NMR-driven identification of anti-amyloidogenic compounds in green and roasted coffee extracts

Carlotta Ciaramelli,[1] Alessandro Palmioli,[1] Ada De Luigi,[2] Laura Colombo,[2] Gessica Sala,[3,4] Chiara Riva,[3,4] Chiara Paola Zoia,[3,4] Mario Salmona,[2] Cristina Airoldi[1,4]*

[1] Department of Biotechnologies and Biosciences, University of Milano-Bicocca, P.zza della Scienza 2, 20126 Milan, Italy; [2] Department of Molecular Biochemistry and Pharmacology, IRCCS- Istituto di Ricerche Farmacologiche "Mario Negri", Via G. La Masa 19, 20156 Milano, Italy; [3] School of Medicine and Surgery, University of Milano-Bicocca, Via Cadore 48, 20900 Monza; [4] Milan Center of Neuroscience (NeuroMI), 20126 Milano (Italy).

Carlotta Ciaramelli carlotta.ciaramelli@unimib.it

Alessandro Palmioli alessandro.palmioli@unimib.it

Ada De Luigi ada.deluigi@marionegri.it

Laura Colombo laura.colombo@marionegri.it

Gessica Sala gessica.sala@unimib.it

Chiara Riva chiara.riva@unimib.it

Chiara Paola Zoia chiarapaola.zoia@unimib.it

Mario Salmona mario.salmona@marionegri.it

*Corresponding author: Cristina Airoldi, cristina.airoldi@unimib.it; tel. +39 02 6448 3303

ABSTRACT

To identify food and beverages that provide the regular intake of natural compounds capable of interfering with toxic amyloidogenic aggregates, we developed an experimental protocol that combines NMR spectroscopy and atomic force microscopy, *in vitro* biochemical and cell assays to detect anti-Aβ molecules in natural edible matrices.

We applied this approach to investigate the potential anti-amyloidogenic properties of coffee and its molecular constituents.

Our data showed that green and roasted coffee extracts and their main components, 5-*O*-caffeoylquinic acid and melanoidins, can hinder Aβ on-pathway aggregation and toxicity in a human neuroblastoma SH-SY5Y cell line. Coffee extracts and melanoidins also counteract hydrogen peroxide- and rotenone-induced cytotoxicity and modulate some autophagic pathways in the same cell line.

KEYWORDS

Coffee, 5-CQA, melanoidins, NMR spectroscopy, anti-amyloidogenic compounds, Aβ peptides, Alzheimer's disease.

CHEMICAL COMPOUNDS STUDIES IN THIS ARTICLE

Chlorogenic acid or 5-*O*-Caffeoylquinic acid (PubChem CID: 1794427)

Neochlorogenic acid or 3-*O*-Caffeoylquinic acid (PubChem CID: 5280633)

Cryptochlorogenic acid or 4-*O*-Caffeoylquinic acid (PubChem CID: 9798666)

1. INTRODUCTION

A growing number of age-related diseases are associated with the aggregation and deposition of incorrectly folded proteins, including neurodegenerative diseases (NDs) (S. J. C. Lee, Nam, Lee, Savelieff, & Lim, 2017) with a major societal burden, such as Alzheimer's (AD) and Parkinson's diseases

(PD). The biochemical events underlying the onset of many NDs take place several years before their clinical manifestations, when neural loss is already irreversible (McDade & Bateman, 2017). These observations suggest that targeting toxic oligomers of misfolded proteins by dis-aggregating ligands and/or ligands able to prevent on-pathway aggregation has potential to be a promising prophylactic strategy against NDs, if implemented early, when neuronal loss is not yet pervasive.

In this context, regular intake in the diet of natural compounds capable of interfering with toxic oligomers could prove very effective. Indeed, interest in nutraceuticals and functional foods has greatly increased in recent years, the potential benefits of their consumption being related to the prevention not only of disorders of aging, but also of certain types of cancer, and cardiovascular diseases (Shahidi & Naczk, 2003).

We have recently set up advanced analytical techniques based on NMR spectroscopy to screen complex mixtures of natural origin for compounds that recognize and bind toxic oligomers of misfolded proteins (Airoldi, Sironi, Dias, Marcelo, Martins, Rauter, et al., 2013; Sironi, Colombo, Lompo, Messa, Bonanomi, Regonesi, et al., 2014). These analyses, combined with other biophysical and biochemical assays, enabled us to assess the neuroprotective activity of extracts from edible plants, particularly sage (Airoldi, et al., 2013) and green tea (Sironi, et al., 2014), and to identify their bioactive components.

Here we applied the same experimental strategy to screen coffee extracts for the presence of ligands and modulators of Aβ peptides, particularly the soluble Aβ oligomers involved in AD etiology which have the highest neurotoxicity among the other Aβ aggregates (Haass & Selkoe, 2007).

The choice to investigate coffee as natural matrix was based on the evidence that it is one of the most popular beverages in the world, amounting to 75% of the non-alcoholic drinks consumed regularly. Recently, interest in green coffee beverages has increased too. A number of beneficial biological effects have been described for coffee and for some classes of molecules it contains, including anti-oxidant, anti-inflammatory and anti-carcinogenic activities (Cano-Marquina, Tarín, & Cano, 2013; Palmioli, Ciaramelli, Tisi, Spinelli, De Sanctis, Sacco, et al., 2017). Recent studies report that moderate coffee

consumption may reduce the risk of NDs, suggesting a neuroprotective role of molecules in coffee extracts, particularly chlorogenic acids (CGAs) (C.-W. Lee, Won, Kim, Lee, Hwang, & Park, 2011). However, the molecular mechanisms of this biological activity remain to be clarified (Gunter, Murphy, Cross, & et al., 2017).

We therefore analyzed extracts of green and roasted coffee beans from six coffee varieties, with different geographical origins. First, we examined the interactions of their principal polyphenolic component, 5-*O*-caffeoylquinic acid (5-CQA), with Aβ1-42 oligomers, by Saturation Transfer Difference (STD)-NMR and transferred (tr)NOESY experiments. Then all the extracts were screened to confirm the binding in the presence of the other coffee constituents. The anti-amyloidogenic activity of green and roasted coffee extracts and their most characteristic components, respectively 5-CQA and melanoidins, was evaluated in biochemical and cellular assays, to validate the correlations among the recognition of the molecular targets and the biological responses. We assessed their ability to inhibit Aβ1-42 on-pathway aggregation *in vitro* by ThT assay, and the effect in prevention of Aβ-induced neurotoxicity was tested in human SH-SY5Y neuroblastoma cells. As several coffee extract components have already been reported as potent antioxidants in the same cell line, we tested the efficacy of green and roasted coffee extracts, 5-CQA and melanoidins in counteracting the cytotoxicity induced by pro-oxidant agents (hydrogen peroxide and rotenone). Then, considering the crucial role of the autophagic-lysosomal system in the clearance of aggregated and aggregate-prone proteins associated with neurodegeneration (Menzies, Fleming, & Rubinsztein, 2015), we checked if coffee extracts, 5-CQA or melanoidins influenced the two main autophagic pathways, macroautophagy and chaperone-mediated autophagy (CMA).

2. MATERIAL AND METHODS

2.1 Green and roasted coffee extraction

Ground green and medium-roasted coffee beans were received from Beyers Koffee, Belgium. Coffee extracts were obtained using a hydro-alcoholic extraction procedure. Briefly, 200 mg of ground sample was extracted with 20 mL of a mixture of acidified (with 0.1 M HCl) water (pH 4.5; 70%) and methanol (30%) by sonication at 30 kHz for 15 min in an ultrasound bath (Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) at 30 °C. After 1 h, the solutions were filtered through cotton wool and 0.45 µm PTFE filters (Pall Corporation, Port Washington, NY, USA), concentrated under reduced pressure at 40 °C and freeze-dried. The lyophilized samples were stored at -20 °C.

2.2 Separation of high and low molecular weight fractions

Freeze-dried samples of roasted coffee extracts were dissolved in MilliQ water (20 mg/mL) and the solution was ultrafiltered using tangential flow ultrafiltration devices with a polyethersulfone membrane and molecular weight cut-off (MWCO) 10 kDa (Vivaspin 500, GE Healthcare, Little Chalfont, Buckinghamshire, UK) through a centrifuge (9425*xg*, 30 min, 5 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). Five ultrafiltration steps were run on each sample, adding MilliQ water each time to the high molecular weight fraction (HMW F) to reach a volume of 500 µL and collecting each low molecular weight fraction separately (5 LMW F samples). The HMW coffee fraction (MW>10 kDa) and the LMW coffee fractions (MW<10 kDa) were freeze-dried and stored at -20 °C.

