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Brief Report

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Scavenger Receptor BI Deficiency in Mice Is Associated With Plasma Ceramide and Sphingomyelin Accumulation and a Reduced Cholesteryl Ester Fatty Acid Length and Unsaturation Degree

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Conflict of Interest
The authors have no conflicts of interest to declare.

ABSTRACT

Objective: Scavenger receptor class B type I (SR-BI) is primarily known for its role in the selective uptake of cholesteryl esters (CEs) from high-density lipoproteins (HDLs). Here we investigated whether SR-BI deficiency is associated with other potentially relevant changes in the plasma lipidome than the established effect of HDL-cholesterol elevation.
Methods: Targeted ultra-high-performance liquid chromatography-tandem mass spectrometry was utilized to measure lipid species in plasma from female wild-type and SR-BI knockout mice.
Results: SR-BI deficiency was associated with a reduction in the average CE fatty acid length (−2%; $p<0.001$) and degree of CE fatty acid unsaturation (−18%; $p<0.001$) due to a relative shift from longer, polyunsaturated CE species CE (20:4), CE (20:5), and CE (22:6) towards the mono-unsaturated CE (18:1) species. Sphingomyelin (SM) levels were 64% higher ($p<0.001$) in SR-BI knockout mice without a parallel change in (lyso)phosphatidylcholine (LPC) concentrations, resulting in an increase in the SM/LPC ratio from 0.102±0.005 to 0.163±0.003 ($p<0.001$). In addition, lower LPC lengths (−5%; $p<0.05$) and fatty acid unsaturation degrees (−20%; $p<0.01$) were detected in SR-BI knockout mice. Furthermore, SR-BI deficiency was associated with a 4.7-fold increase ($p<0.001$) in total plasma ceramide (Cer) levels, with a marked >9-fold rise ($p<0.001$) in Cer (d18:1/24:1) concentrations.
Conclusion: We have shown that SR-BI deficiency in mice not only impacts the CE concentrations, length, and saturation index within the plasma compartment, but is also associated with plasma accumulation of several Cer and SM species that may contribute to the development of specific hematological and metabolic (disease) phenotypes previously detected in SR-BI knockout mice.

Keywords: Scavenger receptor BI; Ceramides; Sphingolipids; Cholesteryl esters; Lipids

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceptualization: Hoekstra M, Zhang Z; Formal analysis: Hoekstra M, Zhang Z; Methodology: Zhang Z, Lindenburg PW; Writing - original draft: Hoekstra M; Writing - review & editing: Lindenburg PW, Van Eck M.

INTRODUCTION

Scavenger receptor class B type I (SR-BI/SCARB1) is an important player in the maintenance of total body homeostasis as judged from the observation that genetic disruption of its functionality is associated with multiple pathologies. Human heterozygous carriers of the loss-of-function P297S mutation in the SCARB1 gene exhibit increased plasma high-density lipoprotein (HDL)-cholesterol levels, an altered platelet morphology and function, and a diminished adrenal steroidogenic capacity.[1] The presence of the human SCARB1 P376L variant, that impairs posttranslational processing of SR-BI, is also associated with relatively high plasma HDL-cholesterol levels as well as an increased susceptibility for the development of atherosclerotic cardiovascular disease.[2] SR-BI deficiency in mice similarly associates with plasma HDL-cholesterol accumulation, adrenal glucocorticoid insufficiency, an increased atherosclerosis susceptibility, and thrombocytopenia.[3-9] Furthermore, SR-BI knockout mice are anemic and display a diminished epidermal skin barrier function.[8,10]

SR-BI is predominantly known for its essential role in the selective uptake of cholesteryl esters (CEs) from HDL, i.e. the removal of HDL-associated CEs from the plasma compartment without parallel cellular uptake of the HDL protein component.[11] However, our recent findings have revealed that total body SR-BI deficiency in mice is also associated with marked changes in ceramide (Cer) and fatty acid levels within the skin.[10] *In vitro* studies have suggested that SR-BI is able to facilitate the cellular uptake of Cers and fatty acid as well as phosphatidylcholine (PC) and sphingomyelin (SM) via both selective and non-selective mechanisms.[12-15] Notably, Cers and fatty acids are bioactive molecules involved in a variety of physiological processes, including cellular and tissue metabolism, cell growth, migration, differentiation, and senescence.[16,17] It can therefore be hypothesized that total body SR-BI deficiency is associated with other significant changes in the plasma lipidome than the established effect on HDL-cholesterol levels that may potentially also contribute to the development of the different (disease) phenotypes detected in SR-BI deficient human subjects and mice. To demonstrate this hypothesis, in this study we quantified the state-of-the-art lipid analysis level of CEs, Cers, (lyso)phosphatidylcholines (LPCs), and SMs in plasma samples obtained from SR-BI knockout mice.

