

ORIGINAL ARTICLE

Blood orange juice inhibits fat accumulation in mice

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Objective: To analyze the effect of the juice obtained from two varieties of sweet orange (*Citrus sinensis* L. Osbeck), *Moro* (a blood orange) and *Navelina* (a blond orange), on fat accumulation in mice fed a standard or a high-fat diet (HFD).

Methods: Obesity was induced in male C57/Bl6 mice by feeding a HFD. *Moro* and *Navelina* juices were provided instead of water. The effect of an anthocyanin-enriched extract from *Moro* oranges or purified cyanidin-3-glucoside (C3G) was also analyzed. Body weight and food intake were measured regularly over a 12-week period. The adipose pads were weighted and analyzed histologically; total RNA was also isolated for microarray analysis.

Results: Dietary supplementation of *Moro* juice, but not *Navelina* juice significantly reduced body weight gain and fat accumulation regardless of the increased energy intake because of sugar content. Furthermore, mice drinking *Moro* juice were resistant to HFD-induced obesity with no alterations in food intake. Only the anthocyanin extract, but not the purified C3G, slightly affected fat accumulation. High-throughput gene expression analysis of fat tissues confirmed that *Moro* juice could entirely rescue the high fat-induced transcriptional reprogramming.

Conclusion: *Moro* juice anti-obesity effect on fat accumulation cannot be explained only by its anthocyanin content. Our findings suggest that multiple components present in the *Moro* orange juice might act synergistically to inhibit fat accumulation. *International Journal of Obesity* (2010) 34, 578–588; doi:10.1038/ijo.2009.266; published online 22 December 2009

Keywords: blood orange; orange juice; fat accumulation; anti-obesity; anthocyanins; cyanidin-3-glucoside.

Introduction

Accumulation of lipid storage in adipose tissue, that is, obesity, is promoted by a high energy and/or a high-fat diet (HFD) together with lack of exercise. Health organizations strongly recommend limiting the consumption of fat- and sugar-enriched foods while promoting the benefit of energy-diluted foods, in particular fruits and vegetables,¹ to prevent obesity. However, the habit of drinking fruit juices to provide water and nutrients in the diet results in an increase in energy intake because of the sugar content of fruit derivatives.^{2,3} Thereby, regardless of the healthy benefits of fruit juices⁴ because of their content of vitamins, carotenoids and polyphenols, their consumption might increase the risk of obesity.⁵

Consumption of orange juice has been boosted in recent years, especially in western countries.⁶ Now, it accounts for almost 50% of the worldwide fruit juice consumption,⁷ equivalent to 15 billion liters per year.

Therefore, it is very important to clearly identify the effect of orange juice on fat accumulation.

The sweet orange (*Citrus sinensis* L. Osbeck) fruit contains a variety of phytochemicals that contribute to the overall flavor and properties of the fruit. These substances, delivered by the juice, include sugars such as sucrose, fructose and glucose; organic acids (primarily citric, malic and isocitric acids); carotenoids such as xanthophylls, and carotenes; vitamins such as vitamin C, A, B₁, B₆ and B₃; flavor compounds, including various esters, alcohols, ketones, lactones and volatile hydrocarbons, and polyphenols such as flavonoids and hydroxycinnamic acids.^{8,9} It is noteworthy that the content of these substances differs significantly among cultivars and it is also affected by fruit maturation and environmental factors such as climate, soil and agricultural procedures.^{10,11}

Blood orange cultivars are the mainstay of Italian orange production. Indeed in Italy 70% of sweet orange production is represented by three blood varieties (*Tarocco*, *Moro* and

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Sanguinello).¹² These varieties differ from the common sweet orange group (*Valencia Late*, *Washington navel* and *Navelina*), for the presence in the flesh and sometimes in the rind, of red pigments belonging to the anthocyanin class.¹⁰ Another peculiar characteristic of blood oranges is the high concentration of vitamin C,¹³ flavanones and hydroxycinnamic acids.^{14,15} The red color of the fruit is an important factor affecting consumer appeal and marketability of both fruit and juices.

Anthocyanins have been reported to have a biological role in mammals. At molecular level, they behave directly as antioxidants thereby providing protection from DNA, protein and lipid damage.¹⁶ It has also been suggested that anthocyanins may indirectly reduce oxidative stress by activating specific detoxification enzymes such as glutathione reductase, glutathione peroxidase, glutathione S-transferase and quinone oxidoreductase.¹⁷

At cellular level, anthocyanins produce different effects: they inhibit tumorigenesis by blocking activation of a mitogen-activated protein kinase pathway,¹⁸ reduce estrogenic activity, induce cytokine production and decrease capillary permeability,¹⁹ whereas when administered to adipocytes, anthocyanins exert a protective role against H₂O₂ or tumor necrosis factor- α -induced insulin resistance.²⁰

Interestingly, two of these processes, oxidative stress and regulation of insulin signalling, are both involved in adipogenesis. In fact, oxidative stress induced by reactive oxygen species (ROS) stimulates fat tissue development both in adipocyte culture²¹ systems and *in vivo*,²¹ whereas ROS scavenging is associated with fat reduction in obese Zucker rats.²² In contrast, induction of insulin receptor signalling, that is, activation of PI3K and Akt followed by re-localization of FOXO1 from the nucleus, has a major role in the regulation of gene transcription involved in adipogenesis.²¹

Importantly, several *in vivo* studies in rodents showed that cyanidin-3-glucoside (C3G), the most abundant anthocyanin present in blood oranges, also reduces body weight, fat accumulation and high fat-induced diabetes.^{23–26}

However, the overall effect of orange juice consumption on weight gain is still controversial mainly owing to its sugar content.

