Concordance and Discordance Between Non-High-Density Lipoprotein Cholesterol and Apolipoprotein B as Cardiovascular Disease Risk Markers over the Full Spectrum of Hypertriglyceridemia: A Cross-sectional Analysis of Lipid Clinic Data

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PREFACE

This thesis is in the format of "article-based thesis", which is composed of one published article, and one manuscript for an article. The thesis topic is introduced in the Background chapter. The published article covers the first two objectives, and the manuscript covers the third objective. The Overall Discussion chapter integrates the implications of findings from both articles.

To conduct the studies for the published article, and the manuscript, we had obtained ethics approval from the Ottawa Health Science Network Research Ethics Board, which is affiliated with The Ottawa Hospital Research Institute and the University of Ottawa.

This research topic stemmed from a research project that I conducted under the supervision of Dr. Teik C. Ooi (University of Ottawa), and guidance from Dr. Daniel Gaudet (University of Montreal). The results of this research project were published in the *Journal of the Endocrine Society* in 2019.

Further research hypotheses were developed and were analysed in the first article in this thesis, which was published in the *Journal of Clinical Lipidology* in 2020. I was the first-author, and I took part in all aspects of this publication including the study design, data analysis, manuscript writing, and revisions. I had ongoing guidance from my co-authors Dr. Teik C. Ooi, Dr. Daniel Gaudet, and Dr. Diane Brisson (University of Montreal). The additional analyses performed on this article were supervised by Dr. Nicholas Birkett (University of Ottawa).

For the second manuscript in this thesis, I am the first-author, and took part in all aspects including study design, data analysis, and manuscript writing. I had ongoing guidance from my co-authors Dr. Teik C. Ooi, Dr. Daniel Gaudet, Dr. Diane Brisson, and Dr. Nicholas Birkett.

ABSTRACT

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Lipid biomarkers are frequently used for prediction of cardiovascular disease risk. Triglycerides are routinely checked in blood work, and triglycerides are a key component of lipoproteins that contribute to atherogenic plaques, which cause cardiovascular disease. High triglycerides are a common condition in the general population. The relative effect of high triglycerides on the lipid biomarkers (non-high-density lipoprotein cholesterol, and apolipoprotein B) for cardiovascular disease risk prediction is the focus of this thesis. Using cross-sectional lipid profile data from a large Lipid Clinic, we compared the correlation and concordance between non-high-density lipoprotein cholesterol and apolipoprotein B as cardiovascular disease risk markers among patients with mild, moderate, and severe hypertriglyceridemia. The findings showed that with higher triglycerides, there is lower agreement between the two biomarkers, which raises caution that they are not interchangeable, and further research is needed.

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The Ottawa Hospital Department of Medicine Academic Scholarship program provided generous support during my Masters studies. I am also grateful for ongoing mentorship from Dr. Alexander Sorisky, and ongoing support from my Division Head Dr. Heather Lochnan.
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LIST OF ABBREVIATIONS

apoB = apolipoprotein B

CAD = coronary artery disease

CVD = cardiovascular disease

HDL = high-density lipoprotein

HDLC = high-density lipoprotein cholesterol

HTG = hypertriglyceridemia

LDL = low-density lipoprotein

LDLC = low-density lipoprotein cholesterol

Non-HDLC = non-high-density lipoprotein cholesterol

NTG = normotriglyceridemia

ROC = receiver operator curve

TC = total cholesterol

T2DM = type 2 diabetes mellitus

TG = triglyceride

TRL = triglyceride-rich lipoprotein

VLDL = very-low-density lipoprotein

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Background Chapter

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Chapter 1B Clarifications and Additional Analyses

GENERAL INTRODUCTION TO THESIS LAYOUT

This thesis is a combination of two articles (one published, and one manuscript) exploring the correlation and concordance between two biomarkers of cardiovascular disease risk categorization, for different triglyceride levels.

Triglycerides (TG) are a component of the lipids contained in the blood. The level of triglycerides is measured as part of a lipid profile that is performed from blood drawn from patients. High triglyceride levels, also referred to as hypertriglyceridemia (HTG), is a lipid disorder that can have various pathophysiologic causes. HTG is a very complex condition.

My research focuses on analysing the differences between two lipid biomarkers, non-high-density lipoprotein cholesterol (non-HDLC) and apolipoprotein B (apoB), in patients with HTG. Non-HDLC and apoB are both widely used as lipid biomarkers for cardiovascular disease (CVD) risk categorization for CVD risk prediction.

Physiologically, the interpretation of non-HDLC and apoB is theoretically expected to be dependent on the TG level. The same HTG level can arise from different situations. For example, it could be comprised of many smaller diameter lipid particles (high apoB) that contain less cholesterol mass per particle (relatively low non-HDLC), or be comprised of larger diameter lipid particles (low apoB) that contain much more cholesterol mass per particle (relatively high non-HDLC).

There is a lack of evidence and guidance on the relative effect of HTG on the reliability of non-HDLC versus apoB for CVD risk prediction. We hypothesized that HTG affects the reliability between non-HDLC and apoB for CVD risk prediction, and can cause clinically significant divergence in CVD risk equivalence categorization.

We used cross-sectional lipid profile data from a large Lipid Clinic database.
For the correlation between non-HDLC and apoB at the level of individual lipid profiles, we used linear regression analyses. For the concordance (reliability/agreement) between non-HDLC and apoB to place lipid profiles into the same CVD risk equivalent category, we used the weighted Kappa statistic.

The Background chapter contains the detailed introduction to this topic area. This is followed by Chapter 1, which is the first article that describes the full Lipid Clinic data with non-HDLC and apoB measurements, and also includes some additional explanations and data analyses.

Chapter 2 is the second manuscript, which delves into the relative effect of type 2 diabetes mellitus, and obesity on the correlation and concordance between non-HDLC and apoB in patients with mild and moderate HTG.

The Overall Discussion and Conclusions chapter integrates the findings from the two articles and discusses clinical contextualization, and future directions.

BACKGROUND CHAPTER

EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is disease of the blood vessels in the body. CVD is comprised of medical conditions that are named based on the location of the diseased blood vessels. CVD can occur in the blood vessels that supply the heart (coronary artery disease), the brain (cerebrovascular disease), and the limbs (peripheral vascular disease). CVD occurring in the major arteries can also be referred to as aortic atherosclerosis, and further sub-divided into thoracic aortic atherosclerosis and abdominal aortic atherosclerosis (Joseph *et al*. 2017).

The prevalence of CVD is estimated to be approximately 15% in the USA in 2018 based on data from the US Centre for Disease Control (CDC web site). The incidence of CVD is difficult to estimate because CVD is a combination of coronary artery disease, cerebrovascular disease, peripheral vascular disease, and various forms of aortic atherosclerosis. CVD is the leading cause of death worldwide. The World Health Organization estimated that about 31% of all deaths (17.9 million deaths) in 2016 were due to CVD (WHO web site).

The major non-modifiable risk factors for CVD are increasing age, and family history of premature CVD in a first-degree relative. The pathophysiological changes that lead to CVD will cumulate over time, and hence increasing age increases CVD risk. Family history of premature CVD is defined as having a first-degree female relative who developed CVD before 65 years of age, or a first-degree male relative who developed CVD before 55 years of age. Positive family history of premature CVD in a first-degree relative will double the individual's CVD risk (CCS 2016).

The potentially modifiable risk factors for CVD include lipid abnormalities (also known as dyslipidemia), hypertension, Type 2 diabetes mellitus, obesity, smoking, and physical inactivity (McQueen *et al*. 2008).

Prediction of CVD risk is important for primary prevention. For the clinician, accurate CVD risk prediction allows for the timely investigations and management of the patient. For the patient, CVD risk prediction can be an encouragement for health behaviour modifications that have a positive impact on the modifiable risk factors. In addition, CVD risk prediction is a major determinant in the decision to start lipid-lowering pharmacotherapy. Commonly used risk calculators for CVD include the Framingham risk calculator, and the ASCVD risk calculator (American College of Cardiology web site).

PATHOPHYSIOLOGY OF CARDIOVASCULAR DISEASE

CVD is the leading cause of mortality worldwide. The pathophysiology underlying cardiovascular disease is atherosclerosis, which is the buildup of atherosclerotic plaques in the intima of arteries. An atherosclerotic plaque is made up of cholesterol from lipoproteins that cross the endothelium, which is the innermost layer of cells in the arterial lumen, and enter into the intima. Over time, cholesterol from lipoproteins is deposited in the intima and builds up the atherosclerotic plaque. Substantial atherosclerotic plaques will narrow and could eventually block the arterial blood supply and cause ischemia due to lack of oxygen delivery to the tissues that are fed by the blocked artery (Insull 2009). Rupture of an atherosclerotic plaque leading to blockage of the artery will also cause ischemia. Furthermore, stasis of blood due to arterial

narrowing can form blood clots that fully occluded the narrowed artery, causing thrombotic cardiovascular disease. If the blood clot travels past the narrowed lumen and blocks a downstream artery to cause ischemia, then this is embolic cardiovascular disease (Insull 2009). Instability of the atherosclerotic plaque is related to many possible mechanisms including thinning of the fibrous cap on the plaque, endothelial dysfunction, necrosis and erosion of the plaque, arterial vasospasm, arterial remodelling as a result of microvascular dysfunction, and pro-inflammatory and pro-thrombotic mediators (Boren *et al*. 2020).

Research into CVD pathophysiology has also shown the importance of oxidative stress, which results in a pro-inflammatory and pro-thrombotic state that facilitates atherosclerotic plaque formation, progression, and potential to rupture (Boren *et al*. 2020). Reactive oxygen species cause oxidative stress, which results in oxidative modification of lipoproteins. Oxidized LDL is more atherogenic than non-oxidized LDL. Oxidized LDL also initiates and sustains an inflammatory response in the human body, which involves the cellular immune response system, and the humoral immune response system (Boren *et al*. 2020). Inflammatory system activation increases adhesion molecules, which also leads to a pro-thrombotic state (Boren *et al*. 2020).

LIPOPROTEINS

The crucial contributor to atherosclerosis is the deposition of cholesterol in the endothelial atherosclerotic plaque. In the bloodstream, cholesterol is hydrophobic, and can only circulate in the form of lipoprotein particles. Each lipoprotein particle is composed of a hydrophobic core of cholesterol esters and triglycerides, which are fully surrounded by a hydrophilic membrane containing phospholipids and apolipoproteins (Gardner *et al.* 2018). The gold standard for

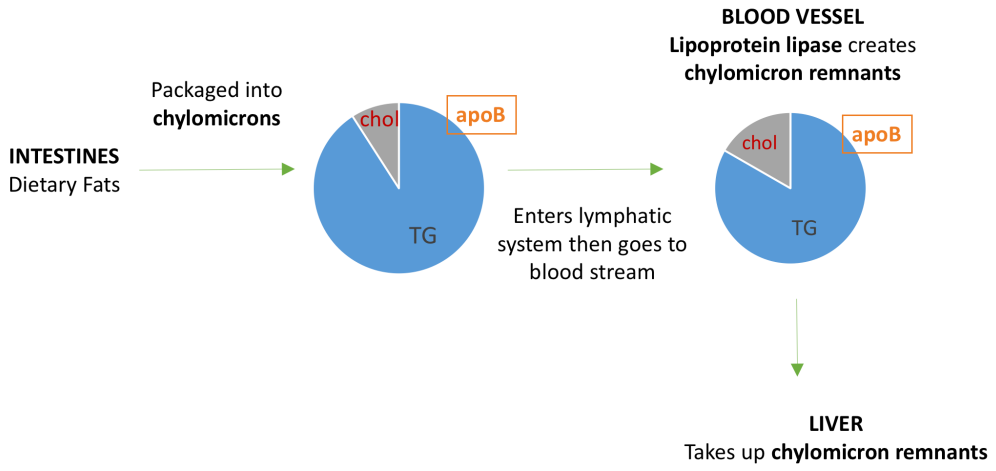
measuring different lipoproteins in the bloodstream traditionally used density-gradient (rate-zonal) ultracentrifugation methodology, which separated lipoproteins along a density gradient. Lipoproteins classes in order of density, from highest to lowest, are the following: high-density lipoproteins (HDL), low-density lipoproteins (LDL), very-low density lipoproteins (VLDL), and chylomicrons (Packard *et al.* 1984; Packard *et al.* 1997; Ooi *et al.* 1998).

Lipoprotein metabolic pathways

There are three lipoprotein metabolism pathways. The exogenous lipoprotein pathway includes chylomicrons and chylomicron remnants. The endogenous lipoprotein pathway includes very-low-density lipoproteins, very-low-density lipoprotein remnants, and low-density lipoproteins. The reverse cholesterol transport pathway includes high-density lipoproteins.

The exogenous lipoprotein pathway

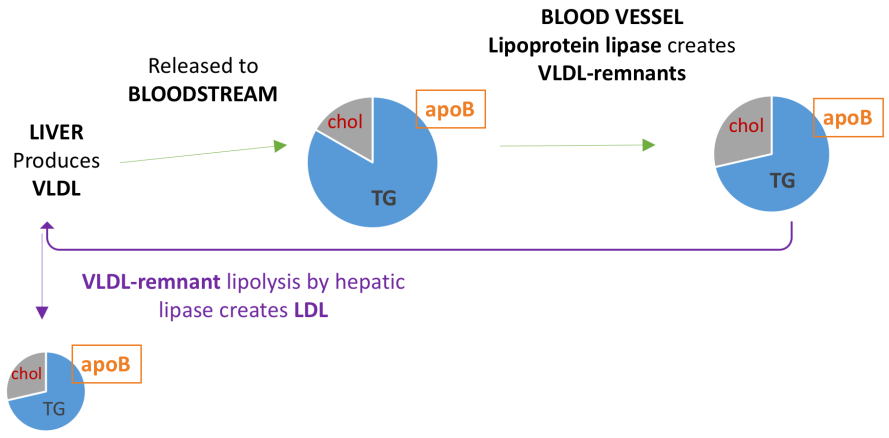
Figure 1 The exogenous lipoprotein pathway



Chylomicrons are part of the "exogenous cholesterol pathway". Dietary fats enter the intestines. All dietary fats are incorporated into lipoproteins called chylomicrons and travel via the lymphatic system before entering the bloodstream (Figure 1). Chylomicrons are the least dense type of lipoproteins because they are the largest in diameter, and, proportionally, contain the highest percentage of triglycerides, and the lowest percentage of cholesterol. Chylomicrons undergo lipolysis by an enzyme called lipoprotein lipase to yield chylomicron remnants, which are smaller in diameter, and have a relatively lower percentage of triglycerides, and a higher percentage of cholesterol. (Packard *et al.* 1984; Goldberg 1996, Ooi *et al.* 1998, Rosenson *et al.* 2014). Chylomicron remnants are transported to the liver as the method of delivery of dietary fats that are then metabolized in the liver (Figure 1).

Endogenous lipoprotein pathway

Figure 2 The endogenous lipoprotein pathway



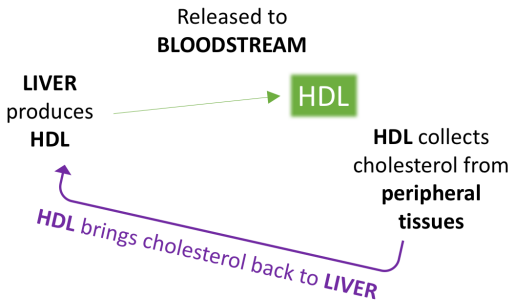
VLDL are produced by the liver (Figure 2). VLDL have high heterogeneity in terms of diameter and relative cholesterol and triglyceride composition. VLDL in the bloodstream will encounter lipoprotein lipase. Lipoprotein lipase performs lipolysis on VLDL whereby the triglycerides are

selectively removed from VLDL. VLDL that have undergone lipolysis will be relatively more cholesterol rich and triglyceride poor, and are referred to as VLDL-remnants, or intermediate-density lipoproteins. VLDL-remnants are smaller in diameter and contain more cholesterol and less triglycerides, when compared with VLDL, which are larger in diameter, and contain less cholesterol and more triglycerides. When compared to HDL or LDL, VLDL and VLDL-remnants contain more triglycerides.

VLDL-remnants can undergo further lipolysis by hepatic lipase, and will be relatively more cholesterol rich and triglyceride poor (Ooi *et al.* 1998), and are referred to as low-density lipoproteins (LDL) (Figure 2). LDL have a smaller diameter, and can more easily cross the arterial endothelial cell layer and enter into the intima. Some biochemical modifications may occur to LDL, which favour LDL and LDL's cholesterol (LDLC) to contribute to the atherosclerotic plaque buildup (Insull 2009). Therefore, LDLC is commonly referred to as "bad cholesterol".

