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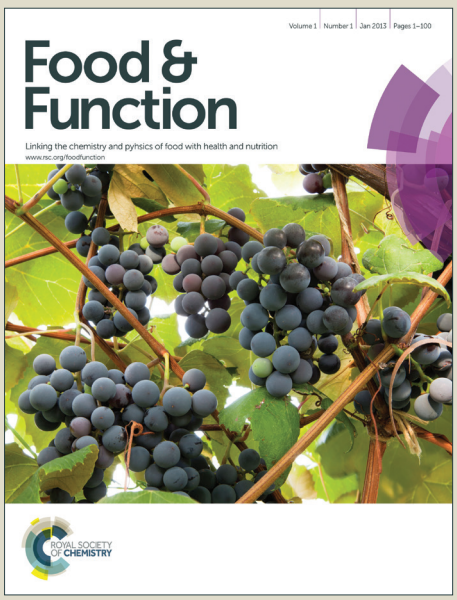

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Modulation of hyperglycemia and TNFα-mediated inflammation by helichrysum and grapefruit extracts in diabetic db/db mice

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ABSTRACT

Type-2 diabetes is associated with a chronic low-grade systemic inflammation accompanying an increased production of adipokines/cytokines by obese adipose tissue. The search of new antidiabetic drugs with different mechanisms of action, such as insulin sensitizers, insulin secretagogues and α-glucosidase inhibitors, have opened the focus for the potential use of flavonoids for 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/kg bw, n=8); helichrysum (1 g/kg bw, n=10) and grapefruit (0.5 g/kg bw, n=10) for 6 weeks of treatment. 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 pro-inflammatory genes (monocyte chemotactic protein-1, tumor necrosis factor-α, cyclooxygenase-2, nuclear factor-kappaB) in liver and epididymal adipose tissue. The site CpG3 of TNFα, 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 liver and the reduction of the expression of proinflammatory genes in liver and visceral fat. The hypermethylation of TNFα in adipose tissue may contribute to reduce the inflammation associated to diabetes and obesity.

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.[1] Insulin resistance (IR) is produced as soon as the pancreatic β-cells cannot compensate a reduced insulin function, leading to elevated circulating glucose levels.[2] Insulin inhibits gluconeogenesis in liver and reduces lipolysis in adipose tissue.[3] Likewise, adipose tissue in diabetes and obesity is characterized by hypertrophy, relative hypoxia, low-grade chronic inflammation and endocrine dysfunctions. [4] In this context, the pro-inflammatory cytokines, many of them secreted by the hypertrophied adipocytes, are controlled through transcription nuclear factor-kappaB (NFkB), whereby the inflammatory response can be down-regulated. [5] In addition, this transcription factor represents a link between inflammation and IR, as it is activated by factors known to promote IR and T2DM.[6] One important downstream target of NFkB is cyclooxygenase 2 (COX2), which catalyzes the production of prostaglandins, the key molecules in inflammation processes of the body.[7] Moreover, NFkB is involved in the expression of many cytokines, including TNFα.[5] 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.[8] Recently, epigenetic modifications have also been implicated in disease-associated changes influencing gene expression.[9]

Targeting the reduction of chronic inflammation is a beneficial strategy to combat several metabolic diseases, including T2DM.[10] Thus, numerous studies have underlined the interest in finding nutritional factors that may help to prevent or treat these diseases.[10, 11] In this sense, flavonoids can act through a variety of mechanisms to prevent and attenuate inflammatory responses.[12] These bioactive compounds can also improve glucose metabolism by stimulating

peripheral glucose uptake in different tissues.[11] In relation to this, grapefruit extract is rich in flavanones (i.e., naringenin-7-*O*-rutinoside) and flavonols (i.e., kaempferol rutinoside).[13] Previous studies have reported that *citrus* flavonoids have many pharmacological activities, including anti-inflammatory properties.[14] Thus, an improvement in hyperglycemia by the hepatic enzymes involved in glucose metabolism was reported in groups of mice, whose diet was supplemented with naringin.[15] Furthermore, a recent study reported that orange juice appears to mediate the inflammatory response, both gene expression and plasma level.[16]

