NEUTROPHILIA

ABSTRACT:

The production and distribution of normally functioning neutrophils is vital to host defenses. In order to understand the normal condition compared to changes that occur in disease states, the development, structure, function, and kinetics of neutrophils will be discussed.

There are a number of causes for increase in neutrophils. In this course we present two cases of neutrophilia and compare the etiology, laboratory findings, and microscopic morphology for each. Other causes of neutrophilia will be discussed.

OBJECTIVES:

After completing this course the participant will be able to:
1. Outline the maturation stages of neutrophils.
2. Discuss how neutrophils protect the body against foreign invaders.
3. Explain the difference between shift neutrophilia and absolute neutrophilia.
4. Compare the microscopic morphology of neutrophils in infections to those in chronic myelocytic leukemia.
5. Discuss the other findings and causes of neutrophilia in infections compared to chronic myelocytic leukemia.
6. List other causes of neutrophilia.

Case #1: A 59-year-old patient with high fever and chills

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>27.2 x 10^3/µL</td>
</tr>
<tr>
<td>Lym</td>
<td>9.3 %</td>
</tr>
<tr>
<td>MLD</td>
<td>2.7 %</td>
</tr>
<tr>
<td>Gran</td>
<td>88.0 %</td>
</tr>
<tr>
<td>PLT</td>
<td>222 x 10^3/µL</td>
</tr>
</tbody>
</table>

MID cells may include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts, and other precursor white cells.

- What is abnormal about the CBC?
- Which parameters can be reported?
- What procedures can be done regarding abnormal result(s)?
Case #2: A 35-year-old male complaining of fatigue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>10^3/μL</td>
<td>4.1 - 11.0 x 10^3/μL</td>
</tr>
<tr>
<td>Lym</td>
<td>5.8 %</td>
<td>13.0 - 48.5 %</td>
</tr>
<tr>
<td>MID</td>
<td>7.1 %</td>
<td>0.1 - 11.0 %</td>
</tr>
<tr>
<td>Gran</td>
<td>87.1 %</td>
<td>46.5 - 82.0 %</td>
</tr>
<tr>
<td>PLT</td>
<td>189 x 10^3/μL</td>
<td>140 - 440 x 10^3/μL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>4.33 x 10^6/μL</td>
<td>4.20 - 6.30 x 10^6/μL</td>
</tr>
<tr>
<td>HGB</td>
<td>13.5 g/dL</td>
<td>12.0 - 18.0 g/dL</td>
</tr>
<tr>
<td>HCT</td>
<td>38.9 %</td>
<td>37.0 - 51.0 %</td>
</tr>
<tr>
<td>MCV</td>
<td>89.9 fL</td>
<td>80.0 - 97 fL</td>
</tr>
<tr>
<td>MCH</td>
<td>31.2 pg</td>
<td>26.0 - 32.0 pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>34.7 g/dL</td>
<td>31.0 - 36.0 g/dL</td>
</tr>
<tr>
<td>RDW</td>
<td>13.6 %</td>
<td>11.5 - 14.5 %</td>
</tr>
</tbody>
</table>

MID cells may include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts, and other precursor white cells.

DISCUSSION:

Neutrophils are one of the three granulocyte types (eosinophils, basophils, and neutrophils) found in peripheral blood. Neutrophils are the most numerous leukocyte, (white blood cell [WBC]), normally 55% to 75% of the leukocytes in the adult. The neutrophil is so named because the granules in mature neutrophils stain with the neutral dyes in Wright’s stain, appearing faint pink-lavender.

Development of Neutrophils:

Neutrophils are produced in the bone marrow from pluripotential hematopoietic stem cells. These hematopoietic stem cells can differentiate into all the blood cell types. One line of differentiation begins with a stem cell (CFU-GEMM) capable of developing into granulocytes, erythrocytes, monocytes, and megakaryocytes. The next differentiation is the CFU-GM (colony forming unit granulocyte, monocyte). This in turn differentiates into CFU-G, the neutrophil cell line. This neutrophil stem cell is no longer capable of producing other blood cell types. Myeloblasts are the first morphologically recognizable cells in the neutrophil cell line. Neutrophils go through six morphologic stages before release into the peripheral blood: myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and polymorphonuclear (PMN) neutrophil. The first three of these stages are capable of replication as well as differentiation (maturation). The last three stages are only able to mature. The maturation stages and morphology of immature neutrophils are shown in the following sequence:
Maturation and Morphology of Immature Granulocytes

- **Myeloblast**: the first and earliest granulocyte
  - Is a large cell (15 µm)
  - High nucleus to cytoplasm (N:C) ratio (5:1)
  - Round or oval nucleus with loose light staining euchromatin
  - 1-2 nucleoli
  - Has minimal light blue cytoplasm
  - Contains no cytoplasmic granules
  - Begins to produce myeloperoxidase granules (MPO)
  - Comprises 1% of the nucleated cells in the bone marrow
  - Takes 18 hours to mature

- **Promyelocyte**: larger than a myeloblast (20 µm)
  - High N:C ratio (3:1)
  - Loose chromatin with nucleoli
  - Dark blue cytoplasm
  - Contains large nonspecific cytoplasmic granules containing myeloperoxidase (MPO)
  - Comprises 3-4% of nucleated bone marrow cells
  - Takes 24 hours to mature

- **Neutrophilic Myelocyte**: medium cell size (12 µm)
  - High N:C ratio (3:1)
  - Round, oval, or slightly indented nucleus with darker blue heterochromatin
  - Last stage of cell division
  - Has active RNA, therefore, the cytoplasm is blue
  - Contains MPO and secondary granules containing leukocyte alkaline phosphatase
  - Comprises 12% of bone marrow nucleated cells
  - Takes 100 hours to mature

- **Neutrophilic Metamyelocyte**: size (11 µm)
  - N: C ratio (2:1)
  - last mononuclear stage, no mitosis
  - Nucleus is kidney or horseshoe shaped, and has condensed heterochromatin
  - Has a prominent Golgi apparatus – clear area located at the indentation site of the nucleus
  - Cytoplasm is similar to the mature cell
  - Comprises 18% of bone marrow cells
  - Takes 72 hours to mature
• Band
  • Same size as a mature neutrophil (10-12 µm)
  • N:C ratio has reversed (1:2)
  • Nucleus is band-or sausage-shaped without segmentation
  • Cytoplasm is filled with small neutrophilic granules
  • Last immature stage
  • Comprises 11% of bone marrow cells and 0-3% of peripheral WBCs
  • Stored in the bone marrow and released when there is an increased demand for neutrophils
  • Shift to the left is an increase in immature cells indicating increased demand for WBCs in peripheral blood
  • Takes 48 hours to mature

Morphology of Mature Granulocytes

• Neutrophils
  • Also known as segmented neutrophils, segs, polymorphonuclear cells, polys, and PMNs
  • N:C ratio is 1:3, and the size is 10-12 µm
  • Average nucleus contains 3-5 segments connected by narrow filaments
  • Hyposegmented is less than 3 segments, and may indicate a shift to the left or an anomaly
  • Hypersegmented is more than 5 segments and may indicate infection or megaloblastic anemia
  • Cytoplasm contains very small neutral granules
  • Granules can become larger upon bacterial infection producing toxic granulation, which are numerous, large, basophilic granules
  • Makes up 55-75% of all peripheral WBCs
  • Average time spent in the blood is 10 hours

• Eosinophils
  • Average size is 13 µm
  • Nucleus is generally bilobed
  • Cytoplasm is bright red or orange which is due to large specific, secretory granules containing peroxidase, acid phosphatase, aryl sulfatase, betaglucuronidase, etc. that stain red with the eosin component of Wright’s stain
  • Makes up 3% of WBCs in the peripheral blood
• **Basophils**

  - Is the smallest granulocyte at 10 µm
  - The nucleus is difficult to see due to heavy granulation
  - Cytoplasm contains large specific, secondary granules that contain **heparin** and **histamines**, which stain purple with Wright's stain. These granules are water-soluble and sometimes appear as holes in the cell if the cells are not fixed well during staining.
  - Makes up to 0.5% of peripheral WBCs
  - Note: **Tissue mast cells** are similar to basophils but are larger and have no developmental relationship with basophils. Mast cells have a mesenchymal (connective tissue) origin and have granules containing **serotonin** (basophilic granules contain no serotonin).

**Function of Neutrophils:**

The major role of neutrophils is to protect the body against infectious agents. Their granules contain substances that are bactericidal, hydrolytic, and activate the complement cascade. Neutrophils have three granule types. The azurophilic or primary granules contain myeloperoxidase, defensins, cathepsin, lysozyme, and several other proteins and enzymes. The specific or secondary granules contain lactoferrin, collagenase, lysozyme, and other factors. The gelatinase or tertiary granules contain gelatinase, acetyltransferase, and lysozyme. In addition, secretory vesicles are present. They contain stores of surface membrane bound receptors. When the neutrophil is activated, the secretory vesicles fuse with the plasma membrane, releasing receptors help prime the neutrophil for antimicrobial action.

