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Hemorphin 7 Reflects Hemoglobin Proteolysis in Abdominal Aortic Aneurysm

Tiphaine Dejouvencel; Delphine Féron; Patrick Rossignol; Marc Sapoval; Claude Kauffmann; Jean-Marie Piot; Jean-Baptiste Michel; Ingrid Fruitier-Arnaudin; Olivier Meilhac

Objective—In human abdominal aortic aneurysm, the accumulation of blood-derived cells and proteases within the mural thrombus plays a pivotal role in the evolution toward vessel wall rupture. We sought to identify peptides released from abdominal aortic aneurysm specimens, characterized by an intraluminal thrombus.

Methods and Results—Intraluminal thrombus were analyzed by differential proteomics, using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. A 1309-Da peptide was detected in larger amounts in the newly formed luminal thrombus layer relative to older layers. It was identified as being LVVYPWTQRF (known as LVV-Hemorphin 7), a peptide generated from hemoglobin by cathepsin D. By immunohistochemical analysis, we showed that Hemorphin 7 (H7) colocalizes with cathepsin D and cathepsin G in the luminal layer of the intraluminal thrombus. In vitro, cathepsin G was able to generate H7 peptides at pH 7.4, whereas cathepsin D was only active in acidic conditions. Finally, H7 peptides were shown to be increased 3- to 4-fold in sera of abdominal aortic aneurysm patients relative to controls, and their levels were positively correlated with the volume of the thrombus.

Conclusion—Our results suggest that circulating H7 peptides may reflect proteolysis of hemoglobin in the aneurysmal intraluminal thrombus and may be used as a biological marker of pathological vascular remodeling. (*Arterioscler Thromb Vasc Biol.* 2010;30:00-00.)

Key Words: ●●●

In human atherothrombosis, the accumulation of blood-derived cells and zymogens contributes to oxidation and proteolysis, together leading to arterial wall destabilization.¹ Depending on the arterial territory, complications associated with atherosclerosis may evolve toward stenosis, frequent in carotid, coronary, and femoral arteries, or outward remodeling, mainly in the aorta. In abdominal aorta, where atherothrombosis frequently develops, evolution toward aneurysm is characterized by the presence of a dynamic intraluminal mural thrombus (ILT), which represents a source of blood-borne proteases participating in extracellular matrix degradation and smooth muscle cell apoptosis, preventing thrombus colonization and cicatrization.^{2,3} In the present study, we aimed to look for biomarkers reflecting the pathological remodeling of aortic atherothrombosis associated with the thrombus in abdominal aortic aneurysm (AAA). For this purpose, we used a differential proteomics approach comparing the different layers of human AAA, samples including the thrombus, and the remaining media. We used surface-enhanced laser desorption/ionization

time-of-flight mass spectrometry (SELDI-TOF-MS), a particularly well-suited technology for detection of peptides and small proteins, including proteolytic fragments. Our hypothesis is that the proteases conveyed by leukocytes (matrix metalloproteases, elastase, cathepsins, etc) or those generated in situ (ie, plasmin, thrombin) may produce protein fragments reflecting the pathological vascular remodeling.⁴ Our strategy was thus to compare the profiles of low-molecular-weight proteins and peptides released by AAA samples. Such peptides could be used as biomarkers of the thrombus activity and aneurysmal progression, and could provide new insights into the biology of atherothrombosis.

After identification of these differentially secreted peptides, we investigated their origin and particularly the proteases responsible for their generation. Finally, we also evaluated their potential diffusion into the blood compartment by a quantitative immunoassay in patients' sera.

Materials and Methods

For expanded Materials and Methods, please see supplemental materials (available online at <http://atvb.ahajournals.org>).

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Sampling

Twenty AAA samples, including the ILT, were collected during surgical repair and rapidly dissected into 5 parts: luminal (at the interface with circulating blood), intermediate and abluminal parts for the thrombus and media, and adventitia for the aneurysmal aortic wall. AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (REflet Sanguin de l'évolutivité des Anévrismes de l'Aorte abdominale, CCPPRB Paris-Cochin number 2095).⁵ All patients gave their informed written consent, and the protocol was approved by a French ethics committee (CCPPRB, Cochin Hospital). Each segment (layers of ILT, media, and adventitia) were cut into small pieces (5 mm³) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (2 mL/g of wet tissue). The conditioned medium (supernatant-containing proteins released by the tissue sample) was obtained after centrifugation at 3000g for 10 minutes at 20°C. Protein concentration of each conditioned medium was measured using the Bradford assay (BioRad).

SELDI-TOF-MS Profiling

Profiling was performed on medium conditioned by incubation with 8 AAA thrombus samples (luminal, intermediate, and abluminal) using ProteinChip arrays (Biorad) with various chromatographic binding conditions: CM10 (cationic exchanger) arrays in 100 mmol/L sodium acetate pH 4; Q10 (anionic exchanger) arrays in 100 mmol/L Tris-HCl, pH 9; and H50 (hydrophobic surface) arrays in H₂O, 0.1% trifluoroacetic acid containing 5% acetonitrile. Twenty micrograms of each sample were added to the corresponding binding buffer (total incubation volume=200 μ L). After 90 minutes of incubation with gentle shaking, the Protein-Chip arrays were washed with the appropriate binding buffer. Finally, arrays were washed with water and allowed to air-dry before adding the matrix, consisting of a saturated solution of α -cyano-4-hydroxy cinnamic acid matrix (Biorad) in 10% v/v acetonitrile and 0.1% v/v trifluoroacetic acid. The *m/z* values of proteins retained on each chromatographic surface were determined from time-of-flight measurements using a Protein-Chip Reader (PCS 4000; Biorad). Data were collected by averaging 795 laser shots for each sample, with laser intensity optimized for each type of ProteinChip array. The peak intensities were normalized by using the total ion current of all spectra and then analyzed by Biomarker Wizard software (Biorad). For validation and quantification of the peaks of interest, H4 ProteinChips (hydrophobic C16 surfaces, particularly suited for peptide detection) were used because it gave a better signal-to-noise ratio than CM10 for detection of the peaks. Analysis of incubation media conditioned by the thrombus or by its corresponding aneurysmal wall medial layer was performed on 8 to 12 additional samples, using 1 μ L of each sample in 5% acetonitrile.

