Lipocalin-2 deficiency or blockade protects against aortic abdominal aneurysm development in mice

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Aims
To study the role of lipocalin-2 (Lcn2) and the effect of Lcn2 blockade via anti-Lcn2 antibody in the development of abdominal aortic aneurysm (AAA).

Methods and results
Expression mRNA and protein levels of Lcn2 and its human orthologue neutrophil gelatinase-associated lipocalin (NGAL) in aortic wall samples from experimental mouse and human AAA samples, respectively, were analysed by real-time PCR and immunohistochemistry. Experimental AAA was induced by aortic elastase perfusion in wild-type mice (WT) and Lcn2-deficient mice (Lcn2−/−). NGAL/Lcn2 mRNA and protein levels in human and murine AAA samples were increased compared with healthy aortas. Decreased AAA incidence and reduced aortic expansion were observed in Lcn2−/− mice or mice preoperative treated with a polyclonal anti-Lcn2 antibody compared with WT mice or mice treated with control IgG, respectively, at Day 14 after elastase perfusion. Moreover, immunohistochemical analysis of AAA tissues from Lcn2−/− or anti-Lcn2-treated mice showed diminished elastin damage, reduced microvessels and polymorphonuclear neutrophil (PMN) infiltration, and enhanced preservation of vascular smooth muscle cells compared with WT aortas. Fluorescent molecular tomography revealed decreased MMP activity in AAA of Lcn2−/− mice compared with WT controls. Therapeutic administration of anti-Lcn2 antibody to WT mice 3 days after elastase perfusion decreased aortic dilatation and PMN infiltration compared with WT mice treated with control IgG.

Conclusion
Either Lcn2 deficiency or anti-Lcn2 antibody blockade limits AAA expansion in mice by decreasing PMN infiltration in the aorta. Lcn2 modulation may therefore be a viable new therapeutic option for the treatment of AAA.

Keywords
Abdominal aortic aneurysm • Lcn2 • PMN • MMP

1. Introduction
Abdominal aortic aneurysm (AAA) is an important disease that occurs in up to 8% of men older than 60 years and has a high mortality rate associated with AAA rupture.1 AAA is a permanent focal dilation of the abdominal aorta that exceeds >50% of the original artery diameter and/or 3 cm. When the aortic diameter is >5.5 cm, AAA patients are treated with surgery to prevent the potential rupture of the wall. In contrast, no treatment effective for patients with AAA between 3 and 5.5 cm currently exists. The pathogenesis of AAA disease is complex and involves multiple processes and cell types.2 A better understanding of the mechanisms and mediators underlying this pathology could lead to the discovery of novel therapeutic targets. Polyomorphonuclear neutrophils (PMNs) play a prominent role both in human and in experimental AAA pathogenesis, because these cells release various causative proteolytic enzymes.2–5 Proteases such as elastase, plasmin, and MMPs enhance aortic dilatation by degrading elastin and interstitial collagens in the extracellular matrix and by...
depleting vascular smooth muscle cells (VSMCs) in the aortic media. This proteolytic injury induces an adventitial response characterized by the presence of immune and inflammatory cells, fibrosis, and neointima.

Significantly, PMN depletion in mice inhibits experimental AAA formation. In human AAA, short-term preoperative doxycycline therapy can decrease MMP expression presumably via an effect on the PMN content of the aortic wall.

Lipocalin-2 (Lcn2) was described as a protein produced and secreted by innate immune cells, which by binding bacterial siderophores, limits bacterial growth. Although initially reported as secreted by PMNs, Lcn2 is now known to be produced by many cell types, including hepatocytes and epithelial, renal tubular, and VSMCs. In addition to the role of Lcn2 in the response to infection, Lcn2 has been described as a growth factor related to lipid metabolism, as necessary for granulocyte function and PMN chemotaxis, and as required for the preservation of neutrophil gelatinase activity (MMP-9).

The human homologue of murine Lcn2 is neutrophil gelatinase-associated lipocalin (NGAL). Over the past decade, it has been shown that systemic levels of NGAL are increased in patients with cardiovascular disorders. We and others have previously demonstrated that systemic levels of NGAL are increased in patients with cardiovas
cular disorders. We and others have previously demonstrated that systemic levels of NGAL are increased in patients with cardiovascular disorders.9 We and others have previously demonstrated that NGAL is increased in PMNs and plasma of AAA patients as well as in human AAA tissue.13 However, to date, there have been no studies reported on whether NGAL plays a driving role in AAA.

