Diagnostic methods in hematology II

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Outline

Specialized hematology tests

- Evaluation of anemia
  - Anemia basics
    - Anemia caused by excessive erythrocyte loss
    - Anemia caused by deficient erythropoiesis
- Diagnostic methods used in leukemia/lymphoma

ANEMIA

- WHO criteria: Hb < 125 g/L in adults
- US criteria:
  - –M: Hb < 135 g/L
  - –F: Hb < 125 g/L

- **Anemia is clinical sign**

Causes of anemia

- **Insufficient RBC production**
  - **deficient erythropoiesis**

- **Excessive RBC loss**
  - **Hemolysis (shortened lifespan of erythrocytes)**
  - **Acute bleeding (chronic bleeding leads to iron depletion and results in iron loss and deficient erythropoiesis)**

Anemia caused by deficient erythropoiesis

Causes of insufficient erythropoiesis?

Anemia caused by deficient erythropoiesis

- **Iron metabolism related (microcytic)**
  - **Iron deficiency**
  - **Chronic diseases**
- **Vitamin B12 or folate deficiency (macrocytic)**
- **Insuficient EPO production and marrow failure (normocytic)**
  - kidney failure
  - aplastic anemia
  - myelodysplasia
  - leukemia

Diagnostic aproaches to iron metabolism related anemia

Iron deficiency
Chronic diseases

Iron deficiency

- microcytic anemia and/or anisocytosis
- ↓ blood reticulocytes
- hypoproliferative BM

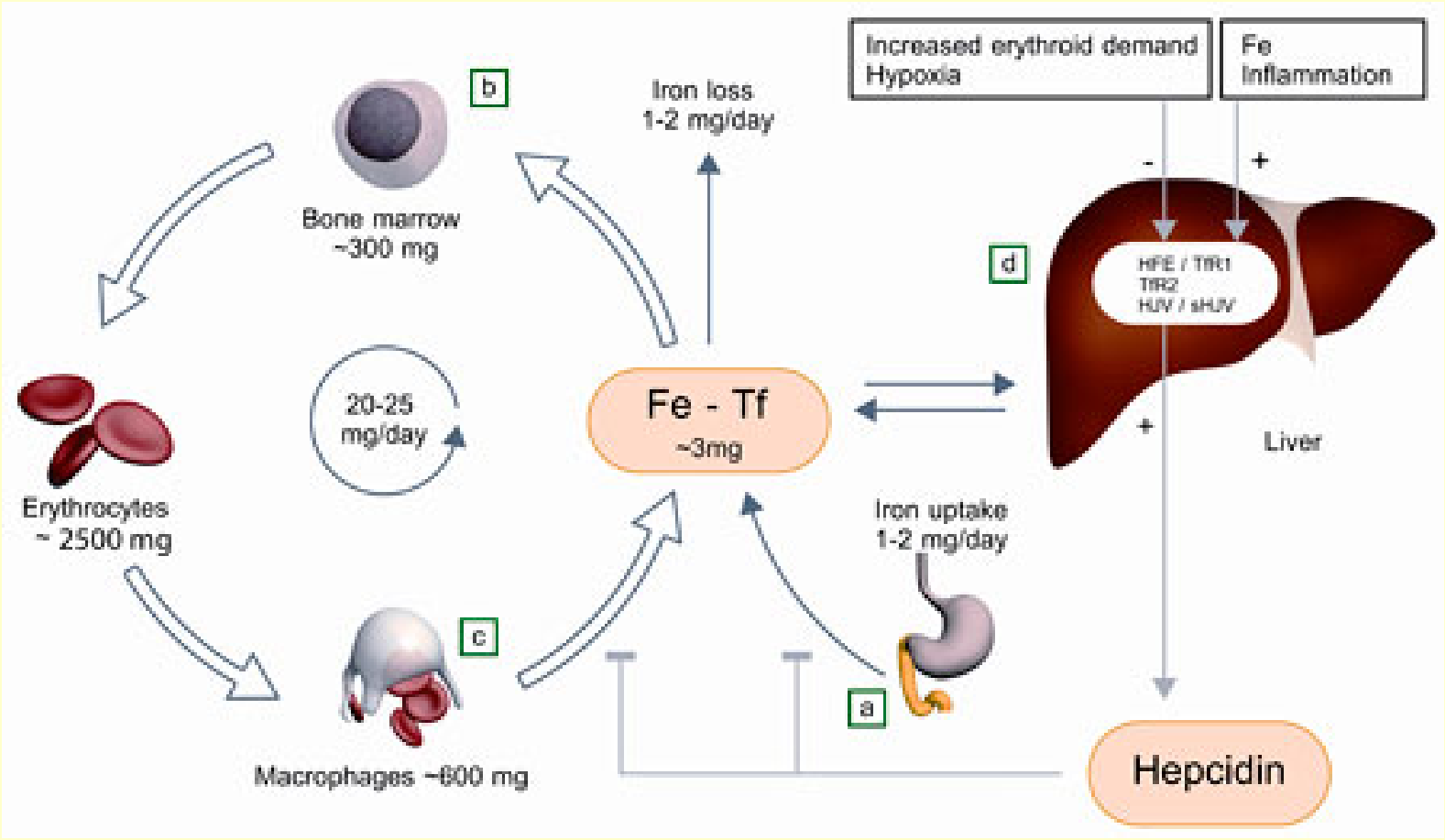
- Blood loss in excess of 10 to 20 mL of blood per day (> 5-10 mg of iron) or iron deficient diet

Anemia from chronic disease

- microcytic anemia and/or anisocytosis
- ↓ blood reticulocytes
- hypoproliferative BM

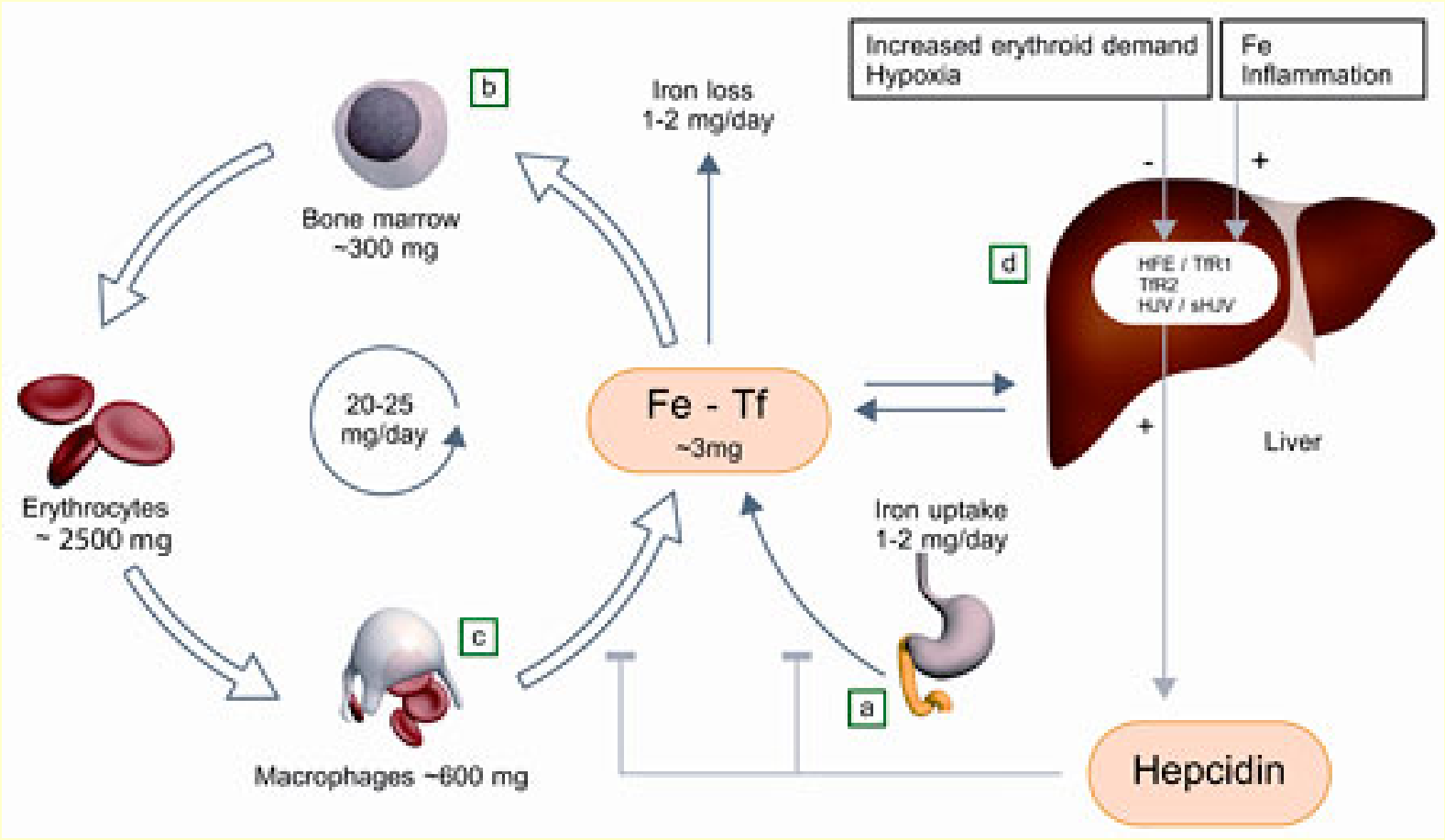
- associated with high levels of proinflammatory cytokines (e.g. chronic inflammation, chronic infection, cancer)
- inadequate iron delivery to the marrow despite of normal or increased iron stores
- Hepcidin plays importat role (transport of iron from the cell)

Iron metabolism



- The lifespan of erythrocyte is ~120 days
- How much blood is replaced every day? (how many RBC?)

Iron metabolism



- The lifespan of erythrocyte is ~120 days
- Daily use of iron in erythropoiesis is ~25 mg (~ 50 mL of blood; ~ 1% RBC)
- The daily need is mostly covered by recycling the iron from dead erythrocytes
- The daily intake of iron is ~ 1-2 mg of iron to maintain the iron stores

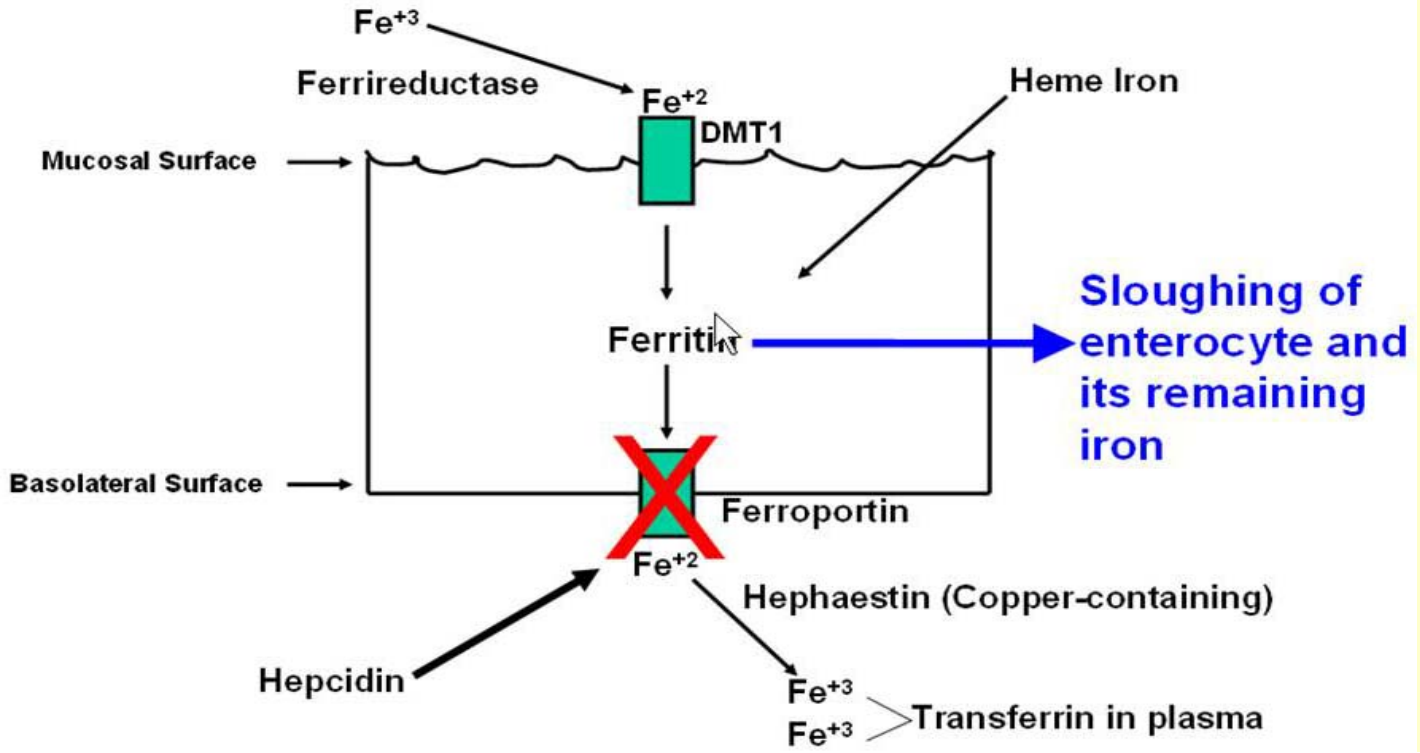
Tests of iron metabolism

Serum iron (SI)

- **~ 6-26 μmol/L** (~ 50 - 150 μg/dL)
- The serum iron level represents the amount of circulating iron bound to transferrin
  - **Low in iron deficiency and chronic disease**
  - High in hemolytic syndromes and iron overload (hemosiderosis, hemochromatosis)

Iron intake

- Iron is reduced to its divalent form (Fe3+ → Fe2+) and than actively transported to enterocytes (**DMT1,** divalent metal transporter 1)
- From enterocyte the divalent iron is transported through **ferroportin** transporter.
- On basal side the iron is oxidized to the trivalent form (Fe2+ → Fe3+) and bound to **transferrin (TF)**



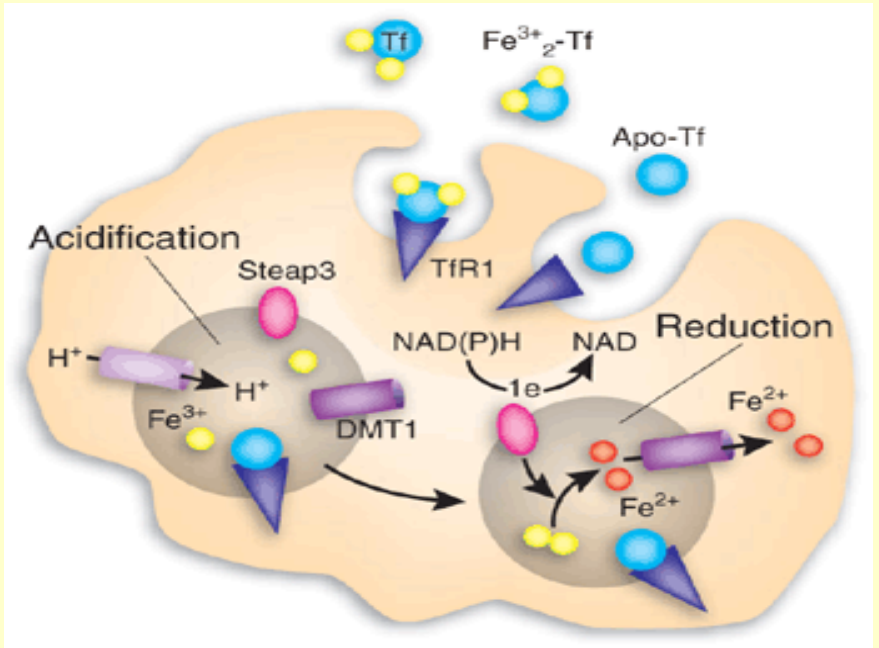
Tests of iron metabolism

Serum transferrin (2-3.6 g/L)

- Acute phase protein (negative reactant)
- Increases in iron deficiency

- Major transporter protein for iron in plasma
- One molecule of transferrin can bind with 2 molecules of divalent iron
- Complex of **Fe2+-TF** binds with transferrin receptors on erythroid progenitors (TFR1)

