Howard A. Morris

School of Pharmacy and Medical Sciences, University of South Australia and Chemical Pathology Directorate, SA Pathology, Australia

# The clinical impact of assay standardization and traceability: A case study of cholesterol

**Corresponding author:**

Prof. Howard Morris
Chemical Pathology Directorate,
SA Pathology,
Box 14 Rundle mall Post Office,
Adelaide, South Australia 5000, Australia
E-mail: howard.morris@unisa.edu.au
Phone: +61 882 223 031

Folia Medica Copernicana 2015;
Volume 3, Number 1, 1–6

Copyright © 2015 Via Medica
ISSN 2300–5432

**ABSTRACT**

Coronary heart disease (CHD) is the major cause of death internationally, and yet most can be prevented using population-wide strategies by addressing behavioral risk factors, including reduction of blood cholesterol levels. Following the definition of clinical decision limits for cholesterol according to risk of CHD, an international effort was undertaken to establish traceability of routine clinical cholesterol assays to improve reproducibility and accuracy. This task has been achieved through the establishment of the Cholesterol Reference Measurement Laboratory Network. This work is associated with a reduction in the number of persons with elevated cholesterol levels and reduction in the death rates from CHD. It has been a major achievement for the discipline of laboratory medicine and a guide for future efforts for the improvement of patient outcomes.

**Key words:** traceability, cholesterol assays, cardiovascular disease, reference measurement procedures

Folia Medica Copernicana 2015; 3 (1): 1–6

## Introduction: Why is traceability of clinical laboratory lipid measurements important for the community?

Coronary heart disease (CHD) remains the leading cause of death worldwide with an estimated 17.5 million deaths in 2012, representing 31% of all global deaths [1]. Current opinion is that most cardiovascular diseases can be prevented using population-wide strategies by addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol. Recognised risk factors include hypertension, diabetes, hyperlipidaemia or already established disease, and such people need early detection and management with counselling and medicines, as appropriate [1]. Therefore, CHD is a major healthcare burden, for which there are at least three modifiable factors including high blood cholesterol levels.

Conclusive evidence from randomized, controlled clinical trials demonstrating that lowering blood cholesterol reduced the risk of CHD was published in 1984 [2], following which the National Cholesterol Education Program (NCEP) was established to reduce the prevalence of increased blood cholesterol [3]. By 1988, the NCEP had identified the following clinical limits for blood cholesterol: less than 5.2 mmol/L (200 mg/dL) was Desirable; 5.2 to 6.2 mmol/L (200 to 239 mg/dL) was Borderline and greater than 6.2 mmol/L (240 mg/dL) was High [4]. NCEP also undertook a high profile publicity campaign under the slogan "Know your number and do something about it" to raise public awareness of the importance of knowing their blood cholesterol level [3]. This campaign generated unprecedented attention on the performance of clinical laboratories and the reliability of clinical cholesterol testing [5]. Therefore, the reliable measurement of blood cholesterol became, initially a US, and later an international public health priority.

## History of standardization of lipid testing

Already in 1957, a requirement for high quality blood cholesterol assays was being raised within the US, e.g. at the Conference on Longitudinal Cardiovascular Studies following the experience gained from researchers exchanging serum specimens amongst themselves [6, 7]. They were concerned by the wide range of results, and there was a general agreement that clinical cholesterol

assays needed to improve reproducibility and comparability. The first Cooperative Cholesterol Standardization Program was established aiming to develop suitable serum-based reference materials for cholesterol with the initial specific tasks to determine if lyophilized human serum was a suitable medium, prepare highly purified cholesterol suitable for the use as a primary standard and to quantify variability of cholesterol testing at that time [8]. After extensive evaluation of reproducibility, the Abell-Kendall method for blood cholesterol measurement was selected as the reference method, which remains today as conducted by or under the supervision of the laboratory of the US Centers for Disease Control and Disease Prevention (CDC). At this time, they found that this method was capable of a coefficient of variation of 1.5% [9].