In this view, the considered choice of a particular orange cultivar might eventually lead to the healthiest effect. Therefore, to identify orange cultivars capable to minimize fat accumulation, we studied the effect of the administration of *Moro* and *Navelina* orange juices on fat development in mice fed a standard diet (SD) or a HFD.

Materials and methods

Mice

In all, 2- to 3-month old *wild-type* mice (129Sv/Ev or C57/Bl6 strains, from Charles River-Italia s.r.l.) were bred in the animal facility of the Department of Experimental Oncology of the European Institute of Oncology, (Ministry of Health authorization: DM No. 86/2005–17/06/2005). Mice were

housed in an air-conditioned room (temperature $21 \pm 1^\circ\text{C}$, relative humidity $60 \pm 10\%$) with a white–red light cycle (lights on from 0830 to 2030 h). Group housing (four animals per cage) was chosen to improve animal's welfare. Home cages were Plexiglas boxes ($42 \times 27 \times 14$ cm) with sawdust as bedding. HFD, SD, orange juices, the *Moro* orange, anthocyanin-enriched, extract (MEX) and the C3G water solutions, as well as tap water, were available *ad libitum* to the mice and bottles replaced every 2 days. Body weight of mice and their liquid and solid food consumption were controlled every 2 days. Duration of all treatments was 12 weeks.

All the experiments with mice were performed in accordance with the Italian Laws (D.L.vo 116/92 and following additions), which enforces EU86/609 directives (Council Directive 86/609/EEC of 24 November 1986 on the approximation of law, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes).

Diets

Mice were fed either the 'SD' (2018S Teklad Global 18% Protein Rodent Diet, provided by Harlan Teklad, Madison, WI, USA) a fixed formula, non-autoclavable diet, or the 'HFD' (The 'Original' HFDs for Diet-Induced Obesity D12492 provided by Research Diets, Inc., New Brunswick, NJ, USA). The energy provided by the macronutrients of the SD was 23% from proteins, 17% from fat and 60% from carbohydrates, for a total of 3.3 kcal g^{-1} (for detailed composition see Supplementary Information). The kilocalories provided by the macronutrients of the HFD were 20% from proteins, 60% from fat and 20% from carbohydrates, for a total of 5.24 kcal g^{-1} .

Fruit juice and extract characterization

Moro and *Navelina* fruits were collected at maturity at Palazzelli (Siracusa) in the experimental farm of the Research Center for Citric culture and Mediterranean Crops (Acireale, Italy). Fruits were immediately stored at 4°C and few days later hand squeezed; the juice obtained was pre-filtered and stored at -20°C in aliquots of 0.5 l.

Every 2 days frozen juice aliquots were thawed, filtered and put in the bottle of each cage.

The anthocyanin-enriched extract from *Moro* oranges was prepared passing centrifuged juice through a styrene-divinylbenzene resin (XAD16, Rohm and Haas, Philadelphia, PA, USA). The resin was washed with H₂O and the absorbed anthocyanins and other polyphenols were eluted with 50:50 ethanol–H₂O solution, containing 1.0% of citric acid. The red-ethanol solution was concentrated by Rotavapor (Büchi, Switzerland) at 35°C in vacuum. The aqueous solution was then diluted and supplied to the mice.

A 0.5 mg ml^{-1} solution of C3G (Extrasynthese, Genay, France) was prepared in tap water and acidified with HCl to pH 3.5. Average daily intake of C3G per mouse was 2.25 mg

(~90 mg kg⁻¹). Every 2 days fresh aliquots were supplied to the mice.

Vitamin C was determined by the 2,6-dichlorophenolindophenol-titrimetric method, modified by Rapisarda and Intelisano.¹³ Total anthocyanins were determined spectrophotometrically by the pH differential method.²⁷ The analysis was carried out by high-performance liquid chromatography using a water 600-E liquid chromatograph equipped with a Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA). The column was a Hypersil ODS (250 × 4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA) and the solvent system was A, water/formic acid (85:15) and B, water/formic acid/acetonitrile (35:15:50). The percentage of B was increased linearly from 10 to 30% in 30 min at a flow rate of 1 ml min⁻¹. Elution was monitored at 520 nm, and the column temperature was maintained at 35 °C.

Flavanone glycosides were determined by high-performance liquid chromatography,²⁸ using the high-performance liquid chromatography-photodiode array detector equipment described above. A sample of centrifuged juice was diluted 1:5 with the mobile phase, 0.45 μm filtered, and injected directly into the column. The eluent was water/acetonitrile/acetic acid (79.5:20:0.5), and the flow rate was 0.8 ml min⁻¹. The column effluent was monitored at 280 nm.

Hydroxycinnamic acids were extracted from the juice by solid phase extraction after the alkaline hydrolysis of hydroxycinnamic esters.¹⁵ Ten milliliters of centrifuged juice was added to 10 ml 2 N NaOH and stored at room temperature in the dark. Complete hydrolysis of bound hydroxycinnamic acids occurred in 4 h. The solution was then acidified with 2 N HCl to pH 2.5 and passed through a C18 Sep-Pak cartridge (Waters). Hydroxycinnamic acids were eluted with 0.1% HCl in methanol. The alcoholic solution was 0.45 μm filtered, and a 20 μl aliquot was analyzed by high-performance liquid chromatography. The mobile phase consisted of water/acetic acid (98:2) (solvent A) and methanol (solvent B). The elution program was 95% A and 5% B for 5 min, ramped down to 70% of A and 30% of B (35 min), and held until the end of the run (45 min) with a flow rate of 1 ml min⁻¹. The detection was performed at 300 nm.

