Journal of the American Heart Association

ORIGINAL RESEARCH

Induction of Heme Oxygenase-1 Is Linked to the Severity of Disease in Human Abdominal Aortic Aneurysm

Anja Hofmann, PhD; Margarete Müglich; Steffen Wolk, MD; Yazan Khorzom, MD; Pamela Sabarstinski, MSc; Irakli Kopaliani, MD; Dmitry Egorov, PhD; Franziska Horn; Coy Brunssen, PhD; Sindy Giebe, PhD; Bianca Hamann, MSc; Andreas Deussen, MD; Henning Morawietz, PhD; David M. Poitz, PhD; Christian Reeps, MD

BACKGROUND: Rupture of abdominal aortic aneurysm (rAAA) is associated with high case fatality rates, and risk of rupture increases with the AAA diameter. Heme oxygenase-1 (gene *HMOX1*, protein HO-1) is a stress-induced protein and induction has protective effects in the vessel wall. *HMOX1*$^{-/-}$ mice are more susceptible to angiotensin II-induced AAA formation, but the regulation in human nonruptured and ruptured AAA is only poorly understood. Our hypothesis proposed that HO-1 is reduced in AAA and lowering is inversely associated with the AAA diameter.

METHODS AND RESULTS: AAA walls from patients undergoing elective open repair (eAAA) or surgery because of rupture (rAAA) were analyzed for aortic *HMOX1*/HO-1 expression by quantitative real-time polymerase chain reaction and Western blot. Aortas from patients with aortic occlusive disease served as controls. *HMOX1*/HO-1 expression was 1.1- to 7.6-fold upregulated in eAAA and rAAA. HO-1 expression was 3-fold higher in eAAA specimen with a diameter >84.4 mm, whereas HO-1 was not different in rAAA. Other variables that are known for associations with AAA and HO-1 induction were tested. In eAAA, HO-1 expression was negatively correlated with aortic collagen content and oxidative stress parameters H_2O_2 release, oxidized proteins, and thiobarbituric acid reactive substances. Serum HO-1 concentrations were analyzed in patients with eAAA, and maximum values were found in an aortic diameter of 55 to 70 mm with no further increase >70 mm, compared with <55 mm.

CONCLUSIONS: Aortic HO-1 expression was increased in eAAA and rAAA. HO-1 increased with the severity of disease but was additionally connected to less oxidative stress and vasoprotective mechanisms.

Key Words: abdominal aortic aneurysm ■ heme oxygenase ■ rupture

Ruptured abdominal aortic aneurysms (rAAA) have high case fatality rates.[1] The risk of rupture increases with AAA diameter. Surgery is the only treatment option for patients with AAA so far.[2] The development of novel nonsurgical therapeutic options requires a detailed understanding of the molecular mechanisms that contribute to the initiation, progression, and rupture of AAA. In brief, AAA are characterized by a progressive dilatation of the vessel wall because of inflammation, loss of vascular smooth muscle cells, and the disruption of extracellular matrix, which is mainly composed of elastin and collagen.[1,3]

A promising novel target for treatment of cardiovascular disease is the stress protein HO-1 (heme oxygenase-1). HO-1 is induced by heme, hypoxia, hyperoxia, heavy metals, nitric oxide, cytokines, chemokines,

Correspondence to: Anja Hofmann, PhD, Division of Vascular and Endovascular Surgery, Department of Visceral, Thoracic and Vascular Surgery, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Fetscherstr. 74, 01307 Dresden, Germany. E-mail: anja.hofmann2@uniklinikum-dresden.de

Supplementary Material for this article is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.121.022747

For Sources of Funding and Disclosures, see page 16.

JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- Serum heme oxygenase-1 (HO-1) concentrations were higher in electively treated patients with abdominal aortic aneurysm (AAA), and peaked in patients with 55 to 70 mm AAA diameter and showed no further increase >70 mm.
- Aortic HO-1 expression was similarly increased in nonruptured and ruptured AAA.
- AAA walls with high HO-1 expression showed a lowering in collagen, a decrease in oxidative stress, and a slight lowering in matrix metalloprotease 9 activity.

What Are the Clinical Implications?

- Analyzing human tissue from patients in advanced stages helps to identify mechanisms that contribute to AAA progression and rupture.
- The potential of HO-1 to prevent AAA progression or rupture should be tested in preclinical animal models and cell cultures and may thus be beneficial in small AAA.
- Serum HO-1 concentrations should be analyzed in ruptured AAA and after surgery to further analyze its potential in being a biomarker for AAA.

Nonstandard Abbreviations and Acronyms

AOD	arterial occlusive disease
CCL2	chemokine (C-C motif) ligand 2
eAAA	electively treated abdominal aortic aneurysm
HMOX1	heme oxygenase-1 gene
HO-1	heme oxygenase-1 protein
IL6	interleukin-6
MMP	matrix metalloprotease
rAAA	ruptured abdominal aortic aneurysm
r_P	Pearson's correlation coefficient
r_S	Spearman's correlation coefficient
TBARS	thiobarbituric acidreactive substances
α-SMA	α-smooth muscle actin

shear stress, growth factors, oxidants, or oxidized low-density lipoprotein (LDL) cholesterol.[4–7] HO-1 catalyzes the degradation of pro-oxidative heme proteins, thus forming carbon monoxide, biliverdin, and ferrous iron ions (Fe^{2+}).[5,7,8] HO-1 affects inflammation, oxidative stress, antioxidant functions, apoptosis, hypoxia, ischemia/reperfusion injury, and angiogenic processes.[5,7,9] A protective role of HO-1 was shown in vascular dysfunction, atherosclerosis, ischemia/reperfusion,[10,11] myocardial infarction,[12] and heart failure.[13]

So far, only a few studies have been conducted to analyze HO-1 in patients with AAA and corresponding mouse models. In AAA, lower frequencies of guanidine thymidine$_n$ dinucleotide repeats were found in the promoter region of the *HMOX1* gene.[7,14,15] Short (<25 guanidine thymidine$_n$) repeats seem to be protective because of an increased HO-1 expression in response to inflammatory stimuli.[14] Mechanistic studies were conducted in preclinical angiotensin II (AngII)-induced mouse models. Mice with genetic deletion of *HMOX1* developed more AAA, showed an increased AAA area, and a higher severity of disease.[16] Although the function of HO-1 is well described in mouse models of AAA, its regulation and associations with the severity at different stages of the human disease remain to be elucidated. Based on previously published mouse models, our research hypothesis proposed that HO-1 is reduced in AAA and lowering is inversely associated with the AAA diameter and the secondary outcome variables vessel wall degeneration, inflammation, and oxidative stress. *HMOX1* mRNA and HO-1 protein expression was analyzed in patients undergoing elective surgical removal (eAAA) or surgery because of rupture (rAAA). HO-1 expression was compared between controls, eAAA, and rAAA and expression was linked with the maximum aneurysm diameter, as a major clinical parameter in AAA. Histopathological features of AAA, oxidative stress, inflammatory cytokines, hemoglobin, and bilirubin were tested as secondary variables. Furthermore, serum HO-1 concentrations were analyzed in eAAA and compared with control patients with venous vessel varicose. Serum HO-1 was correlated with the AAA diameter to assess whether HO-1 is linked to the progression of AAA. In this study, we aimed to assess the regulation of HO-1 in human AAA and to analyze whether it reflects the severity of disease or presents a compensatory mechanism that may limit clinical complications.

METHODS

The authors declare that all supporting data are available within the article and its Data Supplement.

Primary and Secondary Outcome Variables

The maximum AAA diameter was set as the primary outcome variable because it is the most commonly used predictor of AAA rupture.[17] Secondary variables include histopathological vessel wall degeneration, expression of vascular and immune cells, oxidative stress, bilirubin, hemoglobin, inflammation, and activity of matrix metalloproteases (MMPs). These variables were chosen

because they support the primary end point, and are involved in the pathogenesis of AAA and the regulation of HO-1. The following sections describe the quantification of primary and secondary outcome variables.

Abdominal Aortic Wall Collection and Ethical Approval

Aortic specimens were collected from patients undergoing elective open repair (eAAA, n=19) or surgery because of AAA rupture (rAAA, n=11). The eAAA and rAAA groups included specimens that were taken from different sites of the AAA. These biopsies were handled as individual samples without calculating the mean. Because of this, the number of analyzed samples varied from the original included patients. In addition, not all indicated analyses were performed in each tissue, because of the lack of sufficient amounts of material. The aortic samples in the control group were obtained from patients with arterial occlusive disease (AOD, n=4) where the bypass was connected to the abdominal aorta. The mean aortic diameter was assessed by computed tomography before the surgical procedure. Blood lipids, C-reactive protein (CRP) , risk factors, comorbidities, and medical therapies were prospectively evaluated. Smoking was defined as present smoking or any kind of smoking history. Informed consent was obtained from each patient, and the institutional review committee of the Technische Universität (TU) Dresden approved this study (EK 151042017).

Serum Analysis and Control Cohort

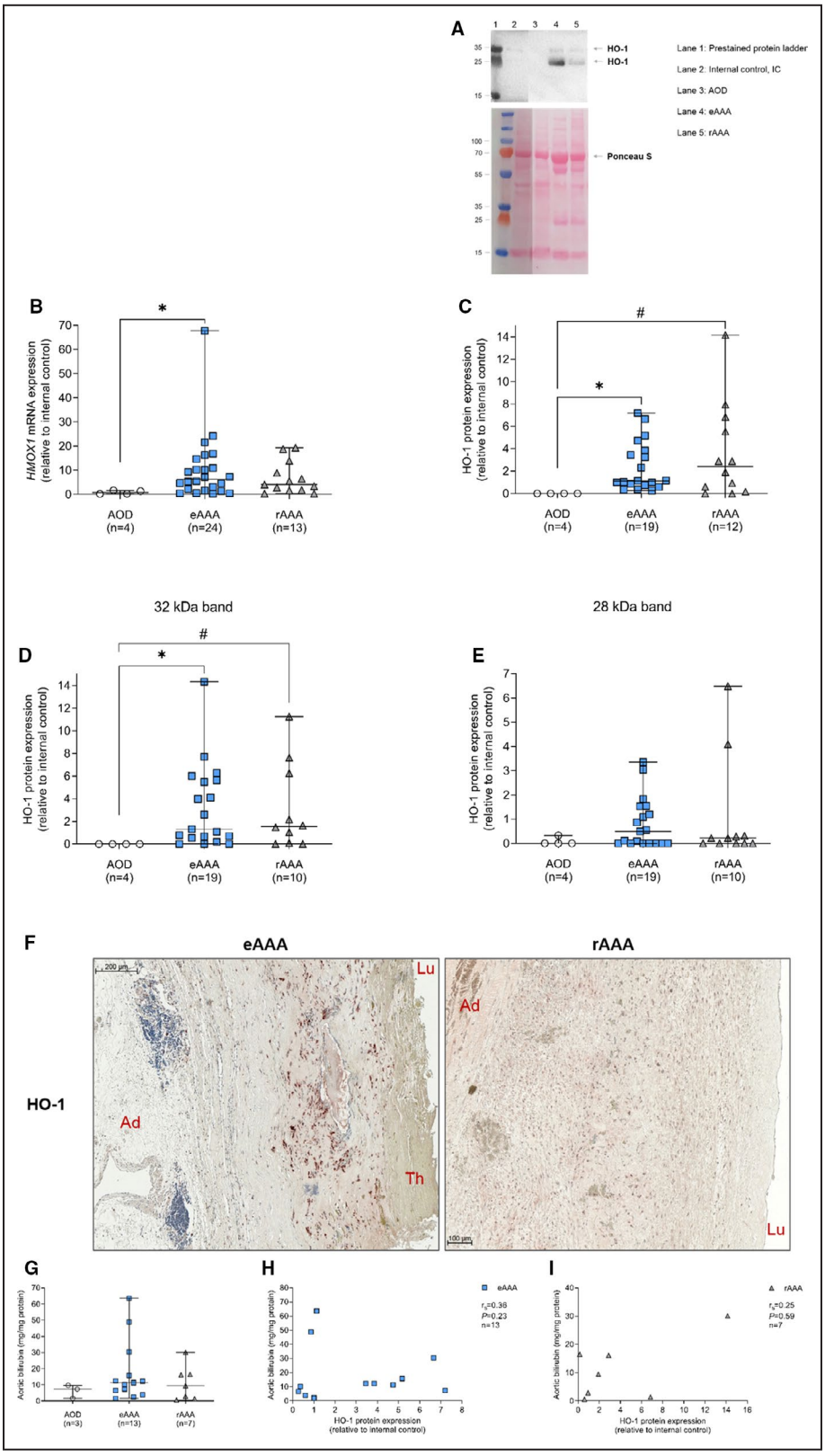
Blood from patients undergoing eAAA repair was collected in the preoperative state. Blood from patients with rAAA was taken during surgery and blood lipids were not measured at the time of emergency surgery. Thus, a comparison between eAAA and rAAA was not possible because of differences in the study protocols. The control group consisted of patients with venous vessel varicose without a history of arterial cardiovascular disease. Inclusion criteria were men and women at age ≥50 years. Serum LDL, high-density lipoprotein (HDL), and total cholesterol, glucose, and CRP were measured at the Institute for Clinical Chemistry and Laboratory Medicine at the TU Dresden using standard laboratory methods. Serum HO-1 (ab207621, Abcam) was determined by ELISA according to the manufacturer's instructions. A dilution of 1:5 was found to be appropriate.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction

Aortic segments were rinsed with 1xDPBS, cleaned from thrombus and the adventitial layer, dissected, and shock frozen in liquid nitrogen. Tissue (30–50 mg) was homogenized in 1 mL TriFast (VWR) using a Precellys 24 homogenizer and RNA was isolated according to the manufacturer's instructions. Afterwards, the RNA Clean and Concentrator Kit (Zymo Research) was used with additional on column digestion of remaining DNA by DNase I treatment. Reverse transcription of mRNA into cDNA was performed with MultiScribe Reverse Transcriptase (Thermo Fisher Scientific) using 0.5 to 1 µg total RNA and random hexamer primers according to the manufacturer's instructions. Quantification of mRNA expression was performed by real-time polymerase chain reaction with GoTaq qPCR Master Mix (Promega) and Step One Plus Real-Time PCR System (Thermo Fisher Scientific). Analysis of raw data was done with Step One Software version 2.3 (Thermo Fisher Scientific) and data are calculated as ΔC_T values. The geometric mean of *RPL32*, *TBP*, and *B2M* was used as reference genes for cDNA content normalization.[18] To ensure comparability of data sets, an internal control was run in every reverse transcription and quantitative polymerase chain reaction and was set to =1. Efficiency was checked for each pair of primers and was >90%. Sequences are summarized in Table S1.

