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# Modulation of LDL receptor expression and promoter methylation in HepG2 cells treated with a *Corylus avellana* L. extract

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ABSTRACT

The aim of our study was to evaluate the impact of an ethanolic extract of *C. avellana* on the molecular pathway (s) regulating the low-density lipoprotein receptor (LDLR) in HepG2 cells, mainly in terms of epigenetics. We demonstrated that viability, proliferation and cell cycle distribution were not affected up to 72 h of treatment, whereas LDLR expression was stimulated as early as 24 h following administration ($P < 0.05$ at 0.04 mg/ml, $P < 0.01$ at 0.4 mg/ml). The level of DNA Methyl Transferase 3A was up-regulated ($P < 0.001$ at 0.004 mg/ml, $P < 0.05$ at 0.4 and 4 mg/ml), without any change in global DNA methylation, whereas the percentage of 5-methyl cytosine was significantly ($P < 0.05$) reduced at LDLR promoter level in response to treatment (0.04 mg/ml). Overall, our data demonstrate that the ethanolic extract of *C. avellana* stimulates the LDLR expression in HepG2 cells by epigenetic mechanisms.

## 1. Introduction

The low-density lipoprotein (LDL) is a major blood cholesterol carrier. In liver, the LDLR triggers the endocytosis of the LDL particles from plasma, thus regulating the blood cholesterol levels (Goldstein & Brown, 2009). Elevated levels of circulating low-density lipoprotein-cholesterol (LDL-C) are associated with an increased risk of developing different cardiovascular diseases (CVDs) (Ridker, 2014). Most commonly prescribed therapies imply the administration of statins to reduce circulating LDL-C, by also positively impacting on the regulation of the LDLR at liver level (Goldstein & Brown, 2009). As statins show different side effects, naturally occurring food components are gaining interest as valuable alternative to the pharmacological options, by acting as modulators of LDLR expression. In this context, antioxidant compounds (including berberine, curcumin, resveratrol, quercetin and quercetin-3-glucoside) have been already reported to be anti-cholesterolemic through stimulation of the hepatic LDLR expression (Kong et al., 2004; Mbikay, Sirois, Simoes, Mayne, & Chrétien, 2014; Moon et al., 2012; Tai et al., 2014). LDLR expression can be affected at multiple levels, including transcriptional (mainly via the sterol regulatory element-binding proteins, SREBP-1a/-1c/-2), post-transcriptional and post-translational levels (Benjannet et al., 2004; Briggs, Yokoyama, Wang, Brown, & Goldstein, 1993; Nakahara, Fujii, Maloney, Shimizu, & Sato, 2002; Yashiro, Yokoi, Shimizu, Inoue, & Sato, 2011; Zelcer, Hong, Boyadjian, & Tontonoz, 2009). Recently, the LDLR gene promoter has been also characterized in terms of epigenetics (Alvarez, Khosroheidari, Eddy, & Done, 2015; Cai et al., 2016). Different CpG (Cytosine/Guanosine) islands in both human and mouse LDLR promoter, as well as the involvement of the DNA Methyl Transferases (DNMTs, the enzymes catalyzing the transfer of the methyl group to the DNA cytosines), have been identified as candidate biomarkers responsive to diet supplementation (Adaikalakoteswari et al., 2015; Hu et al., 2016; Trenteseaux et al., 2017; Xie et al., 2014).

Several epidemiological and clinical studies have demonstrated that a dietary supplementation with nuts (such as hazelnuts, almonds, pistachios, walnuts) can significantly ameliorate the plasma lipids' level, thus lowering morbidity and mortality related to CVDs (Bamberger et al., 2017; Berryman, Fleming, & Kris-Etherton, 2017; Chen, Wan, & Qin, 2016; Del Gobbo, Falk, Feldman, Lewis, & Mozaffarian, 2015; Kelly & Sabaté, 2006; Sabaté, Oda, & Ros, 2010; Sabaté, Ros, & Salas-

*Abbreviations:* 5-mC, 5-methyl cytosine; Alu, short interspersed elements; CVD, cardiovascular disease; DAC, 5-Aza-2-deoxycytidine; DNMT, DNA methyl transferase; HZN, hazelnut; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein-cholesterol; LDLR, low-density lipoprotein receptor; LINE-1, long interspersed nuclear elements-1; Sat-α, satellite DNA alpha; SREBP, sterol regulatory element binding protein

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Salvadó, 2006). Hazelnuts play a major role in nutrition and health as high energetic food, rich in monounsaturated fatty acids (MUFA) and proteins (mainly in the kernel), as well as a valuable source of fiber, phytonutrients, and antioxidants, including the Vitamin E, that have been correlated to the reduced plasma total and LDL-C concentration (Bacchetta et al., 2015; Bacchetta & Di Giovanni, 2013; Orem et al., 2013; Perna et al., 2016; Deon et al., 2018; Pelvan, Olgun, Karadağ, & Alasalvar, 2018). Also our group recently demonstrated that a hazelnut-enriched diet (specifically *Corylus avellana* L. cultivar *Tonda Gentile Romana,* 40 g/day for 14 weeks) can positively impact on blood lipid profile, by significantly decreasing the LDL plasma content, and modulating both uric acid and serum creatinine values in healthy volunteers (Santi, Giorni, Terenzi, Altavista, & Bacchetta, 2017).

Despite consistent clinical findings, the molecular mechanism(s) underlying the plasma lipid benefits due to nuts' consumption are still poorly defined (Domínguez-Avila et al., 2015; Donadio, Rogero, Cockell, Hesketh, & Cozzolino, 2017; Goldstein & Brown, 2009; Lima et al., 2017; Luo et al., 2016). A few experimental evidence demonstrated that pecan supplementation can prevent hyperlipidemia induced by a high fat (HF) diet in Wistar rats, through upregulation of the LDLR transcripts (Domínguez-Avila et al., 2015). In particular, no experimental findings have directly investigated the pathways responsible for hazelnut-driven health-promoting properties. We thus here evaluated the effect of an ethanolic liquid extract of hazelnut *Corylus avellana* L. on human HepG2 hepatocarcinoma cells. The complete metabolomic characterization of the extract has been carried out and recently published by our group (Cappelli et al., 2018). We specifically verified whether hazelnut treatment might affect the expression of the LDLR, and focused on the epigenetic modulation of the LDLR regulatory pathway.

## 2. Materials and methods

### 2.1. Chemicals

Culture media, supplements, trypsin-EDTA, and phosphate buffer saline (PBS) were obtained from Euroclone (Milan, Italy); the foetal bovine serum was purchased from Hyclone (Logan, UT, USA). Aflatoxin B1, 5-aza-2-deoxycytidine (DAC), dimethyl sulfoxide (DMSO), ethylene-diamine-tetra-acetic acid (EDTA), Propidium Iodide (PI), RNAse A, Trypan blue solution (0,4%), Triton X-100 were obtained from SIGMA-Aldrich (St Louis, MO, USA). Ethanol was obtained from CARLO ERBA Reagents (Milan, Italy).