In Vitro Digestion of Hemoglobin by Neutrophil Proteases

Human hemoglobin (60 μ g; Sigma) was incubated with cathepsin D (w/w ratio, 1:182; Sigma-Aldrich) at pH 3.2 and pH 7.4, cathepsin G (250 nM; kindly provided by Dr Dominique Pidard),⁶ elastase (10 nM; Calbiochem), and proteinase 3 (100 nM; Elastin Products) at pH 7.4, for 2 hours at 37°C. Analysis of the fragments was performed by SELDI-TOF-MS using NP20 ProteinChip.

Determination of Serum Hemorphin 7 Peptides Concentrations in AAA Patients

Sera were obtained from patients enrolled in AMETHYST (Aneurysm METalloproteinases and HYPertension STudy), a cohort of patients either with asymptomatic AAA (3- to 5-cm-diameter) or with an aortic diameter >5 cm and therefore scheduled for endovascular repair within 1 month. The study was approved by the ethical committee (Cochin Hospital Comité de Protection des Personnes se Prêtant à la Recherche Biomédicale, approval numbers 1930 and 1931). Exclusion criteria for patients were cancer, infection, and any immuno-mediated disease. Determination of serum Hemorphin 7 (H7) peptide concentration was performed as de-

scribed.⁷ Briefly, microtiter plates were precoated overnight with 2.6×10^{-9} mol/L bovine serum albumin-VV-Hemorphin-7 conjugate and then incubated for 16 hours under gentle shaking at 4°C with rabbit primary antibody against the C-terminal of VV-H7 peptide, appropriately diluted in 1% bovine serum albumin-phosphate-buffered saline and serum (25 μ L of each). Peroxidase-conjugated secondary antibody (1:40 000 in 0.5% bovine serum albumin-phosphate-buffered saline) was added (50 μ L/well) and left for 2 hours at room temperature. After washing the plates 5 times, 100 μ L of ABTS (0.1 mg/1 mL) were added to each well and incubated for 30 minutes under shaking at room temperature. The reaction was stopped by addition of 150 μ L of 1% SDS solution, and the resulting absorbance was measured at 405 nm with an enzyme-linked immunosorbent assay reader (thermo max microplate; MWG BIOTECH).

Statistical Analysis

Differences between the various parts of AAA samples were evaluated by the Wilcoxon paired nonparametric test, and differences between sera of patients with AAA vs those of healthy subjects were evaluated by the Wilcoxon nonpaired nonparametric test (Kaleidagraph version 4.0). Results are expressed as box plots, in which the median is shown. Upper and lower limits of boxes represent interquartiles (25th and 75th), whereas upper and lower bars show percentiles (10th and 90th). Correlations were assessed using the nonparametric Spearman test (Statview software version 4.57). Statistical significance was accepted for $P < 0.05$.

Results

Characterization of AAA Samples and SELDI-TOF-MS Profiling

The ILT (Figure 1A) was dissected into 3 layers (Figure 1B) and the remaining media was separated from the adventitia. Colorimetric quantification of heme (proportional to the presence of hemoglobin) showed a gradient of concentration, decreasing from the luminal thrombus to intermediate and abluminal layers (Figure 1C).

Profiling was performed on conditioned medium obtained from 8 ILT of AAA samples separated into luminal, intermediate, and abluminal layers. We focused on peaks with *m/z* values <3000 for which the sequence could be obtained by direct MS-MS (without purification and trypsin digestion). After automatic detection of the peaks with a signal-to-noise ratio >5, their intensities were compared by Biomarker Wizard software. After analysis, we selected *m/z* 1309 peak, which was found to be more abundant in AAA luminal layer secretomes as compared to other samples. This peptide was retained on the CM10 Protein-Chip (cationic exchanger at pH4) and was found to be differentially released between luminal vs intermediate and abluminal layers of the AAA mural thrombus (n=8; $P=0.0039$; not shown). The presence of the *m/z* 1309 peak was validated and quantified in the different layers (n=10–12 additional samples) using a hydrophobic surface (H4 ProteinChip Arrays) particularly suited for peptide detection. We also quantified the *m/z* 1309 peak in the remaining aortic wall (residual media, n=8). Representative spectra are shown in Figure 2A. Interestingly, the media significantly released larger amounts of LVV-H7, suggesting that it is trapped within this tunica (Figure 2A, B).

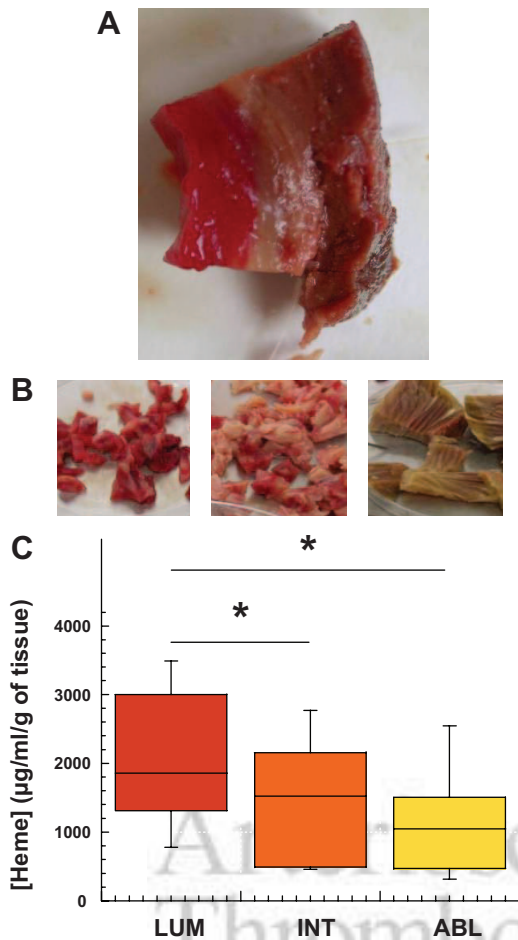


Figure 1. A, An AAA ILT separated into 3 parts: luminal (LUM), intermediate (INT), and abluminal (ABL) layers (B). C, Heme was quantified in the media conditioned by incubation with these samples, (n=8; * $P < 0.05$).

