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ORIGINAL ARTICLE

Microcytic to hypochromic ratio as a discriminant index of thalassaemia trait in subjects with hypochromic anaemia

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Abstract

Introduction: Differentiating between thalassaemia and iron deficiency anaemia (IDA) in hypochromic anaemia is a challenge to pathologists as it influences the choice of subsequent specialized confirmatory tests. In this study, we aimed to evaluate the performance of microcytic to hypochromic ratio (MicroR/Hypo-He, M/H ratio) as a discriminant index in hypochromic anaemia. *Materials and Methods:* A retrospective study was carried out on 318 subjects with hypochromic anaemia, which comprised 162 IDA and 156 thalassaemia trait subjects with α-thalassemia, β-thalassemia and HbE trait. Optimal cut-off value, sensitivity and specificity of M/H ratio for thalassaemia trait discrimination was determined using Receiver Operating Characteristic (ROC) analysis. *Results:* Subjects with thalassaemia trait showed higher MicroR compared to IDA ($p< 0.001$) while subjects with IDA demonstrated higher Hypo-He than thalassaemia trait ($p < 0.001$). M/H ratio was significantly higher in thalassaemia trait compared to IDA, with medians of 3.77 (interquartile range: 2.57 – 6.52) and 1.73 (interquartile range: 1.27 – 2.38), respectively ($p < 0.001$). M/H ratio ≥ 2.25 was the optimal cut-off value for discriminating thalassaemia trait from IDA in hypochromic anaemia, with the area under ROC curve (AUC) of 0.83, sensitivity of 80.8% and specificity of 71.6%. *Conclusions:* M/H ratio is a useful discriminant index to distinguish thalassaemia trait from IDA in hypochromic anaemia prior to diagnostic analysis for thalassaemia confirmation. High M/H ratio is suggestive of thalassaemia trait than of IDA. However, more studies are required to establish the role of M/H ratio as a screening tool for thalassaemia discrimination in hypochromic anaemia.

Keywords: thalassaemia trait, hypochromic anaemia, percentage of microcytic red blood cells, percentage of hypochromic red blood cells, microcytic to hypochromic ratio

INTRODUCTION

Thalassaemia is an inheritable disorder of the haemoglobins secondary to defective synthesis of α- and β-globin chains of haemoglobin. It is the most common monogenic disorder in Malaysia. By the year 2017, there are a total of 7,509 registered thalassaemia patients with 2,623 transfusion-dependent β-thalassaemia major and 2,507 haemoglobin E (HbE)/β-thalassaemia, 754 thalassaemia intermedia and 1,111 haemoglobin H (HbH) diseases.[1] The carrier rates of α- and β-thalassaemia in Malaysia are estimated to be 4.1% and 4%, respectively.[2,3]

National Thalassaemia Screening Program has been established to reduce the incidence and disease burden of transfusion-dependent thalassaemia major and thalassaemia intermedia. One of the aims of the program is to detect thalassaemia carriers, who are often asymptomatic, in preventing and controlling the birth of new cases. Hypochromia, defined by mean corpuscular haemoglobin (MCH) <27 pg, has been incorporated as one of the screening criteria for thalassaemia. However, another major cause of hypochromia, i.e. iron deficiency, is also commonly found among Malaysians, especially among pregnant mothers.[4] Therefore, differentiating iron deficiency and thalassaemia in individuals with hypochromia is important in reducing unnecessary investigations. Iron studies have been recommended prior to specialized confirmatory tests for thalassaemia.

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However, these parameters can be affected by various biological factors, such as recent iron replacement therapy, diurnal variability and concomitant inflammatory status, so they may not reflect the true iron status in the body. Furthermore, they may not be available at the time of full blood count (FBC) review to guide the decision.

