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RESEARCH PAPER

Molecular bases of copper and iron deficiency-associated dyslipidemia: a microarray analysis of the rat intestinal transcriptome

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Abstract As essential cofactor in many proteins and redox enzymes, copper and iron are involved in a wide range of biological processes. Mild dietary deficiency of metals represents an underestimated problem for human health, because it does not cause clear signs and clinical symptoms, but it is associated to long-term deleterious effects in cardiovascular system and alterations in lipid metabolism. The aim of this work was to study the biological processes significantly affected by mild dietary deficiency of both metals in rat intestine, in order to better understand the molecular bases of the systemic metabolic alterations, as hypercholesterolemia and hypertriglyceridemia observed in copper-deficient rats. A gene-microarray differential analysis was carried out on the intestinal transcriptome of copper- and iron-deficient rats, thus highlighting the biological processes significantly modulated by the dietary restrictions. The gene array analysis showed a down-regulation of genes involved in mitochondrial and peroxisomal fatty acids beta-oxidation and an up-regulation of genes involved in plasmatic cholesterol transport (apoprotein E and lecithin:cholesterol acyltransferase) in copper deficiency. Furthermore, a severe down-regulation of ApoH was pointed out in iron-deficient animals.

Keywords Copper deficiency · Iron deficiency · Lipoproteins · ApoH · Lipids metabolism · Microarrays

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Introduction

Copper and iron are essential nutrients in human physiology as their importance is linked to their role as cofactors of many redox enzymes involved in a wide range of biological processes, as well as in oxygen and electron transport. Mild dietary deficiencies of both metals represent an underestimated problem for human health, [1, 2] and references therein, because they are not associated to clear signs and clinical symptoms, even though they may cause long-term deleterious effects in cardiovascular system and alterations in lipid metabolism [3]. Since the first direct experimental evidence about the treatment of chlorosis in young woman, many studies have shown that iron and copper metabolism, absorption and systemic distribution are intimately connected. In fact, molecular mechanisms of iron transport are characterized by crucial redox reactions catalyzed by cuproenzymes, and furthermore iron status has been hypothesized as controlling feedback mechanisms that influence copper metabolism (for a comprehensive review, see Ref. [4]). Moreover, more recent studies reported statistically significant associations of dyslipidemia with marginal copper and iron deficiencies [5, 6], whose molecular bases and regulatory events are still largely unknown. A microarray differential analysis of gene expression, in rat intestine of animals fed on copper- or iron-deficient diets, confirmed significant changes of transcript levels of genes involved in the pathways of fatty

acids (FA) and cholesterol (CL) metabolism, thus leading to the identification of molecular targets affected by the dietary deficiencies. Widely reported models for lipid metabolism investigations are hepatocytes and adipocytes, even though the digestion and absorption of dietary lipids, as well as their re-synthesis and packaging for transport, take place in the intestine. The same tissue, together with the liver, controls the cholesterol homeostasis and plasma cholesterol levels, and contributes to the HDL-CL levels [7, 8]. The interest for a better characterization of the metabolic switches that regulate the onset and the evolution of lipid dismetabolisms in metal deficiencies prompted us to analyze the intestinal transcriptome in order to identify reliable genetic markers for the early diagnosis and, hopefully, for more effective early treatments.

Materials and methods

Animals and diets

As described in our previous paper [9], 24 male weanling, 15 days old, Sprague–Dawley rats were individually housed in stainless steel cages with a 12-h light/dark cycle and had free access to food and to deionized and distilled water. For 21 days, the rats were fed on a pelleted diet (20% protein, 64.8% carbohydrates, 10% fat, supplemented with vitamins and minerals; 15.8 MJ metabolizable energy). Animals were then randomly divided into three dietary groups, aimed at establishing a mild metal deficiency at the end of the treatments: a control group (C), a copper-deficient group (CuD) and an iron-deficient group (FeD). For the next 32 days the CuD group received a copper-deficient diet (Cu, 0.13 μg/g dry weight; Fe, 37.9 μg/g dry weight), the FeD group received an iron-deficient diet (Cu, 0.82 μg/g dry weight; Fe, 14.7 μg/g dry weight) and the C group continued with the control diet (Cu, 0.91 μg/g dry weight; Fe, 38.4 μg/g dry weight). Final body weight and daily food intake were recorded. On day 54, rats were killed by an intraperitoneal injection of pentobarbital (150 mg/kg) and small intestine, liver, kidney, heart, brain and skeletal muscle were explanted from each rat, quickly washed with phosphate-buffered saline (PBS) and immediately frozen in liquid nitrogen.

