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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. Conversely, coffee consumption reduces by ~40% the risk for fibrosis/cirrhosis and HCC, while decaffeinated coffee does not. It is currently unknown whether these protective effects are related to caffeine (CAF), or to its combination with other common and/or highly bioavailable coffee compounds, such 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. Then, male C3H/HeJ mice were submitted to a diethylnitrosamine/carbon tetrachloride-induced model. Animals received CAF (50 mg/kg), CAF+TRI (50 and 25 mg/kg), CAF+CGA (50 and 25 mg/kg) or CAF+TRI+CGA (50, 25 and 25 mg/kg), intragastrically, 5×/week, for 10 weeks. Only CAF+TRI+CGA combination reduced the incidence, number and proliferation (Ki-67) of hepatocellular preneoplastic foci while enhanced apoptosis (cleaved caspase-3) in adjacent parenchyma. CAF+TRI+CGA treatment also decreased hepatic oxidative stress and enhanced the antioxidant Nrf2 axis. CAF+TRI+CGA had the most pronounced effects on decreasing hepatic pro-inflammatory IL-17 and NFκB, contributing to reduce CD68-positive macrophage number, stellate cell activation, and collagen deposition. In agreement, CAF+TRI+CGA upregulated tumor suppressors miR-144-3p, miR-376a-3p and antifibrotic miR-15b-5p, frequently deregulated in human HCC. CAF+TRI+CGA reduced the hepatic protein levels of pro-proliferative EGFR (miR-144-3p target), antiapoptotic Bcl-2 family members (miR-15b-5p targets), and the number of PCNA (miR-376a-3p target) positive hepatocytes in preneoplastic foci. Our results suggest that the combination of most common and highly bioavailable coffee compounds, rather than CAF individually, attenuates fibrosis-associated hepatocarcinogenesis by modulating miRNA expression profile.

Keywords: Caffeine; Trigonelline; Chlorogenic acid; Liver fibrosis; Hepatocarcinogenesis; 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 an 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 (ALD) 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 the human genome are controlled by miRNA expression [7]. A wealth of evidence points out to the pivotal involvement of miRNAs during hepatocarcinogenesis, as either potential tumor suppressors or

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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 fibrogenesis and/or hepatocarcinogenesis risks [15–17]. Overall, coffee consumption (>1 cup/day) reduces by ~40% the risk for fibrosis/cirrhosis and HCC, even upon adjustments for risk factors or highly incident areas [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 (CAF) individually, or, to CAF 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 many compounds of different chemical classes [20,21]. Particularly, the alkaloids 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 presenting beneficial effects individually, the literature lacks 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 and/or bioavailability, and the =concentrations and/or doses applied are usually overestimated. Finally, since naturally occurring phytochemicals are recently proposed to modulate miRNA biosynthesis and downstream targets, contributing to cancer prevention, the modifying effects of common coffee compounds on non-coding RNA expression should be unraveled in the context of liver diseases [15,27].

Thus, we assessed whether CAF administration individually or combined with TRI and/or CGA alleviates fibrosis-associated hepatocarcinogenesis in a diethylnitrosamine (DEN)/carbon tetrachloride (CCl_4)-induced mice model. 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 a human HCC cell line. Our study could provide a translational insight into potential preventive or therapeutic applications based on tumor suppressor or onco-miRNAs modulated by naturally-occuring coffee compounds. This study constitutes the first scientific report on the beneficial effects of coffee compounds on the miRNAomic profile of a common end-stage liver disease mice model, using doses that resemble human exposure to coffee and its main bioactive compounds.

2. Materials and 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=5–10 animals/group) were initiated for liver carcinogenesis by receiving a single intraperitoneal (i.p.) injection of 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) (Fig. 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. increasing CCl_4 doses (0.25–1.50 µl/g b.wt. 10% solution in corn oil, Sigma-Aldrich, USA) from Week 8 to 16 or corn oil vehicle (4–6 p.m.), as previously established by our group [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

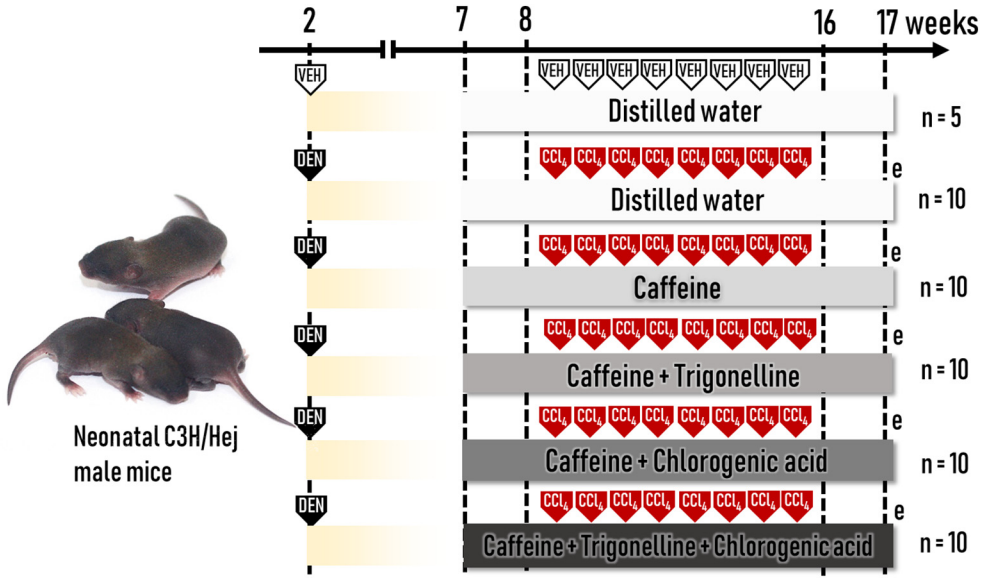

Fig. 1. Animal study diagram. DEN=1× diethyniltrosamine [10 mg/kg body weight (b.wt.), intraperitoneal injections]; CCl_4=3×/week carbon tetrachloride (0.25–1.50 µl/g b.wt., intraperitoneal injections); VEH=saline 0.9% or corn oil vehicle; CAF=caffeine (50 mg/kg b.wt., intragastrically administered, 5×/week); TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., intragastrically administered, 5×/week); n, number of mice/group; e, euthanasia.

mg/kg b.wt./day, respectively) or just distilled water as vehicle (intragastrically, five times *per* week) (8–10 a.m.) (Fig. 1). Solutions containing bioactive coffee compounds were prepared on a daily basis. 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 CCl_4 administration. Blood was collected in heparinized syringes from cardiac puncture, centrifuged (1503×*g*, 10 min.) and serum samples were collected and stored at −80°C for further analysis. At necropsy, the 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/h), relative humidity (45–65%), controlled temperature (20-24ºC) 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 the 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 of bioactive coffee compounds

High coffee consumption, as observed in the USA and in many European countries, leads to an estimated CAF intake range of 200–300 mg/day (~2.8–4.0 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:

$$\text{(I)}\quad HED\,(mg/kg/day) = Animal\ dose\ (mg/kg/day) \times \frac{Mice\ Km*}{Animal\ Km}$$

$$\text{(II)}\quad HED = 50 \times \frac{3}{37}$$

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

*K_m 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 two to three 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. Preneoplastic hepatocyte foci screening and collagen morphometry

Liver samples were fixed in 10% neutral buffered formalin for 24 h at room temperature, stored in 70% ethanol and embedded in paraffin. Five-micrometer-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 in mice. The altered hepatocyte foci (AHF), considered the main endpoint lesions for the 17-week time-point, were identified by the 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, the total number of AHF/liver section area (cm^2), the mean size (mm^2), the relative area (sum of all AHF areas/liver section area, in mm^2/cm^2), 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). 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 periportal and pericentral areas [Collagen area (%)=Sirius red area / total 20× field area analyzed]. Fields presenting vessels comprising>30% field area were discarded. Liver fibrosis was staged according to Ishak et al. [34] criteria, considering stages 0 (no fibrosis), 1 (fibrous expansions in some portal areas), 2 (fibrous expansions in most portal areas), 3 (occasional bridging), 4 (marked bridging), 5 (marked bridging with occasional nodules) or 6 (cirrhosis).

