Lead Article

Progress and perspectives in plant sterol and plant stanol research

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Current evidence indicates that foods with added plant sterols or stanols can lower serum levels of low-density lipoprotein cholesterol. This review summarizes the recent findings and deliberations of 31 experts in the field who participated in a scientific meeting in Winnipeg, Canada, on the health effects of plant sterols and stanols. Participants discussed issues including, but not limited to, the health benefits of plant sterols and stanols beyond cholesterol lowering, the role of plant sterols and stanols as adjuncts to diet and drugs, and the challenges involved in measuring plant sterols and stanols in biological samples. Variations in interindividual responses to plant sterols and stanols, as well as the personalization of lipid-lowering therapies, were addressed. Finally, the clinical aspects and treatment of sitosterolemia were reviewed. Although plant sterols and stanols continue to offer an efficacious and convenient dietary approach to cholesterol management, long-term clinical trials investigating the endpoints of cardiovascular disease are still lacking.

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INTRODUCTION

A meeting of experts in the field of plant sterols and stanols (plant sterols/stanols) was convened September 30 to October 2, 2016, in Winnipeg, Manitoba, to discuss developments and controversies in this active area of functional food science. The first day's sessions were oriented toward understanding contemporary topics surrounding metabolic aspects of dietary supplementation with plant sterols/stanols, while the second day focused on clinical aspects, including the physiology of disorders pertaining to plant sterol/stanol absorption. Case reports of families with sitosterolemia were also presented on the second day. Overall, most of the experts considered plant sterols/stanols to be effective cholesterol-lowering agents that continue to have an important role as functional foods and supplements. It was also apparent, from the data presented, that the understanding of mechanisms through which the cholesterol-lowering actions of plant sterols/stanols occur has improved since the state of the art in 2011.[1] The purpose of this review is to identify the salient points arising from the presentations and ensuing discussions that capture recent developments in the field.

EFFICACY OF PLANT STEROLS/STANOLS IN LOWERING LOW-DENSITY LIPOPROTEIN CHOLESTEROL

Factors that influence the cholesterol-lowering efficacy of plant sterols/stanols

Hundreds of studies have investigated several aspects of the clinical efficacy of plant sterols/stanols for lowering low-density lipoprotein cholesterol (LDL-C), including chemical form (sterol vs stanol), food matrix, and other factors associated with delivery of these compounds.[2–6] First, when plant sterols are compared with plant stanols, consistent evidence demonstrates that both plant sterols and plant stanols lower LDL-C levels by 7.5% to 12% at intakes of 1.5 to 3 g/d.[7] At intakes of up to 3 g/d, which is the current recommended range of intake in most countries, plant sterols and plant stanols produce equal LDL-C–lowering effects. A systematic review of 14 studies showed a nonsignificant weighted mean difference in LDL-C lowering between plant sterols and plant stanols.[8] Moreover, data compiled from 124 studies revealed a clear dose-dependent reduction in LDL-C at plant sterol or plant stanol intakes of up to 4 g/d. In that meta-analysis, an average plant sterol/stanol intake of 2.1 g/d resulted in an 8.4% reduction in LDL-C, while an average intake of 3.3 g/d resulted in a 12.4% reduction.[7] It appeared that, at an intake of 2.1 g/d, there was about a 2% difference between LDL-C lowering with plant sterols and LDL-C lowering with plant stanols. Plant stanols achieved a more pronounced LDL-C lowering, while at higher average intakes of 2.6 and 3.3 g/d, lowering of LDL-C was comparable.[1] These findings persisted in several additional analyses.[9] The consistency of the food format, either solid/edible or liquid/drinkable, is critical for comparing the effects of plant sterols/stanols. Ras et al[7] reported that, in the dose category ranging from 2.0 g/d or more to less than 2.5 g/d, at an average intake of 2.1 g/d, 15 of 40 plant sterol studies used liquid food formats, whereas only 4 of 18 plant stanol studies used this type of food format. Irrespective of the type of plant sterols or stanols used, liquid foods lowered LDL-C concentrations by an average of 6.5%, whereas solid foods lowered LDL-C concentrations by an average of 9.2%.[7] The limited sample size of studies that used the liquid food format as the plant stanol carrier warrants caution in drawing sweeping conclusions. Additional research with head-to-head comparisons between plant sterols and plant stanols is needed.

A second factor influencing the cholesterol-lowering efficacy of plant sterols/stanols is the food matrix. The fat content of the food, the type of fat in the food, the supplement form (capsules or tablet), the use of free or esterified plant sterols/stanols, and the fatty acid used for esterification all may affect cholesterol lowering. In addition, the frequency of administration (eg, single vs multiple daily intakes), intake with vs without a meal, and the time of administration (eg, morning vs later in the day) are all factors contributing to the degree of plant sterol/stanol efficacy. A systematic review of dietary plant sterols/stanols consumed from food vs from tablets showed a similar mean difference in LDL-C lowering.[10] However, most studies that delivered plant sterols/stanols as tablets are missing data on particle size and dissolution activity. Tablet characteristics represent a critical aspect of future research using supplements.

Plant sterols/stanols have been examined across multiple food formats, and there is no apparent difference in their cholesterol-lowering efficacy in high-fat vs lowfat or nonfat foods.[11,12] In terms of the type of carrier fat, a recent study found no difference between different types of carrier fats in the relative reduction in LDL-C levels.[13] Two meta-analyses reported higher efficacy with solid foods (eg, spreads and margarines) than with liquid foods (milk and juices).[7,12] There are no differences between the efficacy of free vs esterified plant sterols,[12,14,15] although the particle size of plant sterols should be considered. Nor does the fatty acid used for esterification affect the cholesterol-lowering efficacy of plant sterols/stanols.[16–18] However, data from meta-analyses show that the frequency of intake matters, and once a day seems suboptimal.[7,12] Lowering of LDL-C

was greater when a yogurt drink was consumed together with a lunch meal than when consumed before breakfast (lowering of 9.4% vs 6.0%).[19] Another study with plant stanol–enriched biscuits also found that biscuits consumed with a meal resulted in a greater cholesterol-lowering effect than biscuits consumed between meals.[20] In a 2000 review of published plant sterol/stanol intervention studies, Law[21] found that the absolute decrease in LDL-C increased with age, although relative changes were comparable across age ranges.

The design of clinical studies is also of interest. In the earliest published research with plant sterols, 9 male study participants consumed 5 to 6 g of β-sitosterol per day, showing mean serum total cholesterol decreases as great as 15% to 20% over 6 weeks.[22] Another early study in which 15 young men, all with previous myocardial infarctions, consumed 12 to 18 g of β-sitosterol per day also showed large declines in serum total cholesterol levels.[23] Neither of these studies, however, was a randomized trial, and the results focused on changes in total cholesterol. Since these initial publications, there have been important advances in trial design and analytical methods. Miettinen et al[24] conducted a landmark 1-year-long study of 153 participants in a double-blind, randomized controlled trial and observed a 14.1% decrease in circulating LDL-C with a plant stanol dosage of 2.6 g/d compared with placebo, without a decrease in high-density lipoprotein-cholesterol (HDL-C).

Overall, data summarized from meta-analyses published from 2000 through 2016 show that most studies reported an LDL-C reduction between 0.3 and 0.4 mmol/L.[7,10–13,21,25] As LDL-C is recognized as an important causal risk factor for coronary heart disease, such a reduction in LDL-C would correspond to a 7% reduction in the risk of heart disease.[26] However, to date, direct evidence of an effect on cardiovascular disease (CVD) is not available, as studies exploring hard endpoints such as CVD events and mortality are lacking, likely because they are expensive and challenging with respect to long-term compliance.

Diversity of natural plant sterols/stanols

Experts agree that a minimum intake of 1 g of plant sterols/stanols per day is necessary to significantly lower circulating LDL-C levels.[27] However, naturally occurring plant sterols in fruits and vegetables range from about 38 to 439 mg/kg of fresh weight, and those in grain range from 329 to 1780 mg/kg. Thus, to consume 1 g of plant sterols, one would need to eat about 2 kg of fruits/vegetables or about 1 kg of grain per day.[28] Plant oils contain higher levels of plant sterols/stanols, but one would need to eat about 100 g of oil per day to reach a daily intake of 1 g. Therefore, fruits/vegetables, grains, and plant oils are not practical sources of dietary plant sterols/stanols, so other approaches need to be considered. Tall oil and vegetable oil deodorizer distillates continue to be major feedstocks for the production of plant sterols/stanols added to functional foods, but other sources are under investigation. For example, corn fiber oil and rice bran oil contain 10% to 15% and 2% total plant sterols, respectively, but have not been used as a commercial feedstock for the production of plant sterols/stanols.[28] In plants, most sterols/stanols are either present in their free unesterified form or are esterified to fatty acids. However, plant sterols/stanols also occur as steryl glucosides and acylated steryl glucosides, with the steryl glucoside esterified to a fatty acid. Unlike sterol esters, steryl glucosides can inhibit cholesterol absorption in their intact form, without being hydrolyzed by digestive enzymes such as pancreatin.[29,30] A future therapeutic option, therefore, could be to clone the gene to produce steryl glucosides, which may be useful if future clinical studies indicate additional benefits of dietary steryl glucosides when compared with common forms of free and esterified plant sterols.[31] Lecithin has been reported to be a valuable organogelator. An organogel is defined as an organic liquid entrapped within a thermoreversible, 3-dimensional gel. Some of the other main organogelators include plant waxes and sitosterol plus oryzanol.[32,33] Hence, further research on organogels is warranted.