Identification of the 1309-Da Peptide

The m/z 1309 peak was purified using a hydrophobic resin (ZipTip C18; Millipore) as described in the online Materials and Methods section. The presence of m/z 1309 peak after elution was checked by SELDI-TOF-MS before MALDI-TOF MS/MS analysis. The deduced amino acid sequences from the MS/MS analysis were PWTQRF, LVV, and Y. Beta-globin was found to match exactly with the PWTQRF sequence when submitted to <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The full-length sequence was extrapolated to be LVVY PWTQRF (m/z 1308.7), a fragment of hemoglobin previously described as LV-H7 (LVV-H7).⁸ The MS/MS spectrum and deduced sequences are presented in Figure 1 (available online at <http://atvb.ahajournals.org>). This 10-amino-acid peptide has been reported to be generated from the hemoglobin- β chain by cathepsin D in a first step, and then VV-H7 (m/z 1195) is produced in a second step.⁹ VV-H7 was also found and exhibited the same distribution as LVV-H7 throughout the different layers of the AAA sample, as determined by SELDI-TOF-MS (Figure 2A). A strong correlation was observed between LVV-H7 and VV-H7, suggesting that they are produced by the same enzyme from the same substrate ($R^2 = 0.839$; $P < 0.001$; Figure 2B).

Cathepsin D Is Contained in and Released by the AAA Thrombus

Because cathepsin D was reported to produce both LVV-H7 and VV-H7, we analyzed its distribution in AAA samples. Enzyme-linked immunosorbent assay for quantification of cathepsin D showed a 3-fold greater release by the luminal layer than by the other thrombus layers (results are expressed in $\mu\text{g/mL/gram}$ of tissue \pm standard error: luminal, n=19, 235.8 ± 31.2 ; intermediary, n=14, 80.5 ± 21.5 ; abluminal, n=19, 71.7 ± 23 ; Figure 3A). The total amount of tissue cathepsin D determined after tissue extraction by Western blot showed a similar trend as compared to conditioned medium (Figure 3B).

Immunohistological Analysis of (L)VV-H7 in AAA Samples

Many publications report variations in LVV-H7 in plasma or serum, as well as in different tissues;^{10–13} however, the origin of LVV-H7 is not clear. Here, we sought to verify whether H7 peptides could be produced in situ by evaluating the presence of free hemoglobin and cathepsin D within the intraluminal thrombus of AAA. For this purpose, we first verified that the quantity of heme determined by colorimetric assay (Figure 1C) was proportional to hemoglobin content in the medium conditioned by incubation with AAA. Western blot for detection of hemoglobin (data not shown) showed the same gradient as that shown in Figure 1 for heme quantification. We performed immunohistochemical analysis on AAA intraluminal thrombus and remaining aortic wall. An antibody directed against the C-terminal of both LVV-H7 and VV-H7 (which does not cross-react with native hemoglobin¹⁴) allowed us to show that H7 peptides were more abundant in the luminal area than in the other thrombus layers (ie, intermediate and abluminal; Figure 3A). Positive staining was mainly observed in cells with polylobed nuclei, likely to be neutrophils. Cathepsin D immunostaining performed on serial sections showed a similar trend (ie, luminal staining), but clearly more cells were positive for H7 than for cathepsin D, suggesting that other enzymes may produce H7 peptides. Double-immunostaining for cathepsin D and (L)VV-H7 confirmed that some cells exhibited a colocalization, but many of them were negative for cathepsin D (Figure 3B, left panel). We therefore performed the simultaneous immunodetection for (L)VV-H7 and cathepsin G, an enzyme known to be produced by neutrophils. Many cells showed a colocalization for cathepsin G and (L)VV-H7 (Figure 3B, right panel), suggesting that this enzyme may participate in H7 peptide generation. In Figure 3, black triangles show cells positive for cathepsin but negative for (L)VVH-7 staining (red), gray triangles show double-staining, and cells positive for either cathepsin D or G are indicated by white triangles (magnification $\times 20$, insets $\times 100$).

Because the medial layer of AAA samples was shown to release large amounts of H7 (Figure 2), we assessed its presence in the residual aortic wall by immunohistochemistry. (L)VV-H7 accumulates in the media, forming a gradient toward the adventitia (Figure 4). This staining was more diffuse than that observed in the luminal thrombus,

