Decreased Nox4 levels in the myocardium of patients with aortic valve stenosis

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Abstract
The NADPH oxidases are a key family of ROS (reactive oxygen species)-producing enzymes which may differentially contribute to cardiac pathophysiology. Animal studies show uncertain results regarding the regulation of cardiac Nox4 by pressure overload and no data are available on human myocardial Nox4. In the present study, we evaluated Nox4 expression and its relationship with myocardial remodelling and LV (left ventricular) function in patients with severe AS (aortic valve stenosis). Endomyocardial biopsies from 34 patients with AS were obtained during aortic valve replacement surgery. LV morphology and function were assessed by echocardiography. Myocardial samples from subjects deceased of non-CVDs (cardiovascular diseases) were analysed as controls. Nox4 localization was evaluated by immunohistochemistry and quantified by Western blot. Myocardial capillary density, fibrosis and cardiomyocyte dimensions and apoptosis were assessed histologically to evaluate myocardial remodelling. Nox4 was present in samples from all subjects and expressed in cardiomyocytes, VSMCs (vascular smooth muscle cells), endothelium and fibroblasts. Nox4 levels were reduced 5-fold in AS patients compared with controls ($P<0.01$). Nox4 levels directly correlated with cardiomyocyte cross-sectional area ($r=0.299, P<0.05$) and diameter ($r=0.406, P<0.05$) and capillary density ($r=0.389, P<0.05$), and inversely with cardiomyocyte apoptosis ($r=-0.316, P<0.05$) in AS patients. In addition, Nox4 levels correlated with echocardiographic parameters (LV ejection fraction: $r=0.353, P<0.05$; midwall fractional shortening: $r=0.355, P<0.05$; deceleration time: $r=-0.345, P<0.05$) in AS patients. Nox4 is expressed in human myocardium and reduced in AS patients suggest a potential role of Nox4 deficiency in the myocardial remodelling present in the human pressure-overloaded heart.

Key words: aortic valve stenosis, myocardial remodelling, NADPH oxidase, Nox4, pressure overload

INTRODUCTION
AS (aortic valve stenosis) is the most common valve disease in developed countries, affecting 2–3% of the population over 65 years, and its prevalence is likely to rise in the future because it increases with advancing age [1]. Chronic pressure overload in AS patients induces a structural remodelling of the LV (left ventricular) myocardium characterized by alterations of cardiomyocytes, the extracellular matrix and the coronary microcirculation, which contributes to LV functional impairment [2,3].

Oxidative stress is implicated in the development of myocardial remodelling [4]. ROS (reactive oxygen species) contribute at the molecular level to alter cardiac structure and function, damaging macromolecules and altering redox-sensitive signalling pathways, thus contributing to myocardial remodelling [4,5]. Although there are multiple cardiac sources of ROS, the NADPH oxidase family of enzymes is the only one whose primary role is ROS generation. In general terms, they consist of a distinct catalytic subunit (Nox1–5, Duox1–2), usually bound to the smaller $p22^{	ext{phox}}$ subunit, and have varying regulation depending upon...
the isoform [6]. Nox4 requires the association of p22phox and is constitutively active [6–8], although its activity is increased by Poldip2 [polymerase (DNA-directed) delta-interacting protein 2] in VSMCs (vascular smooth muscle cells) [9]. Several reports indicate that Nox4 predominantly generates H2O2 [7,8]. In human cells, there is in vitro evidence of Nox4 expression in microvascular endothelial cells [10], cardiac fibroblasts [11] and coronary artery smooth muscle cells [12].

Regarding the modulation of Nox4 expression in experimental cardiac pressure overload, there is no undisputable evidence. Studies in mice subjected to suprarenal constriction show no changes in Nox4 protein levels [13], an increase in Nox4 mRNA levels [14] or an increase in Nox4 protein levels [15]. Models of pressure overload by thoracic aortic constriction report an increase in cardiac Nox4 mRNA [16] or Nox4 protein levels [17].

