



## Low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol measurement in Familial Dysbetalipoproteinemia

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### ABSTRACT

**Aim:** To compare LDL-C concentrations using the Friedewald formula, the Martin-Hopkins formula, a direct assay and polyacrylamide gradient gel electrophoresis (PGGE) to the reference standard density gradient ultracentrifugation in patients with Familial Dysbetalipoproteinemia (FD) patients. We also compared non-HDL-cholesterol concentrations by two methods.

**Methods:** For this study data from 28 patients with genetically confirmed FD from the placebo arm of the EVOLVE-FD trial were used. Four different methods for determining LDL-C were compared with ultracentrifugation. Non-HDL-C was measured with standard assays and compared to ultracentrifugation. Correlation coefficients and Bland-Altman plots were used to compare the methods.

**Results:** Mean age of the 28 FD patients was  $62 \pm 9$  years, 43 % were female and 93 % had an  $\epsilon 2\epsilon 2$  genotype. LDL-C determined by Friedewald ( $R^2 = 0.62, p < 0.01$ ), Martin-Hopkins ( $R^2 = 0.50, p = 0.01$ ) and the direct assay ( $R^2 = 0.41, p = 0.03$ ) correlated with density gradient ultracentrifugation. However, Bland-Altman plots showed considerable over- or underestimation by the four methods compared to ultracentrifugation. Non-HDL-C showed good correlation and agreement.

**Conclusion:** In patients with FD, all four methods investigated over- or underestimated LDL-C concentrations compared with ultracentrifugation. In contrast, standard non-HDL-C assays performed well, emphasizing the use of non-HDL-C in patients with FD.

### Introduction

In clinical practice low-density lipoprotein-cholesterol (LDL-C) is calculated using the Friedewald formula based on measurement of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG). The Friedewald formula assumes a fixed ratio of cholesterol to TG in the very-low-density lipoprotein (VLDL) fraction:  $LDL-C = TC \text{ minus HDL-C minus } TG/2.2$  (in mmol/L) or  $TG/5$  (in mg/dL). [1] The original publication of the Friedewald formula noted three exceptions to its use: non-fasting samples,  $TG > 4.52$  mmol/L and Familial Dysbetalipoproteinemia (FD). FD is the second most common

monogenic lipid disorder, after Familial Hypercholesterolemia (FH). [2] The hallmarks of FD are cholesterol-enriched VLDL together with raised remnant lipoproteins and low LDL-C concentrations. As a result of the cholesterol enrichment of remnants, the fixed ratio of cholesterol to TG in VLDL in Friedewald is invalid. However, this exception to the Friedewald formula is often not appreciated in clinical practice, where LDL-C is still used as treatment goal or risk predictor in FD patients by some physicians. An alternative for the Friedewald formula is the Martin-Hopkins formula, which replaces the fixed ratio by an adjustable factor based on individual non-HDL-C and TG levels ( $LDL-C = TC \text{ minus HDL-C minus } TG/\text{adjustable factor}$ ). [3,4] However, the Martin-Hopkins

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formula is also not recommended in the setting of severe hypertriglyceridemia (>4.52 mmol/L) or FD. [4,5] Although direct (homogeneous) LDL-C assays have shown problems with accuracy and standardization in patients with type 2 diabetes mellitus (T2DM), metabolic syndrome and FD, [6–8] guidelines recommend the use of these assays when standard formulas are not applicable. [5] Another possibility to measure LDL-C concentrations is polyacrylamide gradient gel electrophoresis (PGGE), that separates lipoproteins based on size and stains neutral lipids (i.e. esterified cholesterol and TG) [9] It is not known how well PGGE performs to estimate LDL-C in patients with FD. The reference standard for determining LDL-C is ultracentrifugation, although it is not known whether this is also true in the context of FD. In FD the recommended treatment goal is non-HDL-C. [10] Non-HDL-C is calculated as TC minus HDL-C, with TC and HDL-C measured using standard biochemical assays. The performance of non-HDL-C measured with standard assays compared to non-HDL-C measured with ultracentrifugation in FD is not known. The aim of this study was to compare LDL-C concentrations using the Friedewald formula, the Martin-Hopkins formula, a direct assay and PGGE to ultracentrifugation in FD patients. Furthermore, we compared non-HDL-C concentrations measured by standard assays to ultracentrifugation.

## Methods

### Study design and patients

For this study data from 28 patients with genetically confirmed FD from the placebo arm of the EVOLVE-FD (Effects of EVOLocumab Versus placebo added to standard lipid-lowering therapy on fasting and post fat load lipids in patients with Familial Dysbetalipoproteinemia) trial were used. The design and rationale of the EVOLVE-FD study were previously described. [11] In short, this was a multicenter, randomized, placebo-controlled, double-blind, crossover study (Supplementary Fig. 1). The

study investigated the effect of evolocumab 140 mg on top of standard lipid-lowering medication compared with placebo. A FD genotype (an  $\epsilon 2\epsilon 2$  genotype or a pathogenic dominant APOE variant associated with a FD phenotype) confirmed by genotyping or isoelectric focusing was required for participation. A complete list of in- and exclusion criteria was previously described. [11] During the study patients received an oral fat load that consisted of unsweetened fresh cream. Venous blood samples were collected before and up to 8 h after the oral fat load. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and each patient provided written informed consent. The EVOLVE-FD study was registered at <https://www.clinicaltrials.gov> (NCT03811223).

