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ELECTRONIC SUPPLEMENTARY MATERIAL

Acrolein increases macrophage atherogenicity in association with gut microbiota remodeling in atherosclerotic mice: protective role for the polyphenol-rich pomegranate juice

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MATERIALS AND METHODS

Materials

Acrolein, triglyceride determination kit (TR0100), paraoxon, 2',7'-dichlorofluorescin diacetate (DCFH-DA), fluorescein-isothiocyanate (FITC), trypan blue, protease inhibitor cocktails, N-acetylcysteine (NAC) simvastatin, oleanolic acid, and Oil Red O powder were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rodent chow was purchased from Altromin (Lage, Germany). Cholesterol measurement kit (CHOL), glucose Accu-Chek sensor and test strips were obtained from Roche Diagnostics (Mannheim, Germany). DMEM, PBS, fetal calf serum (FCS), bovine serum albumin (BSA), penicillin, streptomycin, L-glutamine, sodium pyruvate and enzyme-linked chemiluminescence (ECL) solution were obtained from Biological Industries (Beit Haemek, Israel). [^{3}H]-labeled acetate, oleic acid and cholesterol were purchased from PerkinElmer (Waltham, MA, USA). Silica gel plates (60F254) and lactate dehydrogenase (LDH) determination kit were purchased from Merck (Darmstadt, Germany). Primary antibodies against SREBP1, SREBP2, HMGCR and DGAT1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibody against actin was purchased from Milipore (Temecula, CA, USA). HRP-conjugated secondary antibodies were obtained from Jackson Immuno-Research (West Grove, PA, USA). Primary antibody against 4-hydroxynonenal (4-HNE) was purchased from Alexis Biochemicals (San Diego, CA, USA).

PJ preparation and characteristics

Concentrated PJ (65° Brix concentrate) was processed from fresh and whole pomegranate fruits by hydraulically pressing. Large solids were removed by pumping the juice through a rotary screen. Pectinase enzymes (POM Wonderful enzymes, MFG Abitec Enzymes) were then added and the juice was held in tanks for 1 h to assure thorough depectinization. The juice was pre-concentrated in an evaporator where volatiles were stripped and rectified, and was then pasteurized at 96°C. It was then filtered on micro-filtration membranes with a molecular weight cutoff of 4500. Final concentration used an initial heating to 85-92°C and then the juice was cooled to 7°C, and kept in -28°C. PJ characteristics in accordance with the European Medicines Agency (EMA) guideline on declaration of herbal substances and herbal preparations are as follows (Rom and Aviram 2016):

Name of the herbal substance used: Punica granatum fruit

Type of the herbal preparation: Expressed juice from whole punica granatum fruit

Quantity of the genuine expressed juice: 100% genuine expressed juice

DER (drug extract ratio) genuine: 11.4:1

Other excipients: 0%

Quantity of genuine expressed juice in the herbal medicinal product: 1 ml/ml oral liquid

The total polyphenol concentrations of PJ were confirmed spectrophotometrically with phosphomolybdic-phosphotungstic acid reagent and gallic acid as a standard, and were reported as gallic acid equivalents (GAE) (Ben Nasr et al 1996).

ApoE$^{-/-}$ mice study

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH, USA. The protocol for the study was approved by the Committee for Supervision of Animal Experiments of the Technion – Israel Institute of Technology (Approval number: IL0080112). ApoE$^{-/-}$ mice are widely used as animal models for atherosclerosis, since they develop severe hyperlipidemia on a regular chow diet and the histopathology of their lesions closely resemble those in humans (Meir and Leitersdorf 2004). The apoE$^{-/-}$ mice stock (provided in courtesy of Prof. Jan Breslow, Rockefeller University, NY) were bred and housed in pathogen-free conditions at the Animal Care Facility of the Rappaport Faculty of Medicine, Technion.

Acrolein dose and calculation of animal sample size were based on previous reports regarding acrolein effects of exacerbating dyslipidemia and atherosclerotic lesion formation in apoE$^{-/-}$ mice (Srivastava et al. 2011). Sample size calculation was performed using WinPeppi software (version 11.43). The primary measure used for the calculation of sample size was the difference in serum cholesterol between control and acrolein exposed apoE$^{-/-}$ mice, which was previously reported (Srivastava et al. 2011). A sample of 6 mice per group was projected to provide power of 80% ($\alpha = 0.05$) and to detect a difference in serum cholesterol of 90 mg/dl between control and acrolein exposed mice. PJ dose was based on previous studies from our laboratory reporting anti-oxidative/ anti-atherogenic effects of PJ on apoE$^{-/-}$ mice on plasma lipids and peritoneal macrophages of mice (MPM) (Aviram et al. 2000; Kaplan et al. 2001).

Twenty-four male apoE$^{-/-}$ mice aged 7 weeks were divided into four experimental groups (n = 6 per group) for a period of 4 weeks: 1) Control group: received no addition to their drinking water. 2) Acrolein group: received acrolein which dissolves very well in water (Abraham et al. 2011), in their drinking water (3 mg/kg/day). 3) Acrolein + PJ group: received acrolein and PJ in their drinking water (3 mg/kg/day and 7 mg GAE/kg/day, respectively). 4) PJ group: received PJ in their drinking water (7 mg GAE/kg/day). The water intake by the mice was monitored daily to confirm the doses of acrolein and PJ per mouse. Results on group 4 in which mice were treated with PJ alone confirmed the previous reports from our laboratory and showed substantial anti-oxidative/anti-atherogenic effects of PJ on serum, aortas and MPM (Aviram et al. 2000; Kaplan et al. 2001). Thus, these data were not presented in the current report.