Neutrophils protect by migrating to the source of infection or irritation and destroying the foreign substance. They are attracted by chemo-attractants released at the site of infection. The neutrophils follow the concentration gradient of the chemotactic agents, which also prime the neutrophil for subsequent activation. After arriving at the area of infection, the neutrophils adhere to the blood vessel wall and migrate through the wall to the site of infection. The chemo-attractant agents bind to receptors on the neutrophils, setting in motion morphologic changes and metabolic activation.

At the site the neutrophils project pseudopodia that surround the foreign particles, initiating phagocytosis. This process is enhanced if the foreign organism has antibodies or complement factors attached. The organism is engulfed into a phagosome formed by invagination of the neutrophil’s cell membrane.

The contents of the neutrophil’s azurophilic (primary) granules are discharged into the phagosome. The myeloperoxidase catalyzes the production of hypochlorite, a potent anti-microbial. Other oxidative killing mechanisms involve the production of hydrogen peroxide, superoxide anion and singlet oxygen. These substances kill the phagocytized organism. The dead and dying neutrophils, with their contents, form the exudate (pus) seen at the site of the infection.

Specific (secondary) granules are released into the extracellular space. Some of their products activate the complement cascade. Collagenase helps hydrolyze the extracellular matrix, aiding locomotion of the neutrophil through the tissues. Tertiary granules contain gelatinase that plays a similar role in locomotion.
Neutrophil kinetics:

Neutrophils take seven to eleven days to go through the maturation stages in the bone marrow. The last three maturation stages form a resting pool of PMN neutrophils, bands, and metamyelocytes in the bone marrow. The PMNs are released into the peripheral blood to replace those that go into the tissues. When there is increased demand the bone marrow is stimulated to release cells from the neutrophil storage pool: the PMNs, bands and then the metamyelocytes enter the peripheral blood. If the demand is great enough there is stimulus for increased production of neutrophils.

PMNs normally spend ten hours in peripheral blood before migrating into the tissues. Once in the peripheral blood the neutrophils enter the circulating granulocytic pool (CGP) or the marginal granulocytic pool (MGP). The MGP is still in the vascular space but these cells are adherent to the blood vessel walls, especially in small vessels of post-capillary venules. These pools are about equal in size. The cells in these pools can go from one pool to another. For instance, exercise or epinephrine injection can cause demargination and a transient (about 30 min.) increase in the CGP. However the total blood granulocytic pool (TBGP) remains unchanged. The temporary increase in the WBC count at this time should not be confused with an absolute increase in the TBGP. In these demargination cases there is no increase in immature forms in peripheral blood.

NEUTROPHILIA:

Neutrophilia refers to higher than normal numbers of neutrophils (over 11,000/µL) in peripheral blood. This can be a temporary shift of marginal to circulating neutrophils without an increase in TBGP or an absolute increase in the size of the TBGP. Absolute neutrophilia involves an increase in cells from the bone marrow.

Most cases of absolute neutrophilia are associated with infections caused by cocci (e.g. staphylococci, pneumococci, streptococci, meningococci, gonococci), bacilli (e.g. E. coli, Pseudomonas aeruginosa, Actinomyces species), certain fungi, spirochetes, rickettsia, and parasites. Viruses are less likely to cause significant neutrophilia, but rather an increase in lymphocytes. During early infection the neutrophil count can decrease due to increased margination of cells near the site of infection. This is followed by neutrophils entering the circulation from the bone marrow and an increase in the TBGP.

The total leukocyte count in infections is usually below 50,000/µL. A shift to the left (release of younger neutrophils) is due to increased demand and release of band cells and metamyelocytes. If the infection continues there is increased rate of production of neutrophils. As the infection subsides the inflow from the bone marrow decreases and the WBC count falls.
In severe infections there may be such an increase in the leukocyte count, over 30,000 to 50,000/µL, that the blood picture may resemble leukemia. This is called a leukemoid reaction. The differences between a leukemoid reaction and leukemia are:

<table>
<thead>
<tr>
<th>Finding</th>
<th>Leukemoid reaction</th>
<th>Chronic granulocytic leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocyte count</td>
<td>30,000 to &gt;50,000/µL</td>
<td>May be well over 100,000/µL</td>
</tr>
<tr>
<td>Immaturity of neutrophils</td>
<td>No blasts or promyelocytes</td>
<td>Blasts and promyelocytes may occur</td>
</tr>
<tr>
<td>Basophils</td>
<td>Normal %</td>
<td>May be increased</td>
</tr>
<tr>
<td>Toxic granulation</td>
<td>Frequently present</td>
<td>Not usually present</td>
</tr>
<tr>
<td>Döhle bodies</td>
<td>Frequently present</td>
<td>Not usually present</td>
</tr>
<tr>
<td>RBC morphology</td>
<td>Usually normal</td>
<td>May be abnormal</td>
</tr>
<tr>
<td>Leukocyte alkaline phosphatase</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Philadelphia chromosome</td>
<td>Absent</td>
<td>Usually present</td>
</tr>
</tbody>
</table>

Other causes of neutrophilia are:

Hematologic disorders: chronic myelocytic leukemia, polycythemia vera, myelofibrosis, myeloid metaplasia.

In these disorders there are neoplastic transformations in clonal hematopoietic stem cells resulting in excessive production of granulocytes and their precursors. The granulocyte production is not under physiologic control of increased demand but is uncontrolled. In some of these disorders other hematologic cell lines may also be involved.

Non-infectious inflammation: burns, postoperative state, acute myocardial infarction, gout, acute glomerulonephritis, rheumatic fever, hypersensitivity reactions. Neutrophilia in severe burns shows a shift to the left and the presence of degenerative forms, including toxic granulation and Döhle bodies. Neutrophilia post-operatively is caused by release of adrenocortical hormones as a result of tissue injury.

Metabolic: diabetic ketoacidosis, preeclampsia, uremia.

Poisoning: lead, mercury, digitalis, camphor, antipyrine, phenacetin, quinidine, pyrogallol, turpentine, arsenamine, insect venoms.

Acute hemorrhage, especially into peritoneal, pleural, joint or intracranial cavities. Neutrophilia is probably due to pain and release of epinephrine and corticosteroids. During the first one to three hours after an acute hemorrhage, neutrophilia occurs due to a shift from the marginal pool to the circulating pool. This is followed by release of neutrophils from the marrow.

Malignant neoplasms: probably due to tumor necrosis factor or production of neutrophilic growth factors in rapidly growing neoplasms. Acute or long-term administration of corticosteroids.

Physiologic: strenuous exercise and epinephrine cause transient neutrophilia. Can also be seen in pregnancy, labor, and in newborns.
Case #1: 59-year-old patient with high fever and chills
Answers:
1. The WBC count is above normal and the differential is abnormal.
2. The RBC parameters and platelet numbers are normal and can be reported.

<table>
<thead>
<tr>
<th>Manual differential:</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN Neutrophils 26 %</td>
<td>40 – 75 %</td>
</tr>
<tr>
<td>Bands 60 %</td>
<td>0 – 9 %</td>
</tr>
<tr>
<td>Lymphocytes 12 %</td>
<td>16 – 46 %</td>
</tr>
<tr>
<td>Monocytes 2 %</td>
<td>0 – 12 %</td>
</tr>
<tr>
<td>PMN toxic granulation 2+</td>
<td>none</td>
</tr>
<tr>
<td>PMN vacuolation 1+</td>
<td>none</td>
</tr>
<tr>
<td>PMN Döhle bodies 1+</td>
<td>none</td>
</tr>
</tbody>
</table>

The blood picture shows increased neutrophils, 86% total, with a shift to the left but no immaturity beyond bands. The neutrophils also show changes that are characteristic of infection: toxic granulation, vacuolation, and Döhle bodies. Toxic granulation: accumulation of dense azurophilic, peroxide positive granules occurring with rapid production of neutrophils; associated with infection. They may also occur in toxemia of pregnancy, vasculitis, or in patients receiving chemotherapy. They may also be an artifact of staining if the stain is too basic.
Döhle bodies: seen as oval, single, or multiple cytoplasmic inclusions composed of aggregated strands of rough endoplasmic reticulum (RNA). They are associated with rapid production of neutrophils; found in patients with severe infection, burns, aplastic anemia and in patients receiving chemotherapy. In these conditions Döhle bodies represent toxic changes. They are also observed in hereditary disorders: May-Hegglin anomaly and in Chédiak-Higashi syndrome. Vacuolization: round clear unstained areas randomly dispersed in the cytoplasm. When seen in neutrophils, their presence suggests very severe infection.

