

Inhibition of ATP-citrate lyase by bempedoic acid protects against abdominal aortic aneurysm formation in mice

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a prevalent degenerative disease characterized by an exacerbated inflammation and destructive vascular remodeling. Unfortunately, effective pharmacological tools for the treatment of this disease remain a challenge. ATP-citrate lyase (ACLY), the primary enzyme responsible for acetyl-CoA biosynthesis, is a key regulator of inflammatory signaling in macrophages and lymphocytes. Here, we found increased levels of the active (phosphorylated) form of ACLY (p-ACLY) in the inflammatory infiltrate of AAA from patients and in aneurysmal lesions from angiotensin II (Ang II)-infused apolipoprotein E-deficient mice (ApoE^{-/-}). Furthermore, plasma ACLY levels positively correlates with IL6 and IFN γ levels in patients with AAA, while inflammatory stimuli strongly upregulated ACLY expression in macrophages and Jurkat cells. The administration of the ACLY inhibitor bempedoic acid (Bema) protected against Ang II-induced AAA formation in ApoE^{-/-} mice, limiting the progression of aortic dilatation and reducing mortality due to aortic rupture. BMS-303141, another ACLY inhibitor, also ameliorated AAA formation, although to a lesser extent. Bema attenuated vascular remodeling and the disorganization and rupture of elastic fibers induced by Ang II, as well as vascular inflammation, decreasing the recruitment of macrophages (CD68⁺) and neutrophils (Ly-6G⁺) into the aortic wall. Moreover, Bema shifted splenic monocytes toward a functionally anti-inflammatory phenotype, and increased the percentage of CD4⁺CD69⁺ cells. Taken together, these results support the contribution of ACLY to AAA and point to Bema as a promising tool to be considered for future clinical trials addressing the management of this disease which is quite often associated with disorders of lipoprotein metabolism.

Abbreviations: AAA, abdominal aortic aneurysm; Acetyl-CoA, acetyl-coenzyme A; ACLY, ATP citrate lyase; ACSVL1, very long-chain acyl-CoA synthetase; ACTB, β -actin; Ang II, angiotensin II; ApoE^{-/-}, apolipoprotein E-deficient; Bema, bempedoic acid; CXCL2, C-X-C motif chemokine ligand 2; Col, collagen; DAB, 3,3'-diaminobenzidine; ECM, extracellular matrix; FBS, fetal bovine serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GTEX, Adult Genotype Tissue Expression; HFHC, high-fat high-cholesterol; HsCRP, high sensitivity C-reactive protein; HRP, horseradish peroxidase; IFN, interferon; IL, interleukin; LDLR, low-density lipoprotein receptor; MCP1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; NOS2, inducible nitric oxide synthase; P-ACLY, phospho-ATP-citrate lyase Ser455; PMA, phorbol 12-myristate 13-acetate; ScRNA-seq, single cell RNA sequencing; UMAP, Uniform Manifold Approximation and Projection; VSMCs, vascular smooth muscle cells.

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1. Introduction

Abdominal aortic aneurysm (AAA) is a prevalent vascular degenerative disease affecting 4–8 % of men over 65 years of age [1]. AAA is defined as a localized and permanent dilation of the aorta at the abdominal level that exceeds more than 50 % of the normal aortic diameter. The gradual enlargement of the vessel wall and its progressive weakness can eventually lead to aortic rupture, a life-threatening complication with high mortality rates [2]. Key features in the pathogenesis of AAA include chronic vascular inflammation, proteolytic degradation of extracellular matrix (ECM) components, apoptosis-induced loss of vascular smooth muscle cells (VSMCs), neo-vascularization, and oxidative stress [3–7]. Despite AAA remains a substantial public health concern, surgical repair stands as the sole therapeutic option for patients at high risk of AAA rupture. Indeed, there is a lack of effective pharmacological treatments for preventing aortic rupture or slowing aneurysm progression. Consequently, it is imperative to identify novel therapeutic strategies for the medical management of this disease.

Acetyl-coenzyme A (acetyl-CoA) is a critical molecule in cell metabolism, signaling, and epigenetics. Indeed, acetyl-CoA is needed for an important number of metabolic functions, including synthesis of fatty acids, cholesterol, and nucleotide sugars and serves as the acetyl-group donor for both lysine and N-terminal acetylation, regulating histone acetylation and thereby controlling key cellular processes in cardiovascular diseases such as energy metabolism, inflammation or autophagy [8]. ATP-citrate lyase (ACLY) is the primary enzyme responsible for the synthesis of nucleocytosolic acetyl-CoA in mammalian cells [8], and connects metabolism with the epigenetic control of gene expression. The altered expression or activity of ACLY has been linked with aging [9] and with several pathological conditions including cancer [10], fatty liver diseases, such as metabolic-associated steatohepatitis (MASH) [11–13], metabolic disorders and dyslipidemia [14]. Specifically, the cardinal involvement of ACLY in cholesterol and fatty acid synthesis has aroused the interest of the scientific community on this enzyme as an emerging target for pharmacotherapy and prompted the development of ACLY inhibitors as hypolipidemic agents. This is the case of bempedoic acid (BemA), a newly approved drug for the treatment of primary hypercholesterolemia or mixed dyslipidemia, that reduces the risk of major adverse cardiovascular events, as demonstrated by the CLEAR-outcomes trial [14,15].

Recent clinical studies have revealed that BemA not only improves the lipid profile in hypercholesterolemic patients, but also reduces circulating high sensitivity C-reactive protein (hsCRP) levels, suggesting an attenuation of the systemic inflammatory response [16]. Although the reduction of circulating lipid levels could underlie this effect, different experimental evidence indicates that BemA could directly modulate the function of inflammatory cells. Certainly, beyond the importance of ACLY in lipid metabolism, it has been increasingly recognized that in macrophages the ACLY-dependent metabolic reprogramming affects the inflammatory response [17,18] and that this enzyme plays an essential role in the epigenetic regulation of pro-inflammatory cytokines in macrophages and lymphocytes [19–23]. Moreover, ACLY acetylates NF- κ B, further promoting the expression of pro-inflammatory genes [24,25]. Likewise, *in vivo*, ACLY inhibition by BemA has demonstrated to attenuate the low-grade inflammation triggered by a high-fat high-cholesterol (HFHC) diet in murine models [26, 27].

Although inflammation is a key hallmark of AAA, whether ACLY is significantly involved in AAA pathophysiology is fairly unknown, and the potential impact of ACLY inhibition on AAA formation has not been investigated. Here, we demonstrate that the inhibition of ACLY activity with BemA reduces vascular and systemic inflammation and attenuates the destructive remodeling of the arterial wall, thereby preventing aneurysm formation and AAA-rupture associated mortality in an experimental model of this disease. Altogether, these data support the

interest of pharmacological strategies targeting ACLY for the clinical management of AAA.

2. Materials and methods

2.1. Aneurysm and donor sampling preservation

Human aneurysmal samples were collected from patients undergoing open surgery repair of AAA at the Hospital de la Santa Creu i Sant Pau (HSCSP; Barcelona, Spain; n = 84). Healthy aortas were obtained from multi-organ donors (n = 16). Abdominal aortic specimens were collected following strict standard operating procedures and ethical guidelines. Samples from healthy donors had no post-mortem evidence of AAA, atherosclerotic plaques, or other medical conditions that could affect the study. Upon collection, aortas were rapidly stored at -80°C for subsequent RNA extraction, or processed for immunohistochemical analysis. The study was approved by the HSCSP Ethics Committee (IIBSP-NET-2019–40), was conducted in accordance to the Declaration of Helsinki, and patients or legal representatives gave written consent to participate.

2.2. Circulating levels of ACLY, IL6 and IFN γ in human AAA

In a subgroup of AAA patients, for whom plasma samples were available (n = 29), circulating levels of ACLY were determined by ELISA (Ref P53396; RayBiotech, Peachtree Corners, GA, USA;). Interleukin 6 (IL6) and interferon γ (IFN γ) plasma levels were analyzed in a Luminex 200 using xMAP $^{\circledR}$ technology (Millipore Corporation, Billerica, MA) with a MILLIPLEX $^{\circledR}$ MAP multiplexed assay kit (Merck Millipore, Burlington, Massachusetts, USA) following the protocol provided by the manufacturer. The xPONENT version 3.1 (Luminexcorp) and MILLIPLEX $^{\circledR}$ Analyst Version 3.5 (Merck Millipore) were used for acquisition and data analysis, respectively.

2.3. Bioinformatic re-analysis of single cell RNA sequencing data

Single cell transcriptomics datasets of human AAA were retrieved from the Gene Expression Omnibus database (GEO; code GSE237230) and re-analyzed. The Seurat package [28] in R Software version 4.4.0 and RStudio version 2024.04.1 (R Foundation for Statistical Computing Vienna, Austria. <https://www.r-project.org/foundation/>) were applied for the analysis. The CellCycleScoring function [29] was applied to calculate the scores of cell-cycle phases, and the following filter conditions were used: nFeatures_RNA = more than 4000 and less than 100; nCount = more than 20,000, and percent_mt = more than 15 %. A total of 4167 cells were used for the downstream analysis. A data transformation and normalization workflow was adopted to reduce the possible variation due to the technical issues or other variations [30]. Also, cell-cycle genes were regressed as previously described [30]. Uniform Manifold Approximation and Projection (UMAP) was used for converting cells into two-dimensional maps; the Software Loupe Browser version 8.0.0 (10x Genomics) was used for its visualization. The FindAllMarkers function was performed to detect the main features of each cluster with default parameters and the CellMarker web for Annotation of Cell Types (ACT; <http://xteam.xbio.top/CellMarker/#>) [31]. To analyze ACLY gene expression through the different cell clusters identified, the function DotPlot in Seurat was applied. The Adult Genotype Tissue Expression (GTEx; GTEx Analysis Release V8) database was consulted to obtain the bulk tissue gene expression of ACLY (ENSG00000131473.16) in a linear scale.

