

## Enhanced accumulation of the isoprostane, 8-epi-PGF<sub>2α</sub>, in human aortic and pulmonary valves of patients with coronary heart disease

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**Summary.** The aim of the present study was to investigate whether the isoprostane 8-epi-PGF<sub>2α</sub> differently accumulates in semilunar valves of patients suffering from coronary heart disease (CHD, n=19) as compared to valves from healthy heart donors (controls, n=6). Sections from isolated aortic and pulmonary valves were analyzed by semiquantitative immunohistochemistry. The 8-epi-PGF<sub>2α</sub>-content was determined by using a specific radioimmunoassay. The accumulation of 8-epi-PGF<sub>2α</sub> in both valves was higher in CHD-patients in comparison to controls (Aortic valves: 36.49±11.26 % vs. 15.78±3.04 %; pulmonary valves: 46.79±9.80 % vs. 14.99±3.57 %). The results from the radioimmunoassay revealed comparable findings in both groups (CHD vs. controls: 395.95±86.09 vs. 139.50±47.46 pg/mg protein in the aortic valves and 430.47±76.30 vs. 147.33±53.84 pg/mg protein in pulmonary valves). Pulmonary valves seem to be more susceptible to oxidative stress than aortic valves as evidenced by a higher accumulation of 8-epi-PGF<sub>2α</sub> in CHD patients. Considering the data presented in this study, we suggest that 8-epi-PGF<sub>2α</sub> is a valuable indicator of oxidative injury in human semilunar valves.

**Key words:** Atherosclerosis, isoprostane, semilunar valves, oxidative stress, coronary heart disease

### Introduction

Since the early 90s a large body of evidence has been accumulating showing that isoprostanes, products of free-radical-mediated lipid peroxidation, are involved in the initiation and progression of atherosclerosis (Morrow et al., 1990; Patrono and Fitzgerald, 1997).

One of the most prominent isoprostanes so far is 8-epi-PGF<sub>2α</sub> (Wang et al., 1995). The determination of this isoprostane at the tissue level provides an interesting parameter for both pathophysiological understanding and eventual diagnostic assessment of oxidation injury. Previously, it was demonstrated that 8-epi-PGF<sub>2α</sub> can be found in human atherosclerotic lesions (Gniwotta et al., 1997; Pratico et al., 1997). In addition, our group localized this isoprostane in coronary arteries from patients with coronary heart disease (CHD) and to a lesser extent also in vessels derived from patients with dilated cardiomyopathy (CMP) and control patients (Mehrabi et al., 1999).

Oxidation injury of human heart valves has been poorly investigated in the past (Walton et al., 1970; Mehrabi et al., 2000). Previously, it was reported that during early atherogenesis in hypercholesterolemic animals, the heart valves accumulated large amounts of lipid in the increased extracellular matrix (Zahor and Czabanova, 1977; Thurbrigar et al., 1985). Similar findings were described in human heart valves (Walton et al., 1970; Kim and Huang, 1971). It was also demonstrated that the atheromatous lesions are predominant in those valvular areas exposed to low shear stress and high hydrostatic pressure (Mehrabi et al., 2000). Furthermore, ultrastructural studies using thin-sections and freeze-etching in isolated rabbit atrioventricular and aortic valves have provided a visualization of LDL accumulation in the valvular subendothelial space (Nivelstein-Post et al., 1994). In addition, it was demonstrated that valvular LDL accumulation could result in a deformation of the leaflets, thus altering their normal function (Simionescu et al., 1986; Filip et al., 1987). Previously, our group demonstrated an increased accumulation of ox-LDL in the aortic and pulmonary valves of patients with coronary heart disease. In this study, the valvular ox-LDL accumulation was significantly increased in CHD patients as compared to healthy individuals. In addition, more ox-LDL was accumulated in the pulmonary valves

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than in the aortic valves (Mehrabi et al., 2000).

