2 INVESTIGATION OF LIPID METABOLISM & CHOLESTEROL

Topics to be reviewed

Lipids in blood, lipoproteins. Cholesterol – transport and metabolisms. Decision-making analysis, critical difference.

Pre-lab questions

1. Draw formulas of cholesterol and cholesteryl linolate.
2. Explain what the blank is. Which substances are absent in the blank for the determination of serum cholesterol?
3. Which biological samples can be analyzed by Reflotron and Accutrend?
4. Write Friedewald's equation and the two limitations of its usage.
5. Which lipoprotein fraction poses a risk for atherosclerosis? In which serum protein fraction is it comprised? Give its two possible names.

Introduction

Recently, lipid metabolism disorders have come into account in connection with the prevention and treatment of cardiovascular disease.

Basic examination of lipid metabolism involves analysis of triacylglycerols, total cholesterol, HDL-cholesterol and from these values calculated concentrations of LDL-cholesterol and non-HDL cholesterol. The examination is completed with the chylomicrons test and occasionally with the analysis of phospholipids and electrophoresis of lipoproteins.

Hyperlipidaemia is the condition in which the levels of lipids in the blood are too high.

With regard to the development of atherosclerosis we recognize three basic types of hyperlipidaemias*:

I.	Isolated hypercholesterolaemia (isolated increase of the total cholesterol)
II.	Isolated hypertriacylglycerolaemia (isolated increase of triacylglycerols)
III.	Combined hyperlipidaemia (simultaneous increase of cholesterol and triacylglycerols)

* Formerly used classification according to Frederickson, which is based on the determination of total cholesterol, triacylglycerols a electrophoresis of lipoproteins, is no longer used.

Hyperlipoproteinaemia is the condition in which the level of one or more classes of lipoproteins in the blood is increased. Often, the increase in blood lipids is combined with the decrease in the concentration of HDL-cholesterol, therefore, the term **dyslipoproteinaemia** is also used.

Laboratory diagnostics of lipid metabolism disorders must be supported by the examination of blood lipids from at least two blood collections in 2–8 weeks interval, at normal lifestyle. Critical differences of

the individual parameters should be evaluated (see Exp. 1.6.3) and, if they are lower than acceptable, the arithmetic mean is calculated for each analyte. In case that the critical difference is exceeded, the third measurement should be carried out.

The critical differences of lipid metabolism parameters:

Analyte	Critical difference (%)
Total cholesterol	> 20
LDL-cholesterol	> 25
HDL-cholesterol	> 25
Triacylglycerols	> 65

The examination of lipid metabolism is highly influenced by lifestyle (diet, physical activity, and body weight), pharmacotherapy (hormonal contraception, hormonal substitution therapy, etc.) and by acute or non-compensated disease. Therefore, the examination of blood lipids must not be done, when it is expected, that the result will not describe the situation at the normal lifestyle (shortly after vacation, during hospitalization from other reasons, acute diabetes mellitus, gravidity and half a year later, etc.).

2.1 Determination of total cholesterol in serum and blood

Cholesterol is transported in blood as a component of lipoproteins, mainly LDL, less in HDL and VLDL. About two thirds of the cholesterol in the plasma is esterified with long chain unsaturated fatty acids, the remaining one third is present as free (unesterified) cholesterol.

2.1.1 Enzymatic determination of serum cholesterol

Cholesterol esters are hydrolyzed by cholesterol esterase into free cholesterol and fatty acids. The free cholesterol is then oxidized with the aid of cholesterol oxidase and consumption of oxygen giving rise to cholestenon and hydrogen peroxide. The latter, in the peroxidase-catalyzed reaction yields a red dye from of 4-aminophenazon and phenol.

Materials: Kit CHOL (Roche Diagnostics*) containing: *Reagent-cholesterol* (containing Tris buffer 100 mmol/L, pH 7.7; Mg^{2+} 50 mmol/L; 4-aminophenazon 1 mmol/L; sodium cholate 10 mmol/L; phenol 6 mmol/L; 3,4-dichloro-phenol 4 mmol/L, polyglycol ether 0.3 %; cholesterol esterase ≥ 6.67 µkat/L; cholesterol oxidase ≥ 4.17 µkat/L; peroxidase ≥ 3.33µkat/L), *calibrator-cholesterol* (concentration is given on the label of vial). Specimens of blood serum, set of test tubes, micropipettor 20 µL, pipettor adjusted to 2 mL. Spectrophotometer Spekol 1300 and software WinAspect or spectrophotometer Helios Delta and VisionLite Fixed. *Alternatively, the kit of BioVendor, Pliva-Lachema or Human can be used; the composition of reagents may be then different.

