The combination of coffee compounds attenuates early fibrosis-associated hepatocarcinogenesis in mice: involvement of miRNA profile modulation

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Abstract

Aberrant microRNA expression implicates on Hepatocellular carcinoma (HCC) development/progression. Conversely, daily coffee consumption reduces by ~40% the risk for fibrosis/cirrhosis and HCC, while decaffeinated coffee does not. Are these beneficial effects related to caffeine (CAF), or to its combination with other common/highly bioavailable coffee compounds, as trigonelline (TRI) and chlorogenic acid (CGA)? We evaluated whether CAF individually or combined with TRI and/or CGA alleviates fibrosis-associated hepatocarcinogenesis, examining the involvement of miRNA profile modulation. Male C3H/HeJ mice were submitted to a diethylnitrosamine/carbon tetrachloride-induced model. Animals received CAF (50 mg/kg body weight, 5x/week, intragastrically), CAF+TRI (50 and 25 mg/kg), CAF+CGA (50 and 25 mg/kg) or CAF+TRI+CGA (50, 25 and 25 mg/kg) for 10 weeks. Only CAF+TRI+CGA treatment reduced hepatocellular preneoplastic foci development. Moreover, only the combination of all compounds reduced proliferation (Ki-67) in preneoplastic lesions and enhanced apoptosis (cleaved caspase-3) in adjacent tissue. CAF+TRI+CGA also decreased hepatic oxidative stress by enhancing antioxidant Nrf2 axis. CAF+TRI+CGA had the most pronounced effects on decreasing hepatic pro-inflammatory IL-17/NFκB signaling, contributing to reduce CD68-positive macrophage number, stellate cell activation and collagen deposition. The miRNAomic profile revealed that CAF+TRI+CGA upregulated tumor suppressors miR-144-3p and antifibrotic miR-15b-5p, frequently altered in HCC. CAF+TRI+CGA reduced protein levels of pro-proliferative EGFR (miR-144-3p target) and antiapoptotic Bcl-2 family members (Bcl-2, Mcl-1 and Bcl2l2, miR-15b-5p targets). The results suggest that the combination of coffee compounds, rather than CAF individually, attenuates fibrosis-associated hepatocarcinogenesis by modulating miRNA expression profile. Findings provide translational insights on therapeutic approaches based on miRNA profile modulation by coffee compounds.

Key words: Caffeine; trigonelline; chlorogenic acid; liver fibrosis; hepatocarcinogenesis; C3H/HeJ mice; miRNA.
1. Background

Hepatocellular carcinoma (HCC), the main type of primary liver cancer, ranks as the sixth most incident and fourth deadliest cancer worldwide (841,080 new cases and 781,631 deaths per year) [1]. HCC is considered a poor prognosis disease, with overall median survival of 11 months after clinical diagnosis [2]. Most HCC cases (70 - 90%) arise in the setting of liver fibrosis/cirrhosis, mainly caused by chronic hepatitis B (HBV) and C (HCV) virus infections, alcoholic liver disease and non-alcoholic fatty liver disease (NAFLD) [3,4]. This malignancy emerges due to the accumulation of multiple molecular alterations, including the deregulation of microRNA (miRNA) expression [5,6]. miRNAs are noncoding, single-stranded molecules of ~22 nucleotides that constitute a class of gene regulators [7]. In general, miRNAs canonically interact with the 3' untranslated region (3' UTR) of target mRNAs, inducing their degradation or promoting translational repression [7]. It is estimated that ~60% of protein-coding genes in human genome are controlled miRNA expression [7]. A wealth of evidence points out to the pivotal involvement of miRNAs during hepatocarcinogenesis, as either potential tumor suppressors or onco-miRNAs [8]. In order to unveil the molecular aspects involved in liver fibrosis and carcinogenesis, chemically-induced murine models have been established as suitable tools for pre-clinical research [9-11]. These bioassays display striking morphological and molecular similarities to the corresponding human diseases, including aberrant miRNA expression profile [12], enabling the translational screening of preventive and therapeutic strategies for this liver malignancy [9-11].

In contrast, epidemiological and experimental data suggest that nutritional habits and interventions may reduce the incidence of different types of cancer, including HCC [13]. The “common” brewed and espresso coffee beverages, prepared from roasted and grounded seeds of Coffea genus plant species, are widely consumed worldwide and exhibit impressive impact on the economy of producing/exporting countries [14]. Indeed, there is a spectrum of epidemiological data evidencing the clear inverse correlation between coffee consumption and fibrosis/cirrhosis/HCC risks [15-17]. Overall, coffee consumption (>1 cup/day) reduces by ~40% the risk for fibrosis/cirrhosis and HCC, even in adjustments for risk conditions (as chronic HCV/ HBV infections) or highly incident areas (as Asia and Africa) [16,17]. In contrast, decaffeinated coffee intake leads to none or less pronounced risk reduction [18,19]. Thus, could this protection be attributed to caffeine individually, or, to caffeine combination with other common constituents of coffee beverages? The inherent mechanisms and exact compounds involved in this differential response are still unclear.

The brewed and espresso coffees are complex mixtures that include innumerous compounds of different chemical classes [20,21]. Particularly, the alkaloids caffeine (CAF) and trigonelline (TRI), and the polyphenol chlorogenic acid (CGA)
are some of the most abundant bioactive compounds in coffee beverages [20,21]. After coffee consumption, these compounds present high bioavailability and may accumulate in the plasma due to their long half-life times during regular consumption of many coffee cups along the day [22]. In our previous studies, CAF intake attenuated liver fibrosis and carcinogenesis in rats [23,24]. Furthermore, CGA and TRI are also proposed to reduce liver fibrosis and NAFLD in rats, respectively [25,26]. Despite of presenting beneficial effects individually, literature lacks on mechanistical studies concerning the combination of these abundant and highly bioavailable compounds on liver fibrosis/carcinogenesis models. Besides, as recently reviewed by our group [15], many in vivo and in vitro interventions do not resemble human consumption/bioavailability, and concentrations/doses are usually overestimated. Finally, since naturally occurring phytochemicals are recently proposed to modulate epigenetic machinery, contributing to cancer prevention, the modifying effects of common coffee compounds on non-coding RNA expression should be unraveled in the context of liver disease [15,27].

Thus, we assessed whether CAF administration individually or combined with TRI and/or CGA alleviates fibrosis-associated hepatocarcinogenesis in mice. In parallel, hepatic miRNA profiling was evaluated to unveil the underlying involvement of these non-coding RNAs, correlating the changes in miRNA expression with fibrosis-associated hepatocarcinogenesis outcomes. Also considering a physiological plausible approach, we evaluated the cytotoxic effects of the same compounds on an HCC cell line. Our study could provide a translational insight on potential preventive or therapeutic applications based on tumor suppressor or onco-miRNAs modulated by bioactive coffee compounds. This is the first scientific report on the beneficial effects of coffee compounds on the miRNAomic prolife of a common chronic liver disease model.

