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Is plasma apoB48 ELISA determination comparable with apoB48 determined from triglyceride-rich lipoprotein fractions by SDS-PAGE?

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<p>Cholesterol and triglycerides (TG) received from dietary fats are insoluble in plasma, and therefore these compounds must be packed to lipoproteins when transported by the bloodstream. Apolipoproteins are a group of proteins that are bound to lipoprotein particles, serving as cofactors for enzymes or as ligands for cellular binding. Apolipoprotein B exists in two forms: ApoB48 secreted by the intestine and apoB100 synthesized in the liver.</p> <p>Epidemiological studies have shown that elevated postprandial (non-fasting) TG concentrations are a significant risk for cardiovascular diseases (CVD). Previous studies have shown that apoB48 protein serves as a good marker for postprandial triglyceride-rich chylomicrons and VLDL particles. Traditionally apoB48 has been measured with SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) method from lipoprotein fractions separated first by ultracentrifugation (UCF). This SDS-PAGE methodology used to determine the contribution of intestinal triglyceride-rich lipoproteins to postprandial lipemia has been very time consuming. A much less time-consuming method, the ELISA (enzyme-linked immunosorbent assay) method by which apoB48 protein can be determined directly from plasma samples became available about ten years ago. The aim of this thesis is to assess the comparability of these two methods used to measure apoB48.</p> <p>The data for this method comparison was obtained from two clinical studies called the Fructose study and the Gere study. These studies included all-together 199 participants who all went through an 8-hour fat tolerance test at least once. In all, data consisted of 995 apoB48 measurements performed by both methods, SDS-PAGE and ELISA.</p> <p>Current data show that the correlation coefficient of measured apoB48 concentrations (mg/l) between the methods varied from $R = 0.563$, $P < 0.001$ to $R = 0.844$, $P < 0.001$ depending on the concentration of apoB48 in the samples. Data also show that plasma apoB48 concentrations measured by ELISA method are 76 - 85 % higher than the apoB48 concentrations measured by the SDS-PAGE method from lipoprotein fractions. There was a strong positive correlation observed between the two methods $R = 0.765$, $P < 0.001$.</p> <p>This study confirmed the earlier findings made with a smaller amount of data. ApoB48 concentration measured with ELISA directly from plasma reflects well the apoB48 concentration</p>	

measured from the lipoprotein fractions with SDS-PAGE. In the future, the less time-consuming apoB48 ELISA measurement could easily be implemented in clinical laboratories and used as a biological marker of intestinal chylomicron and VLDL particle number when evaluating the risk of CVD especially in hyper triglyceridemic patients.

All the laboratory work and clinical studies were carried out at Biomedicum Helsinki, Research Program Unit, Marja-Riitta Taskinen's study group, Diabetes and Obesity, Clinical Research Institute, HUCH Ltd. The instructors of this thesis are Docent Niina Matikainen, M.D., Ph.D., specialist in endocrinology and internal medicine and Reetta Sihvonen, Lector.

Keywords

Apo B48, lipoproteins, SDS-PAGE, ELISA

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1 Introduction

Cholesterol and triglycerides (TG) received from dietary fats are insoluble in plasma and therefore these compounds must be packed to lipoproteins when transported by the bloodstream. There are five major classes of lipoproteins: Chylomicrons, VLDL (very-low-density lipoprotein), IDL (intermediate-low-density lipoproteins), LDL (low-density lipoproteins) and HDL (high-density lipoproteins). These lipoprotein particles have different densities and they can be separated from fresh-drawn plasma by density gradient ultracentrifugation (UCF) with the help of Svedberg flotation rate (S_i), a unit for sedimentation rate. (Baynes J., Dominiczak M.H. p.215, 2014.)

Every lipoprotein class (Chylomicron, VLDL, IDL, LDL, HDL) has a typical set of apolipoproteins. Apolipoproteins are a group of proteins that compound to lipoprotein particles serving as cofactors for enzymes or as ligands for cellular binding. Apolipoproteins also take part in the exchange of lipid components between lipoprotein particles. Most significant apolipoproteins are apoA, apoB, apoC, apoE and apo(a). (Baynes J., Dominiczak M.H. p.215, 2014.)

Apolipoprotein B exists in two forms, apolipoprotein B 48 (apoB48) and apolipoprotein B 100 (apoB100). ApoB48 is synthesized in enterocytes and secreted by the intestine whereas apoB100 is synthesized in the liver. ApoB48 binds with chylomicrons whereas apoB100 binds with VLDL, IDL and LDL particles. The same gene codes both of these apolipoproteins. (Baynes J., Dominiczak M.H. p.216, 2014.)

After a meal, the intestine secretes chylomicron particles, each containing one copy of apoB48 protein. ApoB48 serves as a good biological marker for the number of chylomicrons and chylomicron remnant particles found in plasma. (Baynes J., Dominiczak M.H. 2014.) (Adiels M. et al. 2012.) ApoB48 is the only known specific marker of the intestinally formed chylomicron particles that largely share the ultracentrifugation properties with hepatic apoB100 containing VLDL particles (Nakajima K et al. 2014).

ApoB48 containing chylomicrons and apoB100 containing VLDLs are highly triglyceride-rich particles. Traditionally TG levels have been measured in fasting condition and guidelines have defined normal TG level to be $< 1.7 \text{ mmol/l} = 150 \text{ mg/dl}$ (Third Report of the

National Cholesterol Education Program (NCEP). 2002). On the other hand, epidemiological studies have shown that elevated postprandial (non-fasting) TG concentrations are a significant risk indicator for cardiovascular diseases (CVD). (Borén J., Matikainen N., Adiels M., Taskinen M-R. 2014.) Measurement of plasma TG does not fully capture the atherogenic potential of so called chylomicron remnants and VLDL remnants that are able to penetrate the vascular endothelium and to form an atherosclerotic plaque (Borén J., Matikainen N., Adiels M., Taskinen M-R. 2014).

