Progress and perspectives in plant sterol and plant stanol research

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Progress and Prospective of Plant Sterol and Plant Stanol Research: Report of the 3rd International Plant Sterols/Stanols, Health and Disease Meeting, Winnipeg 2016

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1. Introduction

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

2. Low Density Lipoprotein-Cholesterol (LDL-C) Efficacy of Plant Sterols/Stanols

2.1 Factors that Influence the Cholesterol-lowering Efficacy of Plant Sterols/Stanols

Hundreds of studies have investigated a variety of aspects of the clinical efficacy of plant sterols and stanols for lowering LDL-C. Firstly, comparing plant sterols with plant stanols, consistent evidence demonstrates that plant sterols/stanols lower LDL-C levels by 7.5 to 12% with intakes of 1.5 to 3 g/d [2]. At intakes of up to 3 g/d, which is the current recommended range of intake in most countries, equal LDL-C lowering effects occur between plant sterols and plant stanols. A systematic review of 14 studies showed a non-significant weighted mean difference in LDL-C lowering [3] between plant sterols and plant stanols. Moreover, compiling data from 124

85 studies revealed a clear dose-dependent reduction in LDL-C at plant sterol and stanol intakes up
86 to 4 g/d. In this meta-analysis, at an average plant sterol/stanol intake of 2.1 g/d, an 8.4%
87 reduction of LDL-C was observed, while with an average intake of 3.3 g/d a 12.4% reduction
88 was found [2]. It appeared that at 2.1 g/day intake, there was about a 2% difference in LDL-C, with
89 plant stanols achieving a more pronounced LDL-C lowering whereas at higher average intakes of
90 2.6 and 3.3 g/d comparable lowering of LDL-C was found [1]. These findings persisted in the
91 results of several additional analyses [4]. The consistency of the food format, either solid/edible or
92 liquid/drinkable, is critical to compare plant sterols/stanols. As described by Ras et al. [2], in the
93 dose category ≥2.0 dose <2.5 g/d, average 2.1 g/d, fifteen of forty plant sterol studies used liquid
94 food formats, whereas only four of eighteen plant stanol studies used this type of food format.
95 Irrespective of the type of plant sterols/stanols used, liquid foods lowered LDL-C concentrations
96 by, on average, 6.5%, whereas solid foods lowered LDL-C concentrations by, on average, 9.2%
97 [2]. So, the limited sample size of studies that used the liquid food formats as plant stanol carrier
98 warrants caution in drawing sweeping conclusions. Additional research with head to head plant
99 sterol vs. stanol comparisons is needed.
100 A second factor influencing the cholesterol-lowering efficacy of plant sterols/stanols is
101 food matrix. Liquid versus solid food matrix, the fat content and fat type of the food, supplement
102 form (capsules or tablet), use of free or esterified plant sterols/stanols and the fatty acid used for
103 esterification, all exist as matrix effects. In addition, frequency of administration, e.g. single vs.
104 multiple daily intakes, intake with or without a meal, as well as the time of administration during
105 the day, e.g. morning vs. later during the day, are factors contributing to the degree of plant sterol
106 or stanol efficacy. A systematic review of dietary plant sterols/stanols coming from food or
107 tablets showed a similar mean difference in LDL-C lowering [5]. However, in most tablet studies,

particle size and dissolution activity data were missing. Tablet characteristics represent a critical aspect for future reported research using tablets.

Plant sterols/stanols have been examined across multiple food formats and there is no apparent difference in efficacy between fat-based and low or non-fat based foods [6,7]. In terms of type of the carrier fat, a recent study found no difference in the relative reduction in LDL-C levels [8]. Higher efficacy of solid (e.g. spreads and margarines) vs liquid food formats (milk and juices) was seen in two meta-analyses [2,7]. No differences exist between the efficacy of free vs esterified plant sterols [7,9,10], however, the particle size of plant sterols should be taken into account. Nor does the fatty acid used for esterification have an impact on the cholesterol-lowering efficacy of plant sterols/stanols [11-13]. However, data from meta-analyses show that intake frequency matters and that once a day seems sub-optimal [2,7]. Larger LDL-C lowering effects of 9.4% were found when a yogurt drink was consumed together with a lunch meal compared to a 6.0% lowering when consumed before breakfast [14]. Another study with plant stanol-enriched biscuits also found that biscuits consumed with a meal resulted in a greater cholesterol-lowering effect compared to biscuits consumed between meals [15]. In 2000, Law found that in the plant sterol and stanol intervention studies published, the absolute decrease in LDL-C increased with age [16], however, 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 males consumed 5-6 g/d of beta-sitosterol showing mean serum total cholesterol decreases of as great as 15 to 20% over 6 weeks [17]. Another early research, in which 15 males all with previous myocardial infarctions consumed 12 to 18 g/d of beta-sitosterol, also showed large declines in serum total cholesterol [18]. Neither of these studies, however, were randomized trials and the results focused on changes in total cholesterol. Since these initial publications, important

advances in trial design and analytical methods have occurred. Miettinen et al. conducted a landmark, one year-long study of 153 subjects in a double-blind, randomized control trial and observed a 14.1% decrease in circulating LDL-C with 2.6 g/d of plant stanols compared to the placebo, without a decrease in high density lipoprotein-cholesterol (HDL-C) [19].

Overall, summarizing data from meta-analyses from 2000 through 2016, most studies report an LDL-C reduction between 0.3 and 0.4 mmol/L [2,5,6,8,16,20]. As LDL-C is recognized as an important causal risk factor for coronary heart disease [21] such a reduction in LDL-C would correspond to a 25% reduction in the risk of heart disease. However, to date, direct evidence on cardiovascular disease (CVD) is not available as studies exploring hard endpoints including CVD events and mortality have not been conducted as they are expensive and challenging like all dietary intervention studies to perform in light of long-term compliance.

2.2 Diversity of Natural Plant Sterols/Stanols

Experts agree that a minimum of 1 g of plant sterols/stanols consumed per day is necessary to significantly lower circulating LDL-C levels [21]. However, plant sterols in fruits and vegetables naturally range from about 38 to 439 mg/kg fresh weight and 329 to 1780 mg/kg in grains, so to consume 1 g of plant sterols, one would need to eat about 2 kg fruits/vegetables or about 1 kg of grains per day [22]. 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 1g. Therefore, fruits/vegetables, grains and plant oils are not practical sources of dietary plant sterols/stanols, so one needs to look at other approaches. Tall oil and vegetable oil deodorizer distillates continue to be major feedstocks for plant sterols/stanols destined for functional foods, but other sources are under investigation. For example, corn fiber oil and rice bran oil contain 10-15% and 2% total plant sterols, respectively, but have not been used as a commercial feedstock for plant sterols/stanols [22]. In

plants, most sterols/stanols occur either in the free un-esterified form or esterified to fatty acids. However, plant sterols/stanols also occur as steryl glucosides (SG) and as acylated steryl glucosides (ASG) with the SG esterified to a fatty acid. Unlike sterol esters, SG can inhibit cholesterol absorption in their intact form, without being hydrolyzed by digestive enzymes such as pancreatin [23,24]. A future option therefore could be cloning the gene to produce SG, which may be useful if future clinical studies indicate additional benefits of dietary SG, when compared to common forms of free and esterified plant sterols [25]. Inclusion of lecithin as a food ingredient, as another strategy, may contribute significant amounts of plant sterols/stanols to the diet. Lecithin also has been reported to be a valuable organogelator. An organogel is defined as an organic liquid entrapped within a thermo-reversible, three-dimensional gel. Some of the other main organogelators include sitosterol plus oryzanol and plant waxes [26,27]. Hence, further research on organogels is warranted.

