IJC Heart & Vasculature 57 (2025) 101647

Contents lists available at ScienceDirect

IJC Heart & Vasculature

journal homepage: www.sciencedirect.com/journal/ijc-heart-and-vasculature

Increased neprilysin expression is linked to atrial fibrotic remodeling in cardiovascular surgery patients

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ARTICLE INFO

Keywords:
Atrial fibrillation
Neprilysin
Left atrium reservoir strain
Atrial fibrosis
Cardiovascular surgery
Collagen
TGFβ1
COL1A1

ABSTRACT

Background: Neprilysin (NEP) is a membrane-bound neutral endopeptidase with various physiological functions. We investigated the roles of NEP in atrial fibrotic remodeling and atrial fibrillation (AF) in patients undergoing cardiovascular surgery.

Methods: Atrial tissue samples were obtained from left atrium (LA) appendages, and mRNA expression level was analyzed by real-time reverse transcription polymerase chain reaction in 61 cases (25 paroxysmal fibrillation (PAF), 36 AF). Adipose tissue (AT) mRNA expression levels were also analyzed. Western blotting and immunohistochemical staining were used for determining tissue protein expression. Serum NEP levels were measured by enzyme-linked immunosorbent assay (ELISA). Two-dimensional speckle tracking echocardiography was performed to measure mean left atrial reservoir strain (mLASr) to evaluate atrial remodeling in pre-operative patients and control participants.

Results: Immunohistochemical staining and western blotting revealed NEP expression in both AT and LA. Serum NEP levels did not correlate with NEP mRNA or protein expression in AT and LA. NEP mRNA expression levels correlated with fibrosis-related gene expression. NEP mRNA, protein, and fibrosis-related gene expression levels increased in PAF patients with low mLASr compared with high mLASr. PAF patients with high NEP mRNA expression showed increased fibrosis-related gene expression compared with those with low NEP expression. Multiple regression analysis revealed that NEP mRNA expression level was an independent variable for predicting fibrosis-related gene expression, whereas NOX4 and NLRP3 were independent variables for predicting NEP expression levels.

Conclusions: Increased atrial expression of NEP is linked to atrial fibrotic remodeling, and the development of AF in patients undergoing cardiovascular surgery.

Abbreviations: AF, (atrial fibrillation); CTGF, (connective tissue growth factor); TGFβ, (transforming growth factor beta); LA, (left atrium); AT, (adipose tissue); LAS, (left atrial strain); LASr, (left atrial reservoir strain); mLASr, (mean left atrial reservoir strain); LV, (left ventricle); ANP, (atrial natriuretic peptide); BNP, (brain natriuretic peptide); 2DSTE, (two-dimensional speckle tracking echocardiography); LAD, (left atrial diameter); LVEF, (left ventricular ejection fraction); LAVI, (left atrial volume index); MR, (mitral regurgitation); MVR, (mitral valve replacement); AVR, (aortic valve replacement); LVEDP, (LV end-diastolic pressure); NOX, (NADPH oxidase); ECM, (extracellular matrix); HFrEF, (heart failure with reduced ejection fraction); HFpEF, (heart failure with preserved ejection function); NEP, (Neprilysin); ACEI, (angiotensin-converting enzyme inhibitors); ARNI, (angiotensin receptor/neprilysin inhibitor); AS, (aortic stenosis); EAT, (epicardial adipose tissue) ; eGFR, (estimated glomerular filtration rate); ELISA, (enzyme-linked immunosorbent assay); LAV, (left atrial volume); LVEDV, (left ventricular end-diastolic volume); PAF, (paroxysmal atrial fibrillation); RT-PCR, (Real-time quantitative reverse transcription-polymerase chain reaction; Sac/Val, (sacubitril/valsartan); SAT, (subcutaneous adipose tissue); BK, (Bradykinin.

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https://doi.org/10.1016/j.ijcha.2025.101647
Received 14 February 2025; Received in revised form 1 March 2025; Accepted 9 March 2025
Available online 17 March 2025
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1. Introduction

Atrial fibrillation (AF) is an increasingly common arrhythmia worldwide, associated with considerable morbidity and mortality. AF results from continuous remodeling of the atria, which involves electrical and structural remodeling secondary to the progression of underlying heart disease and environmental factors [1,2]. Current management aims to prevent the recurrence of AF and includes the prevention of stroke, ventricular rate control, and rhythm control therapies, such as antiarrhythmic drugs and catheter or surgical ablation. On the other hand, the concept of upstream therapy is the use of drugs that aim to counter and/or delay the remodeling process by acting at different levels and with different mechanisms, such as blockade of the renin-angiotensin-aldosterone system and antifibrotic, anti-inflammatory, and antioxidant effects [3]. These drugs may arrest or delay atrial remodeling and cellular processes, leading to AF.

Sacubitril/valsartan (Sac/Val), an angiotensin receptor/neprilysin inhibitor (ARNI), has gained increasing interest in the treatment of heart failure, with the greatest mortality reduction [4]. Neprilysin (NEP), an integral membrane-bound neutral endopeptidase, is widely distributed throughout the body, including the kidney, brain, and heart, and largely contributes to the degradation of the vasodilator and antihypertrophic peptides atrial (A-type) natriuretic peptide (ANP) and brain (B-type) natriuretic peptide (BNP), and the vasodilator and antifibrotic peptide bradykinin [5–7]. ANP, BNP, and bradykinin counteract cardiomyocyte hypertrophy and cardiac fibrosis [8–10]. Clinical and animal studies have shown that ARNI may be effective in ameliorating cardiac maladaptive remodeling and fibrosis [11,12]. Zhu et al. [13] showed that ARNI decreases atrial remodeling in heart failure and plays a potential role in AF prevention. Thus, NEP appears to play a role in atrial fibrosis, remodeling, and development of AF indirectly through neutral endopeptidase action; however, this remains unclear in patients with AF.

Deposition of the extracellular matrix (ECM) leads to fibrosis, which plays an essential role in structural remodeling and functional changes in AF [1,14–16]. The cellular and molecular control of atrial fibrosis is highly complex, and atrial fibrosis includes various individual and multifactorial processes with complicated interactions between cellular and neurohormonal mediators [4]. Several profibrotic signaling pathways [2,17], such as the renin/angiotensin system, and transforming growth factor beta (TGFβ) [18,19] have been implicated in atrial fibrosis and AF. AF usually begins with self-terminating paroxysmal atrial fibrillation (PAF), and progresses to more frequent and long-lasting episodes, followed by persistent AF. Atrial fibrosis was largely regarded as a consequence of AF, so called as “AF begets AF.” However, several studies have shown that increased fibrosis, which is a part of a preexisting substrate, precedes and contributes to AF development in sinus rhythm (SR) patients [20,21]. Two-dimensional speckle tracking echocardiography (2DSTE) is feasible for assessment of myocardial LA function and deformation [22,23]. LA reservoir strain (LASr) has been reported to be directly correlated to the presence of LA fibrosis [24,25]. Schaaf et al. [26] showed that LA remodeling associated with AF is detectable using LASr, even at the PAF stage. These results suggest that LASr may be a marker of atrial fibrosis in patients with cardiovascular diseases, including those with PAF. We recently reported that LASr correlates with atrial expression level of *COL1A1* encoding collagen Type I α1 and that patients with low LASr exhibit increased atrial fibrosis-related gene expression, even those with PAF, in patients undergoing cardiovascular surgery [27]. However, the roles of NEP on atrial fibrosis, LASr, and AF remain unknown.

We investigated the association between NEP and atrial fibrosis-related gene expression, and clarified the roles of NEP in atrial fibrotic remodeling and AF in patients undergoing cardiovascular surgery.

Table 1
Characteristics of patients.

	LA resection patients	Adipose tissue resection patients	Control participants
Number	61	58	109
Male / Female	36 / 25	38 / 20	57/52
Age, y	69.9 ± 11.2	68.3 ± 12.7	72.0 ± 8.0
BMI, kg/m^2	22.6 ± 3.9	24.3 ± 3.8	22.6 ± 3.9
Atrial fibrillation	36	9	0
Risk factors, number			
Hypertension	41	39	54
Diabetes	11	23	23
Dyslipidemia	20	29	42
CKD	36	27	6
Cardiovascular surgery, number			
CABG	4	15	
AS (AVR)	11	6	
AR (AVR)	3	0	
MR (MVR, MVP)	24	12	
MS (MVR)	2	0	
CABG combined with valve procedure (AVR, MVP, MVR)	7	4	
AVR (AS, AR) with MVR or MVP (MR)	4	2	
Aortic disease (AAR, TAR, HAR, et al.)	0	10	
Others	6	9	
Drugs number			
β-blockers	36	33	4
Ca-blockers	23	21	39
ACE-1/ARB	38	32	31
Statin	21	28	34
Anti-diabetic drugs	11	18	18
eGFR, ml/min/1.73 m^2	55.4 ± 24.7	59.4 ± 17.1	
BNP, pg/ml	441 ± 541	292 ± 397	
HbA1C (%)	5.9 ± 0.7	6.4 ± 1.2	
NEP (ng/ml)	0.88 ± 1.21	1.33 ± 1.08	
Echocardiographic data			
LAD, mm	48.4 ± 8.6	43.0 ± 9.7	34.0 ± 5.0
LVEF (bp), %	57.8 ± 11.2	60.0 ± 11.4	64.0 ± 4.0
LAVI (bp), ml/m^2	62.4 ± 23.7	41.1 ± 21.6	25.6 ± 6.5
Mean LASr (mLASr), %	15.4 ± 7.6	21.0 ± 8.6	36.7 ± 3.8

The data are shown as mean ± SD. (Number): number of patients examined. LA, left atrium; BMI, body mass index; CKD, chronic kidney disease; CABG, coronary artery bypass grafting; AS, aortic stenosis; AR, aortic regurgitation; AVR, aortic valve replacement; MVR, mitral valve replacement; MVP, mitral valve plasty; AAR, ascending aorta replacement; TAR, total arch replacement; HAR, hemiarch replacement; NEP, neutral endopeptidase; ACE-1, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; anti-diabetic drugs (i.e. α-glucosidase inhibitor, sulfonylurea, biguanide, dipeptidyl peptidase-4 inhibitor, sodium glucose cotransporter 2 inhibitor); eGFR, estimated glomerular filtration rate; BNP, brain natriuretic peptide; HbA1c, hemoglobin A1c; LAD, left atrial diameter; LVEF (bp), left ventricular ejection fraction measured by the biplane Simpson method; LAVI (bp), left atrial volume index estimated by the biplane area-length method; mean LASr, mean left atrial strain (reservoir).

