

CrossMark  
click for updatesCite this: *Food Funct.*, 2014, 5, 2120

## Modulation of hyperglycemia and TNF $\alpha$ -mediated inflammation by helichrysum and grapefruit extracts in diabetic *db/db* mice

Ana Laura de la Garza,<sup>a</sup> Usune Etxeberria,<sup>a</sup> Sara Palacios-Ortega,<sup>b</sup>  
Alexander G. Haslberger,<sup>c</sup> Eva Aumueller,<sup>c</sup> Fermin I. Milagro<sup>ad</sup>  
and J. Alfredo Martínez<sup>\*ad</sup>

Type-2 diabetes is associated with a chronic low-grade systemic inflammation accompanied by an increased production of adipokines/cytokines by obese adipose tissue. The search for new antidiabetic drugs with different mechanisms of action, such as insulin sensitizers, insulin secretagogues and  $\alpha$ -glucosidase inhibitors, has directed the focus on the potential use of flavonoids in the management of type-2 diabetes. Thirty six diabetic male C57BL/6J *db/db* mice were fed a standard diet and randomly assigned into four experimental groups: non-treated control, ( $n = 8$ ); acarbose (5 mg per kg bw,  $n = 8$ ); helichrysum (1 g per kg bw,  $n = 10$ ) and grapefruit (0.5 g per kg bw,  $n = 10$ ) for 6 weeks. The mRNA expression in pancreas, liver and epididymal adipose tissue was determined by RT-PCR. DNA methylation was quantified in epididymal fat using pyrosequencing. Mice supplemented with helichrysum and grapefruit extracts showed a significant decrease in fasting glucose levels ( $p < 0.05$ ). A possible mechanism of action could be the up-regulation of liver glucokinase ( $p < 0.05$ ). The antihyperglycemic effect of both extracts was accompanied by decreased mRNA expression of some proinflammatory genes (monocyte chemoattractant protein-1, tumor necrosis factor- $\alpha$ , cyclooxygenase-2, nuclear factor-kappaB) in the liver and epididymal adipose tissue. The CpG3 site of TNF $\alpha$ , located 5 bp downstream of the transcription start site, showed increased DNA methylation in the grapefruit group compared with the non-treated group ( $p < 0.01$ ). In conclusion, helichrysum and grapefruit extracts improved hyperglycemia through the regulation of glucose metabolism in the liver and reduction of the expression of proinflammatory genes in the liver and visceral fat. The hypermethylation of TNF $\alpha$  in adipose tissue may contribute to reduce the inflammation associated with diabetes and obesity.

Received 25th February 2014  
Accepted 7th June 2014

DOI: 10.1039/c4fo00154k

www.rsc.org/foodfunction

### Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia as a result of impairments in insulin secretion and insulin action in target tissues.<sup>1</sup> Insulin resistance (IR) is produced as soon as the pancreatic  $\beta$ -cells cannot compensate a reduced insulin function, leading to elevated circulating glucose levels.<sup>2</sup> Insulin inhibits gluconeogenesis in the liver and reduces lipolysis in adipose tissue.<sup>3</sup> Likewise, adipose tissue in diabetes and obesity is characterized by hypertrophy, relative hypoxia, low-grade chronic inflammation and endocrine dysfunctions.<sup>4</sup> In this context, the pro-

inflammatory cytokines, many of them secreted by the hypertrophied adipocytes, are controlled through transcription nuclear factor-kappaB (NF- $\kappa$ B), whereby the inflammatory response can be down-regulated.<sup>5</sup> In addition, this transcription factor represents a link between inflammation and IR, as it is activated by factors known to promote IR and T2DM.<sup>6</sup> One important downstream target of NF- $\kappa$ B is cyclooxygenase 2 (COX2), which catalyzes the production of prostaglandins, the key molecules in inflammation processes of the body.<sup>7</sup> Moreover, NF- $\kappa$ B is involved in the expression of many cytokines, including TNF $\alpha$ .<sup>5</sup> On the other hand, epigenetic changes are heritable yet reversible modifications that occur without alterations in the primary DNA sequence. These modifications may provide a link between the environment (*i.e.* nutrition) and T2DM.<sup>8</sup> Recently, epigenetic modifications have also been implicated in disease-associated changes influencing gene expression.<sup>9</sup>

Targeting the reduction of chronic inflammation is a beneficial strategy to combat several metabolic diseases, including T2DM.<sup>10</sup> Thus, numerous studies have underlined the interest

<sup>a</sup>Department of Nutrition, Food Science and Physiology, University of Navarra, C/Irunlarrea 1, 31008 Pamplona, Navarra, Spain. E-mail: jalfmtz@unav.es; Fax: +34 948425740; Tel: +34 948425600

<sup>b</sup>Department of Biochemistry and Genetics, University of Navarra, Pamplona, Spain

<sup>c</sup>Department of Nutritional Sciences, University of Vienna, Vienna, Austria

<sup>d</sup>Physiopathology of Obesity and Nutrition, CIBERobn, Carlos III Health Research Institute, Madrid, Spain

in finding nutritional factors that may help to prevent or treat these diseases.<sup>10,11</sup> In this sense, flavonoids can act through a variety of mechanisms to prevent and attenuate inflammatory responses.<sup>12</sup> These bioactive compounds can also improve glucose metabolism by stimulating the peripheral glucose uptake in different tissues.<sup>11</sup> In relation to this, grapefruit extract is rich in flavanones (*i.e.*, naringenin-7-*O*-rutoside) and flavonols (*i.e.*, kaempferol rutoside).<sup>13</sup> Previous studies have reported that *citrus* flavonoids have many pharmacological activities, including anti-inflammatory properties.<sup>14</sup> Thus, an improvement in hyperglycemia by the hepatic enzymes involved in glucose metabolism was reported in the groups of mice whose diet was supplemented with naringin.<sup>15</sup> Furthermore, a recent study reported that orange juice appears to mediate the inflammatory response, both at gene expression and plasma levels.<sup>16</sup>

Helichrysum (*Helichrysum italicum*) is a flowering plant that grows around the Mediterranean area and contains naringenin-7-*O*-glucoside, kaempferol-3-*O*-glucoside and other flavonoids.<sup>13</sup> Likewise, plants of the *Helichrysum* genus have been found to have several biological activities, such as anti-inflammatory properties, which have been attributed to different flavonoids.<sup>17</sup> Thus, beneficial roles of kaempferol have been reported in inflammation, hyperglycemia and diabetes using different *in vitro* and *in vivo* models.<sup>18</sup> In addition, some investigations concluded that the anti-inflammatory activity of *Helichrysum italicum* may be explained by enzyme inhibition, free-radical scavenging activity and corticoid-like effects.<sup>19</sup> In this sense, our group previously demonstrated that helichrysum and grapefruit extracts ameliorated hyperglycemia by inhibiting  $\alpha$ -glucosidase (a similar mechanism to acarbose) and  $\alpha$ -amylase enzyme activities and by decreasing the SGLT1-mediated glucose uptake in the gut.<sup>13</sup>

Since inflammation in adipose tissue plays a central role in obesity-related IR and T2DM, our research was conducted using a recognized model of obesity and diabetes, *db/db* mice, displaying characteristics such as overweight, hyperglycemia and hyperinsulinemia due to leptin receptor mutations.<sup>20</sup> Therefore, the aim of this study was to investigate the antihyperglycemic and anti-inflammatory effects of helichrysum and grapefruit extracts, studying the possible involvement of epigenetic mechanisms in *db/db* mice. The effects of both extracts were compared with those of acarbose, an oral anti-diabetic agent whose main mechanism of action is the inhibition of  $\alpha$ -glucosidase.