2.3 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

SDS-PAGE was done in 8% and 10% polyacrylamide separating gels (4% stacking gels) using a Mini-PROTEAN® Tetra vertical electrophoresis system and a PowerPac™ Basic Power Supply (BIO-RAD, Hercules, California, USA). Coffee enriched fractions (HMW F and LMW F), RCEs and GCEs were dissolved in Tris-HCl/SDS sample buffer (pH 6.8) containing 2-mercaptoethanol (50 g/L) and heated in a water bath for 3 min at 90 °C. In a second approach, electrophoresis was done under non-reducing conditions. Sample solutions (10 or 20 mg/mL, 16 µL) and a molecular weight marker solution (BIO-RAD prestained SDS-PAGE Standard, MW range 10-250 kDa,B10 µL) were each applied to a well.

After electrophoresis stacking at 25 mA and run at a constant voltage of 120V for 0.75 h, gels were silver stained according to the method of Heukeshoven and Dernick and interpreted.

2.4 Nuclear magnetic resonance spectroscopy

Freeze-dried samples were suspended in 10 mM deuterated phosphate buffer (PB, pH 7.4) at a final concentration of 5 mg/mL, sonicated (37 kHz, 20 min, Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) and centrifuged (10000 rpm, 10 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, final concentration 1 mM) was added to the supernatant as an internal reference for concentrations and chemical shift. The pH of each sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) for 5 mm NMR tubes and adjusted to 7.4 with NaOD or DCl. All pH values were corrected for the isotope effect. The acquisition temperature was 25 °C. All spectra were acquired on an AVANCE III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and 2H lock) cryogenic probe and a Varian Mercury 400 MHz spectrometer. ^{1}H-NMR spectra were recorded with water suppression (cpmgpr1d pulse sequences in Bruker library) and 64 scans, spectral width 20 ppm, relaxation delay 30 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. Chemical shifts were internally calibrated to the DSS peak at 0.0 ppm. Compound identification and assignment were done with the support of 2D NMR experiments, comparison with reported assignments(Wei, Furihata, Koda, Hu, Miyakawa, & Tanokura, 2012) and the SMA analysis tool integrated in MestreNova Software. The ^{1}H,^{1}H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 48 scans and 512 increments, a mixing time of 80 ms and relaxation delay 2 s. ^{1}H,^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 256 increments, relaxation delay 2 s.

For coffee bean metabolites quantification, was used the global spectrum deconvolution (GSD) algorithm, available in the Mnova software package (MestReNova v 10.0, 2016, Mestrelab Research, Santiago de Compostela, Spain). Overlapping regions were deconvoluted, and absolute quantification

was done also for coffee bean metabolites with resonances in rare crowded spectral areas. For each compound, the mean of the different assigned signals was determined.

2.5 Peptide synthesis and sample preparation

Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) was prepared by Solid Phase Peptide Synthesis (SPPS) on a 433A synthesizer (Applied Biosystems, Foster City, CA) using Fmoc-protected L-amino acid derivatives, NOVASYN-TGA resin and a 0.1 mM scale (Manzoni, Colombo, Bigini, Diana, Cagnotto, Messa, et al., 2011). Peptides were cleaved from the resin as previously described (Manzoni, Colombo, Messa, Cagnotto, Cantù, Del Favero, et al., 2009) and purified by reverse phase HPLC on a semi-preparative C4 column (Waters) using water:acetonitrile gradient elution. Peptide identity was confirmed by MALDI-TOF analyses (model Reflex III, Bruker). The purity of peptides was always above 95%.

2.6 NMR spectroscopy binding studies

NMR data were recorded on a Varian 400 MHz Mercury instrument for experiments on Aβ1–42 and coffee extracts or 5-CQA and on a Bruker Advance III 600 MHz equipped with a cryo-probe for HMW F.

Preparation of a sample containing Aβ oligomers: lyophilized Aβ1–42 was dissolved in 10 mM NaOD (280 µL) then diluted with 20 mM phosphate buffer (140 µL) and the pH adjusted to 7.4. After 1 h incubation at 37 °C, coffee extracts (final concentration 15 mg/mL) or the test compound (5-CQA, 2 mM; HMW, from 50 µg/mL to 1.5 mg/mL) dissolved in 140 µL of 20 mM phosphate buffer (pH 7.4) were added. The pH of each sample was measured with a Microelectrode (Mettler Toledo) for 5 mm NMR tubes and adjusted to pH 7.4 with NaOD and/or DCl. Total sample volumes were 560 µL. Basic sequences were employed for 2D-TOCSY, 2D-NOESY, $^{1}H,^{13}C$-HSQC and STD experiments. For STD, a train of Gaussian-shaped pulses of 50 ms each was employed to selectively saturate the protein envelope; the total saturation time of the protein envelope was adjusted by the number of shaped pulses and varied between 3 and 0.65 s. The on- and off-resonance spectra were acquired in an interleaved mode

with the same number of scans. The STD NMR spectrum was obtained by subtracting the on-resonance spectrum from the off-resonance spectrum. Reference experiments with samples containing only the free compounds were run under the same experimental conditions to verify true ligand binding. The effects observed in the presence of the protein were due to true saturation transfer, because there were no signals in the STD NMR spectra obtained in the reference experiments, except for residues from HDO, indicating that artefacts from the subtraction of compound signals were negligible.

NOESY experiments were carried out using a phase-sensitive pulse program with gradient pulses in the mixing time. Mixing times were set at 800 ms for NOESY and 350 ms for tr-NOESY.

2.7 Thioflavin T binding

Aβ1-42 were dissolved in 10 mM NaOH, H_2O and PB 50 mM (1:1:2) to 2.5 μM with or without coffee extracts (62.5 μg/mL), 5-CQA (100 μM, 35.4 μg/mL) or HMW and LMW fractions (62.5 μg/mL), and were incubated at 37 °C in 20 μM ThT (Sigma, PB 50 mM, pH 7.4) in 96-well black plates (Isoplate, Perkin Elmer). The ThT fluorescence was monitored for 24 hours by a plate reader (Infinite F500 Tecan: excitation 448 nm, emission 485 nm, 37 °C). The corresponding curves are reported in Supplementary Material – Figure S1. Data were expressed as the mean of three replicates, calculated by subtracting the relative control solutions (extracts and compounds alone) and were expressed as the percentage reduction of Aβ1–42 aggregation. The UV-Vis absorption spectra (Varian Cary® 50 UV-Vis spectrophotometer) of control solutions are reported in Supplementary Material – Figure S2.

2.8 Atomic force microscopy (AFM)

Aβ1-42 were dissolved in 10 mM NaOH, H_2O and PB 50 mM (1:1:2) to 2.5 μM with or without coffee extracts (62.5 μg/mL), 5-CQA (100 μM, 35.4 μg/mL) or HMW and LMW fractions (62.5 μg/mL), and were incubated at 37 °C for 24 hours. At different time points, 20 μL of samples were spotted onto a freshly cleaved Muscovite mica disk and incubated for 5 min. The disk was then washed with 5 mL of ddH_2O, and dried under a gentle nitrogen stream. Samples were mounted onto a Multimode AFM with

a NanoScope V system (Veeco/Digital Instruments) operating in Tapping Mode using standard antimony(n)-doped Si probes (T: 3.5–4.5 mm, L: 115–135 mm, W: 30–40 mm, f 0: 313–370 kHz, k: 20–80 N/m) (Bruker). Samples were analysed with the Scanning Probe Image Processor (SPIP Version 5.1.6 (released April 13, 2011) data analysis package. SPIP software was used to analyse the distribution of the molecular assemblies of the different populations in terms of height and diameter profiles, as previously described.

2.9 Aβ-induced cytotoxicity

Human neuroblastoma SH-SY5Y cells was grown in Dulbecco's Modified Eagle's medium (DMEM, Lonza) supplemented with L-glutamine (5 mM, Gibco), antibiotics (penicillin/streptomycin 10000 U, Lonza) and 10% heat-inactivated fetal calf serum (FCS, Gibco). The SH-SY5Y cells was seeded in 96-well plates (10^5 cell/mL) and incubated overnight (37 °C, in a humidified 5% CO_2 atmosphere). After completing planting the medium was replaced with 1% of FCS in DMEM, to reduce cell growth.