3. Effects of Plant Sterols/Stanols Beyond Cholesterol-Lowering

3.1 Plant Sterols/Stanols and Immune Function

Nutrition, whether considered as whole diets, specific nutrients, or bioactive phytochemicals, is a powerful modulator of the immune system, regulating defense against pathogens and the chronic inflammatory response that underlies many disease states [28]. Previous *in vitro* [29], animal [29], and human [30] studies suggest that plant sterols/stanols affect immune response. Calpe-Berdiel et al. reported that, independent of cholesterol-lowering effects, 2% dietary plant sterol supplementation in apolipoprotein E (apoE) deficient mice increased secretion of the type 1 T helper cells (Th1), interleukin (IL-2) and interferon gamma (IFN) from cultured spleen lymphocytes treated with turpentine [29]. An effective biological response to an immune challenge involves the balance of specific patterns of pro- and anti-inflammatory

177 cytokines by Th1 and Th2 helper T cells, respectively [31]. Nashed et al. demonstrated that in
178 addition to cholesterol lowering, 2% dietary plant sterol supplementation in apoE deficient mice
179 for 14 weeks decreased plasma IL-12 concentrations [32]. Brull et al. previously reported evidence
180 that physiological concentrations of both sitosterol and sitostanol induce a Th1 shift in human
181 peripheral blood mononuclear cells [33]. More recently, the same group addressed whether these *in*
182 *vitro* plant sterol/stanol-induced changes could be applied clinically to enhance immune function
183 in asthma patients [34]. In a randomized, double-blind clinical trial, asthma patients receiving plant
184 stanol enriched soy-based yogurts (4.0 g/d plant stanols) vs control demonstrated higher antibody
185 titers against hepatitis A virus vaccination and reductions in plasma total immunoglobulin E,
186 interleukin (IL)-1β, and tumor necrosis factor-α concentrations. Changes in plant stanol
187 concentrations correlated positively with changes in antibody titers and the Th1/Th2 cytokine
188 index and negatively with changes in IL13 concentrations. Although these results are promising,
189 further studies designed to explore clinical benefits in immune compromised populations are
190 required.

191 **3.2 Plant Sterols/Stanols and Triglyceride-Lowering**

192 The rising global obesity epidemic is associated with a characteristic dyslipidemic
193 phenotype that includes elevated serum/plasma cholesterol and triglyceride (TG) concentrations.
194 Previous work suggests that approximately 80% of overweight and obese subjects have serum
195 TG concentrations >150 mg/dL (1.7 mmol/L). Although plant sterols/stanols have a rich history
196 as effective cholesterol-lowering compounds, their benefit in reducing hypertriglyceridemia is a
197 relatively recent discovery. Results of previous randomized controlled studies conducted in
198 normo-triglyceridemic subjects suggest that daily supplementation of plant sterols/stanols (1.6-9
199 g/d) for 1-2 months resulted in a TG-lowering response of 0.8-7%. [35-38]. However, in subjects

200 with elevated serum TG concentrations (>1.7 mmol/L), randomized control trials results suggest
201 that plant sterol/stanol supplementation (1.8-4 g/d) may lower circulating TG concentrations in
202 the range of 11-28% [39-45].

203 Previous animal studies indicate that the TG-lowering effects of plant sterols may be
204 related to altered intestinal fat metabolism including increased fecal fatty acid excretion in plant
205 sterol supplemented mice [46] and reduced postprandial lymphatic transport of TG (5-7 hours
206 following a meal) in thoracic duct–cannulated Sprague-Dawley rats [47]. However, clinical studies
207 investigating postprandial fat handling in normo-triglyceridemic subjects failed to support animal
208 data suggesting that plant sterols can interfere with intestinal fat digestion/absorption [48,49].
209 Studies investigating potential alterations in TG absorption or postprandial handling in response
210 to plant sterol/stanol supplementation in subjects with hypertriglyceridemia are needed.

211 Additionally, previous work implies that plant sterol supplementation may reduce hepatic
212 *de novo* lipogenesis in Golden Syrian hamsters [50], however, species differences have been noted
213 [46]. In support of a TG-lowering mechanism of hepatic origin, Plat et al., reported a reduction in
214 large and medium plasma very low density lipoprotein (VLDL) particles in dyslipidemic
215 metabolic syndrome subjects consuming 2 g/d of plant stanols provided in a yogurt [42]. This was
216 also confirmed in an animal study looking at hepatic VLDL production [51].

217 Future research priorities with respect to plant sterols/stanols and TG metabolism include
218 human intervention studies specifically powered to detect TG responses in hypertriglyceridemic
219 subjects, a direct examination of fatty acid absorption, as well as whole body lipogenesis in
220 response to plant sterol/stanol supplementation. Additionally, identification of both metabolic
221 and genetic factors that determine the magnitude of plant sterol/stanol-induced TG reductions,
222 needs more attention.

3.3 Plant Sterols/Stanols and the Central Nervous System

Consumption of plant sterol-enriched foods increases circulating plant sterol levels and may enhance accumulation of plant sterols in tissues such as aortic valves, liver, but also in the central nervous system (CNS) [52-55]. In a study by Simonen at al consumption of plant sterols/stanols did not enhance accumulation of plant sterols/stanols in stenotic aortic values [56] The mean duration of this intervention was 2.6 ± 0.2 months (range 0.6-5.0 months) [56].

Although sterols are poorly transported across the blood brain barrier (BBB), sterols with a lower molecular side-chain complexity such as cholesterol and campesterol cross the BBB more easily compared to other plant sterols possessing a more complex side chain (e.g. sitosterol and stigmasterol) [57-59]. The exact mechanism by which plant sterols are delivered to the endothelial monolayer of the BBB remains speculative. As ATP-binding cassette sub-family G member 5 and member 8 (*ABCG5/G8)* transporter proteins are not expressed within the brain, or at the BBB [60], this transporter complex would not be expected to modulate plant sterol transport at the level of the BBB. An HDL-mediated plant sterol transport pathway across the BBB has been suggested given that plant sterols are predominantly transported via HDL in wild type and *ABCG5*$^{-/-}$ mice, and scavenger receptor class B member 1 (SR-BI), the major HDL receptor, is highly expressed on the apical membrane of endothelial cells of the BBB [61]. Regardless of the uptake mechanism, animal plant sterol feeding and depletion studies suggest that accumulation of plant sterol in the CNS is virtually irreversible [58]. Although the conversion of cholesterol to 24(*S*)-hydroxycholesterol in neurons accounts for over 60% of cholesterol efflux from the CNS [62-66], once plant sterols enter the CNS, they are not metabolized by the *CYP46A1* gene into 24(*S*)hydroxysterol [58,67], likely due to steric hindrance with respect to the ethyl or methyl group at the C24 position.

Although quantitative data on spatio-temporal accumulation of plant sterols in the human CNS are limited, the total content of plant sterols in the CNS of non-neurologic elderly is estimated at ~75 ng/mg dry tissue, representing about 0.5% of the total amount of sterols in the CNS [54]. Pyramidal cells of the cortex and Purkinje cells of the cerebellum have a cholesterol turnover rate of more than 20%/day [63,68-70]. The high flux of sterols in these metabolically active cells allow fast incorporation of plant sterols in detergent-resistant parts of neuronal membranes, thereby actively modulating CNS cholesterol metabolism [58,71]. A mechanistic study from Burg et al. shows that cleavages of the amyloid precursor protein were beneficially modified by incorporation of plant sterols in neuronal membranes [72]. 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 [73]. 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 [74]. On the other hand, previous studies found that plant extracts have anxiolytic-like effects after intraperitoneal administration in mice [75,76]. Together, data suggest that plant sterols do not enhance cognition in normo-cognitive settings. However, accumulating *in vitro* and *in vivo* findings support a therapeutic potential for plant sterols in a disease-related cognitive impairment.

