ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

Lipoproteins Biochemical examination of lipid metabolism

General Medicine

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1 Lipoproteins

Lipids are poorly soluble in water and therefore must be transported in blood plasma as particulate complexes with proteins, referred to as lipoproteins. Lipoprotein particles provide an efficient mechanism for delivering their lipid content to the tissues.

Each lipoprotein contains an inner lipid core consisting of non-polar triacylglycerols or cholesterol esters or both. An outer shell is formed by polar lipids (phospholipids, free cholesterol), together with specific protein components called apolipoproteins. Apolipoproteins differ in structure and function; and are divided into classes A to H. The bonds between components of lipoprotein particles are non-covalent. Therefore, circulating lipoprotein particles can easily exchange lipids and proteins with each other.

Lipoproteins are heterogeneous: the proportion of lipids and kinds of apolipoproteins varies in different lipoprotein particles. Lipoproteins are usually classified on the basis of their physicochemical characteristics. Two basic methods can be used for the lipoprotein separation: ultracentrifugation and electrophoresis.

The different combination of lipids and proteins produces particles of different densities, which is related to both lipids and protein content. The higher is proportion of lipid content to the protein, the lower is density of the particle, and the larger is particle size. Differences in density of the lipoproteins permit their separation by ultracentrifugation. Five classes are defined by ultracentrifugation:

- chylomicrons
- very low density lipoproteins - VLDL
- intermediate density lipoproteins - IDL
- low density lipoproteins - LDL
- high density lipoproteins - HDL

Another approach used in the routine laboratory analysis of lipoproteins is electrophoresis. The method is similar to the electrophoretic separation of serum proteins; only for the visualisation of separated lipoproteins a lipid-specific stain must be used. The particles move in the electric field in dependence on their surface charge and size; and the pore size of the supporting medium. Electrophoretic mobility of lipoprotein parallels the results obtained when lipoproteins are separated by ultracentrifugation with the exception of intermediate fractions. According to electrophoresis following lipoproteins classes may be distinguished:

- chylomicrons
- pre-β-lipoproteins (corresponding to VLDL)
- β-lipoproteins (corresponding to LDL)
- α-lipoproteins (corresponding to HDL).

1.1 Classes of lipoproteins (Tab. 1)

- **Chylomicrons** are the lipoprotein particles of the largest size and the lowest density. They transport dietary triacylglycerols and some cholesterol from the intestine to the rest of the body, especially muscles and adipose tissue. Chylomicrons are rapidly metabolized by lipoprotein lipase that is present on the endothelial surface of capillaries in adipose tissue and skeletal

muscles. The clearance time from the formation of chylomicrons after a meal until the final removal of remnants by the liver is about 6 hours. Normally, chylomicrons are absent from 12- to 14-hour fasting blood specimens. The fresh plasma containing chylomicrons appears opaque and milky (lipemic). A thick creamy layer floating on the top of a fasting plasma specimen after standing in the refrigerator overnight at 4° C is observed if chylomicrons are present. This finding signals a defect in their clearance. During electrophoresis chylomicrons do not migrate, i.e. remain at the site of sample application.

- **VLDL (Very Low Density Lipoproteins),** other triacylglycerol-rich lipoproteins, are mainly synthesized in and secreted by the liver. They transport triacylglycerols synthesized in the liver. During their circulation, VLDL are gradually delipidated. Following hydrolysis of its triacylglycerols by lipoprotein lipase, VLDL particle is converted to IDL, and then to LDL. VLDL supply peripheral tissues (adipose tissue and skeletal muscle) with fatty acids released from triacylglycerols in the absence of chylomicrons. In addition to triacylglycerols the VLDL contain about 10% of cholesterol, which is derived both from dietary cholesterol and the one synthesised in the liver. Significant elevation of VLDL in serum is evidenced by increased serum triacylglycerol concentration and by its turbid appearance.
- **LDL (Low Density Lipoproteins),** cholesterol-rich lipoproteins arise primarily from the catabolism of IDL after hydrolysis of remaining triacylglycerols by the hepatic lipase; some LDL are also released to the circulation directly by the liver. The LDL are the only lipoprotein class possessing a single apolipoprotein apo B-100. In healthy individuals, LDL cholesterol constitutes about two thirds of the total plasma cholesterol.

