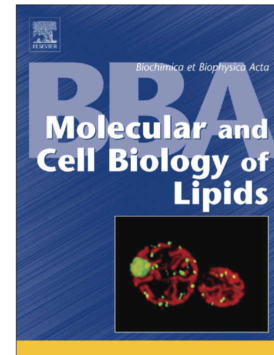
Accepted Manuscript



ATP-binding cassette transporter G1 deficiency is associated with mild glucocorticoid insufficiency in mice

Menno Hoekstra, Amber B. Ouweneel, Joya E. Nahon, Rick van der Geest, Mara J. Kröner, Ronald J. van der Sluis, Miranda Van Eck

PII: S1388-1981(18)30208-7
DOI: https://doi.org/10.1016/j.bbalip.2019.01.003
Reference: BBAMCB 58410

To appear in: *BBA - Molecular and Cell Biology of Lipids*

Received date: 9 August 2018
Revised date: 6 December 2018
Accepted date: 7 January 2019

Please cite this article as: Menno Hoekstra, Amber B. Ouweneel, Joya E. Nahon, Rick van der Geest, Mara J. Kröner, Ronald J. van der Sluis, Miranda Van Eck , ATP-binding cassette transporter G1 deficiency is associated with mild glucocorticoid insufficiency in mice. Bbamcb (2019), https://doi.org/10.1016/j.bbalip.2019.01.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

ATP-binding cassette transporter G1 deficiency is associated with mild glucocorticoid insufficiency in mice

Running title: ABCG1 deficiency diminishes the adrenal steroid function

Menno Hoekstra*[#], Amber B. Ouweneel[#], Joya E. Nahon, Rick van der Geest, Mara J. Kröner, Ronald J. van der Sluis, Miranda Van Eck

Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Gorlaeus Laboratories, Einsteinweg 55, 2333CC Leiden, The Netherlands

* Corresponding author:

Email: hoekstra@lacdr.leidenuniv.nl

Phone: +31-71-5276582

[#] equal contribution

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ABSTRACT

OBJECTIVE: Since cholesterol is the sole precursor for glucocorticoid synthesis, it is hypothesized that genetic defects in proteins that impact the cellular cholesterol pool may underlie glucocorticoid insufficiency in humans. In the current study, we specifically focused on the cholesterol efflux mediator ATP-binding cassette transporter G1 (ABCG1) as gene candidate.

METHODS: The adrenal transcriptional response to fasting stress was measured in wild-type mice to identify putative novel gene candidates. Subsequently, the adrenal glucocorticoid function was compared between ABCG1 knockout mice and wild-type controls.

RESULTS: Overnight food deprivation induced a change in relative mRNA expression levels of cholesterol metabolism-related proteins previously linked to steroidogenesis, i.e. scavenger receptor class B type I (+149%; $P<0.001$), LDL receptor (-70%; $P<0.001$) and apolipoprotein E (-41%; $P<0.01$). Strikingly, ABCG1 transcript levels were also markedly decreased (-61%; $P<0.05$). In contrast to our hypothesis that decreasing cholesterol efflux would increase the adrenal cholesterol pool and enhance glucocorticoid output, ABCG1 knockout mice as compared to wild-type mice exhibited a reduced ability to secrete corticosterone in response to an ACTH challenge (two-way ANOVA: $P<0.001$ for genotype) or fasting stress. As a result, glucocorticoid target gene expression levels in liver and hypothalamus were reduced and blood lymphocyte concentrations and spleen weights increased in ABCG1 knockout mice under fasting stress conditions. This was paralleled by a 48% reduction in adrenal cholesteryl ester stores and stimulation of adrenal NPC intracellular cholesterol transporter 2 (+37%; $P<0.05$) and apolipoprotein E (+59%; $P<0.01$) mRNA expression.

CONCLUSION: ABCG1 deficiency is associated with mild glucocorticoid insufficiency in mice.

ACCEPTED MANUSCRIPT

KEYWORDS: Glucocorticoid; ATP-binding cassette transporter; cholesterol metabolism; adrenal; gene expression; primary adrenal insufficiency

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. INTRODUCTION

The hypothalamus-pituitary-adrenal axis mediates the physiological response to stress. More specifically, upon a physiological or psychological stress trigger, the hypothalamus starts secreting corticotropin releasing hormone that can activate neuronal circuits in the pituitary to produce adrenocorticotropic hormone (ACTH). Subsequently, ACTH travels via the bloodstream to the adrenal glands where it binds to the melanocortin-2 receptor. The ACTH / melanocortin-2 receptor interaction initiates intracellular signaling events within adrenocortical cells that lead to the synthesis of glucocorticoids. Glucocorticoids, i.e. cortisol in humans and corticosterone in rodents, generate the actual biological stress response through modulation of gene transcription in target tissues controlling metabolism and immunity. A failure to secrete proper amounts of glucocorticoids is the underlying cause of Addison's disease (primary adrenal insufficiency), that is characterized by fatigue, muscle weakness, loss of appetite, weight loss, and abdominal pain and a predisposition to loss of consciousness under stress conditions [1].

Glucocorticoids belong to the family of steroid hormones that are derived from the common precursor cholesterol. Genetic association studies in human subjects suffering from primary adrenal insufficiency have suggested that the majority of disease cases is related to a defect in either the melanocortin-2 receptor or proteins involved in the intra-mitochondrial transport of cholesterol towards the steroidogenic machinery or the subsequent conversion of cholesterol into glucocorticoids [2]. However, a significant number of glucocorticoid deficiency cases cannot be explained by known genetic variations [2].

The cellular cholesterol pool is generally maintained through balancing the acquisition of cholesterol, i.e. the novo synthesis and lipoprotein-derived cholesterol uptake by the low-density lipoprotein (LDL) receptor and scavenger receptor class B type I (SR-BI), with the (active) export

ACCEPTED MANUSCRIPT

of cholesterol by locally-produced apolipoprotein E (APOE) and the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1). Notably, studies by Illingworth et al. have shown that disruption of the LDL receptor function in humans is associated with a slightly diminished cortisol response to an ACTH challenge and a reduced basal rate of urinary free cortisol excretion [3]. Our studies in human carriers of a functional mutation in SR-BI have shown that a defect in the cellular uptake of cholesterol from high-density lipoproteins (HDL) is also associated with a diminished adrenal glucocorticoid output [4]. In further support of an essential role for SR-BI in the supply of cholesterol to the glands, ACTH exposure is associated with a marked increase in mRNA levels of SR-BI in adrenocortical cells [5], which translates into a concomitant increase in SR-BI protein levels [6] and an enhanced HDL-cholesteryl ester clearance by the adrenals under stress conditions, i.e. after exposure to lipopolysaccharide [7]. Moreover, adrenals from SR-BI knockout mice are deprived of cholesteryl esters and secrete lower amounts of corticosterone in response to a metabolic or inflammatory stress trigger [8-10]. In contrast to the decrease in glucocorticoid output associated with an impaired cholesterol acquisition from lipoproteins, studies in APOE knockout mice have shown that elimination of APOE-mediated cholesterol efflux is actually associated with an increased ability of the adrenals to secrete glucocorticoids in response to a variety of stress triggers [11,12]. These combined findings (1) highlight that it is of critical importance to maintain the functional pool of cholesterol within the adrenals that is readily available for use in the synthesis of glucocorticoids and (2) underscore that it is possible that genetic defects in other cholesterol metabolism-related proteins may underlie currently unexplained cases of glucocorticoid insufficiency in humans.