Serum analyses

Serum analyses were conducted as previously described (Rosenblat et al. 2015). Blood was collected from the retro-orbital plexus of mice under isoflurane anesthesia (via inhalation). The serum was separated from the clotted blood by centrifugation (1000 g for 15 min) and kept at -80°C. Serum samples were analyzed individually within each group. Serum cholesterol, triglycerides and glucose were measured using commercially

available diagnostic kits. The serum levels of lipid peroxides were measured by the lipid peroxides assay (in non-diluted serum) (el-Saadani et al. 1989), and by the thiobarbituric acid reactive substances (TBARS) assay (diluted serum 1:5), which measures malondialdehyde (MDA) equivalents (Buege et al. 1978). Results were expressed as nmol/ml lipid peroxides or MDA. Serum PON1 activity was determined spectrophotometrically at 412 nm with paraoxon as a substrate. The assay mixture included 10 µl of non-diluted serum, 4 mM paraoxon, 50 mM glycine and 1 mM $CaCl_2$. One unit of paraoxonase activity = 1 nmol of hydrolyzed paraoxon per min/ 1 mL serum.

Aortic analyses

Aortic analyses were conducted as previously described (Rosenblat et al. 2015). Aortas were rapidly removed from isoflurane anesthetized mice and kept at -80°C. Subsequently, aortas were cleared of adhering fat and connective tissue and were then homogenized in 1 ml PBS using Polytron Homogenizer (Kinematica AG, Littau, Switzerland) at 60W for 1 min. Aorta homogenates were then centrifuged at 5000 g for 20 min and the supernatants were analyzed for protein levels by the Lowry assay (Lowry et al. 1951). Aortic lipids were extracted with hexane:isopropanol (3:2, *v:v*), and the hexane phase was evaporated under nitrogen. The amount of aortic cholesterol and triglycerides were determined using commercial kits, and were expressed as µg cholesterol or triglycerides/mg protein. Aortic levels of lipid peroxidation were measured by the lipid peroxides and TBARS assays (Buege et al. 1978; el-Saadani et al. 1989), and were expressed as nmol lipid peroxides or MDA/mg protein.

Mouse peritoneal macrophages (MPM) isolation

Thioglycolate-elicited MPM were isolated as previously described (Rosenblat et al. 2015). MPM were harvested from the peritoneal fluid of isoflurane anesthetized $apoE^{-/-}$ mice, 3 days after intraperitoneal injection of 3 mL of thioglycolate (40 g/L) in saline into each mouse. The cells ($10\text{-}20x10^6$ per mouse) were washed three times with PBS and centrifuged at 1000 g for 10 min. Then, MPM were resuspended in DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FCS. MPM were then plated and incubated in a humidified incubator (37°C, 5% CO_2) for 2 h, washed with DMEM to remove non-adherent cells and the monolayer was further incubated under similar conditions.

***In vitro* acrolein exposure**

J774A.1 murine macrophage-like cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FCS. $1x10^6$, $2x10^6$ and $4x10^6$ cells were plated in 12 wells plates, 6 well plates and T-25 flasks, respectively. J774A.1 macrophages were incubated with increasing concentrations of acrolein (0-100 µM) for 16 h in the absence or presence of PJ (0-40 µM GAE), NAC (10 mM), simvastatin (15 µg/ml), or DGAT1 inhibitor - oleanolic acid (75 µM). The doses of PJ, NAC, simvastatin and oleanolic acid were based on previous studies (Fuhrman et al.

2005; Rosenblat et al. 2009, 2013; Yang et al. 2015). The toxic effects of acrolein (0-100 μM) on J774A.1 macrophages ($1x10^6$ cells) were assessed by trypan blue exclusion, determination of cell protein by the Lowry assay (Lowry et al. 1951), and by the release of LDH into the culture medium.

MPM and J774A.1 macrophage oxidative status

DCFH-DA assay

Intracellular reactive oxygen species (ROS) levels in MPM isolated from $apoE^{-/-}$ mice or in J774A.1 macrophages ($1x10^6$ cells) were determined with DCFH-DA. Cells were washed with PBS and incubated for 40 min with 10 μM DCFH-DA at 37°C. Then, the cells were scraped and measured by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA). Results were expressed as mean fluorescence intensity (MFI).

Cellular lipid peroxidation

Cellular lipids were extracted with hexane:isopropanol (3:2, *v:v*) and the hexane phase was evaporated under nitrogen. Lipid peroxidation was determined by the lipid peroxides assay and was expressed as μmol lipid peroxides /mg protein (el-Saadani et al. 1989).

4-HNE immunofluorescence

Cellular lipid peroxidation was also analyzed by immunofluorescent staining using an antibody against the lipid peroxidation product 4-HNE. J774A.1 macrophages ($1x10^6$ cells) were plated in 12-well glass bottom plates and stimulated with acrolein in the absence or presence of PJ. Then, cells were washed with PBS (x2) and fixed with 4% paraformaldehyde for 15 min. Cells were then washed with PBS (x3) and permeabilized with 0.1% triton for 5 min on ice. Then, cells were incubated with 10% donkey serum overnight at 4°C to prevent nonspecific binding. The next day, cells were washed with PBS (x3) and incubated with an antibody against 4-HNE diluted 1:200 with 1% BSA for 24h at 4°C, then washed with PBS (x3). Negative control cells were not incubated with the primary antibody; rather, they were incubated only with 10% donkey serum. An immunofluorescent reaction was achieved by the addition of a fluorescent goat anti rabbit IgG-Cy3 secondary antibody (1:200) for 45 min in the dark followed by PBS washes (x3). Then, cells were visualized by a fluorescence microscope (Axio Obsrever.Z1; Carl Zeiss, Oberkochen, Germany) and were photographed using a high-resolution monochrome Hamamatsu Orca R2 camera (Iwata, Japan).

