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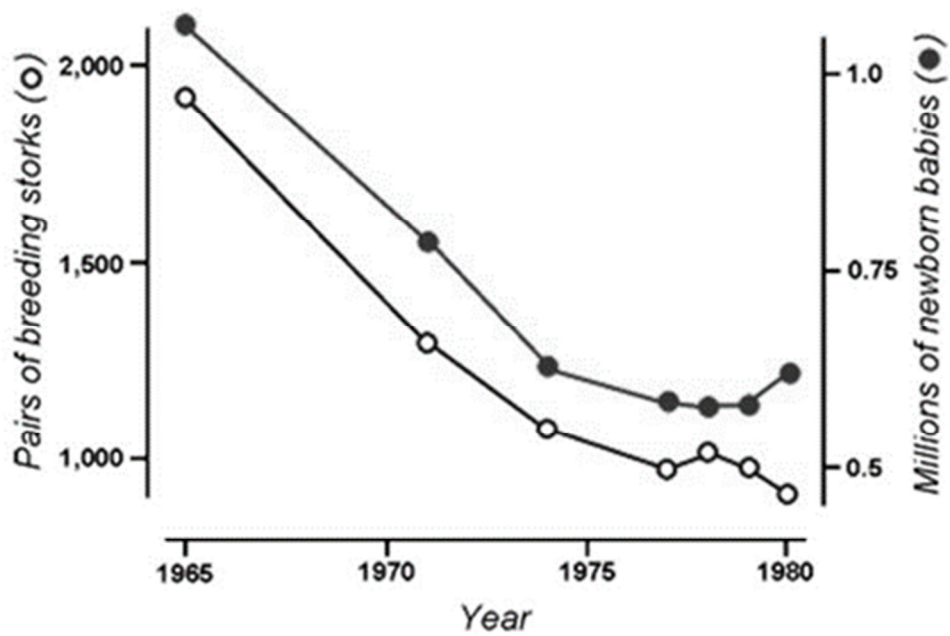
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“Correlation does not imply causation”

- Sies, H. *Nature* (1988) 332:495



1. *Fachserie Gebiet und Bevölkerung* (Statistisches Bundesamt, Kohlhammer, Stuttgart, 1984).
2. Bauer, S. & Thielcke, G. *Die Vogelwarte* **31**, 183-191 (1982).

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Abstract

High density lipoprotein (HDL) has been the focus of intense research since a correlation between low blood levels of HDL-cholesterol and decreased incidence of cardiovascular disease was found. HDL has been shown to be the major player in reverse cholesterol transport, which is transport of cholesterol from peripheral tissues to the liver where it is then secreted in the bile. HDL has also the ability to inhibit the inflammatory response of immune cells by inhibiting cytokine expression and expression of surface receptors. This has attracted the interest of immunologists and the functional assessment of HDL became the prime goal for HDL research.

To assess the impact of composition and purity of HDL samples on immunological experiments we analysed HDL samples from four different laboratories in Austria using liquid chromatography. In three samples we found LDL impurities, and in all four of them high levels of albumin impurities. HDL content was not higher than 67% in any of those samples. In order to optimise HDL sample quality we used a strategic approach optimising and comparing two isolation techniques (gradient ultracentrifugation and sequential ultracentrifugation) in combination with two desalting techniques (dialysis and polydextran desalting columns).

We developed a protocol using gradient ultracentrifugation and polydextran desalting columns that led to the isolation of HDL samples of 98% purity. Consequently, we demonstrated the importance of HDL sample purity for immunological experiments by showing concentration-dependent inhibition of interleukin expression in monocytes and reduced anti-inflammatory properties of samples of less than 93% and less than 83% purity.

This thesis should highlight the importance of HDL purity, be a guide on how to optimise HDL isolation procedures and be a reminder that size exclusion chromatography is a valuable tool to assess HDL sample quality and that it should be applied routinely.

Zusammenfassung

Seit der Erkenntnis, dass niedrige Blutwerte an HDL-C (high density lipoprotein-cholesterol) ein erhöhtes Risiko für kardiovaskuläre Erkrankungen darstellen, ist HDL in den Fokus der Forschung gerückt. HDL spielt nicht nur eine zentrale Rolle im RCT (reverse cholesterol transport), also dem Transport von Cholesterol von peripherem Gewebe zur Leber und in weiterer Folge zur Ausscheidung über die Galle, sondern beeinflusst auch das Immunsystem. Es wurde gezeigt, dass HDL anti-inflammatorische Eigenschaften hat und zum Beispiel die Expression von Interleukinen und Oberflächenrezeptoren in verschiedenen Immunzellen inhibieren kann. Das zentrale Ziel der HDL-Forschung ist nun die Aufklärung verschiedener funktioneller Mechanismen um ein besseres Verständnis der immunologischen Eigenschaften von HDL zu erlangen.

Um den Einfluss von Zusammensetzung und Reinheit isolierter HDL-Proben auf immunologische Experimente zu untersuchen analysierten wir HDL-Proben aus vier Laboratorien in Österreich mittels Ausschlusschromatographie. Wir fanden dabei Anteile an LDL in drei und hohe Anteile an Albumin in allen vier Proben. Keine der uns bereitgestellten Proben hatte einen höheren Anteil als 67% HDL. Um die Qualität der HDL Proben zu verbessern, führten wir eine schrittweise Optimierung der Isolationsprotokolle durch. Desweiteren verglichen wir zwei parallel durchgeführte Isolationsmethoden (Gradientenultrazentrifugation und sequentielle Ultrazentrifugation) kombiniert mit zwei ebenfalls parallel durchgeführten Entsalzungsmethoden (Dialyse und Entsalzung mittels Polydextransäulen).

Wir entwickelten ein Protokoll zur Isolation von HDL mittels Gradientenultrazentrifugation und Entsalzung mit Polydextransäulen und erreichten damit einen HDL-Anteil von 98% mit nur etwa 2% Albumin. Mit diesen Proben zeigten wir die konzentrationsabhängige Inhibierung der Interleukinexpression von Monozyten und demonstrierten die Einschränkung der anti-inflammatorischen Wirkung von HDL-Proben mit weniger als 93% und weniger als 83% Reinheit.

Diese Arbeit soll unterstreichen wie wichtig die Reinheit von HDL-Proben für funktionelle Analysen ist. Auch soll sie als Anleitung zur Optimierung von HDL Isolationsmethoden dienen. Eine wichtige Erkenntnis ist auch, dass Ausschlusschromatographie als wichtiges Instrument zur Qualitätsüberprüfung von HDL-Proben herangezogen werden kann und auch routinemäßig eingesetzt werden sollte.

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List of Abbreviations

ABCA1 – Adenosine triphosphate - binding cassette transporter A-1
ABCG1 – Adenosine triphosphate - binding cassette transporter G-1
Apo – Apolipoprotein
APR-HDL – Acute phase response - high density lipoprotein
BCA – Bicinchoninic acid
BMI – Body mass index
BSA – Bull serum albumin
CE – Cholesterol ester
CETP – Cholesterol ester transfer protein
CRP – C-reactive protein
EDTA – Ethylenediamine tetraacetate
EL – Endothelial lipase
FACS – Fluorescence activated cell sorter
FC – Free cholesterol
FCS – Foetal calf serum
FPLC – Fast protein liquid chromatography
GFP – Green fluorescent protein
glyHDL – Glycated high density lipoprotein
HDL – High density lipoprotein
HDL-C – High density lipoprotein - cholesterol
HL – Hepatic lipase
HSA – Human serum albumin
ICAM-1 – Intercellular adhesion molecule 1
IDL – Intermediate density Lipoprotein
IL – Interleukin
IFN- γ – Interferon γ
LC – Liquid chromatography
LCAT - Lecithin cholesterol acyltransferase
LDL – Low density lipoprotein
LDL-C – Low density lipoprotein cholesterol
LBP – Lipopolysaccharide binding protein
LPL – Lipoprotein lipase
LPS – Lipopolysaccharide
MEM, NEAA – Minimum essential medium, non-essential amino acids
MWCO – molecular weight cutoff

MyD 88 – myeloid differentiation primary response 88
NF κ B – Nuclear factor κ B
oxHDL – oxidized high density lipoprotein
PBS – phosphate buffered saline
PD - Polydextran
PL – Phospholipids
PLPC - 1-palmitoyl-2-linoleyl-phosphatidylcholine
PPAR- γ – Peroxisome proliferator – activated receptor γ
PVDF – Polyvinylidene difluoride
RCT – Reverse cholesterol transport
Rpm – Rounds per minute
SAA – Serum amyloid A
SDS-PAGE – Sodium dodecylsulfate polyacrylamide gel electrophoresis
SR-BI – Scavenger receptor B-I
TBS – tris® buffered saline
TG – Triglycerides
TLR – Toll like receptor
TRAM – TRIF-related adaptor molecule
TRIF – Toll/Interleukin receptor - domain containing adapter-inducing interferon- β
UV₂₄₀ – Ultraviolet light, wavelength 240 nm
VCAM-1 – Vascular cell adhesion molecule 1
vLDL – Very low density lipoprotein
WB – Western blot

1 Introduction

1.1 High density lipoprotein – a brief history

High density lipoprotein (HDL) was first isolated from horse serum in 1929 [1] by Macheboeuf and from human serum in 1949 [2] by Gofman et al using ultracentrifugation. In the following decades HDL was found to be responsible for reverse cholesterol transport (RCT), the transport of cholesterol from peripheral tissues to the liver [3], and correlated with decreased incidence of cardiovascular disease [4]. Hence, HDL-cholesterol (HDL-C) was termed “good cholesterol” whereas low density lipoprotein-cholesterol (LDL-C) was termed “bad cholesterol”. While statins are widely used to lower LDL-C levels by inhibiting cholesterol synthesis in the liver, an emphasis was put on the importance of increasing HDL-C in the early years after 2000 [5]. However, by 2010 a number of compounds tested in clinical trials such as niacin [6], torcetrapib [7] and dalcetrapib [8] successfully elevated HDL-cholesterol but failed to protect against cardiovascular disease. Recent research has highlighted the importance of functionality of HDL rather than pure HDL-C quantity [9, 10]. For example a 30-year follow up cohort study has shown that HDL subfractions HDL 2 and HDL 3 are independently related to coronary heart disease [11]. Proteomic analysis of HDL particles has led to the discovery of more than 70 HDL-associated proteins [12], leading to the discovery of HDL functions not directly related to cholesterol transport. More research and novel approaches to test HDL function will be required to fully understand the role and nature of HDL and its subfractions.

1.2 HDL – Lifecycle in context of cholesterol transport

HDL does not describe one distinct particle but comprises a group of particles with varying size, shape, composition and function. Dynamic interactions of HDL particles with LDL, tissue cells and with each other, give rise to several subpopulation. The complexity of those subpopulations is not well understood and different characterisation techniques (e.g. electrophoresis, chromatography, ion-mobility assays and immunoaffinity) reveal different subpopulation sets [13]. However, it is possible to describe a simplified lifecycle of HDL based on HDLs most studied and best understood function: cholesterol transport.

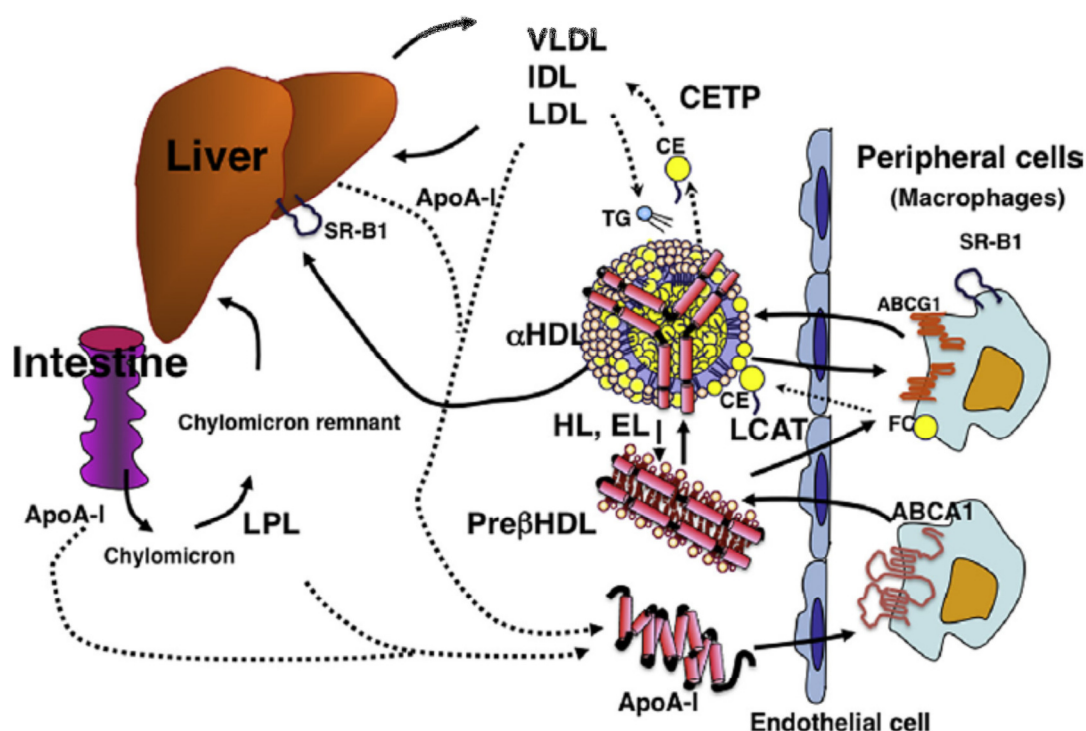


Figure 1 Lifecycle of HDL, replicated from [14]

The most abundant protein found on all HDL particles is apo A-1, it is synthesised and secreted by the liver and the small intestine [15]. Apo A-1 is a surfactant-like molecule that forms α -helices and arranges in an antiparallel dimer belt shape [16]. Given its surfactant properties it can accumulate apolar triglycerides and cholesterol esters as well as other surfactant molecules such as phospholipids or other proteins. Well described is the interaction of apo A-1 with ATP-binding cassette transporter A1 (ABCA1) [17], a transmembrane protein expressed ubiquitously throughout the body. Interaction with ABCA1 allows apo A-1 to take up phospholipids (PL) and free cholesterol (FC) from the cellular membrane forming small (6 – 7 nm) disc shape structures named pre- β HDL [18]. The name pre- β is a derivative of its migration property in isoelectric focusing, the first dimension of a two dimensional non-denaturing electrophoresis. Pre- β HDL then takes up more lipids, PL and FC to form spherical α -HDL particles. Pre- β HDL is also thought to take up lipids from LDL particles mediated by hepatic lipase (HL) and lipoprotein lipase (LPL) [19]. Spherical small α -HDL particles, sometimes referred to as HDL-3 (density ultracentrifugation) or LpA-1 (based on its apo A-1 content) bind lecithin-cholesterol acyltransferase (LCAT), which esterifies cholesterol with fatty acids from phospholipids, the cholesterol esters (CE) move into the hydrophobic core of the HDL-particle, contributing to HDLs spherical shape and micelle-like structure [20]. Through interaction with cell membranes via ATP-binding cassette transporter G1 (ABCG1) [21], scavenger receptor B1 (SR-B1) [22] and by passive diffusion HDL particles take up more free cholesterol. LCAT continues to esterify FC to CE, CE moves

into the core and in turn allowing HDL to take up more FC in its phospholipid layer. This is considered the main mechanism for cholesterol efflux from cells [23]. As HDL takes up FC and TG the particle grows in size (large α -HDL particles also referred to as HDL-2). Figure 2 illustrates different nomenclature of HDL subpopulations depending on the characterisation technique. Large HDL-2 particles have high CE content and associate with cholesterol ester transfer protein CETP [24]. CETP is transferring CE from HDL to LDL in exchange for TG transferred from LDL to HDL. The liver is the main organ for lipid metabolism and has the highest transcription of CETP of all tissues (interestingly, followed by lymph nodes, which is an indication for the importance of cholesterol trafficking for immune function) [25]. In the liver LDL then transports cholesterol to the bile via scavenger receptor BI (SR-BI) where it is excreted in bile acid [26]. The remaining TG loaded HDL particle is now more susceptible to various lipases such as hepatic lipase (HL), lipoprotein lipase (LPL) and endothelial lipase (EL), which break down the HDL particle and recirculate apo A-1 in form of pre- β HDL [13], thus completing the Lifecycle of HDL.

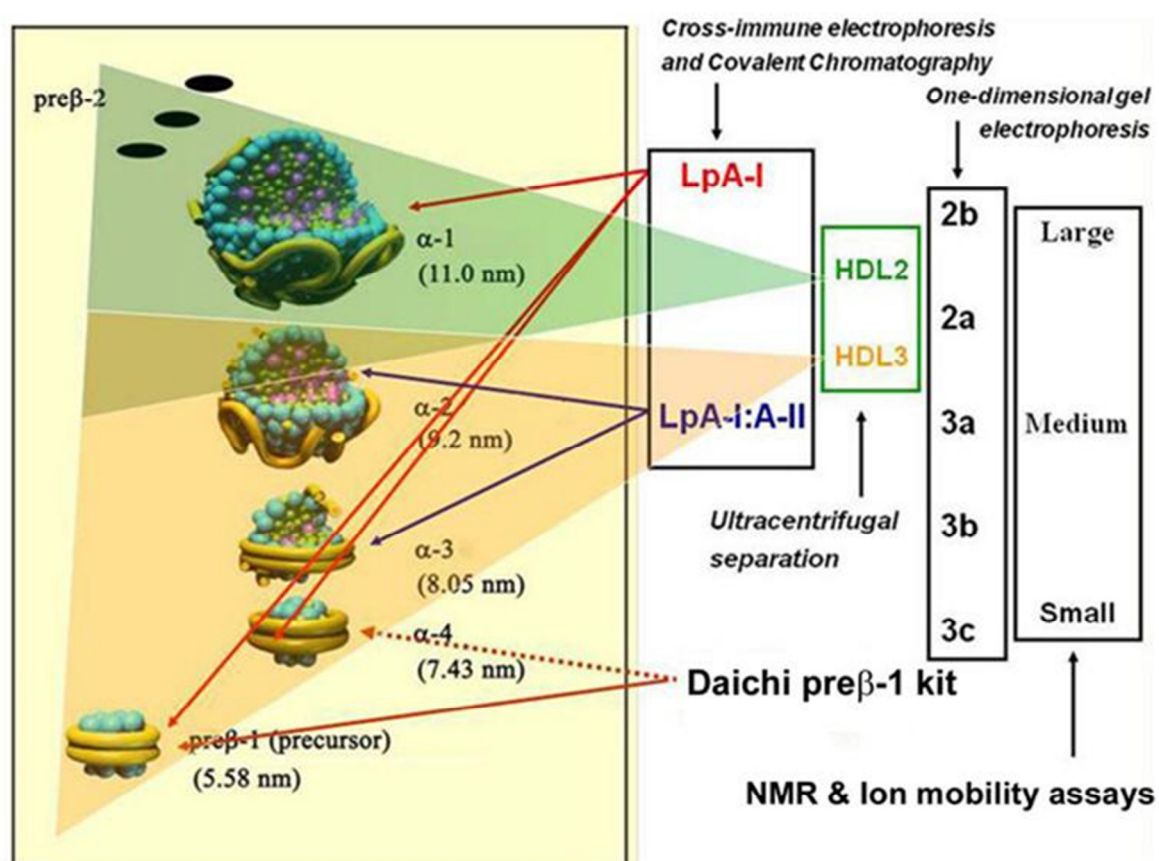


Figure 2 HDL subpopulations in 2D non-denaturing-PAGE and different nomenclature depending on the characterization method. [27]

It has to be stated that this linear circulation image is a very simplified version of the HDL lifecycle. Phospholipid transfer protein (PLTP) for example transfers phospholipids (PL) and

free cholesterol (FC) from very low-density lipoprotein (vLDL) to HDL and among different HDL species. PLTP also remodels various HDL particles by triggering fusion and dissociation processes [28]. Whereas apo A-I is distributed among all HDL particles, other apolipoproteins such as apo A-II, apo A-IV, apo C-I, apo C-III or apo E, are dominantly found in different subsets (Figure 3). Presumably these lipoproteins will differ in their affinity to interaction partners and add enormous complexity to the understanding of HDL.