Reverse cholesterol transport pathway

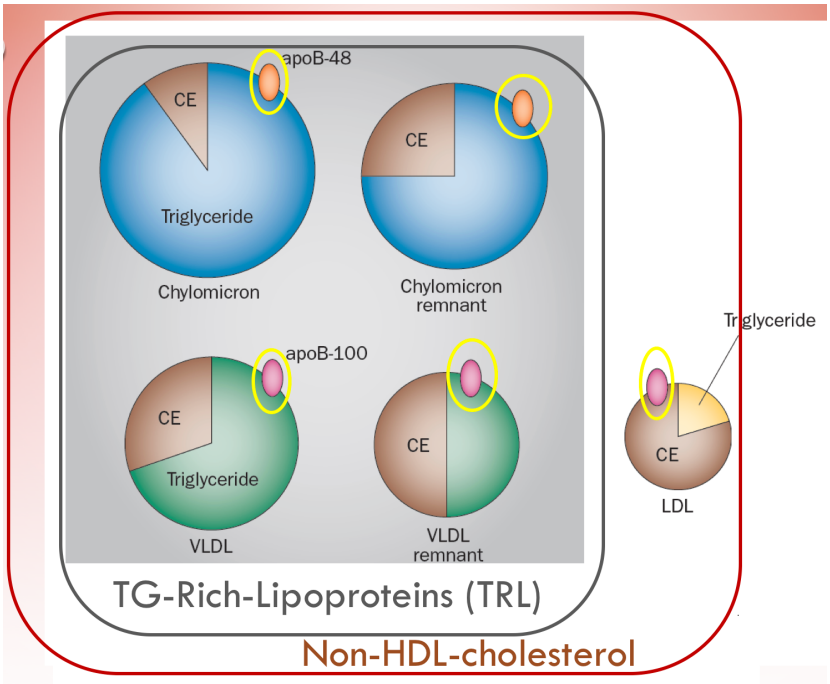
Figure 3 The reverse cholesterol transport pathway



HDL are small in diameter, and are rich in cholesterol, yet contain very little triglycerides. HDL are involved in "reverse cholesterol transport", which is a process that results in bringing excess cholesterol from the peripheral cells back to the liver to be used as a substrate for physiologic processes that are cholesterol dependent (Gardner *et al.* 2018). Since the cholesterol in HDL, called HDLC, does not participate in atherosclerotic plaques, HDLC is therefore commonly referred to as "good cholesterol".

Triglyceride-rich lipoproteins

Figure 4 Triglyceride-rich lipoproteins; non-HDLC



Source: adapted from Watts *et al*. 2013

Triglyceride-rich lipoproteins (TRL) are a collective term that refers to VLDL and VLDL-remnants, and chylomicrons and chylomicron remnants (Figure 4). TRL, due to their larger diameter, are less likely to directly cross the endothelium and enter the intima. However, TRL

can undergo multiple rounds of lipolysis by lipoprotein lipase at the endothelium, and can enter the intima (Ooi *et al.* 1998). Exactly how many rounds of lipolysis are required before TRL can enter the intima has not been studied in humans. Additional theories exist that once TRL enter the intima, because of the larger diameter, TRL are more likely than LDL to remain trapped in the intima (Nordestgaard 2016). Once trapped in the intima, TRL can directly add to atherosclerotic plaque without additional biochemical modifications (Rosenson *et al.* 2014). LDL, being smaller in diameter, are theoretically able to enter and exit the intima.

LABORATORY METHODS TO MEASURE LIPOPROTEINS, LIPOPROTEIN TRIGLYCERIDES, AND LIPOPROTEIN CHOLESTEROL

The gold standard method to measure the concentration of lipoproteins involves density-gradient ultracentrifugation. It is a very expensive and labour-intensive method. As clinical chemistry methodology evolved, biochemical enzymatic methods were developed, which were cheaper, quicker, and able to be upscaled for use in busy clinical laboratories. Biochemical assays have been developed for total cholesterol, HDLC, and triglycerides.

The biochemical assay for total cholesterol quantifies the sum of all cholesterol in lipoproteins. There is a biochemical assay that is selective for HDLC. In addition, there is also a biochemical assay that quantifies the sum of all triglycerides in lipoproteins. Accurate measurement of the other classes of lipoproteins still requires ultracentrifugation.

Friedewald formula for estimated LDLC

As scientific understanding of atherosclerosis developed throughout the twentieth century, and the need to quantify the bad cholesterol, LDLC, without requiring the preparative ultracentrifuge was identified. In 1972, Friedewald and his team developed a formula to estimate LDLC from Total Cholesterol (TC), triglycerides (TG), and HDLC. Their work ignored the contribution to cholesterol from chylomicrons and chylomicron remnants. Furthermore, they lumped the cholesterol from VLDL-remnants into the cholesterol from VLDL.

Their approximate formula for TC: TC = LDLC + HDLC +VLDLC

This provides a method to estimate LDLC:

Estimated LDLC = TC - HDLC - VLDLC

Since TC, TG, and HDLC were already easily measured by biochemistry enzymatic assays, the key component of the Friedewald formula (Friedewald *et al.* 1972) was the development of an approximation for VLDLC. Their research established that VLDLC could be estimated from total triglyceride levels as measured through a biochemistry assay. The average **mass ratio cholesterol to triglycerides** in VLDLs is 1:2.2 in mmol/L units. Therefore, the Friedewald equation is:

Estimated LDLC = TG - HDLC – TG/2.2 [mmol/L]

Friedewald found that the correlation coefficient (r) for the correlation between the scatterplot of estimated LDLC and the ultracentrifuge LDLC was improved (from r=0.85 to r= 0.94 to 0.99) when the data from patients with TG 4.5 mmol/L and above were removed. Consequently, in the landmark 1972 Clinical Chemistry publication, Friedewald published his formula for estimated LDLC, with the limitation that the formula should not be used in patients with TG 4.5 mmol/L

and above. Lipoprotein composition studies (Shen *et al.* 1977, Hatch *et al.* 1968) have shown that "normal" VLDL have the average mass ratio of cholesterol to triglyceride of 1:2.2 mmol/L. However, for the larger VLDL that have a higher percentage of triglycerides, the average mass ratio of cholesterol to triglyceride is smaller than 1:2.2 mmol/L. For example, larger VLDL can have mass ratio of cholesterol to triglyceride of 1:4.4 mmol/L. Chylomicrons and chylomicron remnants can have mass ratio of cholesterol to triglyceride of 1:8.8 mmol/L. Therefore, the Friedewald equation for estimated LDLC does not work well when TG is 4.5 mmol/L and above because the VLDLC is no longer well-estimated with the mass ratio of cholesterol to triglyceride of 1:2.2 mmol/L.

Widespread use of Friedewald formula for LDLC

The simplicity and convenience of the Friedewald formula for estimated LDLC has encouraged its use ever since its publication in 1972. Notably, Friedewald's estimated LDLC served as the outcome measurement in the large clinical trials for lipid-lowering medications, and also in large epidemiological trials assessing prevalence and incidence of cardiovascular disease (Clinical Treatment Trialists' Collaboration 2015).

However, the limitations of the Friedewald formula has led to exclusion of subjects from major studies. Most particularly, subjects with triglycerides 4.5 mmol/L and above are commonly excluded from studies. The large clinical trials for lipid-lowering medications (statins being the most common and most studied class of these medications), excluded patients with TG 4.5 mmol/L and above because Friedewald's formula for estimated LDLC could not be used. For the same reason, patients with TG 4.5 mmol/L and above were also often excluded from

epidemiology studies for incidence and prevalence of cardiovascular disease (Sniderman *et al.* 2011; The Emerging Risk Factors Collaboration 2012; Clinical Treatment Trialists' Collaboration 2015). This commonly occurring exclusion of patients with higher triglyceride levels is a selection bias, which limits the generalizability of these studies' results to patients with triglycerides levels 4.5 mmol/L and above. Therefore, the results from these studies are not to be extrapolated to patients with higher triglyceride levels.

USING LIPID BIOMARKERS TO PREDICT CARDIOVASCULAR DISEASE RISK

Prospective observational studies established total cholesterol's association with increased CVD risk. In 1988, the US National Cholesterol Education Program developed the first guideline that focused on LDLC reduction (Goodman *et al.* 1988). Clinical trials have found that the statins class of medications are highly effective in decreasing LDLC, and meta-analyses have shown that a 1 mmol/L decrease in LDLC is associated with 21% reduction in CVD relative risk (Cholesterol Treatment Trialists' Collaboration 2015). "Discordance analyses of prospective observational studies, which included up to mild HTG, showed that in cases of discordance between apoB and non-HDLC, apoB predicted CVD risk better than non-HDLC (32-34, 59). However, the Emerging Risk Factors Collaboration's analysis of prospective studies (60), and the UK Biobank study (30) did not support superiority of apoB over non-HDLC in CVD risk prediction" (direct quotes from Article 1 – Sun *et al*. 2020) in patients with normal triglyceride levels, and up to mild hypertriglyceridemia.

Complexity of hypertriglyceridemia

The standard laboratory method of triglyceride concentration measurement measures the combination of all triglycerides in lipoproteins. The normal triglyceride level, here on referred to as normotriglyceridemia, is usually defined as TG less than 1.7 mmol/L (Ford *et al.* 2009).

Hypertriglyceridemia (HTG) is defined as TG above 1.7 mmol/L; about 33% of the US general population has HTG, with about 18% of the US general population having TG above 2.3 mmol/L. Mild HTG is defined as triglyceride level between 1.7 mmol/L and 4.5 mmol/L. Moderate HTG is generally accepted as TG > ~ 4.5 mmol/L (Anderson *et al.* 2016; Grundy *et al.* 2019). NHANES data showed that TG above 5.7 mmol/L is present in about 2% of the US general population (Ford *et al.* 2009). Severe HTG is generally accepted as TG above 10 mmol/L and represents about 0.1% of the general population (Benn *et al.* 2007)

The limitations of the Friedewald formula mask the potential importance of HTG. HTG is a highly complex and heterogeneous state because the same value of TG can be obtained from a very different composition of lipoproteins. For example, the same TG might be derived mainly from LDL; this would produce high atherogenicity because there is a high abundance of LDLC contributing to atherosclerotic plaque buildup and proliferation. However, if the same value of TG is derived mainly from larger VLDL and chylomicrons, which are relatively less atherogenic, then it would be expected that this individual would have a lower CVD risk than the first individual.

Concept and widespread use of non-HDLC

In 1987, a new concept called "non-high-density cholesterol" (non-HDLC) was introduced (Frick *et al.* 1987). Conceptually, non-HDLC combines cholesterol from two sources: LDLC, and TRLC (cholesterol from VLDL and VLDL remnants, and chylomicrons and chylomicron remnants). Therefore, non-HDLC is the sum of cholesterol from all atherogenic lipoproteins, including the most atherogenic LDL and small/normal VLDL, and the larger and likely less atherogenic larger VLDL, and the least atherogenic chylomicrons.

Non-HDLC is calculated from two lipid profile components that are routinely measured via biochemical assays in clinical laboratories, and does not require additional laboratory testing. Non-HDLC is defined as 'Total cholesterol – HDLC'.

Non-HDLC was first used in the Helsinki Heart Study in 1987 (Frick *et al.* 1987) because this trial recruited patients with mild and moderate HTG and therefore LDLC estimated from the Friedewald equation could not be used in all these study participants. In the major clinical guidelines published in 2002 by the US National Cholesterol Education Program Adult Treatment Panel III in 2002, non-HDLC was recommended as a lipid biomarker for CVD risk prediction in patients with HTG. In Canada, non-HDLC became a prominent feature of the "2012 Canadian Cardiovascular Society Guidelines for the Diagnosis and Treatment of Dyslipidemia for the Prevention of Cardiovascular Disease in the Adult". Non-HDLC has been a co-primary target for treatment of dyslipidemia in many major clinical guidelines (IAS 2014, NLA 2015, CCS 2016, EAS 2016, AACE 2017), and is often mentioned as the preferred biomarker for CVD risk stratification in patients with HTG, without reference to a TG upper limit (IAS 2014, NLA 2015, CCS 2016, EAS 2016, AACE 2017, AHA 2018).

Concerns about non-HDLC in HTG

Two concerns regarding non-HDLC and its clinical use need to be discussed. The first concern is that major studies of non-HDLC for CVD risk prediction included mainly patients with mild HTG, and either included only a small proportion of participants with moderate or severe HTG (TG 4.5 mmol/L and above), or excluded them completely.

The second concern relates to the change in the mass ratio of cholesterol to triglycerides in VLDLs, as TG increases, which remain with calculated non-HDLC. The derivation of this concern is the following:

Friedewald estimated LDLC = TC – HDLC – **VLDLC**

TC - HDLC = LDLC + **VLDLC**

Non-HDLC = LDLC + **VLDLC**

Non-HDLC = LDLC + **TG/2.2** (in mmol/L)

VLDLC (bolded in the equations above) is an inherent component of non-HDLC. But, VLDLC is estimated in the Friedewald equation based on the mass ratio of cholesterol to triglycerides. For individuals with HTG, the mass ratio is different from the mean value used in the Friedewald equation. This may adversely influence the ability of non-HDLC to selectively represent cholesterol from atherogenic lipoproteins.

Calculated non-HDLC includes cholesterol in larger TRL in HTG (Sun et al. 2019)

Initial work that I have conducted with Dr. Ooi and other colleagues has shown that as TG increases, non-HDLC is less able to selectively represent cholesterol from very atherogenic

lipoproteins. We analyzed cross-sectional lipid profile data from a Lipid Clinic, and a tertiary hospital Biochemistry Laboratory.

Our modelling process included derivation of a lipoprotein composition factor, which is the **median mass ratio of cholesterol to triglyceride** in TRLs. A high lipoprotein composition factor reflects that the median TRLs are mainly the cholesterol-rich and highly atherogenic, including normal VLDLs and their remnant lipoproteins. A low lipoprotein composition factor reflects that the median TRLs are mainly triglyceride-rich, larger and likely less atherogenic, including larger VLDL and chylomicrons, and their respective remnant lipoproteins. We categorized triglycerides into 1 mmol/L groups, ranging from 0.01 mmol/L to 10 mmol/L. Our results showed that there was a steady decline in the lipoprotein composition factor as TG increased. In both sets of data, we showed that non-HDLC included progressively more cholesterol from larger and likely relatively less atherogenic TRLs and chylomicrons at higher triglyceride levels (Sun CJ *et al*. 2019). To explore this further, our next step was to integrate a biomarker for the number of lipoproteins.

Apolipoprotein B – lipid biomarker representative of non-HDL particle number

Lipoproteins also require apolipoproteins, which support lipoproteins' structure and function. Each non-high density lipoprotein contains a single apolipoprotein B (apoB) molecule: each chylomicron and chylomicron remnant contains one molecule of apoB48, and each very-low density lipoprotein and low-density lipoprotein molecule contains one molecule of apoB100. Laboratory assays for apoB detect the total of both apoB48 and apoB100. Whereas non-HDLC is the sum of the cholesterol content of all atherogenic lipoproteins, ranging from the most

atherogenic to the less atherogenic, apoB is the sum of the number of non-HDL particles (Sniderman *et al.* 2018). Therefore, apoB is another lipid biomarker for CVD risk prediction, which is based on the particle number of atherogenic lipoproteins (Figure 4).

In HTG, there is TG-enrichment of TRLs, which makes them larger TRLs. There is a significant amount of cholesterol in larger TRLs that increases the non-HDLC values at higher triglyceride levels (Sun *et al.* 2019). For example, a VLDL and a chylomicron particle can contain up to 10-15 times, and 21-25 times, more cholesterol than an LDL particle, respectively. Therefore, in HTG, non-HDLC could increase disproportionately with respect to apoB (Hatch *et al.* 1968, Shen *et al.* 1977, Sun *et al.* 2019).

IN HTG, WHAT IS THE BEST LIPID BIOMARKER FOR PREDICTION OF CVD?

The literature has not addressed the question of what the best lipid biomarker for prediction of CVD risk is in patients with HTG. Guideline recommendations for patients with TG 4.5 mmol/L and above are commonly extrapolated from data based on patients with TG below 4.5 mmol/L (Sniderman *et al.* 2018). This extrapolation can potentially be misleading, which would influence patient management and consequent cardiovascular outcomes.

Our previous work (Sun *et al.* 2019) has shown that the composition of non-HDLC changes as the severity of HTG increases, incorporating cholesterol from TRL which are larger and likely less atherogenic. This empirical result was theoretically expected based on known lipoprotein physiology. When non-HDLC includes substantial cholesterol from less atherogenic lipoproteins, it decreases the utility of non-HDLC to represent highly atherogenic lipoproteins.

