Dissertation

The role of nuclear magnetic resonance spectroscopy methods in the measurement of lipoprotein subclasses

submitted by

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for the Academic Degree of

Doctor of Medical Science (Dr. scient. med.)

at the

Medical University of Graz

Division of Angiology

under the Supervision of

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2022

DECLARATION

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Martin Rief, 28th September 2022

Disclosures

This study was conducted in collaboration with the Medical University of Graz and the laboratory companies Numares Aktiengesellschaft (AG) in Regensburg, Germany and Labcorp Incorporated (Inc.) in Morrisville, North Carolina, USA.

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Parts of this thesis have been published in the following article:

Rief M, Raggam R, Rief P, Metnitz P, Stojakovic T, Reinthaler M, Brodmann M, März W, Scharnagl H, Silbernagel G. *Comparison of Two Nuclear Magnetic Resonance Spectroscopy Methods for the Measurement of Lipoprotein Particle Concentrations. Biomedicines.* 2022 Jul 21;10(7):1766. doi: 10.3390/biomedicines10071766. PMID: 35885071; PMCID: PMC9312544.

Furthermore, parts of this dissertation were presented in a poster presentation at the *VMAC 2022 congress* in Augsburg, Germany (March 17-19, 2022) and at the *Doctoral Day of the Medical University of Graz*, in Graz, Austria (on February 10, 2022).

Acknowledgements

First of all I want to thank my supervisor Assoz.Prof. PD Dr. Günther Silbernagel and Assoz.Prof. Dr. Hubert Scharnagl for their continuous support and the head of the Department of Clinical Angiology Ao.Univ.Prof.^{in}Dr.^{in} Marianne Brodmann.

Of course i also want to thank the other member of my dissertation committee Univ.Prof.DDr. Philipp Metnitz and PD Dr. Reinhard Raggam and the head of the doctoral school Univ.Prof.^{in}DI^{in} Dr.^{in} Andrea Berghold. I would especially like to emphasize that my department head gave me a lot of time to finish my studies and dissertation, thank you for that.

Last but not least I want to thank my family, my brother PD DDr. Peter Rief for his support throughout the conduction of the study and the thesis, my brother PD DDr. Harald Rief, my parents Dr. Meinhard Rief and Magdalena Rief for making it possible for me to study and for their comprehensive support. I also want to thank my girlfriend DI^{in} Hanna Heimburg for the accompaniment and support in all situations over the last seventeen years.

This thesis was done as a part of the doctoral program at the doctoral school „Sustainable Health Research“.
Financial support in form of a bench fee was provided by the Medical University of Graz to finance various laboratory materials. The Medical University of Graz paid the fee for publication in *Biomedicines*.

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Abbreviations

AG……Aktiengesellschaft

AGES……... Federal Office for Safety in Health Care of Austria

Apo…….apolipoprotein

ß……..beta

BASG……...Bundesamt für Sicherheit im Gesundheitswesen

c ……...cholesterol

C …….celsius

CDC……...Center for Disease Control and Prevention

CE……....Conformite Europeenne

CI…….confidence interval

CVD …….....……...cardiovascular diseases

CTEP……cholesterylester transfer protein

EAS……..European Society of Atherosclerosis

ESC…….European Society of Cardiology

FDA……...…Food and Drug Administration

Fig…….……figure

g……..relative centrifugal force

g/ml…….……..gram per millilitre

GGE……...gradient gel electrophoresis

h…….…….hour

IFCC…….....International Federation of Clinical Chemistry

Inc.…….incorporated

Ho……...null hypothesis

H1…….alternative hypothesis

HDL ……..high density lipoprotein

HMG-CoA…….....3-Hydroxy-3-Methylglutaryl-Coenzym-A-Reduktase

IBM…….....International Business Machines Corporation

IDL ……..…..intermediate density lipoproteins

IRB……..….Institutional Review Board

Labcorp …….....Laboratory Corporation of America Holdings

L ……..……...large

l……..…...litre

LDL ……...…low density lipoprotein

Lp(a)……..…....lipoprotein a

m ……..…….medium

MA……...Massachusetts

ml……..…millilitre

MHz……..…..megahertz

min ……..…minute

mg/dl ……..…milligram per decilitre

ml …….....millilitre

nmol/l…….…...nanomole per litre

nm……nano metre

NMR ……....nuclear magnetic resonance spectroscopy

nr……..…….…..number

p……...……..particles

% ……....percent

P ……....standard deviation

r ……..Pearson correlation coefficient

ref.……...…...reference

rpm …….rounds per minute

s ……...……...small

sdLDL.……...…small dense LDL

TC ……....Total cholesterol

TG ……...…..Triglycerides

USA…….United States of America

VAP…….….vertical auto profile

VLDL ……....very low density lipoprotein

µmol/l……..…….micromole per litre

µL……..……..micro litre

Figures

Tables

Zusammenfassung

Hintergrund.

Lipoproteine sind wesentliche Bestandteile bei Körperfunktionen des menschlichen Organismus und haben in den letzten Jahrzehnten immer mehr an Bedeutung bei der Bestimmung des kardiovaskulären Risikos erlangt. Zur Bestimmung der Lipoproteine und ihrer Subklassen werden verschiedene Methoden eingesetzt wobei die Ultrazentrifugation als Referenzmethode in der klinischen Routine bei der Bestimmung der Standardlipide (Gesamtcholesterin, Triglyceride, Low- und High Density Lipoprotein Cholesterol) eingesetzt wird. Eine weitere praktikable Messmethode ist die Kernspinmagnetresonanzspektroskopie (= nuclear magnetic resonance spectroscopy oder kurz NMR) in der Bestimmung der Lipoprotein Subklassen. Um die Qualität und Quantität von NMR-Methoden bei der Messung von Standardlipiden und Lipoprotein-Subklassen zu untersuchen wurde ein Methodenvergleich von zwei anerkannten NMR Methoden durchgeführt. Diese Dissertation behandelt die Thematik rund um die laborchemische Messung von Lipoproteinen und deren Subklassen mittels NMR.

Methoden.

Im Rahmen einer prospektiven Methodenvergleichsstudie wurde von einhundert-fünfzig Patienten*innen der Universitätsklinik für Innere Medizin (Klinische Abteilung für Angiologie) und der Universitätsklinik für Anästhesiologie und Intensivmedizin (Klinische Abteilung für Allgemeine Anästhesiologie, Notfall- und Intensivmedizin) der Medizinischen Universität Graz, zwischen dem 17. Februar und dem 30. Juni 2020, Serumproben gesammelt. Die Routine-Blutuntersuchungen wurden im ambulanten und stationären Setting durchgeführt. Alle Studienpatienten, die in diese Methodenvergleichsstudie eingeschlossen wurden, gaben vorab ihre schriftliche Zustimmung.

Die Serumproben wurden in drei Laboratorien analysiert: "Numares" (NMR-Methode, Regensburg, Deutschland), "Labcorp" (NMR-Methode, Burlington, North Carolina- USA) und "Referenz- bzw. Standardmethode" (kombinierte Ultrazentrifugation/Fällung, Medizinische Universität Graz, Graz, Österreich). Um die Assoziation zwischen den großen Lipoproteinen und Subklassen zwischen den verschiedenen NMR-Methoden und

der Ultrazentrifugation darzustellen, wurden die Korrelationskoeffizientenanalyse nach Pearson sowie die Regression nach Passing Bablok verwendet.

Ergebnisse.

Der Vergleich der Hauptlipoproteine (Gesamtcholesterin, Triglyzeride, Low Density Lipoprotein Cholesterol und High Density Lipoprotein Cholesterol) zwischen den NMR-Methoden und der Ultrazentrifugation zeigte keine signifikanten Unterschiede ($p<0,001$) und nur geringe Abweichungen im Bereich der Mittelwerte (<5,5%). Bei der Analyse der Lipoprotein Subklassen wurden zwischen den NMR-Methoden große Unterschiede festgestellt. Besonders bei den Partikelkonzentrationen von zum Beispiel Small Low Density Lipoprotein Partikel wurde eine mittlere Differenz von -431 ($r= 0.789$ und slope 0.593) festgestellt. Weitere hohe Abweichungen waren auch zum Beispiel bei den Large Low Density Lipoprotein Partikel (mittlere Differenz von 300 ($r= 0.607$ und slope 1.272) und den High Density Lipoprotein Partikel und Unterklassen zu sehen.

Schlußfolgerungen.

Die Gleichwertigkeit von NMR und Ultrazentrifugation konnte bei der Bestimmung der Standardlipide dargestellt werden. In der laborchemischen Bestimmung der Subklassen konnten einige große Unterschiede zwischen den beiden NMR Methoden dargestellt werden, welche die Vergleichbarkeit der beiden NMR-Methoden im Hinblick auf die Lipoprotein Subklassenbestimmung in Frage stellen. Weitere Untersuchungen sind notwendig um abschließend zu klären, ob beide oder nur eine der NMR Methoden bei der Subklassenbestimmung von der Referenzmethode abweichen. Es kann weiters festgehalten werden, dass NMR Methoden derzeit in der klinischen Routine selten eingesetzt werden und es derzeit keinen dringenden Anlass dafür gibt die Verfügbarkeit von NMR Methoden außerhalb der Forschung weiter auszubauen.

Abstract

Background.

Lipoproteins are essential components in (patho-) physiological functions of the human organism and have gained more and more importance in the determination of cardiovascular risk during the last decades. Various methods are used to determine lipoproteins and their subclasses, with ultracentrifugation as the reference method in routine clinical practice for the measurement of major lipoproteins (total cholesterol, triglycerides, low- and high density lipoprotein cholesterol). Another feasible measurement method is nuclear magnetic resonance (NMR) spectroscopy in the measurement of lipoprotein subclasses. To investigate the quality and quantity of NMR methods in the measurement of standard lipids and lipoprotein subclasses, a method comparison of the two most recognized NMR methods was performed. This dissertation addresses the issue of laboratory chemical measurement of lipoproteins and their subclasses by NMR.

Methods.

In a prospective method comparison study, serum samples were collected from one hundred and fifty patients at the Division of Internal Medicine (Clinical Division of Angiology) and the Department of Anesthesiology and Intensive Care Medicine (Division of General Anesthesiology, Emergency and Intensive Care Medicine) of the Medical University of Graz between February 17th and June 30th, 2020. Routine blood analysis was performed in the outpatient and inpatient settings. All study patients included in this method comparison study provided written informed consent in advance.
Serum samples were analyzed in three laboratories: "Numares" (NMR method, Regensburg, Germany), "Labcorp" (NMR method, Burlington, North Carolina- United States of America), and "reference or standard method" (combined ultracentrifugation/precipitation also called ß-quantification, Medical University of Graz, Graz, Austria). To show the association between the major lipoproteins and subclasses between the different NMR methods and ultracentrifugation, Pearson's correlation coefficient analysis, and Passing Bablok regression were used.

Results.

Comparison of major lipoproteins (total cholesterol, triglycerides, low density lipoprotein cholesterol and high density lipoprotein cholesterol) between NMR methods and ultracentrifugation showed no significant differences ($p<0.001$) and only minor deviations in the range of mean values (<5.5%). When lipoprotein subclasses were analyzed, large differences were found between NMR methods. Particularly for particle concentrations of, for example, small low-density lipoprotein particles, a mean difference of -431 ($r= 0.789$ and slope 0.593) was observed. Further high deviations were also seen for Large Low Density Lipoprotein particles for example (mean difference of 300 ($r= 0.607$ and slope 1.272) and High Density Lipoprotein particles and subclasses.

Conclucions.

The equivalence of NMR and ultracentrifugation could be shown in the determination of standard lipids. In the determination of the lipoprotein subclasses, some major differences between the two NMR methods could be shown, which call into question the comparability of the two NMR methods regarding lipoprotein subclass determination. Further investigations are necessary to finally clarify whether both or only one of the NMR methods deviate from the reference method in subclass determination. It can also be stated that NMR methods are currently rarely used in clinical routine and that there is no urgent need to further expand the availability of NMR methods outside of research due to these findings.

1. Introduction

1.1. Overview

Lipids have become increasingly important in human medicine over the last hundred years, especially in the field of nutrition and cardio- vascular disease research. Already since the middle of the 20th century, the subject of nutrition has been associated with fats (Hilditch and Kraut, 1949). Nutrition or proper nutrition plays a major role today, also due to the fact that fats are common food components that can have good but also bad properties in terms of effects on the body (Diekman et al., 2009). Positive effects would include the maintenance of metabolic processes or fat stores for energy reserve during periods of anaerobic metabolism (Aird, Davies and Carson, 2018) negative effects would include the effects of excessive fat intake such as the development of obesity and various fat maldistributions leading to increased cardiovascular risk and diseases (Maki, Dicklin and Kirkpatrick, 2021; Horlick, 1989). The estimation of increased cardiovascular risk by measuring different compositions of lipoproteins in human blood represents the essential topic of this thesis.

Nevertheless, lipids are important as nutritional components for various body functions, and lipoproteins are required for transport in the various body fluids (cf. Mahley et al., 1984).

In addition to the physiological functions of lipids in the human body, the role of lipids has been increasingly clarified over many years of scientific work.

How lipoproteins are composed, what functions they assume in the body, what conclusions can be drawn of the different lipoproteins, or their components will be presented in the following. Probably the greatest scientific importance has the composition of lipoproteins today since different lipoproteins are associated with the occurrence of cardiovascular diseases. In this review section, I will briefly discuss the different lipids and lipoproteins and their function and the lipoproteins with clinical relevance.

The lipid metabolism or the biochemical composition of the individual lipoproteins will not be discussed in detail. The facts relevant for laboratory medicine with respect of the major lipoproteins and their subclasses will be presented and especially the importance of lipoproteins in medicine today. In the literature section, the various clinical methods of measuring lipids are also discussed in more detail in order to understand the interrelationships of this dissertation.

1.2. Lipids

Mahley and colleagues reported in 1984 that lipids are, a component of tissues, can serve as energy stores (e.g., for periods of hunger) and are involved in various metabolic processes. Lipids (e.g., triglycerides) are mainly absorbed with food in the human body. These lipids (consisting mainly of triglycerides and cholesterol) are then partly absorbed by the body and transferred to their destination. (Mahley et al., 1984)

The transport of lipids in the human body is carried out by transport- proteins because lipids are insoluble in water. However, lipids are not only absorbed through food, but there are also metabolic processes in which lipids are formed, such as the triglycerides formed by carbohydrates. In the blood, triglycerides are transported together with other fats and fat-like substances (e.g., cholesterol) in a special way. (Mahley et al., 1984)

The French doctor and chemist Poulletier de la Salle first identified solid-form cholesterol from gallstones, although only Dr. Michel Eugène gave it the name "cholesterine" in 1815 (Dam, 1958).

The beginning of the first research on lipids started already in the 18th century. Cholesterine (equivalent to cholesterol) is also present in lipoproteins. Cohn *et al.* isolated in the middle of the 20th century a variety of proteins from human plasma and found five major protein families using gradual changes in pH, ionic strength and ethanol concentration. (Cohn and Strong, 1946)

Thus, there was already an interest in subdividing the lipoproteins at that time, which exact intention of Cohn was the basis for this project can only be assumed. Possibly it was the first attempt to show that the different subunits of the lipoproteins have a different medical effect on diseases.

The main function of lipoproteins is to transport lipids (e.g., glycerides) from or to the liver from the body periphery. However, there are also other physiologic functions that are performed or supported by lipoproteins, these are for example: the transport of bacterial endotoxins from areas of invasion and infection (Dam, 1958) or the cholesterol transport (Miller, 1979).

1.2.1. Lipoproteins

Lipoproteins are macromolecule complexes of lipids like triglycerides, cholesterol, phospholipids and proteins as apolipoproteins. As already mentioned above, it is only through the lipoproteins that the hydrophobic lipids can be transported because lipoproteins are water soluble, this represents their main task.
There is not only one type of human plasma lipoproteins, but they are also commonly classified based on their density, other classifications would be the subdivision by size and mobility. (Mahley et al., 1984)

The major classes of human lipoproteins include:

- Chylomicrons,
- very low density lipoproteins (VLDL),
- intermediate density lipoproteins (IDL),
- low density lipoproteins (LDL),
- high density lipoproteins (HDL) and
- lipoprotein a (Lp(a)). (Mahley et al., 1984)

After the initial efforts in the research of lipoproteins of Cohn in the middle of the 20th century, many researchers have worked to find a laboratory chemical subdivision of lipoproteins. A variant of the subdivision of lipoproteins from Lees and Hatch that has been confirmed by several researcher and is still used today is the subdivision based on the density of the different lipoproteins (Lees and Hatch, 1963).
In 1963, Lees and Hatch first separated these four different lipoprotein classes using paper electrophoresis, and divided them according to their mobility:

1) chylomicrons,
2) β-lipoproteins [low-density lipoprotein (LDL); density: 1.006–1.063 g/mL],
3) pre-β-lipoproteins [very low-density lipoprotein (VLDL); density: < 1.006 g/mL], and
4) α-lipoproteins [high-density lipoprotein (HDL), density > 1.063 g/mL]. (Lees and Hatch, 1963)

All lipoproteins basically contain the same lipids including phospholipids, triglycerides, and cholesterol, but the concentrations are vastly different (Mahley et al., 1984).

In *Table 1* the major classes of lipoproteins are summarized for a better overview. This table show a summarized representation of the density and diameter of lipoproteins.

Table 1: Lipoprotein classes with density and diameter

Lipoprotein	**Density range**		**Diameter range**	
	(g/mL)		(nm)	
HDL[1,2,3]	*1.063*	*1.21*	*5*	*12*
LDL[1,4]	*1.019*	*1.063*	*18*	*25*
Lp(a)[5]	*1.055*	*1.085*		*30*
IDL[5]	*1.006*	*1.019*	*25*	*35*
VLDL[1]	*0.930*	*1.006*	*30*	*80*
Chylomicron remnants[5]	*0.930*	*1.006*	*30*	*80*
Chylomicrons[5]		*<0.930*	*75*	*1200*

Legend: *HDL= high density lipoprotein. g/mL= gram per millilitre. IDL= intermediate density lipoprotein. LDL= low density lipoprotein. Lp(a)= lipoprotein a. nm= nanometer. VLDL= very low density lipoprotein. Table summarized from following publications: 1= (Mahley et al., 1984), 2= (Lund-Katz et al., 2003), 3= (Kontush, 2015), 4= (Ivanova et al., 2017), 5= (Feingold, 2000-2021).*

In the following section, the individual major classes of plasma lipoproteins are discussed.

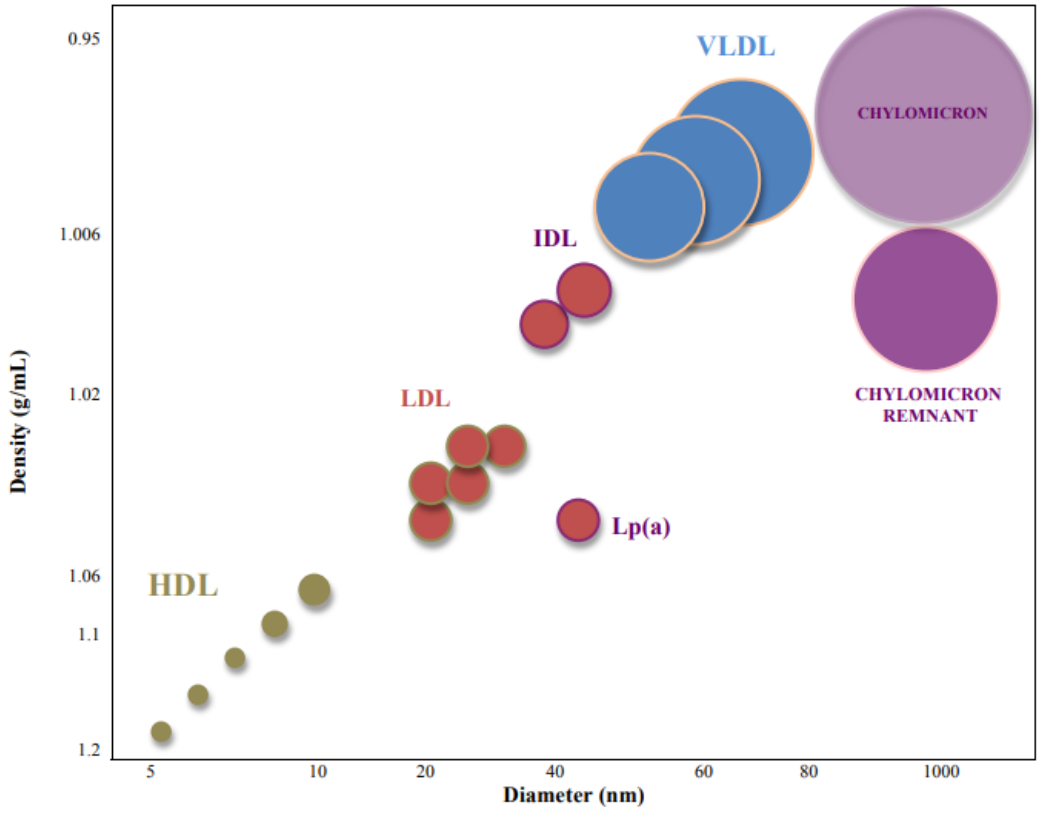
1.2.2. Chylomicrons

Chylomicrons were first discovered as lipoprotein particles in 1924. Gage and Fish showed particles (approximately 1 µm diameter) in blood taken from humans after a fatty meal, and they named such particles as chylomicrons (Gage and Fish, 1924).
Chylomicrons are the largest lipoproteins with a diameter >100 nm (the size ratios of the lipoproteins are shown in *Figure 1* for illustration). Chylomicrons are synthesized in the human intestine to facilitate the transport of dietary triglycerides and cholesterol from the intestine to the various cells in the human organism (lipid-metabolizing tissues like muscle and adipose). Chylomicrons are mainly composed of 85% glycerides. (Miller, 1979)

The fatty acids formed during this metabolic process (hydrolization via lipoprotein lipase) serve as energy suppliers and are taken up by adipocytes and stored as triglycerides. (Mahley et al., 1984)

After the metabolic process (hydrolysis), smaller particles, the so-called chylomicron remnants, are obtained. The chylomicron remnants contain the residual triglyceride and total cholesterol and cholesterol esters. In further biochemical processes, resulting apolipoproteins E are released from the liver via the plasma into the hepatocytes and transferred to the lysosomes. After further metabolization (hydrolysis of cholesterol esters) carbon dioxide is formed from the resulting fatty acids as part of the oxidative metabolism. Furthermore, carbon dioxide could be measured during exhalation to quantify a measure of catabolism of remnants. (Mortimer et al., 1995) This aspect has currently no clinical relevance and is not discussed further.

Figure 1: Illustration of the different lipoprotein particles



Legend: *HDL= high density lipoprotein. g/mL= gram per milliliter. IDL= immediate density lipoprotein. LDL= low density lipoprotein. Lp(a)= lipoprotein a. nm= nanometer. VLDL= very low density lipoprotein. Figure traced from "Vergleich von quantitativen Messmethoden zur Bestimmung von HDL-C und LDL-C im Serum", Dissertation, Universität Greifswald, 2007, Susanne Retter.*

1.2.3. Chylomicron remnants

When triglycerides get removed from chylomicrons by peripheral tissues, the result are smaller particles called chylomicron remnants. The chylomicron releases apolipoprotein C-II to high density lipoproteins but retains apolipoprotein E, thus the chylomicron remnant is only 30-50 nm in size (see *Figure 1*). Apolipoproteins (apolipoprotein B-48 and apolipoprotein E) are further important to identify the chylomicron remnant in the liver for endocytosis and degradation. Chylomicron remnants have an atherogenic feature, this aspect will be discussed in the section "Relevance of lipids in the medicine today".
If these chylomicron remnants accumulate in the plasma (e.g., due to high fat and cholesterol intake), then they are called ß-very low density lipoproteins. (Mahley et al., 1984)

1.2.4. Very low density lipoproteins (VLDL)

The very low density lipoproteins are produced by the liver and are slightly smaller than the chylomicrons and are responsible for the transfer of triglycerides and cholesterol from the liver to peripheral tissues. Triglycerides are hydrolyzed to free fatty acids in plasma again by lipoprotein lipase (and hepatic lipoprotein lipase). This process produces several different lipoproteins such as intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). (Mahley et al., 1984)
That means, very low density lipoproteins get converted into intermediate- and low density lipoproteins. Very low density lipoproteins have an approximate diameter of 30-80 nm (see *Figure 1*) and transport only endogenous products, whereas chylomicrons transport only exogenous (dietary) products. In the research of the last 20 years, lipid composition and by protein composition have been very accurately represented by Dashti (Dashti et al., 2011; Dashty et al., 2014).

1.2.5. Intermediate density lipoproteins (IDL)

Intermediate-density lipoproteins are formed during degradation of very low density- and high density lipoproteins. Intermediate density lipoproteins consist mainly of 35 %

glycerides and 25 % cholesterol ester and have a diameter of 25 to 30 nm (illustrated in *Figure 1*) (Miller, 1979).
Intermediate density lipoproteins transport a variety of triglyceride lipids and cholesterol and, like low density lipoproteins, may promote the growth of atheromas. Intermediate density lipoprotein particles have lost most of their triglyceride content after formation, but they still contain cholesterol esters. Some intermediate density lipoprotein particles are metabolized very rapidly in the liver, but some remain in the bloodstream, where they then undergo further triglyceride hydrolysis by hepatic lipase and are converted to low density lipoprotein particles. Typical of intermediate density lipoproteins is the presence of multiple copies of the receptor ligand apolipoprotein E and apolipoprotein B-100. This allows intermediate density lipoproteins particles to bind to low density lipoproteins with a very high binding affinity. Once conversion from intermediate- to low density lipoproteins has occurred, the apolipoprotein E particles are lost, and apolipoprotein B-100 remain. Subsequently, the affinity at the low density lipoprotein receptor is significantly reduced. (cf. Brown et al., 1986)

1.2.6. Low density lipoproteins (LDL)

The low density lipoproteins are the major cholesterol-transporting lipoproteins in plasma with an approximate size of 20 nm and with density ranging from 1.019 to 1.063 g/ml (Mahley et al., 1984). They have a diameter of 21-25 nm (see *Figure 1*) and mainly consist of 40 % cholesterol ester (Miller, 1979). Especially in familial hypercholesterolemia, low density lipoproteins accumulate in plasma (Mahley et al., 1984).
Each low density lipoprotein particle contains an apolipoprotein B-100 molecule, many other accessory proteins and has a highly hydrophobic core. The core of low density lipoprotein particles consists of linoleate, several hundred cholesterol molecules, a variable number of triglycerides and a shell of phospholipids and unesterified cholesterol surrounded by a single copy of apolipoprotein B-100. Due to the different or changing number of fatty acid molecules contained, the mass and size of the low density lipoprotein particles are different. (Segrest et al., 2001) This fact is discussed further in the chapter on the low density lipoprotein subclasses.
The structure of low density lipoproteins in the human body was recently described by Kumar using cryogenic electron microscopy (Kumar et al., 2011).

1.2.7. High density lipoproteins (HDL)

High density lipoproteins are approximately 8 to 12 nm in size and have a density of 1.063–1.21 g/ml. They represent the smallest lipoproteins (see *Figure 1*). High density lipoproteins consist mainly of 40 % phospholipids and 15 % cholesterol esters. (Miller, 1979)

High density lipoproteins are produced in plasma and are encountered in the liver and intestine. (Mahley et al., 1984) They are also responsible for reverse cholesterol transport, where cholesterol is transported from various body sites to the liver for elimination. (Mahley et al., 1982)

It is possible that high density lipoproteins not only serve to balance excess cholesterol but is also part of the innate immune response. High density lipoproteins probably also attenuate cytokine production triggered by endotoxin, which is relevant in immunomodulatory processes. (Berbee, Havekes and Rensen, 2005; Birjmohun et al., 2007; Levine et al., 1993)

High density lipoproteins have several functions. Although the functions performed by high density- and, moreover, by all other lipoproteins in the human body are still the subject of intensive research and new findings are being generated all the time.

High density lipoprotein cholesterol (HDL-C) is the total cholesterol in high density lipoprotein particles, including cholesterol esters. Low levels of high density lipoprotein cholesterol correlate with higher rates of cardiovascular disease, but a causal risk factor cannot be demonstrated. Raising high density lipoprotein cholesterol levels to prevent cardiovascular disease has so far failed to produce an effect in studies. (Schwartz et al., 2012; Barter et al., 2007; Lincoff et al., 2017)

Although there have been reports in individual studies that a reduction in cardiovascular risk is possible by increasing high density lipoprotein cholesterol levels, these results have always been revised and attributed to changes in low density lipoprotein levels and particle counts (Rubins et al., 1999; Brown et al., 2001; Otvos, Jeyarajah and Cromwell, 2002).