Procedure

☞ Pipette the components into labelled test tubes according to the following table:

Reagens (µL)	Blank	Test	Standard
Reagent-cholesterol	2 000	2 000	2 000
Demi-water	20	-	-
Serum	-	20	-
Calibrator-cholesterol	-	-	20
Mix well and incubate for 10 minutes at room temperature.			
Measure absorbances* of samples (A_x) and standard (A_{STD}) at 500 nm against the blank within 1 h.			

*Measure absorbances according to the enclosed instructions to the spectrophotometers and use relevant software.

Calculation of the concentration of serum total cholesterol: $$c_x = \frac{A_x}{A_{STD}} \times c_{STD}$$

where c_{STD} is concentration of cholesterol in calibrator (it is given on the label of the vial).

Evaluation

According to the recommendation for diagnostics and treatment of hyperlipoproteinaemias of adult people, prepared by the Czech Society for Atherosclerosis, the examination of cholesterol should be performed once in every five years in all adult people from 20 to 75 years old during preventive medical examination. If the concentration of total cholesterol is below 5 mmol/L, next control should follow after five years.

Serum cholesterol above 5 mmol/L should be the reason for further examination, especially the finding of the cholesterol distribution in the lipoprotein fractions. Increased concentration of cholesterol is often found in diabetics or in case of hypothyroidism, decreased (< 2.5 mmol/L) e.g. in case of late hepatic cirrhoses or hyperthyroidism.

2.1.2 Determination of blood cholesterol using analyzer Reflotron®

Reaction zone of the test strip contains enzymes cholesterol esterase, cholesterol oxidase, peroxidase and chromogenous substrate dimethylbenzidine. Determination is based on the sequence of enzymatic reactions described in Exp. 2.1.1. The resulting colouring is evaluated by the reflectance method.

Materials: Analyzer Reflotron® with strips designated for cholesterol, micropipettor 30 µL, lancets or sterile needles, disinfectant Ajatin, cellulose swabs.

Procedure

Follow detailed enclosed instructions to the analyzer Reflotron.

☞ *Preparation of the instrument*. Open flap of measurement chamber.

☞ *Preparation of the strip*. Remove the foil protecting the test strip.

☞ *Capillary blood collection*. Using the pipettor take up exactly 30 μL of capillary blood directly from the puncture site:

Strictly follow the rules for the proper collection of capillary blood (see Exp. 1.1.2):

- *wash* hands in warm water
- *disinfect* the puncture site
- point the puncture using of lancet from *lateral* side of finger pad
- *wipe off* the first drop of blood
- *support* formation of the other drops by gently pressure to the surrounding tissue
- after collection of blood apply a cotton wool wetted in disinfect solution on the puncture site

☞ *Application of blood on the strip*. Apply blood in the centre of the red application zone on the test strip (don't touch the application zone by tip).

☞ *Starting measurement closing flap.* Within 15 seconds after sample application, insert test strip (the magnetic band turned down) into the measuring chamber until it snaps in. Close flap immediately.

☞ *Result reading.* The value CHOL is displayed (a test specific magnetic code recognizes the parameter of interest) and time left to displayed result. Cholesterol value in mmol/L is displayed.

Evaluation

The total cholesterol value should be below 5 mmol/L, see below table „Border value of blood lipids in common population“. Investigation of lipids by „dry chemistry" methods is regarded as screening. The diagnostic and treatment dyslipoproteinaemias must be always supported by examination of lipids from venous blood using the certified method.

2.1.3 Determination of blood cholesterol using analyzer Accutrend®

Determination is based on the sequence of enzymatic reactions described in Exp. 2.1.1. Reaction zone of the test strip contains enzymes cholesterol esterase, cholesterol oxidase, peroxidase and chromogenous substrate dimethylbenzidine. The resulting colouring is evaluated by the reflectance method.

Materials: Analyzer Accutrend® with strips designated for cholesterol, lancets or sterile needles, disinfectant Ajatin, cellulose swabs.

