Novel Automated Hematology Parameters in Clinical Pediatric Practice

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Blood sampling in children is a challenging task, and to extract maximum possible information from these 'precious' samples, the modern-day automated hematology analyzers have been aided with much technological advancement. Various novel blood cell parameters are now available to narrow down the differential diagnoses. However, only few of these are available for routine clinical reporting. Knowledge about their interpretation and reference ranges can prove useful in challenging situations.

Keywords: *Anemia, Blood cell count, Diagnosis, Diagnostic markers*.

Recent technological advancements in the automated hematology analyzers have resulted in generation of many novel parameters to characterize the blood cells. These parameters, though currently for research purposes, hold a promising value to aid clinicians in certain clinical scenarios. The same single run of a blood sample yields many new parameters with no additional cost. These modern-day automated analyzers are coming up with cutting-edge technology; to name a few of them: LH 750, LH 780 and UniCel DxH 800 (Beckman Coulter); Pentra DX Nexus and DX 120 (Horiba Medical); CELL-DYN Sapphire (Abbott diagnostics); Advia 120 and 2120i (Siemens); XE and XN series (Sysmex) and BC-6800 (Mindray).

Extracting useful information from amongst the novel parameters offered by these advanced analyzers, is a challenging task for the clinicians. The current review is an attempt to aid interpretation of the results of these new parameters in appropriate clinical settings. ***Web Table* I** compiles some of these novel parameters along with their reference ranges [1,2], and "where to use this information."

RED BLOOD CELLS (RBC) PARAMETERS

Nucleated Red Blood Cell (NRBC) Count

Nucleated red blood cells (NRBCs) are the erythroid precursors, which can appear in peripheral blood in certain physiological/pathological states. Older versions of analyzers were not able to accurately distinguish NRBCs from small lymphocytes, and thereby gave falsely high white blood cell (WBC) count, ultimately requiring a peripheral blood film (PBF) review. With improved technology, the recent analyzers (***Web Table* I**) can correctly quantify the NRBCs (absolute value and percentage) in blood samples as well as provide the corrected WBC count. Besides, these results are also flagged. Studies have shown a good correlation between manual and automated NRBC counts [3]. The clinical settings in which the NRBC count can aid a clinician are summarized in ***Box* 1**.

Case scenario 1

A 6-month-old boy presented with marked pallor and hepatosplenomegaly. Clinical possibility of acute leukemia was considered and an urgent hemogram was requested. His automated blood cell counts (Beckman Coulter UniCel DxH 800) revealed Hb 6 g/dL, platelet count 210×10^9/L, uncorrected WBC count 35×10^9/L and corrected WBC count of 11.9×10^9/L. The counter gave percentage and absolute NRBC count as 66/100 WBCs and 23.1×10^9/L. Besides, there was reduced MCV (55 fl) and MCH (13 pg) and increased RDW (25%). However, the PBF was not seen, and the automated report was released in which there was no mention about NRBC. The pediatrician got puzzled regarding the corrected WBC count and he finally asked for a PBF that revealed numerous NRBCs along with microcytic hypochromic cells and many poikilocytes suggestive of a hemoglobinopathy. A diagnosis of β-thalassemia major was made after a complete hemolytic work-up.