## Materials and methods

### Chemicals

Mice were fed a standard pelleted chow diet from Harlan Ibérica (Teklad Global, Barcelona, Spain; ref. 2014). Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus x paradisi*) extracts, as well as acarbose®, were provided by “Biosearch S.A.” (Granada, Spain). Plant samples (1–5 g) were pulverized, mixed with washed sea sand and introduced into the extraction cells, where 30 mL of each solvent at 50 °C was added: methanol/water (3 : 1) and methanol–water (1 : 1) for helichrysum and grapefruit,

respectively. The quantification of the phenolic compounds was performed by UPLC-MS/MS.<sup>13</sup> Helichrysum extract contained phenolic acids and flavonoids as flavanones and flavonols subclasses, as previously described.<sup>13</sup> The flavanones found in higher proportions were naringenin-7-*O*-glucoside (3.9 mg g<sup>-1</sup> extract) and naringenin diglycoside (1.2 mg g<sup>-1</sup> extract). Kaempferol-3-*O*-glucoside (13.4 mg g<sup>-1</sup> extract) is the flavonol that was found in a greater proportion. Likewise, the grapefruit extract mainly contained naringenin-7-*O*-rutoside (5.2 mg g<sup>-1</sup> extract) and naringenin (1 mg g<sup>-1</sup> extract) as flavanones, and kaempferol-rutoside (54.2 mg kg<sup>-1</sup> extract) as a flavonol.<sup>13</sup> Glucose was purchased from Sigma-Aldrich (St. Louis, MO, USA) and starch (162.14 g mol<sup>-1</sup>) from Panreac (Barcelona, Spain).

### Experimental animals

Thirty six overweight and diabetic male C57BL/6J *db/db* mice (Charles River, Barcelona, Spain) were randomly assigned into four experimental groups: non-treated control group, *n* = 8; acarbose group (5 mg per kg bw), *n* = 8; helichrysum group (1 g per kg bw), *n* = 10, and grapefruit group (0.5 g per kg bw), *n* = 10. The doses used were calculated by comparing with the acarbose effect and based on the IC<sub>50</sub> of the extracts, as described elsewhere.<sup>13</sup> For 6 weeks, all mice were fed a standard pelleted chow diet from Harlan Ibérica (ref. 2014 S, Barcelona, Spain) containing 20% of energy as proteins (corn and wheat), 67% as carbohydrates (5% sucrose, 62% starch), and 13% as fat by dry weight (2.9 kcal g<sup>-1</sup>). Animals were kept in an isolated room at a constantly regulated temperature between 21 and 23 °C, and controlled humidity (50 ± 10%) under a 12 h : 12 h artificial light/dark cycle. Body weight and food intake were recorded once a week. Body composition was measured at the beginning and at the end of the feeding period. On the 1<sup>st</sup>, 3<sup>rd</sup> and 6<sup>th</sup> weeks, fasting glucose was measured from a drop of blood collected from the tail vein. On the 5<sup>th</sup> week, respiratory quotient (RQ) and energy expenditure (EE) (kg per day per bw<sup>3/4</sup>) measurements were performed by using an Oxylet equipment (Panlab, Barcelona, Spain), as previously reported.<sup>21</sup> This procedure was carried out in groups of four mice daily, introducing each mouse in a box with water and food during 24 hours. At weeks 3 and 6, the oral starch tolerance test (OSTT) and intraperitoneal glucose tolerance test (IPGTT) were carried out, respectively. After 6 weeks of experimental treatment, mice were killed by decapitation and trunk blood was collected to obtain serum for the biochemical measurements. Liver, pancreas, spleen and different adipose depots, such as subcutaneous, retroperitoneal, epididymal and mesenteric, were carefully dissected and weighed. Tissue samples and serum were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. All the procedures were performed according to the guidelines of the Animal Research Ethics Committee of the University of Navarra (04/2011).

### Oral starch tolerance test (OSTT) and intraperitoneal glucose tolerance test (IPGTT)

The OSTT was performed in the 3<sup>rd</sup> week. After a 15 h fast, animals were orally administered by gastric intubation (5 mL

per kg bw) with starch (2 g per kg bw in 30% w/v solution) and acarbose (5 mg per kg bw), helichrysum (1 g per kg bw) and grapefruit (0.5 g per kg bw), respectively. Glycemia was measured before (0') and after the oral administration (30', 60', 120', 180', 240') by venous tail puncture using a glucometer and blood glucose test strips (Optium Plus, Abbott® Diabetes Care, Witney Oxon, UK). The IPGTT was performed in the 6<sup>th</sup> week. After a 15 h fast, mice were injected intraperitoneally with glucose (2 g per kg bw in 30% w/v solution). Blood glucose levels were determined from the tail vein before (0') and after glucose injection (180', 240', 360', 420'). The glucose content was expressed as mmol L<sup>-1</sup>, and the areas under the curve (AUC) were determined by the trapezoidal rule.<sup>22</sup>

### Biochemical measurements

Fasting glucose levels were measured with the HK-CP kit (ABX diagnostic, Montpellier, France), creatinine was determined with the Creatinine-CP kit (ABX Pentra), and triglycerides with the RANDOX triglycerides kit (Randox Laboratories, Crumlin, UK), adapted for the PENTRA C200 equipment (HORIBA Medical, Montpellier, France). The levels of glycated hemoglobin (HbA1C) were determined at the end of the feeding period and measured with the mouse GHbA1C ELISA kit (Cat. no. CSB-E08141m, Cusabio Biotech Co., Ltd., China).

