What are the components of blood?

- Red blood cells: 45%
- White blood cells: 0.1%
- Platelets: 0.17%
- Plasma: 55%
Blood components

Whole blood

- Red cells
  - Hemoglobin
- White cells
  - Types of white cells
- Platelets
- Plasma 55%
  - Water 91%
  - Protein 7%
  - Clotting factors
    - Salt, sugars, Hormones, Vitamins etc. 1.5%
  - Albumin 55%
  - Globulins 38%
  - Fibrinogen 7%
    - Beta
Basic methods for the analysis of blood composition

Measurement of the erythrocyte sedimentation rate (ESR)
Measurement of the hematocrit (centrifuge)
Measurement of whole blood viscosity or plasma viscosity

The results of these methods are affected by blood composition (ratio between cells and plasma, blood composition).
However, except hematocrit they give no results for specific parameters (cells, proteins).
Measurement of the erythrocyte sedimentation rate (ESR)
Principle of the erythrocyte sedimentation rate

- Normal RBC
- RBC and APPs

RBC

Acute phase protein (APP; e.g. fibrinogen)

Sialic acid
Proteins affecting the erythrocyte sedimentation rate (ESR)

Despite the fact that the test in its present form has been in constant use since 1926 the phenomenon of red cell sedimentation is still only partly understood.

In the lag phase of the sedimentation the red cells form a characteristic rouleaux pattern. The size of the rouleaux aggregates formed in the lag Phase is the critical factor affecting the final result of the ESR. The rouleaux itself appears to be influenced mainly by certain plasma proteins including fibrinogen (55 %), α₂-macroglobulin (27 %) immunglobulins (11 %) and albumin (7 %). Opinions vary as to the accelerating and retarding properties of glycoproteins and albumin.
Methods for the determination of the erythrocyte sedimentation rate

Method for the manual determination of the ESR

Automated method for the determination of the ESR
Reference values for the erythrocyte sedimentation rate in adults

<table>
<thead>
<tr>
<th>Age</th>
<th>Upper limit of ESR reference range [mm/hr]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;50 years</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>0 to 15</td>
</tr>
<tr>
<td>Women</td>
<td>0 to 20</td>
</tr>
<tr>
<td>Age &gt;50 years</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>0 to 20</td>
</tr>
<tr>
<td>Women</td>
<td>0 to 30</td>
</tr>
</tbody>
</table>

ESR: Erythrocyte sedimentation rate
Individual factors that may increase erythrocyte sedimentation rate (ESR) - I

Anthropometric factors:
Old age
Female
Pregnancy and menstruation cycle (increase in fibrinogen, anemia)

Inflammatory diseases:
Cytokine driven processes that elevate fibrinogen concentration
(e. g. infection, inflammation, malignancy)

Relative/absolute increase in globuline proteins:
Loss of albumin (e.g. loss of albumin in nephrotic syndrome or increase of globulins in multiple myeloma)
Individual factors that may increase erythrocyte sedimentation rate (ESR) - II

Extensive tissue necrosis:
Myocardial infarction, trauma, tumors

Anemia
E. g. iron deficiency anemia

Red blood cell abnormalities:
Macrocytosis

Medication:
E. g. heparin, plasma expanders, oral contraceptives
Individual factors that may decrease erythrocyte sedimentation rate (ESR)

Factors affecting blood cells:

Extreme leukocytosis
Factors red cell number or shape:
  Polycythemia vera, spherocytosis, acanthocytosis, microcytosis, sickle cell disease

Protein abnormalities:

Increased plasma viscosity (Waldenstrom’s macroglobulinemia)
Decreased plasma proteins (hepatic necrosis, hypofibrinogenemia, hypogammaglobulinemia)
Factors with a questionable effect on erythrocyte sedimentation rate

Factors with no clinically significant effect or questionable effect:

- Obesity
- Body temperature
- Recent meal
- Aspirin
- Nonsteroidal anti-inflammatory drugs (NSAIDs)
Technical factors that may influence the erythrocyte sedimentation rate

Factors that increase ESR:
- Dilutional problem
- Increased temperature of specimen
- Tilted ESR tube

Factors that decrease ESR:
- Dilutional problem
- Inadequate mixing of sample
- Clotting of blood sample
- Short ESR tube
- Vibration during testing
Typical changes of CRP, fibrinogen, ESR and albumin during acute phase reaction

- CRP [mg/l]
- Fibrinogen [% changes]
- ESR [mm/h]
- Albumin [% changes]
Functions and changes of plasma concentrations of acute phase proteins during acute phase response - I

<table>
<thead>
<tr>
<th>Function</th>
<th>Acute phase protein</th>
<th>Increase up to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease inhibitors</td>
<td>$\alpha_1$-antitrypsin</td>
<td>4 fold</td>
</tr>
<tr>
<td></td>
<td>$\alpha_1$-antichymotrypsin</td>
<td>6 fold</td>
</tr>
<tr>
<td>Coagulation proteins</td>
<td>Fibrinogen</td>
<td>8 fold</td>
</tr>
<tr>
<td></td>
<td>Prothrombin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Factor VIII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasminogen</td>
<td></td>
</tr>
<tr>
<td>Complement factors</td>
<td>C1s</td>
<td>2 fold</td>
</tr>
<tr>
<td></td>
<td>C2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C3, C4, C5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C5b</td>
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</table>
Functions and changes of plasma concentrations of acute phase proteins during acute phase response - II

<table>
<thead>
<tr>
<th>Function</th>
<th>Acute phase protein</th>
<th>Increase up to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport proteins</td>
<td>Haptoglobin</td>
<td>8 fold</td>
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<tr>
<td></td>
<td>Haemopexin</td>
<td>2 fold</td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>4 fold</td>
</tr>
<tr>
<td>Scavenger proteins</td>
<td>Ceruloplasmin</td>
<td>4 fold</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>$\alpha_1$-acid glycoprotein</td>
<td>4 fold</td>
</tr>
<tr>
<td></td>
<td>(orosomucoid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum amyloid A protein</td>
<td>1000 fold</td>
</tr>
<tr>
<td></td>
<td>C-reactive protein</td>
<td>1000 fold</td>
</tr>
</tbody>
</table>
Structure and function of C-reactive protein (CRP)

CRP is a cyclic pentamer composed of 5 non-covalently bound 23.5 kD subunits.

CRP was first shown in 1930 by Tillet and Francis who were able to demonstrate that CRP could bind to the C-polysaccharide of pneumococci in acute phase sera.

The main function of CRP is related to the ability to bind biologically significant ligands in vivo.

CRP is found in primitive species like the horse-shoe crab and evolutionary maintained with few structural changes in higher vertebrates like man.

This indicates that CRP has an important function in the host defense system.
Documented and proposed functions and interactions of CRP

- Activation of complement
- Opsonization of bacteria and cell debris
- Blochs activation of macrophages
- Inhibition of platelet aggregation
- Increased hemagglutination
- Blocks superoxide release
- Chemotaxis
- Anti-tumor effect
- Enhanced NK-cell activity
- Binding to LDL
- Clearance of chromatin
- Enzyme inhibition cathepsin, elastase
## Comparison of erythrocyte sedimentation rate and other markers of inflammation

<table>
<thead>
<tr>
<th>Test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR</td>
<td>Inexpensive, quick, simple to perform</td>
<td>Affected by a variety of factors, including anemia and red blood cell size; not sensitive enough for screening</td>
</tr>
<tr>
<td>C-reactive protein (CRP)</td>
<td>Most rapid response to inflammation (complementary to ESR in this regard)</td>
<td>Wide reference may necessitate sequential recording of values; expensive, batch processing may delay individual results</td>
</tr>
<tr>
<td>Plasma viscosity</td>
<td>Unaffected by anemia or red blood cell size</td>
<td>Expensive, not widely available, technically cumbersome to perform</td>
</tr>
</tbody>
</table>
Centrifugation techniques

Different centrifugation techniques are used for:
Measurement of the packed cell volume (hematocrit)
Cytocentrifugation (e.g., cerebrospinal fluid)
Cell isolation (cell culture, preparation of blood components for therapy)
Sedimentation by centrifugation

The settling rate can be increased by centrifugation. In clinical laboratory routine centrifugation techniques are used for:

# Measurement of hematocrit.
# Separation of plasma or serum from blood.
# Cytocentrifugation of cells in special samples (e.g. cerebrospinal fluid, punctation fluid).
# Isolation of blood cells by density gradient centrifugation.
Capillary tubes are designed for both safe blood collection as well as accurate micro hematocrit determinations. Prior to centrifugation they must be closed by a capillary tube sealant.
Measurement of micro-hematocrit by centrifugation - II

Capillaries after centrifugation

Scale for measurement of micro-hematocrit after centrifugation
Measurement of micro-hematocrit by centrifugation - III

- Sealant
- Air
- Plasma
- Red blood cells
In normal samples a thin buffy coat of leukocytes and thrombocytes is visible between red blood cells and plasma.

