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Dietary plant stanol ester supplementation reduces peripheral symptoms in a mouse model of Niemann-Pick type C1 disease^S

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Abstract Niemann-Pick type C (NPC)1 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 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 a *Npc1*-null allele (*Npc1^{nh}*), creating a dysfunctional NPC1 protein. *Npc1^{nh}* mice were fed a 2% or 6% plant stanol ester-enriched diet over the course of 5 weeks. During this period, hepatic and blood lipid and inflammatory profiles were assessed. *Npc1^{nh}* mice fed the plant stanol-enriched diet exhibited lower hepatic cholesterol accumulation, damage, and inflammation than regular chow-fed *Npc1^{nh}* mice. Moreover, plant stanol consumption shifted circulating T-cells and monocytes in particular toward 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.—Magro dos Reis, I., T. Houben, Y. Oligschläger, L. Bücken, H. Steinbusch, D. Cassiman, D. Lütjohann, M. Westerterp, J. Prickaerts, J. Plat, and R. Shiri-Sverdlov. **Dietary plant stanol ester supplementation reduces peripheral symptoms in a mouse model of Niemann-Pick type C1 disease.** *J. Lipid Res.* 2020. 61: 830–839.

Supplementary key words inflammation • cholesterol metabolism • diet • dietary lipids • liver • atherosclerosis • nonalcoholic steatohepatitis • lysosomal storage disease

Niemann-Pick type C (NPC) disease is a rare lysosomal storage disorder caused by deleterious mutations in *NPC1* or *NPC2*. It is estimated that NPC disease affects one in 100,000 live births, with mutations in *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, LDLs merge with late endosomes/lysosomes

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Abbreviations: ALT, alanine aminotransferase; *Arg1*, arginase 1; *Cd36*, cluster of differentiation 36; *Cd68*, cluster of differentiation 68; *Cyp8b1*, cytochrome P450 family 8 subfamily B member 1; LEL, late endosome/lysosome; NPC, Niemann-Pick type C; *Sr-a*, scavenger receptor A.
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(LELs), where lysosomal acid lipases hydrolyze LDL-derived cholesteryl esters. NPC2, a protein located on the luminal surface of LELs, 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 (4). The age of onset and clinical features of NPC1 disease are heterogeneous, likely because of the variability of *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, and liver and pulmonary disease, occur in a significant number 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 that it has a minor impact on systemic symptoms such as splenomegaly (11). Furthermore, intrathecal administration of 2-hydroxypropyl- β -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 therapeutic 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 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 be effectively absorbed in the small intestine (26, 27). Dietary supplementation with plant stanol esters has well-known plasma cholesterol-lowering effects, presumably because 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 ester 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 a NPC1 disease murine model that expresses a *Npc1* allele with a frameshift mutation (*Npc1^{nh}*) that results in the loss of function of the corresponding NPC1 protein (13). While the *Npc1^{nh}* allele was originally discovered and maintained in the BALB/c mouse strain, here we used *Npc1^{nh}* mice with a C57BL/6 genetic background, a model that has been previously described and that results in a more severe NPC1 peripheral disease phenotype (32). To investigate our hypothesis, 2-week-old *Npc1^{nh}* mice received normal chow or a 2% or 6% plant stanol ester-enriched chow diet for 5 weeks. *Npc1^{wt}* mice fed regular chow were included as a control group for NPC1 disease phenotype. *Npc1^{nh}* 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 toward 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 the 6% plant stanol-enriched diet compared with 2% 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^{nh}* mice were derived from heterozygous founders (C57BL/6/*Npc1^{nh}*). Given the reduced lifespan of

Npc1^{nh} 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. Thirteen and 16 *Npc1^{nh}* mice received a 2% and 6% plant stanol ester-enriched diet, respectively (manufactured by Arie Blok B.V., Woerden, The Netherlands). *Npc1^{wt}* and *Npc1^{nh}* 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 supplemental Figs. 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 sterol (sitosterol, campesterol) and plant stanol (sitostanol, campestanol) content in food was analyzed by 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 1,000 rounds per minute on a Thermo Mixer were followed by 45 min of heating at 85°C 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 *gccaaagtaggcgacgact* and *catcactgggtctccatgtat* identified the wild-type allele and primers *gccaaagtaggcgacgact* and *ttccaattgtgatcttccaa* identified the mutant allele. Both PCRs were carried out under the same cycling conditions.