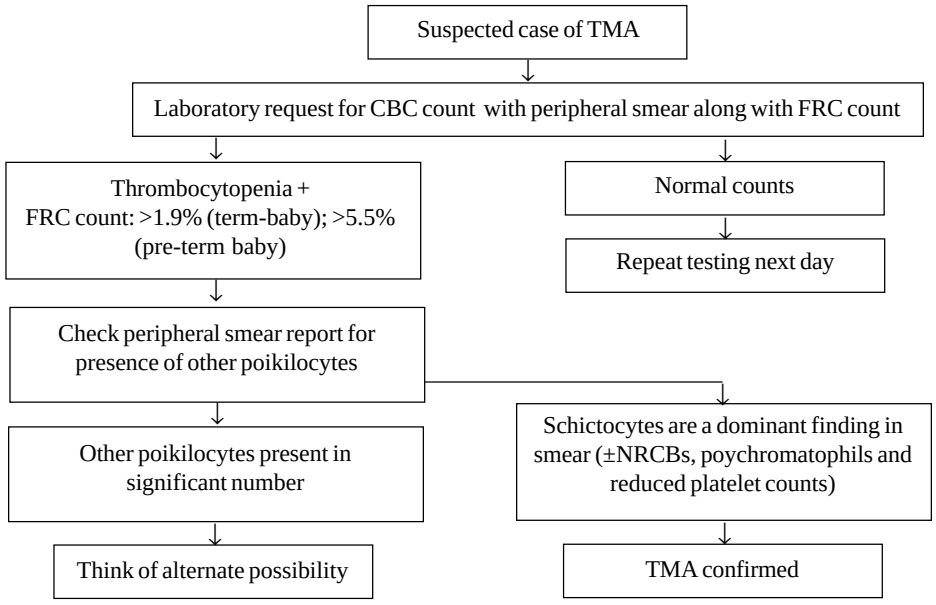
Fragmented Red Cell (schistocytes) Count (FRC)

Schistocytes are red blood cell fragments, which are usually produced as a result of mechanical damage within the vasculature. As per recommendations of 'International Council for Standardization in

Box 1: Nucleated RBC Count: When to Choose and Where to Use?

- 3-7 NRBC/100 WBCs may be normal for a term baby till day 4 and 21 NRBC/100 WBCs in preterm infant till day 10 of life.
- Increased in hemorrhagic, hemolytic and hypoxic states, and in bone marrow infiltrative disorders (leukemia, lymphoma, tumor metastasis, storage disorder or myelofibrosis).
- Assessment of ineffective erythropoiesis and severity of thalassemia or other hemoglobinopathies for optimization of blood transfusion.
- Prognostic marker in intensive care unit patients [4] as well as those undergoing transplantation [5].

Haematology' (ICSH) [6], schistocytes are defined as cell fragments which are smaller than a normal RBC with sharp angles and straight borders, or may be small crescent shaped. Various other morphological forms, like helmet cells, keratocytes, or microspherocytes are also included under this category. Recent hematology analyzers (Advia 2120i and Sysmex XN) have been equipped with various proprietary algorithms to quantify as well as flag these cells by the term 'fragmented red cells' (FRC). Studies have shown a good morphological correlation with these automated counts [7]. This parameter has been shown to have good negative predictive value but a low specificity; hence, all positive cases need to be reviewed on smears. The normal upper limit for a healthy adult is generally set as <1%, though there is no consensus [6]. In case of newborns, schistocytes are more frequently seen with a range of 1.4-1.9% in term babies and 4.9-5.5% in preterm babies [6]. The prime value of detecting significant numbers of schistocytes in pediatric patients is to detect an underlying condition resulting in thrombotic microangiopathy (TMA). In a neonate who presents with jaundice and thrombocytopenia along with an FRC count of >1% (>5% in preterm), an underlying disseminated intravascular coagulation (DIC) secondary to perinatal asphyxia, infection or sepsis should be considered. Besides, other conditions causing such manifestations may be neonatal hemolytic uremic syndrome, congenital form of thrombotic thrombocytopenic purpura (ADAMTS13 deficiency), homozygous protein C deficiency or a giant hemangioma/vascular tumor [8]. There are certain other conditions such as burns, thalassemia syndromes, megaloblastic anemia, congenital sideroblastic and dyserythropoietic anemias and prolonged iron deficient states where schistocytes are found along with other poikilocytes, but associated thrombocytopenia is usually not seen, and clinical symptomatology is different from that seen in TMA or DIC [6] (***Fig.* 1**).