Further work was encouraged when the National Heart Institute initiated collaboration with CDC in 1961 following their recognition that many of the failed heart disease studies were attributable to questionable cholesterol measurements [8], demonstrating once again the well-known management adage “If you can’t measure it (or in this case measure it adequately), you can’t manage it”. In 1962, the World Health Organization (WHO) appointed CDC as a Collaborating Center for Reference and Research in Blood Lipids [8]. CDC’s role was to offer standardization assistance to international research laboratories supporting WHO studies of CHD. By 1965, services were being provided to 65 participating laboratories in the USA and 18 laboratories internationally.

A major milestone in clinical chemistry was the development of the first reference measurement system for cholesterol by the American Association of Clinical Chemistry (AACC) Cholesterol Reference Method Study group formed in 1975. The Abell-Kendall method was selected as the reference method, and isotope dilution-mass spectrometry (IDMC) was selected as the definitive method with the establishment of a further collaboration, this time with the National Institute of Science and Technology (NIST), the USA National Metrology Institute. Thus, the CDC Abell-Kendall reference method, the NIST IDMC definitive method, the NIST Standard Reference Material (SRM) 911 pure cholesterol primary standard and the serum reference materials prepared by CDC and offered by NIST made up the components of the National Reference System for Cholesterol [10]. This development represented the highest level of the application of metrological principles to laboratory medicine at that time, eventually initiating a major activity in standardization of clinical assays that continues today.

Clinicians and laboratory specialists now started collaborating to decide on the clinical requirements for blood cholesterol assays with regard to reproducibility and accuracy. The US National Heart, Lung, and Blood Institute launched the National Cholesterol Education Program (NCEP) aiming to diminish the prevalence of hypercholesterolemia to reduce CHD disease and death. NCEP adopted performance goals for clinical laboratory cholesterol testing such that the coefficient of variation (CV) was less than 3% and bias was not to exceed ± 3% [11]. For clinical laboratories to achieve these recommendations, cholesterol testing in each laboratory had to be traceable to the NIST reference material SRM911.

## Application of traceability of cholesterol assays to routine clinical laboratories

Following the public health campaign of the NCEP, the numbers of cholesterol test requests to clinical laboratories were increasing markedly. To cope with the large number of patient specimens it is impractical for clinical laboratories to perform reference methods on patient specimens. Therefore, a translation of the accuracy of the reference procedures to routine clinical procedures is required. This translation is known as establishing traceability of the routine procedures to the purified NIST SRM911. The problem for clinical assays is that patient cholesterol is in a complex matrix of serum which has the potential to interfere with routine clinical cholesterol assays.

The initial work utilised lyophilized serum specimens for which cholesterol had been measured by the reference method and, therefore, calibrated against SRM911. This was a satisfactory strategy while routine assays employed chemical methods. However, as the clinical requirement for patient analyses increased, the In Vitro Diagnostic industry expanded technology with the application of enzymes for the assay of blood cholesterol. It was now found that the calibration of these assays was not accurate when lyophilised calibrators, controls or reference materials were used. Thus, these lyophilized materials were not commutable for the enzymatic cholesterol assays [12].

## Commutability of assays is an essential requirement for traceability

Non-commutability of an assay is evident when matrix-induced biases (matrix effects) change the analyte’s reactive properties between processed samples, such as the one which occurs with lyophilization of serum, and patient specimens. Primary reference materials are usually purified chemicals, and primary, as well as secondary reference methods are usually independent of matrix effects because they involve extraction procedures. However, when secondary reference materials

are prepared for the assessment of blood, plasma or serum-based assays, they have to be verified as commutable with patient specimens. Such testing involves measuring the secondary reference materials and fresh patient specimens by a comparative method such as the Abell-Kendall reference method for cholesterol, and an evaluated method such as an enzymatic method for cholesterol. If the measured cholesterol levels in the patient pools do not describe the same relationship between the two methods as the fresh patient serum specimens, then secondary reference materials are not commutable.