In leukemia patients the volume of this buffy coat is increased because of the high leukocyte number and the number of erythrocytes is decreased.
Measurement of hematocrit

Manual method:
Capillaries: Length 7.5 cm, lumen 1.15 mm, wall 0.2 mm
Centrifuge: Rotor radius >8 cm, maximum speed < 30 sec., $\omega$: 10000 g - 15000 g

\[
\text{Hct} = \frac{\text{Length of RBC column [mm]}}{\text{Length of Plasma column} + \text{blood cell column [mm]}}
\]

Hematological analyzer:
Erythrocyte count by means of the impedance method or the scatter light method.

\[
\text{Hct} = \frac{\text{MCV [fl]} \cdot \text{erythrocyte concentration [l}^{-1}]}{10^{15}}
\]
## Reference values of hematocrit

<table>
<thead>
<tr>
<th></th>
<th>Hematocrit [l/l]</th>
<th></th>
<th>Hematocrit [l/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord blood</td>
<td>0.48-0.56</td>
<td>Females:</td>
<td></td>
</tr>
<tr>
<td>2 hours</td>
<td>0.49-0.71</td>
<td>14-16 years</td>
<td>0.35-0.43</td>
</tr>
<tr>
<td>6 hours</td>
<td>0.44-0.68</td>
<td>Adults</td>
<td>0.35-0.45</td>
</tr>
<tr>
<td>2.-6. days</td>
<td>0.40-0.70</td>
<td>Males:</td>
<td></td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>0.38-0.70</td>
<td>14-16 years</td>
<td>0.38-0.49</td>
</tr>
<tr>
<td>2-3 weeks</td>
<td>0.38-0.60</td>
<td>Adults</td>
<td>0.36-0.48</td>
</tr>
<tr>
<td>3-7 weeks</td>
<td>0.36-0.46</td>
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<tr>
<td>7-12 weeks</td>
<td>0.30-0.38</td>
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<tr>
<td>10-12 months</td>
<td>0.35-0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5 years</td>
<td>0.32-0.40</td>
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<tr>
<td>6-8 years</td>
<td>0.32-0.41</td>
<td></td>
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<tr>
<td>10-13 years</td>
<td>0.34-0.44</td>
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</tbody>
</table>
Other techniques based on centrifugation

Cytocentrifugation:
Enrichment of cells (prior to microscopy of biological fluids).

Density gradient centrifugation:
Isolation of blood cells.

Density centrifugation:
Isolation of blood cells (e. g. blood products)
Cytocentrifugation of cells

Cytocentrifugation is performed in samples with low cell concentrations. They allow a concentration of cells on slides which can then be stained and analyzed by means of different microscopic methods.

Typical samples are cerebrospinal fluid, urine and punctation fluid.

Cytocentrifugation is used in cytology, immunology, immunochemistry, electronmicroscopy, microbiology, virology and genetics.
Protocol for cytocentrifugation

Prepare a cell suspension of not more than $10^6$ cells/ml of protein-containing medium.
Prepare the slides mounted with the paper pad and the cuvette in the metal holder.
Load up to 200 µl of this suspension in each cuvette and spin at 800 rpm for 3 min.
Extract the slide, paper and cuvette without disarranging. Carefully detach the cuvette and the paper without damaging the fresh cytospin.
Proceed with either immediate fixation or drying. Store unfixed cytospins for max. 2 days at room temperature.
Samples of materials for cytocentrifugation

- Tumor cells in pleural fluid
- Ovarial carcinoma in ascites
- Cells in normal urine
- Cell in normal synovial fluid
- CSF cells of a patient with Cryptococcal meningitis
Principle of isopycnic density gradient centrifugation

In the isopycnic technique, the density gradient column encompasses the whole range of densities of the sample particles. The sample is uniformly mixed with the gradient material. Each particle will sediment only to the position in the centrifuge tube at which the gradient density is equal to its own density, and there it will remain. The isopycnic technique, therefore, separate particles into zone solely on the basis of their density differences, independent of time.
In differential centrifugation the entire tube is filled with sample and centrifuged. Through centrifugation, one obtains a separation of two particles but any particle in the mixture may end up in the supernatant or in the pellet or it may be distributed in both fractions, depending upon it size, shape, density, and conditions of centrifugation. The pellet is a mixture of all of the sedimented components, and it is contaminated with whatever unsedimented particles were in the bottom of the tube initially. The only component which is purified is the slowest sedimenting one. The supernatant can be recentrifuged at higher speed for further purification.
Measurement of viscosity

Half-automated methods allow the determination of static and dynamic viscosity of whole blood or plasma. These parameters play a role in rheology.

Plasma viscosity depends on the protein composition of plasma (high molecular weight proteins (e.g. lipoproteins, $\alpha_2$-macroglobulin) and non-globular proteins (fibrinogen)).

Whole blood viscosity depends on cell number, cell type and cell membrane structure (e.g. rigid leukocytes after activation).
Criteria for the measurement of viscosity and viscoelasticity

A suitable system for the measurement of blood viscoelasticity or plasma viscosity must have several features for clinical applications:

Rapid, reproducible and precise measurements
Small blood or plasma sample volume
Simulate *in vivo* time-varying flow conditions using oscillatory flow in a tube
Precise thermal control
Simple operation
Minimal exposure of operator to blood borne pathogens
Counting chambers for cell quantification

Counting chambers for counting blood cells consist of a thick glass slide having a central polished and ruled platform. The platform is positioned a short distance (typically 0.1 mm) beneath twin polished cover slip supports to create a chamber that can be filled with a precise quantity of fluid. In practice, a clean glass cover slip is placed over the chamber and centrally positioned on the polished supports. The gap between the ruled counting platform and the cover slip equals 100 µm and the engraved face is divided into squares of exact dimension. As a result, the volume of the liquid placed in the chamber can be easily calculated to yield an accurate analysis of the number of particles (cells) per unit volume in a suspension.
Different types of counting chambers

Neubauer Counting chamber:
Designed for counting leukocytes, eosinophils, thrombocytes and cells in cerebrospinal fluid.

Fuchs-Rosenthal Counting Chamber:
Designed for counting leukocytes, eosinophils and cells in cerebrospinal fluid.

Petroff-Hausser Counting Chamber:
Designed for the counting of bacteria, sperm, blood platelets, and the content of vaccines.