Alanine aminotransferase measurements

Plasma alanine aminotransferase (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% H_2O_2 for 5 min. Primary antibodies used were against hepatic macrophages [rat anti-mouse cluster of differentiation 68 (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, 200 \times) and were indicated as number of cells per square millimeter (cells/ mm^2). Immunostainings for hepatic macrophages (CD68) were evalu-

ated by an experienced pathologist and given a score in arbitrary units (A.U.).

Plasma FACS analyses

Tail vein blood was collected from *Npc1^{wt}* and *Npc1^{nh}* 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 min in the dark at 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 min. 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-CyTM7 rat anti-mouse Ly-6G (1:100), PE-CyTM7 rat anti-CD11b (1:300), and APC-H7 rat anti-mouse CD4 (1:100) (BD, San Jose, CA); CD3 monoclonal antibody (1:100) and CD8a monoclonal antibody (1:50) (eBioscienceTM from Thermo Fisher Scientific, San Diego, CA); and anti-mouse Ly-6C-APC (1:10) (Miltenyi, Bergisch Gladbach, Germany). Finally, samples were mixed and incubated in the dark at RT for 15 min with an erythlysis solution [8.4 g NH_4Cl + 0.84 g NaHCO_3 in 1 liter of water (pH 7.2–7.4)]. Sample stainings were quantified within 1 h 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}* versus *Npc1^{nh}* mice fed a regular chow diet ($\#P \leq 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$; $\#\#\#\#P < 0.0001$); *Npc1^{nh}* mice receiving regular chow versus *Npc1^{nh}* mice fed a 2% or 6% stanol-enriched chow diet ($*P \leq 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$). Data were statistically analyzed using GraphPad Prism software (version 6; GraphPad Software Inc, San Diego, CA; www.graphpad.com).

RESULTS

Plant stanol supplementation delays body weight loss in *Npc1^{nh}* mice and reduces relative liver weight

As reduced weight gain reflects NPC1 disease progression in *Npc1^{nh}* mice, we monitored the body weight of *Npc1^{nh}* mice and assessed whether plant stanol supplementation influenced this parameter. From day 12 of the study, which coincided with the weaning period, untreated *Npc1^{nh}* mice were consistently smaller than *Npc1^{wt}* mice (Fig. 1A). *Npc1^{nh}* mice on a 2% plant stanol-enriched diet showed a modest increase in body weight compared with untreated *Npc1^{nh}* mice until day 22 of the study, although this did not reach statistical significance. On the other hand, 6% plant stanol supplementation effectively rescued the weight of *Npc1^{nh}* mice between days 12 and 29 of the study. Furthermore, in the last week of the study, a trend toward increased body weight was observed in *Npc1^{nh}* mice on a 6% plant stanol-enriched diet compared with 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^{nh}* mice

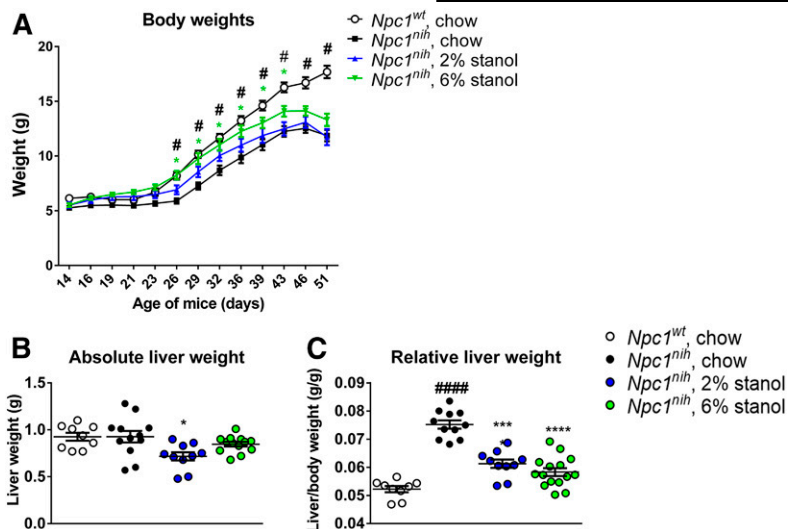


Fig. 1. Effect of dietary stanol supplementation on weight parameters. A: Body weight of *Npc1^{wt}* and *Npc1^{nih}* mice throughout the study period. B, C: Relative liver weight. Statistical analysis was performed by use of two-tailed unpaired *t*-test ($n = 10$ – 16 mice per group). *Npc1^{wt}* versus *Npc1^{nih}* mice fed a regular chow diet ($\#P \leq 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$; $\#\#\#\#P < 0.0001$); *Npc1^{nih}* mice receiving regular chow versus *Npc1^{nih}* fed 2% or 6% stanol-enriched chow diet ($*P \leq 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$). All error bars represent standard error of the mean.