TMA: Thrombotic microangiopathy; FRC Count: Fragmented Red cell count; NRBCs: Nucleated red blood cells

FIG. 1 *Approach to a suspected case of Thrombotic Microangiopathy (TMA) using the FRC count.*

Immature Reticulocyte Fraction (IRF)

Immature reticulocyte fraction (IRF) is defined as the fraction of most immature forms of reticulocyte to the total number of reticulocytes. These reticulocytes have the maximum amount of RNA which can be detected using various RNA binding fluorescent dyes by HAs. HAs usually discriminate the reticulocytes into three population groups based on the intensity of fluorescence; high, medium and low fluorescence reticulocytes (HFR, MFR and LFR). IRF is the sum total of HFR and MFR. Different instruments uses different dyes for their identification; hence, there are different reference ranges for it (***Table* I**). IRF value provides an assessment of the reticulocyte maturation and hence, the degree of effective erythropoiesis. For the diagnosis of various types of anemias, IRF serves as an adjunct to total reticulocyte count but is usually not of much help alone in differentiating them all (***Fig.* 2**). The scenarios in which IRF can be applied clinically are summed up in ***Box* 2**.

Box 2 Immature Reticulocyte Fraction: When to Choose and Where to Use?

- Evaluation of cases of anemia.
- Marker to assess response to iron or vitamin-B_{12}/folate supplementation in nutritional anemias and to monitor erythropoietin (EPO) therapy response as it rises much earlier before the total reticulocyte count [2].
- Alternative for absolute neutrophil count (ANC) for monitoring recovery following bone marrow transplant, as it starts to rise 5-7 days post bone marrow transplant and reaches >10% at 10-14 days and is not affected by infections which are common in such settings [9-10].
- Useful indicator of impaired bone marrow function following chemotherapy-induced bone marrow aplasia in cancer patients [11].

New Parameters for Assessing Functional Iron Deficiency (FID)

Functional iron deficiency (FID) is defined as a state arising due to non-availability of iron for the developing erythroid precursors in the bone marrow in the presence of adequate body iron stores, ultimately resulting in anemia. This is attributed to trapping of iron within the reticuloendothelial system in inflammatory/infectious conditions or in chronic kidney disease patients undergoing regular dialysis sessions. Traditionally, this state was used to be detected with the use of iron profile (serum ferritin, percentage saturation of transferrin). However, their values are often deranged if confounded by inflammation, cancer or infections. Hence, there was a need to identify reliable parameters which could detect the iron deficiency at the very stage of its incorporation into the erythroid precursors (reticulocytes) and at the same time not being affected by the confounders.

Modern-day hematology anlyzers have come up with many new parameters to better characterize the reticulocytes which remain in circulation for two to three days and hence are a better indicator of early changes for the iron-restricted erythropoiesis. The most widely used and clinically important parameters amongst these include, the mean content of hemoglobin within the reticulocytes [CHr-mean reticulocyte hemoglobin content as given on Siemens Advia analyzer] or its equivalent; [Ret-He-reticulocyte hemoglobin equivalent

