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Green tea polyphenols positively impact hepatic metabolism of adiponectin-knockout lean mice

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ABSTRACT

Green tea (GT) has health benefits but the mechanism of its biological effects remains poorly known. Adiponectin has been considered a key adipocytokine for protection against hepatic lipid accumulation. We aimed to assess whether the effects of GT on hepatic lipid metabolism are mediated by adiponectin. For this purpose, we used wild-type lean mice and adiponectin-knockout mice fed with a standard diet and treated with 500 mg/kg of GT extract for 12 weeks. Adiponectin knockout mice had a slight increase in adiposity, and lipid synthesis while an impairment in insulin signaling. These effects were mitigated by GT supplementation independently of adiponectin. GT supplementation was able to stimulate energy expenditure, and oxidative lipid pathways (AdipoR2, SIRT1, AMPK and UCP2 expression) in an adiponectin independent-manner. These results indicate that the hepatoprotective effect of GT against lipid accumulation in non-alcoholic fatty liver disease can be partially mediated by adiponectin.

1. Introduction

Green tea (GT, *Camellia sinensis*) is considered one of the most commonly consumed beverages in the world and its regular consumption in the diet brings health benefits. *Camellia sinensis* plant is rich in polyphenol (Saeed et al., 2017) among them catechins belonging to the flavonoids class. Catechins are known by their key biological activities like antioxidant, anti-inflammatory, antiviral, antitumor, antidiabetic, antiobesity and hepatoprotective activities (Albuquerque, Marinovic, Morandi, Bolin, & Otton, 2016; Marinovic, Morandi, & Otton, 2015; Molina, Bolin, & Otton, 2015; Williamson, 2017).

Data from the literature have shown that GT plays an important role in metabolic tissues such as adipose tissue, muscle, and liver (Onishi et al., 2018; Otton et al., 2018; Rocha, Bolin, Cardoso, & Otton, 2016; Wimmer, Russell, & Schneider, 2015). However, the mechanism by which GT exerts their biological effects remains partially known. Adipocytokines produced by adipose tissue has been extensively studied as potential targets underlying the mechanism of action of GT-polyphenols.

Cho et al. (2007) have demonstrated that *in vitro* treatment of adipocytes with GT-polyphenols increases the expression and secretion of adiponectin and concomitantly suppress Kruppel-like factor 7 (KLF7) protein expression, an adiponectin suppressor (Essex & Mosawy, 2018). *In vivo* studies also support the ability of GT to stimulate adiponectin expression to combat obesity in an animal model (Rocha et al., 2016; Tian et al., 2013). Increased levels of adiponectin can be observed in humans after consumption of GT-catechins associated with the evident improvement of metabolic parameters related to obesity and diabetes (Chen, Liu, Chiu, & Hsu, 2016; Nagao et al., 2009). In addition, the hepatoprotective activity of GT against steatosis has been associated with increased serum adiponectin in mice through the LKB1/AMPK activation pathway (Santamarina et al., 2015).

Adiponectin is also known as Acrp30 and it was discovered (Scherer, Williams, Fogliano, Baldini, & Lodish, 1995) as a protein produced and secreted primarily by adipocytes. It is found as three main oligomeric forms: a low molecular weight trimer, a medium molecular weight hexamer and a high molecular weight multimeric. Currently, it is considered as an important mediator of the endocrine function of adipose tissue. The metabolic actions of adiponectin are mediated by specific receptors AdipoR1 and AdipoR2 activation, and, in addition to AdipoRs, T-cadherin has also been considered as a receptor for adiponectin (Yamauchi & Kadowaki, 2013). Both subtypes of receptors are expressed in the liver, however, AdipoR2 is the mainly expressed one (Combs & Marliss, 2014; Stern, Rutkowski, & Scherer,

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2016).

Plasma adiponectin level is decreased in obese subjects and it has also been observed in obese animal models associated with hepatic lipid accumulation (Stern et al., 2016). For that reason, the adiponectin effects on hepatic metabolism have been widely investigated. Several studies have evidenced that adiponectin reduces lipogenesis and increases β-oxidation through AMPK activation mediated by AdipoR1 (Awazawa et al., 2009; Yamauchi et al., 2002). The increase in lipid oxidation is also mediated by AdipoR2 through activation of PPAR-α (Yamauchi & Kadowaki, 2013). Moreover, both AdipoR1 and AdipoR2 stimulate the activity of intracellular ceramidase, decreasing the content of liver ceramides, while improving insulin sensitivity (Combs & Marliss, 2014; Vasiliauskaité-Brooks et al., 2017). Considering the potential effect of GT in the regulation of adiponectin levels and the essential metabolic role of this adipokine in regulating liver metabolism, in the present study we aimed to assess whether the health beneficial effects of GT extract on hepatic lipid metabolism are dependent on adiponectin by the use of an adiponectin-knockout mouse model (AdipoKO).

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), except those used in the preparation of buffer (Labsynth, Diadema, SP, Brazil). Powdered green tea extract was commercially acquired from Tovani-Benzaquen, São Paulo, SP, Brazil.

2.2. Animals and green tea extract supplementation

Male 8-week old wild-type (WT) C57BL/6J mice and Adiponectin-knockout mice (AdipoKO) (B6;129-Adipoqtm1Chan/J) were obtained from the Jackson Laboratories (Bar Harbor, ME). In AdipoKO mice exon 2 was replaced by a floxed neo cassette inserted by homologous recombination. Transcript was undetected in adipose tissue by Northern blot analysis of homozygous mutant mice (Ma, Cabrero, Saha, Kojima, Li, Chang, & Chan, 2002). All mice weighed 18–21 g at the beginning of the study without signal of illness. The general procedures were performed in accordance to standards established by the Ethics Committee on Animal Experimentation of Cruzeiro do Sul University (CEUA-UNICSUL No. 029/2016). All animals received humane care according to the criteria outlined in the national guidelines “Guide for the Care and Use of Laboratory Animals” used in accordance to the international law on the protection and use of animals (National Institutes of Health guide for the care and use of Laboratory animals - NIH Publications No. 8023, revised 1978). All mice were housed under constant temperature (24 ± 2 °C) and 12-hour light/dark cycle for a one-week period of acclimatization, four mice/cage. A total of 32 mice were randomly divided into 4 groups (8 mice/group); (i) control wild-type (Cont); (ii) green tea wild-type (GT); (iii) control adipoKO (Cont AdipoKO), and (iv) green tea AdipoKO (GT AdipoKO). All mice received a standard rodent diet (Nuvilab CR1 Radiated diet) and water *ad libitum* for 4 weeks. After this period, the animals started oral gavage with 500 mg/kg of body weight (BW) of GT extract (GT WT and GT AdipoKO groups) or water (Cont WT and Cont AdipoKO) and continued for 12 weeks (Monday through Friday) totaling 16 weeks of experimentation. The dosage of GT was based on previous *in vivo* studies of our group (Albuquerque et al., 2016; Otton et al., 2018; Rocha et al., 2016). Allometric scaling assists scientists to exchange doses between species during research, experiments, and clinical trials (Nair & Jacob, 2016). To determine the human equivalent dose (HED) we used the following equation: HED (mg/kg = Animal NOAEL mg/kg) × (Weight animal [kg]/Weight human [kg])$^{(1-0.67)}$. The NOAEL (no adverse effect level) for GTE in mice is = 500 mg/kg BW (Chan et al., 2010). The dose calculated by factor method applies an exponent for body surface area, which account for the difference in metabolic rate, to convert doses between animals and humans. Thus: HED = 500 mg/kg × (0.022 kg/70 kg)$^{0.33}$ = 34.9 mg/kg or 2.44 g for a 70 kg human.

