The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

Establishment of population specific reference intervals in healthy Pakistani adults for 21 routine and special haematology analytes

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ARTICLE INFO

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Key words:
reference interval, Pakistan, hematology

ABSTRACT

Background

The reference interval (RI) is an interval between two limits derived from distribution of the results obtained from a sample of the reference population. These population based RIs are of paramount significance for the accurate clinical understanding of the patient's health status. Haematological RIs are heavily influenced by a variety of geographical and environmental factors. Therefore, accrediting bodies also mandate that each laboratory should establish its own RIs in its own population.

Methods

This cross-sectional study was conducted at the Department of Pathology and Laboratory Medicine, the Aga Khan University Hospital, Pakistan.

Twenty-one routine and special quantitative analytes were measured in adults aged 18-60 years who passed the initial health screening questionnaire. All samples were handled strictly following standard operating procedures. Microsoft Excel and EP Evaluator software were used for statistical analysis. Nonparametric CLSI EP28-A3C method was used to establish upper and lower confidence limits at 90% significance.

Results

A total of 323 participants passed the questionnaire and were short-listed for blood collection. There were 147 males and 176 females. Reference intervals were established in 297 participants after exclusion of 26 outliers with grossly abnormal test results. Analytes included: 8 red, and 12 white blood cell parameters, platelet count, immature platelet fraction, erythrocyte sedimentation levels, haemoglobin A and A2 levels and glucose-6-phosphatase dehydrogenase levels.

Conclusion

Routine and special haematology RIs established in this study reflect significant differences from RIs in Caucasian population. For meaningful interpretation of test results, each haematology laboratory should establish or verify RIs in the population it serves.

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BACKGROUND

As medical laboratory test data are essential for establishing health status as well as determining therapeutic management decisions, provision of accurate results is critical. The concept of a "NORMAL RANGE", coined by Grasbeck and Saris in 1969, is now termed as reference interval (RI) [1,2]. The RI is an interval between, and including, two reference limits, which are values derived from the distribution of the results obtained from a sample of the reference population [3]. This reference population is a healthy group of people for a particular age and gender. The interval represents the values found in 95% of the individuals of that group [4]. These reference population-based RIs are of paramount significance for the accurate clinical understanding of the patient's health status, and are the most commonly applied tool to determine a management protocol for the patients [5]. Haematological RIs in particular are heavily influenced by a variety of confounding factors including gender, ethnicity, geographical predisposition e.g., altitude and genetic make-up [6,7]. With this perspective in mind, the College of American Pathologists (CAP) and Clinical and Laboratory Standards Institute (CLSI) recommends that each laboratory should establish its own RI [3,8].

Since there are strict criteria for developing the RIs, it is highly unlikely for individual labs to set up their reference ranges. Therefore, the laboratories tend to collaborate and obtain the RIs from the published literature, manufacturers' package inserts, textbooks, national or international expert panel recommendations, guidelines, local expert groups or indirect approaches based on data mining [9,10]. Moreover, RIs are also subjected to various criteria that must be met beforehand—one of utmost importance being the population it is catering to should be essentially the same [11].

The Pakistani population has its own unique geographical, physical, and environmental characteristics that shape their physiology. To the best of our knowledge, there is no such study that has attempted to establish haematological RIs in Pakistani adult populations using a direct approach. These population-based RIs are expected to aid in more precise clinical decision making. In our study, we established RIs based on the population we serve and compared them to previously used RIs by our laboratory adopted from Dacie and Lewis Practical Haematology textbook [12].

METHODS

Study setting

This cross-sectional study was conducted at the Section of Haematology, Department of Pathology and Laboratory Medicine, the Aga Khan University Hospital (AKUH) in Karachi, Pakistan from August 1, to December 31, 2018. The city is the largest in the country and located at an altitude of 10 m above sea level on the coast of Arabian Sea. Approximately 90% of its inhabitants are migrants, composed of ethnolinguistic groups from all parts of the country [13]. The section of haematology serves as part of the largest public sector clinical laboratory in the country with a workload of approximately 650,000 routine and special haematology tests per annum. The laboratory adheres to the highest standards of quality and was the first to be accredited by Joint Commission International Accreditation (JCIA) and is the only one with College of American Pathologist (CAP) accreditation in Pakistan.

