**SUPPLEMENTAL MATERIAL**

# Atherogenic lipoprotein(a) increases vascular glycolysis, thereby facilitating inflammation and leukocyte extravasation

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## DETAILED METHODS

*Cell culture*

Primary human aortic endothelial cells were purchased from Lonza (Baltimore, MD, the USA) and seeded on fibronectin (30μg/ml; Merck Chemicals, Amsterdam, The Netherlands)-coated, tissue culture-treated culture T75 flasks. HAECs were maintained in EGM-2 medium (Lonza) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37C°. Cells between passage 3-7 were used for experiments.

*Lp(a) and LDL isolation*

Lipoprotein fractions were isolated from plasma of healthy male and female normolipidemic volunteers. All study subjects provided written informed consent prior to enrolment. The study was conducted according to the principles of the International Conference on Harmonization-Good Clinical Practice guidelines. Blood was collected in EDTA (ethylenediaminetetraacetic acid)-containing 10 mL Vacutainer tubes. Plasma was obtained after centrifugation (3000 rpm, 4°C, 15min). Lp(a) and LDL were isolated from 3.5 mL plasma from subjects with either high or normal levels of Lp(a). Lp(a) was isolated by density gradient ultracentrifugation as previously described [4]. Plasma was adjusted to d=1.25 g/mL with solid KBr solution and a discontinuous gradient was formed by layering 2 mL of d=1.225 g/mL KBr, followed by 4 mL of d=1.100 g/mL KBr and subsequently 3 mL of d=1.006 g/mL KBr. Samples were centrifuged in a Beckman centrifuge for 19 hours at 29.000 rpm at 10°C in a SW 41 Ti rotor without brake (Beckman Coulter Inc., CA). Lp(a) and LDL fractions were separated from each other due to the density gradient, cut and dialyzed at least 3 times against phosphate-buffered saline (PBS). Next, samples were filter sterilized (0.2 μm pore size; Sartorius, Göttingen, Germany) and concentrated using Amicon centrifugal filter units filter (10.000 MWCO; Millipore). Subsequently, Lp(a) and apoB concentrations were measured using commercially available immunoturbidimetric enzymatic assays (Lp(a) 21 FS, cat number: Cat. No. 1 7139 99 10 921, Diasys, Holzheim, Germany) on a Selectra system (Sopachem, Ochten, The Netherlands).

*Lp(a), serum, 17K/17KΔLBS and E06 incubation experiments*

A confluent layer of HAECs was incubated with 100 mg/dL Lp(a), 17K/17KΔLBS (a kind gift of dr. Prof. Koschinsky, Robarts Research Institute, Ontario, Canada) and/or 100μg/mL monoclonal antibody E06 (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA, 330001) in EBM2 medium containing 0.5% FBS after a starvation period of 3h in EBM2 medium supplemented with 0.1% FBS. Cells were incubated for 6h and 18h after which sups and cells were stored for further experiments. For the serum incubation experiment obtained from the IONIS-APO(a)$_{Rx}$ trial [29], human serum samples from were kindly provided by Dr. Prof. Tsimikas (UCSD, La Jolla, USA). HAECs were incubated with serum and EBM-2 medium + 0,5% FBS (1:1 ratio) and cells were harvested after 6h and 18h. Sups were taken for cytokine and lactate measurements. Please see the Major Resources Table in the Supplemental Materials.

*RNA isolation, cDNA synthesis and Real Time quantitative polymerase chain reaction (RT-qPCR)*

HAECs were lysed with TriPure (Roche, Basel, Switzerland) and stored at -80°C prior to RNA isolation. RNA was isolated using manufacturer's instructions. Briefly,1 μg of RNA was used for cDNA synthesis with iScript (BioRad, Veenendaal, The Netherlands). qPCR was performed using Sybr Green Fast (Bioline Meridian Bioscience, Cincinnati, Ohio, USA) on a ViiA7 PCR machine (Applied Biosystems, Bleiswijk, The Netherlands). qPCR was performed for the following genes: *ICAM1, VCAM1, MCP1, IL6, IL8, PFKFB3, SLC2a1, KLF2, HK2, PFKM.* Gene expression was normalized to the housekeeping gene *H36B4*. Primer sequences are outlined in Online Table I. All gene expression graphs indicate fold change of relative gene expression of which values were normalized to the mean of the control group.

*siRNA knockdown*

siRNA duplexes directed against human PFKFB3 (Merck; siRNA sense sequence CAAGAAGUUUGCCAGUGCUdTdT, antisense CAAUGAGGAAGCCAUGAAAdTdT)
and ICAM-1 (Thermo Fisher; trifecta Kit, siRNA sense sequence GGAGCAAGACUCAAGACAUtt, antisense AUGUCUUGAGUCUUGCUCCtt) have been described previously [22]. Scrambled siRNA (Thermo Fisher) was used as a control. Transfection medium was prepared according manufacturer's protocol. In short, siPFKFB3/siICAM-1 and transfection medium were prepared separately. siRNA against PFKFB3/ICAM-1 was added to Opti-MEM (Invitrogen, Waltham, Massachusetts, USA) without antibiotics in a 25nM final concentration. Transfection medium was made adding lipofectamine (Invitrogen) to Opti-MEM in a 1:13.5 dilution. All solutions were incubated for 15 minutes at RT and subsequently carefully mixed. The final transfection solution was added to the cells and incubated overnight in a humidified atmosphere of 95% air and 5% $CO_2$ at 37C°. Next, medium was changed and 60h after transfection, cells were incubated in EBM2 medium containing 0.5% fetal bovine serum (FBS;

ThermoFisher Scientific) for 2h. Finally, cells were stimulated for the timepoints indicated and both the supernatant and cells were stored at -80C° for further analysis.

