A Mix of Chlorogenic and Caffeic Acid Reduces C/EBPß and PPAR-γ1 levels and Counteracts Lipid Accumulation in Macrophages

Mirko Marino[1], Cristian Del Bo'[1,*], Massimiliano Tucci[1], Samuele Venturi[1], Giacomo Mantegazza[1], Valentina Taverniti[1], Peter Møller[2], Patrizia Riso[1], Marisa Porrini[1]

Affiliation

[1]Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), 20133 Milan, Italy.

[2]Department of Public Health, Section of Environmental Health, University of Copenhagen, DK-1014 Copenhagen K, Denmark

*Correspondence: Dr Cristian Del Bo', PhD, Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences - Division of Human Nutrition, Milan, Italy Fax,+39 0250316721; Phone, +39 0250316730; email: cristian.delbo@unimi.it

Abstract

Purpose: Chlorogenic acid (CGA) and caffeic acid (CA) are bioactive compounds in whole grains, berries, apples, some citrus fruits and coffee, which are hypothesized to promote health-beneficial effects on the cardiovascular system. This study aimed to evaluate the capacity of CGA and CA to reduce lipid accumulation in macrophages, recognized as a critical stage in the progression of atherosclerosis. Furtherly, the modulation of CCAAT/enhancer-binding protein β (C/EBPβ) and peroxisome proliferator-activated receptor- γ1 (PPAR-γ1), as transcription factors involved in lipid metabolism, was evaluated.

Methods: THP-1 derived macrophages were treated for 24 h with 0.03, 0.3, 3 and 30 μM of CGA and CA, tested alone or in combination, and a solution of oleic/palmitic acid (500 μM, 2:1 ratio). Lipid storage was assessed spectrophotometrically through fluorescent staining of cells with Nile red. C/EBPβ and PPAR-γ1 mRNA and protein levels were evaluated by RT-PCR and enzyme-linked immunosorbent assay, respectively.

Results: The mix of CGA+CA (1:1 ratio) reduced lipid accumulation at all concentrations tested, except for the highest one. The greatest effect (-65%; $p<0.01$) was observed at the concentration of 0.3 μM for each compound. The same concentration significantly ($p<0.01$) downregulated C/EBPβ and PPAR-γ1 gene expression and reduced their protein levels at 2 h and 24 h, respectively.

Conclusion: The results indicate that the capacity of CGA + CA mix to reduce lipid storage in macrophages is reduced by transcription factors C/EBPβ and PPAR-γ1.

Keywords: polyphenols, THP-1 derived macrophages, lipid accumulation, PPAR-γ1, C/EBPβ, atherosclerosis

Introduction

In the last years, there has been increasing attention on the potential role of dietary polyphenols in the modulation of lipid metabolism and prevention of cardiovascular events [1-8]. Polyphenols are a heterogeneous class of more than 8000 bioactive compounds in plants. The main food sources are vegetables, fruits, whole grains, legumes and chocolate, but they are also abundant in beverages such as tea, coffee, and red wine [9]. Polyphenols are most commonly classified into flavonoids and nonflavonoids. Flavonoids are characterized by a basic structure of diphenyl propane containing several hydroxyl groups on the aromatic rings; while, nonflavonoids share a single aromatic ring as basic structure [10]. Flavanols, anthocyanins, isoflavones, flavones, flavonols and flavanones belong to the group of flavonoids, while phenolic acids, stilbenes and lignans belong to the group of nonflavonoids. Phenolic acids (hydroxycinnamic and hydroxybenzoic acids) such as ferulic, chlorogenic acid (CGA), and its metabolite caffeic acid (CA), are widely distributed in whole grains, apples, some citrus fruits, coffee and berries generally esterified with other molecules such as carbohydrates and organic acids [10]. As reported on Phenol-Explorer, the first and the most exhaustive database on polyphenol content in foods, the concentration of these compounds is variable [11,12]; for example, different types of berries contain from 1 to 200 mg/100g CGA and CA [13], while the total CGA concentration in coffee ranges from 30 to 1000 mg/100 ml of product [14,15].

The absorption of CGA occurs mostly in the intestine by passive diffusion. However, some ex vivo absorption experiments with pig jejunal mucosa showed a role of active efflux in CGA bioavailability and, further, that the mechanism of absorption in the jejunum is governed by physicochemical properties of specific transporters [16]. After absorption, CGA undergoes mainly sulfation and glucuronidation reactions, while the unabsorbed portion is extensively hydrolyzed by gut bacteria [15]. The colonic metabolites can be absorbed and excreted in the urine. For example, after coffee consumption the main metabolites found in urine over a period of 24 hours were: ferulic acid-4-O-sulfate, caffeic acid-3-O-sulfate, isoferulic acid-3-O-glucuronide and dihydrocaffeic acid-3-O-sulfate [17-18]. Due to this extensive metabolism, the in vivo concentrations of CGA and CA are widely variable; the maximum plasma concentration (Cmax) of CGA and metabolites (including CA) ranges from ~2 nM to ~6 μM levels in humans [18-20], depending on the type and magnitude of food administered, individual metabolic and xenobiotic response [21], but also analytical method used for their detection. The role of phenolic acids in the modulation of cardiovascular risk has not been fully investigated. Some studies seem to support their cardiovascular protection by counteracting oxidative stress and inflammation, and by regulating cell adhesion process, migration and lipid accumulation [22-28]. The entrapment of monocytes in the intimal area and their subsequent transformation to macrophage-like foam cells is a pivotal step in atherosclerosis, with a significant impact on plaque inflammation, progression and stability. In particular, lipid accumulation in macrophages determines cell dysfunction and a dysregulation of several transcriptional factors involved in the atherosclerotic process [29]. Lipid overload at the level of arterial tunica intima triggers foam cells development, which is the first event in atherosclerosis that can be noticed [30]. Particularly, the uptake of free fatty acids determines an increase in triglycerides accumulation in macrophages by reducing the beta-oxidation process and by increasing the expression of lipogenic enzymes [31].

Influx, esterification and efflux of lipids are critical stages involved in intracellular lipid droplets generation. Indeed, an alteration in the processes involved in lipid metabolism represents one of the major determinants of the macrophage transformation into a foam cell [32]. Excessive lipid deposition in foam cells leads to persistent endoplasmic reticulum stress which in turn induces apoptosis [33]. The increasing number of dead cells, further worsened by decreased clearance via impaired autophagy, promotes an exacerbation of the inflammatory response and a subsequent establishment of the necrotic core that leads to plaque progression [34]. There are several transcription factors associated with lipid metabolism in foam cells, including peroxisome proliferator-activated family of receptors (PPARs), CCAAT enhancer binding proteins (C/EBPs),

Liver-X family of receptors (LXRs), and sterol regulatory element binding proteins (SREBPs). In addition, although not considered a transcription factor, micro-RNA 33 (miR-33) has also been implicated in regulating lipid metabolism [35]. The peroxisome proliferator-activated receptor-γ (PPAR-γ) and the proliferator-activated receptor-coactivator 1β play a crucial role in the conveyance of lipid metabolism and in the polarization of metabolic profile of macrophages. In particular, PPAR-γ has been described as a sensor of fatty acids for its high responsiveness to these molecules [36]. CCAAT enhancer-binding proteins (C/EBP) are a family of transcription factors involved in adipocyte differentiation with key roles during macrophage polarization [36]. Recent evidence connects C/EBPβ with atherosclerosis since its expression seems to be stimulated by oxidized low-density lipoprotein (oxLDL) in primary human macrophages, suggesting that C/EBPβ may be involved in inflammatory processes triggered by oxLDL during foam cell development [37]. The LXRs belong to the nuclear receptor superfamily of ligand activated transcription factors of which there exist two isoforms, LXRα and LXRβ. These transcription factors are strongly connected to atheroprotection since they promote cholesterol efflux from intimal macrophages, thus reducing atherosclerotic plaques through numerous mechanisms involving ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) [38]. Furthermore, there is evidence that LXRs regulate multiple key metabolic pathways, such as cholesterol homeostasis, inflammation and lipogenesis [38]. The sterol regulatory element binding proteins (SREBPs) are transcription factors that regulate gene expressions of several enzymes involved in lipid and glucose metabolism, known for fostering cholesterol biosynthesis in macrophages through the induction of HMG-CoA reductase [39].