The objective of this study was to address this issue by examining the biological role of Lcn2 in mouse aorta and its potential contribution to experimental AAA progression. Furthermore, we explored whether treatment of mice with polyclonal anti-Lcn2 antibody could mitigate experimental AAA development.

2. Methods

2.1 Human aortic tissue samples

Eleven healthy human aortas were sampled from deceased organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal and devoid of early atheromatous lesions. Seven samples of AAA walls were collected from patients who were undergoing surgical repair of AAA and were enrolled in the RESAA protocol. Informed consent was given for the use of the human samples for research purposes. All of our human studies conformed to the principles outlined in the Declaration of Helsinki.

2.2 Mice

Lcn2 null mice (Lcn2−/−; C57Bl6/J) were provided by Dr Tak W. Mak (Campbell Family Institute for Breast Cancer Research, Toronto). The corresponding WT control mice (C57Bl6/J) were purchased from The Jackson Laboratory (Bar Harbor, USA). No differences in size and weight were detected between WT and Lcn2−/− mice. All animals were housed in isolation rooms in the Animal Facility of our institute. The Ethics Review Board of our institute approved all animal procedures, and the project was authorized by the ILS-FJD-Universidad Autónoma de Madrid (CEI 59-1036-A061) and by the Spanish Authority governing animal experimentation, the Comunidad Autónoma de Madrid (registered approval letter 10/008932.9/15). All animal procedures were performed in accordance with the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.3 Experimental AAA formation

Mice (12 weeks old) were anaesthetized by 2% isoflurane inhalational anesthesia, and a horizontal laparotomy was performed. Using a surgical stereomicroscope, the abdominal aorta was separated from the level of the left renal vein to the bifurcation and temporarily ligated between the renal and iliac arteries. An aortotomy was created with a 30-gauge needle, and the aorta was exsanguinated. A PE-26 polyethylene tube was introduced through the aortotomy, and the aorta was infused for 5 min at 100 mmHg with either saline buffer (control) or type I porcine pancreatic elastase (specific activity 6 U/mg protein; E1250; Sigma Chemical). The aortotomy was then repaired, the ligation was eliminated, and the restoration of blood flow visually confirmed. Incisions were closed and the mice housed under standard conditions. Experimental groups were elastase-infused WT mice (WT, n = 12); elastase-infused Lcn2−/− mice (Lcn2−/−, n = 11); and saline-infused WT and Lcn2−/− mice (sham-operated, n = 6 for each of them). On Day 14 post-surgery, all mice were anaesthetized with a mixture of ketamine/xylazine (100 mg/kg and 10 mg/kg body weight, respectively) and euthanized by cervical dislocation. Pre-perfusion and 14-day post-perfusion aortic diameters were measured to a resolution of 0.01 mm with a calibrated ocular grid while mice were under anaesthesia and physiological blood pressure.

Aortic diameter expansion ≥100% of that before perfusion-defined AAA. Aortic tissue samples were obtained for histological analysis. An additional cohort of elastase-infused WT mice (n = 6) and untreated healthy WT mice (n = 9) were subjected to mRNA isolation from aortic tissues.

2.4 Generation and purification of anti-Lcn2 polyclonal antibody

To obtain a neutralizing anti-Lcn2 polyclonal antibody, a rabbit (8 weeks old, New Zealand White) was immunized once weekly for 6 weeks by intramuscular injection of 100 μg recombinant murine Lcn2 protein (Sino Biological, 50060-M08H4) diluted in 200 μL PBS plus 350 μL of complete Freund’s adjuvant. At the end of the immunization period, the rabbit was bled from the marginal right ear vein, and the blood was collected and incubated at 37°C for 1 h, followed by incubation overnight at 4°C to induce clot formation. Serum was collected by centrifugation at 3000 rpm for 15 min, and the IgG fraction was purified by dialysis in PBS followed by passage over a protein A-Sepharose column (GE Healthcare). Control serum (IgG) was prepared in the same way from a non-immunized rabbit.

After that, 96-Well Flat Bottom Plates (Immunolon 4HBX, Thermo) were coated with 0.5 μg of the recombinant protein, and serial dilutions of the anti-Lcn2 serum (1/500 to 1/1 000 000) were tested. The plates were incubated with the secondary antibody (Goat anti Human IgE/PO, Nordic Covex) diluted 1/5000, and IgG reactivity was detected by adding TMB (Chemicon) and then measuring at 620 nm.

