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7-Hydroxymatairesinol improves body weight, fat and sugar metabolism in C57BJ/6 mice on a high-fat diet

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Abstract

7-Hydroxymatairesinol (7-HMR) is a plant lignan abundant in various concentrations in plant foods. The objective of this study was to test HMRLignan™, a purified form of 7-HMR, and the corresponding *Picea abies* extract (total extract *P. abies*; TEP) as dietary supplements on a background of a high-fat diet (HFD)-induced metabolic syndrome in mice and in the 3T3-L1 adipogenesis model. Mice, 3 weeks old, were fed a HFD for 60 d. Subgroups were treated with 3 mg/kg body weight 7-HMR (HMRLignan™) or 10 mg/kg body weight TEP by oral administration. 7-HMR and TEP limited the increase in body weight (−11 and −13 %) and fat mass (−11 and −18 %) in the HFD-fed mice. Epididymal adipocytes were 19 and −12 % smaller and the liver was less steatotic (−62 and −65 %). Serum lipids decreased in TEP-treated mice (−11 % cholesterol, −23 % LDL and −15 % TAG) and sugar metabolism was ameliorated by both lignan preparations, as shown by a more than 70 % decrease in insulin secretion and insulin resistance. The expression of several metabolic genes was modulated by the HFD with an effect that was reversed by lignan. In 3T3-L1 cells, the 7-HMR metabolites enterolactone (ENL) and enterodiol (END) showed a 40 % inhibition of cell differentiation accompanied by the inhibited expression of the adipogenic genes *PPARγ*, *C/EBPα* and *aP2*. Furthermore, END and ENL caused a 10 % reduction in TAG uptake in HEPA 1–6 hepatoma cells. In conclusion, 7-HMR and TEP reduce metabolic imbalances typical of the metabolic syndrome and obesity in male mice, whereas their metabolites inhibit adipogenesis and lipid uptake *in vitro*.

Key words: Lignan: 7-Hydroxymatairesinol: Obesity: Adipose deposition: Adipocyte differentiation: Metabolism

The high prevalence of the metabolic syndrome and excessive adipose tissue accumulation (in particular, abdominal obesity) is a major threat to public health, being associated with a substantial decrease in health-related quality of life and an increase in economic costs[1–3]. Thus, new strategies related to the long-term prevention and reduction of incidence and severity of CVD and type 2 diabetes need to be initiated. Among these is the identification of beneficial bioactive compounds within many foods, as appropriate dietary strategies that may themselves positively affect disease predisposition and evolution[4].

Lignans are chemicals produced as secondary metabolites[5,6]. They occur in the whole plant kingdom and can also be found in fibre-rich foods and particularly concentrated in oilseeds (especially in flaxseed and sesame)[7–9], in cereal grains (e.g. wheat and rye bran), nuts[10], *Brassica* species, legumes, berries and in many plant-related beverages (tea,

Abbreviations: 7-HMR, 7-hydroxymatairesinol; DMSO, dimethylsulfoxide; END, enterodiol; ENL, enterolactone; HFD, high-fat diet; LFD, low-fat diet; PA, palmitic acid; TEP, total *Picea abies* extract; TFEB, transcription factor EB.

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coffee and so on)[11]. 7-Hydroxymatairesinol (7-HMR) is a dibenzylbutyrolactone plant lignan closely related to matairesinol. It is found at relatively high concentrations in the heartwood of branches and knots of Norway spruce trees (*Picea abies*)[12] and in various concentrations in plant foods[10–14]. The activity and metabolism of 7-HMR has been recently evaluated in rats and in humans, with enterolactone (ENL) being the major metabolite identified[15,16]. Other metabolites detected included hydroxyenterolactone, α-conidendrin, conidendric acid, enterodiol (END), allo-7-HMR and unchanged 7-HMR. All of these minor metabolites, including unchanged 7-HMR, were at concentrations many-fold lower than the primary metabolite ENL[15].

In contrast to other well-studied lignans, which are highly concentrated in oilseeds such as sesamin or secoisolariciresinol[17,18], 7-HMR, although also present in oilseeds, was only recently detected in several cereals by applying improved extraction and detection methodologies[10]. 7-HMR is found to be concentrated in rye, wheat, triticale, oat, barley, millet, maize bran and amaranth whole grain[10]. Thus, as these grains are widely consumed worldwide in various forms, we intended to study 7-HMR, which is the dominant lignan in these foods, in a model of the metabolic syndrome[19] for which the metabolic functions and effects of 7-HMR are still elusive.

In this work, we investigate the effect of 7-HMR on the pathological alterations that occur with the metabolic syndrome following a high-fat diet (HFD), at a dose suggested for nutraceutical use (HMRLignan™) (which provides a high intake of this compound), but that can also produce levels of mammalian metabolites (ENL and END) similar to those reached with a lignan-rich diet.

Methods

Chemicals

7-HMR potassium acetate complex (HMRLignan™) was prepared by co-crystallisation of *P. abies* extract and potassium acetate in a ternary solvent system (ethanol, ethylacetate, water), as previously described[10]. The purity of the preparation HMRLignan™ batch 12809003 is as follows: hydroxymatairesinol potassium acetate 99 % and related substance 4 %. Total *P. abies* extract (TPE) is a mixture of 7-HMR and allo-hydroxymatairesinol, produced by hot ethanolic extraction of *P. abies* knots. TEP is an intermediate in the process to obtain pure 7-HMR. All steps in the 7-HMR purification process are validated, and thus TEP is a standardised and replicable product. The product contains 31 % 7-HMR, 16 % allo-hydroxymatairesinol and other natural constituents of the herbal drug. The balance to 100 % is potassium acetate and water. The chemicals were dissolved in dimethylsulfoxide (DMSO) and further diluted before their oral administration to the mice or the treatment of the cells. The chemicals (HMRLignan™ and TEP) for the experiments were provided by Linnea SA free of charge. The complete chemical analysis of 7-HMR is freely available upon request to the corresponding author. In this work, we use the name 7-HMR throughout the text. HMRLignan™ is the proprietary name of the commercial preparation. Palmitic acid (PA; Sigma-Aldrich) was dissolved in isopropylic alcohol at a 100-mM stock concentration. Stock solution was diluted to 1·25 mM in warm Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS; Gibco) and 10 % bovine serum albumin (BSA; Sigma-Aldrich). The 1·25-mM solution was further diluted in warm growth medium at a final concentration of 125 μM to treat cells. The END and ENL enterolignans were purchased from Sigma-Aldrich and dissolved in DMSO at a stock concentration of 10 mM and further diluted in DMSO for experiments with cells.

Experimental animals

The procedures involving animals and their care were conducted in accordance with institutional guidelines, which comply with national and international laws and policies (National Institutes of Health, *Guide for the Care and Use of Laboratory Animals*, 1996 (7th ed.) (Washington, DC); National Academies Press, National Research Council Guide, www.nap.edu/readingroom/books/labrats). C57BJ/6 mice (Harlan, Udine) were kept in animal rooms maintained at a temperature of 23°C, with natural light/dark cycles in aerated (40 × 25 cm) polycarbonate cages at a density of 8 mice/cage. Chemicals were administered by oral administration in 100 μl of vegetable oil at 10.00 hours with disposable flexible polypropylene tubes in plastic with a soft bulb tip to minimise tissue damage. At the end of the treatments, the animals were killed by cervical dislocation after having received diethyl ether anaesthesia, and the tissues were dissected and immediately frozen on dry ice. Serum was separated from blood cells by centrifugation at 18 928 G-force (13 000 rpm).