MATERIALS AND METHODS

1. Mice

Age-matched C57BL/6 wild-type mice (n=5) and SR-BI knockout mice (n=5) were obtained through in-house breeding and fed a regular chow, low-fat diet ad libitum. Female mice were specifically used for this study as it has become evident from previous experiments that SR-BI deficiency-associated pathologies such as the increased susceptibility to atherosclerotic lesion development and higher bone formation can only be detected in female SR-BI knockout mice and/or are more extremely present in female mice than in their male counterparts.[9,18,19] At 12 weeks of age, mice were anesthetized with a mix of xylazine (70 mg/kg), ketamine (350 mg/kg), and atropine (1.8 mg/kg) and sacrificed by orbital exsanguination. Orbital blood was collected in ethylenediaminetetraacetic acid-containing tubes and centrifuged for 10 minutes at 6,000 rpm to acquire plasma. Plasma samples were stored at −20°C and left otherwise untouched until they were utilized for lipid profiling. The experiment was performed in accordance with the ARRIVE guidelines and approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie voor

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Dierproeven) according to the Dutch Law on laboratory animal experimentation and the EU Directive 2010/63/EU.

2. Plasma lipid profiling

Targeted metabolomic analysis was performed using previously published standard operating procedures.[20-23] Ten microliters of each individual plasma sample were spiked with calibration and internal standards and extracted using isopropyl alcohol. Samples were analyzed using an ACQUITY ultra-high-performance liquid chromatography system (Waters Chromatography Europe BV) coupled to a quadrupole time-of-flight (SCIEX Triple TOF 6600) for the presence of the CE, Cer, LPC, and SM species displayed in **Table 1**. Quality control (QC) procedures were used to monitor the data acquisition quality by adding QC samples that were prepared by pooling equal volumes of each study sample. An in-house developed tool, mzQuality, was used to correct for batch effects and highlight metabolites with high technical noise, calculated as the relative standard deviation observed in the QC samples (QC RSD).

3. Statistical analysis

Statistical analysis was performed using Graphpad Prism software (http://www.graphpad.com). Data were log-transformed before subsequent statistical analysis in order to obtain a normal data distribution. The significance of differences was calculated using a two-tailed unpaired *t*-test or two-way analysis of variance with Bonferroni post-test where appropriate. Probability values less than 0.05 were considered significant.

RESULTS

Five CE variants out of the six species measured could be reliably detected, i.e. with a QC RSD value <15, in plasma samples obtained from female normolipidemic C57BL/6 wild-type mice and the age-matched female SR-BI knockout mice. In accordance with previous findings of Furbee et al.,[24] CE (18:2) and CE (20:4) were the most highly present CE species found in plasma obtained from wild-type mice (**Fig. 1A**). SR-BI deficiency was associated with a significant reduction in the average CE fatty acid length (−2%; $p<0.001$; **Fig. 1B**), favoring 18

Table 1. Individual species included in the targeted plasma lipid profiling

CEs	SMs	LPCs			Cers
CE (18:1)	SM (d18:1/16:1)	LPC (14:0)	PC (34:2)	PC (40:6)	Cer (d18:0/22:0)
CE (18:2)	SM (d18:1/18:0)	LPC (16:0)	PC (34:3)	PC (40:7)	Cer (d18:0/23:0)
CE (18:3)	SM (d18:1/18:1)	LPC (16:1)	PC (34:4)	PC (40:8)	Cer (d18:0/24:0)
CE (20:4)	SM (d18:1/18:2)	LPC (18:0)	PC (36:1)	PC (O-34:1)	Cer (d18:1/16:0)
CE (20:5)	SM (d18:1/20:0)	LPC (18:1)	PC (36:1)	PC (O-34:2)	Cer (d18:1/18:0)
CE (22:6)	SM (d18:1/20:1)	LPC (18:2)	PC (36:2)	PC (O-34:3)	Cer (d18:1/20:0)
	SM (d18:1/21:0)	LPC (18:3)	PC (36:3)	PC (O-36:2)	Cer (d18:1/22:0)
	SM (d18:1/22:0)	LPC (20:3)	PC (36:4)	PC (O-36:3)	Cer (d18:1/22:1)
	SM (d18:1/22:1)	LPC (20:4)	PC (36:5)	PC (O-36:5)	Cer (d18:1/23:0)
	SM (d18:1/23:0)	LPC (20:5)	PC (36:6)	PC (O-36:6)	Cer (d18:1/24:0)
	SM (d18:1/23:1)	LPC (22:6)	PC (38:2)	PC (O-38:4)	Cer (d18:1/24:1)
	SM (d18:1/24:0)	LPC (O-16:0)	PC (38:3)	PC (O-38:5)	
	SM (d18:1/24:1)	LPC (O-16:1)	PC (38:4)	PC (O-38:6)	
	SM (d18:1/24:2)	LPC (O-18:1)	PC (38:5)	PC (O-38:7)	
	SM (d18:1/25:0)	PC (32:0)	PC (38:6)	PC (O-40:6)	
	SM (d18:1/25:1)	PC (32:1)	PC (38:7)	PC (O-42:6)	
		PC (32:2)	PC (40:4)	PC (O-44:5)	
		PC (34:1)	PC (40:5)		