Atomic absorption spectrometry

Copper and iron were determined by atomic absorption spectrometry using a "Model 5100 Perkin Elmer" apparatus equipped with a "Model HGA-600 Zeeman" graphite furnace. Liver, intestine and pelleted diet samples (1 g) were dry-washed at 150°C with 2.5 ml of 70% HNO_3 and 1.0 ml of 50% H_2O_2 added drop wise. The dry residue was dissolved in the appropriate volume of 0.2% HNO_3 for flame analysis. The samples were also diluted and analyzed by the graphite furnace technique. Appropriate amounts of matrix modifier were added following the suggestion of the manufacturer.

Microarray analysis

Total RNA was isolated from small intestine following the method of Chomczynski and Sacchi [10], then treated with Dnase I (Boehringer Mannheim, Germany). The RNA was then retrotranscribed with T7Oligo(dT) and amplified with Illumina® RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) following the instructions of the manufacturer. For each sample, 500 ng of purified RNA was used for biotinylated cRNA synthesis.

The rat intestinal transcriptome of copper- and iron-deficient animals [9] was analyzed on RatRef-12 Expression BeadChip (ILLUMINA™), following the reverse transcription and labeling of 750 ng of RNA per dietary group (Control, CuD, FeD) obtained by mixing equal amounts of nucleic acids from three different animals of each group. Microarray analyses were carried out on six RNA mixtures (biological replicates of Control, Cu and Fe). Each mixture (Control1, Control2, Cu1, Cu2, Fe1, Fe2) was obtained by mixing equal amounts of nucleic acids extracted from groups of three distinct animals (Control1 = Rat 1, Rat 2, Rat 3; Control2 = Rat 4, Rat 5, Rat 6; Cu1 = Rat 7, Rat 8, Rat 9; Cu2 = Rat 10, Rat 11, Rat 12; Fe1 = Rat 13, Rat 14, Rat 15 Fe2 = Rat 16, Rat 17, Rat 18) and was hybridized to the chip in technical triplicates. Furthermore, in the Illumina array, each bead-type (representing one probe for a total of 22,523 sequences) is present in a number of the order of about 30–40 copies and provides in this way an internal technical replica that add to the robustness of the data obtained.

To identify differentially expressed genes, we used the differential analysis algorithm implemented in the BeadStudio software v. 3.0.19.0 (Gene Expression Module v.3.0.14), which takes into account the standard deviation derived from the multiple measurements. The internal DiffScore test of the software was used with a threshold of 30, which corresponds to a p value of 0.001 of the underlying statistical test and only genes satisfying the criteria were considered as differentially expressed genes. The fold difference (Sample/Control) was calculated only for these genes. Data mining was carried out using the online available software David and OntoExpress (http://david.abcc.ncifcrf.gov, http://vortex.cs.wayne.edu/ontoexpress). The RatRef-12 allows the analysis of 21,910 sequences expressed in rat, including annotated genes and EST sequences. The results were filtered by imposing the fold

difference cut-off values as ≥ 1.5 (up-regulated genes) or ≤ -1.5 (down-regulated genes).

Results

Metal deficiencies

Dietary mineral deficiencies are frequently associated with significant reductions in food intake. In order to verify that the obtained results were not affected by possible side effects of the treatments on the appetitive behavior of the animals, the daily food intake of each animal was registered and the group means of rats fed on metal-deficient diets were compared with that of metals adequate control group. As reported in Tables 1 and 2, the dietary administration of low contents of copper and iron did not affect the mean food intakes, and mean body weights were substantially unaffected. Moreover, the animals were fed ad libitum and did not show any evident physiological or behavioral alteration during the period of treatment and before killing.