Erythroid progenitor



Tests of iron metabolism

Serum transferrin (2-3.6 g/L)

- Acute phase protein (negative reactant)
- Increases with iron deficiency

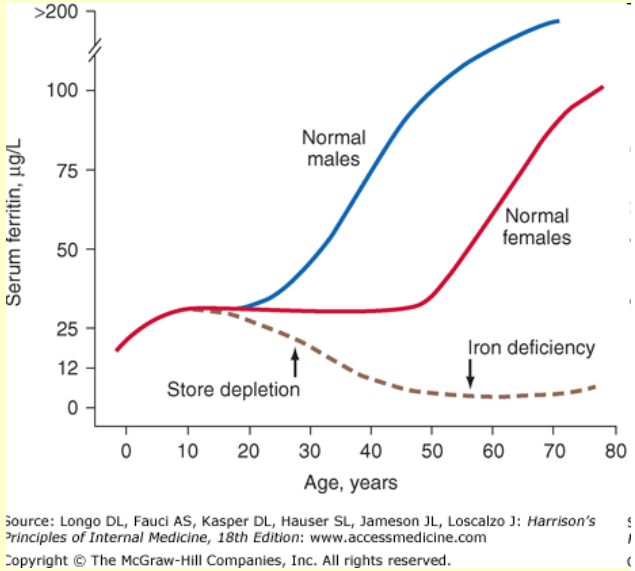
Total iron binding capacity (TIBC) (60-75 μmol/L)

- The amount of iron necessary to reach 100% saturation of transferrin
- Indirect measurement of transferrin
  - High in Fe deficiency
  - Low in chronic disease

Saturation of transferrin

- Is calculated = serum iron x 100 ÷ TIBC
- normally 25–50% (iron deficiency < 20%)

Tests of iron metabolism



Serum ferritin (50-200 µg/L)

- Iron storage glycoprotein
- acute phase protein (positive reactant)
- Closely correlates with total body iron stores
- **1 µg/L of ferritin in serum ~ 8mg of total body iron**
- **< 20 µg/L of serum ferritin** ~ negative iron balance (iron deficiency)
- Elevated in iron overload, liver injury, tumors (Acute phase protein)

Microcytic Hypochromic Anemia (MCV<83; MCHC<31)

	Serum Iron	Total Iron-Binding Capacity (TIBC)	Bone Marrow Iron	Comment
Iron deficiency	⬇	⬆	**0**	Responsive to iron therapy
Anemia from chronic disease	⬇	⬇	**++**	Unresponsive to iron therapy

Iron deficiency anemia

low serum ferritin

Anemia of chronic disease

normal or increased serum ferritin

Other tests of iron metabolism

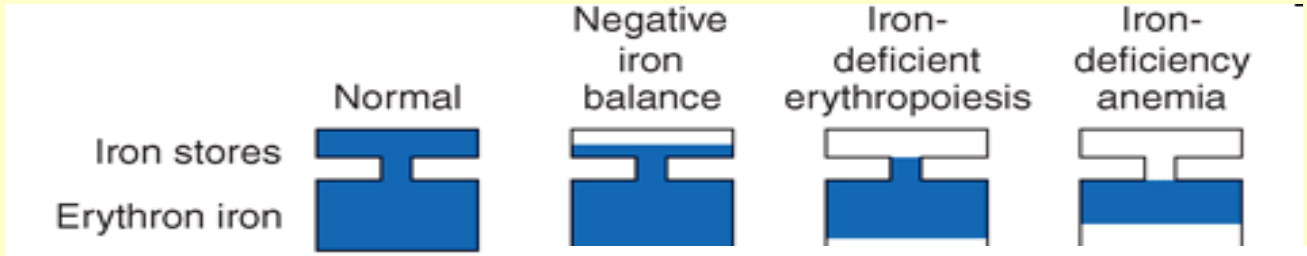
Serum transferrin receptor

- Increase in increased erythropoiesis and early iron deficiency

RBC ferritin

- storage status over the previous 3 month (Fe deficiency/overload)
- unaffected by liver function or acute illness

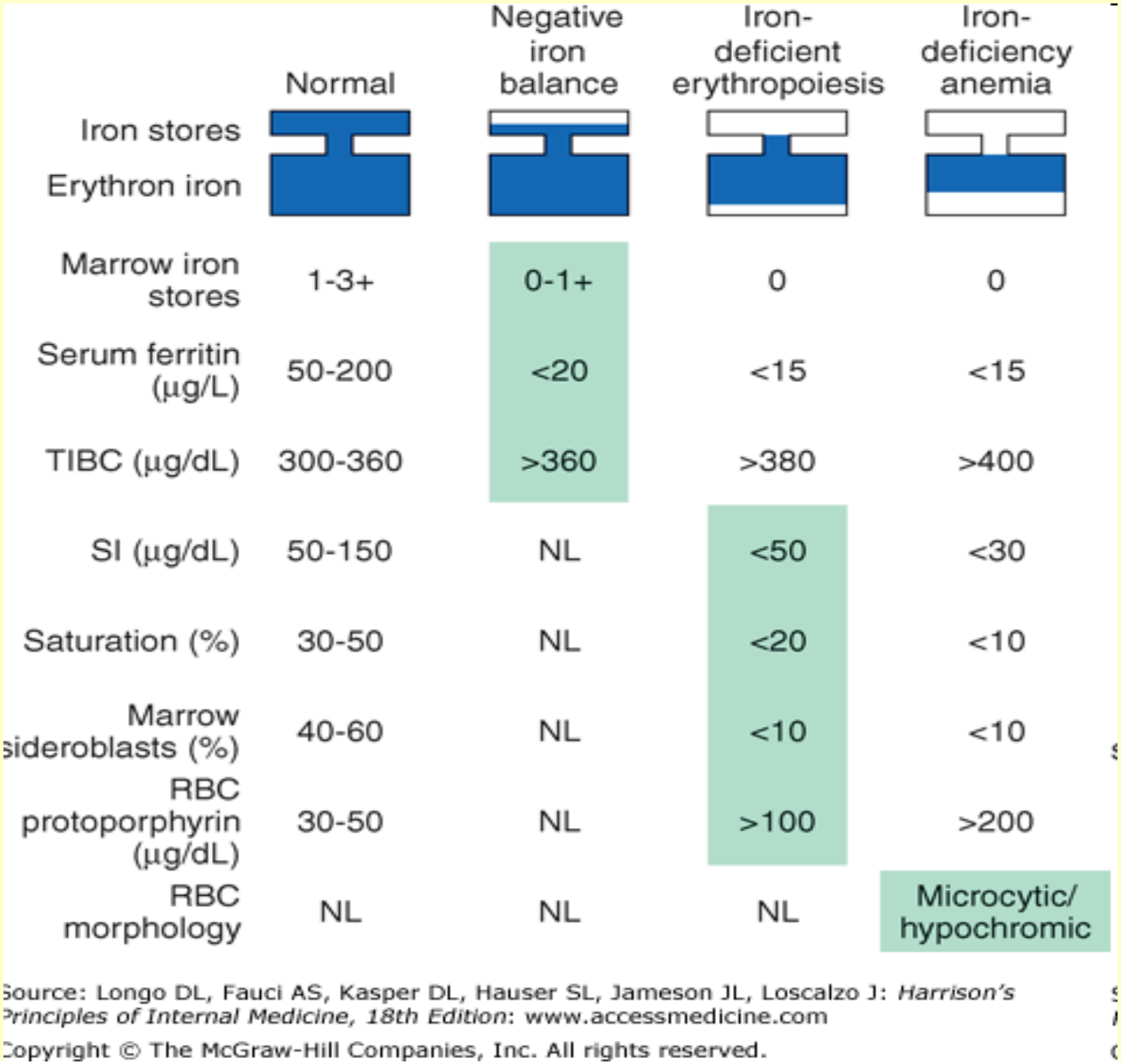
Development of iron deficiency anemia



	Normal	Negative iron balance	Iron-deficient erythropoiesis	Iron-deficiency anemia
Marrow iron stores	1-3+			
Serum ferritin (µg/L)	50-200			
TIBC (µg/dL)	300-360			
SI (µg/dL)	50-150			
Saturation (%)	30-50			
Marrow sideroblasts (%)	40-60			
RBC protoporphyrin (µg/dL)	30-50			
RBC morphology	NL			

Source: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine, 18th Edition*: www.accessmedicine.com

Development of iron deficiency anemia



	Normal	Negative iron balance	Iron-deficient erythropoiesis	Iron-deficiency anemia
Iron stores				
Erythron iron				
Marrow iron stores	1-3+	0-1+	0	0
Serum ferritin (µg/L)	50-200	<20	<15	<15
TIBC (µg/dL)	300-360	>360	>380	>400
SI (µg/dL)	50-150	NL	<50	<30
Saturation (%)	30-50	NL	<20	<10
Marrow sideroblasts (%)	40-60	NL	<10	<10
RBC protoporphyrin (µg/dL)	30-50	NL	>100	>200
RBC morphology	NL	NL	NL	Microcytic/ hypochromic

Source: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine, 18th Edition*: www.accessmedicine.com

RBC protoporphyrin

- Protoporphyrin is converted to heme by ferrocheletase
- RBC protoporphyrin accumulates in:
  - Iron deficiency - impaired heme synthesis
  - Inhibition of ferrocheletase (Lead intoxication)

Iron overload

- **Hereditary hemochromatosis**
  - inborn mutation of genes involved in iron metabolism (HFE, hemojuvelin, hepcidin, ferroportin)
- (Secondary) **hemosiderosis**
  - accumulation of iron secondary to some hematologic diseases or therapy
    - Transfusions (450mL of blood ~ 1 TU ~ 220mg of iron ~5% of total iron stores)
    - Iron loading anemia (e.g. thalasemia, early MDS)
      - nonefective erythropoisis with increased iron uptake

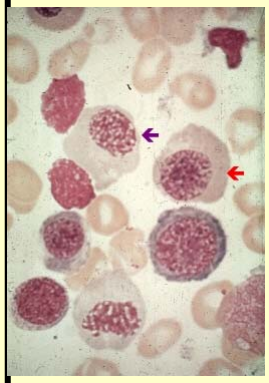
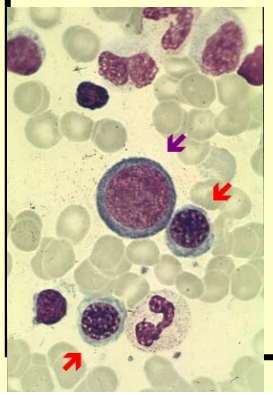
Accumulation of iron in the tissues (e.g. liver, pancreas, heart, skin, joints...) result in organ damage.

Anemia caused by deficient erythropoiesis

- **Iron metabolism related (microcytic)**
  - **Iron deficiency**
  - **Chronic diseases**
- **Vitamin B12 or folate deficiency (macrocytic)**
- **Insuficient EPO production and marrow failure (normocytic)**
  - kidney failure
  - aplastic anemia
  - myelodysplasia
  - leukemia

Diagnostic aproach to vitamin B12 or folate deficiency associated anemia

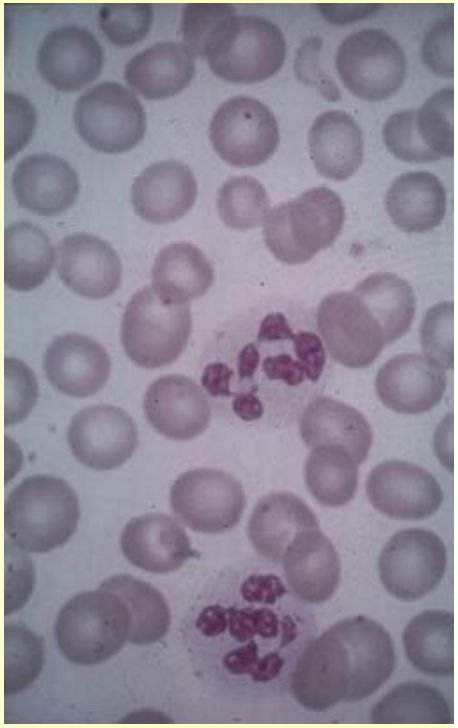
Macrocytic Anemia (MCV, >95)

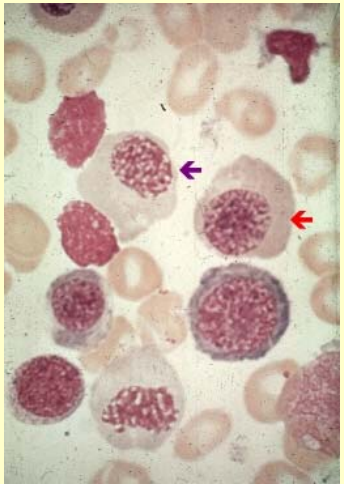
Megaloblastic bone marrow 	**Deficiency of vitamin B-12**
	Deficiency of folic acid
	Drugs affecting DNA synthesis
	Inherited disorders of DNA synthesis
Nonmegaloblastic bone marrow 	Liver disease
	Hypothyroidism and hypopituitarism
	Accelerated erythropoiesis (reticulocytes)
	Hypoplastic and aplastic anemia
	Infiltrated bone marrow

Vitamin B12 or folate deficiency

Megaloblastic anemia

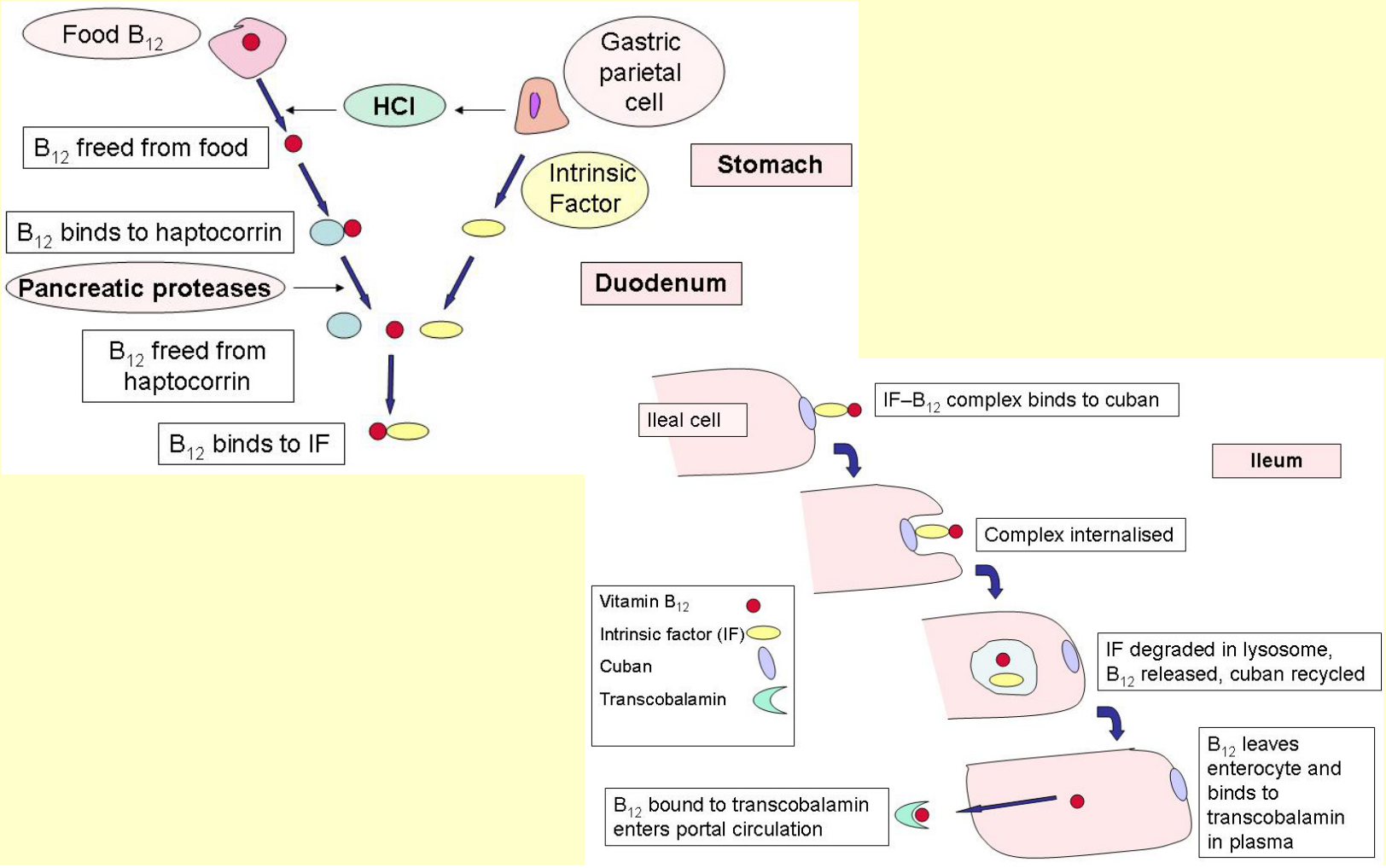
- **Pancytopenia**
- Macrocytosis and/or anisocytosis
- ↓ blood reticulocytes
- Hypersegmented neutophils
- Megaloblastic BM





