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# Endothelium-dependent microvascular vasomotion and its correlation with vasoactive mediators early after cardiac transplantation in humans

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**Abstract** Endothelial dysfunction precedes and predicts transplant vasculopathy. We investigated the relationship between endothelial dysfunction and the vasoactive mediators nitric oxide and endothelin, 33.7 ± 2.0 days after heart transplantation. Coronary flow was measured in 18 patients to determine the endothelial microvascular vasomotor response to acetylcholine. Endomyocardial biopsies were taken to determine the levels of gene expression of isozymes of endothelin and nitric oxide synthases (NOS). Blood samples from the coronary sinus and aorta were withdrawn for measurement of endothelin, nitrite and cytokines. Five patients (30%) showed an impaired coronary flow reserve response to acetylcholine, significantly higher inducible NOS gene expression and significant transcardiac nitrite production. Plasma nitrite correlated with tumour necrosis factor-alpha levels in coronary sinus and a transcardiac net extraction of endothelin was noted in all patients. In conclusion, 30% of patients develop endothelial dysfunction early after heart transplantation; this correlates with the expression and activation of vasoactive and immunomodulatory mediators, which may predict the development of transplant vasculopathy.

**Key words** Nitric oxide · Endothelin · Humans · Heart transplantation · Endothelial function

## Introduction

The integrity of the coronary endothelium plays a key role in the protection of the vasculature from injury; it is a target organ in several disease states such as diabetes, hypercholesterolaemia, hypertension and in patients with chronic, (smoulder) high immunological activity such as after cardiac transplantation. Endothelial injury leads to the development of coronary atherosclerosis, myocardial ischaemia and infarction [1]. The development of transplant vasculopathy (TVP) as a more progressive and accelerating form of coronary atherosclerosis has been observed with the establishment of cardiac transplantation as a therapeutic modality for end stage heart disease. Recently, it has been reported that an early endothelial dysfunction (ED) after cardiac transplantation precedes and predicts the development of TVP [2, 3]. Several vasoactive mediators have been discussed as pathological key factors leading to graft atherosclerosis and dysfunction after cardiac transplantation; these include enhanced endothelial release of the potent vasoconstrictor peptide endothelin-1 (ET) and impaired or increased production of nitric oxide (NO) derived from the constitutive [4–6] and inducible [7–10] nitric oxide synthases (cNOS, iNOS).

The present study investigated the relationship between ED and expression and production of ET and NO in an early but stable phase after cardiac transplantation in humans.

## Materials and methods

The study protocol was reviewed and approved by the Human Subjects Research Committee of the Ludwig-Maximilians University and all participants granted written informed consent. Eighteen patients were included in the study. Exclusion criteria were impaired renal function (serum creatinine levels > 1.5 mg/dl), acute signs of infection 10 days or less prior to blood sample collection and/or rejection grade ≥ 1b (ISHLT) 10 days or less prior to collection of biopsy samples.

Donor hearts were preserved with University of Wisconsin solution and recipients were kept on standard immunosuppressive therapy which included tacrolimus (FK 506; Fujisawa) in combination with azathioprin and prednisolone. Except for the immunosuppressive regime, all medication was discontinued 12–24 h in prior to data and sample collection. All patients were subjected to endomyocardial biopsy and coronary angiography 33.7 ± 2 days after heart transplantation. Two myocardial samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C until reverse transcriptase–polymerase chain reaction (RT–PCR) was performed. Blood was withdrawn from the coronary sinus (CS) and aorta and stored frozen at −80 °C.

### Endothelium-dependent microvascular vasomotion

After a bolus of intravenous heparin (5000 U), microvascular vasomotor response was assessed by flow velocity measurements with an intracoronary Doppler flow wire (0.04 cm; Flo Wire, Cardiometrics, USA). The technical details of the system and its validation for accurate measurements have been described in detail elsewhere [11]. The Doppler flow wire was positioned in the proximal part of the left anterior descending coronary artery. After baseline flow velocity readings were obtained, the endothelium-dependent changes in flow velocity were measured with intracoronary acetylcholine (30.0 μg/min over 5 min). Increases in peak flow velocity by a factor of 2.0 and greater were considered normal. Increases in flow velocity below factor of 2.0 were considered as ED. Heart rate, mean arterial pressure, coronary flow velocity and electrocardiograms were monitored continuously throughout the procedure.