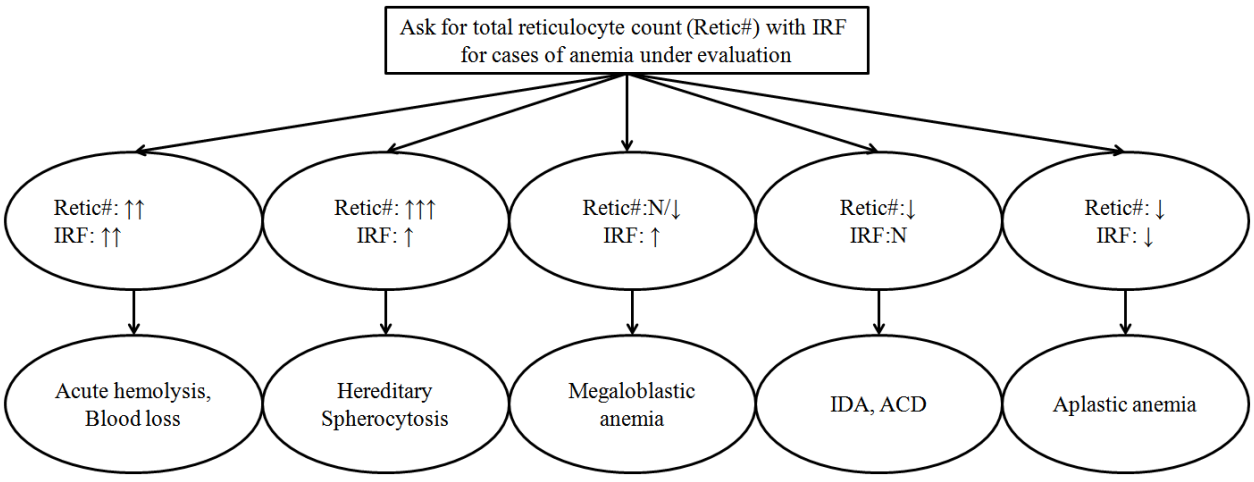


FIG. 2 *Utility of total reticulocyte count and Immature Reticulocyte fraction (IRF) for cases of anemia under evaluation.*

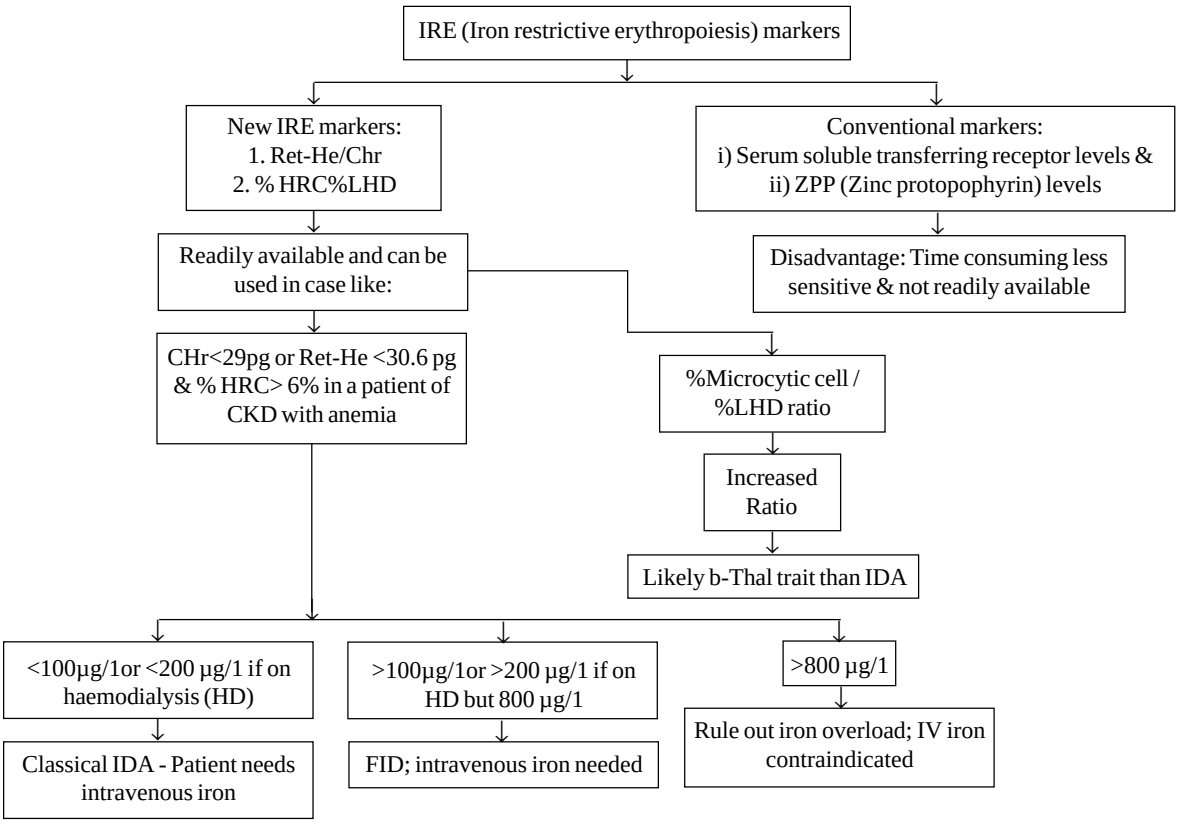
as given on Sysmex analyzer] and percentage hypochromic cells (% HRC; Siemens Advia, Sysmex XE series, CELL-DYN Sapphire) or its equivalent low hemoglobin density (LHD%; Beckman coulter).

