Carla van Heerden, Agnes Magwete and Dineo Mabuza*

Evaluating the need for free glycerol blanking for serum triglyceride measurements at Charlotte Maxeke Johannesburg Academic Hospital

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Abstract

Background: The accurate and precise measurement of triglycerides is important due to the adverse effects associated with hypertriglyceridaemia. Most laboratory methods are based on enzymatic hydrolysis of triglycerides with measurement of the total glycerol. An elevated free glycerol concentration may result in overestimation of triglyceride concentrations. The removal of free glycerol by blanking may therefore be of clinical importance. The aim of this study was to compare the glycerol blanking and non-glycerol blanking triglyceride methods.

Methods: This was a method comparison study of 1518 samples from both in-patients and out-patients at Charlotte Maxeke Johannesburg Academic Hospital. Triglycerides were measured in each sample using both the blanking and the non-blanking methods. Analytical performance was assessed based on the National Cholesterol Education Program (NCEP) goals. Clinical impact was assessed according to the NCEP Adult Treatment Program III (ATP III) risk classification.

Results: The method median was significantly higher in the non-blanking compared to the blanking method (1.33 vs. 1.12 mmol/L, $p < 0.0001$) in all patients. The average bias was above the total allowable error of 15% across all groups. There was a significant change in NCEP ATP III risk classification, with fewer patients classified as normal (67.6% vs. 74.6%, $p < 0.0001$) with the non-blanking method compared to the blanking method.

Conclusions: There was a significant error when glycerol blanking for triglyceride determination was not performed. The non-blanking triglyceride method overestimates triglyceride concentrations. This does not only exceed analytical performance goals, but also impacts on patient categorisation and clinical decision making in all patients.

Keywords: blanking; free glycerol; non-blanking; potential diagnostic error; triglycerides.

***Corresponding author: Dineo Mabuza,** Department of Chemical Pathology, National Health Laboratory Service (NHLS) and University of the Witwatersrand, Charlotte Maxeke Johannesburg Academic Hospital, Wits Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa, Phone: +27(11)489-8762, E-mail: dineo.mabuza@nhls.ac.za.
https://orcid.org/0000-0002-6591-7026
Carla van Heerden and Agnes Magwete: Department of Chemical Pathology, National Health Laboratory Service (NHLS) and University of the Witwatersrand, Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa

Introduction

The accurate and precise measurement of triglycerides in serum is important because of the adverse effects associated with hypertriglyceridaemia. Hypertriglyceridaemia increases the risk of acute pancreatitis and cardiovascular disease (CVD) [1]. Although triglycerides are not directly atherogenic, they are associated with decreased high-density lipoprotein cholesterol (HDL-C) and remnant particles which are atherogenic [2]. Triglycerides may also affect low-density lipoprotein cholesterol (LDL-C) concentration as they are used in the Friedewald equation to indirectly calculate LDL-C [3].

Various studies have shown elevated non-fasting triglyceride concentrations to be associated with increased risk of myocardial infarction, ischaemic heart disease, ischaemic stroke and all-cause mortality [4–6]. Hypertriglyceridaemia is also associated with other metabolic abnormalities related to increased CVD risk, for example, metabolic syndrome, diabetes mellitus, liver- and renal disease [1, 7, 8].

The treatment of hypertriglyceridaemia depends on the cause and severity. The severity is determined by measuring the triglyceride concentration in serum. The National Cholesterol Education Program (NCEP) has established treatment decision points through expert consensus [9, 10]. As per NCEP, hypertriglyceridaemia is defined as a triglyceride concentration ≥1.7 mmol/L and initiation of therapy is recommended at concentrations ≥2.3 mmol/L [9, 10].

The most commonly used automated methods for triglyceride determination are based on the enzymatic

hydrolysis of triglycerides with measurement of the total serum glycerol present. Each glycerol molecule represents one triglyceride molecule [11]. These methods measure total glycerides which include mono-, di- and triglycerides as well as unesterified free glycerol [12]. Mono- and diglycerides have been found to contribute insignificantly to the total glyceride pool [11]. A small amount of free endogenous glycerol is physiologically present in blood where it either gets incorporated into triglycerides or enters other pathways of glucose metabolism [13]. However, high concentrations of free endogenous glycerol will result in overestimation of triglyceride concentrations [11].