*RNA sequencing and library preparation and analysis*

Prior to stimulation, HAECs were starved using EBM-2 medium + 0.1% FBS for 3h at 37C°. Next, Lp(a) was added as indicated using EGM-2 supplemented with 0.5% FBS. After 6h of stimulation, cells were lysed and RNA was isolated as previously mentioned. RNA quality and integrity (RIN-score >8) were determined using Lab-on-a-Chip RNA 6000 Nano on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Processing of all samples was performed on Illumina HumanHT-12 microarray chips by GenomeScan (Leiden, The Netherlands). RNA-seq libraries were generated from total RNA using the "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB #E7420). Briefly, rRNA was depleted from total RNA using the rRNA depletion kit (NEB# E6310). After fragmentation of the depleted RNA, cDNA synthesis was performed. Sequencing adapters were ligated to the cDNA, and the libraries were amplified by PCR. Clustering and DNA sequencing on the Illumina cBot and HiSeq 4000 was performed according to manufacturer's protocols on 2 151-cycle paired-end flow cell lanes. Image analysis, base calling, quality check, and demultiplexing was performed with the Illumina data analysis pipeline RTA (v2.7.7) and Bcl2fastq (v2.20). Reads were aligned to the human genome version hg38 with STAR (v2.5.2b) [44]. Mapped reads were filtered on MAPQ >= 30. Reads were counted in exons and aggregated per gene using HOMER's (v4.9.1) [45] analyzeRepeats.pl script with the following parameters: -count exons -raw. RPKM values were obtained using the same script with the -rpkm flag instead of -raw. Differential expression of genes was assessed with DESeq2 (v1.22.2) [46] in an R (v3.5.1) environment. Briefly, genes were condensed to the highest expressed isoform and filtered to include only genes with median RPKM > 1 in at least one experimental group, after which differential expression was called with the following design formula: '~patient + inhibition + stimulation + inhibition:stimulation'. Results for the relevant contrasts were extracted and visualized using ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.) and pheatmap (Raivo Kolde (2019). pheatmap: Pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap). Pathway and gene ontology analysis was performed using EGSEA (v1.10.1) [47]. P-values were adjusted for multiple testing using DESeq2 as described with an FDR of 0.1 [46]. Significant differentially expressed genes were included if padj < 0.1 and a member of at least one of the following GO terms:
GO_POSITIVE_REGULATION_OF_LEUKOCYTE_MIGRATION;
GO_POSITIVE_REGULATION_OF_LEUKOCYTE_CHEMOTAXIS;
GO_REGULATION_OF_LEUKOCYTE_MIGRATION.GO_REGULATION_OF_LEUKOCYTE_CHEMOTAXIS. R code is available upon request. The RNA sequencing data generated in this study are available at the NCBI Gene Expression Omnibus (GEO) database under accession number GSE145898.

*Cytokine and lactate measurements*

Cytokine production was measured in supernatants of HAECs using commercial enzyme-linked immunosorbent assay kits for IL-6, IL-8 and MCP-1 (all Thermo Fisher) following the manufacturers' instructions. Lactate was quantified by a coupled reaction, where lactate oxidase was used to react with lactate and convert this to pyruvate and hydrogen peroxide. The hydrogen peroxide is used by Horseradish peroxidase (HRP) to react with Amplex Red so the fluorescent product resorufin is formed. Fluorescence was measured (Fluorstar Ex 570nM, EM:585) and a standard curve was used to calculate the concentrations.

*Immunoblotting*

Samples were lysed in reducing (with β-mercaptoethanol) conditions and analyzed on 4-12% poly-acrylamide gels (BioRad) in MOPS running buffer. Proteins were transferred to PVDF membranes (BioRad) and were blocked using 5% BSA in TBS-T (Tris Buffered Saline – Tween-20). Membranes were then incubated with primary antibodies against PFKFB3 (Cell Signaling Technologies, Danvers, Massachusetts, USA, AB2617178), ICAM-1 (Cell Signaling Technologies, AB2280018), HIF-1α (BD, AB398272), and Actin (Abcam, Cambridge, UK, AB306371). Subsequent protein detection was conducted with streptavidin conjugated to horseradish peroxidase (HRP) from (R & D systems, Minneapolis, USA) and visualized with enhanced chemiluminescence and imaged with ChemiDoc MP Imaging System (BioRad) using Image Lab software (BioRad) (GE, Boston, USA). All protein levels were normalized to the loading control. Please see the Major Resources Table in the Supplemental Materials.

*Monocyte isolation*

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples through density centrifugation using Lymphoprep (Stemcell Technologies, Koln, Germany; D=1.077 g/mL) as described in detail elsewhere [48]. In short, blood was diluted in a 1:1 ratio with PBS/2 mM EDTA and subsequently added to a layer of lymphoprep. Next, cells were centrifuged for 20 minutes at 600x g at RT with slow acceleration and no brake. The PBMC fraction was collected and washed twice with PBS 2 mM EDTA. Next, cells were counted using a Casy Counter (Roche Innovatis Casy TT, Bielefeld, Germany). Subsequently, monocytes were isolated using human CD14 magnetic beads and MACS® cell separation columns according to the manufacturers protocol (Miltenyi, Bergisch Gladbach, Germany). In short, cells were resuspended in MACS buffer (0,5% Bovine Serum Albumin (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS 2 mM EDTA). Next, CD14 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands) were added and incubated for 15 minutes on ice. After incubation, the cells were washed with MACS buffer and resuspended in MACS buffer for CD14 bead isolation using

MACS Separation Columns (Miltenyi Biotec). After isolation, cells were counted using a Casy Counter and monocytes were kept at 4°C in RPMI 1640 medium (Thermo Fisher) prior to usage.

*Transendothelial migration assay*

HAEC medium was changed 2h prior to monocyte addition. Next, monocytes ($1*10^5$ monocytes/mL) were added to the monolayer of HAECs and subsequently incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37C° for 30 minutes. Then, cells were fixed with 3.7% formaldehyde (Merck), washed with PBS and at least 5 images in duplicate per condition were obtained using a Zeiss Axiovert 200 inverted-microscope (Planapochromat 10x/0.45 M27 Zeiss objective; Carl Zeiss Inc., Jena, Germany). Transmigrated monocytes were distinguished from adhered monocytes by their transitions from bright to dark morphology. Analysis of transmigrated monocytes was done using ImageJ software and the cell-counter plugin (http://rsb.info.nih.gov/nih-image/).

