Dietary plant stanol ester supplementation reduces peripheral symptoms in a mouse model of Niemann–Pick type C1 disease.

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Abbreviations (in the order of their mention): Niemann-Pick type C (NPC); late endosomes/lysosomes (LEL), alanine aminotransferase (ALT); liver X receptor (LXR)
ABSTRACT

Niemann–Pick type C1 (NPC1) disease is a rare genetic condition in which the function of the lysosomal cholesterol transporter NPC1 protein is impaired. Consequently, sphingolipids and cholesterol accumulate in lysosomes of all tissues, triggering a cascade of pathological events that culminate in severe systemic and neurological symptoms. Lysosomal cholesterol accumulation is also a key-factor in the development of atherosclerosis and non-alcoholic steatohepatitis (NASH). In these two metabolic diseases, the administration of plant stanol esters has been shown to ameliorate cellular cholesterol accumulation and inflammation. Given the overlap of pathological mechanisms among atherosclerosis, NASH and NPC1 disease, we sought to investigate whether dietary supplementation with plant stanol esters improves the peripheral features of NPC1 disease. To this end, we used an NPC1 murine model featuring an Npc1 null allele (Npc1nih), creating a dysfunctional NPC1 protein. Npc1nih mice were fed a two or six percent plant stanol esters–enriched diet over the course of 5 weeks. During this period, hepatic and blood lipid and inflammatory profiles were assessed. Npc1nih mice fed the plant stanol–enriched diet exhibited lower hepatic cholesterol accumulation, damage and inflammation than regular chow–fed Npc1nih mice. Moreover, plant stanol consumption shifted circulating T-cells and monocytes in particular towards an anti-inflammatory profile. Overall, these effects were stronger following dietary supplementation with 6% stanols, suggesting a dose-dependent effect. The findings of our study highlight the potential use of plant stanols as an affordable complementary means to ameliorate disorders in hepatic and blood lipid metabolism and reduce inflammation in NPC1 disease.

Keywords: Niemann-Pick disease; inflammation; cholesterol metabolism; diet; dietary lipids; liver; plant stanols; atherosclerosis; non-alcoholic steatohepatitis (NASH); lysosomal storage disease
INTRODUCTION

Niemann-Pick type C (NPC) disease is a rare lysosomal storage disorder caused by deleterious mutations in \textit{NPC1} or \textit{NPC2}. It is estimated that NPC disease affects one in 100,000 live births, with mutations in \textit{NPC1} occurring in approximately 95% of cases (1). Although caused by different genetic mutations, NPC1 and NPC2 diseases are clinically indistinguishable, as both NPC1 and NPC2 proteins are required for endolysosomal cholesterol efflux. Upon endocytosis, low-density lipoproteins (LDL) merge with late endosomes/lysosomes (LEL), where lysosomal acid lipases hydrolyze LDL-derived cholesteryl esters. NPC2, a protein located on the luminal surface of LEL, binds the resulting free cholesterol and directs it to the luminal domain of NPC1, a LEL transmembrane protein. Studies indicate that the NPC1 protein subsequently delivers the free cholesterol to the plasma membrane and to the endoplasmic reticulum, possibly via membrane contact sites between the endoplasmic reticulum and LELs (2, 3). Due to compromised NPC1 protein function, NPC1 disease (OMIM #257220) is characterized by endolysosomal cholesterol and sphingolipid accumulation in all cells. The age of onset and clinical features of NPC1 disease are heterogeneous, likely because of the variability of \textit{NPC1} mutations among patients (5, 6). Nonetheless, the nervous system of NPC1 disease patients is commonly severely affected, triggering the development of neuropsychiatric disorders and cognitive and motor function degeneration (6). In addition, systemic dysfunctions such as jaundice, cholestatic disease, hepatosplenomegaly, liver and pulmonary disease occur in a significant amount of patients. While such systemic symptoms are most severe in the perinatal and infantile stage of the disease, and tend to become stable in older NPC1 disease patients, in some cases peripheral dysfunction can further progress and result in cirrhosis and hepatocellular carcinoma (6-9). Although awareness of NPC1 disease has increased in recent years, early diagnosis and curative treatments are still lacking. Miglustat, a sphingolipid synthesis inhibitor, was approved in the European Union in 2009 as the first NPC1 disease-targeted drug (10). While Miglustat has been shown to reduce the progression of neurological deterioration in NPC1 disease patients, a report indicates it has a minor impact on systemic symptoms such as splenomegaly (11). Furthermore, intrathecal administration of 2-hydroxypropyl-\(\beta\)-cyclodextrin to reduce neurological symptom progression in NPC1 disease is currently
being evaluated in phase 2/3 clinical trials (ClinicalTrials.gov Identifier: NCT02534844) (12). Despite promising results, the use of 2-hydroxypropyl-β-cyclodextrin as a therapeutical compound in NPC1 disease faces several challenges, including the administration route and side-effects (12). Finally, a different clinical study is currently evaluating the effects of intravenous 2-hydroxypropyl-β-cyclodextrin administration on hepatic NPC1 disease symptoms (ClinicalTrials.gov Identifier: NCT03887533). Overall, considering the limited amount and scope of NPC1 disease treatments, further research is needed to develop a wider range of interventions that can modify NPC1 disease progression (6, 13).

