

Original Paper

Genistein and 17 β -Estradiol Protect Hepatocytes from Fatty Degeneration by Mechanisms Involving Mitochondria, Inflammasome and Kinases Activation

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Key Words

Cell viability • Mitochondria • NAFLD • Phytoestrogens • Triglycerides

Abstract

Background/Aims: Oxidative stress and mitochondria dysfunction could be involved in the onset of non-alcoholic fatty liver disease (NAFLD) and in its progression to non-alcoholic steatohepatitis (NASH). Estrogens/phytoestrogens could counteract liver fat deposition with beneficial effects against NAFLD by unclear mechanisms. We aimed to analyze the protective effects elicited by genistein/estradiol in hepatocytes cultured in NAFLD-like medium on cell viability, triglycerides accumulation, mitochondrial function and oxidative stress and the role of NLRP3 inflammasome, toll like receptors 4 (TLR4), Akt and 5' AMP-activated protein kinase (AMPK) α 1/2. **Methods:** Human primary hepatocytes/hepatoma cell line (Huh7.5 cells) were incubated with a 2 mM mixture of oleate/palmitate in presence/absence of genistein/17 β -estradiol. In some experiments, Huh7.5 cells were exposed to various inhibitors of the above pathways and estrogenic receptors (ERs) and G protein-coupled estrogen receptor (GPER) blockers, before genistein/17 β -estradiol. Cell viability, mitochondrial membrane potential, reactive oxygen species and triglycerides content were examined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA)

and the Triglyceride Colorimetric Assay. The expression/activation of kinases was analyzed by means of Western blot. **Results:** Genistein/17 β -estradiol protected hepatocytes against NAFLD-like medium, by preventing the loss of cell viability and mitochondrial function, triglycerides accumulation and peroxidation. The blocking of kinases, ERs and GPER was able to reduce the above effects, which were potentiated by NLRP3 inflammasome. **Conclusion:** Our findings suggest novel mechanisms underlying the protective effects elicited by phytoestrogens/estrogens against NAFLD/NASH and open novel therapeutic perspectives in the management of NAFLD in postmenopausal women.

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Introduction

Mechanisms related to oxidative stress could be involved in lipid accumulation at the onset of non-alcoholic fatty liver disease (NAFLD) and along its transition towards non-alcoholic steatohepatitis (NASH) [1]. Mitochondria dysfunction has been widely reported to play a significant role in the onset of NAFLD and in its progression, as well [2]. Conceivably, intracellular free fatty acid (FFAs) accumulation could interfere with mitochondria respiratory chain, causing proton leakage, reducing mitochondrial membrane potential and increasing free oxygen radical generation (ROS). In turn, ROS release would potentiate all the above processes [3, 4]. Factors like phosphoinositide 3-kinases (PI3K)/Akt, sterol regulatory element binding protein 1c (SREBP-1C), peroxisome proliferator activating ligand receptors (PPARs) and AMP-activated protein kinase (AMPK) are deeply involved in the control of lipogenesis/beta oxidation [5-7]. Hence, in experimental NAFLD models, the inhibition of Akt has been related to the onset of insulin resistance, whereas the increased expression of both SREBP-1c and PPAR γ seems to contribute to the accumulation of FFAs through the inhibition of beta oxidation [5-8]. Those events could be modulated by the constitutively active AMPK [9].

Furthermore, the triggering of hepatic inflammation would be caused by the activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NLRP3 inflammasome [10]. Consistent with this hypothesis, the stimulation of TLR4 was reported to induce the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-kB), mitogen-activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK), which would eventually trigger the production of proinflammatory cytokines and the activation of hepatic stellate cells (HSC) [11, 12].

It is to note that estrogens have been proposed to counteract fat deposition in liver with beneficial effects against NAFLD [13].

These data might explain, at least in part, the lower incidence of NAFLD in premenopausal women in comparison to men [14] and the reported accelerated progression of NAFLD after the menopause [15]. For those reasons, estrogen supplementation has been suggested as potential treatment for the prevention of NAFLD development and progression in postmenopausal women [14, 16].

Interestingly, genistein, the soybean-derived isoflavone, was shown to ameliorate high-fat-diet-induced NAFLD in mice, and to counteract its progression towards NASH [17, 18]. Taking into account the adverse hepatic effects described for the use of estrogens as hormone replacement therapy (HRT), phytoestrogens may well represent an alternative for the treatment of NAFLD after menopause [15].

Although the involvement of the above reported pathways has been hypothesized, the exact mechanisms implied in the protective effects elicited by estrogens and phytoestrogens remain mostly poor understood.

For this reason, in this study we examined the effects of 17 β -estradiol and genistein against fatty changes in Huh7.5 cells and the related involvement of TLR4, NLRP3 inflammasome, Nf-kB, AMPK, and Akt. The role of estrogenic receptors (ERs) and G protein-coupled estrogen receptor (GPER) was analyzed, as well.

Materials and Methods

Culture of Huh7.5 cells

Huh7.5 cells (male immortalized human hepatoma cell line) were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA), and were maintained in Dulbecco's modified Eagle's medium-F-12 (DMEM/HAM'S F-12; Euroclone, Pero, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Euroclone), 2 mM L-glutamine (Euroclone), 1% penicillin-streptomycin (P/S; Euroclone), at 37°C with 5% CO₂ in incubator.

Isolation of hepatocytes from human liver biopsy specimens

The specimens were acquired from the non-neoplastic portion of the resected liver parenchyma at the of the surgery. The Ethics Committee of the University Hospital Maggiore of Charity of Novara approved the use of biptic samples, remaining after the routine histologic analysis which is executed for the clinical management of patients, for experimental purposes. As required by Ethics Committee of the University Hospital Maggiore of Charity of Novara, all tissue donors gave informed consent for experimental use of clinical data and liver specimen prior to surgery, and biptic samples were handled in anonymous conditions. Then fresh samples were immediately transferred in ice cold physiologic saline solution to Physiology laboratory, and hepatocytes were isolated immediately, as previously performed [19]. The cell suspension was filtered through a nylon mesh, washed three times in Hanks buffer and then, cells were resuspended in complete growth medium DMEM/HAM'S F-12 (Euroclone) supplemented with 10% FBS (Euroclone) and transferred into 75-cm² culture flasks (Euroclone) in incubator under standard conditions. The culturing was performed until passage 15 [19].

