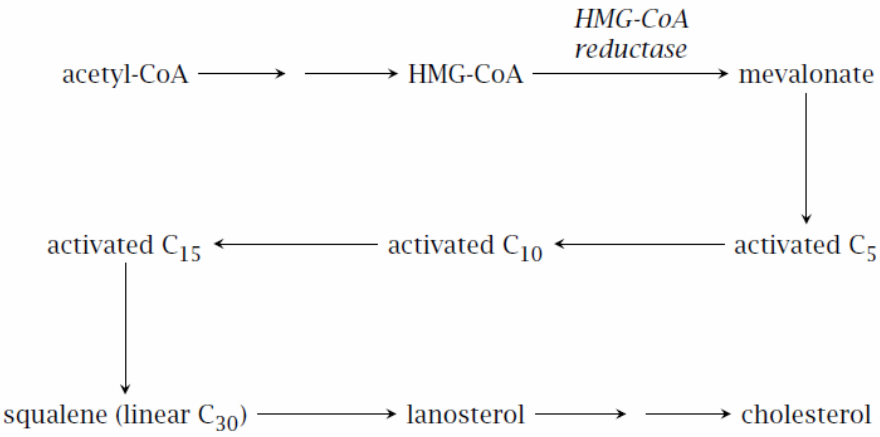
SL Dr. Corina Samoilă corisamoila@umft.ro
As. Dr. Anda Alexa alexa.anda@umft.ro
All questions will be addressed to the corresponding laboratory teaching assistant and guidance will be provided.

Lipid metabolism. Plasma lipoproteins. Determination of total and HDL cholesterol from plasma lipoproteins

Cholesterol is a major constituent of the cell membranes of animal cells. It would be possible for the body to provide its full daily cholesterol requirement (ca. 1 g) by synthesizing it itself. However, with a mixed diet, only about half of the cholesterol is derived from endogenous biosynthesis, which takes place in the intestine and skin, and mainly in the liver (about 50%). The rest is taken up from food. Most of the cholesterol is incorporated into the lipid layer of plasma membranes, or converted into **bile acids**. A very small amount of cholesterol is used for biosynthesis of the **steroid hormones**. In addition, up to 1 g cholesterol per day is released into the bile and thus excreted. Significant amounts of cholesterol only occur in meat, eggs, and milk products.

The pathway of cholesterol synthesis is quite elaborate.



Cholesterol synthesis starts with acetyl-CoA, which is used to synthesize hydroxymethylglutaryl-CoA (HMG-CoA). All steps downstream of HMG-CoA occur in the smooth endoplasmic reticulum. HMG-CoA reductase reduces HMG-CoA to mevalonate; this enzyme is the major target of regulation in the entire pathway. Mevalonate is converted to various isoprene intermediates. Squalene is cyclized to the first sterol intermediate. This molecule, lanosterol, is then converted to cholesterol by several successive modifications. The last biosynthetic precursor of cholesterol, 7-dehydrocholesterol, is also the precursor of vitamin D3 (cholecalciferol).

Regulation of cholesterol synthesis

Biosynthesis of cholesterol is directly regulated by the cholesterol levels present, though the homeostatic mechanisms involved are only partly understood. The amount of cholesterol that is synthesized in the liver is tightly regulated by dietary cholesterol levels. When dietary intake of cholesterol is high, synthesis is decreased and when dietary intake is low, synthesis is increased. The synthesis of endogenous cholesterol is controlled by HMG-CoA reductase.

Lipoproteins

There are several types of lipoproteins in the blood. In order of increasing density, they are: chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Lower protein/lipid ratios make for less dense lipoproteins.

Chylomicrons, the least dense cholesterol transport molecules, contain apolipoprotein B-48, apolipoprotein C, and apolipoprotein E in their shells. Chylomicrons carry fats from the intestine to muscle and other tissues in need of fatty acids for energy or fat production. Unused cholesterol remains in more cholesterol-rich chylomicron remnants, and taken up from here to the bloodstream by the liver.

VLDL molecules are produced by the liver from triacylglycerol and cholesterol which was not used in the synthesis of bile acids. VLDL is converted in the bloodstream to low-density lipoprotein (LDL) and intermediate-density lipoprotein (IDL). VLDL particles have a diameter of 30-80 nm. VLDL transports endogenous products, whereas chylomicrons transport exogenous (dietary) products.

LDL particles are the major blood cholesterol carriers. Each one contains approximately 1,500 molecules of cholesterol ester. LDL molecule shells contain just one molecule of apolipoprotein B100, recognized by LDL receptors in peripheral tissues.

LDL receptors are used up during cholesterol absorption, and its synthesis is regulated by SREBP, the same protein that controls the synthesis of cholesterol *de novo*, according to its presence inside the cell. A cell with abundant cholesterol will have its LDL receptor synthesis blocked, to prevent new cholesterol in LDL molecules from being taken up. Conversely, LDL receptor synthesis proceeds when a cell is deficient in cholesterol.

When this process becomes unregulated, LDL molecules without receptors begin to appear in the blood. These LDL molecules are oxidized and taken up by macrophages, which become engorged and form foam cells. These foam cells often become trapped in the walls of blood vessels and contribute to atherosclerotic plaque formation. Differences in cholesterol homeostasis affect the development of early atherosclerosis (carotid intima-media thickness). These plaques are the main causes of heart attacks, strokes, and other serious medical problems, leading to the association of so-called LDL cholesterol (actually a lipoprotein) with "bad" cholesterol.

