Peer Review File

Article information: https://dx.doi.org/10.21037/jlpm-23-24

Reviewer Comments

Reviewer A

Comment 1: There is limited or no materials that relate to neonates and children

Reply 1: Yes, this probably reflects our wish to include everything whilst not getting bogged down in the very specialist minutiae area of medicine which is neonatal bilirubin problems. We will have a look through and see if we can make it a little more robust whilst making it clear that it does not replace the expertise of a specialist in this more complex area where rare causes may present (note additional information in the introduction).

Comment 2: it is not clear as to who is being guided by the algorithms. They present as a laundry list rather than a strategic decision making too.

Reply 2: this is meant as a general guide to those who are faced with an abnormality in bilirubin and do not know where to start. It most definitely not aimed at hepatologists for example, more for juniors starting out, people who have had little biochemistry training for their clinical role (e.g. nurses, physiotherapists, pharmacists, clinical laboratory scientists) or rarely have to interpret blood tests and are a little rusty or not up to date with modern laboratory tests (or this area e.g. thinking about an orthopaedic surgeon who is more expert on the other tests etc). It is therefore meant to be a starting place to help the jobbing generalist approach an abnormal blood test for which they are not immediately familiar and not to over investigate or have to refer all patients to someone else or to miss important pathology etc. We will try and make that clearer.

Attachment

Comment 3: The algorithms helpful but can be interpreted as a "laundry list" and are not exhaustive. Thus, it is unclear as to whom they would assist and how their practice would be implemented.

Reply 3: thanks I have included a comment that it is meant for the generalist who is not sure what to do with an aberrant bilirubin, not for a specialist etc.

Comment 4: For example: does the suspicion of disease entities lead to the order of a test?

Reply 4: we were trying to present just a laboratory algorithm but of course clinical suspicion should always drive tests however we have limited the algorithm to testing (i.e. the assumption is the clinician is a bit stuck and the cause was not immediately obvious on history and examination). A clinical approach to abnormal results was basically not within our scope. We hope with our discussion about how bilirubin is produced, its sources, the figures etc. that we have provided enough information to help guide the clinician (or at least those with significant clinical training who are competent at patient assessment).

Comment 5: Or are their clinical specific signs that generate a need to order a test?

Reply 5: Completely agree that you would not go down the algorithm if clinically you could already limit the number of possible diagnoses, but as we were avoiding going down that pathway here we have just restricted ourselves to laboratory algorithms. Have added in the conclusion: 'It is hoped the algorithm will be useful in daily clinical practice to help guide investigations and elucidate the reasons for the biochemical derangement but it is not a substitute for clinical assessment and expert guidelines.' Effectively we have meant to provide a prompt to people who are a bit stuck or are sitting with the results and no patient to give them an idea about what sort of things to think about next on the test basis (but hopefully stimulate ideas as to which questions they need to ask and systems they need to examine).

Comment 6: Is the test first ordered by a clinician but then guided by the lab scientist?

Reply 6: This is designed for use by clinicians but is also helpful for lab staff when discussing abnormal results with clinicians (i.e. to give a framework for the sort of tests they may suggest first or to work through it with the clinician). We hope the laboratory scientist may have a good understanding of biochemistry but less so the clinical findings. This article is aimed for both groups effectively but it could be used to inform reflex or reflective testing in the lab.

Comment 7: Or, should the lab scientist guide the clinician on the interpretation of specific tests?

Reply 7: Great point and when writing this we were trying to create an algorithm that would be of use in any health care setting or all users. In some situations there will be easier access to expertise and tests and less so in others. We therefore hope it is helpful for any setting, as much as possible, and it could definitely be employed by lab scientists to inform their practice but also conversely clinicians without access to clinical laboratorians. You are right of course, having such a basic breadth of scope may mean aspects of this are less practical for everyone which is why it is not immediately obvious who it is for perhaps.

Comment 8: Could the authors offer better formats to assist either the clinician or the lab scientist or both?