A popular strategy to address this issue of non-commutability is to use fresh patient specimens. If relative large pools of suitable blood products are available, then this strategy is feasible. The CDC has developed considerable expertise in the preparation of such pools, which can be calibrated by a reference method, and aliquots of the calibrated serum pool can be stored and distributed to appropriate laboratories for calibration of working or routine methods. This strategy overcomes any commutability problems although each pool has to be proven to be commutable with fresh patient specimens before being accepted and proven suitable for distribution as a secondary reference material [12].

The clinical assessment of lipids includes measurements of blood High Density Lipoprotein (HDL) cholesterol and triglyceride with Low Density Lipoprotein cholesterol usually being calculated, using the Friedewald formula [13], from total cholesterol, HDL-cholesterol and triglycerides. Reference systems have been established for these variables, as well as cholesterol. Although there is no primary reference method or material for HDL-cholesterol, a secondary reference method involving ultracentrifugation, Heparin-$Mn^{2+}$ precipitation and quantification of cholesterol by the Abell-Kendall method (supervised by CDC) and secondary reference material of CDC frozen human serum pools (NIST SRM 1951b) are available [14]. Similarly, no primary reference procedure or material is available for LDL-cholesterol, however, a secondary reference procedure involving beta-quantification of cholesterol in the ultracentrifuge bottom fraction (density greater than 1.006 kg/L) by the Abell-Kendall method, and then a calculation as described [14]. NIST has developed a primary reference procedure for triglycerides using liquid chromatography-mass spectrometry technology, and tripalmitin (NIST SRM 1595) is the primary reference material. A secondary reference procedure developed by CDC involves methylene chloride extraction combined with silicic acid treatment, ethanolic-KOH hydrolysis, oxidation by metaperiodate and colour development with chromotropic acid [14]. Lipoprotein(a), Apo-A1 and ApoB also have reference procedures and materials available [14].

## Rolling out traceable clinical cholesterol assays to routine clinical laboratories

The establishment of traceability for clinical laboratory assays is a large project because of the great number of laboratories, wide range of assays and the number of specimens processed by each of the clinical laboratories. For example, in the USA alone there are over 150,000 laboratories providing blood cholesterol assays for patients. Consequently, an approach to individual laboratories, to ensure that their cholesterol assays were traceable to the recognized standard, was not feasible. However, the overwhelming adoption of commercial reagents, with or without automated analytical systems, by clinical laboratories lends itself to a strategy of focusing on the In Vitro Diagnostic manufacturers, which was a much more feasible target [12].

Servicing the international In Vitro Diagnostic industry is still a large task and one which is continuing to grow as many new manufacturers enter the market, particularly from the rapidly developing geographical areas, including Eastern Europe and Asia. This strategy of servicing the In Vitro Diagnostic industry requires considerable resources to meet time deadlines for production, as well as accommodate the quantity of analyses and secondary reference materials that is required. Therefore, it was considered essential to establish a network of reference laboratories distributed internationally. Each reference laboratory was only accepted into the network after demonstrating long-term support for performing the reference assay and expertise to maintain performance of the Abell-Kendall assay to a high standard [12].

Reference laboratories demonstrate their acceptable performance by participating in an external quality assurance program using fresh frozen human serum specimens calibrated by CDC. Requirements for the performance of blood cholesterol assays have been used to set the standard for reference laboratories with an assay CV less than 1% and a Bias of plus or minus 1%. These are significantly tighter limits than required for laboratories providing a clinical service. These CDC accredited reference laboratories are authorized to provide Certificates of Traceability to IVD manufacturers and laboratories [12].