2.5 Western blot

Aliquots (1 μL) of polyclonal antibody (anti-Lcn2) and control antibody (IgG) or mouse serum (1–10 μL) were diluted in PBS to a final volume of 10 μL. The aliquots were resolved on denaturing SDS/12.5% (w/v) polyacrylamide gels. Proteins were then blotted onto PVDF (Immobilon-P Millipore) membranes, and the blots were blocked with 10% (w/v) non-fat dry milk in TBST [0.01 M Tris (pH 7.7), 0.1 M NaCl, and 0.1% Tween 20]. The membrane with mouse serum was incubated with the anti-Lcn2 at a dilution of 1:1000 overnight at 4°C. The membrane with the aliquots of both antibodies and the membrane with mouse serum after primary antibody incubation were incubated with horseradish peroxidase (HRP)-conjugated anti-IgG antibody (Dako) at a dilution of 1:5000. The proteins were then detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences).

2.6 Anti-Lcn2 antibody treatment model

Mice (12 weeks old) were injected intravenously with 100 μg anti-Lcn2 (n = 11) or IgG control antibody (n = 8) 24 h prior to AAA induction (performed as described above) and on Days 5 and 10 post-surgery. Similarly, in a therapeutic design, mice were injected intravenously with 100 μg anti-Lcn2 (n = 8) or IgG control antibody (n = 8) at Day 3 after elastase...
perfusion (performed as described above) and on Day 8 post-surgery. All mice were sacrificed 14 days after surgery, and aortic tissues were isolated for further analysis.

### 2.7 mRNA analysis by real-time PCR

Human or mouse AAAs, as well as aortic wall tissues from healthy human and mouse controls, were snap-frozen in liquid nitrogen, and homogenates (0.2 g) were resuspended in TRizol buffer (Life Technologies), and total RNA was purified. Similarly, lysates from VSMCs were resuspended in TRizol, and total RNA was purified. Duplicate samples were quantified by determining absorbance at 260 nm, and Real-Time PCR was performed as described. The expression of target genes was normalized to housekeeping transcripts (18S and GADPH). The following PCR primers and TaqMan probes were purchased from Applied Biosystems and optimized according to the manufacturer’s protocol: Lcn2 (Mm01324470_m1), GADPH (Mm99999991_g1), NGAL (Hs01080871_m1), and 18S (4310893E), mouse MMP2 (Mm00439498), MMP3 (Mm00440295), MMP9 (Mm00442991), and MMP13 (Mm00439491). All measurements were performed in triplicate. The amount of target mRNA in samples was estimated by the ∆∆CT relative quantification method. The mRNA level values in each experimental sample was expressed as fold increase over control values after normalization.

### 2.8 Immunohistochemistry and histomorphometry

Human and mouse AAA samples were embedded in OCT or paraffin. Serial sections (4 µm) of aortas were cut for histomorphometry, and immunohistochemistry was analysed using ImageJ software. Histomorphometry was performed on Masson trichrome-stained histological sections as previously described. Verhees-van Gieson staining was performed, and elastin was quantified as the percentage of missing elastin area in the media vs. total media area.

Immunohistochemistry to detect PMNs, VSMCs, microvessels, and NGAL in aortic sections was performed using the following primary antibodies: Ly6G (clone 1A8, biotradeg), α-actin (Cy3 conjugated, Clone 1A4; Sigma), CD31 (550274; BD Pharmingen), and anti-NGAL (AF1757, R&D), respectively. For colocalization studies, immunohistochemistry for Lcn2 was followed by IF for α-actin in the same section or by immunohistochemistry for Ly6G in serial sections. Non-specific staining was assessed using control polyclonal IgG antisera. Primary antibodies were visualized by treating sections with the corresponding secondary antibody and ABComplex/HRP. Sections were stained with 3, 3-diaminobenzidine/3-amino-9-ethylcarbazole and mounted in DPX. Vascular SMC loss (α-actin staining) was graded as follows: Grade 1, intact, well-organized cells; Grade 2, minimal aberrations in VSMCs; Grade 3, a few VSMC interruptions and some disorganization; and Grade 4, severe loss of VSMCs and marked disorganization. The VSMCs content was expressed as the % positive α-actin area vs. total area, the PMN content as Ly6G-positive cells vs. total area and CD31+ microvessels as the number of vessels/mm².

### 2.9 Fluorescent molecular tomography

To detect MMP activity associated with remodelled aortic tissue, mice were injected intravenously with the imaging probe MMPSense-680 (Visen; 5 mmol in 150 µL PBS; excitation wavelength 680 ± 10 nm, emission 700 ± 10 nm) on Day 13 after elastase perfusion. On Day 14, mice were euthanized and their aortas subjected to ex vivo imaging by fluorescent molecular tomography (FMT) using a chamber imaging system (FMT 1500 VisEn Medical, PerkinElmer). The amount of MMP activity in the FMT images was evaluated using the FMT1500 software package.