Leica Bright-Line Counting Chamber:
Designed by Leica for accurate counting of blood cells platelets, dust/yeast cluster and specimen samples.
How to use a counting chamber - I

To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with a pasteur or other type of pipet. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power.
## Rulings of the Neubauer counting chamber

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
|            |              | Thrombocytes
Rulings of the Neubauer counting chamber

Leukocytes
Erythrocytes
Thrombocytes
How to use a counting chamber - II

Sperm cells in a counting chamber

Cells lying on the engraved rulings are only once counted
Microscopic measurement of cell size

The ocular micrometers provided are calibrated so that when using 1000x oil immersion microscopy, the distance between any two lines on the scale represents a length of approximately one micrometer. Remember this does not hold true when using other magnifications.
Dimensions of normal erythrocytes

Maximum thickness:
2.58 ± 0.27 μm

Minimum thickness
0.81 ± 0.35 μm

Diameter
7.82 ± 0.62 μm

Surface area
135 ± 16 μm²

Volume
94 ± 14 fl

Dimensions of a cross section of the erythrocyte in isotonic solution. Values are means ± 1SD
Price-Jones curve

Method firstly described 1910 by the London pathologist Cecil Price-Jones (1863-1943)

Determination:
manually: by means of a microscope with a calibrated ocular
automatically: by means of haematological analyzers
Morphologic classification of anemia by Mean Corpuscular Volume (MCV)

Anemia

Erythrocyte Indices
Blood smear

- MCV < 80: Microcytotic
- MCV 80 - 100: Normocytotic
- MCV > 100: Macrocytotic

MCV: [fl]
Erythrocyte volume in different types of anemia

- Microcytic anemia
- Normocytic anemia
- Macrocytic anemia

Cell volume [fl]
Preparation of blood smears

Take a single small drop of whole blood and place it near one end of the microscope slide (A). Bring the end of a second slide (held at a 45 degree angle) up to the drop until the drop disperses along the edge of the second slide (B). Then with the first slide placed on a flat surface, push the second slide quickly and evenly toward the opposite end of the first slide (C). The results should appear with an irregular thin edge, as illustrated (D). Several thick blood smears should also be made by spreading a drop of blood in a small circle on the slides. When the smears are dry, label one end of the slide (with a solvent resistant pen or pencil) with the date, species, animal ID, and location. Slides can then be transported to a laboratory or stained in the field with New Methylene Blue or a Wright's stain kit.
Where to look for cells in a blood smear:

The density of cells varies across the smear. Cells will be "heaped and piled" close to the point where the drop of blood was placed on the slide. White blood cells appear shrunken, and some types are difficult to distinguish from each other. There are fewer cells close to the tip of the smear. In this region, white blood cells are sometimes damaged and erythrocytes may be deformed. The best area to look at is between these two regions. Where it is located exactly and how wide it is will depend on the smear, but the middle of the smear is a good starting point.
The blood smear should be examined at a place of optimal cell density. The meander-like examination allows the analysis of a sufficient cell number (e. g. 100 leukocytes) for the white blood cell analysis without repeated differentiation of any cell.
Microscopic evaluation of red blood cells

Morphological analysis of erythrocytes is usually performed without a fixed number of cells. Analysis considers morphological variations of RBC which can be given semiquantitatively:

Changes of erythrocyte shape (e. g. poikilocytosis, fragmentocytes; +, ++, +++)
Changes of erythrocyte size (microcytosis, macrocytosis; +, ++, +++)
Changes of erythrocyte stain (polychromasia; +, ++, +++)
Erythrocyte inclusions (e. g. malaria)
Microscopic measurement of the white blood cell differential

Morphological analysis of leukocytes is usually performed in a fixed number of cells (e.g. 100 cells)
Analysis considers numerical variations of leukocyte numbers.
Analysis also considers morphological variations of leukocytes (e.g. atypical granula in the cytoplasm, vacuoles in the cytoplasm).
Pathological leukocytes (e.g. blasts) can be described (core-plasma ratio; nucleoli, staining characteristics of core and cytoplasm).
Normal blood cells

- Normochromic normocytic red cells
- Segmented neutrophil
- Lymphocyte
- Monocyte
- Eosinophil
- Basophil
Distribution of monocytes and blasts in the blood smear

Proportion of **monocytes** in a normal sample in different areas of the blood smear (analysis of 100 cells). Microscopic analysis of 1000 cells revealed 67.3 % neutrophils, 3.2 % eosinophils, 0.7 % basophils, 23 % lymphocytes and 5.8 % monocytes.

Proportion of **blasts** in a sample of a patient with an acute lymphatic leukemia (ALL)
Rühmke table of the 95 % CI in microscopic leukocyte differentiation

*a*: % leukocytes of 1 species (e. g. lymphocytes); *n*: number of differentiated leukocytes

<table>
<thead>
<tr>
<th>a</th>
<th>n=100</th>
<th>n=200</th>
<th>n=500</th>
<th>n=1000</th>
<th>n=1000</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0-3.6</td>
<td>0.0-1.8</td>
<td>0.0-0.7</td>
<td>0.0-0.4</td>
<td>0.0-0.1</td>
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<tr>
<td>1</td>
<td>0.0-5.4</td>
<td>0.1-3.6</td>
<td>0.3-2.3</td>
<td>0.5-1.8</td>
<td>0.8-1.3</td>
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<td>2</td>
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<td>1.1-9.9</td>
<td>1.7-7.7</td>
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<td>2.4-9.0</td>
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<td>10</td>
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<td>6.2-15.0</td>
<td>7.5-13.0</td>
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<td>12.8-17.4</td>
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<td>12.7-29.2</td>
<td>14.7-26.2</td>
<td>16.6-23.8</td>
<td>17.6-22.6</td>
<td>19.2-20.8</td>
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<tr>
<td>25</td>
<td>16.9-34.7</td>
<td>19.2-31.6</td>
<td>21.3-29.0</td>
<td>22.3-27.8</td>
<td>24.1-25.9</td>
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<td>30</td>
<td>21.2-40.0</td>
<td>23.7-36.9</td>
<td>26.0-34.2</td>
<td>27.2-32.9</td>
<td>29.1-31.0</td>
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<tr>
<td>40</td>
<td>30.3-50.3</td>
<td>33.2-47.1</td>
<td>35.7-44.4</td>
<td>36.9-43.1</td>
<td>39.0-41.0</td>
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<tr>
<td>50</td>
<td>39.8-60.2</td>
<td>42.9-57.1</td>
<td>45.5-54.5</td>
<td>46.9-53.1</td>
<td>49.0-51.0</td>
</tr>
</tbody>
</table>
Manual differentiation of blood smears

# allows diagnostics of variations in red blood cells and leukocytes
# is tedious and time consuming
# depends strongly on the individual skill of the technologist
# allows analysis of only little cell numbers
# has a low reproducibility depending on the concentration of the different cell populations
# suffers from an uneven distribution of leukocyte populations in the blood smear
# serves as „gold standard“ in cases of „flagged“ results of automated blood cell analyzes e. g. in malaria, immature precursor cells (morphological left shift) and malignant hematological diseases
Determination of reticulocytes

Reticulocytes can be stained with supravital dyes (e. g. new methylene blue). These dyes stain the substancia reticulofilamentosa. Microscopic analysis is performed by counting the number of eticulocytes in 1000 red blood cells.
Fetal hemoglobin (HbF) and adult hemoglobin (HbA) differ in respect to their solubility. HbA but not HbF can be dissolved from erythrocytes prior to RBC staining. The method allows the quantification of fetal blood cells in samples containing maternal and fetal blood.
Methods of cytochemistry - I

Mature leukocytes as well as their immature precursors express specific enzymes which can be detected by chemical reactions using specific substrates.

The reactions are performed after preparation of smears from peripheral blood and/or bone marrow.

Analyzees are performed by means of a microscope (1000x, oil immersion)

Cytochemical methods play a role in the differential diagnosis of leukemias and premalignant hematological diseases.

Some of the reactions can be performed semiquantitatively and an index can be calculated.
Methods of cytochemistry - II

The most important cytochemical studies in the study of acute leukemia are myeloperoxidase (MPO), nonspecific esterase (NSE), PAS, and acid phosphatase (AP).

Myeloperoxidase (MPO):
Myeloperoxidase is an enzyme located in the granules of myeloid and monocytic cells. Myeloperoxidase is never found in lymphoid cells. Thus if positive, is the most important marker distinguishing myeloid from lymphoid blasts.