  LDL transport cholesterol to the cells. The LDL particles are removed from the circulation by cellular uptake mediated by the special LDL receptors found on the surfaces of most cells. By this way cholesterol enters the cell for storage or conversion to other compounds. LDL are heterogeneous in size and density; and may be further separated into 3 subclasses:

  Large LDL: LDL-I

  Intermediate LDL: LDL-II

  Small dense LDL: LDL-III

  The cholesterol carried by LDL is considered the most atherogenic form of serum cholesterol. LDL may enter into the arterial wall, become entrapped in the intima, and undergo modifications of its structure there. This leads to uptake of modified LDL by macrophages, producing foam cells. This is the first stage of atherosclerotic process. The small dense LDL particles are atherogenic the most, because they penetrate the arterial intima more readily and they are more easily oxidised. They have a lower affinity to the LDL receptors. The small dense LDL predominate in patients with elevated triacylglycerol concentration.

  LDL cholesterol is estimated in order to make an accurate assessment of an individual´s coronary heart disease risk, or to monitor a cholesterol-lowering therapy.

- **HDL (High Density Lipoproteins)** are small protein-rich particles. HDL are produced both by the liver and the intestine, or arise intravascularly from the surface components of chylomicrons and VLDL during their catabolism. Nascent (newly forming) HDL are secreted as nascent disc shaped structures, consisting of a phospholipid bilayer and apolipoproteins. In the circulation free cholesterol from the cell membranes of peripheral tissues is picked up by the HDL; and simultaneously converted to cholesterol esters by lecithin:cholesterol acyl transferase (LCAT). The esterified cholesterol moves to the core of HDL and the particle becomes spherical. This cholesterol-rich lipoprotein then returns to the liver. By this way HDL provide the transport of excess cholesterol from the tissues back to the liver – this process is referred to as the reverse cholesterol transport. Liver is the only organ capable of excreting significant amounts of cholesterol in the form of bile acids or neutral sterols into the bile. The antiatherogenic function of HDL is related to its role in reverse cholesterol transport. A strong negative correlation

between coronary heart disease and HDL cholesterol level exists. HDL cholesterol > 1.0 mmol/l counts as "negative" risk factor for atherosclerosis. Serum HDL cholesterol is lowered by smoking, obesity and physical inactivity.

- **Lipoprotein (a) [Lp(a)]** is an LDL-like lipoprotein with one additional molecule of apo (a) linked to apo B-100 by a disulfide bond. Apo (a) has a high degree of homology with plasminogen. The elevated serum levels of Lp(a) is though to be an independent risk factor for the development of premature atheroslerosis. The serum levels of Lp(a) are predominantly genetically determined.

Tab. 1: Characteristics of lipoprotein classes:

Class	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/ml)	< 0.95	0.95 – 1.006	1.006 – 1.019	1.019 – 1.063	1.063 – 1.210
Diameter (nm)	> 75	25 – 70	22 – 24	19 – 23	4 – 10
Electrophoretic mobility	origin	pre-β	slow pre-β	β	α
Composition					
Lipids total (%)	98 – 99	89 – 94	91	75 – 80	50 – 55
Triacylglycerols (%)	86 –94	55 – 65	40	8 – 12	3 – 6
Free cholesterol (%)	0.5 – 1	6 – 8		5 – 10	3 – 5
Cholesterol esters (%)	1–3	12 – 14		35 – 40	14 – 18
Total cholesterol (%)	1.5 – 4	18 – 22	35	40 – 50	17 – 23
Proteins (%)	1 – 2	5 – 10	9	20 – 24	45 –50
Major apoproteins	A I, B-48, C I,II, III, E	A,B-100, C I, II, III, E	B-100,CII, E	B-100	A I, II,C,E
Origin	intestine	liver, intestine	intravascular as catabolic product of VLDL	metabolic end product of VLDL	liver, intestine, intravascular
Function	transport of exogenous triacylglycerols	transport of endogenous triacylglycerols	transport of endogenous triacylglyce-rols and cholesterol esters	transport of cholesterol to peripheral cells	transport of cholesterol from peripheral cells to the liver

