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Plant Sterols Cause Macrothrombocytopenia in a Mouse Model of Sitosterolemia

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Mutations in either ABCG5 or ABCG8 cause sitosterolemia, an inborn error of metabolism characterized by high plasma plant sterol concentrations. Recently, macrothrombocytopenia was described in a number of sitosterolemia patients, linking hematological dysfunction to disturbed sterol metabolism. Here, we demonstrate that macrothrombocytopenia is an intrinsic feature of murine sitosterolemia. Abcg5-deficient (Abcg5\textsuperscript{-/-}) mice showed a 68% reduction in platelet count, and platelets were enlarged compared with wild-type controls. Macrothrombocytopenia was not due to decreased numbers of megakaryocytes or their progenitors, but defective megakaryocyte development with deterioration of the demarcation membrane system was evident. Lethally irradiated wild-type mice transplanted with bone marrow from Abcg5\textsuperscript{-/-} mice displayed normal platelets, whereas Abcg5\textsuperscript{-/-} mice transplanted with wild-type bone marrow still showed macrothrombocytopenia. Treatment with the sterol absorption inhibitor ezetimibe rapidly reversed macrothrombocytopenia in Abcg5\textsuperscript{-/-} mice concomitant with a strong decrease in plasma plant sterols. Thus, accumulation of plant sterols is responsible for development of macrothrombocytopenia in sitosterolemia, and blocking intestinal plant sterol absorption provides an effective means of treatment.

Plant sterols structurally differ from cholesterol only by the presence of an additional methyl or ethyl group at C-24 (campesterol and sitosterol, respectively), in some cases with an additional double bond at C-22 (brassicasterol and stigmas- terol, respectively). These structural differences appear to have a major impact on their handling in the body. Despite the fact that regular Western diets contain similar amounts of cholesterol and plant sterols (intakes of \( \approx 200 – 400 \) mg/day for both) (1), plasma levels of plant sterols are \( \geq 100 \) times lower than those of cholesterol in healthy individuals (2), indicating the presence of effective systems to prevent plant sterol accumulation in the human body. It has become clear that the ATP-binding cassette half-transporters ABCG5 and ABCG8, most abundantly present in intestine and liver, are critically involved herein. ABCG5 and ABCG8 function as an obligate heterodimer and mediate cholesterol and plant sterol transport out of intestinal and hepatic cells (3, 4). The functional heterodimer is present at the apical membranes of enterocytes and hepatocytes, where it transports (plant) sterols back into the intestinal lumen or bile, respectively (4, 5). Expression of ABCG5 and ABCG8 in other organs and tissues is relatively low (6–8), and functionality at sites other than intestine and liver has not been reported.

Mutations in either the ABCG5 or ABCG8 gene cause sitosterolemia (9, 10). Sitosterolemia is a relatively rare autosomal recessive disorder characterized by the accumulation of plant sterols in blood and tissues. Patients frequently develop tendon and cutaneous xanthomas and, most important, are at risk of developing premature coronary atherosclerosis (11–14). A number of associated features, including hemolysis, abnormal platelets, and iron deficiency, have occasionally been reported (12, 15, 16). Recently, a series of subjects with Mediterranean macrothrombocytopenia (low levels of abnormally large platelets) were identified as sitosterolemia patients (17), harboring different known and new mutations in ABCG5 or ABCG8. The mechanism(s) underlying this interesting link between defective sterol metabolism and hematological abnormalities have remained unexplained so far.

Here, we describe for the first time that macrothrombocytopenia is also prominent in a murine model of sitosterolemia, i.e. the Abcg5-deficient mouse. Mice lacking Abcg5 show high levels of dietary plant sterols that appear to be associated with defective megakaryocyte development and disturbed formation of the demarcation membrane system, probably underlying defective platelet formation. Macrothrombocytopenia in these mice could not be cured by transplantation of wild-type bone marrow but was rapidly reversed upon decreasing plasma plant sterol levels by treatment with ezetimibe, a clinically used intestinal sterol absorption inhibitor. These data clearly establish that plant sterols, when present in high concentrations, have profound and deleterious effects on platelet biology and therefore on blood coagulation.
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MATERIALS AND METHODS

Animals and Diet—Abcg5−/− mice were generated by Deltagen, Inc. (Redwood City, CA) using standard gene targeting methods (18). Mice homozygous for disruption of the Abcg5 gene and their heterozygous and wild-type littermates were used as indicated. Mice received standard mouse chow containing 0.017% (w/w) cholesterol (Arie Blok B.V., Woerden, The Netherlands). Where indicated, mice received chow containing 0.005% (w/w) ezetimibe (Ezetrol®, MSD UK, Hertfordshire, UK). All experiments were performed with the approval of the Ethical Committee for Animal Experiments of the University of Groningen.