CE, cholesteryl ester; SM, sphingomyelin; LPC, (lyso)phosphatidylcholine; Cer, ceramide; PC, phosphatidylcholine.

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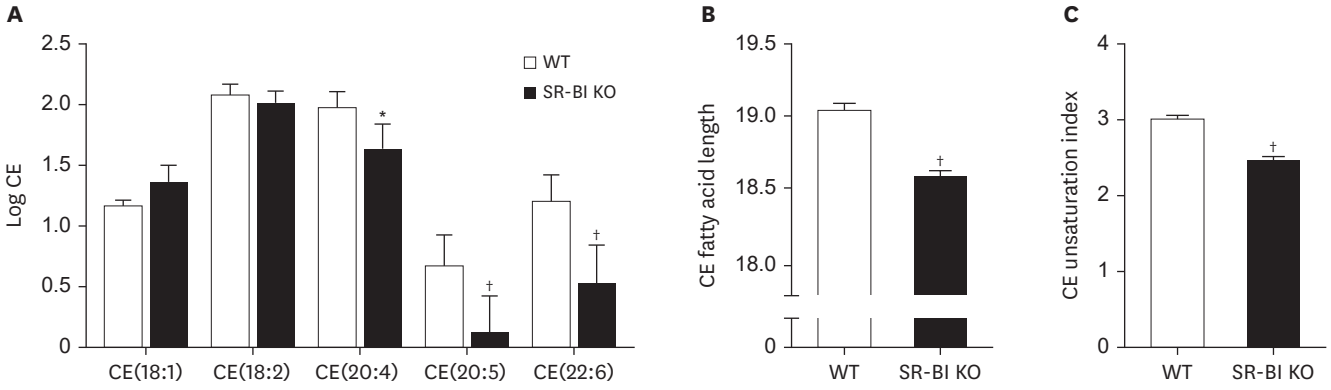

Fig. 1. Effect of SR-BI deficiency on the plasma CE pool. Levels of individual CE species (A) and average CE fatty acid lengths (B) and unsaturation indices (C) in plasma from female WT mice (n=5; white bars) and age-matched female SR-BI KO mice (n=5; black bars).
CE, cholesteryl ester; WT, wild-type; SR-BI KO, scavenger receptor class B type I knockout.
*p<0.05, †p<0.001 vs. WT.

carbon over 20 and 22 carbon fatty acid CE species. As depicted in **Fig. 1C**, SR-BI deficiency was also associated with a reduced degree of CE fatty acid unsaturation (–18%; p<0.001), given that concentrations of longer, polyunsaturated CE species CE (20:4), CE (20:5), and CD (22:6) were significantly reduced in the context of a minor increase in mono-unsaturated CE (18:1) levels (**Fig. 1A**).