2.1.4. Immunohistochemistry

For immunohistochemistry, deparaffinated 5-μm liver sections on silane-covered microscope slides were subject to antigen retrieval in 0.01 M citrate buffer (pH 6.0, 120°C, 5 min) in a Pascal Pressure Chamber (Dako Cytomation, Denmark). After endogenous peroxidase blockade with 1% H_2O_2 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), and anti-cleaved caspase-3 (i.e., apoptosis marker, 5A1E, 1:100 dilution, Cell Signaling, 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.

For the semiquantitative analysis in adjacent liver (avoiding preneoplastic foci), 10 random fields (20× objective) were assessed in left hepatic lobe sections, mainly comprising portal areas. Ki-67 and PCNA positive hepatocytes; cleaved caspase-3 positive cells; and CD68 positive macrophages were counted and divided by the liver area analyzed (mm^2). 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 (mm^2). 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 (ELISA)

Liver samples (~100 mg) of the left medial lobe were homogenized in RIPA buffer (Cell Signaling, USA) containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA), in proportion 30 mg tissue/100 μl buffer, and maintained at 4°C for 2 h. Then, the homogenate was centrifuged (10,000×*g*, 4°C, 30 min.) 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-70 K, 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) [35]. 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 [36]. Glutathione peroxidase (GSH-Px) determination followed the oxidation of 0.16 mM NADPH in the presence of glutathione reductase (GR) [37]. 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 [38]. 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 the left lateral lobe were 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.)] 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 (detecting 600 murine and murine-related viral miRNAs) in an automated system (NanoString Technologies, USA) (Supplementary data). These analyses were performed at the Molecular Oncology Research Center, 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 10 miRNAs presenting the lowest coefficient of variation (low

CV values) using NanostringNorm package [39]. Student *t*-test was used for pair comparison, considering $P<.05$ and fold change (FC) > 1.5. The heat maps were performed using the ComplexHeatmap package in Galaxy computational environment (https://usegalaxy.org/) [40]. 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 the Gene Ontology (GO) Consortium online platform (https://geneontology.org) [41] and KEGG pathway analysis in DAVID Bioinformatics Resources 6.8 online platform (https://david.ncifcrf.gov/) [42]. The main biological process (BP) annotations and KEGG terms were ranked by the lowest adjusted *P* values, considering $P<.05$. STRING v11 (https://string-db.org) was applied for drawing association networks, considering curated databases and experimentally determined interactions among targets [43].

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 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) was 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% CO_2 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). 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, the supernatant was mixed with the 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 $P<.05$. Statistical analyses were performed using GraphPad Prism software 6.0 (GraphPad, USA). Data are presented as mean±standard deviation (S.D.), median (minimum – maximum) or the proportion of lesion-bearing mice (percentage). The number of replicates (n) *per* group for each analysis is determined in the results section.

3. Results

3.1. General findings

All treatments with bioactive coffee compounds did not significantly alter final body weight and food consumption compared to vehicle and DEN/CCl_4 groups (Supplementary Table 2). Indeed, all groups showed similar body weight evolution curves during the 17 weeks of the experiment (Supplementary Fig. 1). As expected, DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis model increased both absolute ($P<.001$) and relative ($P=.019$) liver weights, as well as elevated serum ALT levels ($P=.027$), compared to the untreated counterpart. In contrast, all coffee compound interventions reduced absolute liver weight compared to DEN/CCl_4 model ($P<.001$) (Supplementary Table 2). CAF and CAF+TRI+CGA oral treatments diminished relative liver weight as well ($P=.019$) (Supplementary Table 2). Despite presenting 7% to 20% reductions in ALT levels compared to DEN/CCl_4 group, coffee compound-treated groups did not significantly alter ALT levels (Supplementary Table 2).

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 standardly identified H&E-stained sections as eosinophilic, basophilic and clear cell phenotypes, as represented in Fig. 2A. DEN-induced AHF frequently displays predisposing *Hras* and *Braf* oncogene mutations [44,45], and in the established DEN/CCl_4-induced model, the basophilic phenotype prevails [11]. As expected, the established DEN/CCl_4–induced model increased the incidence of preneoplastic foci compared to the untreated counterpart ($P<.001$) (Table 1). Interestingly, only the CAF+TRI+CGA combination reduced the incidence of clear cell foci compared to DEN/CCl_4 group ($P=.011$) (Table 1). Moreover, only the combination of all coffee compounds reduced by 43% and 38% the number of all AHF types ($P=.024$) and basophilic phenotype ($P=.029$) *per* liver area compared to DEN/CCl_4 group, respectively (Fig. 2B). The decreased number of lesions in the CAF+TRI+CGA group also led to a diminished relative foci area ($P=.032$) (Supplementary Fig. 2A). Our findings indicate that the combination of coffee compounds, rather than CAF individually, may reduce hepatic preneoplasia development.

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

Although all coffee compound treatments did not modulate DEN/CCl_4-induced increase in hepatocyte proliferation in adjacent tissue (Fig. 3), only the combination of CAF+TRI+CGA diminished the number Ki-67+ hepatocytes inside all types of preneoplastic lesions compared to CAF and DEN/CCl_4 groups ($P=.0059$) (Fig. 4A). Sustained cell proliferation into preneoplastic foci could favor the accumulation of molecular alterations, predisposing these lesions to neoplastic progression [46]. In addition, the CAF+TRI+CGA intervention presented significantly more cleaved caspase-3-positive cells in adjacent liver tissue when compared to the other groups ($P<.0001$) (Fig. 3). Treatments did not modulate the number of apoptotic cells inside AHF (Fig. 4B). Representative photomicrographs of Ki-67 and cleaved caspase-3 immunostained sections from all groups are presented in Supplementary Fig. 3.