2. Materials & Methods

2.1 In vivo experiments

2.1.1 Experimental design

The liver-cancer susceptible C3H/HeJ mice strain was submitted to a previously established fibrosis-associated hepatocarcinogenesis model [11]. Briefly, male mice (n = 5 to 10 animals/group) were initiated for liver carcinogenesis by receiving a single intraperitoneal (i.p.) injection of diethylnitrosamine [DEN, 10 mg/kg body weight (b.wt.) in 0.9% saline, Sigma-Aldrich, USA] or just saline vehicle at 14 postnatal day (PND) (week 2) (Figure 1). Mice were weaned at PND 28 (week 4). In order to promote DEN-initiated hepatocytes in a fibrotic background, resembling 70-90% of HCC cases in
humans [3], mice received three weekly i.p. doses of carbon tetrachloride from week 8 to 16 (CCl₄, 10% solution in corn oil, Sigma-Aldrich, USA) or corn oil vehicle (4-6 p.m.). The initial dose of CCl₄ solution was 0.25 µL/g body weight (b.wt.) (corresponding to 0.025 µL/g or 0.040 µg/g of pure CCl₄) and there were 0.25 µL weekly increments to the utmost dose of 1.50 µL/g b.wt. (corresponding to 0.15 µL/g or 0.24 µg/g of pure CCl₄) [11]. In addition, from week 7 to 17, mice received CAF alone (50 mg/kg b.wt.), CAF and TRI (50 and 25 mg/kg b.wt./day, respectively); CAF and CGA (50 and 25 mg/kg b.wt./day, respectively); CAF, TRI and CGA treatments (50, 25 and 25 mg/kg b.wt./day, respectively) or just distilled water vehicle (intragastrically, five times per week) (8-10 a.m.) (Figure 1). Solutions containing coffee compounds were prepared every day. All mice were euthanized by exsanguination under ketamine/xylazine anesthesia (100/16 mg/kg b.wt., i.p.) at 17 weeks of age, a week after the last CCl₄ administration. Blood was collected from cardiac puncture, centrifuged (1503×g, 10 min.) and serum samples were collected in heparinized syringes and stored at -80°C for further analysis. At necropsy, liver was removed, weighted and representative samples from left lateral, right medial and caudate liver lobes were collected for histological analysis, according to previous trimming recommendations [28]. Additional samples from the left lateral and medial lobes were collected, snap-frozen in liquid nitrogen and stored at -80°C.

The animals were obtained from School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ, USP, São Paulo-SP, Brazil) and were kept in Botucatu Medical School of São Paulo State University (FMB, UNESP, São Paulo-SP, Brazil). Mice were kept in a room with continuous ventilation (16-18 air changes/hour), relative humidity (45-65%), controlled temperature (20-24°C) and light/dark cycle 12:12 h and were given water and diet (Nuvital - Nuvilab, Brazil) ad libitum. Body weight and food consumption were recorded once a week during all experimental period. The animal experiment was carried out under protocols approved by Botucatu Medical School/UNESP Ethics Committee on Use of Animals (CEUA) (Protocol number 1186/2016) and all animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” [29].

2.1.2 Dose determination

High coffee consumption, as observed in USA and in many European countries, leads to an estimated CAF intake range of 200-300 mg/day (~2.8 to 4.0 mg/Kg b.wt./day, considering 70 Kg adults) [30,31]. Therefore, the CAF dose (50 mg/Kg b.wt.) was calculated based on the allometric translation of Human Equivalent Dose (HED) [32], considering the dose of 4.0 mg/Kg b.wt./day, as it follows:
(I) \[ \text{HED (mg/kg/day)} = \frac{\text{Animal dose (mg/kg/day)} \times \text{Mice Km}^*}{\text{Animal Km}} \]

(II) \[ \text{HED} = 50 \times \frac{3}{37} \]

(III) \[ \text{HED} = 4 \text{ mg/kg/day or 280 mg/day (70 kg adult)} \]

\(^*\text{Km} = \text{species-related constant based on body weight and surface area [32]}

The administered dose corresponded to the consumption of 280 mg CAF/day, equivalently to 2-3 cups of common coffee brew [15,21]. Moreover, this dose was previously applied and showed no toxic effect on chemically-induced cirrhosis in rodents [33]. Since the epidemiological studies are focused on CAF consumption from coffee beverages, both TRI and CGA doses (25 mg/kg b.wt.) were based on TRI or CGA/CAF ratio found in filtered coffee, as previously determined by our research group [24]. TRI (0.51 mg/mL of coffee), CGA (0.41 mg/mL of coffee) and CAF (1.0 mg/mL of coffee) concentrations in filtered coffee display a ratio of 1/2, resembling previous studies [20,21]. The administration of coffee compounds from the seventh week of age on (sexual maturity) resembles human exposure to coffee/CAF, which starts from puberty and extends over the adult age [31,32].

2.1.3 Proneoplastic hepatocyte foci screening

Liver samples were fixed in 10% buffered formalin for 24 h at room temperature, stored in 70% ethanol and embedded in paraffin. Five-micron thick liver sections from paraffin embedded blocks were obtained and stained with Hematoxylin and Eosin (H&E), the gold standard staining to identify preneoplastic liver lesions. The altered hepatocyte foci (AHF), considered the main endpoint lesions for the 17-week time-point, were identified by blind reading of coded slides, using well-established criteria [11,28]. The analyzed sections had representative fragments of left lateral, right medial and caudate lobes (one slide/animal). Then, total number of AHF/liver section area (cm²), the mean size (mm²), the relative area (sum of all AHF areas/liver section area, in mm²/cm²) and the incidence of these lesions were calculated. The liver section areas were measured by Stemi 2000 stereo zoom microscope (Zeiss, Germany) using a Dino Capture (ANMO Electronics Corporations, USA) image analysis system. The AHF size was measured by Olympus CellSens software (Olympus Corporation, Japan).
2.1.4 Collagen morphometry and Immunohistochemistry

Quantitative analysis of collagen fibers was performed in Sirius red-stained sections using Leica QWin V3 software (Leica Microsystems, Germany), selecting 10 random microscopic fields (20× objective) per section (left lobe), comprising portal areas [Collagen area (%) = Sirius red area / total 20× field area analyzed]. For immunohistochemistry, deparaffinized 5-µm liver sections on silane-covered microscope slides were subject to antigen retrieval in 0.01M citrate buffer (pH 6.0, 120°C, 5 min) in a Pascal Pressure Chamber (Dako Cytomation, Denmark). After endogenous peroxidase blockade with 1% H₂O₂ in phosphate-buffered saline (PBS) (15 min.), the slides were treated with skim milk (60 min.) and incubated in a humidified chamber (4°C, overnight) with anti-α-smooth muscle actin (α-SMA, i.e. hepatic stellate cell marker, ab124964, 1:500 dilution, Abcam, UK), anti-CD68 (i.e. macrophage/Kupffer cell marker, ab125212, 1:1000 dilution, Abcam, UK), anti-Ki-67 (i.e. cell proliferation marker, ab16667, 1:100 dilution, Abcam, UK), anti-Proliferating Cell Nuclear Antigen (PCNA, i.e. cell proliferation marker, PC10, 1:100 dilution, Dako Cytomation, Denmark), anti-cleaved caspase-3 (i.e. apoptosis marker, 5A1E, 1:100 dilution, Cell Signaling, USA), anti-NFκB p65 (sc-372, 1:100 dilution, Santa Cruz Biotechnology, USA) primary antibodies. Then, slides were incubated with one-step horseradish peroxidase (HRP)-polymer (EasyPath - Erviegas, Brazil) (20 min). Reactions were visualized with 3’3-diaminobenzidine (DAB) chromogen (Sigma–Aldrich, USA) and counterstained with Harris hematoxylin.