Currently, there is no information regarding the myocardial localization of Nox4 in humans or of changes of its levels in pressure overload. Therefore the aim of the present study was to evaluate the presence of Nox4 in the myocardium of patients with pressure overload due to AS and assess the associations of Nox4 with myocardial remodelling and L V morphology and function.

MATERIALS AND METHODS

Study population

All patients gave written, informed consent to participate in the present study. The study was carried out in accordance with the Helsinki Declaration and the Ethics Committee of the Virgen de la Victoria University Hospital (Málaga, Spain) approved the protocol.

The study population consisted of 34 patients with clinically diagnosed severe isolated AS, defined in accordance with the following criteria [18]: mean transvalvular pressure gradient ≥40 mmHg, aortic valve area <1 cm² or aortic valve index <0.6 cm²/m². The patients were referred for valve replacement due to the presence of characteristic clinical symptoms (i.e. angina, syncope or heart failure manifestations) and/or LVEF (LV ejection fraction) <50%. Patients with cardiac valve disease other than AS and/or history of acute myocardial infarction were excluded after complete medical examination. Heart failure was clinically diagnosed on the basis of the presence of at least one major and two minor Framingham criteria [19] and was reinforced by either echocardiographic signs of systolic dysfunction (LVEF <50%) or by the presence of elevated levels of the NT-pro-BNP (N-terminal propeptide of brain natriuretic peptide) and alterations in cardiac morphology and function indicative of heart failure with normal ejection fraction according to the European Society of Cardiology [20].

Cardiac samples were collected from 18 subjects who had died from non-cardiovascular-related diseases, nine of which were processed for protein studies [six men and three women, average age 54 (range 46–62) years] and nine processed for histomorphological studies [six men and three women, average age 59 (range 40–68) years], to be used as controls.

Echocardiographic assessment

Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained in each AS patient. LV mass was measured from M-mode recordings according to the American Society of Echocardiography criteria [21], and LVM (LV mass index) was calculated by dividing LV mass by the body surface area. The presence of LV hypertrophy was established when the LVMi was >149 g/m² for men and >122 g/m² for women [22]. The presence of concentric LV hypertrophy was established when relative wall thickness was >0.42 [22]. LV midwall fractional shortening and ejection fraction were recorded. The first sample, for molecular studies, was divided into two pieces, immediately frozen in liquid nitrogen and processed for mRNA and for protein extraction. The second sample was processed for histomorphological and immunohistochemical studies. The biopsies were taken from adjacent areas, so that they were equivalent. The average biopsy size was 21 ± 2 mm³.

Histomorphological and immunohistochemical studies

After formalin fixation, the biopsies were embedded in paraffin and serially sectioned in 4-μm-thick sections. In all the cases, the endogenous peroxidase was inactivated by 30 min incubation in 0.01% H2O2 in methanol. Slides were blocked in 20% (v/v) normal goat serum (Dako) for 30 min. Primary antibodies used were Nox4 (sc-30141 diluted 1:100; SantaCruz Biotechnology), non-commercial Nox4 [14] (diluted 1:100), Poldip2 (ab85364 diluted 1:200; Abcam) and vWF (von Willebrand factor) (A0082 diluted 1:250; Dako). Immunohistochemistry to confirm cell type was performed in serial sections with antibodies against vWF (to detect endothelium), α and β myosin heavy chain (to detect cardiomyocytes) (Ab15 diluted 1:400; Abcam), smooth muscle α-actin (to detect smooth muscle cells) (A5228 diluted 1:400 Sigma) or vimentin (to detect fibroblasts) (sc-6260 diluted 1:500; Santa Cruz Biotechnology). Secondary antibodies were Envision anti-rabbit or anti-mouse, as appropriate, incubated for 30 min. Signal was detected by 3,3′-DAB (diaminobenzidine) (Dako). Slides were counterstained with Harris haematoxylin (Sigma). Negative controls were carried out with primary antibody omission.

Cardiac capillarization was quantified in sections by immunohistochemistry against vWF. Images were captured at ×20 magnification with an Eclipse 80i microscope (Nikon) and the average capillary number was calculated from six fields. Cardiac
capillarization was calculated as the number of capillaries per area (capillaries/mm²) and the capillary-to-cardiomyocyte ratio (by dividing the number of capillaries present in each field by the number of cardiomyocyte nuclei present in each field).