In considering these previous investigations, we wondered whether 8-epi-PGF<sub>2α</sub> enrichment might also be more prevalent in those human semilunar valves which had previously been shown to positively accumulate ox-LDL. Consequently, the aim of the present study was to investigate whether 8-epi-PGF<sub>2α</sub> is also present in human aortic and pulmonary valves. For this purpose, we studied the accumulation of 8-epi-PGF<sub>2α</sub> by semiquantitative immunohistochemistry and radioimmunoassay in human semilunar valves derived from explanted hearts of patients suffering from CHD and from healthy heart donors. In addition, the serum values of this isoprostane were determined in CHD patients.

## Materials and methods

### Patients' characteristics

Patients' characteristics are depicted in Table 1. All patients underwent right and left heart catheterization before heart transplantation including a complete hemodynamic evaluation (Table 1). Furthermore, aortic stenosis was excluded by echocardiography. Chronic heart failure (CHF) was defined as left ventricular ejection fraction <30% and NYHA class IV. Based on a clinical history of myocardial infarction and coronary angiograms, the etiology of CHF was determined as due to CHD. Four CHD patients had a thickening of the aortic valve with mild regurgitation but no gradient, while the remaining patients showed a functionally normal aortic valve in the echocardiography. Similarly, the pulmonary valves were considered normal in all patients. Echocardiograms of controls revealed normal findings.

### Tissue isolation

Semilunar valves were isolated from patients with ischemic (n=19, CHD) cardiomyopathy. Control semilunar valves were harvested from heart donors (n=6), who had been primarily intended for transplantation, but were deferred because of circumstances making the organs not suitable for transplantation, e.g. left bundle branch block or subepicardial bleeding.

### Sample processing

Explanted hearts were immediately immersed into cardioplegic solution containing 1 mM/L EDTA and 50 μmol/L butylated hydroxytoluene at 4 °C to prevent ex vivo formation of 8-epi-PGF<sub>2α</sub>. Tissue harvest was performed three to six hours later. Because all tissues were harvested following the same protocol, the potential of ex-vivo 8-epi-PGF<sub>2α</sub> formation was not likely to interfere with the data. During isolation, the valve leaflets were kept attached to the valve ring, which

mainly consisted of connective tissue. Samples were fixed immediately after removal in formaldehyde (in phosphate buffered saline, pH 7.2).

### Immunoperoxidase staining

Tissue embedded in paraffin wax was cut in 3-5 μm-thick sections, dried at 55 °C for 2 hours and then deparaffinized in xylene for 20 minutes followed by dehydration through graded alcohols. The endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol. Tissue proteolysis was performed by treatment with 0.1% protease (protease XIV, EC 3.4.24.31, Sigma, Vienna, Austria) in 0.05 M Tris-HCl, pH 7.6. After washing in Tris-buffered saline (0.15 M sodium chloride, 0.05 M Tris-hydrochloric acid, pH 7.6), sections were incubated with polyclonal rabbit antibody to 8-epi-PGF<sub>2α</sub> diluted 1:400 (Assay Designs, Inc. Ann Arbor, MI, USA, approx. 18h incubation time at 4 °C), or monoclonal antibody to CD68 diluted 1:100 (Dakopatts a/s, Glostrup, Denmark, 10 μg/ml), followed by the addition of biotinylated goat-anti-rabbit antibody (Dako, Glostrup, Denmark; 15 min, RT). After repeated washes with Tris-HCl buffer (pH 7.4), sections were incubated with streptABComplex/HRP (Dakopatts a/s, Glostrup, Denmark, 15 min, RT). The red reaction product was developed using aminoethylcarbazole (DAKO). Counterstaining was performed with hematoxylin. Sections were cover-slipped in Aquatex