Earlier findings from Lee et al.[25] have suggested that loss of SR-BI functionality is associated with an enrichment of HDL particles with SM, leading to an increase in the plasma SM/PC ratio. In accordance, in our experimental setting we also found plasma total SM levels to be significantly elevated (+64%; p<0.001; **Fig. 2A**) in response to genetic SR-BI deficiency, without a parallel change in LPC concentrations (**Fig. 3A**). As a result, the plasma SM/LPC ratio increased from 0.102±0.005 in wild-type mice to 0.163±0.003 in SR-BI knockout mice (p<0.001). The increase in total SM levels associated with SR-BI deficiency could be attributed to accumulation of SM (d18:1/14:0) (+29%; p<0.05), SM (d18:1/16:0) (+66%; p<0.001), SM (d18:1/18:0) (+78%; p<0.001), SM (d18:1/18:2) (+50%; p<0.001), SM (d18:1/22:0) (+48%; p<0.001), SM (d18:1/24:1) (+85%; p<0.001), and SM (d18:1/24:2) (+64%; p<0.001) within the plasma compartment (**Fig. 2D**). In contrast, plasma levels of SM(d18:1/25:1) were relatively decreased in SR-BI knockout mice as compared to those found in wild-type mice (−44%; p<0.001) (**Fig. 2D**). Overall, the average SM fatty acid length (**Fig. 2B**) and saturation index (**Fig. 2C**) were not influenced by the SR-BI genotype. In contrast, since levels of PC (O-42:6), PC (O-44:5) and various highly unsaturated PC38 and PC40 species were significantly lower and those of LPC (O-16:0), LPC (O-18:1), and PC32 and PCO34 variants higher in plasma from SR-BI knockout mice as compared to that of wild-type mice (**Fig. 3D**), SR-BI deficiency was associated with an average lower LPC length (−5%; p<0.05; **Fig. 3B**) and degree of fatty acid unsaturation (−20%; p<0.01; **Fig. 3C**).

All 11 Cer species available on the analysis platform could be readily detected in the plasma samples from our wild-type and SR-BI knockout mice (QC RSD range: 3.9–7.8). However, as evident from **Fig. 4A**, SR-BI deficiency was associated with a marked 4.7-fold increase (p<0.001) in total plasma Cer levels. Apart from those of Cer (d18:1/22:1), whose levels remained unaffected, amounts of the individual Cer variants were increased by 1.9-fold, i.e. Cer (d18:1/18:0), or more in plasma from SR-BI knockout mice as compared to that of wild-type mice (**Fig. 4D**). Notably, a striking >9-fold rise (p<0.001) in the plasma concentration

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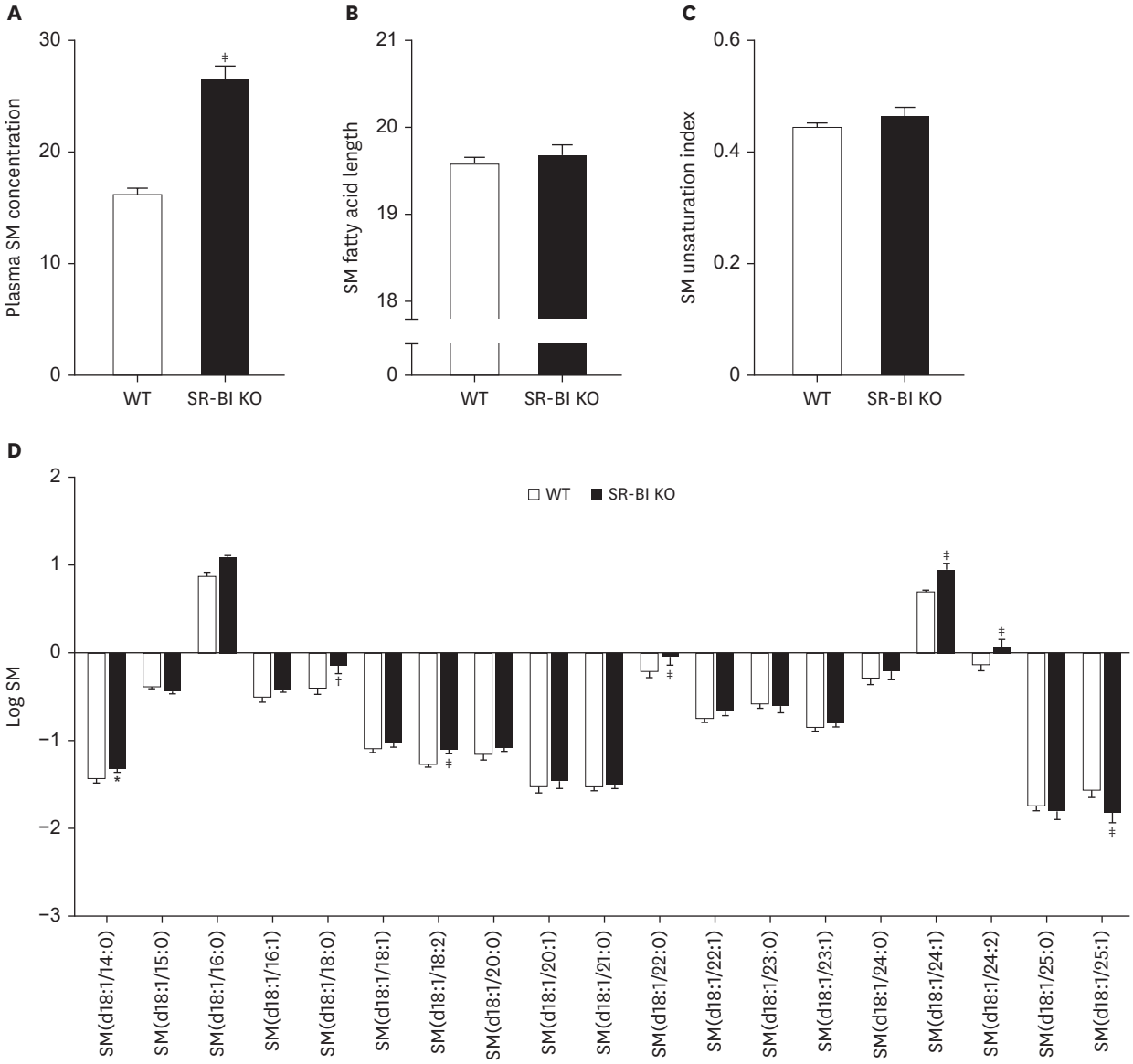