### 2.2. Preparation of the hazelnut liquid extract (HZN)

Raw hazelnuts of *Corylus avellana* L., cultivar *Tonda Gentile Romana*, were provided by the Coopernocciola srl (Vico Matrino, VT, Italy) in 250 g/bag (shipped and maintained under vacuum). Twenty grams of raw materials were homogenized using mortar and pestle (after sterilizing both tools by autoclave for 20 min at 120 °C). When reached a fine particle homogenized material, a 60% aqueous ethanol solution was added at liquid:solid ratio of 1:5 (v/w). After 1 h of continuous stirring, the extraction process was carried out in closed bottle at room temperature (20–22 °C), in dark conditions, for 30 days (Contini, Baccelloni, Massantini, & Anelli, 2008). The hazelnut suspension was mixed by hand each three days. At the end of the maceration procedure, the hazelnut liquid extract (HZN) was collected, passed through a filter of 0.2 μm and stored at −80 °C for all experiments.

The complete metabolomic characterization of our HZN ethanolic liquid extract has been carried out by liquid chromatography–high resolution mass spectrometry, and recently published (Cappelli et al., 2018). In brief, a total number of 85 primary (9 acids, 4 amino acids, 9 sugars, 7 vitamins) and secondary (3 alkaloids, 42 phenylpropanoids, 5 taxanes) metabolites were identified. Overall, phenylpropanoids represented the most abundant metabolic class (49.4% of the detected molecules), followed by acids and sugars (10.6% each). Among the primary metabolites, a very high accumulation of oxaloglutarate (acid), Bis-D-fructose 2′,1:2,1′-dianhydride (sugar) and vitamin B2 was reported. With respect to the secondary metabolism, 3 alkaloids ($C_{26}H_{28}N_2O_5$, being the most abundant; $C_{26}H_{28}N_2O_6$ and $C_{29}H_{25}NO_9$) were identified, and a large group of phenylpropanoids, highly over-represented in the ethanolic hazelnut extract, with flavonoids being the most abundant sub-class. Traces of 4 amino acids (l-*threo*-3-phenylserine, hydroxyl-phenylalanine, phenylalanine and tyrosine) and of 5 taxanes (baccatin III, 7-epi-10-deacetyltaxol, 10-deacetylbaccatin III, 10-deacetyl-7-xylosyltaxol and taxinine M) were also detected.

### 2.3. Cell cultures and treatments with the HZN solution

HepG2 human hepatocellular carcinoma cells were purchased from European Collection of Cell Cultures (ECACC, Sigma-Aldrich), maintained as sub-confluent monolayers in DMEM, with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin (10,000 U/mL), at 37 °C in a 5% $CO_2$ atmosphere in air. HepG2 cell line was selected as a suitable model for liver studies since it maintains several tissue-specific metabolic functions (Nikoloff, Larramendy, & Soloneski, 2014).

In all the experiments (unless otherwise specified) cells were seeded in 60-mm Petri-dishes at $4 \times 10^5$ cells/dish; twenty-four h after plating, cells were treated with either the HZN extract (0.004, 0.04, 0.4, 4 mg/ml), or the corresponding aqueous ethanol solution (0.0006, 0.006, 0.06, 0.6%) for 24 or 72 h (without any medium replacement) (Supplementary Fig. 1A). The solutions were freshly prepared before each experiment in culture media.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.12.024.

The effect on cell proliferation was assessed by counting viable cells at the hemocytometer following Trypan blue staining (0.4% solution). In the experiments with the DAC, the agent was dissolved in DMSO, freshly diluted in PBS before each experiment, and added to cells at final concentration of 1 or 2.5 μM, for 48 h.

### 2.4. CellTiter-Fluor Cell Viability Assay

Viability of HepG2 cells was determined by the CellTiter-Fluor Cell Viability Assay Kit (Promega, Madison, WI, USA), as previously described (Grollino et al., 2017). As positive control, the hepatotoxic agent Aflatoxin B1 was prepared by dissolving the powder in DMSO and used at 10 μM final concentration (Grollino et al., 2017). HepG2 cells were seeded at $1 \times 10^4$ cells/well in black walled 96 well-plates; twenty-four hour after plating, cells were treated with HZN (0.004, 0.04, 0.4, 4 mg/ml) for 24 or 72 h. In all experiments, non-treated cells (negative control), ethanol-treated (0.6%, representing the highest solvent concentration in our study), and cells treated with 10 μM Aflatoxin B1 (positive control) were included. At the end of treatment, cells were processed according to the manufacturer's instructions. Briefly, CellTiter-Fluor reagent was added to each well and plates were incubated 40 min at 37 °C before reading fluorescence (Ex 380–400 nm/Em 505 nm) on a Glomax Discover System (Promega). All experiments were performed in triplicate and the cell viability (%) was expressed as the mean fluorescent intensity of the experimental group/the mean fluorescent intensity of the solvent control group × 100.

### 2.5. Flow-cytometric analysis

A FACScan Flow cytometer (Becton Dickinson, Bedford, MA, USA) equipped with a 488 nm argon laser was used for the flow-cytometric analyses.

#### 2.5.1. Evaluation of cell cycle distribution by DNA content analysis.

The determination of the DNA content for evaluation of the cell

cycle distribution was carried out as previously described (Benassi et al., 2016). Adherent cells were harvested by trypsinization and collected with floating ones; the pool was washed twice in PBS, then fixed in ice-cold ethanol 80% ($1 \times 10^6$ cells/ml) over-night. An aliquot of the suspension (at least $5 \times 10^5$ cells) was then washed twice in PBS, and stained with PI (50 μg/ml) in a mix containing RNAse A (50 μg/ml), Triton X-100 (0.1%), EDTA (0.1 mM) in PBS, in the dark, for 60 min at room temperature, then immediately analyzed. The evaluation of cell cycle distribution by DNA content analysis was performed by the FlowJo software®.

*2.5.2. Evaluation of the LDLR membrane protein expression by fluorescent antibody staining.*

Adherent cells were harvested by cold trypsinization, washed twice in a solution containing ice cold PBS, 10% foetal calf serum, and 1% sodium azide (PSSA solution), and adjusted to a final concentration of $1 \times 10^6$ cells/ml for staining with the antibody. The human LDLR Alexa-Fluor® 488-conjugated monoclonal antibody and the isotype control antibody were purchased from the R&D systems (Minneapolis, USA), and added to cell solution at the concentration of 5 μl/$10^6$ cells; a negative control (cells with no antibody) was also prepared in each experiment. Cells were stained in the dark, at 4 °C for 2 h, then washed three times in polypropylene tubes with the PSSA buffer and kept in the dark on ice until analysis. For acquisition, the forward and side scatters were controlled to virtually exclude cellular debris from the analysis. For fluorescence data analysis, the mean fluorescence intensity (MFI) was calculated as the ratio between the antibody-labelled cells and the unstained ones.