The semiquantitative analysis of α-SMA immunostained sections was performed as described for collagen morphometry. In adjacent liver (avoiding preneoplastic foci), 10 random fields (20× objective) were assessed in left hepatic lobe sections, mainly comprising portal areas. Then, Ki-67 and PCNA positive hepatocytes; cleaved caspase-3 positive cells; and CD68 macrophages were counted and divided by the liver area analyzed (mm²). In preneoplastic foci (considering all types), all Ki-67 and PCNA-positive hepatocytes or cleaved caspase-3-positive cells were counted and divided by the lesion area analyzed (mm²). All analyses were performed in Olympus CellSens (Olympus Corporation, Japan) and Image J software (National Institutes of Health, USA).

2.1.5 Enzyme-Linked Immunosorbent Assay

Liver samples (~100 mg) of the left medial lobe were collected, and homogenized in RIPA buffer (Cell Signaling, USA) containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA), in proportion 30 mg tissue/100 μl buffer (4°C, 2 h.). Then, the homogenate (10,000×g, 4°C, 30 min.) was centrifuged and the supernatant was collected for protein quantification by the Bradford method. The levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and 17 (IL-17) were
determined by the Luminex multiple analyte profiling (xMAP) methodology using a 96-well plate containing specific magnetic beads for each of the cytokines, following the manufacturer's instructions (MCYTOMAG-70K, Millipore, USA). Before normalization by the amount of protein (mg or g), liver samples followed the limits of detection of the kit: IL-6 (1134 - 107 pg/mL), IL-17 (1074 - 103 pg/mL), and TNF-α (1200-110 pg/ml).

2.1.6 TBARS and antioxidant enzymes

For thiobarbituric acid reactive species (TBARS) determination, left medial lobe samples (50 mg) were homogenized with magnetic beads in 0.5 mL of 3% sulfosalicylic acid solution and centrifuged (18,000×g, 4°C, 3 min.) Then, samples were mixed with 0.67% thiobarbituric acid solution (1:1 proportion) [34]. For antioxidant profiling, other samples of the left medial lobe (50 mg) were homogenized in 50 mM phosphate buffer (pH 7.4) using a motor-driven Teflon glass Potter Elvehjem (100×g/min) and centrifuged (12,000×g, 4°C, 15 min). The supernatant was collected for antioxidant enzyme determination. Catalase activity was assayed in sodium and potassium phosphate buffer with 10 mM hydrogen peroxide [35]. Glutathione peroxidase (GSH-Px) determination followed the oxidation of 0.16 mM NADPH in the presence of glutathione reductase (GR) [36]. Superoxide dismutase (SOD) was determined by the reduction of hydroxylamine-generated nitro blue tetrazolium (NBT), in a medium containing 0.1 mM EDTA, 50 mM NTB, 78 mM NADH and 33 mM phenazine methosulfate [37]. All determinations were performed using a microplate reader (25°C) (mQuant-Gen5 2.0 software, Bio-Tec Instruments, USA).

2.1.7 RNA extraction and miRNA global expression assay

Liver samples (~30 mg) of left lateral lobe was homogenized in 1 mL QIAzol (Qiagen, UK). Total RNA was isolated separately using a QIAGEN RNeasy column-based system following the manufacturer’s instructions (Qiagen, UK). RNA quantification and integrity were assessed in Qubit 2.0 Fluorometer (Invitrogen, USA) and Agilent 2100 Bioanalyzer platform (Agilent Technologies, USA), respectively. Samples with RNA integrity number (RIN) >7.0 were profiled for miRNA expression [7.65 ± 0.43, mean ± standard deviation (S.D.)]. Reports on RIN are presented in Supplementary Figure 1. The RNA samples were stored at -80°C until further analysis.

An amount of 100 ng of total RNA (each sample/mouse) was used for nCounter Mouse v1.5 miRNA global expression assay in an automated system (NanoString technologies, USA). These analyses were performed in Barretos Cancer Hospital (Barretos - SP, Brazil). Briefly, total RNA samples were incubated with specific tags that bind the 3’ end
of each mature miRNA, in order to normalize miRNA melting temperatures. The tag excess was removed, and the miRNA-
tag complexes were incubated with 10 µL and 5 µL of reporter and capture probes, respectively, at 64°C for 18 h. Reporter probes had specific fluorescent signals for each miRNA in 5'end and capture probes are biotinylated in 3' end. The mix was purified and then pipetted in a streptavidin-covered cartridge by nCounter Prep Station. Finally, cartridges were analyzed in nCounter Digital Analyzer, which acquired 280 fields of view per sample and counted the miRNA-reporter probe complexes. For miRNA expression analysis, raw counting of miRNA-reporter probe complexes was normalized by the median of the top-ten miRNAs presenting the lowest coefficient of variation (low CV values). Student t test was used for pair comparison, considering $p < 0.05$ and fold change (FC) > 1.5. The heat maps and statistical analysis were performed in Galaxy computational environment (https://usegalaxy.org) [38]. Pair comparisons were crossed using Venn's diagram, distinguishing the differentially expressed miRNAs related to the treatments.

**2.1.8 miRNA target analysis and Gene Ontology/KEGG pathway analysis**

After the identification of differentially expressed miRNAs, Ingenuity Pathway Analysis software (IPA, Qiagen, USA) was applied for miRNA target analysis, restricting outcome target lists to experimentally validated data. The output list was submitted to functional enrichment analysis using Gene ontology (GO) Consortium online platform (https://geneontology.org) [39] and KEGG pathway analysis in DAVID Bioinformatics Resources 6.8 online platform (https://david.ncifcrf.gov) [40]. The main biological process (BP) annotations and KEGG terms were ranked by the lowest adjusted $p$ values, considering $p < 0.05$. STRING v11 (https://string-db.org) was applied for drawing association networks, considering curated databases and experimentally determined interactions among targets [41].

**2.1.9 Immunoblotting**

Aliquots containing 7 µg of total protein (extracted as described in item 2.1.5) were heated (95°C, 5 min) in Laemmli sample buffer (2.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol) and then electrophoretically separated in a 10% SDS–PAGE gel under reducing conditions and transferred to nitrocellulose membranes (Bio-rad Laboratories, USA). Membranes were blocked with skim milk in Tris-Buffered Saline-Tween (TBS-T, 1 M Tris, 5 M NaCl, pH 7.2, 500 µL Tween-20) (1 h). Membranes were subsequently incubated with anti-NFκB p65 (sc-372, 65 kDa, 1:1000 dilution, Santa Cruz Biotechnology, USA), anti-α-SMA (ab124964, 43 kDa, 1:1000 dilution, Abcam, UK), anti-Nrf2 (PA5-27882, ~95–110 kDa, 1:1000, Thermo Fisher Scientific, USA), anti-HIF-1α (PA1-16601, 93 kDa, 1:1000 dilution, Thermo Scientific, USA) and anti-β-actin (A2228, 40 kDa, 1:1000 dilution, Sigma, USA).
dilution, Thermo Fisher Scientific, USA), anti-Bcl2l2 (PA5-78865, 21 kDa, 1:250 dilution, Thermo Fisher Scientific, USA), anti-Mcl-1 (PA5-11389, 37 kDa, 1:4000 dilution, Thermo Fisher Scientific, USA), anti-VEGF (PA5-16754, 37 kDa, 1:700, Thermo Fisher Scientific, USA), anti-Bcl-2 (PA5-20068, 26 kDa, 1:2000, Thermo Fisher Scientific, USA), anti-EGFR (ab2430, 170 kDa, 1:250, Santa Cruz Biotechnology, USA), anti-PCNA (PC10, 36 kDa, 1:1000 dilution, Dako Cytomation, Denmark), or anti-β-actin (sc1615, 43 kDa, 1:1000 dilution, Santa Cruz Biotechnology, USA) primary antibodies diluted in TBS-T overnight. After 5 wash steps with TBS-T, membranes were incubated with specific HRP-conjugated secondary antibodies, according to the primary antibodies used (2 h). Finally, membranes were submitted to immunoreactive protein signals detected using Clarity Max ECL Substrate (Bio-Rad Laboratories, USA). Signals were captured by a G:BOX Chemi system (Syngene, UK) controlled by an automatic software (GeneSys, Syngene, UK). Band intensities were quantified using densitometry analysis Image J software (National Institutes of Health, USA). Finally, protein expression was reported as fold change according to β-actin protein expression used as a normalizer.