## Laboratory measurements

### Density gradient ultracentrifugation

Density gradient ultracentrifugation was performed by the laboratory of Vascular Medicine at the Erasmus University Medical Center, Rotterdam, the Netherlands according to the Proudfoot protocol. [12,13] This method was used to measure the cholesterol content in the chylomicron, VLDL, IDL, LDL and HDL fractions. A detailed description of the procedure is provided in the **Supplementary Methods**.

### Clinical chemistry measurements

Total cholesterol, triglycerides and HDL-C were measured with an Atellica CH Analyzer (Siemens Healthcare Diagnostics). Apolipoprotein B (apoB) was measured by Abbott ARCHITECT. Lipoprotein (a) (Lp(a)) was measured by Atellica neph 360 (Siemens Healthcare Diagnostics). These analyses were performed at the Laboratory Department of the UMC Utrecht according to standard procedures.

### Friedewald formula and Martin-Hopkins formula

The Friedewald and Martin-Hopkins formulas were used to calculate LDL-C based on TC, HDL-C and TG levels. LDL-C concentrations (in mmol/L) based on the Friedewald formula were calculated as follows: TC minus HDL-C minus TG/2.2. [1] LDL-C concentrations based on the Martin-Hopkins formula were calculated as follows: TC minus HDL-C minus TG/adjustable factor. This factor was selected from a previously published table based on the patient's non-HDL-C and TG values in mmol/L. [14].

### Homogeneous direct assay

Homogeneous LDL-C was measured with an enzymatic colorimetric test (Human, Wiesbaden, Germany) and performed at the Laboratory Department of the UMC Utrecht. This assay combined two steps; the first step removed chylomicrons, VLDL and HDL. The second step determined LDL-C by enzymatic reactions, employing specific surfactants for LDL.

### Polyacrylamide gradient gel electrophoresis

The analyses of non-denaturing polyacrylamide gradient gels were performed by the laboratory of Chemical Pathology at the University of Cape Town, South Africa. The preparation of PGGE was previously described. [9] Details with regard to this procedure are provided in the **Supplementary Methods**.

### Non-HDL-C

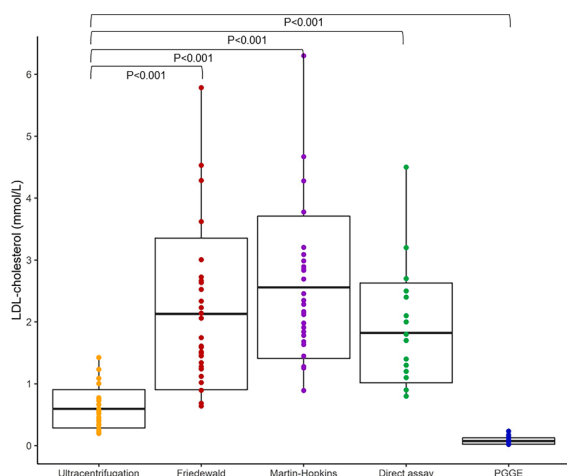
Non-HDL-C was calculated as total cholesterol minus HDL-C. TC and HDL-C were measured with standard clinical chemistry assays and compared to ultracentrifugation with non-HDL-C defined as cholesterol

**Table 1**  
Baseline characteristics.

	Patients (n = 28)
Age (years)	62 ± 9
Female sex (n,%)	12 (43)
APOE genotype (n,%)	
- $\epsilon 2\epsilon 2$	26 (93)
- Dominant APOE variant	3 (11)
Cardiovascular disease (n,%)	7 (25)
Diabetes mellitus type 2 (n,%)	9 (32)
Hypertension (n,%)	22 (79)
Metabolic syndrome (n,%)	21 (75)
Lipid-lowering treatment (n,%)	26 (93)
- Statin only	6 (21)
- Ezetimibe only	2 (7)
- Fibrate only	1 (4)
- Statin + ezetimibe	8 (29)
- Statin + fibrate	8 (29)
- Statin + ezetimibe + fibrate	1 (4)
High intensity statin (n,%)	7 (25)
Current smoking (n,%)	1 (4)
Body mass index (kg/m <sup>2</sup> )	29.5 ± 3.6
<b>Laboratory measurements</b>	
- Total cholesterol (mmol/L)	4.9 ± 1.9
- Triglycerides <sup>a</sup> (mmol/L)	2.8 (1.8 – 3.5)
- Non-HDL-cholesterol (mmol/L)	3.6 ± 1.7
- HDL-cholesterol (mmol/L)	1.3 ± 0.4
- Apolipoprotein B (g/L)	0.8 ± 0.2
- Lipoprotein (a) <sup>a</sup> (mg/dL)	8.2 (3.3 – 31.2)
- Total cholesterol/apoB ratio	6.3 ± 1.2
- Non-HDL-cholesterol/apoB ratio	4.7 ± 1.1

Twenty-six patients had an  $\epsilon 2\epsilon 2$  genotype (93%), two patients had a dominant variant in APOE and one patient had an  $\epsilon 2\epsilon 2$  genotype and a dominant variant in APOE (n = 3, 11%).