MPM and J774A.1 macrophage cholesterol and triglyceride mass

The lipids of MPM from $apoE^{-/-}$ mice or J774A.1 macrophages ($1x10^6$ cells) were extracted with hexane:isopropanol (3:2, *v:v*), and the hexane phase was evaporated under nitrogen. The amount of cellular cholesterol and triglycerides were determined using commercial kits. The remaining cells in the plates were dissolved in 0.1 M NaOH, and an aliquot was taken for the measurement of cellular protein by the Lowry assay (Lowry et al. 1951). Results were expressed as μg cholesterol or triglycerides/mg cell protein.

MPM and J774A.1 macrophage cholesterol biosynthesis rate

Cellular cholesterol biosynthesis was assayed after incubation of cells ($2x10^6$) for 3 h at 37°C with [^{3}H]-acetate (3.3 μCi/ml) in serum-free medium supplemented with 0.2% BSA (Fuhrman et al. 2005). Cellular lipids were extracted with hexane/isopropanol (3:2, *v:v*), and the upper phase was dried under nitrogen. The lipids were then separated by TLC on silica gel plates and developed in hexane/ether/acetic acid (130:30:1.5, *v:v:v*). Unesterified cholesterol spots were visualized by iodine vapor (by using an appropriate standard for identification) and [^{3}H]-labels were counted by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA). Results were expressed as cpm/mg cell protein.

MPM and J774A.1 macrophage triglyceride biosynthesis and hydrolysis rates

Cellular triglyceride biosynthesis was assayed after incubation of cells (2×10^6) for 3 h at 37°C with [^{3}H]-oleic acid (3.3 μCi/ml) in serum-free medium supplemented with 0.2% BSA (Rosenblat et al. 2009). Cellular lipids were extracted, separated, and developed as described above. Triglyceride spots were visualized by iodine vapor (by using an appropriate standard for identification) and [^{3}H]-labels were counted by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA). Results were expressed as cpm/mg cell protein.

Following triglyceride biosynthesis assays, J774A.1 macrophages (2×10^6) were washed with PBS (x2) and incubated for 1, 2 and 4 h with serum-free medium supplemented with 0.2% BSA to determine the rate of triglyceride hydrolysis. After each of the indicated times, cellular lipids were extracted, separated and developed as described above. Triglyceride spots were visualized by iodine vapor and [^{3}H]-labels were counted by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA). Results were expressed as cpm/mg cell protein.

Lipoprotein isolation

LDL and HDL were isolated from fresh plasma derived from healthy normolipidemic healthy subjects by discontinuous density gradient ultracentrifugation as previously described (Rosenblat et al. 2015). The LDL was separated at d=1.063 g/ml, and the HDL at d=1.210 g/ml. Both lipoproteins were dialyzed against 150 mM NaCl, 1 mM Na_2EDTA (pH 7.4) at 4°C, and then sterilized by filtration, kept under nitrogen in the dark at 4°C, and used within 2 weeks. Protein concentration of LDL and HDL was determined by the Lowry assay (Lowry et al. 1951).

Copper ion-induced LDL oxidation

Prior to oxidation, the LDL was dialyzed against EDTA-free PBS solution (pH 7.4), at 4°C. LDL (1 mg of protein/ml) was incubated with 5 μM of $CuSO_4$ for 20 h at 37°C. At the end of the incubation, the extent of LDL oxidation was determined by the lipid peroxide and TBARS assays (Buege et al. 1978; el-Saadani et al. 1989).

MPM and J774A.1 macrophage uptake of LDL or oxidized LDL (Ox-LDL)

LDL and Ox-LDL were conjugated to FITC for cellular lipoprotein uptake studies (Etxebarria et al. 2014; Rosenblat et al. 2015, 2016). Cells ($1x10^6$) were incubated for 3 h at 37°C with FITC-conjugated LDL or FITC-conjugated Ox-LDL at a final concentration of 25 μg of protein/ml in serum-free medium supplemented with 0.2% BSA. The uptake of the lipoproteins was determined by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA). Results were expressed as MFI.

HDL-mediated cholesterol efflux from MPM and J774A.1 macrophages

Cells ($1x10^6$) were incubated with [^{3}H]-labeled cholesterol (2 μCi/ml) in serum-free medium supplemented with 0.2% BSA for 1 h at 37°C. After washing with PBS (x3), the cells were further incubated with DMEM in the presence of HDL (100 μg HDL protein/mL) or in the absence of HDL (basal, non-specific loss of cholesterol from the cells to the medium) for 3 h at 37 °C. Cellular and medium [^{3}H]-labels were quantified by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA), and the basal or HDL-mediated cholesterol efflux was calculated as the ratio of [^{3}H]-label in the medium/([^{3}H]-label in the medium + [3H]-label in the cells). Net HDL-mediated cholesterol efflux data were corrected for the basal.