4. LDL- Responsiveness to Plant Sterols/Stanols

4.1 Increased Cholesterol Excretion as an Alternative Measure of Plant Sterols/Stanols Efficacy

Reduction of cholesterol absorption by plant sterols/stanols is clearly important in their LDL-C lowering action, but it may not be the only mechanism. Plant sterols/stanols also may affect reverse cholesterol transport and whole body cholesterol metabolism[77] , which are emerging areas of interest in cardiovascular risk analysis studies. Plant sterols/stanols exert their principal effects most likely through disruption of the intraluminal solubilization step [78]. In a controlled feeding study with 20 subjects, in which dietary nutrient and plant sterols intakes were measured and carefully controlled, fecal cholesterol excretion rose by 36% as the diet plant sterol content was increased from 59 mg/day to 459 mg/day and by a total of 74% as the plant sterol dose was further increased to 2059 mg/day [79]. In contrast, LDL-C levels were reduced by 5% and 9%, respectively, with each stepwise increase in dose. Additionally, in many studies, plant sterol consumption reduces cholesterol absorption efficiency by 30-45% [80-84], yet circulating levels 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 only LDL-C lowering, but that there should be additional pathways involved. More studies demonstrating enhanced reverse cholesterol transport and reductions in hard cardiovascular outcomes following plant sterol/stanol feeding should improve the ability to make public health recommendations. To successfully achieve this goal better biomarkers to assess plant sterol/stanol consumption precisely are needed. Better biomarkers are needed as measuring plasma plant sterols/stanols alone does not allow a precise estimation of dietary intake because of the large between-individual variation in non-cholesterol sterol handling. Validation of biomarkers of dietary plant sterol/stanol consumption on controlled diets where plant sterol intake is precisely known suggests that a better indicator is the ratio of plasma campesterol (the most avidly absorbed plant

sterols), to 5α-cholestanol (an endogenous cholesterol metabolite). This ratio has been found to be significantly and directly associated with dietary plant sterol intake ($R^2 = 0.79$, $P < 0.0001$[85]).

4.2 The Genetics Behind Plant Sterols/Stanols Responsiveness

Several clinical studies have investigated the genetics behind plant stanol responsiveness. Effects of small amounts of sitosterol, sitostanol and sitostanol esters (< 1 g/day of free sterols) dissolved in rapeseed oil (RSO) were studied on serum lipids and cholesterol metabolism in patients with primary hypercholesterolemia, but with different apolipoprotein E (apo E) phenotypes on a RSO diet. LDL-C reduction was -8% in subjects with apo E epsilon 4 allele and insignificant in those with apo E3/3 phenotype [86]. The relationship of genetic variation in genes encoding apolipoprotein A-IV, scavenger receptor BI, HMG-CoA reductase, CETP and apo E with the response of cholesterol metabolism to plant stanol ester consumption was examined by Plat and Mensink [87]. This group examined 112 non-hypercholesterolemic subjects, 70 of whom consumed 3.8-4.0 g plant stanols in the form of plant stanol esters per day for 8 weeks. No significant differences between the polymorphisms and dietary responsiveness to plant stanol consumption was found, thus indicating it is unlikely that one of the single polymorphisms analyzed in this study was a major factor in explaining the variation in serum LDL-C responses[87]. However, in another study in which changes in serum plant sterol concentrations with *ABCG5/G8* polymorphisms were investigated after consumption of plant stanol esters, cholesterol-standardized serum campesterol and sitosterol concentrations were significantly associated with the *ABCG8* T400K genotype, as were changes in serum plant sterol concentrations after consumption of plant stanols. However, despite the shifts in circulating plant sterol levels, no associations with serum LDL-C levels were found [88]. Gylling et al. also determined whether common polymorphisms of *ABCG5* and *ABCG8* regulate the responses of

serum cholesterol levels and vascular function during long-term inhibition of cholesterol absorption. Here, 282 subjects completed a 1-year study consuming plant stanol or sterol esters (2 g/d plant stanols or sterols) or a control spread. Neither serum cholesterol lowering, nor absorption inhibition, were found to be associated with polymorphic sites of *ABCG5* and *ABCG8*. However, regulation of baseline cholesterol metabolism and vascular function and structure, and intima media thickness (IMT) progression during 1 y seemed to share some common polymorphic sites of these genes, suggesting a gene-regulated interaction between cholesterol metabolism and vascular function and structure [89]. Taken together, although provocative data exist suggesting that genetic architecture influences the response of sterol metabolism to plant sterols/stanols, such mechanisms need further study.

Clinical trials, as shown in **Figure 1,** reveal that substantial inter-individual variability in LDL-C lowering exists in response to plant sterols consumption [40,90], with responses ranging from better than average to non-response or even adverse-responsiveness (please include in citations: Weingärtner O, Bogeski I, Kummerow C et al. Plant sterol ester diet supplementation increases serum plant sterols and markers of cholesterol synthesis, but has no effect on total cholesterol levels. J. Steroid Biochem Mol Biol. 2017; 169: 219-225.) [1,91]. Distinct inter-individual responses to plant sterol consumption have been shown to be reproducible in individuals across repeated plant sterols interventions [92], indicating other potential determinants of responsiveness. Factors responsible for this variability have been investigated. One explanation has focused on individual differences in cholesterol synthesis rates as determined by the circulating lathosterol-to-cholesterol ratio. This was shown to be a biomarker predicting an individual's response of cholesterol biomarkers to plant sterol intervention, as reported by Mackay et al [93]. Response of cholesterol synthesis and plasma cholesterol levels were found

subsequently to be influenced by SNP rs38038607 in CYP7A1- and APOE polymorphisms [94]. In particular, *CYP7A1*-rs3808607 and APOE isoforms were correlated with the extent of reduction in circulating LDL-C levels in response to plant sterol consumption. Thus, these could serve as potential predictive genetic markers to identify individuals who would derive maximum LDL-C lowering with plant sterol consumption [94]. Mackay's study confirmed the results of De Castro-Oros et al [95], which assessed whether a common A to C substitution at position −204 of the promoter of *CYP7A1*-rs3808607 was related to variability in plasma sterol responses to plant sterol supplementation. They found that compared with carriers of the A allele, those bearing the −204C variant had a significantly higher adjusted mean reductions in total cholesterol and increases in lathosterol-to-cholesterol ratios [95].

To investigate if other evidence exists in support of genetic mechanisms explaining inter-individual differences in responsiveness to dietary bioactives, Abdullah et al., reviewed the current knowledge on cholesterol-related genetic variations in association with responses of fasting circulating cholesterol levels in epidemiological and intervention studies [96]. The reviewed studies indicate that carriers of certain genotypes within cholesterol-related genes respond better to a given dietary intervention than others, and the clinical effects of this responsiveness seem to be significant for most cases reported [96]. For example, a 3.9-fold greater reduction in serum LDL-C levels was observed in hypercholesterolemic men carrying the SNP rs4148217-A, but not the other allele, in the *ABCG8* gene when intake of plant sterols was 2.0 g/d for 4 weeks [97]. 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 enhance plant sterol/stanol efficacy in reducing CVD risk factors. Taken together, it has been considered that a tipping point has been reached in understanding that genomic architecture plays

a role in modulating the degree of responsiveness of biomarkers to dietary intervention. A number of cholesterol-related gene-diet interactions have been identified, suggesting that such interactions may represent a further advance for meaningful conclusions that may eventually lead to genetically targeted dietary recommendations in the era of personalized nutrition [96].

5. Challenges in Measuring Plant Sterols/Stanols in Biological Samples and their Use as Surrogate Markers of Cholesterol Metabolism:

5.1 Measuring Plant Sterols/Stanols

Plant sterols/stanols fall broadly into the category of non-cholesterol sterols (NCS), which encompasses a category of biological non-cholesterol and non-steroid hormone sterols. NCS share the steroid skeleton with cholesterol, and are comprised of precursors in the cholesterol synthesis pathway, sterols/stanols of plant origin, and certain cholesterol derivatives [98]. Serum or plasma concentrations of the cholesterol precursors, such as lanosterol, lathosterol, and desmosterol, are widely used as surrogate markers of endogenous cholesterol synthesis [99,100]. Reciprocally, plant sterols, such as campesterol or sitosterol and the cholesterol metabolite 5α-cholestanol, are used as markers of cholesterol absorption [101-103].