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.[13] Likewise, *helichrysum* genus has been found to have several biological activities, such as anti-inflammatory properties, which have been attributed to different flavonoids.[17] Thus, beneficial roles of kaempferol have been reported in inflammation, hyperglycemia and diabetes in different *in vitro* and *in vivo* models.[18] Additionally, some investigations have concluded that the anti-inflammatory activity of *Helichrysum italicum* may be explained by enzyme inhibition, free-radical scavenging activity and corticoid-like effects.[19] In this sense, our group previously demonstrated that helichrysum and grapefruit extracts ameliorated hyperglycemia by inhibiting α-glucosidase (a similar mechanisms as acarbose) and α-amylase enzyme activities and by decreasing SGLT1-mediated glucose uptake in the gut.[13]

Since inflammation in the adipose tissue plays a central role in obesity-related IR and T2DM, our research was conducted in a recognized model of obesity and diabetes, *db/db* mice, displaying characteristics such as overweight, hyperglycemia and hyperinsulinemia due to leptin receptor mutations.[20] 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 α-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.[13] Helichrysum extract contained phenolic acids and flavonoids as flavanones and flavonols subclasses, as previously described.[13] The flavanones found in higher proportion were naringenin-7-*O*-glucoside (3.9 mg/g extract) and naringenin diglycoside (1.2 mg/g extract). Kaempferol-3-*O*-glucoside (13.4 mg/g extract) is the flavonol that was found as a greater proportion. Likewise, grapefruit extract mainly contained naringenin-7-*O*-rutinoside (5.2 mg/g extract) and naringenin (1 mg/g extract) as flavanone, and kaempferol-rutinoside (54.2 mg/kg extract) as flavonol.[13] Glucose was purchased from Sigma Chemicals (St. Louis, MO, USA) and starch (162.14 g/mol) 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/kg bw), n = 8; helichrysum group (1 g/kg bw), n = 10, and grapefruit group (0.5 g/kg bw), n = 10. The doses used were calculated comparing with the acarbose effect and based on the IC_{50} of the extracts, as described elsewhere.[13] 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). Animals were kept in an isolated room under a constantly regulated temperature between 21 and 23 °C, and controlled humidity (50±10%) in a 12h:12h 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 1st, 3rd and 6th weeks, fasting glucose was measured by a drop of blood from a tail vein. On the 5th week, respiratory quotient (RQ) and energy expenditure (EE) (kg/day/$bw^{3/4}$) measurements were performed by using an Oxylet equipment (Panlab, Barcelona, Spain), as previously reported.[21] 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, 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 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 at the 3rd week. After a 15-h fast, animals were orally administered by gastric intubation (5 ml/kg bw) with starch (2 g/kg bw in a 30% w/v solution) and acarbose (5 mg/kg bw), helichrysum (1 g/kg bw) and grapefruit (0.5 g/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 trips (Optium Plus, Abbott® Diabetes Care, Witney Oxon, UK). The IPGTT was performed at the 6th week. After a 15-h fast, mice were injected intraperitoneally with glucose (2 g/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, and the areas under the curve (AUC) were determined by the trapezoidal rule approach.[22]

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). 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 into 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 15 minutes at 4 °C. The complete liquid was transferred to clean tubes and was neutralized with 100 µl 1 M Tris pH 7.5. The pancreatic insulin content was analyzed by enzyme-linked-immunosorbent assay (ELISA) following the protocol described by the manufacturer (Mercodia AB, Uppsala, Sweden). The absorbance was calculated with the appropriate dilution factor. Pancreatic insulin values were corrected for protein concentration, as determined by Bradford assay with bovine

serum albumin as a standard (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Finally, insulin content (ng/mL) was normalized by the protein content (μg/mL).

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 predesigned 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 (NFkB), 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. mRNA levels were normalized with 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 $2^{-\Delta\Delta Ct}$ 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 Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE, SA). The stock solution of DNA samples was 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 (Picodrop Limited, Hinxton, UK). All procedures were carried out according to the manufacturer's protocols.

