Cerebral Accumulation of Dietary Derivable Plant Sterols does not Interfere with Memory and Anxiety Related Behavior in Abcg5−/− Mice

Tim Vanmierlo · Kris Rutten · Leonie C. van Vark · van der Zee · Silvia Friedrichs · Vincent W. Bloks · Arjan Blokland · Frans C. Ramaekers · Eric Sijbrands · Harry Steinbusch · Jos Prickaerts · Folkert Kuipers · Dieter Lütjohann · Monique Mulder

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Abstract Plant sterols such as sitosterol and campesterol are frequently applied as functional food in the prevention of atherosclerosis. Recently, it became clear that plasma derived plant sterols accumulate in murine brains. We questioned whether plant sterols in the brain are associated with alterations in brain cholesterol homeostasis and subsequently with brain functions. ATP binding cassette (Abcg5−/− mice, a phytosterolemia model, were compared to Abcg5+/+ mice for serum and brain plant sterol accumulation and behavioral and cognitive performance. Serum and brain plant sterol concentrations were respectively 35–70-fold and 5–12-fold increased in Abcg5−/− mice (P<0.001). Plant sterol accumulation resulted in decreased levels of desmosterol (P<0.01) and 24(S)-hydroxycholesterol (P<0.01) in the hippocampus, the brain region important for learning and memory functions, and increased lanosterol levels (P<0.01) in the cortex. However, Abcg5−/− and Abcg5+/+ displayed no differences in memory functions or in anxiety and mood related behavior. The swimming speed of the Abcg5−/− mice was slightly higher compared to Abcg5+/+ mice (P<0.001). In conclusion, plant sterols in the brains of Abcg5−/− mice did have consequences for brain cholesterol metabolism, but did not lead to an overt phenotype of memory or anxiety related behavior. Thus, our data provide no contra-indication for nutritional intake of plant sterol enriched nutrition.

Keywords Abcg5 · Anxiety · Cholesterol · Cognition · Phytosterolemia and plant sterol

Abbreviations
ABC ATP binding cassette
BBB blood–brain barrier
CVD cardiovascular diseases
DM distance moved
EZM elevated zero maze
LDL low density lipoprotein
LXR liver x receptor
**Introduction**

Consumption of high amounts of plant sterols leads to reduced serum and LDL cholesterol concentrations, making them attractive compounds in the treatment of atherosclerosis and cardiovascular diseases (CVD) [1]. Besides their natural presence in the diet [2], plant sterols are frequently administered as functional food additives in the prevention of atherosclerosis, although no hard endpoint studies are available [3]. Plant sterols, exclusively derived from the diet, differ from cholesterol only by an additional ethyl or methyl group at C24 and/or a double bond at C22 [4]. Cholesterol in the circulation is prevented from entering the brain by the blood–brain barrier (BBB), and all cholesterol within the brain is synthesized in situ.

Unlike dietary cholesterol, plant sterols can accumulate in the brain [5].

**Materials and Methods**

**Animals**

Male Abcg5−/− mice (n=8) and Abcg5+/+ littermates (n=9), generated on a mixed C57BL/6Jx129/OlaHsd background by Deltagen (Redwood City, CA, USA) as described by Plösch et al. [17] were fed a standard laboratory chow diet. Animals had ad libitum access to the diet and water. Mice were subjected to behavioral tasks on an adult age between 7 and 9 months, conform with previous descriptions [18]. The sequence of behavioral tasks was set to perform the least stressful tasks first. Experimental procedures were approved by the local ethical committee of Maastricht University.

**Object Recognition Task**

The object recognition task (ORT) was performed as described previously [18, 19] with following specifications. Following two habituation days, mice were subjected to a first trial (Tr1) in which the arena contained two identical objects. After 4 min exploration, the mouse was placed back into its home cage. 1 h later, the mouse was returned to the arena for the second trial of 4 min (Tr2), now with two dissimilar objects: the familiar object and a new object. The discrimination index d2 in Tr2 was calculated as measure for object memory (d2 = [(exploration time for the familiar object in Tr2) - (exploration time for the novel object in Tr2)]/[total exploration time in Tr2]).