Reply 8: Not sure we entirely understand, do you mean different versions of the algorithm based on location of clinician? We can see why that may be beneficial but it also infers that each group of user has a standard amount of knowledge and expertise. In our experience there is a great range in confidence within each clinical group as well as between. We would prefer to keep them generic, with the clear limitation therefore that it may be too easy for some, to allow each user to adapt it to their own needs and practice please. It may be that tests need substituting in some health care environments, or other conditions are more or less common, but by providing a suggestion it allows other people to use, adapt, audit, and then improve the strategy, with any luck, and fosters clinical research in the most cost and clinically effective approach and whether this differs based on scope of practice (as indeed you mention this is likely inappropriate for a neonatal physician for example).

Comment 9: Do these algorithms need to be defined by individual diseases, could grouping them by functional defect (defined by metabolic path) is adequate and highlighted by some examples?

Reply 9: Completely understand your point, it means that rarer causes don't all need to be listed for example. We will adjust the figures slightly to ensure it reads more mechanistically rather than disease specific.

Comment 10: Is the article limited to adults?

Reply 10: Yes and no (in the paediatric segment we state 'The focus of this article is hyperbilirubinaemia in adults however a few notable paediatric conditions will be discussed'. We do realise it is a bit confused therefore, effectively it is not meant to be a review for the specialists on rare genetic disorders but a starting point for uncertain generalists. We will see if we can make this a little more definite and make clear that we are being limited in this area.

Comment 11: Neonatal bilirubinemia is mostly unconjugated as compared to pediatric and adult conditions that are primarily conjugated hyperbilirubinemia. Some are associated with concurrent hepatic function (enzyme) disorders and others are not.

Reply 11: yes agreed it is a much more complex case scenario and we have not devoted much attention to this in the current review. We mainly have limited the text to the conditions mentioned in the algorithms and those are primarily directed to adult medicine.

Comment 12: I appreciate their format with certain modifications suggested below. The narrative also has several typographical and factual errors.

Reply 12: thank you we will reread and endeavour to improve the manuscript

Specific Comments

FRAMEWORK REVIEW

Introduction [Essential knowledge to be imparted]. *Bilirubin is the oxidative product of mammalian heme catabolism and, is excreted after its esterification to polar mono- and diconjugated glucuronide derivatives into the biliary canaliculi. The accumulation of unconjugated and conjugated bilirubin in the serum can be caused by several types of hereditary disorder or pre-hepatic and hepatic disease. Increased bilirubin levels occur due to increased production and/ or delayed elimination. A newborn has a characteristic and unique postnatal surge with peaks at 3 to 5 days (specifically, by age in hours and defined by percentiles) that resolve by age 7 to 10 days. Persistence beyond age 2 weeks requires testing for conjugated bilirubinemia and is highly abnormal. The resolution process is delayed due prematurity (for each week of prematurity) or other multifactorial neonatal disorders. Congenital conditions include the Crigler-Najjar syndromes which is caused by a defect in the gene which encodes bilirubin UDP-glucuronosyltransferase (UGT), whereas the Dubin-Johnson syndrome is characterized by a defect in the gene which encodes the canalicular bilirubin conjugate export pump of hepatocytes. Elucidation of both the structure of the UGT1 gene complex, and the Mrp2*

(cMoat) gene which encodes the canalicular conjugate export pump, has led to a greater understanding of the genetic basis of hyperbilirubinemia.

Rudy Schmid and Tony F. McDonagh (The enzymatic formation of bilirubin. Ann N Y Acad Sci. 1975 Apr 15;244:533-52.) have explained the enzymatic formation of bilirubin. Humans produce approximately 300 to 400 mg bilirubin per 24 hour. Approximately 80% of this is derived from hemoglobin of senescent erythrocytes that have been removed from the circulation and destroyed, and the remainder originates from the catabolism of other heme proteins. Under physiological conditions red cell hemoglobin is converted to bilirubin in tissues such as the spleen, liver, bone and macrophage. In subcutaneous bruises, tissue macrophages engulf the extravasated red cells and convert their hemoglobin to bile pigment, which accounts for the characteristic progressive color change from dark purple (heme) to blue-green (biliverdin) and eventually to yellow (bilirubin). In hemolytic states, the sinusoidal cells of the spleen and liver (Kupffer cells) play a major role in the sequestration and degradation of immunologically or chemically damaged erythrocyte. On the other hand, in intravascular hemolysis, when hemoglobin is dissolved in the plasma, the hepatic parenchymal cells and the renal tubules are important sites for the conversion of hemoglobin-heme to bile pigment. The fissure of the heme ring occurs stereo-selectively at the a-meso bridge so that all the bilirubin formed has the IXa configuration. In the course of this ring cleavage, the a-meso carbon atom is converted quantitatively to carbon monoxide.