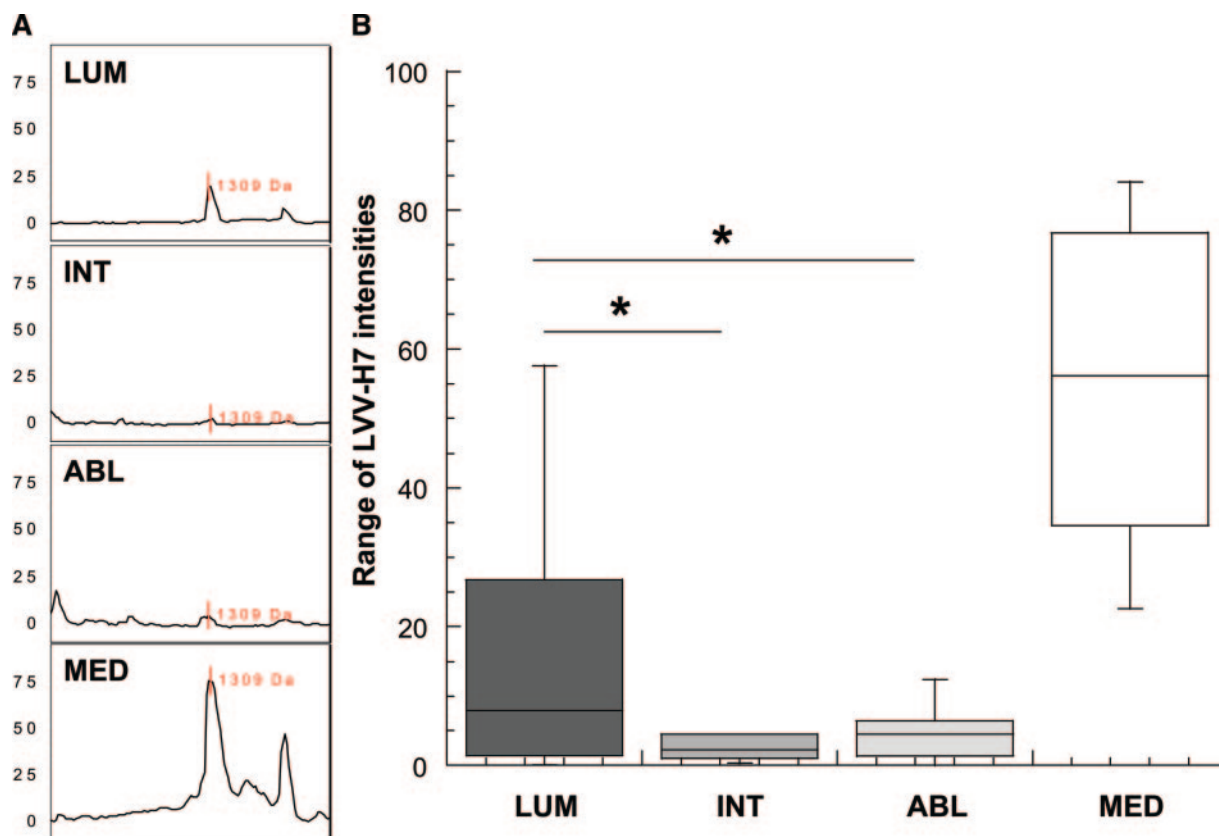


Figure 2. A, Representative mass spectra showing the 1309-Da peak in AAA samples after retention on H4 ProteinChip arrays. B, Quantification of m/z 1309 peak intensities (arbitrary units) in AAA intraluminal thrombus and medial layer (LUM indicates luminal, $n=12$; INT, intermediate, $n=10$; ABL, abluminal, $n=11$; MED, aortic medial layer, $n=8$). $*P<0.05$.

potentially because (L)VV-H7 peptides are not concentrated within cells but are rather trapped by the elastic laminae.

In Vitro Generation of (L)VV-H7 Peptides From Hemoglobin

Human purified hemoglobin was digested in vitro by different neutrophil enzymes (cathepsin G, proteinase 3, and elastase) and cathepsin D for 24 hours at 37°C (Figure 4). Both VV-H7 (m/z 1195) and LVV-H7 (m/z 1309) were detected by SELDI-TOF-MS after incubation with cathepsin D at pH 3 and cathepsin G at pH 7.4. Neither elastase nor proteinase 3 was able to produce H7 peptide, although they generated other hemoglobin fragments. As previously reported,¹⁵ cathepsin D was only active at pH 3, suggesting that its action is limited to the acidic lysosomal compartment. After isolation of peptides on ZipTip C18 (Millipore), and subsequent analysis by MALDI-TOF-MS-MS, m/z 1195 and m/z 1309 were confirmed to be, respectively, VV-H7 and LVV-H7 (Figure V).

Serum Concentration of H7 Peptides in AAA Patients

The release of H7 peptides by the luminal part of AAA thrombus suggests that it may diffuse into the blood compartment. By using a competitive enzyme-linked immunosorbent assay, quantification of H7 peptides in serum of AAA patients vs healthy subjects was performed. Patients were separated into 2 groups, with or without surgical indication,

on the basis of aortic diameter (<5 cm and >5 cm). Results presented in Figure 5 show a 4-fold increase of H7 peptides in sera of patients with large aortic diameters as compared to sex- and age-matched controls, and a 3-fold increase compared to patients with small aneurysms (3 cm< diameter<5 cm). Moreover, maximal aortic diameters in AAA patients were significantly correlated with H7 levels ($\rho=0.293$; $P=0.0402$). Interestingly, a positive correlation was also observed between the thrombus volume and the levels of H7 peptides in serum ($\rho=0.293$; $P=0.0401$).

Biological Effects of LVV-H7

In AAA, neutrophils are attracted to the luminal part of the mural thrombus by known chemokines such as IL-8.¹⁶ Here, we addressed the possibility that LVV-H7 could participate in neutrophil recruitment. For this purpose, PMN were allowed to migrate through a filter into a lower chamber containing potential chemoattractive molecules. IL-8 (0.1 $\mu\text{mol/L}$) was used as a positive control. We studied the effect of LVV-H7 on these cells as compared to the entire hemoglobin protein. PMN migration was expressed as a percentage of the number of neutrophils that migrated without chemoattractant (medium alone), taken as 100%. Neutrophils were attracted more by LVV-H7 than by hemoglobin for concentrations ranging from 3 nM to 12.5 nM (119% \pm 6 vs 99% \pm 11 for LVV-H7 at 3 nM and 122% \pm 7 vs 100% \pm 18 for hemoglobin at 12.5 nM; IL-8 [used as positive control]: 216% \pm 61; $n=3$ independent experiments performed in triplicate; $P<0.05$). This result