EFFECTS OF PLANT STEROLS/STANOLS BEYOND CHOLESTEROL LOWERING

Immune-modulating properties of plant sterols/stanols

Nutrition, whether supplied as whole diets, specific nutrients, or bioactive phytochemicals, is a powerful modulator of the immune system, regulating both defense against pathogens and the chronic inflammatory response that underlies many disease states.[34] Previous in vitro,[35] animal,[35] and human[36] studies suggest that plant sterols/stanols affect the immune response. Calpe-Berdiel et al[35] reported that, independent of cholesterol-lowering effects, 2% dietary plant sterol supplementation in apolipoprotein E–deficient mice treated with turpentine showed increased interleukin 2 and interferon-γ secretion (T-helper type 1 lymphocyte cytokines). An effective biological response to an immune challenge involves a balanced production of specific types of pro- and anti-inflammatory cytokines by Th1 and Th2 helper T cells, respectively.[37] Nashed et al[38] demonstrated that, in addition to lowering cholesterol, 2% dietary plant sterol supplementation in

apolipoprotein E–deficient mice for 14 weeks decreased plasma interleukin 12 concentrations. Brull et al[39] previously reported evidence that physiological concentrations of both sitosterol and sitostanol increased the production of interferon-γ in human peripheral blood mononuclear cells. More recently, the same group addressed whether these in vitro plant sterol/stanol-induced changes could be applied clinically to enhance immune function in patients with asthma.[40] In a randomized, double-blind clinical trial, patients with asthma who consumed a plant stanol–enriched soy-based yogurt (4.0 g of plant stanols daily) vs a control yogurt demonstrated higher antibody titers against hepatitis A virus vaccination and reductions in plasma concentrations of total immunoglobulin E, interleukin 1β, and tumor necrosis factor-α. Although these results are promising, further studies designed to explore clinical benefits in immune-compromised populations are required.

Triglyceride-lowering properties of plant sterols/stanols

The rising global obesity epidemic is associated with a characteristic dyslipidemic phenotype that includes elevated concentrations of serum/plasma cholesterol and triglycerides. Previous work suggests that approximately 80% of overweight and obese individuals have serum triglyceride concentrations of more than 150 mg/dL (1.7 mmol/L). Although plant sterols/stanols have a long history as effective cholesterol-lowering compounds, their benefit in reducing hypertriglyceridemia is a relatively recent discovery. Results of previous randomized controlled studies conducted in normotriglyceridemic individuals suggest that daily supplementation with plant sterols/stanols (1.6–9 g/d) for 1 to 2 months reduces triglycerides by 0.8% to 7%.[15,41–43] However, in individuals with elevated serum triglyceride concentrations ($>$ 1.7 mmol/L), randomized control trials results suggest that plant sterol/stanol supplementation (1.8–4 g/d) may lower circulating triglyceride concentrations by 11% to 28%.[44–50]

Previous animal studies indicate that the triglyceride-lowering effects of plant sterols may be related to altered intestinal fat metabolism, as indicated by increased fecal fatty acid excretion in plant sterol–supplemented mice[51] and reduced postprandial lymphatic transport of triglycerides in thoracic duct–cannulated rats.[52] However, clinical studies investigating postprandial fat handling in normotriglyceridemic individuals failed to support data from animal studies, suggesting that plant sterols can interfere with intestinal fat digestion/absorption.[53,54]

Plant sterol supplementation also may reduce hepatic de novo lipogenesis in golden Syrian hamsters,[55] although results in other species have differed.[51] In support of a triglyceride-lowering mechanism of hepatic origin, Plat and Mensink[47] reported a reduction in large and medium very low-density lipoprotein particles in the plasma of dyslipidemic individuals with metabolic syndrome who consumed 2 g of plant stanols per day. This finding was also confirmed in an animal study that investigated hepatic production of very low-density lipoprotein.[56]

Future research priorities with respect to plant sterols/stanols and triglyceride metabolism include human intervention studies specifically powered to detect triglyceride responses in hypertriglyceridemic individuals, a direct examination of fatty acid absorption, and whole-body lipogenesis in response to plant sterol/stanol supplementation. Additionally, research is needed to identify both metabolic and genetic factors that determine the magnitude of plant sterol/stanol–induced triglyceride reductions.

Plant sterols/stanols and the central nervous system

Consumption of plant sterol–enriched foods increases circulating plant sterol levels and may enhance the accumulation of plant sterols not only in tissues such as aortic valves and liver but also in the central nervous system (CNS).[57–60] In a study by Simonen et al,[61] however, consumption of plant sterols/stanols did not enhance the accumulation of plant sterols/stanols in stenotic aortic values. The mean duration of this intervention was 2.6 $\pm$ 0.2 months (range, 0.6–5.0 months).[61]

Although plant sterols are poorly transported across the blood–brain barrier, those with side chains of lower complexity, such as cholesterol and campesterol, cross the blood–brain barrier more easily than other plant sterols with more complex side chains, such as sitosterol and stigmasterol.[62–64] The exact mechanism by which plant sterols are delivered to the endothelial monolayer of the blood–brain barrier remains speculative. As adenosine triphosphate (ATP)–binding cassette (ABC) subfamily G members 5 and 8 (Abcg5, Abcg8) transporter proteins are not expressed within the brain or at the blood–brain barrier,[65] this transporter complex would not be expected to modulate plant sterol transport at the level of the blood–brain barrier. An HDL-C–mediated plant sterol transport pathway across the blood–brain barrier has been suggested, given that plant sterols are predominantly transported via HDL-C in wild-type and *ABCG5*$^{-/-}$ mice and that scavenger receptor class B member 1, the major HDL-C receptor, is highly expressed on the apical membrane of endothelial cells of the blood–brain barrier.[66] Regardless of the

uptake mechanism, animal studies of plant sterol feeding and depletion suggest that accumulation of plant sterols in the CNS is virtually irreversible.[63] Although the conversion of cholesterol to 24(*S*)-hydroxysterol in neurons accounts for over 60% of the cholesterol efflux from the CNS,[67–71] once plant sterols enter the CNS, they are not metabolized by the *CYP46A1* gene into 24(*S*)-hydroxysterol,[63,72] likely because of steric hindrance caused by the ethyl or methyl group at the C-24 position.

Although quantitative data on spatiotemporal accumulation of plant sterols in the human CNS are limited, the total content of plant sterols in the CNS of elderly individuals without neurological disease is estimated to be approximately 75 ng/mg of dry tissue, representing about 0.5% of the total amount of body sterols.[59] The rate of cholesterol turnover (percentage of cholesterol pool) in pyramidal cells of the cortex and Purkinje cells of the cerebellum exceeds 20% per day.[68,73–75] The high flux of sterols in these metabolically active cells allows fast incorporation into detergent-resistant parts of neuronal membranes, thereby actively modulating cholesterol metabolism in the CNS.[63,76] A mechanistic study from Burg et al[77] shows that cleavages of the amyloid precursor protein were beneficially modified by incorporation of plant sterols into neuronal membranes. To date, it is largely unclear whether accumulation of plant sterols in the CNS has functional implications. Long-term exposure to increased levels of plant sterols in transgenic mice did not lead to an overt cognitive phenotype with respect to memory or anxiety.[78] Similarly, a randomized double-blind placebo-controlled dietary intervention study showed no negative influence of long-term plant sterol or stanol consumption on neurocognitive function or mood in hypercholesterolemic patients receiving statin treatment.[79] On the other hand, previous studies found that plant sterol extracts have anxiolytic-like effects after intraperitoneal administration in mice.[80,81] Together, the data suggest that plant sterols do not enhance cognition in normocognitive settings. However, cumulating in vitro and in vivo findings support a therapeutic potential for plant sterols in disease-related cognitive impairment.