Total phenolic content (TPC) was determined by using Folin-Ciocalteu’s phenol reagent, gallic acid (99% purity, Sigma) and anhydrous sodium carbonate as previously described (Rocha et al., 2016). The concentration of polyphenols and catechins in our GT extract was 39% and 30%, respectively, as obtained by the HPLC analysis described by Rocha (Rocha et al., 2016). Briefly, the GT extract and standards (EC, EGCG, EGC, ECG, catechin, quercetin and caffeine) were analysed in an analytical LC (Varian 210) system with a ternary solvent delivery system equipped with an auto-sampler, a photodiode array detector (PDA) monitored at λ = 200–800 nm. Catechins were quantified at 280 nm, quercetin at 250 nm and caffeine at 270 nm by using external standard calibration curves (0.5–100 μg/mL for catechins and 0.5–20 μg/mL for quercetin and caffeine). The catechins, quercetin, and caffeine were identified in green tea extract by comparing their retention time with those of standard solutions. HPLC analysis showed that the GT extract was a mixture of several catechins (EGC, EC, EGCG, and ECG). The sum of EGC and EGCG contributed with more than 85% of the catechin mixture in the extract. The content of caffeine in the extract was 0.4% by dry weight. GT extract was weighed daily and then solubilized in water at 70 °C to be subsequently administered by gavage to mice prior to the feeding period (18:00–19:00 h) in a final volume of 100 μl. No adverse effects were observed in mice after GT treatment. At the end of the experimental period, the mice were euthanized by decapitation between 9:00 and 12:00 h. A total of 8 mice per group was used for all analysis carried out in this study.

2.3. Assessment of insulin sensitivity

Fasting glucose was measured on the 14th week through the experimental protocol. Animals were fasted overnight (8 h), and in the morning, the blood was withdrawn via a small cut in the distal end of the animal’s tail. Blood glucose was measured using infinity glucose monitors and strips (US Diagnostics, Indianapolis, IN, USA). A week prior to euthanasia glucose tolerance test (GTT) was performed in fasted mice (12 h) by assaying blood glucose at various times before and after i.p. glucose injection (1 g/kg of body weight in a 20% solution of glucose in saline 0.9%). Insulin tolerance test (ITT) was also performed in fasted mice (6 h) by assaying blood glucose at various times before (0 min) and after i.p. insulin injection (Humulin R, Lilly, 0.5 IU/kg of body weight) between 2:00 and 5:00p.m. Blood samples were obtained at various time points (0, 5, 10, 15, 30, and 90 min) from the tail vein, and glucose was measured using infinity glucose monitors and strips. The tests were performed on different days, one on Monday (GTT) and another one (ITT) on Thursday in the same week. Total area under the curve (AUC) was calculated.

2.4. Plasmatic adiponectin and lipid level

Adiponectin levels were measured using Multiplex MAP magnetic bead-based. Mouse Adiponectin Single Plex (Cat. No. MADPN-MAG-70K-01) (Millipore, Billerica, MA, USA) based on the MAGPIX xMAP technology and following the manufactureŕs instruction. Mouse Adiponectin Single Plex was analyzed following 2 h of incubation.

Plasma levels of triglycerides (TG), total cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using commercially available kits (Bioclin, Minas Gerais, Brazil). For liver TG and total cholesterol quantification, samples (approximately 50 mg) were placed into 1.5 ml-tubes containing isopropyl alcohol, lysed by ultrasonication in a Vibra Cell apparatus (Connecticut, USA), centrifuged for 10 min, 10,000*g* at 4 °C and the supernatant was collected and transferred to a new tube, and analyzed using a TG and total cholesterol assay kit. Results were normalized by 50 mg of the liver mass.

2.5. Histological analysis and metabolic measurement

After 16 weeks into the experimental protocol animals were euthanized by decapitation and brown adipose tissue (BAT) and different white adipose tissue (WAT) depots - subcutaneous and epididymal - were rapidly removed. All fat pad depots collected were weighed immediately after removal. Body adiposity index was calculated with the sum of all the fat pad depots per animal and expressed per grams (Rogers & Webb, 2005). Liver samples were removed and rapidly frozen using dry ice, shortly thereafter were stored at −80 °C for posterior analysis of mRNA expression. A small liver sample (10 mg) was also freshly fixed in 4% paraformaldehyde–phosphate-buffered saline (0.137 M NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, pH 7.4) for 24 h. Fixed samples were dehydrated by sequentially increased ethanol concentrations, cleared in xylene, and then embedded in paraffin. The embedded samples were sectioned (5 μM) and stained with hematoxylin and eosin (H&E) and analyzed by optical microscopy (ProgRes, Jenoptik optical system, Germany). The remaining liver samples were immediately frozen and then stored at −80 °C.

Whole-body oxygen consumption was measured using the comprehensive laboratory animal monitoring system (CLAMS, Columbus Instruments). Briefly, mice were housed singly in CLAMS cages and acclimated for 24 h, and data on oxygen consumption (VO2) and respiratory exchange ratio (RER) were recorded during the 24 h period with the temperature at 22 °C.

2.6. RNA preparation and RT-qPCR

The expression levels of key genes in the lipid metabolizing pathway were measured using real-time quantitative PCR (RT-qPCR). RNA extraction, reverse transcription, and RT-qPCR were the same as in our previous study (Otton et al., 2018). Briefly, total RNA (2–5 μg) was treated with 1U DNase I and cDNA was synthesized using random primers in a 20 μl reaction containing 1 mM of each dNTP and 200 U SuperScript II RNase H–reverse transcriptase at 42 °C for 50 min, following the manufacturer's instructions. The quantification of gene expression was performed by RT-qPCR in a total volume of 10 μl, containing 250 nM of each primer (sense and antisense) (Table 1), 30 ng of cDNA and Maxima 1 × EVA-Green Master Mix. Gene expression was assessed in an Agilent AriaMx Real-Time PCR under the following conditions: 95 °C −15 min, and 40 cycles of 95 °C −15 s, 60 °C −20 s, and 72 °C −30 s. Amplicons dissociation curves were used for checking primer specificity. NTCs were included on each plate to detect PCR contamination (NTC > 40 Cq). The expression level of target mRNA was normalized to the mRNA level of reference gene 18S. 18S mRNA level was stably expressed, and their abundances show strong correlation with the total amounts of mRNA present in the samples. The linear amount of the target gene expression to the internal standard was calculated by $2^{-\Delta\Delta Cq}$ Relative and normalized fold expression values were calculated manually in Microsoft Excel from Cq values imported from AriaMx (Agilent). Expression data were imported to GraphPad Prism 6.0 (GraphPad Software, Inc.).