Experimental protocol

To reproduce a condition of human NAFLD *in vitro*, Huh7.5 cells and primary human hepatocytes were treated with palmitic acid (Sigma-Aldrich, Milan, Italy) and oleic acid (Sigma), as previously reported [20]. In preliminary experiments, cells were incubated in serum-free DMEM/HAM'S F-12 (Euroclone) and exposed for 3 h, 24 h and 48 h to increasing concentrations of a fresh mixture of FFAs (0.75 mM, 1.5 mM and 2 mM) in a molar ratio of 2:1 oleic:palmitic acid. This mixture was supplemented with 0.25% bovine serum albumin (BSA; Sigma) in order to increase stability and solubility of FFAs [20-22]. At the end of preliminary experiments performed on cell viability, the concentration of 2 mM of FFAs administered for 3 h of stimulation, was chosen for all subsequent experiments.

Cell viability

Cell viability was examined in Huh7.5 cells and primary human hepatocytes by using the 1% 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT; Life Technologies Italia, Monza, Italy) dye, as previously described [23-26]. For the experiments, 10000 cells/well were plated in 96-well plates.

Huh7.5 cells were stimulated with different concentrations of genistein (10 pM, 10 nM, 100 nM, 1 μM and 10 μM; Sigma) and 17β-estradiol (10 pM, 10 nM and 100 nM, for 30 min) for 30 min. Huh7.5 cells were also stimulated with specific inhibitors administrated for 30 min before the treatment with genistein and 17β-estradiol: the PI3K inhibitor, wortmannin (1 nM; Sigma), the NLRP3 inflammasome inhibitor, MCC950 (1 nM; Aurogene, Rome, Italy), the AMPK inhibitor, dorsomorphin (1 nM; Sigma), the ERs inhibitor, fulvestrant (1 nM; Sigma), the GPER inhibitor, G15 (1 nM; Sigma) and the TLR4 inhibitor, TLR4-IN-C34 (1 nM; Sigma). After the exposure to these agents, the medium was changed and new fresh medium with 2 mM FFAs was added for 3 h [27]. Furthermore, a second pool of cells was also stimulated with tumor necrosis factor α (TNFα) 200 pM administrated for 3 h in co-stimulation with 2 mM FFAs, either alone or before genistein (10 pM and 100 nM) and 17β-estradiol (10 pM and 100 nM). The experimental protocol is shown in Flowchart (Fig. 1).

The same experimental protocol was followed for primary human hepatocytes, that were, however, stimulated with two concentrations of genistein (10 pM and 1 μM for 30 min) and 17β-estradiol (10 pM and 100 nM for 30 min) only, without inhibitors.

At the end of each treatment, the medium was removed, and fresh culture medium without red phenol and FBS and with MTT dye was added in 96-well plates containing the cells and incubated for 2 h at

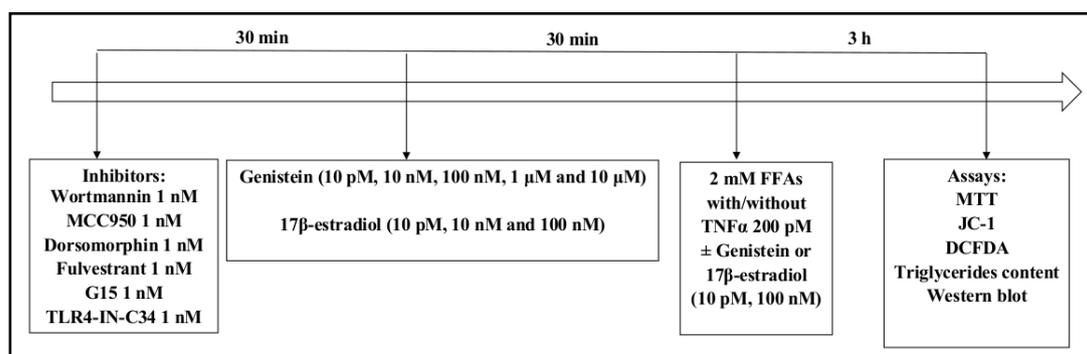


Fig. 1. Flowchart of experimental protocol. Huh7.5 cells and human primary hepatocytes were treated with 2 mM free fatty acids (FFAs) for 3 h, in presence or absence of 200 pM TNF α administered for 3 h in co-stimulation with 2 mM FFAs. In some experiments, genistein and 17 β -estradiol were given for 30 min before FFAs administration, in presence or absence of wortmannin, MCC950, dorsomorphin, fulvestrant, G15 and TLR4-IN-C34, which were administered for 30 min before genistein and 17 β -estradiol. After the exposure to the above agents, specific assays were performed. MTT: 1% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; JC: 5,51,6,61-tetrachloro-1,11,3,31 tetraethylbenzimidazolyl carbocyanine iodide; DCFDA: 2,7-dichlorodihydrofluorescein diacetate; wortmannin: PI3K inhibitor; MCC950: NLRP3 inflammasome inhibitor; dorsomorphin: AMPK inhibitor; fulvestrant: estrogenic receptors inhibitor; G15: G protein-coupled estrogen receptor inhibitor; TLR4-IN-C34: toll like receptor 4 inhibitor.

37°C in an incubator. Thereafter, the medium was removed, and an MTT solubilization solution (dimethyl sulfoxide; DMSO; Sigma) in equal volume to the original culture medium was added and mixed in a gyratory shaker until the complete dissolution of formazan crystals. Cell viability was determined by measuring the absorbance through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) at a wavelength of 570 nm and cell viability was calculated by setting control cells as 100%. Experiments were conducted in triplicate and repeated at least five times.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential measurement in Huh7.5 cells and human primary hepatocytes was performed with JC-1 assay following the same experimental protocol described for MTT assay. After stimulations, the medium of cells plated in starvation medium was removed and incubated with 5, 51, 6,61-tetrachloro-1, 11, 3,31 tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Cayman Chemical; Ann Arbor, Michigan, USA) 1X diluted in Assay Buffer 1X for 15 min at 37°C in an incubator, following the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI, USA; catalogue number 10009172) and as previously performed [23-26]. After incubation, the cells were washed twice with Assay Buffer 1X and then the mitochondrial membrane potential was determined by measuring the red (excitation 550 nm/emission 600 nm) and green (excitation 485 nm/emission 535 nm) fluorescence through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). To identify cells undergoing apoptosis, the ratio of fluorescent intensity of J-aggregates to fluorescent intensity of monomers was used as an indicator of cell health. The data were normalized versus control cells. Experiments were conducted in triplicate and repeated at least five times.

