Effects of Phytosterol Supplementation on miR-33a/b Expression in Human Cell Lines

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Article

Effects of Phytosterol Supplementation on miR-33a/b Expression in Human Cell Lines

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Abstract

Background/ Objectives: Phytosterols (Ps), plant-derived bioactive compounds not synthesized by the organism, reduce intestinal cholesterol absorption and, when consumed regularly, lower plasma cholesterol concentrations. MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression and participate in various physiological processes, with their dysregulation being associated with diseases. Among them, miR-33a/b, intronic microRNAs (miRNAs) located within the sterol regulatory element-binding protein 2 and 1 genes (SREBP-2 and -1), respectively, have recently been shown to regulate lipid homeostasis in concert with their host genes. However, there is a scarcity of studies on the interaction between Ps and miRNAs. We aimed at evaluating the effects of Ps on the expression of miR-33a/b and genes related to cholesterol transport (*ABCA1*, *ABCG1*, *NPC1L1*, *ABCG5*, *ABCG8*) in hepatocytes (Hep-G2), enterocytes (Caco-2), and macrophages (THP-1). **Methods:** Hep-G2, Caco-2, and THP-1 cells were treated with β-sitosterol (Ps), cholesterol (Ch), Ps+Ch (25 μM/24 h), or culture medium only (control). Total RNA, including miRNAs, was extracted with TRIzol™ and the expression of miRNAs was analyzed by RT-qPCR using the Poly-A tailing protocol and the 2-ΔΔCt method. Comparisons were made using ANOVA or Kruskal-Wallis ($p < 0.05$). **Results:** Ps increased miR-33a/b in Hep-G2 ($p < 0.001$), while Ch reduced their expression. In THP-1, Ch elevated miR-33a/b ($p < 0.005$) and Ps reduced them, with a concomitant increase in *ABCA1*. In Caco-2, no significant changes were observed. **Conclusions:** Ps distinctly modulate miR-33a/b in hepatocytes and macrophages, suggesting a role in cholesterol homeostasis and reverse cholesterol transport. These findings reinforce the cardioprotective potential of phytosterols.

Keywords: phytosterols; miR-33a; miR-33b; ABCA1; cholesterol transport

1. Introduction

Cardiovascular diseases (CVDs) constitute the leading cause of morbidity and mortality globally, with dyslipidemia, particularly hypercholesterolemia with elevated low-density lipoprotein cholesterol (LDL-C), being one of the main modifiable risk factors [1]. The maintenance of cholesterol homeostasis depends on a complex balance between intestinal absorption, endogenous hepatic synthesis, and reverse cholesterol transport [2]. In this context, dietary interventions that modulate these metabolic pathways have proven to be effective strategies in the primary and secondary prevention of CVDs.

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Phytosterols (Ps) are plant-derived bioactive compounds, structurally analogous to cholesterol, but not endogenously synthesized by the human organism [3]. Their cholesterol lowering effect is attributed to competition with cholesterol for intestinal micelles, resulting in lower absorption and increased fecal excretion, with an average reduction of 7 to 10% in plasma LDL-C levels upon a daily intake of approximately 2 g [4,5]. Studies indicate that, in addition to their classic effect on intestinal absorption, Ps may modulate the expression of genes related to lipid metabolism, including the transporters Niemann-Pick C1-Like 1 (NPC1L1), ABCG5, ABCG8, and ABCA1 [6,7], suggesting an additional role as molecular modulators.

MicroRNAs (miRNAs) emerge as critical epigenetic regulators of lipid metabolism and atherosclerotic progression. They are small non-coding RNAs that modulate gene expression at the post-transcriptional level, acting in processes that include vascular inflammation, lipid homeostasis, and arterial remodeling [8]. Their stability in biological fluids, either free or encapsulated in exosomes, make them potential biomarkers and therapeutic targets in cardiovascular diseases [9]. Alterations in their expression have been associated with the development of atherosclerosis, insulin resistance, and other cardiometabolic disorders [10].