However, in HTG, the 1:1 ratio of a single apoB per atherogenic lipoprotein particle remains the same. There is a need for more research to explore the impact on apoB at higher levels of TG, and to examine the correlation between non-HDLC and apoB over the full spectrum of mild, moderate, and severe HTG. Our previous work suggests a decreasing correlation and an increasing discordance between non-HDLC and apoB, as TG increases.

IN HTG, HOW DO TYPE 2 DIABETES MELLITUS AND OBESITY AFFECT THE CORRELATION BETWEEN NON-HDLC AND APOB?

Pre-existing medical conditions can affect TG and potentially alter the relationship between non-HDLC and apoB. Type 2 diabetes mellitus (T2DM) and obesity are two medical conditions that are associated with HTG, causing increased TG via increased production of TRL. T2DM also causes decreased clearance of TRL. HTG in T2DM and obesity is related to increased hepatic production of VLDL, and particularly larger VLDL (Goldberg 2001, Feingold 2020). In T2DM, TRL clearance is also reduced due to saturation of clearance mechanisms (Goldberg 2001, Feingold 2020). These mechanisms would theoretically lead to patients with T2DM to have relatively higher non-HDLC and apoB than patients without T2DM, and patients with obesity to have relatively higher non-HDLC and apoB than patients without obesity. Both stronger correlation and stronger levels of agreement between non-HDLC and apoB are plausible hypotheses to explore. T2DM and obesity, when considered as effect modifiers in the association between non-HDLC and apoB, can be studied via stratification into subgroup with T2DM versus subgroup without T2DM, and subgroup with obesity versus subgroup without obesity. Therefore, the effect of T2DM or obesity on the association between non-HDLC and apoB, in HTG, needs to be explored as subgroup analyses. The definition for T2DM and for obesity are included in the

description of the Lipid Clinic database (please refer to the Methods section of Article 1 in this thesis).

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THESIS OBJECTIVES AND HYPOTHESES

Overall objective

To compare the correlation and concordance between non-HDLC and apoB as CVD risk markers among patients with mild, moderate, and severe HTG.

Objective 1

Using cross-sectional lipid profile data from a large Lipid Clinic, we will assess the correlation between individual lipid profiles' non-HDLC and apoB, in TG-intervals of 1 mmol/L.

Objective 2

Using cross-sectional lipid profile data from a large Lipid Clinic, we will assess the concordance (level of agreement) between non-HDLC and apoB to categorize into the same CVD risk equivalent category, as TG levels elevate.

Objective 3

As TG levels elevate, we will analyse the level of concordance between non-HDLC and apoB for CVD risk equivalence categorization in subgroups with T2DM vs without T2DM, and with obesity vs without obesity.

Hypothesis 1

We hypothesize that subjects with higher levels of TG will have a decreased correlation between non-HDLC and apoB.

Hypothesis 2

We hypothesize that subjects with higher levels of TG will have a lower level of concordance between non-HDLC and apoB for CVD risk equivalent categorization.

Hypothesis 3

We hypothesize that, in subjects with higher levels of TG severity, subgroup with Type 2 diabetes mellitus (T2DM), and subgroup with obesity will have a lower level of concordance between non-HDLC and apoB for CVD risk equivalent categorization, when compared with subgroup without T2DM, and subgroup without obesity.

Objectives 1 and 2 are covered in published Article 1.

Objective 3 is covered in manuscript for Article 2.

CHAPTER 1A: ARTICLE 1

Preface to Article 1

Article 1 addresses Objectives 1 and 2.

Contributions of co-authors:

"Contribution Statement: CS and TCO designed the study. DB and DG acquired the data for the study. CS and TCO analyzed the data and drafted the article. CS, DB, DG, and TCO interpreted the data, and revised the article. All authors approved the final article."

*CS = Cathy Sun

Ethics approvals secured: "We received institutional Research Ethics Board (REB) approval (OHSN-REB Protocol ID 20180461-01H)"

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The published version is presented here.
Clarifications, supplementary material, and additional analyses are included after this article.

Relative Effect of Hypertriglyceridemia on Non-HDLC and Apolipoprotein B as Cardiovascular Disease Risk Markers

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ABSTRACT

Background: Non-high density lipoprotein cholesterol (non-HDLC) represents the cholesterol in triglyceride rich lipoproteins (TRL) and low-density lipoproteins (LDL). Apolipoprotein B (apoB) reflects the number of TRL and LDL particles. In hypertriglyceridemia (HTG), there is triglyceride (TG) enrichment of TRLs, and also a substantial increase of cholesterol in larger TRLs that considerably augments the non-HDLC value. Therefore, in HTG, non-HDLC could increase disproportionately with respect to apoB.

Objective: We aimed to compare the relative effect of the full range of mild, moderate, and severe HTG on the status of non-HDLC and apoB as cardiovascular disease (CVD) risk markers.

Methods: Analysis of lipid profile data from 4,347 patients in a Lipid Clinic cohort with baseline fasting lipid profiles documented prior to starting lipid-lowering medications. The correlation between non-HDLC and apoB was assessed in intervals of increasing TG. Non-HDLC and apoB were analyzed at each TG level using comparative CVD risk equivalent categories, and assessed for divergence and discordance.

Results: With increasing TG levels: (1) the correlation between non-HDLC and apoB diminished progressively, (2) non-HDLC levels increased continuously, whereas apoB levels plateaued after an initial increase up to TG of ~ 4.0-5.0 mmol/L (~354-443 mg/dL), (3) there was divergence in stratification of non-HDLC and apoB into CVD risk equivalent categories.

Conclusions: Non-HDLC and apoB should not be viewed as interchangeable CVD risk markers in the presence of severe HTG. This has never been tested. With increasing HTG severity, discordance between non-HDLC and apoB can cause clinically important divergence in CVD risk categorization.

INTRODUCTION

Calculated non-HDL-cholesterol (non-HDLC), which is total cholesterol minus HDLC, is widely recommended as a better cardiovascular disease (CVD) risk marker than low-density lipoprotein cholesterol (LDLC) calculated by Friedewald equation because non-HDLC represents cholesterol in triglyceride-rich lipoproteins (TRL) on top of low-density lipoproteins (LDL), both being atherogenic (1-8). There are newer and better methods to calculate LDLC (9-11); however, they were not used in the major studies that had established LDLC as a CVD risk marker. Furthermore, calculations for LDLC have limited ability to reflect changes in lipoprotein composition related to CETP-mediated lipid exchange in hypertriglyceridemia (HTG). Apolipoprotein B (apoB) is a well-known alternative to non-HDLC, and both are often recommended as almost interchangeable targets for lipid-lowering therapy (3, 12-13). As shown in large population cohort studies, mostly with triglycerides (TG) < 4.5 mmol/L (399 mg/dL), both parameters do not change significantly in the non-fasting state (14-16). Both parameters represent all atherogenic apoB-containing lipoproteins, including TRL, LDL, and lipoprotein (a), but serum apoB reflects the number of these particles while serum non-HDLC encompasses the total cholesterol in them. Lipoprotein compositional changes would therefore alter the CVD risk information derived from these two risk markers.

Studies comparing non-HDLC with apoB as CVD risk markers show that they are highly correlated with each other when there is normal cholesterol content in apoB particles (17-18). As the cholesterol content in apoB particles increases, it can be expected that these two markers would diverge in their prediction of CVD risk. Different strategies have been employed to look

at this issue (7-8; 17-35). Yet, in all these studies, the analyses have not been done specifically as a function of serum TG level.

In normotriglyceridemia (NTG), TRL in blood are mostly normal very-low-density lipoproteins (VLDL). In mild HTG (TG <4.5 mmol/L (399 mg/dL)), TRL in blood consist of VLDL and remnant lipoproteins. It is important to note that on a per mmol/L of cholesterol basis, a lesser reduction in remnant cholesterol than LDL cholesterol is required to achieve the same reduction in a major adverse cardiovascular event, attesting to the greater CVD risk associated with remnants than LDL (36). In moderate HTG (TG 4.5-10.0 mmol/L (399 -886 mg/dL)) and severe hypertriglyceridemia (TG > 10.0 mmol/L (886 mg/dL)), TRL consist more and more of parent VLDL and chylomicrons and their larger remnants (37-39). Despite the varying pools of TRL associated with different degrees of HTG, non-HDLC is often the recommended risk marker regardless of the degree of HTG (1-3, 12-13, 40). In fact, there is preference for use of non-HDLC over LDLC because of the view that LDLC is influenced by TG while non-HDLC is not (1-4, 12-13, 40). There is a significant amount of cholesterol in TRL that augments the non-HDLC value considerably (41). For example, a VLDL and a chylomicron particle can contain up to 10-15 times, and 21-25 times more cholesterol than an LDL particle, respectively (42-43). The atherogenic potential of cholesterol associated with remnants is well documented (44-49) and may even exceed that of LDL (36). However, the relative atherogenic potentials of larger and smaller remnants remain uncertain.

A comparison of the relative effect of the full range of mild, moderate, and severe HTG on the status of non-HDLC and apoB as CVD risk markers has not been studied before. We hypothesized that with increasing HTG, there would be divergence of CVD risk severity categorization indicated by non-HDLC and apoB.

METHODS

Lipid Clinic patient cohort

We received institutional Research Ethics Board (REB) approval (OHSN-REB Protocol ID 20180461-01H) to analyse lipid-profile data from 7,492 patients who were referred to the Chicoutimi Hospital Lipid Clinic from 1990 to 2017. We have previously published on this cohort (41). The Chicoutimi Hospital Lipid Clinic serves patients in the Saguenay-Lac St-Jean region that due to the founder effect, has a higher prevalence of familial hypercholesterolemia (FH) and familial chylomicronemia syndrome (FCS) compared with the general population. This study is based on a subset of 4,347 patients from this cohort who had apoB data on top of standard lipid profile data. Blood samples were taken fasting, and prior to starting on any lipid-lowering medications (including statins, fibrates, cholesterol absorption inhibitors, bile acid sequestrants). All lipid profiles were performed in an accredited clinical laboratory affiliated with the clinic. Total cholesterol, HDLC, and TG were measured by enzymatic assays, and apoB was measured by nephelometry. Although the lab methods have remained very similar over 27 years, the machines and reagents have changed over time. However, coefficients of variation remained between 3 to 5% for intrabatch and interbatch variability.

At the initial visit to the Lipid Clinic, data were collected for baseline coronary artery disease (CAD), type 2 diabetes mellitus (T2DM), and hypertension (HTN). CAD was ascertained on the basis of: (a) clinical and ECG criteria of myocardial infarction according to the consensus document of the joint European Society of Cardiology and the American College of Cardiology committee (50), or, (b) evidence of coronary stenosis of at least 50% in >1 main coronary artery on coronary angiography for the investigation of ischemic heart disease. T2DM

was defined according to the World Health Organization criteria (51) as a 2-hour glucose concentration ≥11.1mmol/L (200 mg/dL) following a 75 g oral glucose load. Hypertension was documented if the average of 2 or more measurements of blood pressure, on at least 2 subsequent visits, was over 140 mmHg for systolic blood pressure or over 90 mm Hg for diastolic blood pressure, or patient was already treated with blood pressure lowering medication.

Correlation between calculated non-HDLC and apoB with increasing TG

We have analyzed the association between calculated non-HDLC and apoB in TG-intervals of 1 mmol/L (89 mg/dL) up to 7 mmol/L (620 mg/dL), and 7- 9 mmol/L (620-797 mg/dL) lumped together to get adequate sample size (Figure 1). Sensitivity analysis was performed where patients with FH and FCS were excluded from the analysis.

For each TG-interval subplot (in Figure 1), we determined the percentile for non-HDLC of 5.7 mmol/L (220 mg/dL), and apoB of 1.4 g/L (140 mg/dL). We compared the trend of percentile change for non-HDLC of 5.7 mmol/L (220 mg/dL) versus apoB, as the TG increased. As TG increased, if the percentile for non-HDLC of 5.7 mmol/L (220 mg/dL) decreased, this indicates that there is an increase in the percentage of lipid profiles with non-HDLC above 5.7 mmol/L (220 mg/dL). Similarly, as TG increased, if the percentile for apoB of 1.4 g/L (140 mg/dL) decreased, this indicates that there is an increase in the percentage of lipid profiles with apoB above 1.4 g/L (140 mg/dL).

Impact of HTG on non-HDLC versus apoB levels for CVD risk equivalent categories

We have determined the association between non-HDLC and TG, and between apoB and TG individually for the full spectrum of mild, moderate, and severe HTG and compared their

trajectories (Figure 2, and Figure 3). Sensitivity analysis was performed where patients with familial hypercholesterolemia (FH), and familial chylomicronemia syndrome (FCS) were excluded from the analysis.

To compare the risk predictions of non-HDLC and apoB at each TG level, their plots were placed against a background of comparative CVD risk equivalent categories. Our CVD risk equivalent categories were based on major Canadian and American guidelines, which recommend a LDLC $\geq$ 4.9 mmol/L (189 mg/dL) (52) or $\geq$ 5.0 mmol/L (193 mg/dL) (12) as high risk. Since the corresponding non-HDLC level is usually around 0.6 to 0.8 mmol/L (23 to 30 mg/dL) above LDLC levels (53), we have used a non-HDLC $\geq$ 5.7 mmol/L (220 mg/dL) to represent high risk. A non-HDLC of 5.7 mmol/L (220 mg/dL) in data from large epidemiological studies has been shown to approximately correspond to an apoB of 1.4 g/L (140 mg/dL) (25, 30-31, 54-56). Low CVD risk equivalent category of non-HDLC <2.6 mmol/L (100 mg/dL) and apoB <0.8 g/L (80 mg/dL) were chosen because they are the treatment co-targets for patients on lipid-lowering therapy (3, 12-13). Intermediate CVD risk equivalent category is the region between non-HDLC 2.6 mmol/L (100 mg/dL) and non-HDLC 5.7 mmol/L (220 mg/dL), and between apoB 0.8 g/L (80 mg/dL) and apoB 1.4 g/L (140 mg/dL).

Subgroup analyses on relationship between mean non-HDLC and mean apoB in mild to moderate HTG

We also performed the dual axes plot of mean non-HDLC vs TG, and mean apoB vs TG in subgroups of patients (Figure 4) based on presence or absence of T2DM, baseline CAD, hypertension, and according to sex.

Discordance between non-HDLC and apoB for high CVD risk stratified by TG level

For each TG interval, we determined the concordance/discordance for high CVD risk categorization at the level of each individual lipid profile. We assessed for discordance where non-HDLC was in the high CVD risk category but apoB was not, and vice versa. For each TG strata, Figure 5 shows the percentage of each for the four categories: discordance non-HDLC $\geq$ 5.7 mmol/L (220 mg/dL) and apoB <1.4 g/L (140 mg/dL); discordance non-HDLC <5.7 mmol/L (220 mg/dL) and apoB $\geq$ 1.4 g/L (140 mg/dL); concordance non-HDLC $\geq$ 5.7 mmol/L (220 mg/dL) and apoB $\geq$ 1.4 g/L (140 mg/dL); concordance non-HDLC <5.7 mmol/L (220 mg/dL) and apoB <1.4 g/L (140 mg/dL).

Using the same method as above, we also assessed for concordance and discordance using median non-HDLC 5.3 mmol/L (205 mg/dL) and median apoB 1.15 g/L (115 mg/dL) as the cut-off values (Figure 6).

Statistical analyses

For the association between apoB vs non-HDLC (Figure 1), non-HDLC vs TG (Figure 2 panel A), and apoB vs TG (Figure 2 panel B), we performed linear regression analysis to determine the coefficient of determination (R^2) for each model. For Figure 2, the mean and median non-HDLC, and mean and median apoB for each TG-interval were calculated and plotted. All statistical analyses were performed using SAS version 9.4 and Microsoft Excel.

RESULTS

Lipid Clinic cohort

In Table 1, we show the baseline characteristics of the patients, and that the subgroup that had apoB measured is generally representative of the Lipid Clinic full cohort.