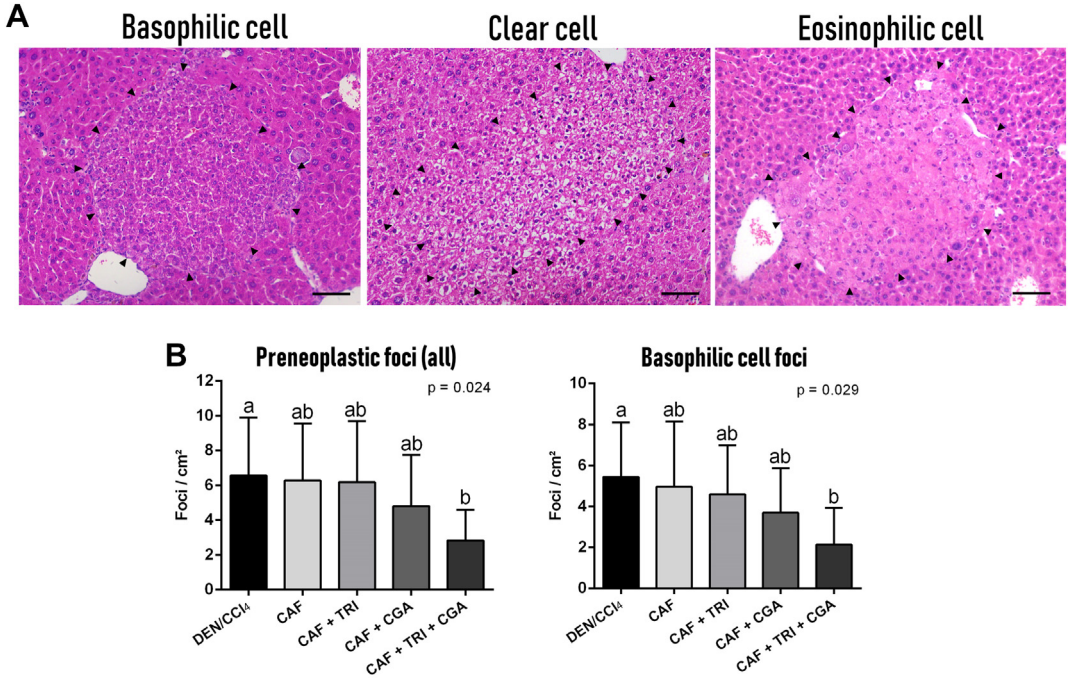

Fig. 2. Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and/or chlorogenic acid (CGA) on preneoplastic foci development during DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis. **(A)** Representative photomicrographs of H&E-stained sections showing basophilic, clear and eosinophilic cell foci (arrowheads, 20× objective, scale bar=50 μm). **(B)** Number of preneoplastic foci (all types) and basophilic foci *per* liver area. Data are mean±S.D. n=5 (untreated) to 10 (other groups) mice/group. DEN/CCl_4= diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25–1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.); TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically administered from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference among groups by ANOVA and *post hoc* Tukey test (P<.05).

3.4. The combination of coffee compounds attenuates fibrosis

In keeping with the increased liver weight and ALT levels, the applied DEN/CCl_4-induced model displayed typical features of chronic liver disease, showing enhanced collagen staging [median stage of 5 (5.0–5.0)], increased collagen area with fibrous expansions and bridging (P<.0001), HSC activation indicated by the increased α-SMA protein expression (P<.0001) and increased number of CD68-positive hepatic macrophages, predominantly concentrated in fibrous expansions (P=.0004) when compared to the untreated counterpart (Fig. 5). In accordance, DEN/CCl_4 model presented 2.4-fold higher hepatic levels of pro-inflammatory cytokine IL-17 compared to the untreated group (P=.007) (Fig. 5B).

Conversely, only the combination of CAF+TRI+CGA, rather than CAF individually, reduced by 25% the collagen area compared to the DEN/CCl_4 group (P<.0001), displaying more delicate collagen fibers in Sirius red-stained liver sections (Fig. 5). CAF+TRI+CGA-treated mice also displayed decreased fibrosis stage [median of 4 (3.0–5.0)] compared to DEN/CCl_4 counterpart (P<.0001), while not differing from the other coffee compound-treated [5 (4.0–5.0)] and untreated groups [0 (0.0–0.0)]. Moreover, only the combination of all coffee compounds decreased the number of CD68-positive macrophages in comparison to CAF, CAF+TRI-treated and DEN/CCl_4 groups (P=.0004) (Fig. 5B). The CAF+TRI, CAF+CGA and, especially, CAF+TRI+CGA treatment reduced IL-17 hepatic levels compared to DEN/CCl_4 group as well (P=.007) (Fig. 5B). IL-6 and TNF-α hepatic levels were similar in all groups (Supplementary Fig. 2B). All coffee compound combinations, including CAF+TRI+CGA, decreased α-SMA protein levels in the liver compared to DEN/CCl_4 (P<.0001), indicating an attenuation in HSC activation by the coffee compound combination that corroborates with reduced collagen deposition in CAF+TRI+CGA group (Fig. 5C). Lastly, only the combination of all coffee compounds significantly reduced pro-inflammatory NFκB p65 protein expression in the liver compared to DEN/CCl_4 group (P=.015) (Fig. 5C). Thus, the combined treatment (CAF+TRI+CGA) more pronounced results on attenuating the pro-fibrogenic IL-17 and NFκB mediators, in keeping with the reduction on CD68 macrophage number, HSC activation and collagen deposition in the liver. Representative photomicrographs of Sirius red-stained, CD68 and α-SMA immunostained sections from all groups are presented as Supplementary Fig. 3.

Table 1
Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and/or chlorogenic acid (CGA) on preneoplastic foci incidence during fibrosis-associated hepatocarcinogenesis

Parameters	Groups[1]					
	Untreated	DEN/CCl_4	CAF	CAF+TRI	CAF+CGA	CAF+TRI+CGA
H&E foci (all)	0/5 (0) b	10/10 (100%)a	10/10 (100%)a	10/10 (100%)a	9/10 (90%)a	8/10 (80%)a
Basophilic foci	-	10/10 (100%)	9/10 (90%)	10/10 (100%)	9/10 (90%)	7/10 (70%)
Eosinophilic foci	-	0/10 (0)	3/10 (30%)	3/10 (30%)	4/10 (40%)	2/10 (20%)
Clear cell foci	-	6/10 (60%)a	3/10 (30%)ab	3/10 (30%)ab	3/10 (30%)ab	0/10 (0)b

Data are the proportion of affected animals (percentage). [1]DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.) intragastrically from Week 7 to 17. TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference by Fisher's Exact test (P<.05).