RESPONSIVENESS OF LDL-C TO PLANT STEROLS/STANOLS

Increased cholesterol excretion as an alternative measure of plant sterol/stanol efficacy

The ability of plant sterols/stanols to reduce cholesterol absorption is clearly an important factor in the LDL-C–lowering action of these compounds, but it may not be the only mechanism. Plant sterols/stanols also may affect reverse cholesterol transport and whole-body cholesterol metabolism,[82] which are emerging areas of interest in studies analyzing cardiovascular risk. Plant sterols/stanols exert their principal effects most likely through disruption of the intraluminal solubilization step.[83] In a controlled feeding study with 20 participants in which dietary intakes of nutrients and plant sterols were measured and carefully controlled, fecal cholesterol excretion rose by 36% when the plant sterol content of the diet was increased from 59 to 459 mg/d and by a total of 74% when the plant sterol dose was further increased to 2059 mg/d.[82] In contrast, these 2 increases in dose resulted in a 5% and a 9% reduction in LDL-C level, respectively. Additionally, in many studies, plant sterol consumption reduces cholesterol absorption by 30% to 45%,[84–88] yet circulating levels of plant sterols are not affected to such a large extent. Taken together, these data emphasize that the effects of plant sterols/stanols on whole-body cholesterol metabolism are broad and not limited to LDL-C lowering but may involve additional pathways. More studies demonstrating enhanced reverse cholesterol transport and reductions in hard cardiovascular endpoints following plant sterol/stanol intake should improve the ability of public health agencies to make recommendations. More studies are also needed to evaluate new biomarkers of plant sterols/stanols consumption, biomarkers that better correlate with hard CVD endpoints. Indeed, because of the large inter-individual variations in non-cholesterol sterol handling, the measurement of plasma plant sterols/stanols alone provides only an inaccurate estimate of dietary intake. Studies in which the dietary intake of plant sterols was controlled have demonstrated a strong correlation between dietary plant sterol intake and the ratio of plasma campesterol (the most avidly absorbed plant sterol) to 5α-cholestanol (an endogenous cholesterol metabolite) ($R^2 = 0.79$, $P < 0.0001$[89]). Such ratio should thus be evaluated as a new biomarker of plant sterol intake in future studies assessing the impact of dietary plant sterol intervention on CVD endpoints.

Genetic basis of plant sterol/stanol responsiveness

Several clinical studies have investigated the genetics behind plant sterol/stanol responsiveness. Miettinen and Vanhanen[90] studied the effects of small amounts of sitosterol, sitostanol, and sitostanol esters (< 1 g of free sterols per day) dissolved in rapeseed oil on serum lipids and cholesterol metabolism in patients with primary hypercholesterolemia but different apolipoprotein E phenotypes who were given a rapeseed oil diet. Low-density lipoprotein cholesterol reduction was reduced by 8% in individuals with the apolipoprotein E4 allele and was insignificant in those with the apolipoprotein E3/3 phenotype.[90]

In another study, the relationship between genetic variations in genes encoding apolipoprotein A-IV, scavenger receptor class B type I, 3-hydroxy-3-methylglutaryl coenzyme A reductase, cholesteryl ester transfer protein, and apolipoprotein E and the response of cholesterol metabolism to consumption of plant stanol esters was examined in 112 nonhypercholesterolemic individuals, 70 of whom consumed 3.8 to 4.0 g of plant stanols (in the form of plant stanol esters) per day for 8 weeks.[91] None of the polymorphisms were associated with the responsiveness of LDL-C concentrations to plant stanol consumption, with the authors concluding that the polymorphisms analyzed were unlikely to be a major factor in explaining the variation in serum LDL-C responses to plant stanols.[91] In another study that investigated changes in serum plant sterol concentrations relative to *ABCG5/G8* polymorphisms after consumption of plant stanol esters, concentrations of cholesterol-standardized serum campesterol and sitosterol were associated with the *ABCG8* T400K genotype. However, despite the shifts in circulating plant sterol concentrations, no associations with serum LDL-C levels were found.[92] Gylling et al[93] also looked at the relationship between *ABCG5/G8* polymorphisms and the responses of serum cholesterol concentrations and vascular function during a longer-term study in which 282 participants consumed plant stanol or sterol esters (2 g of plant stanols or sterols per day) or a control spread for 1 year. Neither serum cholesterol lowering nor inhibition of cholesterol absorption was associated with *ABCG5/G8* polymorphisms.

Clinical trials, as shown in Figure 1,[94] reveal substantial interindividual variability in LDL-C reduction in response to plant sterol consumption,[45,95] with responses ranging from above-average response to nonresponse or even adverse response.[1,96,97] Distinct interindividual responses to plant sterol consumption have been shown to be reproducible in individuals across repeated plant sterol interventions,[98] indicating other potential determinants of responsiveness. Factors thought to be responsible for this variability have been investigated. One explanation has focused on individual differences in the rates of cholesterol synthesis as determined by the ratio of lathosterol to cholesterol. This ratio was shown to be a biomarker predictive of an individual's response to plant sterol intervention.[99] Both the rate of cholesterol synthesis and the plasma cholesterol levels were found subsequently to be influenced by *APOE* polymorphisms and the single-nucleotide polymorphism (SNP) rs38038607 in *CYP7A1*.[100] In particular, the *CYP7A1*-rs3808607 and *APOE* isoforms were correlated with the extent of reduction in circulating LDL-C levels in response to plant sterol consumption. Thus, predictive genetic markers can possibly identify individuals who would derive maximum LDL-C lowering from plant sterol consumption.[100] The study by Mackay et al[100] confirmed results reported by De Castro-Oros et al,[101] who assessed whether a common A>C substitution at position 204 of the promoter of *CYP7A1*-rs3808607 was related to variability in plasma sterol responses to plant sterol supplementation. Compared with carriers of the A allele, individuals bearing the 204C variant had significantly higher adjusted mean reductions in total cholesterol and significantly higher increases in lathosterol to cholesterol ratios.[101]

In another study, a 3.9-fold greater reduction in serum LDL-C levels was observed in hypercholesterolemic men carrying the SNP rs4148217-Ain of the *ABCG8* gene when intake of plant sterols was 2.0 g/d for 4 weeks.[102] However, this association was not replicated by MacKay et al.[100] These findings could represent a first step in evaluating the use of common genetic variations to predict an individual's response to plant sterol/stanol intervention, which would potentially lead to enhanced plant sterol/stanol efficacy in reducing CVD risk.

Taken together, the data suggesting that genetic architecture influences the response of sterol metabolism to plant sterols/stanols are provocative, but such mechanisms need further study. A number of cholesterol-related gene–diet interactions have been associated with plant sterol or stanol response. Further study of such interactions should lead to greater understanding of the interindividual variability in response to plant sterols and stanols.

CHALLENGES IN MEASURING PLANT STEROLS/STANOLS AS MARKERS OF CHOLESTEROL METABOLISM

Measuring plant sterols/stanols

Plant sterols/stanols fall broadly into the category of noncholesterol sterols, which encompasses both noncholesterol and nonsteroid hormone sterols. Noncholesterol sterols share the same steroid skeleton with cholesterol and are comprised of sterols/stanols of plant origin, certain cholesterol derivatives. and precursors in the cholesterol synthesis pathway.[103] Serum or plasma concentrations of the cholesterol precursors, such as lanosterol, lathosterol, and desmosterol, are widely used as surrogate markers of endogenous cholesterol synthesis.[104,105] Reciprocally, plant sterols, such as campesterol or sitosterol and the cholesterol metabolite 5α-cholestanol, have been used as markers of cholesterol absorption.[106–108]

These noncholesterol sterols are often so similar in structure to cholesterol that enzymatic methods to

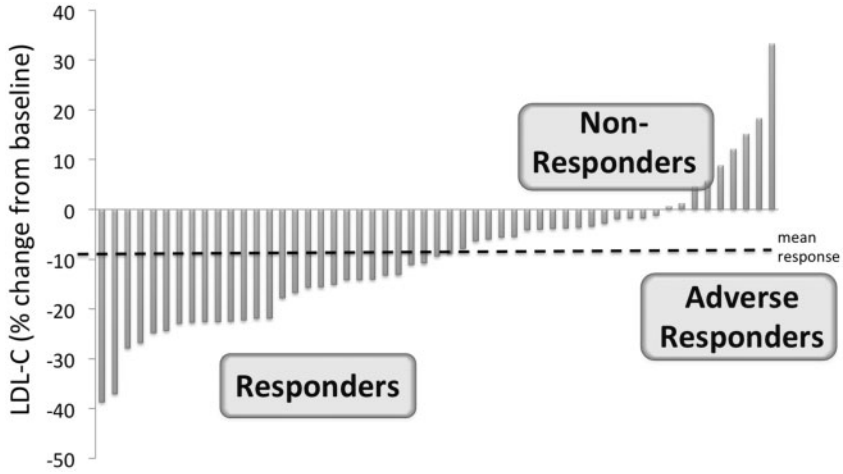

Figure 1 **Percent change in low-density lipoprotein cholesterol (LDL-C) from baseline in response to consumption of a low-fat soy beverage enriched with plant sterols (plant sterol intake, 1.95 g/d).**[94]

quantify cholesterol will actually measure the noncholesterol sterol species as well, artificially inflating the cholesterol concentrations.[109] Conceptually, very little in the quantitation of noncholesterol sterols has changed since Bhattacharyya and Connor[110] measured levels of plant sterols in the first sitosterolemic children identified. The various species of sterols must first be separated chromatographically, often by gas or liquid chromatography, and then measured, typically by using either flame ionization detection or mass spectrometry.[111] Even with precise chromatographic techniques, it can still be impossible to separate certain species of sterols; therefore, these species must be separated using mass-selective detection during mass spectrometry.[112] Noncholesterol sterols are found in biological fluids in concentrations that are profoundly different, ranging from millimole per liter for cholesterol, to micromole per liter for plant sterols/stanols and cholesterol precursors, to picomole per liter or lower for oxidized derivatives of noncholesterol sterols.[113] This wide range of concentrations renders it difficult to capture all concentrations using a single analytical method. As a result, numerous methods for measuring noncholesterol sterols have been specifically developed and compared,[111] but they often use different techniques for chromatographic separation and detection.[112] This variability in methodology is a substantial challenge to the use of noncholesterol sterol concentrations as surrogate measures of cholesterol metabolism because it hinders the comparison of noncholesterol sterol values reported from different laboratories. In fact, variation in measurement methodology has been identified as the greatest contributor to the variability in plant sterol concentrations reported in the scientific literature.[114] In summary, comparing plant sterol or stanol concentrations reported from different laboratories must be done with caution, realizing that measurement methodology, rather than diet or other biological mechanisms, may be the biggest single contributor to differences in results.