**The Morris Water Escape Maze**

Spatial memory performance was measured in the Morris water escape maze (MWEM) as described previously [20].
In brief, the mice were subjected to a total of 24 acquisition trials: 6 days, 4 trials a day, separated by a 5 min inter-trial interval, using 4 different starting positions assigned in a random order between mice to avoid side bias. A gray platform (diameter 5 cm), submerged beneath the water surface, was located at one fixed position for all mice throughout the experiments. Acquisition time (s), distance moved (cm), and swimming speed (cm/s) were measured (Etho Vision™, Noldus, Wageningen, The Netherlands). 1 h after the final acquisition trial (day 6), mice were subjected to a probe trial in which the platform was removed from the pool. Mice were released into the pool, opposite to the previous platform location and allowed to search for the platform over a period of 60 s. The arena was divided into four quadrants, with the target zone being the quadrant where the platform was previously located.

Elevated Zero Maze

The elevated zero maze (EZM), originally described in Shepherd et al., was made of black, infrared-transparent, plastic [21]. It consists of a circular runway (50 cm in diameter), 6 cm path width, 20 cm above floor level) which was divided equally into two opposite open zones (OZ) and two opposite zones, enclosed with 20 cm high side walls (CZ) [22]. Measurements were performed under almost dark conditions for the open area (1–2 lux). The entrance of each OZ and CZ contains at each side respectively two outer border (BO) and two inner border (BI) areas, covering 3 cm of the running way each. A 3 mm high edge surrounded the open zones to prevent falls. Time spent and distance moved in OZ, BI, BO, and CZ during a 5 min trial were measured via an infrared video camera connected to a video tracking system (Etho Vision™, Noldus, Wageningen, The Netherlands).

Tail Suspension Task

The tail suspension task (TST) is designed to test antidepressant-like activity in mice [23]. The test is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by their tail, will develop an immobile posture [24]. Four cages with closed side walls and an open front were used to test the mice in the TST (18 cm of width and 30 cm of height). Each animal was individually hung by its tail to a hook at the ceiling of the chamber, using adhesive tape. Their movements were recorded for 5 min using a video camera device (Etho Vision™, Noldus, Wageningen, The Netherlands). The behavioral variable “immobility” was defined as no movement or no obvious effort to escape from the hook (cutoff: 15% movement). Total activity versus inactivity is regarded as a measure of antidepressant-like activity of plant sterols.

Open Field

The open field test (OF) was conducted over 20 min as described previously [12]. After a mouse was placed in the centre of the OF, the distance moved (DM) and time spent in each zone (TIZ) were registered (Etho Vision™, Noldus, Wageningen, The Netherlands).

Sterol Profile Determination

Following the behavioral experiments, mice were anaesthetized with IP ketamine/xylazine (100 mg/kg ketamine and 5 mg/kg xylazine). Blood was collected prior to perfusion with ice cold PBS by puncturing the right atrium of the heart. Prior to the sterol analysis the cerebellum, right hippocampus and top part of the neo-cortex (Bregma −0.5 mm to −4 mm) were dissected out and snap frozen.

Samples were spun in a speedvac (12 mbar) (Savant AES 1000) for 24 h in order to relate individual sterol concentrations to dry weight. Cholesterol and non-cholesterol sterol concentrations were extracted and quantified by respectively gas-chromatography-FID and gas-chromatography/mass spectrometry as described previously [25].

Statistics

All statistical analyses were performed using GraphPad Prism 4™. Acquisition times, distance covered and speed in the MWEM were analyzed using the two-way ANOVA. The MWEM probe trial, the OF locations and between region brain sterols were analyzed by one-way ANOVA with a post hoc Bonferroni’s multiple comparison test (indicated as “PH”). An unpaired two-sided Student’s t-test was applied to compare the genotypes in the ORT, OF and sterol (between groups) analyses. d2 values (ORT) were compared to 0 by a one sample Student’s t-test. Animals not reaching the minimum of 5 s exploration in ORT were excluded from analysis [19]. Extreme values were excluded by means of Dixon’s principles of exclusion of extreme values.

Results

Cerebral Sterol Metabolism in ABCG5−/− and ABCG5+/+ mice

Corroborating previous results [5, 17], Abcg5−/− mice on normal chow diet displayed strongly increased serum concentrations of sitosterol, campesterol, and stigmasterol.
(30-, 5- and 70-fold, respectively) in comparison with their wild-type littermates, whereas cholesterol concentrations were significantly decreased (2-fold) (data not shown).