*2.6. RNA extraction, reverse transcription and gene expression analysis*

Total RNA was extracted from samples by Trizol® protocol (Invitrogen, Thermo Fisher Scientific). The amount and purity of the extracted RNA was evaluated by fiber optic spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) calculating the 230/260 and the 260/280 absorbance ratios. Five hundred nanograms of total RNA were reverse-transcribed to cDNA with random primers by TaqMan® Reverse Transcription Reagent (Applied Biosystems, Thermo Fisher Scientific), according to manufactures' indications. Analysis of the LDLR, DNMT1 and DNMT3A expression was carried out with 1 μl of cDNA using SYBR Green master mix (Applied Biosystems) and an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA). All reactions were run in quadruplicate and the relative abundance of the transcripts was calculated by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, applying the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). PCR primers were designed by NCBI-Primer Blast free software (https://www.ncbi.nlm.nih.gov/tools/primer-blast), according to gene sequences available in the UCSC database (https://genome.ucsc.edu), and selected to amplify an exon-intron-exon region (≤200 bp) to exclude genomic contamination. PCR primers were synthesized by Eurofins Scientific (Luxembourg). The complete list of primer sequences is reported in Supplementary Fig. 2.

*2.7. DNA extraction and methylation analysis*

DNA methylation analysis was performed by bisulphite conversion and pyrosequencing, as previously detailed (Consales et al., 2017). Briefly, genomic DNA was extracted from liver cells using the *Quick*-DNA™ Miniprep kit (Zymo Research, Irvine, CA, USA). Five hundred nanograms underwent bisulphite modification using the EZ DNA Methylation-Gold™ Kit (Zymo research) according to the manufacturer's protocol. Bisulphite-converted DNA (50 ng in 50 μl reaction) was amplified using the One *Taq*® Quick-Load® 2× Master Mix (New England Biolabs, Ipswich, MA, USA), and pyrosequencing was performed using the Pyromark® Q24 Instrument (Qiagen, Hilden, Germany). The percentage of methylation, expressed as percentage of 5-methyl cytosine (5-mC), was calculated by the provided Qiagen software, and expressed as the number of 5-mC divided by the sum of methylated and unmethylated cytosines. One investigator, blind to all the information regarding the samples except their code, performed the pyrosequencing analyses. Different internal controls were included in every pyrosequencing run to ensure the completion of bisulphite modification, the specificity of PCR amplification, and the success of pyrosequencing reactions. A universal methylated DNA standard (Zymo Research) was used as a positive control for bisulphite conversion.

*2.7.1. Pyrosequencing of human LINE-1 (Long interspersed nuclear elements-1), SAT-α (satellite DNA alpha) and Alu (short interspersed elements)*

LINE-1, SAT-α, and Alu DNA regions have been extensively reported as reliable biomarkers of genomic DNA methylation in response to environment and nutrition (Bollati et al., 2007; Chittur, Sangster-Guity, & McCormick, 2008; Garcia-Lacarte, Milagro, Zulet, Martinez, & Mansego, 2016; Mateo-Fernández, Merinas-Amo, Moreno-Millán, Alonso-Moraga, & Demyda-Peyrás, 2016).

PCR conditions and primers for pyrosequencing have been previously detailed in Consales et al. (2014) (SAT-α, Alu) and in Bollati et al. (2007) (LINE-1). Briefly, PCR and sequences primers (purchased from Eurofins Scientific) are as follows: LINE-1: forward (TTTTGAGT TAGGTGTGGGATATA), reverse (biotin-AAATCAAAAAATTCCCTTTC), sequencing primer (AGTTAGGTGTGGGATATAGT); SAT-α: forward ( TGTAAGTGGATATTTGGATTATTGG), reverse (TTTCCAAAAAAATCTT CAAAAAAAT), sequencing primer (CTCAAAAATTTCTAAAAATACTT CTC); Alu: forward (biotin-TTTTTATTAAAAATATAAAAATT), reverse ( CCCAAACTAAAATACAATAA), sequencing primer (AATAACTAAAATT ACAAAC).

PCR conditions were the following: LINE-1 (95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 (45 cycles); SATα (95 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s (45 cycles); Alu (96 °C for 90 s, 43 °C for 60 s, 72 °C for 120 s (45 cycles). For each repetitive element, the methylation levels of five cytosines for LINE-1, four cytosines for SATα and three cytosines for Alu were measured.

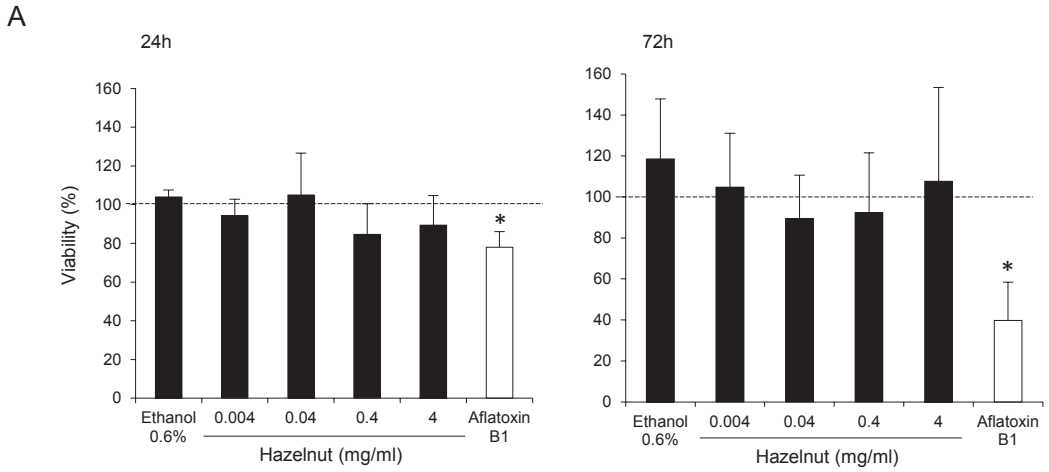
*2.7.2. Human LDLR promoter pyrosequencing*