Protein Isolation and Western Blot

Aortic specimen were grounded in liquid nitrogen using a mortar and lysed in 1×RIPA buffer (10 mg/100 µL) supplemented with 1:100 Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Ultrasonication was applied to remove remaining DNA. Protein concentration was determined by BCA Protein Assay Reagent (Thermo Fisher Scientific). Proteins (15–30 µg) were separated by 4% to 12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against HO-1 (#610713, BD Biosciences). Two bands at ≈32 and ≈28 kDa were quantified. The upper band was described as the complete protein, the lower as the truncated version.[19] The truncated version was described to be enzymatically inactive but activates oxidant-responsive transcription factors.[19] Protein expression was detected with Immobilon Western HRP Substrate (Merck) and band intensity quantified using Image J software. In preliminary experiments, GADPH, β-actin, and α-tubulin were analyzed for their potential as loading controls. All of these proteins varied between the 3 groups and within each group (data not shown). Therefore, protein expression was normalized to a 70 kDa band obtained after Ponceau S staining. To ensure comparability of data, an internal control was run on every Western blot and relative protein expression was normalized to this control (=1). Data are presented in relation to the internal control. The representative Western blot presented in Figure 1C was



cut after Lane 2 (prestained protein ladder and internal control). The full blot with the corresponding Ponceau S staining is presented in Figure S1.

Gelatin Zymography

Gelatin zymography was performed as described in detail elsewhere.[20] In brief, activities of active MMP2,

Figure 1. Aortic *HMOX1* mRNA and HO-1 protein expression, localization within the aortic wall, and bilirubin content in patients undergoing elective surgical repair or surgery because of rupture and AOD controls.
A, Representative Western blot for HO-1 protein expression. The 2 marked HO-1 bands represent native (≈32 kDa) and a truncated (≈28 kDa) isoform of HO-1. **B**, Comparison of *HMOX1* mRNA expression and (**C**) HO-1 protein expression in AOD, eAAA, and rAAA. Data are normalized to an internal control (=1). For *HMOX1* mRNA expression in eAAA, aortic tissues from n=19 patients were available. This group included 2 to 3 different specimen from the same AAA. Because of this, the number of samples was increased to n=24. In rAAA, n=11 aortic specimen were available. This group included 2 different AAA samples from 3 patients and the number of analyzed samples increased to n=13. For HO-1 protein in eAAA, n=17 patients were included and 3 different AAA specimens from the same patient were analyzed. Because of this, the number of samples increased to n=20. In rAAA, n=9 patients were included and 2 different samples from the same AAA were analyzed in 2 patients. **D** and **F**, Separate quantification of the upper (≈32 kDa) and lower (≈28 kDa) HO-1 band. **F**, Representative slides for HO-1 immunohistochemistry in eAAA and rAAA specimen. Redstained areas represent positive HO-1 immunoreactivity. Cell nuclei (blue) were counterstained using Mayer's hemalum. The lumen (Lu), adventitia (Ad), and attaching thrombus (Th) are marked in red. **G**, Comparison of aortic bilirubin content in AOD, eAAA, and rAAA and (**H** and **I**) Spearman's correlation (r_S) with HO-1 protein expression in eAAA and rAAA. In eAAA, pairs of bilirubin and HO-1 protein were available in n=11 patients only. **B–E** and **G**, All data are shown as scatter dot plots. The horizontal line depicts the median with range (B-E, G). The number of analyzed samples is given in the figures. Kruskal–Wallis and Dunn's multiple comparison test. **P*<0.05 AOD vs eAAA. #*P*<0.05 AOD vs rAAA. Ad indicates adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; *HMOX-1*, heme oxygenase-1 gene; Lu, lumen; rAAA, ruptured AAA; and Th, thrombus.

pro-MMP2, active MMP9, and pro-MMP9 were detected in nonreducing 10% SDS-polyacrylamide gels. The gel was exposed to a solution containing essential enzyme co-factors. MMPs within the gel digest gelatin, forming clear bands after staining with Coomassie Brilliant Blue. After partial renaturation, the active site of pro-MMP forms remained exposed to its substrate. Differences in migration within the gel were based on the molecular weight of pro- and active forms and allowed their clear separation. The band intensity was quantified using Image J and normalized to an internal control (set=1) that was run on every gel.

Quantification of Hemoglobin and Bilirubin Concentrations in Aortic Homogenates

Hemoglobin was measured by QuantiChrome Hemoglobin Assay Kit (DIHB-250, Biotrend) and concentrations were normalized to the protein content. To analyze bilirubin concentrations, tissue was homogenized in 1 mL 1×DPBS supplemented with 1:100 Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). After homogenization, samples were centrifuged at 10. 000*g* for 15 minutes and 4 °C. Supernatants were analyzed in duplicates using the QuantiChrom Bilirubin Assay Kit (DIBR-180, Biotrend). Values were normalized to aortic protein content.

Thiobarbituric Acid Reactive Substances Assay for Quantification of Malondialdehyde in AAA Specimen

Aortic segments were homogenized in ice cold 1×DPBS (50 mg/mL) supplemented with 1:100 Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and 50 µmol/L butylated hydroxytoluene. Thiobarbituric acidreactive substances (TBARS) were measured using the TBARS assay kit (700870, Cayman Chemical) according to the manufacturer's instructions. Samples were measured in duplicates and blank wells included the homogenization buffer. Values were normalized to aortic protein content.

Quantification of Extracellular Hydrogen Peroxide by Amplex Red Assay

Extracellular hydrogen peroxide (H_2O_2) production was measured using Amplex Red Assay (Thermo Fisher Scientific). Aortic walls were divided into strips of 2- to 3-mm length and measurements were performed in Krebs-Henseleit buffer. Segments were free of thrombus and were carefully cleaned in 1xDPBS. Segments were incubated in Krebs-Henseleit buffer for 10 minutes at 37 °C and a reaction mix containing 0.1 U/mL horseradish peroxidase (HRP) and 100 µmol/L Amplex Red was added. The tissue was incubated at 37 °C for 30 minutes and fluorescence (relative fluorescence units) was recorded over 60 cycles in a period of 30 minutes using Varioscan LUX (Thermo Fisher Scientific). The sum of all cycles was calculated and normalized to the total protein content. To account for endogenous HRP activity, a separate cohort was analyzed for H_2O_2 formation in the absence of HRP.

Quantification of Carbonyl Residues in Oxidized Proteins by Protein Carbonyl Assay Kit

Carbonyl groups in oxidized proteins were detected by the Protein Carbonyl Assay Kit (ab178020, Abcam). In brief, aortic tissue (30–50 mg) was homogenized in 1 mL of DPBS supplemented with 1:100 Halt Protease and Phosphatase inhibitor using a Precellys 24 homogenizer (VWR). Afterwards, 50 µL of extraction buffer was added to the aortic homogenate and isolation was performed according the manufacturer's instructions. Protein concentrations were determined by bicinchoninic acid assay (BCA) and afterwards, 50 mmol/L

dithiothreitol (DTT) was added to the samples. Proteins (4 µg/µL) were separated by 4% to 12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes. Incubation with primary and secondary antibodies was performed according to the manufacturer's instructions. Negative controls were run in pretests (Figure S2) and an internal control was run on every gel. Data were normalized to this control, which was set to =1.

Elastica van Gieson and Picro-Sirius Red for Assessment of Elastin Degradation and Collagen Content

Aortic wall segments were fixed in 4% formaldehyde solution for a maximum of 24 hours, dehydrated, decalcified, and embedded in paraffin. Serial sections (5 µm) were cut, rehydrated, and stained with Elastica van Gieson and Picro-sirius Red. Sections were photographed with the Axioscan slide scanner microscope (Carl Zeiss, Jena) at a ×10 magnification. Degradation of elastic laminae was classified using a score (1–4) as described previously[21] by 6 people blinded to the experiment. Image J was used to separate red collagen fibers from white background in the media and intima. Adjacent thrombus or adventitia was not included. Data are presented in percent of the total section area. The mean of 2 different sections from 1 specimen was used.

Staining of Ferric Ions (Fe^{3+}) by Perl's Prussian Blue

Staining of ferric ions (mainly in ferritin and hemosiderin) was performed using 10% $K_3Fe(CN)_6$ and 20% HCl in a ratio of 1:1 for 60 minutes at 37 °C. Nuclear fast red was used for counterstaining cell nuclei. Image J quantified the positively stained blue area in the media and intima by separation from violet cell nuclei. Data are presented in percent of the total section area. The mean of 2 different sections from 1 specimen was used for data analysis.

Immunohistochemistry for α-Smooth Muscle Actin, Cleaved Caspase-3, CD68, CD31, and HO-1

Aortic wall segments with attaching luminal thrombus and adventitia were fixed in 4% formaldehyde solution for a maximum of 24 hours, decalcified, embedded in paraffin, and serial sections (5 µm) were cut and rehydrated. Antigen retrieval for α-smooth muscle actin (α-SMA), cleaved caspase-3, CD68, and HO-1 was performed in citrate buffer containing 0.05% Tween 20 at pH 6.0. Antigen retrieval for CD31 was performed in 10 mmol/L Tris buffer containing EDTA (1 mmol/L) and Tween 20 at pH 9.0. Except for CD31, all sections were treated with proteinase K (20 µg/mL). Endogenous peroxidase (S2023, Agilent) and unspecific binding sites were blocked (X0909, Agilent). Primary antibodies with concentrations used are listed in Table S2. Secondary antibodies (Signal Stain Boost IHC Detection Reagent, Cell Signaling) were incubated for 1 hour at room temperature and AEC+ High Sensitivity Substrate Chromogen (K3461, Agilent) and DAB (ImmPACT DAB [HRP] Peroxidase Substrate, Vector Laboratories, SK-4105) were used for color development. Cell nuclei were counterstained with Mayer's hemalum. To exclude unspecific staining, sections were incubated with the same concentration of the corresponding isotype antibody and with the secondary antibody only. Negative controls using the respective isotype control antibody are presented in Figure S3. For α-SMA, cleaved caspase-3, and CD68, red areas in the presence of blue stained cell nuclei were quantified by Image J and data are presented in percent of the total section area. For CD31, positive brown areas were quantified. Only the media and intima were quantified; attaching thrombus and adventitia were excluded. The mean of 2 different sections from 1 patient was analyzed.

Statistical Analysis

The first author had full access to all data in the study and takes responsibility for its integrity and the data analysis. Grubb's test was used to detect significant outliers in all data sets. Outliers within each group are indicated in the figure legends. Data are presented as scatter dot plots. The horizontal line depicts the median or mean with range. Normality was tested by the D'Agostino and Pearson normality test. Non-Gaussian distributed data were analyzed by Kruskal–Wallis and Dunn's multiple comparison test, and Gaussian distributed data by 1-way ANOVA and Tukey's post hoc test. Correlational analysis in non-Gaussian-distributed data was done using Spearman's correlation coefficient (r_S), and Gaussian distributed data were compared by Pearson's correlation coefficient (r_P). Differences in the distribution of cardiovascular risk factors and medical therapies across the 3 independent groups (AOD, eAAA, and rAAA) were compared by the χ^2 test using a contingency table. The null hypothesis (H_0) postulated that distributions of risk factors and medical therapies are independent of the outcome of the disease (AOD, eAAA, or rAAA) and no differences will be observed between all groups. Differences between patients with venous vessel varicose and eAAA were compared by Fisher's exact test. Graph Pad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA) software was used for statistical analysis and $P \leq 0.05$ was considered as significant.

RESULTS

Patients' Characteristics for Quantification of Aortic Heme Oxygenase-1 Gene and Protein Expression

The clinical characteristics of the studied patients are shown in Table 1. The mean age of the AOD (55.8±7.7 years) and eAAA (66.8±9.0 years) group was not different, but patients with rAAA (73.1±7.2 years) were significantly older (P=0.009) than those with AOD. The AAA diameter was lower (P=0.005) in eAAA (median: 59.9 mm) compared with rAAA (median: 90.4 mm). Comparison of cardiovascular risk factors revealed a higher prevalence of atherosclerosis in peripheral and carotid arteries and in smoking in AOD. Patients with eAAA had higher LDL and

Table 1. Clinical Characteristics in Patients Undergoing Electively treated Abdominal Aortic Aneurysm Surgical Repair or Surgery Because of Ruptured Abdominal Aortic Aneurysm and Corresponding Arterial Occlusive Disease Controls

	AOD	eAAA	rAAA	χ^2	*P* value
Clinical characteristics					
N included	4	19	11		
Age, y	55.8±7.7	66.8±9.0	73.1±7.2*		0.0093
Sex, men/women, % men	2/2, 50	16/3, 84	9/2, 82	2.42	0.29
Aortic diameter, mm (n)	…	59.9 (44.0–90.0)	90.4† (52.2–110.0)		0.0046
Cardiovascular risk factors					
Hypertension, n/N total, %	3/4, 75	17/19, 89	9/10, 90	0.72	0.70
Smoking, n/N total, %	4/4, 100	11/19, 58	8/10, 20	8.05	0.02
CRP, mg/L (n)	2.70 (1.20–18.0) 4	3.15 (0.50–127.8) 20	18.8 (1.52–269.4) 11		0.05
LDL cholesterol, mmol/L (n)	1.47 (1.26–1.65) 3	2.94 (0.71–6.79)‡ 19	nd		0.009
HDL cholesterol, mmol/L (n)	1.11 (0.85–1.34) 3	1.13 (0.65–2.43) 19	nd		0.73
Total cholesterol, mmol/L (n)	2.99 (2.88–3.04) 4	4.46‡ (3.04–8.07) 19	nd		0.002
Triglycerides, mmol/L (n)	1.57±0.22, 3	1.91±0.88, 20	nd		0.52
Blood glucose, mmol/L (n)	5.75 (4.40–30.60) 3	5.29 (3.95–7.90) 16	nd		0.43
BMI, kg/m² (n)	25.70±8.00, 4	27.51±4.65, 20	27.18±5.85, 11		0.83
T2D, n/N total, %	1/4, 25	1/19, 5	1/9, 10	1.68	0.43
CAD, n/N, %	2/4, 50	9/19, 47	4/10, 40	0.18	0.91
PAD, carotid artery stenosis, n/N total, %	4/4, 100	6/19, 33	2/9, 22	7.58	0.03
Medical therapy					
Statins, n/N total, %	3/4, 75	15/19, 79	7/10, 70	0.28	0.86
ACE inhibitor, n/N total, %	0/4, 0	8/19, 42	4/10, 40	2.61	0.27
ARB, n/N total, %	1/4, 25	6/19, 36	2/10, 20	0.46	0.79
β-Blocker, n/N total, %	1/4, 25	8/19, 42	5/10, 50	0.73	0.69
CCB, n/N total, %	0/4, 0	7/19, 37	2/10, 20	2.64	0.26
Diuretics, n/N total, %	0/4, 0	8/19, 42	3/10, 30	2.71	0.25
ASA, n/N total, %	4/4, 100	12/19, 63	7/10, 70	2.12	0.35
Anticoagulants, n/N total, %	2/4, 50	5/19, 26	3/10, 30	0.87	0.64
Insulin, n/N total, %	1/4, 25	1/19, 5	0/10, 0	3.18	0.20

In rAAA, blood lipids were not measured at the time of emergency surgery. Thus, a comparison between eAAA and rAAA was not possible. No data on cardiovascular risk factors were available from 1 patient in rAAA. All data are presented as the median with minimum and maximum (range) or as mean±SD depending on the result of normality testing. Comparison of controls and eAAA was done using Mann–Whitney U test and AOD, eAAA, and rAAA Kruskal–Wallis and Dunn's post hoc test or 1-way ANOVA and Tukey's post hoc test. Prevalence of risk factors (sex, hypertension, smoking, T2D, CAD, and PAD/carotid artery stenosis) and medical therapies was analyzed by χ^2 test. ACE indicates angiotensin-converting enzyme; AOD, arterial occlusive disease; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blocker; CRP, C-reactive protein; eAAA, electively treated abdominal aortic aneurysm; HDL, high-density lipoprotein; LDL, low-density lipoprotein; nd, not determined; PAD, peripheral artery disease; rAAA, ruptured abdominal aortic aneurysm; and T2D, type 2 diabetes.