Future studies about the impact of high postprandial chylomicron and VLDL levels to CVD risk are needed. However, time consuming and costly laboratory methods have been an obstacle for larger studies. Separation of plasma samples to lipoprotein fractions by ultracentrifugation and the determination of apoB48 and apoB100 by the SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) method has taken all together approximately 5 laboratory working days per one patient with fasting and postprandial samples.

Measuring apoB48 directly from plasma samples by the ELISA (Enzyme Linked Immuno Sorbent Assay) method is much simpler and faster to perform. It takes only one day for the results to be ready and one measurement kit can include samples from 16 different fat load tests (Kinoshita M. et al. 2005). In this study, we compare these two methods in the determination of apoB48: SDS-PAGE and ELISA.

Within this thesis the term “SDS-PAGE” is used in the meaning that the SDS-PAGE method contains both, the lipoprotein separation by ultracentrifugation (UCF) and the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2 Background

For decades, apoB48 has been measured by the SDS-PAGE method from lipoprotein fractions separated from plasma samples by ultracentrifugation with the help of NaCl density gradient (Karpe F., Hamsten A. 1994). A much less time-consuming method, the ELISA (enzyme-linked immunosorbent assay) method for measuring apoB48 protein directly from plasma became available about ten years ago. (Kinoshita M. et al. 2005). In our study group, we have used this ELISA method simultaneously with our traditional

SDS-PAGE method since the year 2010. Some comparisons of these two methods have been made but a thorough comparison with adequate sample number and concentration range of the methods still needs to be performed (Borén J., Matikainen N., Adiels M., Taskinen M-R. 2014).

The later adapted ELISA method is much faster and simpler to use compared to the SDS-PAGE method. ELISA is a very widely used laboratory method and many laboratories already have the facilities to perform it. Ultracentrifuges necessary for the lipoprotein fraction separation are a big investment for a research group. Because there is no need to separate lipoproteins to lipoprotein fractions before ELISA measurement, the analysis of apoB48 can be at the reach of far more laboratories than it used to be using SDS-PAGE method. From the economical point-of-view, ELISA is a better choice compared to SDS-PAGE also because it is so much less time consuming, and gives the possibility to get the results in timely manner after the last patient has finished in a clinical study.

Up to now our study group has carried out a comparison of apoB48 results for 10 subjects measured at time points (0, 2, 3, 4, 6, 8 h) after a standardized fat-rich test meal. Meaning that a total of 60 apoB48 results were compared. This small-scale comparison showed that the plasma values measured by ELISA correlated well with apoB48 results measured from the triglyceride-rich lipoprotein particles by the SDS-PAGE method. (Borén J., Matikainen N., Adiels M., Taskinen M-R. 2014.) In the present study we performed a large-scale comparison of the methods after we had collected more samples and data in our clinical studies.

3 Research questions

The aim of this thesis is to study the comparability of two methods, SDS-PAGE and ELISA, for analysing the apoB48 protein from fasting and postprandial blood samples.

The two research questions in this thesis are: 1) Is plasma apoB48 ELISA determination comparable with apoB48 determined from triglyceride-rich lipoprotein fractions by SDS-PAGE? and 2) Does the apoB48 protein concentration range have an influence on the correlation coefficient between the methods?

4 Analytical methods

All the study samples have been collected and the laboratory analyses performed during years 2012 to 2016. Samples were coded with patient numbers so that no subject participating in the studies can be identified. The author has personally analysed most of the SDS-PAGE samples and has performed all the data collection for this study. She has also taken part in analysing the apoB48 by the ELISA method and in clinical work with the study subjects during the fat load tests.

Laboratory work and clinical studies were performed at Biomedicum Helsinki, Research Program's Unit, Marja-Riitta Taskinen's study group, Diabetes and Obesity, Clinical Research Institute, HUCH Ltd. GCP (good clinical practice) and GLP (good laboratory practice) were followed closely in all the work.

4.1 Study participants

In this work, we compare apoB48 results measured in two studies called the Fructose-study and the Gere-study. Before taking part in our clinical studies, all participants were informed about the study procedures and asked to give a written informed consent. The clinical studies, from which all the data is collected, were ethically approved by the Ethics Committee of Helsinki University Hospital. All study procedures were conducted following the ethics rules stated by the Declaration of Helsinki.

In the Fructose-study, we recruited 51 non-smoking, obese men (mean body mass index, BMI 30.6 kg/m²) to visit our clinic for a fat tolerance test before and after consuming 75 g of a fructose-sweetened beverage daily for three months. (Matikainen N. et al. 2017). The GERE-study consisted of 58 participants, men and women, from families identified with hereditary lipid dysfunction and 87 healthy control subjects. 109 of these GERE-study patients went through a fat tolerance test once. During the performed fat tolerance tests, we took apoB48 samples at time points 0h, 3h, 4h, 6h and 8h. Samples for both methods (gradient ultracentrifugation + SDS-PAGE and ELISA) were taken at the same time points during the test days.

Table 1. The number of participants in both studies, the number of fat load tests performed and the number of fat load tests with complete apoB48 data available for the method comparison.

