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RESEARCH ARTICLE

Beta-carotene affects gene expression in lungs of male and female $Bcmo1^{-/-}$ mice in opposite directions

**Yvonne G. J. van Helden · Roger W. L. Godschalk ·
Hans J. M. Swarts · Peter C. H. Hollman ·
Frederik J. van Schooten · Jaap Keijer**

Abstract Molecular mechanisms triggered by high dietary beta-carotene (BC) intake in lung are largely unknown. We performed microarray gene expression analysis on lung tissue of BC supplemented *beta-carotene 15,15′-monooxygenase 1* knockout ($Bcmo1^{-/-}$) mice, which are—like humans—able to accumulate BC. Our main observation was that the genes were regulated in an opposite direction in male and female $Bcmo1^{-/-}$ mice by BC. The steroid biosynthetic pathway was overrepresented in BC-supplemented male $Bcmo1^{-/-}$ mice. Testosterone levels were higher after BC supplementation only in $Bcmo1^{-/-}$ mice, which had, unlike wild-type ($Bcmo1^{+/+}$) mice, large variations. We hypothesize that BC possibly affects hormone synthesis or metabolism. Since sex hormones influence lung cancer risk, these data might contribute to an explanation for the previously found increased lung cancer risk after BC supplementation (ATBC and CARET studies). Moreover, effects of BC may depend on the presence of frequent human BCMO1 polymorphisms, since these effects were not found in wild-type mice.

Introduction

Beta-carotene (BC) is a lipid-soluble provitamin, and a major dietary source of vitamin A. BC itself is thought to behave as a health-promoting agent. This has been attributed to its ability to efficiently scavenge radicals and thereby prevent macromolecular damage [1]. Furthermore, epidemiological studies focusing on dietary intakes of BC have indicated that a high BC intake is associated with a reduced risk for the development of cardiovascular diseases and several types of cancer [2, 3]. Moreover, BC is a provitamin A, and therefore its consumption prevents vitamin A deficiency, which is of importance even in Western society. For example, pregnant and lactating women are at risk for vitamin A deficiency in the Western society due to the discouragement of the consumption of food containing high amounts of vitamin A, in combination with their increased requirements [4].

Although BC is mainly considered to be a health-promoting agent, large-scale intervention trials failed to confirm its beneficial properties. Two intervention trials even showed an increased lung cancer risk and increased mortality because of lung cancer in smokers after BC supplementation (CARET study and ATBC study) [5, 6]. A 6-year follow-up of the CARET study showed differences in lung cancer risk in response to BC supplementation between male and female volunteers, while the ATBC study was only executed in male volunteers. In the CARET study, male subjects had an increased lung cancer risk upon BC supplementation, but only for the duration of the BC

Y. G. J. van Helden · H. J. M. Swarts · J. Keijer (✉)
Human and Animal Physiology, Wageningen University,
P.O. Box 338, 6700 AH Wageningen, The Netherlands
e-mail: Jaap.Keijer@wur.nl

Y. G. J. van Helden · R. W. L. Godschalk · F. J. van Schooten
Department of Health Risk Analysis and Toxicology,
Research Institute NUTRIM, Maastricht University,
Maastricht, The Netherlands

Y. G. J. van Helden · P. C. H. Hollman
RIKILT-Institute of Food Safety, Wageningen, The Netherlands

intervention. On the other hand, BC-supplemented females had an increased lung cancer risk that persisted even after the intervention had stopped, and it took several years before the lung cancer risk in females reached the levels observed in the controls [7]. The BC-induced increase in lung cancer risk is thought to occur only in smokers or asbestos-exposed subjects since one large-scale intervention trial with mainly non-smokers showed no effect of BC on lung cancer [8, 9]. Although the effect of BC on lung carcinogenesis has been studied extensively, the mechanism by which BC increases lung cancer risk is still not precisely known. The main reason for this is that research on molecular pathways influenced by BC is hampered by the lack of an appropriate animal model that also allows for the use of state-of-the-art functional genomic tools, such as commercially available microarrays. Mice and rats are animal models for which these tools are commercially available, but they differ greatly in BC metabolizing activity compared to humans. The main cause for this is the presence of a more active beta-carotene 15, 15′-monooxygenase 1 (Bcmo1) enzyme variant in rodents [10]. Bcmo1 is the key enzyme in BC metabolism, and in rodents, Bcmo1 cleaves almost all absorbed BC. Since rodents metabolize BC to a high degree, BC supplementation results in an increase in BC metabolites rather than the accumulation of BC, and rodents are therefore inadequate animals to study the effects of BC in humans [10].

Not only inter-species differences in Bcmo1 activity have been described, but also large inter-individual differences in BCMO1 activity are present between humans. Around 25–45% of the volunteers participating in BC conversion studies have been classified as poor converters [11–13]. These inter-individual differences in BC metabolism are at least partly related to polymorphisms in the BCMO1 gene, which results in a reduced BCMO1 activity [14, 15]. Of the reported polymorphisms, there are two frequently occurring polymorphisms with variant allele frequencies of 42 and 24%. As a result, individuals with these polymorphisms have relatively low vitamin A and high BC plasma concentrations compared to their wild-type counterparts [14]. Since differences in activity of BCMO1 result in different BC metabolite concentrations, the activity of BCMO1 might therefore have an effect on previously reported BC-induced effects.

The aim of our study was to investigate gene expression changes induced by dietary BC to try to explain the previously found harmful effects of BC in the lung. For this purpose we used a recently described mouse model, which has no functional Bcmo1 enzyme and which therefore displays increased BC plasma concentrations upon BC supplementation [16]. We performed whole-genome microarray gene expression analysis on lung tissue of both female and male wild-type ($Bcmo1^{+/+}$) and Bcmol

knockout ($Bcmo1^{-/-}$) mice with or without 14 weeks of 150 mg/kg BC supplementation. We previously analyzed the effects of BC in female $Bcmo1^{-/-}$ mice, and the major effect observed was that BC supplementation counteracted inflammation associated gene expression effects, which were most likely caused by an increased vitamin A demand in $Bcmo1^{-/-}$ mice [17]. This effect was not observed in male $Bcmo1^{-/-}$ mice, where two genes, Frizzled 6 (Fzd6) and Collagen triple helix receptor containing 1 (Cthrc1) were strongly downregulated by BC supplementation, which was not observed in female mice [18]. Thus, the major BC-affected processes seemed different between both genders. However, both females and males displayed increased cancer risk after BC supplementation during the intervention of the CARET study. Therefore, we aimed to specifically focus on those genes that are commonly regulated by BC supplementation in both genders, i.e., in male as well as female $Bcmo1^{-/-}$ mice.

Materials and methods

Animals and treatment

The experimental setup has been described previously (females, [17] and males [18]). Twelve female (fe) and 12 male (ma) B6129SF1 ($Bcmo1^{+/+}$) and 12 female and 12 male B6;129S-$Bcmo1^{tm1dnp}$ ($Bcmo1^{-/-}$) mice [16] were used for the dietary intervention, which was conducted in accordance with the German animal protection laws by the guidelines of the local veterinary authorities. During the breeding and weaning periods of the mice, mothers were maintained on KLIBA 3430 chow containing 14,000 IU vitamin A/kg diet (Provima Kliba AG, Kaiseraugst, Switzerland). Five-week-old female and male $Bcmo1^{+/+}$ and $Bcmo1^{-/-}$ mice were caged in groups containing two to four siblings per group and were maintained under environmentally controlled conditions (temperature 24°C, 12 h/12 h light/dark cycle). Mice had ad libitum access to feed and water. Basic feed consisted of the pelletized diet D12450B (Research Diets Inc, USA) with a fat content of 10%. The diet was modified to contain 1,500 IU vitamin A/kg of diet, which is a vitamin A-sufficient diet, and the control diet (control) was supplemented with water-soluble vehicle beadlets (DSM Nutritional Products Ltd., Basel, Switzerland) containing DL-alpha-tocopherol and ascorbyl palmitate as stabilizers, as well as carriers such as gelatin, corn oil sucrose and starch. The BC diet (BC) was supplemented with identical water-soluble beadlets containing BC (DSM Nutritional Products Ltd., Basel, Switzerland) to generate 150 mg BC/kg diet. Beadlets were added by the manufacturer before low temperature pelletting. Feed pellets were color marked and stored at 4°C in the dark.