To uncover a potential influence on the adrenal glucocorticoid output of other proteins that are generally involved in the control of cellular cholesterol homeostasis, we therefore first investigated the transcriptional response to stress in C57BL/6 wild-type mice. In addition to the anticipated changes in adrenal SR-BI and APOE transcript levels, a marked fasting stress-

ACCEPTED MANUSCRIPT

associated decrease in the adrenal mRNA expression level of the cholesterol efflux protein ATP-binding cassette transporter G1 (ABCG1) was detected. We followed up on this interesting initial observation by studying the effect of total body ABCG1 deficiency on adrenal cholesterol levels and glucocorticoid output in mice.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

2. MATERIALS AND METHODS

2.1 Animals

All mice used in the experiments were generated from in-house breedings. Throughout the experiments all mice were housed in the same climate controlled stable with a 12h/12h dark-light cycle and handled identically. All animal work was approved by the Leiden University Animal Ethics committee and performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament.

To study the effect of fasting stress on adrenal glucocorticoid levels, ~12 week old C57BL/6 wild-type mice were either fed a regular chow diet ab libitum (N=6) or deprived of food from 05:00 PM onwards (N=6). At 9:00 AM the next morning, blood was drawn from the tail to obtain a basal plasma corticosterone measurement. Subsequently, mice were sacrificed via cervical dislocation and adrenals harvested for gene expression analysis.

To evaluate the effect of ABCG1 deficiency on adrenal cholesterol homeostasis and glucocorticoid output, we utilized male ABcG1 knockout mice and C57BL/6 wild-type controls. At the age of 8-12 weeks old, groups of age-matched wild-type (N=9) and ABCG1 knockout (N=6) mice were single-housed for 2 weeks. At 9:00 AM, blood was drawn from the tail to obtain a basal plasma corticosterone measurement. Subsequently, mice were injected intraperitoneally with 200 µg adrenocorticotropic hormone (ACTH; Tetracosactide; MT-Diagnostics Netherlands BV) and bled at 1, 2, and 3 hours following the ACTH injection to measure the maximal plasma corticosterone response [13-15]. After a two week recovery period, mice were subjected to food deprivation from 5:00 PM to 9:00 AM the next morning. After anesthesia by subcutaneous injection with a mix of 70 mg/kg body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine, mice were bled via retro-orbital bleeding, sacrificed, and subjected

ACCEPTED MANUSCRIPT

to whole body perfusion with PBS to facilitate collection of liver, spleen, brains, and adrenals. During sacrifice, the hypothalamus was directly isolated from the brain tissue. Tissues were stored at -20ºC and/or fixed overnight in 3.7% neutral-buffered formalin solution (Formalfixx; Shandon Scientific Ltd, UK). Additional groups of 10-12 week old age-matched male wild-type (N=7) and ABCG1 knockout mice (N=7) were group-housed for the complete duration of their life and sacrificed in the overnight fasted state for the purpose of obtaining more adrenal gland specimens.

2.2 Blood and plasma analyses

Total white blood cells counts and the distribution over different subclasses of white blood cells were routinely measured using an automated SYSMEX XT-2000iV Veterinary Heamatology analyzer (SYSMEX Corporation). Corticosterone levels in tail blood plasma were determined using the corticosterone ^{3}H RIA Kit from ICN Biomedicals according to the protocol from the supplier.

2.3 Adrenal lipid composition and histology

Lipids from adrenals were extracted using the method of Bligh and Dyer [16]. After dissolving the lipids in 1% Triton X-100, unesterified cholesterol and cholesteryl ester contents were determined using enzymatic colorimetric assays and expressed as micrograms per milligram of protein. Seven micrometer cryosections were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with hematoxylin (Sigma) and Oil red O (Sigma) for neutral lipid visualization.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

2.4 Real-time quantitative PCR

Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction. Equal amounts of RNA were reverse transcribed and subsequently real-time quantitative PCR analysis was executed on the cDNA using an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Acidic ribosomal phosphoprotein P0 (36B4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), hypoxanthine guanine phosphoribosyl transferase (HPRT), and ribosomal protein L27 (RPL27) were used as housekeeping genes for normalization.

2.5 Data analysis

Statistical analysis was performed using Graphpad Instat software (San Diego, USA, http://www.graphpad.com). Normality of the experimental groups was confirmed using the method of Kolmogorov and Smirnov. The significance of differences was calculated using a two-tailed unpaired t-test or two-way analysis of variance (ANOVA) where appropriate. Probability values less than 0.05 were considered significant.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

3. RESULTS

To uncover the possible relevance of individual proteins in the maintenance of the adrenal steroidogenic cholesterol pool, we investigated the effect of stress induction on adrenal mRNA expression levels of proteins that are generally recognized as active players in cellular cholesterol metabolism. Our previous studies in mice have indicated that overnight food deprivation is an important metabolic stress trigger, as fasting is associated with a highly reproducible increase in plasma corticosterone levels [8,9,10,13,14,17]. We therefore quantified adrenal relative mRNA expression levels in C57BL/6 wild-type mice that were either subjected to overnight fasting or, as a control, were provided a regular chow diet ad libitum. Overnight fasting did effectively increase plasma corticosterone levels (319±69 ng/ml for fasted mice versus 140±35 ng/ml for controls; $P<0.05$). In accordance with the notion that SR-BI-mediated uptake of cholesteryl esters from HDL is a quantitatively important cholesterol acquisition route under high steroidogenic pressure conditions, the increase in adrenal glucocorticoid output was paralleled by a significant rise in adrenal SR-BI relative mRNA expression levels (+149%; $P<0.001$; Figure 1A). The expression of the low-density lipoprotein receptor (LDLR) was, however, markedly decreased (-70%; $P<0.001$; Figure 1A) in response to food deprivation. No apparent change was detected in the expression levels of HMG-CoA reductase (HMGCR) that facilitates rate-limiting step in de novo cholesterol synthesis (Figure 1A). This fits with the previous observations by Illingworth et al. and Laue et al. that treatment of human hypercholesterolemic subjects with mevinolin, an inhibitor of HMGCR activity, does not affect corticosteroid production by the adrenal cortex during prolonged ACTH stimulation [18,19]. As evident from Figure 1B, adrenal APOE expression levels were significantly lower in mice in the fasting state (-41%; $P<0.01$). A fasting-associated minor decrease in ABCA1 expression levels failed to reach significance (Figure 1B). In contrast, fasting stress was associated with a marked >2.5-fold decrease in

ACCEPTED MANUSCRIPT

adrenal ABCG1 transcript levels (P<0.05; Figure 1B). It can thus be suggested that adrenocortical cells, in response to a stress trigger, lower their APOE-mediated (passive) efflux of cholesterol and predominantly block their active efflux of cholesterol via ABCG1 to guarantee that sufficient levels of unesterified cholesterol are available for the production of glucocorticoids.

