# EVALUATION OF TOTAL SERUM LIPIDS USING ENZYMATIC METHODS

Adrian Covaci[1], Stefan Voorspoels[1], Cathrine Thomsen[2], Bert van Bavel[3], Paul Schepens[1]

[1]University Of Antwerp
[2]Norwegian Institute of Public Health
[3]Örebro University

## Introduction

The concentration of lipid-soluble persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), may often be better expressed relative to the total lipid content than to the samples wet weight. These pollutants are partitioning among various tissues depending on their lipid content, lipid composition and on the characteristics of the tissue. Therefore, the reporting of POPs concentrations in ng/g lipid weight depends on the lipid content in the tissue and on the method used to measure the amounts of lipids.

Historically, gravimetrical methods[1-3] have been used for the measurement of the total lipid (TL) content in serum. The measured TL depends strongly on the solvent mixture used for extraction and on the analyst's skills. Recently, enzymatic methods have become increasingly popular for the determination of lipids in serum[4-6]. While performing measurements of all individual classes of lipids (triglycerides (TG), cholesterol (CHOL) and phospholipids (PL)), the enzymatic methods offer a more accurate way of measuring the TL content. However, enzymatic lipid measurements are often done in clinical laboratories where PL measurements are not routinely performed and therefore, the TL needs to be predicted from measurements of only TG and CHOL. Several formulas are presented in the literature[4,7], but these have been derived from a relatively low number of subjects (n=81 and 47, respectively).

The goal of the present work was to compute a formula for predicting TL from TG and CHOL. This formula was obtained using a data set (n=483) that includes TG, CHOL and PL measurements. Other aims were to compare results obtained for TL with different formulas in the literature and to estimate the errors made on expressing PCBs in ng/g lipid weight when using different formulas to predict TL.

## Materials and Methods

*Description of data sets*

Lipid measurements in human serum and plasma were available from 4 populations from Belgium, Sweden and Norway. The first group (G1) consisted of young women from Belgium[6] (n=98, 32 ± 4 years, range 24 – 42 years, sampled in 1996-1998. The second group (G2) consisted of older women from Belgium[8] (n=47 pooled samples derived from 200 individual serum samples, 58 ± 4 years, range 50 – 65 years, sampled in 1999). The third group (G3) contained females from Sweden with a high dietary intake of fish (n=141, mean 40 years, range 19 - 56 years, sampled in 2001). The fourth group (G4) consisted of Norwegian males and females (n=197, mean 56 years, range 21-88 years, sampled in 2003). For these groups, individual measurements of TG, CHOL (sum of free and esterified cholesterol) and PL were available. The serum TL was calculated by summation of concentrations of TG, CHOL, and PL.

In each case, blood was collected in a vacuum heparintubes, centrifuged (15 min, 2000 g) within 24 hafter collection. Serum was separated and stored at -20°C until analyzed.

To compare the newly computed formula for the prediction of TL with other formulas described in the literature, three other groups of subjects from Belgium (C1, n=132; C2, n=499 and C3, n=200) were used. For groups C1-3, only TG and CHOL individual measurements were available. PCB levels[9] were available for group C1.

*Statistics*

A formula for predicting TL from independent measurements of TG and CHOL was derived by multiple linear regression analysis conducted on data sets containing measurements of TG, CHOL and PL. Correlations between the TL predicted with different formulas and TL summed from TG, CHOL and PL were calculated using Pearson correlation coefficients. A finding was considered statistically significant if its *p*-value was less than 0.05. ANOVA with Scheffe's post-hoc test was used to compare the TL results obtained with different formulas. All statistical calculations were performed with Statistica v.5 for Windows (Statsoft, Tulsa, OK, USA).

**Results and Discussion**

The use of enzymatic techniques for the measurement of lipid classes in serum offers a good standardized way of expressing the TL content. Considerably lower volumes of serum are needed compared to gravimetric techniques. In addition, the assays for determination of individual classes of lipids are automated, offer a good reproducibility, are regularly calibrated and do not rely simply on the analyst's skills or the extraction solvents used. The assays for each lipid class follow the same principle and are based on the spectrophotometric detection of one of the products formed after specific enzymatic hydrolysis of lipids.

Ideally, the TL concentration should be calculated by the summation of concentrations of TG, CHOL and PL. However, PL are not often measured in clinical laboratories and therefore, several formulas[4,7] for the prediction of TL from measurements of only TG and CHOL are available in the literature.Using 4 groups (G1-G4) from Belgium, Sweden and Norway (n=483) for which measurements of TG, CHOL and PL were available, a new formula for predicting TL was deducted using multiple linear regression.