Collagen was identified by Picosirisius Red staining and quantified as described previously [24], which allowed us to calculate the area occupied by collagen [CVF (collagen volume fraction)].

Cell apoptosis was assessed by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) as described previously [25].

Cardiomyocyte mean cross-sectional diameter and area were assessed in Masson's trichrome-stained sections, captured at ×40 magnification, with the AnalySIS 3.1 software (Soft Imaging System).

Quantification of Nox4 immunostaining was carried out by quantitative morphometry with an automated image analysis system (AnalySYS; Soft Imaging System).

Biochemical determinations
Venous blood samples were obtained in each patient from the left antecubital vein. The NT-pro-BNP was measured in plasma samples by an ELISA method (Biomedica Gruppe) [26].

Protein studies
Protein was extracted in lysis buffer [7 mol/l urea, 2 mol/l thiourea, 4% (w/v) CHAPS and 1% (w/v) DTT (dithiothreitol)] and quantified (Pierce BCA Assay Kit; Thermo Scientific). Then, 10 μg of the protein homogenate were mixed with adequate volumes of Laemmli buffer and subjected to SDS/PAGE (10% gel). Gels were blotted on to nitrocellulose membranes (Hybond ECL; GE Healthcare). Membranes were then washed in TBST [Tris-buffered saline with 1% (v/v) Tween 20] for 5 min at room temperature. Afterwards, the blots were incubated with the primary antibody in blocking solution [Nox4: sc-30141 diluted 1:1000 (Santa Cruz Biotechnology); and Poldip2: ab85364 diluted 1:1000 (Abcam)] overnight at 4°C with slow rocking.

The blots were then washed five times for 5 min in TBST and incubated with an HRP (horseradish peroxidase)-conjugated secondary antibody in blocking solution. After washing five times for 5 min in TBST, bands were identified by chemiluminescence with the ECL Advanced kit (GE Healthcare). The membranes were stripped using Restore Western Blot Stripping Buffer (Pierce) according to instructions and rebotted for α-tubulin levels.

The specificity of the Nox4 band was confirmed with a non-commercial antibody against Nox4 (diluted 1:2000) that had been validated using Nox4-transfected cells and Nox4-knockout mice as positive and negative controls respectively [14,27].

RNA studies
Extraction of total RNA was performed with TRIzol® (Invitrogen) according to the manufacturer's instructions. RNA quantity and quality were assessed by absorbance (Ultraspec spectrophotometer). Retrotranscription of 1 μg of total RNA was carried out with random hexamers and Superscript III (Invitrogen). Quantification of the cDNA was performed by Real time PCR with TaqMan probes (4333760F for 18S, Applied Biosystems) and SYBR green technology (Promega) with primers 5′-GGGCTGATGCATGAGCCT-3′ and 5′- CCAAGATGGTGACTTGTGCT-3′ for eNOS (endothelial nitric oxide synthase) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Data are expressed as AU (arbitrary units) relative to 18S ribosomal RNA.

Statistical analysis
Results are expressed as means ± S.E.M. Differences between the groups were evaluated using a Student’s t test for unpaired data or Mann–Whitney U test, as appropriate after evaluating normality (Shapiro–Wilks test). Correlations between continuously

