Name number Study group

Theoretical part
Red blood cell count

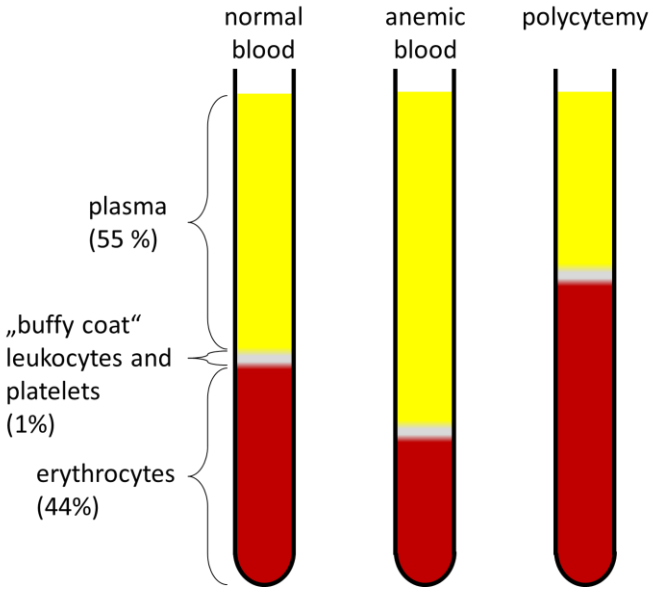
Blood and its components

Blood, one of the bodily fluids, consists of blood elements (so-called formed elements or corpuscles) suspended in blood plasma. Blood is highly specialised tissue, representing approximately 7–8% of body mass. The volume of blood circulating within the cardiovascular system in an average adult is roughly 4–6 litres, depending on gender, weight, body consistency, etc.

Blood carries out a wide range of essential functions. It represents the main transporting system supplying oxygen and nutrients throughout the body. Furthermore, blood removes metabolic waste products such as lactic acid, carbon dioxide and so forth. Blood also has an essential role in maintaining homeostasis, thermoregulation, immune reactions as well as hormonal signalling and regulation.

Blood plasma is composed of water and organic (proteins, lipids, carbohydrates, etc.) and inorganic substances (electrolytes) dissolved in the water. Blood plasma is obtained by centrifugation of anticoagulated blood with consequent separation of its components, separated based on their molecular weight. Anticoagulated blood is obtained by addition of an anticoagulant agent into the blood which must be used in the production of blood plasma. Inversely, blood serum lacking clotting factors is produced by centrifugation of blood without any anticoagulation agent (clotted blood). The anticoagulant agents prevent a coagulation cascade in any of its steps. Sodium citrate, EDTA and sodium oxalate bind calcium ions, crucial reactants in the coagulation cascade and hence are used *in vitro*, in contrast to heparin, which is used only *in vivo*. Heparin promotes the action of the anticoagulation systems, especially antitrombin III.

Blood is ranked among the non-Newtonian fluids and thus its viscosity (the measure of a fluid's ability to resist gradual deformation by shear or tensile stresses) is dependent on the shear rate as well as its content, which highly influences its flow. The haematocrit represents the volume percentage of red blood cells in blood and is examined by centrifugation or sedimentation. The physiological range is normally 39–51% in men and 35–46% in women.