Given that the extent of fasting stress-induced lowering in the expression of ABCG1 (-61%) was even greater than in that of APOE (-41%) and that ABCG1 is expressed at relatively high levels in human adrenal glands [20], we verified whether ABCG1 deficiency impacts the adrenal glucocorticoid output. As can be appreciated from Figure 2A, total body ABCG1 knockout mice as compared to C57BL/6 wild-type mice exhibited a reduced overall glucocorticoid response to an ACTH challenge (two-way ANOVA: P<0.001 for genotype). Plasma corticosterone levels increased to ~600 ng/ml after intraperitoneal ACTH administration and remained at the similar high level during the full course of the challenge (3 hours) in wild-type mice (Figure 2A). The plateau concentration reached in ABCG1 knockout was, however, only ~450 ng/ml. Furthermore, ABCG1 knockout mice were not able to maintain this maximal level of glucocorticoid output until the end of the test (Figure 2A). More specifically, at three hours after ACTH administration, plasma corticosterone levels in ABCG1 knockout mice were only 62% of those observed in C57BL/6 mice (P<0.01; Figure 2A). To further validate that ABCG1 knockout mice suffer from mild glucocorticoid insufficiency, we also subjected the two types of mice to overnight fasting stress. In line with our ACTH response outcome, plasma corticosterone levels also tended to be lower in overnight fasted ABCG1 knockout mice than in overnight fasted wild-type mice (P=0.13; Figure 2B). The 22% lowering in plasma corticosterone levels under fasting stress conditions coincided with a similar decrease in hepatic mRNA expression levels of the metabolic glucocorticoid-sensitive gene 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2; -20%; P<0.01; Figure 2C). In addition, the hepatic expression of the glucocorticoid receptor target gene fibroblast growth factor 21 (FGF21) was 57% lower in ABCG1 knockout mice (P<0.01;

ACCEPTED MANUSCRIPT

Figure 2C). Glucocorticoids stimulate hypothalamic neuropeptide Y (NPY) mRNA levels during food deprivation [21]. In accordance with a general reduction in the extent of cellular glucocorticoid signaling as a result of adrenal ABCG1 deficiency, NPY mRNA expression was significantly lower (-23%; $P<0.05$) in hypothalamus specimens from ABCG1 knockout mice as compared to those isolated from wild-type mice (Figure 2D). Blood lymphocyte concentrations as well as relative spleen weights were markedly increased in ABCG1 knockout mice (Figures 2E & 2F), which highlights that ABCG1 deficiency is probably not only associated with a diminished metabolic glucocorticoid action but also a reduction in the immunosuppressive glucocorticoid activity.

To provide insight into the potential mechanism underlying the impaired ability of the ABCG1 knockout adrenals to secrete an equally high level of corticosterone as compared to their wild-type counterparts, we isolated adrenals from wild-type and ABCG1 knockout mice, in the overnight fasting stressed state, for biochemical analysis. ABCG1 deficiency did not have an effect on absolute adrenal weights (Figure 3A). Since total body weights also did not differ between ABCG1 knockout mice and wild-type controls (data not shown), the body weight corrected adrenal weights were also virtually identical in the two groups of mice, 0.030±0.003% for ABCG1 knockout mice (N=13) versus 0.030±0.004% for wild-type mice (N=16), respectively. Given that intra-mitochondrial transport of cholesterol and the subsequent step-wise conversion of cholesterol into glucocorticoids are considered rate-limiting for the secretion of corticosterone, a potential effect of ABCG1 deficiency on the mRNA expression of genes crucially involved in these processes was determined. Relative mRNA expression levels of proteins that respectively facilitate the intra-mitochondrial cholesterol flux (STARD1) and subsequent conversions of cholesterol into pregnenolone (CYP11A1), pregnenolone into progesterone (HSD3B2), progesterone into deoxycorticosterone (CYP21A1), and deoxycorticosterone into corticosterone (CYP11B1) were all within the normal range in ABCG1 knockout adrenals (Figure 3B). As such,

it seems that a defect in the actual steroidogenic process probably does not underlie the ABCG1 deficiency-associated glucocorticoid insufficiency.

Given that we hypothesized that ABCG1 plays a quantitative role in maintaining cellular cholesterol homeostasis, we subsequently determined the impact of total body ABCG1 deficiency on adrenal cholesterol homeostasis. As evident from Figure 4A, quantification of tissue cholesterol levels showed that ABCG1 deficiency did not execute a major effect on adrenal unesterified cholesterol levels. Strikingly, a marked 48% reduction in the adrenal cholesteryl ester pool could be observed in ABCG1 knockout mice. The difference in adrenal levels of esterified cholesterol, however, failed to reach statistical significance due to the large intra-group variation ($P=0.10$; Figure 4B). Importantly, histochemical staining of adrenal sections with Oil red O could verify that ABCG1 deficiency was indeed associated with a significant change in neutral lipid accumulation. Sections of wild-type adrenals contained large Oil red O-positive lipid vacuoles, whilst only relatively small lipid droplets were visible in sections of ABCG1 knockout adrenals (Figure 4C).

Additional mRNA expression analysis was employed to delineate why a genetic defect in the cholesterol efflux gene ABCG1 was not paralleled by an increase in cellular cholesterol levels, but rather decreased adrenal cholesterol stores. Adrenal relative mRNA expression levels of HMGCR, SR-BI, and the LDL receptor were not different between ABCG1 knockout and wild-type mice (Figure 5A), which implies that the reduction in adrenal cholesterol stores was not secondary to a change in the rate of adrenal cholesterol acquisition. Cholesterol (de-)esterification rates were likely also not affected as judged from the unchanged mRNA expression levels of ACAT1 and HSL (Figure 5B). Interestingly, APOE levels were significantly elevated in ABCG1 knockout adrenals (+59%; $P<0.01$; Figure 5C), suggesting a possibly higher rate of adrenocortical cell cholesterol efflux. In agreement, the expression levels of the major cholesterol efflux protein ABCA1 also tended to increase in response to ABCG1 deficiency

ACCEPTED MANUSCRIPT

(+48%; P=0.10; Figure 5C). Several studies have indicated that ABCG1, similarly to ABCA1 and APOE, plays a role in the plasma membrane cholesterol flux [22-25]. The upregulation of APOE and ABCA1 expression may thus serve as a compensatory response to restore the cholesterol flux across the plasma membrane. However, findings from Tarling et al. have highlighted that murine ABCG1 is probably primarily acting intracellularly, i.e. on early and late endosomes and lysosomes [26,27]. We therefore also investigated a potential effect of ABCG1 deficiency on the expression levels of lysosomal cholesterol transport genes. ABCG1 deficiency was not associated with a change the adrenal relative mRNA expression levels of NPC intracellular cholesterol transporter 1 (NPC1) that is regarded the main transporter of cholesterol across the lysosomal limiting membrane (Figure 5D). Strikingly, ABCG1 knockout adrenals did – however – exhibit a significant 37% increase ($P<0.05$; Figure 5D) as compared to wild-type adrenals in the relative mRNA expression levels of NPC2 that plays an obligate role in transport cholesterol through the lysosomal lumen [28].