Atomic absorption spectrometry carried out on mineralized diets and on liver and small intestine samples showed that the lower content of copper and iron in the diets mirrored a significantly lower amount of each metal in CuD and FeD rat tissues (Table 2). Furthermore, to confirm the homeostatic response to low dietary iron, we analyzed the expression of the divalent metal transporter 1 (DMT1) [9]. This protein is able to transport dietary non-heme iron, and other divalent metal cations, and is localized on the apical surface of absorptive enterocytes. Its expression is positively regulated in the proximal duodenum of mice fed on iron-deficient diet. Real-time PCR analysis showed a positive regulation of DMT1 in the intestines of both FeD and CuD rats. Finally, microarray data confirmed the up-regulation of DCytb (FeD = +6.25; CuD = +1.70) and ferroportin (FeD = +1.29; CuD = n.d.) genes. On the other hand, current copper biomarkers are not reliable to detect minor but biologically significant variations of copper status [11].

Microarray analysis

As reported in Table 3, taking into account the generally accepted cut-off value of ±1.5 for the fold induction, both metal deficiencies cause the down-regulation of the most part of the affected genes. Furthermore, by comparing the effects of dietary treatments, the data show that the intestinal transcriptome is considerably more sensitive to the iron deprivation.

As it can be inferred by the summary of the regulated genes ordered by biological process (Fig. 1a, b), both dietary deficiencies affect the energetic metabolism of the cell and modulate the FA and CL metabolism. Moreover, other processes are affected by both metals (e.g. intracellular transport of protein, G-protein coupled receptor protein signaling pathway, phospholipids transport, etc.), thus confirming their biological importance and entwined metabolic relationship.

Tables 4 and 5 show that genes involved in the lipid metabolism generally present smaller fold values (threshold set to ±1.2), although they are still statistically significant.

Table 1 Daily mean food intake of rats during dietary treatments

Dietary treatment	Mean food intake (gram of diet/rat/day)[a]
Control	12.91 ± 1.26
Fe deficient	13.29 ± 1.15
Cu deficient	13.06 ± 1.11

Data are the mean ± SD of eight rats for each group

Table 2 Body weight, liver and intestine metals content of control (C), copper-deficient (CuD) and iron-deficient (FeD) rats

	C group	CuD group[a]	FeD group[b]
Final body weight (g)	351 ± 28	360 ± 30	361 ± 20
Liver copper content (μg/g)	14.6 ± 1.2	6.7 ± 0.4 $p < 1.0 \times 10^{-10}$	13.9 ± 1.1 $p < 0.3$
Intestine copper content (μg/g)	6.9 ± 0.7	4.7 ± 0.3 $p < 0.002$	7.0 ± 0.8 $p < 0.011$
Liver iron content (μg/g)	268.6 ± 26.5	263.4 ± 37.9 $p < 0.8$	102.7 ± 11.5 $p < 2.0 \times 10^{-10}$
Intestine iron content (μg/g)	79.3 ± 22.7	111.8 ± 13.9 $p < 0.02$	31.6 ± 5.3 $p < 0.016$

Data were analyzed using unpaired, two-tailed *t* test comparing two variables and are presented as mean ± SD. *p* values <0.05 were considered as significant

[a] *p* values CuD versus C group

[b] *p* values FeD versus C group

Discussion

The overall results of the microarray analysis show that copper deficiency down-regulates the mitochondrial and peroxisomal beta-oxidation of FA. In fact, Acyl-CoA synthetase (*Acsl1*), carnitine-palmitoyltransferase (*Cpta1*) and L-3-hydroxyacyl CoA dehydrogenase participate in the CoA activation and carnitine-bound transport of FA, on the outer and the inner mitochondrial membrane (*Acsl1*; *Cpta1*). Also, the down-regulation of CD36 protein, acting as fatty acid translocase on the plasma membrane and as a scavenger receptor on macrophages, reduces the availability of intracellular FA. Both steps channel the FA