Among the miRNAs implicated in cholesterol metabolism, miR-33a and miR-33b stand out. They are encoded in the introns of the sterol-responsive element-binding regulatory genes (SREBP-2 and SREBP-1, respectively) [11]. These miRNAs repress transporters such as ABCA1 and ABCG1, reducing high-density lipoprotein (HDL) biogenesis and reverse cholesterol transport [12]. Experimental evidence demonstrates that the pharmacological inhibition of miR-33 significantly increases plasma HDL-C levels and induces the regression of atherosclerotic lesions in animal models [13], consolidating it as a potential target for therapeutic interventions.

Although the effects of Ps in reducing cholesterol absorption and the role of miR-33a/b in lipid regulation are well-established, the possible interaction between these bioactive compounds and miRNA modulation remains little explored [6,14]. The elucidation of this mechanism can contribute to the understanding of the cardioprotective effects of Ps and provide support for new therapeutic approaches in CVDs. Thus, the present study aimed to evaluate the effects of phytosterol supplementation on the expression of miR-33a/b and genes related to cholesterol transport in hepatic, intestinal, and macrophage cellular models.

2. Materials and Methods

2.1. Cell Lines

Human cell lines HepG2 (ATCC® HB-8065™, RRID:CVCL_0027), Caco-2 (ATCC® HTB-37™, RRID:CVCL_0025), and THP-1 (ATCC® TIB-202™, RRID:CVCL_0006) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 is a human hepatocellular carcinoma cell line, Caco-2 is a human colorectal adenocarcinoma-derived epithelial cell line, and THP-1 is a human monocytic cell line derived from acute monocytic leukemia. Cell line identity and genetic background information were verified using publicly available databases, including Cellosaurus (https://www.cellosaurus.org), with accession numbers CVCL_0027 (HepG2), CVCL_0025 (Caco-2), and CVCL_0006 (THP-1). All cell lines were handled according to the supplier's recommendations and standard cell culture practices.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hep-G2 and Caco-2) or RPMI-1640 (THP-1), supplemented with 10% to 20% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL), in an incubator at 37 °C with 5% CO_2. After reaching approximately 80% confluence, they were treated with β-sitosterol (Ps), cholesterol (Ch), or a combination of both (Ps+Ch), at a final concentration of 25 µM for 24 hours. The control group received only the vehicle (ethanol, 0.4% v/v). All assays were performed in biological and technical triplicate.

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2.2. RNA Extraction, Quantification, and Quality Control

At the end of the treatment, total RNA, including the microRNA fraction, was extracted using TRIzol™ reagent (Invitrogen, USA), strictly following the manufacturer's instructions. The integrity of the RNA was verified by 1% agarose gel electrophoresis, observing distinct bands corresponding to 28S and 18S rRNAs. Purity and concentration were determined by spectrophotometry (NanoDrop™ 2000, Thermo Scientific), with samples considered adequate if the A260/A280 ratio was between 1.8 and 2.0 and A260/A230 was above 1.8. Only samples with adequate integrity and purity were used in subsequent steps.

2.3. cDNA Synthesis, Gene Selection, and Primer Design

cDNA synthesis for miRNAs was performed using the Poly(A) tailing method (EpiS-cript™, Lucigen), followed by reverse transcription with oligodT primers specific for non-coding RNAs. For mRNAs, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. Genes involved in cholesterol metabolism with proven functional relevance were selected: *ABCA1* and *ABCG1* (cholesterol efflux and reverse cholesterol transport), *NPC1L1* (intestinal absorption), and the transporters *ABCG5* and *ABCG8* (biliary excretion). Specific primers were designed based on validated sequences in Primer-BLAST (NCBI) and miRBase v22, prioritizing products of 80–150 bp (Table 1). As endogenous controls, RNU48 was used for miRNAs and GAPDH for mRNAs. Amplification efficiency was confirmed by standard curves (acceptable between 90% and 110%) and melting curve analysis, ensuring product specificity.

