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Reviewer A

Comment 1: Did the patient have a history (personal or family) of diabetes?
Reply 1: The patient did not have neither a personal nor family history of diabetes.

Comment 2: Is it routine to have HbA1c as part of the screening package? Afterall fasting glucose was quite normal.

Reply 2: Measurement of HbA1c for the diagnosis of diabetes was recommended by the Australian Diabetes Society in 2012 and the Australian Federal Government began public funding for HbA1c in May 2014 in asymptomatic patients considered to be at high risk according to the Australia Type 2 Diabetes Risk Assessment Tool (AUSDRISK).
It is routine practice that HbA1c is requested simultaneously with a fasting blood glucose, in case of an abnormal result. In which case, diagnosis of diabetes can be made more timely. If either the fasting FPG or HbA1c falls within the diabetic range, then that test gets repeated to confirm diabetes. An oral glucose tolerance test (OGTT) would be order if FPG is between 5.5-6.9 mmol/L or if screening for gestational diabetes.

Comment 3: Sentence in line 58 would be more appropriate at line 67.

Reply 3: Noted with thanks.

Changes in the text:
Abstract, Line 68: ‘ An interference was detected in the measurement of HbA1c and the sample was sent to a reference laboratory for confirmation’ is now on line 71 of the abstract.

Comment 4: Some additional pointers in the discussion would be helpful and appreciated by readers:
- How frequent does you lab encounter Hb variants during HbA1c testing?
- Do you have an algorithm to alert technologists to the presence of variants in the sample (eg HbA1c < 4% or > 15%)? A short paragraph on this in the discussion would be useful.
Some more updated references would be nice especially to underscore the fact that labs can still encounter unknown or uncommon Hb variants in their practice.

Reply 4: As suggested, we have now included the detection rate of haemoglobin variants seen in our hospital. In terms of an algorithm for laboratory staff, we have recently (within the last 3 months) implemented a statewide testing and reporting algorithm for laboratory scientists and technician to follow. This algorithm is not yet published and so has not been included in this report.

Changes to the text:
Discussion, line 199:
'Incidence of haemoglobinopathies.
Our laboratory is located in a large teaching hospital, providing specialised health care service for more than 700,000 people living in the centre and inner west of Sydney. In addition to our acute services, we are also a reference laboratory and often receive test referrals for HbA1c confirmation from private pathology laboratories. Our laboratory has a haemoglobin variant detection rate of 2.0% per year (December 2019) and this has progressively increased due to the implementation of a state-wide harmonisation of HbA1c testing and reporting (not published).'

Reviewer B

Comment 1: Suggest to report the HbA1c to a single decimal point.
Reply 1: HbA1c in the abstract is now reported to a single decimal place.
Changes to the text:
Abstract, line 16: '…abnormality is a HbA1c of 53.9% (566 mmol/mol) on the Biorad D-100…'

Comment 2: Suggest to provide HbA1c measurements for Siemens DCA Vantage, Abbott Architect and Sebia Capillary 2 in parenthesis.
Reply 2: Noted with thanks.
Changes to the text:
Abstract, line 16 – 19: ' HbA1c was further measured on the Siemens DCA Vantage (3.6% or 15 mmol/mol), Abbott Architect (3.8% or 81 mmol/mol) and Sebia Capillarys 2 Flexi Piercing (5.4% or 36mmol/mol), which gave discordant results.'

Comment 3: Some of the names of the instruments appears inconsistent in the Abstract and the main text – suggest to standardise.
Reply: Suggestion was noted.
Changes to the text:
Results, line 61-63: ' However, when analysed by HbA1c capillary electrophoresis (Sebia Capillarys 2 Flexi Piercing)…'
Results, line 75 : '… electrophoresis instrument (Sebia Minicap Flexi Piercing)…'

Comment 4: A HbA1c of 53.9% is supraphysiological and should also rouse suspicion of interference, other than a discrepant result between D100 and Capillarys.
Reply 4: Noted with thanks

