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The phenolic compounds tyrosol and hydroxytyrosol counteract liver fibrogenesis *via* the transcriptional modulation of NADPH oxidases and oxidative stress-related miRNAs

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ABSTRACT

Liver fibrosis is the result of a chronic pathological condition caused by the activation of hepatic stellate cells (HSCs), which induces the excessive deposition of extracellular matrix. Fibrogenesis is sustained by an exaggerated production of reactive oxidative species (ROS) by NADPH oxidases (NOXs), which are overactivated in hepatic inflammation. In this study, we investigated the antifibrotic properties of two phenolic compounds of natural origin, tyrosol (Tyr) and hydroxytyrosol (HTyr), known for their antioxidant and anti-inflammatory effects. We assessed Tyr and HTyr antifibrotic and antioxidant activity both *in vitro,* by a co-culture of LX2, HepG2 and THP1-derived Mϕ macrophages, set up to simulate the hepatic microenvironment, and *in vivo,* in a mouse model of liver fibrosis obtained by carbon tetrachloride treatment. We evaluated the mRNA and protein expression of profibrotic and oxidative markers (α-SMA, COL1A1, NOX1/4) by qPCR and/or immunocytochemistry or immunohistochemistry. The expression of selected miRNAs in mouse livers were measured by qPCR. Tyr and HTyr reduces fibrogenesis *in vitro* and *in vivo,* by downregulating all fibrotic markers. Notably, they also modulated oxidative stress by restoring the physiological levels of NOX1 and NOX4. *In vivo*, this effect was accompanied by a transcriptional regulation of inflammatory genes and of 2 miRNAs involved in the control of oxidative stress damage (miR-181-5p and miR-29b-3p). In conclusion, Tyr and HTyr exert antifibrotic and anti-inflammatory effects in preclinical *in vitro* and *in vivo* models of liver fibrosis, by modulating hepatic oxidative stress, representing promising candidates for further development.

1. Introduction

Liver fibrosis is a chronic pathological condition characterized by the excessive deposition of extracellular matrix (ECM), mainly collagen, and increased tissue stiffness. Fibrosis may regress, but this mainly occurs at earlier stages. Advanced stages of fibrosis may progress to cirrhosis and eventually hepatocellular carcinoma [1]. Liver fibrosis is the result of hepatic diseases of different etiology, including non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD), hepatitis virus infection (HBV and HCV), and autoimmune liver diseases. Although many pathological mechanisms have been carefully described and several candidate drugs are currently under clinical investigation, to date no

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anti-fibrotic treatment has been approved for liver fibrosis, and all the available therapies are focused on the management of the disease causing the fibrotic damage. Thus, many efforts have been addressed to gain a deeper comprehension of the molecular mechanisms of fibrogenesis, with the final aim of identifying new drug targets and therapies active against fibrotic damage, irrespective of its origin.

Many studies have demonstrated that an exaggerated production of reactive oxidative species (ROS) promptly promotes hepatic inflammation and fibrogenesis, sustaining the activation of hepatic stellate cells (HSCs), which are the pivotal actors in the production of extracellular matrix and fibrogenesis [2]. Since hepatic NADPH oxidases (NOXs) are the main hepatic enzymes mainly devoted to ROS production, efforts have been directed toward elucidating their role in fibrogenesis [3]. NOXs are heterogeneous membrane-bound enzymes catalyzing the production of ROS from oxygen, that are expressed in several types of hepatic cells, i.e., hepatocytes, Kupffer cells (KCs) and HSCs. An imbalanced activity of the three hepatic isoforms NOX1, NOX2 and NOX4 has been linked to the progression of non-alcoholic steatohepatitis (NASH) to hepatocellular carcinoma (HCC), and, accordingly, it is well known that an exaggerated NOX activation was able to induce HSC activation, hepatocyte apoptosis, and KC-related inflammation responses, three main processes of hepatic fibrogenesis [4,5].

Emerging active players in the regulation of fibrinogenesis are microRNAs (miRNAs), small non-coding RNAs (20–24 nucleotides) able to negatively regulate gene expression by several mechanisms including repression of translation or mRNA cleavage and degradation (by binding to the 3′-untranslated regions of mRNA) [6]. miRNAs contribute to the regulation of a wide variety of biological pathways involve in liver disease and also participate in the control of HSC transdifferentiation [7]. An example of this are miR-29b-3p, which reduces TGF-β1 [8] and has among its validated targets COL1A thus considered an anti-fibrotic miRNA, and miR-181-5p, which, on the opposite, exerts pro-fibrotic effects [9].

In this study, we investigated the effect of the treatment with two phenolic compounds present in extra virgin olive oil (EVOO), tyrosol (Tyr) and hydroxytyrosol (HTyr), on *in vitro* and *in vivo* models of liver fibrosis, focusing on their antioxidant, antifibrotic and anti-inflammatory activities.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Dulbecco's phosphate-buffered saline (DPBS), Streptomycin-penicillin, trypsin-EDTA, L-glutamine, matrigel matrix and fetal bovine serum (FBS) were purchased from Corning (Corning, NY, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Luis, MO, USA). Tyrosol (Tyr) and hydroxytyrosol (HTyr) were purchased from TCI (Belgium).

2.2. Cell culture maintenance and viability assessment

Human LX2 and HepG2 cells were maintained in a complete DMEM medium, with L-glutamine (1 %) and streptomycin/penicillin (1 %) and 3 % or 10 % FBS, respectively. THP-1 monocyte-like cells were maintained in a complete RPMI medium (containing L-glutamine (1 %), added with 1 % streptomycin/penicillin and 10 % FBS. All cell lines were kept at 37 °C with 5 % CO_2 in the humified atmosphere. To assess Tyr and HTyr effect on their viability, LX2 were seeded at 5000 cells/well in a 96-well plate. After 24 h-treatment with the two phenolic compounds (0.2–25 μM, n = 5), cell viability was assessed using MTT assay, as previously described by [10]. Briefly, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide MTT solution (0.5 mg/mL) was added into each well and incubated at 37 °C. After 4 h, the produced crystals of formazan were dissolved in acidified isopropanol and absorbance was measured at 570 nm using a VictorNivo multiplate reader (Perkin Elmer). Cells treated with medium (0.01 % DMSO) were used as a control to calculate the percentage of cell viability.

2.3. In vitro models of HSC activation: TGF-β1 treatment and LX2 cells co-cultured with HepG2 and THP1-derived MΦ

To assess Tyr and HTyr effect on HSC activation, two cellular models were set up. To set up the first model, human hepatic stellate cell line (LX2) cells were seeded in a 6-well plate (15 * 10^4 cell/well) and were treated for 24 h with transforming growth factor-β1 (TGF-β1) (2 ng/mL), a prototypical stimulus inducing HSC activation, for 24 h in presence or absence of 2 μM Tyr and HTyr. Additionally, a second *in vitro* model was set up to simulate the hepatic microenvironment (Fig. 1). LX2 were seeded in a 6-well matrigel-coated plate and co-cultured in a transwell system with human liver cancer cell line (HepG2) cells and MΦ macrophages, obtained by differentiating THP1 cells with phorbol 12-myristate 13-acetate (PMA) 320 nM for 24 h, as previously reported [11].