2 Dyslipidemias

Disorders associated with an abnormal serum lipid concentration are referred to as dyslipidemias. Abnormalities in serum lipids arise due to a disorder in the synthesis, transport or catabolism of the lipoproteins. Dyslipidemias include hyperlipoproteinemias (HLP) and hypolipoproteinemias. The importance of serum lipid disturbances lies in their participation in the development of atherosclerosis. Complications of atherosclerosis (myocardial infarction, strokes) are the leading causes of morbidity and mortality in many Western developed countries.

2.1 Classification of hyperlipoproteinemias

Several classifications of hyperlipoproteinemias exist.

2.1.1 Fredrickson´s classification (Tab. 2)

Fredrickson introduced the first classification of hyperlipidemia. It is based on a combination of lipid analysis and lipoprotein electrophoresis, which allows the categorisation of patterns into five abnormal lipoprotein phenotypes. Each such form of hyperlipoproteinemias does not, however represent a homogeneous entity from a genetic, clinical, and pathological point of view, rather, it is a group of conditions with different metabolic causes having the same electrophoretic pattern. On the other hand, the lipoprotein phenotype of an individual can change from one pattern to another in response to the diet or treatment. For these disadvantages other classifications of HLP have been introduced.

Tab. 2: Fredrickson´s classification of hyperlipoproteinemias:

Phenotype	**Total cholesterol**	**LDL cholesterol**	**Triacylglyce-rols**	**Lipoproteins abnormality**	**Occurren-ce**	**Clinical associations**
I	normal or increased	low or normal	increased	excess chylomicrons	rare	pancreatitis
IIa	increased	increased above 5 mmol/l	normal	excess LDL	common	markedly increased risk of atherosclerosis
IIb	increased	increased	increased	excess LDL and VLDL	common	markedly increased risk of atherosclerosis
III	increased	normal or low	increased	excess chylomicron remnants and IDL, broad β-band in ELFO	rare	increased risk of atherosclerosis
IV	normal or increased	normal	increased	excess VLDL	very common	increased risk of atherosclerosis
V	increased	normal	markedly increased	excess chylomicrons and VLDL	rare	pancreatitis

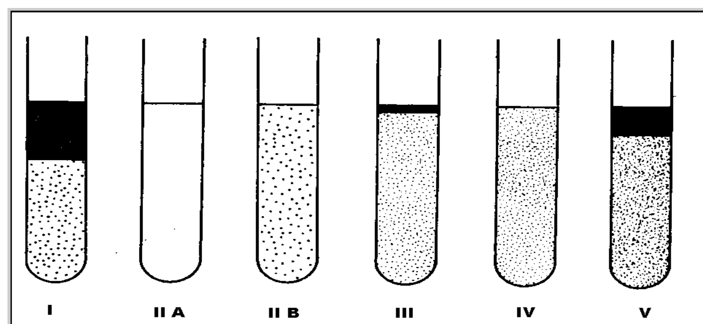


Fig. 1: Appearance of serum sample (chylomicron test) in various hyperlipoproteinemia types. Fresh well-mixed serum or plasma taken after 10-12-h fasting is allowed to stay overnight (16 – 18 h) at 4 °C. The tube is than carefully visually evaluated in a strong light against a black background. Every normal serum should be clear after 12-h fasting.