2.4. Cell culture

The RAW264.7 cell line was purchased from ATCC (Manassas, Virginia, USA), while JurkatE6.1 cells were kindly provided by Dr. F Sánchez Madrid (Instituto de Investigación Sanitaria Hospital de la

Princesa, Madrid, Spain). RAW264.7 cells were grown in DMEM high glucose (Biowest, Nuaille, France) supplemented with 10 % fetal bovine serum (FBS; Biological Industries, Beit HaEmek, Israel), sodium pyruvate (1 mM; Biological Industries), and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin; Gibco, Waltham, MA, USA) at 37 °C with 5 % CO₂. Cells were seeded in multi-well plates, exposed to a brief period of serum starvation in serum-free DMEM and treated with 20 ng/mL IFN γ (Merck-Millipore, Burlington, MA, USA) for different times. Jurkat cells (1·10⁶ cells/mL) were cultured in RPMI 1640 + GlutaMAXTM supplemented with 10 % FBS (Biological Industries) and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin; Gibco) and incubated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) and 2 μ M ionomycin (Sigma-Aldrich).

2.5. Animal handling

The study was carried out in apolipoprotein E deficient mice (ApoE^{-/-}, B6.129P2-ApoE^{tm1Unc}/J; Charles River, Sant Cugat del Vallès, Spain), which are susceptible to angiotensin II (Ang II)-induced AAA [32–34]. Animals were kept in the Animal Experimentation Unit (Institut de Recerca HSCSP, Barcelona, Spain) under a 12 h light/12 h dark cycle, controlled temperature and humidity conditions, and provided with food and water *ad libitum*. All procedures were approved by the local ethical committee (Law 5/June 21, 1995; Generalitat de Catalunya) and were performed in accordance with the principles and guidelines established by the Spanish Policy for Animal Protection RD53/2013 and the European Union Directive 2010/63/UE.

Due to the higher prevalence of AAA in males, both in humans and in animal models, the studies were exclusively performed in male mice. AAA was induced in 12-week-old mice by subcutaneous infusion of Ang II [1000 ng/kg body weight (BW)/min, Sigma-Aldrich] via osmotic pumps (model 1004, Alzet; Durect Corporation, Cupertino, CA, USA) for 28 days [34,35]. Briefly, mice were anesthetized with inhaled isoflurane (2 %) in oxygen (0.8 l/min). Anesthetic depth was confirmed by the lack of response to foot pinch. An incision at the interscapular level was made, and pumps were implanted subcutaneously. The wound was sutured and then animals were kept warm in a heating pad until awake and closely supervised to evaluate their well-being throughout the post-surgery period. Animals infused with saline solution for the same period of time were used as controls. The procedure was carried out using aseptic techniques in a dedicated and approved surgical area. Mice were randomly distributed into three experimental groups: (1) Ang II-infused mice receiving vehicle (0.5 % carboxymethyl cellulose and 0.025 % Tween-20; n = 19), (2) Ang II-infused mice treated with a daily oral dose of the ACLY inhibitor BemA (n = 20) oral gavage; 30 mg/kg; MedChemExpress, Monmouth Junction, NJ, USA), and (3) saline-infused animals receiving vehicle, used as controls; n = 10. All treatments started 24 h before Ang II infusion. Analysis were also performed in a early stage of AAA pathogenesis, corresponding to ApoE^{-/-} mice infused with AngII for 5 days, as described [32]. Sample size was calculated based on previous studies [34,35]. In a second experimental approach Ang II-infused ApoE^{-/-} mice were treated or not with BMS-303141 (Saline, n = 10; AngII, n = 12; AngII+BMS, n = 13) suspended in 0.5 % carboxymethyl cellulose; oral gavage) at 50 mg/kg twice daily due to its short half-life [36].

2.6. Non-invasive measurement of systolic blood pressure

Systolic blood pressure was non-invasively assessed in conscious mice, prior to minipump implantation and weekly throughout the experimental period, using the tail-cuff plethysmography method (CODA® tail-cuff blood pressure system, Kent Scientific Corporation, Torrington, CT, USA). Mice were trained to the blood pressure measurement protocol over a period of one week prior to conducting the experimental procedure. To avoid the influence of the circadian cycle,

measurements were recorded at the same time (between 9 am and 12 am). For each animal and day, at least 6 measurements were recorded and averaged [34,35].

2.7. Aortic diameter determination by ultrasonography and sample collection

Abdominal aortic diameter was measured by ultrasound imaging before Ang II infusion, and weekly until the end of the experimental protocol. Shortly, anesthetized mice (2 % inhaled isoflurane) were laid supine on a warming platform. The abdominal region was shaved, and an echography was performed to quantify abdominal aortic diameter using a Vevo 2100 ultrasound with a 30 MHz transducer (VisualSonics, Toronto, ON, Canada) [34,35]. The aorta was recorded at longitudinal and transversal views. The aortic diameter was measured at systole and at the suprarenal region. An external diameter of the abdominal aorta greater than 1.5 mm was considered as aneurysm. After 28 days of Ang II infusion, mice were deeply anesthetized via intraperitoneal injection with ketamine (150 mg/kg) and xylazine (1 mg/kg). When anesthetic depth was confirmed, unconscious animals were euthanized by exsanguination via direct cardiac puncture, and 0.5–1 mL of blood was collected. After that, the aorta was perfused with saline solution to remove clots and blood cells. The aorta was harvested, frozen in liquid nitrogen, and stored at –80 °C or directly processed for histological analysis. The severity of the aneurysm was established on the basis of a 0–4-point grading scale previously described: type 0, lack of aneurysm; type I, dilated lumen of the suprarenal aorta without thrombus; type II, vascular remodeling of suprarenal aorta that frequently contained thrombus; type III, a pronounced bulbous aneurysmal form of type II that contained thrombus, and type IV, characterized by the presence of multiple AAAs containing thrombus [37].

2.8. Plasma analysis

Heparinized blood from 4h-fasting mice was collected through by cardiac puncture at the end of the experimental procedure. Plasma levels of total cholesterol (Wako Cholesterol E, Wako Pure Chemicals, Osaka, Japan) and triglycerides (L-type triglyceride M, Wako Pure Chemicals) were determined. Murine IL6 plasma levels were determined using the Quantikine Mouse IL-6 ELISA kit.

2.9. ACLY activity assay

Protein from mouse liver was extracted on ice for 10 min with a lysis buffer containing KH₂PO₄ 50 mM pH 7.4, NaCl 150 mM, DTT 1 mM, EDTA 30 mM and sucrose 0.25 M. Lysates were centrifuged for 10 min at 16,000 x g and the supernatant was then collected. ACLY activity was determined using the malate dehydrogenase-coupled method as described [38]. Briefly, 250 μ g of liver extracts were incubated in a reaction buffer containing 100 mM Tris-HCl pH 8.7, 10 mM MgCl₂, 20 mM citrate, 10 mM dithiothreitol (DTT), 300 μ M CoA, 0.5 U/mL MDH and 140 μ M NADH, with or without 5 mM ATP. The yield of oxaloacetate generated by ACLY reaction in the presence or absence of ATP was measured as the change in absorbance at 340 nm at the beginning and after 15 min incubation at 37 °C using the VarioskanTM LUX microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA). Values were normalized to total protein concentrations.

2.10. Analysis of mRNA levels

Total RNA was isolated from both human and mouse aortas using the TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocol. 500 ng of total RNA were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in the presence of random hexamers. Quantification of mRNA levels was performed

using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) and specific primers and probes provided by the Assay-on-Demand system (Applied Biosystems) for human *ACLY* (Hs00982738_m1). Alternatively, oligonucleotides for the specific amplification of human very long-chain acyl-CoA synthetase (ACSVL1), encoded by *SLC27A2*, by real-time PCR with SYBR-Green were also applied (5'-AGCGGATTGAAGGCAGATGA-3' and 5'-AGTCCGCAAGGCAAGAGTAG-3'). In order to analyze mRNA levels in murine samples, the following primers and probes (provided by Applied Biosystems or Integrated DNA Technologies, Coralville, IA, USA) were employed for: *Acly* (Mm01302282_m1), *Slc27a2* (Mm00449517_m1), inducible nitric oxide synthase (*Nos2*; Mm00440502_m1), collagen type I $\alpha 1$ chain (*Col1a1*; Mm.PT.58.7562513), matrix metalloproteinase 2 (*Mmp2*; Mm.PT.58.9606100), *Mmp9* (Mm.PT.58.10100097), *Il6* (Mm.PT.58.10005566), monocyte chemoattractant protein 1 (*Mcp1*; Mm.PT.58.42151692), and C-X-C motif chemokine ligand 2 (*Cxcl2*; Mm.PT.58.10456839). β -actin (*ACTB*; HS99999903_m1) and glyceraldehyde-3-phosphate dehydrogenase (*Gadph*; Mm.PT.58.39a.1) were used as housekeeping genes for human and mouse tissues respectively, while *GAPDH* (Hs99999905_m1) and TATA-binding protein (*Tbp*; Mm00446973_m1) were employed as housekeeping genes for the quantification of mRNA levels in Jurkat and RAW264.7 cells, respectively. Relative mRNA levels were determined using the $2^{-\Delta\Delta C_t}$ method.

2.11. Western blotting

Jurkat cells were lysed for 10 min on ice in a buffer containing 20 mM Tris pH 8.0, 100 mM NaCl, 70 mM KCl, 1 mM MgCl₂, 0.2 mM EDTA, 2 mM DTT and 0.5 % NP-40. Cell lysates were then centrifuged for 10 min at 16,000 x g and the supernatant was collected and stored at -20 °C until further analyses. Protein from mouse liver was isolated by using a buffer containing 0.5 mM EGTA, 100 mM NaCl, 100 mM glycerol-2-phosphate, 50 mM HEPES, 10 % glycerol, 0.1 % Tween 20 supplemented with 1X Protease Inhibitor Cocktail (Merck-Millipore). Protein lysates were resolved using dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon, Merck-Millipore). After that, blots were blocked with 5 % non-fat dry milk in Tris-buffered saline, 0.05 % Tween 20 (TBST) buffer at room temperature for 1 h. Membranes were then incubated at 4 °C overnight with primary antibodies directed against phospho-ATP-citrate lyase (Ser455; p-ACLY; #4331, Cell Signaling Technology, Danvers, MA, USA), ACSVL1 (14,048-1AP, Proteintech, Planegg-Martinsried, Germany) or the low-density lipoprotein receptor (LDLR; 3839, BioVision, Milpitas, CA, USA). Subsequently, the appropriate horseradish peroxidase-conjugated secondary antibodies (Dako Products, Agilent, Santa Clara, CA, USA) and the Luminata™ horseradish peroxidase (HRP) Substrate (Immobilon, Merck-Millipore) were applied to detect bound antibodies. The size of the detected proteins was estimated using protein molecular-mass standards (Hyperpage Prestained Protein Marker; Bioline, Paris, France or Protein Marker VI (10 – 245) prestained; PanReac, Applichem, Barcelona, Spain). β -actin (A5441, Merck) was used as a loading control to verify equal loading in each lane.