Lipoprotein lipase, which regulates triglyceride breakdown into free fatty acids and glycerol, is under hormonal control. Epinephrine, thyroxine, growth hormone and glucagon increase its activity. This explains why emotional stress, surgery, critical illness and certain metabolic conditions will increase the endogenous release of glycerol [14]. Other causes of elevated endogenous glycerol are uncontrolled diabetes mellitus, hyperthyroidism, chronic alcohol use, liver disease, glycerol-containing alcoholic beverages, recent vigorous exercise and rarely glycerol kinase deficiency [11, 14–17]. Glycerolaemia exceeding the renal threshold is excreted in urine; therefore, impaired renal function may also contribute to hyperglycerolaemia. Exogenous causes of elevated free glycerol include contamination with skin care products, for example, hand cream, laboratory detergents, collection tubes with glycerol-coated stoppers and equipment used in the preparation of quality control materials [11, 14, 18]. Prolonged storage of whole blood at room temperature results in continuous triglyceride hydrolysis or the release of glycerol from phospholipids in erythrocyte cell membranes [11, 19].

To prevent pseudohypertriglyceridaemia, a glycerol blanking step can be performed. This involves either enzymatic consumption of free glycerol prior to triglyceride hydrolysis, i.e. internal blanking or measurement of free glycerol in order to subtract it from the total glycerol measured, i.e. external blanking [11, 19]. Internal blanking is more cost-effective than the external blanking method [17]. The American Association for Clinical Chemistry (AACC) set out recommendations on glycerol blanking with the aim of reducing triglyceride measurement errors [11, 19]. Their recommendations include: (1) laboratories use platforms with glycerol blanking capabilities; (2) blanking when the triglyceride concentration exceeds 2.3 mmol/L; (3) laboratory reports should state if the method incorporated glycerol blanking; (4) centres that conduct research or have specialised lipid units should employ glycerol blanking; (5) routine blanking on all hospital in-patient specimens; (6) blanking on outpatient specimens from specialised clinics (endocrine or diabetic); (7) refrigerate all specimens until analysis; (8) blanking for non-turbid hypertriglyceridaemia specimens [11, 19]. Despite their recommendations, blanking is not commonly incorporated into routine laboratory methods. In the USA less than 5% of laboratories routinely correct for endogenous glycerol [12]. Of all the participating laboratories of the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Program in 2018 only two laboratories using the Roche cobas® analyser corrected for free glycerol. Japan is one of the few countries that incorporate nationwide glycerol blanking and report net triglyceride concentrations [20].

In 1985, Naito [14] recommended that glycerol blanking is necessary for the accurate determination of triglycerides. Jeon et al. [21] were in support of this recommendation while Jessen et al. [16] concluded that in most circumstances routine glycerol blanking was not clinically necessary. Although the concept of glycerol blanking is not new, there is a paucity of studies in South Africa. In resource limited countries, like South Africa, the cost implications of additional analytical steps, further investigations and unnecessary medical treatment are of critical importance. This necessitates the need for studies that investigate if blanking is essential. This study aimed to compare the difference in serum triglyceride concentrations using both a glycerol blanking and a non-glycerol blanking method and to determine the magnitude of bias according to patient location, i.e. in-patients and outpatients. The clinical impact on patient classification was assessed using NCEP Adult Treatment Program III (ATP III) triglyceride cut-offs for risk.

Materials and methods

A total of 1518 patient samples collected between 7 January and 9 February 2019 at the Charlotte Maxeke Academic Johannesburg Hospital as part of routine care were included. All samples were analysed at the National Health Laboratory Service (NHLS) laboratory within the hospital. Clinical and demographic data was collected from the NHLS TrakCare™ laboratory information system.

Ethical approval

This study complied with the relevant institutional policies and was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificate No.M181102) and performed in accordance with the Declaration of Helsinki.