Tab. 3 Appearance of serum sample (chylomicron test) in hyperlipoproteinemia´s types:

Appearance of serum	**Lipoproteins abnormalities**	**Corresponding phenotype**
Clear	normal serum or increased LDL	II a
Creamy layer, infranatant clear or slightly turbid	chylomicrons float to the upper surface and form a distinct opaque band during incubation,	I
Cream layer and infranatant turbid to opaque	increased chylomicrons and VLDL	V
Turbid to opaque	increased VLDL , the intensity of turbidity depends on the content of TG in VLDL	IV or II b
Thin cream layer, infranatant turbid to opaque	increased chylomicron remnants and IDL	III

2.1.2 Primary and secondary hyperlipoproteinemias

HLP may occur either as primary conditions, or as secondary to other diseases.

- **Primary hyperlipoproteinemias** may be caused directly by genetic abnormalities in the lipoprotein metabolism. Most HLP are supposed to be primary (for example familial hypercholesterolemia, familial hypertriacylglycerolemia).
- **Secondary hyperlipoproteinemias** are abnormalities of lipid metabolism that occur as secondary to another disease. The causes of secondary dyslipidemia include e.g. diabetes mellitus, hypothyroidism, obstructive liver disease, chronic renal failure, alcoholism and obesity.

2.1.3 Classification of hyperlipoproteinemias according to The European Atherosclerosis Society (1992)

It is a simple and practical method for the HLP classification, which categorises the type of lipid abnormalities on the basis of quantitative measurement of serum cholesterol and triacylglycerol alone. Three types of HLP are distinguished – hypercholesterolemia, hypertriacylglycerolemia and, if both parameters are raised, combined hyperlipidemia. The choice of therapeutic agents is based largely on this classification.

1. Hypercholesterolemia – ***increased serum levels of total cholesterol, predominantly LDL cholesterol.***

- Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by a lack or a deficient number of functional LDL receptors. In homozygous FH there is a total absence of functional LDL receptors. Consequently with lack of uptake of LDL via the receptor-dependent pathway LDL cholesterol concentration increases in the circulation. In the heterozygous form, the LDL cholesterol levels are between 7 – 10 mmol/l and in the homozygotes cholesterol levels are in range 15 – 30 mmol/l. A population gene frequency in heterozygous form is estimated at 1 in 500. Homozygous state is much rarer (frequency 1 : 1 million). Both heterozygotes and homozygotes suffer from premature heart diseases, in homozygotes may develop before the age of 10 years. Electrophoresis shows an increase in β-lipoprotein (type II a); sometimes also pre-β-lipoprotein may be elevated (type II b).
- Polygenic hypercholesterolemia is commoner than familial hypercholesterolemia. The interaction of genetic and environment factors contribute to elevated LDL cholesterol levels and may explain this form of hypercholesterolemia. Total cholesterol levels are usually about 8 mmol/l. The risk of atherosclerosis is increased. The majority of patients exhibit a type II a, but a type II b may develop as well.
- Secondary hypercholesterolemia is often found in hypothyroidism and nephrotic syndrome.

II. Combined hyperlipidemia – ***increased serum levels of both cholesterol and triacylglycerols.***

- Familial combined hypertriacylglycerolemia is one of the commonest genetic hyperlipoproteinemic disorders in lipoprotein metabolism. Its prevalence is estimated about 1 % to 2 %. It is caused by overproduction of lipoproteins containing apo B-100. It can be manifested as increased plasma levels of VLDL and LDL corresponding mainly to the phenotype II b, but the phenotype IIa, IIb, IV and V may be also observed. The patients have an increase incidence of coronary artery disease.
- Secondary forms are seen in the hypothyroidism or due to administration of corticosteroids.

III. Hypertriacylglycerolemia – ***increased serum levels of triacylglycerols only***.

- Familial hypertriacylglycerolemia is a common autosomal dominant disorder with prevalence approximately 0.2 – 0.3 % population. It is characterised by elevated VLDL (phenotype IV), probably resulting from their overproduction. Serum triacylglycerol levels are usually less than 6.0 mmol/l, total cholesterol levels are typically normal, but the levels of HDL cholesterol tend to be lower. The risk of myocardial infarction is increased.
- Familial hyperlipoproteinemia type I is a rare disorder characterised by hyperchylomicronemia. Triacylglycerol levels often exceed 20 mmol/l. The patients are in danger of developing acute pancreatitis.
- Secondary form of hypertriacylglycerolmia is often associated with diabetes mellitus, obesity or excessive alcohol consumption.