	Number of participants	Number of fat load tests performed	Number of fat load tests with complete apoB48 data
FUCTOSE-study	51	100	100
GERE-study	145	109	99
FRUCTOSE and GERE	196	209	199

Two participants in the Fructose-study withdrew their consent before the end of the study, and they did not come back to the clinic for the second fat load test. In the Gere-study, we had to exclude ten patients' fat load test results from the data analysis because there had been difficulties in blood sampling leading to missing apoB48 results for some time points. All together, we had 199 fat load tests with complete data (including all time points 0h, 3h, 4h, 6h and 8h) for the apoB48 method comparison as shown in Table 1. In the present study, the total number of apoB48 measurements for the method comparison was 995.

4.2 The Fat Tolerance Test

On the morning of the fat tolerance test, study subjects came to the clinic after 12 h of fasting. Blood samples were taken before the fat containing test meal at 0 h and at 3 h, 4 h, 6 h and 8 hours after the meal. The test meal contained bread, butter, cheese, sliced sausage, boiled egg, paprika, soured milk, orange juice and coffee or tea all together 72 grams of fat. (Fig. 1) Detailed composition of the test meal is described in Appendix 1.



Figure 1. Fat tolerance test meal contains 72 g of fat, 50g of carbohydrates and 38 g of proteins. All together 1000 kcal per serving.

Besides the fat load test meal, study subjects were permitted to drink only water during the test day. (Mero-Matikainen N. 1999).

4.3 Lipoprotein separation by ultracentrifugation

The lipoproteins were divided to subclasses as follows; $S_f > 400$ Chylomicrons, $S_f > 60 - 400$ VLDL1, $S_f 20 - 60$ VLDL2, $S_f 12 - 20$ IDL and $S_f 0 - 12$ LDL. S_f stands for Svedberg's flotation rate, a unit for sedimentation rate. During the fat load test, 10 ml of EDTA-plasma was collected at each time point (0h, 3h, 4h, 6h and 8h). Samples were kept on ice before centrifugation at $+4^\circ\text{C}$ with 2000 G for 10 min. To adjust the plasma density to 1.10 g/ml, 4.5 ml of centrifuged plasma was pipetted on top of 0.6315 g NaCl. Then 4.5 μl of PMSF (phenylmethylsulfonyl fluoride 10 mmol/l in isopropanol) and 22.5 μl of aprotinin (Trasylo1 1400 $\mu\text{g/l}$) was added to avoid apoB proteolytic degradation. After this, the plasma tube was mixed well and left at $+4^\circ\text{C}$ overnight. This lipoprotein fraction isolation must be performed from unfrozen EDTA-plasma samples.

The next day the plasma tubes were centrifuged lightly for 2 min at 1000 G and 4 ml of plasma with a density of 1.10 g/ml was pipetted at the bottom of Ultra-Clear Beckman SW40 ultracentrifuge tubes. Salt solutions of different densities were carefully overlaid

with a pump on top of the plasma as follows: first layer 3 ml of 1.065 g/ml, second layer 3 ml of 1.020 g/ml and third layer 2.8 ml of 1.006 g/ml NaCl solution.

Ultracentrifugation (UCF) was performed with a Beckman L8-55 ultracentrifuge using a SW 40 Ti swinging bucket rotor with a speed of 40,000 rpm at +15°C temperature. Lipoprotein fractions were collected at three different time points: 32 min ($S_f > 400$, Chylomicrons), 3 h 28 min ($S_f > 60 - 400$, VLDL1) and 17 h ($S_f 20 - 60$, VLDL2, $S_f 12 - 20$ IDL and $S_f 0 - 12$ LDL). At these time points, 1 ml of the lipoprotein fraction was collected carefully from the top of each tube using a long-form glass pipette (Pasteur). Always before the next ultracentrifugation, 1 ml of NaCl solution with a density of 1.006 g/ml was added on top of the UCF tube to replace the collected lipoprotein fraction.

4.4 SDS-PAGE

By the SDS-PAGE method, it is possible to measure simultaneously both apoB48 and apoB100 protein concentrations. These two forms of apolipoprotein B have different molecular weights (apoB48 260 kDa and apoB100 550 kDa) and they get separated at the same gel electrophoresis run by their different molecular weight. Before the SDS-PAGE method is used, all the collected EDTA-plasma samples must be separated to lipoprotein fractions by density gradient ultracentrifugation (UCF) as described in chapter 3.3. In this study, the SDS-PAGE method for measuring apoB48 was performed according to the method of Karpe F. and Hamsten A. (1994).

4.4.1 Sample preparation

After the lipoprotein fraction isolation, the fractions were delipidated in a test tube to ice-cold methanol-diethyl ether solvent. Then the test tubes were spun three times for 30 min at 4000 rpm in a centrifuge set to + 4°C, aspirating and adding ice cold diethyl ether in between the centrifugations. At the end, the delipidated and dried protein material in the bottom of the glass tubes was dissolved into DTT (Dithiotreitol) containing phosphate buffer, and samples were stored at - 80°C for the time preceding the SDS-PAGE electrophoresis. Before pipetting the samples for gel electrophoresis, all samples were kept for 10 minutes at +80°C and then centrifuged 2 minutes at 1500 G.

In this study only chylomicron, VLDL 1 and VLDL 2 fractions were delipidated for the apoB48 measurement. This means that all possible apoB48 protein in IDL and LDL fractions was not measured by the SDS-PAGE method.

4.4.2 Gel casting and electrophoresis

All the polyacrylamide gels were casted manually in our laboratory because no commercially sold gels are available. To separate apoB48 and apoB100 in a polyacrylamide gel, we prepared a gel consisting linear gradient from 3% of acrylamide to 20% of acrylamide. The protein particles were separated based on their molecular weight, apoB48 being 260 kDa and apoB100 being 550 kDa (Jackson and Williams. 2004). Figure 2. Electrophoresis was run by using a vertical Hoefer Mighty Small II electrophoresis apparatus connected to a power supply.

