




Establishment of haemoglobin A₂ reference intervals in Pretoria, South Africa: A retrospective secondary data analysis

**Authors:**

Cailin Nieuwenhuizen^{1,2} 
Tshiphiri Netshidzivhani^{1,2} 
Johan Potgieter^{1,2} 

Affiliations:

¹Department of Haematology,
Faculty of Health Sciences,
University of Pretoria,
Pretoria, South Africa

²Department of Haematology,
Tshwane Academic Division,
National Health Laboratory
Service, Pretoria, South Africa

Corresponding author:

Cailin Nieuwenhuizen,
coetzeecailin@gmail.com

Dates:

Received: 28 Jan. 2022

Accepted: 20 Apr. 2022

Published: 12 Aug. 2022

How to cite this article:

Nieuwenhuizen C,
Netshidzivhani T, Potgieter J.
Establishment of haemoglobin
A₂ reference intervals in
Pretoria, South Africa: A
retrospective secondary data
analysis. Afr J Lab Med.
2022;11(1), a1841. <https://doi.org/10.4102/ajlm.v11i1.1841>

Copyright:

© 2022. The Authors.
Licensee: AOSIS. This work
is licensed under the
Creative Commons
Attribution License.

Read online:

Scan this QR
code with your
smart phone or
mobile device
to read online.

Background: Haemoglobinopathies are one of the most common inherited diseases worldwide. Quantification of haemoglobin A₂ is necessary for the diagnosis of the beta thalassaemia trait. In this context, it is important to have a reliable reference interval for haemoglobin A₂ and a local reference range for South Africa has not been established.

Objective: This study aimed to establish reference intervals for haemoglobin A₂ using stored patient laboratory data.

Methods: This descriptive study used retrospective data to evaluate haemoglobin A₂ levels determined using high-performance liquid chromatography at the National Health Laboratory Service haematology laboratory in Pretoria, South Africa. All tests performed from 01 October 2012 to 31 December 2020 were screened for inclusion; of these, 144 patients' data met the selection criteria. The reference interval was calculated using descriptive statistics (mean and standard deviation) with a 95% confidence interval.

Results: Analysed data from enrolled patients showed a normal distribution. The mean age of the patients was 40 years (range: 3–84 years). The reference interval for haemoglobin A₂ calculated from this data was 2.3% – 3.6%. The minimum haemoglobin A₂ was 2.3% and the maximum was 3.9% with a mean of 2.95% and a standard deviation of 0.357%.

Conclusion: A normal reference interval has been established for the population served by the laboratory that will assist with accurate diagnosis of the beta thalassaemia trait. This reference interval may also be useful to other laboratories that employ the same technology, especially smaller laboratories where obtaining a sufficiently large number of normal controls may be challenging.

Keywords: Haemoglobin A₂; reference range; reference interval; beta thalassaemia; high-performance liquid chromatography.

Introduction

Reference intervals are an integral part of any functioning clinical laboratory. Reference intervals are often the endpoint used by clinicians in the management of patients and in clinical decision-making. It has been estimated that 80% of medical decisions are made from laboratory results.¹ The value of the results produced by a laboratory is largely influenced by the quality of the reference interval used in the interpretation of that result.²

An example of where reference intervals are of diagnostic utility is in the diagnosis of the beta thalassaemia trait. The increase in haemoglobin A₂ (HbA₂) level is the most important laboratory parameter for the identification of carriers of beta thalassaemia, and is considered diagnostic in the appropriate clinical context.³ Accurate quantification of HbA₂ in the haematology laboratory is therefore essential to allow for routine diagnosis of the beta thalassaemia trait.⁴ This highlights the value of reliable reference intervals.

Beta thalassaemia is inherited in an autosomal recessive pattern and the beta thalassaemia trait is estimated to have a prevalence of 1.5% worldwide, affecting approximately 80–90 million people.⁵ Africa has a considerable disease burden in terms of haemoglobinopathies and in particular beta thalassaemia with 1520 conceptions affected annually, Western Africa accounting for the majority of cases.⁶

Reference intervals are established through a validation process with a statistically adequate number, ideally 120, of healthy reference individuals.⁷ This needs to be done for all reagents and instrument combinations. However, even the Clinical and Laboratory Standards Institute guidelines recognise that this is not feasible for many laboratories and finding a cohort of 120 healthy individuals is not feasible for every test.⁸ Alternatively, reference intervals may be verified, with only 20 samples needed, or transferred provided that the analytic system and the test population are comparable.⁸ However, verification and transfer of reference intervals is not ideal and these methods have their own disadvantages.⁸

Every laboratory that performs HbA₂ testing is responsible for establishing its own reference interval. This is done by quantifying the HbA₂ percentage in a cohort of healthy adults who do not have iron deficiency or the thalassaemia trait.³ The reference interval is a range that should be calculated including individuals with characteristics that are comparable to the reference group so that the reference interval can be correctly applied to the population serviced by the laboratory.⁷ Given the importance of HbA₂ reference ranges, considerable work has been done internationally on normal reference intervals for HbA₂. However, there is a paucity of literature from Africa with no published reference intervals for HbA₂.

