Original Article

Ubiquinol affects the expression of genes involved in PPARα signalling and lipid metabolism without changes in methylation of CpG promoter islands in the liver of mice

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Coenzyme Q_{10} is an essential cofactor in the respiratory chain and serves as a potent antioxidant in biological membranes. Recent studies *in vitro* and *in vivo* provide evidence that Coenzyme Q_{10} is involved in inflammatory processes and lipid metabolism via gene expression. To study these effects at the epigenomic level, C57BL6J mice were supplemented for one week with reduced Coenzyme Q_{10} (ubiquinol). Afterwards, gene expression signatures and DNA promoter methylation patterns of selected genes were analysed. Genome-wide transcript profiling in the liver identified 1112 up-regulated and 571 down-regulated transcripts as differentially regulated between ubiquinol-treated and control animals. Text mining and GeneOntology analysis revealed that the "top 20" ubiquinol-regulated genes play a role in lipid metabolism and are functionally connected by the PPARα signalling pathway. With regard to the ubiquinol-induced changes in gene expression of about +3.14-fold ($p \leq 0.05$), +2.18-fold ($p \leq 0.01$), and –2.13-fold ($p \leq 0.05$) for ABCA1, ACYP1, and ACSL1 genes, respectively, hepatic DNA methylation analysis of 282 (sense orientation) and 271 (antisense) CpG units in the respective promoter islands revealed no significant effect of ubiquinol. In conclusion, ubiquinol affects the expression of genes involved in PPARα signalling and lipid metabolism without changing the promoter DNA methylation status in the liver of mice.

***Key Words*:** **ubiquinol, inflammation, lipid metabolism, methylation, gene expression**

Coenzyme Q_{10} (CoQ_{10}) is an important cofactor in the respiratory chain. The reduced form of Coenzyme Q_{10} (ubiquinol) serves as a potent antioxidant in mitochondria and lipid.[1] More recently, studies *in vitro*,[2,3] in SAMP1 mice[4] and in humans[5] provide evidence that ubiquinol is involved in inflammatory processes and lipid metabolism via gene expression. Moreover, reducing effects of coenzyme Q_{10} (ubiquinone) have been also described on genes involved in inflammation and hepatic stress-associated processes in the liver of diet-induced obese mice.[6] However, when related to the oxidized form of coenzyme Q_{10} (ubiquinone), stronger effects have been observed for the reduced form (ubiquinol) both on the transcriptional[4,7] or protein level[8,9] *in vitro* and *in vivo*. To study the observed transcriptional effects of reduced coenzyme Q_{10} (ubiquinol) in more mechanistic detail, C57BL6J mice were supplemented for one week with 250 mg/kg BW/d ubiquinol. Afterwards, the resulting gene expression signatures were analyzed in the liver of mice, and DNA methylation patterns within the promoters of regulated genes were evaluated. Epigenetic mechanisms such as DNA methylation have been shown to entail heritable changes in gene expression.[10,11] Of note, impacts on DNA methylation processes have been already described for various dietary supplements and food nutrients so far.[12,13] DNA methylation consists of the addition of a methyl group to the fifth carbon-position of the cytosine pyrimidine ring in the context of a CpG dinucleotide. Although most genomic DNA in mammals is deficient in CpG sites, clusters of CpG dinucleotides (CpG islands) were described to be primarily located in promoter regions of genes.[14] Thus, the present study postulates that ubiquinol affects gene expression by modulating DNA methylation patterns in the respective CpG island promoter regions of the selected genes.

Materials and Methods

Animals and diet. C57BL6J mice were reared in the Biochemical and Medical Research Laboratories, KANEKA Corp. (Takasago, Japan), at 22 ± 2°C and a 12 h-light-dark cycle. Water and food intake were available ad libitum. At the beginning of the short-term supplementation study, eight 10-week old male C57BL6J mice were purchased from Charles River Lab. Inc. (Yokohama, Japan). To avoid fighting, mice were housed separately throughout 1-week acclimation period and ubiquinol-supplementation study. In the initial phase of the experiment, C57BL6J mice were randomly assigned to either the ubiquinol ($n = 4$) or control group ($n = 4$). Ubiquinol (KANEKA, QH™) was added to a standard laboratory mouse diet (powdered CE-2, CLEA, Osaka, Japan) at a concentration of 0.2% with corn oil (1%, v/w). The only corn oil (1%, v/w) was used as a vehicle in the control diet. The mixture was incorporated in pellet-type chow by adding 30% ethanol solution (v/v), pressure shaping and drying. In general, body weights and food consumption of the mice were calculated every second day, while mice were inspected daily. After 7-day supplementation period with ubiquinol or the respective control diet, animals were sacrificed by anesthesia using isoflurane gas. Livers were removed and the outer left lobes were stored in RNA-*later* Storage Solution (Qiagen, Tokyo, Japan) until use for gene expression and methylation analyses.