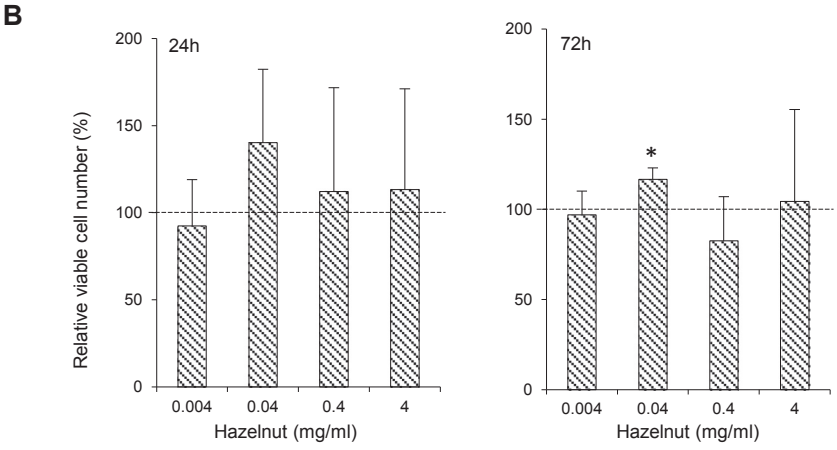
To study the DNA methylation of the human LDLR promoter, we retrieved the promoter sequence from the Eukaryotic Promoter Data Base (http://epd.vital-it.ch/index.php). We selected the −2000/ +200 bp region (relative to the transcription starting site, TSS) and uploaded it to the DBCAT software (DataBase of Cpg island & Analytical tools, http://dbcat.cgm.ntu.edu.tw) to identify putative CpG rich regions. The DBCAT-generated output (Supplementary Fig. 3A) recognized a dense CpG island located at position −221/+174 (fragment #1) that was used for searching pyrosequencing primers through the Pyromark® assay design software (Qiagen) (Supplementary Fig. 3B). The assay was designed for pyrosequencing nine cytosines (identified by Y1-Y9).

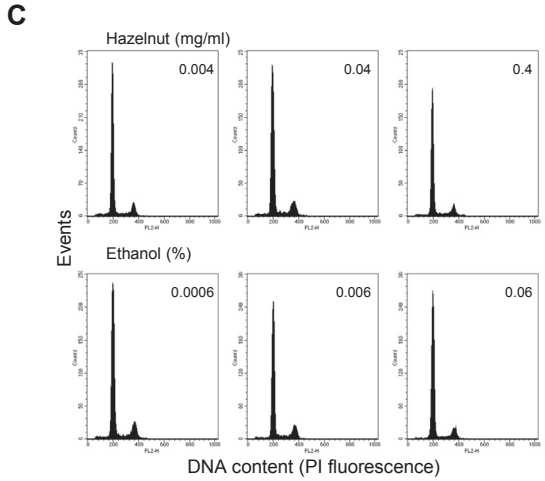
A second region to be pyrosequenced (fragment #2) was selected according to the literature (Adaikalakoteswari et al., 2015). By blast search (http://danio.mgh.harvard.edu/blast/wblast2.html), we mapped this region at position −739/-548 on the LDLR promoter gene. This sequence was also uploaded into the Pyromark® assay design software for primer design (Supplementary Fig. 3C); the assay was designed for analyzing the methylation status of four cytosines (Y1–Y4).

*2.8. Statistical analysis*

The variations of samples values are reported as Mean ± S.D. calculated in N ≥ 3 replicates. The statistical differences were analysed trough the KailedaGraph program (Synergy Software, Reading PA, USA) by applying either (i) one-way ANOVA followed by Dunnett post-hoc test, or (ii) two-sided Student's *t*-test when each single HZN




D

| | Ethanol (%) | | |
|---|---|---|---|
| | 0.0006 | 0.006 | 0.06 |
| sub $G_1$ | 2.2 ± 1.8 | 2.1 ± 0.9 | 1.8 ± 0.3 |
| $G_0/G_1$ | 80.5 ± 1.2 | 80.4 ± 1.5 | 81.5 ± 0.7 |
| S | 4.7 ± 0.7 | 4.5 ± 1.3 | 3.9 ± 1.5 |
| $G_2$/M | 13.6 ± 1.7 | 14.6 ± 1.2 | 11.8 ± 2.3 |

| | Hazelnut (mg/ml) | | |
|---|---|---|---|
| | 0.004 | 0.04 | 0.4 |
| sub $G_1$ | 2.8 ± 0.4 | 2.4 ± 1.3 | 1.2 ± 0.3 |
| $G_0/G_1$ | 79.2 ± 1.0 | 81.1 ± 3.8 | 82.6 ± 0.6 |
| S | 4.8 ± 0.6 | 4.4 ± 1.8 | 4.1 ± 0.7 |
| $G_2$/M | 14.6 ± 1.2 | 12.6 ± 1.3 | 12.2 ± 1.0 |

**Fig. 1.** Treatment with HZN extract does not affect viability, proliferation and cell cycle phases distribution of HepG2 cells. (A) Assessment of cell viability performed in response to ethanol (0.6%) and hazelnut extract at the dose range reported. Aflatoxin B1 (10 μM) was added as positive control of the test. Values are normalized to the value of the untreated control group (dashed line), and statistically analyzed by one-way ANOVA followed by post-hoc Dunnet test ($^{*}P < 0.05$ for aflatoxin B1 compared to control untreated cells); N = 3. (B) Evaluation of cell proliferation carried out by hemocytometer counting of trypan blue negative cells. The value represents the percent number of cells in hazelnut extract-treated vs corresponding ethanol-treated cultures (dashed line). $^{*}P < 0.05$ (0.04 mg/ml HZN vs 0.006% ethanol), as determined by two-sided Student's *t*-test; N = 5. (C) Representative DNA content histograms of PI-stained cells (flow cytometric analysis), and (D) corresponding evaluation of cell cycle phase percentages carried out by FlowJo software®; statistical analysis has been carried out by one-way ANOVA followed by post-hoc Dunnet test; N = 3.

concentration has been compared to the corresponding ethanol solution. The statistical test applied, the number of experimental replicates, as well as the experimental groups that have been compared for statistical analysis have detailed in each figure legend. P values < 0.05 were considered statistically significant and indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

## 3. Results

### 3.1. The Corylus avellana L. extract is not toxic to HepG2 cells

The viability and proliferation rate of HepG2 cells following treatment with the ethanolic extract of *C. avellana* (HZN at 0.004–4 mg/ml) were evaluated. As reported in Fig. 1A, no change in the viability was observed following 24 and 72 h of continuous HZN treatment at all the