2.2 In vitro experiments

2.2.1 Cell culture, treatments and cytotoxicity assay

HCC C3A cell line (clonal derivative of human HepG2 cells) (ATCC, CRL-10741TM, USA) were grown in Eagle’s Minimum Essential Medium (MEM) (Vitrocell, Brazil) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) in a humidified atmosphere of 5% CO2 at 37°C. C3A cells were seeded in 96-well plates at a density of 7 × 10^4 cells/mL. Twenty-four hours after seeding, tumor cells were treated with medium supplemented with varying concentrations of CAF alone or combined with TRI and/or CGA (Sigma-Aldrich, USA) (6 subcultures per treatment) for 24 or 48 h, according to the Supplementary Table 1. All compounds were diluted with MEM to the desired concentrations. CAF concentrations were based on serum peak (~40 µM) after the ingestion of 280 mg of CAF, equivalently to 2-3 cups of common coffee brew [15,21,22].

For cytotoxicity assessment, lactate dehydrogenase (LDH) levels were measured using a colorimetric kit (Roche Diagnostics, Germany), based on the reduction of tetrazolium salt to formazan by NADH generated during LDH-mediated conversion of lactate to pyruvate. A positive control for cell lysis was established by adding 100 µL of 2% triton X-100 solution (Thermo Fisher Scientific, USA). Plates were centrifuged (250 ×g, 10 min.) in order to obtain a cell-free supernatant. Then, the supernatant (100 µL/well) was transferred from the top of all the wells to the LDH assay plate. Next, supernatant was mixed with kit working solution (100 µL/well) and assay plates were then incubated at room temperature in the dark for
20 min. The absorbance (abs.) was measured at 340 nm using an automated ELISA plate reader (Varioskan Flash, Thermo Scientific, USA). The results were calculated by the following relation: (sample abs. value – untreated control abs. value) / (positive control abs. value – untreated control abs. value) × 100. Three independent experiments were performed.

2.3 Statistical analysis

Data were analyzed by One-way ANOVA or Kruskal-Wallis and post hoc Tukey’s test. Data on incidence were analyzed by Fisher’s Exact test. Differences were considered significant when p<0.05. Statistical analyses were performed using GraphPad Prism software 6.0 (GraphPad, USA). Data are presented as mean ± standard deviation (S.D.), box plot or the proportion of affected animals (percentage). The number of replicates (n) per group for each analysis is determined in results section.

3. Results

3.1 General findings

All interventions with bioactive coffee compounds did not significantly alter final body weight and food consumption compared to vehicle and DEN/CCl₄ groups (Table 1). Indeed, all groups showed similar body weight evolution curves during the 17 weeks of experiment (Supplementary Figure 2). As expected, DEN/CCl₄-induced fibrosis-associated hepatocarcinogenesis model increased absolute (p<0.001) and relative (p=0.019) liver weights, as well as elevated serum ALT levels (p=0.027), compared to the untreated counterpart (Table 1). In contrast, all coffee compound interventions reduced liver absolute weight compared to DEN/CCl₄ model (p<0.001) (Table 1). CAF and CAF+TRI+CGA oral treatments diminished liver relative weight as well (p=0.019) (Table 1). Despite of presenting 7% to 20% reductions in ALT levels compared to DEN/CCl₄ group, coffee compound-treated groups did not significantly alter ALT levels (Table 1).

3.2 The combination of coffee compounds attenuates preneoplastic lesion development

In the early stages of mouse hepatocarcinogenesis, the AHF are considered putative preneoplastic lesions and were identified in H&E-stained sections as eosinophilic, basophilic and clear cell phenotypes (Figure 2A). DEN-induced AHF frequently display Hras and Braf (97.3% of basophilic foci) oncogene mutations, predisposing these hepatocellular lesions to neoplastic progression under promoting stimuli [42,43]. In the established DEN/CCl₄-induced model, basophilic phenotype prevails [11]. As expected, the established DEN/CCl₄–induced model increased the incidence preneoplastic foci
compared to the untreated counterpart (p<0.001) (Table 2). Interestingly, only the CAF+TRI+CGA combination reduced the incidence of clear cell foci (H&E) compared to DEN/CCl4 group (p=0.011) (Table 2). Moreover, only the combination of all coffee compounds reduced by 43% and 38% the number of all foci (p=0.024) and basophilic phenotype (p=0.029) per liver area compared to DEN/CCl4 group, respectively (Figure 2B). The decreased number of lesions also led to a diminished relative foci area (%) in CAF+TRI+CGA group (p=0.032) (Supplementary Figure 3). Due to carcinogen effect on glycogen metabolism, the glycogenotic eosinophilic and clear cell foci phenotypes are the first to arise in hepatocarcinogenesis [44]. Progressively, these lesions tend to a “metabolic turnover”, giving rise to the basophilic foci with a glycogenolytic profile [44]. These metabolic changes, as well as Braf and Hras mutations, are the stimulus to promote cell proliferation, a cancer hallmark [44, 45]. Thus, our findings indicate that the combination of coffee compounds, rather than CAF individually, may modulate different steps of hepatic preneoplasia development.

### 3.3 The combination of coffee compounds reduces proliferation in preneoplastic foci and increases apoptosis in adjacent tissue

Sustained cell proliferation into preneoplastic lesions could favor the accumulation of genetic and epigenetic alterations, predisposing AHF to neoplastic progression [45]. DEN/CCl4 model induced an increase (p<0.0001) in hepatocyte proliferation (Ki-67-positive cells) in adjacent tissue, compared to the untreated counterpart (Figure 3). Although all coffee compound treatments did not modulate cell proliferation in adjacent tissue, only the combination of all coffee compounds diminished hepatocyte proliferation (Ki-67+ cells) inside preneoplastic lesions (considering all types) compared to CAF and DEN/CCl4 groups (p=0.0059) (Figure 4A). A negative modulation of cell proliferation into AHF can slow lesion development [46], resulting in diminished incidence, number of lesions per liver area and relative area as previously indicated in item 3.2. In addition, the CAF+TRI+CGA intervention presented significantly more apoptotic cells (cleaved caspase-3-positive cells) in adjacent liver tissue when compared to the other groups (p<0.0001) (Figure 3). Treatments did not modulate the number of apoptotic cells inside preneoplastic foci (Figure 4B).