ROS quantification

The oxidation of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) into 2,7-dichlorodihydrofluorescein (DCF) was used to assess ROS generation, following the manufacturer's instructions (Abcam, Cambridge, United Kingdom). For ROS production, 25000 Huh7.5 cells or primary human hepatocytes per well were plated in 96-well plates, and the same experimental protocol used for MTT and JC-1 assays was followed, although using only two concentrations of genistein (10 pM and 1 μM, for 30 min) and 17 β -estradiol (10 pM and 100 nM, for 30 min), without TNF α .

After treatments, the reactions were stopped by removing the medium and washing with phosphate buffer saline (PBS) followed by staining with 10 μM H2DCFDA for 20 min at 37°C. The fluorescence intensity of DCF was measured at an excitation and emission wavelength of 485 nm and 530 nm by using a

spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) [24-26]. The amount of intracellular ROS was proportional to the intensity of DCF fluorescence, and the fluorescence intensity was recorded directly to indicate the relative amount of ROS. Results were expressed as DCF fluorescence intensity, which is proportional to the amount of intracellular ROS. Experiments were conducted in triplicate and repeated at least five times.

Triglycerides content

For intracellular quantification of triglycerides in Huh7.5 cells, the Triglyceride Colorimetric Assay was used according to the manufacturer's instructions (Cayman Chemical), using 1800000 cells/well. The same experimental protocol described for ROS production was adopted. Briefly, after treatments, cells were lysed in cold Standard Diluent, by using a rubber policeman (not using proteolytic enzymes) and then sonicated. The cell pellet was centrifuged at 10 000 g for 10 min at 4°C and the supernatant was collected and diluted 1:2 before the assay.

Ten µl of each dilute sample was transferred to a 96-well plate and 150 µl of Enzyme Mixture solution was added to each well in order to start the reaction. The plate was covered and incubated for 15 min at room temperature. The absorbance at 530 nm-550 nm, was read by using a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). The value of each sample was quantified in respect to triglyceride standard curve and expressed as triglyceride content (mg/dl). Experiments were conducted in triplicate and repeated at least five times.

Cell lysates

For protein expression/activation examination, Huh7.5 cells were stimulated with genistein 10 pM and 17β-estradiol 10 pM for 30 min before the treatment with 2 mM FFAs for 3 h. At the end of stimulation, Huh7.5 cells were lysed in iced Ripa buffer supplemented with sodium orthovanadate (2 mM; Sigma) and protease inhibitors cocktail (1 mM; Thermo Fisher Scientific) and phenylmethanesulfonyl fluoride (1 mM; Sigma). The extracted proteins were quantified through bicinchoninic acid protein (BCA, Pierce) and used for electrophoresis and immunoblotting studies. Experiments were conducted in triplicate and repeated at least five times

Western blot analysis

Cell lysates (30 µg protein each sample) were dissolved in 5X Laemmli buffer, boiled for 5 min and resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories). After electrophoresis they were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories), which were incubated overnight at 4°C with specific primary antibodies: anti Nf-kB p50 (1:1000; Santa Cruz Biotechnology), anti PPAR-γ (1:1000; Santa Cruz Biotechnology; catalogue number sc-271392), anti p-AMPKα1/2 (1:1000; Thr 172; Santa Cruz Biotechnology), anti AMPKα1/2 (1:1000; Santa Cruz Biotechnology), anti phospho-Akt (1:1000; Ser 473, Santa Cruz Biotechnology), anti Akt (1:1000; Santa Cruz Biotechnology).

The membranes were washed and then incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma), peroxidase-coupled rabbit anti-goat IgG and horseradish peroxidase-coupled goat anti-mouse IgG (Sigma) for 45 min and were developed through a non-radioactive method using Western Lightning Chemiluminescence (PerkinElmer Life and Analytical Sciences). Phosphorylated protein expression was calculated as a ratio towards specific total protein expression or β-actin (1:5000; Santa Cruz Biotechnology) detection.

Statistical analysis

All data were recorded using the Institution's database. Statistical analysis was performed by using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc., Cary NC, USA). Data were checked for normality before statistical analysis. All the results obtained were examined through one-way ANOVA followed by Bonferroni post hoc tests. All data are presented as means ± standard deviation (SD) of five independent experiments for each experimental protocol. A value of P <0.05 was considered statistically significant.

Results

In the first part of the study, we aimed to examine the protective effects elicited by genistein and 17 β -estradiol on cell viability, mitochondrial function, triglycerides accumulation and oxidative stress of hepatocytes cultured in oleate/palmitate medium. A dose-response effect analysis was performed in order to choose the optimal dosage of both estrogens/phytoestrogens to be adopted in the second part of the study.

As shown in Fig. 2, genistein and 17 β -estradiol were able to increase cell viability and mitochondrial membrane potential in Huh7.5 cells treated with FFAs. Moreover, both agents were able to counteract the increased ROS release and triglycerides overaccumulation caused by FFAs in Huh7.5 cells (Fig. 3). The protective effects elicited by genistein and 17 β -estradiol in Huh7.5 cells were also confirmed by experiments performed in presence of TNF α . Hence, both estrogens and phytoestrogens reduced the deleterious effects elicited by TNF α on cell viability and mitochondrial membrane potential (Fig. 4). Also, in primary human hepatocytes, both genistein and 17 β -estradiol were able to prevent the damages caused by FFAs (Fig. 5).

Note that no differences in the effects of genistein from 10 pM up to 1 μ M were observed on Huh7.5 cells. On cell viability and mitochondrial membrane potential the lowest response was found at 10 μ M genistein. For this reason, all subsequent experiments were performed with 1 μ M genistein. Similarly, as regarding estradiol, no grading was observed with 10 pM-100 nM. Thus, 10 nM estradiol was used to execute all other experiments.