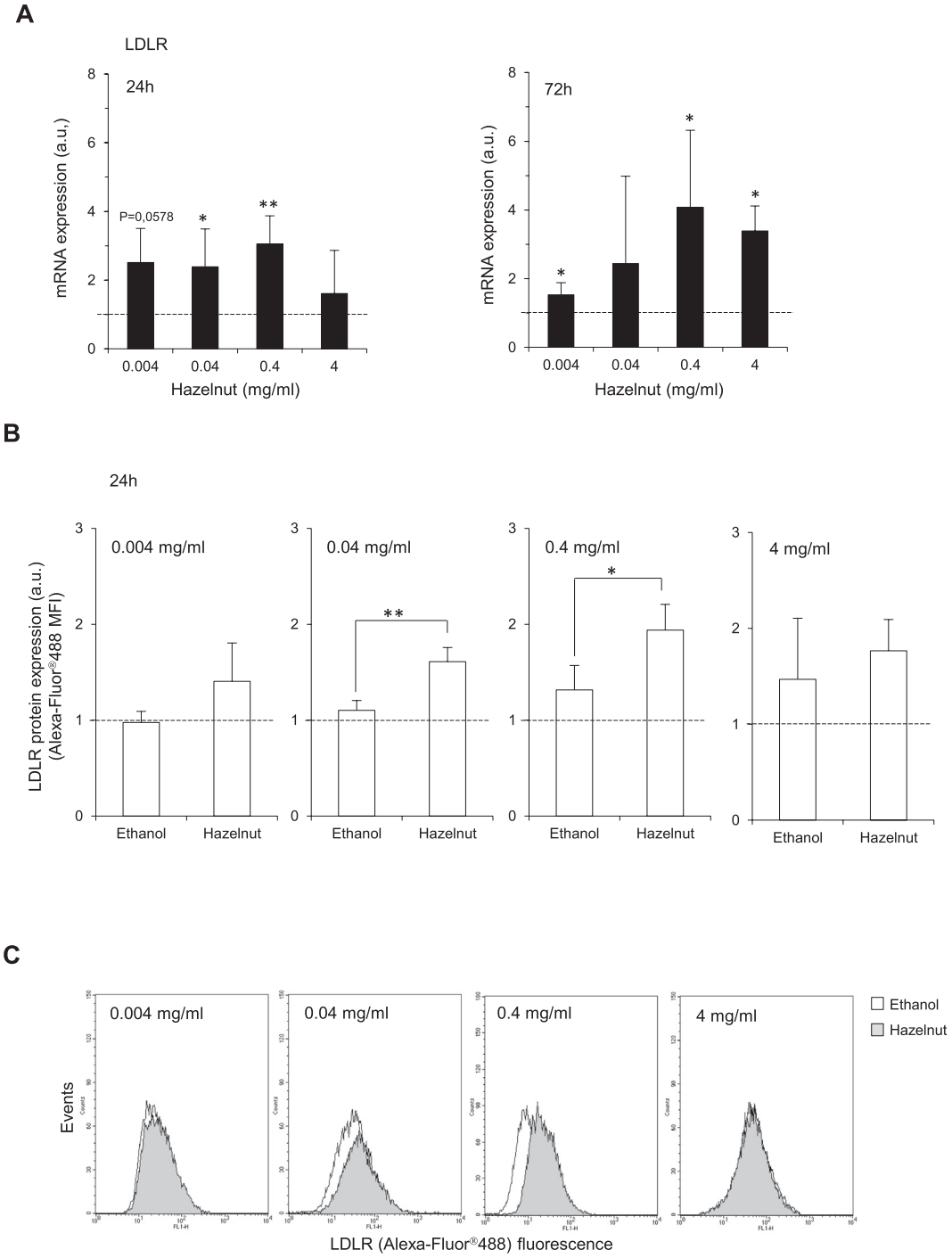



**Fig. 2.** Treatment with the HZN extract stimulates the LDLR expression in HepG2 cells. (A) Evaluation of LDLR transcript expression carried out by real-time PCR at 24 and 72 h of HZN treatment. The values represent mRNA expression levels at each HZN extract concentration normalized to the value of the corresponding ethanol-treated group (dashed line). $^{*}P < 0.05$, $^{**}P < 0.01$ (HZN-treated vs ethanol-treated cells), as calculated by two-sided Student's *t*-test; N = 5. (B) Analysis of the LDLR protein levels (normalized to the untreated control value, dashed line) determined by flow cytometric analysis at 24 h of treatment with the reported HZN extract or the corresponding solvent concentrations. $^{*}P < 0.05$, $^{**}P < 0.01$, as determined by two-sided Student's *t*-test; N = 3. (C) Representative histograms of LDLR expression assayed in cells stained with the LDLR-Alexa-Fluor®488 antibody and analyzed by Flow Cytometry.

doses selected (treatment with 10 μM Aflatoxin B1 was included as positive control). In agreement, no significant modulation of cell proliferation was reported in the HZN-treated cells if compared to corresponding ethanol solvent solution (Fig. 1B); a significant increase in viable cell number was exclusively reported at the 0.04 mg/ml HZN dose at 72 h of treatment (Fig. 1B). The effect of the aqueous ethanol solution was preliminary assessed in HepG2 cells to exclude that solvent might affect the growth rate of liver cells over the 24–72 h window (Supplementary Fig. 1B).

The cell cycle distribution was then evaluated in HepG2 cells following 72 h of HZN extract treatment. By flow cytometric analysis of PI-stained cells, we demonstrated that no cell cycle phases' perturbation was detectable in cells treated with the HZN extract (Fig. 1C), the percentages of cell cycle phases perfectly overlapping between HZN- and ethanol-treated liver cells (Fig. 1D). The DNA profile of the control (untreated) sample is reported in Supplementary Fig. 1C.