Microarray analysis. Microarray analysis was conducted on

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four samples for each group, respectively, by using GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, High Wycombe, United Kingdom) containing 45,100 probe sets. Initially, total RNA was extracted from liver tissues with the miRNeasy kit (Qiagen, Hilden, Germany). The following procedure was performed according to manufacturer's instructions using Poly-A RNA Control Kit (Affymetrix) and One-Cycle cDNA Synthesis Kit (Affymetrix) for cDNA synthesis, Sample Cleanup Module (Affymetrix) for purification, and IVT Labeling Kit (Affymetrix) for synthesis of biotin-labeled cRNA. Fifteen µg of fragmented cRNA was hybridized to a Mouse Genome 430 2.0 Array for 16 h at 45°C at 60 rpm. Subsequently thereafter, arrays were washed and stained using Fluidics Station 450. Hybridization, washing and staining solutions were obtained from analogous GeneChip® kits. After hybridization and washing procedures, microarrays were scanned with the GeneChip® scanner 3000, using GCOS software. If not stated otherwise, all kits and equipment were purchased from Affymetrix. Fluorescence data were obtained in CEL file format. Quality control and normalization procedure of the files was performed with R software 2.7.1 and BioConductor 2.0.1 provided by the MADMAX database (https://madmax.bioinformatics.nl). Data were normalized with the GC-RMA algorithm. Only probe sets showing present calls for all arrays at one experimental group (intervention or control) were considered for further analysis. The complete datasets will be submitted to NCBI Gene Expression Omnibus (GEO).

qRT-PCR. Primer sequences for real-time quantitative RT-PCR (qRT-PCR) experiments were designed with Primer Express® Software 3.0 (Applied Biosystems, Darmstadt, Germany). Primer pairs (5'-3') for NELF (forward: GAACCCCGAGCCGAATG, reverse: CCGTTAGGGTTCCCCAGAAT) and GPX3 (forward: ACAGGAGCCAGGCGAGAA, reverse: CCACCTGGTCGAACATACTTGA) were obtained from MWG Biotech AG (Ebersberg, Germany). cDNA synthesis was initially carried out with the reverse transcriptase core kit (Eurogentec, Köln, Germany) on a thermocycler (Biometra, Göttingen, Germany). qRT-PCR amplification was performed with the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) on an Applied Biosystem 7300 qRT-PCR system. Ct-values of target genes were related to those of the corresponding housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Text mining analysis. For analysis of common pathways between regulated genes, Genomatix software 2010 (www.genomatix.de) was used. Probe set IDs of the selected genes were uploaded to BibliospherePathwayEdition (BSPE) software. This text mining tool identifies putative functional connections of genes based on co-citations with transcription factors and other genes in the network from NCBI PubMed. The most stringent co-citation filter restricted to sentences with expert curated information (level B4) was applied.

Promoter DNA-methylation analysis. The presence of CpG islands within the ABCA1, ACSL1 and ACYP1 gene promoters was predicted by EMBOSS CpGplot program. Quantitative methylation analysis of the three genes was performed with the MassARRAY® system (Sequenom, Hamburg, Germany). The MassCLEAVE™ biochemistry was applied after bisulfite treatment of DNA samples and MALDI-TOF mass spectrometry for analyte detection according to the standard protocols recommended by the supplier. Genomic DNA was extracted from mice liver with the DNeasy Kit (Qiagen). One microgram DNA was treated with sodium bisulfate (DNA Bisulfite Treatment Kit, Sequenom, Hamburg, Germany) and target regions of the modified nucleic acid were amplified by PCR using methylation independent primers, designed by the MassARRAY platform specific EpiDesigner software (Table 2). The PCR products were then subjected to *in vitro* transcription with RNase A cleavage being used for the T-reverse reaction (Sequenom). The generated fragments were displayed based on their molecular weight in the mass spectrum, which was acquired after sample conditioning with a MassARRAY® Analyzer Compact. The resulting methylation calls were analyzed with EpiTyper Software (Sequenom) to generate quantitative results for each CpG site.

Statistics. If not stated otherwise, data are expressed as means ± SEM of four mice per each group, respectively. Differences between ubiquinol-treated and non-treated animals were analyzed by an unpaired two-tailed Students *t* test. *p* values less than or equal to 0.05 were considered as significant.

Results and Discussion

Ubiquinol supplementation reveals no impact on body weight changes. Eight male C57BL6J mice were received at the age of 10 weeks, and initially maintained for 1-week acclimation period. Immediately thereafter, mice were randomly grouped ($n = 4$ for each group) and either supplemented with ubiquinol (250 mg/kg/d) or a respective control diet for one week. As indicated in Fig. 1, body weights slightly increased both in the subsequently treated ubiquinol group and in control animals during acclimation period from day 1 (–6) to day 7 (0) from 22.15 ± 0.26 g and 22.68 ± 0.44 g to 22.9 ± 0.29 g ($p = 0.0032$) and 23.53 ± 0.60 g, respectively. Increases in body weights were obtained during grouping period (from day 0 to day 1) in both groups with total values of 24.43 ± 0.25 g in the ubiquinol group and 24.38 ± 0.11 g in control animals. No significant changes of body weights have been found during supplementation period from day 1 until sacrifice of mice (day 8) both in ubiquinol-treated and control animals. The final body weights were 25.15 ± 0.15 g and 24.73 ± 0.34 for treatment and control animals, respectively, and are thus comparable to age-matched C57BL6J mice described in the literature.[6] Finally, at any indicated time point, no significant differences in body weights were found between treatment and control group. Hence, subsequently described effects of ubiquinol supplementation on gene expression and promoter methylation patterns cannot be simply ascribed to changes in weight.