Lysosomal lipid accumulation is at the core of NPC1 disease pathology and triggers a series of events that culminate in tissue and organ dysfunction. Such events include disturbed lysosomal function and lipid metabolism, as well as increased oxidative stress, inflammation and apoptosis (14-17). The aforementioned pathological mechanisms mirror those observed, though to a lesser extent, in atherosclerosis and non-alcoholic steatohepatitis (NASH). Similarly to NPC1 disease, these metabolic disorders are characterized by lysosomal lipid accumulation in macrophages, which has been shown to be a key factor in disease severity and development (18-22).

Notably, in vitro and in vivo studies have shown plant stanol ester supplementation to be beneficial in both NASH and atherosclerosis (23, 24). Plant stanols are essential components of plant cells derived from the saturation of plant sterols, which share a similar chemical structure and biochemical functions as the mammalian cholesterol (25). The average human daily intake of plant stanols is 20-50 mg, of which up to 0.15% is estimated to effectively be absorbed in the small intestine (26, 27). Dietary supplementation with plant stanol esters has well-known plasma cholesterol-lowering effects, presumably since they interfere with intestinal cholesterol absorption (27-29). Specifically, dietary plant stanol supplementation has been shown to reduce cellular cholesterol accumulation in NASH and atherosclerosis models. In addition, the aforementioned studies indicate that plant stanol esters supplementation ameliorates hepatic inflammation, a mechanism that also contributes to NPC1 disease severity (23, 30, 31).
Considering the parallels between the pathological mechanisms of NPC1 disease, atherosclerosis and NASH, the aim of this study was to investigate whether dietary supplementation with plant stanol esters also improves peripheral features in NPC1 disease. To this end, we used an NPC1 disease murine model which expresses an Npc1 allele with a frameshift mutation (Npc1nih) that results in the loss of function of the corresponding NPC1 protein (13). While the Npc1nih allele was originally discovered and maintained in the BALB/c mouse strain, here we used Npc1nih mice with a C57BL/6 genetic background, a model which has been previously described and which results in a more severe NPC1 peripheral disease phenotype (32).

To investigate our hypothesis, two-weeks-old Npc1nih mice received a normal chow or a two or six percent plant stanol esters-enriched chow diet for five weeks. Npc1wt mice fed a regular chow were included as a control group for NPC1 disease phenotype. Npc1nih mice fed a plant stanol-enriched diet showed decreased hepatic cholesterol accumulation, as well as reduced hepatic damage and inflammation. In addition to the localized effects in the liver, plant stanol administration led to a systemic immune shift towards an anti-inflammatory profile, as assessed by FACS analysis of white blood cells. Of note, the effect of plant stanol esters on peripheral NPC1 disease symptoms was overall more pronounced after supplementation of six percent plant stanol-enriched diet compared with two percent enriched diet, proving the beneficial effect of plant stanols to be dose-dependent.