Haematology test menu at AKUH

The routine testing menu in haematology section includes, complete blood count (CBC), reticulocyte count, immature platelet fraction (IPF), erythrocyte sedimentation rate (ESR), malaria/filaria microscopy and body fluid cell count enumeration. Special tests include haemoglobin variant analysis by high performance liquid chromatography (HPLC), glucose-6-phosphate dehydrogenase quantification, osmotic fragility test, bone marrow examination, haemoglobin F staining by Kleihauer-Betke method, Sudan Black B, Periodic acid-Schiff and Perl's Prussian blue staining for iron. Strict QC measures are practiced at all phases of testing to ensure reliability of test results. All tests are validated before final inclusion in the testing menu. This validation process includes verification of accuracy, precision (repeatability), analytical measurement range/linearity, carry over and reference interval.

Participant recruitment

Non-probability consecutive sampling technique was used for participant recruitment. In accordance with CLSI recommendations, samples were collected from a sufficient number of qualified reference individuals to yield a minimum of 120 for analysis. A pre-designed health screening questionnaire (Table 1) was administered before collecting blood samples from participants aged between 18-60 years. The questionnaire included all pertinent attributes that provide insight into overall health of an individual. Since malaria, dengue fever, hepatitis B and C are endemic in our country, questions about these illnesses were specifically included. Participants were excluded from the analysis if one or more criteria were not fulfilled. Since red blood cell indices tend to vary in males and females, these (and white blood cells) were calculated separately in both the cohorts; all other parameters were calculated in combination (Figure 1). Serum ferritin level was also checked in all females to rule out sub-clinical iron deficiency.

Sample handling

All the quantitative tests were included for reference interval study. All pre-analytical, analytical and post-analytical standard operating procedures were followed strictly as per laboratory policy. Complete blood count analysis was performed using fully automated XN-1000™ haematology analyser (Sysmex Corporation, Kobe, Japan). Reticulocyte count and immature platelet fraction (IPF) were also done on the same analyser. Haemoglobin subtypes (Haemoglobin A and A2) were measured using BIO-RAD VARIANT™ II Haemoglobin testing system (BIO-RAD Laboratories Inc, Hercules, CA, USA). Glucose-6-phosphate dehydrogenase

Table 1 Health screening questionnaire administered before blood sample collection*

	Questionnaire	Yes	No
1	Currently taking any antibiotic/medication?		
2	Had any vaccinations within 4 weeks?		
3	Fever, cold, flu or sore throat within the last 2 weeks?		
4	Had dengue fever in past 4 weeks?		
5	Had malaria in past 1 year (any malaria species confirmed by test)?		
6	Pregnancy within 6 weeks or are you pregnant now?		
7	Had a major surgery/procedure in past 1 year?		
8	Had a blood and blood component transfusion within 12 months?		
9	Any history of jaundice in past 1 year?		
10	Tested positive for the Hepatitis B surface Antigen or Hepatitis C?		
11	Known iron deficiency or Thalassemia Minor?		
12	Any chronic disease such as hypertension, diabetes mellitus, lung disease, kidney disease, liver disease, heart disease, acquired immunodeficiency syndrome, cancer, convulsions/fits, etc.?		
13	Have you ever had bleeding condition or a blood disease?		