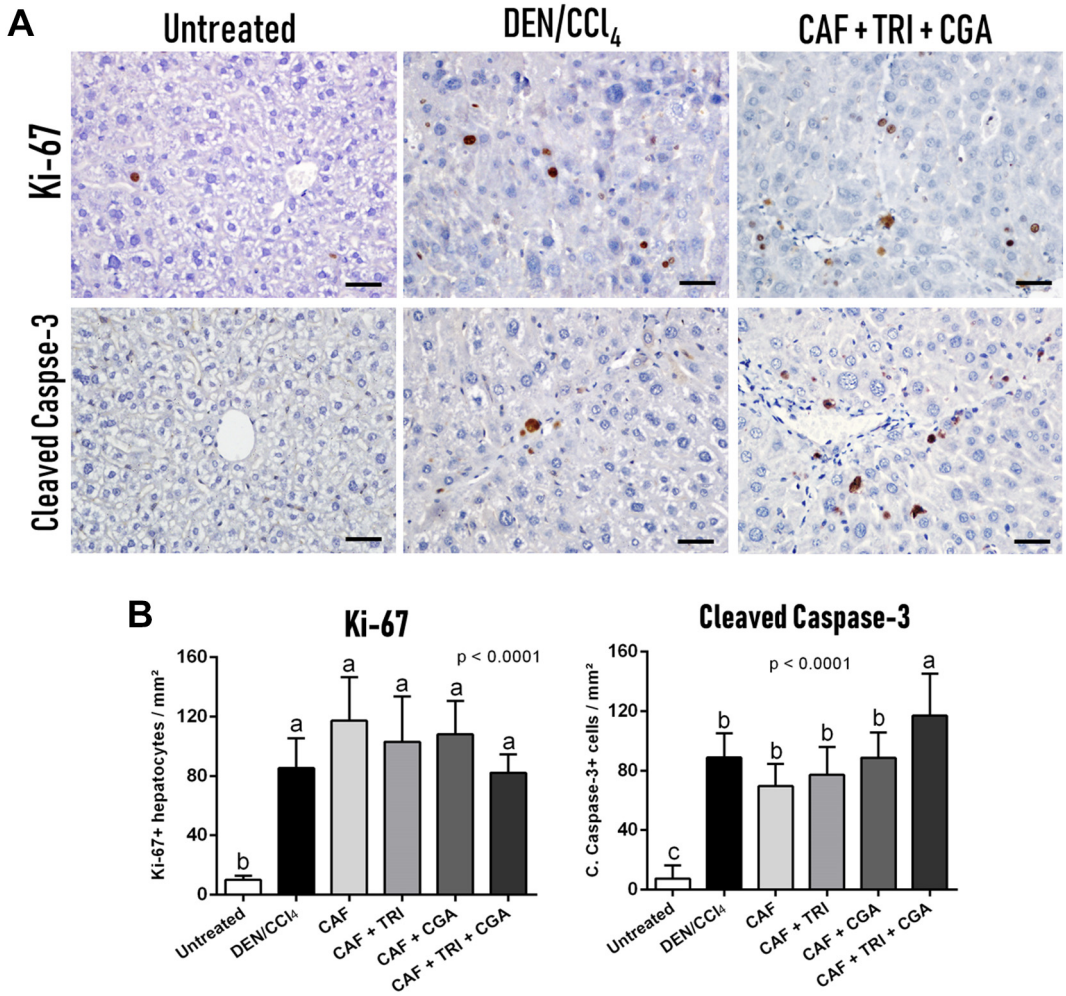

Fig. 3. Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and/or chlorogenic acid (CGA) on cell proliferation (Ki-67) and apoptosis (Cleaved Caspase-3) in adjacent liver tissue during DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis. **(A)** Representative photomicrographs (40× objective, scale bar=25 μm). **(B)** Semiquantitative analysis. Data are mean±S.D. n=5 (untreated) to 8 (other groups) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.), and TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically administered from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference among groups by ANOVA and *post hoc* Tukey test (P<.05).

3.5. The combination of coffee compounds attenuates oxidative stress and induces antioxidant response

Oxidative stress is positively implicated in both human and chemically-induced liver fibrosis and carcinogenesis outcomes [10,11,47]. Only CAF+TRI+CGA treatment significantly decreased TBARS levels, markers of oxidative stress [48], compared to DEN/CCl_4 counterpart (P=.013 respectively). Although all coffee compound treatments did not alter DEN/CCl_4-induced decrease in catalase activity, all treatments increased GSH-Px activity compared to DEN/CCl_4 (P<.0001). Of note, only CAF+TRI+CGA treatment increased SOD activity compared to DEN/CCl_4 and CAF+TRI groups (P=.0011). In addition, only the combination of all coffee compounds increased antioxidant Nrf2 protein levels compared to DEN/CCl_4–treated and untreated counterparts (P=.010) (Supplementary Fig. 4). These findings suggest that the combination all coffee compounds had more noticeable response on reducing carcinogen-induced oxidative stress and increasing hepatic endogenous antioxidant agents.

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 outcomes in comparison to DEN/CCl_4 group, we evaluated the global miRNA profile in this group. We also analyzed the global miRNA expression in the CAF-treated group to identify CAF-modulated miRNAs in the CAF+TRI+CGA-related miRNA signature. DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis model displayed 19 differentially expressed miRNAs compared to the untreated group (14 up and 5 downregulated) (Table 2). Interestingly, miR-144-3p (FC=0.57; P=.0054) and miR-376a-3p (FC=0.53; P=.0008) were downregulated in the liver of DEN/CCl_4-submitted mice. Of note, both miR-144-3p and miR-376a-3p are considered tumor suppressor miRNAs, found to be downregulated in human and experimental liver carcinogenesis [49–52].

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=.001), miR-376a-3p (FC=1.62, P=.0035) and miR-15b-5p (FC=1.52, P=.0011) (Table 3, Fig. 6A, Supplementary Fig. 5). CAF treatment upregulated only 5/19 (26%) (Fig. 6A, Supplementary Table 3). Using Venn's diagram (Fig. 6A), we observed that CAF+TRI+CGA and CAF comparisons to DEN/CCl_4 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

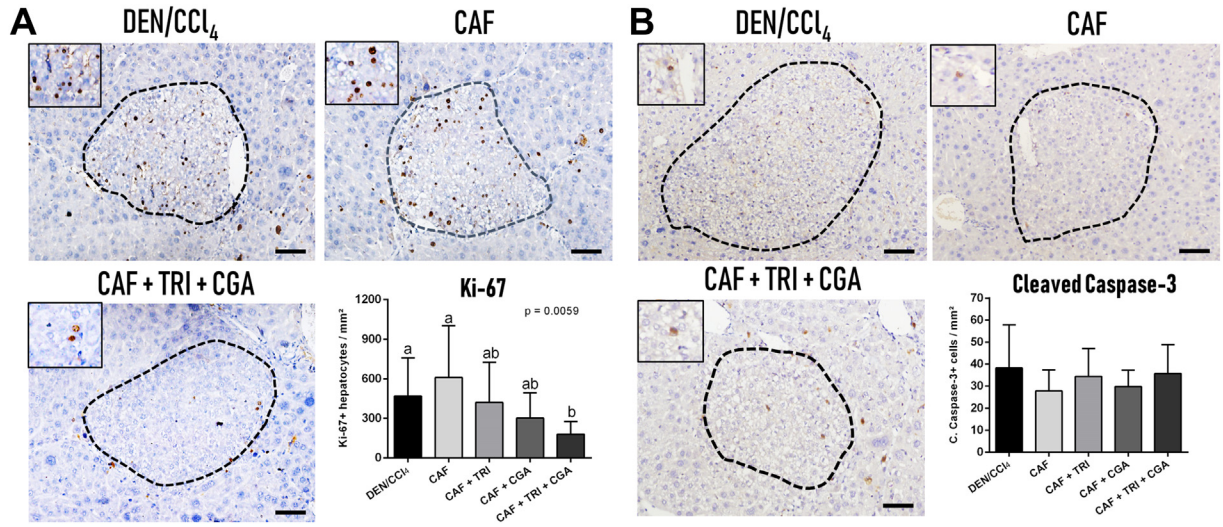

Fig. 4. Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and/or chlorogenic acid (CGA) on **(A)** cell proliferation (Ki-67) and **(B)** apoptosis (Cleaved Caspase-3) in preneoplastic foci during DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis. Representative photomicrographs (20× objective, scale bar=50 μm) and semiquantitative analysis are presented. Data are mean±S.D. n=5 (untreated) to 8 (other groups) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.); TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically administered from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference among groups by ANOVA and *post hoc* Tukey test (P<.05).