Table 1. Formulas for prediction of TL from measurements of TG and CHOL (all expressed in mg/dl).

| Nr. | Formula | Reference |
|---|---|---|
| 1 | TL = 2.27*CHOL + TG + 62 | Philips et al.[4] |
| 2 | TL = 1.28*(CHOL + TG) + 96 | Thuresson et al.[7] |
| 3 | **TL = 1.33*TG + 1.12*CHOL + 148** | Present study |

For the G1-G4 group, a good correlation (r=0.949, p<0.001) was computed between the measured TL (from summation of TG, CHOL and PL) and the TL values predicted by Formula 3. Furthermore, a similar correlation between the measured TL and the TL values predicted with Formula 2 was found (r=0.947, p< 0.001), while the corresponding correlation using Formula 1 was weaker (r=0.897, p< 0.001).

Table 2. Mean values (standard deviation) for TL (mg/dl) predicted by applying Formulas 1-3 to independent data sets for which TG and CHOL measurements were available. Values followed by different letters are significantly different (p<0.01)

| | | TL measured | TL predicted | | |
|---|---|---|---|---|---|
| | N | Enzymatic | Formula 1 | Formula 2 | Formula 3 |
| Group C1 | 132 | n.a. | 642 (131)$^{A}$ | 505 (114) $^{B}$ | 531 (113) $^{B}$ |
| Group C2 | 499 | n.a. | 598 (118) $^{A}$ | 464 (96) $^{B}$ | 489 (94) $^{C}$ |
| Group C3 | 200 | n.a. | 539 (95) $^{A}$ | 435 (79) $^{B}$ | 465 (76) $^{C}$ |
| Groups G1-4 | 483 | 602 (151) $^{A}$ | 788 (224) $^{B}$ | 587 (153) $^{A}$ | 599 (143) $^{A}$ |

TL values predicted for different groups of subjects using Formulas 1-3 were compared using ANOVA (Table 2). For each studied group, TL values predicted with Formulas 2 and 3 were relatively similar and were significantly lower than TL values produced with Formula 1 (Table 2). The obtained TL mean values always increased in the order: Formula 2 < Formula 3 < Formula 1. The results obtained using Formula 1 (derived in 1989 from a group of 81 adults[4] from Missouri, USA) may suggest that its use should be revised.

Differences in results are exclusively due to the prediction of PL from TG and CHOL measurements. PL are derived by Philips et al.[4] using the following formula PL = 0.766*CHOL + 62, while PL = 0.28*(TG + CHOL) + 96 and PL =

0.33*TG + 0.12*CHOL + 148 (all units in mg/dl) are derived using Formulas 2 and 3, respectively. These formulas were applied for calculating PL for the selected populations and results are presented in Table 3.PL levels are highly dependent on the dietary habits of the studied population and are subject to high variation between individuals. Furthermore, they are not in strict relation with TG and CHOL ($r < 0.48$ for Formulas 1-3 applied to Group G1-G4).

Table 3. Mean (standard deviation) for PL (mg/dl) predicted by applying Formulas 1-3 to independent data sets for which only TG and CHOL measurements were available. Values followed by different letters are significantly different ($p<0.01$).

| | N | Measured PL | PL-Formula 1 | PL-Formula 2 | PL-Formula 3 |
|---|---|---|---|---|---|
| Group C1 | 132 | n.a. | 215 (31) $^{A}$ | 185 (25) $^{B}$ | 211 (24) $^{A}$ |
| Group C2 | 499 | n.a. | 212 (31) $^{A}$ | 176 (21) $^{B}$ | 201 (19) $^{C}$ |
| Group C3 | 198 | n.a. | 190 (23) $^{A}$ | 170 (17) $^{B}$ | 200 (16) $^{C}$ |
| Groups G1-4 | 483 | 218 (54) $^{A}$ | 268 (68) $^{B}$ | 203 (34) $^{C}$ | 218 (26) $^{A}$ |

Several studies[5-7] have shown moderate to good correlations between TL calculated by summation of enzymatically measured TG, CHOL and PL and gravimetrically measured TL ($r=0.82$, 0.75 and 0.98, respectively). For groups C3, G1 and G2, for which TG and CHOL, but also gravimetrically measured TL were available, correlations between the predicted TL and gravimetric TL are presented in Table 4.

Table 4. Mean (standard deviation) for TL (mg/dl) predicted by applying Formulas 1-3 to independent data sets for which only TG and CHOL measurements were available. Pearson's correlation coefficients are calculated between TL obtained gravimetrically and predicted TL. Values followed by different letters are significantly different (*$p<0.05$ for A-B, **$p<0.05$ for C-D, otherwise $p<0.01$).

| | | TL measured | TL predicted | | |
|---|---|---|---|---|---|
| | N | Gravimetric | Formula 1 | Formula 2 | Formula 3 |
| Group C3 | 200 | 511 (117) $^{A*}$ | 539 (95) $^{B*}$, r=0.69 | 435 (79) $^{C**}$, r=0.69 | 465 (78) $^{D**}$, r=0.68 |
| Groups G1 | 98 | 724 (217) $^{A}$ | 540 (122) $^{B}$, r=0.68 | 436 (106) $^{C}$, r=0.63 | 466 (104) $^{C}$, r=0.62 |
| Groups G2 | 47 | 662 (70) $^{A}$ | 784 (72) $^{B}$, r=0.85 | 628 (66) $^{A}$, r=0.85 | 650 (66) $^{A}$, r=0.85 |