In vivo analysis

Analysis on feces: animal feces were collected in metabolic cages, dried at 55 °C overnight and stored at -20 °C until assayed. The gross energy content of the fecal pellets was determined using a calorimetric bomb (C5000; IKA, Werke, Staufen, Germany) as described earlier.²⁹

Histological analysis: after killing the mice by CO₂ asphyxia, total body fat mass was evaluated: inguinal, interscapular and abdominal fat tissues were weighed and fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Analysis on blood samples: triglycerides, total cholesterol and low-density lipoproteins were measured using Reflotron Plus system from Roche Diagnostic (Milan, Italy).

Glucose tolerance tests were performed on animals fasted for 15 h. Glycemia was measured immediately before and 20, 40, 60 and 120 min after intraperitoneal injection of D-glucose (2 g kg⁻¹). Insulin sensitivity tests were performed on 5 h starved mice, and glycemia measured immediately before and 15, 30 and 60 min after intraperitoneal injection of human recombinant insulin, 0.4 U kg⁻¹.

In vitro analysis

Primary brown pre-adipocytes were isolated from Brown Adipose Tissue interscapular fat pads from 2-day old 129Sv/Ev newborns, as described earlier²¹ (see Supplementary Information).

For the experiments on insulin-induced activation (serine phosphorylation) of Akt kinase, primary adipocytes were serum starved for 15 h and then treated with 0.1 μg ml⁻¹ of insulin, 100 μM C3G or 20% v/v MEX for 90, 30 and 10 min or 1-min pulse. Antibodies against total Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the antibody against phosphorylated Akt was from Cell Signalling Technology (Danvers, MA, USA).

Western blotting was conducted according to standard procedures (see Supplementary Information). To determine intracellular ROS levels, cells were incubated with 20 μM 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) for 45 min in the dark and DCF fluorescence was quantified by fluorescence-activated cell sorting analysis.²¹

Gene expression profiling

Abdominal white adipose tissue (20–30 mg) was collected from mice supplied with *Moro* orange Juice, *Navelina* orange juice and water, and fed with a SD or HFD; it was immediately frozen and stored in liquid nitrogen. Total RNA was extracted using RNeasy (Qiagen, Hilden, Germany) following manufacturer's instructions.

For each condition, biotinylated complementary RNA targets were synthesized starting from 3 μg of total RNA, pooled from three mice, and hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 (Affymetrix, Santa Clara, CA, USA), according to Affymetrix protocols (see Supplementary Information). Arrays examine the expression of approximately 39 000 murine transcripts. Absolute and comparative analyses were performed using Affymetrix Microarray Partek Genomics Suite version 6.4 software, using RMA summarization for all arrays. An analysis was carried out on functional information concerning the lists of retrieved genes with Gene Ontology available sources (David Bioinformatics resources 2008).^{30,31} Microarray results for selected genes were further validated by assessing expression levels using quantitative reverse transcription PCR.

Data analysis

Data are represented as mean ± s.e.m. Statistical analyses were performed using the STATISTICA software (Statsoft Inc.,

Tulsa, OK, USA). Data were analyzed using two-way (treatment group \times time) analysis of variance; the Fisher LSD (Least Significant Difference) test was used as *post hoc* comparison. Probability values lower than 0.05 were accepted as significant. Histological analysis data were analyzed using *t*-test, probability values lower than 0.05 were accepted as significant.

Results

Animals and diets

Before starting the *in vivo* experiments we verified whether orange juice was well tolerated by the different mouse strains used in the experiments (129Sv, C57/Bl6 and Balb-c) when fed SD. Animals from all our strains appear to perfectly tolerate the replacement of drinking water with orange juice (100% orange juice, as the only drinking source) and did not show any gross behavior alteration (activity, social interactions and self-care). To assess the effect of orange juice on food and liquid intake, C57/Bl6 and 129Sv mice were provided food and either water or one of the varieties of orange juice *ad libitum*. Under these conditions the average consumption of the different orange juices was 4.05 ± 1.25 ml day⁻¹ per mouse, comparable in both genders and strains and, notably, comparable to the daily water

intake that was 4.00 ± 0.50 ml day⁻¹ per mouse (Figure 1a). As orange juice has higher energy content compared with water, the consumption of approximately 4 ml day⁻¹ of *Navelina* or *Moro* juice corresponds to an excess of energy intake of around 1.6 kcal day⁻¹. In spite of this, none of the orange juices provided altered food consumption, as indicated by the daily food intake that was approximately 3.00 ± 0.65 g day⁻¹ per mouse in all the conditions examined (Figure 1b). As replacement of water with orange juice (100% orange juice, as the only drinking source) *per se* was neither affecting animal behavior nor drinking and eating habits, we replaced water with 100% orange juice in our experimental setting.

We used C57/Bl6 young adult male mice (2- to 3-month old to avoid both developmental and aging effects) as they are more sensitive to HFD than their female counterparts or the 129Sv strain. Food and beverage consumption was measured twice a week for the entire duration of the experiments (12 weeks). Animals fed the HFD or the SD and offered freshly squeezed orange juice did not alter either liquid consumption (as compared with drinking water) (Figure 1c) or food intake (~ 3 g day⁻¹ per mouse, equivalent to a energy intake of approximately 16 kcal day⁻¹ per mouse) (Figure 1d). Thus, the energy intake provided by orange juice was 1.6 kcal day⁻¹ per mouse corresponding to a 10% energy increase compared with water. We then examined whether