Data shown as mean with standard deviation (SD) or number (n) with percentage (%) unless stated otherwise. <sup>a</sup> median with interquartile range.



**Fig. 1.** LDL-C concentration in patients with FD (n = 28) Box represents mean with standard deviation.

levels in the chylomicron, VLDL, IDL and LDL fractions.

**Data analyses**

Paired *t*-tests were used to evaluate the differences between the four methods and gradient density ultracentrifugation. Furthermore, differences between the four methods and ultracentrifugation were analyzed and Pearson correlation coefficients were used to determine their

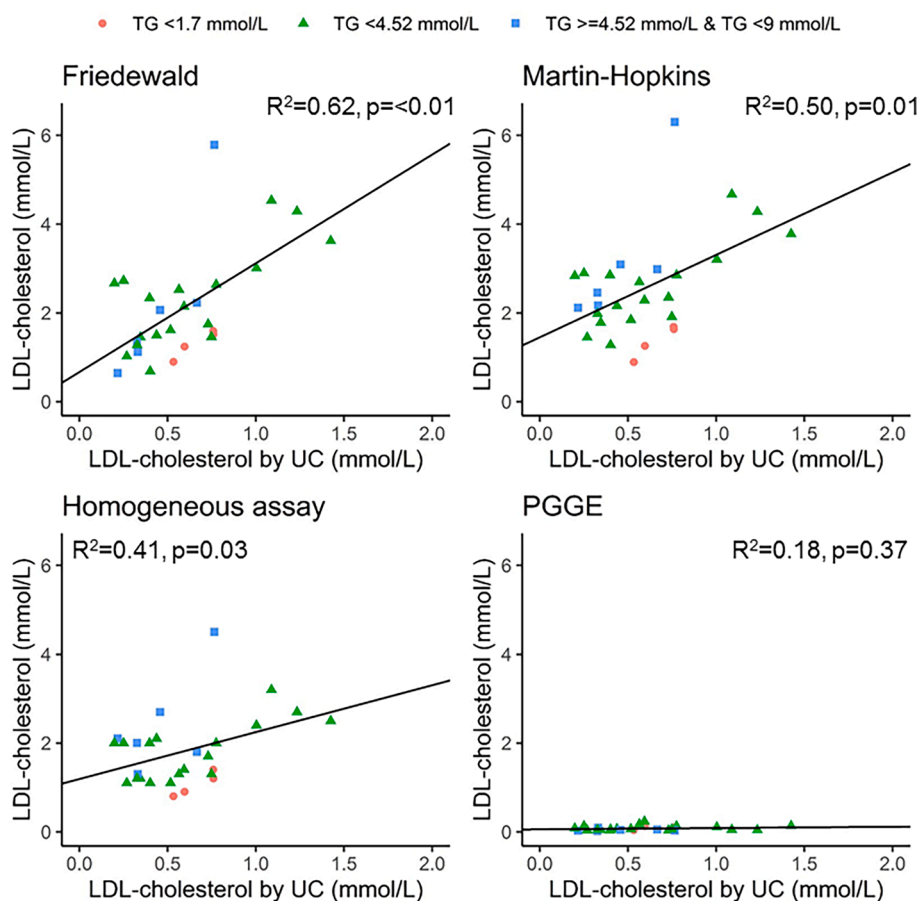
correlation. Linear regression analyses were used to fit regression lines in the correlation plots. The correlation and differences were stratified by TG levels. A TG concentration < 1.7 mmol/L was defined as normotriglyceridemia, TG < 4.52 mmol/L is often used as the cut-off for using the Friedewald and Martin-Hopkins formula and TG < 9 mmol/L was the maximum concentration for the total study population (based on the exclusion criteria of the study). Bland-Altman plots were used to visually assess the agreement between the investigated methods and ultracentrifugation. Similar analyses were performed for non-HDL-C by comparing standard assays and ultracentrifugation.

In addition, fasting and non-fasting LDL-C and non-HDL-C concentrations up to eight hours after the oral fat load were compared. There were no missing values for standard laboratory, ultracentrifugation or PGGE samples. All analyses were performed with R statistical software (Version 3.5.1; R foundation for Statistical Computing, Vienna, Austria). All *p*-values were two-tailed, with statistical significance set at 0.05.