Table 1. Specific primers designed for validated sequences in Primer-BLAST (NCBI) and miRBase v22, prioritizing products of 80–150 bp.

miRNA	Sequence
hsa-miR-33a	5 -'GTG CAT TGT AGT TGC ATT GCA- 3'
hsa-miR-33b	5 -'GTG CAT TGC TGT TGC ATT GC-3'
hsa-miR-21	5 -'TAG CTT ACT AGA CTG ATG TTG A-3'
RNU48	5-'GCA GGG ATG CCA TCA CGC CAG C-3'
Universal primer	5-'AAG CAG TGG TAA CAA CGC AGA GT-3'
ABCA1	Forward: 5-'CAA TGC CCC TCT TCA TGA CT-3'
	Reverse: 5-'TGC AGT GGT GAG ATT GAA GC-3'
ABCG1	Forward: 5-' TCT TGT GCC ATA TTT GAG GGA T-3'
	Reverse: 5-' CTG AGT CAC ACA TGC CCT C - 3'
ABCG5	Forward: 5-' GCG TAG GTC TCC TTT ACC AGT TTG-3'
	Reverse: 5-' GGA AAC AGA TTC ACA GCG TTC A-3'
ABCG8	Forward: 5-' CCA GTA TTT CAC AGC CAT CGG-3'
	Reverse: 5-' GCGAGTGACTGAGCCTTCT-3'
NPC1L1	Forward: 5-' CAC TGG ATC ACT CGA GGT GTT G-3'
	Reverse: 5-' CCA GTC CCA CGC TGA TGT G-3'
GAPDH	Forward: 5-' TGC ACC ACC AAC TGC TTA GC – 3'
	Reverse: 5-' GGC ATG GAC TGT GGT CAT GAG – 3'

2.4. Quantification by RT-qPCR and Statistical Analysis

The expression of miR-33a/b and target genes was quantified in real-time by Quantitative reverse transcription-PCR (RT-qPCR) (StepOnePlus™, Applied Biosystems), using SYBR™ Green PCR Master Mix in 96-well plates. All reactions were performed in trip-licate, including negative controls without template (NTC). Relative expression was calcu-lated using the 2^-ΔΔCt method, using the control group as a calibrator. Data were ex-pressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism v8.0 software, applying one-way ANOVA

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with Tukey's post-test for parametric variables or Kruskal-Wallis followed by Dunn for non-parametric variables. *p*-values < *0.05* were considered statistically significant.

3. Results

3.1. Modulation of the Expression of miR-33a and miR-33b in Different Cell Types

Treatment with phytosterols (Ps) distinctly modulated the expression of miR-33a and miR-33b in the different cell lines. In Hep-G2, a significant increase in the expression of miR-33a and miR-33b was observed in the Ps-treated group compared to the control and the cholesterol (Ch) treated group (p <0.001), while Ch reduced these levels (Figure 1). In the Ps+Ch group, the levels of miR-33a/b were slightly increased compared to the control, but lower than those observed with isolated Ps.

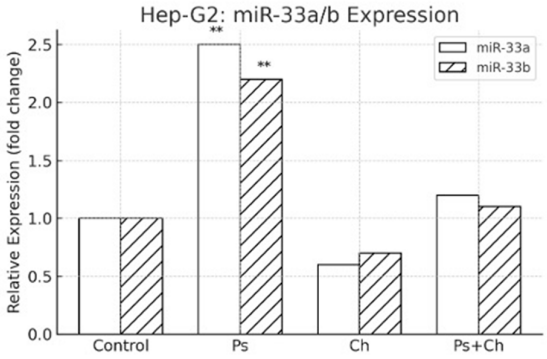

Figure 1. Relative expression of miR-33a and miR-33b in Hep-G2 cells treated for 24 h with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch).Ps significantly increased miR-33a/b expression compared with Ch (p <0.001), whereas Ch reduced their expression. Data are presented as fold change relative to control.

In THP-1 macrophages, treatment with Ch induced a significant increase in the expression of miR-33a and miR-33b (p <0.005), while Ps promoted a significant reduction of these miRNAs compared to the control and the Ch-treated group (Figure 2). The Ps+Ch group showed intermediate expression, suggesting a modulatory effect of Ps on the stimulus induced by cholesterol.