1.2.8. Lipoprotein (a) (Lp(a))

Lipoprotein (a) consists of a low density lipoprotein molecule and a unique apolipoprotein (a). It is also atherogenic, the functionality has not yet been clarified. (Mahley et al., 1982) Several laboratory methods do not have the ability to map lipoprotein a, therefore this lipoprotein is then included in the analysis in other values such as high- or low density lipoproteins. There is, however, a method where the antibodies against apolipoprotein (a) are measured and then the conclusion is drawn about the number of lipoprotein (a). The results are then used to determine the number of lipoproteins. Hoogeveen currently published an excellend overview of the determination methods of lipoproteins in 2021. (Hoogeveen and Ballantyne, 2021)

1.2.9. Apolipoproteins

The functions and activities of lipoproteins are mainly defined by the combination of apolipoproteins they contain, which direct their interaction with receptors as well as containing a variety of enzymatic activities. The apolipoproteins can act as ligands for receptors, build the formation of lipoproteins and are involved in the lipoprotein metabolism. (Afonso and Spickett, 2019)
The different lipoproteins have different associated apolipoproteins, which are as follows:

- chylomicrons (apolipoprotein A, -B and -C),
- very low density lipoproteins (apolipoprotein B, -C and -E),
- intermediate density lipoproteins (apolipoprotein B,-C and E),
- low density lipoproteins (apolipoprotein B) and
- high density lipoproteins (apolipoprotein A,-C, and -E) (Miller, 1979).

There are many apolipoproteins, but since there are only incidental to understanding this thesis and since they do not apply in this work, they will not be discussed further.
An important point regarding apolipoproteins and high density lipoproteins will be discussed to understand why no direct conclusion can be drawn from apolipoprotein number to particle number of lipoproteins, in this case high density lipoprotein particles.

Apolipoprotein A-I (ApoA-I) is the major apolipoprotein of high density lipoproteins. However, the number of apolipoprotein A-I per high density lipoprotein particle can range from one to four copies, so that no direct conclusion can be drawn from apolipoprotein A-I to the particle number of high density lipoproteins (the estimation of apolipoprotein B particle is however possible). In addition, significantly more copies have been found in individual cases (up to ten). (Davidson, 2014; Hutchins et al., 2014)

As described above, the transport of lipids in the human body are complicated processes that are interrelated and can be influenced by other physiologic or pathologic processes. Nowadays not only the major lipid particles like e.g., high- or low density lipoprotein cholesterol or triglycerides are of clinical relevance, especially the subclasses of the different lipoproteins are of great importance. (Afonso and Spickett, 2019)
For a better understanding of the size relationship of the different lipoproteins, all the lipoproteins are graphically presented in *Figure 1*.
To understand the terminology of this dissertation, it is noted at this point that major lipoproteins such as total cholesterol, triglycerides, low- and high density lipoprotein cholesterol are also referred to as standard lipids or major lipids.

1.3. Lipoprotein Subclasses

1.3.1. Low density lipoprotein subclasses

The precise origins of low density lipoprotein subclasses are not yet understood. Nevertheless, low density lipoprotein particles can be divided into up to 4 different subclasses based on the different measurement methods:

- large low density lipoprotein particles,
- intermediate low density lipoprotein particles,
- small low density lipoprotein particles and
- very small low density lipoprotein particles.

The different measurement methods of the low density lipoprotein subclasses differ not only in the measurement form but also at the target variable. Historically, the first method

to show a subdivision of low density lipoprotein subclasses was analytical ultracentrifugation, which can subdivide low density lipoprotein subclasses by their density:

- large low density lipoproteins 1.019-1.023 g/ml,
- intermediate low density lipoproteins 1.023-1.034 g/ml,
- small low density lipoproteins 1.034-1.044 g/ml,
- very small low density lipoproteins 1.044-1.060 g/ml). (Bickerstaffe and Desmond, 1982)

In a later chapter, the different measurement methods for the determination of lipoproteins and their subclasses will be discussed in more detail.

1.3.2. High density lipoprotein subclasses

There are similar to the low density lipoprotein subclasses a variety of different high density lipoprotein subclasses. According to their isolation/separation method, size, density etc. there are different subdivision forms.
The three main subclasses, which are also relevant in this work, are

- small (7.3 - 8.2 nm) high density lipoprotein particles
- medium (8.2 - 8.8 nm) high density lipoprotein particles and
- large (8.8 - 13 nm) high density lipoprotein particles

and differ in size. (Lund-Katz et al., 2003)
These three subclasses can be measured by the nuclear magnetic resonance spectroscopy (NMR) method such as very- and low density lipoprotein subclasses and particle sizes. Further subclasses would be high density lipoproteins- 2 a and b, 3 a, b and c - which can be measured by the gradient gel electrophoresis (GGE) method - which also differs by different nm size (7.2 - 12 nm). Another method mentioned here is the agarose gel method, which differentiates by surface charge and shape, and the reference method for such analyses - ultracentrifugation, which differentiates by density (high density lipoprotein 2 (1.063-1.125 g/mL) and high density lipoprotein 3 (1.125-1.21 g/mL)). As a consequence

of the variety of the measurement methods, there are only few comparisons between the different methods. (Kontush, 2015; Lund-Katz et al., 2003)
In 2011, an expert panel issued a recommendation on the classification of high density lipoprotein subclasses to standardize the differentiation of subclasses for future research projects with five different subclasses:

- very large-,
- large-,
- medium-,
- small- and
- very small high density lipoproteins. (Rosenson et al., 2011)

However, this classification has not yet been adopted in all research projects or even by laboratories in this way.

1.4. Emergence of relevance of lipids in medicine

Lemoine hypothesized at the beginning of the 20th century that the presence of hypercholesterolemia may have an atheromatous effect (Page, Kirk and Van Slycke, 1936). This could be shown in animal experiments and later in several other studies (Mahley et al., 1984).
Building on these findings, countless further studies were conducted in the years that followed.

1.4.1. Relevance in the medicine today

Atherosclerosis and associated cardiovascular disease, as one of the leading causes of death in the Western world, demand investigation of the causes and emergence of risk factors. (Page, 1954) Cardiovascular diseases are based on the formation of atherosclerotic plaques, which leads to vascular constriction and subsequently to tissue undersupply with blood and the corresponding development of symptoms. A prime example of this is coronary artery disease, which is caused by vasoconstriction of the coronary vessels (the

vasoconstriction is caused by the build-up of atherosclerotic plaques). (Wynands et al., 1970)

Another example would be peripheral arterial disease, where atherosclerotic plaques accumulate in peripheral vessels until blood flow to extremities has stopped or is restricted (Spitell, 1994). Currently, it is known that the growth of atherosclerotic plaques depends on the uptake of circulating cholesterol by subendothelial cells. This means that cholesterol plays an essential role in the development of atherosclerotic plaques and thus in the development of cardiovascular diseases. The development of atherosclerosis is illustrated in *Figure 2*. A major finding in the late 20th century was that high low density lipoprotein levels were associated with increased rates of coronary heart disease in studies. This subsequently led to an effort to treat these high low density lipoprotein levels in the population with lipid-lowering drugs. (Goldstein and Brown, 1982)

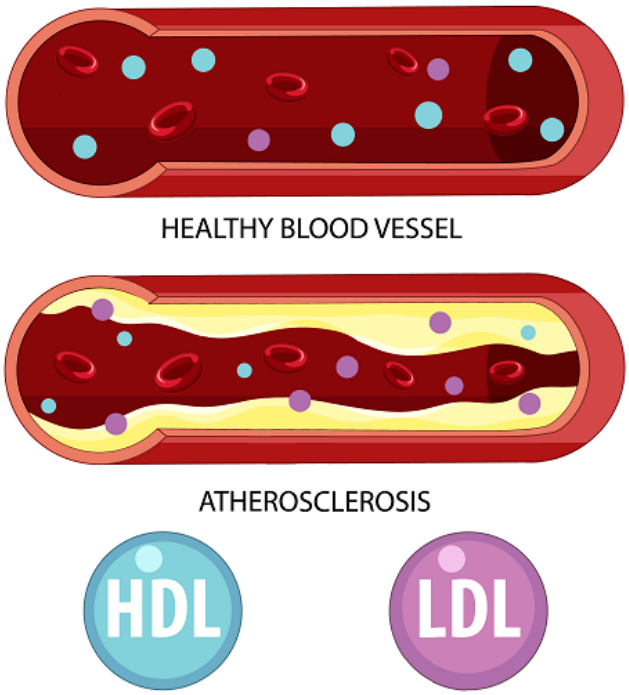
From low density lipoprotein cholesterol, one cannot infer the actual low density lipoprotein particles, i.e., low density lipoprotein cholesterol is only an estimate that is not directly measured. It is an estimate of how much cholesterol is transported by all low density lipoprotein particles, i.e., either a low concentration of large particles or a high concentration of small particles. Low density lipoprotein particles contain many molecules of cholesterol, triglycerides, phospholipids, etc. This subsequently also led to investigations into the particle composition of the various lipoproteins. Evidence has also been found that the particle number of lipoproteins influences the significance of the main lipoproteins with regard to cardiovascular risk and is clinically relevant.

In addition, hypercholesterolemia is a risk factor for atherosclerosis, therefore lipid-lowering therapy is also commonly used in clinical practice to treat the risk for cardiovascular disease. (Ivanova et al., 2017)

Several studies have found an association between low high density lipoprotein levels and an increased risk of atherosclerotic cardiovascular disease (Reiner et al., 2011; Linsel-Nitschke and Tall, 2005).

Thus, in modern times, efforts to achieve higher high density lipoprotein levels and lower low density lipoprotein levels have always resulted in the prevention of cardiovascular disease in the general population (Heiss et al., 1980). It is hard to imagine today's medicine without lipid-lowering drug therapy.

Figure 2: Presentation of the development of atherosclerosis in combination with high- and low density lipoprotein particles



Legend: *HDL= high density lipoprotein. LDL= low density lipoprotein. License free provided by: Hintergrund Vektor erstellt von brgfx - de.freepik.com.*

Also as described above, chylomicron remnants may play a role in atherosclerosis.
The difficulty in measuring chylomicrons has always hampered previous understanding of their metabolism. Plasma concentrations of chylomicrons and their remnants do not allow further conclusions about respective effects by chylomicrons. To date, it is thought that impaired clearance of chylomicron remnants is likely to indicate impaired lipoprotein metabolism. There is also evidence that the same clearance of chylomicron remnants may play a role in the progression of atherosclerosis (Redgrave, 2004).

1.4.2. Current findings

Several studies have identified high low density lipoprotein cholesterol levels and low high density lipoprotein cholesterol levels as cardiovascular risk factors (Stampfer et al., 1996; Chapman and Bruckert, 1996; Baigent et al., 2005).

In the past, evidence on the role of lipoproteins in atherogenesis was drawn from large population studies. The difficulty is that at that time only lipids were measured, not the corresponding lipoproteins (Otvos, 1999). Subsequently, it was the specific directly measured particles of the lipoprotein subclasses that became increasingly important, challenging the findings of the earlier large studies. This was of course only due to the great efforts of the laboratories that nowadays perform detailed lipoprotein subclass determinations worldwide.

Also, ratios of individual lipoproteins to each other, more specifically the ratio of high density lipoprotein cholesterol to apolipoprotein A-1, were identified as risk factors for cardiovascular disease (Rhee, Byrne and Sung, 2017).

Even the composition of the individual subfractions of high density lipoprotein could be shown to be related to cardiovascular disease (Superko et al., 2012; Silbernagel et al., 2017).

Furthermore, high density lipoprotein particles have been shown to be a better predictor of cardiovascular disease than high density lipoprotein cholesterol (Hutchins et al., 2014; Kontush, 2015; Mackey et al., 2012)

The small dense low density lipoprotein particles (sdLDL) are considered to be the most atherogenic fraction (Campos et al., 1992; Coresh and Kwiterovich, 1996; Kathiresan et al., 2006).

Small and medium high density lipoprotein particles, on the other hand, are considered to be particularly protective in cardio- and cerebrovascular diseases (Ditah et al., 2016; May et al., 2016; Austin et al., 1988; Kim et al., 2016).

High density lipoproteins have protective effect due to it’s role in reverse cholesterol transport as mentioned above. Furthermore, high density lipoprotein associated enzymes are limiting the oxidative modification of low density lipoproteins. (Lund-Katz et al., 2003)

1.5. Measurement methods

The reference method for the determination of low- and high density lipoprotein subclasses is ultracentrifugation. This means actually only that the scientific society has agreed that it is recognized as a reference method or standard method in lipoprotein analysis.
Other methods in the measurement of lipoproteins are gradient gel electrophoresis, nuclear magnetic resonance spectroscopy (NMR) and specific precipitation. Nevertheless, it could already be demonstrated that the accuracy of conventional lipoprotein analysis methods other than ultracentrifugation and NMR is not sufficient for the measurement of subclasses. (Otvos, 1999; Otvos et al., 2006; Davies, Graham and Griffith, 2003)
Though ultracentrifugation methods have the disadvantage that they require a high expenditure of time and personnel and are therefore not suitable for patient care and studies with large numbers of patients. Furthermore, currently the only clinically feasible alternative method in determination of lipoprotein subclasses is the technique of NMR. Nowadays, NMR methods are widely used for research purposes but relatively rarely in clinical routine. NMR is a direct and efficient method to determine the concentrations of very low density lipoproteins, low density lipoproteins, and high density lipoprotein subclasses. Lipoprotein subclass determination of the NMR method works on the principle that the spectral properties of the lipids contained in the particles vary depending on the diameter of the phospholipid membrane. (Otvos et al., 1992; Otvos, 1997; Otvos, 1999)
Thus far, no investigations have yet been carried out to determine whether NMR methods of different laboratories produce comparable results. Williams et al. compared gradient gel electrophoresis (GGE), vertical autoprofile ultracentrifugation (VAP-II) and NMR in determination of lipoprotein subfraction concentrations for atherosclerotic progression, but this study did not compare the four methods with one another (Williams et al., 2014).

1.5.1. Measurement methods over time

In 1949, Gofman and colleagues fist mentioned the density gradient ultracentrifugation method for the analysis of lipoprotein classes (Gofman, Lindgren and Elliott, 1949).
In 1955, Havel and colleagues established a sequential flotation ultracentrifugation and separated three major lipoprotein classes by their density (Havel, Eder and Bragdon, 1955)

and a couple of years later in 1960, further subdivisions could be made by density from Baxter, Goodman, and Havel (Baxter, Goodman and Havel, 1960).
In the end of the 20th century the NMR method was first reported and also investigated in various studies until today, where it is a reputable method in the determination of lipoprotein subclasses.
In the following years, many further developments and attempts to replace ultracentrifugation as a reference method with simpler and faster methods (e.g., NMR) were made, but these have not yet been successful.

1.6. Ultracentrifugation

In the following lines, ultracentrifugation will now be discussed in more detail, and it will be shown how the process has developed, which different methods are counted as ultracentrifugation and the advantages and disadvantages as well as the limitations will be presented. The ultracentrifugation methods have a high ability for the separation of lipoprotein classes and are considered as the reference method in the determination of lipoprotein but require a long time and high personnel effort in the determination. Also, these methods are inferior to ultracentrifugation in measuring many different samples, a measurement of large numbers of different samples is accomplished much faster by NMR.

1.6.1. Beta quantification (specific precipitation ultracentrifugation)

ß-quantification is in our study the reference method for the analysis of high- and low-density lipoprotein cholesterol, this has also been assured by the Center for Disease Control and Prevention (CDC = agency of the Department of Health and Human Services based in Druid Hills/USA) (Cole et al., 2001; Nakamura et al., 2014; Hainline, 1995). It is a very widely used method and the most clinically used method nowadays.
This method uses ultracentrifugation steps to separate apolipoprotein B particles according to the hydrated density at a density of 1.006 g/mL (Hainline, 1995).

1.6.2. Analytical Ultracentrifugation

Historically, the first method that allowed subdivision of low density lipoprotein fractions was analytical ultracentrifugation (Bickerstaffe and Desmond, 1982.
This method is based on density gradient flotation using Schlieren optics. The analytical ultracentrifugation was used since the mid 20th century for characterizing lipoproteins. Furthermore, by sepraration with sequential flotation, lipoproteins could be divided into:

- high-,
- intermediate-,
- low-,
- very- low density lipoproteins and
- chylomicrons. (De Lalla and Gofman, 1954; Rosenson et al., 2011)

In the analytical ultracentrifugation procedure, the plasma is increased to a density of 1.063 g/ml based on the initial hydration density range (1.006 g/ml) with various neutral salts. (De Lalla and Gofman, 1954; Rosenson et al., 2011)

1.6.3. Density gradient ultracentrifugation

This method is based on the isopycnic equilibrium approach (Chapman et al., 1981).
In this method, there is a single ultracentrifugation step that facilitates the isolation of high density lipoproteins and avoids major contamination with plasma proteins present at the bottom of the tube (Rosenson et al., 2011).
Chapman mentioned in 1981, that the density gradient ultracentrifugation method reduces centrifugation steps and preparation time (Chapman et al., 1981), which is necessary for isolating lipoprotein subspecies (Guerin et al., 2020).
A special feature of this ultracentrifugation method is that the simultaneous isolation of low density lipoprotein subfractions and high density lipoproteins is possible, thus the cholesterol content of all five lipoprotein classes can be measured in sequence (Chung et al., 1981; Kulkarni, 2006).

1.6.4. Vertical auto profile (VAP) ultracentrifugation

The measurement to separate all lipoproteins of the vertical auto profile method takes less than one hour (Kulkarni et al., 1997; Kulkarni, 2006; Chung et al., 1986).
This test is so sensitive that only very small amounts of plasma or serum are required (< 50 µL) (Movva and Rader, 2008), is economical, relatively widely available (Rosenson et al., 2011) and of high reliability (Kulkarni, 2006).
This method was compared with only a few techniques for measuring lipoprotein subfractions (e.g.: NMR). It was shown that in comparison with the NMR method no high correlations could be presented (Chung et al., 1980).
While the main field of application of ultracentrifugation is in research and is considered the "gold standard" or "reference" technique for the separation of lipoproteins and their subclasses, it is not practical for routine analytical measurements in clinical practice especially due to the reasons of personnel and time already mentioned above. (Rosenson et al., 2011)

1.7. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy is used to determine very low-, low-, high density lipoproteins of different sizes precisely (Ala-Korpela et al., 2007; Otvos, 1999).
This method distinguishes between subclasses based on spectral properties of lipids contained in the particles, which vary depending on the diameter of the phospholipid membrane.
Meaning each lipoprotein particle of a given size has its own characteristic lipid methyl group NMR signal (Kontush et al., 2015).
This technique uses proton spectroscopy for direct determination of the different sizes of lipoprotein subfractions rapidly (Jeyarajah, Cromwell and Otvos, 2006).
NMR is widely used in special lipid reference laboratories (Cole et al., 2013; Jeyarajah, Cromwell and Otvos, 2006). By NMR method, high density lipoproteins can be divided into (see *Table 2 and 3*):

- large (9.4-14 nm),
- medium (8.2-9.4 nm) and

- small (7.3-8.2 nm) high density lipoprotein particles (Rosenson et al., 2011; Movva and Rader, 2008).

Low density lipoproteins can be divided by NMR into (see *Table 2 and 3*):

- large-,
- medium- and
- small- low density lipoprotein particles (Otvos, 1999).

Furthermore, very low density lipoproteins can be divided by NMR into (see *Table 2*):

- very large-,
- large-,
- medium-,
- small- and
- very small- very low density lipoprotein particles (Otvos, 1999).

However, NMR methods can image a variety of other subunits of high density lipoprotein particles (~26 subclasses) (Kontush, Lhomme and Chapman, 2013), but which currently have no further significance in clinical medicine.

Although there are already many scientific papers on the good applicability of NMR methods in clinical routine, there are also always publications that contradict this.

The most important limitation of this method is the requirement for specialized equipment not found in routine clinical laboratories (de la Llera Moya, 2012).

NMR spectroscopy measures lipoprotein subclasses directly and efficiently and produces information that may improve the assessment and management of cardiovascular disease risk (Otvos, 1999).

There are several competing methods for measuring the concentration and size of lipoprotein particles (concentration and size). The NMR methodology (initially developed by Jim Otvos) promises to greatly reduce costs while improving accuracy. NMR has reported an up to 25 % reduction in cardiovascular events within a year, in contrast to claims by many others that its superiority over existing methods has not been clearly demonstrated. For about 30 years it has now been possible to use NMR measurements to determine lipoprotein particles at a significantly lower cost (< 100 US dollars) compared to the previous costs (> 400 US dollars) and with greater accuracy. (Otvos et al., 2006)

There are also other assays for low density lipoprotein particles, but they also only estimate low density lipoprotein cholesterol and thus only indirectly infer low density lipoprotein

particle concentrations. Direct low density lipoprotein particle measurement by NMR has also been mentioned by professional societies as being beneficial in predicting individual risk of atherosclerotic disease events. However, there is still a debate as to whether particle size measurements add value to the measurement of low density lipoprotein particle concentrations.

The different NMR measurement methods consider different density or diameter values, in the following table a short overview of the different density values and diameters is shown. The values not marked with asterisks are taken from the literature, the others were provided by the laboratories Labcorp and Numares. The table should also illustrate how the high-, low- and very low density lipoprotein particles are distributed and what density and diameter they have.

Table 2: Lipoproteins and subclasses with diameter values determined by nuclear magnetic resonance (NMR) spectroscopy

Lipoprotein	Subclasses	Density range (g/mL)		Diameter (nm)	
HDL[1,2,3]	total	1.063	1.21	-8 (7.4*)	-12 (13*)
	small			7.3 (7.4*, 7.3**)	8.2 (8.0*, 8.8**)
	medium			8.2 (8.1*)	8.8 (9.5*)
	large			8.8 (9.6*, 8.8**)	13 (13*, 13**)
LDL[1,4]	total	1.019	1.063	19*	23*
	Very small	1.044	1.060		
	small	1.034	1.044	19* (18**)	20.4* (21.2**)
	medium	1.023	1.034	20.5*	21.4*
	large	1.019	1.023	21.5* (8.8**)	23* (13**)
VLDL[1]	total		<1.006	30 (24*)	90 (240*)
	Very small			24*	29*
	Small			30*	36*
	Medium			37*	49*
	Large			50* (60**)	89* (200*)
	Very large			90*	240*

Legend: **Diameter provided by Labcorp.** Diameter provided by NUMARES. nm= nanometer. g/mL= gram per milliliter. Table summarized from following publications: 1= (Mahley et al., 1984), 2=(Lund-Katz et al., 2003), 3= (Kontush, 2015), 4=(Ivanova et al., 2017).*

1.8. Further methods in lipoprotein diagnostic and therapeutic use

Another method in the determination of lipoprotein subclasses is for example the gradient gel electrophoresis (GGE).
With this method the measurement of low density lipoprotein subfraction analysis is possible, where subclasses are separated by their electrophoretic mobility and is determined by the size and shape of the lipoproteins.
Different subclasses were defined in gradient gel electrophoresis technique: low density lipoprotein I-IV (22.0–28.5 nm) (Ivanova et al., 2017).
There is a strong correlation between by ultracentrifugation and gradient gel electrophoresis in the measurement of low density lipoprotein particles, where the comparison of particle sizes of gradient gel electrophoresis and NMR could not present significant correlations.
Other notable methods for low density lipoprotein subclass analysis include high-performance liquid chromatography with gel filtration columns, dynamic light scattering, ion mobility analysis, and homogeneous assay analysis (Ivanova et al., 2017).
The basic purpose of lipid research is to minimize the associated risk of disease. The high lipoprotein concentrations, which should or can lead to a high cardiovascular risk, can then be reduced with medication or lifestyle changes. Furthermore, the various drug groups that are currently used in the treatment of high lipid levels in human medicine are discussed.
Statins are one of the most commonly used lipid-lowering drugs. They reduce high low density lipoprotein particle concentrations by inhibiting HMG-CoA reductase in cells (a step in cholesterol synthesis). In order to subsequently compensate for the reduced cholesterol availability, the density of low density lipoprotein receptors is increased, which leads to the clearance of low density lipoprotein particles. (Sirtori, 2014)
Ezetimibe inhibits the absorption of cholesterol from the gut and can therefore reduce low density lipoprotein particle concentrations in combination with statins (Lamb, 2020).
Vitamin B3 (niacin) inhibits the hepatic diacylglycerol acyltransferase 2, thereby triglyceride synthesis and very low density lipoprotein secretion and thus a reduction in the low density lipoprotein concentration (Malik and Kashyap, 2013).
A relatively new group of drugs are the PCSK9 inhibitors, for which large studies are currently investigating whether the effectiveness is more effective than that of statins and

the combination of statins and ezetimibe in lowering low density lipoprotein (Silbernagel et al., 2019).

Cholesterylester transfer protein (CETP) inhibitors should increase the high density lipoprotein cholesterol concentration and have shown an increase in the high density lipoprotein cholesterol level in previous studies, but no significant reduction in the risk of atherosclerosis. In addition, it should be noted that it was also shown that the mortality rate was increased compared to placebo. Fibrates, such as clofibrate, are also used to lower cholesterol levels (Morton and Liu, 2020). Because studies have linked clofibrate to significantly increased cancer and stroke mortality, it has lost its clinical importance. Newer fibrates such as fenofibric acid are primarily used to reduce very low density lipoprotein particles (triglycerides) (House and Motsinger-Reif, 2020). Delta- and gamma-tocotrienols are described as over-the-counter statin alternatives for treating high cholesterol. Gamma-tocotrienol might also inhibit HMG-CoA reductase and thus reduce cholesterol production. Similar to statins, the fall in intrahepatic low density lipoprotein levels can cause upregulation of the hepatic low density lipoprotein receptor, which also causes plasma low density lipoprotein levels to fall.

Phytosterols have also been shown to lower low density lipoprotein cholesterol and are recommended in various guidelines for lipid lowering therapy (Micallef and Garg, 2009). There are many other drug groups for lipid lowering that are currently being researched. Our research group also deals with various questions of lipid research, which subsequently also indirectly estimates the benefit of various drug groups in lipid lowering.

The introduction should give an overview of the current laboratory methods in lipoprotein determination as well as a short overview of the relevant lipoproteins and a brief insight into the development history of lipoproteins and their relevance in medicine. In the following, the methodological aspects and questions of the method comparison study and the dissertation will be presented and then discussed.

1.9. Aims and limitations of the study

Hypothesis: Different NMR methods yield equivalent results in the determination of lipoproteins and lipoprotein subclasses.

H-0 hypothesis: Two different NMR methods yield equivalent results in the determination of standard lipids (e.g. total cholesterol, tiglycerides, low density lipoprotein and high density lipoprotein cholesterol) and lipoprotein subclasses.

H-1 hypothesis: Two different NMR methods yield different results with ultracentrifugation in the determination of standard lipids (e.g. total cholesterol, tiglycerides, low density lipoprotein and high density lipoprotein cholesterol) and lipoprotein subclasses.

To evaluate whether the quality and quantity of the methods remains the same, the following points were also investigated:

- Comparison of mean concentrations of major lipoproteins between NMR methods and ultracentrifugation
- Comparison of the measured values between NMR methods by Pearson correlation, Bland Altman plots and Passing Bablok regression analysis
- Graphical presentation and comparison of the measured lipoprotein values

1.9.1. Specific aim 1

The first aim of our analysis was to compare two established NMR methods in determination of major lipoproteins.

1.9.2. Specific aim 2

The second aim of our analysis was to compare two established NMR methods in determination of major lipoproteins with routine laboratory tests (ß-quantification/ultracentrifugation specific precipitation).

1.9.3. Specific aim 3

The third aim of our analysis was to compare two established NMR methods in determination of lipoprotein subclasses with each other.

1.9.4. Novelty of the question

Up to now, individual NMR methods have never been directly compared with each other, as this is primarily a direct evaluation for the companies or laboratories performing the analyses. This evaluation, if done by us as independent scientists, can of course also result in a negative evaluation of individual methods, which can also be detrimental to the reputation of the respective companies.
Accordingly, this method comparison represents special novelty value, whether the NMR methods compared with each other yield comparable values in the determination of lipoproteins and their subclasses as well as whether the NMR methods yield comparable values regarding standard procedures in the measurement of major lipoproteins.
Furthermore, it should be noted that the two NMR methods or laboratories we considered in our study are among the most respected NMR methods laboratories.