We have previously reported the capacity of anthocyanin- and phenolic acid-rich fractions, from a wild blueberry powder, to reduce lipid accumulation in human THP-1 derived macrophages [27]. We documented that the effects for anthocyanin-rich fraction, the single anthocyanins and their derivatives were compound-dependent. Regarding the phenolic acid-rich fraction, rich mainly in CGA, we have shown that the lipid-lowering effect was most effective at low-medium concentrations, which are physiologically relevant. Based on these results, the aim of this study was to evaluate the effect of CGA and its metabolite (i.e. CA) on the reduction of fatty acids accumulation in monocyte-derived macrophages. The compounds were tested alone and in combination both at physiological and supraphysiological concentrations. Moreover, the impact of CGA and CA on the expression of PPAR-γ1 and C/EBPβ has been investigated. In particular, PPAR-γ represents an interesting molecular target for atherosclerosis treatment, as pivotal transcription factor involved in lipid metabolism of macrophages and atherosclerotic plaque development, while C/EBPβ is a key regulator of metabolism, adipocyte differentiation, and macrophage activation [40-43].

Materials and methods

Chemicals and reagents

Standard of CGA (CAS No. 327-97-9), also called 3-O-caffeoylquinic acid, and CA (CAS No. 331-39-5), bovine serum albumin (BSA), palmitic acid, oleic acid, fetal bovine serum (FBS), Hanks balanced salt solution, Trypan Blue, Pluronic F127, Nile Red, phorbol 12-myristate 13-acetate (PMA), DNase I amplification grade kit, hydrochloric acid, methanol and ethanol were purchased from Merck (Darmstadt, Germany). Water was obtained from Milli-Q apparatus (Millipore, Milford, MA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate, gentamicin, RPMI-1640 medium, and trypsin–EDTA were obtained from Life Technologies (Monza Brianza, Italy). RNeasy Mini kit was purchased from QIAGEN (Hilden, Germany). iScript cDNA Synthesis Kit and SsoFast EvaGreen Supermix were purchased from BIORAD (Segrate, Milano, Italy). Forward and reverse primers of PPAR-γ1 and C/EBPβ genes were obtained from biomers.net (Ulm, Germany). Forward and reverse primers of 18S were acquired from Primmbiotech (Cambridge, MA, USA).

Preparation of chlorogenic and caffeic acid

A stock solution of CGA and CA standards (**Figure 1**) was prepared. These phenolic acids were selected based on our previous publication in which CGA and CA were the most representative compounds found in a phenolic-rich fraction obtained from wild blueberry [27]. Lyophilized standards (50 mg) were dissolved in acidified methanol (5 ml; HCl 0.05 mM). The final concentration of methanol in exposure medium was 0.0025%. Aliquots (1 mL) of standard were prepared, quantified spectrophotometrically, and finally stored at −20 °C until use.

Preparation of fatty acids and control solution

The stock solution of free fatty acids (FFA; 0.2 M oleic/palmitic acid; 2:1 ratio) was prepared in EtOH and stored at −20 °C. On the day of use, 5 mM of FFA water-soluble solution (FFA/BSA solution) was generated by incubating the FFA in Hanks solution containing 10% BSA at 37 °C for about 30 minutes with occasional shaking. The FFA/BSA solution was added to the medium to obtain final 500 μM FFA concentration for the exposure. Equal volumes of the Hanks solution/EtOH/FFA-free BSA were applied to control cells. The final concentrations of EtOH and BSA in exposure media were 0.25 and 0.1%, respectively.

Cell culture

The monocytic THP-1 cell line (human monocytic leukemia) was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were maintained in culture in RPMI-1640 medium containing 10% of heat inactivated FBS and supplemented with 1% HEPES, 1% sodium pyruvate and 0.1% gentamicin. The cells were subcultured every 3 days during cell growth by directly diluting cell culture with fresh medium, or by withdrawing a portion of the cells from the culture flask and diluting the remaining cells to an appropriate seeding density ($3x10^5$ cells/ml). According to Tsuchiya et al [44], the cells maintain their monocytic characteristics for over 14 months of continuous growth. In our study, cells were used for the experiments up to 2 months of growth which correspond to a passage number between 5 and 15.

Viability assay

The toxicity of the compounds was tested on THP-1 derived macrophages by Trypan blue using a TC20TM automated cell counter and dual-chamber cell counting slides (BIORAD, Segrate, Milano, Italy). THP-1 cells were differentiated to macrophages and treated with CGA, CA and the mix of CGA and CA at the maximum concentration tested (30 μM of each compound) for 24 hours and in presence of FFA/BSA solution (500 μM). Subsequently cells were trypsinized, resuspended and used for the Trypan blue assay. Three independent experiments were performed in which each compound was tested in triplicate.

Lipid accumulation in THP-1 derived macrophages

Cells were cultured in complete RPMI cell medium at 37 °C and 5% CO_2. After growth, cells were differentiated into macrophages by treatment with 5 ng/mL PMA for 72 hours. Macrophages become adherent to the surface of the culture flasks; thus, cells were washed with Hanks Solution to remove all non-adherent cells. Subsequently, 3 mL trypsin (0.05%)–EDTA (0.53 mM) was added and the cells were incubated for 2 minutes at 37 °C and 5% CO_2 in order to release the attachment to the flask. After incubation, 2 mL of complete RPMI-1640 medium was added to inactivate the trypsin. Cells were collected in Falcon tubes, quantified in a TC20TM automated cell counter and then centrifuged (mod. Eppendorf 5804R Centrifuge, Milano, MI, Italy) for 5 minutes at 250×g. After centrifugation, cells were resuspended in a new complete RPMI-1640 medium (without PMA) in order to reach a final concentration of 2.5×10^5 cells/mL. Two hundred microliters of cell suspension (5×10^4 cells) were added into each well of a black 96-well plate and incubated for 24 hours at 37 °C and 5% CO_2, in order to allow the cell adhesion to the surface of the plate. Medium was removed and 200

μL of new complete RPMI-1640 medium, containing 500 μM FFA and different concentrations of CA and CGA alone or in combination, was added. All these compounds were prepared in acidified methanol (0.05 mM HCl) and then diluted in the culture medium before use. The highest methanol content was <0.03% while the concentration of HCl in cell media was less than 0.001% without impact on pH and cell viability, as also documented in our previous papers [27]. The concentrations for each compound were 0.03, 0.3, 3 and 30 μM tested alone or in combination. These concentrations were selected in order to mimic both physiological (ranges from ~2 nM to ~6 μM) and supraphysiological conditions in accordance with data found after oral administrations of foods or single compounds, as previously reported [18-20]. Cells were incubated for 24 hours at 37 °C and 5% CO_2. The lipid accumulation was measured by using the fluorescent dye Nile red [45]. Nile red is not fluorescent in aqueous solution but can be extensively fluorescent in lipid rich environments. Nile red is thus suitable for measuring lipid accumulation both in cells and in tissue. To measure the lipid accumulation in macrophages, the cells were washed with Hanks solution and then stained in Hanks solution containing 0.5 μg/mL Nile red and 0.01% Pluronic F127 for 15 min at 37 °C and 5–7% CO_2. Later, cells were washed twice with 200 μL of Hanks solution and then added 100 μL Hanks solution. The fluorescence (excitation: 544 nm, emission: 590 nm) was measured in a fluorescence spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy), and the fold increase compared to the control (without FFA) was calculated.