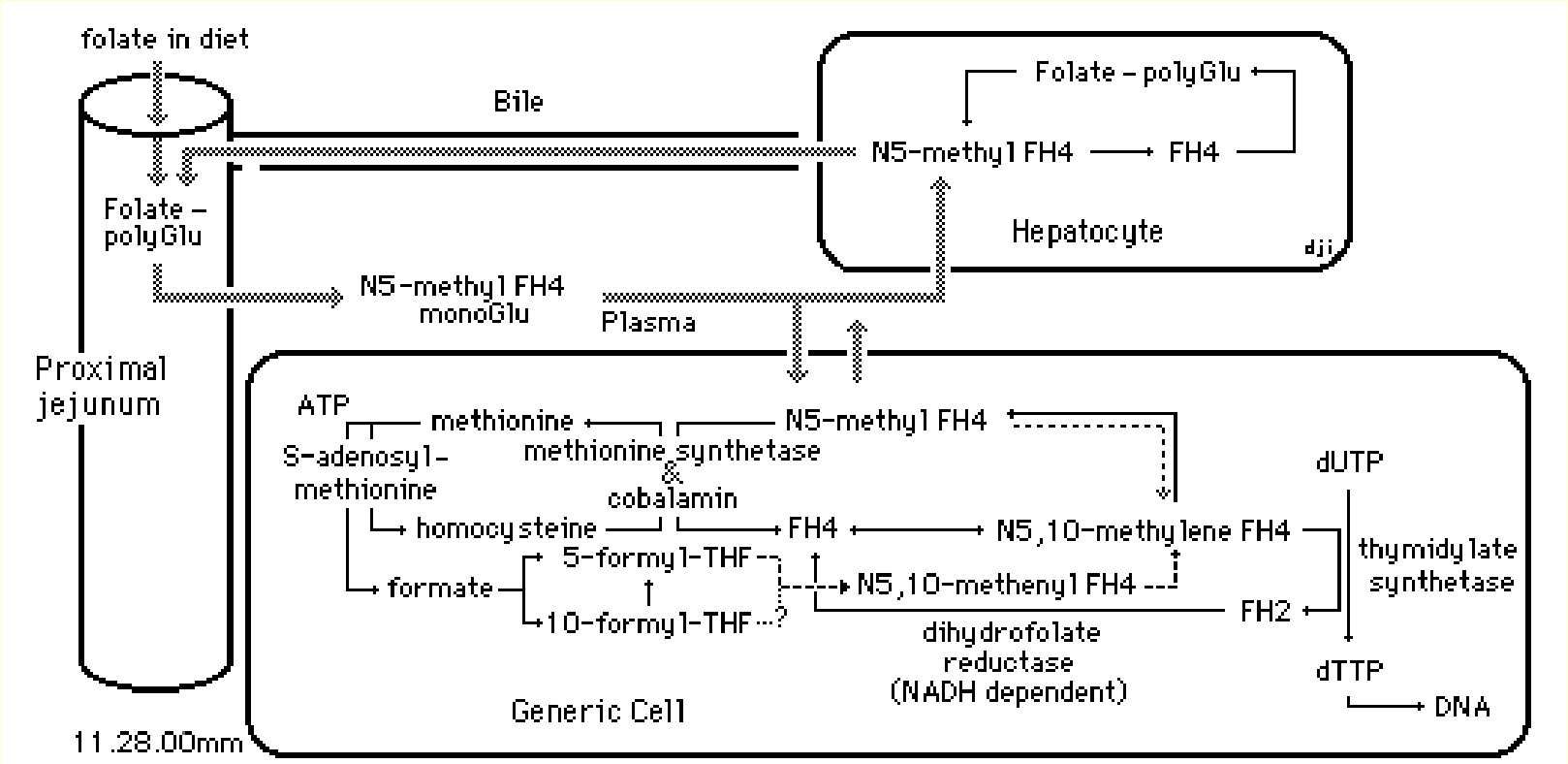
Uptake of vitamin B12 (cobalamin)

- Passive transport < 1% of ingested (GI mucosa)
- Active transport

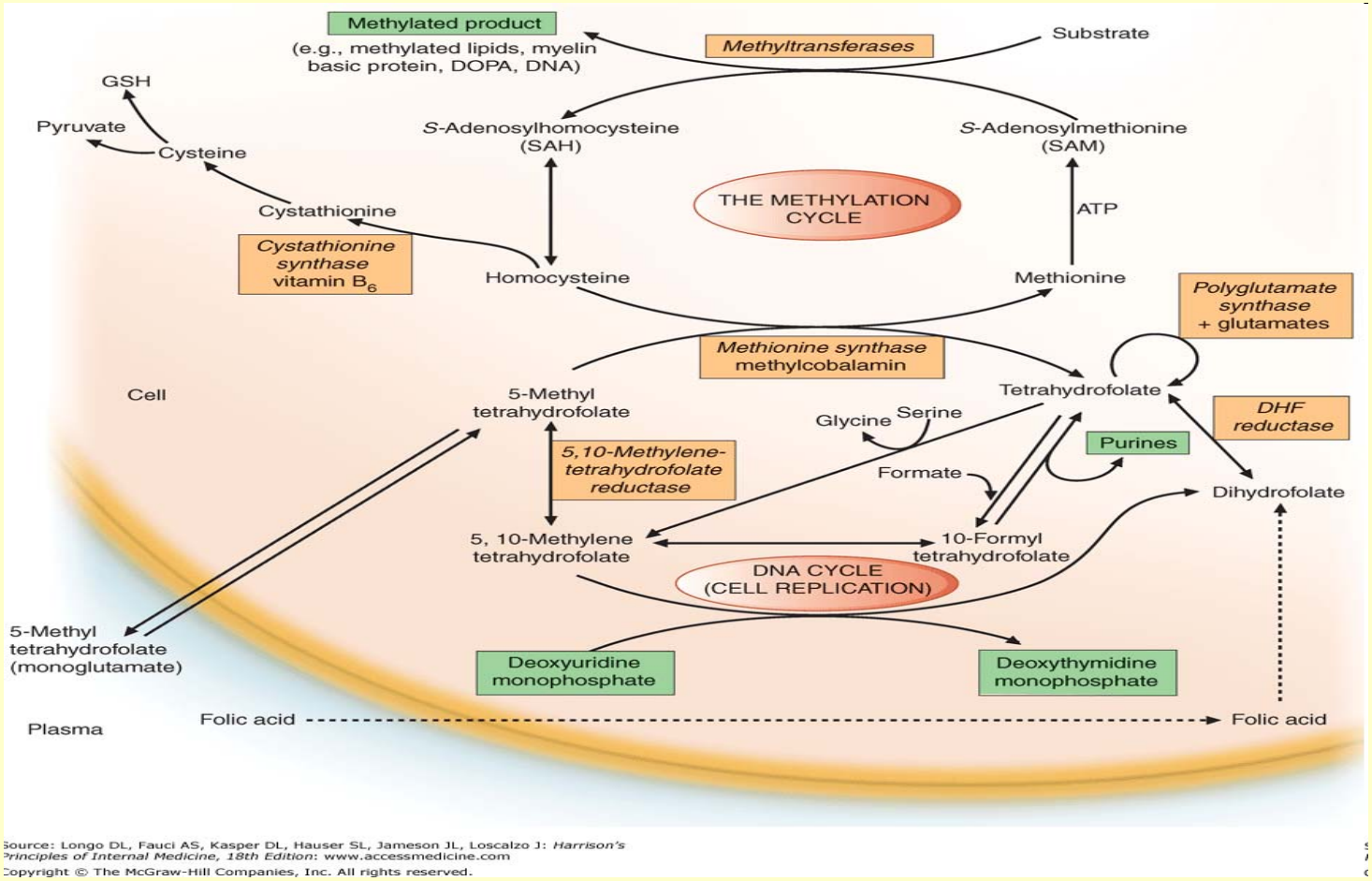


Uptake of folic acid (folate)

- Folates are absorbed rapidly from the upper small intestine
- Dietary folates are converted to 5-methylTHF (5-MTHF) within the small-intestinal mucosa before entering portal plasma
- About one-third is loosely bound to albumin and two-thirds is unbound



- Folates act as coenzymes in the transfer of single-carbon units.
- Two of these reactions are involved in purine synthesis and one in pyrimidine synthesis necessary for DNA and RNA replication.



Mechanism of anemia development in B12 or folate deficiency

Defect in DNA synthesis that affects rapidly dividing cells in the bone marrow

- Failure to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), the precursor of dTTP

Alternatively

- Misincorporation of uracil into DNA because of a buildup of deoxyuridine triphosphate (dUTP) at the DNA replication fork as a consequence of the block in conversion of dUMP to dTMP

Tests for megaloblastic anemia

- Serum level of **vitamin B12 (**190-660 ng/L)
- Serum level of **folic acid** (3-17μg/L)
- Folic acid in erythrocytes (180- 600μg/L)

Vitamin B12 (ng/L)	Folic acid (μg/L)	Deficit
>300	>4	Probably no deficit
<200	>4	Vitaminu B12 deficiency
>300	<2	Folic acid deficiency

Schilling test

- Test for pernicious anemia
  - Is B12 deficiency caused by defect in B12 resorption - intrinsic factor deficiency (in atrophic gastritis)?
- p.o. administration of radio-labeled vitamin B12 (Co57 or Co58)
- An intramuscular injection of unlabeled vitamin B12
  - to temporarily saturate B12 receptors to prevent radioactive vitamin B12 binding in body tissues
- Measurement of B12 radio-activity in urine or blood

Schilling test - modifications

- To explore other possibilities of B12 deficiency
  - p.o. administration of radio-labeled vitamin B12 together with
    - intrinsic factor
      - should correct the B12 resorption in case of intrinsic factor deficiency in atrophic gastritis
    - with antibiotics
      - **malapsorption caused by small bowel bacterial overgrowth**
    - with pancreatic enzymes
      - B12 malabsorption caused by pancreatic exocrine insuficiency

Anemia caused by deficient erythropoiesis

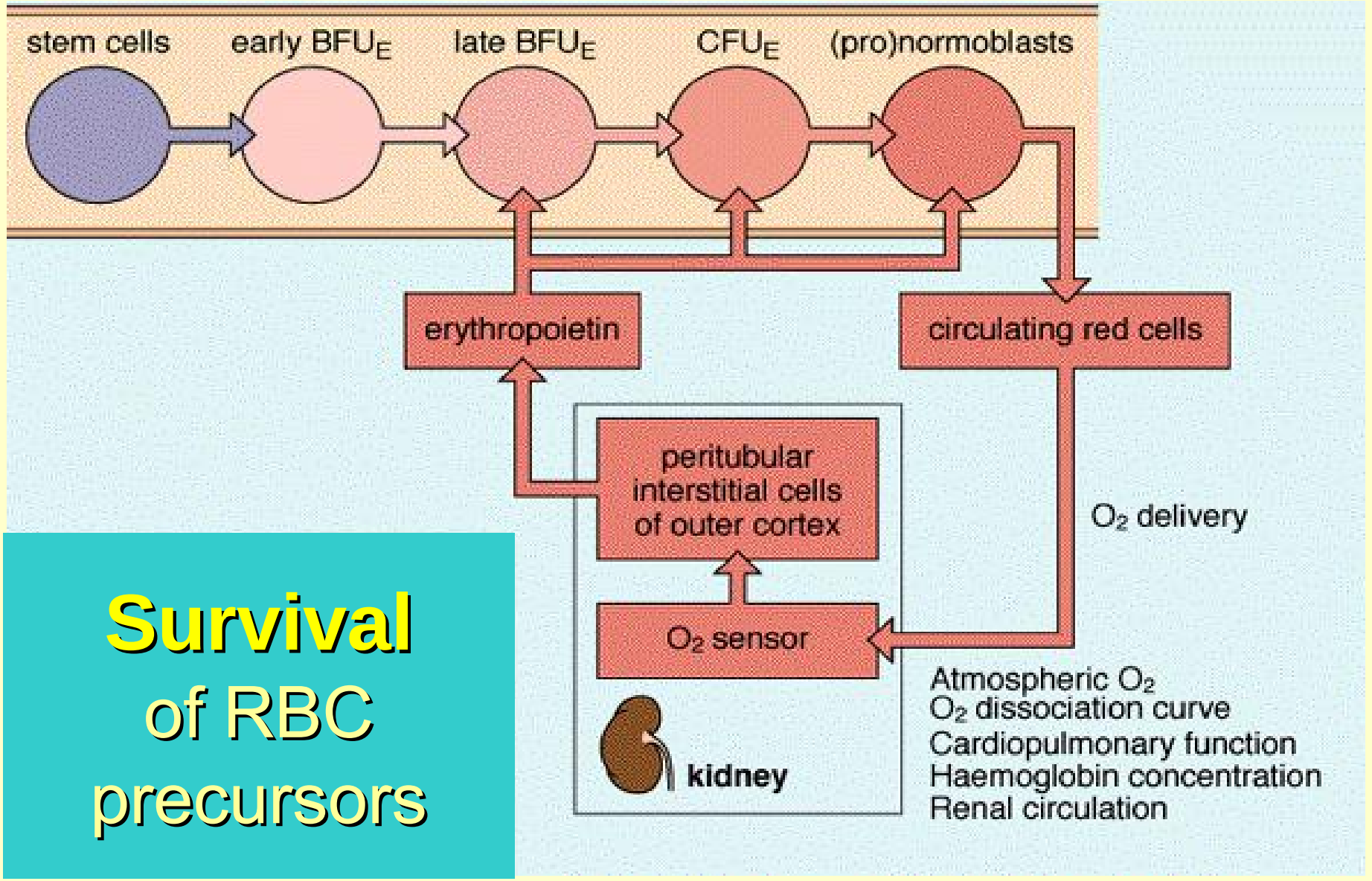
- **Iron metabolism related (microcytic)**
  - **Iron deficiency**
  - **Chronic diseases**
- **Vitamin B12 or folate deficiency (macrocytic)**
- **Insufficient EPO production and marrow failure (normocytic)**
  - kidney failure
  - aplastic anemia
  - myelodysplasia
  - leukemia

Anemia caused by insufficient EPO production

Kidney failure and anemia

- Kidney - main source of erythropoietin (but some other tissues produce EPO as well)
- EPO expression
  - **increase** by hypoxia (e.g. anemia, high altitude)
  - **decrease**
    - kidney disease (ClCr < 45 mL/min)
    - other chronic diseases

Role of Erythropoietin in RBC Production

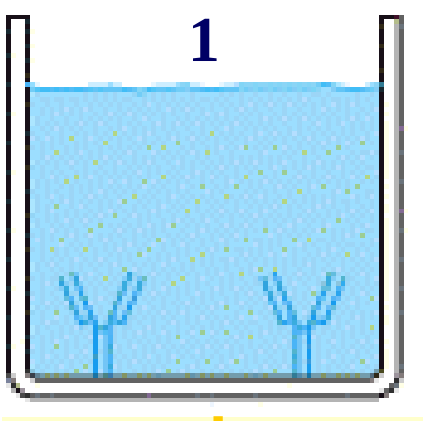


Survival of RBC precursors

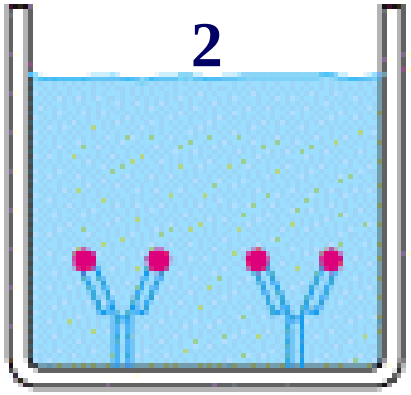
Serum erythropoietin (EPO)