1.9.5. Limitations

The limitations of this study relate mainly to the fact that the blood samples were collected in Austria and were further analyzed in other countries.
Although the samples were immediately centrifuged and frozen as it is good scientific practice, it cannot be excluded that possibly deviating values would have been obtained if the samples had been analyzed directly by the laboratories without freezing.
It was clarified in advance with the participating laboratories which tubes should be used for the blood collection and how much serum must be available per analysis in order to create equal conditions.
It was also clarified with the laboratories in advance which values are comparable with the respective other values, since the designations and limits of individual values were

different (e.g., large and very large very low density lipoproteins from Labcorp correspond to large very low density lipoproteins from Numares, etc.). Furthermore, it should be mentioned that the standard lipids of the NMR methods could be compared with the reference method, but the subclasses were only compared between the two NMR methods and no ß-quantification/ ultracentrifugation specific precipitation for subclass determination was performed.
These circumstances are also presented in more detail in the methods section and will be dealt with again in the discussion.

2 Methods

The following data and information were already published in *Biomedicines (Rief et al, 2022) (see Appendix A.8.)*, as part of my dissertation and are reproduced here accordingly and with partially identically content.

2.1. Registration and Insitutional Review Board

The study was planned prior to initiation and commencement of patient recruitment in accordance with Good Scientific Practice and submitted to the relevant ethics committee for review.
After a positive review by the responsible ethics committee, a corresponding expert opinion was obtained from the Federal Office for Safety in Health Care of Austria (AGES). This was done because three different laboratory methods were used in the determination of lipoprotein subclasses in this method comparison. The first method is a recognized standard method in lipid analysis (ultracentrifugation), the second method (NMR) of Numares AG in Regensburg, Germany, is a CE certified method, but the third method (NMR) of Labcorp Corp. (Liposcience) has accordingly no CE certification but is FDA (Food and Drug Administration of the United States) cleared. For this reason, we decided to submit an application to the Federal Office for Safety in Health Care of Austria, which was subsequently also positively assessed.
The study was approved by the institutional review board of the Medical University of Graz (29-479 ex 16/17; Ethics Committee of the Medical University of Graz, IRB00002556, chairperson Prof. Josef Haas) and the Federal Office for Safety in Health Care of Austria (Bundesministerium für Sicherheit im Gesundheitswesen - BASG; Agency for Health and Food Security -AGES reference number (ref.nr.) 11458092) on May 15th, 2019 before commencement of patient recruitment.

2.2. Study design

The study discussed in this dissertation is prospective. The study was planned and completed according to the study protocol. This study protocol can be found in the

Appendix (*see Appendix A.1.*). In the following text, the specific values determined during the lipid measurement will be discussed in more detail.

2.3. Study setting

This study was performed at the University Medical Centre in Graz, an academic teaching hospital with about 1,500 beds. Graz University Hospital is home to all standard medical departments and represents a supra-regional trauma and burn center, as well as high expertise in the various specialties of internal medicine and laboratory medicine. The hospital is located in the capital of the southern Austrian province of Styria.

2.4. Patient recruitment

This method comparison study was performed at the Department of Internal Medicine (Division of Angiology) and the Department of Anaesthesiology and Intensive Care Medicine (Division of General Anaesthesiology, Emergency- and Intensive Care Medicine) of the Medical University of Graz.
A total of one hundred-fifty consecutive patients, who were admitted to the outpatient clinic of the angiology department, the preoperative anesthesia outpatient clinic or the angiology ward of the university hospital in Graz, were included in this study. Patients were included according to the study protocol (*see Appendix A.1.*).

2.4.1. Inclusion criteria

Following inclusion criteria was provided:

- Adult patients (minimum age of 18 years for inclusion in the study),
- male and female sex,
- subordinated consent form.

2.4.2. Exclusion criteria

- Persons below the age of 18,
- Patients unable to give informed consent.

2.5. Informed consent

If the patients confirmed in participating in the study, they were informed extensively according to the informed consent (*see Appendix A.2.*).
All participants provided their written informed consent beforehand, then they underwent venous blood sampling. Our included patients were not fasting at blood draw. According to a recent study, it is no longer necessary to ensure overnight fasting when measuring plasma lipid profiles (Nordestgaard et al., 2016). The only aim of the present analyses was to compare the two methods. Therefore, there is no possible confounding by the fasting state.

2.6. Blood samples

We collected blood samples as a part of blood withdrawal in clinical routine from one hundred and fifty patients at the wards and outpatient clinics listed above between February 17th and June 30th, 2020.

2.6.1. Specimen material

From each patient, two tubes of nine ml whole blood were collected with Greiner bio-one ® Vacuette Z Serum (red, 9.0 ml; 455092) according to the study protocol.
Serum was revovered by centrifugation (10 min at 6490 rpm and 15° C) and aliquoted in cryotubes (Nunc Universal ® 1.8 ml).
Between five and seven milliliter of serum could be obtained from each patient, this serum samples were stored within two hours after blood withdrawal.

2.6.2. Use or anonymisation of the samples

After blood withdrawal, the samples were made anonymous, i.e., patients were identified with a consecutive identification number from one to one hundred and fifty (neither name nor initials, date of birth, laboratory request number were recorded).

2.6.3. Storage of samples and shipping

The cryotubes were cooled down and stored at minus eighty degrees celsius at the laboratory of the Department of Angiology at the Medical University of Graz. These one hundred and fifty serum samples of one millilitre serum each, were sent via airmail in accordance with international security regulations for medical specimens to the laboratories in frozen condition.

2.7. Laboratories and laboratory methods

In this study, three different laboratories were set for method comparison.

2.7.1. Labcorp

Labcorp Inc. in Morrisville (100 Perimeter Park, 27560 North Carolina, USA) performed nuclear magnetic resonance spectroscopy (NMR) analysis using NMR LipoProfile ® (*see Appendix A.6.*). Particle concentrations of lipoproteins of different sizes were calculated from the measured amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Weighted-average lipoprotein particle sizes are derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal.

2.7.2. Numares

The Numares Aktiengesellschaft (AG) in Regensburg (Am Biopark 9, 93053 Regensburg, Germany) performed nuclear magnetic resonance spectroscopy (NMR) analysis using AXINON ® lipoFIT ® (*see Appendix A.5.*). with an Avance III HD nuclear magnetic resonance spectrometer (Bruker; Billerica, MA, USA), an Ascend 600 MHz magnet (Bruker), and using TopSpin 3.2 (Bruker) and Axinon Suite 1.0.0.1 (Numares, Regensburg, Germany) software.

2.7.3. Medical University of Graz

At the Clinical Institute for Medical and Chemical Laboratory Diagnostics of the Medical University of Graz (Auenbruggerplatz 15, 8036 Graz, Austria) specific precipitation ultracentrifugation (ß- quantification) was performed. The very low density lipoprotein fraction (density < 1.006 g/mL) was removed after ultracentrifugation (18 h, 10 °C, 98,000× *g*). Apolipoprotein B containing lipoproteins in the resulting bottom fraction were precipitated using phosphotungstic acid with the high density lipoprotein particles remaining in solution. Low density lipoprotein cholesterol was calculated by subtracting cholesterol after precipitation from the respective concentrations before precipitation. Cholesterol and triglycerides were measured with enzymatic reagents from Diasys (Holzheim, Germany) on an Olympus AU680 analyzer (März et al., 2001). This method is continuously referred to as ß-quantification or ultracentrifugation in this thesis.

2.8. Data acquisition and statistical methods

The anonymized data was provided by the laboratories in a Microsoft ® Excel ® file after laboratory determination in February 2021.
The following parameters were provided by the laboratories (and have been considered in the further statistical analysis according to the study protocol), among others:

2.8.1. Labcorp (NMR method)

Standard lipids:

- Total cholesterol (TC),
- triglycerides (TG),
- high density lipoprotein-cholesterol (HDL-c),
- low density lipoprotein-cholesterol (LDL-c).

Lipoprotein subclasses:

- large very low density lipoprotein particles (lVLDLp),
- very large very low density lipoprotein particles (vlVLDLp),
- low density lipoprotein particles (LDLp),
- small low density lipoprotein particles (sLDLp),
- medium low density lipoprotein particles (mLDLp),
- large low density lipoprotein particles (lLDLp),
- high density lipoprotein particles (HDLp),
- high density lipoprotein particles (H1-H7 HDL particles).

2.8.2. Numares (NMR method)

Standard lipids:

- Total cholesterol (TC),
- triglycerides (TG),
- high density lipoprotein-cholesterol (HDL-c),
- low density lipoprotein-cholesterol (LDL-c).

Lipoprotein subclasses:

- large very low density lipoprotein particles (lVLDLp),
- low density lipoprotein particles (LDLp),
- small low density lipoprotein particles (sLDLp),
- large low density lipoprotein particles (lLDLp),
- high density lipoprotein particles (HDLp),

- large high density lipoprotein particles (lHDLp),
- small high density lipoprotein particles (sHDLp).

2.8.3. Medical University of Graz (Ultracentrifugation (ß-quantification))

Standard lipids:

- Total cholesterol (TC),
- triglycerides (TG),
- high density lipoprotein-cholesterol (HDL-c),
- low density lipoprotein-cholesterol (LDL-c).

2.9. General patient characteristics

Baseline demographic patient data were not recorded or further considered in this study, as this study was solely concerned with the measured values and no comparison with patient data such as age, gender, etc. was further carried out. In the initial planning phase of the study, it was planned that the patients should also take a questionnaire to record an anamnesis of their illness or that this anamnesis should be carried out by the investigator. However, we rejected this again because the extended anamnesis (apart from gender, age, etc.) would not have provided us with any added value for our questioning. Since our study was exclusively a comparison of methods.

2.9.1. Comparability of the values

In the study protocol, before the start of the study, the laboratories and us (the investigators) defined which parameters should be compared with which parameters. The parameters that were compared (indicating the different units) are shown in *Table 4* and the corresponding size ranges of the parameters are shown in *Table 5*. Additionally, the corresponding diameter sizes of the Labcorp and Numares NMR methods are illustrated in *Table 3* (provided by the companies).

Table 3: Lipoproteins and subclasses with diameter values by different NMR methods

Lipoprotein	**Subclasses**	**Diameter size range**(nm)			
		Labcorp		**Numares**	
HDL	*total*	7.4	13		
	small	7.4	8.0	7.3	8.8
	medium	8.1	9.5		
	large	9.6	13	8.8	13
LDL	*total*	19	23		
	small	19	20.4	18	21.2
	medium	20.5	21.4		
	large	21.5	23	21.2	23
VLDL	*total*	24	240		
	very small	24	29		
	small	30	36		
	medium	37	49		
	large	50	89	60	200
	very large	90	240		

Legend: *nm= nanometer. HDL= high density lipoprotein. LDL= low density lipoprotein. VLDL= very low density lipoprotein. Table modified from (Rief et al., 2022) -permission with CC BY 4.0 license*.

.

Table 4: Corresponding parameters and units

	Labcorp (NMR)	***Numares (NMR)***	***Standard method (Ultracentrifugation)***	***Unit***
Major lipoproteins	TC	TC	TC	mg/dl
	TG	TG	TG	mg/dl
	HDL-C	HDL-C	HDL-C	mg/dl
	LDL-C	LDL-C	LDL-C	mg/dl
Lipoprotein sublasses	Large + very large VLDL	Large VLDL	No value	nmol/l
	LDL-p	LDL-p	No value	nmol/l
	Small + medium LDL-p	Small LDL-p	No value	nmol/l
	Large LDL-p	Large LDL-p	No value	nmol/l
	HDL-p	HDL-p	No value	µmol/l
	HDL-p (H4-H7)	Large HDL-p	No value	µmol/l
	HDL-p (H1-H3)	Small HDL-p	No value	µmol/l

Legend: *C= cholesterol. HDL= high density lipoproteins. l= liter. LDL= low density lipoprotein. mg/dl= milligram per deciliter. µmol/l= micromoles per liter. nmol/l= nanomoles per liter. NMR= nuclear magnetic resonance spectroscopy. p= particles. TC= total cholesterol. TG= triglycerides. VLDL= very low density lipoproteins. Table modified from (Rief et al., 2022) -permission with CC BY 4.0 license*.

Table 5: NMR values compared due to their size

Labcorp (Sub)Class	*Labcorp size (nm)*	*Numares (Sub)Class*	*Numares size (nm)*
Total VLDL-p	24-240		
Very large VLDL-p	90-240	***Large VLDL-p***	60-200
Large VLDL-p	50-89		
Medium VLDL-p	37-49		
Small VLDL-p	30-36		
Very small VLDL-p	24-29		
Total LDL-p	19-23	***Total LDL-p***	18-23
Large LDL-p	21.5-23	***Large LDL-p***	21.2-23
Medium LDL-p	20.5-21.4	***Small LDL-p***	18-21.2
Small LDL-p	19-20.4		
Total HDL-p	7.4-13	***Total HDL-p***	7.3-13
Large HDL-p	9.6-13	***Large HDL-p***	8.8-13
H7-p	12		
H6-p	10.8		
H5-p	10.3		
H4-p	9.5		
Medium HDL-p	8.1-9.5	***Small HDL-p***	7.3-8.8
H3-p	8.7		
Small HDL-p	7.4-8		
H2-p	7.8		
H1-p	7.4		

Legend: *HDL= high density lipoproteins. l= liter. LDL= low density lipoproteins.* µmol/l= micromoles per liter. *nmol/l= nanomoles per liter. p= particles. VLDL= very low density lipoproteins. Table from (Rief et al., 2022) -permission with CC BY 4.0 license.*

2.9.2. Statistical methods

Statistical analyses were performed descriptively-statistically.

To present association among the standard lipids and lipoprotein subclasses between the different NMR methods and ultracentrifugation Pearson correlations and Passing Bablok

regression was used. Subsequently, Bland Altman plots were used to show the deviations numerically and also graphically.

For statistical analysis, the Statistical Package for the Social Sciences ® from IBM ® (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp) was used. Passing–Bablok regression was calculated with the Analyse-it Method Validation Edition for Microsoft ® Excel ® 5.90 (Analyse-it Software Ltd., Leeds, UK).

3 Results

The following data and information were already published in *Biomedicines* (Rief et al., 2022) as part of my dissertation and are reproduced here accordingly and with partially identically content. After performing laboratory analyses, the laboratories provided the values accordingly and the statistical analysis and presentation was performed by us.
In the data sets provided by Numares, there were individual values that we could only include in our analysis to a limited extent. For individual values we received either values less than or equal to (e.g., <1.5 etc.) or values with 0 or missing values.
These values can be seen in *Table 6*.
If we have not received any value we have considered it as 0, values less than or equal to specifications (e.g.: <1.5) we have used as 1.5.
It was particularly striking in the data analysis that only Numares provided individual values with gaps. The dataset provided by Labcorp and the Medical University of Graz was complete.

Table 6: Numares values under the detection limit

Parameter	***Value***	***Quantity***
SLDL-p	<152 nmol/l	4
LHDL-p	<2780 µmol/l	32
SHDL-p	<6034 µmol/l	2
TG	<44 mg/dl	4
HDL-C	<17 mg/dl	1

Legend: *C= cholesterol. HDL= high density lipoproteins. LHDL= large high density lipoproteins. mg/dl= milligram per decilitre. µmol/l= micromoles per liter. nmol/l= nanomoles per liter. p= particles. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TG= triglycerides. Table from (Rief et al., 2022)* -permission with CC BY 4.0 license.

3.1. Comparison of major lipids

The mean concentrations of the standard lipids – triglycerides, total-, low- and high density lipoprotein cholesterol showed uniformity among all three methods (*Table 7*). The

measured mean concentrations of the standard lipids of the NMR methods did not deviate more than about 5% from the reference or standard method. More precisely the deviation from ß-quantification for Labcorp were for total cholesterol about 4,3%, for triglycerides 2,3%, for low density lipoprotein cholesterol 4,4% and for high density lipoprotein cholesterol 1,2%. The deviations of mean concentrations of the Numares method and ß-quantification were for total cholesterol 2,7%, for triglycerides 2,3%, for low density lipoprotein cholesterol 2,7% and for high density lipoprotein cholesterol about 5,5%.
In a direct comparison of the mean concentrations of the two NMR methods, percentage deviations of 1,6 % of total cholesterol, 0 % of triglycerides, 1,9 % of low density lipoprotein cholesterol and 6,4 % of high density lipoprotein cholesterol were shown.
Larger deviations can be seen in the mean concentrations of lipoprotein particle concentrations (see Table 7).

Table 7: Mean concentrations of standard lipids and lipoprotein (sub-)classes

Parameter	***Units***	***Labcorp***	***Numares***	***Standard method***
TC	*mg/dl*	180 (± 45)	183 (± 46)	188 (± 51)
TG	*mg/dl*	135 (± 81)	135 (± 78)	132 (± 84)
LDL-C	*mg/dl*	105 (± 37)	107 (± 38)	110 (± 38)
HDL-C	*mg/dl*	50,1 (± 13,2)	53,5 (± 12,7)	50,7 (± 17,7)
LVLDL-p	*nmol/l*	3,42 (± 4,18)*	4,94 (± 5,6)	
LDL-p	*nmol/l*	1330 (± 444)	1176 (±465)	
LDL-s	*nm*	21,1 (± 0,56)	21,2 (± 0,37)	
LLDL-p	*nmol/l*	377 (± 241)	677 (± 289)	
SLDL-p	*nmol/l*	953 (± 407)**	528 (± 270)	
HDL-p	*µmol/l*	18,4 (± 4,4)	33,2 (± 7,1)	
HDL-s	*nm*	9,19 (± 0,51)	9,10 (± 0,47)	
LHDL-p	*µmol/l*	4,37 (± 1,87)†	6,08 (± 3,31)	
SHDL-p	*µmol/l*	14,0 (± 4,0)‡	26,8 (± 7,6)	

Legend: * *large + very large VLDL.* ** *small + medium LDL-p.* † *H4-H7 HDL-p.* ‡ *H1-H3 HDL-p. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. p= particles. s= size. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides. Table modified from (Rief et al., 2022) -permission with CC BY 4.0 license.*

Correlations between NMR methods and ß-quantification showed high significances, with HDL-C levels showing the least agreement.
According to the mean concentrations where for high density lipoprotein cholesterol about 1.2% lower values were seen with the Labcorp method and about 5% higher values with Numares.

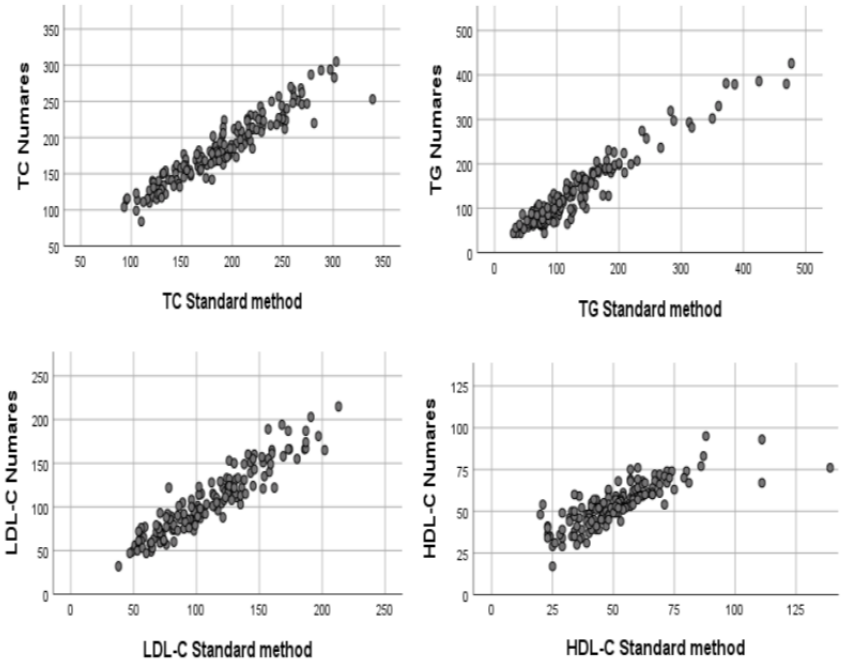
Table 8: Comparison of standard lipids between ß-quantification and the Numares NMR and Labcorp NMR methods

Parameter	***Labcorp***			***Numares***		
	r-correlation	***Passing Bablok***	***Bland Altman***	***r- correlation***	***Passing Bablok***	***Bland Altman***
		Slope (95%CI)	***MD (95%CI)***		***Slope (95%CI)***	***MD (95%CI)***
TC	0.964	0.889 (0.861 – 0.921)	- 8.2 (-10.5 – -5.91)	0.947	0.914 (0.864 – 0.963)	-4.56 (-7.21 – -1.91)
TG	0.979	1.027 (1.000 – 1.045)	2.58 (-0.184 – 5.34)	0.961	0.983 (0.924 – 1.046)	3.19 (-0.571 – 6.94)
LDL-C	0.941	0.960 (0.921 – 1.000)	-4.65 (-6.76 – -2.55)	0.935	0.973 (0.909 – 1.034)	-3.25 (-5.47 – -1.03)
HDL-C	0.837	0.830 (0.756 – 0.897)	-0.593 (-2.18 – 0.991)	0.805	0.786 (0.700 – 0.885)	2.79 (1.07 – 4.5)

Legend: *C= cholesterol. CI= confidence interval. HDL= high density lipoproteins. MD= mean difference. LDL= low density lipoproteins. LoA= limit of agreement. TC= total cholesterol. TG= triglycerides. Table modified from (Rief et al., 2022) -permission with CC BY 4.0 license.*

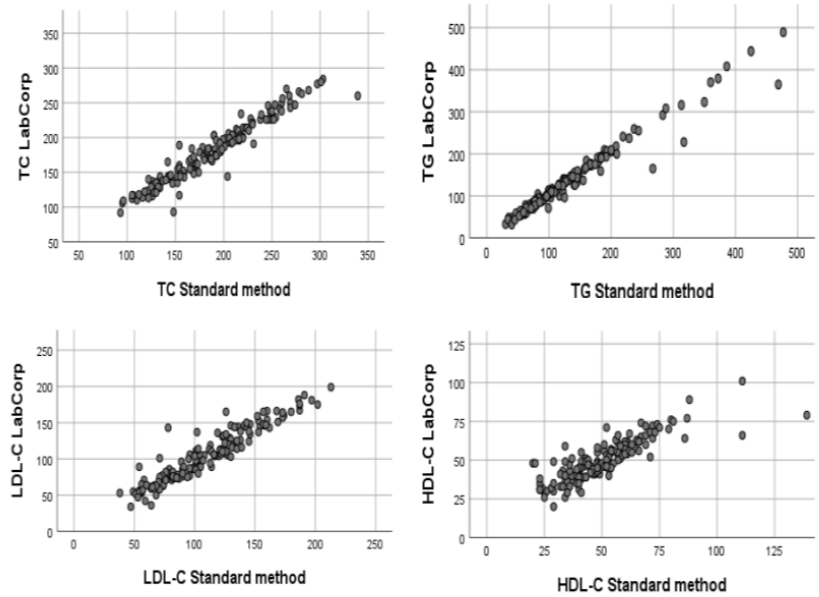
Subsequently, the calculated correlations are shown in the point diagram and according to the Passing Bablok analysis and the Bland Altman plots (see *Figure 3a and 3b*).

Figure 3a: Comparison of Numares NMR and standard method (point diagram)



Legend: *C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. TC= total cholesterol. TG= triglycerides.*

Figure 3b: Comparison of standard lipids between ß-quantification and Labcorp NMR (point diagram)



Legend: *C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. TC= total cholesterol. TG= triglycerides.*

Figure 3a and *3b* directly show the measured values of the respective parameters (total cholesterol, triglycerides, low density lipoprotein cholesterol and high density lipoprotein cholesterol) and are compared graphically. The line of identity and the corresponding slopes, which are shown in *Figure 4a* and *4b* attached further below, are missing in the point diagrams presented here.

Figure 4: Comparison of standard lipids between ß-quantification and Numares NMR (4a) and Labcorp NMR (4b)

Figure 4a:

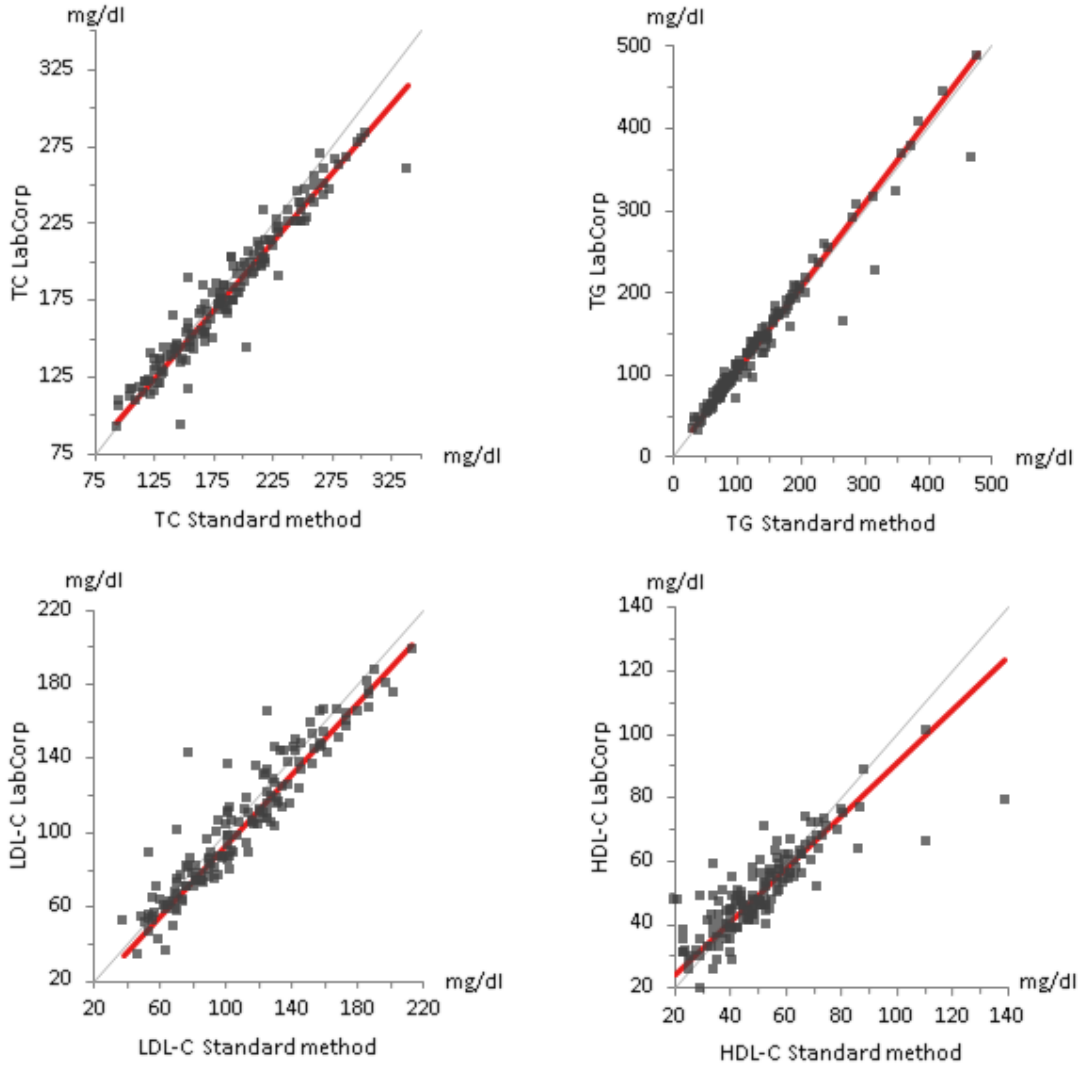
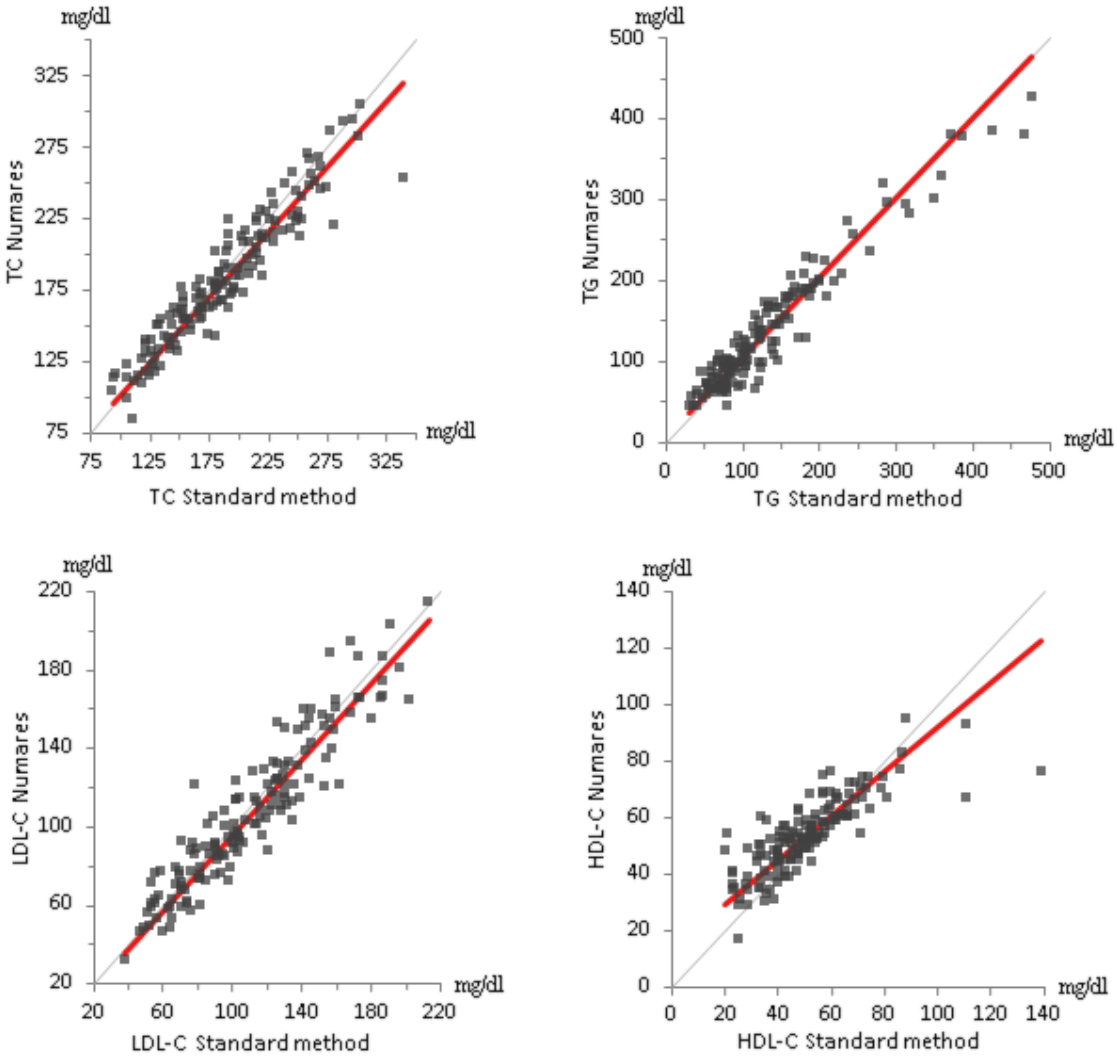