The pancreatic insulin content was determined by acid-ethanol extraction. Briefly, the pancreas was placed in 5 mL acid-ethanol (1.5% HCl in 70% EtOH) overnight at -20 °C, homogenized and incubated overnight at -20 °C. Samples were centrifuged at 2000 rpm for 15 minutes at 4 °C. The supernatant was transferred to clean tubes and neutralized with 100 µL 1 M Tris, pH 7.5. The pancreatic insulin content was analyzed by the enzyme-linked-immunosorbent assay (ELISA) following the protocol described by the manufacturer (Mercodia AB, Uppsala, Sweden). The absorbance was calculated with an appropriate dilution factor. Pancreatic insulin values were corrected for the protein concentration, as determined by the Bradford assay with bovine serum albumin as a standard (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Finally, the insulin content (ng mL<sup>-1</sup>) was normalized by the protein content (µg mL<sup>-1</sup>).

### RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from pancreas, liver and epididymal adipose tissue using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were measured with a Nanodrop Spectrophotometer 1000 (Thermo Scientific, Delaware, USA). Then, RNA (2 µg) was reverse-transcribed to cDNA using MMLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen). RT-PCR assays were performed following the manufacturer's recommendations using an ABI PRISM 7000 HT Sequence Detection System and pre-designed TaqMan® Assays-on-Demand by Applied Biosystems (Texas, USA). Glucokinase (GCK), Mm00439129\_m1; glucose 6-phosphatase (G6Pase), Mm00839363\_m1; phosphoenolpyruvate carboxykinase

(PEPCK), Mm01247058\_m1; monocyte chemotactic protein 1 (MCP1), Mm00656886\_g1; nuclear factor-kappaB (NF-κB), Mm00476361\_m1; cyclooxygenase 2 (COX2), Mm00478374\_m1; tumor necrosis factor α (TNFα), Mm00443260\_g1; betatrophin, Mm01175863\_g1; Insulin (Ins1), Mm019550294\_s1; Insulin receptor (InsR), Mm01211875\_m1; glucose transporter 4 (GLUT4), Mm00436615\_m1 and Taqman Universal Master Mix were also provided by Applied Biosystems. The mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm 99999915\_g1 and beta actin (ActB), Mm 00607939\_s1, as housekeeping genes. All samples were analyzed in triplicate. The relative expression level of each gene was calculated by the 2<sup>-ΔΔCt</sup> method.

### DNA extraction and bisulfite conversion

Genomic DNA was isolated from epididymal adipose tissue using the DNA purification protocol for tissues of the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA). DNA concentration and quality were measured by using a Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE, SA). The stock solutions of DNA samples were stored at -80 °C until use. For epigenetic analysis, all DNA samples were bisulfite-treated using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), resulting in the deamination of unmethylated cytosine to uracil. The concentration of DNA was measured on a Pico100 (Pico-drop Limited, Hinxton, UK). All procedures were carried out according to the manufacturer's instructions.

### PCR and methylation analysis by DNA pyrosequencing

Quantitative methylation analyses were performed by pyrosequencing of bisulfite-converted DNA using PyroMark Q24 (Qiagen). PCR was carried out in 25 µL reaction mixtures containing 12.5 µL PyroMark 2× PCR master mix, 0.15 nM of each primer for TNFα, 5'-GGAAGTTTITAGAGGGTTGAATGAGA-3' (forward) and 5'-CTAACTAATCCCTTACTATCCT-3' (reverse), 2.5 µL CoralLoad Concentrate 10× (Qiagen) and 1 µL of DNA samples after bisulfite conversion, at a concentration of 10 ng µL<sup>-1</sup>. PCR conditions were 95 °C for 15 minutes; 45 cycles of 94 °C for 30 s, 55.5 °C for 45 s, 72 °C for 45 s; final elongation at 72 °C for 10 minutes. PCR products were checked by 2% agarose gel electrophoresis. A total of 22 µL of the PCR product was used for subsequent pyrosequencing using a PyroMark Q24 System (Qiagen). All procedures for quantification of CpG methylation levels were performed based on a protocol described elsewhere.<sup>23</sup> For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient bisulfite DNA conversion.

### Statistical analysis

All the results are expressed as mean ± standard deviation (SD) of the mean. Statistical significance of differences among the groups was evaluated using the One-Way ANOVA test followed by the Dunnett's post-hoc test. The two-tailed Pearson test was used to assess selected correlations among variables. A level of probability of *p* < 0.05 was set as statistically significant. All

analyses were performed using SPSS 15.0 packages for Windows (Chicago, IL).

## Results

### Food intake, body weight gain and body fat mass

At the end of the supplementation period, the grapefruit group gained more body weight ( $p < 0.05$ ) than the non-treated group (Table 1). Although not statistically significant, the percentage of total adipose tissue (WAT) was slightly higher in the treated groups (Table 1). Furthermore, significant differences were found in spleen weight between the acarbose ( $p < 0.05$ ) and helichrysum ( $p < 0.01$ ) groups when compared with the non-treated group, whereas liver weights were similar in all groups (Table 1).

Regarding food efficiency, the average daily food intake throughout the experimental period remained unaltered in the acarbose group and after helichrysum and grapefruit extract administration (Table 1).

### Respiratory quotient and energy expenditure

The respiratory quotient (RQ) assessment, which is used to evaluate the relative oxidation of substrates, evidenced that the grapefruit group ( $p < 0.05$ ) improved carbohydrate oxidation when compared with the non-treated group (Table 1). Otherwise, there were no differences among groups with respect to energy expenditure (EE), suggesting that the possible effect of helichrysum and grapefruit extracts on glucose metabolism did not significantly affect thermogenesis (Table 1).

### Blood glucose and serum parameters

Glycemia levels at baseline and at the end of the supplementation period are shown in Table 1. All mice were diabetic when the experiment began ( $x = 10 \pm 3 \text{ mmol L}^{-1}$ ). Although no significant differences were found in the acarbose group, both supplemented groups showed significantly lower levels of glycemia ( $p < 0.05$ ) at the end of the 6-week treatment when

compared with the non-treated group (Table 1). The grapefruit group showed a decreased glucose AUC in the OSTT ( $p < 0.05$ ) (Fig. 1A). Likewise, both supplemented groups showed lower AUC than the non-treated group in the IPGTT ( $p < 0.05$ ) (Fig. 1B).

No statistically significant differences between groups were found in fasting triglyceride levels. Conversely, creatinine serum levels were slightly lower in the acarbose and grapefruit groups, but did not reach statistical significance in comparison with the non-treated group (Table 1).

The long-term glucose control was also evaluated by measuring HbA1C (Table 1), but no relevant differences were found among the experimental groups.

### Determinations in the pancreas

Pancreatic insulin content was analyzed to determine whether the use of both extracts might have beneficial effects on glucose metabolism *via* the insulin secretory capacity of the pancreas. There were no differences in the pancreatic insulin content among the experimental groups (Table 1). However, the mRNA expression of *Ins1* was decreased in the pancreas from the acarbose group when compared with the non-treated group (data not shown). No statistical differences were found between groups in the mRNA expression of *GCK* in the pancreas (data not shown).