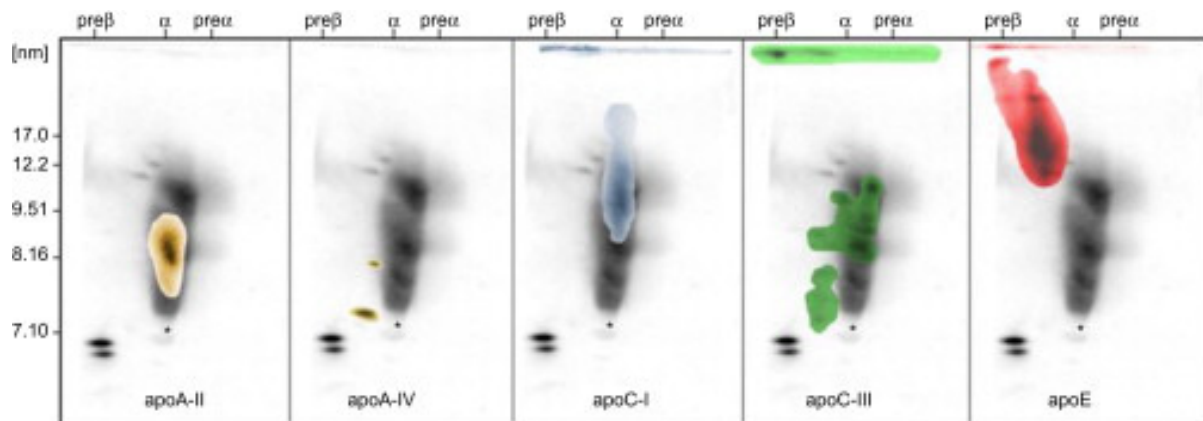


Figure 3 Distribution of major apolipoproteins in HDL [18]

1.3 HDL – An Anti-Inflammatory Agent

The well demonstrated correlation of high HDL - cholesterol levels and low cardiovascular disease has made HDL a popular target for scientists doing research on atherogenesis. In the process of atherogenesis HDL has been shown to inhibit cytokine induced expression of adhesion molecules VCAM-1, ICAM-1 and E-selectin on endothelial cells, important for recruitment of leukocytes[29, 30]. HDL also attenuates secretion of chemokine MCP-1 in endothelial cells, which attracts monocytes [13]. Later, HDL was found to inhibit expression of cytokines, chemokines and surface receptors as well as reducing antigen presentation on monocytes, macrophages and monocyte derived dendritic cells [31-33]. Many speculate that the anti-inflammatory properties of HDL are mainly attributed to its well established role in RCT thereby reducing lipid rafts, which are essential for immune cell signalling [34, 35]. On the other hand, the following table (Table 1) summarizes a variety of described mechanisms through which HDL may exhibit its anti-inflammatory potential.

Table 1 Summary of mechanisms for pro and anti-inflammatory HDL

Antiinflammatory HDL	Proinflammatory HDL (APR-HDL, oxHDL, glyHDL)
<i>RCT reduces lipid rafts [35]</i> Lipid rafts essential for TLR signalling	<i>Missing beneficial properties [33]</i> SAA, replaces ApoA-1
<i>Inhibit TLR signalling [36]</i> TLR4 MyD88 (C-dependent) TRAM/TRIF (C-Independent)	<i>SAA loading [37]</i> activates NFκB through TLR-4 activation
<i>LPS binding [38]</i> HDL through LBP - binds LPS	<i>oxHDL [39]</i> In Inflammatory conditions loses antioxidative properties
<i>Phospholipids [40]</i> HDL transports special phospholipids to cell membrane of immune cells like PLPC PLPC – activates PPARγ	
<i>Antioxidant [41, 42]</i> Paraoxogenase 1, ApoA-1, LCAT hydrolyse oxidized lipids	

HDL can exhibit its antioxidant function by clearing oxidized lipids, which are initiators of multiple inflammatory pathways [42]. HDL-associated enzymes like Paraoxigenase 1 and LCAT have been reported to hydrolyse oxidized lipids [41, 42]. Perrin-Cocon [40] found that the phospholipid fraction of HDL was able to retain the inhibition of IFN-γ secretion in dendritic cells (DC). They identified phospholipid, 1-palmitoyl-2-linoleyl-phosphatidylcholine (PLPC) as the most active PL for inhibiting NFκB activation, showing that only one single phospholipid present in HDL can exhibit anti-inflammatory properties. Using ABCA-1 and ABCG-1 deficient mice (to demonstrate cholesterol-independent mechanisms) it was shown that HDL inhibits inflammatory signals by inhibiting both the TRAM/TRIF and the MyD88 arm of TLR-4 signalling [36]. LPS binding protein (LBP), binds preferably to HDL [43], thus enabling HDL to attenuate effects of the endotoxin LPS. Intravenous HDL injection has been shown to protect mice from toxic effects induced by LPS [38]. HDL has also been reported as a pro-inflammatory agent, but the pro-inflammatory nature was mostly linked to missing beneficial anti-inflammatory properties, in acute phase response SAA replaces Apo A-1 [44, 45]. SAA can also activate NFκB through TLR-4 activation [37]. Similar oxidized HDL is

believed to have lost its beneficial antioxidant properties [39]. Many of the findings about HDLs immunoregulatory function are controversial and it is hard to formulate conclusive statements. To find a number of different mechanisms for one observed HDL ability is not very surprising for a particle as complex and heterogeneous as HDL. After all more than 70 proteins have been found on HDL (proteins common in at least 3 proteomic studies) [12].

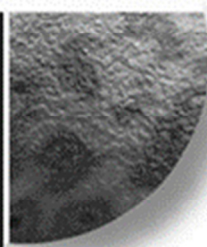
1.4 Methods in use for HDL isolation, desalting and characterisation

In this section the basic principles and theoretic background for methods used for HDL isolation, desalting and characterisation are described briefly.

1.4.1 HDL Isolation using ultracentrifugation

Table 2 gives an overview of different lipoprotein species found in human blood plasma. The general assumption is that size is indirectly correlated with density since lipoprotein particles increase in size as they are loaded with low density compounds such as triglycerides and cholesterol esters. This assumption is supported by electron microscope pictures of density isolated lipoprotein fractions [46].

Table 2 Classification of plasma lipoproteins [47]

Mol Wt (Daltons)	Size (nm)	Lipoprotein name / Apo Content	Percent Lipid Content		
			Trig	Chol	PL
400 x 10 ⁶	75-1200	 Chylomicrons --- Apos B-48, A-I, A-II, A-IV, C-I, C-II, C-III, E ---	80-95	2-7	3-9
10-80 x 10 ⁶	30-80	 --- VLDL --- Apos B-100, C-I, C-II, C-III, E ---	55-80	5-15	10-20
5-10 x 10 ⁶	25-35	 --- IDL --- Apos B-100, C-I, C-II, C-III, E ---	20-50	20-40	15-25
2.3 x 10 ⁶	18-25	 --- LDL --- Apo B-100 ---	5-15	40-50	20-25
1.7 - 3.6 x 10 ⁵	5-12	 --- HDL --- Apos A-I, A-II, A-IV, C-I, C-II, C-III, E ---	5-10	15-25	20-30
		0.95 0.95-1.006 1.006-1.019 1.019 - 1.063 1.063 - 1.21			
		Flotation Density (Ultracentrifugation)			

A number of different approaches on how to isolate HDL and other Lipoproteins based on their different densities are available [46, 48]. All require the use of ultracentrifugation to achieve complete separation and high salt concentrations (usually KBr due to its high solubility). The different approaches include sequential centrifugation steps with adjustments of density, use of density gradients or combination of both. Ultracentrifugation is the most

commonly used preparative method to isolate HDL particles but the influence of high salt concentration, high G-force and thus high shear forces is considered a disadvantage of the method [49].

1.4.2 HDL desalting using dialysis

Dialysis is a classical laboratory technique for desalting, buffer exchange or other applications. It relies on selective diffusion of molecules through a semi permeable membrane. The membrane (usually a sheet of regenerated cellulose [50]) contains pores of a defined size distribution. A sample and a buffer solution (called the dialysate) are placed on opposite sides of the membrane. Particles like salt ions or small molecules are smaller than the pores and will pass through the membrane while bigger particles are retained. This will result in net influx or efflux of small molecules or salt ions from one side to the other until a concentration equilibrium is reached.

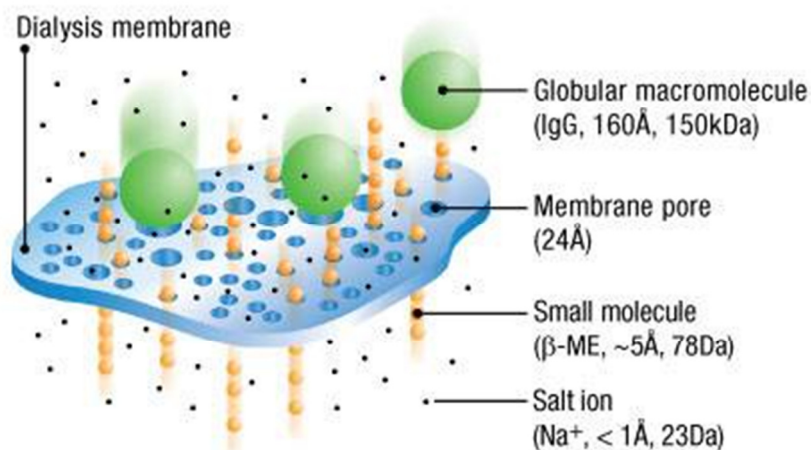


Figure 4 Separation principle of dialysis membranes [50]

HDL samples that are isolated using ultracentrifugation techniques contain KBr concentrations of 2 – 3 mol/L. Physiological potassium concentration is 3,5 – 5 mmol/L [51]. In order to decrease the sample salt content by a factor of 1000, sufficiently large dialysate volume and multiple dialysis steps are required. Dialysis is a slow process that usually has to be carried out overnight.

1.4.3 HDL desalting using desalting columns

An alternative to dialysis is desalting using desalting columns based on the gel filtration principle also called size exclusion. The solid matrix packed in the column is a disperse carbohydrate polymer like dextran (e.g. in SephadexTM). Porous beads allow the entrance of small molecules or salt ions, which are then retained, while larger molecules like proteins or particles containing many macromolecules such as lipoproteins avoid the inner matrix of the

beads and elute earlier. In other words the volume that each component of the sample has to pass before it is eluted from the column (V_e elution volume) depends on the size of the molecule. Large molecules pass only the void volume V_0 ($V_e=V_0$), solvent molecules (water) and salt ions pass the void volume V_0 and the interstitial volume V_i ($V_e=V_0+V_i$) see Figure 5.

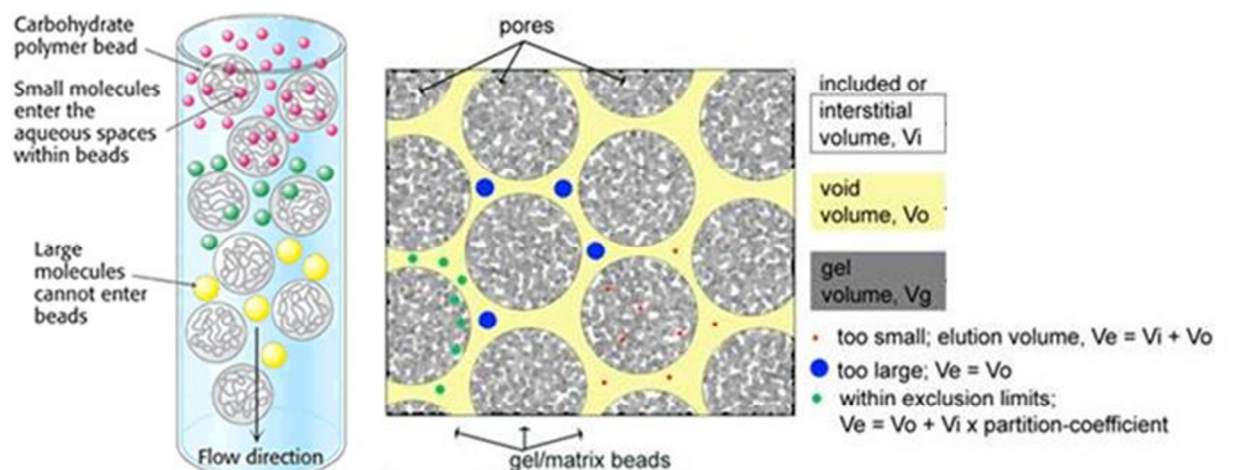


Figure 5 Separation principle of gel filtration and size exclusion desalting columns [52, 53]

To remove the high quantities of salt in HDL samples isolated by ultracentrifugation size exclusion columns offer an alternative to lengthy dialysis procedures. However the desalting capacity is a critical parameter. Therefore it is important to choose a small elution volume V_e , which in turn could reduce sample recovery.

1.4.4 HDL characterisation using size exclusion chromatography

The principle of gel filtration used for size exclusion desalting can also be applied for chromatographic analysis. For chromatographic application the pore size of the matrix beads has to be larger than for desalting procedures, the size of the analytes should be within the exclusion limits of the bead matrix. The principle of separation is solely based on size as described before (chapter 1.4.3). Figure 6 shows an image of an Äkta™ fast protein liquid chromatography (FPLC) system and a simple schematic diagram of a basic LC system. The solvent reservoir contains degassed solvent. The pump provides a constant flow rate (the limit of the flow rate is usually the maximum pressure that can be applied to the columns or the system). The injection valve contains an injection loop that allows precise sample injection. Different particles in the column are separated based on their size. The detector quantifies eluting analytes, most commonly via UV-absorption or conductivity measurement. Fractions can be collected for further analysis using a fraction collector.

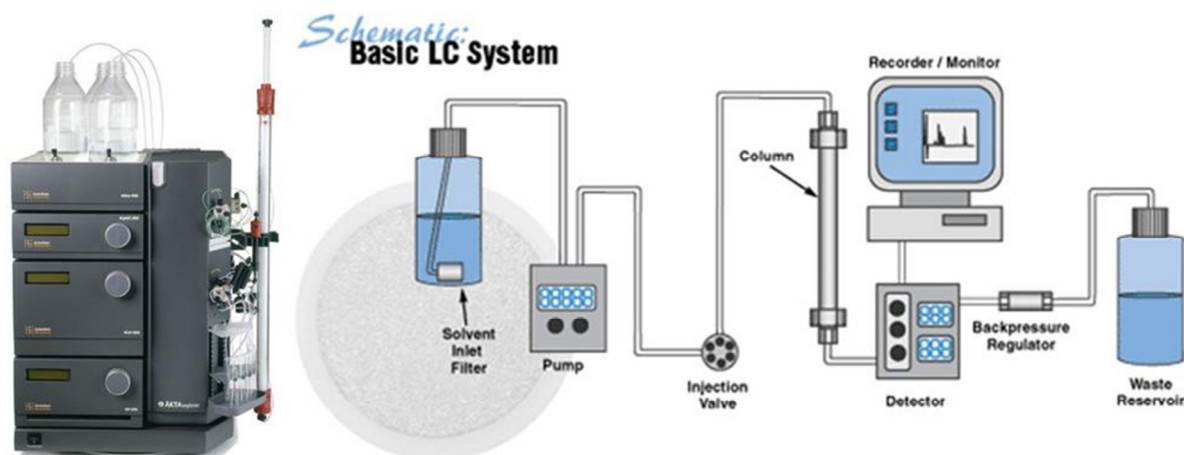


Figure 6 Äkta FPLC explorer system and simple schematic illustration of LC system[54, 55]

Separation of particles based on size is an ideal approach to analyse Lipoprotein samples. Although size exclusion FPLC is lacking resolution power and cannot distinguish between HDL or LDL subspecies, it is an optimal tool to determine purity of HDL samples.

1.4.5 HDL characterisation using Immunodetection

Lipoproteins are traditionally divided in classes based on size and density distribution. However, many believe that classification based on apolipoprotein content would be more appropriate [13]. Apolipoprotein content of HDL samples can be analysed in various ways, the most common used technique to compare different HDL samples is western blot (WB). It involves protein quantification using Lowry, Bradford or bicinchoninic acid (BCA) assays, followed by reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate HDL proteins depending on size, transfer of proteins from the gel onto a carrier membrane (nitrocellulose or polyvinylidene difluoride (PVDF) membrane) and detection using peroxidase conjugated antibodies and a photosensitive film to visualise a chemiluminescent reaction.

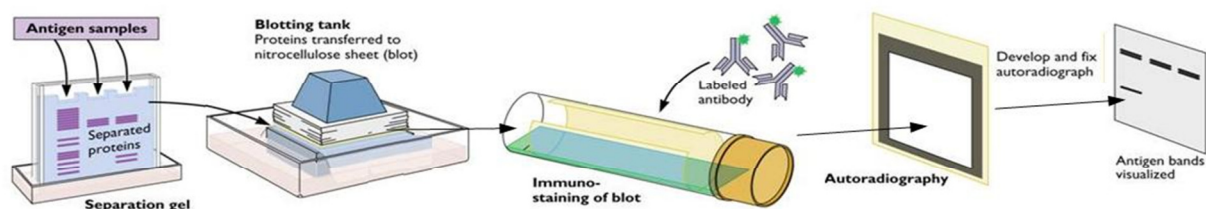


Figure 7 Western blot procedure scheme [56]

2 Methods

In this section the methods that were used in this thesis are described briefly. Detailed and user friendly lab protocols can be found in the attachments of this thesis.

2.1 HDL Isolation

2.1.1 Density gradient ultracentrifugation

For HDL isolation blood samples were taken in 9 mL EDTA tubes. The blood was centrifuged at 400 g, 15°C, 20 min to obtain 4 mL plasma. The density of the plasma was adjusted to 1,24 g/mL by the addition of KBr (Potassium bromide BioUltra, SIGMA 60089). 4 mL density adjusted plasma was then layered under 11 mL PBS. After ultracentrifugation, 4 h at 50.000 rpm at 15°C, using Beckman type 75 Ti rotor (fixed angle rotor), HDL and LDL fractions can be withdrawn from the centrifugation tube by penetration of the tube wall with a 18 ga needle. HDL samples were desalted using Polyacrylamid desalting columns (PD-10 desalting columns 8,3 mL from GE Healthcare) or alternatively using Slide-A-lyzer dialysis chambers (3 kDa MWCO from Thermo Fisher / Pierce Science). Samples are stored under N₂-atmosphere at 4°C. For detailed laboratory protocol see Appendix 1.1.