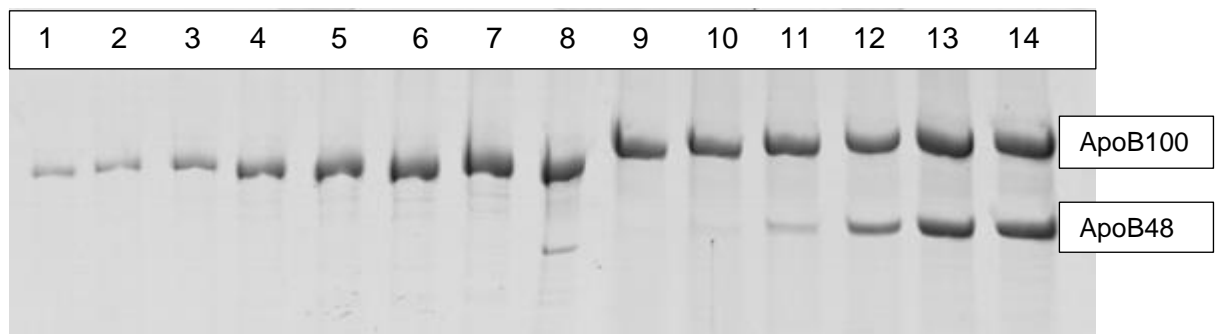


Figure 2. This picture shows a typical apoB48 SDS-PAGE gel after Coomassie blue staining. Exact amounts of isolated protein samples have been pipetted to 14 lanes. First 7 lanes from the left contain apoB100 standards pipetted as follows 0.120, 0.120, 0.240, 0.480, 0.720, 0.960 and 1.2 mg/l. The 8th lane is with locally prepared qualitative control for apoB100 and apoB48. The lanes 9 to 14 contain isolated chylomicron fractions pipetted from time points 0, 3, 4, 6 and 8 hours. Larger apoB100 particles form a protein band closer to the top of the gel and smaller apoB48 particles form the protein band closer to the bottom of the gel.

Every gel run included one 6-point standard curve, and every gel had an in-house control. Gels were run for 2h 20min and fixed for 1 h before 2 h staining with Coomassie blue G-250 protein stain in a solution of methanol-acetic acid. Finally, gels were destained three times with the methanol-acetic acid solution.

4.4.3 Gel imaging and analysing

After the staining process, gels were scanned and analysed by using Amersham Pharmacia's Image Quant TL program. The apoB48 and apoB100 protein concentrations were obtained from a standard curve included in every gel run. Coomassie blue G-250 protein dye used for staining the gels stains apoB48 and apoB100 protein particles equally and therefore it is possible to use apoB100 standard curve also to measure the apoB48 concentrations (Karpe F., Hamsten A. 1994).

To get the final concentrations of apoB48 in mg/l, the results had to be multiplied by different dilution factors originating from UCF, delipidating, sample buffer volume added on top of the dry protein material and sample volume pipetted on the gel for electrophoresis. All these calculations demanding great precision were performed using Excel. The inter-assay CV % for apoB48 results using this method varied between 14 – 16%.

4.5 ApoB48 ELISA

ApoB48 has a high structural homology with the hepatic apoB100. Therefore, an assay using specific anti-apoB48 was not easy to develop. (Kinoshita M. et al. 2005). A reliable ELISA (Enzyme Linked Immuno Sorbent Assay) method was finally brought for commercial use by the Japanese company Shibayagi. The antibody used in this sandwich ELISA method has a cross-reaction to human apoB100 less than the lower detection limit (2.5 ng/ml).

Precision and reproducibility of the ELISA method are good, with the average CV being between 2.8 to 8.6 %. (Shibayagi. Human Apo B-48 Elisa kit. Web publication.) There are also commercial controls available for this ELISA method which is an advantage compared to the traditional SDS-PAGE method where only in-house controls can be used. The principle of the sandwich enzyme linked immune sorbent assay is described in figure 3.

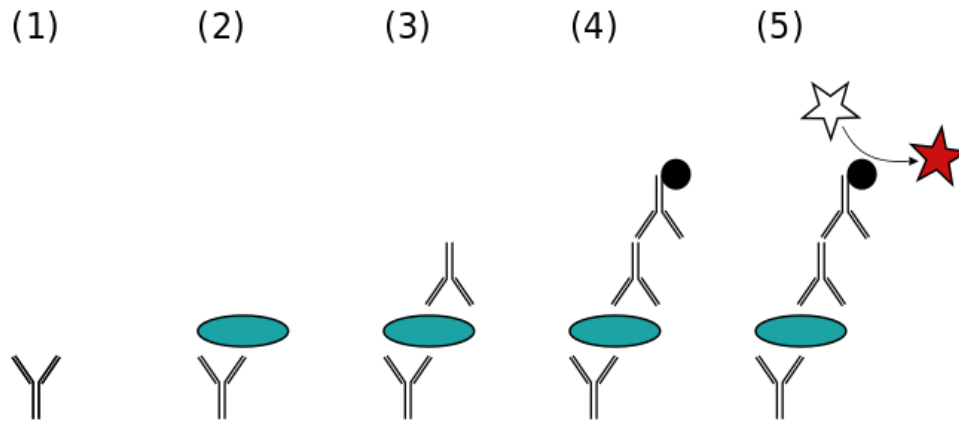


Figure 3. This picture explains the principle of sandwich ELISA method. **(1)** anti-apoB48 antibody is pre-coated on the surface of the microplate **(2)** apoB48 protein attaches to the anti-apoB48 antibody **(3)** biotin-conjugated anti-apoB48 attaches to the apoB48 protein particle **(4)** and **(5)** peroxidase-conjugated streptavidin solution reacts with biotin-conjugated molecules forming a blue colour after chromogen substrate reagent addition. Reaction is stopped with acid (1M H₂SO₄) and blue colour turns to yellow. The amount of colour produced is directly comparable with the amount of apoB48 protein in the sample. (Picture source: https://de.wikipedia.org/wiki/Enzyme-linked_Immunsorbent_Assay#/media/File:ELISA-sandwich.svg)