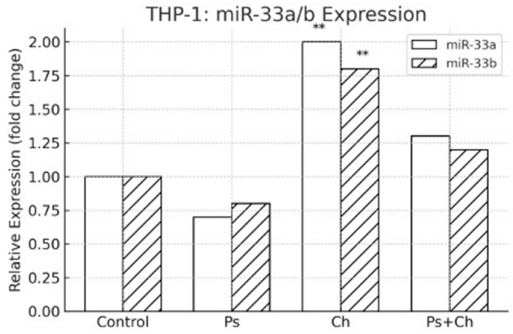

Figure 2. Relative expression of miR-33a and miR-33b in THP-1 cells treated for 24 h with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). Ch significantly increased miR-33a/b levels (p <0.005), whereas Ps reduced their expression. Data are presented as fold change relative to control.

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In the Caco-2 lineage, no significant changes were observed in the expression of miR-33a and miR-33b among the experimental groups (Figure 3), indicating that in enterocytes, the modulation of lipid metabolism genes by Ps may occur predominantly through miRNA-independent pathways.

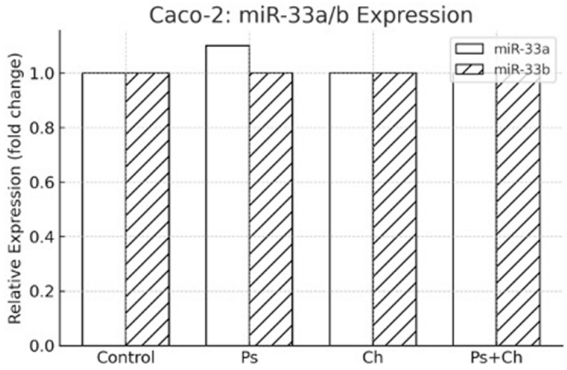

Figure 3. Relative expression of miR-33a and miR-33b in Caco-2 cells treated for 24 h with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). No significant differences were observed among groups. Data are presented as fold change relative to control.

3.2. Expression of Target Genes in Different Cell Types

3.2.1. Expression of ABCA1 in Hep-G2, THP-1, and Caco-2 Cells

Regarding the target genes, the expression of *ABCA1* showed a profile dependent on the cell type. In Hep-G2, Ps slightly reduced *ABCA1* compared to the control, while Ch did not promote significant changes (Figure 4). In THP-1, Ps significantly increased the expression of *ABCA1* (p <0.05), corroborating the reduction of miR-33a/b levels. In Caco-2, Ps promoted a slight increase in the expression of *ABCA1*, without statistical significance.

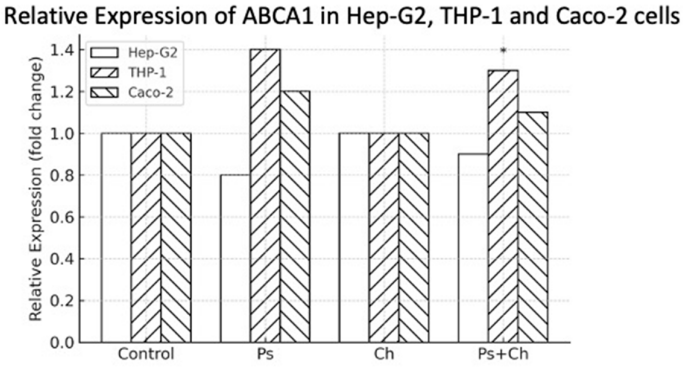

Figure 4. Relative expression of *ABCA1* in Hep-G2, THP-1, and Caco-2 cells after 24 h treatment with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). Ps slightly reduced *ABCA1* expression in Hep-G2, significantly increased it in THP-1 (p <0.05), and caused a mild increase in Caco-2. Data are presented as fold change relative to control.

3.2.2. Expression of ABCG1 in THP-1 Cells

The expression of *ABCG1* showed a trend towards increase in THP-1 treated with Ps, although without statistical significance (Figure 5).