In the second part of the study, the involvement of PI3K/Akt, AMPK, TLR4, NLRP3 inflammasome, ERs and GPER was examined by performing the same experiments of the first part in presence of specific inhibitors. In addition, Western Blot was executed in order to better address the role of Akt, AMPK, Nf-kB and PPAR γ .

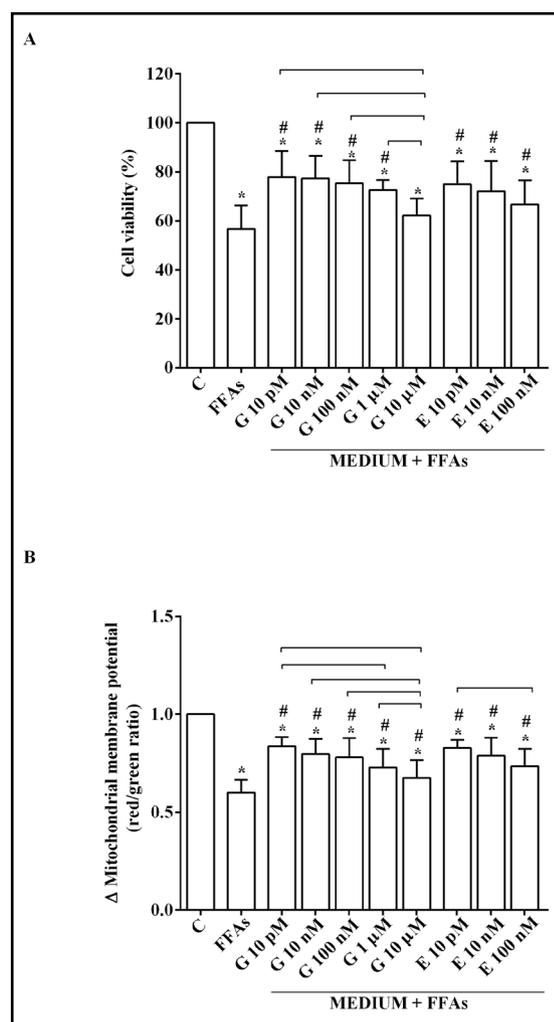


Fig. 2. Effects of genistein and 17 β -estradiol on cell viability (A) and mitochondrial membrane potential (B) in Huh7.5 cells. In A and B, the effects of genistein (G) and 17 β -estradiol (E), are shown. Data were normalized versus related control values. C=control (non-treated cells); FFAs=free fatty acids (2 mM for 3 h). Reported data are means \pm SD of five independent experiments for each protocol. Significance between groups: *P<0.05 vs. C; # P <0.05 vs FFAs. Short square brackets indicate significance between groups (P<0.05).

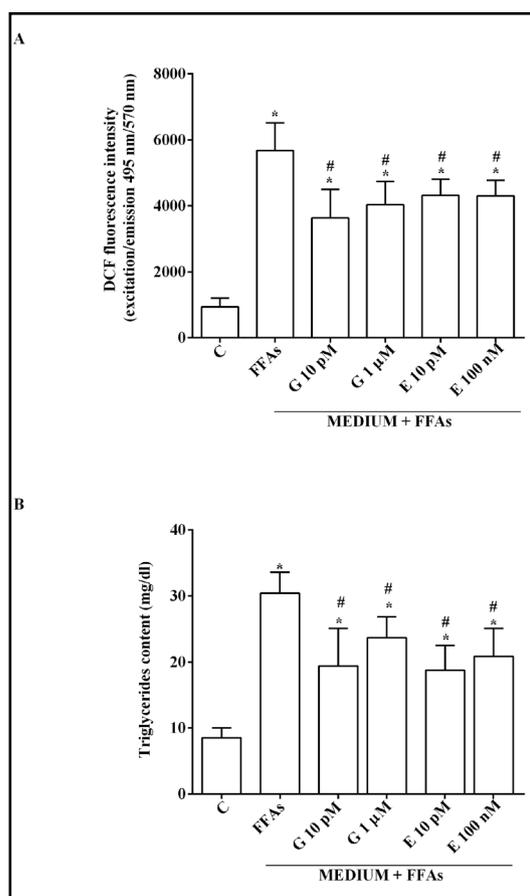


Fig. 3. Effects of genistein and 17 β -estradiol on ROS production (A) and triglycerides content (B) in Huh7.5 cells. In A, results are expressed as DCF fluorescence intensity, which is proportional to the amount of intracellular ROS. In B, results are expressed as triglycerides content (mg/dl) produced after each stimulation. G: genistein; E: 17 β -estradiol. C=control (non-treated cells); FFAs=free fatty acids. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: *P<0.05 vs C; #p<0.05 vs. FFAs.

