

Research Communication

Ubiquinol-Induced Gene Expression Signatures are Translated into Altered Parameters of Erythropoiesis and Reduced Low Density Lipoprotein Cholesterol Levels in Humans

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Summary

Studies *in vitro* and in mice indicate a role for Coenzyme Q₁₀ (CoQ₁₀) in gene expression. To determine this function in relationship to physiological readouts, a 2-week supplementation study with the reduced form of CoQ₁₀ (ubiquinol, Q₁₀H₂, 150 mg/d) was performed in 53 healthy males. Mean CoQ₁₀ plasma levels increased 4.8-fold after supplementation. Transcriptomic and bioinformatic approaches identified a gene–gene interaction network in CD14-positive monocytes, which functions in inflammation, cell differentiation, and peroxisome proliferator-activated receptor-signaling. These Q₁₀H₂-induced gene expression signatures were also described previously in liver tissues of SAMP1 mice. Biochemical and NMR-based analyses showed a reduction of low density lipoprotein (LDL) cholesterol plasma levels after Q₁₀H₂ supplementation. This effect was especially pronounced in atherogenic small dense LDL particles (19–21 nm, 1.045 g/L). In agreement with gene expression signatures, Q₁₀H₂ reduces the number of erythrocytes but increases the concentration of reticulocytes. In conclusion, Q₁₀H₂ induces characteristic gene expression patterns, which are translated into reduced LDL cholesterol levels and altered parameters of erythropoiesis in humans. © 2011 IUBMB

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INTRODUCTION

Coenzyme Q₁₀ (CoQ₁₀) acts as a cofactor in electron transport in the respiratory chain and is required for the biosynthesis of pyrimidine nucleotides and the function of uncoupling proteins (1). The reduced form of CoQ₁₀ (ubiquinol, Q₁₀H₂) serves as a potent antioxidant in mitochondria and lipid membranes as well as a regenerator of other lipid soluble antioxidants (1). Our group and others observed anti-inflammatory (2–4) and antiapoptotic (5, 6) effects of CoQ₁₀ *in vitro*. These effects are hypothesized to be mediated by the antioxidant properties of Q₁₀H₂ (7). Based on transcriptomic approaches, CoQ₁₀ has been identified as a modulator of gene expression in HeLa cells (8), intestinal CaCo-2 cells (9), and monocytic THP-1 cells (10). An influence of CoQ₁₀ on gene expression was also confirmed in the heart of C57BL/6 mice (11) and several tissues of SAMP1 mice (12). Here, we investigated the effects of ubiquinol on gene expression in relationship to physiological readouts in humans.

SUBJECTS AND METHODS

Q₁₀H₂ Formulation

Q₁₀H₂ capsules (Q₁₀H₂, rapeseed oil, diglyceryl monooleate, bee wax, and lecithin) were obtained from KANEKA Corporation, Japan. Capsule stabilization was ensured by a method disclosed in patent applications (WO 03/06408, WO 03/06409, WO 03/06410, WO 03/06411, WO 03/06412, WO 03/08363, and WO 03/32967).

Subjects and Study Design

Fifty-three healthy male volunteers, 21–48 years of age, received 150 mg Q₁₀H₂ daily in the form of three softgel

capsules with each principal meal (a 50-mg) for 14 days. Fasting blood samples (54 mL each) were taken before (T_0) and after (T_{14}) supplementation with Q₁₀H₂ from all study participants and 4 weeks after study completion (T_{42} , washout period) from nine subjects (Supporting Information Supplemental Methods section). The study was approved by the ethics committee of the Medical Faculty of Kiel University, Germany, and was conformed to Helsinki Declaration. All volunteers gave written informed consent.

High-Pressure Liquid Chromatography Analysis

Analysis was based on the method of high-pressure liquid chromatography with electrochemical detection and internal standardisation using ubihydroquinone-9 and ubiquinone-9 as standards and is described elsewhere (13).

Monocyte Isolation and RNA Extraction

CD14-positive monocytes were isolated from ethylenediamine tetraacetic acid (EDTA)-blood samples (36 mL) of volunteers at each indicated time point (T_0 , T_{14} , and T_{42}) using microbead-conjugated anti-CD14 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (14).