upregulated by CAF+TRI+CGA treatment (miR-15b-5p, miR-342-3p, miR-350-3p and miR-335-5p) (Fig. 6A). Therefore, we considered all 9 miRNAs as part of the CAF+TRI+CGA-related miRNA signature for further target analysis. Although miR-15b-5p is upregulated in our DEN/CCl_4-induced model (Table 2), as human in HCC samples and cell lines, findings suggest that this miRNA may act a tumor suppressor/antifibrotic miRNA when the expression is way up increased by mimetic transfection [53,54]. 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 with negative regulation of apoptosis and positive regulation of proliferation

The target analysis of the nine miRNAs modulated by CAF+TRI+CGA revealed an output of 232 validated genes. Interestingly, most of these validated targets are modulated by miR-15b-5p (197/232) (Supplementary Table 4). 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 (Fig. 6B). Especially, 20% (47/232) were associated with "negative regulation of cell death", while~18% (43/232) were associated with "positive regulation of cell population proliferation" annotation (Fig. 6B). 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 (Fig. 6B). 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, in keeping with the proliferation-related annotations in GO analysis (Fig. 6B).

Network analysis using STRING depicted many experimentally determined interactions among the proteins coded by the target genes (Supplementary Fig. 6). Among interactions regarding cell death annotations, we observed key members of the antiapoptotic 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 Fig. 6 and Supplementary Table 4). 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 Tables 4 and 5). Network analysis also evidenced the central role of the epidermal growth factor receptor (*Egfr*) on target interactions in cell proliferation annotations (Supplementary Fig. 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 4 and 5), activates of PI3K-Akt and Ras pathways (Fig. 6B), promoting cell proliferation in these lesions [55]. 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 [55]. This interaction was also depicted in our network analysis (Supplementary Fig. 6). It is worthy of note that HIF-1α and VEGF are miR-144-3p and miR-15b-5p targets, respectively (Supplementary Tables 4 and 5). The EGFR-mediated HIF-1α activation may be crucial in the expansion of preneoplastic hepatocyte population and neoplastic progression as well as EGFR-mediated PI3IK-Akt/Ras induction [55]. Indeed, *Egfr* shared both positive regulation of cell population proliferation and hepatocellular carcinoma annotations in network analysis (Supplementary Fig. 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 line with target analysis, we observed that the combination of all coffee compounds reduced the protein levels of Bcl-2 family members evaluated (Fig. 7A). In the CAF+TRI+CGA group, Mcl-1 was significantly reduced compared to untreated and DEN/CCl_4 groups (P=.019), whereas Bcl-2 and Bcl2l2 were significantly reduced compared to the untreated group (P=.017 and P=.032, respectively) (Fig. 7A). As expected, CAF treatment did not reduce the protein levels 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 anti-apoptotic proteins, contributing to an increased number of apoptotic cells (cleaved caspase-3 positive) in the

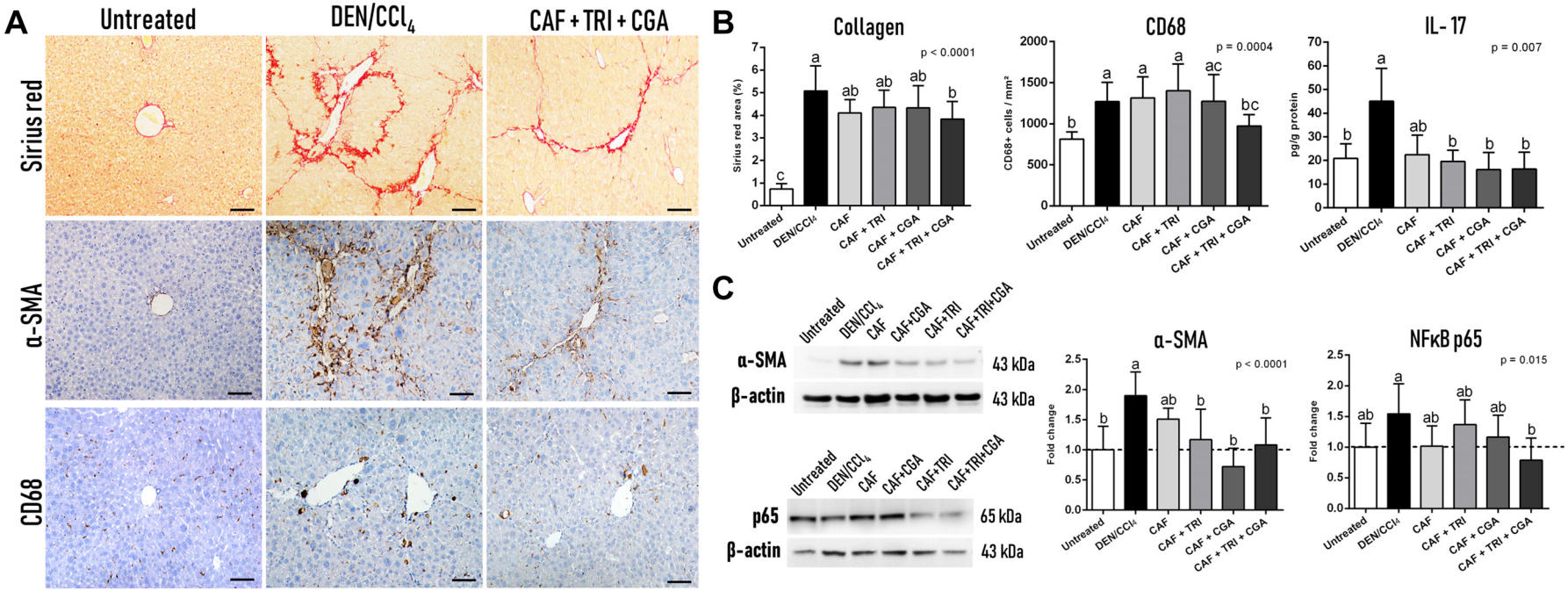

Fig. 5. Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and/or chlorogenic acid (CGA) on fibrosis markers during DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis. **(A)** Representative photomicrographs of collagen fibers in Sirius red-stained sections and α-smooth muscle actin (α-SMA) and CD68 immunostaining (20× objective, scale bar=50 μm). **(B)** Collagen area (Sirius red-stained sections), number of CD68-positive macrophages and interleukin-17 (IL-17) levels (ELISA). **(C)** Representative blot bands and semiquantitative analysis of α-SMA and NFκB p65 protein levels. Data are mean±S.D. n=5 (untreated) to 8 (other groups) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.); TRI, CGA=trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically administered from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference among groups by ANOVA and *post hoc* Tukey test (P<.05).

