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RESEARCH PAPER

The nutrigenomic investigation of C57BL/6N mice fed a short-term high-fat diet highlights early changes in clock genes expression

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Abstract Mice fed long-term high-fat diets (HFD) are an established model for human metabolic disorders, such as obesity and diabetes. However, also the effects of short-term HFD feeding should be investigated to understand which are the first events that trigger the onset of a pre-disease condition, the so-called *metabolic syndrome*, that increases the risk of developing clinical diseases. In this study, C57BL/6N mice were fed a control diet (CTR) or a HFD for 1 (T1) or 2 weeks (T2). Metabolic and histological effects were examined. Cecum transcriptomes of HFD and CTR mice were compared at T2 by microarray analysis. Differentially expressed genes were validated by real-time PCR in the cecum and in the liver. After 2 weeks of diet administration, HFD mice showed an altered expression pattern in only seven genes, four of which are involved in the circadian clock regulatory pathway. Real-time PCR confirmed microarray results of the cecum and revealed the same trend of clock gene expression changes in the liver. These findings suggest that clock genes may play an important role in early controlling gut output systems in response to HFD in mice and that their expression change may also represent an early signaling of the development of an intestinal pro-inflammatory status.

Keywords Cecum · Clock genes · Microarray analysis · Nutrigenomics · Short-term high-fat diet

Introduction

High-fat (HF) feeding treatments have been described in the mouse by many authors, above all as a model to study obesity, hyperglycemic, hypercholesterolemic and hyperinsulinemic symptoms (Jump 2011), which are major risk factors for chronic diseases in humans, including type-2 diabetes, cardiovascular disease, hypertension, stroke and certain forms of cancer (Anderson et al. 2005). Most studies were based on long-term HF feeding treatments; however, also short-term effects should be evaluated to understand which are the first "alarm bells" for the development of a pre-disease condition, the so-called

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metabolic syndrome (de Wilde et al. 2008; Sasaki et al. 2010; Isken et al. 2010). This syndrome has attracted much attention, because it seems to be caused by an excess of nutrients (Kimokoti and Brown 2011). Most of these studies focused on the physiology and underlying molecular mechanisms in liver (Kreeft et al. 2005; Akagiri et al. 2008), skeletal muscle (Anderson et al. 2008) and adipose tissue (Akagiri et al. 2008; Kintscher et al. 2008), as these organs are targets of insulin-modulated metabolism. However, increasing evidence links the intake of a HF diet (HFD) to gut local inflammatory events, being gut the first organ to be exposed to dietary components. In particular, the mammalian small intestine quickly responds to HFD by undergoing pronounced morphological and functional adaptations to changes in the diet. de Wit and co-workers examined by microarray analysis the role of small intestine in the development of dietary fat–induced obesity and insulin resistance in C57BL/6J mice. They found that the biological processes influenced by feeding a high-fat diet were related to lipid metabolism, cell-cycle regulation and inflammation/immune response. For this last process, they could not draw a definitive conclusion on inflammatory status of the small intestine as a whole, because almost no gene showed a consistent up- or down-regulation in all the segments of the small intestine (de Wit et al. 2008). In the study conducted by Ding and co-workers, C57BL/6 mice were exposed to high-fat or low-fat diets and assessed for the expression of multiple pro-inflammatory cytokines in the small intestine and in the colon. Only TNFα was found to be up-regulated by the HF diet and this occurred specifically in the ileum and within a 2–6-week time frame from the beginning of HF diet administration, before diet-induced weight gain and increased fat mass could be observed (Ding et al. 2010). In this study, no detectable increase was identified in TNFα or other cytokines in plasma. Most of these studies focused on the small bowel. However, also the large bowel, so far not intensively explored, can be influenced by diet variation and is a potential target of inflammation (Chapman-Kiddell et al. 2010). In fact, a recent work of Patrone et al. (Patrone et al. 2012) reported that a short-term HF diet rich in soy oil modified the cecum microbiota of C57BL/6 mice. In light of this finding, in the present study we investigate whether a short-term HF diet also induces early changes in gene expression in the cecum of C57BL/6N mice.