Table 1: Baseline characteristics of the Full Lipid Clinic cohort; Study participants (subgroup with apolipoprotein (apoB)); subgroup without apoB

	Study Participants (Subgroup with apoB)	**Subgroup without apoB**	**Full Lipid Clinic Cohort**
Number of patients	4347	2871	7218
Age (years, mean [SD])	49 [12.5]	51 [14.6]	49 [13.4]
Female (N, %)	1999, 45.99	1334, 46.46	3333, 46.20
Male (N, %)	2348, 54.01	1537, 53.54	3885, 53.80
With Type 2 Diabetes mellitus (N, %)	631, 14.52	301, 10.48	932, 12.93
With Coronary Artery Disease (N, %)	1159, 26.66	912, 31.77	2071, 28.69
With Hypertension (N, %)	1651, 37.98	1015, 35.35	2666, 36.94
With Familial Hypercholesterolemia (N, %)	1021, 23.49	531, 18.5	1552, 21.5
With Familial Chylomicronemia Syndrome (N, %)	28, 0.64	12, 0.42	40, 0.55
Non-HDLC			
Mean [SD] in mmol/L; mg/dL	5.68 [2.46]; 219.65 [95.13]	5.35 [1.94]; 206.88 [75.02]	5.55 [2.27]; 214.62 [87.78]
10th %ile in mmol/L; mg/dL	3.26; 126.06	3.40; 131.48	3.30; 127.61
25th %ile in mmol/L; mg/dL	4.10; 158.55	4.11; 158.93	4.10; 158.55
Median in mmol/L; mg/dL	5.30; 204.95	5.10; 197.22	5.20; 201.08
75th %ile in mmol/L; mg/dL	6.70; 259.09	6.21; 240.14	6.50; 251.36
90th %ile in mmol/L; mg/dL	8.40; 324.83	7.30; 282.29	7.93; 306.65
apoB			
Mean [SD] in g/L; mg/dL	1.17 [0.35]; 117 [35]		1.17 [0.35]; 117 [35]
10th %ile in g/L; mg/dL	0.77; 77		0.77; 77
25th %ile in g/L; mg/dL	0.94; 94		0.94; 94
Median in g/L; mg/dL	1.15; 115		1.15; 115
75th %ile in g/L; mg/dL	1.37; 137		1.37; 137
90th %ile in g/L; mg/dL	1.59; 159		1.59; 159

Triglycerides			
Mean [SD] in mmol/L; mg/dL	3.50; 309.99	2.94; 260.40	3.28; 290.51
10th %ile in mmol/L; mg/dL	0.89; 78.83	0.90; 78.83	0.90; 78.83
25th %ile in mmol/L; mg/dL	1.20; 106.28	1.20; 106.28	1.20; 106.28
Median in mmol/L; mg/dL	1.90; 168.28	1.80; 159.43	1.90; 168.28
75th %ile in mmol/L; mg/dL	3.30; 292.28	2.80; 247.99	3.10; 274.57
90th %ile in mmol/L; mg/dL	6.40; 566.85	5.00; 442.85	5.80; 513.71

Correlation between calculated non-HDLC and apoB with increasing TG

In Figure 1, we show the association between calculated non-HDLC and apoB at each TG-interval of 1 mmol/L. As TG increase, there was a progressive decrease in the coefficient of determination (R^2) for the relationship between non-HDLC and apoB, with the R^2 value dropping progressively from 0.64 for TG 0.01-1.0 mmol/L (0.9 to 89 mg/dL) to 0.06 for TG 7.0-9.0 mmol/L (620-797 mg/dL). Sensitivity analysis when patients with FH and FCS were excluded (figure not shown) also showed a progressive decrease in R^2 value from 0.48 for TG 0.01-1.0 mmol/L (0.9 to 89 mg/dL) to 0.02 for TG 7.0-9.0 mmol/L (620-797 mg/dL).

Using these plots, we determined that the percentile for non-HDLC level of 5.7 mmol/L (220 mg/dL) decreased more rapidly with increasing TG (from 79 %ile for TG of 0.01-1.0 mmol/L (0.9 to 89 mg/dL) to 18 %ile for TG of 7.0-9.0 mmol/L (620-797 mg/dL)) than the percentile for apoB level of 1.4 g/L (140 mg/dL) (from 90 %ile for TG of 0.01-1.0 mmol/L (0.9 to 89 mg/dL) to 61 %ile for TG of 7.0-9.0 mmol/L (620-797 mg/dL)).

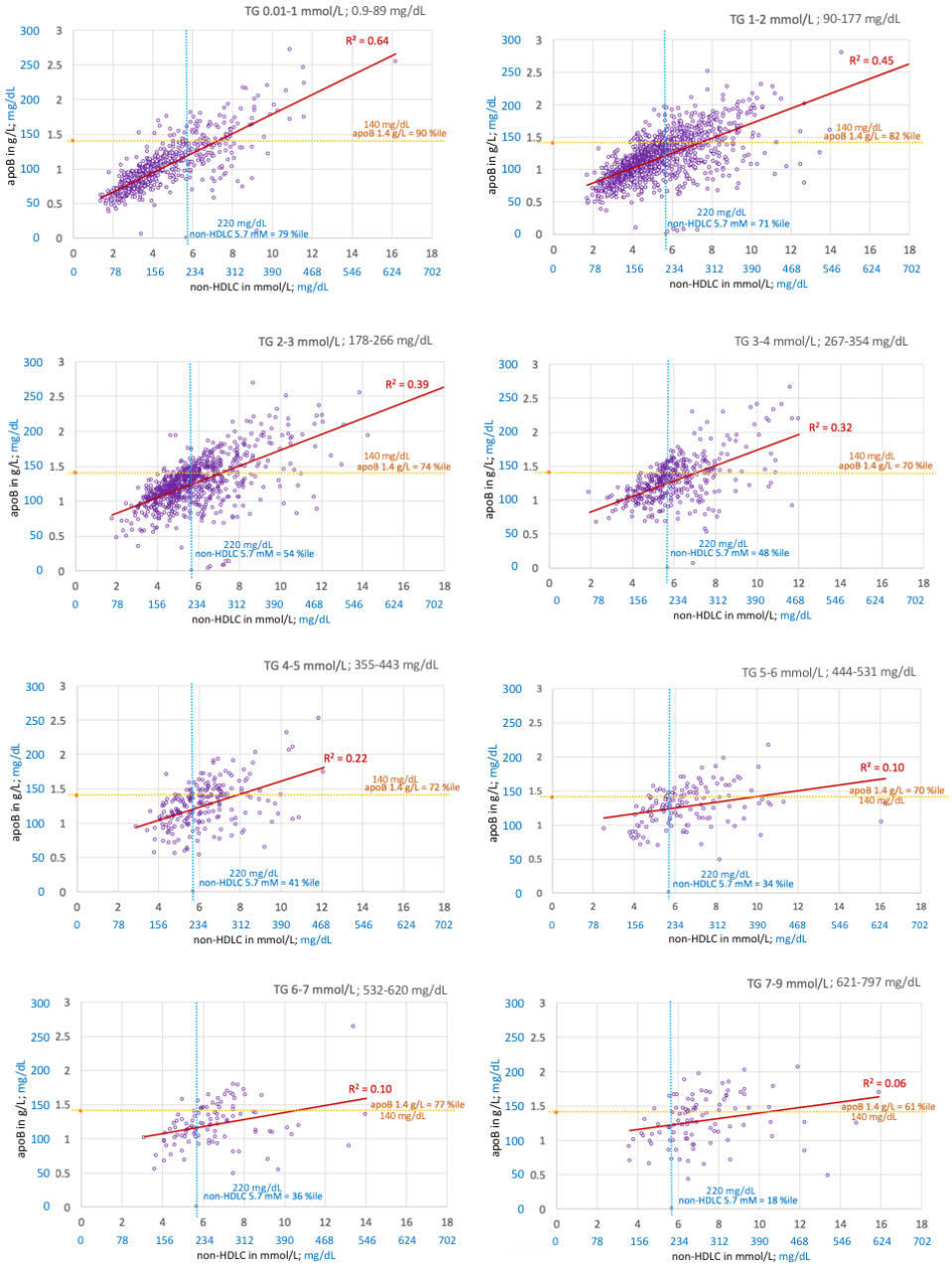
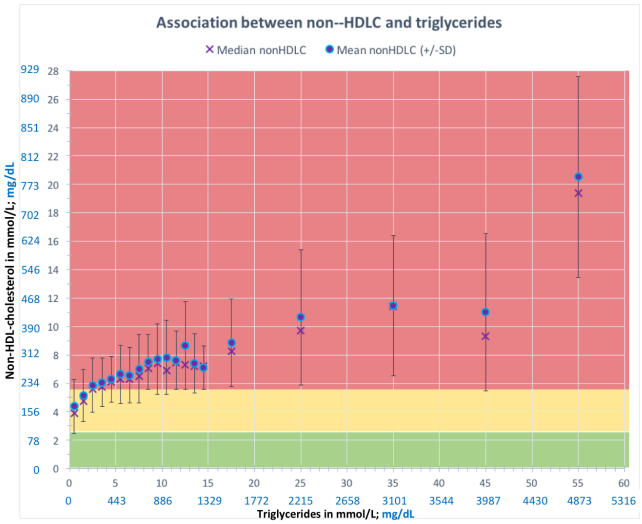


Figure 1 caption: The association between apoB and non-HDLC for TG-intervals. TG-intervals of 1 mmol/L (89 mg/dL), up to 7 mmol/L (620 mg/dL); then TG 7-9 mmol/L (620-797 mg/dL). The linear regression and coefficient of determination (R^2) are indicated in red. Coefficients of determination are significant, with $p<0.0001$ except for TG 7-9 mmol/L (620-797 mg/dL) where $p = 0.02$. The percentile for apoB 1.4 g/L (140 mg/dL) and non-HDLC 5.7 mmol/L (220 mg/dL) are shown with dashed lines. TG 0.01 to 1 mmol/L (0.9-89 mg/dL) N = 753; TG 1.01-2 mmol/L (90 - 177 mg/dL) N = 1598; TG 2.01-3 mmol/L (178 -266 mg/dL) N =791; TG 3.01-4 mmol/L (267-354 mg/dL) N = 379; TG 4.01-5 mmol/L (355-443 mg/dL) N = 211; TG 5.01-6 mmol/L (444-531 mg/dL) N = 142; TG 6.01-7 mmol/L (532-620 mg/dL) N = 105; TG 7.01-9 mmol/L (621-797 mg/dL) N =100. (*2 column colour figure*)

Impact of HTG on non-HDLC versus apoB levels for cardiovascular disease risk equivalent categories

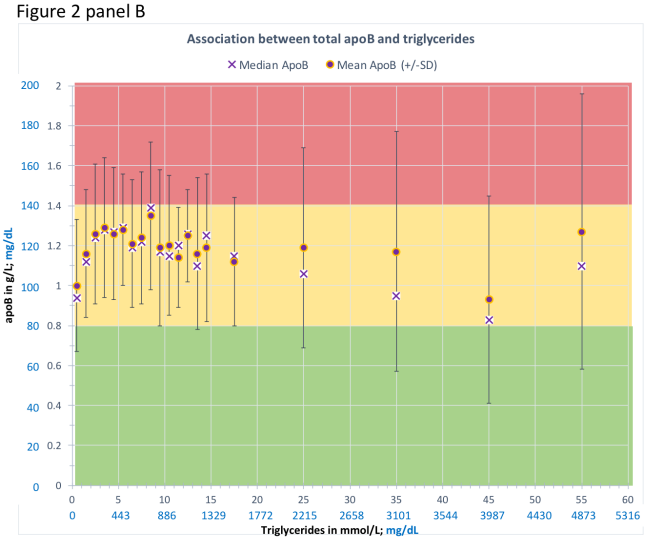
In Figure 2 panel A, from TG interval of 2-3 mmol/L (177-266 mg/dL) and above, both the mean and median non-HDLC are in the high-risk zone, i.e. above 5.7 mmol/L (220 mg/dL). The linear regression analyses' coefficient of determination (inset table) show a very high degree of correlation for the trend of increasing non-HDLC over the entire TG range up to ~ 55.0 mmol/L (4,871 mg/dL). In Figure 2 panel B, for the full range of TG, both the mean and median apoB are in the intermediate-risk zone (yellow shaded area) between 0.80 g/L (80 mg/dL) and 1.4 g/L (140 mg/dL). The standard deviation of mean apoB does extend into the red area of high CVD risk equivalence. The linear regression analyses' coefficient of determination (inset table) shows an increase in mean apoB from TG 0.01 mmol/L (0.9 mg/dL) to around TG 6 mmol/L (531 mg/dL), beyond which the correlation weakens and gradually ceases to exist. Sensitivity analysis when patients with FH and FCS were excluded showed similar results (data not shown).

Figure 2 panel A



Correlation between Non-HDLC and TG-Intervals	
TG -Interval (mmol/L; mg/dL)	R^2
0.01-4; 0.9-354	0.95
0.01-5; 0.9-443	0.92
0.01-6; 0.9-531	0.93
0.01-7; 0.9-620	0.88
0.01-8; 0.9-709	0.90
0.01-9; 0.9-797	0.93
0.01-10; 0.9-886	0.94
4-10; 354-886	0.95
5-10; 443-886	0.94
6-10; 531-886	0.92
7-10; 620-886	0.97
7-11; 620-974	0.93
10-55; 886-4,871	0.79

Figure 2 panel B



Correlation between ApoB and TG-Intervals	
TG -Interval (mmol/L; mg/dL)	R^2
0.01-4; 0.9-354	0.92
0.01-5; 0.9-443	0.74
0.01-6; 0.9-531	0.68
0.01-7; 0.9-620	0.43
0.01-8; 0.9-709	0.36
0.01-9; 0.9-797	0.48
0.01-10; 0.9-886	0.27
4-10; 354-886	0.05
5-10; 443-886	0.01
6-10; 531-886	0.01
7-10; 620-886	0.01
7-11; 620-974	0.03
10-55; 886-4,871	0.05

Figure 2 caption:
Panel A: The correlation between median non-HDLC and mean non-HDLC (+/- one standard deviation) and triglycerides.
Panel B: The correlation between median apoB and mean apoB (+/- one standard deviation) and triglycerides.
Triglycerides are in increments of 1 mmol/L (89 mg/dL) up to 15 mmol/L (1,329 mg/dL), and then increasing increments due to sparsity of lipid profile data. Data for TG 50.01 mmol/L (4,429 mg/dL) to 120 mmol/L (10,628 mg/dL) are plotted at TG 55 mmol/L (4,871 mg/dL). Panel A and Panel B are both plotted against a background colour scheme representing cardiovascular disease risk equivalence categories: red represents high risk (non-HDLC >/= 5.7 mmol/L (220 mg/dL); apoB >/= 1.4 g/L (140 mg/dL); yellow represents intermediate risk (non-HDLC 2.6-5.7 mmol/L (100-220 mg/dL); apoB 0.8-1.4 g/L (80-140 mg/dL)); green represents low risk (non-HDLC less than 2.6 mmol/L (100 mg/dL)); apoB less than 0.8 g/L (80 mg/dL). The inset table shows linear regression analysis results as the coefficient of determination (R^2) for TG-intervals.
(2 column colour figure)

In Figure 3, as TG increase up to 10 mmol/L (886 mg/dL), the mean non-HDLC increases whereas the mean apoB initially increases, but plateaus. In normotriglyceridemia, and up to TG~4 mmol/L (354 mg/dL), there is a trend towards relatively lower apoB than non-HDLC. Starting around moderate HTG, non-HDLC and apoB diverge enough so that they show discordant cardiovascular disease risk.

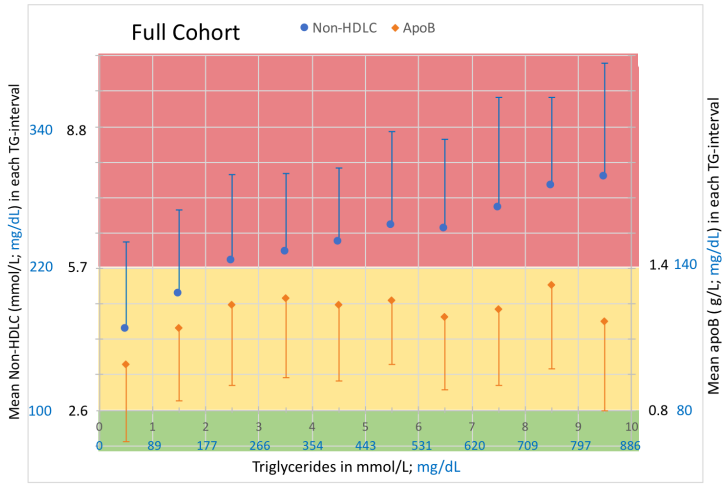


Figure 3 caption: The association between mean non-HDLC (SD) vs TG and mean apoB (SD) versus TG plotted on the same graph using dual axes, up to TG 10 mmol/L (886 mg/dL). The mean non-HDLC and mean apoB levels for each TG-interval of 1 mmol/L (89 mg/dL) are plotted at the mid-point of the TG-interval. *(1 column colour figure)*

Relationship between mean non-HDLC and mean apoB in mild to moderate HTG in sub-groups

Figure 4 shows that the overall trend of divergence between mean non-HDLC and mean apoB in mild and moderate HTG is present irrespective of patient's T2DM status, baseline CAD status, gender, and hypertension status. All subgroups show a similar trend starting around moderate HTG where non-HDLC leaps into the high CVD risk equivalent zone while the mean apoB remains in the intermediate CVD risk equivalent zone.