The apoB48 ELISA measurements for this study were performed as described in publication “Determination of apoB48 in serum by a sandwich ELISA” by Kinoshita M. et al. 2005. EDTA-plasma samples for ELISA measurements were collected during the fat load tests at the same time as the samples for the SDS-PAGE method. After sample collection, samples were frozen and kept at -80°C before analysing.

Samples were diluted prior to pipetting on the microplate, pre-coated with anti-apoB48. Standards and controls were pipetted at the same time with patient samples on the plate. Then the microplate was incubated for 1 h at room temperature (RT) to allow the apoB48 molecules in the pipetted samples to connect with the anti-apoB48 molecules attached to the bottom of the wells.

After one hour, all the free-floating material was discarded, and the plate was washed four times with ELISA plate washer. After which biotin-conjugated anti-apoB48 was added and the plate incubated for one hour and washed again four times. Then peroxidase-conjugated streptavidin solution was added and after 30 min incubation, the plate was washed again four times.

At the end, chromogen substrate reagent causing blue colour was added to the wells, and after 20 min incubation, the reaction stopper (1M H₂SO₄) causing yellow colour was

added to stop the reaction. The absorbance of each well on the microplate was measured at 450 nm, and the apoB48 protein concentration results were obtained from a standard curve. For the final concentration of apoB48, results were multiplied by the dilution factor.

5 Statistical methods

All study data has been made available to the author in Excel format and statistical analyses were performed using SPSS 22.0 for Windows and Excel 2013. iAUC (incremental area under the curve) values were used to evaluate the methods.

5.1 iAUC value

The incremental area under the curve (iAUC) values for both methods were calculated using apoB48 concentrations obtained from the 199 fat tolerance tests performed to our study subjects. iAUC measured by the Trapezoid method excludes the area left under the starting values, i.e. the background, from the total area under the curve (AUC) value. The iAUC values were determined by Excel 2013. Figure 4.

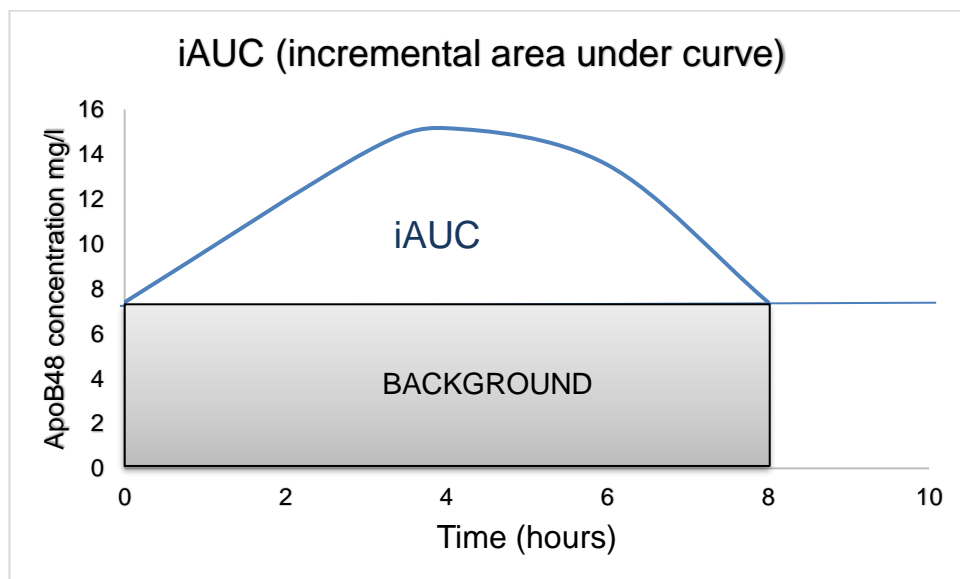


Figure 4. iAUC (incremental area under curve). The unit used for iAUC in this study is mg/l*h.

In this study, the iAUC values represent apoB48 protein concentration changes in blood circulation during a fat tolerance test from 0 to 8 hours.

5.2 Normality testing

Kolmogorov-Smirnov and Shapiro-Wilk tests in SPSS were used to determine whether the data is normally distributed or not. Normality testing was performed because the statistical method chosen for the statistical analysis depends on the distribution of the data. The null hypothesis was that the data is normally distributed and the hypothesis would be rejected with a P value less than 0.05. In the analysis it was found that the P value was less than 0.05 for three tested parameters and at the lower limit of true significance for one test parameter. Table 2.

Table 2. The results of normality testing by Kolmogorov-Smirnov and Shapiro-Wilk tests. P value equals to Sig. in this table.