### Table 1 Clinical characteristics of the patients

Results are expressed as means ± S.E.M or percentage of subjects. Vc/Va, maximal early transmitral diastolic velocity/maximal late transmitral diastolic velocity ratio.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>71 ± 1</td>
</tr>
<tr>
<td>Sex (male/female) (n)</td>
<td>10/24</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.2 ± 0.9</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>71</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>35</td>
</tr>
<tr>
<td>Coronary artery disease (%)</td>
<td>35</td>
</tr>
<tr>
<td>Heart failure (%)</td>
<td>56</td>
</tr>
<tr>
<td>Atrial fibrillation (%)</td>
<td>32</td>
</tr>
<tr>
<td>Treatment (%)</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>21</td>
</tr>
<tr>
<td>Diuretics</td>
<td>70</td>
</tr>
<tr>
<td>Digitalis</td>
<td>9</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>18</td>
</tr>
<tr>
<td>AngII type1 receptor antagonists</td>
<td>15</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>36</td>
</tr>
<tr>
<td>Aldosterone antagonists</td>
<td>6</td>
</tr>
<tr>
<td>Ca²⁺ antagonists</td>
<td>18</td>
</tr>
<tr>
<td>Aortic valve area (cm²)</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>Aortic valve area index (cm²/m²)</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>Mean transvalvular pressure gradient (mmHg)</td>
<td>53.45 ± 2.89</td>
</tr>
<tr>
<td>Maximal transvalvular pressure gradient (mmHg)</td>
<td>82.24 ± 4.37</td>
</tr>
<tr>
<td>LVMI (g/m²)</td>
<td>147.7 ± 7.5</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.612 ± 0.025</td>
</tr>
<tr>
<td>Concentric LV hypertrophy (%)</td>
<td>58</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>66.15 ± 2.84</td>
</tr>
<tr>
<td>LV midwall fractional shortening (%)</td>
<td>38.96 ± 1.91</td>
</tr>
<tr>
<td>End-systolic wall stress (kdyn/cm²)</td>
<td>59.66 ± 4.28</td>
</tr>
<tr>
<td>Vc/Va</td>
<td>1.05 ± 0.18</td>
</tr>
<tr>
<td>Isovolumic relaxation time (ms)</td>
<td>97.73 ± 7.37</td>
</tr>
<tr>
<td>Deceleration time (ms)</td>
<td>291.24 ± 20.50</td>
</tr>
<tr>
<td>NT-pro-BNP (fmol/ml)</td>
<td>829 ± 72</td>
</tr>
</tbody>
</table>

Statistical analysis
Results are expressed as means ± S.E.M. Differences between the groups were evaluated using a Student's t test for unpaired data or Mann–Whitney U test, as appropriate after evaluating normality (Shapiro–Wilks test). Correlations between continuously
distributed variables were tested by bivariate association and univariate and multivariate regression analysis. A $P$ value $<0.05$ was considered statistically significant. Analyses were performed with the SPSS 15.0 statistical package.

**RESULTS**

**Characteristics of patients**
The clinical characteristics of patients are summarized in Table 1. Around half of them had clinical heart failure and almost one-third exhibited atrial fibrillation.

**Myocardial remodelling**
Compared with controls, patients had increased cardiomyocyte cross-sectional area ($498.48 \pm 95.91$ compared with $203.09 \pm 17.36 \, \mu m^2; \ P < 0.001$) and diameter ($26.13 \pm 0.44$ compared with $16.61 \pm 0.69 \, \mu m; \ P < 0.001$). The cardiomyocyte apoptotic index was increased in patients compared with controls ($0.101 \pm 0.016$ compared with $0.0045 \pm 0.0016 \% ; \ P < 0.001$). The cardiac capillary density was reduced in patients compared with controls, whether calculated as the capillaries/mm$^2$ ($522 \pm 48$ compared with $1531 \pm 78; \ P < 0.001$) or the capillary-to-cardiomyocyte ratio ($1.18 \pm 0.08$ compared with $2.63 \pm 0.15; \ P < 0.001$). Patients showed greater CVF than controls ($17.33 \pm 1.90 \%$ compared with $1.95 \pm 0.07 \% ; \ P < 0.001$).

**Nox4 and Poldip2 expression**
Detection of Nox4 by immunohistochemistry showed that it is expressed in the human heart, both in controls and AS patients, although with greater stained area in controls ($23.3 \pm 2.2$ compared with $9.5 \pm 2.5 \% ; \ P < 0.01$) (Figure 1). Serial immunohistochemistry with cell-type markers confirmed the expression of Nox4 in cardiomyocytes, VSMCs, endothelial cells and fibroblasts, both in controls and in AS patients (Figure 2). In cardiomyocytes, Nox4 staining was present in the cytoplasm and some nuclei (Figure 3A).