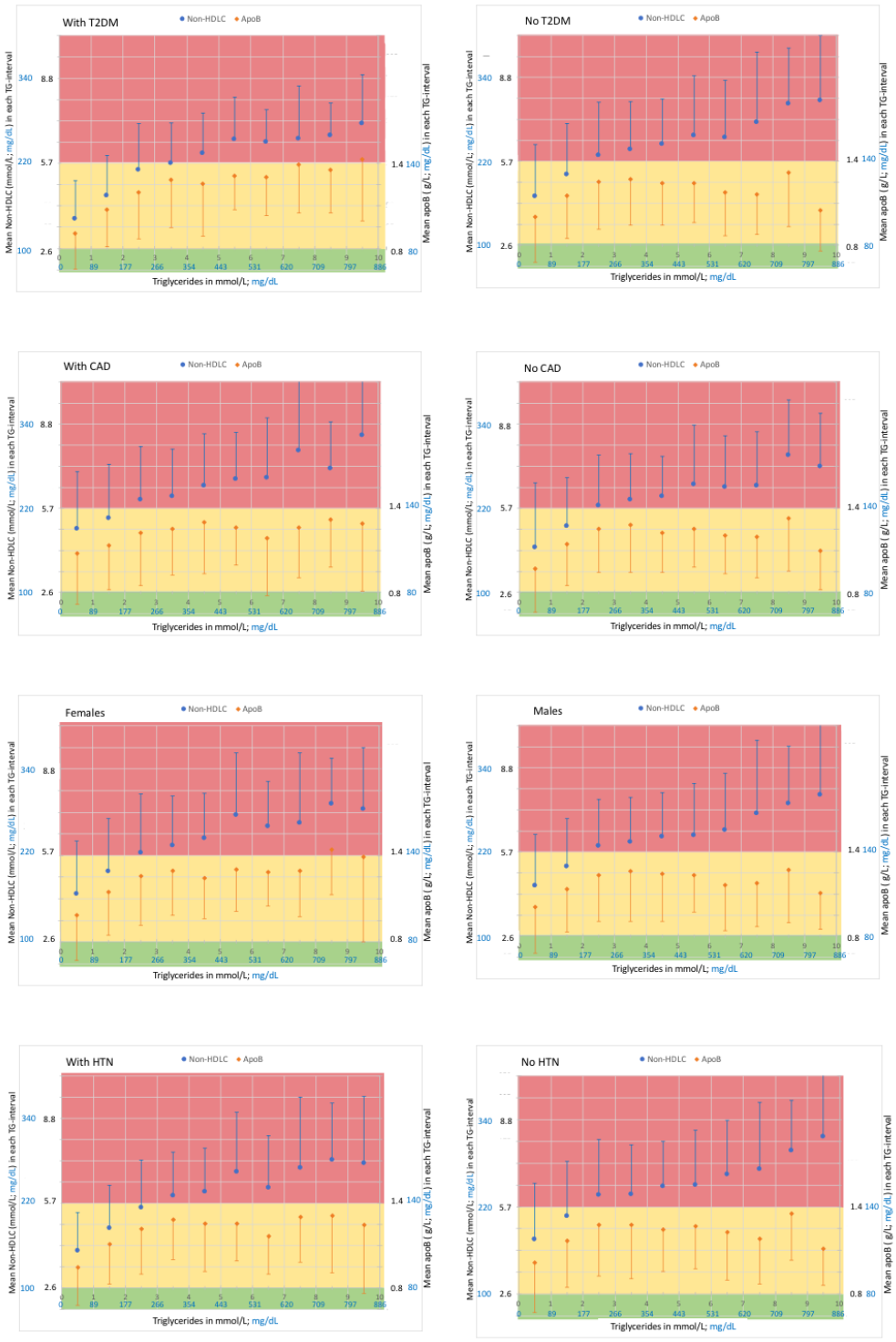


Figure 4 caption: The association between mean non-HDLC (SD) vs TG and mean apoB (SD) versus TG plotted on the same graph using dual axes, up to TG 10 mmol/L (886 mg/dL), for the following subgroups: With Type 2 Diabetes mellitus (T2DM), No T2DM, With Coronary Artery Disease (CAD), No CAD, Females, Males, With Hypertension (HTN), No HTN. *(2 column colour figure)*

Discordance between non-HDLC and apoB for high CVD risk stratified by TG level

Figure 5 shows that in HTG, there is increasing discordance due to non-HDLC ≥ 5.7 mmol/L (220 mg/dL) yet apoB < 1.4 g/L (140 mg/dL). This substantial discordance is depicted by the rise in the red line for all levels of TG. The other possible discordance, depicted by the orange line, is due to non-HDLC < 5.7 mmol/L (220 mg/dL) and apoB ≥ 1.4 g/L (140 mg/dL), which occurs in a smaller proportion of patients as TG increases. When median non-HDLC and median apoB are used as the cut-points (Figure 6), the overall trend of increasing discordance as TG increases is generally similar to when we used our high CVD risk cut-points (Figure 5).

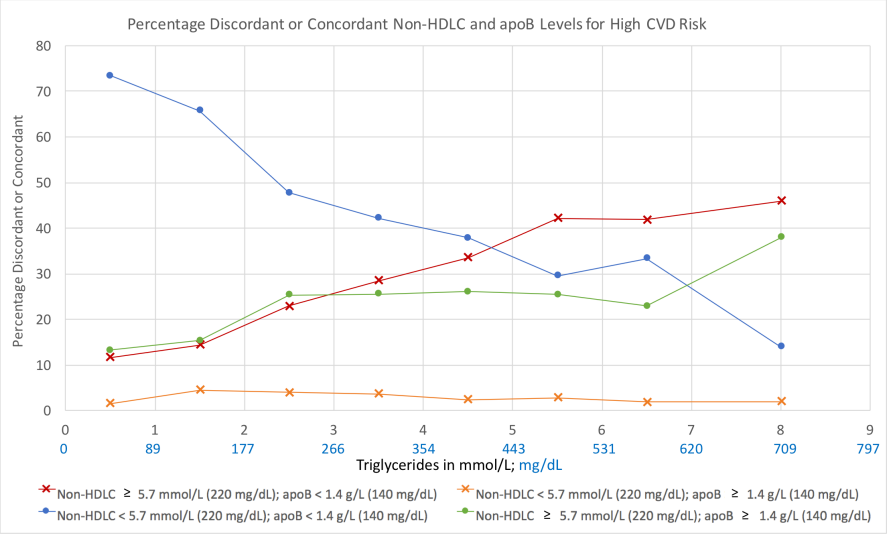


Figure 5 caption: For each TG stratum, the percentage of each for the four categories are presented: discordance non-HDLC ≥ 5.7 mmol/L (220 mg/dL) and apoB <1.4 g/L (140 mg/dL); discordance non-HDLC <5.7 mmol/L (220 mg/dL) and apoB ≥ 1.4 g/L (140 mg/dL); concordance non-HDLC ≥ 5.7 mmol/L (220 mg/dL) and apoB ≥ 1.4 g/L (140 mg/dL); concordance non-HDLC <5.7 mmol/L (220 mg/dL) and apoB <1.4 g/L (140 mg/dL). The percentage for each TG interval is plotted at the mid-point of the TG-interval. *(1 column colour figure)*

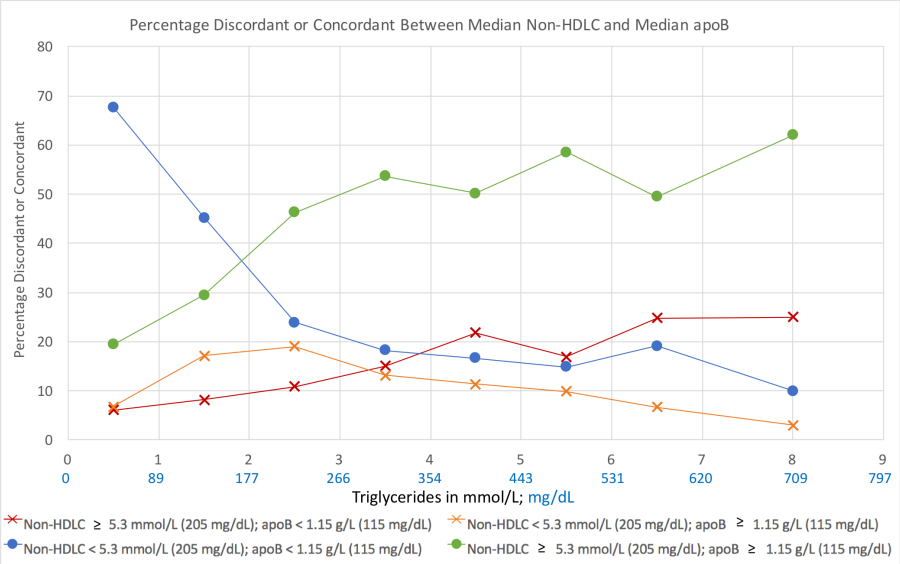


Figure 6 caption: For each TG stratum, the percentage of each for the four categories are presented: discordance non-HDLC ≥ 5.3 mmol/L (205 mg/dL) and apoB <1.15g/L (115 mg/dL); discordance non-HDLC <5.3 mmol/L (205 mg/dL) and apoB ≥ 1.15 g/L (115 mg/dL); concordance non-HDLC ≥ 5.3 mmol/L (205 mg/dL) and apoB ≥ 1.15 g/L (115 mg/dL); concordance non-HDLC <5.3 mmol/L (205 mg/dL) and apoB <1.15 g/L (115 mg/dL). The percentage for each TG interval is plotted at the mid-point of the TG-interval. *(1 column colour figure)*

DISCUSSION

We have used lipid profile data from a large Lipid Clinic cohort to compare the status of non-HDLC and apoB as CVD risk markers across the full spectrum of serum TG levels, from NTG to severe HTG. The main findings of our study were that, with increasing TG levels: (1) the correlation between non-HDLC and apoB diminished progressively, (2) non-HDLC levels increased progressively well into TG levels of ~55 mmol/L (4,871 mg/dL) while apoB levels plateaued after an initial progressive increase up to TG of ~ 4.0-5.0 mmol/L (354-443 mg/dL), (3) there was divergence in stratification of non-HDLC and apoB into CVD risk equivalent categories. Findings 2 and 3 were demonstrated in the whole cohort as well as in all sub-groups of males and females, and those with and without T2DM, CAD, and hypertension.

It is well recognized that non-HDLC and apoB are highly concordant in the presence of a normal amount of cholesterol in apoB-containing lipoproteins (17-18, 57). They become progressively discordant with increasing cholesterol enrichment of these particles (57). In this study, we have focused on HTG, which is a common clinical situation in which there is cholesterol enrichment of apoB-containing particles. Yet, the relationship between non-HDLC and apoB has not been systematically and specifically examined in relation to TG status. To the best of our knowledge, this is the first analysis of this kind.

Since our study focused on the relative effect of the full spectrum of mild, moderate, and severe hypertriglyceridemia, inferences cannot be made from previous studies that have compared apoB with non-HDLC for CVD risk prediction only in people with NTG and mild HTG up to TG ~ 4.5 mmol/L (399 mg/dL), and without stratification according to TG levels (22, 24-25, 27-29; 58). Stratification by TG level in two studies, which had participants with NTG and mild HTG (23, 26), did not significantly affect the CVD relative risk for apoB or non-HDLC. Discordance analyses of prospective observational studies, which included up to mild HTG, showed that in cases of discordance between apoB and non-HDLC, apoB predicted CVD risk better than non-HDLC (32-34, 59). However, the Emerging Risk Factors Collaboration's analysis of prospective studies (60), and the UK Biobank study (30) did not support superiority of apoB over non-HDLC in CVD risk prediction.

Our data predictably confirmed that cholesterol enrichment of apoB-containing particles associated with HTG caused discordance between non-HDLC and apoB. Our findings provide an important message that non-HDLC and apoB should not be viewed as interchangeable CVD risk markers in the presence of HTG. Their discordance clearly indicates that they predict CVD risk differently.

When we view our data from the perspective of the individual status of non-HDLC and apoB as a function of TG, we see a different pattern for the 2 markers. Non-HDLC is higher when TG is higher while apoB plateaus beyond a TG of 4-5 mmol/L (354-443 mg/dL). Since both markers show progressive increase with each mmol/L (89 mg/dL) increase in TG from NTG up to ~4-5 mmol/L (354-443 mg/dL), our data may be taken to indicate that divergence starts to show only above this TG level. In fact, our correlation data suggest to us that divergence very likely starts much earlier since the coefficient of determination for the relationship between non-HDLC and apoB shows a progressive decline with every 1 mmol/L (89 mg/dL) increase in TG levels above 1.0 mmol/L (89 mg/dL). Above TG 4-5 mmol/L (354-443 mg/dL), the divergence becomes more obvious.

By placing the TG-related trajectories of non-HDLC and apoB onto comparative risk equivalent categories, we have demonstrated that the 2 markers in our cohort share the same intermediate-risk category for TG up to 2-3 mmol/L (177-266 mg/dL), beyond which the 2 markers occupy different risk zones. Our selection of non-HDLC $\geq$ 5.7 mmol/L (220 mg/dL), and apoB $\geq$ 1.4 g/L (140 mg/dL) to be high CVD risk equivalents is based on observational epidemiological studies (25, 30-31, 54-55). As is the case with many cut-off values, they are not absolute, but are useful for general reference. Non-HDLC moves into the high-risk zone while apoB remains in the intermediate-risk zone. If particle number is a better CVD risk predictor than the cholesterol content of apoB-containing lipoproteins, as has been suggested in multiple studies (23, 26-27, 29, 32-34), our data could be interpreted to indicate that non-HDLC provides a falsely higher index of risk, as TG increases. This is consistent with our previous work which examined this issue from a lipoprotein composition perspective (41). We showed that as TG increased up to TG ~ 7 mmol/L (620 mg/dL), the calculated non-HDLC was progressively more

inclusive of cholesterol from lipoproteins with higher triglyceride:cholesterol ratios (41). These lipoproteins may be less atherogenic than LDL and smaller remnant lipoproteins (37-39, 61-63). On the other hand, if the cholesterol content of apoB-containing atherogenic lipoproteins (non-HDLC) is a better CVD risk predictor, as indicated by many studies (45-49), our data would be interpreted to indicate that apoB provides a falsely lower index of risk with HTG. This may be due to the failure of apoB to capture risk associated with the higher and more atherogenic cholesterol content of remnants.

Similar to our previous study (41), this study shows no TG inflection point where non-HDLC shows a sudden deviation from apoB. Instead, the divergence of risk information from these two markers shows a gradation beginning from TG >1.0 mmol/L (89 mg/dL), picking up to a more obvious divergence with TG > 4-5 mmol/L (354-443 mg/dL). We present the first empirical data showing the extent to which non-HDLC can increase disproportionately with respect to apoB in moderate and severe HTG.

Figure 5 shows that the percentage of discordance between non-HDLC and apoB increases with HTG. This discordance is predominantly due to non-HDLC ≥5.7 mmol/L (220 mg/dL), while apoB remains below 1.4 g/L (140 mg/dL). This is likely due to non-HDLC encompassing progressively more cholesterol from larger TRL (37-39, 41-43).

A limitation of our study is that our data are from a Lipid Clinic cohort in a region with a relatively higher genetic homogeneity due to the founder effect. Our findings may therefore not be generalizable to the general population. We expect that our study results would be more representative of Lipid Clinic patient populations. Also, our study does not include age and other medical factors. Moreover, this is a single centre study with cross-sectional data from lab tests collected over 27 years. While the lab methods have remained very similar, the machines and

reagents have changed over the years. However, the average bias has remained between 3 to 5% for all lipid profile test results. Overall, the large size of this cohort has allowed us to see the big picture of discordance between non-HDLC and apoB in HTG, and future studies could explore the impact of additional patient characteristics.

Future studies on lipid biomarkers in CVD risk prediction in people with moderate and severe hypertriglyceridemia, which affects up to 2% of the general population (64-65), are needed. In addition, in-process and future studies on the effects of medications on TG and CVD risk reduction can include the effects of non-HDLC and apoB in the analyses (66-67).

