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Morphologic Evaluation of Anemia – I

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Abstract

Anaemia is a feature of many tropical diseases. Anaemia diagnosis therefore remains a crucial intervention among physicians in developing countries. A barrage of laboratory test (anaemic work-up) is usually deployed in differentiating its underlying cause. However, central to anaemia evaluation is the morphology of the red cells and other cell lines. Conventionally, initial laboratory tests include full blood count, reticulocyte count and peripheral blood film (PBF). PBF is often a clinical request, performed by skilled technologist and reported by haematologist/haematomorphologist. Findings from PBF are reviewed and reported in the light of patient's clinical history and examination findings. This article therefore aims to promote PBF evaluation in anaemic patients, facilitate laboratory communication of morphologic findings among clinicians, particularly in developing nations where advanced investigations such as flow cytometry and molecular diagnosis may not be readily available, invariably improving patient care and treatment outcomes.

Keywords: Anaemia; Diagnosis; Full blood count; Reticulocyte count; Peripheral blood film (PBF)

Introduction

Anaemia has a significant public health burden in developing nations [1,2]. Anaemia is never a diagnosis - it occurs secondary to an underlying disease process. Technically, anaemia defines a state in which an individual's haemoglobin concentration (red cell mass) falls two standard deviations below the reference intervals in a particular population (individuals of similar age, gender and geographical location) [3,4]. In other words, anaemia cut off depends on variables such as biologic age, sex, race, and altitude above sea level, pregnancy, smoking status and others [5]. According to World Health Organisation (WHO), anaemia cut off for individuals of non-African extraction, non smokers, non pregnant, living at an altitude of 1000 meters below sea level, is defined in Table 1 below [5-8]. However, in individuals of African origin, a cut off of 1 g/dl lower is recommended [6]. In pregnancy, Center for Disease Control and prevention (CDC) in the US defines anaemia with a cut-off of 11 g/dl in the first and third trimester, 10.5 g/dl in the second trimester [9].

Various epidemiologic studies both locally and in other developing nations have highlighted the burden, distribution and risk factors of anaemia. According to WHO estimates, more than a third of the world population (2 billion) is affected by anaemia [1,2]. Developing nations in Africa and Asia bear its highest burden, especially women of child bearing age and children [1,2,10]. Over 30% of all women and 52.8 to 61.3% of women in developing countries are anaemic [1,2]. The prevalence of anaemia is as high as 26.8–27.3% among hospitalized patients in Nigeria [11,12]. Investigation of anaemia therefore remains a crucial aspect of diagnostic formulations in developing nations. As such, there is a need for practicing physicians to be conversant with current approaches to anaemia diagnosis. Despite advances in haematology automations, flow cytometry/immunophenotyping and molecular biology techniques, red cell morphology remains a vital aspect of anaemia work up [13]. In the present communication an attempt has been made to review the morphologic evaluation of anaemias and their clinical interpretations. Invariably, this will engender better communication between practicing clinicians and haemato-morphologists regarding diagnosis of anaemia. Emphasis is placed on the clinical utility of PBF and other morphologic tests used in the evaluation of anaemia. The clinical significance of major PBF findings is highlighted. This first treatise is limited to morphology of the peripheral blood (excluding bone marrow evaluations).

Age Groups:
Adult males above 15 years: Less than 13 g/dl
Non-Pregnant females above 15 years: Less than 12 g/dl
Teens aged 12 to 14.99 years: Less than 12 g/dl
Children aged 5 to 11.99 years: Less than 11.5 g/dl
Children aged 6 months to 4.99 years: Less than 11 g/dl
Pregnant women: Less than 11 g/dl
SEVERITY OF ANAEMIA
Mild (10 to 10.9 g/dl)
Moderate (7 to 9.9 g/dl)
Severe (less than 7 g/dl)

Table 1: WHO anaemia categories (haemoglobin cut-offs in g/dl).

All the relevant articles including original research and review papers were searched on major databases including pubmed and google scholar. Key words used in the search include anaemia, anaemia diagnosis, red cell morphology, epidemiology, peripheral blood films.

Result/findings were collated, analyzed and presented in the different sections of this manuscript.

