# Chapter 5

# Assaying Low-Density-Lipoprotein (LDL) Uptake into Cells

**Anke Loregger, Jessica K. Nelson, and Noam Zelcer**

## Abstract

Determination of LDL particle uptake into cells is a valuable technique in the field of cholesterol metabolism. This allows assessment of LDL uptake capacity in different adherent and non-adherent cells types, as well as the effect of cellular, genetic, or pharmacological perturbations on this process. Here, we detail a general procedure that describes the production of fluorescently-labeled LDL particles and quantitative and non-quantitative assays for determining cellular LDL uptake.

**Key words** Cholesterol metabolism, LDL, LDLR, Endocytosis

## 1 Introduction

Cholesterol is vital for mammalian life [1]. Apart from its important function as an integral component of cellular membranes, cholesterol is essential for diverse cellular processes and signaling pathways. However, cholesterol's physiochemical properties, particularly its inherent insolubility in water, pose a challenge when it comes to transporting this molecule throughout the body. For this reason, cholesterol and other lipids are packaged into lipoprotein particles, which are trafficked systemically. Lipoproteins are particles that contain triacylglycerol, phospholipids, cholesterol, and amphipathic proteins called apolipoproteins (for a detailed review of lipoproteins see Hegele et al. [2]). The four major classes of lipoproteins are chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), which vary in their triacylglycerol, phospholipid, cholesterol, and protein composition. Amongst these, LDL constitutes ~50% of the total lipoprotein mass in the plasma. It contains a single protein, $ApoB_{100}$, and lipids (~25 and ~75% of mass, respectively), the latter consisting of ~6–8% free cholesterol, ~45–50% cholesteryl ester, ~18–24% phospholipid, and ~4–8% triacylglycerols [2]. LDL is a major carrier of cholesterol to peripheral cells, accounting for over 60% of the total cholesterol in plasma.

Ingrid C. Gelissen and Andrew J. Brown (eds.), *Cholesterol Homeostasis: Methods and Protocols*, Methods in Molecular Biology, vol. 1583, DOI 10.1007/978-1-4939-6875-6_5, 

Extensive epidemiological studies have shown that elevated levels of circulating LDL-cholesterol is a well-established risk factor for atherosclerosis, which is the primary cause for coronary artery disease and ensuing cardiovascular complications that account for ~25% of world-wide deaths [3]. As such, the transport and uptake of LDL to hepatic and non-hepatic tissues is extensively coordinated and is an important determinant of LDL levels in the circulation. Endocytosis of LDL depends on the interactions of the sole protein in LDL, $ApoB_{100}$ with a dedicated plasma membrane receptor, the LDL receptor (LDLR) [4]. The LDLR-LDL complex is internalized from the plasma membrane by clathrin-mediated endocytosis where it dissociates as a result of the decreasing pH in the endosomal transport system. Subsequently, the LDL particle is delivered to lysosomes from which cholesterol can be salvaged for cellular use, whereas the receptor is recycled back to the cell surface for a new round of uptake.

Hepatic LDLR activity is a central determinant of circulating levels of LDL. Accordingly, mutations in the *LDLR* are the leading cause of autosomal dominant hypercholesterolemia [5]. In line with its crucial function in cholesterol homeostasis, LDLR abundance is tightly regulated at the transcriptional as well as post-transcriptional level. Transcriptional levels of the *LDLR* are primarily determined by the sterol response element-binding protein (SREBP) transcription factors [6]. On a post-translational level, the SREBP target gene proprotein convertase subtilisin/kexin 9 (PCSK9) and the Liver X receptors (LXR) target gene inducible degrader of the LDLR (IDOL) control LDLR abundance [7–10]. While both PCSK9 and IDOL increase lysosomal degradation of the LDLR, the underlying mechanism they use for doing so is distinct [8]. Acting as an E3 ubiquitin ligase, IDOL promotes ubiquitination of the intracellular tail of LDLR, thereby targeting it for lysosomal degradation. In contrast, PCSK9 is a secreted protein that binds to the extracellular domain of LDLR, and after internalization prevents receptor recycling back to the plasma membrane, thereby increasing its lysosomal degradation. These pathways are also of clinical relevance. Recently, antibodies that target PCSK9 and thereby increase hepatic LDLR levels and LDL clearance have shown strong LDL-lowering potency [11, 12], and are being currently introduced into clinical practice [13]. Strategies to pharmacologically inhibit IDOL activity with small molecules are also being investigated, but are at an earlier phase of development.