Bone Marrow Transplantation Study—Twenty-four hours after lethal irradiation (9.5 grayS, IBL 637 137Cs γ-ray source; CIS Biointernational, Gif-sur-Yvette, France), Abcg5−/− mice and control littermates (11–13 weeks of age) were transplanted with 6 × 10⁶ bone marrow cells isolated from either Abcg5−/− mice or control littermates. PCR analysis of colonies of bone marrow cells revealed that all transplanted mice had reached ≥87.5% chimeras at 12 weeks after transplantation. Age-matched, non-irradiated, non-transplanted wild-type and Abcg5−/− littermates were used as controls.

Blood Analysis—Blood was collected in EDTA-coated tubes upon heart puncture. Subsequently, blood was analyzed using an automated hematology analyzer (Sysmex XE 2100) for red blood cell counts, mean cellular volume, red blood cell distribution width, hemoglobin concentration, and platelet count. For histology, blood smears were prepared from undiluted blood and stained with Wright-Giemsa stains. Flow cytometry analysis was performed on a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences). Whole blood was diluted 10 times in Hanks’ balanced salt solution containing 10 units/ml EDTA and centrifuged at 200 × g for 7 min to spin down red blood cells. Platelet-rich supernatant was aspirated and centrifuged. Platelets were labeled with fluorescein isothiocyanate-labeled anti-mouse CD41 antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in phosphate-buffered saline for 30 min in the dark. Platelets were washed, and size was measured by flow cytometry. In the ezetimibe study (see Fig. 4), platelet count were determined by flow cytometry using reference beads. Plasma thrombopoietin concentration was measured by mouse thrombopoietin (TPO)3 immunoassay (R&D Systems, Minneapolis, MN). Plasma plant sterol levels were determined by gas chromatography-mass spectrometry as described (18). The resistance of erythrocytes to hemolytic actions of micellar bile acids and erythrocyte osmotic fragility were determined following the methods of Van der Meer et al. (19) and Orcutt et al. (20), respectively.

Assay for Colony-forming Cells—For megakaryocyte colony-forming unit assays, bone marrow mononucleated cells (1 × 10⁶) were cultured in duplicate with MegaCult-C medium (StemCell Technologies, Vancouver, British Columbia, Canada) in the presence of 50 ng/ml thrombopoietin and 10 ng/ml each interleukin-3, interleukin-6, and interleukin-11. After 8 days of culture, the colonies were stained with acetylcholine esterase. For granulocyte/macrophage colony-forming units, bone marrow mononucleated cells (2 × 10⁵) were cultured in duplicate following the methylcellulose method of Iscove and Sieber (21) in the presence of 100 ng/ml recombinant rat stem cell factor, 10 ng/ml recombinant mouse granulocyte/macrophage colony-stimulating factor (supplied by Behringwerke, Marburg, Germany), and 2 units/ml recombinant human erythropoietin. Colonies were scored at day 8.

In Vitro Culture of Murine Bone Marrow and Megakaryocytes—Isolated bone marrow cells (1 × 10⁶ cells/ml) were cultured in serum-free hematopoietic progenitor growth medium (Cambrex, Walkersville, MD) supplemented with 100 ng/ml thrombopoietin. After 8 days of culture, proplatelet formation was examined by phase-contrast microscopy.

Real-time PCR—The CD41⁺ cell fraction was purified from spleen cells by MoFlo sorting (DakoCytomation, Glostrup, Denmark). Dead cells were excluded from analysis by gating on forward and sideward light scatter. A total of 100.000 CD41⁺ cells/mouse (n = 6) were sorted. Total RNA was extracted with an RNeasy minikit (Qiagen, Hilden, Germany). cDNA synthesis was performed as described previously (22). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector according to the manufacturer’s instructions. The primers and probes used in this study have been described (23–25). All expression data were subsequently standardized for 18 S RNA, which was analyzed in separate runs.