PCR and methylation analysis by DNA pyrosequencing. Quantitative methylation analyses were performed by pyrosequencing of bisulfite-converted DNA using the PyroMark Q24 (Qiagen). PCR was carried out in 25 µl reaction mixtures with 12.5 µl PyroMark 2x PCR master mix, 0.15 nM of primers for TNFα, 5'-GGAAGTTTTAGAGGGTTGAATGAGA- 3' (forward), 5'-CTAACTAATCCCTTACTATCCT-3' (reverse), 2.5 µl CoralLoad Concentrate 10x (Qiagen) and 1 µl of DNA samples after bisulfite conversion, at concentration of 10 ng/µl. 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; and a 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 of quantification of CpG methylation levels were performed based on a protocol described elsewhere.[23] 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 One-Way ANOVA test followed by Dunnett's post hoc test. The two-tailed Pearson test was used to assess selected

206 correlations among variables. A level of probability of $p<0.05$ was set as statistically significant.
207 All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).
208
209
210 **Results**
211 **Food intake, body weight gain and body fat mass.** After the end of the supplementation
212 period, the grapefruit group gained more body weight ($p < 0.05$) than the non-treated group
213 (**Table 1**). Although not statistically significant, the percentage of total adipose tissue (WAT)
214 was slightly higher in the treated groups (**Table 1**). Furthermore, significant differences were
215 found in spleen weight between the acarbose ($p < 0.05$) and helichrysum ($p < 0.01$) groups when
216 compared to the non-treated group, whereas liver weights were similar in all groups (**Table 1)**.
217 Regarding food efficiency, the average daily food intake throughout the experimental period
218 remained unaltered in the acarbose group and after helichrysum and grapefruit extract
219 administration (**Table 1**).
220 **Respiratory quotient and energy expenditure.** The respiratory quotient (RQ) assessment,
221 which is used to evaluate the relative oxidation of substrates, evidenced that the grapefruit group
222 ($p < 0.05$) improved carbohydrate oxidation when compared with the non-treated group (**Table**
223 **1**). Otherwise, there were no differences among groups with respect to energy expenditure (EE),
224 suggesting that the possible effect of helichrysum and grapefruit extracts in glucose metabolism
225 did not significantly affect thermogenesis (**Table 1**).
226 **Blood glucose and serum parameters.** Glycemia levels at baseline and at the end of the
227 supplementation period are shown in **Table 1**. All mice were diabetic when the experiment
228 began ($x = 10 \pm 3$ mmol/L). 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 decreased the 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 statistical 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 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 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 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 to the non-treated group. No statistical differences were found in G6Pase, PEPCK and betatrophin mRNA levels in liver (**Table 2**). Interestingly, mRNA expression levels

of GCK in liver showed a negative correlation ($r = -0.692$, $p < 0.001$) with final blood glucose levels (mmol/L) (**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 liver and epididymal adipose tissue. Thus, the hepatic mRNA levels of TNFα, MCP1, COX2 and NFkB decreased in the acarbose group and after the supplementation with both helichrysum and grapefruit extracts (**Table 2**). Statistical differences in the mRNA expression of TNFα, 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 TNFα was measured in epididymal adipose tissue (**Fig. 3**). Interestingly, a hypermethylation (Δ of methylation: 2.5%) was detected in the CpG 3 (CpG site + 5 bp) after supplementation with grapefruit extract ($p < 0.01$) (**Fig. 3B**). Moreover, TNFα 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 a widespread presence in nature and their potential pharmacological effects.[11] Flavonoids are

bioactive constituents abundant in the grapefruit and helichrysum extracts. Different *in vitro* and *in vivo* studies have shown beneficial roles of flavonoids in inflammation,[6,10] hyperlipidemia[24, 25] and diabetes.[11] 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 to 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. This 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 the adipose tissue.[26, 27] GLUT4, an insulin sensitive glucose transporter, plays an important role in glucose transport in peripheral tissues.[28] Thus, hesperidin and naringin enhanced GLUT4 expression in WAT in type-2 diabetic mice.[29] Likewise, naringenin improved insulin-stimulated glucose uptake in 3T3-L1 cells.[30] Kaempferol and kaempferol 3-neohesperidoside (the flavonoid glycoside) showed insulinomimetic effects and stimulation of glucose uptake in differentiated 3T3-L1 adipocytes.[31, 32] 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 no statistically significant, a slight increase in betatrophin gene expression of supplemented groups was found. The expression of betatrophin in adipose tissue may be an indicator of the action of pancreatic β-cells,[33] but the mechanisms involved in the control of the proliferation of pancreatic β-cells are still unclear.[33]

