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Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

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Chapter

6

CXCL8 HYPERSIGNALING IN THE AORTIC ABDOMINAL ANEURYSM: THE ORAL CXCR2 ANTAGONIST DF2156A FULLY ABROGATES EXPERIMENTAL ANEURYSM FORMATION

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ABSTRACT

Background

The chemokine CXCL8 is concurrently involved in neutrophil-mediated inflammation and angiogenesis, two prominent pathological characteristics of abdominal aneurysm disease (AAA). Previous work identified CXCL8 hyperexpression as a discriminative feature of AAA disease. In the absence of molecular targets for pharmaceutical aneurysm stabilization we considered an evaluation of the CXCL8 signaling cascades in human AAA and the potential of interfering with CXCL8 signaling relevant.

Methods

ELISA's, Western blot analysis, real time PCR and array analysis were used to explore CXCL8 expression and signaling in aneurysm wall samples from patients undergoing elective AAA repair. A role for CXCL8 in AAA disease was tested through the oral CXCR1/2 antagonist DF2156A in the elastase model of AAA disease.

Results

There is an extreme disparity in aortic wall CXCL8 content between AAA and atherosclerotic disease (median [IQR] aortic wall CXCL8 content: 425 [141 - 1261] (AAA) vs. 23 [2.8 - 89] $\mu\text{g/g}$ protein (atherosclerotic aorta), ($P < 1.5 \cdot 10^{-15}$)) and abundant expression of the CXCR1 and 2 receptors in AAA. Array analysis followed by pathway analysis showed that CXCL8 hyper-expression in AAA is followed increased by IL-8 signaling ($p < 0.000039$).

Interference with CXCL8 signaling through DF2156A fully abrogated AAA formation in the murine elastase model of AAA disease ($p < 0.001$).

Conclusion

Activation of the CXCL8-signaling pathway is a prominent and distinctive feature of AAA. Interference with the pathway constitutes a promising target for medical stabilization of AAA.

INTRODUCTION

An Abdominal Aortic Aneurysm (AAA) is a common pathology and a major cause of death due to rupture¹. Most AAAs are asymptomatic and remain undetected until rupture¹. Hence, some countries instigated nationwide screening programs for the identification of AAA. These programs resulted in a major increase in patients with an identified AAA, most of them small in size.

In accordance to prevailing guidelines these patients with smaller AAAs are kept under surveillance until the AAA reach the threshold for repair at 55 mm. It is estimated that up to 70% of the patients in the watch and follow up program will eventually reach the 55 mm intervention threshold². Accordingly, it has been pointed out that pharmaceutical intervention reducing or inhibiting progression of small AAA, and thus reducing the need for surgical repair could have major advantages; both from a patients' as from a socio-economical perspective³. Despite clear preclinical successes, no pharmaceutical intervention has been proven to be effective so far⁴.

The pathology of growing AAAs is thought to be a localized chronic inflammatory response that is accompanied and perpetuated by exaggerated angiogenesis and a proteolytic imbalance; the latter is being held responsible for a progressive weakening of the aortic wall⁵. The actual molecular basis has not been identified.

We previously documented CXCL8 hyper-expression as a clear distinctive and unique feature of AAA with 300-fold higher CXCL8 protein levels in the aneurysm wall than in advanced aortic atherosclerotic wall samples^{5,6}. CXCL8 has comprehensive chemotactic effects on a wide-variety of immune cells, in particular *but* not-exclusively on neutrophils; a cell type that is explicitly implicated in AAA disease^{5,7,8}. Moreover, CXCL8 stimulates protease expression and inflammation⁹, and exerts strong pro-angiogenic effects by promoting chemotaxis and proliferation of endothelial cells¹⁰⁻¹².

In this context, we considered further examination of a putative role for CXCL8 signaling as a potential therapeutic target in AAA disease relevant. The present study confirms the CXCL8 hyper-expression and exaggerated activation of the CXCL8 downstream pathways in human aneurysms, and shows that interference with CXCL8 signaling through the oral CXCR1/2 antagonist DF2156A fully abrogates aneurysm formation in an accepted model of AAA disease (the murine elastase model).