LX2 activation was evaluated in both models by measuring a prototypical marker, i.e., the increase of alpha-smooth muscle actin (αSMA) protein expression by immunocytochemistry, performed as previously described by using an anti-αSMA primary antibody (Cell Marques, Sigma-Aldrich, Darmstadt, GE) [12]. Images were acquired at 63 × magnification with a T-lapse Zeiss LSM800 microscope and then analyzed with ImageJ software.

To analyze the mRNA expression of NADPH oxidases (NOXs), αSMA and collagen type I alpha 1 chain (COL1A1), total RNA was extracted from cell cultures with TRIzol and Directzol RNA MiniPrep (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. qRT-PCR on extracted RNA was performed using QuantiNova SYBR Green RT-PCR Kit (Qiagen, Germantown, MD, USA) and Eco Illumina Real-Time PCR system (San Diego, CA, USA). The primer sequences used for the analysis of gene expression in human cell lines are reported in Table 1. Relative mRNA quantification was performed according to the ΔΔCt method using GAPDH as housekeeping gene.

2.4. In vivo study on a mouse model of liver fibrosis

Animal studies were performed in compliance with national and European guidelines for the handling and use of laboratory animals (Authorization no. 201/2019-PR) and all necessary measures were taken to minimize their pain and discomfort. To set up the animal model of liver fibrosis, 6-week aged Balb/C male mice were randomly divided into 2 experimental groups, one treated with carbon tetrachloride (CCl_4) *via* intraperitoneal injection (IP) (0.5 mL/kg) 3 times a week, the other (control) treated with vehicle (corn oil) for 4 weeks, as previously described [9]. The daily oral administration of the phenolic compounds Tyr and HTyr (at the dose of 10 mg/Kg) started after the first week of CCl_4-treatment and was performed by intragastric gavage for the following 3 weeks. At sacrifice, liver tissue was collected, rinsed in saline buffer, nitrogen frozen and stored at − 80 °C for further analyses. After collection, a small portion of the hepatic tissue was fixed in 4 % neutral buffered formalin and paraffin-embedded to perform histological analysis. Hematoxylin-eosin and Masson's trichrome stains were used to assess the extent of fibrosis. Liver sections were examined by the same pathologist, blinded to any information about the treatment administered to the animals. The hepatic content of Aspartate aminotransferase (AST) was evaluated by means of a commercially available kit (AST Activity Assay kit, Sigma-Aldrich, St. Luis, Mo, USA), following the manufacturer's instructions.

2.5. Quantification of gene expression in hepatic tissue

Total RNA was extracted from frozen liver tissue as previously reported [13]. One step qRT-PCR was performed with an Eco Real-Time

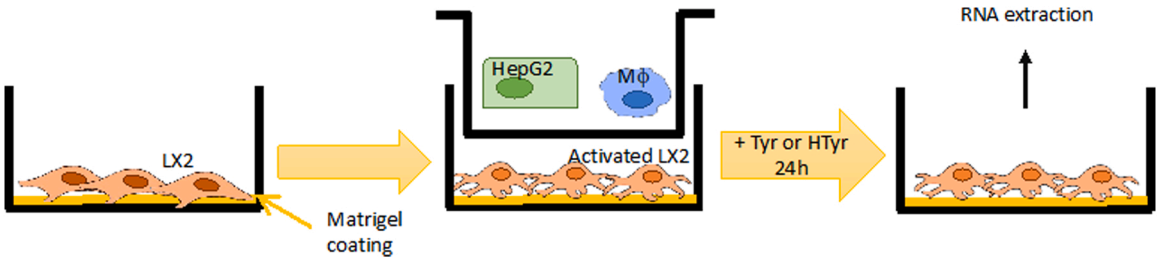

Fig. 1. *In vitro* model of the hepatic microenvironment. Scheme of co-culture of LX2, HepG2 cells and THP1-derived MΦ macrophages (described in the text).

Table 1
Primers for qRT-PCR analysis performed on human cell lines.

Target gene	Forward	Reverse
α-SMA	CCTTTGGCTTGGCTTGTCAG	CGGACAGGAATTGAAGCGGA
COL1A1	ACGTCCTGGTGAAGTTGGT	CAGGGAAGCCTCTCTCTCCT
NOX1	CTGGTTGTTTGGTTAGGGCTG	TTCAAGCAGAGAGCAGACGC
NOX4	AGATGTTGGGGCTAGGATTGTGT	AATCTCCTGGTTCTCCTGCTTG
GAPDH	ACATCAAGAAGGTGGTGAAGCA	GTCAAAGGTGGAGGAGTGGTT

PCR system (Illumina, San Diego, CA, USA) on extracted total mRNA using the specific probe primers reported in Table 2 by means of the One Step TB Green PrimeScript RT-PCR Kit II (Takara, Mountain View, CA, USA). Relative mRNA quantification was performed by using $2^{-\Delta\Delta Ct}$ method, with β-actin as the housekeeping gene [14].

2.6. Quantification of hepatic miRNAs expression

Total miRNAs were extracted from frozen hepatic tissue using miRNeasy Mini Kit (Qiagen, Germany). Extracted miRNAs were reverse transcripted with miScript Reverse Transcription Kit (Qiagen, Germany) and the obtained cDNA diluted in RNase-free water as previously described [9]. qPCR was performed on the MiniOpticon CFX 48 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA), running samples in triplicate using the miScript SYBR-Green PCR kit (Qiagen, Germany). The specific probes MiScript Primer for miRNA quantification were obtained from Qiagen: mmu-miR-181-5p (MIMAT0000210), (MIMAT0000669), mmu-miR-29b-3p (MIMAT0000127), and mmu-RNU6, used as housekeeping. Relative miRNA quantification was performed using Ct method, normalizing miRNA expression to that of RNU6.

2.7. Immunohistochemical analyses of NOXs, aSMA and COL1A1

To analyze the localization and expression of NOX isoforms and the fibrotic markers aSMA and COL1A1, immunohistochemical analyses were performed on 5-μm formaldehyde-fixed paraffin-embedded hepatic tissue sections, as previously described [12]. A heat-induced epitope retrieval was conducted for 20 min in citrate buffer (pH 6) for NOXs and COL1A1, and in Tris-EDTA buffer (pH 7.6) for αSMA. A permeabilization step was performed for 10 min with 0.2 % Triton in phosphate saline buffer with Tween 20 (PBS-T) in humidified atmosphere, followed by a blocking step with 5 % FBS and 1 % bovine albumin serum (BSA) in PBS-T for 30 min at room temperature. Liver tissue sections were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal αSMA antibody (Cell Marque, Sigma-Aldrich, Darmstadt, GE), mouse monoclonal COL1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal NOX4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal NOX1 antibody (GeneTex, Alton Pkwy Irvine, CA). To block the activity of endogenous peroxidase, tissue sections were incubated for 20 min with 3 % H_2O_2. The following secondary antibodies were used (1 h at 37 °C), i.e., goat anti-rabbit HRP-conjugated IgG (KPL, Gaithersburg, MD) and goat anti-mouse HRP-conjugated IgG (Abcam, Cambridge, UK). Liver sections were incubated with 3,3'-diaminobenzidine (DAB) substrate for HRP-conjugated antibodies (Sigma-Aldrich, Milan, Italy) and then counterstained with hematoxylin, dehydrated and mounted with the EUKITT® Quick-hardening mounting medium (Sigma-Aldrich, Darmstadt, GE). Images were acquired at 10X magnification with a Nikon Eclipse Ti-S microscope and then analyzed with ImageJ software.