*P<0.01 rAAA vs AOD.

†P<0.01 eAAA vs rAAA.

‡P<0.01 eAAA vs AOD.

Figure 2. Linkage of HO-1 expression with AAA diameter and histopathological vessel wall degeneration in patients undergoing elective surgical repair or surgery because of AAA rupture and AOD controls.
A, Comparison of HO-1 protein expression in eAAA samples with a median diameter less or higher than 84.5 mm. The minimum value was 56.0 mm, the maximum 90.0 mm. Only patients with a diameter higher than 55.0 mm were included because this diameter is chosen in the clinics for surgical removal of the AAA. **B**, Comparison of HO-1 protein expression in rAAA samples with a diameter less or higher than 94.0 mm. The minimum value was 52.2 mm, the maximum value 110.0 mm. **D** and **E**, Elastin fiber degradation was graded into a score of 1 to 4 and HO-1 protein expression was grouped. **G**, Comparison of aortic collagen content in AOD, eAAA, and rAAA and (**H** and **I**) Spearman's correlation (r_S) with HO-1 protein expression in eAAA and rAAA. Representative slides for (**C**) Elastica-van-Gieson and (**F**) Picro-sirius Red staining. The black rectangles represent enlargements of the lower magnifications. All data are presented as scatter dot plots. The horizontal line depicts the median with range (A, B, D, E, and G). The number of analyzed samples is given in the figures. **A**, **B**, **D**, and **E**, Mann–Whitney *U* test. **G**, Kruskal–Wallis and Dunn's post hoc test. *P<0.05 56.0 to 84.4 vs 84.5 to 90.0 mm. Ad indicates adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; and Th, thrombus.

total cholesterol concentrations when compared with AOD. Medical therapies did not differ among all groups (Table 1).

HMOX1 mRNA and HO-1 Protein Expression Are Increased in eAAA and rAAA

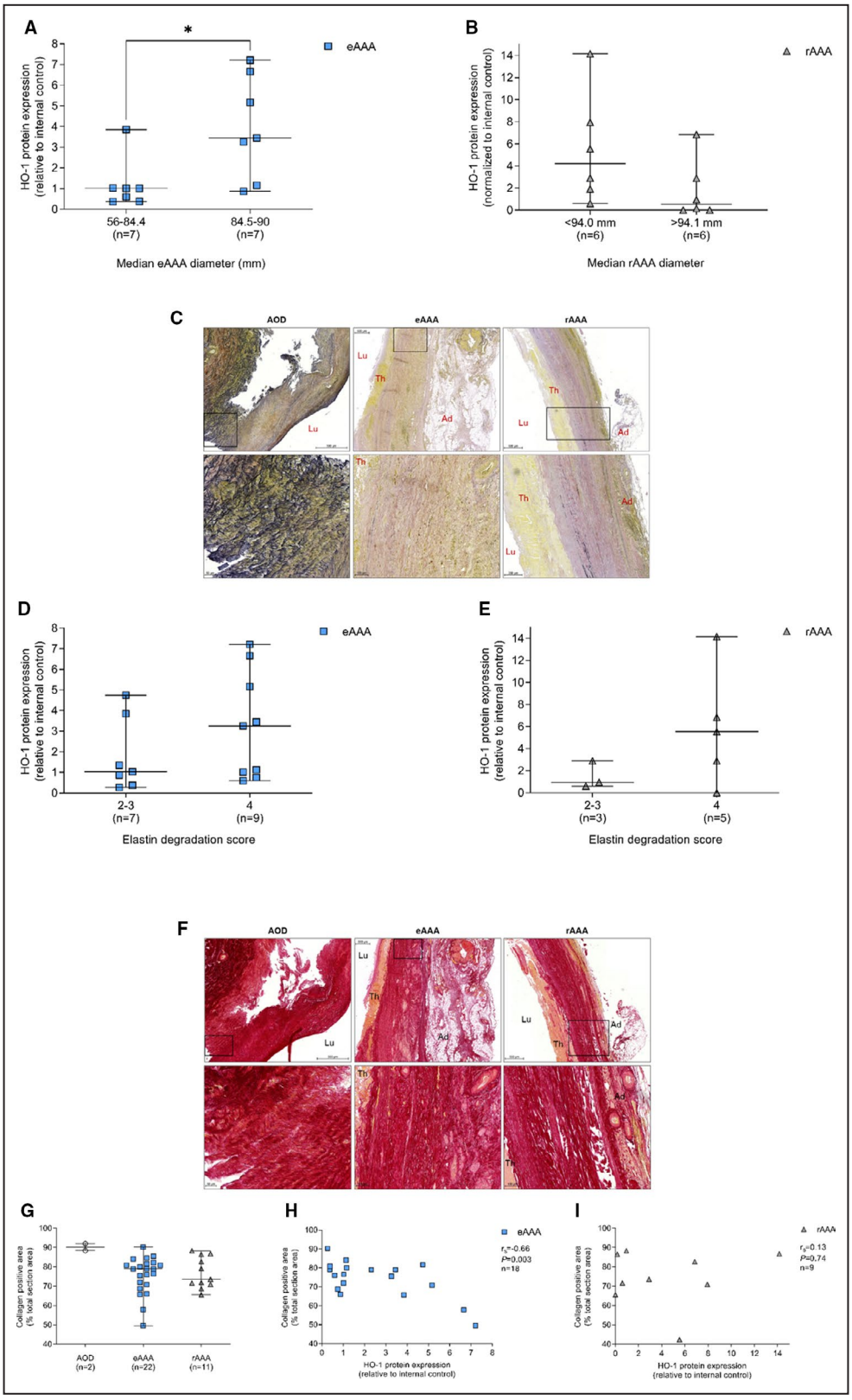
HMOX1 mRNA expression was compared between patients with eAAA, rAAA, and AOD. Gene expression was 7.6-fold higher (P=0.03) in eAAA than in AOD. *HMOX1* mRNA was elevated (P=0.10) in rAAA when compared with controls but did not reach significance because of scatter. Aortic walls from eAAA and rAAA showed a similar mRNA expression. HO-1 protein was nearly undetectable in AOD aortas and increased in eAAA (P=0.01). Protein expression was higher in rAAA (P=0.02) and similar between eAAA and rAAA (Figure 1A through 1C). The HO-1 antibody used detects different HO-1 isoforms, and protein expression of the upper and lower band was separated. Quantification of the ≈32-kDa band revealed a significant increase in eAAA (P=0.03) and rAAA (P=0.04) when compared with AOD. The lower band (≈28 kDa) was comparable among all groups (Figure 1D and 1E). To analyze HO-1 localization within aortic walls, immunohistochemistry of HO-1 protein was performed. In eAAA, HO-1 was mainly located in the media, near the border with the intima or adventitia. Representative slides are shown in Figure 1F. Furthermore, the HO-1 downstream reaction product bilirubin was quantified in the AAA specimen. Bilirubin was not related to HO-1 expression (r_S=0.36, P=0.23) in eAAA and rAAA (r_S=0.25, P=0.59) (Figure 1G through 1I).

Associations of HO-1 Protein Expression With the AAA Diameter and Histopathological Vessel Wall Degeneration

The median AAA diameter was calculated to assess HO-1 expression in different stages of the disease. In eAAA, only patients with a diameter >55 mm were analyzed because this diameter is used for surgical intervention. HO-1 expression was significantly higher in AAA samples with a diameter >84.5 mm (P=0.02). In the rAAA group, HO-1 expression was slightly lower in samples with a diameter >94.1 mm (Figure 2A and 2B). To analyze whether changes in aortic HO-1 expression are linked to the histopathological features of AAA, elastin fiber degradation, collagen, endothelial and vascular smooth muscle cells content, and apoptosis were quantified. In eAAA and rAAA, HO-1 expression was similar in aortic walls showing an elastin degradation score of 2/3 and 4 (Figure 2C through 2E). A negative association between HO-1 and aortic collagen was found in eAAA (r_S=–0.66, P=0.003) (Figure 2F through 2I). No associations with cleaved caspase-3-positive areas were demonstrated (Figure S4A through S4D). A lowering in α-SMA was found in eAAA (P=0.04), and HO-1 expression was not associated with α-SMA-positive areas in eAAA (r_S=0.37, P=0.13) and rAAA (r_S=0.50, P=0.17) (Figure S5A through S5D). Assessment of CD31 positive areas revealed a trend towards an increase (P=0.07) in eAAA when compared with AOD. Furthermore, CD31 positive staining was lower (P=0.04) in rAAA when compared with eAAA. In eAAA, CD31 positive areas were enlarged in aortic walls with a higher HO-1 expression (r_S=0.52, P=0.02). This was not found in rAAA (Figure S6A through S6D). Representative slides for assessment of elastin degradation, collagen, cleaved caspase-3, α-SMA, and CD31 positive areas are shown above the corresponding figures.

Aortic HO-1 Expression and Activity of MMP9 and MMP2

Because of their matrix degrading function, MMP9 and MMP2 are linked to AAA wall dilation. Expression and activity of their respective pro- and mature forms were assessed in order to evaluate relations with HO-1 expression. Pro-MMP9 expression was positively correlated with aortic HO-1 expression in eAAA (r_S=0.52, P=0.02) and rAAA (r_S=0.60, P=0.04) (Figure 3A through



3C). The activity of mature MMP9 tended to be higher in eAAA samples with a lower HO-1 protein expression (r_S=−0.41, P=0.07) without being significant (Figure 3D through 3F). Pro-MMP2 and MMP2 were not linked to aortic HO-1 protein expression (Figure S7A through S7F).