Food consumption was monitored every 2 d as the difference between the weight of the supplied and the consumed pellets. Spilled food, if any, was collected in apposite trays underneath the food containers, measured and taken into account.

Diets

Low-fat diet (LFD) and HFD containing 10 and 50 % energy content from fat, respectively, were purchased from Piccioni (www.totofood.it/). The diet was prepared in pellets. The composition is reported in Table 1. Assay of 7-HMR or TEP or analogues in the diets did not reveal any presence of these

Table 1. Composition of the diets*

LFD (10 % energy content from fat)	HFD (50 % energy content from fat)
20 % casein	25 % casein
0·3 % L-cystine	0·3 % cystine
25 % maize starch	10·8 % maize starch
8·5 % maltodextrin	5·0 % maltodextrin
31·5 % sucrose	20·5 % sucrose
5·0 % cellulose	5·0 % cellulose
2·5 % maize oil	2·5 % maize oil
2·0 % lard	13·0 % lard plus 12·5 % hydrogenated coconut oil
4·0 % mineral mix	4·0 % mineral mix
1·0 % vitamin mix	1·0 % vitamin mix
0·2 % choline bitartrate	0·4 % choline chloride

LFD, low-fat diet; HFD, high-fat diet.
* Amounts of components in the diets: LFD and HFD containing 10 and 50 % fat, respectively, were purchased from Piccioni.

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chemicals. Quantification was performed by the method reported in Smeds *et al.*[10,20,21]. Food consumption was monitored every 2 d as difference between the weight of the furnished and the consumed pellets (see the 'Results' section). Mean daily water intake was 3·5 (SE 0·2) ml/mouse for mice on the LFD and 3·8 (SE 0·4) ml/mouse for mice on the HFD. 7-HMR or TEP administration did not change the average water intake.

The LFD was composed of 15·6 % SFA, 45·2 % MUFA and 39·2 % PUFA, whereas the HFD was composed of 60·3 % SFA, 32·9 % MUFA and 6·7 % PUFA.

Echo MRI analysis

The Echo MRI system (EchoMRI Corporation Pte Ltd) was used as already reported[22].

Determination of 7-hydroxymatairesinol and metabolites in mouse serum

7-HMR, END and ENL were determined by HPLC–MS/MS in the mouse serum samples after enzymatic hydrolysis and solid-phase extraction according to a previously described method[20]. The method was slightly modified by taking 50 µl of serum instead of 600 µl and 290 units of β-glucuronidase/sulphatase dissolved in 0·5 ml of 10 mM sodium acetate buffer (pH 5·0) for each sample (for the enzymatic hydrolysis). The glucuronidase used was β-glucuronidase/sulphatase (type H-1, from *Helix pomatia*) (Sigma-Aldrich Co.). The samples were incubated at 37°C for 19 h and cleaned after incubation by solid-phase extraction. For details, see Smeds *et al.*[21]. The possible presence of residual lignans in the glucuronidase preparation was not determined, as the method has been validated and found reliable. The small amounts of ENL and END measured in mice on the LFD and HFD reflect the consumption of a certain amount of sawdust as no other sources of lignans were used in this work.

Biochemical assays

Blood was collected from each mouse, stored at 37°C for 30 min and centrifuged at 3500 ***g*** at room temperature for 10 min to obtain serum. TAG, HDL and cholesterol concentrations were measured spectrophotometrically with a biochemical multi-analyser ILab Aries (Instrumentation Laboratory Company) using kits by the same manufacturer. The parameters are as follows: TAG analytical range, 0·07–11·3 mmol/l; precision, CV (%) 1·5; cholesterol analytic range, 0·1–23·3 mmol/l; precision, CV (%) 2·0; HDL-cholesterol analytic range, 0·03–6·5 mmol/l; precision, CV (%) 3·1. Species-specific kits are not necessary for the determination of these parameters. Our value ranges are in agreement with data from the literature on the same analytes. Insulin concentration was assayed by a standardised mouse insulin assay (catalogue no. EZRMI-13K; Millipore) and insulin sensitivity was determined by the homoeostasis model assessment (Diabetes Trials Unit, The Oxford Centre for Diabetes). The oral glucose tolerance test was performed as previously described[22]. Mice were deprived of food overnight and then orally given glucose (1 mg/g body weight). Tail vein blood samples were taken at 0, 15, 30, 45 and 60 min after glucose administration to measure blood glucose concentrations. Blood glucose concentrations were measured by a portable Accu-check compact glucometer (Roche Diagnostic GmbH). AUC was calculated by using the trapezoid rule[23].

For immunoblot analysis, cell lysates were obtained as previously described[23]. Equal protein amounts, separated by SDS-PAGE, were transferred to polyvinylidene difluoride (PVDF) membranes. Filters were incubated with diluted 1:1000 primary antibodies (mouse anti-*PPARγ* and mouse anti-fatty acid binding protein 4, *aP2/FABP4*) and then with peroxidase-labelled goat anti-mouse secondary antibody. All antibodies were purchased from Santa Cruz Biotechnology. Bound activity was revealed by chemiluminescence (SuperSignal West Femto Substrate; Thermo Fisher Scientific). The chemiluminescent signal was captured and quantified by the use of GENE-GNOME (Syngene).

RNA extraction and gene expression analysis

Total RNA was extracted from 10 to 30 mg of tissue or from treated cells using 1 ml of TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Tissues in TRIzol reagent were pre-homogenised at 30 Hz for 3 min using the homogeniser MM301 (Qiagen). RNA for each sample was treated with RQ1-DNaseI (Promega) and reversed-transcribed using Improm II Reverse Transcriptase (Promega) and random hexamer primers. For quantitative RT-PCR, 2 µl of single-stranded complementary DNA was mixed with TaqMan Universal PCR Master (Thermo Fisher Scientific) and assay-on-demand gene-specific products, using *HPRT1* as the normalising gene (Integrated DNA Technology), and analysed on a ABI PRISM 7700 Sequence Detection System (Life Technologies). The calculation of threshold cycle (C_t) values was performed using the SDS 2.2 software (Applied Biosystems), after automatically setting the baseline and the threshold. Data were analysed using the $2^{-\Delta\Delta C_t}$ method, as previously described[24].

Histological examination

Epididymal adipose tissue and liver were fixed in formaldehyde and embedded in paraffin. Sections measuring 3 µm were dewaxed and rehydrated through decreasing alcohol series up to distilled water and stained with haematoxylin–eosin. Original magnification was 20×. Analyses were conducted in a blinded manner by two independent pathologists.

Cell culture and treatments. Mouse 3T3-L1 confluent cells (cells with a fibroblast-like morphology, differentiating into an adipocyte-like phenotype) were cultured in basal or differentiation medium (3-isobutyl-1-methylxanthine, dexamethasone, insulin; MDI) as described[22,25]. On day 9 after induction of differentiation, the cells were treated with different doses of 7-HMR, TEP, ENL and END (0·01, 0·1, 1 µM) for two further days. On day 9 after differentiation induction, lipid content was quantified as described[22]. For mRNA quantification the confluent cells were treated for 48 h with vehicle or defined concentrations of the molecules, and RNA was extracted using the TriReagent Solution (Ambion), treated with DNaseI (Promega) and reverse-

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transcribed. Quantitative PCR analyses were performed using Assay on Demand kits on an ABI PRISM 7000 (Applied Biosystems)[26]. *HPRT1* was used as the reference gene.