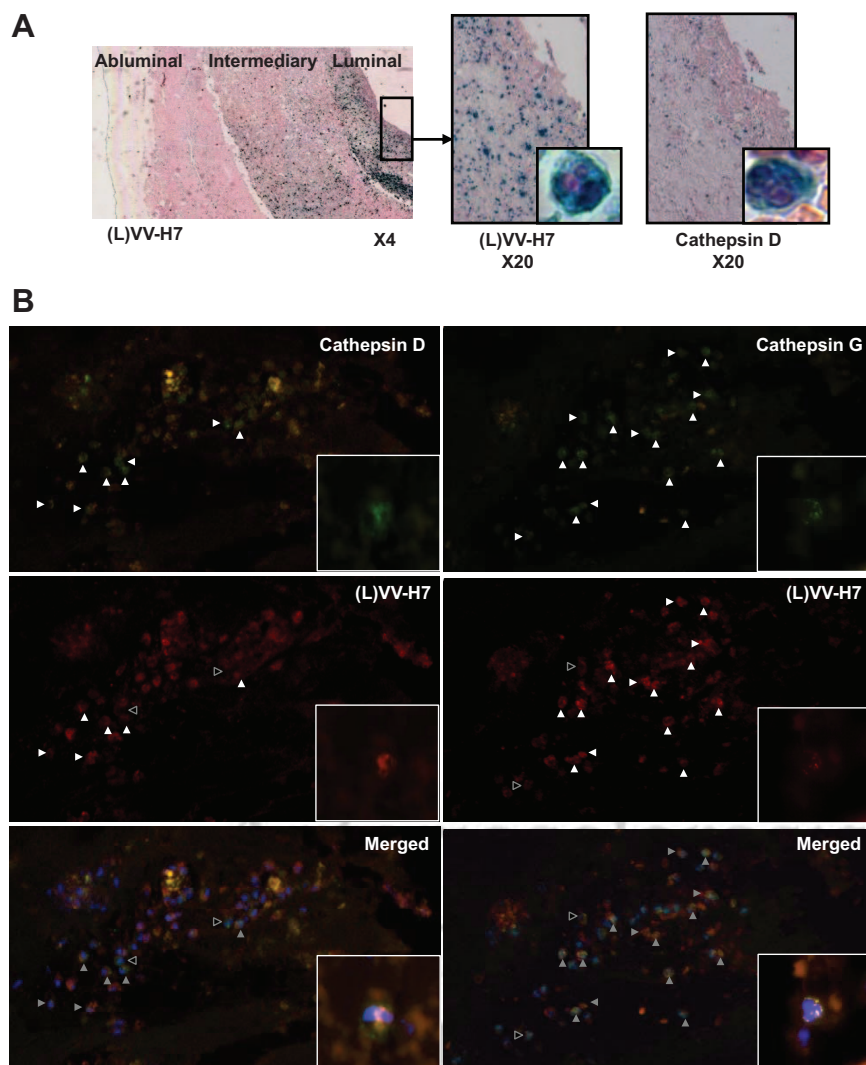


Figure 3. Immunohistochemistry for detection of (L)VVH-7, cathepsin D, and cathepsin G. A, Immunoreactivity is shown in green. Counterstaining of nuclei appears in red. B, Double-immunostaining on serial sections (cathepsin D or G in green and -white triangles, (L)VVH-7 in red, nuclei in blue). Gray triangles show double-staining.



suggests that LVV-H7 may have a moderate but significant effect on neutrophil recruitment relative to intact hemoglobin.

Discussion

AAA represents an evolution of the atherothrombotic plaque toward outward remodeling, characterized in most cases by the formation of ILT, in which red blood cells and neutrophils are entrapped.² We and others have shown the ILT to be a major source of blood-borne free hemoglobin and proteases, including those of the fibrinolytic systems, matrix metalloproteinases, and neutrophil proteases.^{2,3,16}

In the present study, we explored the peptides and low-molecular-weight proteins able to be released by the different layers of abdominal aortic aneurysm thrombus. For this purpose, we used differential proteomics by SELDI-TOF mass spectrometry, a particularly sensitive method for the detection of polypeptides <20 kDa. Two peaks at 1195 and 1309 Da were found to be more abundantly released by the recently formed luminal layer of AAA thrombus relative to intermediate and abluminal older layers. These peptides were identified from their amino acid sequence by tandem mass spectrometry as VV-H-7 (VVYPWTQRF) and LVV-H7 (LVVYPWTQRF), respectively.¹⁴ These peptides are re-

ported to be mainly generated by digestion of the hemoglobin β -chain by cathepsin D.⁹ They were isolated from brain,¹⁷ lung,¹⁸ and biological fluids,¹³ and they display various biological activities such as angiotensin-converting enzyme inhibition,¹⁹ opioid-like functions,²⁰ and modulation of inflammation.¹² They also possess analgesic properties.¹³ Serum H7 peptides have been already assayed and found to be decreased in patients with breast cancer²¹ and in patients with diabetes.⁷

Cathepsin D is a lysosomal enzyme present in macrophages.¹⁵ In neutrophils, cathepsin D colocalizes with myeloperoxidase in azurophilic granules and participates in apoptosis initiation.²² Neutrophils are the most abundant leukocytes in the luminal thrombus of AAA.³ Here, for the first time to our knowledge, we provide evidence of the simultaneous presence of hemoglobin, cathepsin D, cathepsin G, and H7 peptides in the same pathological tissue sample. In AAA, this staining was associated with cells and showed a gradient from the luminal to the abluminal layer. The polylobed aspect of the nuclei and positive elastase staining (not shown) in the luminal layer of the AAA thrombus strongly suggest that H7 peptides were produced by neutrophils. Double-immunostaining for cathepsin D and (L)VV-H7 showed that some,



Figure 4. A, SELDI-TOF mass spectra of hemoglobin (Hb) digested by cathepsin D in acidic conditions (pH 3.2) and at neutral pH (7.4), elastase, proteinase 3 (PR3), and cathepsin G at neutral pH for 2 hours at 37°C.

but not all, cells were double-stained, suggesting that another enzyme was able to generate H7 peptides. We report for the first time to our knowledge that cathepsin G colocalized with (L)VV-H7 and may produce H7 peptides at physiological pH. In contrast, cathepsin D is only active at pH 3, suggesting that hemoglobin needs to be taken up into lysosomes before H7 peptide could be generated. The fact that cathepsin G is able to generate H7 peptides is of particular importance because this phenomenon therefore can take place in the extracellular compartment, after neutrophil degranulation, in areas rich in free hemoglobin. This could facilitate the diffusion of H7 peptide through the thrombus. Interestingly, the medial wall of AAA released large amounts of H7 peptides but no

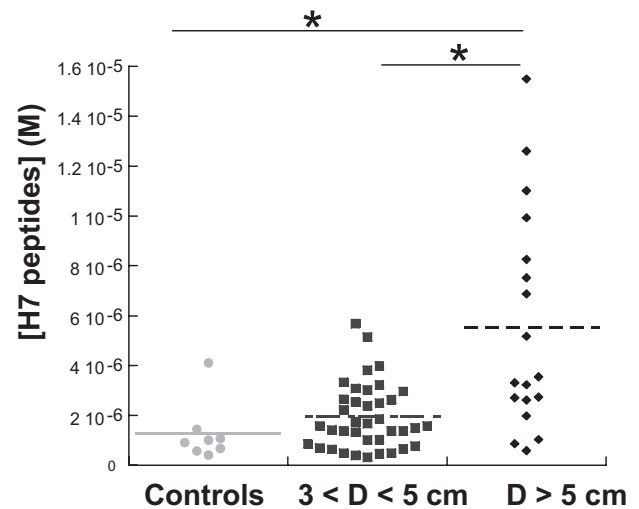


Figure 5. H7 peptide concentration in serum of patients with AAA as compared to healthy subjects. Controls are healthy subjects (n=9); 3 cm<D<5 cm indicates patients with an abdominal aortic diameter <5 cm (n=38); D>5 cm, patients with an abdominal aortic diameter >5 cm (n=18). *P<0.05. D, aortic diameter.

hemoglobin. Immunostaining confirmed that these peptides are trapped in the aneurysmal media in the absence of staining for cathepsin D and hemoglobin (not shown). This suggests that H7 peptides produced by the luminal layer of the thrombus are conveyed centrifugally by mass transport toward the adventitia and become trapped in the aneurysmal media. Our results illustrate this concept of hydraulic conductance of protein fragments across the ILT, recently reviewed by Michel et al.²³ This raises the question of the potential biological function of H7 peptides on smooth muscle cells and whether they are able to bind them.