These NCS are often so similar in structure to cholesterol that enzymatic methods to quantify cholesterol will actually measure the NCS species as well, artificially inflating cholesterol concentrations [104]. Conceptually, very little in the quantitation of NCS has changed since they were measured by Bhattacharyya and Connor in the first sitosterolemic children identified [105]. The various species of sterols must be separated chromatographically, often by gas or liquid chromatography and then measured, which typically either uses flame ionization detection or mass spectrometry [106]. Even with careful chromatographic techniques it can still be impossible to separate certain species of sterols; therefore, separation of these species must occur

during the detection using mass spectrometry with mass selective detection [107]. While NCS may share a similar chemical structure as cholesterol, they are found in biological fluids in concentrations which are profoundly different, ranging from mmol/L for cholesterol, umol/L for plant sterols/stanols and cholesterol precursors, down to pmol/L or lower for their oxidized sterol derivatives [108]. The large range of concentrations in NCS renders it difficult to capture all using a single analytical method, which have contributed to the numerous methods which have been specifically developed for measuring NCS [106]. These methods for NCS measurement often vary in chromatographic separation techniques and detection methods [107]. This variability in methodology used to measure NCS is a substantial challenge to their use as surrogate measures of cholesterol metabolism because it hinders the ability to compare NCS values reported from different laboratories. In fact, measurement methodology has been identified as the greatest contributor to variability in plant sterols concentrations reported in the scientific literature [109]. This variability has led to an attempt by researchers in the field to work towards harmonizing NCS measurement and to conduct ring-trials to measure the amount of variability across various laboratories [106]. In summary, comparing plant sterol or stanol concentrations reported from different laboratories must be done with caution, realizing that methodology may be the biggest single contributor to differences, rather than diet or other biological mechanisms.

5.2 Plant Sterols/stanols as Surrogate Markers of Cholesterol Metabolism

As mentioned above, circulating plant sterol/stanol levels are often used as surrogate measures of cholesterol absorption [102]. Compared to direct and indirect methods of measuring whole body cholesterol absorption or synthesis, measuring NCS is faster, affordable and less invasive. However, occasions occur when using plant sterols or stanols as surrogate markers of cholesterol absorption is not appropriate and may not accurately represent intestinal sterol

absorption even in the absence of supplemental intake of plant sterols/stanols. When intakes of plant sterols or stanols are changing, such as in a trial involving plant sterol/stanol supplementation, use of concentrations of those compounds as surrogate measures of cholesterol absorption is invalidated [110]. When plant sterols, or other NCS, are to be used as surrogate measures, they should be expressed as ratios to total cholesterol, which standardizes for variations in sterol transport protein concentrations [101] and show even stronger correlations with cholesterol absorption and synthesis. Plant sterols and other NCS, as surrogates for cholesterol absorption, have been associated with CVD risk [111,112]. NCS have also been used to differentiate between different types of dyslipidemias [98,113,114]; predict response to statin therapy [115,116]; and could be used to guide lipid lowering therapy [117,118, 190; (please include this citation)]. Beyond their use individually as markers of cholesterol absorption or synthesis, the ratios of cholesterol synthesis to cholesterol absorption surrogates, such as the lathosterol to campesterol ratio, are also utilized to assess the overall balance of cholesterol metabolism, with higher values representing more synthesis and lower absorption [119]. However, due to the inherent nature of ratios, 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 fit well with the actual impact of these different values on biology. To overcome this limitation it is possible to arrange the synthesis and absorption markers in a Cartesian plane and relate an outcome in a third plain as was done by Qi et al. [120]. A new approach of using both absorption and synthesis markers together as a method of measuring cholesterol metabolism was proposed (**Figure 2**). By taking the length of the hypotenuse of a

triangle created by graphing cholesterol absorption surrogates against synthesis surrogates, a potential overall measure of cholesterol metabolism is obtained.

Due to their ease of use, measuring plant sterol or other NCS, as surrogates of cholesterol metabolism is not likely to become less common. Improvements and standardization in the measurements of NCS and how they are used as surrogate markers of cholesterol metabolism will further improve their utility.

6. Plant Sterols/Stanols as Adjuncts with Diet and Drugs

6.1 Lipid Lowering Drugs and Plant Sterols: Ezetimibe

Ezetimibe (Zetia, Ezetrol) is a selective cholesterol absorption inhibitor that potently inhibits the uptake and absorption of biliary and dietary cholesterol and non-cholesterol sterols from the intestinal lumen without affecting the absorption of other nutrients. Clinically, ezetimibe reduced fractional cholesterol absorption and this was accompanied by an LDL-C lowering of 20.4% in 18 patients with mild hypercholesterolemia [121]. Ezetimibe alone reduces plasma total cholesterol and LDL-C levels by18% in patients with primary hypercholesterolemia, and when ezetimibe was added to on-going statin treatment, an additional 25% reduction in LDL-C levels occurred [122]. On the other hand, ezetimibe also blocks plant sterol absorption. In clinical studies, after just two weeks of ezetimibe at 10 mg/day, plasma sitosterol and campesterol were reduced 41% to 48%, respectively. Ezetimibe also reduced serum plant sterol levels by about 50% in combination with statins (simvastatin and atorvastatin) [123].

Sitosterolemia is caused by mutations in the ATP-binding cassette (ABC) co-transporters, either *ABCG5* and/or *ABCG8*, leading to an accumulation of plant sterols in plasma and tissues which, in turn, results in accelerated cardiovascular disease, anemia, platelet defects, and other disorders. Case studies have examined ezetimibe treatment for sitosterolemia, and in some

instances ezetimibe treatment caused xanthomas to resolve, platelet counts to increase, and cardiovascular symptoms to improve [124]. Ezetimibe reduced the serum levels of the atherogenic sterols campesterol and sitosterol in 37 patients with sitosterolemia [125].

The intestinal transporter for cholesterol and plant sterols is Niemann Pick C1 Like 1 (NPC1L1) [126]. Ezetimibe works by inhibiting the NCP1L1 mediated uptake of sterols into the enterocyte and it also blocks the re-uptake of sterols from the bile back into hepatocytes in humans [127]. This blockage results in enhanced excretion of fecal neutral sterols and a reduction of both plasma and tissue cholesterol and plant sterol levels.

Pre-clinically, ezetimibe treatment or the lack of NPC1L1 in mice has been shown to reduce atherosclerosis [128]. The effect of NPC1L1 mutations on human atherosclerosis was not known. Sekar Kathiresan et al led a study where they exon-sequenced >22,000 individuals and found 15 inactivating mutations of NPC1L1. Then they screened for these inactivating NPC1L1 mutations in >100,000 individuals and looked at their 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 fall in the risk of coronary heart disease (CHD) by 53% [129]. Since these are heterozygotes, this is a lifelong 50% inhibition of NPC1L1. So, whether the use of ezetimibe to inhibit NPC1L1 will cause a similar large decrease in CHD in a hard outcomes trial needed to be addressed.

The IMPROVE-IT was an acute coronary syndrome (ACS) secondary prevention outcomes trial in over 18,000 patients [130]. The objective was to reduce LDL-C levels to either 70 mg/dl with simvastatin alone or to 55 mg/dl by adding ezetimibe, seeing if even lower than the 70 mg/dl LDL-C guideline recommendations is better with the combination. The baseline LDL-C levels were 94 mg/dl at the start of this trial. In contrast to previous data [131], there was about a

16 or 17 mg/dl difference between the treatment groups; with simvastatin alone, LDL-C levels were 70 vs 53 mg/dl with the combination with ezetimibe. There was a significant reduction of 6.4% treatment effect on top of simvastatin with ezetimibe for the primary CVD outcome endpoints in the intention to treat population [124]. In another study, the addition of plant sterols to ezetimibe improved the effects of ezetimibe on whole-body cholesterol metabolism and plasma LDL-C as shown by Lin et al, [132] . Recently, Gomez et al., reported that the combination of plant sterols and ezetimibe was associated with lower LDL-C levels [133]. In that regard, long-term use of sitostanol-ester margarine as a substitute for part of normal dietary fat had a favorable effect in subjects with mild hypercholesterolemia in lowering serum total cholesterol and LDL-C levels [19]. Therefore, this indicates that LDL-C lowering with ezetimibe is probably causing the reduction in CV events. These data help emphasize the primacy of LDL-C lowering as 'a strategy to prevent coronary heart disease' [134].