### RNA extraction and cDNA preparation

Samples were homogenised with an OMNI 200 homogeniser (Süd-Laborbedarf, Germany) in 600 μl of lysis buffer (Quiagen, Germany). Insoluble material was separated from the lysate by centrifugation at 10000 *g* for 3 min. Total RNA was extracted from the supernatant using spin columns with a selective binding silica-based membrane (RNeasy kit, Quiagen). Total RNA was quantified by measuring the optical density at $A_{260}$ and was confirmed by gel electrophoresis. cDNA was prepared from 2 μg of total RNA in 30 μl reverse transcription buffer (Gibco BRL, UK) supplemented with 0.6 mM each of dATP, dGTP, dCTP and dTTP (New England Biolabs, Germany), 32 U RNase inhibitor (Boehringer, Germany), 400 U of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; Gibco BRL), 10 mM dithiothreitol and 1.5 μM $(p(dt)_{15}$ primer (Boehringer) at 37 °C for 60 min. Subsequently, the reaction mixture was heat inactivated for 10 min at 95 °C.

**Table 1** Sequences of the primers used in this study are shown. The various primers were designed to result in specific detection of products from the cDNA and not from the genomic DNA. (*GAPDH* Glyceraldehyde-3-phosphate dehydrogenase, *iNOS* inducible nitric oxide synthase, *ecNOS* endothelial constitutive nitric oxide synthase, *ET-1* endothelin-1)

| Primer | Sequence (5' to 3') | Product size (bp) |
|---|---|---|
| GAPDH | TGAAGGTCGGAGTCAACGGATTTGGT<br>CATGTGGGCCATGAGGTCCACCAC | 983 |
| iNOS | GGCCTGGAAACGCACAAGCTG<br>TTGGGGTTGAAGGCACAGCTG | 506 |
| ecNOS | GAAGAGGAAGGAGTCCAGTAACAC<br>GGTGGCCCTCGTGGACTTGCTG | 451 |
| ET-1 | GTCAACACTCCCGAGCACGTT<br>CTGGTTTGTCTTAGGTGTTCCTC | 350 |

### PCR procedure

An aliquot (3 μl) of cDNA was amplified by PCR with a DNA thermal cycler (Perkin Elmer 480, Cetus, USA). The amplification reaction was carried out in a total volume of 50 μl of PCR buffer containing 10 mM TRIS-HCl (pH 8.3), 1,5 mM MgCl2, 50 mM KCl, 200 μM each of dATP, dGTP, dCTP and dTTP, 400 nM each of 3' and 5' primers, and 1 U *Taq* DNA polymerase (Boehringer) The oligonucleotide primers had been synthesised on a DNA synthesiser (Applied Biosystems, USA) and purified using Sephadex columns (NAP 5; Pharmacia, Germany).

The primers were designed such that only the expected products were obtained from the cDNA and not from genomic DNA [12]. The glyceraldehyde-3-phosphate dehydrogenase transcript was used as an internal control of the processed RNA. The nucleotide sequences of the primers used in the amplification reaction are shown in Table 1. The PCR reaction mixture was covered with 50 μl of light mineral oil (Sigma Chemie, Germany). After 1 min predenaturation at 94 °C, the PCR conditions were as follows: denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s and extension at 72 °C for 1 min. To ensure detection of low-abundance mRNA, 35 amplification cycles were performed. A sample (10 μl) of each amplified product was subjected to electrophoresis in a 1 % agarose gel (Promega, USA), stained with ethidium bromide and visualised by UV illumination.

### Determination of plasma ET levels

Plasma (ca 5.0 ml) was frozen at −80 °C. Plasma ET concentration was determined by radioimmunoassay (RIA), as described previously [13]. In brief, samples were lyophilised and redissolved in 350 μl of RIA buffer (containing 13.75 g $NaH_2PO_4$, 43.8 g NaCl, 0.5 % bovine serum albumin, 5 g gelatin and 0.5 % Triton X-100 in 5 l deionised water at pH 7.3) to which 100 μl ET-1 antiserum (RAS 6901 N, rabbit; Peninsula, Germany) was added. Cross-reactivity of the antiserum was 100 % to the human antigen, as specified by the manufacturer. [3-$^{125}$I-Tyr-]-ET-1 (50 μl) was added to the assay reaction and samples were incubated overnight. Separation of bound and unbound tracer was achieved by addition of 500 μl charcoal suspension (containing 0.5 g bovine serum albumin and 1.25 g activated charcoal in 100 ml of RIA buffer without Triton X-100). Bound radioactivity was measured in a gamma counter and calculated in fmol/ml.