Plant sterols/stanols as surrogate markers of cholesterol metabolism

As mentioned in the section *Measuring Plant Sterols/Stanols*, circulating plant sterol/stanol levels are often used as surrogate measures of cholesterol absorption.[107] Compared with direct and indirect methods of measuring whole-body cholesterol absorption or synthesis, the measurement of noncholesterol sterols is faster, more affordable, and less invasive. However, in some circumstances, the measurement of plant sterols or stanols as surrogate markers of cholesterol absorption is not appropriate and may not accurately reflect intestinal sterol absorption, even in the absence of supplemental intake of plant sterols/stanols. When intakes of plant sterols or stanols are changing, as in a trial of plant sterol/stanol supplementation, the use of plant sterol/stanol concentrations as surrogate measures of cholesterol absorption is invalidated.[115]

When plant sterols or other noncholesterol sterols are to be used as surrogate measures, they should be expressed as ratios relative to the total cholesterol level, which standardizes for variations in concentrations of sterol transport proteins.[106] Such ratios show even stronger correlations with cholesterol absorption and synthesis. Concentrations of plant sterols and other noncholesterol sterols, as surrogate markers of cholesterol absorption, have been associated with CVD risk.[116,117] Levels of noncholesterol sterols have also been used to differentiate between different types of dyslipidemia[103,118,119] and to predict response to statin therapy[120,121] and might be useful to guide lipid-

lowering therapy.[122–124] Beyond the use of plant sterol or other noncholesterol sterol levels individually as surrogate markers of cholesterol absorption or synthesis, the ratios of cholesterol synthesis to cholesterol absorption of these sterols, such as the ratio of lathosterol synthesis to campesterol absorption, are also utilized to assess overall cholesterol metabolism, with higher values representing greater synthesis and less absorption.[125] However, owing to the inherent nature of ratios, the use of the ratio of synthesis to absorption markers does not take into account the absolute values of each marker. This hypothetically means that an individual with the unlikely scenario of high concentrations of both synthesis and absorption surrogate markers could have the same ratio as someone with very low values, which likely does not accurately reflect the actual biological impact of these different values. To overcome this limitation, the synthesis and absorption markers can be arranged in a Cartesian plane and related to an outcome in a third plane, as was done by Qi et al,[126] who proposed a new approach of using both absorption and synthesis markers together (Figure 2). By taking the length of the hypotenuse of a triangle created by graphing cholesterol absorption surrogates against cholesterol synthesis surrogates, a potential overall measure of cholesterol metabolism is obtained.

Since concentrations of plant sterols or other noncholesterol sterols are easy to measure, they are likely to remain in common use as surrogate markers of cholesterol metabolism. Their usefulness will be enhanced by improving and standardizing the measurement of noncholesterol sterols.

PLANT STEROLS/STANOLS AS ADJUNCTS TO DIET AND DRUGS

Lipid-lowering drugs and plant sterols/stanols: ezetimibe

Ezetimibe (Zetia, Ezetrol) is a selective cholesterol absorption inhibitor that potently inhibits the uptake and absorption of biliary and dietary cholesterol and noncholesterol sterols from the intestinal lumen without affecting the absorption of other nutrients. Clinically, ezetimibe reduced fractional cholesterol absorption, which was accompanied by a 20.4% reduction in LDL-C in 18 patients with mild hypercholesterolemia.[127] Ezetimibe alone reduces plasma total cholesterol and LDL-C levels by 18% in patients with primary hypercholesterolemia and, when added to ongoing statin treatment, reduces LDL-C by an additional 25%.[128] On the other hand, it also blocks plant sterol absorption. In clinical studies, plasma sitosterol and campesterol were reduced 41% and 48%, respectively, after just 2 weeks of

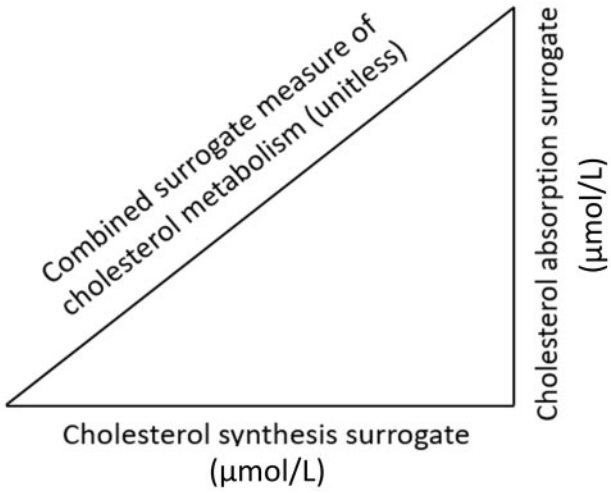

Figure 2 **Proposed surrogate measure of cholesterol metabolism that could overcome issues related to the use of ratios of markers of surrogate synthesis to markers of absorption.**[126]

ezetimibe at a dosage of 10 mg/d. Ezetimibe also reduced serum plant sterol levels by about 50% when used in combination with the statins simvastatin and atorvastatin.[129]

Sitosterolemia is caused by mutations in the ATP-binding cassette (ABC) cotransporters, either *ABCG5* and/or *ABCG8*, leading to an accumulation of plant sterols in plasma and tissues that, in turn, results in accelerated development of CVD, anemia, platelet defects, and other disorders. Ezetimibe treatment of sitosterolemia has been examined in case studies, and in some instances it resulted in resolution of xanthomas, increased platelet counts, and improved cardiovascular symptoms.[130] It also reduced the serum levels of the atherogenic sterols campesterol and sitosterol in 37 patients with sitosterolemia.[131]

The intestinal transporter for cholesterol and plant sterols is Niemann-Pick C1-Like 1 (NPC1L1) protein.[132] Ezetimibe works by inhibiting the NCP1L1-mediated uptake of sterols into the enterocyte and by blocking the re-uptake of sterols from the bile back into hepatocytes in humans.[133] This blockage results in enhanced excretion of fecal neutral sterols and a reduction in levels of plasma and tissue cholesterol and plant sterols.

In preclinical studies, ezetimibe treatment or the lack of NPC1L1 in mice has been shown to reduce atherosclerosis.[134] The Myocardial Infarction Genetics Consortium[135] sequenced the exons of *NPC1L1* in more than 22 000 individuals and found 15 inactivating mutations of *NPC1L1*. They subsequently screened more than 100 000 individuals for these inactivating *NPC1L1* mutations as well as for CVD risk and found that being heterozygous for an inactivating mutation of

NPC1L1 was associated with an average plasma LDL-C reduction of about 12 mg/dL and a decline in the risk of coronary heart disease by 53%. Since these individuals are heterozygotes, they have a lifelong 50% inhibition of NPC1L1. Whether the use of ezetimibe to inhibit NPC1L1 function causes a similar large decrease in coronary heart disease needs to be addressed in a trial investigating hard cardiovascular endpoints.

The IMPROVE-IT (IMProved Reduction of Outcomes: Vytorin Efficacy International Trial) investigated the prevention of secondary outcomes following acute coronary syndrome in over 18 000 patients.[136] The objective was to reduce LDL-C levels to either 70 mg/dL with simvastatin alone or to 55 mg/dL by combination treatment with simvastatin and ezetimibe and to see if LDL-C levels even lower than the recommended 70 mg/dL can be reached with the combination. The mean baseline LDL-C level was 94 mg/dL at the start of this trial. In contrast to previous data,[137] there was about a 16- or 17-mg/dL difference between the treatment groups: LDL-C levels were 70 mg/dL with simvastatin alone vs 53 mg/dL with the combination of simvastatin plus ezetimibe. Weingärtner et al[123] reported a significant LDL-C reduction of 6.4% when ezetimibe was added to simvastatin for the primary CVD outcomes in the intention-to-treat population. In another study, the addition of plant sterols to ezetimibe improved the effects of ezetimibe on plasma LDL-C, as shown by Lin et al.[138] Recently, Gomes et al[139] reported that combination therapy with plant sterols and ezetimibe was associated with lower LDL-C levels. Similarly, long-term use of sitostanol ester margarine as a substitute for part of normal dietary fat had a favorable effect of lowering serum total cholesterol and LDL-C levels in individuals with mild hypercholesterolemia.[24] Therefore, this indicates that LDL-C lowering with ezetimibe probably caused the reduction in cardiovascular events. These data help emphasize the primacy of LDL-C lowering as a strategy to prevent coronary heart disease.[140]

