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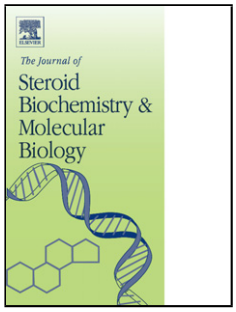

Title: The Isoflavones Genistein and Daidzein Increase Hepatic Concentration of Thyroid Hormones and Affect Cholesterol Metabolism in Middle-Aged Male Rats

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The Isoflavones Genistein and Daidzein Increase Hepatic Concentration of Thyroid Hormones and Affect Cholesterol Metabolism in Middle-Aged Male Rats

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Highlights:

- We examined whether isoflavones increase hepatic thyroid hormone concentrations and affect cholesterol metabolism in middle-aged rats
- Serum T_3 was not affected while hepatic T_3 was almost doubled, which supports increased local T_3 availability.
- Obtained results are compatible with displacement of TH from TTR, major transport protein in rodent blood and human CSF.
- Hepatic increase of T_3 correlated with up-regulated expression of the Cyp7a1 gene and elevated 7α-hydroxycholesterol
- IF also lowered 24-hydroxycholesterol and desmosterol in liver and serum, while the total cholesterol levels remained unchanged.

Abstract

We examined whether isoflavones interfere with thyroid homeostasis, increase hepatic thyroid hormone concentrations and affect cholesterol metabolism in middle-aged (MA) male rats. Thirteen-month-old Wistar rats were injected subcutaneously with 35 mg/kg b.w./day of genistein, daidzein or vehicle (controls) for four weeks. Hepatic *Dio1* gene expression was up-regulated by 70% ($p<0.001$ for both) and Dio1 enzyme activity increased by 64% after genistein ($p<0.001$) and 73% after daidzein treatment ($p<0.0001$). Hepatic T_3 was 75% higher ($p<0.05$ for both), while T_4 increased only after genistein treatment. Serum T_4 concentrations were 31% lower in genistein- and 49% lower in dadzein-treated rats ($p<0.001$ for both) compared with controls. Hepatic *Cyp7a1* gene expression was up-regulated by 40% after genistein and 32% after daidzein treatment ($p<0.05$ for both), in agreement with a 7α-hydroxycholesterol increase of 50% ($p<0.01$) and 88% ($p<0.001$), respectively. Serum 24- and 27-hydroxycholesterol were 30% lower ($p<0.05$ for both), while only 24-hydroxycholesterol was decreased in the liver by 45% after genistein ($p<0.05$) and 39% ($p<0.01$) after dadzein treatment. Serum concentration of the cholesterol precursor desmosterol was 32% ($p<0.05$) lower only after dadzein treatment alone, while both isoflavones elevated this parameter in the liver by 45% ($p<0.01$). In conclusion, isoflavones increased T_3 availability in the liver of MA males, despite decreasing serum T_4. Hepatic increase of T_3 possibly contributes to activation of the neutral pathway of

cholesterol degradation into bile acids in the liver. While isoflavones obviously have the potential to trigger multiple mechanisms involved in cholesterol metabolism and oxysterol production, they failed to induce any hypocholesterolemic effect.

Keywords: genistein; daidzein; thyroid homeostasis; liver; cholesterol metabolism; middle -aged rats

1. Introduction

Genistein and daidzein are isoflavones (IF) present in largest quantities in plant species of the bean family (*Leguminosa*e or *Fabaceae*), particularly soybean. These are potent bioactive compounds with antioxidant, anticarcinogenic, osteoprotective and hormone-like actions [1].

Despite potential health benefits, consumption of soy and IF-enriched food may not be safe for everyone [2]. Supplementing the diet with IF was reported to triple the risk of overt hypothyroidism in patients with subclinical hypothyroidism [3]. Isoflavones may interact with various molecular targets of the thyroid hormone system: both genistein and daidzein can act as competitive substrates for thyroid peroxidase (TPO) [4, 5]; genistein, and with a lower potency daidzein, inhibit binding of transthyretin (TTR) to thyroxin (T_4) and triiodothyronine (T_3) [6], while only genistein act as an inhibitor of type 1 deiodinase (Dio1) in vitro [7]. However, the goitrogenic potential of IF in vivo depends on numerous factors, including insufficient iodine in the diet or co-exposure with other goitrogen [8]. Apart from these factors, the effect of IF on the thyroid hormone (TH) status in rodent models depends on animal age and endogenous gonadal steroid status [4, 8, 9,10]. Comprehensive analyses of the pituitary-thyroid axis and peripheral tissue (liver) in an orchidectomized (Orx) middle-aged (MA) rat model after genistein and daidzein treatments revealed decreased intrathyroidal and serum TH concentrations, accompanied by higher TSH (indices of systemic hypothyroidism). However, in the liver, expression of T_3– regulated genes *Dio1* and *Thrsp* was up-regulated, while Dio1 enzyme activity increased, indicating local hepatic increase of T_3 [9]. The obtained hepatic changes were compatible with displacement of TH from TTR, the major carrier protein in rodent blood [6], resulting in higher hepatic TH uptake.

The liver is the organ where cholesterol homeostasis is maintained by a complex network of tightly controlled cellular processes. Most recent data analyses confirmed a hypocholesterolemic effect of soy protein isolate and IF-enriched soy, but the effect of IF extracts alone is not completely clear [11]. Apart from a well-known interference with estrogen receptors [12], IF were shown to regulate farnesoid (FXR) and liver nuclear receptor (LXR) activity, which are the key regulators of cholesterol metabolism [13, 14]. A significant amount of work has demonstrated effects of IF on expression and activity of various cytochrome P_{450} enzymes (CYP) in the liver, including CYP7A1 [15]. Data regarding changes in concentration of the endpoints of these enzyme activities, cholesterol precursors and oxidation products, are scarce.

TH plays an important role in the regulation of cholesterol metabolism in the liver, mainly through its nuclear receptor β actions [16]. T_3 mimetics and analogues, which accumulate in the liver and mediate metabolic effects of TH, show high efficacy in reducing serum cholesterol and liver steatosis [16, 17]. Lifetime exposure to high doses of genistein (250mg/kg) increased hepatic Dio1 expression and activity and mildly reduced lipid droplets in the liver of healthy young adult male rats [18]. A recent *in vitro* study of Ariyani et al. [19] demonstrated that high doses of genistein and daidzein enhance thyroid receptor (TR)-mediated transcription in the absence of T_3. However, IF treatments of acyclic middle-aged female rats did not affect serum TH status, nor concentrations of total cholesterol, its precursors or oxidative metabolites, except 27-hydroxycholesterol, which was decreased in serum and liver [10]. However, female rodents, unlike humans, are less susceptible to disruption of thyroid homeostasis after exposure to xenobiotics in comparison to males [20].

The aim of this investigation was to directly measure the effect of IF on local tissue concentration of TH in the liver of testes-intact MA rats. Moreover, our goal was to examine

whether IF, under the same experimental conditions as applied in our previous study with MA females, would affect cholesterol metabolism more prominently, and whether supposed changes in hepatic T_3 correlate with changes of examined steroidal precursors and/or oxidation products of cholesterol metabolism. To the best of our knowledge, such investigation had not been performed to date.

Materials and Methods

2.1. Animals

Male Wistar rats were housed in the unit for experimental animals at the Institute for biological research "Siniša Stanković". They were kept in groups of two - three per cage at 22 ± 2 ˚C with a 12:12-h dark-light cycle. Animal experiments were performed in accordance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research ''Siniša Stanković'', University of Belgrade, Belgrade, Serbia.

At the age of 13 months, two weeks prior to the experiments, male rats were put on a semi-purified *soy*-free diet, to avoid any additional goitrogenic and estrogenic stimulus. This diet was prepared in cooperation with the Department of Animal Nutrition and Botany, Faculty of Veterinary Medicine, Belgrade, Serbia, exactly as previously described [9, 10].