### Glucose metabolism

In order to investigate the mechanisms through which flavonoid-rich extracts ameliorate hyperglycemia in *db/db* mice, the mRNA expression of different genes that regulate glucose homeostasis in the liver was examined (Table 2). *GCK* expression levels were statistically higher in the acarbose group ( $p < 0.001$ ) and both supplemented groups ( $p < 0.05$ ) when compared with the non-treated group. No statistically significant differences were found in *G6Pase*, *PEPCK* and *betatrophin* mRNA levels in the liver (Table 2). Interestingly, mRNA expression levels of *GCK* in the liver showed a negative

Table 1 Effects of flavonoid-containing extracts from helichrysum and grapefruit on body weight and tissues, and biochemical measurements<sup>a</sup>

	Non-treated	Acarbose	Helichrysum	Grapefruit
Weight gain (g)	9.6 ± 3.5	10.6 ± 2.2	11.6 ± 2.0	13.6 ± 2.8*
Food efficiency (g/100 kcal)	0.75 ± 0.04	0.72 ± 0.01	0.68 ± 0.02	0.80 ± 0.01
Total WAT (%)	51 ± 0.9	50 ± 1.1	52 ± 0.6	53 ± 0.4
Liver (g per bw)	4.4 ± 0.5	4.5 ± 0.2	4.3 ± 0.2	4.2 ± 0.2
RQ 24 h	0.78 ± 0.02	0.75 ± 0.03	0.79 ± 0.02	0.81 ± 0.03*
EE 24 h (kg per day per bw <sup>3/4</sup> )	122 ± 16	112 ± 4	111 ± 9	114 ± 15
Blood glucose (mmol L <sup>-1</sup> )				
Initial	10.5 ± 2.0	10.2 ± 1.1	9.4 ± 0.7	9.4 ± 1.3
Final	27.3 ± 1.5	24.5 ± 1.7	20.0 ± 1.4*	20.1 ± 1.8*
Pancreatic insulin (µg mL <sup>-1</sup> * mg protein)	0.78 ± 0.00	0.79 ± 0.01	0.80 ± 0.01	0.80 ± 0.01
HbA1C (ng mL <sup>-1</sup> )	2.31 ± 0.12	2.23 ± 0.14	2.13 ± 0.15	2.17 ± 0.13
Triglycerides (mg dL <sup>-1</sup> )	136 ± 10	127 ± 10	145 ± 9	139 ± 7
Creatinine (mg dL <sup>-1</sup> )	0.42 ± 0.08	0.33 ± 0.11	0.41 ± 0.08	0.34 ± 0.06

<sup>a</sup> Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA, and the Dunnett's test was used to analyze differences in the mean of each group compared with the non-treated group. Non-treated and acarbose groups ( $n = 6$ ); helichrysum and grapefruit groups ( $n = 8$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ .

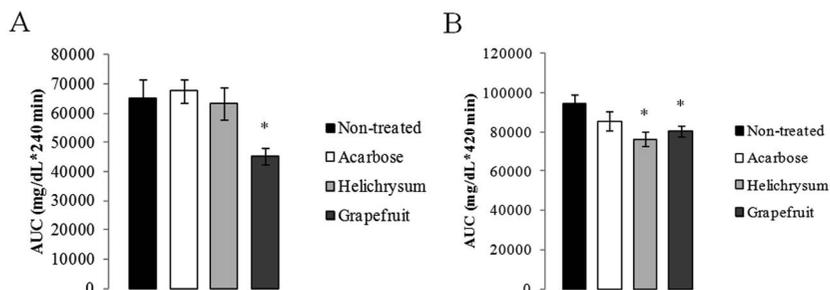


Fig. 1 Area under the curve (AUC) after the oral starch tolerance test – OSTT (A) and the intraperitoneal glucose tolerance test – IPGTT (B) in *db/db* mice. Results are expressed as mean  $\pm$  SD. Statistical analysis was performed using ANOVA, and the Dunnett's test was used to analyze differences in the mean of each group compared with the non-treated group. Non-treated and acarbose groups ( $n = 6$ ); helichrysum and grapefruit groups ( $n = 8$ ). \* $p < 0.05$ .

Table 2 Effects of flavonoid-containing extracts from helichrysum and grapefruit on mRNA expression levels in the liver. Genes related to glucose metabolism and inflammation<sup>a</sup>

Fold change					
Metabolism	Gene name	Non-treated	Acarbose	Helichrysum	Grapefruit
Glucose	GCK	1.0 $\pm$ 0.2	2.8 $\pm$ 0.2***	1.8 $\pm$ 0.2*	1.8 $\pm$ 0.1*
	G6Pase	1.0 $\pm$ 0.3	1.3 $\pm$ 0.3	1.2 $\pm$ 0.2	1.3 $\pm$ 0.2
	PEPCK	1.0 $\pm$ 0.2	1.5 $\pm$ 0.4	1.3 $\pm$ 0.3	0.9 $\pm$ 0.2
	Betatrophin	1.0 $\pm$ 0.2	1.4 $\pm$ 0.4	1.2 $\pm$ 0.5	0.9 $\pm$ 0.2
Inflammation	TNF $\alpha$	1.0 $\pm$ 0.2	0.3 $\pm$ 0.1*	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1*
	MCP1	1.0 $\pm$ 0.1	0.4 $\pm$ 0.3*	0.2 $\pm$ 0.1**	0.3 $\pm$ 0.2*
	COX2	1.0 $\pm$ 0.2	0.1 $\pm$ 0.1***	0.1 $\pm$ 0.1***	0.1 $\pm$ 0.1***
	NF- $\kappa$ B	1.0 $\pm$ 0.2	0.5 $\pm$ 0.2*	0.4 $\pm$ 0.1**	0.4 $\pm$ 0.1**

<sup>a</sup> Results are expressed as fold changes compared to the housekeeping (GAPDH) gene, and are shown as mean  $\pm$  SD. Statistical analysis was performed using ANOVA, and the Dunnett's test was used to analyze differences in the mean of each group compared with the non-treated group (normalized to 1). ( $n = 6$ ) \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

correlation ( $r = -0.692$ ,  $p < 0.001$ ) with final blood glucose levels ( $\text{mmol L}^{-1}$ ) (Fig. 2).

Moreover, mRNA expression levels of betatrophin, InsR and GLUT4 were measured in epididymal adipose tissue, although no differences were found among the experimental groups (Table 3).

### Inflammatory markers

The expression of several pro-inflammatory markers was analyzed in the liver and epididymal adipose tissue. Thus, the hepatic mRNA levels of TNF $\alpha$ , MCP1, COX2 and NF- $\kappa$ B decreased in the acarbose group and after supplementation with both the helichrysum and grapefruit extract (Table 2). Statistically significant differences in the mRNA expression of TNF $\alpha$ , MCP1 and COX2 were also found in epididymal adipose tissue, but only in the groups supplemented with the natural extracts (Table 3).