A question still remains whether it is just LDL-C reduction with ezetimibe that lowers the CV event rates. Ezetimibe also blocks plant sterol absorption, and possibly oxysterol absorption, which may add to the anti-atherosclerotic activity of ezetimibe, but this requires further investigation.

6.2 Guidelines for Lowering Serum Cholesterol Levels: Is There a Place for Plant Sterols/Stanols?

There has been a long-standing argument over the "statin hypothesis" - the idea that statins have a 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 treatment 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 per liter (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 [135].

In this regard, the recent development of PCSK9 inhibitors is also of note. These agents reduce LDL-receptor degradation, thereby enhancing LDL clearance from the circulation, and reducing LDL-C levels by as much as 60% [136]. Definitive clinical outcomes trials with these agents are ongoing. Sabatine et al found that PCSK9 inhibition with the PCSK9 inhibitor Evolocumab on a background of statin therapy reduced LDL-C levels and the risk of CVD [137].

6.3 Plant Sterols and Other Dietary Agents

Like fiber, plant sterol intake appears to have contracted substantially in modern diets. It has been estimated from studies of early ancestral diets that one would have consumed ~1 g/d of plant sterols 4-5 million years ago when splitting genealogically from the gorillas and chimpanzees.

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 30-35% were observed [138]. This fall in cholesterol was related to increased intakes of fiber, vegetables, vegetable proteins, nuts and plant sterols in the diet that was very low in saturated fat with zero cholesterol content. It can be reasoned that the lack of these components in the current diet, together with the consumption of significant amounts of animal products, high in saturated fat, cholesterol and animal proteins, was responsible for the current elevated LDL-C levels seen in humans consuming Western-type diets. This current intake has resulted in the need to take statin drugs instead of employing diet modification to improve cholesterol levels.

The key elements of the ancestral dishes, which were individually been approved by FDA for cholesterol reduction claims, were taken to create a new diet, which required consumption of

519 a very large volume of plant foods. Elements included vegetable protein (soy); nuts; viscous
520 fibers (oats, barley and psyllium); and plant sterols, incorporated in standardized amounts into a
521 single diet termed the "dietary portfolio". This portfolio diet lowered LDL-C and CRP levels by
522 20-35% in hyperlipidemic participants on metabolic diets [139]. In an ad libitum study over 6
523 month on a self-selected dietary portfolio in a cross-Canada multicenter trial of 335 participants,
524 LDL-C levels were decreased by 13-14%, and by~20% on the West Coast [140]! It is believed that
525 plant sterols were a major reason for the dietary portfolio's LDL-C reducing effect, since a 10-
526 15% reduction can be seen with 2 g/d intake and isotopic studies have shown that both plant
527 stanols and sterols reduce cholesterol absorption comparably. Plant sterols therefore appear to
528 have a very useful role in maintaining healthy cholesterol levels.

529 **7. Plant Sterols/Stanols and Cardiovascular Disease (CVD) Risk**

530 **7.1 Vascular Function Effects of Plant Sterols/Stanols**

531 The LDL-C lowering effect of plant sterols/stanols is well established [2,7,84]. Nevertheless,
532 direct evidence linking the intake of foods with added plant sterols/stanols and CVD risk is still
533 lacking. As mentioned earlier, CVD endpoint trials with plant sterols/stanols are prohibitively
534 expensive and challenging to perform. Depending on the length of follow-up and the annual risk
535 level, 36,000 to 636,000 subjects would be needed to have enough power to show a LDL-C
536 lowering benefit. A typical CVD endpoint study was deemed therefore not feasible for foods
537 with added plant sterols/stanols due to the large sample size required, compliance aspects and
538 costs. Therefore, surrogate endpoint markers will remain to serve as an alternative to study the
539 direct effect of plant sterols/stanols on CVD risk. As atherosclerosis progression occurs from an
540 early age onwards, the function and structure of the arterial wall is influenced. Endothelial

function may be impaired, arteries may become stiffer, and thickness of the arterial wall may increase and low-grade inflammation may occur.

7.2 Plant Sterols/stanols and Endothelial Function

Several types of evidence support a link between LDL-C and endothelial function, including data from patients with familial hypercholesterolemia [141], LDL apheresis [142] and other LDL-C lowering treatments such as statins [143,144] and ezetimibe [145,146]. Furthermore, a significant inverse association between flow-mediated dilation (FMD) and CVD risk seems to exist, so people with a higher FMD possess a lower risk of CVD [147].

After consumption of plant sterols their concentrations in plasma and tissues increases. This raises the question of whether this may affect surrogate endpoint markers in a beneficial or perhaps detrimental way. The change in plasma plant sterols after an intake of plant sterol-enriched foods was investigated in a meta-analysis including 41 studies [148]. On an absolute scale, sitosterol and campesterol were increased modestly, on average by 2.2-5.0 µmol/L especially compared to the average change in LDL-C (-0.33 mmol/L). However, on a relative scale, increases were considerable, on average 31-37%. Plasma plant sterol concentrations have been linked to increased CVD risk in homozygous sitosterolemic patients [149] and in some, but not all, observational studies [150]. However, there are also controversial findings as demonstrated by the results of another study in five sitosterolemic subjects. In spite of massive hypercholesterolemia and high plant sterol/stanol levels, none of these individuals had symptoms of CVD or positive clinical markers of atherosclerosis [151]. It should be realized that intake of foods with added plant stanols, the saturated form of plant sterols, increases plasma plant stanol concentrations despite a lower absorption rate compared to plant sterols. A randomized trial with a 4-week intake of 3 g/d of plant stanols showed increased plasma plant stanol concentrations by about 400% [152]. On the

564 absolute scale, however, these increases were minor, being far less than those in plant sterols
565 when their intake was increased.

566 The effects of plant sterols/stanols on endothelial function have been investigated in
567 several animal and human studies. In wild-type mice fed for 4 weeks extremely high doses of
568 plant sterol esters (2%; ~100 times higher than the 2 g/d recommended dose for lowering LDL-C
569 in humans), intake of plant sterols increased plasma plant sterol concentrations and impaired
570 endothelial-dependent vasodilatation, as measured by vascular relaxation of aortic rings [53].
571 Furthermore, cerebral lesion size increased after plant sterol intake. However, plasma cholesterol
572 concentrations in these mice were not affected, questioning whether these wild-type mice were
573 suitable for studying the effects of plant sterols. In another animal study with an atherogenic
574 apoE-/- mouse model, plant sterol and plant stanol supplementation reduced serum cholesterol
575 and increased plant sterol and plant stanol concentrations, as expected [153]. Elevated levels of
576 plant sterols/stanols were associated with impaired endothelial function. Atherosclerotic lesion
577 retardation was more pronounced in response to plant stanol compared to plant sterol
578 supplementations, however, this effect was not significant [153]. Diet supplementation with plant
579 sterols and ezetimibe, alone and in combination reduced the atherosclerotic lesion compared to
580 control, however the reduction was significantly greater in the ezetimibe versus the plant sterol
581 fed group [53]. Contrary to the findings in mice studies, 6-week intake of sitosterol and
582 stigmasterol in hamsters improved aortic functioning as measured by acetylcholine induced
583 endothelium-dependent relaxation [154]. Taken together, animal studies reporting effects of plant
584 sterol/stanol intake on endothelial function show conflicting results.

585 A few human studies have investigated the effect of plant sterol/stanol intake on FMD as
586 summarized by Plat et al. [1]. Despite significant reductions in LDL-C in these studies, none

showed statistically significant effects on FMD. However, when the effects seen in five of these studies were combined, an indication for a modest improvement in FMD was found [89,131,155-157].