**Diagnosis:** Neutrophilia due to bacterial infection. Neutrophils show a ‘shift to the left’— increased bands, toxic granulation, vacuolation, and Döhle bodies, all associated with increased demand due to the infection.

**Case #2: 35-year-old male complaining of fatigue**

**Answers:**
1. The WBC count is above normal and the differential is abnormal.
2. Although the Hgb and Hct are slightly low for a male, the RBC parameters and platelet numbers are normal and can be reported.
3. **a.** The WBC count is too high to be reported by the automated hematology instrument: do a 1:1 dilution of whole blood and re-run WBC count. The WBC count on the diluted blood was 68.9 x 10^3/µL. Correct for the dilution: 68.9 x 2 = 137.8 x 10^3/µL.

   **b.** Perform a manual WBC differential because of the abnormal automated differential and to corroborate the high WBC count.

   ![Image 5: CML](image5.png)

**Diagnosis:** The very high white count and the immaturity of the neutrophils that goes back to blast cells, but with predominance of more mature forms, are characteristic of chronic myelocytic leukemia (CML). The near normal RBC numbers and normal morphology as well as normal platelet numbers also are indicative of CML rather than acute myelocytic leukemia (AML), which has anemia, sometimes severe, and decreased platelets. CML may have anemia but it is mild. Platelet numbers in CML are normal to increased.
In chronic myelocytic leukemia there are other characteristics that can sometimes be observed on the blood smear: increased numbers of basophils, eosinophils and/or platelets, including giant platelets or megakaryocytic fragments. Occasionally nucleated RBC may be found along with RBC abnormalities—aniso-poikilocytosis, basophilic stippling, and polychromasia.

Other laboratory findings are
1. The presence of the Philadelphia (Ph) chromosome. This is a cytogenetic abnormality involving a translocation between the long arms of chromosomes 22 and 9. The translocation results in a shortened chromosome 22, called the Philadelphia chromosome, named after the city in which the discovery was made. The Ph chromosome is found in 90 to 95% of the cases of typical CML. Its presence is diagnostic of the disease.
2. Low levels of leukocyte alkaline phosphatase. This is in contrast to neutrophilia caused by infection in which there are high levels of leukocyte alkaline phosphatase.

CONCLUSION:
Neutrophil function, development, and kinetics were discussed as a background for understanding neutrophilia. Photomicrographs illustrated the maturation morphology in the neutrophil line. The two cases illustrate the differences associated with neutrophilia due to infection compared to neutrophilia due to chronic myelocytic leukemia. Other causes of neutrophilia were also discussed.

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REFERENCES:
HEMATOLOGY CASE STUDY:
A HYPOCHROMIC, MICROCYTIC ANEMIA

CASE: A 19 year old man (C.C.) had been competing as an amateur boxer. He was successful enough to turn professional. He found his stamina decreased in the longer pro-boxing bouts, making him less competitive in the professional ranks. Concern regarding his boxing future caused him to consult a physician. The physical exam was normal. His CBC yielded the following results:

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULTS</th>
<th>SI UNITS</th>
<th>CONVENT. UNITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>9.5 x 10³/ml</td>
<td>9.5 x 10⁹/l</td>
<td>4.8-10.8 x 10⁹/ml</td>
</tr>
<tr>
<td>RBC</td>
<td>5.35 x 10⁶/ml</td>
<td>5.35 x 10¹²/l</td>
<td>4.2-5.4 x 10⁶/ml</td>
</tr>
<tr>
<td>Hgb</td>
<td>10.5 g/dl</td>
<td>105 g/l</td>
<td>14.0-18.0 g/dl (male)</td>
</tr>
<tr>
<td>Hct</td>
<td>36.0%</td>
<td>360 l/l</td>
<td>42-52% (male)</td>
</tr>
<tr>
<td>MCV:</td>
<td>67 fl</td>
<td>67 fl</td>
<td>80-99 fl</td>
</tr>
<tr>
<td>MCH:</td>
<td>19.6 pg</td>
<td>19.6 pg</td>
<td>27-31 pg</td>
</tr>
<tr>
<td>MCHC:</td>
<td>29.2 g/dl</td>
<td>292 g/l</td>
<td>33-37 g/dl</td>
</tr>
<tr>
<td>RDW:</td>
<td>14.2%</td>
<td></td>
<td>11.5-14.5%</td>
</tr>
<tr>
<td>Platelets</td>
<td>260 x 10³/ml</td>
<td>260 x 10⁹/l</td>
<td>130-400 x 10⁹/ml</td>
</tr>
<tr>
<td>MPV</td>
<td>10.5 fl</td>
<td></td>
<td>7.4-10.4 fl</td>
</tr>
</tbody>
</table>

*Harmening (Ref. 1)

The RBC morphology on the peripheral blood smear showed microcytosis with slight hypochromia. A few target cells and slight anisocytosis were noted.

The physician, noting the low hemoglobin and hematocrit, prescribed oral iron and ordered a test for the most common source of unknown bleeding in adult males, a stool occult blood. The test was negative. After 2 months of iron therapy C.C. reported no improvement in his endurance. A repeat CBC at this time showed similar results to the first one. At this point the physician consulted with the Hematology Clinical Laboratory Scientist before ordering additional tests.
Consider the questions
1. What are the causes of hypochromic, microcytic anemias?
2. What tests are used to differentiate them?

**COURSE OBJECTIVES:** at the end of the course the participant will be able to:

1. List the causes of hypochromic, microcytic anemias
2. Identify tests used to differentiate among hypochromic, microcytic anemias
3. State the globin chain composition of the various hemoglobins mentioned
4. Differentiate between the genetic causes of alpha thalassemia and beta thalassemia
5. Discuss the clinical manifestations of homozygous versus heterozygous states of the defective genes
6. Describe the red cell morphology associated with thalassemia minor
7. Differentiate between alpha thalassemia minor and beta thalassemia minor
8. Evaluate and compare laboratory tests and morphology between thalassemia minor and iron deficiency anemia

**DISCUSSION:** The causes of hypochromic, microcytic anemias are iron deficiency (the most common), anemia of chronic disease, thalassemia, sideroblastic anemia and lead poisoning. They may be differentiated by the tests in the following Table 1:

<table>
<thead>
<tr>
<th></th>
<th>RDW</th>
<th>Serum Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
<th>FEP</th>
<th>A2 Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td>Inc</td>
<td>Dec</td>
<td>Inc</td>
<td>Dec</td>
<td>Inc</td>
<td>Nor</td>
</tr>
<tr>
<td>Chronic disease</td>
<td>Nor</td>
<td>Dec</td>
<td>Dec</td>
<td>Inc</td>
<td>Inc</td>
<td>Nor</td>
</tr>
<tr>
<td>αThalassemia trait</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
</tr>
<tr>
<td>βThalassemia trait</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Inc</td>
</tr>
<tr>
<td>Sideroblastic</td>
<td>Inc</td>
<td>Inc</td>
<td>Nor</td>
<td>Inc</td>
<td>Inc</td>
<td>Nor</td>
</tr>
<tr>
<td>Lead Poisoning</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Nor</td>
<td>Inc</td>
<td>Nor</td>
</tr>
</tbody>
</table>

RDW = red cell distribution width, TIBC = total iron binding capacity, FEP = free erythrocyte protoporphyrin
*Adapted from Harmening (Ref. 1)

The physician ordered serum iron, ferritin and FEP. The results were within reference ranges, eliminating iron deficiency, anemia of chronic disease, sideroblastic anemia and lead poisoning. This resulted in a provisional diagnosis of Thalassemia minor. At this point, what other information would be useful to confirm the diagnosis?