In keeping with good laboratory practice, a need was identified to determine HbA₂ reference intervals in a local African population for the Department of Haematology of the National Health Laboratory Service (NHLS), Tshwane Academic Division (TAD), Pretoria, South Africa. Using a sufficient number of results from medical records would provide a healthy cohort for establishing a reference interval without the need to recruit healthy individuals. This is particularly useful for a test that is not routinely requested and only offered by specialised laboratories. In order to establish a reference interval for HbA₂ without the limitations inherent to traditional methods of establishing reference intervals, we made use of previously reported normal high-performance liquid chromatography (HPLC) results from the NHLS Department of Haematology.

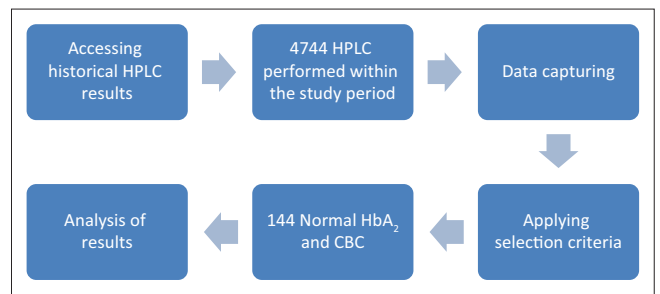
Methods

Ethical considerations

Approval was obtained from the Academic Affairs, Research and Quality Assurance Department of the NHLS. The study protocol was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (protocol number 518/2019). Patient consent was waived by the ethics committee as the study was conducted using historical data. Participants' information was treated with confidentiality. Each participant was allocated a unique study number to ensure anonymity.

Study design and setting

This was a descriptive study using retrospective data from blood test results stored in the Laboratory Information



HPLC, high-performance liquid chromatography; CBC, complete blood counts; Hb, haemoglobin.

FIGURE 1: Flow diagram of the study design, Pretoria, South Africa, 01 October 2012 – 31 December 2020.

System of the NHLS at the Department of Haematology, TAD, and Vermaak and Partners Path Care Pathology group.

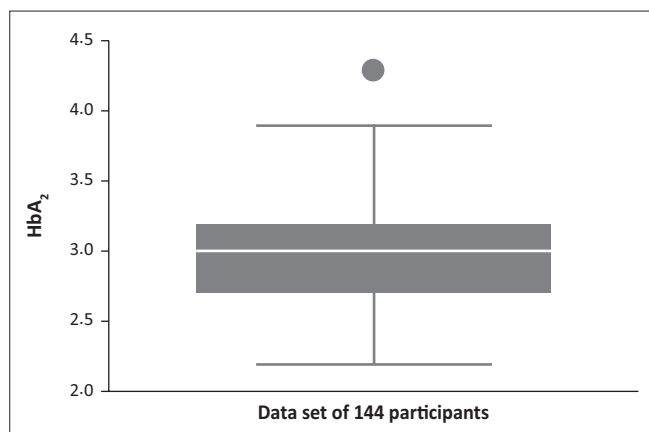
Study population and sampling strategy

The study population comprised patients investigated by TAD, NHLS, and included patients from both high-income and low-income settings and represented a variety of ethnicities. All HPLC results of tests performed at the NHLS TAD from 01 October 2012 to 31 December 2020 were screened for inclusion (Figure 1). The following selection criteria were applied: HPLC performed within the study period and normal HPLC results of patients who also had a corresponding complete blood counts (CBC) were included in this study. Results of individual patients were excluded if they were aged two years or younger, were anaemic (haemoglobin < 12.5 g/dL), had a mean cell volume < 75 fL or > 100 fL, had a mean corpuscular haemoglobin below 27 pg, had a red cell distribution width of > 15% or had variant haemoglobin, inclusive of haemoglobin S, detected in their haemoglobin electrophoretic result. Participants that did not meet the selection criteria or met the exclusion criteria were excluded from the study and subsequent analysis. A minimum sample size of 120 patient is required in order to establish a reference interval.⁸

Data collection

Apart from HPLC reports, corresponding results of CBC, thyroid stimulating hormone, serum folate, serum vitamin B12 and ferritin level, performed within a week of the taking of the HPLC specimen, were evaluated. Only normal HPLC results were included in the analysis.