Quantification in cardiac homogenates showed that Nox4 protein levels were 5-fold lower in AS patients than in controls ($0.48 \pm 0.03$ compared with $2.45 \pm 0.50$ AU; $P < 0.01$) (Figure 3B). We observed in patients that Nox4 levels were higher in women than in men ($0.50 \pm 0.04$ compared with $0.34 \pm 0.02$ AU; $P < 0.05$). However, there were no differences in Nox4 levels between patients without ($n = 14$, $0.437 \pm 0.057$ AU) and with ($n = 20$, $0.502 \pm 0.036$ AU) LV hypertrophy, between patients without ($n = 20$, $0.508 \pm 0.046$ AU) and with heart failure ($n = 14$, $0.428 \pm 0.037$ AU), between patients without...
Decreased Nox4 in human aortic stenosis

Figure 2 Co-expression of Nox4 and cell-type markers
Serial immunohistochemical staining of Nox4 and cell-type markers α- and β-myosin heavy chain for cardiomyocyte (×20 magnification), vWF for endothelium (×40 magnification), α-actin for vascular smooth muscle (×40 magnification) and vimentin for fibroblasts (×100 magnification) showed that Nox4 was expressed in these cell types in myocardial samples from (A) controls and (B) patients with AS (n = 23, 0.482 ± 0.045 AU) and with (n = 11, 0.461 ± 0.030 AU) arrhythmias, between patients without (n = 22, 0.459 ± 0.037 AU) and with (n = 12, 0.505 ± 0.061 AU) coronary artery disease, between patients without (n = 10, 0.457 ± 0.055 AU) and with (n = 24, 0.483 ± 0.039 AU) hypertension, or between patients without (n = 22, 0.471 ± 0.039 AU) and with (n = 12, 0.482 ± 0.057 AU) diabetes. There were no differences in Nox4 levels between patients taking medications interfering with the renin–angiotensin–aldosterone system (n = 13, 0.498 ± 0.041 AU) and those who were not taking such medication (n = 20, 0.462 ± 0.047 AU), or between patients under statin treatment (n = 21, 0.451 ± 0.031 AU) and those who were not (n = 12, 0.521 ± 0.072 AU).

In addition, we assessed Poldip2, a regulator of Nox4 activity [9]. Immunohistochemical studies showed that it was expressed in the human heart, in the cytoplasm and nucleus of cardiomyocytes (Figure 3C). Quantification in homogenates showed that Poldip2 expression was 8-fold lower in AS patients than in controls (0.29 ± 0.02 compared with 2.33 ± 0.51 AU; P < 0.01) (Figure 3D).

Association studies
Nox4 levels directly correlated with the cardiomyocyte cross-sectional area (Figure 4A) and diameter (Figure 4B) in AS patients. Furthermore, Nox4 levels correlated inversely with the apoptotic index in AS patients (Figure 4C). There was a direct correlation between Nox4 levels and the degree of capillary density expressed as the capillary-to-cardiomyocyte ratio (Figure 4D) in AS patients. These associations were independent of age, sex and the severity of AS (transvalvular gradient and valve area). Nox4 levels did not correlate with the CVF (r = −0.181, P = not significant).

It has been proposed that Nox4 may regulate eNOS expression and contribute to vessel generation [28,29]. We observed in AS patients that Nox4 levels directly correlated with the mRNA levels of eNOS (r = 0.402, P < 0.05).
**Figure 3** Localization of Nox4 and Poldip2 within the myocardium

(A) Immunohistochemistry of Nox4 in patients with AS. Nox4 was detected mainly in cardiomyocyte cytoplasm and some nuclei (arrows) (left, ×10 magnification; right, ×40 magnification). (B) Representative Western blot images and histograms quantifying Nox4 in homogenates. Nox4 expression was lower in patients with AS than in controls. (C) Immunohistochemistry of Poldip2 in patients with AS. Poldip2 was detected in cardiomyocyte cytoplasm, by the plasma membrane (dotted arrows) and some nuclei (closed arrows) (left, ×10 magnification; right, ×40 magnification). (D) Representative Western blot images and histograms quantifying Poldip2 in homogenates. Poldip2 expression was lower in patients with AS than in controls. *P<0.01.