**Table 1.** Patients' characteristics.

PARAMETERS	DIAGNOSTIC CATEGORY	
	CHD (n = 19)	Controls (n = 6)
Age (years)	60.2±7.17	51.3±15.5
Sex(male/female)	18/1	4/2
Hypertension# (y/n)	12/7	0/6
Diabetes (y/n)	9/10	0/6
Smoker (y/n)	15/4	4/2
Cholesterol (mg%)	266.5±64.3	209.0±38.2
Triglycerides (mg%)	202.0±55.2	140.0±44.1
NYHA (1-4) classification	3.5±0.6	Not applicable
Condition of AV	Thickened (n=4)	Normal
LVEF (%)	16.9±6.5	Not done
CO (L/min)	4.1±1.4	Not done
PAMP (mmHg)	36.4±5.6	Not done
PCWP (mmHg)	25.0±5.8	Not done
RVEDP (mmHg)	14.6±6.3	Not done
PVR (dynes.cm.sec-1)	248±104	Not done

#: patients who had a systolic or diastolic blood pressure above 140/90 mmHg or had an antihypertensive medication were regarded as hypertensives. Hemodynamic data were measured during right and left heart catheterization. Values are presented as means ± SD. No statistical differences were observed for any value within the groups. LVEF: left ventricular ejection fraction; CO: cardiac output; PAMP: pulmonary arterial mean pressure; PCWP: pulmonary capillary wedge pressure; RVEDP: right ventricular end diastolic pressure; PVR: pulmonary vascular resistance.

## Isoprostane accumulation in heart valves

(Merck, Darmstadt, Germany). To exclude unspecific binding, IgG-control tissues were treated overnight at 4 °C with polyclonal rabbit anti-mouse interleukin-3 (Genzyme, Cambridge, UK), followed by the addition of biotinylated goat-anti-rabbit antibody (Dako, Glostrup, Denmark) for 15 minutes at room temperature to stop the reaction. Cross reactivities of the 8-epi-PGF<sub>2α</sub> antibody with other prostaglandins including, PGE<sub>2</sub>, 6-oxo-PGF<sub>1α</sub> did not contribute significantly to the 8-epi-PGF<sub>2α</sub> immunohistochemical staining as described previously (Mehrabi et al., 1999).

### Computer-assisted quantitative histological evaluation of the specimens

All specimens were digitized in their full size utilizing a slide scanner (Nikon 6.0 35 mm film scanner, LS-20, Nikon Corporation, Tokyo, Japan). Images were processed and the color contrast enhanced using the Adobe Photoshop 3.0 software package (Adobe Systems Inc., San Jose, CA). Planimetric analysis of the area positively staining with anti-8-epi-PGF<sub>2α</sub> in percent (%) of total specimen area was performed using computer-based planimetry (NIH Image 1.61/ppc, Bethesda, Maryland, Table 1). In addition, staining intensity was analyzed using a score, ranging from 0-4 estimated units (eU) as follows: (0= no immunoreactivity, 1= minimal staining, 2= light staining, 3= intense staining and 4= very intense staining). At least three sections from the aortic and pulmonary valves were analyzed from each patient. The mean values of these determinations were used for statistical calculations.

Masson-Goldner stains were utilized to assess general specimen histology. Using this technique erythrocytes stain yellow, fibrin red, collagen green and elastic fibers dark-blue.

### Radioimmunoassay of 8-epi-PGF<sub>2α</sub>

The amount of the isoprostane 8-epi-PGF<sub>2α</sub> was determined in the semilunar valves in both groups by RIA. For this purpose, freshly isolated samples were rinsed with ice-cold buffer and then immediately placed into an isotonic NaCl solution containing 1 mM EDTA and 50 μmol/L butylated hydroxytoluene at 4 °C to prevent *ex vivo* formation of 8-epi-PGF<sub>2α</sub>. Afterwards, the samples were homogenized by Ultraturax and then stored at -70 °C. Addition of EDTA in combination with antioxidative substances and storage at -70 °C were done to prevent *ex-vivo* formation of 8-epi-PGF<sub>2α</sub>. Total lipids were extracted with an ice-cold Folch solution, chloroform/methanol (2:1, vol/vol). Afterwards, 8-epi-PGF<sub>2α</sub> was liberated in the organic phase after evaporation under N<sub>2</sub> and hydrolysis with KOH. Subsequently, the RIA procedure was performed according to Wang et al. (1995). The detection limit of the RIA was 2 pg/mg wet weight. The interassay variability was 8.3±2.4 % while the intraassay variability amounted to 5.0±2.3%.