Aβ1–42 was dissolved in 10 mM NaOH, H_2O and PBS (1:1:2) and added to coffee extracts (250 μg/mL), 5-CQA (100 μM) or HMW and LMW fractions (250 μg/mL), before treatment of the SH-SY5Y cells, to obtain a final concentration of 10 μM for Aβ1–42 in the well. Cytotoxicity was evaluated after 24 h incubation, using the MTT reduction assay. Tetrazolium solution (20 μL of 5 mg/mL, Sigma Aldrich) was added to each well and incubated for 4 h. The medium was replaced with acidified isopropanol (0.04 M HCl) to dissolve the purple precipitate and the absorbance intensity was measured at 570 nm, using a plate reader (Infinite M200, Tecan). Data were expressed as percentages of controls (vehicle) for three separate replicates.

2.10 Oxidant-induced cytotoxicity

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium-F12 (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 100 U/mL penicillin (EuroClone),

100 μg/mL streptomycin (EuroClone) and 2 mM L-glutamine (EuroClone), at 37 °C in an atmosphere of 5% CO_2 in air.

SH-SY5Y cells were treated with 250 μg /mL coffee extracts, 100 μM 5-CQA or 250 μg/mL high- and low-molecular-weight (HMW and LMW) fractions for 1 h then co-treated with 100 μM hydrogen peroxide or 400 nM rotenone for 24 h. After these treatments, cell viability was evaluated by MTT assay. Cells were incubated with 0.5 mg/mL MTT in standard medium for 45 min at 37 °C in an atmosphere of 5% CO_2 in air. After the cells has been dissolved with DMSO, absorbance was quantified (wavelength 570 nm) using a multi-mode microplate reader (FLUOstar Omega, BMG LABTECH) and cell viability was expressed as % the percentage of vehicle-treated cells.

2.11 RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically at 260 nm. RNA (2 μg) was retro-transcribed into cDNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) at the following conditions: 10 min at 25 °C and 60 min at 42 °C. The reaction was terminated at 85 °C for 5 min and cDNAs were stored at -20 °C.

2.12 Real-time quantitative PCR (qPCR)

cDNAs from total RNA (50 ng for beclin-1, LC3 and hsc70 and 100 ng for lamp2A) were amplified in triplicate in the ABI Prism 7500 HTSequence Detection System (Applied Biosystems). 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) was used at the following conditions: 95 °C for 15 min, 40 cycles of: 95 °C for 15 s, 62.5 °C for 20 s, 72 °C for 20 s. The following primer pairs (Sigma-Aldrich) were used: beclin-1-F (ATCTCGAGAAGGTCCAGGCT) and beclin-1-R (CTGTCCACTGTGCCAGATGT); LC3-F (CAGCATCCAACCAAAATCCC) and LC3-R (GTTGACATGGTCAGGTACAAG); hsc70-F (CAGGTTTATGAAGGCGAGCGTGCC) and hsc70-R (GGGTGCAGGAGGTATGCCTGTGA); lamp2A-F (CACAAGGAAAGTATTCTACAGCTCA) and

lamp2A-R (CAGCATGATGGTGCTTGAGAC); β-actin-F (TGTGGCATCCACGAAACTAC) and β -actin-R (GGAGCAATGATCTTGATCTTCA). For quantification of each target in relation to β-actin mRNA, the comparative C_T method was used.

2.13 Statistical analysis

All data are shown as mean ± standard deviation (SD). Statistical analysis was done using GraphPad Prism 4.0. Repeated measures ANOVA, followed by Dunnett's multiple comparison test, was used to assess the significance of differences between groups.

3. RESULTS

3.1 Green coffee extracts and their main polyphenolic components inhibit Aβ1-42 peptide on-pathway aggregation and reduce Aβ1-42-induced neurotoxicity.

The coffee bean metabolites most studied for their potential biological activities are chlorogenic acids (CGAs) (Ludwig, Clifford, Lean, Ashihara, & Crozier, 2014), esters formed between hydroxycinnamic derivatives – mainly caffeic and ferulic acid – and quinic acid (Figure 1). CGAs are the most significant family of polyphenols in green coffee beans, with 5-*O*-caffeoylquinic acid (5-CQA) being the most abundant (Esquivel & Jiménez, 2012).

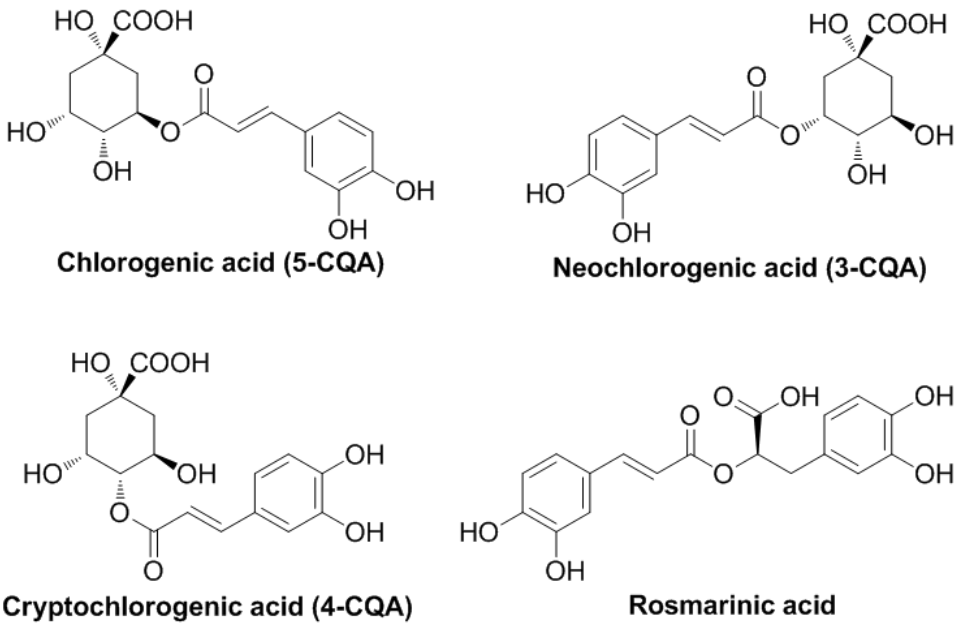


Figure 1. CGAs and rosmarinic acid chemical structures.

In 2011 Lee and co-workers reported preliminary data on the protective effect of 5-CQA against Aβ-induced neurotoxicity, so we started our investigation from this compound (C.-W. Lee, Won, Kim, Lee, Hwang, & Park, 2011). They suggested that 5-CQA attenuated Aβ-induced neurotoxicity by reducing the apoptotic effect and inhibiting calcium influx. However, moving from its structural similarity to another polyphenolic compound, rosmarinic acid (Figure 1), already reported as an Aβ1-42 ligand and aggregation inhibitor by our group (Airoldi, et al., 2013), we speculated on 5-CQA's ability to recognize and bind Aβ1-42 peptide, reducing its on-pathway aggregation.

5-CQA binding to Aβ1-42 oligomers, the most toxic form of the peptide (Lambert, Barlow, Chromy, Edwards, Freed, Liosatos, et al., 1998), was assessed by NMR spectroscopy, in particular STD-NMR (Airoldi, Merlo, & Sironi, 2015; Airoldi, Sommaruga, Merlo, Sperandeo, Cipolla, Polissi, et al., 2011; Mayer & Meyer, 1999) and tr-NOESY experiments (Airoldi, Merlo, & Sironi, 2015; Meyer & Peters, 2003), whose robustness and reliability for this purpose has already been demonstrated through numerous examples (Ahmed, VanSchouwen, Jafari, Ni, Ortega, & Melacini, 2017; Airoldi, Cardona, Sironi, Colombo, Salmona, Silva, et al., 2011; Airoldi, Colombo, Manzoni, Sironi, Natalello, Doglia, et al.,

2011; Airoldi, D'Orazio, Richichi, Guzzi, Baldoneschi, Colombo, et al., 2016; Airoldi, Mourtas, Cardona, Zona, Sironi, D'Orazio, et al., 2014; Airoldi, et al., 2013; Guzzi, Colombo, Luigi, Salmona, Nicotra, & Airoldi, 2017; Jesus, Dias, Matos, de Almeida, Viana, Marcelo, et al., 2014; Merlo, Sironi, Colombo, Cardona, Martorana, Salmona, et al., 2014; Sironi, et al., 2014). STD NMR experiments allow revealing the interaction between a small molecule and a high molecular weight biomolecule, such as a protein or, as in this case, peptide/protein oligomers, through the transfer of magnetization from the receptor to the ligand that can take place only if the two molecular entities bind each other. Some receptor resonances are selectively saturated and, after the binding, the magnetization transferred from the receptor to the ligand.