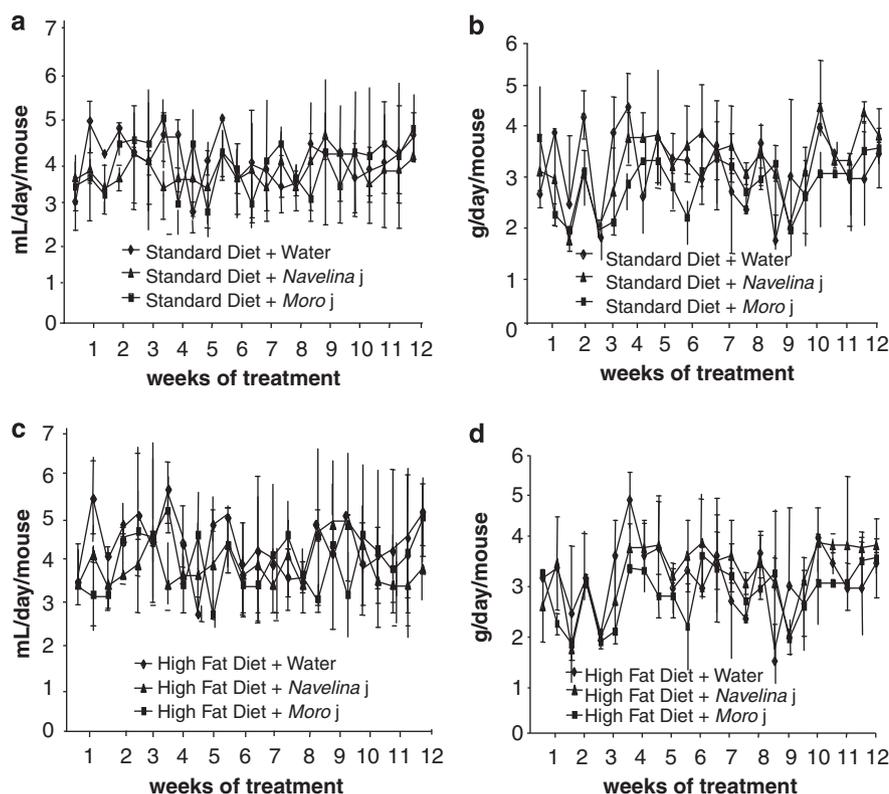


Figure 1 Drink and food consumption of mice is not affected by orange juice supply. Liquids intake (a) and food intake (b) of C57Bl/6 mice fed the standard diet (SD) for 12 weeks. Liquids intake (c) and food intake (d) of C57Bl/6 mice fed the high-fat diet (HFD) for 12 weeks. Values are mean \pm s.e.m., $n = 10$.

Table 1 Characteristics of orange juices

Measurements	Moro juice	Naveline juice
pH	3.19	3.50
Ascorbic acid (mg l ⁻¹)	433.50	578.30
Total anthocyanins (mg l ⁻¹)	80.71	0.00
Total flavanones (mg l ⁻¹)	201.17	312.29
Total OH-cinnamic acid (mg l ⁻¹)	81.01	85.25

orange juice administration altered nutrient absorption measuring the gross energy content of feces by a calorimetric bomb. Neither orange juice altered fecal energy content in mice fed either SD (16.1 ± 0.13 kcal g⁻¹, 15.65 ± 0.20 kcal g⁻¹, 16.11 ± 0.02 kcal g⁻¹ drinking *Moro*, *Navelina* or water, respectively) or HFD (15.84 ± 0.10 kcal g⁻¹, 16.42 ± 0.03 kcal g⁻¹, 15.84 ± 0.24 kcal g⁻¹ drinking *Moro*, *Navelina* or water, respectively). In the same experimental set-up plasma levels of triglycerides, free fatty acids, total cholesterol, low-density lipoproteins together with basal glycemia, glucose tolerance and insulin sensitivity were determined. Again, drinking orange juice, either *Navelina* or *Moro*, did not change lipid profiles or glucose metabolism (data not shown).

Juice characterization

We processed and tested oranges produced during 3 consecutive years. The composition of the *Moro* and *Navelina* juices obtained from the 2008 production is reported in Table 1. As expected, anthocyanins were detected (approximately 80 mg l⁻¹) only in *Moro* orange juice.

Effect on fat accumulation

Then, we determined whether orange juice administration could affect body weight gain in mice fed SD and HFD. Regardless of any increased energy intake, SD mice drinking either type of orange juice did not become overweight (Figure 2a). Indeed, SD mice drinking *Moro* juice gained less weight ($P < 0.005$) than mice drinking water or *Navelina* juice. In particular, analysis of variance confirmed that weight gain was affected by treatment ($P < 0.005$), time ($P < 0.00001$) and interaction 'treatment \times time' ($P < 0.001$). Different batches of juice prepared from oranges harvested in three different years gave similar results (data not shown).

Furthermore, *Moro* juice supplementation reduced significantly, almost abolishing it, the weight gain induced by HFD ($P < 0.00001$, analysis of variance) (see Figure 2b). This result was confirmed in all the experiments, independent of the year or the *Moro* juice batch used.

To assess whether orange juice intake could alter lipid metabolism, inguinal and abdominal fat deposits were isolated and weighed. *Moro* juice treatment significantly reduced the abdominal and inguinal fat mass by approximately 50% ($P < 0.05$) (Figure 2c).

The HFD induces hypertrophy of mouse adipocytes after few weeks. Indeed, histological examination of the adipose tissue from mice treated with *Moro* juice showed a marked reduction in adipocyte cell size with decreased lipid accumulation (Figure 2d).

Gene expression profile

A more extensive examination of the molecular effects of both juices on fat was carried out through the analysis of gene expression profiles in fat tissues from mice drinking water, *Navelina* or *Moro* juice while fed SD or HFD.

Cross comparison results identified 697 genes of which 569 up- (fold change > 3) and 128 down- (fold change < -3) regulated by HFD, and which represented 4% of the total regulated genes in each sample. Therefore, a large cohort of genes, encompassing most cellular biological functions (Gene Ontology chart, Figure 3a), appeared to be regulated by HFD. We next analyzed the effect of both juices on the two diets and noticed that drinking *Moro* but not *Navelina* juice counteracted the effect of the HFD on adipose tissue gene expression. Indeed, the gene expression profile of fat tissue from mice eating HFD and drinking *Moro* juice strikingly resembled that of mice fed SD. The expression drifts of 21% of the 569 genes upregulated by the HFD and of 55% of the 128 genes downregulated were abrogated by *Moro* juice supplementation, as shown in the dendrogram of Figure 3b.