Methods

Animals and diets

C57BL/6N mice (14 females and 13 males, 3 weeks old) were obtained from the breeding colony of the Centro Ricerche Biotecnologiche of the Università Cattolica del Sacro Cuore (Cremona, Italy). The experimental facility was maintained at a temperature of 23 °C (±0.5 °C). The light cycle was set at 14/10 h (light/dark). Animals were given ad libitum access to food and water. The experimental procedures were carried out in agreement with Italian regulations (D. M. 116/92) and EU guidelines. The control diet (CTR) was obtained by mixing 70 % of ground standard mouse diet (4RF18, Mucedola srl, Settimo Milanese, Milan, Italy) with 30 % of a mixture with similar composition but lacking the mineral–vitamin components. The HF diet was prepared by mixing 66.5 % of ground 4RF18 with 25 % of soy oil and 8.5 % of potato protein flour, to obtain an isoproteic diet with the control one (16.52 vs. 16.56 % on dry matter basis respectively for CTR and HF). The soy oil contains about 50 % of linoleic acid, the main precursor of the arachidonic acid, reported to drive the metabolism to the synthesis of pro-inflammatory mediators (Calder 2002, 2003). The exact composition of the diets is reported in Table 1. Mice were divided into five groups (males and females equally divided). The experiment started at weaning (T0), with the killing of the first group (five mice), set as reference. On the same day, the other four groups started to be fed the experimental diets: two received the control diet and two the high-fat diet. After one (T1) and two weeks (T2) of such dietary regimens, one control diet group and one high-fat diet group were killed (five mice per diet group at T1 and six mice per diet group at T2). Mice were weighed at each time point, and the weight gain of each animal was calculated.

Tissue harvest

Mice were anaesthetized with a mixture of Zoletil 100® (Virbac) and Rompun (Bayer) diluted in PBS at a ratio of 1:2:37. For each time point, animals were killed in the morning to avoid diurnal variability. In addition, to minimize possible temporal effects on analytical measurements, mice to be killed were chosen in turn from the two experimental groups. Following deep anesthesia, blood was collected by intracardiac puncture using a heparinized syringe. Plasma was obtained by centrifugation and stored at –20 °C until use. The large intestine was harvested via midline laparotomy and rinsed with PBS to remove luminal contents. Next, three consecutive segments were collected. The first 0.5-cm segments were processed for histological analysis. The last 1-cm segment was snap-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA isolation.

Blood analyses

Blood metabolites were analyzed at 37 °C by a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory,

Table 1 Composition of the experimental diets

Ingredients	CTR g/kg of diet	HF
Barley flour	474.4	312.7
Corn flour	256.0	146.3
Forage flour	85.0	56.5
Soybean meal	84.0	79.8
Wheat bran	35.0	33.3
Potatoes protein flour	27.0	85.0
Mineral and vitamin mix[a]	21.1	21.1
Meat meal	10.5	10.0
Soy oil	5.0	253.3
Amino acid mix[b]	2.0	2.0
Fatty acids composition[c]		
C16:0	4.6	30.2
C16:1	0.1	0.1
C18:0	0.8	10.4
C18:1	6.1	63.3
C18:2	15.5	143.5
C18:3	2.0	18.6
Gross energy, kJ/g	10.93	17.53
Protein, % energy	21.9	14.3
Carbohydrates, % energy	67.7	27.6
Lipid, % energy	10.4	58.1

Amount of energy is 16.7 kJ/g for protein and carbohydrate and 37.6 kJ/g for fat

[a] U/kg diet: vitamin A 9,828 U.I.; cholecalciferol 860 U.I.; thiamin 9.2 mg; riboflavin 4.6 mg; vitamin B6 4.1 mg; vitamin B12 0.018 mg; vitamin E (alpha tocopherol) 33.8 mg; menadione sodium bisulfite 1.9 mg; niacin 37.5 mg; folic acid 1.31 mg; pantothenic acid 9.8 mg; biotin 0.190 mg; choline 686; calcium 2,688 mg; phosphorus 1,276 mg; sodium 1,329 mg; chloride 2,050 mg; manganese (manganous sulfate monohydrate) 33 mg; iron (ferrous sulfate heptahydrate) 109 mg; zinc (zinc sulate monohydrate) 41.0 mg; copper 7.1 mg; iodine (calcium iodate) 0.5 mg; cobalt (basic cobalt carbonate monohydrate) 0.38 mg

[b] Composition of amino acid mix (%): arginine 25.2; cystine 10.2; lysine 24.1; methionine 12.2; tryptophan 7.2; glycine 21.0

[c] Calculated parameters

Lexington, MA). Glucose, total protein, albumin, total cholesterol, triglycerides, urea and aspartate aminotransferase (AST/GOT) were determined using kits purchased from Instrumentation Laboratory (IL Test). Globulin was calculated as the difference between total protein and albumin. Non-esterified fatty acids (NEFA) were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). Ceruloplasmin was analyzed using the method described by Sunderman and Nomoto (1970). Myeloperoxidase analysis was performed using the method described by Bradley et al. (1982). Serum amyloid A protein was assayed using an ELISA commercial kit (SAA, by ELISA, Tridelta, Ireland) and a microplate reader (The Synergy™ 2 BioTek Instruments, Inc., Winooski, Vermont, USA).