With the establishment of the network of reference laboratories the CDC-National Heart, Lung and Blood Institute (National Institutes of Health) Lipid Standardization Program was created [11]. The overall structure and relationships between stakeholders of the Choles-

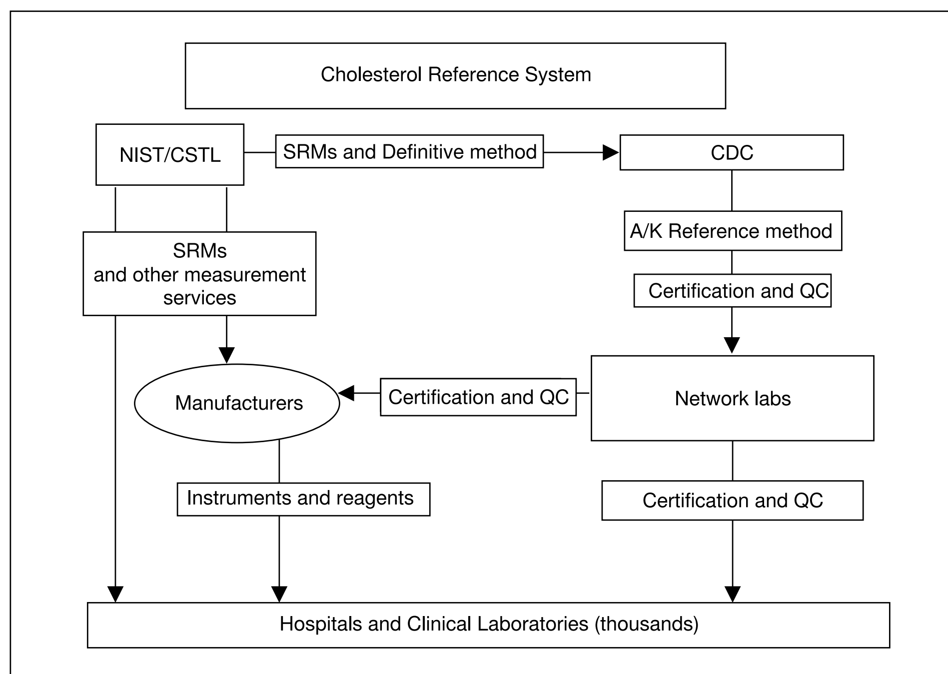


**Figure 1.** Structure of the Cholesterol Reference Measurement Laboratory Network and relationships between each of the stakeholders ([12] and Dr David Bunk, NIST personal communication). NIST — National Institute of Science and Technology; CSTL — Center for Science Teaching and Learning; CDC — Centers for Disease Control and Prevention; SRMs — Standard Reference Materials; A/K — Reference method: Abell-Kendall reference method for cholesterol assay; QC — quality control

terol Reference Measurement Laboratory Network for establishing traceability of clinical cholesterol assays in practice is summarized in Figure 1. The calibration hierarchy and metrological traceability for blood cholesterol measurement, upon which traceability of cholesterols is based, is presented in Figure 2. The number and types of institutions accessing the cholesterol reference measurement procedure, including the network of reference laboratories, involves some 100 laboratories, 60% of which are located in the US and make up about 60% of all users. Another 27% of users are academic hospitals and institutes and commercial clinical trial laboratories, whereas government and public health laboratories make up a further 9% and the IVD industry about 2% of users [15].

## Clinical impact of the lipid standardization program

### CVD death rates and blood cholesterol levels

The current death rates (2011) from cardiovascular disease (CVD) for a number of selected countries around the world are presented in Table 1. They indicate a wide range amongst various countries, with levels in Russia and Eastern Europe amongst the highest rates, and the lowest rates observed in Sweden, Australia and France [17]. Death rates have varied markedly with time, especially in many of those countries which currently have the lower rates. It has been estimated that during the period 1965 to 1990, CVD related mortality fell by some 50% in Australia, Canada, France, and the United States, and by 60% in Japan [18]. The variation in death rates for the US is presented in Figure 3 demonstrating a peak around the 1950's and a relatively constant reduction which continues to this day [19].