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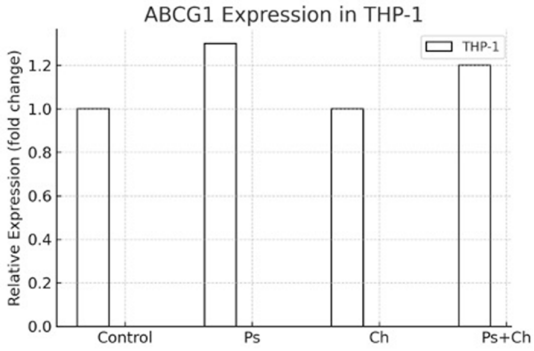

Figure 5. Relative expression of *ABCG1* in THP-1 cells after 24 h treatment with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). No statistically significant differences were detected among treatments.

3.2.3. Expression of NPC1L1 in CaCo-2 and Hep-G2 Cells

For the *NPC1L1* gene, a significant reduction was observed in Caco-2 treated with Ps+Ch (p <0.01) and in Hep-G2 treated with Ps and Ch (p <0.05 for both), suggesting a direct effect of Ps in decreasing cholesterol uptake (Figure 6).

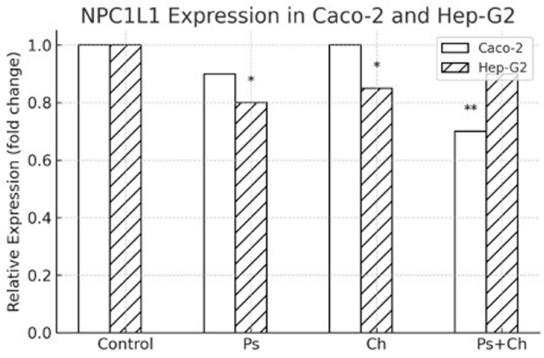

Figure 6. Relative expression of *NPC1L1* in Caco-2 and Hep-G2 cells after 24 h treatment with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). Significant reduction was observed in Caco-2 treated with Ps+Ch (p <0.01) and in Hep-G2 treated with Ps (p <0.05) and Ch (p <0.05).

3.2.4. Expression of ABCG5 in CaCo-2 and Hep-G2 Cells

The expression of *ABCG5* was significantly reduced in Caco-2 treated with Ps (p <0.05) and Ch (p <0.01), while in Hep-G2, the changes were slight and not statistically significant (Figure 7).

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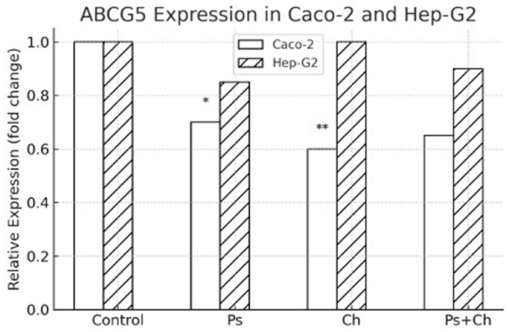

Figure 7. Relative expression of *ABCG5* in Caco-2 and Hep-G2 cells after 24 h treatment with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). Significant downregulation was observed in Caco-2 with Ps ($p < 0.05$) and Ch ($p < 0.01$), whereas Hep-G2 showed no significant differences.

3.2.5. Expression of ABCG8 in CaCo-2 and Hep-G2 Cells

Finally, *ABCG8* showed no significant differences in any of the analyzed cell lines (Figure 8).

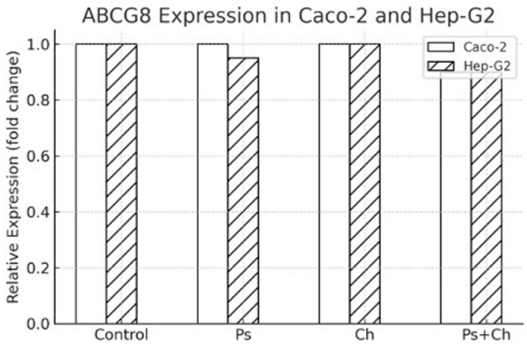

Figure 8. Relative expression of *ABCG8* in Caco-2 and Hep-G2 cells after 24 h treatment with phytosterol (Ps), cholesterol (Ch), and phytosterol + cholesterol (Ps+Ch). No statistically significant differences were observed.