Various red blood cell (RBC) parameters and derivative formulas have been proposed for the discrimination between thalassaemia and iron deficiency in the setting of RBC hypochromia and/or microcytosis. These RBC parameters are readily available or can be made available, along with routine FBC. However, none of these discriminant functions is accurate enough in classifying the thalassemia trait subjects, especially in those with other concomitant causes of anaemia.[5]

Over the years, newer RBC parameters have been introduced and their roles in differentiating thalassaemia trait and iron deficiency have been extensively studied. Percentage of microcytic RBCs and percentage of hypochromic RBCs were first introduced in Technicon haematology analyzers (now Advia, by Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) and similar parameters were subsequently being developed by other manufacturers. Previous studies showed that RBCs were more microcytic in thalassaemia, while in iron deficient, they were more hypochromic.[6] These findings provide the basis for the application of percentage of microcytic to hypochromic RBCs (microcytic to hypochromic ratio or M/H ratio) in discriminating between the two conditions. In a meta-analysis, M/H ratio demonstrated the best performance among other discriminant indices in distinguishing between thalassaemia and iron deficiency in patients with microcytic anaemia.[6]

Most of the studies on M/H ratio published previously discriminated between β-thalassaemia trait and iron deficiency in microcytosis or microcytic anaemia. The proposed cut-off points varied across the analysers.[7] In this current study, we included α-thalassaemia trait and HbE trait, besides β-thalassaemia trait, for the assessment of M/H ratio as a discriminant index to differentiate between thalassaemia trait and IDA in hypochromic anaemia.

MATERIALS AND METHODS

This was a retrospective study carried out on subjects who had their haemoglobin analysis performed at Haematology Unit, Department of Pathology, Hospital Kuala Lumpur, from August 2015 to October 2016. Demographic data and clinical history of the subjects were obtained from laboratory request forms. Results from haematological, biochemical and molecular analysis were retrieved from the hospital laboratory information system. The study protocol was reviewed and approved by the Medical Research and Ethics Committee Malaysia (MREC), Ministry of Health Malaysia (NMRR-16-2448-33603 (IIR)), Hospital Research Review Committee HKL (HCRC.IIR-2017-01-002) and Research Ethics Committee University Malaysia (Code: FF-2017-142).

Samples for haemoglobin analysis were collected in EDTA anticoagulant tubes. Full blood count (FBC) including extended RBC parameters were run in the Sysmex XN3000 analyzer (Sysmex Corporation, Kobe, Japan).

Haemoglobin analysis was subsequently performed using high-performance liquid chromatography (HPLC) on Bio-Rad Variant II system (Bio-Rad Laboratories, Hercules, CA, USA) and/or capillary electrophoresis (CE) on Sebia Capillarys 2 Flex Piercing (Sebia, Paris, France). Classical β-thalassaemia trait was defined by HbA2 level ≥ 4.0% according to the laboratory's protocol. Cases with borderline HbA2 level (3.4%-3.9%) were excluded from our study as the data on molecular diagnosis was not available. HbE trait was demonstrated by high HbA2 level as determined by HPLC coinciding with the presence of a peak at zone 4 in CE. Confirmatory diagnosis of deletional and non-deletional α-thalassaemia trait was done by PCR-based DNA analysis using multiplex gap PCR and multiplex amplification refractory mutation system (MARMS), respectively.

The calculated sample size was 318, with equal distribution for thalassaemia trait and IDA study groups. Cases with concomitant thalassaemia trait and IDA were excluded from this study. Subjects were first screened for hypochromic anaemia. Anaemia was defined as < 12 g/dL in non-pregnant females, < 11 g/dL in pregnant females and < 13 g/dL in males.[8] Hypochromia was defined as MCH < 27 pg. Iron deficiency was defined as serum ferritin < 13 μg/L in female, < 30 μg/L in male and / or transferrin saturation < 15%. Only subjects ≥ 18 years old were selected. Subjects with Hb < 9 g/dL were excluded as they are usually not to be confused with pure thalassaemia trait in daily practice. Based on the information available,

subjects with ongoing infection, chronic illness, malignancy and history of transfusion within 3 months from the date of haemoglobin analysis were also excluded.

For the thalassaemia trait group, consecutive subjects diagnosed with α-thalassaemia trait, β-thalassaemia trait or HbE trait by methods aforementioned and with iron deficiency ruled out were first selected. These subjects were subsequently subcategorized into each thalassaemia subtype for further analysis. For the IDA group, consecutive subjects with iron deficiency identified using criteria described in the previous section and with normal HPLC and/or CE findings were selected.