Table 2
Specific primers for qRT-PCR analysis on liver tissues.

Target	Forward	Reverse
α-SMA	GCTACGAACTGCCTGACGG	GCTGTTATAGGTGGTTTCGTGGA
COL1A1	AGCACGTCTGGTTTGGAGAG	GACATTAGGCGCAGGAAGGT
IL-6	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCCAGAGAAC
IL-17A	ACCCTGATAGATATCCCT	TGAGAACAGAATTCATGT
IL-23	GAGCAACTTCACACCTCCCT	CTGCCACTGCTGACTAGAAC
NOX1	GTTTCTCTCCCGAAGGACCTC	TTCAGCCCCAACCAGGAAA
NOX4	CTGCTCATTTGGCTGTCCCT	CCTAGGCCCAACATTTGGTGA
β-actin	ATGTGGATCAGCAAGCAGGA	AAGGGTGTAAAACGCAGCTCA

2.8. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by the appropriate post-hoc test using GraphPad Prism software ver. 8.0 (San Diego, CA, USA). $p < 0.05$ was considered statistically significant. If not otherwise specified, data are presented as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. In vitro evaluation of Tyr and HTyr antifibrotic activity

Since HSC activation is a key step in the triggering of hepatic fibrogenesis and collagen extracellular matrix deposition, we firstly investigated the effect of Tyr and HTyr on two *in vitro* activated HSC-like models, one obtained by treating LX2 cells with the classical fibrotic stimulus TGF-β1, and the other by coculturing LX2 cells in a transwell system with HepG2 cells and THP1-derived MΦ macrophages to simulate a fibrotic microenvironment. To exclude any cytotoxic effect on LX2 cells at the concentration used for assessing *in vitro* anti-fibrotic potential, we evaluated LX2 cell viability after 24 h-treatment with the two phenolic compounds. No cytotoxic effect was detected in the tested range of concentrations (data not shown).

In TGF-β1-activated LX2 cells, the mRNA expression of α-SMA and COL1A1, two markers of HSC activation, was significantly increased compared to control cells (Fig. 2 A–B). Tyr and HTyr significantly counteracted this upregulation, restoring the basal levels of these two markers of HSC activation. To further confirm the antifibrotic potential of the two phenolic compounds, we assessed their effect on α-SMA activation also at the protein level, clearly confirming that LX2 activation is reduced by both Tyr and HTyr (Fig. 2C–D). Moreover, TGF-β1 treatment induced a significant upregulation also of the two pro-oxidant genes NOX1 and NOX4, which was restored to physiological levels by

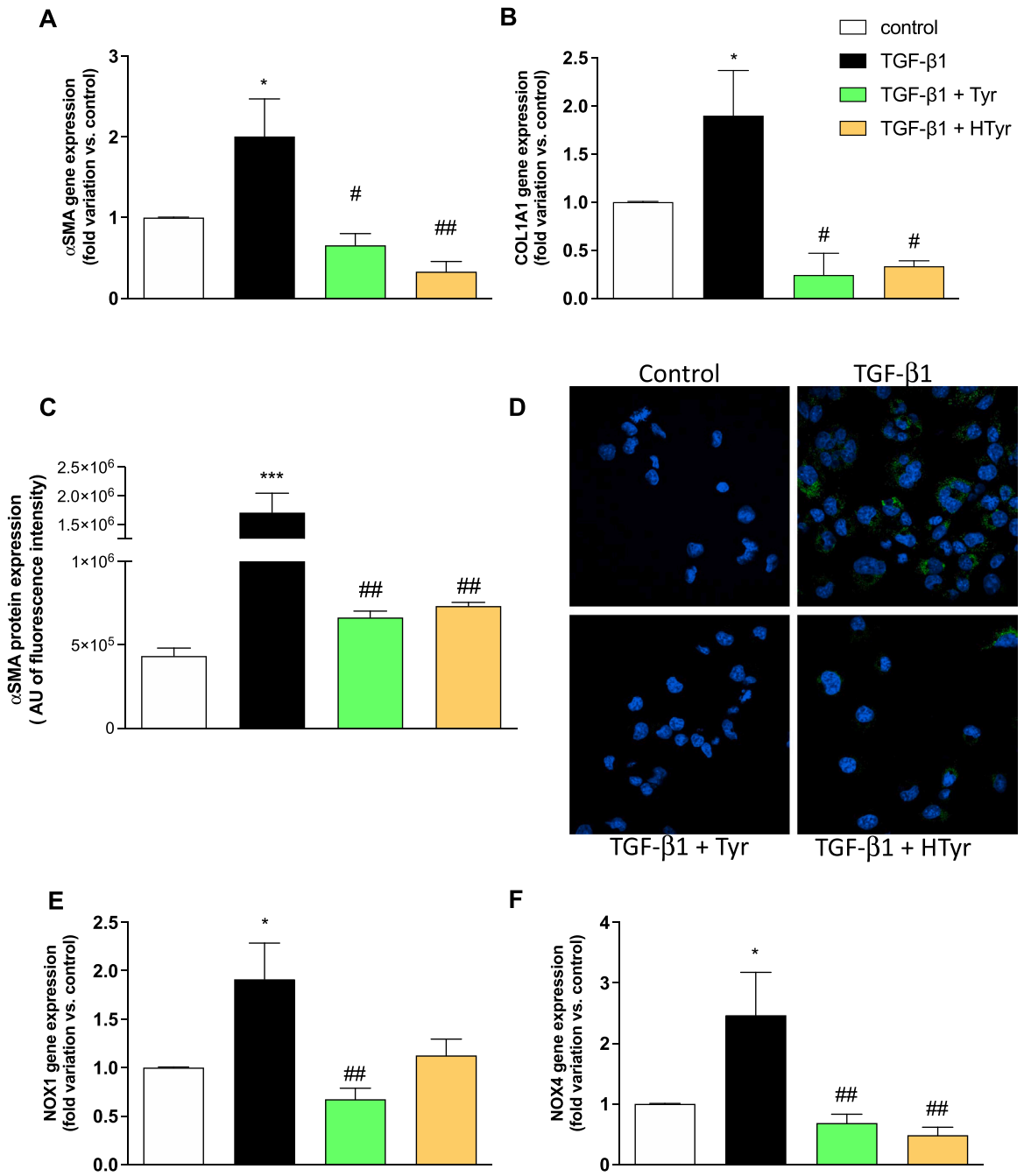

Fig. 2. Effect of Tyr and HTyr on the main markers of HSC activation and NOX enzymes in TGFβ1-activated LX2 cells. The mRNA expression of αSMA and COL1A1 (A, B) and the protein expression of αSMA (C, D) are decreased by the treatment with Tyr and HTyr, as well as the mRNA expression of the NADPH oxidases isoforms NOX1 and NOX4 (E, F). Representative images (63 × magnification) of αSMA-related green fluorescence are shown. Cell nuclei are stained blue with Hoechst. At least 5 fields per coverslip have been analyzed to obtain the quantification of fluorescence. Data are expressed as mean ± SEM of 3 independent experiments. *P < 0.05 *vs.* control, $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 *vs.* TGF-β1-treated cells.