These results indicate that Ps modulate the expression of both miR-33a/b and genes related to cholesterol transport in a cell type-dependent manner, highlighting the increase of *ABCA1* expression in macrophages, improving cholesterol efflux from arterial wall, and the reduction of *NPC1L1* expression in enterocytes, reflecting less cholesterol absorption.

4. Discussion

The results of this study demonstrate that phytosterols (Ps) distinctly modulate the expression of miR-33a/b and genes related to cholesterol transport in different cell types, suggesting specific and complementary mechanisms in the regulation of lipid homeostasis.

In Hep-G2, the significant increase of miR-33a/b observed with Ps supports the hypothesis that, in hepatocytes, the concomitant activation of the host genes *SREBP-2* and *SREBP-1* may induce the expression of these miRNAs under conditions of lower intracellular cholesterol availability [11–13]. This response aligns with studies that demonstrated co-expression of miR-33 with *SREBP-2* under cholesterol depletion conditions [14,15,19]. The slight reduction of *ABCA1* in these same groups reinforces the inverse relationship between miR-33 and its targets, as previously described [15,16].

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In THP-1, the reduction of miR-33a/b and the significant increase of *ABCA1* after treatment with Ps indicate a possible stimulus to reverse cholesterol transport (RCT). This finding is consistent with studies showing that miR-33 inhibition increases *ABCA1* expression and promotes cholesterol efflux in macrophages, favoring HDL formation and regression of atherosclerotic lesions [17,18,21]. The opposite modulation observed with Ch, which elevated miR-33a/b, reinforces the atheroprotective role of Ps by antagonizing the pro-atherogenic effect of cholesterol in macrophages.

In Caco-2, the absence of significant changes in miR-33a/b suggests that the regulation of Ps in the intestine occurs predominantly through direct modulation of transporters. The significant reduction of *NPC1L1* and *ABCG5* in the enterocytes corroborates previous findings that Ps decrease cholesterol uptake and increase intestinal cholesterol excretion independently of miRNA-mediated epigenetic pathways [6,7,25,26]. These effects are clinically relevant, as NPC1L1 is the main transporter responsible for intestinal cholesterol absorption and a target of pharmacological therapies such as ezetimibe.

Regarding hepatic transporters, the absence of significant differences in *ABCG5/8* gene expression suggests that Ps, in the model used, exert a more pronounced effect on intestinal uptake and macrophage efflux than on biliary excretion. This response may be related to the exposure time or concentration of Ps, which requires further investigation.

The set of findings reinforces the hypothesis that Ps exert complementary effects on cholesterol homeostasis: in hepatocytes, possibly acting on synthesis and intracellular regulation via miR-33; in macrophages, promoting cholesterol efflux through the increase of *ABCA1* gene expression and reduction of miR-33; and in enterocytes, reducing intestinal absorption via *NPC1L1*. These mechanisms may explain, at least in part, the cardioprotective effects of Ps observed in clinical studies [22,23].

The results of this study corroborate evidence that phytosterols (Ps) specifically and cell type-dependently modulate crucial genes in cholesterol homeostasis, including *ABCA1*, *ABCG1*, *NPC1L1*, and *ABCG5/8*. Classical studies have shown that Ps inhibit SREBP-2 processing, reducing hepatic cholesterol synthesis [24]. Considering the central role of NPC1L1 in intestinal cholesterol absorption, the significant reduction of this transporter in Caco-2 and Hep-G2 treated with Ps aligns with findings describing beneficial effects on lowering plasma cholesterol levels through modulation of this protein [25,26]. Although the expression of miR-33 has been related to the repression of Niemann-Pick C1 (NPC1) protein in human cells [27], there is still no direct evidence linking miR-33 to the regulation of NPC1L1, suggesting that the action of Ps on this transporter occurs predominantly through miRNA-independent pathways.