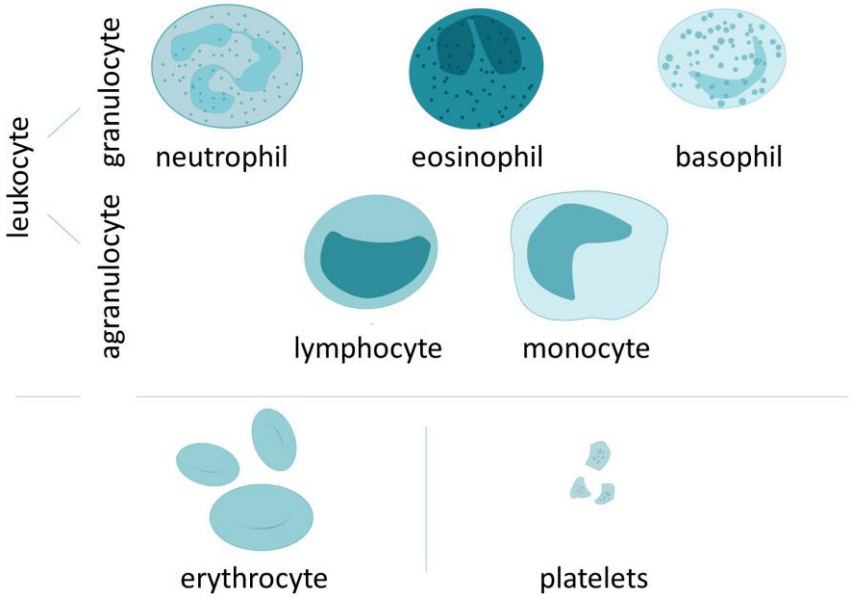
Name number Study group

Leukocytes

Leukocytes, also called white blood cells, are blood elements with a nucleus providing the body's immune response. The white cell count ranges under physiological conditions between 4000 and 10,000 leukocytes per 1 µl of blood, far less than the number of erythrocytes. Commonly white blood cells are divided into two major groups, granulocytes and agranulocytes. Granulocytes are further subdivided into neutrophils, eosinophils and basophils, all representing the cellular subsystem of non-specific immunity. The agranulocytes group comprises lymphocytes and macrophages.

Thrombocytes

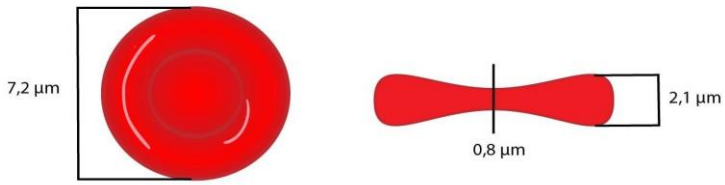
Platelets (thrombocytes) are nuclei lacking discoid blood elements. They circulate in the blood in the range of 150,000 to 450,000 per 1 µl of blood. Thrombocytes are essential in haemostasis, a sophisticated mechanism preventing blood loss during injury. Moreover, platelets play a crucial role also in coagulation. Platelets are produced in the bone narrow as fragments of the megacaryocyte's cytoplasm into circulation and after 7–10 days are eliminated.



Erythrocytes

Erythrocytes, also called red blood cells, are blood elements produced in the bone marrow. As they mature, erythrocytes lose their nucleus and take on a specific shape: oval biconcave discs. Under the microscope one observes a brighter middle disc surrounded by a reddish oval. This colour discrepancy is based on differences in haemoglobin concentration within the erythrocyte. The bright central part of the erythrocyte is thinner (0.8 µm) and contains far less haemoglobin in comparison to the periphery (2.2 µm). A red blood cell (RBC) with a 7.2 µm diameter is called a normocyte. Larger RBCs are called macrocytes and smaller ones are termed microcytes. The condition when there are a lot of RBCs of differing diameter is known as anisocytosis. Poikilocytosis, on the other hand, stands for a condition where there is an excessive amount of improperly shaped RBCs. 1 µl of blood contains $4.3–5.3 * 10^6$

RBCs in healthy men and 3.8–4.8 * 10^6 RBCs in women. A decreased number of RBCs is called anaemia while an increased number is known as erythrocytosis or polyglobulia.



Erythropoiesis

Erythropoiesis is the specific process taking place within the bone marrow of adults which leads to production of erythrocytes. It starts with a pluripotent stem cell that, under the effect of erythropoietin, the red blood cell growth factor produced mainly in kidneys, differentiates into a reticulocyte. The reticulocyte is eventually washed out into the blood, where 48 hours later it develops into a mature erythrocyte. Physiologically the level of reticulocytes in the blood is 0.5–1.5%, but this might be increased after an injury or other blood loss (reticulocytosis). The normal life span of an erythrocyte is approximately 120 days. An old or damaged erythrocyte is eliminated in the spleen.