2.1.2 Sequential ultracentrifugation

For HDL isolation blood samples were taken in 9 mL EDTA tubes. The blood was centrifuged, at 400 g, 15°C, 20 min to obtain 4 mL plasma. The density of the plasma was adjusted to 1,063 g/mL by the addition of KBr (Potassium bromide BioUltra, SIGMA 60089). After the first ultracentrifugation step, 4 h at 50.000 rpm at 15°C, using Beckman SW 60 Ti rotor, the top layer, containing vLDL, LDL and TG was removed. The bottom layer was collected and adjusted to a density of 1,24 g/mL by further addition of KBr. After a second ultracentrifugation step, 4 h at 50.000 rpm at 15°C, using Beckman SW 60 Ti rotor, HDL can be withdrawn from the top layer. HDL samples were desalted using Polyacrylamid desalting columns (PD-10 desalting columns 8,3 mL from GE Healthcare) or alternatively using Slide-A-lyzer dialysis chambers (3 kDa MWCO from Thermo Fisher / Pierce Science). Samples are stored under N₂-atmosphere at 4°C. For detailed laboratory protocol see Appendix 1.3.

2.2 HDL Desalting

2.2.1 Slide-A-lyzer dialysis

Slide-A-lyzer dialyses cassettes with a molecular weight cut-off of 3,5 kDa were purchased from Pierce. Up to 4 samples of 2,5 mL each were dialyzed twice against 5 L PBS at 4°C overnight with mild stirring. For detailed laboratory protocol see Appendix 1.4.

2.2.2 Desalting using PD-10 Sephadex™ desalting columns

PD-10 Sephadex™ desalting columns were purchased from GE Lifesciences. Desalting was carried out according to the manufacturer's recommendations. Samples were eluted with PBS. For detailed laboratory protocol see Appendix 1.5.

2.3 FPLC Analysis

Size exclusion chromatography using a Superdex™ 200 HR 10/30 column (GE, Amersham) and the Äkta Explorer 10 FPLC System (GE, Amersham) was carried out to analyse particle size of HDL, LDL and whole serum samples. 125 mM NaCl, 1 mM EDTA in PBS was used as an isocratic running buffer, a flow rate of 0,7 mL/min and maximum pressure of 10 MPa was applied. Detection was carried out using a UV-900 detector at 214 nm and 280 nm and a pH/C-900 pH/conductivity detector. Fractions of 0,5 mL were collected using Frac-900 fraction collector. For detailed laboratory protocol see Appendix 1.6.

2.4 WB

If not stated otherwise samples are separated by size using reducing SDS – polyacrylamide gel electrophoresis (SDS-PAGE). Gels with 12% acrylamide content are usually applied. Samples are blotted on polyvinylidene difluoride (PVDF) membranes using a BIO-RAD semi-dry blotting system. Bands are visualised with Ponceau-S staining. Antibody detection is carried out with primary and secondary antibodies as listed in Table 3. Apo E-1 bionylated primary antibodies are detected with streptavidin-peroxidase conjugate. Amersham ECL™ detection using photosensitive Amersham Hyperfilm™ is applied for visualisation. For detailed laboratory protocols see Appendix 1.9 (BCA Assay for protein quantification) and Appendix 1.10 (WB standard procedure).

Table 3 List of primary antibodies applied for WB analysis.

Antibody	Target size	Incubation buffer	Company
Ms - α - apo B-100	550 kDa	1:3000, 2% milk in TBS-T, 0,02% NaN ₃	Cell signalling
Ms - α - apo A-1	28 kDa	1:5000; 5% milk in TBS-T, 0,02% NaN ₃	Cell signalling
Ms - α – SAA	12 kDa	1:1000; 1% BSA in TBS-T, 0,02% NaN ₃	Cell signalling
Ms - α - apo E-1 - biotinylated	35 kDa	1:5000; 5% milk in TBS-T, 0,02% NaN ₃	Cell signalling

Table 4 List of secondary antibodies applied for WB analysis and strepavidin-peroxidase conjugate for detection of biotinylated antibodies.

Target	Modification	Incubation buffer	Company
Rb IgG	Peroxidase conjugated	1:10000, 2% milk in TBS-T	Amersham, Rb-ECL
Ms IgG,	Peroxidase conjugated	1:10000, 2% milk in TBS-T	Amersham, NIF 825
Ms IgG,	Human IgG cross absorbed, peroxidase conjugated	1:10000, 2% milk in TBS-T	Amersham, bethyl A90-216P
Biotin, biotinylated antibodies	Strepavidin-peroxidase conjugate	1:20000, 2% milk in TBS-T	Roche 11089153001

2.5 Cell culture

THP-1 cells were grown in THP-medium containing RPMI 1640, 10% FCS, 100 µg/mL streptomycin, 100 U/mL penicillin, MEM NEAA (100x stock from GIBCO 11140), 1mM sodium pyruvate (all from GIBCO®) and 0,05 mM β - mercaptoethanol. Cells were maintained at $0,1 - 1 \times 10^6$ cells/mL by splitting every 3 – 4 days. Cells were incubated at 37°C and 5% CO₂ and had a doubling time of 35 to 50 h.

U937 cells were grown in RPMI 1640 medium (GIBCO) with 10% FCS (GIBCO), 100 µg/mL streptomycin, 100 U/mL penicillin (penstrep 100x stock from GIBCO). U937 cells were kept a density of $0,5 - 1,5 \times 10^6$ cells/mL. Cells were incubated at 37°C and 5% CO₂.

2.6 IL expression analysis using Luminex Assay

THP-1 cells were grown to a density of 10^6 cells/mL and stimulated with HDL for 1h. Inflammatory cytokine response was triggered by addition of 100 ng/mL LPS. Cell free supernatants were collected after 18 h of incubation. IL-6, IL-10, IL-12p40 and TNF-α concentrations were determined using a Luminex bead system. For detailed laboratory protocols see Appendix 1.7.

2.7 NFκB activation assay using a GFP – reporter U937 cellline

U937 cells containing a GFP-reporter for NFκB activation were grown to a density of 10^6 cells/mL. Cells were preincubated with HDL or reference blank solutions for 1 h before addition of 100 ng/mL LPS. After 18h cells NFκB activation was quantified directly from the cell suspension using FACS analysis on a BD-DIVA FACS Instrument from BD-Bioscience. For detailed laboratory protocols see Appendix 1.8.

3 Results

This section first describes the results obtained from optimized HDL isolation, desalting and characterisation procedures (chapters 3.1 - 3.3), namely density gradient ultracentrifugation, sequential ultracentrifugation, desalting using dialysis, desalting using PD-10 desalting columns and size exclusion FPLC.

Then in chapter 3.4 differences in purity of HDL-samples from different laboratories in Austria are revealed, followed by a comparison of different isolation and desalting techniques and their effect on obtained HDL-sample purity. At last in chapter 3.5 the importance of sample purity on immunological assays is highlighted by showing how the immunological effect of HDL is attenuated with decreased sample purity.

3.1 HDL Isolation

Two methods available for HDL isolation, density gradient ultracentrifugation and sequential ultracentrifugation were carried out. The following sections show results obtained by the two methods.

3.1.1 Density gradient ultracentrifugation

Using the one-step density gradient ultracentrifugation, HDL isolation can be carried out and finished on the same day blood samples are taken from individuals. HDL Isolation of up to 8 samples takes about 7 to 9 hours. HDL and LDL fractions separate according to density and form visible yellow bands as seen in Figure 8. Both the size and colour intensity of the fractions are largely dependent on the individual donor, and do not necessarily correlate with HDL or LDL content, neither did band size and colour correlate with inflammation status CRP or body mass index BMI (Figure 8 and Figure 9).

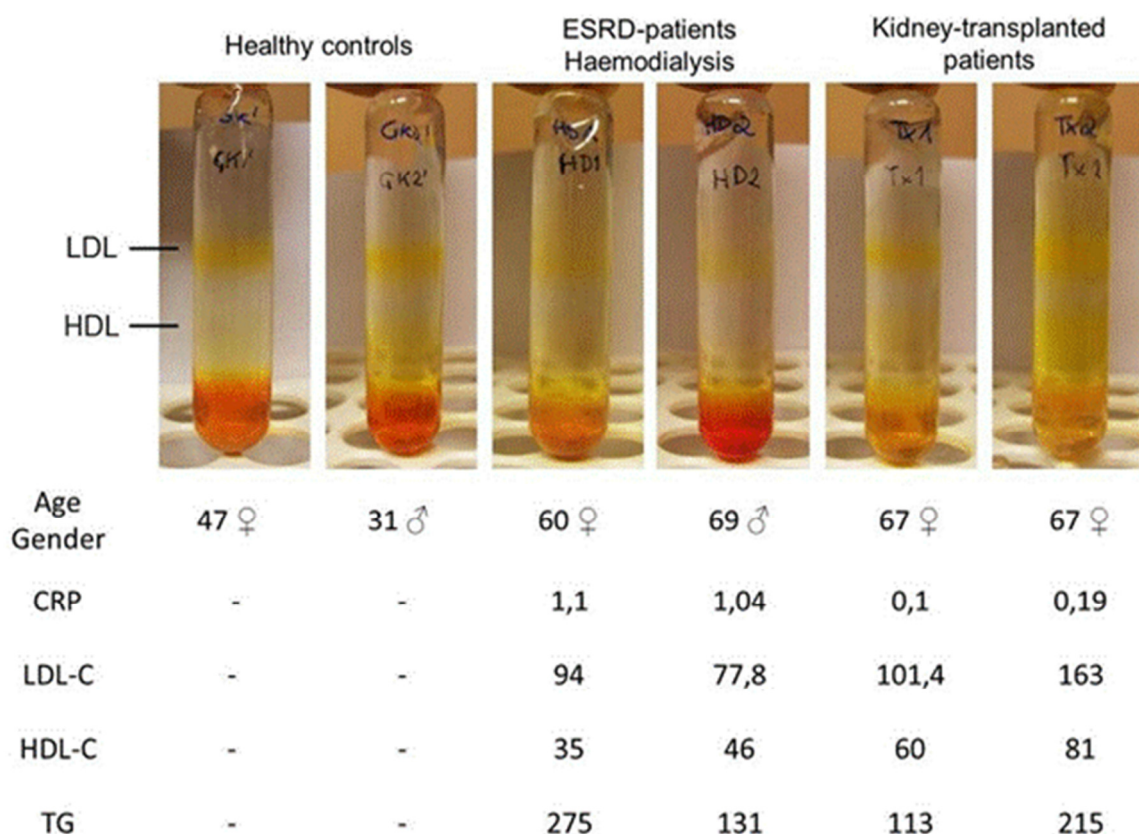


Figure 8 HDL samples after gradient ultracentrifugation, units for CRP, LDL-C, HDL-C and TG are mg/dL

In patients with high body mass index >30 and high blood level of triglycerids a white fat fraction is visible above the LDL and vLDL fraction. In a different batch of HDL isolation (Figure 9) the very high load of triglycerides of sample 4 in a 35 year old male also caused a shift in the location of the LDL band in contrast to samples with normal blood triglyceride levels. The triglyceride fraction in the sample 4 was large enough (about 1 to 2 mL in volume) to cause an unusual density gradient. The achieved HDL concentrations highly depend on the individual sample and the total volume withdrawn from the centrifugation tubes, it typically ranges from about 1 mg/mL to 3 mg/mL. The HDL is found in the zone where the density is about 1,21 g/mL, which corresponds to a KBr concentration of about 2,5 mol/L. For immunological experiments the sample has to be desalted using dialysis or size exclusion chromatography.

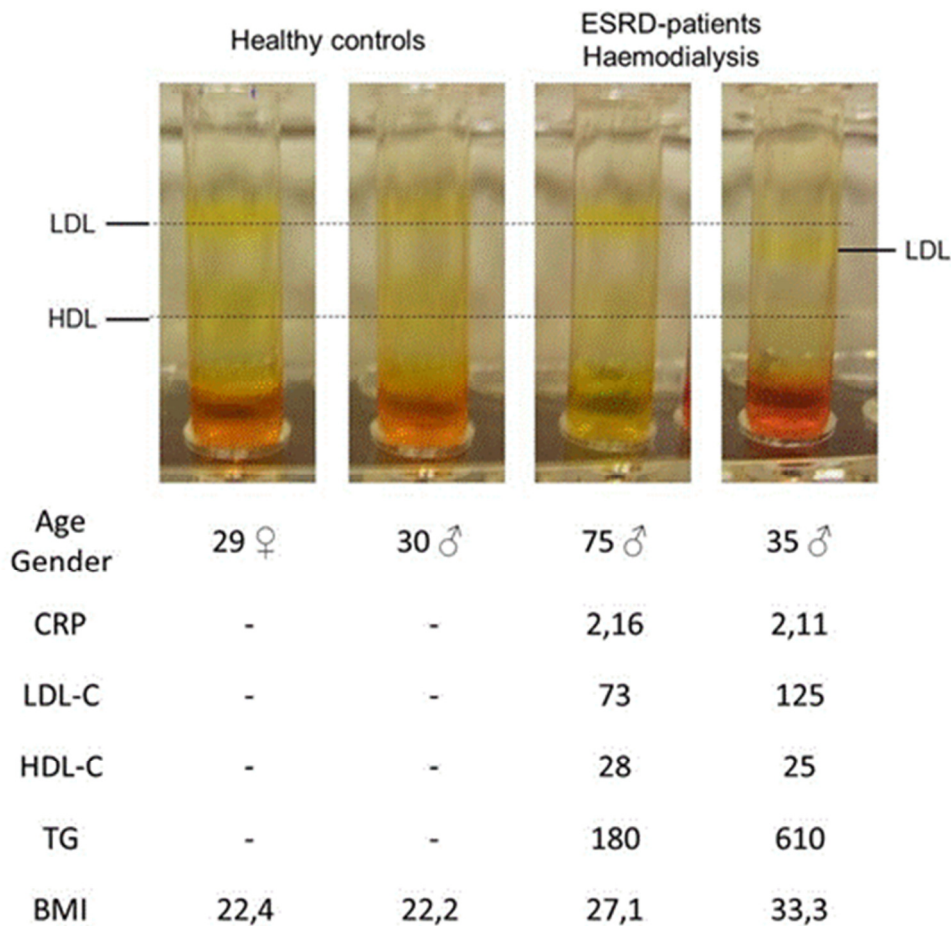


Figure 9 HDL samples after gradient ultracentrifugation, LDL band shift of lipemic sample. Units for CRP, LDL-C, HDL-C and TG are mg/dL

3.1.2 Sequential ultracentrifugation

HDL isolation using the two step sequential ultracentrifugation method takes about 11 – 14 h, and has to be carried out overnight. This method results in significantly, 5 to 10 folds, higher sample concentrations because no density solution is added and samples are pooled for the second centrifugation step. HDL is withdrawn as the top fraction from a density adjusted plasma solution (Figure 10). Centrifugation occurs in density adjusted plasma, which results in an enrichment of HDL. Table 5 presents typically achieved HDL concentrations in samples isolated by sequential ultracentrifugation. In the last centrifugation step the density is brought to 1,24 g/mL by addition of KBr. As a result samples contain 3,3 mol/L KBr, which has to be removed by dialysis or size exclusion chromatography.

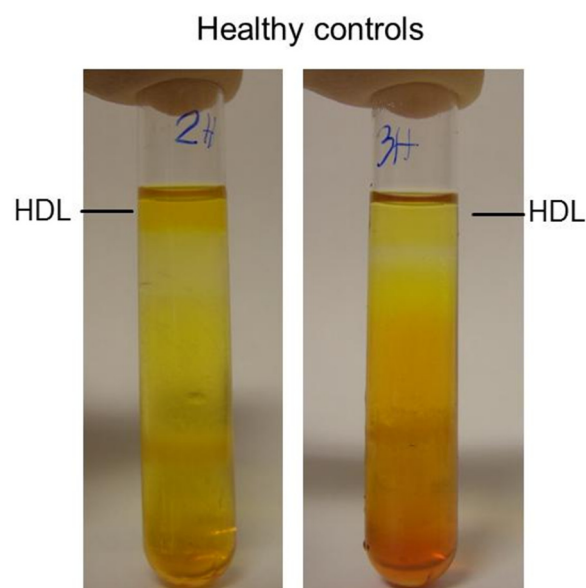


Figure 10 HDL samples after sequential ultracentrifugation

Table 5 HDL sample concentrations using sequential ultracentrifugation

HDL sample	Concentration
HD-patient C2	12,61 mg/mL
Healthy control C9	15,45 mg/mL
HD-patient D7	35 mg/mL
Healthy control D13	24 mg/mL
Healthy control E10	9,98 mg/mL
Healthy control E11	9,90 mg/mL

3.2 Desalting of HDL samples

HDL isolation using ultracentrifugation methods always requires the addition of KBr to increase the density to 1,24 g/L and leads to KBr concentrations of about 2 - 3 mol/L in the sample. For immunological experiments HDL samples have to be desalted. Two methods for desalting were carried out: dialysis and desalting using size exclusion columns.

3.2.1 Slide-A-lyzer dialysis

Dialysis is a classical method for desalting samples. In the applied experimental setup up to 4 HDL samples of 2,5 ml each are dialysed against 5 L of PBS. To achieve sufficient removal of KBr dialysis has to be carried out twice overnight adding up to a total time of 40 h. Assuming that equilibrium is reached in both dialysis steps the final concentration of KBr would be about 10 $\mu\text{mol/L}$, making dialysis a very efficient method for desalting. Average potassium levels in serum are about 400 times higher ranging from 3,5 – 5 mmol/L. In reverence experiments dialysed KBr density solution did not show any significant effects on immunological experiments carried out. Dialysis leads to dilution of sample, exemplary results are shown in Table 6.

Table 6 Dialysis of HDL samples, loss of concentration

HDL Sample reference	HDL conc. (BCA) before dialysis [mg/mL]	HDL conc. (BCA) after dialysis [mg/mL]	Relative HDL conc. after dialysis
D13	2,99	2,49	83%
D7	2,27	1,65	73%
E8	1	0,83	83%
P1	3,55	2,51	71%
P2	3,46	2,56	74%
S1	4,08	3,16	77%
S2	3,61	3,21	89%

Dilution of sample can be critical since isolation of HDL using gradient ultracentrifugation does not effectively enrich the concentration of HDL from the plasma. Removal of the sample from the dialysis cassette always leaves some residue sample in the cassette, which can be significant if low amounts of sample are dialysed. Therefore it is recommended to dilute low volumes of sample to a concentration of about 3 mg/mL (HDL isolation using sequential ultracentrifugation leads to a sample volume of 500 μL).

3.2.2 Desalting using PD-10 SephadexTM desalting columns

An alternative to dialysis is desalting using desalting columns, which work on the principle of size exclusion chromatography and can be operated in gravity flow. Conditioning of the columns takes about 1 h, the actual desalting step of the sample happens in only a few minutes, and can easily be carried out directly after Isolation. Figure 11 illustrated the removal of low molecular compounds in a sample containing HDL and excess of methylene blue dye.