Microarray Analysis

Experiments were performed as described previously (10) with RNA samples from monocytes of three volunteers at the indicated time points (T_0 , T_{14} , and T_{42}), including a total of nine human genome U133 Plus 2.0 GeneChip[®] microarrays. Sample selection from volunteers for microarray analysis was based on quality and quantity of isolated RNA (Supporting Information Supplemental Methods section).

Quantitative Real-Time 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 (Supporting Information Table 1) were obtained from MWG Biotech AG (Ebersberg, Germany). cDNA synthesis with subsequent PCR amplification procedure is described in Supporting Information Supplemental Methods section.

In silico Analysis

For analysis of common pathways between regulated genes, Ingenuity Pathways Analysis 2010 (www.ingenuity.com) was used (Supporting Information Supplemental Methods section).

Ex vivo Whole Blood Stimulation

Venous blood from study participants was diluted 1:5 with cell culture medium (RPMI 1640, Invitrogen, Karlsruhe, Germany) and plated in a 24-well format. Subsequently after 1 h, blood samples were stimulated with LPS (*Salmonella*

Table 1
Effects of 2-week Q₁₀H₂ supplementation on basic characteristics of study subjects ($n = 53$), as well as parameters involved in erythropoiesis, lipid metabolism, and inflammation

Basic characteristics	T_0	T_{14}
Age (years)	30.13 ± 6.71	30.13 ± 6.71
Weight (kg)	79.11 ± 10.17	79.18 ± 10.01
Height (m)	1.81 ± 0.06	1.81 ± 0.06
BMI (kg/m ²)	24.12 ± 2.50	24.14 ± 2.44
Glucose (mg/dL)	86.47 ± 10.68	84.26 ± 10.10
Creatinine (mg/dL)	1.05 ± 0.10	1.07 ± 0.12
GOT ^a (U/L)	30.09 ± 8.67	33.06 ± 35.95
GPT ^b (U/L)	37.79 ± 14.73	37.26 ± 16.52
γ-GT ^c (U/L)	20.49 ± 10.36	17.79 ± 7.68*
Blood pressure (mm Hg)		
Systolic	126.79 ± 12.00	126.11 ± 12.29
Diastolic	82.74 ± 9.31	81.79 ± 8.46
ADMA (μmol/L)	0.98 ± 0.27	1.11 ± 0.50
Erythropoiesis		
Leukocytes (nL)	5.65 ± 1.43	5.74 ± 1.28
Erythrocytes (pL)	4.95 ± 0.29	4.89 ± 0.31**
Haemoglobin (g/dL)	15.16 ± 0.82	15.04 ± 0.85
Haematocrit (%)	44.48 ± 2.36	43.78 ± 2.40***
MCV ^a (fL)	89.82 ± 2.87	89.76 ± 2.78
MCH ^b (pg)	30.62 ± 1.12	30.82 ± 1.17**
MCHC ^c (g/dL)	34.09 ± 0.53	34.33 ± 0.53**
Reticulocytes (per mill)	10.94 ± 3.03	12.09 ± 2.46*
Lipid metabolism		
Cholesterol (mg/dL)	166.09 ± 29.79	163.85 ± 27.85
HDL cholesterol (mg/dL)	51.13 ± 12.93	51.3 ± 11.9
LDL cholesterol (mg/dL)	95.51 ± 28.89	90.60 ± 27.21**
OxLDL (U/mL)	20.62 ± 14.67	20.43 ± 14.46
Triglycerides (mg/dL)	97.40 ± 49.04	112.32 ± 73.13
Inflammation		
TNFα (pg/mL)	139.37 ± 122.24	144.60 ± 140.00
CXCL2 (pg/mL)	251.97 ± 68.78	252.02 ± 69.47
MCP-1 (ng/mL)	184.88 ± 108.55	183.23 ± 107.59
CRP (mg/dL)	0.30 ± 0.01	0.31 ± 0.05

Data are described as means ± SD.

* T_0 versus T_{14} , $P \leq 0.001$; ** T_0 versus T_{14} , $P \leq 0.05$; *** T_0 versus T_{14} , $P \leq 0.01$.

^aGlutamate oxalacetate transferase (ASAT, aspartate aminotransferase).

^bGlutamate pyruvate transaminase (ALAT, alanine aminotransferase).

^cGamma glutamyl transferase.

enteritidis, 20 ng/mL). Unstimulated (−LPS) samples served as controls. Plates were further incubated for 6 h at 37 °C in humidified atmosphere. Subsequently, thereafter, plates were centrifuged and supernatants were removed. Samples were stored at −80 °C until further analysis.