The HbA₂ levels were determined in the haematology laboratory using the HPLC D10 instrument (Bio-Rad® Laboratories, Hercules, California, United States). The D10 instrument uses ion-exchange HPLC technology to analyse haemoglobin. All analyses were performed according to good laboratory practice and the manufacturer's recommendations. The haematology laboratory at the NHLS TAD has been accredited by the South African National Accreditation System. Appropriate controls and calibrators were used throughout the study. The Lyphochek® Hemoglobin A₂ Control Level 1 and 2 (Bio-Rad Laboratories,



HbA₂, haemoglobin A₂.

FIGURE 2: Box-plot of haemoglobin A₂ distribution, Pretoria, South Africa, 01 October 2012 – 31 December 2020.

Irvine, California, United States) were used as controls and the D10™ Dual Program HbA₂/F/A_{1C} Calibrator/Diluent Set (Bio-Rad® Laboratories, Hercules, California, United States) was used for calibration.

The blood test results listed above were captured in a Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States) spreadsheet. Exclusion criteria were applied after which 144 samples with normal HbA₂ levels were identified, and these were used for calculation of reference intervals.

Data analysis

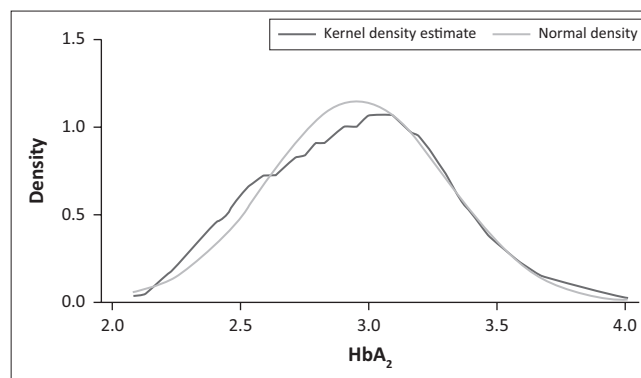
The descriptive statistics mean, median, standard deviation and inter-quartile range, with 95% confidence interval, and the 2.5th and 97.5th percentiles were used to describe the continuous variables such as HbA₂ levels. The two-sample *t*-test, or non-parametric alternative were used to compare group means. Pearson's correlation was used to measure correlations between HbA₂ levels and age, as well as other continuous variables such as CBC parameters. Tests were evaluated at 5% level of significance. All analyses were done using Stata 15 (StataCorp, College Station, Texas, United States) software.

Results

The mean age of the 144 patients included in this data analysis was 40 years (range 3–84 years). The study population comprised 67 female and 77 male patients. After a single outlier of 4.3% was excluded from the analysis, the mean HbA₂ value was 2.95% with the range between 2.2% and 3.9%. The standard deviation was 0.357% (Figure 2).

Data were normally distributed as indicated in the Kernel density estimation (Figure 3). The HbA₂ reference interval established from this data set was 2.3% – 3.6%.

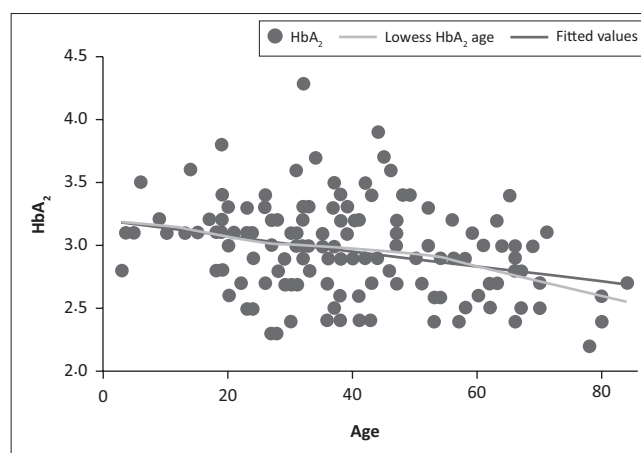
A sex comparison was performed by *t*-test to compare the mean HbA₂ of male patients to that of female patients in an attempt to identify possible bias. No significant difference



Note: Kernel = epanechnikov, bandwidth = 0.1157.

HbA₂, haemoglobin A₂.

FIGURE 3: Kernel density estimate demonstrating the normal distribution of data, Pretoria, South Africa, 01 October 2012 – 31 December 2020.



HbA₂, haemoglobin A₂.