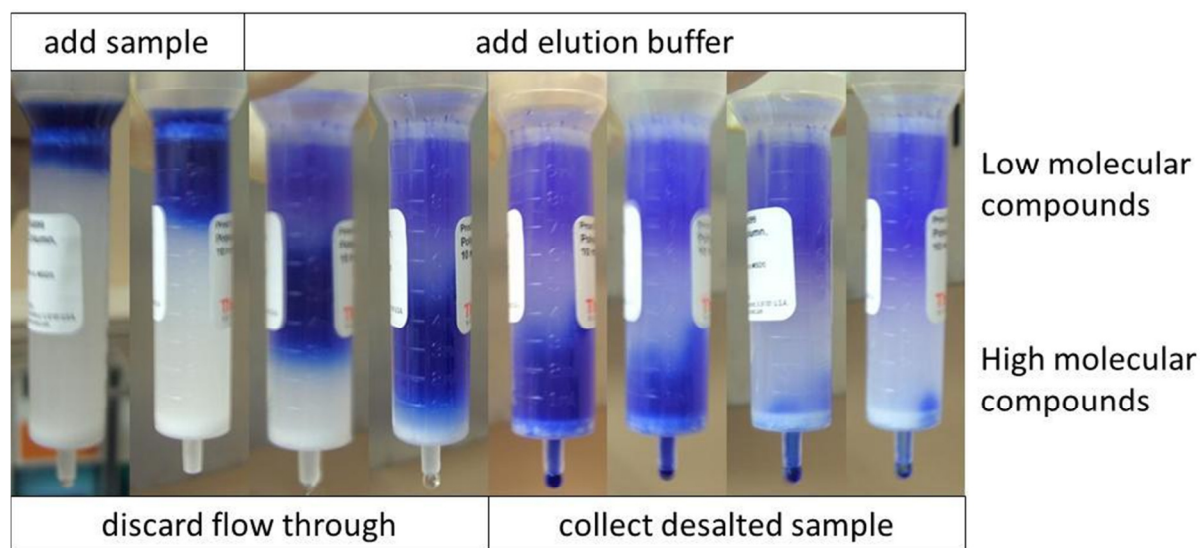


Figure 11 Visual illustration of desalting efficiency of PD-10 Sephadex desalting columns

The last column in Figure 11 shows the endpoint of the desalting procedure. It is visible that irregularities in the column packaging caused loss of sample. Nevertheless, to ensure efficient desalting it is important not to elute too much sample. Like dialysis this method always leads to dilution of sample. Dilution factor, sample recovery and remaining salt concentration depend largely on the fractions, which are collected during the desalting procedure. Figure 12 shows results of PD-10 desalting column elution experiments with BSA solution in four columns. In Figure 12 (a), (b), (c) and (d) bars indicate the start and end point of sample collection. In example (a) the sample collection is optimized for recovery, example (b) is optimized for low salt content and example (c) is optimized for high sample concentration. Example (d) shows sample collection as it was carried out for HDL samples, where dilution rates are ranging from 1.3 to 1.5, sample recovery is about 90% and remaining salt concentration is minimized. In reference experiments PD-10-column desalted KBr density solution did not show any significant effects on immunological experiments carried out (see results chapter 3.5 Figure 23).

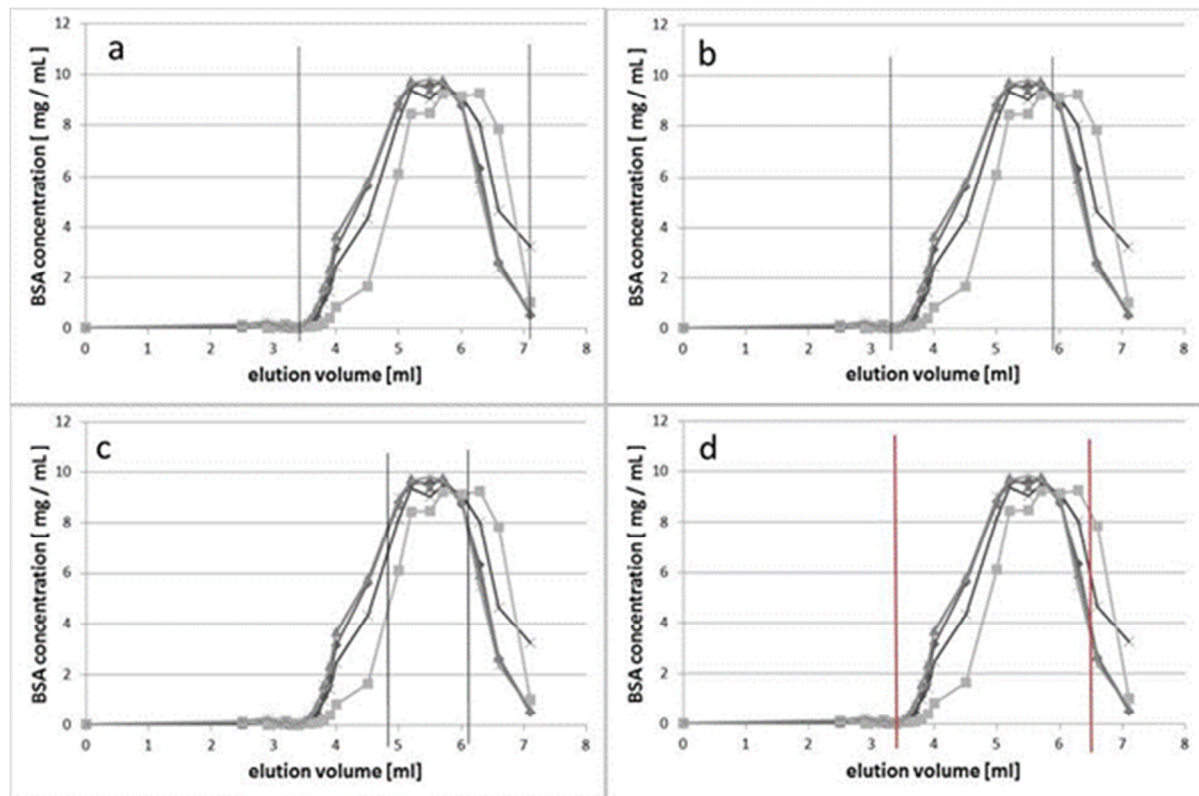


Figure 12 BSA elution experiments; PD-10 Sephadex® desalting of 2 mL BSA solution with concentration of 10 mg/mL, the bars indicate start and end point of sample collection.

3.3 Size exclusion FPLC – Analysis of Lipoproteins

Size exclusion chromatography allows quantitative and qualitative analysis of HDL, LDL or whole serum samples. Size exclusion chromatography can distinguish between Lipoprotein species, and UV detection at 214 nm or 280 nm gives a concentration dependent signal. A method with a run time of 42 min effectively separates LDL, HDL and albumin. Figure 13 shows a chromatogram of serum (black), and in overlay chromatograms of isolated vLDL (red), LDL (orange), HDL (green) and, albumin-rich HDL containing 70% albumin (blue).

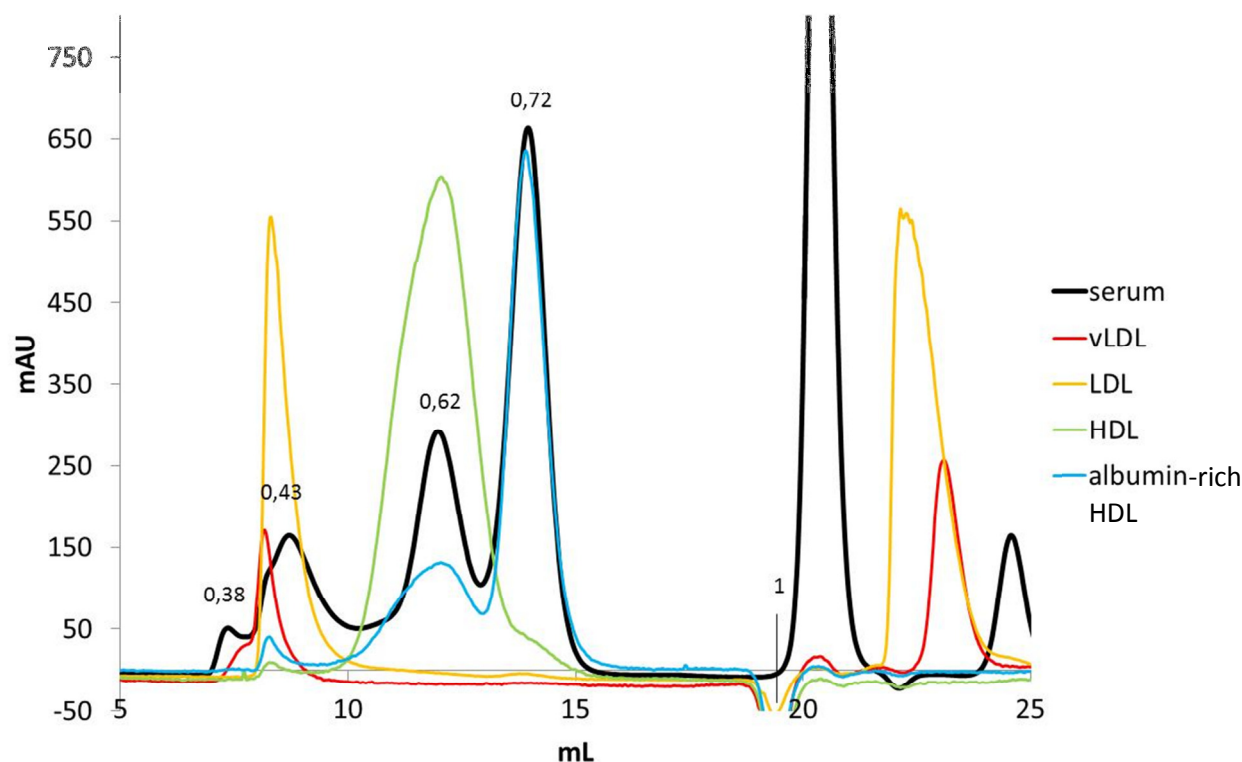


Figure 13 size exclusion FPLC chromatograms of serum, vLDL, LDL, HDL and albumin-rich HDL. Detection at UV_{214 nm}.

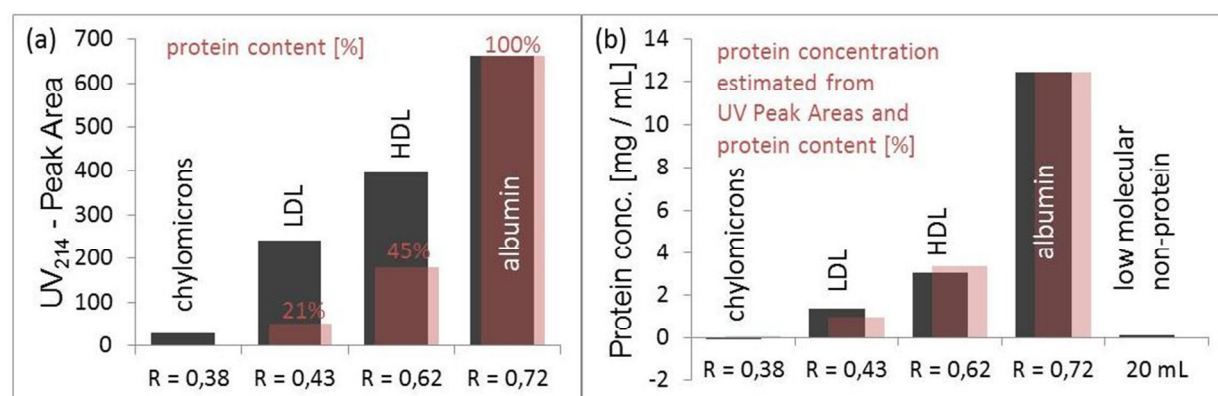


Figure 14 Comparison of UV₂₁₄ chromatogram peak areas (Figure 13) and BCA protein quantification of fractions collected from serum sample. In red the protein content of LDL, HDL and albumin [12].

In Figure 13 the retention factor is written above the peaks, it is the factor of elution time of the sample and elution time of the negative injection peak, which is located at 19,38 mL. Interestingly the injection peak of whole serum is not visible as a negative peak, but a slightly retained peak with strong UV absorbance indicating a low molecular compound found in serum. This low molecular compound can also be found in very small quantities in isolated LDL and HDL samples. The other retained peaks of vLDL, LDL and serum with elution times later than 20 mL could represent free cholesterol or triglycerides, identification would require further investigation. The early peak in serum, with retention factor 0,38 likely represents chylomicrons. Size exclusion FPLC fractions were collected and the fractions representing

the individual peaks analysed for protein content using the BCA assay. Peak identification is supported by comparison of UV – peak area and BCA protein quantification. Chylomicrons with very low protein (1 - 2%) content give no detectable signal in BCA assay. Likewise, the low molecular non-protein compound in serum, peak at 20 mL, gives very low BCA signal although UV absorbance in the chromatogram is very high, confirming that it does not represent protein or peptide. UV₂₁₄ absorbance in contrast to UV₂₈₀ absorbance gives a more uniform signal for all components of HDL and LDL. In Figure 14 (a), the UV₂₁₄ peak areas of LDL and HDL are shown in black bars and represent total LDL and total HDL. According to literature data, LDL consist of only about 21% protein, whereas HDL consists of about 45% protein [12], those percentages are indicated by red bars in Figure 14 (a). In contrast to Figure 14 (a), Figure 14 (b) shows protein concentration determined by BCA assay. Indeed, the protein concentration found for HDL and LDL fits the predicted values (red bars). This strongly supports the suggested peak identification. Table 7 shows retention factors and corresponding peak identification.

Table 7 Peak identification by retention factors. * In some samples HDL appears in subfractions, HDL-2 at R=0,57, and HDL-3 at R=0,62

	R
Chylomicrons	0,38
vLDL	0,41
LDL	0,43
HDL*	0,62*
Albumin	0,72

3.4 Comparison of HDL-sample purities

Different methods are available for HDL isolation and are routinely performed in numerous laboratories. In the first approach (3.4.1) an assessment of HDL-sample quality from samples isolated in 4 different laboratories in Austria was carried out. In a second approach (3.4.2) different isolation techniques were carried out parallel in one laboratory to determine the optimal isolation and desalting procedure.

3.4.1 Assessment of HDL-sample quality in different laboratories

Blood samples were taken from two healthy individuals, A and B. HDL was isolated simultaneously in “laboratory 1” at Universität Graz and “laboratory 2” at LKH Graz using a

different approach (Table 8). One week later HDL was isolated in “laboratory 3” at AKH Wien from fresh blood samples of individuals B and C. “Laboratory 4” at Medical University of Vienna provided a fourth independently isolated HDL-sample, sample X. Western Blot analysis of the HDL samples (Figure 15), revealed presence of apo B-100, a protein typically found in LDL, in the samples of “laboratory 2” isolated by sequential ultracentrifugation. The SAA – levels vary among individuals but show consistency throughout the different isolation and purification techniques. apo A-1 was consistently found in all samples. Ponceau-S stain reveals strong bands around 25 kDa representing apo A-1, weaker bands around 70 kDa are likely to represent serum albumin.

Table 8 HDL Isolation from different laboratories in Austria

Lab group	Samples from Individuals	Isolation method	Purification method
laboratory 1 Uni Graz	A, B	gradient centrifugation	desalting column
laboratory 2 LKH Graz	A, B	sequential centrifugation	dialysis
Laboratory 3 AKH Wien	C, B	gradient centrifugation	dialysis
Laboratory 4 Med Uni Vienna	X	n.d.	n.d.

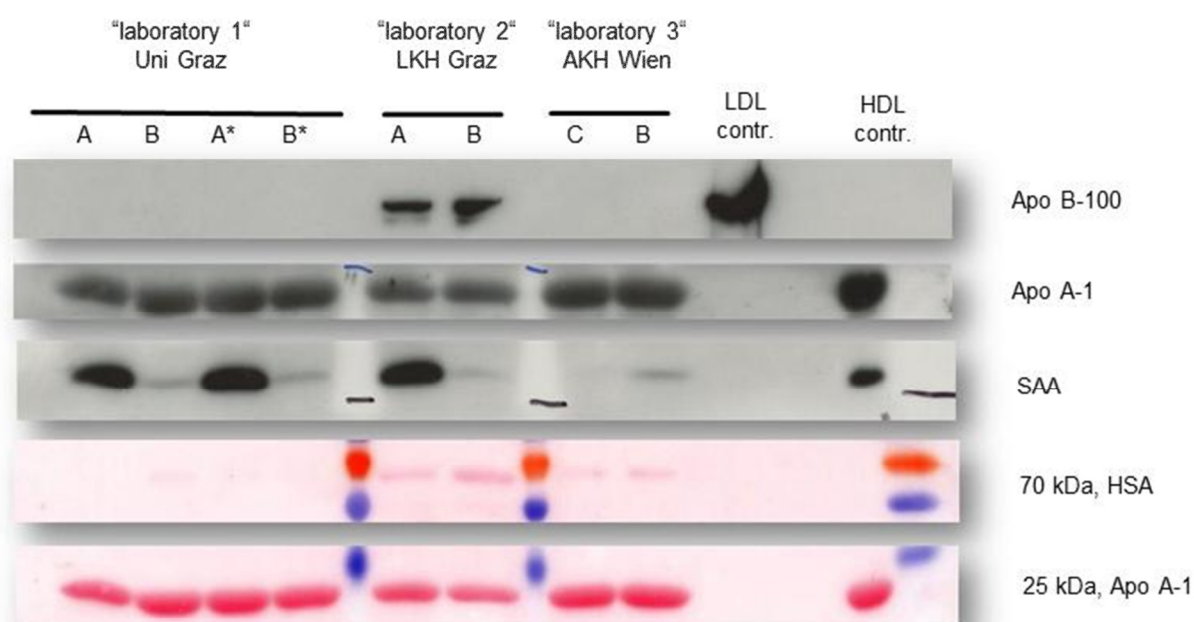


Figure 15 WB analysis and Ponceau-S stain of HDL samples from laboratories 1 - 3. A*, B* Isolation using PBS instead of 1,063 g/ml KBr density solution for gradient centrifugation.

