Coronary flow reserve and nitric oxide synthases after cardiac transplantation in humans

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Abstract

Objective: Coronary endothelial dysfunction may precede morphological changes in both the epicardial conduit and microvascular resistance vessels in heart transplant recipients. Since the development of transplant atherosclerosis is the major limiting factor for long-term survival, the identification of early mediators of vasomotor dysfunction may be of therapeutic interest. We therefore investigated the potential relationship between the expression of nitric oxide synthases (NOS) and coronary endothelial function in human cardiac transplant recipients over time.

Methods: Forty-two human cardiac transplant recipients were studied at 1 and 12 months after heart transplantation (HTx). The microvascular coronary flow velocity reserve (CFVR) was tested for endothelium-dependent (acetylcholine) and -independent (adenosine) stimuli by intravascular Doppler flow-wire. Epicardial diameter changes were evaluated by quantitative coronary angiography. Endomyocardial inducible (iNOS) and endothelial constitutive nitric oxide synthase were determined by RT-PCR. Nitric oxide production (nitrite and nitrate (NOx)) and TNF-α were measured in plasma samples from the aorta and coronary sinus.

Results: CFVR was impaired in 26.1% (n = 11) of patients at 1 month and in 31% (n = 13) 12 months after HTx. iNOS-mRNA levels were significantly higher in patients with impaired endothelium-dependent CFVR. In addition, only in these patients were TNF-α levels higher and these correlated with plasma NOx levels at 1 and 12 months post-HTx (1 month: r = 0.81, P < 0.001; 12 months: r = 0.62, P = 0.04).

Conclusions: Coronary microcirculatory dysfunction in response to acetylcholine is present in nearly 30% of patients during the first year following transplantation. These patients present with higher iNOS-mRNA expression and TNF-α plasma levels. Selective modulation of the TNF-α/iNOS-pathway may be of therapeutic value to improve coronary endothelial dysfunction in cardiac transplant recipients. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Heart transplantation; Endothelial function; Inducible nitric oxide synthase; Cytokines; Humans

1. Introduction

Coronary endothelial dysfunction is an initial event in the development of transplant coronary artery disease (TxCAD) which is the major limiting factor for long-term survival in patients after heart transplantation (HTx) [1,2]. A number of immunological and vasoactive factors may contribute to impaired endothelial function early after HTx [3–5]. The expression and activation of nitric oxide synthase (NOS) has been shown in transplanted hearts during acute rejection episodes. In particular, the inducible nitric oxide synthase (iNOS), which is cytokine regulated and calcium-insensitive, is involved in the inflammatory process and associated with contractile dysfunction and vascular barrier dysfunction under these conditions [6]. Acute rejection episodes correlated with the amount of myocyte apoptosis in human transplanted hearts and were associated with an up-regulation of iNOS expression [7]. Moreover, Russell and co-workers demonstrated that the early and persistent up-regulation of iNOS in rat cardiac allografts contributes to the inflammatory response, mediating transplant arteriosclerosis [8]. iNOS up-regulation was associated with left ventricular contractile dysfunction as shown by Lewis and colleagues [9].

Based on these observations, we determined the relationship between coronary vasomotor function in epicardial and microvascular compartments in response to endothelium-
dependent and independent stimuli and the expression of iNOS and endothelial constitutive nitric oxide synthase (ecNOS) in simultaneously procured endomyocardial biopsies. In addition, the production/release of total NOx and TNF-α was measured in aortic and coronary sinus (CS) blood samples.

2. Methods

A total of 42 transplant recipients were included in this prospective, longitudinally designed study. The study group consisted of 37 male and five female patients (mean age, 50 years; range, 16–67 years) who received orthotopic HTx in our center. Triple drug immunosuppression (Table 1) was initiated immediately after organ implantation and was maintained throughout the study. Other medications, including ace-inhibitors, calcium-antagonists, statins and diuretics, were discontinued 24–48 h prior to each examination. All patients agreed to participate in the study and gave written informed consent. The study protocol was approved by the Ethics Committee of the Ludwig-Maximilians University, Munich, Germany. No study-related complications or deaths occurred during the 1-year follow-up of patients.