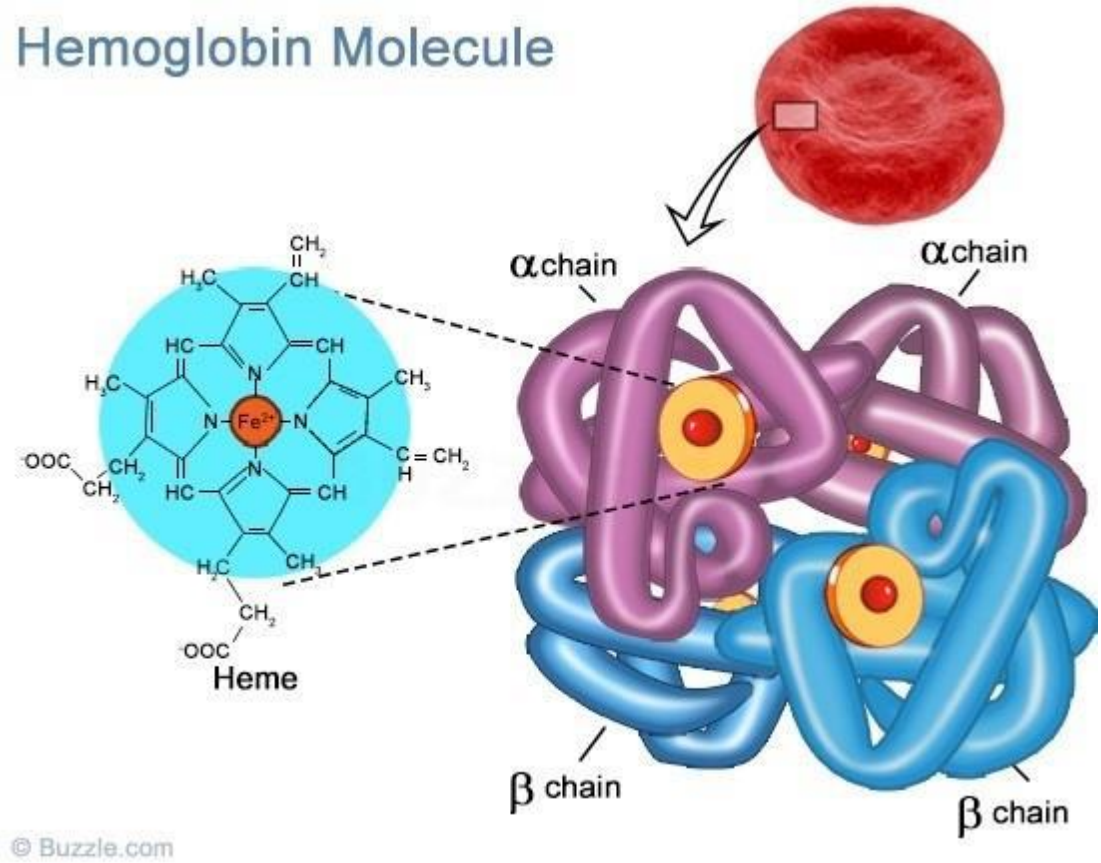
Erythropoiesis requires several absolutely essential substances, such as:

1. Amino acids – an essential part of the globin part of haemoglobin as well as of porphyrin
2. Iron – invaluable for hem production; a lack of it leads to iron deficiency anaemia
3. Vitamin B12 and folic acid – both crucial for nuclide acid synthesis and a lack of them causes pernicious anaemia

Haemoglobin

Haemoglobin is a protein compounded from four heme molecules (a porphyrin ring containing Fe^{2+} ion in the centre) and four globin molecules (two alpha and two beta chains). Haemoglobin not only represents the most essential transport mechanism for oxygen, but it also participates in CO_2 transport. Each globin molecule creates a hydrophobic pocket protecting a Fe^{2+} ion against its oxidation into a Fe^{3+} ion that would be incapable of further transporting oxygen. Haemoglobin is produced during the evolution of an erythrocyte from its precursors in the bone marrow from glycine and succinyl-CoA.