### DNA methylation analysis

The methylation pattern of TNF $\alpha$  was analysed in epididymal adipose tissue (Fig. 3). Interestingly, a hypermethylation ( $\Delta$  of methylation: 2.5%) was detected at CpG3 (CpG site + 5 bp) after supplementation with the grapefruit extract ( $p < 0.01$ ) (Fig. 3B).

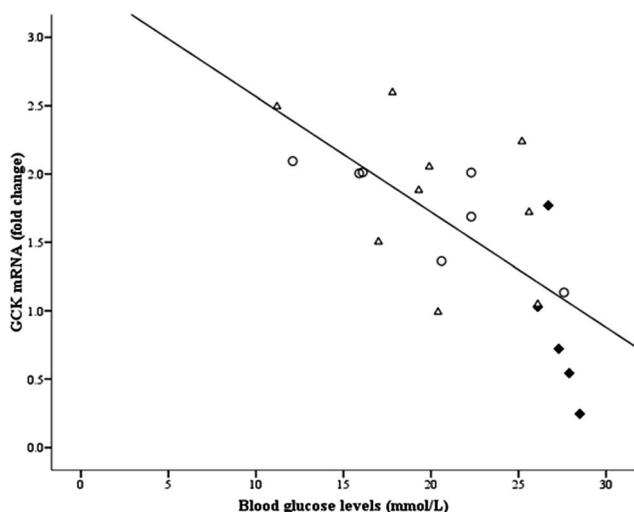


Fig. 2 Correlation analysis between GCK gene expression in the liver (fold change) and final blood glucose level ( $\text{mmol L}^{-1}$ ).  $R$ , Pearson's correlation coefficient. Results are expressed as mean. Non-treated ( $n = 5$ ); helichrysum and grapefruit groups ( $n = 8$ ). (◆ non-treated group,  $\Delta$  helichrysum group, and  $\circ$  grapefruit group).

**Table 3** Effects of flavonoid-containing extracts from helichrysum and grapefruit on mRNA expression levels in epididymal adipose tissue. Genes related to glucose metabolism and inflammation<sup>a</sup>

Fold change					
Metabolism	Gene name	Non-treated	Acarbose	Helichrysum	Grapefruit
Glucose	InsR	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
	GLUT4	1.0 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
	Betatrophin	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.1
Inflammation	TNF $\alpha$	1.0 ± 0.2	0.8 ± 0.2	0.5 ± 0.1***	0.7 ± 0.2**
	MCP1	1.0 ± 0.1	0.8 ± 0.4	0.5 ± 0.2*	0.6 ± 0.4
	COX2	1.0 ± 0.3	0.7 ± 0.1	0.5 ± 0.3**	0.5 ± 0.2**
	NF- $\kappa$ B	1.0 ± 0.3	0.9 ± 0.2	0.7 ± 0.3	0.8 ± 0.3

<sup>a</sup> Results are expressed as fold changes compared to the housekeeping (GAPDH) gene, and are shown as mean  $\pm$  SD. Statistical analysis was performed using ANOVA, and the Dunnett's test was used to analyze differences in the mean of each group compared with the non-treated group (normalized to 1). ( $n = 6$ ) \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Moreover, TNF $\alpha$  CpG3 methylation levels (%) showed a positive correlation with body weight gain (g) ( $r = 0.562$ ,  $p < 0.05$ ) and WAT (%) ( $r = 0.706$ ,  $p < 0.01$ ) (Fig. 3C), suggesting a link between DNA methylation, inflammation and adipose tissue mass.

## Discussion

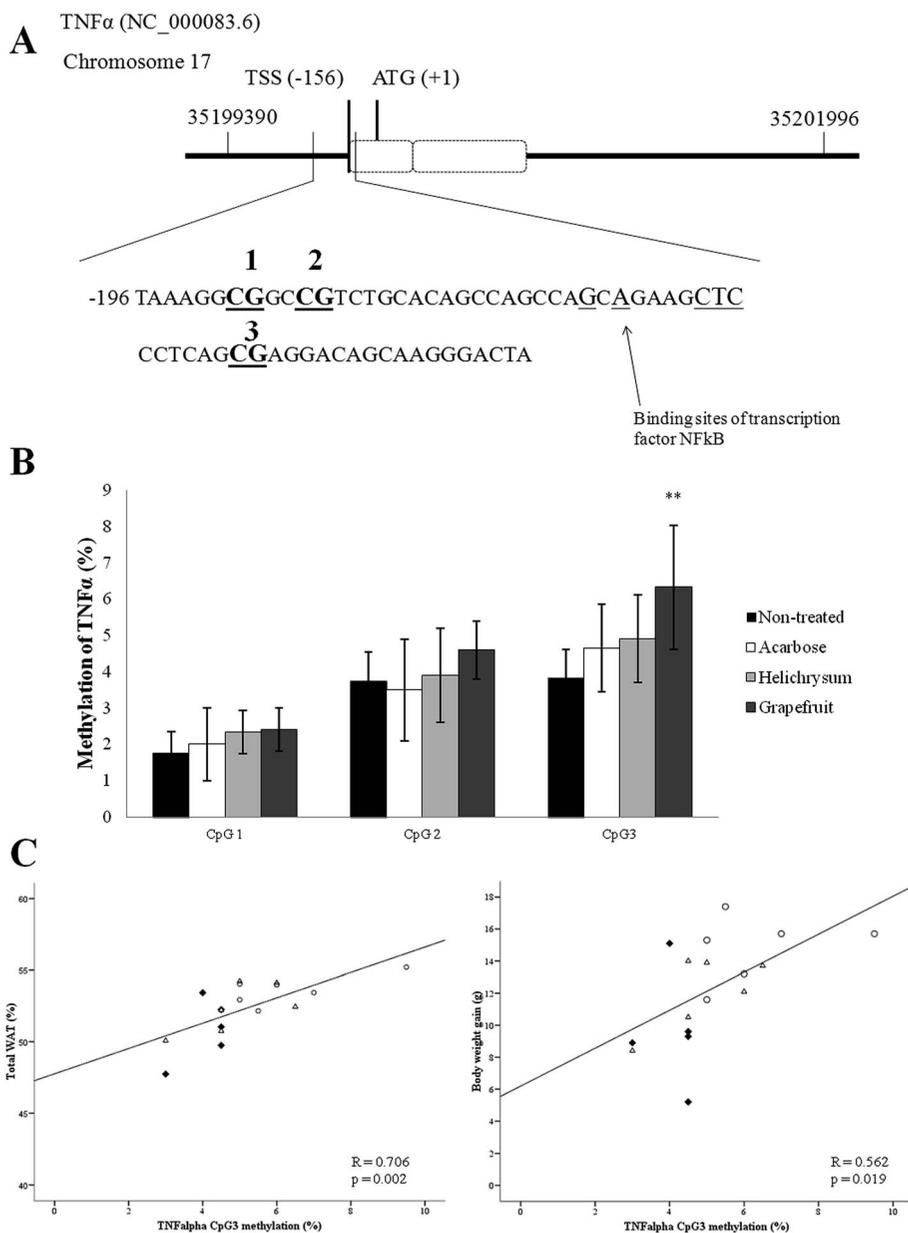
Persistent efforts to identify potential compounds that can be useful in the control and treatment of T2DM have been devoted. In this sense, flavonoids are attractive candidates because of their widespread presence in nature and their potential pharmacological effects.<sup>11</sup> Flavonoids are bioactive constituents abundantly present in the grapefruit and helichrysum extracts. Different *in vitro* and *in vivo* studies have shown beneficial roles of flavonoids in inflammation,<sup>6,10</sup> hyperlipidemia<sup>24,25</sup> and diabetes.<sup>11</sup> With regard to the antidiabetic effects of the 6 week supplementation with grapefruit and helichrysum extracts, lower fasting blood glucose levels were found when compared with the non-treated *db/db* mice. At the end of the experimental period, we noted that the mice were already in a state of diabetes with symptoms that caused severe metabolic disturbances. However, the grapefruit extract administration apparently delayed cachexia associated with diabetes and showed slightly higher levels of RQ, suggesting a better management of the carbohydrate metabolism. The improvement in metabolic glucose utilization as an energy source was significantly correlated with the results obtained from the OSTT. Concerning the molecular mechanisms implicated, previous studies have shown that flavonoids can improve glucose metabolism by stimulating peripheral glucose uptake in adipose tissue.<sup>26,27</sup> GLUT4, an insulin sensitive glucose transporter, plays an important role in glucose transport in peripheral tissues.<sup>28</sup> Thus, hesperidin and naringin enhanced GLUT4 expression in WAT in type-2 diabetic mice.<sup>29</sup> Likewise, naringenin improved insulin-stimulated glucose uptake in 3T3-L1 cells.<sup>30</sup> Kaempferol and kaempferol 3-neohesperidoside (the flavonoid glycoside) showed insulinomimetic effects and stimulation of glucose uptake in differentiated 3T3-L1 adipocytes.<sup>31,32</sup> Conversely, in our study no significant differences