2.1. Study protocol

Patients were examined at months 1 (37 ± 8 days), 6 (205 ± 12 days) and 12 (370 ± 17 days) after transplantation.

Left heart catheterization was performed at 1 and 12 months after HTx and consisted of a measurement of hemodynamics and routine coronary angiography to exclude

<table>
<thead>
<tr>
<th>Table 1 Donor- and recipient-dependent criteriaa,b,c</th>
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<tr>
<td><strong>Impaired CFVR (n = 11)</strong></td>
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<td><strong>Donors</strong></td>
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<td>Immunomsuppressive regime</td>
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<td>Tacrolimus + azathioprine + prednisolone</td>
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<td>Cyclosporine + azathioprine + prednisolone</td>
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<td>Number of treated rejection episodes/patient</td>
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a Relevant donor- and recipient-dependent criteria at time of transplantation (HTx). Patients are grouped according to microvascular CFVR in response to acetylcholine as determined 1 month after HTx.

b CMV, cytomegaly virus; PVR, pulmonary vascular resistance; CAD, coronary artery disease.

c Figures in parentheses represent percentage values.
donor transmitted coronary artery disease. In addition, an assessment of endothelium-dependent and -independent epicardial (quantitative coronary angiography) and microvascular (Doppler flow-wire) coronary vasomotor function was performed.

At each follow-up appointment, endomyocardial biopsy samples were obtained from the interventricular septum for determination of iNOS- and ecNOS-messenger RNA (mRNA) expression. To account for differences in sample quality, we always used homogenates from two biopsy samples for each reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. Blood samples from the aorta and CS were withdrawn for measurement of TNF-α (RT-PCR) analysis. Blood samples from the aorta and CS for each reverse-transcriptase polymerase chain reaction expression. To account for differences in sample quality, were obtained from the interventricular septum for determination.

2.2. Functional assessment of coronary vasomotor function

2.2.1. Endothelium-dependent and -independent epicardial vasomotion

Quantitative coronary angiography with a computerized automatic-analysis system (Hicor, Siemens) was used to assess the coronary vasomotor response (epicardial luminal diameter changes (%)) to the following stimuli: endothelium-dependent with intracoronary administration of acetylcholine (Ach; 1.0 and 30.0 μg/min for 5 min each) and endothelium-independent with intracoronary infusion of adenosine (80.0 and 160.0 μg/min for 5 min each) as described elsewhere. No pathological vasoconstriction was observed during the investigation. Since autoregulation maintains constant coronary flow over a range of perfusion pressures, changes observed in the microvasculature were not due to epicardial vasoconstriction [10].

2.2.2. Endothelium-dependent and -independent microvascular vasomotion

Microvascular vasomotor response was assessed by flow velocity measurements with an intracoronary Doppler flow-wire (0.018 inch (0.04 cm); Flo Wire, Cardiometrics, Inc., USA). The flow-wire was introduced in a 6 F Judkins catheter and positioned in the proximal part of the left anterior descending or circumflex coronary artery. After baseline flow velocity readings were obtained, hyperemic flow velocity data were determined with intracoronary adenosine infusion (Ad; 80.0 and 160.0 μg/min over 5 min each). Endothelium-dependent changes in flow velocity were measured with intracoronary infusion of acetylcarnine (Ach; 1.0 and 30.0 μg/min over 5 min each). The coronary flow velocity reserve (CFVR) was expressed as the ratio of peak to baseline blood flow velocity. Heart rate, mean arterial pressure, coronary flow velocity and electrocardiogram were monitored continuously throughout the procedure. It was assured that measurements of the flow velocity reserve of the microvascular bed were not altered by epicardial vasoconstriction during acetylcarnine infusion. An increase in the flow velocity below factor 2.0 was considered pathological [11].