Fig. 2. Effect of SR-BI deficiency on the plasma SM pool. Total SM levels (A), average SM fatty acid lengths (B), unsaturation indices (C), and levels of individual SM variants (D) in plasma from female WT mice (n=5; white bars) and age-matched female SR-BI KO mice (n=5; black bars) (D).
SM, sphingomyelin; WT, wild-type; SR-BI KO, scavenger receptor class B type I knockout.
*p<0.05, †p<0.01, ‡p<0.001 vs. WT.

of the highly present unsaturated Cer species Cer (d18:1/24:1) was detected in response to the genetic lack of SR-BI (**Fig. 4D**). As a result, the average degree of fatty acid unsaturation within the Cer pool was increased by 71% (p<0.001) (**Fig. 4C**), whilst also a minor, but significant increase in the Cer fatty acid length was detected (+3%; p<0.001; **Fig. 4B**).

DISCUSSION

In the current study we tested the hypothesis that total body SR-BI deficiency is associated with additional pathological changes in the plasma lipidome than the firmly established cholesterol raising effect. We have shown that scavenger receptor BI deficiency in mice is

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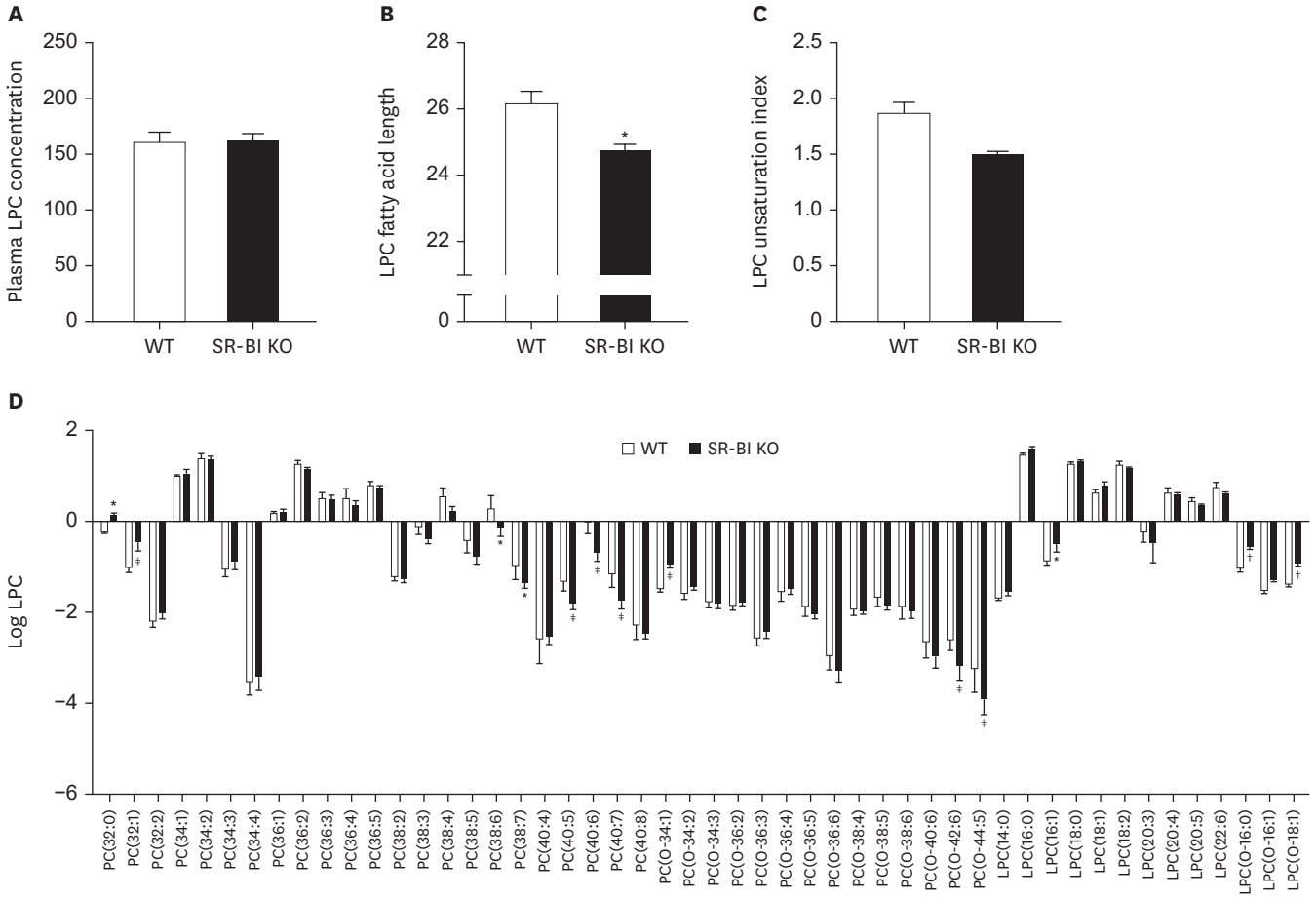