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR)

Cells were obtained using the same conditions as the lipid accumulation assay, except the plating of THP-1 derived macrophages was done in a 24-well plate (5×10^5 cells) instead of 96 well plate (5×10^4 cells) in order to have a suitable extraction of RNA. RNA from THP-1 macrophages was extracted immediately after the experiment by RNeasy Mini kit according to manufacturer's protocol. DNase treatment, using DNase I amplification grade kit (Merck), was used to degrade DNA in the RNA preparations in a 15-minute digestion at room temperature. The reaction was then stopped adding 1 μL of Stop Solution and DNase I was heat inactivated for 10 min at 70°C. Purified RNA was quantified through spectrophotometer (Power Wave XS2, BioTek, Winooski, VT, USA) by loading each sample in duplicate; the A260/A280 ratio was verified to be above 1.8 as sufficient quality of RNA purity. An equal amount of RNA for each sample was immediately reverse transcribed using iScript cDNA Synthesis Kit and Mastercycler nexus gradient (mod. Eppendorf, Milano, MI, Italy) by using the following thermal cycle: 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. Controls without reverse transcriptase (No-RT) and without RNA template (no RNA) were performed to be used in qPCR in order to rule out residual genomic DNA contamination in RNA samples and reagents contamination with nucleic acids. Afterwards, the obtained cDNA was diluted with nuclease free water to a concentration of 3 ng/μL. qRT-PCR was performed by using C1000 Thermal Cycler / CFX96 Real-Time System (BIORAD, Segrate, Milano, Italy) and SsoFast EvaGreen Supermix according to manufacturer's protocol. A total amount of 15 ng of cDNA was used. Controls included No-RT, no RNA and a control sample without cDNA for each primer pair. Thermal cycling conditions for the analysis of PPAR-γ1 were 15 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C, 20 seconds at 55°C, and 10 seconds at 72°C with the primer pairs indicated in **Table 1**. The cycling program for the analysis of C/EBPβ was 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds with the primer pairs indicated in **Table 1**. Primer pairs have been used at a concentration of 0.5 μM. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product. The expression levels of all target genes were normalized against the expression of the ribosomal 18S housekeeping gene, which served as the endogenous control was used as the most suitable reference gene in the cell model employed. Relative fold changes between different experimental conditions were calculated with the comparative Ct method ($2^{-\Delta\Delta Ct}$ Method). RT-PCR was performed in line with MIQE guidelines, including relevant QA information on quality and amount of RNA extract, for detailed information refer to Online Resource 1.

Enzyme-linked immunosorbent assay (ELISA)

Cells were obtained using the same conditions as the lipid accumulation assay, except the plating of THP-1 derived macrophages was done in a 6-well plate (1×10^6 cells) instead of 96 well plate (5×10^4 cells) in order to have a suitable amount of proteins. According to manufacturer's protocol, cell culture supernatant was collected for PPAR-γ1 analysis and cell extract was collected for C/EBPβ analysis. Cell culture supernatant was obtained by centrifuging at 500 g for 10 min at 4°C and aliquots of samples were immediately stored at -80°C. Protein extraction from THP-1 derived macrophages was performed immediately after the experiment by placing the plate on ice, washing with cold PBS and incubating on ice for 30 min with extraction buffer. Successively, lysed cells were centrifuged at 4,500 g for 20 min at 4°C to pellet insoluble content and aliquots of cell extracts were immediately stored at -80°C. Enzyme-linked immunosorbent assay (ELISA) was employed to determine the contents of PPAR-γ1 and C/EBPβ (Cat No. were MBS263089 and MBS2020315, respectively; MyBioSource, Inc. San Diego, USA).

Data analysis

STATISTICA software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis. All the results are expressed as means ± standard error of the mean (SEM). One-way ANOVA was applied to verify the effect of CGA and CA supplementation on cell viability, lipid accumulation process, gene expression and protein levels of PPAR-γ1 and C/EBPβ. The least significant difference (LSD) test was used to assess differences between treatments by setting the level of statistical significance at $p < 0.05$.

Results

Effect of CGA and CA on Cell Viability

Table 2 presents the effects of the compounds on the cellular viability measured by Trypan blue assay at all the concentrations tested for 24 h and in presence of FFA/BSA solution (500 μM). The control condition is represented by cells in their normal growth medium without phenolic acids and fatty acids solution. CGA, CA and the mix of CGA and CA did not affect the cell viability that remained higher than 90%.

Effect of CGA and CA on lipid accumulation in THP-1 derived macrophages

The results of lipid accumulation in THP-1 derived macrophages after incubation with CGA and CA are shown in **Figure 2a** (CGA) and **2b** (CA). Stimulation with 500 μM of FFA significantly increased ($p < 0.05$) the lipid accumulation in macrophages compared to cells without stimulation (No FFA). No effect was observed after treatment with different concentrations of CGA and CA, tested alone, compared to the FFA treatment.

Effect of the mix CGA + CA on lipid accumulation in THP-1 derived macrophages

The results of lipid accumulation in THP-1 derived macrophages after incubation with the mix CGA + CA are shown in **Figure 3**. Stimulation with 500 μM of FFA significantly increased ($p < 0.05$) the lipid accumulation process in macrophages compared to cells without stimulation (No FFA). The treatment with CGA and CA tested in combination significantly decreased ($p < 0.01$) the lipid accumulation in macrophages compared to the FFA treatment, except for the maximum concentration. In particular, the size of the effect was similar between the different concentrations tested, -56%, -65% and -48% respectively for the mix CGA + CA at 0.03 μM, 0.3 μM and 3 μM (concentration of each compound).

Effect of the mix CGA + CA on C/EBPβ gene expression and protein levels

The results of gene expression kinetic and protein levels of C/EBPβ after the administration of the mix of CGA + CA at the physiological concentration of 0.3 μM (i.e. the most effective concentration in counteracting the

lipid accumulation) are shown in **Figure 4a-b**. Gene expression and protein levels have been evaluated at five different time points (1, 2, 4, 8 and 24 hours) in three independent experiments in which every experimental condition has been tested in triplicate. There was a statistically significant increase of C/EBPβ gene expression (**Fig. 4a**) following 2 hours ($p < 0.01$) from the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA induced, after 2 hours, a statistically significant ($p < 0.01$) reduction in C/EBPβ gene expression compared to the positive control (only FFA administration); these results were in line and not different from those obtained in the negative control (No FFA). Regarding protein levels (additional data are given in Online Resource 2), there was a statistically significant increase of C/EBPβ concentration (**Fig. 4b**) following 2 hours (58.4%; $p < 0.01$) from the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA induced, after 2 hours, a statistically significant reduction in C/EBPβ protein level (58%; $p < 0.01$) compared to the positive control (only FFA administration), while no difference has been reported compared to the negative control (No FFA).
No difference was documented for the other time points analyzed, in terms of both gene expression and protein levels.