2.12. Histological and immunohistochemical analysis

Arteries were fixed in 4 % paraformaldehyde/0.1 M PBS (pH 7.4) for 24 h, and paraffin-embedded. Tissue Section (5 μ m-thick) were deparaffinized in xylene and rehydrated in graded ethanol solutions. The slides were treated with 3 % hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Further and to avoid non-specific binding of the antibodies, slides were treated with PBS containing 0.1 % Tween 20 and 10 % serum of the species in which the secondary antibody was generated. Endogenous avidin and biotin were blocked using a commercial kit (Vector Laboratories, Burlingame, CA, USA). Then, slides were incubated overnight at 4 °C with primary antibodies

against p-ACLY (Ser455; SAB4504020, Sigma-Aldrich), ACSVL1 (14048-1AP, Proteintech), Ly6G (87048, Cell signaling) or CD68 (ab125212, Abcam, Cambridge, UK). Afterwards, samples were washed three times in PBS and incubated with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After extensive washing in PBS, samples were incubated with the avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. Color was developed after incubation with 3,3'-diaminobenzidine (DAB Substrate, Roche, Basel, Switzerland) which is oxidized by hydrogen peroxide in a reaction catalyzed by HRP. Finally, sections were counterstained with hematoxylin, dehydrated and mounted. Samples in which the primary antibody was omitted were used as negative controls (Supplementary Fig. S1).

The histological characterization of aortic samples was carried out by hematoxylin and eosin staining or Masson trichrome, while elastic fiber integrity was assessed by orcein staining (Casa Álvarez, Madrid, Spain) [39].

2.13. In situ zymography

Aortic gelatinolytic activity was assessed in OCT-embedded unfixed frozen sample Section (8 μ m) using fluorescein-labeled gelatin as a fluorogenic substrate (Quenched Fluorogenic DQ™ gelatin; D-12054, Thermo Fisher Scientific). Briefly, DQ-gelatin was dissolved in water at 1 mg/mL and then diluted 1:10 in 1 % (w/v) low gelling temperature agarose (A9414, Sigma-Aldrich). The mixture was applied onto each aortic section and cover-slipped. To allow gelatin gelling, sections were incubated at 4 °C for 30 min and, then, maintained at room temperature for 24 h protected from light. FITC fluorescence was visualized using a Leica TCS SP5 confocal microscopy (excitation wavelength: 495 nm; emission wavelength: 515 nm) and the Leica LAS AF Lite software (Leica Microsystems S.L.U.). The fluorescence intensity of each section, excluding elastin fiber autofluorescence, was determined using Image J software. Negative controls in which samples were pre-incubated with 20 mM EDTA before the addition of the labelled substrate were included.

2.14. Flow cytometry

The immune response of mice receiving BemA was evaluated in spleen by flow cytometry. In brief, spleens were excised and tissue disrupted to release a single-cell suspension, which was passed through a 40- μ m sterile strainer. Cells were centrifuged (300 x g) and incubated for 5 min in Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) at 1:10. After this, cells were washed in PBS and counted. Then, 10⁶ cells were protected from light and incubated for 10 min, with the following conjugated antibodies: Viogreen-CD45 (130-110-803, Miltenyi Biotec), PE-Vio770-CD11b (130-113-808, Miltenyi Biotec), BV605-Ly6c (128,035, Biolegend, San Diego, CA, USA), PE-CCR2 (130-117-548, Miltenyi Biotec), APC-CXCR1 (149,007, Biolegend), PerCP-Vio700-CD3 ϵ (130-119-656, Miltenyi Biotec), Vio Bright R720-CD4 (130-127-473, Miltenyi Biotec), APC-Vio770-CD44 (130-118-695, Miltenyi Biotec), PE-Vio615-CD62L (139-112-842, Miltenyi Biotec), FITC-CD69 (130-115-574, Miltenyi Biotec), Vioblue-Ly6g (130-119-986, Miltenyi Biotec). After washing, samples were analyzed with the MACSQuant Analyser 16 cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) using MacsQuantify software version 2.13.1 (Miltenyi Biotec).

2.15. Statistical analysis

Data are expressed as mean $\pm$ SEM. Testing data for normality by Kolmogorov-Smirnov test was systematically performed before selecting the appropriate statistical test. When data followed a normal distribution, the Student's *t*-test was used to compare two groups and one-way ANOVA, two-way ANOVA, or two-way ANOVA with repeated

measures for comparing three or more groups. When the distribution of data failed the normality test we applied the Mann-Whitney *U* test or the Kruskal-Wallis test followed by the Benjamini, Krieger and Yekutieli post hoc test. The differences in AAA incidence were analyzed by the χ^2 test. Data were analyzed with GraphPad Prism version 9.01 (GraphPad Software, San Diego, USA). Statistical significance was set as $p < 0.05$.

3. Results

3.1. *p*-ACLY and ACSVL1 are upregulated in human and experimental AAA

The knowledge about the expression and regulation of ACLY in the vascular wall is scarce. Data from the Genotype-Tissue Expression (GTEx) project evidences the widespread tissue expression of ACLY which includes the aorta and other vascular beds (Supplementary Fig. S2A). Concerning AAA, no significant differences in aortic ACLY mRNA levels were found between patients and donors samples (Fig. 1A). However, in human aneurysmal lesions, immunohistochemical analysis detected high levels of ACLY phosphorylated at serine 455 (*p*-ACLY), a key post-translational modification that tightly increases ACLY enzymatic activity [40]. In AAA, *p*-ACLY was specifically located in areas of inflammatory infiltration, and barely detected in healthy aortas (Fig. 1B). Analysis of consecutive aortic sections showed that *p*-ACLY is highly expressed by macrophages (CD68 + cells) and neutrophils (ELANE+ cells) (Fig. 1C). Accordingly, a re-analysis of scRNA-seq raw data of aortic samples from patients with AAA (GSE237230) also uncovered the high expression of ACLY in infiltrated inflammatory cells (Supplementary Fig. S2B and C). Interestingly, plasma ACLY levels positively correlated with those of IFN γ and IL6 in patients with AAA (Supplementary Fig. S3). Clinical data from patients and donors are shown in Table S1 in the Data supplement.

We further addressed whether ACLY phosphorylation was similarly

upregulated in experimental AAA. Immunohistochemical analysis of aneurysmal lesions from ApoE^{-/-} mice subjected to a chronic Ang II-challenge revealed a pronounced expression of the active form of ACLY within inflammatory cells, particularly, in areas rich in infiltrating macrophages and neutrophils (Fig. 2A and B). Of note, a significant rise in *Acly* expression was detected in the pre-aneurysmal lesions formed few days after Ang II infusion (Fig. 2C), in parallel to the induction of IL6 and other inflammatory mediators (Fig. 2D).

Likewise, the expression of ACSVL1 (encoded by *SLC27A2*), the enzyme that processes BemA to its active form, was found to be upregulated in the inflammatory infiltrate of human and murine aneurysmal lesions (Fig. 3A and B) colocalizing with *p*-ACLY (Supplementary Fig. 3 C and D).

3.2. Inflammatory stimuli induce ACLY and ACSVL1 in macrophages and lymphocytes

In view of these results, we aimed to characterize the regulation of ACLY and ACSVL1 in inflammatory cells. We observed that IFN γ early induced the expression of *Acly* in the murine macrophage cell line RAW264.7 (3 h after stimulation) (Supplementary Fig. S4A), and concomitantly increased ACSVL1 mRNA and protein levels (Supplementary Fig. S4B and C). Moreover, in Jurkat cells, an immortalized line of human T lymphocytes that expresses 20-fold more *ACLY* mRNA levels than RAW264.7 (data not shown), the inflammatory response induced by PMA and ionomycin [41], did not early alter *ACLY* mRNA levels (Supplementary Fig. S4D), but markedly increased both *p*-ACLY protein levels (Supplementary Fig. S4E) and ACSVL1 expression (Supplementary Fig. S4F and G).

3.3. BemA prevents AAA formation induced by Ang II in ApoE^{-/-} mice

The increased expression of *p*-ACLY found in human and

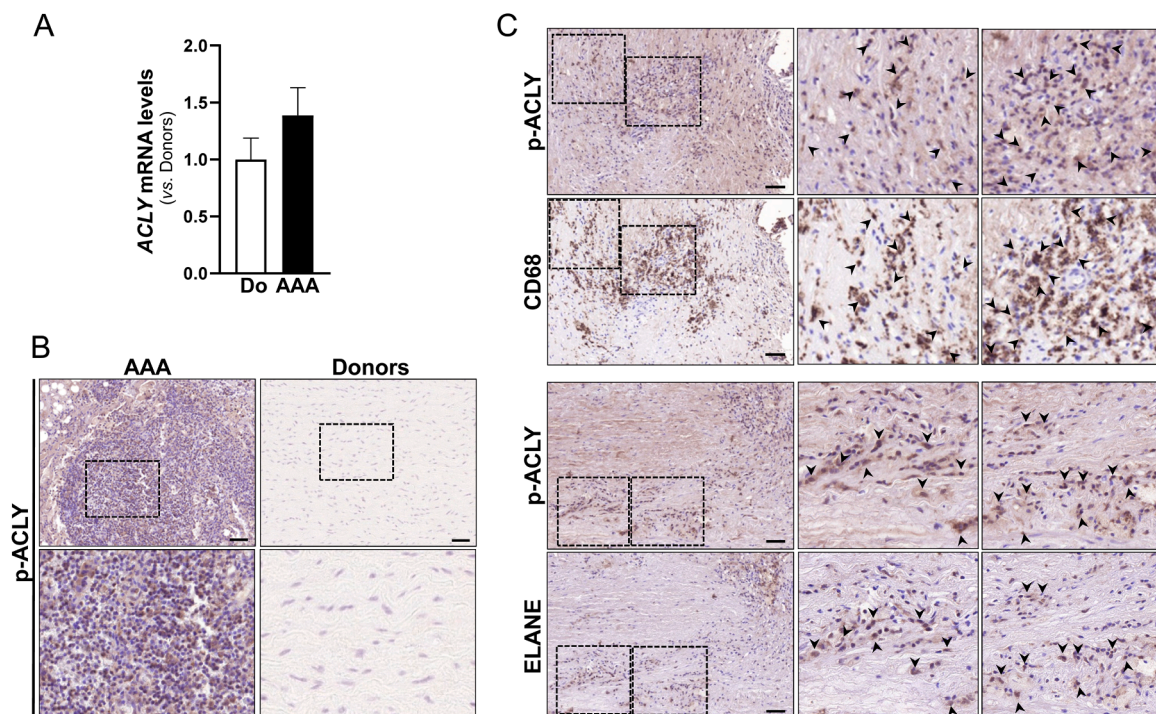


Fig. 1. Phospho-ATP-citrate lyase (*p*-ACLY) is increased in human abdominal aortic aneurysm (AAA). A) *ACLY* mRNA levels were analyzed in the abdominal aorta from patients with AAA ($n = 86$) and healthy donors (Do; $n = 16$). Data, normalized to β -actin expression, are expressed as mean $\pm$ SEM. B) Representative *p*-ACLY immunostainings in human abdominal aortas from patients with AAA and healthy donors (bars: 50 μ m). The boxed areas are shown at a higher magnification below. C) Representative immunohistochemical analysis for *p*-ACLY and CD68 or *p*-ACLY and ELANE in consecutive sections from human AAA (bars: 50 μ m). The boxed areas are shown at a higher magnification, and the arrowheads indicate positive cells for *p*-ACLY and the corresponding cell marker.