Overall, these findings highlight the potential of plant stanol esters as a widely available and affordable additional tool to ameliorate hepatic symptoms and the phenotype of blood monocytes and T-cells in NPC1 disease patients, in combination with other therapies, such as Miglustat and 2-hydroxypropyl-β-cyclodextrin.
MATERIALS AND METHODS

Mice

Male and female \( Npc1^{nih} \) mice were derived from heterozygous founders (C57BL/6 / \( Npc1^{nih} \)). Given the reduced lifespan of \( Npc1^{nih} \) mice, as soon as the genotypes of the mice were known, the experimental diets were administered to the mothers, who would transfer the experimental diet to the pups via breastmilk (week 0 of the experiment). After the weaning period, at 14 days of age, mice began being fed the appropriate diet as solid chow. 13 and 16 \( Npc1^{nih} \) mice received a two percent and six percent plant stanol esters-enriched diet, respectively (manufactured by Arie Blok B.V., Woerden, The Netherlands). \( Npc1^{wt} \) and \( Npc1^{nih} \) mice fed a regular chow diet (n=10 and 13, respectively) were included as controls. Mice were housed under standard conditions and given free access to food and water. For an overview of the study setup and dietary plant stanol and sterol composition, please refer to Fig. S1 and S2, respectively. Blood from the tail vein was collected on weeks 3 and 5 of the experiment, when mice were 35 and 49 days old, respectively. All tissues were isolated and snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/PBS. The collection of blood and tissue specimens, biochemical determination of lipids in plasma and liver, RNA isolation, cDNA synthesis and qPCR were performed as described previously (33-35). All experiments were performed according to Dutch laws and approved by the Animal Experiment Committee of Maastricht University.

GC-MS

Plant sterols (sitosterol, campesterol) and plant stanols (sitostanol, campestanol) content in food was analyzed by gas–liquid chromatography–mass spectroscopy (GC–MS) as described previously (36).

Genotyping

Genotypes of animals were determined by PCR analysis of tail DNA. Toes were clipped at postnatal day 2 and homogenized in DirectPCR-Tail (Peqlab, Erlangen, Germany) supplemented with a tenth part Proteinase K (Qiagen, Hilden, Germany). Three hours of incubation at 56 °C and agitation at 1000 rounds
per minute on a Thermo Mixer were followed by 45 min of heating at 85ºC to inactivate the proteinase. Samples were then spun at full speed in a benchtop centrifuge for 1 min. The PCR reactions were performed with 0.5 ml of the obtained extracts. Each lysate underwent two PCRs. Primers gccaagttaggcagact and catctactggctccatatgtat identified the wild-type allele and primers gccaagttaggcagact and ttccaattgtgatctttccaa identified the mutant allele. Both PCRs were carried out under the same cycling conditions.

**Alanine aminotransferase (ALT) measurements**

Plasma ALT levels were measured with the Reflotron® test strips (Roche, Germany) according to manufacturer’s instructions, using the Reflotron® apparatus.

**Immunohistochemistry**

Frozen liver sections (7 μm) were fixed in acetone and blocked for endogenous peroxidase by incubation with 0.25% of 0.03% H₂O₂ for 5 minutes. Primary antibodies used were against hepatic macrophages (rat anti-mouse CD68, clone FA11) and infiltrated macrophages and neutrophils (rat anti-mouse Mac-1 [M1/70]). 3-Amino-9-ethylcarbazole (AEC) was applied as color substrate and hematoxylin for nuclear counterstain. Sections were enclosed with Faramount aqueous mounting medium. Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands). Infiltrated macrophages and neutrophil cells (Mac-1) were counted by two blinded researchers in six microscopical views (original magnification, 200x) and were indicated as number of cells per square millimeter (cells/mm²). Immunostainings for hepatic macrophages (CD68) were evaluated by an experienced pathologist and given a score in arbitrary units (A.U.).

**Plasma FACS analyses**

Tail vein blood was collected from Npc1wt and Npc1nha mice on weeks 3 and 5 of the experiment, when mice were 35 and 49 days old, respectively. Stainings were performed using Trucount tubes (BD Biosciences,
Breda, The Netherlands), according to the manufacturer’s instructions, to detect the following populations: monocytes (NK1.1-Ly6G-CD11b+; Ly6C) and T-cells (CD3+; CD4+; CD8+). Briefly, heparinized blood samples were mixed and incubated for 10 minutes in the dark at room temperature (RT) with CD16/32 antibody (eBioscience, Halle-Zoersel, Belgium) to block Fc receptor. Samples were then gently vortexed with the appropriate antibodies and incubated in the dark at RT for 20 minutes. All antibodies were diluted in FACS buffer (PBS, 0.1% BSA, 0.01% sodium azide). In this study, the following antibodies were used: PE Mouse Anti-Mouse NK-1.1 (1:100); APC-Cy™7 Rat Anti-Mouse Ly-6G (1:100); PE-Cy™7 Rat Anti-CD11b (1:300); APC-H7 Rat anti-Mouse CD4 (1:100) (BD, San Jose, USA); CD3 Monoclonal Antibody (1:100), CD8a Monoclonal Antibody (1:50) (eBioscience™ from Thermo Fisher Scientific, San Diego, USA); Anti-Mouse Ly-6C-APC (1:10) (Miltenyi, Bergisch Gladbach, Germany). Finally, samples were mixed and incubated in the dark at RT for 15 minutes with an erylysis solution (8.4g NH₄Cl + 0.84g NAHCO₃ in 1 liter H₂O, pH 7.2-7.4). Sample stainings were quantified within 1 hour using BD FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Data are expressed as the group mean and standard error of the mean. Three sets of data comparisons were performed via two tailed unpaired t-test: Npc1∗wt vs Npc1∗nih mice fed a regular chow diet (# p ≤ 0.05; ## p < 0.01; ### p < 0.001; #### p < 0.0001); Npc1∗nih mice receiving regular chow vs Npc1∗nih mice fed a two percent or six percent stanol-enriched chow diet (* p ≤ 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). Data were statistically analyzed using GraphPad Prism software (version 6, GraphPad Software Inc, San Diego, CA, U.S; www.graphpad.com).
RESULTS