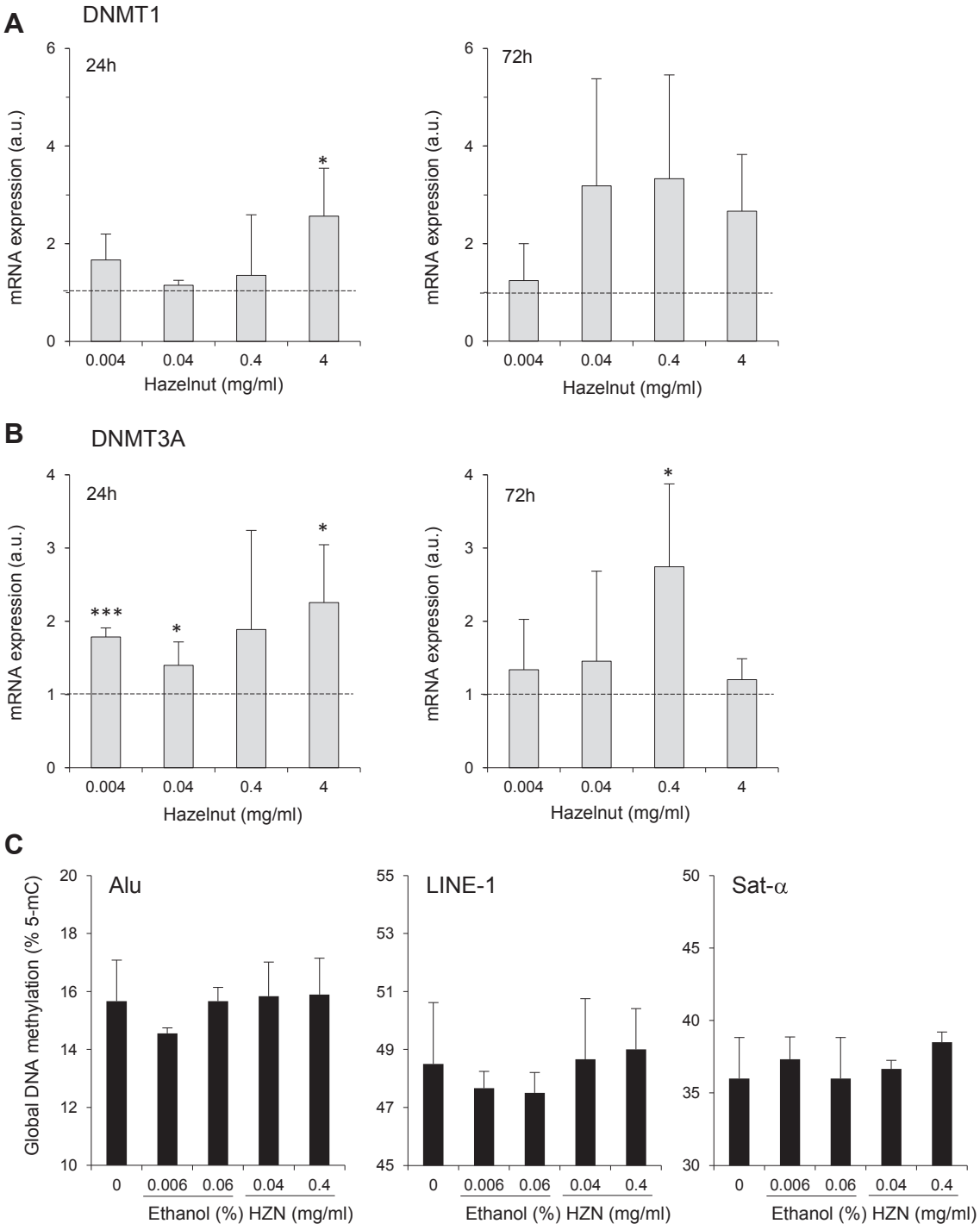
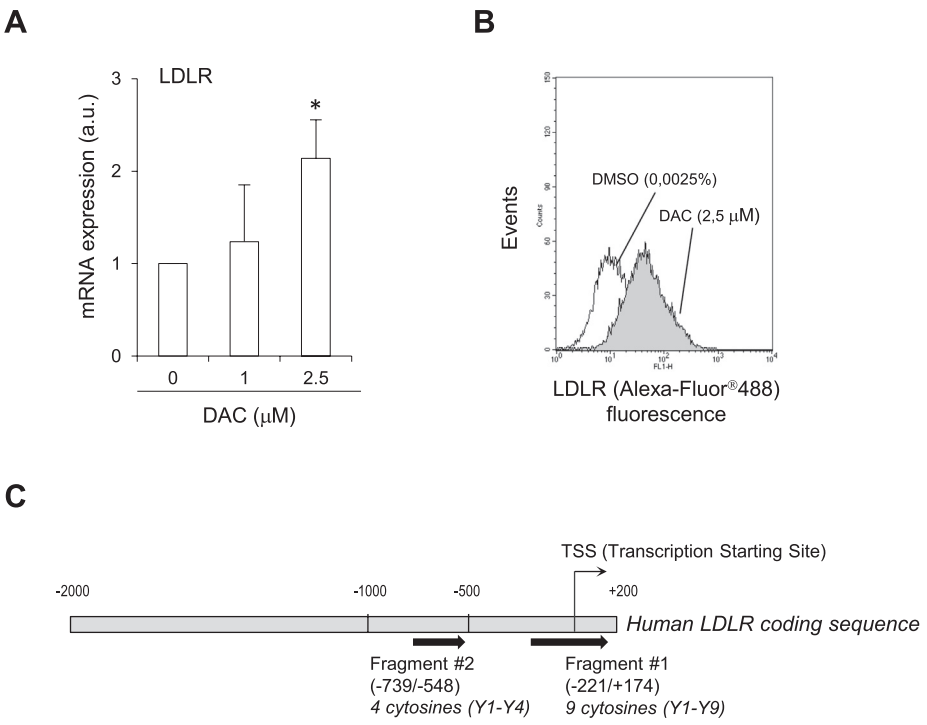
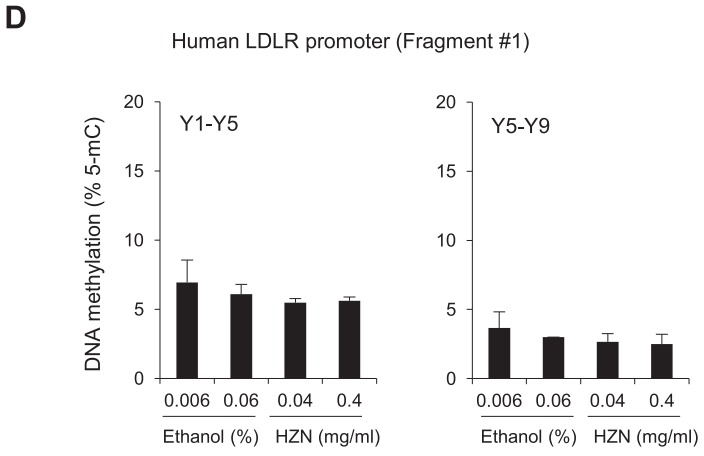



**Fig. 3.** Effect of the HZN treatment on DNMTs expression and global DNA methylation. Evaluation of DNMT1 (A) and DNMT3A (B) mRNA expression carried out by real-time PCR at 24 and 72 h of HZN treatment. The values represent mRNA expression levels at each HZN extract concentration normalized to the value of the corresponding ethanol-treated group (dashed line). $^{*}P < 0.05$, $^{***}P < 0.001$ (HZN-treated vs ethanol-treated cells), as determined by two-sided Student's *t*-test; N = 4. (C) Analysis of global DNA methylation (expressed in percentage of 5-mC) carried out by bisulphite conversion and pyrosequencing of the Alu, LINE-1 and Sat-α repetitive intersperse sequences. N = 3.