Electron Microscopy—For ultrastructural analyses of megakaryocytes, spleen was collected and fixed overnight at 4 °C in 2% glutaraldehyde and processed for electron microscopy as described previously (26).

Statistics—Differences between groups were compared using the Mann-Whitney U test or one-way analysis of variance using GraphPad Prism software. The level of significance was set at p < 0.05. Data are presented as the means ± S.D.

RESULTS

Characterization of Macrothrombocytopenia in Abcg5−/− Mice—An Abcg5 deficiency in mice leads to 8- and 37-fold elevated plasma concentrations of campesterol and β-sitosterol, respectively, when the animals are fed normal chow, thus providing a murine model for sitosterolemia (18). Interestingly, we observed that Abcg5 deficiency was associated with a strong bleeding tendency during surgery for gallbladder cannulation or jugular vein catheterization. To assess whether the hematological phenotype reported in patients with sitosterolemia (17, 27) is also present in Abcg5−/− mice, we analyzed several hematological parameters. Chow-fed Abcg5−/− mice showed severe thrombocytopenia, as blood platelet counts were only 32% of those observed in wild-type littermates (Fig. 1A). Platelet size, based on forward scatter, was strongly increased in Abcg5−/− mice (Fig. 1B). Histological analysis confirmed the increased platelet size (Fig. 1C), establishing that Abcg5−/− mice indeed suffer from macrothrombocytopenia.

Although no significant differences in red blood cell count and mean cell volume were observed between Abcg5−/− and control mice, these cells were slightly more heterogenous in size in the Abcg5−/− mice (supplemental Table 1). Strongly

3 The abbreviations used are: TPO, thrombopoietin; SR-BI, scavenger receptor class B, type I; DMS, demarcation membrane system; WT, wild-type; TPN, total parenteral nutrition.
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FIGURE 1. Platelet count and size and measures of megakaryocyte differentiation in wild-type and Abcg5−/− mice.

A, platelet count in wild-type and Abcg5−/− mice (n = 16/group). *p < 0.01. B, platelet size as measured by flow cytometry in wild-type and Abcg5−/− mice (n = 6/group). *, p < 0.01. A.U., arbitrary units. C, Wright-Giemsa staining of blood cells from wild-type and Abcg5−/− mice. D, demonstration of in vitro differentiation of bone marrow cells from wild-type and Abcg5−/− mice (representative picture from three independent experiments) into proplatelet-forming mature megakaryocytes. The arrows indicate proplatelet structures. E, expression levels of genes involved in cholesterol homeostasis in spleen megakaryocytes from control and Abcg5−/− mice. Values are standardized for 18S RNA, and expression in control mice was set at 1 (n = 5/group). *, p < 0.05.

Elevated concentrations of all plant sterols and stanols were detected in washed red blood cell fractions from Abcg5−/− mice compared with control mice, e.g. 16- and 117-fold for campesterol and β-sitosterol, respectively (supplemental Table 2). Despite this, the vulnerability of red blood cells from Abcg5−/− mice to hemolytic actions of micellar bile acid concentrations (supplemental Fig. 1A) and their osmotic fragility (supplemental Fig. 1B) were not affected.

Megakaryocyte Differentiation ex Vivo Is Not Affected by Abcg5 Deficiency—Decreased platelet levels in Abcg5−/− mice can theoretically be explained by impaired megakaryocyte differentiation. The decreased platelet levels in Abcg5−/− mice were associated with increased levels of TPO (528 ± 136 versus 338 ± 57 pg/ml in wild-type mice, p < 0.05), which is an important cytokine involved in megakaryocyte proliferation and differentiation (28). Increased TPO levels in Abcg5−/− mice corresponded with increased numbers of megakaryocyte progenitor cells in their bone marrow (36 ± 1 versus 17 ± 2 megakaryocyte colony-forming units/1 × 10⁷ cells in wild-type mice, p < 0.05). Along with the megakaryocyte progenitors, the granulocyte/monocyte progenitor compartment was slightly increased in Abcg5−/− mice (26 ± 4 versus 20 ± 2 granulocyte/macrophage colony-forming units/1 × 10⁷ cells in wild-type mice, n = 3). In addition to bone marrow, megakaryocytes are also present in spleen. The relative weights of the spleens of Abcg5−/− mice were significantly higher than in control mice (5.6 ± 0.5 versus 3.0 ± 0.4 mg/g of body weight in wild-type mice, p < 0.05). Histological analysis revealed increased numbers of megakaryocytes to be present in the spleens of Abcg5−/− mice (data not shown), which was confirmed by flow cytometry (19.2 ± 4.7% versus 11.0 ± 6.2% CD41⁺ cells in wild-type mice, p < 0.05). Analysis of spleen megakaryocytes (CD41⁺ cells) showed no difference in DNA ploidy distribution (data not shown). In vitro culture of these megakaryocytes in the presence of TPO but in the absence of plant sterols showed that megakaryocytes from Abcg5−/− mice were capable of forming proplatelet structures within 8 days, similar to cells from wild-type mice (Fig. 1D).