Whole genome expression data reveal effects of ubiquinol supplementation on liver lipid metabolism. Microarray-based whole genome expression profiles were analyzed from liver samples of mice either supplemented with ubiquinol (250 mg/kg

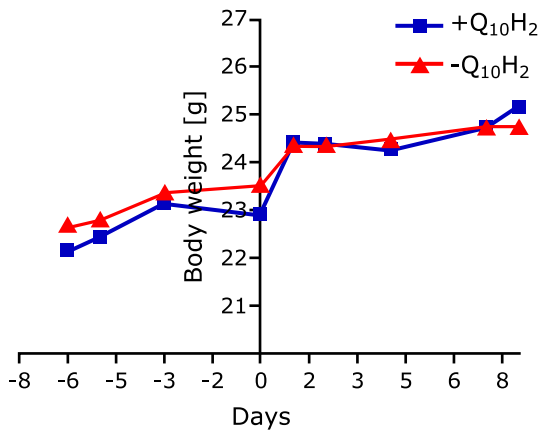

Fig. 1. Body weight development of 10-week old C57BL6J mice during acclimation period and one-week supplementation period with ubiquinol (250 mg/kg/d) or a respective control diet. Body weights of $Q_{10}H_2$-treated and control animals increased slightly during acclimation period (from day 1 [–6] to day 7 [0]). Strong increases in body weights were obtained during grouping period (from day 0 to day 1) in both groups. No significant changes of body weights have been found during supplementation period from day 1 until sacrifice of mice (day 8) both in the ubquinol-treated ($+Q_{10}H_2$) and control group ($–Q_{10}H_2$).

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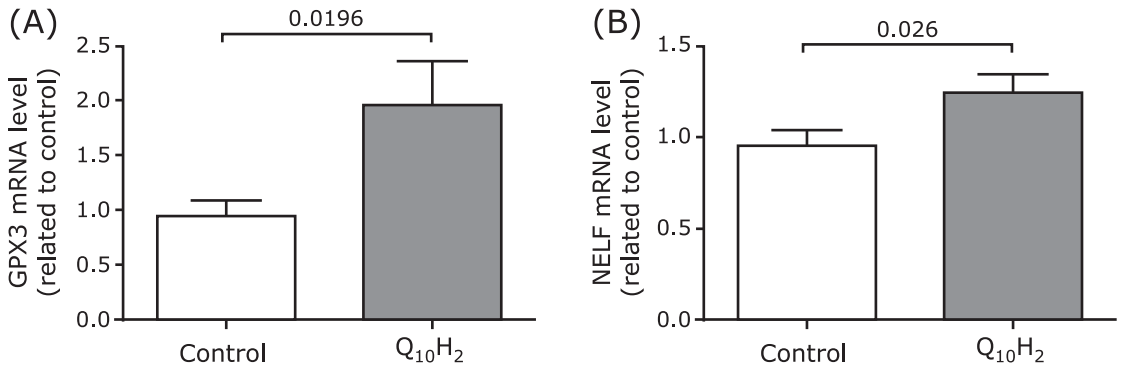

Fig. 2. Verification of selected "top 20" genes from microarray experiments by qRT-PCR. For verification of microarray data, two "top 20" up-regulated genes (GPX3, NELF) were further selected for qRT-PCR experiments. Finally, GPX3 (A) and NELF (B) genes were up-regulated about 2.0-fold (p = 0.0196) and 1.3-fold (p = 0.026) in the ubiquinol-treated (+$Q_{10}H_2$) animals, respectively, when related to control mice (–$Q_{10}H_2$).

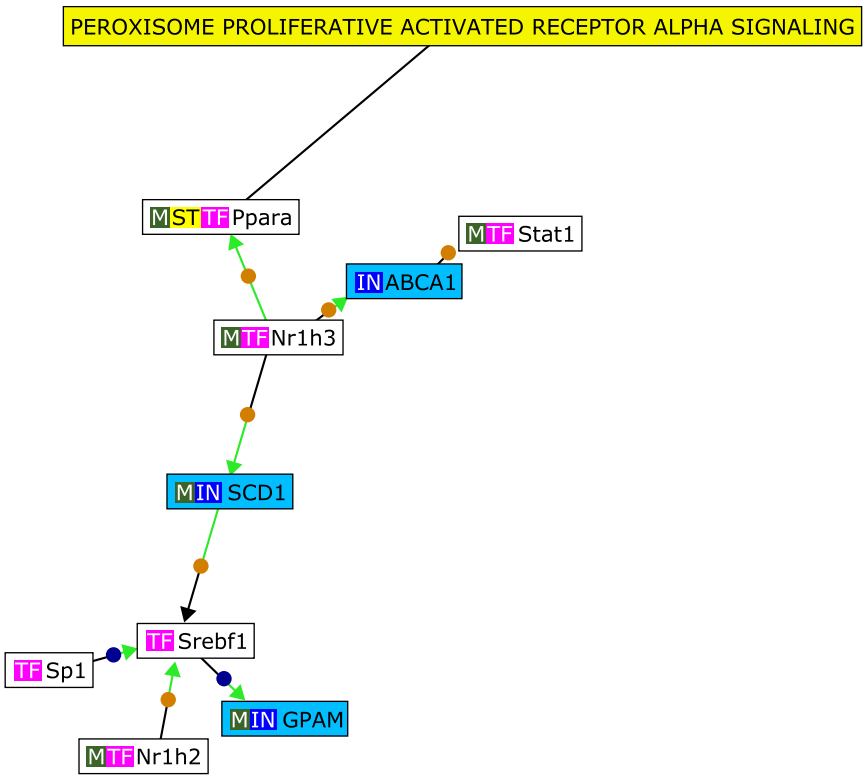

Fig. 3. Bibliosphere network of ubiquinol-sensitive genes regulated in liver tissues of C57BL6J mice. Based on co-citations with transcription factors and other genes in the network (GFG level B4), 3 ubiquinol-inducible genes were connected with each other by BibliospherePathwayEdition Software. According to this, the uploaded genes seem to play a key role in PPAR-α signalling pathways. IN, input gene; TF, transcription factor; M, gene product is part of a metabolic pathway; ST, gene product is part of a Genomatix signal transduction pathway.