After 14 weeks of dietary intervention, six female and six male $Bcmo1^{+/+}$ mice on the control diet ($Bcmo1^{+/+}$ Co), six female and six male $Bcmo1^{+/+}$ mice on the BC diet ($Bcmo1^{+/+}$ BC), three female and six male $Bcmo1^{-/-}$ mice on the control diet ($Bcmo1^{-/-}$ Co), and three female and six male $Bcmo1^{-/-}$ mice on the BC diet ($Bcmo1^{-/-}$ BC) were randomly killed on three subsequent mornings. Due to an insufficient number of female $Bcmo1^{-/-}$ mice in the original breeding pool, six additional female $Bcmo1^{-/-}$ mice were used that were born 2 weeks later from an identical experiment, three were fed the control diet and three the BC diet, to generate $n = 6$ for each group. These mice were killed 2 weeks after the first group of mice. Blood was collected from the vena cava after isoflurane and ketamin anesthesia. Blood was coagulated for at least 20 min at room temperature, cooled to 4°C and centrifuged. Lung tissue was removed, rinsed in phosphate-buffered saline (PBS) and snap frozen in liquid nitrogen. The lung tissues were stored at −80°C.

RNA isolation

Left lung lobes were homogenized in liquid nitrogen using a cooled mortar and pestle. Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands) followed by purification using RNeasy columns (Qiagen, Venlo, The Netherlands) using the instructions of the manufacturer. RNA concentration and purity were measured using the Nanodrop system (IsoGen Life Science, Maarsen, The Netherlands). Approximately 30 μg of total RNA was isolated with A_{260}/A_{280} ratios above 2 and A_{260}/A_{230} ratios above 1.9 for all samples, indicating good RNA purity. RNA degradation was checked on the Experion (Bio-Rad, Veenendaal, The Netherlands) using Experion StdSense chips (Bio-Rad). Two samples did not meet RNA quality (female $Bcmo1^{+/+}$ mice, one on BC diet and one on control diet) and were omitted from the experiment.

Microarray hybridization procedure

The 4×44 k Agilent whole mouse genome microarrays (G4122F, Agilent Technologies, Inc. Santa Clara, CA) were used. Preparation of the sample and the microarray hybridization were carried out according to the manufacturer's protocol with a few exceptions as described previously [19, 20]. In brief, cDNA was synthesized from 1 μg lung RNA using the Agilent Low RNA Input Fluorescent Linear Amplification Kit for each animal without addition of spikes. Thereafter, samples were split in two equal amounts to synthesize Cyanine 3-CTP (Cy3) and Cyanine 5-CTP (Cy5) labeled cRNA using half the amounts per dye as indicated by the manufacturer (Agilent Technologies). Labeled cRNA was purified using RNeasy columns

(Qiagen). Yield, A_{260}/A_{280} ratio, and Cy3 or Cy5 incorporation were examined for every sample using the nanodrop. All samples met the criteria of a cRNA yield higher than 825 ng and a specific activity of at least 8.0 pmol Cy3 or Cy5. Then 1,200 ng of every Cy3-labeled cRNA sample was pooled and used as a common reference pool. Individual 825 ng Cy5-labeled cRNA and 825 ng pooled Cy3-labeled cRNA were fragmented in $1\times$ fragmentation and $1\times$ blocking agent (Agilent Technologies) at 60°C for 30 min and thereafter mixed with GEx Hybridization Buffer HI-RPM (Agilent Technologies) and hybridized in a 1:1 ratio at 65°C for 17 h in an Agilent Microarray hybridization Chamber rotating at 4 rpm. After hybridization, slides were washed according to the wash protocol with Stabilization and Drying solution (Agilent Technologies). Arrays were scanned with an Agilent scanner with 10 and 100% laser power intensities (Agilent Technologies).

Data analyses of microarray results

Signal intensities for each spot were quantified using Feature Extraction 9.1 (Agilent Technologies). Median density values and background values of each spot were extracted for both the experimental samples (Cy5) and the reference samples (Cy3). Quality control for every microarray was performed visually, by using quality control graphs from Feature Extraction, M-A plots and boxplots that were made using limmaGUI in R (Bioconductor) [21]. Data were imported into GeneMaths XT 2.0 (Applied Maths, Sint-Martens-Latem, Belgium).

The median Cy5 signal intensity per spot per array was used for the individual gene expression, while the median Cy3 signal intensity was used for normalization. Since values close to the background may give aberrant fold changes between groups, spots with an average Cy5 or Cy3 median density value below two times the average of the background density were discarded. Spots with an average of the median Cy5 and Cy3 densities twice above the average of the background density were selected and log transformed. Thereafter, the log transformed Cy5 median density value was normalized against the fold change in the log-transformed Cy3 median density value compared to the average log-transformed Cy3 median density value for each spot as described before [22]. Pathway analysis was performed using Gene-Ontology (GO) overrepresentation analysis (ErmineJ) [23], and classification of genes was based on OMIM (www.ncbi.nlm.nih.gov), genecards (www.genecards.org) and literature mining.

Analysis of mRNA expression by real-time Q-PCR

Differential gene expression of *lecithin-retinol acyltransferase* (*phosphatidylcholine-retinol-O-acyltransferase*)

(*Lrat*), *glutamate receptor, ionotropic, AMPA1 (alpha 1)* (*Gria1*) and *endothelin 1* (*Edn1*) was analyzed using quantitative PCR (Q-PCR) to validate microarray results. Then 1 µg RNA isolated from lung of individual animals was converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). One sample was taken along without reverse transcriptase to examine the presence of DNA (without RT reaction). An equal amount of cDNA of all individual samples was pooled and diluted ($10\times$, $31.6\times$ $100\times$, $316\times$ $1,000\times$, $3,160\times$, $10,000\times$) for the generation of a calibration curve to test the efficiency of the Q-PCR reaction. Each PCR reaction (25 µl) contained $1\times$ iQ SYBR green supermix (Bio-Rad), 10 µM sense primer, 10 µM antisense primer and 2 µl 100 times diluted cDNA. Primers for *Lrat* were sense 5′-GCTCTCGGATCAGTCCACAGGC-3′ and antisense 5′-TCCCAAGACAGCCGAAGCAAGA-3′, for *Gria1* sense 5′-TGGTGGTGGTGGACTGTGAATC-3′ and antisense 5′-CAGGTTGGCGAGGATGTAGTGG-3′ and for *Edn1* sense 5′-AGACCAGGCAGTTAGATGTCAGTG and antisense 5′-TCGTAGTTTCCTTCCTTCCACCAG. For the amplification of the reference genes we used *syntaxin 5a* (*Stx5a*) with sense 5′-TTAAAGAACAGGAGG AAACGATTCAGAG-3′ and antisense 5′-CAGGCAAGG AAGACCACAAAGATG-3′, and *ring finger protein 130* (*Rnf130*) with sense 5′-ACAGGAACCAGCGTCGTCT TG-3′ and antisense 5′-ACCCGAACAACATCATTCTGC TTATAG-3′, which showed equal gene expression levels for all individual animals on the microarray. These intron-spanning primers were designed using Beacon designer 7.00 (Premier Biosoft International, Palo Alto, CA) or using primer-BLAST (http://www.ncbi.nlm.nih.gov/). Amplification was performed in duplicate using the MyIQ single-color real-time PCR detection system (Bio-Rad) using the following temperature cycles: $1\times$ 3 min at 95°C, $40\times$ 2-step amplification (15 s 95°C, 45 s 60°C), $1\times$ 1 min 95°C, and $1\times$ 1 min 65°C followed by melting curve analysis ($60\times$ 10 s 65°C with an increase of 0.5°C/10 s. A negative control without cDNA template and the minus reverse transcriptase sample were taken along with every assay. Using the standard curves for every gene, the relative level of expression of all genes was calculated. Normalized Gene Expression ($\Delta\Delta$Ct) analysis was calculated using the IQ5 software version 2.0 (Bio-Rad).

Testosterone measurement

Testosterone concentrations were measured using radio immunoassays (DSL 4100, Diagnostic Systems Laboratories, Beckman Coulter, Assendelft, The Netherlands) and were performed according to the manufacturer's protocol using quarters of all volumes as has been described previously [24]. Gamma-radiation was measured on a Wallac 1470 Wizard Gamma Counter (Perkin-Elmer). The recovery of testosterone spikes and linearity of the dilution were tested before use.