2. Methods

2.1. Tissue preparation

Samples of left atrium (LA) appendages were obtained from 61 patients who underwent cardiovascular surgery at Dokkyo Medical Hospital. Twenty-five patients experienced PAF that spontaneously stopped within 7 days of onset and SR on admission and during cardiac surgery. Thirty-six patients had AF. Thirty-six patients were male. The mean age

was 69.9 ± 11.2 years. The tissue samples were prepared for pathological and molecular assessments. Twenty-four patients had mitral regurgitation (MR) and underwent mitral valve replacement (MVR) or mitral valve plasty (MVP). Eleven patients had aortic stenosis (AS) and underwent aortic valve replacement (AVR). Four patients underwent coronary artery bypass graft (CABG), and seven patients received CABG combined with valve replacement or plasty. Four patients underwent AVR with valve replacement or plasty. The baseline patient characteristics, risk factors, medical treatments, and other operative procedures are shown in Table 1. All patients underwent medical treatment as shown in Table 1. None of the patients received treatment with NEP inhibitors.

Adipose tissue (AT) samples were also obtained after the initiation of cardiopulmonary bypass, as previously reported [28]. Subcutaneous adipose tissue (SAT) samples were obtained from 44 patients, and epicardial adipose tissue (EAT) samples were obtained from 58 patients. SAT samples were obtained around the xiphoid process of the sternum and EAT samples were collected near the proximal right coronary artery. LA and AT samples were obtained from nine patients. Nine patients had AF, and the remaining 49 had SR. Thirty-eight patients were male. The mean age was 68.3 ± 12.7 years. Twelve patients had MR and underwent MVR or MVP. Six patients had AS and underwent AVR. Fifteen patients underwent CABG and four patients received CABG combined with valve replacement or plasty.

The study protocol conformed to the Declaration of Helsinki and was approved by the Institutional Human Research Committee. The study was approved by the Bioethics Committee of Dokkyo Medical University Hospital (No. 27077), and written informed consent was obtained from all participants.

2.2. Blood sampling

Biochemical data were collected at the time of admission and analyzed using routine chemical methods in the clinical laboratory of Dokkyo Medical University Hospital. Hemoglobin A1c (HbA1c), BNP, and estimated glomerular filtration rate (eGFR) were measured preoperatively. Samples for measuring serum NEP concentration were collected on the morning of cardiovascular surgery. Blood samples were drawn into pyrogen-free tubes without EDTA. Serum was collected by centrifugation at 3000 rpm for 10 min at 4 °C and by centrifugation at 1000 rpm for 10 min at room temperature, and stored in aliquots at 80 °C for enzyme-linked immunosorbent assay (ELISA).

2.3. Measurement of transthoracic echocardiography

Each patient underwent preoperative transthoracic echocardiography and two-dimensional (2D) imaging according to the recommendations of the American Society of Echocardiography. The left atrial diameter (LAD), left ventricular end-diastolic diameter, and left ventricular end-systolic diameter were measured in the parasternal long axis view. The left ventricular end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were measured from the apical view using the biplane method. The left ventricular ejection fraction (LVEF) was calculated using Simpson's method.

$$LVEF(bp) = 100 \times (LVEDV - LVESV)/LVEDV$$

Left atrial volume (LAV) was measured using the biplane Simpson's method of disks. The left atrium (LA) was divided into a pile of disks perpendicular to the longitudinal direction in both four- and two-chamber views. The radius of each disk was measured from the longitudinal axis to the LA contour in two perpendicular planes. The volume of each disk was calculated automatically, and the LAV was calculated by the summation of the disk volume:

$$LAV = \pi/4\Sigma(i = 1to20)ai \times bi \times L/N,$$

where ai and bi are 20 discs obtained in the two orthogonal incidences (the four- and two-chamber views). The formula for the biplane A/L method is as follows:

$$LAV = (8/3)\pi(A1 \times A2)/([L1 + L2]/2)$$

The LAV index (LAVI (bp)) was obtained by indexing LAV to body surface area.

2.4. 2DSTE

To obtain the preoperative LAS, 2DSTE analysis was performed using an image analysis software (TOMTEC-ARENA) (TomTec Imaging Systems GmbH, Munich, Germany). Automatic analyses (AutoStrain LA) of four-chamber (4CH) and two-chamber (2CH) apical view images of the LA were performed as previously reported [27]. Three consecutive heart cycles were recorded during a single breath-hold using a frame rate of > 80 fps for offline analysis. The LA endocardial border was traced, and the area of interest was manually adjusted to include the thickness of the entire LA wall. The software then selected stable speckles within the LA wall and tracked them in a frame-by-frame manner throughout the cardiac cycle. The entire LA tracking in the 4CH and 2CH apical view images was divided into six segments using the software. Speckle-tracking analysis was not performed in patients with unacceptable image quality. To calculate the LAS, we used the upslope of the R-wave as the reference point for strain on the electrocardiogram. The following LA strain components were defined: LASr = peak (maximal) longitudinal LA strain. Measurements of LASr were performed in the 4CH and 2CH views, and the mean values for both the 4CH and 2CH views were calculated as recommended by the European Association of Cardiovascular Imaging [29]. The values were averaged for all 12 segments, 6 in the apical 4CH view and 6 in the apical 2CH view, and the mean LASr (mLASr) value was obtained.

In addition, as shown in Table 1, 109 participants who underwent echocardiography and met the following criteria were included in the control group: 1) No history of PAF or AF; 2) obvious cardiac diseases (e.g., myocardial infarction, angina pectoris, cardiomyopathy [hypertrophic cardiomyopathy, dilated cardiomyopathy]) or valvular abnormalities; or 3) abnormal LVEF (bp) (<60 %), LAVI (>34 ml/m^2), or mean E/e' (> 14). Fifty-seven participants were males. The mean age was 72.0 ± 8.0 years. Some control participants underwent medical treatment, as shown in Table 1. None of the control participants received NEP inhibitors.

2.5. ELISA

Serum NEP levels were determined using a Human Neprilysin ELISA Kit (ELH-Neprilysin, RayBiotech Life, Inc., Peachtree Corners, GA, USA). The mean intra-assay coefficient of variation (CV) was < 10 %, and the mean inter-assay CV was < 11 %. The samples, reagents, and buffers were prepared according to the manufacturer's recommendations. The NEP detection threshold was 0.4 ng/ml.

2.6. Western blotting

NEP expression in the SAT, EAT, and LA was assessed by western blotting. The samples (100–150 mg) were homogenized in ice-cold lysis buffer (500 μL protein elution buffer containing 8 μL protease inhibitor cocktail) and centrifuged at 14,000 rpm/25 min at 4 °C, and the supernatant was aliquoted. The specimens were stored at − 80 °C. The protein concentration of each sample was measured using a Pierce BCA Protein Assay Kit (23227, Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, SDS-polyacrylamide gel electrophoresis using sample buffer (39001, Thermo Fisher Scientific Inc.) and polyvinylidene difluoride membrane (Amersham™ Hybond™ P PVDF, Cytiva, Marlborough, MA, USA), followed by blocking primary antibody reactions, were performed overnight at 4 °C. Mouse anti-NEP monoclonal antibodies (ab114033, Abcam plc, Cambridge, UK) for NEP were used. Secondary antibody