Assessment of macrophage lipid droplets by Oil Red O staining

J774A.1 macrophages were plated in Costar™ 12-well plates. The cells ($1x10^6$) were incubated without or with acrolein (25 μM) for 16 h in the absence or presence of PJ (40 μM GAE). Macrophages were also incubated with Ox-LDL (50 μg/ml) as positive control. Following the treatments, macrophage lipid droplets were stained by Oil Red O as previously described (Sengupta et al. 2013). Oil Red O stock solution was prepared by dissolving 0.35 g of Oil Red O powder in 100 ml of isopropanol (100%). The next day, the stock solution was filtered (0.2 μm) and the Oil Red O working solution was prepared by diluting the stock solution with DDW (6:4 *v:v*), allowing to stand for 10 min at room temperature and then filtered (0.2 μm). Following the cell treatments, macrophages were washed with PBS followed by cell fixation in 10% phosphate buffered formalin for 1 h. Then, macrophages were rinsed in DDW and then rinsed in 60% isopropanol for 5 min to facilitate the lipid staining. Next, the cells were stained with the Oil Red O working solution for 10 min, followed by rinsing with DDW (x4). Images were taken under 20× objective using a light microscope (Carl Zeiss, Oberkochen, Germany) with a Hamamatsu Orca R2 camera (Iwata, Japan). Oil Red O-stained lipid droplets were further quantified by elution of the stain with 100% isopropanol for 10 min at room temperature. After a thorough pipetting to ensure complete elution, the absorbance was measured at 500 nm.

Cell lysates and Western blotting

Following experiments, cells ($4x10^6$) were washed twice by PBS and lysed with RIPA buffer supplemented with protease inhibitor. Total protein concentrations in lysates were measured by the Bradford assay, using BSA as a standard. A total protein of 25 μg per lane was loaded and separated by standard SDS-PAGE (Rom et al. 2013). Then, proteins were transferred to nitrocellulose membranes. Membranes were then blocked with 5% non-fat milk powder in TBS-T (0.125% Tween) for 1 h and exposed overnight to primary antibody at 4°C. Primary antibodies were diluted as follows: SREBP1 (1:500), SREBP2 (1:500), HMGCR (1:500), DGAT1 (1:500), actin (1:4000). The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with appropriate secondary antibodies. Detection was performed with ECL using ImageQuant LAS 4000 digital imager system (GE Healthcare, Bucks, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software V2006C (Nonlinear Dynamics, Newcastle-on-Tyne, UK). Actin was used for normalization of protein quantities as a loading control. Results were expressed as relative protein levels vs. control.

Microbiota composition analysis

Fecal samples were collected from each mouse individually before and after the treatment period and stored at -80°C until processing. Total fecal DNA was extracted from the samples using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). Genomic DNA was PCR amplified using primers 341F and 806R, targeting the V3-V4 region of the bacterial and archaeal 16S rDNA (Green et al. 2015). The primers contained 5' common sequence linkers (CS1 and CS2) as previously described (Moonsamy et al. 2013). The forward primer, CS1_341F (ACACTGACGACATGGTTCTACACCTACGGGAGGCAGCAG) and CS2_806R (TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT) were synthesized by Sigma–Aldrich (St. Louis, MO, USA) as standard oligonucleotides. Each PCR mixture contained 0.2 mM deoxynucleoside triphosphates, 0.4 μM forward and reverse primers, 0.02 U of Taq polymerase (SuperNova, JMR Holding, Kent, England) per μl, 1×reaction buffer (containing 1.5 mM MgCl2) and 5 μl of DNA (5 ng per reaction). The reaction carried out in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as follows: 95˚C/15 min, 35x (95˚C/45 sec, 60˚C/45 sec, 72˚C/1 min), 72˚C/10 min, as previously described (Green et al. 2015). PCR amplification products were verified by gel (1.2%) electrophoresis and observed by UV fluorescence. A second PCR reaction was established, with 1 μl of amplification product from the first stage used as input to the second reaction. The primers for the second stage amplifications were the AccessArray barcoding system primers (Fluidigm, South San Francisco, CA, USA), containing Illumina sequencing adapters, sample-specific barcodes (10 bases, with a minimum hamming distance of 3), and CS1 and CS2 linkers (Green et al. 2015). The reaction carried out in a MyFi 2X PCR mastermix (Bioline, Taunton, MA, USA) as follows: 95°C/5 min, 8x (95°C/30 sec, 60°C/30 sec, 72°C/1 min). Final PCR products were purified and equalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples were pooled in equimolar ratio and quantified using a Qubit 2.0 fluorometer

with the dsDNA BR Assay (Life Technologies, Grand Island, NY, USA). Sequencing was performed on an Illumina MiSeq sequencer using standard V3 chemistry with paired-end, 300 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Demultiplexing of reads was performed on instrument. Library preparation and sequencing was performed at the DNA Services Facility at the University of Illinois at Chicago.

The resulting paired-end FASTQ files were merged using PEAR software package (Zhang et al. 2014). The software package CLC genomics workbench (v7; CLC Bio, Qiagen, Boston, MA, USA) was used for primer, quality (Q20) and length trimming (sequences <390 bp were removed). A chimera check (USEARCH61) was performed and putative chimeras were removed from the dataset (Edgar et al. 2011). Sequence data was processed using quantitative insight into microbial ecology (QIIME) 1.8.0 pipeline against Greengenes database (v13_8) as reference (Caporaso et al. 2010). For phylogenetic analyses, operational taxonomic units (OTUs) were defined based on 97% similarity clustering. Sequence reads are available under NCBI BioProject SUB1471256 (http://www.ncbi.nlm.nih.gov/bioproject/).