Abcg5 Deficiency Leads to Altered Cholesterol Homeostasis in the Megakaryocyte—Although Abcg5−/− mice showed no alterations in in vitro megakaryocyte differentiation, expression of cholesterol transporter genes in freshly harvested CD41⁺ cells from the spleen was significantly affected. The mRNA expression levels of the ATP-binding cassette transporter genes Abca1 and Abcg1, both involved in the efflux of cholesterol and phospholipids to high density lipoprotein particles, were decreased in megakaryocytes from Abcg5−/− mice by 60 and 45%, respectively (Fig. 1E). Abcg5 and Abcg8 mRNA levels were below detection limits (Ct > 40) in megakaryocytes from wild-type mice. Expression of SR-BI, involved in cholesterol ester uptake from high density lipoprotein, and of the low density lipoprotein receptor, involved in low density lipoprotein particle uptake and therefore in cholesterol internalization, was increased by 68 and 185%, respectively, in megakaryocytes from Abcg5−/− mice. The megakaryocyte mRNA levels of the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase, a key enzyme in cholesterol synthesis, were not affected by Abcg5 deficiency.

Loss of Abcg5 Leads to Altered Megakaryocyte Structure—Electron microscopic evaluation was performed on spleen megakaryocytes from wild-type and Abcg5−/− mice (Fig. 2, A–F). Spleen megakaryocytes from wild-type mice showed the characteristic morphological features (Fig. 2A) with an extensive, open demarcation membrane system (DMS) (Fig. 2B). Megakaryocytes from Abcg5−/− mice demonstrated an immature appearance with swollen mitochondria and multivesicular bodies (Fig. 2, D and E) and a clear perturbation of the DMS (Fig. 2, cf. B and E). In addition, in Abcg5−/− mice, the presence
of extremely large platelets, which were frequently without granulae, was noted at their site of formation in spleen (Fig. 2, cf. C and F).

High Plant Sterol Levels Are the Cause of Macrothrombocytopenia—To determine whether the macrothrombocytopenia in the Abcg5<sup>−/−</sup> mice resulted directly from Abcg5 deficiency or indirectly from the prevailing high plant sterol levels, bone marrow transplantation experiments were performed. Wild-type and Abcg5<sup>−/−</sup> mice were lethally irradiated and received 1-day post-irradiation bone marrow cells from either Abcg5<sup>−/−</sup> (Abcg5<sup>−/−</sup> → WT) or wild-type (WT → Abcg5<sup>−/−</sup>) donor mice. To ensure full recovery from the transplantation, blood, bone marrow, and spleen cells were analyzed at 12 weeks after transplantation. No weight differences were noted between the transplanted Abcg5<sup>−/−</sup> (WT → Abcg5<sup>−/−</sup>) and wild-type (Abcg5<sup>−/−</sup> → WT) mice or when the transplanted animals were compared with non-irradiated, non-transplanted wild-type and Abcg5<sup>−/−</sup> mice. Transplanted wild-type mice showed a small decrease in platelet numbers compared with non-transplanted wild-type mice (Fig. 3A); however, the platelets did not significantly differ in size (Fig. 3B). Transplantation of wild-type bone marrow cells could not rescue the macrothrombocytopenia seen in Abcg5<sup>−/−</sup> mice, as platelet levels were severely decreased, and platelet size was markedly increased in transplanted Abcg5<sup>−/−</sup> mice at 12 weeks after transplantation (Fig. 3, A and B), although not to the same extent as observed in Abcg5<sup>−/−</sup> control mice. These data strongly indicate that the absence of Abcg5 is not the primary cause of macrothrombocytopenia but that the high plant sterol levels as such affect megakaryocyte development.