body weight/d) or a respective control diet for one week. Samples were taken from four mice per each group respectively, resulting in a total of eight microarrays. Differentially expressed genes between ubiquinol-treated and control animals were selected as follows: Initially transcripts with at least four present calls (100%) in one group (treatment or control) were chosen for further analysis. Secondly, transcripts showing at least a 1.5-fold increase or decrease in the ubiquinol-treated animals when related to controls ($p \leq 0.05$, Students t test) were selected. Based on these criteria, 1112 and 571 transcripts were up- and down-regulated by ubiquinol, respectively. To study the observed effects of ubiquinol supplementation on gene expression in more detail, transcripts with the highest fold change values ("Top 20", $p \leq 0.05$) were selected. However, before unravelling putative functional connections among these genes, the accuracy of microarray data was verified by qRT-PCR for two selected "top 20" genes, namely glutathione peroxidase 3 (GPX3) and nasal embryonic LHRH factor (NELF) (Fig. 2). In brief, GPX3 has been described to mediate PPARγ-associated antioxidant effects in human muscle cells and plasma.[15] Additionally, an influence on the regulation of

Table 1. Display of the "top 20" up- and down-regulated genes in liver samples of mice supplemented with ubiquinol for 7 days

Affymetrix Probe Set ID	FC	*p* value	Gene symbol	Gene name	GeneOntology Process/Function
up-regulated genes					
1451488_at	6.67	0.04	FITM1	fat storage-inducing transmembrane protein 1	Lipid particle organization, positive regulation of sequestering of triglyceride
1439398_x_at	5.82	0.0001	Nelf	Nasal embryonic LHRH factor	
1452637_a_at	5.09	0.02	Bola1	Bola-like 1 (*E. coli*)	biological process, molecular function
1428554_a_at	4.59	0.04	1810035L17Rik	RIKEN cDNA 1810035L17 gene	Biological process, regulation of transcription, RNA-binding, molecular function, nucleic acid binding, nucleotide binding
1416439_at	4.57	0.01	Dctpp1	dCTP pyrophosphatase 1	Nucleoside triphosphate catabolic process, protein homotetramerization, dCTP diphosphatase activity, hydrolase activity, metal ion binding
1428464_at	4.48	0.05	Ndufa3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3	Biological process, electron transport chain, transport, molecular function
1420952_at	4.26	0.04	Son	Son cell proliferation protein	DNA binding, RNA binding, nucleic acid binding, protein binding
1438403_s_at	4.24	0.04	—	—	
1422608_at	4.14	0.02	Arpp19	cAMP-regulated phosphoprotein 19	positive regulation of Ras protein signal transduction, molecular function
1449106_at	4.1	0.05	Gpx3	glutathione peroxidase 3	Glutathione metabolic process, hydrogen peroxide metabolic process, oxidation reduction, response to oxidative stress, glutathione binding, peroxidase activity, oxidoreductase activity
1421374_a_at	4.01	0.03	Fxyd1	FXYD domain-containing ion transport regulator 1	Cellular calcium ion homeostasis, ion transport, muscle contraction, transport, chloride channel activity, ion channel activity
1416217_a_at	4	0.05	Rpl37a	ribosomal protein L37a	biological process, molecular function
1434823_x_at	3.91	0.02	Myeov2	myeloma overexpressed 2	biological process, molecular function
1436757_a_at	3.91	0.03	Cox6b1	cytochrome c oxidase, subunit VIb polypeptide 1	cytochrome-c oxidase activity
1420642_a_at	3.91	0.05	Romo1	reactive oxygen species modulator 1	biological process, molecular function
1448685_at	3.88	0.04	2900010M23Rik	RIKEN cDNA 2900010M23 gene	biological process, molecular function
1438655_a_at	3.86	0.04	Rpl34	ribosomal protein L34	biological process, molecular function
1431199_at	3.84	0.04	Ggnbp1	gametogenetin binding protein 1	Cell differentiation, mitochondrial fission, multicellular organismal development, spermatogenesis, protein binding
1416285_at	3.82	0.04	Ndufc1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1	biological process, electron transport chain, transport, molecular function
1429453_a_at	3.8	0.02	Mrpl55	mitochondrial ribosomal protein L55	biological process, molecular function
down-regulated genes					
1452391_at	–3.37	0.002	Cxadr	Coxsackie virus and adenovirus receptor	cardiac muscle fiber development, cell adhesion, cell-cell junction organization, heart development, mitochondrial organization, negative regulation of cardiac muscle cell proliferation, protein binding, receptor activity
1450392_at	–3.15	0.02	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	cholesterol efflux, cholesterol transport, lipoprotein biosynthetic process, phospholipids efflux, positive regulation of cholesterol efflux, protein amino acid lipidation, ATP binding, ATPase activity, cholesterol transporter activity
1427408_a_at	–2.91	0.02	Thrap3	thyroid hormone receptor associated protein 3	positive regulation of transcription from RNA polymerase II promoter, transcription coactivator activity
1417015_at	–2.84	0.04	Rassf3	Ras association (RalGDS/AF-6) domain family 3	biological process, signal transduction, molecular function, protein binding
AFFX-PyruCarbMur/ L09192_5_at	–2.7	0.04	Pcx	pyruvate carboxylase	gluconeogenesis, lipid biosynthetic process, metabolic process, oxaloacetate metabolic process, pyruvate metabolic process, ATP binding, biotin binding, metal ion binding, pyruvate carboxylase activity, nucleotide binding
1425461_at	–2.67	0.02	Fbxw11	F-box and WD-40 domain protein 11	Wnt receptor signalling pathway, biological process, cell cycle, rhythmic process, molecular function
1420948_s_at	–2.61	0.002	Atrx	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	DNA repair, forebrain development, response to DNA damage stimulus, ATP binding, DNA binding, chromatin binding, metal ion binding
1433515_s_at	–2.6	0.01	Etnk1	ethanolamine kinase 1	biological process, phospholipid biosynthetic process, ATP binding, ethanolamine kinase activity, kinase activity, molecular function, nucleotide binding, transferase activity
1425834_a_at	–2.6	0.05	Gpam	glycerol-3-phosphate acyltransferase, mitochondrial	Acyl-CoA metabolic process, cellular response to insulin stimulus, defense response, fatty acid homeostasis, glycerophospholipid metabolic process, triglyceride metabolic process, phospholipid homeostasis, regulation of cytokine secretion, acyltransferase activity
1430991_at	–2.56	0.03	1810014B01Rik	RIKEN cDNA 1810014B01 gene	biological process, molecular function
1424484_at	–2.55	0.04	Mobkl1b	MOB1, Mps One Binder kinase activator-like 1B (yeast)	metal ion binding, protein binding
1448158_at	–2.49	0.04	Sdc1	syndecan 1	canonical Wnt receptor signaling pathway, myoblast development, cytoskeletal protein binding, glycoprotein binding
1422862_at	–2.45	0.02	LOC669660	similar to PDZ and LIM domain protein 5 (Enigma homolog) (Enigma-like PDZ and LIM domains protein)	
1420864_at	–2.43	0.0003	Zfp161	zinc finger protein 161	Regulation of transcription, DNA binding, metal ion binding, nucleic acid binding, protein binding, zinc ion binding
1415965_at	–2.4	0.004	Scd1	stearoyl-Coenzyme A desaturase 1	Brown fat cell differentiation, cholesterol esterification, fatty acid biosynthetic process, lipid metabolic process, oxidation reduction, white fat cell differentiation, oxidoreductase activity, stearoyl-CoA9-desaturase activity, iron ion binding
1431096_at	–2.4	0.04	Ints8	integrator complex subunit 8	biological process, molecular function
1419816_s_at	–2.39	0.004	Errfi1	ERBB receptor feedback inhibitor 1	lung alveolus development, negative regulation of epidermal growth factor receptor activity, regulation of keratinocyte differentiation, stress-activated protein kinase signalling cascade, kinase binding, protein binding, receptor activity
1420927_at	–2.38	0.03	St6gal1	beta galactoside alpha 2,6 sialyltransferase 1	metabolic process, protein amino acid glycosylation, sialyltransferase activity
1448607_at	–2.37	0.004	Nampt	nicotinamide phosphoribosyltransferase	NAD biosynthetic process, positive regulation of muscle cell proliferation, pyridine nucleotide biosynthetic process, cytokine activity, nicotinamide phosphoribosyltransferase activity, protein homodimerization activity
1418279_a_at	–2.36	0.01	Akap1	A kinase (PRKA) anchor protein 1	Negative regulation of NFAT protein import into nucleus, negative regulation of protein amino acid dephosphorylation, RNA binding, kinase activity, nucleic acid binding