### 2.10 In vitro experiments

Aortic VSMCs were isolated from aorta of wild-type and Lcn2−/− mice. All mice were anaesthetized with a mixture of ketamine/xylazine (100 and 10 mg/kg body weight, respectively) and euthanized by cervical dislocation. Briefly, adhering fat and connective tissue were removed by blunt dissection from the thoracic aorta. Vessels were minced into 1 mm pieces, incubated in DMEM (Whitaker) containing 1 mg/mL collagenase (type II, 290 U/mg), penicillin (100 U/mL), streptomycin (100 Lg/mL), and glutamine (2 mmol/L) (Sigma) for 30 min at 37 °C in 95% air/5% CO2. Then aortas were rinsed twice with DMEM (Whitaker) containing 20% FBS penicillin (100 U/mL), streptomycin (100 µg/mL), and glutamine (2 mmol/L) (Sigma) to remove the cells, which were counted and seeded at a concentration of 104 cells/cm² in plastic culture flasks (Costar) in DMEM with 20% FBS. Cells were harvested for passaging at 2- to 3-day intervals and used between the second and seventh passages.

### 2.11 Chemotaxis

Leucocytes were isolated from blood of wild-type mice (3 days after elastase perfusion as described in the Experimental AAA formation section, n = 6). All mice were anaesthetized with a mixture of ketamine/xylazine (100 and 10 mg/kg body weight, respectively) and euthanized by cervical dislocation. Briefly, 0.8 mL blood obtained in EDTA tubes are incubated with 10 volumes of erythrocyte lysis buffer. After several centrifugation steps of 5 min at 1500 rpm, we keep the pellet fraction containing leucocytes until it is clean, resuspended in RPMI, and then labelled with Ly6G (1:50, 30 min, clone 1A8, biotradeg).

Transwell migration assays were performed using 96-well disposable chemotaxis chambers with an 8 µm polycarbonate filter (ChemoTX, Neuroprobe). Briefly, 29 µL of conditioned media from VSMC (wt and Lcn2−/− mice, n = 3 each) were added to the lower compartment and tested against leucocytes isolated from surgery operated mice (n = 6) added to the upper compartment (30 000 cells/well). The chamber was then incubated at 37 °C in a humidified atmosphere (5% CO2) for 2 h. A standard curve, consisting of a 1:2 dilution cascade of leucocytes, was constructed. After incubation, the framed filter was carefully removed, and the number of cells that had migrated was determined by flow cytometer analysis. Each experiment was performed in duplicate. Values are presented as a percentage of Ly6G+ migrated cells.

### 2.12 Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS 20.0 software and the Mann–Whitney test. The incidence of aneurysm was calculated by χ² analysis. A P-value of <0.05 was considered statistically significant.

### 3. Results

#### 3.1 NGAL/Lcn2 is up-regulated in human and experimental AAA

Although previous studies have documented positive immunostaining for NGAL in human AAA thrombus and wall, whether NGAL/Lcn2 mRNA is up-regulated in pathological AAA tissue compared with healthy tissue has not been addressed. We analysed the expression of NGAL/Lcn2 mRNA and protein in human and mouse wall aneurysms and compared levels with those in healthy aortic wall samples. We found that, in either human AAA or mouse elastase-induced experimental AAA, the NGAL/Lcn2 mRNA expression in AAA walls was elevated compared with healthy control aortas (Figure 1A and C, P < 0.01 and P < 0.05, respectively). Similar results were observed at the protein level by immunohistochemistry in human and mice tissues (Figure 1B and D, respectively). Moreover, we observed that Lcn2 is expressed in neutrophils and VSMCs of AAA wall (see Supplementary material online, Figure S1). Moreover, as previously observed in humans, Lcn2 plasma levels were increased in AAA (both at Day 3
Figure 1  NGAL/Lcn2 mRNA is up-regulated in human AAA and experimental murine AAA tissues. (A) Quantitation of NGAL mRNA in wall of human AAA tissues ($n = 7$) vs. healthy human control aortic samples ($n = 11$). Data are the mean ± SEM and are expressed relative to healthy control values, **$P < 0.01$. (B) Representative immunohistochemistry of NGAL in wall of human control samples and AAA. (C) Quantitation of Lcn2 mRNA in elastase-induced AAA of WT mice ($n = 6$) compared with aortic tissues of untreated healthy WT controls ($n = 9$). Data are the mean ± SEM and are expressed relative to healthy control values, *$P < 0.05$. (D) Representative immunohistochemistry of Lcn2 in wall of mice control samples and elastase-induced AAA. All scale bars = 100 μM.
(n = 7) and at Day 14 (n = 14)] compared with healthy (n = 15) mice (435.5 ± 85.9 vs. 376.7 ± 50.6 vs. 259 ± 31.2 ng/mL, P < 0.01).