Tests of Normality						
	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
ELISA iAUC	0.046	199	0.200*	0.982	199	0.010
SDS-page iAUC	0.071	199	0.017	0.931	199	0.001
*. This is a lower bound of the true significance.						
a. Lilliefors Significance Correction						

By these Kolmogorov-Smirnov and Shapiro-Wilk normality tests the null hypothesis was rejected and the data was found not normally distributed. Because of this finding only nonparametric tests were used for the statistical calculations.

5.3 Significance testing

The Wilcoxon signed-rank test was used to test the significance between the results obtained by the two different methods (SDS-PAGE and ELISA). This test is a nonparametric test, and it can be used to analyse data which is not normally distributed like the data in this study (Satake E. 2015). The null hypothesis was that the difference between the results measured by the two methods was obtained by chance, and this hypothesis would be rejected with a P value less than 0.05.

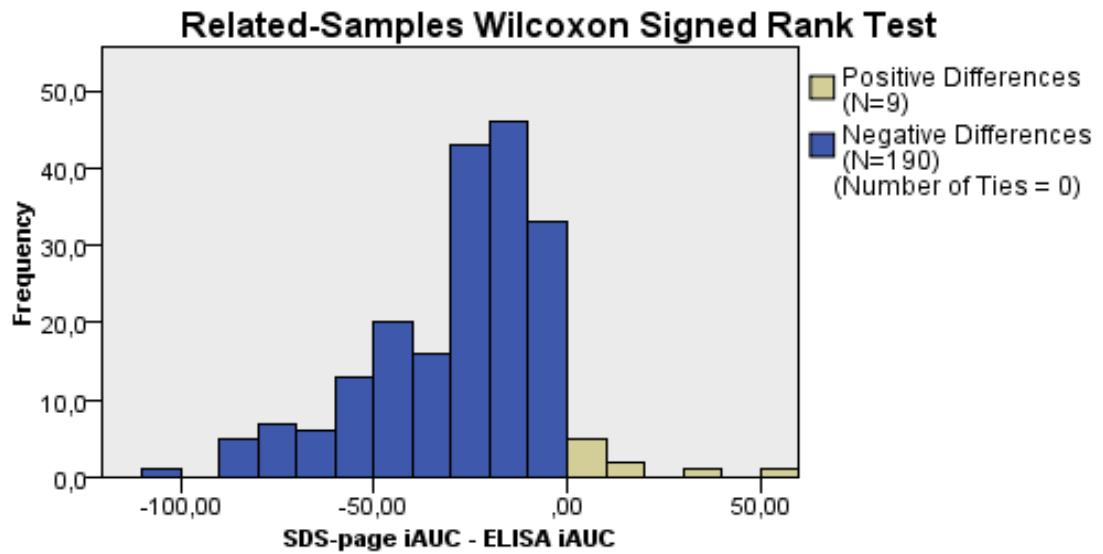


Figure 5. Wilcoxon signed rank test results. In 190 cases out of 199 the apoB48 concentration measured by SDS-page method was found lower than the concentration measured by ELISA.

After analyzing the data by the Wilcoxon signed-rank test, the null hypothesis was rejected with a P value of 0.001, meaning that the difference between the results obtained by the two different methods was statistically significant, not coincidental. Figure 5.

5.4 Correlation coefficient testing

The Spearman's rank correlation test was used to determine the correlation coefficient between the results obtained by the two different methods. Spearman's rank correlation test was chosen for analysing the data because it is a nonparametric test designed for analysing data which is not normally distributed (Satake E. 2015).

The null hypothesis was that there was no correlation between the results obtained by the two different methods, and this hypothesis would be rejected with a P value less than 0.05. After Spearman's rank correlation testing the null hypothesis was rejected with a P value less than 0.001. A statistically significant positive correlation was observed between the used methods with R value of 0.765. Figure 7.

Spearman's rank correlation coefficient testing was also performed using five different time points: 0, 3, 4, 6 and 8 hours by calculating the correlation individually for each time point. In these calculations we used the apoB48 concentrations measured in mg/l instead

of iAUC values. The mean concentration of apoB48 protein at each time point (0, 3, 4, 6 and 8h) was also determined. Table 3.

6 Results

Current data show that the correlation coefficient of measured apoB48 concentrations (mg/l) between the methods varied from $R = 0.563$, $P < 0.001$ to $R = 0.844$, $P < 0.001$ depending on the concentration of apoB48 in the samples, with weaker correlation coefficient with samples containing lower apoB48 protein concentration i.e. fasting samples. Respectively, the stronger correlations coefficients were observed in the samples with higher apoB48 concentrations i.e. 3, 4, 6 and 8 hours after the fat-rich test meal. The results of these correlations are shown in Table 3.

Table 3. Correlations (R), P-values and mean values of ELISA and SDS-PAGE methods measured at five different time points 0, 3, 4, 6 and 8 hours. (fat load test, n = 199)

TIME (hours)	R	P-value	ELISA mean mg/l	SDS-PAGE mean mg/l
0	0.563	0.001	7.4	1.1
3	0.693	0.001	14.1	3.4
4	0.763	0.001	15.2	3.6
6	0.844	0.001	13.5	3.1
8	0.799	0.001	10.4	2.0

Data also show that plasma apoB48 concentrations measured by ELISA method are 76 - 85 % higher than the apoB48 concentrations measured by the SDS-PAGE method from lipoprotein fractions. This difference in the apoB48 concentration levels between the two methods is shown in Figure 6.