After two weeks of adaptation to soy-free diet, rats were randomly divided into three groups (n=6/group). Two groups ere subcutaneously (s.c.) injected with 35 mg/kg of genistein (Genistein; LC Laboratories, MA, USA) or daidzein (Daidzein; LC Laboratories, MA, USA), respectively, daily for four weeks. In a third group, animals received vehicle only (olive oil was sterilized and then mixed and absolute ethanol, ratio 9:1) under the same regime and served as

controls (Control). The volume injected was 0.2 ml per animal. Animal age, route and duration of isoflavone administration, were the same as in a previous study on acyclic ovary-intact MA female rats [10]; the s.c. route of administration was chosen to precisely regulate the dosage of IF and to avoid metabolism of dietary daidzein to equol by intestinal microflora [21]. All rats were decapitated 24h after the last treatment. Livers were perfused with ice cold physiological saline, excised and weighed. Blood was collected from the trunk and the serum stored at -80°C. One liver portion was immediately frozen in liquid nitrogen and stored at -80°C. Prior to the analyses, frozen tissue was pulverized under dry ice (solid CO2) in pre-cooled Teflon containers, using a micro – dismembrator (B. Braun Biotech International GmbH, Melsungen, Germany).

2.2. Quantification of IF in serum

Analyses of bioactive aglycones and total isoflavones (aglycones + glucuronides) in serum samples (200 µl; n=6) were determined by gas chromatography–mass spectrometry (GC-MS) and quantitative nuclear magnetic resonance methods, as previously described [22]. In brief, IF levels were determined after drying of serum samples using Freeze Dryers Rotational-Vacuum Concentrator (GAMMA 1–16 LSC, Germany).

Prior to GC–MS analysis, glucuronides in an acidified lyophilized sample were enzymatically hydrolyzed by a mixture of β-glucuronidase from Escherichia coli (Sigma-Aldrich, Saint Louis, MO, USA). Following a usual treatment and extraction of the hydrolysates, the aglycones were silylated with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich, Saint Louis, MO, USA) and subsequently analyzed by GC-MS [9]. The initial analyses were run in full scan to verify the presence and identity of IF and their metabolites, which was followed by a single ion monitoring quantitation. The ions monitored for identification were the following: m/z

425, 482 for daidzein, m/z 470, 234 for equol, m/z 555, 612 for genistein and m/z 685 for 3´-hydroxygenistein. GC–MS analyses were performed on a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 °C and 320 °C, respectively. Oven temperature was raised from 70 to 315 °C at a heating rate of 5 °C/min and then isothermally held for 20 min. As a carrier gas helium at 1.0 ml/min was used. The mass selective detector was operated at the ionization energy of 70 eV, and in full scan mode in the 35–750 amu range and scanning speed of 0.32 s.

Quantitative NMR experiments were performed according to a previously described procedure [23]. Lyophilized samples of known mass were completely dissolved in dimethyl sulfoxide-d6 and a known amount of an internal standard - sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 (0.75%, w/w) was added (upon the addition of the standard compound no changes to the appearance of the spectra were noted). 13C-decoupled 1H NMR spectra (a large data set was collected; 10 points per Hz digital resolution) were recorded with a signal to noise ratio of at least 1000:1. Parameters were as follows: number of points in the time domain=32k, spectral width=10 ppm, O1=6.0 ppm, p1=45° 1H transmitter pulse, acquisition time=5 s and number of scans=1024. After zero-filling and phase and baseline corrections, integration of signals (0.00 ppm for the internal standard; non-overlapping signals in the region 6.4–7.3 ppm for IF or IF-metabolites) was performed. The ratio of the signal integrals was used to calculate the amount of IF or IF-metabolites in the samples.

2.3. Quantitative real-time-PCR (qRT-PCR)

Total RNA was extracted from the liver (50mg; n=5 for control, n=6 for treatment groups) using the TRIzol reagent (Invitrogen, Karlsruhe, Germany), following the manufacturer's instructions. Quality and quantity of extracted RNA were assessed by measurement of absorbance ratio at 260:280 nm on Eppendorf BioPhotometar, Hamburg, Germany. Complementary DNA (cDNA) was synthesized from 500ng of total RNA, using the cDNA Reverse Transcription kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA). Reactions were carried out under RNase - free conditions at 25 °C for 10 min followed by 37°C for 2 h and final denaturation at 85°C for 5 min. The cDNA was diluted (1:10) with RNAase-free water and stored at -80°C until further use. Gene specific primers for RT-qPCR were designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The assay was performed using the ABI Prism 7000 System (Applied Biosystems, CA, USA) and Power SYBR® Green PCR master mix (Applied Biosystems, CA, USA). The program included 3 min at 95°C for initial denaturation of cDNA, followed by 40 cycles, each consisting of 15 sec of denaturation at 95°C, 30 sec at 69°C for annealing step, and 30 sec at 72°C for elongation step. The primer sequences were: *Dio 1*-f: 5′- f TTTAAGAACAACGTGGACATCAGG-3′; *Dio* 1-r: 5′- GGTTTACCCTTGTAGCAGATCCT -3′; *Cyp7a1*- f: 5′-CACCATTCCTGCAACCTTTT -3′; *Cyp7a1*-r: 5′-GTACCGGCAGGTCATTCAGT -3′; *Hprt*–f: 5′-TATGGACAGGACTGAAAGACTTG -3′; *Hprt*– r: 5′-CAGCAGGTCAGCAAAGAACTTATA -3′. Gene expression was calculated by the delta-delta CT method, using *Hprt* as endogenous control for normalization, based on the previous housekeeping gene validation and analysis [9].

2.4. Dio1 enzyme activity measurement

Liver protein samples (40 µg of microsomal proteins; n=5 for control, n=6 for treatment groups) were prepared and activity of type 1 Dio assayed exactly as previously described [10, 24].

2.5. Quantification of thyroid hormones in the liver

Extraction of TH from liver was performed using a liquid-liquid extraction procedure [25]. Liver tissue (n=5 for control, n=6 for treatment groups) was homogenized followed by protein precipitation, acidification, and delipidation. For the extraction, ethyl acetate served as organic solvent. Samples were subjected to protein precipitation followed by removal of phospholipids by transferring the supernatant to HybridSPE-phospholipid cartridge (Sigma-Aldrich Co., Munich, Germany). The resulting organic layers of liver extraction were evaporated to dryness (Eppendorf concentrator 5301 at 45°C), and reconstituted in 100 µl of 50/50 methanol/water (v/v; containing 0.1% formic acid) by vortex-mixing for 20 sec. Samples were then centrifuged at 14,000 rpm for 5 min and stored at –20°C until liquid chromatography/mass spectrometry (LC-MS/MS) analysis. Identification and quantification of T_4 and T_3 were performed using a binary pump HPLC system (Agilent Technologies GmbH, Waldbronn, Germany) and a QTrap 6500 (AB SCIEX Germany GmbH, Darmstadt, Germany) fitted with a TurboIonSprayinterface. The stable isotope standards were used as internal controls during the extraction procedure: $^{13}C_6$-T_4 and $^{13}C_6$ -T_3 , (Isoscience LLC, King of Prussia, Pa., USA).

2.6. Measurement of total TH and TSH in serum

Serum total T_4 and thyroid-stimulating hormone (TSH) were measured using commercial rat ELISA kits according to the manufacturer's instructions (Cusabio Biotech Co., Wuhan, China and IBL International GmbH; Hamburg, Germany, respectively), while total T_3 was quantified using electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim,

Germany). All samples (n=6/group) were measured in duplicate within one run. The intra-assay CV for T_4 measurements was <15%, for TSH 10%, while for T_3 it was 4%.