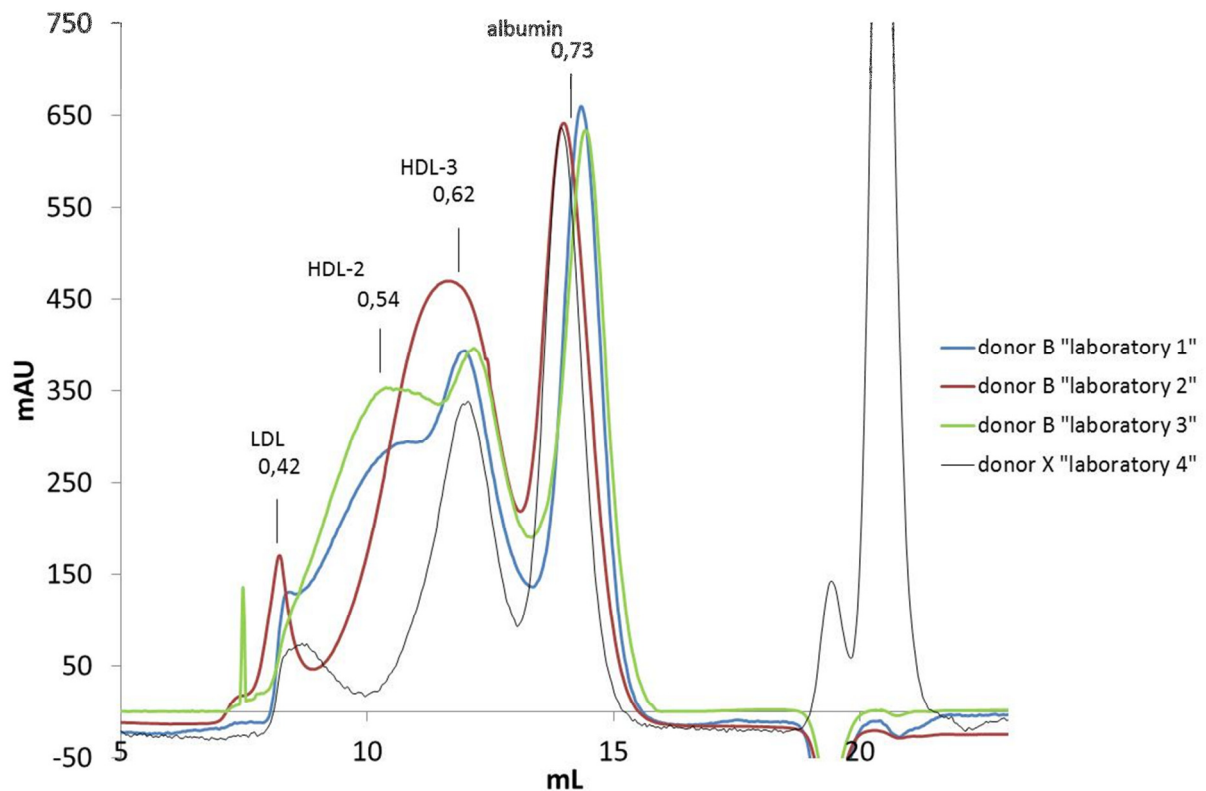


Figure 16 Size exclusion FPLC chromatograms of HDL samples isolated in laboratories 1 - 4

Figure 16 shows the size exclusion FPLC chromatogram of HDL samples from the same donor (donor B) isolated in 3 different laboratories and a fourth sample, labelled donor X, isolated from "laboratory 4" (Medical University of Vienna). The HDL-sample from laboratory four looks very similar to serum and probably represents apo B-100 depleted serum by PEG precipitation. The chromatogram reveals varying amounts of LDL, HDL and albumin in the different samples. Interestingly, samples "laboratory 1" and "laboratory 3" show two distinct peaks for HDL, whereas in samples "laboratory 2" and "laboratory 4" HDL elutes in one peak. LDL-Peaks are found in samples of laboratories 1, 2 and 4. Notably in the WB analysis of sample "laboratory 1" no apo-B-100 was detected in the sample (Figure 15). Strong albumin peaks ranging from 33% - 51% of total peak area were found in all samples. The unspecific Ponceau-S bands around 70 kDa were stronger in samples "laboratory 2" and only slightly visible for sample donor B of "laboratory 1". To further support peak identification size exclusion FPLC fractions representative of peaks LDL, HDL-2, HDL-3 and albumin were taken from sample "Eller" donor A and subjected to WB analysis (Figure 17).

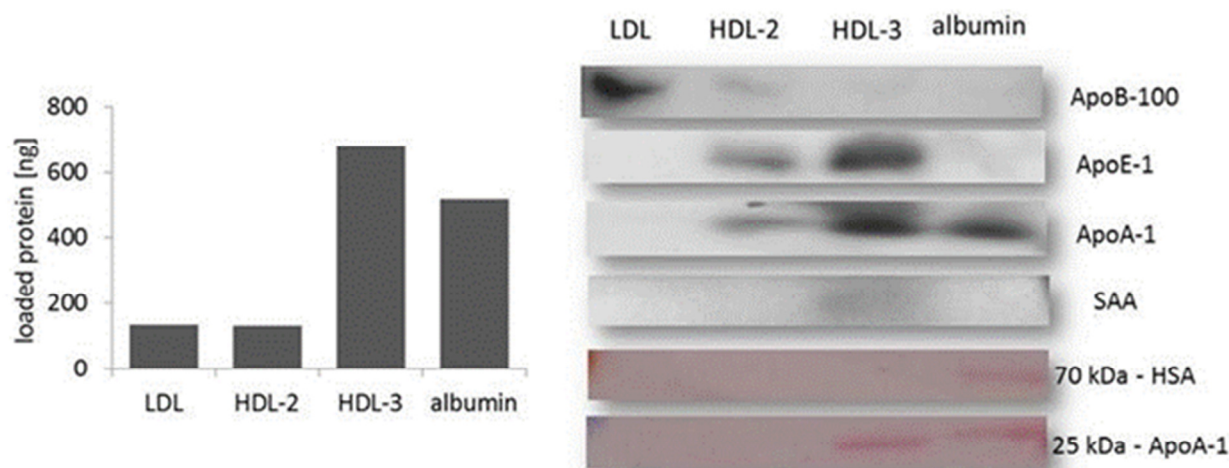


Figure 17 Figure 10 WB of FPLC fractions, R=0,45 LDL, R=0,54 HDL-2, R=0,63 HDL-3, R=0,72 albumin of sample donor B “laboratory 2”. Bar diagram shows amount of Protein loaded for WB analysis (equal volumes of FPLC fractions).

LDL was confirmed by the apo-B-100 Peak. As expected HDL fractions contained apo E-1 and apo A-1, difference in HDL-2 and HDL-3 band intensities result from different amounts of loaded protein. The fraction of the albumin peak shows signals for apo A-1 (representing free apo A-1) and the Ponceau-S band at 70 kDa confirms presense of albumin. In accordance with the analysis Table 9 presents the composition of isolated HDL samples based on UV₂₁₄ peak areas of the chromatograms in Figure 16.

Table 9 Composition of isolated HDL-samples from laboratories 1 - 4.

	LDL	HDL	albumin
donor B “laboratory 1”	3%	63%	34%
donor B “laboratory 2”	6%	58%	36%
donor B “laboratory 3”	<1%	67%	33%
donor X “laboratory 4”	7%	42%	51%

3.4.2 Assessment of HDL-sample purity depending on isolation and purification methods

Plasma samples were taken from 3 healthy individuals. As illustrated in Table 10, HDL was isolated using gradient ultracentrifugation and sequential ultracentrifugation. The isolated HDL samples were divided and then desalted using PD-10 Sephadex desalting columns or Slide-A-Lyzer dialysis. Isolation and desalting of all samples was carried out parallel in one laboratory to allow direct comparison of the applied methods.

Table 10 HDL isolation and desalting methods used for HDL-samples M1 - M10

Donor 1		Donor 2				Donor 3			
gradient centrifugation		gradient centrifugation		sequential centrifugation		gradient centrifugation		sequential centrifugation	
PD-10	Dialysis	PD-10	dialysis	PD-10	dialysis	PD-10	dialysis	PD-10	dialysis
M1	M6	M2	M7	M4	M9	M3	M8	M5	M10

Figure 18 shows WB analysis of isolated and desalted samples. The lanes on the left hand side (M1-M5, PD-10 desalting columns) and the lanes on the right hand side (M6-M10, dialysis) show the same band pattern in Ponceau-S and WB stains, hence the choice of desalting method has no effect on protein composition of the sample. However looking at gradient centrifugation versus sequential centrifugation some differences are quite obvious. Samples isolated using the sequential centrifugation show stronger Ponceau-S bands around 70 kDa. This is true for both samples but is especially pronounced in the sample isolated from donor 3. Strong bands in Ponceau-S around 70 kDa usually represent the most abundant protein in human plasma, human serum albumin. Since all the samples were diluted to a total protein concentration of 1 mg/mL higher albumin content means lower HDL concentration. This effect is clearly visible for apo A-1 (The most abundant lipoprotein in HDL) in the Ponceau-S stain band at 25kDa and the according WB stain of apo A-1 but also for the other HDL-lipoproteins apo E-1 and apo C-2. The WB stain of apo E-1 reveals individual differences between donor 1 and donors 2 and 3. Interestingly apo E-1 is slightly elevated in donor 2 in the sample isolated with sequential ultracentrifugation, in spite of lower HDL content. apo C-2 content is also lower in donor 1 as compared to donor 2 and donor 3. SAA concentration varies greatly between individuals, here donor 2 exhibits the highest content of SAA in HDL and donor 3 the lowest.

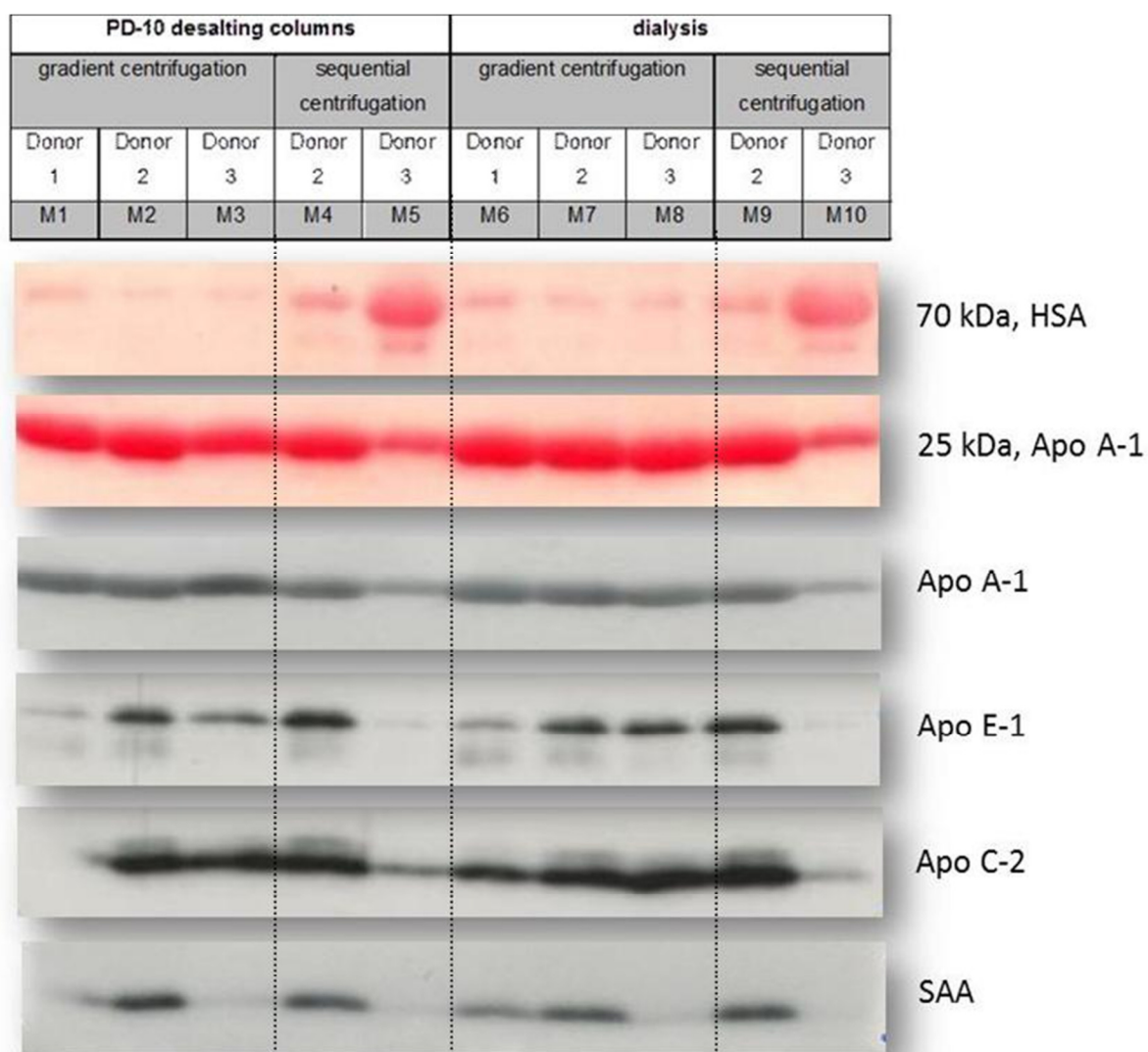


Figure 18 Ponceau-S stains and WB analysis of HDL samples comparing different isolation and desalting techniques

Samples were also subjected to size exclusion FPLC analysis. Figure 19 -Figure 21 show sample composition of each donor using the different isolation and purification techniques.

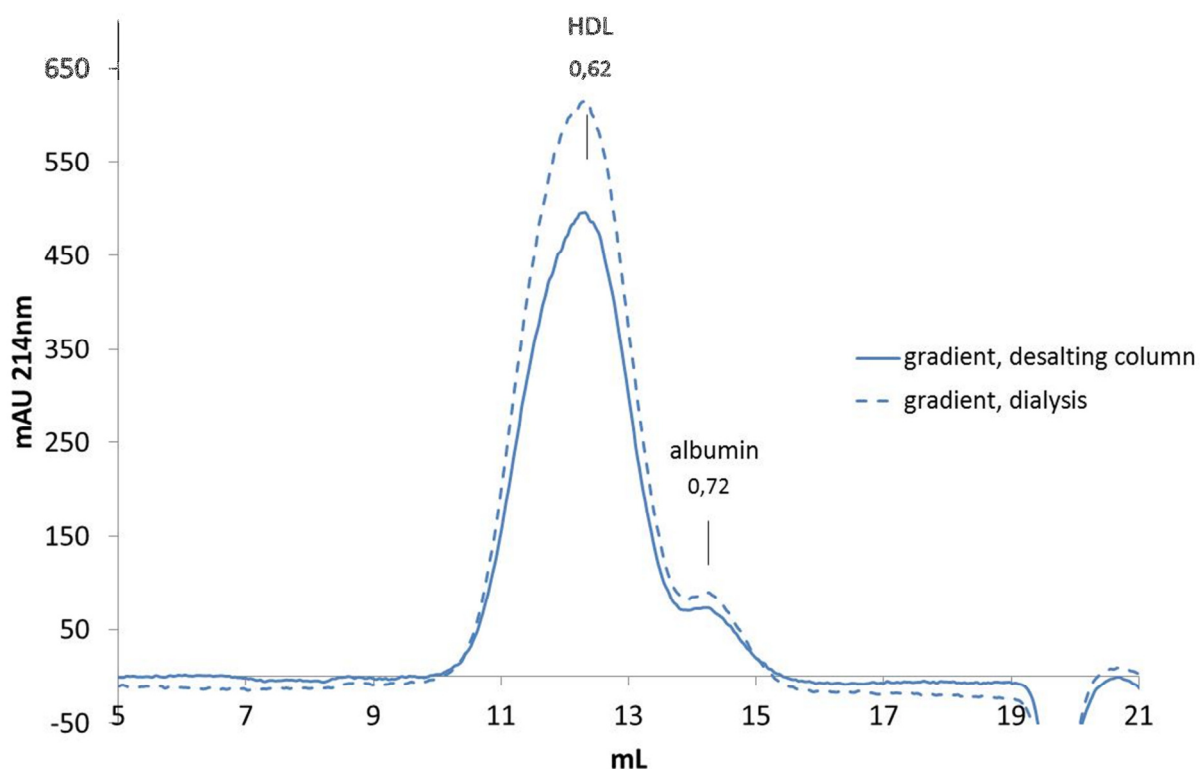


Figure 19 Size exclusion FPLC chromatograms of HDL samples of donor 1, comparing different desalting techniques

Table 11 HDL sample composition according to chromatogram peak areas, donor 1

Donor 1 HDL sample composition	PD-10 desalting columns			Slide-A-Lyzer Dialysis		
	LDL	HDL	albumin	LDL	HDL	albumin
gradient ultracentrifugation	<1%	94%	6%	<1%	94%	6%

HDL samples from donor 1 isolated by gradient ultracentrifugation contain no detectable LDL, the concentration of free protein, labelled as albumin, is about 6%. There is no observable difference between the two desalting methods, (desalting columns and dialysis). The difference in Peak height is probably due to inaccuracies in sample loading but does not affect the interpretation of the chromatograms,

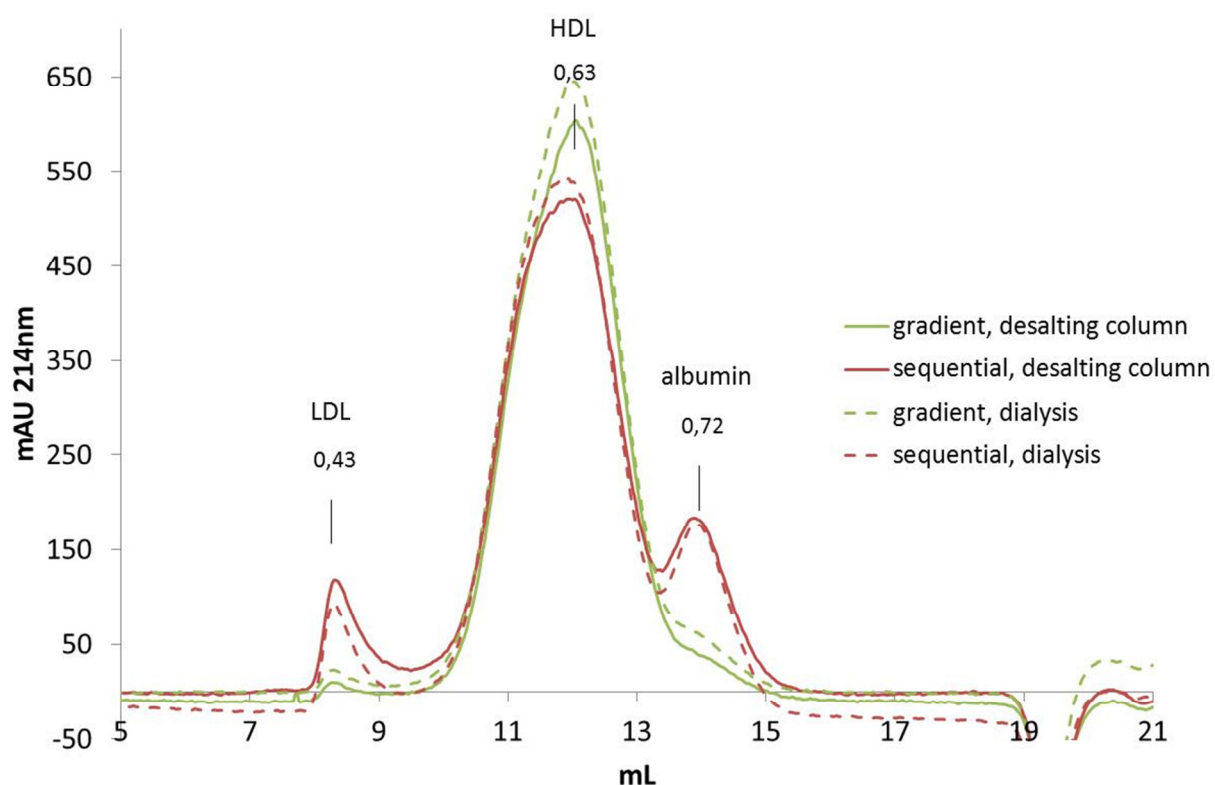


Figure 20 Size exclusion FPLC chromatograms of HDL samples of donor 2, comparing different isolation and desalting techniques

Table 12 HDL sample composition according to chromatogram peak areas, donor 2

Donor 2 HDL sample composition	PD-10 desalting columns			Slide-A-Lyzer Dialysis		
	LDL	HDL	albumin	LDL	HDL	albumin
gradient ultracentrifugation	<1%	98%	2%	<1%	97%	3%
sequential ultracentrifugation	7%	79%	14%	4%	83%	14%

HDL samples from donor 2 isolated by gradient ultracentrifugation have much less impurities than samples isolated by sequential ultracentrifugation. The red lines in Figure 20 (isolation by sequential ultracentrifugation) show clear LDL and free protein (albumin) peaks. In consistency with results for donor 1 there is no observable difference between the two desalting methods, (desalting columns and dialysis).