2.7. Western blotting

Proteins from liver were extracted (n = 4 per group) by homogenizing in solubilization buffer [1% Triton X-100, 100 mM Tris–HCl, (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride] at 4 °C with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY). Insoluble material was removed by centrifugation for 20 min at 12.000*g*. The protein concentration in the supernatants was determined by the Bradford dye-binding method. Samples containing 30–60 μg of protein extract were separated by 10–12% SDS-PAGE gel, transferred to nitrocellulose membranes (250 mA for 2 h), blocked in 5% non-fat milk diluted in TBS-T buffer for 1 h at room temperature and incubated overnight at 4 °C with the following primary antibodies: SIRT1 (1:1000, sc-74465, Santa Cruz Biotechnology), UCP2 (1:1000, sc-390189, Santa Cruz Biotechnology), PPARα (1:1000, sc-398394, Santa Cruz Biotechnology), p-AMPK (1:1000, sc-33524, Santa Cruz Biotechnology), AMPK (1:1000, sc-74461, Santa Cruz Biotechnology), IRβ (1:1000, sc-711, Santa Cruz Biotechnology), PI3 Kinase p85 (1:1000, #4282, Cell Signaling Technology), Phospho-Akt (Ser473) (1:1000, #9271, Cell Signaling Technology) AKT (1:1000, #9272, Cell Signaling Technology). Detection was performed by Amersham Imager 680 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) after incubation with peroxidase-labeled secondary antibody (1:5000 for 1 h at room

Table 1
Nucleotide sequences of primers used for RT-qPCR amplification.

Gene (MUS Musculus)	Primer sense (5′ – 3′)	Primer anti-sense (3′-5′)	NCBI reference sequence
18S rRNA	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG	NR_003278.3
Pparg	TCAGCTCTGTGGACCTCTCC	ACCCTTGCATCCTTCACAAG	NM_001127330.2
Ppargc1a	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC	NM_008904.2
Sirt1	TCTCCTGTGGGATTCCTGAC	ACACAGAGACGGCTGGAACT	NM_019812.3
Ppard	TCAGCTCTGTGGACCTCTCC	ACCCTTGCATCCTTCACAAG	NM_011145.3
Pik3r1	AAAAATGGCGACGACTTACG	TTGCACTGGATTTGCATGAT	NM_001024955.2
Irs2	CTGCGTCCTCTCCCAAAGTG	GGGGTCATGGGCATGTAGC	NM_010719.5
Acaca - Acc	GAGAGGGGTCAAGTCCTTCC	TGCTGCCGTCATAAGACAAG	NM_133360.2
Fasn	GAGGACACTCAAGTGGCTGA	GTGAGGTTGCTGTCGTCTGT	NM_007988.3
Srebp1c	GGAGGCAGAGAGCAGAGATG	CACAGGTTCCCCATAGACAAA	NM_001358314.1
Hmgcs2	AGGACATCAACTCCCTGTGC	TCAGTGTTGCCTGAATCCTG	NM_008256.4
Fgf21	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCATGTTCC	NM_020013.4
Adipor1	AGGCTGAGGAAGATCAAGCA	CGTTGTCTTTCAGCCAGTCA	NM_001306069.1
Adipor2	CCAATCCGGTAGCACATCGT	TCCTACAGGCCCATCATGCTA	NM_001355692.1
Ucp2	GGTCGGAGATACCAGAGCAC	TGTCATGAGGTTGGCTTTCA	NM_011671.5
Foxo1	GTGAACACCATGCCTCACAC	ACTTGGGAGCTTCTCCTGGT	NM_019739.3
Hmgcr	CTCCTCTCCACAAAGCTTGC	CTGGTACTCCTTCCCATCCA	NM_001313979.1
Prkaa2	GTGATCAGCACTCCGACAGA	TCTCTGGCTTCAGGTCCCTA	NM_001356568.1
Insr	GAATGTGACAGCCACCACAC	CTGGGGATTCTTGATTGCAT	NP_001316985.1

18S rRNA (18S ribosomal RNA); *Pparg* (Peroxisome Proliferator Activated Receptor Gamma); *Ppargc1a* (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha); *Sirt1* (sirtuin 1); *Ppard* (Peroxisome Proliferator Activated Receptor delta); *Pik3r1* (Phosphoinositide-3-Kinase Regulatory Subunit 1); *Irs2* (Insulin Receptor Substrate 2); *Acaca* (Acetyl-CoA Carboxylase Alpha); Fasn (Fatty Acid Synthase); Srebp1c (Sterol Regulatory Element Binding Transcription Factor 1); *Foxo1* (Forkhead Box O1); *Fgf21* (Fibroblast Growth Factor 21); *Hmgcs2* (3-Hydroxy-3-Methylglutaryl-CoA Synthase 2); *Adipor1* (Adiponectin Receptor 1); *Adipor2* (Adiponectin Receptor 2); *Ucp2* (uncoupling protein 2); *Hmgcr* (3-Hydroxy-3-Methylglutaryl-CoA Reductase); *Prkaa2* (Protein Kinase AMP-Activated Catalytic Subunit Alpha 2); *Insr* (insulin receptor).