Statistical Analysis

Statistical software package SPSS version 25.0 (IBM, Armonk, USA) was used for statistical analysis. Normality of the data was tested using the Kolmogorov-Smirnov test. Mann-Whitney U test and Kruskal-Wallis H test were performed to compare parameters between study groups. *P* values < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) analysis was utilized to illustrate the diagnostic performance of the studied parameters in discriminating thalassaemia trait from IDA in hypochromic anaemia.

RESULTS

The age of the subjects ranged from 18 to 75 years. The subjects consisted of Malays (84.6%), Indians (8.2%), Chinese (6.3%) and other ethnicities (0.9%). Distribution of ethnicity in each study group is illustrated in Fig. 1. The subjects were predominantly women (98.1%).

Among 318 subjects with hypochromic anaemia, 156 (49.1%) were subjects with thalassaemia trait and 162 (50.9%) were subjects with IDA. Of the thalassaemia trait group, 55 (35.3%) were subjects with β-thalassaemia trait, 51 (32.7%) showed α-thalassaemia trait and 50 (32.1%) showed HbE trait. α-thalassaemia trait in the study was due to heterozygous SEA deletion (35.3%), heterozygous Hb Constant Spring (29.4%), heterozygous 3.7 deletion (23.5%), compound heterozygous 3.7 deletion and Hb Constant Spring (5.9%), homozygous 3.7 deletion (3.9%) and heterozygous Filipino deletion (2%).

Haematological parameters of the subjects with thalassaemia trait and IDA are summarized in Table 1. Subjects with thalassaemia trait showed significantly higher median Hb and RBC with lower median MCV than subjects with IDA (*P* <0.001). Median RDW-CV was significantly increased in subjects with IDA (*P* <0.05). For extended RBC parameters, Ret-He and MicroR were significantly higher in subjects with thalassaemia trait (25.7 pg and 19.2%) than subjects with IDA (24.2 pg and 15.0%; *P* <0.001). Conversely, Hypo-He was significantly raised in subjects with IDA (8.3%) than in thalassaemia trait (4.9%; *P* <0.001). This gave rise to a significantly higher M/H ratio observed in subjects with thalassaemia trait (3.77) than in IDA (1.73; *P* <0.001).

Further analysis of the thalassaemia trait subtypes showed that subjects with β-thalassaemia trait showed significantly lower MCV, MCH and Ret-He than the non-β-thalassaemia trait (*P* <0.001). On the other hand, Hypo-He and MicroR were significantly

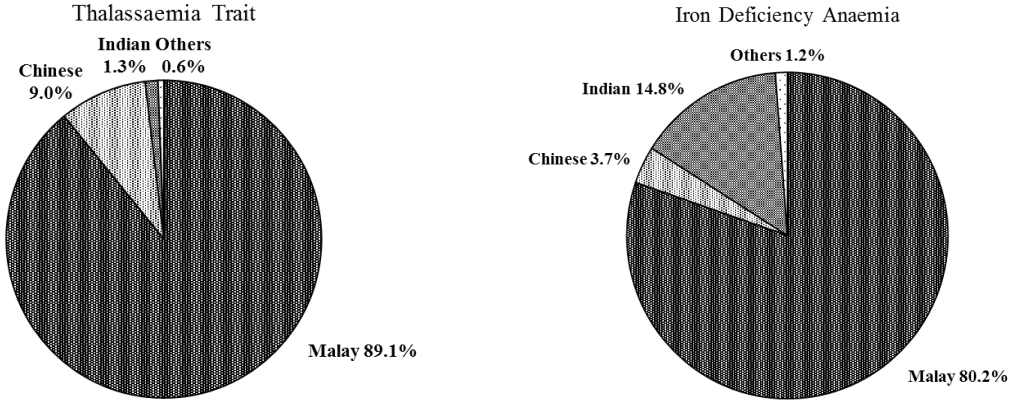

FIG 1: Distribution of ethnicity in each study group. A) Thalassaemia Trait and B) Iron Deficiency Anaemia

TABLE 1: Haematological parameters of the subjects with thalassaemia trait and IDA. Data shown are median with interquartile range in parentheses

