The variability of measured and calculated low-density lipoprotein (LDL) cholesterol in statin-treated diabetes patients.

Running Title: LDL-cholesterol variability in statin-treated patients

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DECLARATIONS

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List of abbreviations: LDL-C, low density lipoprotein cholesterol; BV, biological variation; dLDL-C, directly measured LDL cholesterol; T2DM, type 2 diabetes; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides.

Abstract

Background

The Sampson-NIH and Martin-Hopkins low-density lipoprotein cholesterol (LDL-C) equations are advocated as being superior to the Friedewald calculation. However, their mathematical complexity means they may have different biological and analytical variation when tracking LDL-C in the same patient. This study has established the biological variation (BV) of calculated and directly measured LDL-C (dLDL-C) in patients taking equivalent doses of a long (atorvastatin) and short (simvastatin) half-life statin. It also modelled how analytical imprecision might add to these BVs.

Methods

In a cross-over study of lipid BV involving 26 patients with type 2 diabetes (T2DM) initially taking either simvastatin 40mg or atorvastatin 10mg, fasting lipids were measured 10 times over 5 weeks after a 3 month run-in. The same procedure was then followed for the alternate statin. Outlier removal and CV-ANOVA established the BV of dLDL and each formula. Analytical measurement uncertainty was estimated from 6 months of real-world data.

Results

The intraindividual BV of dLDL-C measurement was considerably lower with atorvastatin than simvastatin (CV 1.3%(95% CI 1.1-1.5%) vs. 11.1%(10.2-12.2%) respectively). No equation could distinguish this difference (Friedewald 11.0%(95%CI 10.0-12.1%) vs. 12.9%(11.8-14.2%), Sampson-NIH 10.4%(9.5-11.5%) vs. 11.7% (10.7-12.8%), Martin-Hopkins 9.3%(8.5-10.3%) vs. 11.3%(10.3-12.4%)). Real-world analytical CVs were 2.6% (Sampson-NIH), 2.6% (Martin-Hopkins) 2.8% (Friedewald) and 2.0% (dLDL-C).

Conclusions

Inherent biological LDL-C variability using these formulae is substantially greater than direct measurement in T2DM patients taking atorvastatin. Typical analytical imprecision was also greater. Together, this may fundamentally limit these equations' ability to track true LDL-C changes in patients taking popular statin treatments.

Keywords:

Cholesterol; low-density-lipoprotein cholesterol; biological variation; LDL formula

Introduction

Assessing the low-density lipoprotein cholesterol (LDL-C) concentrations of patients remains a central tenet of cardiovascular risk assessment and monitoring.[1] Traditionally, measurement of LDL-C has involved laborious and costly techniques of β-quantitation involving ultracentrifugation.[2] As a consequence, numerous formulae have been suggested to estimate LDL-C based on the concentrations of other blood lipids which are more easily measured, namely total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG).[3] One of the first equations, the 'Friedewald formula', has gained widespread adoption but also comes with limitations, including being particularly unreliable in patients who are not fasting or have high (>4.5mmol/L) triglyceride concentrations.[4]

More recently, two formulae derived from large datasets have been proposed which help address some of the disadvantages of the Friedewald formula. The 'Martin-Hopkins' equation, based on over 1 million participants in the Very Large Database for Lipids, was first published in 2013, bringing with it apparent accuracy benefits at low LDL-C concentrations, especially in the presence of high triglyceride levels.[5] The subsequent 'Sampson-National Institutes of Health (NIH)' equation in 2020 was based on over 250,000 patients who had their LDL-C measured either directly or by β-quantitation and claimed even better accuracy, both at low LDL-C concentrations and in hypertriglyceridemic subjects up to a concentration of 9.0 mmol/L.[6] There was an extension to the Martin-Hopkins equation in 2021 which also made it more reliable in non-fasting subjects and in hypertriglyceridemia up to similar concentrations as the Sampson-NIH formula.[7] However, fundamental assumptions are made with any estimation formula which means that even if a large dataset is used in a regression analysis there is still no guarantee that the calculated quantity will show the same biological characteristics as the mimicked (true) quantity. Since causality is not equal to regression, the calculated quantity must be tested for each diagnostic feature of any other measured or calculated quantity. In other words, the patient or cohort of patients must be a representative of the dataset used in the original regression analysis.