presented by ≥2 probe set Ids in Top 20 gene list.

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Table 2. PCR primers for analysis of the methylation status of (A) ABCA1, (B) ACSL1 and (C) ACYP1 gene promoters. All positions are relative to the entire input sequences

(A) ABCA1

	amplicon				primer	
	start	end	length	CpGs	left	right
forward direction						
ABCA1_amp01	50	506	457	8	GGTTTTTGGAAGGTAGAGATTTTTT	CCCTAACCCTAACCTACTAACCTTC
ABCA1_amp02	482	1101	620	40	GAAGGTTAGTAGGTTAGGGTTAGGG	CCCTATACTATTTACATCCCCAAAAA
ABCA1_amp03	1076	1515	440	21	TTTTTGGGGATGTAAATAGTATAGGG	CCCAAAAAACTCAAACAACAATAAC
ABCA1_amp04	1424	1967	544	22	GATTGGGATTGTATGTTTTTGTTTT	CACCAATTTTAACCAAATTCACAAT
reverse direction						
ABCA1_amp05	115	516	402	8	TTTTTTGTAGTTTTGGTTTTGATTTG	AAACTAATATTCACTATCCATTCCACA
ABCA1_amp06	491	891	401	36	GGGGGAAATAGGGAGTAGAGTAGTT	CAAATCAAAACCAAAACTACAAAAA
ABCA1_amp07	865	1367	503	23	AGGATTTAGATGTGATATTTGTGGG	AAAACTACTCTACTCCCTATTTCCCC
ABCA1_amp08	1343	1669	327	7	AGGGTGGATTGGGTATTTTAGTTT	CCCACAAATATCACATCTAAATCCT
ABCA1_amp09	1646	2084	439	17	GGTTTGGGAGTTAGGAAATAAATAAA	AAACTAAAATACCCAATCCACCCT

(B) ACSL1

	amplicon				primer	
	start	end	length	CpGs	left	right
forward direction						
ACSL1_amp01	27	390	364	13	AGGATGGAAGAGTTAAAGGGTATTT	TATCTAAACTAATCCAAACCCCTCA
ACSL1_amp02	366	1078	713	78	TGAGGGGTTTGGATTAGTTTAGATA	CAAAATCCCCAACAAACAAAA
ACSL1_amp03	1049	1278	230	22	GAGAGGTGGTTTTGTTTGTTGG	TCTAAAAACTCCTCTTAAAAACACC
reverse direction						
ACSL1_amp04	87	387	301	13	TTGGGTTGATTTAAGTTTTTTAGATT	AAAACTTCCAAATTTACCCTTTACC
ACSL1_amp05	364	1077	714	78	AGGGTTTTTAGTAAGTAGGATTATTTTT	TCTAAAAAACTTAAATCAACCCAAA
ACSL1_amp06	1050	1290	241	22	GTGTGTAGTGGTTTTGGGAGTTT	AAAAATAATCCTACTTACTAAAAACCCT