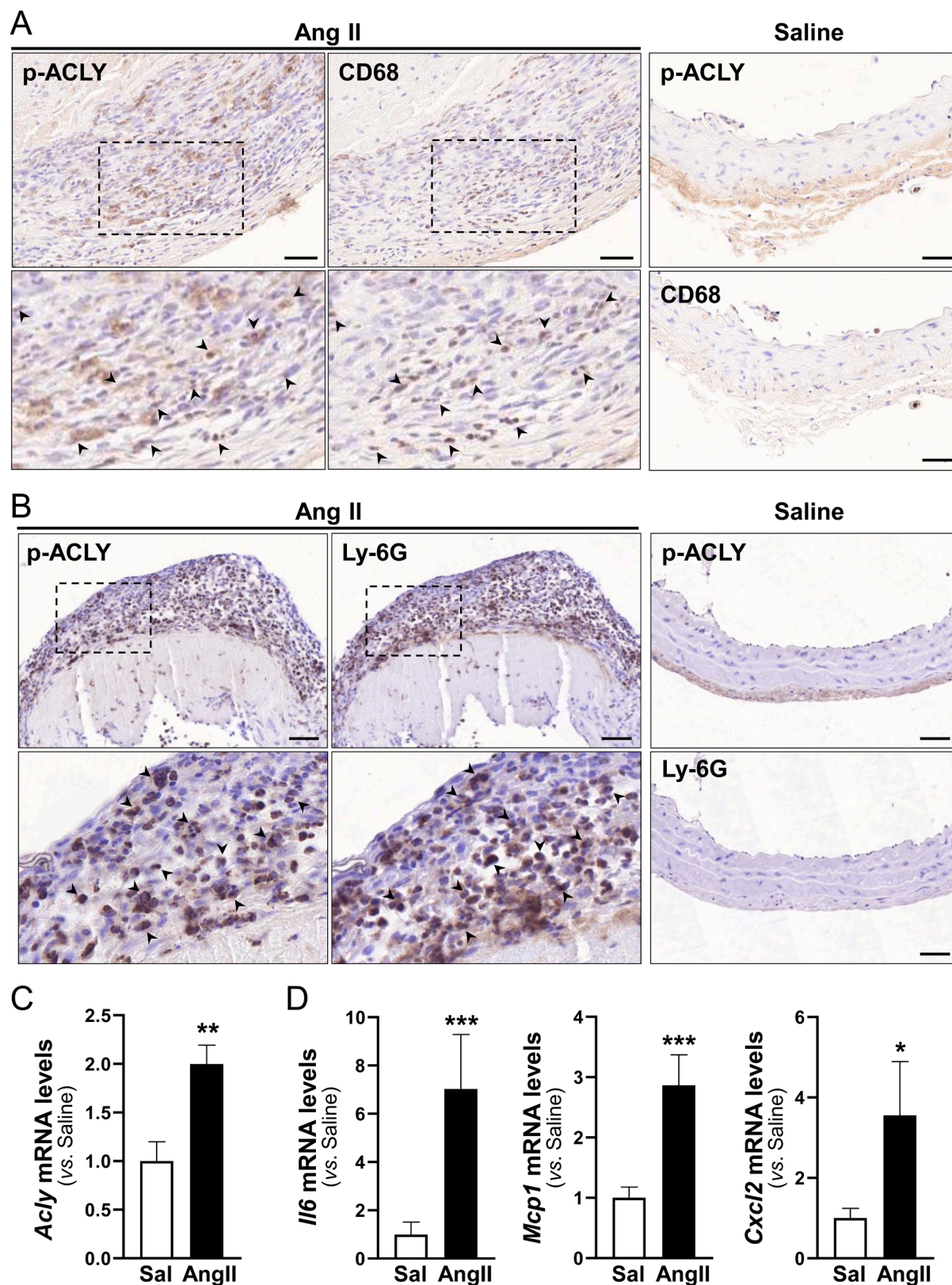


Fig. 2. High phospho-ATP-citrate lyase (p-ACLY) levels in aneurysms from Ang II-infused ApoE^{-/-} mice. (A and B) Representative immunohistochemical analysis for p-ACLY and CD68 (A) and p-ACLY and Ly-6G (B) in consecutive sections from Ang II-induced AAA (1000 ng/kg/min; left panels) and in abdominal aortas from saline-infused mice (right panels) (bars: 50 μ m). The boxed areas are shown at a higher magnification below, and the arrowheads indicate positive cells for p-ACLY and the corresponding cell marker. C) Aortic *Acly* mRNA levels in ApoE^{-/-} mice infused with saline solution or Ang II (1000 ng/kg/min) for 5 days (saline, n = 14; Ang II, n = 15). mRNA levels were assessed by real time-PCR. Data are mean $\pm$ SEM. ** P < 0.01 vs. Saline (two-way ANOVA). D) Aortic mRNA levels of *Il6*, *Mcp1* and *Cxcl2* in aorta from Ang II-infused mice as indicated in C. * P < 0.05, *** P < 0.001 vs. Saline (Mann-Whitney U test).

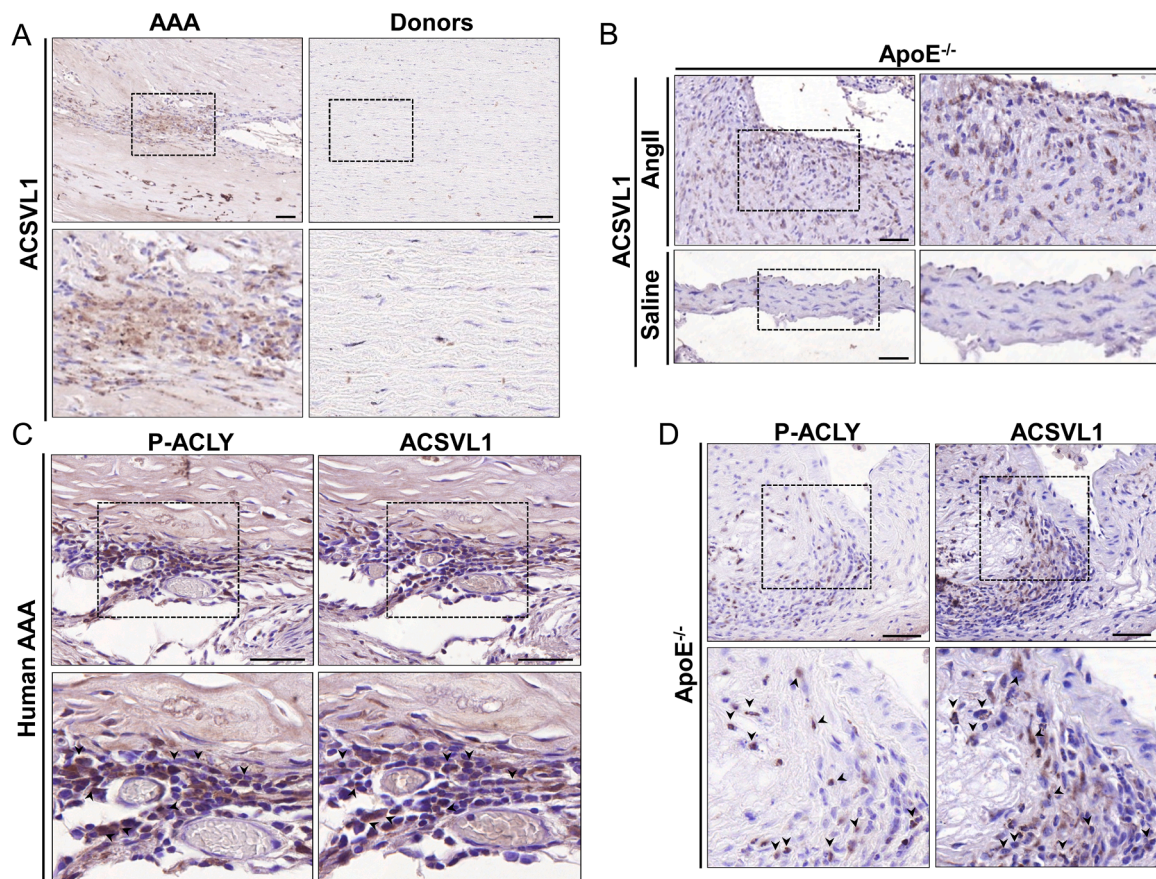


Fig. 3. ACSVL1 is increased in human and experimental abdominal aortic aneurysms (AAA) and colocalizes with p-ACLY. A and B) Immunohistochemical analysis of ACSVL1 assessed in the abdominal aorta from patients with AAA and healthy donors (A) and in abdominal aorta samples from saline- or Ang II-infused ApoE^{-/-} mice (B). C and D) Representative immunohistochemical analysis for p-ACLY and ACSVL1 in consecutive sections from human AAA (C) and murine aneurysmal lesions (D). The arrowheads indicate positive cells for p-ACLY and ACSVL1. Bars: 50 μ m. The boxed areas are shown at a higher magnification.