### 3.4 The combination of coffee compounds attenuates fibrosis by decreasing IL-17/ NFκB p65 axis

In keeping with the increased liver weight and ALT levels, the applied DEN/CCl4 model displayed typical liver fibrosis, featuring enhanced collagen area (fibrous expansions and bridging) (p<0.0001), HSC activation (increased α-SMA protein expression) (p<0.0001) and increased number of CD68-positive macrophages (predominantly concentrated in
fibrous expansions) (p=0.0004) when compared to the untreated counterpart (Figure 5A, B and C). In accordance, DEN/CCl4 model presented 2.4-fold higher hepatic levels of pro-inflammatory cytokine IL-17 compared to the untreated group (p=0.007) (Figure 5B). Conversely, only the combination of CAF+TRI+CGA, rather than CAF individually, reduced by 25% the collagen area compared to the DEN/CCl4 group (p<0.0001), displaying more delicate collagen fibers in Sirius red-stained liver sections (Figures 5A and B). Moreover, only the combination of all coffee compounds decreased the number of CD68-positive macrophages in comparison to CAF, CAF+TRI-treated and DEN/CCl4 groups (p=0.0004) (Figure 5B). The CAF+TRI, CAF+CGA and specially, CAF+TRI+CGA treatment reduced IL-17 hepatic levels compared to DEN/CCl4 group as well (p=0.007) (Figure 5B). IL-6 and TNF-α hepatic levels were similar in all groups (Supplementary Figure 3). All coffee compound combinations, including CAF+TRI+CGA, decreased α-SMA protein expression in the liver compared to DEN/CCl4 (p<0.0001), indicating an attenuation in HSC activation by coffee compound combination that corroborates with reduced collagen deposition in CAF+TRI+CGA group (Figure 5C). Lastly, only the combination of all coffee compounds significantly reduced pro-inflammatory NFκB p65 protein expression in the liver compared to DEN/CCl4 group (p=0.015) (Figure 5C).

Liver resident (Kupffer cells, KC) and recruited macrophages play pivotal roles on inducing pro-inflammatory and pro-fibrogenic responses in hepatic stellate cells (HSC) through the production of cytokines and subsequent paracrine signaling [46]. The pro-inflammatory IL-17 production, mainly mediated by KC, directly contributes to HSC activation and collagen production, being essential on undergoing liver fibrosis [47]. The IL-17 signaling, as well as increased oxidative stress, are stimuli for NFκB transcription factor (including p65 subunit) upregulation and nuclear translocation, eliciting pro-survival and pro-inflammatory and responses in both HSC and KC [47]. NFκB signaling may not only contribute to the profibrogenic response but is also proposed to induce a pro-survival response in hepatocytes, promoting preneoplastic and neoplastic lesion development [48]. Indeed, immunohistochemistry for NFκB p65 revealed both cytoplasmic/nuclear staining in hepatocytes of basophilic lesions and nuclear staining in adjacent hepatocytes, mainly in DEN/CCl4 group (Supplementary Figure 4). On the other hand, IL-17 receptor α knockout (IL17RA−/−) or antagonism, as well as the selective inactivation of NFκB using a decoy, are proposed to ameliorate CCl4-induced liver fibrosis [47,49]. Thus, when combined (CAF+TRI+CGA) and administered in physiological applicable doses, coffee compounds showed more pronounced results on attenuating the pro-fibrogenic IL-17/NFκB axis in the liver, in keeping with the reduction on HSC activation and collagen deposition.
3.5 The combination of coffee compounds attenuates oxidative stress and induces antioxidant response

Oxidative stress plays pivotal roles on both human and experimental fibrosis/cirrhosis-associated hepatocarcinogenesis [10,11,50]. DEN and CCl₄ hepatic metabolisms generate reactive oxygen species (ROS) and nucleophilic metabolites [51,52]. The DEN-induced DNA oxidative damage is implicated on genomic instability, predisposing hepatocytes to preneoplastic and neoplastic lesion development [51]. Moreover, the byproducts of CCl₄ metabolism, as well as ROS produced by CD68-positive macrophages, contribute to oxidative stress-mediated signaling that leads HSC activation and collagen deposition in fibrosis [47,52,53]. TBARS, formed as byproducts of lipid peroxidation, are well-accepted markers of oxidative stress [54]. All coffee compound treatments showed a trend on decreasing TBARS and increasing Nrf2 protein levels, but only CAF+TRI+CGA reached significance when compared to untreated and/or DEN/CCl₄ groups (p=0.013 and p=0.010, respectively) (Figure 6A and B). DEN/CCl₄ model significantly decreased catalase and GSH-Px activities while presented a trend on reducing SOD activity (Figure 6C). Although all coffee compound interventions did not alter catalase activity, all interventions GSH-Px activity compared to DEN/CCl₄ (p<0.0001) (Figure 6C). Of note, only CAF+TRI+CGA treatment increased SOD activity compared to DEN/CCl₄ and CAF+TRI groups (p=0.0011) (Figure 6C).

The nuclear factor erythroid-related factor 2 (Nrf2) is a transcription factor that controls the expression enzymatic antioxidant agents, including GSH system, catalase and SOD, which are accounted for ROS and lipid hydroperoxide neutralization [55,56]. In vitro, both caffeinated (containing CAF, TRI and CGA) and decaffeinated coffee (containing TRI and CGA) treatments similarly induced antioxidant response through the upregulation of Nrf2 and downstream antioxidant response element (ARE) axis in HCC cells (HepG2) [57]. Other in vitro findings suggest that this antioxidant effect is related to the hydroxycinnamic acid fraction of the beverage, including CGA, and not to CAF [58]. Nonetheless, several in vivo reports have shown the individual ability of CAF, TRI or CGA to induce Nrf2 and/or downstream antioxidant agents [26,59], although the exact mechanisms remain to be fully elucidated. Here, findings suggest that combination of the most common and bioavailable coffee compounds (as seen in caffeinated coffee beverages), rather than caffeine alone, is responsible for reducing oxidative stress and increasing hepatic endogenous antioxidant Nrf2 axis. The individual free radical scavenging capacity of these molecules, mainly CAF and CGA, and their metabolites should be considered to understand the decrease in MDA levels as well [60]. Thus, decreased oxidative stress in CAF+TRI+CGA group may be in part attributed to the induction of Nrf2 axis and SOD/GSH-Px enzymes, ultimately contributing to reduce fibrosis and to decrease the incidence/number of preneoplastic lesion in this group.
3.6 The combination of coffee compounds upregulates miR-144-3p, miR-376a-3p and miR-15b-5p

Since CAF+TRI+CGA treatment displayed the most pronounced effects on attenuating preneoplastic lesion development and liver fibrosis, we assessed the miRNA expression signature of this group in order to unveil the potential implications/correlations of miRNA modulation on the previously observed outcomes. We also performed global miRNA expression in CAF-treated group to identify CAF-modulated miRNAs in CAF+TRI+CGA-related miRNA signature. DEN/CCl₄-induced fibrosis-associated hepatocarcinogenesis model displayed 19 differentially expressed miRNAs compared to the untreated group (14 up and 5 downregulated) (Table 3). Interestingly, both miR-144-3p (FC=0.57; p=0.0054) and miR-376a-3p (FC=0.53; p=0.0008) were downregulated in the liver of DEN/CCl₄-submitted mice. In keeping with our data, miR-144-3p levels were decreased in HCC in a similar DEN-induced hepatocarcinogenesis model in neonatal C3H mice [61]. In samples of human HCC and fibrotic liver, miR-144-3p is downregulated as well, being inversely correlated with tumor staging and profibrotic HSC-related transforming growth factor β1 marker, respectively [62,63]. Moreover, miR-144-3p (HepG2, Hep3B and Huh7) and miR-376a-3p (HepG2 and Huh7) expressions are decreased in classical human HCC cell lines [62,64].