*2-NBDG uptake assays (flow cytometry and confocal microscopy)*

For flow cytometry, HAECs were incubated for 3h with 0.1% FBS in EBM-2 medium prior addition of Lp(a) in 0.5% FBS in EBM-2 medium. Cells were incubated for 18h in a humidified atmosphere of 95% air and 5% $CO_2$ at 37C°. Next, Lp(a)-rich medium was replaced with 0.1% FBS in EBM-2 medium and was incubated for 1h. 2-NBDG ((2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; Thermo Fisher) was added in a final concentration of 50µM for all conditions. After 2h of incubation, the cells were washed twice with pre-cooled PBS and centrifuged at 1200 rpm. Cells remained on ice prior flow cytometric measurements. Fluorescence was measured in the FITC channel with a FACS Canto II (BD) and analyzed with FlowJo software version 7.6.5. (FlowJo, LLC, Ashland, OR). For confocal imaging, HAECs were added to fibronectin-coated (30 µg/mL) glass coverslips and incubated at 37°C, 5% $CO_2$, until they reached full confluency. Then cells were treated as described after which they were fixed with a final concentration of 3.7% formaldehyde and washed with PBS. Imaging was performed on a Leica TCS SP8 Confocal laser scanning microscope.

*Breeding of Lp(a) and LBS- Lp(a) transgenic mice*

Lp(a) and LBS-Lp(a) transgenic mice were bred in the Prof. Tsimikas Laboratory at UCSD. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Transgenic mice expressing both apo(a) and human apoB, which assemble to form Lp(a), and mice that express apoB and mutant apo(a) with 2 key point mutations in its lysine binding site (LBS) lacking OxPL binding (LBS-Lp(a)) have been previously described [23,49]. Briefly, Lp(a) transgenic (Lp(a)-Tg) mice express recombinant human apo(a) cDNA encoding kringles IV-1, IV-2, a fusion of IV-3 and IV-5, IV-6 to IV-10, V, and the protease domain (8K-IV apo(a)). LBS-Lp(a)-Tg mice express mutant 8K-IV apo(a) with residues $Asp^{55}$ and $Asp^{57}$ in kringle IV-10 replaced by $Ala^{55}$ and $Ala^{57}$, losing

its ability to covalently bind OxPL as verified by lack of E06 immunoreactivity[23]. In this study, all mice were on a *Ldlr*$^{-/-}$ background, fed a regular chow diet, and were 9-12 months of age. A total of 6 (4 males and 2 females) Lp(a)-Tg mice and 6 (3 males and 3 females) LBS-Lp(a)-Tg mice were used in this study.

*Immunofluorescent staining of murine aortas*

All animal experiments were conducted at the Animal Facility of the AMC and approved by the Committee for Animal Welfare (DLV102141-1) of the AMC, Amsterdam, The Netherlands. Aortas of apo(a) transgenic- and wild type mice were dissected using a dissection microscope. The aorta was dissected until 3-5mm before the iliac bifurcation. Directly after sacrifice, aortas were isolated and stored in room temperature EGM-2 medium. Next, every aorta was excised in 5 pieces of approximately 3-5mm. Finally, the aorta rings were incubated in EGM-2 medium in the presence or absence of 100 mg/dL Lp(a) and 5 μM PFK158 for 24h (Cayman Chemical, Ann Arbor, Michigan, USA). Please see the Major Resources Table in the Supplemental Materials. Subsequently, aortas were fixed in a final concentration of 3.7% formaldehyde and stored at 4°C. Next, aortas were embedded in paraffin and cut into 10 μm slices. Before staining, the aortas were washed 3x with tris-buffered saline (TBS) and stained for PFKFB3 (Abcam, AB181661), PECAM-1 (Thermo Fisher, AB2631039) and nuclei/DAPI for 2h in blocking buffer (5% BSA in TBS). After primary antibody incubation, samples were washed 3x with TBS prior to 60 min incubation at RT with secondary antibodies (Alexa Fluor 568 (AB2534121) for PFKFB3 and Alexa 633 (AB 2535731) for PECAM-1, Thermo Fisher). The aortas were the fixed on a glass bottom culture dish 35/10mm (GreinerBioOne, Kremsmunster, Austria) and mounted using DAKO mounting medium containing DAPI (Agilent, Santa Clara, USA). Next, samples were visualized on a Leica TCS SP8 Confocal laser scanning microscope and fluorescent intensity was quantified using ImageJ.

*Immunofluorescent staining of human carotid plaques*

The Athero-Express Biobank study design and plaque processing has been previously reported [50]. All patients provided written informed consent. The Athero-Express study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans. Briefly, this is a prospective ongoing biobank study that includes all patients undergoing carotid or iliofemoral endarterectomy in two referral hospitals in the Netherlands. The sample size of atherosclerotic plaques (Athero-Express) was determined by our stringent selection criteria. We estimated that with a minimum of seven subjects per group, we would be able to identify changes with $p = 0.05$ and a power of 0.9. To minimize for possible influential variables only men, without diabetes and no statin use were included. Plaque sections were dewaxed using xylene and dehydrated in graduated concentration of ethanol (100%, 96% and 70% ethanol). Next, heat-induced epitope retrieval was performed prior blocking with 5% BSA in TBS for 20 min. Sections were subsequently incubated

with primary antibody: PFKFB3 rabbit anti-human (Abcam, AB2617178), ICAM-1 mouse anti-human (Abcam, AB2213), von Willebrand Factor mouse anti-human (Agilent, AB2216702). After 2h of incubation at RT, sections were washed and incubated for 1h in the dark at RT with biotinylated secondary antibody: goat anti-rabbit Alexa 488 (ThermoFisher, AB 143165), goat anti-mouse Alexa 488 (ThermoFisher, AB2536161) and goat anti-mouse Alexa 647 (ThermoFisher, AB 2535804). Sections were washed and mounted with DAKO mounting medium containing DAPI (Agilent). Next, sections were imaged on a Leica TCS SP8 Confocal laser scanning microscope and quantified using Leica LAS-X software (Leica Camera, Wetzlar, Germany).