Nox4 levels correlated with echocardiographic parameters in AS patients. Nox4 levels directly correlated with the LVEF (Figure 5A) and the LV midwall fractional shortening (Figure 5B) and inversely with the deceleration time (Figure 5C). These associations were independent of age, sex and the severity of AS (transvalvular gradient and valve area).

Poldip2 levels did not correlate with Nox4 levels, parameters of myocardial remodelling or parameters of cardiac function.

**DISCUSSION**

The main novel findings of our study are the following: (i) Nox4 is expressed in human cardiac cells; (ii) Nox4 levels are abnormally decreased in patients with severe AS; (iii) Nox4 levels are associated with alterations in parameters assessing myocardial structure in AS patients; and (iv) Nox4 levels are associated with parameters of LV systolic and diastolic function in these patients. Taken together, these results suggest that Nox4 deficiency may contribute to the remodelling of the myocardium present in human cardiac pressure overload.

In the present study, we provide the first data on the expression of Nox4 in the human myocardium. Regarding its localization, we detected Nox4 expression in cardiomyocytes, as well as in VSMCs, endothelial cells and fibroblasts, in both controls and AS patients. These data expand previous observations in animal studies showing Nox4 expression in cardiac cells [30]. Moreover, we observed Nox4 in both the cytoplasm and the nucleus of cardiomyocytes, supporting data from other studies identifying Nox4 in the nuclei [31] and cellular organelles (i.e. ER (endoplasmic reticulum [30,32] and mitochondria [17,30]).

Currently, there is conflicting evidence on the myocardial changes of Nox4 in experimental pressure overload, with studies showing no changes in Nox4 protein levels [13], an increase in Nox4 mRNA levels [14,16], or an increase in Nox4 protein levels [15,17]. Surprisingly, our data indicate that patients with pressure overload secondary to AS present a marked reduction of myocardial Nox4 levels compared with controls. Nox4 seems to be modulated by a number of factors that may also be of relevance for myocardial remodelling in the setting of pressure overload, for instance, AngII (angiotensin II) [30,33]. Although some authors have shown that AngII administration decreases Nox4 expression in VSMCs [34], others failed to show any effect [35], and yet others reported increased Nox4 expression in VSMCs [36] and endothelial cells [37]. In this regard, no relationship was found between Nox4 expression and the treatment with drugs interfering with the renin–angiotensin–aldosterone system in the patients from the present study. In addition, down-regulation of Nox4 expression in the vasculature has been reported following...
Decreased Nox4 in human aortic stenosis

Figure 4 Correlations of Nox4 with parameters assessing myocardial remodelling

Myocardial Nox4 expression correlated with (A) the cardiomyocyte cross-sectional area ($y = 103.6x + 434.59$), (B) the cardiomyocyte cross-sectional diameter ($y = 6.6682x + 23.335$), (C) the cardiomyocyte apoptotic index ($y = -0.0988x + 0.1252$) and (D) the capillary-to-cardiomyocyte ratio ($y = 0.9479x + 0.7299$) in patients with AS.

pathophysiological shear stress [38,39], although the potential contribution of this factor to reduced Nox4 expression in the myocardium of AS patients remains to be investigated.

Nox4 seems to be constitutively active [7] and thus Nox4 levels may be a good indicator of the Nox4-dependent NADPH oxidase activity. In addition, we also evaluated the levels of Poldip2, an enhancer of Nox4 activity [9]. We identified for the first time that Poldip2 is expressed in human myocardium and that Poldip2 levels were lower in AS patients than in controls, thus suggesting that besides reduced Nox4 availability, the activity of the enzyme dependent on this factor may also be reduced in the myocardium of AS patients.