How have these reductions in death rates been achieved? It has been viewed that identification of major risk factors through population-based studies and clinical trials, and effective control strategies combining community education and targeted management of high risk individuals have contributed to the fall in CVD mortality rates [16]. A range of medical procedures have been introduced to improve the care of patients with CVD. More specifically, for blood cholesterol levels data are available indicating a fall in average serum cholesterol levels in representative samples of the US population as recruited for the National Health and Nutrition Examination Surveys (NHANES), as well as the number of people with the highest levels of cholesterol. Between 1988–1994 and 1999–2002, the average

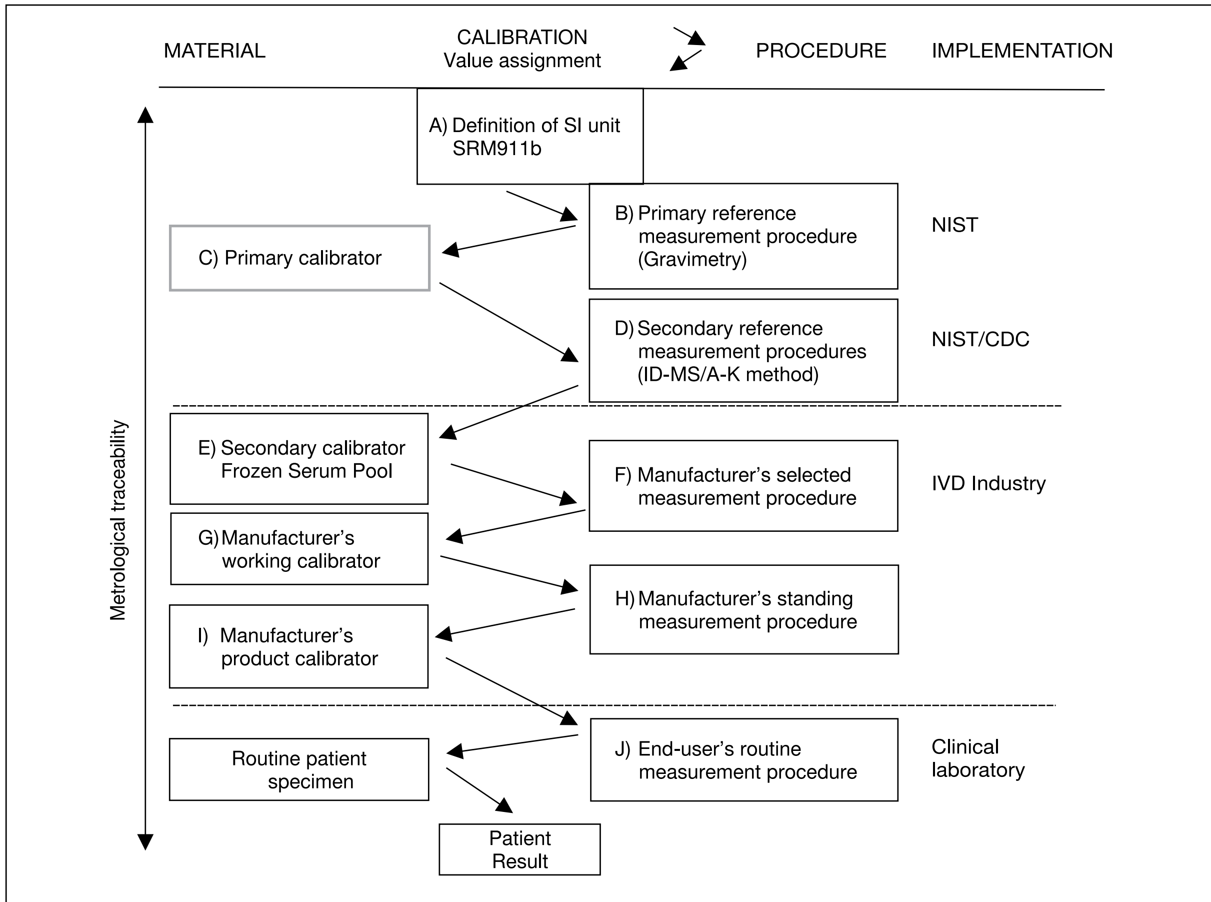


**Figure 2.** Extensive calibration hierarchy and metrological traceability to SI for cholesterol. ID-MS — isotope dilution-mass spectroscopy definitive method for cholesterol assay

**Table 1.** International cardiovascular disease statistics (WHO, 2011) [16]

| Country | Deaths/100,000 persons |
|---|---|
| Ukraine | 400 |
| Russia | 297 |
| India | 166 |
| Poland | 122 |
| USA | 81 |
| China | 80 |
| Sweden | 71 |
| United Kingdom | 69 |
| Australia | 60 |
| France | 29 |

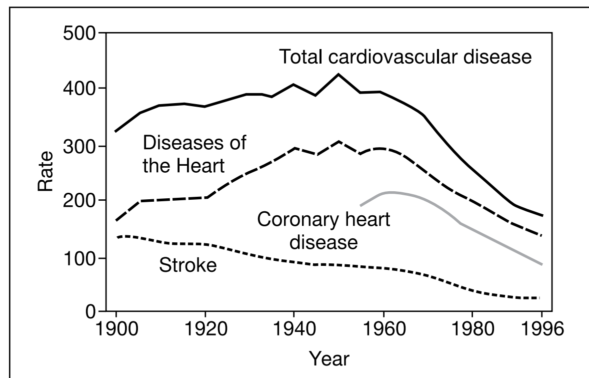


**Figure 3.** Age-adjusted death rates for total cardiovascular disease in the United States by year; death rates are per 100,000 persons [19]

serum cholesterol level of adults aged 20 years or older decreased from 206 mg/dL (5.34 mmol/L) to 203 mg/dL (5.26 mmol/L) ($p = 0.009$), and LDL cholesterol levels decreased from 129 mg/dL (3.34 mmol/L) to 123 mg/dL (3.19 mmol/L) ($p < 0.001$) [19]. Importantly greater decreases were observed in men 60 years or older and in women 50 years or older. Perhaps most importantly, the percentage of adults with a total cholesterol level at