Statistical analysis

General effects of diet, gender and genotype on concentrations of BC, retinol and retinyl esters in lung and serum were analyzed using $2 \times 2 \times 2$ factorial univariate ANOVA (SPSS version 15.0) and considered statistically significant when $p < 0.05$. Effects of BC compared to control diet were tested using a Student's *t*-test and considered statistically significant at $p < 0.05$). Fold changes for both microarray gene expression and Q-PCR gene expression were calculated using mean log signal intensities. P-values for differential expressions were calculated between two groups using two-tailed Student's *t*-test statistics on log intensity values. Changes were considered statistically different at $p < 0.05$. Regression analysis of gene expression in males versus females was performed in GraphPad Prism software (version 5.0; GraphPad Software, Inc, San Diego, CA) using nonlinear regression analysis.

Results

BC supplementation and knockout of *Bcmo1* results in changes in BC and BC metabolites

BC concentrations in lung and serum were increased after 14 weeks of BC treatment compared to control diet-fed mice, and as expected, to a higher level in $Bcmo1^{-/-}$ mice as in $Bcmo1^{+/+}$ mice (as has been described separately for female [17] and male [18] mice). There were no significant differences in retinol serum concentrations between the different groups. In lung tissue, retinol and retinyl ester concentrations were increased upon BC supplementation compared to the control diet fed mice, but there was no interaction between BC and the *Bcmo1* genotype concerning the concentration of these metabolites (Fig. 1).

Gene expression of all groups was analyzed, and of the 43,379 spots (number of spots minus control spots) on the whole genome microarrays, 31,128 spots had an average signal twice above background and were regarded as positive spots and included in the analysis. There were two genes involved in downstream BC metabolism that were significantly regulated by BC in $Bcmo1^{+/+}$ mice (Table 1). The biggest fold change was found for *lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase (Lrat;* 1.6 fold increase after BC supplementation). Five genes involved in downstream BC metabolism were regulated because of the knock-out of *Bcmo1*. Most striking was the high fold change up regulation of *Lrat* (1.7–2.1 fold increase in $Bcmo1^{-/-}$ mice) and the high fold change
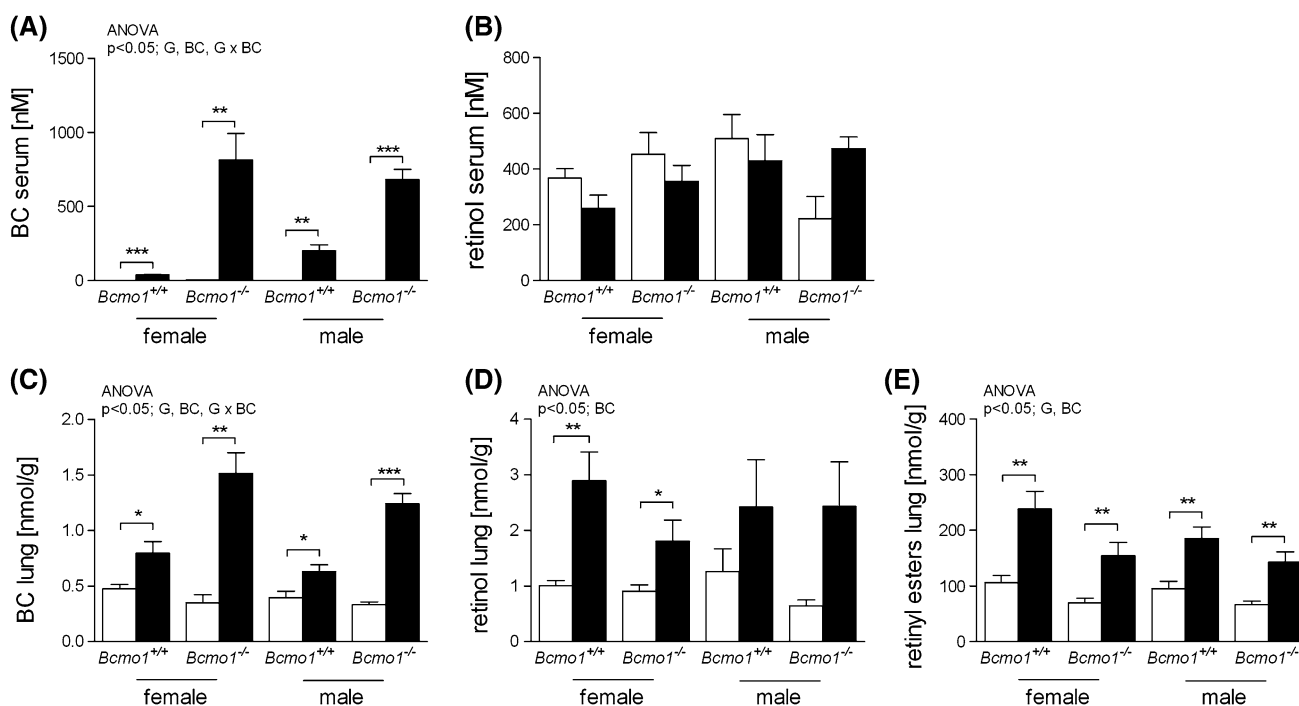
Fig. 1 Beta-carotene (BC) (**a**, **c**), retinol (**b**, **d**) concentrations in serum and lung tissue, respectively, and retinyl ester concentrations in lung tissue (**e**) of female and male $Bcmo1^{+/+}$ mice and $Bcmo1^{-/-}$ mice fed a control diet (*white bars*) or a BC diet (*black bars*). Data are expressed as mean $\pm$ SEM; significance was tested for diet (BC), gender (S) and genotype (G) using ANOVA and considered significant at $p < 0.05$. Significant effects for the different factors or any interaction between those factors are displayed on top of each figure. Student's *t*-test was used between BC and control groups when there was a significant diet effect by ANOVA and considered significant when $p < 0.05$. Using Student's *t*-test statistics: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

down regulation of *alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide (Adh7*; 1.6–2.7 fold decrease in $Bcmo1^{-/-}$ mice).

Gene expression is changed in the opposite direction by BC in male and female $Bcmo1^{-/-}$ mice

BC supplementation resulted in a considerably higher number of significantly regulated spots in the $Bcmo1^{-/-}$ mice than in the $Bcmo1^{+/+}$ mice (Fig. 2a, b). Only 20 regulated genes overlapped in male and female $Bcmo1^{+/+}$ mice and 89 genes in $Bcmo1^{-/-}$ mice. These two groups of regulated genes commonly affected in both genders showed no overlap between $Bcmo1^{-/-}$ and $Bcmo1^{+/+}$ mice. To further determine whether these genes were similarly regulated by BC in both genders, fold changes of gender-independent BC-regulated genes were plotted for female mice against the fold changes in male mice. The 20 BC-regulated genes in male and female $Bcmo1^{+/+}$ mice were distributed in all four quadrants of the plot (Fig. 2c). Regression analysis resulted in a significant correlation [$R = 0.66$; slope $= 0.787$; 95% CI slope $= (0.2988–1.276)$]. This correlation was however based on only two data points $(-1.93; -1.87)$ and $(1.34; 1.56)$. Without these two points, there was no significant correlation [$R = 0.14$;

slope $= -0.193$; 95% CI slope $= (-0.8832; 0.4960)$]. To our surprise, genes regulated by BC in female $Bcmo1^{-/-}$ were regulated in opposite directions compared to BC-regulated genes in male $Bcmo1^{-/-}$ mice (upwards regulated genes in males were down in females and vice versa), with only 4 out of the 89 probes being regulated in the same direction (in italics in Table 3). We found >95% of the probes being regulated in the opposite direction by BC between males and females, suggesting that this is a real effect of BC. Randomly affected gene expression would theoretically result in 50% of the probes being regulated in a similar direction and 50% in opposite directions. Regression analysis resulted in a significant negative regression [$R = 0.76$; slope $= -0.436$; 95% CI slope $= (-0.5144; -0.3582)$]. Genes regulated by BC in both male and female $Bcmo1^{+/+}$ mice (Table 2) or $Bcmo1^{-/-}$ mice (Table 3) were categorized into biological processes, with endothelial functioning, immune response and nervous response being most prominently regulated by BC in $Bcmo1^{-/-}$ mice (Fig. 3).