Name number Study group



Haemoglobin forms	*Bound molecule*
Oxyhaemoglobin	O_2
Carbaminohaemoglobin	CO_2
Methaemoglobin	Heme contains Fe^{3+} instead of Fe^{2+}
Carboxyhaemoglobin	CO

The initial phase of haemoglobin degradation is located within macrophages following phagocytosis of a disrupted erythrocyte or the haemoglobin molecule leaking into plasma and bound to haptoglobin, a protein binding free haemoglobin molecules floating in plasma. Degradation of haemoglobin is caused by its oxidation into verdoglobin. Verdoglobin is transformed to green biliverdin by splitting the globin molecule off, which is eventually chopped off into amine acids. Biliverdin is enzymatically modified and, as bilirubin, it is released into the blood stream. Bilirubin as a lipophilic molecule is bound to albumin, transporting it to the liver where it is further modified by conjugation with glucuronic acid. Conjugated bilirubin, so-called direct, is excreted into the gall and consequently mixed with chymus. An increased bilirubin concentration in plasma is known as jaundice or icterus and typically first appears as yellowish discolouration of sclera and eventually mucosa and skin accompanied by itching.

Sex differences

	Men	Women
total red blood cells	4.3–5.3 x 10^{12}/l	3.8–4.8 x 10^{12}/l
hemoglobin	140–180 g/l	120–160 g/l
hematocrit	0.39–0.51	0.35–0.46

Sex differences are caused by sex hormones. Testosterone stimulates erythropoiesis by increasing erythropoietin production in kidneys, while oestrogen decreases its production.

Name number Study group

Anaemia

Anaemia is a medical condition where haemoglobin concentration is decreased, causing insufficient oxygen transport throughout the body. Anaemia develops when the production of red blood cells is impaired or loss of them increases. Anaemia might present as pale and cold skin, shortness of breath, tiredness and poor physical endurance. The body compensates for anaemia by increasing the heart rate.

Iron deficiency anaemia

Iron deficiency anaemia is the most common anaemia in humans, predominantly occurring in women and caused by a lack of iron necessary for haemoglobin production. Hypoxemia caused by anaemia stimulates massive erythropoietin production and thus the erythrocytes released from the bone marrow are small and have a decreased haemoglobin content (microcytic hypochromic anaemia).

Pernicious anaemia

Pernicious anaemia is a medical condition that develops due to a lack of vitamin B12 or folic acid. Both of these vitamins are crucial for the nuclide acid production, namely thymine. Because there is not enough thymine for DNA synthesis, erythrocytes produced in the bone marrow are great and contain a lot of haemoglobin (macrocytic, hyperchromic anaemia).

Name number Study group

Protocol
Red blood cell count

Methods

Red blood cell count

Equipment: Bürker's counting chamber with cover glass, microscope with lamp, a cup of approx. 10–12 ml with stopper, micropipette with adjustable range of 1–5 ml and 10–100 µl, dropper with a fine tip, blood sample, Hayem's solution – beware! POISON! (Na2SO3 – 5 g, NaCl – 1 g, HgCl2 – 0.5 g, distilled water 200 ml), a vessel with disinfecting solution.

Note:
1. Hayem's solution is a highly toxic solution due to its HgCl2 content, which causes burns after ingestion and is harmful also after long-lasting skin exposure. Before handling, read the safety rules carefully!
2. Always use gloves when working with blood!

Procedure:

1. Place 4950 µl of Hayem's solution into the cup by using the micropipette.
2. Add 25 µl of blood using the other micropipette: the chosen volume (25 µl) is set on the micropipette and can be checked on its display. Fix the tip by slightly pressing the bottom of the micropipette, immerse it (approx. 1 cm) into the blood sample and gently press the button (position 1). Pull the tip from the blood – any excess of blood should be carefully removed using a pad of cotton wool. Then, the blood is expelled into the cup with Hayem's solution. The button of the micropipette is pressed completely (position 2) when the tip is immersed in the solution – the whole volume of blood is expelled. Blood is slowly expelled into the solution, and near the wall of the vessel so that it is not mixed yet. The used tip is then disposed into the vessel with disinfecting solution by pressing the ejector of the micropipette, and the device is then placed back onto the stand.
3. The cup is closed by a stopper and its content mixed by whirling movements for 1–2 min in order to get a homogenous suspension. The solution should not come into contact with the stopper.
4. Prepare the Bürker's counting chamber. On the middle prism, two grids are engraved constituting a network of smaller (1/400 mm^2) and larger (1/25 mm^2) squares for counting red and white blood cells, respectively.
5. Take a sample of the suspension from the cup into a fine dropper. The tip of the dropper is placed between the cover glass and the bottom of the chamber where the suspension is drawn by capillarity. When filling the chamber, we have to prevent the solution from overflowing into the grooves between the prisms or to the space over the other grid where the red blood cell count should be performed. If this happens, the chamber must be cleaned and refilled. Then, put the filled chamber on the microscope plate and, under steady visual control, bring the objective near to the cover glass.
6. Red blood cells are counted in a total of 40 (or 80 if a higher precision is desired) small squares. If some cells touch the margin of the square, only those lying on the upper and the right side of the square are counted, irrespective of whether they are within or outside the square, as shown in Fig. 2.