Nonspecific esterase (NSE):
Alpha naphthyl acetate esterase is an enzyme found in large amounts in monocytic cells, but in only minor concentrations in myeloid or lymphoid cells. Useful to identify monocytes.
Methods of cytochemistry - III

PAS: The periodic acid Schiff reaction for glycogen is usually strongly positive immature granulocytes. Myeloid or monocytic blasts are typically weakly positive or negative. A granular (may be fine or coarse or block) PAS pattern with a negative background is characteristic of lymphoblastic leukemia.

Acid phosphatase (AP): Although the acid phosphatase enzyme is ubiquitous, T-lymphocytes and lymphoblasts have a characteristic "dot-like" focus of intense positivity, whereas the activity in most cells is diffuse.

Tartrate-resistant acid phosphatase (TRAP): It is an important diagnostic feature of hairy cell leukemia.

Leukocyte alkaline phosphatase (LAP): It is useful in evaluation of acute and chronic leukemias and myeloproliferative disorders.
Methods of cytochemistry - IV
Peroxidase and alpha-Naphthylacetate esterase

Panoptic stain of the bone marrow (left; 1000x) and peripheral blood (right; 500x)

Left: Peroxidase reaction. Strongly positive granulocyte at left with negative or a few weakly positive leukemia cells. Right: alpha-Naphthylacetate esterase reaction. Very high activity of the leukemic cells in a patient with acute monocytic leukemia.
Methods of cytochemistry - V
Peroxidase

Left: Blast of an acute promyelocytic leukemia (M3) in peripheral blood.
Right: M3 blasts are strongly positive in the peroxidase reaction
Normal cells (PMNs) contain the enzyme leukocyte alkaline phosphatase (LAP). In CML the PMNs lack or contain a decreased amount of LAP. Both peripheral blood samples shown above were stained for LAP. The sample on the left is a normal blood sample, and shows positive LAP staining. The sample on the right is from a CML patient. LAP staining is characteristically low or absent in CML.
Acid phosphatase demonstrates a specific activity in almost all hemopoetic cell elements and is characteristically strong in T-lymphoblast cells. Principle: Acid phosphatase catalyses the hydrolysis of phosphate esters in an acid milieu. In the test system, naphthol-AS-Bl is released from naphthol-AS-OL phosphate and is coupled to diazo-salt to form a red-brown azo-dye that precipitates in the cell. Addition of tartrate inhibits normal phosphatase activity with the result that either no or very weak staining takes place in the blood and bone marrow cells. Acid phosphatase (isoenzyme 5) alone is „tartrate-resistant" in the characteristic cells of hair cell leukaemia and can thus be used as a diagnostic parameter. Sample material: Thin, air-dried, maximum 3-days old blood or bone marrow smears. Native cell sample only may be used, as the coagulation inhibitor EDTA weakens the reaction considerably.
Methods of cytochemistry - VIII
Leukocyte acid phosphatase

Staining: Pararosaniline method
Reaction product: Red granules

Occurrence:
Neutrophils: Strong positive reaction at the early maturation stage, positive reaction in mature forms.
Basophils: Strong positive reaction
Eosinophils: Strong positive reaction
Monocytes: Positive reaction
Lymphocytes: Positive reaction.
Erythroblasts: Focal positive reaction.
Megakaryocytes: Strong positive reaction.

The indicated erythroblast shows typical perinuclear activity of acid phosphatase. Also a strongly positive megakaryocyte (middle) and a macrophage can be seen.
Fluorescence microscopy - I

Left: Neutrophil granulocyte stained with fluorescein.
Right: Cells stained with fluorescein and propidium iodide.
Fluorescence microscopy - II

Apoptosis mediates rapid and efficient deletion of unwanted or surplus cells without damaging surrounding tissue. Intact cells undergoing this "programmed" type of cells death are swiftly recognized and ingested by phagocytes (Figure left), preventing leakage of potentially noxious contents from dying cells. Efficient phagocytic clearance of neutrophils undergoing apoptosis is likely to be of particular importance in the resolution of inflammation. A number of molecules are implicated in supporting the process. These molecules are CD14, avb3, CD36, phosphatidylserine receptor, the ATP-binding cassette transporter 1, as well as scavenger receptors.

Phase contrast microscopy

Polymorph neutrophil granulocytes. The left cell is undergoing apoptosis.
Spectroscopic measurement of hemoglobin

Spectroscopic methods for the quantification of hemoglobin are used for:

Measurement of hemoglobin in routine blood samples (manual methods and automated analyzers)

Measurement of hemoglobin for stability testing of erythrocytes

Quantification of hemoglobin derivatives (e.g. carboxy-hemoglobin (COHb))
Hemoglobin and its derivatives can be determined by spectrometry. Derivatives of hemoglobin (e.g. deoxy-hemoglobin, oxyhemoglobin, carboxyhemoglobin, NO-hemoglobin, methemoglobin) differ in their spectra.
Measurement of hemoglobine
by spectrometry - II

Blood cells are lysed by means of a detergent. Measurements are performed in an 1:250 dilution of blood.

Fe$^{2+}$ of hemoglobin is oxidized to Fe$^{3+}$ by K$_3$Fe(CN)$_6$.

The produced hemoglobin (HI) reacts with CN$^-$ from KCN which is also part of the solution. The produced HiCN as a broad absorption maximum at $\lambda = 540$ nm.

The absorbance is proportional to the hemoglobin concentration.

Analyzers are calibrated with a secondary HiCN standard.

The method serves as a reference method.
In patients with hereditary spherocytosis the osmotic fragility test demonstrates increased hemolysis when the red cells are placed in hypotonic saline. Hemolysis is greatly increased if the cells are first incubated without glucose at body temperature for 24 hours.
The HAM test (acidified serum lysis) establishes the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) demonstrating a characteristic abnormality of PNH red cells by acidified fresh normal serum. Here is a PNH patient's red cells lysed by normal serum at room temperature (RT) and at 37 °C compared to normal red cells (no hemolysis). Heated serum at 56 °C inactivates complement and prevents hemolysis in PNH cells.
Solubility test for sickle cell hemoglobin

When blood containing a significant amount of HS is dissolved in a mixture containing lysing agent, high phosphate buffer and a deoxygenating agent HbS is precipitated and forms a cloudy solution as in the tube on the right.

False positive test: Exceedingly rare.
False negative test: Quite common.
Measurement of hemoglobin derivatives

Spectroscopic methods allow the quantification of:
- oxygenated hemoglobin
- desoxygenated hemoglobin
- carboxyhemoglobin
- methemoglobin
- sulfhemoglobin

based on the different absorption spectra of these derivatives.

The different hemoglobin derivatives can be analyzed in parallel by measurement of the absorption at different wavelengths.
## Spectral characteristics of hemoglobin and its derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>λ#)</th>
<th>e§</th>
<th>λ#)</th>
<th>e</th>
<th>λ#)</th>
<th>e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyhemoglobin</td>
<td>430</td>
<td>133</td>
<td>555</td>
<td>12.5</td>
<td>----</td>
<td>---</td>
</tr>
<tr>
<td>Oxyhemoglobin</td>
<td>415</td>
<td>125</td>
<td>541</td>
<td>13.8</td>
<td>577</td>
<td>14.6</td>
</tr>
<tr>
<td>Acid methemoglobin</td>
<td>405</td>
<td>179</td>
<td>500</td>
<td>10.0</td>
<td>631</td>
<td>4.4</td>
</tr>
<tr>
<td>Alkaline methemoglobin</td>
<td>410</td>
<td>120</td>
<td>540</td>
<td>11.0</td>
<td>575</td>
<td>9.2</td>
</tr>
<tr>
<td>Cyanomethemoglobin</td>
<td>419</td>
<td>124</td>
<td>540</td>
<td>12.5</td>
<td>----</td>
<td>---</td>
</tr>
<tr>
<td>Hemichromes</td>
<td>411</td>
<td>105</td>
<td>534</td>
<td>14.7</td>
<td>565</td>
<td>12.5</td>
</tr>
<tr>
<td>Carboxyhemoglobin</td>
<td>419</td>
<td>191</td>
<td>569</td>
<td>13.4</td>
<td>569</td>
<td>13.4</td>
</tr>
<tr>
<td>Sulfhemoglobin</td>
<td>421</td>
<td>86</td>
<td>618</td>
<td>24.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#) λ: wavelength in nm
§) e: millimolar extinction coefficient
Automated blood analysis