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

4. DISCUSSION

In the current study we show for the first time that ABCG1 knockout mice suffer from mild glucocorticoid insufficiency, which is associated with smaller lipid-droplets in the adrenocortical cells. The observation that the amounts of cholesterol used for steroidogenesis and storage in the cholesteryl ester pool are reduced as a result of ABCG1 deficiency can be conceived as remarkable, since ABCG1 is generally thought to play a role in the efflux of cholesterol from cells.

We observed that fasting stress is associated with an increase in adrenal relative mRNA expression levels of SR-BI, whilst adrenal mRNA expression levels of genes associated with cholesterol efflux (i.e. APOE and ABCG1) are significantly reduced in response to food deprivation. In light of the fact that the steroidogenic machinery needs a relatively high amount of unesterified cholesterol to produce glucocorticoids under (fasting) stress conditions, it is not surprising that adrenals increase their expression levels of SR-BI to facilitate uptake of exogenous cholesterol from HDL, the primary lipoprotein circulating in mice. Furthermore, a decrease in the rate of cellular cholesterol efflux would theoretically also translate into a higher availability of cholesterol for use in steroidogenesis. The parallel 70% decrease in mRNA expression levels of the LDL receptor may in this light seem counterintuitive as this is a primary receptor for cellular uptake of cholesterol from non-HDL lipoproteins, i.e. very-low-density lipoprotein (VLDL) and LDL. However, our previous adrenal transplantation studies in total body LDL receptor knockout mice have shown that LDL receptor-mediated cholesterol uptake is actually linked to a reduction in the overall glucocorticoid output [14].

An unexpected finding from our studies was that deletion of the cholesterol efflux transporter ABCG1 in mice did not increase adrenal cholesterol levels, but rather decreased adrenal

ACCEPTED MANUSCRIPT

cholesterol stores and lowered the ability of adrenals to secrete glucocorticoids in response to ACTH exposure or fasting stress. Given that we did not observe a parallel change in the expression of key genes involved in steroidogenesis and cholesterol (de-)esterification it is anticipated that the ABCG1 deficiency-associated glucocorticoid insufficiency and cholesteryl ester deprivation are secondary to a reduction in the relevant intracellular (unesterified) cholesterol substrate pool. Based upon the fact that gene expression levels of SR-BI, HMGCR, and the LDL receptor as well as plasma cholesterol levels (data not shown) were not different between wild-type and ABCG1 knockout mice, it is also unlikely that the glucocorticoid insufficiency is related to disrupted cholesterol acquisition by adrenocortical cells. We detected a significant increase in the mRNA expression levels of the lysosomal cholesterol transport protein NPC2 in ABCG1 knockout mice. In fibroblasts in culture, NPC2 facilitates cellular cholesterol efflux [29]. Mechanistic studies by Boadu et al. have indicated that NPC2 specifically facilitates the ABCA1-mediated flux of cholesterol from the (endo)lysosomal compartment towards the plasma membrane for subsequent removal by exogenous acceptors APOA1 and HDL [30]. In agreement with a critical role for NPC2 in lysosomal cholesterol efflux, deletion of NPC2 functionality is associated with accumulation of cholesterol in the lysosomal compartment [31,32]. NPC2 is a regulatory target of the nuclear oxysterol receptor liver X receptor (LXR), that plays an important role in the pathway for the removal of excess cholesterol from the body (reviewed in [33-35]). Rigamonti et al. have shown that exposure to the synthetic LXR agonists T0901317 and GW3965 stimulates the expression of NPC2 in human macrophages [36]. Interestingly, in addition to the observed increase in mRNA expression of NPC2, also relative mRNA expression levels of respectively APOE and ABCA1, two other LXR-responsive genes, were significantly higher or tended be increased in ABCG1 knockout adrenals as compared to wild-type adrenals. It is therefore tempting to speculate that absence of ABCG1 leads to activation of LXR in the adrenals. Importantly, previous in vitro and in vivo findings have suggested that the LXR activation status is negatively correlated with the rate of cholesterol

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

storage and hormone formation in steroidogenic cells and tissues. More specifically, disruption of in vivo LXR functionality in LXRalpha/beta double knockout mice is associated with adrenal accumulation of both unesterified and esterified cholesterol, an increased adrenocortical cell glucocorticoid secretion and higher plasma corticosterone levels [37]. In contrast, treatment with LXR agonists reduces cellular cholesterol levels and lowers progesterone secretion in cultured human luteinized granulosa cells [38]. Notably, studies in which the function of the membrane-associated (late) endosomal cholesterol transport protein StAR related lipid transfer domain containing 3 (MLN64/ STARD3) was eliminated have proven that mitochondria rely for a large part on lysosomes for the acquisition of the cholesterol substrate used for steroidogenesis [39]. When taking these combined findings into account, it is hypothesized that, within ABCG1 knockout adrenals, LXR activation stimulates the NPC2 / ABCA1-mediated transport of cholesterol from lysosomes towards the plasma membrane for subsequent efflux, thereby depriving the lysosomal cholesterol pool that can be used for cholesteryl ester storage or MLN64-mediated transfer to the mitochondria for subsequent use in steroidogenesis. Studies by Karuna et al. have suggested that ABCG1 mediates the cellular efflux of the endogenous LXR ligand 27-hydroxycholesterol [40]. As such, the apparent ABCG1 deficiency-associated activation of LXR and reduction in the glucocorticoid output can theoretically be secondary to adrenal 27-hydroxycholesterol accumulation. However, in light of the imaging findings from Tarling et al. that - in mice - ABCG1 primary localizes to early and late lysosomes [26,27], it is also tempting to speculate that, while NPC2 seems to donate cholesterol to ABCA1 for trafficking towards the plasma membrane and efflux to exogenous acceptors, ABCG1 actively supplies lysosomal cholesterol to MLN64 for subsequent mitochondrial delivery. It should be acknowledged that our current working model regarding the impact of ABCG1 deficiency on adrenal cholesterol homeostasis is based solely on changes in the mRNA expression levels of a set of cholesterol metabolism-related genes. Although studies by Koussounadis et al. have implied that differentially expressed mRNAs are more likely than non-differentially expressed

ACCEPTED MANUSCRIPT

mRNAs to translate into concordant behavior at the protein level [41], it is clear that dedicated (more mechanistically oriented) cholesterol flux follow-up studies are warranted to uncover how ABCG1 deficiency exactly impacts adrenocortical cell cholesterol homeostasis.

In conclusion, we have shown that ABCG1 deficiency is associated with reduced adrenal cholesterol levels and mild glucocorticoid insufficiency in mice. Since recent studies by de Aguiar Vallim et al. have shown that within the lungs the function of ABCG1 is similar in mice and humans [42], our mouse data highlight that is of interest to investigate whether variations in the activity of the ABCG1 gene/protein also associate with an altered adrenal glucocorticoid function in humans.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

5. REFERENCES

1. https://www.niddk.nih.gov/health-information/endocrine-diseases/adrenal-insufficiency-addisons-disease/definition-facts; visited on 30/11/2018.