For over 2 decades, there has also been the option of directly measuring LDL-C in blood but the adoption of these assays has not been as extensive as that of direct HDL-C measurement, presumably partly because of the cost implications compared to calculating

LDL-C and partly because of a concern around the specificity of some assays towards other lipoproteins.[8]

Measurement of LDL-C is not just to identify individuals with lipid disorders but to gauge success in achieving lipid treatment targets while taking lipid lowering treatment. A key component when tracking LDL-C in this situation is its inherent intra-individual biological variation (BV) whilst on treatment. Two of the most commonly prescribed lipid lowering agents are atorvastatin and simvastatin, with atorvastatin being England's most prescribed drug of any type and simvastatin the second most prescribed lipid lowering drug.[9] These two statins have different properties, with the half-life of simvastatin in the bloodstream being much shorter (circa 1-2 hours) than atorvastatin (approximately 14 hours)[10], raising the possibility that they could demonstrate different intra-individual LDL-C BVs.

We have already found that patients with type 2 diabetes can have markedly higher LDL-C variability when a patient takes simvastatin rather than atorvastatin but that this difference was only apparent with direct LDL-C measurement and not when the Friedewald formula was used.[11] This is presumably at least in part because the LDL-C equation combines the biological variability of all three lipid components rather than just one if LDL-C is measured directly. The Friedewald, Martin-Hopkins and Sampson-NIH equations are of respectively increasing mathematical complexity, and so it is possible that they may exhibit different LDL-C biological variation to one another. Also, when lipids are routinely measured in a clinical laboratory, these three formulae may combine the three lipid sources of analytical imprecision differently as well, thereby further influencing how they compare both to one another and with directly measured LDL-C.

This study has extended our previous work to establish the inherent biological variation of LDL-C using the Friedewald, Martin-Hopkins and Sampson-NIH equations, and also by directly measuring LDL-C in type 2 diabetes patients taking equivalent doses of atorvastatin and simvastatin in a cross-over study. It has also modelled how analytical imprecision might add to these biological variations in routine clinical practice.

Methods

The patient cohort has been described previously.[12] Briefly, 30 patients with type 2 diabetes for at least 3 years and HbA1c values between 6 and 9% were recruited into the study, excluding any individuals with untreated hypothyroidism or nephrotic syndrome. Nineteen patients were taking 10 mg atorvastatin before bed while 11 further patients took simvastatin 40 mg before bed. All the patients had been taking these doses of statin for at least 3 months and none were prescribed any additional lipid lowering therapy. The insulin doses of patients who took insulin were not changed by >10% throughout the study.

Blood sampling and analysis

The biological variation of LDL-C was assessed by measuring 12-h fasting blood samples at 4-day intervals on 10 consecutive occasions. Thereafter, the patients on simvastatin were changed to the equivalent dose of atorvastatin and vice versa.[13] After 3 months, the biological variation of lipid parameters was again assessed by measuring fasting blood samples at 4-day intervals on 10 consecutive occasions in these patients. Fasting venous blood was collected into serum gel tubes (Becton Dickinson, Oxford, UK) at the same time each day (08:00–09:00 hours) after the patient was seated for at least 5 minutes with the tourniquet applied for no more than 1 min. Samples were separated by centrifugation at 2000 g for 15 min at 4◦C, and two aliquots of the serum were stored at −20◦C within 1 h of collection. The serum samples were split before assay. According to our previous studies,[11,12] duplicate samples (i.e. two per visit) were randomized and then analysed using a single batch of Beckman reagents for direct LDL-C using a Synchron DxC analyser (Beckman-Coulter, High Wycombe, UK) using LDL-C reagents and calibrators.