Murine HEPA 1–6 hepatocarcinoma cells (IZSLER Brescia) were used as the steatosis *in vitro* model. Cells were cultured in DMEM medium containing 10 % FBS. For PA treatment, cells were seeded at a density of 2×10^4/well in ninety-six-well plates in growth medium for 24 h. Then, medium containing the SFA PA at 125 μM was applied for 24 h in the presence of vehicle (isopropylic alcohol 0·1 %, plus BSA 1 %, plus DMSO 0·05 %) or 7-HMR, TEP, END or ENL in the dose range of 0·001–1 μM.

Statistical analysis

Sample size was calculated in order to be able to detect differences of about 5 g in total body weight between HFD mice using one-way repeated-measures ANOVA at 80 % power. Calculations were performed using the G*Power 3.1.5 software (available at http://www.gpower.hhu.de/en.html). With a β level of 0·20, an α level of 0·05 and a small effect size, the sample size was thirty-nine mice in each of the four groups (then increased to forty, to account for possible dropouts). Statistical analysis was performed by ANOVA, either one-way or two-way with repeated measures on one factor, according to the data type, followed by *post hoc* Bonferroni analysis. All the analyses were conducted in a blinded manner and were performed using the Prism 5.0 software (GraphPad Software, www.graphpad.com).

Results

Effects of 7-hydroxymatairesinol and total Picea abies extract on body fat deposition, lipid profile and sugar metabolism in male mice fed a high-fat diet

Peripubertal male mice, 3 weeks old, were randomised into four groups (LFD, HFD, HFD + 7-HMR and HFD + TEP). The compounds were given daily by oral administration to mimic dietary assumption and to allow microbiota metabolism. The mice were fed the LFD or the HFD plus or minus the oral lignan for 60 d. Average food consumption was 2·99 (SE 0·5) g/mouse per d for the mice on the LFD (46·65 kJ/mouse per d (11·15 kcal/mouse per d)) and 2·61 (SE 0·4) g/mouse per d for the mice on the HFD (51·46 kJ/mouse per d (12·30 kcal/mouse per d)). Food intake of the mice on the HFD plus oral administration with vegetable oil with dissolved 7-HMR or TEP was 2·70 (SE 0·6) g/mouse per d and 2·65 (SE 0·6) g/mouse per d, respectively, showing that vegetable oil plus the chemicals did not have any effect compared with vegetable oil alone.

HFD-fed mice were treated daily with 3 mg/kg body weight (bw) 7-HMR (doses of 1 up to 22·5 mg/kg body weight have been studied in humans and showed no toxic effects)[16] (HORMOS study number: 3000-4302 and one manuscript in preparation) or 10 mg/kg body weight TEP (an experimental dose based on previous unpublished results). Total body weight was measured periodically for 60 d and total body fat was measured at the end of the experiment. From day 30 until the end of the experiment the body weight started to be significantly lower in mice treated with 7-HMR and TEP (−7 and −6 %, respectively, at day 30, and −11 and −13 %, respectively, at day 60) compared with the mice on the HFD alone (Fig. 1(a)).

We also found that total fat and isolated epididymal and gluteal fat pads were significantly smaller in the 7-HMR- and TEP-treated mice compared with mice on the HFD alone (Fig. 1(b)–(d)). Serum glycaemia decreased (fasting glucose: −12 % 7-HMR and −14 % TEP. AUC −33 % 7-HMR and −27 % TEP), as well as insulin secretion (−74 % 7-HMR and −89 % TEP) and insulin resistance (−71 % 7-HMR and −78 % TEP), in the 7-HMR- and TEP-treated mice (Fig. 2(a)–(e)). Histological examination of the adipocyte size in epididymal fat revealed that the decrease in fat weight of mice fed 7-HMR or TEP corresponds to a decrease in cellular dimensions (Fig. 3(a) and (b)). Mice on the HFD developed accumulation of lipid droplets in the liver (liver steatosis), which were measured by histological analysis (Fig. 3(c)) and the fat content quantified (Fig. 3(d)). 7-HMR and TEP lowered the lipid content in the liver of the treated mice.

Effects of 7-hydroxymatairesinol and total Picea abies extract on serum lipids profile in male mice fed a high-fat diet

After 10 weeks on the HFD, we observed a decrease in total serum cholesterol levels (−11 %) in TAG (−15 %) and LDL (−23 %) in mice fed the TEP-supplemented diets compared with those in HFD-fed mice. HDL did not change in both groups of treated mice (Table 2). No significant changes were visible in the serum of mice fed the 7-HMR-supplemented diet ($P < 0·05$).

Concentration of enterolactone and enterodiol in mouse serum

The major 7-HMR metabolites ENL and END (Fig. 4), produced by the intestinal microbiota from the dietary precursor, were measured in mouse serum. The sera of mice treated with 7-HMR have significantly higher levels of both metabolites, whereas TEP generated only significant levels of ENL (Table 3), according to a previous work performed in rats with the same precursor[15]. The small amount of metabolites measured in mice on the control LFD and HFD reflects the consumption of a certain amount of sawdust as no lignans were detectable in the administered diets.

Changes in metabolic pathways in the visceral fat and the liver

Increased adipose accumulation is associated with changes in several metabolic parameters in visceral fat and in the liver. We analysed the deregulation of the expression of sets of genes involved in fat accumulation and metabolism, autophagy, inflammation and antioxidant defences.

The genes that were found to be modulated code for members of the inflammatory response pathway. *TNF-α* and *IL6* were induced by the HFD and inhibited by 7-HMR and TEP. The antioxidant defence enzyme *glutathione-S-transferase 1* was inhibited in the high-fat-treated mice and established at higher levels by TEP. The *transcription factor EB* (*TFEB*) was inhibited in the high-fat group and was re-expressed in 7-HMR and TEP-treated mice. The fat metabolism genes *LPL*, *PLA2G7* and *leptin*