2.2 Laboratory examination of patients with hyperlipoproteinemia

The routine laboratory examinations of patients with hyperlipoproteinemia include the determinations of total serum cholesterol and triacylglycerol. Furthermore LDL and HDL cholesterol may be assayed. Lipoprotein electrophoresis and estimation of apolipoproteins levels are required in a few patients for a more detailed diagnostics. The methods of molecular genetic methods are also introduced to the diagnostic investigation of lipid metabolism.

2.2.1 Estimation of serum triacylglycerols

Principle:

The recommended methods for the estimation of serum triacylglycerols include several enzymatic reactions: Triacylglycerols are first hydrolyzed by lipoprotein lipase to produce glycerol and free fatty acids. Glycerol is converted to glycerol-3 phosphate by glycerol kinase in the presence of ATP. In the next enzymatic reaction catalysed by glycerol-3-phosphate oxidase glycerol-3 phosphate is oxidised to produce dihydroxyacetone phosphate and H_2O_2. Finally, horseradish peroxidase uses the hydrogen peroxide for oxidation of a chromogen to yield a colour product, measurable spectrophotometrically (Fig. 2).

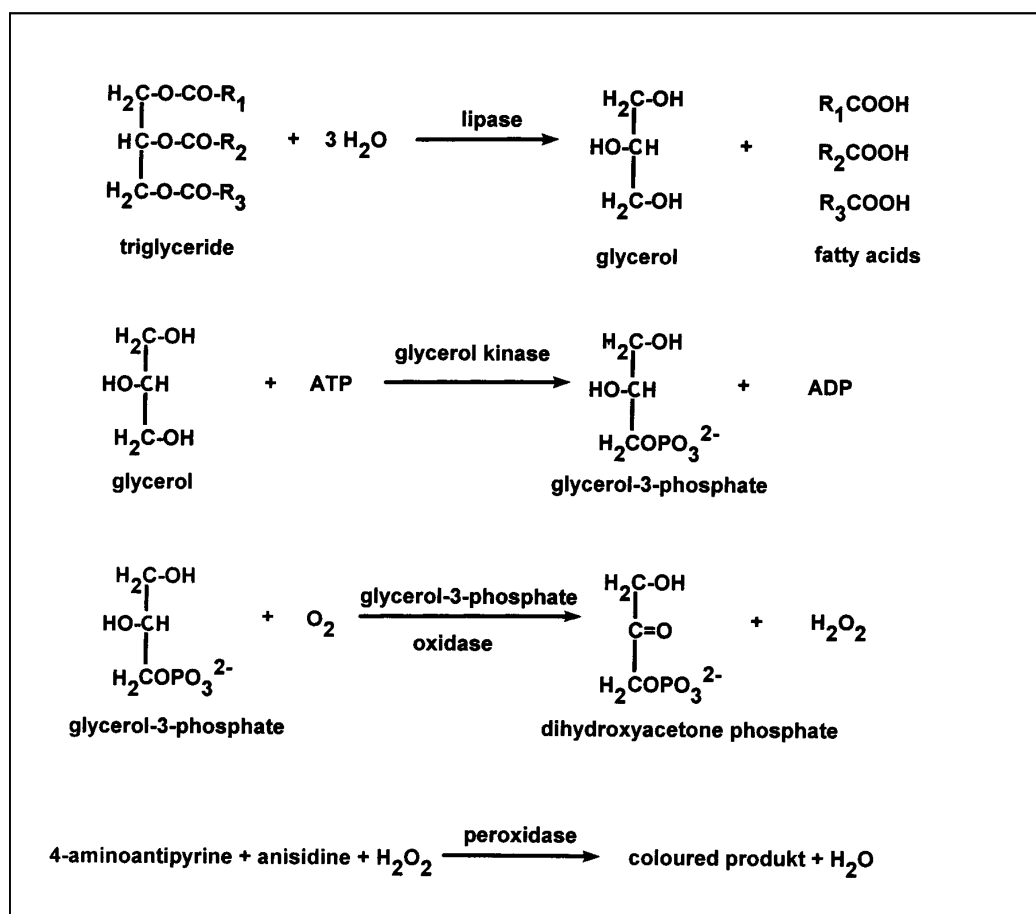


Fig.2: Enzymatic estimation of triacylglycerols

Reference values of serum triacylglycerols: 0.45 – 1.7 mmol/l

Triacylglycerol values depend on the prandial state. Higher levels occur after meal, therefore, blood for triacylglycerol determination should be taken after a 14-hour fasting.

Evaluation of serum triacylglycerol concentration with respect to the risk of atherosclerosis:

Elevated triacylglycerols > 1.7 mmol/l (fasting) are an independent risk factor for atherosclerosis. Increased triacylglycerol concentration tends to be associated with decreased HDL cholesterol.

2.2.2 Estimation of serum total cholesterol

Principle:

The commonest methods for estimation of cholesterol are enzymatic assays. The first step in the enzymatic methods is hydrolysis of cholesterol esters by cholesterol esterase to produce free cholesterol and fatty acids. In the presence of oxygen the released free cholesterol together with the one initially present in the sample is then oxidised by cholesterol oxidase to 4-cholesten-3-one and H_2O_2.

The final step again uses the ability of produced H_2O_2 to oxidise various compounds to coloured products in the presence of peroxidase. The approach most widely used for routine cholesterol testing is based on the reaction of phenol with 4-aminoantipyrine forming a quinonimine dye (red colour). The intensity of a red dye is proportional to the cholesterol concentration in the sample (Fig. 3).