Enzyme-Linked Immuno Sorbent Assay (ELISA)

Supernatants of native and/or *ex vivo* stimulated whole blood samples were measured with commercially available ELISA kits (Supporting Information Supplemental Methods section).

¹H NMR Analysis to Determine Lipoproteins

For analysis of low density lipoprotein (LDL) cholesterol levels in serum samples of study subjects, ¹H NMR spectroscopy was carried out on a Bruker AVANCE II spectrometer operating at 600 MHz ¹H resonance frequency (Supporting Information Supplemental Methods section).

Statistical Analysis

Based on power analysis including F-value (95% power, $P \leq 0.05$) of 12.99, a standard deviation of tumour necrosis factor alpha (TNF α) levels of $\sigma = 0.7$ and literature-based differences in TNF α levels between controls and treatment groups of $d = 0.5$, the minimum number of study subjects was determined as 51. Thus, 53 study subjects were finally included in the study. Statistics were calculated with SPSS 11.5 software (SPSS GmbH Software, München, Germany), Microsoft Excel 2003, and GraphPad Prism 4.0. Before statistical analysis, normal distribution of the parameters was tested. Results were analyzed by a two-sided, paired Students *t*-test if not stated otherwise. Levels of statistical significance were set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Studies *in vitro* and in mice indicate a role of Coenzyme Q₁₀ (CoQ₁₀) in gene expression. To determine this function in relationship to physiological readouts in humans, a 2-week supplementation study with the reduced form of CoQ₁₀ (ubiquinol, Q₁₀H₂, 150 mg/d) was performed in 53 healthy males.

Q₁₀H₂ Supplementation Leads to a 4.8-Fold Increase in CoQ₁₀ Plasma Levels

Fasting blood samples were taken before (T_0) and after (T_{14}) supplementation with Q₁₀H₂. Basic characteristics such as body mass index (BMI) or fasting glucose levels of the subjects were in accordance with the inclusion criteria of the study and show values within the physiological range for healthy men (Table 1). Because studies with CoQ₁₀ indicated effects on markers of endothelial dysfunction (15, 16), blood pressure and asymmetric dimethylarginine were determined. We found no effect of Q₁₀H₂ on these parameters (Table 1). With regard to the pre-condition of the study, Q₁₀H₂ supplementation elevates cholesterol-related plasma CoQ₁₀ levels from $229.2 \pm 61.3 \mu\text{mol/mol}$ (T_0) to $1109.8 \pm 343.8 \mu\text{mol/mol}$ (T_{14} ; Fig. 1A) and reduces the oxidized form of CoQ₁₀ (Fig. 1B). Four weeks after study completion (T_{42} , washout period, $n = 9$), plasma levels and redox state of CoQ₁₀ returned to baseline levels. Thus, we were able to increase CoQ₁₀ plasma levels about 4.8-fold in Q₁₀H₂-supplemented persons.

Q₁₀H₂ Supplementation Induces Gene Expression Signatures that Function in Inflammation, Cell Death, and Cell Differentiation

For the identification of Q₁₀H₂-sensitive genes, mRNA steady state levels of 25,044 probe sets were determined in CD14-positive monocytes of three study subjects before and after Q₁₀H₂ supplementation. Fifty-six probe sets were up-regulated and 216 probe sets were downregulated with a fold change ranging from 1.52 to 2.85 and -1.51 to -27.30 , respectively (Supporting Information Tables 2 and 3). Technical and biological verification ($n = 9$) of nine selected genes confirmed the microarray data (Supporting Information Table 4). For additional confirmation, samples 4 weeks after discontinuation of Q₁₀H₂-supplementation ("washout") were used. With exception of the *CCL3* gene, all other selected genes returned essentially to pretreatment levels after washout period (Figs. 1C–1I).