Fig. 3. Effect of SR-BI deficiency on the plasma LPC pool. Total LPC levels (A), average LPC fatty acid lengths (B) and unsaturation indices (C), and levels of individual LPC variants (D) in plasma from female WT mice (n=5; white bars) and age-matched female SR-BI KO mice (n=5; black bars) (D).
LPC, (lyso)phosphatidylcholine; WT, wild-type; SR-BI KO, scavenger receptor class B type I knockout; PC, phosphatidylcholine.
*p<0.05, †p<0.01, ‡p<0.001 vs. WT.

associated with plasma Cer and SM accumulation and a reduced CE fatty acid length and unsaturation degree.

The observation that SR-BI deficiency is associated with a reduction in the CE length and unsaturation index concurs with the earlier finding from Lee et al.[25] that the activity of the CE (20:4) and CE (22:6)-producing enzyme lecithin-cholesterol acyltransferase (LCAT) is decreased in SR-BI knockout mice. Thacker et al.[26] have shown that normalization of LCAT functionality in SR-BI knockout mice through transgenic LCAT overexpression is able to reverse the SR-BI deficiency associated decrease in the circulating blood platelet concentration and increase in atherosclerosis susceptibility. Given that findings from Martins Cardoso et al.[10] have suggested that the accumulation of CE (18:1) is likely underlying the SR-BI deficiency-associated change in skin lipid levels and skin barrier disruption, it will be of interest to investigate whether skin morphology and functionality is also normalized in LCAT overexpressing SR-BI knockout mice.

The combined accumulation of SMs and Cers in the plasma compartment of SR-BI knockout mice fuels the notion, derived from *in vitro* studies by Urban et al.[27] and Li et al.,[12] that SR-BI is an important player in the cellular uptake of (HDL particle-associated) SMs and Cers. *In*