2. Guran T, Buonocore F, Saka N, Ozbek MN, Aycan Z, Bereket A, Bas F, Darcan S, Bideci A, Guven A, Demir K, Akinci A, Buyukinan M, Aydin BK, Turan S, Agladioglu SY, Atay Z, Abali ZY, Tarim O, Catli G, Yuksel B, Akcay T, Yildiz M, Ozen S, Doger E, Demirbilek H, Ucar A, Isik E, Ozhan B, Bolu S, Ozgen IT, Suntharalingham JP, Achermann JC. Rare Causes of Primary Adrenal Insufficiency: Genetic and Clinical Characterization of a Large Nationwide Cohort. J Clin Endocrinol Metab. 2016 Jan;101(1):284-92. doi: 10.1210/jc.2015-3250.

3. Illingworth DR, Lees AM, Lees RS. Adrenal cortical function in homozygous familial hypercholesterolemia. Metabolism. 1983 Nov;32(11):1045-52.

4.. Vergeer M, Korporaal SJ, Franssen R, Meurs I, Out R, Hovingh GK, Hoekstra M, Sierts JA, Dallinga-Thie GM, Motazacker MM, Holleboom AG, Van Berkel TJ, Kastelein JJ, Van Eck M, Kuivenhoven JA. Genetic variant of the scavenger receptor BI in humans. N Engl J Med. 2011 Jan 13;364(2):136-45. doi: 10.1056/NEJMoa0907687

5. Liu J, Voutilainen R, Heikkilä P, Kahri AI. Ribonucleic acid expression of the CLA-1 gene, a human homolog to mouse high density lipoprotein receptor SR-BI, in human adrenal tumors and cultured adrenal cells. J Clin Endocrinol Metab. 1997 Aug;82(8):2522-7.

6. Rigotti A, Edelman ER, Seifert P, Iqbal SN, DeMattos RB, Temel RE, Krieger M, Williams DL. Regulation by adrenocorticotropic hormone of the in vivo expression of scavenger receptor class

ACCEPTED MANUSCRIPT

B type I (SR-BI), a high density lipoprotein receptor, in steroidogenic cells of the murine adrenal gland. J Biol Chem. 1996 Dec 27;271(52):33545-9.

7. Hoekstra M, Korporaal SJ, Li Z, Zhao Y, Van Eck M, Van Berkel TJ. Plasma lipoproteins are required for both basal and stress-induced adrenal glucocorticoid synthesis and protection against endotoxemia in mice. Am J Physiol Endocrinol Metab. 2010 Dec;299(6):E1038-43. doi: 10.1152/ajpendo.00431.2010.

8. Hoekstra M, Meurs I, Koenders M, Out R, Hildebrand RB, Kruijt JK, Van Eck M, Van Berkel TJ. Absence of HDL cholesteryl ester uptake in mice via SR-BI impairs an adequate adrenal glucocorticoid-mediated stress response to fasting. J Lipid Res. 2008 Apr;49(4):738-45. doi: 10.1194/jlr.M700475-JLR200.

9. Hoekstra M, Ye D, Hildebrand RB, Zhao Y, Lammers B, Stitzinger M, Kuiper J, Van Berkel TJ, Van Eck M. Scavenger receptor class B type I-mediated uptake of serum cholesterol is essential for optimal adrenal glucocorticoid production. J Lipid Res. 2009 Jun;50(6):1039-46. doi: 10.1194/jlr.M800410-JLR200.

10. Hoekstra M, van der Sluis RJ, Van Eck M, Van Berkel TJ. Adrenal-specific scavenger receptor BI deficiency induces glucocorticoid insufficiency and lowers plasma very-low-density and low-density lipoprotein levels in mice. Arterioscler Thromb Vasc Biol. 2013 Feb;33(2):e39-46. doi: 10.1161/ATVBAHA.112.300784.

11. Thorngate FE, Strockbine PA, Erickson SK, Williams DL. Altered adrenal gland cholesterol metabolism in the apoE-deficient mouse. J Lipid Res. 2002 Nov;43(11):1920-6.

12. Raber J, Akana SF, Bhatnagar S, Dallman MF, Wong D, Mucke L. Hypothalamic-pituitary-adrenal dysfunction in Apoe(-/-) mice: possible role in behavioral and metabolic alterations. J Neurosci. 2000 Mar 1;20(5):2064-71.

ACCEPTED MANUSCRIPT

13. Hoekstra M, Korporaal SJ, van der Sluis RJ, Hirsch-Reinshagen V, Bochem AE, Wellington CL, Van Berkel TJ, Kuivenhoven JA, Van Eck M. LCAT deficiency in mice is associated with a diminished adrenal glucocorticoid function. J Lipid Res. 2013 Feb;54(2):358-64. doi: 10.1194/jlr.M030080.

14. van der Sluis RJ, Van Eck M, Hoekstra M. Adrenocortical LDL receptor function negatively influences glucocorticoid output. J Endocrinol. 2015 Sep;226(3):145-54. doi: 10.1530/JOE-15-0023.

15. Ouweneel AB, van der Sluis RJ, Nahon JE, Van Eck M, Hoekstra M. Simvastatin treatment aggravates the glucocorticoid insufficiency associated with hypocholesterolemia in mice. Atherosclerosis. 2017 Jun;261:99-104. doi: 10.1016/j.atherosclerosis.2017.02.014.

16. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959 Aug;37(8):911-7.

17. Hoekstra M, Van Eck M. HDL is redundant for adrenal steroidogenesis in LDLR knockout mice with a human-like lipoprotein profile. J Lipid Res. 2016 Apr;57(4):631-7. doi: 10.1194/jlr.M066019.

18. Illingworth DR, Corbin D. The influence of mevinolin on the adrenal cortical response to corticotropin in heterozygous familial hypercholesterolemia. Proc Natl Acad Sci U S A. 1985 Sep;82(18):6291-4.

19. Laue L, Hoeg JM, Barnes K, Loriaux DL, Chrousos GP. The effect of mevinolin on steroidogenesis in patients with defects in the low density lipoprotein receptor pathway. J Clin Endocrinol Metab. 1987 Mar;64(3):531-5.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

20. Klucken J, Büchler C, Orsó E, Kaminski WE, Porsch-Ozcürümez M, Liebisch G, Kapinsky M, Diederich W, Drobnik W, Dean M, Allikmets R, Schmitz G. ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. Proc Natl Acad Sci U S A. 2000 Jan 18;97(2):817-22.

21. Ponsalle P, Srivastava LS, Uht RM, White JD. Glucocorticoids are Required for Food Deprivation-Induced Increases in Hypothalamic Neuropeptide Y Expression. J Neuroendocrinol. 1992 Oct;4(5):585-91. doi: 10.1111/j.1365-2826.1992.tb00207.x.

22. Xie Q, Engel T, Schnoor M, Niehaus J, Hofnagel O, Buers I, Cullen P, Seedorf U, Assmann G, Lorkowski S. Cell surface localization of ABCG1 does not require LXR activation. Arterioscler Thromb Vasc Biol. 2006 Nov;26(11):e143-4

23. Seres L, Cserepes J, Elkind NB, Törocsik D, Nagy L, Sarkadi B, Homolya L. Functional ABCG1 expression induces apoptosis in macrophages and other cell types. Biochim Biophys Acta. 2008 Oct;1778(10):2378-87. doi: 10.1016/j.bbamem.2008.06.010.