Effect of the mix CGA + CA on PPAR-γ1 gene expression and protein levels

The results of gene expression kinetic and protein levels of PPAR-γ1 after the administration of the mix of CGA + CA at the physiological concentration of 0.3 μM are shown in **Figure 5a-b**. Gene expression and protein levels have been evaluated at five different time points (1, 2, 4, 8 and 24 hours) through three independent experiments in which every experimental condition has been tested in triplicate. There was a statistically significant increase ($p < 0.01$) of PPAR-γ1 gene expression (**Fig. 5a**) at 2 and 24 hours following the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA increased PPAR-γ1 gene expression; the magnitude of increase was comparable with the positive control (only FFA administration). Conversely, no significant effect was observed after 24 hours; here, the levels of expression were lower compared to the positive control and not statistically different from the negative control. Regarding protein levels (additional data are given in Online Resource 2), there was a statistically significant increase at 24 hours (68%; $p < 0.01$) of PPAR-γ1 protein production (**Fig. 5b**) following the administration of FFA (positive control) compared to negative control (No FFA). The treatment with the mix of CGA + CA induced, after 24 hours, a statistically significant reduction in PPAR-γ1 protein level (63%; $p < 0.01$) compared to the positive control (only FFA administration), while no difference has been reported compared to the negative control (No FFA).
No difference was documented for the other time points analyzed, in terms of both gene expression and protein levels.

Discussion

In the present manuscript, we documented the ability of CGA and CA to counteract lipid accumulation in monocyte-derived macrophages and to positively modulate the levels of PPAR-γ1 and C/EBPβ as important transcription factors involved in lipid metabolism.

The role of (poly)phenols in the modulation of lipid accumulation and metabolism has been evaluated in different in vitro studies. Results are equivocal probably due to the different experimental conditions used including cell lines, phenolic components tested, concentrations, and duration of exposure. Here, we found that physiologically relevant concentrations of CGA and CA alone did not reduce lipid accumulation in THP-1 derived macrophages. Other studies reported a beneficial effect when polyphenols were tested at supraphysiological concentrations. For example, Aranaz et al [49] observed that p-coumaric, ellagic and ferulic acids when tested at 100 μM reduced the content of triglycerides in 3T3-L1 preadipocytes, while no effect was observed at the concentrations of 10 and 50 μM. Analogous results were obtained by Zhao et al [50] reporting the capacity of 25 and 50 μg/mL, but not 5 μg/mL, of a pomegranate peel polyphenol-rich extract to counteract

lipid accumulation in RAW264.7 macrophages. In another in vitro model, Yeh and colleagues [51] showed the ability of high concentrations of a Chinese olive polyphenol-rich extract (100, 200 and 400 μg/mL) to reduce lipid content in FL83B hepatocytes. Finally, Liu and coworkers [52] documented the reduction of lipid accumulation in RAW264.7 macrophages following incubation with 80 μg/mL of a wild blueberry extract rich in CGA. Conversely, Liu et al [53] observed an increase of lipid droplets in HepG2 cells supplemented with 0.5-2 mM of CGA and these effects were attributable to the induction of the expression of SREBP-1c and PNPLA3, two important pathways involved in adipogenesis.

Interestingly, we found that CGA and CA when tested in combination reduced the amount of intracellular lipids in our in vitro model, suggesting a possible synergistic effect of the two compounds. This synergy has been observed also by other authors in several in vitro studies after the administration of different polyphenols [54-57]. We also documented that the positive effect of the mix was observed only at low-medium concentrations (0.03-3 μM), while no effect was reported at the maximum concentration tested (30 μM). This result is in line with the findings obtained in our previous study when testing an anthocyanin- and a phenolic acid-rich fraction from a wild blueberry extract [27]. Specifically, we showed that the administration of high concentrations (5 and 10 μg/mL) of a wild blueberry phenolic acid-rich fraction, rich in CGA, was not able to reduce lipid accumulation in THP-1 derived macrophages, while the low-medium concentrations (0.05-0.3 μg/mL) were effective [27]. By excluding a cytotoxic effect, the absence of a modulation could be attributed to other factors. For example, we postulated a possible involvement of the hormetic response in which biological systems exhibit a reaction when exposed to low concentration of a stimulus, while they are inhibited at high concentrations [58-60]. In addition, we cannot exclude interactions of polyphenols with components in the cell culture medium, as well as problems related to the possible formation of CGA-CA complex, when tested at maximum concentration, with a consequent loss of bioactivity of the molecules.

Another important aspect evaluated within this study was the identification of the potential mechanisms of action through which CGA and CA can modulate lipid accumulation. In particular, we tested the gene expression and protein production of C/EBPβ and PPAR-γ1, two important transcription factors that are critical orchestrators of macrophage lipid homeostasis. Numerous studies reported a connection between the expression of C/EBPβ and PPAR-γ1; for example, the differentiation of preadipocytes is regulated by a specific pathway in which, after hormonal induction, C/EBPβ activates the expression of C/EBPα and PPAR-γ1 [61-64]. There is evidence showing that PPAR-γ activation upregulates CD-36-mediated lipid accumulation in macrophages [65]. The role of phenolics in the modulation of C/EBPβ and PPAR-γ1 has been poorly evaluated. The main results regard the potential role of epigallocatechin, genistein and resveratrol in adipogenesis [66], while the contribution of CGA and CA deserves investigation. In our experimental conditions, we found that FFA induced C/EBPβ (at 2 h) and PPAR-γ1 (at 24 h) gene expression and protein levels. On the other hand, we observed a significant increase in PPAR-γ1 gene expression at 2 h after the administration of FFA that was not reflected by a significant production of PPAR-γ1 protein. This discrepancy could be attributed to the timing of sample collection, not sufficient to determine a complete translation of mRNA levels for this time point. Conversely, the administration of CGA + CA was able to counteract C/EBPβ and PPAR-γ1 gene expression and protein levels compared to the positive control (only FFA administration). Our results on PPAR-γ1 are different from those reported by Wu and colleagues [67], who found an increment (+50%) of PPAR-γ1 expression after 24 h stimulation with ox-LDL (50 mg/mL) in relation to the administration of two different concentrations of CGA (1 and 10 μM) in RAW264.7 macrophages. This discrepancy in the results could be related to the different CGA concentrations tested (more physiological in our study) and/or to the different cell models used, including species differences. For instance, RAW264.7 cells are actively proliferating macrophages which is an unusual characteristic of these cells. In fact, THP-1 cells are monocytes that must be treated (e.g. with PMA) to develop a macrophage-like characteristic and cease to proliferate. The treatment of THP-1 cells with PMA is known to induce protein kinase C [68]. Protein kinase C stimulates signaling pathways involved in sensing of intracellular energy resources [69]. However, the uptake of lipids in activated THP-1 cells is not affected by the protein kinase C activation [70]. Thus, murine macrophages such as RAW264.7 and human PMA-stimulated monocytes may respond differently to polyphenols due to differences in species background and regulation of energy sources such as lipids. In addition, we cannot exclude that lower level of PPAR-γ1 gene expression observed in our experimental conditions is due to an effective reduction of intracellular lipids at 24 h, after the administration of the mix CGA + CA. Another

possibility is that the primary expression of C/EBPβ at 2 h, found after administration of FFA, led to the second peak of PPAR-γ1 at 24 h after its transcription and translation. Thus, it is possible that the CGA + CA mix acted on PPAR-γ1 by reducing its expression through an indirect way. This hypothesis seems in line with the observations reported by Murase and coworkers [71], who documented that a coffee phenolic extract, rich in CGA, did not modulate the expression of PPAR-γ. This data could support the hypothesis of an involvement of other pathways not directly related to PPAR-γ.