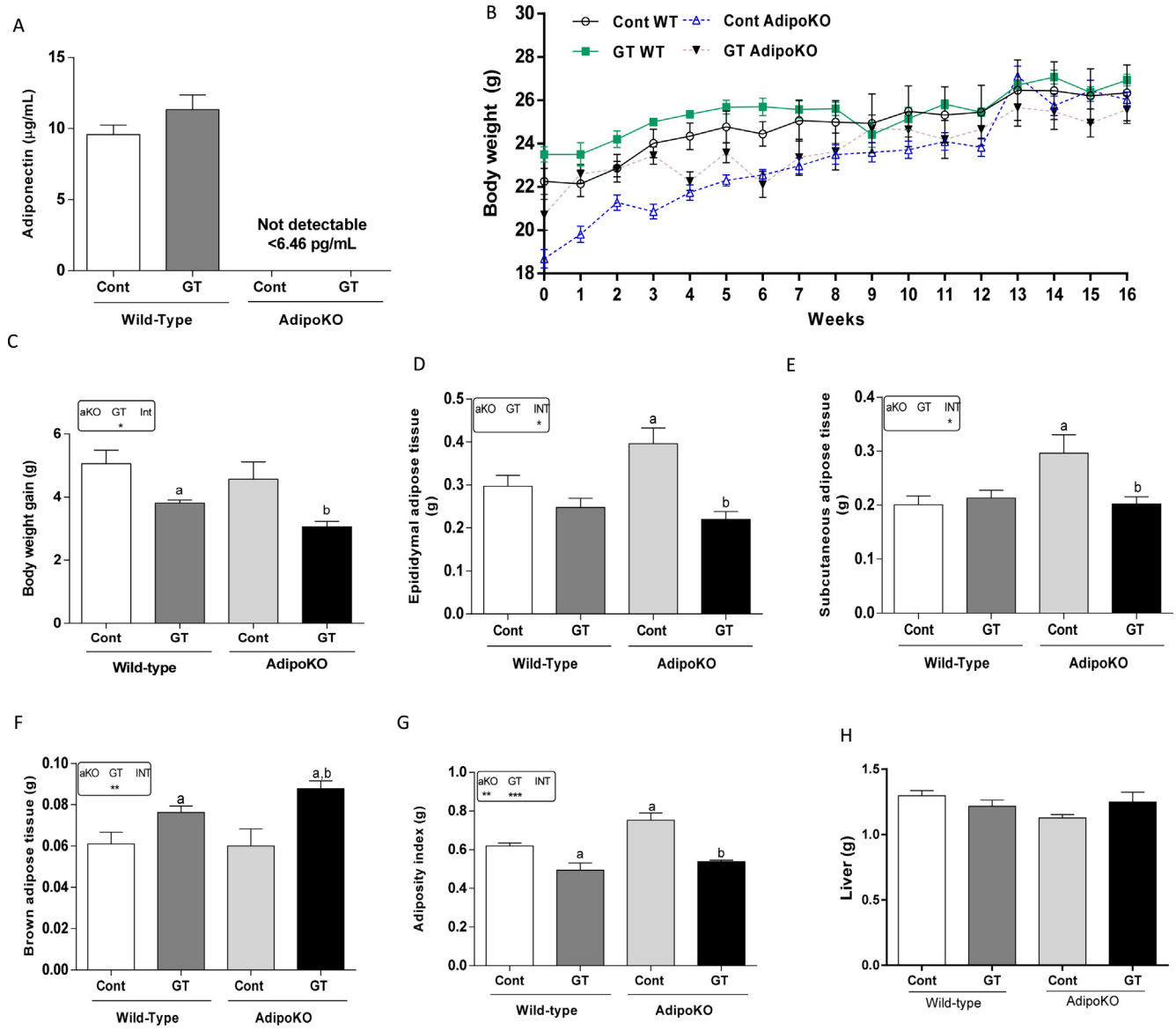

Fig. 1. Green tea extract modulates fat depots in AdipoKO mice. Plasma adiponectin (A), weekly weight (B), body weight gain (C), the weight of epididymal fatty deposits (grams) (D), subcutaneous (E), brown adipose tissue (F), adiposity index (G), liver weight (H). Results are presented as mean ± SEM of at least 05 animals per group. For statistical analysis, the two-way ANOVA was used to determine the effects of genotype (aKO), treatment with green tea (GT), and their potential interaction (INT). When the difference was statistically significant the following overwritten letters were used: a = statistically different compared to the Cont-WT mice, b = statistically different compared to the Cont-AdipoKO mice, c = statistically different compared to the GT-WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

temperature), and Pierce ECL western blotting substrate detection system (Thermo Scientific). Signals were quantified using UN-SCAN-IT gel version 6.1 Software using β-actin as a reference.

2.8. Statistical analysis

Results are given as mean ± SEM. The Levene test was used to verify the variance of the data. When the Levene test was $p > 0.05$, the interaction (INT) was evaluated through a factorial two-way ANOVA (genotype, aKO, × GT-treatment, GT) followed by the Tukey post-hoc test. When the interaction was not statistically significant, the main effect (aKO and/or GT) was assessed by a factorial one-way ANOVA. A significant p-value was considered below 0.05 ($p < 0.05$). The minimum sample size per group for each parameter analyzed was defined by a n sufficient to perform the analysis of distribution of samples through the "D'Agostino and Pearson omnibus normality test" recommended by the GraphPad Prism version 6.0 Software. The statistical power of the study was 80%. The statistical analysis was performed using the GraphPad Prism statistics software package version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) and SPSS/Windows version 22 statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Green tea extract modulates fat depots in AdipoKO mice

Initially to validate the model used in this study we quantified plasma adiponectin level. Plasma levels of adiponectin were undetectable in AdipoKO mice (Fig. 1A) while were similar among groups of wild-type mice as expected. To determine the influence of adiponectin knockdown and GT supplementation on the body composition we evaluated some parameters related to weight and adiposity. There