All brain regions of the Abcg5−/− mice, displayed higher concentrations of sitosterol (12-fold), campesterol (7-fold), and stigmasterol (5-fold) than those of Abcg5+/+ mice (Table 1). Both Abcg5+/+ and Abcg5−/− mice display the highest absolute concentrations of sitosterol ($P<0.01$) and campesterol ($P<0.01$) in the cerebellum in comparison with the cortex and the hippocampus (Table 1). Also the ratio of sitosterol and campesterol over cholesterol was highest in the cerebellum in Abcg5−/− mice, but not in Abcg5+/+ mice. No regional absolute or relative differences were found for stigmasterol in Abcg5−/− group. Thus, Abcg5−/− mice on a normal laboratory chow diet were prone to accumulate plant sterols in their brain, in particular in the cerebellum. In Abcg5−/− mice, plant sterol concentrations in the brain were not directly related to their serum concentrations. Abcg5−/− mice displayed serum sitosterol concentrations that were twice as high as campesterol (data not shown), while campesterol concentrations were higher in the brain. Despite overall increases in brain plant sterol concentrations, the concentrations of endogenous sterols were affected to a lesser extent also in the hippocampus, in line with increased concentrations in serum (Table 1). Desmosterol ($P<0.01$) and 24(S)-OHcholesterol ($P<0.01$) concentrations were significantly reduced (to 72% and 68% of the control levels, respectively) exclusively in the hippocampus.

Learning and Memory Functions in Abcg5−/− and Abcg5+/+ Mice

Abcg5−/− and Abcg5+/+ mice displayed no detectable differences in general behavior. To examine possible effects of elevated brain plant sterols on learning and memory functions, Abcg5−/− and Abcg5+/+ littermates were subjected to the ORT and MWEM.

d2 values were significantly higher than 0 for both the Abcg5−/− ($P<0.01$) and Abcg5+/+ mice ($P<0.01$), indicating good memory performance (Fig. 1). Exploration times were comparable (data not shown), whereas the d2 values for Abcg5−/− mice were slightly, but not significantly ($P=0.28$) higher in comparison with the Abcg5+/+ mice (Fig. 1). Hence, increased plant sterol levels in the brain of the Abcg5−/− mice did not result in detectable influences on object memory performance at the 1 h intertrial interval.

Subsequently, both groups were subjected to MWEM. Both the Abcg5−/− and the Abcg5+/+ mice located the platform well (acquisition time: $F(5,90)=31.58$; $P<0.001$ (Fig. 2a) and acquisition distance: $F(5,90)=22.02$; $P<0.001$; data not shown), but there were no significant differences in the acquisition times and distances covered to reach the platform at the different experimental days between the two groups. At day 6, 1 h after the last

### Table 1 Sterol profiles in brain regions of Abcg5−/− and Abcg5+/+ mice on normal chow diet

| Brain   | Abcg5   | HC       | CTX      | CB        |
|---------|---------|----------|----------|-----------|
| Cholesterol (µg/mg) | +/+     | 71.5 (3.4) #  | 61.3 (3.4) ###  | 91.2 (3.6)  |
|         | −/−     | 66.8 (5.9) ## | 63.3 (4.2) ##  | 89.0 (3.6)  |
| Sitosterol (ng/mg)   | +/+     | 35.6 (6.2) *** #  | 32.2 (3.7) *** #  | 61.4 (7.4) *** |
|         | −/−     | 422.9 (71.0) ## | 381.1 (44.3) ##  | 772.7 (91.2) |
| Campesterol (ng/mg)  | +/+     | 71.3 (10.6) *** #  | 60.9 (7.4) *** #  | 143.6 (26.7) *** |
|         | −/−     | 504.2 (82.1) ## | 465.9 (54.3) ##  | 947.6 (109.0) |
| Stigmasterol (ng/mg) | +/+     | 3.7 (0.7) *** ### | 4.2 (0.5) *** #  | 8.4 (0.9) *** |
|         | −/−     | 25.5 (3.8) #  | 23.9 (2.1) #  | 34.4 (2.6)  |
| Lathosterol (ng/mg)  | +/+     | 68.6 (3.3) #  | 70.0 (2.1) #  | 78.2 (5.8)  |
|         | −/−     | 83.1 (18.4) #  | 88.6 (9.0) #  | 87.7 (5.9)  |
| Desmosterol (ng/mg)  | +/+     | 422.0 (14.6) ** §§  | 277.9 (9.0) ###  | 406.6 (30.2) |
|         | −/−     | 305.8 (28.6) #  | 312.1 (19.7) #  | 400.8 (25.4) |
| Lanosterol (ng/mg)   | +/+     | 13.0 (2.0) #  | 12.8 (0.6) #  | 17.8 (1.0)  |
|         | −/−     | 16.1 (2.8) #  | 17.1 (1.1) #  | 17.0 (2.2)  |
| 27-OHchol (ng/mg)    | +/+     | 6.1 (2.0) §  | 1.1 (0.2) §  | 2.7 (0.5)  |
|         | −/−     | 6.7 (1.3) §§ §§ #  | 1.0 (0.16) §§ #  | 2.4 (0.2)  |
| 24-OHchol (ng/mg)    | +/+     | 300.7 (20.0) ** §§ #  | 244.3 (11.4) ###  | 56.6 (4.3)  |
|         | −/−     | 205.8 (16.5) § §§ #  | 266.6 (8.5) ###  | 48.3 (3.3)  |