*Seahorse Flux Analysis*

A Seahorse XFe 96 analyzer (Seahorse Bioscience, Billerica, USA) was used to analyze cellular respiration. Prior to stimulation, HAECs were seeded in 80 µL EGM-2 medium at a density of 50,000 cells per well on fibronectin-coated XFe96 microplates (Seahorse Bioscience) and incubated for 48h. The plate was incubated in unbuffered DMEM assay medium (Merck) for 1.5h in a non-$CO_2$ incubator at 37°C. Extracellular acidification rates (ECAR) were measured by three baseline recordings, followed by sequential injection of glucose (10mM), the mitochondrial/ATP synthase inhibitor oligomycin (1.5µM), and the glycolysis inhibitor 2-Deoxy-D-glucose (2-DG; 100mM). Glycolytic rate is the ECAR of the cell population after the addition of glucose. Glycolytic capacity is the maximum ECAR rate after oligomycin addition, which inhibits oxidative phosphorylation and forces the cell to fully use glycolysis to its maximum capacity. OXPHOS was determined by measuring OCR. In short, cells were stimulated as aforementioned (ECAR determination) and OCR changes were measured in response to oligomycin (1.5µM), FCCP (1.5µM) and rotenone (1.25µM) + antimycin A (2.5µM) injection. Values were corrected for cell count.

*Isotopic labeling of polar metabolites and metabolic flux analysis*

HAECs were seeded 48h prior the start of the experiment in FN coated 6-wells tissue culture plates (Merck, Darmstadt, Germany). Cells (p3) were cultured until a confluent mono layer was formed. After overnight stimulation (20h) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C with 100 mg/dL Lp(a) derived from three different donors and 20 mM PFK158 (1h pre-incubation), medium was removed and stored. Cells were washed with PBS before adding glucose free RPMI supplemented with EGM2 singlequots (endothelial growth factors) and 0.5% dialyzed and heat-inactivated FBS and 1mM of labeled [U-$^{13}$C]-glucose (Cambridge Isotope Laboratories, Cambridge, UK) for 30 min. Samples were harvested by two-phase methanol-water/chloroform extraction as described in [51]. Briefly, medium was removed, cells were washed twice with ice-cold 0.9% NaCl, and metabolism was quenched by the addition of 1 mL ice-cold methanol-water (1:1, v/v). Thereafter, the cells were scraped from the well and collected in a 2 mL centrifuge tube. One mL of chloroform was added to the mixture,

followed by tip sonication and centrifugation at 10,000 x g for 10 min. After collection of the aqueous phase, the insoluble pellets were re-extracted with 1 mL methanol-water (1:1, v/v). The aqueous phases of both extractions were collected and evaporated. The metabolite residue was dissolved in 100µL 60% (v/v) methanol and analyzed by ultra-high-pressure liquid chromatography system (Thermo Scientific) with a SeQuant ZIC-cHILIC column (100 x 2.1mm, 3 µm particle size; Merck) coupled to a Thermo Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific). The column was kept at 15°C and the flow rate was 0.250 mL/min. The mobile phase was composed of (A) 9:1 acetonitrile/water with 5 mM ammonium acetate; pH 6.8 and (B) 1:9 acetonitrile/water with 5 mM ammonium acetate; pH 6.8, respectively. The LC gradient program was: start with 100% (A) hold 0-3 min; ramping 3-20 min to 36% (A); ramping from 20-24 min to 20% (A); hold from 24-27 min at 20% (A); ramping from 27-28 min to 100% (A); and re-equilibrate from 28-35 min with 100% (A). Data was acquired in full-scan negative ionization mode. Data interpretation was performed using the Xcalibur software (Thermo Scientific). $^{13}C$ enrichment was calculated based on mass isotopomer distribution analysis (MIDA), all results were corrected for their natural $^{13}C$ abundance as described in [52].

*Proliferation and apoptosis assay*

HAECs were incubated with 10mM BrdU for 45 min. After incubation, cells were stained using the BrdU kit (Abcam, Cambridge, United Kingdom) according manufacturer's instruction and analyzed using a FACS Canto II (BD) and analyzed with FlowJo software version 7.6.5. (FlowJo). For the 7-AAD apoptosis assay, HAECs were incubated with PFK158 in an increasing dosage as indicated. Then cells were detached using accutase (Stemcell Technologies) and stained for 7-aminoactinomycin D (7-AAD) according manufacturer's protocol (Abcam). Fluorescence was subsequently determined with a FACS Canto II (BD) and data was analyzed with FlowJo software version 7.6.5. (FlowJo).

*Lp(a) spiking experiments*

Human serum was added 1:1 with EBM-2 medium containing 0.5% FBS to HAECs and was subsequently spiked with 100 mg/dL Lp(a) and incubated for 6h at 37°C, 5% $CO_2$. Both supernatant and cell lysates were stored at -80°C for further experiments.

*Quantification and statistical analysis*

Data are presented as the mean ± standard error of the mean (SEM), unless stated otherwise. To avoid observer bias, the human serum experiments as well as the murine and human plaque analysis were blinded. In brief, an external colleague blinded the samples and after analysis/quantification, samples were deblinded. We tested for normality using the Shapiro-Wilk test and tested for outliers using Grubb's tests. Experiments were assessed with (repeated measures) one- or two-way ANOVA with Tukey's correction and other experiments were analyzed using two-tailed Student unpaired T-test as