Comment 5: Please mention that D100 is a cation-exchange HPLC method – this is important since it is liable to variant Hb interference compared to boronate HPLC, which is generally thought to be resilient against variant Hb interference.
Reply 5: Noted and suggested changes is now made to the report.
Changes to the text:
Results, line 60: ‘ Cation exchange high performance liquid chromatography (HPLC)…’

Comment 6: Please provide formal annotation of the mutation, i.e. HBB c.8A>C (p.His3Pro)
Reply 6: Noted with thanks. Suggested changes is now made to the report.
Changes to the text:
Diagnosis of Haemoglobin Agrigente, line 103: ‘ A HBB.8A>C (pHis2Pro) mutation was detected…’
Discussion, line 121-122: ‘... a point mutation at codon 2 of the haemoglobin beta chain molecule resulting in the translation of proline instead of histidine (Figure 6).’

Comment 7: Typo: “sometimes above the physiologically range” to “sometimes above the **physiological range”**
Reply 7: Noted with thanks
Changes to the text:
Discussion, line 157: ‘Consequently, the % HbA1c is higher, sometimes above the physiological range.’

Comment 8: Typo: “a ‘normal’ HbA1c peak in the electropherogram (Figure 2) contradict this” to “a ‘normal’ HbA1c peak in the electropherogram (Figure 3) contradict this”.
Reply 8: The reference to the electropherogram in figure 2 is correct. Figure 3 is a chromatogram produced from the Biorad Variant II. As another reviewer had pointed out, the legend/caption for figure 2 was lost in the formatting.
Changes to the text: Reformatted all images and inserted image caption in results section, line 87 : ‘ Figure 2: Electropherogram from the Sebia Capillarys 2 Flexi Piercing showing a normal HbA1c result of 5.4% and a high HbA0 peak of 91.0%.’

Comment 9: Perhaps the authors can suggest general alternate means of diagnosing (fasting glucose or OGTT) and monitoring (home glucose monitoring, fructosamine) in the presence of analytically unreliable HbA1c.

Reply 9: An addition of fasting blood glucose and oral glucose tolerance tests has been added to line 218 of the conclusion. Exclusion of other markers such as fructosamine and glycated albumin has been deliberately omitted as these are yet to be endorsed in clinical guidelines as alternative biomarkers for diagnosis of diabetes.
Changes to the text:
Conclusion, line 214 : '..diabetes and measurement of alternative markers (fasting glucose or oral glucose tolerance test) may…'

Reviewer C

Comment 1: Tracked changes are still visible on the page numbers

Reply 1: Noted with thanks.
Changes to the text: Tracked changes in the page numbers were noted and this is no longer visible in version 2.

Comment 2: All Biochemistry results are reported in a table, including normal ranges. As all except one were unremarkable and within the normal ranges, it seems unnecessary to report so much information. At the very least the normal ranges column could be excluded, alternatively, the biochemistry results could be summarised in a line or 2 of text as was reported for the haematology profile.
Reply: For standardised analytes such as eletrolytes, variations in the reference intervals are small. However, for other analytes such as AST, ALT, lipid profiles, there is still considerable difference reported world-wide. The intention to include the reference intervals in the report was to allow readers to compare the results against the reference values adopted by our laboratory, as these might be slightly different to theirs.
Nonetheless, the reviewer's comments and suggestion is noted and the table has now been removed and replaced with text.

Changes to the text:
Results, line 54 – 56: ' The biochemistry results were unremarkable except for a mildly elevated non-HDL-cholesterol of 2.9 mmol/L (<2.5 mmol/L). Fasting blood glucose was also normal at 4.9 mmol/L (3.0-5.4 mmol/L).'

Comment 3: The authors rightly recommend an OGTT in this case for diagnosis of diabetes rather than HbA1c, was an OGTT performed? This would be interesting information to be presented.
Reply 3: An OGTT was not performed at the time of presentation nor at subsequent follow up when being managed by the geneticist as it was not deemed to be relevant given the normal fasting blood glucose. It has been communicated to the general

practitioner that for future assessment of glycemic status, that an OGTT is the best test for this patient.