Figure 4b:

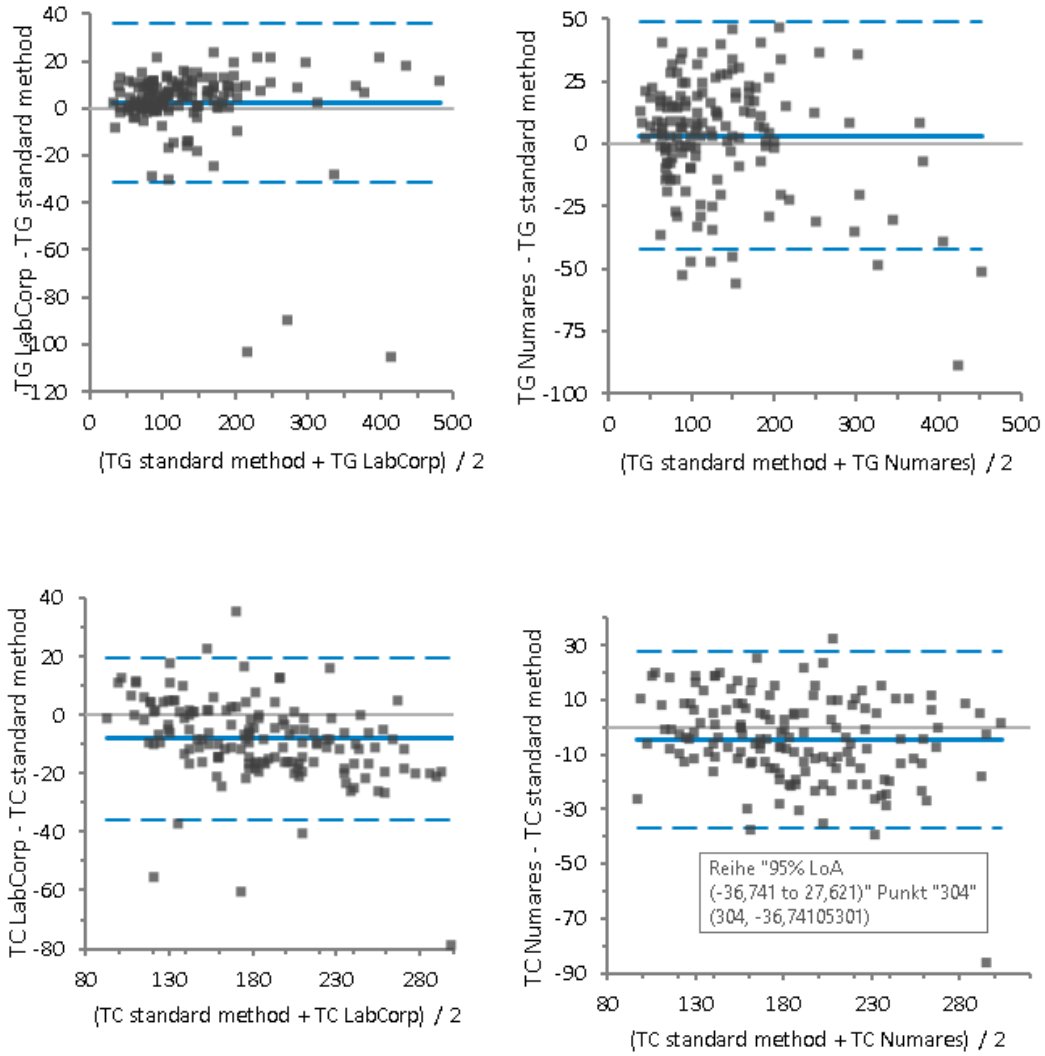


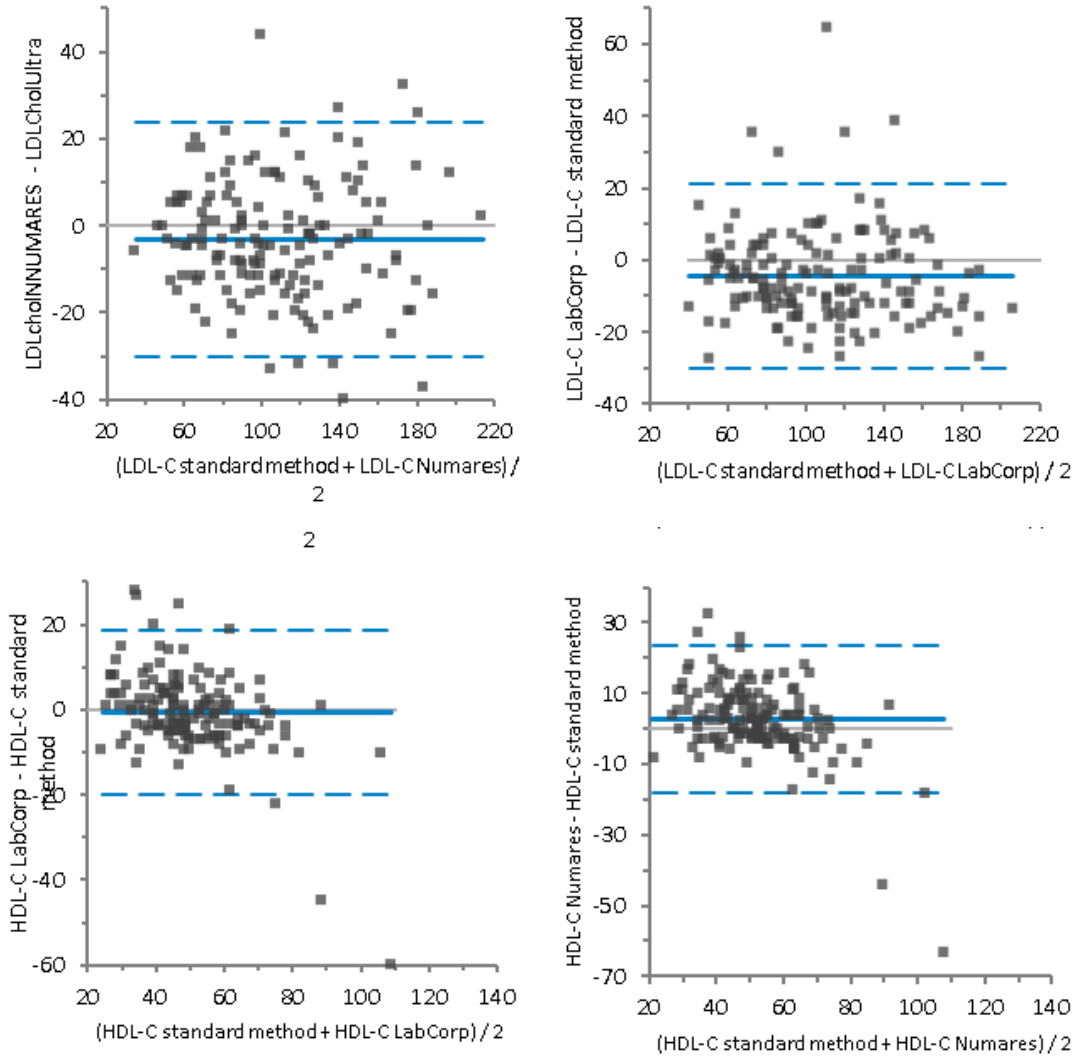
Legend: *C= cholesterol. HDL= high density lipoproteins. mg/dl= milligram per decilitre. LDL= low density lipoproteins. TC= total cholesterol. TG= triglycerides. Figure slightly modified from* (*Rief et al., 2022) - permission with CC BY 4.0 license.*

The *Figures 4a* and *4b* show the Passing-Bablok regression for total cholesterol (top left), triglycerides (top right), low density lipoprotein cholesterol (bottom left), and high density lipoprotein cholesterol (bottom right). The respective slopes of the regression lines (red) were 0.889 (total cholesterol), 1.027 (triglycerides), 0.960 (low density lipoprotein cholesterol), and 0.830 (high density lipoprotein cholesterol) for Labcorp and 0.914 (total cholesterol), 0.983 (triglycerides), 0.973 (low density lipoprotein cholesterol), and 0.786 (high density lipoprotein cholesterol) for Numares, respectively. The grey line represents the line of identity.

Subsequently, the Bland Altman plots are also given here, in which the comparison with the NMR methods is shown for each parameter of the standard lipids (total cholesterol, triglycerides, low density lipoprotein cholesterol and high density lipoprotein cholesterol). This graphical representation can also be seen numerically further below in *Table 9*.

Figure 5: Bland Altman plots in comparison of Labcorp and Numares NMR with ß-quantification





Legend: *dashed line = limits of agreement. continuous horizontal line = mean difference. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. TC= total cholesterol. TG= triglycerides.*

3.2. Comparison of lipoprotein subclasses

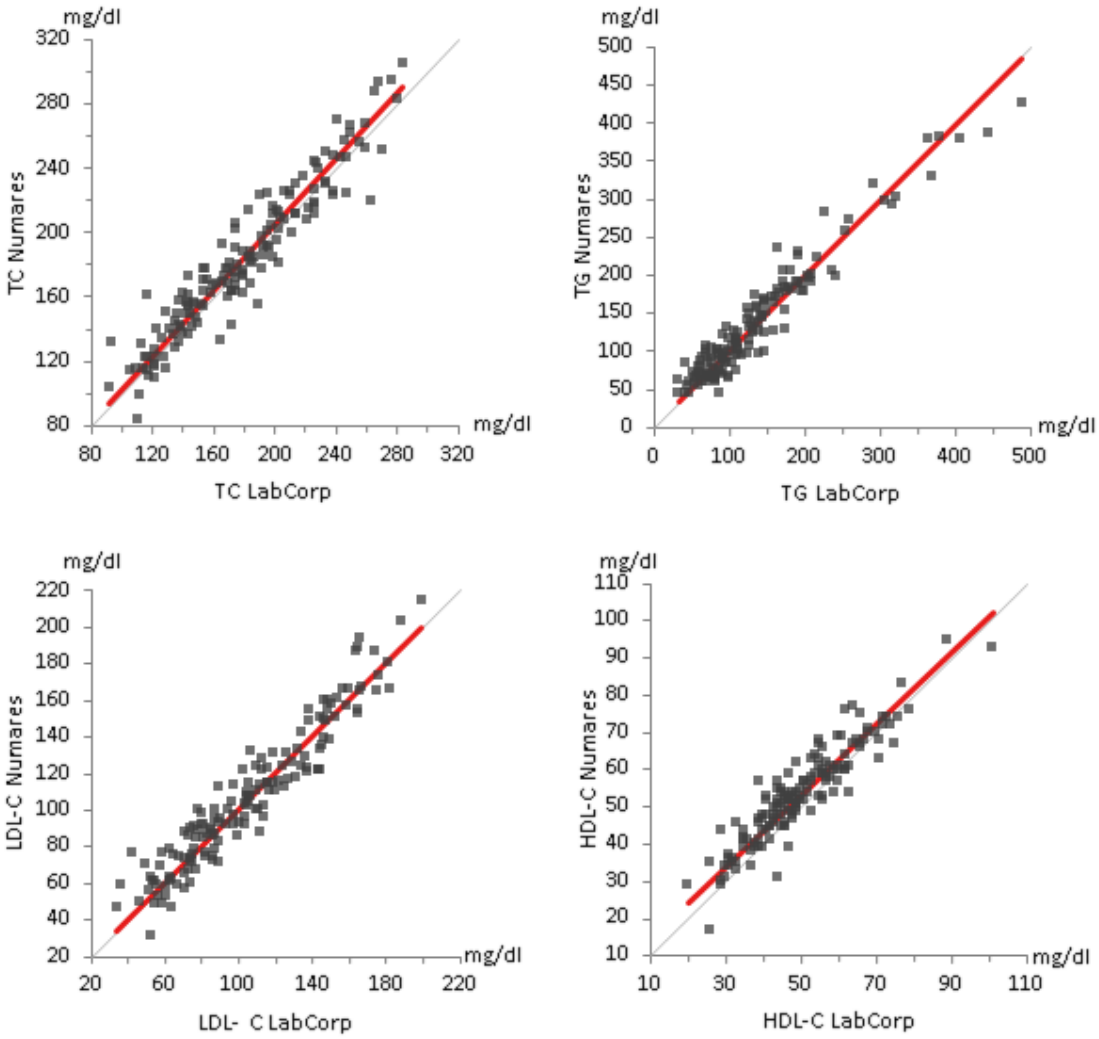
Table 9: Comparison between the Numares NMR and Labcorp NMR methods

Parameter	***r-correlation***	***Passing Bablok regression***		***Bland Altman analysis***	
		slope	***95% CI***	***MD***	***95% CI***
TC	0.950	1.026	0.966 – 1.082	3.6	1.3 – 5.94
TG	0.964	0.988	0.932 – 1.050	0.6	-2.87 – 4.08
LDL-C	0.953	1.000	0.946 – 1.067	1.4	-0.46 – 3.26
HDL-C	0.921	0.958	0.889 – 1.000	3.4	2.55 – 4.21
LVLDL-p*	0.898	0.980	0.865 – 1.157	1.52	1.1 – 1.94
LDL-p	0.908	1.057	0.984 – 1.138	-153.8	-185 – -122
LLDL-p	0.607	1.272	1.077 – 1.468	299.6	261 – 338
SLDL-p*	0.789	0.593	0.519 – 0.668	-431	-472 – -389
HDL-p	0.934	1.637	1.532 – 1.770	14.8	14.22 – 15.3
LHDL-p†	0.869	1.722	1.540 – 1.903	1.72	1.41 – 2.03
SHDL-p‡	0.735	1.817	1.642 – 2.058	12.8	11.9 – 13.7
LDL-s	0.677	0.860	0.738–1.002	-0.001	-0.05–0.048
HDL-s	0.843	1.014	0.916–1.099	-0.13	-0.175–-0.086

Legend: * *compared with Labcorp large + very large VLDL-p.* ** *compared with Labcorp small + medium LDL-p.* † *compared with Labcorp H4-H7 HDL-p.* ‡ *compared with Labcorp H1-H3 HDL-p. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. MD= mean difference. p= particles. S=size. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides. Table from (Rief et al., 2022)-permission with CC BY 4.0 license.*

In a direct comparison between the two NMR methods, high correlations were found for the standard lipids (all correlations >0.92) and some larger differences for the lipoprotein subclasses. For example, particularly low levels of agreement were found for large low density lipoprotein particles (r=0.607, slope 1.272 with a mean difference of approximately 300). Especially the small and large low density lipoprotein particles and all high density lipoprotein particle subclasses showed large deviations (see *Table 9*).

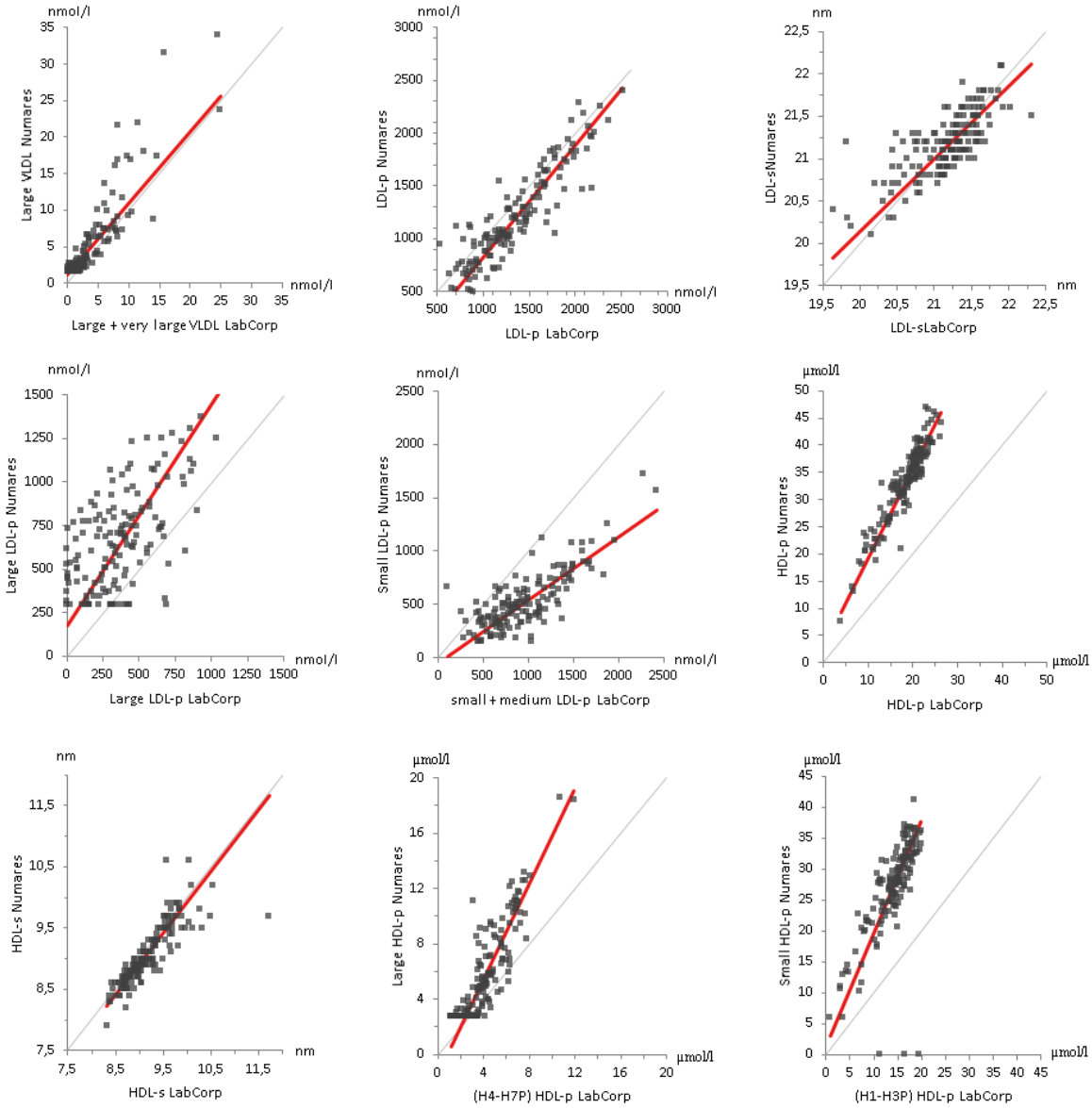
Figure 6: Comparison of standard lipids between the Labcorp and Numares NMR methods



Legend: *C= cholesterol. HDL= high density lipoproteins. mg/dl= milligram per decilitre. LDL= low density lipoproteins. TC= total cholesterol. TG= triglycerides. Figure slightly modified from (Rief et al., 2022) - permission with CC BY 4.0 license.*

The figures show the Passing-Bablok regression for total cholesterol (top left), triglycerides (top right), low density lipoprotein cholesterol (bottom left, and high density lipoprotein cholesterol (bottom right). The respective slopes of the regression lines (red) were 1.026 (total cholesterol), 0.988 (triglycerides), 1.000 (low density lipoprotein cholesterol), and 0.958 (high density lipoprotein cholesterol), respectively. The grey line represents the line of identity.

Figure 7: Comparison of lipoprotein particles between the Labcorp NMR and Numares NMR methods (Passing Bablok regression)

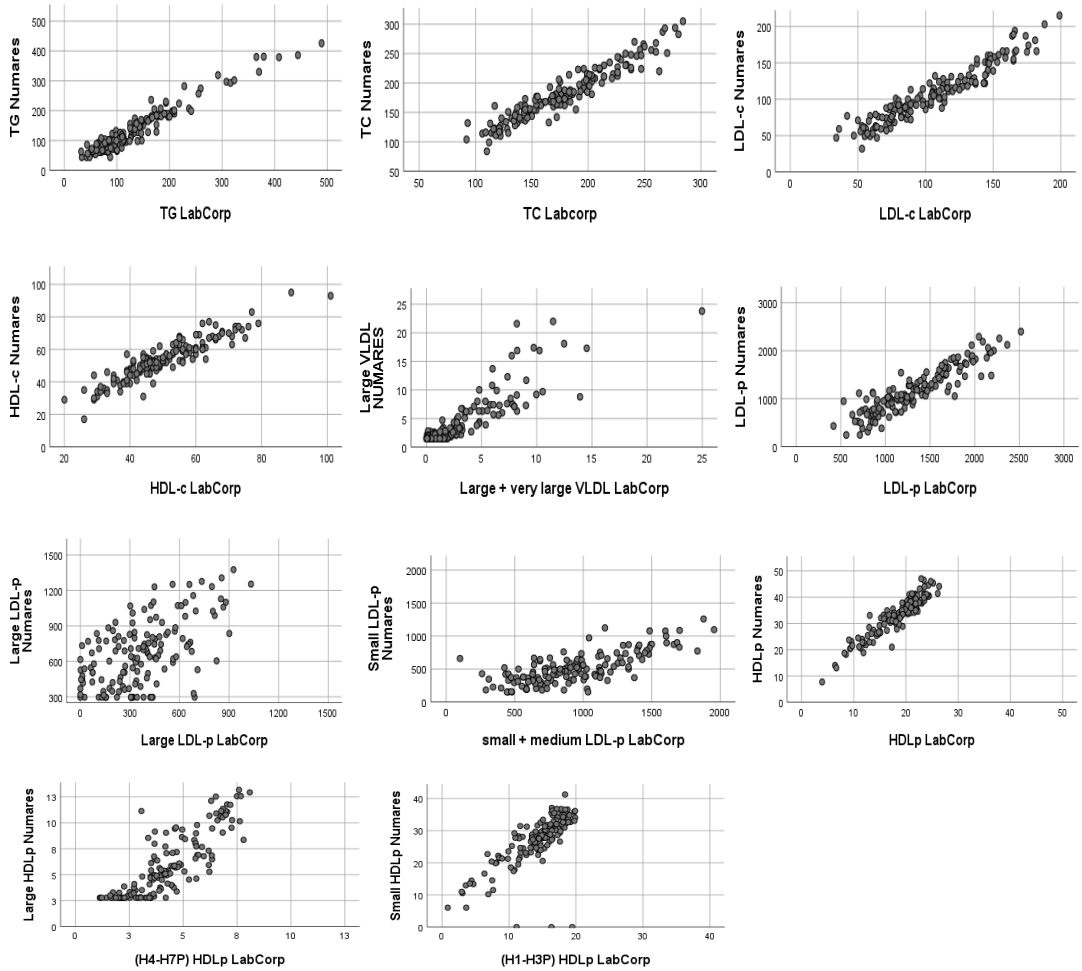


Legend: *C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. MD= mean difference.nmol/l= nanomole per litre.* p= *particles. s=size. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides. µmol/l= micromoles per litre; Figure modified from (Rief et al., 2022) -permission with CC BY 4.0 license.*

The figures show the Passing-Bablok regression for large and very large very low density lipoproteins (top left), low density lipoprotein particles (top middle), low density lipoprotein size (top right), large low density lipoprotein particles (middle left), small and

medium low density lipoprotein particles (middle), high density lipoprotein particles (middle right), high density lipoprotein size (bottom left), large high density lipoprotein particles (bottom middle), and small high density lipoprotein particles (bottom right). The respective slopes of the regression lines (red) were 0.980 (large very low density lipoprotein particles), 1.272 (large low density lipoprotein particles), 0.593 (small and medium low density lipoprotein particles), 1.817 (small high density lipoprotein particles) and 1.722 (large high density lipoprotein particles), respectively. The grey line represents the line of identity.