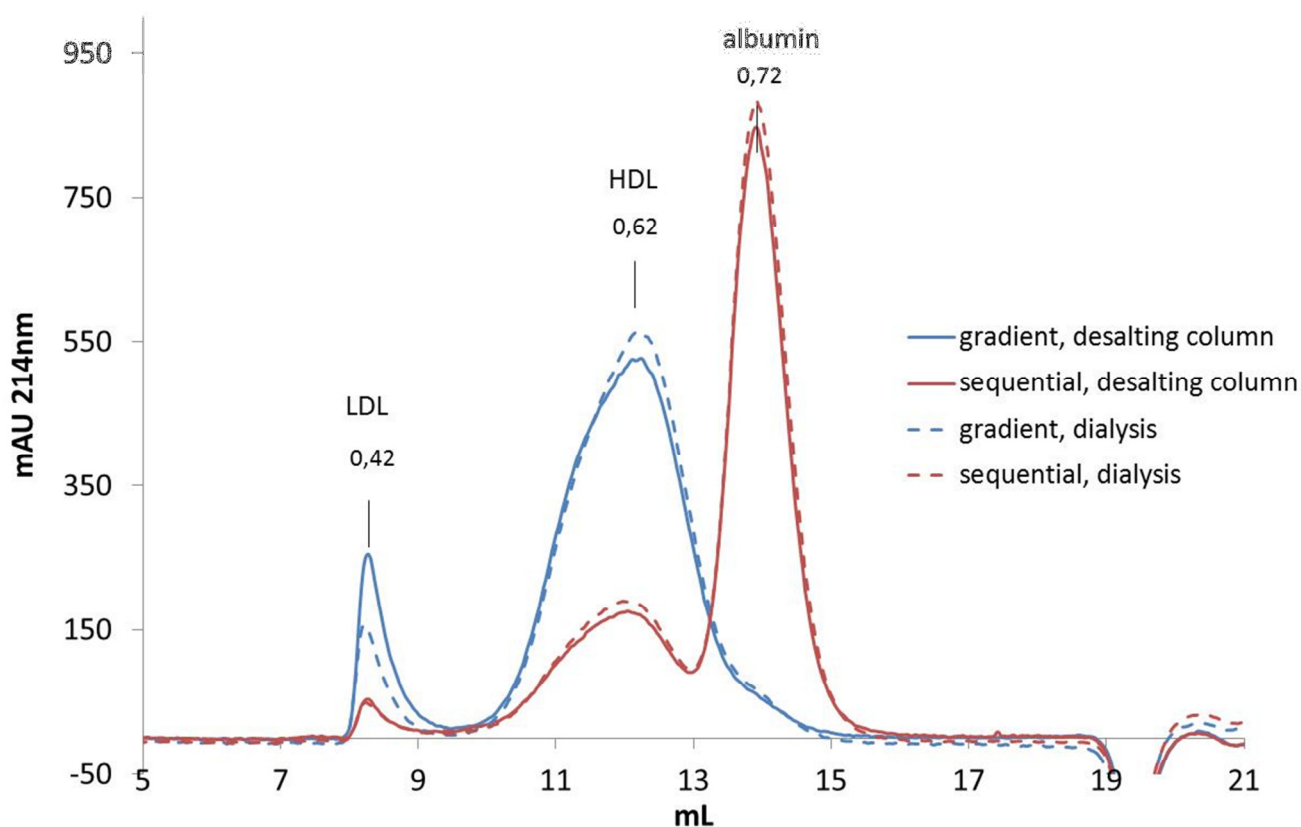


Figure 21 Size exclusion FPLC chromatograms of HDL samples of donor 3, comparing different isolation and desalting techniques

Table 13 HDL sample composition according to chromatogram peak areas, donor 3

Donor 3 HDL sample composition	PD-10 desalting columns			Slide-A-Lyzer Dialysis		
	LDL	HDL	albumin	LDL	HDL	albumin
gradient ultracentrifugation	11%	88%	1%	6%	93%	1%
sequential ultracentrifugation	2%	28%	70%	2%	28%	70%

HDL samples isolated by donor 3 show the highest amount of LDL and albumin impurities thus were isolated poorly. However, these samples give a good insight in what is happening during isolation and help explain why impurities are frequently found in sequentially isolated samples. According to Figure 21 the dominant component in donor 3 HDL samples, isolated by sequential ultracentrifugation (red lines), is albumin. This is also confirmed by a strong Ponceau-S band at 70 kDa (not visible in samples of high purity) and a strong decrease in apo A-1, apo E-1 and apo C-2 band intensities in WB analysis of the according HDL samples (samples M5 and M10 - Figure 18). Together with the results of donor 1 and donor 2 samples it appears that LDL and albumin impurities are inversely correlated. An improved

gradient and high precision in sample removal after gradient isolation will optimize HDL purity.

3.5 HDL sample purity and the effect on immunological experiments

As described earlier (Introduction chapter 1.3) HDL exhibits a number of anti-inflammatory properties, including inhibition of cytokine secretion by various immune cells. From the HDL samples isolated for comparison of isolation and desalting techniques, the purest sample with a HDL content of 98%, is the sample labelled M2 (donor 2, gradient ultracentrifugation, PD desalting columns). M2 HDL was used for cytokine inhibition experiments. THP-1 cells were grown to a density of 1×10^6 cells/mL and preincubated with M2 HDL at concentration of 10, 100 and 400 $\mu\text{g/mL}$ for one hour. Then an inflammatory cytokine response was triggered by the addition of LPS to a concentration of 100 ng/mL. Cells were incubated at 37°C overnight. Cytokine concentration in the supernatant was determined using Luminex IL – Assay. Figure 22 shows concentration dependent inhibition of IL-10, IL-6 and IL-12. Cells stimulated with 400 $\mu\text{g/mL}$ HDL gave no detectable signal for IL-12 expression. TNF- α expression was not influenced by HDL incubation.

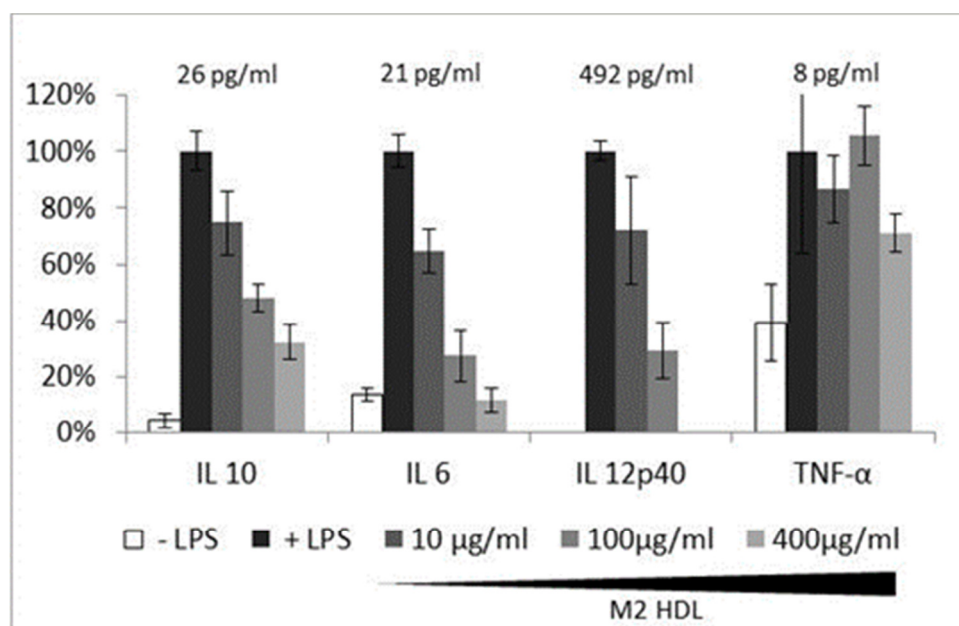


Figure 22 Concentration dependent cytokine inhibition of LPS stimulated THP-1 cells by M2 HDL

In another experiment U937 cells with a GFP-reporter for NF κ B activation were stimulated the same way as the THP-1 cells before. Analysis of the cell suspension using a FACS system revealed inhibition of NF κ B activation similar to interleukin inhibition (Figure 23). Figure 23 also shows that a solution of 3,3 M KBr, the KBr concentration that is necessary to achieve a density of 1,24 g/mL, if desalted using PD-10 desalting columns, has no significant effect on NF κ B activation following LPS stimulation.

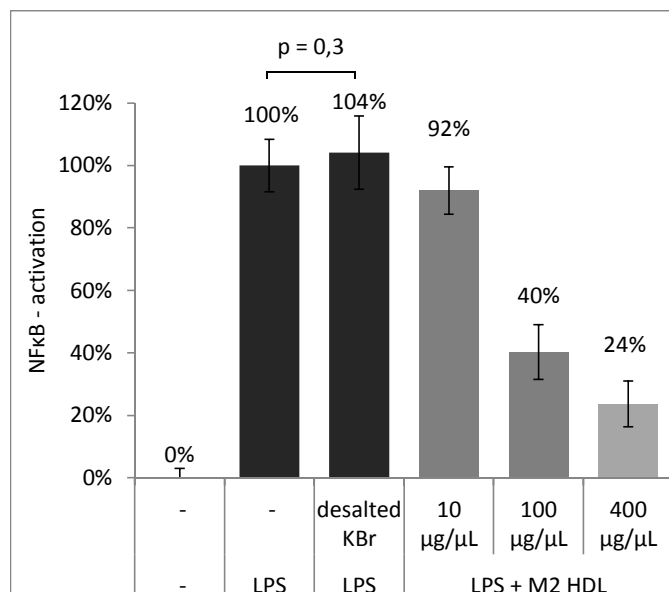


Figure 23 Concentration dependent inhibition of NFκB activation by M2 HDL using U937 cells. P was calculated using the one-sided, student's t-test function "t.test" in Microsoft Excel 2010.

The above experiments were also carried out with all other samples from batch M (different isolation techniques, different purities) using a HDL concentration of 100 µg/µL. Comparing the inhibition efficiency of high purity HDL with LDL and albumin containing HDL samples highlights the importance of high quality HDL samples (Figure 24).

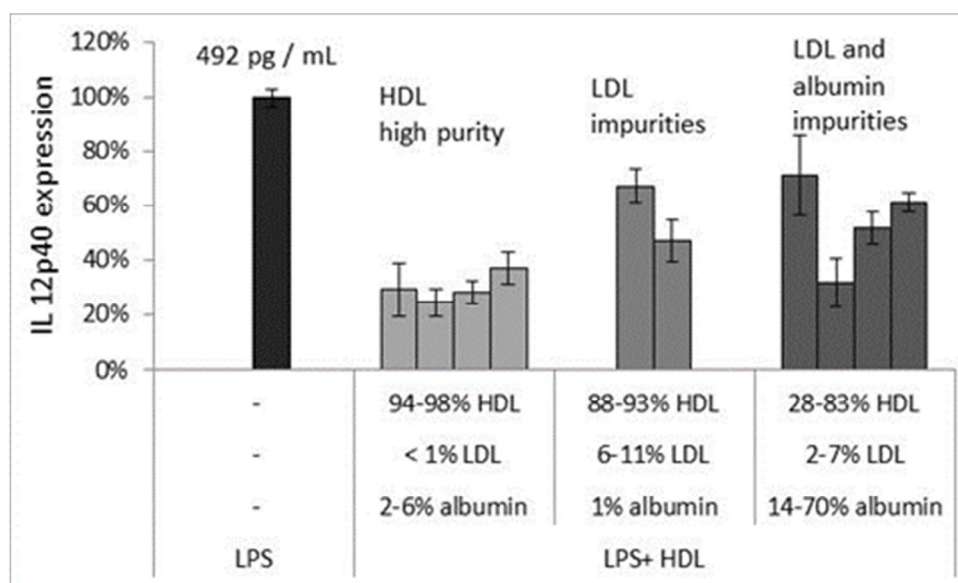


Figure 24 Effect of HDL impurities on inhibition of IL 12p40 expression in THP-1 cells

It is worth noticing that the 10 samples represented in Figure 24 contain different amounts of SAA as shown in WB analysis Figure 18 (page 27). SAA affects the anti-inflammatory capacities of HDL as described earlier (Introduction chapter 1.3). However, SAA loading is quite low in the SAA containing samples of this batch, compared to samples of APR-HDL

where high levels of SAA in HDL affect its anti-inflammatory capacity. This was shown with HDL samples of end stage renal disease (ESRD) patients [9]. The HDL-samples used in this experiment are all isolated from healthy individuals and the individual levels of SAA loading did not affect inhibition of IL-12 expression in Figure 24.

4 Discussion

4.1 LDL and albumin impurities consistently found in HDL samples

FPLC and WB analysis of HDL samples obtained from 4 different laboratories in Vienna and Graz revealed surprisingly high levels of LDL and free non HDL-bound protein, presumably albumin. Both impurities have a big impact on functional analysis of HDL. LDL is a protein that counteracts HDL in cholesterol transport delivering cholesterol to peripheral tissues via LDL-receptors [57] while HDL removes cholesterol from peripheral tissues via the ABCA-1 and ABCG-1 receptors. Thus, LDL is likely to also counteract functional effects of HDL that are dependent on reverse cholesterol transport. Also LDL is susceptible to oxidation and oxidized LDL found in atherosclerotic plaques is pro-inflammatory [58]. Hence, it is quite obvious that LDL impurities have to be minimized when studying HDL. On the other hand impurities of non HDL-bound free plasma protein albumin were found. The sheer quantity of albumin in plasma and protein bands with an approximate mass of 70 kDa at the ponceau-S stains are indications for the identification of those impurities. Of course, all impurities should be avoided as much as possible, but albumin doesn't strike the biochemistry student as a protein interfering with immunological experiments. Not functional interference, but wrong estimates of HDL concentration could be the major problem caused by varying albumin impurities. While impurities of LDL were between 1 and 10% (% of total Peak area in a UV₂₁₄ absorbance chromatogram), albumin impurities accounted for 1 to 70% of total UV₂₁₄ absorbance in a size exclusion FPLC chromatogram.

4.2 Source of LDL and albumin impurities in sequential ultracentrifugation

The quantity of LDL in serum is about 2 or 3 times the quantity of HDL (as a measure of cholesterol) but varies greatly between individuals. For example a 35 year old lipaemic haemodialysis patient had LDL-C of 125 mg/dL and HDL-C of only 25 mg/dL, whereas a 69 year old male patient had much better cholesterol levels: LDL-C of 78 mg/dL and HDL-C of 46 mg/dL. In the isolation protocol for HDL using sequential centrifugation the top 0,5 mL fraction is withdrawn to remove LDL, vLDL and triglycerides. It seems virtually impossible to withdraw the top fraction without leaving some small residual LDL in the centrifugation tube, and withdrawing 0,5 mL might not be enough for lipaemic samples. When withdrawing the bottom fraction after the first centrifugation step without penetrating the tube wall, it seems unavoidable to transfer some LDL into the tubes for the second centrifugation step. LDL impurities accounting for 5 - 10% of total UV₂₄₀ absorption were found consistently in samples isolated using the described isolation procedure (in "Methods" chapter 2.1.2 and "Protocols" in appendix 1.3). Albumin is the most abundant protein in human plasma protein.

After the second centrifugation step in plasma at a density of 1,24 g/mL HDL is withdrawn from the top layer. Presumably just below the HDL layer a lot of albumin is present in the density adjusted serum. Again when withdrawing the top layer it appears to be very challenging to withdraw only HDL and not albumin lying right underneath the HDL layer. Especially if the HDL content (depending on the individual sample) varies and the visual difference of layers in yellow shading is not obvious. See pictures of centrifugation tubes after second centrifugation step in “Results” 3.1.2 Figure 10. That hypothesis can also explain the extreme content of albumin in the samples M5 and M10 (donor 3 sequential ultracentrifugation). If the pipette tip was inserted just a little too deep and reached below the HDL layer, the extreme value of 70% albumin content is explainable, and at the same time the lower LDL content of only 2% (as compared to the 5 - 10% usually found for sequentially isolated samples.)

4.3 Possible improvements for sequential HDL isolation

The purity of HDL samples isolated using sequential isolation could be improved if the bottom layer containing HDL after the first centrifugation step would be removed by penetration the tube wall from the bottom, this would eliminate LDL impurities. Albumin on the other hand seems to be a more persistent by-product of the isolation. Critical is the removal of the top HDL layer after the second isolation step. Possibly HDL purity could be improved at the cost of sample quantity if only 250 µl instead of the usual 500 µL were withdrawn. If the top HDL layer forms a clearly visible band very careful handling by the scientist might be enough to hold albumin contamination under 10%. Either way, quality control of the HDL samples after isolation using FPLC chromatography should be a routinely performed analysis. If albumin contamination remains a problem, purification processes like affinity chromatography should be considered.

4.4 Impurities in HDL samples isolated by gradient centrifugation.

Gradient ultracentrifugation is superior over sequential ultracentrifugation resulting in HDL purities over 90% opposing to less than 80%. This can be explained quite easily by the more elegant setup of a gradient where density adjusted plasma is layered under PBS. During the centrifugation process HDL fractions emerge from the plasma and travel in the gradient to the according density layer. As a result PBS solution is now shielding LDL, HDL, and free plasma protein fraction from one another and sample can be withdrawn by penetrating the tube walls from the side. The hydrodynamic density of albumin and other free plasma proteins at salt concentration around 2 mol/L is hard to predict and it cannot be excluded that they may reside close to the HDL fraction. However FPLC analysis of isolated HDL sample revealed only low amounts of free protein. Low concentrations of free protein in HDL

samples could also result from HDL bound protein that dissociated from the protein-lipid particle.

4.5 Possible Improvements for gradient HDL isolation

HDL Isolation using gradient ultracentrifugation can be difficult, especially when the HDL band is not bright yellow in colour but barely visible (e.g. sample HD2 in Figure 8 in Chapter Results 3.1.1). Impurities from fractions lying above and below the HDL fraction could be minimised by withdrawing a smaller quantity of HDL, possibly reducing sample recovery but improving sample purity. Also by adjusting the densities for the gradient HDL sample purity and concentration might be influenced. If for example density adjusted PBS (addition of KBr to $\rho = 1,063 \text{ g/mL}$) instead of “normal” PBS is used for gradient isolation, LDL will float up to the top of the centrifugation tube and LDL impurities will be diminished. The disadvantage of density adjusted plasma is a “wider” gradient and HDL will be recovered at lower concentrations. It seems that the impurities of LDL and albumin found in gradient ultracentrifugation (Chapter Results 3.4.2) are inversely correlated (see Table 14). That supports the hypothesis that albumin impurities result from layers just underneath the HDL fraction, whereas the LDL fraction obviously lies above the HDL fraction. According to that hypothesis albumin impurities could be diminished by choosing a higher point (lower density) for sample withdrawal of HDL, but that might influence HDL composition.