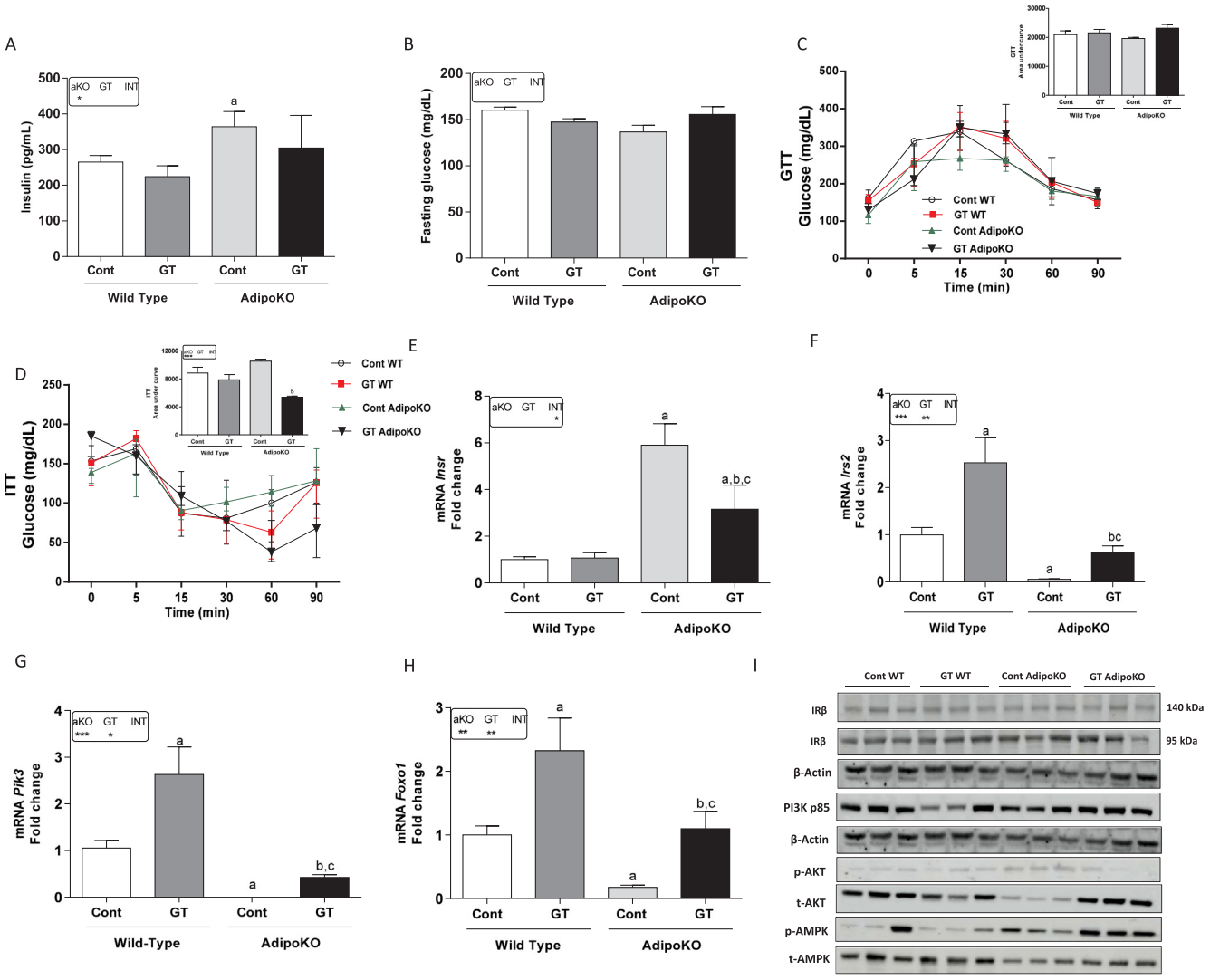

Fig. 2. Green tea extract modulates liver insulin-related genes independently of adiponectin. Plasma insulin concentration (A) Fasting glycemia (B), glucose tolerance test (GTT) (C), insulin tolerance test (ITT) (D), Hepatic gene expression of *Ir, Irs2, Pi3k,* and *Foxo1* (E-H), representative blots of IRβ (140 kDa/95 kDa), Pi3K, pAkt/t-Akt, p-AMPK/t-AMPK of 3 mice/group (I). The GTT and ITT assessment was performed at 0, 5, 15, 30, 60 and 90 min after intraperitoneal administration of glucose or insulin, respectively. Results are presented as mean ± SEM of at least 05 animals per group. For statistical analysis, the two-way ANOVA was used to determine the effects of genotype (aKO), treatment with green tea (GT), and their potential interaction (INT). When the difference was statistically significant the following overwritten letters were used: a = statistically different compared to the Cont-WT mice, b = statistically different compared to the Cont-AdipoKO mice, c = statistically different compared to the GT-WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

was no difference in the BW gain in Cont-AdipoKO mice compared to the Cont-WT group (Fig. 1B–C), however, GT-treatment (GT main effect, $p < 0.05$) reduced BW gain in both KO and WT mice. In spite of not differing in final BW, Cont-AdipoKO mice presented greater epididymal and subcutaneous fat depots resulting in a higher adiposity index compared to the wild-type control mice (interaction effect, $p < 0.05$). GT-AdipoKO mice presented lower epididymal and subcutaneous fat pad weight and adiposity index when compared to the Cont-AdipoKO group. Brown adipose tissue was greater in mice supplemented with GT (GT main effect, $p < 0.05$) in both WT and AdipoKO mice (Fig. 1D–G) while liver weight was not different among the groups. These data suggest that the lack of adiponectin leads to increased adiposity and treatment with GT is able to reduce adiposity independently of adiponectin suggesting that GT has potent metabolic actions even in the absence of obesity or high-fat diet.

3.2. Green tea extract modulates insulin-related genes independently of adiponectin

To understand the impact of adiponectin knockdown and GT supplementation on insulin sensitivity we evaluated some systemic and hepatic parameters. Adiponectin-knockout increases plasma insulin levels (aKO main effect) without altering fasting glucose and GTT (Fig. 2A–C). However, the AdipoKO mice (aKO main effect) have shown better tolerance for insulin (Fig. 2D). To determine if the best insulin sensitivity reflects any change in liver insulin signaling pathway we evaluated some proteins of the insulin signaling pathway. AdipoKO mice exhibit greater mRNA level but not protein level of insulin receptor (*Insr*) that was similar among the groups (Fig. 2I, Fig. S1A). Also, there was lower mRNA levels of the downstream genes *Irs2, Pik3,* and *Foxo1* in KO mice (aKO main effect, $p < 0.01$). Expression of Pi3k at protein level was similar among the groups while phospho-Akt was greater only in Cont-AdipoKO mice (Fig. 2I, Fig. S1C, D). Phospho-AMPK protein level showed a tendency ($p < 0.07$) to increase in