Ezetimibe Corrects Sitosterolemia-associated Macrothrombocytopenia in Mice—Treatment of sitosterolemia subjects (29, 30) or Abcg<sup>5</sup><sup>−/−</sup>/Abcg<sup>8</sup><sup>−/−</sup> double knock-out mice (31) with the sterol absorption inhibitor ezetimibe was recently shown to reduce their elevated plasma plant sterol levels (23) for an 18-day treatment period. The latter coincided with a strong decrease (−64%) of plasma β-sitosterol sterol levels (Fig. 4C). Elevated plasma concentrations of other plant sterols and
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TABLE 1

Comparison of plasma cholesterol, plant sterol, and plant stanol concentrations in the plasma of Abcg5+/− and Abcg5−/− mice before and after treatment with ezetimibe for 18 days. Values represent the means ± S.D. for n = four and n = six animals/group, respectively. Cholesterol is measured in mmol/liter, and all others are measured in μmol/liter.

|          | Abcg5+/− | Abcg5−/− |
|----------|----------|----------|
|          | −Ezetimibe | +Ezetimibe | −Ezetimibe | +Ezetimibe |
| Cholesterol | 2.22 ± 0.43 | 2.15 ± 0.28 | 1.42 ± 0.23 | 1.73 ± 0.27 |
| Campesterol | 76.8 ± 34.9 | 41.1 ± 14.4 | 632.5 ± 92.1 | 140.9 ± 19.7 |
| Sitosterol | 25.5 ± 9.0 | 2.3 ± 0.3 | 975.8 ± 185.6 | 348.0 ± 52.4 |
| Stigmasterol | 0.93 ± 0.37 | 0.16 ± 0.02 | 24.3 ± 5.2 | 9.9 ± 0.9 |
| Stigmasterol | 0.41 ± 0.10 | 0.13 ± 0.04 | 24.8 ± 4.0 | 13.0 ± 1.9 |
| Campestanol | 0.38 ± 0.29 | 0.20 ± 0.14 | 127.2 ± 27.4 | 68.4 ± 8.3 |
| Sitostanol | 0.14 ± 0.14 | 0.06 ± 0.04 | 137.7 ± 24.7 | 61.7 ± 8.3 |
| Stigmasterol | 0.27 | 0.19 | 1.73 | 1.73 |
| Stigmasterol | 0.02 | 0.01 | 0.02 |

* p < 0.05 indicates significant effect of ezetimibe.

Using an established mouse model of sitosterolemia (18), we have shown that high levels of dietary plant sterols have profound and deleterious effects on platelet formation. Abcg5-deficient mice showed severely decreased platelet counts and a grossly increased platelet size, implying that these mice suffer from macrothrombocytopenia analogous to the situation described recently in sitosterolemia patients (17, 27). Macrothrombocytopenia was not due to decreased numbers of megakaryocytes or their progenitors, as these cells were abundantly present in the bone marrow and spleens of Abcg5−/− mice, presumably as a consequence of high TPO levels. The disturbed platelet formation is likely caused by plant sterol-induced changes in megakaryocyte development, including deterioration of the DMS. We have documented that this phenotype is related to the prevailing high plant sterol levels rather than to the loss of Abcg5. First, megakaryocytes isolated from Abcg5-deficient mice were fully capable of proplatelet formation when cultured in vitro in the absence of plant steroids. Second, lethally irradiated wild-type mice that received bone marrow from Abcg5−/− mice displayed normal platelets, whereas Abcg5−/− mice harboring wild-type bone marrow still showed macrothrombocytopenia. Third and most important, treatment with the sterol absorption inhibitor ezetimibe rapidly reversed macrothrombocytopenia in Abcg5−/− mice concomitant with a strong reduction in plasma plant sterol levels. These data unequivocally demonstrate that the accumulation of plant sterols in sitosterolemia is responsible for development of macrothrombocytopenia and that prevention of intestinal plant sterol absorption provides an effective means to treat this complication of the disease.