Samples

Samples were collected in BD Vacutainer® SST™ II *Advance* serum separating blood collection tubes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and stored at 2–8 °C until analysis. All samples were analysed within the stability period of 10 days. Samples containing insufficient volume for analysis by both methods were excluded.

Triglyceride measurement

Triglyceride measurements were performed on the Roche cobas® 8000 automated analyser (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Triglycerides were measured in each sample using the routine blanking method (TRIGB) and the non-blanking method (TRIGL). Verification studies were performed for both methods and the performance was within the manufacturer's claims.

The TRIGB method involves the elimination of free glycerol through coupled enzymatic reactions. Glycerol is phosphorylated to glycerol-3-phosphate by glycerol kinase in the presence of ATP. Glycerol-3-phosphate is subsequently oxidised to dihydroxyacetone phosphate and peroxide by glycerol-3-phosphate oxidase. In the final step peroxidase catalyses the oxidation of 4-chlorophenol by hydrogen peroxide to form an oxidation product [22].

The TRIGL method does not eliminate free glycerol in the sample prior to enzymatic hydrolysis of triglycerides. In both the TRIGB and the TRIGL methods triglycerides are hydrolysed by microbial lipoprotein lipase to yield glycerol and fatty acids. The glycerol undergoes further coupled enzymatic reactions, as described above, except that the final step includes 4-chlorophenol and 4-aminophenazone as substrates. A red dye, 4-(p-benzoquinone-monoimino)-phenazone, is formed in the Trinder endpoint reaction [22, 23]. The intensity of this coloured product is determined photometrically based on its absorbance which is directly proportional to the triglyceride concentration. TRIGB has been standardised against standard reference material (SRM) 909b and triolein standards [22]. TRIGL has been standardised against the isotope dilution mass spectrometry (IDMS) method [23].

Statistical analysis

Data was captured in Microsoft Office Excel (Microsoft, Seattle, WA, USA) and analysed using MedCalc®, version 18.11.6 (MedCalc Software, Ostend, Belgium) and Analyse-it®, version 5.30 (Analyse-it Software, Ltd, Leeds, United Kingdom). The Grubbs test was performed for outlier detection and one result was excluded which resulted in a total of 1518 results. Triglyceride concentrations from both methods were categorised according to NCEP ATP III cut-points as follows: Normal (<1.70 mmol/L), borderline-high (1.70–2.25 mmol/L), high (2.26–5.64 mmol/L) and very high (>5.64 mmol/L) [9, 10]. In addition, the data was grouped and analysed according to patient location, i.e. in-patient or out-patient.

Descriptive statistics were used to analyse data in terms of distribution, minimum and maximum values, mean, standard deviation, median and interquartile range (IQR). TRIGB was used as the comparative (X) method and TRIGL as the test (Y) method. Passing-Bablok regression and Bland-Altman plots were used to assess method agreement and bias. The bias was determined at the medical decision limits (MDL) of 1.7, 2.3 and 10 mmol/L. The NCEP goals for analytical performance of triglyceride measurements were used with allowable limits of ±5% and ±15% for bias and total error, respectively [24]. The McNemar test was used to assess if there were significant differences, between methods, in each NCEP category. A p-value of <0.05 was considered statistically significant.

Results

A total of 1518 patient samples were analysed and the majority (81.9%) were from out-patients (Table 1). The triglyceride concentrations ranged from 0.18–16.90 mmol/L and 0.37–18.45 mmol/L for TRIGB and TRIGL methods, respectively. Table 1 shows the serum triglyceride medians and IQR for the different groups. The median triglyceride concentration of TRIGB was significantly different compared to TRIGL (1.12 vs. 1.33 mmol/L, $p < 0.0001$) for all patients (Table 1).