297 Furthermore, it has been reported that flavonoids may directly act on pancreatic β-cells.[34] In an
298 *in vitro* study, naringenin downregulated the expression of GCK and Ins1, suggesting an
299 enhancement of glucose-stimulated insulin secretion and glucose sensitivity in INS-1E cells.[35] In
300 the present study, no significant differences were found in the expression of GCK and Ins1 in
301 pancreas, which might be due to different factors like the dose used, the time or the period of
302 supplementation.
303 In liver, glucose is phosphorylated by glucokinase (GCK) and, depending on the cell's
304 requirements, can be stored via glycogenesis activation (PEPCK) or oxidized to generate ATP
305 (glycolysis). In this sense, previous studies showed that dietary supplementation with hesperidin
306 and naringin improved hyperglycemia by altering the expression of genes involved in glycolysis
307 and gluconeogenesis in liver.[14,15] Jung et al. [29] showed increased liver expression of GCK after
308 administering hesperidin and naringin in *db/db* mice, whereas naringin reduced the expression of
309 PEPCK and G6Pase. Moreover, the inhibition of PEPCK decreased the hepatic glycogen content
310 and finally improved the glucose metabolism. Park et al. [14] found a significantly lower
311 expression of PEPCK in the liver of *db/db* mice supplemented with *citrus* extract. However, they
312 did not find significant differences in G6Pase expression. In our study, no significant differences
313 were obtained in the expression of PEPCK and G6Pase in the liver. Meanwhile, liver GCK
314 expression was significantly higher in the mice supplemented with grapefruit and helichrysum
315 extracts, suggesting that the antidiabetic effects may occur in the liver by affecting the enzymes
316 involved in glycolysis and gluconeogenesis. Thus, there is a negative correlation between blood
317 glucose levels and liver GCK expression ($p < 0.001$), proposing that the decrease of glucose
318 levels may be related to an increase of liver glucose sensitivity.

Several studies reported that down-regulation of inflammatory cytokine genes, including TNFα or MCP1, protect against the development of insulin resistance and hyperglycemia in obese mice.[36-38] Flavonoids might also act by interfering with the secretion of pro-inflammatory cytokines, improving thus the state of T2DM and obesity.[10] In this sense, mice supplemented with kaempferol showed an inhibition of proinflammatory gene expression by modulating the NF-kB signaling cascade.[39] Likewise, Park et al.[40] showed 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 in *db/db* mice. In cultured cells, lipopolysaccharide (LPS)-stimulated macrophages treated with naringenin presented lower expression of TNFα and IL-6.[41] Several studies in animals analyzing the effects of *citrus* flavonoids have also shown a preventive effect on obesity- and diabetes-associated inflammation.[11,24,25] Thus, mice treated with naringin showed lower serum TNFα levels,[42] whereas naringenin and naringin suppressed the activation of NFkB.[43] Although the inflammatory pathways regulated by these flavonoids have not been fully elucidated, a recent study suggested that local upregulation of TNFα in intestine was more sensitive than circulating cytokine levels.[44] Recent studies have found that TNFα 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 NFkB.[45] TNFα is usually overexpressed in the adipose tissue of different animal models of obesity and insulin resistance.[46]

Concerning epigenetic modifications, DNA methylation may influence the pathogenesis of T2DM and inflammation[1,47] and dietary factors are a major aspect of the environment that may influence DNA methylation.[48] One of the epigenetic modifications of the TNFα gene is an

increase in DNA methylation.[49] In this sense, we measured the methylation pattern of the promoter and first exon of TNFα. The results suggest that the DNA methylation levels of TNFα were higher in the *db/db* mice supplemented with grapefruit extract. Interestingly, we have found correlations between DNA methylation in the CpG3 and body weight gain and the percentage of WAT. Previous studies of our group have evidenced a role of dietary factors on the modulation of TNFα DNA methylation[50] and have reported that the promoter methylation levels of TNFα could be used as an epigenetic biomarker concerning the response to a low-calorie diet in obese women.[50]

To date, no study with *citrus* flavonoids and kaempferol have 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.[9,51]

These results suggest that epigenetic changes in TNFα 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 associated to diabetes and obesity 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,[52] flavonoids could be an interesting therapeutic tool in the management of this situation. Thus, defining the role of epigenetic regulation of TNFα may lead to new therapeutic strategies for these metabolic diseases through the modulation of the inflammatory status.[53] 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 more effect.