The Thalassemias are a heterogeneous group of hereditary diseases of hemoglobin synthesis involving decreased production of one of the hemoglobin globin chain types. Normal adult hemoglobin is composed of 95 - 97% Hb A (2α and 2β chains), 2 – 3% Hb A2 (2α and 2δ chains), and 2% Hb F (fetal hemoglobin, 2α and 2γ chains).
The 2 principal types of Thalassemia are alpha Thalassemia and beta Thalassemia depending on which chains are affected. The following shows a general classification:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genotype</th>
<th>Clinical Feature</th>
<th>Newborn Hb pattern</th>
<th>&gt;First Year Hb pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrops fetalis</td>
<td>-/-</td>
<td>Fetal or neonatal death</td>
<td>Hb Bart’s &gt;80%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hb H, Hb Portland</td>
<td></td>
</tr>
<tr>
<td>Hb H disease</td>
<td>-/-α</td>
<td>Chronic hemolytic anemia</td>
<td>Hb Bart’s 20-40%</td>
<td>Hb H 5-30% Hb Bart’s--</td>
</tr>
<tr>
<td>Thalassemia minor</td>
<td>-/-aa or -α/-α</td>
<td>Little anemia, Micro, hypo RBC</td>
<td>Hb Bart’s 2-10%</td>
<td>Normal</td>
</tr>
<tr>
<td>Silent Carrier</td>
<td>αα/-α</td>
<td>No hematologic or clinical abnormal.</td>
<td>Hb Bart’s 1%</td>
<td>Normal</td>
</tr>
<tr>
<td>Normal</td>
<td>αα/αα</td>
<td>No hematologic or clinical abnormal.</td>
<td>Hb Bart’s 0-trace</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**NORMAL**

<table>
<thead>
<tr>
<th>HEMOGLOBIN F</th>
<th>HEMOGLOBIN A</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2γ2</td>
<td>α2β2</td>
</tr>
</tbody>
</table>

**ALPHA THALASSEMIA**

α2γ2 excess gamma chains, Hb Bart’s

α2β2 excess beta chains, Hb H

**BETA THALASSEMIA**

α2γ2 Hb F persists beyond ppt. as inclusions

α2β2 excess alpha chains infancy

β2 or α2 indicates decreased production

**ALPHA THALASSEMIA**

Alpha thalassemia is decreased production of alpha chains. Alpha chain production is controlled by 4 genes, 2 on each chromosome 16. The genetic mechanism is gene deletion. Alpha thalassemia is evident at birth because alpha chains are required for all hemoglobins, fetal, A2 as well as A. Thus Hb F, usually comprising 50-85% the hemoglobin at birth, is not present to carry O2 at this time. The severity of alpha thalassemia depends on the number of genes deleted as seen in Table 2.

**TABLE 2**
Deletion of all 4 genes is incompatible with life. Hb H disease has some production of Hb A but Hb H (b4) is unstable and precipitates in the cells causing increased hemolysis of RBCs.

Alpha thalassemia minor can be caused by both genes deleted on one chromosome or 1 gene deleted on both chromosomes. Deletion of only one gene causes no apparent consequences, a condition called a silent carrier.

Alpha thalassemia is more commonly found in Southeast Asia, less commonly in the Mediterranean and sporadically in other parts of the world.

Other genetic abnormalities which cause alpha chain elongation, such as Hb Constant Spring, Hb Seal Rock, Hb Koya Dora or Hb Icaria, result in decreased alpha chain production with effects similar to alpha gene deletion. Other genetic causes of decrease in alpha chain production have been identified, giving geneticists much fodder for investigation.

**BETA THALASSEMIA**

There are 2 genes for production of beta chains, one on each chromosome 11. The genetics of decreased production of beta chains is more complex than that found in alpha thalassemia. There may be various mutations in introns (non-coding intervening sequences in the beta gene) causing decreased production of mRNA, a mutation in the promoter area or other mechanisms. A number of different genetic backgrounds have been described, usually associated with a different geographic area. Beta thalassemia is commonly found in the Mediterranean Sea area (‘thalassa’ means sea). It is particularly common in No. Italy, Greece, Algeria and Saudi Arabia and can also be found across southern Asia to Southeast Asia. The clinical severity of beta thalassemia is variable, depending on the type of genetic defect or the combination of defects. Severe beta thalassemia is not evident until the infant is several months old since Hb F is produced in adequate quantities until then. The main categories of genetic defects are $\beta^a$ and $\beta^+$. $\beta^a$ gene produces no beta chains. $\beta^+$ gene produces variable amounts of beta chains depending on the specific genetic inheritance. There are several other genetic defects, Hb Lepore, which results from unequal crossover between delta and beta genes, and $\delta\beta$Thal, a combined defect of delta and beta chain synthesis. These are less common and will not be discussed further. The following table gives a brief overview of beta thalassemias:

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Genotype</th>
<th>Hb pattern</th>
<th>Clinical feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta^a$ thalassemia</td>
<td>$\beta^a\beta^a$</td>
<td>No HbA, var. Hb A2, Rest is HbF</td>
<td>Thalassemia major</td>
</tr>
<tr>
<td>$\beta^+$ thalassemia</td>
<td>$\beta^+\beta^+$</td>
<td>decreased Hb A, increased Hb F, variable Hb A2</td>
<td>Thalassemia major or intermedia</td>
</tr>
<tr>
<td>$\beta^a\beta^+$ heterozygote</td>
<td>$\beta^a\beta^+$</td>
<td>Marked decreased Hb A, increased Hb F, variable Hb A2</td>
<td>Thalassemia major</td>
</tr>
</tbody>
</table>
TABLE III-B Thalassemia minor

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Genotype</th>
<th>Hb pattern</th>
<th>Clinical feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>βα thal minor</td>
<td>βαβ</td>
<td>Hb A, increased Hb A2, slight increase Hb F</td>
<td>Thalassemia minor</td>
</tr>
<tr>
<td>β+</td>
<td>β+β</td>
<td>Hb A, increased Hb A2, slight increase Hb F</td>
<td>Thalassemia minor</td>
</tr>
</tbody>
</table>

LABORATORY FINDINGS IN THALASSEMIA

The main focus of this course is thalassemia minor, but a brief discussion of the more severe thalassemias follows:

**Thalassemia major:**
Anemia is profound – Hb 2-3 g/dl (Hb 20 to 30 g/L). Hematocrit and RBC count are also decreased, hence the indices are uniformly depressed. The MCV, MCH and MCHC are all decreased. The RDW is increased due to anisocytosis.

The blood smear shows marked hypochromia and microcytosis with extreme anisocytosis and poikilocytosis with bizarre shapes, target cells, ovalocytosis, Cabot rings, Howell Jolly bodies, nuclear fragments, basophilic stippling, siderocytes and often large number of nucleated RBCs.

**Hemoglobin H disease:** the peripheral smear shows hypochromia and microcytosis, target cells, mild to moderate anisopoikilocytosis. Incubation of blood with brilliant cresyl blue supravital stain will cause precipitation of Hb H in the erythrocytes as multiple “golf ball like” inclusion bodies.

**Thalassemia minor:**
Thalassemia minor is common, particularly in areas where there are people of Mediterranean, Southeast Asian and African ancestry. As was illustrated by the case study, the causes of a low hemoglobin and hematocrit must be differentiated. In the absence of clinical symptoms, giving a course of oral iron therapy and evaluating the result is not a recommended procedure to assure quality patient outcomes. Rather, assessment of serum iron, ferritin, TIBC and FEP will better determine the probable cause.

Decreased serum iron would indicate iron deficiency or anemia of chronic disease; increased serum iron would indicate sideroblastic anemia and increased FEP along with normal serum iron would be characteristic of lead poisoning. Again, referring to the question posed at the end of the case study, what other information would be useful to confirm the diagnosis? In this patient, a healthy active young man, anemia of chronic disease and lead poisoning are unlikely. Iron deficiency is ruled out by the unresponsiveness to iron therapy. Knowledge of the individual’s racial background might be useful. In this case, he was of Italian descent. This particular ancestry coupled with the decreased MCV and microcytic red cells that are not corrected with iron therapy targets a diagnosis of Thalassemia minor. The next step is to determine the type of thalassemia. Hemoglobin electrophoresis may be useful in demonstrating the type of thalassemia by showing the presence of Hb A2, Hb F, Hb H, Hb Constant Spring, Hb Lepore or other structurally abnormal hemoglobins. In this case Hb A2 was increased (5%) and Hb F was 4.5%. Thus, there is corroboration of beta thalassemia minor.
Diagnosis of thalassemia minor is important in order to reassure the patient that the levels of hemoglobin and hematocrit are normal for him and he should not be placed on iron therapy, which could lead to iron overload. Also the patient needs to be counseled that if he marries a woman who is a carrier of beta thalassemia, hemoglobin E or hemoglobin S, there may be significant consequences in their children.

Alpha thalassemia minor is harder to diagnose than beta thalassemia minor because the levels of Hb A2 and Hb F are not increased. Frequently it is a diagnosis made by exclusion. Again knowing the patient’s racial background is useful. The hematological values along with other clues will help.