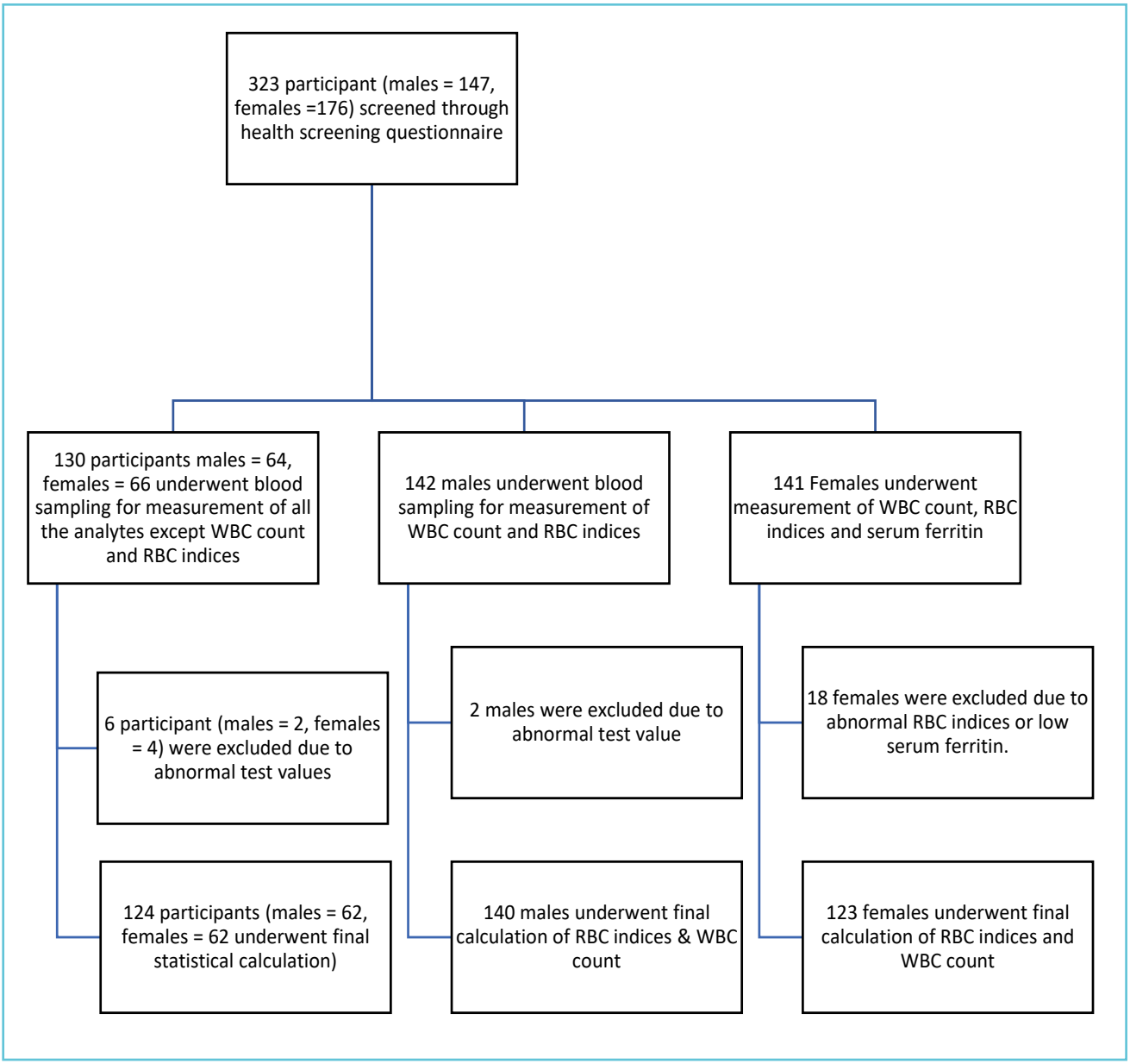
**Blood sample was collected only if the response to all of the above questions was "NO".*

(G6PD) enzyme levels were quantified spectrophotometrically using the Pointe Scientific reagent set (Pointe Scientific Inc, Canton, MI, USA). Erythrocyte sedimentation rate was measured on Sed Rate Screener 20/II (SRS 20/II; Greiner Bio-One, Kremsmünster, Austria).

Statistical analysis

Statistical analysis was done using MS Excel (Microsoft Corporation, Washington, United States) and EP Evaluator version 10.3.0.556 (Data Innovations, LLC, VT, US). Nonparametric CLSI EP28-A3C method was used to establish

Figure 1 Flow chart depicting inclusion and exclusion of participants in the study



upper and lower confidence limits at 90% significance [3].

Ethical aspects

The study was started after obtaining approval from the institutional ethical review board (5140-Pat-ERC-17). Informed consent was taken from each participant before collecting blood samples. In case abnormal test results were found, participants were appropriately guided about consulting their physicians.