the treatment with the two phenolic compounds (Fig. 2 E–F).

Since ROS-mediated HSC activation could be influenced not only in an autocrine but also in a paracrine manner, by the ROS released by other cells of the hepatic microenvironment, such as macrophages and hepatocytes, we set up a co-culture with LX2, HepG2 cells and THP1-derived MΦ macrophages. This *in vitro* system was proved to be fibrogenic, since a significantly increased gene expression of α-SMA, COL1A1, NOX1 and NOX4 was observed compared to LX2 cultured alone, indicating that this system caused the activation of LX-2 and the concomitant upregulation of NOXs (Fig. 3). This activation was significantly counteracted by both Tyr and HTyr treatments, confirming the antifibrotic activity of the two compounds also in HSC simulated by a profibrotic hepatic microenvironment. Moreover, also in this setting, the anti-fibrotic effect of Tyr and HTyr occurs in concomitance to a reduction of the upregulation of NOX1 and NOX4 observed in activated-LX2.

3.2. Assessment of Tyr and HTyr effect in a murine model of hepatic fibrosis

To confirm the anti-fibrotic effect observed *in vitro*, a murine model of liver fibrosis was set up. After 4 weeks of CCl_4-treatment *via* IP injection, significant deposition of fibrotic tissue was observed, as

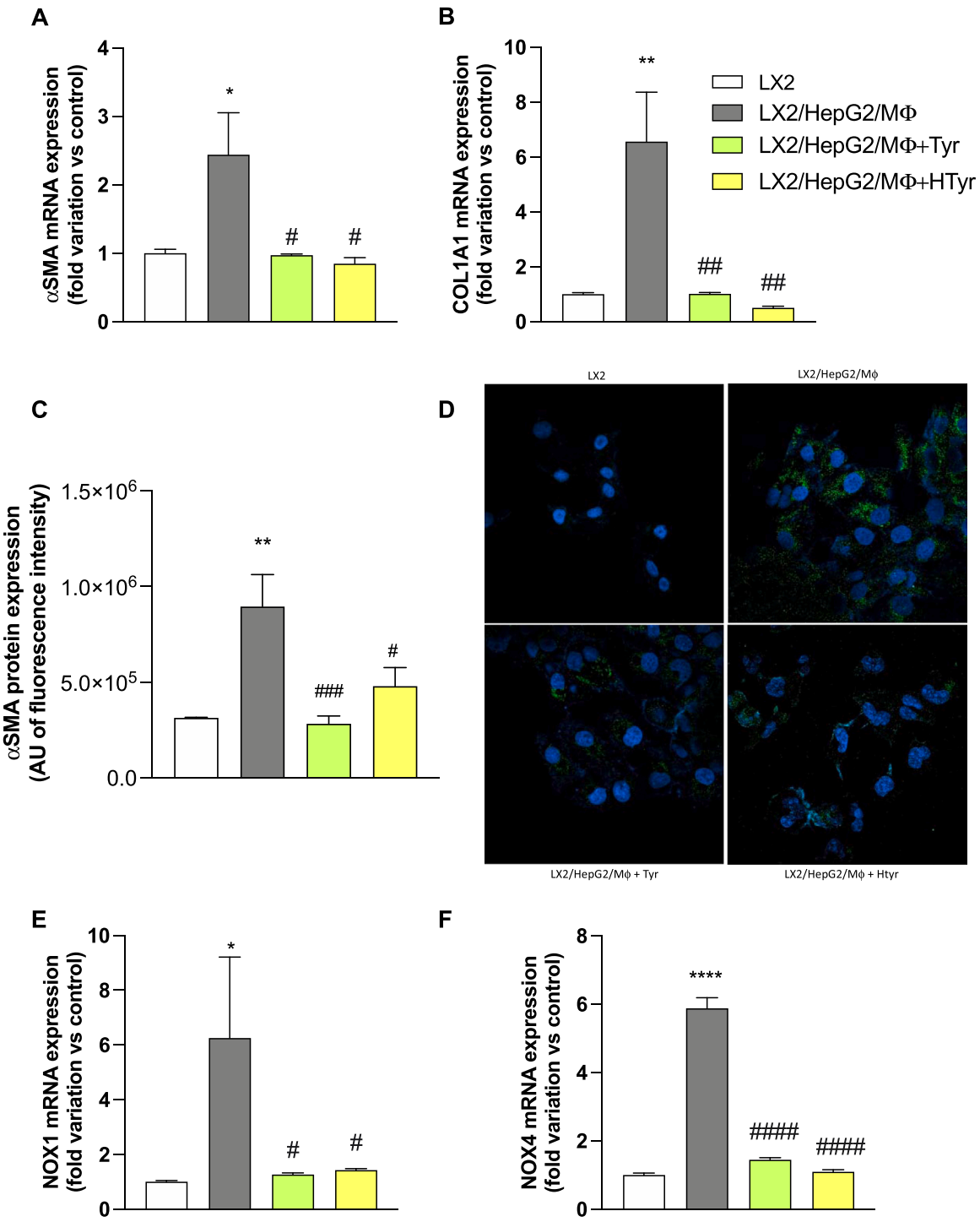

Fig. 3. Effect of Tyr and HTyr on the main markers of HSC activation and NOX enzymes in LX2 cells in co-culture with HepG2 cells and THP1-derived MΦ macrophages. The mRNA expression of αSMA and COL1A1 (A, B) and the protein expression of αSMA (C, D) are decreased by the treatment with Tyr and HTyr, as well as the mRNA expression of NOX1 and NOX4 (E, F). Gene expression was normalized to that of quiescent LX2 cells in matrigel-coated single culture, considered as control. Representative images (63 × magnification) of αSMA-related green fluorescence are shown. Cell nuclei are stained blue with Hoechst. At least 5 fields per coverslip have been analyzed to obtain the quantification of fluorescence. Data are expressed as mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01 and ****P < 0.0001 *vs.* LX2, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001 *vs.* co-cultured HepG2/MΦ LX2 cells.

demonstrated by the histological staining of Masson's Trichrome, staining, showing a high deposition of collagen fibers, with development of cirrhosis, and hepatic damage (Fig. 4). Tyr and HTyr were able to counteract CCl_4-induced hepatic fibrosis improving liver histological damage, since none to only a few thin fibrous septa could be seen in the livers of treated mice.