A knowledge-based pathway construction approach was applied for the identified 272 Q₁₀H₂-sensitive genes. The resulting overrepresented and functionally connected Q₁₀H₂-sensitive genes (Figs. 2A and 2B) are, among others, involved in inflammation [*e.g.*, TNF α , chemokine (C-X-C motif) ligand 2 (*CXCL2*), and *CCL3*], cell death, and cell differentiation (*e.g.*, *PMAIP1*, *NR4A2*, *CFLAR*, and *MMD*). Gene ontology classification and Genomatix Bibliosphere pathway analysis of the Q₁₀H₂-induced gene expression signatures identified six genes (*TNF*, *CXCL2*, *CCL3*, *NR4A2*, *BRE*, and *PMAIP1*), which are regulated by the peroxisome proliferator-activated receptors (PPAR) and cell proliferation (TP53) pathways connected by NF κ B (Supporting Information Fig. 1). A PPAR α gene expression signature was also identified in liver samples of Q₁₀H₂-supplemented SAMP1 mice (12). PPAR α activators have been also described to prevent cell differentiation processes *in vivo* (17, 18). A distinct role of Q₁₀H₂ in PPAR α -mediated cell differentiation processes is suggested by the downregulation of the *PMAIP1* (Fig. 1I) and *MMD* gene (Supporting Information Table 3) in human monocytes, probably mediated by PPAR α -mediated inhibition of NF κ B. Taken together, Q₁₀H₂ supplementation in humans induces gene expression patterns that are involved in inflammation, cell death, and cell differentiation.

Q₁₀H₂-Induced Changes in Gene Expression are Translated into Decreased Erythropoiesis

We found that Q₁₀H₂ supplementation reduces the expression of the *MMD* gene (monocyte to macrophage differentiation) and increases *CFLAR* mRNA (caspase 8 and FADD-like apoptosis regulator). Both genes are part of the deduced network of gene-gene interactions (Fig. 2B) and are involved in various cell differentiation processes including erythropoiesis (19). Moreover, a significant ($P \leq 0.05$) downregulation of *MMD2* (-3.17 -fold) and upregulation of *CFLAR* (1.77-fold) was also found in liver tissues of Q₁₀H₂-supplemented mice (unpublished data).