### ELISA

Blood was withdrawn from CHD patients shortly before orthotopic heart transplantation, centrifuged immediately and finally serum was frozen at -70 °C. Serum concentrations of 8-epi-PGF<sub>2α</sub> were determined using an enzyme linked immunosorbent assay (ELISA).

### Statistical analysis and data presentation

Values are presented as mean ± SD. Analysis of variance (ANOVA) with the Scheffé procedure as a post-hoc test was used to compare the means.

## Results

### Anti-8-epi-PGF<sub>2α</sub> -staining

Strong positive immunoreactivity for 8-epi-PGF<sub>2α</sub> was detected around atherosclerotic plaques (Fig. 1A, B). This colocalized with immunostaining for an antibody direct against the specific monocyte/macrophage epitope CD68 (Fig. 1G,H) suggesting that areas positive for 8-epi-PGF<sub>2α</sub> also contained macrophages. The Masson-Goldner stains clearly showed degenerative processes in those areas of the valvular tissues where inflammatory processes took place (Fig. 1D,E).

The percentage of the areas showing 8-epi-PGF<sub>2α</sub> enrichment in the aortic valves was higher in the CHD group (36.49±11.26 %) than in controls (15.78±3.04 %). The differences between both groups were significant (p < 0.008, Fig. 2A). Accumulation of 8-epi-PGF<sub>2α</sub> in pulmonary valves was highest in CHD- (46.73±9.80%) as compared to control valves (14.99±3.57%). Again, the differences between both groups were significant (p < 0.001, Fig. 2A).

The staining-intensity values in the aortic valves were also significantly higher (p < 0.03) in CHD patients (2.24±0.79 eU) than in the control group (0.67±0.26 eU, Fig. 2B). The differences in the intensity values between both groups revealed similar findings in the pulmonary valves (CHD vs. controls: 2.61±0.70 vs. 0.83 ±0.41 eU, p < 0.001).

Furthermore, accumulation and intensity of 8-epi-PGF<sub>2α</sub> in the pulmonary valves of CHD patients were considerably higher than in the aortic valves (Fig. 2A,B).

### RIA of 8-epi-PGF<sub>2α</sub>

Determining 8-epi-PGF<sub>2α</sub> radioimmunologically in extracts of the respective valves, the highest values were found in valves from CHD patients (395.95±86.09 pg/mg protein in the aortic valves and 430.47±76.30 pg/mg protein in pulmonary valves).