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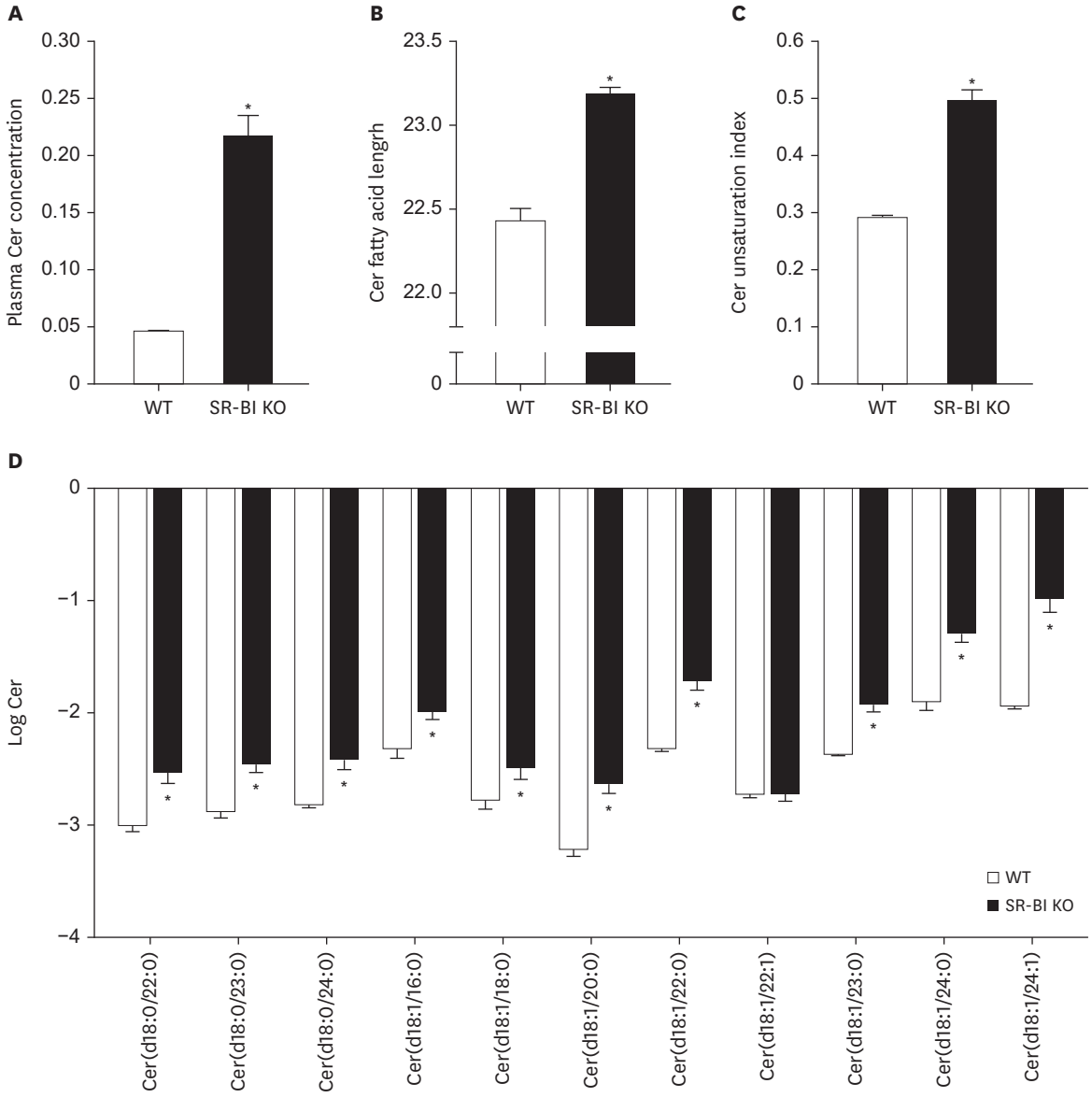

Fig. 4. Effect of SR-BI deficiency on the plasma Cer pool. Total Cer levels (A), average Cer fatty acid lengths (B) and unsaturation indices (C), and levels of individual Cer variants (D) in plasma from female WT mice (n=5; white bars) and age-matched female SR-BI KO mice (n=5; black bars).
Cer, ceramide; WT, wild-type; SR-BI KO, scavenger receptor class B type I knockout.
*p<0.001 vs. WT.

vitro studies have suggested that only a limited amount of SM is selectively taken up into cells by SR-BI.[14] However, since levels of SM (d18:1/24:1) are substantially elevated in plasma of SR-BI knockout mice (+85% vs. wild-type plasma), we anticipate that the relative rates of selective and non-selective lipid uptake may differ between *in vitro* cultured cells and the *in vivo* setting. In this context, it good to appreciate that findings from Tsukamoto et al.[28] have already highlighted that significant differences exist in the mechanism behind PDZ domain containing adaptor protein PDZK1-dependent expression of SR-BI (and associated CE uptake) between cultured cells and hepatocytes *in vivo*.

High levels of Cers have previously been associated with induction of oxidative stress i.e. the production of reactive oxygen species.[29] Cer accumulation may therefore contribute to the