Adipocyte treatment with cyanidin-3-glucoside and Moro orange extract

To determine the intracellular levels of ROS, after starvation primary adipocytes were treated for 8 days in the absence of insulin, then incubated with H2-DCFDA and analyzed by fluorescence-activated cell sorting. Although insulin addition ($0.1 \mu\text{g ml}^{-1}$) increased intracellular levels of ROS, the further addition of 20% v/v solution of MEX or $100 \mu\text{M}$ C3G decreased the insulin-induced ROS production (Figure 4a).

As explained in the introduction, induction of insulin signalling has a major role in the regulation of gene transcription involved in adipogenesis.²¹

To analyze the effect of anthocyanins on this process, primary adipocytes were serum starved for 15 h and then treated for 90, 30 and 10 min or 1-min pulse with control medium, $100 \mu\text{M}$ C3G or 20% v/v MEX, in the absence or presence of $0.1 \mu\text{g ml}^{-1}$ insulin. Proteins were then extracted from cells to evaluate the phosphorylation level of Akt, a downstream kinase of the insulin receptor signalling cascade. As shown in Figure 4b, phosphorylation of Akt was decreased in the primary adipocytes treated with either the *Moro* extract or C3G.

In vivo supplementation of cyanidin-3-glucoside and Moro orange extract

We thus analyzed *in vivo* the role of *Moro* juice anthocyanins in the reduction of fat deposits supplying mice fed HFD with

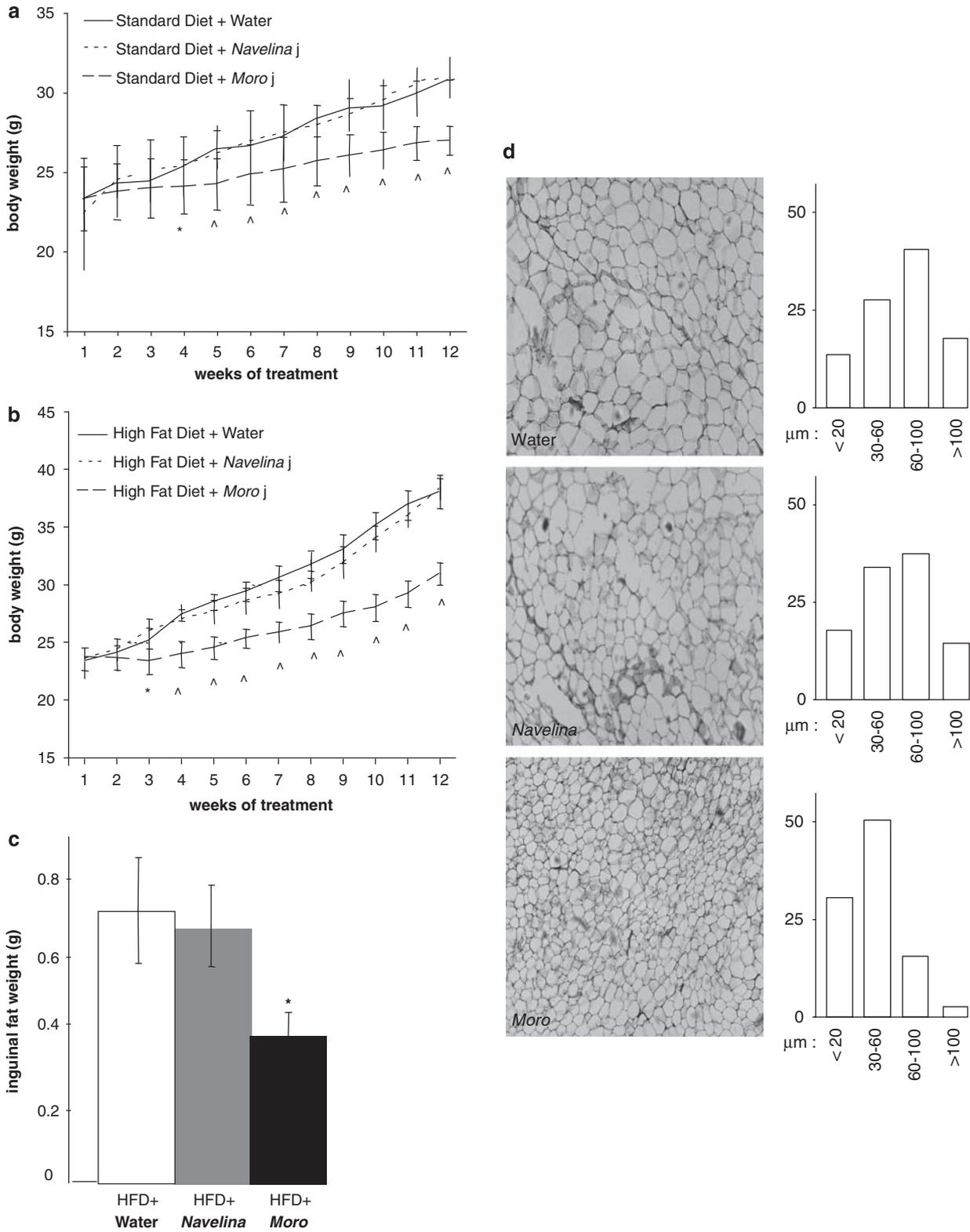


Figure 2 *Moro* orange juice consumption reduces fat development. Effects of *Moro* and *Navelina* orange juices, during the 12-weeks period, on body weight of mice fed standard diet (SD), $^{\wedge}P < 0.005$ (a) high-fat diet (HFD), $^{\wedge}P < 0.00001$ (b), inguinal and abdominal fat weight, $*P < 0.05$ (c) and adipocyte size (% distribution) (d) in HFD fed C57BL/6 mice. Data are means \pm s.e.m. $n = 10$.

