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Published in:
Journal of endocrinology

DOI:
10.1530/JOE-19-0079

Publication date:
2019

RESEARCH

Cholestasis-associated glucocorticoid overexposure does not increase atherogenesis

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Abstract

Chronic glucocorticoid overexposure predisposes to the development of atherosclerotic cardiovascular disease in humans. Cholestatic liver disease is associated with increased plasma glucocorticoid levels. Here, we determined – in a preclinical setting – whether the chronic presence of cholestatic liver disease also induces a concomitant negative impact on atherosclerosis susceptibility. Hereto, regular chow diet-fed atherosclerosis-susceptible hypercholesterolemic apolipoprotein E (APOE)-knockout mice were treated with the bile duct toxicant alpha-naphthylisothiocyanate (ANIT) for 8 weeks. ANIT exposure induced the development of fibrotic cholestatic liver disease as evident from collagen deposits and compensatory bile duct hyperproliferation within the liver and the rise in plasma levels of bilirubin (+60%; $P < 0.01$) and bile acids (10-fold higher; $P < 0.01$). Adrenal weights (+22%; $P < 0.01$) and plasma corticosterone levels (+72%; $P < 0.01$) were increased in ANIT-treated mice. In contrast, atherosclerosis susceptibility was not increased in response to ANIT feeding, despite the concomitant increase in plasma free cholesterol (+30%; $P < 0.01$) and cholesteryl ester (+42%; $P < 0.001$) levels. The ANIT-induced hypercorticosteronemia coincided with marked immunosuppression as judged from the 50% reduction ($P < 0.001$) in circulating lymphocyte numbers. However, hepatic glucocorticoid signaling was not enhanced after ANIT treatment. It thus appears that the immunosuppressive effect of glucocorticoids is uncoupled from their metabolic effect under cholestatic disease conditions. In conclusion, we have shown that cholestatic liver disease-associated endogenous glucocorticoid overexposure does not increase atherosclerosis susceptibility in APOE-knockout mice. Our studies provide novel preclinical evidence for the observations that the hypercholesterolemia seen in cholestatic human subjects does not translate into a higher risk for atherosclerotic cardiovascular disease.

Key Words

- cholestatic liver disease
- glucocorticoid
- atherosclerosis
- mouse model
- lymphocyte
- hepatic gene expression

Journal of Endocrinology (2019) **242**, 1–12

Introduction

Glucocorticoids, members of the steroid hormone superfamily, are secreted into the bloodstream by the adrenals in response to stress-induced activation of the hypothalamus–pituitary–adrenal axis. Through an interaction with their cognate nuclear receptor in target tissues, glucocorticoids are able to impact many physiological processes to facilitate the body's response to stress. Activation of the glucocorticoid receptor (GR) leads to a change in the cellular expression of GR target genes involved in the body's metabolic and inflammatory

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responses to stress, which translates in – for example – an increase in the hepatic glucose production rate and immunosuppression (Kadmiel & Cidlowski 2013). In accordance with the notion that glucocorticoids play an essential role in restoring homeostasis after a stressful event, it is essential that plasma glucocorticoid levels remain within normal (physiological) ranges. More specifically, glucocorticoid insufficiency is associated with fatigue, muscle weakness, loss of appetite, weight loss and abdominal pain and a predisposition to loss of consciousness under stress conditions (Hannah-Shmouni & Stratakis 2018). Conversely, chronic glucocorticoid overexposure, for example in response to treatment of inflammatory disease patients with synthetic glucocorticoids such as dexamethasone or as a result of the presence of an adrenocorticotropic hormone-producing pituitary adenoma, represents the underlying cause of Cushing's syndrome or disease. Key features of Cushing's include the development of central obesity, hypertension and dyslipidemia and an increased predisposition to the development of atherosclerotic cardiovascular disease (Faggiano *et al.* 2003, Vettori *et al.* 2010, Giles *et al.* 2011, Lupoli *et al.* 2017).

Cholestasis is a pathological condition of the liver that results from a disruption in the flow of bile from the liver toward the intestine, that is due to gallstones. Initially, cholestatic liver disease is only associated with pruritus as a result of an increase in plasma bile acid levels. However, when left untreated, the pathology can evolve toward the development of cholangitis (bile duct inflammation) and primary biliary cirrhosis, a life-threatening condition that is characterized by hepatic fibrosis and liver dysfunction resulting in yellow discoloration of the skin (jaundice), abdominal pain, fevers and rigors. Although primary sclerosing cholangitis occurs somewhat more frequently in males (Lazaridis & LaRusso 2016), it should be noted that women make up to 90% of the primary biliary cirrhosis patient population (Kaplan & Gershwin 2005, Hohenester *et al.* 2009). Our previous studies in mice have shown that alpha-naphthylisothiocyanate (ANIT)-mediated induction of acute cholestatic liver disease is associated with a concomitant rise in the plasma level of corticosterone, the primary glucocorticoid species circulating in mice (van der Geest *et al.* 2016). These relatively high levels of glucocorticoids appear to contribute to the liver injury and hypercholesterolemia associated with the induction of cholestasis (van der Geest *et al.* 2016). Importantly, studies by Bell *et al.* (2015) have shown that serum levels of the physiologically active 11β-hydroxyl glucocorticoid, cortisol and the inactive 11-keto glucocorticoid, cortisone, are also significantly increased as compared to healthy controls in human subjects suffering from primary biliary cirrhosis and primary sclerosing cholangitis. As such, it can be suggested that stimulation of the adrenal glucocorticoid function is a general phenotype associated with the occurrence of cholestasis. Since a long-term increase in plasma glucocorticoid levels is generally associated with a higher risk for atherosclerotic cardiovascular disease in humans, we determined – in a preclinical setting – whether the chronic presence of cholestatic liver disease also induces a concomitant negative impact on atherosclerosis susceptibility.