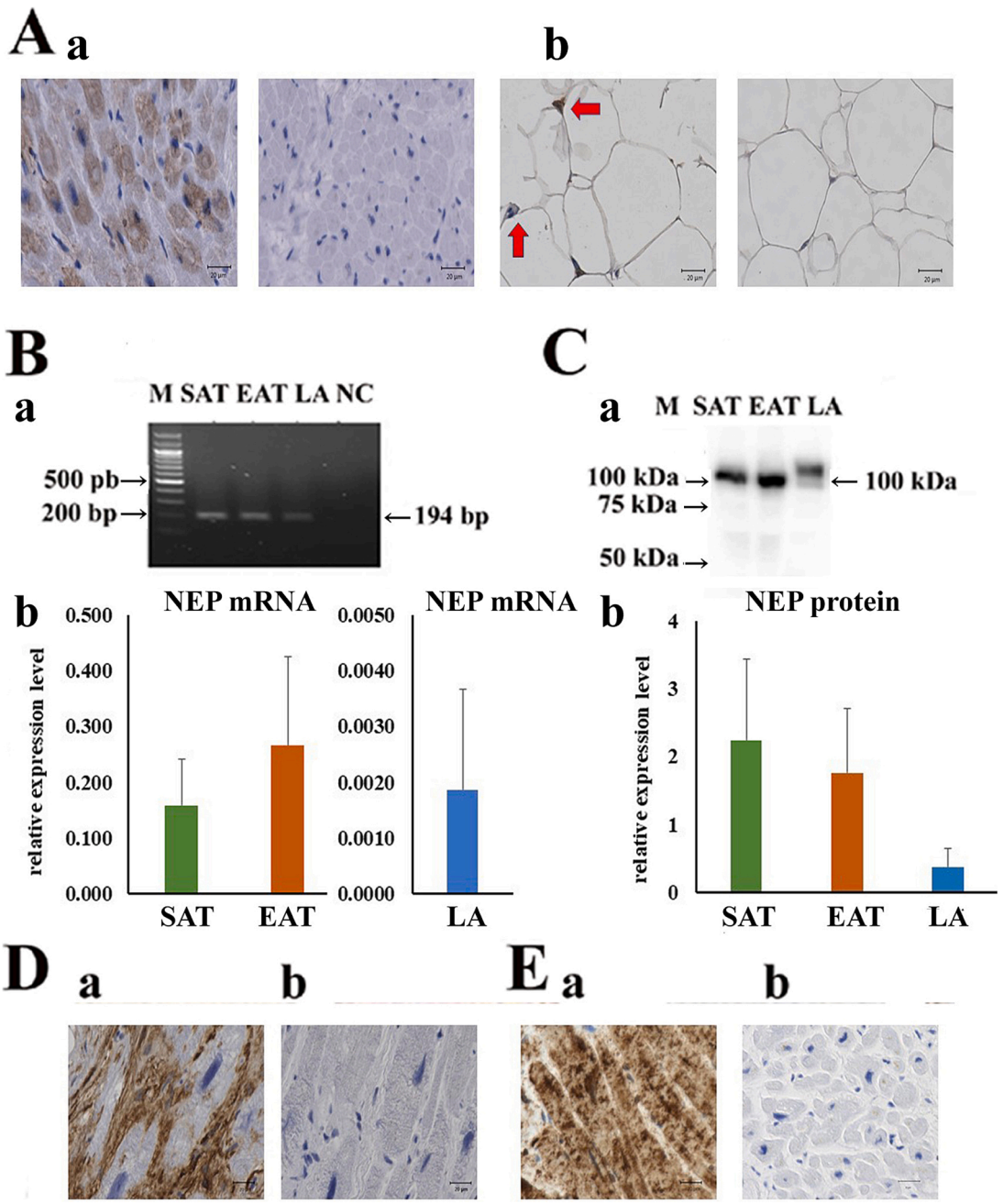

Fig. 1. Expression of NEP mRNA and protein in both LA tissue and adipose tissue. A: Immunohistochemical staining of NEP in LA tissue (LA, a) and epicardial adipose tissue (EAT, b). Negative control without antibodies (anti-NEP) is also shown (right panel). Immunocytochemical studies showed that both the LA and adipose tissues (indicated by red arrows) were positively immunostained with anti-NEP. B: NEP mRNA expression detected by RT-PCR in the SAT (n = 44), EAT (n = 58), and LA (n = 61). M: marker, NC: negative control (a). The relative expression levels of NEP mRNA, normalized to GAPDH as an internal control, are shown in b. C: NEP protein expression measured by western blot analysis. The relative expression levels of NEP protein (SAT (n = 44), EAT (n = 58), and LA (n = 61)), normalized with reference to GAPDH as an internal control, are shown in b. D and E: Immunohistochemical staining of collagen type I α1 (Da) and ANP (Ea) in LA tissues. A negative control without antibodies (anti-collagen type I α1 or anti-ANP) is also shown (Db, Eb).

reactions were performed at room temperature for 1 h. Immunoreactive bands were visualized by chemiluminescence using Chemi-Lumi One Super (Chemi-Lumi One Super [02230–30, Nacalai Tesque, Kyoto, Japan]). To quantify the expression of NEP protein, the levels were corrected with GAPDH levels measured using a GAPDH antibody (GT 239, GeneTex, CA).

2.7. Histological staining and Immunohistochemistry

The specimens were fixed with 4 % paraformaldehyde phosphate buffer solution (NACALAI TESQUE, Inc., Kyoto, JAPAN) and embedded in paraffin. Formalin-fixed paraffin-embedded (FFPE) atrial tissues were sectioned at 5 μm thickness and stained with Elastica van Gieson to visualize collagen fibers. For immunohistochemical staining, the FFPE atrial tissue sections were deparaffinized and rinsed. The sections were treated with DAKO EnVision™ FLEX Target Retrieval Solution High pH (Agilent Technologies, Inc., Santa Clara, CA, USA) at 97 °C for 20 min for antigen retrieval, and then with DAKO EnVision™ FLEX Peroxidase-Blocking Reagent (Agilent) for 5 min at room temperature. The slides were then stained using a DAKO EnVision™ FLEX System (Agilent). The

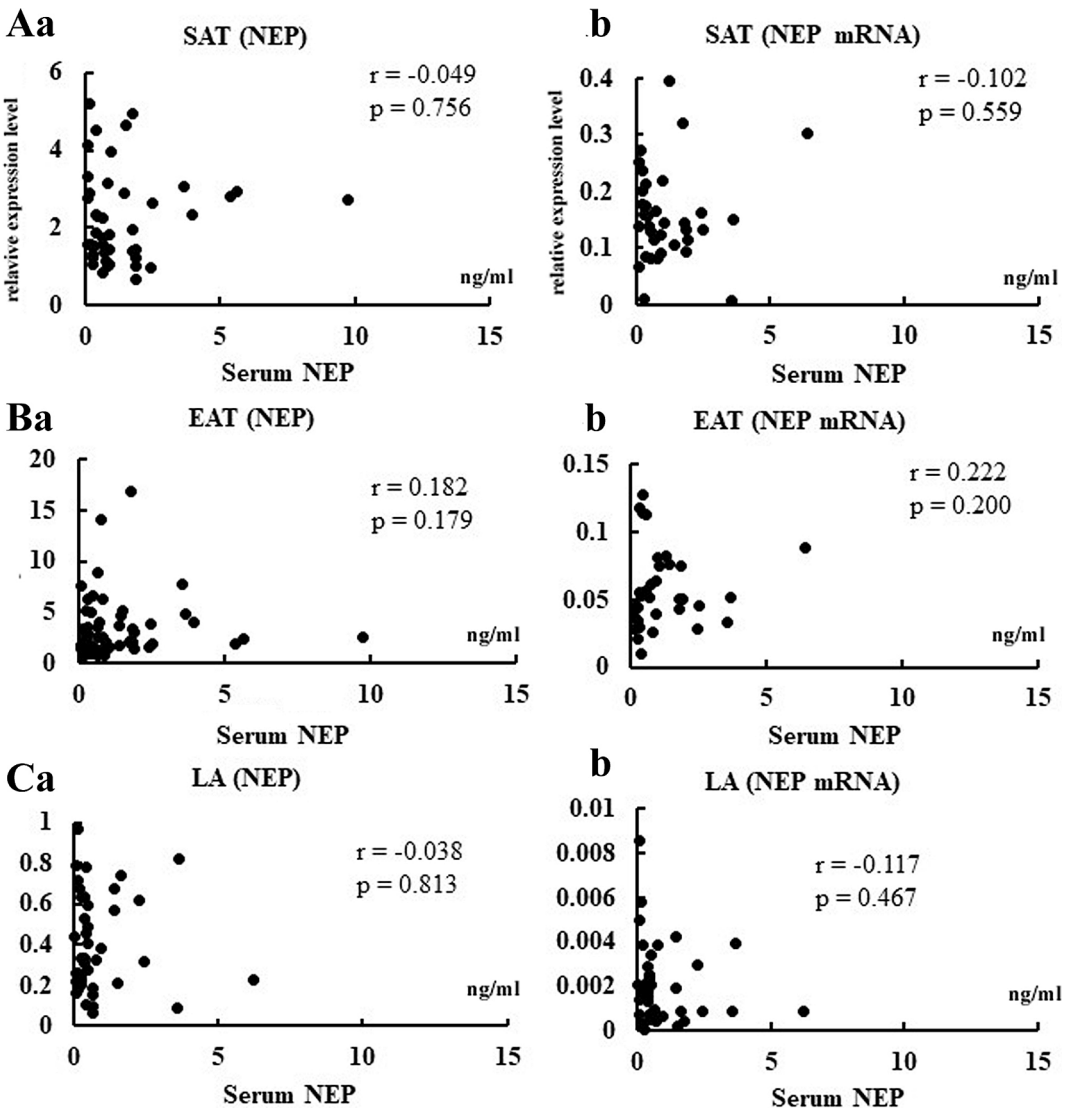

Fig. 2. Relationships between serum NEP levels and tissue NEP expression levels (adipose and LA tissues). A: Relationship between serum NEP levels and NEP protein (a) and mRNA expression (b) in the SAT (n = 44). B: Relationship between serum NEP levels and NEP protein (a) and mRNA expression (b) in EAT (n = 58). C: Relationship between serum NEP levels and NEP protein (a) and mRNA expression (b) in the LA (n = 61). The r- and p-values are shown.

first antibody reaction was performed using rabbit monoclonal anti-collagen type I α1 antibody (ab138492, Abcam plc, Cambridge, UK), anti-CD10/neprilysin antibody (65534 s, Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-ANP (rabbit polyclonal anti-ANP antibody, GTX55513, GeneTex, Inc., Irvine, CA, USA). Specificity was controlled by the addition of normal rabbit IgG (I-1000, VECTOR LABORATORIES, Inc., Burlingame, CA, USA) as the primary antibody.

2.8. RNA Extraction and Real-Time quantitative reverse Transcriptase/ Polymerase chain reaction (RT-PCR)

Cellular RNA was extracted from each LAA muscle sample or AT using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan). For RT-PCR, complementary DNA (cDNA) was synthesized from 40 ng/10 μl reaction of total RNA using a ReverTra Ace qPCR RT Master Mix (TOYOBO Co., Ltd., Osaka, Japan). Quantitative RT-PCR was performed using the KOD SYBR qPCR Mix (TOYOBO) and an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), as previously reported [27]. The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 40 cycles of heat denaturation, annealing, and extension. The PCR products were size-fractionated on 2 % agarose gels and controlled under blue LED light. Primers were selected based on the sequences of human genes, as shown in Supplemental Table 1. The RNA level was analyzed as an internal control (GAPDH) and used to normalize the values for the transcript quantity of mRNA. The analyzed genes were as follows: 1) ECM-related genes: *COL1A1* encoding collagen Type I α1, *COL3A1* encoding collagen Type III α1, and *FN1* encoding (fibronectin 1); 2) ECM degradation-related genes: *MMP2* encoding matrix metallopeptidase, *TIMP1* and *TIMP2* encoding tissue inhibitor of metalloprotease; 3) pro-fibrogenic cytokine or substance-related genes: *TGFB1* encoding TGFβ1, *EDN1* encoding endothelin-1, *PDGFD* encoding platelet derived growth factor D, and *CTGF* encoding connective tissue growth factor (CTGF), *ACE* encoding angiotensin I converting enzyme; 4) oxidant stress-related genes: *NOX2* and *NOX4* encoding NADPH oxidase; 5) inflammation-related genes: *IL1B* encoding interleukin 1β, *TNF* encoding TNFα, and *NLRP3* encoding NLR family pyrin domain containing 3; 6) natriuretic peptide A: *NPPA* encoding ANP, 7) natriuretic peptide B: *NPPB* encoding BNP, 8) *MME* encoding NEP (also known as CD10 or NEP), and 9) *GAPDH* encoding glyceraldehyde-3-phosphate dehydrogenase as an internal control (Supplemental Table 1) as partly described in recent papers [27].