STD NMR experiments were carried out on ligand/peptide mixtures dissolved in deuterated phosphate buffer (pH 7.4, 25 °C) (Figure 2). To assure its oligomeric state, Aβ1–42 peptide was dissolved in aqueous buffer according to the procedure previously described (Airoldi, Colombo, et al., 2011). Briefly, lyophilized Aβ1-42 is dissolved in 10 mM NaOD to allow the disaggregation of any preformed seeds, leading to Aβ peptide solutions containing monomers and some low-molecular-weight oligomers. The sample is then diluted with phosphate buffer and the pH adjusted to 7.4. Because of this pH shift, the peptide aggregation starts immediately, affording the sample enrichment in Aβ oligomers. After 1h of incubation at 37° C the sample is made mainly of soluble Aβ oligomers heterogeneous in dimensions.
5-CQA was added after its dissolution in phosphate buffer, pH 7.4. The selective saturation of some aliphatic protons of Aβ oligomers was achieved by irradiating at -1.00 ppm (on-resonance frequency). When irradiation conditions are set properly to ensure there is no direct irradiation of the test compound (verified by blank experiments on a sample containing test molecule only), the detection of its NMR signals in the STD spectrum is unequivocal evidence of its interaction with the receptor. Any signal from non-binding compounds is erased in the difference spectrum (STD spectrum), thus demonstrating that the molecule is not a ligand. Thus, the 5-CQA resonances in the STD spectrum (Figure 2A2) are a non-ambiguous demonstration of the interaction with Aβ1-42 oligomers.

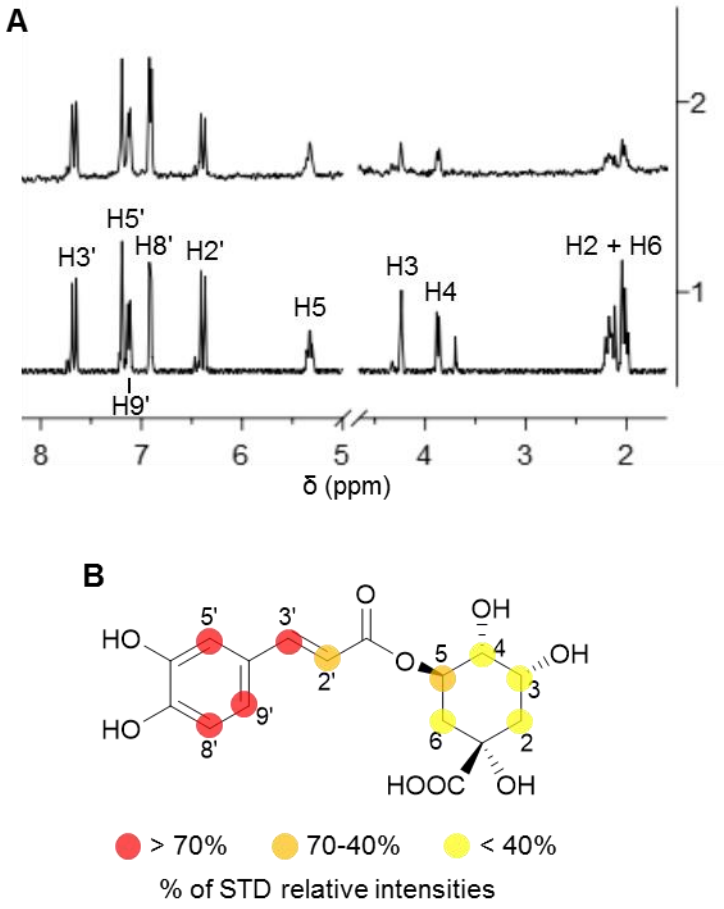


Figure 2. **STD-NMR characterization of 5-CQA interaction with Aβ oligomers. A**) ^{1}H-NMR spectrum of 2 mM (708 µg/mL) 5-CQA in deuterated phosphate buffer (**1**) and STD NMR spectrum (**2**) recorded on a mixture containing 2 mM (708 µg/mL) 5-CQA and 80 µM Aβ1-42 peptide. The STD spectrum was acquired with 1024 scans and 2 s saturation time, at 25 °C and 400 MHz. **B)**. 5-CQA binding epitope to Aβ1-42 oligomers obtained for 1.5 s saturation time. The largest relative STD intensity was scaled to 100%.

STD experiments were acquired with five different saturation times (0.65, 1.0, 1.5, 2.0 and 3.0 s), to obtain the relative STD intensity for each 5-CQA proton giving STD signals (Supplementary Material - Figure S3 and Table S1). From a qualitative point of view, stronger intensity of a ligand signal in the STD NMR spectrum indicates shorter inter-proton distances between that ligand proton and the receptor surface in the bound state. Thus, from the intensity distribution of STD signals, we obtain information

on the ligand binding epitope. The STD-based ligand epitope is depicted in Figure 2B. According to the relative intensities of STD signals, the region of the ligand mainly involved in the interaction with Aβ1-42 oligomers is the aromatic moiety, while protons of the quinic acid ring show less intense STD signals.

Further confirmation of the binding was obtained from tr-NOESY experiments (Figure S4) acquired on the same ligand/peptide mixtures used for STD NMR. Comparing the 2D-NOESY spectrum acquired in the absence of oligomers (Figure S4A) with the tr-NOESY spectrum obtained in the presence of Aβ1-42 (80 μM) (Figure S4B), the sign of 5-CQA NOE cross peaks resulted inverted. This reflects a dramatic change in 5-CQA correlation time due to its interaction with a higher molecular weight entity, Aβ oligomers. In addition, providing tr-NOESY conformational information on the bound state of the ligand, it indicates that binding to the receptor does not induce changes in the ligand conformation. Indeed, no significant differences were detected between the NOE fingerprints for free and bound ligand, as is clear from the spectra (Figure S4).

Collectively, our NMR data clearly demonstrate the ability of 5-CQA to recognize and bind amyloid oligomers, suggesting that also its neuroprotective activity in cells may depend, at least in part, on its direct interaction with Aβ peptides.

To investigate the efficacy of 5-CQA present in complex edible matrixes, and based on its significant content in green coffee, we verified its ability to interact with Aβ oligomers also in the presence of the other components of green coffee extracts (GCEs).

We prepared GCEs from ground green coffee beans by ultrasound-assisted hydroalcoholic extraction. Six coffee varieties of different origins were selected (Brazil, Burundi, Colombia, Tanzania, Uganda and Vietnam). The varieties from the first three countries belong to the Arabica species and the last three to the Robusta. ^{1}H-NMR-based metabolic profiling was obtained for each GCE (Supplementary Material – Figure S5A). A representative spectrum acquired on GCE from Vietnam beans is depicted in Figure 3A.

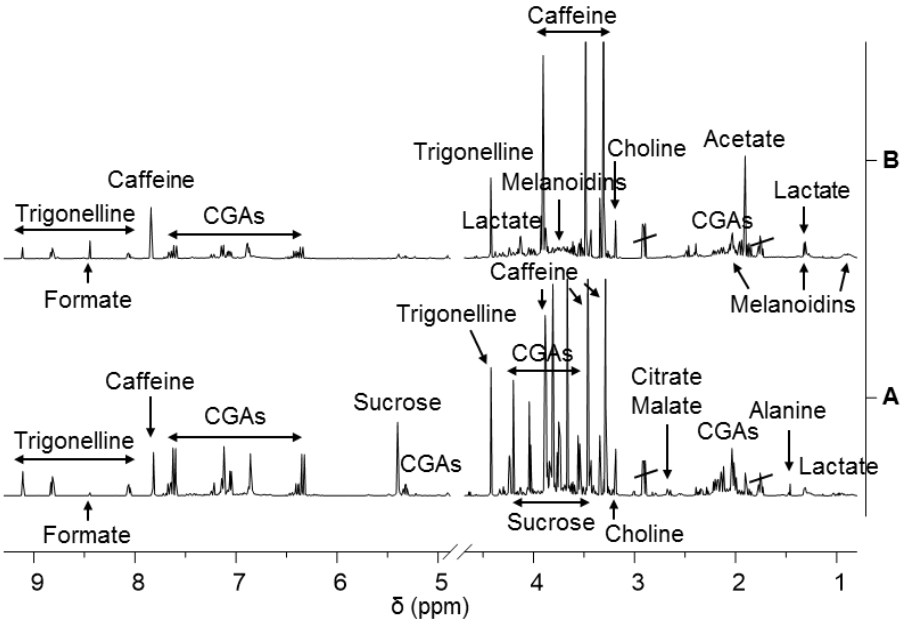


Figure 3. **NMR profiling of green and roasted coffee extracts from Vietnam**. ^{1}H-NMR spectra of green (**A**) and roasted (**B**) coffee extracts from Vietnam. Each sample, containing 5 mg/mL of extract, was dissolved in 10 mM deuterated phosphate buffer, pH 7.4, DSS 1 mM. Spectra were acquired at 25 °C and 600 MHz. Assignments for the most important coffee bean metabolites are shown.