RESULTS

A total of 323 participants passed the initial questionnaire (Table 1, Figure 1) and were short-listed for blood collection. There were 147 males and 176 females. One hundred and twenty-four participants underwent calculation of all the

Table 2 Instrument quality control and tolerance limits before analysis

Instrument	Parameter		Acceptable limits	Our value Acceptable yes/no
Sysmex XN-1000™	Background checks*	RBC (x 10^{12}/L)	0.02 or less	Acceptable
		Haemoglobin (g/dL)	0.1 or less	Acceptable
		WBC (x 10^{9}/L)	0.1 or less	Acceptable
		Platelets (x 10^{9}/L)	10 or less	Acceptable
	XN CHECK™ Control	Level I (Low abnormal)	#	Acceptable
		Level II (Normal)	#	Acceptable
		Level III (High abnormal)	#	Acceptable
	Pneumatic Unit Pressures (Megapascals)		0.25 ± 0.04	Acceptable
			0.16 ±0.16	Acceptable
			0.07 ± 0.01	Acceptable
	Pneumatic Unit Vacuum (Megapascals)		-0.037 – (-0.054)	Acceptable
	Aspiration Sensor Span		6500 ± 500	Acceptable
	Ambient temperature (Celsius)		15-30	Acceptable
BIO-RAD VARIANT™ II TURBO	Lyphochek® Haemoglobin A2 Control	Level I (Normal)	#	Acceptable
		Level II (High abnormal)	#	Acceptable
	Maximum Pump pressure (kg/cm^2)		<280	Acceptable
	Chromatographic station ambient operating temperature (Celsius)		15-35	Acceptable

Sed Rate Screener 20/II	Accu-Sed® Plus Control	Normal	#	Acceptable
		High abnormal	#	Acceptable
	Internal temperature (Celsius)		15-32	Acceptable
	Mechanical calibration reference		300 ± 10	Acceptable
	Delta value between reading cycles		± 2	Acceptable
Thermo Fisher Scientific GENESYS 150 UV-Vis Spectrometer	Trinity Biotech G-6-PDH Control	Normal	#	Acceptable
		Deficient	#	Acceptable
	Absorbance with water (Blank)		Zero	Acceptable
	Wavelength (nanometres)		340	Acceptable

#More than 1 control lots (with variable acceptable limits) were used during the study period.
**Analysis without aspirating the samples to verify the effects of auto rinse.*

analytes with WBC count and RBC indices, calculated separately in male and female cohorts (Figure 1, Table 3 & 4). The median age in male and female participants was 27 and 31 years, respectively.

All the quality control and instrument tolerance limits were acceptable before sample analysis (Table 2). Clinical and Laboratory Standards Institute recommends that only extreme outliers be removed—an extreme outlier being defined as one for which the distance to the adjacent value exceeds one-third of the total sample range [3]. Based on this criterion, a fraction of participants (n = 26; males = 4, females = 22) were excluded as depicted in Figure 1. Table 3 shows RBC indices in males and females. Table 4 shows white blood cell counts with differential counts in absolute numbers as well as in percentages. Table 5 shows platelet count, IPF, G6PD, ESR, haemoglobin A (HbA), haemoglobin A_2 ($HbA_{2)}$ and reticulocyte counts in the study population.

DISCUSSION

Most regulatory bodies including CAP advocate the use of population specific RIs in clinical laboratory reports to make them comprehensible and clinically useful [8]. To the best of our knowledge and belief, current study is the most comprehensive adult haematology RI data to date in Pakistan. Firstly, participant recruitment criteria were very stringent; both in terms of pre-sampling health screening of participants (Table 1) as well as monitoring of pre-analytical and analytical aspects of quality assurance (Table 2). Secondly, the study does not only include routine parameters but also advanced clinical parameters such as IPF, that has been approved later by the United States Food and Drug Administration (FDA, US) and The National Institutes of Health Clinical Centre (CC NIH) [14]. Furthermore, inclusion of specialized tests such as haemoglobin A, A2 and G6PD makes this data a complete reference package for regional haematology laboratories.

In scenarios where establishing reference intervals by including at least 120 individuals is not possible either due to cost constraints or any other reason, CLSI recommends verification of pre-existing reference interval data. This verification can be achieved using 20 samples instead of 120 samples. However, the existing reference interval data should be from a population that is as close as possible to the population being catered by the verifying laboratory. Therefore, our data can serve as database that can be utilized by other laboratories in Pakistan and in the region which intend to verify reference intervals in their laboratories.