Statistical analysis

Results are presented as mean ± SEM of at least three independent observations. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc. IBM, Chicago, IL, USA) by one-way ANOVA followed by Bonferroni post-hoc tests to compare the means between the groups. $p<0.05$ was considered statistically significant. For microbiota data, diversity analyses were performed with QIIME pipeline (Caporaso et al. 2010), using sampling depth of 25,000 reads per sample. Linear discriminant analysis coupled with effect size measurements (LEfSe) was used to identify differences in relative abundance within each experimental group separately, using an alpha value of 0.05 following by the Kruskal – Wallis test with a threshold of 2.5 for logarithmic linear discriminant analysis (LDA) scores (Segata et al. 2011). Taxa which showed LDA>2.5 following acrolein treatment were further tested for correlation calculations with nine atherosclerosis-related markers: levels of cholesterol, triglycerides and lipid peroxides in serum, aorta and in MPM. The Spearman correlation coefficients were calculated using R (R Foundation for Statistical Computing, Vienna, Austria). Several pairs of taxa appeared to have very similar relative abundance values across all 18 samples (3 groups x 6 mice). Therefore, 'equivalence groups' were created using the following rule: if for markers *i* and *k* we have

$$max_{j=1,\dots 18}\{|y_{ij} - y_{kj}|\} < 10^{-5}$$

then these two markers are considered equivalent for the purpose of analyzing their correlation with the atherosclerosis-related markers. Among the 58 taxa which were identified by LEfSe, 36 equivalence groups were found. For each representative from a group of taxa its correlation with the nine markers was calculated, and the null hypothesis that the mean correlation coefficient was zero was tested. To account for multiple testing, the 36 p-values were adjusted using Holm's method.

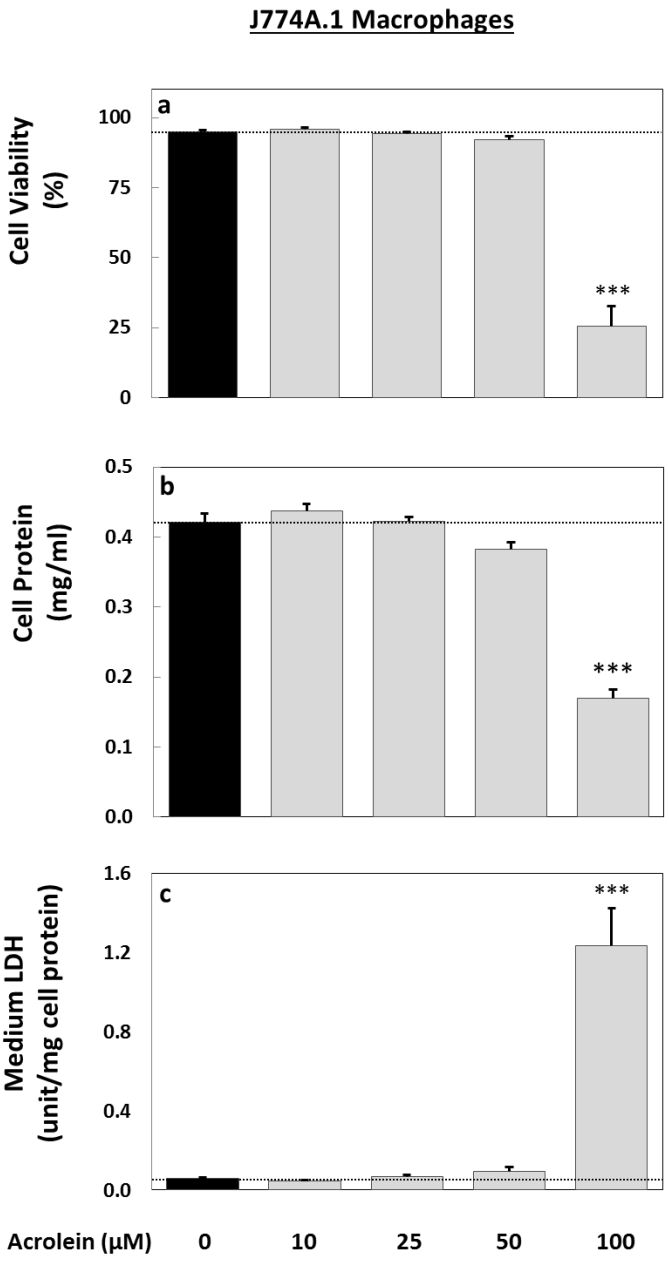

Fig. S1 Cytotoxic effects of acrolein on J774A.1 macrophages. J774A.1 macrophages were incubated without acrolein (control) or with increasing concentrations of acrolein (10-100 µM) for 16 h, followed by assessments of **(a)** cell viability with trypan blue exclusion, **(b)** cell protein, and **(c)** LDH levels in the culture medium, as described under Materials and Methods. Results are expressed as mean ± SEM (n=3-6). ***$p<0.001$ vs. control group