and *Npc1^{wt}* mice, liver weights relative to body weight of untreated *Npc1^{nih}* mice were higher compared with *Npc1^{wt}* mice (Fig. 1B, C). Relevantly, relative liver weight of *Npc1^{nih}* mice fed a 2% and 6% plant stanol-enriched diet was reduced. In addition, absolute liver weights were reduced in *Npc1^{nih}* mice on a 2% plant stanol-supplemented diet. Overall, these results indicate that dietary supplementation with plant stanol esters ameliorates body weight gain and hepatomegaly in *Npc1^{nih}* mice.

Decreased plasma and hepatic total cholesterol levels in *Npc1^{nih}* mice fed a plant stanol-enriched diet

To assess the effect of dietary plant stanol supplementation on lipid metabolism of *Npc1^{nih}* mice, biochemical analyses of plasma and liver lipids were performed. In line with plant stanols' well-known plasma cholesterol lowering effect, *Npc1^{nih}* 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 with untreated *Npc1^{nih}* mice. Of note, these effects were more pronounced following a 6% stanol-enriched diet. Next, we analyzed hepatic lipid accumulation, a prominent systemic feature of NPC1 disease. As expected, untreated *Npc1^{nih}* mice displayed higher levels of hepatic cholesterol compared with *Npc1^{wt}* mice (Fig. 2C). *Npc1^{nih}* mice that received plant stanol supplementation, particularly at 6%, showed prominently lower levels of hepatic total cholesterol than untreated *Npc1^{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^{nih}* mice, who displayed lower levels of liver triglycerides than *Npc1^{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 *Npc2*, a protein that transfers free cholesterol

within LELs to NPC1; on *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 (Fig. 2E–J, respectively). Following 2% plant stanol administration, expression of *Sr-a* and *Npc2* decreased in the livers of *Npc1^{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^{nih}* mice. Furthermore, *Npc1^{nih}* mice on a 2% plant stanol-enriched diet displayed higher expression of *Cyp8b1*, suggesting increased conversion of excess hepatic cholesterol into bile acids. Likewise, *Npc1^{nih}* mice on a 6% 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, 6% 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^{nih}* mice. Finally, *Npc1^{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^{nih}* mice in a dose-dependent manner.

Dietary plant stanol supplementation improves hepatic damage and inflammation in *Npc1^{nih}* mice

Following the observed improvements in hepatic cholesterol metabolism, we next investigated the effects of plant stanol supplementation on hepatic damage and inflammation. Plasma ALT levels of untreated *Npc1^{nih}* mice showed a near 4-fold increase in relation to *Npc1^{wt}* mice, indicating increased liver damage in *Npc1^{nih}* mice (Fig. 3A). Remarkably, after dietary plant stanol supplementation, plasma ALT levels of *Npc1^{nih}* mice were comparable to *Npc1^{wt}* mice, indicating a strong decrease in overall liver damage. We further looked into hepatic inflammatory status via immunohistochemistry by measuring CD68 and Mac-1