24. Sano O, Ito S, Kato R, Shimizu Y, Kobayashi A, Kimura Y, Kioka N, Hanada K, Ueda K, Matsuo M. ABCA1, ABCG1, and ABCG4 are distributed to distinct membrane meso-domains and disturb detergent-resistant domains on the plasma membrane. PLoS One. 2014 Oct 10;9(10):e109886. doi: 10.1371/journal.pone.0109886.

25. Pandzic E, Gelissen IC, Whan R, Barter PJ, Sviridov D, Gaus K, Rye KA, Cochran BJ. The ATP binding cassette transporter, ABCG1, localizes to cortical actin filaments. Sci Rep. 2017 Feb 6;7:42025. doi: 10.1038/srep42025.

26. Tarling EJ, Edwards PA. ATP binding cassette transporter G1 (ABCG1) is an intracellular sterol transporter. Proc Natl Acad Sci U S A. 2011 Dec 6;108(49):19719-24. doi: 10.1073/pnas.1113021108.

ACCEPTED MANUSCRIPT

27. Tarling EJ, Edwards PA. Intracellular Localization of Endogenous Mouse ABCG1 Is Mimicked by Both ABCG1-L550 and ABCG1-P550-Brief Report. Arterioscler Thromb Vasc Biol. 2016 Jul;36(7):1323-7. doi: 10.1161/ATVBAHA.116.307414.

28. Liscum L, Sturley SL. Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. Biochim Biophys Acta. 2004 Oct 11;1685(1-3):22-7.

29. Berzina Z, Solanko LM, Mehadi AS, Jensen MLV, Lund FW, Modzel M, Szomek M, Solanko KA, Dupont A, Nielsen GK, Heegaard CW, Ejsing CS, Wüstner D. Niemann-Pick C2 protein regulates sterol transport between plasma membrane and late endosomes in human fibroblasts. Chem Phys Lipids. 2018 Jul;213:48-61. doi: 10.1016/j.chemphyslip.2018.03.006.

30. Boadu E, Nelson RC, Francis GA. ABCA1-dependent mobilization of lysosomal cholesterol requires functional Niemann-Pick C2 but not Niemann-Pick C1 protein. Biochim Biophys Acta. 2012 Mar;1821(3):396-404. doi: 10.1016/j.bbalip.2011.11.013.

31. Ko DC, Binkley J, Sidow A, Scott MP. The integrity of a cholesterol-binding pocket in Niemann-Pick C2 protein is necessary to control lysosome cholesterol levels. Proc Natl Acad Sci U S A. 2003 Mar 4;100(5):2518-25.

32. Sleat DE, Wiseman JA, El-Banna M, Price SM, Verot L, Shen MM, Tint GS, Vanier MT, Walkley SU, Lobel P. Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. Proc Natl Acad Sci U S A. 2004 Apr 20;101(16):5886-91.

33. Millatt LJ, Bocher V, Fruchart JC, Staels B. Liver X receptors and the control of cholesterol homeostasis: potential therapeutic targets for the treatment of atherosclerosis. Biochim Biophys Acta. 2003 Mar 17;1631(2):107-18.

34. Zhao C, Dahlman-Wright K. Liver X receptor in cholesterol metabolism. J Endocrinol. 2010 Mar;204(3):233-40. doi: 10.1677/JOE-09-0271.

ACCEPTED MANUSCRIPT

35. Hong C, Tontonoz P. Liver X receptors in lipid metabolism: opportunities for drug discovery. Nat Rev Drug Discov. 2014 Jun;13(6):433-44. doi: 10.1038/nrd4280.

36. Rigamonti E, Helin L, Lestavel S, Mutka AL, Lepore M, Fontaine C, Bouhlel MA, Bultel S, Fruchart JC, Ikonen E, Clavey V, Staels B, Chinetti-Gbaguidi G. Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. Circ Res. 2005 Sep 30;97(7):682-9.

37. Cummins CL, Volle DH, Zhang Y, McDonald JG, Sion B, Lefrançois-Martinez AM, Caira F, Veyssière G, Mangelsdorf DJ, Lobaccaro JM. Liver X receptors regulate adrenal cholesterol balance. J Clin Invest. 2006 Jul;116(7):1902-12.

38. Drouineaud V, Sagot P, Garrido C, Logette E, Deckert V, Gambert P, Jimenez C, Staels B, Lagrost L, Masson D. Inhibition of progesterone production in human luteinized granulosa cells treated with LXR agonists. Mol Hum Reprod. 2007 Jun;13(6):373-9.

39. Zhang M, Liu P, Dwyer NK, Christenson LK, Fujimoto T, Martinez F, Comly M, Hanover JA, Blanchette-Mackie EJ, Strauss JF 3rd. MLN64 mediates mobilization of lysosomal cholesterol to steroidogenic mitochondria. J Biol Chem. 2002 Sep 6;277(36):33300-10.

40. Karuna R, Holleboom AG, Motazacker MM, Kuivenhoven JA, Frikke-Schmidt R, Tybjaerg-Hansen A, Georgopoulos S, van Eck M, van Berkel TJ, von Eckardstein A, Rentsch KM. Plasma levels of 27-hydroxycholesterol in humans and mice with monogenic disturbances of high density lipoprotein metabolism. Atherosclerosis. 2011 Feb;214(2):448-55. doi: 10.1016/j.atherosclerosis.2010.10.042.

41. Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. Sci Rep. 2015 Jun 8;5:10775. doi: 10.1038/srep10775.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

42. de Aguiar Vallim TQ, Lee E, Merriott DJ, Goulbourne CN, Cheng J, Cheng A, Gonen A, Allen RM, Palladino END, Ford DA, Wang T, Baldán Á, Tarling EJ. ABCG1 regulates pulmonary surfactant metabolism in mice and men. J Lipid Res. 2017 May;58(5):941-954. doi: 10.1194/jlr.M075101.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

FUNDING SOURCES

This study was supported by ‘the Netherlands CardioVascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences’ for the GENIUS project ‘Generating the best evidence-based pharmaceutical targets for atherosclerosis’ [CVON2011] and the Netherlands Organization for Scientific Research [VICI Grant 91813603]. Miranda Van Eck is an Established Investigator of the Netherlands Heart Foundation [Grant 2007T056].

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

FIGURE LEGENDS

Figure 1: Fasting stress alters the adrenal mRNA expression profile of cholesterol metabolism-related proteins in C57Bl/6 wild-type mice. Relative mRNA expression levels of proteins involved in the acquisition (*A*) and efflux (*B*) of cholesterol were measured in adrenals of C57BL/6 mice subjected to overnight food deprivation (black bars) and compared to those of ad libitum-fed control mice (white bars). Data represent means+SEM of 6 mice per group. * P<0.05, ** P<0.01, *** P<0.001.