Plant stanol supplementation delays body weight loss in Npc1<sup>nih</sup> mice and reduces relative liver weight

As reduced weight gain reflects NPC1 disease progression in Npc1<sup>nih</sup> mice, we monitored the body weight of Npc1<sup>nih</sup> mice and assessed whether plant stanol supplementation influenced this parameter. From day 12 of the study, which coincided with the weaning period, untreated Npc1<sup>nih</sup> mice were consistently smaller than Npc1<sup>wt</sup> mice (Fig. 1A). Npc1<sup>nih</sup> mice on a two percent plant stanol-enriched diet showed a modest increase in body weight compared to untreated Npc1<sup>nih</sup> mice until day 22 of the study, although this did not reach statistical significance. On the other hand, six percent plant stanol supplementation effectively rescued Npc1<sup>nih</sup> mice’s weight between days 12 and 29 of the study. Furthermore, in the last week of the study, a trend towards increased body weight was observed in Npc1<sup>nih</sup> mice on a six percent plant stanol-enriched diet compared to their untreated counterparts.

Additionally, liver weights were analyzed, as hepatomegaly is a prominent systemic feature of NPC1 disease. Although absolute liver weight was comparable between Npc1<sup>nih</sup> mice and Npc1<sup>wt</sup> mice, liver weights relative to body weight of untreated Npc1<sup>nih</sup> mice were higher compared to Npc1<sup>wt</sup> mice (Fig. 1B-C). Relevantly, relative liver weight of Npc1<sup>nih</sup> mice fed a two percent and six percent plant stanol-enriched diet was reduced. In addition, absolute liver weights were reduced in Npc1<sup>nih</sup> mice on a two percent plant stanol-supplemented diet. Overall, these results indicate that dietary supplementation with plant stanol esters ameliorates body weight gain and hepatomegaly in Npc1<sup>nih</sup> mice.

Decreased plasma and hepatic total cholesterol levels in Npc1<sup>nih</sup> mice fed a plant stanol-enriched diet

To assess the effect of dietary plant stanol supplementation on lipid metabolism of Npc1<sup>nih</sup> mice, biochemical analyses of plasma and liver lipids were performed. In line with plant stanols’ well-known plasma cholesterol lowering effect, Npc1<sup>nih</sup> mice fed a plant stanol-supplemented diet displayed lower levels of plasma total cholesterol (Fig. 2A), but not of plasma total triglycerides (Fig. 2B) compared to untreated
Npc1\textsuperscript{nih} mice. Of note, these effects were more pronounced following a six percent stanol enriched diet. Next, we analyzed hepatic lipid accumulation, a prominent systemic feature of NPC1 disease. As expected, untreated Npc1\textsuperscript{nih} mice displayed higher levels of hepatic cholesterol compared to Npc1\textsuperscript{wt} mice (Fig. 2C). Npc1\textsuperscript{nih} mice that received plant stanol supplementation, particularly at six percent, showed prominently lower levels of hepatic total cholesterol than untreated Npc1\textsuperscript{nih} mice, indicating that plant stanol supplementation reduced hepatic cholesterol levels in a dose-dependent manner (Fig. 2C). In contrast, plant stanol supplementation showed no effects on hepatic triglyceride accumulation of Npc1\textsuperscript{nih} mice, who displayed lower levels of liver triglycerides than Npc1\textsuperscript{wt} mice (Fig. 2D).