Studies have found a good correlation among CHr and Ret-He as they can provide the status of functional iron available for the erythropoiesis during the last 3-4 days [12-13]. Together with % HRC or % LHD parameter (defined as RBC's with cellular Hb <28g/dL and volume < 60 fL), they are termed as iron restricted erythropoiesis (IRE) markers, and are helpful in various clinical settings. The reference range for adults for Ret-He is approximately 28-35 pg (below 28 pg is considered iron deficient) and % HRC is <2.5% [13]. The main clinical utility of these IRE markers is in differentiating actual iron deficiency anemia (IDA) *versus* FID state (IDA- CHr or Ret-He <25 pg and % LHD >6% *versus* FID- CHr or Ret-He <28 pg and % LHD >2.5%). It is also useful in planning need for iron supplementation in cases of anemia of chronic disease (ACD) or for parenteral iron in chronic kidney disease (CKD) cases on erythropoietin stimulating agents (ESA) (when CHr or Ret-He <28 pg and % LHD >6% with a reduced to normal ferritin level) [15] (***Fig.* 3**).

% LHD along with % microcytic cells (given by Sysmex XN-1000 series), is also used to distinguish between IDA and β-thalassemia trait [14]. A high ratio of % microcytic cells to % LHD is a better marker suggestive for β-thalassaemia trait [15] (***Fig.* 3**).

Other less important but novel research based HA parameters for RBC include mean reticulocyte volume (MRV) and mean sphered cell volume (MSCV). Studies have generated algorithms involving both these parameters and a MCV-MSCV >10 and MRV-MSCV <25 shows a good accuracy as a screen for cases of hereditary spherocytosis, and helps differentiate the condition from other common causes of spherocytosis *i.e.* autoimmune hemolytic anemia (AIHA) and ABO incompatibility [16].

Percentage Unghosted cells (UGC) is also a relatively new parameter, though less well studied. It is



RetHe:- Reticulated Hemoglobin equivalent; rCH:- Mean reticulocyte hemoglobin content; LDH:- Low density hemoglobin; CKD:- Chronic kidney disease; HRC:- Hypochromic red cells; IDA:- Iron deficiency anemia; FID:- Functional iron deficiency.

FIG. 3 *Utility of Iron Restricted erythropoiesis (IRE) markers in clinical case scenarios.*

only available on Beckman Coulter UniCel DxH 800 and the count reflects the percentage target cells and hence can be used as a marker to screen for β-thalassemia trait or other hemoglobinopathies [17].

WBC Parameters

White Blood Cell Volume, Conductivity and Scatter (VCS)

*V*olume, *C*onductivity and *S*catter (VCS) technology of the Beckman Coulter series is an approach to WBC analysis where in addition to numerically quantifying and sub-classifying these cells, it also yields a large amount of information on their physical, electrical, optical and hence, structural properties. The numerical VCS data or coordinates are visualized graphically in the form of a 3-D cube. A total of 24 parameters (mean positions on the 3-axes of Figure, and standard deviation for each cell type) are thus available with every routine differential leukocyte count without any further analyses or increased cost, and regardless of clinical suspicion. Various authors have utilized VCS parameters (mean neutrophil volume, MNV) for early identification of neonatal sepsis by formulating the regression equation which combines other laboratory parameter [18-19]. Other uses of VCS technology are detection of chorio-amnionitis, malaria and dengue as well as malignancies like myelodysplastic syndromes, myeloproliferative neoplasms, acute leukemias and lymphoproliferative disorders [20]. Various authors have devised their own algorithmic approaches so as to characterize a particular disease state.