We used Q-PCR to validate the microarray experiment. For validation of the effects in $Bcmo1^{+/+}$ mice, *lecitin retinol acyltransferase (phosphatidylcholine-retinol O-acyltransferase) (Lrat)*, an enzyme involved in downstream BC metabolism, was selected. For validation of effects induced

Table 1 Genes involved in BC metabolism or retinoic acid catabolism that were significantly regulated after BC supplementation or *Bcmo1* knockout

Symbol	Name	Probe name	Systematic name	Fold change induced by BC supplementation				Fold change induced by *Bcmo1* knockout			
				Female		Male		Female		Male	
				$Bcmo1^{+/+}$	$Bcmo1^{-/-}$	$Bcmo1^{+/+}$	$Bcmo1^{-/-}$	Co	BC	Co	BC
Aldh1a2	Aldehyde dehydrogenase family 1, subfamily A2	A_52_P58145	NM_009022	−1.04	−1.03	−1.02	−1.02	1.25*	1.25**	1.35***	1.35**
Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3	A_52_P87843	NM_053080	−1.25**	1.00	−1.11	−1.12	−1.43***	−1.14	−1.14	−1.14
Aldh2	Aldehyde dehydrogenase 2, mitochondrial	A_52_P116134	NM_009656	1.05	1.17*	−1.03	1.01	−1.18*	−1.07	−1.10	−1.05
Lrat	Lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	A_52_P669005	NM_023624	1.58**	1.34	1.57*	1.49	2.58*	1.88*	1.80*	1.71**
Adh7	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	A_51_P233797	NM_009626	−1.11	1.23	1.01	1.13	−2.67***	−1.91***	−1.83***	−1.64**

* Significant difference ($p < 0.05$)

** Significant difference ($p < 0.01$)

*** Significant difference ($p < 0.001$)

by BC supplementation in $Bcmo1^{-/-}$ mice, the genes *glutamate receptor, ionotropic, AMPA1 (alpha 1) (Gria1)* and *endothelin 1 (Edn1)* were chosen because of their relatively high expression level and relatively high fold changes. All three selected genes showed an identical expression using the microarray technique and Q-PCR (Fig. 4).

BC supplementation results in the overrepresentation of the steroid biosynthetic process in male $Bcmo1^{-/-}$ mice

Gene-ontology processes affected by BC supplementation in male and female $Bcmo1^{-/-}$ mice were examined to further explain the opposite direction of gene expression induced by BC. This overrepresentation analysis was performed using ErmineJ overrepresentation analysis [23] on all genes and their corresponding *p*-value. We found a significant overrepresentation of the steroid biosynthetic process (GO:0006694) in male $Bcmo1^{-/-}$ mice by BC supplementation ($p = 4.6 \times 10^{-6}$), but not in female $Bcmo1^{-/-}$ mice ($p = 0.17$). Genes regulated by BC resulting in the overrepresentation of the steroid biosynthetic process in male $Bcmo1^{-/-}$ mice are listed in Table 4 (genes presented until $p = 0.1$).
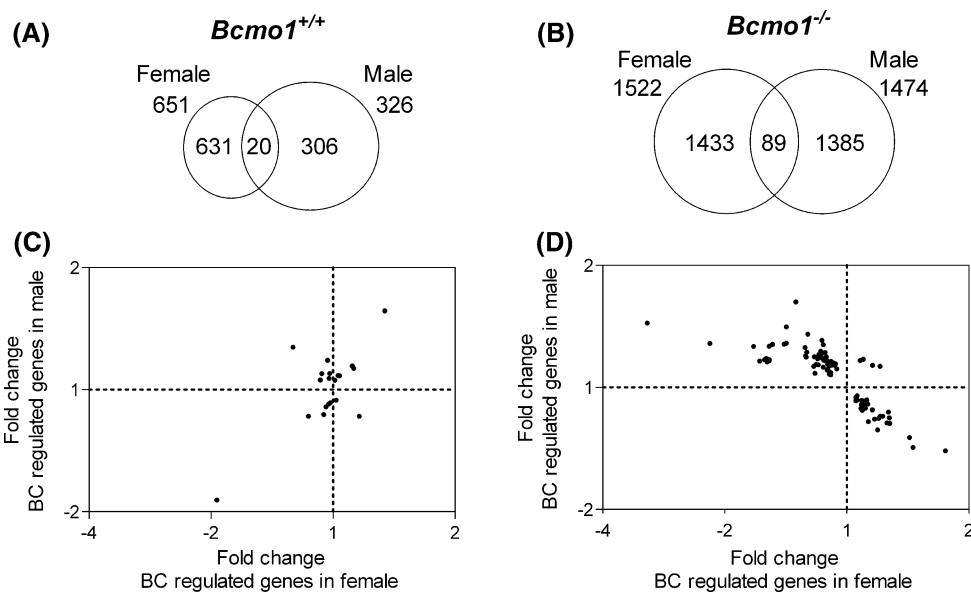
Effect of BC on serum testosterone levels

We determined serum concentrations of the sex hormone testosterone. Since only limited amounts of serum were available, other sex hormones requiring bigger serum amounts could not be determined. As expected, testosterone levels were significantly higher in $Bcmo1^{+/+}$ males than in $Bcmo1^{+/+}$ females (Fig. 5a). In $Bcmo1^{-/-}$ mice, however, testosterone levels showed a high inter-individual variability (Fig. 5b). Due to this large variation, there was no statistically significant difference in testosterone levels between male and female $Bcmo1^{-/-}$ mice. However, BC increased testosterone levels in both $Bcmo1^{-/-}$ female and male mice with a fold change of 4.7 and 4.0, respectively (Fig. 5c), although, because of the high variation, differences did not reach statistical significance (ANOVA; effect of diet $p = 0.12$ and interaction effect of diet with *Bcmo1* genotype $p = 0.14$).

Discussion

We showed for the first time that BC supplementation to $Bcmo1^{-/-}$ mice resulted in the regulation of gene expression in opposite directions in male and female mice. We hypothesize that BC alters systemic hormone production, which was supported by the overrepresentation of the

Fig. 2 Venn diagrams representing the number of genes significantly ($p < 0.05$) regulated by BC in female and male mice and the number of genes regulated by BC in both females as well as males (*overlap*) in *Bcmo1*$^{+/+}$ (**a**) and *Bcmo1*$^{-/-}$ (**b**) mice. The scatter plots represent the gene expression fold changes of the genes regulated by BC independent of gender in females (*x*-axis) and males (*y*-axis) in *Bcmo1*$^{+/+}$ (**c**) and in *Bcmo1*$^{-/-}$ (**d**) mice

steroid biosynthetic process (GO:0006694) in male *Bcmo1*$^{-/-}$ mice upon consumption of a BC diet ($p = 4.6 \times 10^{-6}$). Moreover, testosterone concentrations were increased, but highly variable after BC supplementation in *Bcmo1*$^{-/-}$ mice. The presence of an active *Bcmo1* enzyme was of importance in the observed opposite direction of gene expression changes induced by BC supplementation in male and female mice, since these effects were not observed in *Bcmo1*$^{+/+}$ mice.

BC supplementation to *Bcmo1*$^{-/-}$ mice resulted in an opposite direction of gene regulation in males and females. A hypothesis to explain this is that BC supplementation induces alterations in sex hormone synthesis or metabolism in male and female mice. Interestingly, the biosynthetic pathway for BC production in plants partly overlaps with the steroid biosynthetic pathway in animals [25]. To support this hypothesis, we measured testosterone levels in serum, which were highly variable in BC-supplemented *Bcmo1*$^{-/-}$ mice, but not in the other groups. Another important sex hormone that might have been changed after BC supplementation to *Bcmo1*$^{-/-}$ is 17β-estradiol, but the amount of serum left did not allow us to measure this. Although we were not able to technically prove a change in sex hormones, a change in sex hormones would result in a gender-specific change since the lung contains all necessary elements for sex hormone action, such as enzymes involved in androgen and estrogen metabolism as well as estrogen and androgen receptors [26–28]. In line with this, the GO process for steroid biosynthesis was significantly regulated in male lung tissue, as can be seen in Table 4. Several enzymes involved in steroid conversion were shown to be significantly regulated in male *Bcmo1*$^{-/-}$ mice. For example, testosterone can be converted in the

lung into estrone and 17β-estradiol. 17β-Estradiol can bind to the estrogen receptor to induce estrogen-related responses [29]. Interestingly, the gene expression of *17-beta-hydroxysteroid dehydrogenase 12* (*Hsd17b12*), which converts estrone into estradiol, was significantly upregulated [30], and *steroid 5 alpha-reductase 1* (*Srd5a1*), an important enzyme in the inactivation of testosterone [27], was downregulated ($p = 0.055$) in lungs of male *Bcmo1*$^{-/-}$ mice upon BC supplementation. Moreover, *17-beta-hydroxysteroid dehydrogenase 7* (*Hsd17b7*), which oxidizes or reduces estradiol, and thus decreases the estrogenic potency, was significantly downregulated by BC in female *Bcmo1*$^{-/-}$ mice. These data indicate that BC might have had an effect on sex hormone synthesis and had an effect on hormone conversion in male *Bcmo1*$^{-/-}$ mice. Further research is needed to elucidate the exact mechanism underlying the observed opposite change in gene regulation induced by BC supplementation in male and female *Bcmo1*$^{-/-}$ mice.