Erythrocyte Morphology and General Aspects of Anaemia

Erythrocytes are anucleate, discoid blood cells packed with haemoglobin molecules (oxygen carrier molecule). Proliferation and differentiation of red cells takes place in the bone marrow (erythron) prior to their release into the peripheral circulation [14,15]. Final maturation takes about 1–2 days in the peripheral circulation through the pitting action of the spleen [15,16]. The life span of a normal red cell ranges about 100-120 days [17,18]. In normal physiologic state, production and senescence of erythrocytes is in a constant balance that occurs at a rate of about 1%, which represents about 250 billion erythrocytes in a healthy adult [17,18]. A tilt in this balance, either red cell underproduction, excessive destruction or both, triggers anaemia. The resulting functional consequence is decreased oxygen carrying capacity of the blood leading to tissue hypoxia. Symptomatology of anaemia, which may be specific or non-specific, depends on factors such as age of the individual, cardio-vascular reserve, the chronicity of the anaemia, co-morbidities and others [7,15]. Non-specific features of anaemia are hypoxia related effects on organ systems especially the heart, brain and muscles. They include easy fatiguability, dizziness, fainting spells, malaise, tinnitus, palpitations, paraesthesia, dyspnoea and angina of efforts (pre-existing cardiac disease) [19]. Other notable signs of anaemia include paleness of skin and mucous membrane Specific features are related to specific causes such as koilonychia in iron deficiency, atrophic glossitis in megaloblastic anaemia [4].

Quantitative and qualitative aberrations in circulating red cell or marrow precursor triggers anaemia. Almost all anaemias are associated with abnormalities in the size, shape, color, distribution or intracytoplasmic content of the red cells. In general, red cells have a fairly uniform variation in size, with a red cell distribution width of 11–15% in normal individuals. Abnormal variations in sizes and shape are termed aniso-cytosis and poikilocytosis respectively [14].

Manual techniques such as PBF microscopy and automated complete blood analysis (FBC) provide clinicians a good fenestra to evaluate anaemia in affected persons and both complement each other. Despite advances in automation of blood examinations, PBF/morphology remains sine qua non in medical parlance as regards anaemia diagnosis. Interestingly, automated blood film review is now possible using advanced microscopy such as the advanced RBC application of the Cellavision automated microscope which is able to pre-classify and pre-characterise almost all morphologic alterations in red cell morphology before validation [20]. However, manual PBF review still remains invaluable in developing nations where such technology is unavailable. Automated haematology analyzers (particle counters), which are readily available, are designed to flag off abnormal counts or morphology, thereby necessitating a need for morphologic evaluation of the circulating cells. In contrast to making a PBF slide (which may be quite cumbersome), automated analysis have the advantages of faster output, less manpower, less observer dependent variations, as well as, better accuracy and precision (if well calibrated). Clinical indications to request a peripheral blood film are highlighted in Table 2 [21,22]. A haemato-morphologist/haematologist relies on a well-made PBF slide, coupled with relevant clinical details and other laboratory test results, to make an individualized interpretation of the PBF request [21]. In situations where anaemia diagnosis remains cryptic after requisite peripheral blood analysis, bone marrow examination (needle aspiration and trephine biopsy) may be a necessary follow-up. Studies have shown that most common bone marrow diagnoses in developing nations are nutritional anaemias and leukaemias [23-27].

Unexplained peripheral blood cytopenias	Anaemia
	Leucopenia
	Thrombocytopenia
Unexplained high leucocyte counts	
Jaundice or haemolysis	
Features suggestive of an inherited haemolytic anaemia	
Suspected leukaemias or lymphomas	
Liver or renal failures	
Severe bacterial sepsis and parasitic infections	
Advanced cancers with possible bone marrow involvement	
Cases of nutritional anaemias	
Others	

Table 2: Indications for peripheral blood film.

Anisocytosis

Normal red cells (normocytes) are about 7–8 microns in diameter [16]. Reduced size is termed microcytosis. Increase in red cell diameter above normal is called macrocytosis. Red cell sizes form a basis for morphologic or cytometric classification of anaemia [28]. Anaemia could be associated with a microcytic, normocytic or macrocytic picture (Figure 1). On morphology, the size of a normocyte is compared to the nucleus of a small lymphocyte (Figure 2 - Slide A/B). The reference range for mean red cell volume (MCV) is 80-95 femtoliter [15,29]. Red cell size less than 6 micrometer and MCV less than 80fl is termed microcytic [30]. MCV greater than 95 fl is termed macrocytic. In terms of aetiology, microcytic anaemias are usually associated with iron deficiency, thalassemias, sideroblastic anaemia and anaemia of chronic inflammation (20% of cases). Usually, furthers test such as serum ferritin, total iron binding capacity (TIBC), haemoglobin electrophoresis with quantification helps to differentiate [19,29].