There are several ways a laboratorian may suspect that the patient has a thalassemia minor from the initial CBC. In particular it is important to differentiate between thalassemia minor and iron deficiency. In thalassemia minor the hemoglobin and hematocrit are decreased but the RBC count is not correspondingly low and frequently is in the normal range, resulting in discordance in the indices. (The MCV is slightly decreased and the MCH is decreased but the MCHC is near normal). Also the cells tend to be a similar size so the RDW is normal. In contrast, in iron deficiency the RBC count is usually relatively lower and there is significantly more anisocytosis, thus the indices are in concordance and the RDW is increased. A mathematical manipulation of the indices has been used to help differentiate between thalassemia minor and iron deficiency. One of the formulas is Mentzer’s, as follows:

\[
\text{If the result is } <13, \text{ then thalassemia minor} \\
\text{If the result is } >13, \text{ then iron deficiency}
\]

The red cell morphology on the blood smear may also give indication of whether the patient has thalassemia minor or iron deficiency. The morphology seen in thalassemia minor is hypochromic, microcytic with slight anisocytosis, mild to moderate poikilocytosis, target cells and frequently basophilic stippling. The smear is characterized by having a majority of similar appearing cells. The morphology in iron deficiency shows hypochromia, microcytosis, moderate anisocytosis, mild to moderate poikilocytosis—ovalocytes, “pencil cells” (long elliptical forms), folded cells, usually no basophilic stippling.

The differences are that thalassemia minor has similar sized cells, usually no pencil shaped cells and may show basophilic stippling while iron deficiency has moderate anisocytosis, more poikilocytosis, especially pencil shaped cells, and no basophilic stippling. An individual’s iron stores must be determined. Serum ferritin is a good indicator of the level of stored iron. In iron deficiency there are decreased stores of iron as indicated by decreased serum ferritin. In thalassemia minor there are normal iron stores. (refer to Table I)

CONCLUSION

In this course we have discussed the causes of hypochromic, microcytic anemias: iron deficiency, a thalassemia, b thalassemia, anemia of chronic disease, sideroblastic anemia and lead poisoning. These anemias may be differentiated by the laboratory tests shown in Table I along with clinical history. Emphasis was placed on the causes and identification of thalassemias, in particular the types of thalassemia minor. Identifying and differentiating thalassemia minor from iron deficiency anemia may be done by evaluating the serum iron and ferritin levels, the FEP and TIBC. Cellulose acetate electrophoresis will differentiate between a thalassemia and b thalassemia.
Megaloblastic Anemia

OBJECTIVES: At the end of this course the participant will be able to:
1. discuss the biochemical basis of megaloblastic anemia
2. outline the requirements and dietary sources of vitamin B12
3. outline the requirements and dietary sources of folic acid
4. list the causes of megaloblastic anemias
5. outline the symptoms of megaloblastic anemias
6. describe the blood smear morphology characteristic of megaloblastic anemia
7. discuss the tests used to differentiate among the different kinds of megaloblastic anemias
8. interpret the abnormal results in the case study

CASE STUDY: A fourteen year old white male was referred to a large medical center for profound macrocytic anemia. He was seen in the Pediatric Hematology clinic where blood was drawn for CBC and Reticulocyte count. The CBC and differential results were as follows:

**CBC: Patient Normal**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>1.14 x 10⁶/µL</td>
<td>4.7-6.1 x 10⁶/µL</td>
</tr>
<tr>
<td>WBC</td>
<td>3.6 x 10³/µL</td>
<td>4.8-10.9/µL</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>12.7%</td>
<td>42-52%</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>4.4 g/dl</td>
<td>14-18 g/dl</td>
</tr>
<tr>
<td>MCV</td>
<td>111.4 fl</td>
<td>80-94 fl</td>
</tr>
<tr>
<td>MCH</td>
<td>38.7 pg</td>
<td>27-31 pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>34.7 g/dl</td>
<td>33-37 g/dl</td>
</tr>
<tr>
<td>RDW</td>
<td>22.1%</td>
<td>11.5-14.5%</td>
</tr>
<tr>
<td>Platelets</td>
<td>76 x 10⁵/µL</td>
<td>130-400 x 10⁵/µL</td>
</tr>
<tr>
<td>MPV</td>
<td>9.3 fl</td>
<td>7.4-10.4 fl</td>
</tr>
</tbody>
</table>

**Differential: Patient Normal**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percentage</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN</td>
<td>29%</td>
<td>39-68%</td>
</tr>
<tr>
<td>Bands</td>
<td>1</td>
<td>2-10</td>
</tr>
<tr>
<td>Lymphs</td>
<td>67</td>
<td>16-45</td>
</tr>
<tr>
<td>Monos</td>
<td>3</td>
<td>3-14</td>
</tr>
<tr>
<td>NRBCs/100 wbc</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

**Suspect flags:** dimorphic RBC; microcytic RBC, RBC fragments
**Definitive flags:** neutropenia, lymphocytosis, 2+ anisocytosis, 3+ macrocytosis, thrombocytopenia

**Reticulocyte count: Patient Normal**

| Reticulocytes | 2.6% | 0.5-1.5% |
| Retic. Absolute | 29.0 x 10³/µL | 22.5-88.5 x 10³/µL |
| Retic. Corrected | 0.7% | 0.4-1.7% |

**B12/Folate Studies: Patient Normal**

<table>
<thead>
<tr>
<th>Test</th>
<th>Value</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12</td>
<td>&lt;100 pg/ml</td>
<td>200-950 pg/ml</td>
</tr>
<tr>
<td>Folate</td>
<td>12.7 ng/ml</td>
<td>3.7-19.0 ng/ml</td>
</tr>
</tbody>
</table>
INTRODUCTION:
Megaloblastic anemias are a heterogeneous group of disorders that have common blood abnormalities and symptoms. The characteristic blood picture consists of large oval erythrocytes, hypersegmented neutrophils and large abnormal platelets. Bone marrow RBC precursors show abnormally high nuclear to cytoplasmic ratio and abnormal megakaryocytes. Nuclear maturation is delayed while cytoplasmic development is normal.

The diseases associated with megaloblastosis are primarily pernicious anemia (associated with vitamin B12 deficiency) and folic acid deficiency. Although the number of megaloblastic anemia cases has decreased in recent years, the incidence remains between 0.25-0.5 cases per 1000 in older individuals.

HISTORY:
Paul Ehrlich in 1880 first used the term megaloblast to describe the abnormal cells in the bone marrow of a patient with pernicious anemia. He felt the very large basophilic RBC precursor was a cell type unique to the disease. We now know that this cell is a morphologically and functionally abnormal counterpart of the normal erythroid precursor cell.

Thomas Addison first described pernicious anemia, the best known of the megaloblastic anemias, in 1855. For a number of years the disease was known as Addisonian anemia. Anton Biermer in 1872 first used the term, pernicious anemia.

Treatment for pernicious anemia was instituted in 1926 when it was found that giving patients large amounts of liver reversed the disease process. The active liver principle, vitamin B12, was discovered in 1948 but it took 25 years to develop a synthetic vitamin B12.

BIOCHEMICAL BASIS OF MEGALOBLASTIC ANEMIAS:
The etiology of the megaloblastic anemias is impaired DNA synthesis and assembly. The most common causes are vitamin B12 (cobalamin) or folic acid deficiencies. These substances are essential in the DNA synthesis pathway. Impaired DNA production causes disruption of cell division and maturation. Rapidly proliferating cells of the bone marrow and epithelial surfaces are primarily affected, leading to the characteristic blood picture and symptoms. Cobalamin is also necessary for normal function of cells of the nervous system.

Cobalamin and folate metabolisms are related and deficiency of either results in the deoxyuridylate–deoxythymidy late pathway impairment (see Figure 1 - on next page). This pathway forms deoxythymidylate from deoxyuridylate and methionine from homocysteine. Folic acid is required for synthesis of thymidylate. Since thymidylate is the precursor to thymine, essential for DNA, a limitation in the supply of thymidylate impairs DNA synthesis and leads to the morphologic manifestation of megaloblastic maturation.

Cobalamin catalyzes the conversion of homocysteine to methionine. When this reaction is impaired, folate metabolism is deranged. Since folic acid is required for synthesis of thymidylate, this derangement underlies the defect in DNA synthesis and the megaloblastic maturation in patients who are cobalamin deficient. Impairment in the conversion of homocysteine to methionine may also be partly responsible for the neurologic complications of cobalamin deficiency. Methionine is needed for the production of choline and choline-containing phospholipids, which are required in the nervous system.
METABOLISM:

**Cobalamin** (Cyanocobalamin, Vitamin B12): This substance is a complex organometallic compound, in which a cobalt atom is situated within a corrin ring, a structure similar to the porphyrin ring from which heme is formed with the addition of a ferrous atom. Unlike heme, cobalamin cannot be synthesized in the human body and must be supplied by the diet. Microorganisms manufacture cyanocobalamin. These bacteria are found in the intestinal tract, but below the area where vitamin B12 is absorbed, vitamin B12 enters the food chain through animals that eat their feces. Therefore it is found only in animal products, not in plants. The minimum daily requirement is about 2.5 µg. Normally about 2 mg is stored in the liver and 2 mg is stored elsewhere in the body. If absorption ceases abruptly, such as when a person becomes a vegan with no supplementation, it takes about three to six years to use up the body stores and become deficient. Intrinsic factor (IF), produced by parietal cells in the stomach, is necessary for absorption of vitamin B12. The secretion of IF generally parallels that of hydrochloric acid. The cobalamin-IF complex is bound to and absorbed by specific receptors in a portion of the distal ileum.