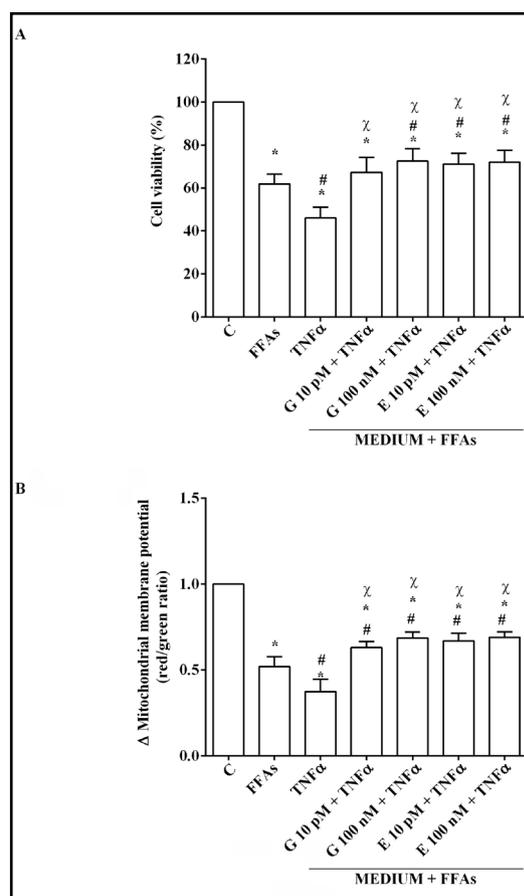
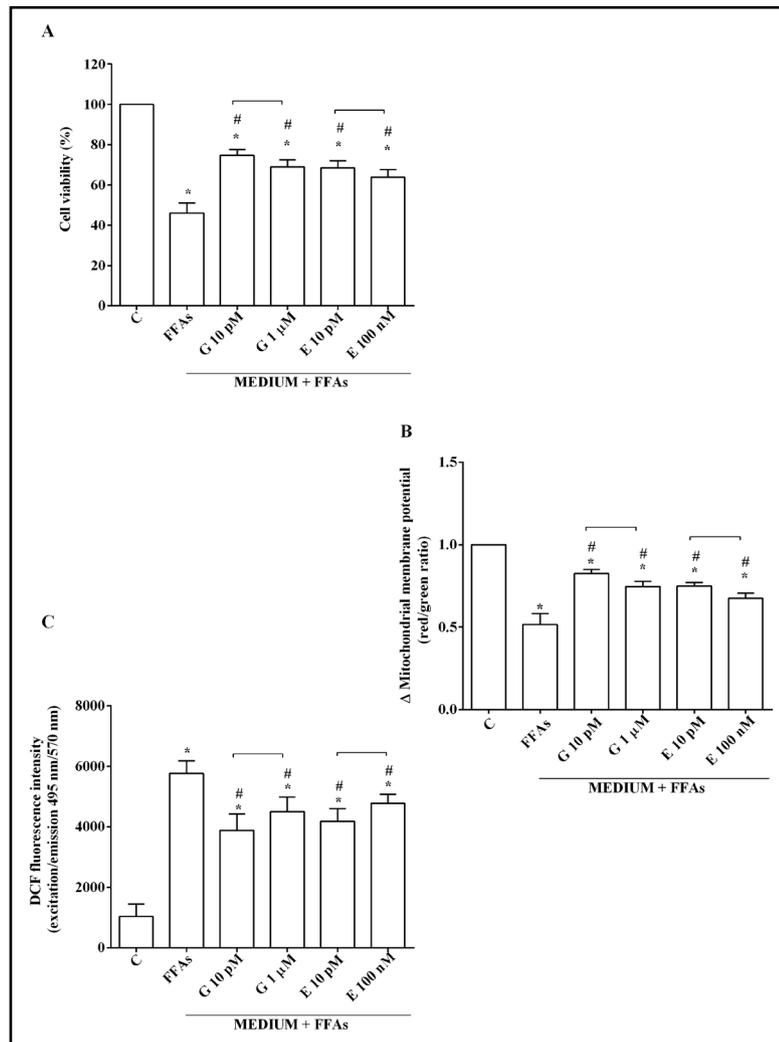


Fig. 4. Effects of genistein and 17 β -estradiol on cell viability (A) and mitochondrial membrane potential (B) in Huh7.5 cells in presence of tumor necrosis factor (TNF α). Data were normalized against the control value. G: genistein; E: 17 β -estradiol. C=control (non-treated cells); FFAs=free fatty acids. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * P <0.05 vs. C; # P <0.05 vs FFAs; χ P <0.05 vs. TNF α .

Fig. 5. Effects of genistein and 17 β -estradiol on cell viability (A), mitochondrial membrane potential (B) and ROS production (C) on primary human hepatocytes. G: genistein; E: 17 β -estradiol. C=control (non-treated cells); FFAs=free fatty acids. Reported data are means \pm SD of at least five independent experiments for each experimental protocol. Significance between groups: * P <0.05 vs. C; # P <0.05 vs. FFAs. Short square brackets indicate significance among groups (P <0.05).

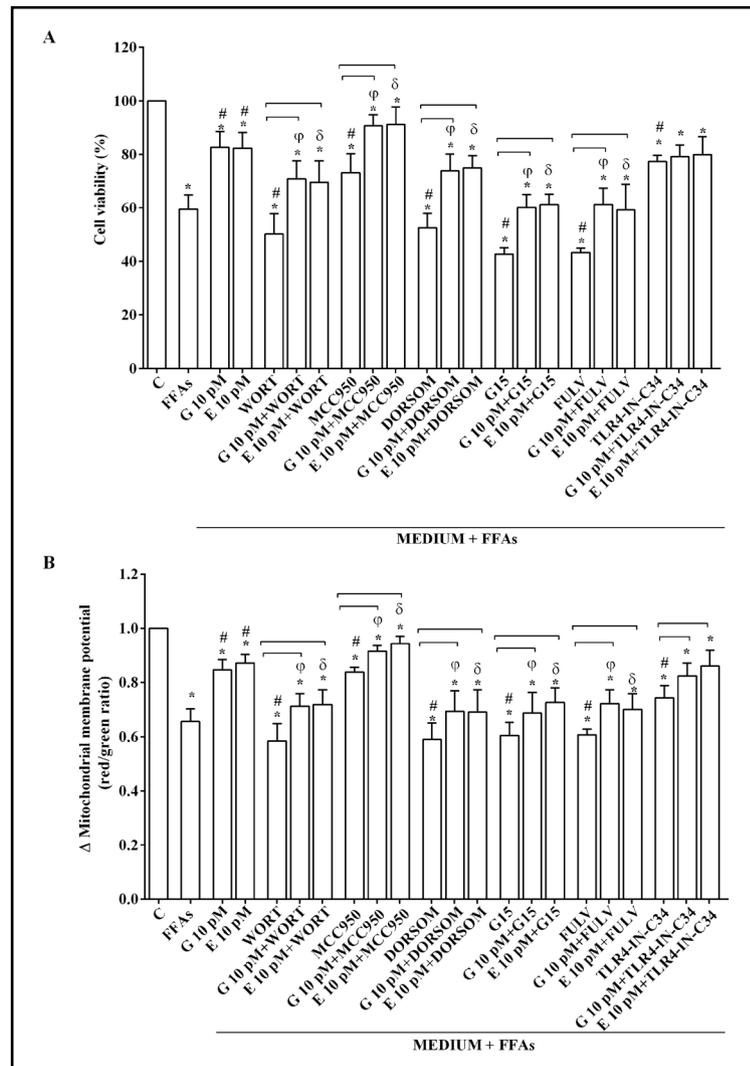


In presence of wortmannin, dorsomorphin, G15 and fulvestrant, the effects of genistein and 17 β -estradiol on cell viability, mitochondrial membrane potential, ROS release and triglycerides accumulation caused by FFAs in Huh7.5 cells were generally counteracted (Fig. 6 and 7).