To better understand changes in hepatic cholesterol accumulation following plant stanol supplementation, hepatic gene expression analysis was performed on cluster of differentiation 36 (Cd36) and scavenger receptor A (Sr-a), which mediate the uptake of modified lipoproteins in macrophages, such as those increased in NPC1 disease patients (37, 38); on Niemann-Pick type C2 (Npc2), a protein that transfers free cholesterol within LELs to NPC1; on ATP-binding cassette sub-family G1 and G8 (Abcg1 and Abcg8), which mediate excess sterol efflux from leukocytes and hepatocytes, respectively (39); and on cytochrome P450 family 8 subfamily B member 1 (Cyp8b1), which promotes excess cholesterol excretion by mediating the synthesis of bile acids (Figs. 2E-J, respectively). Following two percent plant stanol administration, expression of Sr-a and Npc2 decreased in the livers of Npc1\textsuperscript{nih} mice, suggesting a reduction in the uptake of pro-inflammatory modified lipoproteins by macrophages and lower build-up of free cholesterol in LELs of Npc1\textsuperscript{nih} mice. Furthermore, Npc1\textsuperscript{nih} mice on a two percent plant stanol-enriched diet displayed higher expression of Cyp8b1, suggesting increased conversion of excess hepatic cholesterol into bile acids. Likewise, Npc1\textsuperscript{nih} mice on a six percent plant stanol-enriched diet displayed lower hepatic expression of Sr-a and Npc2 and increased expression of Cyp8b1 than their untreated counterparts. In addition to improving expression of the aforementioned genes, six percent plant stanol supplementation reduced hepatic expression of Cd36 and increased expression of Abcg8, suggesting reduced uptake of modified lipoproteins and increased excretion of excess cholesterol in hepatocytes of Npc1\textsuperscript{nih} mice. Finally, Npc1\textsuperscript{nih} mice displayed
lower hepatic expression of Abcg1, suggesting reduced efflux of cholesterol and oxysterols in macrophages. Overall, these findings indicate that, besides lowering plasma cholesterol levels, plant stanols reduce cholesterol accumulation in the liver of Npc1<sup>nth</sup> mice in a dose-dependent manner.

**Dietary plant stanol supplementation improves hepatic damage and inflammation in Npc1<sup>nth</sup> mice**

Following the observed improvements in hepatic cholesterol metabolism, we next investigated the effects of plant stanol supplementation on hepatic damage and inflammation. Plasma alanine transaminase (ALT) levels of untreated Npc1<sup>nth</sup> mice showed a near four-fold increase in relation to Npc1<sup>wt</sup> mice, indicating increased liver damage in Npc1<sup>nth</sup> mice (Fig. 3A). Remarkably, after dietary plant stanol supplementation, plasma ALT levels of Npc1<sup>nth</sup> mice were comparable to Npc1<sup>wt</sup> mice, indicating a strong decrease in overall liver damage. We further looked into hepatic inflammatory status via immunohistochemistry by targeting measuring CD68 and Mac-1 proteins, which identify resident hepatic macrophages and infiltrated neutrophils and macrophages, respectively (Figs. 3B-E). Untreated Npc1<sup>nth</sup> mice displayed higher levels of immune cells in both immunostainings, indicating prominent hepatic inflammation in Npc1<sup>nth</sup> mice. The number of hepatic immune cells, particularly in the case of infiltrated neutrophils and macrophages, was reduced following plant stanol supplementation. To further assess the effects of plant stanol supplementation on liver inflammation, hepatic gene expression analyses were performed on tumor necrosis factor alpha (Tnf-α), cluster of differentiation 68 (Cd68), cathepsin D (Ctsd), macrophage inflammatory protein 2 (Mip2), chemokine (C-C motif) ligand 3 (Ccl3) and arginase 1 (Arg1) (Figs. 3F-K). Hepatic gene expression of inflammatory markers was consistently increased in untreated Npc1<sup>nth</sup> mice compared to Npc1<sup>wt</sup> mice, and decreased in the case of Arg1, a marker for alternatively activated macrophages. Both two and six percent plant stanol supplementation reversed these observations, supporting the aforementioned findings that Npc1<sup>nth</sup> mice fed a plant stanol esters-supplemented diet display lower hepatic inflammation and damage.