CONCLUSIONS

Our study showed the extent to which non-HDLC can increase disproportionately with respect to apoB in moderate and severe HTG. With increasing TG levels, the correlation between non-HDLC and apoB decreased. With increasing HTG severity, the discordance between non-HDLC and apoB can cause clinically important divergence in CVD risk categorization. More studies are needed, and the ultimate test of the relative merit of non-HDLC versus apoB as a CVD risk marker in the presence of the full spectrum of mild, moderate, and severe HTG would be a prospective endpoint study. Pending such data, we suggest that with TG above 4-5 mmol/L (354-443 mg/dL), both non-HDLC and apoB could be measured to benefit from their complementary information in cardiovascular disease risk stratification.

Declarations of interest: None

Contribution Statement: CS and TCO designed the study. DB and DG acquired the data for the study. CS and TCO analyzed the data and drafted the article. CS, DB, DG, and TCO interpreted the data, and revised the article. All authors approved the final article.

HIGHLIGHTS

- As TG increases, the correlation between non-HDLC and apoB decreases progressively
- In HTG, discordant non-HDLC and apoB causes divergence in CVD risk categorization
- Non-HDLC and apoB should not be viewed as interchangeable CVD risk markers in HTG

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CHAPTER 1B: CLARIFICATIONS FOR ARTICLE 1 IN CONTEXT OF THIS THESIS, AND ADDITIONAL ANALYSES

Clarifications for Article 1

Regarding the "Lipid Clinic cohort"

In this article, I have used the term "Lipid Clinic cohort", and intent was a general use of "cohort" to refer to a collective group of Lipid Clinic patients. I realize that this "cohort" term, could be mis-interpreted to imply longitudinal follow-up. However, in this article, I had also clearly indicated that these are cross-sectional data with data analyses and interpretation within the confines of the nature of these data.

We do not have follow-up data for these Lipid Clinic patients. This database was created to document the first lipid profile. Not all patients were followed in this clinic after the initial assessment.

The study design to stratify by TG-intervals of 1 mmol/L

HTG is a complex condition. Historically, this TG component of all lipoproteins has been analysed in spectrum of severity. Because the main focus of this thesis is the relative effect of HTG on the association between non-HDLC and apoB, our study design necessitates stratification into TG-intervals. Therefore, we chose to stratify by TG-intervals of 1 mmol/L.

Defining correlation between apoB vs non-HDLC in this thesis

Correlation in this thesis refers to the association between apoB vs non-HDLC, using individual lipid profiles. Linear regression was the method, and the regression coefficients and coefficients

of determination were analysed over the TG-intervals. Assessment of correlation between apoB and non-HDLC has been used in studies that have compared apoB to non-HDLC for CVD risk prediction (Sniderman *et al.* 2003; Sniderman *et al.* 2012; Lawler *et al.* 2017). Since apoB and non-HDLC are fundamentally different entities with different units, this correlation assessment is used for comparison purposes. In addition, the coefficient of determination is expected to decrease as TG levels get higher. This is because there would be an increasing number of possible combinations of quantities and classes of lipoprotein particles, and hence a wider variation in apoB levels are possible.

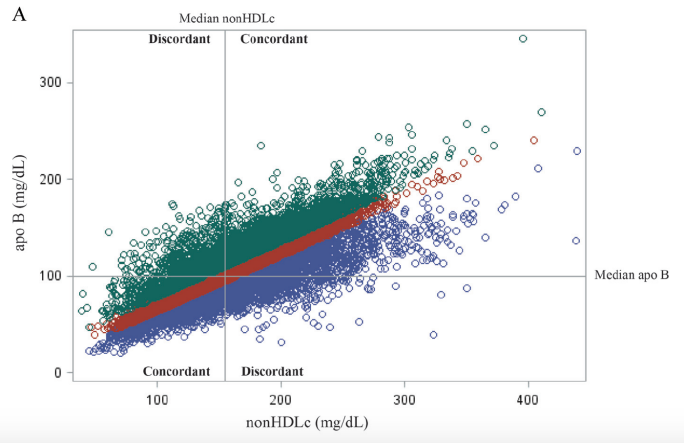
Clarification for the inset tables in Figure 2 panel A and panel B

The inset table for Figure 2 panel A, and the inset table for Figure 2 panel B, both show that coefficient of determination for various ranges of triglyceride levels. The purpose of these analyses was to determine if there was a more continuous association between non-HDLC or apoB over a broader range of triglyceride levels.

Concordance and discordance between apoB vs non-HDLC in the literature

Concordance and discordance between non-HDLC and apoB is an established concept, and this has been applied to analyse data from prospective observational studies. More often, the concordance and discordance concept has been based on four quadrants created from median non-HDLC, and median apoB. We had used this definition in our Article 1 Figure 6. The following Figure 1 is taken from Lawler *et al.* 2017, and is shown here to demonstrate that discordance, even in normotriglyceridemia to mild hypertriglyceridemima, is a common finding.

Figure 1: Baseline concordant versus discordant non-HDLC and apoB in the Women's Health Study taken from Lawler *et al.* 2017



Source: Lawler *et al.* 2017 Figure 1 panel A

Defining concordance between apoB vs non-HDLC in this Article 1

Concordance is the reliability (agreement) of apoB and non-HDLC to place the lipid profile into the same CVD risk equivalence category. Discordance is when apoB and non-HDLC place the lipid profile into different CVD risk equivalence categories.

Supplemental Information - Data Table for Article 1 Figure 1

Table 1: Linear regression for correlation between apoB and non-HDLC in the full Lipid Clinic

TG Interval (mmol/L)	N	Regression coefficient	Standard Error	95% CI		p-value	R^2	R^2
				Lower	Upper			interpretation
0.01 to 1	753	0.140	0.004	0.132	0.148	<0.0001	0.64	moderate
1.01 to 2	1598	0.114	0.003	0.108	0.120	<0.0001	0.45	weak
2.01 to 3	791	0.113	0.005	0.103	0.123	<0.0001	0.39	weak
3.01 to 4	379	0.116	0.009	0.098	0.134	<0.0001	0.31	very weak
4.01 to 5	211	0.095	0.013	0.069	0.121	<0.0001	0.22	very weak
5.01 to 6	142	0.043	0.011	0.021	0.065	<0.0001	0.10	very weak
6.01 to 7	105	0.053	0.015	0.023	0.083	<0.0001	0.10	very weak
7.01 to 9	100	0.041	0.016	0.009	0.073	0.02	0.06	very weak

The potential application of decreasing slope as "delta apoB / delta non-HDLC"

The regression coefficient is the slope for the association between apoB (dependent variable) and non-HDLC (independent variable). Therefore, the regression coefficient is the slope that represents delta apoB divided by delta non-HDLC. For comparison purposes, if we set delta non-HDLC to "one", then we see that the delta apoB declines as TG levels are higher.

The interpretation would therefore be that the relative elevation in apoB decreases with respect to the elevation in non-HDLC as TG levels get higher.

The limitations to using the delta apoB / delta non-HDLC as a clinical index would be that it has not been used in prior studies. In addition, the 95% confidence intervals of this delta apoB / delta non-HDLC index are quite wide. The main advantage is that this index does not require any additional resources in studies that have already assessed participants' apoB and non-HDLC levels. Future studies in this field should explore the relative merits of this index in longitudinal studies.

Decline in coefficient of determination at higher TG levels

The decline in the coefficient of determination at higher TG levels reflects the increased variability in apoB levels for each non-HDLC level. Theoretically, this is expected and can be explained by the same value of non-HDLC that can be comprised of a large possibility of apoB values. For example, the same non-HDLC level can be coming from a few large triglyceride-rich

lipoproteins (relatively lower apoB), or the same value of non-HDLC can also be comprised of many smaller triglyceride-rich lipoproteins (relatively higher apoB).

Additional Analyses

METHODS

Assessment of age and sex as confounders

To assess for age and sex as potential confounders of the association between apoB and non-HDLC, linear regression models with and without age, and linear regression models with and without sex were performed. The regression co-efficient for non-HDLC (independent variable) was compared in linear regression models with and without age, and with and without sex. For age or sex to be a confounder, there needs to be a significant change in regression co-efficient, which was defined as 10% difference, and had to be present across all TG intervals.

Assuming apoB 1.4 g/L to be reference standard for prediction of high CVD risk, assessment of sensitivity and specificity via receiver-operator curves for various non-HDLC cut-offs

To assess for the sensitivity and specificity of non-HDLC to predict high CVD risk, we needed to assume that apoB 1.4 g/L is the reference standard for prediction of high CVD risk. Receiver-operator curves (ROC) were generated and the c-statistic was used to assess for strength of the model for high CVD risk prediction. The goal was to find a non-HDLC cut-off that would meet c-statistic 0.7 for all TG-intervals, because c-statistic 0.7 and above is generally considered a good model.

Assuming non-HDLC 5.7 mmol/L to be reference standard for prediction of high CVD risk, assessment of sensitivity and specificity via receiver-operator curves for various apoB cut-offs

To assess for the sensitivity and specificity of apoB to predict high CVD risk, we needed to assume that non-HDLC 5.7 mmol/L is the reference standard for prediction of high CVD risk. Receiver-operator curves (ROC) were generated and the c-statistic was used to assess for strength of the model for high CVD risk prediction. The goal was to find an apoB cut-off that would meet c-statistic 0.7 for all TG-intervals, because c-statistic 0.7 and above is generally considered a good model.

Limitation of the c-statistic in risk prediction

The c-statistic is used here for its discriminative ability based on the sensitivity and specificity of various cut-off values of non-HDLC and apoB for high CVD risk equivalence categorization. The main limitation of this c-statistic method is that the calibration for these models cannot be determined (Cook 2007). Assessment of indices of calibration requires knowing how well the prediction risk agrees with the observed (real) risk (Cook 2007). The cross-sectional nature of this dataset did not allow for longitudinal prediction of risk, and therefore the calibration was not feasible.

RESULTS

Analysis for age and sex as confounders for the association between apoB vs non-HDLC

Table 2: Assessment of age as potential confounder for correlation between apoB and non-HDLC in full Lipid Clinic

TG Interval (mmol/L)	N	Without AGE				WITH AGE			
		Regression coefficient	Standard Error	95% CI Lower	95% CI Upper	Regression coefficient	Standard Error	95% CI Lower	95% CI Upper
0.01 to 1	753	0.140	0.004	0.132	0.148	0.141	0.004	0.133	0.149
1.01 to 2	1598	0.114	0.003	0.108	0.120	0.115	0.003	0.109	0.121
2.01 to 3	791	0.113	0.005	0.103	0.123	0.112	0.005	0.102	0.122
3.01 to 4	379	0.116	0.009	0.098	0.134	0.115	0.009	0.097	0.133
4.01 to 5	211	0.095	0.013	0.069	0.121	0.095	0.013	0.069	0.121
5.01 to 6	142	0.043	0.011	0.021	0.065	0.043	0.011	0.021	0.065
6.01 to 7	105	0.053	0.015	0.023	0.083	0.051	0.015	0.021	0.081
7.01 to 8	57	0.043	0.018	0.007	0.079	0.044	0.018	0.008	0.080
8.01 to 9	43	0.031	0.030	-0.029	0.091	0.034	0.030	-0.026	0.094
9.01 to 10	33	0.041	0.028	-0.015	0.097	0.039	0.028	-0.017	0.095

Over the TG-intervals, the regression coefficients for the association between apoB vs non-HDLC are not significantly different. Therefore, age is not a confounder for this relationship.

Table 3: Assessment of sex as potential confounder for correlation between apoB and non-HDLC in full Lipid Clinic

TG Interval (mmol/L)	N	Without SEX				WITH SEX			
		Regression coefficient	Standard Error	95% CI Lower	95% CI Upper	Regression coefficient	Standard Error	95% CI Lower	95% CI Upper
0.01 to 1	753	0.140	0.004	0.132	0.148	0.140	0.004	0.132	0.148
1.01 to 2	1598	0.114	0.003	0.108	0.120	0.115	0.003	0.109	0.121
2.01 to 3	791	0.113	0.005	0.103	0.123	0.113	0.005	0.103	0.123
3.01 to 4	379	0.116	0.009	0.098	0.134	0.116	0.009	0.098	0.134
4.01 to 5	211	0.095	0.013	0.069	0.121	0.095	0.013	0.069	0.121
5.01 to 6	142	0.043	0.011	0.021	0.065	0.041	0.011	0.019	0.063
6.01 to 7	105	0.053	0.015	0.023	0.083	0.051	0.015	0.021	0.081
7.01 to 8	57	0.043	0.018	0.007	0.079	0.044	0.017	0.010	0.078
8.01 to 9	43	0.031	0.030	-0.029	0.091	0.031	0.029	-0.027	0.089
9.01 to 10	33	0.041	0.028	-0.015	0.097	0.046	0.026	-0.006	0.098

Over the TG-intervals, the regression coefficients for the association between apoB vs non-HDLC are not significantly different. Therefore, sex is not a confounder for this relationship.

Table 4: ROC curves (maximizing c-statistic) for apoB 1.4 g/L corresponding to varying levels of non-HDLC

	c statistic							
TG Interval (mmol/L)	Non-HDLC 5.3 mmol/L	Non-HDLC 5.5 mmol/L	Non-HDLC 5.7 mmol/L	Non-HDLC 6.0 mmol/L	Non-HDLC 6.5 mmol/L	Non-HDLC 7.0 mmol/L	Non-HDLC 7.5 mmol/L	Non-HDLC 8.0 mmol/L
0.01 to 1	0.51	0.52	0.51	0.51	0.52	0.54	0.52	0.54
1.01 to 2	0.57	0.57	0.59	0.58	0.59	0.61	0.59	0.58
2.01 to 3	0.51	0.50	0.52	0.50	0.52	0.50	0.52	0.51
3.01 to 4	0.56	0.53	0.53	0.53	0.53	0.51	0.51	0.51
4.01 to 5	0.57	0.53	0.51	0.51	0.55	0.56	0.56	0.58
5.01 to 6	0.56	0.55	0.52	0.53	0.51	0.51	0.52	0.51
6.01 to 7	0.50	0.52	0.58	0.50	0.56	0.53	0.51	0.55
7.01 to 8	0.65	0.65	0.59	0.50	0.56	0.64	0.59	0.62
8.01 to 9	0.58	0.58	0.57	0.56	0.62	0.55	0.53	0.62
9.01 to 10	0.70	0.69	0.65	0.65	0.62	0.58	0.58	0.57

Despite varying non-HDLC cut-offs from 5.3 mmol/L to 8.0 mmol/L, the concordance with apoB 1.4 g/L is overall similar, and does not meet c-statistic 0.7 for a good model for high CVD risk prediction.

Table 5: ROC curves (maximizing c-statistic) for non-HDLC 5.7 mmol/L corresponding to varying levels of apoB

	c statistic						
TG Interval (mmol/L)	apoB 1.0 g/L	apoB 1.1 g/L	apoB 1.2 g/L	apoB 1.3 g/L	apoB 1.4 g/L	apoB 1.6 g/L	apoB 1.8 g/L
0.01 to 1	0.54	0.57	0.54	0.53	0.51	0.50	0.51
1.01 to 2	0.54	0.57	0.57	0.58	0.59	0.57	0.57
2.01 to 3	0.51	0.52	0.53	0.52	0.52	0.51	0.51
3.01 to 4	0.57	0.56	0.51	0.50	0.53	0.56	0.57
4.01 to 5	0.55	0.58	0.55	0.53	0.51	0.52	0.50
5.01 to 6	0.53	0.54	0.57	0.54	0.52	0.53	0.50
6.01 to 7	0.59	0.51	0.54	0.62	0.58	0.55	0.55
7.01 to 8	0.53	0.56	0.52	0.56	0.59	0.62	0.61
8.01 to 9	0.52	0.51	0.51	0.56	0.57	0.59	0.66
9.01 to 10	0.56	0.61	0.62	0.62	0.65	0.59	0.61

Despite varying apoB cut-offs from 1.0 g/L to 1.8 g/L, the concordance with non-HDLC 5.7 mmol/L is overall similar, and does not meet c-statistic 0.7 for a good model for high CVD risk prediction.

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CHAPTER 2: MANUSCRIPT FOR ARTICLE 2

Preface

This article manuscript addresses Objective 3.

This manuscript has not been submitted for publication at the time of this thesis submission.

Contributions of co-authors: Cathy Sun designed the study, analysed the data, and drafted the article. Daniel Gaudet (DG) and Diane Brisson (DB) acquired the data for the study. Cathy Sun, DB, DG, Teik C. Ooi (TCO), and Nicholas Birkett (NB) interpreted the data and revised the article.

Ethics approvals secured: We received approval from the Ottawa Health Science Network Research Ethics Board (OHSN-REB Protocol ID 20180461-01H).