Procedure

Follow detailed instructions of the analyzer Accutrend®:

☞ *Switch the instrument on*. To start a measurement, switch the meter on. The display shows the stored code number. If the meter is already coded skip next step.

☞ *Coding.* Insert the check strip into the instrument and the code is saved.

☞ *Insert test strip*. Insert a cholesterol test strip until it snaps in. The meter recognizes the type of test strip and confirms correct placement by an acoustic signal. A blank value of the test strip is taken automatically.

☞ *Capillary blood collection*. Strictly follow the rules for the proper collection of capillary blood (see Exp. 1.1.2)

- *wash* hands in warm water
- *disinfect* place of puncture site
- point the puncture using of lancet from *lateral* side of finger pad
- *wipe off* the first drop of blood
- *support* formation of the other drops by gently pressure to the surrounding tissue
- after collection of blood apply a cotton wool wetted in disinfect solution on the puncture site

☞ *Application of the blood on the strip.* Open the flap and apply the drop of blood directly from the punctured finger onto the yellow mesh of the test strip. If the application field is not completely covered with blood, repeat this step and put a drop of blood on a new test strip.

☞ *Initiation of measurement.* Close the flap immediately. After having applied a drop of blood, close the flap immediately. Only now the counting of the time starts. The cholesterol value is displayed after 180 seconds and stored automatically with time and date.

☞ *Alternatively result reading.* The round colour field on the reverse side of the test strip has to be stained uniformly. If not, repeat the measurement with a new test strip. In addition, please compare the colour field with the colour scale on the test strip package.

Evaluation

The concentration of total cholesterol should be below 5 mmol/L, see below table "Cut-off values of blood lipids in common population". Generally, the examination of lipids by "dry chemistry" methods is regarded as screening. The diagnostics and treatment of dyslipoproteinaemias must be always supported by the examination of lipids from venous blood using the certified method.

2.2 Determination of blood triacylglycerols using analyzer Reflotron®

Determination of triacylglycerols belongs among basic methods for the examination of lipid metabolism. The Reflotron® analyzer and capillary blood are used for testing in the practical lesson.

Principle: After placing the blood sample on the test strip, the erythrocytes and other cell elements are separated. Consecutively, a sequence of enzymatic reactions takes place in the reaction zone of the test strip. The triacylglycerols (TG) are hydrolyzed by esterase into fatty acids and glycerol. Then glycerol is phosphorylated by glycerol kinase to glycerol-3-phosphate, which is oxidised by glycerol-phosphate oxidase in presence of oxygen. Arising hydrogen peroxide reacts with the redox indicator catalysed by peroxidase to form coloured product. The resulting coloration is evaluated by the reflectance method. Methods used in the automatic analyzers are based on the similar principle.

Materials: Analyzer Reflotron® with strips designated for triacylglycerols, micropipettor 30 μL, lancets or sterile needles, disinfectant Ajatin, cellulose swabs.

Procedure

☞ Follow detailed instructions of the analyzer Reflotron®.

☞ The procedure is analogous as in Exp. 2.1.2.

Evaluation

Serum triacylglycerols in the healthy population (the cut-off level) should be less than 2 mmol/L. Samples of serum with the concentration of TG above 3.4 mmol/L are opalescent, TG concentrations over 11.3 mmol/L indicate the presence of chylomicrons and serum has milky turbidity. Therapy is strictly based on the diet with low content of fats and sugars, increased physical activity, sufficient amount of antioxidants in the nutrition and the overall hypolipidemic treatment.

2.3 Determination of HDL-cholesterol and LDL-cholesterol

A high proportion of serum total cholesterol incorporated in HDL is considered as a sign of the satisfactory ability of an organism to eliminate undesirable excess cholesterol. On the contrary, an increased concentration of LDL-cholesterol represents the high coronary risk involved in hypercholesterolaemia.

The determination of HDL-cholesterol (HDL_{Chol}) is based on the procedure, in which chylomicrons, VLDL and LDL are precipitated with a suitable precipitating agent (e.g. phosphotungstic acid and $MgCl_2$ or heparin and $MnCl_2$). The supernatant after centrifugation contains cholesterol associated with soluble HDL. As the concentration of cholesterol in HDL is very low, a sensitive enzymatic method must be used for the determination (see Exp. 2.1.1).

The recent methods are able to determine HDL-cholesterol directly in the serum without the previous precipitation of the other lipoprotein particles. Enzymes cholesterol esterase and cholesterol oxidase modified by polyethylene glycol in presence of Mg^{2+} ions and cyclodextrin sulphate catalyse selectively only the transformation of HDL-cholesterol.