2.3. Intravascular ultrasound

Immediately after Doppler flow measurement, intracoronary ultrasound (ICUS) was performed to detect intimal hyperplasia not detectable by angiography at 1 month post-transplant. Before intravascular positioning, 200 μg of nitroglycerin was injected into the left coronary artery. The imaging system consisted of a 30-MHz ultrasound transducer enclosed within an acoustic housing on the tip of a 2.9 F flexible, rapid-exchange catheter (CVIS, Inc., Sunnyvale, CA, USA). The catheter was advanced to the distal left anterior descending or circumflex artery, with careful observance of the lumen–ICUS catheter diameter ratio of >1.5. During the subsequent standardized pullback maneuver, images were documented on SVHS videotape for further off-line analysis. The three sites with the most severe intimal proliferation were evaluated semiquantitatively concerning the radial and circumferential extent of intimal hyperplasia, and the averaged maximal intimal thickness was calculated. The mean maximal intimal thickening (MIT) was determined using a minimum of five randomized proximal to distal sites. The data are expressed as: 1, normal vessel morphology; 2, mild MIT (<300 μg); 3, moderate MIT (300–600 μg); 4, severe MIT (>600 μg). The predominant MIT localization is separated in three types: (a), proximal; (b), distal; (c), diffuse localization.

2.4. Detection of iNOS- and ecNOS-mRNA by RT-PCR

Endomyocardial biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C. For RNA extraction and cDNA preparation, the technique described previously was used.

An aliquot (3 μl) of cDNA was amplified by PCR with a DNA thermal cycler (Perkin–Elmer 480, Cetus Corp., Norwalk, CT). The amplification reaction was carried out as described. The nucleotide sequences of the chosen primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGAAGGTGGAGTCAA-CCGATTGTG-3' for sense and 5'-CATGTGGGCACAT-GAGTCCACAC-3' for antisense (product size, 983 bp); iNOS, 5'-GGGCGTTGAAAACGCAACAGCTG-3' for sense and 5'-TGGGGGTTGAAAGGCACAGCTG-3' for antisense (product size, 506 bp); ecNOS, 5'-GAAGAGGAAGGGATCCAGTAACAC-3' for sense and 5'-GGTGGCCCTCGTGACTTG-3' for antisense (product size, 451 bp). Semiquantitative analysis was performed using a densitometric analysis system. It was assured that the amplification was within the linear range of the particular primer studied. Each signal was normalized for the DNA standard of the same gel to account for variances between gels. Normalization for the housekeeping gene, GAPDH, was performed to account for the variability in sample quality.
2.5. Measurement of plasma NOx and TNF-α

Total NOx levels were measured by the Griess reaction as described previously [12]. In brief, plasma was deproteinated by ultrafiltration (Centrifree micropartition system, Amicon, Beverly, MA). The nitrate content of the sample was reduced to nitrite with a nitrate reductase. Samples were measured by spectrophotometric analysis at 540 nm. A standard curve was performed in each experiment. The NOx content of the samples was calculated from the standard curve, which was linear within this range.

TNF-α was measured by a commercially available, enzyme-linked immunoabsorbant assay (ELISA, Medgenix, Ratingen, Germany). All samples were run in duplicate and the optical density was read at the appropriate wavelength.

2.6. Data analysis

The CFVR to acetylcholine was considered normal when the flow increase was above factor 2.0 from the baseline level. Nominal data were analyzed with Fisher’s Exact test. Normally distributed data were analyzed with either the paired or unpaired Student’s t-test as appropriate, and correlations were determined with single regression analysis. Multiple comparisons were performed with ANOVA and corrected with Bonferroni-post-hoc analysis. Non-normally distributed data were analyzed using the Mann–Whitney U-test. The data are expressed as means ± SD. P values of <0.05 were considered statistically significant. For multiple comparisons, a P value of <0.002 was considered statistically significant.

3. Results

3.1. Donor and recipient demographics

Important donor- and recipient-dependent criteria are shown in Table 1. None of the patients included in the study showed significant donor transmitted disease, visible during angiography 1 month post-HTx.

None of the patients included in the investigation had clinical signs of infection or acute rejection episodes of ISHLT grade 1b or greater during the time of sample collection and functional assessment of the coronary vasculature. Moreover, no significant differences with regard to the number of acute cellular rejection episodes were found between the two groups (Table 1).