Regardless of the geographical origin, sucrose, caffeine, CGAs and trigonelline were the most abundant coffee bean metabolites in hydroalcoholic GCEs. Among CGAs, 5-CQA had the highest concentration in all the samples (Supplementary Material – Figure S6). These findings are in full agreement with previous data (Amigoni, Stuknytė, Ciaramelli, Magoni, Bruni, De Noni, et al., 2017; Palmioli, et al., 2017; Perrone, Farah, & Donangelo, 2012; Wei, Furihata, Koda, Hu, Miyakawa, & Tanokura, 2012).

STD NMR experiments were repeated on each of the six GCEs (15 mg/mL) in a mixture with Aβ1-42 (80 μM), to verify the possible interference of other coffee bean metabolites in the extract with 5-CQA binding to oligomers. Figure 4 shows representative STD NMR spectra for Arabica Burundi (panel A, spectrum 2) and Robusta Vietnam (panel B, spectrum 2) GCEs.

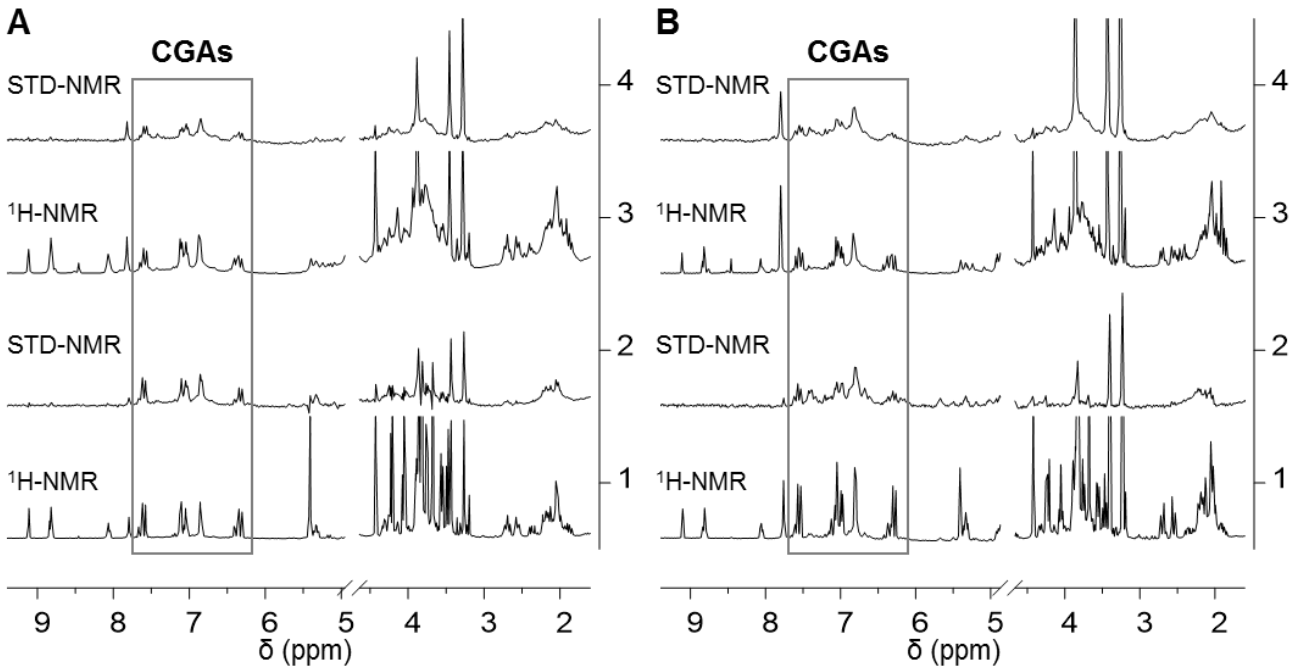


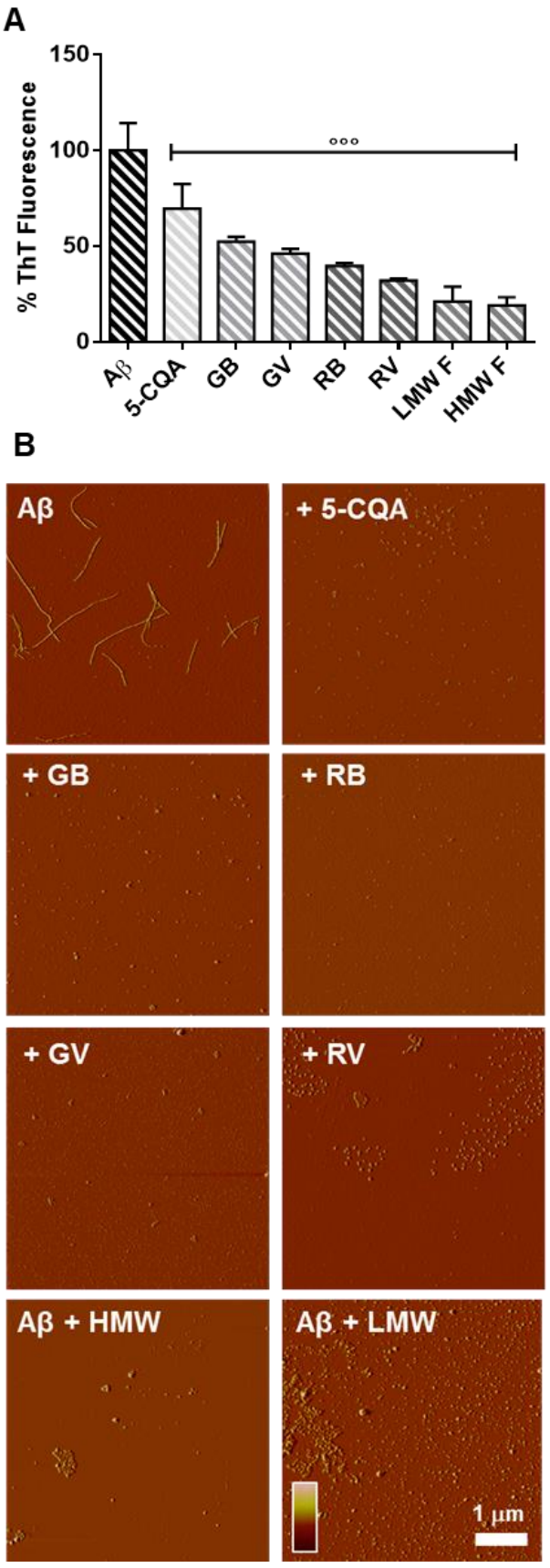
Figure 4. A) ^{1}H-NMR and STD-NMR spectra acquired on a mixture of green (1 and 2) and roasted (3 and 4) Arabica Burundi coffee extracts (15 mg/mL) and Aβ1-42 protein (80 μM) in deuterated phosphate buffer, pH 7.4. **B)** ^{1}H-NMR and STD-NMR spectra acquired on a mixture of green (1 and 2) and roasted (3 and 4) Robusta Vietnam coffee extracts (15 mg/mL) and Aβ1-42 protein (80 μM) in deuterated phosphate buffer, pH 7.4. STD spectra were acquired with 1024 scans and 3 s saturation time at 400 MHz, 25 °C.

5-CQA resonances appeared on both STD spectra (Figure 4, spectrum A2 for Burundi and B2 for Vietnam), assessing its binding to the peptide oligomers. The result was the same when ligand-receptor interaction studies were done in the presence of the other four GCEs (data not shown). Thus, 5-CQA binding to Aβ oligomers is not hindered by the other constituents of GCEs. Caffeine signals appear in STD spectra recorded in the presence of Aβ oligomers (Figure 4, spectra A2 and B2); however, blank STD spectra acquired on coffee extracts in the absence of Aβ peptide contained the same resonances (data not shown), suggesting an interaction between caffeine and other GCE components.