Moreover, there is also a new turbidimetric method for the direct determination of LDL_{Chol} (without any preparatory steps and calculations), which uses a polyanionic detergent. LDL particles form insoluble complexes with this detergent. The resulting turbidity is measured by the turbidimetric method.

Procedure and evaluation

In spite of lack of time, the task is carried out theoretically.

☞ Evaluate the concentrations of HDL_{Chol} provided by the assistant in accordance with the reference values given below.

2.4 Calculation of LDL-cholesterol and derived parameters

The concentration of LDL-cholesterol (LDL_{Chol}) is usually calculated from the difference between concentration of total cholesterol ($Chol_{Tot}$) and the concentration of HDL-cholesterol and triacylglycerols (TG) (all values are in mmol/L) using **Friedewald's formula**:

$$\mathbf{LDL_{Chol} = Chol_{Tot} - TG/2.2 - HDL_{Chol}} \qquad \text{(mmol/L)}$$

This approximately formula is based on the assumption that in most cases chylomicrons are absent after overnight fasting. Serum triacylglycerols originate mostly from VLDL, in which the molar ratio TG/cholesterol is usually a constant having the value approximately of 2.2. This formula may be used only if serum TG concentration is less than 4.5 mmol/L.

Additional derived parameters of the lipid metabolism

The aim of these calculations is to make the determination of the risk, resulting from the measured concentrations of blood lipids, more accurate.

- **$Chol_{Tot}/HDL_{Chol}$ index**

This index takes into account that higher level of HDL_{Chol} is a "negative risk factor", which lowers the risk of the atherosclerotic cardiovascular disease. Concurrent increase of the total and HDL-cholesterol may not increase the risk of atherosclerotic cardiovascular disease.

- **Non-HDL_{Chol}**

$$\mathbf{Non\text{-}HDL_{Chol} = Chol_{Tot} - HDL_{Chol}} \qquad \text{(mmol/L)}$$

This parameter takes into account the concentrations of all atherogenic lipoproteins. It is used in case, when the calculation of LDL cholesterol cannot be carried out because of the increased concentration of triacylglycerols.

Cut-off values of blood lipids in common population:

$Chol_{Tot}$ (mmol/L)	LDL_{Chol} (mmol/L)	TG (mmol/L)	HDL_{Chol} (mmol/L)	Index $Chol_{Tot}/HDL_{Chol}$	Non-HDL_{Chol} (mmol/L)
< 5.0	< 3.0	< 2.0	> 1.0	< 5	< 3.8

If any of the measured concentrations exceeds the cut-off value, additional examinations must be performed to reveal the disorder of lipid metabolism and to evaluate the risk of atherosclerotic cardiovascular disease.

Procedure and evaluation

☞ Using Friedewald's formula and the given values of $Chol_{Tot}$, TG and HDL_{Chol} calculate the value of LDL_{Chol} and compare all of them with the desired ones (see above).

2.5 Electrophoresis of serum lipoproteins

The lipoproteins can be separated electrophoretically at an alkaline pH on agarose gel according to their different mobility. The separated lipoproteins are stained with a lipid specific Sudan Black.

Materials: Kit Hydragel LIPO + Lp(a) (Sebia) containing: *Tris-barbital buffer, pH 8.75* (0.05 mol/L**,** prepared by a dilution of 22.5 mL of the stock solution from the kit with demi-water to final volume of 300 mL), *Sudan black solution* (2 mL of the 6% solution from the kit in 160 mL of ethanol and 140 mL of demi-water), *destaining solution* (45 % ethanol), *agarose gels* (ready to use). Micropipettor 5 µL, applicator, strips of filter paper, migration chamber CUVE K20, source of voltage, hair dryer. Specimens of serum.

Procedure

☞ *Preparation of the gel.* Unpack the HYDRAGEL agarose gel plate and place it on the filtration paper (the gel side up). Place one thin filter paper onto the gel surface (in the application point, on the plane with arrows) to absorb the excess of liquid. Remove the paper immediately. Do not leave the filter paper for a too long contact with the gel to avoid its dehydration.

☞ *Application of the samples.* Place the applicator with the notches on a flat surface. Apply gradually 5 µL of serum samples into the applicator wells and let it seep for 5–10 minutes. Remove the non-seeped serum by a strip of the filtration paper and take away the applicator.