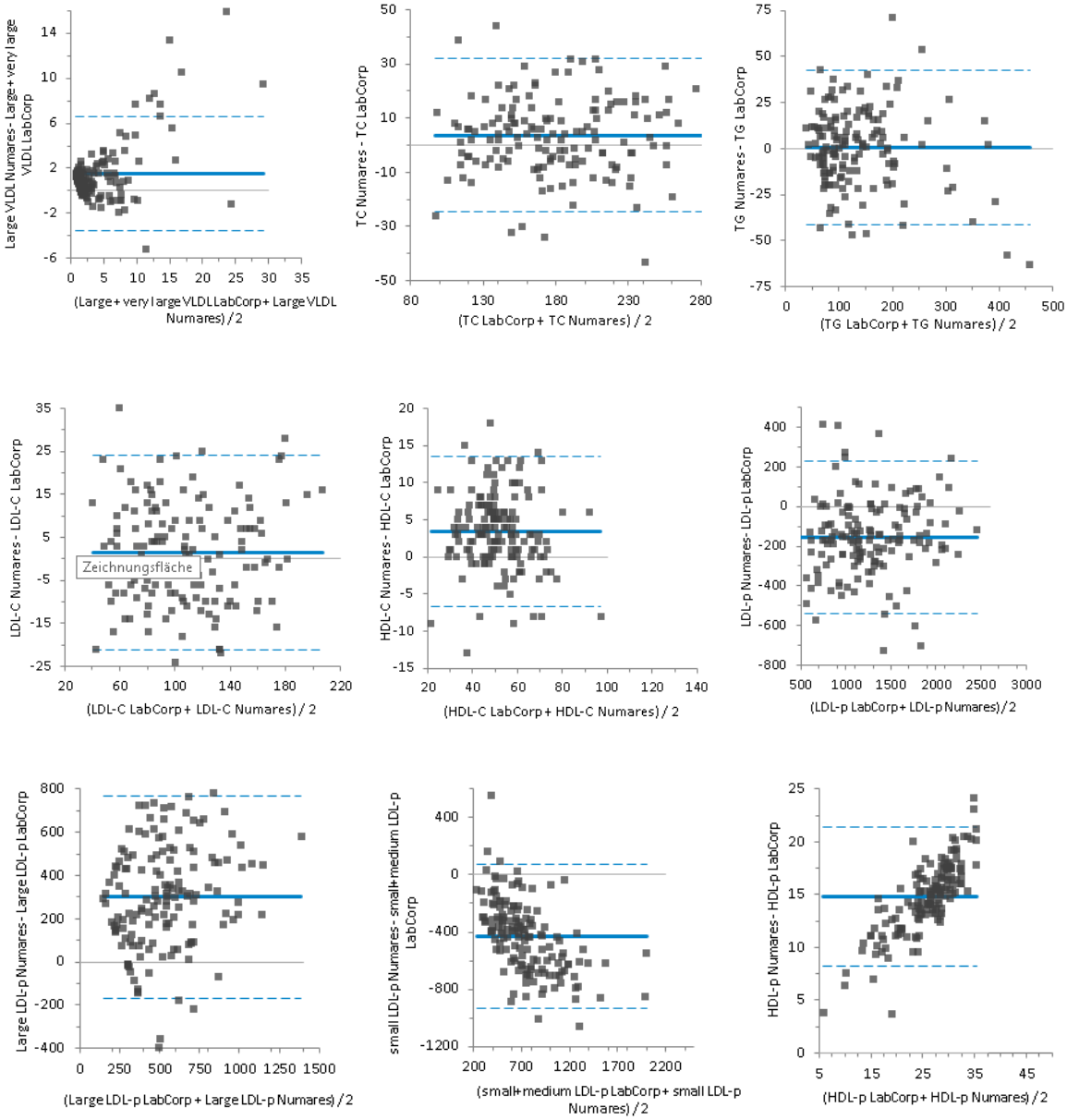
Figure 8: Comparison of lipoprotein particles between the Labcorp NMR and Numares NMR methods (dot plot)

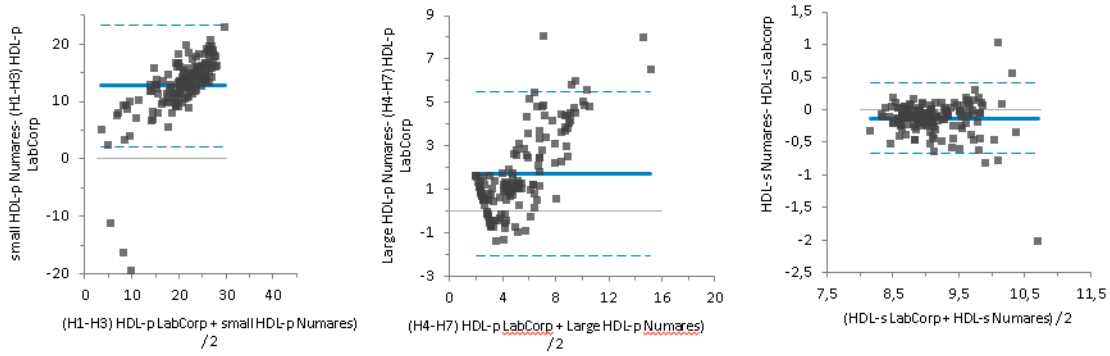


Legend: *C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins.* p= *particles. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides.*

In order to also see the absolute distribution of the measured values for the standard lipids and lipoprotein subclasses, the point diagrams are shown here.

Figure 9: Comparison of lipoprotein particles between the Labcorp NMR and Numares NMR methods (Bland Altman plots)





Legend: *dashed line = limits of agreement. continuous horizontal line = mean difference. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. MD= mean difference.* p= *particles. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides.*

The Bland Altman plots comparing the two NMR methods for the standard lipids and lipoprotein subclasses are also given.

3.3. Internal correlations and comparison within the methods

We also calculated the internal correlations of the two NMR methods, whereby the connection between the individual parameters was illustrated (see *Table 10*).

Table 10: Internal correlation matrix for the Numares method

Numares	LVLDL-p	p	LDL-p	p	LLDL-p*	p	SLDL-p*	p	HDL-p	p	LHDL-p	p	SHDL-p**	p	TC	p	TG	p	HDL-C	p	LDL-C	p
LVLDL-p	1		0,34	<0,001	-0,01	0,928	0,56	<0,001	0,07	0,365	-0,29	<0,001	0,23	0,005	0,15	0,07	0,9	<0,001	-0,28	<0,001	0,05	0,582
LDL-p	0,34	<0,001	1		0,8	<0,001	0,74	<0,001	0,32	<0,001	-0,15	0,069	0,42	<0,001	0,87	<0,001	0,54	<0,001	0,06	0,485	0,91	<0,001
LLDL-p*	-0,01	0,928	0,8	<0,001	1		0,21	<0,011	0,42	<0,001	0,26	<0,002	0,31	<0,001	0,85	<0,001	0,18	0,029	0,41	<0,001	0,83	<0,001
SLDL-p*	0,56	<0,001	0,74	<0,001	0,21	0,011	1		0,02	0,859	-0,61	<0,001	0,36	<0,001	0,49	<0,001	0,69	<0,001	-0,4	<0,001	0,56	<0,001
HDL-p	0,07	0,365	0,32	<0,001	0,42	<0,001	0,02	0,859	1		0,3	<0,001	0,86	<0,001	0,49	<0,001	0,11	0,17	0,7	<0,001	0,32	<0,001
LHDL-p	-0,29	<0,001	-0,15	0,069	0,26	0,002	-0,61	<0,001	0,3	<0,001	1		-0,22	0,009	0,15	0,069	-0,38	<0,001	0,81	<0,001	-0,02	0,841
SHDL-p	0,23	0,005	0,42	<0,001	0,31	<0,001	0,36	<0,001	0,86	<0,001	-0,22	0,009	1		0,42	<0,001	0,33	<0,001	0,29	<0,001	0,35	<0,001
TC	0,15	0,07	0,87	<0,001	0,85	<0,001	0,49	<0,001	0,49	<0,001	0,15	0,069	0,42	<0,001	1		0,36	<0,001	0,44	<0,001	0,96	<0,001
TG	0,9	<0,001	0,54	<0,001	0,18	0,029	0,69	<0,001	0,11	0,17	-0,38	<0,001	0,33	<0,001	0,36	<0,001	1		-0,29	<0,001	0,28	<0,001
HDL-C	-0,28	<0,001	0,06	0,485	0,41	<0,001	-0,4	<0,001	0,7	<0,001	0,81	<0,001	0,29	<0,001	0,44	<0,001	-0,29	<0,001	1		0,25	0,002
LDL-C	0,045	0,582	0,91	<0,001	0,83	<0,001	0,56	<0,001	0,32	<0,001	-0,2	0,841	0,35	<0,001	0,96	<0,001	0,28	<0,001	0,25	0,002	1	

*148,**147

Legend: **148 values. **147 values. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. p= particles. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides. Table modified from (Rief et al., 2022) - permission with CC BY 4.0 license.*

Table 11: Internal correlation matrix among lipids and lipoprotein particles for the Labcorp method

LabCorp	LVLDL-p	p	LDL-p	p	LLDL-p*	p	SLDL-p*	p	HDL-p	p	LHDL-p	p	SHDL-p**	p	TC	p	TG	p	HDL-C	p	LDL-C	p
LVLDL-p	1		0,17	*0,043*	-0,37	*<0,001*	0,4	*<0,001*	0,1	*0,244*	-0,3	*<0,001*	0,24	*0,003*	0,19	*0,023*	0,85	*<0,001*	-0,33	*<0,001*	0,04	*0,646*
LDL-p	0,17	*0,043*	1		0,42	*<0,001*	0,84	*<0,001*	0,3	*<0,001*	-0,11	*0,198*	0,38	*<0,001*	0,9	*<0,001*	0,35	*<0,001*	0,07	*0,368*	0,92	*<0,001*
LLDL-p*	-0,37	*<0,001*	0,42	*<0,001*	1		-0,13	*0,106*	0,01	*0,902*	0,25	*0,002*	-0,11	*0,198*	0,52	*<0,001*	-0,28	*0,001*	0,32	*<0,001*	0,61	*<0,001*
SLDL-p*	0,4	*<0,001*	0,84	*<0,001*	-0,13	*0,11*	1		0,33	*<0,001*	-0,26	*0,001*	0,48	*<0,001*	0,67	*<0,001*	0,54	*<0,001*	-0,11	*0,198*	0,64	*<0,001*
HDL-p	0,1	*0,244*	0,3	*<0,001*	0,01	*0,902*	0,33	*<0,001*	1		0,41	*<0,001*	0,91	*<0,001*	0,48	*<0,001*	0,07	*0,375*	0,64	*<0,001*	0,32	*<0,001*
LHDL-p	-0,3	*<0,001*	-0,11	*0,198*	0,25	*0,002*	-0,26	*0,001*	0,41	*<0,001*	1		-0,02	*0,812*	0,14	*0,091*	-0,31	*<0,001*	0,86	*<0,001*	-0,02	*0,799*
SHDL-p	0,24	*0,003*	0,38	*<0,001*	-0,11	*0,198*	0,48	*<0,001*	0,91	*<0,001*	-0,02	*0,812*	1		0,46	*<0,001*	0,22	*0,006*	0,3	*<0,001*	0,36	*<0,001*
TC	0,19	*0,023*	0,9	*<0,001*	0,52	*<0,001*	0,67	*<0,001*	0,48	*<0,001*	0,14	*0,091*	0,46	*<0,001*	1		0,35	*<0,001*	0,34	*<0,001*	0,95	*<0,001*
TG	0,85	*<0,001*	0,35	*<0,001*	-0,28	*0,001*	0,54	*<0,001*	0,07	*0,375*	-0,31	*<0,001*	0,22	*0,006*	0,35	*<0,001*	1		-0,35	*<0,001*	0,19	*0,021*
HDL-C	-0,33	*<0,001*	0,07	*0,368*	0,32	*<0,001*	-0,11	*0,198*	0,64	*<0,001*	0,86	*<0,001*	0,3	*<0,001*	0,34	*<0,001*	-0,35	*<0,001*	1		0,18	*0,026*
LDL-C	0,04	*0,646*	0,92	*<0,001*	0,61	*<0,001*	0,64	*<0,001*	0,32	*<0,001*	-0,02	*0,799*	0,36	*<0,001*	0,95	*<0,001*	0,19	*0,021*	0,18	*0,026*	1	

Legend: **148 values. **147 values. C= cholesterol. HDL= high density lipoproteins. LDL= low density lipoproteins. LHDL= large high density lipoproteins. LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. p= particles. SHDL= small high density lipoproteins. SLDL= small low density lipoproteins. TC= total cholesterol. TG= triglycerides. Table modified from (Rief et al., 2022) - permission with CC BY 4.0 license.*

The internal correlation matrices appeared consistent comparing the Labcorp and Numares methods (*Table 10 and 11*). With the Numares method, low density lipoprotein particles were positively associated with total cholesterol and triglycerides. They were also positively associated with large very low density lipoprotein particles and high density lipoprotein particles. Large low density lipoprotein particles were only weakly associated with small low density lipoprotein particles and positively associated with high density lipoprotein particles. Small low density lipoprotein particles were inversely related to low density lipoprotein size. High density lipoprotein particles were not associated with triglycerides. Large very low density lipoprotein particles were inversely related to high density lipoprotein cholesterol. Low density lipoprotein size was positively associated with high density lipoprotein size (*Table 10*).

With the Labcorp method, low density lipoprotein particles were also positively associated with total cholesterol and triglycerides. They were modestly and positively associated with large very low density lipoprotein particles and high density lipoprotein particles.

Large low density lipoprotein particles were not significantly associated with small low density lipoprotein particles and high density lipoprotein particles. Small low density lipoprotein particles were inversely related to low density lipoprotein size. High density lipoprotein particles were also not associated with triglycerides. Large very low density

lipoprotein particles were also inversely related to high density lipoprotein cholesterol. Low density lipoprotein size was also positively associated with high density lipoprotein size (*Table 11*). (Rief et al., 2022)

4 DISCUSSION

To our knowledge, this is the first independent and systematic comparison of the two most wide-spread NMR methods for the quantification of lipoprotein particle concentrations.
Why there has been no comparison of different NMR methods so far cannot be answered, we assume that there were company political motives that have not allowed such a comparison so far.
Overall, however, it can be stated that it was a great concern for both laboratories to make an independent scientifically comparison and of course the laboratories were also curious how their methods performed, compared to competing laboratory methods.

Regarding the data sets provided by the laboratories, we can state that Labcorp NMR and the ultracentrifugation method performed at the Medical University of Graz provided us with complete data sets as shown in *Table 6*. For whatever reason, the NMR data set provided by Numares was not complete or several values were given below the detection limit. Especially the large high density lipoprotein particles with thirty-two values below the detection limit are to be emphasized, the other values like e.g. small low density lipoprotein particles or triglycerides had only a few (four) values below the detection limit. This may have an impact on the results, especially for the values where many were below the detection limit, i.e. large high density lipoprotein particles.

4.1. Standard lipids

The mean concentrations of the standard lipids showed only minor differences between the NMR methods and also the reference method. The highest deviations were still evident in the high density lipoprotein cholesterol values of Numares, where with 53.5 (± 12.7) mg/dl compared to Labcorp with 50.1 (± 13.2) mg/dl and the reference method 50.7 (± 17.7) mg/dl, but also this deviation is below five percent and thus represents only a very small deviation from the reference method. (*see Table 7*)
Both, the Numares and the Labcorp methods showed high correlations with ß-quantification for total-, low and high density lipoprotein cholesterol, and for triglycerides. In the correlation analysis and Passing Bablok regression analysis, a consistent picture was also presented for the standard lipids, both for Labcorp and Numares. The largest deviation

was also detected for high density lipoprotein cholesterol with a correlation of 0.837 and a slope of 0.830 for Labcorp and a correlation of 0.805 and a slope of 0.786 for Numares. The mean differences also showed deviations of maximum 8.2 in the absolute values.
A few high values for high density lipoprotein cholesterol measured with ß-quantification compared with both NMR methods may be due to limitations of the precipitation step (Otvos, 1999). All other slope values of the two methods were >0.889 and correlation >0.935 for the standard lipids which underlines a very good agreement or the agreement in the absolute numbers. A few downward outliers for triglyceride measured with the Labcorp NMR method may be due to the effects of freezing on triglyceride rich particles (Jeyarajah, Cromwell and Otvos, 2006). In addition, as described in *Table 6* at the beginning of the results, four triglyceride values were not reported for the Numares method.

In summary, the differences of mean concentrations compared to ß-quantification derived measurements were under five percent for all standard lipids, for both NMR methods. Hence, the two NMR methods appear to provide reliable information on the concentration of standard lipids.

A direct comparison of the two NMR methods also showed a very high agreement between the two NMR methods in the determination of the standard lipids (see *Table 9*).
All correlation values were above 0.921. The worst agreement was again found for the high density lipoprotein cholesterol values with a correlation of 0.921 and a slope of 0.958. Especially the low density lipoprotein cholesterol values showed a nearly perfect agreement with correlation of 0.953 and slope of 1.000. From this it can be deduced that the low density lipoprotein cholesterol values are almost identical under the two methods and that there is a greater deviation in comparison with the reference method. Because in the comparison with the reference method regarding the low density lipoprotein cholesterol values slopes of 0.941 (Labcorp) and 0.935 (Numares) were to be seen, which however also represents a very good agreement and/or only a probably insignificant deviation. Especially clinically such small deviations are not relevant and have academic character.

It can be stated that the standard lipids give comparable or acceptable results not only in the comparison of the two NMR methods but also in the comparison with the reference method.

This finding is clinically relevant because the two methods differ not only in the specific method but also in other factors.

4.2. Advantages and disadvantages of NMR methods compared to the reference method

There are, of course, several advantages and disadvantages of NMR methods over the reference method (Ultracentrifugation or equivalent) that should be considered. It is an advantage that the NMR measurements offer a comprehensive picture of lipoprotein metabolism.
The NMR methods have also the advantage that a much higher number of tests can be performed in a short time. Furthermore, with the NMR methods it is possible to work in a resource-saving way due to a lower personnel expenditure. This makes them useful for scientific purpose considering that standard procedures to analyze lipoprotein metabolism such as analytical ultracentrifugation are very time-consuming and expensive. The NMR method is also cheaper compared to other methods such as ultracentrifugation.

Based on the findings of this study, NMR methods could also be used equivalently in the future in clinical routine and not only for research purposes in the measurement of standard lipids. However, there are currently no indications that it would be necessary to perform such precise determinations (e.g., ultracentrifugation or NMR) in clinical routine for the determination of standard lipids. Therefore, the application of NMR and ultracentrifugation is nowadays limited to the determination of subclasses in clinical routine. To date, mainly scientific reports on the value of NMR methods have been published with the major bias that the authors have or had a close economic relationship to the NMR companies. Especially the publications of Jim Otvos and colleagues who is considered as the founder of the NMR method has covered most of the scientific papers on this topic (Otvos, Jayarajah and Cromwell, 2002; Otvos, 1997; Otvos, 1999; Otvos et al., 1992).

In our work we were able for the first time as non-biased scientists to address the issue of whether the NMR methods provide comparable values with respect to the reference method or whether the NMR methods are comparable with each other.

A disadvantage of the NMR methods is that they are not routinely available in standard laboratories because they require special equipment. Moreover, large sample sizes are necessary (about 0.5-1 mg that is dissolved in 0.5 mL of solvent) for analysis. The lack of harmonization between the different providers also makes it difficult to interpret and compare certain results. Moreover, the associations of certain lipoprotein particle concentrations with cardiovascular endpoints have been inconsistent (Coresh and Kwiterovich, 1996). Hence certain particle concentrations from NMR measurements are currently also not recommended as therapeutic targets in the guidelines endorsed by the European Atherosclerosis Society (EAS) and European Society of Cardiology (ESC) (Boren et al., 2020).

However, if clinical institutes or laboratories in the clinical field want to acquire new methods that can be used not only for standard lipid diagnostics but also for the more detailed investigation of lipoprotein subfractions, NMR would be a suitable method for standard lipid analyses.

4.3. Lipoprotein subclasses

Furthermore, the main objective of this study was to compare the results of the two NMR methods regarding lipoprotein particle concentrations. In fact, there were acceptable correlations of the low- and high density lipoprotein particle concentrations between the Numares and Labcorp methods. Whereas low density lipoprotein particle concentrations were similar, there were considerable differences for the mean concentrations of high density lipoprotein particles with the Numares method reporting markedly higher values (~14 µmol/l). The mean concentrations of the low density lipoprotein particles were 1330 nmol/l for Labcorp and 1176 nmol/l for Numares. This deviation is not particularly large, and further statistical analysis confirmed this with correlations of 0.908 and a slope 1.057. But even such a deviation should make the laboratories think about what this deviation could be. The high density lipoprotein particle numbers showed a clear deviation, and this was also evident in the regression analysis with a slope of 1.637.
Regarding lipoprotein subclass particle concentrations, it must be considered that the Numares and Labcorp categorizations differ. It is quite reasonable to expect that if the

subfractions of the two NMR methods were to include the exact same sizes, different values would result.

In our study, for example, Labcorp included sizes from 19 to 21.4 nm for the small low density lipoprotein particles, i.e., small and medium low density lipoprotein particles, and Numares included 18 to 21.2 nm.

This shows that there are small deviations in the allocation, which can be seen in *Table 7*.

Why these allocations have been made by the laboratories and how the limits have arisen, about this no information can be given from our side. Meeusen reported in the context of another method comparison in measuring high density lipoprotein particles that the definitions of large medium and small high density lipoproteins are not consistent. (Meeusen, 2018)

We assume that harmonization of the different cutoff values could be a first step in the approximation of the different NMR methods. In addition, further work would have to clarify whether the harmonization of the cutoff values yields different results or whether and to what extent the mathematical algorithm underlying the different NMR methods has an influence on the deviating results.

A harmomization effort would necessarily be voluntary until such time as some sort of regulatory framework, like FDA, is imposed. So near-term prospects are not great. However, the need for such regulation is at least recognized by the International Federation of Clinical Chemistry (IFCC) Metabolomics Working Group. Still, any such implementation appears years away.

Hence, “apple to apple” comparisons were not feasible. Rather, we aimed to compare roughly corresponding, partly combined lipoprotein subclass categories. Weak concordance between the two NMR methods was particularly observed for small and large low density lipoprotein particle concentrations, with the Labcorp method showing a higher proportion of small low density lipoprotein particles and the Numares method showing a higher proportion of large low density lipoprotein particles.

In the statistical comparison, the small low density lipoprotein particles showed low agreement between the two NMR methods (*Table 9*; r=0.789, slope 0.593 and mean difference in absolute values of 431). Comparably lower agreements were seen for the large low density lipoprotein particles. For example, large low density lipoprotein particles

differed in mean concentrations by 300, which was also confirmed in the regression analysis with slope 1.272 and r=0.607.

Figure 7 also shows a scatter far from the line of identity for the small and large low density lipoprotein particles.

This may have to do with categorization issues since the results were more consistent between the two methods when medium and large low density lipoprotein particles of the Labcorp method were compared with large Numares low density lipoprotein particles instead of combining medium with small Labcorp low density lipoprotein particles.

As observed for total high density lipoprotein particles, small and large high density lipoprotein particle concentrations were markedly higher with the Numares method.

These considerable differences may also be due to calibration but cannot be definitely explained.

The concentration of total very low density lipoprotein particles is not provided by the Numares method so that only large very low density lipoprotein particles could be compared.

Although there appeared to be good correlation, the mean particle concentration of large very low density lipoprotein particles was markedly higher for the Numares method and with a good agreement in the regression analysis with r=0.898 and slope 0.980.

In the statistical comparison of the two NMR methods concerning the high density lipoprotein particles, strong correlations were found. For the total, large and small high density lipoprotein particles, the correlations between the two NMR methods were strong, but the regression analysis also showed significant deviations (Table 9) with a slope >1.637.

Again, the reason for the difference remains unclear.

The enormously high deviations of the absolute numbers (e.g., mean difference high density lipoprotein particles 14.8, correlation of 0,934 and a slope 1,637) of the two NMR methods suggest that the high density lipoprotein particle determination with these two methods or one of the two methods is not reliable. High deviations in absolute numbers and r values or passing bablok regression were also seen in the other subclasses of high density lipoprotein particles. As in the case of large high density lipoprotein particles with a correlation of 0.869 and a slope of 1.722 or small high density lipoprotein particles with a correlation of 0.735 and a slope of 1.817.

For this, further studies would have to compare which method provides more reliable results in comparison with the reference method.

However, in synopsis with our measured results, we have to state here that the comparison of the two NMR methods in the subclass determination of the lipoproteins delivered very poor results.
Neither the high- nor the low density lipoprotein mean particle concentrations and any statistical methods such as regression analysis or correlations could show acceptable values in the comparison.
Our conclusion is that these two NMR methods in the lipoprotein subclass determination show very large deviations.

4.4. Evaluation of low-, high- and very low density lipoprotein size and size ranges

Nearly perfect concordance between the Numares and Labcorp methods was observed for the measured mean values of the low- and high density lipoprotein sizes.
On the other hand, correlations were somewhat weaker. This was calculated by us to show whether the two laboratories might have different analysing limits. Then it could have been explained why the two NMR methods showed such large deviations in the subclass analysis.
In principle, not all sizes were given to us by the laboratories (see *Table 7*), although Labcorp gave a complete listing of size ranges. In *Table 4* you will find the compared parameters and in *Table 5* the size ranges that were given to us. There it is obvious that the parameters of the different NMR methods partly consider other size ranges. As an example, the size ranges of the small high density lipoprotein particles, which include 7.3 to 8.8 nm at Numares and 7.4 to 8.0 nm at Labcorp. The range for the small low density lipoprotein particles of 18 to 21.2 nm is considered for Numares and a range of 19 to 20.4 nm for Labcorp. For large very low density lipoprotein particles, a range of 60 to 200 nm is given for Numares and a range of 50 to 89 nm for Labcorp. This is the reason why the compared parameters of the two NMR methods often differ from the nomenclature (in comparison Labcorp large and very large very low density lipoproteins 50 to 240 nm and large very low density lipoprotein particles from Numares with 60 to 200 nm), because the measured size ranges are often different. This could be improved only approximately by other comparison, the identical size ranges could not be compared nevertheless we assume

that it is method-specific reasons why one cannot compare identically here, from the laboratories no statement could be made to us concerning this. The best illustration of the opposing size ranges is shown in *Table 5*.

4.5. Further analyses

Comparing the Numares and Labcorp analyte panel, Labcorp provides a more comprehensive list of parameters (*see Appendix A.7.*). It includes a more detailed separation of lipoprotein subclasses and provides information on apolipoprotein B.
The internal correlation matrix among major lipids and lipoprotein particles gave similar results for the Numares and the Labcorp methods.
This supports that measurement of lipoprotein particles with these methods is qualitatively comparable.
Most importantly, there should not be a strong positive correlation between large low density lipoprotein particles and small low density lipoprotein particles (Remaley and Otvos, 2020). No such correlation was seen with the lipoprofile and lipofit methods. This contrasts with a recent analysis with the Nightingale method which showed strong, positive correlations among all low density lipoprotein subclasses ($r > 0.8$) (Balling et al., 2019). Still, the difference in mean particle concentrations requires further investigation. This is of particular relevance as a more precise characterization of the lipoprotein profile may help to improve risk classification reflecting different pathophysiological features of the various lipoprotein subclasses (Boren et al., 2020).

4.5.1. Further issues in lipoprotein analysis

Our research group around Prof. Silbernagel, Prof. März and Prof. Scharnagl deals with various questions around lipoprotein analysis as well as in combination with the risk stratification of lipoproteins for internal diseases. In a recent work we could show that increased low density lipoprotein and apolipoprotein B/low density lipoprotein cholesterol ratios can be associated with increased cardiovascular mortality. Furthermore, the use of the low density lipoprotein and apolipoprotein B/low density lipoprotein cholesterol ratio allows an estimation of the atherogenic risk. (Silbernagel et al., 2022)

Further data concerning apolipoproteins (Apolipoprotein C-II) will be published soon and will certainly become even more important in science than before.

To previous findings, Tamura et al showed that elevated apolipoprotein C-II has already been associated with an increased incidence of Takayasu arteritis, and even further associations are to be expected (Tamura et al., 2021; Wolska, Reimund and Remaley, 2020).

In recent years, apolipoprotein C-II has not been adequately studied. However, in a new paper, we can provide further insights into this apolipoprotein C-II. The results are currently unpublished but will be published soon. Given the broad spectrum of its biochemical activity and its role in atherogenesis, much emphasis has always understandably been placed on understanding apolipoprotein C-III. Very low-density lipoproteins and residual lipoproteins are now implicated in atherogenesis. This has led to increased interest in the regulation of lipoprotein lipase, the enzyme responsible for triglyceride lipolysis in triglyceride-enriched lipoproteins. Many gene variants and insulin resistance lead to genetic and functional deficiencies of lipoprotein lipase. Apolipoprotein C-II is a critical activator of lipoprotein lipase. Silbernagel et al evaluate several functional characteristics of apolipoprotein C-II using the prospective longitudinal cohort Ludwigshafen RIsk and Cardiovascular Health (LURIC) study. Importantly, this study included approximately 3100 participants who were followed for nearly 10 years. (Silbernagel et al., 2022)

Unlike many biochemical activators, apolipoprotein C-II activation of lipoprotein lipase peaks and appears to be inhibitory at high concentrations. This helps to explain the previous observation that overexpression of apolipoprotein C-II inhibits lipoprotein lipase. Because lipolytic activity would be expected to correlate with serum concentrations of triglyceride-enriched lipoproteins, the relationship between cardiovascular mortality and apolipoprotein C-II levels is inverse to the triglyceride-enriched lipoproteins lipolysis profile. This also implies that apolipoprotein C-II is a viable therapeutic target.

A better understanding of the biochemistry and physiology/pathophysiology of apolipoproteins is critical for the development of sophisticated approaches to treat the many forms of dyslipidemia currently affecting the world population.

4.6. Statistical analyses

The statistical methods applied such as Pearson correlation and Passing Bablok regression analysis were the appropriate methods to compare the different parameters and represent the statistical method of choice in a method comparison such as ours. The Bland Altman plots also graphically depict the comparability of the methods well.
Since this comparison of methods does not deal with significant results, we must clarify that we are dealing with two identical methods and that they are identical in the best case and highly significant anyway, therefore mainly no p values (<0.05) are given.

4.7. Strengths of the study

The very strong aspect of this study is that we performed an independent method comparison of the two most wide-spread NMR methods for the quantification of lipoprotein particle concentrations.
The standard biochemical analyses were performed by a very experienced laboratory at the Medical University of Graz and the NMR analyses were performed by very experienced and recognized laboratories of Numares in Regensburg (Germany) and Labcorp in North Carolina (USA).
Another strong aspect is that ß-quantification, the standard method, was used to measure standard lipids. In all aspects of this study, the study protocol, which was written prior to the study, was followed exactly and there were no deviations from it. Data analysis was also performed by experienced lipid researchers at the Medical University of Graz (Austria).