RESULTS

CXCL8 expression in human abdominal aortic aneurysms

We first performed a validation of our previous observations of CXCL8 hyper-expression in 238 AAA wall samples and control aorta samples from the Aneurysm-Express Biobank. Results confirmed previous observations and showed a several hundred-fold increase CXCL8 protein content in aneurysm wall samples ($P < 1.5 \cdot 10^{-15}$, Figure 1A) and an approximately 16-fold higher CXCL8 mRNA expression ($P < 0.01$, Figure 1B). Immunohistological staining for CXCL-8 in the aneurysm wall shows comprehensively expression in macrophages, neutrophils, and smooth muscle cells; as well as in a subpopulation of lymphocytes, and occasional endothelial cells (Figure 1C). Expression in advanced aortic atherosclerotic disease on the other hand is essentially confined to foam cells, macrophages, and occasional smooth muscle cells the intimal layer and intimal border zone of the medial layer of the aortic wall, and incidental lymphocytes (Figure 1C).

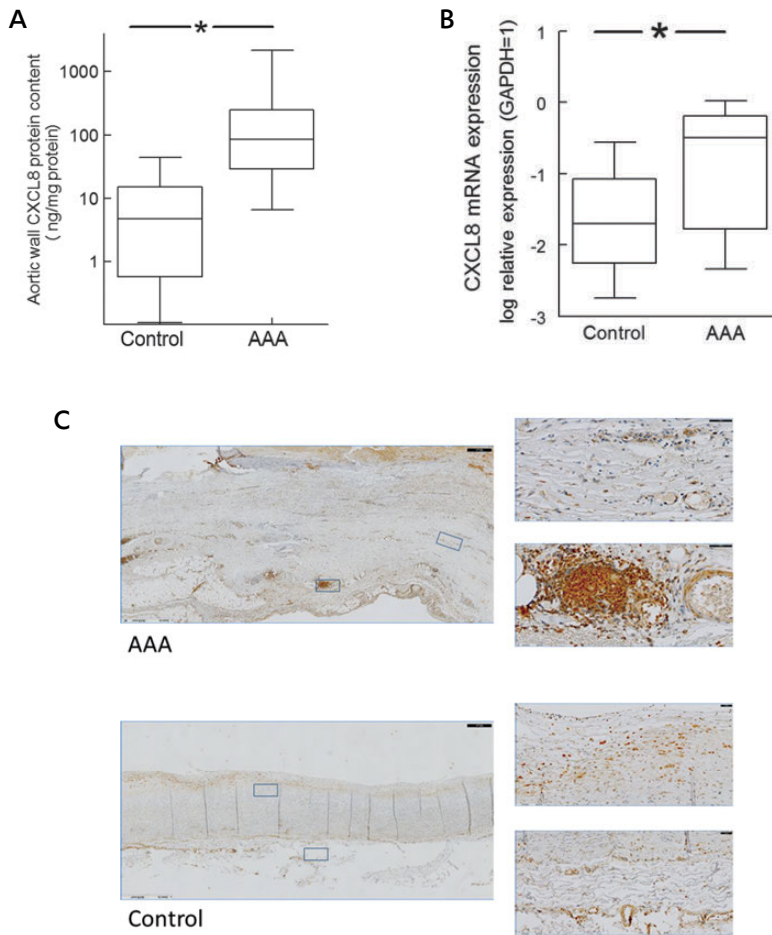


Figure 1. CXCL expression in human AAA and controls (atherosclerotic aorta). A) Extreme disparity in aortic wall CXCL8 protein content. AAA vs control: $P < 1.5 \cdot 10^{-15}$. B) Aortic wall CXCL8 mRNA content. AAA vs Control $P < 0.01$. C) Representative samples showing aortic wall CXCL-8 distribution in AAA and control aorta. Overview 5X, detail 40x.

Abundant presence and activation of the CXCL8- pathway in human abdominal aortic aneurysms

CXCL8 signaling in humans is through its receptors: CXCR1 and 2. CXCL8 binding to these receptors results in activation of multiple effector pathways such as phosphorylation of ERK (extracellular signal regulated protein kinase) 1/2. Immunohistochemical analysis shows abundant expression of both the CXCR1 and CXCR2 receptors (Figure 2) and enhanced ERK phosphorylation in AAA compared to aortic atherosclerotic disease. CXCL8 signaling was further explored through Ingenuity-based transcriptomics analysis. This analysis identified CXCL8 (IL-8) signaling pathway and Agranulocyte Adhesion and Diapedesis among the top upregulated pathways in AAA disease ($p < 0.000039$ and < 0.000000049 respectively).