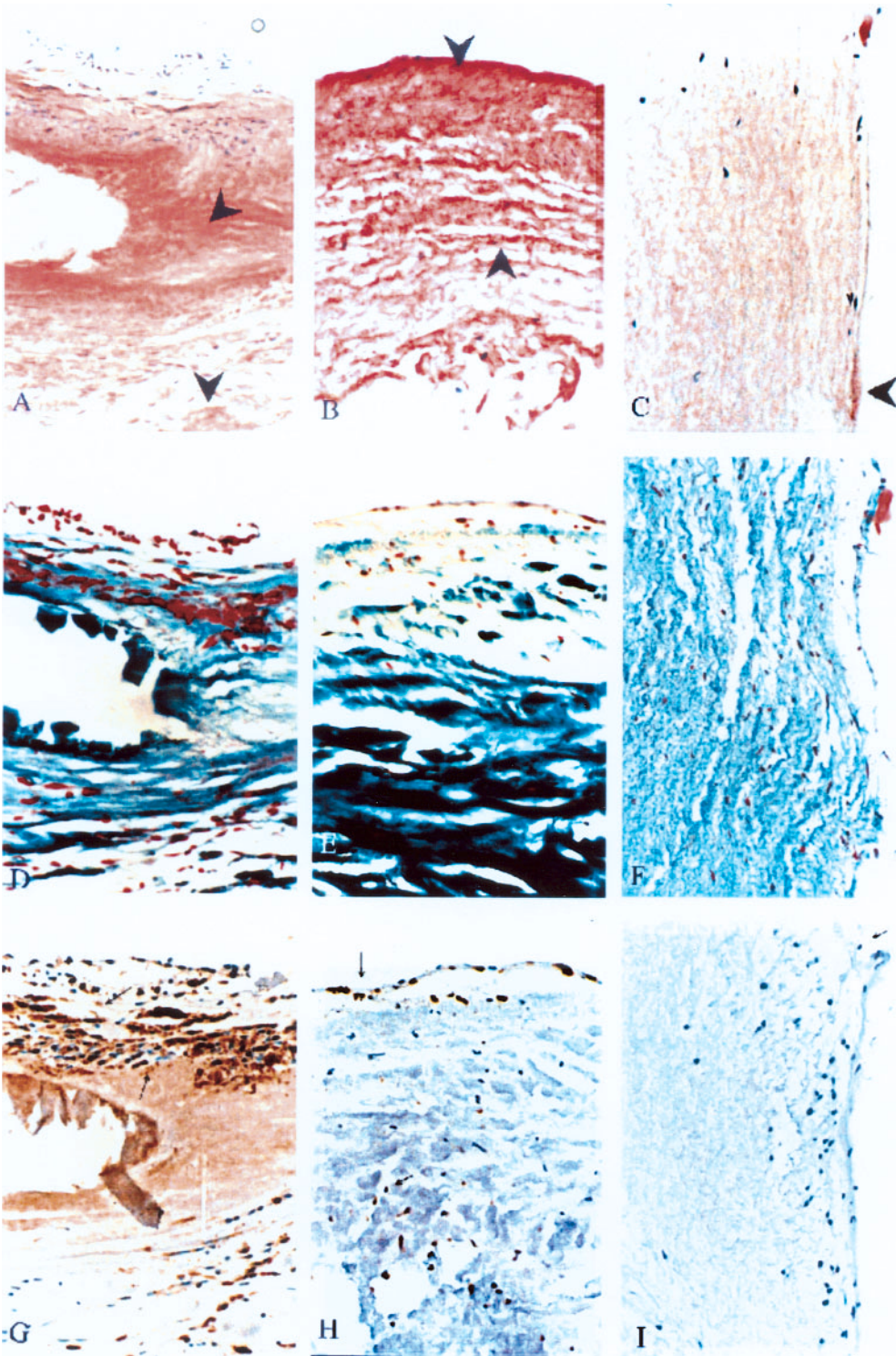
In controls, significantly less 8-epi-PGF<sub>2α</sub> was detected in the aortic valves (139.50±47.46 pg/mg protein, p < 0.006) and also in the pulmonary valves (147.33±53.84 pg/mg protein, p < 0.001) (Fig. 2C).



### ELISA of 8-epi-PGF<sub>2α</sub>

CHD patients had high serum concentrations of 8-epi-PGF<sub>2α</sub> (550.21±168.87 pg/ml) when compared with

reference values of healthy individuals (150-250 pg/ml), reported in a previous manuscript, where the same method was applied (Oguogho et al., 1999).



**Fig. 1.** Immunohistochemical detection of 8-epi-PGF<sub>2α</sub> in an aortic valve (**panel A**) and pulmonary valve (**panel B**) of a representative patient with coronary heart disease as compared to a pulmonary valve derived from a control subject (**panel C**). Red colored deposits resulting from 8-epi-PGF<sub>2α</sub> accumulation represent positive immunoreactivity (arrowheads). **Panels D** (CHD aortic valve), **E** (CHD, pulmonary valve) and **F** (control, pulmonary valve) show parallel sections stained with a modified trichrome stain where green represents collagen and red delineates smooth muscle cells. **Panels G** (CHD aortic valve), **H** (CHD, pulmonary valve) and **I** (control pulmonary valve) are parallel sections stained with CD68 for the visualization of monocytes and macrophages (arrow). Note the colocalization of 8-epi-PGF<sub>2α</sub> staining and macrophages. x 200