Figure 2: ABCG1 deficiency is associated with mild glucocorticoid insufficiency in mice. (*A*) Plasma corticosterone levels measured over time after an intraperitoneal injection with ACTH. (*B*) Plasma corticosterone levels measured in the overnight fasted state. (*C*) Hepatic relative mRNA expression levels of the glucocorticoid-sensitive genes HMGCS2 and FGF21. (*D*) Relative mRNA expression levels of the glucocorticoid target gene NPY in hypothalamus. (*E*) Blood lymphocyte concentrations. (*F*) Spleen weights as percentage of total body weight. White bars/symbols represent data from wild-type mice and black bars/symbols those from ABCG1 knockout mice. Data represent means+SEM of respectively 9 and 6 (panels *A-D*) and 16 and 13 (panels *E* and *F*) wild-type and ABCG1 knockout mice per group. * P<0.05, ** P<0.01, *** P<0.001.

Figure 3: ABCG1 deficiency does not alter adrenal weights or adrenal relative mRNA expression levels of steroidogenic genes. Adrenal weights (*A*) and relative mRNA expression levels of the indicated genes involved in adrenal steroidogenesis (*B*) were measured in the overnight fasting state. White bars represent data from wild-type mice and black bars those from

ACCEPTED MANUSCRIPT

ABCG1 knockout mice. Data represent means+SEM of respectively 16 and 13 (panel *A*) and 9 and 6 (panel *B*) wild-type and ABCG1 knockout mice per group.

Figure 4: ABCG1 deficiency is associated with a trend to a reduction in adrenal cholesteryl ester stores. (*A*) Adrenal free cholesterol (FC) and cholesteryl ester (CE) levels. Data represent means+SEM of 9 wild-type mice (WT; white bars) and 6 ABCG1 knockout mice (ABCG1 KO; black bars). (*B*) Representative images of adrenal sections showing Oil red O-stained neutral lipid stores in adrenocortical cells (400x magnification). Note that ABCG1 deficiency is associated with the presence of only relatively small cholesteryl ester-containing lipid droplets.

Figure 5: ABCG1 deficiency is associated with significant alterations in the adrenal mRNA expression profile of cholesterol metabolism-related proteins. Relative mRNA expression levels of genes involved in cholesterol acquisition (*A*), cholesterol (de-)esterification (*B*), cholesterol efflux (*C*), and lysosomal cholesterol handling (*D*) were measured in adrenals of mice under fasting stress conditions. Data represent means+SEM of 9 wild-type mice (white bars) and 6 ABCG1 knockout mice (black bars). * $P<0.05$, ** $P<0.01$.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

HIGHLIGHTS

- ABCG1 deficiency is associated with reduced adrenal cholesterol ester storage
- ABCG1 knockout adrenals exhibit an increase in (lysosomal) cholesterol efflux genes
- ABCG1 deficiency is associated with mild glucocorticoid insufficiency in mice

ACCEPTED MANUSCRIPT

A

SR-BI

Relative mRNA expression

0.0
0.5
1.0
1.5
2.0
2.5
3.0

***

Fed
Fasting

LDLR

0.00
0.25
0.50
0.75
1.00
1.25

***

Fed
Fasting

HMGCR

0.00
0.25
0.50
0.75
1.00
1.25
1.50

Fed
Fasting

B

APOE

Relative mRNA expression

0.00
0.25
0.50
0.75
1.00
1.25

**

Fed
Fasting

ABCA1

0.00
0.25
0.50
0.75
1.00
1.25

Fed
Fasting

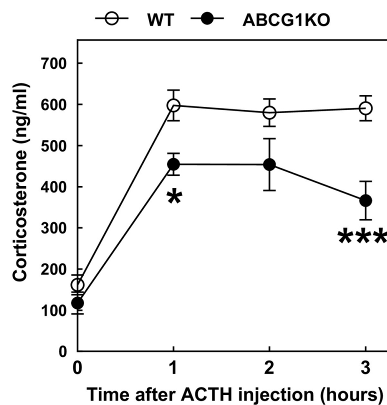
ABCG1

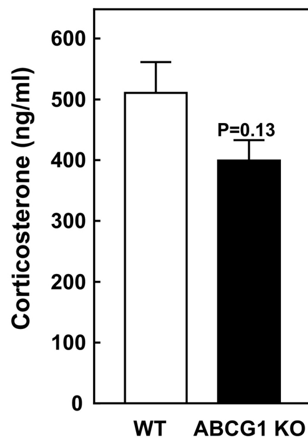
0.00
0.25
0.50
0.75
1.00
1.25

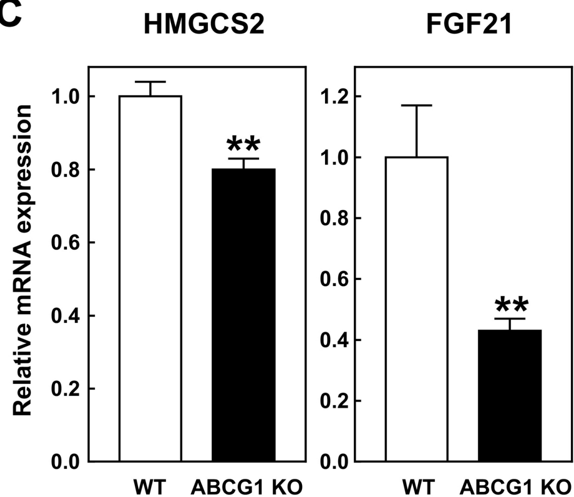
*

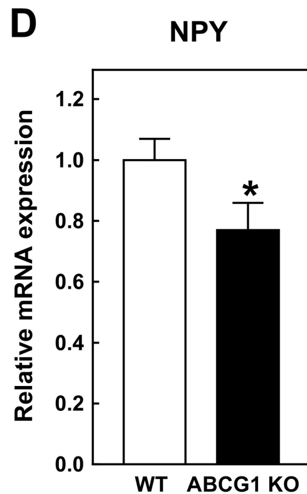
Fed
Fasting

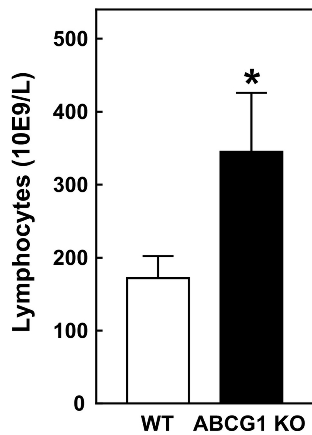
Figure 1











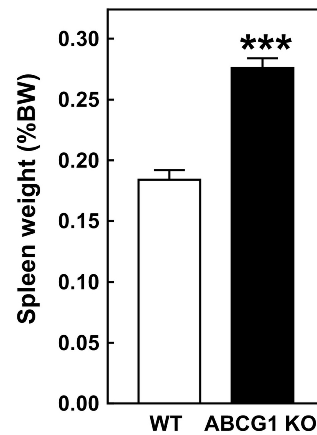
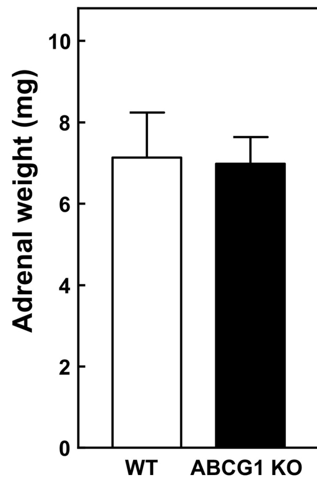