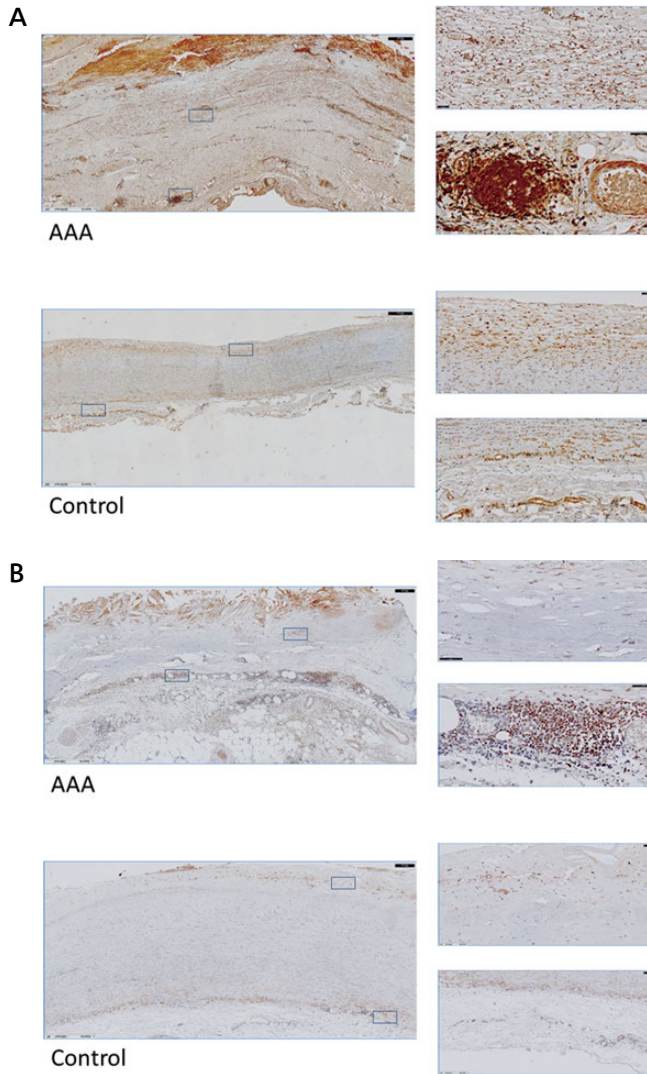
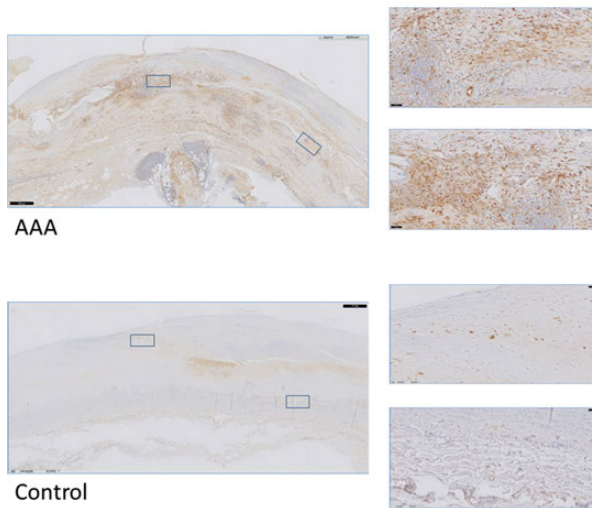


Figure 2. CXCR1 and CXCR2 expression in human aortic wall. A) Representative samples showing aortic wall CXCR1 distribution in AAA and control aorta. Overview 5X, detail 40X. B) Representative samples showing aortic wall CXCR2 distribution in AAA and control aorta. Overview 5X, detail 40X.