The regression analysis equation was calculated as $y = 1.012x + 0.1623$ (Figure 1). The average bias for all patients was 0.193 (16.7%, $p < 0.0001$) (Figures 2 and 3). The average bias for in-patients and out-patients was 0.207 (16.7%, $p < 0.0001$) and 0.190 (16.8%, $p < 0.0001$) respectively. The bias at MDL of 1.7, 2.3 and 10 for all patients was 10.7%, 8.2% and 2.8%, respectively (Table 2). The correlation coefficient (r) was 0.99 across all groups (Table 2).

Table 3 shows the different categories of serum triglyceride concentrations based on the NCEP ATP III classification. Fewer patients were classified as normal (67.6% vs. 74.6%, $p < 0.0001$) with TRIGL compared to TRIGB (Table 3). There was no significant difference in the

Table 1: Serum triglyceride medians and IQRs.

Grouping	n (%)	Median, mmol/L	IQR, mmol/L	p-Value
All patients	1518 (100%)			<0.0001
TRIGB		1.12	0.76–1.71	
TRIGL		1.33	0.94–1.92	
In-patients	275 (18.1%)			<0.0001
TRIGB		1.25	0.81–1.93	
TRIGL		1.44	0.96–2.20	
Out-patients	1243 (81.9%)			<0.0001
TRIGB		1.11	0.75–1.68	
TRIGL		1.30	0.94–1.88	

IQR, interquartile range; TRIGB, blanked triglyceride method; TRIGL, non-blanked triglyceride method.

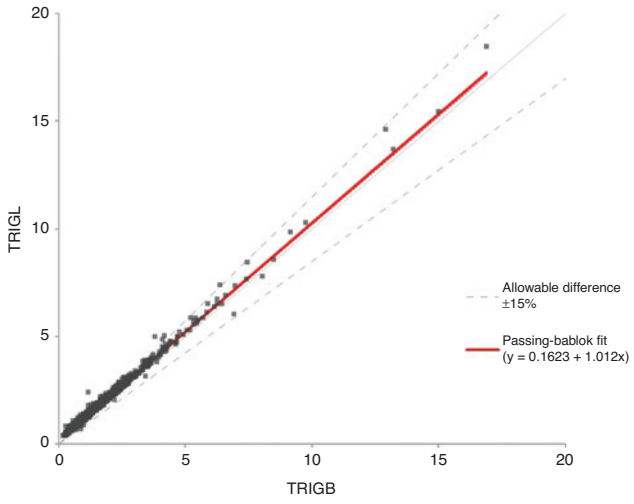

Figure 1: Scatter plot and regression analysis for TRIGB and TRIGL methods.

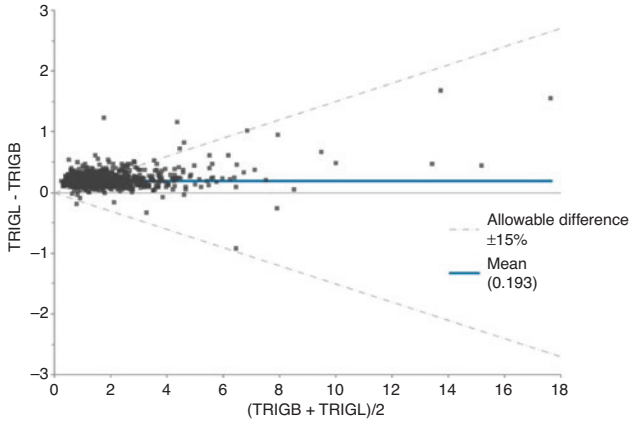

Figure 2: The absolute difference plot of TRIGB and TRIGL methods.

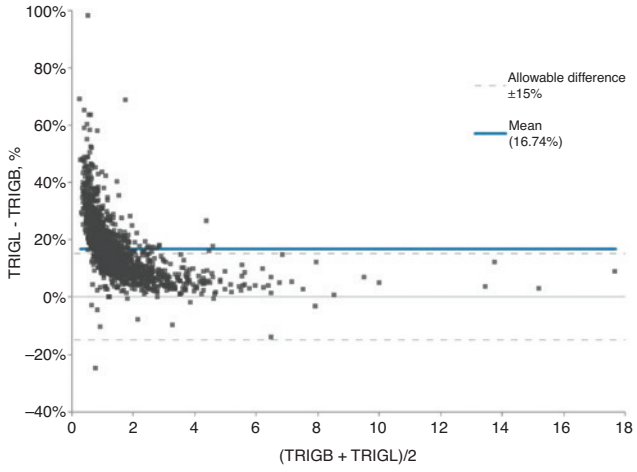

Figure 3: The percentage difference plot of TRIGB and TRIGL methods.

patient classification between the methods for triglyceride concentrations of >5.64 mmol/L (Table 3).