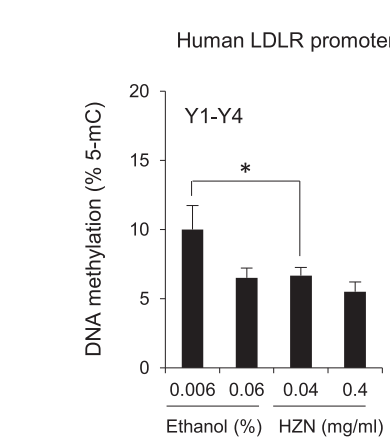




**Fig. 4.** The HZN extract induces a hypo-methylation of the LDLR promoter in HepG2 cells. Evaluation of LDLR expression carried out at both (A) transcript (values are normalized to that of the untreated control) and (B) protein level in cells exposed to the demethylating agent DAC. DAC-untreated cells correspond to those administered with the solvent (DMSO, 0.0025% final concentration). $^{*}P < 0.05$ (DAC-treated vs untreated cells), as calculated by one-way ANOVA followed by post-hoc Dunnet test; N = 3. (C) Schematic representation of the human LDLR coding sequence ( − 2000/ + 200 bp relative to the TSS). Fragment#1 and fragment #2 represent the target regions of the DNA methylation analysis carried out by bisulphite conversion and pyrosequencing. (D and E) Analysis of the DNA methylation level (expressed in percentage of 5-mC) carried out by bisulphite conversion and pyrosequencing of two fragments mapping in the human LDLR promoter region. $^{*}P < 0.05$; as determined by two-sided Student's *t*-test by comparing each HZN dose to the corresponding ethanol concentration; N = 3.

### 3.2. Treatment with the Corylus avellana L. extract stimulates the LDLR expression in the HepG2 cells

The analysis of the LDLR mRNA level was carried out at 24 and 72 h of HZN treatment (ranging from 0.004 to 4 mg/ml). As reported in Fig. 2A, a significant increase in the LDLR transcript expression was observed in cells upon HZN extract treatment. At 24 h of treatment, the highest and statistically significant stimulation was reported at the 0.04 and 0.4 mg/ml doses. After 72 h of continuous exposure, almost all the concentrations triggered a significant increase in the LDLR gene expression. In Supplementary Fig. 4A, the effect exerted by the ethanol on the LDLR expression is shown, and demonstrates that the solvent did not affect LDLR mRNA level in the dose-range applied in the experiments. A significant perturbation was only reported after 24 exposure to the highest ethanol dilution (0.6%) that completely recovered within the following 72 h.

As assessed by staining cells with a specific LDLR Alexa-Fluor® 488-conjugated antibody, the mRNA level increase led to a correspondent rise in the LDLR membrane protein expression in the HZN- compared to ethanol-treated cells (Fig. 2B and C), demonstrating that the HZN extract treatment is able to actively stimulate the LDLR expression in liver cells at both mRNA and protein level.

### 3.3. The Corylus avellana L. extract influences liver cells at epigenetic level, and affects the DNA methylation status of the LDLR promoter

The expression level of both the DNMT1 and DNMT3A genes was evaluated in HepG2 cells. Preliminary assessment of their expression was carried out in liver cells in response to the ethanol; we demonstrated that the solvent did not affect the DNMTs levels after 24 and 72 h of continuous exposure (Supplementary Fig. 4B and C). In response to HZN, we reported that DNMT1 was significantly stimulated after 24 h of treatment at the highest dose (4 mg/ml), whereas no statistically significant change was observed following 72 h exposure (Fig. 3A). Also the DNMT3A transcript expression significantly increased as early as 24 h h after treatment (Fig. 3B); in this case, all doses were shown to be effective, with the exception of 0.4 mg/ml that apparently induced a delayed stimulation at 72 h (Fig. 3B). Given the stimulation of DNMTs, we verified whether global DNA methylation might be altered by HZN treatment in HepG2 cells. By bisulphite conversion and DNA pyrosequencing, we analyzed the methylation status of Alu, LINE-1 and SAT-α repetitive interspersed sequences. As shown in Fig. 3C, no change in DNA methylation was triggered by HZN administration to liver cells, as none of the three biomarkers underwent any modification in response to the extract.

To assess the involvement of DNA methylation in the regulation of the LDLR expression, we treated the HepG2 cells with the demethylating agent DAC, at 1 and 2.5 μM for 48 h. We demonstrated that basal level expression of the LDLR gene in liver cells depends on epigenetic regulation, as forced hypo-methylation by DAC (at the highest dose administered) significantly increased the LDLR expression, at both mRNA and protein level (Fig. 4A and B). To verify the hypothesis that change in the gene-specific DNA methylation might be responsible for the increased LDLR expression observed following HZN administration, we evaluated the DNA methylation status of the LDLR promoter. We analysed two sequences in the 5′ regulatory region of the human LDLR coding region (Fig. 4C), by bisulphite conversion and pyrosequencing (Supplementary Fig. 3A and B). We demonstrated that both regions display a low degree of methylation in HepG2 cells at basal level (evaluated at day 2 and 4 after seeding cells in plates), the 5-mC percentage being about 5% (fragment #1) and 8% (fragment #2) in control untreated cells (data not shown). Following 24 h of treatment, no difference in promoter DNA methylation of fragment #1 was observed in response to HZN extract if compared to corresponding ethanol solution (Fig. 4D). The pyrosequencing of the 4 cytosines located in fragment #2 revealed instead the ability of the 0.04 mg/ml HZN dose to modify the methylation status of the regulatory regions of the LDLR gene, since the percentage of the 5-mC underwent a significant reduction in the HZN-treated cells compared to the ethanol-treated counterpart (Fig. 4E).

## 4. Discussion

We here demonstrate that treatment of the HepG2 human hepatocellular carcinoma cells with an ethanolic extract of *Corylus avellana* L. *(Tonda Gentile Romana)* stimulates the expression of the LDLR gene, without impairing viability, cell proliferation and cell cycle distribution of liver cells *in vitro*. The LDLR is a master regulator of the lipoprotein turnover, and its modulation has been studied in response to different drugs and nutrients, including pecan nuts, with evidence of positive effects on blood cholesterol levels (Choi, Gwon, Ahn, Jung, & Ha, 2013; Domínguez-Avila et al., 2015; Goldstein & Brown, 2009; Kong et al., 2004). We also provide the first experimental evidence that hazelnuts can affect the epigenetic machinery in human liver cells, as the treatment can (i) affect the expression of genes involved in the epigenetic control such as DNMTs and (ii) decrease DNA methylation of specific regions of the LDLR promoter (at 0.04 mg/ml dose). The latter finding could be, at least in part, responsible for the increase in LDLR expression in hazelnut-treated cells. To the best of our knowledge, these are the first experimental findings investigating the molecular mechanism (s) triggered by the administration of a hazelnut extract to HepG2 cells.

Our hazelnut ethanolic extract was obtained from the kernel of the *Corylus avellana* L., and shows no effect on viability and proliferation of HepG2 cells. Interestingly, Gallego and colleagues (Gallego et al., 2017) demonstrated that both leaf and stem extracts from *Corylus avellana* L. hazel trees (at doses ranging from 1.66 to 3.33 mg/ml) significantly reduced the viability of HepG2 cells after maceration with methanol, suggesting a potential use of these extracts in the pharmacotherapy of cancers. These findings indicate that the functional properties of hazelnuts strongly depend on the portion of the plant selected for studies, as well as on the extraction method.