4.8. Limitations of the study

It may be seen as a limitation of this study that analytical ultracentrifugation was not performed.
It is difficult to say whether the subclass determination would have yielded different results with analytical ultracentrifugation. If we had also done this investigation, then it would

have been possible to compare the subclasses of the NMR methods with ultracentrifugation.

However, this was not considered by us in the initial planning of the study, it was simply planned to compare only the standard lipids and the purpose of the study was to compare the two NMR methods. In further studies, it would be interesting to see how analytical ultracentrifugation compares to the two NMR methods investigated with respect to the lipoprotein subclasses.

Hence, we were not able to perform an independent comparison with the standard method for the analysis of lipoprotein subclass particle concentrations.

However, the primary focus was to address the comparability of the two wide-spread NMR methods.

Another limitation could be the different cutoff values of the NMR methods. It was taken into account before the study that there are different cutoff values from the two NMR method laboratories, so this was also taken into account, and it was clarified in advance with the two companies which parameters should be compared with each other. *Table 4* shows which parameters were compared with each other. After completion of the study and with the available results, several other variants of comparability were discussed, the results were of course not changed because this would have meant a deviation from our study protocol. We have also calculated this other comparability and included it as a side note in our results section, in case the readers of this paper ask why we did not compare the subclasses (more precisely e.g.: small and medium high density lipoprotein particles of one company against small particles of the other company) differently.

Probably the most important limitation of the study is that in the context of this study the NMR methods are mainly comparable based on the standard lipids, the subclasses were determined, but there were quite large differences to identify. Thus, the core statement of the study that the two NMR methods show comparable values in the standard lipid analysis is only of limited use. The recognized reference method for the determination of standard lipids is at least a method with a step of ultracentrifugation. NMR methods have advantages over ultracentrifugation, but also many disadvantages over ultracentrifugation. Ultracentrifugation is currently certainly more often clinically available than NMR methods and therefore there is no good reason to change this.

5 Conclusions

To sum up, the present study shows that standard lipids can be reliably measured with these two NMR methods. However, the purpose of this work was not to compare the standard lipids but the lipoprotein subclasses - determination of the different NMR methods. Lipoprotein particle concentrations of low- and high density lipoprotein subfractions showed suboptimal congruency between the Numares and Labcorp NMR methods.

The differences in mean concentrations especially of total high density lipoprotein particles and of all lipoprotein subclass particle concentrations comparing the Numares with the Labcorp method require further investigations.

Also, possibly the results of lipoprotein subclass analysis of NMR methods should be compared with those of analytical ultracentrifugation in a new study to check or verify the results.

Such studies are highly encouraged as the use of NMR methods to measure lipoprotein classes and is expected to increase.

Efforts should also be made to harmonize the NMR methods in order to subsequently be able to speak of "one" NMR method. The goal should certainly be that different laboratory methods worldwide can provide the same or appropriately comparable values. In this work it could be shown that this is not always the case. The fact that the NMR method delivered good results in the determination of the standard lipids in this work should not be overestimated, since the NMR methods are used in principle for the determination of the subclasses. And for this subclass determination one cannot assume that two different NMR methods or performed by different laboratories or used different test kits deliver comparable results.

The most accurate possible test methods are necessary to accurately reflect the researched lipoprotein value targets. The risk stratification of e.g., cardiovascular risk can only be implemented clinically if good laboratory methods are available to measure it accurately. Currently, it can be assumed that the standard methods provide reliable values and NMR in the standard lipid determination. In the measurement of lipoprotein subclasses, the NMR method still needs to be standardized and to pass the test of providing globally comparable values. Based on our findings, the current most interesting further investigation would be

the comparison of the Labcorp and Numares NMR methods and the ultracentrifugation method in the measurement of lipoprotein subclasses.

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Appendix

A.1. Study protocol

PROTOKOLL

Medizinische Universität Graz

Titel: Methodenvergleich zur Bestimmung von Lipoprotein-Subklassen

Gesamtseitenanzahl: 3

Prüfer:

Ass.Prof.PD.Dr.Günther Silbernagel
Universitätsklinik für Innere Medizin, Abteilung für Angiologie, Medizinische Universität Graz

Mitarbeiter an der Studie:

Dr. Martin Rief
Universitätsklinik für Anästhesiologie und Intensivmedizin, Klinische Abteilung für Allgemeine Anästhesiologie, Notfall- und Intensivmedizin, Medizinische Universität Graz

Assoz. Prof. Hubert Scharnagl, PhD
Klinisches Institut für Medizinische und Chemische Labordiagnostik, Medizinische Universität Graz

Hintergrund:

Erhöhte Konzentrationen von low density lipoprotein (LDL)-Cholesterin und niedrige Konzentrationen von high density lipoprotein (HDL)-Cholesterin gelten als kardiovaskuläre Risikofaktoren. Allerdings sind LDL und HDL eine heterogene Gruppe von Lipoproteinpartikeln. Die kleinen, dichten LDL werden als die am stärksten atherogene Fraktion angesehen. Kleine HDL hingegen werden als besonders protektiv angesehen.

Der Goldstandard für die Bestimmung von LDL- und HDL-Subklassen ist die Ultrazentrifugation. Ultrazentrifugationsmethoden haben allerdings den Nachteil, dass sie einen hohen Aufwand an Zeit und Personal erfordern und daher für die Patientenversorgung und für Studien mit großer Patienten/innenzahl nicht geeignet sind.

Eine Alternative zur Bestimmung der Lipoprotein-Subklassen mittels Ultrazentrifugation stellt die Technik der Nuklearmagnetresonanz (NMR)-Spektroskopie dar. Die NMR Methoden von NUMARES und LabCorp werden zu Forschungszwecken und in der Klinischen Routine häufig eingesetzt.

Es wurden bisher allerdings keine Untersuchungen durchgeführt, ob die Methoden von NUMARES und LabCorp vergleichbare Ergebnisse bringen.

Ziele:

Ziel unserer Studie ist es daher, neben der Routine-Labordiagnostik die Bestimmung der Lipoprotein-Subklassen mit den beiden oben erwähnten NMR-Methoden bei 150 Patienten/innen durchzuführen.

Ziel unserer Analyse ist es, einen Vergleich von zwei etablierten NMR-Methoden zur Bestimmung von Lipoprotein-Subklassen untereinander und mit Routine-Laboruntersuchungen durchzuführen. Die verwendeten statistischen Methoden sind Bland-Altman und Regression nach Passing/Bablok.

Design:

Bei diesem multizentrischen Methodenvergleich wird Probenmaterial von 150 PatientInnen der Universitätsklinik für Innere Medizin der Medizinischen Universität Graz verwendet. Es sind folgende Einschlußkriterien vorgesehen:

- Personen ab dem vollendeten 18. Lebensjahr
- männliches und weibliches Geschlecht
- unterschiebene Einverständniserklärung

Proben/Blutabnahme:

Zusätzlich zur Blutabnahme für die Routineanalytik werden den Patienten/innen 2 Röhrchen zu je 9 ml Vollblut abgenommen (Greiner bio-one Vacuette Z Serum, red, 9.0 ml; 455092). Aus den 18 ml Vollblut werden ca. 7 ml Serum gewonnen, die wie folgt verwendet werden:

- zwei NMR-Methoden: jeweils 2 ml Serum
- Restmaterial (2-3 ml) wird für eventuell notwendige Wiederholungsmessungen

Verwendung bzw. Anonymisierung der Proben:

Nach der Blutabnahme werden die Proben anonymisiert, d.h. PatientInnen werden mit einer fortlaufenden ID-Nummer bezeichnet (weder Name noch Initialen, Geburtsdatum, Laboranforderungsnummer werden festgehalten).

Lagerung der Proben und Versand:

Die Proben werden auf -80°C abgekühlt und nach Abschluss der Probensammlung an die beiden Laboratorien zur Auswertung gesendet.

Verwendung klinischer Daten:

Für die statistische Auswertung des Methodenvergleichs werden keine klinischen Daten verwendet. Zur Interpretation der Ergebnisse kann es unter Umständen notwendig sein, auch klinische Daten zu berücksichtigen. Sollten beim Methodenvergleich Ergebnispaare deutlich voneinander abweichen, könnten Patientendaten verwendet werden um die klinische Wertigkeit dieser Ausreißer zu beurteilen (z.B. Diagnose, Begleiterkrankungen, Therapie, Medikation, Rauchen, Alter etc.).

Zentren:

Routineanalytik:

Assoz. Prof. Scharnagl, PhD
Klinisches Institut für Medizinische und Chemische Labordiagnostik
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NMR Methode 1 „Labcorp“:

Prof. James D. Otvos, PhD
Strategic Director, NMR Diagnostics
100 Perimeter Park (Suite C)
Morrisville, NC 27560
Adj. Prof. Molecular & Structural Biochemistry, NC State Univ.
Adj. Prof. Medicine, UNC-Chapel Hill
(919) 388-5530 (direct)
otvosj@labcorp.com

NMR Methode 2 “Numares”:

Philipp Pagel, PhD
Vice President, CMO
NUMARES Regensburg AG
Am Biopark 9, 93053 Regensburg, Deutschland
Tel: +49 941 280 949-00
Fax: +49 941 280 949-50
Email: philipp.pagel@numares.com

Ethische Aspekte/Risikoabschätzung:

Für die Studie wird zusätzlich zur geplanten Blutabnahme im Rahmen der Patientenversorgung 40 ml Blut abgenommen. Für die PatientInnen besteht somit kein zusätzliches Risiko. Außer der Blutabnahme entstehen für die PatientInnen keine zusätzlichen Belastungen.

A.2. Informed consent form

PatientInneninformation[1] und Einwilligungserklärung zur Teilnahme an der klinischen Studie

Methodenvergleich zur laborchemischen Bestimmung von Lipoprotein Subklassen

Sehr geehrte Teilnehmerin, sehr geehrter Teilnehmer!

Wir laden Sie ein an der oben genannten klinischen Studie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

Ihre Teilnahme an dieser klinischen Studie erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.

Klinische Studien sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer klinischen Studie ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser klinischen Studie schriftlich erklären. Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Arzt sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur

- wenn Sie Art und Ablauf der klinischen Studie vollständig verstanden haben,
- wenn Sie bereit sind, der Teilnahme zuzustimmen und
- wenn Sie sich über Ihre Rechte als Teilnehmer an dieser klinischen Studie im Klaren sind.

Zu dieser klinischen Studie, sowie zur Patienteninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

1. Was ist der Zweck der klinischen Studie?

Der Zweck dieser klinischen Studie ist der Vergleich verschiedener Labormethoden bei der Bestimmung von Fettmolekülen im Blut.

1 Wegen der besseren Lesbarkeit wird im weiteren Text zum Teil auf die gleichzeitige Verwendung weiblicher und männlicher Personenbegriffe verzichtet. Gemeint und angesprochen sind – sofern zutreffend – immer beide Geschlechter.

2. Wie läuft die klinische Studie ab?

Diese klinische Studie wird an der Universitätsklinik Graz durchgeführt, und es werden insgesamt ungefähr 100 Personen daran teilnehmen. Ihre Teilnahme an dieser klinischen Studie ist nach dem Aufklärungsgespräch, dem Ausfüllen des Erhebungsbogens bzw. der Blutabnahme abgeschlossen.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:

Während dieser klinischen Studie wird bei Ihnen eine Blutabnahme durchgeführt, sowie ein Umfragebogen von einem Studienarzt ausgefüllt. Die Blutabnahme wird entweder während der Katheteruntersuchung oder im Rahmen einer anderen Routine-Blutabnahme durchgeführt. Das heißt, es ist keine zusätzliche Blutabnahme notwendig, sondern während einer Routine-Blutabnahme werden einfach zusätzlich Blutröhrchen abgenommen (insg. 30ml).

3. Worin liegt der Nutzen einer Teilnahme an der Klinischen Studie?

Es ist möglich, dass Sie durch Ihre Teilnahme an dieser klinischen Studie keinen direkten Nutzen für Ihre Gesundheit ziehen. Das heißt, das von Ihnen abgenommene Blut wird genau hinsichtlich der Fettzusammensetzung untersucht, sollten irgendwelche interessanten Erkenntnisse aus der Blutfettzusammensetzung gewonnen werden - werden diese Ihnen mitgeteilt.

4. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

Es werden bei dieser Studie keine zusätzlichen Risiken und Beschwerden auf die Teilnehmer zukommen.

5. Hat die Teilnahme an der klinischen Studie sonstige Auswirkungen auf die Lebensführung und welche Verpflichtungen ergeben sich daraus?

Es ergeben sich keine Auswirkungen auf die Lebensführung und es ergeben sich aus der Studienteilnahme keine Verpflichtungen.

6. Wann wird die klinische Studie vorzeitig beendet?

Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der klinischen Studie ausscheiden ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr Studienarzt wird Sie über alle neuen Erkenntnisse, die in Bezug auf diese klinische Studie bekannt werden, und für Sie wesentlich werden könnten, umgehend informieren. Auf dieser Basis können Sie dann Ihre Entscheidung zur **weiteren** Teilnahme an dieser klinischen Studie neu überdenken.

Es ist aber auch möglich, dass Ihr Studienarzt entscheidet, Ihre Teilnahme an der klinischen Studie vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen.

7. In welcher Weise werden die im Rahmen dieser klinischen Studie gesammelten Daten verwendet?

Sofern gesetzlich nicht etwas anderes vorgesehen ist, haben nur die Studienärzte und deren Mitarbeiter Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden. Diese Personen unterliegen der Schweigepflicht. Die Weitergabe der Daten erfolgt ausschließlich zu statistischen Zwecken und Sie werden ausnahmslos nicht namentlich genannt. Auch in etwaigen Veröffentlichungen der Daten dieser klinischen Studie werden Sie nicht namentlich genannt.

8. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser klinischen Studie entstehen für Sie keine zusätzlichen Kosten. Eine Vergütung ist nicht vorgesehen.

9. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser klinischen Studie stehen Ihnen Ihr Studienarzt und seine Mitarbeiter gern zur Verfügung. Auch Fragen, die Ihre Rechte als Patient und Teilnehmer an dieser klinischen Studie betreffen, werden Ihnen gerne beantwortet. Sobald allgemeine Ergebnisse dieser klinischen Studie vorliegen, können Sie ebenfalls darüber informiert werden, falls Sie dieses wünschen.

Name der Kontaktperson: Dr. Martin Rief

Ständig erreichbar unter: 0316/385 84661

Name der Kontaktperson: Ass.Prof.PD.Dr. Günther Silbernagel

Ständig erreichbar unter: 0316/385 30050

10. Einwilligungserklärung

Name des Patienten in Druckbuchstaben: .....

Geb.Datum: ..... Code: .....

Ich erkläre mich bereit, an der klinischen Studie **Methodenvergleich zur laborchemischen Bestimmung von Lipoprotein Subklassen** teilzunehmen.

Ich bin von Herrn _____ ausführlich und verständlich über mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der klinischen Studie, sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser Patientenaufklärung und Einwilligungserklärung, die insgesamt 4 Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Studienarzt verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.

Ich werde den ärztlichen Anordnungen, die für die Durchführung der klinischen Studie erforderlich sind, Folge leisten, behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung jederzeit zu beenden, ohne dass mir daraus Nachteile für meine weitere medizinische Betreuung entstehen.

Beim Umgang der im Rahmen der "Methodenvergleich zur laborchemischen Bestimmung von Lipoprotein Subklassen" erhobenen Daten werden die Bestimmungen des Datenschutzgesetzes 2000 beachtet. Alle Personen, die auf Grund ihrer beruflichen Tätigkeit Zugang zu diesen Daten haben, sind - unbeschadet anderer gesetzlicher Verpflichtungen - gemäß § 15 DSG 2000 an das Datengeheimnis gebunden.

Nach dem DSG 2000 sind „personenbezogene Daten" Angaben über Studienteilnehmer/-innen, durch die deren Identität bestimmt oder bestimmbar ist. Unter „indirekt personenbezogenen Daten" versteht das DSG 2000 Daten, deren Personenbezug derart ist, dass die Identität der Studienteilnehmer/-innen mit rechtlich zulässigen Mitteln nicht ermittelt werden kann. Ich stimme zu, dass meine im Rahmen und zum Zweck dieser Studie ermittelten personenbezogenen Daten (Name, Anschrift, Alter, Angaben über die Gesundheit wie z.B.: Vorerkrankungen, Körpergröße, Körpergewicht) verarbeitet werden und in indirekt personenbezogener (pseudonymisierter bzw. verschlüsselter) Form an, Studienmitarbeiter sowie Statistiker zum Zweck der Datenauswertung übermittelt werden. Mir ist bekannt, dass zur Überprüfung der Richtigkeit der Datenaufzeichnung Beauftragte der zuständigen Behörden, der Ethikkommissionen und des Auftragsgebers der Prüfung beim Prüfarzt Einblick in die Daten nehmen dürfen.

Mir ist auch bekannt, dass ich meine Zustimmung zur Datenverwendung ohne Angabe von Gründen und ohne nachteilige Folgen für meine medizinische Behandlung jederzeit widerrufen kann, wobei ein Widerruf grundsätzlich die Unzulässigkeit der weiteren Verwendung der Daten bewirkt, sofern nicht andere gesetzliche Vorschriften oder überwiegende berechtigte Interessen die Datenverwendung weiterhin zulässig machen.

Darüber hinaus bin ich für den Fall, dass ich aus der Studie ausscheide oder die Studie beendet wird, damit einverstanden, dass etwaige Restproben für weitere Forschungsprojekte auf dem Gebiet (Forschungsgebiet) verwendet werden. Für jedes dieser Forschungsprojekte wird zuvor die Zustimmung der Ethikkommission eingeholt. Mir ist bekannt, dass ich jederzeit verlangen kann, dass meine Proben vernichtet werden. Verantwortlich für die Lagerung und die Vernichtung der Proben ist (Dr. Martin Rief, Studienarzt). Eine Kopie dieser Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Studienarzt.

.....

(Datum und Unterschrift des Patienten)

.....

(Datum, Name und Unterschrift des verantwortlichen Arztes)

(Der Patient erhält eine unterschriebene Kopie der Patienteninformation und Einwilligungserklärung, das Original verbleibt im Studienordner des Studienarztes.)

A.3. Statement of the institutional review board

Medizinische Universität Graz
Ethikkommission

Auenbruggerplatz 2, A-8036 Graz
ethikkommission@medunigraz.at
Tel.: +43 / 316 / 385-13928, Fax: -14348

FOLGEVOTUM
gültig bis 18.09.2020

EK-Nummer:	29-479 ex 16/17
Studientitel:	Comparison of different laboratory methods for the measurement of lipoprotein subclasses
Prüfer:	Ass.-Prof. Dr.med Günther Silbernagel Univ. Klinik für Innere Medizin
Sponsor:	Medizinische Universität Graz
Ansprechpartner:	Ass.Prof.Dr. Günther Silbernagel, 8036 Graz, Auenbruggerplatz
CRO:	-
Antragsteller:	Medizinische Universität Graz, Univ. Klinik für Anästhesiologie und Intensivmedizin
Ansprechpartner:	Dr. Martin Rief, 8036 Graz, Auenbruggerplatz 29/I

Die o.a. Studie wurde von der Ethikkommission erstmals im 'expedited Review' am 02.06.2017 behandelt. Die Ethikkommission ist zu folgendem Schluss gekommen:

Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: keine

Zur Beurteilung vorliegende Dokumente:

Dokumente eingegangen am 22.05.2017, begutachtet im 'expedited Review' am 02.06.2017	
✓ Antragsformular ECS Teil A unterschrieben	22.05.2017
✓ Antragsformular ECS	22.05.2017
Originalprotokoll Studienprotokoll Methodenvergleich Lipoproteine_V1 1	22.05.2017
Informed Consent Form Einwilligungserklärung 1	22.05.2017
✓ Sonstiges: Patientenfragebogen Version 3.0 1	22.05.2017
Dokumente eingegangen am 23.05.2017, begutachtet im 'expedited Review' am 02.06.2017	
✓ Antrag Teil B ECS unterschrieben, Silbernagel	23.05.2017
Dokumente eingegangen am 21.06.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Originalprotokoll 2	13.06.2017
✓ Informed Consent Form 2	13.06.2017
✓ Sonstiges: Stellungnahme zum Sponsor	20.06.2017
✓ Sonstiges: Stellungnahme zu den Datierungen	13.06.2017
Dokumente eingegangen am 04.08.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Sonstiges: E-Mail - Stellungnahme zur Bearbeitungsmitteilung	04.08.2017
Dokumente eingegangen am 13.09.2017, begutachtet im 'expedited Review' am 18.09.2017	
✓ Letter of Authorization	13.09.2017

Dokumente eingegangen am 10.07.2018, begutachtet im 'expedited Review' am 18.07.2018	
✓ Originalprotokoll 3	09.07.2018
✓ Informed Consent Form 3.0	09.07.2018
✓ Sonstiges: EK-Meldeformular Unterschriftenseite	09.07.2018
✓ Sonstiges: EK-Meldeformular undatiert	
Dokumente eingegangen am 19.08.2018, begutachtet im 'expedited Review' am 03.10.2018	
✓ Zwischenbericht (e-mail)	19.09.2018
Dokumente eingegangen am 19.12.2019, begutachtet im 'expedited Review' am 07.01.2020	
✓ Zwischenbericht	19.12.2019

Datum Erstvotum: 18.09.2017

Die Ethikkommission geht – rechtlich unverbindlich – davon aus, dass es sich um eine Leistungsbewertungsprüfung nach MPG handelt und macht darauf aufmerksam, dass vor Beginn der Prüfung eine ordnungsgemäße Meldung an das Bundesamt für Sicherheit im Gesundheitswesen zu erstatten ist.

Es handelt sich um eine Studie im Rahmen einer Dissertation.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

Graz, 07. Jänner 2020

Univ.Prof.DI Dr.Josef Haas
Vorsitzender

Univ.Prof.Dr.Hans Dimai
Stv. Vorsitzender

Achtung: Bitte bei allen das Projekt betreffende Schreiben oder telefonischen Anfragen die EK-Nummer angeben!

A.4. Statement of the Bundesamt für Sicherheit im Gesundheitswesen

Bundesamt für Sicherheit im Gesundheitswesen
BASG

BASG/AGES
Institut Überwachung
Traisengasse 5, 1200 Wien, Österreich

Medizinische Universität Graz
Dr. Martin Rief
Auenbruggerplatz 15
A-8036 Graz

date: 15.05.2019
department: clinical trials - CLTR
phone: +43(0)5 0555 36441
e-mail: clinicaltrials@ages.at
reference: 11458091

Bestätigung der ordnungsgemäßen Erstmeldung einer Leistungsbewertungsprüfung gemäß § 40 Abs. 3 MPG idgF

Sehr geehrter Herr Hirschmann, MSc, BSc, sehr geehrter Herr Professor Silbernagel,

das Bundesamt für Sicherheit im Gesundheitswesen (BASG) bestätigt hiermit die formale Vollständigkeit der Leistungsbewertungsprüfung **„Comparison of two nuclear magnetic resonance (NMR-) methods in the measurement of lipoprotein subclasses"** mit Datum **02.05.2019.**

Gemäß § 40 Abs. 3 MPG idgF kann mit der klinischen Prüfung nach Erhalt des gegenständlichen Schreibens begonnen werden, sofern eine befürwortende Stellungnahme der zuständigen Ethikkommission vorliegt.

Die Klinische Prüfung wird unter der Referenznummer **11458092** geführt. Dieser Code ist bei weiterem Schriftverkehr zu dieser Studie als Referenz anzuführen.

Den Leitfaden einschließlich der Meldeverpflichtungen sowie Formulare sind auf folgender Website zu finden: www.basg.gv.at/medizinprodukte/klinische-pruefung-von-medizinprodukten/.

Für das Bundesamt

Zmuda Violetta
am 15.5.2019

BUNDESAMT FÜR SICHERHEIT IM GESUNDHEITSWESEN @ AMTSSIGNATUR	Dieses Dokument wurde amtssigniert. Informationen zur Prüfung der elektronischen Signatur und des Ausdrucks finden Sie unter http://www.basg.gv.at/amtssignatur. Bundesamt für Sicherheit im Gesundheitswesen Traisengasse 5, 1200 Wien	
	Signaturwert	Wiuf1W1Bmzbsm/0/STDPSs0I5Gwi5 tmTwt/mt0tdIomTrGSGt0oa02os DbfSdSkID2fvkBDs1eSkGWDfdmul5d diht1SrPT5bS0ADBiepbipkgAdv z0sg/fvu/osg2IdsIzpgDWfa0ovsI1e 1nIowAwAuW5InhDmPDorInnbIzlei P1dk/stzziBrttacrtgu/tvPv2IaPW5

A.5. Labcorp method description

NMR LipoProfile® With Lipids

TEST: 123810; CPT: 80061; 83704

Test Includes

Color graphical report; historical reporting (LDL-P, LDL-C); insulin-resistance score; lipoprotein particle number (LDL-P); particle concentration and size (total HDL-P, small LDL-P, LDL size); standard lipid panel (total cholesterol, calculated LDL cholesterol, HDL cholesterol, triglycerides).

Expected Turnaround Time

2 - 3 days; Turnaround time is defined as the usual number of days from the date of pickup of a specimen for testing to when the result is released to the ordering provider. In some cases, additional time should be allowed for additional confirmatory or additional reflex tests. Testing schedules may vary.

Specimen Requirements

Specimen

Spun NMR LipoTube (preferred), serum from a plain red-top tube, plasma from a lavender-top (EDTA-no gel), or green-top (heparin-no gel) tube. Keep refrigerated and ship with frozen cool packs.

Volume

2 mL

Minimum Volume

1 mL

Container

NMR LipoTube (black-and-yellow-top tube) is the preferred container, plain red-top tube, lavender-top (EDTA-no gel) tube, or green-top (heparin-no gel) tube.

Collection

Collect specimen in NMR LipoTube (black-and-yellow-top tube), which is the preferred container. Plain red-top, green-top (heparin-no gel), or lavender-top (EDTA-no gel) tubes are also acceptable. Serum or plasma drawn in gel-barrier collection tubes other than the NMR LipoTube should not be used. The LipoTube is the only acceptable gel-barrier tube. Gently invert tube 8 to 10 times to mix contents and allow specimen to clot for 30 minutes upright at room temperature prior to centrifugation (Plasma tubes should not clot). Centrifuge specimen within two hours of collection at 1800xg for 10 to 15 minutes to separate serum/plasma from the red cells and to avoid red cell contamination during shipment. If the sample cannot be centrifuged immediately, the sample should be refrigerated (at 2ºC to 8ºC) and centrifuged within 24 hours of collection. Note: Centrifuging the specimen while still cold may negatively affect the migration of the gel to the serum/red cell interface and may increase the likelihood of specimens being contaminated with red cells during shipment. All specimens should be centrifuged by the client, prior to shipment to Labcorp, to ensure sample integrity. Do not open NMR LipoTube (black-and-yellow-top). Immediately after centrifugation, pipette separated red-top serum or green-top/lavender-top plasma into a transport tube and label accordingly (serum, heparin plasma, EDTA plasma). Keep samples refrigerated until shipment to the laboratory, and ship with frozen cool packs.

Storage Instructions

Refrigerate all acceptable tube types as soon as possible after centrifugation and within 24 hours of collection. Keep refrigerated prior to shipment, and ship on frozen cool packs. Do not store at room temperature. Do not freeze the sample. Sample is stable refrigerated for six days.

Patient Preparation

Patient should be fasting for eight hours.

Causes for Rejection

Unspun specimens; plasma/serum contaminated with red cells; citrated plasma (light blue-top tube); gross hemolysis; specimen received in inappropriate container; specimen stored at room temperature for more than a total preanalytical time of 24 hours; specimen more than six days old

Test Details

Use

NMR LDL-P is a management tool used in appropriate high-risk patients (type 2 diabetes mellitus, metabolic syndrome, CVD risk equivalent, statin-treated patients) to adjudicate response to treatment and guide adjustment in therapy. It is used in conjunction with other lipid measurements and clinical evaluation to aid in the management of lipoprotein disorders associated with cardiovascular disease.

Limitations

If triglyceride level is >800 mg/dL, LDL cholesterol will not be calculated.