- **Enzyme-Linked ImmunoSorbent Assay** (ELISA)

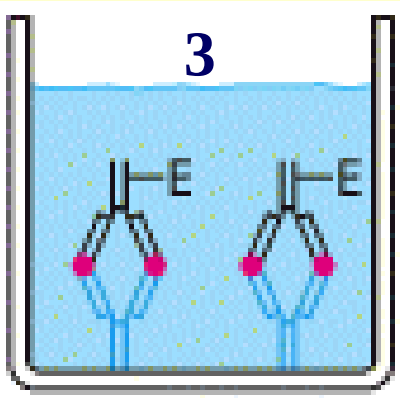
Sandwich ELISA



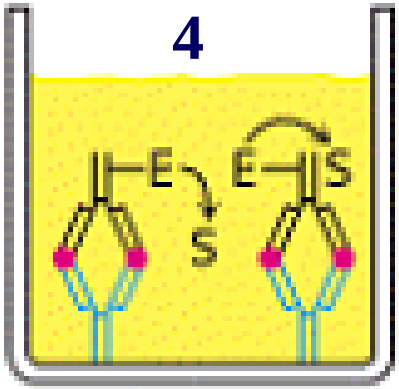
Antibody coated wells
e.g. anti-EPO



Antigen binds to antibody
e.g. EPO from human plasma/serum



Second monoclonal antibody binds to immobilized antigen (e.g. EPO)



A substrate is added and converted by enzyme to colored product

Diagnostic approach to marrow failure associated anemia

aplastic anemia
myelodysplasia
leukemia/lymphoma

Marrow failure

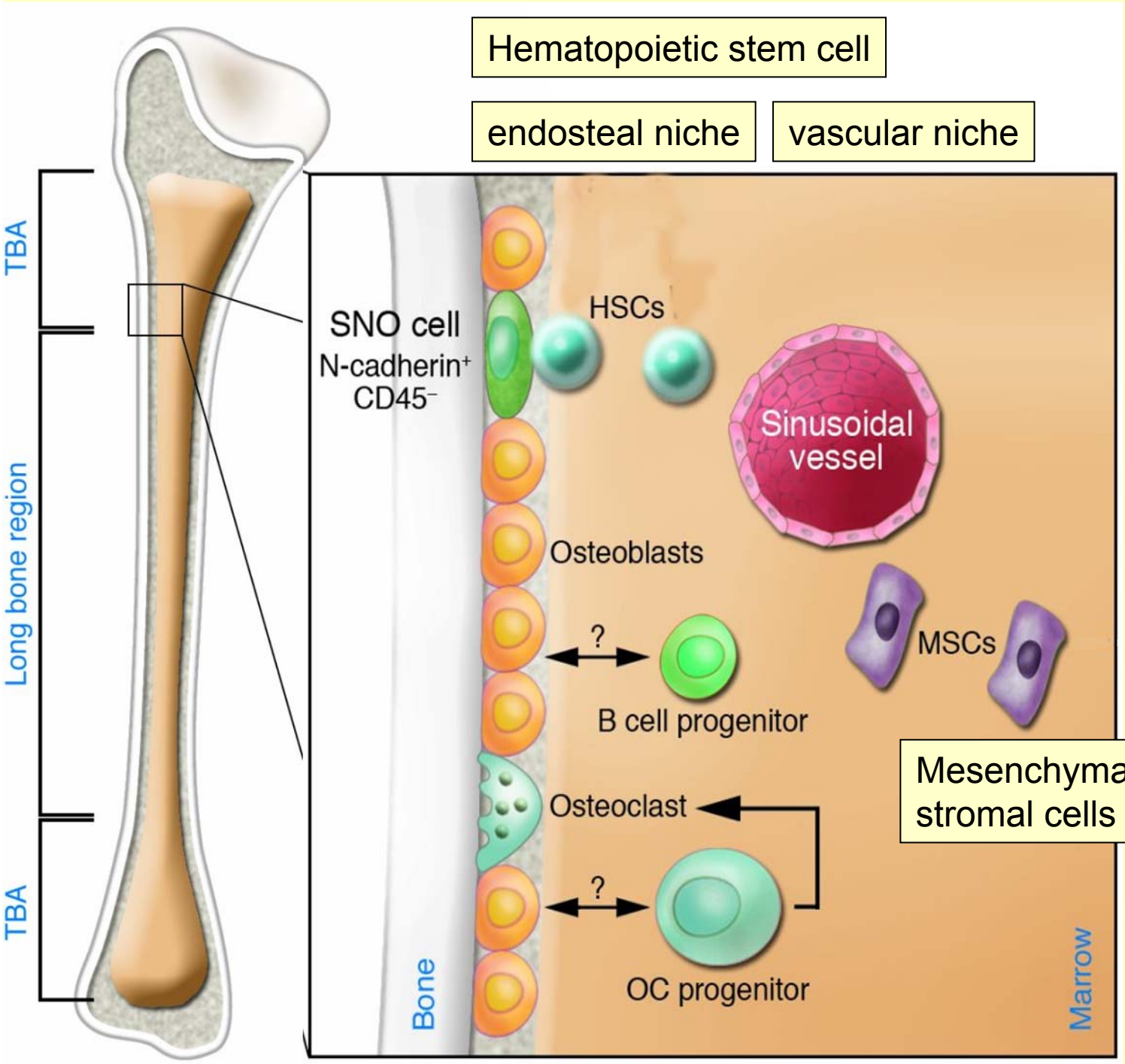
- aplastic anemia
- myelodysplasia
- leukemia / (lymphoma)

  – normochromatosis - normocytosis
  – BM hypoplasia or hyperplasia

Damage of BM

May affect hematopoietic stem cells or hematopoiesis supporting microenvironment (stroma, niche)

- Failure of all hematopoietic lineages
- Failure of individual lineages
  - Myeloid lineage failure
    - Erythroid
    - Megakaryocytic
  - Lymphoid – imunodeficiency / autoimunity

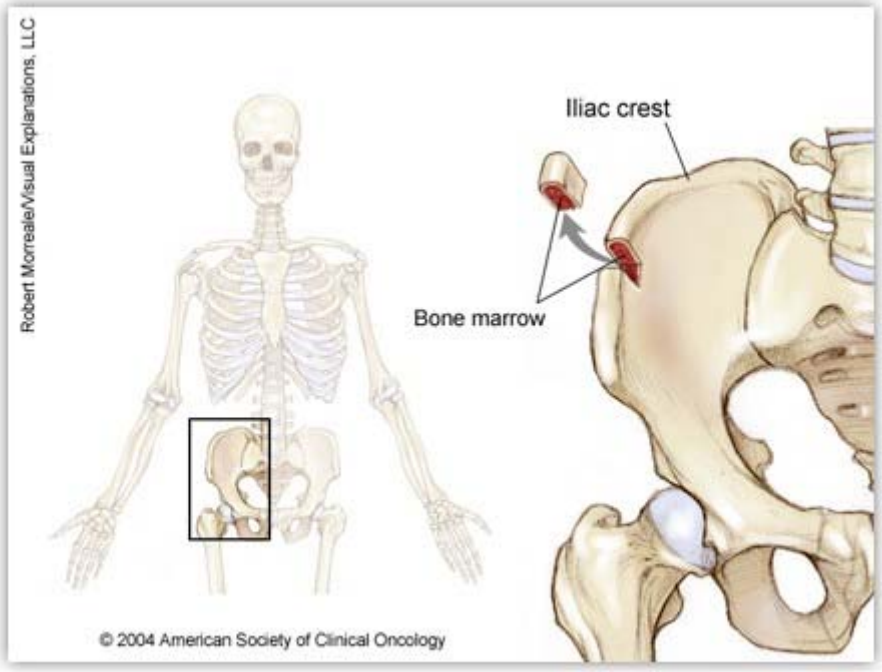


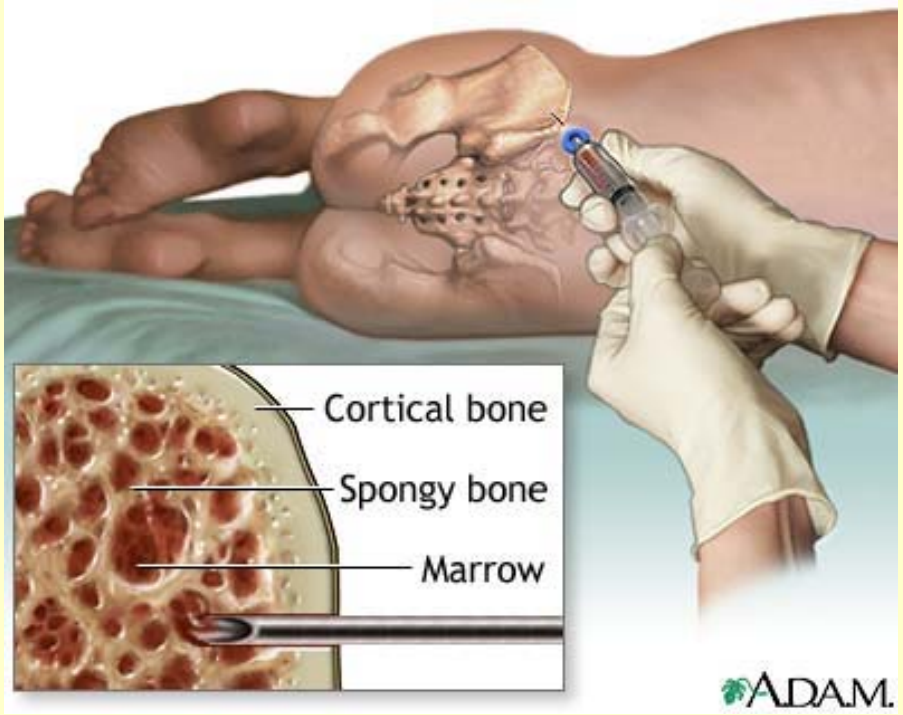
Mesenchymal stem cell: origin of stromal cells

Bone Marrow (BM) Analysis

- **Bone marrow biopsy**

- **Aspiration of bone marrow**
  - **usually from posterior iliac crest or sternum 0.5-2.0 mL**



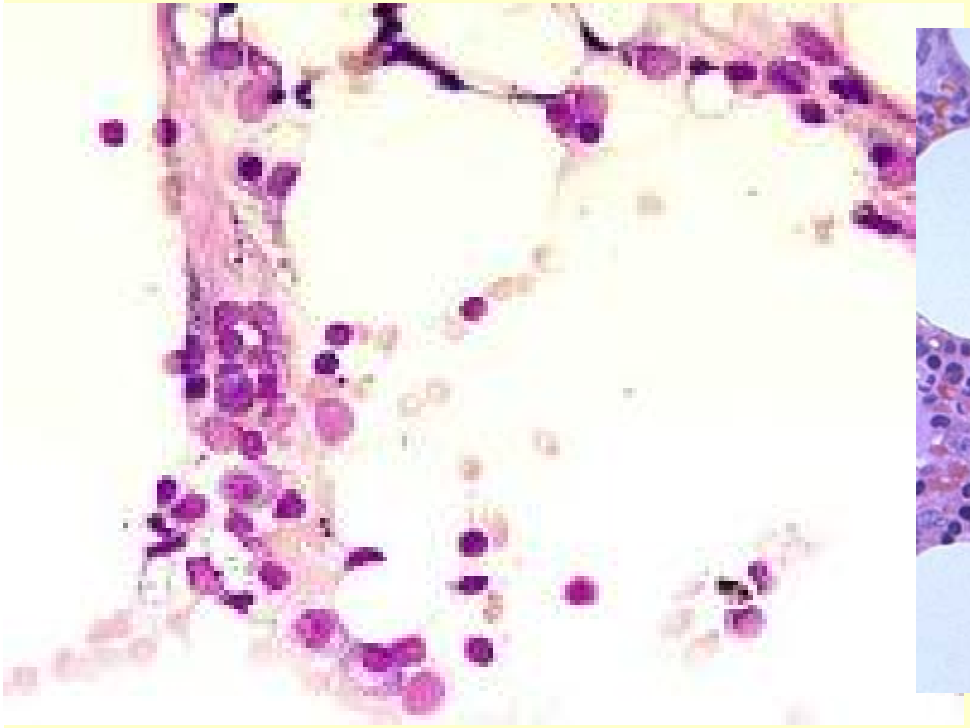


Direct observation of bone marrow activity

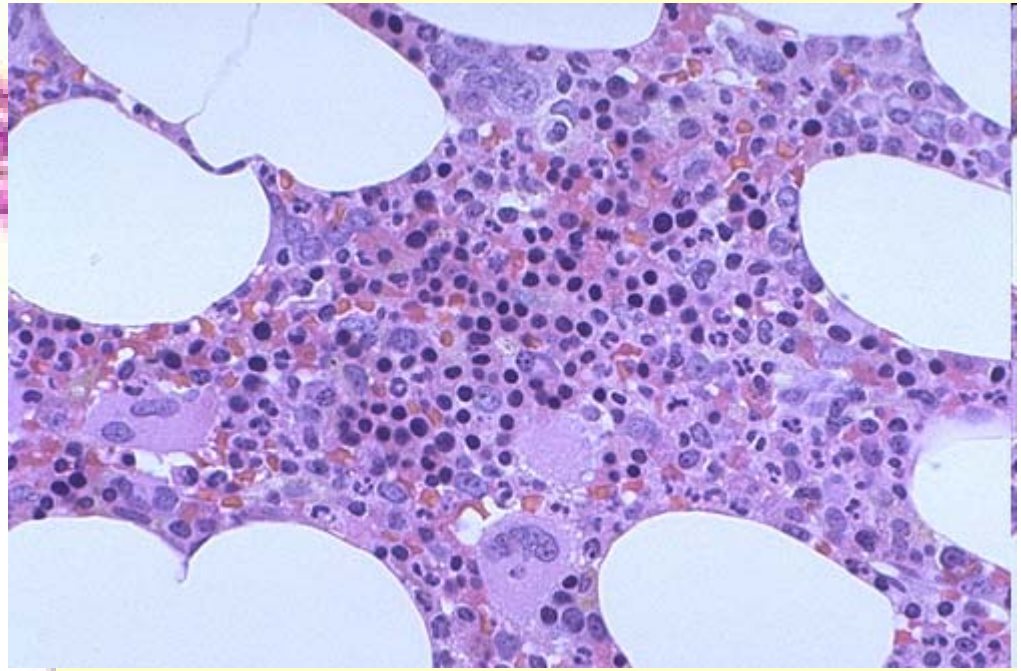
Bone Marrow (BM) Analysis

- Indication
  - Unexplained anemia and other cytopenias
  - Unexplained leukocytosis and thrombocytosis
  - Suspicion of leukemia and myeloproliferative diseases
- Histologic, cytologic, cytogenetic, and molecular biologic analysis

BM biopsy



BM failure Aplastic anemia



BM normal

Anemia caused by excessive RBC loss

– Causes of RBC loss?