The recruitment of neutrophils by the ILT is potentially a major process impeding cicatrization. We thus tested whether LVV-H7 could promote chemoattraction of neutrophils *in vitro*. By recruiting neutrophils from circulating blood, H7 peptides present in the luminal pole of the thrombus, and, in particular LVV-H7, may participate in thrombus renewal/progression, whereas H7 peptides, which have accumulated in the residual aortic wall as a result of centrifugal convection, may foster inflammatory cell infiltration. Albeit moderate, the LVV-H7 effect on neutrophil chemoattraction was significantly higher than that of native hemoglobin. The release of H7 peptides could be interpreted as a signal that the capacity for clearance of hemoglobin is overwhelmed and recruitment of additional phagocytic cells is required.

Finally, we addressed the possibility that H7 peptides produced in the thrombus may reach the blood stream and reflect the progression of the disease, as we have previously described for markers of platelet activation.¹ Serum H7 peptides were increased in patients with AAA relative to controls and were positively correlated with the AAA diameter and thrombus volume. These hemoglobin-derived peptides represent an interesting biological marker reflecting the presence of hemoglobin and protease activity associated with neutrophils. Whether LVV-H7 may be used for evaluation of thrombus-associated aneurysmal progression in AAA would

need further investigation. However, the half-life of small peptides in the circulation depends on several factors, such as their elimination rate in urine and their trapping by potential receptors (ie, angiotensin receptors in brain²⁴ or opioid receptors¹⁸).

In conclusion, we have identified, by a differential proteomic approach, LVV-H7 and VV-H7 peptides as being abundantly released by the hemoglobin–leukocyte-rich luminal layer of AAA thrombus. Assessment of the biological role of these peptides and their use as potential biomarkers will require additional studies.

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Disclosure

None.

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Supplemental Material

Determination of AAA thrombus characteristics

Semi-automated AAA diameter and thrombus volume measurements were performed using dedicated software ¹, by a single observer blinded to the serum H7 levels. Briefly, the main steps consisted of: 1) user identification of AAA lumen entry and exit points located near the celiac trunk and iliac bifurcation, respectively; 2) automatic segmentation of 3D lumen; 3) automatic curved multiplanar reformation computation of lumen path; 4) semi-automated aneurysm wall segmentation on curved multiplanar reformations based on active contour processing; 5) interactive contour validation and editing if needed. Finally, 3D mathematical models of the AAA components were reconstructed and automatic calculation of maximal AAA diameter, and thrombus volume were performed.

MALDI-TOF/MS-MS peak identification

For purification and identification of the biomarker discovered on CM10 and H4 ProteinChips by SELDI-TOF-MS, a volume of 10 µL of medium conditioned by luminal thrombus layer containing large amounts of the peak of interest was purified by a hydrophobic resin (ZipTip C18, Millipore). Briefly, after wetting (100% acetonitrile) and equilibration of the resin with 0.1% TFA, 10 µL of sample containing 0.1% acetonitrile were allowed to bind to the ZipTip. Elution was achieved in 50% acetonitrile – 0.1% TFA solution. The resulting eluate was analyzed by SELDI-TOF-MS on normal phase ProteinChips (NP20) and 1 µL containing the biomarker was analysed by MALDI-TOF MS. For this purpose, the sample was mixed 1:2 (v:v) with 2.5 mg/mL of α -cyano-4-hydroxycinnamic acid (HCCA, Laser Biolabs) in 50% ACN (VWR – HPLC Grade) and 0.1 % TFA (Pierce). The analysis was performed using a MALDI TOF/TOF ABI 4800 + (Applied Biosystems) equipped with YAG-200 Hz laser (355 nm). Spectra acquisition and processing was performed using the

4000 Series Explorer software (ABI) version 3.5.1. (Proteomics platform, Institut Claude Bernard and Institut Jacques Monod, Paris, France). The deduced amino-acid sequence was submitted to <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Determination of heme/hemoglobin content

Heme content was assessed by addition of formic acid to the conditioned media (v/v 10/90), OD was monitored at 405 nm². Hemoglobin from human erythrocytes (Sigma Aldrich) was used as a standard. In conditioned media, heme was considered as being proportional to hemoglobin release.

Determination of cathepsin D content

Concentrations of cathepsin D in conditioned medium of AAA were determined using an ELISA kit, according to the manufacturer's instructions (Calbiochem). Comparison between the amount of cathepsin D content in conditioned medium and the corresponding tissue extract was evaluated by western blot. Briefly, the thrombus layers were dissected out and separated into equal fragments for tissue extraction and preparation of conditioned medium for each case. Proteins from luminal, intermediary and abluminal parts of the thrombus were extracted in 7M urea, 2M thiourea, 1% CHAPS containing a cocktail of antiproteases (Sigma-Aldrich), using a Dounce tissue grinder (borosilicate glass pestle). Twenty micrograms of proteins extracted from snap-frozen thrombus samples and 20 µg of the conditioned medium obtained from the same samples were resolved by SDS-12% PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes, blocked with 5% milk powder in Tris-buffer saline, pH 7.4 containing 0.1% Tween 20, and then probed with mouse monoclonal anti-cathepsin D (20 ng/mL, Calbiochem) and peroxidase-conjugated

secondary antibody (dilution 1:25,000 Jackson ImmunoResearch Laboratories) followed by ECL detection.