**Plant stanol supplementation shifts plasma profile of immune cells towards an anti-inflammatory phenotype**
To better understand the effects of plant stanol supplementation on systemic inflammation, we investigated monocyte and T-cell populations by FACS analysis in the blood of 35 and 49 days old Npc1\(^{nih}\) mice. To analyse the profile of circulating monocytes, we targeted Ly6C, a protein highly expressed in pro-inflammatory monocytes (30, 40, 41). Untreated Npc1\(^{nih}\) mice displayed higher relative levels of Ly6C\(^{hi}\) monocytes and lower relative levels of Ly6C\(^{lo}\) monocytes in the blood than Npc1\(^{wt}\) mice on both time points (Fig. 4A-B), suggesting higher amounts of pro-inflammatory monocytes and lower levels of anti-inflammatory monocytes, respectively. While Npc1\(^{nih}\) mice on a two percent plant stanol diet displayed lower levels of Ly6C\(^{hi}\) monocytes at 49 days old alone, six percent plant stanol supplementation reduced the relative amount of circulating Ly6C\(^{hi}\) monocytes on both timepoints, suggesting a reduction in circulating pro-inflammatory monocytes. Furthermore, for two and six percent plant stanol supplementation, a trend towards an increase in blood Ly6C\(^{lo}\) monocytes was observed in 49 days old Npc1\(^{nih}\) mice. In addition, six percent plant stanol supplementation effectively triggered an increase in circulating Ly6C\(^{lo}\) monocytes in 35 days old Npc1\(^{nih}\) mice. Concerning blood T-cell populations, untreated Npc1\(^{nih}\) mice displayed higher levels of CD8\(^+\) T-cells compared to Npc1\(^{wt}\) mice at 35 days of age, but not at 49 days old, whereas levels of CD4\(^+\) T-cells were lower in untreated Npc1\(^{nih}\) mice at both time points (Fig. 4C-D). Although plant stanol supplementation had no effect on CD8\(^+\) T-cells on 35 days old Npc1\(^{nih}\) mice, Npc1\(^{nih}\) mice following six percent plant stanol supplementation displayed lower levels of CD8\(^+\) T-cells at 49 days of age. Finally, although plant stanol supplementation did not significantly increase circulating helper T-cells in Npc1\(^{nih}\) mice, a trend was observed suggesting this effect for six percent plant stanol supplementation in 35 days old Npc1\(^{nih}\) mice. Overall, these results indicate that dietary plant stanol supplementation shifted the ratio of pro- and anti-inflammatory circulating monocytes and T-cells towards a more anti-inflammatory phenotype.

Altogether, these findings indicate that dietary plant stanol esters supplementation improves hepatic lipid metabolism and reduces damage and inflammation in NPC1 disease. In addition, plant stanol
supplementation shifts the phenotype of blood immune cells towards a more anti-inflammatory profile in NPC1 disease, particularly at higher concentrations.
DISCUSSION

In NPC1 disease, whole-body lysosomal lipid accumulation triggers a cascade of pathological events that culminates in a wide range of peripheral and neurological symptoms. In addition, early diagnosis and effective therapeutic tools are currently lacking for NPC1 disease, making it a severe and lethal condition that warrants further research in order to improve quality of life and lifespan of patients. In this study, we show that dietary plant stanol esters supplementation improves progressive weight loss, as well as hepatic cholesterol accumulation and damage in a murine model for NPC1 disease. In addition, the current study shows that dietary plant stanol supplementation shifts the profile of blood immune cells towards a more anti-inflammatory phenotype. Based on these findings, we propose that dietary plant stanol supplementation should be further investigated as a complementary tool to ameliorate hepatic symptoms and the phenotype of blood immune cells in NPC1 disease patients.