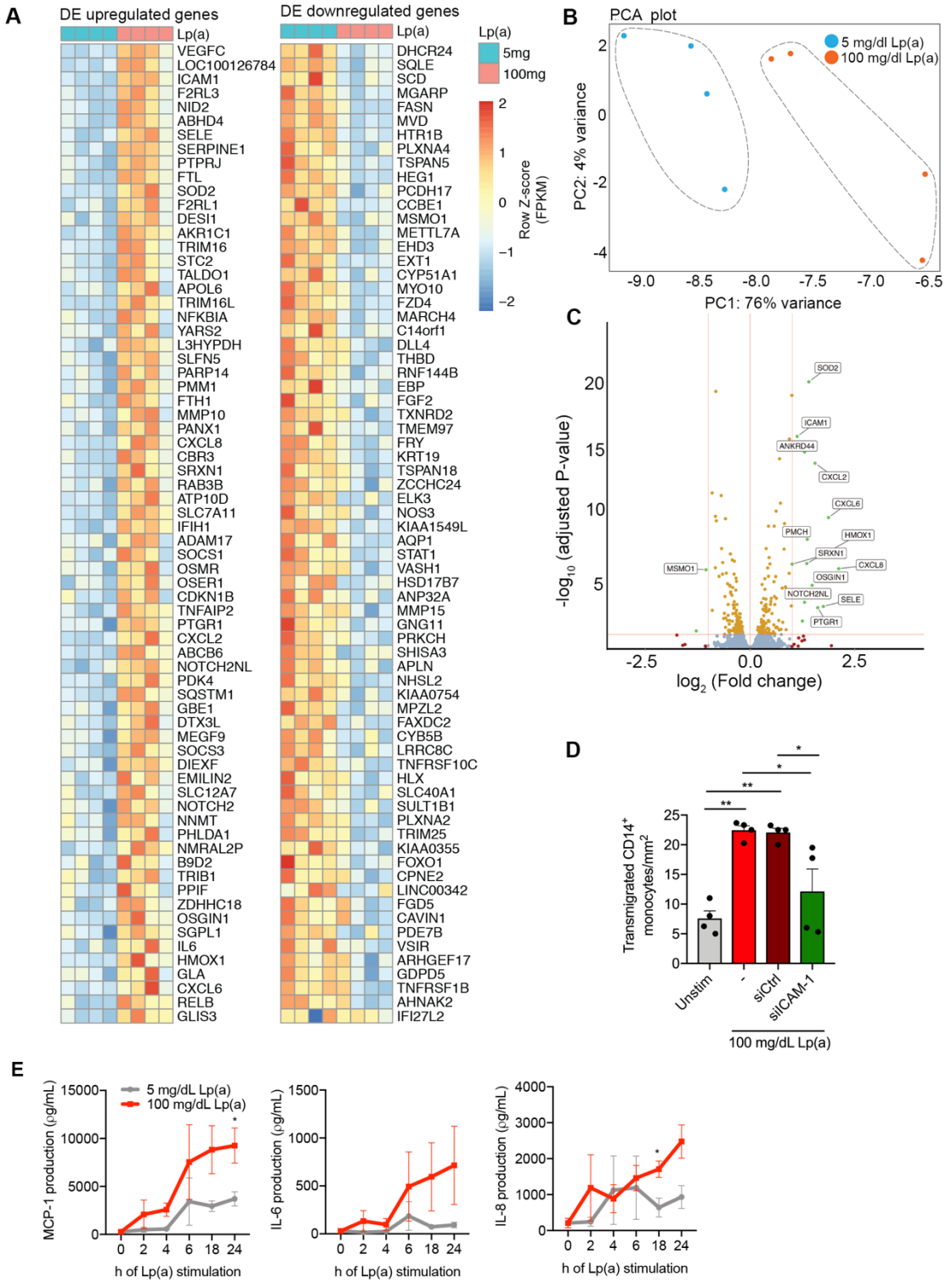
indicated in the figure legends. No corrections for multiple testing were made across tests, only within-test corrections. Patients were matched using 1-sided Chi-Square test. Representative images were selected based on the value closest to the mean value per group. Statistics were performed using Graphpad Prism (v8.0h; La Jolla, CA) and statistical significance was reported as follows: *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

**Online Table I: Primer sequences**

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| *H36B4* | ACGGGTACAAACGAGTCCTG | GCCTTGACCTTTTCAGCAAG |
| *ICAM1* | ATGGCAACGACTCCTTCTCG | GCCGGAAAGCTGTAATG GT |
| *VCAM1* | TGTCAATGTTGCCCCCAGA | TGCTCCACAGGATTTTCGGA |
| *MCP1* | TGTCCCAAAGAAGCTGTGATC | ATTCTTGGGTTGTGGAGTGAG |
| *IL6* | CTGCAGAAAAAGGCAAAGAATCTA | GTTGTCATGTCCTGCAGCC |
| *IL8* | TGTTCCACTGTGCCTTGGTTTCTCC | TGCTTCCACATGTCCTCACAACATC AC |
| *PFKFB3* | GCAGCTGCCTGGACAAAACA | GAGGGCAGGACACAAGCTAA |
| *KLF2* | CATCTGAAGGCGCATCTG | CGTGTGCTTTCGGTAGTGG |
| *SLC2a1* | CGGGCCAAGAGTGTGCTAAA | TCTTCTCCCGCATCATCTGC |
| *HK2* | CCCCTGCCACCAGACTAAAC | CAAAGTCCCCTCTCCTCTGG |
| *PFKM* | TTGGGGGCTTTGAGGCTTAC | GAGCCAGGGACATTGTTGGA |