Knowledge framework

1. Why should be a bilirubin test be ordered?
2. Population: Neonate, Pediatric, Adult.
3. Natural biologic pigment
4. Benign or toxic
5. Indicator of normal physiology or pathology.
6. What are normal values?
7. Types of bilirubin?
    a. Total
    b. Unbound
    c. Unconjugated
    d. Conjugated
    e. Photo-isomers
    f. Urobilin and stercobilin
8. Source: heme catabolism
9. Metabolism of bilirubin
    a. Hemolysis
    b. Stepwise process

    c. Conjugation
    d. Elimination
    e. Genetics of metabolism and consequent disorders
10. Techniques to Assay
    a. Pigment color
    b. Chemical assay
    c. Spectrophotometry
    d. HPLC
    e. Site: blood, plasma, serum, amniotic fluid, urine, fecal, others
11. Pigment photosensitivity impact
12. Accuracy and precision of assay
13. Comparison of common devices
14. Standardization of the "gold standard" for plasma/serum bilirubin.
15. Algorithms for diseases

1. Hyperbilirubinemia (thresholds?)
    a. Neonate (by age in hours)
    b. Post-neonatal (days/weeks)
    c. Adulthood
2. Hypobilirubinemia:
    a. Low bilirubinemia (threshold?)
    b. Anbilirubinemia: does it exist?
3. Bilirubin-albumin binding and the associated consequences.

Reviewer B

Comment 1: This is a somewhat antiquated review of bilirubin disorders. It contains mostly established information with little new information regarding bilirubin.

Reply 1: Thank you for your time looking at the article. This invited article's purpose was to provide an online open access and modern approach to the laboratory investigation of an aberrant human plasma bilirubin result for the jobbing general clinician rather than to be a critical appraisal of new developments but indeed we haven't aimed to be old fashioned. Such algorithms do exist in textbooks and are very useful to the trainee doctors or generalists who are not familiar with how to approach certain blood abnormalities. The problem with a lot of the published algorithms are that some of the textbook in which they were contained are out of print and unavailable to the new trainees coming through but in addition a lot of them are out of date regarding the laboratory tests currently available (and this will continue to change no doubt as genotyping, mass spec and biomarkers become more affordable and universally available). We aimed to write an algorithm that could be used by generalists now anywhere in the world and therefore have deliberately limited ourselves to tests that are widely available and affordable rather than suggests new diagnostic modalities that may not be accessible to many.

Comment 2: For example, the authors write (line 185) that "low bilirubin is not a clinical concern because firstly normal ranges will often include zero but also because it does not cause any

pathological symptoms or illness." This statement is patently false as several studies have documented the pathological effects of low bilirubin (PMID: 33284088; PMID: 36828710; PMID: 37445792; PMID: 23323254; PMID: 29447261). This section needs to be modified according to the significant amount of literature on this subject.

Reply 2: The aim of this paper is for a current doctor to use to look up what tests are currently widely available now when investigating a bilirubin disturbance and whilst we have talked about diseases here to a limited extent the point is to help a clinician with a patient a high bilirubin to confidently identify the cause without doing incorrect tests or missing important ones out. As yet we see no clinical indication to investigate low bilirubin as none of the papers you have kindly suggested actually tell us why the bilirubin might be low and what we should do about it and therefore at this current point in modern medicine we feel we cannot justify unnecessary health resources and patient anxiety to investigate further. In these series we have actually deliberately shied away from discussing conditions associated with low or high biomarkers but deliberately limited ourselves to 'what other tests might you consider'.