Histological analysis

Sections of cecum, collected from all mice at each time point, were fixed in 10 % phosphate-buffered PFA at 4 °C for 24 h. PFA-fixed intestinal tissues were embedded in paraffin, sectioned to a thickness of 5 μm, stained with hematoxylin and eosin and observed by a light microscope. Inflammation was evaluated on stained sections *in blind* by two independent researchers on the basis of the following factors: leukocytes infiltration, tissue alteration (edema and mucosal injury) and presence of mucus in the lumen. A rating score ranging between 0 (no change from normal tissue) and 5 (lesions involved most areas and all layers of the intestinal section including mucosa and muscle) was applied for each factor. The sum of scores for inflammatory cell infiltration, tissue alteration and presence of mucus in the lumen was considered as the total inflammatory score (TIS) for each specimen. The crypt depth was also measured in 10 cryptae per mouse. Only complete cryptae with an intact layer of epithelial cells were chosen for the measurement. All evaluations were conducted with a micrometric ocular at a 100× magnification.

RNA extraction

Total RNA was extracted from cecum samples collected at T2 using the Chomczynski–Sacchi method (Chomczynski and Sacchi 1987) and quantified using Qubit™ fluorometer (Invitrogen, Milan, Italy). The quality of RNA samples was ascertained using Experion (Bio-Rad Laboratories, Segrate, Milan, Italy).

Microarray hybridization

Gene expression was measured on cecum samples of individual CTR and HF mice killed at T2, in a total of 6 biological replicates per group. In addition, two samples per group were hybridized twice on different arrays and treated as technical replicates. Custom Array™ 90 K arrays were produced by the Functional Genomics Center at the University of Verona using a Custom Array Synthesizer (CombiMatrix, Irvine, CA, USA). A total of 29,435 non-redundant probes were synthesized in triplicate, representing all the available mouse (*Mus musculus*) genes in GenBank database. Total RNA (2 μg) was amplified and labeled with Cy5 using RNA Ampulse amplification and labeling kit for CombiMatrix arrays (Kreatech Diagnostics, The Netherlands), according to the manufacturer's recommendations. In short, the

double-strand cDNA was obtained by reverse-transcribing the total RNA using T7 Oligo(dT) primer. Thereafter, cDNA was transcribed in vitro, to generate amplified antisense RNA (aRNA). aRNA was labeled with cyanine 5 fluorophore using a chemical ligation. After purification, labeled aRNA was quantified by Varian Cary spectrophotometer (Agilent Technologies—Life Sciences and Chemical) and 4 μg hybridized to the array, according to the standard protocol supplied with Custom Array™ 90 K (CombiMatrix, Irvine, CA, USA). Fluorescent signals were collected on a GenePix 4,000B scanner (Molecular Devices, Sunnyvale, CA, USA).

Data analysis of microarray experiment

After image processing, signal intensity for each spot was quantified using GenePix Pro software v7. Cy5 signal quality was checked with an implemented R pipeline that takes advantage of limma package functions from Bioconductor (Bolstad et al. 2003). The reproducibility of the experiments was assessed calculating the average Spearman's rank correlation coefficient among all technical and biological replicates ($r = 0.96$) (range 0.99–0.91). After background correction, data were normalized (Gentleman et al. 2004) using the normal–exponential convolution (McGee and Chen 2006). Statistically significant differential expression between HF and control groups was tested using the limma package (Smyth 2004) on $\log_2$-transformed data. To increase the power of statistical analysis, we assigned weights to each array using a heteroscedastic linear model with shared array variance terms. In experiments with few replicate arrays, this approach outperforms the standard method of filtering data (Ritchie et al. 2006). The limma package was used to apply a Bayes model to moderate the standard errors of the estimated log-fold changes. This moderated t-statistic is computed for each probe and for each contrast. This has the same interpretation as an ordinary t-statistic, except that standard errors have been moderated across genes, that is, shrunk toward a common value, improving the statistical power of analyses, particularly in the case of small-size samples. Benjamini and Hochberg 5 % false discovery rate (q value ≤ 0.05) was used as threshold, to identify significant differentially expressed genes. Hierarchical clustering of all samples was computed based on genes differentially expressed with a q value ≤ 0.05.