Table 2
Differentially expressed miRNAs in DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis group compared to untreated group

miRNAs	*P* value	FC
Upregulated (14)		
miR-199a-3p	.0011	2.29
miR-125a-5p	9.90E-06	2.14
miR-883b-3p	.0470	2.14
miR-199a-5p	.0200	2.00
miR-342-3p	.0040	2.00
miR-466a-3p	.0330	1.86
miR-223-3p	.0140	1.74
miR-132-3p	3.50E-05	1.62
miR-34a-5p	.0003	1.62
miR-669a-5p	.0250	1.62
miR-150-5p	.0350	1.51
miR-15b-5p	.0039	1.51
miR-335-5p	.0150	1.51
miR-350-3p	.0001	1.51
Downregulated (5)		
miR-451a	.0140	0.61
miR-496a-3p	.0130	0.61
miR-144-3p	.0054	0.57
miR-486a-5p	.0067	0.57
miR-376a-3p	.0008	0.53

$p<0.05$ and fold change (FC) >1.5. $n=5$ (Untreated) or 6 (DEN/CCl_4) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16.

adjacent liver (Fig. 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 [54], a coffee compound-induced pro-apoptotic signal in HSC may contribute on alleviating fibrosis in this intervention group, as observed (Fig. 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 Fig. 8).

Moreover, CAF+TRI+CGA treatment reduced by 54% the DEN/CCl_4-mediated increase in EGFR protein levels ($P=.018$) (Fig. 7B). Our results indicate that coffee compound combination-mediated upregulation of miR-144-3p may alleviate EGFR signaling activation during the early stages of mouse hepatocarcinogenesis, contributing to decrease proliferation (Ki-67-positive hepatocytes) inside preneoplastic foci (Fig. 4A), ultimately attenuating preneoplastic foci development (Fig. 2B, Table 1). 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 expressions were not modified by CAF+TRI+CGA treatment (Supplementary Fig. 9). The downstream modulation of the PI3K-Akt/Ras axis is hypothesized (Fig. 6B), but complementary studies are warranted. Regarding cell proliferation, we also evaluated the beneficial effects of the coffee compound combination on proliferating nuclear cell antigen (PCNA), a miR-376a-3p target widely accepted as a proliferation marker [56] (Supplementary Tables 4 and 5). Although CAF+TRI+CGA intervention failed on reducing the number of PCNA-positive hepatocytes in adjacent liver, also presenting a statistical trend ($P=.051$) on decreasing hepatic PCNA protein levels (Supplementary Fig. 9), only CAF+TRI+CGA reduced the number of PCNA- positive hepatocytes in preneoplastic foci ($P=.039$) (Fig. 7B), in keeping with Ki-67 findings (Fig. 3B). Despite 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 (Fig. 7B). Therefore, we hypothesize that other mechanisms, independently from miR-144-3p and miR-376a-3p modulation, may contribute to decreasing the number of preneoplastic lesions in the CAF+TRI+CGA group, as reduced oxidative stress (Supplementary Fig. 4).

Table 3
miRNAs modulated by CAF+TRI+CGA treatment during fibrosis-associated hepatocarcinogenesis (vs. DEN/CCl_4 group)

miRNAs	*P* value	FC
Upregulated (9)		
miR-199a-5p	.00008	2.30
miR-144-3p	.00100	2.14
miR-199a-3p	.00001	1.87
miR-335-5p	.00610	1.62
miR-342-3p	.03400	1.62
miR-376a-3p	.00350	1.62
miR-132-3p	.00037	1.52
miR-15b-5p	.00110	1.52
miR-350-3p	.00011	1.52

$p<.05$ and fold change (FC) >1.5. $n=6$ (DEN/CCl_4) or 7 (CAF+TRI+CGA) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25 to 1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.) intragastrically from Week 7 to 17. TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically from Week 7 to 17 (see Materials and Methods section).

3.9. The combination of coffee compounds enhances cytotoxicity in human HCC cells

The exposure to all concentrations of CAF individually (160, 80, 40 and 20 μM) significantly increased cytotoxicity in C3A cells after 48 h of exposure ($P<.001$), not after 24 h (Supplementary Fig. 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 48 h of exposure ($P<.001$) (Supplementary Fig. 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 [57]. 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 [53]. 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. The modulation of miRNA profile by these compounds was also investigated, correlating changes in expression with liver fibrosis/carcinogenesis outcomes. In summary, the combination of all compounds displayed the most pronounced effects on alleviating preneoplastic foci development. This treatment also reduced hepatocyte proliferation in preneoplastic lesions and enhanced apoptosis in adjacent tissue. In addition, CAF+TRI+CGA combination alleviated fibrosis and HSC activation, reducing hepatic pro-inflammatory IL-17 and NFκB mediators. Moreover, CAF+-TRI+CGA decreased hepatic oxidative stress and enhanced endogenous antioxidant agents. The miRNAomic profile showed the upregulation of miR-144-3p and miR-15b-5p, which were in accordance with the reduction of 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 downregulation of miR-376a-3p was also in accordance with the reduction in number of PCNA (miR-376a-3p targets)-positive hepatocytes inside preneoplastic foci. Noteworthy, this is the first report on the modulation of miRNA expression by bioactive coffee compounds administered in