**ONLINE FIGURES**

## Online Figure I




***Online Figure I. Up- and down regulated EC genes with and without Lp(a) stimulation***

**(A)** Heatmap of differentially expressed genes of 100 mg/dL vs 5 mg/dL Lp(a)-incubated ECs. (incubation 6h; n=4)

**(B)** PCA plot of EC stimulated with 5 mg/dL (blue) vs 100 mg/dL Lp(a) (red) for 6h (n=4).

**(C)** Volcano plot of differentially expressed gene transcripts upon Lp(a) stimulation for 6h. (n=4)

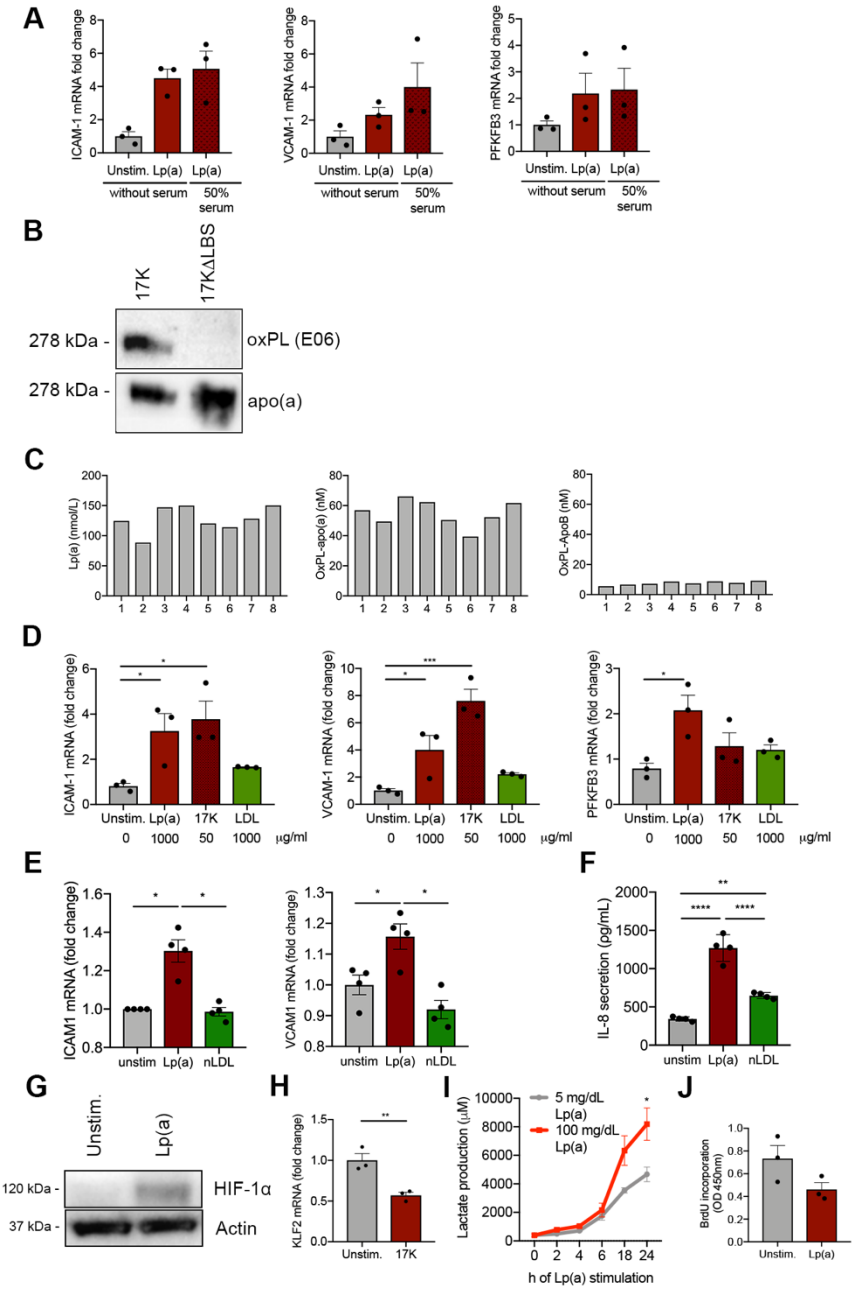
**(D)** Monocyte TEM was increased when ECs were incubated with 100 mg/dL Lp(a) (red bars) and decreased after knockdown of ICAM-1 (green bar). Data were analyzed using two-way ANOVA with Tukey's correction. P=0.0054 unstimulated vs Lp(a); P=0.0064 unstimulated vs siCtrl; P=0.0435 Lp(a) vs siICAM-1; P=0.0495 siCtrl vs siICAM-1 (18h stimulation; n=5).

**(E)** MCP-1, IL-6 and IL-8 secretion of ECs in medium over time (grey line = ECs stimulated with 5 mg/dL and red line = ECs stimulated with 100 mg/dL Lp(a). Data were analyzed using repeated measures two-way ANOVA with Tukey's correction. For MCP-1 P=0.0481 and for IL-8 P=0.0212. (n=4).

Lp(a), lipoprotein(a); PCA, principal component analysis; ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; IL-6, interleukin 6; IL-8, interleukin 8.

All data are mean ± SEM. *P<0.05; **P<0.005.

## Online Figure II




***Online Figure II. Serum and LDL does not affect Lp(a)-EC phenotype and Lp(a)-ECs does not induce proliferation.***

**(A)** Gene expression of Lp(a)-ECs with (right red bar) and without 50% serum addition (left red bar). Data were analyzed using one-way ANOVA with Tukey's correction. For *ICAM1* P=0.0296 unstimulated vs lp(a) and P=0.0155 unstimulated vs Lp(a)+50% serum (6h incubation; n=3)

**(B)** Representative immunoblot of E06-detectable OxPLs on r-apo(a) constructs.

**(C)** Lp(a) measurement of 8 donors used in this study (left graph). OxPL-apo(a) and OxPL-apoB measurements in Lp(a) fractions after ultracentrifugation (middle and right graph, respectively; n=8).

**(D)** *ICAM1*, *VCAM1*, *PFKFB3* expression of ECs stimulated with 100 mg/dL Lp(a) (measured based on apo(a); red bars), 50μM 17K (bordeaux bars) and 100 mg/dL LDL (green bars). Data were analyzed using two-way ANOVA with Tukey's correction. For *ICAM1* P=0.0439 unstimulated vs Lp(a), P=0.0171 unstimulated vs 17K; for *VCAM1* P=0.0451 unstimulated vs Lp(a), P=0.0004 unstimulated vs 17K; for *PFKFB3* P=0.0125 unstimulated vs Lp(a). (6h incubation; n=3)

**(E)** *ICAM1* and *VCAM1* expression of ECs stimulated with similar concentration of Lp(a) (measured based on apoB; red bars) and LDL (green bars). Data were analyzed using one-way ANOVA with Tukey's correction. For *ICAM1* P=0.0283 unstimulated vs Lp(a), P=0.0167 Lp(a) vs nLDL; for *VCAM1* P=0.0266 unstimulated vs Lp(a), P=0.0240 Lp(a) vs nLDL (6h incubation; n=4).

**(F)** IL-8 secretion of ECs stimulated with the same concentration of Lp(a) (based on apoB; red bars) and LDL (green bars). P<0.0001 unstimulated vs Lp(a), P=0.0067 unstimulated vs nLDL, P<0.0001 Lp(a) vs nLDL (6h incubation; n=4).

**(G)** Representative immunoblot of HIF-1α in ECs incubated with 100 mg/dL Lp(a) (same experiment as showed in Fig. 5D).