Manuscript Title

Relative effect of type 2 diabetes mellitus and obesity on discordance between non-HDLC and apoB for CVD risk categorization, in hypertriglyceridemia

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ABSTRACT

Calculated non-high-density lipoprotein cholesterol (non-HDLC) encompasses the cholesterol content in low-density lipoproteins and triglyceride-rich lipoproteins, whereas apolipoprotein B (apoB) represents the number of non-high-density lipoprotein particles. We have previously shown that, in hypertriglyceridemia, the discordance between non-HDLC and apoB at higher triglyceride (TG) levels can result in divergence in cardiovascular disease risk equivalent categories.

We aimed to test the hypothesis that there is decreased correlation, and increased level of discordance between apoB and non-HDLC associated with Type 2 diabetes (T2DM) and obesity, in patients with mild and moderate HTG.

We analysed cross-sectional lipid profile data from 3,098 patients from the Chicoutimi Hospital Lipid Clinic with TG 0.01 to 10 mmol/L and without monogenic dyslipidemia. The following categorized sub-groups were studied: with T2DM; without T2DM; with obesity; without obesity. To examine the impact of TG levels on correlation and concordance, we categorized TG into intervals and conducted separate analyses in each subgroup.

Within each subgroup, we used simple linear regression to assess the correlation between apoB and non-HDLC. To assess the reliability of non-HDLC and apoB to risk stratify patients into the same CVD risk equivalent category, we calculated weighted Kappa statistics.

Overall, the correlation between apoB versus non-HDLC declined with higher TG levels in the

subgroup without T2DM, subgroup with obesity, and subgroup without obesity. In addition, there was overall similar level of discordance between patients with T2DM and without T2DM, and between patients with obesity and without obesity.

A unifying conclusion from our results would be that irrespective of how the HTG level was pathophysiologically achieved in T2DM or obesity, the high TG level itself is an indicator of increased level of discordance between non-HDLC and apoB in CVD risk equivalence categorization.

INTRODUCTION

Calculated non-high-density lipoprotein cholesterol (non-HDLC) and apolipoprotein B (apoB) have both been widely used as biomarkers for cardiovascular disease (CVD) risk prediction (Anderson *et al.* 2016; Grundy *et al.* 2019; Catapano *et al.* 2016). Non-HDLC encompasses the cholesterol content in low-density lipoproteins (LDL) and triglyceride-rich lipoproteins, whereas apoB represents the number of atherogenic lipoproteins. The concordance and discordance between non-HDLC and apoB to categorize patients into CVD risk equivalent categories can be influenced by hypertriglyceridemia (HTG) (Sun *et al.* 2019; article 1 = Sun *et al.* 2020).

Hypertriglyceridemia is a spectrum whereby with increasing HTG severity, there is increased abundance of larger triglyceride-rich lipoproteins, which are composed of the larger very-low-density lipoproteins and their remnants, and of chylomicrons and their remnants. HTG is a complex condition. The same HTG level can be comprised of numerous combinations of the various types of lipoproteins, which will result in different non-HDLC and apoB levels. Our previous study (article 1) has shown that there is discordance between non-HDLC and apoB in HTG. The discordance between non-HDLC and apoB in HTG can result in divergence in CVD risk equivalent categories (article 1).

The influence of type 2 diabetes mellitus (T2DM) and obesity status on the concordance and discordance between non-HDLC and apoB as CVD risk markers in mild and moderate HTG has not been studied before. T2DM and obesity (body mass index >= 30 kg/m^2) both have pathophysiologic effects of increased production of triglyceride-rich lipoproteins, and increased prevalence of hypertriglyceridemia (Goldberg 2001; Feingold 2020). Patients with T2DM also

have decreased clearance of triglyceride-rich lipoproteins due to relative insulin insufficiency, which decreases the expression and function of lipoprotein lipase, a key enzyme in the metabolism of triglyceride-rich lipoproteins (Goldberg 2001; Feingold 2020). We aim to test the hypothesis that there is decreased correlation, and increased level of discordance between non-HDLC and apoB associated with T2DM and obesity, in patients with mild and moderate HTG.

METHODS

Lipid Clinic patients – cross-sectional lipid profile data

With institutional Research Ethics Board approval (OHSN-REB Protocol ID 20180461-01H), we analysed lipid profile data from subgroups of patients who were referred to the Chicoutimi Hospital Lipid Clinic. The data have been previously described in detail (article 1). Briefly, the Chicoutimi Hospital Lipid Clinic cross-sectional data contained 4,347 lipid profiles with laboratory measures of TG, non-HDLC, and apoB. For this subgroup analysis, we included patients with TG levels between 0.01 up to 10 mmol/L (4,112 patients). We excluded the 1,014 patients with monogenic dyslipidemias (familial hypercholesterolemia and familial chylomicronemia syndrome). The final study sample included 3,098 patients with TG 0.01 to 10 mmol/L and without monogenic dyslipidemia. The following categorized sub-groups were studied: with T2DM; without T2DM; with obesity; without obesity. To examine the impact of TG levels on concordance, we categorized TG into intervals of 1 mmol/L and conducted separate analyses in each subgroup. Due to decreased sample size at higher TG levels, in order to attain a sample size of at least 30 for each TG interval, we had to increase the TG interval range. A TG interval of 6.01 to 10 mmol/L was required for the subgroups with T2DM and without T2DM,

and TG interval of 7.01 to 10 mmol/L was required for the subgroups with obesity and without obesity.

Analysis methods

The impact of TG on the correlation between apoB and non-HDLC

Within each subgroup, we used simple linear regression to assess the correlation between apoB (dependent variable) and non-HDLC (independent variable), in strata of TG intervals. Non-HDLC was chosen as the independent variable because it is more widely used for cardiovascular disease risk stratification and as treatment target for patients on lipid-lowering medications. Furthermore, an assessment of the variability of apoB with respect to the non-HDLC level also shows the influence of the increased combinations of different lipoprotein classes, and hence different apoB levels, that can arise from a similar non-HDLC level.

We assessed for differences in the regression coefficient, and assessed the strength of correlation with the coefficient of determination (R^2) (Taylor 1990, D'Agostino *et al.* 2006). We also included a qualitative interpretation of the coefficient of determination (Table 1).

Table 1: Qualitative interpretation of coefficient of determination (R^2)

Coefficient of determination (R^2)	**Interpretation**
$R^2 < 0.30$	Very weak correlation
$0.31 \leq R^2 \leq 0.50$	Weak correlation
$0.51 \leq R^2 \leq 0.70$	Moderate correlation
$R^2 > 0.70$	Strong correlation

Impact of HTG on non-HDLC versus apoB for concordance/discordance in CVD risk equivalent categories

Within each TG subgroup, we compared the classification of non-HDLC and apoB into CVD risk equivalent categories, in strata of TG intervals. The low, intermediate, and high CVD risk equivalent categories have been previously described (article 1 quoted below), and were based on major American and Canadian guidelines, and large epidemiological studies. The same cut-offs for non-HDLC were also used by Brunner *et al.* 2019.

Direct quote from Article 1 Methods

"Our CVD risk equivalent categories were based on major Canadian and American guidelines, which recommend a LDLC $\geq$ 4.9 mmol/L (189 mg/dL) (52) or $\geq$ 5.0 mmol/L (193 mg/dL) (12) as high risk. Since the corresponding non-HDLC level is usually around 0.6 to 0.8 mmol/L (23 to 30 mg/dL) above LDLC levels (53), we have used a non-HDLC $\geq$ 5.7 mmol/L (220 mg/dL) to represent high risk. A non-HDLC of 5.7 mmol/L (220 mg/dL) in data from large epidemiological studies has been shown to approximately correspond to an apoB of 1.4 g/L (140 mg/dL) (25, 30-31, 54-56). Low CVD risk equivalent category of non-HDLC <2.6 mmol/L (100 mg/dL) and apoB <0.8 g/L (80 mg/dL) were chosen because they are the treatment co-targets for patients on lipid-lowering therapy (3, 12-13). Intermediate CVD risk equivalent category is the region between non-HDLC 2.6 mmol/L (100 mg/dL) and non-HDLC 5.7 mmol/L (220 mg/dL), and between apoB 0.8 g/L (80 mg/dL) and apoB 1.4 g/L (140 mg/dL)."

To assess the reliability of non-HDLC and apoB to risk stratify patients into the same CVD risk equivalent category (low, intermediate, high), we calculated weighted Kappa statistics. For the weighted Kappa (Watson *et al.* 2010), the weighting is shown in Table 2. We have three full concordance categories weighted with factor one; four categories weighted with factor 0.5, and two categories weighted with factor zero. For the discordance interpretation, the lower the weighted Kappa, the greater the discordance between apoB and non-HDLC (Table 3).

Table 2: weighted Kappa for non-HDLC and apoB categories

	non-HDLC			
apoB	<2.60	2.60-5.69	>=5.70	Row total
<0.80	Weight 1	Weight 0.5	Weight 0	R1
0.80-1.39	Weight 0.5	Weight 1	Weight 0.5	R2
>=1.40	Weight 0	Weight 0.5	Weight 1	R3
Column total	C1	C2	C3	Total N

Table 3: Qualitative interpretation of weighted Kappa

Weighted Kappa Range	Interpretation
$\kappa < 0.00$	Less than chance agreement
$0.00 \leq \kappa \leq 0.20$	Slight agreement
$0.21 \leq \kappa \leq 0.40$	Fair agreement
$0.41 \leq \kappa \leq 0.60$	Moderate agreement
$0.61 \leq \kappa \leq 0.80$	Substantial agreement
$0.81 \leq \kappa \leq 0.99$	Almost perfect agreement

Source Viera *et al.* 2005

Software

Statistical analyses were performed with SAS version 9.4 and Microsoft Excel. Figures were generated in Microsoft Excel.

RESULTS

Table 4: Characteristics of the subgroups with TG 0.01 to 10 mmol/L and without monogenic dyslipidemia

	3098 patients	Subgroup with T2DM	Subgroup without T2DM	Subgroup with Obesity	Subgroup without Obesity
Number of patients	3098	493	2605	802	2173
Non-HDLC (mmol/L)					
Mean (95% CI)	4.72 (1.76-7.68)	4.90 (2.08-7.72)	4.68 (1.70-7.66)	5.00 (1.92-8.08)	4.63 (1.71-7.55)
Median	4.61	4.80	4.59	4.81	4.52
apoB (g/L)					
Mean (95% CI)	1.09 (0.55-1.63)	1.14 (0.56-1.72)	1.08 (0.56-1.60)	1.13 (0.57-1.69)	1.07 (0.53-1.61)
Median	1.08	1.15	1.07	1.11	1.07
				*Obesity status is missing for 123 patients	

The mean and median values for non-HDLC and for apoB in each of the subgroups is shown in Table 4. The BMI was missing from 123 patients. There were no significant differences in the mean non-HDLC and mean apoB between the four subgroups.

Subgroup with T2DM vs without T2DM

Table 5: Linear regression for correlation between apoB and non-HDLC in subgroup with T2DM, and subgroup without T2DM

	With T2DM								Without T2DM							
TG Interval (mmol/L)	N	Regression Coefficient	Standard Error	95% CI		p-value	R^2	R^2 Interpretation	N	Regression Coefficient	Standard Error	95% CI		p-value	R^2	R^2 interpretation
				Lower	Upper							Lower	Upper			
0.01 to 1	37	0.176	0.035	0.106	0.246	<0.0001	0.41	weak	541	0.176	0.006	0.164	0.188	<0.0001	0.59	moderate
1.01 to 2	165	0.170	0.012	0.146	0.194	<0.0001	0.54	moderate	1014	0.161	0.006	0.149	0.173	<0.0001	0.44	weak
2.01 to 3	94	0.145	0.020	0.105	0.185	<0.0001	0.37	weak	451	0.118	0.011	0.096	0.140	<0.0001	0.21	very weak
3.01 to 4	67	0.178	0.027	0.124	0.232	<0.0001	0.38	weak	209	0.085	0.015	0.055	0.115	<0.0001	0.13	very weak
4.01 to 5	42	0.158	0.046	0.066	0.250	0.001	0.21	very weak	128	0.049	0.019	0.011	0.087	0.009	0.05	very weak
5.01 to 6	34	0.056	0.028	0.000	0.112	0.0007	0.09	very weak	94	0.039	0.015	0.009	0.069	0.01	0.06	very weak
6.01 to 10	54	0.175	0.026	0.123	0.227	<0.0001	0.46	weak	168	0.017	0.010	-0.003	0.037	0.10 = NS	0.02	very weak; NS

Subgroup with T2DM

In the subgroup with T2DM, the regression coefficient remains similar from TG 0.01 to 5 mmol/L. There is a significant decline in the regression coefficient for TG 5.01 to 6 mmol/L, and then an increase in regression coefficient back to a similar level as TG 0.01 to 5 mmol/L, when the TG is 6.01 to 10 mmol/L. With the exception of moderate strength coefficient of determination for TG 1.01 to 2 mmol/L, the coefficients of determination were all in the weak to very weak categories.

Subgroup without T2DM

In the subgroup without T2DM, the regression coefficients decreased steadily from TG 0.01 to 10 mmol/L. The coefficient of determination also decreased from TG 0.01 to 10 mmol/L, and was very weak starting at TG 2.01 mmol/L. This indicates that with higher TG levels, there is increased variability in the apoB, for any value of non-HDLC.

Table 6: Kappa for agreement between apoB and non-HDLC in subgroup with T2DM, and subgroup without T2DM

	With T2DM						Without T2DM					
TG Interval (mmol/L)	N	Weighted Kappa (κ_w)	SE for κ_w	95% CI		κ_w interpretation	N	Weighted Kappa (κ_w)	SE for κ_w	95% CI		κ_w interpretation
				Lower	Upper					Lower	Upper	
0.01 to 1	37	0.43	0.23	-0.03	0.89	moderate	541	0.40	0.07	0.26	0.54	fair
1.01 to 2	165	0.29	0.16	-0.03	0.61	fair	1014	0.32	0.07	0.18	0.46	fair
2.01 to 3	94	0.18	0.19	-0.20	0.56	slight	451	0.30	0.09	0.12	0.48	fair
3.01 to 4	67	0.20	0.21	-0.22	0.62	slight	209	0.30	0.11	0.08	0.52	fair
4.01 to 5	42	0.44	0.19	0.06	0.82	moderate	128	0.20	0.13	-0.06	0.46	slight
5.01 to 6	34	0.24	0.24	-0.24	0.72	fair	94	0.15	0.15	-0.15	0.45	slight
6.01 to 10	54	0.22	0.19	-0.16	0.60	fair	168	0.13	0.10	-0.07	0.33	slight

Subgroup with T2DM

For reliability assessment of discordance in CVD risk equivalence categorization, there was overall a rather high degree of discordance. The weighted Kappa was only moderate concordance for TG 0.01 to 1 mmol//L, and TG 4.01 to 5 mmol/L. Over the entire TG 0.01 to 10 mmol/L range, with the exception of TG 4.01 to 5 mmol/L, the weighted Kappas had 95% confidence intervals that reached less than zero, which means “less than chance agreement” (Viera *et al*. 2005).

Subgroup without T2DM

Similarly, in the subgroup without T2DM, there was also discordance present since there was only a fair level of agreement in the weighted Kappas from TG 0.01 to 4 mmol/L, and only slight agreement from TG 4.01 to 10 mmol/L. From the weighted Kappa values alone, there was a steady decline from TG 0.01 to 10 mmol/L. For TG 4.01 to 10 mmol/L, the weighted Kappas had 95% confidence intervals that included “less than chance agreement” (Viera *et al.* 2005).

Subgroup with obesity vs without obesity

Table 7: Linear regression for correlation between apoB and non-HDLC in subgroup with obesity, and subgroup without obesity

	With Obesity								Without Obesity							
TG Interval (mmol/L)	N	Regression Coefficient	Standard Error	95% CI		p-value	R^2	R^2 Interpretation	N	Regression Coefficient	Standard Error	95% CI		p-value	R^2	R^2 interpretation
				Lower	Upper							Lower	Upper			
0.01 to 1	62	0.196	0.016	0.164	0.228	<0.0001	0.70	moderate	485	0.176	0.007	0.162	0.190	<0.0001	0.57	moderate
1.01 to 2	266	0.160	0.010	0.140	0.180	<0.0001	0.47	weak	852	0.164	0.006	0.152	0.176	<0.0001	0.45	weak
2.01 to 3	179	0.114	0.017	0.080	0.148	<0.0001	0.20	very weak	347	0.125	0.012	0.101	0.149	<0.0001	0.23	very weak
3.01 to 4	98	0.104	0.022	0.060	0.148	<0.0001	0.17	very weak	173	0.099	0.018	0.063	0.135	<0.0001	0.15	very weak
4.01 to 5	61	0.077	0.033	0.011	0.143	0.02	0.07	very weak	103	0.067	0.022	0.023	0.111	0.004	0.07	very weak
5.01 to 6	50	0.074	0.020	0.034	0.114	0.0007	0.20	very weak	77	0.030	0.017	-0.004	0.064	0.07 = NS	0.03	very weak; NS
6.01 to 7	41	-0.008	0.028	-0.064	0.048	0.77 = NS	0.00	very weak; NS	58	0.052	0.017	0.018	0.086	0.004	0.12	very weak
7.01 to 10	45	0.029	0.023	-0.017	0.075	0.21 = NS	0.04	very weak; NS	78	0.016	0.017	-0.018	0.050	0.33 = NS	0.01	very weak; NS

Subgroup with obesity

In the subgroup with obesity, the regression coefficient declines steadily from TG 0.01 to 6 mmol/L, above which it is no longer significantly different from zero. With the exception of moderate strength coefficient of determination for TG 0.01 to 1 mmol/L, the coefficients of determination were all in the weak to very weak categories.