Under our experimental conditions, the ethanolic extract of *Corylus avellana* L. stimulates the expression of the LDLR gene at transcription level as early as 24 h after administration, allowing a stable over-expression of such protein on cell surface. At 24 h, the main stimulatory effect is observed at 0.04 and 0.4 mg/ml hazelnut concentration (without a dose-dependent effect), whereas almost all doses trigger a strong and significant stimulatory effect following a prolonged 72 h exposition. This variability might be in part explained by the very rich composition of the whole hazelnut extract, whose different components might exert different and/or opposite effects on LDLR, according to their concentration. Furthermore, hazelnut components might affect in different ways, and with different kinetics, other liver pathways that, in turn, might modulate LDLR expression. By a metabolomics approach, we profiled the hazelnut ethanolic extract and identified a complex composition of bioactive molecules, including vitamins, alkaloids and phenylpropanoids (Cappelli et al., 2018). These biomolecules might be in future individually correlated to hazelnuts' beneficial properties as done for other food components (Dwivedi et al., 2016), to better identify the major responsible for LDLR stimulation. Along with LDLR stimulation, it is likely that hazelnut might modulate additional pathways controlling lipid metabolism at liver level. Moreover, future work may be devoted to simulate the digestion process of *Corylus avellana* L. as whole fruit, to better mimic the bio-availability and bioactivity of hazelnut components that are actually delivered to human blood, and eventually to liver.

The epigenetic control of LDLR level has recently emerged, mainly through DNA hypo-methylation of CpG sites and/or modulation of DNMTs expression, as reported in both human adipocytes and mouse livers in response to dietary regimens (Adaikalakoteswari et al., 2015; Alvarez et al., 2015; Cai et al., 2016; Chittur et al., 2008; Fernandes et al., 2017; Hardy & Tollefsbol, 2011; Hu et al., 2016; Khan,

Aumsuwan, Khan, Walker, & Dasmahapatra, 2012; Trenteseaux et al., 2017). We here report that the *Corylus avellana* L. ethanolic extract induces an early but transient change in the DNMT3A expression, that progressively recovers within the 72 h of treatment. Still, a great variability has been reported in DNMT expression according to dose and time of exposure that, as much as LDLR expression, might reflect the very rich composition of the hazelnut extract. Among the mammalian DNMTs, the DNMT3 family is responsible for *de novo* methylation (Goll & Bestor, 2005), and its role varies according to tissue or pathological conditions (Chaudry & Chevassut, 2017; Tajrishi, Shin, Hetman, & Kumar, 2014; You et al., 2017). The stimulation of DNMT3A might appear surprising, as polyphenol-rich foods (including nuts) have been shown to revert aberrant epigenetic patterns by inhibiting DNMTs and histone methyltransferases in cancers (Kanwal, Datt, Liu, & Gupta, 2016; Zwergel, Valente, & Mai, 2016). Our data suggest that treatment with the hazelnut ethanolic extract can affect the epigenetic machinery by tuning the expression of DNMTs, although such effect does not correspond to any change in global DNA methylation. The DNMT3A/(CpG) dinucleotides complex has been recently resolved by crystal structure (Zhang et al., 2018), and it will hopefully provide new input to *in silico* analysis for better understanding the biological significance of DNMT3A activity in response to nutrients.

The lack of global DNA methylation variation does not necessary correspond to the absence of *locus*-specific methylation change. We indeed demonstrate that the LDLR promoter is hypo-methylated in response to hazelnut treatment, which agrees with the observed increase of LDLR transcription and protein level. We pyro-sequenced two specific regions on LDLR promoter and verified that they show a low pattern of methylation in HepG2 cells at basal level (percentage of 5-mC about 5–8%). Adaikalakoteswari et al. (2015) reported a much higher basal methylation percentage in promoter fragment #2, but they pyrosequenced human adipocytes. Low DNA methylation might be consistent with the functions of liver tissue that physiologically needs to sustain LDLR expression. In fact, the high methylation status of CpG islands is associated with silencing of gene expression, whereas unmethylated CpG islands are typical of housekeeping genes (Vinson & Chatterjee, 2012). We demonstrate that *Corylus avellana* L. ethanolic extract is able to reduce the percentage of 5-mC, exclusively at the dose of 0.04 mg/ml, i.e. the concentration that drives the higher LDLR transcription stimulation. This suggests that LDLR increase depends on additional converging molecular mechanism(s) that trigger the LDLR transcription at all the doses and times analyzed. The DAC treatment suggests that LDLR expression relies on DNA methylation pathways; therefore, other regions on LDLR promoter might be putative candidates of DNA hypo-methylation for further analyses. Alternatively, the methylation-dependent effect on LDLR transcription might be indirect, and protein regulators of LDLR transcription, such as SREBPs, might be target of epigenetic activation and consequently drive LDLR stimulation.

The hypo-methylation of LDLR promoter upon hazelnut treatment might be in apparent contrast to the increased DNMT3A expression, the latter enzymes directly devoted to the transfer of methyl groups from the S-adenosylmethionine to cytosine residue in DNA. Different hypotheses can be developed to explain our experimental findings. First, the two processes might be unrelated; DNMT3A stimulation might increase methylation at promoter sites other than LDLR, and be thus responsible for the regulation of cellular processes by *Corylus avellana L* still to be identified. A whole genome methylation analysis in response to hazelnut treatment might help address this issue. Alternatively, there might be a direct or indirect inhibition of DNMT enzyme activity by one or more *Corylus avellana* L. biomolecules, that might in turn lead to a negative feedback regulation at DNMT transcription level to sustain the impaired enzymatic function (Slack, Cervoni, Pinard, & Szyf, 1999). Finally, DNMT3A might drive the hyper-methylation, and consequent gene silencing, of protein repressors specifically devoted to LDLR promoter regulation.

## 5. Conclusions

Overall, our data identify in the LDLR a direct target of the ethanolic extract of *Corylus avellana* L. in HepG2 cells that is affected, at least in part, through a promoter DNA methylation-dependent mechanism.

The involvement of DNA methylation changes by food components in liver cells is definitely novel and put the bases for further studies aimed at clarifying the pathways responsible for the hazelnut-driven health-promoting properties through epigenetic mechanism(s). However, our molecular findings are preliminary and limited to an *in vitro* experimental model that, although maintaining several liver-specific metabolic functions, is a neoplastic cell line. In perspectives, primary hepatocytes might represent a more reliable *in vitro* liver experimental model for extending the molecular characterization of LDLR regulation before moving to *in vivo* systems.

## 6. Ethics statements

We declare that our research did not include any human subjects and animal experiments.

## Conflict of interest statement

The authors confirm that there are no conflicts of interest associated with this publication.

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