experimental AAA prompted us to investigate whether the activation of ACLY might play an active role in the pathophysiology of this disease. To address this, BemA, was administered to Ang II-infused ApoE^{-/-} mice and aneurysm progression was monitored by ultrasonography throughout the experimental protocol. As shown in Fig. 3, BemA protected against AAA formation in this model, limiting the increase in aortic diameter induced by Ang II infusion (Fig. 4A–D). Certainly, BemA exerted an early benefit on aneurysm development in Ang II-challenged mice with a significant decrease of aortic diameter already detected after 7 days of Ang II infusion (Fig. 4B). This effect was associated with a reduction in aneurysm incidence. Roughly 70 % of Ang II-infused ApoE^{-/-} mice developed aneurysms, but BemA decreased this percentage to 20 % ($P < 0.01$) (Fig. 4E). Similarly, BemA prevented the development of the most severe and complicated forms of AAA (Fig. 4F). Importantly, a significant improvement in the survival rate was detected in BemA-treated mice compared to those receiving Ang II alone. In fact, Ang II infusion caused the death of 40 % of mice, whereas 95 % of the BemA-treated animals survived at the end of the experimental procedure (Fig. 4G). Concerning vascular phenotype, hematoxylin-eosin staining revealed the striking disturbance of the vascular wall morphology in Ang II-challenged mice, *versus* the improved vascular remodeling observed in mice treated with BemA (Fig. 4H). The impact of BemA on AAA development was independent of hemodynamic effects, since this drug did not significantly affect the increase in blood pressure levels evoked by Ang II (Supplementary Fig. S5A). Likewise, body weight was not modified by this intervention (Supplementary Table S2). As expected, BemA significantly reduced the liver activity of ACLY and enhanced the hepatic levels of the LDLR, thereby confirming the effectiveness of the drug targeting the hepatic enzyme (Supplementary Fig. S6A, S6B and S7).

However, in these ApoE^{-/-} mice, fed a normal chow diet, circulating triglyceride levels were similar in all groups, while total cholesterol levels increased in BemA-treated animals (Supplementary Table S2).

In Ang II-infused ApoE^{-/-} mice, BemA treatment preserved most features of saline-infused animals, including elastin integrity (Fig. 5A), and reduced Ang II-induced collagen deposition (assessed by Mason's trichrome staining) (Fig. 5B). BemA also attenuated the compensatory increase in aortic collagen I expression (Fig. 5C), decreased the enhanced vascular expression of *Mmp-2* induced by Ang II and lowered *Mmp-9* mRNA levels compared with mice receiving Ang II alone (Fig. 5D). Furthermore, *in situ* zymography showed that BemA-treated mice exhibit a similar MMP activity than that of saline-infused mice (Fig. 5E). Interestingly, BemA ameliorated the vascular inflammatory response in Ang II-challenged mice, limiting the recruitment of macrophages and neutrophils into the vessel wall (Fig. 6A). Likewise, this drug significantly curtailed the Ang II-mediated increase of *Il6*, *Nos2* and *Cxcl2* aortic expression, while AngII was unable to increase *Mcp1* mRNA levels in BemA-treated mice (Fig. 6B). Further, BemA significantly reduced circulating IL6 levels compared with Ang II-infused animals (Fig. 6C).

3.4. BemA alters inflammatory cell subsets in the spleen from Ang II-infused ApoE^{-/-} mice

Next, we sought to investigate the influence of Ang II on splenic immune cell populations in ApoE^{-/-} mice and to assess whether BemA could modify this profile. Regarding CD4⁺ cells, flow cytometry data revealed that BemA administration led to a marked increase in effector memory (CD44^{hi} CD62L⁻) CD4⁺ T cells, accompanied by a concurrent

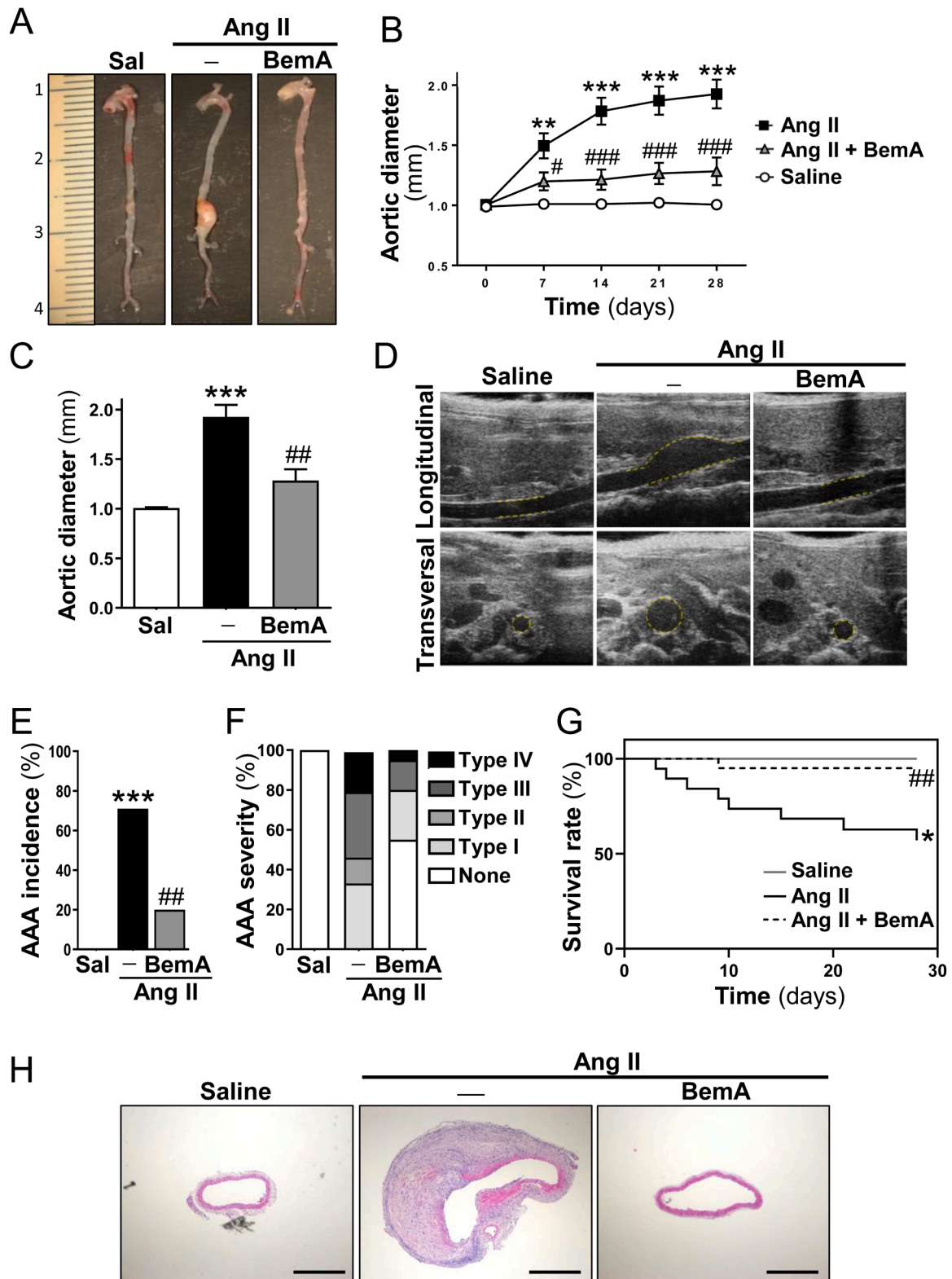
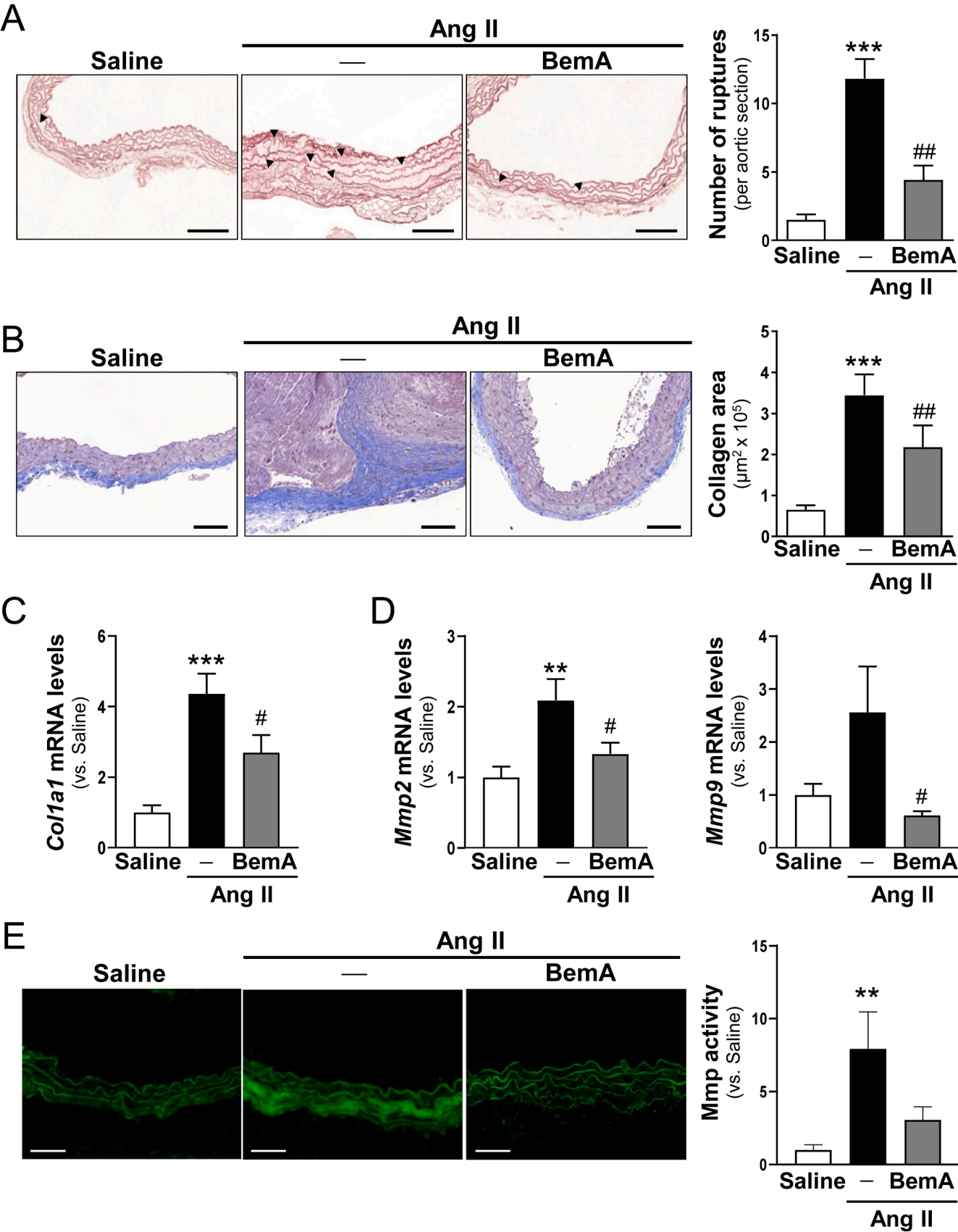