Conversely, we found that CAF+TRI+CGA treatment upregulated 9/19 (~50%) of the differentially expressed miRNAs in fibrosis-associated hepatocarcinogenesis model, including miR-144-3p (FC=2.14, p=0.001), miR-376a-3p (FC=1.62, p=0.0035) and miR-15b-5p (FC=1.52, p=0.0011) (Table 4, Figure 7A, Supplementary Figure 5). CAF treatment upregulated only 5/19 (26%) (Supplementary Table 2, Figure 7A). Using Venn’s diagram (Figure 7A), we observed that CAF+TRI+CGA and CAF comparisons to DEN/CCl₄ group shared the upregulation of 5 miRNAs (mmu-miR-199a-3p, miR-199a-5p, miR-132-3p, miR-144-3p, miR-376a-3p). Since CAF+TRI+CGA vs. CAF comparison showed no statistical contrast regarding these 5 miRNAs, these are probably modulated by CAF administration. The other 4 miRNAs were exclusively upregulated by CAF+TRI+CGA treatment (miR-15b-5p, miR-342-3p, miR-350-3p and miR-335-5p) (Figure 7A). Therefore, we considered all 9 miRNAs as part of the CAF+TRI+CGA-related miRNA signature for further target analysis.

Noteworthy, treatment with miR-144-3p by intravenous administration diminished HCC size in a DEN-induced neonatal hepatocarcinogenesis model in C3H mice by targeting and decreasing the protein expression of proliferation-related growth factor receptor (EGFR) [61]. Similar findings on attenuating cell proliferation were observed after miR-144-3p or miR-376a-3p mimic transfection to human HCC cells and xenograft mouse model [63,64]. Although miR-15b-5p is upregulated in our DEN/CCl₄-induced model (Table 3), as well as in HCC samples and tumor cell lines, previous findings suggest that this miRNA may act a tumor suppressor/antifibrotic miRNA when the expression is way up increased by mimetic
transfection. Indeed, miR-15b-5p transfection induced endoplasmic reticulum stress and apoptosis in HCC cells and xenograft mouse model [65]. Moreover, miR-15b-5p transfection induced apoptosis in activated rat HSC by targeting antiapoptotic Bcl-2 and enhancing caspase axis [66]. Thus, the upregulation of miR-144-3p, miR-376a-3p, miR-15b-5p by CAF+TRI+CGA may have direct implications on the attenuation of both fibrosis and preneoplastic lesion development.

3.7 Target genes are associated to negative regulation of apoptosis and positive regulation of proliferation

The target analysis of the 9 miRNAs modulated by CAF+TRI+CGA revealed an output of 232 validated genes (Supplementary Table 3). Interestingly, according to IPA analysis, most of these validated targets are modulated by miR-15b-5p (197/232) (Supplementary Table 3). Gene enrichment analysis regarding biological processes (BP) evidenced that 18-25% of the target genes were significantly associated with cell death or proliferation functional annotations (Figure 7B). Especially, 20% (47/232) were associated with “negative regulation of cell death”, while ~18% (43/232) were associated to “positive regulation of cell population proliferation” annotation (Figure 7B). In addition, KEGG pathway analysis revealed that ~18% (43/232), 12% (28/232), and 7% (17/232) were linked to “pathways in cancer”, “microRNAs in cancer” and “Hepatocellular Carcinoma”, respectively (Figure 7B). Among other significant terms, we observed that ~14% (32/232), ~9% (21/232) and ~6% (14/232) of genes were associated to the pro-proliferative PI3K-Akt, Ras and HIF-1 pathways, respectively, corroborating with proliferation-related annotations in GO analysis (Figure 7B).

Network analysis using STRING depicted many experimentally determined interactions among the proteins coded by the target genes (Supplementary Figure 6). Among interactions regarding cell death annotations, we observed key members of the Bcl-2 family, including myeloid cell leukemia sequence 1 (Mcl1), B cell leukemia/lymphoma 2 (Bcl2) and BCL2-like 2 (Bcl2l2), which are responsible for a strong anti-apoptotic (pro-survival) signaling (Supplementary Figure 6 and Supplementary Table 3). These Bcl-2 family members are experimentally validated targets of miR-15b-5p in different tissues, including mouse liver, rat HSCs and human HCC cell lines (Supplementary Table 3 and 4). Network analysis also evidenced central role of epidermal growth factor receptor (Egfr) on target interactions in cell proliferation annotations (Supplementary Figure 6). In the early stages of DEN-induced hepatocarcinogenesis, particularly in preneoplastic foci and adenomas, the downstream signaling mediated by EGFR, which is a target of miR-144-3p (Supplementary Tables 3 and 4), activates of PI3K-Akt and Ras pathways (Figure 7B), promoting cell proliferation in these lesions [67]. EGFR activation is also proposed to induce the hypoxia-independent overexpression of hypoxia-inducible factor 1 (HIF-1α) transcription factor and its downstream targets, including vascular endothelial growth factor (VEGF) and c-Met [67]. This interaction was also depicted
in our network analysis (Supplementary Figure 6). It is worthy of note that HIF-1α and VEGF are miR-144-3p and miR-15b-5p targets, respectively (Supplementary Tables 3 and 4). The EGFR-mediated HIF-1α activation may be crucial in the expansion of preneoplastic hepatocyte population and neoplastic progression as well as EGFR-mediated PI3K-Akt/Ras induction [67]. Indeed, Egfr shared both positive regulation of cell population proliferation and hepatocellular carcinoma annotations in network analysis (Supplementary Figure 7), eliciting the importance of this miR-144-3p target on hepatocarcinogenesis.

3.8 The combination of coffee compounds reduces protein levels of Bcl-2 family members and EGFR

In keeping with target analysis, we observed that the combination of all coffee compounds reduced the protein levels of all Bcl-2 family members evaluated (Figure 8A). In CAF+TRI+CGA group, Mcl-1 was significantly reduced compared to untreated and DEN/CCl4 groups (p=0.019), whereas Bcl-2 and Bcl2l2 were significantly reduced compared to untreated group (p=0.017 and p=0.032, respectively) (Figure 8A). As expected, CAF treatment did not reduce protein expression of Bcl-2 family members. These findings could indicate coffee compound-mediated upregulation of miR-15b-5p may reduce the protein expression of these key antiapoptotic proteins, contributing to increased number of apoptotic cells (cleaved caspase-3 positive) in adjacent liver (Figure 3A). Since a miR-15b-5p mimic induced apoptosis in activated HSC in rats by diminishing Bcl-2 mRNA and protein expressions and increasing caspase axis [66], a coffee compound-induced pro-apoptotic signal in HSC may contribute on alleviating fibrosis in this intervention group, as observed (Figure 5). Indeed, as seen in Sirius red-stained, α-SMA and cleaved caspase-3 immunostained sections, apoptotic cells were found near the regions of increased α-SMA expression (activated HSCs) and collagen accumulation (Supplementary Figure 8).