Table 3 Summary of the regulated genes

Sample versus sample	Detected p value = 0.05	Diff score = 30	Fold difference ≥1.5 (up)	Fold difference ≤−1.5 (down)
Control versus CuD	11,377	1,321	65	133
Control versus FeD	11,429	2,515	81	783
CuD versus FeD	11,120	1,221	56	286

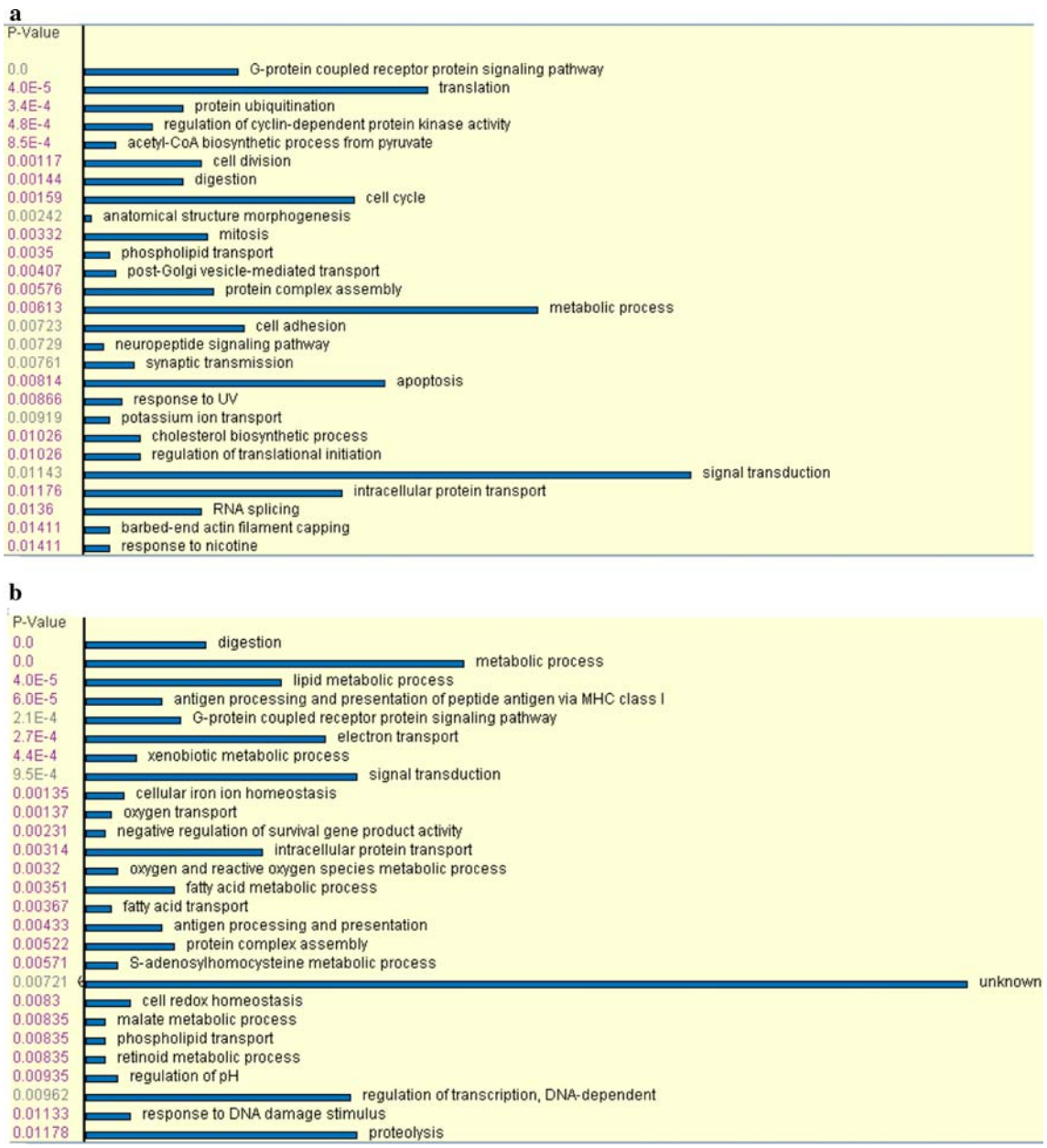

Fig. 1 **a** Biological processes affected by copper deficiency. **b** Biological processes affected by iron deficiency