Malaria factor has been derived using VCS parameters like SD of the mean volume for lymphocytes (MLV-SD) and Monocytes (MMV-SD) for the possible presence of malarial parasites. A cut-off value for the Malaria Factor greater than 3.7 is an indicator of malaria infection with the specificity of 94% and sensitivity 98% [21].

Immature Granulocytes (IGs)

This parameter identifies and quantifies immature myeloid cells which combine promyelocytes, myelocytes and metamyelocytes and helps overcome the possibility of missing these cells in a manual 100-cell differential leukocyte count, especially in leukopenic patients. Their presence in the peripheral blood is indicative of a systemic inflammation and sepsis, a hematological disorder like myeloproliferative neoplasm or acute myeloid leukemia, or a bone marrow infiltrative disorder (where peripheral blood smear may show leuco-erythroblastic picture). In fact, an immature to total (I: T) granulocytic cell ratio of >0.2 or an immature to mature (I:M) granulocytic cell ratio of >0.3 is a 100% sensitive marker for diagnosis of sepsis in an appropriate clinical setting [22].

Atypical/Immature Lymphocytes

The detection and quantification of atypical lymphocytes has been provided with the Sysmex XE series (High fluorescent lymphocytes, HFL), Horiba Pentra (Atypical lymphocytes, ALY%) and Siemens Advia 2120 (Large unstained cells, %LUC). This parameter can quantify various morphologies of atypical lymphocytes like activated lymphocytes in viral infections (*e.g.,* in infectious mononucleosis), lymphoma cells as well as small blasts. Hence, it can also be used in the monitoring of sepsis because of viral infections [20].

Neutrophil Granulation (NEUT-X/NEUT-Y)

NEUT-X is a measure of granularity of neutrophils based on their side-scatter property whereas NEUT-Y indicates cellular content of nucleic acid and protein. These parameters are provided by Sysmex XN/XE series. Both these parameters have been shown to have an increased value in sepsis and a low value in cases of myelodysplastic syndromes or myelodysplastic/ myeloproliferative neoplasms (chronic myelomonocytic leukemia) [23,24].

Hematopoietic Progenitor Cell Count

The quantitative hematopoietic progenitor cell (HPC) count is offered by Sysmex XE-2100 and XN-2000. This can be used for determining the optimal time for cell harvest in cases of hematopoietic stem cell transplant. HPC count provided by the instrument is substantially equivalent to CD34+ count by flow cytometry [25].

PLATELET PARAMETERS

Mean Platelet Volume (MPV)

Mean platelet volume is derived from the impedance platelet size distribution curve. MPV is calculated by dividing the plateletcrit with platelet count. The normal reference range is 7-12 fL. It is provided by almost all latest analyzers. A high MPV is seen in inherited macrothrombocytopenia (like Bernard-Soulier Syndrome) and myeloproliferative neoplasms. MPV is also high in immune mediated pathologies of thrombocytopenia like immune thrombocytopenic purpura (ITP) as compared to primary bone marrow pathologies resulting in thrombocytopenia [26]. As large platelets are functionally more active than smaller ones, high MPV has been observed to predict higher risk of and following myocardial infarction and/or stroke in combination with other risk factors in various studies,

especially in pediatric cases with type I diabetes mellitus [27].

Reticulated Platelets and Immature Platelet Fraction (IPF)

Reticulated platelets are the young platelets with a higher RNA content. HAs like Sysmex XE/XN series, Abbott CELL-DYN Sapphire and BC-6800 Mindray quantify the reticulated platelets and IPF. The reference range is 1.1-6.1% of the platelet count [28]. IPF is raised in patients with peripheral destruction of platelets (ITP and thrombotic thrombocytopenic purpura, TTP) and is normal or low in patients with bone marrow failure [28]. IPF is also shown to be useful following a peripheral blood stem cell transplant where it has been shown to increase 1-2 days prior to the increase of platelet count [29]. They are increased in the circulation following recovery of thrombopoiesis in dengue fever and it has been shown in studies that a single value of >10% is indicative of platelet recovery within 24-48 hours, thereby reducing load of unnecessary platelet transfusions in clinical setting [30].