among the experimental groups were found in the expression of GLUT4 and InsR in adipose tissue.

However, although not statistically significant, a slight increase in the betatrophin gene expression of supplemented groups was found. The expression of betatrophin in adipose tissue may be an indicator of the action of pancreatic  $\beta$ -cells,<sup>33</sup> but the mechanisms involved in the control of the proliferation of pancreatic  $\beta$ -cells are still unclear.<sup>33</sup>

Furthermore, it has been reported that flavonoids may directly act on pancreatic  $\beta$ -cells.<sup>34</sup> In an *in vitro* study, naringenin downregulated the expression of GCK and Ins1, suggesting an enhancement of glucose-stimulated insulin secretion and glucose sensitivity in INS-1E cells.<sup>35</sup> In the present study, no significant differences were found in the expression of GCK and Ins1 in the pancreas, which might be due to different factors like the dosage used, and the time or the period of supplementation.

In the liver, glucose is phosphorylated by glucokinase (GCK) and, depending on the cell's requirements, can be stored *via* glycogenesis activation (PEPCK) or oxidized to generate ATP (glycolysis). In this sense, previous studies have shown that dietary supplementation with hesperidin and naringin improved hyperglycemia by altering the expression of genes involved in glycolysis and gluconeogenesis in the liver.<sup>14,15</sup> Jung *et al.*<sup>29</sup> have shown an increased liver expression of GCK after administering hesperidin and naringin in *db/db* mice, whereas naringin reduced the expression of PEPCK and G6Pase. Moreover, the inhibition of PEPCK decreased the hepatic glycogen content and finally improved the glucose metabolism. Park *et al.*<sup>14</sup> have found a significantly lower expression of PEPCK in the liver of *db/db* mice supplemented with *citrus* extract. However, they did not find significant differences in the G6Pase expression. In our study, no significant differences were observed in the expression of PEPCK and G6Pase in the liver. Meanwhile, the liver GCK expression was significantly higher in the mice supplemented with grapefruit and helichrysum extracts, suggesting that the antidiabetic effects may occur in the liver by modulating the enzymes involved in glycolysis and gluconeogenesis. Thus, there is a negative correlation between blood glucose levels and liver GCK expression ( $p < 0.001$ ),



**Fig. 3** (A) Nucleotide sequences of the CpG island in the TNF $\alpha$  promoter and exon regions showing individual CpG dinucleotides. (B) Effect of helichrysum and grapefruit extracts on the methylation levels of individual CpG dinucleotides in the TNF $\alpha$  promoter region in adipose tissue. Correlation analysis between percentage of DNA methylation and (C) Total WAT (%) and (D) body weight gain (g). Results are expressed as mean  $\pm$  SD. Statistical analysis was performed using ANOVA, and the Dunnett's test was used to analyze differences in the mean of each group compared with the non-treated group. *R*, Pearson's correlation coefficient. ( $n = 6$ ). \*\* $p < 0.01$ . ( $\blacklozenge$  non-treated group,  $\Delta$  helichrysum group, and  $\circ$  grapefruit group).

suggesting that the decrease of glucose levels may be related to the increase of liver glucose sensitivity.

Several studies have reported that the down-regulation of inflammatory cytokine genes, including TNF $\alpha$  and MCP1, protects against the development of insulin resistance and hyperglycemia in obese mice.<sup>36–38</sup> Flavonoids might also act by interfering with the secretion of pro-inflammatory cytokines, thus improving the state of T2DM and obesity.<sup>10</sup> In this sense, mice supplemented with kaempferol showed an inhibition of proinflammatory gene expression by modulating the NF- $\kappa$ B

signaling cascade.<sup>39</sup> Likewise, Park *et al.*<sup>40</sup> have shown that kaempferol also inhibited COX2, iNOS and MCP1 gene expression in the kidney of aged Sprague-Dawley rats. Our data indicates that the supplementation with grapefruit and helichrysum extracts seems to have a favorable effect on the inflammatory status of *db/db* mice. In cultured cells, lipopolysaccharide (LPS)-stimulated macrophages treated with naringenin presented lower expression of TNF $\alpha$  and IL-6.<sup>41</sup> Several studies in animals analyzing the effects of *citrus* flavonoids have also shown a preventive effect on obesity- and diabetes-associated

inflammation.<sup>11,24,25</sup> Thus, mice treated with naringin showed lower serum TNF $\alpha$  levels,<sup>42</sup> whereas naringenin and naringin suppressed the activation of NF- $\kappa$ B.<sup>43</sup> Although the inflammatory pathways regulated by these flavonoids have not been fully elucidated, a recent study suggested that local upregulation of TNF $\alpha$  in the intestine was more sensitive than circulating cytokine levels.<sup>44</sup> Recent studies have found that TNF $\alpha$  is a key player in adipose tissue chronic inflammation, inducing the activation/inhibition of signaling cascades that perpetuate the inflammatory status and cause insulin resistance and hyperlipidemia by activating NF- $\kappa$ B.<sup>45</sup> TNF $\alpha$  is usually overexpressed in adipose tissue in different animal models of obesity and insulin resistance.<sup>46</sup>

