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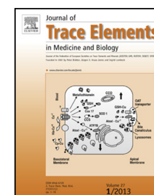

Aberrant expression of copper associated genes after copper accumulation in COMMD1-deficient dogs

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ABSTRACT

Background: COMMD1-deficient dogs progressively develop copper-induced chronic hepatitis. Since high copper leads to oxidative damage, we measured copper metabolism and oxidative stress related gene products during development of the disease.
Methods: Five COMMD1-deficient dogs were studied from 6 months of age over a period of five years. Every 6 months blood was analysed and liver biopsies were taken for routine histological evaluation (grading of hepatitis), rubeanic acid copper staining and quantitative copper analysis. Expression of genes involved in copper metabolism (*COX17, CCS, ATOX1, MT1A, CP, ATP7A, ATP7B,*) and oxidative stress (*SOD1, catalase, GPX1*) was measured by qPCR. Due to a sudden death of two animals, the remaining three dogs were treated with d-penicillamine from 43 months of age till the end of the study. Presented data for time points 48, 54, and 60 months was descriptive only.
Results: A progressive trend from slight to marked hepatitis was observed at histology, which was clearly preceded by an increase in semi-quantitative copper levels starting at 12 months until 42 months of age. During the progression of hepatitis most gene products measured were transiently increased. Most prominent was the rapid increase in the copper binding gene product *MT1A* mRNA levels. This was followed by a transient increase in *ATP7A* and *ATP7B* mRNA levels.
Conclusions: In the sequence of events, copper accumulation induced progressive hepatitis followed by a transient increase in gene products associated with intracellular copper trafficking and temporal activation of anti-oxidative stress mechanisms.

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Introduction

The trace element copper is actively taken up by cells via the copper transporter CTR1, and subsequently integrated in enzymes involved in several vital biologic processes [1–3]. Free copper is toxic due to its ability to generate reactive oxygen species, including the highly reactive hydroxyl radicals (•OH) [4]. Consequently, free intracellular copper levels are maintained at very low levels due to the presence of copper chaperones (ATOX1, CCS, COX17), sequestering proteins (MT1A, GSH), and efflux pumps (ATP7A, ATP7B). ATOX1 delivers copper to copper-dependent ATPases, CCS distributes copper to Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1), and COX17 delivers copper to cytochrome c oxidase in the mitochondria. Furthermore, free intracellular copper is sequestered by GSH and metallothioneins (MT) and MT1A mRNA and protein levels are rapidly increased when intracellular free copper levels rise [5]. Copper efflux occurs via the copper ATPase pumps encoded by the Wilson's disease gene ATP7B and Menkes disease gene ATP7A [6]. Mutations in the copper transporter ATP7A lead to Menkes disease, an X-linked recessive disease leading to copper deficiency [7–9]. ATP7A and ATP7B are involved in the transfer of copper from the Golgi network to the apical membrane under circumstances of increased intracellular copper [10,11]. ATP7B

Abbreviations: ALT, alanine aminotransferase; ATOX1, anti-oxidant protein 1; ATP7A, ATPase Cu ($^{2+}$)–transporting alpha polypeptide; ATP7B, ATPase Cu ($^{2+}$)–transporting beta polypeptide; CAT, catalase; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism; MURR1, domain-containing protein 1; COX17, cytochrome c oxidase assembly homolog; CP, ceruloplasmin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX1, glutathione peroxidase 1; HPRT, hypoxanthine phosphoribosyl transferase; LEC, long Evans Cinnamon; MT1A, metallothionein 1 A; qPCR, quantitative reversed-transcriptase polymerase chain reaction; ROS, reactive oxygen species; RPS5, ribosomal protein S5; RPS19, ribosomal protein S19; SOD1, Cu/Znsuperoxide dismutase; WD, Wilson's disease.

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deficiency results in a loss of biliary excretion of copper as well as a lack of copper incorporation into secreted proteins produced in the liver, such as ceruloplasmin (CP) [12].

Positional cloning in a population of Bedlington terriers led to the discovery of a new gene product involved in copper handling, *copper metabolism (Murr1) domain containing protein1 (COMMD1)* , which is ubiquitously expressed in a large number of tissues [13,14]. Dogs with a deletion encompassing exon-2 of *COMMD1* , develop copper-induced chronic hepatitis. *In vitro* studies revealed increased copper accumulation in several cell types after *COMMD1* gene silencing [15,16]. Two *in vivo* studies, one in mice with a hepatic deletion of the *COMMD1* gene and one in *COMMD1*-deficient dogs confirmed hepatic copper accumulation due to a deletion of this gene [17,18]. Although the details of how COMMD1 regulates intracellular copper remain enigmatic, a functional relationship has been demonstrated between COMMD1 and ATP7B and ATP7A [19].

Several model animals are used to investigate the human copper storage disorder Wilson's disease (WD), such as toxic milk mice [20], Long-Evans Cinnamon (LEC) rats [21,22], and *ATP7B* knockout mice [23]. In these models the effects of copper accumulation on the liver are in most cases observed when pathology has fully developed. As body size and lifespan of rodents clearly hampers long-term sequential liver biopsies, longitudinal studies on liver samples following individual animals in time until chronic hepatitis has developed are not available. In contrast to the impossibility to acquire sequential mouse liver biopsies urinary copper excretion could be measured longitudinally as described in a recent study on ATP7b $^{-/-}$ mice [24].