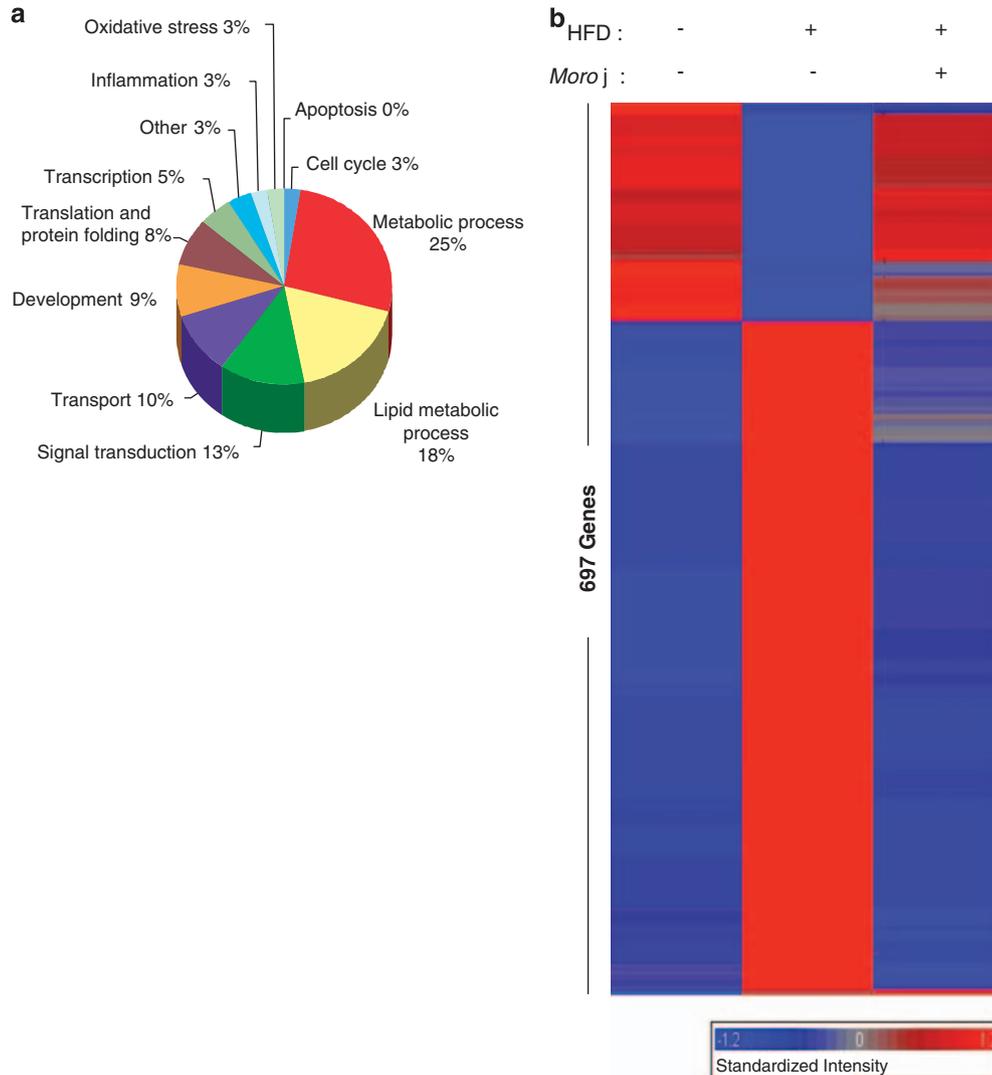


Figure 3 High fat-induced gene expression is rescued by *Moro* orange juice. (a) Gene Ontology chart on the highly regulated genes targeted by the high-fat diet (HFD versus the standard diet (SD) in adipose tissue obtained from C57/Bl6 male mice. (b) Cluster of regulated genes in adipose tissues of C57/Bl6 mice fed SD and water, HFD and water, and HFD and *Moro* orange juice. Each column represents the mean ratio of an experimental condition in three subjects. The ratio of the abundance of the transcripts of each gene to the mean abundance across the experimental conditions is represented by the color of the corresponding cell in the matrix file. Blue, red and gray lines represent transcripts below, above or equal to the median, respectively.

water-diluted MEX or a solution of purified C3G (Figure 5a). Both treatments were adjusted to provide 90 mg kg^{-1} per mouse per day of anthocyanins, a threefold excess amount compared with what provided by the *Moro* orange juice. Analysis of variance on body weight indicated a consistent effect of the duration of the treatment (time; $P < 0.00001$) and of the interaction (treatment group \times time, $P < 0.0001$), with a statistically significant reducing effect of MEX on body weight gain from week 6 ($P < 0.05$). These findings were supported by a significant difference at 12 weeks in inguinal and abdominal fat weights in the same mice ($P < 0.05$) compared with mice drinking water (Figure 5b). On the contrary, the C3G solution did not significantly affect body weight. No significant 'treatment group \times time' interaction

was observed and, in particular, from weeks 9 to 12 body weight was comparable (Figure 6a). Finally, inguinal and abdominal fat weight determinations did not significantly differ in the two groups of mice (Figure 6b).