Duplicate measurements of total cholesterol, HDL-C cholesterol and fasting triglycerides were used to estimate LDL-C twice for each patient visit by using the Friedewald, Martin-Hopkins and Sampson-NIH equations. While the Friedewald and Sampson-NIH equations were calculated directly, the Martin-Hopkins calculations were performed using their own validated Excel spreadsheet (https://ldlcalculator.com/).

Statistical Analysis

Biological variation

Outlier points which were imprecise for any duplicate were identified by the Cochran C test applied to the variance of duplicate measurements. These points, as well as all other measured/calculated LDL-C values for that patient's same timepoint were removed in a stepwise manner until all remaining data was below the Critical C values of $p<0.05$ significance. Thereafter, the CV-ANOVA method was used to establish the biological variation.[14,15] This involves transforming each LDL-C value into a coefficient of variation by dividing each data point by that person's mean value. ANOVA was subsequently performed in the traditional way by subtracting the analytical variance of duplicate measurements from the variance of transformed results.[16] Confidence intervals for biological variance were calculated using the statistical package referred to below which used the techniques described in Burdick and Graybill.[17]

Comparisons of biological variances between the atorvastatin and simvastatin groups using the same direct method or estimated LDL-C formula used 95% confidence interval lack of overlap to indicate statistical significance, and was also used to compare any differences between direct or estimated LDL-C methods within each group.

Analytical variation

To estimate the effect of routine analytical imprecision on measured and estimated LDL-C, the analytical CVs of total cholesterol, triglycerides, HDL cholesterol and directly measured LDL-C were derived from quality control material results collected over a six month period using standard Beckman-Coulter AU assays at mean values of 4.0mmol/L, 1.7mmol/L, 1mmol/L and 1.7mmol/L respectively. From this data, 100,000 randomised points for each of the four tests were created using the Excel NORMINV(RAND()) function so as to follow each test's respective mean and SD distributions. In turn, this allowed the overall analytical CV of each LDL-C formula to be calculated based on the resultant 100,000 lipid profiles. Again, the Martin-Hopkins LDL-C values were calculated using their own spreadsheet.

Statistical analysis was performed using the Analyse-it software add on to Microsoft Excel (https://analyse-it.com/). All subjects gave their written informed consent before entering

the study, which had been approved by the South Humber Local Research Ethics Committee (Ref: 04/Q1105/40). The study was funded by an unrestricted educational grant from Pfizer.

Results

Table 1 describes the baseline characteristics of the patients. Four patients discontinued the trial for various reasons.[12] None of the patients developed elevated liver transaminases or creatine kinase (CK). There was no significant change in glycaemic control during the course of the study in any patients (median ± SD) (61 ± 11 beginning vs. 61 ± 10 mmol/mol end, P = 0.60).

Figure 1 shows the mean and range of LDL-C values obtained during the period when each patient was taking atorvastatin, comprising 250 visits in total. These are derived from the first of the duplicate direct measurements and from the first calculation of the three formulae. Figure 2 shows the equivalent data while the same patients were taking simvastatin, containing 255 visits in total.

The Cochran C test eliminated two imprecise visit results from each of the statin groups. Table 2 shows the mean LDL-C and intraindividual biological coefficient of variations using all four methods of LDL-C assessment. The 95% CI of biological CV showed there was only a statistically significant difference in biological variation between statins when the direct LDL-C method of assessment was used, with atorvastatin being much less variable than simvastatin (CV 1.3% vs. 11.1%). All three formulae could not detect any significant difference, although the Martin-Hopkins formula was closest in doing so. Within the atorvastatin group there was a significant difference in BV between the direct LDL-C and all three formulae which was not present when just comparing the three formulae with one another. Within the simvastatin group, there was no significant difference in intraindividual BV between any of the four LDL-C assessments.