CXCL8 has particularly strong effects on neutrophil chemotaxis, stabilization and activation, and is described to be the dominant promoter of CXC chemokine-mediated angiogenesis. Histologic evaluation shows abundant and dispersed neutrophils (MPO staining) in human aneurysms, while neutrophils are absent in control atherosclerotic samples (Figure 4). Occasional neutrophils in the vaso vasora confirmed the validity of the staining. This characterizes neutrophils abundance as a clearly distinctive feature of human AAA.

A



B

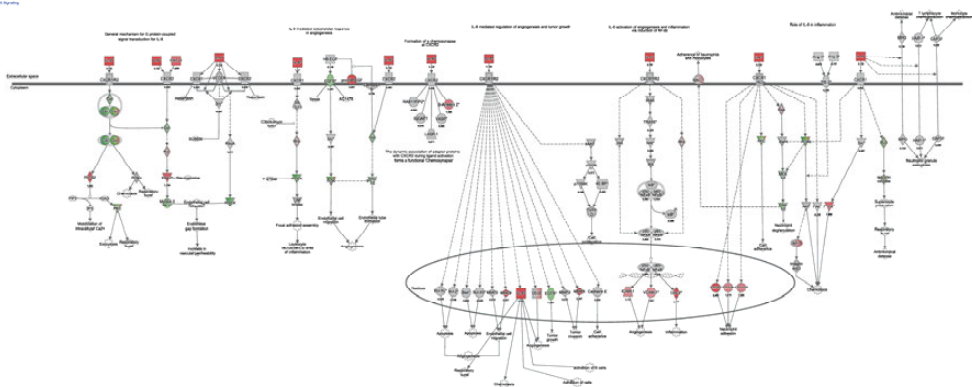


Figure 3. CXCL-8 signaling in human aorta. A) Representative samples showing aortic wall pERK1/2 distribution in AAA and control aorta. Overview 5X, detail 40X. B) CXCL-8 signaling in AAA (Ingenuity Pathway Analysis).

CXCR1/2 inhibition abrogates aneurysm formation

To evaluate possible involvement of the CXCL8 in aneurysm formation, we tested whether the oral CXCR1/CXCR2 inhibitor (DF2156A) influences aneurysm formation in the established murine elastase model of the disease. Mice ($n=10$) received DF2156A during two weeks via daily oral gavage starting from the day before elastase perfusion. Control animals ($n=10$) received daily oral gavage with saline. At the day before elastase perfusion, 7 days and 14 days after perfusion the aortic diameter was measured via ultrasound. Aortic dilatation at day 7 was similar in both groups (12.7% SD $\pm 8.5\%$ (DF2156A) and 21.5 % SD $\pm 14.9\%$ (vehicle), $p=0.161$). A daily gavage completely abolished aneurysm formation, measured at day 14, in all animals (17.7% dilatation SD $\pm 9.6\%$, (DF2156A) and 71.9% SD $\pm 26.7\%$ (vehicle) $p<0.001$) (Figure 5).

DF2156A treatment almost completely quenched vascular inflammation and preserved the integrity of the vessel wall as shown by an increased collagen content and less elastin breaks

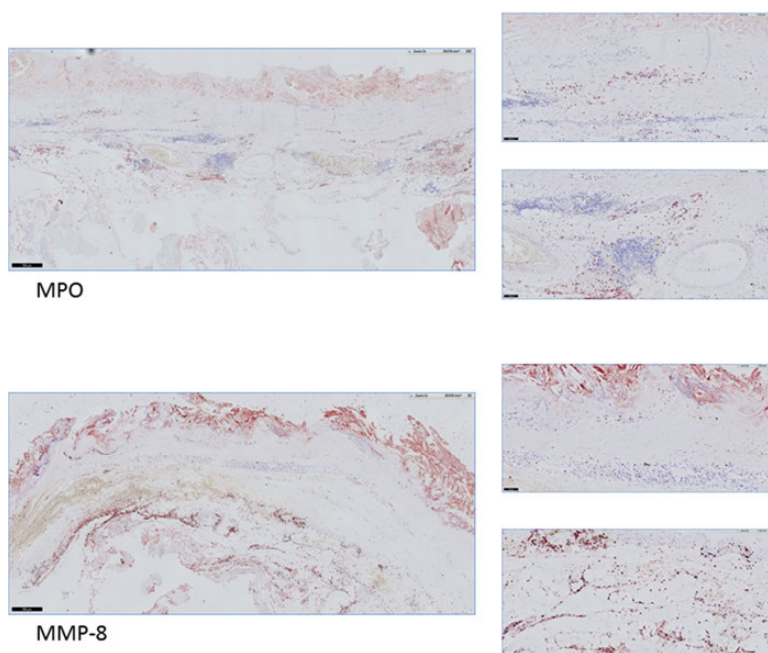


Figure 4. Neutrophil abundance in AAA.