While the mechanisms underlying the beneficial effects of stanols are yet to be fully elucidated, cumulative evidence indicates that these molecules interfere with cholesterol micellar solubilization in the intestines and may further inhibit cholesterol absorption and stimulate cholesterol excretion by activation of liver X receptor (LXR) transcription factor (42). As such, clinical benefits of increased plant stanol ester consumption are largely attributed to reduced dietary cholesterol absorption and consequent lowering of plasma cholesterol levels. In this study, increased plant stanol ester consumption induced a reduction in plasma and liver cholesterol levels in Npc1<sup>nih</sup> mice, in line with results from a previous NASH study (43). In addition to the effects of plant stanols on cholesterol absorption, a growing body of findings indicates that these molecules have anti-inflammatory and immunomodulatory properties (44). In a previous ex vivo study, sitostanol administration to mouse bone marrow-derived macrophages was shown to induce an anti-inflammatory effect independent of LXR activation. It should be noted that, since diets were not supplemented with cholesterol, mice in this study consumed low amounts of cholesterol. As such, although we cannot exclude a beneficial effect from reduced intestinal cholesterol absorption in Npc1<sup>nih</sup> mice following increased plant stanol consumption, it is likely that plant stanols’ anti-inflammatory properties
also contributed to the observed improvement in hepatic inflammation and damage. Furthermore, in a previous study, pharmacological LXR activation increased brain cholesterol excretion and ameliorated disease burden in Npc1<sup>−/−</sup> mice (45). As previously mentioned, plant stanol molecules are known LXR activators. As such, it is likely that increased plant stanol molecules improved hepatic pathology in Npc1<sup>−/−</sup> mice via a variety of mechanisms, namely reduced intestinal cholesterol absorption, anti-inflammatory effects and LXR activation. In addition to a local effect on hepatic inflammation, dietary plant stanol ester supplementation shifted the profile of plasma monocytes and T-cells in Npc1<sup>−/−</sup> mice towards a more anti-inflammatory phenotype, particularly in the former population. Previously, Brüll et al. have demonstrated that sitostanol administration elicits a TLR2-dependent T-helper 1 shift in human peripheral blood mononuclear cells cultures, even at very low concentrations. Further studies on asthma patients-derived peripheral blood mononuclear cells cultures confirmed the findings that sitostanol administration induces a T-helper 1 cell response and, in addition, lead to an increase in numbers and activity of regulatory T cells (46). It is thus possible that the shift in phenotype of circulating immune cells of Npc1<sup>−/−</sup> mice following plant stanol ester supplementation is derived from the direct effect of plant stanols on circulating immune cell populations. Of note, previous studies found that phytosterol supplementation ameliorates inflammation and oxidative stress in Crohn disease, a disorder which occurs in several NPC1 disease patients (47, 48). Considering the anti-inflammatory effects attributed to plant stanols, it is possible that a plant stanol-enriched diet could also ameliorate the intestinal problems of NPC1 disease patients. If so, this would further enhance the application of plant stanol supplementation as an additional therapeutical tool in NPC1 disease.

Of note, unlike stanols, phytosterol molecules are prone to oxidation (49, 50), and may therefore have pro-inflammatory effects if consumed in high amounts. Given the importance of inflammation in NPC1 disease burden (30, 51, 52), phytosterol supplementation should thus be regarded with caution.

Currently, Miglustat is the only approved drug for the treatment of NPC1 disease symptoms. While clinical observations indicate that Miglustat delays progression of neurological deterioration, the effects of Miglustat on systemic features of NPC1 disease remain largely unexplored, as it is specifically prescribed for
amelioration of neurological symptoms (10, 11). On the other hand, 2-hydroxypropyl-β-cyclodextrin, which previously has been shown to improve systemic symptoms of NPC1 disease in murine models, is currently being evaluated in clinical trials regarding its efficacy on neurological and systemic symptoms (12, 53). While neuroinflammation and degeneration are the largest contributors to reduced quality of life and lifespan of NPC1 disease patients, hepatic, splenic, intestinal and lung dysfunction are also observed in a significant amount of NPC1 disease patients, particularly in early onset NPC1 disease cases (6, 7, 47, 48). As such, further strategies to reduce systemic manifestations of NPC1 disease and to complement neurological-targeted treatments are required. In the past, cholesterol-lowering therapeutic strategies such as dietary cholesterol restriction and statin administration have been explored in murine NPC1 disease models and patients and found to ameliorate hepatic symptoms (54-56). Of note, combined use of dietary plant stanol supplementation and statins amplifies the cholesterol-lowering properties of each intervention in hypercholesterolemic patients (57, 58). As such, it is possible that the administration of statins and plant stanol esters simultaneously has additional benefits to systemic manifestations of NPC1 disease and to improving life quality of NPC1 disease patients. On the other hand, combining several methods to reduce peripheral cholesterol has been controversial in the clinical setting, demanding the need for additional clinical trials to assess the clinical use of combinational approaches to reduce plasma cholesterol (59).