**(H)** KLF2 gene expression of ECs stimulated with 100 mg/dL Lp(a). Data were analyzed using two-tailed Student unpaired T-test, P=0.0092. (6h incubation; n=3)

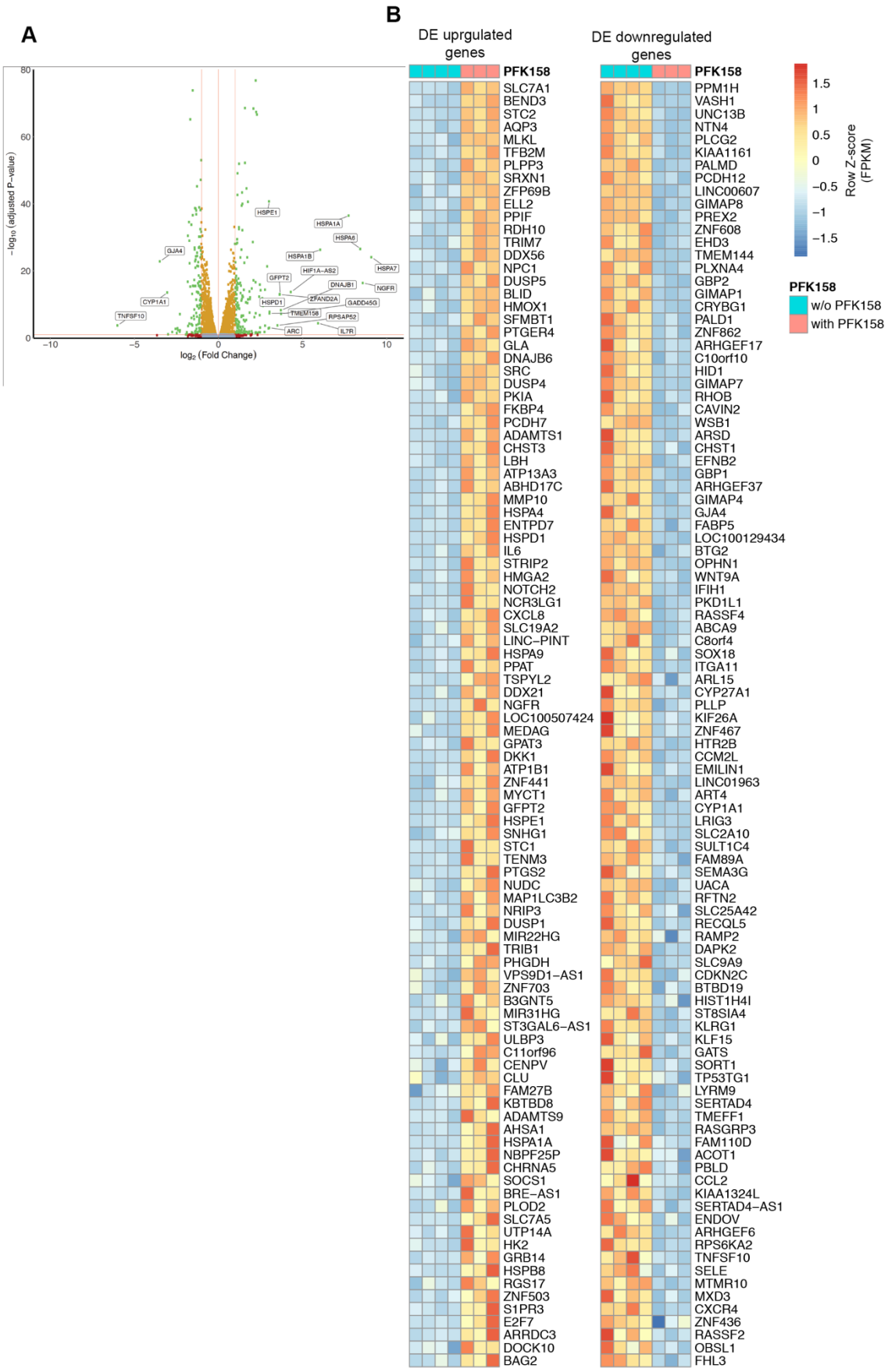
**(I)** Lactate production of 5 mg/dL-stimulated ECs (grey line) compared to 100 mg/dL Lp(a)-stimulated ECs (red line) over time. Data were analyzed using repeated measures two-way ANOVA with Tukey's correction, P=0.0305 (n=4).

**(J)** BrdU-incorporation in unstimulated ECs (grey bar) compared to Lp(a)-ECs (red bar). (24h incubation; n=3)

Lp(a), lipoprotein(a); ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular adhesion molecule 1; PFKFB3, 6-phophofructo-2-kinase/fructose-2,6-biphosphatase; 17K, 17K recombinant apolipoprotein(a); 17KΔLBS, 17K recombinant apolipoprotein(a) with a mutation in the lysine binding site; OxPL, oxidized phospholipid; E06, E06, murine IgM monoclonal antibody E06 which binds the PC moiety of OxPLs; apo(a), apolipoprotein(a); IL-8, interleukin 8; HIF-1α, hypoxia inducible factor 1α; KLF2, Kruppel like factor 2; BrdU, bromo deoxyuridine; OD, optic density.

All data are mean ± SEM. *P< 0.05; **P< 0.005; ***P< 0.0005; ****P<0.00005.