Close inspection of the GCEs ^{1}H-NMR spectra recorded at different times indicated a decrease of 5-CQA resonance intensities and a concomitant increase of signals from its regioisomers 3-CQA and 4-CQA. This is in agreement with earlier data (Pauli, Poetsch, & Nahrstedt, 1998; Xie, Yu, Zhong, Yuan, Ye, Jarrell, et al., 2011). 5-CQA isomerization under our experimental conditions was monitored for seven days by acquiring several ^{1}H-NMR spectra of a solution of 2 mM (708 μg/mL) 5-CQA dissolved in 10 mM deuterated phosphate buffer, pH 7.4, 25 °C (Figure S7). The spectrum recorded seven days after sample incubation (Figure S7F) showed 3-CQA and 5-CQA at a same concentrations, while 4-CQA was about half of the others, as expected from their relative stabilities (Perrone, Farah, & Donangelo, 2012; Wei, Furihata, Koda, Hu, Miyakawa, & Tanokura, 2012; Xie, et al., 2011).

Aβ1-42 peptide was added to this mixture and an STD experiment was run to assess the ability of 3-CQA and 4-CQA to interact with Aβ oligomers. As their resonances, together with those of 5-CQA, appeared in the corresponding STD spectrum (Figure S7G), we can conclude that all three molecules are Aβ ligands. This indicates that the position at which quinic acid esterification occurs does not influence the molecular recognition and fits with the prominent role of the aromatic moiety in the binding (Figure 2B).

Once we had evaluated the molecular interaction with Aβ oligomers from the structural point of view, we tested 5-CQA and GCEs anti-fibrillogenic activity in the Thioflavin T (ThT) assay (Hawe, Sutter, & Jiskoot, 2008). ThT is a benzothiazole dye that gives enhanced fluorescence upon binding to amyloid aggregates *in vitro* and *ex vivo* and is usually employed to monitor the on-pathway aggregation of Aβ peptides and quantify the formation of amyloid aggregates in the absence or presence of anti-amyloidogenic compounds. We monitored ThT fluorescence for 24 hours after the addition of Aβ peptide co-incubated with 5-CQA or GCEs and compared it with a control containing only ThT and Aβ. Figure 5A reports the results for 5-CQA and GCEs from Burundi (GB) and Vietnam (GV), as representative of Arabica and Robusta species respectively.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Aβ + LMW
1 µm

1

2

Figure 5. Effects of 5-CQA, selected coffee extracts and melanoidins on Aβ1-42 fibrillation. The effect of co-incubation (24 h at 37 °C) of 5-CQA (50 μM, 17.7 μg/mL), coffee extracts (62.5 μg/mL) or LMW and HMW fractions (62.5 μg/mL) on Aβ1-42 (2.5 μM) aggregation was determined by ThT fluorescence assay (**A**) and by AFM (**B**, Scale bar: 1 μm). Values are mean ± standard deviation of three replicates, after subtraction of their relative control solutions (extracts alone). °°°$p < 0.001$ vs. Aβ1-42 alone. One-way ANOVA followed by Dunnett's multiple comparisons test. GB: Green Burundi; GV: Green Vietnam; RB: Roasted Burundi; RV: Roasted Vietnam; LMW F: Low Molecular Weight Fraction; HMW F: High Molecular Weight Fraction.

Data for all the other GCEs are depicted in Supplementary Material - Figure S8. Co-incubating the peptide with 50 μM (17.7 μg/mL) 5-CQA for 24 h reduced Aβ1–42 on-pathway aggregation by 30% (Figure 5A). Arabica appeared less potent (green Burundi (GB) -48%, green Brazil (GR) -39%, green Colombia (GC) -38%) than Robusta (green Tanzania (GT) -55%, green Uganda (GU) -58% and green Vietnam (GV) -54%). These data correlated with the different CGAs concentrations in the samples, higher in Robusta species (Supplementary Material - Figure S6).

In addition, an AFM morphological analysis of Aβ peptide after co-incubation with 5-CQA or Burundi (GB) and Vietnam (GV) GCEs was done. AFM images (Figure 5B) showed inhibition of Aβ1–42 fibrillation in all three cases. Aβ1–42 peptide alone showed typical fibrillar structures with characteristic twisted morphology, while samples co-incubated with 5-CQA or GCEs had no fibrils but had round structures of different sizes (Figure 5B). The cumulative frequency graph reports the diameter and height distribution (Supplementary Material - Figure S9).

We then studied the ability of 5-CQA and GCEs to prevent Aβ-induced neurotoxicity by MTT assay (Airoldi, Colombo, et al., 2011) on a human neuroblastoma SH-SY5Y cell line treated with 10 μM peptide in oligomeric form. As shown in Figure 6A and Supplementary Material S10, co-treatment of SH-SY5Y cells with 100 μM (35.4 μg/mL) of 5-CQA for 24 h increased cell survival by 33.3%.

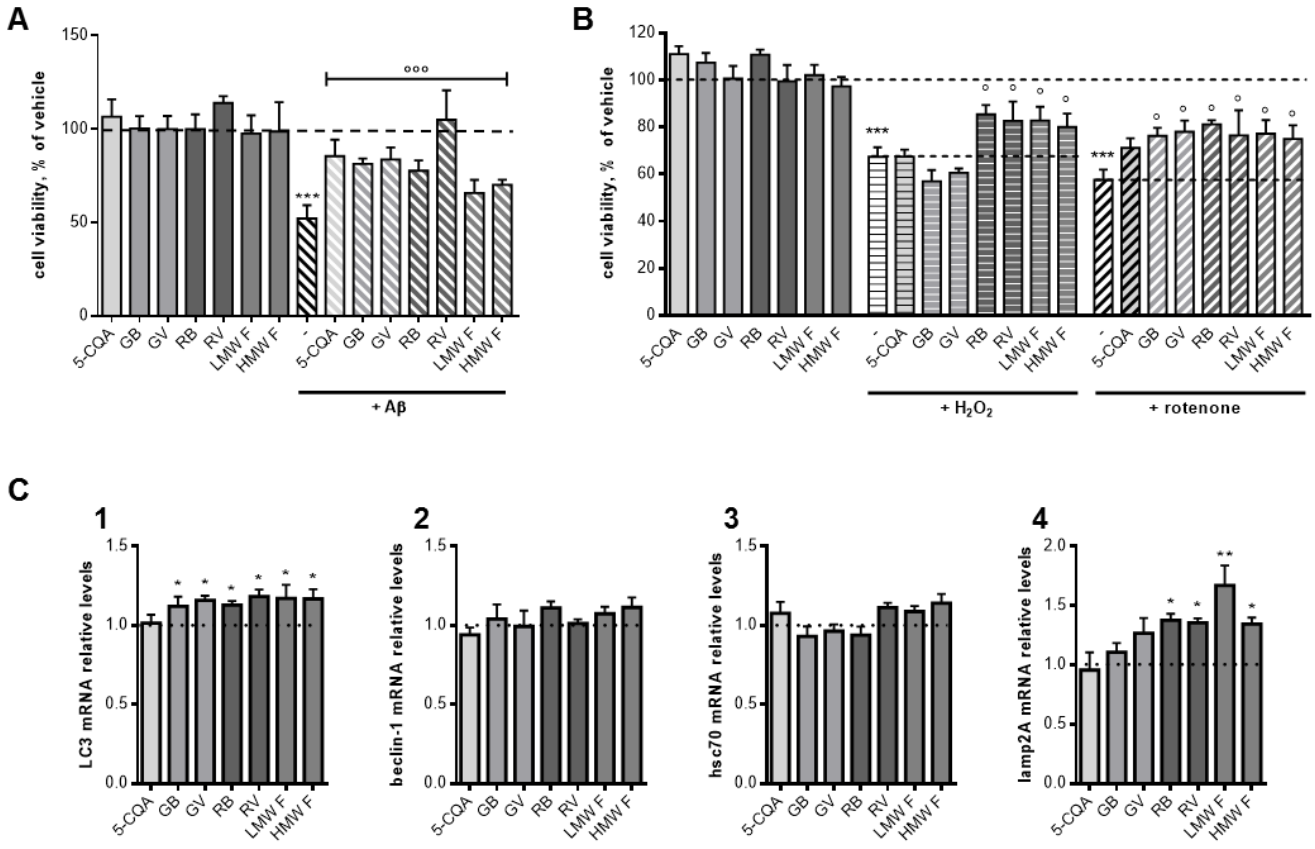

Figure 6. **Effects of 5-CQA, selected coffee extracts and melanoidins on Aβ-induced neurotoxicity, oxidative stress and autophagy on a human neuroblastoma SH-SY5Y cell line**. **A**) Effects of 5-CQA, selected coffee extracts and melanoidins on Aβ-induced neurotoxicity, by MTT assay. Cells were treated with Aβ1-42 oligomers and incubated with or without 100 µM (35.4 µg/mL) 5-CQA, coffee extracts (250 µg/mL) or LMW and HMW fractions (250 µg/mL) for 24 h. **B**) Effects of 5-CQA, selected coffee extracts and melanoidins on oxidant-induced cytotoxicity, by MTT assay. After 1 hour pre-treatment with 250 µg/mL coffee extracts, 100 µM (35.4 µg/mL) 5-CQA or 250 µg/mL HMW and LMW fractions, cells were co-treated with 100 µM hydrogen peroxide (H_2O_2) or 400 nM rotenone for 24 h. **C**) Effects of coffee extracts, 5-CQA or melanoidins on autophagy markers in human SH-SY5Y cells. Relative quantification, as the ratio to beta-actin of LC3 (**1**), beclin-1 (**2**), hsc70 (**3**) and lamp2A (**4**) mRNA after 24 h treatment with coffee extracts (250 µg/mL), 5-CQA (100 µM, 35.4 µg/mL) or HMW and LMW fractions (250 µg/mL).