### 3.2 Lcn2 deficiency diminishes aneurysmal development and growth

Because we observed that Lcn2 mRNA was increased in experimental AAA induced by elastase perfusion in WT mice, we subjected Lcn2−/− mice to elastase perfusion to investigate Lcn2’s role as a mediator of AAA. Analysis on Day 14 after elastase perfusion showed a decreased incidence of AAA in Lcn2−/− mice compared with WT mice. Only 11/11 (9.1%) of Lcn2−/− mice developed AAA (defined as an increase of 100% over the initial aortic diameter), whereas 10/12 (83.3%) of WT mice developed AAA (P < 0.001). Furthermore, the increase in aortic diameter in Lcn2−/− mice was significantly lower than that in WT mice (Figure 2A, P < 0.001). No sham-operated mouse, either Lcn2−/− or WT, developed AAA, and the modest increase in aortic diameter observed in both groups was equivalent [WT-Sham (n = 6) 32.7 ± 3.5 vs. Lcn2-Sham (n = 6) 32.5 ± 7.1%; P > 0.05].

Three main characteristics of aneurysmal wall injury are elastin degradation and VSMC loss in the media, as well as neoangiogenesis in the adventitia. Using Verhoeff-van Gieson staining, we showed that elastin degradation was reduced in Lcn2−/− mice compared with WT mice on Day 14 after elastase perfusion (Figure 2B, P < 0.01). In addition, α-actin staining revealed that VSMC loss was higher (not shown), whereas VSMC content was lower in WT mice vs. Lcn2−/− mice (Figure 2C, P < 0.05). We found that the number of neovessels in the adventitia was reduced in Lcn2−/− mice compared with WT controls (Figure 2D, P < 0.05). These data reinforce our hypothesis that AAA injury is reduced in the absence of Lcn2.

### 3.3 Treatment of WT mice with anti-Lcn2 antibody limits AAA expansion

To investigate Lcn2 as a potential therapeutic target for the treatment of AAA, we generated a polyclonal anti-Lcn2 antibody. The antibody characterization and its presence in mice are displayed in Supplementary material online, Figure S2. Significantly, WT mice that received anti-Lcn2 antibody did not develop AAA on Day 14 after elastase perfusion, unlike WT mice treated with control IgG antibody (P < 0.05). In addition, the increase in aortic diameter induced by elastase perfusion was lower in anti-Lcn2-treated WT mice than those treated with control IgG antibody (Figure 3A, P < 0.001). Anti-Lcn2-treated WT mice also showed a slight decrease in elastin layer damage compared with IgG-treated WT mice (Figure 3B, P < 0.05). Moreover, anti-Lcn2-treated mice showed lower VSMC loss compared with the IgG-treated group (not shown), while no differences were observed in total VSMC content (Figure 3C). Finally, treatment with anti-Lcn2 antibody diminished the number of neovessels (Figure 3D, P < 0.05).

### 3.4 Lcn2 deficiency or anti-Lcn2 treatment decreases PMN infiltration and MMP activity

To understand why deletion or blockade of Lcn2 protects against AAA formation, we investigated whether the diminished structural damage associated with Lcn2 deficiency was linked to decreased immune/inflammatory responses. We found that the number of PMNs was reduced in Lcn2−/− mice compared with WT controls (Figure 4A, P < 0.001) and in AAA mice preoperative treated with anti-Lcn2 antibody (Figure 5A). No differences were detected in either T lymphocytes (CD3+ cells) or monocytes/macrophages (MOMA2) in wild-type compared with Lcn2−/− mice (WT = 119.00 ± 29.08 vs. Lcn2−/− = 99.85 ± 18.12 CD3+ cells/mm², P > 0.05). WT = 32.45 ± 1.84 vs. Lcn2−/− = 27.09 ± 4.44% MOMA2-positive area/total area, P > 0.05).