HDL particles are thought to transport cholesterol back to the liver, either for excretion or for other tissues that synthesize hormones, in a process known as reverse cholesterol transport (RCT). Large numbers of HDL particles correlates with better health outcomes, whereas low numbers of HDL particles is associated with atheromatous disease progression in the arteries.

Methods for plasma lipoprotein fraction separation

Plasma lipoproteins can be separated using different methods:

1. Physico-chemical methods:

Ultracentrifugation is based on the density difference of plasma lipoproteins according to their lipids and proteins content. Using high gravity fields (approx. 100000g) and according to the medium density, lipoproteins will sediment (if their densities are higher than the medium one) or float (if their densities are lower). Using this method, in a buffer with a density of 1.063 g/cm^3, four fractions can be separated:

- chylomicrons $d \leq 0.96$ g/cm^3
- VLDL (very low density lipoproteins) d = 0.96-1.006 g/cm^3
- LDL (low density lipoproteins) d = 1.006-1.063 g/cm^3
- HDL (high density lipoproteins) d = 1.063-1.21 g/cm^3

Electrophoresis – plasma lipoproteins migrate in an electric field because proteins and phospholipids bear electrical charges. The separation is done at a pH = 8.6 and the fractions are visualized with specific dyes. The separated fractions are:

- chylomicrons - practically do not migrate due to their low content in proteins and phospholipids
- β-liporpoteins – correspond to LDL
- pre- β-liporpoteins – correspond to VLDL
- α-lipoproteins – correspond to HDL

In a normal serum, collected after 8-10 fasting hours, chylomicrons do not appear, and VLDL (pre- β) appears only in traces.

2. Chemical methods

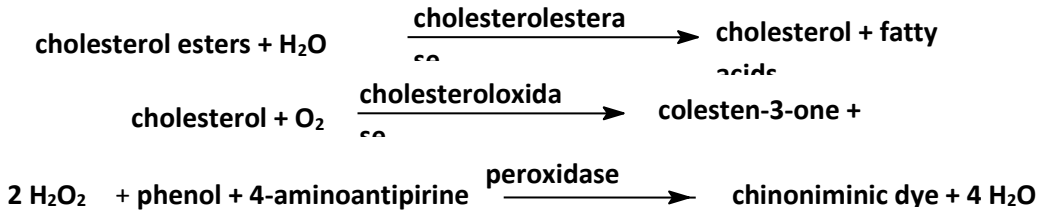
The separation of lipoprotein fractions can be realized using cations (2^+), polyanions or surface active substances. The cholesterol in the separated fractions can be determined using the same method as for total cholesterol.

HDL fraction is separated by precipitation with a phosphotungstic reagent and the HDL + LDL fractions are precipitated with sodium dodecylsulfate.

Determination of total cholesterol

Principle

Cholesterol and its esters are liberated from lipoproteins by the action of tensioactive agents. Cholesterolesterase hydrolyses the esters and cholesteroloxidase oxidizes the substrate resulting hydrogen peroxide.



Procedure

Pipette according to the table:

Reagents, µl	**Sample**	**Standard**	**Blank**
Standard	-	100	-
Serum	100	-	-
Distilled water	-	-	100
Working reagent	1000	1000	1000

Mix vigorously, and then incubate 5 min. at 37^0C. Measure the sample and standard extinctions at 546 nm zeroing the spectrophotometer with the blank (the color is stabile for 60 minutes).

Calculation

mg cholesterol/100 ml = (E_S/E_{St}) x 200

Normal values

140 - 220 mg%

Pathological values

Hypercholesterolemia occurs in familial hypercholesterolemia, diabetes mellitus, nephrotic syndrome, alcoholism, pregnancy.

Low values are found in hepatic cirrhosis, hyperthyroidism.

Determination of HDL cholesterol

Direct enzymatic method

In the first step, chylomicrons, LDL and VLDL fractions are eliminated by a specific reaction. In the second step, the reactions are identical with those of total cholesterol determination.

Procedure

Pipette according to the table:

Reagents, µl	**Sample**	**Standard**	**Blank**
Standard	-	120	-
Serum	120	-	-
Distilled water	-	-	120
R1 reagent	600	600	600
Mix and incubate 5 min at 37^0C, then add:			
R2 reagent	200	200	200

Incubate at 37^0C for 5 minutes.
Read the extinction of sample and standard at 600 nm against the blank. Calculate:

Calculation

mg HDL cholesterol/100 ml = (E_S/E_{St}) x 50.5

Normal values

Men: 35-55 mg HDL cholesterol/100 ml serum
Women: 45-65 mg HDL cholesterol/100 ml serum

Atherogenic risk	**Men**	**Women**
low	> 55 mg/100 ml	> 65 mg/100 ml
high	< 35 mg/100 ml	< 45 mg/100 ml

The atherogenic risk can be also appreciated by the calculation of the ratio C_T/C_{HDL}.
Normal ratio $C_T/ C_{HDL} = 3.5 – 5$

$C_T/ C_{HDL} < 3.5$ low atherogenic risk
$C_T/ C_{HDL} > 5$ high atherogenic risk

Calculation of LDL cholesterol (Friedewald equation):

mg LDL cholesterol/100 mg serum = total cholesterol – (HDL+VLDL) cholesterol

Normal values

<150 mg LDL/100 ml: no medical treatment needed
150-190 mg LDL/100 ml: medium atherosclerosis risk
>190 mg LDL/100 ml: high atherosclerosis risk-treatment needed