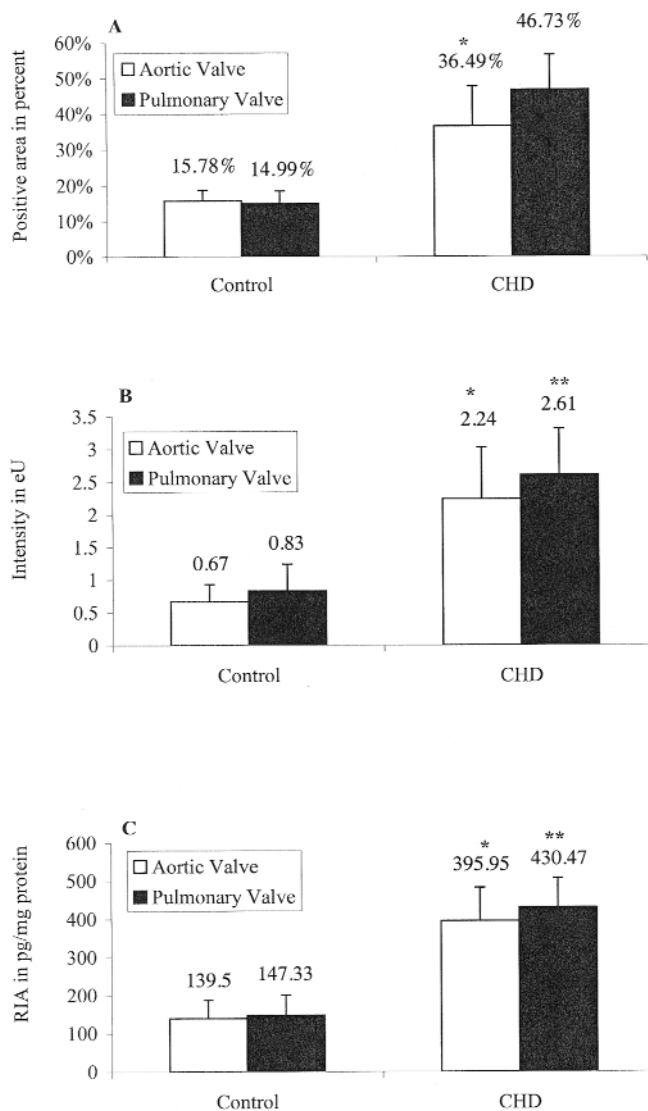
## Isoprostane accumulation in heart valves

### Correlation between the analytical methods

Our results indicate a significant correlation between the radioimmunoassay vs. the immunohistochemical analysis in both valves (Fig. 3).