As regarding TLR4, the administration of TLR4-IN-C34 reduced the damages caused by FFAs but was not able to affect the response of Huh7.5 cells to neither genistein nor 17 β -estradiol (Fig. 6 and 7). Conversely, the NLRP3 inflammasome inhibitor, MCC950, was able to potentiate the protective effects elicited on Huh7.5 cells by both agents (Fig. 6 and 7).

The involvement of Akt and AMPK in the protective effects elicited by both estrogens and phytoestrogens in Huh7.5 cells treated with FFAs was confirmed by Western blot, as well (Fig. 8A and B). In addition, also NF- κ B and PPAR γ were found to play a role. As shown in Fig. 8C and D, both genistein and 17 β -estradiol not only increased the activation of Akt and AMPK but were also able to reduce the expression of NF- κ B and PPAR γ .

Fig. 6. Effects of genistein and 17 β -estradiol on cell viability (A) and mitochondrial membrane potential (B) in Huh7.5 cells in presence/absence of inhibitors. G: genistein; E: 17 β -estradiol. C=control (untreated cells); FFAs=free fatty acids. WORT (1 nM wortmannin for 30 min); MCC950 (NLRP3 inhibitor; 1 nM for 30 min); DORSOM (1 nM dorsomorphin for 30 min); G15 (1 nM for 30 min); FULV (fulvestrant; 1 nM for 30 min); TLR4-IN-C34 (TLR4 inhibitor; 1 nM for 30 min). Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * P <0.05 vs C; # P <0.05 vs FFAs; ϕ P <0.05 vs G 10 pM; δ P <0.05 vs. E 10 pM. Short square brackets indicate significance among groups (P <0.05).

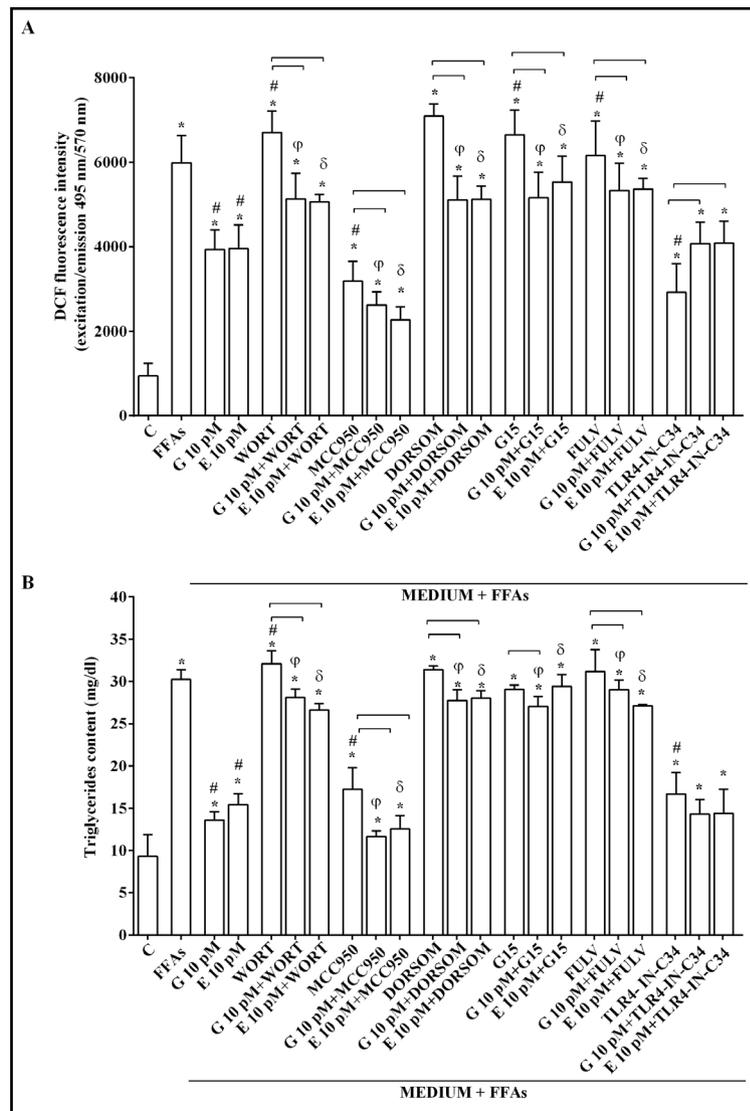


Discussion

The results of this study show that both genistein and 17 β -estradiol protect both Huh7.5 cells and human primary hepatocytes against intracellular fat overaccumulation by preserving mitochondrial function through the modulation of the oxidants/antioxidants balance. These effects are seen to involve pathways underlying the mechanisms of triglycerides accumulation and hepatic inflammation.

In our study, we employed an established human hepatoma cell model of NAFLD and related progression to NASH made of Huh7.5 cells exposed to a mixture of oleate/palmitate [19-22, 28], which are common long chains fatty acids in the Western diet and the most abundant fatty acids in normal and steatotic liver [20]. As previously shown, treatment with a 2:1 ratio oleic/palmitic acids mixture induced steatosis in both Huh7.5 cells and primary human hepatocytes [29]. It is to note that we used a fatty acids concentration similar to the plasmatic levels found in patients with metabolic syndrome [20]. Moreover, we observed that the fat overaccumulation in Huh7.5 cells was accompanied by a diminution of the mitochondrial membrane potential associated with an increased in ROS release, which ended in reduced cell viability. Similar deleterious effects were observed in primary human hepatocytes. Both genistein and 17 β -estradiol were able to prevent the ectopic accumulation of fat in Huh7.5 cells and primary human hepatocytes where, consequently, a