This effect was also confirmed by qRT-PCR and immunohistochemical analyses on αSMA and COL1A1, as shown in Fig. 5. Gene and protein expression of these two markers of liver fibrosis were significantly increased in fibrotic mice with respect to healthy mice, indicating HSC activation and collagen deposition. The two phenolic compounds were able to restore the basal levels of expression of both αSMA and COL1A1.

Moreover, a significant increase of the hepatic content of aspartate

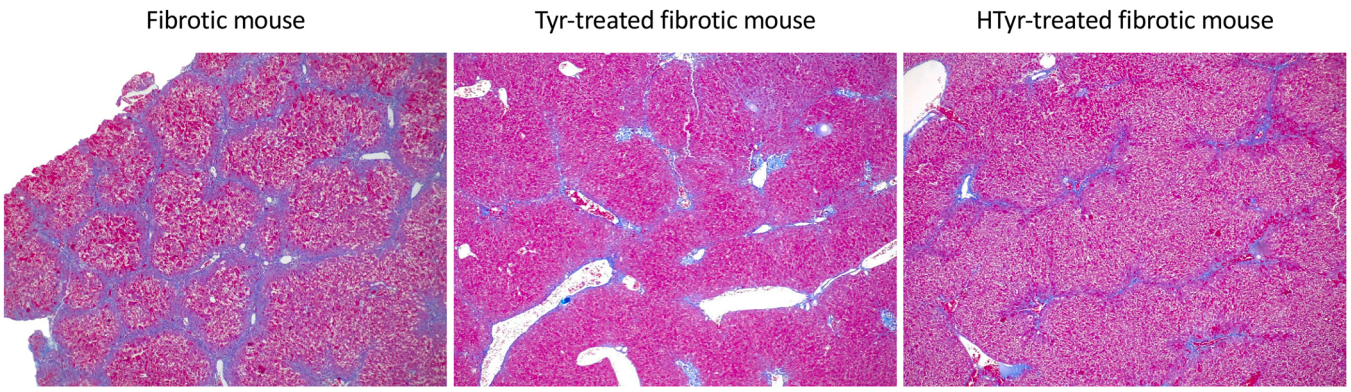

Fig. 4. Histological evaluation of hepatic tissue in CCl_4-treated mice, with and without Tyr/Htyr treatment. As reported in the text, CCl_4-treated mice showed liver cirrhosis, while none or only a few thin fibrous septa can be seen in livers of Tyr/Htyr-treated mice (Masson's trichrome stain; original magnification 5 ×).

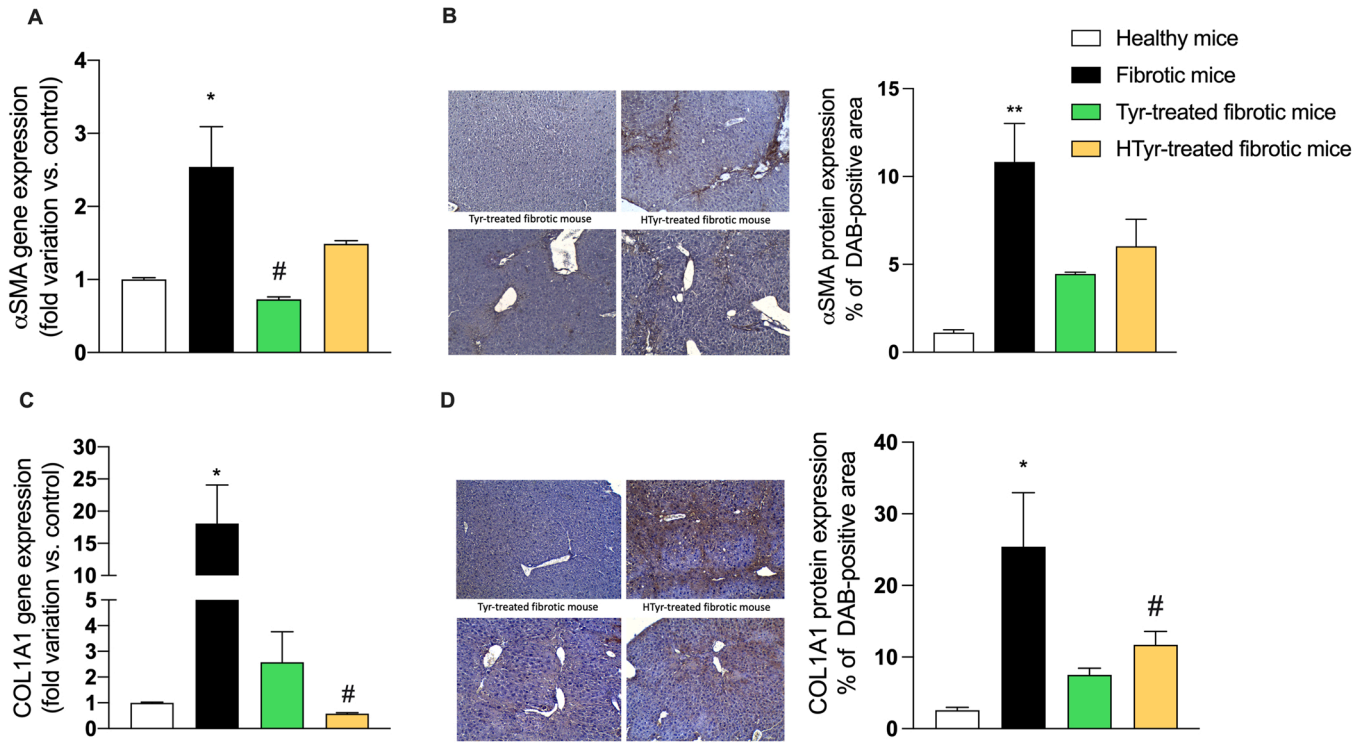

Fig. 5. Effect of Tyr and HTyr on hepatic gene expression of fibrotic markers, αSMA (A) and COL1A1 (C) in fibrotic mice. Representative microphotographs of hepatic tissues reported protein expression as DAB-positive brown area and protein expression quantification of the two fibrotic markers αSMA (B) and COL1A1 (D). Original magnification 10 ×. Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05 and **P < 0.01 *vs.* Healthy mice, ♯P < 0.05 *vs.* Fibrotic mice.