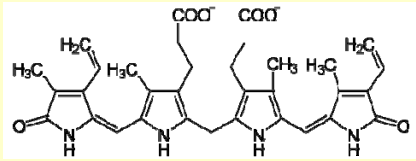
Anemia caused by excessive RBC loss

- **Hemolysis (shortened lifespan of erythrocytes)**
  - **extrinsic**
  - **intrinsic**
- **Acute bleeding**

Anemia caused by hemolysis (RBC destruction)

Typical laboratory finding

- Anemia
- Reticulocytosis
- Increased plasma LDH activity
- Increased unconjugated bilirubin level



- Decrease of hemolysis markers
  - **Haptoglobin** (binds hemoglobin)
  - **Hemopexin** (binds hem)

Anemia caused by hemolysis (RBC destruction)

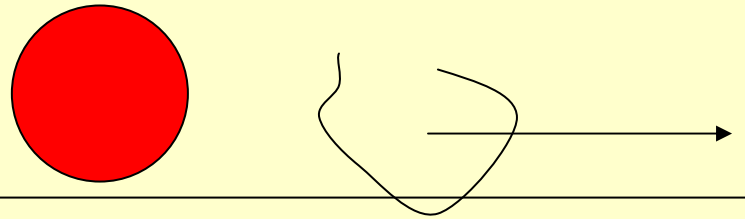
Reticulocytosis, LDH is increased, increase of unconjugated bilirubin

Extrinsic cause (normocytic-normochromic RBC)

- Immunologic abnormalities (AIHA, PNH)
- Mechanical injury (trauma, infection)

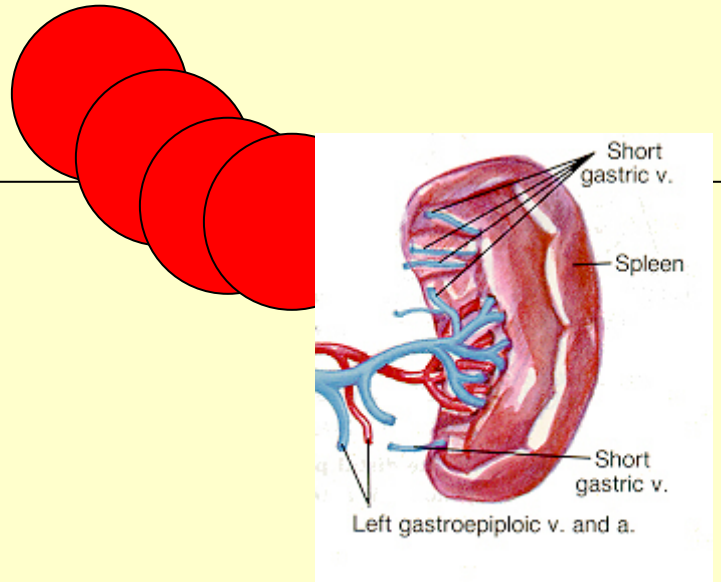
HEMOLYSIS

INTRAVASCULAR



haptoglobin
kidneys

EXTRAVASCULAR

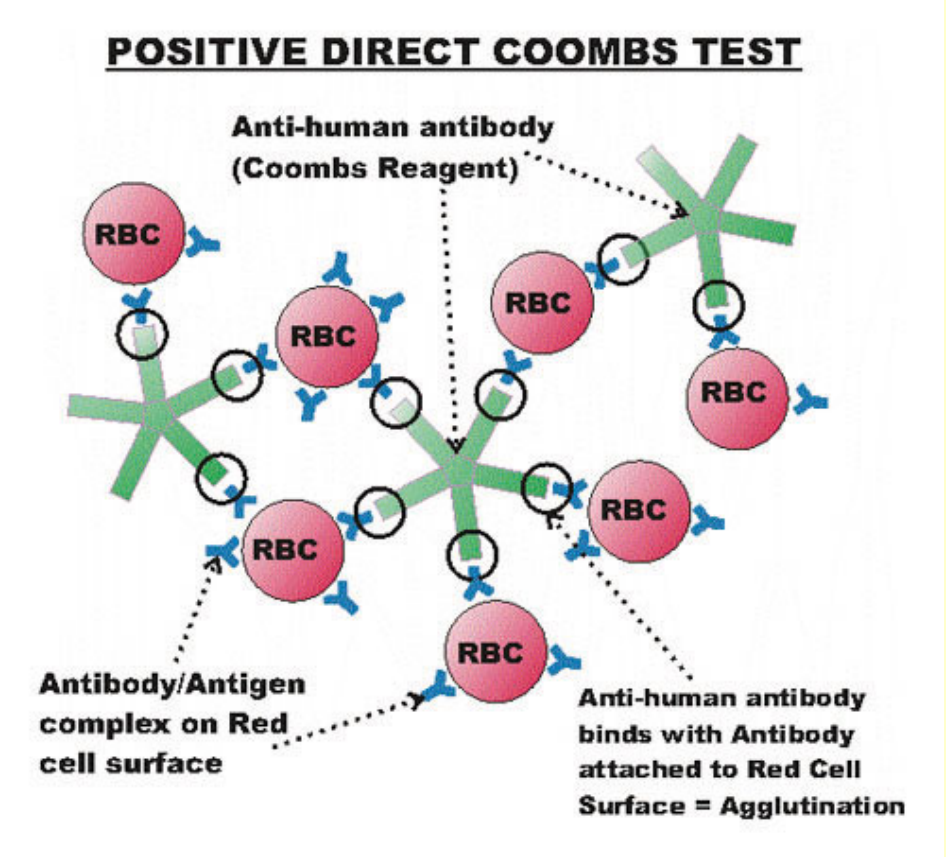


spleen, bone marrow, liver (macrophages)

AIHA

Hemolysis caused by immune mechanisms – Direct Coombs (antiglobulin) test

- *Detection of antibodies to erythrocyte surface antigens*
- *Antibodies other than to AB antigens*
- *These Abs are responsible for hemolysis*

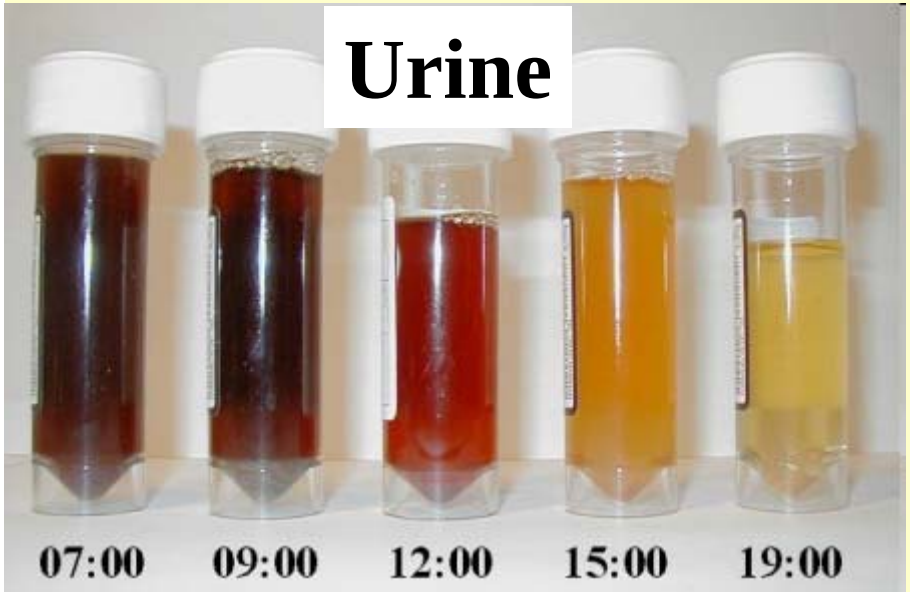


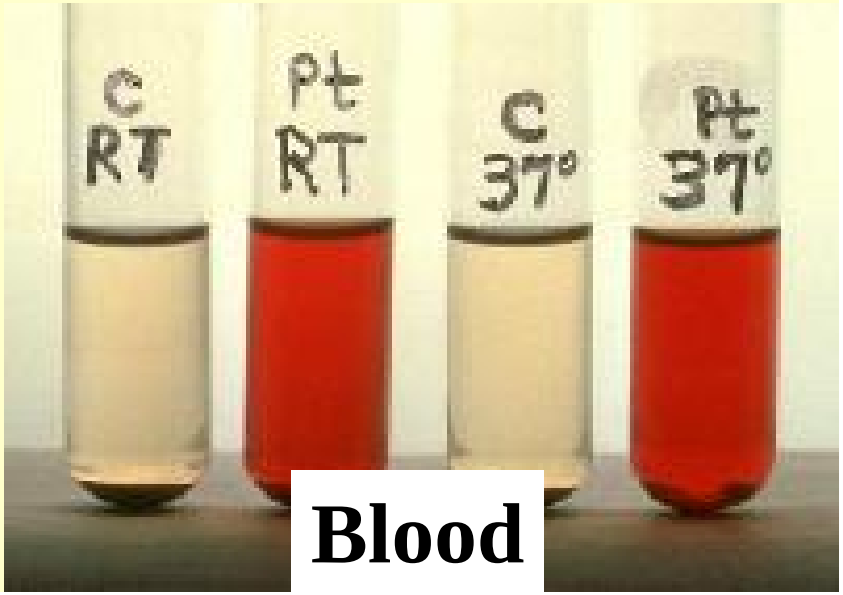
Antiglobulin serum (anti-hu Ig) is added to washed patients RBC: agglutination indicates presence of immunoglobulins bound to RBC

PNH

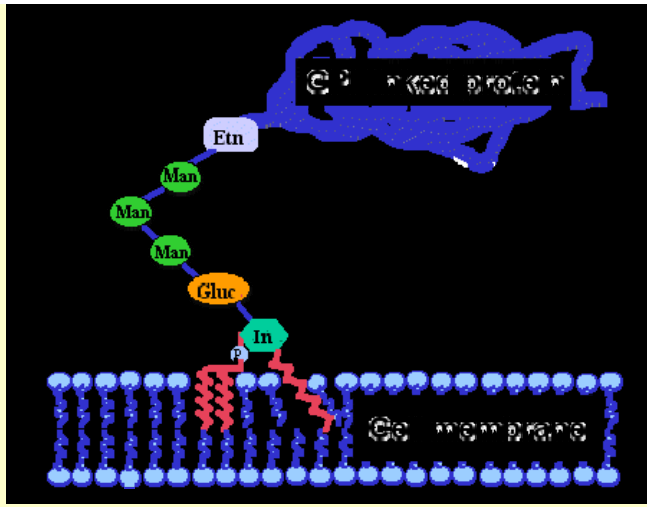
Acid hemolysis (Hams') test

- Diagnostic test for paroxysmal nocturnal hemoglobinuria (PNH)
- Acidification of blood (HCl) result in hemolysis due to complement activation in PNH patients (Ham) test





Diagnosis of Paroxysmal nocturnal hemoglobinuria (PNH) flow cytometry



- Acquired hemolytic anemia due to a hematopoietic stem cell mutation defect
- Glycosyl-phosphatidylinositol anchor abnormality caused by the PIG-A gene mutation
- Clinical manifestation result from the lack of GPI dependent proteins on the surface of a portion of leukocytes and erythrocytes
- Flow cytometry analysis for CD55 and CD59 is used to diagnose PNH

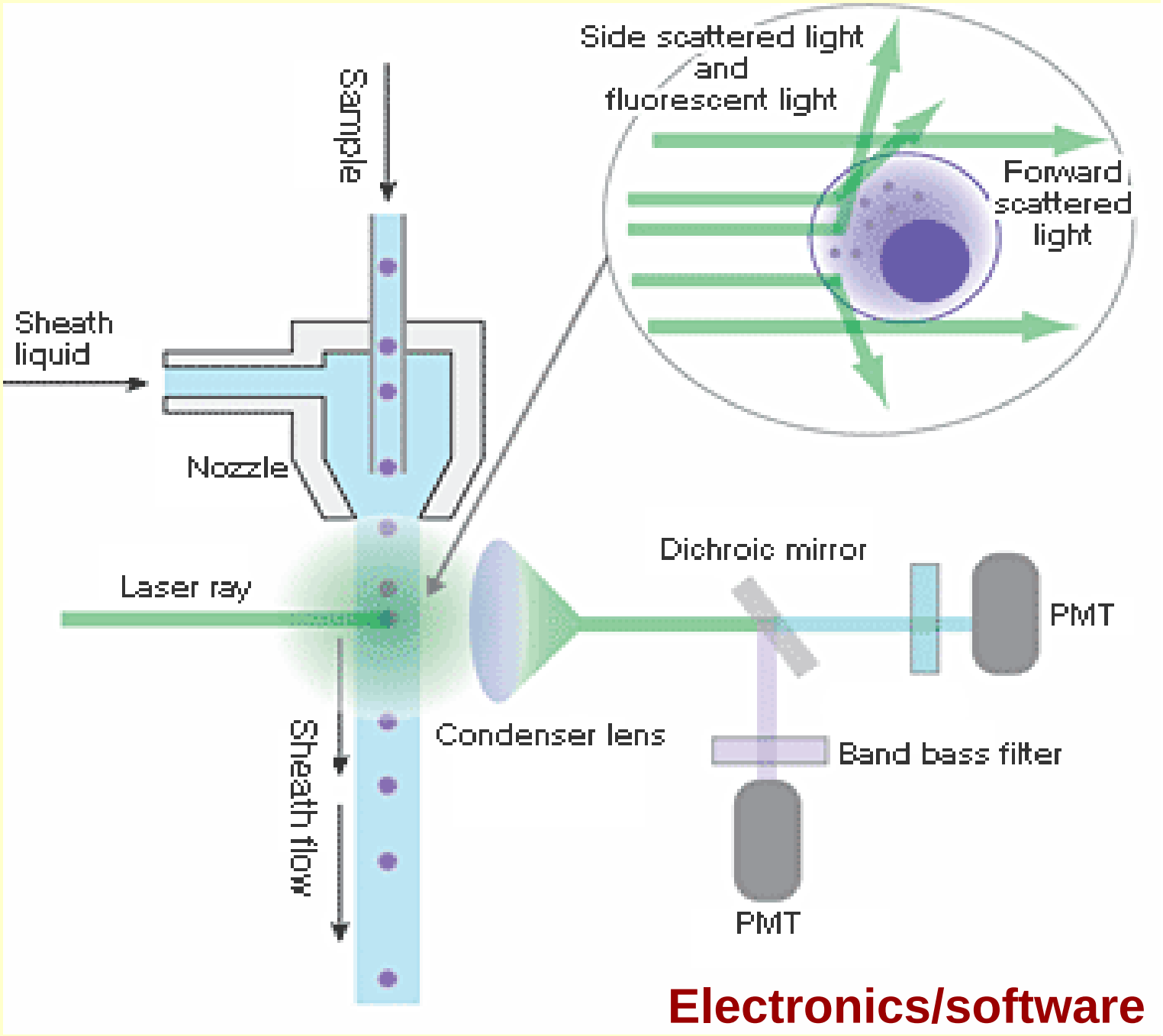
Flow cytometry

- Technique which allows quantitative and qualitative analysis of cells in suspension.

- **FACS** (Fluorescence-activated-cell-sorting)
  - analysis and sorting of cells in suspension based on the differences in light scattering and fluorescence of the cell or cell label.