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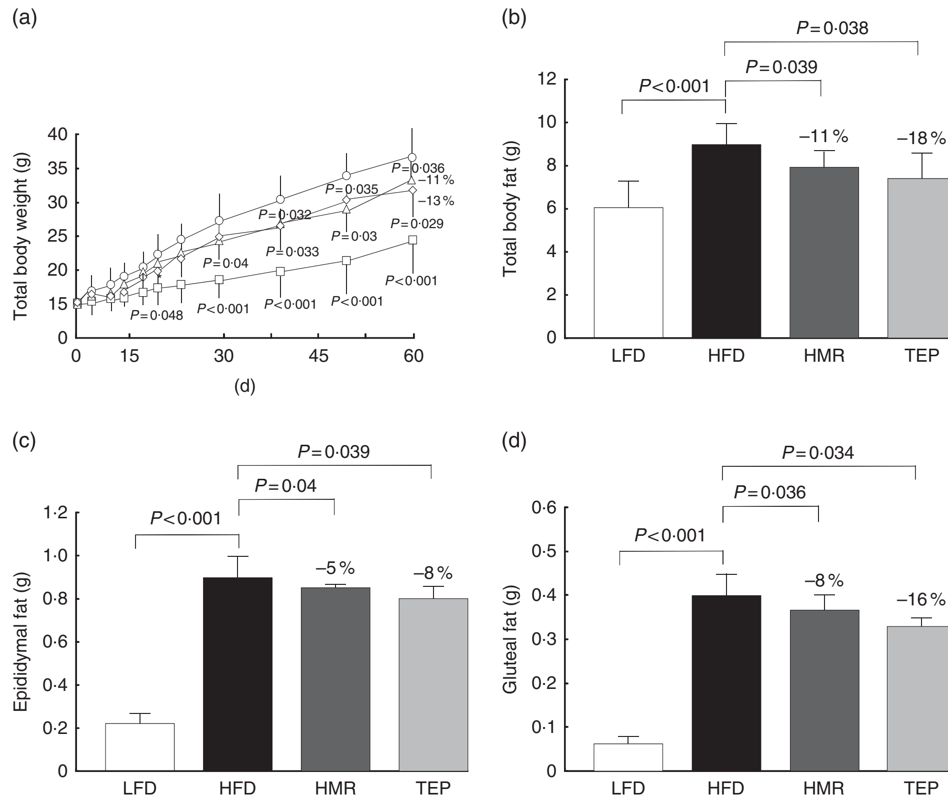

Fig. 1. Effect of 7-hydroxymatairesinol (7-HMR)- and total *Picea abies* extract (TEP)-medicated diets on fat development in male mice. Time-course effects of control – low-fat diet (LFD), control – high-fat diet (HFD), HFD + 7-HMR and HFD + TEP on body weight and total adipose tissue deposition in male C57BL/6J mice. Total adipose mass was analysed using an EchoMRI system. (a) Total body weight, (b) total body fat (g), (c) weight of epididymal fat and (d) weight of gluteal fat. Time of treatments was 60 d. Fat pads were excised immediately after euthanasia. a: □, LFD; ○, HFD; △, HFD + 7-HMR; ◇, HFD + TEP. Percentage values (11 and 13 % less weight) in the graph represent the extent of the change on the HFD value. (b)–(d): □, LFD; ■, HFD; ■, HFD + 7-HMR; ■, HFD + TEP at the end of the 60-d treatments (b–d). Percentage values on the histograms represent the extent of the change on the HFD value. Values are means (*n* 40 per group), with their standard errors represented by vertical bars. *P* = actual value or *P* < 0·001.

were induced by the HFD and repressed by 7-HMR and TEP; the *ATGL* and adiponectin genes were inhibited by the HFD and restored by TEP (Fig. 5). The *PPARγ1* gene, *C/EBPα* and *aP2*, which are key factors in adipocyte differentiation and fat metabolism, were up-regulated in both fat and liver and were significantly reduced in TEP-treated mice.

Activity of 7-hydroxymatairesinol and total Picea abies *extract on 3T3-L1 cell differentiation and regulation of the PPARγ pathway*

To understand whether the increase in fat accumulation observed *in vivo* could be similarly replicated *in vitro* and to verify whether it was dependent on fat cell accumulation, we challenged the 3T3-L1 *in vitro* model of cell differentiation with our compounds. We first characterised the effects of 7-HMR, TEP and the major metabolites ENL and END on 3T3-L1 fat cell accumulation. This was performed in cells maintained in differentiation medium and treated with different doses of 7-HMR, TEP, ENL and END (0·01, 0·1, 1 μM). The Oil-Red-O staining of the treated cells revealed that the lignan metabolites ENL and END inhibited lipid accumulation in differentiating cells. 7-HMR showed less potency and TEP was not active (Fig. 6). To understand whether the modulation of adipogenesis was occurring at a molecular level through key adipogenic factors, we studied the regulation of *PPARγ*, *C/EBPα* and *aP2* from an array of genes induced in differentiating 3T3-L1 cells (Fig 7(a)). *PPARγ* and *aP2* were maximally expressed at day 9 of the differentiation schedule (Fig. 7(b) and (b1)), and thus for the following experiment we used this end point. At day 9 the lignans 7-HMR and ENL inhibited the activity of *PPARγ*, whereas all four lignans efficiently inhibited *C/EBPα* and *aP2* in differentiating cells. The *aP2* protein was slightly inhibited in the 7-HMR- and TEP-treated cells (Fig. 8(c)).

Quantification of TAG accumulation in HEPA 1–6 hepatocytes

In the treated mice, the liver appeared to be responsive to lignan through the inhibition of the diet-induced steatosis, and thus we used a model of liver steatosis *in vitro*, the HEPA 1–6 hepatoma cells, to identify direct mechanisms of action of the lignans. The cells were challenged with the SFA PA for 24 h and the accumulated lipids were measured by the Oil-Red-O assay. Fig. 8 shows that TAG content was significantly higher in PA-treated cells. The addition of ENL and END decreased PA-induced TAG accumulation starting at the concentration of 0·001 μM, whereas 7-HMR and TEP were effective only at supraphysiological doses (10 μM) (not shown).

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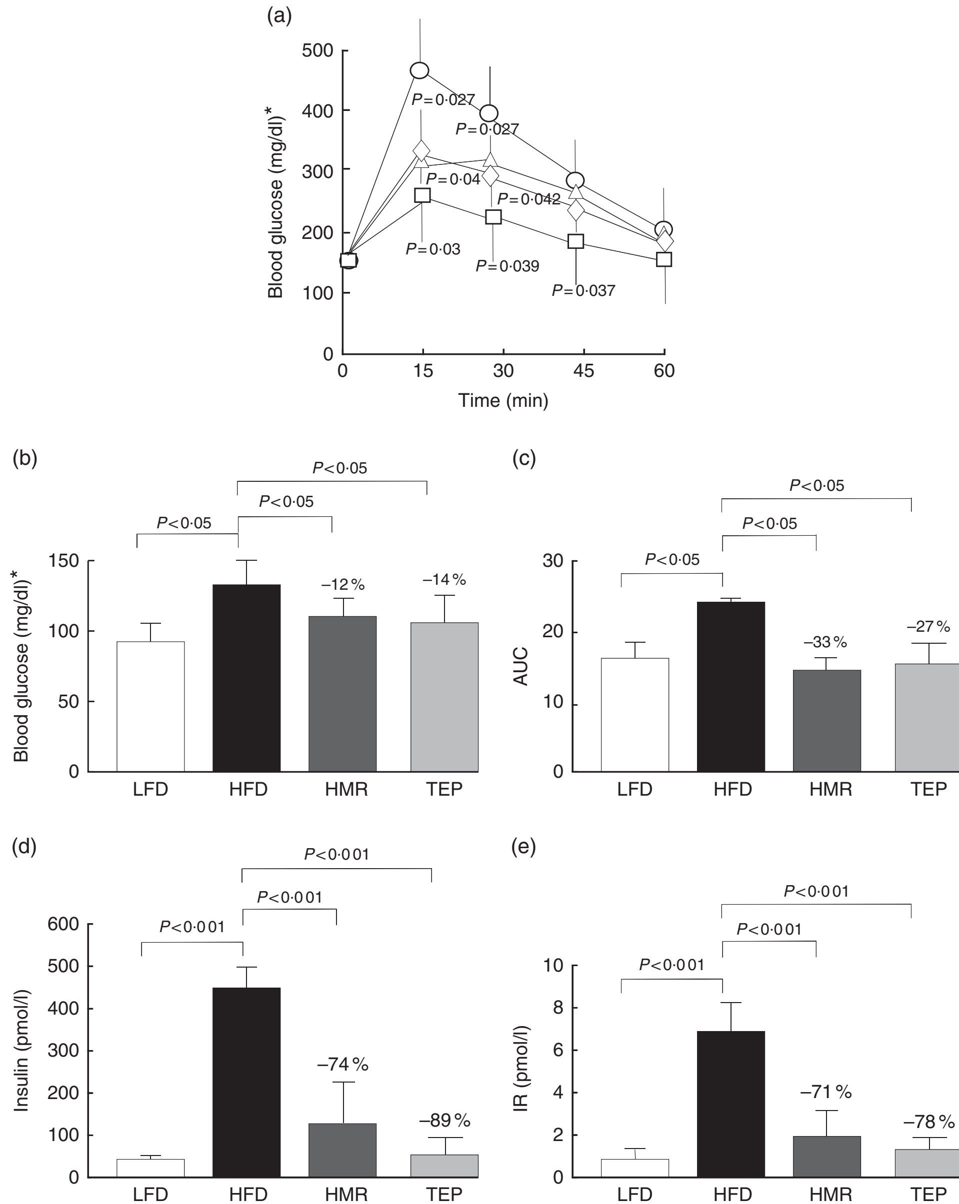