Concerning epigenetic modifications, DNA methylation may influence the pathogenesis of T2DM and inflammation,<sup>1,47</sup> and dietary factors are a major aspect of the environment that may influence DNA methylation.<sup>48</sup> One of the epigenetic modifications of the TNF $\alpha$  gene is DNA methylation.<sup>49</sup> In this sense, we measured the methylation pattern of the promoter and first exon of TNF $\alpha$ . The results suggest that the DNA methylation levels of TNF $\alpha$  were higher in the *db/db* mice supplemented with the grapefruit extract. Interestingly, we have found correlations between DNA methylation in the CpG3, body weight gain and the percentage of WAT. Previous studies of our group have evidenced the role of dietary factors on the modulation of TNF $\alpha$  DNA methylation,<sup>50</sup> and have reported that the promoter methylation levels of TNF $\alpha$  could be used as an epigenetic biomarker for identifying the response to a low-calorie diet in obese women.<sup>50</sup>

To date, no study with *citrus* flavonoids and kaempferol has analyzed their effects on DNA methylation. However, other bioflavonoids, such as quercetin, fisetin, myricetin and tea catechins, have been reported to exert an effect on this epigenetic mechanism.<sup>9,51</sup>

These results suggest that epigenetic changes in TNF $\alpha$  could subsequently contribute to ameliorate inflammation and finally improve insulin resistance-induced hyperglycemia. The supplementation with helichrysum and grapefruit extracts shows beneficial effects against diabetes and obesity associated inflammation in *db/db* mice. These changes may be due, at least in part, to small epigenetic modifications that can be induced by the flavonoids and other compounds found in the natural extracts. Regarding the implication of inflammation in DNA methylation patterns,<sup>52</sup> flavonoids could be an interesting therapeutic tool in the management of this situation. Thus, defining the role of epigenetic regulation of TNF $\alpha$  may lead to new therapeutic strategies for these metabolic diseases through modulation of the inflammatory status.<sup>53</sup> However, more detailed studies at the molecular and cellular levels are needed to determine how both extracts exert their antidiabetic activity as well as the individual compounds with an increased effect.

In summary, helichrysum and grapefruit extracts modulate hyperglycemia and TNF $\alpha$ -mediated inflammation in a diabetic model. Advances in this area may open the door to recognize the epigenetic regulatory role of different bioactive compounds involved in the metabolic control and the conditions that facilitate DNA methylation.

## Abbreviations

ActB	beta-Actin
AUC	Area under the curve
COX2	Cyclooxygenase-2
EE	Energy expenditure
ELISA	Enzyme-linked immunosorbent assay
G6Pase	Glucose 6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCK	Glucokinase
GLUT4	Glucose transporter-4
InsR	Insulin receptor
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin resistance
MCP1	Monocyte chemoattractant protein-1
NF- $\kappa$ B	Nuclear factor-kappaB
OSTT	Oral starch tolerance test
PEPCK	Phosphoenolpyruvate carboxykinase
RQ	Respiratory quotient
RT-PCR	Reverse transcription and quantitative real-time polymerase chain reaction
SGLT1	Sodium-dependent glucose transporter-1
T2DM	Type 2 diabetes mellitus
TNF $\alpha$	Tumor necrosis factor- $\alpha$
WAT	White adipose tissue

## Acknowledgements

We thank Línea Especial (LE/97) from the University of Navarra (Spain), CIBERobn from Madrid (Spain) and Biosearch S.A. within the framework of the CENIT PRONAOS Program granted by the Center for Industrial Technological Development (CDTI, initiative INGENIO 2010, Spain). We also thank “Asociación de Amigos” of the University of Navarra for the predoctoral grant awarded to A. L. de la Garza and Sara Palacios-Ortega. U. Etxeberria holds a predoctoral grant from the Department of Education, Universities and Research, Basque Government.

## References

- 1 B. T. Yang, T. A. Dayeh, C. L. Kirkpatrick, J. Taneera, R. Kumar, L. Groop, C. B. Wollheim, M. D. Nitert and C. Ling, *Diabetologia*, 2011, **54**, 360.
- 2 R. G. Baker, M. S. Hayden and S. Ghosh, *Cell Metab.*, 2011, **13**, 11.
- 3 M. Bajaj and R. A. Defronzo, *J. Nucl. Cardiol.*, 2003, **10**, 311.
- 4 M. Pasarica, O. R. Sereda, L. M. Redman, D. C. Albarado, D. T. Hymel, L. E. Roan, J. C. Rood, D. H. Burk and S. R. Smith, *Diabetes*, 2009, **58**, 718.
- 5 A. Jungbauer and S. Medjakovic, *Maturitas*, 2012, **71**, 227.
- 6 A. Leisherer, A. Mundlein and H. Drexel, *Vasc. Pharmacol.*, 2013, **58**, 3.
- 7 E. Ricciotti and G. A. FitzGerald, *Arterioscler., Thromb., Vasc. Biol.*, 2011, **31**, 986.
- 8 J. H. Park, D. A. Stoffers, R. D. Nicholls and R. A. Simmons, *J. Clin. Invest.*, 2008, **118**, 2316.