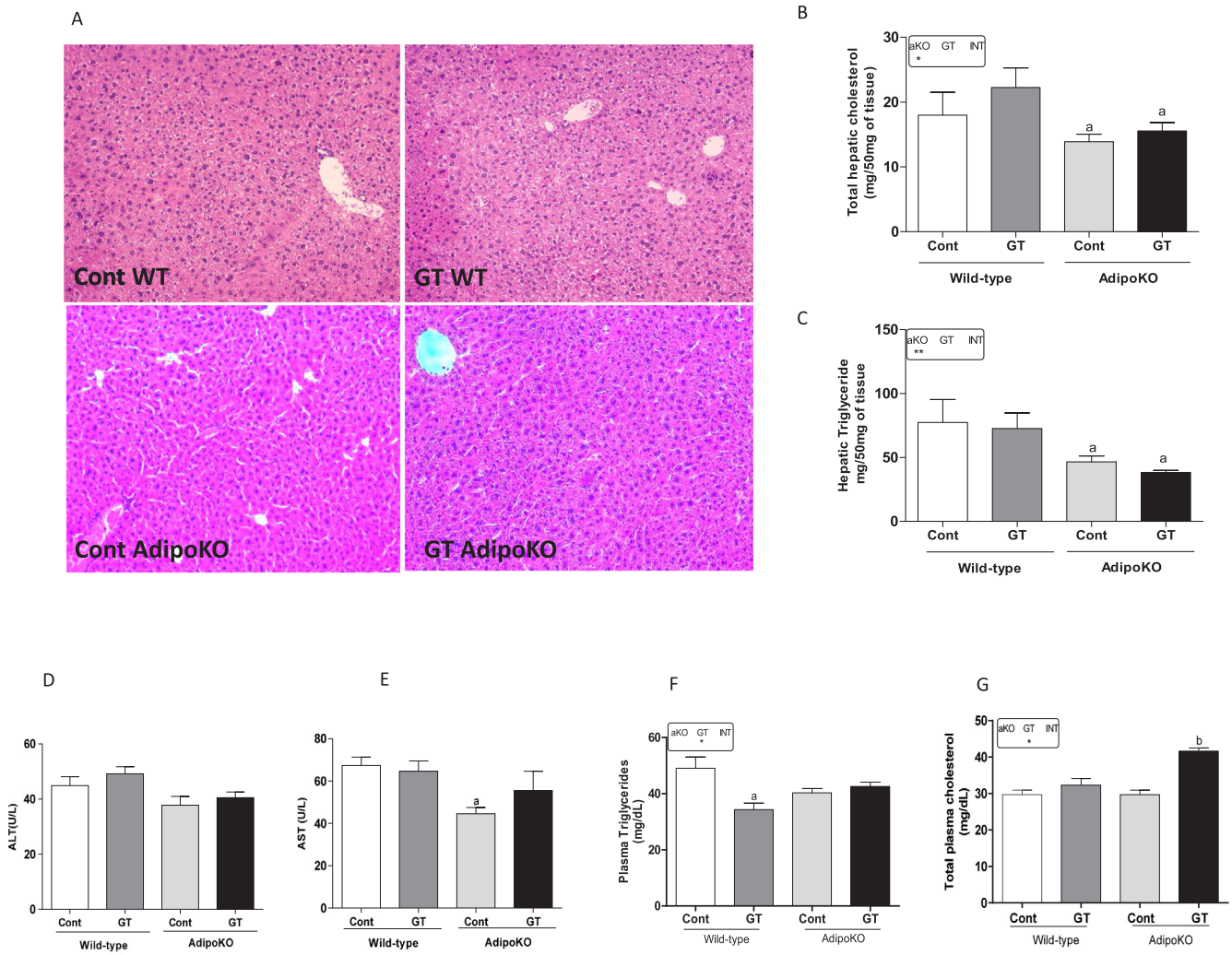

Fig. 3. Lack of adiponectin does not damage the liver. Liver histological analysis (A), total hepatic cholesterol, triglyceride content (B and C), ALT and AST plasma level (D and E), plasma levels of triglyceride and total cholesterol (F and G). Results are presented as mean ± SEM of at least 05 animals per group. For statistical analysis, the two-way ANOVA was used to determine the effects of genotype (aKO), treatment with green tea (GT), and their potential interaction (INT). When the difference was statistically significant the following overwritten letters were used a = statistically different compared to the Cont-WT mice, b = statistically different compared to the Cont-AdipoKO mice, c = statistically different compared to the GT-WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

AdipoKO mice. GT extract supplementation only decreased the mRNA expression of the insulin receptor in AdipoKO mice whereas it increases the expression of the all downstream genes of the insulin signaling pathway compared with Cont-AdipoKO group. GT-supplementation also promoted a higher mRNA expression of *Irs2, Pik3,* and *Foxo1* in wild-type mice (GT main effect, Fig. 2E–H). Taken together, these data have shown that the lack of adiponectin modulates the mRNA level of hepatic insulin signaling genes also increasing the phosphorylation level of Akt and AMPK while GT supplementation modulates mRNA but not protein level independently of adiponectin.

3.3. Lack of adiponectin does not damage the liver

Histological analysis of the liver revealed no differences between the experimental groups in both wild-type and knockout mice (Fig. 3A). The assessment of hepatic TG content (Fig. 3C) showed that the lack of adiponectin decreases liver TG content (aKO main effect) while plasma TG was reduced by GT-treatment only in WT mice (Fig. 3F). Liver total cholesterol content was not different among the groups (Fig. 3B) but was higher in the plasma of GT-AdipoKO mice (Fig. 3G). Plasma activity of ALT and AST enzymes were similar among the groups with a small decrease of AST level in Cont-AdipoKO mice (Fig. 3D–E).

3.4. Effects of green tea extract and adiponectin on hepatic lipid metabolism

In order to determine if the effects of GT-supplementation in hepatic lipid metabolism are dependent on adiponectin, we have evaluated mRNA levels of some key metabolic genes. In WT mice the GT-supplementation increases the expression of lipolytic genes such as *Adipor2*, *Fgf21*, *Sirt1*, *Prkaa2* (AMPK-α), *Ppard* (PPARδ), and *Ucp2* compared with WT-control mice (Fig. 4A–G). Protein level of Ucp2 was also increased in WT mice treated with GT while Sirt1 and PPARα were not changed (Fig. 4P, Fig. S1F–H). It seems that in WT-condition GT activates lipid oxidation pathways. AdipoKO control mice presented lower expression of the *Fgf21, Prkaa2, Ppard,* and *Hmgcs* genes whereas they presented increased expression of *Sirt1, Ucp2, Hmgcr, Pparg* (PPARγ), *Srebp1*, *Acc,* and *Fasn* compared with WT control mice. Protein level of UCP2, Sirt1, and PPARα were increased in Cont-AdipoKO mice. In AdipoKO GT-treated mice, unlike the wild-type mice, GT was not able to alter the expression of *Adipor2, Fgf21, Sirt1, Prkaa2, Ucp2, and Hmgcr* lipolytic genes whereas it has increased UCP2, and Sirt1 protein levels (Fig. 4A–P, Fig. S1F–G). *Adipor1* expression was not