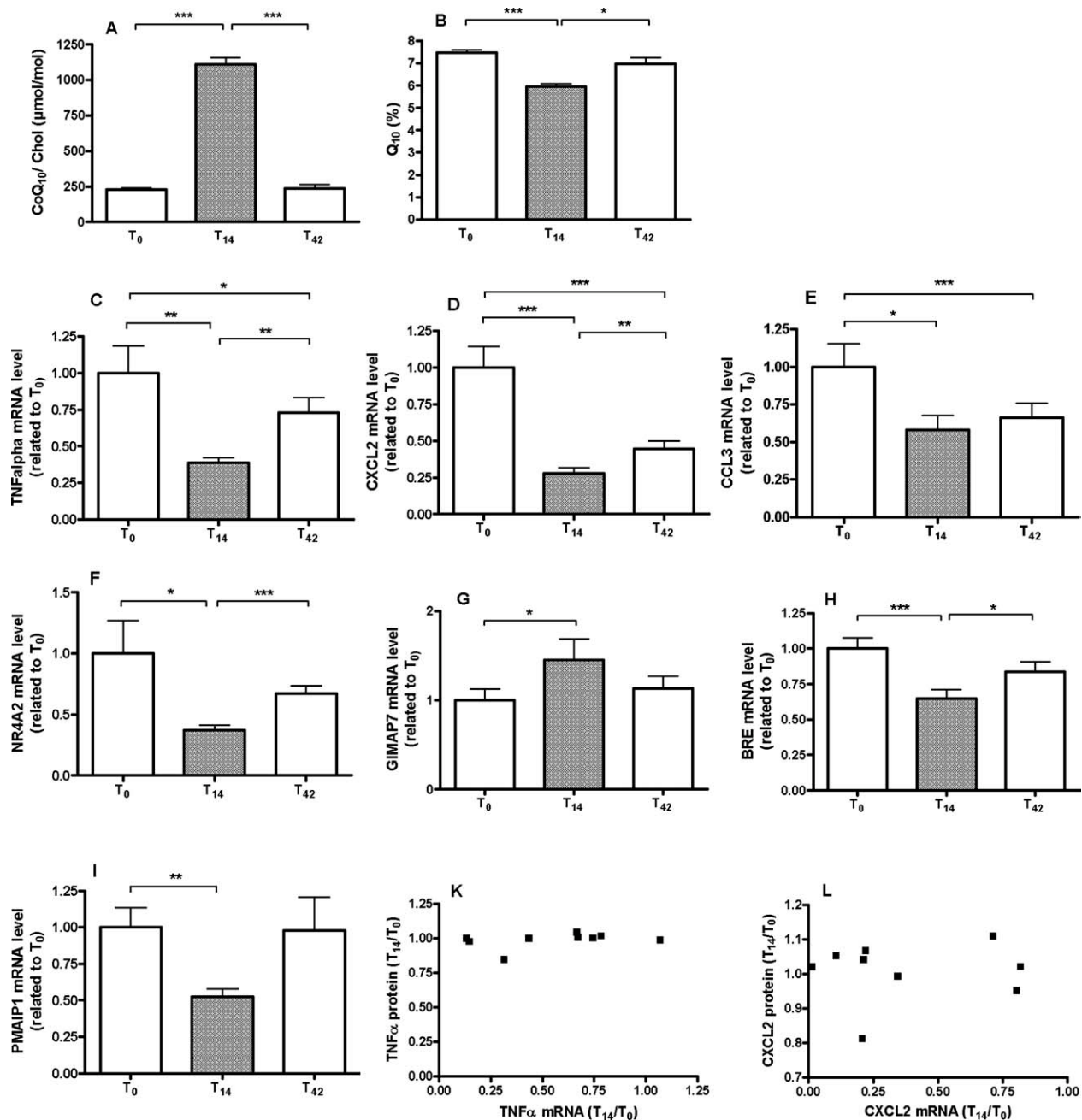


Figure 1. The impact of Q₁₀H₂ supplementation on CoQ₁₀ plasma levels (A, B), and signalling molecules in human monocytes involved in inflammatory and apoptotic processes on the transcriptional (C–L) and protein level (K, L). Data show effects on plasma CoQ₁₀ levels (A), and its redox state (B) before (T₀) and after Q₁₀H₂ supplementation (T₁₄), as well as after 4-week washout period (T₄₂). Data are calculated from means (\pm SEM) of 53 measurements for T₀ and T₁₄, as well as 9 measurements for T₄₂, respectively. Technical as well as biological verification experiments [quantitative real-time polymerase chain reaction (qRT-PCR)] of monocyte samples revealed the expression of 7 Q₁₀H₂-sensitive genes primary related to inflammatory and apoptotic processes: *TNF α* (C), *CXCL2* (D), *CCL3* (E), *NR4A2* (F), *GIMAP7* (G), *BRE* (H) and *PMAIP1* (I). Data are means (\pm SEM) of samples from 9 volunteers, respectively. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Relative mRNA levels for TNF α (K) and CXCL2 (L) were correlated to respective serum protein levels of study subjects ($n = 9$) after Q₁₀H₂ supplementation. No correlation was found between these parameters.