Fig. 2. Glucose tolerance. For glucose tolerance tests animals were fasted overnight for 12 h and blood samples were obtained from the tail vein. (a) Animals were then injected intraperitoneally with 2 g/kg body weight of glucose and blood samples were taken at the indicated intervals. (b) Fasting glucose levels, (c) the AUC (AUC from the glucose tolerance test) and (d) plasma insulin levels were measured with an insulin ELISA kit (Millipore, mouse insulin ninety-six-well plate assay; catalogue no. EZRMI-13K). (e) Determination of homoeostasis model assessment of insulin resistance (IR). Calculations were carried out using glucose and insulin concentrations obtained after 6 h of food withdrawal, using the HOMA Calculator (Diabetes Trials Unit), the Oxford Centre for Diabetes, Endocrinology and Metabolism: homa.calculator@dtu.ox.ac.uk. a: low-fat diet (□, control – low-fat diet (LFD); ○, control – high-fat diet (HFD); ◇, HFD + 7-hydroxymatairesinol (7-HMR); △, HFD + total *Picea abies* extract (TEP); (b)–(e): □, LFD; □, HFD; ■, HFD + 7-HMR; ■, HFD + TEP. Percentage values on the histograms represent the extent of the change on the HFD value. Values are means (*n* 10 per group), with their standard errors represented by vertical bars. *P* = actual value or *P* < 0·001. * To convert glucose in mg/dl to mmol/l, multiply by 0·0555.

Discussion

Lignans are expected to have beneficial effects in humans after fermentative conversion in the colon to bioactive metabolites that may favourably influence parameters related to the metabolic syndrome[27]. Here we studied the effects of 7-HMR and TEP (a mixture of 7-HMR and allo-HMR) from *P. abies* as anti-obesity compounds at doses that can be reached through nutraceutical supplementation or approached with a lignan-rich diet[28]. The study was performed in C57BJ/6 mice, which, among the mouse models of obesity, show the best similarities to human obesity[29–32]. Moreover, similar amounts of the ENL metabolite are observed in our 7-HMR-treated mice and in people on lignan-rich diets, supporting the use of this rodent model for metabolic studies.

The novelty of our results is the demonstration that 7-HMR and TEP exhibit marked effects in limiting body weight, total fat mass deposition, liver steatosis and serum lipids (total cholesterol, LDL-cholesterol and TAG) in mice on a HFD and improve sugar metabolism controlling blood sugar level, insulin concentration

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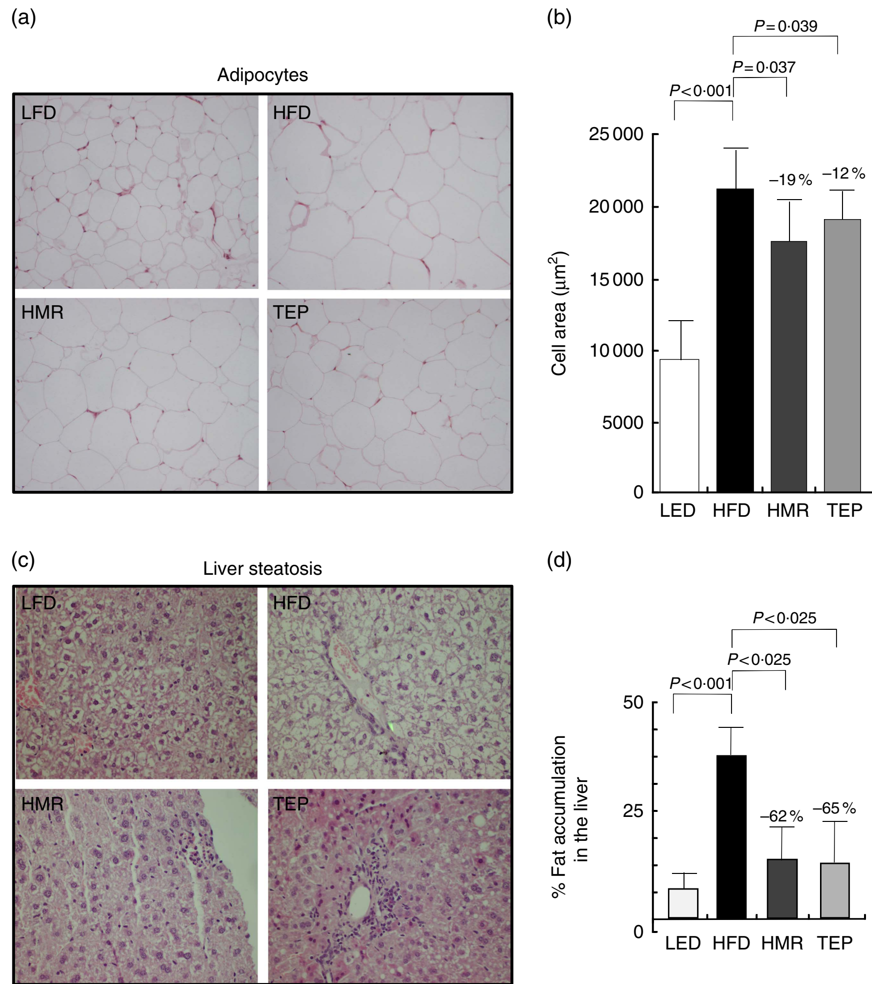

Fig. 3. Histological examination epididymal adipose tissue and liver parenchyma were fixed in formaldehyde and paraffin embedded. Sections (3 µm) were stained with haematoxylin–eosin. Cells were photographed at 20× magnification with a digital camera (Nikon Digital Camera DMX 1200). (a, b) Adipocyte size was measured using dedicated software (Image Pro Plus; Imaging and Computer). At least four different fields of three different tissue sections were evaluated for each sample. (c) Liver steatosis. Representative liver sections stained with haematoxylin–eosin. (d) Liver tissue was processed to quantify the fat content. Percentage fat content was determined relative to fat content in the liver of mice on the high-fat diet (HFD). Data represent the average result of the analysis of tissues from five different mice (*n* 5). Percentage values on the histograms represent the extent of the change on the HFD value. Values are means with their standard errors represented by vertical bars. LFD, low-fat diet; HMR, hydroxymatairesinol; TEP, total *Picea abies* extract. P = actual value, or $P < 0·025$, or $P < 0·001$.