Lowering serum cholesterol levels: potential role of plant sterols/stanols

There has been a long-standing argument over the so-called statin hypothesis—the idea that statins have unique efficacy in atherosclerotic vascular disease not shared by other lipid-modifying agents, and that reductions in LDL-C levels are not the only basis for the beneficial effect of statins. The efficacy and safety of statin therapy was explored in a prospective meta-analysis of data from over 90 000 individuals in 14 randomized trials. The study concluded that, on average, a reduction of 1 mmol/L (38.7 mg/dL) in LDL-C levels by statin therapy yields a consistent 23% reduction in the risk of major coronary events over 5 years.[141]

In this regard, the recent development of PCSK9 (proprotein convertase subtilsin/kexin type 9) inhibitors is also of note. These agents reduce the degradation of LDL receptor, thereby enhancing LDL-C clearance from the circulation and reducing LDL-C levels by as much as 60%.[142] Definitive clinical outcomes trials with these agents are ongoing. While the data are still too preliminary to be conclusive, it appears that plant sterol/stanol supplements efficiently decrease plasma LDL-C levels and intestinal cholesterol absorption by influencing PCSK9 expression.[143] Sabatine et al[144] found that the PCSK9 inhibitor evolocumab, when administered in combination with background statin therapy, reduced LDL-C levels by a median of 30 mg/dL and lowered the risk of cardiovascular events.[144]

Plant sterols/stanols and other dietary agents

Like fiber intake, plant sterol intake appears to have contracted substantially in modern diets. It has been estimated from studies of early ancestral diets that, 4 to 5 million years ago, when humans split genealogically from gorillas and chimpanzees, they would have consumed approximately 1 g of plant sterols per day.

When this early diet was recreated and fed to healthy volunteers, major increases in fecal output (1 kg/d) and marked reductions in circulating LDL-C levels of 30% to 35% were observed.[145] This decline in cholesterol was attributed to increased dietary intakes of fiber, vegetables, vegetable proteins, nuts, and plant sterols and very low intakes of saturated fat and cholesterol. It can be reasoned that the lack of these components in the current diet, together with the consumption of significant amounts of animal products that are high in saturated fat, cholesterol, and animal proteins, is responsible for the current elevated LDL-C levels seen in humans consuming Western-type diets. This current intake pattern has led to many patients taking statin drugs instead of employing diet modification to improve cholesterol levels.

The key elements of ancestral dishes, which were individually approved by the US Food and Drug Administration (FDA) for cholesterol reduction claims, were used to create a new diet that required consumption of a very large volume of plant foods. Elements included vegetable protein (soy), nuts, viscous fibers (oats, barley, and psyllium), and plant sterols, all incorporated at standardized amounts into a single diet termed the *dietary portfolio*. This portfolio diet lowered LDL-C and C-reactive protein levels by 20% to 35% in hyperlipidemic participants on metabolic diets.[146] In an ad libitum cross-Canada multicenter trial conducted

over 6 months in 335 participants on a self-selected dietary portfolio, LDL-C levels decreased by 13% to 14% overall, and by approximately 20% on the West Coast![146] It is believed that plant sterols were a major reason for the dietary portfolio's LDL-C–reducing effect, since a reduction of 10% to 15% can be seen with a plant sterol intake of 2 g/d. Therefore, when combined with other dietary factors, plant sterols appear to have a very useful role in maintaining healthy cholesterol levels.

PLANT STEROLS/STANOLS AND CVD RISK

Effect of plant sterols/stanols on vascular function

The LDL-C–lowering effect of plant sterols/stanols is well established.[7,12,88] Nevertheless, direct evidence linking the intake of foods containing added plant sterols/stanols to CVD risk is still lacking. Cardiovascular disease endpoint trials with plant sterols/stanols are prohibitively expensive and exceedingly challenging to perform. Depending on the length of follow-up and the annual risk level, 36 000 to 636 000 individuals would be needed to have enough statistical power to show a beneficial effect on CVD risk. As a result, a typical CVD endpoint study investigating the intake of foods with added plant sterols/stanols could be deemed not feasible because of the large sample size required, the concerns about compliance, and the costs. Therefore, surrogate endpoint markers will continue to serve as alternatives for studying the direct effect of plant sterols/stanols on CVD risk. Since atherosclerosis progresses from an early age, the function and structure of the arterial wall is influenced. Endothelial function may be impaired, arteries may become stiffer, arterial wall thickness may increase, and low-grade inflammation may ensue.

Plant sterols/stanols and endothelial function

Evidence supports a link between LDL-C and endothelial function in children with familial hypercholesterolemia[147] and in patients treated with LDL apheresis[148] and other LDL-C–lowering therapies such as statins[149,150] and ezetimibe.[151,152] Furthermore, there seems to be a significant inverse association between flow-mediated dilation and CVD risk, so people with a higher flow-mediated dilation have a lower risk of CVD.[153]

After plant sterols are consumed, their concentrations in plasma and tissues increase. This raises the question of whether surrogate endpoint markers are affected beneficially or, perhaps, detrimentally. The change in plasma concentrations of plant sterols after intake of plant sterol–enriched foods was investigated in a meta-analysis of 41 studies.[154] On an absolute scale, increases in sitosterol and campesterol levels were modest, on average 2.2 to 5.0 µmol/L, especially compared with the average change in LDL-C (−0.33 mmol/L). However, on a relative scale, increases were considerable, on average 31% to 37%. Plasma plant sterol concentrations have been linked to increased CVD risk in homozygous sitosterolemic patients[155] and in some, but not all, observational studies.[156] However, controversial findings have also been reported, as demonstrated by a study in 5 sitosterolemic individuals. Despite severe hypercholesterolemia and high plant sterol/stanol levels, none of these individuals had symptoms of CVD or positive clinical markers of atherosclerosis.[157] It should be noted that intake of foods with added plant stanols increases plasma concentrations of stanols despite stanols having a lower absorption rate than plant sterols. A randomized trial of plant stanol intake of 3 g/d for 4 weeks showed that plasma plant stanol concentrations increased by about 400%.[158] On an absolute scale, however, these increases were minor, being far less than those observed with plant sterols when their intake was increased.

The effects of plant sterols/stanols on endothelial function have been investigated in several animal and human studies. In wild-type mice fed extremely high doses of plant sterol esters (2%; ≈ 100 times higher than the dosage of 2 g/d recommended for lowering LDL-C in humans) for 4 weeks, intake of plant sterols increased plasma concentrations of plant sterols and impaired endothelial-dependent vasodilatation as measured by vascular relaxation of aortic rings.[58] Furthermore, cerebral lesion size increased after plant sterol intake. However, plasma cholesterol concentrations in these mice were not affected, which raises doubt about whether these wild-type mice were suitable for studying the effects of plant sterols. In another animal study in an atherogenic *apoE*$^{-/-}$ mouse model, plant sterol and plant stanol supplementation reduced serum cholesterol and increased plant sterol and plant stanol concentrations, as expected.[159] Elevated levels of plant sterols/stanols were associated with impaired endothelial function. Dietary supplementation with plant sterols and ezetimibe, individually and in combination, reduced atherosclerotic lesions compared with the control diet, although the reduction was significantly greater in the ezetimibe group than in the group fed plant sterols.[58] Contrary to the findings in mouse studies, a 6-week intake of sitosterol and stigmasterol in hamsters improved aortic functioning as measured by acetylcholine-induced endothelium-dependent relaxation.[160] Taken together, animal studies of the effects of plant sterol/stanol intake on endothelial function show conflicting results.

A few human studies have investigated the effect of plant sterol/stanol intake on flow-mediated dilation, as summarized by Plat et al.[1] Despite showing significant reductions in LDL-C, none of these studies showed statistically significant effects on flow-mediated dilation. However, when data from 5 of these studies were combined, a modest improvement in flow-mediated dilation was demonstrated.[93,137,161–163]

Recently, a large randomized trial investigating the effects of plant sterols on vascular function (INVEST [INternational VErapamil SR Trandolapril Study]) examined the influence of plant sterol intake on flow-mediated dilation as a primary outcome measure, together with other markers of vascular function.[164] The study included 240 participants who consumed enriched margarine (3 g of plant sterols per day) for 3 months. Plant sterol intake had a neutral effect on endothelial function as assessed by a placebo-corrected change in flow-mediated dilation of 0.01 percentage point (95%CI, 20.73–20.75). Arterial stiffness, as measured by pulse wave velocity and augmentation index, was also unaffected. It should be noted that the reduction in LDL-C was only −0.26 mmol/L (95%CI, −0.46 to −0.07) or −7% compared with control, which was less than anticipated for a plant sterol intake of 3 g/d. In general, it is estimated that a plant sterol intake of 3 g/d would lower LDL-C by approximately 12%, so the implications of these results for vascular function are difficult to assess. Table 1[94,162–165,167,168] summarizes studies that investigated the effect of plant sterol/stanol intake on endothelial function. Overall, there was no statistically significant improvement in flow-mediated dilation, despite a significant effect on cholesterol lowering.