Methods for automated blood analysis allow the:

- Quantification of hemoglobin
- Quantification of blood cells
- Quantification of blood cell volume
- Calculation of erythrocyte indices
- Analysis of cell volume distribution curves
- Differentiation of leukocyte species
- Quantification of reticulocytes
The sample is first diluted, then counting is performed by drawing the cells through an aperture of the instrument. Each cell causes a change in electrical resistance as it passes the aperture, and this pulse is detected and amplified by the instrument. The sensitivity can be adjusted so that platelets (smaller than red cells) are not counted; WBC are counted as well as RBC, but white cell numbers (in thousands/µL) are too low to cause significant error in the red cell count (in millions/µL). The amplitude of the pulse is proportional to cell size and, in some analyzers (e.g. in Coulter analyzers), this is used for determining the MCV.
Automated measurement of erythrocytes, thrombocytes and leukocytes

Blood cells are electrically analyzed while passing the aperture of the analyzer.

The obtained signal is proportional to cell size.

Cells are characterized in respect to their size and sorted into histograms.

Thrombocytes are much smaller than erythrocytes and leukocytes and are determined as a specific cell population.

In the analysis of the RBC concentration, leukocytes are counted as „erythrocytes“; however, their number is too small to cause a relevant increase of the RBC number.

Leukocytes are measured after lysis of RBC but there is no differentiation of the different leukocyte species.
Price-Jones curve

Method firstly described 1910 by the London pathologist Cecil Price-Jones (1863-1943)

**Determination:**

- manually: by means of a microscope with a calibrated ocular
- automatically: by means of haematological analyzers

![Price-Jones curve diagram](image)
Red blood cell volume distribution curve

**Determination:**
automatically: by means of haematological analyzers
Morphologic classification of anemia by Mean Corpuscular Volume (MCV)

Anemia

Erythrocyte Indices
Blood smear

MCV: [fl]

- MCV < 80: Microcytotic
- MCV 80 - 100: Normocytotic
- MCV > 100: Macrocytotic
Erythrocyte volume in different types of anemia

Cell volume [fl]

Number of cells [%]

Microcytic anemia
Normocytic anemia
Macrocytic anemia
In Coulter analyzers the erythrocyte gate is between 36 fl and 360 fl. The thrombocyte gate includes cells with volumes between 2 fl and 20 fl. In hematological analyses the value of the mean platelet volume (MPV) is used to characterize morphologically pathologic platelets.
Measurement of hematocrit

Manual method:
Capillaries: Length 7.5 cm, lumen 1.15 mm, wall 0.2 mm
Centrifuge: Rotor radius >8 cm, maximum speed < 30 sec., ω: 10000 g - 15000 g

\[
\text{Hct} = \frac{\text{Length of RBC column [mm]}}{\text{Length of Plasma column} + \text{blood cell column [mm]}}
\]

Hematological analyzer:
Erythrocyte count by means of the impedance method or the scatter light method.

\[
\text{Hct} = \frac{\text{MCV [fl] \cdot erythrocyte concentration [l}^{-1}]}{10^{15}}
\]
Automated measurement of the white blood cell differential

White blood cells are differentiated by means of a flow cytometer after lysis of erythrocytes in the sample. In consequence incomplete lysis of erythrocytes results in false leukocyte values.

**VCS-principle:**

By means of this principle cells are characterized by determination of their volume (V), conductivity (C) and laser light scatter (S). The principle is used in Coulter analyzers.

**Staining methods and specific lysis:**

Cells are characterized by laser light scatter before and/or after staining for specific granules (e. g. peroxidase in granulocytes and their precursors; eosinophil granula) or lysis of specific cell species (e. g. all except basophils). Analyzers from Bayer/Siemens (Advia) and ABX (e. g. Pentra) are based on these methods.
VCS-principle of white blood cell measurement in coulter analyzers - I

Cells are hydrodynamically focussed and pass a flow cell in which measurements of volume, conductivity and laser light scatter are performed. The technique allows the analysis of more than 8000 cells per sample.
Volume:
As opposed to using the light loss to estimate cell size, VCS utilizes the Coulter Principle. Impedance is used to physically measure the volume that the entire cell displaces in an isotonic diluent. This method accurately sizes all cell types regardless of their orientation in the light path.
Conductivity:

Alternating current in the radio frequency (RF) range short circuits the bipolar lipid layer of a cell’s membrane, allowing the energy to penetrate the cell. This powerful probe is used to collect information about the internal structure of the cell, including chemical composition and nuclear volume.
VCS-principle of white blood cell measurement in coulter analyzers - IV

Scatter:
When a cell is struck by the coherent light of a LASER beam, the scattered light spreads out in all directions. Using a proprietary new detector, median angle light scatter signals are collected to obtain information about cellular granularity, nuclear lobularity and cell surface structure.
VCS-principle of white blood cell measurement in coulter analyzers - V

Simultaneous measurements:
VCS is the only single channel analysis that uses 3 independent energy sources to probe approximately 8,192 cells in their near native state. Working in concert with each other, these three measurements are taken simultaneously, each providing 256 channels of resolution - over 16,700,000 channels in all.
VCS-principle of white blood cell measurement in coulter analyzers - VI

Leukocyte populations
Different compensation mechanisms are used for minimizing the bias of the different methods and the interference between the used techniques. This allows the VCS technology to accurately separate what would normally be mixed cell types (such as neutrophils and eosinophils) into distinct clusters without mathematical manipulation. It also enhances the separation between the non granular cell types.
VCS-principle of white blood cell measurement in coulter analyzers - VII
Reticulocyte analysis by means of the Coulter analyzer

Reticulocytes in various stages of maturity are pictured at the left. The younger cells have more residual RNA and therefore stain more heavily with the supravital dye new methylene blue. When illuminated by a HeNe Laser, these cells scatter the most light. Older reticulocytes have little residual RNA and stain less intensely. They are easily separated from mature erythrocytes by light scatter. Analyzers from Coulter characterize more than 30000 cells per sample.
Hemoglobin measurement in the ADVIA analyzer

With the Advia analyzer (Bayer), red cells are sphered in a diluent and then passed through a laser. The cells scatter light (at different angles) which is detected by the instrument. The laser detects the number of cells, cell volume (using low angle scatter) and internal content, i.e. hemoglobin concentration (using high angle scatter) by light scatter.
White blood cell measurement in the ADVIA analyzer - I

The Advia counts white cells in 2 ways, both of which use flow cytometry.

In the first method, called the peroxidase method, the white cells are stained with peroxidase and the cells are counted based on size and staining characteristics. This method also provides an automated differential cell count by separating the cells into clusters.