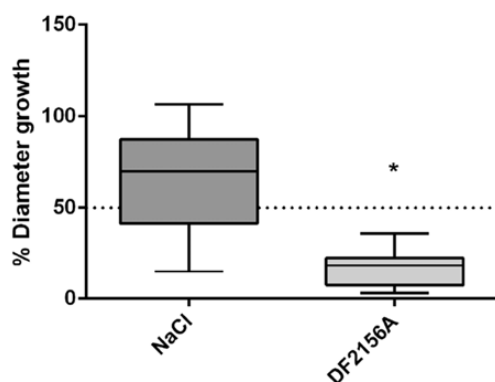


Figure 5. The CXCR1/2 antagonist DF2156A fully abrogates AAA formation. The oral CXCR1/2 antagonist DF2156A (repertaxim) fully abrogates aneurysm development in the elastase model of AAA disease. Percentage given is the increase in aorta diameter from baseline at day 14. $P < 0.001$.

(Figure 6d). Furthermore, treatment with the CXCR1/CXCR2 inhibitor resulted in significantly less leucocytes ($p < 0.05$) and limited the MMP9 expression ($p < 0.05$) compared to the controls (Figure 6a and b). While all mice revealed similar macrophage counts ($p = 0.98$) (Figure 6b), which reveals that the effect on vascular inflammation is highly selective.

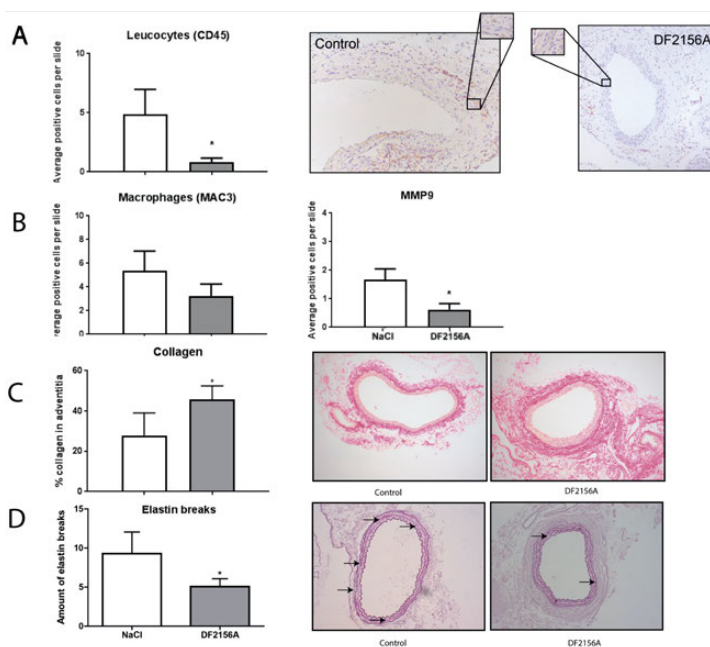


Figure 6. The CXCR1/2 antagonist DF2156A quenches vascular inflammation and preserves adventitial collagen, and medial elastin. The oral CXCR1/2 antagonist DF2156A reduces aortic wall leucocyte content (CD45 staining) and MMP-9 expression (both $P < 0.05$) but does not influence aortic wall macrophage content (MAC3) staining, adventitial collagen and reduces medial elastin breaks. All data shown is for day-14.

DISCUSSION

This study confirms CXCL8 hyper-expression and enhanced activation of the CXCL8 axis as a distinctive feature of AAA. Interference with the oral CXCL8 antagonist fully abrogated AAA formation, characterizing this axis as a potential pharmaceutical target for AAA.