RT and real-time PCR

Real-time PCR of selected genes (Table 2) was performed to validate microarray results of cecum samples and to analyze the expression of the same genes in liver tissue. Samples (1 μg of total RNA) were reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Monza, Italy). The reaction was carried out in a total volume of 20 μL containing 2 μL of cDNA sample diluted 1:5, 500 nmol/L of each primer and 1x Kapa Sybr® Fast Universal Master Mix (Kapa Biosystem, Inc., Woburn, MA). The PCR program used for all primers was as follows: 95 °C for 2 min; 95 °C for 15 s, 55 °C for 20 s, 72 °C for 20 s, 40 cycles; 95 °C for 1 min; 65 °C for 30 s; 95 °C for 30 s. Amplification efficiencies for each gene were calculated by generating a standard curve using serial dilutions of two pooled samples of cDNA of CTR and HF mice. The housekeeping mouse *Hprt* (hypoxanthine phosphoribosyltransferase) and *Gusb* (beta-glucuronidase) gene expression was assessed to confirm that their expression was unaffected by the experimental treatment (Livak and Schmittgen 2001) (data not shown) and used to normalize expression. Primer sequences (Table 2) were designed across consecutive exons using Primer3 software (http://frodo.wi.mit.edu/). Samples were assayed in triplicate on a 96-well plate along with an inter-run calibrator (a CTR mouse cDNA) and a no-template control for one target gene and the two housekeeping genes, thus eliminating inter-plate variability. Dissociation curves were assessed to ensure the presence of a single amplicon. Relative gene expression was calculated using the DataAssist™ v2.7 beta software (Applied Biosystems) and REST 2008 (V2.0.7; Corbett Research and Pfaffl; (Pfaffl et al. 2002)). For each target gene, expression data were expressed as fold change between HF group and CTR group. Differences were considered as significant at $P \leq 0.05$.

Statistical analysis

Statistical analysis of blood parameters was carried out by ANOVA using the GLM procedure of SAS® (SAS Institute Inc., Cary, NC, USA, release 9.1), including the effects of time (T0, T1 and T2), sex (male and female), diet (CTR and HF) and their interactions. Significance threshold was set at $P \leq 0.05$.

Results

Growth and metabolic parameters

At the end of the experiment (T2), neither diets nor sex significantly influenced the growth of the animals. The

Table 2 Real-time PCR primers

Gene symbol	Sense	Antisense
PPARα	AGGCTGTAAGGGCTTCTTTC	ACGGCAGTACTGGCATTTGT
Bmal1	GTCACAGGCAAGTTTTACAGAC	CTGAACCATCGACTTCGTAGC
Nfil3	AGCAGAACCACGATAACCCAT	TGCATTTTTCTCAGCTGCAT
Tef	AAGAAGGCAGATAAGGAAAAG	CATCGTAGGGGATGGTCTTG
Nr1d2	GATGCATCTGGTGTGTCCTA	CTTTGCAAATTCCACCACCT
Hlf	TGCCAGCAAACCGCAAT	CATACCCGACTGGTACCTGGAT
Cry1	ATGTCCCCCTGGTTGAGCTA	GGCATAGGTTCTGTCCATTGTG
Clock	CGCGAGAAAGATGGACAAGTC	CCTGTCGAATCTCACTAGCATCTG
Hprt	ATTATGGACAGGACTGAAAGAC	TGTAATCCAGCAGGTCAGCA
Gusb	CGAACCAGTCACCGCTGAGA	CTTCCGAAACACTGGGTTCT

Table 3 Plasma glucose, total cholesterol, triglycerides, urea, SAA levels in mice fed CTR or HF diet, after 7 (T1) and 14 days of treatment (T2)

	7 days (T1)			14 days (T2)		
	CTR	HF	SEM	CTR	HF	SEM
N°	5	5		6	6	
Glucose, mmol/l	13.5	14.3	0.757	13.3	15.1	0.691
Total cholesterol, mmol/l	1.60**	2.26	0.112	1.55**	2.37	0.103
Triglycerides, mmol/l	0.82*	0.54	0.069	0.68*	0.40	0.063
Urea, mmol/l	4.3	4.6	0.407	5.5*	5.0	0.372
SAA, μg/ml	7.6	8.3	0.898	5.7**	11.1	0.900

Values are mean ± SEM

Significant differences are * $P \leq 0.05$; ** $P \leq 0.01$

value of biochemical and metabolic parameters measured in mouse plasma are reported in Table 3. High-fat mice had a higher level of total cholesterol ($P \leq 0.01$) and glucose (N.S.) and a lower level of triglycerides ($P \leq 0.05$) compared with control mice, both at T1 and T2. Urea was lower in HF than in CTR at T2 ($P \leq 0.05$). NEFA, total proteins, albumins, globulins and AST/GOT showed no significant difference between the two groups. The serum amyloid A (SAA) levels (Table 3) were much lower than those detected in the case of an acute inflammation but consistently higher in HF compared with CTR and significantly different at T2 (11.1 mg/L in HF vs. 5.7 mg/L in CTR; $P \leq 0.01$).