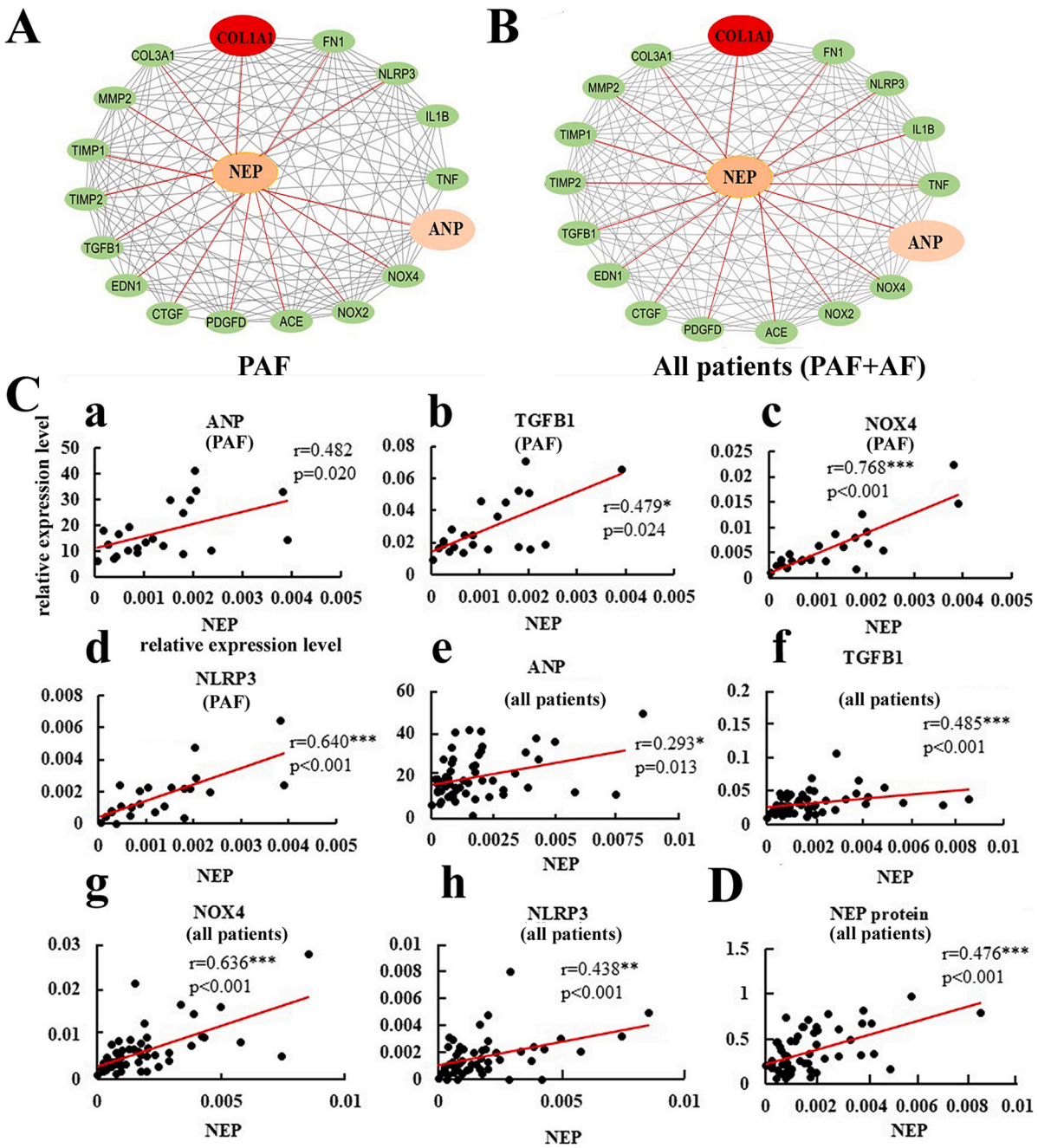

Fig. 3. Correlations among NEP, atrial fibrosis-related gene expression level, and NEP protein in LA. A and B: Correlations between NEP/ANP mRNA levels and atrial fibrosis-related gene expression level in PAF patients (A, n = 24–25) and all patients (B, n = 59–61). Significant correlations among ATP, and fibrosis-related gene expression are illustrated by black lines in PAF (A) and in all patients (B). Significant positive correlations among NEP, and fibrosis-related gene expression are illustrated by brown lines in PAF (A) and in all patients (B). C: Correlations among fibrosis-related gene expression level (ANP, TGFB1, NOX4, NLRP3) and NEP in PAF patients (a-d) and all patients (e-h) receiving LA resection. *p < 0.05, **p < 0.01, ***p < 0.001 D: Correlations between atrial NEP mRNA and protein expression level in all patients. Note that significant correlations were observed between atrial NEP mRNA and protein expression level.

2.9. Statistical analysis

All data are presented as the mean ± standard deviation. After testing for normality (Kolmogorov–Smirnov test or Shapiro–Wilk test), the comparison of means between groups was analyzed using a two-sided, unpaired Student's *t*-test in the case of normally distributed parameters or the Mann–Whitney *U* test in the case of non-normally distributed parameters. Associations between parameters were evaluated using Pearson's or Spearman's correlation coefficients. The univariate regression analysis with atrial genes such as *NEP* expression level as the dependent variable and fibrosis-related gene expression as the independent variable was performed in all patients, and the adjusted R-squared value (R^2), β-value, and *p*-value were obtained. Furthermore, multiple linear regression analyses with atrial genes such as COL1A1 and NEP expression levels as dependent variables were performed to identify the independent factors of mRNA expression levels (i.e. TGFB1, NOX4). Age, sex, and BMI were used as covariates for all patients. Since the value of events per variable = 10 seems most prudent for regression analysis [30], 6 variable factors including covariates factors were acceptably for multiple linear regression analyses with atrial genes (n = 61). When the actual measured values for both dependent and independent variables did not follow a normal distribution, the data were

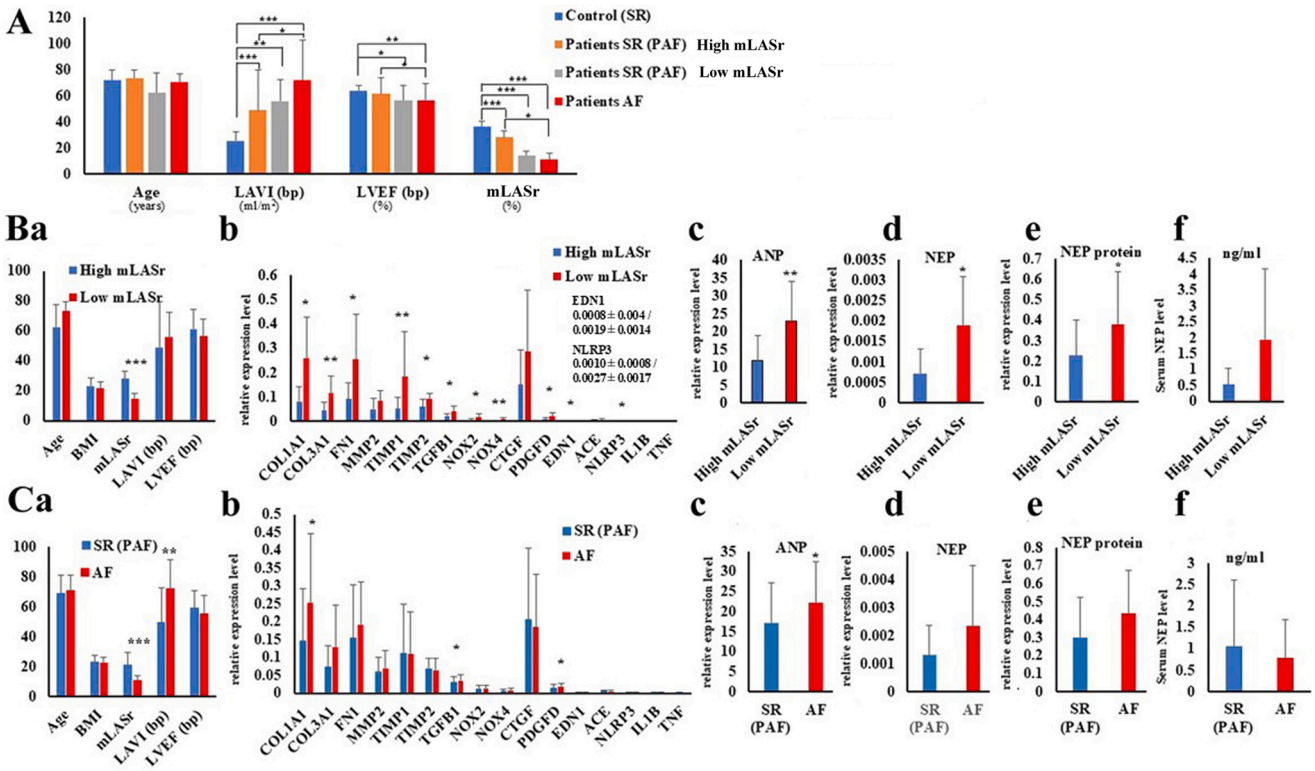

Fig. 4. Comparison of echocardiographic parameters and fibrosis-related gene expression level including NEP mRNA and protein between PAF with low and high mLASr and AF. A: Age and echocardiographic differences between control participants (n = 109) and PAF patients (n = 21) with low and high mLASr and AF (n = 34). PAF patients (n = 21) were divided into two groups (low mLASr (n = 10, male 5, female 5) and high mLASr (n = 11, male 7, female 4) based on the median mLASr value. B: Comparisons of echocardiographic findings, gene expression levels, and NEP protein, and serum NEP level between PAF patients with high and low LASr. (a) Age, BMI, and echocardiographic findings; (b) fibrosis-related gene expression, (c) ANP mRNA, (d) NEP mRNA, (e) NEP protein, and (f) serum concentration of NEP. Blue bar, high mLASr (n = 10–11); red bar, low mLASr (n = 9–10). C: Comparisons of echocardiographic findings, gene expression levels, and NEP protein, and serum NEP level between PAF and AF patients. (a) Age, BMI, and echocardiographic findings. Blue bar, SR (PAF, n = 21); red bar, AF (n = 34). (b-f) fibrosis-related gene expression (b), ANP mRNA (c), NEP mRNA (d), NEP protein (e), and serum concentration of NEP (f). For some genes, actual data (mean ± standard deviation) are shown in the figure. Blue bar, SR (PAF, n = 24–25); red bar, AF (n = 34–36). *p < 0.05, **p < 0.01, ***p < 0.001.

logarithmically transformed to achieve a normal distribution. Multicollinearity between variables was defined as a VIF (Variance Inflation Factor) < 5. All statistical analyses were performed using SPSS version 26 (IBM Corp., Armonk, NY, USA). $p < 0.05$ was regarded as significant. Partial correlation diagrams were created using JMP18 software (SAS Institute Japan, Tokyo, Japan). Partial correlation coefficients were calculated for the logarithmic values of the variables shown in the diagram, and partial correlation diagrams were created. The lines connecting each index, which had a partial correlation coefficient of 0.25 or more were shown.