Regarding PMID papers 33284088 – I found this review difficult to follow but essentially we can dismiss bilirubin's role in redox states as this area of study, many times, has been disproven (i.e. that by giving antioxidants we can change patient outcomes) and should have no place as a hypothesis until we know we can alter the status in current literature. The argument that bilirubin is a hormone is not convincing and whilst certainly it would make sense that a metabolite has feedback mechanisms in its metabolic pathway this does not, in our mind, meet the definition of a hormone. Bilirubin nanoparticles are not part of accepted medical treatment yet as evidence is lacking in humans and this is not the subject of the current article, which is what disease should I look for when I find bilirubin abnormalities in human blood results. The problem with association studies and hyperbilirubinemia is that there may be associations but that is not a the same as proof of cause and we do not know of any cardiology guideline that would suggest we should do screening for cardiovascular diseases in those with low bilirubin

Pmid 284088 is another review detailing the same arguments

PMID 37445792 data on bilirubin is in medians, ranges etc. Suggesting data are not normally distributed, ranges completely cross over and I think they have overstated their findings. The difference in bilirubin levels here are unlikely to be clinically significant and are well within normal ranges. Also outside the scope of this article

PMID 23323254 is another association study I am afraid, not a cause nor can it have a protective role attributed to it from this type of study. It is also interesting to note what they have missed out in this comparison e.g. no other source of bilirubin e.g. diet, red cell count, haemolysis, drugs etc are included.

PMID 29447261, like a previous paper, interesting but again an association study. Essentially our paper is not about what benefits bilirubin may or may not have but how to work out why it is out of the normal range.

Comment 3: Lines 311-312- What is meant by "only using 1% of its full potential?" Potential for what?

Reply 3: thank you we have added metabolic to this sentence

Reviewer C

Thank you for taking the time to read our paper and your comments to help us improve it.

Comment 1: "…in diagnosing the cause of various plasma abnormalities", I would suggest adding "bilirubin plasma abnormalities", when writing "plasma abnormalities" only it includes a very wide range of possibilities, please be specific.

Reply 1: Thanks, I have altered in a slightly different way as we are submitting several articles, all on different plasma abnormalities, and this one just happens to be on bilirubin so I hope I have made that clearer

Comment 2: Key content and Findings are empty

Reply 2: Thanks so much for spotting, I have rectified.

Comment 3: Conclusion of the abstract

Reply 3: you are quite right, we missed this, thanks for pointing out, now more specific

Comment 4: key words

Reply 4: we have added abnormalities. I have not removed high or low as I want people to be able to find our paper even if their English is not terrific, as this paper, if accepted, will be open access, and therefore we wanted to keep the terms as simple as possible

Comment 5: introduction about bilirubin metabolism

Reply 5: I have opted for your clear statement as we have figures later to show what happens so best to keep the prose short and not confusing, thanks for pointing out.

Comment 6: methods

Reply 6: Thanks for spotting this, I have checked and changed it to May, the abstract was correct in this instance.

Comment 7: water insoluble bilirubin

Reply 7: thanks I have clarified. We bring in the term "indirect" later so that anyone who does not understand the distinction or why that word is used can hopefully follow.

Comment 8: change "in health"

Reply 8: changed to "in physiological conditions"

Comment 9: full stop before however

Reply 9: done, thanks.

Comment 10: alternatives methods

Reply 10: changed to alternative methods

Comment 11: This indicates

Reply 11: this likely indicates

Comment 12: effected

Reply 12: affected

Comment 13: phenobarbital as treatment

Reply 13: ensured it was clear that only in selected cases

Comment 14: regarding coherence in using ALT and AST.

Reply 14: I am not sure what you mean here. I have gone through to check that I have explained what an abbreviation is before using it and I think they are correct throughout, sorry if I have missed something, but we had already defined AST earlier.

Comment 15: too many ideas within the very same statement, please rewrite

Reply 15: I think this is about child to adult liver and reachign full potential???

Comment 16: too many ideas within the very same statement, please rewrite

Reply 16: yes we had missed out a few essential words to make this sentence work, thanks for spotting.

Of note the nomenclature of fatty liver disease has changed so we have also taken the liberty to update the nomenclature throughout.