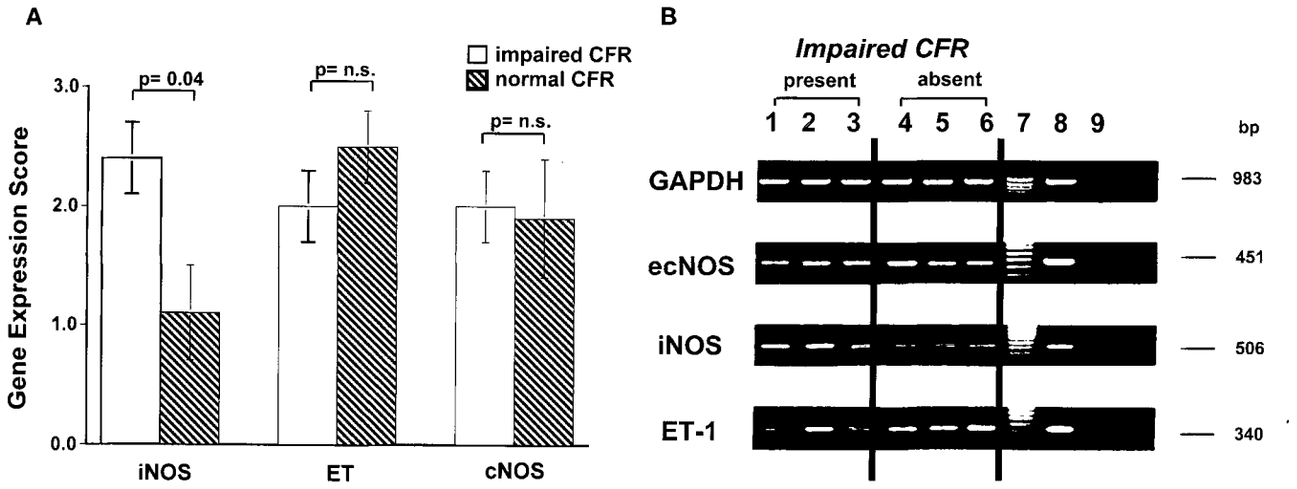


**Fig. 1 A, B** Myocardial gene expression of inducible *(iNOS)* and constitutive nitric oxide synthases *(ecNOS)* and endothelin *(ET-1)* were semiquantitatively determined by reverse transcriptase–polymerase chain reaction using specific primers. As shown in **A** a significantly higher iNOS gene expression was noted in patients with impaired coronary flow reserve *(CFR)*. No significant differences were noted with respect to cNOS and ET. **B** Gene expression for three different patients in each group. Patients 1–3 have impaired CFR, 4–6 have normal CFR. *Lane 7* DNA marker, *lanes 8, 9* positive and negative controls, respectively. Glyceraldehyde dehydrogenase *(GAPDH)* served as a control gene

### Determination of total plasma nitrate/nitrite oxide and cytokine levels

Total nitrate/nitrite levels were measured by the Gries reaction. In brief, plasma was deproteinised by ultrafiltration (Centrifree micropartition system; Amicon, USA). The nitrate content of the sample was reduced to nitrite with nitrate reductase. Sample aliquots were incubated in the presence of 0.1 U/ml nitrate reductase in 2.5 mM phosphate buffer containing 5.0 μM FAD and 50 μM NADPH (all from Boehringer-Mannheim) in a final sample volume of 106.7 μl and incubated for 30 min at 37 °C. NADPH was oxidized by adding 6.7 μl of a reaction mix containing lactate dehydrogenase (dilution 1 : 10; 60 μl) and pyruvate (140 μl) followed by 5 min incubation at 37 °C. Finally, the Gries reagent was added followed by incubation at 37 °C for 10 min. The reaction was stopped by adding 333.0 μl cold TCA (1.2 M). Samples were measured by spectrophotometric analysis at 540 nm. A standard curve was performed in each experiment. The nitrite content of the samples was calculated from the standard curve, which was linear within this range.

The pro-inflammatory cytokine tumour necrosis factor (TNF)-alpha and its specific receptors TNF-Rp1, TNF-Rp2 was well as interleukin (IL)-6 and sIL-2r were measured by enzyme immunoassay and ELISA, was described in detail elsewhere [14].