Discussion

To date dietary recommendations aimed to prevent obesity are limited to generic advice for the reduction of energy and fat intake rather than suggesting the consumption of specific 'anti-obesity' foods. Regardless, phyto-therapy approaches against obesity promise to be effective. However, the

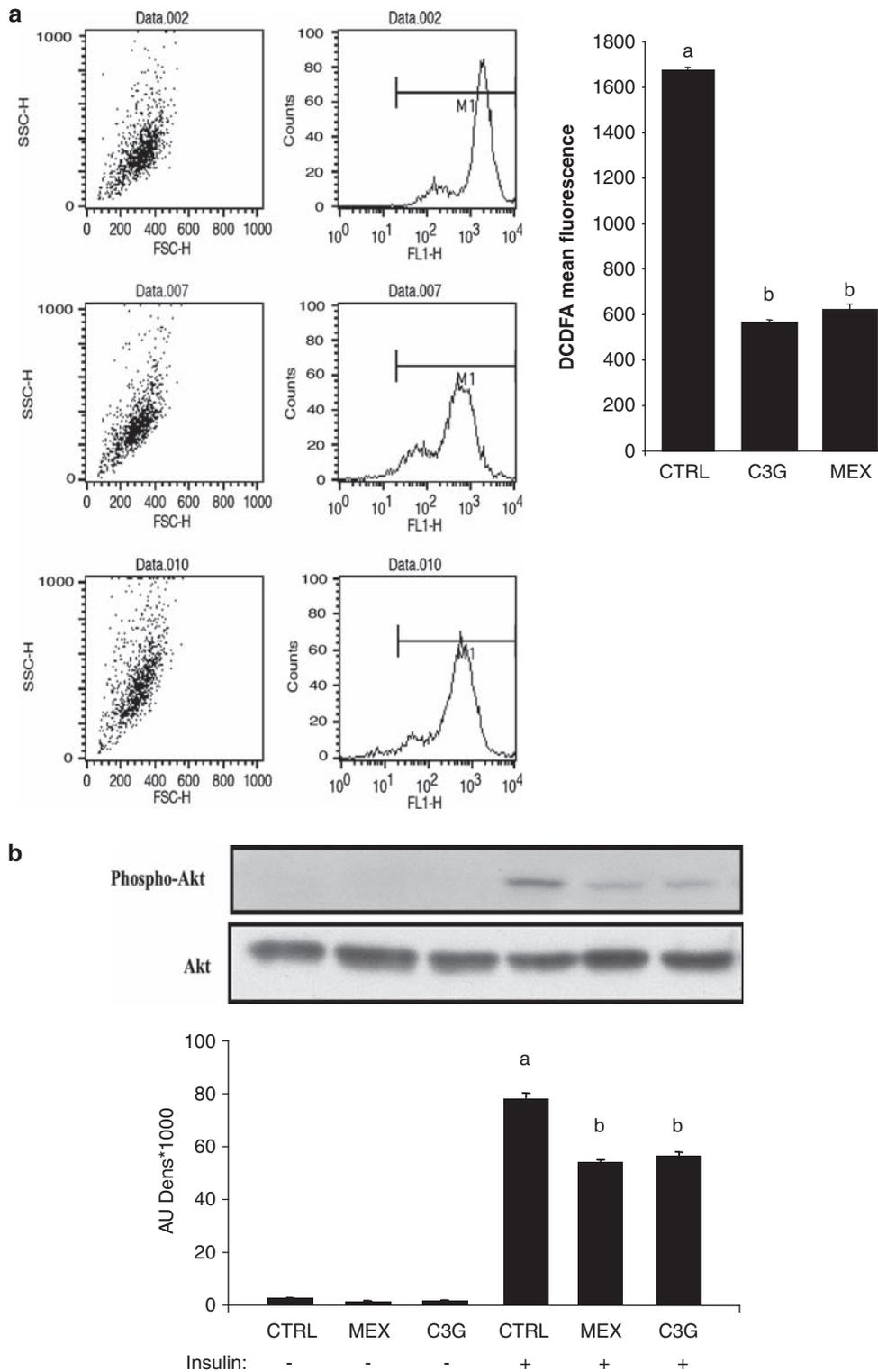


Figure 4 Anthocyanins decrease oxidative stress and insulin signalling. (a) Primary adipocytes treated with 100 μM cyanidin-3-glucoside (C3G) and 20% v/v *Moro* orange extract (MEX) for 8 days, stained with 40 mM DCDFDA and analyzed by fluorescence-activated cell sorting (FACS). Left panels: physic parameters, control (top), C3G treated cells (middle) and MEX treated cells (bottom). Right graph: fluorescence analysis. Data are means \pm s.e.m. 'a' compared with 'b', $P < 0.01$. (b) C3G and MEX inhibit tyrosine phosphorylation of Akt in primary adipocytes. Cells were serum starved for 24 h and then stimulated with either 0.1 $\mu\text{g ml}^{-1}$ insulin in control medium (CTRL), 100 μM C3G or 20% v/v MEX. Top: western blot showing phosphorylated and total Akt. Bottom: graph showing results expressed as relative densitometry units (means \pm s.d.); $n = 3$, $P < 0.05$.

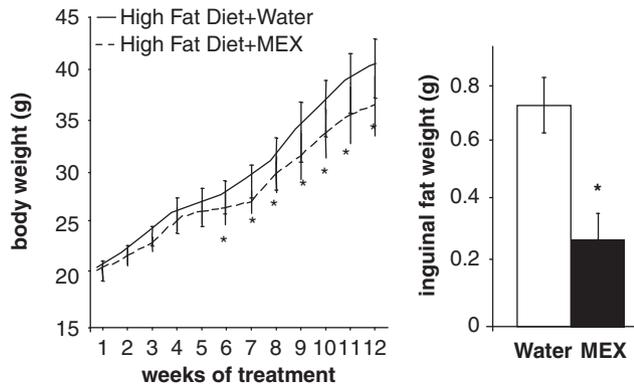


Figure 5 *Moro* extract inhibits fat accumulation. (a) Effect of *Moro* orange extract (MEX) supply on body weight, * $P < 0.05$ and (b) inguinal and abdominal fat weight, * $P < 0.05$ in C57/BL6 mice fed HFD. Data are means \pm s.e.m.; $n = 10$.