FIGURE 4: Scatterplot of haemoglobin A₂ and age distribution, Pretoria, South Africa, 01 October 2012 – 31 December 2020.

was found ($p = 0.1328$). The correlation between age and HbA₂ was also assessed. A trend towards lower HbA₂ values with increased age was appreciated (Figure 4), with a Pearson correlation coefficient of -0.2826 .

Discussion

The HbA₂ reference interval established from this data set was 2.3% – 3.6%. Normal reference intervals for HbA₂ have been published for other patient populations. A study performed at the Leiden University in the Netherlands, using the Variant Classic HPLC (Bio-Rad®) platform, reported a HbA₂ reference interval of 2.3% – 3.5%.³ Despite the difference in study population, the reference interval determined in this current study is comparable to that of the Leiden group which used a similar method, that is, HPLC (Bio-Rad®) technology. Han et al. reported a reference interval of 2.3% – 3.1% for HbA₂ in a Chinese population of reproductive age.⁹ However, these investigators used a CAPILLARYS2 instrument (Sebia, France) to generate their data.⁹

Most evidence suggests that HbA₂ of $> 4\%$ is indicative of beta thalassaemia trait with almost 100% sensitivity and 90% specificity.¹⁰ A grey zone between 3.1 and 3.9 is generally

accepted as reported in a comprehensive review.¹¹ This often poses a diagnostic challenge. Studies have been conducted to identify the presence of mutations in these individuals.^{10,11,12,13,14} Giambona et al. found that 80% of patients in this group in an Italian population were negative for molecular defects, and the most significant finding was the presence of beta thalassaemia gene mutations found mostly in patients with HbA₂ in the region of 3.5–3.9 and mean cell volume < 80 fL.¹¹

The upper limit of the normal reference interval established in this current study does fall within the previously described 'grey zone'. However, in the presence of a normal CBC, the possibility of an underlying carrier state in these patients remains small. This highlights the importance of interpreting HbA₂ within the clinical context, taking into consideration the CBC parameters and iron studies.

There appears to be a weak association between a decreasing HbA₂ value and increasing age; this was true even when looking at subsets of age and when excluding patients aged 70 years or older. Although this trend was seen, when calculating the reference interval by age, the reference interval remained 2.3–3.6 when rounded to one decimal place. Therefore, this was not a significant finding.

In this current study we used data available on the laboratory information system in order to establish a reference interval. This represents a novel approach in our setting. Data mining is emerging as an alternative to the traditional direct a priori method. Data mining makes use of electronic data records and statistical techniques to determine the healthy population within a data set in order to establish a reference interval.¹⁵ The electronic data records may be obtained from insurance claims, electronic health records as well as a variety of other sources.¹⁶ All sources require data capturing platforms which allow for database management in order to deal with the enormous volume of information currently being generated as well as the complexity of analysing and interpreting the data.

The analytical methods that have been employed to establish reference intervals include The Hoffmann method, Bhattacharya method and more recently the Truncated Maximum Likelihood method.¹⁵ The Truncated Maximum Likelihood method employs complex statistical algorithms that make use of maximum likelihood estimation and require 4000 data points in order to establish a robust reference interval.¹⁵ Currently there is still hesitancy regarding the use of indirect methods but it is likely to be used to establish many reference intervals in the future. Indirect methods may be particularly useful for tests that are not routinely performed as screening tests in the healthy population.¹⁷ Data mining has many advantages: it is less costly as the blood results of large cohorts of individuals are readily available, it is faster, and it can even be considered more ethical.¹⁵ A study conducted by Katayev et al. showed that reference intervals could be reliably and reproducibly established using data mining. Reference intervals were calculated for eight analytes and were found to be comparable to already accepted published reference intervals.¹⁷ Although our study was

small and the data were captured manually, it does highlight the potential of using laboratory information records to glean valuable data with relatively low cost and fewer limitations than are inherent to establishing reference intervals in the conventional manner by direct population sampling.

Limitations

One of the limitations of this study was the inability to exclude all confounding factors. Although our study could be improved on by only including patients who have been tested for all confounding factors for HbA₂, this would only be feasible if a large number of data sets were included. Another option would be to use a larger cohort and an algorithm that does not require the exclusion of all confounders. A normal CBC was used as a surrogate marker of a nutritional deficiency, and this remains a limitation. It should be noted that all published confounders are not routinely considered when establishing reference intervals for HbA₂ or when interpreting HPLC results.