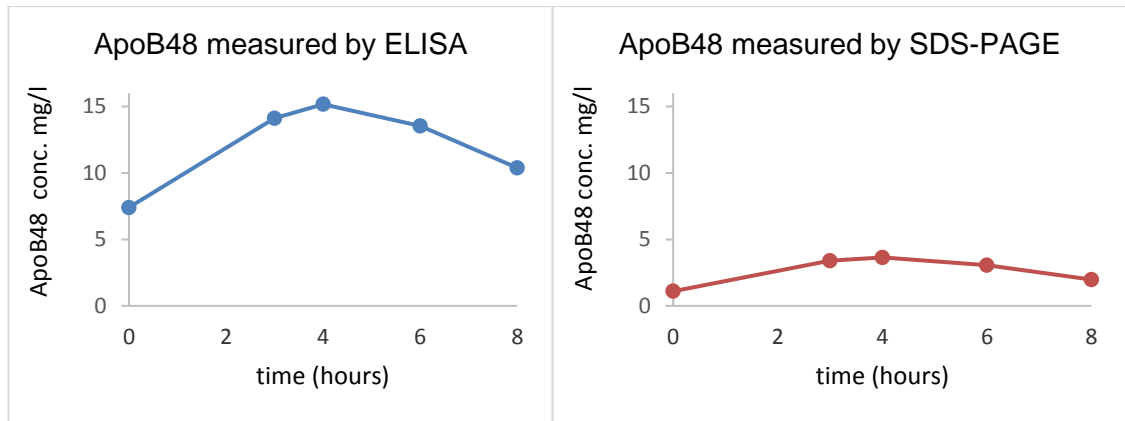


Figure 6. This figure shows mean apoB48 protein concentrations at time points 0, 3, 4, 6 and 8 hours measured by both, ELISA (blue line) and SDS-PAGE (red line) methods. Time points (hours) are set at the x-axis and apoB48 concentration in (mg/l) at the y-axis.

The calculated incremental area under the curve (iAUC) values for apoB48 concentrations during the 199 fat tolerance tests performed showed a strong positive correlation between the two methods $R = 0.765$, $P < 0.001$ as shown in Figure 7.

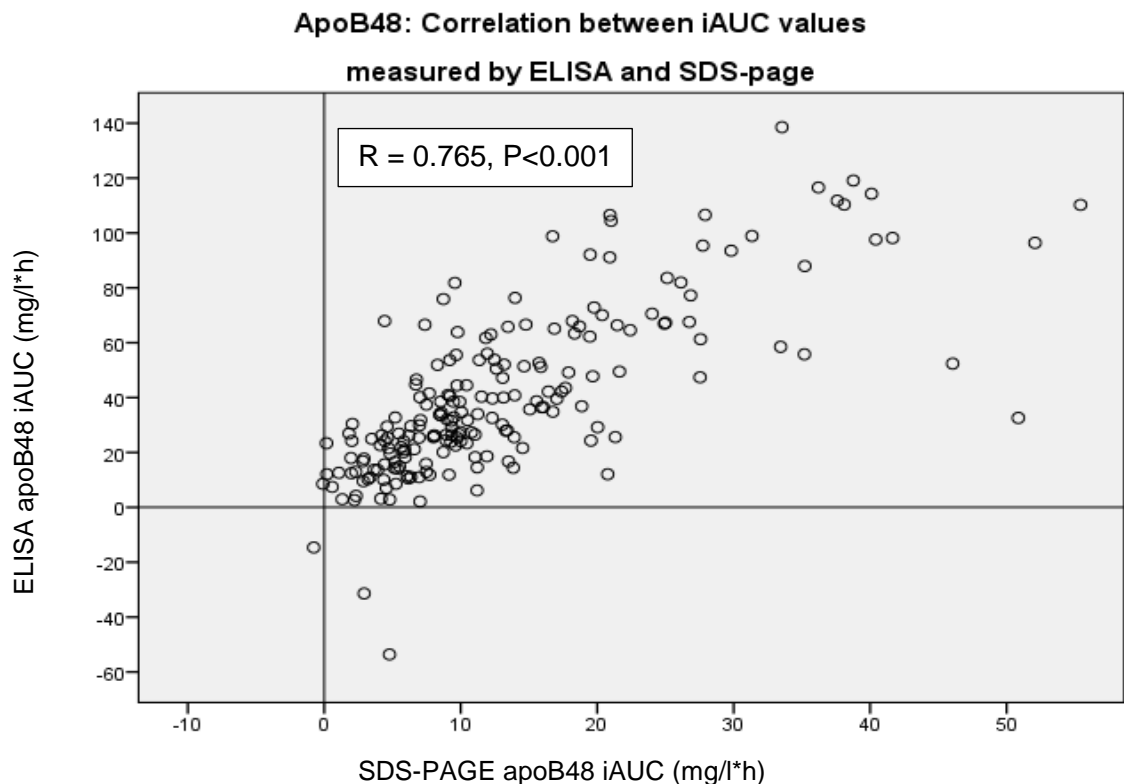


Figure 7. Correlation $R = 0.765$, $P < 0.001$ between the iAUC values measured by ELISA and the iAUC values measured by SDS-PAGE method. ELISA iAUC values on y-axis and SDS-PAGE iAUC values on x-axis. The apoB48 concentration change during the fat load test is expressed as mg/l*h.

It is normal that in some cases iAUC values are negative. This may occur when the apoB48 protein concentration is lower at the end of the fat tolerance test (8 hours) than in the beginning of the test (0 hours).

7 Discussion

In the present study, we aimed to assess the comparability of the two methods. We asked the question if it is possible to reliably compare data from our previous studies in which apoB48 protein was measured by SDS-PAGE to our ongoing studies where apoB48 protein is measured by the ELISA method.