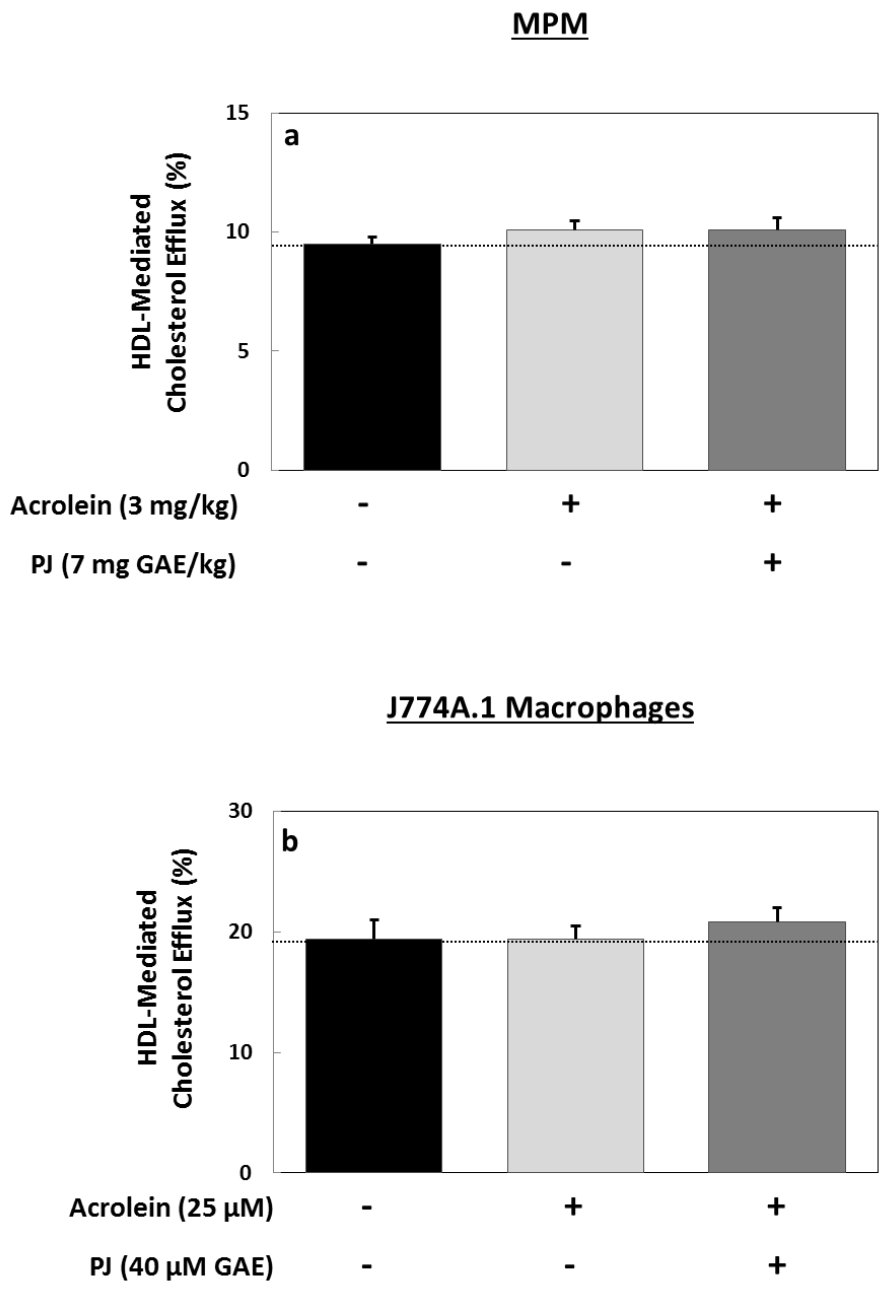

Fig. S2 Acrolein effects on HDL-mediated cholesterol efflux from MPM and J774A.1 macrophages. **(a)** MPM were isolated from apoE$^{-/-}$ mice, followed by assessments of HDL-mediated cholesterol efflux from MPM, as described under Materials and Methods, (n=6 mice per group). **(b)** J774A.1 macrophages were incubated without or with acrolein (25 µM) for 16 h in the absence or presence of PJ (40 µM GAE) followed by assessments of HDL-mediated cholesterol efflux from the cells (n=3).