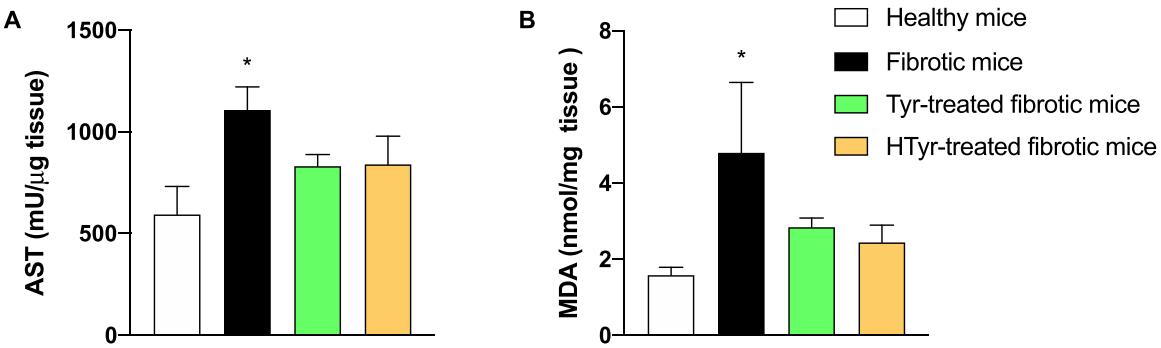

Fig. 6. Effect of Tyr and HTyr on AST hepatic content (A) and lipid peroxidation measured as MDA content in hepatic tissue (B). Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05 *vs.* healthy mice.

aminotransferase (AST), a validated biomarker of liver damage (Fig. 6A), was observed in fibrotic animals, whereas mice treated with Tyr and Htyr had AST levels similar to those of healthy controls. Furthermore, also lipid peroxidation, measured as malondialdehyde (MDA) hepatic content, was increased in fibrotic mice compared to healthy controls. Tyr and HTyr treatments return the MDA hepatic levels to baseline, confirming the antioxidant effect of these two compounds (Fig. 6B).

During fibrogenesis, a panel of hepatic interleukins (IL), among which IL-6, IL-17A and IL-23, play a pivotal role, promoting an inflammatory environment that sustains fibrosis development and progression. In our mouse model of fibrosis, a significant increase in the mRNA expression of these three ILs was observed (Fig. 7). The physiological levels of IL-6 mRNA expression were restored by Tyr and HTyr treatments. Moreover, both phenolic compounds were able to significantly downregulate the expression of IL-17A and IL-23 genes, demonstrating they are useful in attenuating the pro-inflammatory hepatic environment typical of fibrogenesis onset and progression.

Since the involvement of NOXs has been demonstrated in fibrotic processes occurring in the liver and other organs, we investigated the effect of Tyr and HTyr on the mRNA and protein expression of NOX1 and NOX4. Both qRT-PCR and immunohistochemical analyses confirmed the increase of NOX expression in the hepatic tissues of fibrotic mice with respect to healthy ones (Fig. 8). Notably, both phenolic compounds were able to counteract this fibrosis-induced NOX overexpression.

Since the ability of HTyr to modulate miRNA expression has been already demonstrated [15], and miRNAs are involved in HSC activation and fibrogenesis [7], we measured the expression of two miRNAs involved in this pathological condition. As shown in Fig. 9, the profibrotic miR-181-5p was significantly upregulated in fibrotic mice with respect to controls. The treatment with Tyr and HTyr counteracted this effect, restoring the physiological expression of this miRNA. Conversely, the expression of the antifibrotic miR-29b-3p was significantly lower in fibrotic mice compared to healthy animals, and both Tyr and HTyr increased its level.

4. Discussion

Liver fibrosis remains a global health issue due to its multifaceted pathological mechanisms and etiologies that involve multiple dysregulated pathways [1,2]. HSCs are key cells in triggering fibrogenesis. Upon proinflammatory stimuli, they switch to an activated phenotype, characterized by a loss of lipid droplets storing vitamin A and a high proliferative and migratory potential. In this activated phase, they transdifferentiate to myofibroblasts and start the production of collagen-rich fibrotic extracellular matrix, two key steps involved in fibrosis progression. These HSC-derived myofibroblasts, characterized by high expression of mesenchymal markers, *e.g.* αSMA or COL1A1, are recruited to the site of inflammation and produce a huge and constant amount of ECM that disrupts hepatic and vascular architecture. If prolongated, this may evolve into threatening conditions, such as cirrhosis and hepatocarcinoma [16].

Since the liver is exposed to many xenobiotics and intestinal antigens, the hepatic microenvironment is characterized by a balance between tolerance and inflammation, fundamental for the maintenance of its physiology [17]. These intestinal antigens are generally neutralized by resident macrophages and hepatocytes upon their recognition by pattern recognition receptors (PRRs) located on the cell surface, which avoid the massive release of pro-inflammatory mediators and favor a tolerogenic status and tissue regeneration [18,19]. After liver injury, the hepatic immunotolerance is disrupted, and the release of massive proinflammatory cytokines leads to inflammation, hepatocyte apoptosis, recruitment of circulating macrophages and the activation of HSC. In this context, the increase of hepatic oxidative stress has been described as a further promoting factor in fibrotic damage progression [5]. NADPH oxidases are the main hepatic enzymes devoted to ROS production. Their upregulation has been observed in many fibrotic diseases, thus suggesting that this family of enzymes could be effectively targeted to improve fibrosis and prevent its progression to more severe stages of fibrosis, culminating in cirrhosis [4].

Many compounds of natural origin have been investigated for their ability to exert beneficial effects on human health, thanks to their antioxidant activity [9]. In this study, we investigated two phenolic compounds, i.e., Tyr and its hydroxylated derivative HTyr, that have already been proved to reduce oxidative stress by modulation of multiple cellular pathways, and regulate some miRNAs involved in chronic diseases [15,20,21]. In the light of these considerations, we investigated whether these compounds could exert an antifibrotic effect thanks to the above-described mechanisms in both *in vitro* and *in vivo* models of liver fibrosis.

Since HSC activation plays a key role in fibrogenesis, we study the effect of Tyr and HTyr treatment on two *in vitro* models, i.e., a) HSC-like LX2 cells activated by TGF-β1, a prototypical fibrotic stimulus; b) a more complex system in which LX2 cells have been co-cultured with HepG2 and THP-1-derived Mθ macrophages, simulating a profibrotic microenvironment. In both cellular models, Tyr and HTyr demonstrated an antifibrotic activity, by significantly downregulating the gene and protein expression of αSMA and COL1A1, well-recognized markers of HSC activation. Moreover, both phenolic compounds effectively downregulate the two NOX isoforms NOX 1 and NOX4, restoring their physiological levels, thus suggesting the antifibrotic effect exerted by Tyr and HTyr is accompanied by the modulation of oxidative stress and ROS production.