Immunohistological analysis

AAA samples were fixed in 3.7% paraformaldehyde, embedded in paraffin and sectioned at 6 μ m. Immunohistochemistry was performed using a polyclonal antibody directed against the C-terminal of Hemorphin-7 peptides (H7 peptides) (University of La Rochelle), rabbit anti-cathepsin D (2 μ g/mL; SantaCruz Biotechnology, sc-10725), anti-CD68 (macrophages, 0.4 μ g/mL; PG-M1, Dako) and anti-elastase (dilution 1 μ g/mL; SantaCruz Biotechnology) as primary antibodies. Peroxidase LSAB Dako kit (Dako) followed by Histogreen substrate (AbCys SA, France) were used for detection. Sections were then counterstained with Nuclear Red. Double staining was performed on similar samples using mouse monoclonal anti-cathepsin D (Calbiochem, IM03), rabbit polyclonal anti hemorphin-7 and appropriate secondary antibodies: anti-mouse conjugated to Alexa 488 (1:500, Invitrogen) and an anti-rabbit conjugated to Alexa 555 (1:500, Invitrogen). 4'-6-Diamidino-2-phenylindole (DAPI) was used at 0.1 μ g/mL for nuclear staining. Control irrelevant antibodies (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

Cell isolation and chemotaxis assay

Neutrophils were isolated from venous blood of healthy volunteers (with informed consent), sampled on EDTA. Red blood cells were aggregated by addition of 2% dextran for 20 minutes at 20°C and the upper phase containing leukocytes was centrifuged on Ficoll (20 minutes at 600g, 20°C) (PAA Laboratories GmbH, LSM 1077). The pellet containing neutrophils was submitted to a hypo-osmotic shock to eliminate residual erythrocytes.

Cells were stained using 0.5µg/mL Calcein AM (Invitrogen) in PBS for 30 minutes at 37°C and then rinsed once. Transwell migration assays were performed as described³ with slight modifications, using 96-well disposable chemotaxis chambers with a 8 µm polycarbonate filter (ChemoTX, Neuroprobe, Cabin John, MA, USA). Briefly, 29 µL of RPMI medium containing or not chemoattractant (Interleukin-8, LVV-H7, hemoglobin) were added to the lower compartment of each well. Polymorphonuclear cells (75,000) in 25 µL of RPMI were added to the upper compartment. The chamber was then incubated at 37 °C in a humidified atmosphere (5% CO₂) for 2 h. A standard curve, consisting of a 1:2 dilution cascade of polymorphonuclear cells (top standard, 50,000 cells in 29 µL), was constructed. After incubation, the framed filter was carefully removed and the number of cells that had migrated was determined reading fluorescence at 485ex/530em by and comparison with the standard curve. The experiment was performed 3 times in triplicate.

Figure legends

Supplemental Figure I. MS-MS spectrum of *m/z* 1309 confirming the sequence of LVV-Hemorphin 7 from medium conditioned by incubation with luminal part of the thrombus.

Supplemental Figure II. A, VV-Hemorphin 7 distribution in medium conditioned by AAA samples (thrombus and media). Results are presented as intensities of the 1309 Da peak detected after retention on ProteinChip H4 (reverse phase) and SELDI-TOF-MS reading (arbitrary intensity units). LUM: luminal n=12, INT: intermediate n=10, ABL: abluminal n=11, MED: aortic medial layer n=8, *p<0.05. B, Correlation between LVV-H7 and VV-H7 intensities detected by mass spectrometry.

Supplemental Figure III. A, Concentration of cathepsin D in media conditioned by incubation with different layers of AAA thrombus, determined by ELISA. LUM: luminal n=19, INT: intermediate n=14, ABL : abluminal n= 19, *P<0.05. B, Representative Western-blot for detection of cathepsin D (34 kDa) released from incubated thrombus (conditioned medium) or extracted by homogenisation in lysis buffer (tissue extract), n=4.

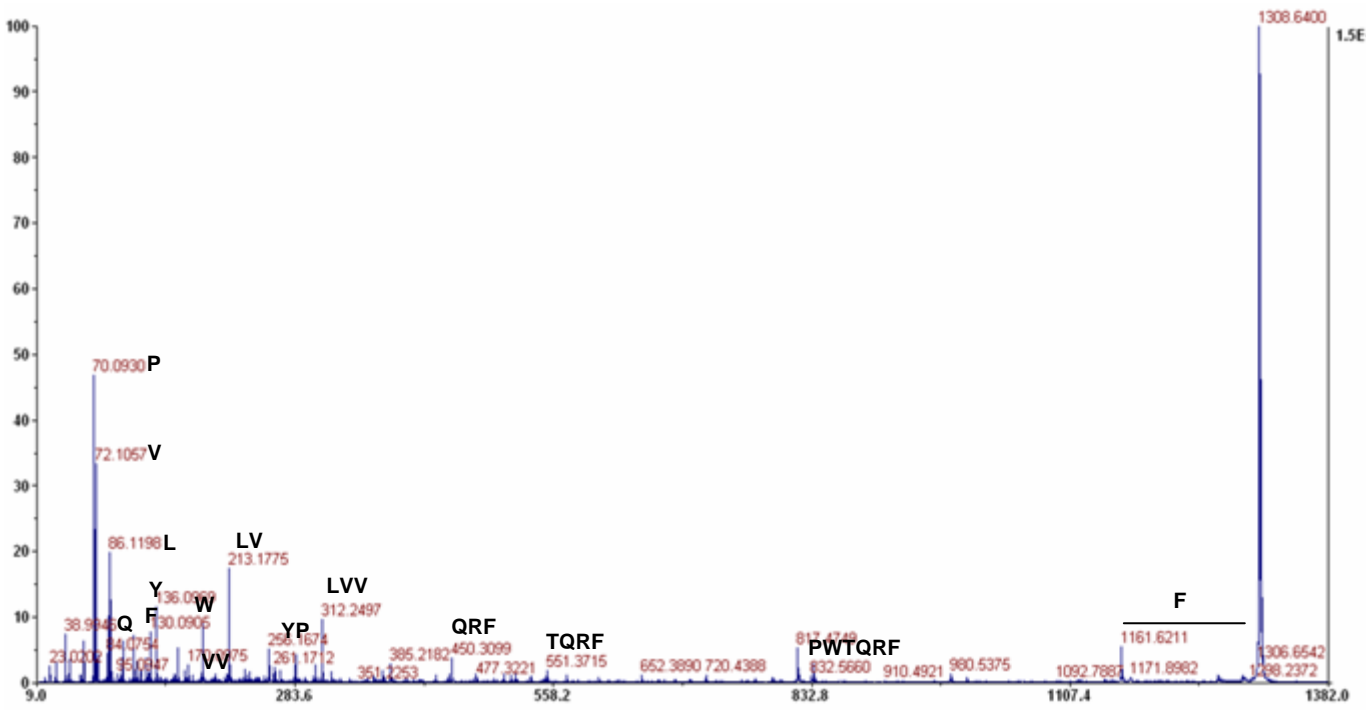
Supplemental Figure IV. Immunostaining of (L)VVH-7 in the residual arterial wall of AAA (red). Nuclei are stained by DAPI (blue). Media and adventitia are indicated in the merged image.