Moreover, CAF+TRI+CGA treatment reduced by 54% the DEN/CCl4-mediated increase in EGFR protein levels (p=0.018) (Figure 8B). Our results indicate that coffee compound combination-mediated upregulation of miR-144-3p may alleviate EGFR signaling activation during early stages of mouse hepatocarcinogenesis, contributing to decrease proliferation (Ki-67-positive hepatocytes) inside preneoplastic foci (Figure 4A), ultimately attenuating preneoplastic foci development (Figure 2B, Table 2). Our findings suggest that EGFR-mediated activation of HIF-1α transcription factor may not be involved in this effect, since HIF-1α and VEGF protein expression were not modified by CAF+TRI+CGA treatment (Supplementary Figure 9). The downstream modulation of PI3K-Akt/Ras axis is hypothesized (Figure 7A), but future studies are warranted. Regarding cell proliferation, we also evaluated the beneficial effects of coffee compound combination on proliferating nuclear cell antigen (PCNA), a miR-376a-3p target widely accepted as a proliferation marker [68].
Although CAF+TRI+CGA intervention failed on reducing the number of PCNA-positive hepatocytes in adjacent liver, presenting a statistical trend (p=0.051) on decreasing hepatic PCNA protein expression (Supplementary Figure 9), only CAF+TRI+CGA reduced the number of PCNA-positive hepatocytes in preneoplastic foci (p=0.039) (Figure 8B), in keeping with Ki-67 findings (Figure 3B). Despite of sharing the upregulation of miR-144-3p and miR-376a-3p, CAF treatment failed on reducing EGFR protein levels and the number of PCNA-positive hepatocytes inside preneoplastic foci (Figure 8B). Therefore, we hypothesize that other mechanisms, independently from miR-144-3p and miR-376a-3p modulation, may contribute to decrease the number of preneoplastic lesions in CAF+TRI+CGA group, as reduced oxidative stress (Figure 6A).

3.9 The combination of coffee compounds enhances cytotoxicity in C3A cells

The exposure to all concentrations of CAF individually (160, 80, 40 and 20 μM) significantly increased cytotoxicity in C3A cells after 48h of exposure (p<0.001), not after 24h (Supplementary Figure 10). In general, the combination of all coffee compounds (CAF+TRI+CGA) in all tested concentrations displayed more pronounced results on enhancing cytotoxicity compared to untreated-, CAF-treated and/or two drug-treated cells after both 24 and 48h of exposure (p<0.001) (Supplementary Figure 10). LDH in vitro findings are in keeping with the in vivo results suggesting that the combination of coffee compounds, rather than CAF individually, may attenuate HCC. Increased LDH levels may indicate a disruption of the cell membrane which occurs in necrosis or in late stages of apoptosis [69]. Pro-apoptotic effects of CAF, TRI or CGA are not well-described in HCC cell lines [15], but miR-15b-5p transfection increased the number of late apoptotic cells in human HCC Hep3B cell line by targeting and suppressing Rab1A oncogene [65]. Nonetheless, the correlation between coffee compound-induced cytotoxicity and miR-15b-5p expression in vitro needs further evaluation.

4. Discussion

In the current study, we aimed at evaluating the effects of CAF individually or combined with TRI and/or CGA on a well-established chemically-induced model of fibrosis-associated hepatocarcinogenesis in C3H/HeJ mice. A potential modulation of miRNA profile by these compounds was also investigated, correlating changes in expression with liver fibrosis/carcinogenesis outcomes. In summary (Figure 9), the combination of all compounds displayed the most pronounced effects on alleviating preneoplastic foci development. This treatment also reduced proliferation (Ki-67) in preneoplastic lesions and enhanced apoptosis (cleaved caspase-3) in adjacent tissue. In addition, CAF+TRI+CGA combination alleviated...
fibrosis, reducing hepatic pro-inflammatory IL-17/NFκB signaling. Moreover, CAF+TRI+CGA decreased hepatic oxidative stress and enhanced antioxidant Nrf2 axis. miRNAomic profile showed the upregulation of miR-144-3p and miR-15b-5p. In accordance with these findings, the protein levels of pro-proliferative EGFR (miR-144-3p target) and antiapoptotic Bcl-2 family members (Bcl-2, Mcl-1 and Bcl2l2, miR-15b-5p targets) were reduced in this group. Noteworthy, this is the first scientific report on the modulation of miRNA expression by coffee compounds administered in physiologically-based doses during fibrosis-associated hepatocarcinogenesis. In special, results indicate that miR-15b-5p and miR-144-3p upregulation are potentially implicated on liver fibrosis/carcinogenesis outcomes.

Globally, coffee beverage consumption is a popular and safe dietary habit, presenting a growing cultural and economic impact [14,15]. In fact, from 1960 to 2017, global coffee bean production increased ~200% [70]. There is a wealth of epidemiological evidence pointing out to the hepatoprotective effects of coffee consumption on liver fibrosis and cancer [15-17]. On the other hand, recent data evidenced that decaffeinated coffee, compositionally identical to caffeinated coffee apart from not having CAF, showed none or less pronounced protection [18,19]. In this context, some authors support the “caffeine hypothesis”, directly correlating the hepatoprotective effects of coffee beverages to the widely reported bioactive properties of this xanthine [71]. Nonetheless, coffee is a complex pharmacopoeia, and potential effects of other abundant compounds, as TRI and CGA, were also considered in the present investigation. In previous chemically-induced models of fibrosis and hepatocarcinogenesis in rats, caffeinated coffee resulted in more pronounced attenuation of preneoplastic foci development and collagen III mRNA expression than decaffeinated coffee and CAF alone [23], suggesting that the combination of CAF with other common coffee compounds may account for this protective effect. However, the mechanisms and exact compounds involved in this differential response were still to be unveiled. As highlighted before, both in vivo and in vitro experimental approaches designed herein reflected this widespread dietary habit. CAF dose was based on average CAF consumption from coffee in the USA and European countries, which are top coffee consuming nations, corresponding to ~280 mg CAF/day (2-3 cups) [30,31]. As reviewed by our research group, allometric HED calculation approach for CAF was not applied in previous fibrosis/hepatocarcinogenesis bioassays, and doses/concentrations are usually above human intake [15]. In vitro, the corresponding serum peak of the same CAF intake was chosen (~40 µM) [22]. Regarding TRI and CGA doses, since epidemiological data on human consumption of these compounds from coffee are scant, we considered the concentration ratio compared with CAF found in coffee beverages [15,22].

As hypothesized, only the combination of the most common and highly bioavailable coffee compounds attenuated collagen deposition and preneoplastic foci development (Figure 9), the main outcomes observed in the DEN-initiated/CCl4-
promoted mouse model at this timepoint (week 17). DEN is an initiating agent for liver carcinogenesis that causes DNA damage and genomic instability in hepatocytes, while multiple CCl₄ administrations lead to lipid peroxidation and cell death, providing the necessary necro inflammatory background for HSC activation, collagen deposition and preneoplastic lesion growth [11,51,52]. This short/medium-term bioassay combining DEN and CCl₄ regimens, rather than only DEN-induced models, resembles molecular and morphological features of the corresponding human disease in its early stages, since most HCC cases (70 - 90%) are set up on fibrosis/cirrhosis context [3]. Although coffee compounds (8-10 a.m.) and CCl₄ (4-6 p.m) were concomitantly administered for 8 weeks, CCl₄ is biotransformed by cytochrome (CYP) 2E1, while CAF is majorly metabolized (90%) by CYP1A2, TRI is methylated by nicotinamide N-methyltransferase (NNMT), and CGA is heavily metabolized by colonic microbiota [15, 52]. Thus, it is unlikely that coffee compound intervention interfered with CCl₄ phase I metabolism.