2.7. Quantification of sterols and oxysterols in liver and serum

Characteristic sterols and oxysterols were quantified in order to describe cholesterol metabolism in liver and in serum (n=5 for control, n=6 for treatment groups). The O-trimethylsilylated sterol and -di-trimethylsilylated oxysterol ethers were separated by gas chromatography from the same lipid liver extract or serum sample in analogy to [26]. Cholesterol was detected by less sensitive flame-ionization detection (FID) (5α-cholestane, internal standard, ISTD), the non-cholesterol sterols (epicoprostanol, ISTD) and the oxysterols (^2H_x-oxysterols, ISTD) by highly specific and sensitive mass spectrometry in the selected ion monitoring mode (MS-SIM).

Gas chromatographic separation and detection of cholesterol and 5α-cholestane (ISTD) was performed on a DB-XLB 30 m x 0.25 mm i.d. x 0.25 µm film thickness (J&W Scientific Alltech, Folsom, CA, U.S.A.) in an Hewlett-Packard (HP) 6890 Series GC-system (Agilent Technologies, Palo Alto, CA, U.S.A), equipped with an FID.

Authentic sterols and deuterium labeled oxysterols were separated on another DB-XLB column (30 m x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific Alltech, Folsom, CA, U.S.A.) in a HP 6890N Network GC system (Agilent Technologies, Waldbronn, Germany) connected with a direct capillary inlet system to a quadruple mass selective detector HP5975B inert MSD (Agilent Technologies, Waldbronn, Germany). Both GC systems were equipped with HP 7687 series auto samplers and HP 7683 series injectors (Agilent Technologies, Waldbronn, Germany).

To determine the serum and hepatic concentrations of cholesterol, its main steroidal precursors as well as oxysterols, 50 µg 5α-cholestane (Serva, Heidelberg, Germany) (50 µl from a stock solution of 5α-cholestane in cyclohexane (Merck KGaA, Darmstadt, Germany; 1 mg/ml), one µg

epicoprostanol (Sigma, Deisenhofen, Germany) (10 µl from a stock solution epicoprostanol in cyclohexane; 100 µg/ml) and 50 ng racemic [23,23,24,25-$^{2}H_4$]24(R,S)-hydroxycholesterol (Medical Isotopes Inc., Pelham, NH, USA), 100 ng 26.26.26.27.27.27-[$^{2}H_6$]-7α-hydroxycholesterol, and 100 ng [16,16,17,20,22-$^{2}H_5$]-(25R)27-hydroxycholesterol (Medical Isotopes Inc. Pelham, NH, USA) (50 µl from a stock solution in toluene (Merck KGaA, Darmstadt, Germany; 2 µg/ml), respectively, were added as internal standards to 100 µl plasma or 100 µl of an chloroform/methanol liver extract (5 ml chloroform/methanol, 2:1, v/v) per 10 mg dry liver tissue). To avoid autoxidation 50 µl of a 2.6.-di-tert.-butylmethylphenol/methanol solution (mg/mL) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was added.

After saponification with 2 mL 1M 95% ethanolic sodium hydroxide solution (Merck KGaA, Darmstadt, Germany) at 60°C for one hour, the free sterols and oxysterols were extracted three times with 3 mL cyclohexane each. The organic solvent was evaporated by a gentle stream of nitrogen at 60°C on a heating block. The residue was dissolved in 80 µL n-decane (Merck KGaA, Darmstadt, Germany). An aliquot of 40 µl was incubated (1h at 70°C on a heating block) by addition of 20 µl of the trimethylsilylating (TMSi) reagent (chlortrimethylsilane, Merck KGaA, Darmstadt, Germany)/1.1.1.3.3.3-Hexamethyldisilasane (Sigma Aldrich, Co., St. Louis, MO, U.S.A)/pyridine (Merck KGaA, Darmstadt, Germany), 9:3:1) in a GC vial for GC-MSD non-cholesterol and oxysterol analysis. Another aliquot of 40µl was incubated by addition of 40 µl of the TMSi-reagent and dilution with 300 µl n-decane in a GC vial for GC-FID cholesterol analysis [27].

An aliquot of 2 µl was injected by automated injection in a splitless mode using helium (1ml/min) as carrier gas for GC-MS-SIM and hydrogen (1ml/min) for GC-FID analysis at an injection temperature of 280°C. The temperature program for GC was as follows: 150°C for

three minutes, followed by 20°C/min up to 290°C keeping for 34 minutes. For MSD electron impact ionization was applied with 70 eV. SIM was performed by cycling the quadruple mass filter between different *m/z* at a rate of 3.7 cycles/sec. Non-cholesterol sterols were monitored as their TMSi-, the oxysterols as their di-TMSi-derivatives using the following masses: epicoprostanol m/z 370 (M^+-90, M^+-OTMSi), lathosterol at m/z 458 (M^+), desmosterol at m/z 441 (M^+-15, M^+-CH_3), lanosterol at m/z 393 (M^+-90-15, M^+-OTMSi-CH_3), 26.26.26.27.27.27-[$^2H_6$]-7α-hydroxycholesterol at m/z 462 (M^+-90), 7α-hydroxycholesterol at m/z 456 (M^+-90), [23,23,24,25-$^2H_4$]24(R,S)-hydroxycholesterol at m/z 416 (M^+-90-44, M^+-OTMSi-$CD(CH_3)_2$), 24(S)-hydroxycholesterol at m/z 413 (M^+-90-43, M^+-OTMSi-$CH(CH_3)_2$, [16,16,17,20,22-$^2H_5$]-(25R)27-hydroxycholesterol at 461 (M^+-90), (25R)27-hydroxycholesterol at 456 (M^+-90).

Peak integration was performed manually. Cholesterol was directly quantified by multiplying the ratios of the area under the curve of cholesterol to 5α-cholestane by 50 µg (ISTD amount). Non-cholesterol sterols and oxysterols were quantified from the ratios of the areas under the curve of the respective non-cholesterol sterols/oxysterol after SIM analyses against internal standards using standard curves for the listed sterols/oxysterols. Identity of all sterols was proven by comparison with the full-scan mass spectra of authentic compounds. Additional qualifiers (characteristic fragment ions) were used for structural identification (m/z values not shown).

2.8. Liver histology

For histology, liver pieces from each animal (n=6/group) were deparaffinized and rehydrated and then dehydrated in graded series of ethanol (30%-100%) and xylene and then embedded in Histowax (Histolab Product Ab, Göteborg, Sweden). Serial 5 µm thick sections from each liver piece were stained with hematoxylin and eosin and histologically analyzed. Digital images of the

liver sections were made on a DM RB Photomicroscope with a DFC 320 CCD Camera (Leica, Wetzlar, Germany).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA). Normality of distribution and the equality of variance were tested by Bartlett's and Brown-Forsythe test, respectively. Statistical significance of the data was determined by one-way ANOVA (Dunnet's post hoc test), while correlation analysis was performed using Pearson's coefficient. Data are presented as mean ± SD. P-values less than 0.05 were considered significant.

3. Results

3.1. Concentration of IF in serum

Serum samples of the genistein-treated group contained 5.05 ± 0.11nmol/l of free aglycones and 47.4 ± 0.8 nmol/l of 3′-hydroxygenistein, while the total amount of genistein (free aglycones plus glucuronides) was 116.4 ± 2.1 nmol/l, with marked domination of the conjugated form (>95%). The ^{1}H NMR spectra of the serum samples of daidzein-treated animals provided detection of 7-O-glucuronides and free aglycones, while the presence of equol in the samples was not confirmed. The average serum concentration of daidzein was 0.28 ± 0.08 nmol/l and these concentrations were lower than those of genistein, while the bound daidzein (plus the low amount of the free one) was 121.0 ± 0.7 nmol/l, which was comparable to that of genistein.