Case scenario 2

A 10-year-old girl presented with history of high-grade fever, arthralgia along with petechial rash and nose bleed for 2 days. There was an ongoing outbreak of dengue fever in the area and the resident doctor ordered complete blood counts (CBC) along with NS1 antigen test for dengue virus. Investigations revealed thrombocytopenia of $15x10^9$/L and the NS1 antigen test was positive. Her IPF value on third day of admission (***Fig.* 5**) was 26.1%. The patient was managed as dengue hemorrhagic fever with adequate hydration and antipyretic therapy and not given unnecessary platelet transfusions as IPF was > 10% and patient was likely to recover platelet count within 24-48 hrs as per studies. Subsequently the patient improved with a fall in hematocrit, and stable blood pressure and pulse rate. Subsequent days IPFs were 13.9%, !3.3%, 7.7%, 6.5%. The platelet count too showed a rising trend to a maximum of $134x10^9$/L.

Platelet Distribution Width (PDW), Plateletcrit and Platelet Large Cell Ratio (P-LCR)

Platelet distribution width measures platelet anisocytosis and the 'plateletcrit' is the product of the MPV and platelet count and may be seen as indicative of the volume of circulating platelets in a unit volume of blood. The platelet large cell ratio (P-LCR) is the ratio of number of platelets falling above the 20 fL threshold on the platelet size histogram divided by the total number of platelets. A high PLCR or PDW may be indicative of peripheral immune destruction of platelets [33]. PDW can be useful for distinguishing essential thrombocythaemia (PDW increased) from reactive thrombocytosis (PDW normal) [31]. The plateletcrit has been shown to be as a marker of active Crohn's disease [32]. All derived platelet parameters are highly specific to the individual technologies, with different HAs having different normal ranges.

TAKE HOME MESSAGE

- Automated NRBCs help give corrected WBC counts wherever necessary, avoiding errors of spuriously high WBC count. The IRF provides earliest information on bone marrow regeneration post-transplant or chemotherapy and is also a sensitive marker to monitor response to Iron/B12 therapy. Ret-He and % HRC help in rapid and sensitive differentiation of FID *vs* IDA and or ACD-IDA and especially useful in guiding parenteral iron therapy in CKD patients on ESA.
- IGs provide useful information on I:T and I:M ratio for diagnosing early onset neonatal sepsis and are very sensitive and precise than manual counts especially in leukopenic cases.
- % FRC and IPF if used together in clinically challenging cases can help in differentiating TTP from ITP cases. % FRC also has 100% sensitivity in ruling out microangiopathic haemolytic anemia.
- IPF better defines pathogenesis of thrombocytopenia than MPV. Moreover, it is a sensitive marker for assessing regenerating bone marrow post chemotherapy or transplant and is also a useful marker to predict recovery of platelet counts in dengue cases.

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TABLE I NOVEL COMPLETE BLOOD COUNT PARAMETERS WITH THEIR REFERENCE RANGE AND CLINICAL UTILITY