**Folic acid**: This is the common name for pteroylmonoglutamic acid. It is formed of three basic components: a pteridine derivative, para-aminobenzoic acid and L-glutamic acid. Many different plants and bacteria synthesize it. Fruits and vegetables form the primary dietary source of the compound but prolonged cooking destroys it. The minimum daily requirement is about 50 µg but this may be increased several-fold during times of increased metabolic demand such as pregnancy or in hemolytic anemias. Normally body stores range from 5 to 20 mg, with half stored in the liver. The large minimum daily requirement means that a deficiency will occur within months if dietary intake or intestinal absorption is curtailed. It is readily absorbed in the duodenum and proximal jejunum.
SYMPTOMS OF MEGALOBLASTIC ANEMIA:

The anemia symptoms of both folic acid deficiency and vitamin B12 deficiency are the same. In both there are often gastrointestinal tract symptoms such as anorexia and diarrhea because the epithelial cells lining the intestines also have a rapid turnover. Frequently the patient does not seek Technology medical help until some of the following conditions occur: weakness, light-headedness, palpitations, angina, and symptoms of congenital heart failure. Folic acid deficiency results in anemia and intestinal manifestations but not neurological symptoms.

In pernicious anemia neurologic manifestations also occur. The onset is insidious as destruction of parietal cells increases and the body stores of vitamin B12 are gradually consumed. Neurologic symptoms begin after demyelination followed by axonal degeneration. The most common manifestations are numbness and paraesthesias in the extremities, weakness and ataxia. The patient experiences tingling of the extremities and has an unsteady gait. Some have deranged mental states ranging from irritability and forgetfulness to severe dementia and psychosis. Although administering vitamin B12 by injection may cure the anemia, the neurologic symptoms may persist if there has been death of neurons. Differentiation between folic acid deficiency and vitamin B12 deficiency is important. If large amounts of folic acid are given to a pernicious anemia patient, the anemia may be improved but the neurologic symptoms become worse.

DEFICIENCY STATES: (See Table I, Classification of Deficiency States)

Causes of megaloblastic anemia differ in various parts of the world. In the temperate zones folate deficiency in alcoholics and pernicious anemia predominate. In the equatorial areas tropical sprue is an important cause. In Scandinavia the fish tapeworm, *Diphyllobothrium latum*, may contribute to the incidence.

In the United States, supplementation of bread and cereal products with folic acid and vitamin B12 has decreased the incidence of megaloblastic anemia due to inadequate intake. Only people with very poor diets, such as alcoholics who get most of their calories from drinks, have inadequate intake of folic acid. Defective absorption, increased folic acid requirements and use of drugs that impair DNA metabolism continue to cause megaloblastosis in the U.S.

**Cobalamin (Vitamin B12) Deficiency:**

Deficiency of vitamin B12 in the U.S. is almost always due to malabsorption. Several different conditions may cause malabsorption—decreased production of Intrinsic Factor (IF) (pernicious anemia), disorders of the terminal ileum, and competition in the intestinal tract for vitamin B12. *Pernicious Anemia:*

This disease is caused by inadequate secretion of IF by the parietal cells in the stomach—due to atrophy of the gastric mucosa or autoimmune destruction of the parietal cells. It is found most often in older individuals, particularly in those of northern European descent or in African Americans. It is less common in southern Europeans and Asians. There is an increased incidence of pernicious anemia in patients with autoimmune diseases such as Hashimoto’s thyroiditis, Grave’s Disease, myxedema, or hypoparathyroidism. About 90% of pernicious anemia patients have abnormal circulating antibodies against parietal cells. About 60% have anti-intrinsic factor antibodies. Relatives of pernicious anemia patients have increased incidence of the disease. *Disorders of the terminal ileum:*

Any abnormality that compromises the absorptive capacity of the terminal ileum can result in cobalamin deficiency. The conditions include ileitis, surgical resection of the small intestine and sprue.
Competition for Vitamin B12 by intestinal organisms:

*D. latum*, the fish tapeworm, may cause vitamin B12 deficiency by competition for vitamin B12 in the intestinal tract. Elimination of the parasite cures the problem.

Blind loops and other intestinal anatomic lesions may harbor bacteria that consume cobalamin before absorption.

Folic Acid Deficiency:

Folic acid deficiency can be due to dietary deficiency, increased requirement, or defective absorption. *Dietary deficiency*:

Dietary supplementation has decreased the incidence of dietary deficiency since the required addition of folic acid to enriched grain products was instituted in 1998. However, it can still occur in individuals with markedly inadequate diets such as alcoholics and the elderly who eat no fresh food. *Increased demand*:

Pregnant women, hemolytic anemia patients, and some infants and teenagers undergoing growth spurts have increased demand for folic acid. Folic acid deficiency in the first weeks of pregnancy, usually before the pregnancy is known, can cause neural tube defects in the fetus. Dietary supplementation has decreased the incidence of this defect by 50%. Patients with markedly increased hematopoiesis as occurs in hemolytic anemias require additional folic acid. *Defective absorption*:

Tropical sprue, a poorly understood disease with malabsorption, and non-tropical (celiac) sprue (gluten sensitivity) may result in decreased absorption of folic acid. Other small bowel disorders may also cause malabsorption. *Other causes of Deficiency States*:

Drugs that impair DNA synthesis or metabolism may cause megaloblastic anemia. These are primarily drugs used in the treatment of malignancies, particularly leukemias, including DNA inhibitors (purine and pyrimidine analogues) and folate antagonists (methotrexate). Zidovudine, an anti-viral drug used in HIV treatment, may also cause megaloblastic anemia.

**TABLE I: CLASSIFICATION OF DEFICIENCY STATES:**

**Cobalamin Deficiency:**
1. Inadequate intake (vegans—strict vegetarians)
2. Defective absorption
   - Inadequate production of intrinsic factor
     - Pernicious anemia
     - Gastrectomy
     - Congenital absence or abnormality of intrinsic factor
   - Disorders of terminal ileum
     - Tropical sprue
     - Non-tropical sprue
     - Ileal resection
     - Regional enteritis
     - Neoplasms of ileum
   - Competition for Cobalamin
     - Fish tapeworm (*Diphyllobothrium latum*)
     - Bacteria (blind loop syndrome)
   - Drugs: para amino salicylic acid, colchicines, neomycin
3. Impaired utilization of cobalamin
   Nitrous oxide
   Inborn errors of metabolism (rare)

**Folic Acid Deficiency**
Inadequate intake
Increased requirements
   Pregnancy
   Infancy
   Malignancy
   Increased hematopoiesis (hemolytic anemias)
Defective absorption
   Intestinal disorders (Tropical sprue, Non-tropical sprue)
   Drugs: phenytoin, barbiturates, alcohol
Impaired metabolism
   Folic acid antagonist therapy (methotrexate, trimethoprim, pyrimethamine)
   Enzyme deficiencies

**Other:**
Drugs that impair DNA metabolism
   Purine and pyrimidine antagonists, acyclovir, zidovudine, hydroxyurea
Marrow stem cell disorders
   Dysmyelopoietic syndromes
   Erythroleukemia (DiGuglielmo’s syndrome)
Metabolic disorders (rare)

**LABORATORY TESTS:**
The finding of significant macrocytosis (MCV>100fL) suggests the presence of a megaloblastic anemia. Other causes of macrocytes are hemolysis with increased reticulocytes, liver disease, alcoholism, hypothyroidism, and aplastic anemia; however, the macrocytes in these conditions are round, not oval. If the MCV is over 110fL, the patient is most likely to have a megaloblastic anemia. Other findings in the blood count are decreased hemoglobin and RBC count, decreased numbers of WBC and platelets.

The blood smear characteristically shows well-hemoglobinized oval macrocytes and anisopoikilocytosis. Occasional nucleated RBC may be present. The neutrophils may show hypersegmentation. The finding of one neutrophil with 6 lobes signifies megaloblastic anemia (normal neutrophils have 2-4 lobes). Large or bizarre misshapen platelets are usually present.

Bone marrow shows hypercellularity with decreased myeloid:erythroid ratio. The erythroid precursors are abnormally large with the nucleus less mature than the cytoplasm. The nuclear chromatin is more dispersed than normal and shows a characteristic megaloblastic pattern. Granulocyte precursors are larger than normal, particularly bands and metamyelocytes. Megakaryocytes are decreased with abnormal morphology.