Comment 4: In the discussion, the authors discuss the similarity in HPLC results for Hb Agrigente as well as HB Marseille and Hb Long-Island Marseille. Are the discrepancies seen in this case report for the other methods (capillary electrophoresis, latex agglutination, enzymatic) common to Hb Marseille and Hb Long-Island Marseille?

Reply 4:
Yes. In other published cases of Hb Marseilles and Hb Long-Island Marseilles, only the 1 (reported by Florkowski et al 2003) found similar discrepant results on other HbA1c methods. In their report, they had measured HbA1c ranging from 45% (468 mmol/mol) by cation- exchange HPLC, 2.8% (7.1 mmol/mol) by immunoassay Bayer DCA 2000 and 4.6% (25 mmol/mol) by affinity chromatography.
The 2 other sources referenced in the paper were back in 1989 and 1984, when availabilities of commercial assays were limited.

Comment 5: The final line of the conclusion- HPLC falsely high while CE, enzymatic and latex agglutination are falsely low with HB Agrigente. Is this true for the results presented? The CE value was considered a normal HbA1c value in line 63 and indeed a value of 5.4% would fit well with the reported FPG of 4.9mmol/L.
Reply 5:
Although the CE result of 5.4% is considered to be normal, the HbA1c is calculated as a ratio of the HbA1c peak over the HbA peak. The HbA peak on the CE was 97.8% (Figure 2). Substituting 97.8% in the denominator gives a ‘normal’ HbA1c peak. However, if the HbA0 peak from the Biorad D-100 and Biorad Variant II is used (41.46 and 47.8% respectively) (Figure 1 and 3) and if the assumption is correct in that the difference in the HbA0 peak is due the variant, the HbA1c is expected to be higher.

The relationship between fasting plasma glucose and HbA1c has been contentious. Results from DCCT Trial (Diabetes care. 2002 Feb 1;25(2):275-8), suggests there is an overall linear relationship, where FPG can be predicted using the regression equation : FPG =1.98*HbA1c (%) – 4.29, however, the authors of the study cautioned the use of FPG alone as it underestimates HbA1c. We know that an isolated FPG cannot (and should not be used) to make an assessment of average exposure to glucose due to the large intra-individual variation of glucose. So while the ‘normal’ HbA1c result fits with the FPG of 4.9 mmol/L, there is lacking evidence of the relationship in the presence of a haemoglobin variant.

Changes to the text:
Additional text to clarify the reviewer's comments.
Discussion, line 174-177 : '… the Hb Agrigente has co-eluted with the Hb A in the CE method. Since HbA1c is calculated as a ratio of the HbA1c peak over the HbA, a lower HbA as the denominator (41.46%) will give a higher HbA1c result, and conversely, high denominator (91%) results in a lower HbA1c.'

Comment 6: I am slightly confused by the figures- Fig 1 is the D100 chromoatogram, Fig 2 is the Variant II chromatogram, between the 2 is an electrophoresis trace but no legend- this may be a formatting issue

Reply 7: Noted with thanks. Images are now reformatted and include captions/ legends.

Reviewer D

Comment 1: Hemoglobinopathies are a common public health problem worldwide, It is estimated that around 7% of the world's population are carriers of hemoglobinopathies. Originally they were mainly found in tropical areas of subSahara Africa, Asia and in the Mediterranean area, but with migration these diseases are now found globally.
In a general Laboratory the incidentally detection Hb variants in the course of HbA1c analysis is a common finding. HbA1c is used to monitor the glycemic control of patients with diabetes mellitus and to assess the risk of development of diabetic complications. Interference in HbA1c measurement can lead to misdagnosis and incorrect treatment.
For most variants, interference was seen with 1 or more of the ion-exchange methods, but the effect of different Hb variants in the diverse analytical methods are diverse
The authors report the effect of this rare variant on different methods
: HPLC Biorad D-100, CE Capillarys 2 Flexi Piercing and chemical methods (Siemens DCA Vantage, Abbott Architect) , which gave discordant results

The case is interesting, the results well presented and the rationale of the effect on Hb variants on HbA1c analysis well documented.

Reply 1: Noted with thanks.