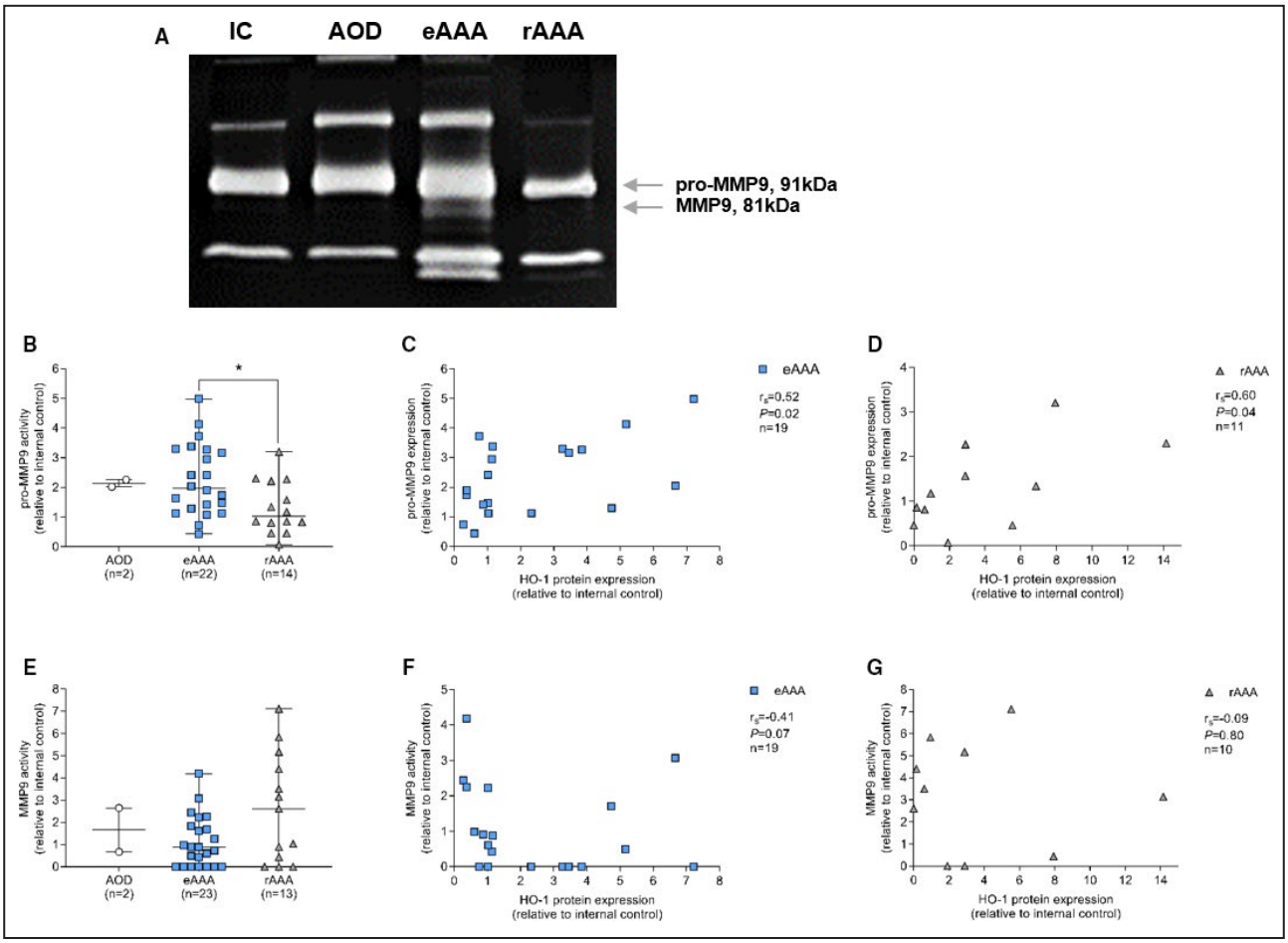
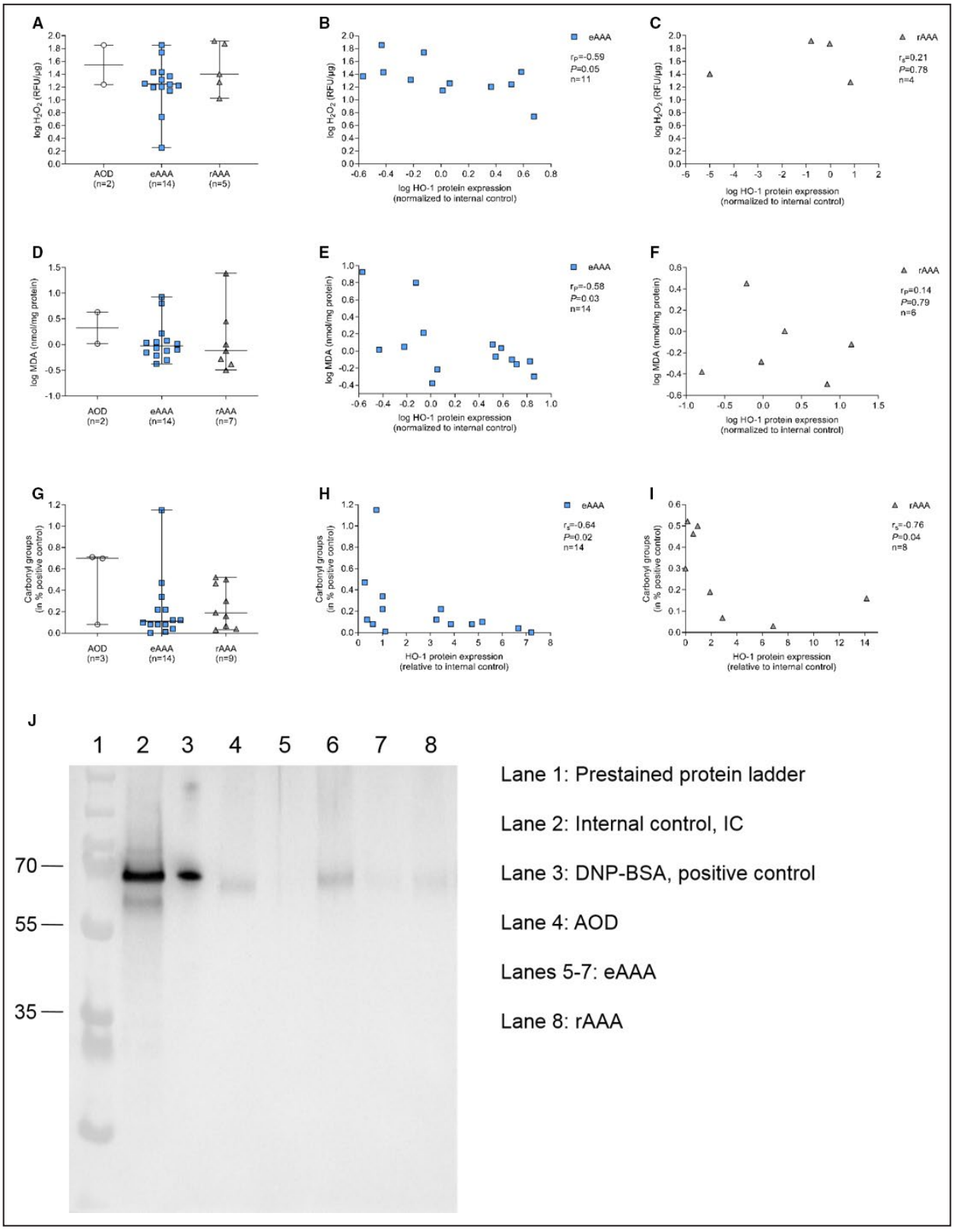


Figure 3. Activity of matrix metalloprotease 9 and correlation with HO-1 in patients undergoing elective surgical repair or surgery because of AAA rupture and AOD controls.
A, Representative zymograms for detection of pro-MMP9 and MMP9 in AOD, eAAA, and rAAA. **B**, Comparison of aortic pro-MMP9 expression in AOD, eAAA, and rAAA and (**C** and **D**) Spearman's correlation (r_S) with HO-1 expression in eAAA and rAAA. **E**, Comparison of aortic MMP9 activity in AOD, eAAA, and rAAA and (**F** and **G**) Spearman's correlation (r_S) with HO-1 protein in eAAA and rAAA. Grubb test was performed and 1 significant outlier was detected in the data set for MMP9 (**B**, rAAA). Data are presented as scatter dot plots. The horizontal line depicts the median with range (B and E). The number of analyzed samples is given in the figures. Kruskal–Wallis and Dunn's multiple comparison test. *P<0.05 eAAA vs rAAA. AOD indicates arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; MMP, matrix metalloprotease; and rAAA, ruptured AAA.

Increased HO-1 Expression Is Linked to a Lowering in Oxidative Stress

Oxidative stress is known to play a pivotal role in AAA.[22] To analyze whether reactive oxygen species and oxidative stress might be connected to the increase in HO-1, extracellular H_2O_2 release, TBARS, and carbonyl groups in oxidized proteins were quantified. Concentrations of H_2O_2 (r_P=−0.59, P=0.05), TBARS (r_P=−0.58, P=0.03), and carbonyl residues (r_S=−0.64,

Figure 4. Oxidative stress and correlation with HO-1 expression in patients undergoing elective surgical repair or surgery because of ruptured AAA and AOD controls.
A, Comparison of aortic H_2O_2 release in AOD, eAAA, and rAAA. Extracellular H_2O_2 was measured by Amplex Red assay and fluorescence was normalized to the protein content. **B** and **C**, Pearson's correlation (r_P) of H_2O_2 with HO-1 expression in eAAA and rAAA. **D**, Comparison of aortic thiobarbituric acid reactive substances (TBARS) in AOD, eAAA, and rAAA samples. Values were normalized to the protein content. **E** and **F**, Pearson's correlation (r_P) of TBARS with HO-1 expression in eAAA and rAAA. **G**, Comparison of carbonyl residues in oxidized proteins in AOD, eAAA, and rAAA. Carbonyl residues were derivatized and quantified by Western blot. **H** and **I**, Spearman's correlation (r_P) of oxidized proteins with HO-1 expression in eAAA and rAAA. **J**, Representative Western blot for detection of carbonyl groups in AOD, eAAA, and rAAA. **A** and **D**, Because of scatter, data were log transformed. Data are shown as scatter dot plots. The horizontal line depicts the median with range (A, D, and G). The number of analyzed samples is given in the figures. Data were compared by Kruskal–Wallis and Dunn's multiple comparison test. AOD indicates arterial occlusive disease; DNP-BSA, 2,4-dinitrophenyl conjugated to BSA; eAAA, electively treated AAA; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; MDA, malondialdehyde; rAAA, ruptured AAA; and RFU, relative fluorescence units.



P=0.02) were lower in eAAA samples with higher HO-1 expression. In rAAA, the amount of oxidized proteins was also inversely correlated with HO-1 expression (r_S=−0.76, *P*=0.04) (Figure 4A through 4J).

HMOX1 Expression Is Linked to Inflammatory Cytokines and Chemokines

Because *HMOX1*/HO-1 can be induced by inflammatory stimuli, aortic chemokine (C-C motif) ligand 2

(CCL2) and interleukin-6 (*IL6*) mRNA were quantified to assess associations with *HMOX1* expression. In eAAA, *HMOX1* expression increased with *CCL2* (r_P=0.60, *P*=0.003) and *IL6* (r_P=0.46, *P*=0.03) (Figure S8A through S8F). To validate a potential link between HO-1 and inflammation, the overall macrophage marker CD68 was stained and quantified. CD68 was not linked with HO-1, nor in eAAA (r_S=−0.21, *P*=0.43), nor in rAAA (r_S=0.48, *P*=0.24) (Figure S9A through S9D).

HO-1 Expression Is Not Associated With Aortic Fe^{3+} Storage and Hemoglobin Content

Activation of HO-1 enzyme forms Fe^{2+}, which can be stored as Fe^{3+} in ferritin or hemosiderin. Hemosiderin positive areas were similar and no correlations with HO-1 expression were found in eAAA (r_S=0.04, *P*=0.88) and rAAA (r_S=−0.50, *P*=0.45) (Figure S10A through S10D). Heme proteins are known inducers of HO-1 expression.[7] Hemoglobin did not differ between AOD, eAAA, and rAAA and no correlations with HO-1 expression were found in eAAA (r_S=0.30, *P*=0.26) and rAAA (r_S=0.47, *P*=0.30) (Figure S10E through S10G).

Serum HO-1 in Patients With eAAA and Correlations With the AAA Diameter

Serum HO-1 concentrations were analyzed in patients with eAAA and compared with control patients who were treated because of venous vessel varicose. Comparison of the clinical characteristics revealed a significantly lower age (64.0±8.4 versus 73.8±8.6 years, *P*<0.0001), a higher number of women, and difference in LDL, HDL, and total cholesterol and CRP. Prevalence of smoking, coronary artery disease, and atherosclerosis in peripheral and carotid arteries was higher in patients with eAAA. The frequency of treatment with statins and acetylsalicylic acid (ASA) was significantly different compared with controls (Table 2).

Serum HO-1 concentrations were 2.3-fold higher in eAAA (median 1482 versus 3156 pg/mL, *P*<0.0001) and tended to be increased with the AAA diameter (r_S=0.21, *P*=0.08, n=69). Correlational analysis revealed a cluster of patients with an AAA diameter >70 mm that showed different associations with serum HO-1. Therefore, the AAA diameter was divided into 3 different groups. Serum HO-1 was higher (*P*=0.04) in the second group (AAA diameter >55.0 to ≤70.0 mm, median 3477 pg/mL) when compared with the first (AAA diameter ≤55.0 mm, median 2932 pg/mL). A further elevation was not found in the third group (AAA diameter >70.0 mm, median 3581 pg/mL). Serum HO-1 was correlated with CRP concentrations because AAA is an inflammatory disease and associations of *IL6* and *CCL2* with *HMOX1* were found. Serum HO-1 was positively correlated with CRP concentrations (r_S=0.38, *P*=0.002, n=68) (Figure 5A through 5D).

DISCUSSION

In this study, the regulation of HO-1 expression in electively treated and ruptured AAA was analyzed. HO-1 expression was increased in eAAA compared with controls and similar in eAAA and rAAA. The highest HO-1 expression was found in eAAA tissues with a diameter >84.4 mm. eAAA samples with a high HO-1 expression showed a lower collagen content, reductions in oxidative stress, H_2O_2 release, and partly in MMP9 activity. The increase in *HMOX1* gene expression was connected with increases in proinflammatory IL6 and CCL2. Analysis of serum HO-1 concentrations revealed an elevation in patients with eAAA when compared with venous vessel varicose controls. Serum HO-1 was highest in patients with eAAA with a diameter of 55 to 70 mm but not further increased above this diameter.

Within the present study, HO-1 protein expression was quantified using an antibody directed against epitopes between amino acids 150 to 286. A cleavage site for the generation of a truncated HO-1 isoform was described at amino acids 275/276.[23] The estimated size of the truncated protein is ≈28 kDa.[24] HO-1 expression in the present study includes the complete and a truncated version of the protein. Truncated HO-1 can translocate into the nucleus but has less or no enzymatic activity.[19] It promotes cytoprotection by activation of oxidative stress responsive transcription factors, protecting against H_2O_2-induced injury.[19] Despite this, assessing HO-1 enzyme activity would have been more appropriate and is one limitation of the present study.