$HC$ hippocampus, $CTX$ cortex, and $CB$ cerebellum.

$Abcg5^{+/+}$ ($n=7$) $Abcg5^{−/−}$ ($n=8$), mean ± SEM.

$*= $Between group 2 sided unpaired Student’s t-test; # = ANOVA within group Bonferroni post hoc; HC/CTX vs CB; $§= $ANOVA within group Bonferroni post hoc; HC vs CTX. $* , # = P<0.05$; $**, §§, ## = P<0.01$; $*** , §§§ , ### = P<0.001$.
acquisition trial, a 1 h probe trial was performed. Both genotypes spent significantly more time in the target quadrant compared to the other quadrants indicating both groups memorized the prior location of the platform (Fig. 2c). However, since no differences between the two groups were detected, it can be concluded that neither object- nor spatial memory was significantly affected by increased brain plant sterol concentrations. Remarkably, Abcg5−/− (15.03±1.07 cm/s) displayed a significantly higher swimming speed than their wild-type littermates (12.87±0.88 cm/s) ($P<0.001$, Fig. 2b).

Performance of Abcg5−/− and Abcg5+/+ in the EZM, TST and OF

In order to examine the effects of plant sterol accumulation on anxiety- and mood- related behavior, mice were subjected to the elevated zero maze (EZM), tail suspension task (TST), and the open field (OF).

In the EZM, a model for anxiety in rodents, the latency of first entrance of the closed zone ($P=0.35$), time spent in the closed zone ($P=0.24$), open zone ($P=0.71$), inner borders ($P=0.66$) and outer borders ($P=0.52$) as well as the frequency of entrances did not significantly differ between Abcg5−/− and Abcg5+/+ mice (Fig. 3a-c). Furthermore, the TST did not reveal differences in depression related behavior, measured by immobility ($P=0.37$; Fig. 3d). In the OF task both Abcg5−/− ($F(2,20)=14.65$; $P<0.001$) and Abcg5+/+ ($F(2,26)=50.14$; $P<0.001$) mice spent most of their time in the corners and near the walls, whereas the center of the arena was avoided (data not shown). No significant differences were detected between the Abcg5−/− and Abcg5+/+ mice with respect to the time spent in the center ($P=0.14$), the corners ($P=0.54$), or near the walls ($P=0.19$). Although, there was a difference in swimming speed in MWEM, the locomotor activity in the OF task was comparable between the two groups (Abcg5+/+: 8.8±1.2 m versus Abcg5−/−: 7.1±0.5 m).

Discussion

Plant sterols are massively applied as functional nutrition in the prevention of atherosclerosis. It has become clear that plant sterols can accumulate in the brain. Therefore, our observations that accumulation of plant sterols in the brain to a limited extent affected brain cholesterol metabolism, but did not have major effects on memory functions or on anxiety and mood related behavior in Abcg5−/− mice in the applied tasks are of major importance. The Abcg5−/− mice did display an increased swimming speed in the MWEM.