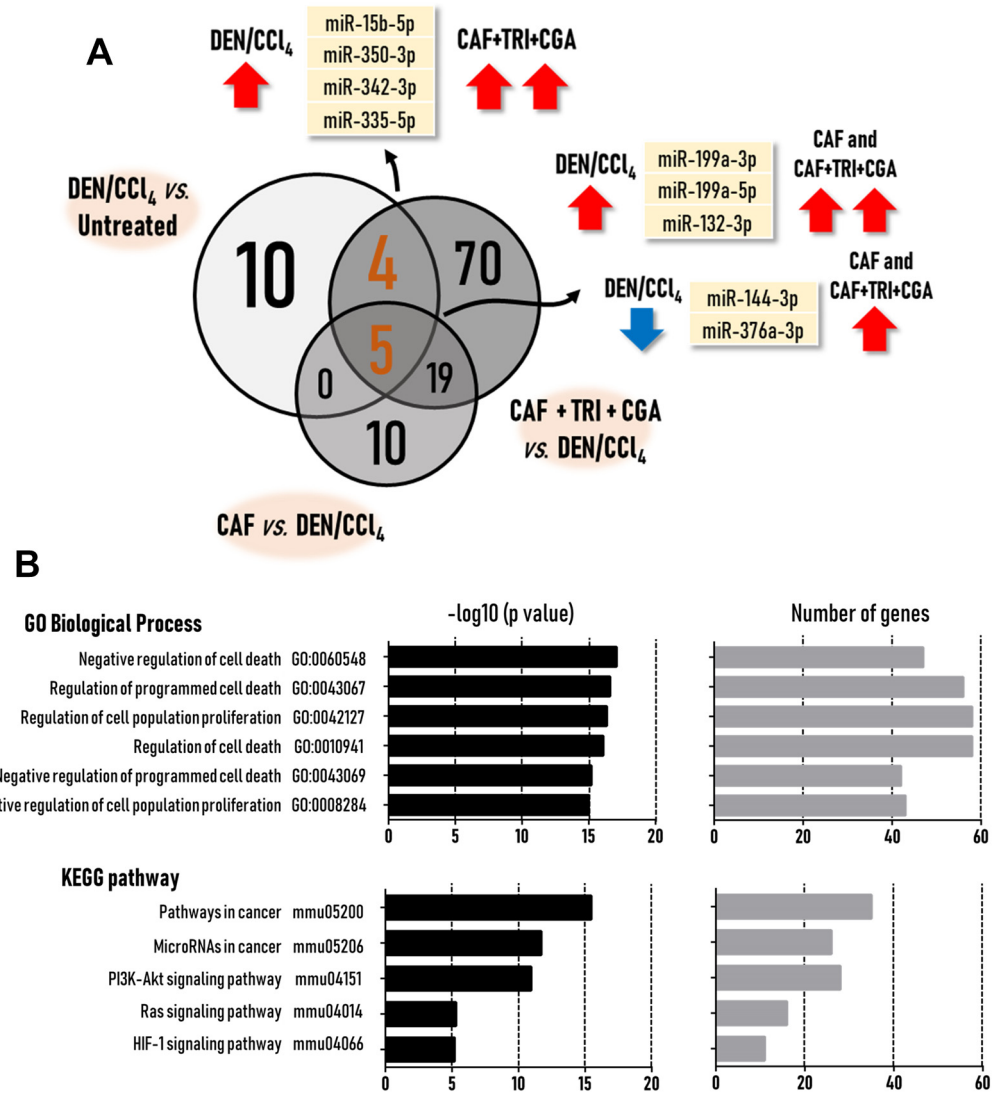

Fig. 6. **(A)** Venn's diagram analysis of global miRNA expression by NanoString. Nineteen miRNAs were differentially expressed in DEN/CCl_4-induced fibrosis associated carcinogenesis hepatocarcinogenesis, including the downregulation of tumor suppressor miR-144-3p and miR-376a-3p. CAF+TRI+CGA treatment upregulated 9/19 (~50%) of the differentially expressed miRNAs in DEN/CCl_4 group, including the tumor suppressors miR-144-3p, miR-376a-3p and antifibrotic miR-15b-5p. CAF+TRI+CGA-related miRNA signature (9 miRNAs) involved 4 miRNAs exclusively upregulated in this treatment and 5 miRNAs also upregulated in CAF-treated group. ↑: upregulation; ↓: downregulation. **(B)** Gene ontology and KEGG pathway analysis of the 232 experimentally validated target genes associated with CAF+TRI+CGA treatment miRNA signature, ranked by adjusted -log10 (*P* value).

physiologically based doses during fibrosis-associated hepatocarcinogenesis. Especially, the findings indicate that miR-15b-5p and miR-144-3p upregulation are potentially implicated in liver fibrosis and 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 by~100% [58]. 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 [59]. Nonetheless, coffee is a complex pharmacopeia, and the 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 group, the 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

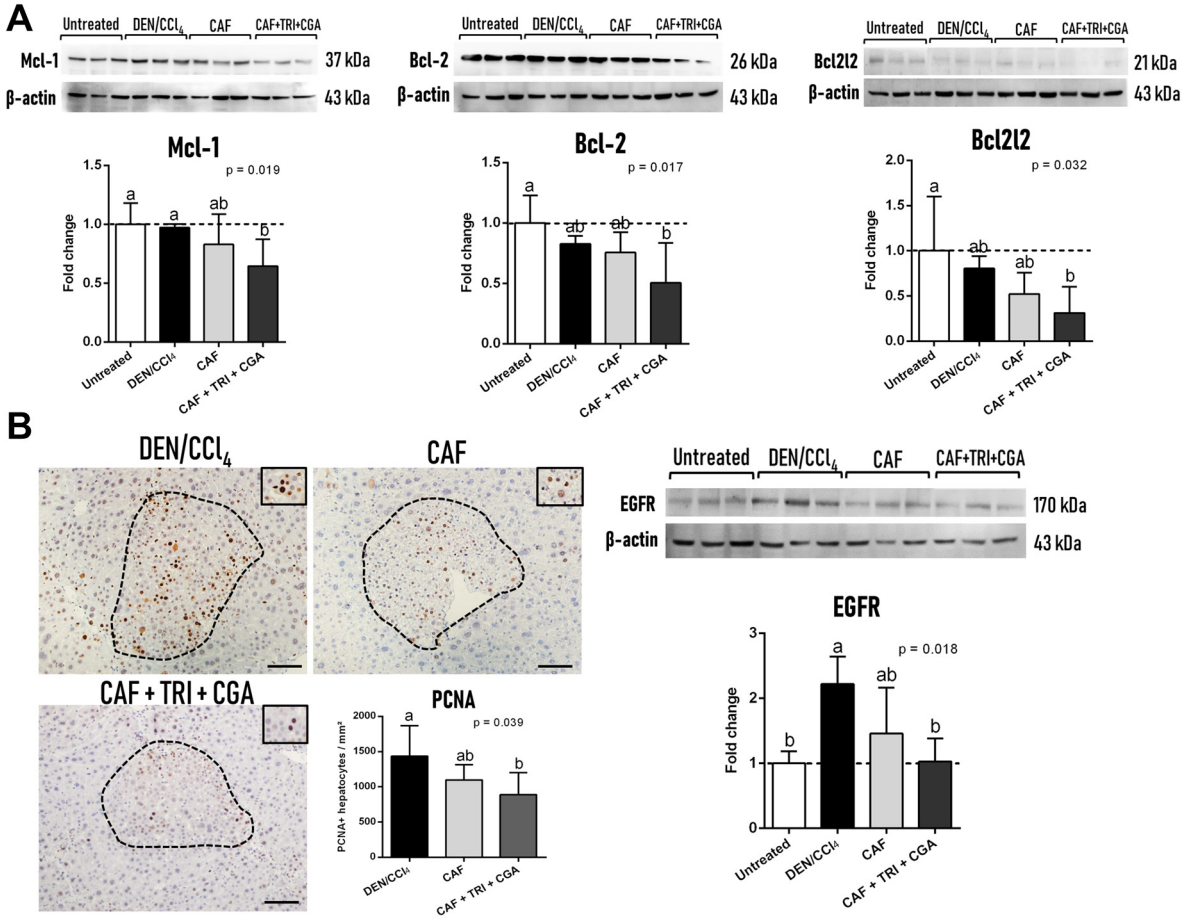

Fig. 7. Effects of caffeine (CAF) individually or combined to trigonelline (TRI) and chlorogenic acid (CGA) on **(A)** Mcl-1, Bcl-2, Bcl2l2 (miR-15b-5p targets) hepatic protein levels, **(B)** PCNA (miR-376a-3p target) immunostaining in preneoplastic foci and EGFR (miR-144-3p target) hepatic protein levels during DEN/CCl_4-induced fibrosis-associated hepatocarcinogenesis. Representative blot bands and semiquantitative analysis are presented. 20× objective, scale bar=50 μm. Data are mean±S.D. n=5 (untreated) to 6 (other groups) mice/group. DEN/CCl_4, diethylnitrosamine 10 mg/kg b.wt. i.p. at Week 2 and carbon tetrachloride 0.25–1.50 μl/g b.wt. i.p. from Week 8 to 16. CAF, caffeine (50 mg/kg b.wt.); TRI, CGA, trigonelline and/or chlorogenic acid (25 mg/kg b.wt., both) intragastrically administered from Week 7 to 17 (see Materials and Methods section). Different letters correspond to statistical difference among groups by ANOVA and *post hoc* Tukey test (P<.05).

and preneoplastic foci development, the main outcomes observed in the DEN-initiated/CCl_4-promoted mouse model at this time point (Week 17). DEN is an initiating agent for liver carcinogenesis that causes DNA damage and genomic instability in hepatocytes, while multiple CCl_4 administrations lead to lipid peroxidation and cell death, providing the necessary necroinflammatory background for HSC activation, collagen deposition and preneoplastic lesion growth [11,60,61]. This short/medium-term bioassay combining DEN and CCl_4 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]. Concerning the inbred strain applied, C3H/HeJ mice display increased susceptibility to (pre)neoplastic liver lesion emergence compared to other classically applied strains (C57BL/6 J and B6C3F1) [62]. This feature is predominantly linked to the occurrence of different hepatocarcinogen sensitivity loci (*Hcs*), conferring increased hepatocyte proliferation especially in chemically-induced preneoplastic foci [63]. Although coffee compounds (8–10 a.m.) and CCl_4 (4–6 p.m.) were concomitantly administered for 8 weeks, CCl_4 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 [15,60]. Thus, it is unlikely that coffee compound intervention interfered with CCl_4 phase I metabolism.

