Supplemental Material for the Manuscript:

The effects of endurance exercise and diet on atherosclerosis in young and aged ApoE$^{-/-}$ and WT mice

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Materials and Methods

Animals and exercise program

Female C57BL/6 wild-type (WT) and apolipoprotein E knock-out, on a C57BL/6 background, ($ApoE^{-/-}$) mice were purchased from Charles River (Sulzfeld, Germany) and kept and bred under specific pathogen-free conditions at the Central Laboratory Animal Facility at the Medical University of Innsbruck on a 12-hour light/dark cycle with water and food *ad libitum*. The mice were put on a treadmill (Columbus Instruments, Columbus, OH, USA) exercise program at the age of 14 weeks (young) or 49-52 weeks (aged), which lasted for 5 weeks. The standard conventional chow diet (CD) was simultaneously changed to a high-cholesterol (21.2% fat) Western diet (WD) (Sniff GmbH, Soest, Germany) at the beginning of the experiment. 6 mice per group were used and control groups were sedentary mice on CD or WD. Two methods of calculation of sample size were used. To test the null hypothesis that plaque regression is estimated in 90% of exercised- and 10% of not exercised-mice, we used a software available online (http://clincalc.com/stats/samplesize.aspx). According to that, the software predicted that -at least- a sample size of 10 mice (5 mice/group) will have 80% power to detect a significant difference of atherosclerotic plaque reduction in exercised group in comparison with not exercised mice, using a two group t-test with a 0.05 two-sided significance level. Moreover, we also used a second method, called “resource equation method” and according to that, 6 mice for 4 groups could be considered as appropriate sample size [1]. The exercise program consisted of 1 hour of running at a speed of 16 meters per minute at a 5° uphill angle, for 5 days per week for 5 consecutive weeks (See Supplemental Video). The exercise program was designed based on previous studies showing reduced plaque formation and improved plaque stability after a 5-week treadmill intervention [2, 3]. 2 days after the last training, the mice were weighed and sacrificed by terminal CO_2 inhalation and with cervical dislocation. Blood, aorta, axillary, brachial and inguinal lymph nodes and the anterior tibialis muscles were collected for further processing. Animal experiments were approved by the National Animal Experimentation Committee (Ethical approval numbers BMWF-66.011/0099-II/10b/208 and the amendments BMWF-66.011/0115-II/10b/2009 and BMWF-66.011/0030-II/10b/2010).

En face plaque assessment

The aortae were cleaned from fat and surrounding muscle tissue, cut longitudinally and stained with Sudan IV (Roth, Karlsruhe, Germany), for 15 minutes according to manufacturers instructions. Aortal plaques were photographed *en face* with ProgRes CapturePro 2.5 (JENOPTIK, Jena, Germany) with a fixed magnification and the total plaque area was calculated using ImageJ (Version 1.43, NIH, USA), by manually selecting the area of the total aorta and the areas where plaque was visible. The area of the selected region is then measured by ImageJ and given as units of pixels. Percentage of the aorta that is covered by plaque is calculated by dividing the total area of the plaques with the total area of the aorta. 6 aortas per group were analyzed.

T regulatory (T_{REG}) cell flow cytometry

Lymph node cells (pooled) from each group (n=6) were and stained for T_{REGs} using the Mouse Regulatory T Cell Staining Kit #2 (eBioscience), according to manufacturer instructions, immediately after sacrification. Briefly, the cells were stained with anti-mouse CD4 FITC, anti-mouse CD25 PE and anti-mouse CD8 PE-Cy7 antibodies (Biolegend), followed by permeabilization and staining with anti-mouse FoxP3 APC antibody (eBioscience). Samples were acquired using a FACS Calibur (BD) and analysed with the CellQuest software (BD).

Enzyme linked immunosorbent assay (ELISA) for anti-HSP60 antibodies and lipids

Anti-HSP60 antibodies were analyzed in plasma samples, as described previously [4], with 6 samples per group being analyzed.

Serial dilutions of Precinorm L and Precinorm U (Roche Diagnostics, Indianapolis, IN, USA) of standards for the cholesterol and triglycerides, respectively, were prepared. Standards and plasma samples were mixed with Chol-Reagent (Roche) and incubated for 10 minutes. The samples were measured at OD_{459} using the PowerWave XS (Bio-Tek, Winooski, VT, USA) and the results were analyzed with KC Junor Software (Bio-Tek). 6 samples per group were analyzed.

Luminex cytokine measurements

Luminex panels for interleukin (IL)-1β, IL-6, IL-10, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 (Bio-Rad, München, Germany) were used to measure plasma cytokine levels according to manufacturers instructions. Briefly, the plasma samples were added in duplicates, and bead standards were added in four-fold serial dilutions on 96-well filter plates. Thereafter, the samples were incubated with a detection antibody followed by streptavidin-PE. After washing, the samples were measured on the Bio-Plex 200 System (Bio-Rad). 3 samples per group were analyzed. Results are presented as pg/ml.