The antifibrotic potential of Tyr and HTyr was also evaluated *in vivo* using a widely used mouse model of liver fibrosis, obtained by injecting CCL_4 intraperitoneally for 4 weeks. The two compounds (at the dose of 10 mg/kg) were administered daily by oral gavage starting after one week of CCL_4-treatment. The histological analyses confirmed the antifibrotic effect of Tyr and HTyr observed *in vitro*. Furthermore, since an increased expression of αSMA and COL1A1 is observed in the liver, due

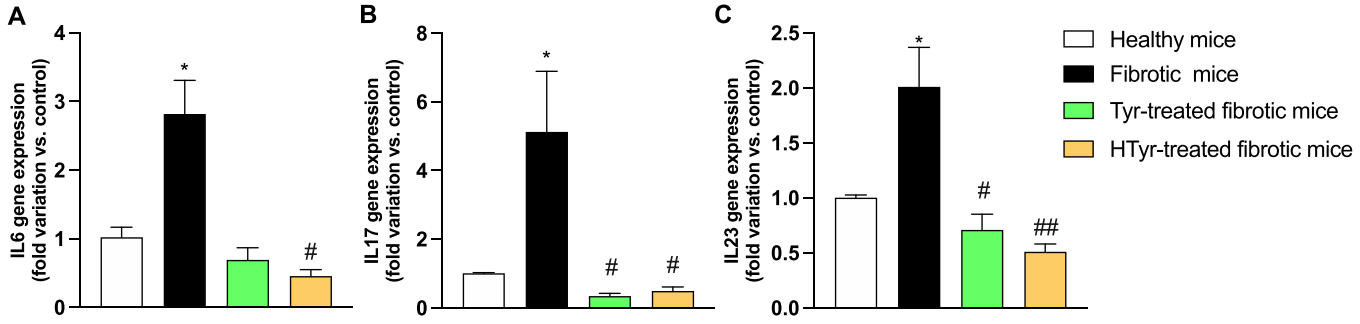

Fig. 7. Effect of Tyr and HTyr on gene expression of the hepatic interleukins IL-6, IL-17A and IL-23, in the fibrotic model. Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05 *vs.* Healthy mice, $^{\sharp}$P < 0.05, $^{\sharp\sharp}$ P < 0.01 *vs.* fibrotic mice.

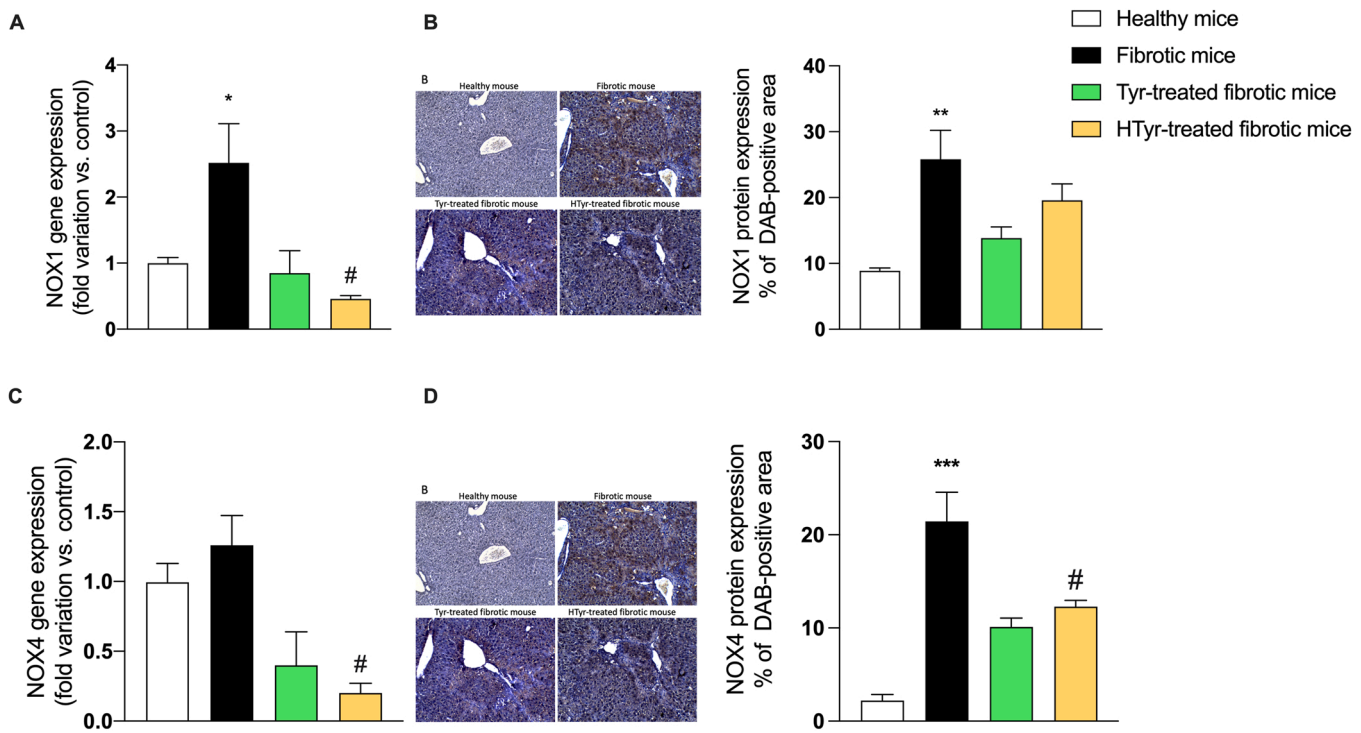

Fig. 8. Effect of Tyr and HTyr on hepatic gene expression of NADPH oxidase isoforms, NOX1 (A) and NOX4 (C), in the fibrotic murine model. Representative microphotographs of hepatic tissues and quantification reporting protein expression of the two isoforms NOX1 (B) and NOX4 (D) reported as DAB-positive brown area. Original magnification 20 ×. Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 *vs.* Healthy mice, ♯P < 0.05 vs. Fibrotic mice.

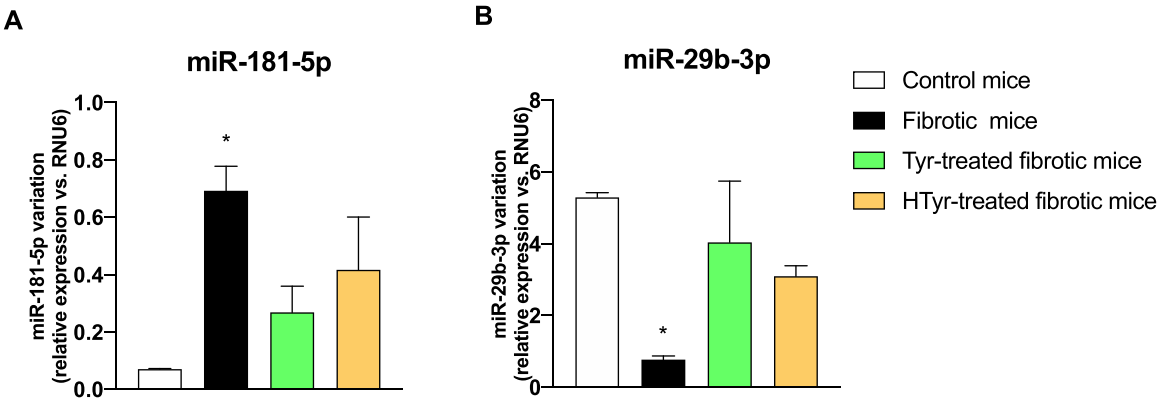

Fig. 9. Effect of Tyr and HTyr on miR-181-5p and miR-29b-3p expression in hepatic tissue of fibrotic mice. Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05 *vs.* Healthy mice.

to the activation of HSCs, we performed a qRT-PCR and IHC analyses of liver tissues, that revealed that both Tyr and HTyr were able to counteract the HSC activation and collagen deposition observed in fibrotic animals, further confirming their anti-fibrotic effect.