Table 2. Lipid profile in serum*
(Mean values and standard deviation; *n* 10 samples/group)

	Control (LFD)		Control (HFD) (Group a)			HFD plus 7-HMR (Group b)		HFD plus TEP (Group c)		
	Mean	SD	Mean	SD	*P*	Mean	SD	Mean	SD	*P*
Total cholesterol (mg/dl)†	178	28	219	49	0·02	208	53	196	37	0·01
LDL (mg/dl)†	37	11	57	23	0·02	53	20	43	15	0·02
HDL (mg/dl)†	112	21	133	26	0·04	139	35	141	68	
TAG (mg/dl)†	118	17	153	36	0·03	140	51	129	41	0·03

LFD, low-fat diet; HFD, high-fat diet; 7-HMR, 7-hydroxymatairesinol; TEP, total *Picea abies* extract.
* Serum lipids were measured in the different groups of mice. From the left column: control mice on the LFD, mice on the HFD, mice on the HFD plus 7-HMR; mice on the HFD plus TEP. Group a, mice on the HFD *v.* mice on the LFD; group b, 7-HMR-treated mice *v.* mice on the HFD alone; group c, TEP-treated mice *v.* mice on the HFD alone. P = actual value.
† To convert cholesterol in mg/dl to mmol/l, multiply by 0·0259. To convert TAG in mg/dl to mmol/l, multiply by 0·0113.

and insulin resistance, all strongly altered parameters in the obese mice. The activity of these compounds on metabolic pathways in fat tissue was evidenced at the level of the expression of modulated genes such as *PPARγ* and *C/EBPα*, which are master regulators of fat metabolism whose activity is critical for progression to the final stages of adipocyte differentiation[33–35].

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In the mouse liver, the fat-rich diet inhibited regulators of lysosomal biogenesis and autophagy pathways such as the master gene *TFEB* in parallel to an increase in fat accumulation and the induction of severe steatosis, in line with the observations of Settembre *et al.*[36] who showed that autophagy is required for lipid degradation via the *TFEB* pathway. 7-HMR and TEP increased *TFEB* to levels found in the liver of mice on the LFD (10 % fat), suggesting that the *TFEB*-regulated pathway may be one of the mechanisms through which lignan can prevent liver steatosis. Amelioration of liver steatosis was also investigated *in vitro* by using the HEPA 1–6 model. Both the metabolites ENL and END were effective in limiting fat accumulation experimentally induced in these cells, already at the concentration of 1 nM, whereas 7-HMR and TEP showed no effect, indicating that the inhibition of lipid accumulation in the liver is exerted by these dietary compounds in their metabolised form at concentrations as can be found in human plasma of subjects consuming a lignan-rich diet[20,21].

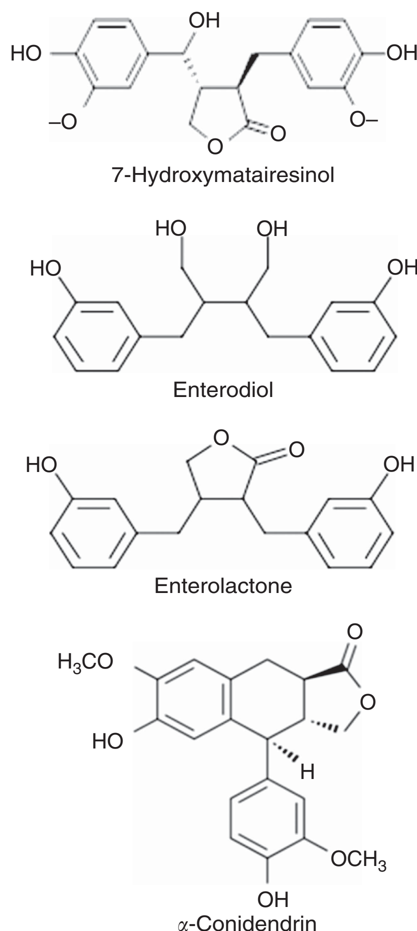

Fig. 4. Structure of 7-hydroxymatairesinol (7-HMR) and mammalian lignan. The molecular structure of 7-HMR and mammalian metabolites.

In the attempt to further delineate mechanisms at fat-cell level, we studied our chemicals in 3T3-L1 cells, a model of adipocyte differentiation. 7-HMR exerted anti-adipogenic effects, although both metabolites ENL and END showed much higher efficiency. At the molecular level, all the lignans effectively inhibited the *C/EBPα* and *aP2* transcription factors, whereas only 7-HMR and ENL modulated *PPARγ* expression, suggesting that inhibition of lipid accumulation may probably involve an indirect control of *PPARγ* through *C/EBP* family members. *C/EBPα* (*C/EBP homologous protein*) is a factor that directly or indirectly can affect *PPARγ* expression, stability and activity and consequently adipogenesis. This can occur through co-activators of the *C/EBP* family, possibly *CHOP*, a dominant negative form of the *C/EBP* members[37] that blocks adipogenesis[38] through inhibition of *C/EBPβ*, which is expressed early in the differentiation programme and is the transcriptional activator of *PPARγ* and *C/EBPα*. These *in vitro* data, however, do not unequivocally illustrate what might occur *in vivo*. Although several studies confirm our data regarding the up-regulation of *PPARγ* during the anabolic course of lipid storage in HFD-fed mice[39], a recent work by Soccio *et al.*[40] with genomic experiments showed a reduced level of *PPARγ* and *PPARγ* DNA/chromatin occupancy in obese mice consistent with earlier reports[41]. In the attempt to interpret these apparently contrasting data, we can consider that *PPARγ* may perform different functions in metabolically sick rodents compared with healthy ones, as recently discussed by Bandera Merchan *et al.*[39]. Similarly, in healthy subjects, a HFD induces *PPARγ*, whereas the same diet decreases *PPARγ* in morbidly obese patients[42]. In our healthy mice fed the HFD, we also registered an increase in *PPARγ* levels and, following the observations of the cited authors, it would be informative to study the levels of *PPARγ* in this model when the disease aggravates towards morbid obesity after longer periods on the HFD.

We just mentioned above that 7-HMR metabolites show higher efficiency in inhibiting cell differentiation in 3T3-L1 cells already at nM doses. However, knowing that metabolism of 7-HMR takes 24–48 h to be 95 % complete, that C_{max} for ENL is reached at 24 h and that ENL appearance continues up

Table 3. Concentration of enterolactone and enterodiol in mouse serum*
(Mean values and standard deviations; *n* 16 samples/group)

	LFD		HFD		HFD plus 7-HMR			HFD plus TEP		
	Mean	SD	Mean	SD	Mean	SD	*P*	Mean	SD	*P*
Enterolactone (ng/ml) (MW: 298·338)	ND		10	2·5	39	7	0·044	28	5	0·048
Enterodiol (ng/ml) (MW: 302·37)	6	2·9	16	2·9	22	4	0·049	19	5	

LFD, low-fat diet; HFD, high-fat diet; 7-HMR, 7-hydroxymatairesinol; TEP, total *Picea abies* extract; MW, molecular weight.
* Lignan metabolites were measured by HPLC–MS/MS in mouse serum. From the left column: control mice on the LFD, mice on the HFD, mice on the HFD plus 7-HMR; mice on the HFD plus TEP. *P* = actual value.