Materials and methods

Animals

Apolipoprotein E (APOE)-knockout mice on a C57BL6/J genetic background were bred in the animal facilities at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research. Mice were held in rooms with controlled temperature and a 12 h/12 h darkness–light cycle. Given that female mice are generally more susceptible to develop atherosclerotic lesions as compared to male mice and, in humans, females are more susceptible to suffer from primary biliary cholestasis (Kaplan & Gershwin 2005, Hohenester *et al.* 2009), we chose to specifically use female mice to study the impact of cholestasis on atherosclerosis outcome. Hereto, age-matched 6-week-old female APOE-knockout mice were provided a regular chow diet supplemented with (N=10) or without (N=11) 0.025% w/w alpha-naphthylisothiocyanate (ANIT; Sigma-Aldrich) *ad libitum*. No apparent change in food intake was noted upon supplementation of diet with ANIT and average bodyweights were similar between control and ANIT-treated mice throughout the experiment. After 8 weeks of ANIT feeding, mice were anesthetized with a single subcutaneous injection of a mixture of xylazine (70 mg/kg), ketamine (350 mg/kg) and atropine (1.8 mg/kg) at 09:00 h. Upon anesthesia, blood was collected in EDTA-coated tubes through retro-orbital bleeding. Subsequently, the chest was opened and organs were perfused with PBS, isolated, weighed and frozen or stored in Shandon Formal-Fixx Neutral Buffered Formalin (Thermo Fisher Scientific). Experiments were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All experimental protocols were approved by the Ethics

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Committee for Animal Experiments of Leiden University (protocol #10217).

Plasma measurements

All measurements were performed in the *ad libitum* fed state. Triglyceride levels were measured using a colorimetric assay from Roche (Roche Diagnostics). Cholesterol levels were determined via an enzymatic colorimetric assay. In short, free cholesterol levels were determined by incubating plasma with cholesterol oxidase (0.025 U/mL; Calbiochem) and cholesterol peroxidase (0.065 U/mL; Sigma-Aldrich) in 1.0 M potassium phosphate buffer, containing 0.01 M phenol, 1 mM 4-aminoantipyrine, 7.5% methanol and 1% polyoxyethylene-9-laurylether. Plasma cholesterol ester levels were determined by adding cholesteryl esterase (0.003 U/mL; Calbiochem). Absorbance at 490 nm was measured using a spectrophotometer. The lipoprotein distribution was analyzed by fractionation of 30 µL pooled plasma using fast protein liquid chromatography (ÄKTAFPLC, GE Healthcare Life Sciences). Cholesterol content of the resulting fractions was determined using the enzymatic colorimetric assay described earlier, taking the recovery of the column into account. Plasma glucose levels were measured with an Accu-Check glucometer (Roche Diagnostics). Corticosterone levels were determined using the ImmuChem ^{125}I Corticosterone Radioimmunoassay Kit (MP Biomedicals, Santa Ana, CA, USA) according to manufacturer's instruction. Total bilirubin levels were measured using a Reflotron Plus clinical chemistry system and Reflotron bilirubin test strips according to manufacturer's instruction (Roche Diagnostics). Bile acid levels in plasma and in liver specimens were determined using liquid chromatography tandem mass spectrometry as described previously (Alnouti *et al.* 2008).

Blood cell analysis

Hematological analysis was performed on eye blood collected in EDTA-containing tubes using a XT-2000i Automated hematology Analyzer (Sysmex, Etten-Leur, The Netherlands) to determine the amount of circulating white blood cells and the subpopulation distribution.

Liver histology

The left lateral lobe of livers was fixed in formalin for approximately 24 h. After that, the lower part of the left lobe was embedded in paraffin and subsequently sectioned using a RM2235 rotary microtome (Leica Microsystems) at 8 µm intervals. Collagen content of the sections was analyzed using the Masson's Trichrome staining kit (Sigma-Aldrich). To visualize cholangiocyte proliferation, sections were incubated with a rabbit anti-mouse keratin 19 primary antibody (diluted 1:500; Novus Biologicals) and Dako goat anti-rabbit IgG-HRP secondary antibody (diluted 1:200; Agilent Technologies). The NovaRED peroxidase substrate kit (Vector Laboratories, Peterborough, United Kingdom) was used to develop the sections. Slides were counterstained with Mayer's Hematoxylin Solution (Sigma-Aldrich).

Gene expression analysis

Total RNA was isolated by guanidine thiocyanate-phenol-chloroform extraction and subsequently reverse transcribed into cDNA using RevertAid M-MulV reverse transcriptase (Thermo Fisher Scientific). Gene expression analysis was performed using SYBR Green technology on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are displayed in Table 1. Control samples without reverse transcriptase were included in the analysis to exclude false-positive signals. Relative gene expression levels were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of the housekeeping genes beta actin (ACTB), ATP synthase, H+ transporting Mitochondrial Fo complex subunit F (ATP5J), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex, subunit A, flavoprotein (SDHA) and raising two to the power of this difference.

Atherosclerotic lesion analysis

Hearts were fixed in formalin for approximately 24 h. Subsequently, hearts were embedded in O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and serial sections of the aortic root were made using a CM3050 S cryostat (Leica Microsystems) at 10 µm intervals. Sections were stained with Oil red O (Sigma-Aldrich) to visualize the lipid accumulation in the arterial wall. Atherosclerotic lesion size was quantified using QWin V3 software (Leica Microsystems) as the average Oil red O-positive lesion area (µm^2) in five consecutive sections of the aortic root per mouse, starting from the appearance of the three aortic valve leaflets. To determine the lesional macrophage content, three sections per slide were incubated with a rat anti-mouse MOMA-2 primary

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Table 1 Primers used for real-time PCR analysis.