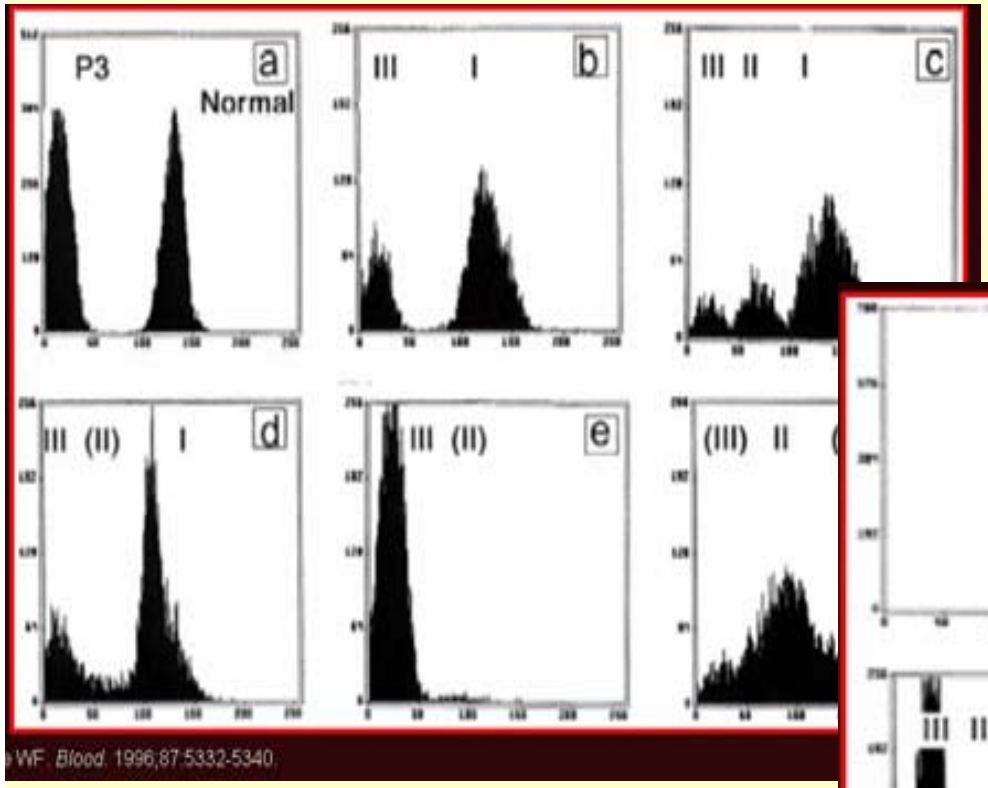
Fluidics

Optics



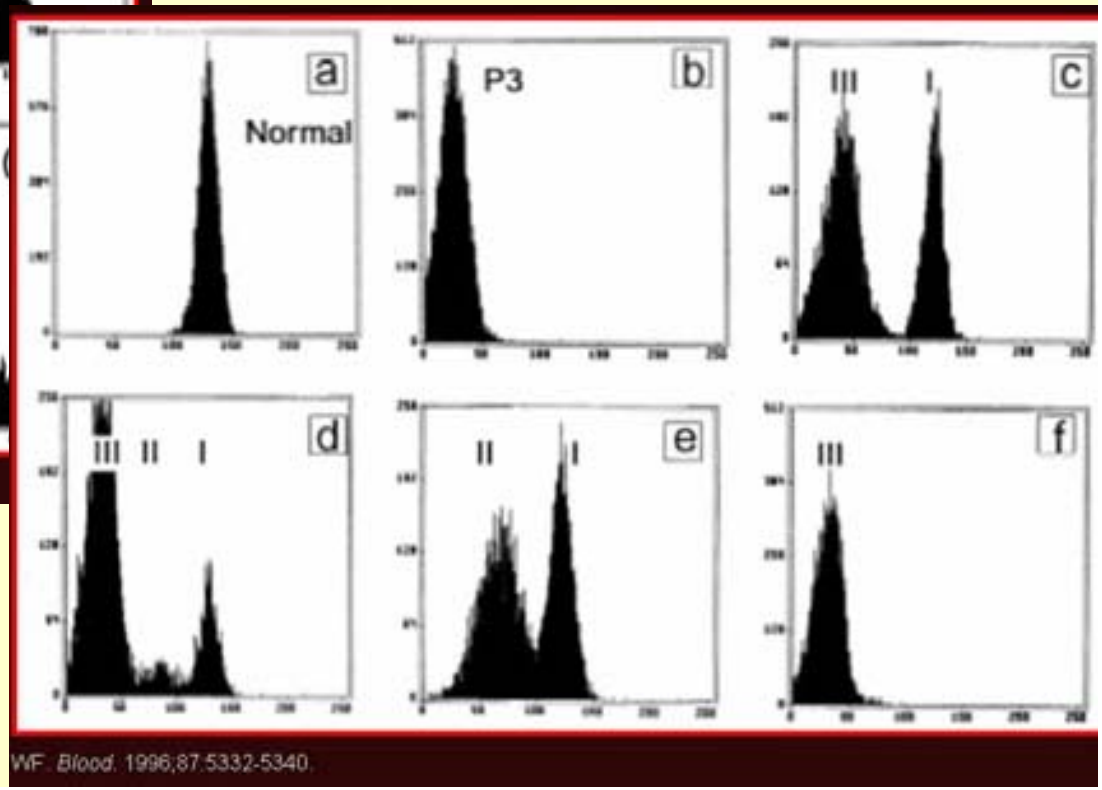
Electronics/software

Expression of CD59 on the surface of erythrocyte and granulocyte in PNH patients



Erythrocytes

Granulocytes

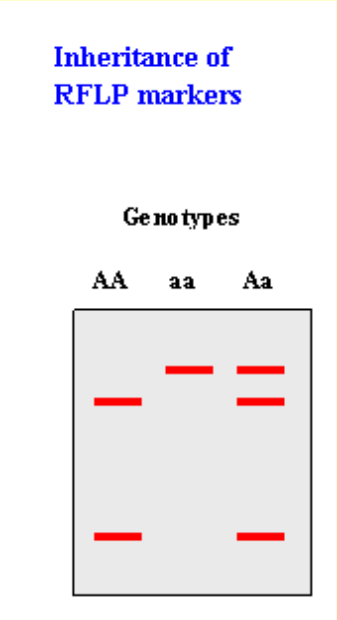


Anemia caused by hemolysis (RBC destruction)

reticulocytosis, LDH is increased, unconjugated bilirubin accumulate

- Intrinsic cause
  - Congenital
    - Membrane alterations (spherocytosis, elliptocytosis)
    - Metabolic disorders (G6PD deficiency)
    - Hemoglobinopaties (Sicle cell disease, Thalassemia)
  - Aquired
    - hypophosphatemia

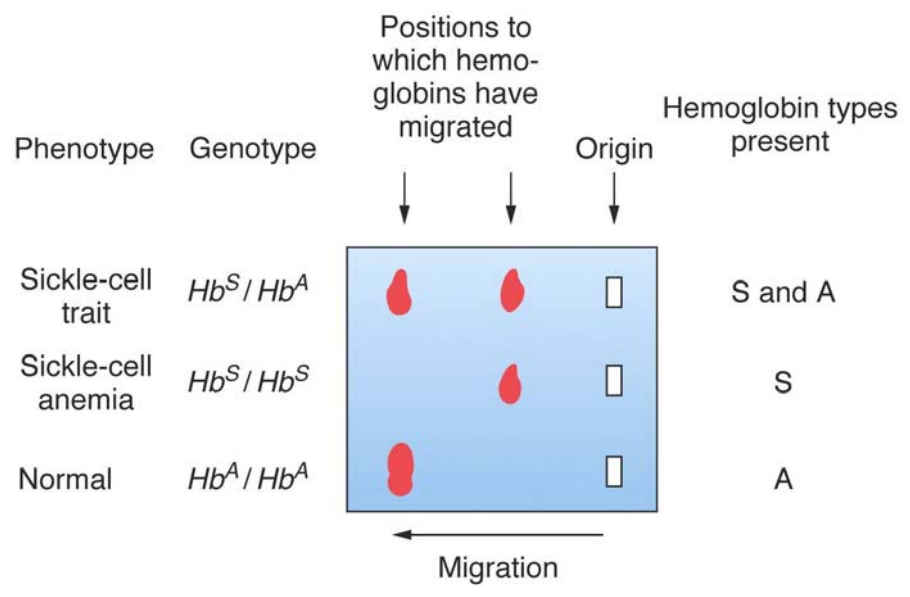
Mutation analysis

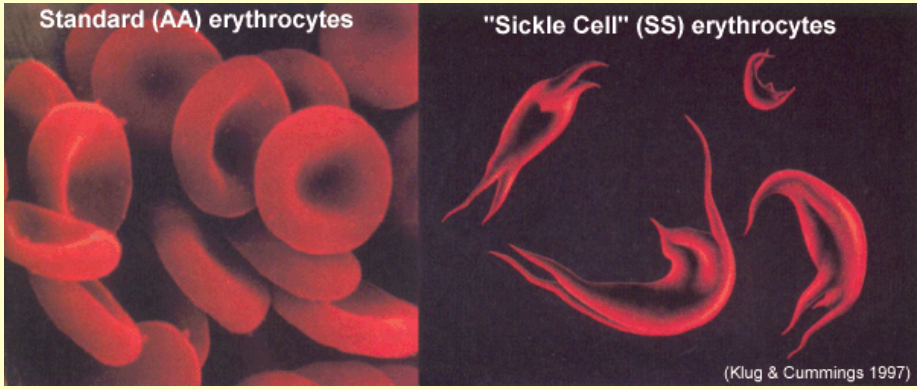


- RFLP (restriction fragment length polymorphism)
- ARMS (Amplification-refractory mutation system)
- Sequencing

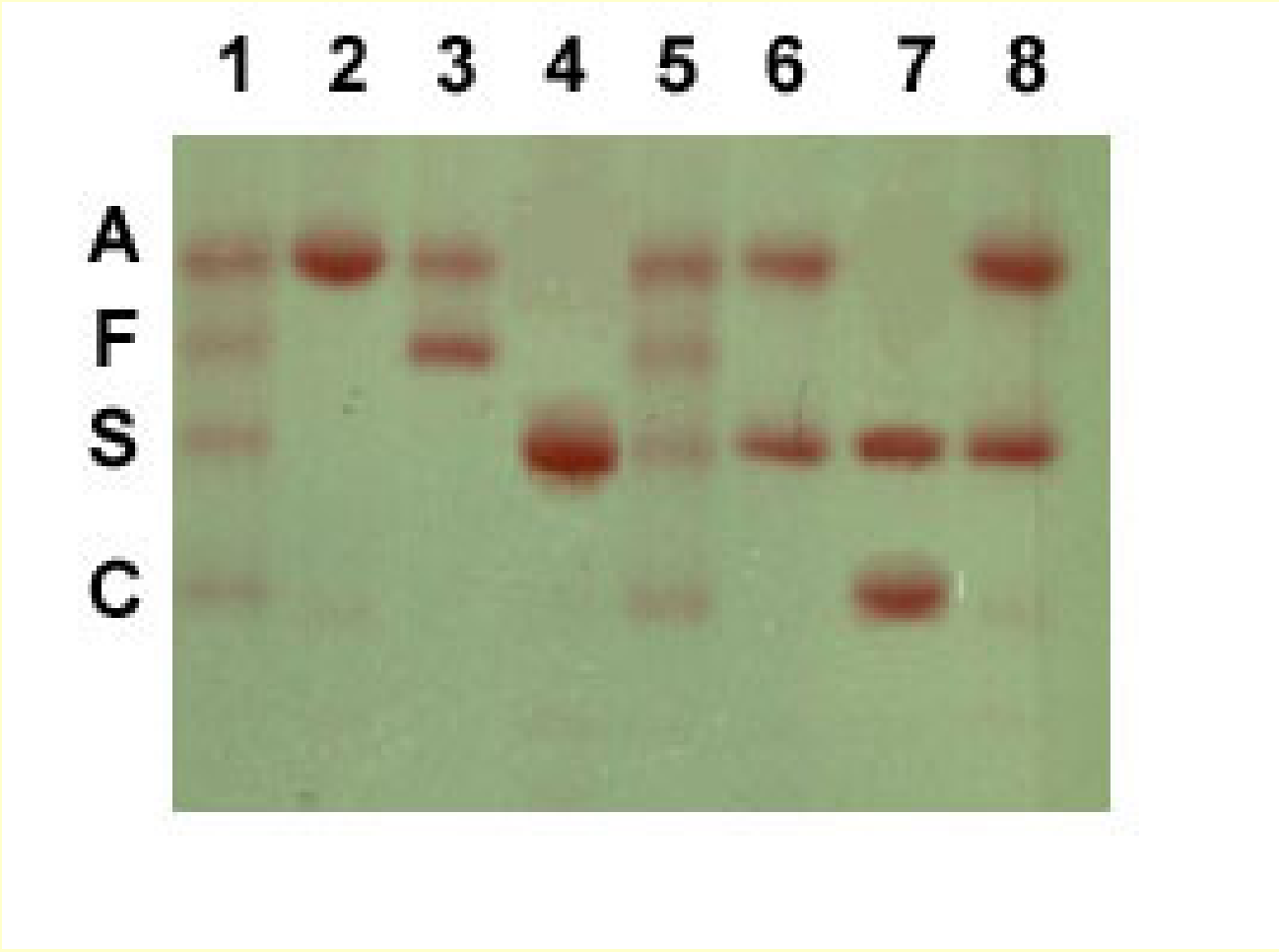
Evaluation of hemoglobin

- Hemoglobin electrophoresis
  - identification of abnormal hemoglobins
  - e.g. hemoglobin S
    - replacement of a negatively-charged glu in the HbA beta-globin by a neutral val in HbS results in a protein with a slightly negative charge





Hemoglobin electrophoresis



Pattern of hemoglobin electrophoresis from several different individuals. Lanes 1 and 5 are hemoglobin standards. Lane 2 is a normal adult. Lane 3 is a normal neonate. Lane 4 is a homozygous HbS individual. Lanes 6 and 8 are heterozygous sickle individuals. Lane 7 is a SC disease individual.

Malignant Hematopoiesis
Diagnostic Approach

Malignant hematopoiesis

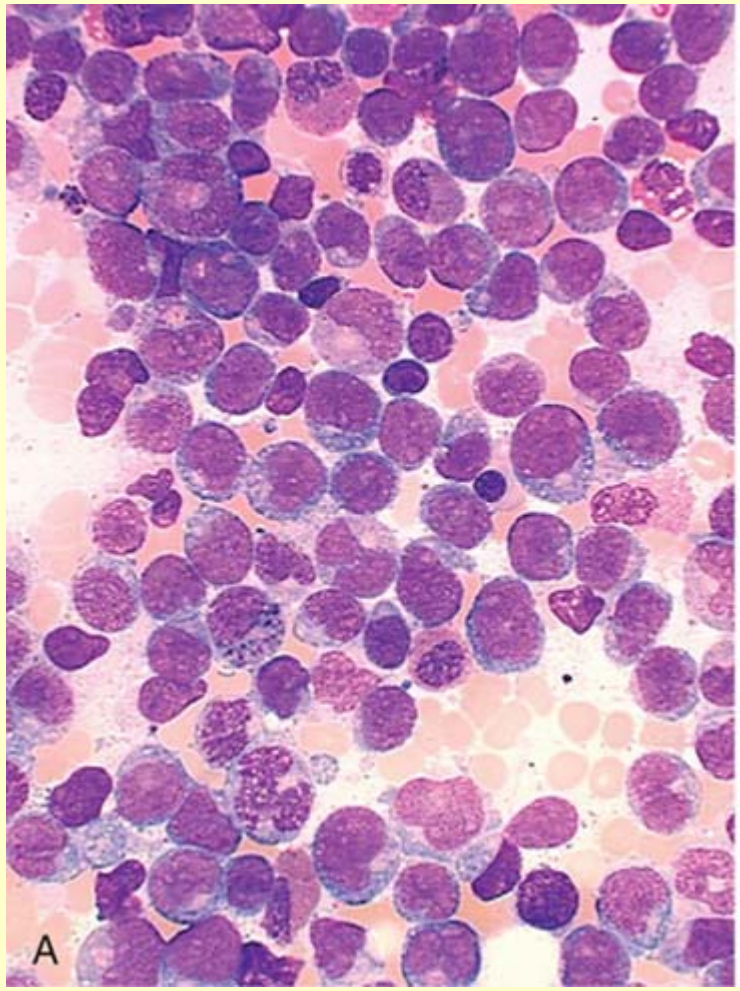
- Chronic myeloproliferative diseases
  - CML, PV, idiopatic myelofibrosis, ...
- Chronic lymphoproliferative diseases
  - CLL
- Acute myeloproliferative diseases
  - AML
- Acute lymfoproliferative diseases
  - Lymphoma (NHL x HL)

Peripheral Blood / Bone Marrow Analysis

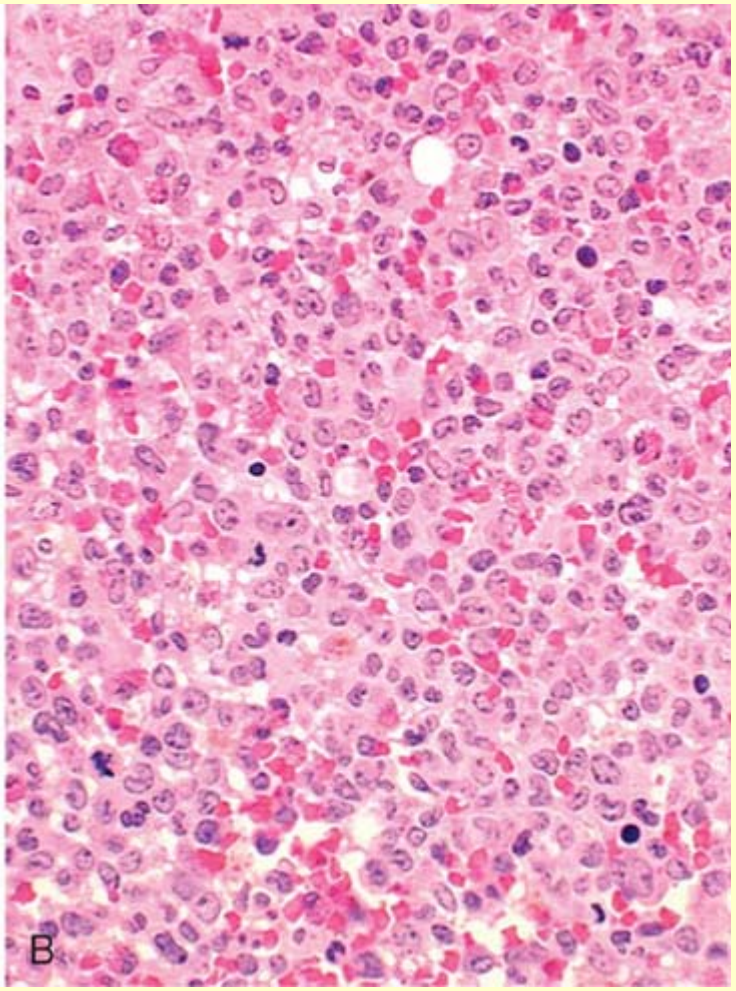
- Morphology of cells
- Cytogenetic and molecular analysis
- Immunophenotyping
- Histochemistry
- Cell phenotype