Table 14 Inverse correlation of LDL and albumin impurities found in HDL samples isolated by gradient ultracentrifugation

	LDL	albumin
HDL donor 1	Not detected	6%
HDL donor 2	>1%	3%
HDL donor 3	8%	1%

4.6 Desalting of HDL using PD-10 desalting columns – concerns about desalting capacity

For HDL isolation using ultracentrifugation high salt concentration up to 3 mol/L KBr are used to generate high density or a density gradient. Removal of KBr is crucial for further immunological analysis of HDL properties as high potassium concentration will influence membrane potential and interfere with cell function. Whereas remaining levels of KBr in the sample after 2 rounds of dialysis are of no concern (according to the protocol in use Appendix Protocols 1.4). The desalting capacity of PD-10 columns with gravity flow remains

an issue to be addressed. According to the manufacturers manuals desalting capacity ranges from 95% to >99%. Potassium concentrations would thus range from 150 mmol to <30 mmol in the sample. The use of HDL samples as 10 x stocks in immunological experiments would then lead to concentrations ranging from 15 to <3 mmol/L, potentially three times higher than average potassium concentration in plasma of 3,5 - 5 mmol/L. To minimize potassium concentration in the sample while optimising dilution factor and sample recovery the desalting procedure was optimized as described (Results chapter 3.2.2), and Appendix Protocols 1.5. To ensure desalting using one-step desalting with gravity flow desalting columns is sufficient a desalted solution of 3 mol/L KBr was used in control experiments. Desalted KBr did not interfere with NF κ B activation (Results chapter 3.5).

5 Conclusion

Analysis of HDL samples from various laboratories in Austria has revealed high levels of LDL and albumin impurities. LDL counteracts most HDL functions and varying levels of albumin lead to wrong HDL concentrations determined by protein quantification assays. Comparison of isolation methods led to the conclusion that gradient ultracentrifugation is superior to sequential ultracentrifugation because it results in higher sample purity. Gradient ultracentrifugation is also less labour intensive and time consuming, but leads to lower HDL concentrations. Comparison of desalting methods did not reveal differences in sample quality. Both methods are suitable for HDL desalting. However the use of PD-10 desalting columns is less time consuming than dialysis and, in combination with gradient ultracentrifugation, allows isolation and desalting of samples in one day. Nevertheless, even an optimised isolation procedure is no guarantee for pure samples as each individual sample behaves differently during isolation processes (e.g. samples from lipaemic patients). Just like people vary in size their lipoproteins vary in concentration and that ultimately affects HDL isolation. Liquid chromatography analysis proved to be a very valuable tool to determine sample purity, and should be carried out routinely to ensure correct interpretation of immunological or any other functional experiments on HDL.

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Appendix

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1 **Protocols**

1.1 *Density Gradient Ultracentrifugation for isolation of HDL and LDL*

Protocol suitable for up to 8 samples, duration 7-9h

Materials

- Centrifuge tubes Quick-Seal (Beckman Tubes Quick-Seal 344322, 16x76 mm)
- Ultracentrifuge rotor (Beckman Type 75 Ti rotor)
- Size exclusion columns (PD-10 Columns GE, Sephadex™ G-25M 17-0815-01)
- KBr (BioUltra, SIGMA 60089)
- PBS ($\rho=1,006$ g/ml)

Preparation of the gradient:

- Take a blood sample (to obtain 4ml Plasma), using EDTA tubes.
- Centrifuge at 400g, 20min, 15°C, no Brake.
- Transfer the plasma to a 15ml tube
- Add KBr to plasma (382 mg KBr / ml Plasma) to achieve ($\rho=1,24$ g/ml)
Note: dissolve KBr gently, vigorous mixing will disrupt Lipoprotein integrity.
- Check plasma density by weighing (adjust density if necessary by addition of PBS or KBr)
- Transfer 10ml PBS ($\rho=1,006$ g/ml) to a centrifuge tube
- Prepare a two-step density gradient by layering 4ml of the density-adjusted plasma ($\rho=1,24$ g/ml) underneath PBS ($\rho=1,006$ g/ml). Note: Use a long needle and add Plasma in an upright position, excess liquid will flood the centrifuge tube.
- Remove liquid from the sealing capillary.
- Seal the centrifuge tube.

Centrifugation:

- Type 75 Ti rotor, fixed angle, 4hours at 50.000rpm

Sample recovery:

- Penetrate the top of the centrifuge tube with a needle.
- Use a needle and syringe and penetrate the side wall of the centrifuge tube (right underneath the layer you want to recover) and recover the fraction (about 1,5mL).
Note: If you want to recover 2 layers recover upper layer first, then withdraw excess liquid, then recover lower layer).

- Store the sample under N₂ atmosphere at 4°C.

1.2 Density Gradient Ultracentrifugation for isolation of HDL₂ and HDL₃

Protocol suitable for up to 8 samples, duration 7-9h

Materials:

- Centrifuge tubes Quick-Seal (Beckman Tubes Quick-Seal 344322, 16x76 mm)
- Ultracentrifuge rotor (Beckman Type 75 Ti rotor)
- KBr (BioUltra, SIGMA 60089)
- Density solution, KBr 83 mg/ml in PBS ($\rho=1,063$ g/ml)

Preparation of the gradient:

- Take a blood sample (to obtain 4ml Plasma), using EDTA tubes.
- Centrifuge at 400 g, 20 min, 15°C, no Brake.
- Transfer the plasma to a 15ml tube
- add KBr to plasma (382 mg KBr / ml Plasma) to achieve ($\rho=1,24$ g/ml)
Note: dissolve KBr gently, vigorous mixing will disrupt Lipoprotein integrity.
- Check plasma density by weighing (adjust density if necessary by addition of PBS or KBr)
- transfer 10ml density solution ($\rho=1,063$ g/ml) to a centrifuge tube
- prepare a two-step density gradient by layering 4 ml of the density-adjusted plasma ($\rho=1,24$ g/ml) underneath density solution ($\rho=1,063$ g/ml). Note: Use a long needle and add Plasma in an upright position, excess liquid will flood the centrifuge tube.
- remove liquid from the sealing capillary.
- Seal the centrifuge tube.

Centrifugation:

- Type 75 Ti rotor, fixed angle, 4 h at 50.000rpm.

Sample recovery:

- Penetrate the top of the centrifuge tube with a needle.
- Use a needle and syringe and penetrate the side wall of the centrifuge tube (right underneath the layer you want to recover) and recover the fraction (about 1,5mL).
Note: If you want to recover 2 layers recover upper layer first, then withdraw excess liquid, then recover lower layer).
- Store the sample under N₂ atmosphere at 4°C.

1.2.1 Calculations for density solutions and centrifugal force:

Calculate KBr for gradient:

$$gKBr = \frac{V(\rho_f - \rho_i)}{1 - (0,312 * \rho_f)}$$

V plasma Volume

ρ_f final density (1,24 g/ml)

ρ_i initial density (check by weighing, usually about 1,025 g/ml)

0,312... partial specific volume of KBr

0,236... partial specific volume of NaBr

1,24 g/ml Plasma: 382 mg KBr / ml Plasma

1,063 g/ml PBS: 83 mg KBr / ml PBS

Calculation for relative centrifugal force:

Use rotor calculation tool at Beckman – homepage:

<https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/centrifugation/rotors/index.htm?t=3> access date January 2014.

$$rcf (g) = 1,118 \times 10^{-5} RS^2$$

R Radius [cm] (from sample to center of rotor), can vary by factor 2 within a tube.

S Speed of centrifuge in rpm.

1.3 Sequential Ultracentrifugation for isolation of HDL

Protocol for 3 samples, duration approximately 11-14h

Materials:

- Centrifuge tubes Thickwall, Polycarbonate, 3 mL
- Ultracentrifuge rotor (Beckman SW 60 Ti rotor)
- KBr (BioUltra, SIGMA 60089)

Preparation Centrifugation step 1:

- Take a blood sample (to obtain 5ml Plasma), using EDTA tubes.
- Centrifuge at 400 g, 20 min, 15°C, no Brake.
- Transfer the plasma to a 15mL tube
- add KBr to plasma (83 mg KBr / ml Plasma) to achieve $\rho=1,063$ g/mL
Note: dissolve KBr gently, vigorous mixing will disrupt Lipoprotein integrity.
- Check plasma density by weighing (adjust density if necessary by addition of PBS or KBr)
- Transfer 2,5ml of the density adjusted plasma $\rho=1,063$ g/mL to a centrifuge tube (gives two tubes per sample)

Centrifugation step 1:

- Type SW 60 Ti rotor, fixed angle, 4 h at 50.000 rpm, 4°C.

Preparation centrifugation step 2:

- Withdraw the top 0,5 mL to remove glycerides, vLDL and LDL fractions
- Collect the bottom fraction of 1ml and pool one sample into a 15 mL tube
- add KBr to plasma (289 mg KBr / ml Plasma) to achieve $\rho=1,24$ g/mL
Note: dissolve KBr gently, vigorous mixing will disrupt Lipoprotein integrity.
- Check plasma density by weighing (adjust density if necessary by addition of PBS or KBr)
- Transfer 2,5ml of the density adjusted plasma $\rho=1,063$ g/mL to a centrifuge tube (gives two tubes per sample)

Centrifugation step 2:

- Type SW 60 Ti rotor, fixed angle, 4 h at 50.000 rpm, 4°C.

Sample recovery:

- Withdraw the top 0,5 mL to achieve the HDL fraction.
- Store the sample under N₂ atmosphere at 4°C.

1.4 *Slide-A-Lyzer Dialysis*

Protocol for 4 samples (0,5 ml – 3 ml), duration 2 days, preparation time 1 day.

Reagents and Equipment:

- Slide-A-Lyzer Dialysis Cassettes Molecular weight cut-off 3,5 kDa, size 0,5 ml - 3 ml (purchased from Pierce)
- Syringes 5 ml, with 18 ga, 1 inch beveled needle
- Phosphate buffered saline

Preparation:

- One day prior to the dialysis prepare 2 beakers with 5 l PBS (4,5 l deionised water + 500ml 10x PBS) and keep them at 4 °C overnight.

Dialysis:

- Filling and removal of samples has to be carried out in a sterile laminar hood.
- Take up sample (0,5 ml – 3 ml) in a syringe.
- Insert syringe through one of the corner ports, inject the sample and withdraw any excess air. Mark the position of the corner port.
- Attach cassette to a float buoy and dialyze against 5 l PBS at 4°C for 20h with mild stirring.
- Transfer cassettes into a beaker with fresh 5 l PBS, dialyze at 4°C for 20h with mild stirring.

Removal of sample:

- Insert empty syringe at a second corner port, inject some air to expand the cassette chamber, then withdraw the dialyzed sample.
- Store the sample under N₂ Atmosphere at 4 °C.

1.5 Desalting using PD-10 Sephadex™ columns

Protocol suitable for 10 samples, preparation time: 1 h, desalting time: 10min

Reagents and Equipment:

- Size exclusion columns (PD-10 Columns GE, Sephadex™ G-25M 17-0815-01)
- Phosphate buffered saline

Sample Purification:

- Equilibrate PD-10 size exclusion columns with 30ml ice cold PBS
- Put 2ml sample on the column (if sample volume is lower dilute sample with PBS)
- Add 1,5ml ice cold PBS (discard flow through)
- Elute sample with 3ml ice cold PBS (now collect flow through)
- Wash columns with 30ml PBS.

Dilution factor, sample recovery and remaining salt concentration depend largely on the fractions, which are collected during the desalting procedure. Figure 12 shows results of PD-10 desalting column elution experiments with BSA solution in four columns. In figures a, b, c and d bars indicate the start and end point of sample collection. In example a the sample collection is optimized for recovery, example b is optimized for low salt content and example c is optimized for high sample concentration. Example d shows sample collection as it was carried out for HDL samples, where dilution rates are ranging from 1.3 to 1.5, sample recovery is about 90% and remaining salt concentration is minimized.

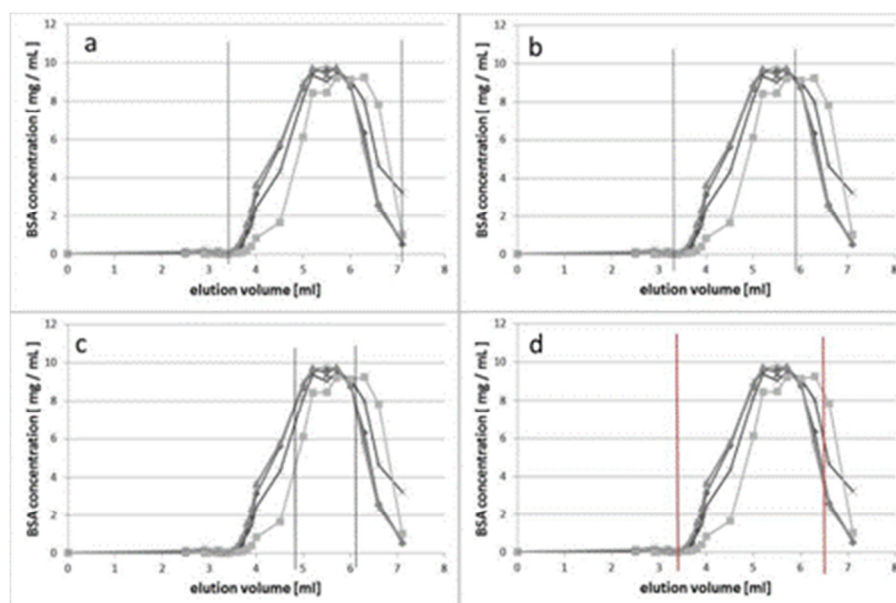


Figure 25 BSA elution experiments; Sample 2 mL BSA solution with concentration of 10 mg/mL, the bars indicate what sample fractions are collected

1.6 Size exclusion FPLC analysis

Preparation time: 2h, Analysis time: 45min per sample

Reagents and Equipment:

- Akta Explorer 10 FPLC (GE, Amersham) equipped with UV-900 detector, conductivity detector, FRAC-900 fraction collector, 6-port valve system.
- Column: Superdex 200 HR 10/30 (GE, Amersham)
- Running buffer, elution buffer, 125 mM NaCl, 1 mM EDTA, PBS
- Unicorn v3.21 Software

Preparation:

- Degas the elution buffer using a vacuum pump
- Insert the buffer into a vacuum chamber and apply a vacuum until the buffer starts boiling.
- Condition the system manual using a flow rate of 0,7ml/min, or pressure up to 10MPa, condition the column until the baseline is stable (30min – 1h). If the column was stored in EtOH previously a flow rate of 0,7 mL/min will not be possible due to pressure increase, instead use a flow rate of 0,1 - 0,2 mL/min, in this case conditioning can take up to 4h.
- Set the flow rate 0,7 mL/min (or maximum flow rate possible to keep pressure under 10MPa), use the same flow rate in the FPLC program Unicorn v3.21

Table 15 FPLC program

Step	Flow rate	Total Volume
1 Conditioning	0,7 mL/min	0,1 mL
2 Injection	0,7 mL/min	1 mL
3 Elution	0,7 mL/min	30 mL

- Enable the UV detector using wavelengths 214nm and 280nm, enable conductivity detector.
- Autozero the UV detector

- If desired enable the automated fractioning using the FRAC-900 fraction collector, collection of 0,5 mL fractions is recommended.

Running the FPLC Analysis:

- Set the 6-port-valve position to load
- Using a 1 mL syringe inject 300µl of sample into the 100µl loop (300µl sample are required due to handling and dead volume).
- Run the FPLC program

1.7 IL-expression Luminex Assay

Reagents and Equipment:

- Luminex analyzer for 96-well plats (e.g. Luminex™ 200 analyzer R&D Systems)
- Luminex 96-well filter plater
- Antibody conjugated beads (IL - 6, IL - 10, IL-12p40 and TNF – α)
- Fluorophore-conjugated secondary antibody (IL - 6, IL - 10, IL-12p40 and TNF – α) or biotinylated secondary antibody and streptavidin-fluorophore conjugate.

Procedure adapted from online resource 8 ¹

- Prepare a standard solution containing 10.000 pg/mL of IL - 6, IL - 10, IL-12p40 and TNF – α .
- Prepare serial dilutions to cover the concentration range from 10 pg/mL to 10.000 pg/mL for every analyte.
- Centrifuge sample supernatants at 16.000 rpm for 1 min to ensure complete removal of cells and cell-debris.
- Pre-wet wells with 200 μ L PBS, aspire the working solution (PBS), an adhesive plate cover may be used to seal the unused wells
- Pipette 25 μ L of the antibody conjugated beads into the wells and aspire the solution, the plate should now be protected from light.
- Pipette standards and samples into the 96 well plates
- Incubate for 2 hours at room temperature on an orbital shaker
- Wash the wells twice with 200 μ L wash solution (PBS).
- Add the fluorophore- conjugated secondary antibody or biotinylated secondary antibody, and incubate for 1 hour at room temperature on an orbital shaker.
- Wash the wells three times with 200 μ L washing solution
- If biotinylated secondary antibody is used, incubate with streptavidin-fluorophore conjugate for 30min at room temperature and wash wells 3 times with 200 μ L washing solution.
- Add 100 μ L working solution, resuspend beads on an orbital shaker (500-600 rpm, 3 min)
- Uncover the plate and insert plate into the XY platform of the Luminex analyzer.

¹ http://www.ucalgary.ca/snyder_chair/luminexgeneral

1.8 U937 NFκB-GFP reporter Assay using FACS Analysis

U937 cells containing a GFP-reporter for NFκB activation were grown to a density of 10^6 cells/mL. Cells were preincubated with HDL or reference blank solutions for 1 h before addition of 100 ng/mL LPS. After 18h cells NFκB activation was quantified directly from the cell suspension using FACS analysis on da BD-DIVA FACS Instrument from BD-Bioscience.

1.9 Quantification using BCATM Protein Assay

Reagents and Equipment:

- BCATM Protein Assay Reagent A Thermo Scientific 23223
- BCATM Protein Assay Reagent B Thermo Scientific 23224
- Bull serum albumin BSA
- Sonificator
- 96-well ELISA plate
- 96-well ELISA plate reader

BCATM Protein Assay:

- Samples should be sonificated to ensure homogenisation of protein
- BSA standard solutions are prepared according to Table 16.

Table 16 Preparation of BSA standard solutions for BCATM Protein Assay

	Concentration	Preparation
BSA stock solution	20 mg/mL	Dissolve 1g BSA in 50mL PBS
Standard 5	2,0 mg/mL	100 µL BSA stock + 900 µL PBS
Standard 4	1,0 mg/mL	100 µL Standard 5 + 100 µL PBS
Standard 3	0,5 mg/mL	100 µL Standard 4 + 100 µL PBS
Standard 2	0,25 mg/mL	100 µL Standard 3 + 100 µL PBS
Standard 1	0,125 mg/mL	100 µL Standard 2 + 100 µL PBS

- Pipette 10µl of standard solution or sample into a 96-well ELISA plate, pipette all samples as duplicates as illustrated in Table 17

Table 17 Pipetting table for BCA™ Protein Assay ELISA plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS		Standard 1		Standard 2		Standard 3		Standard 4		Standard 5	
B	Sample 1		Sample 2		...		...		...		...	
C	...		...		...		...		...		...	
D	...		...		...		...		...		...	
E	...		...		...		...		...		...	
F	...		...		...		...		...		...	
G	...		...		...		...		...		...	
H	...		...		...		...		...		Sample 42	

- Mix BCA™ reagents (50 parts reagent A : 1 part reagent B), 2,5 mL BCA™ reagent mix is sufficient per row.
- Using a multipath pipette, add 200µl BCA™ reagent mix to each well.
- Incubate the plate for 30min at 37°C, turn on ELISA reader (lamps need a couple of minutes to heat up and give constant light intensity).
- Measure absorbance at a wavelength of 550nm.
- Calculate the concentration of samples using linear regression analysis.

To determine the protein concentration of the sample, it has to be lower than 2 mg/mL (concentration of the highest standard), for a sample of unknown concentration dilutions 1:10 and 1:100 should also be measured.