Name	Gene ID	Forward	Reverse
ABCB4	NM_008830	GATAGCTATCTTTGGCCCTGGGGATG	TCTCTCCAGCTTTCCCAAACGTGAAG
ACTB	NM_007393	CTTCTTTGCAGCTCCTTCGTTGCCG	AATACAGCCCGGGGAGCATCGTC
ATP5J	NM_016755	CAGGGCCGGAAGTAGAACGG	AAGGACAGAGGAGAGCCTGAAGA
COL1A1	NM_007742	CATCCCTGGACAGCCTGGACTT	AGCCATAGGACATCTGGGAAGCAA
CYP11A1	NM_019779	AGAACATCCAGGCCAACATTACCGAG	AGGACTTCAGCCCGCAGCATC
CYP11B1	NM_001033229	TGCCATCCAGGCTAACTCAATGGAACT	AGGGCCTGCTGAACATCTGGGT
CYP21A2	NM_009995	GGGAACTGCCCAGCAAGTT	AGGATGGTGTTCTGGGATTCTTC
CYP7A1	NM_007824	GCTAAGACGCACCTCGTGATCC	GCTGCTTTCATTGCTTCAGGGC
FKBP5	NM_010220	GCTGGCAAACAACACGAGAG	GAGGAGGGCCGAGTTCATT
GAPDH	NM_008084	ATCCTGCACCACCAACTGCTTA	CATCACGCCACAGCTTTCCAG
HSD3B2	NM_153193	AGCCTTCCTGTGCCCCTACT	CAGGAGGAAGCTCACAGTTTCC
KRT19	NM_008471	GACCATCGAGGACTTGCGCGAC	AAGGCGTGTTCTGTCTCAAACTTGGT
NTCP	NM_001177561	GCCACACTATGTACCCTACGTCC	GTTGCCCACATTGATGACAGACAG
PEPCK	NM_011044	CATCTTTGGTGGCCGTAGACCTGAA	CTGCTCCTACAAACACCCATGCTG
PER1	NM_011065	TGGCTCAAGTGGCAATGAGTC	GGCTCGAGCTGACTGTTCACT
SDHA	NM_023281	TATAGGTGCAGAAGCTCGGAAGG	CCTGGATGGGCTTGGAGTAATCA
SHP	NM_011850	CCAAGGAGTATGCGTACCTGAAGGG	AAGACTTCACACAGTGCCCAGTGAG

antibody (diluted 1:200; Bio-Connect Diagnostics, Huissen, The Netherlands) and a goat anti-rat IgG-alkaline phosphatase secondary antibody (diluted 1:100; Sigma-Aldrich). The Dako BCIP/NBT Substrate System (Agilent Technologies) was used to visualize the macrophage-positive area (black color). The Trichrome Stain (Masson) Kit (Sigma-Aldrich) was used to visualize lesional collagen content. A Dako rabbit anti-mouse CD3 primary antibody (diluted 1:100; Agilent Technologies) and BrightVision poly-HRP-anti-rabbit secondary antibody (Immunologic, Gelderland, The Netherlands) were used to detect lesional T cell infiltration in two sections per mouse. The NovaRED peroxidase substrate kit (Vector Laboratories, Peterborough, United Kingdom) was used to visualize the T cells. Individual macrophage and collagen areas and T cell counts were corrected for respective total lesion areas. Quantification of these lesion indices was performed by an observed that was blinded to the group identity.

Data analysis

Statistical analyses were performed with Prism 6 Software (Graphpad Software). Normal distribution was verified for all data sets and Grubbs' test was used to detect outliers. The statistical significance of differences between groups was determined using a two-tailed Student's *t*-test or Mann–Whitney test in case of non-normal distributions. The level of statistical significance was set at $P<0.05$.

Results

To test our hypothesis that the presence of cholestasis is associated with high glucocorticoid levels that exacerbate atherosclerosis susceptibility, atherosclerosis-susceptible hypercholesterolemic apolipoprotein E (APOE)-knockout mice were subjected to dietary administration of the bile duct toxicant ANIT for 8 weeks. Previous studies have indicated that chronic administration (≥2 weeks) of the similar 0.025% (w/w) dose of ANIT induces the development of fibrotic cholestatic liver disease in normolipidemic wild-type mice (Sullivan *et al.* 2010, 2012). In accordance, substantial collagen deposits were visible in liver sections of our ANIT-treated APOE-knockout mice after Masson's Trichrome staining (Fig. 1A). In contrast, as can be appreciated from Fig. 1A, liver sections of APOE-knockout mice that had received the regular chow diet devoid of ANIT stained negative for collagen (no blue staining visible). We also could effectively reproduce the findings of Sullivan *et al.* (2012) that the chronic presence of cholestasis is paralleled by an increase in hepatic transcript levels of alpha-1 type I collagen (COL1A1). More specifically, we observed that relative mRNA expression levels COL1A1 were respectively 2.6-fold ($P<0.05$) higher in livers of ANIT-treated mice as compared to controls (Fig. 1B). Glaser *et al.* (2009) have previously shown that the surgery-mediated induction of cholestasis in C57BL/6 wild-type mice, that is through bile duct ligation, is associated with a bile duct hyperproliferation as evidenced by the presence of a relatively high number of keratin 19 (KRT19)-positive cholangiocytes. In agreement, a significant 3.7-fold increase ($P<0.001$) in liver KRT19 mRNA expression levels was detected in ANIT-treated APOE-knockout mice (Fig. 1C). Immunohistochemical staining verified the presence of many KRT19-positive bile ducts in liver sections from ANIT-treated mice, which were virtually