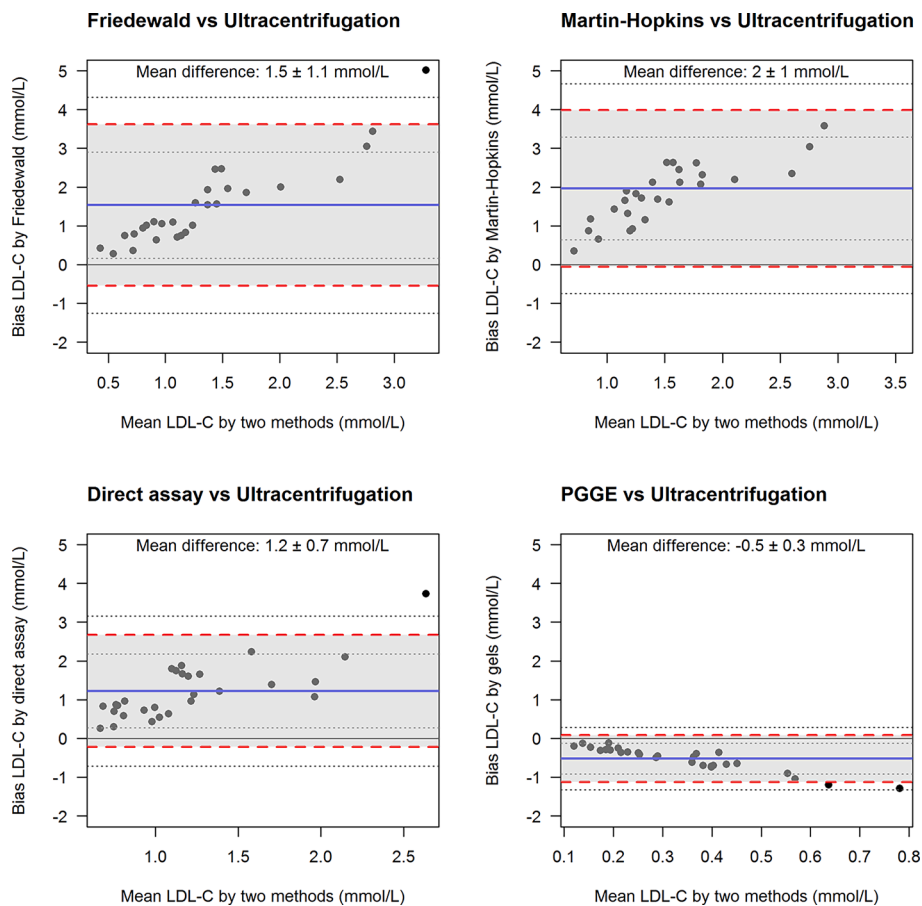
**Results**

*Baseline characteristics*

The baseline characteristics of the 28 FD patients are presented in Table 1. The mean age was 62 ± 9 years and 12 patients (43 %) were female. Overall, 25 % had CVD and 32 % had T2DM. Twenty-six patients (93 %) used lipid-lowering therapy; most patients used a combination of a statin and ezetimibe (29 %) or a statin and a fibrate (29 %). In addition, 25 % patients used a high-intensity statin. At baseline, mean total cholesterol was 4.9 ± 1.9 mmol/L, median TG 2.8 (IQR 1.8–3.5) mmol/L, mean apoB was 0.8 ± 0.2 g/L and mean HDL-C was 1.3 ± 0.4 mmol/L



**Fig. 2.** Correlation between diagnostic methods and ultracentrifugation Scatter plots with regression lines and correlation coefficients ( $R^2$ ), stratified for triglyceride levels. PGGE = polyacrylamide gradient gel electrophoresis, TG = triglycerides, UC = ultracentrifugation,  $R^2$  = correlation coefficient, LDL = low-density lipoprotein.



**Fig. 3.** Agreement between diagnostic methods and ultracentrifugation (Bland-Altman plots) Bland-Altman plots comparing four diagnostic methods versus ultracentrifugation to measure LDL-C in patients with FD. The blue line is the mean difference between the two methods. The upper and lower limits of agreement (red dashed lines) are the mean difference  $\pm 1.96 \times$  standard deviation. Mean LDL-C (x-axis) is calculated per patient by adding the LDL-C values from both methods and dividing by 2. The LDL-bias (y-axis) is calculated per patient by subtracting the LDL-C value of the diagnostic method from the LDL-C value measured by ultracentrifugation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

L. [Supplementary Fig. 2](#) shows the distribution of fasting TG across the study population. Median Lp(a) concentrations for this study population were 8.2 (IQR 3.3–31.2) mg/dL. Three patients had Lp(a) concentrations  $> 50$  mg/dL. The distribution of Lp(a) is provided in [Supplementary Fig. 3](#).