Fig. 4. Bempedoic acid (BemA) ameliorates the formation of AAA in Ang II-infused ApoE^{-/-} mice. ApoE^{-/-} mice were infused with saline solution (Sal) or Ang II (1000 ng/kg/min) for 28 days. Ang II-challenged mice were treated or not with bempedoic acid (BemA, 30 mg/kg/day). **A)** Representative macroscopic images of the aortas from each experimental group. **B)** Time-course analysis of the abdominal aortic diameter by echocardiography. The data are mean $\pm$ SEM (saline, n = 10; Ang II, n = 17; Ang II+BemA, n = 20). *** P < 0.01, *** P < 0.001 vs. Saline, # P < 0.05, ### P < 0.001 vs. Ang II (two-way ANOVA). **C)** Quantitative analysis of the aortic diameter at the end of the experimental protocol (28 days). The data are mean $\pm$ SEM. *** P < 0.001 vs. Saline, ## P < 0.01 vs. Ang II (Kruskal-Wallis test). **D)** Representative ultrasonographic frames after 28 days of Ang II infusion. Longitudinal and transversal images taken at the level of the suprarenal aorta are shown. The aortic diameter is indicated with a yellow line. **E)** Incidence of AAA in each experimental group. *** P < 0.001 vs. Saline, ## P < 0.01 vs. Ang II (Chi-square test). **F)** Aneurysm severity based on the Manning scale. **G)** Percentage of animal survival in the three study groups. * P < 0.05 vs. Saline, ## P < 0.01 vs. Ang II (Chi-square test). **H)** Representative hematoxylin-eosin staining of abdominal aortic sections from Ang II-infused mice (bars: 500 μ m).



(caption on next page)

Fig. 5. Bempedoic acid (BemA) preserves elastin integrity and prevents collagen deposition in the aorta from Ang II-infused ApoE^{-/-} mice. ApoE^{-/-} mice were infused with saline solution or Ang II (1000 ng/kg/min) for 28 days. Mice challenged with Ang II were treated or not with bempedoic acid (BemA, 30 mg/kg/day). A) Representative images showing the structure of elastin fibers in the abdominal aortas after orcein staining. Arrowheads mark elastin fibers ruptures (bars: 100 μ m). The graph on the right shows the quantitative analysis of the number of ruptures of elastic fibers per aortic section. The data are mean $\pm$ SEM (saline-infused mice, n = 6; Ang II, n = 14; Ang II+BemA, n = 19). ***P < 0.001 vs. Saline, ##P < 0.01 vs. Ang II (Kruskal-Wallis test). B) Representative images of Masson trichrome staining of the abdominal aortas from each group (bars: 100 μ m). The histogram shows the quantitative analysis of the collagen positive area. The data are mean $\pm$ SEM (saline, n = 6; Ang II, n = 14; Ang II+BemA, n = 19). ***P < 0.001 vs. Saline; ##P < 0.01 vs. Ang II (Kruskal-Wallis test). C-D) Aortic mRNA levels of *Col1a1* (C), *Mmp2*, and *Mmp9* (D) were analyzed by real-time PCR. Data, normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression, are expressed as mean $\pm$ SEM (saline, n = 6; Ang II, n = 12; Ang II+BemA, n = 10). **P < 0.01, ***P < 0.01 vs. Saline; # vs. Ang II (one-way ANOVA). E) Aortic MMP activity per aortic section was analyzed by *in situ* zymography. Representative images are shown in the left panel. Bars: 50 μ m. Data, normalized by area, is expressed as mean $\pm$ SEM (saline, n = 6; Ang II, n = 7; Ang II+BemA, n = 7). **P < 0.01 vs. Saline (Kruskal-Wallis test).

decrease in naive CD4⁺ T (CD44⁺ CD62L⁺) cells, compared to both saline- and Ang II-infused mice (Fig. 7A). Similarly, BemA significantly increased early activated (CD69⁺) CD4⁺ lymphocytes, as opposed to saline- and Ang II-infused animals (Fig. 7B). The infusion of Ang II augmented the percentage of pro-inflammatory monocytes (Ly6C^{hi} CCR2⁺) as well as triggered a decline of anti-inflammatory monocytes (Ly6C^{hi} CXCR1⁺). Notably, BemA restored the proportion of these two monocyte populations to values similar to those found in saline-infused mice (Fig. 7C).

3.5. BMS-303141 prevents AAA development in Ang II-infused ApoE^{-/-} mice

We further aimed to assess the effect of a second ACLY inhibitor (BMS-303141). Under our experimental conditions, this drug was less effective inhibiting ACLY. In fact, in Ang II-infused ApoE^{-/-} mice, BMS-303141 did not significantly reduce hepatic ACLY activity (by 23 %, *p* = 0.07; Supplementary Fig. S6C) and only slightly increased hepatic LDLR protein levels (Supplementary Fig. S6D and S7). BMS-303141, however, attenuated the progressive enlargement of aortic diameter induced by Ang II (Fig. 8A-D), although this response was weaker than that elicited by BemA (Fig. 8C). BMS-303141 also showed a tendency to reduce AAA incidence (from 80 % to a 41,7 %; *p* = 0.099; Fig. 8E) and improved aneurysm severity (low incidence of type III lesions) (Fig. 8F). In accordance, this drug improved vascular remodeling, decreasing the number of elastin fiber breaks and reducing vascular inflammation (Supplementary Fig. S8A-C); however, no essential differences in survival were found (Fig. 8G). Finally, BMS-303141 did not affect body weight, plasma lipids (Supplementary Table S3) or blood pressure levels (Supplementary Fig. S5B).

4. Discussion

Effective pharmacological approaches to treat or prevent AAA progression and rupture are not yet available [7,42]. Drug repurposing could be a valuable alternative for diseases such as AAA for which there are no effective treatments [43]. In the last years, several studies including our own research have delved into the mechanisms involved in the destructive remodeling of the vascular wall in human AAA [32–35], postulating new therapeutic targets and pharmacological strategies. The present study, unveils, for the first time, the instrumental role of ACLY on the pathophysiology of this disease and evidences that the ACLY inhibitor BemA increases survival and protects against experimental AAA development, attenuating vascular remodeling and limiting local and systemic inflammation.

Disturbances in ACLY expression might represent a new pathogenic mechanism underlying numerous diseases not only dyslipidemia [14], but also hepatic steatosis [11,13], diabetes [44] and several malignancies [10]. ACLY phosphorylation at Ser 455 (p-ACLY Ser455) strongly increases ACLY catalytic activity [40]; in fact p-ACLY Ser455 is generally considered as the active form of this enzyme [45]. Enhanced p-ACLY Ser455 levels have been reported in human osteoarthritis [46], cancer [47–49] and sepsis-associated organ injury [45]. Regarding vascular diseases, the information is scarce but p-ACLY has been

detected in unstable human atherosclerotic lesions [50]. Similarly, we have uncovered a substantial increase of the active form of ACLY in human AAA and in the aneurysmal lesions from Ang II-challenged ApoE^{-/-} mice. P-ACLY was particularly detected within inflammatory cells, and more precisely in regions enriched in neutrophils and macrophages. The high levels of p-ACLY detected in established AAAs and the early up-regulation of its expression in dilated or pre-aneurysmal abdominal aorta in the preclinical model, strongly stress the role of ACLY on the pathophysiology of AAA.

These findings prompted us to assess whether an ACLY inhibitor already used to treat hypercholesterolemia (bempedoic acid, BemA) could prevent the formation of AAA in an experimental model. It should be noted that, both in human and murine AAAs, we observed a concomitant upregulation of pACLY and ACSVL1, the enzyme responsible for the activation of the prodrug BemA to its CoA derivative, which is the active form that actually inhibits ACLY. Although ACSVL1 has been considered essentially a hepatic enzyme, our data revealed that this protein is located in the inflammatory infiltrate of aneurysmal lesions and is upregulated by inflammatory stimuli in T cells and macrophages in culture, in agreement with previous studies reporting high expression of ACSVL1 in activated populations of myeloid cells in cancer [51,52]. Therefore, it is plausible to speculate that BemA could be activated in inflammatory cells. Irrespective of this, it should be highlighted that BemA could exert anti-inflammatory responses in macrophages through AMPK activation and NF- κ B inhibition [53,54], although we have not observed any influence of BemA on aortic AMPK activation in our experimental model (data not shown).

The administration of BemA to Ang II-infused ApoE^{-/-} mice, a common model for preclinical AAA research and drug testing, at a dosage that reduced plasma cholesterol levels in hypercholesterolemic mouse models of atherosclerosis and non-alcoholic steatohepatitis [11, 26], attenuated hepatic ACLY activity and enhanced LDLR levels, indicating that this drug efficiently targeted the liver. Under these conditions, BemA largely protected from Ang II-induced AAA in ApoE^{-/-} mice, limiting aortic growth, as assessed by ultrasonography, while reducing the incidence and severity of aneurysms. The development of AAA was also attenuated by another ACLY inhibitor, BMS-303141, although to a lesser extent. The short half-life of BMS-303141 [36] could explain the marginal impact of this compound on hepatic ACLY activity and LDLR levels and also its lower capability to limit AAA compared to BemA, despite it was administered twice daily at a dose previously reported [55].