To study the activity of the LDLR we describe below a general approach to determine LDL uptake into cells, using fluorescently-labeled LDL. Originally, studies of LDLR function were carried out using radioactive assays based on the measurement of binding,

uptake and degradation of $^{125}$I-labeled LDL. Although radioactive assays have the advantage of being very sensitive, iodination of LDL is expensive, requires specific expertise and equipment, and handling radioactive material is subject to strict regulations. In contrast, performing these assays using fluorescently-labeled LDL, as described below, is an inexpensive and simple alternative that is suitable for both adherent and non-adherent cells and can be used to provide qualitative or quantitative information.

## 2 Materials

Prepare all solutions using ultrapure water ($dH_2O$) and analytical grade reagents and follow all waste disposal regulations when disposing of waste materials.

### 2.1 Isolation of Human LDL

Human LDL can be purchased from different commercial suppliers. Alternatively, the isolation procedure is included in this Chapter and reagents required listed below.

1. EDTA stock solution 0.5 M (pH 8.0): 186.1 g of disodium EDTA ($Na_2EDTA$) in 800 mL $dH_2O$; adjust the pH to 8.0 with NaOH (~50 mL): bring volume to 1 L with $dH_2O$; stir vigorously on a magnetic stirrer (*see* **Note 1**).
2. NaCl/EDTA solution (0.9% (w/v) NaCl/1 mM EDTA): Dissolve 9 g NaCl in 900 mL $dH_2O$, add 2 mL of 0.5 M EDTA stock and bring volume to 1 L with $dH_2O$.
3. Make density solutions with KBr (1.225 and 1.100 g/mL) in NaCl/EDTA solution.
4. EDTA-containing blood collection tubes.
5. Beckman L-70 ultracentrifuge with a SW-40 rotor or equivalent equipment.
6. Suitable ultracentrifuge tubes.

### 2.2 Labeling LDL

1. DyLight 488 NHS-Ester can be purchased. Alternatively, other DyLight Dyes, with absorption spectra ranging from 350 to 770 nm are commercially available and can be substituted. Dissolve reagent in DMF to 10 mg/mL.
2. PBS: To prepare 1 L of 1× PBS, dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$ and 0.24 g $KH_2PO_4$ in 800 mL of $dH_2O$. Adjust the pH to 7.4 with HCl, and add $dH_2O$ to a final volume of 1 L.
3. Dialysis tubing or dialysis slides with a molecular weight cutoff of 14 kDa.

### 2.3 LDL-Cholesterol Uptake Assay

1. Simvastatin stock solution 5 mg/mL: Dilute commercially available simvastatin sodium salt in DMSO. Use as 2000× solution (final concentration: 2.5 μg/mL).
2. 200 mM mevalonic acid: Dissolve 1 g of mevalonolactone in 20 mL of 1 N NaOH. Stir overnight at RT and then transfer to 50 mL conical tube. Add 385 μL of 2 M HEPES (pH 7.4). Adjust the pH to 7.4 by drop-wise addition of concentrated HCl. Incubate at 37 °C overnight maintaining the pH at 7.4. Bring the volume to 38.4 mL with $dH_2O$, then sterilize by filtration. Aliquot and store at −20 °C. Use as 2000× solution (final concentration: 100 μM).
3. Sterol-depletion medium: Culture medium supplemented with 10% (v/v) lipoprotein-deficient FCS (*see* **Note 2**), 2.5 μg/mL simvastatin and 100 μM mevalonic acid.
4. DMEM-BSA (0.5% (w/v)): Dissolve 0.5 g BSA per 100 mL DMEM.
5. PBS-BSA (0.5% (w/v)): Dissolve 0.5 g BSA per 100 mL PBS.

### 2.4 Quantification of LDL-Cholesterol Uptake in Total Cell Lysates

1. RIPA (radioimmunoprecipitation assay) buffer: 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) SDS (sodium dodecyl sulphate), 50 mM Tris–HCl; pH 8.0. Protease inhibitors should be added freshly before use.
2. Fluorescence microplate reader: Typhoon FLA-9500 imager or equivalent.
3. Black 384-well microplates with a flat and transparent bottom or equivalent.
4. BCA protein assay kit.