Values are mean ± standard deviation of three replicates. One-way ANOVA followed by Dunnett's multiple comparison test (**A**, **B**, **C**); * $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. vehicle; ° $p<0.05$, °°°$p<0.001$ vs. Aβ1-42 alone (**A**) and H_2O_2 or rotenone (**B**). GB: Green Burundi; GV: Green Vietnam; RB: Roasted Burundi; RV: Roasted Vietnam; LMW F: Low Molecular Weight Fraction; HMW F: High Molecular Weight Fraction.

Co-treatment with all GCEs improved cell survival. (Figure 6A and Supplementary Material – S10).

In order to exclude a possible biological effect of sucrose and caffeine, the other two most abundant components of GCEs, these compounds too were tested and were completely unable to prevent Aβ fibrillation (data not shown) and Aβ oligomer cytotoxicity (Supplementary Material – Figure S11). This finding is in agreement with data previously reported (Chan, Kantham, Rao, Palanivelu, Pham, Shaw, et al., 2016; Shukitt-Hale, Miller, Chu, Lyle, & Joseph, 2013).

Our data clearly demonstrate that GCEs exert inhibitory activity on Aβ1-42 peptide on-pathway aggregation and neurotoxicity. The same biological effects were shown by 5-CQA, the most abundant polyphenolic component of GCEs, whose ability to recognize and bind Aβ oligomers was assessed by NMR spectroscopy, suggesting that its interaction with the target could be at the basis of its mechanism of action.

In addition, at an equal 5-CQA content, GCEs had greater activities than 5-CQA alone in all the biological assays. We can thus speculate that GCE biological activity cannot be ascribed only to 5-CQA. For example, 3-CQA and 4-CQA, already proved to bind Aβ oligomers (Figure S5), and other chlorogenic constituents, i.e. 3-, 4-, 5-FQA and 3,4-, 3,5-, 4,5-diCQA previously identified in GCE (Amigoni, et al., 2017), might exert an additive and/or synergistic biological effect.

Nevertheless, as worldwide consumption of roasted coffee is much higher than green, we extended our investigation to roasted coffee extracts (RCEs) too.

3.2 Roasted coffee extracts and melanoidins inhibit Aβ1-42 peptide on-pathway aggregation and lower Aβ1-42-induced neurotoxicity

RCEs were obtained with the procedure described for GCEs and from roasted beans with the same six geographical origins. All the coffee analyzed had the same medium degree of roasting. In this case too, a ^{1}H-NMR-based metabolic profile was obtained for each RCE (Supplementary Material – Figure S5B). A representative spectrum, acquired on RCE from Vietnam beans, is depicted in Figure 3B. The comparison of GCE (Figure 3A) and RCE (Figure 3B) NMR profiles shows the almost complete disappearance of sucrose in RCE with a significant reduction of CGAs, as a consequence of melanoidin formation during the roasting (Perrone, Farah, & Donangelo, 2012). The total CGA content fell from 19% (Burundi) and 27% (Vietnam) in GCEs to 13% w/w of solid extract in the RCEs from both origins. Moving from GCEs to RCEs the 5-CQA concentration varied from 11% to 4% w/w (Burundi) and 13% to 3% w/w (Vietnam) of solid extract (Supplementary Material - Figure S6).

STD experiments in the presence of Aβ oligomers (Figure 4, spectra A4 and B4) confirmed that 5-CQA in RCEs retained its ability to recognize and bind the peptide oligomers. Also in this case, blank STD spectra acquired on RCEs in the absence of Aβ peptide contained caffeine resonances (data not shown), suggesting an interaction between this molecule and other RCE components.

The ThT assay, AFM analysis and MTT assay results in the experimental conditions described for GCEs are reported in Figures 5A, 5B and 6A respectively. RCEs prevented Aβ on-pathway aggregation and counteracted Aβ-mediated neurotoxicity, with potencies comparable to those of GCEs. In the light of the reduction in CGA content, this finding was unexpected and suggested the presence of other anti-amyloidogenic species in RCEs.

As mentioned earlier, during the roasting of coffee beans, flavours and coloured compounds are formed because of pyrolysis and Maillard reaction. These are melanoidins, a class of heterogeneous brown macromolecules, whose chemical structure is complex and largely unknown (Nunes & Coimbra, 2007). They have been studied in recent years on account of their nutritional, biological and health implications,

such as antioxidant activity, metal chelating properties, antimicrobial and *in vitro* antihypertensive activity (Moreira, Nunes, Domingues, & Coimbra, 2012).

Considering their structural heterogeneity and high molecular weight (MW range 3-22 kDa, with non-covalently linked species reaching MW>100 kDa)(Gniechwitz, Reichardt, Ralph, Blaut, Steinhart, & Bunzel, 2008) we speculated that melanoidins might be able, at least in part, to sequester Aβ1-42 peptide, hindering its on-pathway aggregation and neurotoxic effects. To verify this, RCE samples enriched in melanoidins were prepared by ultrafiltration using tangential flow ultrafiltration devices with a polyethersulfone membrane and 10 kDa molecular weight cut-off (MWCO). This gives a coffee fraction enriched in high-molecular-weight melanoidins (HMW F, MW>10 kDa) and a fraction enriched in low-molecular-weight compounds - including free CGAs - (LMW F, MW<10 kDa). Their molecular weight distribution was verified by SDS-PAGE (Supplementary Material - Figure S12). For the HMW fraction, preliminary ^{1}H NMR characterization clearly showed broadened resonance compatible with the presence of macromolecules (Supplementary Material – Figure S13).

To see whether melanoidins (HMW fraction) affected the oligomerization equilibria of Aβ1-42, we acquired ^{1}H NMR spectra of the peptide (80 μM Aβ1-42 in 10 mM deuterated phosphate buffer pH 7.4) in the absence (Supplementary Material - Figure S14A) and presence of HMW fraction at different concentrations (from 50 μg/mL to 1.5 mg/mL, Supplementary Material - Figure S14B-G). The spectrum of Aβ1-42 without HMW F (Supplementary Material - Figure S14A) was line-broadened beyond the line width expected for these peptides. When the HMW fraction containing melanoidin was added to the sample, there was a reduction of peptide signal intensities and their line broadening increased. Both effects became more evident at higher concentrations of the HMW fraction in the sample. These observations are consistent with the hypothesis that melanoidins interact with the Aβ1-42 peptide.

We assessed melanoidin's ability to interfere with Aβ on-pathway aggregation and toxicity by ThT (Figure 5A), AFM analysis (Figure 5B) and toxicity assays (Figure 6A), as previously shown for the

other tested samples. The on-pathway aggregation of Aβ1–42, determined by ThT assay, was affected by co-incubation for 24 hours with the HMW fraction (-80.7%, Figure 5A). AFM analysis clearly showed the anti-fibrillation activity of melanoidins: co-incubation with the HMW fraction led to the formation of few macro-aggregates, whose morphology was completely different from fibrils formed by the peptide alone (Figure 5B). Finally, in Aβ1–42-induced cell toxicity assays the HMW fraction promoted cell survival, improving it by 17.9%. As control, also the LMW fraction, enriched in CGAs and the other small molecules characteristic of RCEs (Supplementary Material - Figure S13C), was tested. As expected on the basis of its molecular composition, it hindered both Aβ fibrillation (Figure 5) and cytotoxicity (Figure 6A).

The finding that both RCEs and melanoidins, one of their characteristic components, can exert effective action against Aβ peptide toxicity sounds very interesting and, together with the results reported for GCEs and CGAs, confirms that coffee might really serve as a source of molecules with anti-amyloidogenic activity. To further dissect coffee's biological activity, we investigated the ability of the previously tested samples to prevent oxidative stresses and modulate autophagy, in view of the vital role of these mechanisms in AD onset.