Anaemia may also be associated with normocytic picture, as in cases of acute blood loss, aplasia and endocrinopathies. Macrocytes are either oval or round. Oval macrocytosis (Figure 2 - Slide B) is seen in megaloblastic anaemias, myelodysplasia and drugs such as hydroxyurea [31]. Round macrocytes are seen in liver disease and alcoholism [32]. In combined (substrate) deficiency states, MCV may appear normal with the automated particle counter. However, the blood picture will reveal marked anisopoikilocytosis. The red cell distribution width (RDW) is a measure of size distribution/heterogeneity of the red cells. RDW is the percentage coefficient of variation of the individual red cell volumes of total number of red cells enumerated by the particle counter [33]. Normally, RDW ranges between 11–15%. Increased RDW is associated with iron deficiency anaemia, megaloblastic anaemia (folate and cobalamin deficiency), haemolytic anaemia, recent blood transfusion, hereditary

spherocytosis and sickle cell syndromes [33,34]. RDW is useful in interpreting apparently normal MCV since it will be quite high in combined deficiency state.

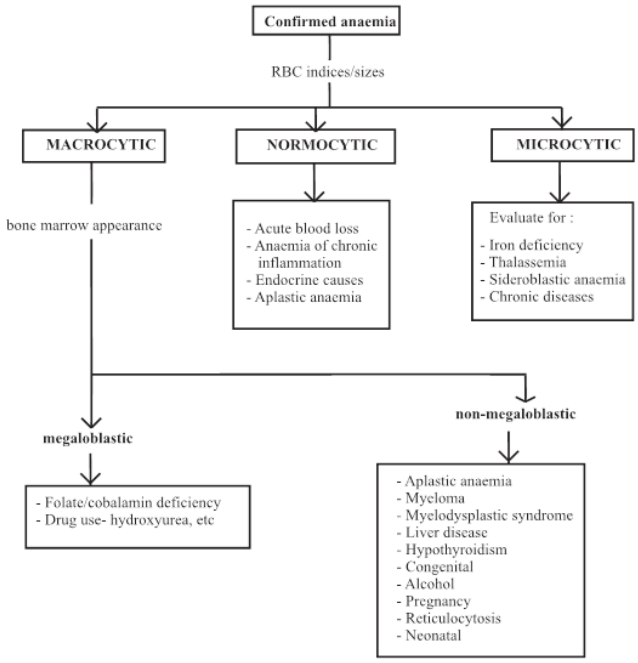

Figure 1: Aetio-morphological classification of anaemia.

Poikilocytosis

Shape anomalies (poikilocytes) are pointers to specific diagnosis. It is important to point out that shape anomalies may also occur in vitro (artefactual causes). As such, there is a need to control pre-analytic and analytic factors that can affect morphology, leading to wrong impressions. Blood specimens for PBF are best collected in EDTA bottles through venipuncture. Optimal blood:anticoagulant ratio should be observed. Samples should be dispatched immediately to the haematology laboratory. Prolonged delay in analysis allows for cellular degeneration and artefactual changes such as pseudo-thrombocytopenia [22]. Ideally, samples should be analysed within 2 hours of blood collection.

Poikilocytes may be categorized as spiculated or non-speculated. Spiculated forms includes fragmented red cells, burr cells, acanthocytes, tear drop red cells and sickle cell. Non-spiculated variants include target cells, ovalocytes and stomatocytes. Spiculated red cells have at least one pointed projection from the cell surface [30]. Various mechanical, biochemical and molecular processes underlie pathologic changes in red cell shape. Some occur as a result of disturbances in the haematopoietic system.

Teardrop cells (dacrocytes) are associated with an abnormal spleen or bone marrow as in primary myelofibrosis, where the red cells must stretch out in order to navigate its way into the periphery (Figure 2 – Slide C). Tearing of the red cell either occurs as the normal red cell attempts to pass through a distorted intermedullary vasculature of the spleen/bone marrow or as a result of stretching from the pitting action of the spleen, when red cells with inclusions such as heinz bodies navigates the splenic cords into the sinuses [30].