### 2.5 Quantification of LDL-Cholesterol Uptake Using Fluorescence Assisted Cell Sorting (FACS)

1. FACS buffer: 2 mM EDTA, 0.5% (w/v) BSA (added freshly) in PBS; pH 7.4.
2. Non-enzymatic dissociation reagent to release the cells from the cell surface, for example Trypsin or Trypsin-EDTA.
3. FACS machine: Any FACS equipped with a laser capable of excitation at 488 nm and emission filters at 518 nm (for DyLight 488) can be used.

### 2.6 Imaging of Cellular LDL-Cholesterol Uptake

1. 4% paraformaldehyde: Dilute 16% paraformaldehyde (w/v; Methanol-free; commercially available) with PBS.
2. Mounting medium with DAPI (4′,6-diamidino-2-phenylindole; commercially available) (*see* **Note 3**).
3. Glass coverslips.
4. Fluorescent microscope with compatible filters or a confocal laser-scanning microscope, equipped with a 488-nm laser (for DyLight 488-labeled LDL) and a 405-nm laser (for DAPI).

## 3 Methods

### 3.1 Isolation of Human LDL

Serum lipoproteins comprise a heterogeneous population of lipid–protein complexes that can be grouped into different classes: very low (VLDL), low (LDL), and high (HDL) density, each having a different protein and lipid composition as also reflected in their density. The density of VLDL is <1.006 g/mL, of LDL 1.019–1.063 g/mL, and that of HDL is 1.063–1.21 g/mL. This difference in density can be used to specifically isolate any given lipoprotein. To isolate human LDL (d 1.019–1.063 g/mL) from plasma, a one-step ultracentrifugation procedure [14] is described here. *Important*: As human plasma is potentially a source of blood-borne disease, wear gloves and eye protection during the whole procedure.

1. Draw blood from a normolipidemic adult volunteer, and collect blood into EDTA-containing tubes.
2. Separate plasma from whole blood by centrifugation at 1000 × *g* for 20 min at 4 °C and use freshly. Alternatively, purchase human plasma.
3. Add 2.695 g KBr to 7 mL of plasma (final density of 1.25 g/L).
4. Transfer 3.5 mL density-adjusted plasma to a centrifuge tube. Gently overlay this with KBr solutions (Subheading 2.1, **item 3**) as follows: Add first 2 mL of 1.225 g/mL solution, followed by 4 mL of 1.100 g/mL solution, and then 3 mL of NaCl/EDTA solution (Subheading 2.1, **item 2**).
5. Centrifuge the gradient using a swing-out bucket rotor (SW-40) at 105,000 × *g* for 20 h at 10 °C (with brakes set to "off") in a Beckman L-70 ultracentrifuge (or equivalent).
6. After centrifugation, collect the LDL fraction by slicing the tube using a tube slicer (*see* **Note 4**). To do so, firmly position the tube between two rubber rings and with care make a cut between the LDL fraction (above) and the HDL fraction (below) (Fig. 1). Subsequently, from the cut tube collect the LDL band with a pipette.
7. To confirm whether one-step ultracentrifugation provides LDL of acceptable purity, one can subject the isolated LDL fraction to high-performance gel-permeation chromatography (HPGC). To do so, run 60 μL of isolated LDL on a Superose 6 HR 10/30 column [15].
8. Determine the LDL concentration by measuring ApoB using a commercially available nephelometric assay on an auto-analyzer system. ApoB is the only protein constituent of LDL and its levels can be directly used to standardize the uptake assay

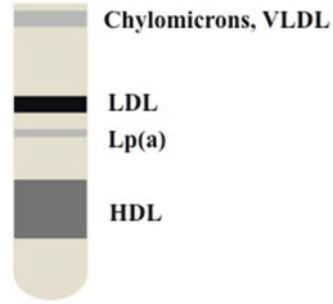


**Fig. 1** Schematic diagram of lipoprotein-subclass separation after gradient density centrifugation

according to the equivalent number of LDL particles. Alternatively, use a BCA assay to determine the protein content of the isolated LDL and use this to standardize the uptake assay.

9. LDL can be stored at 4 °C for up to 1 month. However, it is preferable to directly continue with the LDL labeling procedure.

### 3.2 Fluorescent Labeling of Isolated LDL

DyLight 488-labeled LDL can be produced as previously described [16].