3. Results

3.1. Patients

The baseline characteristics of the patients and control participants are shown in Table 1. Thirty-six patients who underwent LA resection had AF, and 25 patients had PAF. The mean values for LA resection patients were as follows: eGFR, 55.4 ± 24.7 ml/min/1.73 m^2; BNP, 441 ± 541 pg/mL; and HbA1c, 5.9 ± 0.7 %. In patients who underwent LA resection, the serum NEP concentration was 0.88 ± 1.21 ng/ml. The LAD, LAVI (bp), and LVEF (bp) values in the control group were 34.0 ± 5.0 ml, 25.6 ± 6.5 ml/m^2, and 64.0 ± 4.0 %, respectively. The mLASr in the control group was 36.7 ± 3.8 %. The LAD, LAVI (bp), and LVEF (bp) values in patients receiving LA resection were 48.5 ± 8.7 ml, 62.0 ± 24.0 ml/m^2, and 57.6 ± 11.3 %, respectively. The mLASr was 15.6 ± 7.7 %, which was significantly lower than that in the control group ($p < 0.001$). The mean values for AT resection patients were as follows: eGFR, 59.4 ± 17.1 ml/min/1.73 m^2; BNP, 292 ± 397 pg/mL; and HbA1c, 6.4 ± 1.2 %. The serum NEP level was 1.33 ± 1.08 ng/ml. The LAD, LAVI (bp), LVEF (bp), and mLASr values were 43.0 ± 9.7 ml, 41.1 ± 21.6 ml/m^2, 60.0 ± 11.4 %, and 21.0 ± 8.6 %, respectively.

3.2. Expression of NEP mRNA and protein in both LA and AT

Fig. 1 shows the immunohistochemical staining of NEP in LA tissue (a) and EAT (b). Immunocytochemical studies showed that both LA and EAT were positively immunostained for anti-NEP. RT-PCR and western blot analyses also showed the presence of NEP mRNA and protein expression in both adipose (SAT, EAT) and LA tissues, as shown in Fig. 1B and 1C. The relative expression levels of NEP mRNA and protein are shown in Fig. 1Bb and 1Cb. The expression of NEP mRNA and protein in AT was higher than that in the LA.

3.3. Relationships between serum NEP levels and NEP mRNA and protein expression levels in adipose and LA tissues

Fig. 2 shows the relationship between the tissue NEP expression (mRNA and protein) and serum NEP levels. The NEP protein levels at EAT, SAT and LA were not significantly correlated with BMI (data not shown). Serum NEP levels did not significantly correlate with NEP protein or mRNA expression in the SAT (Fig. 2Aa and 2Ab). Similar results were observed for the EAT (Fig. 2Ba and 2Bb) and LA (Fig. 2Ca and 2Cb). These results suggest that serum NEP levels do not reflect NEP mRNA and protein expression levels in adipose tissues (SAT and EAT) and LA.

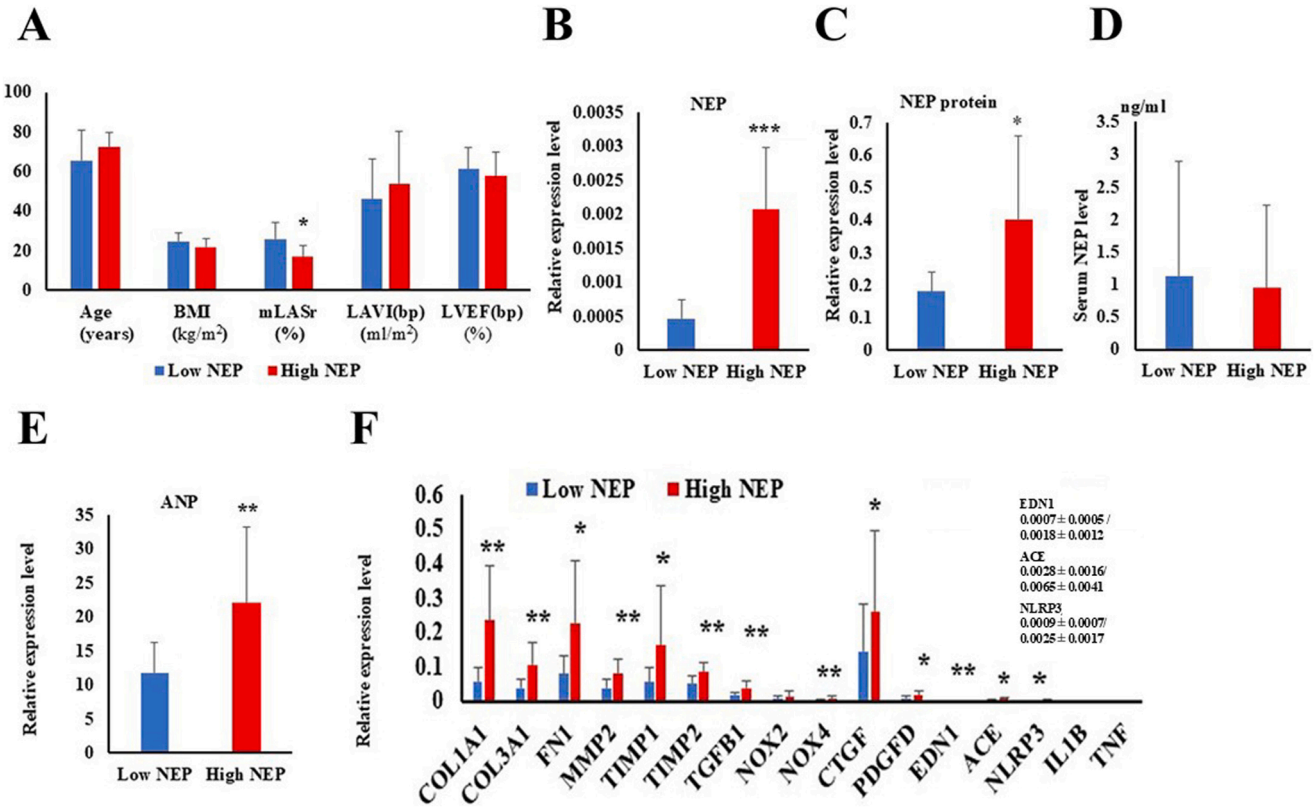

Fig. 5. Comparison of echocardiographic findings and gene expression levels between PAF patients with atrial low NEP and high NEP expression. A: Age, BMI, and echocardiographic findings in PAF patients (n = 21) with low or high atrial NEP mRNA expression. B-F: Comparison of NEP mRNA (B), NEP protein level (C), serum concentration of NEP (D), ANP mRNA (E), and fibrosis-related gene expression (F) between PAF patients (n = 25) with low and high NEP mRNA expression. For some genes, actual data (mean ± standard deviation) are shown in the figure. Red bar, high NEP (n = 12–13); blue bar, low NEP (n = 11–12). *p < 0.05, **p < 0.01.

3.4. Correlations among NEP, ANP, and fibrosis-related gene expression level in LA

We investigated the relationships between ANP and NEP mRNA expression levels and atrial fibrosis-related gene expression in PAF patients (A) and all patients (B) in the LA, as shown in Fig. 3 and Supplemental Fig. 1. Significant positive correlations illustrated by lines were observed between ANP mRNA and ECM-related genes (*COL1A1*, *COL3A1*, *FN1*), ECM degradation-related genes (*TIPM1*), oxidant-stress-related genes (*NOX4*), pro-fibrogenic cytokine or substance-related genes (*TGFB1*, *EDN1*, *CTGF*, *PDGFD, ACE*), and an inflammation-related gene (*NLRP3*) mRNA in PAF and in all patients. Significant positive correlations were also observed among the fibrosis-related gene expression in PAF (A) and in all patients (B) as shown in black lines (Fig. 3) and blue areas (Supplemental Fig. 1).

NEP mRNA expression levels correlated with ANP mRNA levels in PAF (Fig. 3A, Supplemental Fig. 1A) and in all patients (Fig. 3B, Supplemental Fig. 1B). NEP mRNA expression was also correlated with ECM-related genes (*COL1A1*, *COL3A1*, and *FN1*), ECM degradation-related genes (*TIPM1*, *TIMP2*, and *MMP2*), oxidant stress-related genes (*NOX2* and *NOX4*), pro-fibrogenic cytokine or substance-related genes (*TGFB1*, *EDN1*, *CTGF*, *PDGFD*, and *ACE*), and inflammation-related gene (*NLRP3*) mRNA as shown in brown lines. These results were observed in patients with PAF (Fig. 3A, Supplemental Fig. 1A) and all patients (Fig. 3B, Supplemental Fig. 1B). Fig. 3C (a-d) shows the relationships between NEP and ANP (r = 0.482, p = 0.020, Fig. 3Ca), TGFB1 (r = 0.479, p = 0.024, Fig. 3Cb), NOX4 (r = 0.768, p < 0.001, Fig. 3Cc), and NLRP3 (r = 0.640, p < 0.001, Fig. 3Cd) in PAF patients. Similar results were observed in all patients, as shown in Fig. 3C (e-h). Significant correlations were observed in NEP and ANP mRNA expression (r = 0.293, p = 0.013, Fig. 3Ce), TGFB1 (r = 0.485, p < 0.001, Fig. 3Cf), NOX4 (r = 0.636, p < 0.001, Fig. 3Cg), and NLRP3 (r = 0.438, p < 0.001, Fig. 3Ch) mRNA expression level. Fig. 3D shows the relationships between NEP mRNA and NEP protein. Significant correlations were observed between NEP mRNA and protein expression level in LA (r = 0.476, p < 0.001) in all patients.