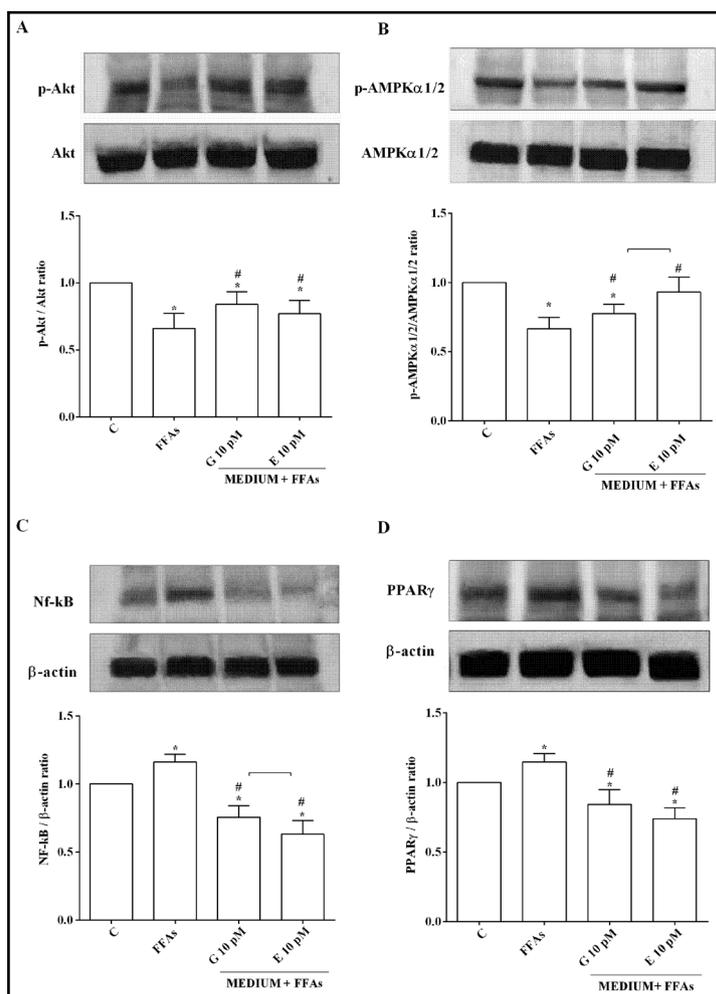
Fig. 7. Effects of genistein and 17 β -estradiol on ROS production (A) and triglycerides content (B) in Huh7.5 cells in presence/absence of inhibitors. G: genistein; E: 17 β -estradiol. C=control (untreated cells); FFAs=free fatty acids. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * P <0.05 vs C; # P <0.05 vs FFAs; ϕ P <0.05 vs G 10 pM; δ P <0.05 vs. E 10 pM. Short square brackets indicate significance among groups (P <0.05).



reduction in the release of ROS and the recovery of the mitochondrial membrane potential were also observed. Note that the doses of genistein used for the study were in the range of nutritional concentrations and comparable to those administrated in previous works using cellular models [30, 31]. 17 β -estradiol was also used at concentrations referable to those of menstrual or menopausal women and comparable to those given to Huh7.5 cells in previous studies [32-34]. Both genistein and 17 β -estradiol were also able to limit the deleterious effects of TNF α , which was used to induce inflammatory stress in Huh7.5 cells.

NAFLD is characterized by a diffused fat accumulation in vesicles that displace the cytoplasm of hepatocytes, i.e., steatosis. So far, the underlying mechanism behind the development of NAFLD and its progression towards fibrosis and NASH has not been fully elucidated. Historically, “two-hit” hypothesis was used to explain the pathogenesis of NAFLD. However, this view was considered to be too simplistic to summarize the synergy of multiple stimulating factors in the occurrence of NAFLD. Presently, NAFLD is rather inclined to be a “multiple hit” disease [35]. Such hits involve genetic, metabolic and environmental factors including epigenetic modifications, dietary intake, hormones (leptin, adiponectin) secreted from adipose tissue, crosstalk between different organs or tissues and so on. Among these factors, at cellular level oxidative stress and mitochondria play a primary role as starting point [2, 36].

Fig. 8. Changes in Akt and AMPK α 1/2 phosphorylation (A and C) and Nf-kB and PPAR γ expression (B and D) in Huh7.5 cells. Densitometric analysis and an example of Western blot taken from 5 different experiments are shown. AMPK α 1/2: AMP-activated protein kinase α 1/2; Nf-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; PPAR γ : peroxisome proliferator activating ligand receptors γ ; G: genistein; E: 17 β -estradiol. C=control (untreated cells); FFAs=free fatty acids (2 mM for 3 h). The data were normalized versus control value. Reported data are means \pm SD of three independent experiments for each experimental protocol. Significance among groups: * P < 0.05 vs. C; # P < 0.05 vs FFAs.



In this regard, it's well known that fat and energy homeostasis in hepatocytes are regulated by mitochondrial activities, including β -oxidation and production of ATP [4]. Mitochondrial dysfunction modifies the balance between oxidants/antioxidants, leading to an increase of non-metabolized FFAs in the cytosol and the consequent induction of ROS release. In turn, an increased overload of FFAs into mitochondria would augment the permeability of the inner mitochondrial membrane leading to dissipation of the membrane potential. Moreover, the increase of fatty acid oxidation could generate an "electron leakage" ensuring a direct reaction between electrons and oxygen. All above events would result in an increased release of ROS [3]. Given these roles of mitochondria in energy homeostasis and ROS production, it is reasonable to suppose that any mitochondrial defect in hepatocyte can flow to NAFLD [21].

Our results showing that both genistein and 17 β -estradiol act by preserving the mitochondrial membrane potential in both Huh7.5 cells and primary human hepatocytes is a novel finding with potential clinical relevance.

Besides to oxidative stress, inflammation also plays a relevant role in the pathophysiology of NAFLD and its progression toward NASH and cancer. Hepatic inflammation is considered not only the main driver of hepatic tissue damage, but also the factor that triggers the progression from NAFLD to severe fibrogenesis and, ultimately, hepatocellular carcinoma [10].