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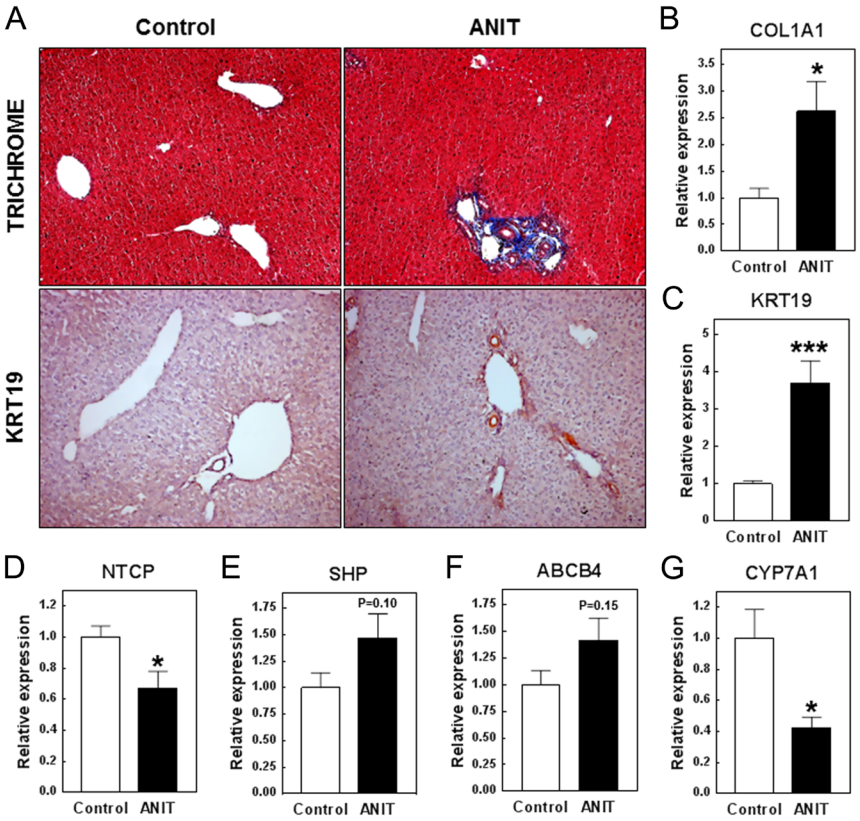

Figure 1
(A) Representative images show the increased keratin 19 protein (red staining; bottom panels) in ductular structures and the presence of periportal collagen deposits (blue staining; top panels) in sections from ANIT-treated mice versus reduced levels and absence, respectively, in controls. (B, C, D, E, F and G) Effect of ANIT treatment on hepatic mRNA expression levels of collagen type I alpha 1 chain (COL1A1), keratin 19 (KRT19) and several genes involved in bile acid metabolism. Data represent means + S.E.M. of 10 ANIT-treated and 11 control mice. *$P < 0.05$, ***$P < 0.001$ versus control. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0079.

absent in liver sections from regular chow diet-fed APOE-knockout control mice (Fig. 1A).

Humans subjects that suffer from cholestasis generally exhibit an increase in blood levels of the biochemical hepatocyte toxicity marker alanine transaminase (ALT), bilirubin and bile acids (Sugita *et al.* 2015). In accordance with the initial observation that ANIT-treated APOE-knockout mice display bile duct hyperproliferation, probably in an attempt to compensate the cholestasis-associated disruption of bile duct functionality, their plasma ALT levels were also 2.7-fold higher as compared to those of non-cholestatic mice (110 ± 34 U/L for ANIT-treated mice vs 40 ± 2 U/L for controls; $P<0.05$; Table 2). In addition, significant accumulation of bilirubin was detected in plasma of ANIT-treated APOE-knockout mice (+60%; $P<0.01$; Table 2). Although the difference in the

Table 2 Bilirubin and bile acid levels in plasma and liver samples of APOE-knockout mice fed a regular chow control diet or a chow diet supplemented with 0.025% ANIT for 8 weeks.

	Control ($N = 11$)	**ANIT** ($N = 10$)	***P* value** (*t*-test)
Plasma (μM)			
Bilirubin	9.00 ± 0.35	14.43 ± 1.27	<**0.01**
Cholic acid	0.12 ± 0.03	0.80 ± 0.56	0.21
Deoxycholic acid	0.18 ± 0.04	0.36 ± 0.16	0.19
Taurocholic acid	0.099 ± 0.008	2.4 ± 1.0	<**0.01**
Liver (μmol/100 g tissue)			
Alpha-muricholic acid	5.3 ± 0.6	4.5 ± 0.7	0.38
Beta-muricholic acid	7.3 ± 1.2	4.7 ± 0.8	0.11
Cholic acid	11.9 ± 2.8	5.7 ± 1.9	0.16
Chenodeoxycholic acid	0.54 ± 0.11	0.39 ± 0.08	0.32
Deoxycholic acid	2.0 ± 0.3	2.7 ± 0.3	0.073
Hyodeoxycholic acid	0.41 ± 0.09	0.62 ± 0.14	0.21
Omega-muricholic acid	2.8 ± 0.5	2.2 ± 0.5	0.39
Ursodeoxycholic acid	1.2 ± 0.3	1.0 ± 0.2	0.67

Data represent means ± S.E.M. Bold indicates statistical significance.

plasma levels of cholic acid and deoxycholic acid failed to reach significance (Table 2), combined levels of the three major bile acids species found in plasma of APOE-knockout mice, that is taurocholic acid, cholic acid and deoxycholic acid, were almost 10-fold higher after ANIT exposure (3.62±1.60 µM for ANIT-treated mice versus 0.36±0.06 µM for controls; $P<0.05$). In line with the observations from Sugita *et al.* (2015) that among all cholestatic liver disease subtypes biliary tract diseases are specifically associated with an increase in blood taurocholic acid levels, the overall increase in plasma bile acid concentrations could primarily be attributed to a marked increase in plasma taurocholic acid levels (24-fold higher; $P<0.01$; Table 2). The rise in plasma taurocholic acid levels coincided with as significant decrease (–27%; $P<0.05$) in the hepatic relative mRNA expression levels of the Na^+-taurocholate cotransporting protein (NTCP; Fig. 1D). This suggests that the ANIT-induced increase in plasma taurocholic acid levels was probably secondary to a reduced hepatic re-uptake of bile acids. NTCP transcription is a regulatory target of the nuclear bile acid receptor farnesoid X receptor/small heterodimer partner (FXR/SHP) pathway that aims to overcome cellular bile acid accumulation by reducing bile acid synthesis and re-uptake (Chiang 2009). As evident from Table 2, total bile acid concentrations were not significantly higher in livers of ANIT-treated mice. However, FXR did tend to be activated to a higher extent in response to ANIT treatment as judged from the trend toward an increase in the relative expression levels of the FXR target genes SHP (+47%) and ATP-binding cassette transport B4/multidrug resistance protein 2 (ABCB4/MDR2; +41%) (Fig. 1E and F). In further support of an active response to overcome hepatic bile acid overload, relative mRNA expression levels of cholesterol 7alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in the classical bile acid synthesis pathway, were significantly reduced (–58%; $P<0.05$) in livers of ANIT-treated mice as compared to those of control mice (Fig. 1G).