3.5. Comparison of echocardiographic parameters and fibrosis-related gene expression levels, including NEP mRNA and protein between PAF patients with low and high mLASr, and AF

Fig. 4 shows the comparisons of echocardiographic parameters and fibrosis-related gene expression levels, including NEP mRNA and protein levels, between PAF with low and high mLASr and AF. PAF patients (n = 21) were divided into two groups (low mLASr (n = 10, Male 5, female 5) and high mLASr (n = 11, male 7, female 4)) based on the median mLASr value (mLASr = 20.1 %). Fig. 4A shows the echocardiographic differences between control participants and PAF patients with low and high mLASr and AF. There were no significant differences in age among the four groups. PAF patients with high and low mLASr and AF patients had higher LAVI (bp) and lower mLASr than the control participants. AF patients had a higher LAVI (bp), lower LVEF (bp), and lower mLASr than PAF patients with high mLASr and control participants. No significant differences in LAVI (bp), LVEF (bp), or mLASr were observed between PAF patients with low mLASr and AF patients. These results suggest that the structural remodeling, as reflected by the LAVI (bp) and mLASr, was almost established during PAF before AF development in our cardiovascular surgery patients.

Fig. 4B shows a comparison of gene expression levels between PAF patients with high and low mLASr. There were no significant differences

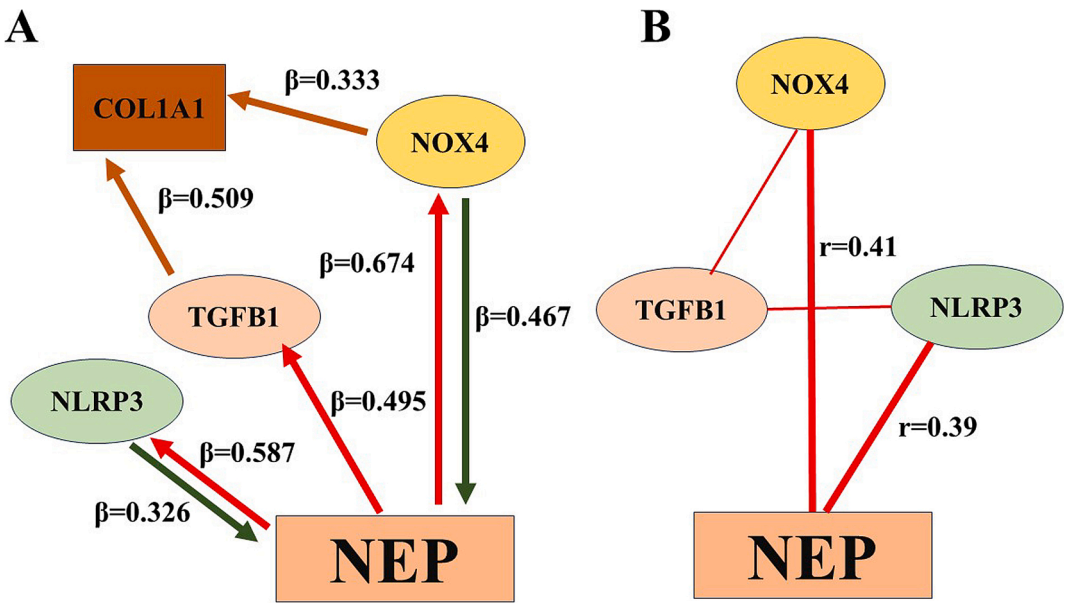

Fig. 6. Multiple linear regression analysis and partial correlation analysis between atrial NEP mRNA expression level and fibrosis-related gene expression level. A: Multiple regression analysis. Multivariate regression analysis showed that 1) log (TGFB1) and log (NOX4) were independent variables for predicting log (COL1A1) expression (brown arrow). 2) Log (NOX4) and log (NLRP3) were independent variables for predicting log (NEP) expression levels (green arrow). 3) Log (NEP) was an independent variable for predicting log (NLRP3), log (TGFB1), and log (NOX4) (red arrow). β-values are shown. B: Partial correlation analysis of NOX4, TGFB1, NLRP3, and NEP. Log (NEP) expression level was significantly positively correlated with log (NOX4) (r = 0.41) and log (NLRP4) (r = 0.39) expression level, but not with log (TGFB1). The partial correlation coefficients (r) are also shown. The lines connecting each index have a partial correlation coefficient of 0.25 or more.

in age, BMI, LAVI (bp), or LVEF (bp) between them (Fig. 4Ba). However, the expression of *COL1A1*, *COL3A1*, *FN1*, *TIPM1*, *TIMP2*, *TGFB1*, *ACE*, *EDN1*, *PDGFD*, *NOX2*, *NOX4*, and *NLRP3* mRNA was significantly higher in PAF patients with low mLASr than in PAF patients with high mLASr (Fig. 4Bb). The expression of ANP (Fig. 4Bc) and NEP mRNA (Fig. 5Bd) was also significantly increased in PAF patients with low mLASr. NEP protein expression significantly increased in PAF patients with low mLAS (Fig. 4Be); however, serum concentration of NEP did not differ between PAF patients with low and high mLASr (Fig. 4Bf).

Fig. 4C shows a comparison of echocardiographic parameters and fibrosis-related gene expression levels, including NEP mRNA and protein, between PAF and AF. AF patients had a higher LAVI (bp) and lower mLASr than patients with PAF. No significant differences in LVEF (bp) were observed between PAF and AF patients (Fig. 4Ca). The expression of *COL1A1*, *TGFB1*, *PDGFD*, and *ANP* mRNA was significantly higher in patients with AF than in those with PAF (Fig. 4Cb and 4Cc). This was consistent with the immunohistochemical staining for collagen type I α1 (Fig. 1), as marked staining of collagen was observed in patients with AF (Fig. 1D). Immunohistochemical staining also revealed significant ANP staining (Fig. 1E). However, no significant differences in NEP mRNA (Fig. 4Cd), NEP protein (Fig. 4Ce), or serum NEP concentration (Fig. 4Cf) were observed between PAF and AF patients. The expression levels of other genes, including *COL3A1*, *FN1*, and *NOX4*, were also not statistically different between PAF and AF patients.

3.6. Comparison of echocardiographic findings and gene expression levels between PAF patients with low and high atrial NEP expression

Next, we compared the parameters between PAF patients with low and high atrial NEP expression. PAF patients were divided into two groups based on the median value of NEP expression (relative expression level of 0.001). Age, BMI, LAVI (bp), and LVEF (bp) were not significantly different between the two groups (Fig. 5A). PAF patients with high NEP had a lower mLASr compared with PAF patients with low NEP. PAF patients with high NEP had higher atrial NEP mRNA expression (Fig. 6B) and protein levels (Fig. 5C). However, the serum concentration of NEP was not significantly different between PAF patients with low and high NEP expression (Fig. 5D). We compared the gene expression level between PAF patients with low NEP and high NEP as shown in Fig. 5E and F. Patients with high NEP showed higher expression of ANP (Fig. 5E), ECM-related genes (*COL1A1*, *COL3A1*, *FN1*), ECM degradation-related genes (*MMP2*, *TIMP1*, *TIMP2*), pro-fibrogenic cytokine or substance-related genes (*TGFB1*, *CTGF*, *PDGFD*, *EDN1*, *ACE*), oxidant stress-related genes (*NOX4*), and inflammation-related genes (*NLRP3*) mRNA, compared with those with low NEP as shown in Fig. 5F.

3.7. Relationships between atrial COL1A1 / NEP and other fibrosis-related gene expression

We investigated the relationship between NEP and atrial fibrosis-related gene expression by performing multivariate regression analysis. First, multiple linear regression analysis was performed with COL1A1 expression level as the dependent variable and TGFB1, NOX4, and NLRP3 as independent variables, as shown in Table 2A. After adjustments for age, log (TGFB1) ($\beta = 0.509$, $p < 0.001$) and log (NOX4) ($\beta = 0.333$, $p = 0.001$) were independent variables for predicting log (COL1A1) expression level (adjusted $R^2 = 0.787$, Table 2A and Fig. 6A [as shown by brown arrow]). Similarly, multiple linear regression analysis with COL1A1 expression level as the dependent variable and TGFB1, NOX4, NLRP3, and NEP as the independent variables showed that even after adjustments for age, log (TGFB1) ($\beta = 0.510$, $p < 0.001$) and log (NOX4) ($\beta = 0.296$, $p = 0.006$), but not log (NEP) or log (NLRP3), were independent variables for predicting log (COL1A1) expression levels (adjusted $R^2 = 0.792$, Table 2A). These results suggest that the expression level of TGFB1 and NOX4, but not NEP, were independent variables for predicting COL1A1 expression levels, Alternatively, multiple linear regression analysis with log (TGFB1), log (NOX4), or log (NLRP3) expression level as the dependent variable and NEP as the independent variable showed that even after adjustments for age, sex, and BMI, log (NEP) was an independent variable for predicting log (TGFB1) (adjusted $R^2 = 0.293$, $\beta = 0.495$, $p < 0.001$), log (NOX4)

Table 2
Regression analysis of COL1A1, NEP, and fibrosis-related gene expression level.