Erythroid precursors show increased destruction (ineffective erythropoiesis). This increased destruction results in increased unconjugated bilirubin and lactic acid dehydrogenase in the plasma. The patient may show jaundice from the bilirubin.
The reticulocyte count helps differentiate between other causes of macrocytosis and megaloblastosis. If the reticulocyte count is sufficiently increased to be the cause of macrocytosis, a possible hemolytic anemia needs to be investigated. Reticulocytes may show polychromatophilia on the blood smear, and are round, not oval. If the reticulocyte count is decreased/normal then tests need to be done to determine which deficiency is present. Serum folic acid and vitamin B12 levels are performed. If the serum folate is less than the normal range, this is the cause of the megaloblastic anemia. If the serum cobalamin level is decreased below 100pg/ml, then further tests need to be done to find the cause. The Schilling test can determine whether the cause is pernicious anemia, some other cause of defective absorption, or dietary deficiency.

The Schilling test: A patient is given radioactive cobalamin by mouth, followed shortly thereafter by an intramuscular injection of unlabeled cobalamin. This unlabeled cobalamin is given to fill the deficient body sites so that absorbed radioactive B12 will be excreted in the urine rather than be retained to fulfill the deficiency. The proportion of the administered radioactivity excreted in the urine during the next 24 hours provides a measure of absorption of cobalamin (making sure the urine collection is complete). Since vitamin B12 deficiency is almost always due to deficient absorption, this stage of the Schilling test should be abnormal, i.e., small amounts of radioactivity in the urine. Next the patient is given radiolabeled cobalamin bound to intrinsic factor. Absorption of the radiolabeled B12 bound to intrinsic factor will be normal if the patient has pernicious anemia. If cobalamin absorption is still decreased, the patient may have ileal disease or bacterial overgrowth (blind loop) syndrome. Bacterial overgrowth may be corrected by antibiotic administration. Ileal disease and pernicious anemia require regular vitamin B12 injections.

CASE STUDY: Refer to the case at the beginning of the article.

The patient’s cell counts reflected a slight pancytopenia. Inspection of the Coulter printout showed a relatively normal WBC histogram. (The histograms are not shown in this article).

The RBC histogram showed a very broad, distorted bell-shaped curve. The mode is shifted to the right, which correlates with the MCV of 111. The left side of the histogram, which normally comes down to the baseline, showed a significant population of small cells, which are most probably The histogram is very wide. This is reflected by the red cell distribution width (RDW) of 22.1.

The platelet histogram, which usually shows a normal distribution, does not come down to the baseline on the right side, which correlates with the presence of schistocytes. These RBC fragments are in the size range of platelets and are included in the platelet count.

The Definitive and Suspect Flags indicated all of these abnormalities.

On the manual differential, RBC morphology was markedly abnormal with marked aniso-poikilocytosis including macro-ovalocytes, target cells, teardrop RBCs and schistocytes. Nucleated RBCs were present. A few hypersegmented neutrophils were also seen. The reticulocyte % and the absolute reticulocyte count were slightly elevated and the corrected reticulocyte count was normal, but inappropriately low for the degree of anemia present.

A vitamin B12 assay was performed which showed the patient to be markedly deficient. Folate levels were normal. History: The patient was born in 1977 at 28 weeks gestation, weighing 2 lbs., 7 oz. He had problems with hyaline membrane disease and developed necrotizing enterocolitis at five days of age. The bowel perforated on the eighth day and 100 cm. of his bowel, including the ileocecal valve, was removed. This was estimated to be approximately 50% of his ileum.
He was eventually released from the hospital and placed on multi-vitamins with folate and on vitamin B12 replacement therapy by intramuscular injection. The family had numerous employment and insurance difficulties during the ensuing years and the vitamin B12 injections were not given regularly.

A Schilling test (without Intrinsic Factor) had been performed in 1984, which established that the patient was definitely unable to absorb vitamin B12 normally, as excretion of radiolabelled Co-58 was less than 0.6% (normal >7%). The next step of the Schilling test was not done, since the patient history indicated that a large portion of the ileum had been removed, thus the remaining area was shown to be unable to absorb the vitamin.

Finally, vitamin B12 injections were discontinued 2-3 years ago because the family lacked medical insurance and could not afford the treatments. This time frame correlates with the amount of time necessary to deplete stored vitamin B12 in a child this age and with the onset of his clinical symptoms.

**Case Study Conclusions:**

The patient was given an injection of vitamin B12 and re-tested a week later. There was marked improvement in his clinical symptoms, CBC and a significant reticulocyte response.

This patient requires ongoing vitamin B12 replacement therapy for the rest of his life. Failure to comply will result in reappearance of symptoms and possible irreversible damage to his nervous system.

**REFERENCES**

1. Case study furnished by Cannon J. UCDavis Medical Center
   Accessed 2/14/2006
HEMATOLOGY CASE STUDIES: PLATELETS

OBJECTIVES:
After completing this course the participant will be able to:
1. Differentiate among the causes of thrombocytopenia.
2. Explain how to determine the platelet count when the count is above the upper reportable range of the analyzer.
3. Estimate the platelet count from the blood smear.
4. List the signs and symptoms of Essential Thrombocythemia.
5. Enumerate the causes of thrombocytopenia.
6. Discuss the causes of pseudothrombocytopenia.
7. Explain the methods of determining the causes of pseudothrombocytopenia.

Case #1
A 44-year-old woman comes in for a complete blood count (CBC) as part of a routine physical exam. The results from the hematology analyzer, Cell-Dyn 1700 ® (Abbott Diagnostics), are:

<table>
<thead>
<tr>
<th>WBC</th>
<th>7.5 K/μL</th>
<th>RBC</th>
<th>4.22 M/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lym</td>
<td>28.7 %</td>
<td>HGB</td>
<td>12.4 g/dL</td>
</tr>
<tr>
<td>MID</td>
<td>10.4 %</td>
<td>HCT</td>
<td>38.6 %</td>
</tr>
<tr>
<td>Gran</td>
<td>60.9 %</td>
<td>MCV</td>
<td>91.4 fL</td>
</tr>
<tr>
<td>PLT</td>
<td>&gt;&gt;&gt;&gt;&gt; K/μL</td>
<td>MCHC</td>
<td>32.0 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RDW</td>
<td>13.5 %</td>
</tr>
</tbody>
</table>

MID cells may include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts, and other precursor white cells.

Questions:
1. What is abnormal about her CBC?
2. Which parts can be reported?
3. What procedures can be done regarding the abnormal result?

Answers:
1. The platelet count is above the upper reportable range.
2. The WBC histogram and 3-part differential are normal and can be reported. The RBC histogram is normal and can be reported.
3. To determine the platelet count:
   a. Make a 1:1 dilution of the whole blood and re-run the platelet count. Correct the platelet count for the dilution.
   b. Make a smear of the whole blood and examine for platelet morphology and numbers.
Discussion:

The platelet count on 1:1 diluted blood was 534, so the platelet count is $2 \times 534 = 1,068$ K/µL (normal is 150-400 K/µL).

On blood smears made from EDTA-blood and stained with a Romanowsky stain, platelets are round or oval, 2-4 µm in diameter, and separated from one another. The platelet count can be estimated from the smear. At 1000x magnification (oil immersion), this is equivalent to about 730 platelets per oil immersion field (OIF). Count the number of platelets in 10 oil immersion fields. Divide the total by 10 to get the average number of platelets per field. Each platelet seen on the smear equates to approximately 15,000/µL. Multiply the average number per OIF to get the platelet estimate. See Image #1. In this case the average number of platelets per field was 70. The estimate equals $70 \times 15,000 = 1,050$ K/µL. Thus the platelet estimate derived from the smear in Images #1 and #2 correlates with the corrected platelet count of 1,068 K/µL.

The causes of increased platelet counts include:

- Inflammatory disorders
- Iron deficiency anemia
- Splenectomy
- Chronic granulocytic leukemia
- Polycythemia vera
- Undetected cancer
- Essential (primary) thrombocythemia

Since the patient had no symptoms, no history of splenectomy, and normal WBC and RBC hemograms, all except essential (primary) thrombocythemia can be eliminated or are unlikely.

Essential (Primary) Thrombocythemia

Essential thrombocythemia (ET) is a myeloproliferative disease. These diseases are a group of disorders that share features that include the clonal overproduction of one or more blood cell lines. Clonal diseases begin with a mutation in one or more bone marrow cell lines. Myeloproliferative diseases include polycythemia vera, myelofibrosis, chronic granulocytic leukemia, and essential thrombocythemia.
In ET there is overproduction of megakaryocytes, the precursor to platelets (thrombocytes). Abnormalities in platelet aggregation and adhesiveness tests suggest defective platelet function. In about half the patients with ET there is a mutation of the JAK2 (Janus kinase 2) gene in their blood cells. In the others the cause is unknown.