Regarding C/EBPβ gene expression, Zhao et al [72] observed that palmitate induced lipid accumulation in HepG2 cells, also increasing mRNA expression of C/EBPβ. Knockdown of C/EBPβ inhibited lipid storage and promoted lipolysis in palmitate-treated cells, highlighting a key role of this transcription factor in lipid metabolism. Also, Ma et al [73] reported the capacity of oxLDL (100 μg/mL) to stimulate C/EBPβ gene expression at 12, 24 and 48 hours in RAW 264.7 macrophages, that in turn led to the induction of *p65* gene (i.e. a subunit in NF-kB), and consequently the production of inflammatory markers such as IL-1β.

The role of polyphenols in the modulation of C/EBPβ has been poorly investigated. To the best of our knowledge, only Mosqueda-Solis et al [74] tested the effect of polyphenols (luteolin, genistein, hesperidin, kaempferol, pterostilbene, or vanillic acid at a concentration of 25 μM) in the modulation of C/EBPβ and other genes (e.g. PPAR-γ) in pre-adipocytes treated from day 0 to day 8. The authors found that genistein, kaempferol and pterostilbene were effective in reducing the expression of C/EBPβ (about -55%) and PPARγ (about -65%). However, these results are difficult to compare with our findings due to the different compounds and concentrations tested.

The diverse effect on C/EBPβ and PPAR-γ1 levels could be dependent by several factors including for example the type of cells, lipid stimulation, duration of exposure as well as different polyphenols tested. A possible hypothesis regards the association of the rapid modulation of C/EBPβ and PPAR-γ1 production and the reduced lipid accumulation observed at 24 h; this effect could be attributed to the inhibition of C/EBPβ by CGA and CA, which in turn would result in a lower activation of PPAR-γ1, as demonstrated by other authors [61-64]. Consequently, the inhibition of PPAR-γ1 is supposed to downregulate CD-36-mediated lipid accumulation in macrophages, as reported by Poznyak and colleagues [65]. However, downstream targets have not been assessed in our experiments; thus, the results do not cover the array of pathways through which CGA and CA reduce lipid accumulation. Another potential explanation is represented by the inhibition of inflammatory pathway due to the suppression of C/EBPβ, that is strongly demonstrated to be involved in lipid accumulation [75,76]. In this case, PPAR would not be involved in the modulation observed and the reduction at 24 h would be essentially caused by the absence of a lipid excess in the cells, which represents a stimulus in the expression of this transcriptional factor.

Furthermore, the limited effects observed on C/EBPβ and PPAR-γ1 in relation to lipid accumulation data opens up for alternative hypotheses. For example, it is known that the liver X receptors, LXRA and LXRB, or rather a subfamily of the nuclear receptor superfamily, are important regulators of macrophage function involved in the control of lipid homeostasis and inflammation [76]. The inducible LXRA is highly expressed in macrophages and tissues such as lung and kidney, whereas LXRB is ubiquitously expressed. Ligand-activated LXRs form heterodimers with retinoid X receptors and regulate expression of target genes containing LXR response elements [77]. Tangirala et al [78] documented the crucial role of LXRs in atherosclerosis through the elimination of LXR activity in bone marrow-derived cells, which caused lipid accumulation in macrophages. Joseph et al [79] reported that LXRs and their ligands were negative regulators of macrophage inflammatory gene expression with impact on lipid metabolism and innate immune response. Finally, Zelcer et al [80] showed that LXRs can modulate lipid metabolism, thereby limiting cholesterol uptake and enhancing cholesterol efflux through ABC transporters. Based on these premises, we cannot exclude that further pathways, other than PPAR-γ and C/EBPβ, can have contributed to the modulation observed. A limitation in our findings is related to the choice of only two transcriptional factors instead of performing a complete downstream transcriptional pathway of the different genes involved in the lipid metabolism. Moreover, since CGA and CA are also rapidly metabolized in vivo mainly in the liver, the contribution of their metabolites in the modulation of PPAR-γ and C/EBPβ, or other genes, cannot be excluded.

In conclusion, the mix of CGA and CA reduced lipid accumulation in monocytes-derived macrophages probably through the modulation of PPAR-γ1 and C/EBPβ. These results are promising since the modulation of these transcription factors was obtained at concentrations of CGA and CA close to physiological conditions and thus achievable in vivo through diet. Moreover, lipid accumulation represents an important step in the process of atherogenesis and blunting its progression by dietary intervention can be considered crucial for the prevention of cardiovascular events. However, further studies are necessary in order to corroborate these findings and to verify the contribution of CGA and CA also in the regulation of other transcription factors potentially involved in macrophages lipid metabolism and atherogenesis.

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S.V. performed the experiments on lipid accumulation supervised by M.M.
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References

1. Marino M, Del Bo' C, Martini M, Porrini M, Riso P (2020) A Review of Registered Clinical Trials on Dietary (Poly)Phenols: Past Efforts and Possible Future Directions. Foods 9(11), 1606; https://doi.org/10.3390/foods9111606.
2. Martini D, Marino M, Angelino D, Del Bo' C, Del Rio D, Riso P, Porrini M (2020) Role of berries in vascular function: a systematic review of human intervention studies. Nutr Rev 78(3):189-206. doi: 10.1093/nutrit/nuz053
3. Widmer RJ, Freund MA, Flammer AJ, Sexton J, Lennon R, Romani A, Mulinacci N, Vinceri FF, Lerman LO, Lerman A (2012) Beneficial effects of polyphenol-rich olive oil in patients with early atherosclerosis. Eur J Nutr 52(3):1223-31. doi: 10.1007/s00394-012-0433-2
4. Del Bo' C, Deon V, Campolo J, Lanti C, Parolini M, Porrini M, Klimis-Zacas D, Riso P (2017) A serving of blueberry (V. corymbosum) acutely improves peripheral arterial dysfunction in young smokers and non-smokers: two randomized, controlled, crossover pilot studies. Food Funct 8(11):4108-4117. doi: 10.1039/c7fo00861a
5. Wood E, Hein S, Heiss C, Williams C, Rodriguez-Mateos A (2019) Blueberries and cardiovascular disease prevention. Food Functn 10(12):7621-7633. doi: 10.1039/c9fo02291k
6. Lutz M, Fuentes E, Ávila F, Alarcón M, Palomo I (2019) Roles of Phenolic Compounds in the Reduction of Risk Factors of Cardiovascular Diseases. Molecules 24(2). pii: E366. doi: 10.3390/molecules24020366
7. Godos J, Sinatra D, Blanco I, Mulè S, La Verde M, Marranzano M (2017) Association between Dietary Phenolic Acids and Hypertension in a Mediterranean Cohort. Nutrients. 9(10). pii: E1069. doi: 10.3390/nu9101069
8. Tajik N, Tajik M, Mack I, Enck P (2017) The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. Eur J Nutr 56(7):2215-2244. doi: 10.1007/s00394-017-1379-1
9. Tsao R (2010) Chemistry and biochemistry of dietary polyphenols Nutrients. 2(12):1231-46. doi: 10.3390/nu2121231