In addition to the reduction on liver fibrosis and preneoplastic foci development, miRNomic profile of CAF+TRI+CGA group revealed an upregulation of the tumor suppressors miR-144-3p and miR-376a-3p, and antifibrotic/tumor suppressor miR-15b-5p (Figure 9). While miRNAs are directly implicated in physiological processes in the liver, including postnatal growth, regeneration and function, their abnormal expression is pivotal in different stages of fibrosis/cirrhosis-associated hepatocarcinogenesis in humans, contributing to sustained cell proliferation, epithelial mesenchymal transition, invasion, metastasis, angiogenesis, and drug resistance hallmarks [8]. In chemically-induced rodent models of fibrosis and hepatocarcinogenesis, the importance of miRNA deregulation is also reported [6, 72]. Especially, the DEN/CCl₄ approach used recapitulated some miRNomic features of the corresponding human disease, as the downregulation of miR-144-3p and miR-376a-3p, eliciting the translational value of the present short/medium term bioassay. As briefly discussed before, miR-144-3p downregulation is also a feature of more advanced stages of DEN-induced mouse hepatocarcinogenesis since it is also observed in HCC [61]. He et al., 2017 [61] showed that the intravenous treatment with miR-144-3p increased its levels in tumor and adjacent tissue. When enhanced, miR-144-3p targeted EGFR and decreased downstream pro-proliferative Akt signaling pathway, acting as a tumor suppressor by reducing HCC size. In contrast, miR-144-3p knockout increased EGFR/Akt axis [61]. Noteworthy, 85% of human HCC cases present miR-144-3p downregulation, while 32–66% and 45% display EGFR overexpression and amplification, correspondingly, evoking the importance of this pathway in human liver tumorigenesis as well [63,73]. Here, we reported that the combination of coffee compounds upregulated miR-144-3p while decreased EGFR protein levels (Figure 9). In line with these findings, CAF+TRI+CGA combination reduced cell proliferation inside preneoplastic foci. These findings suggest that even in the early stages of hepatocarcinogenesis, miR-
144-3p upregulation may target EGFR, decreasing cell proliferation and resulting in lower preneoplastic foci development (Figure 9).

The roles of miR-367a-3p on rodent models of liver fibrosis and carcinogenesis are not described. However, the downregulation of this miRNA is featured in both physiological and pathological liver contexts. Around 80% of human HCC samples displayed decreased levels of this miRNA, and similar findings were observed in HCC cell lines [64]. Interestingly, this non-coding RNA was also downregulated during liver regeneration after partial hepatectomy in mice, suggesting underlying roles in controlling hepatocyte proliferation [64]. Our findings are the first to report the downregulation of this miRNA during the early stages of chemically-induced fibrosis-associated hepatocarcinogenesis. In vitro, the transfection of miR-376a-3p mimics exerted a tumor-suppressive effect in Huh7 HCC cell line by targeting p85α [64]. In mice, the only experimentally validated target of this miRNA is PCNA, which is an auxiliary protein of DNA polymerase δ, an enzyme necessary for DNA synthesis during cell replication [68]. We found that CAF+TRI+CGA combination upregulated miR-376a-3p while decreased the number of PCNA-positive hepatocytes in preneoplastic foci, indicating that, along with miR-144-3p, the modulation this miRNA could result on reduced number of preneoplastic foci (Figure 9). Although we found a reduction in cell proliferation indexes (Ki-67-and PCNA-positive hepatocytes) inside preneoplastic foci, CAF+TRI+CGA treatment did not alter proliferation in adjacent tissue (Ki-67- and PCNA-positive hepatocytes and PCNA protein levels). Since preneoplastic foci display increased cell proliferation compared to adjacent tissue, in part due to mutations in Brf and Hras [42,43], our findings could indicate that these lesions may be sensitive to CAF+TRI+CGA-mediated modulation of these tumor suppressor miRNAs. While speculative, further studies using laser microdissection methodology could evaluate the events underlying the differences between preneoplastic foci and adjacent hepatocytes regarding proliferative responses.

The combination of all coffee compounds upregulated miR-15b-5p in the liver as well. In active HSC isolated from rat liver, the upregulation of miR-15 family by the transfection of mimics (including miR-15a, miR-15b and miR-16) contributed to cell death by targeting anti-apoptotic proteins, such as Bcl-2, and subsequently increasing caspase levels (caspase-3, -8 and -9) [66]. Hence, the modulation of miR-15 family is proposed as a novel therapeutic strategy for liver fibrosis, considering that HSC death could attenuate collagen deposition [66]. In line with increased miR-15b-5p expression, we observed that protein levels of antiapoptotic Bcl-2 family members were reduced while apoptosis (cleaved caspase-3-positive cells) was increased in adjacent tissue in CAF+TRI+CGA treatment (Figure 9). Bcl-2, Mcl-1 and Bcl2l2, proteins placed in mitochondrial membrane, bind to pro-apoptotic BH3 sensitizers, initiators, or pore formers, avoiding mitochondrial permeability, cytochrome release and activation of caspase cascade [74]. Then, results indicate that CAF+TRI+CGA-
mediated upregulation of miR-15b-5p and decrease of Bcl-2 family may lead to HSC apoptotic cell death (thus reduced α-SMA protein expression), contributing to decreased collagen deposition (Figure 9). Nonetheless, the negative modulation of oxidative stress and proinflammatory IL-17/NF-κB axis should also be accounted for decreased liver fibrosis, since it appears to be independent from miRNA modulation (Figure 9). Along with miR-144-3p and miR-376a-3p modulation, the amelioration of hepatic fibrotic context by CAF+TRI+CGA may indirectly contribute to slow preneoplastic foci development (Figure 9).

Finally, none is reported on how alkaloids and polyphenols could directly alter canonical and non-canonical miRNA biogenesis, but the modulation of the crosstalk between cellular pathways and miRNA biogenesis is speculated. The activation of pro-inflammatory NF-κB transcription factor is responsible for upregulating some anti-inflammatory miRNA families in a negative feedback [75]. EGFR upregulation is also responsible for argonaute 2 (AGO2) phosphorylation, reducing its binding to Dicer and thus inhibiting miRNA processing from precursor to mature miRNAs, what could hinder the maturation of specific tumor-suppressor-like miRNAs [76]. In addition, oxidative stress is proposed to cause miRNA deregulation at the level of transcription, processing, cellular localization and functioning [75]. Thus, the relationship between CAF+TRI+CGA-mediated effects on oxidative stress, NF-κB, EGFR and miRNA biogenesis should be evaluated on future investigations.

Therefore, findings suggest that the combination of the most common and bioavailable coffee compounds (as seen in coffee beverages), rather than CAF alone, attenuates chemically-induced fibrosis-associated hepatocarcinogenesis. Results also indicate that these beneficial effects may be mechanistically mediated by alterations in miRNA expression. The modulation of tumor suppressor and antifibrotic miRNAs by naturally occurring compounds may open a therapeutic avenue for fibrosis-associated hepatocarcinogenesis, as these non-coding RNAs should be considered for further translational investigations. The results also provide a mechanistical insight on the hepatoprotective population-level effects attributed to caffeinated coffee consumption. However, in order to have a wider landscape on this matter, other targets of the differentially expressed miRNAs should also be evaluated in future bioassays considering our network analysis.

5. Conflicts of interest

The authors declare no conflict of interest.
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8. References