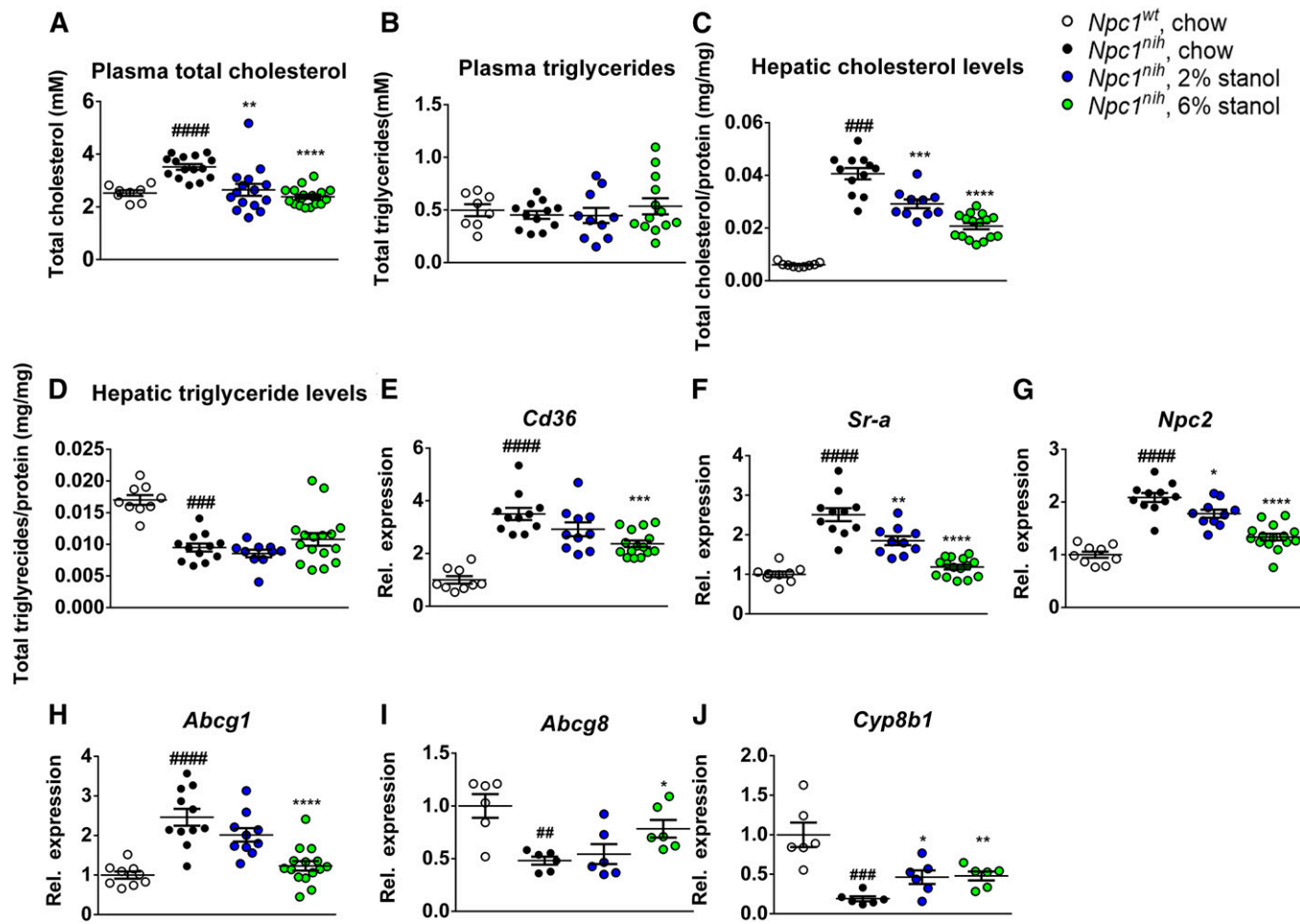


Fig. 2. Lipid metabolism parameters. A, B: Total plasma cholesterol and triglyceride levels of *Npc1^{wt}* and *Npc1^{nih}* mice on a regular and stanol-supplemented chow diet. C, D: Liver cholesterol and triglyceride levels. E–J: Hepatic lipid metabolism-related gene expression of *Cd36*, *Sr-a*, *Npc2*, *Abcg1*, *Abcg8*, and *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^{wt}* versus *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 versus *Npc1^{nih}* mice fed 2% or 6% 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.

proteins, which identify resident hepatic macrophages and infiltrated neutrophils and macrophages, respectively (Fig. 3B–E). Untreated *Npc1^{nih}* mice displayed higher levels of immune cells in both immunostainings, indicating prominent hepatic inflammation in *Npc1^{nih}* 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 *Tnf-α*, *Cd68*, cathepsin D (*Ctsd*), macrophage inflammatory protein 2 (*Mip2*), chemokine (C-C motif) ligand 3 (*Ccl3*), and arginase 1 (*Arg1*) (Fig. 3F–K). Hepatic gene expression of inflammatory markers was consistently increased in untreated *Npc1^{nih}* mice compared with *Npc1^{wt}* mice, and decreased in the case of *Arg1*, a marker for alternatively activated macrophages. Both 2% and 6% plant stanol supplementation reversed these observations, supporting the aforementioned findings that *Npc1^{nih}* mice fed a plant stanol ester-supplemented diet display lower hepatic inflammation and damage.