7. Estimate the average number of RBCs in one square from the value obtained by counting 40 or 80 squares. Since the volume above the square is 1/4000 mm^3, multiply the average number by 4.109. Finally, multiply by the dilution ratio, i.e. 199 to get the number of RBCs in 1 litre of blood. The margin of error of such estimation of RBCs is ± 200,000 mm^3.

The Bürker's counting chamber and basic rules for counting RBCs

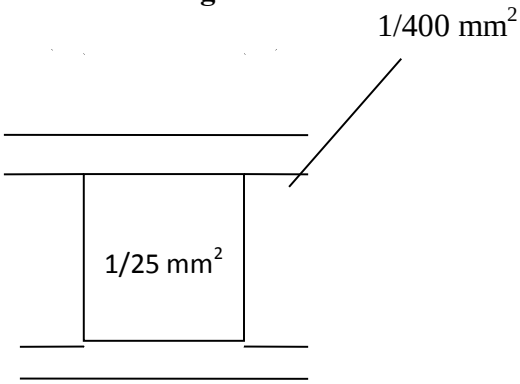


Fig. 1

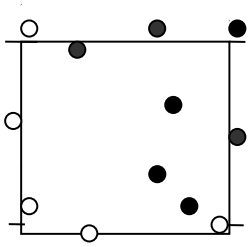


Fig. 2

Estimation of haemoglobin concentration

Equipment: Spectrophotometer Spekol, funnel and 5 ml cylinder for transforming solution, micropipette with adjustable range 10–100 µl, vessel with disinfection solution, and transforming solution (solution by van Kempen-Zijlstra: K3Fe(CN)6 – 0.2 g, KCN – 0.05 g, KH2PO4 – 0.14 g, distilled water to 1000 ml; in Drabkin's solution, KH2PO4 is replaced by NaHCO3 – 1.0 g), automatic analyser Mythic 18

Note:
1. Transforming solution is a highly toxic solution due its KCN content, which is highly toxic when inhaled, ingested or in contact with skin. Before handling, read the safety rules carefully!
2. Always use gloves when working with blood!

Procedure:

1. Switch the apparatus on and wait at least 15 minutes before the measurement. For estimation of haemoglobin concentration, a wavelength of 540 nm is used. The lower lever has to be turned to the left position.
2. Prepare the measured solution: put 5 ml of transforming solution into a test tube by using the funnel and 5 ml cylinder and add 20 µl of blood taken from the blood sample by means of an adjustable micropipette (for the procedure, see Exercise I.1). The absorbance is measured 10 min after mixing the solution.
3. Fill the cuvette with distilled water (blind experiment) and move cuvette inside the apparatus. Press C. Press FAKT – either 1.000 or last factor will appear on the display. Insert the correct factor for your measurement (for haemoglobin this is 22.8) using POS and INC. Press FAKT again (this inserts the value into the memory of the Spekol). Press R. Now you can read the concentration of the blind experiment.

4. After this setting, insert the cuvette with the measured sample and read the concentration of haemoglobin (mmol/l). Multiply this value by the coefficient 16.11 to obtain the concentration of Hb in g/l.
5. Check the values obtained by the Spekol by using the Mythic 18 automatic analyser.

Note:
Cuvettes must be clean and their outer surface dry. If some solution gets onto the surface while filling the cuvette, it must be wiped off (the instrument would be damaged if the aggressive solution reaches its inside). Cuvettes may be taken only by their upper parts, optimally in lateral ground areas, never in areas where the measuring light beam passes. The meter and the fragile cuvettes are the most sensitive parts of the instrument: avoid any shock or shaking.