Based upon our previous studies regarding the effect of acute cholestasis on the adrenal glucocorticoid function (van der Geest *et al.* 2016) we hypothesized that chronic ANIT treatment would be associated with an enhanced glucocorticoid output. Basal plasma corticosterone levels in our regular chow diet-fed APOE knockout mice were already higher than those normally found in chow diet-fed normolipidemic wild-type mice (~200 ng/mL vs 50–150 ng/mL) (Hoekstra *et al.* 2008, 2009, 2010, 2013), which fits with previous findings from others (Thorngate *et al.* 2002, Grootendorst *et al.* 2004). However, the presence of fibrotic cholestatic liver disease in ANIT-treated mice was still paralleled by a significant rise in plasma corticosterone levels (+72%; $P<0.01$; Fig. 2A). As a result, plasma corticosterone in ANIT-treated APOE-knockout mice reached levels close to those previously found in wild-type mice subjected to fasting stress or an inflammatory stress trigger, that is lipopolysaccharide exposure (Hoekstra *et al.* 2009, 2010, 2013, van der Sluis *et al.* 2015, Hoekstra & Van Eck 2016). The basal rate of glucocorticoid production is essentially determined by the ability of adrenocortical cells to convert cholesterol into corticosterone. As can be appreciated from Fig. 2B, no significant changes were observed in the adrenal relative mRNA expression levels of CYP11A1, HSD3B2, CYP21A1 or CYP11B1 that respectively facilitate the conversion of cholesterol into pregnenolone, pregnenolone into progesterone, progesterone into deoxycorticosterone and deoxycorticosterone into corticosterone. However, the weight of the adrenals was significantly higher in ANIT-treated mice as compared to controls (0.060±0.003% vs 0.049±0.002%; $P<0.01$; Fig. 2C). These observations suggest that the ANIT treatment-associated increase in plasma glucocorticoid levels resulted from adrenal hyperplasia without a change in the steroidogenic activity of the adrenocortical cells.

Strikingly, the cholestasis-associated increase in plasma glucocorticoid levels did not translate into an increased susceptibility for the development of atherosclerotic lesions. The extent of atherosclerosis in aortic root area was actually a bit decreased in ANIT-treated mice as compared to control chow diet-fed mice. The aortic root of control mice exhibited early staged, macrophage-rich atherosclerotic lesions of an average total size of $191\pm19\times10^3$ µm², while lesion sizes were only $138\pm16\times10^3$ µm² in ANIT-treated mice ($P<0.05$; Fig. 3A and B). Lesional macrophage and collagen contents were highly similar in the two groups of mice (Fig. 3A and B). Given that the effect of a particular intervention on atherosclerosis outcome can theoretically depend on the anatomical location studied, we also determined the expression levels of the macrophage gene marker CD68 in aortic arch specimens as surrogate measure for the aortic atherosclerosis extent. Aortic CD68 mRNA expression levels were only marginally lowered in ANIT-treated mice (–16%; $P>0.05$; data not shown). It thus appears that the chronic presence of cholestasis does not induce a major effect on overall atherosclerosis susceptibility in APOE-knockout mice.

Hyperlipidemia is an established risk factor for the development of atherosclerotic lesions both in humans

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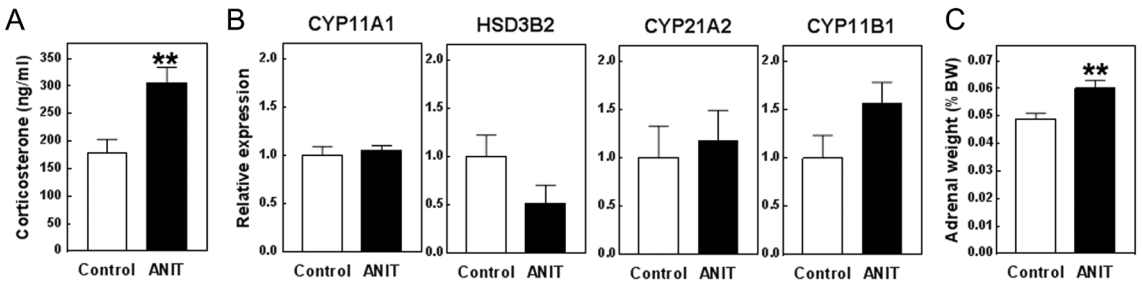

Figure 2
Effect of ANIT treatment on plasma corticosterone levels (A), adrenal relative mRNA expression levels of steroidogenic genes involved in the conversion of cholesterol into corticosterone (B), and body weight-corrected adrenal weights (C). Data represent means + S.E.M. of 10 ANIT-treated and 11 control mice. $^{**}P < 0.01$ versus control.

and mice (Emerging Risk Factors Collaboration *et al.* 2009, Silverman *et al.* 2016). Plasma lipid levels were measured to exclude the possibility that the suggested detrimental effect of hypercorticosteronemia on atherosclerosis outcome was compensated for by an unanticipated reduction in the hyperlipidemia extent. Plasma triglyceride levels were not significantly different between the two groups (Fig. 4A). However, ANIT treatment was actually associated with a significant increase in plasma levels of both free cholesterol (+31%; $P<0.01$) and cholesteryl esters (+42%; $P<0.001$) (Fig. 4A). Lipoprotein distribution analysis, performed after 8 weeks of ANIT treatment, revealed that the higher cholesterol levels in ANIT-treated mice could be attributed to an increase in the level of