It has been already described that neutrophils from Lcn2−/− mice are defective in different functions, among them a decrease in capacity to migrate. In the other hand, Lcn2 is chemotactic for neutrophils, so we hypothesized that VSMCs from Lcn2−/− mice could have a decrease in chemotactic capacity to attract neutrophils, also contributing to decrease in neutrophil infiltration observed in the vascular wall. Then, we tested the capacity of neutrophils from small AAA mice (isolated from mice perfused by elastase and killed at Day 3) to migrate against supernatant of VSMCs from wild-type mice and Lcn2−/− mice. As observed in Figure 4B, VSMCs from Lcn2−/− mice showed a decrease in capacity to attract neutrophils compared with VSMCs from wild-type mice (P < 0.05).

In addition, Lcn2 may enhance MMP-9 activation. MMP activation is known to contribute to the development, growth, and rupture of aneurysms. To determine whether the decreased aortic remodeling observed in AAA of Lcn2−/− mice was associated with decreased MMP activation, we performed a new series of surgeries to quantify MMP activity in AAA using FMT. Lcn2−/− mice showed a significant decrease in MMP activity in elastase-induced AAA compared with WT mice (Figure 4C, P < 0.01). Similarly, there was less aortic MMP activity in the anti-Lcn2-treated group compared with the control IgG-treated group (Figure 5C, P < 0.01). Finally, to test a potential direct effect of Lcn2 in MMP expression, MMP-2, −3, −9, and −13 was analysed in murine VSMCs from both wild-type or Lcn2−/− mice. However, no differences between VSMCs of wild type or Lcn2−/− were observed for MMP-2 (5.7 × 10−5 ± 8 × 10−6 vs. 2.9 × 10−5 ± 1 × 10−6), MMP-3 (1.5 × 10−8 ± 2 × 10−9 vs. 1.1 × 10−8 ± 2 × 10−9), MMP-9 (2.9 × 10−8 ± 4 × 10−9 vs. 3.1 × 10−8 ± 2 × 10−9), or MMP-13 (2.1 × 10−6 ± 3 × 10−7 vs. 1.4 × 10−6 ± 2 × 10−7) expression (P > 0.05 in all cases).

### 3.5 Anti-Lcn2 treatment reduces progression of small AAA

To investigate Lcn2 as a potential therapeutic target for the treatment of small-developed AAA, animals were treated with anti-Lcn2 antibody at Day 3 post-elastase perfusion (coinciding with the initial increase of neutrophils in this model). Significantly, the increase in aortic diameter induced by elastase perfusion was lower in anti-Lcn2-treated WT mice than those treated with control IgG antibody (Figure 6A, P < 0.005). Anti-Lcn2-treated WT mice also showed a decrease in elastin layer damage and VSMC loss (not shown), and increased vascular SMC content, compared with IgG-treated WT mice, although these differences were not statistically significant (Figure 6B and C, P = 0.1). Interestingly, treatment with anti-Lcn2 antibody diminished the content of PMN in AAs compared with IgG-treated mice (Figure 6D, P < 0.05). These observations indicate that anti-Lcn2 antibody may have therapeutic potential for the treatment of small AAA.

### 4. Discussion

In recent years, the potential utility of NGAL as a biomarker of cardiovascular disease has gained considerable interest. Increased systemic

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C. Tarin et al.
levels of NGAL have been observed in patients with myocardial infarction, atherosclerosis, or AAA.\textsuperscript{12,22,23} Moreover, high NGAL levels are linked to an increased risk of major adverse cardiovascular events and to all-cause mortality.\textsuperscript{24} In this study, we have demonstrated that NGAL/Lcn2 is not only a biomarker of AAA but also a player involved in the development of the disease.

Previous work has shown that NGAL is up-regulated in atherosclerotic plaques, and that this up-regulation correlates with characteristics of unstable plaques, such as the presence of intraplaque hemorrhage.\textsuperscript{25,26} Other work has established the presence of positive NGAL immunostaining in human AAA thrombus and wall that colocalizes with areas of MMP activation.\textsuperscript{12,13} In our study, we have