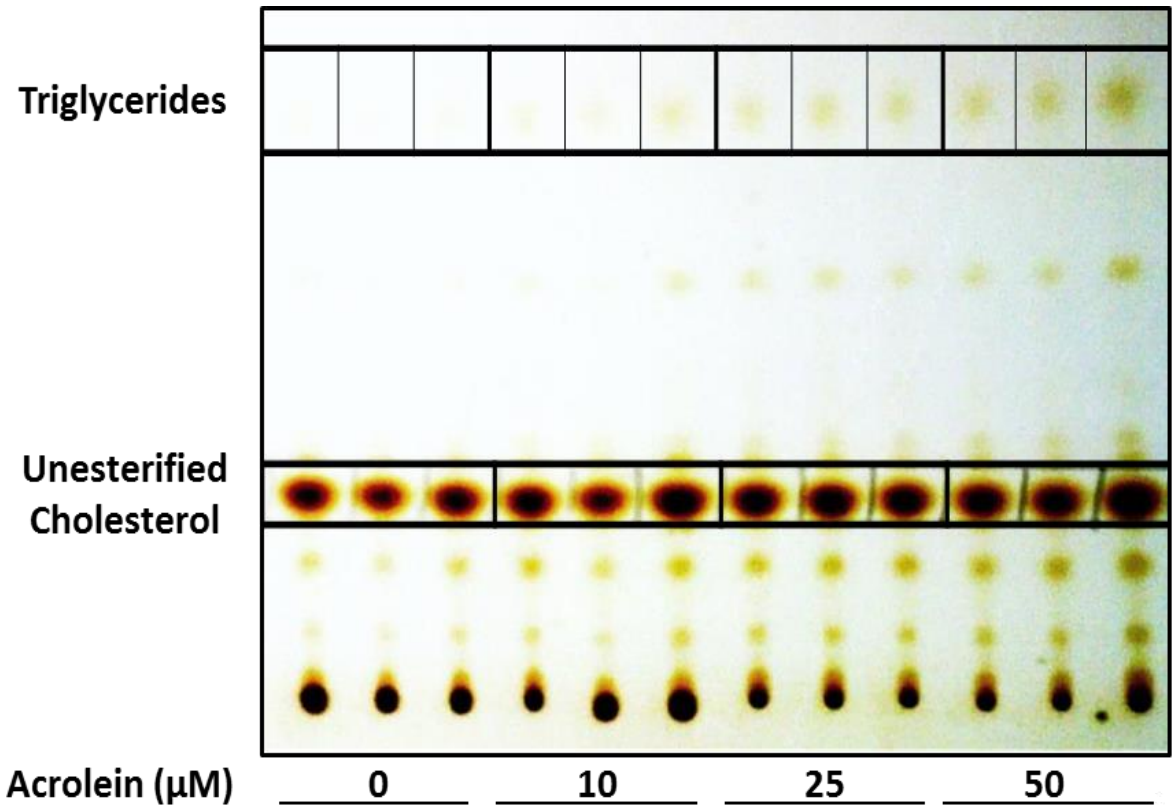

Fig. S3 Acrolein induces a dose-dependent lipid accumulation in J774A.1 macrophages. J774A.1 macrophages were incubated with increasing concentrations of acrolein (0-50 µM) for 16 h, followed by cellular lipid extraction and separation by TLC as describe under Materials and Methods. A representative TLC plate is presented showing dose-dependent effects of acrolein on cholesterol and triglyceride accumulation (n=3).

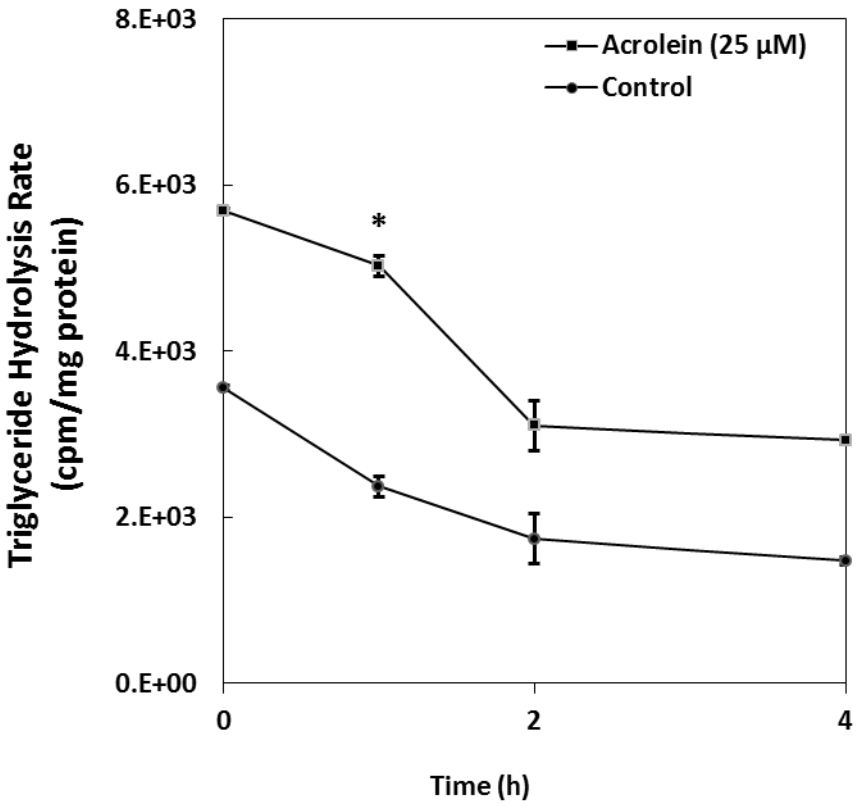

Fig. S4 Acrolein effects on triglyceride hydrolysis rate in J774A.1 macrophages. J774A.1 macrophages were incubated without or with acrolein (25 µM) for 16 h, followed by assessment of triglyceride hydrolysis rate for t up to 4 h, as described under Materials and Methods. Results are expressed as mean ± SEM (n=3). *$p<0.05$, vs. control group