In summary, helichrysum and grapefruit extracts modulate hyperglycemia and TNFα-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.

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Abbreviations

ActB, beta actin; AUC, area under curve; COX2, ciclooxigenase-2; EE, energy expenditure; ELISA, enzyme-linked-immunosorbent assay; G6Pase, glucose 6-phosphatase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GCK, glucokinase; GLUT4, glucose transporter-4; InsR, insulin receptor; IPGTT, intraperitoneal glucose tolerance test; IR, insulin resistance;

MCP1, monocyte chemotactic protein-1; NFkB, nuclear factor-kappaB; OSTT, oral starch torelance 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; TNFα, tumor necrosis factor-α; WAT, white adipose tissue.

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Table 1. Effects of flavonoid-containing extracts from helichrysum and grapefruit on body weight, tissues, and biochemical measurements.

	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/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/day/bw3/4)	122 ± 16	112 ± 4	111 ± 9	114 ± 15
Blood glucose (mmol/L)				
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 * mg protein)	0.78 ± 0.00	0.79 ± 0.01	0.80 ± 0.01	0.80 ± 0.01
HbA1C (ng/mL)	2.31 ± 0.12	2.23 ± 0.14	2.13 ± 0.15	2.17 ± 0.13
Triglycerides (mg/dL)	136 ± 10	127 ± 10	145 ± 9	139 ± 7
Creatinine (mg/dL)	0.42 ± 0.08	0.33 ± 0.11	0.41 ± 0.08	0.34 ± 0.06

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

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

		FOLD CHANGE			
Metabolism	**Gene description**	**Non-treated**	**Acarbose**	**Helichrysum**	**Grapefruit**
Glucose	GCK	1.0 ± 0.2	2.8 ± 0.2 ***	1.8 ± 0.2 *	1.8 ± 0.1 *
	G6Pase	1.0 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	1.3 ± 0.2
	PEPCK	1.0 ± 0.2	1.5 ± 0.4	1.3 ± 0.3	0.9 ± 0.2
	Betatrophin	1.0 ± 0.2	1.4 ± 0.4	1.2 ± 0.5	0.9 ± 0.2
Inflammation	TNFα	1.0 ± 0.2	0.3 ± 0.1 *	0.6 ± 0.2	0.4 ± 0.1 *
	MCP1	1.0 ± 0.1	0.4 ± 0.3 *	0.2 ± 0.1 **	0.3 ± 0.2 *
	COX2	1.0 ± 0.2	0.1 ± 0.1 ***	0.1 ± 0.1 ***	0.1 ± 0.1 ***
	NFkB	1.0 ± 0.2	0.5 ± 0.2 *	0.4 ± 0.1 **	0.4 ± 0.1 **

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

Table 3. Effects of flavonoid-containing extracts from helichrysum and grapefruit on mRNA expression in the epididymal adipose tissue. Genes related to glucose metabolism and inflammation.

		FOLD CHANGE			
Metabolism	**Gene description**	**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	TNFα	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 **
	NFkB	1.0 ± 0.3	0.9 ± 0.2	0.7 ± 0.3	0.8 ± 0.3