ET occurs mostly in adults. There are about 0.1 to 2.4 new cases per 100,000 in the U.S. each year. The disease does not ordinarily shorten life expectancy, but serious complications can occur, so the patient needs to be followed by a physician.

Many patients have no symptoms. In others signs, symptoms and complications of ET result from the increased numbers of platelets in the peripheral blood. Since platelets are involved in the process of clot formation in response to blood vessel injury, the most common complication of ET is blockage of blood vessels by excess platelets (thrombosis). Less often the increased platelets cause bleeding.

Signs, symptoms, and complications include:
- Burning or throbbing in the feet
- Headache, dizziness, and weakness or numbness on one side to the body or other signs of inadequate blood flow to the brain
- Thrombosis (abnormal clotting)
- Unexpected or exaggerated bleeding (infrequent, associated with very high platelet count)
- Enlarged spleen
- Complications of pregnancy

Diagnosis of ET may occur when a higher than normal platelet count occurs on a routine blood count (as with this patient), or on a blood count that is ordered on a patient who has a blood clot, unexpected bleeding, or an enlarged spleen and there is no other cause for the increased numbers of platelets. In ET the platelet count is over 600 K/µL blood and remains high in subsequent counts. Although the diagnosis cannot be made by laboratory tests alone, the following may be useful: JAK2 mutation in blood cells, slightly lower than normal blood hemoglobin and slightly higher WBC count, no evidence of other myeloproliferative diseases, and an examination of the bone marrow. The bone marrow will show a significant increase in megakaryocytes and masses of platelets.

Treatment of patients with ET is based on the risk of clotting or bleeding complications. If there are no signs or symptoms, the patient is seen for regular checkups. If the patient has high risk as determined by previous clotting or bleeding episodes, a history of a clot, cardiovascular risk factors—diabetes, high cholesterol, smoking, hypertension, obesity—therapy may be considered.

Drug therapy may include aspirin, hydroxyurea, anagrelide, or interferon alfa. Aspirin, although decreasing clotting, may increase the risk of bleeding. When the platelet count is very high and the patient suffers acute clotting, platelethpheresis may be done on an emergency basis.

This patient had no symptoms and was given follow-up appointments.
Case #2
A 38-year-old female inpatient has the following results on her initial complete blood count on Coulter Gen-S® (Beckman-Coulter):

<table>
<thead>
<tr>
<th>WBC</th>
<th>8.9 K/μL</th>
<th>RBC</th>
<th>4.86 M/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>57.9 %</td>
<td>HGB</td>
<td>14.4 g/dL</td>
</tr>
<tr>
<td>LY</td>
<td>33.4 %</td>
<td>HCT</td>
<td>42.5 %</td>
</tr>
<tr>
<td>MO</td>
<td>6.3 %</td>
<td>MCV</td>
<td>87.4 fL</td>
</tr>
<tr>
<td>EO</td>
<td>1.9 %</td>
<td>MCH</td>
<td>29.8 pg</td>
</tr>
<tr>
<td>BA</td>
<td>0.5 %</td>
<td>MCHC</td>
<td>34.0 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RDW</td>
<td>12.5 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLT</td>
<td>64 K/fL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MPV</td>
<td>6.9 fL</td>
</tr>
</tbody>
</table>

Suspect/Definitive Messages/Flags:
- Micro/Fragmented Red Cells
- Giant Platelets
- Platelet clumps

R flag on Platelet Count & MPV

Comments:
- Do not verify platelets; review first and redraw if necessary

Questions
1. What is abnormal about the blood count?
2. Which parts of the CBC can be reported?
3. What would you do to investigate the abnormal result?

Answers:
1. The platelet count is abnormally low and there are flags for microcytic or fragmented RBC, giant platelets, or platelet clumps.
2. The WBC histogram and differential are normal and can be reported.
3. The platelet and RBC histogram patterns are consistent with platelet clumps, fragmented red cells, or microcytic red cells. Make and review the smear (See Image #3) for platelet clumps, fragmented red cells, or small red cells before verifying the platelet count.
Discussion:

The platelet count was below normal, a condition known as thrombocytopenia. The causes of decreased platelet counts are:

- **Decreased Production**
  - Leukemia or lymphoma
  - Cancer treatments such as radiation or chemotherapy
  - Various anemias
  - Toxic chemicals
  - Medications: diuretics, chloramphenicol
  - Viruses: chickenpox, mumps, Epstein-Barr, parvovirus, AIDS
  - Alcohol in excess
  - Genetic conditions: Wiskott-Aldrich, May-Hegglin, Bernard-Soulier syndromes

- **Abnormal distribution**
  - Splenomegaly with sequestration in the spleen

- **Increased destruction**
  - Autoimmune diseases: Idiopathic (immune) thrombocytopenic purpura
  - Medications: quinine, antibiotics containing sulfonamides, Dilantin®, vancomycin, rifampin, heparin-induced thrombocytopenia
  - Surgery: man-made heart valves, blood vessel grafts, bypass machines
  - Infection: septicemia
  - Pregnancy: about 5% of pregnant women develop mild decrease
  - Thrombotic thrombocytopenic purpura
  - Disseminated intravascular coagulation

- **Pseudothrombocytopenia**
  - Partial clotting of specimen
  - EDTA-platelet clumping
  - Platelet satellitism around WBCs
  - Cold agglutinins
  - Giant platelets
Results of the blood smear evaluation (Case #2, Image #3):

The smear showed numerous platelet clumps (make sure to examine the edges of the smear since the clumps may migrate there; Images #4 and #5). There were no giant platelets, fragmented RBC, or small RBC. To obtain an automated platelet count, obtain a blood specimen drawn into Sodium Citrate (NaCitrate).

Results of the platelet count on the NaCitrate specimen (Case #2, Image #6):

There were no flags or error messages. The platelet count of 289 K/µL needs to be corrected for the dilution of the blood by liquid NaCitrate as follows:

\[ 289 \times 1.1 \text{ (dilution factor)} = 318 \text{ K/µL} \]

The diagnosis is EDTA-platelet clumping. This condition may persist for decades without any evidence of abnormal hemostasis. EDTA-platelet clumping needs to be recognized and documented in the patient’s chart to prevent unnecessary treatment for thrombocytopenia, and to guide future laboratory tests.
Causes of pseudothrombocytopenia are as follows:

Partial clotting of the specimen:

With a low platelet count the first procedure is to examine the specimen for evidence of clotting as well as to make a smear and look for evidence of platelet clumping. When blood clots, platelets adhere to the clot and are removed from the fluid blood. If evidence of micro-clots or clumping is seen, obtain a new specimen.

EDTA-Induced Platelet Agglutination (EIPA) (EDTA-platelet clumping):

EIPA is an in-vitro phenomenon due to the presence of naturally occurring autoantibody against a cryptantigen on the GPIIb/IIIa platelet receptor. Under normal in vivo conditions this antigen is not accessible for antibody binding (crypt or hidden antigen). When calcium is chelated by EDTA, the GPIIb protein undergoes a structural change that exposes the cryptantigen. The antibody can then bind to the exposed site and crosslink to other platelets causing agglutination. The condition occurs in 0.1 to 2% of hospitalized patients5.

Platelet satellitism

In this phenomenon platelets rosette around neutrophils or rarely around other cells. The satellite platelets are not counted by automated cell counters, resulting in spurious thrombocytopenia. Platelet satellitism is caused by EDTA-dependent antiplatelet and antineutrophil IgG antibodies in the patient’s plasma (5).

The phenomenon has not been associated with any disease state or drug and is thought to be benign.

The diagnosis is made by making a blood smear and looking for platelet rosettes: Images #7 and #8. This needs to be documented in the patient’s chart.
Cold agglutinins
Spontaneous EDTA-independent agglutination associated with cold antibodies is rare. The condition should be considered when agglutination occurs in citrate and heparin as well as EDTA anticoagulants. This phenomenon is temperature dependent. The specimen should be maintained at 37° C or warmed to 37° C to obtain an accurate platelet count6.

Giant platelets
Giant platelets that are 36 fL or larger will be counted as red cells (See Images #9 and #10) in most automated electronic platelet counters, resulting in spuriously low platelet counts. Low platelet counts along with instrument flagging of giant platelets should prompt the operator to confirm the abnormal platelet count by blood smear review/platelet estimate or perform a manual platelet count. The confirmatory method of choice employs a manual platelet count using phase-contrast microscopy. Manual platelet counts include three steps: dilution of the blood with simultaneous lysis of RBCs with ammonium oxalate; sampling the diluted suspension into a measured volume using a hemocytometer; and counting the platelets in that volume1. When significant numbers of giant platelets are counted as red cells, spuriously low platelet counts cannot be reported. The platelet estimate or manual platelet count must be reported in the place of automated platelet count.
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REFERENCES

2. www.ils.org