Calculated parameters of red blood cells

Based on the values obtained by measurements, further parameters commonly used in clinical practice can be reached by calculation:

1. average volume of red blood cell (MCV = mean corpuscular volume)
MCV = Hct / number of red blood cells (physiological values: 80–95 fl)

2. average weight of Hb in red blood cell (MCH = mean corpuscular haemoglobin)
MCH = Hb / number of red blood cells (physiological values: 27–32 pg)

3. average concentration of haemoglobin in red blood cell (MCHC = mean corpuscular haemoglobin concentration)
MCHC = Hb / Hct (physiological values: 310–360 g Hb / litre of red blood cell)

Name number Study group

Results

Red blood cell count

Describe the method:

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……
……
……

Fill in the number of red blood cells (counted in 40 small squares)

....

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....

....

Average number of red blood cells per square

.....

The volume of one small square: 1/4000 μl

The number of red blood cells in one small square is multiplied by $4 * 10^9$

.....

Obtained value is multiplied by * 200

Total red blood cell /l:.....

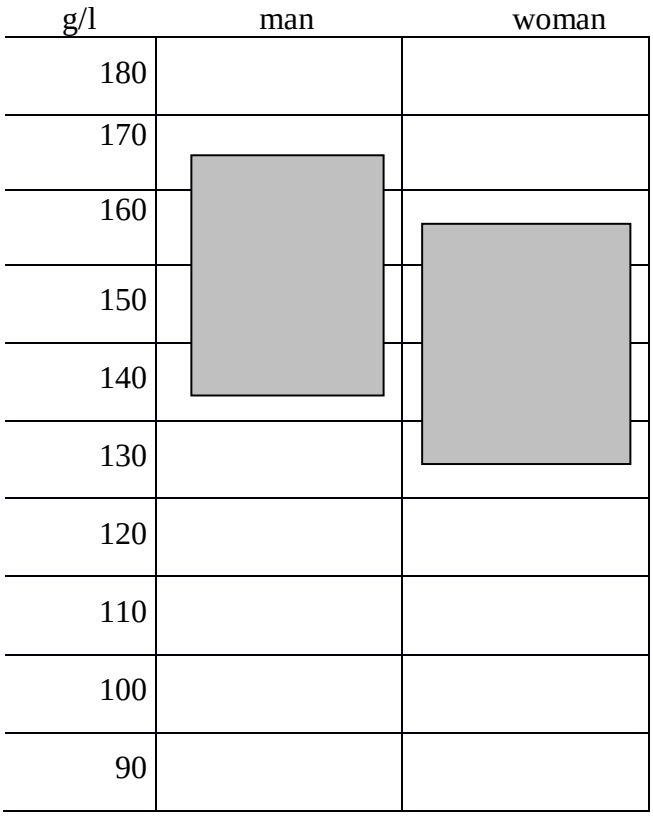
Estimation of haemoglobin concentration

In Graph 1 note the haemoglobin concentration that you have found. The physiological range of haemoglobin concentration in men 130–175 g/l [Hb (1Fe) 8.07 – 10.9 mmol/l], and in women 120–165 g/l [Hb (1Fe) 7,45 – 10,2 mmol/l], is marked in the graph.

Declined values: anaemia; raised values: dehydration, polycythaemia

Name number Study group

Graph 1. Haemoglobin concentration (g/l)



Calculated parameters of red blood cells

1) **Average volume of red blood cell (MCV = mean corpuscular volume)**

MCV = Haematocrit / number of red blood cells (physiological values: 80–95 fl)
Calculation: MCV =

2) **Average weight of Hb in red blood cell (MCH = mean corpuscular haemoglobin)**

MCH = haemoglobin / number of red blood cells (physiological values: 27–33 pg)
Calculation: MCH =

3) **Average concentration of haemoglobin in red blood cell (MCHC = mean corpuscular haemoglobin concentration)**

MCHC = haemoglobin / haematocrit (physiological values: 310–360 g/l)
Calculation: MCHC =

Conclusion

According to your results, draw a conclusion about your specimen. Suggest the gender of your unknown patient and attempt to diagnose him/her.

……

……

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