Supplemental Figure V: MS-MS spectrum of m/z 1309, confirming the sequence of LVV-H7 after hemoglobin digestion by cathepsin D.

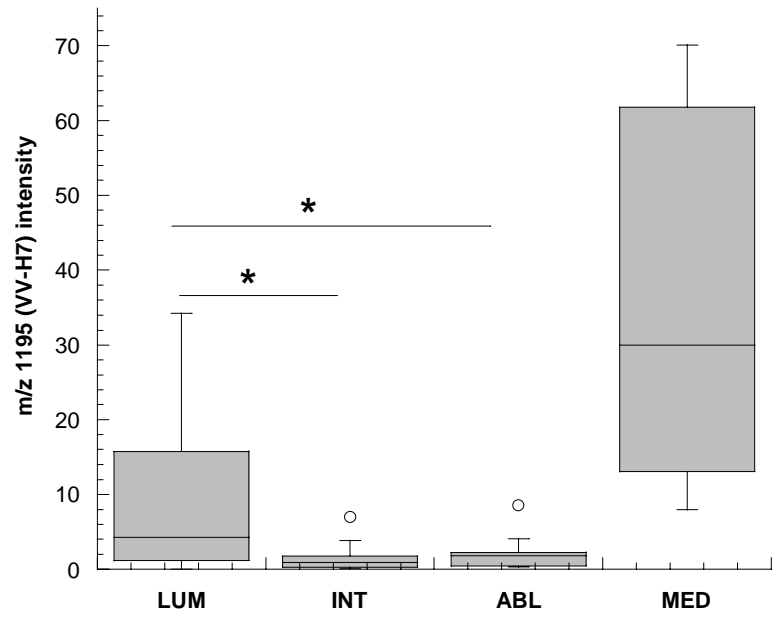
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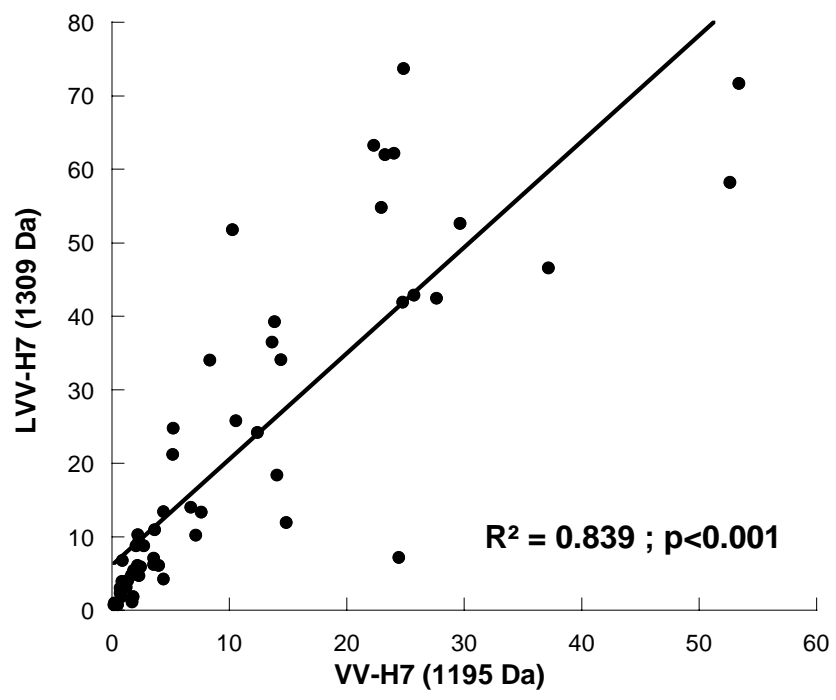
Supplemental Figure I



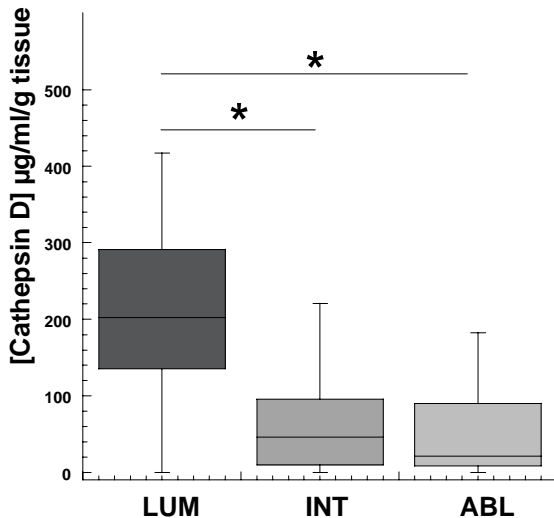
A



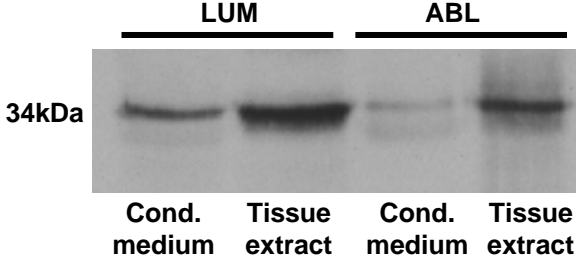
B



A



B



Supplemental Figure IV