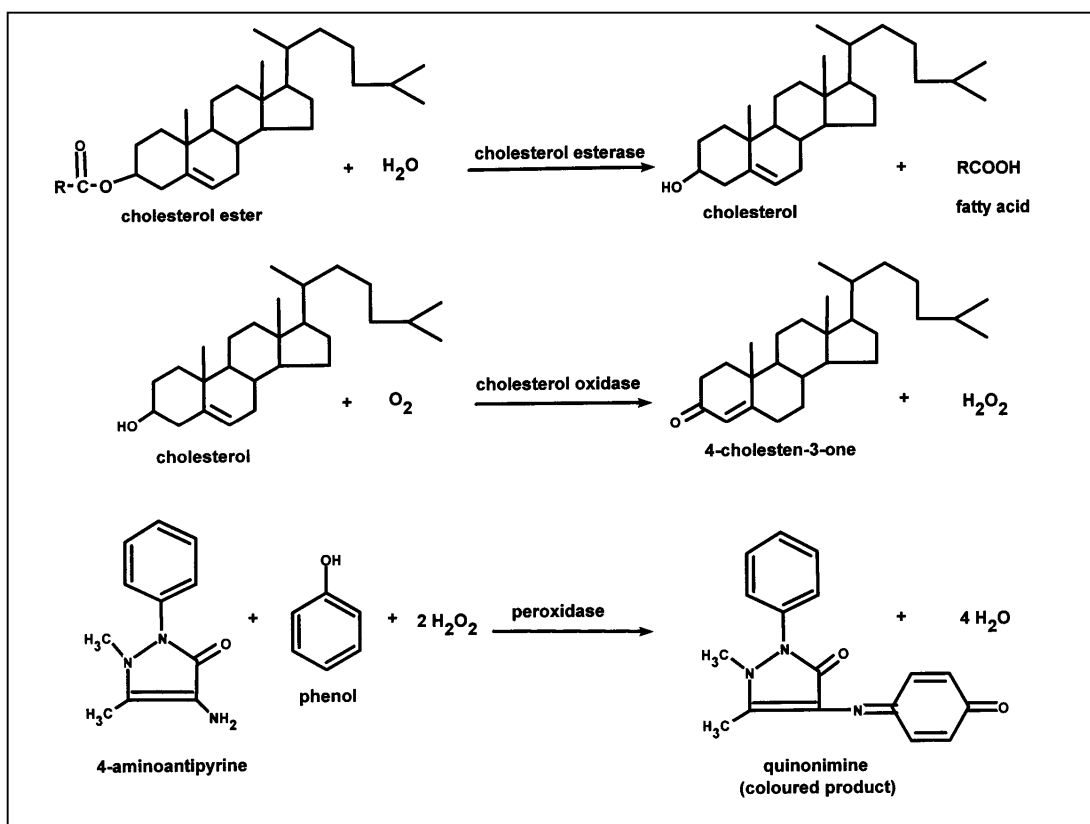


Fig.3: Enzymatic estimation of total cholesterol

Reference values: 2.9 – 5.0 mmol/l

Evaluation of total cholesterol concentration with respect to the risk of atherosclerosis:

Cholesterol values in serum < 5.0 mmol/l indicate the risk of coronary heart disease is low. The determination of total cholesterol is sufficient for screening purposes. If the concentration of total cholesterol is above 5.0 mmol/l, it is necessary to measure LDL and HDL cholesterol.

Fasting is not required if only total cholesterol is measured.

2.2.3 Estimation of HDL cholesterol

Principle:

Methods used for estimation of HDL cholesterol (HDL-C) are based on the measurement of cholesterol following removal of all non-HDL particles by precipitation.

The determination of HDL-C proceeds in two steps:

- Selective precipitation of major lipoproteins (VLDL, LDL, IDL and chylomicrons) is achieved by formation of insoluble complexes of the lipoproteins with appropriately chosen polyanions and metal ions. Dextran sulfate-magnesium chloride or sodium phosphotungstate-magnesium chloride are the most widely used precipitating agents. The precipitated lipoproteins are sedimented by centrifugation.
- The HDL cholesterol is then quantified in the supernatant by one of the enzymatic assays as for total cholesterol.

There are some newer methods that estimate HDL-C directly without precipitation.

Evaluation of HDL cholesterol concentrations with respect to the risk of atherosclerosis:

HDL cholesterol concentration	Evaluation
> 1.0 mmol/l (men)	lower risk