Effect of high-fat diet on histological parameters

Histological analyses showed that either the total inflammatory scores nor the crypt depth in the cecum sections evidenced any significant difference between mice fed with HF and control diets (Table 4).

Identification of genes in the cecum affected by high-fat diet

Whole-transcriptome microarray analysis was carried out on cecum samples from individual CTR and HF mice killed at T2. After logarithmic transformation, the fold changes of significantly differentially expressed genes spanned between −1.17 and +1.96. Only 7 genes across the whole transcriptome differed statistically (q value ≤0.05) in expression levels comparing the two groups (Table 5). Three (*Fst, Tspan4*, *H2-Q10*) are involved in the inflammatory process. Follistatins inactivate activins and pro-inflammatory proteins, and modulate the resolution of ensuing tissue damage (de Kretser et al. 2012). Tetraspanins modulate the function of adhesion receptors involved in inflammation, lymphocyte activation, cancer and pathogen infection (Yàñez-Mó et al. 2009). The histocompatibility 2, Q region locus 10 (H2-Q10) is one of the MHC class I molecules that mediate the interactions of leukocytes with other immune system and somatic cells. At T2, *Fst* and *H2-Q10* were down-regulated in HFD, while *Tspan4* was up-regulated.

The other four significant genes are directly or indirectly participating in the regulation of the circadian clock system. Two (*Bmal1*, *Nr1d2*) are involved in the transcriptional/translational feedback loops controlling the circadian clock system and two (*Tef*, *Hlf*) are genes coding for PAR bZip (proline and acidic amino-acid-rich basic leucine zipper) transcription factors, whose circadian expression is driven by the core clock. In the list of the top ten genes sorted by adjusted p value, we also found *Nfil3* (q value ≤0.2) (Table 5) also involved in the modulation of the circadian clock system.

At T2, the HF diet decreased the expression of *Bmal1* and *Nfil3* and increased *Nr1d2*, *Hlf* and *Tef* (Table 5). As shown in Fig. 1, hierarchical clustering analysis of these genes identified two groups corresponding to the group of mice fed respectively the HF diet (on the right side of the HeatMap, C22–C27) and the control diet (on the left side of the HeatMap, C16–C21).

Table 4 Effect of high-fat diet on the histology of the cecum of mice

	T0 ($n = 5$)	T1 CTR ($n = 5$)	T1 HF ($n = 6$)	T2 CTR ($n = 6$)	T2 HF ($n = 6$)
Crypt depth, μm	150.00 ± 30.00	156.00 ± 26.08	155.00 ± 30.00	161.67 ± 20.41	156.67 ± 22.51
TIS	4.20 ± 2.69	6.60 ± 4.39	4.47 ± 2.77	5.16 ± 3.09	6.3 ± 3.54

Values are mean ± SEM

TIS Total Inflammatory Score

Table 5 Top 10 of the differentially expressed genes in the cecum of mice at T2, in response to high-fat diet

Gene symbol[a]	Accession number	Definition	logFC[b]	q value
Bmal1	NM_007489.3	Mus musculus aryl hydrocarbon receptor nuclear translocator–like (Arntl) or brain muscle Arnt-like-1 (Bmal1), mRNA	−1.17	0.00032
Tef	NM_153484.2	Mus musculus thyrotroph embryonic factor (Tef), transcript, mRNA	+1.96	0.00241
Fst	NM_008046.2	Mus musculus follistatin (Fst), mRNA	−0.82	0.00430
Nr1d2	NM_011584.3	Mus musculus nuclear receptor subfamily 1, group D, member 2 (Nr1d2), mRNA	+1.41	0.01701
H2-Q10	NM_010391.4	Mus musculus histocompatibility 2, Q region locus 10 (H2-Q10), mRNA	−1.11	0.01719
Tspan4	NM_053082.2	Mus musculus tetraspanin 4 (Tspan4), mRNA	+0.66	0.02231
Hlf	NM_172563.3	Mus musculus hepatic leukemia factor (Hlf), mRNA	+0.85	0.03847
Kif26b	NM_177757.3	Mus musculus kinesin family member 26B (Kif26b), mRNA	−0.57	0.16457
Slc30a10	NM_001033286.2	Mus musculus solute carrier family 30, member 10 (Slc30a10), mRNA	−1.12	0.18149
Nfil3	NM_017373.3	Mus musculus nuclear factor, interleukin 3, regulated (Nfil3), mRNA	−0.52	0.18149

