemia. And they were damaging variants. Otherwise, every single case would have non-pathogenic variants.

o Why only 9 of 36 were validated by Sanger?
Reply: Yes, here we would like to show the detail of genetic results in the supplementary material.

o You are reporting information about a genetic panel, so… what was the affected gene in Alagille syndrome cases?
Reply: We would like to show the detail of genetic results in the supplementary material.

o What was the affected gene in Niemann-Pick? The disease was an Acid Sphingomyelinase Deficiency or a Niemann-Pick type C?
Reply: We would like to show the detail of genetic results in the supplementary material.

o The found variants are new or previously reported? Do you found some VUS? How you evaluated them?
Reply: We would like to show the detail of genetic results in the supplementary material.

o It is mandatory to write genes in italics, and strongly recommended include the gene ID
Reply: Thanks for the warm suggestion. We will change the format, and would like to show the detail of genetic results in the supplementary material.

Discussion:
- It is mandatory to write genes in italics

Reply: Thanks for the warm suggestion. We will change the format, and would like to show the detail of genetic results in the supplementary material.

- Some acronyms are not defined (PN, IUGR, BW, CMV, MRI, IVIG)

Reply: Thanks. **IUGR= intrauterine growth retardation**, has been defined in the manuscript. **PN=Parenteral nutrition** will be added, was considered as part of PNAC. **BW=birth weight** defined in the wrong place not the first time. **CMV= cytomegalovirus**, will be added. **MRI= magnetic resonance imaging**, will be added. **IVIG= intravenous immune globulin** will be added.

Figures:
- The SROBE Statement is nor referenced on the manuscript

Thanks. The STROBE Statement was shown in the last page of the manuscript.

Changes in the text:

We have changed the wrong number and added the abbreviations definition (see line149, 153, 155, 162, 165, 166, 290, 308, 311, 312).
We have changed the paragraph of genetic results (line 188~195).
We have added the detail of gene test as a supplementary material, we are not sure whether we should add it in the manuscript.

Reviewer C

The Authors presented a very interesting study on the etiology of neonatal cholestasis and the usefulness of NGS in this clinical setting.

The manuscript is worth publishing.
I have some minor issues to be revised before publication.

Reply C:
verse 84-86: The latest (2017) definition by ESPGHAN/NASPGHAN of cholestasis was referred as direct serum bilirubin equal or more than 1 mg/dl (total serum bilirubin was defined as irrelevant to recognition of cholestasis). The Authors cited the reference [4] published in 2013.
Reply: Thanks, it's true, but neonate has a special period of jaundice (unconjugated bilirubinemia), so this definition of "direct serum bilirubin equal or more than 1 mg/dl" may be not suitable for neonatal population. As a result, we use the one from 2013, and lots of literature also use it, such as reference [8] in 2019.

verse 96: Regarding clinical exome sequencing, I found in the latter part of manuscript that it was a panel of genes (targeted NGS). Please, define which genes were included in the panel.
Reply: The technology of genetic tests was described in the manuscript. There were 2742 genes in the panel kit. And the detail could be found in our previous work (reference 13).
The genetic tests were done using 2 mL of EDTA-anticoagulated blood sample. DNA was extracted from peripheral blood. Clinical exome sequencing (CES) using the Agilent ClearSeq Inherited Disease panel kit **(including 2742 genes**) was enriched from the fragments of patients' genomic DNA and performed on an Illumina HiSeq X10 (Illumina). The average on-target sequencing depth was 200× for CES[13]. Variants of interest were validated by Sanger sequencing.

verse 174: Please, define the exact Niemann-Pick disease type - A, A/B, B, C
Reply: We would like to show the detail of genetic results in the supplementary material. This case is Niemann-Pick disease type C1.

Changes in the text:
We have changed the paragraph of genetic results (line 188~195).
We have added the detail of gene test as a supplementary material.

We also add Acknowledgements.