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**Figure 2** Lcn2 deficiency diminishes aneurysmal development and growth. (A) Representative Masson’s trichrome staining (left) and quantitation of increment in aortic diameter (right) at 14 days post-perfusion of elastase into WT (n = 12) and Lcn2−/− (n = 11) mice. Values shown are aortic diameter increase for individual mice, with mean and SEM indicated. (B) Representative Verhoeff-van Gieson staining (left). Right, quantitation of missing elastin area (see methods). WT (n = 10) and Lcn2−/− (n = 11) mice. Data are the mean ± SEM. (C) Representative α-actin (red) staining. Nuclei were counterstained with DAPI (blue). Right, quantitation of vascular SMA content. WT (n = 11) and Lcn2−/− (n = 11) mice. (D) Representative immunostaining (left) and quantitation (right) of CD31\textsuperscript{+} neovessels in aortic walls of wild-type (WT; n = 10) and Lcn2−/− (n = 11) mice at 14 days post-perfusion. Data are the mean ± SEM. *P < 0.05 and ***P < 0.001 vs. WT. All scale bars = 100 μM except α-actin (50 μM).
demonstrated that NGAL mRNA expression and protein is increased in human AAA wall compared with healthy aortic wall. At the experimental level, it has been previously shown that Lcn2 is highly induced in the intima after angioplasty in a rat carotid artery injury model. In our study, we observed that Lcn2 is increased in murine aortic wall and plasma in response to elastase-induced AAA formation, indicating that Lcn2 expression is up-regulated following vascular injury. However, whether the increased levels of NGAL/Lcn2 expression in human

Figure 3 Treatment with polyclonal anti-Lcn2 antibody decreases AAA formation and expansion. (A) Representative Masson’s trichrome staining (left) and quantitation of increment in aortic diameter (right) at 14 days post-perfusion of elastase in WT mice treated with control IgG antibody (n = 11) or anti-Lcn2 antibody (n = 8). Values shown are aortic diameter increase for individual mice, with mean and SEM indicated. (B) Representative Verhoeff-van Gieson staining (left). Right, quantitation of missing elastin area (see Methods). Control IgG antibody (n = 11) or anti-Lcn2 antibody (n = 8). Data are the mean ± SEM. (C) Representative α-actin (red) staining. Nuclei were counterstained with DAPI (blue). Right, quantitation of vascular SMA content. Control IgG antibody (n = 10) or anti-Lcn2 antibody (n = 8). (D) Representative immunostaining (left) and quantitation (right) of CD31+ neovessels in aortic walls of WT mice treated with control IgG antibody (n = 11) or anti-Lcn2 antibody (n = 7) at 14 days post-perfusion. Data are mean ± SEM. *P < 0.05 and ***P < 0.001 vs. IgG. All scale bars = 100 μM.
and experimental AAA tissue reflect the increased recruitment of leucocytes, or are due to NGAL/Lcn2’s potential role as a mediator of AAA, cannot as yet be distinguished.

In our mouse model of elastase-induced experimental AAA, Lcn2 deficiency or anti-Lcn2 preoperative treatment resulted in lower AAA incidence and much less AAA expansion compared with controls. Elastin layers are one of the main structural components of the aorta, and consequently, the degradation of these elastin layers is a key initial step that allows the dilatation of the vessel. The observed decrease in aortic diameter in AAA of Lcn2−/− or anti-Lcn2 preoperative treated
mice was accompanied by reduced degradation of elastin layer. Two other processes related to aneurysmal wall expansion are VSMC deple- tion in the aortic media and neoangiogenesis in the adventitia.29,30 The loss of VSMCs by apoptosis is one of the critical steps of aneurysmal destabilization.31 The role of Lcn2 in apoptosis is still controversial, with some studies supporting the hypothesis that Lcn2 inhibits apop- tosis and others indicating that it enhances it.32–34 In our present paper, we observed that VSMC loss was lower in the medial layer of Lcn2−/− or anti-Lcn2 preoperative treated mice than in their corre- sponding controls. Similarly, there are conflicting data suggesting that Lcn2 both inhibits and promotes angiogenesis, depending on the cell type or disease under study.35–38 In our present paper, we observed that VSMCs loss was lower in the medial layer of Lcn2−/− or anti-Lcn2 preoperative treated mice than in their corre- sponding controls. Similarly, there are conflicting data suggesting that Lcn2 both inhibits and promotes angiogenesis, depending on the cell type or disease under study.35–38 In our present paper, we observed that there were fewer neovessels in the adventitia layer of Lcn2−/− mice or anti-Lcn2 preoperative treated mice compared with controls. While these data could support a pro-apoptotic and pro-angiogenic effect of Lcn2 under the conditions present in the AAA wall, we cannot discount the possibility that the observed VSMC preservation and decreased neovascularization are indirect effects of Lcn2 deletion that reflect decreased wall injury. In any case, we can confidently conclude that Lcn2 is a mediator involved in aneurysm formation and expansion.