As previously reported, in the absence of the Abcg5 gene, plant sterols accumulated not only in serum, but also in the brain [5]. In addition to Plösch et al. [17] and Jansen et al. [5] who report increased plant sterol levels in serum and total brain hemispheres homogenates respectively, we now demonstrate that campesterol and sitosterol, relative to the local cholesterol content, accumulate predominantly in the
cerebellum of the Abcg5−/− mice. However, the high plant sterol concentrations in the brain were associated with increased changes in cholesterol metabolism particularly in the hippocampus. Lanosterol concentrations were increased in the cortex, and desmosterol- and 24(S)-OHcholesterol concentrations were decreased in the hippocampus. Since we previously found no significant differences in the expression of genes involved in the brain cholesterol homeostasis of Abcg5−/− versus Abcg5+/+ mice on normal chow [5], we examined gene expression in the brains of Abcg5+/+ (n=6) and Abcg5−/− (n=3) mice, further challenged with a high plant sterol diet for a period of three months (data not shown). The expression of the LXR target genes Abca1 (P<0.05) and Abcg1 (P<0.05) were significantly increased in the hippocampus of Abcg5−/− mice in comparison to Abcg5+/+ mice on a high plant sterol diet. However, other LXR responsive genes, Srebplc, Apoe, Apoc1 and LXRα which are known to be significantly upregulated by the synthetic LXR-activator T0901317 [13] remained unaffected (data not shown), suggesting the mechanism for their upregulation is not via the LXR-pathway. Together, these data indicate that elevated plant sterol concentrations in the brain modestly affect cholesterol metabolism in a brain region specific manner, with the strongest effect in the hippocampus.

Our data show that despite the substantially increased brain plant sterol concentrations in the Abcg5−/− mice, neither spatial nor object memory were significantly affected, showing no overt negative influences of plant sterols. In the present study, the ORT was performed using a 1 h inter-trial interval in order to reveal a strong overt phenotype. However, both groups approached ceiling d2 values in the ORT, indicating hardly improvable memory [19]. In parallel to a T0901317 treated Alzheimer mice...
showing improved memory in the ORT, whereas control littermates remained on ceiling levels after T0901317 administration, a beneficial role for plant sterols on memory in neurodegenerative diseases cannot be excluded. In line herewith, patients with familial hypercholesterolemia (FH) on statin therapy in combination with plant sterol supplementation displayed a higher incidence of mild cognitive impairment [26]. In contrast, a recent study showed that 85 weeks of dietary plant sterol supplementation of 43–69 year old statin-treated hypercholesterolemic individuals did not affect cognitive functions [27]. Yet, both studies, however, did not take possible effects of statins on cognition into account [28, 29]. In another study, no correlation between serum sitosterol and campesterol concentrations and cognitive performance in a healthy aging population could be detected, neither at baseline nor after 6 years of follow-up [30]. Although Abcg5−/− mice differ at some points from FH and phytosterolemic patients; e.g. Abcg5−/− mice do not develop premature atherosclerosis [31], they provide a good model to study functional consequences of brain plant sterol accumulation by bearing high serum plant sterol concentrations comparable to FH and phytosterolemic patients.

Although no differences in running speed were recorded in the unforced movement tasks such as the OF and EZM, Abcg5−/− mice displayed a significantly increased swimming speed during the acquisition trials in the MWEM compared with their Abcg5+/+ littermates. However, in contrast, administering extracts of the plant species Leucas inflata Labiatae, containing high concentrations of stigmasterol, significantly reduced performance on a rotarod assay in mice [32]. Therefore, plant sterols might influence other, none CNS related parameters in the MWEM, e.g., thermoregulation, which can affect swimming speed outcome [33].

Decreased serum cholesterol concentrations have been associated with severe depressive behavior [14]. However, the markedly suppressed serum cholesterol concentrations caused by elevated serum plant sterol concentrations in the Abcg5−/− mice did not affect anxiety and mood related behavior as indicated by their performance in the applied tasks. Plant sterols from the plant species, Tilia americana var. mexicana, have been reported to possess anxiolitic properties, with sitosterol as the identified bioactive anxiolytic compound, having a dose dependent sedative response resembling diazepam [34]. Nonetheless, the strongly increased serum and brain sitosterol concentrations in the Abcg5−/− mice were not associated with aberrant behavior in anxiety or mood related tasks. Therefore, our results show no overt effects of strongly elevated brain plant sterol levels on unforced mood or anxiety related behavior in mice. Further studies (e.g., conditioned anxiety tests) may elucidate if other cognitive and emotive domains are similarly unaffected.

Since high concentrations of plant sterols (2.25 g/day) are advised without prescriptions as functional food in the prevention of atherosclerosis, it is of major importance that we demonstrate that even strongly elevated serum and brain plant sterol concentrations in a phytosterolemia mouse model, Abcg5−/− mice, did not result in an overt phenotype in behavioror, e.g., memory, anxiety and mood in the applied behavioral tasks. Therefore, this study does not provide a contra-indication for nutritional intake of plant sterol enriched nutrition.

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Conflicts of Interest There are no actual conflicts of interest.

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