RNA isolation and cDNA preparation

Total RNA was extracted from the tibialis anterior muscles from 3 mice per group following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chlorofrom extraction (Trizol, Invitrogen) followed by a RNeasy clean-up procedure (Qiagen). RNA concentration and purity were assessed in a spectrophotometer (Nanodrop) and $A_{260/280}$ and $A_{260/230}$ ratios were determined. The integrity of the RNA was assessed by the Agilent Bioanalyzer (Agilent Technologies). For the reverse transcription reaction, 0.1µg of total RNA for each sample was used, using the WT expression kit (Ambion) starting with the T7 promoter-tailed random hexamers. cRNA was synthesized using a T7 polymerase catalyzed reaction containing unmodified ribonucleoside triphosphates. Single cDNA was synthesized from the cRNA template using random primers and dNTPs. The cRNA was hydrolyzed by RNase H and the single-stranded cDNA was purified. Fragmentation of the single-stranded cDNA was done with endonuclease I at uracil-containing positions. The fragmented cDNA is end-labeled with a biotinylated nucleotide analog.

Microarray expression profiling

Three independent microarray analyses were preformed for each group of samples. Total RNA was processed for the use on mouse GeneChip® Gene 1.1 ST Array Strip (Affymetrix, Santa Clara, CA, USA) containing 700.000 murine probe sets which interrogates 29.000 transcripts. Sequences used in the design of the array were selected from GenBank®, dbEST and RefSeq. The fragmented cDNA was added to a hybridization solution and incubated to a microarray chip overnight at 45°C. The chips

were then transferred to a fluidics instrument to wash away unhybridized cDNA. Bound cDNA was then labeled with phycoerythrin-conjugated streptavidin (SAPE). Additional flours were then added using a biotinylated anti-streptavidin antibody and additional SAPE. The chip fluorescence was then measured using confocal laser scanning and the images were analyzed using the Affymetrix GCOD software version 1.4.

Analysis of microarray data

Microarray data were analyzed using the R/Bioconductor statistical software environment [5]. Preprocessing and quantile normalization were done by the robust multi-array average method (RMA) [6]. Probesets were annotated following a recent Affymetrix annotation file and only probesets with mRNA Refseq annotation were considered. Differentially expressed genes (DEG) were identified by using linear models and the empirical Bayes methods in the package *limma* [7] and p-values were adjusted for multiple testing based on the false discovery rate according to the Benjamini-Hochberg method [8]. All of the 48 possible individual comparisons (4 factors age, genotype, exercise, diet with 2 levels each) were done and two-way analyses (exercise x diet) were performed on the 4 experimental conditions young/wt, young/ApoE$^{-/-}$, old/wt, old/ApoE$^{-/-}$. Genes were considered differentially expressed with adjusted P-values <0.05 and >1.3 fold change (or where stated>2 fold change). Gene ontology (GO) and pathway analyses for differentially expressed genes were performed using DAVID [9, 10]. Hierarchical clustering and heat map visualization were done using Genesis [11]. Accession number: E-MTAB-5685.

Quantitative RT-PCR (qPCR)

The qPCR reactions were performed using the SsoAdvanced™ Universal SYBR® Green Supermix kit (Biorad, Germany) in a CFX96 (Biorad, Germany) using following protocol: 95°C for 2 min, 40 cycles of 95°C (15s), 60°C (15s), and 72°C (10s). Gene expression was determined by using the Bio-Rad CFX Manager 3.1 software and CT values were normalized to the expression of the Hypoxanthin-Guanin-Phosphoribosyltransferase gene (HPRT). Referenced and newly designed primers used in this study were

synthesized by Microsynth Austria (Table 1) and specificity was tested by the assessment of the melting curve. 3 mice per group were used.

Gene	Sense primer	Antisense primer
mouse PGC-1a1[12]	5'-GGACATGTGCAGCCAAGACTCT-3'	5'-CACTTCAATCCACCCAGAAAGCT-3'
mouse PGC-1a2[12]	5'-CCACCAGAATGAGTGACATGGA-3'	5'-GTTCAGCAAGATCTGGGCAAA-3'
mouse PGC-1a3[12]	5'-AAGTGAGTAACCGGAGGCATTC-3'	5'-TTCAGGAAGATCTGGGCAAAGA-3'
mouse PGC-1a4[12]	5'-TCACACCAAACCCACAGAAA-3'	5'-CAGTGTGTGTATGAGGGTTGG-3'
mouse IL10[13]	5'-AGTGGAGCAGGTGAAGAGTG-3'	5'-TTCGGAGAGAGGTACAAACG -3'
mouse HSP60[12]	5'-ACGATCTATTGCCAAGGAGG-3'	5'-TCAGGGGTTGTCACAGGTTT -3'
mouse HSP90[14]	5'-CATCAAGTTGTATGTTCGC-3'	5'-GCTCTGAAAGCTTCTTCCG-3'
mouse TGFB1[13]	5'-GCTACCATGCCAACTTCTGT-3'	5'-CGTAGTAGACGATGGGCAGT-3'
mouse HPRT[15]	5'-GTCATGCCGACCCGCAGTC-3'	5'-GTCCTTCCATAATAGTCCATGAGGAATAAAC-3'

Table1: Primer pairs used for mRNA determination

Statistics

All values are shown as mean ± SEM. One-way ANOVA test, followed by Bonferroni's Multiple Comparison Test and two-way unpaired Student´s t-test were used. P-values <0.05 were considered as significant. The data were analysed with GraphPad Prism version 5.0c. The analysis of microarray data was done as stated above.

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