In the present study, an increase in *HMOX1* mRNA and protein expression was found in vessel walls obtained from eAAA and rAAA. Our data are in line with studies in wild-type mice subjected to Ang II-infusion.[16] Only a few studies compared human AAA in the terminal and ruptured state. A different regulation of genes involved in tissue remodeling, angiogenesis, and adipogenesis was demonstrated.[25] With respect to the biomechanical properties of AAA,[26] alterations in wall shear stress might have contributed to the HO-1 increase. Pulsatile laminar shear is known to increase the HO-1-mediated cytoprotection against oxidative stress in endothelial cells.[27,28] Differences in HO-1 expression between rAAA and controls could be because of the higher age in rAAA. Studies demonstrated decreases and increases in HO-1 in different organs with aging.[29,30] Another explanation for the scatter of data in eAAA and rAAA is *HMOX1* promoter polymorphisms.[14]

One limitation of the present study is the control AOD aortas that were used for comparison of tissue expression. An increased HO-1 expression was demonstrated in atherosclerotic aortas,[31] but HO-1

Table 2. Clinical Characteristics in Patients With eAAA and Controls Who Were Analyzed for Serum HO-1 Concentrations

	Varicose	eAAA	*P* value
N total	32	69	
Clinical characteristics			
Age, y (n)	64.00±8.43, 32	73.75±8.58, 69	<0.0001
Sex, men/women, % men	15/17, 47	62/7, 90	<0.0001
Aortic diameter, mm (n)	nd	57.0 (42.0–90.0), 69	
Cardiovascular risk factors			
LDL cholesterol, mmol/L (n)	3.41 (1.70–5.62), 28	2.49 (0.71–6.79), 62	0.0002
HDL cholesterol, mmol/L (n)	1.71 (1.13–3.41), 28	1.25 (0.39–2.43), 62	<0.0001
Total cholesterol, mmol/L (n)	5.39 (3.14–7.74), 28	4.24 (2.34–8.07), 62	<0.0001
Triglycerides, mmol/L (n)	1.35 (0.66–3.05), 28	1.43 (0.64–4.50), 63	0.18
Blood glucose, mmol/L (n)	5.21 (4.08–10.19), 30	5.26 (2.93–14.88), 68	0.93
CRP, mg/L (n)	1.99 (0.30–6.60), 30	3.40 (0.50–113.4), 68	0.0008
Smoking, n/N total, %	3/30, 10	32/67, 48	0.0002
Hypertension, n/N total, %	26/30, 86	57/68, 84	>0.999
CAD, n/N total, %	1/32, 3	22/68, 32	0.0008
PAD, carotid artery stenosis, n/N total, %	0/30, 0	19/67, 28	0.0005
T2D, n/N total, %	5/30, 17	11/66, 17	0.999
BMI, kg/m² (n)	27.99±4.45, 30	26.99±4.17, 66	0.27
Medical therapy			
Statins, n/N total, %	7/30, 18	50/69, 86	<0.0001
ACE inhibitors, n/N total, %	6/30, 20	25/69, 36	0.16
ARB, n/N total, %	10/30, 33	26/69, 38	0.82
β-Blocker, n/N total, %	12/30, 40	31/69, 45	0.67
CCB, n/N total, %	8/30, 27	27/69, 39	0.26
Diuretics, n/N total, %	8/30, 27	27/69, 39	0.26
Anticoagulants, n/N total, %	10/30, 33	14/69, 20	0.20
ASA, n/N total, %	3/30, 10	45/69, 65	<0.0001
Insulin, n/N total, %	2/30, 7	3/69, 4	0.64

All data are presented as median with minimum and maximum (range) or mean±SD, depending on the results of normality testing. Comparison of Gaussian distributed data was done using unpaired *t* test, non-Gaussian distributed by Mann–Whitney *U* test. Comparison of prevalence for risk factors and medical therapies was analyzed by Fisher exact test. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blocker; eAAA, electively treated abdominal aortic aneurysm; HDL, high-density lipoprotein; HO-1, heme oxygenase-1; LDL, low-density lipoprotein; n, number of available data from all included patients (n/N); N, total included patients; nd, not determined; PAD, peripheral artery disease; and T2D, type 2 diabetes.

expression in eAAA and rAAA was even greater than that in AOD vessels, suggesting that AOD is less atherosclerotic and the induction in AAA is higher than in peripheral artery disease. Analysis of HO-1 by immunohistochemistry revealed a primary localization in the media, at the border of the adventitia or intima. Our data are supported by preclinical animal models, where HO-1 was mainly located in the media and extended to the adventitia at later stages.[16]

HO-1 expression correlated with endothelial marker CD31 in eAAA. In atherosclerotic lesions, positive HO-1 immunostaining was detected in endothelial and medial smooth muscle cells.[32] Another possible explanation is the HO-1 expression by a macrophage subpopulation (hemorrhage-associated mac) that is differentiated in response to intraplaque hemorrhage.[33] Cell culture studies demonstrated that heme and hemoglobin induce these atheroprotective macrophages in an HO-1 dependent manner.[34] However, immunohistological markers within the present study were assessed semiquantitatively. Costaining of HO-1 and cellular markers or isolation of single cells would have allowed detailed conclusions on cells that express HO-1.

In eAAA, HO-1 expression was highest in AAA with a median diameter >84.4 mm. Previously published studies in carotid artery plaques showed an increased HO-1 expression with features of plaque vulnerability, and induction promoted a more stable plaque phenotype.[35] The authors conclude that the rise in HO-1 reflects a protective response against atherosclerosis.[36]

HO-1 expression was similar in samples with less or higher elastin breakdown, in eAAA and rAAA.

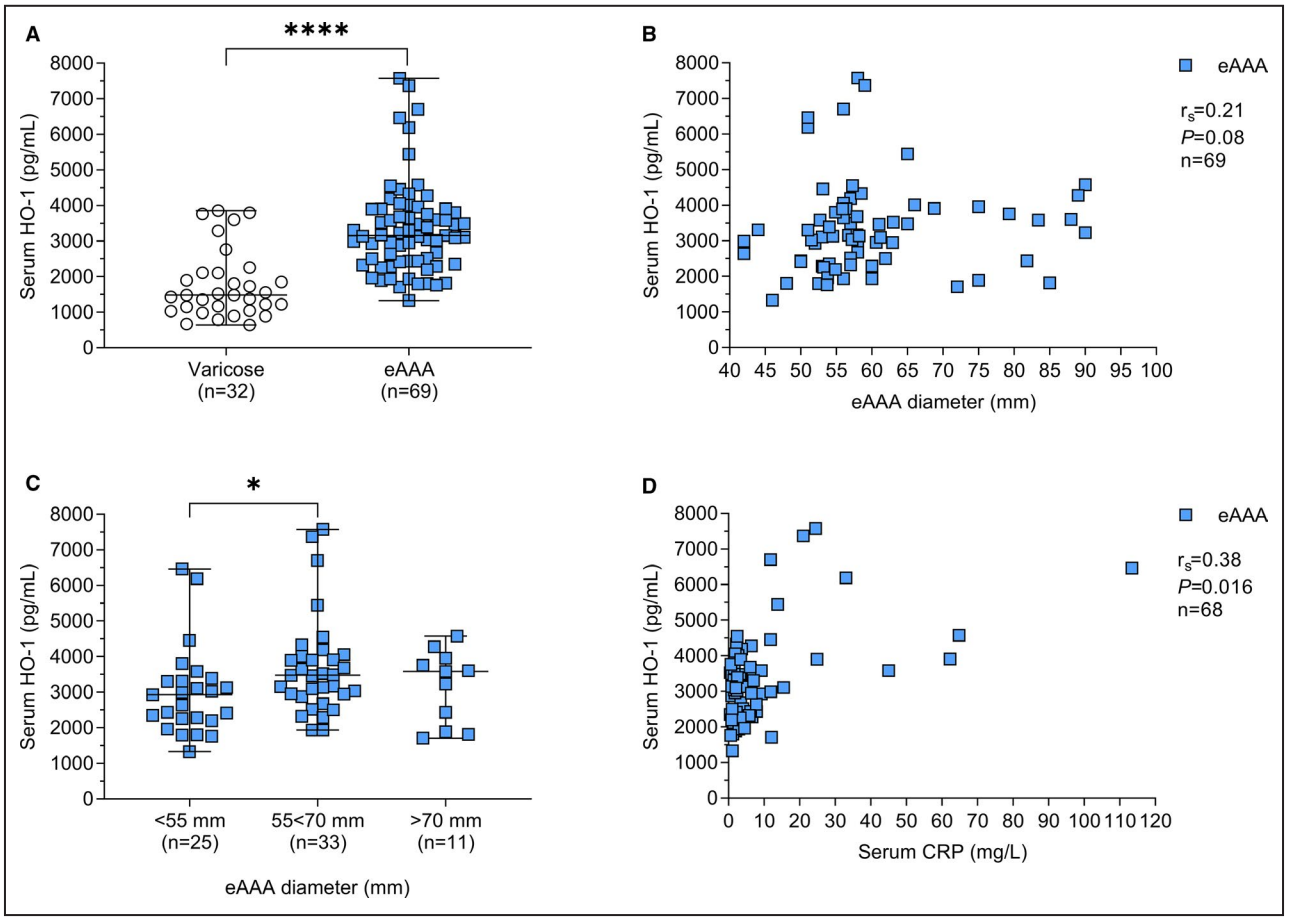


Figure 5. Analysis of serum HO-1 concentrations in patients undergoing elective surgical repair and patients with varicose. **A**, Serum HO-1 concentrations were quantified in patients with eAAA and in venous vessel varicose in the preoperative state. Surgery because of rAAA was always an emergency procedure and blood was collected intraoperatively. To exclude effects caused by differences in the study protocol, serum HO-1 was not determined in rAAA samples. **C**, The AAA diameter was grouped as <55.0, 55.0 to 70.0, and >70.0 mm. **C** and **D**, Spearman's correlation coefficient (r_S) between serum HO-1 and the AAA diameter and CRP concentrations. The horizontal line depicts the median with range (A and C) . The number of analyzed samples is given in the figures. **A**, Mann–Whitney *U* test. **B**, Kruskal–Wallis and Dunn's post hoc test. *P<0.05 <55.0 vs 55.0 to 70.0 mm. ****P<0.0001 varicose vs eAAA. CRP indicates C-reactive protein; eAAA, electively treated abdominal aortic aneurysm; HO-1, heme oxygenase-1; and rAAA, ruptured abdominal aortic aneurysm.

Studies in HO-1$^{-/-}$ mice revealed higher elastin degradation after AngII-infusion[16] Differences in the pathology of AAA in animal models and humans, especially at later stages,[37] may have contributed to observed differences. The increase in HO-1 expression correlated with a lowering in collagen. An induction of HO-1 was described with antifibrogenic properties.[38] The linkage between HO-1 and collagen could point towards an increase of HO-1 with the severity of the disease.

MMPs are major extracellular matrix degrading enzymes. In the present study, HO-1 expression was positively correlated with pro-MMP9 and negatively with MMP9, at least in part. Studies revealed a predominant role of pro-MMP9 in human AAA[39] and deletion of *HMOX1* increases the activity of MMP9 in macrophages.[16]

Biliverdin is a HO-1 reaction product and is reduced to bilirubin. Vasoprotective effects of HO-1 action are ascribed to biliverdin and bilirubin because of their anti inflammatory and anti oxidative properties.[40] In eAAA and rAAA, aortic bilirubin was not associated with an increased HO-1 expression. However, direct analysis of HO-1 activity and the use of a bilirubin assay that detects unconjugated bilirubin would be more appropriate and may explain the missing link.

Activation of HO-1 forms ferrous iron (Fe^{2+}), which can be stored as Fe^{3+} in ferritin or hemosiderin. In the present study, hemosiderin staining was not associated with HO-1 expression. This is an unexpected finding because the increase in HO-1 in eAAA and rAAA could be accompanied by an elevation in Fe^{2+} and its storage in hemosiderin. Quantification of hemosiderin was semiquantitative and analysis of ferritin would be

another opportunity. In addition, micro bleedings are most likely invisible in hemosiderin staining, and the small amount of samples may have contributed to the missing association.

Because the majority of AAA are covered by an intraluminal thrombus,[41] an increased hemolysis with the release of heme proteins could be associated with the induction in HO-1. However, aortic hemoglobin content was not linked to HO-1 expression, nor in eAAA nor in rAAA. Most likely, the methods used lack sensitivity to detect existing differences.

Within the present study, HO-1 expression was higher in eAAA walls showing less oxidative stress. An activation of HO-1 releases Fe^{2+} ions that act in the Fenton reaction with H_2O_2 to produce highly reactive hydroxyl radicals.[42] In the present study, an inverse correlation between H_2O_2 release and HO-1 was found. It might be speculated whether this is because of the increased reaction of H_2O_2 with Fe^{2+} that are produced by HO-1 protein in eAAA or reflects a general lowering in oxidative stress. Malondialdehyde is a stable end product of lipid peroxidation and is known for its oxidative modifications of proteins and DNA.[43] In the present study, malondialdehyde, as measured by TBARS, was higher in eAAA samples with less HO-1 expression. These findings are in line with reductions in malondialdehyde that have been described in rats with myocyte-specific HO-1 overexpression.[13]

In the present study, *HMOX1* positively correlated with pro inflammatory *IL6* and *CCL2* mRNA in eAAA. An induction of HO-1 in response to inflammatory stimuli has been shown in several cell types.[44] A higher number of immune cells, secreting chemokines and cytokines and expressing HO-1, have been previously demonstrated in AngII-infused mice[16] with large amounts of HO-1-positive macrophages.[6]

Serum HO-1 concentrations were higher in eAAA compared with control patients with venous vessel varicose. Patients with varicose were chosen because they presented with no history of arterial cardiovascular disease. Studies in peripheral artery disease demonstrated a lowering in plasma HO-1, whereas HO-1 was elevated in coronary artery disease and carotid artery stenosis.[36,45] Some limitations in the present study are the differences in age, sex, presence of cardiovascular risk factors, and medical therapies because these parameters might have contributed to the HO-1 increase in eAAA. Multivariate logistic regression analysis using a higher number of controls and patients with eAAA would be appropriate. Up to now, the data are only exploratory.

Our data demonstrate a trend towards an increase of serum HO-1 with the AAA diameter by using Spearman's correlational analysis. Analyzing these data revealed a cluster of patients with a diameter >70 mm that was differentially regulated. We therefore grouped serum HO-1 according to a diameter ≤55.0, >55.0 to ≤70.0, and >70.0 mm. Serum HO-1 was higher in the second group but not further elevated in the third group. This is an interesting finding that (1) could point towards a lowering of HO-1 with the progression of the disease and (2) needs the analysis of patients with rupture to clarify whether HO-1 has a diagnostic potential to detect patients prone to rupture. Furthermore, a positive correlation of CRP with HO-1 levels was shown in eAAA, pointing towards a link to inflammation in the induction or potentiation of HO-1.[46]

In conclusion, HO-1 expression was increased in nonruptured and ruptured AAA, suggesting that HO-1 is not differentially regulated in the transition towards rupture, thus rejecting our proposed research hypothesis. HO-1 did not show a clear association with the maximum AAA diameter but was highest in samples >84.4 mm. By evaluating associations with secondary outcome variables, data of the present study point towards a protective role of an HO-1 induction in the nonruptured state. In eAAA, serum HO-1 concentrations increase with the diameter until 70.0 mm with no further increase at higher diameters. Until now, these associations have only been explorative and need further evaluation in a greater number of subjects.

Limitations of the Present Study

The present study is a complete descriptive study and does not allow conclusions on the mechanisms contributing to HO-1 induction. Another limitation is the small number of aortic walls in the AOD and rAAA groups as well as the difference in their age. A strong scatter of data was noticeable in the rAAA group. Furthermore, the varicose control group for comparison of serum HO-1 concentrations was significantly younger, included more women, and differed in their cardiovascular risk profile and medical therapies. Nevertheless, analyzing human tissues from patients with advanced and ruptured AAA helps to identify mechanisms that contribute to the progression and transition towards AAA rupture. The analysis of aortic tissues and serum from patients with AAA provides information about a stage of the disease, where AAA typically are identified and surgically treated.

ARTICLE INFORMATION

Received June 4, 2021; accepted August 23, 2021.

Affiliations

Division of Vascular and Endovascular Surgery, Department of Visceral, Thoracic and Vascular Surgery, University Hospital and Medical Faculty Carl Gustav Carus (A.H., M.M., S.W., Y.K., P.S., F.H., B.H., C.R.); Department of Physiology, Medical Faculty Carl Gustav Carus Dresden (I.K., D.E., A.D.); Division of Vascular Endothelium and Microcirculation, Department of Medicine III, University Hospital and Medical Faculty Carl Gustav Carus (C.B., S.G., H.M.) and Institute for Clinical Chemistry and Laboratory Medicine, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany (D.M.P.).