A: Multivariate regression analysis of COL1A1 and fibrosis-related gene expression level			
Dependent variable: log COL1A1	Model 1	Model 2	VIF
Adjusted R^2	0.749	0.787	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
TGFB1 (log)	**0.469 (<0.001)** ***	**0.509 (<0.001)** ***	2.526/2.566
NOX4 (log)	**0.364 (0.001)** **	**0.333 (0.001)** **	2.158/2.183
NLRP3 (log)	0.133 (0.214)	0.159 (0.110)	2.463/2.480
Dependent variable: log COL1A1	Model 1	Model 2	VIF
Adjusted R^2	0.754	0.792	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
TGFB1 (log)	**0.467 (<0.001)** ***	**0.510 (<0.001)** ***	2.535/2.583
NOX4 (log)	**0.346 (0.003)** **	**0.296 (0.006)** **	2.660/2.722
NLRP3 (log)	0.097 (0.386)	0.117 (0.259)	2.715/2.725
NEP (log)	0.077 (0.427)	0.104 (0.249)	2.050/2.068
B. Univariate regression analysis of fibrosis-related gene and NEP expression level			
Dependent variable: log TGFB1	Model 1	Model 2	VIF
Adjusted R^2	0.262	0.293	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
NEP (log)	**0.524 (<0.001)*****	**0.495 (<0.001)*****	1.000/1.031
Dependent variable: log NOX4	Model 1	Model 2	VIF
Adjusted R^2	0.452	0.444	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
NEP (log)	**0.679 (<0.001)*****	**0.674 (<0.001)*****	1.000/1.034
Dependent variable: log NLRP3	Model 1	Model 2	
Adjusted R^2	0.403	0.467	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
NEP (log)	**0.643 (<0.001)*****	**0.587 (<0.001)*****	1.000/1.044
C. Multivariate regression analysis of NEP and fibrosis-related gene expression level			
Dependent variable: log NEP	Model 1	Model 2	VIF
Adjusted R^2	0.504	0.483	
Independent variable	β-value (p)	β-value (p)	Model1/Model2
NOX4 (log)	**0.463 (0.001)** **	**0.467 (0.002)** **	1.989/2.082
NLRP3 (log)	**0.317 (0.022)** *	**0.326 (0.036)** *	1.989/2.424

Model 1, multivariate analysis, unadjusted; Model 2, adjusted for age. Data are shown as β-values (p-values). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
Model 1, univariate analysis, unadjusted; Model 2, adjusted for age, sex, and BMI. Data are shown as β-values (p-values). *** $p < 0.001$.
Model 1, univariate analysis, unadjusted; Model 2, adjusted for age, sex, and BMI. Data are shown as β-values (p-values). *** $p < 0.001$.
Model 1, univariate analysis, unadjusted; Model 2, adjusted for age, sex, and BMI. Data are shown as β-values (p-values). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(adjusted $R^2 = 0.444$, $\beta = 0.674$, $p < 0.001$), and log (NLRP3) (adjusted $R^2 = 0.467$, $\beta = 0.587$, $p < 0.001$), respectively (Table 2B and Fig. 6A [indicated by red arrow]). Similar results were observed in other fibrosis-related genes. These results suggest that atrial expression level of NEP is a determinant of the fibrosis-related gene expression level in LA.

Multiple linear regression analysis was performed with log (NEP) expression level as the dependent variable and fibrosis-related gene expression as the independent variable. The univariate analysis showed that log (NOX4) [$\beta = 0.671$, $p < 0.001$] and log (NLRP3) [$\beta = 0.638$, $p < 0.001$] were potent independent variables for predicting log(NEP) expression level (log (ACE) [$\beta = 0.627$, $p < 0.001$], log (NOX2) [$\beta = 0.585$, $p < 0.001$], log (EDN1) [$\beta = 0.581$, $p < 0.001$], log (PDGFD) [$\beta = 0.568$, $p < 0.001$], log (TGFB1) [$\beta = 0.512$, $p < 0.001$], and log (CTGF) [$\beta = 0.438$, $p < 0.001$]), log (IL1B) [$\beta = 0.368$, $p = 0.006$], and log (TNF) [$\beta = 0.428$, $p = 0.002$]), after adjustments for age. In addition, multiple linear regression analysis with log (NEP) expression level as the dependent variable and pro-fibrotic cytokine or substance-related gene expression (TGFB1, EDN1, ACE, PDGFD, and CTGF) as the independent variable was performed. After adjustments for age, multivariate analysis showed that log (EDN1) ($\beta = 0.313$, $p = 0.021$) and log (ACE) ($\beta = 0.361$, $p = 0.021$) were independent variables for predicting log (NEP) expression level (Supplemental Table 2Aa). It also showed that NOX4 ($\beta = 0.524$, $p = 0.001$) among oxidant stress-related genes (NOX2, NOX4) and NLRP3 ($\beta = 0.845$, $p = 0.002$) among inflammation-related genes (IL1B, TNF, NLRP3) were the most potent independent variables to predict log (NEP) expression level (Supplemental Table 2Ab and Ac).

Furthermore, the correlation and partial correlation between NEP mRNA expression level and variable gene expression level were examined, as shown in Supplemental Table 2B. The correlations between NEP and TGFB1, EDN1, ACE, PDGFD (all $p < 0.001$), and CTGF ($p = 0.001$) expression level were statistically significant. In contrast, when NOX4 expression level was used as a control factor, the partial correlation analysis showed that the statistical significance of the correlation between NEP and TFGB1, EDN1, ACE, PDGFD, and CTGF expression level ($p > 0.05$) was abolished, suggesting that TGFB1, EDN1, ACE, PDGFD, and CTGF were indirectly correlated with NEP through NOX4. Therefore, we examined the partial correlation between NOX4, NLRP3, and NEP. It showed that log (NEP) was significantly correlated with log (NOX4) ($r = 0.41$, $p < 0.001$) and log (NLRP4) ($r = 0.39$, $p < 0.001$) but not with log (TGFB1), as shown in Fig. 6B. In addition, multiple linear regression analysis with log (NEP) expression level as the dependent variable and TGFB1, NOX4, and NLRP3 as independent variables was performed, as shown in Table 2C. After adjustments for age, sex, and BMI, multivariate regression analysis found that log (NOX4) ($\beta = 0.467$, $p = 0.002$), and log (NLRP3) ($\beta = 0.326$, $p = 0.036$) were independent variables for predicting log (NEP) expression (adjusted $R^2 = 0.483$, Table 2C and Fig. 6A, indicated by green arrow). These results suggest that the expression level of NOX4 and NLRP3 is a determinant of the expression level of NEP in LA.

4. Discussion

NEP is widely distributed in various organs, including AT and the heart [5,7]. In the present study, RT-PCR analysis, immunohistochemical staining, and western blotting revealed NEP expression in both AT and LA. The expression of NEP in AT was much higher than that in LA. Serum NEP concentration has been reported to increase with BMI, and identified weight loss via a hypocaloric low-fat diet as physiological intervention in humans reduces NEP in plasma and AT [31]. However, in the present study using non-obese cardiovascular surgery patients (BMI 22.6 ± 3.9 kg/m^2), NEP protein level in AT did not significantly correlate with BMI. Furthermore, serum NEP levels did not reflect NEP protein expression level in AT and LA.

Several profibrotic signaling pathways have been implicated in atrial fibrosis and AF [1,2]. TGF-β1 plays a critical role in matrix remodeling and enhancing collagen synthesis, and increased expression of TGF-β1 causes myocardial fibrosis [1,2,18,19]. In addition, the role of oxidative stress in the promotion of structural substrates for AF is well established [1,2,15,17], and the activation of NOX plays a causal role [32,33]. Cardiac ECM primarily consists of type I and III collagen, and type I collagen constitutes approximately 85 % of the total myocardial collagen [34,35]. Therefore, as previously reported [27], we investigated the relationship between COL1A1 / NEP and atrial gene expression by performing multivariate regression analysis. In the present study, multiple regression analysis with COL1A1 expression level as the dependent variable and TGFB1, NOX4, NLRP3, and NEP as independent variables showed that TGFB1 and NOX4, but not NEP, were independent predictors of COL1A1 expression levels. These results suggest that TGFB1 and NOX4 are major determinants of atrial *COL1A1* mRNA expression in patients undergoing cardiovascular surgery. Meanwhile, in our study, although the adjusted R^2 was low, atrial NEP expression level was identified as factors related to fibrosis-related gene expression, such as TGFB1, NOX4, and NLRP3 mRNA levels in the LA. Several mechanisms have been proposed to explain the phenomenon. NEP catalyzes the degradation of antifibrotic peptides, such as ANP, BNP, and bradykinin [13,36], which counteract cardiomyocyte hypertrophy and cardiac fibrosis. Helske et al. [37] showed that NEP activity is increased in stenotic aortic valves and that NEP catalyzes the degradation of BK in parallel with the upregulation of BK-1R to promote valvular fibrosis and remodeling. In addition, treatment of cultured myofibroblasts with an NEP inhibitor downregulated the expression of TGFβ1 mRNA [37]. ANP also directly affects cardiac tissue by inhibiting cardiac hypertrophy and fibrosis [8–10,38]. This is achieved by inhibition of fibroblast proliferation through attenuation of TGFβ1, collagen 1, fibronectin, and tissue inhibitor metalloproteinase 3 (TIMP3) expression [38]. Nakagawa et al. [39] showed that transgenic mice overexpressing NEP in cardiomyocytes exhibited an exaggerated response of hypertrophy and fibrosis in the TAC pressure overload model, probably due to the locally promoted degradation of ANP in the heart. Thus, it is very likely that NEP potentiates atrial fibrosis and remodeling by degrading these antifibrotic peptides (BK, BNP, and ANP), resulting in an increase of the expression of fibrosis-related genes, as observed in the present study.