Discussion

The clinical relevance of free glycerol and the need for glycerol blanking remains a topic of debate. The presence of free glycerol, endogenous or exogenous, contributes to the total triglyceride concentration and may affect interpretation of results. Reasons that preclude laboratories from performing routine glycerol blanking include inconvenience, technical effort, limited availability of commercial blanking assays, increased turn-around time and increased analytical cost due to additional reagents, supplies and consumables [14, 16]. It is also generally assumed that a consistently small and insignificant amount of free glycerol is present in most patients [14]. This study compared a blanked triglyceride method (TRIGB) with a non-blanked triglyceride method (TRIGL) using NCEP analytical performance goals. The impact on patient classification was assessed by evaluating patient NCEP ATP III subclass categorisation in both methods. We also assessed if there were any differences between in-patients and out-patients.

We found that the TRIGB and TRIGL methods correlate well, with a correlation coefficient of 0.99 across all groups. Although a good correlation shows an association between the methods, it does not equate to agreement between methods [25]. The correlation coefficient does not also detect bias, which was invariably found in this study [26]. It does, however, indicate the usefulness of further statistics derived using linear regression [25]. The Bland-Altman difference plot demonstrated the bias between the two methods, especially in the low concentration range. The mean bias percentage found was significant as it exceeds the NCEP analytical goal of 15% for total allowable error [24].

In all patients, TRIGL was found to have a higher median triglyceride concentration compared to TRIGB. This finding is in keeping with studies by Choi et al. [27] and Jeon et al. [21] that found triglyceride concentrations measured by a non-glycerol blanking method were significantly higher compared to those measured by a blanking method. This is in contrast to Jessen et al. [16] who found that blanking would not significantly influence triglyceride concentration as all 339 of their out-patient samples had a free glycerol concentration of <0.28 mmol/L.

We found a higher median triglyceride concentration in the in-patient group compared to the out-patient group with both methods. Similarly, Jessen et al. [16] reported a higher incidence of elevated free glycerol in their in-patient group compared to their out-patient group. Choi et al. [27]

Table 2: Regression analysis and bias at medical decision limits.

	Slope	Intercept	r-Value	Bias % at medical decision levels		
				1.7 mmol/L	2.3 mmol/L	10 mmol/L
All patients (n = 1518)	1.012	0.1623	0.99	10.7	8.2	2.8
In-patients (n = 275)	1.007	0.1683	0.99	10.6	8.0	2.4
Out-patients (n = 1243)	1.012	0.1611	0.99	10.7	8.2	2.8

Table 3: Categories of serum triglyceride concentrations according to the NCEP ATP III classification.

Category	TRIGB, n (%)	TRIGL, n (%)	p-Value
All patients (n = 1518)			
Normal (<1.70 mmol/L)	1133 (74.6%)	1026 (67.6%)	<0.0001
Borderline-high (1.70–2.25 mmol/L)	155 (10.2%)	203 (13.4%)	0.0002
High (2.26–5.64 mmol/L)	209 (13.8%	263 (17.3%)	<0.0001
Very high (>5.64 mmol/L)	21 (1.4%)	26 (1.7%)	0.0625
In-patients (n = 275)			
Normal (<1.70 mmol/L)	193 (70.2%)	173 (62.9%)	<0.0001
Borderline-high (1.70–2.25 mmol/L)	27 (9.8%)	36 (13.1%)	0.1496
High (2.26–5.64 mmol/L)	48 (17.5%)	58 (21.1%)	0.0063
Very high (>5.64 mmol/L)	7 (2.5%)	8 (2.9%)	0.9999
Out-patients (n = 1243)			
Normal (<1.70 mmol/L)	940 (75.6%)	853 (68.6%)	<0.0001
Borderline-high (1.70–2.25 mmol/L)	128 (10.3%)	167 (13.4%)	0.0009
High (2.26–5.64 mmol/L)	161 (13%)	205 (16.5%)	<0.0001
Very high (>5.64 mmol/L)	14 (1.1%)	18 (1.4%)	0.1250