Target cells have a central of haemoglobinisation (hyperchromic bull eyes) surrounded by a halo of pallor (Figure 2- Slide D). Target cell have increased red cell surface area to volume ratio due to its redundant membrane which accounts for the targetoid shape. Target cell is believed to be due to decreased red cell volume as seen in haemoglobinopathies and iron deficiency or increased red cell membrane as in liver cholestasis, lecithin:cholesterol acyltransferase (LCAT) deficiency and post splenectomy state.

Stomatocytes are erythrocytes with slit like central pallor (fish mouth appearance) (Figure 2- Slide D). Stomatocytes mostly results from increase in red cell permeability, leading to increased red cell volume. Stomatocytes are either inherited or acquired. Hereditary sherocytosis is associated with the Rh null phenotype. Acquired stomatocytosis is associated with recent excessive alcohol and resolves within two weeks of alcohol withdrawal. Stomatocyte may also be artefactual and should be suspected when less than 10% of red cells are stomatocytes.

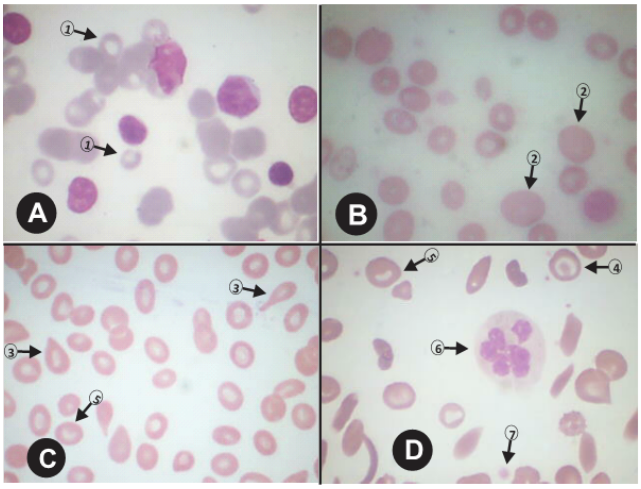

Figure 2: Anaemia morphology slides A-D. SLIDE A (Magnification x 1000): 1=microcyte. SLIDE B (Magnification x 1000): 2=oval macrocyte (compared to the nucleus of a mature lymphocyte). SLIDE C (Magnification x 1000): 3=tear drop red cell; 5=stomatocyte. SLIDE D (Magnification x 1000): 4=target red cell, 5=stomatocyte, 6=neutrophil hypersegmentation, 7=platelet/thrombocyte.

Fragmented red cells or schistocytes (Figure 3 – Slide E) often results from mechanical break-up/resealing of red cells in the periphery by microthrombi [35]. Pencil cells – (Figure 3 – Slide F) are shaped like pencil or cigar and are seen in iron deficiency anaemia. Elliptocytes (Figure 3 – Slide G) have elliptical shape and may reflect inherited defect (hereditary elliptocytosis). Elliptocytes are also seen in iron deficiency, myelodysplasia, megaloblastic anemia and thalassemias.

Another genetic cause of poikilocytosis is the irreversible sickle cells seen in sickle syndromes (Figure 3 - Slide H). In sickle cell syndromes, the primary event is intra-erythrocytic haemoglobin precipitation (gelation), with resultant formation of tactoids, which deforms the discoid red cell to sickle or crescent morphology [36].

Importantly, poikilocytes may also be artefactual. With experience, the morphologist will be able to identify artefactual causes. Great care should be exercised in preparation of blood smears to reduce artefactual poikilocytes such as crenated/burr cells; these cells may be due to poor fixation/high humidity in the ambience. Artefactual tear drop cells should be suspected if the tails line up in the same direction. Table 3 itemizes common poikilocytes and its differentials [13,14,21,30,37,38].

Red cell shapes	Differential diagnosis
Irreversibly sickled red cells (drepanocytes)	Sickle cell syndromes(SS, SC, Sβthalassemia)
Target cells (codocytes, mexican hat cells)	Sickle cell disease, haemoglobin C trait, haemoglobin CC disease, thalassemias, iron deficiency, Liver disease (cholestasis), asplenia,
Fragmented red cells (schistocytes, helmet cells, keratocytes)	Thrombotic micro-angiopathic haemolytic anaemias such as Disseminated intravascular coagulopathy (DIC), thrombotic thrombocytopenic purpura, haemolytic uraemic syndrome.
Pencil cells	Iron deficiency
Stomatocytes	Artifact(due to slow drying in humid environment), Liver disease, alcoholism, Rh-null disease, Obstructive lung disease
Elliptocytes	Hereditary Elliptocytosis (>25%)
Bite cells (degmacytes)	G6PD deficiency, Oxidative stress, unstable haemoglobins, congenital heinz body anaemia
Basket cells (half ghost cells/Blister cells)	Oxidant damage, G6PD deficiency, Unstable haemoglobins
Spherocytes	Hereditary spherocytosis, ABO incompatibility, Autoimmune hemolytic anemia (warm antibody type), Severe burns
Teardrop red cell (dacrocytes, lacrymocytes)	Idiopathic myelofibrosis, myelophthisic anaemia, thalassemias