Although the number of studies that have assessed effects of BC on hormone production is limited, these studies actually support a relation between BC and steroid hormone alterations. BC supplementation to female dogs resulted in increased plasma progesterone concentrations [31], and cats supplemented with BC had increased estradiol concentrations [32]. In addition, it has been reported that downstream BC metabolites, such as vitamin A and the bioactive metabolite retinoic acid, also have an effect on steroidogenesis and that these effects are, at least in part, due to an increased expression of enzymes involved in steroidogenesis [33, 34]. It is not known whether previously described effects of downstream BC metabolites on steroidogenesis are regulated by binding of retinoic acid to

Table 2 Classification of genes that were significantly regulated by BC in both female and male Bcmo1+/+ mice

Gene symbol	Gene name	Probe name	Systematic name	BC-induced fold change			
				Female		Male	
				Bcmo1+/+	Bcmo1−/−	Bcmo1+/+	Bcmo1−/−
Immune response							
Cd207	CD 207 antigen	A_51_P110381	NM_144943	−1.40**	1.16 (ns)	−1.16*	−1.21 (ns)
Cd209b	CD209b antigen	A_52_P267717	NM_026972	−1.29***	−1.05 (ns)	−1.15*	1.10 (ns)
Cd5 l	CD5 antigen-like	A_51_P205779	NM_009690	−2.02*	−1.93 (ns)	−1.87*	−1.15 (ns)
Csf2rb1	Colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte–macrophage)	A_52_P664853	NM_007780	−1.14*	1.03 (ns)	1.08*	1.01 (ns)
Ly6g5b	Lymphocyte antigen 6 complex, locus G5B	A_51_P369443	NM_148939	−1.09*	−1.08 (ns)	1.06*	1.03 (ns)
Xpnpep2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	A_51_P152585	NM_133213	1.15*	1.12 (ns)	1.13*	1.09*
Apoptosis							
Dnase1l3	Deoxyribonuclease 1-like 3	A_52_P165657	NM_007870	−1.26*	−1.15 (ns)	−1.16*	−1.43*
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1	A_51_P127297	NM_008288	1.22*	−1.07 (ns)	1.09*	−1.05 (ns)
Retinol metabolism							
Lrat	Lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	A_52_P669005	NM_023624	1.58**	1.34 (ns)	1.57*	1.49 (ns)
Rdh12	Retinol dehydrogenase 12	A_51_P141960	NM_030017	−1.07*	1.01 (ns)	1.05**	1.02 (ns)
Mucus production							
Muc4	Mucin 4	A_51_P392277	AF218265	1.23*	−1.25 (ns)	1.27*	1.06 (ns)
Mitochondrial functioning							
Sirt4	Sirtuin 4 (silent mating type information regulation 2 homolog) 4 (S. cerevisiae)	A_51_P424641	BC022653	1.06*	1.02 (ns)	−1.06*	1.02 (ns)
Trit1	tRNA isopentenyltransferase 1	A_51_P137317	NM_025873	1.08*	−1.03 (ns)	−1.09*	1.08 (ns)
Intracellular trafficking							
Tom1	Target of myb1 homolog (chicken)	A_52_P1480	NM_011622	−1.13*	1.04 (ns)	1.08*	1.02 (ns)
Ion chanel							
Tpcn2	Two pore segment channel 2	A_51_P517952	NM_146206	−1.23*	−1.02 (ns)	1.10*	1.19 (ns)
Protein modification							
Ube1l2	Ubiquitin-activating enzyme E1-like 2	A_52_P671062	NM_172712	−1.13*	−1.03 (ns)	1.18*	1.11 (ns)
Unknown							
Phgdhl1	Phosphoglycerate dehydrogenase like 1	A_52_P82229	NM_026861	−1.11*	−1.02 (ns)	1.07*	1.04 (ns)
Zmynd10	Zinc finger, MYND domain containing 10	A_51_P392967	NM_053253	1.16*	−1.02 (ns)	−1.08*	−1.02 (ns)
AI508575		A_52_P724015	AI508575	1.12*	−1.04 (ns)	−1.10*	−1.06 (ns)
AK080164		A_52_P33067	AK080164	−1.18*	1.12 (ns)	1.14*	1.02 (ns)

* Significant difference ($p < 0.05$) between BC and control diet fed mice

** Significant difference ($p < 0.01$) between BC and control diet fed mice

*** Significant difference ($p < 0.001$) between BC and control diet fed mice

ns no significant difference ($p > 0.05$) between BC and control diet fed mice

Table 3 Classification of genes that were significantly regulated by BC in both female and male $Bcmol^{-/-}$ mice

Gene symbol	Gene name	Probe name	Systematic name	BC-induced fold change			
				Female		Male	
				$Bcmol^{+/+}$	$Bcmol^{-/-}$	$Bcmol^{+/+}$	$Bcmol^{-/-}$
Immune response							
B2m	Beta-2 microglobulin	A_51_P129006	NM_009735	1.00 (ns)	−1.48*	−1.00 (ns)	1.17*
B2m	Beta-2 microglobulin	A_51_P129006	NM_009735	1.01 (ns)	−1.50*	1.01 (ns)	1.18*
B2m	Beta-2 microglobulin	A_51_P129006	NM_009735	1.01 (ns)	−1.48*	−1.02 (ns)	1.18*
B2m	Beta-2 microglobulin	A_51_P129006	NM_009735	1.02 (ns)	−1.47*	1.02 (ns)	1.17*
B2m	Beta-2 microglobulin	A_51_P129006	NM_009735	1.01 (ns)	−1.48*	1.03 (ns)	1.16*
Cd247	CD247 antigen	A_52_P173555	NM_031162	−1.06 (ns)	−1.14*	1.13 (ns)	1.12*
Dappl	Dual adaptor for phosphotyrosine and 3-phosphoinositides 1	A_52_P422172	NM_011932	−1.03 (ns)	−1.11*	−1.05 (ns)	1.14*
Gja5	Gap junction membrane channel protein alpha 5	A_52_P612636	AK082993	1.04 (ns)	−1.17*	1.21 (ns)	1.14*
Gvin1	GTPase, very large interferon inducible 1	A_52_P535484	NM_029000	−1.14 (ns)	−1.56*	1.01 (ns)	1.16*
H2-Q8	Histocompatibility 2, Q region locus 8	A_52_P152133	NM_023124	−1.01 (ns)	−1.56*	−1.11*	1.26**
Il18bp	Interleukin 18 binding protein	A_52_P577384	NM_010531	1.11 (ns)	−1.49*	−1.04 (ns)	1.18*
Ifi44	Interferon-induced protein 44	A_52_P550858	AK085407	1.25 (ns)	−2.36*	−1.03 (ns)	1.47*
Nfam1	Nfat activating molecule with ITAM motif 1	A_52_P686701	NM_028728	−1.02 (ns)	−1.13***	1.09 (ns)	1.21*
LOC668139	Similar to very large inducible GTPase 1 isoform A	A_52_P666442	XR_001627	−1.10 (ns)	−2.14**	1.00 (ns)	1.31*
LOC675624	Similar to very large inducible GTPase 1 isoform A	A_52_P764477	XM_983705	−1.14 (ns)	−1.53**	−1.05 (ns)	1.26*
Oasld	2'-5' oligoadenylate synthetase 1D	A_51_P183025	NM_133893	−1.10 (ns)	−1.48*	1.05 (ns)	1.27***
Psme2	Proteasome (prosome, macropain) 28 subunit, beta	A_52_P305289	NM_011190	1.04 (ns)	−1.19*	−1.02 (ns)	1.08*
Endothelial functioning							
Aebp1	AE binding protein 1	A_51_P336770	NM_009636	−1.04 (ns)	1.12*	−1.05 (ns)	−1.07*
Col25a1	Procollagen, type XXV, alpha 1	A_51_P333929	NM_029838	−1.09	−1.14*	−1.07 (ns)	1.16*
Darc	Duffy blood group, chemokine receptor	A_52_P110052	NM_010045	−1.16 (ns)	−1.36*	1.05 (ns)	1.29*
Ecgf1	Endothelial cell growth factor 1 (platelet-derived)	A_51_P267080	NM_138302	1.11 (ns)	1.11*	−1.03 (ns)	−1.11*
Edn1	Endothelin 1	A_51_P115005	NM_010104	−1.33 (ns)	1.44*	1.22 (ns)	−1.33*
Gata1	GATA binding protein 1	A_52_P670095	NM_008089	−1.03 (ns)	−1.26*	1.02 (ns)	1.25***
Gata1	GATA binding protein 1	A_52_P670095	NM_008089	−1.07 (ns)	−1.17*	1.06 (ns)	1.18*
Habp4	Hyaluronic acid binding protein 4	A_51_P269078	NM_019986	1.03	1.10*	1.01 (ns)	−1.10*
Pthlh	Parathyroid hormone-like peptide	A_51_P129363	NM_008970	−1.15	−1.15**	1.01 (ns)	1.20*
Selp	Selectin, platelet	A_51_P211854	NM_011347	−1.03 (ns)	−1.14**	−1.01 (ns)	1.17*
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	A_52_P639522	NM_011111	−1.37 (ns)	−1.25**	−1.06 (ns)	1.23*
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	A_51_P101146	NM_011111	−1.46 (ns)	−1.39*	1.06 (ns)	1.38*
Nervous response							
Ahnak	AHNAK nucleoprotein (desmoyokin)	A_51_P224534	NM_009643	1.01 (ns)	1.16*	−1.03 (ns)	−1.14*
Atxn7l2	Ataxin 7-like 2	A_52_P287195	NM_175183	−1.00 (ns)	1.05*	1.00 (ns)	−1.06*
Dyrk4	Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 4	A_51_P307463	NM_207210	−1.01 (ns)	−1.25*	1.02 (ns)	1.18*