Parameter	*Reference values/range for pediatric age group*	*Where to use*	*Remarks*
RBC Parameters			
Nucleated RBC (NRBC)	3-7% (till day 4 of life for term infant); <21% (till day 10 of life for pre-term infant); <1% beyond the above time period	• Acute hemolytic episodes • Severe hypoxic states • To assess severity of thalassemia/ hemoglobinopathies and determine transfusion requirement • To suspect bone marrow infiltrative disorder • Prognostic marker in ICU/transplant patients	Indicates increased erythroid turnover
Fragmented red cells (FRC %)	1.4-1.9% (term infant);4.9-5.5% (preterm infant); <1% (beyond infancy)	Diagnosis of thrombotic microangiopathy in • DIC (perinatal asphyxia, infection, sepsis) • Neonatal hemolytic uremic syndrome • Congenital ADAMTS13 deficiency; Homozygous protein C deficiency; Giant hemangioma	• Good negative predictive value but low specificity (get a smear reviewed if FRCs are more) • Case with high MCV may show false negative results
Immature reticulocyte fraction (IRF %)	9.0-24.0 (6 months – 5yr); 7.5-23.4 (6-11yr); 6.5-26.7 (12-17 yr, F); 6.9-23.0 (12-17, M)	• To distinguish various types of anemias • Monitoring recovery following bone marrow transplant • Early diagnoses of chemotherapy-induced bone marrow aplasia • To assess response to iron or Vitamin-B12/ folate supplementation • To assess response to ESAs	Indicator of degree of effective erythropoiesis
Mean content of hemoglobin within the reticulocytes (CHr –pg)	For CHr-27.5-33.4 (6 months–5yr); 28.3-33.1 (6-11yr); 29.1-34.5 (12-17 yr, F); 28.8-35.2 (12-17 yr, M)	• Diagnosis of anemia of renal failure, anemia of chronic disease and iron deficiency anemia (Ret-He <25pg in IDA*) • To assess early response to iron supplementation (Ret-He <30.6pg*) or for monitoring EPO therapy (CHr value <29 pg and Ret-He value <25pg predicts FID in patients receiving EPO therapy*)	• Provide the status of functional iron available for the erythropoiesis during the last 3-4 days • Also reduced in hemoglobinopathies • Increased in macrocytosis
Mean reticulocyte volume(MRV-fl)	MRV-LH 750- 93-117.8 fl*MCVr-Advia 120- 98-115 fl*Cell Dyn Sapphire- 92-116 fl*	• Similar clinical utility as of mean content of hemoglobin within the reticulocytes • To monitor the response to iron or vitamin-B12/folate therapy • Screening cases of hereditary spherocytosis	Reference intervals should be determined according to the use of specific methods or analyzers
Percentage hypo-chromic cells (% HRC)	0.1-3.7 (6 months -5 yr); 0.1-2.9 (6-11 yr) 0.2-2.1 (12-17 yr, F)0.1-2.2 (12-17 yr, M)	• Assessment of iron restricted erythropoiesis (during last three months)	• Limited value if there is coexistent a-thalassemia

continued....

Mean sphered cell volume (MSCV)	Not available	• Used in conjunction with other parameters for screening of hereditary spherocytosis.	MCV-MSCV > 10 and MRV-MSCV <25 have good sensitivity and specificity
WBC Parameters			
WBCs volume, conductivity and scatter (VCS)	Not available	• Alters as per the diagnosis of diseases which causes changes in WBC populations	Various regression equations/ algorithms proposed for specific conditions
Immature granulocytes (IGs)	Not applicable	• Present in systemic inflammation and sepsis, hematological disorder like myeloproliferative neoplasm or acute myeloid leukemia, or a bone marrow infiltrative disorder	Includes promyelocytes, myelocytes and metamyelocytes. • Indicative of viral infection, lymphoma or leukemia.
Atypical lymphocytes	Not applicable	• Monitoring of sepsis because of viral infections	Warrants a smear review.
Neutrophil granulation	Not available	• Increased value in sepsis and low in cases of MDS or MDS/MPN (CMML)	NEUT-X lower than 1,315 and NEUT-Y lower than 400 may indicate MDS*.
Platelet Parameters			
Mean platelet volume (MPV)	7-12 fL*	• To assess bleeding disorders and thrombocytopenia	-
Immature platelet fraction(IPF)	1-5%*	• Increased in ITP/TTP and low to normal in bone marrow failure.	-

**Indicates cut-off values/reference range for adult patients; ICU: Intensive care unit; DIC: Disseminated intravascular coagulation; ADAMTS13: A disintegrin and metalloproteinase with thrombospondin like domain 13; MCV: Mean corpuscular volume; ESAs: Erythropoiesis stimulating agents; EPO: Erythropoietin; FID: Functional iron deficiency; MDS: Myelodysplastic syndrome; MDS/MPN: Myelodysplastic syndrome/Myeloproliferative neoplasm overlap; ITP: Immune thrombocytopenic purpura; TTP: Thrombotic thrombocytopenic purpura.*