In the INVEST study, plasma plant sterol concentrations were significantly increased in the plant sterol group, as expected, but the increases were not related to changes in flow-mediated dilation (Figure 3[164]). On the other hand, a larger reduction in LDL-C was significantly correlated with an increase in flow-mediated dilation, suggesting that lowering LDL-C could lead to improvements in endothelial function. In addition, several plasma biomarkers of endothelial dysfunction, ie, E-selectin, soluble vascular cell adhesion molecule 1 (sVCAM-1), and soluble intercellular adhesion molecule-1 (sICAM-1), also measured in INVEST, were not significantly affected by intake of plant sterols vs control.[168] Taken together, plant sterols/stanols have not been shown to consistently improve endothelial function, despite producing significant reductions in LDL-C. This could be because the plant sterol/stanol doses used were below the threshold needed to trigger measurable differences in endothelial function. Furthermore, populations used in studies thus far may

Table 1 **Overview of human studies that measured flow-mediated dilation to investigate the effect of interventions with plant sterols or stanols on endothelial function**

Reference	Study population	No of participants	Study design	Study duration	Dose (g/d)	Average change in serum cholesterol vs placebo[a]	Average absolute change in FMD vs placebo[b]
de Jongh et al (2003)[163]	Children with familial hypercholesterolemia	41	Crossover	4 wk	Plant sterols: 2.3	TC: −11% LDL-C: −14%	+0.50%
Jakulj et al (2006)[165]	Children with familial hypercholesterolemia	41	Crossover	4 wk	Plant stanols: 2.0	TC: −7.5% LDL-C: −9.2%	+0.05%
Hallikainen et al (2006)[162]	Individuals with hypercholesterolemia	76	Parallel crossover	10 wk (intervention) 20 wk (control)	Plant sterols: 1.93 Plant stanols: 1.98	TC: −9% LDL-C: −12% TC: −6% LDL-C: −9%	+0.90% +0.12%
Hallikainen et al (2008)[166]	Patients with type 1 diabetes	19	Parallel	12 wk	Plant stanols: 2.15	TC: −10.8% LDL-C: −16.1%	−2.47%
Raitakari et al (2008)[167] Gylling et al (2009)[93]	Individuals with hypercholesterolemia Individuals with hypercholesterolemia	190 282	Parallel Parallel	12 wk 52 wk	Plant stanols: 2.0 Plant sterols: 2.15 Plant stanols: 2.13	LDL-C: −9.3% TC: −4.4% TC: −4.2%	+0.91% +0.37% +0.58%
Ras et al (2015)[164]	Healthy individuals with hypercholesterolemia	232 (199 for FMD analysis)	Parallel	12 wk	Plant sterols: 3.0	TC: −4.5% LDL-C: −6.7%	+0.01%

Abbreviations: FMD, flow-mediated dilation; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol.
[a]All studies showed a statistically significant reduction in total cholesterol and low-density lipoprotein cholesterol.
[b]None of the studies showed statistically significant effects on flow-mediated dilation.

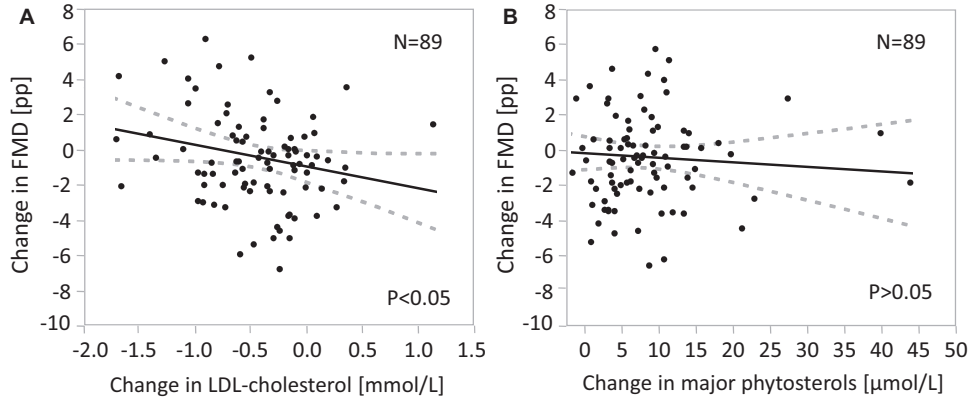

Figure 3 **Correlations between changes in serum low-density lipoprotein cholesterol (LDL-C) and plasma plant sterols and changes in flow-mediated dilation (used with permission from Ras et al[164]).** *Abbreviations*: FMD, flow-mediated dilation; pp, percentage points

have been too healthy. Improvements in endothelial function may only be detectable in individuals with impairment. A longer intervention period might also be needed to detect effects on the endothelium. Importantly, as the evidence shows, plant sterol intake does not compromise endothelial function even though it increases concentrations of plasma plant sterols.

Plant sterols/stanols and other surrogate markers of arterial health

Recently, a few other studies with plant sterols/stanols investigated surrogate endpoint markers, including arterial stiffness, intima media thickness, and inflammation. Gylling et al[169] investigated the effects of plant stanols on arterial stiffness in a randomized controlled study. They found that lowering LDL-C by approximately 10% with plant stanol esters reduced arterial stiffness in small arteries, with some indications of a beneficial effect in large arteries only in men. It should be noted, however, that these effects were driven mainly by increases in arterial stiffness in the control group. Endothelial function, as measured by the reactive hyperemia index, overall was not improved with plant stanol intake. However, changes in LDL-C correlated significantly with changes in the reactive hyperemia index in the plant stanol group, which is consistent with the findings of INVEST.

In an observational study with Old Order Amish people who are prone to be heterozygous for sitosterolemia,[170] carriers of a specific *ABCG8* variant had higher plasma sitosterol concentrations compared with noncarriers of this variant, whereas LDL-C levels did not differ between groups. Compared with noncarriers, carriers had decreased carotid intima-media thickness, suggesting less plaque formation in their vessels with increased concentrations of plasma plant sterols.

Inflammation is also involved in the process of atherosclerosis. Recently, a meta-analysis summarized the effects of plant sterol/stanol intake on markers of inflammation, particularly C-reactive protein.[171] No beneficial effect on this marker was observed.

Evidence regarding effects on surrogate markers of CVD risk, such as endothelial function, is still inconclusive. Notably, no worsening of endothelial function with elevated concentrations of plasma plant sterols has been shown thus far.

Personalizing and optimizing lipid-lowering therapies

Statins reduce cardiovascular morbidity and mortality in primary and secondary prevention trials.[172–174] However, studies have shown that statin efficacy can vary between individuals, which can be attributable to variations in cholesterol metabolism,[141,175–177] with some individuals demonstrating genetically determined high cholesterol synthesis and others showing patterns of higher cholesterol absorption.[123] In individuals with high cholesterol synthesis, statins are potent cholesterol-lowering drugs, but in those who are high absorbers, statins are less effective than cholesterol absorption inhibitors in lowering LDL-C.[178–180] This contrasts, however, with the findings of some studies. For instance, Lakoski et al[181] reported that combination therapy using ezetimibe and simvastatin lowered LDL-C by 15% or greater in more than 95% of participants. Moreover, inhibition of cholesterol synthesis results in increased cholesterol absorption, along with increased uptake of plant sterols.[182] As a consequence, in patients with high cholesterol absorption, statins have been shown to increase rates of cardiovascular events.[183] These findings suggest that individuals with low synthesis and high absorption of cholesterol should be treated with a combination of cholesterol-lowering agents, ie, a statin and a cholesterol absorption inhibitor such as plant sterols/stanols.[183]

Genetic studies have shown that lifelong lower cholesterol levels are associated with lower CVD risk.[184] In

individuals with inactivating mutations of *NPC1L1*, a reduction in cholesterol of 12 mg/dL lowered CVD risk dramatically, by 53%.[135] Moreover, it has been shown that defects in the sterol transporter gene *ABCG8* are associated with higher plant sterol levels and increased cardiovascular risk in the general population.[117,155] Other studies have demonstrated that high cholesterol absorption is associated with increased severity of coronary artery disease[185] and higher cardiovascular mortality.[186] Interestingly, the ratio of cholesterol absorption to cholesterol synthesis has been shown to be associated with severity of coronary artery disease.[187] These results have been verified in the Framingham Offspring Study, with the ratio of cholesterol absorption to cholesterol synthesis shown to be the best lipid parameter to predict cardiovascular risk.[188] New studies using intravascular optical devices show the same finding. In patients with stable or unstable angina pectoris, those with high cholesterol absorption markers and low cholesterol synthesis demonstrated thinner fibrous caps and larger lipid cores.[189] In another study, the effect of atorvastatin treatment on lesion progression in patients with coronary heart disease was assessed with intravascular ultrasound. Atherosclerotic plaque progression was most pronounced in patients with an inadequate response to statin treatment.[190] In the PRECISE-IVUS trial (Plaque REgression with Cholesterol absorption Inhibition or Synthesis inhibition Evaluated by IntraVascular UltraSound), statin monotherapy was compared with a combination of the lipid-lowering drugs statin and ezetimibe in patients with suspected coronary heart disease.[191] After 9 to 12 months, the reduction in LDL-C was greater in patients who received the combination treatment than in those who received statin monotherapy (63 mg/dL vs 73 mg/dL). Moreover, intravascular ultrasound demonstrated a more pronounced regression of atherosclerotic plaques in patients who received the combination treatment. In the GLAGOV (GLobal Assessment of Plaque reGression With a PCSK9 antibOdy as Measured by intraVascular Ultrasound) study, an ezetimibe/statin combination had a more pronounced effect on lesion regression than a statin/PSCK9 inhibitor combination.[192]