Table 3 continued

Gene symbol	Gene name	Probe name	Systematic name	BC-induced fold change			
				Female		Male	
				$Bcmo1^{+/+}$	$Bcmo1^{-/-}$	$Bcmo1^{+/+}$	$Bcmo1^{-/-}$
Gad1	Glutamic acid decarboxylase 1	A_51_P282538	NM_008077	−1.12 (ns)	−1.17**	−1.01 (ns)	1.21**
Gria1	Glutamate receptor, ionotropic, AMPA1 (alpha 1)	A_52_P39575	NM_008165	−1.03 (ns)	1.28*	−1.02 (ns)	−1.24**
Kcnn2	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	A_51_P309854	NM_080465	1.06	1.22*	−1.03 (ns)	1.12*
Olfml1	Olfactomedin-like 1	A_51_P476879	NM_172907	1.00 (ns)	−1.20*	−1.01 (ns)	1.19***
Plcl1	Phospholipase C-like 1	A_52_P191567	AK140530	1.01 (ns)	1.06*	1.00 (ns)	−1.07*
Reln	Reelin	A_51_P365369	NM_011261	1.15*	1.20*	−1.05 (ns)	−1.20*
Reln	Reelin	A_51_P365369	NM_011261	1.09*	1.21*	1.02 (ns)	−1.17*
Reln	Reelin	A_51_P365369	NM_011261	1.08*	1.27*	−1.06 (ns)	−1.15*
Reln	Reelin	A_51_P365369	NM_011261	1.13**	1.24*	−1.00 (ns)	−1.17*
Reln	Reelin	A_51_P365369	NM_011261	1.09 (ns)	1.26**	1.03 (ns)	−1.22*
Slc6a17	Solute carrier family 6 (neurotransmitter transporter), member 17	A_52_P373637	NM_172271	−1.07 (ns)	−1.12*	1.07 (ns)	1.19*
Cell structure							
Elmo1	Engulfment and cell motility 1, ced-12 homolog (C. elegans)	A_51_P306129	NM_080288	1.00	−1.06*	1.02 (ns)	1.14*
Mmp13	Matrix metallopeptidase 13	A_51_P184484	NM_008607	1.06	−1.40*	−1.06 (ns)	1.28*
Mmp24	Matrix metallopeptidase 24	A_52_P605500	NM_010808	−1.20	1.10**	1.04 (ns)	1.17*
Twf1	Twinfilin, actin-binding protein, homolog 1 (Drosophila)	A_52_P463365	NM_008971	1.01	−1.06*	−1.00 (ns)	1.11*
Transport							
Slc12a7	Solute carrier family 12, member 7	A_51_P421734	NM_011390	1.07	1.09**	1.02 (ns)	−1.08*
Slc35a5	Solute carrier family 35, member A5	A_52_P618379	NM_028756	−1.02	−1.08*	1.01 (ns)	1.15*
Slc5a11	Solute carrier family 5 (sodium/glucose cotransporter), member 11	A_52_P271085	NM_146198	−1.20	−1.14*	1.04 (ns)	1.21*
Rab6	RAB6, member RAS oncogene family	A_51_P209873	NM_024287	1.11	1.77**	1.08 (ns)	−1.39*
Cell cycle							
Ccnb1	Cyclin B1	A_52_P558401	NM_172301	−1.02 (ns)	−1.15*	1.01 (ns)	1.18*
Esco2	Establishment of cohesion 1 homolog 2 (S. cerevisiae)	A_51_P195034	NM_028039	1.11 (ns)	−1.15*	−1.04 (ns)	1.28*
Fbxw2	F-box and WD-40 domain protein 2	A_52_P643001	BC003834	1.26 (ns)	−1.11*	−1.04 (ns)	1.16***
Hspa2	Heat shock protein 2	A_51_P453149	NM_008301	−1.08 (ns)	1.27**	−1.00 (ns)	−1.19*
DNA damage							
Ankrd13a	Ankyrin repeat domain 13a	A_51_P190886	NM_026718	1.03 (ns)	1.13***	−1.02 (ns)	−1.10*
Ankrd13a	Ankyrin repeat domain 13a	A_52_P665309	NM_026718	1.26 (ns)	1.09*	1.03 (ns)	−1.09*
Oxr1	Oxidation resistance 1	A_51_P161893	NM_130885	1.15 (ns)	1.13*	−1.03 (ns)	−1.22***
Sf3b2	Splicing factor 3b, subunit 2	A_51_P280158	NM_030109	1.02 (ns)	1.06*	−1.01 (ns)	−1.05*
Mitochonrial energy							
Acsl4	Acyl-CoA synthetase long-chain family member 4	A_51_P268154	NM_019477	1.03	−1.10*	1.07 (ns)	1.09*
Atp5g3	ATP synthase, H + transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3	A_51_P294849	NM_175015	1.06	1.09*	−1.02 (ns)	−1.13*