> 1.2 mmol/l (women)	lower risk

Reference values:

HDL cholesterol concentration (men): **1.0 – 2.1 mmol/l**

HDL cholesterol concentration (women): **1.2 – 2.7 mmol/l**

2.2.4 Estimation of LDL cholesterol

Principle:

Several methods for LDL cholesterol (LDL-C) estimation have been developed.

LDL-C can be calculated mathematically from the serum concentration of total cholesterol, triacylglycerols and HDL cholesterol using the Friedewald formula:

$$\text{LDL cholesterol (mmol/l)} = \text{Total cholesterol (mmol/l)} - \text{HDL cholesterol (mmol/l)} - \frac{\text{Triacylglycerols (mmol/l)}}{2.2}$$

This formula is used almost universally in routine clinical practise. Total cholesterol and HDL cholesterol can be measured by the methods described above, and the cholesterol content in VLDL is indirectly estimated on the assumption that the VLDL mass ratio of cholesterol: triacylglycerols is 1 : 5 (molar ratio 1 : 2.2). The Friedewald formula is applicable only for fasting serum without chylomicrons and triacylglycerol concentration less than 4.5 mmol/l.

But the Friedewald calculation for LDL-C only approximates LDL cholesterol, therefore the methods enabling direct measurement of LDL-C, if available, are nowadays preferred. One of the direct assays employs monoclonal antibodies immobilized on latex beads, reacting specifically against epitopes on the apolipoproteins of VLDL, IDL and HDL. Precipitated lipoproteins are then separated by microfiltration and centrifugation; and cholesterol in separated LDL particles is measured by enzymatic methods.