Genes differentially expressed with a q value ≤0.2 in the cecum of HF mice ($n = 6$) compared with CTR mice ($n = 6$)

[a] Genes are sorted based on q value

[b] The fold change is expressed in log2

Confirmation of clock genes differential expression by real-time PCR in cecum and liver

The differential expression of *Bmal1*, *Nr1d2*, *Nfil3, Tef* and *Hlf* was validated by real-time PCR. Also *Cry1* and *Clock*, two other important "clock genes," and *PPARα*, playing a key role in lipids metabolism, detected by microarray analysis as not differently modulated, were included into real-time PCR analysis to verify the robustness of microarray data. Real-time PCR analysis confirmed microarray data for all genes (Table 6). The expression of *Cry1* ($P = 0.89$), *Clock* ($P = 0.343$) and *PPARα* ($P = 0.73$) was not different in animals fed CTR and HF diets. The same genes were analyzed in mice liver samples at T2. The expression of clock genes differentially expressed in the cecum followed the same pattern in the liver. Also, the liver expression of *Cry1* ($P = 0.185$) did not differ between groups, as observed in the cecum. Conversely, *PPARα* was significantly up-regulated and *Clock* down-regulated in the liver (Table 6), whereas the expression of these two genes was similar between the groups in the cecum.

Discussion

The present study investigated the early response of C57BL/6N mice to an increased consumption of dietary fat. The unusual high-fat diet was enriched in the main precursor of the arachidonic acid, reported to drive the metabolism to the synthesis of pro-inflammatory mediators (Calder 2002, 2003).

No increase in body weight gain was evident in mice fed the HF diet compared with CTR, in spite of the higher energy content. Also, histological parameters of intestinal tissue samples collected from HF mice showed neither morphological alterations nor leukocytes infiltration. It is quite likely that the short period of dietary treatment was not sufficient to produce visible effects on growth and to induce a detectable tissue alteration in the gut.

Cholesterol

Total plasma cholesterol concentration displayed a significant increase in the HF group both at T1 and T2, as already described in previous studies (Paigen 1995;

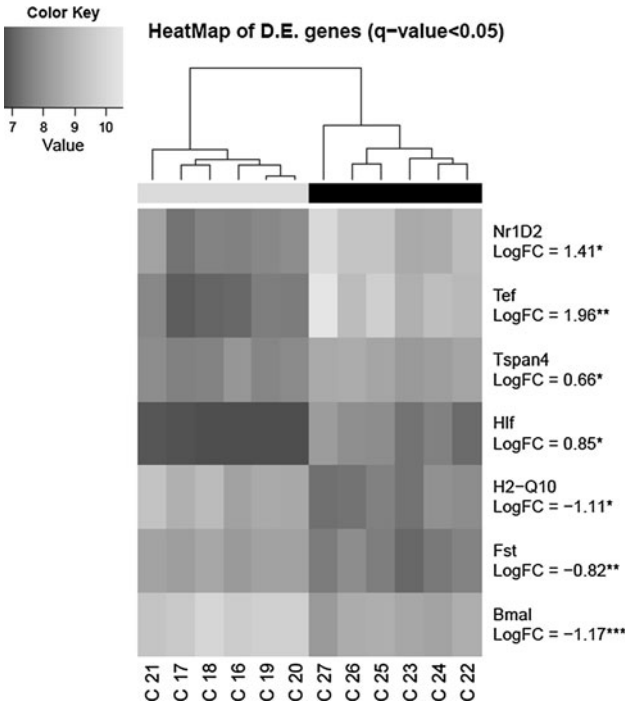

Fig. 1 Hierarchical clustering of all the samples based on genes differentially expressed with a q value ≤ 0.05. Relative levels of gene expression are *gray-scale*-coded: the *lightest gray* represents the highest level of expression, and the *darkest gray* represents the lowest one. Unsupervised clustering identified 2 subsets of samples, one including all mice fed the high-fat diet ($n = 6$; C22–C27) and the other all mice fed the control diet ($n = 6$; C16–C21)

Gallou-Kabani et al. 2007). Moreover, Paigen (1995) observed that the increased cholesterol level was mostly due to the non-HDL fraction of cholesterol, thus reproducing the situation observed in studies on human metabolic syndrome.