Immune/inflammatory processes are hallmarks of the pathological vascular remodelling taking place in AAA. Our analysis of the immu-inoflammatory profile of AAA in the absence of Lcn2 showed that only the PMN content was significantly decreased in the AAA walls of Lcn2−/− mice or anti-Lcn2 preoperative treated mice compared with their corresponding controls. It has been already described that neutrophils from Lcn2−/− mice are defective in different functions, among them a decrease capacity to migrate.19 In addition, Lcn2 modu- lates PMN function and migration,9,11 and we have shown that VSMCs from Lcn2−/− mice have a decrease chemotactic capacity to attract neutrophils from AAA mice. On the whole, both defective neutrophils and decrease in chemotactic activity of VSMCs in Lcn2−/− mice could contribute to decrease in neutrophil infiltration observed in the AAA wall. In addition, Lcn2 regulates MMP-9 stability and activation. Although multiple enzymes execute the extensive alterations to the vessel that occur during AAA development and growth, the MMP family is a crucial mediator of this remodelling process.19–41

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**Figure 5** Treatment with polyclonal anti-Lcn2 antibody reduces PMN infiltration and MMP activity. (A) Representative immunostaining (left) and quantitation (right) of Ly-6G+ PMNs in the aortic tissues of WT mice treated with control IgG antibody (n = 10) or anti-Lcn2 antibody (n = 7) mice at 14 days post-perfusion. (B) Left, Representative FMT analysis to detect MMP activity in ex vivo AAA tissues acquired from WT mice treated with control IgG antibody (n = 6) or anti-Lcn2 antibody (n = 5) at 14 days post-perfusion. Right, quantitation of MMP activity in the aortic tissues in the left panel. **P < 0.01 vs. IgG. For A and B, data shown are the mean ± SEM. *P < 0.05 and **P < 0.01 vs. IgG. Scale bars = 100 μM.
We have shown that Lcn2-deficient mice display a decrease in MMP activity in AAA tissue, which may explain the reduced elastin layer degradation also observed in the aortic wall. However, no differences in MMP expression were observed between VSMCs isolated from wild-type and Lcn2−/− mice, suggesting that the effect on aortic MMP activity observed could be derived from the reduced neutrophil recruitment. Our data support an important role for Lcn2 in AAA pathogenesis through PMN chemotaxis.

Experimental therapeutic approaches in AAA have been designed to modulate cells/mediators of known pathogenic pathways and so have mainly targeted proteolysis and immune/inflammatory processes. Since immune/inflammatory cells and inflammatory mediators are abundantly present in human and experimental murine AAA, modulation of T and B lymphocytes, monocytes, mast cells and PMNs, as well as their associated cytokines, have been investigated as potential therapies. Various tactics that directly or indirectly inhibit PMNs,
either their numbers, activation, or infiltration capacity.\textsuperscript{6,44–46} have demonstrably decreased aneurysmal expansion.\textsuperscript{7} Similarly, strategies aimed at targeting proteases (mainly MMP) have been pursued.\textsuperscript{47–49} However, the transfer of these approaches into the clinic has not been satisfactory. For example, two recent clinical trials have shown that neither mast cell inhibition by pemirolast treatment nor MMP inhibition by doxycycline reduces AAA growth in patients.\textsuperscript{50,51}

At present, no effective treatment for patients with AAA between 3 and 5.5 cm currently exists. We hypothesized that an experimental therapy based on anti-Lcn2 antibody treatment might protect against AAA progression. We treated mice at Day 3 post-elastase perfusion to model small AAA and based on time course studies that suggested that neutrophil changes occur prominently during this post-surgical period in the elastase model.\textsuperscript{52–54} We have shown that therapeutic administration of polyclonal anti-Lcn2 antibody to WT mice 3 days post-elastase perfusion successfully reduces aneurysmal growth by decreasing PMN infiltration. Thus, our results demonstrate that treatment with a single agent, anti-Lcn2 antibody, can prevent AAA development/growth (at least in mice) through inhibition of PMN infiltration.

In conclusion, we have demonstrated that Lcn2 is involved in AAA pathogenesis by modulating PMN infiltration. The administration of anti-Lcn2 antibody diminishes the development of experimental AAA in mice, suggesting its potential usefulness as a therapeutic strategy for the prevention of AAA progression in humans.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: Some authors (C.T., E.B., C.P., P.L.-G., J.E., L.M.B.-C., and J.L.M.-V.) are named as co-inventors on a pending patent that has been filed by the IIS-Fundacion Jimenez Diaz and Autonoma University, and relates to the use of anti-Lcn2 antibody as a potential therapy for abdominal aortic aneurysm.

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