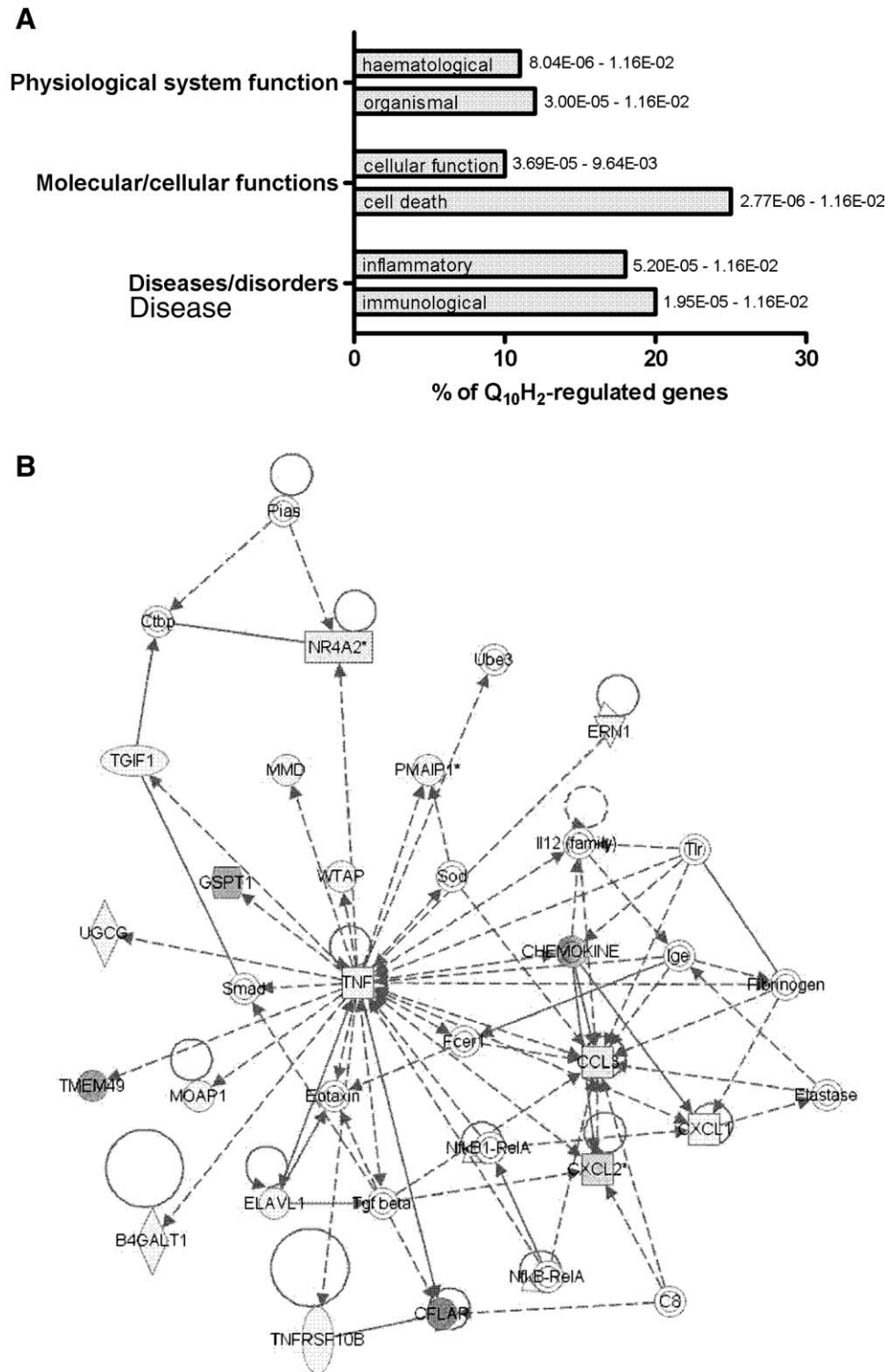


Figure 2. *In silico* classification of the identified $Q_{10}H_2$ -sensitive genes in human monocytes. Based on Ingenuity knowledge base analysis tools, the 272 $Q_{10}H_2$ -sensitive genes play a relevant role in haematological and inflammatory processes, as well as cell death (A). Results are related to a total of 210 mapped transcripts. *P*-values are depicted in the figure bars. A connection of the $Q_{10}H_2$ -regulated genes by signaling pathways of inflammation (e.g., *TNF α* , *CXCL2* and *CCL3*), cell death and cell differentiation (e.g., *PMAIP1*, *NR4A2*, *CFLAR*, *MMD*) is additionally indicated by Ingenuity pathway network (B).

Because processes such as liver hematopoietic stem cell proliferation and red blood cell differentiation are also related to caspase 8-dependent mechanisms (19, 20), effects on hematological parameters were additionally determined. The number of erythrocyte and hematocrit values decreased significantly from 4.95 ± 0.29 to 4.89 ± 0.31 ($P = 0.02$) and 44.48 ± 2.36 to 43.78 ± 2.40 ($P = 0.01$), respectively, for T_0 and T_{14} (Table 1). Mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), and reticulocyte count increased significantly from 30.62 ± 1.12 to 30.82 ± 1.17 ($P = 0.02$), 34.09 ± 0.53 to 34.33 ± 0.53 ($P = 0.02$), and 10.94 ± 3.03 to 12.09 ± 2.46 ($P = 0.0006$), respectively, at T_0 and T_{14} (Table 1). No effects were found for leukocyte count and hemoglobin concentration (Table 1). In conclusion, Q₁₀H₂-supplementation affects erythroid differentiation processes both on the transcriptional and cellular level.