Importantly, our study further evidences that BemA significantly reduced mouse mortality, triggering an overall improvement of the survival rate, and drastically ameliorating the destructive remodeling of the vascular wall. Certainly, BemA-treated mice retained most features of saline-infused mice. This drug preserved elastin integrity and attenuated the enhanced expression of MMPs and the compensatory increase of collagen levels triggered by Ang II. Little information is available regarding the impact of BemA on ECM remodeling and even less in relation to the vascular wall. Previous studies reported that the pharmacological inhibition of ACLY normalized the expression of MMPs induced by inflammatory stimuli in chondrocytes [46] and that BemA exerted anti-fibrotic effects in models of non-alcoholic steatohepatitis

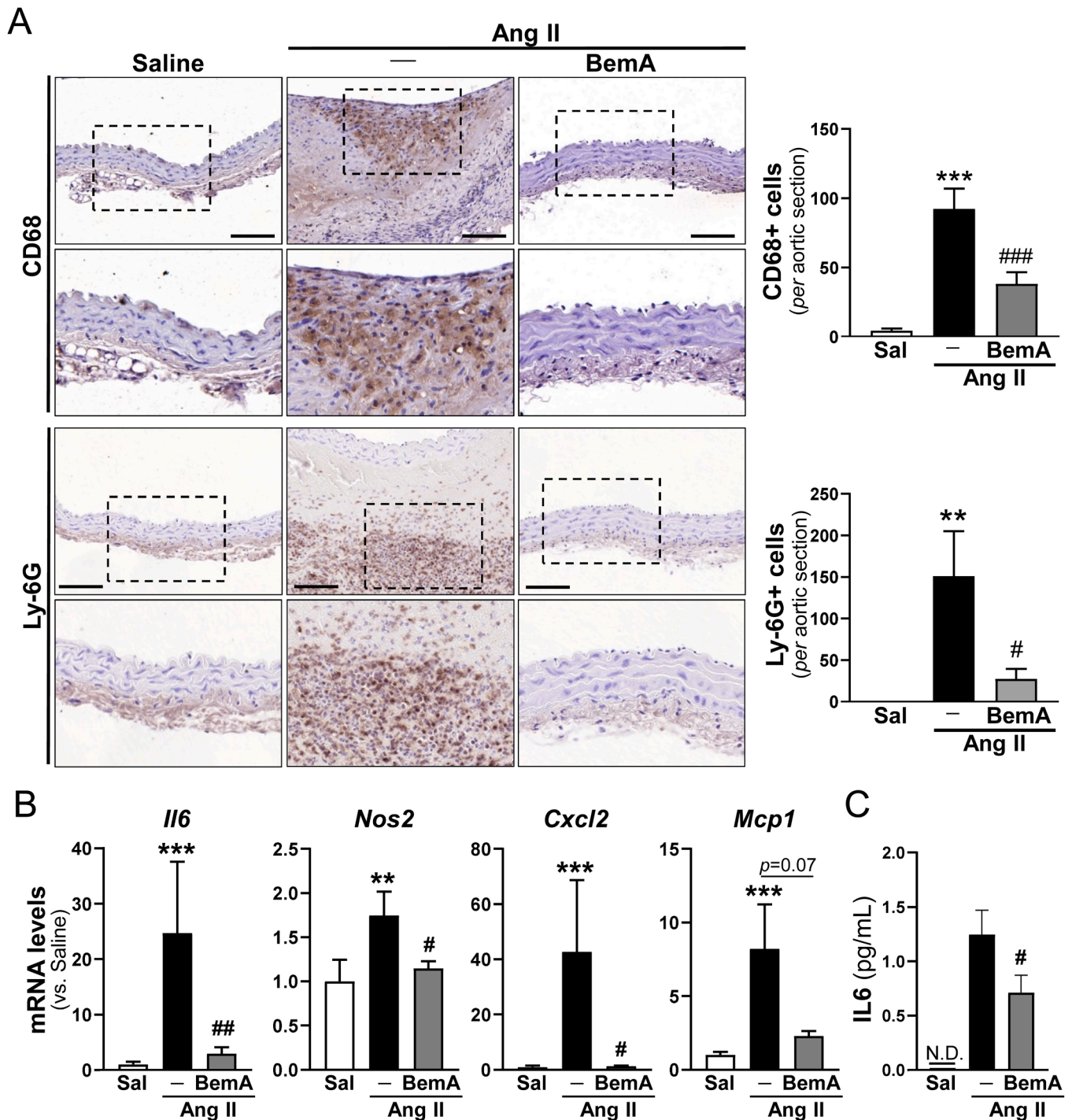


Fig. 6. Bempedoic acid (BemA) attenuates aortic inflammation in Ang II-infused ApoE^{-/-} mice. ApoE^{-/-} mice were infused with saline solution (Sal) or Ang II (1000 ng/kg/min) for 28 days. Mice challenged with Ang II were treated or not with bempedoic acid (BemA, 30 mg/kg/day). A) Representative images corresponding to infiltrated macrophages (CD68 + cells) and neutrophils (Ly-6G+ cells) (bars: 100 μ m). The boxed areas are shown at a higher magnification below. The histograms on the right show the quantification of the number of CD68 and Ly-6G positive cells per aortic section. The data are mean $\pm$ SEM (saline, n = 6; Ang II, n = 13; Ang II+BemA, n = 19). ** P < 0.01, *** P < 0.01 vs. Saline; # P < 0.05, ### P < 0.001 vs. Ang II (Kruskal-Wallis test). B) *Il6*, *Mcp1*, *Nos2* and *Cxcl2* mRNA levels were analyzed by real-time PCR in abdominal aortas. Data, normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression, are expressed as mean $\pm$ SEM (saline, n = 6; Ang II, n = 12; Ang II+BemA, n = 10). ** P < 0.01, *** P < 0.01 vs. Saline; # P < 0.05 vs. Ang II (Kruskal-Wallis test). C) IL6 plasma levels in AngII-infused mice treated or not with BemA. Data are expressed as mean $\pm$ SEM (Ang II, n = 13; Ang II+BemA, n = 18). # P < 0.05 vs. Ang II (*t*-test after log transformation).

(NASH) affecting pathways implicated in collagen biosynthesis and ECM homeostasis [11,56]. Further, BemA limits the induction of TGF- β , MMP-2 and collagen type-1 in mesenteric arteries in a rat model of renal artery ligation, an approach that induces a hypertensive response [57].

Although in that study the benefit provided by BemA was attributed to an antihypertensive response, we did not observe any impact of BemA on the Ang II-mediated increase of blood pressure, excluding the involvement of hemodynamic effects on the attenuation of experimental

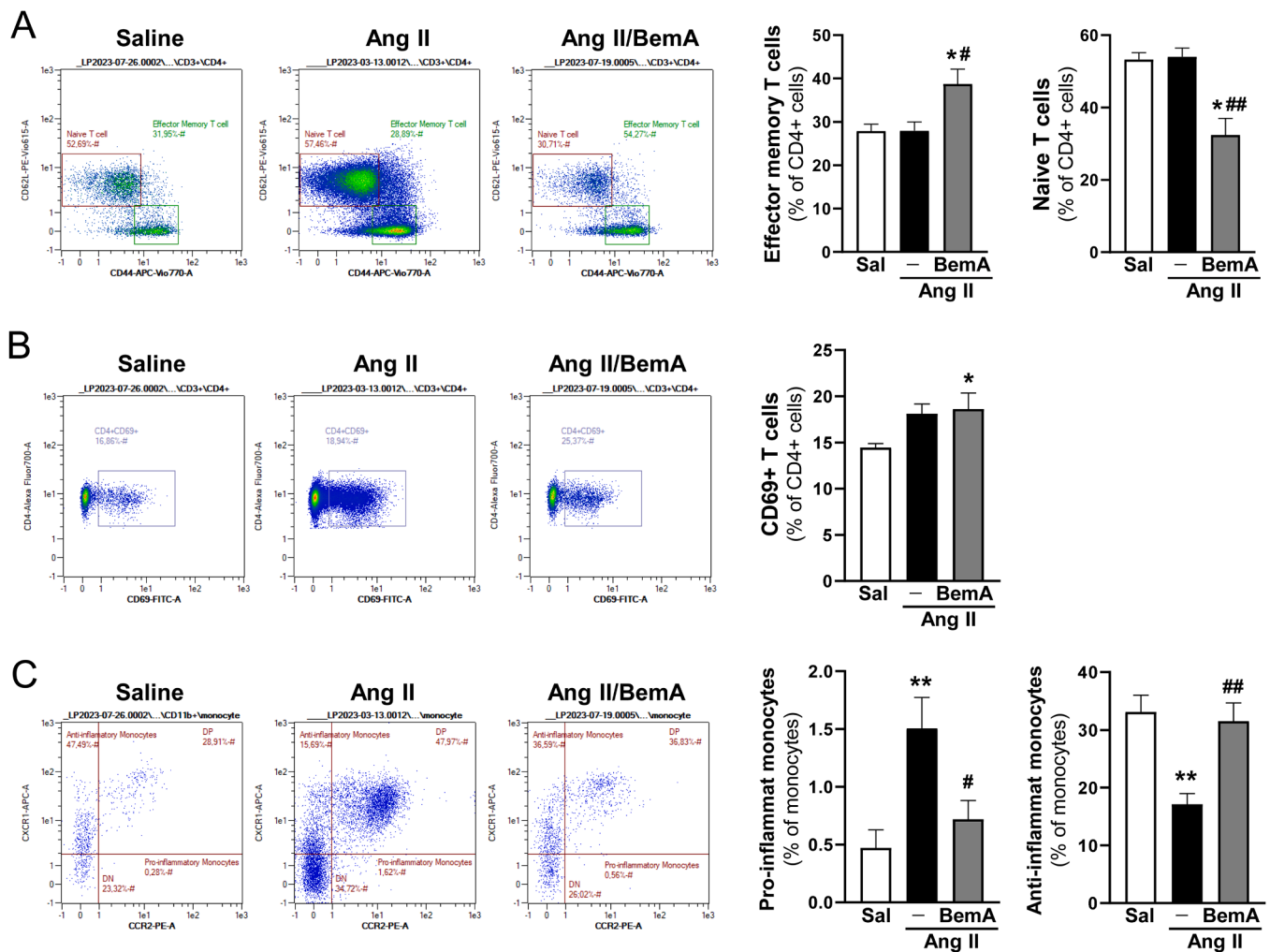


Fig. 7. Effect of bempedoic acid (Bema) on splenic immune cells from Ang II-infused ApoE^{-/-} mice. ApoE^{-/-} mice were infused with saline solution or Ang II (1000 ng/kg/min) for 28 days. Mice challenged with Ang II were treated or not with bempedoic acid (Bema, 30 mg/kg/day). The percentage of effector memory CD4⁺ lymphocytes and naive CD4⁺ cells (A), CD69 + CD4⁺ lymphocytes (B), and pro-inflammatory and anti-inflammatory monocytes (C) for each group is shown. Representative FACS plots of each experimental condition are included. The data are mean $\pm$ SEM (saline, n = 6; Ang II, n = 9; Ang II+Bema, n = 7). * P < 0.05, ** P < 0.01 vs. Saline; # P < 0.05, ## P < 0.05 vs. Ang II (one-way ANOVA).

