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Review

Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis

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ABSTRACT

Non-cholesterol sterols (NCS) are used as surrogate markers of cholesterol metabolism which can be measured from a single blood sample. Cholesterol precursors are used as markers of endogenous cholesterol synthesis and plant sterols are used as markers of cholesterol absorption. However, most aspects of NCS analysis show wide variability among researchers within the area of biomedical research. This variability in methodology is a significant contributor to variation between reported NCS values and hampers the confidence in comparing NCS values across different research groups, as well as the ability to conduct meta-analyses. This paper summarizes the considerations and conclusions of a workshop where academic and industrial experts met to discuss NCS measurement. Highlighted is why each step in the analysis of NCS merits critical consideration, with the hopes of moving toward more standardized and comparable NCS analysis methodologies. Alkaline hydrolysis and liquid–liquid extraction of NCS followed by parallel detection on GC-FID and GC–MS is proposed as an ideal methodology for the bio-analysis of NCS. Furthermore the importance of cross-comparison or round robin testing between various groups who measure NCS is critical to the standardization of NCS measurement.

Contents

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1. Introduction

Non-cholesterol sterols (NCS), which encompass endogenous cholesterol precursors and exogenous phytosterols and cholesterol metabolites, are widely used in biomedical research as surrogate markers for estimating rates of cholesterol absorption and synthesis [1–3] (Fig. 1 surrogates). Cholesterol precursors such as desmosterol and lathosterol are typically found in serum at up to 15 μmol/L range [4], while phytosterols, such as plant sterols are typically in the up to 30 μmol/L range [3] and plant stanols are in the 0.3 μmol/L range [5]. Tilvis and Miettinen demonstrated that serum phytosterols, i.e. campesterol and sitosterol, correlated positively with fractional cholesterol absorption measured by radio-isotopic tracers in a Finnish population on a regular Finnish diet [6]. Cholesterol precursors, such as desmosterol and lathosterol, have been shown to directly correlate with cholesterol synthesis measured by cholesterol balance [7] and by deuterium incorporation [8]. These NCS show even stronger correlation with cholesterol absorption or synthesis when expressed as ratios relative to total circulating cholesterol concentrations, enabling standardization for variations in sterol transport protein concentrations [1]. Specifically, when reporting NCS as a ratio to cholesterol, the cholesterol measurement should ideally be from the same sample preparation as the NCS.

Assessment of NCS levels in conjunction with cholesterol measurement can provide an estimate of cholesterol metabolism from a single blood sample, therefore, offers considerable advantages relative to isotopic or whole body balance approaches. As such, these surrogates have been widely used to study the impact of pharmaceutical, dietary, physiological and genetic factors on cholesterol trafficking and are ideal for use in even very large epidemiological trials [2,9]. In fact, the ratios of cholesterol to NCS levels have been used as surrogate markers of cholesterol absorption or synthesis in relation to coronary heart disease risk; however, questions remain regarding the validity for this purpose. Coronary heart disease and its severity have been associated with NCS markers reflecting high basal cholesterol absorption and lower cholesterol synthesis [4,10–13]. This finding has led to the suggestion that elevated phytosterols in the normal range may be atherogenic [14–16]. However, it has also been postulated that an association between plasma phytosterols and risk of coronary heart disease may reflect atherogenic effects stemming from increased cholesterol absorption. A recent meta-analysis by Genser et al. [17] failed to reveal any evidence of an association between serum campesterol and sitosterol concentrations in terms of cardiovascular disease risk.