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

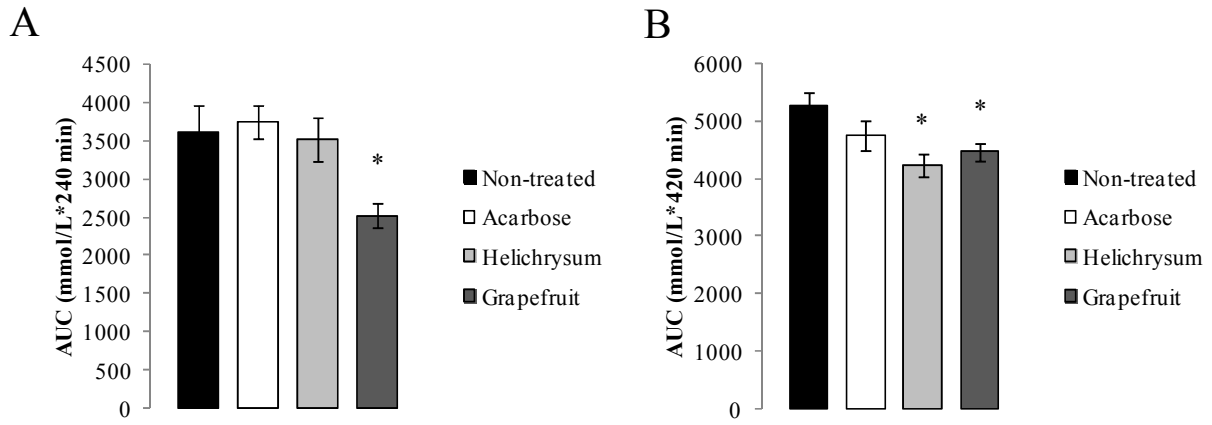


Fig. 1 Area under the curve (AUC) after the oral starch tolerance test - OSTT (A) and the intraperitoneal glucose tolerance test - IPGTT (B) in *db/db* mice. Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the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

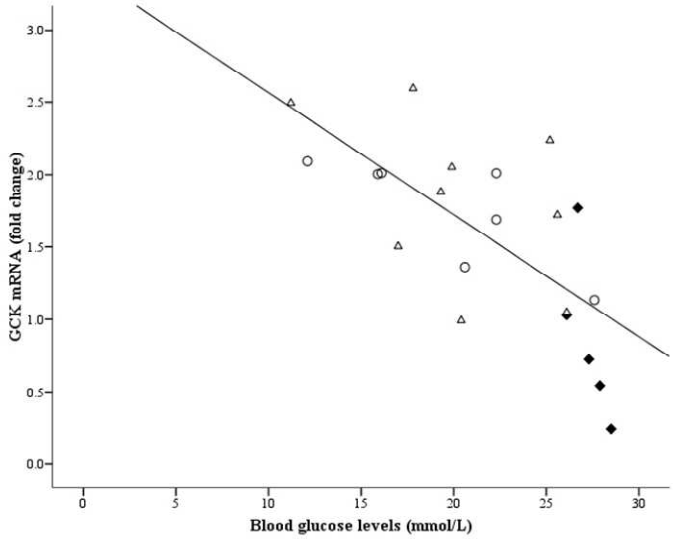


Fig. 2 Correlation analyses between GCK gene expression in liver (fold change) and final blood glucose (mmol/L). R, Pearson's correlation coefficient. Results are expressed as mean. Non-treated (n = 5); helichrysum and grapefruit groups (n = 8). (♦ non-treated group, ∆ helichrysum group and ○ grapefruit group)