Acknowledgments

We thank Michael Gerlach, PhD from the Core Facility Cellular Imaging (CFCI) at the TU Dresden (Head: Prof Dr Thomas Müller-Reichert) for the outstanding help in the generation of macros for analyzing the immunohistochemistry. We thank Ellen Geibelt from the Light Microscopy Facility, a Core Facility of the CMCB Technology Platform at TU Dresden, for her support with the slide scanner.

Author contributions: Hofmann designed the study; Hofmann, Müglich, Horn, Egorov, Hamann, and Sabarstinski performed the experiments; Hofmann and Müglich analyzed the data; Wolk, Khorzom, and Reeps collected the samples; Hofmann wrote the draft manuscript; and Wolk, Brunssen, Giebe, Poitz, Kopaliani, Deussen, Morawietz, and Reeps edited the draft manuscript. All authors reviewed and approved the final manuscript.

Sources of Funding

The research was supported by funds of the Medical Faculty of the TU Dresden. Müglich and Horn received funding by the "Carus Promotionskolleg" fellowship from the Medical Faculty of the TU Dresden.

Disclosures

None.

Supplementary Material

Tables S1–S2
Figures S1–S10

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SUPPLEMENTAL MATERIAL

An induction of heme oxygenase-1 is linked to the severity of disease in human abdominal aortic aneurysm

Running Title: Regulation of HO-1 in human late stage AAA

Anja Hofmann, PhD[1#], Margarete Müglich[1], Steffen Wolk, MD[1], Yazan Khorzom[1], Pamela Sabarstinski[1], Irakli Kopaliani, MD[2], Dmitry Egorov, PhD[2], Franziska Horn[1], Coy Brunssen, PhD[3], Sindy Giebe, PhD[3], Bianca Hamann[1], Andreas Deussen, MD[2], Henning Morawietz, PhD[3], David M. Poitz, PhD[4] and Christian Reeps, MD[1]

[1]Division of Vascular and Endovascular Surgery, Department of Visceral, Thoracic and Vascular Surgery, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Germany;
[2]Department of Physiology, Medical Faculty Carl Gustav Carus Dresden, Technische Universität Dresden, Germany;
[3]Division of Vascular Endothelium and Microcirculation, Department of Medicine III, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;
[4]Institute for Clinical Chemistry and Laboratory Medicine; University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;

[#]Corresponding author:
Anja Hofmann, PhD, Division of Vascular and Endovascular Surgery, Department of Visceral-, Thoracic and Vascular Surgery, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Fetscherstr. 74, 01307 Dresden, Germany
Tel: +49 351 458 16607
Fax: +49 351 458 7292
Email: Anja.Hofmann2@uniklinikum-dresden.de

Subject term list: Vascular disease, Aneurysm

SUPPLEMENTAL TABLES

Supplemental Table 1. Primers Used for Gene Expression Analysis by Quantitative Real-Time PCR.

Gene	Primers	Sequence, 5'-3'
HMOX1	Forward	AGTCTTCGCCCCTGTCTACT
	Reverse	CTTCACATAGCGCTGCATGG
RPL32	Forward	CACCGTCCCTTCTCTCTTCCT
	Reverse	TCTTGGGCTTCACAAGGGGT
TBP	Forward	CGCCGGCTGTTTAACTTCG
	Reverse	AGAGCATCTCCAGCACACTC
B2M	Forward	GATGAGTATGCCTGCCGTGT
	Reverse	CATGATGCTGCTTACATGTCTCG

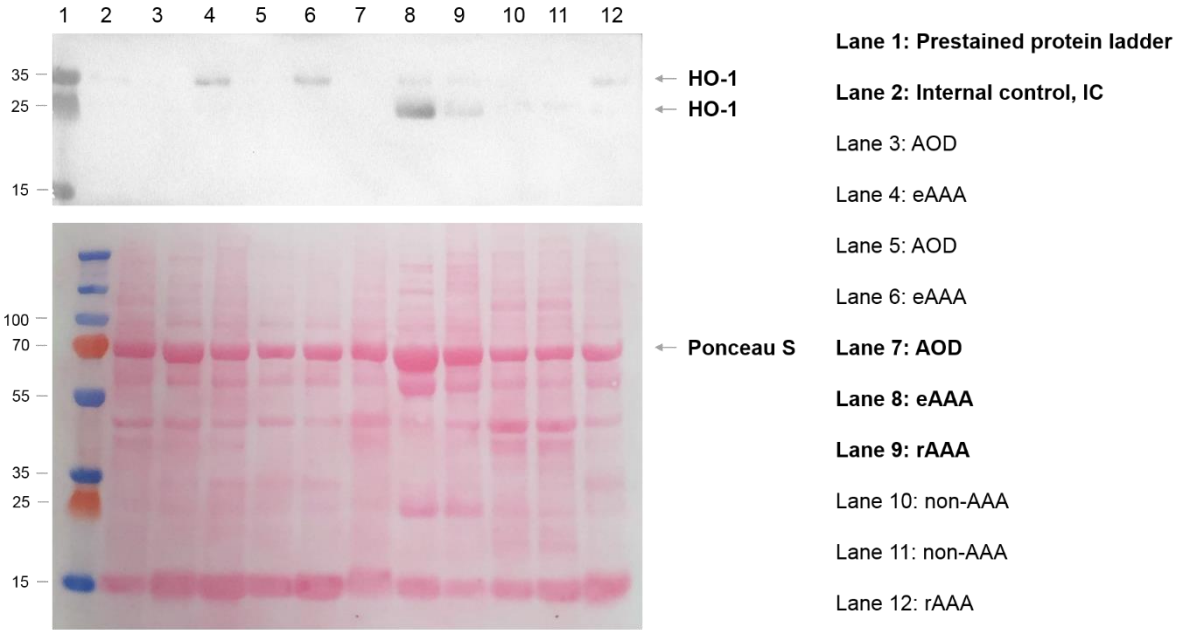
Abbreviations: *B2M*, beta-2-microglobulin; *HMOX1*, heme oxygenase-1; *RPL32*, ribosomal protein L32; *TBP*, TATA-box binding protein.

Supplemental Table 2: Primary Antibodies used for Immunohistochemistry.

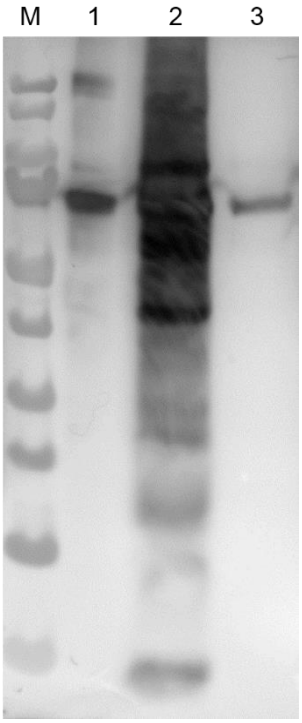
Abbreviations: RT, room temperature.

Protein	**Supplier**	**Concentration**	**Incubation time**
α-SMA	Sigma Aldrich, A5228, Clone 1A4	2 µg/mL	1h, RT
Cleaved caspase-3	Cell Signaling, #9661	0.59 µg/mL	1h, RT
CD68	Agilent, M0814, Clone KP1	4 µg/mL	Overnight, 4°C
HO-1	Enzo, ADI-OSA-110-D	10 µg/mL	Overnight, 4°C
CD31	Agilent, M0823, Clone JC70A	4.1 mg/mL	1h, RT

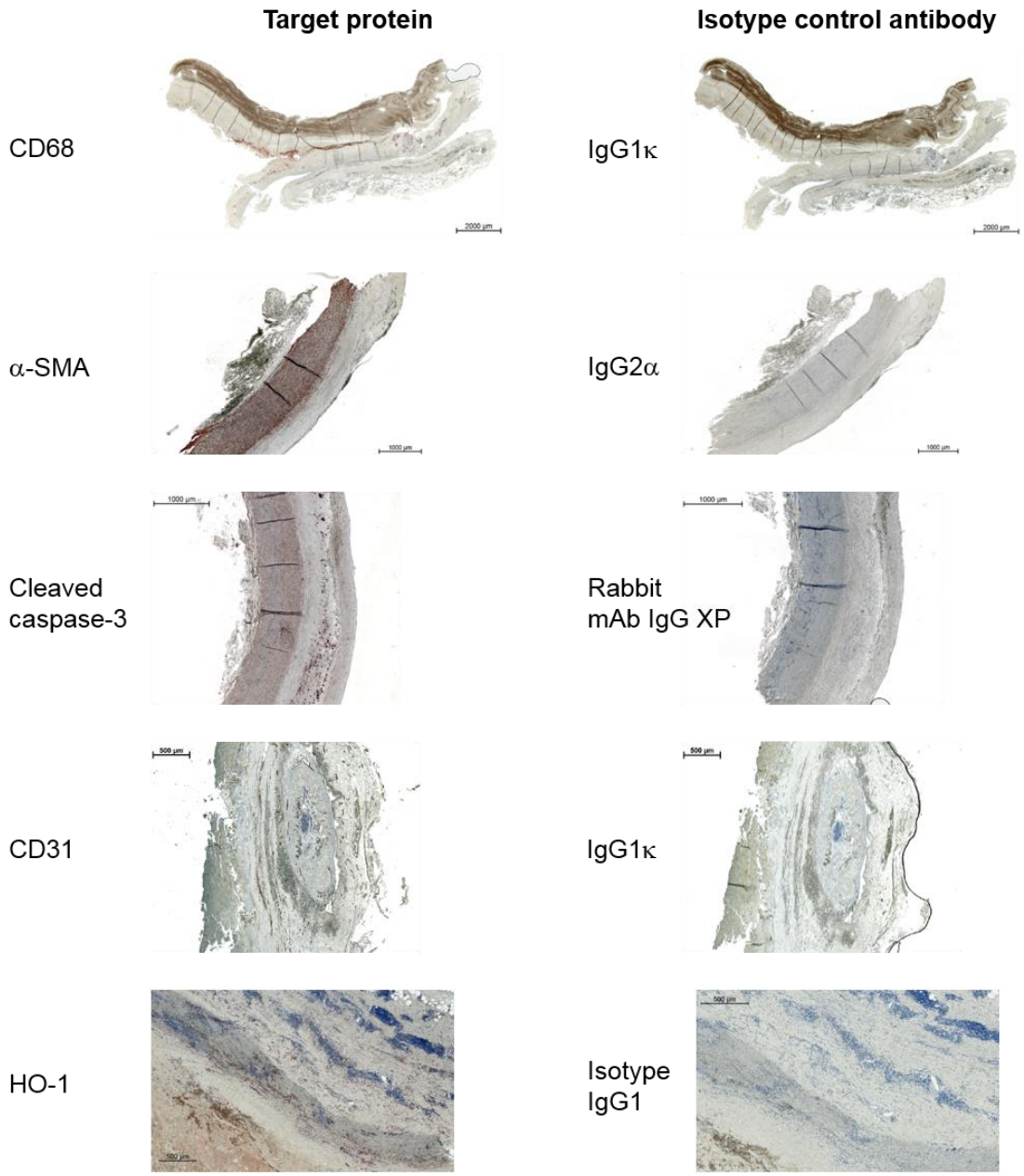
SUPPLEMENTARY FIGURES



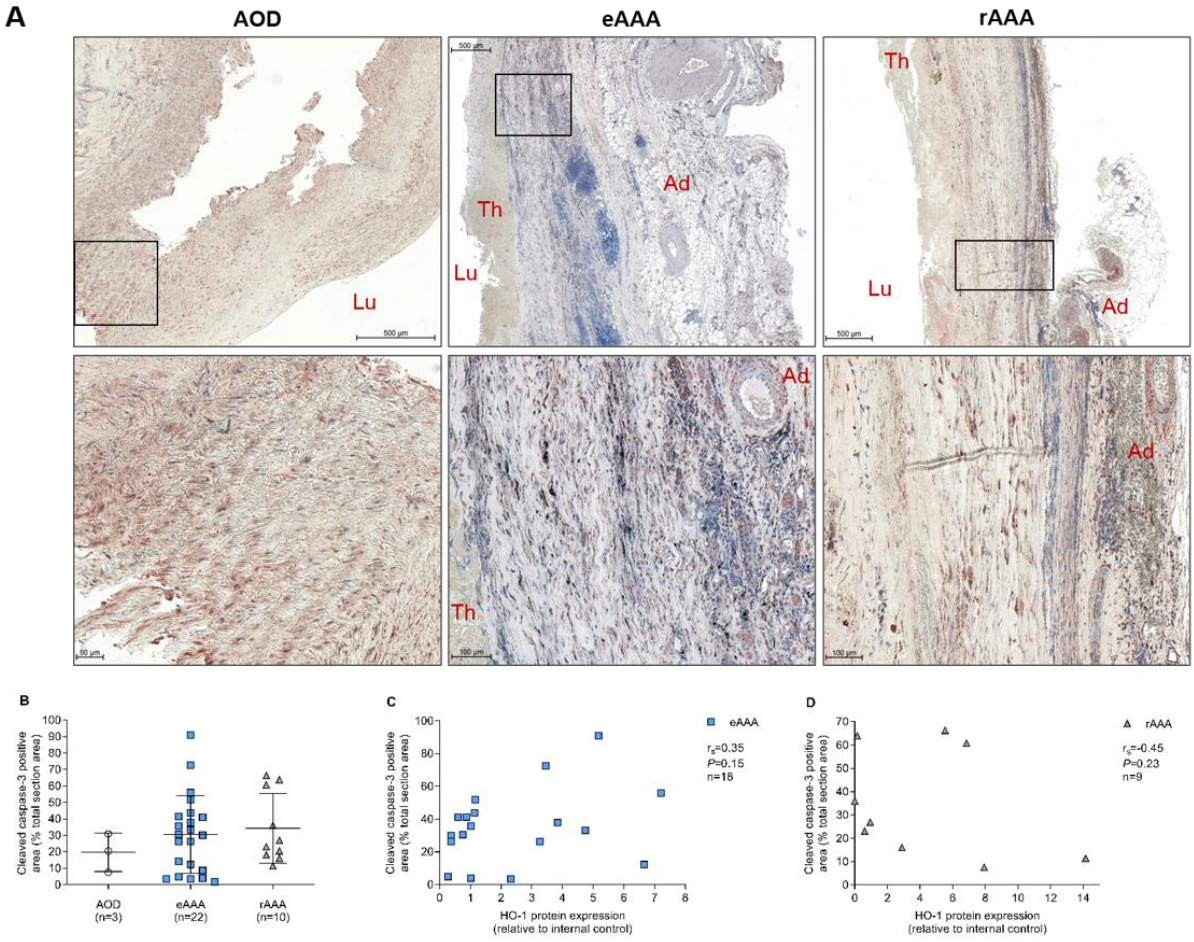
Supplemental Figure 1: Full Western Blot Used for Representative HO-1 Protein Expression in Patients Undergoing Elective Surgical Repair or Surgery due to Ruptured AAA and AOD Controls. The two marked HO-1 bands represent the native HO-1 (~32 kDa) and a truncated (~25 kDa) isoform. The band intensities within lanes 7–9 are AOD, eAAA and rAAA samples, showing representative values for HO-1 protein expression. These bands were used for representative Figure 1D. The prestained protein ladder (lane 1) and the corresponding controls (lane 2) were cut and added to the other lines. The full Western blot for HO-1, showing all lines and the corresponding Ponceau S stain, is presented in this figure. **Abbreviations:** AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; rAAA, ruptured AAA.