Recently, the large randomized trial focusing on vascular function effects of plant sterols (the INVEST study), investigated the influence of plant sterol intake on FMD as a primary outcome measure together with other vascular function markers [158]. The study included 240 subjects who consumed margarine enriched with 3 g/d of plant sterols for 3 months. The INVEST study showed that plant sterol intake had a neutral effect on endothelial function based on a placebo-corrected change in FMD of 0.01 percentage points (95% CI: 20.73, 0.75). Also, arterial stiffness as measured by pulse wave velocity and augmentation index, was not affected. This neutral effect supports neither a worsened nor an improved vascular function with plant sterol intake. It should be realized that the LDL-C lowering effect observed in this study was only -0.26 mmol/L (95% CI: -0.46; -0.07) or -7% compared to control, which is smaller than anticipated for a plant sterol intake of 3 g/d. In general, it is estimated that 3 g/d of plant sterols would lower LDL-C by ~12%.

In the INVEST study, plasma plant sterol concentrations were significantly increased in the plant sterol group as expected, but these increases were not related to changes in FMD (**Figure 3** permission to re-use required). On the other hand, although not very strong, a larger reduction in LDL-C was significantly correlated with an increase in FMD, suggesting that lowering LDL-C could lead to improvements in endothelial function.

Also, several plasma biomarkers of endothelial dysfunction, E-selectin, soluble vascular cell adhesion molecule-1 (sVCAM-1), and soluble intercellular adhesion molecule-1 (sICAM-1), measured as well in the INVEST study, were not significantly affected by plant sterol intake compared to control [159].

610 Taken together, plant sterols/stanols have not been shown to consistently improve
611 endothelial function, despite significant reductions in LDL-C. This could be because the plant
612 sterols/stanols doses used were below the threshold needed to trigger measurable differences in
613 endothelial function. Furthermore, populations used in studies so far may have been too healthy.
614 Improvements in endothelial function may only be detectable in individuals with impaired
615 endothelial function. Furthermore, a longer intervention period is perhaps needed to detect
616 effects on the endothelium. Importantly, the evidence shows that plant sterol intake does not
617 weaken endothelial function, despite increases in plasma plant sterol concentrations.

618 **7.3 Plant Sterols/Stanols and Other Surrogate Markers of Arterial Health**

619 Recently a few other studies with plant sterols/stanols investigated surrogate endpoint
620 markers including arterial stiffness, intima media thickness (IMT) and inflammation. In a
621 randomized controlled study by Gylling et al., the effects of plant stanols on arterial stiffness
622 were investigated [160]. The study found that lowering LDL-C by ~10% with plant stanol esters
623 reduced arterial stiffness in small arteries with some indications of a beneficial effect on that in
624 large arteries only in men. It should, however, be noted that these effects were mainly driven by
625 increases in arterial stiffness in the control group. Endothelial function, as measured by reactive
626 hyperemia index (RHI), was overall not improved with plant stanol intervention. However,
627 changes in LDL-C correlated significantly with changes in RHI in the plant stanol group, which
628 is consistent with the findings of the INVEST study.

629 In an observational study with Old Order Amish people who are prone to be heterozygous
630 for sitosterolemia [161], carriers of a specific *ABCG8* variant had higher plasma sitosterol
631 concentrations compared to non-carriers of this variant, whereas LDL-C levels did not differ
632 between groups. Compared to non-carriers, carriers had decreased carotid intima-media wall

633 thickness, suggesting less plaque formation in their vessels with increased plasma plant sterol
634 concentrations.

635 Inflammation is also involved in the process of atherosclerosis. Recently, a meta-analysis
636 was published that summarized the effects of plant sterol/stanol intake on inflammation markers,
637 and particularly on C-reactive protein (CRP) [162]. A beneficial effect on this marker was not seen.

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

641 **7.4 Personalizing and Optimizing Lipid-Lowering Therapies**

642 Statins reduce cardiovascular morbidity and mortality in primary and secondary prevention
643 trials [163-165]. However, statin efficacy shows individual differences which can be because of the
644 cholesterol metabolism variations between individuals [135,166,167,168], with some subjects
645 demonstrating a genetically determined rather high cholesterol synthesis and others a higher
646 cholesterol absorption [118]. In subjects with high cholesterol synthesis, statins are potent
647 cholesterol lowering drugs, but in those who are high absorbers, statins are less effective than
648 cholesterol absorption inhibitors in lowering LDL-C [169-171]. However, some studies have found
649 controversial results. For instance, Lakoski et al reported that combination therapy using
650 ezetimibe and simvastatin lowered LDL-C by 15% or greater in more than 95% of participants
651 [172] . Moreover, inhibition of cholesterol synthesis results in increased cholesterol absorption,
652 with increased uptake of plant sterols [173]. As a consequence, in patients with high cholesterol
653 absorption, statins have been shown to increase cardiovascular event rates [174]. These findings
654 suggest that individuals with low synthesis and high absorption of cholesterol should be treated
655 with combined cholesterol lowering using a statin and a cholesterol absorption inhibitor [174].

Genetic studies have shown that life-long lower cholesterol levels are associated with lower CVD risk [175]. In individuals with inactivating mutations of NPC1L1 a minor cholesterol lowering of 12 mg/dl reduced cardiovascular risk dramatically by 53% [129]. Moreover, it has been shown for the sterol transporter gene *ABCG8* that plant sterol levels are associated with cardiovascular risk in the general population [112,149]. Other studies have demonstrated that high cholesterol absorption is associated with coronary artery disease severity [176], and high cholesterol absorption is associated with higher cardiovascular mortality [177]. Interestingly, the ratio of cholesterol absorption to cholesterol synthesis has been shown to be associated with coronary artery disease severity [178]. These results have been verified in the Framingham-offspring-study, with the ratio of cholesterol absorption to cholesterol synthesis being the best lipid parameter to predict cardiovascular risk [179]. New studies using intravascular optical devices show the same direction. In patients with stable and unstable angina pectoris, those with high cholesterol absorption markers and low cholesterol synthesis demonstrated thinner fibrous caps and larger lipid cores [180]. In patients with coronary heart disease, the atorvastatin treatment effect on lesion progression was assessed with intravascular ultrasound. In those patients not responding adequately to statin treatment, atherosclerotic plaque progression was most pronounced [181]. In the PRECISE-IVUS trail, statin monotherapy was compared to combined lipid-lowering with a statin and ezetimibe combination in patients with suspected coronary heart disease [182]. After a study period of 9-12 months, LDL-C lowering was greater with combined lipid-lowering than with statin monotherapy (63 mg/dl vs. 73 mg/dl). Moreover, intravascular ultrasound demonstrated a more pronounced atherosclerotic plaque regression with combined lipid lowering. The effect of an ezetimibe-statin combination on lesion regression was more pronounced than the effect of a combination of a statin with a PSCK9-inhibitor in the GLAGOV study [183].

In patients on dialysis, statins did not show any effect on cardiovascular mortality [184,185]. A possible explanation for this is that patients on dialysis are characterized by high cholesterol absorption and low cholesterol synthesis, with high cholesterol absorption being associated with greater mortality [186]. This may also explain why in the study of heart and renal protection (SHARP) a comparably less effective LDL-C lowering resulted in a significant reduction of cardiovascular events with combined lipid-lowering [187]. A post-hoc analysis of the AURORA study (a study to evaluate the use of rosuvastatin in subjects on regular hemodialysis: an assessment of survival and cardiovascular events) points in the same direction. In this analysis only patients on dialysis who were known to be high cholesterol synthesizers showed a reduction in cardiovascular mortality on statins [188]. Since the publication of the IMPROVE-IT trial additional evidence has surfaced that a combined lipid-lowering in high risk patients can reduce cardiovascular mortality [130]. With these risk calculations in mind, one can speculate that a combined lipid-lowering approach – assessed on an individual basis on differences in cholesterol metabolism – can further reduce cardiovascular risk [189,190].

8. Sitosterolemia: Clinical Perspective, Diagnosis, Treatment, Screening Programs

8.1 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" [191]. Many studies have shown that nutrition can affect gut microbiota [192,193]. Some studies show associations between microbiome and serum lipid levels [194]. The composition of the microbiome was recently evaluated during early stages of sitosterolemia. Those 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 only a mild form of it. Furthermore, differences in the microbial population across

groups were identified [195]. Specifically, levels of lactobacillus were found to be down-regulated in those with severe experimental autoimmune encephalomyelitis (EAE) [195]. Lactobacillus is a big component of all of those probiotics in the market. Could one use a probiotic 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 [196]. However, a recent study could not confirm this finding in human volunteers [197].