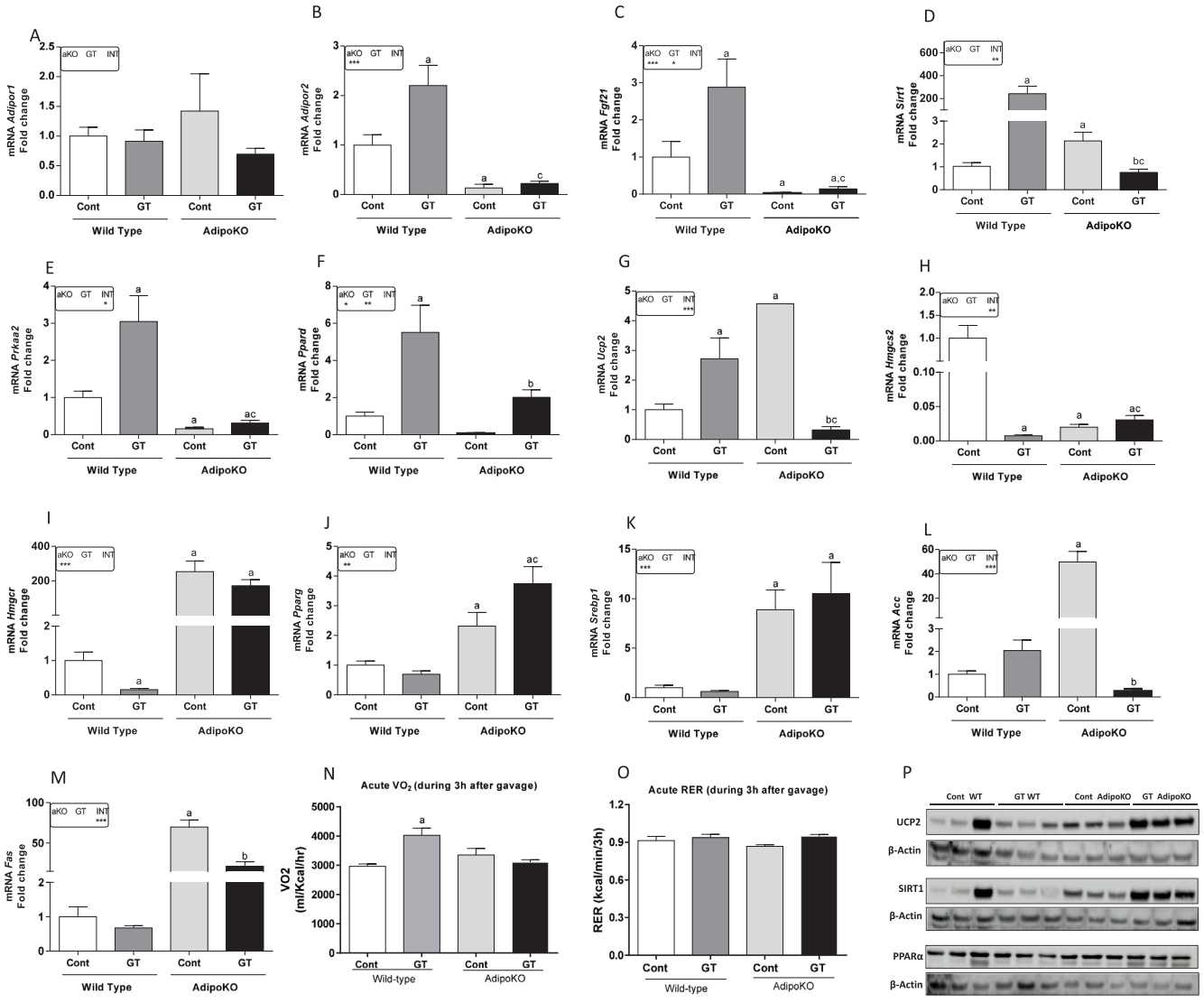

Fig. 4. Effects of green tea extract and adiponectin on hepatic lipid metabolism Analysis of gene expression of *Adipor1, Adipor2, Fgf21r, Sirt1, Prkaa2, Ppard, Ucp2, Hmgcs2, Hmgcr, Pparg, Srebp1, Acc* and *Fasn* (A-M), Acute VO2 volume (N), Acute RER (O), representative blots of UCP2, Sirt1 and PPAR α of 3 mice/group. Results are presented as mean ± SEM of at least 05 animals per group. For statistical analysis, the two-way ANOVA was used to determine the effects of genotype (aKO), treatment with green tea (GT) and their potential interaction (INT). When the difference was statistically significant the following overwritten letters were used: a = statistically different compared to the Cont-WT mice, b = statistically different compared to the Cont-AdipoKO mice, c = statistically different compared to the GT-WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

different between groups (Fig. 4A). Thus the modulation of those genes by GT seems to occur through the activation of the adiponectin pathway instead of being by a direct effect of green tea. In WT mice GT-supplementation does not modulate the expression of *Pparg*, *Srebp1, Acc*, and *Fasn* while it does decrease *Acc* and *Fasn* expression in the adipoKO GT-treated mice (Fig. 4J–M). In addition, the lack of adiponectin stimulates hepatic lipid synthesis and GT minimizes this effect independently of adiponectin. These results could be reinforced when we analyzed the acute energy expenditure in mice (VO2 data calculated during 5 h after GT-gavage - Fig. 4N) which was increased only in wild-type mice treated with GT. However, increased energy expenditure in wild-type mice did not impact systemically fatty acid oxidation as indicated by RER outcomes that were not different among the groups (Fig. 4O).

4. Discussion

In our study, we have shown that GT is able to stimulate lipid oxidation pathways, and brown adipose tissue while decrease WAT depot even in the lack of adiponectin. In adiponectin knockout mice it seems that hepatic insulin signaling is slightly impaired while lipid synthesis is stimulated, which can be mitigated by GT supplementation independently of adiponectin.

Adiponectin is one of the main hormone derived from adipose tissue capable of regulating lipid and glucose metabolism, influencing the development of metabolic diseases such as obesity, type 2 diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD) (Nigro et al., 2014). Hypoadiponectinemia is associated with an increased ALT levels in patients with NAFLD (Lee et al., 2006). In our study, ALT and AST levels were similar among AdipoKO and WT-mice as well as the liver of mice from both background did not have steatosis. Therefore, the lack of adiponectin may not represent an independent risk factor for NAFLD development i.e., livers exposed to lower adiponectin levels are not more susceptible to damage. Unpublished data from our group have shown that a high-fat diet (HFD) consumption exacerbate steatosis in AdipoKO mice compared with HFD-fed mice in