(C) ACYP1

	amplicon				primer	
	start	end	length	CpGs	left	right
Region 01						
forward direction						
ACYP1_amp01	142	729	588	29	GAGAGGGGGTGATAGATATTTGAG	AATATAACCCCAAAAAAACCTCTCC
ACYP1_amp02	767	945	179	7	AGATTTTAGGGTGTGTTTTTGTTTG	CCTAAAACTCTTACATAACCCTACCT
reverse direction						
ACYP1_amp03	76	355	280	12	TGGGGATTTTTTTTGAGTTGGAG	TCAAACCCTCATACAAAAATACTCTAT
ACYP1_amp04	344	943	600	26	TGGAGTTTTTATATAGTTTTATTTGGGG	AAAAAATCCCCAAAACCTAATTTTC
Region 02						
forward direction						
ACYP1_amp05	11	394	384	16	TTTTAGGTATTTAGTAGGGTGAGTGGA	ATTTCTAAAACTTCCCCAAAAACC
ACYP1_amp06	195	599	405	26	ATTTGTTTTGAAGTTTTATTTTTGGG	CTCCAATACCATAAAAACCTCTCC
reverse direction						
ACYP1_amp07	1	601	601	29	TTTTTTAATATTATGGGGGTTTTTT	ATACTAACAACCCTAAACACCCAA

local and systemic oxidative stress processes has been described.[16] In this context, effects of ubiquinol supplementation on hepatic oxidative stress-associated gene regulation has been already described previously.[6] The NELF gene is primarily expressed in olfactory sensory cells and LHRH cells during embryonic development,[17] and is therefore ascribed as an important factor in the developmental migration process. In adult tissue, NELF has been also shown to be present in the liver.[17] In summary we could show that the fold change values of the two selected "top 20" genes glutathione peroxidase 3 (GPX3) and nasal embryonic LHRH factor (NELF) (Fig. 2) were in accordance to the microarray data (Table 1). To determine functional connections between the "top 20" up- and down-regulated genes, text mining analysis was subsequently performed by the use of Genomatix BibliospherePathwayEdition Software including GFG level B4. Based on these stringent criteria, three ubiquinol-sensitive genes [ATP-binding cassette, sub-family A (ABC1), member (ABCA1), stearoyl-Coenzyme A desaturase 1 (SCD1) and glycerol-3-phosphate acyltransferase (GPAM)] were functionally connected with each other in the PPARα signalling pathway (Fig. 3). Moreover, when related to GeneOntology terms (Table 1), 6 (15%) of the "top 20" up- and down-regulated genes were related to cholesterol or lipid metabolism. These results suggest a functional role of ubiquinol in cholesterol and/or lipid metabolism and are, inter alia in agreement to a previous performed supplementation study with ubiquinol in senescence accelerated mice prone (SAMP1). In this