Before this study, our group has shown in a small study with data of 60 samples the correlation coefficient of AUC values between these two methods to be good (is $R = 0.88$, $P < 0.001$) (Borén J., Matikainen N., Adiels M., Taskinen M-R. 2014). This study expands the previous knowledge by showing that the apoB48 concentration measured with ELISA directly from plasma reflects well the apoB48 concentration measured from the lipoprotein fractions by SDS-PAGE within a wide apoB48 concentration range.

The correlation coefficient of measured apoB48 concentrations between the ELISA and SDS-PAGE method varied from $R = 0.563$, $P < 0.001$ to $R = 0.844$, $P < 0.001$ depending on the concentration of apoB48 in the samples. The iAUC values during the 199 fat tolerance test performed showed a strong positive correlation between the two methods ($R = 0.765$, $P < 0.001$) We had data of 995 samples measured by both methods. Our data had a wide concentration range of apoB48, including a lot of samples with low, medium and high concentrations of apoB48 protein, and we were able to look at correlation coefficient values at different protein concentration levels on a larger scale.

It would be ideal if we could find a factor to convert the SDS-PAGE results to be equivalent with measurements performed by the ELISA method. What makes this specific factor determination very complicated is the fact that the apoB48 concentrations measured by the SDS-PAGE method are 76 - 85 % lower than those measured by the ELISA method, and correlation coefficient values depend on the concentration of the apoB48 protein in the samples. The correlation coefficient decreases simultaneously with the decreasing concentration of the apoB48 protein.

Several factors may explain the observed difference between the apoB48 protein concentration levels measured with SDS-PAGE or ELISA methods. This observation may partly be explained by the difference in the sensitivity of the compared methods and also by the difference in the sample material used for the methods. In this study only chylomicron and VLDL fractions were delipidated for the apoB48 measurements performed by SDS-PAGE method. The apoB48 concentrations used for measuring iAUC values for SDS-PAGE method were obtained by adding together the apoB48 results measured from the different lipoprotein fractions (Chylomicrons and VLDLs). This means that all apoB48 protein potentially in IDL and LDL fractions was not measured by the SDS-PAGE method. Furthermore, we also must take into consideration that during the collection of these lipoprotein fractions used for the SDS-PAGE method and during the sample preparation some material is always lost no matter how carefully the instructions are followed.

By using the ELISA method directly on plasma samples, there is no fear of losing sample material. Therefore, all possible apoB48 protein in the samples is determined and the measured concentrations represent complete apoB48 protein concentration in plasma. This and methodological factors may explain the higher protein concentrations obtained by ELISA method. On the other hand, the obvious weakness of the ELISA method is that only total plasma apoB48 concentration is measured, and investigators are left without the valuable information about the apoB48 concentration in each individual lipoprotein fraction.

In addition to the two methods compared in this study, there have been some experiments to analyse apoB48 protein by immunoblotting. However, these experiments have not succeeded because there has been too much intra- and inter-assay variability in the results due to antibody interactions and the number of laboratory steps involved. (Jackson K. and Williams C. 2004).

For this study the data was collected during four years (2012 – 2016). Especially SDS-PAGE laboratory analysis was laborious and required the contribution of one laboratory technologist over the years. During all these years, when working with the study subjects, the principles of GCP were followed carefully. The subjects were always well informed both orally and in writing about the study procedures before participating in the clinical studies, and they all gave a written informed consent at the first clinic visit. The work in the laboratory was performed in accordance with GLP and the personnel taking part to

the laboratory work were well qualified. The data of this study were recorded reliably and traceably in the laboratory to enable the data management afterwards. All these study procedures followed during the years make the data used for this method comparison reliable and accurate.

In conclusion, this study confirms our earlier findings made with a smaller amount of data. Our study also expands the previous knowledge by showing that the apoB48 concentration measured with ELISA directly from plasma reflects well the apoB48 concentration measured from the lipoprotein fractions by SDS-PAGE within a wide apoB48 concentration range. In the future, the less time-consuming apoB48 ELISA measurement can easily be implemented in clinical laboratories and used as a biological marker of intestinal chylomicron and VLDL particle number when evaluating the risk of CVD, especially in hypertriglyceridemic patients.

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ABBREVIATIONS

ApoB Apolipoprotein B

ApoB48 Apolipoprotein B 48

ApoB100 Apolipoprotein B 100

AUC Area under curve

iAUC Area under incremental curve

BMI Body mass index

CV Coefficient of variation

CVD Cardio vascular disease

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme Linked Immuno Sorbent Assay

GCP Good clinical practice

GLP Good laboratory practice

HDL High-density lipoprotein

HUCH Helsinki university central hospital

IDL Intermediate-low-density lipoprotein

LDL Low-density lipoprotein

PMSF Phenylmethylsulfonylfluoride

R Correlation

Rpm Revolutions per minute

RT Room temperature

SDS-PAGE *) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

S_r Svedberg flotation rate, a unit for sedimentation rate

TG Triglyceride

UCF Ultracentrifugation

VLDL Very-low-density lipoprotein

*) Within this thesis the term “SDS-PAGE” is used in the meaning that the SDS-PAGE method contains both, the lipoprotein separation by ultracentrifugation (UCF) and the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Fat load test meal contents

Bread	65 g
Butter	35 g
Cheese	50 g
Sliced sausage	40 g
Boiled eggs	70 g
Fresh paprika	50 g
Soured whole milk 1 %	200 g
Orange juice	1.25 dl
Coffee	

The fatty acids of the meal

65%	SFA
30 %	MUFA
5 %	PUFA

Fat	65%	→	72 g
Carbohydrate	20%	→	50 g
Protein	15%	→	38 g

Cholesterol	490 mg
Energy	1000 kcal