For sitosterolemia management, ezetimibe is the standard treatment. Although it has been shown to reduce plasma sitosterol levels by about 30-40%, this may not be sufficient to treat severe symptoms of the disorder. Could one modify the abundance and the function of the microbiome in order to treat sitosterolemia? How about using a genetically modified vector as a delivery system? Can one deliver a probiotic that proliferates in the gut, and which is able to carry a gene that might actually be able to be transferred into the epithelial cells of the gut? 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 [198,199]. This shows that such delivery systems can be effective in carrying human genes and transferring them into cells. How about using a genetically modified probiotic that can target the *ABCG5* and *ABCG8* genes in enterocytes? 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.

8.2 Clinical Perspective: When to Add Sitosterolemia to the Differential Diagnosis List

In 1974 Drs. William Connor and Ashim Bhattacharyya reported the first cases of sitosterolemia [105]. The index patients were two young adult sisters who had onset of tendon

xanthomas at the ages of 7 and 8 years, progressing at 13-14 years, which led to medical evaluation. They otherwise had normal development, including "normal" plasma cholesterol concentrations. The total circulating cholesterol levels in both subjects were around 200 mg/dl [105], which at the time was considered an oddity in the context of prominent tendon xanthomas because the level is much lower than what one would expect to see due to a disorder such as familial hypercholesterolemia (FH). FH 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. FH is caused by defects in the LDL receptor, apolipoprotein B (apo-B), proprotein convertase subtilisin/kexin type 9 (PCSK9), and homozygous defects in the LDL receptor adaptor protein. Roughly one- third of patients with a clinical diagnosis of FH do not have an identifiable mutation even when all of the known genes are sequenced, suggesting other genes involved [200]. At the time these sisters were evaluated, one would have expected a total cholesterol concentration of 350 mg/dl to 400 mg/dl or higher in a patient with FH. Furthermore, at the time, the presence of tendon xanthomas was usually consistent with a diagnosis of FH, or rarely cerebrotendinous xanthomatosis (CTX) caused by mutations in CYP27A1 that encodes sterol 27-hydroxylase, a key enzyme in the bile acid synthetic pathway [201]. However, it has been suggested that some individuals with undiagnosed sitosterolemia may masquerade as pseudo-FH as a consequence of marked diet-induced hypercholesterolemia that may be seen in some patients with sitosterolemia in response to high intake of dietary cholesterol and plant sterols [202]. The proportion of patients with a clinical diagnosis of presumed FH who actually have sitosterolemia is unknown.

Sitosterolemia is caused by mutations in sterol transporter genes *ABCG5* and/or *ABCG8*, resulting in several consequences, including intestinal hyper-absorption of all dietary sterols,

impaired hepatic excretion of sterols into bile, increased tissue content of plant sterols, and the development of extensor tendon xanthomas and atherosclerosis.

An important question in relation to clinical practice relates to when a diagnosis of sitosterolemia should be considered. It is a rare disorder, so random screening of patients is not indicated or useful, but there are 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 Drs. Connor and Bhattacharyya, sitosterolemia should be considered when tendon xanthomas are present in the absence of severe hypercholesterolemia [105]. Another situation that may be a sign of occult sitosterolemia is the development of extreme hypercholesterolemia after consumption of high cholesterol/saturated fat diets. As a consequence of mutations in *ABCG5* or *ABCG8,* patients with sitosterolemia hyper-absorb dietary cholesterol and plant sterols/stanols, resulting in exaggerated diet-induced hypercholesterolemia. One patient was identified with sitosterolemia on the basis of an increase in the LDL-C concentration from 120 mg/dl to 295 mg/dl during consumption of a diet high in saturated fat and cholesterol. Other conditions that may be suggestive of a diagnosis of sitosterolemia include a paradoxical hypercholesterolemia in response to pharmacological treatment with plant sterols. Unlike normal individuals who may achieve an 8-10% decrease in the plasma concentration of LDL-C because of plant sterol-mediated inhibition of micelle formation resulting in inhibition of cholesterol absorption, patients with sitosterolemia will hyper-absorb the plant sterols, and may actually have a hypercholesterolemic response. Hypo-responsiveness to the LDL-C lowering efficacy of statins is another indicator that the patient may have sitosterolemia, but this finding may be confounded by noncompliance with statin treatment, gain of function mutations in PCSK9, or other factors

unrelated to sitosterolemia. Hence, the vast majority of patients who are hypo-responsive to the LDL-C lowering efficacy of statins are unlikely to have sitosterolemia.

A key step in the diagnosis of sitosterolemia is measurement of serum/plasma plant sterols using gas chromatography/ mass spectrometry. Some patient groups have false positive elevations in the concentration of plasma sitosterol equivalent to 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 found to be completely reversible after cessation of parenteral administration of plant sterols. Clinical features that may facilitate with diagnosis of sitosterolemia can 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, but the absence of these features does not exclude the diagnosis [203]. Management of sitosterolemia includes decreasing dietary intake of plant sterols and cholesterol, as well as treatment with ezetimibe, possibly bile acid binding resins, and treatment of hypercholesterolemia with statins as indicated.

In summary, the diagnosis of sitosterolemia should be considered in a variety of clinical settings, including hyper-responsiveness to dietary sterol intake, paradoxical responses to treatment with plant sterols, the presence of tendon xanthomas in the absence of hypercholesterolemia, hypo-responsiveness to statins, findings of platelet and red blood cell abnormalities, as well as early onset coronary artery disease without significant hypercholesterolemia.

8.3 Sterol Metabolism in Sitosterolemia

Although the clinical symptoms of sitosterolemia may vary across individuals, a consistently important diagnosis of the disorder is highly elevated circulating levels of plant

sterols. Abnormal sterol homeostasis has been observed in individuals with sitosterolemia [204]. It is characterized by increased retention of plant sterols and cholesterol, reduced removal, and expanded whole body pools which compensate for the reduced cholesterol synthesis in sitosterolemia [204]. Using *in vivo* radiolabeled isotopic techniques, Salen et al. [204] observed that the turnover rates of plasma cholesterol and sitosterol in sitosterolemia patients were similar and significantly slower compared to a control subject. It has been shown that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and synthase, and other key enzymes involved in cholesterol synthesis, are down regulated in sitosterolemia patients [205-207]. Accumulation of plant sterols may account for the low cholesterol synthesis rates observed in sitosterolemia [208]. Strategies such as feeding either the cholesterol precursor mevalonic acid, or low sterol diets [207] failed to stimulate *de novo* cholesterol synthesis in patients with sitosterolemia. While ezetimibe is the current standard therapy for sitosterolemia, its effect on the rates of cholesterol synthesis and sterol turnover in sitosterolemic patients are undefined and need further investigation.

9. Intravenous Plant Sterols and Pediatric Intestinal Failure Associated Liver Disease

When enteral nutrition is limited due to insufficient intestinal length and/or poor function, intestinal failure develops. In order to prevent dehydration and malnutrition, patients with intestinal failure are prescribed parenteral nutrition (PN), or intravenous nutrition. PN serves as an important source of water, electrolytes, and macro- and micronutrients. While PN is life sustaining for intestinal failure patients, it can lead to intestinal-failure associated liver disease (IFALD), a potentially fatal liver disorder. IFALD is defined by the presence of intestinal failure, or prolonged PN use, and liver dysfunction, which includes elevated serum transaminases and/or a conjugated hyperbilirubinemia. On liver biopsy, IFALD is characterized by cholestasis,

816 inflammation, and steatosis. After a short course of PN, liver fibrosis can develop. In some
817 patients, IFALD culminates in cirrhosis, liver failure, and death. Once liver failure develops, a
818 liver transplant is the only life-saving option.