3.2. Body mass and liver weight

The body mass and liver weights are shown in Table 1. There was no difference in body mass, absolute or relative liver weight between controls and the IF – treated groups.

3.3. Thyroid status in liver and serum

In the liver, expression of *Dio1* gene was found to be up-regulated in the genistein- and the daidzein-treated group (by 70%, $p<0.001$ for both treatments; Fig. 1A), and Dio1 enzyme activity had increased (by 64%, $p<0.001$ and 73%, $p<0.0001$, respectively; Fig.1B) in comparison to controls.

Only the genistein-treated group had higher T_4 level (by 57%, $p<0.05$; Fig. 1C), while both IF increased concentration of T_3 (by 75%, $p<0.05$; Fig. 1D) in comparison to controls.

Concentration of total T_4 in serum of genistein- and daidzein- treated groups was lower (by 31%, $p<0.001$ and 49%, $p<0.0001$, respectively), while total T_3 and TSH remained unaltered in comparison to the corresponding control values (Fig. 2A-C).

3.4. Cholesterol metabolism in liver and serum

In the liver, concentration of 7α-hydroxycholesterol was increased in the genistein- and the daidzein- treated group (by 50%, $p<0.01$, and 88%, $p<0.001$, respectively; Fig. 3A), while 24-hydroxycholesterol was decreased (by 45%, $p<0.01$, and 39%, $p<0.05$, respectively; Fig. 4C) in comparison to controls by both isoflavones.

Concentration of 7α-hydroxycholesterol in serum was unchanged (Fig.3D), while levels of 27- and 24- hydroxycholesterol were lower in the genistein- and the daidzein-treated group (by about 30%, $p < 0.05$ for both treatments) in comparison to controls (Fig. 3E-F).

There was no difference in concentration of the cholesterol precursors lanosterol and lathosterol in the liver (Fig. 4A-B) and hepatic desmosterol decreased after genistein and daidzein treatments (by 46% and 43%, $p < 0.01$ for both, respectively; Fig. 4C) in comparison to controls. Serum concentration of lanosterol and lathosterol also remained unchanged (Fig. 4E-F). Desmosterol level in serum was decreased only after daidzein treatment (by 32%, $p<0.05$; Fig. 4G). However, concentrations of total cholesterol in liver or serum remained unaltered after IF treatments (Fig. 4D, H).

Expression of the *Cyp7a1* gene in the liver, which encodes Chol 7 α-hydroxylase, the enzyme that catalyzes the first and initial step of the main neutral pathway of Chol degradation to bile acids, was higher after genistein and daidzein treatments (by, 40% and 32%, $p< 0.05$, respectively; Fig. 5A).

Moreover, increases in hepatic T_3 correlated positively with hepatic *Cyp7a1* gene expression ($r=0.5190$, $p<0.05$; Fig. 5B) and 7α-hydroxycholesterol ($r=0.5093$, $p<0.05$; Fig. 5C).

3.5. Liver histology

Liver histology of 13-month-old males was characterized by normal morphology of lobules and usual arrangement of hepatocytes surrounding central vein (Fig. 6A1, A2). However, mild degenerative changes were clearly visible and included hepatocyte hypertrophy and ballooning, being present in all examined animals. Microvesicular steatosis was present in <10% of the tissue, which may be considered not significant (n=3/6; Fig. 6A2).

No evidence of fibrosis, inflammation or other histopathological changes was observed in the liver of IF-treated rats (Fig. 6B, C). Both IF reduced the occurrence of hepatocyte hypertrophy,

while ballooning was less pronounced in genistein-treated animals. With regard to microvesicular steatosis, after IF treatments, its occurrence was even less than in controls.

Discussion

In this study, we clearly demonstrated that IF elevated the availability of T_3 in the liver, despite decreasing serum concentration of T_4 in male MA rats. Serum T_3 was not affected while hepatic T_3 was almost doubled, which supports our experimental approach to target hepatic local T_3 formation and action. Hepatic increase of T_3 correlated with up-regulated expression of the *Cyp7a1* gene and elevated 7α-hydroxycholesterol in the liver of MA males. IF also lowered 24-hydroxycholesterol and desmosterol in the liver and serum, and decreased 27-hydroxycholesterol in serum, while the total cholesterol levels remained unchanged.

Concentration of IF and their metabolites in serum of genistein- and daidzein- treated males were similar to equally treated MA females [10], and conformed to concentrations in blood of healthy people upon ingestion of similar doses of purified IF [28] or natural food sources rich in IF [5]. Our data confirmed that S(-)-equol was, as expected, not present in the serum because of the subcutaneous route of administration which consequently avoided any metabolism of dietary daidzein to S(-)-equol by gut microbiota [21]. In contrast to rodents, only 20-30% of human soy consumers in Western countries have the possibility to produce S(-)-equol, the most potent phytoestrogen and antioxidant of all isoflavones [21].

T_3 concentrations and Dio1 enzyme activity in the liver were higher in both IF-treated groups, while T_4 was elevated only in genistein-treated rats. The obtained results are in line with displacement of both TH from TTR by IF as previously demonstrated in vitro [6]. TTR binding of both isoflavones might transiently elevate free TH and make them more easily available for peripheral tissues, especially liver [29, 30]. Similar results were also obtained for rats treated

with synthetic flavonoid F 21388, which is a highly selective competitor for T_4 binding to TTR [31, 32]. Increased hepatic Dio1 expression and activity, in the context of decreased or unchanged serum T_4 and/or T_3 concentrations, has been reported in rodents for several conditions where endocrine disrupting compounds were administered [8, 33] and is compatible with elevated hepatic T_3 concentrations observed for both genistein and daidzein.

Aside from elevated T_3 concentration, increased hepatic T_4 was detected only in case of genistein-treated animals, which might be explained by a higher serum concentration of the genistein aglycone and the lower affinity of daidzein in competing for T_4 binding to TTR [6]. As daidzein is a weaker competitor at TTR and has lower serum concentration in comparison to genistein, probably not so much T_4 could be accumulated in the liver. Dio1 can function as either phenolic ring or tyrosyl ring iodothyronine Dio enzyme, thus contributing to both T_3 production and, according to substrate affinity, even more to metabolism of TH in peripheral organs such as liver and kidney [34]. Moreover, differences in inhibition of TH efflux from the liver may also contribute to its increased intracellular availability [35, 36]. In the mouse liver, MCT 8 and 10 seems to be more involved in facilitating the efflux of TH, whereas other unidirectional transport systems are critical for the influx of TH [37]. A limited number of papers are available regarding IF interference with TH transporters. Genistein was reported to act as an inhibitor of MCT8 at high concentrations but is a pharmacological chaperone of mutated human MCT8 at low concentrations [38].

Extrapolation of our data obtained in rodents to humans is uncertain as thyroxin - binding globulin (TBG) is the major serum transport protein in humans and IF do not compete with THs for binding to TBG or albumin *in vitro* [6]. However, we should bear in mind that TTR is the only transport protein of TH in cerebrospinal fluid, and also that the biological importance of

transport proteins is related to the delivery of TH to cells, which makes TTR responsible for much of the immediate delivery of TH to human tissues [30, 39].