least 240 mg/dL (greater than or equal to 6.22 mmol/L) decreased from 20% during 1988–1994 to 17% during 1999–2002 ($p < 0.001$). These later effects are likely to have a greater effect of reducing deaths from CVD than changes to the population average serum cholesterol level, which is a blunt variable. These figures must be considered with a caveat since the data were generated by different assays across the various surveys although this was a period when traceability of blood cholesterol assays was being implemented.

### Improved cholesterol measurement accuracy saves health care dollars

Another key performance indicator of healthcare is cost and calculations regarding savings to the healthcare costs in the USA alone arising from the improvement of performance of blood cholesterol assays which have been made (Dr David Bunk, personal communication). Based on known performances of cholesterol assays in 1949, there was a false positive and false negative rate for diagnosis of hypercholesterolemia of 24% in the US. Such a figure means that 24% of patients were placed on treatment when they did not need it, and 24% of patients did not receive treatment when they needed it, which consequently increased their risk of a CVD event. This rate reduced to 19% by 1969, to 11% by 1980, 6.4% by 1986 and from 1990 to 1994 it was between 5.5 and 7.2%. It was estimated that the improvement of blood cholesterol measurements between 1969 and the end of the 20$^{th}$ century saved $100 M per year in the USA alone for unneeded treatment costs.