A: Neutrophils       B: Monocytes       C: Lymphocytes
D: Eosinophils       E: Large unstained cells   F: Platelets/noise
G: Debris/noise
White blood cell measurement in the ADVIA analyzer - II

The second method, called the basophil method, involves stripping the cells of cytoplasm and counting nuclei. In this cytogram, the cells are displayed as a worm, with the mononuclear cells (lymphocytes and monocytes) in the head and the granulocytes (neutrophils) in the body of the worm. This is a far more accurate method for counting white cells and is the default method on the Advia.
Complete blood count (CBC) in a normal blood sample

<table>
<thead>
<tr>
<th>CBC</th>
<th>MORPHOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0 x10^9/L WBC</td>
<td>ANISO +</td>
</tr>
<tr>
<td>4.29 x10^{12}/L RBC</td>
<td>MICRO +</td>
</tr>
<tr>
<td>150 g/L Hgb</td>
<td>MACRO +</td>
</tr>
<tr>
<td>.45 Hct</td>
<td>YAR</td>
</tr>
<tr>
<td>89.5 fL MCV</td>
<td></td>
</tr>
<tr>
<td>31.3 pg MCH</td>
<td></td>
</tr>
<tr>
<td>33.3 g/L MCHC</td>
<td></td>
</tr>
<tr>
<td>14.5 % RDW</td>
<td></td>
</tr>
<tr>
<td>350. x10^9/uL PLT</td>
<td></td>
</tr>
</tbody>
</table>
Automated and manual blood cell analysis

Automated blood cell analyzers allow

# a rapid analysis of a high number of blood cells
# highly standardized analysis of blood samples
# analysis of blood samples with a low variability
# are a valuable tool in clinical routine analysis
# flag the results of pathological analyses
# allow poor differentiation of pathological blood cells
## Reproducibility of blood counting procedures

<table>
<thead>
<tr>
<th>Cell type counted</th>
<th>Two coefficients of variation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemacytometer(^#)</td>
<td>Automated analyzer(^##)</td>
</tr>
<tr>
<td>Red cells</td>
<td>± 11 %</td>
<td>± 1 %</td>
</tr>
<tr>
<td>White cells</td>
<td>± 16 %</td>
<td>± 1.5 %</td>
</tr>
<tr>
<td>Platelets</td>
<td>± 22 %</td>
<td>± 2 %</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>± 34 %</td>
<td>± 5 %</td>
</tr>
</tbody>
</table>

\(^\#\) Minimum error \(^\##\) Usual error

The error may be greater with low (<35 • 10\(^9\)/l) or very high (>450 • 10\(^9\)/l) platelet counts.
## Reproducibility of red cell indices

<table>
<thead>
<tr>
<th>Index</th>
<th>Method used</th>
<th>% error (+2CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-concentration</td>
<td>Spectrophotometric</td>
<td>1.0 % - 2.0 %</td>
</tr>
<tr>
<td></td>
<td>Automated</td>
<td>&lt;1.0 %</td>
</tr>
<tr>
<td>MCV</td>
<td>Hemacytometer</td>
<td>9.5 %</td>
</tr>
<tr>
<td></td>
<td>Automated</td>
<td>&lt;1.0 %</td>
</tr>
<tr>
<td>MCH</td>
<td>Hemacytometer</td>
<td>10.0 %</td>
</tr>
<tr>
<td></td>
<td>Automated</td>
<td>0.6-1.2 %</td>
</tr>
<tr>
<td>MCHC</td>
<td>Automated</td>
<td>1.0 - 1.5 %</td>
</tr>
</tbody>
</table>

CV: Coefficient of variation  
Hb: Hemoglobin  
MCV: Mean corpuscular volume  
MCH: Mean corpuscular hemoglobin  
MCHC: Mean corpuscular hemoglobin concentration
Disorders and conditions that may reduce the accuracy of blood cell counting - I

## Red blood cells:

<table>
<thead>
<tr>
<th>Disorder/Condition</th>
<th>Effect on cell count</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcytosis or schistocytes</td>
<td>May underestimate RBC</td>
<td>Lower threshold of RBC counting window is greater than microcyte size</td>
</tr>
<tr>
<td>Howel-Jolly bodies</td>
<td>May spuriously elevate platelet count (in whole blood platelet counters only)</td>
<td>Howel-Jolly bodies are similar in size to platelets</td>
</tr>
<tr>
<td>Polycythemia</td>
<td>May underestimate RBC</td>
<td>Increased coincidence counting</td>
</tr>
</tbody>
</table>
## Disorders and conditions that may reduce the accuracy of blood cell counting - II

### White blood cells:

<table>
<thead>
<tr>
<th>Disorder/Condition</th>
<th>Effect on cell count</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytosis</td>
<td>Overestimate RBC</td>
<td>Increased coincidence counting</td>
</tr>
<tr>
<td>Acute leukemia and chronic lymphocytic leukemia</td>
<td>May spuriously lower WBC</td>
<td>Increased fragility of leukocytes including immature forms</td>
</tr>
<tr>
<td>Chemotherapy of acute leukemia</td>
<td>May artifically increase platelet count</td>
<td>Leukemic cell nuclear or cytoplasmic fragments identified as platelets</td>
</tr>
</tbody>
</table>
## Disorders and conditions that may reduce the accuracy of blood cell counting - III

### Platelets and plasma:

<table>
<thead>
<tr>
<th>Disorder/Condition</th>
<th>Effect on cell count</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet agglutinins</td>
<td>May underestimate platelet count sometimes with spurious increase in WBC</td>
<td>Platelet clumping; aggregates may be identified as leukocytes</td>
</tr>
<tr>
<td>Cold agglutinins</td>
<td>May underestimate RBC with spurious macrocytosis</td>
<td>Red cell doublets, triplets etc. have increased volume</td>
</tr>
<tr>
<td>Cryoglobulins</td>
<td>Variation in platelet count</td>
<td>Protein precipitates may be identified as platelets</td>
</tr>
<tr>
<td>cryofibrinogens</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Interpreting blood counts
The blood count (CBC)

Hemoglobin (Hb) [g/l]
Number of red blood cells (RBC) [\(10^{12}/l\)]
Size of the red blood cells [fl]
Number of white blood cells (WBC) [\(10^{9}/l\)]
Number of platelets [\(10^{9}/l\)]
## Reference values of erythrocyte concentration

<table>
<thead>
<tr>
<th></th>
<th>RBC concentration $[10^6/\mu l]$ or $[10^{12}/l]$</th>
<th>RBC concentration $[10^6/\mu l]$ or $[10^{12}/l]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord blood</td>
<td>3.6-5.6</td>
<td>Females:</td>
</tr>
<tr>
<td>1 day</td>
<td>4.3-6.3</td>
<td>13-16 years</td>
</tr>
<tr>
<td>2-6 days</td>
<td>4.0-6.8</td>
<td>Adults</td>
</tr>
<tr>
<td>14-23 days</td>
<td>3.7-6.1</td>
<td>Males:</td>
</tr>
<tr>
<td>24-37 days</td>
<td>3.2-5.4</td>
<td>13 years</td>
</tr>
<tr>
<td>40-50 days</td>
<td>3.1-5.1</td>
<td>14-16 years</td>
</tr>
<tr>
<td>2.0-2.5 months</td>
<td>2.8-4.8</td>
<td>Adults</td>
</tr>
<tr>
<td>3.0-3.5 months</td>
<td>3.1-4.7</td>
<td>4.2-5.2</td>
</tr>
<tr>
<td>5-7 months</td>
<td>3.2-5.2</td>
<td>4.3-5.6</td>
</tr>
<tr>
<td>8-10 months</td>
<td>3.6-5.2</td>
<td>4.5-5.9</td>
</tr>
<tr>
<td>11-13.5 months</td>
<td>3.6-5.2</td>
<td></td>
</tr>
<tr>
<td>1.5-3.0 years</td>
<td>3.7-5.3</td>
<td></td>
</tr>
<tr>
<td>4-9 years</td>
<td>3.9-5.1</td>
<td></td>
</tr>
<tr>
<td>Age Group</td>
<td>Hb concentration [g/dl]</td>
<td>Age Group</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Cord blood</td>
<td>13.5-20.7</td>
<td>5 years</td>
</tr>
<tr>
<td>1 day</td>
<td>15.2-23.5</td>
<td>10 years</td>
</tr>
<tr>
<td>2-6 days</td>
<td>15.0-24.0</td>
<td>12 years</td>
</tr>
<tr>
<td>14-23 days</td>
<td>12.7-18.7</td>
<td>15 years</td>
</tr>
<tr>
<td>24-37 days</td>
<td>10.3-17.9</td>
<td></td>
</tr>
<tr>
<td>40-50 days</td>
<td>9.0-16.6</td>
<td></td>
</tr>
<tr>
<td>2.0-2.5 months</td>
<td>9.2-15.0</td>
<td>Adults:</td>
</tr>
<tr>
<td>3.0-3.5 months</td>
<td>9.6-12.8</td>
<td>Females</td>
</tr>
<tr>
<td>5-7 months</td>
<td>10.1-12.9</td>
<td>Males</td>
</tr>
<tr>
<td>8-10 months</td>
<td>10.5-12.9</td>
<td></td>
</tr>
<tr>
<td>11-13.5 months</td>
<td>10.7-13.1</td>
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<tr>
<td>1.5-3.0 years</td>
<td>10.8-12.8</td>
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</table>
## Reference values of hematocrit