AAA triggered by this drug.

The positive response on AAA evoked by Bema was also related to a reduction of inflammation. Certainly, Bema significantly attenuated both vascular immune infiltration and the expression of inflammatory markers, further improving systemic inflammation. *In vivo* and *in vitro* data have previously suggested that ACLY could be involved in both M1 and M2 macrophage polarization [19,22]. Monocytes mobilization from the spleen in Ang II-infused ApoE^{-/-} mice contributes to AAA development [58]. Interestingly, our flow cytometry analysis of splenic immune cells in Ang II-treated mice, showed that Bema shifts monocytes toward a functionally anti-inflammatory phenotype. Further, we have found an increased number of CD4⁺ CD69⁺ cells in the spleen of Bema-treated ApoE^{-/-} mice. CD69, limits T cell egress, inducing lymphocyte retention [59]. Thus, our data suggest that Bema might enhance T cell retention in secondary lymphoid organs, which in turn, could contribute, at least in part, to the favorable effect of Bema limiting aneurysm progression. Of note, in human AAA, we found a significant relationship between the plasma levels of ACLY and those of IFN γ and IL6, cytokines strongly increased in aneurysmal disease and critical pathophysiological regulators of AAA [60]. This is consistent with recent studies that uncovered the link between plasma ACLY and the severity of inflammation in sepsis [45]. Thus, our data strength the notion about the close relationship between ACLY and systemic

inflammation.

Preclinical studies analyzing the consequences of ACLY inhibition on AAA expansion had not been previously addressed and its impact on other vascular diseases insufficiently analyzed. Former experimental studies showed the ability of Bema to prevent HFHC-induced atherosclerosis in murine models, including ApoE^{-/-} mice [26,27]. In these studies Bema significantly diminishes the increase of total cholesterol levels achieved in HFHC-fed mice, although occasionally the effect was modest [26]. In our present research, however, this drug not only did not cause a hypocholesterolemic response, but actually aggravated hyperlipidemia, in agreement with previous studies reporting increased circulating cholesterol levels in Bema-treated rodents [12,61]. It should be highlighted that, in our study, circulating cholesterol levels were about 5- to 8-fold lower than those reached in ApoE^{-/-} mice fed a HFHC, a condition that significantly disturbs hepatic lipid metabolism [26]. This could explain the different impact of Bema on the lipid profile in HFHC- and chow-fed ApoE^{-/-} mice. In any case, our data in ApoE^{-/-} mice support that the impact of Bema on lipid levels does not contribute to the beneficial response observed on AAA.

Overall, our study sheds light on the potential role of ACLY in the pathophysiology of AAA, and highlights its role in vascular remodeling and inflammation. Previous studies have shown that the depletion in inflammatory cells or in cytokine production prevent experimental

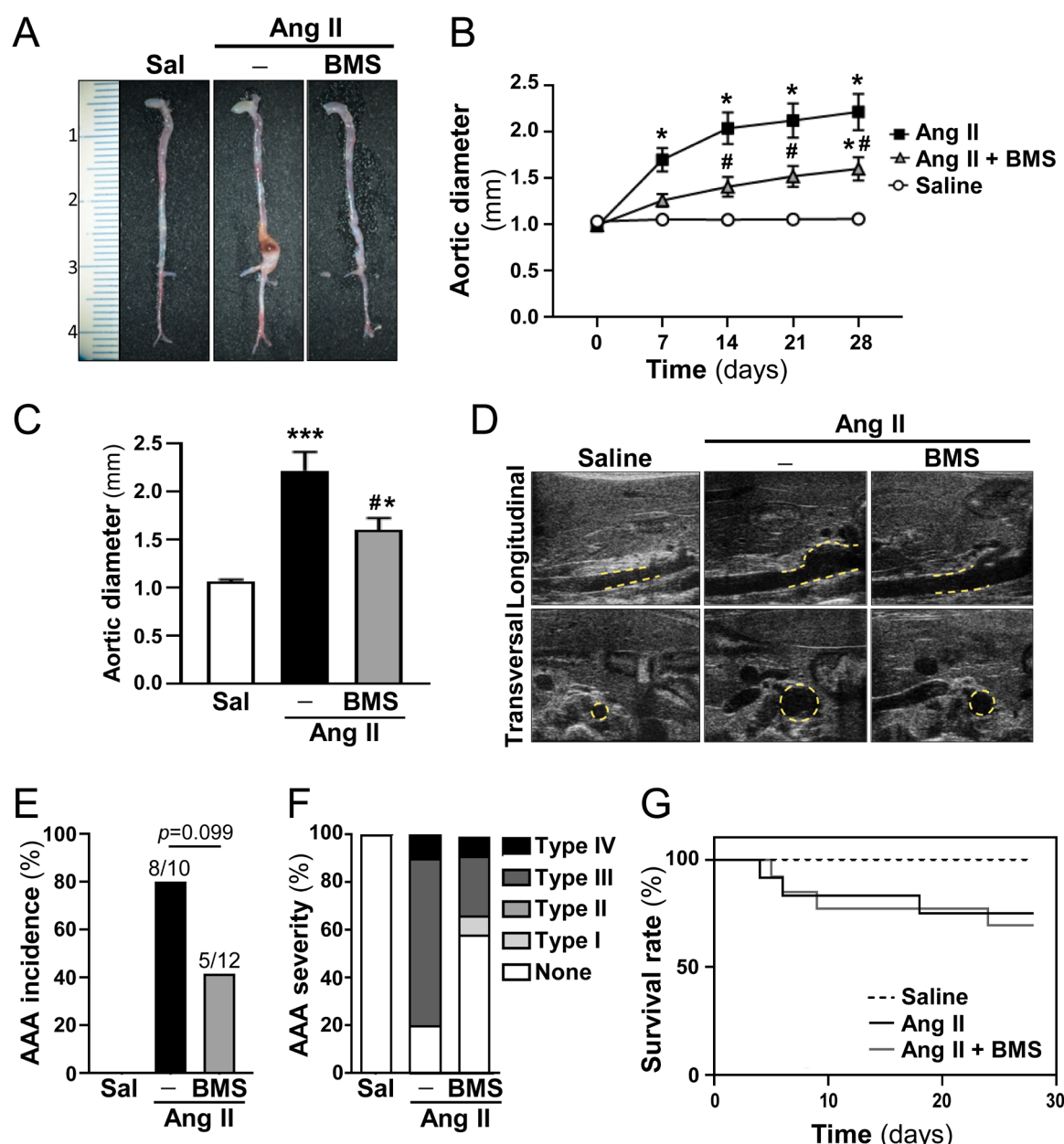


Fig. 8. BMS-303141 attenuates AAA development in AngII-infused ApoE^{-/-} mice. ApoE^{-/-} mice were infused with saline solution or Ang II (1000 ng/kg/min) for 28 days. Mice challenged with Ang II were treated or not with BMS-303141 (BMS, 50 mg/kg twice daily). A) Representative images of the aortas from each experimental group (at 28 days). B) Data corresponding to the ultrasonographic follow-up of the abdominal aortic diameter. C) Quantitative analysis of the aortic diameter at the end of the experimental period. In B) and C), the data are mean $\pm$ SEM (saline, n = 10; Ang II, n = 10; Ang II+BMS, n = 12). * P < 0.05, *** P < 0.001 vs. Saline-infused mice; # vs. Ang II-challenged mice (two-way ANOVA). D) Representative ultrasonographic frames after 28 days of Ang II infusion. Longitudinal and transversal images taken at the level of the suprarenal aorta are shown. E) and F) Incidence (E) and severity of AAA (F; based on the Manning scale). G) Graph showing the survival rate in each experimental group.

aneurysms [62–64], and that BemA is able to attenuate hyperlipemia-induced systemic inflammation by reducing hsCRP levels [16]. Taken in this context, our results suggest a potential of BemA as a treatment for AAA that allows us to postulate the pharmacological repositioning of this drug as a plausible working hypothesis. Since effective pharmacological interventions for AAA have not yet been found, this should be an incentive to justify further research and to implement clinical trials addressing the benefit of BemA in this disease, in order to set the grounds for translating these experimental insights into clinical practice.

Authors contribution

C.R. and J.M.-G. conceived the study, designed the experiments, acquired the financial support and wrote the manuscript. L.P.-U, J.A., L. B.-C, A.R.-S. N.R. and J.C.L., performed the *in vivo* and *in vitro* studies and acquired data. L.P.-U, and M.C. participated in patient's plasma analysis and supervised the clinical aspects of the research. L.P.-U, C.R., M.G. and L.B.-C performed the immunohistochemical analysis and R.A.-P the bioinformatic analysis. All the authors have critically revised the manuscript and approved its final version.

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Roglans Nuria: Writing – review & editing, Methodology, Investigation, Formal analysis. **Galán María:** Writing – review & editing, Methodology, Investigation. **Rodríguez-Sinovas Antonio:** Writing – review & editing, Methodology, Investigation. **Camacho Mercedes:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Almendra-Pegueros Rafael:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis. **Blanco-Casoliva Laia:** Writing – review & editing, Methodology, Investigation. **Alonso Judith:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Puertas-Umbert Lidia:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Cristina Rodriguez:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Martínez-González José:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Data curation, Conceptualization. **Laguna Juan Carlos:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cristina Rodriguez reports financial support was provided by Instituto de Salud Carlos III. Cristina Rodriguez reports financial support was provided by Fundación Eugenio Rodríguez Pascual. Cristina Rodriguez reports financial support was provided by Fundación Española de Arteriosclerosis. Cristina Rodriguez reports financial support was provided by Fundación Española de Cardiología. Jose Martinez-Gonzalez reports financial support was provided by Spain Ministry of Science and Innovation. Juan Carlos Laguna reports financial support was provided by Spain Ministry of Science and Innovation. Jose Martinez-Gonzalez reports financial support was provided by Fundació La Marató de TV3. Juan Carlos Laguna reports financial support was provided by AGAUR. Lidia Puertas-Umbert reports financial support was provided by Instituto de Salud Carlos III. Rafael Almendra Pegueros reports financial support was provided by Instituto de Salud Carlos III. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2025.117876](https://doi.org/10.1016/j.biopha.2025.117876).

Data availability

Data will be made available on request.

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