As stated above, increased oxidative stress and inflammation are related to fibrosis progression. In particular, the pro-inflammatory hepatic environment that sustains fibrogenesis is favored by the release of some interleukins, among which IL-6, IL-17A and IL-23 [22]. These ILs are released in the liver by different immune cells, such as resident Kupffer cells and T helper 17 cells (Th17), a subset of pro-inflammatory T helper cells producing IL17. The existence of a Th17/IL-17A axis plays an active role in the pathogenesis of many chronic liver diseases characterized by the development of fibrosis, such as NASH [23,24], obstructive cholestasis [25,26], primary biliary cholangitis [27–29], primary sclerosing cholangitis [30], drug-induced liver injury [31,32], and viral hepatitis [33–35], and also in their worsening towards hepatocarcinoma (HCC) [36–38]. Moreover, IL-6 and IL-23, which are usually released after the onset of hepatic inflammation, further promote the Th17 differentiation and the subsequent production of IL-17A in peripheral blood [39–42]. It has been demonstrated that the Th17/IL-17A axis can induce HSC activation in the liver, increase the expression of TGF-β, metalloproteinases, and collagen and promote the recruitment of inflammatory cells [22,43,44]. In our model of fibrosis, a significant upregulation of the expression of these three ILs was observed, which was counteracted by Tyr and HTyr, that restored their physiological mRNA expression. Thus, we can hypothesize that they display their antifibrotic effect on HSC activation by modulating the Th17/IL17A axis *via* the reduction of IL6, IL17 and IL23 expression. The positive effect on inflammatory and oxidative liver status induced by the phenolic compounds with tyrosol-like structure was also confirmed by

measuring oxidative stress in hepatic tissue of fibrotic animals. CCL_4-induced fibrosis increased oxidative stress in hepatic tissue, as demonstrated by the malondialdehyde (MDA) content [45]. Therefore, we concluded that Tyr and HTyr reduced hepatic oxidative stress, by decreasing MDA content in the liver compared to untreated fibrotic animals. Since NOXs are enzymes mainly devoted to ROS production and their upregulation was observed in many fibrotic and inflammatory processes, we investigated the effect of Tyr and HTyr on NOX expression in our fibrotic model. Previous studies have demonstrated that NOX1 could induce HSC activation in consequence of angiotensin II stimulation and, consistently, that NOX1 deficiency decreased liver inflammation and fibrosis in two models obtained by CCL_4 administration and bile duct ligation (BDL) [46,47]. At variance with NOX1, NOX4 is a constitutively activated isoform that does not require costimulatory subunits to produce ROS and usually acts *via* TGF-β1 induction [48]. This isoform has been demonstrated to be crucial for myofibroblast activation and transdifferentiation in lung and kidney fibrosis [48,49]. Moreover, this isoform results to be overexpressed in HCV hepatitis, and its role in fibrosis has been demonstrated a NOX4-deficient bile duct-ligated mouse model [50]. In our model of hepatic fibrosis, we observed a significant increase of both NOX1 and NOX 4 compared to healthy animals. Tyr and HTyr restored physiological levels of both NOX isoforms. This observation further confirms that the antifibrotic and antioxidant effects exerted by the two phenolic compounds are linked to a transcriptional modulation of hepatic NOXs. Interestingly, some studies have demonstrated a link between oxidative stress and miRNAs [7]. For example, Luna et al. [51] induced chronic oxidative stress in human trabecular meshwork cells [51], and consequently observed a drop in miR-29b expression. Therefore, we evaluated the expression of this miRNA in our experimental *in vivo* model, also considering that these authors also demonstrated that miR-29b negatively regulates the expression of multiple genes involved in the synthesis and deposition of ECM in these cells [51] and some studies have already demonstrated that Tyr and HTyr can modulate the expression of some miRNAs [15,21]. We demonstrated that, in our model, miR-29b-3p, which is involved in the inhibition of HSC activation [52], was decreased in fibrotic mice with respect to controls. Both Tyr and HTyr were able to increase miR-29b-3p expression to physiological levels. Recently, another miRNA, i.e., miR-181-5p, has been related to cardiac oxidative stress [53], and interestingly also to liver fibrosis and inflammation [54]. In our *in vivo* model, we observed that miR-181-5p was significantly upregulated in fibrotic mice [9], and Tyr and HTyr restored its physiological levels. Notably, the role of some miRNA in multiple signaling cascades involved in fibrogenesis has been demonstrated [55], including the MAPK, Wnt, PI3K/AKT, Hedgehog and NF-kB, which are involved in the progression of hepatic fibrosis [55]. Accordingly, the therapeutic use of miRNAs as RNA interference agents to modulate gene transcription has raised considerable interest in the research on anti-fibrotic agents [56]. Our observations, besides strengthening the postulated relationship between oxidative stress, inflammation, and miRNA in fibrogenesis, indicate that compounds of natural origin like Tyr and HTyr can target these complex mechanisms to exert their beneficial activity. Notably, our results are in line with recent observations obtained in both preclinical and clinical settings, indicating that the association of HTyr and vitamin E was able to reduce fibrosis in animal models and to modulate the expression of pro-oxidant genes and inflammatory mediators in children with biopsy-proven NAFLD by targeting TGFβ-dependent signaling pathways [57,58]. Thus, we shed new light on the role of Tyr and HTyr in counteracting fibrogenesis, by acting against two hallmarks of fibrosis, i.e., inflammation and oxidative stress. However, to obtain a complete picture of Tyr and HTyr pharmacodynamics, mechanistic studies are needed to describe in detail the pharmacological mechanisms responsible for their beneficial effects.

In conclusion, we demonstrated that the two phenolic compounds Tyr and HTyr exert NOX-related antifibrotic and antioxidant effects in preclinical models of liver fibrosis, *via* the modulation of ILs and miRNAs, known to regulate HSC activation. Therefore, Tyr and Htyr deserve interest for further development as antifibrotic agents.

CRediT authorship contribution statement

Conceptualization, DG and SDM; Methodology, MG, SC, MD, MM, PN and FPR; Investigation, DG, SC, SS, KS, IZ, BP; Data curation, DG, SDM; Writing – original draft, DG, SC, IZ; Writing – review & editing, DG, SDM, MC; Supervision, SDM; Project administration, DG, SDM; Funding acquisition, SDM. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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