Overall, considering the promising results here described, we propose that dietary plant stanol esters supplementation should be further investigated as a complementary therapeutical tool to ameliorate hepatic symptoms and the phenotype of blood immune cells in NPC1 disease. Plant stanol esters are widely available in functional foods, such as margarine spreads, and have been studied in human populations where it showed very minor side-effects, even when consumed in higher concentrations (24, 60, 61). Nonetheless, it should be further investigated whether increased stanol consumption bears so far unknown side-effects in NPC1 disease, as well as plant stanols effects on the nervous system.
DATA AVAILABILITY STATEMENT

All data pertaining to the findings of this study are available upon request from the corresponding author:

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**FIGURES**

Figure 1: Effect of dietary stanol supplementation on weight parameters. (A) Body weight of *Npc1<sup>wt</sup>* and *Npc1<sup>nih</sup>* mice throughout the study period. (B, C) Relative liver weight. Statistical analysis was performed by use of two-tailed unpaired t test. n=10-16 mice per group. *Npc1<sup>wt</sup>* vs *Npc1<sup>nih</sup>* mice fed a regular chow diet (# *p* ≤ 0.05; ## *p* < 0.01; ### *p* < 0.001; #### *p* < 0.0001); *Npc1<sup>nih</sup>* mice receiving regular chow vs *Npc1<sup>nih</sup>* fed two percent or six percent stanol-enriched chow diet (* *p* ≤ 0.05; ** *p* < 0.01; *** *p* < 0.001; **** *p* < 0.0001). All error bars represent standard error of the mean.
Figure 2: Lipid metabolism parameters. (A, B) Total plasma cholesterol and triglyceride levels of Npc1<sup>wt</sup> and Npc1<sup>nih</sup> mice on a regular and stanol-supplemented chow diet. (C, D) Liver cholesterol and triglyceride levels. (E-J) Hepatic lipid metabolism-related gene expression of Cd36, Sr-a, Npc2, Abcg1, Abcg8, Cyp8b1. Statistical analysis was performed by use of two-tailed unpaired t test. n=9-15 mice per group for liver gene expression analyses. Npc1<sup>wt</sup> vs Npc1<sup>nih</sup> mice fed a regular chow diet (# p ≤ 0.05; ## p < 0.01; ### p < 0.001; #### p < 0.0001); Npc1<sup>nih</sup> mice receiving regular chow vs Npc1<sup>nih</sup> mice fed two percent or six percent stanol-enriched chow diet (* p ≤ 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). All error bars represent standard error of the mean.
Figure 3: Effect of dietary stanol supplementation on hepatic inflammation. (A) Plasma ALT levels. (B-E) Representative pictures of liver sections stained for hepatic macrophages (CD68) and infiltrated macrophages and neutrophils (Mac-1). CD68 immunostainings were scored (B,C), whereas Mac-1 positive cells were counted (D,E). (F-K) Hepatic gene expression of inflammatory markers Tnf-α, Cd68, Ctsd, Mip2, Ccl3 and Arg1. Statistical analysis was performed by use of two-tailed unpaired t test. n=9-15 mice per group for liver gene expression analyses. Npc1<sup>wt</sup> vs Npc1<sup>nih</sup> mice fed a regular chow diet (# p ≤ 0.05; ## p < 0.01; ### p < 0.001; #### p < 0.0001); Npc1<sup>nih</sup> mice receiving regular chow vs Npc1<sup>nih</sup> mice fed two percent or six percent stanol-enriched chow diet (* p ≤ 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). All error bars represent standard error of the mean.
Figure 4: Effect of dietary stanol supplementation on plasma monocytes and T-cells phenotype. (A-H) Relative levels of plasma pro-inflammatory (LyC6\textsuperscript{high}) and anti-inflammatory (LyC6\textsuperscript{low}) monocytes, as well as cytotoxic (CD8\textsuperscript{+}) and helper T-cells (CD4\textsuperscript{+}) were measured by FACS analysis on week 3 and 5 of the study, when mice were 35 and 49 days old. Statistical analysis was performed by use of two-tailed unpaired t test. n=5 mice per group. Npc1\textsuperscript{wt} vs Npc1\textsuperscript{nih} mice fed a regular chow diet (# p ≤ 0.05; ## p < 0.01; ### p < 0.001; #### p < 0.0001); Npc1\textsuperscript{nih} mice receiving regular chow vs Npc1\textsuperscript{nih} mice fed two percent or six percent stanol-enriched chow diet (* p ≤ 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). All error bars represent standard error of the mean.