Cell morphology

Acute myelogenous leukemia

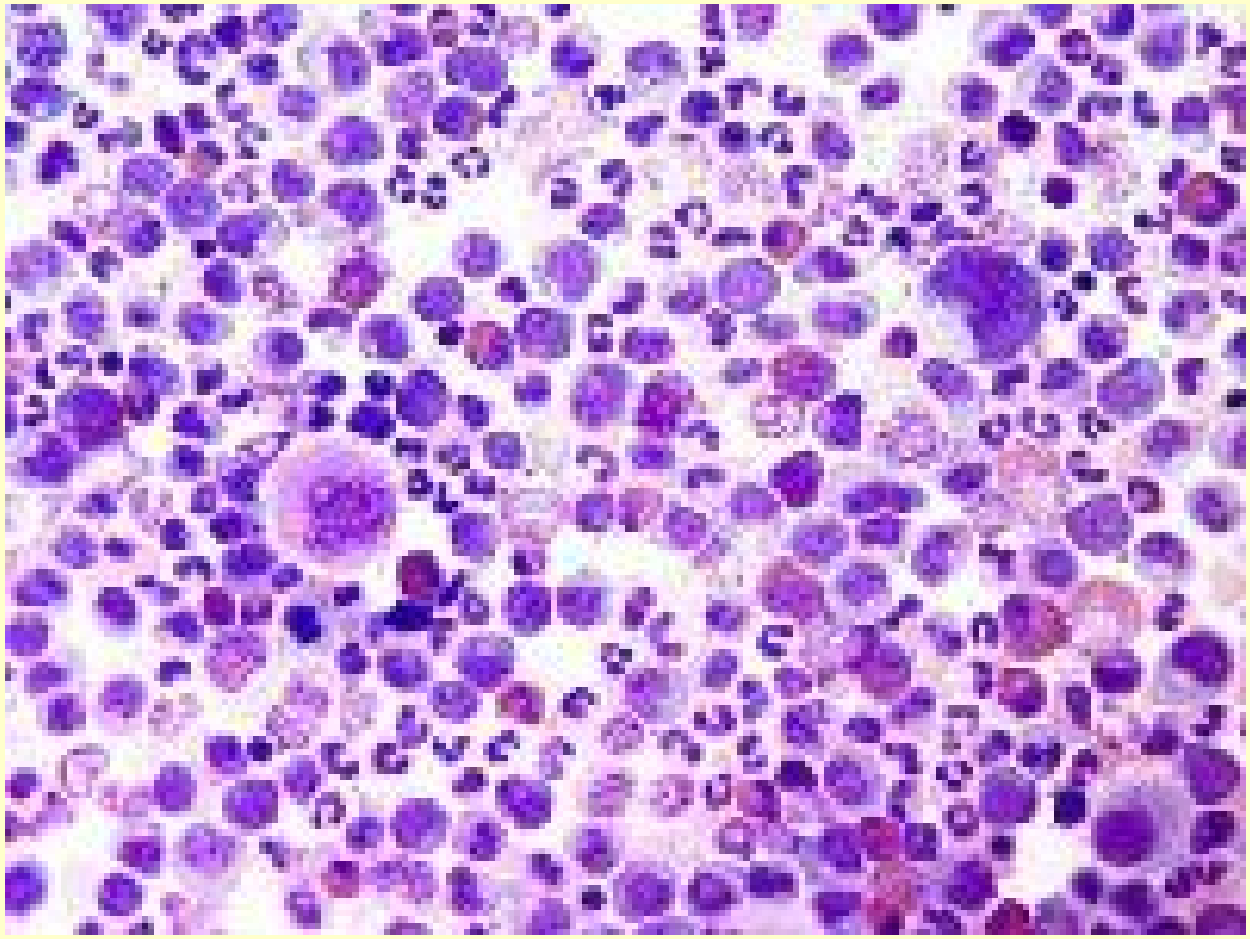


Bone marrow aspirate x50
> blasts



Bone marrow biopsy x40

BM cell aspirate: CML



BM cells (400X) demonstrates clear dominance of granulopoiesis

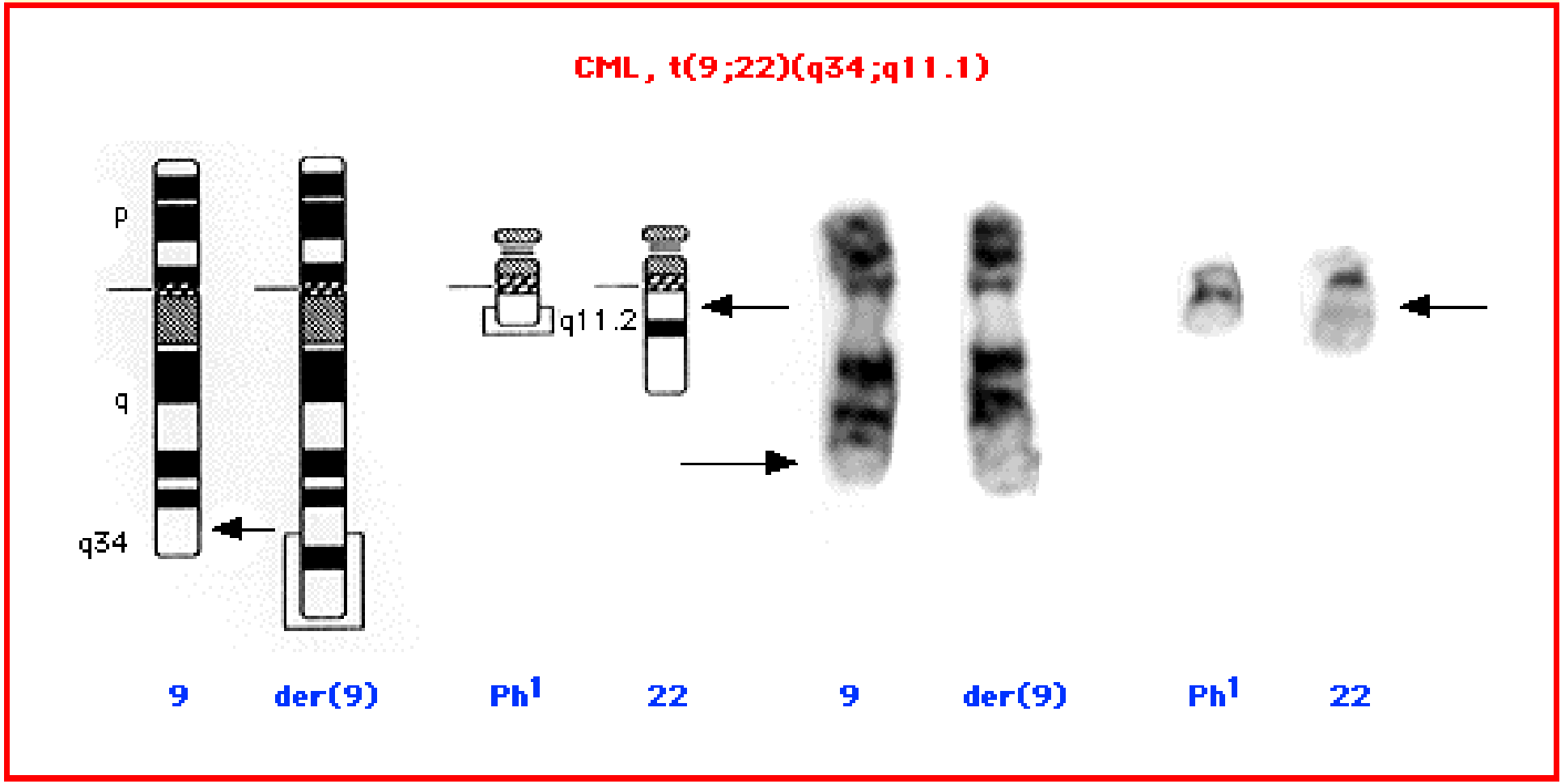
Cytogenetic and molecular analysis
Analysis of PB and BM

Chromosomal abnormalities

Gene mutations

Philadelphia chromosome

- Chromosome 22 often referred as Ph (>95% of pts with CML)
- Reciprocal translocation t(9;22) – piece of chromosome 9 (c-abl) translocate to chromosome 22 (bcr) [BCR-ABL] and piece of 22 to 9 [ABL-BCR].
- BCR-ABL - constitutively active tyrosin-kinase
  - target for therapy (STI571, imatinib mesylate, Gleevec – approved by FDA 2001)
  - prognostic value
  - therapy response - Minimal Residual Disease

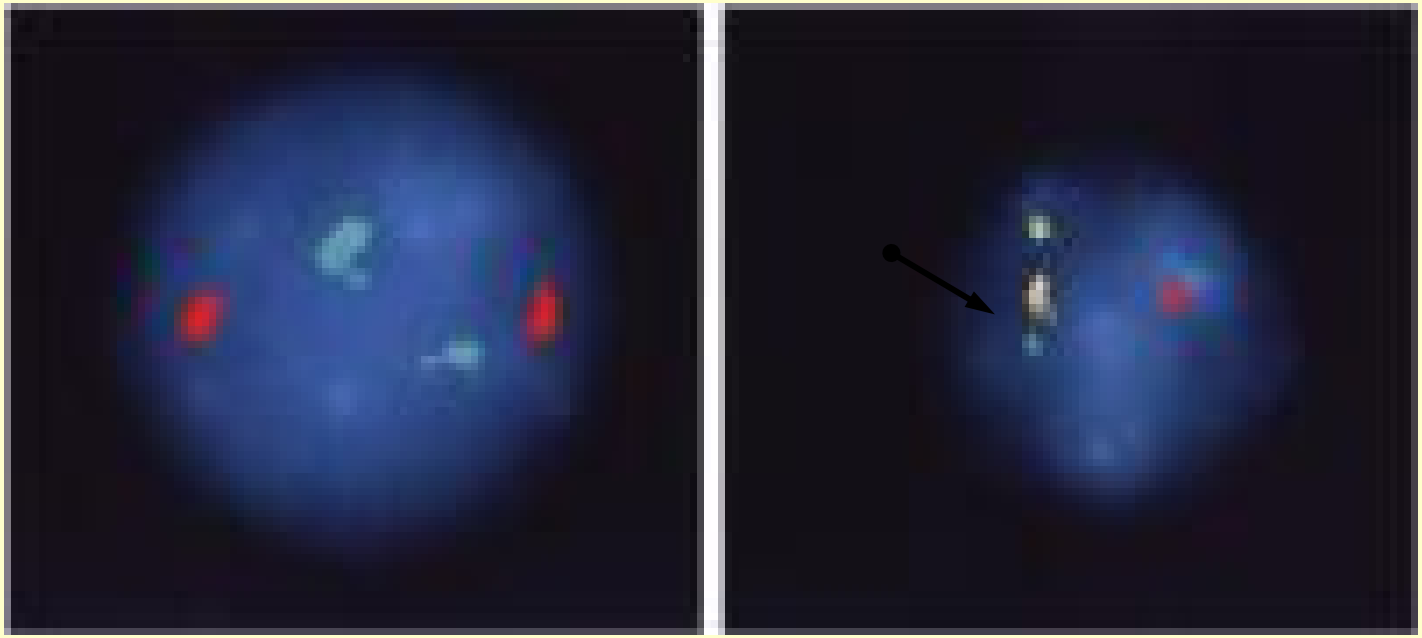


Genesis of Philadelphia chromosome G-band ideograms (left) and partial karyotype (right) of the CML-associated chromosome translocation t(9;22)(q34;q11). Breakpoints are indicated with arrows on the normal chromosome homologs. Translocated segments are framed on the der(9) and Ph ideograms. The translocation results in a slightly longer chromosome 9 [der(9)] and a shorter chromosome 22 [der(22)], which is termed the Philadelphia chromosome. Courtesy of Athena Cherry, PhD.

CML - FISH

control

bcr/abl fusion present



DNA probes:

- *bcr* (22q11.2) in red
- c-*abl* (9q34) in green.

Molecular analysis of BM and PB cells

- PCR measurement of gene expression or identification of mutations
  - Confirmation of diagnosis
    - Jak2 mutation = polycythemia vera
  - Prognostic markers
  - Monitoring of therapy (MRD):
    - AML: e.g. AML-ETO
    - CML: BCR-ABL

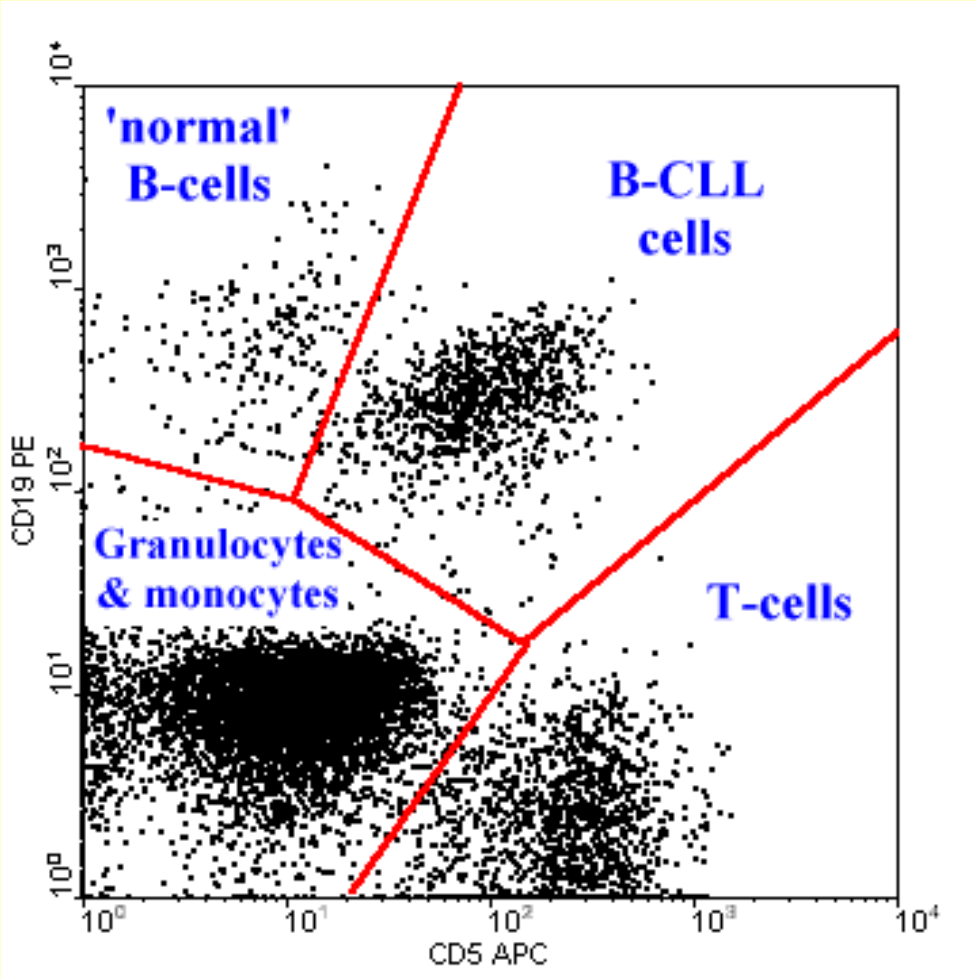
Immunophenotyping of BM or PB cells

Flow cytometry (FACS):

- suspected lymphoproliferative and myeloproliferative diseases (AML, CML)