Triglycerides

Triglycerides levels in HF group were 34 % (T1) and 41 % (T2), lower than those of mice in the CTR groups. These results are in line with many studies in the murine species (Paigen 1995; Tsunoda et al. 1998) and in humans (Lichtenstein 2006). The decrease in triglyceride levels following fish oil administration has been in part ascribed by some authors to a decrease in the hepatic production of triglyceride-rich particles (VLDL, the lipoprotein responsible for transporting triglycerides for subsequent delipidation by peripheral tissue) or to an increase in the conversion rate of VLDL to LDL (Balk et al. 2006).

Serum amyloid A protein

Serum amyloid A is the major acute-phase protein in vertebrates (Uhlar and Whitehead 1999). Its level can increase 100- to 1,000-fold in response to acute inflammatory stimuli. Our results indicate a significant increase in SAA level at T2 in animals fed HF diet. However, the increase recorded was inconsistent with the presence of acute inflammation, since HF group displayed approximately twice the level of SAA compared with the CTR group. This observation was consistent with data from Lewis et al. (2004) that noticed that SAA level in atherosclerosis susceptible $LDLR^{-/-}$ mice fed high-fat diet was higher than in control mice but much lower than the one observed during

Table 6 Relative expression ratio of the eight genes validated by real-time PCR in mice at T2 in response to the HF diet

Gene name	Cecum			Liver		
	Fold change[a] (HF/CTR)	SE range	*P* value	Fold change[a] (HF/CTR)	SE range	*P* value
Aryl hydrocarbon receptor nuclear translocator–like (*Arntl*) or brain muscle Arnt-like-1 (*Bmal1*)	↓↓ (0.303)	0.202–0.584	0.002	↓↓↓ (0.053)	0.026–0.089	<0.001
Thyrotroph embryonic factor (*Tef*)	↑↑↑ (4.926)	3.538–7.028	<0.001	↑↑ (11.146)	6.505–18.958	0.002
Hepatic leukemia factor (*Hlf*)	↑↑ (4.533)	3.228–9.696	0.001	↑↑ (2.920)	2.048–4.620	0.003
Nuclear receptor subfamily 1, group D, member 2 (*Nr1d2*) or *Rev-erbβ*	↑↑ (3.112)	2.073–5.300	0.001	↑↑ (4.666)	2.284–8.304	0.003
Nuclear factor, interleukin 3, regulated (*Nfil3*) or E4 promoter-binding protein 4 (*E4BP4*)	↓↓↓ (0.352)	0.167–0.674	<0.001	↓↓↓ (0.180)	0.112–0.294	<0.001
Circadian locomotor output cycles kaput (*Clock*)	–[b]	–	0.343	↓↓↓ (0.401)	0.292–0.546	<0.001
Cryptochrome 1 (photolyase-like) (*Cry1*)	–[b]	–	0.890	–[b]	–	0.185
Peroxisome proliferator activated receptor alpha (*PPARα*)	–[b]	–	0.73	↑ (1.620)	1.007–2.322	0.021

Data were statistically analyzed by the software REST 2008 (relative expression software tool; [26]; http://rest.gene-quantification.info/)

[a] Values are means, $n = 6$. Arrows indicate the direction and significance level of the difference from control: 1 arrow, $0.01 \leq P < 0.05$; 2 arrows, $0.001 \leq P < 0.01$; 3 arrows, $P < 0.001$. Fold changes indicate the gene expression difference in mice at T2 induced by the high-fat diet relative to the control diet

[b] Not different from the CTR, $P > 0.05$

an acute inflammatory response. Interestingly, in clinical studies in humans, Ridker et al.(2000) and Jousilahti et al. (2001) reported that small increases in SAA, if compared with the SAA level in an acute inflammation, have proven to be predictive values for cardiovascular disease events, hence indicating SAA as an early marker for inflammation increasing before the appearance of any evidence for distress.

Metabolic data, cholesterol and SAA in particular, suggest at T2 the beginning of a possible onset of inflammation.

Gene expression

Gene expression in gut was assessed at T2 by microarray analysis. The study revealed seven genes differentially expressed in HF mice compared with CTR mice (q value ≤ 0.05).

The *Fst, H2-Q10* and *Tspan4* are genes implicated in inflammatory processes. During an acute systemic inflammation activin A is released concurrently with follistatin as part of the circulatory cytokine cascade (Jones et al. 2004). Follistatin is down-regulated in HFD and consequently less able to counteract the pro-inflammatory activity of activins. *H2-Q10* is down-regulated as well. The gene is part of the MHC complex; however, its specific function is poorly known. Conversely, *Tspan4* is up-regulated by HFD. Among the functions of the tetraspanin trans-membrane protein, there is the modulation of the avidity of adhesion receptors involved in inflammation, and the regulation of cell migration and invasion (Yàñez-Mó et al. 2009). The HFD seems therefore on the one side to pave the way to an inflammatory response and on the other to weaken the potential reaction to the onset of an inflammatory status.