Statins showed no effect on cardiovascular mortality in patients on dialysis.[193,194] A possible explanation is that patients on dialysis are characterized by high cholesterol absorption and low cholesterol synthesis, with high cholesterol absorption being associated with greater mortality.[195] This may also explain why, in the SHARP (Study of Heart And Renal Protection), a comparably smaller decrease in LDL-C resulted in a significant reduction of cardiovascular events in patients treated with a combination of lipid-lowering drugs.[196] A post hoc analysis of the AURORA study (A study to evaluate the Use of Rosuvastatin in subjects On Regular haemodialysis: an Assessment of survival and cardiovascular events) points in the same direction: only patients on dialysis who were known to be high cholesterol synthesizers showed a reduction in cardiovascular mortality with statin treatment.[197] Since the publication of IMPROVE-IT, additional evidence has shown that a combination of lipid-lowering drugs in high-risk patients can reduce cardiovascular mortality.[136] With these risk calculations in mind, one can speculate that the use of combined lipid-lowering approaches—assessed on an individual basis to account for differences in cholesterol metabolism—can further reduce cardiovascular risk.[124,198]

SITOSTEROLEMIA: CLINICAL PERSPECTIVE, DIAGNOSIS, TREATMENT, AND SCREENING

Microbiota therapeutics: perspectives on management of sitosterolemia

The gut microbiome is "the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space."[199] Many studies have shown that nutrition can affect gut microbiota.[200,201] Some studies show associations between the microbiome and serum lipid levels.[202] The composition of the microbiome was recently evaluated during the early stages of sitosterolemia. Animals that developed severe forms of the disease had an overall different composition of the microbiome compared with those that either did not develop the disease or had only a mild form of it. Furthermore, differences in the microbial population across groups were identified.[203] Specifically, levels of lactobacilli were found to be downregulated in those with severe experimental autoimmune encephalomyelitis.[203] *Lactobacillus* is a major component of all commercially available probiotics. Could a probiotic be used to treat something so specific such as sitosterolemia? Some studies show that plant sterols can affect the microbiome. As an example, dietary supplementation with 5% plant sterol esters induced alterations in the fecal microbiota of hamsters.[204] However, a recent study could not confirm this finding in human volunteers.[205]

Ezetimibe is the standard treatment for sitosterolemia management. Although it has been shown to reduce plasma sitosterol levels by about 30% to 40%, that may not be sufficient to treat severe symptoms of sitosterolemia. Could the diversity and the function of the microbiome be modified in order to treat sitosterolemia? Or, could a genetically modified vector be used as a delivery system? Can a probiotic that proliferates in the gut and is able to carry a gene that might be transferable into the epithelial cells of the gut be delivered?

Bacterial vectors have been used in the past to induce protective peripheral immunity. For example, *Salmonella* has been successfully adapted for live-vector vaccine delivery.[206,207] Perhaps a genetically modified probiotic that can target the *ABCG5* and *ABCG8* genes in enterocytes could be developed. Many issues require consideration, including the pathogenic factors of potential vectors; however, these are provocative concepts to explore as potential adjunctive treatment options for sitosterolemia.

Clinical perspective: when to consider sitosterolemia within the differential diagnosis

In 1974, William Connor and Ashim Bhattacharyya[110] reported the first cases of sitosterolemia. Two sisters who had onset of tendon xanthomas at the ages of 7 and 8 years, with progression of the condition at 13 to 14 years, were medically evaluated as young adults. They otherwise had normal development, including normal plasma cholesterol concentrations. Total circulating cholesterol levels in both sisters were around 200 mg/dL,[110] which, at the time, was considered an oddity in the context of prominent tendon xanthomas because that level was much lower than what would be expected with a disorder such as familial hypercholesterolemia. Familial hypercholesterolemia is an autosomal dominant disorder that affects about 1 in 250 individuals in the general population, is associated with severe hypercholesterolemia, and is the most common cause of tendon xanthomas. Roughly one-third of patients with a clinical diagnosis of familial hypercholesterolemia do not have an identifiable mutation, even when all of the known genes are sequenced, suggesting the involvement of other genes.[208] At the time these sisters were evaluated, a total cholesterol concentration of 350 to 400 mg/dL or higher would have been expected in a patient with familial hypercholesterolemia. Furthermore, at the time, the presence of tendon xanthomas was usually consistent with a diagnosis of familial hypercholesterolemia or, rarely, cerebrotendinous xanthomatosis caused by mutations in the *CYP27A1* gene, which encodes sterol 27-hydroxylase, a key enzyme in the bile acid synthesis pathway.[209] However, it has been suggested that some individuals could have undiagnosed sitosterolemia masquerading as pseudo–familial hypercholesterolemia as a consequence of the marked diet-induced hypercholesterolemia sometimes seen in patients with sitosterolemia in response to high intakes of dietary cholesterol and plant sterols.[210] The proportion of patients with a clinical diagnosis of presumed familial hypercholesterolemia who actually have sitosterolemia is unknown.

Sitosterolemia is caused by mutations in the sterol transporter genes *ABCG5* and/or *ABCG8*, resulting in intestinal hyperabsorption of all dietary sterols, impaired hepatic excretion of sterols into bile, increased content of plant sterols in tissue, and development of extensor tendon xanthomas and atherosclerosis.

An important question in clinical practice is when to consider a diagnosis of sitosterolemia. As sitsterolemia is a rare disorder, random screening of patients is not indicated or useful. There are, however, several situations in which it is reasonable to consider the diagnosis of sitosterolemia. In line with the clinical presentation of the index patients described by Connor and Bhattacharyya,[110] sitosterolemia should be considered when tendon xanthomas are present in the absence of severe hypercholesterolemia. Another possible sign of occult sitosterolemia is the development of extreme hypercholesterolemia after consumption of diets high in cholesterol or saturated fat. As a consequence of mutations in *ABCG5* or *ABCG8*, patients with sitosterolemia hyperabsorb dietary cholesterol and plant sterols/stanols, resulting in exaggerated diet-induced hypercholesterolemia. Other conditions that may suggest sitosterolemia include paradoxical hypercholesterolemia in response to pharmacological treatment with plant sterols. In normocholesterolemic individuals, plasma LDL-C may decrease as much as 8% to 10% because of plant sterol–mediated inhibition of micelle formation, resulting in inhibition of cholesterol absorption. In contrast, patients with sitosterolemia will hyperabsorb the plant sterols and may actually have a hypercholesterolemic response. Hyporesponsiveness to the LDL-C–lowering effect of statins is another potential indicator of sitosterolemia, but this finding may be confounded by noncompliance with statin treatment, loss-of-function mutations in *PCSK9*, or other factors unrelated to sitosterolemia. Hence, the vast majority of patients who are hyporesponsive to the LDL-C–lowering effect of statins are unlikely to have sitosterolemia.

A key step in the diagnosis of sitosterolemia is measurement of plant sterols in serum or plasma using gas chromatography–mass spectrometry. Some patient populations have plasma sitosterol levels that exceed the cutoff for sitosterolemia, such as babies and patients with severe liver disease who are treated with soy-based parenteral nutrition high in plant sterols. In these individuals, the sitosterolemia is completely reversible after parenteral administration of plant sterols is ceased. Clinical features that may facilitate the diagnosis of sitosterolemia include extensor tendon xanthomas (rarely, tuberous xanthomas), normal to elevated plasma cholesterol, thrombocytopenia, chronic hemolytic anemia and stomatocytosis, and, occasionally, elevated liver enzymes and acute liver failure. However,

the absence of these features does not exclude the diagnosis.[211] Management of sitosterolemia includes decreasing the dietary intake of plant sterols and cholesterol, treatment with ezetimibe and, possibly, bile acid sequestrants, and treatment of hypercholesterolemia with statins, as indicated.

In summary, a diagnosis of sitosterolemia should be considered in individuals presenting with hyperresponsiveness to dietary sterol intake, paradoxical responses to treatment with plant sterols, the presence of tendon xanthomas in the absence of hypercholesterolemia, hyporesponsiveness to statin treatment, platelet and red blood cell abnormalities, or early-onset coronary artery disease without significant hypercholesterolemia.