Parameters	Thalassaemia trait (n = 156)	IDA (n = 162)	*P* value
Hb (g/dL)	10.3 (9.8 – 10.7)	9.9 (9.4 – 10.4)	<0.001*
RBC (x10^{12}/L)	4.5 (4.1 – 4.8)	4.3 (4.0 – 4.5)	<0.001*
MCV (fL)	73.0 (67.0 – 78.0)	76.0 (73.0 – 79.0)	<0.001*
MCH (pg)	23.1 (21.0 – 25.1)	23.5 (22.2 – 24.6)	0.169
RDW-CV (%)	15.8 (14.5 – 17.2)	16.6 (15.0-18.7)	0.002*
Ret-He (pg)	25.7 (22.9 – 28.6)	24.2 (22.4-26.5)	<0.001*
Hypo-He (%)	4.9 (1.6-14.4)	8.3 (4.6 – 14.8)	<0.001*
MicroR (%)	19.2 (10.2 – 39.3)	15.0 (9.8 – 22.2)	<0.001*
M/H Ratio	3.77 (2.57 – 6.52)	1.73 (1.27 – 2.38)	<0.001*

Abbreviation: Hb, haemoglobin; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; RDW-CV, red cell distribution width - coefficient of variation; Ret-He, reticulocyte haemoglobin equivalent; Hypo-He, percentage of hypochromic red blood cells, %MicroR; percentage of microcytic red blood cells; *Significant *P* value < 0.05

increased in subjects with β-thalassaemia trait (14.2% and 42.3%) than in the non-β-thalassaemia trait (2.1% and 12.7%; *P* <0.001). The M/H ratio was significantly higher in the non-β-thalassaemia trait group (5.36) than in the β-thalassaemia trait group (3.09; *P* <0.001) (Table 2). Analysis of the individual subtypes showed that β-thalassaemia trait had the highest Hypo-He and MicroR. HbE trait had the highest median M/H ratio (8.58) compared to α- and β-thalassaemia trait (3.42 and 3.09; *P* <0.001) (Table 3).

At M/H ratio cut-off value of 2.25, ROC analysis yielded an AUC of 0.83, sensitivity value of 80.8% and specificity value of 71.6% in identifying thalassaemia trait from IDA (Figure 2). Sensitivity and specificity of the M/H ratio at various cut-off values are illustrated in Table 4. Comparatively, MicroR and Hypo-He yielded lower AUC values at 0.61 and 0.62, respectively.

TABLE 2: Haematological parameters of the subjects with β-thalassaemia trait and non-β-thalassaemia trait. Data shown are median with interquartile range in parentheses

Parameters	β-thalassaemia trait (n = 55)	Non-β-thalassaemia trait (n = 101)	*P* value
Hb (g/dL)	10.1 (9.6 – 10.5)	10.4 (10.0 – 10.8)	0.007*
RBC (x10^{12}/L)	4.8 (4.6 – 5.2)	4.3 (4.0 – 4.6)	<0.001*
MCV (fL)	66.0 (63.0 – 69.0)	76.0 (72.0 – 79.0)	<0.001*
MCH (pg)	20.7 (19.8 – 21.8)	24.6 (22.7 – 25.4)	<0.001*
RDW-CV (%)	17.2 (16.3 – 18.9)	14.9 (14.2 – 16.2)	<0.001*
Ret-He (pg)	22.6 (21.8 – 24.4)	27.9 (24.9 – 29.2)	<0.001*
Hypo-He (%)	14.2 (6.8 – 21.0)	2.1 (1.1 – 7.0)	<0.001*
MicroR (%)	42.3 (31.4 – 50.7)	12.7 (8.6 – 22.3)	<0.001*
M/H Ratio	3.09 (2.27 – 4.28)	5.36 (2.61 – 8.99)	<0.001*

Abbreviation: Hb, haemoglobin; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; RDW-CV, red cell distribution width - coefficient of variation; Ret-He, reticulocyte haemoglobin equivalent; Hypo-He, percentage of hypochromic red cells; MicroR, percentage of microcytic red cells; *Significant *P* value < 0.05

TABLE 3: Comparison of haematological parameters across different thalassaemia trait subgroups