Q₁₀H₂ Supplementation Causes a Characteristic PPAR α Gene Expression Pattern and Reduces LDL Cholesterol Levels

With regard to present gene expression data and our recent findings in SAMP1 mice (12), Q₁₀H₂-mediated effects on PPAR α signaling pathways were hypothesized. Because PPARs are known key players in lipid and cholesterol metabolism (21), effects on serum lipid parameters were analyzed. As determined by routine laboratory tests, we found no significant effects of Q₁₀H₂ on triglycerides, total cholesterol, high density lipoprotein (HDL), and oxidized LDL (Table 1). A significant reduction of LDL cholesterol levels was found in serum samples of study subjects (from 95.51 ± 28.89 mg/dL to 90.60 ± 27.21 mg/dL, $P = 0.022$). On the basis of ¹H NMR analysis, Q₁₀H₂ supplementation shows significant reductions of LDL cholesterol of $\sim 12.71\%$ ($P = 1.21E-07$). The observed effects on total LDL cholesterol levels are mediated through significant reductions of the LDL subfractions B, C, and E with relative reductions of 33.02% ($P = 0.00002$), 14.62% ($P = 0.0098$), and 16.52% ($P = 0.008$), respectively (Fig. 3). In contrast, no Q₁₀H₂-mediated alterations were found for the LDL subfractions A and D. On the basis of ¹H NMR analysis, Q₁₀H₂ is comparable to the described effects of plant sterols in different cohorts. It has been shown that a usual daily intake of 2–2.5 g plant sterols or stanols results in an average reduction of LDL cholesterol levels of $\sim 14\%$ (22, 23). Taken together, Q₁₀H₂ supplementation in humans mediates distinct reducing effects on LDL cholesterol levels with a pronounced effect on atherogenic small dense LDL particles (19–21 nm, 1.045 g/L).

Q₁₀H₂ Dependent Reduction of Proinflammatory Cytokines at mRNA Level is Not Found at Protein and Functional Level

Because our gene expression approach suggested a reduction of anti-inflammatory cytokines by Q₁₀H₂, we examined these inflammatory markers at the protein level in serum samples. No significant effects of Q₁₀H₂ supplementation were observed for

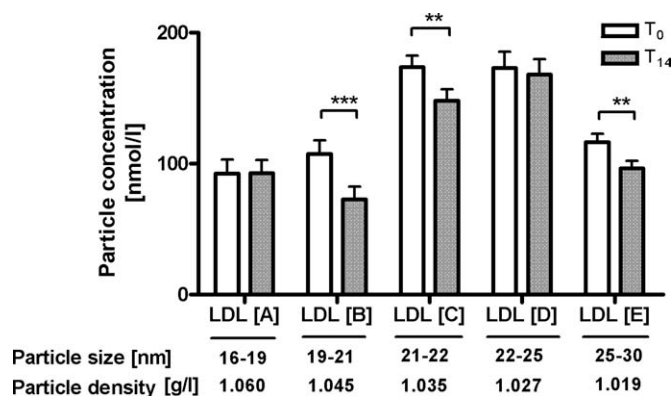


Figure 3. Effects of Q₁₀H₂ supplementation on LDL subfractions in serum samples of study subjects. Based on ¹H NMR analysis, effects of Q₁₀H₂ supplementation on concentrations of LDL subfractions were analyzed in serum samples of study subjects ($n = 53$). Significant effects have been found for the LDL subfractions B, C and E with relative reductions of 33.02%, 14.62% and 16.52%, respectively. ** $P \leq 0.01$; *** $P \leq 0.001$

TNF α , monocyte chemoattractant protein-1 (MCP-1), CXCL2, and C-reactive protein (CRP; Table 1). Comparison between mRNA and protein levels of TNF α and CXCL2 revealed no correlation (Figs. 1K and 1L). As a functional readout for inflammation, blood samples were stimulated *ex vivo* with LPS and the resulting secretion levels of TNF α and MCP-1 were determined. The stimulation values showed no differences between T_0 and T_{14} (Supporting Information Table 5). Therefore, Q₁₀H₂-mediated effects on inflammatory markers are considered to occur at the transcriptional level. This is in contrast to the described effect of Q₁₀H₂ in THP-1 cells (3). This study showed that Q₁₀H₂ reduces the secretion of several proinflammatory cytokines. One explanation for the observed discrepancies of Q₁₀H₂ supplementation from *in vitro* and *in vivo* studies might be the heterogeneity of blood plasma and serum matrices when compared with isolated cell culture systems. Post-transcriptional (*e.g.*, micro-RNAs) (24) and post-translational mechanisms (25) may also account for the different effects of Q₁₀H₂ on inflammation from *in vitro* and *in vivo* studies.

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