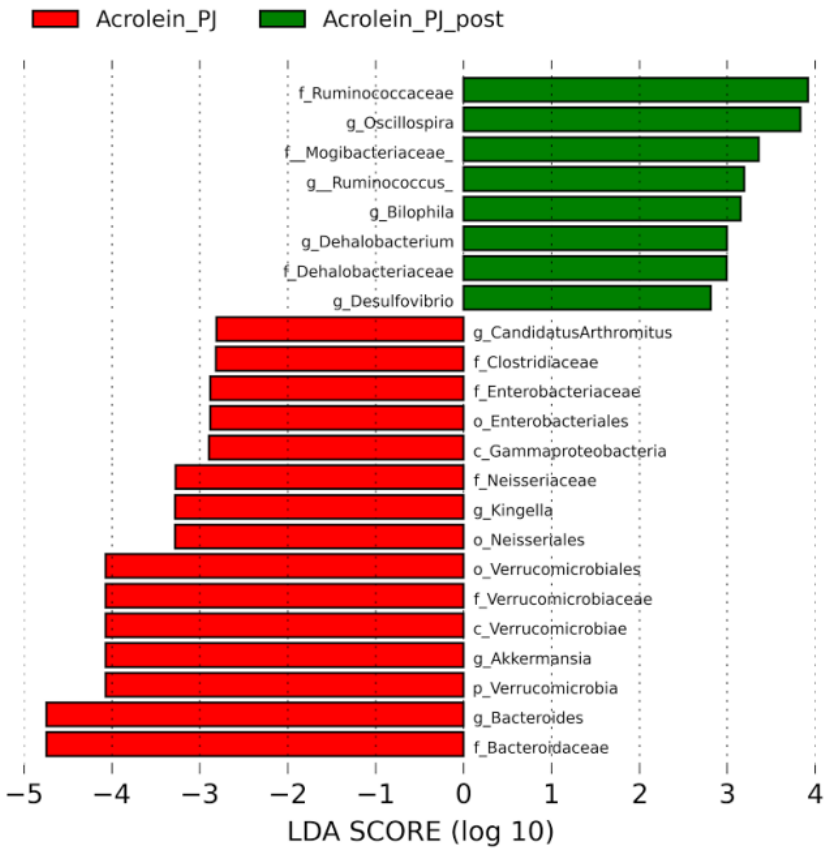

Fig. S5 LDA scores for differently abundant taxa preceding (red) and following (green) acrolein + PJ treatment to apoE$^{-/-}$ mice (only taxa meeting LDA≥2.5 are shown)

REFERENCES

Abraham K, Andres S, Palavinskas R et al (2011) Toxicology and risk assessment of acrolein in food. Mol Nutr Food Res 55:1277-1290

Aviram M, Dornfeld L, Rosenblat M et al (2000) Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am J Clin Nutr 71:1062-1076

Ben Nasr C, Ayed N, Metche M (1996) Quantitative determination of the polyphenolic content of pomegranate peel. Z Lebensm Unters Forsch 203: 374-378

Buege JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymol 52: 302-310

Caporaso JG, Kuczynski J, Stombaugh J, et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335-336

Edgar RC, Haas BJ, Clemente JC, et al (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194-200

el-Saadani M, Esterbauer H, el-Sayed M, et al (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. J Lipid Res 30:627-630

Etxebarria A, Benito-Vicente A, Alves AC et al (2014) Advantages and versatility of fluorescence-based methodology to characterize the functionality of LDLR and class mutation assignment. PLoS One. 9:e112677

Fuhrman B, Volkova N, Aviram M (2005) Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. J Nutr Biochem 16:570-766

Green SJ, Venkatramanan R, Naqib A (2015) Deconstructing the polymerase chain reaction: understanding and correcting bias associated with primer degeneracies and primer-template mismatches. PLoS One 10: e0128122

Kaplan M, Hayek T, Raz A, et al (2001) Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. J Nutr 131:2082-2089

Lowry OH, Rosebrough NJ, Farr AL, et al (1951) Protein measurement with the Folin phenol reagent. J biol Chem 193: 265-275

Meir KS, Leitersdorf E (2004) Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24:1006-1014

Moonsamy PV, Williams T, Bonella P, et al (2013) High throughput HLA genotyping using 454 sequencing and the Fluidigm Access Array™ System for simplified amplicon library preparation. Tissue Antigens 81:141-149

Rom O, Aviram M (2016) Paraoxsonase2 (PON2) and oxidative stress involvement in pomegranate juice protection against cigarette smoke-induced macrophage cholesterol accumulation. Chem Biol Interact in press doi: 10.1016/j.cbi.2016.05.009

Rom O, Kaisari S, Aizenbud D, et al (2013) The effects of acetaldehyde and acrolein on muscle catabolism in C2 myotubes. Free Radic Biol Med 65:190-200

Rosenblat M, Coleman R, Reddy ST, et al (2009) Paraoxonase 2 attenuates macrophage triglyceride accumulation via inhibition of diacylglycerol acyltransferase1. J Lipid Res 50:870-879

Rosenblat M, Rom O, Volkova N, et al (2016) Nitro-Oleic Acid Reduces J774A.1 Macrophage Oxidative Status and Triglyceride Mass: Involvement of Paraoxonase2 and Triglyceride Metabolizing Enzymes. Lipids 51:941-953

Rosenblat M, Volkova N, Aviram M (2013) Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protected against macrophage foam cells formation. Atherosclerosis 226:110-117

Rosenblat M, Volkova N, Borochov-Neori H, et al (2015) Anti-atherogenic properties of date vs. pomegranate polyphenols: the benefits of the combination. Food Funct 6: 1496-1509

Sengupta B, Narasimhulu CA, Parthasarathy S (2013) Novel technique for generating macrophage foam cells for in vitro reverse cholesterol transport studies. J Lipid Res 54:3358-3372

Srivastava S, Sithu SD, Vladykovskaya E, et al (2011) Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. Atherosclerosis 215:301-318

Yang X, Yao H, Chen Y, et al (2015) Inhibition of Glutathione Production Induces Macrophage CD36 Expression and Enhances Cellular-oxidized Low Density Lipoprotein (oxLDL) Uptake. J Biol Chem 290:21788-99

Zhang J, Kobert K, Flouri T, et al (2014) PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30: 614-620