#### LDL-C concentrations according to different methods

With density gradient ultracentrifugation the mean LDL-C concentration was  $0.6 \pm 0.3$  mmol/L. With the Friedewald formula, the mean LDL-C concentration was significantly higher with  $2.1 \pm 1.2$  mmol/L ( $p < 0.001$ ). The mean LDL-C concentration calculated with the Martin-Hopkins formula was  $2.6 \pm 1.1$  ( $p < 0.001$  compared to ultracentrifugation). The mean LDL-C concentration measured by a direct assay was  $1.8 \pm 0.8$  mmol/L ( $p < 0.001$ ). Lastly, the mean LDL-C concentration measured with PGGE, was  $0.07 \pm 0.05$  mmol/L, which was significantly lower compared to ultracentrifugation ( $p < 0.001$ ) ([Fig. 1](#)).

#### Over- and underestimation of LDL-C compared to reference standard

Friedewald, Martin-Hopkins and the direct assay all overestimated mean LDL-C by on average at least 1 mmol/L compared to ultracentrifugation. In contrast, PGGE underestimated mean LDL-C concentration by approximately 0.5 mmol/L on average. Including only patients with TG  $< 4.52$  mmol/L ( $n = 22$ ) did not change the results. When including only patients with normal TG ( $< 1.7$  mmol/L) ( $n = 4$ ) there were fewer outliers, but there was still an overestimation of LDL-C concentrations by Friedewald, Martin-Hopkins and the direct assay and an underestimation by PGGE ([Supplementary Fig. 4](#)).

#### Correlation and agreement

The Friedewald formula ( $R^2 = 0.62$ ,  $p < 0.01$ ), Martin-Hopkins formula ( $R^2 = 0.50$ ,  $p = 0.01$ ), and direct assay ( $R^2 = 0.41$ ,  $p = 0.03$ ) were significantly correlated with density gradient ultracentrifugation, and PGGE was not ( $R^2 = 0.18$ ,  $p = 0.37$ ) ([Fig. 2](#)). To evaluate agreement, the difference between the four diagnostic methods and ultracentrifugation (defined as LDL-C bias) was plotted against their mean in the Bland-Altman plots ([Fig. 3](#)). All four methods over- or, in case of PGGE, underestimated LDL-C compared with ultracentrifugation. This difference depended on the mean value between the two measurements, reflecting proportional bias, indicating there was no systematic under- or overestimation for any of the methods compared to ultracentrifugation.

#### Non-HDL-C

Mean non-HDL-C was  $3.6 \pm 1.4$  mmol/L and  $3.5 \pm 1.4$  mmol/L ( $p = 0.43$ ) measured with standard assays and UC, respectively ([Fig. 4](#)). Stratification by TG levels did not change the results ([Supplementary Fig. 5](#)). Non-HDL-C measured with standard assays and ultracentrifugation showed good correlation ( $R^2 = 0.81$ ,  $p < 0.001$ ) and agreement, without over- or underestimation or proportional bias in the Bland-Altman plots ([Fig. 5A and 5B](#)).

#### Sensitivity analyses

LDL-C values measured with ultracentrifugation were the same after an oral fat load compared to the fasting values. The direct assay and PGGE also show very stable LDL-C concentrations before and after the oral fat load, while the LDL-C concentrations calculated with the

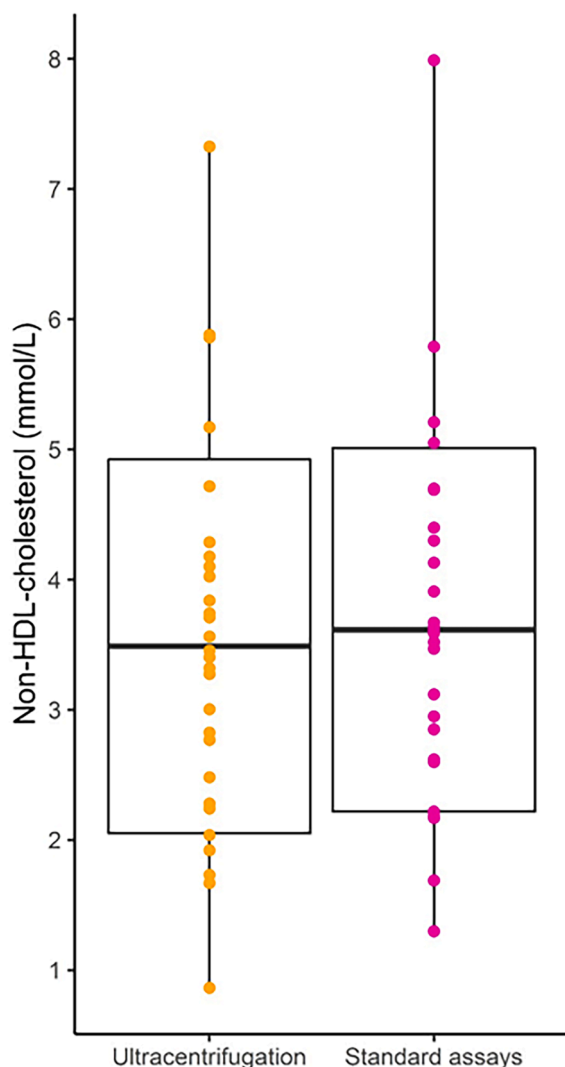


Fig. 4. Non-HDL-C concentration in patients with FD (n = 28) Box represents mean with standard deviation.