## Online Figure III

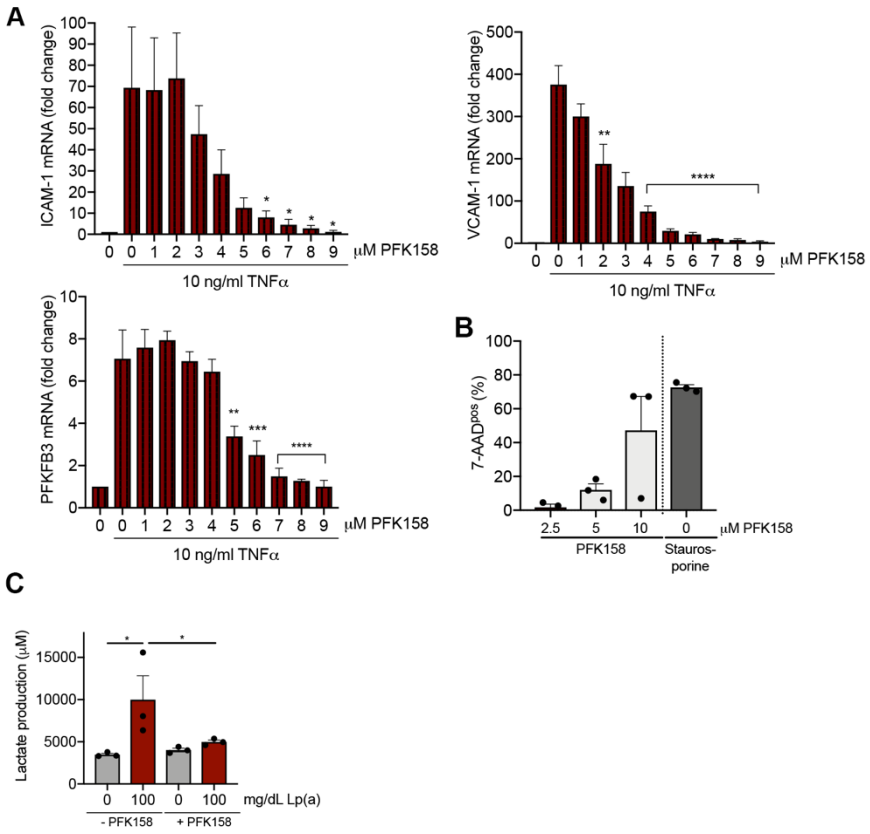


***Online Figure III. Differentially regulated genes in Lp(a)-ECs co-incubated with and without PFK158.***

**(A)** Volcano plot of statistical significance against fold change between Lp(a)-ECs (n=4) and PFK158-treated Lp(a)-ECs (n=3). (6h incubation)

**(B)** Heatmap of all differentially expressed genes in Lp(a)-ECs (n=4) and PFK158-treated Lp(a)-ECs (n=3). (6h incubation)

**Online Figure IV**




***Online Figure IV. Toxicity of PFK158 in ECs.***

**(A)** Gene expression of ECs incubated with 10 ng/mL TNFα and an increasing dosage of 0-9 μM PFK158. Data was analyzed using repeated measures two-way ANOVA with Tukey's correction. (6h incubation; n=3)

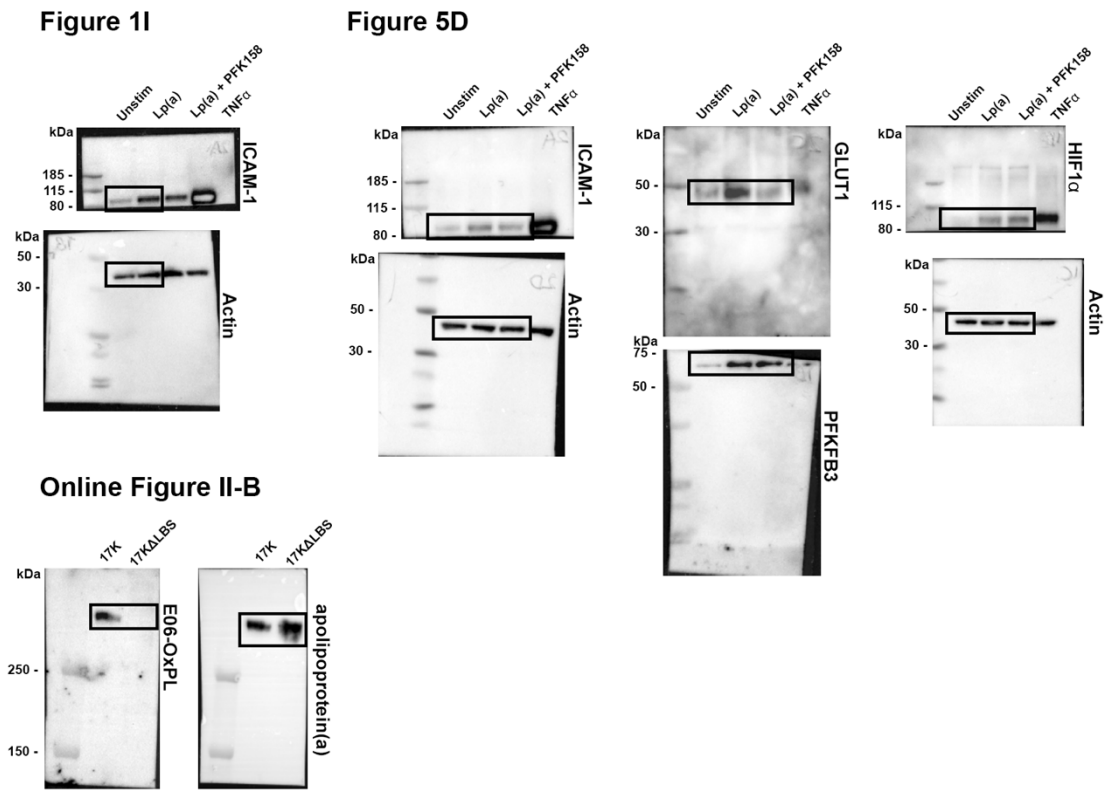
**(B)** 7-AAD analysis to indicate apoptotic ECs stimulated with an increasing dosage PFK158. (18h incubation; staurosporine used as positive control; n=3).

**(C)** Lactate graph: ECs were stimulated with 100 mg/dL Lp(a) (red bars) and co-incubated with glycolytic inhibitor PFK158 (5 μM). Data was analyzed using repeated measures two-way ANOVA with Tukey's correction. P=0.0386 0 vs 100 mg/dL Lp(a), P=0.0487 100 mg/dL Lp(a) vs 100 mg/dL Lp(a) (+PFK158) (n=3).

TNFα, tumor necrosis factor α; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular adhesion molecule 1; PFKFB3, 6-phophofructo-2-kinase/fructose-2,6-biphosphatase; 7-AAD, 7 aminoactinomycin D; Lp(a), lipoprotein(a).

All data are mean ± SEM *P<0.05; **P<0.005; ***P<0.0005; ****P<0.00005

## Online Figure V




***Online Figure V: whole Western blots.***