### Statistical analysis

Group data are presented as arithmetic mean values ± SEM. One-way analysis of variance was performed to compare patients with and without ED. A paired *t*-test was used to assess differences between each individual. Correlation coefficients were determined by simple regression analysis. *P* values of 0.05 or less were considered statistically significant.

## Results

All patients presented with normal renal function parameters (serum creatinine 1.1 ± 0.3 mg/dl) and absence of acute rejection episodes of grade ≥ 1b (ISHLT) for at least 10 days prior to examination. In addition, no signs of acute bacterial or viral infection were observed or had had to be treated within 10 days prior to examination.

Thirteen patients showed normal, endothelium-dependent coronary flow reserve (CFR 2.8 ± 0.2). However, in five patients (29 %), an impaired flow increase in response to the endothelium-dependent vasodilator acetylcholine was observed, suggesting an ED (CFR 1.5 ± 0.9). None of the patients presented with angiographic evidence of macroscopic coronary atherosclerosis.

Patients with impaired CFR showed a significantly higher score for myocardial iNOS gene expression and an equal or lower cNOS gene expression than patients with normal CFR (Fig. 1). In addition, CS plasma nitrite levels tended to be higher than the aortic concentration in patients with ED, suggesting transcardiac NO production (Fig. 2). Moreover, CS nitrite levels correlated with CS TNF-alpha concentration (Fig. 2).

ET gene expression was noted in all patients (Fig. 1). However, the plasma ET concentration was significantly lower in the CS than in the aorta, suggesting a transcardiac ET extraction (Fig. 3). Moreover, plasma ET levels correlated with CFR in response to acetylcholine (Fig. 3).

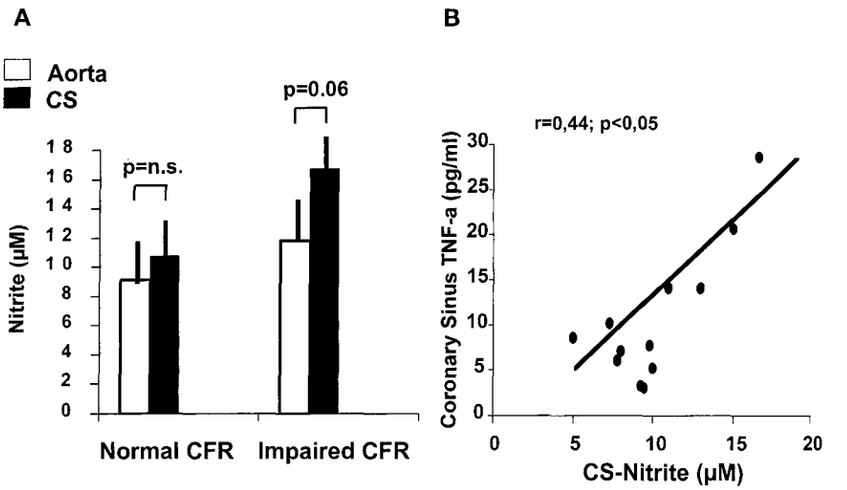


**Fig. 2 A, B** In patients with normal CFR no significant changes in plasma nitrite levels between aortic and coronary sinus *(CS)* were observed. In contrast, a strong trend for transcardiac nitrite release was found in patients with impaired CFR ($P = 0.06$; **A**). In addition, a significant correlation between the pro-inflammatory cytokine, tumour necrosis factor-alpha (*TNF-α*) and plasma nitrite concentration in CS blood was shown ($r = 0.44$, $P < 0.05$), supporting the potential role for iNOS in cardiac production of nitric oxide (**B**)

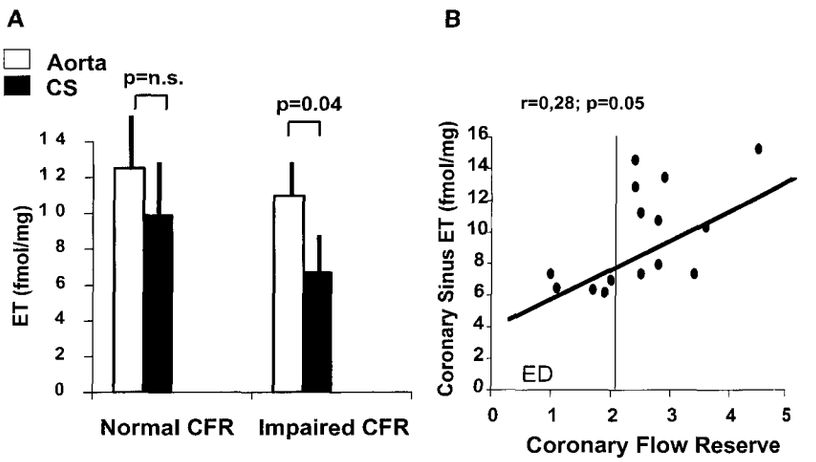


**Fig. 3 A, B** Patients with impaired CFR showed a significant transcardiac endothelin *(ET)* extraction (**A**). No significant differences between aortic and CS plasma ET concentration were noted in patients with normal CFR. Interestingly, a significant correlation between CFR and CS ET levels was noted ($r = 0.28$, $P = 0.05$; **B**)