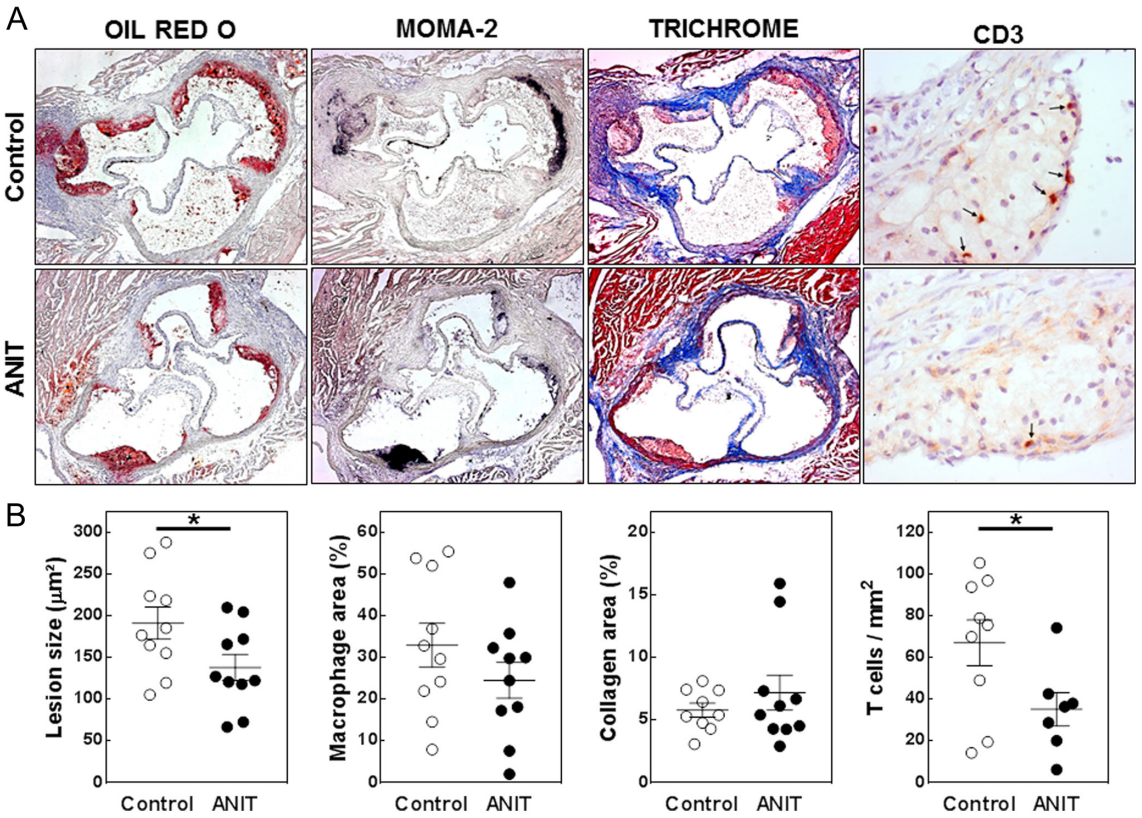

Figure 3
(A) Representative images showing Oil red O stainings (red; left panels) for total lesion size, MOMA-2 stainings (black; left-middle panels) for macrophage content, Trichrome stainings (blue; right-middle panels) for collagen content and CD3 stainings (arrows; right panels) for T cell content on sections from ANIT-treated mice and controls. (B) Quantification of absolute lesion sizes and relative lesional macrophage, collagen and T cell contents. Data in graphs represent individual data points and group means + S.E.M. (horizontal line). $^{*}P < 0.05$ versus control. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0079.

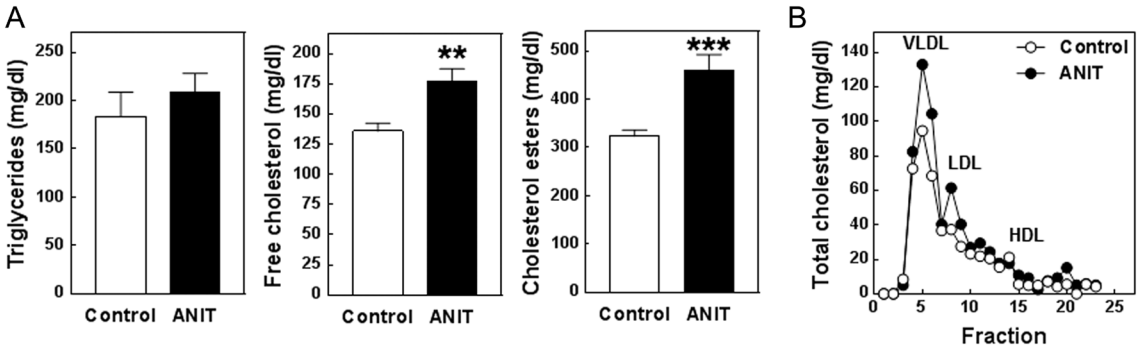

Figure 4
Effect of ANIT treatment on non-fasting plasma triglyceride, free cholesterol and cholesterol ester levels (A), and the cholesterol distribution over the different lipoprotein fractions (B). Data represent means + S.E.M. of 10 ANIT-treated and 11 control mice. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

cholesterol associated with lipoprotein subclasses with a relatively lower density, since no change was detected in the amount of cholesterol associated with high-density lipoproteins (HDL) (Fig. 4B).

To possibly uncover why ANIT-treated mice did not show an enhanced atherosclerosis susceptibility in the context of hypercorticosteronemia as well as exacerbated hypercholesterolemia, we also investigated the effect of ANIT treatment on the different downstream (signaling) effects of glucocorticoids. In accordance with an enhanced immunosuppressive action as a result of the higher glucocorticoid levels, the number of circulating white blood cells was 44% lower in cholestatic mice as compared to non-diseased mice ($4.25 \pm 0.38 \times 10^9$/L vs $7.52 \pm 0.35 \times 10^9$/L; $P < 0.001$). The decrease in total leukocyte numbers found in response to ANIT treatment was exclusively related to a 50% decrease ($P < 0.001$) in blood lymphocyte numbers, since blood concentrations of neutrophils, monocytes and eosinophils were virtually identical in the two groups of mice (Fig. 5A). Notably, the decrease in blood lymphocyte numbers was paralleled by a 48% decrease in lesional T cell content ($P < 0.05$; Fig. 3A and B). In sharp contrast to the evident immunosuppression in ANIT-treated mice, the presence of cholestasis did not seem to impact the extent of hepatic (metabolism-related) GR signaling. Relative mRNA expression levels of the sensitive glucocorticoid/GR target genes *PEPCK*, *PER1* and *FKBP5* were unaltered in liver after chronic ANIT exposure (Fig. 5B). In further support of an unchanged metabolic status, plasma glucose levels were also not different between ANIT-treated mice and regular chow diet-fed controls (Fig. 5C). From these combined