- 9 H. J. Kim, S. H. Kim and J. M. Yun, *J. Evidence-Based Complementary Altern. Med.*, 2012, 1.
- 10 M. H. Pan, C. S. Lai and C. T. Ho, *Food Funct.*, 2010, 1, 15.
- 11 P. V. Babu, D. Liu and E. R. Gilbert, *J. Nutr. Biochem.*, 2013, 24, 1777.
- 12 A. Garcia-Lafuente, E. Guillaumon, A. Villares, M. A. Rostagno and J. A. Martinez, *Inflammation Res.*, 2009, 58, 537.
- 13 A. L. de la Garza, U. Etxeberria, M. P. Lostao, B. San Roman, J. Barrenetxe, J. A. Martinez and F. I. Milagro, *J. Agric. Food Chem.*, 2013, 61, 12012.
- 14 H. J. Park, U. J. Jung, S. J. Cho, H. K. Jung, S. Shim and M. S. Choi, *J. Nutr. Biochem.*, 2013, 24, 419.
- 15 U. J. Jung, M. K. Lee, K. S. Jeong and M. S. Choi, *J. Nutr.*, 2004, 134, 2499.
- 16 R. C. Coelho, H. H. Hermsdorff and J. Bressan, *Plant Foods Hum. Nutr.*, 2013, 68, 1.
- 17 S. C. Gouveia and P. C. Castilho, *Rapid Commun. Mass Spectrom.*, 2010, 24, 1851.
- 18 J. M. Calderon-Montano, E. Burgos-Moron, C. Perez-Guerrero and M. Lopez-Lazaro, *Mini Rev. Med. Chem.*, 2011, 11, 298.
- 19 A. Sala, M. Recio, R. M. Giner, S. Manez, H. Tournier, G. Schinella and J. L. Rios, *J. Pharm. Pharmacol.*, 2002, 54, 365.
- 20 L. Herberg and D. L. Coleman, *Metabolism*, 1977, 26, 59.
- 21 D. F. Garcia-Diaz, J. Campion, F. I. Milagro, A. Lomba, F. Marzo and J. A. Martinez, *J. Physiol. Biochem.*, 2007, 63, 337.
- 22 M. M. Tai, *Diabetes Care*, 1994, 17, 152.
- 23 O. J. Switzeny, E. Mullner, K. H. Wagner, H. Brath, E. Aumuller and A. G. Haslberger, *Clin. Epigenet.*, 2012, 4, 19.
- 24 N. Siriwardhana, N. S. Kalupahana, M. Cekanova, M. LeMieux, B. Greer and N. Moustaid-Moussa, *J. Nutr. Biochem.*, 2013, 24, 613–623.
- 25 J. M. Assini, E. E. Mulvihill and M. W. Huff, *Curr. Opin. Lipidol.*, 2013, 24, 34.
- 26 M. Claussnitzer, T. Skurk, H. Hauner, H. Daniel and M. J. Rist, *Mol. Nutr. Food Res.*, 2011, 55(suppl. 1), S26.
- 27 P. H. Ducluzeau, L. M. Fletcher, H. Vidal, M. Laville and J. M. Tavares, *Diabetes Metab.*, 2002, 28, 85.
- 28 S. Huang and M. P. Czech, *Cell Metab.*, 2007, 5, 237.
- 29 U. J. Jung, M. K. Lee, Y. B. Park, M. A. Kang and M. S. Choi, *Int. J. Biochem. Cell Biol.*, 2006, 38, 1134.
- 30 A. W. Harmon and Y. M. Patel, *Biochem. Biophys. Res. Commun.*, 2003, 305, 229.
- 31 X. K. Fang, J. Gao and D. N. Zhu, *Life Sci.*, 2008, 82, 615.
- 32 C. N. Vishnu Prasad, S. Suma Mohan, A. Banerji and A. Gopalakrishnapillai, *Biochem. Biophys. Res. Commun.*, 2009, 380, 39.
- 33 P. Yi, J. S. Park and D. A. Melton, *Cell*, 2013, 153, 747.
- 34 K. Hanhineva, R. Torronen, I. Bondia-Pons, J. Pekkinen, M. Kolehmainen, H. Mykkanen and K. Poutanen, *Int. J. Mol. Sci.*, 2010, 11, 1365.
- 35 S. Bhattacharya, N. Oksbjerg, J. F. Young and P. B. Jeppesen, *Diabetes, Obes. Metab.*, 2014, 16, 602.
- 36 G. S. Hotamisligil, N. S. Shargill and B. M. Spiegelman, *Science.*, 1993, 259, 87.
- 37 K. T. Uysal, S. M. Wiesbrock, M. W. Marino and G. S. Hotamisligil, *Nature.*, 1997, 389, 610.
- 38 C. C. Chuang, A. Bumrungpert, A. Kennedy, A. Overman, T. West, B. Dawson and M. K. McIntosh, *J. Nutr. Biochem.*, 2011, 22, 89.
- 39 J. M. Kim, E. K. Lee, D. H. Kim, B. P. Yu and H. Y. Chung, *Age*, 2010, 32, 197.
- 40 M. J. Park, E. K. Lee, H. S. Heo, M. S. Kim, B. Sung, M. K. Kim, J. Lee, N. D. Kim, S. Anton, J. S. Choi, B. P. Yu and H. Y. Chung, *J. Med. Food.*, 2009, 12, 351.
- 41 L. W. Soromou, Z. Zhang, R. Li, N. Chen, W. Guo, M. Huo, S. Guan, J. Lu and X. Deng, *Molecules*, 2012, 17, 3574.
- 42 P. Pu, D. M. Gao, S. Mohamed, J. Chen, J. Zhang, X. Y. Zhou, N. J. Zhou, J. Xie and H. Jiang, *Arch. Biochem. Biophys.*, 2012, 518, 61.
- 43 S. J. Tsai, C. S. Huang, M. C. Mong, W. Y. Kam, H. Y. Huang and M. C. Yin, *J. Agric. Food Chem.*, 2012, 60, 514.
- 44 S. Ding, M. M. Chi, B. P. Scull, R. Rigby, N. M. Schwerbrock, S. Magness, C. Jobin and P. K. Lund, *PLoS One*, 2010, 5, 1.
- 45 M. A. Vazquez-Prieto, A. Bettaieb, F. G. Haj, C. G. Fraga and P. I. Oteiza, *Arch. Biochem. Biophys.*, 2012, 527, 113.
- 46 G. S. Hotamisligil, P. Arner, J. F. Caro, R. L. Atkinson and B. M. Spiegelman, *J. Clin. Invest.*, 1995, 95, 2409.
- 47 F. I. Milagro, M. L. Mansego, C. De Miguel and J. A. Martinez, *Mol. Aspects Med.*, 2013, 34, 782.
- 48 I. T. Johnson and N. J. Belshaw, *Food Chem. Toxicol.*, 2008, 46, 1346.
- 49 V. Kruys, P. Thompson and B. Beutler, *J. Exp. Med.*, 1993, 177, 1383.
- 50 P. Cordero, J. Campion, F. I. Milagro, E. Goyenechea, T. Steemburgo, B. M. Javierre and J. A. Martinez, *J. Physiol. Biochem.*, 2011, 67, 463.
- 51 W. Vanden Berghe, *Pharmacol. Res.*, 2012, 65, 565.
- 52 P. Stenvinkel, M. Karimi, S. Johansson, J. Axelsson, M. Suliman, B. Lindholm, O. Heimbürger, P. Barany, A. Alvestrand, L. Nordfors, A. R. Qureshi, T. J. Ekstrom and M. Schalling, *J. Intern. Med.*, 2007, 261, 488.
- 53 K. E. Sullivan, A. B. Reddy, K. Dietzmann, A. R. Suriano, V. P. Kocieda, M. Stewart and M. Bhatia, *Mol. Cell. Biol.*, 2007, 27, 5147.