through the mitochondrial matrix where beta oxidative enzymes catalyze the formation of acetyl-CoA. Along the oxidative pathway, L-3-hydroxyacyl CoA dehydrogenase (*Hadhb*) catalyzes the third step of each oxidative cycle, which gives rise to the corresponding beta-ketoacyl-CoA derivative. A lower expression of *Hadhb* concurs with the

Table 4 Copper deficiency: down-regulated (A) and up-regulated (B) sequences in fatty acids and cholesterol metabolic pathways

		Fold induction
A		
PhlpB	Phospholipase B	−2.01
Cd36	cd36 antigen	−1.50
RGD1310224	Similar to RIKEN CDNA 1810022C23	−1.43
Peci	Peroxisomial delta2-enoyl-CoA-isomerase	−1.42
Plcl2_predicted	Phospholipase C-like 2 (predicted)	−1.39
Hsd17b4	Hydroxysteroid (17-beta) dehydrogenase 4	−1.38
Hpgd	15-Hydroxyprostalandin dehydrogenase	−1.37
Cept1	Choline/ethanolamine phosphotransferase 1	−1.36
Sult1b1	Sulfotransferase family 1B, member 1	−1.35
Pitpn	Phosphatidylinositol transfer protein	−1.33
Ugt2b10_predic.	UDP glycosyltransferase 2 family, polypeptide B10 (predicted)	−1.32
Acsl1	Acyl-CoA synthetase long-chain family member 1	−1.32
Crot	Carnitine O-octanoyltransferase	−1.30
Cpt1a	Carnitine-palmitoyltransferase 1, liver	−1.29
Insig2	Insulin induced gene 2	−1.27
Acadvl	Acyl-coenzyme A dehydrogenase, very long chain	−1.25
Asah2	*N*-Acylsphingosine amidohydrolase 2	−1.23
Aadac	Arylacetamide deacetylase (esterase)	−1.22
Cds1	CDP-diacylglycerol synthase 1	−1.22
Scd2	Stearoyl-coenzyme A desaturase 2	−1.22
Hadhb	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A	−1.21
	Thiolase/enoyl-coenzyme A hydratase (trifunctional protein)	−1.21
	Beta subunit	
B		
Lcat	Lecithin cholesterol acyltransferase	1.56
Pnliprp2	Pancreatic lipase-related protein 2	1.34
Pmvk	Phosphomevalonate kinase	1.24
ApoE	Apolipoprotein E	1.20

above-mentioned enzymes in slowing down the process, with a reduced production of acetyl-CoA and energy, and a consequent cytoplasmic accumulation of FA. The down-regulation of acyl-CoA synthetase, delta-2-enoyl-CoA isomerase (*Peci*), carnitine-octanoyl transferase (*Crot*) and hydroxysteroid-17-beta dehydrogenase (*Hsd17b4*) mirrors the analogue trend of mitochondrial beta-oxidation, thus coherently reducing also the peroxisomal catabolism of longer fatty acid chain (≥C18).