Sterol metabolism in sitosterolemia

Abnormal sterol homeostasis is a consistent feature in individuals with sitosterolemia.[212] It is characterized by both increased retention and reduced elimination of plant sterols and cholesterol, as well as expanded whole-body cholesterol pools that compensate for the reduced cholesterol synthesis in sitosterolemia.[212] Using in vivo radiolabeled isotopic techniques, Salen et al[212] observed that the turnover rates of plasma cholesterol and sitosterol in patients with sitosterolemia were similar and significantly slower than those in a control individual. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and synthase, as well as other key enzymes involved in cholesterol synthesis, are downregulated in patients with sitosterolemia.[213–215]

Accumulation of plant sterols may account for the low rates of cholesterol synthesis observed in sitosterolemia.[216] Strategies such as feeding either the cholesterol precursor mevalonic acid or a low-cholesterol diet[215] failed to stimulate de novo cholesterol synthesis in patients with sitosterolemia. While ezetimibe is currently the standard therapy for sitosterolemia, its effects on cholesterol synthesis and sterol turnover in sitosterolemic patients are undefined and need further investigation.

Sisterolemia: the challenge of researching rare diseases

The National Institutes of Health has defined a rare disease as one that affects fewer than 200 000 people in the US population, which corresponds to a prevalence ranging from 1 in 16 000 to fewer than 1 in 500 000 individuals, depending on the disease. However, the prevalence of various rare diseases is highly variable, with some diseases being very infrequent. Currently, 7000 separate diseases have been identified as rare, with many of these being hereditary. The study of rare diseases presents multiple challenges, including limited numbers of patients available for recruitment, the unknown natural history of a rare disease, and the considerable phenotypic variability in these diseases. This adds to the complications of investigating not only the disease itself but also the therapeutic approaches. Most physicians fail to recognize rare diseases in clinical practice because they have never seen a case of a disease that occurs in 1 in 100 000 individuals. The National Institutes of Health recognizes the challenges in diagnosing and treating the very large constellation of rare diseases, as demonstrated by its establishment of the Rare Disease Clinical Research Network, which now specifically targets 22 diseases. The Sterol and Isoprenoid Research Consortium, 1 of the 22 in the Rare Disease Clinical Research Network, is a consortium focused on sterol metabolism disorders. The idea behind Rare Disease Clinical Research Network is that no center alone will encounter enough patients with a rare disease to be able to conduct a valid clinical study, and therefore efforts should be pooled in carrying out multicenter studies.

Clinical aspects and treatment of sitosterolemia: observations from the Manitoba Sitosterolemia Cohort

The Manitoba Sitosterolemia Cohort is a kindred of Hutterite patients living mostly in Manitoba, Canada. They are a religious isolate based in rural communities. Within this cohort was a 5-year-old girl who died suddenly and was found at autopsy to have extensive aortic and coronary atheroma.[217] Her medical history included anemia and recurring abdominal pain.[218] This led to a search for a diagnosis and, eventually, a determination of sitosterolemia before the specific mutation was identified.[217,218] Subsequent cascade screening over a period of some 16 years has identified a cohort of 21 patients, all having the *ABCG8* S107X mutation. All 20 survivors have responded very favorably to ezetimibe therapy.[219,220]

INTRAVENOUS PLANT STEROLS/STANOLS AND PEDIATRIC INTESTINAL FAILURE–ASSOCIATED LIVER DISEASE

When enteral nutrition is limited because of insufficient intestinal length and/or poor intestinal function, intestinal failure develops. In order to prevent dehydration and malnutrition, patients with intestinal failure are prescribed parenteral (intravenous) nutrition. Parenteral nutrition serves as an important source of water, electrolytes, and macro- and micronutrients.

While parenteral nutrition is life sustaining for patients with intestinal failure, it can lead to intestinal failure–associated liver disease (IFALD), a potentially fatal liver disorder. Intestinal failure–associated liver disease is defined by the presence of intestinal failure or prolonged use of parenteral nutrition in conjunction with liver dysfunction, characterized by elevated serum transaminases and/or conjugated hyperbilirubinemia. On liver biopsy, IFALD is characterized by cholestasis, inflammation, and steatosis. Liver fibrosis can develop after a short course of parenteral nutrition. In some patients, IFALD culminates in cirrhosis, liver failure, and death. Once liver failure develops, a liver transplant is the only life-saving option.

Intestinal failure–associated liver disease and sepsis are the top 2 causes of mortality in children with intestinal failure.[221,222] For several reasons, IFALD is more common in children than in adults. Gestational age, birth weight, underlying gastrointestinal disorders, and duration of parenteral nutrition are important risk factors for IFALD. Seventy percent of infants who have received more than 60 days of parenteral nutrition will develop IFALD.[221] Moreover, gestational age and birth weight are inversely correlated with the incidence of IFALD. Premature neonates and low-birth-weight neonates are at high risk of IFALD.[221] Lastly, children with gastroschisis, volvulus, distal intestinal atresias, and short bowel syndrome commonly develop IFALD.[221]

Intravenous lipids are prescribed with parenteral nutrition as a source of nonprotein calories and essential fatty acids. In the United States, the only FDA-approved intravenous lipid emulsion for children is entirely soy based (Intralipid, Fresenius Kabi, Uppsala, Sweden). Soy oil–based lipid emulsions have a long-standing association with IFALD.[221–227] Intravenous soybean oil contains high concentrations of plant sterols ($>$ 350–400 mg/L).[222] In contrast to intravenous soybean oil, a non–FDA-approved fish oil–based lipid emulsion (Omegaven, Fresenius Kabi, Bad Homburg, Germany) contains a negligible amount of plant sterols. Fish oil–based lipid emulsions are prescribed in the United States under compassionate-use protocols to treat pediatric IFALD.[222–224] Intravenous fish oil is a safe, effective treatment for IFALD; studies have demonstrated that IFALD resolves in approximately 75% of children treated with fish oil and is associated with a decrease in both the incidence of liver failure and the need for liver transplantation.[222–224]

Levels of plant sterols in soybean oil and fish oil differ substantially. When infants with IFALD are compared with children with IFALD, the infants have higher plant sterol concentrations.[225,226] Furthermore, plasma sterol concentrations correlate with hepatic sterol concentrations and histological changes in liver biopsy.[227] In addition, when intravenous soybean oil was replaced with intravenous fish oil in children with IFALD, not only did plasma sterol concentrations decrease dramatically, but early changes in plasma stigmasterol predicted later changes in conjugated bilirubin.[223] This suggests that stigmasterol levels may serve as surrogate marker of disease severity and treatment response.

Animal experiments provide mechanistic evidence that stigmasterol may be one of the main culprits driving IFALD. Mice infused with parenteral nutrition and intravenous soybean oil have decreased expression of hepatic nuclear transcription factors, liver X receptor, and farnesoid X receptor as well as decreased mRNA expression of bilirubin, bile acid, and sterol liver transporters. In addition, mice exposed to parenteral nutrition plus intravenous soybean oil developed cholestasis and showed elevated liver function tests, mimicking pediatric IFALD.[228] In contrast, mice infused with parenteral nutrition plus intravenous fish oil showed expression of farnesoid X receptor, liver X receptor, and sterol transporters similar to that observed in control mice, and they were protected against IFALD.[228]

These studies demonstrate that the type of intravenous lipid emulsion and, more specifically, the type of intravenous plant sterols, are important players in the pathogenesis of IFALD. With the advent of new lipid formulations, it is important to consider sterol content. It is unknown whether specific sterols are safer than others or whether there is a safe sterol content for lipid emulsions. Further research is needed to resolve these uncertainties.

CONCLUSION

The present review provides an overview of past and recent developments in the basic biology of plant sterols and stanols, largely within the context of therapy and management of dyslipidemia in the general population. Also presented is guidance for the clinical management of rare disorders that result from mutations in regulators of sterol metabolism that lead to the retention of cholesterol, plant sterols and stanols, and other types of noncholesterol sterols in serum and tissues. Particularly novel in the area of plant sterol/stanol physiology is the recognition that even low levels of intake of plant sterols or stanols can influence both the efficiency of cholesterol absorption and the circulating cholesterol pool in both adults and infants. Furthermore, the reciprocity between cholesterol synthesis and absorption and the impact of the ratio of cholesterol synthesis to cholesterol absorption on the efficacy of plant sterols/stanols in lowering LDL-C is being increasingly recognized. Recent studies provide better understanding about how

polymorphisms within genes coding for enzymes active in lipid pathways affect LDL-C lowering. In addition, the advantages of combining plant sterols/stanols with other dietary elements such as fiber, soy protein, and nuts are becoming more widely recognized. The overall importance of LDL-C lowering in reducing CVD risk has been further established in trials investigating combinations of drugs, such as IMPROVE-IT[136] and the FOURIER trial.[144] Additionally, a clear association between LDL-C and atherosclerotic CVD has been identified recently from multiple clinical and genetic studies.[229] In best approaches to clinical management of sitosterolemia, ezetimibe continues to prevail as the drug of choice. The disparity in the degree of sitosterolemia severity across patients was emphasized, as was the importance of proper screening using as diagnostic criteria both the levels of circulating plant sterols and confirmation of the specific gene mutation. It is important to rely on these tools for correct identification of patients with sitosterolemia to avoid confusing them with patients with familial hypercholesterolemia. In summary, plant sterols and stanols continue to offer an efficacious and convenient dietary approach to cholesterol management and serve as important natural health products as well as ingredients of functional foods. Their clinical benefits to vascular function, however, have not yet been established in long-term studies with predefined CVD endpoints.

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