1.10 WB Standard procedure

Reagents and Equipment:

- 30% Acrylamide/Bis solution 29:1 (3.3%C) BIO-RAD 161-0400
- Tris: Trizma® base T1503 – Sigma
- Glycin (Fluka 50052)
- Glycerol
- Methanol (Fisher Chemical M/4000/17)
- Tween 20 BIO-RAD 170-6531
- Blotting grade blocker, nonfat dry milk BIO-RAD 170-6404
- SDS – Merck 3311901
- Triton X-100 Sigma 9002-93-1
- HEPES SIGMA 7365-45-9
- Dithiothreitol: DL – DTT SIGMA 1001253868
- TEMED (BioRad)
- APS
- Protein Marker – PeqLab Protein Marker IV
- Bull serum albumin BSA
- NaF
- Na₃VO₄
- Glycerophosphate
- Iso-propanol Fluka 59319
- Amersham ECL™ Western Blotting Detection Reagents, GE Healthcare RPN2106
- Ponceau-S

- Gel pouring stands (BioRad)
- Power supply
- Sonificator
- Test tube thermo block or PCR cyclor
- SDS electrophoresis apparatus (BioRad)
- PVDF-membrane (Amersham)
- Blotting paper
- Amersham Hyperfilm ECL™ – GE Healthcare
- Film development system
- Semi-dry blotting apparatus – BIO-RAD 170-3940

Stock solutions:

5x Laemmli buffer (pH 8,3 1000 mL): 125 mM (15,14 g) Tris, 960 mM (72,07 g) Glycin, 0,5% (5 g) SDS, add H₂O (milli-Q grade)

5x Transfer buffer (pH 8,3 1000 mL): 125mM (15,14 g) Tris, 960 mM (72,07 g) Glycin, add H₂O (milli-Q grade)

10x TBS – Tris buffered saline (pH 7,4 1000 mL): 250mM (30,3 g) Tris, 1550 mM (90,6 g) NaCl, dissolve in H₂O (milli-Q grade) add hydrochloric acid to reach pH 7,4 (about 17 mL of 37% HCl), add H₂O (milli-Q grade)

10x Phosphatase Inhibitor Cocktail (10 mL): 500 mM (210 mg) NaF, 100 mM (446 mg) Na₄P₂O₇, 100 mM (216 mg) Glycerophosphat, 10 mM (18 mg) Na₃VO₄

50x Protease Inhibitor cocktail (Protease Inhibitor cocktail tablet complete, EDTA-free – Roche 1187358001)

Triton-X-100 Lysis buffer (2 mL): 200µl 10x Phosphatase Inhibitor, 40µl 50x Protease Inhibitor, 200µl 10% Triton-X-100, 1mL 2x basic buffer (buffer of choice, HEPES pH 7,5 or 1x TBS pH 7,4), 560µl H₂O (milli-Q grade),

4x Sample buffer (12 mL): 6mL 0,5 M Tris-HCl (pH 6,8), 40% (4,8 mL) glycerol, 8% SDS (0,96 g), stain with bromphenolblue, add H₂O (1,2 mL). For reducing sample buffer add 4x sample buffer to the sample and add DTT (62 mg/ml) (400 mM DTT).

Working solutions:

1x Laemmli buffer (pH 8,3 1000 mL): 200 mL 5x Laemmli buffer, add H₂O (milli-Q grade)

1x Transfer buffer (pH 8,3 1000 mL): 200 mL 5x Transfer buffer, 200 mL Methanol, add H₂O (milli-Q grade)

1x TBS-T – Tris buffered saline + tween (1000 mL): 100mL 10x TBS, 0,05% (500µl) Tween-20, add H₂O (milli-Q grade)

0,5 M Tris-HCl (pH 6,8) – stacking gel buffer (500 mL): 500 mM Tris (30,3g) Tris, dissolve in H₂O (milli-Q grade) add hydrochloric acid to reach pH 6,8 then add H₂O (milli-Q grade)

1,5 M Tris-HCl (pH 8,8) – resolving gel buffer (500 mL): 1500 mM Tris (90,9g) Tris, dissolve in H₂O (milli-Q grade) add hydrochloric acid to reach pH 8,8 then add H₂O (milli-Q grade)

Stripping buffer (50 mL): 6,25 mL 0,5 M Tris-HCl (pH 6,8), 2% (1 g) SDS, 100 mM (350 µL) β-Mercaptoethanol, add H₂O (milli-Q grade)

Blocking solution (50 mL): 5% (2,5 g) nonfat dry milk or 5% (2,5g) BSA, dissolve in 1x TBS-T

Antibody solution (10 mL): 1:500 – 1:10000 (20 µl – 1 µL) Antibody of choice, dissolve in Blocking solution.

Ponceau-S staining solution (50mL): 0,1% (0,05 g) Ponceau-S, 5% (2,5 mL) acetic acid, add H₂O (milli-Q grade)

Pouring the Gel:

For pouring a polyacrylamide gel the concentration of acrylamide in the resolving gel is critical. High concentrations of 15% acrylamide will provide good separation for small proteins (<20 kDa), whereas low concentrations of 7,5% will result in better resolution for larger proteins (>70 kDa). Mix the solutions according to Table 18, after addition of TEMED and APS polymerisation starts. The quantities in Table 18 are sufficient for pouring two gels (of about 12 x 7 cm).

Table 18 Mixing table for pouring SDS acrylamide gels (reproduced from laboratory protocols by Christopher Kaltenecker, BSc)

	stacking gel (%)	resolving gel (%)			
	4	7,5	10	12	15
volume	2,5 mL	10 mL	10 mL	10 mL	10 mL
30% cis-acrylamid	0,33 mL	0,33 mL	0,33 mL	0,33 mL	0,33 mL
0,5 M Tris-HCl pH 6,8	0,63 mL	0,63 mL	0,63 mL	0,63 mL	0,63 mL
1,5 M Tris-HCl pH 8,8					
10% SDS	0,03 mL	0,03 mL	0,03 mL	0,03 mL	0,03 mL
H ₂ O (milli-Q grade)	1,52 mL	1,52 mL	1,52 mL	1,52 mL	1,52 mL
TEMED	3 µL	10 µL	10 µL	10 µL	10 µL
10% APS	13 µL	50 µL	50 µL	50 µL	50 µL

- Wear protective gloves while pouring gels, acrylamide is highly toxic
- Set up the gel pouring apparatus according to the manufacturers manual
- Mix solutions for a resolving gel, add TEMED and APS as the last step, after addition of TEMED and APS work quickly to avoid inconsistent polymerisation.
- Mix the solution (resolving gel only) by pushing it through a pipette 5 times
- Pipette the resolving gel into the gel apparatus until it is about ¾ full
- Add 1 mL iso-propanol (creates a flat and smooth surface)
- Wait 20 min until polymerisation is complete
- Remove iso-propanol by pouring it out of the gel-apparatus, use filter-paper to remove residue iso-propanol
- Mix solutions for the stacking gel, add TEMED and APS and mix like before

- Pipette the solution on top of the resolution gel until the apparatus is full
- Insert the comb into the liquid stacking gel (avoid bubbles, make sure there is sufficient stacking gel between the slots)
- Wait 20 min until polymerisation is complete.
- Use a permanent marker and mark the positions of the slots on the glass
- Before use, carefully remove the comb (avoid rupture of the slots).
- Gels can be stored at 4°C for several weeks if wrapped into wet paper-tissue.

Reducing SDS polyacrylamide electrophoresis (SDS-PAGE):

- Samples and 4x sample buffer are combined (e.g. 30µl sample + 10µl 4x sample buffer), consider the high viscosity of the sample buffer (cut pipette tip and pipette slowly). Add DTT to the 4x sample buffer before use (see stock solutions). Also prepare a blank solution combined with 4x sample buffer.
- Samples are sonicated to ensure homogenisation
- Samples are heated to 95°C for 5 min to ensure complete reduction and denaturation
- The gel is placed in the electrophoresis apparatus (mark the slots for loading with permanent marker)
- Fill the apparatus with 1x Laemmli buffer
- Using a syringe rinse the slots of the gel with Laemmli buffer (small dust particles can prevent uniformity of the electrophoresis)
- Load the samples and the protein marker, load empty slots with blank solution containing sample buffer (this will also increase uniformity of the electrophoresis).
- Run the electrophoresis, typical settings are 90V, with a maximum current of 15mA per Gel. (Higher voltages increase migration speed but cause high current flow and heat the system, lower voltages increase the run time but can improve the resolution).
- After removal of the gel separate the stacking gel from the resolution gel. Cut one corner of the gel (e.g. top left corner) to know the orientation of the gel.

Semi dry blotting:

- Cut blotting paper to the size of the electrophoresis gel
- Cut PVDF membrane to the size of the gel (PVDF membranes are highly adsorbent, wear gloves and avoid contamination of the membrane, minimize contacts with anything but the electrophoresis gel and clean blotting paper to avoid artefacts.).
- Cut one corner of the membrane (e.g. top left corner) to know the orientation of the gel.
- Soak the PVDF membrane in pure methanol for a few seconds to moisture it

- Soak the PVDF membrane, the blotting paper and the polyacrylamide electrophoresis gel in transfer buffer for 5 min.
- Assemble the blotting sandwich according to manufacturer's instructions
- Run the semi dry blotting at 25 V, do not exceed 3 mA/cm². Run the semi dry blot for 30 min (50 min for large proteins >150 kDa).
- Disassemble the blotting sandwich and rinse the membrane with TBS-T.

Ponceau-S stain:

- Soak the PVDF membrane in Ponceau-S staining solution for 5 min.
- Rinse the membrane with H₂O (milli-Q grade), until protein bands are clearly visible.
- Destain by washing in TBS-T, 3 x 5 min

Antibody detection:

- Incubate membrane in blocking solution, 1 h at room temperature or overnight at 4°C
- Incubate membrane with primary antibody solution (antibody against your target protein)
- Rinse membrane with TBS-T then wash membrane 3 x 10 min with TBS-T
- If the primary antibody is not peroxidase conjugated, incubate membrane with secondary antibody (peroxidase conjugated antibody against primary antibody) 1 h at room temperature or overnight at 4°C. Then rinse and wash the membrane 3 x 10 min with TBS-T
- Place the membrane on transparent foil
- Mix equal volumes of Amersham ECLTM reagent 1 and Amersham ECLTM reagent 2 and cover the membrane (about 2 mL per gel)
- Incubate the membrane for 5 min in the dark at room temperature.
- Place the membrane between two transparent foils into a film cassette.
- To develop the film work in a darkroom, never turn on the light when working with photosensitive films.
- Place a sheet of autoradiography film on top of the membrane, close the cassette and expose for 15sec – 10min
- Develop the film in a dark room using a film development system

Stripping and new detection

- After detection incubate the membrane in stripping buffer for 20 min at 56°C
- Rinse membrane with water, wash with TBS-T 3 x 10 min
- Incubate membrane in blocking solution, 1 h at room temperature or overnight at 4°C

- continue with new antibody detection as above

Curriculum Vitae

Georg Michlits, BSc.

School and University Education

Dates	October 2009 – currently
Name of the Institute	University of Vienna Währinger Straße 42, 1090 Vienna, Austria
Main Subjects	Biological Chemistry (MSc) Bioanalytical Chemistry, Biophysical Chemistry, Cellbiology, Immunology, Structural Biochemistry
Qualification	Master of Science Results: N.A.
Dates	October 2010 – January 2011
Name of the Institute	University of Gothenburg Medicinaregatan 5, 413 90 Gothenburg, Sweden
Main Subjects	Advanced Immunology, Advanced Structural Biochemistry, Swedish, (Erasmus exchange program)
Qualification	Master of Science (30 out of 120 ECTS) Results: Distinction on all courses
Dates	September 2007 – June 2008
Name of the Institute	Dublin Institute of Technology DIT Kevin Street, Dublin 8, Ireland
Main Subjects	Physical and Life Sciences / Chemistry (BSc) Analytical Chemistry, Inorganic Chemistry, Organic Chemistry, Spectroscopy, Physical Chemistry, Chemical Technology, Environmental Chemistry, Pharmaceutical Chemistry.
Qualification	Bachelor of Science - 25.06.2008 Results: Distinction

Dates	September 2002 – June 2007
Name of the Institute	College of Chemistry Specialising in Biochemistry, Biotechnology and Genetic Engineering Rosensteingasse 79, 1170 Vienna, Austria
Main Subjects	Analytical Chemistry, Biochemistry, Biotechnology, Business, Genetic Engineering, Organic Chemistry, Physical Chemistry, Process Engineering
Qualification	School leaving certificate / A-level – 15.06.2007 Results: Distinction

Dates	September 1998 – June 2002
Name of the Institute	GRG 23, Secondary school Anton Baumgartnerstraße 123, 1230 Vienna, Austria

Dates	September 1994 – June 1998
Name of the Institute	Volksschule Alt Erlaa, Primary school Anton Baumgartnerstraße 44-II, 1230 Vienna, Austria

Work experience

Dates	June 2008 – December 2008
Status of employment	Research Assistant
Main activities and responsibilities	• Assay validation (on antimicrobial efficacy) • Nanoparticle development • Evaluation of antimicrobial properties
Name and address of employer	Dublin Institute of Technology CREST – Centre for Research in Engineering Surface Technology 143-149 Rathmines Road, Dublin 6, Ireland
Working field	Antimicrobial Nanoparticle Coatings

Dates	July 2006 – August 2006
Status of employment	Internship - Analytical Chemistry Quality Control Laboratory
Main activities and responsibilities	Performing analytical standard techniques • Microscopic analysis of the activated sludge • Spectrophotometric analysis of various compounds • HPLC Control of the plants settling and aeration tanks Servicing of online oxygen sensors
Name and address of employer	Entsorgungsbetriebe Simmering Ges.m.b.H Haidequerstraße 6, 1110 Vienna, Austria
Working field	Analytical and environmental chemistry

Dates	August 2004 – September 2004
Status of employment	Internship – Analytical Laboratory Environmental Chemistry
Main activities and responsibilities	Routine analysis of ashes from waste incineration. Storage, identification and disposal of toxic chemicals and heavy metals. • AAS, TOC • GC, HPLC • Spectrophotometric analysis • Electrochemical analysis
Name and address of employer	Gemeinde Wien MA48 - ABA Percostraße 2, 1220 Vienna, Austria
Working field	Analytical and environmental chemistry
Dates	January 2009 – Juli 2009
Status of employment	Mandatory military service, Research Assistant
Main activities and responsibilities	Basic military training, Research activities on • European security policy • Conflict South Ossetia (Georgia, Russia) • Conflict Transnistria (Moldova) Event Management and Organisation of • United Nations – Senior Mission Leader Course • Symposium on post-conflict Bosnia
Name and address of employer	National Defence Academy Institute for Peace- and Conflict-Management Stiftgasse 2 a, 1070 Vienna, Austria
Working field	Ethics and social sciences, Event Management
Dates	May 2006 – June 2006
Status of employment	Educational Work experience
Main activities and lectures	Quality Control Laboratories – Baxter Bioscience Introduction into various activity areas of a pharmaceutical company. Work in various Departments of Quality Control • QC of vaccines, ELISA techniques • Coagulation Analysis • QC of fibrin sealing • QC of clinical devices Advanced training in QA, GMP and Validation procedures.
Name and address of employer	Baxter Vaccine plm. Industriestraße 67, 1220 Vienna, Austria
Working field	BioSience QC / GMP

Abstract

High density lipoprotein (HDL) has been the focus of intense research since a correlation between low blood levels of HDL-cholesterol and decreased incidence of cardiovascular disease was found. HDL has been shown to be the major player in reverse cholesterol transport, which is transport of cholesterol from peripheral tissues to the liver where it is then secreted in the bile. HDL has also the ability to inhibit the inflammatory response of immune cells by inhibiting cytokine expression and expression of surface receptors. This has attracted the interest of immunologists and the functional assessment of HDL became the prime goal for HDL research.

To assess the impact of composition and purity of HDL samples on immunological experiments we analysed HDL samples from four different laboratories in Austria using liquid chromatography. In three samples we found LDL impurities, and in all four of them high levels of albumin impurities. HDL content was not higher than 67% in any of those samples. In order to optimise HDL sample quality we used a strategic approach optimising and comparing two isolation techniques (gradient ultracentrifugation and sequential ultracentrifugation) in combination with two desalting techniques (dialysis and polydextran desalting columns).

We developed a protocol using gradient ultracentrifugation and polydextran desalting columns that led to the isolation of HDL samples of 98% purity. Consequently, we demonstrated the importance of HDL sample purity for immunological experiments by showing concentration-dependent inhibition of interleukin expression in monocytes and reduced anti-inflammatory properties of samples of less than 93% and less than 83% purity.

This thesis should highlight the importance of HDL purity, be a guide on how to optimise HDL isolation procedures and be a reminder that size exclusion chromatography is a valuable tool to assess HDL sample quality and that it should be applied routinely.

Zusammenfassung

Seit der Erkenntnis, dass niedrige Blutwerte an HDL-C (high density lipoprotein-cholesterol) ein erhöhtes Risiko für kardiovaskuläre Erkrankungen darstellen, ist HDL in den Fokus der Forschung gerückt. HDL spielt nicht nur eine zentrale Rolle im RCT (reverse cholesterol transport), also dem Transport von Cholesterol von peripherem Gewebe zur Leber und in weiterer Folge zur Ausscheidung über die Galle, sondern beeinflusst auch das Immunsystem. Es wurde gezeigt, dass HDL anti-inflammatorische Eigenschaften hat und zum Beispiel die Expression von Interleukinen und Oberflächenrezeptoren in verschiedenen Immunzellen inhibieren kann. Das zentrale Ziel der HDL-Forschung ist nun die Aufklärung verschiedener funktioneller Mechanismen um ein besseres Verständnis der immunologischen Eigenschaften von HDL zu erlangen.

Um den Einfluss von Zusammensetzung und Reinheit isolierter HDL-Proben auf immunologische Experimente zu untersuchen analysierten wir HDL-Proben aus vier Laboratorien in Österreich mittels Ausschlusschromatographie. Wir fanden dabei Anteile an LDL in drei und hohe Anteile an Albumin in allen vier Proben. Keine der uns bereitgestellten Proben hatte einen höheren Anteil als 67% HDL. Um die Qualität der HDL Proben zu verbessern, führten wir eine schrittweise Optimierung der Isolationsprotokolle durch. Desweiteren verglichen wir zwei parallel durchgeführte Isolationsmethoden (Gradientenultrazentrifugation und sequentielle Ultrazentrifugation) kombiniert mit zwei ebenfalls parallel durchgeführten Entsalzungsmethoden (Dialyse und Entsalzung mittels Polydextransäulen).

Wir entwickelten ein Protokoll zur Isolation von HDL mittels Gradientenultrazentrifugation und Entsalzung mit Polydextransäulen und erreichten damit einen HDL-Anteil von 98% mit nur etwa 2% Albumin. Mit diesen Proben zeigten wir die konzentrationsabhängige Inhibierung der Interleukinexpression von Monozyten und demonstrierten die Einschränkung der anti-inflammatorischen Wirkung von HDL-Proben mit weniger als 93% und weniger als 83% Reinheit.

Diese Arbeit soll unterstreichen wie wichtig die Reinheit von HDL-Proben für funktionelle Analysen ist. Auch soll sie als Anleitung zur Optimierung von HDL Isolationsmethoden dienen. Eine wichtige Erkenntnis ist auch, dass Ausschlusschromatographie als wichtiges Instrument zur Qualitätsüberprüfung von HDL-Proben herangezogen werden kann und auch routinemäßig eingesetzt werden sollte.