Inflammation is also an important factor that contributes to AF development [40,41]. Previous studies have revealed that NLRP3 inflammasome, which contributes to formation of caspase-1 to produce the active forms of IL-1β, plays a role as an essential mediator in cardiac remodeling by inducing inflammation and activating the profibrotic pathway [42,43]. NLRP3/IL-1β activation is associated with the formation of cardiac fibrosis [44]. The activity of the NLRP3 inflammasome has also been reported to be elevated in atrial myocytes of patients with paroxysmal and chronic AF [45]. Thus, inhibition of the NLRP3 inflammasome has been implicated as a potential strategy to prevent pathological cardiac remodeling, including cardiac inflammation and fibrosis. Li et al. [43] reported that Sac/Val inhibits cardiac fibrosis, remodeling, and inflammation following pressure unloading surgery after aortic banding, probably by suppressing NLRP3 inflammasome activation. Ishii et al. [46] also reported that Sac/Val suppresses proinflammatory cytokine expression and inflammatory cell infiltration compared to ACEI, contributing to improved cardiac outcomes. We showed that atrial expression level of NEP is a determinant of NLRP3 in LA. Thus, NEP inhibitors may be potential targets for the inhibition of NLRP inflammatory activation. Alternatively, NEP expression may also be dynamically regulated by hypoxia, oxidative stress [47], and inflammation [48]. Helske et al. [37] reported that TNFα induced the expression of NEP in cultured myofibroblasts. In the present study, we showed that NOX4 and NLRP3 are independent variables for predicting atrial NEP expression levels. Several studies have reported that NOX4 and NLRP3 induce various diseases and have upstream and downstream regulatory relationships. In lipopolysaccharide-induced acute kidney injury, dexmedetomidine inhibited NLRP3 activation by regulating the TLR4/NOX4/NF-κB pathway, thereby reducing disease development [45]. The NOX4/NLRP3 inflammasome signaling pathway is also essential for the development of liver fibrosis and is an important target for the regulation of intestinal bacteria [49]. Thus, the NOX4/NLRP3 inflammasome pathway appears to be involved in atrial fibrosis and remodeling, where NEP activation may be involved. However, the further basic studies are required to clarify the molecular mechanisms of NEP and NOX4/NLRP3 inflammasome signaling pathways in atrial fibrosis and AF.

LA remodeling is caused by an altered atrial structure following pressure and volume overload, leading to AF [50]. An increase in atrial pressure and mechanical stretching induces the activation of profibrotic signaling pathways, leading to fibroblast proliferation, collagen synthesis, and fibrosis, contributing to the development of AF [1,51]. In the present study, fibrosis-related gene expression is positively associated with atrial ANP mRNA expression, even in the PAF group, reflecting increased mechanical stretching [52,53]. LA reservoir function is altered secondary to increased left ventricle (LV) filling pressure and LV end-diastolic pressure (LVEDP), with consequent mechanical stress on the LA, leading to reduced reservoir function [54,55]. Several studies have reported that low LASr correlates with the pathophysiology of AS [56]. As AS progresses, chronic increases in LV afterload and LV hypertrophy develop, which causes decreased LV compliance and an increase in LV filling pressure, resulting in LA dilatation, reduced LA compliance, and LA reservoir function [57]. Atrial remodeling in AS may serve as a substrate for AF [58]. We showed that the value of mLASr was 15.6 ± 7.7 % in PAF patients including AS, which was significantly lower than that in the control participants (36.7 ± 3.8 %). Moreover, AF patients had lower mLASr, compared with PAF patients with high mLASr and control participants. Helske et al. [37] showed that NEP activity is increased in stenotic aortic valves and that the NEP protein is localized to valvular endothelial cells and myofibroblasts. Fielitz et al. [59] also described increased myocardial NEP mRNA expression and NEP activity in the LV of patients with severe heart failure, and AS, probably due to increased LV filling pressures and LVEDP. They also showed that NEP was localized in myocytic and non-myocytic areas or cells, with the latter most likely representing fibroblasts. In the present study including AS patients, atrial NEP mRNA expression correlated with fibrosis-related gene expression. In PAF patients with low mLASr, the expression of fibrosis-related genes was significantly increased compared with that in those with high mLASr. The expression of ANP, NEP mRNA, and NEP protein level also significantly increased. In addition, PAF patients with high NEP mRNA expression showed increased fibrosis-related gene expression compared with those with low NEP expression. Thus, in cardiovascular surgery patients, atrial remodeling associated with increased atrial fibrosis-related gene expression including NEP as well as COL1A1 occurs before AF. This is compatible with the PREDICT-AF study showing that atrial remodeling associated with increased gene expression such as COL1A1 occurs before incident AF in patients receiving CABG [60].

In patients with heart failure with reduced ejection fraction (HFrEF), plasma concentrations of NEP were found to be elevated and were a risk

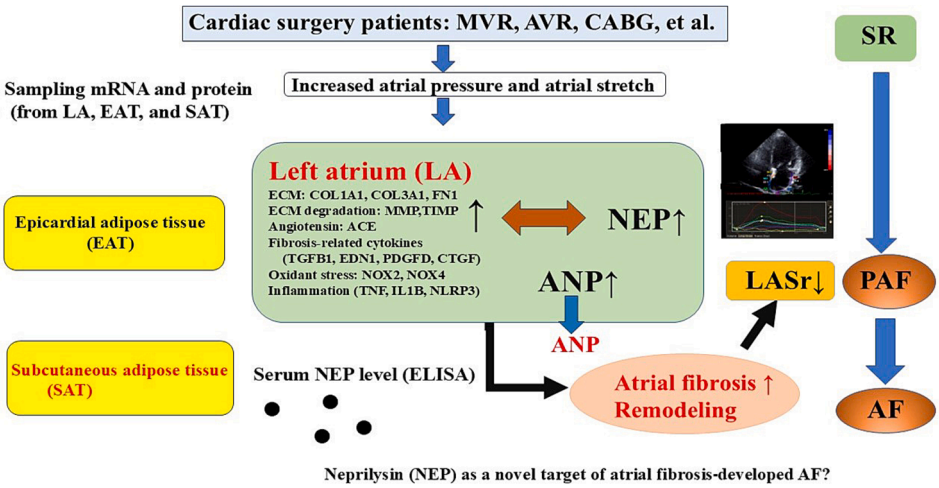

Fig. 7. Graphic abstract of the study.

factor for cardiovascular death and HF hospitalization [7]. A recent study investigated NEP release and expression in myocardial tissue samples from patients with HFrEF and found that plasma NEP levels were higher in the coronary sinus than in samples from the cubital vein [61]. On the other hand, Pavo et al. [62] reported that tissue NEP expression is downregulated and translates into reduced tissue protein concentrations and activity in patients with HFrEF, and therefore, plasma NEP level is not related to tissue NEP activity. In addition, patients with heart failure with preserved ejection function (HFpEF) have lower serum NEP levels than controls [63]. In the present study, serum NEP levels did not correlate with NEP mRNA or protein expression in the AT and LA. The kidneys may play a crucial role in regulating systemic NEP actions, as they have 20 to 100 times higher NEP content and activity than any other organ, including the heart [62]. Thus, neither serum NEP concentrations nor activities appear to reflect heart NEP regulation, and using NEP as a circulating biomarker seems to be inappropriate in patients undergoing cardiovascular surgery, including those with AF.

The present study showed that increased atrial expression of NEP is linked to atrial fibrotic remodeling and the development of AF in cardiovascular surgery patients. Thus, NEP appears to be an early novel target of atrial fibrotic remodeling, and subsequent AF. However, serum NEP levels did not reflect atrial NEP mRNA or protein expression in the LA and AT. Increased mechanical stretching owing to atrial volume and pressure overload induces atrial ANP mRNA expression, which subsequently releases ANP from the LA. Therefore, the concentration of local atrial ANP in the heart with PAF or AF is much higher than that in the peripheral plasma. Taken together, atrial NEP may play an important role in ANP metabolism and, consequently, in atrial fibrosis and remodeling, as illustrated in graphic abstract (Fig. 7). ARNI use showed greater effects on LA reverse remodeling and was associated with a better prognosis than ACEI/ARB use in HFrEF [64]. De Vecchis et al. [65] reported a case in which the SR was restored after ARNI application. Thus, upstream therapy for AF might have an NEP antagonist as the ideal drug because this molecule acts in a balanced way on the electroanatomic remodeling of the atria via the amplification of the beneficial role of the ANP obtained by the inhibition of NEP, which is responsible for its degradation.

This study had several limitations that must be addressed. First, LA appendages were obtained from PAF or AF patients, and no healthy controls for ethical reasons. Furthermore, all patients included in our study underwent cardiac surgery, and many had valve diseases (MR and AS). The pathophysiological mechanisms of AF in lone AF patients may differ. Therefore, our findings are not necessarily applicable to the general population of patients undergoing cardiovascular surgery or to patients with lone AF. Most participants had risk factors and received medical treatment, and the use of drugs, including ACE-1/ARB, might have affected atrial fibrosis in AF. The cardiovascular medications by themselves may also affect NEP expression. And, we could not exclude the interplay between these drugs such as ACE and NEP on atrial fibrosis. Finally, although the pathogenesis of AF is affected by many factors, we examined the expression of several major genes selected, and investigated the relationship between NEP and COL1A1 and atrial gene expression by performing multivariate regression analysis. Therefore, the further studies are required to clarify the detailed molecular mechanisms of NEP on atrial fibrosis, remodeling, and AF.

4. Conclusions

This is the first study to show that increased atrial expression of NEP, possibly involving NOX4 and NLRP3, is linked to atrial fibrotic remodeling, reflecting a decrease in LASr and the development of AF in patients undergoing cardiovascular surgery. However, serum NEP levels do not reflect atrial NEP expression in the LA. Thus, NEP appears to be a novel target of atrial fibrosis, the development of atrial remodeling, and subsequent AF.

CRediT authorship contribution statement

Toshiaki Nakajima: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Formal analysis. **Akiko Haruyama:** Methodology, Data curation. **Taira Fukuda:** Formal analysis. **Kentaro Minami:** Methodology. **Takafumi Nakajima:** Methodology. **Takaaki Hasegawa:** Methodology, Data curation. **Seiko Tokoi:** Methodology, Data curation, Conceptualization. **Syotaro Obi:** Methodology. **Gaku Oguri:** Methodology. **Masashi Sakuma:** Methodology. **Ikuko Shibasaki:** Data curation. **Hirotsugu Fukuda:** Supervision. **Shigeru Toyoda:** Supervision.

Funding

This study was supported in part by JSPS KAKENHI Grant Numbers 19H03981 and 23 K24715 (to T.N.) and the Vehicle Racing Commemorative Foundation (to T.N.). The funding sources for this study had no roles in study design, data collection, analysis, or interpretation. The opinions expressed herein are those of the authors. No additional external funding was received for this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank the cardiovascular surgeons and the staff members who collected the blood samples and atrial tissue samples.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcha.2025.101647.

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