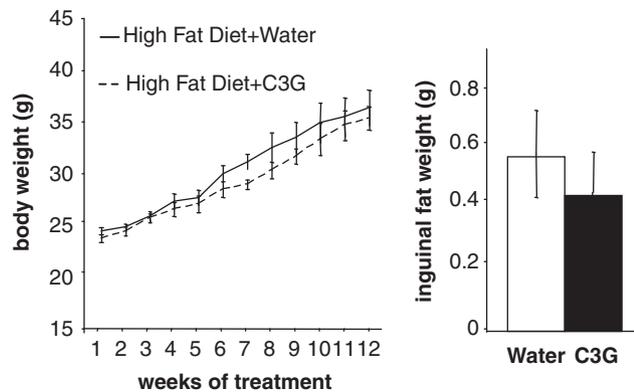


Figure 6 Cyanidin-3-glucoside (C3G) ($90 \text{ mg kg}^{-1} \text{ day}^{-1}$) does not reduce fat accumulation. Effect of C3G on body weight (a) and inguinal fat weight (b) in HFD fed C57BL/6 mice. Data are means \pm s.e.m.; $n = 5$.

consumption of plant foods does not always guarantee an effective reduction in energy intake, at least when a high amount of sugar is present.

Orange juice contains an array of potent bioactive compounds including flavonoids (hesperidin and narirutin, predominantly as glycosides), carotenoids (xanthophylls and carotenes), vitamin C and other beneficial phytochemicals, such as folates. It is noteworthy that blood oranges are characterized by a high content in anthocyanins, in particular C3G, reported to attenuate obesity and ameliorate insulin resistance when supplemented to HFD fed mice.²³ However, a large consumption of orange juice, despite its healthy effects, has the potential to contribute to overweight because of its sugar (10%~ w/v), in particular fructose (~2.5%w/v) content.

We have found that providing *Moro* juice or a *Moro* anthocyanin-enriched extract to mice prevents body weight gain, fat development and inhibits high fat-induced obesity without decreasing total energy intake. Furthermore, glucose

as well as fatty acid and triglyceride blood levels were not altered by supplementing the diet with *Moro* juice or with the extract (not shown).

Therefore, *Moro* juice seems to directly target the ability of adipocytes to accumulate fat. Indeed, the study of the fat tissue transcriptome revealed that *Moro* juice can effectively counteract the consequences of the HFD on adipocyte gene expression.

Analytically, it is difficult to determine a particular *Moro* juice component singly responsible for the anti-obesity effect. Previous reports^{23,24} have shown that supplementation of $320 \text{ mg day}^{-1} \text{ kg}^{-1}$ ²³ of C3G or $160 \text{ mg day}^{-1} \text{ kg}^{-1}$ ²⁴ of anthocyanins through the food pellets reduces fat accumulation in mice.

However, we found that the consumption of crude *Moro* juice, which provided 20 mg of total anthocyanins/ $\text{day}^{-1} \text{ kg}^{-1}$, was more effective in preventing fat accumulation than the consumption of the *Moro* anthocyanin-enriched extract, which ensured 90 mg of total anthocyanins/ $\text{day}^{-1} \text{ kg}^{-1}$. Moreover, administration of purified C3G did not show any effect. It is noteworthy that both circulating levels and kinetics of C3G in mice on administration of a single bolus of C3G solution or *Moro* juice by gavage were similar (data not shown), indicating that the juice does not affect the absorption and pharmacokinetic properties of C3G.

All these findings suggest that other components of the *Moro* Juice rather than or in addition to anthocyanins may contribute to the anti-obesity effects. Indeed, other substances, which are typical constituents of crude juices without directly affecting fat development, might boost the activity of anthocyanins. It has been already shown that the food matrix, that is, the dietary source, might be also relevant to determine the effect of anthocyanins on fat accumulation.^{23,24} Finally, administration modalities might also influence compound intake. For example, administration of ascorbic acid was more effective in counteracting oxidative stress when provided through orange juice than when supplemented as a simple water solution.³²

A few studies have shown the lipolytic activity of another component of orange juice, synephrine,^{33,34} an adrenergic compound that could have adverse health effects.^{35,36} Nevertheless, no difference in the amount of synephrine was found between *Moro* and *Naveline* juices. We cannot exclude, however, that anthocyanins synergize with synephrine, thus amplifying their activities on fat tissue. In contrast, traces of molecules potentially active on adipocytes, although barely detectable in orange juices but abundant in the orange leaf or peel, may have a role in combination with anthocyanins. Among them isoflavones, shown to potentiate epinephrine-induced lipolysis in primary rat adipocytes³⁷ and to lower lipidemia,³⁸ and quercetin, reported to block insulin-mediated lipogenesis by preventing the insulin receptor tyrosine kinase from phosphorylating its substrate.³⁹ It is noteworthy that the synergistic effects of isoflavones and quercetin on adipogenesis has been also shown.⁴⁰

Further studies are required to evaluate the relationship among key components of the *Moro* juice responsible for the inhibition of fat accumulation. However, regardless of the nature of these factors, our results on the effect of blood orange juice on obesity corroborate the benefit of a phytotherapy approach against obesity.

Conflict of interest

LT, IB, MS, KP, CT, P Riso, MP, P Rapisarda and GRR have no conflict of interest. MT is an employer of Congenia S.r.l. SM, PGP and MG are share holders of Congenia S.r.l. The remaining authors declare no conflict of interest.

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