Table 3: Poikilocytosis and differentials.

Anisochromia/polychromasia

Anisochromia refers to increased or decreased haemoglobinization of the red cells. The most common form is hypochromia (when the central pallor exceeds one third of the entire red cell diameter). Hypochromia usually follows microcytosis. Occasionally, severe hypochromia is associated with macrocytic red cells, termed leptocytes. Leptocytes are seen in severe iron deficiency, thalassemia and liver diseases [37].

Increased haemoglobination are associated with shape abnormalities such as (micro)-spherocytes and sickled red cells. Increased haemoglobinization (dense red cells) obliterates central pallor.

Polychromasia on PBF is synonymous with reticulocytosis. Polychromasia literally means 'many colours', i.e. the red cells bears another shade of colour than pink (eosinophilic). Polychromatic red cells are macrocytic (young red cells) and have a bluish tinge. The bluish tinge denotes the presence of rRNA which eventually undergo the pitting action of the spleen to become mature erythrocytes [14]. Normally, polychromatic red cells are not obvious on PBF – adult reticulocyte population is about 0.5–2.5% [15]. However, polychromatic red cells in excess of 1–2% in the periphery should be considered significant since normal daily rate of red cell turnover is about 1–2% [18]. In situations of acute haemorhage, haemolysis, and high altitude, hypoxia induces increased erythroid activity, hence polychromasia. Polychromasia is also seen in extramedullary haemopoiesis due to myeloid metaplasia in reticulo-endothelial tissue. Polychromatic red cells are also seen as a response to haematinic therapy in substrate (nutritional) deficiency anaemias [14].

In severe situations, nucleated red cells (normoblastemia) also appear in the periphery (Figure 3 - Slide H) [38]. Conditions associated with normoblastemia includes severe anaemia, asplenic/hyposplenic state as in sickle cell disease, severe hypoxia, marrow replacements or infiltrations and extramedullary haemopoiesis [39,40]. Nucleated red cells may be seen normally in neonates [38].

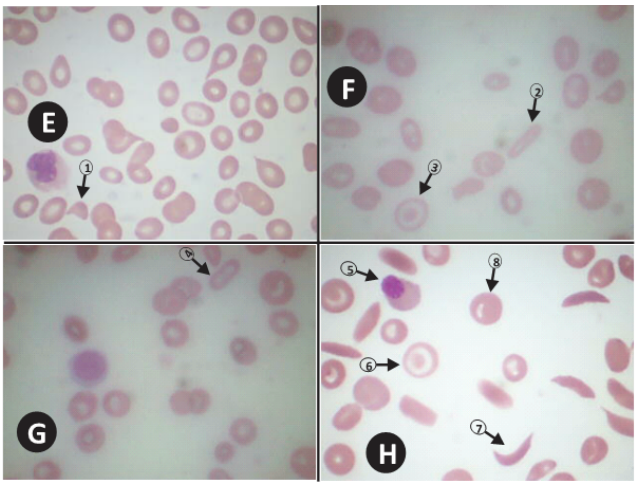

Figure 3: Anaemia morphology slides E-H. SLIDE E (Magnification x 1000): 1=fragmented red cell. SLIDE F (Magnification x 1000): 2=pencil cell, 3=target cell, SLIDE G (Magnification x 1000): 4=elliptocyte, SLIDE H (Magnification x1000): 5=nucleated red cell (orthochromatic erythroblast); 6=target cell; 7=irreversibly sickle red cell.