Gravimetric methods are more prone to weighing errors due to low lipid content and strongly depend on the analyst's skills and on the solvent extraction mixture used. For serum, which contains lipids of different polarity, the solvent mixtures used for extraction will largely be responsible for the measured lipid content. While often being part of the procedure used for the chemical analysis of POPs and not requiring additional work for lipid determination, the gravimetric methods may affect the detection limit of certain analytes (when an aliquot of extract is used for lipid determination) or may lead to the loss of volatile analytes during the extract evaporation to dryness. Mixtures consisting of a polar solvent and a non-polar solvent (methanol:chloroform[1,2] or iso-propanol:cyclohexane[3]) were shown to give the most accurate results. The Folch method[1] was shown to yield 15-40% higher lipid amounts than a mixture of hexane:acetone probably due to only partial extraction of polar lipids (e.g. PL) in the latter method[10]. However, long experience is usually required to obtain high reproducibility with gravimetric methods, demonstrated in intercalibration studies for POPs where the lipid content was requested to be additionally measured[11].

*Errors in determination of POPs*

The quantitative determination of PCBs in serum requires accuracy at below-ng range and precision within ± 10%. If serum concentrations are lipid normalized, additional uncertainty is added by the lack of consistency in gravimetric lipid measurements or in the lipid values predicted with different formulas. Since the concentration of lipids in plasma is low, an error in such a number can change the lipid normalized pollutant concentrations dramatically.

Compared to results obtained applying Formula 3 (computed in the present study), differences of -16% and +5% in the concentration of PCBs were obtained using TL predicted with Formula 1 and 2, respectively. A similar trend was observed also for p,p'-DDE (-17% and +5%, respectively). However, in both cases, results obtained using Formulas 2 and 3 were statistically not different (Table 5).

Table 5. Mean (standard deviation) concentrations of PCBs (ng/g lipid weight) obtained after applying Formulas 1-3 to an independent data set for which only TG and CHOL measurements were available. Values followed by the different letters are significantly different (*p<0.05, otherwise p<0.01).

| | N | Formula 1 | Formula 2 | Formula 3 |
|---|---|---|---|---|
| p,p'-DDE | 130 | 187 (121) $^{A*}$ | 237 (154) $^{B*C}$ | 225 (147) $^{AC}$ |
| Sum 7 marker PCBs | 132 | 235 (89) $^{A}$ | 296 (112) $^{B}$ | 281 (106) $^{B}$ |

## Conclusions

Our new formula (**TL = 1.33*TG + 1.12*CHOL + 148** (mg/dl)) based on a large data set (n=483) from independent European populations was computed for the calculation of TL based on measurements of TG and CHOL. TL levels derived using Formula 3 were found to be similar to TL levels obtained using the formula of Thuresson[7], but lower than TL levels obtained using the formula of Philips[4]. The use of the latter formula will therefore result in the underestimation of lipid-normalized concentrations of POPs compared to values produced using Formula 3.However, TG and CHOL are not good predictors of PL and therefore, the TL levels derived using a mathematical formula which includes TG and CHOL will not be as precise as the TL levels summed for measurements of TG, CHOL and PL.

## Acknowledgments

Dr. Gudrun Koppen (VITO, Belgium), Dr. Hugo Neels (AZ Stuivenberg, Belgium) and Ingrid Ericson (Örebro University, Sweden) for sharing data containing lipid measurements.

## References

1. Folch J., Lees M. and Stanley S. (1957) *J Biol Chem.* 226: 497-509.

2. Bligh E.G. and Dyer W.J. (1959) *Can J BiochemPhysiol.* 37: 911-917.

3. Smedes F. (1999) *Analyst* 124: 1711-1718.

4. Philips D.L., Pirkle J.L., Burse V.W., et al. (1989) *Arch Environ ContamToxicol.* 18: 495-500.

5. Grimvall E., Rylander L., Nilsson-Ehle P., et al. (1997) *Arch Environ ContamToxicol*. 32: 329-336.

6. Pauwels A., Covaci A., et al. (2000) *Arch Environ ContamToxicol.* 39: 265-270.

7. Thuresson K., Bergman A. and Jakobsson K. (2005) *Environ SciTechnol.* 39: 1980-1986.

8. Koppen G., Covaci A., Van Cleubenbergen R. et al. (2002) *Chemosphere* 48: 811-825.

9. Voorspoels S., Covaci A., Maervoet J. and Schepens P. (2002) *Bull Environ ContamToxicol.* 69: 22-29.

10. Sandau C.D., Ph D Thesis, Carleton University, Canada, 2000.

11. AMAP Ring Test, Institut Nationale de Santé Publique Quebéc, Centre de Toxicologie, Report 2004.