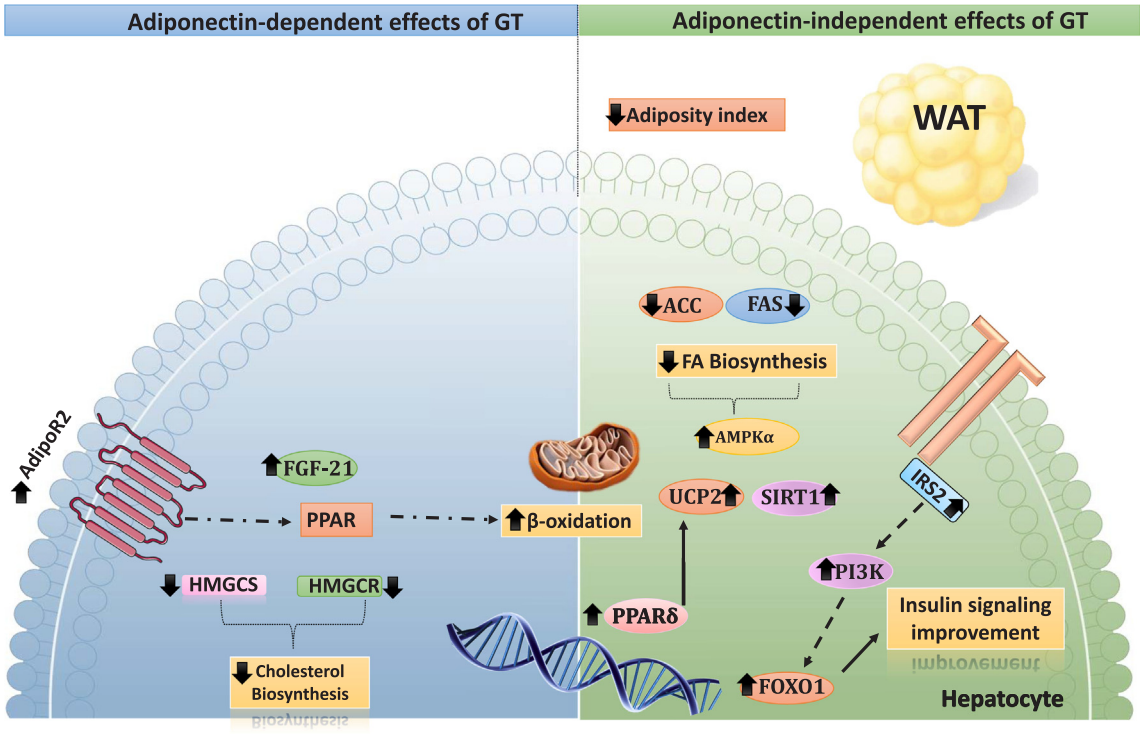

Fig. 5. Green tea extract action on hepatic metabolism. The GT extract activates the lipid oxidation pathway dependent on adiponectin by inducing *Adipor2* and *Fgf-21* mRNA, increasing *Pparδ* and VO2 consumption and decreasing cholesterol synthesis. Furthermore, GT independent of adiponectin decreases the WAT depot while it increases brown adipose depot, increasing the protein expression of AMPK/Sirt1/UCP2, suggesting a possible contribution to the activation of β-oxidation. Also, independent of adiponectin the green tea extract decreases the expression of enzymes responsible for lipogenesis (*Acc* and *Fasn*).

WT condition.

The increament in circulating levels of insulin to compensate the loss of adiponectin promotes the storage of lipids which can lead to insulin resistance (Combs & Marliss, 2014). Similarly, here we show that mice knockout of adiponectin presents a higher adiposity index, accompanied by a higher plasma level of insulin as well as increased *Insr* mRNA level, however without affecting IR protein level. Also, the lack of adiponectin decreases *Irs2* gene expression in the liver, as well as the downstream genes. In parallel indicators of high synthesis of fatty acids (FA) were observed in KO mice. Currently, decreased adiponectin in serum is considered a strong predictor for metabolic syndrome (Ghadge, Khaire, & Kuvalekar, 2018). This is due to the biological effects of adiponectin leading to an improvement in glucose uptake, increased lipid oxidation and decreased hepatic gluconeogenesis.

Adiponectin is known to modulate liver function, especially lipid metabolism (Ghadge et al., 2018). Adiponectin is also able to reduce hepatic lipid content through two main mechanisms; indirectly, by inhibiting hepatic glucose production also reducing circulating levels of insulin, which decreases insulin-mediated lipogenesis and inhibition of insulin-mediated fatty acid oxidation. On the other hand, adiponectin also stimulates the oxidation of fatty acids through the activation of SIRT1/AMPK/PPARα (Awazawa et al., 2011; Combs & Marliss, 2014; Jiang et al., 2015; Yamauchi & Kadowaki, 2013). Both AMPK and SIRT1 are responsible for the phosphorylation and deacetylation of PGC1-α, respectively, leading to its activation. PGC1-α is an important regulator of mitochondrial biogenesis and activator of PPAR-α and PPAR-δ, nuclear receptors that coordinate lipid oxidation by targeting enzymes responsible for mitochondrial and peroximal fuel oxidation, such as UCP2 (Villarroya, Iglesias, & Giralt, 2007). Similar mechanisms to those of adiponectin GT-polyphenols are known to decrease the accumulation of lipids in the liver through AMPK activation (Huang, Zhou, Wan, Wang, & Wan, 2017). As expected, our results show an increment in the mRNA of AMPKα, SIRT1, PPAR-δ and UCP2 in wild-type mice supplemented with GT corroborating with previous data in the literature. Interestingly, the GT-mediated effect on AMPKα, SIRT1, and UCP2 expression was shown to be adiponectin independent since in AdipoKO mice GT also increased the levels of these proteins while mRNA levels were decreased.

In the last years, several authors have described that GT polyphenols and EGCG are effective in increasing the serum adiponectin level and the protein expression of their receptors (AdipoR1 and AdipoR2) in humans and mice fed with a high-fat diet (Derdemezis et al., 2010; Wang et al., 2014; Wu & Butler, 2011). It is totally possible to transpose the dose of GT extract from our study to the human being aiming to achieve those similar beneficial effects observed in mice, however, it needs some caution. By using an allometric scale we can reach the dose of 2.44 g/day/70 kg of BW. Then, it is possible to suggest that GT should be administrated as a pharmaceutical or nutraceutical intervention using a standardized extract supplementing the diet. We expect in a future clinical trial using the green tea extract provided as capsules, we can confirm these findings in humans, so reinforcing the beneficial effects of GT to promote liver health.

Recently, Santamarina (Santamarina et al., 2015) demonstrated that supplementation with decaffeinated green tea extract prevents hepatic steatosis by activation of AMPK via LKB1 accompanied by the increment of AdipoR2. However, it has not been determined yet whether the effects of GT polyphenols are mediated through adiponectin or whether the increase of adiponectin and its receptors in response to GT are reciprocal effects. Our results strongly suggest that the effects of GT on the stimulation of lipid oxidation are independent on adiponectin since the increase in the expression of AMPK/Sirt1/UCP2 protein level was also observed in AdipoKO-mice while we have observed an increase in EE due to the GT-treatment just in WT-mice. Thus, it seems that there is a switch in energy metabolism when adiponectin is not present. Based on our outcomes it seems that the liver of KO mice treated with GT has increased oxidation of energy substrates, whereas in the WT-mice

treated with GT the brown adipose tissue seems to be responsible for the increased EE. It is important to note that both, WT and KO mice have lost weight after GT treatment.

In a previous study from our group (Ferreira et al., data not yet published), we observed that green tea extract supplementation prevented the development of NAFLD in obese mice accompanied by an increase in plasma adiponectin levels and an increase in the expression of both AdipoR1 and AdipoR2 in the liver of the mice. Possibly, the beneficial effects of GT against NAFLD of obese mice are mediated by adiponectin, evidencing the central role of adiponectin in the regulation of hepatic metabolism.

In summary, our study demonstrates that the lack of adiponectin leads to increased adiposity leading to increased lipogenic genes. GT supplementation decreases adiposity and improves insulin signaling and consequently decreases lipogenic genes independently of adiponectin. In addition, we are providing for the first time strong evidence that GT-polyphenols active lipid oxidation pathway independent of adiponectin. These findings may contribute to understanding the mechanism of action of GT in the liver (Fig. 5).

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103679.

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