Subgroup without obesity

In the subgroup without obesity, the regression coefficients decreased steadily from TG 0.01 to 10 mmol/L, and was not significantly different from zero at TG 5.01 to 6 mmol/L, and TG 7.01 to 10 mmol/L. With the exception of moderate strength coefficient of determination for TG 0.01 to 1 mmol/L, the coefficients of determination were all in the weak to very weak categories. This indicates that with higher TG levels, there is increased variability in the apoB, for any value of non-HDLC.

Table 8: Kappa for agreement between apoB and non-HDLC in subgroup with obesity, and subgroup without obesity

	With Obesity						Without Obesity					
TG Interval (mmol/L)	N	Weighted Kappa (κ_w)	SE for κ_w	95% CI		κ_w interpretation	N	Weighted Kappa (κ_w)	SE for κ_w	95% CI		κ_w interpretation
				Lower	Upper					Lower	Upper	
0.01 to 1	62	0.46	0.18	0.10	0.82	moderate	485	0.39	0.07	0.25	0.53	fair
1.01 to 2	266	0.31	0.13	0.05	0.57	fair	852	0.31	0.07	0.17	0.45	fair
2.01 to 3	179	0.32	0.14	0.04	0.60	slight	347	0.25	0.10	0.05	0.45	fair
3.01 to 4	98	0.23	0.16	-0.09	0.55	slight	173	0.28	0.12	0.04	0.52	fair
4.01 to 5	61	0.27	0.18	-0.09	0.63	moderate	103	0.24	0.14	-0.04	0.52	slight
5.01 to 6	50	0.35	0.19	-0.03	0.73	fair	77	0.10	0.17	-0.24	0.44	slight
6.01 to 7	41	0.16	0.20	-0.24	0.56	fair	58	0.24	0.18	-0.12	0.6	slight
7.01 to 10	45	0.28	0.22	-0.16	0.72	fair	78	0.04	0.14	-0.24	0.32	slight

Subgroup with obesity

For reliability assessment of discordance in CVD risk equivalence categorization, there was overall a rather high degree of discordance. The weighted Kappa was only moderate concordance for TG 0.01 to 1 mmol//L, and TG 4.01 to 5 mmol/L. For TG 3.01 to 10 mmol/L, the weighted Kappas had 95% confidence intervals that reached less than zero, which means "less than chance agreement" (Viera *et al.* 2005).

Subgroup without obesity

Similarly, in the subgroup without obesity, there was also discordance present since there was only a fair level of agreement in the weighted Kappas from TG 0.01 to 4 mmol/L, and only slight agreement from TG 4.01 to 10 mmol/L. From the weighted Kappa values alone, there was a steady decline from TG 0.01 to 10 mmol/L. For TG 4.01 to 10 mmol/L, the weighted Kappas had 95% confidence intervals that included "less than chance agreement" (Viera *et al.* 2005).

DISCUSSION

This is the first study for the T2DM- and obesity-specific correlation between individual lipid profiles, and discordance in CVD risk equivalence categorization, in mild and moderate HTG. Overall, the correlation between apoB versus non-HDLC declined with higher TG levels in the subgroup without T2DM, subgroup with obesity, and subgroup without obesity. However, for the subgroup with T2DM, there was a rebound in the regression coefficient and coefficient of determination for the TG interval of 6.01 to 10 mmol/L. In addition, there was overall similar level of discordance between patients with T2DM and without T2DM, and between patients with obesity and without obesity. For the subgroup with T2DM, although there was similar variability in apoB at TG 6.01 to 10 mmol/L as TG 0.01 to 5 mmol/L (Table 5), there was a steady decline in the reliability for apoB and non-HDLC to categorize patients into the same CVD risk equivalent category (Table 6). In fact, the level for discordant apoB and non-HDLC to categorize patients into different CVD risk equivalence category was overall quite high. A unifying conclusion from our results would be that irrespective of how the HTG level was pathophysiologically achieved in T2DM or obesity, the high TG level itself is an indicator of increased level of discordance between non-HDLC and apoB in CVD risk equivalence categorization.

For assessment of reliability between apoB and non-HDLC, the weighted Kappas were all less than 0.5 with wide 95% confidence intervals that often included Kappas less than expected from chance alone, and this was not dependent on T2DM status, or obesity status. A potential explanation for this study's results is that T2DM and obesity are both diseases with a spectrum of severity, and the diagnoses are based on cut-offs (Punthakee *et al.* 2018; Wharton *et al.* 2020).

While the diagnostic criteria may be slightly arbitrary in the choice of cut-offs, they are required for clinical practice and international standardization. Therefore, patients with pre-diabetes and who are overweight, were compared with patients with T2DM and obesity. The lipid metabolism abnormalities in T2DM and in obesity are often already occurring in patients with prediabetes and who are overweight (Abbasi *et al.* 2016, Tenenbaum *et al.* 2014). A limitation of this study is that we were unable to account for this concern.

In our previous study (article 1) we found decreasing correlation and increasing discordance between non-HDLC and apoB, with higher TG levels. Moreover, we performed a graphical analysis in patients with T2DM vs without T2DM whereby the mean non-HDLC and mean apoB in each 1 mmol/L TG-interval (up to 10 mmol/L), diverged into discordant CVD risk categories (article 1, Figure 4). In this study, we aimed to be more representative of the general population by excluding patients with monogenic dyslipidemia.

This study is the first to use weighted Kappa to assess the reliability of non-HDLC versus apoB for classification into the low, intermediate, and high CVD risk equivalence categories. Our definition of discordance as the decreased reliability between non-HDLC and apoB in CVD risk categorization builds on previous studies of discordance between non-HDLC and apoB. Large prospective observational studies (Sniderman *et al.* 2012, Lawler *et al.* 2017) have often used median non-HDLC and median apoB to define concordant categories, and discordant categories. Our results that discordance is increased with higher TG levels, is consistent with previous studies (Sniderman *et al.* 2012, Lawler *et al.* 2017) that also detected similar discordance between non-HDLC and apoB in CVD risk prediction, both in normotriglyceridemia, and mild

hypertriglyceridemia (TG up to ~ 4.5 mmol/L). However, prospective studies for CVD risk prediction in patients with moderate HTG (TG ~ 4.5 mmol/L to 10 mmol/L) and severe HTG (TG > 10 mmol/L) are lacking. This is a challenging yet important patient population for future studies. Even in our cross-sectional data collected over 27 years at the tertiary care Chicoutimi Hospital Lipid Clinic, the sample sizes are decreased at higher TG levels, and this decreased sample size is itself a limitation in our data analyses.

A limitation of this study is that it is based on cross-sectional data. Therefore, we do not know whether non-HDLC or apoB is more accurate in CVD risk prediction in the patients with hypertriglyceridemia in this Lipid Clinic population. For patients with normal triglyceride levels, and up to mild hypertriglyceridemia, there have been numerous prospective studies from which the CVD risk equivalence category cut-offs for non-HDLC and apoB were based (NCEP 2002; Sniderman *et al.* 2003; Ridker *et al.* 2005; Contois *et al*. 2009; Parish *et al*. 2009; Anderson *et al.* 2016; Catapano *et al.* 2016; Jellinger *et al.* 2017; Brunner *et al.* 2019; Grundy *et al.* 2019; Sniderman 2019; Welsh *et al.* 2019). In addition, clinical CVD risk stratification is often based on a single lipid-profile prior to starting lipid-lowering pharmacotherapy (Anderson *et al.* 2016; Catapano *et al.* 2016; Grundy *et al.* 2019). Future work on CVD outcomes in patients with HTG, especially triglyceride levels 4.5 mmol/L and above, would be very helpful in elucidating which lipid biomarker is more accurate in CVD risk prediction.

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OVERALL DISCUSSION AND CONCLUSIONS CHAPTER

Stratification into mild, moderate, and severe hypertriglyceridemia

Hypertriglyceridemia as a clinical condition, has traditionally been stratified into categories based on the extent (severity) of triglyceride elevation. For clinical conditions, it is often the case where categorization is used to guide diagnosis and management based on the category into which the patient fits. Since both studies in this thesis aimed to analyse the relative effect of the full spectrum of hypertriglyceridemia on cardiovascular disease risk equivalence categorization, stratification based on triglyceride levels has a sound basis in the research methodology. We acknowledge that other methods exist, such as checking for the effect of interaction between triglycerides and the lipid biomarkers. Other methods could potentially be explored in future studies.

Methodological considerations

For the correlation analyses between non-HDLC and apoB, we used linear regression because a moderate to strong linear relationship has been shown in previous studies, in patients with normotriglyceridemia and up to mild hypertriglyceridemia (Sniderman *et al*. 2003, Barkas *et al*. 2016). We aimed to detect how this known linear relationship changes at higher triglyceride levels. A potential direction for future work in this area could be exploring non-linear relationships between non-HDLC and apoB at higher triglyceride levels. This will require an abundance of data for higher triglyceride levels. In addition, separate data sources would be needed to validate non-linear models for this relationship.

A limitation of the cross-sectional nature of the Lipid Clinic data is that we were unable to examine variables as predictors of cardiovascular disease.

Potential impact of measurement error

For the Lipid Clinic lipid profile measurements, the intra-assay variation and inter-assay variation of the lab assays for total cholesterol, high-density lipoprotein cholesterol, triglycerides, and apoB are all between 3 to 5% (Sun CJ *et al*. 2020). In addition, all lipid profiles were drawn in the fasting state, which helps with reducing variability in lipids related to meals (CCS 2016). Variability of non-HDLC, triglycerides, and apoB in the fasting period in the same individual has not been systematically studied, and could be studied if samples were taken in duplicate or triplicate and run on the same equipment.

For the correlation analyses of the association between apoB versus non-HDLC, measurement error in the independent variable tends to bias the linear regression slopes towards the null (Meijer *et al*. 2021). Furthermore, measurement error in dependent and independent variable likely results in under-estimate of R-square statistic (Meijer *et al*. 2021). Nevertheless, measurement error is unavoidable in studies of lipid biomarkers since all lab assays have measurement error, and the measurement error in our data is well within the acceptable range for biochemical assays for clinical use.

Clinical contextualization

Clinical decision making is often based on risk categorization. Cardiovascular disease risk prediction is a broad field, and there are various risk calculators, including the most widely used

Framingham risk calculator, which uses cardiovascular disease risk factors determined from epidemiological studies, in addition to lipid biomarker parameters (CCS 2016). In this thesis, since the focus is on lipid biomarkers (non-HDLC and apoB) for cardiovascular disease risk equivalence categorization, we chose to focus on the clinical scenario where the non-HDLC 5.7 mmol/L or above, or apoB 1.4 g/L or above, is equivalent to the low-density lipoprotein cholesterol of 5 mmol/L or above. Low-density lipoprotein cholesterol of 5 mmol/L or above is itself sufficient to classify the patient into the high cardiovascular disease risk category based on the 2016 Canadian Cardiovascular Society clinical guidelines, and the 2018 American Heart Association and American College of Cardiology guidelines (CCS 2016, AHA/ACC 2018). Therefore, non-HDLC or apoB levels that fit into the high cardiovascular disease risk equivalence category has the clinical implication where lipid-lowering pharmacotherapy is strongly recommended. However, if the patient's non-HDLC or apoB levels are in the intermediate cardiovascular disease risk equivalence category, then lipid-lowering pharmacotherapy could be considered in conjunction with management of modifiable risk factors. As previously mentioned, low-density lipoprotein cholesterol cannot be estimated via the Friedewald equation once triglycerides are 4.5 mmol/L or higher. Therefore, for patients with moderate or severe hypertriglyceridemia, non-HDLC and/or apoB will be assessed to see if the patient fits into the high cardiovascular disease risk equivalence category based on the lipid biomarker criterion. Our studies' findings of low agreement between non-HDLC and apoB to risk stratify the patient into the same cardiovascular disease risk equivalence category has important clinical implications since one lipid biomarker may indicate that lipid-lowering pharmacotherapy should be initiated, whereas the other lipid biomarker may not make this indication. This highlights the need for longitudinal studies to assess for the validity of non-

HDLC versus apoB to stratify patients into cardiovascular disease risk equivalent categories in patients over the full spectrum of hypertriglyceridemia.

Generalizability

The generalizability of the results of our studies are limited because the lipid profile data came from a dedicated Lipid Clinic, and are therefore representative of patients who had an indication for referral to a specialist-led Lipid Clinic. Referral bias and survival bias will both affect the generalizability because the referred patient also needed to survive at least until the initial clinic visit. Article 1 used data on patients who had apoB measured, and therefore introduces a selection bias in the results. However, we do not have information on the reasons for patients to have apoB measured and therefore we are unable to predict the effect of this selection bias on our results. Furthermore, generalizability is impacted by our lipid data coming from a location with higher genetic homogeneity for lipid disorders due to the founder effect in the Saguenay-Lac St-Jean region. The higher prevalence of familial hypercholesterolemia in this region could have increased the mean non-HDLC and the mean apoB in the normotriglyceridemia range (Brunham *et al*. 2018). However, since hypertriglyceridemia is not a known characteristic of familial hypercholesterolemia, we would not expect our results on hypertriglyceridemia in Article 1 (Sun CJ *et al* 2020) to be significantly affected. Moreover, the increased prevalence of familial chylomicronemia syndrome (Baass *et al*. 2020) is a strength of our Article 1 because it allows us to see the extent that non-HDLC can increase disproportionately with respect to apoB, in severe hypertriglyceridemia. Of note, patients with familial hypercholesterolemia and patients with familial chylomicronemia were excluded in our Article 2 manuscript in order for those results to have better generalizability to the general population.

Overall conclusions

The overall conclusion from these two articles is that higher levels of hypertriglyceridemia are related to decreasing correlation and decreasing concordance between non-HDLC and apoB. This is clinically important because decreased concordance between non-HDLC and apoB can place patients into different cardiovascular disease risk equivalence categories. Although our studies are based on cross-sectional lipid profile data, clinically, cardiovascular disease risk stratification is often based on a single lipid profile result.

We are the first to use weighted Kappa to study reliability between these two lipid biomarkers. The weighted Kappa-statistic, even in patients with normal triglyceride levels, showed only moderate agreement between non-HDLC and apoB. Theoretically, we would have expected a higher level of agreement. Empirically, our study results are similar with previous studies (Sniderman *et al.* 2003; Sniderman *et al.* 2012; Lawler *et al.* 2017) that have shown a lower than theoretically-expected degree of correlation and concordance between non-HDLC and apoB in normal triglyceride levels and mild triglyceride elevation.

In mild hypertriglyceridemia, there is a notable discordance between non-HDLC and apoB for CVD risk prediction and actual incidence of CVD events (Sniderman *et al.* 2003; Sniderman *et al.* 2012; Lawler *et al.* 2017). Although many discordance analysis studies have noted the superiority of apoB over non-HDLC for CVD risk prediction up to *mild* HTG (Sniderman *et al.* 2012; Sniderman *et al.* 2014; Pencina *et al.* 2015; Lawler *et al.* 2017), the largest prospective observational studies (which included up to mild HTG) did not find apoB to be superior to non-

HDLC in models of CVD risk prediction (The Emerging Risk Factors Collaboration 2012; Welsh *et al.* 2019).

We have demonstrated concerns regarding the decreased reliability of non-HDLC versus apoB in hypertriglyceridemia for CVD risk equivalence categorization. However, the validity of non-HDLC versus apoB in hypertriglyceridemia for CVD risk categorization has not been explored, and will require prospective longitudinal cohort studies to determine CVD outcomes. Therefore, our research highlights a pressing need for CVD outcomes studies in patients over the full spectrum of mild, moderate, and severe hypertriglyceridemia. Future research is needed to assess the validity of non-HDLC versus apoB for CVD risk prediction in patients with hypertriglyceridemia, which is common in the general population.

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