Plant stanol supplementation shifts plasma profile of immune cells toward 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-day-old *Npc1^{nih}* mice. To analyze 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^{high} monocytes and lower relative levels of Ly6C^{low} 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 2% plant stanol diet displayed lower levels of Ly6C^{high} monocytes at 49 days old alone, 6% plant stanol supplementation reduced the relative amount of circulating Ly6C^{high} monocytes at both time points, suggesting a reduction in circulating pro-inflammatory monocytes. Furthermore, for 2% and 6% plant stanol supplementation, a trend toward

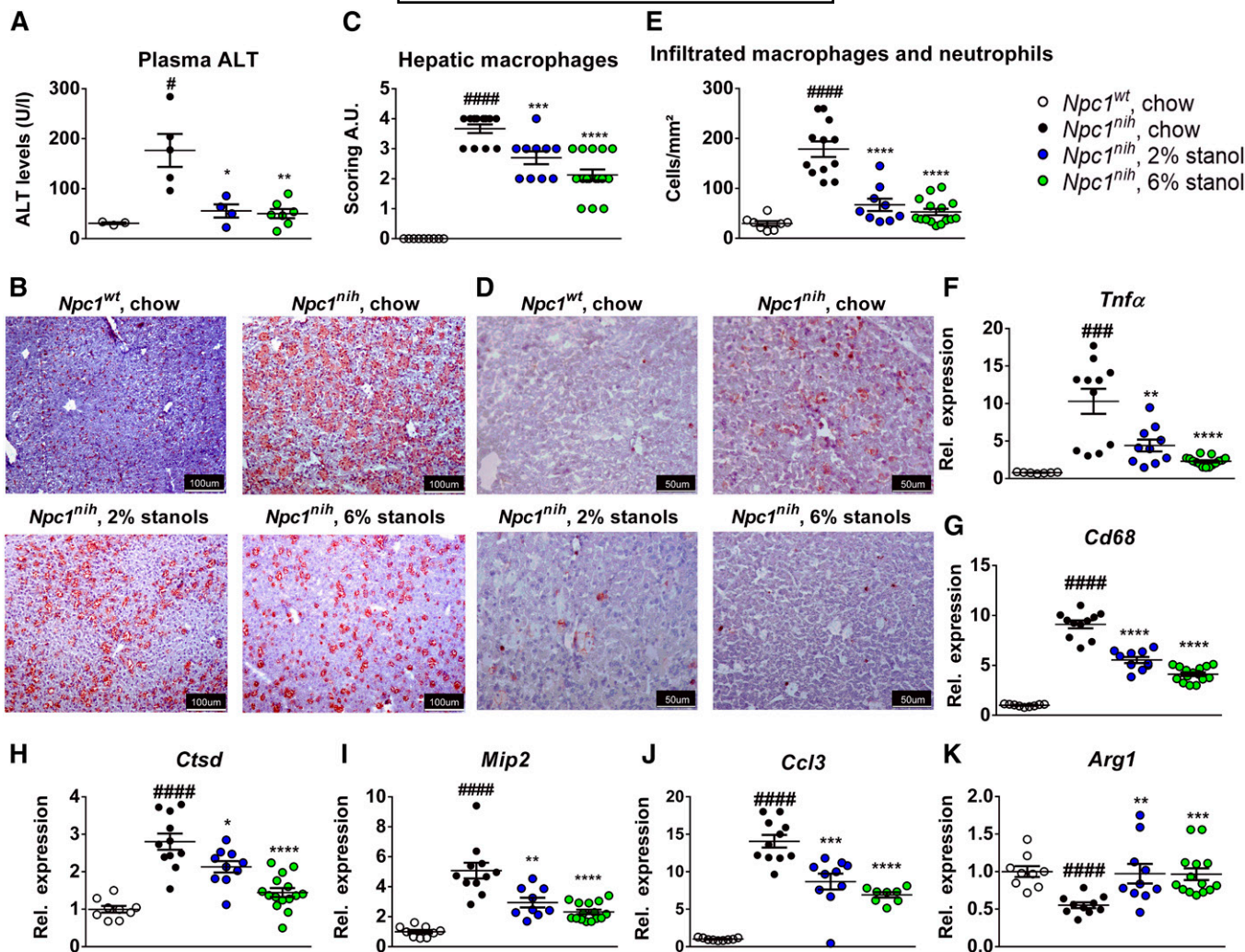


Fig. 3. Effect of dietary stanol supplementation on hepatic inflammation. A: Plasma ALT levels. B–E: Representative pictures of liver sections stained for hepatic macrophages (CD68) and infiltrated macrophages and neutrophils (Mac-1). CD68 immunostainings were scored (B, C), whereas Mac-1-positive cells were counted (D, E). F–K: Hepatic gene expression of inflammatory markers *Tnfa*, *Cd68*, *Ctss*, *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*^{wt} versus *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 versus *Npc1*^{nih} mice fed 2% or 6% 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.

an increase in blood Ly6C^{low} monocytes was observed in 49-day-old *Npc1*^{nih} mice. In addition, 6% plant stanol supplementation effectively triggered an increase in circulating Ly6C^{low} monocytes in 35-day-old *Npc1*^{nih} mice. Concerning blood T-cell populations, untreated *Npc1*^{nih} mice displayed higher levels of CD8⁺ T-cells compared with *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 in 35-day-old *Npc1*^{nih} mice, *Npc1*^{nih} mice following 6% 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 6% plant stanol supplementation in 35-day-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 toward a more anti-inflammatory phenotype.

Altogether, these findings indicate that dietary plant stanol ester 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 toward 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