1. Mix 1–10 mg of purified LDL with DyLight 488 NHS-Ester in a volume of 100 μL–1 mL. The amount of fluorescent-labeling reagent to use for each reaction depends on the amount of protein in the LDL fraction. Follow the manufacturer's instructions and calculate the appropriate amount of fluorophore needed. For example, we label ~1 mg of LDL (as determined by ApoB mass) with ~20 μg of the DyLight 488 NHS-Ester in a total volume of 1 mL.
2. Mix well by inverting the tube and incubate the reaction at room temperature for 1 h in the dark. Mix regularly. Do not vortex the tube as this can lead to LDL aggregation.
3. Subsequently, to remove excess non-reacted dye, dialyze the reaction mixture 4 h for at least four times against 1 L PBS.
4. Store DyLight488-labeled LDL in a light-protective tube in the dark at 4 °C for up to 1 month. Discard when formation of precipitates is observed.

### 3.3 Cellular LDL-Cholesterol Uptake Assay

We describe a general strategy for conducting the LDL uptake assay. Depending on the specific experiment, the cell type, number of cells, well size, and other experimental parameters may be adjusted. After performing the assay, several readouts to evaluate LDL uptake are available.

*Day 1*

1. Seed cells in a 12-well plate and allow them to reach 70% confluency (e.g. for human hepatic HepG2 cells, seed 250,000 cells per well).

*Day 2*

1. Wash cells twice with pre-warmed PBS.
2. When grown in standard growth medium containing 10% (v/v) serum, most cells have low LDLR levels and low basal LDL uptake activity. Therefore, to increase LDLR abundance, cells should be incubated in sterol-depletion medium for 16–24 h (*see* **Note 5**).

*Day 3*

1. Wash cells twice with pre-warmed PBS.
2. Incubate cells with 5 μg/mL DyLight488-labeled LDL in DMEM-BSA for 1 h at 37 °C. As controls, include cells that are treated as above, but with addition of excess (100 μg/mL) unlabeled LDL, and cells incubated with DMEM-BSA only. These controls can be later used to determine the specific LDL uptake into cells.
3. To terminate the assay, wash cells twice with ice-cold PBS-BSA followed by one wash with ice-cold PBS (*see* **Note 6**).

At this point, cellular LDL uptake can be assessed as described under Subheadings 3.4–3.6, either by determining (readout 1) fluorescence in total cell lysates, (readout 2) cellular fluorescence by FACS, (readout 3) cellular fluorescence by imaging. Note that the first two readouts are quantitative, whereas the latter is qualitative in nature.

### 3.4 Quantification of LDL-Cholesterol Uptake in Total Cell Lysates

Perform the LDL-uptake assay as described in Subheading 3.3.

1. To cells from **step 6**, add ice-cold RIPA buffer (1 mL per $5 \times 10^6$ cells) supplemented with protease inhibitors (*see* **Note 7**).
2. Gently rock plate for 30 min at 4 °C in the dark to facilitate cell lysis.
3. Collect total cell lysates by quantitatively transferring the lysate to a pre-chilled microcentrifuge tube.
4. Centrifuge the tube at 4 °C for 10 min at 14,000 × *g* to remove cell debris.
5. Transfer the supernatant to a fresh pre-chilled tube on ice and discard the original tube.
6. The fluorescent signal in the total cell lysate can now be measured. Transfer 30 μL of each lysate to a well of a black 384-well microplate with a flat and transparent bottom (*see* **Note 8**).

7. To measure fluorescence on the Typhoon imager, set excitation to 473 nm and filter LPB. Determine the scan area, sensitivity, and resolution and scan plate. Once scan is completed, save the associated image for further analysis.
8. Using image analysis software (e.g. Image J) to quantify the fluorescent signal in each well. Keep the size and position of the measured area consistent between wells. Preferably, use a grid-based measurement procedure. For each measured value, subtract the background (i.e. fluorescence measured from cells that have been incubated with an excess of non-labeled LDL).
9. For each cell lysate, determine protein concentration using a BCA kit following the manufacturer's instructions. Normalize the corrected fluorescence signal to protein level, and plot averages (*see* **Note 9**).

### 3.5 Quantification of Cellular LDL-Cholesterol Uptake by Fluorescence Activated Cell Sorting (FACS)

Perform the LDL-uptake assay as described in Subheading 3.3.