T_4 was lower, while T_3 and TSH concentrations remained unaltered in serum of IF-treated MA rats. Lower serum T_4 may be caused by decreased synthesis and secretion via the thyroid gland and/or increased hepatic TH conjugation and elimination. Both genistein and daidzein were shown to markedly lower activity of TPO, a key enzyme in TH biosynthesis in vitro and in vivo [4]. As we detected elevated or unchanged hepatic T_4 in case of genistein- and daidzein-treated rats, respectively, it seems logical to assume that thyroid production of both T_4 and T_3 was reduced upon IF treatments. In rats, all of the f T_4 and approximately 40% of serum T_3 in rats is produced by the thyroid gland, while approximately 60% originates from extrathyroid deiodination pathways mediated by Dio1 (30%) and Dio2 (30%) enzymes [40]. Keeping in mind demonstrated hepatic elevation of T_3 and its rapid exchange with the circulation, unchanged concentration of T_3 and consequently unchanged TSH in serum might be expected. However, considering serum TH status, young adult and age-matched MA female rats remained euthyroxinemic [4, 5, 10]. This age- and sex-related difference is probably due to a lower capacity of the thyroid hormone endocrine system to compensate adverse effects of xenobiotics in males with advanced age [20, 41, 42]. In contrast to observations reported here, lower serum T_4 and T_3, as well as higher TSH were obtained in Orx MA model [9], in accordance with decreased activity of pituitary Dio2 and hepatic Dio1 enzymes, which indicated local tissue hypothyroidism upon orchidectomy [43].

Next, we supposed that an elevated hepatic T_3 concentration might potentiate cholesterol degradation to bile acids in the liver. Our assumption was supported by the fact that T_3, among several other factors, up-regulates transcription of the P_{450} enzyme cholesterol 7 α-hydroxylase

(*Cyp7a1*) gene in rats and humans [44, 45]. This is the rate-limiting enzyme in the main neutral pathway for elimination of cholesterol from the liver [46]. Indeed, we demonstrated an up-regulated expression of *Cyp7a1* gene as well as increased concentration of CYP7A1 end product, 7α- hydroxycholesterol after IF treatments. In line with our data, genistein and daidzein elevated hepatic expression of *Cyp7a1* in adult male Sprague-Dawley rats [15]. However, when we treated MA females with IF under the same regime, concentration of 7 α-hydroxycholesterol remained unchanged [10]. MA females did not respond to IF treatments with regard to serum T_4, while hepatic Dio1 activity was moderately elevated only after genistein treatment. The thyroid endocrine system in females is less susceptible to xenobiotics than in males [20], and our results are in line with indications that hepatic regulation of cholesterol metabolism in males is more sensitive to TH manipulation compared to female rodents [47, 48].

However, it is important to add that regulation of *Cyp7a1* is subject to a complex transcriptional regulation by the flux of bile acids and cholesterol through the liver, and by a great number of dietary and hormonal factors [46] that can be also influenced by IF. Apart from the hereby examined interaction with thyroid homeostasis, IF were shown to directly bind to estrogen and FXR receptors [12, 13], and to indirectly regulate LXR-mediated actions, possibly by mediating receptor phosphorylation via adenosine monophosphate-activated protein kinase (AMPK) [14].

Lower hepatic and serum concentration of 24-hydroxycholesterol and desmosterol concentrations as well as serum 27-hydroxycholesterol, were obtained in both IF-treated groups compared to controls. 24-hydroxycholesterol is formed by cholesterol 24-hydroxylase (CYP46a1) mainly in the brain, but also in the rat liver [49] in quantities comparable to those obtained in the present study. In contrast to *Cyp7a1*, the expression of the *Cyp46a1* gene seems

to be independent of exposure to factors known to modulate cholesterol homeostasis, including nuclear receptor ligands [50]. However, it is possible that IF affected this enzyme activity at the posttranscriptional level, considering their free radical scavenging potential [50]. This mechanism might also contribute to lower desmosterol levels, as concentration of desmosterol and its parallel metabolite 24S,25-epoxycholesterol were reduced in the*Cyp46a1*$^{-/-}$ mouse [50, 52]. However, it cannot be excluded that the lower concentration of 24- hydroxycholesterol may be also due to its elevated degradation, which is mediated by cholesterol 7α- hydroxylase [53] and substrate- specific 7α- hydroxylase, CYP39A1 [54]. However, 27-hydroxycholesterol was decreased only in the serum of IF-treated groups, indicating its lower production outside of the liver [55]. In acyclic MA females, hepatic as well as serum concentrations of this oxysterol were lower [10]. Keeping in mind that elevation of 27-hydroxycholesterol promotes atherosclerosis in *Apoe*$^{-/-}$ mice without altering lipid status [56], the obtained change may be also considered beneficial. Overall, our obtained results indicate a strong capacity of IF to trigger multiple mechanisms involved in the control of oxysterol production. Further studies are needed to clarify the molecular interactions underlying the observed changes.

However, IF treatments and the elevated availability of TH in the liver failed to disturb cholesterol homeostasis and to induce a hypocholesterolemic effect in MA males, despite significant changes in various oxysterol and desmosterol levels. This is in agreement with researchers who claim that IF significantly reduce serum cholesterol only in combination with other soy component, such as soy protein isolate (SPI) [11]. As the percentage of aglycone relative to total IF in serum (aglycone + conjugated forms) was less than 5% in this and in our previous study with age-matched females [10], rapid systemic phase II metabolism might attenuate internal exposures to IF and their subsequent pharmacological action if not bound to

SPI as a carrier [57]. The aglycone is the available chemical form for rapid intestinal absorption, but also for phase II metabolism in the intestine [57]. Thus, as we administered isoflavones subcutaneously to avoid species differences in S(-)-equol production by intestinal microflora, their bioavailability may be different in comparison to oral administration, which is a potential limitation of this study. However, genistein exerts similar estrogenic biological effect on female reproductive system independently of administration route [58] and inhibitory effect on TPO activity was confirmed upon oral IF administration [4, 5]. These biological effects in target tissues are primarily related to aglycone form and not to genistein metabolites [59]. However, for a number of endpoints conjugation does not always decrease the biological activity of flavonoids [60]. Examinations of hepatic concentration of IF and their metabolites, and direct comparisons with oral treatment in case of genistein, may be a good approach to address this issue in the future.

Hepatic elevation of TH under our experimental conditions may also contribute to the anti-steatotic effects reported for IF [18]. Histological examinations revealed that IF only slightly reduced age-related degenerative changes and the occurrence of microvesicular steatosis in the liver. However, our experimental design did not provoke fatty liver pathogenesis.

In conclusion, the obtained results directly indicated that IF increased T_3 availability in the liver of MA male rats, apparently by displacing TH from serum TTR binding. Hepatic increase of T_3 may contribute to IF-triggered degradation of cholesterol to bile acids, based on elevated level of *Cyp7a1* gene transcript and of 7α-hydroxycholesterol in the liver. Isoflavones also decreased hepatic and serum 24-hydroxycholesterol and desmosterol, as well as serum 27-hydrohycholesterol levels. Purified IF seem to have the ability to affect multiple targets involved

in the regulation of cholesterol metabolism and oxysterol production in a sex-specific manner, but fail to induce a hypocholesterolemic effect.

Declaration of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends

Figure 1. Thyroid state in the liver of middle-aged vehicle (Control)-, genistein (Genistein) - and daidzein (Daidzein) - treated male rats. Expression of Dio1 gene (A), Dio1 enzyme activity (B), concentration of T_3 (C) and T_4 (D) in liver. Data are presented as mean ± SD (n=5 for control, n=6 for treatment groups; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Figure 2. Concentrations of total thyroxin (T_4; A), 3,3',5-triiodothyronine (T_3; B) and thyroid stimulating hormone (TSH; C) in serum of middle-aged vehicle (Control)-, genistein (Genistein)- and daidzein (Daidzein)- treated male rats. Data are presented as mean ± SD (n=6/group; ** $p<0.01$, **** $p<0.0001$).

Figure 3. Concentration of bile acid precursors 7α-, 27-, and 24-hydroxycholesterol in liver (A-C, respectively) and serum (D-F, respectively) of middle-aged vehicle (Control)-, genistein (Genistein)- or daidzein (Daidzein)- treated male rats. Data are presented as mean ± SD (n=5 for control, n=6 for treatment groups; * $p<0.05$, ** $p<0.01$).