Our previous work identified CXCL8 hyperexpression as a unique feature of human AAA.¹³ These observations were validated in this study in an independent and large patient cohort (Aneurysm Express) using a different analysis platform. Immunohistochemistry for CXCL8 showed comprehensive expression in both leucocytes as well as mesenchymal cells. This pattern was clearly distinct from advanced atherosclerotic disease in which expression was predominantly confined to the intima, in particular to foam cells.

In humans CXCL8 signals through the CXCR1 and -2 receptors. These receptors have different affinities, suggesting distinct responses at varying CXCL8 levels. The activities are thought to be partially overlapping activities, but they also appear to mediate distinct aspects of CXCL-8 mediated inflammation²². The expression pattern is broad, and includes a wide variety of leucocytes, mesenchymal cells (smooth muscle cells, fibroblasts) and endothelial cells. Immunohistochemical staining for CXCR1 and 2 followed by morphological analysis showed abundant receptor expression in leucocytes as well as smooth muscle cells and (myo)fibroblasts of the aneurysm wall. Observations above not only identify CXCL8 hyperexpression as a clear distinctive feature between AAA and

atherosclerotic disease, but also show that the transcriptional machinery required for CXCL8 signaling is present in AAA. Prominent ERK1/2 phosphorylation, and Ingenuity based pathway analysis confirmed exaggerated CXCL8 signaling as a distinctive feature of AAA disease.

CXCL8 classically associates with neutrophil influx and neutrophil-mediated inflammation. CXCL8 not only acts as a strong chemo-attracted for neutrophils, but it also increases neutrophil half-life by preventing apoptosis, thereby further contributing to neutrophil abundance in AAA disease.²³ These neutrophils may contribute to the proteolytic imbalance through release of multiple proteases such as the serine protease neutrophil elastase and metalloproteinases MMP8 (neutrophil collagenase) and MMP9 (neutrophil gelatinase), but also through protease mediate degradation of protease inhibitors such as TIMPs (through the action of neutrophil elastase) and cystatin C (through MMP9 and neutrophil elastase)⁵. A crucial role for neutrophils in AAA disease is emphasized by several animal studies in which interference with either neutrophil activation or infiltration alleviates AAA formation and/or progression^{24;25}. Apart from its effects on neutrophils, CXCL8 also has potent pro-angiogenic effects and influences other leucocytes (in particular M1 macrophages²⁶) thereby contributing to a proinflammatory environment²⁷. Angiogenesis is a characteristic feature that has been linked to vascular inflammation and AAA rupture, and as such has been brought forward as a therapeutic target for pharmaceutical AAA stabilization²⁸.

The CXCL8-pathway has long been identified as potential pharmacological target for several acute and chronic inflammatory conditions^{21;29}. Combined CXCR1/2^{30;31;33} and selective CXCR2 inhibitors are currently under clinical evaluation^{32;33}.

We tested the ability of the combined CXCR1/2 antagonist DF2156A to inhibit AAA formation in an established murine model of the disease. A single daily dose strongly reduced AAA formation; in fact the minimal dilatation observed presumably reflects the effect of pressure-perfusion and/or the loss of elastic recoil by the elastase treatment, and not the influx of any inflammatory cells. The effects exerted by DF2156A in the model by far exceed the effects reported for other established anti-inflammatory agents, such as doxycycline³⁴, indomethacin³⁵ or cyclosporin³⁶.

Considering the apparent failure of medical stabilization of small AAA so far, the potency of CXCR1/2 inhibition in vivo is remarkable and merits clinical evaluation. As mice only express CXCR2, we could not test the contribution of each receptor to the process of clinical AAA formation. Consequently it is unclear whether clinical trial with selective CXCR2 inhibitors would be equally effective as a combined CXCR1/2 antagonist.

In conclusion, to our knowledge, this study is the first to demonstrate full abrogation of aneurysm formation in the murine elastase model, emphasizing the critical role of the CXCR2-axis in aneurysm formation in the model. This and along with the clinical data, identifies activation of the CXCL8-pathway as a distinctive feature of AAA and characterizes this pathway as a promising (possibly the most promising) target for the medical stabilization of growing AAA.