Conclusion

A normal HbA₂ reference interval of 2.3% – 3.6% has been established for the population served by the laboratory. This will assist with the interpretation of results. The reference interval could also be useful to other laboratories, especially smaller laboratories where obtaining a sufficiently large number of normal controls may be challenging. The inability to exclude all of the confounding factors that influence HbA₂ levels needs further research.

Acknowledgements

We thank Prof. R. Pool for his insight and guidance and Dr D. Toi for his assistance in proofreading the article.

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

J.P. and T.N. conceived of the presented idea. C.N. developed the theory and performed the computations. J.P., C.N. and T.N. verified the analytical methods. J.P. supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

Sources of support

Funding has been provided by the University of Pretoria Development Fund.

Data availability

The data that support the findings of this study are available on request from the corresponding author, C.N.

Disclaimer

The views expressed in the submitted article are the authors' own and not an official position of the institution.

References

1. Castellone DD. Establishing reference intervals in the coagulation laboratory. *Int J Lab Hematol*. 2017;39(S1):121–127. <https://doi.org/10.1111/ijlh.12661>
2. Jones G, Barker A. Reference intervals. *Clin Biochem Rev*. 2008;29(Suppl 1):S93–S97.
3. Mosca A, Paleari R, Ivaldi G, Galanello R, Giordano P. The role of haemoglobin A2 testing in the diagnosis of thalassaemias and related haemoglobinopathies. *J Clin Pathol*. 2009;62(1):13–17. <https://doi.org/10.1136/jcp.2008.056945>
4. Stephens AD, Angastiniotis M, Baysal E, et al. ICSH recommendations for the measurement of haemoglobin A₂. *Int J Lab Hematol*. 2012;34(1):1–13. <https://doi.org/10.1111/j.1751-553X.2011.01368.x>
5. Galanello R, Origa R. Beta-thalassemia. *Orphanet J Rare Dis*. 2010;5(1):11. <https://doi.org/10.1186/1750-1172-5-11>
6. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ*. 2008;86(6):480–487. <https://doi.org/10.2471/BLT.06.036673>
7. Katayev A, Balciza C, Seccombe DW. Establishing reference intervals for clinical laboratory test results: Is there a better way? *Am J Clin Pathol*. 2010;133(2):180–186. <https://doi.org/10.1309/AJCPNSBMTSF1CDYP>
8. Boyd J. Defining, establishing, and verifying reference intervals in the clinical laboratory: Approved guidelines. Document C28-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.
9. Han WP, Huang L, Li YY, et al. Reference intervals for HbA₂ and HbF and cut-off value of HbA₂ for β -thalassemia carrier screening in a Guizhou population of reproductive age. *Clin Biochem*. 2019;65:24–28. <https://doi.org/10.1016/j.clinbiochem.2018.11.007>
10. Greene DN, Vaughn CP, Crews BO, Agarwal AM. Advances in detection of hemoglobinopathies. *Clin Chim Acta*. 2015;439:50–57. <https://doi.org/10.1016/j.cca.2014.10.006>
11. Giambona A, Passarello C, Renda D, Maggio A. The significance of the hemoglobin A₂ value in screening for hemoglobinopathies. *Clin Biochem*. 2009;42(18):1786–1796. <https://doi.org/10.1016/j.clinbiochem.2009.06.026>
12. Giambona A, Passarello C, Vinciguerra M, et al. Significance of borderline hemoglobin A₂ values in an Italian population with a high prevalence of beta-thalassemia. *Haematologica*. 2008;93(9):1380–1384.
13. Tamagnini GP, Gonçalves P, Ribeiro ML, et al. Beta-thalassemia mutations in the Portuguese; high frequencies of two alleles in restricted populations. *Hemoglobin*. 1993;17(1):31–40.
14. Efreinov DG, Dimovski AJ, Baysal E, et al. Possible factors influencing the haemoglobin and fetal haemoglobin levels in patients with beta-thalassaemia due to a homozygosity for the IVS-1-6 (T→C) mutation. *British journal of haematology*. 1994;86(4):824–830.
15. Bohn MK, Adeli K. Application of the TML method to big data analytics and reference interval harmonization. *J Lab Med*. 2021;45(2):79–85. <https://doi.org/10.1515/labmed-2020-0133>
16. Yang J, Li Y, Liu Q, et al. Brief introduction of medical database and data mining technology in big data era. *J Evid Based Med*. 2020;13(1):57–69. <https://doi.org/10.1111/jebm.12373>
17. Katayev A, Fleming JK, Luo D, Fisher AH, Sharp TM. Reference intervals data mining: No longer a probability paper method. *Am J Clin Pathol*. 2015;143(1):134–142. <https://doi.org/10.1309/AJCPQPRNIB54WFKJ>