In up to 60 % of all patients, a significant transcardiac cytokine release was observed (IL-6) 58.8 %, TNF-Rp2 58.8 %, TNF-alpha 53.3 %, sIL-2R 50 %, TNF-Rp1 41.6 %9.

## Discussion

The results of the present study indicate that nearly 30 % of patients have an impaired endothelium-dependent CFR in response to acetylcholine 1 month after heart transplantation. This finding may be of importance since recent data suggest that epicardial ED early after cardiac transplantation predicts the development of TVP at 1 year posttransplant [2]. However, a possible relationship between ED and expression and activation of vasoactive mediators early after cardiac transplantation has not been addressed to date. The new finding of the present study is the increased iNOS gene expression in patients with impaired microvascular endothelial function. In addition, there was a trend for transcardiac nitrite production in patients with impaired CFR, indicating cardiac NO production. Which isoenzyme has actually produced NO within the heart remains uncertain. However, increased iNOS gene expression may be a potential source of cardiac NO production and might cause the impairment of endothelial response to acetylcholine. In this context, iNOS activation might deprive the endothelium of substrate for NO production and might explain the compromised endothelium-dependent vasodilatation observed in these patients. Similar findings have been shown recently in a rabbit model of atherosclerosis [15]. In addition, upregulation of iNOS activity has been shown in acute cardiac allograft rejection. Selective modulation of this isozyme by aminoguanidine prolonged graft survival and prevented vascular barrier dysfunction in rats [7, 16]. Moreover, Lewis et al. [9] showed an association

between vascular and contractile dysfunction of the left ventricle and induction of iNOS in the human cardiac allograft. In the present study, endothelial cNOS gene expression appeared to be unchanged or impaired in patients with ED. Similar findings were reported by Akyürek et al. [17] who observed unchanged cNOS expression during development of TVP in rat aortic grafts. Decreased endothelium-derived NO may contribute to ED due to enhanced leucocyte and platelet adhesion to the vascular wall, as shown recently after ischaemia and reperfusion [18–20]. This is supported by Drexler et al. [21], who reported beneficial effects of intracoronary L-arginine in cardiac transplant recipients with severely depressed blood flow response to acetylcholine, supporting the notion that ED in those patients was improved by administration of the NO precursor. Moreover, decreased cNOS activity may enhance the expression of adhesion molecules, contributing to further endothelial cell injury and subsequent development of TVP. In fact, it has been shown recently that enhanced arteriolar endothelial ICAM-1 expression correlated with early angiographically visible TVP in humans [22, 23].

An important finding in this study is the correlation of CS nitrite levels and CS TNF-alpha concentration Fig. 2). TNF-alpha and other cytokines are known to upregulate iNOS gene expression and activation [24]. High amounts of NO produced by NOS could contribute to endothelial and myocardial dysfunction, as previously reported [9, 16, 25].

In addition to alterations of the NO pathway, a significant transcardiac extraction of ET was noted in most patients. The functional significance of this finding remains elusive but similar findings have been reported by others [26]. Whether or not the transcardiac net extraction of ET reflects myocardial activation of ET receptors cannot be answered in the present setting. However, ET may contribute to ED and impaired CFR due to enhanced vasoconstriction and mitogenic effects, as shown previously in early atherosclerosis [5]. The positive correlation between TNF-specific receptors and CS ET levels is noteworthy and reflects an immunological process in the development of ED.

In conclusion, the present data indicate that approximately 30% of patients develop ED within 1 month after cardiac transplantation, which is associated with alterations of vasoactive and immunomodulatory mediators such as NO, endothelin and cytokines. Since transplant vasculopathy is a major limiting constituent of lifetime after heart transplantation, the contribution of vasoactive factors to the development of ED in the early phase after heart transplantation should not be underestimated and may be an important therapeutic target in these patients.

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