The patients did not differ with regard to the number of classic atherosclerotic risk factors, such as hypertension, hyperlipidemia, diabetes or history of smoking.

In addition, no significant differences with regard to cardiac hemodynamics were observed at both follow-up appointments. Moreover, we found no correlation between NOS-gene expression or NOx levels and cardiac hemodynamics at any time point (data not shown).

3.2. Epicardial vasomotor function

The administration of acetylcholine resulted in epicardial coronary diameter changes of −5 ± 2% in proximal and −11 ± 5% in distal segments at 1 month, and −7 ± 3% in proximal and −12 ± 4% in distal segments 12 months after HTx.

The administration of adenosine resulted in epicardial vasodilator changes of 7 ± 3% in proximal segments and 17 ± 6% in distal segments at 1 month, and 6 ± 2% in proximal and 15 ± 8% in distal segments 12 months after HTx.

Fig. 1. Endothelium-dependent and -independent CFVR as measured by intracoronary Doppler flow-wire is shown at 1 and 12 months after HTx. Patients are grouped according to endothelium-dependent CFVR in response to acetylcholine (30 μg/min × 5 min). An impaired CFVR was defined as the flow velocity increase from baseline levels of less than factor 2.0. Eleven patients showed an impaired CFVR early after HTx (1 month post-HTx). However, endothelium-independent CFVR to adenosine (160 μg/min × 5 min) was not different 1 month after HTx. At 12 months post-HTx, 13 patients presented with an impaired CFVR to acetylcholine. These patients also showed a significant impairment of endothelium-independent vasomotor function in response to adenosine (160 μg/min × 5 min). Data are shown in box plots. The box indicates 50% of the observed data points between the 1st and 3rd quartiles. The line within the box represents the median. The whiskers show the data between the 10th and 90th percentiles. Extreme data points below and above the 10th and 90th percentile are shown as dots.
transplantation. Epicardial vasomotor responsiveness to both acetylcholine and adenosine was not associated with endomyocardial expression of both iNOS and ecNOS-mRNA.

### 3.3. Microvascular vasomotor function

Eleven out of 42 patients (26.1%) showed an impaired CFVR in response to acetylcholine 1 month after HTx which was significantly different from those patients with a normal increase in CFVR (Fig. 1). At 1 year post-transplant, 13 patients (31%) presented with an impaired CFVR. These patients also showed a significantly reduced endothelium-independent microvascular vasomotor function in response to adenosine 12 months after HTx (Fig. 1).

Importantly, seven out of 12 patients (58.3%) who showed an impaired CFVR in response to acetylcholine at 1 month following HTx significantly improved over time, having normal responsiveness of the coronary microvasculature at 12 months after HTx.

Vice versa, nine out of 13 patients (69.2%) with an impaired CFVR in response to acetylcholine at 12 months following HTx demonstrated a normal CFVR at 1 month after HTx.

### 3.4. Coronary morphological alterations

At 1 month following HTx, no differences concerning the extent of coronary intimal thickening were observed between patients with impaired and normal endothelium-dependent CFVR (Table 2).

### 3.5. NOS-mRNA expression

No significant differences in overall ecNOS-mRNA expression over time were noted between patients with and without an impairment of endothelium-dependent CFVR at 1 and 12 months post-HTx (Fig. 2A).

In contrast, there was a significant increase in iNOS-gene expression over time in patients with dysfunctional CFVR (Fig. 2B). Patients who remained with normal vasomotor function over the first year had the lowest iNOS-mRNA levels. Patients who developed vasomotor dysfunction over the first year showed a significant increase in iNOS-gene expression (Fig. 2B). Those patients with initial impairment of CFVR 1 month post-HTx had higher levels when compared with values obtained for patients with normal CFVR. Their levels were higher than those of patients with normal CFVR and those with an initial impairment of CFVR but who improved over time (Fig. 2B).

### 3.6. Plasma TNF-α and NOx levels

The overall TNF-α levels (pg/ml) remained elevated throughout the first year following HTx (1 month, 19.5 ± 2.3; 6 months, 21.5 ± 4.8; 12 months, 23.6 ± 4.5; P = 0.02 for 1 vs. 12 months). Patients with impaired CFVR had significantly higher plasma levels of TNF-α at 1 and 12 months after HTx (Fig. 3A).