Table 3 Red blood cell indices in study population

Analyte	Gender	Lower		Upper		Previously used RIs
		Value	95% confidence interval	Value	95% confidence interval	
RBC (x10^{12}/L)	Male	4.25	4.1-4.39	6.02	5.81-6.25	5.0± 0.5
	Female	3.61	3.6-3.9	5.2	5.09-5.40	4.3 ± 0.5
Haemoglobin (g/dL)	Male	12.3	12.0-12.7	16.6	16.2-16.8	15 ± 2.0
	Female	11.0	11.0-11.1	14.5	14.2-15.1	13.5 ± 2.0
Haematocrit (%)	Male	38.4	36.9-39.5	50.7	49.6-51.4	45 ± 5.0
	Female	34.5	33.9-34.8	45.4	44.1-47.7	41 ± 5.0
Mean cell volume (fL)	Male	78.7	73.9-80.4	96.3	94.7-98.5	92 ± 9.0
	Female	78.1	76.2-79.3	95.3	94.2-99	92 ± 9.0
Mean corpuscular haemoglobin (pG)	Male	25.1	23.7-25.5	31.6	31.1-32.6	29.5 ± 2.5
	Female	25.3	25.0-25.5	31.7	31.0-32.5	29.5 ± 2.5
Mean corpuscular haemoglobin concentration (g/dL)	Male	30.0	29.2-30.6	35.5	34.8-36.6	33 ± 1.5
	Female	30.3	30.30.4	34.4	33.9-34.6	33 ± 1.5
Red cell distribution width (%)	Male	12.0	12.0-12.1	16.0	15.3-16.5	12.8 ± 1.2
	Female	12.1	11.5-12.3	16.9	16.7-18	

Table 4 White blood cells (WBC) and differential counts in study population

Analyte		Lower		Upper		Previously used RIs
		Value	90% confidence interval	Value	90% confidence interval	
White blood cell ($x10^9$/L)	Male	4.88	4.6-5.48	11.38	10.56-12.4	4.0-10
	Female	4.6	4.0-5.09	10.8	10.2-10.9	4.0-10
Neutrophils	Absolute	1.81	1.6-2.2	7.59	6.9-7.9	2.0-7.0
	Percentage	34.9	29.4-38.7	76.2	71.3-78.8	40-80
Lymphocytes	Absolute	1.1	1.07-1.5	4.75	4.0-5.0	1.0-3.0
	Percentage	17.5	11.9-19.6	45	44.5-46.4	20-40
Monocytes	Absolute	0.20	0.14-0.2	1.0	0.9-1.1	0.2-1.0
	Percentage	3.9	1.7-4.5	10.0	9.8-10.3	2.0-10
Eosinophils	Absolute	0.02	0.0-0.04	0.6	0.51-0.79	0.02-0.5
	percentage	0.3	0.2-0.6	7.4	7.1-8.4	1.0-6.0%
Basophils	Absolute	0.01	0.0-0.01	0.09	0.08-0.10	0.02-0.1
	Percentage	0.10	0.03-0.20	1.0	1-1.2	< 1-2 %
Neutrophil Lymphocyte ratio	Ratio	1.0	0.9-1.1	4.0	3.2-4.6	#

**Absolute values are in billion cells per litre (x 10^9/L).*
 # Recently included in test menu; our own established RI is reported since the initiation of test in our laboratory.

Table 5 Platelet count, IPF, ESR, G6PD, HbA, HbA_2 and reticulocyte count in study population

Analyte	Lower		Upper		Previously used RIs
	Value	90% confidence interval	Value	90% confidence interval	
Platelet ($x10^9$/L)	154	142-182	433	384-488	280 ± 130
IPF (%)	1.2	0.6-1.4	8.3	7.7-9.2	#
ESR (mm/hr)	2	1-4	15	12-18	<10
G6PD (U/gHb)	6.0	5.4-6.4	12.4	11.3-13.9	8.83 ± 1.59
Haemoglobin A (%)	86.5	85.0-87.5	97.9	97.6-98.3	96.0-97.8
Haemoglobin A2 (%)	2.4	2.3-2.4	3.2	3.1-3.5	2.2-3.5
Reticulocyte Count (%)	0.6	0.4-0.7	2.4	2.1-2.5	0.5-2.5

 # Recently included in test menu; our own established RI is reported since the initiation of test in our laboratory.

Besides being the most comprehensive Pakistani data, the other significant strength of our study is utilization of appropriate statistical calculation as per CLSI guidelines. We utilized non-parametric test to establish RI. One Pakistani study reported mean ± SD values as RI which is simplest example of parametric method [15]. In this study, only routine CBC parameters were reported. Use of mean ± SD is feasible only when one is confident that the study population follows Gaussian distribution. A parametric method based on false assumption may be unreliable; not only is the estimate unreliable, the 90% confidence interval is also overly optimistic [16]. Therefore, it is not recommended to use mean ± SD unless it is very clear that the curve really is Gaussian.