A substantial challenge of studies using NCS as cholesterol trafficking surrogates is the considerable variability in methodology of measurement of NCS and cholesterol. In the general population, circulating plasma NCS concentrations are 200–1000 fold lower than cholesterol concentrations [3], thus some researchers have chosen to employ different methods for quantitation of each categorical level. Most often NCS levels are measured by gas chromatography (GC), however, in rapid chromatographic separation methods the large quantity of cholesterol in the sample may exceed the maximum detection level or interfere with separation of some NCS, therefore, fail to accurately measure certain sterol values. Thus some researchers use enzymatic methods to measure cholesterol concentrations. When cholesterol values from an enzymatic method are used it should be specifically reported. Indeed, in the review of NCS levels as surrogates, Miettinen et al. [1] highlighted that cholesterol standardized values are the markers of choice. Specifically, one needs to standardize for the GC cholesterol data and not the enzymatic cholesterol data. This variability in methodology requires normalization between methods before use in meta-analyses, as used by Genser et al. [17]. Otherwise this could reduce the potential to detect real associations, whereas failure to normalize could produce differences that are based only on methodology and not on biology [2]. To effectively assess whether specific phenotypes based on high or low NCS concentrations are associated with cardiovascular risk requires researchers to combine results of cohort studies using comparable sterol methodology. Moreover, in these combined analyses individual serum sterol levels, expressed as absolute values or as a ratio to cholesterol, should be used instead of mean values of populations.

More recently the use of phytosterols as markers of cholesterol absorption has been challenged. Jakulj et al. [18] compared plant sterol to cholesterol ratios to "gold-standard stable isotope-determined cholesterol absorption". Campesterol to cholesterol and sitosterol to cholesterol ratios as well as absolute levels of campesterol and sitosterol failed to significantly correlate with cholesterol absorption assessed directly. Interestingly, cholestanol to cholesterol ratio did associate with measured cholesterol absorption ($\beta = 0.26$, $p = 0.035$). The authors concluded that their results do not support the use of plant sterols as markers of cholesterol absorption. However, values provided by the "gold-standard stable isotope-determined cholesterol absorption" measured by Jakulj et al. ($24\% \pm 14\%$) were much lower than expected (40–50%) and as were reported in other trials using similar methodology [19,20], and included values for cholesterol absorption as low as 1%. A recent editorial by Grundy commenting on the study by Jakulj et al. noted that the cholesterol synthesis precursor lathosterol was not measured, which would helped to validate the use of NCS for cholesterol absorption as well as synthesis [21]. The review by Miettinen et al. [1] acknowledged that limitations exist with use of NCS for estimating cholesterol absorption or synthesis.

Methodological issues associated with the study by Jakulj et al. were also highlighted by Grundy, noticeably the fact that the heptadeuterated cholesterol was not dissolved in oil prior to administration, which could create a solubility problem and reduce the bioavailability of the oral tracer; thereby requiring larger dose of isotope to label the body pools [21]. Also, the isotopic tracer given intravenously in the Jakulj et al. trial was [3,4-^{13}C]cholesterol, which has insufficient mass difference from natural cholesterol to allow for easy GC–MS discrimination, as opposed to the recommended [23,24,25,26,27-^{13}C]cholesterol [22]. These factors could account for the lack of association seen between plant sterols and cholesterol absorption in the study by Jakulj et al., and certainly should be evaluated before dismissing the validity of plant sterols as markers of cholesterol absorption.

Certain circumstances do exist where plant sterols cannot be used as markers of cholesterol absorption, for instance, when intake levels of plant sterols are being manipulated, as plant sterol levels not only reflect cholesterol absorption but also plant sterol intake [1,23,24]. Plant sterols do work well as absorption markers in trials where plant stanols are administered, because the plant stanol preparations contain little to no plant sterols [1]. During plant sterol consumption cholestanol can still be used as a marker of intestinal cholesterol absorption as it is not found in typical plant sterol preparations. While some cholestanol is produced as a by-product of bile acid synthesis and some can also be taken up from meat consumption, serum cholestanol concentrations correlate well with intestinal cholesterol absorption [25]. The validity of using NCS as a measure of cholesterol metabolism has been reviewed and compared to absolute measures across various conditions by Miettinen et al. [1]. Here it was concluded that the validity of use of NCS as markers of cholesterol metabolism should not be considered self-evident and should be verified by absolute measurements whenever possible [1]. The use of multiple instead of only one precursor and absorption marker is worthwhile when assessing alterations in synthesis or absorption of cholesterol was also recommended. Studies evaluating the validity of NCS as surrogate markers during different interventions and in different