819 IFALD and sepsis are the top two causes of mortality for children with intestinal failure [207].
820 For several reasons, IFALD is more common in children than adults. PN duration, gestational
821 age, birth weight, and underlying gastrointestinal disorders are important risk factors for IFALD.
822 70% percent of infants who have received greater than 60 days of PN will develop IFALD [209].
823 Moreover, gestational age and birth weight are inversely correlated to the incidence of IFALD.
824 Premature neonates and low birth weight neonates are at high risk for IFALD due to prolonged
825 PN courses, immature livers, feeding intolerance, and a high incidence of necrotizing
826 enterocolitis [207]. Last, children with gastroschisis, volvulus, distal intestinal atresias, and short
827 bowel syndrome commonly develop IFALD [207].

828 Intravenous lipids are prescribed with PN as a source of non-protein calories and essential
829 fatty acids. In the US, the only FDA-approved intravenous lipid emulsion for children is entirely
830 soy-based (Intralipid™(Fresenius Kabi, Uppsala, Sweden). SO-based lipid emulsions have a
831 long-standing association with IFALD [195,196,210,211]. Intravenous soybean oil contains a high
832 concentration of plant sterols (>350-400 mg/L) [195,196,210,211]. In contrast to intravenous soybean
833 oil, a non-FDA approved fish oil-based lipid emulsion (Omegaven™, Fresenius Kabi, Bad
834 Homburg, Germany) contains a negligible amount of plant sterols. Fish oil-based lipid emulsions
835 are prescribed in the US under compassionate use protocols and serves as an important rescue
836 treatment for children with advanced IFALD [209,210,211,212]. Studies have demonstrated that
837 intravenous fish oil is a safe, effective treatment for IFALD; 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 need for liver transplantation [210,211].

While there are several differences between soybean and fish oil lipid emulsions, the plant sterol concentration cannot be overlooked. In comparison to healthy controls, infants with IFALD have higher circulating concentrations of various plant sterols. When IFALD infants are compared to IFALD children, IFALD infants have higher plant sterol concentrations [213,214]. Furthermore, plasma sterol concentrations correlate with hepatic sterol concentrations and histological changes on liver biopsy [215]. Last, in IFALD children whose intravenous soybean oil was replaced with intravenous fish oil, plasma sterol concentrations not only dramatically decreased, but early changes in plasma stigmasterol predicted later changes in conjugated bilirubin [210] . This suggests that stigmasterol may serve as surrogate for disease severity and treatment response.

Animal experiments provide mechanistic evidence that stigmasterol may be one of the main culprits driving IFALD. Mice infused with PN and intravenous soybean oil have decreased expression of hepatic nuclear transcription factors, liver X receptor (LXR) and farnesoid X receptor (FXR), and decreased mRNA expression of bilirubin, bile acid, and sterol liver transporters. Also, mice exposed to PN plus intravenous soybean oil developed cholestasis and elevated liver function tests, mimicking pediatric IFALD [216]. In contrast, when mice were infused with PN plus intravenous fish oil, FXR, LXR, and transporter expression were similar to control mice, and they were protected against IFALD [216]. However, when stigmasterol was added to fish oil, FXR, LXR and transporter expression were similar to the soybean oil group and the mice developed IFALD [216].

From these studies, it can be concluded that the type of intravenous lipid emulsion and, more specifically, intravenous plant sterols are important players in IFALD pathogenesis. With the advent of new lipid formulations, careful attention should be paid to sterol content. It remains unknown if specific sterols are safer than others, and if there is a "safe" sterol content for lipid emulsions. Further research is needed to answer these questions.

10. Plant Sterols: Patients' Perspectives

10.1 Introductory Remarks

The National Institutes of Health (NIH) has defined a rare disease as one that affects less than 200,000 people in the US population, which corresponds to 1 in 16,000 to <1 in 500,000 individuals. However, the prevalence of various rare diseases is quite variable, with some incidences being highly infrequent. Currently, 7,000 separate diseases have been identified as rare, with many of these being inherited. Multiple challenges exist with studying rare diseases, including limited recruitment of patients, unknown natural history of the disorder and considerable phenotypic variability in these diseases. This adds to the complications in investigating not only the disease itself, but also therapeutic approaches to these diseases. Very few investigators are trained specifically in rare disease research largely because of the rarity of most of these disorders. Most physicians fail to recognize diseases when they encounter them because they have never seen a case of a disease that occurs one in 100,000 incidents. So many challenges exist. The NIH well recognizes the challenges in diagnosing and treating the very large constellation of rare diseases that exists. This is demonstrated by its establishment of a rare disease clinical research network (RDCRN) which now specifically targets 22 diseases. The Sterol and Isoprenoid Research Consortium (STAIR), one of the 22 in the RDCRN network, is a consortium that is focused on sterol metabolism disorders. The consortium itself has a number of

advantages, such as including recruitment of patients. The idea behind it is that no center will encounter enough patients with a rare disease to be able to conduct a valid clinical study alone, and therefore efforts should be pooled in carrying out multi-center studies on these diseases.

10.2 Sitosterolemia, Clinical and Treatment Aspects. Observations from the Manitoba Cohort

The Manitoba Sitosterolemia Cohort is a kindred of Hutterite patients living mostly in Manitoba. They are a religious isolate based in rural communities. A specific case was a five-year- old girl who died suddenly and was found at autopsy to have extensive aortic and coronary atheroma [217]. Her medical history was anemia and recurring abdominal pain [218]. This led to searching 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 sixteen years has built up a cohort of 21 patients all having the *ABCG8* S107X mutation. All 20 survivors have responded very favorably to ezetimibe therapy [219,220].

11. Summary and Conclusions

The present review provides a comprehensive overview of past and recent developments in the basic biology of plant sterols and stanols, largely in the context of their value as therapeutic agents for dyslipidemia management in the general population. It also presents guidance for the clinical management of rare disorders resulting from mutations in sterol metabolism at various levels that lead to the retention in the circulation and tissues of cholesterol, plant sterols and stanols, as well as other types of non-cholesterol sterols. 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 cholesterol absorption efficiency and circulatory pools in both adults and infants. Also, the reciprocity between cholesterol synthesis and absorption and how

that ratio impacts the efficacy of plant sterol/stanol action in LDL-C lowering is being increasingly recognized. How polymorphisms within genes coding for enzymes active in lipid pathways affect the LDL-C lowering action are now better understood. Advantages of combining plant sterols/stanols with other dietary elements such as fiber, soy protein and nuts have been recognized. Overall importance of LDL-C lowering in CVD risk has been further established from combined drug trials such as IMPROVE-IT [130], FOURIER [137]. Additionally, Ference et al., recently found a clear association between LDL and atherosclerotic cardiovascular disease, from investigating numerous and multiple clinical and genetic studies [221]. In best approaches to clinical management of sitosterolemia, ezetimibe continues to prevail as the drug of choice. The disparity in degree of severity of this disorder across patients was emphasized, as well as the importance of proper screening using both levels of circulatory plant sterols as well as confirmation of the specific mutation as diagnostic criteria. It is considered important to rely on these tools for correct identification of patients with sitosterolemia so as not to confuse them with FH. In summary, plant sterols and stanols continue to offer an efficacious and convenient dietary approach to cholesterol management and serve as an important natural health product as well as functional food ingredient. Their clinical benefit through long-term studies addressing CVD endpoints has however not been established.

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929 (Appendix 1).

930 **Declaration of Interest**

931 **Oliver Weingärtner has received speaker honoraria from AMGEN, Berlin-Chemie**
932 **Menarini, MERCK, Sanofi and serves on advisory boards for AMGEN, MERCK and**
933 **Berlin-Chemie Menarini.**

934

935 **Appendix 1**

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Figures Legends

Figure 1. Percentage change in LDL-C in individuals from baseline in response to the consumption of a low-fat plant sterol enriched soy beverage (1.95 g plant sterols /d) [222].

Figure 2. Proposed surrogate measure of cholesterol metabolism which could overcome issues related to using ratios of surrogate synthesis to absorption markers.

Figure 3. Correlations between changes in serum LDL-C and plasma plant sterols and changes in flow-mediated dilation (copied from Ras et al.[158]; permission to re-use required)