Evaluation of LDL cholesterol concentrations with respect to the risk of atherosclerosis:

- Increased concentration of LDL-cholesterol is significantly involved in ***development of atherosclerosis***. The recommended concentration of LDL-cholesterol is **< 3.0 mmol/l** according to the current Czech and European guidelines.

Reference values:

LDL cholesterol concentration **1.2 – 3.0 mmol/l**

2.2.5 Other calculated parameters of lipid metabolism

Atherogenic index:

The estimated parameters of cholesterol can be utilized for calculation of various atherogenic indexes. The recommended variant of atherogenic index takes into account effects of total cholesterol as well as HDL-C on the risk of cardiovascular disease. HDL-C acts as a "negative risk factor", actually decreasing risk of ischemic heart disease. Thus, simultaneous increase in total and HDL cholesterol need not increase the risk of cardiovascular disease.

$$\textbf{Atherogenic index} = \frac{\textbf{Total cholesterol (mmol/l)}}{\textbf{HDL cholesterol (mmol/l)}}$$

Recommended value of the **atherogenic index: < 5.0**

Non-HDL cholesterol:

- Is used for estimation of risk of atherosclerosis in cases when calculation of LDL cholesterol concentration is not possible. It consists of cholesterol present in all potentially atherogenic lipoprotein particles LDL, IDL, VLDL, and chylomicron remnants.

  Non-HDL cholesterol (mmol/l) = Total cholesterol (mmol/l) – HDL cholesterol (mmol/l)

  Recommended value of non-HDL cholesterol: **< 3.8 mmol/l**

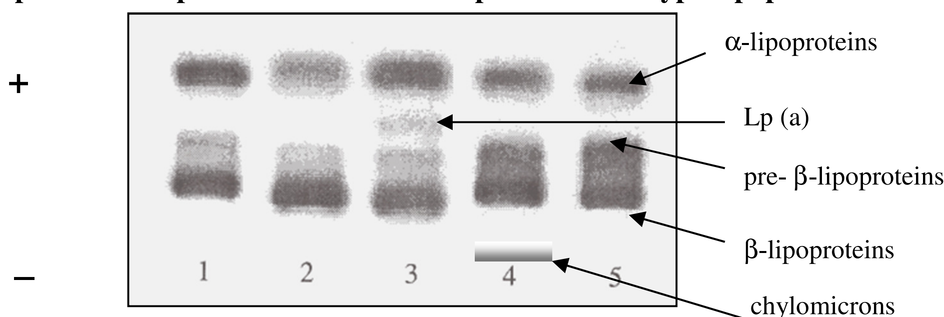
2.2.6 Electrophoresis of lipoproteins

The electrophoretic separation of lipoproteins provides information on an individual lipoprotein profile, including detection of abnormal lipoproteins [LP(a)], and diagnosis of the rare Fredrickson´s type III. The electrophoretic mobility of major classes of lipoproteins is dependent on their charge, size, shape and interaction with the support medium. Agarose is the most widely used support medium for this kind of electrophoresis, but agar, polyacrylamide and cellulose acetate may also serve this purpose.

HDL migrate most rapidly toward the anode and are conventionally associated with α-globulin region, hence called α-lipoproteins. HDL are followed by VLDL as pre- β-lipoproteins. The next prominent band with β-electrophoretic mobility is LDL, referred to as β-lipoproteins. Chylomicrons do not migrate well, because they are too large to enter the gel and usually remain at the origin. The lipoprotein (a) moves between α-lipoproteins and pre-β-lipoproteins ("sinking pre- β-lipoprotein").

The lipoprotein electrophoresis may be evaluated visually or by means of densitometry.

Example of electrophoresis of serum from patients with hyperlipoproteinemias:



1 normal
2 lipoprotein phenotype II a

3 elevated Lp(a)
4 lipoprotein phenotype V
5 lipoprotein phenotype IV