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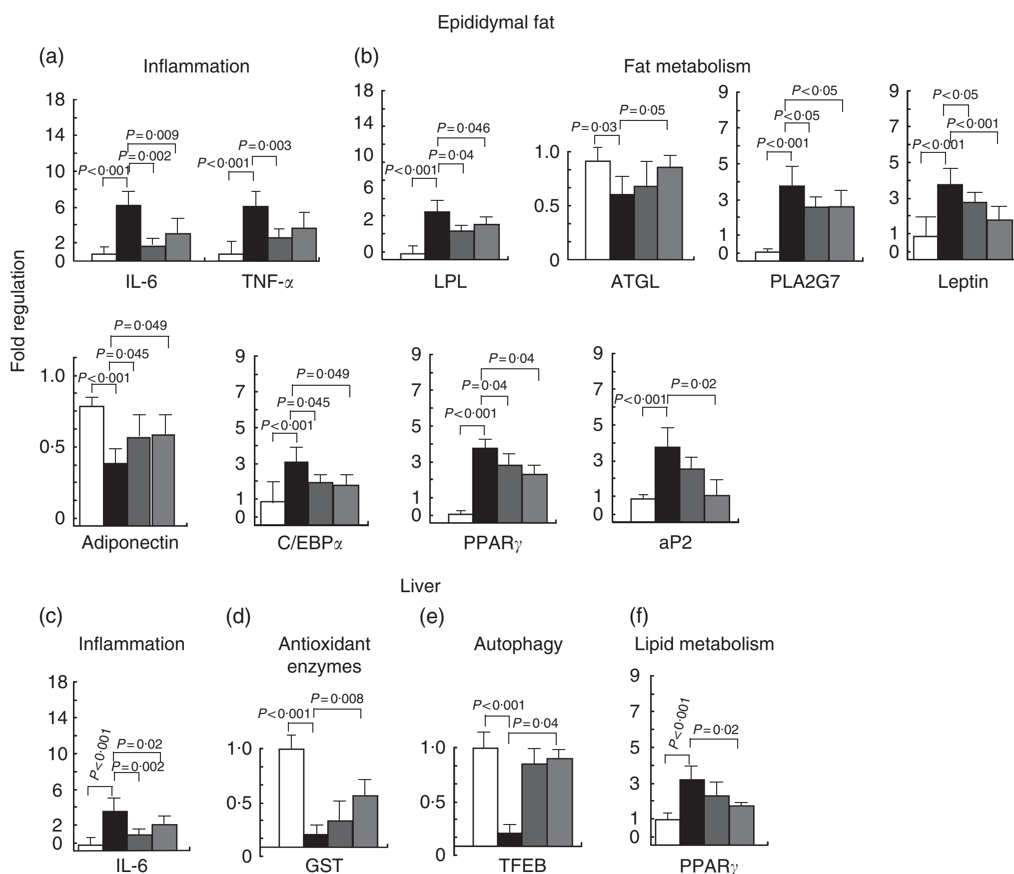

Fig. 5. Gene expression analysis in epididymal fat and the liver. The expression of a set of genes involved in (a), (c) inflammation and (b), (f) fat metabolism (d), antioxidant defences and (e) autophagy were quantified in epididymal fat and liver tissues of the treated mice. Values are mean fold change (*n* 8), with their standard errors represented by vertical bars. The experiments were performed by the use of Taqman LDA microfluidic cards with *HPRT1* RNA used as the normalising gene. □, Control; ■, high-fat diet (HFD); ■, HFD + 7-hydroxymatairesinol (7-HMR); ■, HFD + total *Picea abies* extract (TEP). *P* = actual value or *P* < 0·001.

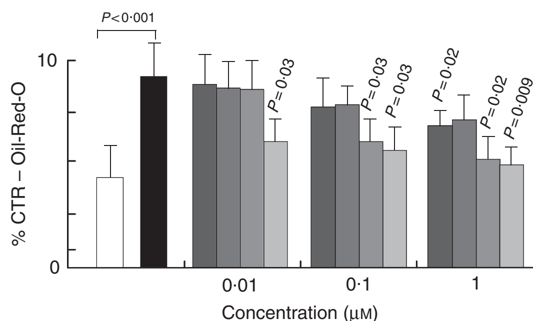

Fig. 6. Regulation of cell differentiation by lignin. 3T3-L1 cells were either maintained in undifferentiated (basal) or differentiated state (3-isobutyl-1-methylxanthine, dexamethasone, insulin; MDI) and stimulated for 9 d with vehicle as control (C). During MDI-induced differentiation the cells were treated with 7-hydroxymatairesinol (7-HMR), total *Picea abies* extract (TEP), enterolactone (ENL) and enterodiol (END) at increasing concentrations (0·01, 0·1, 1 μM). The effect of these compounds on cell differentiation was measured by the Oil-Red-O staining 9 d after transfection. Values were expressed as percentage of control undifferentiated and untreated (vehicle) cells. Differentiated cells *v.* control undifferentiated cells; 7-HMR, TEP, ENL, END *v.* MDI-treated cells. Values are means (*n* 4), with their standard errors represented by vertical bars. □, C; ■, MDI; ■, MDI + 7-hydroxymatairesinol (7-HMR); ■, MDI + TEP; ■, MDI + ENL; □, MDI + END. *P* = actual value, compared with controls (cells in basal medium). *P* = actual value or *P* < 0·001 compared with controls (MDI-treated cells).

to 72 h and more[16], we also included 7-HMR and TEP in our *in vitro* experiments. The results show that unmodified 7-HMR was effective only at 1 μM, indicating that its metabolism is required for full biological activity and that in its unmodified form it only marginally contributes to the observed effects. Consistently with this, in HEPA cells a significant inhibition of TAG accumulation is achieved only by the metabolised lignans. In contrast, at the molecular level all the lignans were effective on the expression of *C/EBPα* and *aP2* in 3T3-L1 cells, probably indicating that other factors involved in the differentiation process are differentially targeted by the specific lignans[43].

This represents the first comprehensive study of 7-HMR on metabolic functions altered by HFD at doses that can be reached through nutraceutical supplementation and dietary intake. The highest amount of 7-HMR can be assumed in a nutraceutical form. HMRLignan™ can provide an intake of about 2–3 mg/kg, depending on the formulation (36–144 mg/d)[16,44]. 7-HMR is also the most abundant lignan in cereals and the dominant lignan in wheat, triticale, oat and millet bran (from 3 up to 7–9 mg/100 g)[10]. It is present at a high concentration in sesame (17 mg/100 g)[45], which is often consumed daily as sesame paste (tahin) in the Middle Eastern countries and it is widespread in several plants (i.e. 1·3 mg/100 g cranberry seeds)[45]. Consumption of a