The overall CS plasma NOx levels (μM) remained elevated during the first year (1 month, 40.6 ± 4.2; 6 months, 38.0 ± 4.3; 12 months, 43.3 ± 6.3; P = ns). However, at 1 and 12 months after HTx, a significant transcardiac NOx release was observed (Fig. 3B). In addition, NOx levels were associated with TNF-α levels only in patients with an impaired CFVR to acetylcholine at both 1 and 12 months following HTx (Fig. 3C,D). No correlation was found in patients with normal CFVR in response to acetylcholine at either time point (1 month: r = 0.22, P = 0.28; 12 months: r = 0.19, P = 0.67).

### 4. Discussion

The novel finding of the present study is that patients with higher expression of endomyocardial iNOS have an impaired coronary microvascular flow reserve (CFR) at 1 and 12 months after HTx (Fig. 3). This coincides with an enhanced TNF-α production at 1 and 12 months following HTx and occurs in the absence of macroscopic lesions of the coronary arteries, as well as acute infection and rejection episodes.

The data demonstrate that under immunosuppressive therapy sufficient to prevent acute rejection episodes, a considerable inflammatory process is active within the allograft. This inflammatory process may not be limited to an

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Table 2

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<tr>
<th>Normal morphology (n)</th>
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<td>11 (35.4)</td>
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<td>Diffuse</td>
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<th>Normal morphology (n)</th>
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<th>Normal CF VR (n = 31)</th>
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* Figures in parentheses represent percentage values.
Fig. 2. Endomyocardial NOS mRNA expression as determined by RT-PCR is shown. Signals were normalized for the housekeeping gene, GAPDH. (A) No significant differences were observed for endothelial NOS (eNOS)-mRNA expression over time in either patient group. The subgroup of patients who remained with normal CFVRs (group A; increase in CFVR of factor >2.0 in response to acetylcholine) over the first year (n = 22) did not show changes in iNOS-mRNA levels. Patients with normal CFVR who developed endothelium-dependent vasomotor dysfunction (Dysf; increase in CFVR of factor <2.0 in response to acetylcholine) over the first year (group B; n = 9) showed a significant increase in iNOS-gene expression. Patients with initial impairment of CFVR 1 month post-HTx who improved over the first year (group C; n = 7) did not show significant differences in iNOS-mRNA levels over time. Patients with a persistent impairment of CFVR over time (group D; n = 4) showed iNOS-mRNA levels which were significantly higher when compared with 1 month levels in groups A and B. bp, base pairs. Data are shown in box blots. The box indicates 50% of the observed data points between the 1st and 3rd quartiles. The line within the box represents the median. The whiskers show the data between the 10th and 90th percentiles. Extreme data points below and above the 10th and 90th percentiles are shown as dots.

early phase (1 month post-HTx), but is persistent as shown by the 6 and 12 month levels for TNF-α and iNOS-mRNA. iNOS-gene expression may be involved in the functional alteration of the microvascular endothelium since, in these patients, its expression was significantly higher at 1 and 12 months post-HTx. It demonstrates, in the clinical setting, that the persistent up-regulation of inflammatory mediators contributes to endothelial dysfunction. It is of importance to note that there was no correlation between NOS-gene expression or NOx levels and cardiac hemodynamics at any time point. This is in line with data from Birks et al., who studied prospectively the potential role of nitric oxide (as measured by plasma nitrates) on myocardial function following human HTx [13].

It is known that plasma NOx varies between individuals and may depend on food intake, renal function and medication. We believe that in this clinical study, an association between the two parameters, plasma NOx and TNF-α, more accurately reflects a certain pathophysiological condition than one alone (i.e. plasma NOx). The subgroup analysis on plasma NOx levels between patients with and without impaired CFVR did not reveal significant differences. However, the interaction between NOx and TNF-α in the subgroup of patients with impaired microvascular function at 1 and 12 months suggests that the inflammatory system is activated.