Parameters	β-thalassaemia trait (n = 55)	α-thalassaemia trait (n = 51)	HbE Trait (n = 50)	*P* value
Hb (g/dL)	10.1 (9.6 – 10.5)	10.3 (9.8 – 10.8)	10.4 (10.2 – 10.9)	0.008*
RBC (x10^{12}/L)	4.8 (4.6 – 5.2)	4.4 (4.0 – 4.8)	4.2 (4.0 – 4.4)	<0.001*
MCV (fL)	66.0 (63.0 – 69.0)	75.0 (69.0 – 80.0)	76.0 (74.0 – 78.0)	<0.001*
MCH (pg)	20.7 (19.8 – 21.8)	23.0 (21.4 – 25.3)	25.1 (24.3 – 25.7)	<0.001*
RDW-CV (%)	17.2 (16.3 – 18.9)	15.4 (14.0 – 17.0)	14.6 (14.3 – 15.3)	<0.001*
Ret-He (pg)	22.6 (21.8 – 24.4)	26.1 (24.4 – 29.2)	28.5 (27.5 – 29.1)	<0.001*
Hypo-He (%)	14.2 (6.8 – 21.0)	5.0 (1.7 – 12.0)	1.5 (1.0 – 2.1)	<0.001*
MicroR (%)	42.3 (31.4 – 50.7)	16.20 (6.7 – 30.8)	12.1 (9.6 – 15.4)	<0.001*
M/H Ratio	3.09 (2.27 – 4.28)	3.42 (2.27 – 5.40)	8.58 (4.90 – 10.81)	<0.001*

Abbreviation: Hb, haemoglobin; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; RDW-CV, red cell distribution width - coefficient of variation; Ret-He, reticulocyte haemoglobin equivalent; Hypo-He, percentage of hypochromic red cells; MicroR, percentage of microcytic red cells; *Significant *P* value < 0.05

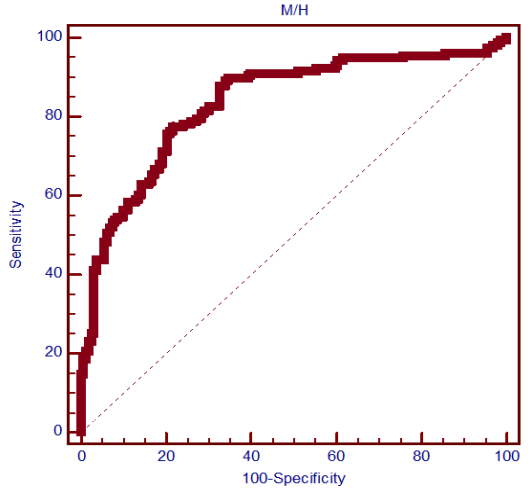

FIG. 2: ROC curve of M/H ratio in discriminating thalassaemia trait from IDA in subjects with hypochromic anaemia showing AUC value = 0.83 (Red line)

TABLE 4: Sensitivity and specificity of the M/H ratio at various cut-off values

Cut-off value	Sensitivity (%)	Specificity (%)
≥ 1.00	96.2	14.2
≥ 1.71	91.0	50.0
≥ 2.00	89.7	62.4
≥ 2.25	80.8	71.6
≥ 2.65	71.2	80.9
≥ 3.00	65.4	83.3
≥ 3.51	56.4	90.1

DISCUSSION

Results from the current study demonstrated that thalassaemia trait yielded higher MicroR and lower Hypo-He as compared to IDA, resulting in a higher M/H ratio. Specifically, subjects with β-thalassaemia trait had the highest MicroR and Hypo-He, while subjects with HbE trait demonstrated the highest M/H ratio compared to other subtypes. A study by Winichagoon *et al*. also showed similar differences among subjects with different thalassaemia subtypes in terms of percentage of microcytic and hypochromic RBCs.[9] The study also showed the HbE trait possessed the highest M/H ratio (>7.3) among subtypes of thalassaemia trait. Although a low level of HypoHe and/or very high MicroR are indicative of thalassaemia trait in patients with mild hypochromic anaemia, they did not perform as good as M/H ratio in differentiating thalassaemia from IDA in this study.