## Conclusions

The standardization of lipid measurements, including procedures for cholesterol, HDL-cholesterol and triglycerides, has been successfully achieved internationally. This has provided traceability of routine clinical assays to internationally recognized standard reference materials. The accuracy and precision of routine clinical assays for cholesterol, HDL-C, LDL-C and triglycerides have been markedly improved and clinical decision limits defined. These developments have been associated with reduction in mean population cholesterol levels in countries where laboratories have ensured the use of assays traceable to internationally recognized standard reference materials. Most importantly, these developments have been associated with a decline in the death rate from CVD. Many improvements in the treatment of cardiovascular disease have been adopted over this period and, therefore, it is impossible to assign a specific level of reduction in the death rate for CVD as a result of the adoption of traceability of clinical cholesterol assays although it is likely that this activity has made an important contribution.

## References


1. http://www.who.int/mediacentre/factsheets/fs317/en/ (viewed 17 Feb 2015).
2. Lowering blood cholesterol to prevent heart disease. NIH Consensus Statement Online 1984 Dec 10-12 [cited 2015 Feb 28]; 5: 1–11.
3. Lenfant C. A new challenge for America: the National Cholesterol Education Program. Circulation 1986; 73: 855–856.
4. The Expert Panel. National cholesterol education program expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. Arch Intern Med 1988; 148: 36–39.
5. Naito HK. Reliability of lipid, lipoprotein, and apolipoprotein measurements. Clin Chem 1988; 34: 884–894.
6. http://www.epi.umn.edu/cvdepi/essay/brookline-conference-on-longitudinal-cardiovascular-studies/ (cited 23 Feb 2015)
7. The Pooling Project Research Group. Relationship of blood pressure, serum cholesterol, smoking habit, relative weight and ECG abnormalities to incidence of major coronary events: Final report of the pooling project. J Chron Disease 1978; 31: 201–306.
8. http://www.epi.umn.edu/cvdepi/oral-history/cooper-gerald/ (cited 18 Feb 2015).
9. Cooper GR, Smith SJ, Duncan IW et al. Interlaboratory testing of the transferability of a candidate reference method for total cholesterol in serum. Clin Chem 1986; 32: 921–929.
10. Cooper GR, Myers GL, Henderson LO. Establishment of reference methods for lipids, lipoproteins and apolipoproteins. Eur J Clin Chem Clin Biochem 1991; 29: 269–275.
11. Laboratory Standardization Panel. Current status of blood cholesterol measurement in clinical laboratories in the United States: a report from the Laboratory Standardization Panel of the National Cholesterol Education Program. Clin Chem 1988; 34: 193–201.
12. Myers GL, Kimberly MM, Waymack PP, Smith J, Cooper GR, Sampson EJ. A reference method laboratory network for cholesterol: A model for standardization and improvement of clinical laboratory measurements. Clin Chem 2000; 46: 1762–1772.
13. http://www.mdcalc.com/ldl-calculated/ (viewed 28 Feb 2015).
14. Warnick GR, Kimberly MM, Waymack PP, Leary ET, Myers GL. Standardization of measurements for cholesterol, triglycerides and major lipoproteins. Labmedicine 2008; 39: 481–490 (http://labmed.ascpjournals.org/content/39/8/481.full.pdf, cited 15 Feb 2015).
15. http://www.cdc.gov/labstandards/crmln.html (cited 18 Feb 2015).
16. Lopez AD. Assessing the burden of mortality from cardiovascular disease. World Health Stat Q 1993; 46: 91–96.
17. http://www.worldlifeexpectancy.com/cause-of-death/coronary--heart-disease/by-country/ (cited 25 Feb 2015).
18. http://www.aihw.gov.au/cardiovascular-health/prevalence/ (cited 22 Feb 2015).
19. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4830a1.htm (viewed 28 Feb 2015).
20. Carroll MD, Lacher DA, Sorlie PD et al. Trends in serum lipids and lipoproteins of adults, 1960–2002. JAMA 2005; 294: 1773–1781.