Friedewald and Martin-Hopkins formulas decreased after an oral fat load, due to increasing TG concentrations (Supplementary Fig. 6A). Non-HDL-C concentrations measured with standard assays and ultracentrifugation were the same before and after an oral fat load (Supplementary Fig. 6B). Including only patients with Lp(a) < 50 did not change the results (data not shown).

## Discussion

In patients with FD the four methods under evaluation (i.e. Friedewald formula, Martin-Hopkins formula, direct homogenous assay and PGGE) either over- or underestimated LDL-C concentrations compared to density gradient ultracentrifugation. The results showed that neither the Friedewald formula nor the Martin-Hopkins formula nor the direct homogeneous assay can be used in patients with FD. To the contrary, non-HDL-C measured with standard assays performed well compared to density gradient ultracentrifugation, underscoring the importance of using non-HDL-C instead of LDL-C in the treatment of FD.

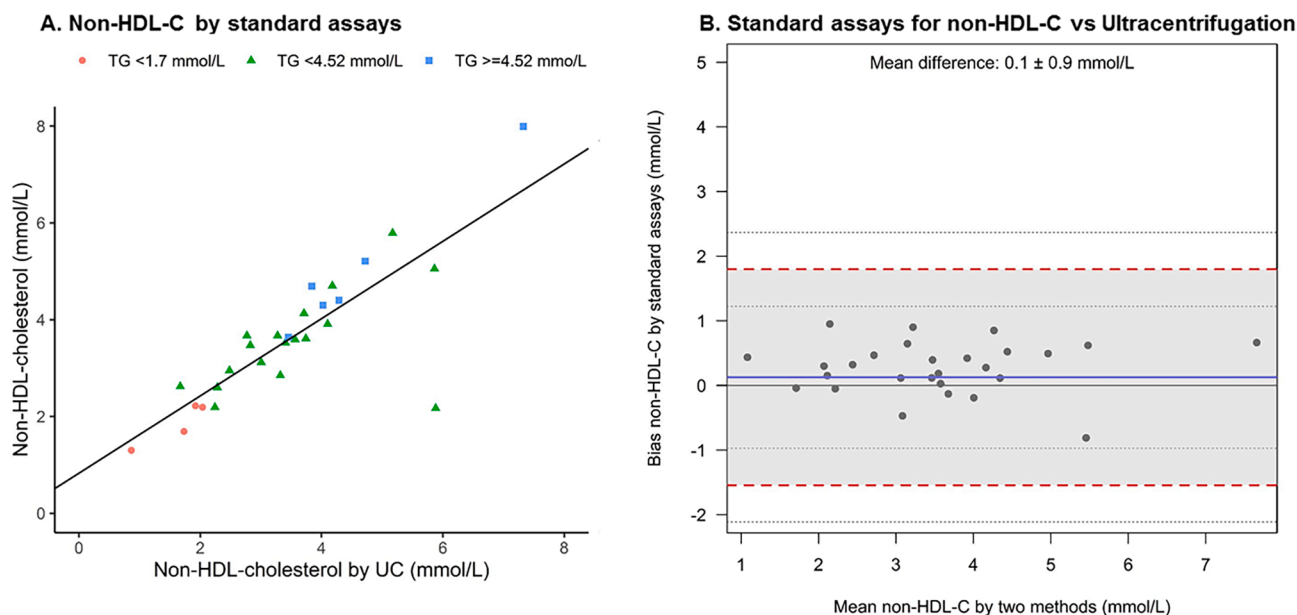
The use of LDL-C is not recommended in FD, for several reasons. First, as was shown in this study, in FD LDL-C cannot be reliably estimated or measured in routine clinical laboratories. Second, although LDL-C is usually low or absent in FD patients, they have a very high CVD risk. Therefore, LDL-C is not a reliable marker to estimate risk nor an appropriate treatment goal in FD. There are two mechanisms that

contribute to the relatively low plasma concentrations of LDL in FD patients. First, it is a consequence of the impaired lipolysis from the VLDL delipidation cascade to LDL, as apolipoprotein E2 (apoE2) displaces apolipoprotein C2, the cofactor of lipoprotein lipase, and the action of hepatic lipase on remnants is impaired by apoE2, by mechanisms yet unknown. [15] Second, the very low binding affinity of the apoE2 protein to the low-density lipoprotein-receptor (LDL-R) leads to a reduced influx of remnants into the liver, which leads to an upregulation of LDL-R, resulting in a greater internalization of LDL, which requires apoB100 for uptake by the LDL-R. [15,16].