3.3 Coffee extracts and melanoidins counteract hydrogen peroxide- and rotenone-induced cytotoxicity in SH-SY5Y cells

The antioxidant activity (AOC) of coffee and melanoidins has been amply reported in the literature (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Ludwig, Clifford, Lean, Ashihara, & Crozier, 2014). Here we briefly characterized the *in vitro* antioxidant activity of our samples, measuring the total reducing capacity (Folin-Ciocalteau assay) and the ability to scavenge radical species (ABTS and DPPH assay), confirming that the effect is correlated with both the total amount of CGAs and the roasting process (Figure S15). As reported, with the exception of the Uganda and Vietnam coffees, there was a significant increase of AOC for RCEs in comparison to GCEs, Tanzania RCE being the most effective.

It was clear that the antioxidant potential of RCEs is mainly exerted by melanoidins (HMW fraction) and partially retained by the LMW fraction.

We therefore investigated the possible protective effect of coffee extracts, 5-CQA and melanoidins against oxidative stress-induced cell death by cytotoxicity assays in SH-SY5Y cells. We used hydrogen peroxide, a well-known oxidative stress donor, and rotenone, a specific mitochondrial complex I inhibitor that secondarily results in cell damage by promoting generation of peroxides, to induce a pro-oxidant milieu. Cells were pre-treated for 1 hour with green (GB and GV) or roasted (RB and RV) coffee extracts (250 µg /mL), 5-CQA (100 µM, 35.4 µg/mL) or LMW and HMW fractions (250 µg/mL), then co-treated with hydrogen peroxide (100 µM) or rotenone (400 nM) for 24 h.

Exposure to all the coffee extracts, 5-CQA or melanoidins alone did not significantly alter cell viability, while hydrogen peroxide and rotenone significantly reduced cell viability, by about 35 and 40% respectively ($p<0.001$) (Figure 6B). Both RCEs (RB and RV), melanoidins (HMW fraction) and the LMW fraction were able to partially counteracted ($p<0.05$) the hydrogen peroxide-induced cell death, while GCEs (GB and GV) and 5-CQA had no significant cytoprotective effect (Figure 6B). Furthermore, all tested coffee extracts and melanoidines, but not CGA, showed a significant protection ($p<0.05$) against rotenone-induced cell death (Figure 6B).

3.4 Effects of coffee extracts, 5-CQA and melanoidins on autophagy markers in human SH-SY5Y cells

A link between autophagy dysfunction and Aβ generation and clearance has been reported in AD (Wolfe, Lee, Kumar, Lee, Orenstein, & Nixon, 2013). Stimulation of autophagy can therefore be expected to enhance the clearance of Aβ peptide and the APP-derived fragment in neurons and provide protective effects in cellular and animal models of AD (Jiang, Yu, Zhu, Tan, Wang, Cao, et al., 2014). Both the AD-associated APP and tau proteins bear chaperone-mediated autophagy (CMA)-targeting motifs, making them potential CMA substrates.

On the basis of these considerations, we investigated a possible direct influence of coffee extracts, 5-CQA or melanoidins on autophagy.

SH-SY5Y cells were exposed to these samples for 24 h and mRNA levels of two macroautophagy (beclin-1 and LC3) and two CMA markers (hsc70 and lamp2A) were quantified by real-time PCR. Exposure to GCEs, RCEs and enriched LMW and HMW fractions, but not 5-CQA, resulted in a significant increase of LC3 mRNA levels (Figure 6C1), with a similar, although not significant, trend observed for beclin-1 mRNA levels (Figure 6C2). None of the compounds affected hsc70 mRNA levels (Figure 6C3), but RCEs (RB and RV), the LMW fraction and melanoidins (HMW F) markedly raised lamp2A mRNA levels (Figure 6C4).

The specific molecular mechanisms of this effect are still not defined and will be investigated in further studies. However, the finding that coffee extracts and melanoidins, besides inhibiting Aβ neurotoxicity and cell death due to oxidative stresses, can induce some autophagic pathways expands the panel of beneficial biological activities related to AD found in this beverage.

Our results suggest that further studies on coffee extracts hold out promise for the identification of new preventive approaches for AD.

4. CONCLUSIONS

As very recently highlighted (McDade & Bateman, 2017), there is an urgent need for effective strategies for the prevention of AD (and other NDs) instead of symptom treatments, as at this stage of the disease neuronal damage is irreversible. Aβ peptides are an ideal target for primary prevention; numerous findings indicate that their abnormal metabolism is also the cause of dominantly inherited AD (DIAD) (McDade & Bateman, 2017). However, they have to be targeted and inhibited as soon as possible. Primary prevention treatments are therefore needed, even exploiting diets rich (or "enriched") in compounds that interfere with amyloids.

Here we investigated the possible anti-amyloidogenic activity of coffee and its main molecular constituents. In view of the complexity of AD, we studied a broad panel of biological properties related to the disease: the ability to target Aβ oligomers and prevent their neurotoxicity, *in vitro* antioxidant activity and action on chaperone-mediated autophagy and macroautophagy. This experimental approach permitted a finer dissection of the neuroprotective effects of coffee extracts and their main constituents.

We found significant biological activities for green and roasted coffee, identifying the molecular components mainly responsible for their neuroprotective action, i.e. CQAs and melanoidins respectively. We also demonstrated that CQAs and melanoidins are Aβ oligomer ligands, suggesting that their ability to hinder peptide fibrillation and neurotoxicity depends on direct interaction with the target. In addition, GCEs, RCEs and melanoidins prevented cell death due to oxidative stresses and induced some autophagic pathways. Our *in vitro* assays to verify the samples' ability to inhibit neurotoxicity, counteract hydrogen peroxide- and rotenone-induced cytotoxicity, and modulate autophagy indicated that GCE and RCE were more effective than the single components, suggesting additive and/or synergistic effect of other minority compounds too. This argues in favour of the use of coffee extracts rather than the isolated bioactive compounds.

Thus, although green coffee has so far been considered the most beneficial to health, our experimental results indicate interesting biological properties also for roasted coffee, whose worldwide consumption is much greater and could therefore have more impact from the nutraceutical point of view.

One of the possible limitation to the use of coffee as a functional food for AD prophylaxis arises from the relative low biological activity of CGAs and its other bioactive components. However, CGAs average content in a cup of Espresso coffee (30 mL) is about 200 mg (40 mg for 5-CQA), so that CGAs daily intake in coffee drinkers is 0.5–1 g (Olthof, Hollman, & Katan, 2001). According to Fogliano and Morales (Fogliano & Morales, 2011) the intake of coffee melanoidins ranged between 0.5 to 2.0 g per day for moderate and heavy consumers, respectively. In a recent study the three CQA isomers and diCQA (*di*-caffeoylquinic acid) isomers were identified in the plasma of all tested subjects after roasted coffee

consumption (Monteiro, Farah, Perrone, Trugo, & Donangelo, 2007). Similarly, the major CGA compounds present in green coffee are highly absorbed and bioavailable in human plasma several hours after green coffee consumption (Farah, Monteiro, Donangelo, & Lafay, 2008). To the best of our knowledge, no definitive information about the ability of coffee bioactive compounds to cross the blood-brain barrier is available. Nevertheless, the identification of coffee bioactive components in plasma is very important since an alternative approach to reduce Aβ levels in the brain is based on lowering Aβ peptides in peripheral organs, via the so-called "peripheral sink-effect" (DeMattos, Bales, Cummins, Dodart, Paul, & Holtzman, 2001; Zhang & Lee, 2011). As Aβ peptides in the brain and periphery are in equilibrium, the removal of Aβ in the periphery, followed by its passive diffusion down a concentration gradient, would afford in a reduction of Aβ in the brain. The peripheral administration of Aβ-binding agents can promote this clearance process.

The considerable caffeine content of coffee (especially roasted coffee) might be another limitation to its use for prophylactic purposes; nevertheless, here we showed that caffeine does not hinder Aβ aggregation and neurotoxicity and is therefore not required for the coffee extract's neuroprotective activity, suggesting that decaffeinated coffee could be used for this purpose.

No significant differences were observed related to the coffee harvest areas. This is another important finding, since commercially available coffee blends are very heterogeneous as regards the geographical origins of the beans.

From the methodological point of view, our results demonstrate the power and robustness of NMR spectroscopy for screening complex mixtures of natural compounds, aimed at the identification of ligands of relevant pharmacological targets, such as amyloid aggregates.

Our approach also provided new insights into the ligand structural requirements for binding to Aβ oligomers, allowing the identification of a 5-CQA binding-epitope by STD NMR spectroscopy.

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Authors declare no conflicts of interest.

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