NCEP ATP III, national cholesterol education program adult treatment program III; TRIGB, blanked triglyceride method; TRIGL, non-blanked triglyceride method.

found a similar mean free glycerol concentration in both in-patients and out-patients. Despite finding higher triglyceride concentrations in the in-patient group in this study, comparison of the two methods does not indicate a preferential need for blanking in this group. Our findings are not surprising as hospital in-patients have been found to have a higher incidence of free endogenous glycerol. This may be related to critical illness, biliary obstruction, small bowel infarction, peritoneal- or haemodialysis solutions, total parenteral nutrition, enteral feeding, mannitol infusions, heparin infusions or glycerol-containing medication like nitro-glycerine, glycerine suppositories and propofol [11, 14, 16–18, 24, 28, 29].

Using a non-blanking method, the triglyceride concentration was overestimated in all samples. If a falsely elevated triglyceride concentration is used in the Friedewald equation, it can lead to the underestimation of CVD risk using calculated LDL-C. The Friedewald equation calculates LDL-C using very low density lipoprotein (VLDL-C), which in turn is calculated from triglycerides: calculated LDL-C = total cholesterol–HDL-C–VLDL-C (triglycerides/2.2) [3]. The equation becomes unreliable when the triglyceride concentration exceeds 2.8 mmol/L and does not apply when triglycerides exceed 4.5 mmol/L [11]. Interestingly, the Friedewald equation was derived using "net" or "true" triglyceride values obtained from a fluorometric assay [3, 14, 20]. Fortunately, in our laboratory, the LDL-C is measured, and therefore there is no effect on LDL-C irrespective of the method used for triglyceride measurement.

Although different classification systems are available for triglycerides, they all use 1.7 mmol/L as the cut-off value for a normal triglyceride concentration [8]. This concentration is lower than the preceding NCEP ATP II cut-off value of 2.3 mmol/L [24]. The NCEP ATP III Triglyceride Classification for risk uses 1.7 mmol/L based on prospective observational studies [1]. This cut-off is also used as part of the diagnostic criteria for metabolic syndrome [7]. To assist in the evaluation of cardiovascular risk, the Endocrine Society Clinical Practice Guideline recommends that patients with triglyceride concentrations between 1.7 and 2.3 mmol/L should be identified [1]. The 2018 South African Heart Association and the Lipid and Atherosclerosis Society of Southern Africa's Dyslipidaemia Guidelines [30] are based on the

2016 guidelines from the European Society of Cardiology and the European Society of Atherosclerosis [31]. In their 2018 consensus statement the South African guidelines recommend medical therapy for patients with triglyceride concentrations greater than 2.3 mmol/L or those at high risk of cardiovascular disease or pancreatitis [30]. Triglyceride concentrations greater than 5.6 mmol/L, especially if greater than 10 mmol/L, is associated with an increased risk of acute pancreatitis [30]. We analysed bias at MDL of 1.7 mmol/L, 2.3 mmol/L and 10 mmol/L. The bias at MDL of 1.7 mmol/L and 2.3 mmol/L exceeded the NCEP analytical goal of 5% for bias across all patient sub-groups.

With the TRIGL method, we classified 13.4%, 17.3% and 1.7% of results as borderline-high, high and very high, respectively. These findings are similar to the 1999–2004 National Health and Nutrition Examination Survey's results of 14%, 16% and 2%, respectively [32]. We found that, for all patient groups, there is a significant change in NCEP ATP III risk classification between the two methods. When the non-blanking method is employed, fewer patients are classified as normal compared to the blanking method. This results in more patients being eligible for treatment when using the non-blanking method. Although it is more expensive to blank for free glycerol, the cumulative cost of medical therapy resulting from such misclassification should also be considered. Our finding that there was no significant difference in the patient classification for triglyceride concentrations of >5.64 mmol/L may be due to the small number of patients within this category.