Supplemental Figure 2: Detection of Carbonyl Residues in Oxidized Proteins by Using DNP. Carbonyl groups in side chains of proteins are derivatized with 2,4-dinitrophenylhydrazine (DNPH) to 2,4-dintrophenylhydrazone (DNP-hydrazone). DNP derivatized proteins were separated on Bis-Tris protein gels, transferred to nitrocellulose membranes and DNP moieties were detected using an anti-DNP antibody. Lanes 1-4: Prestained protein ladder, positive control DNP-BSA, aortic tissue extract treated with DNPH, aortic tissue extract treated without DNPH. **Abbreviations:** DNP-BSA, 2,4-Dinitrophenyl conjugated to BSA.

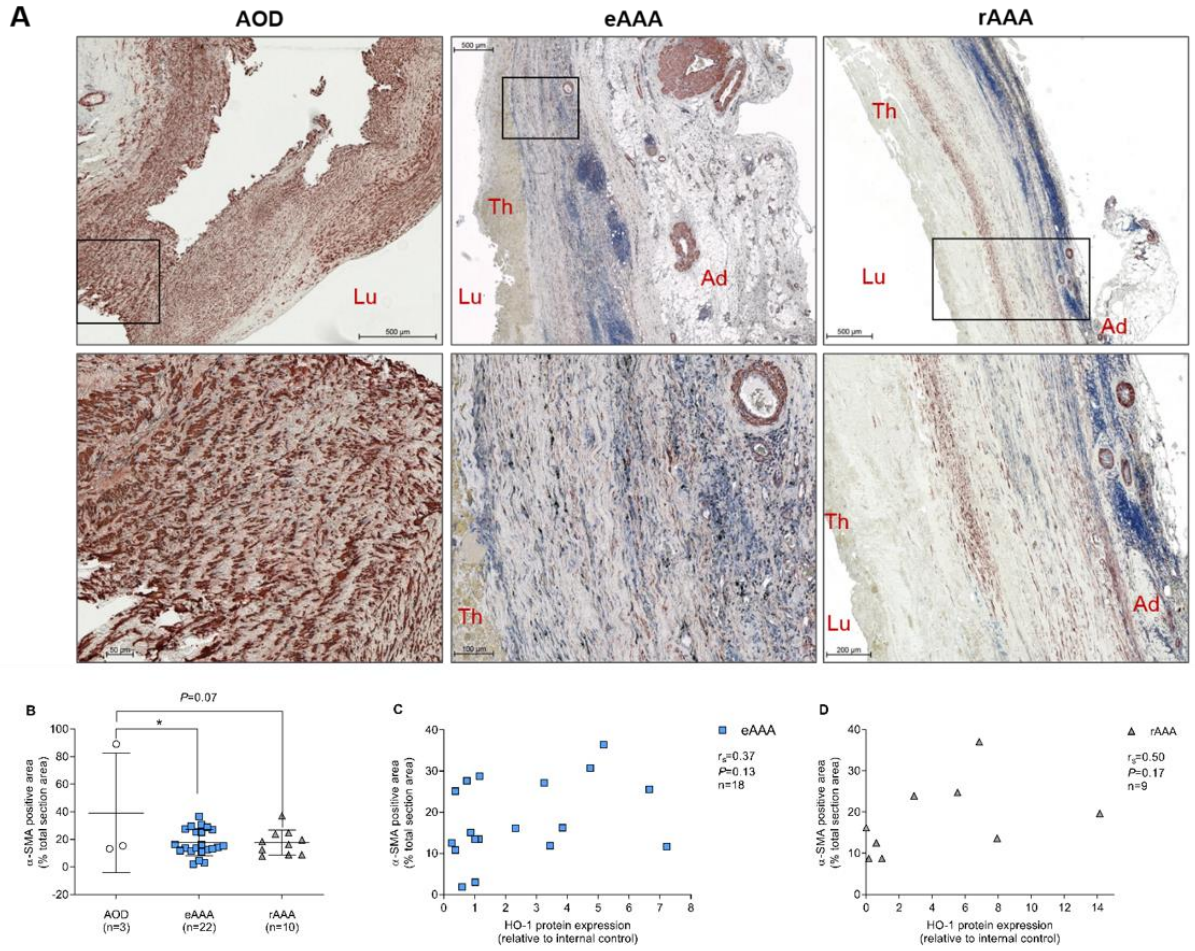


Supplementary Figure 3: Specificity of CD68, α-SMA, Cleaved caspase-3, CD31 and HO-1 Signals by Using the Corresponding Isotype Control Antibodies. Immunohistochemistry for **A,** CD68 and the IgG1κ, **B,** α-SMA and IgG2α, **C,** cleaved caspase-3 and rabbit (DA1E) mAb IgG XP, **D,** CD31 and mouse IgG1κ and **E,** HO-1 and IgG1. The negative control antibody was used instead of the primary antibody. Red and brown areas present positively stained areas whereas isotype antibodies revealed no staining. Cell nuclei (blue) were counterstained with Mayer's hemalum.



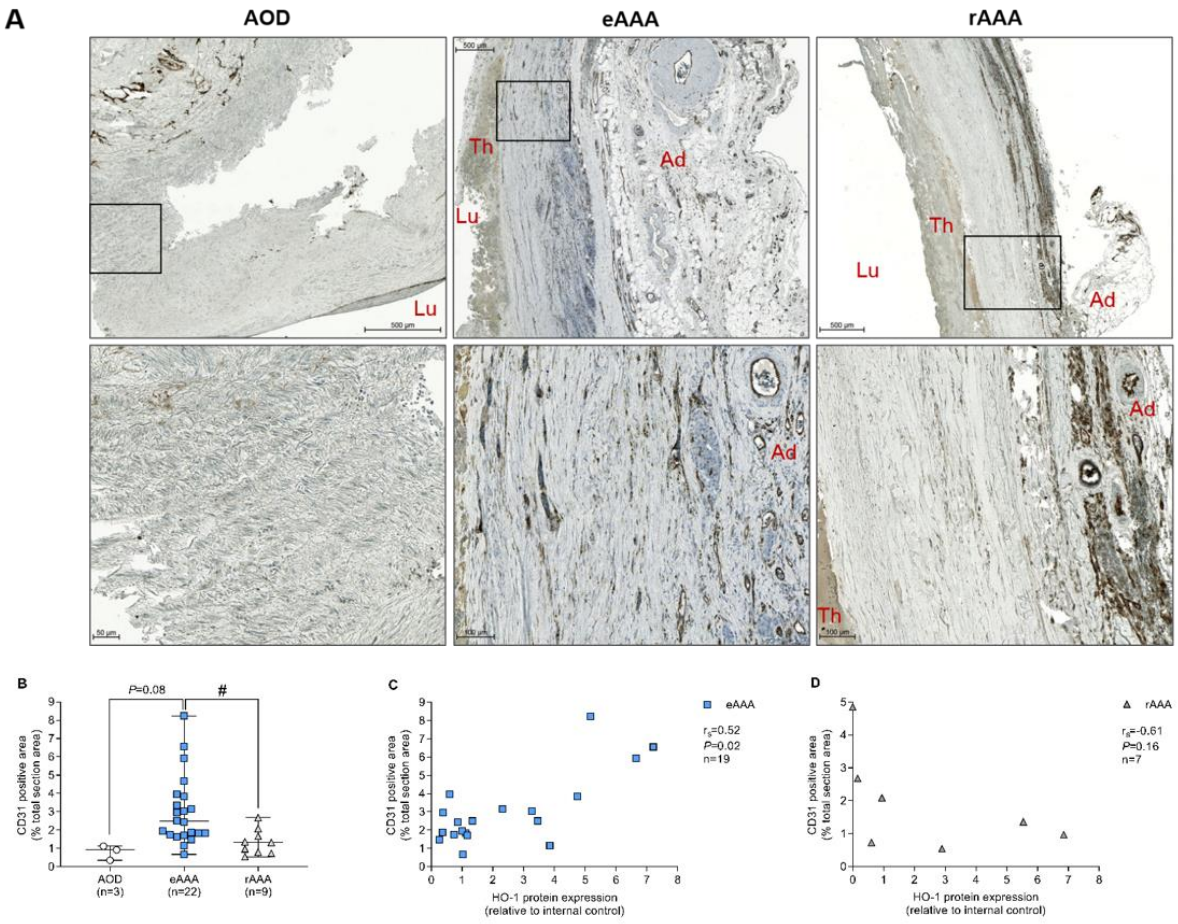
Supplementary Figure 4: Linkage of HO-1 Expression with Cleaved Caspase-3 Positive Areas in Patients Undergoing Elective Surgical Repair or Surgery due to AAA Rupture and AOD Controls. B, Comparison of aortic cleaved caspase-3 positive areas in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (r_S) with HO-1 protein expression in eAAA and rAAA. **A,** Representative slides for cleaved caspase-3. Positive, red positive areas were quantified and data are shown in relation to the total section area consisting of media and intima. Cell nuclei (blue) were counterstained with Mayer's hemalum. The black rectangles represent enlargements of the lower magnifications. The lumen (Lu), adventitia (Ad) and attaching thrombus (Th) are marked in red. **Statistics: B,** Data are presented as scatter dot plots. The horizontal line depicts the mean with standard deviation. The number of analyzed samples is given in the figures. One-Way ANOVA and Tukey's post hoc test. **Abbreviations:** Ad,

adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; Th, thrombus.



Supplementary Figure 5: Linkage of HO-1 Expression with α-SMA Positive Areas in Patients Undergoing Elective Surgical Repair or Surgery due to AAA Rupture and Controls. B, Comparison of aortic alpha-smooth muscle actin (α-SMA) positive areas in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (r_S) with HO-1 protein expression in eAAA and rAAA. **A,** Representative slides for α-SMA. Positive, red positive areas were quantified and data are shown in relation to the total section area consisting of media and intima. Cell nuclei (blue) were counterstained with Mayer's hemalum. The black rectangles represent enlargements of the lower magnifications. The lumen (Lu), adventitia (Ad) and attaching thrombus (Th) are marked in red. **Statistics: B,** Data are presented as scatter dot plots. The horizontal line depicts the mean with standard deviation. The number of analyzed samples is given in the figures. One-Way ANOVA and Tukey's post hoc test. **P*<0.05 AOD *vs.* eAAA. **Abbreviations:** α-SMA, alpha-smooth muscle actin; Ad, adventitia; AOD, arterial occlusive

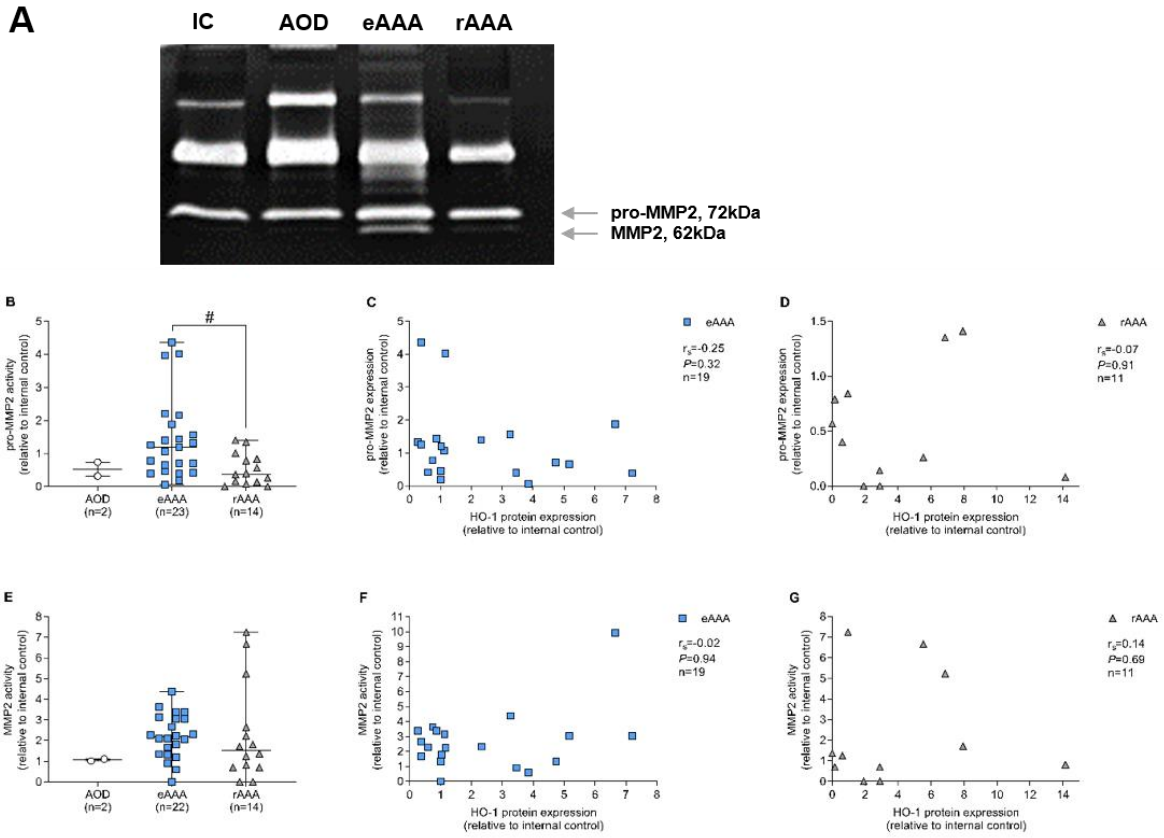
disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; Th, thrombus.