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increased oxidative stress status previously detected in SR-BI knockout mice.[30] In addition, increased Cer-induced eryptosis, i.e. Cer-mediated changes in membrane permeability and concomitant suicidal death of erythrocytosis,[31] can be considered a potential driver of the SR-BI deficiency-associated increase in erythrocyte turnover and anemia development.[8] Given that findings from Yamaguchi et al.[32] have suggested that a higher erythrocyte SM content is associated with an increased hypotonic hemolysis rate, the plasma SM accumulation may potentially also contribute to the SR-BI deficiency-associated anemia development. Studies using SM synthase 2 deficiency platelets have indicated that SMs facilitate the aggregation of platelets in response to thrombin and collagen.[33] Plasma SM accumulation in SR-BI knockout mice would thus be expected to stimulate platelet aggregation. However, Simon and Gear[34] have shown—using isolated human platelets—that the cell-permeable short-chain analogue C2-Cer causes the formation of irregular pseudopodia, leading to a diminished platelet aggregation potential. We therefore anticipate that the previously observed decrease in the ability of SR-BI knockout platelets to aggregate[1,7,35] can be attributed to the fact that the relative impact of SR-BI deficiency on total plasma levels of SMs/platelet activators (1.6-fold) is much less pronounced than that on plasma levels of Cers/platelet activity inhibitors (4.7-fold). Studies in rats have shown that an increase in hepatic Cer stores is a phenomenon rapidly detected in response to infusion with lard oil.[36] An impaired ability of SR-BI deficient hepatocytes to take up Cers may potentially also underlie the protection of total body SR-BI knockout mice against high fat (lard) diet-induced fatty liver development.[37]

An interesting observation in our study was that levels of Cer (d18:1/24:1) were >9-fold elevated in plasma of SR-BI knockout mice. High plasma levels of Cer (d18:1/24:1) have consistently been associated with a higher systemic inflammatory state and an increased atherosclerotic cardiovascular disease risk in humans.[38-41] One could thus perhaps anticipate that chow-fed SR-BI knockout mice also display a high susceptibility to develop atherosclerotic lesions. However, to our knowledge, no atherosclerotic lesions have ever been detected in regular chow diet-fed (aged) SR-BI knockout mice. We therefore perceive the increase in Cer (d18:1/24:1) concentrations rather a signal that SR-BI knockout mice exhibit a higher baseline inflammatory state. This corroborates our earlier findings that liver macrophages (i.e. Kupffer cells) from chow diet-fed SR-BI knockout mice exhibit a more inflammatory phenotype as exemplified by relatively high gene expression levels of the pro-inflammatory cytokines interleukin-6 and tumor necrosis factor-alpha.[4] Dihydroceramide desaturase 1 (DEGS1) appears to play an essential role in the synthesis of Cer (d18:1/24:1) as the L175Q loss-of-function mutation in DEGS1 in humans is associated with a reduction in plasma Cer (d18:1/24:1) levels in the context of elevated Cer (d18:1/24:0) levels.[42] Dogra et al.[43] have shown that pro-inflammatory polarization of macrophages is associated with an increase in DEGS1 expression. These combined findings imply that the increase in plasma Cer (d18:1/24:1) levels observed in SR-BI knockout mice may actually be secondary to an increase in the number and/or activity of pro-inflammatory macrophages. To provide proof for an important contribution of macrophages to plasma Cer (d18:1/24:1) levels and uncover the actual relevance for atherosclerosis susceptibility, selective elimination of DEGS1 function in macrophages in SR-BI knockout mice as well as atherosclerosis-susceptible hypercholesterolemic mice, i.e. low-density lipoprotein receptor knockout or apolipoprotein E knockout mice, should be pursued.

In conclusion, we have shown that SR-BI deficiency in mice not only impacts the CE concentrations, length, and saturation index within the plasma compartment, but is also associated with plasma accumulation of several Cer and SM species that may contribute to the

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development of specific hematological and metabolic (disease) phenotypes previously detected in SR-BI knockout mice. A strong point of our findings is that we have been able to complement earlier *in vitro* evidence with *in vivo* proof for the role of SR-BI as mediator of SM and Cer uptake. Furthermore, these novel observations highlight that it is of interest to determine the impact of plasma Cer level normalization on SR-BI deficiency-associated phenotypes. Given that the enlarged, lipid-rich HDL particles from SR-BI knockout mice are commonly regarded as being less functional,[44] the individual changes in the plasma lipidome detected in these mice can potentially be used as novel biomarkers for HDL dysfunction in the human clinical (atherosclerotic cardiovascular disease) setting. In this context, it will be important to further validate the relevance of our findings in mouse models with a more human-like lipoprotein profile, i.e. in mice that do express human CE transfer protein, as well as in human subjects carrying a functional mutation in the SR-BI gene. In follow-up studies special attention should also be given to the potential impact of sex on genotype-associated changes in the HDL/plasma lipidome, i.e. the current study was limited to the use of female mice, since significant SR-BI gene by sex interaction has been seen before in the clinical setting.[45] In addition, in future studies it will be important to monitor the plasma lipidome and composition and function of blood cells in the same test animals/human subjects to provide more clear evidence for a causal relationship between changes in the two individual parameters.

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