Laboratories' decision whether to use a blanking or non-blanking method is further complicated by calibrator traceability. In 2011, the National Institute of Standards and Technology (NIST) developed the primary reference measurement procedure for triglyceride determination based on gas chromatographic IDMS (GC-IDMS) [12]. NIST went on to develop standard reference materials (SRMs) with assignment of target values based on IDMS. NIST SRM1951b, which is lipids in frozen human serum, and NIST SRM909b, as lipids in lyophilised human serum, includes both glycerol blanked and non-blanked triglyceride concentrations. However, SRM1951c, as lipids in frozen human serum, and SRM909c, as frozen human serum, that were subsequently developed, include only non-blanked triglyceride concentrations (total glycerides) [17]. The Advisory Committee of the Australasian Association of Clinical Biochemists (AACB) Working Party on Accuracy and Standardisation assigns sample target values for the RCPA Quality Assurance Programs. For triglycerides NIST909c is used as the primary reference material. Considering that most laboratories are not blanking, the Centres for Disease Control and Prevention (CDC) also use an IDMS reference method that measures total glycerides [17]. As a consequence, laboratories utilising glycerol-blanking methods are unable to compare their results to the CDC reference method. The glycerol content in calibrators, control materials and survey samples may therefore be variable [12, 24]. The Roche TRIGB calibrator has appropriately been standardised against SRM909b. However, the results obtained from this assay might not be comparable to those of laboratories that use calibrators standardised against SRM909c or SRM1951c.

From previous surveys and proficiency scheme data it is clear that the recommendations on glycerol blanking, as set out by the AACC, have been poorly incorporated into standard laboratory practice. As with most guidelines, the implementation is optional and the majority of laboratories choose not to blank as it appears too expensive and laborious. Our study indicated the need for blanking across all triglyceride concentrations and although blanking will increase cost, the accuracy of triglyceride measurement directly affects the assessment of patients. Unfortunately, even major clinical lipid guidelines do not mention the utility or advocate the use of glycerol blanking. In this regard, the introduction of blanking into clinical practice guidelines may educate clinicians on its importance and encourage the incorporation of blanking across more laboratories.

This study had a few limitations. Individual disease states and clinical histories, including medication use, were not assessed. Therefore, possible causes for elevated free glycerol cannot be derived and recommendations on the need for glycerol blanking in specific disease groups cannot be made. Additionally, neither of the methods used were compared against the IDMS reference method due to financial constraints. A further limitation is that the fasting status of patients was not documented. Evidence supports the use of non-fasting triglyceride measurement in screening, however, the NCEP classification is based on concentrations following a 12-h fast and the Endocrine Society Clinical Practice Guideline Task Force recommends fasting concentrations for the diagnosis of hypertriglyceridaemia [1]. Lastly, the results of this study may not be applicable to other patient populations or transferrable across different analytical platforms. A multicentre study is required to establish the need for glycerol blanking in the general South African population. Such a multicentre study should also include a larger number of patients in the >5.64 mmol/L category. Further studies to evaluate the effect of triglyceride blanking on calculated LDL-C might also be of clinical interest.

To the best of our knowledge this is the first study assessing the need for glycerol blanking in a South African population. It included a large number of specimens from

a diverse population assumed to have various medical conditions. Results spanned the measuring range which is appropriate for a method comparison study.

In conclusion, we found that a statistically and clinically significant error was introduced when blanking for free glycerol was not performed for triglyceride determination. The non-blanking triglyceride method overestimates triglyceride concentrations. This overestimation not only exceeds analytical performance goals, but also impacts on patient categorisation and clinical decision making in both out-patients and hospital patients. Based on these findings, we support the AACC guidelines and recommend glycerol blanking with all triglyceride measurements.

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