The analytical coefficient of variation for 6 months of quality control material (same QC lot) data at levels closest to the mean cholesterol, triglycerides, HDL cholesterol and measured LDL-C cholesterol found in the current study were 1.5%, 1.5%, 1.5% and 2.0% respectively. Modelling with 100,000 examples showed that the analytical CV for Friedewald could be estimated as being 2.84%, for Martin-Hopkins 2.65% and for Sampson NIH 2.66% with measured LDL-C being the native 2.0%.

Discussion

Estimating LDL cholesterol concentrations from other test parameters is perhaps unique amongst routinely reported biochemistry assays because, in contrast to estimated glomerular filtration rate (eGFR) or estimated average glucose (eAG), a direct measurement of the analyte is as readily available as the calculated value. This would not be an issue if the clinical utility of estimated and measured LDL cholesterol proved to be identical. However, this study has shown that while there are obvious differences in the inherent biological variation of directly measured LDL-C in type 2 diabetes patients treated with simvastatin as opposed to atorvastatin, neither the traditional Friedewald nor the more recent Martin-Hopkins and Sampson-NIH LDL-C formulae could make this distinction.

The magnitude of the differences between directly measured LDL-C biological variation and the three formulae whilst taking atorvastatin were not inconsiderable. If the CV is regarded as a measure of the square root of the variance then the Martin-Hopkins, Sampson-NIH and Friedewald LDL-C concentrations were respectively 52 times, 64 times and 72 times more biologically variable than directly measured LDL-C. This contrasts with the non-significant differences between direct and calculated LDL-C variability we found with simvastatin and also with a previous study looking at individuals not taking lipid lowering treatment.[18]

Amongst the three LDL-C formulae, there appeared to be a trend towards LDL-C with the Martin-Hopkins equation being the least biologically variable followed by Sampson-NIH followed by Friedewald being the most variable with either statin (LDL-C CV 9.3% vs. 10.4% vs. 11.0% respectively for atorvastatin, CV 11.3% vs. 11.7% vs. 12.9% for simvastatin) although this did not meet statistical significance. It is of note that while it could have been expected that the more mathematically complex formulae, especially Sampson-NIH, might exhibit greater LDL-C variability than the simpler ones this does not obviously appear to be the case. Thus, for each formula it seems the three components of biological variability (total cholesterol, HDL cholesterol, triglycerides) together contribute to similar imprecision, at least for this group of patients. As regards which of the three test components contributes most to the overall variability, the Cordova LDL-C equation,[19] which only uses total and HDL cholesterol in its calculation, was in contrast found to be able to distinguish between the biological variation of simvastatin and atorvastatin variability.[20]

This study's original hypothesis was that the pharmacokinetics of the short half-life statin simvastatin as opposed to the long half-life atorvastatin might mean that the variability of LDL-C could be greater in patients taking simvastatin.[12] The crossover of type 2 diabetes patients showed this to be the case with directly measured LDL-C for both the study group as a whole and, indeed, for every single patient who participated, even when all samples were collected in the morning fasting state. This pharmacokinetic explanation thus remains the most likely reason for our findings. Of the other commonly prescribed statins, pravastatin and fluvastatin have similar elimination half-lives to simvastatin while rosuvastatin joins atorvastatin in having a longer half-life, being approximately 20 hours.[10]

Beyond biological variation, analytical variation will independently contribute to the imprecision of a reported LDL-C result. With each formula having 3 sources of analytical imprecision it might again be expected that this would favour direct measurement and, with the homogeneous assay used here, would seem to be the case with it having an intra-instrument CV of approximately 2.0% in comparison to the nearly identical CVs of Martin-Hopkins and Sampson-NIH (2.65% and 2.66%). Like for biological variation, the Friedewald formula also trended to be most analytically imprecise (CV 2.84%).