458 10. Singla RK, Dubey AK, Garg A, Sharma RK, Fiorino M, Ameen SM, Haddad MA, Al-Hiary M (2019) Natural
459 Polyphenols: Chemical Classification, Definition of Classes, Subcategories, and Structures. J AOAC Int 102(5):1397-1400.
460 doi: 10.5740/jaoacint.19-0133
461 11. Rothwell JA, Perez-Jimenez J, Neveu V, Medina-Remón A, M'Hiri N, García-Lobato P, Manach C, Knox C, Eisner R,
462 Wishart DS, Scalbert A (2013) Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on
463 the effects of food processing on polyphenol content, Database, Volume 2013, bat070, DOI: 10.1093/database/bat070.
464 12. Neveu V, Perez-Jiménez J, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, Scalbert A
465 (2010) Phenol-Explorer: an online comprehensive database on polyphenol contents in foods, Database, Volume 2010,
466 bap024, https://doi.org/10.1093/database/bap024.
467 13. Jakobek L, Seruga M (2012) Influence of Anthocyanins.; Flavonols and Phenolic Acids on the Antiradical Activity of
468 Berries and Small Fruits. Int J Food Prop 15:1.; 122-133.; DOI: 10.1080/10942911003754684.
469 14. Liang ND, Kitts D (2016) Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions.
470 Nutrients. 8(1): 16. doi: 10.3390/nu8010016
471 15. Farah A, de Paula Lima J (2019) Consumption of Chlorogenic Acids through Coffee and Health Implications.
472 Beverages.; 5(1).; 11; DOI: 10.3390/ beverages5010011.
473 16. Erk T, Hauser J, Williamson G, Renouf M, Steiling H, Dionisi F, Richling E (2014) Structure- and dose-absorption
474 relationships of coffee polyphenols. Biofactors 40(1):103-12. doi:10.1002/biof.1101
475 17. Stalmach AC, Steiling H, Williamson G, Crozier A (2010) Bioavailability of chlorogenic acids following acute ingestion
476 of coffee by humans with an ileostomy. Arch Biochem Biophys 501(1):98-105. doi: 10.1016/j.abb.2010.03.005.
477 18. Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C, Steiling H, Williamson G, Crozier A (2009) Metabolite
478 profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of
479 biomarkers of coffee consumption. Drug Metab Dispos 37.; 1749–1758. DOI: 10.1124/dmd.109.028019.
480 19. Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C (2007) Chlorogenic acid compounds from coffee are
481 differentially absorbed and metabolized in humans. J Nutr 137.; 2196–2221. DOI: 10.1093/jn/137.10.2196.
482 20. Farah A, Monteiro M, Donangelo CM, Lafay S (2008) Chlorogenic acids from green coffee extract are highly bioavailable
483 in humans. J Nutr 138.; 2309–2315. DOI: 10.3945/jn.108.095554.
484 21. Scherbl D, Renouf M, Marmet C, Poquet L, Cristian I, Dahbane S, Emady-Azar S, Sauser J, Galan J, Dionisi F, Richling
485 E (2017) Breakfast consumption induces retarded release of chlorogenic acid metabolites in humans. Eur Food Res Technol
486 243, 791–806. https://doi.org/10.1007/s00217-016-2793-y.
487 22. Sobhani M, Farzaei MH, Kiani S, Khodarahmi S (2020) Immunomodulatory; Anti-inflammatory/antioxidant Effects of
488 Polyphenols: A Comparative Review on the Parental Compounds and Their Metabolites. Food Rev Int doi:
489 10.1080/87559129.2020.1717523
490 23. Carullo G, Governa P, Spizzirri UG, Biagi M, Sciubba F, Giorgi G, Loizzo MR, Di Cocco ME, Aiello F, Restuccia D (2020)
491 Sangiovese cv Pomace Seeds Extract-Fortified Kefir Exerts Anti-Inflammatory Activity in an In Vitro Model of Intestinal
492 Epithelium Using Caco-2 Cells. Antioxidants (Basel) 9(1). pii: E54. doi: 10.3390/antiox9010054
493 24. Krga I, Tamaian R, Mercier S, Boby C, Monfoulet LE, Glibetic M, Morand C, Milenkovic D (2018) Anthocyanins and
494 their gut metabolites attenuate monocyte adhesion and transendothelial migration through nutrigenomic mechanisms
495 regulating endothelial cell permeability. Free Radic Biol Med 124:364-379. doi: 10.1016/j.freeradbiomed.2018.06.027
496 25. Marino M, Del Bo' C, Tucci M, Klimis-Zacas D, Riso P, Porrini M (2020) Modulation of Adhesion Process, E-Selectin
497 and VEGF Production by Anthocyanins and Their Metabolites in an in vitro Model of Atherosclerosis. Nutrients 12(3). pii:
498 E655. doi: 10.3390/nu12030655
499 26. Del Bo' C, Marino M, Riso P, Møller P, Porrini M (2019) Anthocyanins and metabolites resolve TNF-α-mediated
500 production of E-selectin and adhesion of monocytes to endothelial cells. Chem Biol Interact 300:49-55. doi:
501 10.1016/j.cbi.2019.01.002
502 27.Del Bo' C, Cao Y, Roursgaard M, Riso P, Porrini M, Loft S, Møller P (2016) Anthocyanins and phenolic acids from a wild
503 blueberry (Vaccinium angustifolium) powder counteract lipid accumulation in THP-1-derived macrophages. Eur J Nutr
504 55(1):171-82. doi: 10.1007/s00394-015-0835-z
505 28. Tsakiroglou P, Weber J, Ashworth S, Del Bo C, Klimis-Zacas D (2019) Phenolic and anthocyanin fractions from wild
506 blueberries (V. angustifolium) differentially modulate endothelial cell migration partially through RHOA and RAC1. J
507 Cell Biochem. doi: 10.1002/jcb.28383.
508 29. Gibson MS, Domingues N, Vieira OV (2018) Lipid and Non-lipid Factors Affecting Macrophage Dysfunction and
509 Inflammation in Atherosclerosis. Front Physiol 9:654. doi: 10.3389/fphys.2018.00654
510 30. Orekhov AN, Myasoedova VA (2019) Low density lipoprotein-induced lipid accumulation is a key phenomenon of
511 atherogenesis at the arterial cell level. Vessel Plus 3:3. doi: 10.20517/2574-1209.2018.80
512 31. Feingold KR, Shigenaga JK, Kazemi MR, McDonald CM, Patzek SM, Cross AS, Moser A, Grunfeld C (2012) Mechanisms
513 of triglyceride accumulation in activated macrophages. J Leukoc Biol 10, Vol.92 (4), p.829-839 doi: 10.1189/jlb.1111537.
514 32. Yu XH1, Fu YC, Zhang DW, Yin K, Tang CK (2013) Foam cells in atherosclerosis. Clin Chim Acta 424:245-52. doi:
515 10.1016/j.cca.2013.06.006