In this regard, experimental studies with iNOS-deficient mice suggest that the development of TxCAD is augmented in the absence of iNOS [14]. In addition, it has been demonstrated by Shears and colleagues that transfection of the iNOS-gene protects against the development of TxCAD by inhibition of intimal and medial thickening [15].

In contrast, Akyürek and colleagues found iNOS expression in infiltrating cells and vascular smooth muscle cells in neointima and media during the development of experimental transplant arteriosclerosis in rats [16]. Skarsgard and colleagues demonstrated direct vasodilation and inhibition of the myogenic tone of vascular smooth muscle cells by both ecNOS and iNOS-based NO-production in allograft resistance vessels [17]. Indeed, as shown in Fig. 1, patients with an impaired CFVR and high iNOS-mRNA levels also developed endothelium-independent vasomotor function in response to adenosine, suggesting an alteration of vasomotor function downstream to the coronary endothelium. Since acetylcholine-mediated vasodilation in the coronary circulation is, in part, mediated by NO, the functional alteration of this pathway may be involved. In addition, supporting evidence that iNOS is involved in the development of TxCAD in humans has been reported by Ravalli and colleagues. They studied tissue from 15 patients with TxCAD and compared their findings with ten patients having normal coronary arteries. They found a significantly higher expression of both iNOS and nitrotyrosine in the TxCAD group. iNOS was expressed in macrophages and smooth muscle cells and was co-localized with nitrotyrosine formation, suggesting that iNOS, and possibly, peroxynitrite, contribute to the progression of TxCAD in humans [18].

What are the underlying mechanisms for iNOS-mediated endothelial dysfunction of the coronary microcirculation before morphological changes occur?

One possibility is that higher (iNOS-derived) NO concentrations (or its cytotoxic reaction product, peroxynitrite) trigger cellular activation, possibly by NO-dependent formation of secondary oxidants such as peroxynitrite [19,20]. It has been shown that the extent of apoptotic cell death by cytokine-induced NO production is modulated by the availability of oxygen free radicals and by alterations in the cellular balance of Bak and Bcl-xL [21].
Other factors may be synergetic with an enhanced iNOS expression contributing to endothelial dysfunction. Bauer-sachs and colleagues demonstrated that increased superoxide production by a NADH-dependent oxidase plays an important role in the development of endothelial dysfunction in chronic myocardial infarction [22]. This may occur by increased formation of peroxynitrite (in the presence of high NO levels) and reduced NO-bioactivity due to its inactivation by superoxide [23,24]. In this regard, it has been shown that peroxynitrite inactivates manganese superoxide dismutase, thereby promoting irreversible oxidative injury in chronic rejection of human renal allografts [25].

In conclusion, the present study demonstrates for the first time that in human cardiac transplant recipients, endothelial dysfunction of the coronary microcirculation parallels an enhanced endomyocardial iNOS-mRNA expression which may be regulated by the proinflammatory cytokine, TNF-α. Selective modulation of the TNF-α/iNOS-pathway may be a novel therapeutic target in cardiac transplant recipients and may help to prevent the transition from endothelial dysfunction to coronary morphological changes.

Acknowledgements

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References


Appendix A. Conference discussion

Dr P. Macchiarini (Hannover, Germany): What do you think would be the potential clinical implications or applications practically?

Dr Wildhirt: We would suggest to test the potential benefit of selective iNOS inhibitors with regard to the development of transplant atherosclerosis in both experimental and clinical studies. This follows experimental studies, one recently published in Circulation by Behr-Rousse and colleagues from the group in Paris showing that the chronic treatment with a highly-selective iNOS inhibitor, and it was L-NIL in this case, and actually prevented the development of morphological changes in the native atherosclerosis model using hypercholesterolemic rabbits. We currently study, in a rat model of heterotopic allograft transplantation, selective iNOS inhibitors of various forms, and there will be more and more specific selective inhibitors in the future, and simultaneous inhibition of superoxide production on a longitudinal basis to see whether or not we can prevent the development of transplant atherosclerosis. Of course, it would be interesting clinically to add those inhibitors to the preservation solutions and finally treat those patients on a long-term basis.