Other red cell anomalies

These include presence of inclusion bodies and pathologic distribution of red cells on smear. A mature erythrocyte lacks inclusion bodies. Red cell inclusion bodies include nuclear products RNA/DNA, haemoglobin or iron pigments. Some, such as haemoglobin H inclusions and Heinz bodies can only be appreciated with supravital staining [14]. Red cell inclusions result from oxidant stress, severe infections and dyserythropoiesis (maturation defects). Basophilic stipplings or punctuate basophilia are denatured RNA fragments dispersed within the cytoplasm. Basophilic stipplings may be fine, blue stipplings or coarse granules. They are non-specific and are generally related to disorders in haem biosynthetic pathways [14,41]. Differentials include haemoglobinopathies (thalassemias), lead or arsenic poisoning, unstable haemoglobins, severe infections, sideroblastic anaemia, megaloblastic anaemia and a rare inherited condition, pyrimidine 5' nucleotidase deficiency [14,22,42].

Clinically insignificant, fine basophilic stippling may be associated with polychromasia/accelerated erythropoiesis/reticulocytosis. Coarse stipplings are clinically significant and indicates impaired haemoglobin synthesis as seen in megaloblastic anaemia, thalassemias, sideroblastic anaemias and lead poisoning [14,41]. Unlike other basophilic inclusions such as Howell jolly bodies and Pappenheimer bodies which tend to be displaced to the periphery, basophilic stipplings are diffusely dispersed throughout the red cell cytoplasm. Howell jolly bodies are DNA remnants seen in post-splenectomy patients, anatomical or functional asplenia (Figure 4-Slide J). Siderotic granules or Pappenheimer bodies appear purple on Rowmanosky stain, blue on perl's stain and are seen in disorders of iron utilization like sideroblastic anaemias.

Parasites such as *Plasmodium* spp. or *Babesia* spp. may also be seen on peripheral blood smear [43]. Both parasites invade the red cells. Their identification requires some level of knowledge and experience. Several species of *Plasmodium* spp. exist. *Plasmodium* spp. may exist in different forms such as ring forms (trophozoites), gametocytes and schizonts. *Babesia* spp. appear in small ring forms (like *Plasmodium falciparum*) but schizonts and gametocytes are not formed [14,43]. Unlike *Plasmodium* spp., *Babesia* spp. do not produce pigments. However, *Babesia* spp. may appear in groups outside the erythrocyte. Clinical history and travel history is also helpful in differentiating the two parasites. Other red cell inclusions such as Heinz bodies and Haemoglobin H inclusions can only be appreciated with supravital staining (reticulocyte preparations). Heinz bodies are denatured haemoglobin (seen in oxidant injury, G6PD deficiency). Haemoglobin H inclusions are seen in alpha-thalassemias giving rise to the characteristic 'golf ball' appearance of the erythrocytes [13,14,36].

Rouleaux formation refers to stacking of red cells like coins in a single file. Rouleaux is seen in hyperproteinaemias. Elevated plasma fibrinogen or globulins reduces the zeta potential (repulsive force) between circulating red cells, facilitating their stacking effect. Rouleaux is associated with myeloma/paraproteinaemias, other plasma cell disorders as well as B cell lymphomas (Figure 4 – Slide I). On the other hand, agglutination refers to clumping or aggregation of red cells into clusters or masses and is usually antibody mediated [14]. Agglutination of red cells may be seen in cold haemagglutinin disease and waldenstroms macroglobulinaemia [14,36]. Agglutination is associated with falsely reduced red cell count and high MCV. Pre warming the specimen with heating block helps to disperse the red cells prior to making of a blood smear and automated cell counts.