METHODS

Human Samples

The investigation conforms the principles outlined in the Declaration of Helsinki (2013). Sample collection and handling was performed in accordance with the guidelines of the medical ethical

committee of the Leiden University Medical Center. We obtained tissue from anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (>5.5 cm or larger). Aortic tissue samples removed along with the renal artery during kidney ex-plantation from brain-dead, heart-beating, adult organ donor, were used as control samples. Aortic wall samples were divided in two parts. One half was immediately snap-frozen in CO₂-cooled isopentane or liquid N₂ and stored at -80°C for later analysis. The other half was fixed in 4% formaline for 12 hours and decalcified. The latter segments were paraffin embedded and 4 µm sections were processed into slices. For immunohistochemistry, sections (n=10 AAA, n=10 control atherosclerotic aortic wall samples) were deparaffinized, treated for 10 minutes with H₂O₂ to block endogenous peroxidase activity, and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibodies were used: human myeloperoxidase (DAKO), CXCL8 (bs-078012, Bioss, Huissen, The Netherlands), CXCR1 (ab124344, Abcam, Cambridge UK), CXCR2 (bs-1629R, Bioss), pERK1/2 (1481-1 Epitomics, Leiden, The Netherlands). CXCL8 mRNA expression was quantified by semi quantitative RT, to that end a total RNA extraction was performed according to manufacturer's instructions. cDNA was prepared by using a Promega kit (Proega, Leiden, the Netherlands) for RT-PCR. For the determination of mRNA expression we used an established CXCL8 primer/probe set (Thermo Fisher Scientific, Bleiswijk, The Netherlands), the mastermix (Eurogentec, Maastricht, the Netherlands) and the ABI-7700 system (Thermo Fisher Scientific) as previously described¹³. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Thermo Fisher Scientific) was used for normalization. Aortic wall CXCL8 protein content was determined using the Aneurysm-Express Biobank¹⁴ (n=238 AAA samples and n=26 control atherosclerotic samples) via ELISA, employing Luminex multianalyte profiling technology^{15,16}, using a bio-plex system (Bio-Rad, Veenendaal, the Netherlands). Total protein concentration of every sample was quantified via a BCA protein measurement method (Thermo Fisher Scientific). All measured concentrations were related to the protein concentrate of every sample. Inter-assay coefficient of variation was <10%. Microarrays: RNA extraction was performed from full thickness aortic wall samples from 31 AAA patients (mean age 69.5 yrs. mean diameter 62.3±12.1 mm) collected during elective aneurysm repair and 9 control samples (infra renal aorta obtained during kidney procurement for donation).

RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips (Illumina, Eindhoven, the Netherlands). Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

Analysis of array data: Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software.

Association of genome-wide expression data with AAA phenotype revealed 11486 transcripts with P<0.05. These differentially expressed transcripts were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (<http://www.ingenuity.com>, accessed 2016). Levels of significance were determined using Fisher's exact tests implemented in the software.

Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines. Eight-to-ten weeks old, male, C57BL/6 mice were obtained from Charles River, France. The aneurysms were created

via porcine pancreatic elastase (PPE) infusion as previously described¹⁷⁻²⁰. After the elastase infusion 0.05-0.1 mg/kg/12hrs buprenorphine was given and the mice recovered with free access to food and water. The oral CXCR1/2 antagonist DF2156A (6 mg/kg), a generous gift from Dompé Pharma, Milan, Italy²¹ was given (n=10) daily via oral gavage in 100 microliter of 0.25% carboxymethylcellulose diluted in PBS. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals (n=10) received daily oral gavage of 100 microliter of 0.25% carboxymethylcellulose diluted in PBS for 15 days. To compare the aortic growth rates of the different groups we measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, CA). At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in PBS -1% albumin. The sections were incubated with CD45 (BD Pharmingen, Breda, The Netherlands), MAC3 (BD Pharmingen), MMP9 (Santa Cruz Biotechnology) and MPO (Abcam). Additional sections were stained with Sirius Red for collagen and Weigert's elastin stain to visualize elastic laminae. Six slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on six slices was calculated for each animal.

Statistical Analysis

All values are shown as mean (SD) and probability values of $P < 0.05$ were considered statistically significant. After performing an ANOVA test to explore the difference between human AAA and human atherosclerotic samples, an unpaired t-test was performed. The Mann-Whitney U test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice. All analysis were performed using SPSS 23.0 (SPSS Inc. Chicago).

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