1. To cells from **step 6**, add a non-enzymatic dissociation reagent to release the cells from the cell surface. Note that dissociation of some cell types may require the use of Trypsin or Trypsin-EDTA (*see* **Note 10**). Cover the cell monolayer and incubate at 37 °C until cells detach from the surface.
2. Add 1 mL FACS-buffer to dissociated cells. If using trypsin, add medium containing serum to the cell suspension to inhibit further trypsinisation, which may damage cells. Alternatively, when culturing in serum-free conditions, soybean trypsin inhibitor can be used to stop trypsinisation.
3. Transfer cell suspension to a microcentrifuge tube. From this point on, keep cells and all solutions cold.
4. Centrifuge cells at 500 × *g* for 5 min at 4 °C.
5. Wash cells once with cold FACS buffer.
6. Resuspend cells in FACS buffer to a density of 1–3 × $10^6$ cells per mL (typically 200–400 μL of buffer is required).
7. Transfer cells to a FACS compatible tube and measure the fluorescent signal in the cells by FACS (Fig. 2).

### 3.6 Imaging of Cellular LDL-Uptake

If a qualitative assessment of LDL uptake in cells is sufficient, imaging can be used instead of the quantitative assessments described under Subheadings 3.4 and 3.5 (*see* **Note 11**).

1. Grow cells on glass coverslips to a confluence of approximately 70%.
2. Perform the LDL-uptake assay as described above in Subheading 3.3.

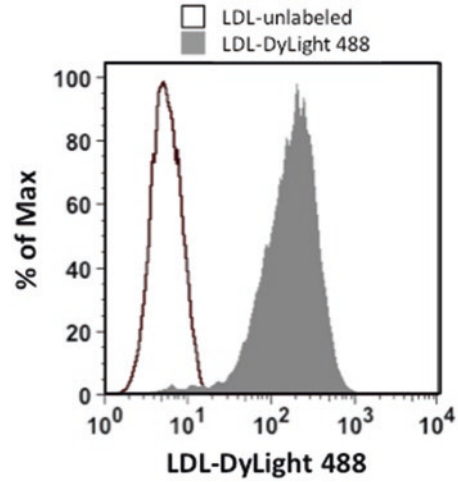


**Fig. 2** FACS analysis of LDL uptake in A431 cells. Cells were cultured in sterol-depletion medium for 16 h to induce expression of the LDLR. Subsequently, cells were incubated with 5 μg/mL LDL-DyLight 488 (*grey*) or unlabeled LDL (*white*) for 1 h at 37 °C, after which cells were washed and prepared for FACS analysis as described in the text. Note the large increase in cellular LDL content following uptake of DyLight488-labeled LDL

3. Following the wash steps, fix the cells with 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature in the dark.
4. Wash the fixed cells three times with PBS.
5. For long-term storage and visualization of the nucleus, mount the cells on microscope slides using mounting medium containing DAPI (fluorescence excitation/emission maxima: 358/461 nm).
6. Examine the uptake of DyLight 488-labeled LDL into cells using a fluorescent microscope with compatible filters. Alternatively, a confocal laser scanning microscope equipped with a 488-nm laser (for DyLight 488-labeled LDL) and a 405-nm laser (for DAPI) can be used (Fig. 3).
7. Store microscope slides at 4 °C in the dark.

## 4 Notes

1. The di-sodium salt of EDTA will not dissolve until the pH of the solution is adjusted to 8.0 by addition of NaOH.
2. Lipoprotein-deficient FCS is commercially available or can be prepared following the procedure described by Redgrave et al. [17].
3. DAPI fluoresces when bound to DNA and is used as a nuclear counterstain.

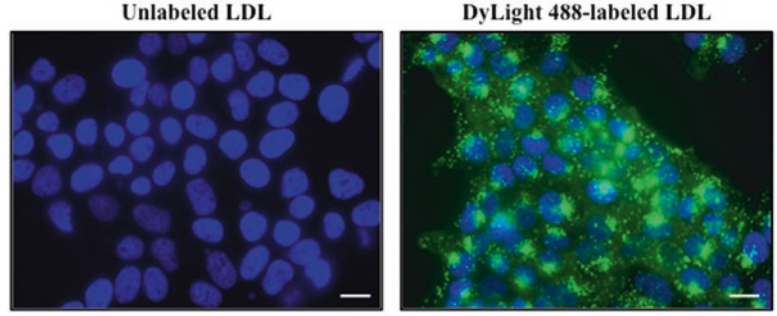


**Fig. 3** Imaging of fluorescent LDL uptake in A431 cells. Cells were grown on glass coverslips and cultured for 16 h in sterol-depletion medium to induce expression of the LDLR. Subsequently, cells were incubated with 5 μg/mL LDL-DyLight 488 or unlabeled LDL for 1 h at 37 °C, washed, fixed and prepared for fluorescent microscopy. Size bar is 10 μm