☞ *Electrophoretic separation.* Measure about 150 mL of Tris-barbital buffer into each compartment of the migration chamber. Place the gel plate on the bridge with the gel side facing down and according to the polarity indicated on the gel (the application side of the gel on the cathodic side); the gel should be dip about 1 cm into the buffer on each side. Plug the chamber to the power supply. Migration takes place for 90 minutes at the constant voltage of 50 V (initial current 11±2 mA per gel). After migration, unplug the chamber and remove the gel plate.

☞ *Fixation and staining.* Dry the gel with hot air (max. temperature 80 °C). The gel must be perfectly dry. Immerse the dried and cooled gel in the staining solution <u>for exactly 15 min</u>. Destain the gel in destaining solution <u>for exactly 5 min</u>. Wash gel by immersing into demi-water for several minutes.

☞ *Drying of the gel.* Drain vertically excess liquid on the gel surface and dry the gel with hot air.

Evaluation

After the electrophoretic separation the chylomicrons stay at the start, LDL (β-lipoproteins) migrates at the position of β_2-globulins, VLDL (pre-β-lipoproteins) at the position of β_1-globulins. HDL particles have the highest mobility and they appear at the position of α_2-globulins.

A fraction characteristic for lipoprotein (a) – Lp(a) – a risk factor of atherosclerosis and coronary heart disease – may appear between α- and β-lipoproteins (i.e. HDL and LDL). It may be determined quantitatively by immunoassays or electroimmunodiffusion.

Electrophoregram can be evaluated densitometrically at 580 nm. The results are then expressed in percentages of the optical density for the individual fractions with respect to the whole coloured area. The measured values together with other indicators (e.g. total cholesterol, triacylglycerols) are used to characterize hyperlipoproteinaemias.

Reference intervals:	α-lipoproteins	23–46 %
	pre-β-lipoproteins	3–18 %
	β-lipoproteins	42–63 %

In practice, electrophoregram is evaluated visually. Individual serum fraction is compared with the reference one and evaluated as normal/increase/decrease lipoprotein fraction.

🕮 The other tests of lipid metabolism

Chylomicrons test

Measurement of chylomicrons in serum is not commonly indicated. Samples collected after overnight fasting (12 h at least) should be free of chylomicrons. Their presence in serum is indicated by severe chylosity. The chylomicrons test may be used for their detection. Chylomicrons presence is indicated by an opalescent or white ring after 12hours standing of serum at 4 °C. In this time, the possibly present chylomicrons are floating to the surface of the sample.

Related problems

► 1. Mr. J. (45 years) had the concentration of cholesterol 6.0 mmol/L and after 8 weeks 5.2 mmol/L. The critical difference (CD) for cholesterol is 20 %. a) What does the CD value express? b) What conclusion could be made from both examinations? Should they be followed by the third examination, or can we conclude, that the mean value from both examinations is 5.6 mmol/L?

► 2. Mr. P. (36 years old) had repeatedly the level of cholesterol about 8.1 mmol/L, LDL-cholesterol 4 mmol/L. His daily intake of cholesterol was about 600 mg. He was recommended a diet with low cholesterol content and a change of lifestyle. After three months, the mean value of cholesterol was 7.4 mmol/L. Then, man had prescribed Colestid, which bounds bile acids in the small intestine and increases their excretion in urine. Taking of this drug lead to decrease of cholesterol level to the range of 5.7–6.4 mmol/L.

a) How the first findings of cholesterolaemia should be interpreted?

b) Which types of food should be excluded and which should prevailed in the diet of Mr. P.?

c) Is the cholesterol intake in food the only source of cholesterol in the organism?

d) What is the relation between cholesterol and bile acids? Why could the taking of Colestid lead to the decrease of the cholesterol concentration?

► 3. Mr. Z. (40 years old) had confirmed high serum concentrations of the total and LDL-cholesterol (10 mmol/L and 8.6 mmol/L, respectively). Clinical examination proved characteristic xanthomas on tendons. Mr. Z.'s father died of myocardium infarction at the age of 55. It was concluded, that the patient suffers from familiar hypercholesterolaemia (the cause is the defective gene for the LDL receptor).

a) What can explain the increased level of cholesterol in this patient?

b) How could we positively confirm the diagnosis?