33. Tabas I (2009 Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. Antioxid Redox Signal 11(9):2333-9. doi: 10.1089/ARS.2009.2469
34. Brophy ML, Dong Y, Wu H, Rahman HN, Song K, Chen H (2017) Eating the Dead to Keep Atherosclerosis at Bay. Front Cardiovasc Med 4:2. doi: 10.3389/fcvm.2017.00002
35. Remmerie A, Scott CL (2018) Macrophages and lipid metabolism. Cell Immunol 330:27-42. doi: 10.1016/j.cellimm.2018.01.020
36. Batista-Gonzalez A, Vidal R, Criollo A, Carreño LJ (2020) New Insights on the Role of Lipid Metabolism in the Metabolic Reprogramming of Macrophages. Front Immunol 10:2993. doi: 10.3389/fimmu.2019.02993
37. Reschen ME, Gaulton KJ, Lin D, Soilleux EJ, Morris AJ, Smyth SS, O'Callaghan CA (2015) Lipid-induced epigenomic changes in human macrophages identify a coronary artery disease-associated variant that regulates PPAP2B Expression through Altered C/EBP-beta binding. PLoS Genet 11(4):e1005061. doi: 10.1371/journal.pgen.1005061
38. Rasheed A, Cummins CL (2018) Beyond the Foam Cell: The Role of LXRs in Preventing Atherogenesis. Int J Mol Sci 19(8). pii: E2307. doi: 10.3390/ijms19082307
39. Kaplan M, Aviram M, Hayek T (2012) Oxidative stress and macrophage foam cell formation during diabetes mellitus-induced atherogenesis: role of insulin therapy. Pharmacol Ther 136(2):175-85. doi: 10.1016/j.pharmthera.2012.08.002
40. Pineda Torra I, Chinetti G, Duval C, Fruchart JC, Staels B (2001) Peroxisome proliferator-activated receptors: from transcriptional control to clinical practice. Curr Opin Lipidol 12: 245 254. DOI: 10.1097/00041433-200106000-00002.
41. Li AC, Palinski W (2006) Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis. Annu Rev Pharmacol Toxicol 46: 1–39. DOI: 10.1146/annurev.pharmtox.46.120604.141247.
42. Duval C, Chinetti G, Trottein F, Fruchart JC, Staels B (2002) The role of PPARs in atherosclerosis. Trends Mol Med 8: 422–430. DOI: 10.1016/s1471-4914(02)02385-7.
43. Rahman SM, Janssen RC, Choudhury M, Baquero KC, Aikens RM, de la Houssaye BA, Friedman JE (2012) CCAAT/Enhancer-binding Protein β (C/EBPβ) Expression Regulates Dietary-induced Inflammation in Macrophages and Adipose Tissue in Mice. J Biol Chem 287(41):34349-34360. DOI: 10.1074/jbc.m112.410613.
44. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 26:171–176 doi: 10.1002/ijc.2910260208
45. Vesterdal LK, Danielsen PH, Folkmann JK, Jespersen LF, Agu- ilar-Pelaez K, Roursgaard M, Loft S, Møller P (2014) Accumulation of lipids and oxidatively damaged DNA in hepatocytes exposed to particles. Toxicol Appl Pharmacol 274:350–360 doi: 10.1016/j.taap.2013.10.001
46. Valli V, Heilmann K, Danesi F, Bordoni A, Gerhäuser C (2018) Modulation of Adipocyte Differentiation and Proadipogenic Gene Expression by Sulforaphane, Genistein, and Docosahexaenoic Acid as a First Step to Counteract Obesity. Oxid Med Cell Longev 2018:1617202. doi: 10.1155/2018/1617202
47. Mohan M, Aye PP, Borda JT, Alvarez X, Lackner AA (2008) CCAAT/enhancer binding protein beta is a major mediator of inflammation and viral replication in the gastrointestinal tract of simian immunodeficiency virus-infected rhesus macaques. Am J Pathol 173(1):106-18. doi: 10.2353/ajpath.2008.080108
48. Taverniti V, Minuzzo M, Arioli S, Junttila I, Hämäläinen S, Turpeinen H, Mora D, Karp M, Pesu M, Guglielmetti S (2012) In vitro functional and immunomodulatory properties of the Lactobacillus helveticus MIMLh5-Streptococcus salivarius ST3 association that are relevant to the development of a pharyngeal probiotic product. Appl Environ Microbiol 78(12):4209-16. doi: 10.1128/AEM.00325-12
49. Aranaz P, Navarro-Herrera D, Zabala M, Miguéliz I, Romo-Hualde A, López-Yoldi M, Martínez JA, Vizmanos JL, Milagro FI, González-Navarro CJ (2019) Phenolic Compounds Inhibit 3T3-L1 Adipogenesis Depending on the Stage of Differentiation and Their Binding Affinity to PPARγ. Molecules 24(6). pii: E1045. doi: 10.3390/molecules24061045
50. Zhao S, Li J, Wang L, Wu X (2016) Pomegranate peel polyphenols inhibit lipid accumulation and enhance cholesterol efflux in raw264.7 macrophages. Food Funct 7(7):3201-10. doi: 10.1039/c6fo00347h
51. Yeh YT, Cho YY, Hsieh SC, Chiang AN (2018) Chinese olive extract ameliorates hepatic lipid accumulation in vitro and in vivo by regulating lipid metabolism. Sci Rep 8(1):1057. doi: 10.1038/s41598-018-19553-1
52. Liu S, Sui Q, Zhao Y, Chang X (2019) Lonicera caerulea Berry Polyphenols Activate SIRT1, Enhancing Inhibition of Raw264.7 Macrophage Foam Cell Formation and Promoting Cholesterol Efflux. J Agric Food Chem 67(25):7157-7166. doi: 10.1021/acs.jafc.9b02045
53. Liu Y, Zhai T, Yu Q, Zhu J, Chen Y (2018) Effect of high exposure of chlorogenic acid on lipid accumulation and oxidative stress in oleic acid-treated HepG2 cells. Chin Herb Med 000 (2018) 1–7. https://doi.org/10.1016/j.chmed.2018.03.005
54. Chen L, Teng H, Cao H (2019) Chlorogenic acid and caffeic acid from Sonchus oleraceus Linn synergistically attenuate insulin resistance and modulate glucose uptake in HepG2 cells. Food Chem Toxicol 127:182-187. doi: 10.1016/j.fct.2019.03.038