Phospholipase B (*PhlpB*) (or Lysolecithinase B) catalyzes the hydrolysis of lysolecithins to glycerophosphorilcholine and FA. Oxidized LDL lipoproteins, that play a pivotal role in the development of atherosclerosis, contain lysolecithins that also cause the alteration of the vasal tone by impairing the endothelium-dependent relaxation [12]. On the other hand, lysolecithin concentration may significantly increase in the atherosclerotic arterial walls, and its cytotoxicity at high concentration may contribute to the cell death observed in the atheromatous lesions. Lysolecithinase B reduces the concentration of this bioactive phospholipid in the modified LDL, thus lowering the risk of its release at the atheromatous plaques. The significant down-regulation of *PhlpB* (twofold) could lead to the hypothesis of a long-term pro-atherogenic effect of copper deficiency associated to the accumulation of the oxidized fraction of LDL lipoproteins.

Several studies showed a clear correlation among copper deficiency and dyslipidemia. The main alterations concern higher plasma CL and triglyceride (TG) concentrations, increased VLDL-LDL to HDL lipoproteins ratio, and the shape alteration of HDL lipoproteins. Lipid composition of HDL copper-deficient rats does not differ from that of copper adequate animals, unlike the ApoE fraction that shows a considerable enrichment [13, 14]. Physiological concentrations of ApoE ensure the lipid homeostasis and its anti-atherogenic effects, while higher concentrations of ApoE are responsible for the spherical to discoidal transformation of HDL, which makes them unable to bind to the

Table 5 Iron deficiency: down-regulated (A) and up-regulated (B) sequences in fatty acids and cholesterol metabolic pathways

		Fold induction
A		
Apoh	Apolipoprotein H	−20.35
Pla2g4a	Phospholipase A2, group IVA	−2.80
Cd36	cd36 antigen	−2.29
Prkaa1	Protein kinase, AMP-activated, alpha 1 subunit	−1.88
Lypla1	Lysophospholipase 1	−1.84
Edg2	Endothelial differentiation, G-protein coupled recap.	−1.75
Plaa	Phospholipase A2, activating protein	−1.73
Plscr1	Phospholipid scramblase 1	−1.70
Acsl3	Acyl-CoA synthetase long-chain family member 3	−1.59
Idi1	Isopentenyl-diphosphate delta isomerase	−1.57
Scp2	Sterol carrier protein 2	−1.56
Anxa1	Annexin A1	−1.56
Acsl1	Acyl-CoA synthetase long-chain family member 1	−1.47
Pitpn	Phosphatidylinositol transfer protein	−1.44
Cd1d1	CD1d1 antigen	−1.34
Scd2	Stearoyl-coenzyme A desaturase 2	−1.27
Cds1	CDP-diacylglycerol synthase 1	−1.25
Cnbp1	Cellular nucleic acid binding protein 1	−1.26
Cdipt	CDP-diacylglycerol-inositol3-phosphatidyltransferase	−1.22
Pctp	Phosphatidylcholine transfer protein (Pctp), mRNA.	−1.22
B		
Dgka	Diacylglycerol kinase, alpha (80 kDa)	1.22
Clps	Colipase, pancreatic	1.27
Cel	Carboxyl ester lipase	1.30
Fabp6	Fatty acid binding protein 6 (gastrotropin)	1.31
Pnliprp1	Pancreatic lipase-related protein 1	1.32
Pmvk	Phosphomevalonate kinase	1.32
Fabp2	Fatty acid binding protein 2, intestinal	1.32
Pnlip	Pancreatic lipase	1.33
Pla2g1b	Phospholipase A2, group IB	1.38
Mvd	Mevalonate (diphospho) decarboxylase	1.38

liver LDL receptors and slow down the retrograde transport of CL [15]. On the other hand, lecithin:cholesterol acyltransferase (LCAT) catalyzes the esterification of free CL in the HDL, contributing to the lipoprotein maturation and to the shape transition from the discoidal to the spherical form, but plasma LCAT activity in copper-deficient rats is considerably reduced [16]. In such metabolic context the observed up-regulation of phosphomevalonate kinase (*Pmvk*) and Apo E genes correlates with the hypercholesterolemia and hypertriglyceridemia in copper-deficient animals, while LCAT up-regulation may account for a homeostatic response to the decreased enzymatic activity in the plasma.