Table 3 continued

Gene symbol	Gene name	Probe name	Systematic name	BC-induced fold change			
				Female		Male	
				$Bcmo1^{+/+}$	$Bcmo1^{-/-}$	$Bcmo1^{+/+}$	$Bcmo1^{-/-}$
Rhot1	Ras homolog gene family, member T1	A_52_P232663	NM_021536	−1.04	1.08*	1.04 (ns)	1.16**
DNA regulation							
Dpys	Dihydropyrimidinase	A_51_P244950	BC029718	−1.06 (ns)	−1.34**	−1.05 (ns)	1.60**
Rrm1	Ribonucleotide reductase M1	A_51_P502082	NM_009103	1.05 (ns)	−1.19*	−1.04 (ns)	1.14*
Apoptosis							
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	A_52_P158527	NM_207680	1.02 (ns)	1.09*	−1.00 (ns)	−1.10*
Zc3h12a	Zinc finger CCCH type containing 12A	A_51_P297925	NM_153159	−1.06 (ns)	−1.10*	1.01 (ns)	1.13*
Transcription/translation							
Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	A_52_P426768	NM_019563	−1.23 (ns)	−1.21***	1.06 (ns)	1.13*
Eif2s1	Eukaryotic translation initiation factor 2, subunit 1 alpha	A_51_P448334	NM_026114	1.01 (ns)	−1.15*	−1.07 (ns)	1.27**
Snrpd3	Small nuclear ribonucleoprotein D3	A_52_P150588	AK163643	−1.07 (ns)	−1.09*	−1.04 (ns)	1.13*
Other							
Mlana	Melan-A	A_51_P499623	AK020928	1.09	1.20	−1.15 (ns)	−1.27*
Rex2	Reduced expression 2	A_52_P412109	NM_009051	−1.01	−1.10**	1.05 (ns)	1.07*
Rnaset2b	Ribonuclease T2B	A_51_P310100	AK029149	−1.08 (ns)	−1.12*	1.01 (ns)	1.11*
Wdr33	WD repeat domain 33	A_52_P280899	NM_028866	1.00	1.10**	−1.01 (ns)	−1.08*
Unannotated							
1700029I01Rik	RIKEN cDNA 1700029I01 gene	A_51_P109496	NM_027285	−1.05 (ns)	−1.26*	1.07 (ns)	1.19**
4932432K03Rik	RIKEN cDNA 4932432K03 gene	A_52_P413907	NM_144535	−1.07 (ns)	−1.09*	−1.01 (ns)	1.16*
AU020772	Expressed sequence AU020772	A_52_P564951	NM_001017985	−1.02 (ns)	1.05*	1.03 (ns)	−1.08***
AU042671	Expressed sequence AU042671	A_51_P450764	AK037367	1.04 (ns)	1.12*	1.01 (ns)	−1.13*
D14Ertd426e	DNA segment, Chr 14, ERATO Doi 426, expressed	A_52_P527268	AK139611	−1.09 (ns)	−1.07**	1.08 (ns)	1.13*
LOC435271	Similar to schlafen 3	A_52_P322927	XM_487182	−1.05 (ns)	−1.18***	1.01 (ns)	1.19*
LOC631232	Similar to zinc finger protein 665	A_52_P17746	XM_904987	−1.01 (ns)	−1.12*	−1.00 (ns)	1.11*
LOC631624	Similar to reduced expression 2	A_52_P501447	XM_919663	−1.03 (ns)	−1.10**	1.04 (ns)	1.08
Zc3h12a	Zinc finger CCCH type containing 12A	A_51_P297925	NM_153159	−1.06 (ns)	−1.10*	1.01(ns)	1.13*
	Glycerol-3-phosphate acyltransferase mRNA, complete cds	A_51_P471819	M77003	1.08 (ns)	1.16**	−1.04 (ns)	1.13*
	Q3WEM2_9ACTO (Q3WEM2) Parallel beta-helix repeat precursor, partial (6%)	A_52_P214241	TC1678456	1.01 (ns)	−1.11*	1.01 (ns)	1.10*
	7 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:A730019A03 product:unclassifiable, full insert sequence	A_51_P482265	AK163132	−1.03 (ns)	−1.16	1.03 (ns)	1.23**
	Adult male diencephalon cDNA, RIKEN full-length enriched library, clone:9330176N07 product:unclassifiable, full insert sequence	A_52_P448829	AK034318	1.07 (ns)	1.52*	−1.02 (ns)	−1.36*
	G protein-coupled receptor associated sorting protein 2	A_51_P362373	ENSMUST00000096320	−1.13 (ns)	1.18*	−1.05 (ns)	−1.19*
BB736773	BB736773 RIKEN full-length enriched, 6 days neonate spleen Mus musculus cDNA clone F430010B14 3', mRNA sequence	A_52_P700030	BB736773	−1.19 (ns)	−1.23*	1.02 (ns)	1.34**

Table 3 continued

Gene symbol	Gene name	Probe name	Systematic name	BC-induced fold change			
				Female		Male	
				$Bcmo1^{+/+}$	$Bcmo1^{-/-}$	$Bcmo1^{+/+}$	$Bcmo1^{-/-}$
	K0288C02-5 N NIA Mouse Unfertilized Egg cDNA Library (Long) Mus musculus cDNA clone NIA:K0288C02 IMAGE:30053113 5', mRNA sequence	A_52_P174014	CA561520	1.01 (ns)	−1.18*	1.01 (ns)	1.18*

* Significant difference ($p < 0.05$) between BC and control diet fed mice

** Significant difference ($p < 0.01$) between BC and control diet fed mice

*** Significant difference ($p < 0.001$) between BC and control diet fed mice

ns no significant difference ($p > 0.05$) between BC and control diet fed mice

Fig. 3 Classification of genes that are regulated by BC ($p < 0.05$) in male as well as in female $Bcmo1^{-/-}$ mice into different biological process categories

the transcription factors retinoic acid receptor (RAR) or rexinoid X receptor (RXR).

Our results suggest that the BC-induced opposite direction of gene expression in the different genders of $Bcmo1^{-/-}$ mice was due to an increase in BC concentration and not due to a change in BC metabolites such as retinoic acid, since this opposite direction of gene expression regulation was only observed in $Bcmo1^{-/-}$ mice. Compared to $Bcmo1^{-/-}$ mice, male and female $Bcmo1^{+/+}$ mice had higher concentrations of the BC metabolites; retinol and retinyl esters accumulated in lung tissue after BC supplementation. Furthermore, although still not fully understood, it is generally believed that retinoic acid concentrations are well regulated [35]. Nevertheless, it may be that the concentration of the bioactive metabolite retinoic acid, able to bind to RAR and RXR, is altered only in $Bcmo1^{-/-}$ mice and not in $Bcmo1^{+/+}$ mice. We previously reported that the $Bcmo1^{-/-}$ mice were more prone to a phenotypic vitamin A deficiency than $Bcmo1^{+/+}$ mice due to alterations in the expression of downstream BC-metabolizing enzymes important in the production of retinoic acid [17]. Supplemental BC was able to restore this phenotype. We similarly show in this study that downstream BC metabolism was altered in $Bcmo1^{-/-}$ mice. Most striking was the upregulation of Lrat combined with the downregulation of Adh7 in $Bcmo1^{-/-}$ mice. Lrat is involved in the storage of retinol by the formation of retinyl esters [36], while Adh7 is involved in the conversion of retinol into retinal, which is the precursor for retinoic acid [37]. This therefore implicates a more vitamin A-storing phenotype of $Bcmo1^{-/-}$ mice compared to $Bcmo1^{+/+}$ mice. Moreover, more enzymes involved in downstream BC metabolism were altered, and altogether these data indicate that also a change in retinoic acid, possibly because of a less tight regulation of retinoic acid stores in $Bcmo1^{-/-}$ mice compared to $Bcmo1^{+/+}$ mice, might explain the observed alterations in hormone production.

Another question is whether the processes that were regulated by BC supplementation in lung tissue of $Bcmo1^{-/-}$ mice are known to respond to hormonal
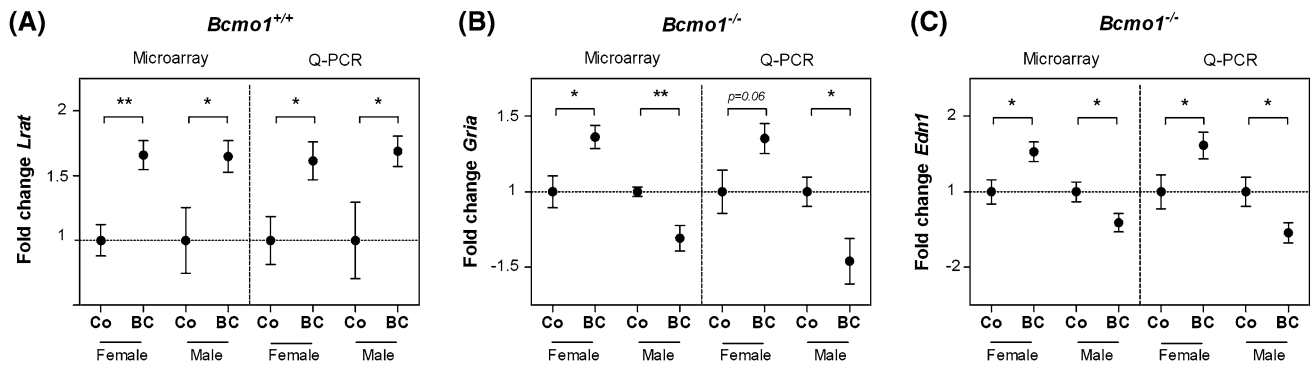
Fig. 4 The fold change gene expression of *Lrat* in *Bcmo1*$^{+/+}$ mice (**a**), *Gria* in *Bcmo1*$^{-/-}$ mice (**b**) and *Edn1* in *Bcmo1*$^{-/-}$ mice (**c**) in BC supplementated mice relative to the control diet fed mice in the microarray, validated by Q-PCR using the reference genes *Stx5a* and *Rnf130*. Data represent the average gene expression $\pm$ SEM compared to control diet fed mice. Using Student's *t*-test statistics on log transformed data: *$p < 0.05$ and **$p < 0.01$