### Discussion

Isoprostanes are a novel family of bioactive

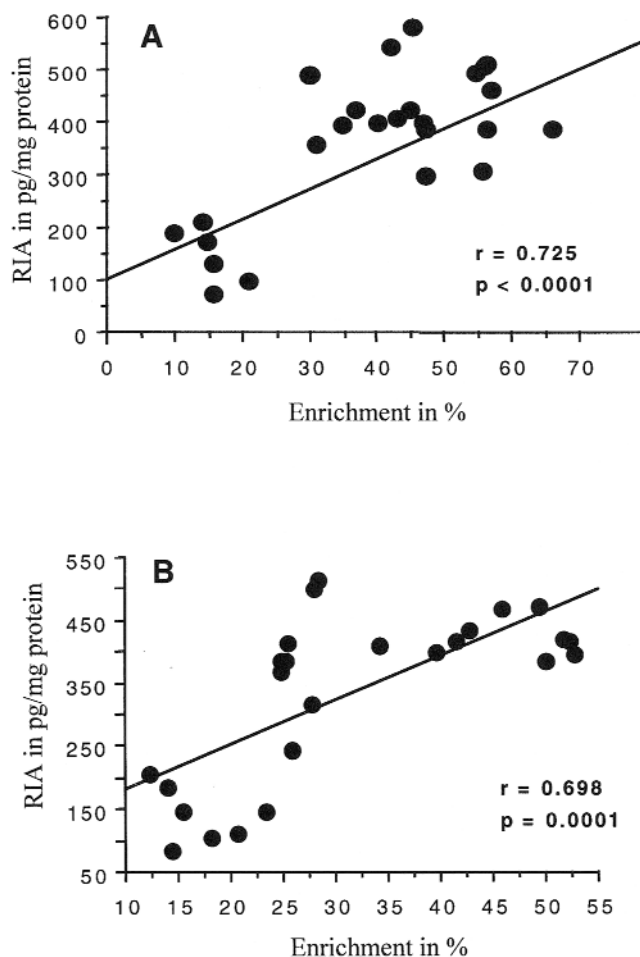


**Fig. 2.** Enrichment, intensity and concentration of 8-epi-PGF<sub>2α</sub> in the aortic and pulmonary valves from CHD patients compared to controls. **A.** The percent positive areas of 8-epi-PGF<sub>2α</sub> in the aortic and in the pulmonary valves from control and CHD patients. Data are presented as mean ± SD. \* p<0.008, \*\* p<0.001. **B.** The intensity of 8-epi-PGF<sub>2α</sub> in the aortic and in the pulmonary valves from control and CHD patients. Data are presented as mean ± SD. \* p<0.03, \*\* p<0.001. **C.** Radioimmunological determination of 8-epi-PGF<sub>2α</sub> in semilunar valves from control and CHD patients. Values are presented as mean ± SD. \* p<0.006, \*\* p<0.001.

prostaglandin-like compounds quickly gaining relevance in the understanding of atherosclerosis (Liu et al., 1999). They are generated in vivo independent of the cyclooxygenase as products of free-radical-catalyzed peroxidation of LDL and subsequently cleared in blood and urine (Wang et al., 1995).

One of the most prominent isoprostanes, namely 8-epi-PGF<sub>2α</sub>, exerts proliferative and vasoconstrictory activity and moreover, makes it an interesting parameter for both pathophysiological understanding and eventual diagnostic assessment of oxidation injury in tissue extracts, serum and/or urine (Liu et al., 1999). Previously, other groups also demonstrated that the isoprostanes 8-epi-PGF<sub>2α</sub> and IPF<sub>2α</sub>-I are present in human atherosclerotic lesions and may therefore contribute to the pathogenesis of atherosclerosis (Gniwotta et al., 1997; Pratico et al., 1997; Mehrabi et al., 1999).

The oxidation injury of human heart valves has been poorly investigated in the past. Our previous



**Fig. 3.** Linear regression analysis between RIA vs. immunohistochemical results in the pulmonary (A) and aortic valves (B) of both study groups.

investigations clearly revealed the cytotoxic effect of the oxidative stress marker ox-LDL in human aortic and pulmonary valves reflecting significant relevance to the atherosclerotic process in patients suffering from coronary heart disease (Mehrabi et al., 2000). The present study extends these observations by demonstrating the presence of the isoprostane 8-epi-PGF<sub>2α</sub> in human semilunar valves in which oxidative modification of ox-LDL occurs.

Human semilunar valves are composed chiefly of an extracellular matrix surrounded by an endothelial cell monolayer and a few scattered cells, which are known to have characteristics intermediary between fibroblasts and smooth muscle cells. In diet-induced hyperlipidemia they become loaded with large lipid droplets (Zahor and Czabanova, 1977; Filip et al., 1987; Nievelstein-Post et al., 1994) as smooth muscle cells of the arterial wall, providing an excellent model of the atherosclerotic alterations of the vascular intima (Hughes et al., 1994).