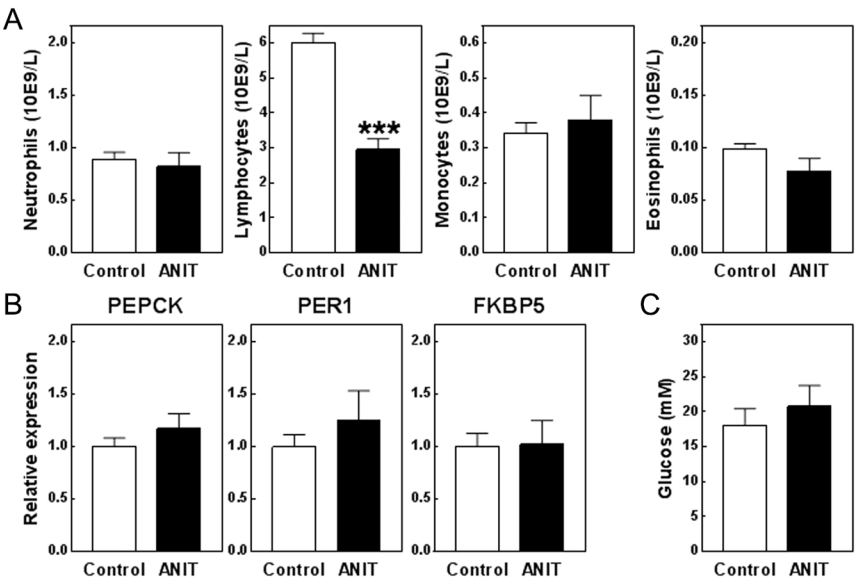

Figure 5
Effect of ANIT treatment on blood leukocyte concentrations (A), hepatic relative mRNA expression levels of established glucocorticoid target genes (B) and plasma glucose levels (C). Data represent means + S.E.M. of 10 ANIT-treated and 11 control mice. $^{***}P < 0.001$ versus control.

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findings, it appears that the immunosuppressive effect of glucocorticoids is uncoupled from their metabolic effect under cholestatic disease conditions.

Discussion

Here we have shown that the chronic presence of cholestasis in regular chow diet-fed APOE-knockout mice is not associated with an increased atherosclerosis susceptibility despite the fact that plasma levels of both glucocorticoids and cholesterol were significantly higher in cholestatic than in non-cholestatic APOE-knockout mice.

The unchanged atherosclerosis susceptibility in the context of exacerbated hypercholesterolemia in ANIT-treated mice contrasts the general view that plasma cholesterol levels are directly correlated to the (atherosclerotic) cardiovascular disease risk (Emerging Risk Factors Collaboration *et al.* 2009, Silverman *et al.* 2016). However, our finding that the extent of hypercholesterolemia and the susceptibility for the development of atherosclerosis are uncoupled in under cholestatic conditions does fit with several previous clinical observations. A small-scale study by Crippin *et al.* has suggested that the hyperlipidemia associated with primary biliary cirrhosis does not increase the risk for atherosclerotic death (Crippin *et al.* 1992) In agreement, Allocca *et al.* (2006) observed that the carotid intima media thickness, a surrogate measure for the extent of atherosclerotic cardiovascular disease, is significantly lower in (hypercholesterolemic) primary biliary cirrhosis patients than in non-cholestatic hypercholesterolemic human subjects. Furthermore, Longo *et al.* (2002) have also observed that the hypercholesterolemia associated with longstanding cholestasis does not translate into an excess risk of cardiovascular disease. Studies by Van Dam & Gips (1997) even detected a reduced risk for the development of fatal and non-fatal myocardial infarctions and other cardiovascular events in primary biliary cirrhosis patients. The appearance of lipoprotein X (LpX), an abnormal low-density lipoprotein particle that is poor in cholesteryl esters and triglycerides, is a common feature in human cholestatic subjects as well as in a variety of cholestasis animal models (Elferink *et al.* 1998). A study by Chang *et al.* (2004) has suggested that that LpX can reduce the atherogenicity of LDL particles. We have performed gel electrophoresis and silver staining on plasma samples of the two groups of mice to visualize LpX. However, we have not been able to provide conclusive evidence whether LpX was indeed present in our cholestatic samples. Despite the fact that the plasma free cholesterol/cholesterol ester ratio was not different between ANIT-treated mice and controls, it cannot be excluded that the exacerbated hypercholesterolemia in our ANIT-treated mice is, at least in part, due to the appearance of free cholesterol-containing LpX particles. Given that our remaining resources, for example plasma amounts, of the current study are too limited, additional (more mechanistically oriented) studies are warranted to uncover the possible relevance of the presence of LpX in the apparent resistance of ANIT-treated mice to the expected hypercorticosteronemia-induced rise in atherosclerosis susceptibility.

ANIT treatment was associated with a marked decrease in the number of circulating white blood cells, probably as a result of an enhanced immunomodulatory action related to the rise in plasma glucocorticoid levels. The clinical relevance of this particular finding is corroborated by the fact that a decrease in blood lymphocytes is a well-recognized finding in human primary biliary cirrhosis patients. Sandilands *et al.* (1977) detected a ~40% decrease in total blood leukocyte numbers in primary biliary cirrhosis patients, which could be primarily attributed to a decrease in the absolute number of (activated) T cells and natural killers cells. Studies by Bhan *et al.* (1982) have further highlighted that during the early stages of the disease, the decrease in blood lymphocyte concentrations in primary biliary cirrhosis is mainly driven by a decrease in the percentage of $CD3^+$ inducer T cells. Importantly, Ensrud & Grimm (1992) and Kannel *et al.* (1992) have observed that a higher number of total circulating white blood cells, that is lymphocytes, is associated with a relatively higher risk of cardiovascular events and death. In support, studies in recombination-activating gene 1 (RAG1) × APOE double-knockout mice have shown that lymphocyte deficiency lowers the atherosclerosis susceptibility in chow diet-fed APOE-knockout mice (Dansky *et al.* 1997). In this light, it can be suggested that the decrease in white blood cell/lymphocyte counts and the lower number of lesional T cells in ANIT-treated mice may have counterbalanced the anticipated increase in atherosclerosis susceptibility resulting from the exacerbated hypercholesterolemia.