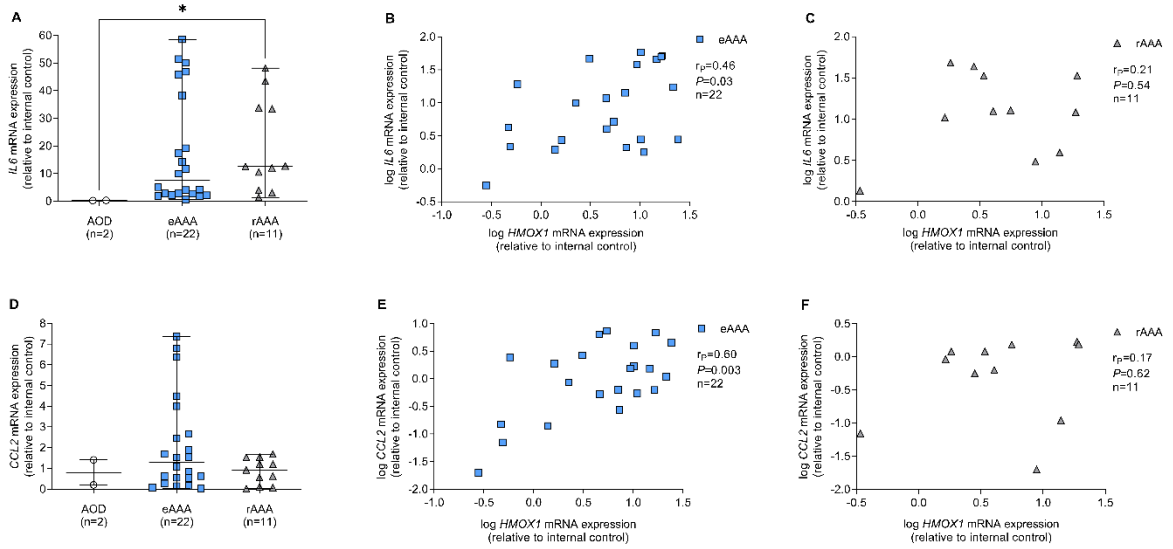
Supplementary Figure 6: Linkage of HO-1 Expression with CD31 Positive Areas in Patients Undergoing Elective Surgical Repair or Surgery due to AAA Rupture and AOD Controls. B, Comparison of CD31 positive areas in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (rs) with HO-1 protein expression in eAAA and rAAA. **A,** Representative slides for CD31 are shown above the figures. The black rectangles represent enlargements of the lower magnifications. The lumen (Lu), adventitia (Ad) and attaching thrombus (Th) are marked in red. Positive, brown positive areas were quantified and data are shown in relation to the total section area consisting of media and intima. Cell nuclei (blue) were counterstained with Mayer's hemalum. **Statistics:** Grubb's test was performed and one significant outlier was detected in the data set for CD31 (B, rAAA). **B,** Data are presented as scatter dot plots. The horizontal line depicts the median with range. The number of analyzed samples is given in the figures. Grubbs test was performed and one significant outlier was

detected in rAAA. One-Way ANOVA and Tukey's post hoc test. $^{\#}P<0.05$ eAAA *vs.* rAAA.

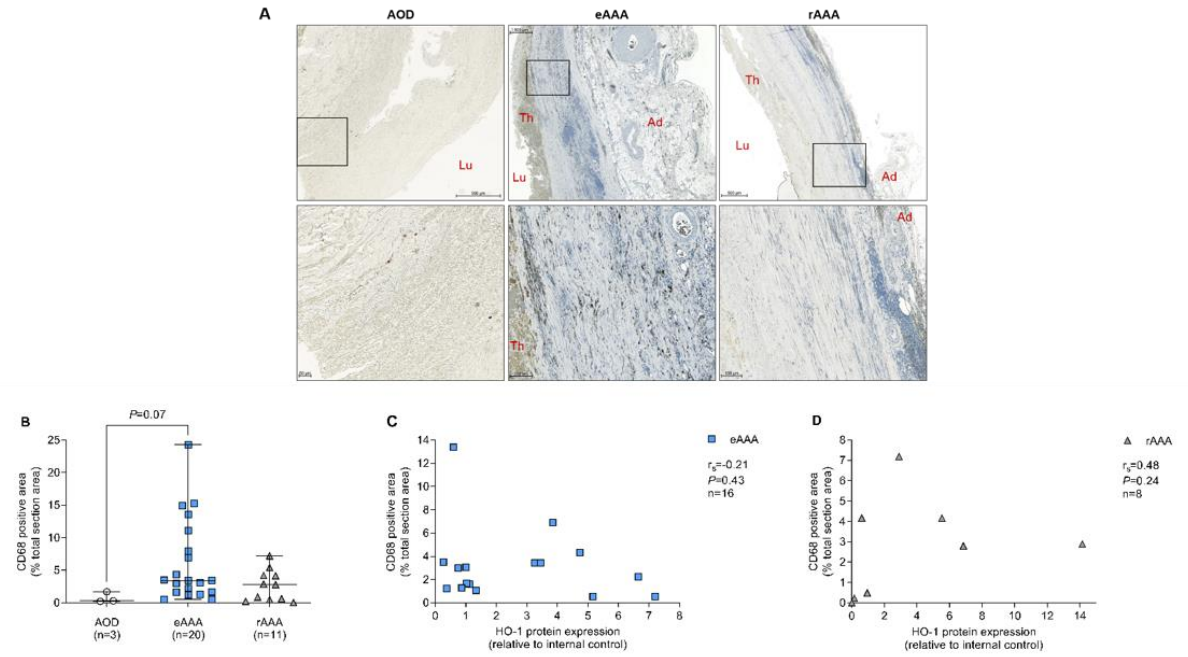
Abbreviations: Ad, adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; Th, thrombus.



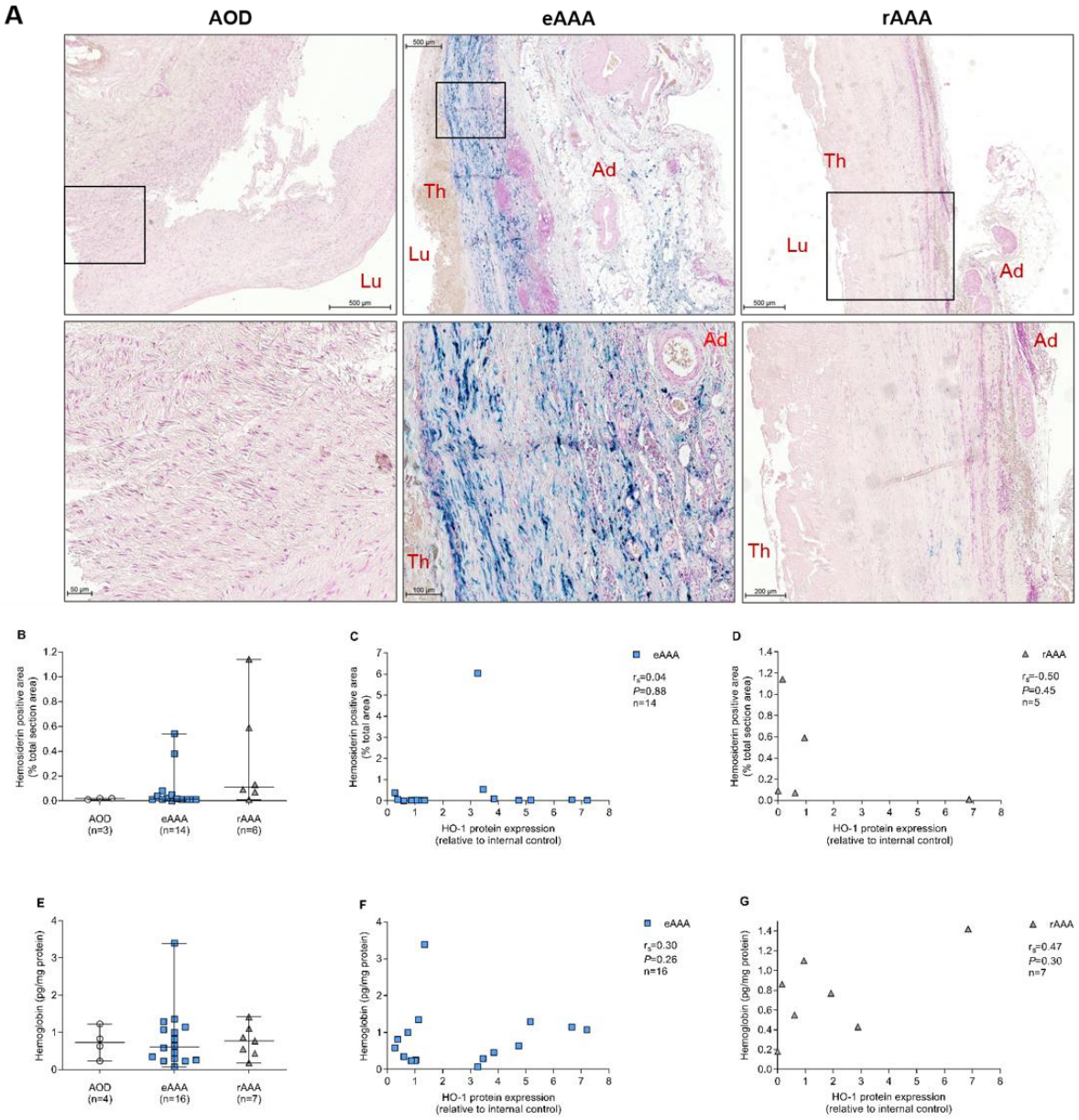
Supplementary Figure 7: Activity of Matrixmetalloprotease 2 and Correlation with HO-1 in Patients Undergoing Elective Surgical Repair or Surgery due to AAA Rupture and AOD Controls. A, Representative gelatin zymogram for pro-MMP2 and MMP2 in AOD, eAAA and rAAA samples. IC, Internal control. **B,** Comparison of aortic pro-MMP2 expression in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (rs) with HO-1 expression in eAAA and rAAA. **E,** Comparison of aortic MMP2 activity in AOD, eAAA and rAAA and **F, G,** Spearman's correlation (rs) with HO-1 protein in eAAA and rAAA. **Statistics:** Grubb's test was performed and one significant outlier was detected in the data set for MMP2 (E, eAAA). **A, D,** All data are presented as scatter dot plots. The horizontal line depicts the median with range. The number of analyzed samples is given in the figures. Kruskal-Wallis and Dunn's multiple comparison test. $^{\#}P<0.05$ eAAA *vs.* rAAA. **Abbreviations:** AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; MMP, matrixmetalloprotease; rAAA, ruptured AAA.



Supplementary Figure 8: Inflammatory Markers and Correlation with *HMOX1* Expression in Patients Undergoing Elective Surgical Repair or Surgery due to Ruptured AAA and AOD Controls. A, Comparison of aortic interleukin-6 (*IL6*) mRNA expression in AOD, eAAA and rAAA and **B, C,** Pearson's correlation (r_P) with *HMOX1* mRNA expression in eAAA and rAAA. **D,** Comparison of aortic chemokine (C-C motif) ligand 2 (*CCL2*) mRNA expression in AOD, eAAA and rAAA and **E, F,** Pearson's correlation (r_P) with *HMOX1* mRNA expression in eAAA and rAAA. All data are normalized to an internal control (=1). Due to scatter, data were log transformed. **Statistics: A, D,** Data are shown as scatter dot plots. The horizontal line depicts the median with range. The number of analyzed samples is given in the figures. Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05 AOD *vs.* rAAA. **Abbreviations:** AOD, arterial occlusive disease; *CCL2*, chemokine (C-C motif) ligand 2 gene; eAAA, electively treated AAA; *HMOX1*, heme oxygenase-1 gene; *IL6*, interleukin-6 gene; rAAA, ruptured AAA.



Supplementary Figure 9: Correlation of CD68 Positive Areas with HO-1 Expression in Patients Undergoing Elective Surgical Repair or Surgery due to Ruptured AAA and AOD Controls. A, Representative slides for CD68 immunohistochemistry. Positive, red areas in the intima and media were quantified and set in relation to the total section area. Cell nuclei (blue) were counterstained with Mayer's hemalum. The black rectangles represent enlargements of the lower magnifications. The lumen (Lu), adventitia (Ad) and attaching thrombus (Th) are marked in red. **B,** Comparison of CD68-positive areas in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (r_S) with HO-1 protein expression in eAAA and rAAA. **Statistics:** Grubb's test was performed and one subject in the data set of CD68 (rAAA) was identified as a significant outlier. **B,** Data are shown as scatter dot plots. The horizontal line depicts the median with range. The number of analyzed samples is given in the figures. Kruskal-Wallis and Dunn's multiple comparison test. **Abbreviations:** Ad, adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; Th, thrombus.



Supplementary Figure 10: Aortic Hemosiderin Staining and Hemoglobin Content in Patients Undergoing Elective Surgical Repair or Surgery due to Ruptured AAA and AOD Controls. A, Representative slides of hemosiderin staining by Perl's Prussian blue. Blue positive areas were quantified and data are shown in relation to the total section area consisting of media and intima. Cell nuclei (pink) were counterstained with Nuclear fast red. The black rectangles represent enlargements of the lower magnifications. The lumen (Lu), adventitia (Ad) and attaching thrombus (Th) are marked in red. **B,** Comparison of hemosiderin positive areas in AOD, eAAA and rAAA and **C, D,** Spearman's correlation (r_S) with HO-1 expression in

eAAA and rAAA. **E,** Comparison of aortic hemoglobin concentration in AOD, eAAA and rAAA and **F, G,** Spearman's correlation (rs) with HO-1 expression. Hemoglobin was analyzed in aortic homogenates and normalized to the protein content. **Statistics:** Grubb's test was performed and one sample in the eAAA data set for hemosiderin (eAAA) was identified as a significant outlier. **B, F,** All data are presented as scatter dot plots. The horizontal line depicts the median with range. The number of analyzed samples is given in the figures. Kruskal-Wallis and Dunn's multiple comparisons test. **Abbreviations:** Ad, adventitia; AOD, arterial occlusive disease; eAAA, electively treated AAA; HO-1, heme oxygenase-1; Lu, lumen; rAAA, ruptured AAA; Th, thrombus.