Tracking the LDL-C of the same patient has recently become even more important than previously given the advent of more expensive injectable lipid lowering treatment classes such as the proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and the gene-silencing technologies which inhibit PCSK9 formation.[21] In some health care systems, such as in parts of the UK's National Health Service, continuation with PCSK9 inhibitor or gene-silencing treatments can be contingent on a minimum 30% reduction in LDL-C after 12 weeks of treatment.[22] The requirement, therefore, to be able to reliably track LDL-C changes in the same patient can mean the difference between continuing to receive a treatment or not. While this present study has not investigated the biological variation of LDL-C in patients taking these injectable agents, it is known that their half-life in circulation is far in excess of even atorvastatin, for example being 11-17 days for evolocumab.[23] It is therefore possible that LDL-C in PCSK9 inhibitor treated patients could be as stable as found here with atorvastatin while estimation using a formula remains much more variable.

Being able to reliably assess reduced LDL-C variability may have clinical consequences distinct from treatment eligibility. The lowering of C-reactive protein (CRP) with statin treatment is associated with reduced cardiovascular risk, independently of LDL-C reductions[24] This current study group of patients was previously found to have lower and less variable C-reactive protein (CRP) while taking atorvastatin rather than simvastatin, irrespective of which statin they were taking initially and despite them having similar LDL cholesterols when receiving each treatment.[25] While it cannot be excluded that this was a pleiomorphic effect associated with atorvastatin, it likewise cannot be excluded that this reflected the reduced LDL-C variability that was only apparent when the direct LDL-C assay was used.

This study is not without its limitations. The results were obtained in a group of subjects with type 2 diabetes and so may not be relevant to all patients, or even the cohorts used in establishing the algorithms. This caveat is valid for all calculated quantities which are defined by a regression analysis such as all the different algorithms for eGFR or albumin-corrected calcium concentration.

In addition, only one direct LDL-C assay was used and so our findings may not be applicable to all homogeneous assays. Indeed, differences in the comparability between assays from different manufacturers has likely been a deterrent to their more widespread adoption[26] and it is of note that the direct LDL-C values obtained in this study were uniformly lower than all three estimating formulae and so while dLDL-C variability may have been lower in patients taking atorvastatin, accuracy cannot be assured. Notwithstanding these points, and the fact that more patient participants and more collection periods would always be preferable, the crossover design of the study can give confidence that the observed difference in direct LDL-C variability found between statins is genuine.

In conclusion, while the newer Martin-Hopkins and Sampson NIH estimated LDL-C formulae have been shown to have several potential advantages over the traditional Friedewald formula,[27,28] this study has confirmed that there may be limitations when using any of these calculations to track the progress of patients taking long-acting lipid lowering treatments.

Conflicts of Interest

None declared

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Table 1. Baseline characteristics of the study participants.

	Simvastatin crossed over to atorvastatin group	Atorvastatin crossed over to simvastatin group
Total number of patients completing the study	10	16
Duration of diabetes median (interquartile range) months	84 (108)	108 (108)
Number of patients taking insulin	4	6
HbA1c (mmol/mol) (mean ± SD)	61 ± 11	61 ± 10
Sex (male : female)	7 : 3	10 : 6
Age (years) median (range)	58 (48–76)	64 (46–73)
Body mass index (mean ± SD) kg/m2	34.7 ± 6.6	34.5 ± 7.2
Waist circumference (mean ± SD) cm	120 ± 16.0	115 ± 17.3
Baseline TC (mean ± SD) mmol/L	4.1 ± 0.71	4.0 ± 0.49
Baseline LDL-C (mean ± SD) mmol/L, Friedewald	2.2 ± 0.4	2.2 ± 0.44
Baseline HDL-C (mean ± SD) mmol/L	1.1 ± 0.23	1.1 ± 0.24
Baseline TG (median ± IQR) mmol/L	1.9 ± 0.6	1.7 ± 0.7

Table 2. Intraindividual biological coefficients of variation using LDL established from direct measurement and 3 formulae.