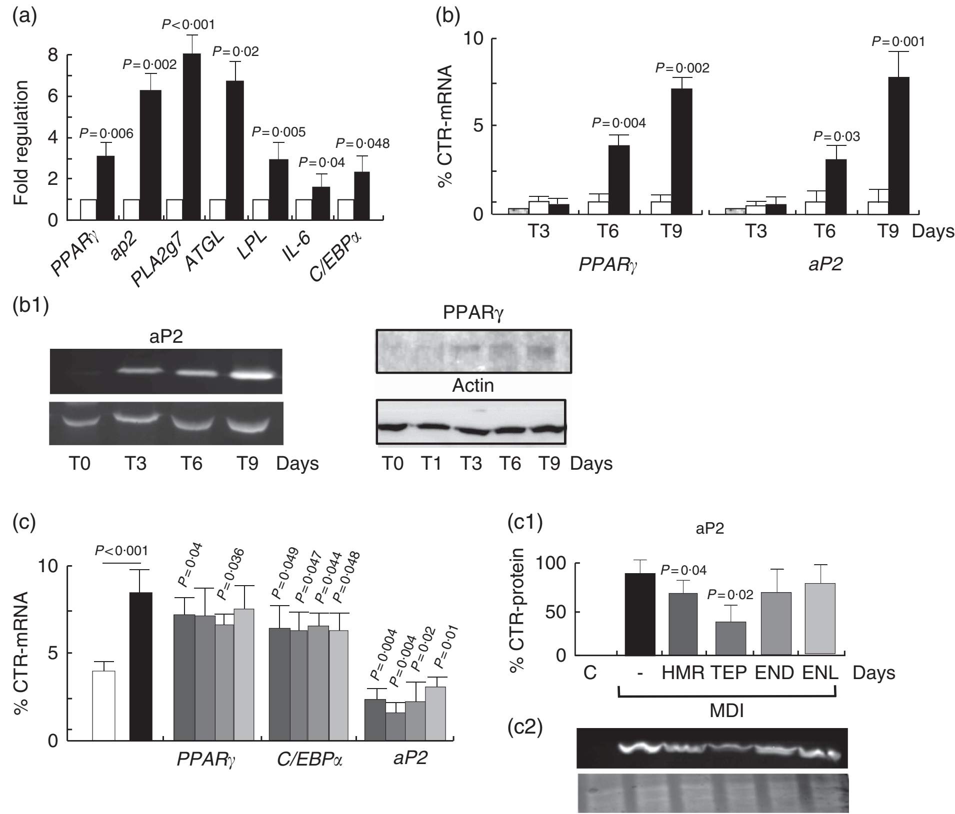

Fig. 7. Regulation of adipogenic factors in 3T3-L1 cells. (a) Regulation of genes of fat metabolism and inflammation in 3T3-L1 cells. Values are means (*n* 4), with their standard errors represented by vertical bars. □, Control; ■, MDI (3-isobutyl-1-methylxanthine, dexamethasone, insulin). *P* = actual value, compared with controls (cells in basal medium). (b) Time course of *PPARγ* and *aP2* mRNA expression and protein synthesis in differentiating 3T3-L1 cells. 3T3-L1 cells were treated with MDI for 3, 6 and 9 d and then mRNA and proteins were harvested for the measurement of *PPARγ* and *aP2* mRNA and protein (b1) as molecular factors of cell differentiation. Data are shown as mean values of at least three experiments. Values are means (*n* 3), with standard errors represented by vertical bars. *P* = actual value, compared with controls (cells in basal medium). (c) Differentiating cells were treated for 9 d with vehicle as control (C) or 7-hydroxymatairesinol, total *Picea abies* extract (TEP), enterolactone and enterodiol at the active concentration of 1 μM in the differentiation (MDI) medium. mRNA expression levels of *PPARγ*, *C/EBPα* and *aP2* mRNA and protein were evaluated by real-time RT-PCR. mRNA expression of control cells was arbitrarily set at 1. (c1) Densitometric scanning of aP2 protein level measured by Western blotting (c2). Values are means (*n* 5), with their standard errors represented by vertical bars. □, C; ■, MDI; ▪, MDI + 7-hydroxymatairesinol (7-HMR); ▪, MDI + TEP; ▪, MDI + ENL; ▪, MDI + END. *P* = actual value, compared with controls (cells in basal medium). *P* = actual value, compared with controls (MDI-treated cells).

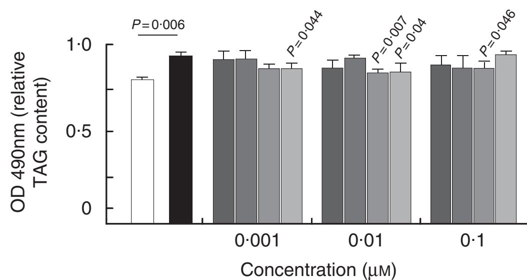

Fig. 8. Quantification of TAG in HEPA 1–6 hepatocytes. Cells were stimulated with palmitic acid alone (PA) or plus 7-hydroxymatairesinol (7-HMR), total *Picea abies* extract (TEP), enterolactone (ENL) and enterodiol (END) in the concentration range of 0·001, 0·01 and 0·1 μM for 24 h and the accumulated TAG were measured by the Oil-Red-O assay. Values are means of six experiments (*n* 12 per dose), with their standard errors represented by vertical bars. □, Control; ■, PA; ▪, PA + 7-HMR; ▪, PA + TEP; ▪, PA + END; ▪, PA + ENL. PA-treated cells *v.* control untreated cells, *P* = actual value.

diet rich in these foods may bring 7-HMR daily intake to as much as 30 mg/d, very close to nutraceutical doses. Moreover, the ENL levels (39 ng/ml) reached in plasma of our mice treated with 3 mg/kg of 7-HMR are quite close to the concentration of ENL that can be found in people consuming conventional northern European diets (14·8 and 12 ng/ml)[20,46] and lignan-fortified diets (21 ng/ml)[47], but we can realistically hypothesise that it can be found at a higher concentration in people consuming lignan-richer diets with the food components cited above (i.e. Middle Eastern or Mediterranean diets)[48].

In conclusion, this work adds novel information to what has been found for other lignans in similar models[4,13,49,50] and highlights the physiological relevance of 7-HMR from nutraceutical and nutritional sources.

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G. B. and I. Z. performed animal treatments, autopsy, tissue collection, detailed experimental design, Echo MRI analysis and *in vitro* assays (3T3-L1, HEPA6 cells). F. P. performed tissue extraction and qRNA analysis, gene expression analysis and *in vitro* assays (3T3-L1). M. C. performed histology. I. A. carried out blood collection from the mice, analysis of biochemical parameters, monitoring of animal health and animal care and quantification of food and water consumption. M. L. and B. P. contributed to preparation of chemicals to be administered, title and analytical control of chemical purity and performed quality control. E. M. carried out adipocytic differentiation *in vitro* and gene expression profiles from cell cultures (3T3-L1). P. M. and R. A. carried out cell culture maintenance; animal maintenance, care, feeding and control; and DNA extraction and amplification. (Moreover, they performed HPLC and MS analysis on several markers that, although not present in this article, greatly contribute to understand the efficiency of the studied chemicals.) G. D. P. performed data analysis, linearity, accuracy and precision, and contributed in writing the paper for his part of competence. F. S. carried out statistical analysis of the *in vivo* experiments and contributed to writing the paper for his part of competence. A. S. performed determination of 7-HMR and metabolites in mouse serum. D. D. L. contributed to study conception and design, data analysis and in writing the paper.

Two scientists from the company that manufactures and markets the studied chemicals contributed to this work (M. L. and B. P., Linnea SA).

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