Our findings showed that the combination of coffee compounds, rather than CAF individually, had the most pronounced effects on reducing the incidence (clear foci), number (all types and basophilic foci) and cell proliferation (Ki-67) of hepatic preneoplastic foci. Due to the carcinogen effect on glycogen metabolism, the glycogenotic eosinophilic and clear cell foci phenotypes are the first to arise in hepatocarcinogenesis [64]. Progressively, these lesions tend to a "metabolic turnover", giving rise to the basophilic foci with a glycogenolytic profile [64]. These metabolic changes, as well as the frequently featured *Braf* and *Hras* mutations, are the stimulus to promote cell proliferation, a cancer hallmark, predisposing these lesions to neoplastic progression [44,45]. Therefore, a negative modulation of cell proliferation into AHF can slow lesion development [46], resulting in diminished incidence and number of lesions *per* liver area in CAF+TRI+CGA group. In addition, the combination of coffee compounds had the most noticeable results on decreasing pro-inflammatory IL-17 and p65 mediators, attenuating CD68 macrophage number, HSC activation and collagen accumulation. Liver resident (Kupffer cells, KC) and recruited macrophages play pivotal roles in inducing pro-inflammatory and pro-fibrogenic responses in HSC through the production of cytokines and subsequent paracrine signaling [65]. IL-17 synthesis, mainly mediated by KC, directly contributes to HSC activation and collagen production [66]. 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 [66]. 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 CCl_4-induced liver fibrosis [66,67]. Thus, CAF+TRI+CGA treatment is proposed to attenuate pro-fibrogenic IL-17/NFκB axis, contributing to decrease liver fibrosis.

Moreover, CAF+TRI+CGA reduced hepatic oxidative stress, inducing endogenous antioxidant Nrf2 transcription factor and GSH-Px and SOD enzymes. In DEN/CCl_4 model, reflecting human hepatocarcinogenesis, oxidative stress is implicated in collagen accumulation and preneoplastic lesion emergence [10,11,47]. The nuclear factor erythroid-related factor 2 (Nrf2) controls the expression enzymatic antioxidant agents, including GSH system, catalase, and SOD, which are accounted for ROS and lipid hydroperoxide neutralization [68,69]. *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 HepG2 cells [70]. 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 [71]. Nonetheless, several *in vivo* reports have shown the individual ability of CAF, TRI or CGA to induce Nrf2 and/or downstream antioxidant agents [26,72], although the exact mechanisms remain to be fully elucidated. Here, findings suggest that the combination of 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, should be considered on these terms as well [73]. Thus, decreased TBARS in the 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.

In addition to the reduction in liver fibrosis and preneoplastic foci outcomes, the miRNomic profile of the 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. Especially, the DEN/CCl_4 regimen 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 our bioassay. In keeping with our data, miR-144-3p levels were decreased in HCC in a similar DEN-induced hepatocarcinogenesis model in neonatal C3H mice [49]. In 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 (TGF)-β1 marker, respectively [50,51]. Moreover, human HCC cell lines presented decreased expressions of miR-144-3p (HepG2, Hep3B, and Huh7) and miR-376a-3p (HepG2 and Huh7) [50,52]. Noteworthy, the upregulation of 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) and downstream Akt signaling pathway [49]. In contrast, miR-144-3p knockout increased EGFR/Akt axis [49]. 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 [51,74]. Here, we reported that the combination of coffee compounds upregulated miR-144-3p while decreased EGFR protein levels. 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. In previous studies, individual CAF administration in low doses decreased Akt pathway in HepG2 cells [75], while CGA alone attenuated ERK1/2 pathway in HepG2 cells and in a xenograft HCC mice model [76]. Data on the individual effects of TRI on this pathway during liver carcinogenesis is absent. Thus, synergistic or/and additive effects may be considered on understanding the effects of CAF+TRI+CGA on miR-144/EGFR modulation, since ERK and Akt are downstream members of EGFR signaling, yet further studies are warranted.

The roles of miR-367a-3p on rodent models of liver fibrosis and carcinogenesis are not described yet. 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 [52]. Interestingly, this non-coding RNA was also downregulated during liver regeneration after partial hepatectomy in mice, suggesting underlying roles in controlling hepatocyte proliferation [52]. 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 the Huh7 HCC cell line by targeting p85α [52]. 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 [56]. 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 a reduced number of preneoplastic foci. Interestingly, we found a reduction in cell proliferation indexes (Ki-67-and PCNA-positive hepatocytes) just inside preneoplastic foci, whereas CAF+TRI+CGA treatment did not alter cell proliferation in adjacent tissue. Since preneoplastic foci display increased cell proliferation compared to adjacent tissue, in part due to mutations in *Braf* and *Hras* [44,45], our findings could indicate that these lesions may be sensitive to CAF+TRI+CGA-mediated modulation of these tumor suppressor miRNAs.

The combination of all coffee compounds upregulated miR-15b-5p in the liver as well. On activated 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) [54]. Hence, the modulation of the miR-15 family is proposed as a novel therapeutic strategy for liver fibrosis, considering that HSC death could attenuate collagen deposition [54]. 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. Bcl-2, Mcl-1 and Bcl2l2, proteins placed in the mitochondrial membrane, bind to pro-apoptotic BH3 sensitizers, initiators, or pore formers, avoiding mitochondrial permeability, cytochrome release and activation of caspase cascade [77]. 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. 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 of miRNA modulation. 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.

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 the pro-inflammatory NF-κB transcription factor is responsible for upregulating some anti-inflammatory miRNA families in negative feedback [78]. 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, which could hinder the maturation of specific tumor-suppressor-like miRNAs [79]. In addition, oxidative stress is proposed to cause miRNA deregulation at the level of transcription, processing, cellular localization and functioning [78]. Thus, the relationship between CAF+TRI+CGA-mediated effects on oxidative stress, NF-kB, EGFR and miRNA biogenesis should be evaluated on future investigations.

In conclusion, 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 and hepatocarcinogenesis. Results also indicate that these beneficial effects may be mediated by alterations in miRNA expression, providing mechanistical insights on the hepatoprotective population-level effects attributed to caffeinated coffee consumption. Finally, the modulation of tumor suppressor and antifibrotic miRNAs by naturally occurring compounds may open a chemopreventive avenue, inspiring further translational investigations.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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