As depicted in Tables 3, 4 and 5, the results obtained in this study are considerably different from the previously used RIs. For instance, MCV and MCH values obtained in current study are lower than previously used Caucasian values. It follows that should our local laboratories continue to use previously adopted values, patients with microcytic hypochromic anemia can falsely be missed and this variation can lead physicians to think about alternate diagnosis and hence wastage of important human efforts and financial resources. Similarly, upper limit of ESR in our study is 15mm (in first hour) as compared to previously used value of 10mm. Unfortunately, ESR values falling in between 10-15 mm would falsely have misled physicians to believe that their patients had some kind of

inflammatory condition. This further supplements the fact that population-specific RIs are imminently needed in the Pakistani population which has a unique genetic framework compared to Caucasian population, from which most of the currently used RIs by various laboratories are adopted.

Moreover, a noteworthy strength is gender partitioning for WBC count and red cell indices which was missing in previously used RIs. Even though, owing to the financial constraints, gender-based partitioning was not undertaken for RIs of all parameters, this fact doesn't add substantial limitation as these parameters are unlikely to vary significantly between genders. Except for red cell parameters, no significant data for other parameters were found on literature search indicating gender-based differences in RIs. Of note, total allowable error (TEa) values of most analytes other than RBC indices are significantly higher. Based on biological variations, TEa is a widely accepted concept in laboratory medicine that expresses the degree of error in a test result that can be tolerated without negatively impacting patient care. For instance, acceptable CLIA values of TEa for haemoglobin, haematocrit, red blood cells, white blood cells and platelets are 7%, 6%, 6%, 15% and 25%, respectively. This fact further supplements improbability of gender-based variation for analytes other than RBC indices. Although with significant overlap, red cell parameters in alpha thalassemia carriers might differ from normal subjects; this possibility was kept in check by monitoring fast moving peaks (before 1 minute) on high performance liquid chromatography. One limitation of our study was the lack of evaluation of dietary and environmental impacts such as sub-clinical iron deficiency. In the overall design of the study, including completely voluntary inclusion of participants, the administered health screening questionnaire did include evaluation of iron deficiency (Table 1). Additionally, serum ferritin levels were checked in all females and reactive increase in serum ferritin was ruled out by correlating with health screening questionnaire, normal white blood cell counts and erythrocyte sedimentation rates.

The confounding effect of variation in altitude is an important factor that must be addressed in RI studies. Ideally, blood samples from individuals residing in different cities of the country at different altitudes must be included in the study for comparative analysis. This was not possible in our situation principally because of cost constraints. This study was a special effort by our laboratory's management in terms of financial support. Nevertheless, we have good data at hand to start off which we consider as a first ray of light in complete darkness. In future, we intend to collaborate with laboratories situated at different altitudes in the country for measurement of the same parameters using the same reagent/equipment and study design. The samples will be added on to our laboratory's daily routine runs to remove errors arising from inter-laboratory differences linked to methodologies, operator competency and statistical compilation of the RIs. This expanded project design will produce data of vastly incremental clinical and education value not just for the people of Pakistan but also for the readership of manuscript. Future studies are required to further explore these points. Nonetheless, despite the possible highlighted short comings, the established RIs reported herein have the potential to serve as a vital resource for diagnostic laboratories in Pakistan and neighbouring regions.

CONCLUSION

Routine and special haematology RIs established in this study reflect significant differences from RIs in Caucasian populations. For meaningful interpretation of test results, each haematology

laboratory should establish or verify RIs in the population it serves.

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Ethics approval

This study was approved by Aga Khan University ethical review committee (AKU-ERC).

Consent for publication

Not applicable as this manuscript contains no individually identifiable details or images.

Competing interests

The authors declare that they have no competing interests.

Funding: Not applicable.

Authors' contributions

MSS designed the study, collected/analysed data and wrote the initial draft. AK, SA and MHH contributed to writing of the manuscript. US and NA critically reviewed the manuscript. All authors contributed to conception of the study and approved the final version of the manuscript.

Acknowledgements

The authors are grateful to laboratory staff who helped with handling of specimens and analysis of data.

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