An interesting finding of our studies was that the inflammatory and metabolic functions of glucocorticoids seem to be uncoupled under cholestatic conditions. Based upon our previous findings in adrenalectomized/adrenal transplanted mice (van der Sluis *et al.* 2012), we anticipated that the increase in plasma corticosterone levels observed

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in response to ANIT feeding would result in a (over) stimulation of hepatic GR signaling and a reduction in blood leukocyte concentrations. In contrast, the apparent immunosuppressive action in ANIT-treated mice was not paralleled by a concomitant increase in metabolic GR target gene expression levels or change in plasma glucose levels. We did not perform dedicated studies to uncover the reason behind this discrepancy. However, we assume that the failure of the high plasma glucocorticoid levels to increase liver GR signaling resulted from the fact that bile acid-induced cellular signaling is enhanced in hepatocytes under cholestatic conditions. Borgius *et al.* (2002) have shown that the nuclear receptor SHP can act as a functional inhibitor of GR transcriptional activity. As such, bile acid-induced activation of the FXR/SHP pathway may have alleviated the hepatic GR hyperactivation and overcome the expected hypercorticosteronemia-associated metabolic disturbances. Importantly, the negative effect of hypercortisolemia on global metabolism has been suggested to serve as the primary underlying cause of the increased cardiovascular disease risk in Cushing's disease patients (Pivonello *et al.* 2017). The absence of an impact on metabolism can thus perhaps already explain why atherosclerosis susceptibility was not increased after ANIT treatment.

Clinical studies have suggested that subjects suffering from (end-stage) cholestatic liver disease exhibit a diminished adrenal glucocorticoid function. More specifically, 72% of a cohort of patients with liver disease who were admitted to a liver transplant intensive care unit met the criteria for adrenal insufficiency (Marik *et al.* 2005). Furthermore, patients with end-stage liver disease awaiting transplantation also generally display an impaired ability to increase their plasma cortisol levels in response to an established steroidogenic trigger, that is, adrenocorticotropic hormone exposure (McDonald *et al.* 1993). When taking these findings into account, our observation from the current and previous study (van der Geest *et al.* 2016) that corticosterone levels are increased in ANIT-treated mice may be conceived as contradictory. However, it should be noted that we have measured corticosterone levels under the non-stressed/non-stimulated conditions. As such, it is very well possible that adrenals from ANIT-treated mice also would display a diminished capacity to induce their glucocorticoid output in response to a steroidogenic (stress) trigger. In accordance with the notion that the induction of cholestasis is able to disrupt the adrenal glucocorticoid responsiveness to stress also in rodents, bile duct resection for only 5 days already reduced maximal plasma corticosterone levels in rats (Swain *et al.* 1993). The adrenal insufficiency detected in liver disease patients appears to be related to a decrease in HDL cholesterol levels (Spadaro *et al.* 2015), which suggests that the availability of exogenous cholesterol substrate used for steroidogenesis is too limited in these human subjects. In accordance, plasma HDL cholesterol levels are also reduced in rats after bile duct ligation (Sewnath *et al.* 2000). In contrast, we did not observe a marked change in the plasma level of cholesterol in the HDL fraction. The absence of an effect in our current experimental setting could be explained by the fact that we utilized mice genetically lacking APOE, since a specific enrichment of HDL fractions with APOE has previously been observed under cholestatic conditions (Mitamura 1984). Notably, the observed increase in basal glucocorticoid levels did not appear to be secondary to changes in adrenocortical cell functionality, but rather the result of adrenal growth as judged from the increased adrenal weight detected in ANIT-treated mice. Studies by Palmer & Heywood (1974) have shown that, in monkeys, chronic oral treatment of pregnant females with the bile acid species chenodeoxycholic acid is associated with an increased fetal adrenal weight. Given that the overall exposure of adrenals to bile acids was stimulated in our cholestatic mice as a result of the ~10-fold higher plasma bile acid level, it can be suggested that this contributed, at least in part, to the apparent adrenal growth. However, the exact underlying reason for the development of adrenomegaly in cholestatic mice remains to be determined.

In conclusion, we have shown that the cholestatic liver disease-associated endogenous glucocorticoid overexposure does not increase atherosclerosis susceptibility in regular chow diet-fed APOE-knockout mice. Follow-up studies that focus at different vascular beds (i.e. both the aortic sinus and the thoracic artery) in mice fed an atherogenic diet Western-type diet are warranted. However, our current findings do already provide the first preclinical evidence for the hypothesis, originally derived from several small-scale clinical studies (Van Dam & Gips 1997, Longo *et al.* 2002, Allocca *et al.* 2006), that the exacerbated hypercholesterolemia in cholestatic subjects does not translate into a higher risk for atherosclerotic cardiovascular disease. Furthermore, our data imply that activation of the FXR/SHP pathway can uncouple the immunomodulatory and metabolic actions of glucocorticoids. Since preclinical studies have suggested an anti-atherogenic effect of FXR agonist treatment (Hartman *et al.* 2009, Hambruch *et al.* 2012), we consider it of interest to evaluate whether chronic treatment of inflammatory disease patients with synthetic

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glucocorticoids (i.e. dexamethasone) in combination with a FXR agonist is able to reverse the associated cardiovascular disease risk in the context of efficient glucocorticoid-driven immunosuppression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Dutch Heart Foundation (Grants 2008T070 and 2012T080 to Menno Hoekstra) and the Netherlands Organization for Scientific Research (VICI Grant 91813603 to Miranda Van Eck). Miranda Van Eck is an Established Investigator of the Dutch Heart Foundation (Grant 2007T056).

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Received in final form 26 April 2019
Accepted 29 April 2019
Accepted Preprint published online 29 April 2019