Ultracentrifugation is considered the reference standard to determine LDL-C and diagnose FD. However, cholesterol in remnants and Lp (a) can be included in the LDL-C fraction when the cut-off between remnants and LDL is not clear, which is often the case in FD. So although ultracentrifugation is and will remain the reference standard to diagnose FD based on the specific lipoprotein pattern (based on the VLDL-C/VLDL-TG or VLDL-C/TG ratio), the validity of ultracentrifugation to determine LDL-C in FD needs consideration because it can overestimate 'true' LDL-C concentrations.

PGGE, which separates lipoproteins based on size, might be an alternative to estimate 'true' LDL-C in FD. Although there is still potential overestimation in the determination of LDL-C with PGGE, since this technique stains all neutral lipids in the LDL fraction, this effect is expected to be limited. Especially, once TG concentrations are lowered, the estimation of LDL-C with PGGE becomes more reliable. The results of the present study are consistent with previous findings from a study in 64 patients with ultracentrifugally proven FD of whom 43 % had no detectable LDL on PGGE. In patients that were untreated (n = 39) this was 72 %. [9] In conclusion, it is very likely that PGGE is less prone to error than calculation of LDL-C by inferring the composition of VLDL. However, whether PGGE is a more appropriate measurement of LDL-C in FD than ultracentrifugation is very difficult to test due to lack of a suitable reference standard. Moreover, the use of non-HDL-C makes an accurate measurement of LDL-C unnecessary.

Although it has been known since 1972 that in FD the Friedewald formula underestimates VLDL-C and subsequently overestimates LDL-C, many laboratories today still report LDL-C concentrations in patients with FD and physicians use it to estimate cardiovascular risk and as treatment goal. The Martin-Hopkins formula was developed in 2013 for patients with low LDL-C and/or (mildly) increased TG. [5] Although low LDL-C and increased TG are also found in dysbetalipoproteinemia, the present study showed that the Martin-Hopkins formula resulted in overestimation of LDL-C concentrations compared to density gradient ultracentrifugation in FD patients. The Martin-Hopkins formula was not validated in patients with TG > 4.52 mmol/L but we found that the overestimation in FD patients was irrespective of TG concentration. These results suggest that the VLDL-C to VLDL-TG ratio changes differently in FD than is assumed by the Martin-Hopkins formula or that other mechanisms may play a role in estimating LDL-C in FD. The latter is supported by the original publication of the Martin-Hopkins formula [4] that showed that one-third of the variance in the VLDL-C to VLDL-TG ratio is not explained by the standard lipid profile. Of the total validation dataset of the Martin-Hopkins formula, which included 1.35 million people, 446 had a dysbetalipoproteinemia phenotype (based on ultracentrifugation, defined as VLDL-C/TG ratio > 0.3, TG > 130 mg/dL, and LDL-C < 90th percentile, but not genetically confirmed). They found that the largest discordance of all types of hyperlipoproteinemia was found in a dysbetalipoproteinemia phenotype and therefore acknowledge limitations of the use of the Martin-Hopkins formula in the setting of FD. [4] However, the original paper only compared concordance with the Friedewald formula, which is not informative in this context knowing that the Friedewald formula is not accurate in patients with FD. One other study used the Martin-Hopkins formula to estimate LDL-C concentrations in a cohort with FD patients (with TG levels < 4.5 mmol/L), and found median LDL-C concentrations between 2.6 (2.0–3.5) and 3.6 (2.6–4.5) mmol/L (depending on the definition of FD



**Fig. 5.** Correlation and agreement of non-HDL-cholesterol. A. Scatter plot with regression line and correlation coefficient of non-HDL-C (measured with standard assays for total cholesterol and HDL-C) versus non-HDL-C measured by ultracentrifugation (defined as cholesterol content in the chylomicron, VLDL, IDL and LDL fraction), stratified for triglyceride levels. B. Bland-Altman plot showing non-HDL-C measured with direct assay versus non-HDL-C measured by ultracentrifugation. The blue line is the mean difference. The upper and lower limits of agreement (red dashed lines) are the mean difference  $\pm 1.96 \times$  standard deviation. UC = ultracentrifugation, non-HDL-C = non-HDL-cholesterol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used). [17] The EAS/EFLM guidelines endorse that the Martin-Hopkins formula is preferred to the Friedewald formula in patients with TG levels between 2.0 and 4.5 mmol/L, [5] but does not mention FD as an exception to this rule.