Table 4 Genes regulated by BC in male *Bcmo1*$^{-/-}$ mice resulting in a significant overrepresentation of the steroid biosynthetic process (GO: 0006694)[a]

Probe namer	Source symbol	Gene name	BC-induced fold change gene expression *Bcmo1*$^{-/-}$ mice	
			Female	Male
A_52_P137371	*Hmgcr*	*3-Hydroxy-3-methylglutaryl-Coenzyme A reductase*	−1.08 (0.29)	1.14 (0.01)
A_51_P413238	*Pbx1*	*Pre B-cell leukemia transcription factor 1*	1.02 (0.68)	−1.11 (0.02)
A_51_P309733	*Cyp11a1*	*Cytochrome P450, family 11, subfamily a, polypeptide 1*	1.06 (0.50)	1.20 (0.02)
A_51_P169527	*Mvk*	*Mevalonate kinase*	−1.05 (0.48)	1.11 (0.03)
A_52_P165654	*Star*	*Steroidogenic acute regulatory protein*	1.09 (0.41)	1.12 (0.03)
A_52_P384574	*Stard4*	*StAR-related lipid transfer (START) domain containing 4*	1.07 (0.54)	−1.27 (0.04)
A_52_P223224	*Dhrs8*	*Dehydrogenase/reductase (SDR family) member 8*	−1.02 (0.63)	1.11 (0.04)
A_51_P515446	*Cyp39a1*	*Cytochrome P450, family 39, subfamily a, polypeptide 1*	1.01 (0.83)	1.08 (0.05)
A_52_P164570	*Hsd17b12*	*Hydroxysteroid (17-beta) dehydrogenase 12*	1.00 (0.95)	−1.15 (0.05)
A_52_P85765	*Stard6*	*StAR-related lipid transfer (START) domain containing 6*	−1.05 (0.51)	1.11 (0.05)
A_51_P420415	*Srd5a1*	*Steroid 5 alpha-reductase 1*	−1.00 (0.98)	1.12 (0.06)
A_51_P485791	*Cyp51*	*Cytochrome P450, family 51*	−1.10 (0.14)	1.14 (0.06)
A_52_P566605	*Hsd17b7*	*Hydroxysteroid (17-beta) dehydrogenase 7*	−1.18 (0.03)	1.09 (0.07)
A_52_P136138	*Fdft1*	*Farnesyl diphosphate farnesyl transferase 1*	−1.08 (0.33)	1.18 (0.07)
A_52_P388072	*Hmgcs1*	*3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1*	−1.10 (0.22)	1.16 (0.08)
A_52_P636752	*Cyp51*	*Cytochrome P450, family 51*	−1.13 (0.19)	1.15 (0.08)
A_51_P379798	*Fdps*	*Farnesyl diphosphate synthetase*	−1.04 (0.65)	1.14 (0.08)
A_51_P485946	*Fdft1*	*Farnesyl diphosphate farnesyl transferase 1*	−1.15 (0.20)	1.07 (0.10)

[a] Genes presented: BC versus control in male *Bcmo1*$^{-/-}$ mice $p < 0.1$; between brackets the actual *p*-value

changes. The BC-regulated genes in lungs of *Bcmo1*$^{-/-}$ mice in this study were mainly categorized into three biological categories: *endothelial functioning*, *immune response* and *nervous response*. A literature search revealed that these three categories are all affected by one gaseous mediator: nitric oxide (NO). NO is a radical and can be produced endogenously by the iron-containing enzyme *nitric oxide synthase (Nos)*. Three distinct NOS isoforms have been characterized, neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII) and endothelial NOS (eNOS or NOSIII), and all three NOS isoforms have been detected in the airway epithelium [38, 39]. The functions of NO in the airway include neurotransmission, vascular tone homeostasis and regulation of immune functioning [40]. In our microarray, only NOSIII had an expression twice above background, and there were no
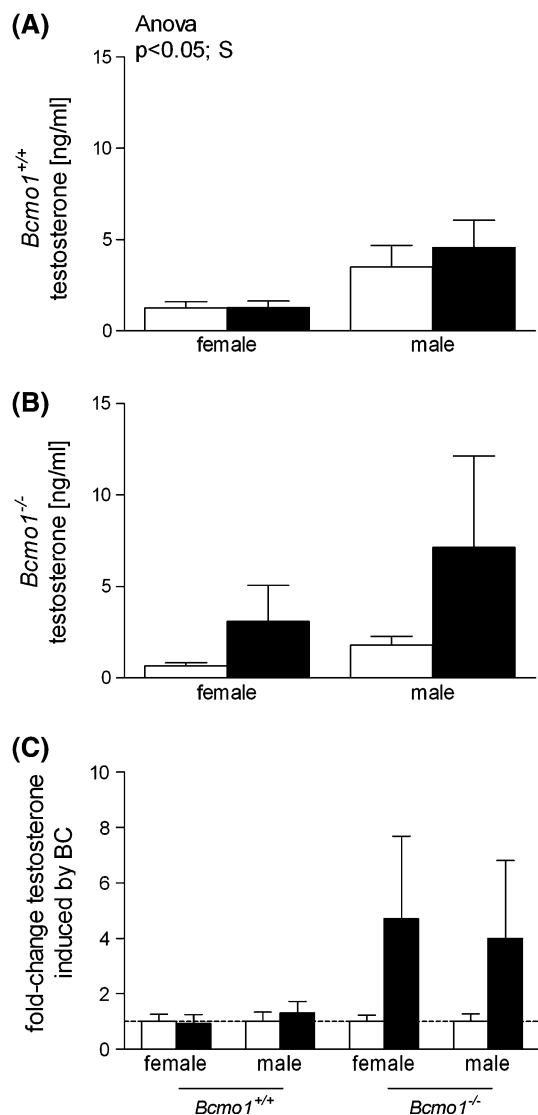
Fig. 5 Testosterone levels in serum of *Bcmo1*$^{+/+}$ mice (**a**), *Bcmo1*$^{-/-}$ mice (**b**) and the corresponding fold change in testosterone (**c**) induced by BC supplementation (*black bars*) as compared to control mice (*white bars*). Data are expressed as mean ± SEM; significance was tested for diet (BC), gender (S) and genotype (G) using ANOVA and considered significant at $p < 0.05$. Significant effects using ANOVA are displayed on top of the figure

significant differences in gene expression after BC supplementation. It is known that estrogen and testosterone can have opposite effects on NO production resulting from NOS activity [41]. An effect of BC on sex hormone production may therefore result in a change in expression of genes that are mediated by NO in opposite directions in male and female lungs. Thus, when BC-induced changes in hormone production, this possibly results in differences in NO production, which might explain that particularly genes involved in immune response, nervous response and endothelial functioning were changed after BC supplementation to male and female *Bcmo1*$^{-/-}$ mice.

A last point that we would like to address is whether changes in hormone production or metabolism induced by BC could possibly explain an increase in lung cancer risk as observed in the CARET and ATBC studies. If this is the case, than hormones are a determining factor in lung carcinogenesis, resulting in gender-related differences in lung cancer risk. Indeed, females are thought to have a higher risk for the development of smoke-induced lung cancer than males [42, 43], and lung cancer is more frequent in never-smoking females than in never-smoking males [44]. It has been hypothesized that especially estrogens might be an important contributor to the differences in lung cancer risk between the different genders. For example, estrogen replacement therapy increases lung cancer risk, and early menopause, which results in decreased estrogen levels, which is associated with a reduced risk for lung cancer in women [45]. Therefore, BC-induced changes in hormone production could be an explanation for part of the increases in lung cancer risk in the CARET and ATBC studies.

In summary, we used *Bcmo1*$^{-/-}$ mice to investigate BC-induced alterations in gene expression in lung. We unexpectedly found that BC supplementation resulted in an opposite direction of gene expression in male and female *Bcmo1*$^{-/-}$ mice. We hypothesize that BC supplementation to *Bcmo1*$^{-/-}$ mice resulted in changes in hormone production or metabolism, which would explain our opposite effects on lung gene expression. To fully understand and explain these effects, further research is needed specifically focusing on reproductive organs as potential target organs for BC and on quantitative measurements of sex hormones in response to BC supplementation in males and in females.

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