Figure 4. Concentration of cholesterol precursors lanosterol, lathosterol and desmosterol, as well as total cholesterol in liver (A-D, respectively) and serum (E-H, respectively) of middle-aged vehicle (Control)-, genistein (Genistein)- and daidzein (Daidzein)- treated male rats. Data are presented as mean ± SD (n=5 for control, n=6 for treatment groups; * $p<0.05$, ** $p<0.01$).

Figure 5. Expression of *Cyp7a1* gene (A; * $p<0.05$), correlation between: T_3 and *Cyp7a1* (B; $r=0.5190$, $p<0.05$), T_3 and 7α-hydroxycholesterol (C; $r=0.5093$, $p<0.05$) in liver of rats of the middle-aged vehicle (Control)-, genistein (Genistein)- and daidzein (Daidzein)- treated group (n=5 for control, n=6 for treatment groups).

Figure 6. Histological examination of hematoxylin- and eosin-stained liver sections of middle-aged vehicle (A1, A2)-, genistein (B)- and daidzein (C)- treated rats; ms-microvesicular steatosis, b- ballooning hepatocytes.

Fig 1

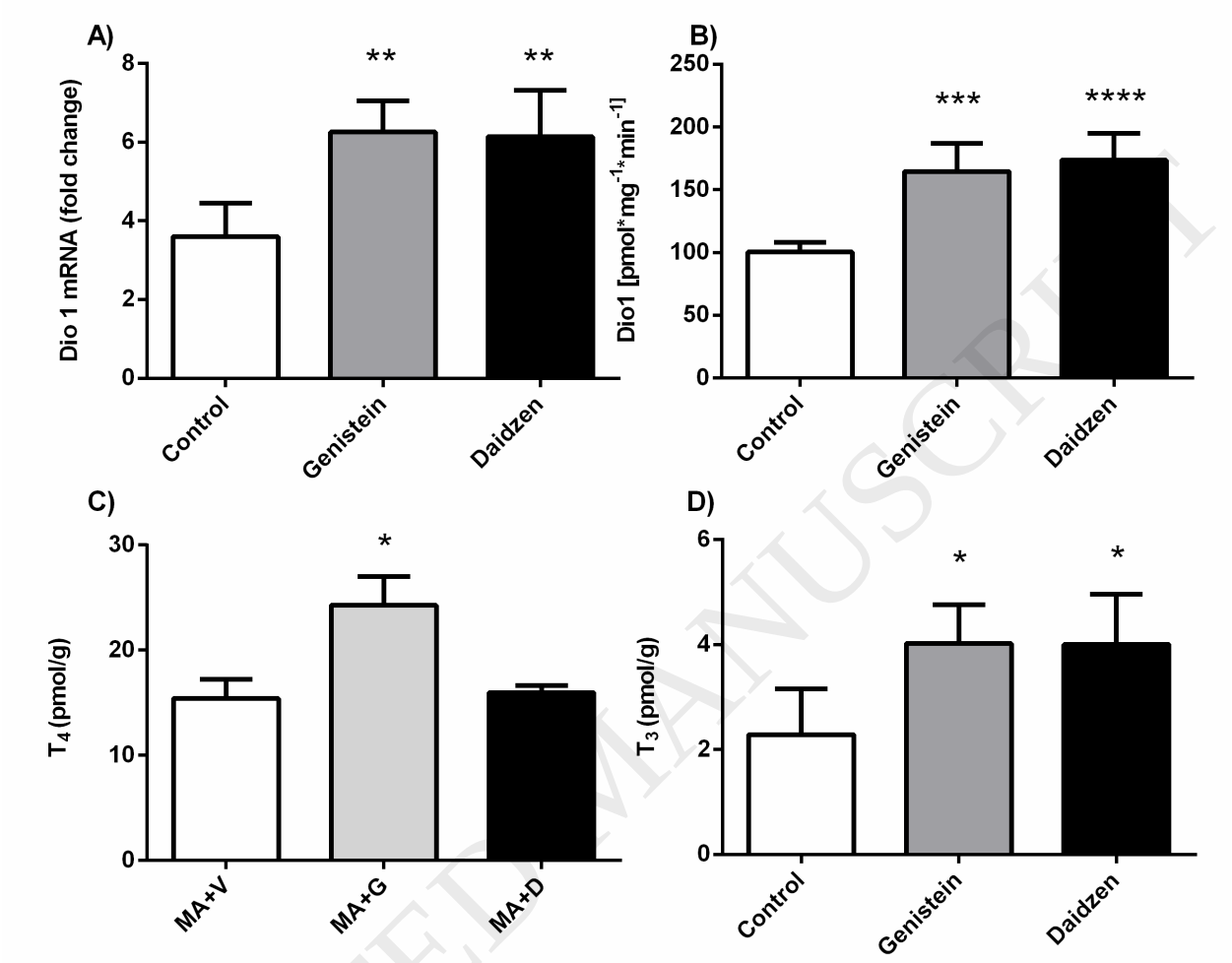

Fig 2

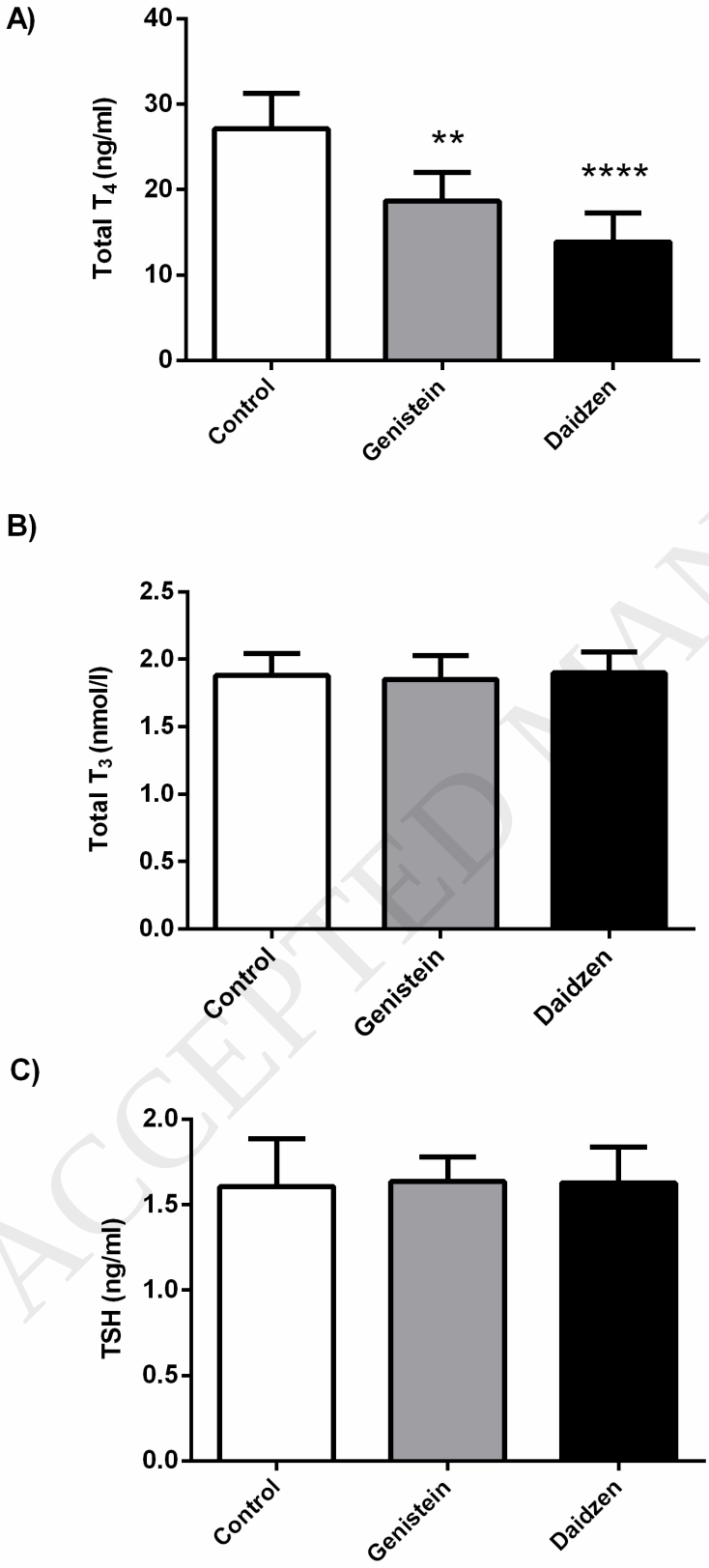

Fig 3

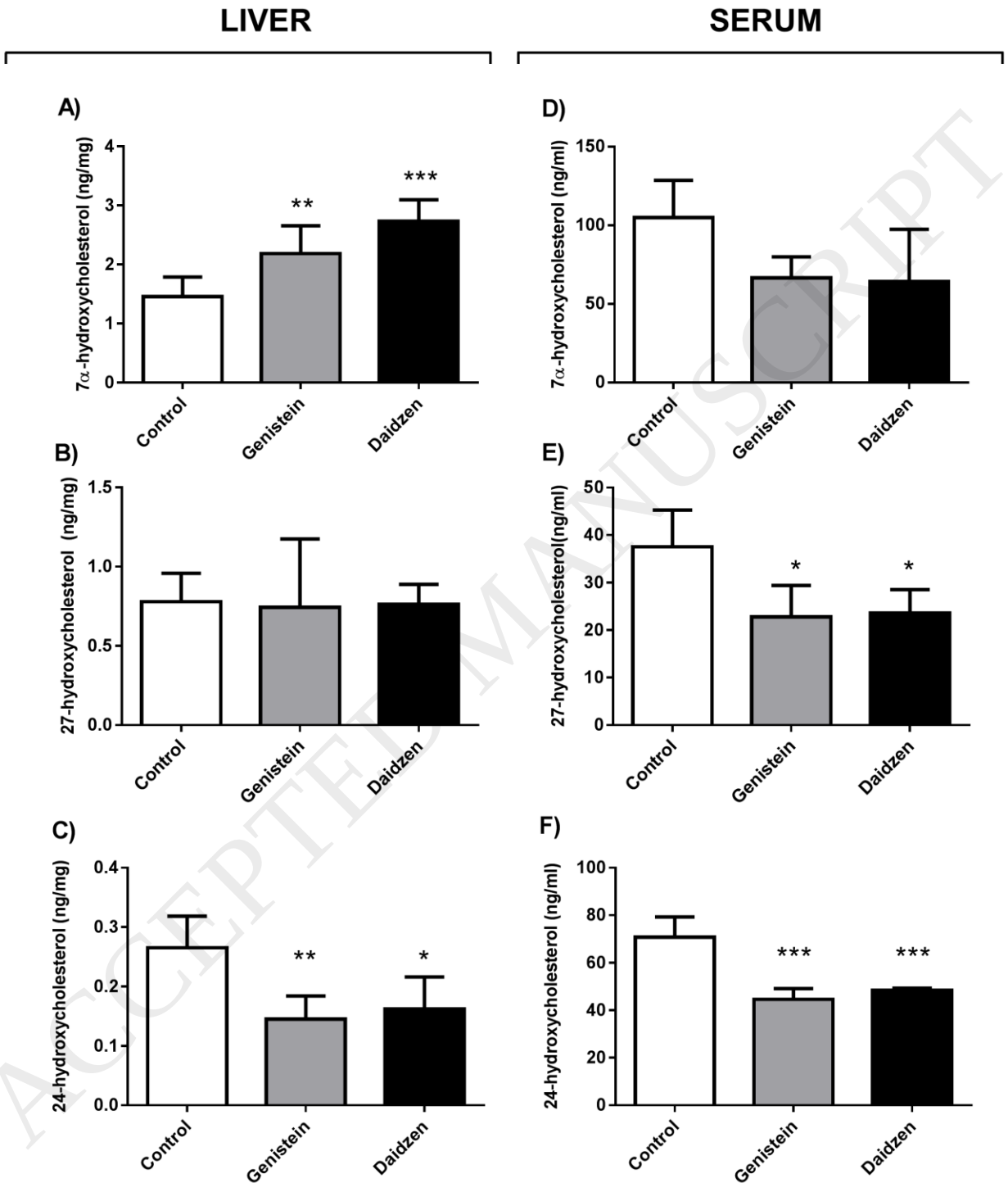