The heterodimer transporters ABCG5 and ABCG8, expressed at the brush border of enterocytes and in the canalicular membranes of hepatocytes [28], are crucial for intestinal cholesterol efflux and biliary excretion of phytosterols [29]. The expression of these transporters is regulated by multiple transcription factors, including nuclear receptors such as FXR and, primarily, LXRα, the major transcriptional regulator of ABCG5/8 [30,31]. Considering that Ps act as natural ligands for LXR [32], an increase in the expression of these transporters would be expected. However, in this study, a slight reduction in the expression of *ABCG5* and *ABCG8* was observed in Caco-2 and Hep-G2 treated with Ps, which may reflect a regulatory effect dependent on cell type, exposure time, or concentration of the compound.

The transporters ABCA1 and ABCG1 play complementary roles in reverse cholesterol transport: ABCA1 promotes cholesterol efflux to apoA-I and the formation of nascent HDL particles, while ABCG1 facilitates the transfer of cholesterol to mature HDL particles [33,34]. The inverse relationship between miR-33 and these transporters has been previously demonstrated in cellular models [35] and was also observed in this study, in which THP-1 treated with Ps showed a reduction of miR-33a/b associated with a significant increase of ABCA1. Experimental inhibition of miR-33 has been associated with increased cholesterol efflux and elevated HDL levels [36], while its overexpression in macrophages is associated with endoplasmic reticulum stress and lipid accumulation [37]. Indeed,

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the suppression of miR-33 results in increased expression of *ABCA1* and *ABCG1*, preventing foam cell formation and reducing plaque progression [6,38].

Therefore, the findings of this study reinforce that Ps act at multiple levels of lipid metabolism: (i) inhibiting intestinal cholesterol absorption via reduction of NPC1L1; (ii) stimulating reverse transport in macrophages through increased ABCA1 and potentially ABCG1; and (iii) discretely modulating ABCG5/8, possibly in response to LXR-dependent mechanisms. This integrated action contributes to the cardioprotective effects attributed to Ps and suggests that their combination with epigenetic modulators, such as miR-33 inhibition, may represent a therapeutic strategy.

Finally, some limitations should be considered. The in vitro model does not fully replicate the physiological environment, and the observed effects may be concentration-dependent and time of exposure to Ps. In vivo studies, as well as clinical trials, are needed to confirm the proposed mechanisms and explore the potential of Ps as epigenetic modulators in the context of atherosclerosis.

5. Conclusions

The results of this study demonstrate that phytosterols (Ps) modulate the expression of miR-33a/b and genes involved in cholesterol homeostasis in a cell type-dependent manner, with a role in cholesterol homeostasis and reverse cholesterol transport. These findings reinforce the role of Ps as cardioprotective agents, as they act complementarily in the synthesis, efflux, and absorption of cholesterol. Furthermore, Ps may exert epigenetic effects by modulating the expression of miRNAs related to lipid metabolism.

Author Contributions: Conceptualization, C.M.M., M.C.I., and M.G.C.; methodology, C.M.M., M.C.I., and M.G.C.; validation, C.M.M., and M.C.I.; formal analysis, C.M.M, V.A.M., and M.C.I.; investigation, C.M.M.; resources, A.M.F.N.; data curation, C.M.M., M.C.I., and M.G.C.; writing—original draft preparation, C.M.M., F.A.H.F., V.A.M., A.G., and M.C.I.; writing—review and editing, C.M.M., M.C.I., F.A.H.F., V.A.M., A.G. and A.M.F.N.; visualization, A.G. and M.C.I.; supervision, M.C.I.; project administration, M.C.I.; funding acquisition, A.M.F.N. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

2-ΔΔCt	2 to the power of minus delta delta Ct

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ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
AHA	American Heart Association
ApoA-I	Apolipoprotein A-I
CaCo-2	Human colon carcinoma cell line
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HepG-2	Human hepatic carcinoma cell line
HDL-C	High-Density Lipoprotein Cholesterol
HMG-CoA	Hydroxymethylglutaryl Coenzyme A
LCAT	Lecithin:cholesterol acyltransferase
LDL-C	Low-Density Lipoprotein Cholesterol
LXRα	Liver X Receptor alpha
miRNA	microRNA
NPC1L1	Niemann-Pick C1-Like 1
Ps	Phytosterols
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RNA	Ribonucleic Acid
SREBP	Sterol regulatory element-binding protein
THP-1	Human monocytic leukemia cell line

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