Results of this study showed that M/H ratio of ≥2.25 was the optimal cut-off value to distinguish thalassaemia trait from IDA in hypochromic anaemia, with AUC of 0.83, sensitivity of 80.8% and specificity of 71.6%. A higher M/H ratio improved the specificity but reduced the sensitivity to detect thalassaemia carriers. The cut-off value from this study approximates the value of 2.0 proposed by the study of Urrechaga *et al*. using Sysmex XE-5000 analyzer.[10] In their study, M/H ratio yielded AUC of 0.928, sensitivity of 89.9% and specificity of 84.1% in the discrimination of β-thalassaemia trait from IDA in the microcytic anaemia. However, the cut-off value of the M/H ratio varies between different analyzers. It was proposed to be 1.0 in Technicon H*1 analyzer when M/H ratio was first introduced;[11] 3.7 and 3.5 in Advia 2120;[12,13] and 6.4 in CELL-DYN Sapphire.[7]

In another study, M/H ratio demonstrated an AUC of 0.984, with 100% sensitivity, 87.5% specificity and discriminant efficiency of 91.5% for β-thalassaemia trait screening in microcytic anaemia[13]. However, when only patients with mild IDA (Hb 9–11 g/dl) were considered, the performance of M/H ratio reduced, with AUC of 0.923, sensitivity of 97.0%, specificity of 77.5% and discriminant efficiency of 87.5% for β-thalassaemia trait screening. In a separate study, 91.1% of microcytic patients were correctly classified as thalassaemia trait and IDA using the M/H ratio.[7] The study also observed that M/H ratio was not as highly sensitive in detecting α-thalassaemia trait, but still performed very well in β-thalassaemia trait with concomitant iron deficiency. Since the current study also included subjects with α-thalassaemia trait and HbE, the sensitivity and specificity were also likely to be different.

Overall, The AUC, sensitivity and specificity of M/H ratio for thalassaemia trait discrimination obtained from our study were lower compared to other studies previously performed. This may be partly explained by the different inclusion criteria applied in our study as compared to previous studies. The previous studies were mostly carried out in the setting of microcytosis rather than hypochromia. In addition, most of the studies recruited exclusively subjects with β-thalassaemia trait and only a few studies included α-thalassaemia trait. However, in view of the high prevalence of HbE trait and the significant number of HbE/β-thalassaemia patients in Malaysia, HbE trait subjects were included in our study.

More recently, new discriminant index using %MicroR and %HypoHe have been proposed, i.e. %MicroR - %HypoHe (M-H) and %MicroR - %HypoHe – RDW (M-H-RDW).[10,14] Performed on Sysmex analyzers, both formulas delivered the highest Youden Index as compared to other discriminant formulas in differentiating thalassaemia trait from IDA in microcytic anaemia. This may serve the basis for similar research in the future using MicroR and Hypo-He.

Several limitations of this study need to be addressed. IDA in our study was defined according to transferrin saturation and ferritin levels. Exclusion of concomitant thalassaemia trait in this group of subjects was mainly by normal HPLC and/or CE findings, where only a small proportion underwent molecular study for α-thalassaemia. Therefore, concomitant α-thalassaemia cannot be fully excluded in this group of subjects. Other than this, we did not include thalassaemia trait with concomitant IDA in our study. Hence, the efficiency of the M/H ratio to detect or exclude concomitant thalassaemia trait in patients with IDA was not being looked into.

Besides, the definition of hypochromic RBCs varies between different analyzers. Sysmex analyzers, used in this study, derived Hypo-He from the percentage of hypochromic RBCs with a Hb content <17 pg and MicroR from the percentage of microcytic RBCs with a volume <60 fL. On the other hand, Advia (Siemens Medical Solutions Diagnostics, Tarrytown,

NY, USA) and CELL-DYN Sapphire (Abbott Diagnostics, Santa Clara, CA, USA) analyzers defined RBCs with Hb concentration <28 g/dl as hypochromic, whereas the definition of RBCs with volume <60 fL as microcytic is the same as Sysmex analyzers. In a local study conducted using Sysmex XE-5000 analyzer, %MicroR and %HypoHe (similar to MicroR and Hypo-He in the XN series) measured among the healthy populations ranged from 0 - 3.8 % and 0 - 1.3%, respectively.[15] This would prevent the generalization of the results in this study to laboratories using different instruments.

CONCLUSION

M/H ratio is useful in distinguishing between thalassaemia trait and IDA in subjects with hypochromic anaemia to guide the decision for subsequent diagnostic investigations for thalassaemia. A higher M/H ratio is more indicative of thalassaemia trait than of IDA. However, more studies are required to establish the role of the M/H ratio for thalassaemia screening in hypochromic anaemia.

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Conflict of interest: The authors declare they have no conflict of interest.

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