Among the genes with altered expression in iron deficiency, ApoH results the most severely down-regulated gene (fold −20, 35). Apolipoprotein H (*ApoH*) (or beta-2-glycoprotein) is present in TG-enriched lipoproteins (chylomicrons, VLDL, LDL), and its role, still not completely characterized, was formerly related to the TG "clean-up" of plasma [17]. ApoH influences the balance of the cell uptake and efflux of CL and causes a net reduction of its intracellular concentration. Moreover, it has an anti-atherogenic effect by inhibiting the extracellular cholesterol translocation into the macrophages, and in addition, low levels of plasma ApoH are often associated to the increase of oxidized LDL [18]. Several reports correlate the iron deficiency with metabolic alterations of lipids. An increase in plasma concentration of TG and lipoproteins was observed, especially VLDL and LDL, along with low levels of CL in LDL, VLDL an HDL lipoproteins in mild deficiency rats [6]. This first evidence of a consistent and substantial down-regulation of ApoH can give good reason

for the clinical reports. It may be of great impact in the comprehension of the molecular bases of dyslipidemia and for the prophylaxis of cardiovascular diseases potentially associated the wide diffusion of sub-clinical iron deficiencies. Further evidences are provided by the parallel up-regulation of genes encoding enzymes involved in the intestinal absorption of TG and cholesterol, as pancreatic lipase (*Pnlip*), colipase (*Clps*), pancreatic lipase-related protein 1 (*Pnliprp1*), phospholipase A2-IB (*Pla2g1b*) and carboxyl ester lipase (*Cel*), or key enzymes in the CL biosynthesis, as phosphomevalonate kinase (*Pmvk*) and mevalonate (diphospho) decarboxylase. In human, the positive correlation among plasma LDL-CL and its intestinal absorption may be one of the main factors responsible for hypertriglyceridemia and hypercholesterolemia [19]. Furthermore, as observed in copper deficiency, the down-regulation of genes involved in the mitochondrial beta-oxidation (*Acsl1, Acsl3* and *CD36*) decrease the efficiency in the uptake and utilization of FA.

A final consideration has to be dedicated to the combined down-regulation of genes involved in the arachidonic acid pathway [phospholipase A_2IVA (*Pla2g4a*), scramblase 1 (*Plscr1*), phospholipase A_2 activating protein (*Plaa*), annexin 1 (*Anxa1*)], leading to the biosynthesis of mediators (prostaglandins, leucotriens, eicosanoids, eicosapentaenoic acid) that modulate the intensity and the duration of the inflammatory response. The intestinal immune system is constantly challenged by pathogens and luminal content, which stimulate the immune and inflammatory response aimed at neutralizing the infectious agents and repair the intestinal mucosa. The reduction of inflammation mediators and their biosynthetic enzymes can impair the physiological response to harmful stimuli, and alters the gastrointestinal milieu through the decrease of cytoprotective metabolites that preserve the mucosa against necrotizing agents [20, 21]. In agreement to these data, we previously reported the up-regulation in copper deficiency of the TFF1 peptide, belonging to the "Trefoil factor" family [22]. TFF1 stabilizes the mucous layer that protects the gastrointestinal mucosa and acts as a motogenic factor during the epithelial restitution, in which endogenous prostaglandins play an important regulatory role. The microarray analysis confirmed the up-regulation of TFF1 and showed a similar behavior for TFF3 peptides in copper deficiency and for TFF2 in iron deficiency (data not shown).

Even though the microarray analyses do not provide accurate quantitative information about the regulatory relationship between single genes, they give back a very powerful "instant view" that allows to evaluate the integrated molecular response to different stimuli, especially in the cases of complex stress responses as for dietary treatments. Far from describing the details of the single gene regulation, we obtained a useful map of candidate genes involved in the cross talk of apparently distant and unrelated metabolic pathways. A better understanding of the molecular mechanisms leading to dyslipidemia, associated to the evolution of the dietary habits in western countries, contributes to the present efforts of scientific community aimed at reducing the high incidence of cardiovascular disease, obesity and diabetes that still remain prevailing causes of morbidity and mortality.

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