Several findings reported in the present study deal with the potential consequences of Nox4 deficiency. On the one hand, we find an association between reduced Nox4 levels and decreased capillarization in patients with AS. This mirrors the data from Zhang et al. [14] showing that cardiac Nox4 overexpression is associated with increased capillary density in mice subjected to pressure overload. Craigie et al. [28] demonstrated that an augmented endothelial Nox4 expression promotes angiogenesis in an eNOS-dependent manner, which was recently corroborated by Schröder et al. [29] in a study on the role of endogenous Nox4 during ischaemic or AngII-induced stress. In this regard, we have detected in AS patients an association between reduced Nox4 levels and decreased eNOS mRNA expression. Thus, it can be hypothesized that Nox4 deficiency contributes to diminished capillarization via reduced eNOS availability among other mechanisms in the myocardium of AS patients. On the other hand, we detect an association between reduced Nox4 levels and reduced cardiomyocyte hypertrophy and increased cardiomyocyte apoptosis. It is known that H$_2$O$_2$ may determine cardiomyocyte fate, either death (apoptosis) or survival (hypertrophy), in a dose-dependent manner [40–42], with the involvement of Nrf2 (nuclear factor-erythroid 2-related factor 2) and antioxidants [41,43]. Interestingly, Brewer et al. [44] in cardiomyocytes and Schröder et al. [29] in blood vessels, showed that Nox4 contributes to the antioxidant defences via the Nrf2 transcription factor, supporting a beneficial role of Nox4. In this conceptual framework, it can be suggested that reduced Nox4 may contribute to the cardiomyocyte alterations seen in AS patients.

Some considerations suggest that the association of Nox4 with myocardial remodelling reported in the present study may be of potential pathophysiological relevance. First, there is evidence of reduced capillarization in AS [45] and of its negative impact on cardiac function [46,47]. Similarly, there is evidence of increased apoptosis in AS, which is associated with the preoperative functional class and valve-replacement postoperative outcome [48]. These findings fit well with our results showing that Nox4 levels are associated with parameters of LV systolic and diastolic function, suggesting that reduced Nox4 levels may contribute to the functional alterations of the left ventricle in AS patients.

Several limitations of our study must be acknowledged. First, this is a clinical study involving relatively small amounts of tissue obtained from patients, making it difficult to undertake an extensive range of analyses as would be possible in an animal-based study. Secondly, we studied AS patients at the time of...
Myocardial Nox4 expression correlated with (A) ejection fraction ($y = 31.769x + 51.056$), (B) midwall fractional shortening ($y = 20.499x + 29.079$) and (C) deceleration time ($y = -1.5546x + 3.5834$) in patients with AS.

valve replacement, hence we lack information regarding the progression of the disease. Thus, although novel and valuable, the information provided in the present study regarding Nox4 expression is rather a snapshot of the late state of the valve disease. In addition, the study involved a relatively small number of patients, but because of the nature of the aims of the study, the design was appropriate. Moreover, we are aware that patients were under treatment, which may influence the results, although Nox4 levels did not seem to be influenced by therapy. Finally, as there was no material available for mRNA studies in controls, we could not evaluate if the decrease in Nox4 levels in AS patients was due to decreased Nox4 transcription.

**CLINICAL PERSPECTIVES**

- NADPH oxidases are a key family of ROS-producing enzymes, but there is currently no information regarding the presence of Nox4 in the myocardium of patients with pressure overload due to aortic valve stenosis (AS) or changes in its levels during pressure overload.
- In the present study, we provide novel information on cardiac Nox4 in humans. In particular, our findings suggest that a decrease in Nox4 levels is associated with myocardial remodelling in patients with severe AS.
- Although descriptive in nature, these findings set the stage for future investigations to assess the role of Nox4 in the development and progression of myocardial remodelling in human cardiac pressure overload, as well as to explore its modulation as a potential therapeutic target to prevent myocardial remodelling.

**AUTHOR CONTRIBUTION**

María Moreno, Javier Díez, Guillermo Zalba conceived, designed, analysed and interpreted the data, drafted and revised the paper critically for important intellectual content, and gave final approval. Idoia Gallego, Begoña López and Arantxa González conceived, designed, analysed and interpreted the data, and revised the paper critically for important intellectual content and gave final approval. Ana Fortuño, Gorka San José, Félix Valencia, Juan Gómez-Doblas, Eduardo de Teresa and Ajay Shah revised the paper critically for important intellectual content and gave final approval.

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