<table>
<thead>
<tr>
<th></th>
<th>Hematocrit [l/l]</th>
<th></th>
<th></th>
<th>Hematocrit [l/l]</th>
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<tbody>
<tr>
<td>Cord blood</td>
<td>0.48-0.56</td>
<td>Females:</td>
<td></td>
<td>0.35-0.43</td>
</tr>
<tr>
<td>2 hours</td>
<td>0.49-0.71</td>
<td>14-16 years</td>
<td></td>
<td>0.35-0.45</td>
</tr>
<tr>
<td>6 hours</td>
<td>0.44-0.68</td>
<td>Adults</td>
<td></td>
<td>0.35-0.45</td>
</tr>
<tr>
<td>2.-6. days</td>
<td>0.40-0.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>0.38-0.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3 weeks</td>
<td>0.38-0.60</td>
<td>Males:</td>
<td></td>
<td>0.38-0.49</td>
</tr>
<tr>
<td>3-7 weeks</td>
<td>0.36-0.46</td>
<td>14-16 years</td>
<td></td>
<td>0.38-0.49</td>
</tr>
<tr>
<td>7-12 weeks</td>
<td>0.30-0.38</td>
<td>Adults</td>
<td></td>
<td>0.36-0.48</td>
</tr>
<tr>
<td>10-12 months</td>
<td>0.35-0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5 years</td>
<td>0.32-0.40</td>
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<td></td>
<td></td>
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<tr>
<td>6-8 years</td>
<td>0.32-0.41</td>
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<tr>
<td>10-13 years</td>
<td>0.34-0.44</td>
<td></td>
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</tr>
</tbody>
</table>
Erythrocyte indices

**MCV: Mean cellular volume**

\[
MCV = \frac{\text{Hematocrit (as fraction)}}{\text{Number of erythrocytes per litre}}
\]

**MCH: Mean cellular hemoglobin**

\[
MCH = \frac{\text{Hemoglobin concentration [g/l]}}{\text{Number of erythrocytes [10}^{12}/\text{l}]}
\]

**MCV: Mean cellular hemoglobin concentration**

\[
MCHC = \frac{\text{Hemoglobin concentration [g/l]}}{\text{Hematocrit (as fraction)}}
\]
<table>
<thead>
<tr>
<th>Age Category</th>
<th>MCV [µm³]</th>
<th>MCH [pg/cell]</th>
<th>MCHC [g/dl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord blood</td>
<td>101-125</td>
<td>33-41</td>
<td>31-35</td>
</tr>
<tr>
<td>1 day</td>
<td>98-122</td>
<td>33-41</td>
<td>31-35</td>
</tr>
<tr>
<td>2-6 days</td>
<td>94-135</td>
<td>29-41</td>
<td>24-36</td>
</tr>
<tr>
<td>14-23 days</td>
<td>84-128</td>
<td>26-38</td>
<td>26-34</td>
</tr>
<tr>
<td>24-37 days</td>
<td>82-126</td>
<td>26-38</td>
<td>25-34</td>
</tr>
<tr>
<td>40-50 days</td>
<td>81-125</td>
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<td>2.0-2.5 months</td>
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<td>26-34</td>
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<tr>
<td>3.0-3.5 months</td>
<td>77-113</td>
<td>23-36</td>
<td>26-34</td>
</tr>
<tr>
<td>5-7 months</td>
<td>73-109</td>
<td>21-33</td>
<td>26-34</td>
</tr>
<tr>
<td>8-10 months</td>
<td>74-106</td>
<td>21-33</td>
<td>28-32</td>
</tr>
<tr>
<td>11-13.5 months</td>
<td>74-102</td>
<td>23-31</td>
<td>28-32</td>
</tr>
<tr>
<td>1.5-3.0 years</td>
<td>73-101</td>
<td>23-31</td>
<td>26-34</td>
</tr>
<tr>
<td>4-12 years</td>
<td>77-89</td>
<td>25-31</td>
<td>32-36</td>
</tr>
<tr>
<td>13-16 years</td>
<td>79-92</td>
<td>26-32</td>
<td>32-36</td>
</tr>
<tr>
<td>Adults</td>
<td>80-96</td>
<td>28-33</td>
<td>33-36</td>
</tr>
</tbody>
</table>
The red cell distribution width (RDW) describes the distribution pattern of the MCV. It is the value of the standard deviation of the relative frequency curve of MCV.
Flow cytometry

In hematology flow cytometry is used for:

# Characterization of leukocyte subsets (e.g. T-cells, B cells, helper cells, suppressor cells)
# Characterization of leukemias (e.g. ALL)
# Measurement of cell activation (e.g. activated T-cells, activated monocytes)
# Detection of organ rejection in transplant recipients
# Measurement of reticulocytes
# Analysis of the cell cycle
Methods in flow cytometry

The different methods in flow cytometry allow:

# Immunological measurement of molecules on cell surface
# Immunological measurement of proteins and signal substances (e. g. IgG, cytokins, Calcium) in cell cytoplasm
# Cell cycle analysis (measurement of DNA)
# Cell maturation assays (measurement of RNA in reticulated RBCs and platelets)
# Differentiation between dead and living cells
# Cell function assays:
  
  Respiratory burst of leukocytes
  Phagocytosis of leukocytes
  Degranulation of basophils
  Systemic receptor defects (e. g. apoB-receptor)
  Cell specific receptor defect (e. g. on thrombocytes)

Isolation of specific cells (cell sorting)
Relation between fluorescence microscopy and flow cytometry

Fluorescence microscopy allows the characterization of cells after staining with fluorescent dyes by means of a microscope. The distribution of fluorescence within the cell (surface or cytoplasm can be determined). However, microscopy allows neither quantification of fluorescent cells nor cell sorting.

Flow cytometry allows analysis of a great number of stained cells. The intensity of the fluorescence can be quantified and cells can be sorted after analysis. However, the distribution profile of fluorescence within the cells cannot be analyzed.

Principles of flow cytometry

Cells are embedded in sheath fluid for hydrodynamic focusing and are rapidly passing a thin channel. When passing they are characterized by means of a laser beam.

Cells are characterized for forward scatter (cell size) and sideward scatter (granularity) of the laser beam as well as fluorescence intensities at different wavelengths.
Schematic diagram of a flow cytometer
Immune phenotyping of cells:
Cells of interest are incubated with monoclonal antibodies labelled with fluorescent dyes. The antibodies bind to the antigens expressed on cell surface. Cells of interest then can be detected (and isolated) by means of a flow cytometer (and sorter).
Methods in flow cytometry - II

Forward Scatter/Side Scatter plot of a normal sample. The analysis gate for fluorescence has been set on the lymphocyte population.