Methodology

Nuclear magnetic resonance (NMR)

Additional Information

The NMR LipoProfile® test is an FDA-cleared blood test that directly measures the amount of LDL circulating in the body. “LDL” is low-density lipoprotein and has long been recognized as a major causal factor in the development of heart disease. Although the relationship of increased LDL particle number and plaque buildup in the artery wall has been known since the 1950s, a diagnostic test did not exist to measure LDL particle number (LDL-P). Historically, LDL cholesterol, or LDL-C, has been used to estimate LDL levels to assess a patient’s LDL-related cardiovascular risk and judge an individual’s response to LDL-lowering therapy. Today, a more reliable measure of LDL exists that directly counts the number of LDL particles a patient has using NMR technology.

A.6. Numares method description

AXINON® lipoFIT®*

Advanced Lipoprotein Testing for Cardiovascular Risk Assessment

numares HEALTH

AXINON® lipoFIT® – Lipoprotein Analysis for CVD Risk Management

- Determination of lipoprotein particle numbers & sizes for advanced cardiovascular risk assessment in a single measurement
- Standardized on-site NMR system
- Easy usability and automated platform with QC standards for reliable, high-throughput results

Cardiovascular disease (CVD) is the number one cause of death worldwide [1]. Clinical CV risk factors include hypertension, diabetes mellitus, chronic kidney disease, obesity, cigarette smoking and family history. The most important biomarkers for CV risk determination are lipids including total cholesterol, triglycerides, LDL and HDL cholesterol. However, about 50% of patients hospitalized for coronary artery disease (CAD) have LDL cholesterol (LDL-C) levels within the normal range [2].

50% of patients hospitalized for coronary artery disease (CAD) do not have elevated LDL-C.[2]

Particle Number and Size Matter

Considerable discordance between LDL-C and the number of LDL particles (LDL-p) has been observed, especially in those individuals with other comorbid conditions such as diabetes mellitus [3-5]. Despite having the same level of LDL-C, patients can have different concentrations of LDL-p (Fig. 1). Small, dense LDL particles are described to be more atherogenic than large ones [6-9].

Multiple studies have shown that CV risk is more closely associated with LDL particle concentration rather than cholesterol content [4, 5, 10-12]. Recent studies demonstrated the cost-effectiveness of LDL-p guided therapy [13-16].

Furthermore, lower concentrations of high-density lipoprotein particles (HDL-p) are associated with increased risk of CVD [17], while large HDL particles (LHDL-p) seem to have a protective effect [18, 19]. Thus, the determination of lipoprotein parameters beyond the conventional lipid panel can offer a more accurate risk assessment.

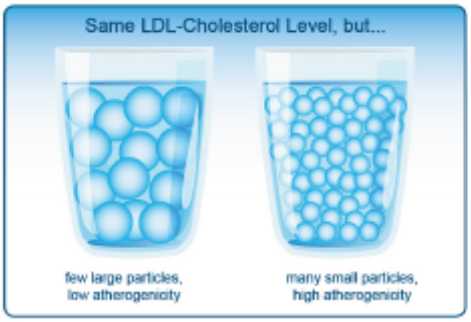

Fig. 1: Different LDL-p can sum up to the same LDL-C.

AXINON® lipoFIT® provides a detailed analysis of lipoprotein subclasses allowing further CV risk stratification. In addition to the standard lipid panel, the test measures concentration and size of lipoprotein particles.

Standard lipid panel +	Particle concentrations +	Particle sizes
Total cholesterol	LDL-p	VLDL-s
LDL cholesterol	HDL-p	LDL-s
HDL cholesterol	LVLDL-p	HDL-s
Triglycerides	SLDL-p	
	LHDL-p	

In particular, *AXINON® lipoFIT®* parameters include

■ **LDL-p: Concentration of LDL particles.**
Strong cardiovascular risk marker beyond LDL cholesterol [4,5].

■ **Small LDL-p: Concentration of small LDL particles.**
Elevated concentrations are associated with increased risk for coronary heart disease [11].

■ **HDL-p: Concentration of HDL particles.**
Reduced levels are strongly and independently linked to atherosclerotic risk [17].

** Available as a CE-labeled in vitro diagnostic product in the European Union and as Research-Use-Only product in the United States. numares' products have not yet been approved or cleared by the U.S. Food and Drug Administration.*

Expert Panels Recommend LDL-P

AXINON® lipoFIT® can help to identify those at increased risk despite normal LDL-C levels. Several expert panels recommend use of LDL-p to optimize treatment of intermediate and high risk patients [20-25].

Year	Expert Panel
2019	**AACE/ACE Diabetes Management Algorithm [20]** American Association of Clinical Endocrinologists/ American College of Endocrinology' Comprehensive Diabetes Management Algorithm
2017	**AACE/ACE Guidelines [21]** American Association of Clinical Endocrinologists' and American College of Endocrinology Guidelines for Management of Dyslipidemia and Prevention of Cardiovascular Disease
2015	**NLA Recommendations [22]** National Lipid Association recommendations for patient-centered management of dyslipidemia
2013	**AACC Assessment [23]** Association of apolipoprotein B and nuclear magnetic resonance spectroscopy-derived LDL particle number with outcomes in 25 clinical studies: assessment by the AACC Lipoprotein and Vascular Diseases Division Working Group on Best Practices
2011	**National Lipid Association (NLA) [24]** Clinical utility of inflammatory markers and advanced lipoprotein testing: advice from an expert panel of lipid specialists

AXINON® lipoFIT® Workflow

1) One-step sample preparation

2) Automated processing - 24/7

3) Output into LIS

How to Use the Test

The combination of *Magnetic Group Signaling™ (MGS®)* and the *AXINON®* System allows for spectral analysis with highly-reproducible results. The system is fast and efficient with automation capabilities allowing short hands-on-time, minimal operator interaction and high walk-away capability.

Test Features

Capacity: Five racks hold positions for up to 93 analytical samples each. Samples are processed in batches. The test system can be continuously loaded over just a few minutes.

Throughput: ~ 300 samples/24 h.

Walk-away operation: *AXINON®* supports the fully-automated measurement of up to 465 samples.

Hands-on Time: ~ 1 min. per sample (shorter when an automatic pipetting system is used)

Specimen collection, storage and transport

AXINON® lipoFIT® is performed on human serum samples collected according to standard techniques for laboratory testing. Appropriate tubes without anti-coagulation additives must be used. Specimens can be stored at 2-8°C for up to one week or can be frozen at -20°C or below.

Test Principle

Samples are prepared with the *AXINON®* serum kit and measured using a qualified *AXINON®* 600 MHz NMR system. The high magnetic field strength of 600 MHz enhances signal resolution and sensitivity. The test parameters are calculated by fitting the broad methyl group signals of lipoproteins using mathematical functions.

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A.7. Additional analyses from Labcorp

LabCorp	VLDL-p	VLVLDL-p	LVLDL-p	MVLDL-p	SVLDL-p	VSVLDL-p	LDL-p	LLDL-p	MLDL-p	SLDL-p	HDL-p	HDL-p H1	HDL-p H2	HDL-p H3	HDL-p H4	HDL-p H5	HDL-p H6	HDL-p H7	LHDL-p	MHDL-p	SHDL-p	TC	TG	HDL-C	LDL-C	apoB
VLDL-p	1	0.16	0.47‡	0.57‡	0.59‡	0.69‡	0.53‡	0.19*	0.42‡	0.17‡	-0.02	-0.12	0.13	0.19*	-0.18*	-0.27‡	0.09	-0.38‡	-0.23†	0.03	0.03	0.6‡	0.59‡	-0.25‡	0.59‡	0.6‡
VLVLDL-p	0.16	1	0.54‡	0.39‡	-0.11	0.17*	0.05	-0.32‡	0.07	0.29‡	0.08	0.22†	0.11	-0.17*	0.01	-0.06	-0.18*	-0.18*	-0.21†	-0.15	0.21*	0.06	0.72‡	-0.23‡	-0.09	0.21*
LVLDL-p	0.47‡	0.54‡	1	0.76‡	0.04	0.35‡	0.18*	-0.35‡	0.22†	0.32‡	0.09	0.09	0.24‡	-0.02	-0.08	-0.22†	-0.15	-0.35‡	-0.35‡	-0.08	0.23†	0.2*	0.81‡	-0.32‡	0.06	0.25†
MVLDL-p	0.57‡	0.39‡	0.76‡	1	0.31‡	0.21†	0.29†	-0.29‡	0.34‡	0.29‡	0.22	0.26‡	0.3†	-0.12	-0.02	-0.15	-0.15	-0.37‡	-0.32‡	-0.14	0.38‡	0.34‡	0.76‡	-0.22‡	0.21†	0.35‡
SVLDL-p	0.59‡	-0.11*	0.04	0.31‡	1	-0.16	0.6‡	0.45‡	0.46‡	-0.01	0.21*	0.03	0.28‡	0.13	-0.14	-0.11	0.14	-0.3†	-0.08	0.01	0.23†	0.67‡	0.22†	0.01	0.71‡	0.58‡
VSVLDL-p	0.69‡	0.17*	0.65‡	0.21†	-0.16	1	0.12	-0.04	0.08	0.14	-0.28‡	-0.27†	-0.15	0.18*	-0.12	-0.22†	0.05	-0.15	-0.15	0.08	-0.27‡	0.14	0.37†	-0.26‡	0.12	0.2
LDL-p	0.53‡	0.05	0.18*	0.29†	0.6‡	0.12	1	0.42‡	0.75‡	0.31‡	0.3†	0.12	0.31‡	0.15	-0.03	-0.01	-0.02	-0.28‡	-0.12	0.12	0.3†	0.9‡	0.35‡	0.07	0.92‡	0.95‡
LLDL-p	0.19*	0.32‡	0.35‡	-0.29‡	0.45‡	-0.04	0.42‡	1	0.09	-0.32‡	0.01	-0.17*	-0.1	0.19*	0.06	0.05	0.32‡	0.15	0.3‡	0.23†	-0.17*	0.52‡	-0.28‡	0.32‡	0.61‡	0.4‡
MLDL-p	0.42‡	0.07	0.22†	0.34‡	0.46‡	0.08	0.75‡	0.09	1	-0.15	0.41‡	0.17*	0.49‡	-0.04	0.08	0.02	-0.17*	-0.22†	-0.18*	0.03	0.46‡	0.71‡	0.33‡	0.12	0.68‡	0.7†
SLDL-p	0.17*	0.29†	0.32‡	0.29†	-0.01	0.14	0.31‡	-0.32‡	-0.15	1	-0.04	0.14	-0.04	0.12	-0.21†	-0.09	-0.1	-0.33‡	-0.23†	-0.05	0.05	0.09	0.39†	-0.32‡	0.08	0.3‡
HDL-p	-0.02	0.08	0.09	0.22†	0.21*	-0.28‡	0.3†	0.01	0.41‡	-0.04	1	0.6‡	0.71‡	-0.1	0.38‡	0.33‡	-0.02	0.01	0.18*	0.21†	0.88‡	0.48‡	0.07	0.64‡	0.32‡	0.26†
HDL-p H1	-0.12	0.22†	0.09	0.26‡	0.03	-0.27‡	0.12	-0.17*	0.17*	0.14	0.6‡	1	0.13	-0.4‡	0.16*	0.45‡	-0.16	0.06	0.18*	-0.26‡	0.66‡	0.2*	0.15	0.28‡	0.1	0.18*
HDL-p H2	0.13	0.11	0.24†	0.3‡	0.28‡	-0.15	0.3‡1	-0.1	0.49‡	-0.04	0.71‡	0.13	1	-0.19*	0.17*	-0.08	-0.2†	-0.3†	-0.29‡	-0.05	0.83‡	0.36‡	0.21†	0.16*	0.29‡	0.24†
HDL-p H3	0.19*	-0.17*	-0.02	-0.12	0.13	0.18*	0.15	0.19*	-0.04	0.12	-0.1	-0.4‡	-0.19*	1	-0.37‡	-0.11	0.24†	-0.22†	-0.37‡	0.67‡	-0.37‡	0.1	-0.1	-0.02	0.16*	0.07
HDL-p H4	-0.18*	0.01	-0.08	-0.02	-0.14	-0.12	-0.029	0.06	0.08	-0.21‡	0.38‡	0.17*	-0.37‡	1	0.02	1	-0.18*	0.22†	-0.03	0.45‡	0.22†	0.09	-0.08	0.47‡	-0.03	-0.04
HDL-p H5	-0.27‡	-0.06	-0.22†	-0.15	-0.11	-0.22†	-0.01	0.05	0.02	-0.9	0.33‡	0.45‡	-0.08	-0.11	0.02	1	-0.18*	0.37‡	0.6‡	-0.09	0.19*	0.09	-0.19*	0.48‡	0.01	-0.01
HDL-p H6	0.09	-0.18*	-0.15	-0.15	0.14	0.05	-0.02	0.32‡	-0.17*	-0.1	-0.02	-0.16	-0.2*	0.24†	-0.18*	-0.18*	1	0.15	0.61‡	0.09	-0.24†	0.12	-0.15	0.374‡	0.06	-0.06
HDL-p H7	-0.38‡	-0.18*	-0.35‡	-0.37‡	-0.3†	-0.15	-0.28‡	0.15	-0.22†	-0.33‡	0.01	0.06	-0.3†	-0.22†	0.22†	0.37‡	0.15	1	0.67‡	-0.03	-0.19*	-0.63	-0.43‡	0.36‡	-0.12	-0.2*
LHDL-p	0.19*	0.21†	0.35‡	-0.32‡	-0.08	-0.15	-0.12	0.3†	-0.18*	-0.23†	0.18*	0.66‡	-0.29‡	0.02	-0.03	0.6‡	0.61‡	0.67‡	1	-0.01	-0.12	0.11	-0.36‡	0.73‡	0.01	-0.12
MHDL-p	0.42†	-0.15	-0.08	-0.14*	0.01	0.08	0.12	0.26†	0.03	-0.05	0.21†	-0.26‡	-0.05	0.67‡	0.45‡	-0.09	0.09	-0.03	-0.01	1	-0.18*	0.17*	-0.16	0.36‡	0.13	0.04
SHDL-p	0.17*	0.21*	0.23†	0.38‡	0.23†	-0.27‡	0.3†	-0.17*	0.46‡	0.05	0.88‡	0.18*	0.83‡	-0.37‡	0.22†	0.19*	-0.24‡	-0.19*	-0.12	-0.18*	1	0.38‡	0.25†	0.28‡	0.28‡	0.28‡
TC	0.6‡	0.06	0.2*	0.34‡	0.67‡	0.14	0.89†	0.52‡	0.71‡	0.09	0.48‡	0.2*	0.36‡	0.1	0.09	0.09	0.12	-0.63	0.11	0.17*	0.38‡	1	0.35‡	0.34‡	0.95‡	0.91‡
TG	0.59‡	0.72‡	0.81‡	0.76‡	0.22†	0.37	0.35‡	-0.28‡	0.33‡	0.39‡	0.07	0.15	0.21‡	-0.1	-0.08	-0.19*	-0.15	-0.43‡	-0.36‡	-0.16	0.25‡	0.35‡	1	-0.35‡	0.19*	0.5‡
HDL-C	-0.25‡	0.23†	-0.32‡	-0.22†	0.01	-0.26‡	0.07	0.32‡	0.12	-0.32‡	0.64‡	0.28‡	0.16*	-0.02	0.47‡	0.48‡	0.37‡	0.56‡	0.73‡	0.36‡	0.28‡	0.34‡	-0.35‡	1	0.18*	0.03
LDL-C	0.59‡	-0.09	0.06	0.21†	0.71‡	0.12	0.92‡	0.61‡	0.68‡	0.08	0.32‡	0.1	0.29‡	0.16*	-0.03	0.01	0.06	-0.12	0.01	0.13	0.28‡	0.95‡	0.19*	0.18*	1	0.89‡
apoB	0.6‡	0.21*	0.25†	0.35‡	0.58‡	0.2*	0.95‡	0.4‡	0.7†	0.3‡	0.26†	0.18*	0.24‡	0.07	-0.04	-0.01	-0.06	-0.2*	-0.12	0.04	0.25‡	0.91‡	0.5‡	0.03	0.89‡	1

Legend: **p<0.05. †p<0.01. ‡p<0.001. apoB= apolipoprotein B. C= cholesterol. HDL= high density lipoproteins. l= liter. LDL= low density lipoproteins. LHDL= large high density lipoproteins . LLDL= large low density lipoproteins. LVLDL= large very low density lipoproteins. MHDL= medium high density lipoproteins. MLDL= medium low density lipoproteins. MVLDL= medium very low density lipoproteins. p= particles. SHDL= small high density lipoproteins . SLDL= small low density lipoproteins . SVLDL= small very low density lipoproteins . TC= total cholesterol. TG= triglycerides. VLDL= very low density lipoproteins. VLVLDL= very large very low density lipoproteins. VSVLDL= small very low density lipoproteins. Table from (Rief et al., 2022) -permission with CC BY 4.0 license.*

A.8. Published article in Biomedicines

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Comparison of Two Nuclear Magnetic Resonance Spectroscopy Methods for the Measurement of Lipoprotein Particle Concentrations

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Citation: Rief, M.; Raggam, R.; Rief, P.; Metnitz, P.; Stojakovic, T.; Reinthaler, M.; Brodmann, M.; März, W.; Scharnagl, H.; Silbernagel, G. Comparison of Two Nuclear Magnetic Resonance Spectroscopy Methods for the Measurement of Lipoprotein Particle Concentrations. *Biomedicines* **2022**, *10*, 1766. https://doi.org/10.3390/biomedicines10071766

Academic Editors: Ilenia Calcaterra and Alessandro Di Minno

Received: 13 May 2022
Accepted: 16 July 2022
Published: 21 July 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Background: Measuring lipoprotein particle concentrations may help to improve cardiovascular risk stratification. Both the lipofit (Numares) and lipoprofile (LabCorp) NMR methods are widely used for the quantification of lipoprotein particle concentrations. **Objective:** The aim of the present work was to perform a method comparison between the lipofit and lipoprofile NMR methods. In addition, there was the objective to compare lipofit and lipoprofile measurements of standard lipids with clinical chemistry-based results. **Methods:** Total, LDL, and HDL cholesterol and triglycerides were measured with ß-quantification in serum samples from 150 individuals. NMR measurements of standard lipids and lipoprotein particle concentrations were performed by Numares and LabCorp. **Results:** For both NMR methods, differences of mean concentrations compared to ß-quantification-derived measurements were ≤5.5% for all standard lipids. There was a strong correlation between ß-quantification- and NMR-derived measurements of total and LDL cholesterol and triglycerides (all $r > 0.93$). For both, the lipofit ($r = 0.81$) and lipoprofile ($r = 0.84$) methods, correlation coefficients were lower for HDL cholesterol. There was a reasonable correlation between LDL and HDL lipoprotein particle concentrations measured with both NMR methods (both $r > 0.9$). However, mean concentrations of major and subclass lipoprotein particle concentrations were not as strong. **Conclusions:** The present analysis suggests that reliable measurement of standard lipids is possible with these two NMR methods. Harmonization efforts would be needed for better comparability of particle concentration data.

Keywords: nuclear magnetic resonance spectroscopy; ultracentrifugation; lipoproteins; lipoprotein subclasses; methods; analysis; very-low-density lipoproteins; low-density lipoproteins; high-density lipoproteins

1. Introduction

Measurement of lipoprotein particle concentrations in addition to standard lipids may help to estimate cardiovascular risk [1–7]. Historically, lipoprotein particle concentrations were analyzed with methods based on ultracentrifugation [8]. Another established

method to measure lipoprotein particle concentrations is gradient gel electrophoresis [9,10]. However, ultracentrifugation and gradient gel electrophoresis are time-consuming and expensive. Therefore, ultracentrifugation and gradient gel electrophoresis are predominantly used for scientific purposes and not common in clinical routine. Today, several nuclear magnetic resonance (NMR)-based methods are available to measure lipoprotein particle concentrations [11,12]. The two most widespread methods for NMR-based quantification of lipoprotein particle concentrations have been introduced by LabCorp and Numares. There is broad evidence that high-density lipoprotein (HDL) particle concentrations measured with NMR may help to estimate cardiovascular risk [6,13]. Less consistently, low-density lipoprotein (LDL) particle concentrations have been suggested to be independently related to cardiovascular risk [5].

The comparability of different, advanced analytical methods for lipids and lipoproteins remains an issue [14,15]. So far, the comparability between different NMR methods has not been systematically investigated.

The aim of the present investigation was to provide an independent comparison of the two most widespread NMR-based methods for the quantification of lipoprotein particle concentrations. In addition to a comparison of the particle concentrations of the major lipoprotein particle classes, we aimed to compare NMR-based measurements of the major lipids (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides) with standard clinical chemistry-based measurements (ß-quantification). Moreover, we aimed to analyze internal correlation matrices for the two NMR methods. Finally, we aimed to perform a comparison of corresponding lipoprotein subclass particle concentrations.

2. Methods

2.1. Study Participants

Recruitment of one hundred fifty study participants was performed at the outpatient clinic of the Division of Angiology (of the Department of Internal Medicine) and the preoperative anaesthesia outpatient clinic of the Division of General Anaesthesiology, Emergency and Intensive Care Medicine (of the Department of Anaesthesiology and Intensive Care Medicine) at the University Hospital Graz, Austria, between 17 February and 30 June 2020. The inclusion criteria were age $\geq$ 18 years. There were no exclusion criteria. Written informed consent was obtained from each participant. Total cholesterol ranged between 93 and 339 mg/dL.

2.2. Blood Sampling

We collected blood samples as a part of blood withdrawal in clinical routine. Overnight fasting was not required, as the samples were only used for a method comparison. In addition, fasting is not required according to the current recommendations of the European Atherosclerosis Society and the European Federation of Clinical Chemistry and Laboratory Medicine [16].

2.3. Specimen Material

From each patient, two tubes of 9 mL of whole blood were collected with Greiner bio-one® Vacuette Z Serum (red, 9.0 mL; 455092). Serum was separated from blood cells by centrifugation (10 min at 6490 rpm and 15 °C) and aliquoted in cryotubes (Nunc Universal® 1.8 mL).

2.4. Storage of Samples and Shipping

The cryotubes were cooled down and stored at −80 °C. The 150 samples of 1 mL serum each were sent in accordance with international security regulations for medical specimens to the laboratories in frozen condition.

2.5. Laboratory Measurements

In this study, three different laboratories performed the analyses. Standard lipid measurements were performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics of the Medical University of Graz, Austria. Lipoproteins were separated using a combined ultracentrifugation–precipitation method (ß-quantification). The VLDL fraction (d < 1.006 g/mL) was removed after ultracentrifugation (18 h, 10 °C, 98,000× *g*). ApoB-containing lipoproteins in the resulting bottom fraction were precipitated using phosphotungstic acid with the HDL particles remaining in solution. LDLC was calculated by subtracting cholesterol after precipitation from the respective concentrations before precipitation. Cholesterol and triglycerides were measured with enzymatic reagents from Diasys (Holzheim, Germany) on an Olympus AU680 analyzer [8]. The LabCorp Corp. (100 Perimeter Park, Morrisville, 27560 North Carolina, USA) performed NMR analysis using the NMR LipoProfile® LP4 method (in the further text briefly lipoprofile) in Raleigh [11]. Particle concentrations of lipoproteins of different sizes were calculated from the measured amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Weighted-average lipoprotein particle sizes are derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal [4]. The Numares AG (Am Biopark 9, 93053 Regensburg, Germany) performed NMR analysis using the AXINON® lipoFIT® method (in the further text briefly lipofit) in Regensburg with an Avance III HD nuclear magnetic resonance spectrometer (Bruker; Billerica, MA, USA), an Ascend 600 MHz magnet (Bruker), and using TopSpin 3.2 (Bruker) and Axinon Suite 1.0.0.1 (Numares, Regensburg, Germany) software [12,17].

2.6. Statistical Methods

LabCorp provided duplicate measurements, of which the first measurements were used for analyses. The measurements are given as means and standard deviations. Associations among clinical chemistry and NMR-based standard lipid measurements are given as Pearson and Spearman correlations. Likewise, comparisons between the Numares (lipofit) and LabCorp (lipoprofile) measurements were analyzed using Pearson correlation coefficients and non-parametric Passing–Bablok regression. The analysis plan has been prespecified. The statistical package from IBM® (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA: IBM Corp) was used. Passing–Bablok regression was calculated with the Analyse-it Method Validation Edition for Microsoft Excel 5.90 (Analyse-it Software Ltd., Leeds, UK)

2.7. Ethical and Regulatory Aspects

The study was approved by the ethics committee of the Medical University of Graz (29-479 ex 16/17) and the Federal Office for Safety in Health Care of Austria (Bundesministerium für Sicherheit im Gesundheitswesen—BASG); Agency for Health and Food Security (AGES) on 15 May 2019 (ref. No. 11458092). The study was performed in accordance with the Declaration of Helsinki, and all participants gave written, informed consent.

3. Results

3.1. Raw Data

The entire anonymized raw data file is provided in the online supplements. All measurements were complete with the lipoprofile NMR method. In two samples, the values for small LDL particles, and in three samples, the values for small HDL particles were missing with the lipofit method. For several parameters, few values were below the detection limit with the Numares NMR (lipofit) method (Supplemental Table S1).

3.2. Standard Lipid Measured with ß-Quantification, Lipofit NMR and Lipoprofile NMR

For both NMR methods, differences of mean concentrations compared to ß-quantification-derived measurements were ≤5.5% for all standard lipids. Total cholesterol was lower with both NMR methods compared with the enzymatic assay. Triglycerides were modestly

higher with both NMR methods compared with the enzymatic assay (Table 1). Correlations between the standard method and both the lipoprofile and lipofit methods were strong for total cholesterol, LDL cholesterol and triglycerides with marginally higher correlation coefficients for the lipoprofile method. Correlations with ß-quantification-derived HDL cholesterol were weaker for both the lipofit and the lipoprofile methods (Figure 1a,b, Supplemental Table S5). It can also be seen from Figure 1b that there are few downward outliers with the lipoprofile NMR triglyceride measurement. Correlations of standard lipids measurements between the lipoprofile and lipofit methods were strong (Supplemental Table S6 and Figure S3).

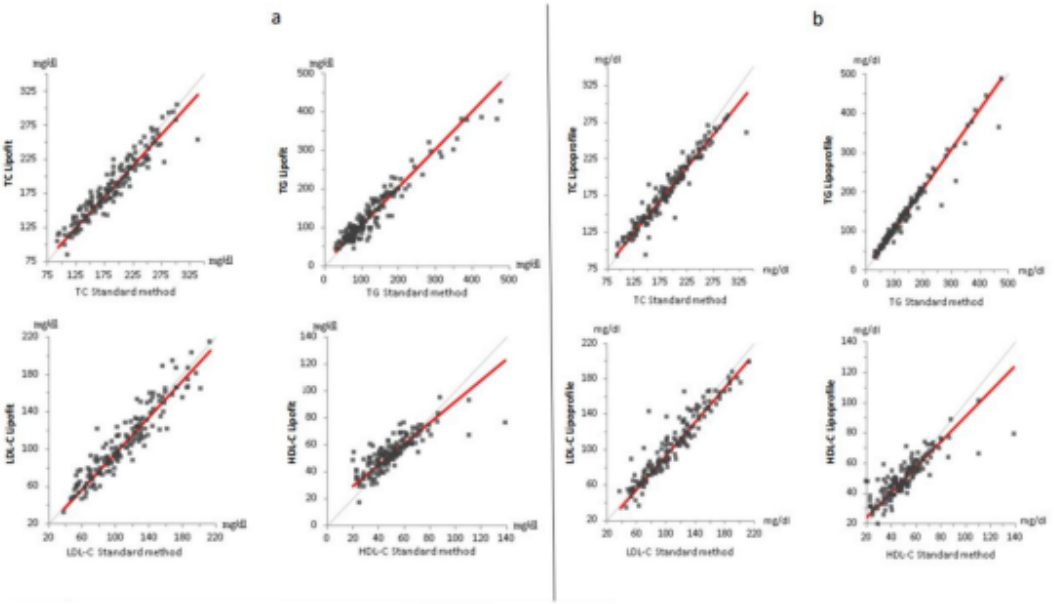

Figure 1. Comparison of standard lipids between ß-quantification and lipofit NMR (**a**) and lipoprofile NMR (**b**). The (**a**,**b**) show the Passing–Bablok regression for total cholesterol (top left), triglycerides (top right), low-density lipoprotein cholesterol (bottom left), and high-density lipoprotein cholesterol (bottom right). The respective slopes of the regression lines (red) were 0.889 (TC), 1.027 (TG), 0.960 (LDL-C), and 0.830 (HDL-C) for lipofit and 0.914 (TC), 0.983 (TG), 0.973 (LDL-C), and 0.786 (HDL-C) for lipoprofile, respectively. The grey line represents the line of identity. (C = cholesterol. HDL = high-density lipoproteins. LDL = low-density lipoproteins. TC = total cholesterol. TG = triglycerides).