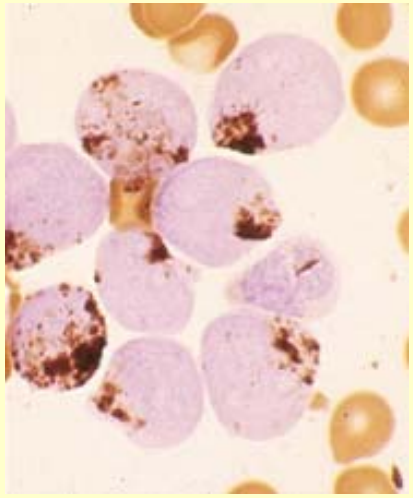
Flow cytometry use in hematology:

- immunophenotyping of myeloproliferative and lymphoproliferative diseases using fluorescently labeled antibodies



Histochemical analysis of enzymatic activity in BM blasts

- Alcaline phosphatase (ALP)
  - high in normal blasts / low in leukemia blasts

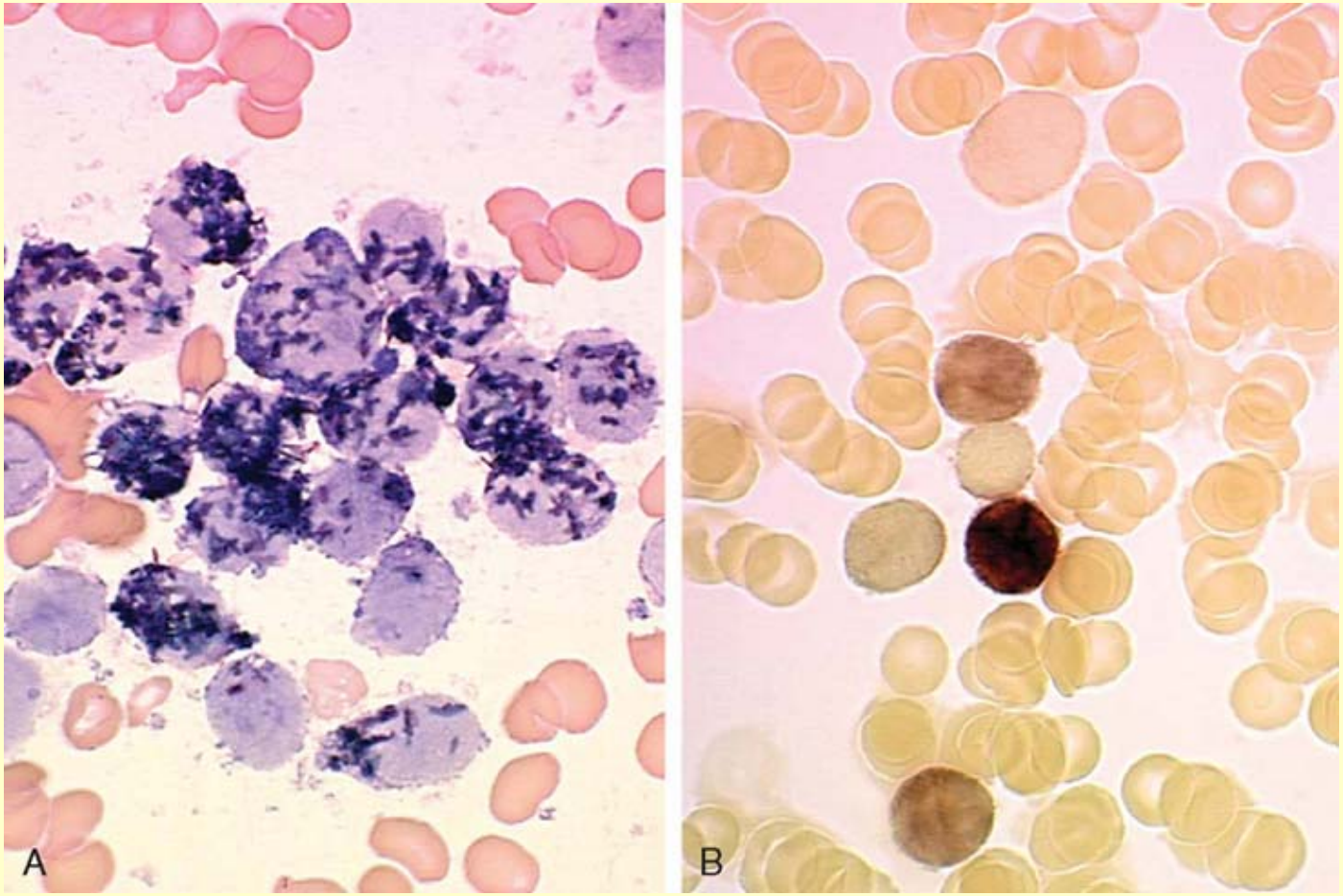


- Myeloperoxidase activity
  - blasts of myeloid origin

- Nonspecific esterase –
  - monocytes
  - positive eg. in AMML

Myeloperoxidase

Nonspecific esterase



• stain is positive in blasts and appears as blue-black cytoplasmic granularity –
• positive in blasts of myeloid origin

•useful for the identification of monocytic differentiation

•is positive in some blasts in this case of acute myelomonocytic leukemia.

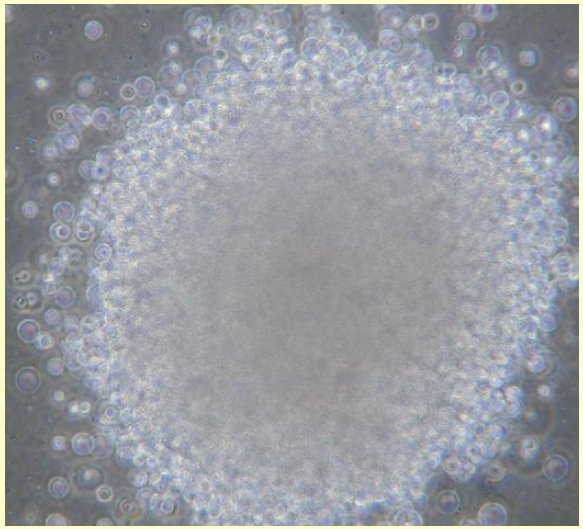
Clonogenic assay: Functional test for hematopoietic progenitors

PV, Leukemia, MDS

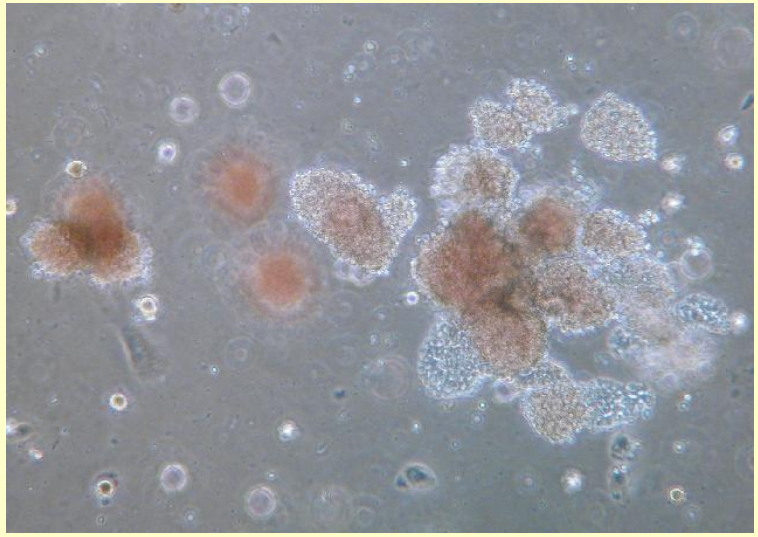
- Cultivation of BM cells in semisolid media with growth factors
- EPO independent BFU-E colonies P. Vera
- CFU-GM/cluster ratio – decrease in leukemic hematopoiesis

Clonogenic assay

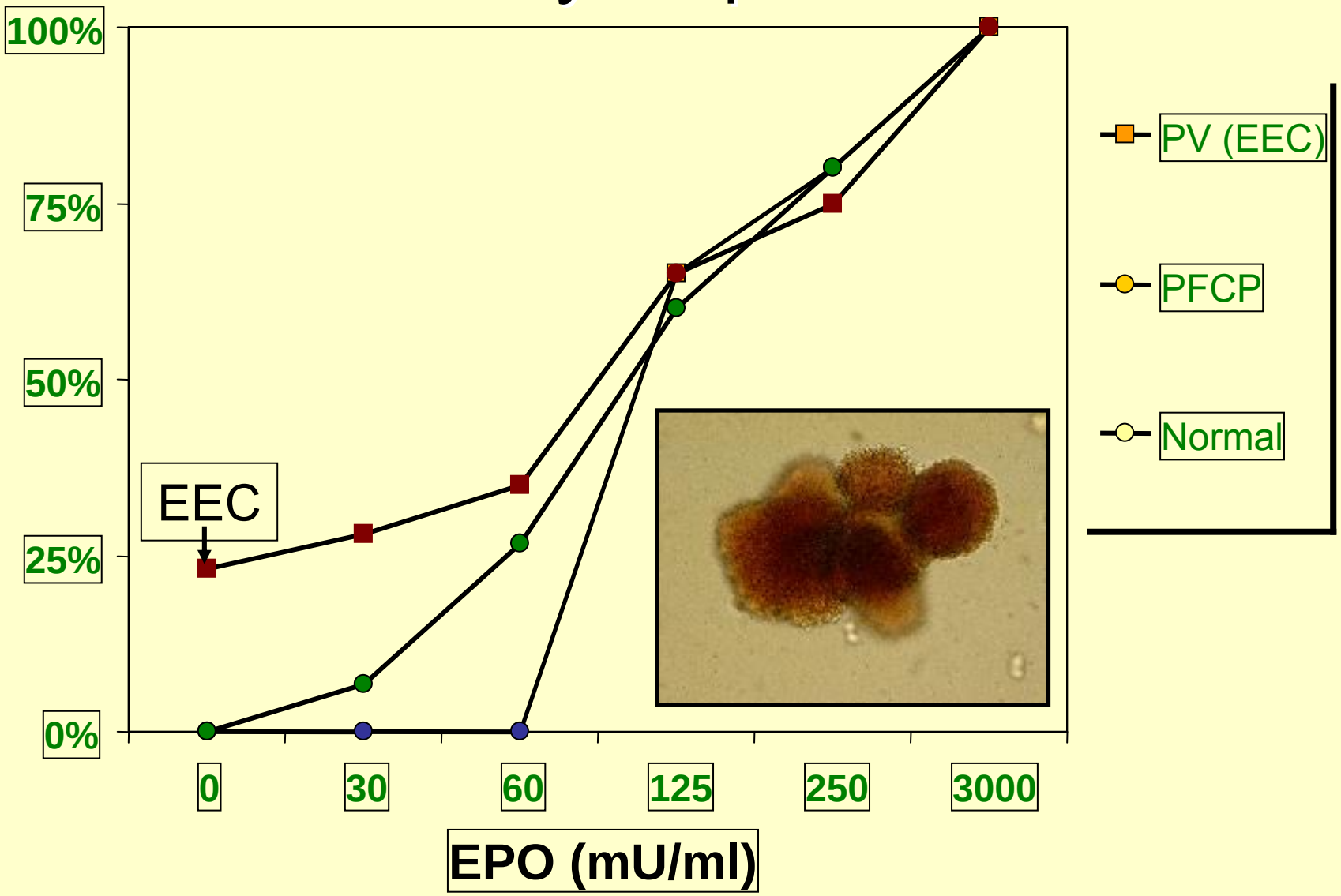
CFU-GM



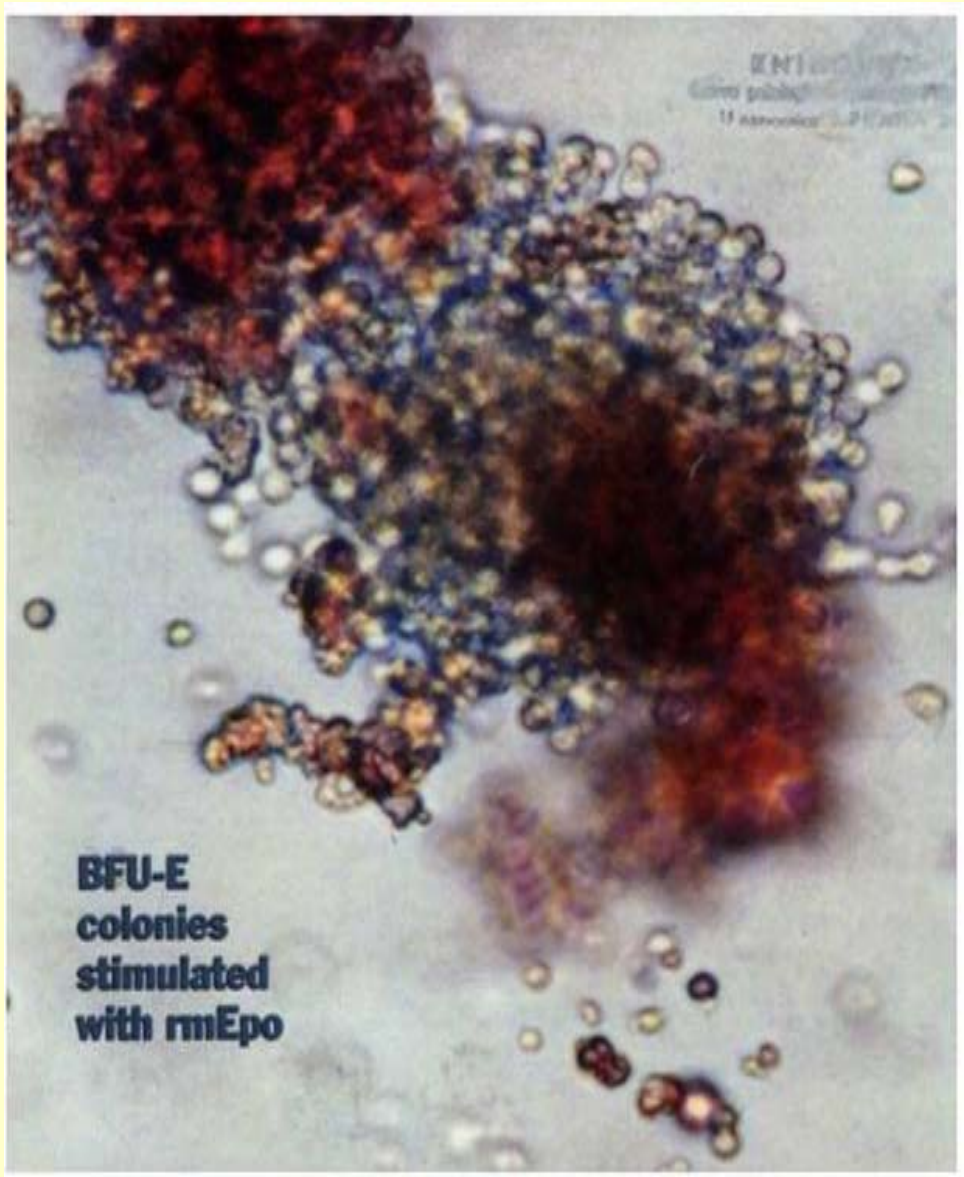
BFU-E



Sensitivity of BFU-E progenitors to erythropoetin



Burst Forming Unit - Erythroid colony



Stopka T, Zivny J, Goldwasser E *et al.* Experimental Hematology 26:831-834 (1998)

Questions

What is the major pathophysiological consequence of anemia?

a. decrease in partial pressure of oxygen in blood

b. worsening of rheological properties of blood and clotting of blood in microcirculation

c. decrease in oxygen delivery to the tissues

d. bone marrow failure

Questions

What is the definition of anemia?

a. decrease in red blood cell count
b. decrease in hematocrite below 30%
c. decrease in blood hemoglobine concentration
d. bone marrow failure

Questions

What happens with partial pressure of oxygen in arterial blood of anemia patient?

a. Is often slightly increased (compensation)

b. Partial pressure of oxygen in arterial blood is independent on hemoglobin level

c. Is decreased

d. Is significantly increased

Questions

What happens to the partial pressure of oxygen in venous blood of patient with severe anemia?

a. Is often slightly increased (compensation)
b. Is decreased
c. Is significantly increased
d. Similar to individuals w/o anemia

Thank you