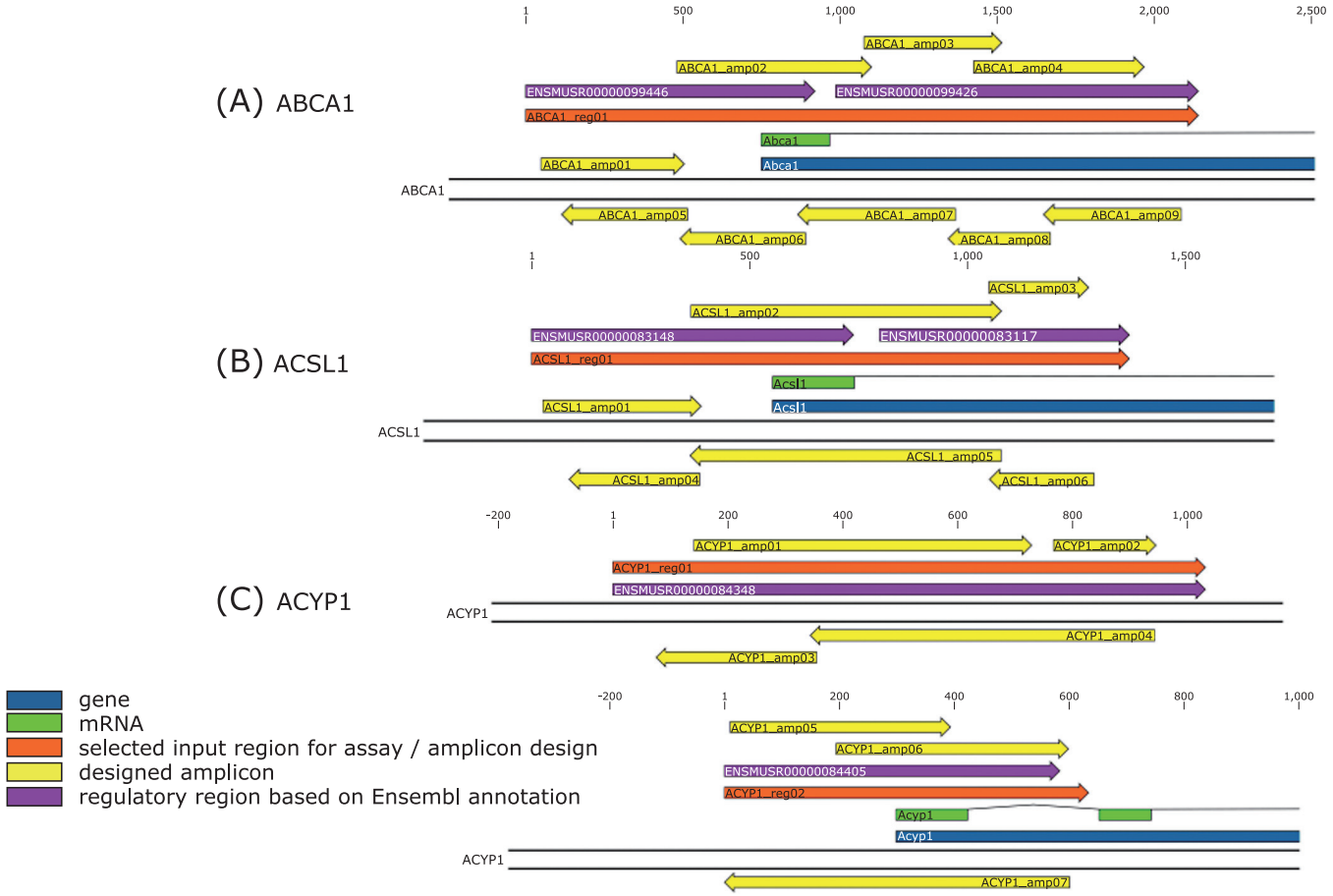

Fig. 4. Schematic overview of amplicons processed for ABCA1 (A), ACSL1 (B) and ACYP1 (C) analyses in the genomic context. Positions are relative to the selected input region (indicated as reg) and correspond to Table 2 for each respective gene. The regions are located for ABCA1 (A) and ACSL1 (B) gene at position –748 to 1391 and –552 to 819, respectively, when related to the gene start position. For ACYP1 gene (C), region 01 (upper portion) and region 02 (lower portion) are located at position –6863 to –7663 and –298 to 336, respectively, when related to the gene start position. All genes are shown in their transcribed orientation, which is located for ABCA1 and ACYP1 on the antisense strand of chromosome 4 and 12, respectively. For ACSL1 gene, the transcribed orientation is shown on the sense strand of chromosome 8.

study, various genes with a functional role in cholesterol synthesis, fat assimilation and lipoprotein metabolism have been shown to be affected.[4] Moreover, these transcriptional effects were also translated into physiological readouts of cholesterol metabolism *in vivo*.[18] In summary, our present and previous data show both short and long-term effects of ubiquinol supplementation on genes involved in liver lipid and/or cholesterol metabolism.

Ubiquinol-induced effects on gene expression are not mediated by changes in promoter methylation. With regard to the functional analysis of the microarray data from liver tissues of ubiquinol-supplemented mice, distinct effects on genes related to lipid and/or cholesterol metabolism were found (Table 1, Fig. 3). Because transcriptional variation has been correlated with CpG island variation[19] and previous literature reports have documented distinct roles of various micronutrients in the epigenetic regulation of gene expression,[20–23] CpG island regions in promoter regions of three regulated genes strongly related to lipid metabolism were analyzed for putative changes by using base-specific cleavage and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. In general, as an essential precondition, all three genes show CpG islands in their promoter regions and could be therefore selected for DNA methylation analysis. Based on these criteria and the results from microarray experiments, the following ubiquinol-regulated genes ($p \leq 0.05$) were finally selected for subsequent methylation analysis: ABCA1 (ATP-binding cassette, sub-family A, member 1), ACSL1 (Acyl-CoA Synthetase long-chain family member 1) and ACYP1 (Acylphosphatase 1). These genes have been shown to be regulated about +3.14-fold ($p \leq 0.05$), –2.13-fold ($p \leq 0.05$) and +2.18-fold ($p \leq 0.01$), respectively, when related to liver samples of control mice. In brief, the ABCA1 gene is a LXR target gene and plays an important role in reverse cholesterol transport.[24] ACSL1 plays an important role in fatty acid metabolism and triacylglycerol synthesis.[25] Disturbances of these pathways may result in e.g. dyslipidemia, one hallmark of the metabolic syndrome.[26] ACYP1 primarily mediates hydrolytic activity on e.g. acyl phosphates and aryl phosphate monoesters.[27] Overall, our DNA methylation analysis at 282 (sense orientation) and 271 (antisense orientation) CpG units, spanning nucleotides relatively to the gene start position from –748 to 1,391 for ABCA1 gene (Fig. 4A), from –552 to 819 for ACSL1 gene (Fig. 4B), and from –6,863 to –7,663 as well as –298 to 336 for ACYP1 gene (Fig. 4C) revealed no differences between $Q_{10}H_2$-treated and control animals. From these results, no significant differences in the promoter methylation patterns of ABCA1, ACSL1 and ACYP1 genes could be detected with regard to a short term supplementation period with ubiquinol in C57BL6J mice.