In two samples, HDL cholesterol measured with the enzymatic method was considerably higher compared with the two NMR methods (Supplemental Table S5, Figure 1b).

Table 1. Mean concentrations of standard lipids and lipoprotein (sub)classes.

Parameter	Units	Lipoprofile	Lipofit	Standard Method
TC	mg/dL	180 (±45)	183 (±46)	188 (±51)
TG	mg/dL	135 (±81)	135 (±78)	132 (±84)
LDL-C	mg/dL	105 (±37)	107 (±38)	110 (±38)
HDL-C	mg/dL	50.1 (±13.2)	53.5 (±12.7)	50.7 (±17.7)
LVLDL-p	nmol/L	3.42 (±4.18) [a]	4.94 (±5.6)	
LDL-p	nmol/L	1330 (±444)	1176 (±465)	
LDL-size	nm	21,1 (±0,56)	21.2 (±0.37)	
LLDL-p	nmol/L	377 (±241)	677 (±289)	
SLDL-p	nmol/L	953 (±407) [b]	528 (±270)	
HDL-p	µmol/L	18.4 (±4.4)	33.2 (±7.1)	
HDL-size	nm	9.19 (±0.51)	9.10 (±0.47)	
LHDL-p	µmol/L	4.37 (±1.87) [c]	6.08 (±3.31)	
SHDL-p	µmol/L	14.0 (±4.0) [d]	26.8 (±7.6)	

([a] large + very large VLDL-p. [b] small + medium LDL-p. [c] H4-H7 HDL-p. [d] H1-H3 HDL-p. C = cholesterol. HDL = high-density lipoproteins. LDL = low-density lipoproteins. LHDL = large high-density lipoproteins. LLDL = large low-density lipoproteins. LVLDL = large very-low-density lipoproteins. p = particles. s = size. SHDL = small high-density lipoproteins. SLDL = small low-density lipoproteins. TC = total cholesterol. TG = triglycerides).

3.3. Correlations among Lipids and Lipoprotein Particles within the Lipofit NMR Method and within the Lipoprofile NMR Method

Overall, internal correlation matrices appeared consistent when comparing the lipoprofile and lipofit methods (Tables 2 and 3). With the lipofit method, LDL particles were positively associated with total cholesterol and triglycerides. They were also positively associated with large VLDL particles and HDL particles. Large LDL particles were only weakly associated with small LDL particles and positively associated with HDL particles. Small LDL particles were inversely related to LDL size. HDL particles were not associated with triglycerides. Large VLDL particles were inversely related to HDL cholesterol. LDL size was positively associated with HDL size (Table 2). With the lipoprofile method, LDL particles were also positively associated with total cholesterol and triglycerides. They were modestly and positively associated with large VLDL particles and HDL particles. Large LDL particles were not significantly associated with small LDL particles and HDL particles. Small LDL particles were inversely related to LDL size. HDL particles were also not associated with triglycerides. Large VLDL particles were also inversely related to HDL cholesterol. LDL size was also positively associated with HDL size (Table 3 and Supplemental Table S2).

3.4. Lipoprotein Particles Measured with Lipofit NMR and Lipoprofile NMR

The correlations of the LDL and HDL lipoprotein particle concentrations were strong between the lipofit and lipoprofile methods (Supplemental Table S6 and Figure S3). The mean LDL particle concentration was 13% higher with the lipoprofile method, whereas the HDL particle concentration was markedly higher with the lipofit method (+55%). Total VLDL particles could not be compared as this parameter is not available for the lipofit method. There was good agreement between mean LDL size measured with the lipofit and lipoprofile methods (Table 1). Correlations between the two NMR methods were moderate for LDL size but stronger for HDL size (Supplemental Table S6).

Table 2. Internal correlation matrix among lipids and lipoprotein particles for the lipofit method.

Lipofit	LVLDL-p	p	LDL-p	p	LLDL-p [a]	p	SLDL-p [a]	p	HDL-p	p	LHDL-p	p	SHDL-p [b]	p	TC	p	TG	p	HDL-C	p	LDL-C	p	LDLsize	p	HDLsize	p
LVLDL-p	1		0.34	<0.001	−0.01	0.928	0.56	<0.001	0.07	0.365	−0.29	<0.001	0.23	0.005	0.15	0.07	0.9	<0.001	−0.28	<0.001	0.05	0.582	−0.47	<0.001	−0.34	<0.001
LDL-p	0.34	<0.001	1		0.8	<0.001	0.74	<0.001	0.32	<0.001	−0.15	0.069	0.42	<0.001	0.87	<0.001	0.54	<0.001	0.06	0.485	0.91	<0.001	−0.11	0.188	−0.34	<0.001
LLDL-p [a]	−0.01	0.928	0.8	<0.001	1		0.21	<0.001	0.42	<0.001	0.26	<0.002	0.31	<0.001	0.85	<0.001	0.38	0.029	0.41	<0.001	0.83	<0.001	0.35	<0.001	0.04	0.634
SLDL-p [a]	0.56	<0.001	0.74	<0.001	0.21	0.011	1		0.02	0.859	−0.61	<0.001	0.36	<0.001	0.49	<0.001	0.69	<0.001	−0.4	<0.001	0.56	<0.001	−0.56	<0.001	−0.69	<0.001
HDL-p	0.07	0.365	0.32	<0.001	0.42	<0.001	0.02	0.859	1		0.3	<0.001	0.86	<0.001	0.49	<0.001	0.11	0.17	0.7	<0.001	0.32	<0.001	0.04	0.661	−0.01	0.877
LHDL-p	−0.29	<0.001	−0.15	0.069	0.26	0.002	−0.61	<0.001	0.3	<0.001	1		−0.22	0.009	0.15	0.069	−0.38	<0.001	0.81	<0.001	−0.02	0.841	0.51	<0.001	0.92	<0.001
SHDL-p [b]	0.23	0.005	0.42	<0.001	0.31	<0.001	0.36	<0.001	0.86	<0.001	−0.22	0.009	1		0.42	<0.001	0.33	<0.001	0.29	<0.001	0.35	<0.001	−0.23	0.005	−0.52	<0.001
TC	0.15	0.07	0.87	<0.001	0.85	<0.001	0.49	<0.001	0.49	<0.001	0.15	0.069	0.42	<0.001	1		0.36	<0.001	0.44	<0.001	0.96	<0.001	0.25	0.003	−0.03	0.717
TG	0.9	<0.001	0.54	<0.001	0.38	0.029	0.69	<0.001	0.11	0.17	−0.38	<0.001	0.33	<0.001	0.36	<0.001	1		−0.29	<0.001	0.28	<0.001	−0.42	<0.001	−0.5	<0.001
HDL-C	−0.28	<0.001	0.06	0.485	0.41	<0.001	−0.4	<0.001	0.7	<0.001	0.81	<0.001	0.29	<0.001	0.44	<0.001	−0.29	<0.001	1		0.25	0.002	0.51	<0.001	0.64	<0.001
LDL-C	0.05	0.582	0.91	<0.001	0.83	<0.001	0.56	<0.001	0.32	<0.001	−0.2	0.841	0.35	<0.001	0.96	<0.001	0.28	<0.001	0.25	0.002	1		0.19	0.021	−0.16	0.053
LDL size	−0.47	<0.001	−0.11	0.188	0.35	<0.001	−0.56	<0.001	0.04	0.661	0.51	<0.001	−0.23	0.005	0.25	0.003	−0.42	<0.001	0.51	<0.001	0.19	0.021	1		0.56	<0.001
HDL size	−0.36	0.1	−0.34	<0.001	0.04	0.634	−0.69	<0.001	−0.01	0.877	0.92	<0.001	−0.52	<0.001	−0.03	0.717	−0.52	<0.001	0.65	<0.001	−0.16	0.034	0.56	<0.001	1	

([a] 148 values. [b] 147 values. C = cholesterol. HDL = high-density lipoproteins. LDL = low-density lipoproteins. LHDL = large high-density lipoproteins. LLDL = large low-density lipoproteins. LVLDL = large very-low-density lipoproteins. *p* = particles. SHDL = small high-density lipoproteins. SLDL = small low-density lipoproteins. TC = total cholesterol. TG = triglycerides).

Table 3. Internal correlation matrix among lipids and lipoprotein particles for the lipoprofile method.

Lipoprofile	LVLDL-p	p	LDL-p	p	LLDL-p	p	SLDL-p	p	HDL-p	p	LHDL-p	p	SHDL-p	p	TC	p	TG	p	HDL-C	p	LDL-C	p	LDL-s	p	HDL-s	p
LVLDL-p	1		0.18	0.031	−0.35	<0.001	0.4	<0.001	0.09	0.267	−0.35	<0.001	0.23	0.003	0.2	0.016	0.81	<0.001	−0.32	<0.001	0.06	0.451	−0.46	<0.001	−0.42	<0.001
LDL-p	0.18	0.031	1		0.42	<0.001	0.31	<0.001	0.3	<0.001	−0.12	0.14	0.3	<0.001	0.9	<0.001	0.35	<0.001	0.07	0.368	0.92	<0.001	0.08	0.345	−0.32	<0.001
LLDL-p	−0.35	<0.001	0.42	<0.001	1		−0.13	0.106	0.01	0.902	0.25	0.002	−0.11	0.198	0.52	<0.001	−0.28	0.001	0.32	<0.001	0.61	<0.001	0.79	<0.001	0.3	<0.001
SLDL-p	0.4	<0.001	0.31	<0.001	−0.13	0.11	1		0.33	<0.001	−0.26	0.001	0.48	<0.001	0.67	<0.001	0.54	<0.001	−0.11	0.198	0.64	<0.001	−0.67	<0.001	−0.29	<0.001
HDL-p	0.09	0.267	0.3	<0.001	0.01	0.902	0.33	<0.001	1		0.41	<0.001	0.91	<0.001	0.48	<0.001	0.07	0.375	0.64	<0.001	0.32	<0.001	0.05	0.527	−0.35	<0.001
LHDL-p	−0.35	<0.001	−0.12	0.14	0.25	0.002	−0.26	0.001	0.41	<0.001	1		−0.02	0.812	0.14	0.091	−0.31	<0.001	0.86	<0.001	−0.02	0.799	0.32	<0.001	0.73	<0.001
SHDL-p	0.23	0.003	0.3	<0.001	−0.11	0.198	0.48	<0.001	0.91	<0.001	−0.02	0.812	1		0.46	<0.001	0.22	0.006	0.3	<0.001	0.36	<0.001	−0.13	0.122	−0.6	<0.001
TC	0.2	0.016	0.9	<0.001	0.52	<0.001	0.67	<0.001	0.48	<0.001	0.14	0.091	0.46	<0.001	1		0.35	<0.001	0.34	<0.001	0.95	<0.001	0.25	0.002	−0.16	0.048
TG	0.81	<0.001	0.35	<0.001	−0.28	0.001	0.54	<0.001	0.07	0.375	−0.31	<0.001	0.22	0.006	0.35	<0.001	1		−0.35	<0.001	0.19	0.021	−0.5	<0.001	−0.46	<0.001
HDL-C	−0.32	<0.001	0.07	0.368	0.32	<0.001	−0.11	0.198	0.64	<0.001	0.86	<0.001	0.3	<0.001	0.34	<0.001	−0.35	<0.001	1		0.18	0.026	0.36	<0.001	0.4	<0.001
LDL-C	0.06	0.451	0.92	<0.001	0.61	<0.001	0.64	<0.001	0.32	<0.001	−0.02	0.799	0.36	<0.001	0.95	<0.001	0.19	0.021	0.18	0.026	1		0.34	<0.001	−0.17	0.039
LDL-s	−0.46	<0.001	0.08	0.345	0.79	<0.001	−0.67	<0.001	0.05	0.527	0.32	<0.001	−0.13	0.122	0.25	0.002	−0.5	<0.001	0.36	<0.001	0.34	<0.001	1		0.38	<0.001
HDL-s	−0.42	<0.001	−0.32	<0.001	0.3	<0.001	−0.29	<0.001	−0.35	<0.001	0.73	<0.001	−0.6	<0.001	−0.16	0.048	−0.46	<0.001	0.4	<0.001	−0.17	0.039	0.38	<0.001	1	

(C = cholesterol. HDL = high-density lipoproteins. LDL = low-density lipoproteins. LHDL = large high-density lipoproteins. LLDL = large low-density lipoproteins. LVLDL = large very-low-density lipoproteins. p = particles. SHDL = small high-density lipoproteins. SLDL = small low-density lipoproteins. TC = total cholesterol. TG = triglycerides).

Based on size categorization, small and medium lipoprofile LDL particles were compared with small lipofit LDL particles (Supplemental Table S6). With the lipofit method, the mean concentration of large LDL particles was higher than the mean concentration of small LDL particles. With the lipoprofile method, on the other hand, small LDL particles were the predominant subclass (Table 1). The correlations of the LDL subclass particle concentrations between the two NMR methods were only moderate, especially for large LDL particles (Supplemental Table S6, Figures 2 and 3). We have performed further calculations with alternative size categories, which did not correspond to the predefined study protocol. Combining medium and large LDL particles with the lipoprofile method to compare them with large lipofit LDL particles resulted in mean values of 909 (±442) nmol/L for the lipoprofile method and 677 (±289) nmol/L for the lipofit method and a correlation of 0.771. Comparing small lipoprofile to small lipofit LDL particles, there was better agreement for mean concentrations with 528 (±270) nmol/L for lipoprofile and 421 (±266) nmol/L for lipofit. However, the correlation coefficient was only 0.555.

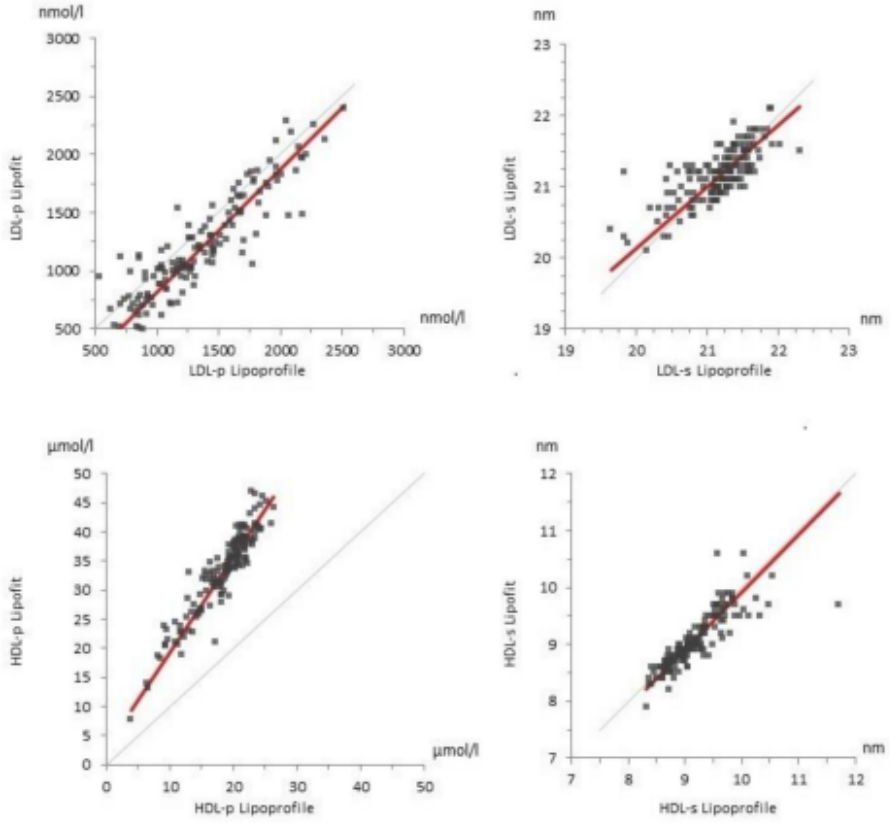

Figure 2. Comparison of lipoprotein particles between the lipoprofile NMR and lipofit NMR methods. The figures show the Passing–Bablok regression for LDL-p (top **left**), LDL-s (top right), HDL-p (bottom left, and HDL-s (bottom **right**). The respective slopes of the regression lines (red) were 1.057 (LDL-p), 0.860 (LDL-s), 1.637 (HDL-p), and 1.014 (HDL-s), respectively. The grey line represents the line of identity. (HDL = high-density lipoproteins. LDL = low-density lipoproteins. p = particles. s = size).

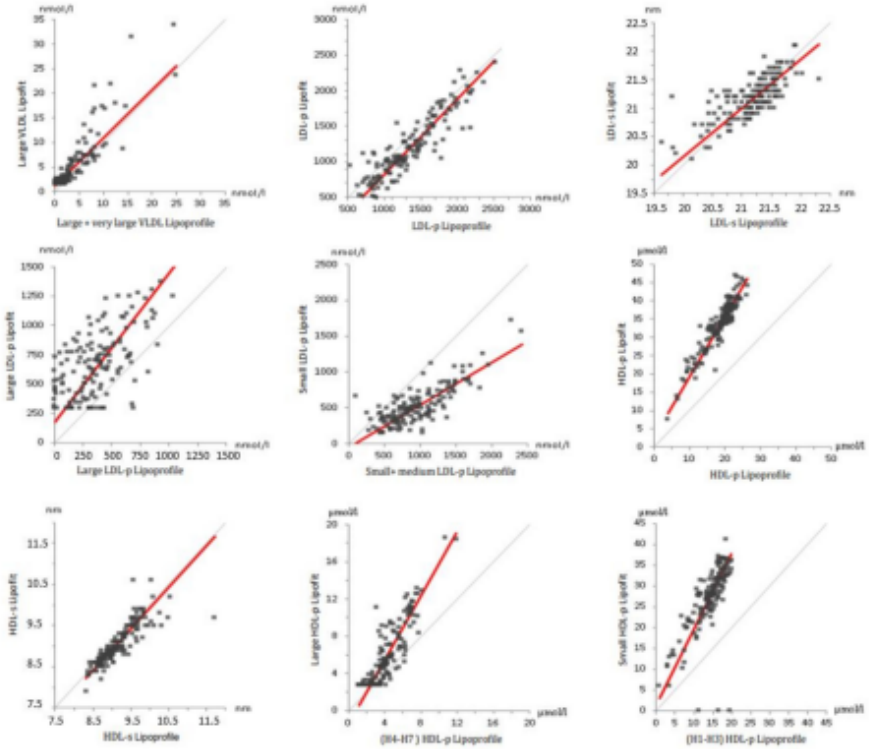

Figure 3. Comparison of lipoprotein particles between the lipoprofile and lipofit methods. The figures show the Passing–Bablok regression for large and very large VLDL (top **left**), LDL-p (top middle), LDL-s (top **right**), large LDL-p (middle **left**), small and medium LDL-p (**middle**), HDL-p (middle right), HDL-s (bottom **left**), large HDL-p (bottom middle), and small HDL-p (bottom **right**). The respective slopes of the regression lines (red) were 0.980 (large VLDL-p), 1.272 (large LDL-p), 0.593 (small and medium LDL-p), 1.817 (small HDL-p) and 1.722 (large HDL-p), respectively. The grey line represents the line of identity. (HDL = high-density lipoproteins. LDL = low-density lipoproteins. p = particles. VLDL = very-low-density lipoproteins).

Consistent with the lower total HDL particle concentration, the concentrations of HDL subclass particles were also substantially lower with the lipoprofile compared to lipofit method (Table 1). The correlations were stronger for large HDL particles than for small HDL particles (Supplemental Table S6, Figures 2 and 3).

The concentration of large VLDL particles was lower with the lipoprofile method compared with the lipofit method, with weak correlations between the two methods (Table 1).

4. Discussion

This is the first independent and systematic comparison of the two most widespread NMR methods for the quantification of lipoprotein particle concentrations. Both the lipofit and the lipoprofile methods showed strong correlations with ß-quantification for total, LDL, and HDL cholesterol, and for triglycerides. A few downward outliers for triglycerides measured with the lipoprofile NMR method may be due to the effects of freezing on triglyceride-rich particles [11]. The relatively high difference in HDL cholesterol values between the enzymatic method and both NMR methods in some samples may be due to

limitations of the precipitation step. The differences of mean concentrations compared to ß-quantification-derived measurements were ≤5.5% for all standard lipids, both for the lipofit and the lipoprofile methods. Hence, the two NMR methods appear to provide reliable information on the concentration of standard lipids.

The main objective of the present study was to compare the results of the two NMR methods with regard to lipoprotein particle concentrations. In fact, there were acceptable correlations of the LDL and HDL particle concentrations between the two NMR methods. Whereas LDL particle concentrations were similar, there were differences for the mean concentrations of HDL particles with the lipofit method reporting higher values.

Regarding lipoprotein subclass particle concentrations, it has to be considered that the lipofit and lipoprofile categorizations differ considerably. Hence, direct comparisons were not feasible. Rather, we aimed to compare roughly corresponding, partly combined lipoprotein subclass categories. Relatively weak concordance between the two NMR methods was particularly observed for small and large LDL particle concentrations, with the lipoprofile NMR method showing a higher proportion of small LDL particles and the lipofit NMR method showing a higher proportion of large LDL particles. This may be due to differences in classifications, since the results were more consistent when medium and large LDL particles of the lipoprofile method were compared with large lipofit LDL particles instead of combining medium with small lipoprofile LDL particles. As observed for total HDL particles, small and large HDL particle concentrations were higher with the lipofit method. These differences may also be due to calibration but cannot be definitely explained.

The concentration of total VLDL particles is not provided by the lipofit method so that only large VLDL particles could be compared. Although there appeared to be a strong correlation, the mean particle concentration of large VLDL particles was higher for the lipofit method.

High concordance between the two NMR methods was observed for the mean values of the LDL and HDL sizes. However, the correlations were only moderate.

Comparing the lipofit and lipoprofile analyte panel, lipoprofile provides a more comprehensive list of parameters. It includes a more detailed separation of lipoprotein subclasses and also provides information on apolipoprotein B.

The internal correlation matrix among major lipids and lipoprotein particles gave similar results for the lipofit and the lipoprofile methods. This supports that measurement of lipoprotein particles with these methods is comparable. Most importantly, there should not be a strong positive correlation between large LDL particles and small LDL particles [18]. No such correlation was seen with the lipoprofile and lipofit methods. This is in contrast to a recent analysis with the Nightingale method, which showed strong, positive correlations among all LDL subclasses ($r > 0.8$) [19]. Still, the differences in mean particle concentrations between the lipofit and lipoprofile methods require further investigations. This is of particular relevance, as a more precise characterization of the lipoprotein profile with NMR may help to improve risk classification reflecting different pathophysiological features of the various lipoprotein subclasses [20]. This may also be of relevance in times when an increasing number of drugs is available to treat distinct lipid disorders, e.g., therapies addressing apolipoprotein C-III for familial hyperchylomicronaemia [21]. It is also an advantage that the NMR methods are not time-consuming and a large number of samples can be analyzed in a relatively short period of time. This makes them useful for scientific purposes, considering that standard procedures to analyze lipoprotein metabolism such as analytical ultracentrifugation are very time-consuming and expensive. It is a disadvantage of the NMR methods that they are not routinely available in standard laboratories because they require special equipment. Moreover, a large sample size is necessary (~0.5–1 mg that is dissolved in ~0.5 mL of solvent) for the analysis. The lack of harmonization between the different providers also makes it difficult to interpret and compare certain results. Moreover, the associations of certain lipoprotein particle concentrations with cardiovascular endpoints have been inconsistent [1–8]. Therefore, particle concentrations from NMR

measurements are currently also not recommended as therapeutic targets in the guidelines for the treatment of dyslipidaemia endorsed by the European Atherosclerosis Society and the European Society of Cardiology [21].

Regarding the harmonization of NMR methods, using the same particle size cutoff values could be a first step. In addition, further work would have to clarify whether the harmonization of the cutoff values yields different results or whether and to what extent the mathematical algorithm underlying the different NMR methods may be the primary cause of the deviating results. However, a harmonization effort would necessarily be voluntary until some sort of regulatory framework is imposed, for example, by the Food and Drug Administration. So near-term prospects are not great. Nevertheless, the need for such regulation, which appears years away, is at least recognized by the IFCC Metabolomics Working Group.

The strong aspect of this study is that we performed an independent method comparison of the newest versions of the two most widespread NMR methods for the quantification of lipoprotein particle concentrations.

One limitation of the study is that the ß-quantification method provides results for the main lipoprotein fractions (VLDL, LDL, HDL) only. Hence, we were not able to provide an independent method comparison for the analysis of LDL and HDL subclass particles. However, the primary focus was to address the comparability of the two widespread NMR methods.

5. Conclusions

To sum up, the present study shows that standard lipids can be reliably measured with NMR methods. Harmonization efforts for better comparability of lipoprotein particle concentrations measured with NMR are encouraged.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10071766/s1, Table S1: Lipofit values under the detection limit; Table S2: Additional analyses from lipoprofile; Table S3: Compared parameters and units of the corresponding parameters; Table S4: NMR values compared due to their size; Table S5: Comparison of standard lipids between ß-quantification and the lipofit and lipoprofile methods; Table S6: Comparison between the lipofit and lipoprofile methods; Figure S1: Comparison of standard lipids between ß-quantification and NMR (Bland Altman plots); Figure S2: Comparison of lipoprotein particles between the lipoprofile and lipofit methods (Bland Altman plots); Figure S3: Comparison of standard lipids between the lipoprofile and lipofit methods.

Author Contributions: G.S. and H.S. designed the study. M.R. (Martin Rief), R.R. and P.R. recruited the participants, performed ultracentrifugation and stored the samples. T.S. and H.S. performed standard lipid analyses in Graz. G.S., H.S. and M.R. (Martin Rief); performed the statistical analyses and wrote the manuscript. W.M., M.R. (Markus Reinthaler), P.M. and M.B. and all other authors critically reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the Medical University of Graz, Austria. Numares provided the NMR measurements for free.

Institutional Review Board Statement: Ethics commission of the Medical University of Graz ref. 29-479 ex 16/17, 18 July 2018; BASG/AGES ref. 11458092, 15 May 2019.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We highly appreciate James D. Otvos from LabCorp and Philipp Pagel, Torsten Winkler, Katja Barthelmes, and Volker Pfahlert from Numares for their open cooperation. Finally, we thank all the study participants. Without their cooperation, this article would not have been written.

Conflicts of Interest: M. Rief reports a bench fee for laboratory materials from the Medical University of Graz. H. Scharnagl reports research grants from Amgen and Abbott, personal fees from Amgen and Sanofi outside the submitted work. G. Silbernagel reports a research grant from Numares and grants and personal fees from Amgen, Sanofi, Daiichi Sankyo and Bayer outside the submitted work. All other authors report no conflicts of interest. W. März reports grants and personal fees from AMGEN, and grants from Synlab Holding Deutschland, Siemens Healthineers, Astra Zeneca, Bayer Vital GmbH, Bestbion dx GmbH, Boehringer Ingelheim, Immundiagnostik GmbH, Merck Chemicals, MSD Sharp and Dohme, Novartis, and Olink Proteomics, grants and personal fees from Aegerion Pharmaceuticals, AMGEN, Sanofi, Alexion Pharmaceuticals, BASF, Abbott Diagnostics, Numares AG, Berlin-Chemie, and Akzea Therapeutics, outside the submitted work. W. März is an employee of Synlab Holding Deutschland GmbH. M.E.K. reports personal fees from Bayer, outside the submitted work.

Abbreviations

apoB apolipoprotein B; C cholesterol; d diameter; h hour; HDL high-density lipoprotein; l liter; LDL low-density lipoprotein; LHDL large high-density lipoproteins; LLDL large low-density lipoproteins; LVLDL large very-low-density lipoproteins; mg/dL milligram per deciliter; MHDL medium high-density lipoproteins; min minute; mL millilitre; MLDL medium low-density lipoproteins; MVLDL medium very-low-density lipoproteins; nmol/L nanomol per liter; NMR nuclear magnetic resonance spectroscopy; p particles; p significance value; r correlation coefficient; rpm rounds per minute; SHDL small high-density lipoproteins; SLDL small low-density lipoproteins; SVLDL small very-low-density lipoproteins; TC Total cholesterol; TG Triglycerides; VLDL very-low-density lipoprotein; VLVLDL very large very-low-density lipoproteins; VSVLDL very small very-low-density lipoproteins.

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