4. If a tube slicer is not available, the LDL band can be drawn out with a syringe and fine needle. Ensure that the ultracentrifugation tube has been pierced with a small needle at the top for air to be drawn in when the LDL band is removed.
5. Dependent on the cell type, cells can be cultured in sterol-depletion medium for 12 to 30 h. This should be determined for each cell type.
6. To assess LDL binding to cells (i.e. a proxy for LDL-LDLR association at the cell surface), LDL uptake assays can be conducted at 4 °C, as this will prevent subsequent endocytosis of LDL. Keep buffers cold as well.
7. Other lysis buffers are compatible with this protocol. The advantage of using RIPA is that this buffer allows protein extraction of cytoplasmic, membrane and nuclear proteins and is compatible with many downstream applications, including immunoblotting.
8. Any instrument compatible with measuring DyLight 488 (excitation: 493 nm; emission: 518 nm) can be used. We use a Typhoon FLA-9500 imager (GE Healthcare), a laser scanner with multiple imaging applications including sensitive and quantitative measurements of fluorescence. Subheading 3.4, **steps 7** and **8** can be adjusted when using other instruments.
9. Culturing cells in sterol-depletion medium should increase LDL uptake several fold. Treatment of cells with recombinant PCSK9 or with an IDOL-inducing LXR agonist (e.g. GW3695) will result in decreased LDLR abundance and LDL uptake. If required, these treatments can be included as controls in LDL uptake experiments.
10. Trypsin solutions can range from 0.025 to 0.5% (w/v). Different cell lines require varying trypsin concentrations and

incubation times for detachment. These parameters should be determined for each cell line. If trypsin must be used to dissociate the cells, limit the treatment to the minimum required time.

11. In parallel, immunofluorescence staining can be performed in order to immune-localize additional proteins of interest (e.g., LDLR).

## Acknowledgment

We thank Dr. Geesje M. Dallinga-Thie (AMC, The Netherlands) for her advice and comments. AL is supported by a Dekker grant from the Dutch Heart Foundation (2016T015). NZ is an Established Investigator of the Dutch Heart Foundation (2013T111) and is supported by an ERC Consolidator grant (617376) from the European Research Council.

### References

1. Maxfield FR, van Meer G (2010) Cholesterol, the central lipid of mammalian cells. Curr Opin Cell Biol 22:422–429
2. Hegele RA (2009) Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 10:109–121
3. Mozaffarian D et al (2015) Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation 131:e29–322
4. Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232:34–47
5. Hobbs HH, Russell DW, Brown MS, Goldstein JL (1990) The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet 24:133–170
6. Goldstein JL, DeBose-Boyd RA, Brown MS (2006) Protein sensors for membrane sterols. Cell 124:35–46
7. Zelcer N, Hong C, Boyadjian R, Tontonoz P (2009) LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science 325:100–104
8. Sorrentino V, Zelcer N (2012) Post-transcriptional regulation of lipoprotein receptors by the E3-ubiquitin ligase inducible degrader of the low-density lipoprotein receptor. Curr Opin Lipidol 23: 213–219
9. Abifadel M et al (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 34:154–156
10. Cohen J et al (2005) Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet 37:161–165
11. Blom DJ et al (2014) A 52-week placebo-controlled trial of evolocumab in hyperlipidemia. N Engl J Med 370:1809–1819
12. Stein EA et al (2012) Effect of a monoclonal antibody to PCSK9 on LDL cholesterol. N Engl J Med 366:1108–1118
13. Stein EA, Raal F (2014) Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of PCSK9. Annu Rev Med 65:417–431
14. Kleinveld HA, Duif PF, Pekelharing HL, van Rijn HJ (1996) Oxidation of lipoprotein(a) and low density lipoprotein containing density gradient ultracentrifugation fractions. Biochim Biophys Acta 1303:15–21
15. Levels JHM, Lemaire LCJM, van den Ende AE, van Deventer SJH, van Lanschot JJB (2003) Lipid composition and lipopolysaccharide binding capacity of lipoproteins in plasma and lymph of patients with systemic inflammatory response syndrome and multiple organ failure. Crit Care Med 31:1647–1653
16. Motazacker MM et al (2012) Advances in genetics show the need for extending screening strategies for autosomal dominant hypercholesterolaemia. Eur Heart J 33:1360–1366
17. Redgrave TG, Roberts DC, West CE (1975) Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem 65:42–49