CD4/CD8 plot of the gated lymphocyte population. 31% of the cells are CD4⁻/CD8⁻, resembling B-cells and NK-cells.
Measurement of platelet activation:
Glycoproteins GPIa/IIa, GPIb/IX and GPIIb/IIIa are essential receptors for platelet function and expressed on the surface of normal resting platelets. After cell activation granula migrate to the surface and degranulate. At this time further antigens are expressed on the surface of the platelets which can be quantified as a marker for cell activation.
Schematic diagram of flow cytometric cell cycle analysis.
Methods in flow cytometry - V

Cell Sorting:

Some flow cytometers are also equipped to separate and collect user-specified single cells or beads from a sample. With these instruments, the flow cytometer nozzle is vibrated at a high frequency by a piezoelectric transducer that causes the microscopic fluid stream exiting the flow chamber to break into discrete droplets. As a cell or bead of interest reaches the droplet break-off point, it receives a positive or negative charge. As the droplets pass individually through two vertical deflection plates, the electric field created by those plates directs them toward the appropriate, user-specified collection receptacles. Uncharged droplets flow into a waste receptacle.
Electrophoretic and immunological methods

Electrophoretic and immunological methods are used for:

- Characterization of protein mutations (e.g. hemoglobin)
- Detection of atypical proteins in serum and/or urine (e.g. M-gradient, Bence-Jones proteins)
- Quantification of serum proteins
Hemoglobin electrophoresis

Top figure:
Healthy African-American child. Hb electrophoresis, cellulose acetate pH 8.4; the patient (*) appears to have Hb A and Hb S.
Serum protein electrophoresis

A drop of serum is applied in a band to a thin sheet of supporting material, like paper, that has been soaked in a slightly-alkaline salt solution. At pH 8.6, which is commonly used, all the proteins are negatively charged, but some more strongly than others. A direct current can flow through the paper because of the conductivity of the buffer with which it is moistened. As the current flows, the serum proteins move toward the positive electrode. The stronger the negative charge on a protein, the faster it migrates. After a time (typically 20 min), the current is turned off and the proteins stained to make them visible (most are otherwise colorless). The separated proteins appear as distinct bands.

Apply sample
Normal serum protein electrophoresis

Density profile of a normal serum protein electrophoresis

Distribution profile of some plasma proteins in the normal serum electrophoresis
Pathological serum electrophoreses

Polyclonal gammopathy usually occurs secondary to many chronic diseases. This patient was a 39 year old male with sarcordosis. The sequential increase of the globulin fractions illustrated "sarcoid stepping." IFE excluded the possibility of a monoclonal protein.

Monoclonal gammopathy (M-gradient) in a patient with multiple myeloma. The diagnosis of can be proven my IFE which shows a monoclonal fraction of IgG chains
Mobility of immunoglobulins in serum electrophoresis

Electrophoretic mobility

Relative amounts

- Albumin

- IgG

- IgA

- IgM

- IgD
Serum electrophoresis in patients with multiple myeloma

The electrophoresis shows the mobility pattern of samples from different patients with multiple myeloma (indicated samples) and a normal reference sample (right). The location and intensity of the M-gradient differs strongly in the 5 patients.
Immunological methods are operating in antibody excess.
In a first step serum proteins are separated electrophoretically (red arrows). Then, the gel is incubated with antibodies against human serum proteins which were added into the indicated spline of the gel (black arrow). Antigen-antibody complexes are developing and can be detected by protein staining.
Pathological immunoelectrophoresis

- Patient
- Control
- Patient
- Control
- Patient

Antibodies:
- Anti IgA
- Anti IgG
- Anti IgM
- Anti NHS
- Anti kappa
- Anti lambda
In a first step serum proteins are separated electrophoretically. Then, the different strips are incubated with (monoclonal) antibodies against human serum proteins (1), IgG (2), IgA (3), IgM (4), kappa (5) and lambda (5). After formation of immune complexes the gel is washed and stained for proteins. Immune complexes become visible after protein staining. The method allows the detection of specific immunoglobulins or their fragments in myeloma patients.
Immunological methods are operating in antibody excess.
Radial immunodiffusion (Mancini)

The RID assay is performed in (ready-for-use) agar plates, containing a specific antibody against ferritin. Test samples, standard sera and control sera are prepared and added to the plates. After 48-64 hours incubation at room temperature the diameters of the immunoprecipitation rings are measured. The ferritin concentrations in the test samples may be quantified in two ways:

Calibration curve method: Ring diameters and concentrations of the standards are plotted and the values of the test sample are determined by interpolation.

Tabular method: Ring diameters of the calibration curve are listed and the values of the test sample are read from a table. It is not necessary to make a calibration curve. The control serum is assayed to check the validity of the calibration curves and also the accuracy of the test.
Antigen is electrophoresed into gel containing antibody. The distance from the starting well to the front of the rocket shaped arc is related to antigen concentration.
Turbidimetry and nephelometry

Turbidimetry: Measurement of forward light absorption about two hours after begin of antigen-antibody reaction.

Nephelometry: Measurement of sideward light scatter after end of the antigen-antibody reaction or within the reaction (kinetic nephelometry)
ELISA methods

Principle of a sandwich elisa
Molecular analysis

Methods of molecular biology are used for:
Mutation screening in risk groups
Characterization of leukemias
Analysis of regulatory processes
Molecular diagnostics of HbS - I

Detect nucleotide difference:

Single base change disrupts restriction endonuclease site

Cvn I       CCTNAGG

Normal DNA  CCTGAGG

Mutant DNA  CCTGTGG
Molecular diagnostics of HbS - II

Loss of restriction endonuclease site is a convenient method for assaying for a genetic defect.

However, the majority of mutations won’t alter restriction endonuclease site

Need for other strategies for mutation detection
Molecular diagnostics of HbS - III

A

Primer

Normal sequence

Primer

Primer

Sickle-cell anemia sequence

B

Amplified normal sequence

Amplified sickle-cell anemia sequence

CvnI  CvnI  CvnI

256 bp  201 bp  181 bp  88 bp

CvnI  CvnI

256 bp  382 bp  88 bp
Molecular diagnostics of HbS - IV

Amplified normal sequence

<table>
<thead>
<tr>
<th>CvnI</th>
<th>CvnI</th>
<th>CvnI</th>
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</thead>
<tbody>
<tr>
<td>256 bp</td>
<td>201 bp</td>
<td>181 bp</td>
</tr>
</tbody>
</table>

Amplified sickle-cell anemia sequence

<table>
<thead>
<tr>
<th>CvnI</th>
<th>CvnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>256 bp</td>
<td>382 bp</td>
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<table>
<thead>
<tr>
<th>Size (bp)</th>
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<th>AS</th>
<th>SS</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>88</td>
<td></td>
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</tbody>
</table>
Immature cells from the marrow of a patient with M4 showing both granulocytic and monocytic features.

Shown in this karyotype is the translocation (6;9) (p22;q34) seen in AML M4 (or M2) and often associated with marrow basophilia: break-points are indicated by arrows.

In the schematic presentation of the translocation, the genes involved are DEK-CAN.
Coagulation analyzes

Coagulation analyzes include in vitro and in vivo analyzes:

In vitro analyzes:
Global analyzes of the clotting system (e.g. Quick test)
Functional analyzes of single clotting factors (e.g. factors VIII and IX (Hemophilia A and B, respectively)
Immunological quantification of clotting factors in plasma
Immunological tests for antibody detection (e.g. Lupus anti-coagulants, heparin induced thrombocytopenia - II (HIT-II)
Thrombocyte count
Analyzes of thrombocyte function

In vivo analyzes:
In-vivo bleeding time
The clotting system

The blood clotting or coagulation system is a proteolytic cascade. Each enzyme of the pathway is present in plasma in an inactive (zymogen) form, which, when activated, releases the active factor from the precursor molecule. The mechanism functions as a series of positive and negative feedback loops to effectively control the process. The main aim is to produce thrombin, which can convert soluble fibrinogen into fibrin, and forms a clot.

There are three phases of coagulation, the intrinsic and extrinsic pathways that provide alternative routes for the generation of factor X, and the final common pathway which results in thrombin formation.
In-vitro analysis of the clotting system

Automated analyzers allow the global characterization of the clotting system (e.g. thrombin time, PTT, Quick test) and the (functional) quantification of single clotting factors. Analyzes of the latter are performed by means of factor deficient plasma.
The bleeding time test is used to evaluate how well a person's blood is clotting. The test evaluates how long it takes the vessels cut to constrict and how long it takes for platelets in the blood to seal off the hole. Blood vessel defects, platelet function defects, along with many other conditions can result in prolonged bleeding time.