Direct chemical assays are often used to measure LDL-C when standard formulas are not applicable. Although it is recommended by the EAS/EFLM guidelines that direct assays for LDL-C should be used when TG levels are  $> 4.52$  mmol/L (which is the limit of use of Friedewald and Martin-Hopkins formulas), they acknowledge that direct assays do not necessarily yield accurate measurements of LDL-C in every patient. [5] In clinical practice, a direct LDL-C assay is often used to measure LDL-C in patients with FD but the ‘lipoprotein specific’ surfactant might not discriminate perfectly between LDL and remnant lipoproteins. [7] This is also the reason why direct assays have limited accuracy in patients with high TG and mixed dyslipidemia (which often includes remnant lipoproteins). [5,8,18] In line with the findings in the present study, two studies evaluated different direct LDL-C assays in 348 patients with and without several types of dyslipidemia, including 6 patients with FD. Both studies showed that LDL-C concentrations were overestimated with most direct assays compared with beta quantification in FD patients. [7,8] Taken together, these results suggest that direct assays should not be used to measure LDL-C in FD and underline that these assays are not an alternative for the formulas to determine LDL-C in clinical practice in FD. Non-HDL-C calculated based on standard assays of TC and HDL-C showed good correlation and agreement compared to ultracentrifugation and confirmed that non-HDL-C can be reliably measured in FD. This better agreement is caused by the fact that non-HDL-C measured with both standard assays and ultracentrifugation includes cholesterol in remnant lipoproteins beyond the LDL-C fraction. In line with this, the TC/apoB and non-HDL-C/apoB were increased, confirming the presence of VLDL and remnants [19,20] which is reflected in non-HDL-C but not LDL-C. Non-HDL-C is therefore the lipid measurement of choice to use as treatment goal in FD.

#### Strengths and limitations

The strengths of this study include the well-characterized and relatively large FD population, the systematic measurement of LDL-C and non-HDL-C with different laboratory methods, with extensive measurement of postprandial lipids in patients on different (combinations of) lipid-lowering medication.

Some limitations should be considered. Firstly, only one homogeneous assay for the direct measurement of LDL-C was evaluated, while the results might be highly dependent on the manufacturer. [7] Although there is no evidence that other direct assays would perform very differently compared to the homogeneous LDL-C assay evaluated in this study, the results should be applied to other direct assays with care. This is also the case for non-HDL-C calculated using HDL-C. In this study HDL-C was measured using the Siemens Atellica HDL-C assay. Since there is a clear difference between methods and manufacturers for HDL-C assays in dyslipidemic patients, the results of this study should not be considered representative to all HDL-C assays. [21,22].

Second, patient samples were analyzed after different freezing periods. The direct assay was usually analyzed within 24 h, while ultracentrifugation and PGGE were analyzed after variable intervals of up to three months. Although freezing could have variable influence on the different lipoprotein classes [23], it is not known whether this happened in our samples and if so, whether this influenced the results. Finally, for this study density gradient ultracentrifugation was used, although the Center for Disease Control recommends beta-quantification ultracentrifugation as reference standard for LDL-C. The difference between these two methods is very small. With beta-quantification the chylomicron, VLDL and IDL fractions are cut out, after which LDL, IDL and Lp (a) are precipitated and cholesterol in this fraction is measured; whereas in density gradient ultracentrifugation the tube is fractionated and the fractions HDL, LDL, IDL and VLDL are pooled and analyzed separately. The latter method requires a few more steps and is therefore more prone to error, which might have influenced the results.

## Conclusions

All four methods to determine LDL-C in patients with FD investigated here, either over- or underestimated LDL-C concentrations compared with density gradient ultracentrifugation, and even ultracentrifugation can overestimate LDL-C by including remnant cholesterol, especially in FD. Therefore the use of LDL-C is not recommended in the management of FD. In contrast, non-HDL-C performed well compared to ultracentrifugation, emphasizing the use of non-HDL-C in the management of FD instead of LDL-C.

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## CRediT authorship contribution statement

**Britt E. Heidemann:** Conceptualization, Methodology, Project administration, Visualization, Writing – original draft. **Charlotte Koopal:** Conceptualization, Supervision, Writing – review & editing. **Jeanine E. Roeters van Lennep:** Writing – review & editing. **Erik S. Stroes:** Writing – review & editing. **Niels P. Riksen:** Writing – review & editing. **Monique T. Mulder:** Investigation, Writing – review & editing. **Leonie C. van Vark – van der Zee:** Investigation, Writing – review & editing. **Dee M. Blackhurst:** Investigation, Writing – review & editing. **Frank L.J. Visseren:** Conceptualization, Supervision, Writing – review & editing. **A. David Marais:** Conceptualization, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: BEH declares no conflicts of interest. CK declares no conflicts of interest. JRvL has received a research grant by Amryt. Ad-board speaker fees have been paid to institution of ES by: Amgen, Sanofi, Esperion, Novo-Nordisk, Akcea/Ionis, Regeneron. NR declares no conflict of interest. MM declares no conflicts of interest LvZ declares no conflicts of interest. DMB declares no conflict of interest. ADM declares no conflict of interest. FV declares no conflict of interest.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2022.11.035>.

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