Plant sterols are currently widely employed as a dietary supplement to lower plasma low density lipoprotein cholesterol levels in hypercholesterolemia subjects through interference with intestinal cholesterol absorption (32). Unlike originally thought, it is now evident that these sterols enter the enterocytes (33) via the recently identified intestinal cholesterol uptake protein NPC1L1 (34). Because plant sterols are poor...
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substrates for acyl-CoA:cholesterol acyltransferase 2 (ACAT2) (35), these sterols remain in their unesterified form and, in contrast to cholesteryl esters, are not effectively incorporated into chylomicrons. As a consequence, plant sterols are effectively resecreted into the intestinal lumen through the actions of ABCG5/ABCG8 (9, 10). Increased intake of dietary plant sterols in humans and mice with functional ABCG5/ABCG8 results in only slight increases in their plasma concentrations, delineating the effectiveness of the system. However, polymorphisms in both ABCG5 and ABCG8 that do affect plasma plant sterol levels have been described (36), and in view of the present results, it may be prudent to check for potential consequences of increased dietary plant sterol intake on platelet morphology and function in these subjects as well as in obligate heterozygotes.

High plasma plant sterol levels and thrombocytopenia have also been reported in patients receiving total parenteral nutrition (TPN) who developed liver dysfunction (37). TPN based on soybean oil is, by definition, rich in plant sterols (38), but not all TPN formulations share this feature. Children on long-term TPN have been reported to develop hematological disorders (39), including thrombocytopenia. The cause of TPN-associated thrombocytopenia is obviously difficult to determine directly in severely ill patients, yet based on the results of this study, it is tempting to speculate that plant sterols can be involved here.

An obvious question is why platelet formation is so severely affected by plant sterols, whereas functional properties of red blood cells and their resistance to hemolytic stress are not or only mildly affected in mice because studies in humans (17, 19) strongly emphasize the coexistence of platelet and red blood cell abnormalities in sitosterolemia. Whereas the underlying cause of this remarkable species difference remains unclear, it does demonstrate that platelet and red blood cell abnormalities represent independent features of this condition. We have tried to compare plant sterol levels in washed red blood cells and isolated platelets from wild-type and Abcg5−/− mice. Red blood cells from the latter showed the expected enrichment with plant sterols/stanols (supplemental Table 2). Unfortunately, only the most abundant plant sterols, i.e. sitosterol and campesterol, could be detected in platelet fractions isolated from ∼1 ml of mouse Abcg5−/− blood, whereas all plant sterols/stanols were below the detection limit of our gas chromatography-mass spectrometry method in control platelet fractions.

Platelet formation is thought to be highly dependent on correct formation of the DMS in the megakaryocyte, as this represents the source of proplatelet and platelet membranes (40). The DMS originates from tubular invaginations of the plasma membrane and is readily accessible from the extracellular space (40). The markedly disturbed formation of the DMS in megakaryocytes from Abcg5−/− mice implies that plant sterols have a profound effect on the formation of this extensive and highly dynamic membrane system. Incorporation of plant sterols in biological and artificial membranes has been shown to alter membrane fluidity (41–44), and it may well be that this is the underlying cause of malfunctioning of the platelet formation process. Gene expression studies indicated that cholesterol metabolism is perturbed in megakaryocytes from Abcg5−/− mice, with reduced expression of cholesterol efflux transporters (Abca1 and Abcg1) and increased expression of lipoprotein (cholesterol) uptake systems (SR-BI and low density lipoprotein receptor). It is likely that this reflects an adaptation aimed to spare cellular cholesterol for adequate membrane formation. Surprisingly in this context, expression of the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase was not affected in megakaryocytes from Abcg5−/− mice, suggesting that cellular cholesterol synthesis was unchanged. High plant sterol levels have previously been reported to be associated with increased Abca1 expression in adrenal glands in Abcg5−/−/Abcg8−/− double knock-out mice due to activation of the liver X receptor (45). However, this clearly does not happen in megakaryocytes, indicating that cholesterol homeostasis is differentially affected by plant sterols in different cell types.