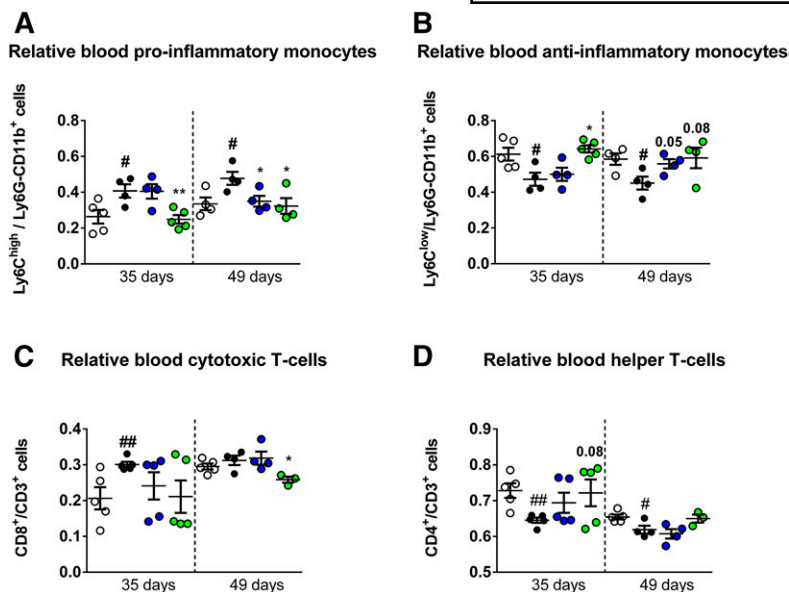


Fig. 4. Effect of dietary stanol supplementation on plasma monocyte and T-cell phenotype. A–H: Relative levels of plasma pro-inflammatory (LyC6^{high}) and anti-inflammatory (LyC6^{low}) monocytes, as well as cytotoxic (CD8⁺) and helper T-cells (CD4⁺) were measured by FACS analysis on weeks 3 and 5 of 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*^{wt} versus *Npc1*^{nh} mice fed a regular chow diet (#*P* ≤ 0.05; ##*P* < 0.01; ###*P* < 0.001; ####*P* < 0.0001); *Npc1*^{nh} mice receiving regular chow versus *Npc1*^{nh} mice fed 2% or 6% 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.

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 ester 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 toward 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 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*^{nh} 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, because 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*^{nh} 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*^{-/-} 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*^{nh} 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*^{nh} mice toward a more anti-inflammatory phenotype, particularly in the former population. Previously, Brüll et al. (46) 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 patient-derived peripheral blood mononuclear cell cultures confirmed the findings that sitostanol administration induces a T-helper 1 cell response and, in addition, leads 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*^{nh} 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 therapeutic 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 neurologically 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 described here, we propose that dietary plant stanol ester supplementation should be further investigated as a complementary therapeutic 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 they 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

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

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