Our findings clearly demonstrate the accumulation of 8-epi-PGF<sub>2α</sub> in human cardiac valves of patients with coronary atherosclerosis. Strong positive immunoreactivity for 8-epi-PGF<sub>2α</sub> was detected in and around atherosclerotic plaques. This colocalized with immunostaining for an antibody directed against the specific monocyte/macrophage epitope CD 68 suggesting that areas positive for 8-epi-PGF<sub>2α</sub> also contained macrophages. Consequently, it can be assumed that 8-epi-PGF<sub>2α</sub> in combination with the macrophage-mediated inflammatory process are most likely to be responsible for necrosis and degeneration of valvular tissue which is afterwards successively replaced by collagen fibers. These findings are supported by the immunohistochemical analysis showing that 8-epi-PGF<sub>2α</sub> staining was most intense in areas highly positive for Masson-Goldner staining indicating the presence of collagen. In addition, our observations are in accordance with previous data, where accumulation of LDL was shown to be associated with extracellular collagen fibers in cardiac valves of rabbits after a high cholesterol high-lipid diet (Nievelstein-Post et al., 1994).

The 8-epi-PGF<sub>2α</sub> content was significantly higher in the atherosclerotic segments than in uninvolved areas. Previously, it was reported that aortic valve sclerosis, defined as valve thickening, without restriction of leaflet motion on echocardiography, is associated with an increased risk of death from cardiovascular causes (Otto et al., 1999). Since aortic valve disease and CHD share similar risk factors (Aronow et al., 1987; Mohler et al., 1991; Gotoh et al., 1995), our findings lend strong support to the argument that 8-epi-PGF<sub>2α</sub> accumulation plays an important role in the atherosclerotic development and pathophysiological alterations observed in the semilunar heart valves. This observation is strongly supported by the fact that enrichment of 8-epi-PGF<sub>2α</sub> in the aortic valves was highest in those patients with severe CHD, suggesting that accumulation of this isoprostane in cardiac valves especially in subendothelial areas is an additional mechanism

accelerating valvular degeneration in these patients (Mehrabi et al., 1999). In contrast, in healthy controls, only trace amounts of 8-epi-PGF<sub>2α</sub> accumulation were evident (Fig. 1C).

The immunohistochemical findings were confirmed by a radioimmunoassay, revealing significantly higher values of the isoprostane 8-epi-PGF<sub>2α</sub> in extracts of respective valves of CHD patients. By using a linear regression analysis we additionally demonstrated that the data obtained from the immunohistochemistry and the radioimmunoassay significantly correlate, verifying the accuracy and validity of both methods. Additionally, it was demonstrated that CHD-patients had higher levels of 8-epi-PGF<sub>2α</sub> in serum when compared with normal values reported previously in healthy individuals (Oguogho et al., 1999).

Of particular interest was the fact that pulmonary valves accumulated more 8-epi-PGF<sub>2α</sub> than aortic valves in patients with CHD. One possible explanation could be the elevated right ventricular systolic pressure in the presence of a decrease in left ventricular systolic pressure in chronic heart failure. Additionally, the lower shear stress in the right ventricle could have favoured 8-epi-PGF<sub>2α</sub> accumulation in the pulmonary valves. Locally, the accumulation of cytokines and altered release of NO and prostaglandins from valvular endothelium could also serve as mechanisms explaining the increased accumulation of 8-epi-PGF<sub>2α</sub> in the pulmonary valves (Belov et al., 1999; Dibbs et al., 1999; Martin, 1999).

In the present study, we did not perform staining for other prostaglandins generated largely via the cyclooxygenase pathway. Previously, it was reported that blocking COX non-specifically with aspirin did not impair urinary 8-epi-PGF<sub>2α</sub> excretion as compared to untreated patients (Davi et al., 1997). No significant difference was found in the urinary 8-epi-PGF<sub>2α</sub> content between CHD patients taking aspirin versus those who were not (Pratico et al., 1997; Mehrabi et al., 1999).

In summary, we have provided evidence that accumulation of the isoprostane 8-epi-PGF<sub>2α</sub> in human semilunar valves provides, due to its oxidative ability, valuable insights into potential mechanisms underlying valvular atherosclerosis. As a therapeutical consequence, we suggest that these patients could benefit from a valve-protecting anti-oxidative therapy.

However, it has to be noted that also other members of the large family of isoprostanes could be involved in the process of atherosclerosis, requiring future studies to be performed to clarify their role in the development of atherosclerosis.

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*Isoprostane accumulation in heart valves*

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