Recent animal studies (Ref. 31 and this study), a case report in a single sitosterolemia patient (30), and a placebo-controlled study in sitosterolemia subjects (29) employing the NPC1L1 inhibitor ezetimibe have demonstrated that interference at the level of intestinal absorption provides a means to lower plasma plant sterols. In the latter study (29), treatment for 8 weeks at a dose of 10 mg/day reduced plasma plant sterols by ∼25%. Within this time frame, platelet numbers in treated subjects increased slightly, but because of the fact that placebo-treated subjects also showed a small increase in platelet count, the between-group difference did not reach statistical significance. No information on platelet size was provided in the previous study. Intriguingly, the 65% reduction in plant sterol levels by ezetimibe in the present study rapidly normalized platelet numbers and strongly reduced their size within 18 days in sitosterolemia mice. This time frame of normalization corresponds well with the estimated turnover of platelets in mice, i.e. 4–5 days (46), which implies that reduction in plant sterol absorption must have resulted in a virtually immediate restoration of megakaryocyte function. Differences in platelet turnover rate between humans (47) and mice (46) as well as the different dosages of ezetimibe employed (∼0.14 mg/kg/day in humans versus ∼0.60 mg/kg/day in mice) may have contributed to the differential outcome of ezetimibe treatment between humans and mice. Interestingly, it was very recently reported that addition of ezetimibe to the “standard” cholestyramine therapy led to a doubling of platelet numbers in a single sitosterolemia patient after 1 year, in addition to a complete disappearance of xanthomata (30). We conclude that although the underlying mechanism remains to be defined, this work establishes that macrothrombocytopenia is a direct consequence of excessively high plant sterol concentrations in sitosterolemia in mice and provides a “physiological rationale” for the existence of a highly effective transporter system in the intestinal tract and liver to prevent accumulation in the body of these ubiquitous diet components.

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**Supplemental Table 1.** Comparison of blood parameters in wild-type and \textit{Abcg5^{-/-}} mice.

* indicates significant difference (Mann-Whitney U tests, P<0.01)

RBC: red blood cell count; MCV: mean cell volume; RDW-SD: red cell distribution width-standard deviation, HB: hemoglobin concentration; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration

| Mouse       | n  | RBC (E12/L) | MCV (fL) | RDW-SD (fL) | HB (mmol/L) | MCH (fmol) | MCHC (mmol/L) |
|-------------|----|-------------|----------|-------------|-------------|------------|--------------|
| Wildtype    | 20 | 8.9±0.7     | 49.1±1.5 | 23.5±1.1    | 8.4±0.7     | 944±27     | 19.3±0.5     |
| \textit{Abcg5^{-/-}} | 20 | 8.7±0.8     | 48.4±0.7 | 26.1±1.6*   | 7.8±0.2*    | 886±12*    | 18.3±0.4*    |
**Supplemental Table 2.** Comparison of plant sterol/stanol levels in red blood cells and platelets isolated from wild-type and *Abcg5*−/− mice.

* indicates significant difference (Mann-Whitney U tests, P<0.05)

Values in µg determined in lipid extracts from 200 µL washed RBC or from platelet isolations on 500 µL plasma; ND, non-detectable (limit 0.01 µg)

|          | RBC                  | Platelets             |
|----------|----------------------|-----------------------|
|          | * Abcg5+/+          | * Abcg5−/−            | * Abcg5+/+          | * Abcg5−/−          |
| Campesterol | 0.93±0.15          | 14.76±3.26*          | ND                  | 0.02±0.01*          |
| Sitosterol   | 0.12±0.01          | 13.99±5.35*          | ND                  | 0.04±0.03*          |
| Brassicasterol | ND                   | 0.49±0.29*          | ND                  | ND                  |
| Stigmastanol   | ND                   | 0.14±0.06*          | ND                  | ND                  |
| Campestanol   | ND                   | 3.45±1.53*          | ND                  | ND                  |
| Sitostanol   | ND                   | 1.02±2.35*          | ND                  | ND                  |
Supplemental Figure 1: Sensitivity of red blood cells from wild-type (open symbols) and Abcg5−/− mice (closed symbols) to hemolytic effects of a mixture of primary bile acids (cholic acid and chenodeoxycholic acid, molar ratio 3:1) (A) and their osmotic fragility (B) were tested exactly as described in references 40 and 41, respectively. Data represent mean values ± SD for 3 independent experiments. Percent hemolysis was calculated by comparing the absorption at 540 nm with that of an identical amount of red blood cells lysed in double-distilled water.