Fig 4

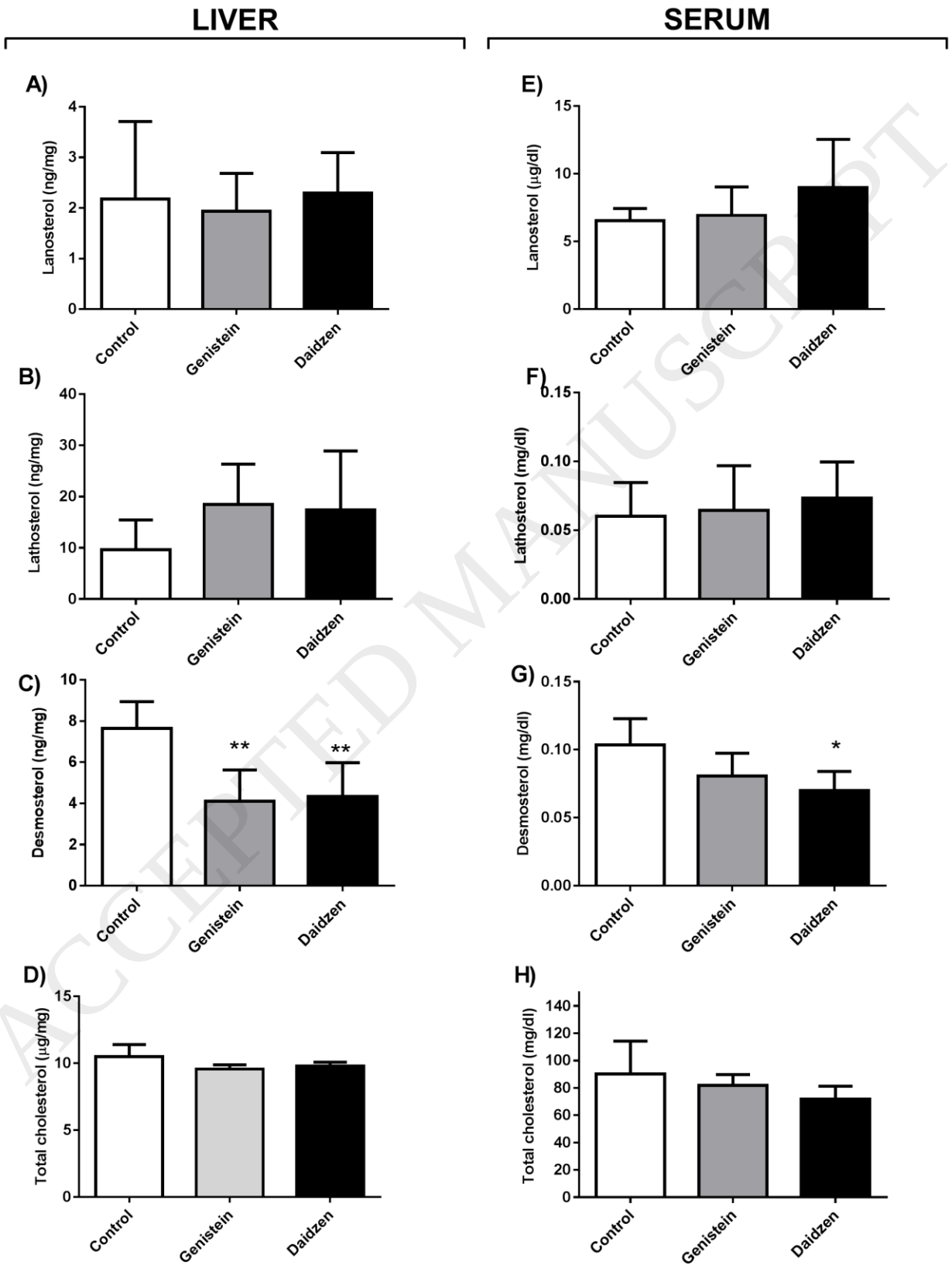

Fig 5

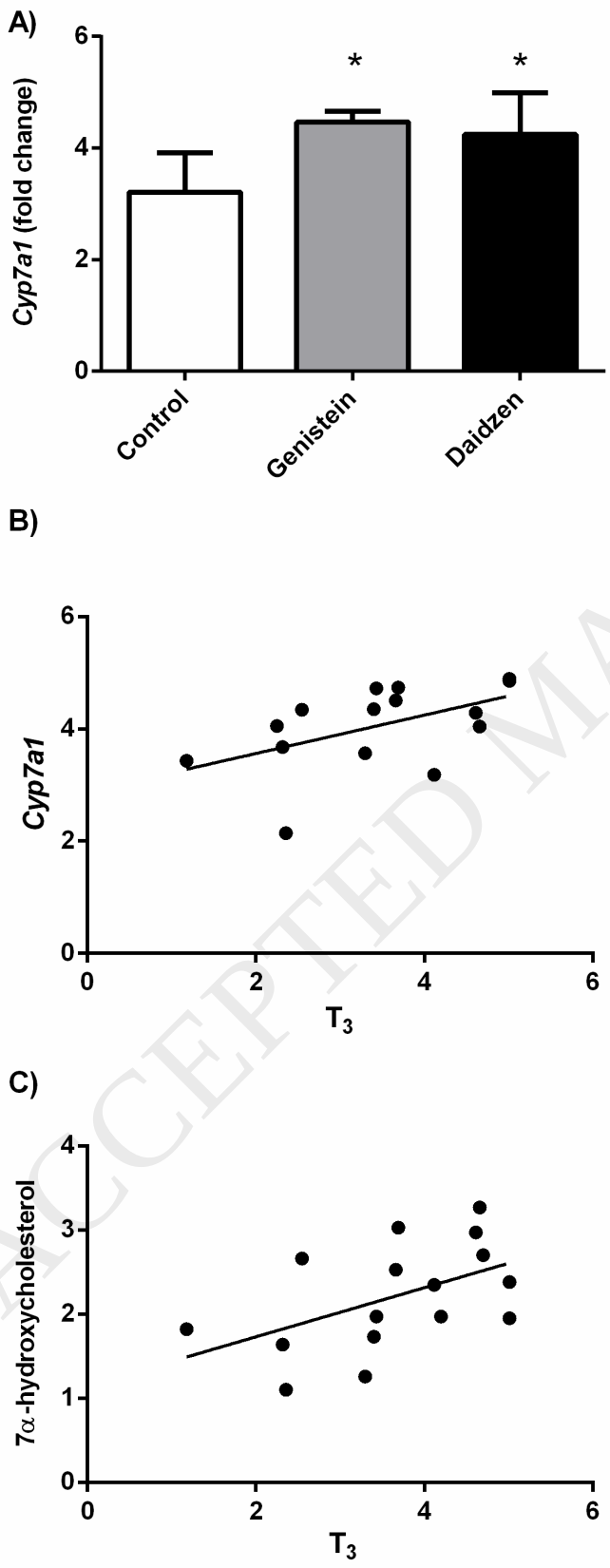

Fig 6

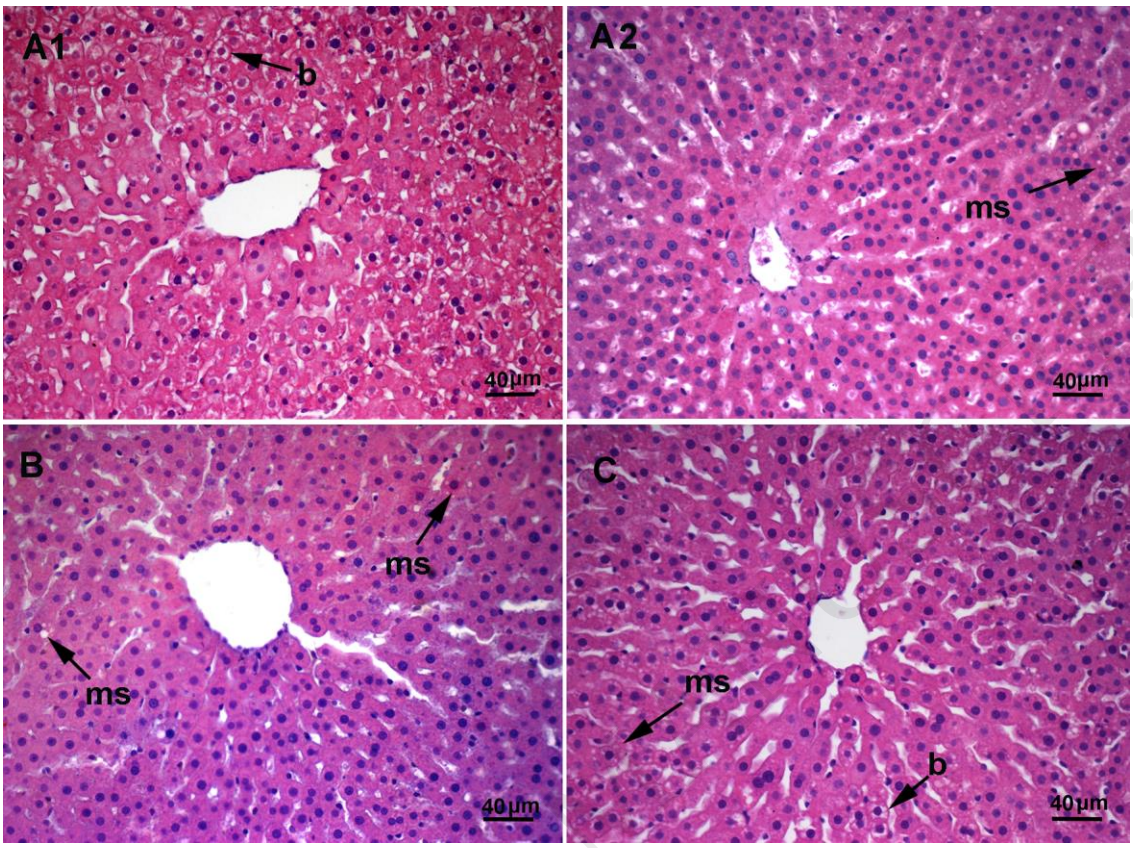

Table 1. Body mass, absolute and relative liver weights of middle-aged vehicle (Control)-, genistein (Genistein)- and daidzein (Daidzein)- treated rats

Group	Body mass (g)	Absolute liver weight (g)	Relative liver weight (% b.w.)
Control	546 ± 30	16.5 ± 1.5	3.1 ± 0.2
Genistein	494± 42	14.9 ± 0.6	2.9 ± 0.2
Daidzein	498± 58	15.2 ± 1.9	2.8 ± 0.1

The values are mean ± SD (n=6)