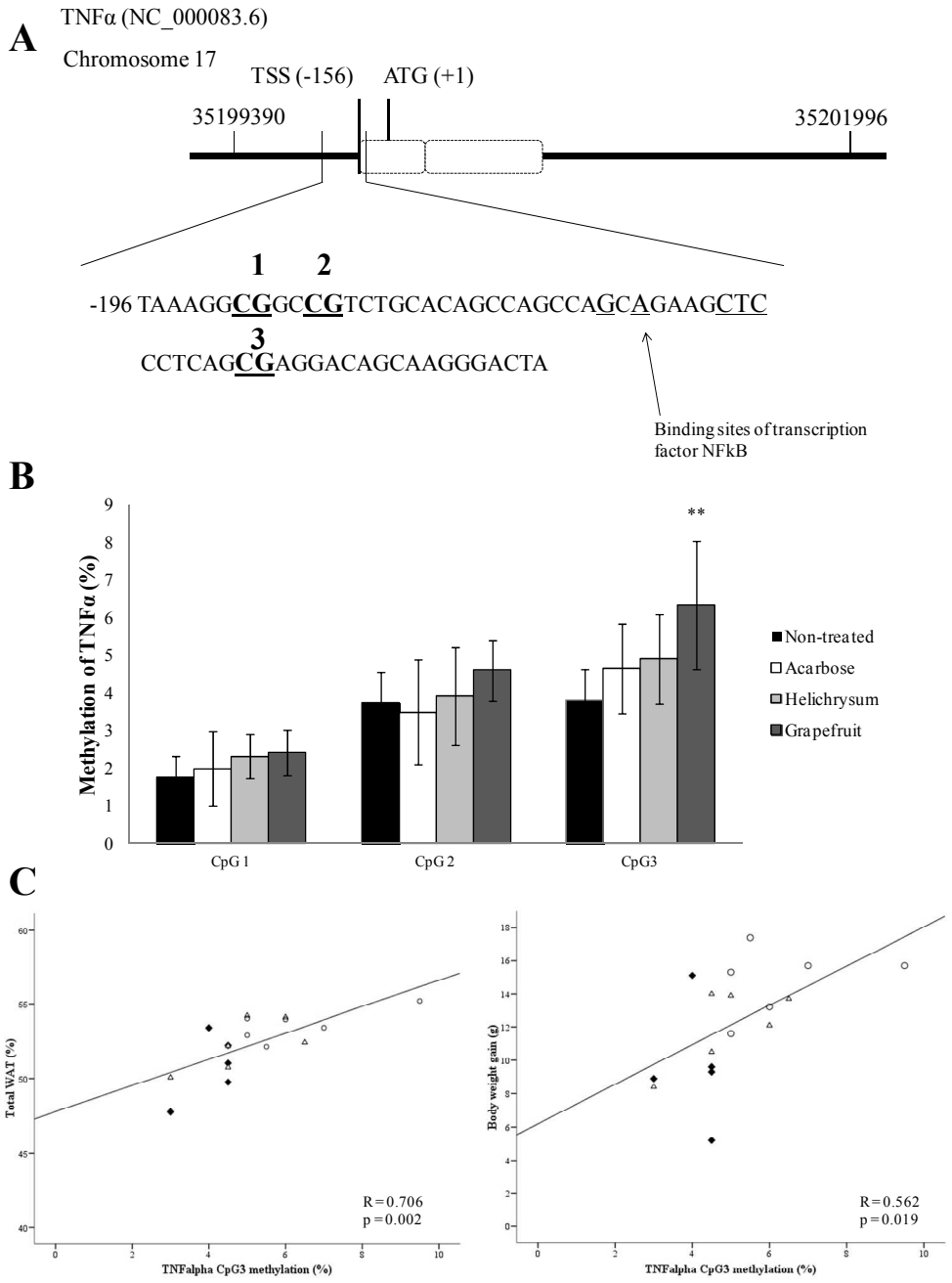
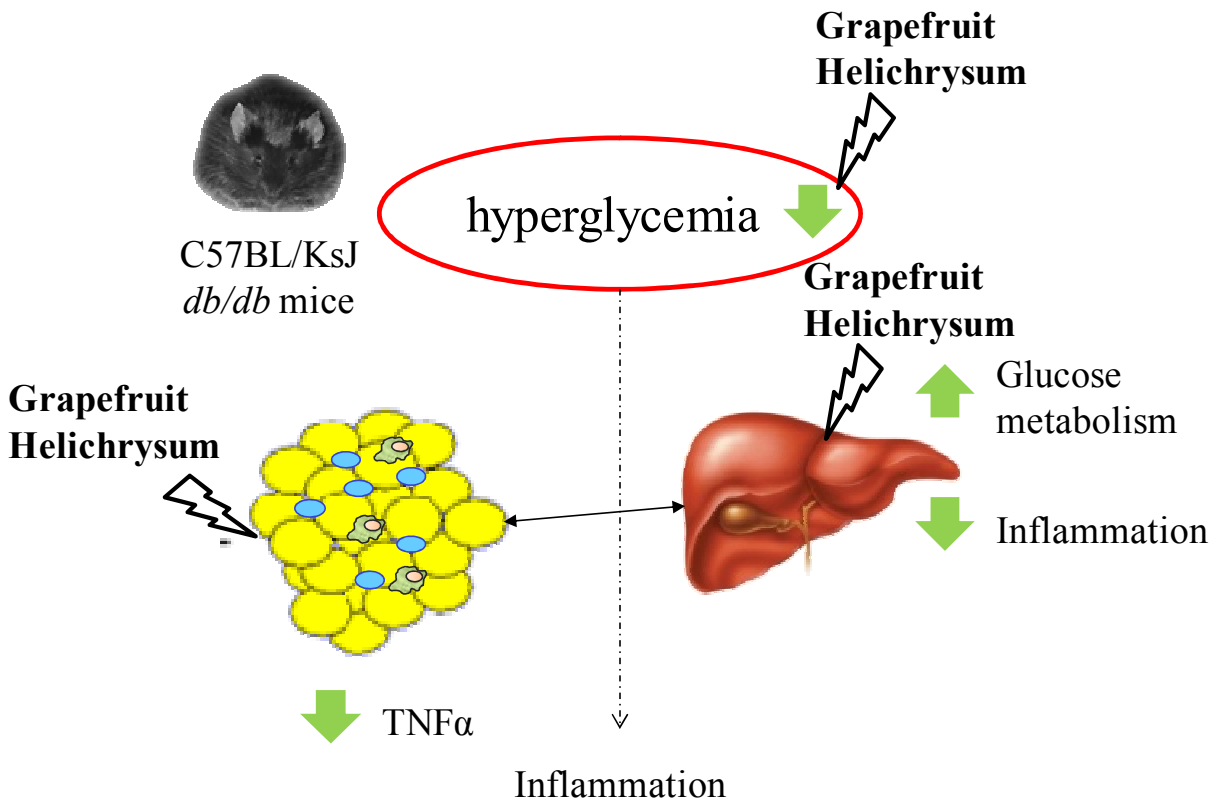
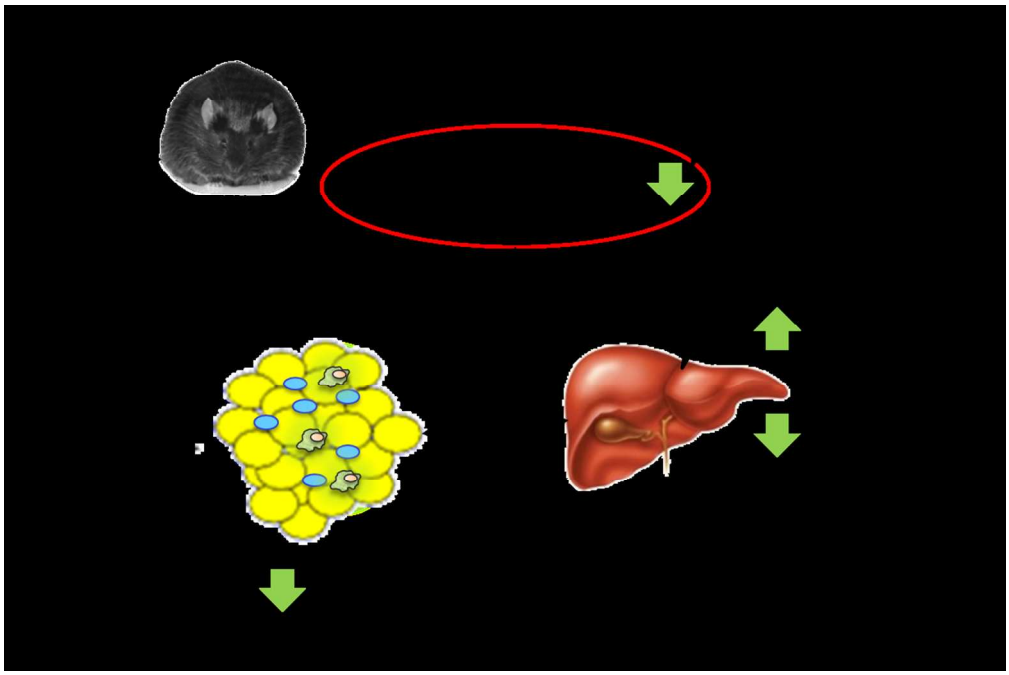


Fig. 3 Nucleotide sequence of the CpG island in the TNFα promoter and exon regions showing individual CpG dinucleotides **(A)**. Effect of helichrysum and grapefruit extracts in the methylation levels of individual CpG dinucleotides in the TNFα promoter in adipose tissue **(B)**. Correlation analyses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



528
529 **Graphical Abstract.**



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