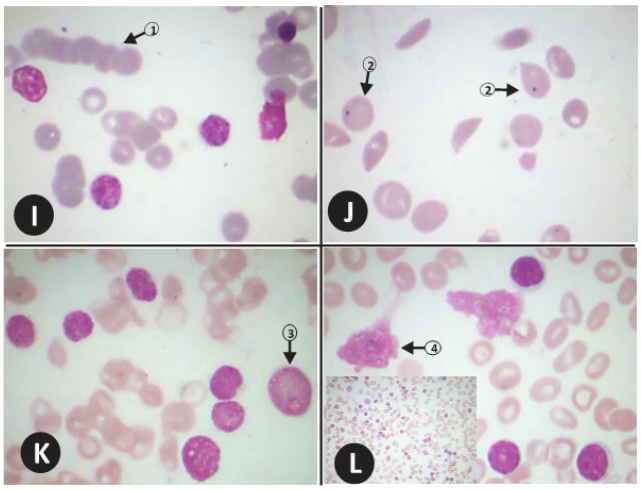

Figure 4: Anaemia morphology slides I-L. SLIDE I (Magnification x 1000): 1=rouleaux formation. SLIDE J (Magnification x 1000): 2=Howell jolly inclusion bodies in the cytoplasm of red cells of a 36 year old haemoglobin SS disease patient. SLIDE K (Magnification x 1000): 6=lymphoid blast (moderate size blast with scanty cytoplasm, high nuclear-cytoplasmic ratio, single prominent nucleolus). SLIDE L (Magnification x 1000, Inset x 400): 4=small (mature looking) lymphocyte; 5=Smear cells; Inset: Sea of mature looking, monomorphic lymphoid cells and smear cells. PBF features are in keeping with chronic lymphocytic leukaemia.

Other Cell Lines

Haemopoiesis is trilineage and closely linked. As such, anaemia may be associated with abnormalities in leucocytes and platelets, which are also evident on blood film. Though anaemia is the main focus of this writing, it is important to mention some commonly associated abnormalities in other cell lineages.

Cytopenia involving two or more cell lines (anaemia, leucopenia or thrombocytopenia) often suggests central marrow suppression or failure [4,44]. This is often associated with hypoproliferative anaemia. On a well-made blood smear, pancytopenia is seen as general paucity of cells. Causes of pancytopenia are listed in Figure 5 below [15]. Constant cytopenias with marked dysplasia (>10%) in one or more cell lines suggests myelodysplastic syndrome [45] and should warrant further investigation including bone marrow evaluation, cytogenetics and molecular studies [45].

Anaemia may also be associated with increased proliferation in other cell lines. For instance, acute leukaemias typically show marked increases in white cell population on morphology, alongside anaemia and thrombocytopenia (Figure 4 - Slide K). Broken cells (otherwise called smear or smudge cells) are seen in chronic lymphocytic leukaemias (Figure 4 - Slide L).

The term, 'leucoerythroblastic' is used when left shift (immature leucocytes) are seen in the background of nucleated red cells. Leucoerythroblastosis occurs in the setting of bone marrow stress such as severe sepsis, severe anaemia, hypoxia or even extramedullary haemopoiesis. Other causes of leucoerythroblastic picture include

marrow fibrosis, marrow infiltrations (particularly secondary metastasis) and marrow challenge with growth factors such as G-CSF.

Neutrophil hypersegmentation is often seen in megaloblastic anaemia [29,31]. Less commonly, hypersegmented neutrophil occurs in severe iron deficiency, renal disease and familial causes.

Reactive thrombocytosis is associated with acute haemorhage or haemolysis, iron deficiency, connective tissue diseases, corticosteroid use, major surgery and preterms. On morphology, platelets are increased in counts, with platelet anisocytosis (large platelets).

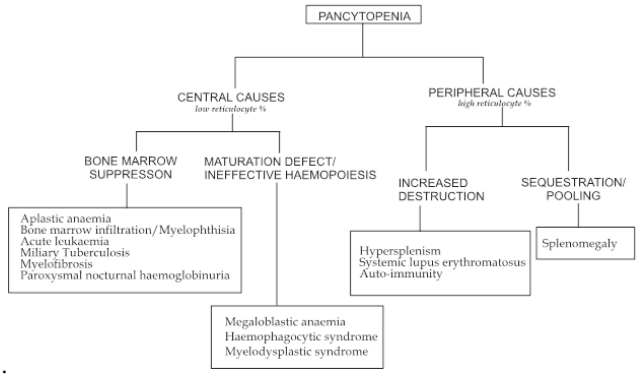

Figure 5: Causes of Pancytopenia.

Conclusion

Though investigation of primary haemopathies especially anaemias and haematological malignancies goes beyond cellular haematology (morphology) to include immunophenotyping/flow cytometry and molecular assays, morphology remains the initial (baseline) tool for diagnostic evaluations. Again, morphologic evaluation of anaemia remains relatively sine qua non in developing nations where flow cytometry immunophenotyping and molecular diagnosis may not be available.

Specific morphologic abnormalities of red cells corresponds to numerous differential diagnosis, which are best interpreted by trained haemato-morphologists (haematologists with laboratory background) in light of the individual's clinical history, physical findings and ancillary laboratory results.

Anaemia is never normal. Its cause should be sought and treated appropriately. Clinicians should request PBF and engage the haematologist when necessary, especially in cases of moderate-severe anaemia.

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