55. Skroza D, Mekinić IG, Svilović S, Šimat V, Katalinić V (2015) Investigation of the potential synergistic effect of resveratrol with other phenolic compounds: A case of binary phenolic mixtures. J Food Compost Anal 38:13-18 https://doi.org/10.1016/j.jfca.2014.06.013
56. Aslam S, Jahan N, Rahman K, Zafar F, Ashraf MY (2017) Synergistic interactions of polyphenols and their effect on antiradical potential. Pak J Pharm Sci 30(4):1297-1304. PMID: 29039328
57. Mikstacka R, Rimando AM, Ignatowicz E (2010) Antioxidant effect of trans-resveratrol, pterostilbene, quercetin and their combinations in human erythrocytes in vitro. Plant Foods Hum Nutr 65(1):57-63. doi: 10.1007/s11130-010-0154-8
58. Calabrese EJ, Bachmann KA, Bailer AJ, Bolger PM, Borak J, Cai L, Cedergreen N, Cherian MG, Chiueh CC, Clarkson TW, Cook RR, Diamond DM, Doolittle DJ, Dorato MA, Duke SO, Feinendegen L, Gardner DE, Hart RW, Hastings KL, Hayes AW, Hoffmann GR, Ives JA, Jaworowski Z, Johnson TE, Jonas WB, Kaminski NE, Keller JG, Klaunig JE, Knudsen TB, Kozumbo WJ, Lettieri T, Liu SZ, Maisseu A, Maynard KI, Masoro EJ, McClellan RO, Mehendale HM, Mothersill C, Newlin DB, Nigg HN, Oehme FW, Phalen RF, Philbert MA, Rattan SI, Riviere JE, Rodricks J, Sapolsky RM, Scott BR, Seymour C, Sinclair DA, Smith-Sonneborn J, Snow ET, Spear L, Stevenson DE, Thomas Y, Tubiana M, Williams GM, Mattson MP (2007) Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. Toxicol Appl Pharmacol 222(1):122-8
59. Speciale A, Chirafisi J, Saija A, Cimino F (2011) Nutritional antioxidants and adaptive cell responses: an update. Curr Mol Med 11(9):770-89. doi: 10.2174/156652411798062395
60. Noyan-Ashraf MH, Wu L, Wang R, Juurlink BH (2006) Dietary approaches to positively influence fetal determinants of adult health. FASEB J 20(2):371-3. doi: 10.1096/fj.05-4889fje
61. Zhang JW, Tang QQ, Vinson C, Lane MD (2004) Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci U S A 101(1):43-7 doi: 10.1073/pnas.0307229101
62. Farmer SR (2005) Regulation of PPARgamma activity during adipogenesis. Int J Obes (Lond) 29 Suppl 1:S13-6. doi: 10.1038/sj.ijo.0802907
63. Tanaka T, Yoshida N, Kishimoto T, Akira S. (1997) Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. EMBO J16(24):7432-43. doi: 10.1093/emboj/16.24.7432
64. Zuo Y, Qiang L, Farmer SR (2006) Activation of CCAAT/enhancer-binding protein (C/EBP) alpha expression by C/EBP beta during adipogenesis requires a peroxisome proliferator-activated receptor-gamma-associated repression of HDAC1 at the C/ebp alpha gene promoter. J Biol Chem. 2006 Mar 24;281(12):7960-7. doi: 10.1074/jbc.M510682200
65. Poznyak AV, Wu WK, Melnichenko AA, Wetzker R, Sukhorukov V, Markin AM, Khotina VA, Orekhov AN (2020) Signaling Pathways and Key Genes Involved in Regulation of foam Cell Formation in Atherosclerosis. Cells 9(3). pii: E584. doi: 10.3390/cells9030584
66. Moseti D, Regassa A, Kim WK (2016) Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules. Int J Mol Sci 17(1). pii: E124. doi: 10.3390/ijms17010124
67. Wu C, Luan H, Zhang X, Wang S, Zhang X, Sun X, Guo P (2014) Chlorogenic acid protects against atherosclerosis in ApoE-/- mice and promotes cholesterol efflux from RAW264.7 macrophages. PLoS One 9(9):e95452. doi: 10.1371/journal.pone.0095452
68. Schwende H, Fitzke E, Ambs P, Dieter P (1996) Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. J Leukoc Biol 59(4):555-61. PMID: 8613704
69. Chang MY, Huang DY, Ho FM, Huang KC, Lin WW (2012) PKC-dependent human monocyte adhesion requires AMPK and Syk activation. PLoS One 7(7):e40999. doi: 10.1371/journal.pone.0040999
70. Szilagyi K, Meijer AB, Neele AE, Verkuijlen P, Leitges M, Dabernat S, Förster-Waldl E, Boztug K, Belot A, Kuijpers TW, Kraal G, de Winther MP, van den Berg TK (2014) PKCδ is dispensible for oxLDL uptake and foam cell formation by human and murine macrophages. Cardiovasc Res 104(3):467-76. doi: 10.1093/cvr/cvu213
71. Murase T, Misawa K, Minegishi Y, Aoki M, Ominami H, Suzuki Y, Shibuya Y, Hase T (2011) Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. Am J Physiol Endocrinol Metab 300(1):E122-33. doi: 10.1152/ajpendo.00441.2010
72. Zhao NQ, Li XY, Wang L, Feng ZL, Li XF, Wen YF, Han JX (2017) Palmitate induces fat accumulation by activating C/EBPβ-mediated G0S2 expression in HepG2 cells. World J Gastroenterol 23(43):7705-7715. doi: 10.3748/wjg.v23.i43.7705
73. Ma J, Liu C, Yang Y, Yu J, Yang J, Yu S, Zhang J, Huang L (2018) C/EBPβ Acts Upstream of NF-κB P65 Subunit in Ox-LDL-Induced IL-1β Production by Macrophages. Cell Physiol Biochem 48(4):1605-1615. doi: 10.1159/000492282
74. Mosqueda-Solís A, Lasa A, Gómez-Zorita S, Eseberri I, Picó C, Portillo MP (2017) Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes. Food Funct 8(10):3576-3586. doi: 10.1039/c7fo00679a
75. Lu Y, Guo J, Di Y, Zong Y, Qu S, Tian J (2009) Proteomic Analysis of the Triglyceride-Rich Lipoprotein-Laden Foam Cells.; Mol Cells 28(3):175–181.doi: 10.1007/s10059-009-0120-1.
76. Korf H, Vander Beken S, Romano M, Steffensen KR, Stijlemans B, Gustafsson JA, Grooten J, Huygen K (2009) Liver X receptors contribute to the protective immune response against Mycobacterium tuberculosis in mice. J Clin Invest 119(6):1626–1637. doi: 10.1172/JCI35288.1626.

630 77. Rasheed A, Cummins CL (2018) Beyond the foam cell: The role of LXRs in preventing atherogenesis. Int J Mol Sci
631 19(8):2307. doi: 10.3390/ijms19082307.
632 78. Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA,
633 Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG (2002) Identification of macrophage liver X receptors as
634 inhibitors of atherosclerosis. Proc Natl Acad Sci U S A 99(18):11896-901. doi: 10.1073/pnas.182199799.
635 79. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P (2003) Reciprocal regulation of inflammation and lipid
636 metabolism by liver X receptors. Nature Medicine 9:213-219. doi: 10.1038/nm820.
637 80. Zelcer N, Hong C, Boyadjian R, Tontonoz P (2009) LXR regulates cholesterol uptake through Idol-dependent
638 ubiquitination of the LDL receptor.; Science 325(5936):100-4. doi: 10.1126/science.1168974.

Fig. 1 Chemical structure of chlorogenic and caffeic acid.

Fig. 2 Effect of different concentrations (0.03 – 30 μM) of CGA (a) and CA (b) on lipid accumulation in THP-1 derived macrophages. Results are expressed as mean ± standard error of mean. Each value represents the mean of 7 values ± SEM from 3 individual experiments. [a,b] Bar graphs reporting different letters are significantly different ($p \leq 0.05$). No FFA: control without free fatty acids; FFA: free fatty acids (500μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 3 Effect of different concentrations (0.03 – 30 μM) of the mix CGA + CA on lipid accumulation in THP-1 derived macrophages. Results are expressed as mean ± standard error of mean. Each value represents the mean of 7 values ± SEM from 3 individual experiments. [a,b,c,d] Bar graphs reporting different letters are significantly different ($p \leq 0.01$). No FFA: control without free fatty acids; FFA: free fatty acids (500μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 4 Effect of the mix of CGA + CA in the modulation of the levels of expression of C/EBPβ (**a**) and protein levels (**b**) in macrophages. Results are expressed as mean of fold increase ± standard error of mean. Each value represents the mean of 3 values ± SEM from 3 individual experiments. ** shows a statistically significant difference ($p < 0.01$). No FFA: control without free fatty acids; FFA: free fatty acids (500 μM); CGA: chlorogenic acid; CA: caffeic acid

Fig. 5 Effect of the mix of CGA + CA in the modulation of the levels of expression of PPAR-γ1 (**a**) and protein levels (**b**) in macrophages. Results are expressed as mean of fold increase ± standard error of mean. Each value represents the mean of 3 values ± SEM from 3 individual experiments. ** shows a statistically significant difference ($p < 0.01$). No FFA: control without free fatty acids; FFA: free fatty acids (500 μM); CGA: chlorogenic acid; CA: caffeic acid