	Atorvastatin		Simvastatin	
LDL Parameters	Mean LDL concentration (mmol/L	CV_I (%) (95% CI)	Mean LDL concentration (mmol/L)	CV_I (%) (95% CI)
Direct LDL	1.65	1.3 (1.1-1.5)	1.70	11.1 (10.2-12.2)
Friedewald LDL	2.25	11.0 (10.0-12.1)	1.98	12.9 (11.8-14.2)
Martin-Hopkins LDL	2.37	9.3 (8.5-10.3)	2.12	11.3 (10.3-12.4)
Sampson-NIH LDL	2.33	10.4 (9.5-11.5)	2.07	11.7 (10.7-12.8)

Figure 1. Mean and range of LDL cholesterol when assessed using direct measurement and 3 formulae in 26 type 2 diabetes subjects taking atorvastatin

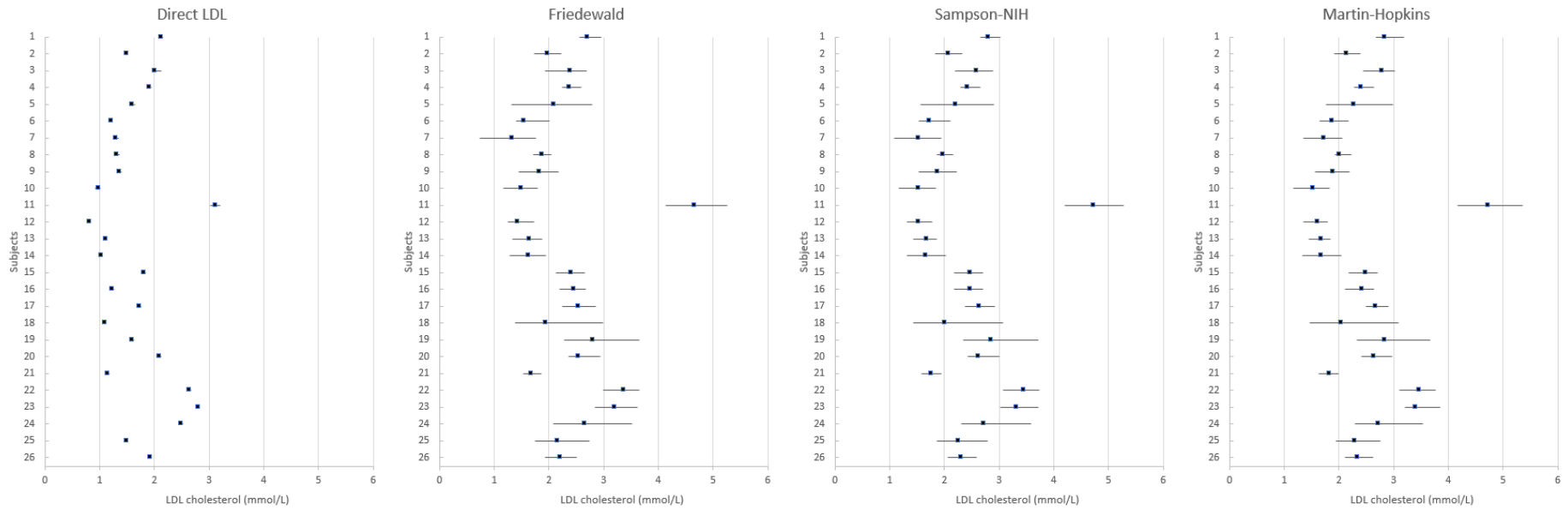

Figure 2. Mean and range of LDL cholesterol when assessed using direct measurement and 3 formulae in 26 type 2 diabetes subjects taking simvastatin