Figure 2



B

Relative mRNA expression

2.0

1.5

1.0

0.5

0.0

STARD1

CYP11A1

HSD3B2

CYP21A1

CYP11B1

cholesterol

CYP11A1

pregnenolone

HSD3B2

progesterone

CYP21A1

11-deoxycorticosterone

CYP11B1

corticosterone

Figure 3

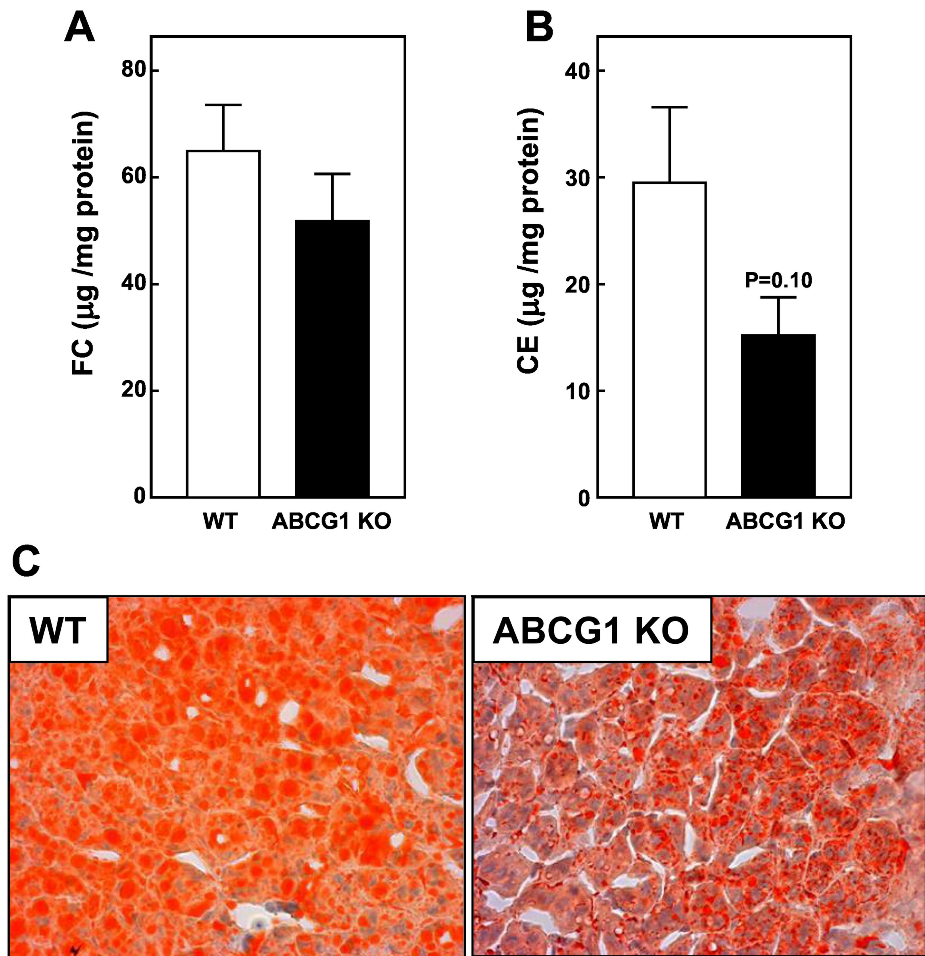


Figure 4

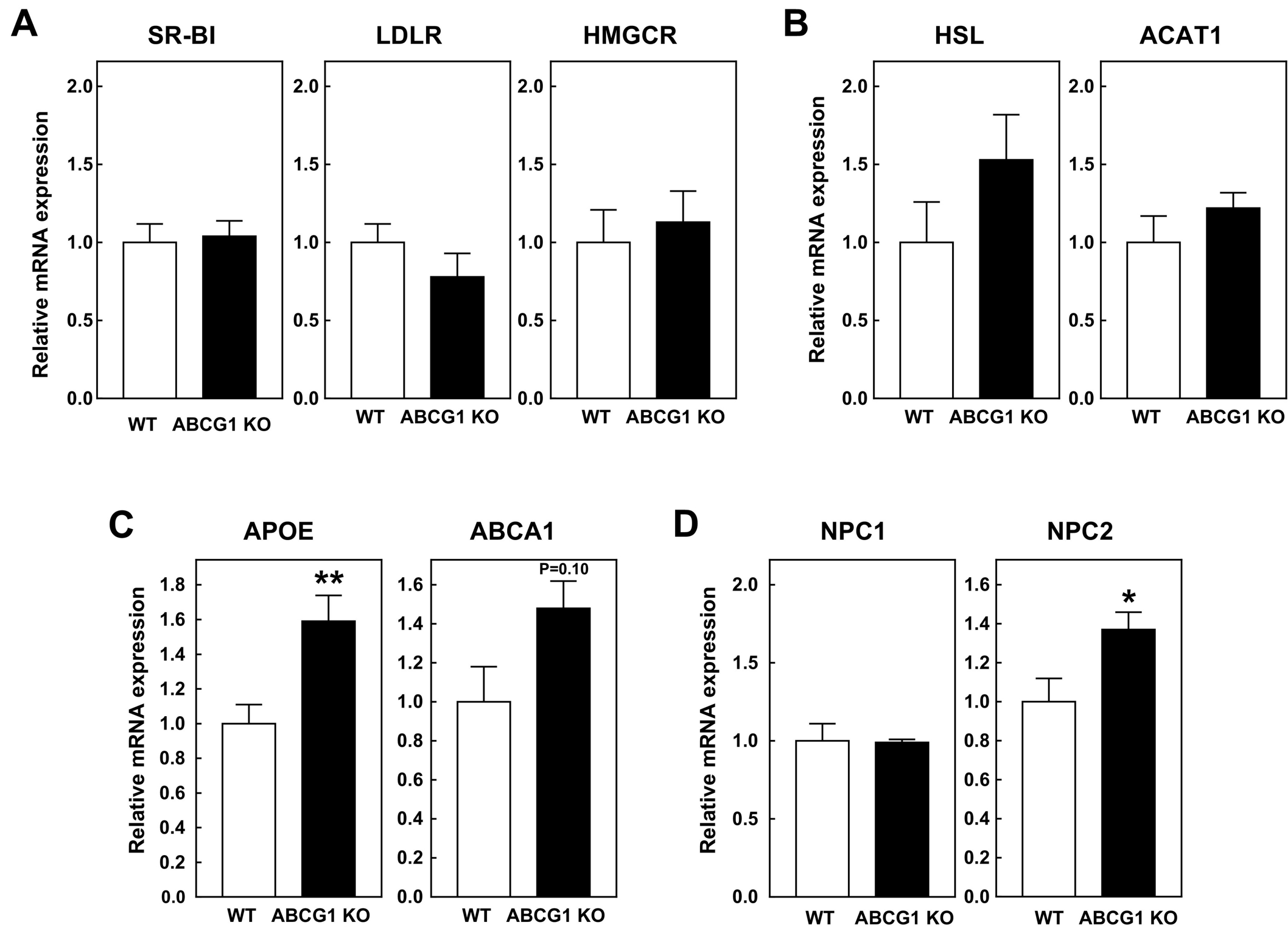


Figure 5