The other four differentially expressed genes were involved in molecular mechanisms regulating circadian rhythms. The *Bmal1* gene, down-regulated in HF, is a transcription factor playing a central role in the regulation of circadian rhythms and seems to contribute to the regulation of adipogenesis and adipocyte functions (Shimba et al. 2005). The *Nr1d2* gene, up-regulated in HF, is highly expressed during daylight and represses *Bmal1* transcription (Guillaumond et al. 2005). It is also implicated in the regulation of many genes involved in lipid homeostasis in skeletal muscle cells (Ramakrishnan et al. 2005). The *Tef* and the *Hlf* gene, both up-regulated in HF, serve as mediators in transmitting circadian signals and in controlling various metabolic pathways and physiological functions. Finally, it is worth mentioning the *Nfil3* gene coding for the E4BP4 protein that, although not significantly (q value $= 0.18$), appears down-regulated in HF. *Nfil3* plays a regulatory role in the circadian transcriptional circuitry (Ueda et al. 2005).

Microarray data were validated by real-time PCR in the cecum and assessed in the liver, assumed to be the most responsive organs to diet interventions. Results confirmed all cecum microarray data and revealed the same trend of gene expression modulation in the liver, with the exception of *PPARα* expression, up-regulated in the liver of HF-fed mice, and of *Clock* expression, down-regulated in the liver in response to HF treatment.

In the last years, metabolic diseases have been shown to be associated with altered temporal organization of many physiological functions. Therefore, attention has been directed toward the investigation of links between circadian rhythms and functions, in particular of the gastrointestinal tract (GI). A number of investigations have been conducted in transgenic mice with disruptions or deficiencies of some clock genes fed high-fat diets or obesity-induced diets and have demonstrated that disruption of circadian rhythms leads to metabolic disorders (Hemmeryckx et al. 2011; Dallmann and Weaver 2010; Marcheva et al. 2010). By contrast, only a few studies have investigated the relationship existing between different diets and circadian clock regulation in wild-type mice. The effect of administering high-fat diet on the expression of clock genes has so far been studied in different tissues (Kohsaka et al. 2007; Barnea et al. 2009; Hsieh et al. 2010; Yanagihara et al. 2006), but to date, alterations of clock gene expression in the gut following administration of high-fat diet in wild-type mice have never been reported.

In the present work, we show for the first time that the administration of a high-fat diet leads to a change in the expression of clock genes in the cecum of C57BL/6N mice. Out of 30,000 genes investigated, only 7 genes appear to respond very early (after 2 weeks of treatment) to the administration of HF diets, in the presence of only slight alterations of metabolic parameters and no evidence of clear inflammation, and four of these genes were involved in the regulation of the circadian clock system. The variations we observed in cecum and liver gene expression are in accordance with known regulatory circuits (Kohsaka et al. 2007; Mitsui et al. 2001). *Nr1d2*, *Tef* and *Hlf* expression alterations in peripheral tissues following administration of high-fat diet had never been described by other authors.

Even if most of the complex molecular interactions underlying the circadian systems remain poorly understood, it is now evident that the circadian clock plays an important role in regulating metabolism and energy homeostasis in peripheral tissues by mediating the expression and the activity of certain metabolic enzymes and transport systems. This study suggests that also in the cecum of mice, as well as in the liver, diet directly influences clock genes expression and it is conceivable that their up- or down-regulation mediates alterations in metabolic processes (i.e., energy utilization and/or storage) by

means of subtle transcriptional–translational feedback loops. The internal clock system is thought to allow organs to quickly adapt to environmental changes, and this might explain the changes in clock genes expression in the cecum and liver following high-fat-diet administration. Although the molecular mechanisms underlying the results observed remain unknown, we can suppose that cecum might be one of the first organs to be subject to changes in clock genes expression following a diet intervention.

Further studies are needed to better understand whether the low fold changes and the lack of inflammation might be ascribed to the short-term high-fat-diet treatment, as stated in previous works, or to our unusual high-fat diet model including soy oil, rich in unsaturated fatty acids, as fat source. For this reason, it could also be interesting to understand whether there is a correlation between the changes in the gut microbiota (Patrone et al. 2012) and in the gut gene expression following the HFD administration, al least in this model. Finally, it remains to be elucidated whether there is an association between the small increase in SAA and the change in some clock genes expression levels in the cecum and liver of HF-fed mice at T2 as an early signaling of the development of an intestinal pro-inflammatory status. In this view, clock genes could be very useful as "early markers" of pre-disease conditions for metabolic disorders.

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