Recently we described a molecular time-lapse of the development of copper-accumulation and hepatitis in COMMD1-deficient dogs [18]. In contrast to COMMD1$^{-/-}$ mice, that die during gestation [25], COMMD1-deficient dogs are born healthy but develop copper-associated progressive chronic hepatitis later in life [18]. Copper-associated hepatitis is usually treated with D-penicillamine in man [26] and dogs [27,28].

In the present longitudinal study we provide a detailed time-course analysis on the mRNA levels of various proteins involved in intracellular copper metabolism as a direct or indirect consequence of COMMD1 deficiency. Insight in the genomic consequences of COMMD1-deficiency in a naturally occurring large animal (canine) disease model may render new leads to interfere in diseases associated with copper overload.

Materials and methods

Animals

All procedures were approved by Utrecht University's Ethical Committee, as required under Dutch legislation (DEC-number ID 2007.III.06.080). Five COMMD1-deficient dogs (two males, three females) were used for longitudinal follow-up. COMMD1 deficiency status was confirmed as described previously based on the deletion of exon-2 [29]. The dogs were examined every 6 months up to the age of 60 months (10 examination points). At each occasion the clinical status was monitored, physical and blood examination was performed, and five liver biopsies were taken per animal. At 43 months of age D-penicillamine treatment (20 mg/kg/day) was started in the remaining three dogs. The dogs were housed individually, fed once a day a normal commercial dog food not restricted for copper (Noblesse, Purina, St Louis, Missouri), and had free access to tap water.

Sampling,histopathology and copper levels

Liver biopsies were obtained using a 14 G Menghini needle [30]. Five biopsies taken one time- point were stored differently to acquire optimal material for various analyses [31]. Two biopsies were formalin-fixed and paraffin-embedded, and 4 μm thick paraffin sections were stained with hematoxylin and eosin (HE) and rubeanic acid (RA). Copper accumulation was evaluated semi-quantitatively using a scale from 0 to 5 as described previously [18,32,33]. Grading of hepatitis was performed by one board-certified veterinary pathologist (TvdI) according to the WSAVA classification [18,34]. The presence and activity of hepatitis was graded as 0 (absent), 1 (slight), 2 (mild), 3 (moderate), and 4 (marked). Other biopsies were fixed in RNAlater (Ambion, Austin, TX, USA) for a maximum of 24 h and stored at −70 °C. The quantitative copper analysis was performed on biopsies stored in copper-free cassettes and freeze-dried prior to instrumental neutron activation analysis [35]. Normal copper concentration in dogs is considered to be ≤400 ppm [36].

Serum enzyme activity and bile acid concentration

Serum alanine transaminase (ALT) and bile acids were determined in heparin plasma using a DXC-600 Beckman (Beckman Coulter, Woerden, the Netherlands). Reference intervals for ALT and bile acids measured on this device are 0–54 U/l and 0–10 μmol/l, respectively.

RNA isolation and quantitative RT-PCR (qPCR)

RNA was isolated from RNAlater fixed biopsies, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions, including a 15 min on-column DNaseI treatment. RNA concentration was measured with a Nanodrop (Isogen Life Sciences, IJsselstein, the Netherlands), RNA quality was analysed with the Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). For all samples, cDNA was synthesized using the iScript TM cDNA Synthesis Kit (Biorad, Veenendaal, the Netherlands) with a mix of oligo-dT and random hexamer primers. qPCR was performed on a total of seven gene products involved in copper homeostasis (*ATP7A*, *ATP7B*, ceruloplasmin (*CP*), metallothionein 1 A (*MT1A*), antioxidant protein 1 homolog (*ATOX1*), COX17 cytochrome c oxidase assembly homolog (*COX17*), copper chaperone for superoxide dismutase (*CCS*)) and three gene products involved in oxidative stress response (superoxide dismutase (*SOD1*), Catalase (*CAT*), and Glutathione peroxidase 1 (*GPX1*)). Perlprimer v1.1.14 was used for primer design with Ensembl annotated transcripts, and MFold was used to test for secondary structures in the amplicons [37].

Primer specificity was obtained in silico (BLAST analysis) and by sequencing and melt curve analysis. qPCR was performed using primers as described in Table 1 and was based on the high affinity double-stranded (ds) DNA-binding dye SYBR green I (iQSYBR Green Supermix, BioRad Veenendaal, the Netherlands) [38]. PCR conditions were a 3-minute Taq polymerase activation step at 95 °C, followed by 45 cycles consisting of 10 s at 95 °C (denaturation), 30 s at the amplicon specific temperature. Reactions were performed in 25 μl volume containing 0.5X SYBR Green Supermix (Biorad), 20 pmol primer, 1 μl cDNA and 20 μM dNTPs. Normalisation was performed using the geometric mean of four reference-genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl transferase (*HPRT*), ribosomal protein S5 (*RPS5*) and S19 (*RPS19*)) [39]. This combination revealed stable expression of the normalizing reference genes according to the GeNorm algorithm previously and in the Beagleton liver samples analysed [39,40]. qPCR results were related to the expression of the gene products measured in liver samples of six control Beagle dogs (one to three years of age). Minus-RT and no-template controls were negative indicating no contaminations.

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