Although we could not found any significant effects of

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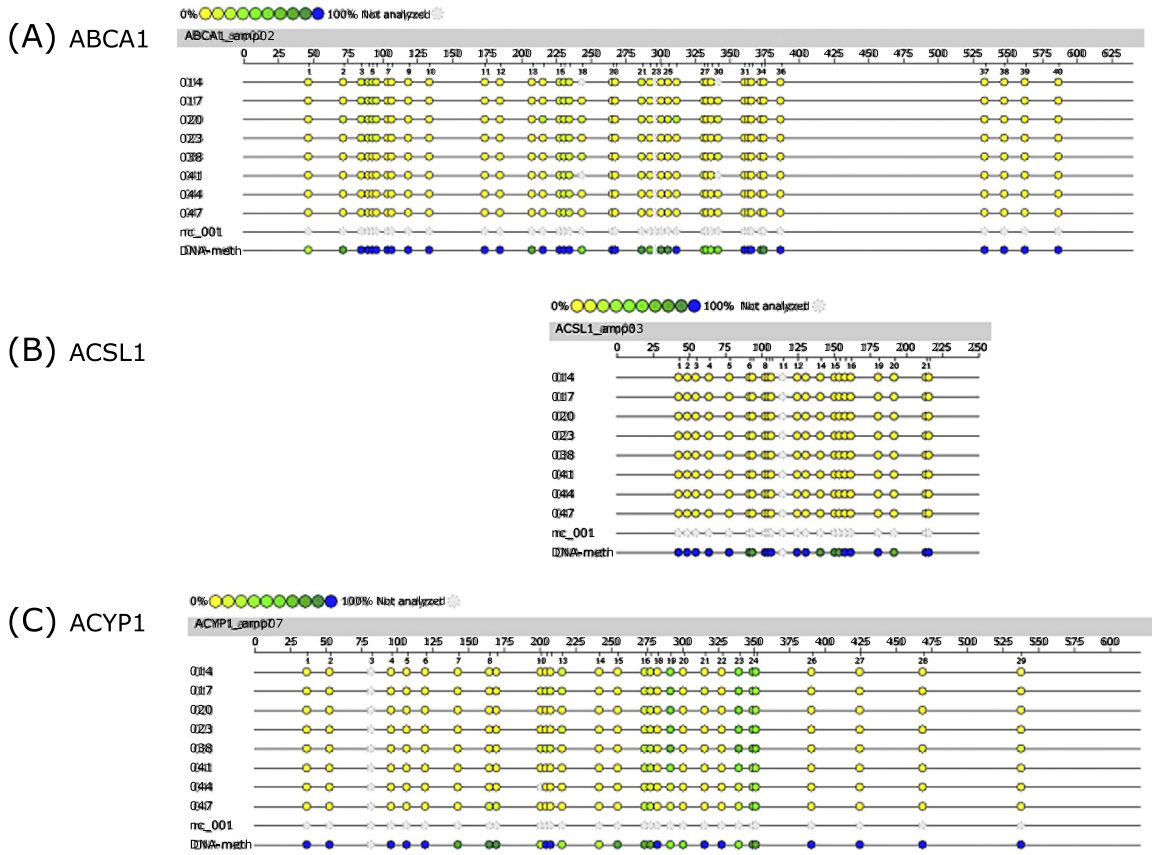

Fig. 5. Epigram of quantitative methylation analysis of promoter regions in ABCA1, ACSL1 and ACYP1 genes. Genomic DNA isolated from liver samples of $Q_{10}H_2$ supplemented (14, 17, 20, 23) and non-supplemented control mice (38, 41, 44, 47) was analyzed for methylation status of 553 (sense and antisense) CpG sites of the (A) ABCA1, (B) ACSL1 and (C) ACYP1 gene promoter. Exemplary data for one amplicon of each gene is shown. The coloured dots indicate the software determined methylation ratio at each analyzed CpG-unit for each sample. nc_001 represents the reaction negative control (water) and DNA-meth the artificially completely methylated control DNA. The reference sequence above the epigram corresponds to the genomic sequence of the analyzed strand. Displayed is the sense orientation in 5' → 3' direction. Base numbering in the epigram refers to the analyzed amplicon.

ubiquinol supplementation on CpG island promoter region methylation patterns of ABCA1, ACSL1 and ACYP1 genes, previous results in the literature show that coenzyme Q_{10} containing coenzyme supplementation is able to induce the disappearance of tamoxifen-induced RASSF1A DNA methylation patterns in breast cancer patients.[28] Moreover, this supplementary therapy has also been shown to counteract the tamoxifen-induced increased lipoprotein and lipid levels in these patients.[28] By the way, ubiquinol-induced reductions of LDL cholesterol levels have been also observed previously in healthy male volunteers.[5] From these results it seems that ubiquinol supplementation may modulate lipid metabolism through an impact on DNA methylation patterns. Although we could not find any ubiquinol-induced supplementary effects in the present study, it can not be ruled out that changes in the methylation pattern other than in the promoter region of ABCA1, ACSL1 and ACYP1 genes have led to the observed differences on gene expression. In fact, effects on global methylation patterns have been already described for several other dietary supplements and micronutrients in the literature.[20,21,29–32] Thus, a global methylation analysis would be required to finally answer the question of a ubiquinol-dependent epigenetic regulation of ABCA1, ACSL1 and ACYP1 genes. Moreover, the observed changes in gene expression might be also mediated through modifications of histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation or isomerisation.[22,23] The ability of dietary compounds to influence epigenetic processes via histone modification has been already described *in vitro* and *in vivo*.[32,33] However, the translation of these findings into clinical health applications is still a remaining challenge for further studies in the future.

In summary, we could show that one-week supplementation with ubiquinol (250 mg/kg BW/d) induces a gene expression pattern in liver tissues of C57BL6J mice with a functional role in PPARα signalling, lipid and/or cholesterol metabolism. Because transcriptional variation has been correlated with CpG island variation, CpG island regions in promoter regions of three regulated genes (ABCA1, ACSL1 and ACYP1) with a relevant impact in lipid and/or cholesterol metabolism were analyzed. However, promoter DNA methylation analysis of ABCA1, ACSL1 and ACYP1 promoters in the liver of C57BL6J mice revealed no differences between ubiquinol-treated and control mice. Thus, ubiquinol affects the expression of genes involved in PPARα signalling and lipid metabolism without changes in promoter DNA methylation in the liver of mice.

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