In this context, the activation of proinflammatory pathways via hepatic receptors such as TLRs and NOD-like receptors has longstanding recognition [37, 38]. Among various TLRs and NOD-like receptors, TLR4 and inflammasome NLRP3 are widely considered to have

relevance in liver inflammation and fibrogenesis [39, 40]. Downstream TLR4, there would be the activation of NF- κ B, MAPK [41] and extracellular signal regulated kinase-1 (ERK1), which finally would trigger the production of proinflammatory cytokines [10, 42]. This activation, in turn, would up-regulate the transcription of NLRP3 inflammasome-related components, which would potentiate cytokines release and liver damage

Our observation that FFAs treatment of Huh7.5 cells leads to an increase of the expression of the Nf- κ B, is an important acquisition. TLR4 and inflammasome NLRP3 are also found to be involved in mediating the damages caused by fatty acids as shown by the experiments in presence or absence of specific inhibitors. Hence, while the administration of MCC950 was able to improve cell viability and mitochondrial potential and reduce ROS release and triglycerides accumulation, TLR4-IN-C34 caused the opposite effect. Furthermore, in Huh7.5 cells treated with MCC950 a potentiation of the protective effects elicited by genistein and 17 β -estradiol was observed. This suggests, for the first time, involvement of NLRP3 inflammasome pathway in the mechanism with which estrogens and phytoestrogens act to reduce the ectopic accumulation of fat within hepatocytes.

Both FFAs synthesis and catabolism are regulated by factors like SREBP-1c, the carbohydrate response element binding protein (ChREBP) and PPARs, among which PPAR- γ , whose expression in conditions of liver damage is usually high, that would particularly contribute to the accumulation of FFAs [43-45]. Since, the use of PPAR- γ agonists has been shown to reduce lipotoxicity in the liver [46], PPAR- γ is considered as a drug target for type 2 diabetes and other components of metabolic syndrome.

NAFLD pathogenesis also involves AMPK that acts as a sensor of the energy levels, stimulating mitochondrial β -oxidation and lipogenesis [47]. Following phosphorylation at Thr 172, AMPK would inhibit rate-limiting steps in lipogenesis, such as SREBP-1C, through mammalian target of rapamycin in hepatic cells. Conversely, AMPK inhibition has been shown to counteract those effects, accelerating fatty liver development [9].

As concerning the mechanisms through which genistein and 17 β -estradiol could hinder the harmful effects of FFAs in Huh7.5 cells, the results of the present study suggest a role for both PPAR- γ and AMPK. Hence, both agents were able to reduce the increased expression of PPAR- γ and the reduced phosphorylation of AMPK at Thr 172, caused by fatty acids. In this context, the actions of genistein and 17 β -estradiol on AMPK resemble those elicited by the anti-diabetic drug metformin [48]. It is to note that also metformin was able to preserve mitochondrial membrane potential in HepG2 cells and primary rat hepatocytes treated with palmitate [49].

Overall, our observations are consistent with *in vitro* studies performed with HepG2 cells showing an increase of the expression of PPARs in response to 10 μ M genistein [50]. Also, in BRL cells treated with a mixture of fatty acids, genistein was capable to counteract fat overaccumulation through regulation of fatty acids metabolism mediated by AMPK [51].

As regarding 17 β -estradiol, the experimental results acquired with the present study are in line with the known beneficial effects exerted by estrogens in liver metabolic dys-homeostasis [52]. Estrogens act suppressing lipid accumulation, inflammation, and fibrosis slowing down NAFLD/NASH progression in premenopausal.

Liver has both nuclear ERs and GPER, too. Previous findings have reported that the activation of ERK1/2, Akt, MAPK could act as downstream signaling of both ERs and GPER stimulation [53-56]. As also shown in Huh7.5 cells and HSC [33], fulvestrant and G15 were able to reduce the damages caused by the ectopic accumulation of fat in hepatocytes and to prevent the beneficial effects exerted by both estrogens and phytoestrogens.

Although not clearly examined, it could be speculated that genistein and 17 β -estradiol counteract the effects of FFAs in hepatocytes by the activation of signalling pathways, downstream ERs and GPER, involving AMPK and PPAR- γ . This would be followed by the reduction of the oxidative stress and inflammation and preservation of the mitochondrial membrane potential. Finally, both phytoestrogens and estrogens may protect hepatocytes against NAFLD by activating kinases engaged in the modulation of the insulin pathway such as Akt.

Conclusion

Taken together, our findings would highlight novel mechanisms of action through which both genistein and 17 β -estradiol could exert protective effects against NAFLD with potential clinical implications. While the prevalence of NAFLD is increasing worldwide, no therapies have been approved for this condition. In addition, the higher prevalence of NAFLD/NASH in men and postmenopausal women compared to premenopausal women, and experimental data showing the protective effects of estrogen in the liver, would indicate that estrogens are involved in NAFLD. Very few studies have shown a beneficial effect of HRT on NAFLD, although adverse hepatic effects have been attributed to progesterone. In this context, phytoestrogens could represent alternatives to HRT, although their long-term efficacy and safety remain to be shown [15]. Further studies are needed to better address those issues and improve the current knowledge of the mechanisms of action involving various pathways and related cross talks.

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Author Contributions

Conceptualization, D.S., M.P. and E.G.; Methodology, S.F., G.C., P.M., R.R. and E.G.; Software, S.F., G.C., P.M. and E.G.; Validation, S.F., G.C., P.M., R.R., D.S., G.Ca., M.B., M.P. and E.G.; Formal Analysis, S.F., M.B. and E.G.; Investigation, S.F., G.C., P.M., R.R. and M.B.; Resources, D.S., M.B., M.P. and E.G.; Data Curation, D.S., G.C., M.B., M.P. and E.G.; Writing – Original Draft Preparation, R.R., G.C., M.P. and E.G.; Writing – Review & Editing, S.F., G.C., P.M., R.R., D.S., G.Ca., M.B., M.P. and E.G.; Visualization, S.F., G.C., P.M., R.R., D.S., G.Ca., M.B., M.P. and E.G.; Supervision, S.F., G.C., P.M., R.R., D.S., G.Ca., M.B., M.P. and E.G.; Project Administration, M.P. and E.G.; Funding Acquisition, D.S., M.B., M.P. and E.G. We thank Azienda Ospedaliero-Universitaria of Novara for its help.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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