Glycine preconditioning to ameliorate pulmonary ischemia reperfusion injury in rats†

Sebastian-Patrick Sommera,b,*, Stefanie Sommera, Bhanu Sinhaa,b and Rainer G. Leyha

a Department of Cardiothoracic and Thoracic Vascular Surgery, University Hospital Würzburg, Würzburg, Germany
b Interdisciplinary Center for Clinical Research, University Hospital Würzburg, Würzburg, Germany
 Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, Germany

* Corresponding author: Department of Cardiothoracic and Thoracic Vascular Surgery, University Hospital Würzburg, Oberdürrbacher Straße 6, 97078 Würzburg, Germany. Tel: +49-931-20133111; fax: +49-031-20133008; e-mail: sommer_s1@klinik.uni-wuerzburg.de or sommersebastianpatrick@freenet.de (S.-P. Sommer).

Received 2 September 2011; received in revised form 3 January 2012; accepted 4 January 2012

Abstract
This study examines the impact of glycine (Gly) preconditioning on ischemia reperfusion (IR)-induced pulmonary mitochondrial injury to research the previously, in pig lungs, demonstrated Gly-dependent amelioration of pulmonary IR injury. IR injury was induced in rat lungs by 30 min pulmonary hilum clamping followed by 60 min reperfusion time. Rats were subjected to controls, shams and two study groups (IR30/60, Gly-IR30/60) receiving 37.5 mg Gly i.v. or not before IR induction. The wet/dry-weight ratio, mitochondria viabil-
ity (MV), membrane integrity (MI), respiratory chain complex (RCC) activities, mitochondrial membrane potential ($\Delta\Psi_m$) and cyto-
chrome C (Cyt C) content were analysed. In IR30/60, RCC and MV were impaired; Cyt C loss and MI combined with matrix metalloproteinase-9 (MMP-9) activation and $\Delta\Psi_m$ alteration were observed when compared with controls. In Gly-IR30/60, complex II function and mitochondrial viability were protected during IR, and MMP-9 activation combined with tissue-water content accumulation and $\Delta\Psi_m$ alteration were ameliorated. Cyt C loss, mitochondrial membranes damage, tissue GSH oxidation or neutrophil sequestration was not extenuated in Gly-IR30/60. Gly ameliorates IR-associated mitochondrial dysfunction and decay of viability and normalizes $\Delta\Psi_m$ but does not protect from Cyt C liberation and mitochondrial membrane damage. Our data suggest that the previously described effect of Gly preconditioning results at least partially from mitochondrial protection. A dose-finding study is necessary to improve results of Gly preconditioning.

Keywords: Mitochondria • Ischemia reperfusion injury • Lung • Glycine

INTRODUCTION
Ischemia reperfusion (IR) injury causative for graft failure contributes to mortality after pulmonary transplantation (LTX). We have demonstrated amelioration of pulmonary IR injury after glycine (Gly) preconditioning in pigs [1]. The mechanism of Gly-mediated pulmonary protection remained unclear. Gly is a chloride (Cl) channel opener and inhibits cellular depolarization during stress [2]. Gly also acts as a radical scavenger, protecting from reactive oxygen species [3]. This study focuses on the effect of Gly preconditioning on mitochondrial integrity and function, tissue degradation, oedema formation and neutrophil sequestration after pulmonary IR. We hypothesize that Gly preconditioning (i) protects the mitochondrial electron transport chain, (ii) improves mitochondrial viability, (iii) inhibits mitochondrial-induced apoptosis, and (iv) ameliorates tissue degradation, oedema formation and neutrophil sequestration after IR.

MATERIALS AND METHODS
Experimental setup and animals
All studies were conducted as described previously [4, 5]. Male Wistar rats (180–250 g) were obtained from Harlan-Winkelmann (Borchen, Germany). Chemicals were obtained from Sigma-Aldrich GmbH (Munich, Germany), unless stated otherwise. Buffers were described earlier [4, 5].

Surgery and isolation of mitochondria
Animals were subjected to controls, sham, to IR (IR30/60) and to IR after preconditioning with Gly (Gly-IR30/60) with six animals in each group. In Gly-IR30/60, 37.5 mg Gly dissolved in 200 µl saline was applied i.v. immediately before surgery. The Gly dose was chosen in analogy to the previously conducted experiment in pigs [1]. Isoflurane® maintained anaesthesia. The left lung was accessed via thoracotomy. Thirty minutes in-situ pulmonary hilum clamping followed by 60 min reperfusion time induced IR injury before lungs were harvested. Lung-harvested native tissue served as control. Sham animals received thoracotomy and

†Presented at the 25th Annual Meeting of the European Association for Cardio-Thoracic Surgery, Lisbon, Portugal, October 1–5, 2011.
90 min of ventilation without pulmonary clamping. The two study groups (IR30/60, Gly-IR30/60) received IR with or without Gly preconditioning. Pulmonary mitochondria isolation was described before [4, 5]. Briefly, lung tissue immersed in buffer was minced. The suspension was centrifuged. The filtered supernatant was centrifuged. The sub-cellular fraction containing supernatant was stored at −80°C. The pellet was washed. Protein content was quantified by the BCA test (Thermo Fischer Scientific, Germany). Mitochondria were kept suspended in buffer. In preparation for MPO assay and matrix metalloproteinase-9 (MMP-9) zymography, lung tissue was stored at −80°C.

Assay of respiratory chain complexes

Analyses were performed using an Oxytherm® Clark-type electrode (Hansatech Instruments Ltd, Norfolk, UK). Mitochondria suspended in buffer were equilibrated at 25°C. The energetic substrates were added followed by ADP. State2 respiration was measured before and State3 respiration after ADP addition. Differential respiratory chain complex (RCC) analysis was described before [4, 5].

Mitochondrial viability testing

Ca2+-induced swelling of energized mitochondria was determined according to the Halestrap and Davidson [6]. Mitochondria were suspended in buffer containing rotenone and succinate. Twenty-five millimolar Ca2+ induced mitochondrial swelling. The decrease in optical absorption at 540 nm was determined.

FACS analysis of mitochondrial membrane potential (ΔΨm)

In the presence of rotenone and succinate, mitochondria were stained by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyanobenzimidazolyl-carbocyanine iodide (JC1, Enzo Life Sciences GmbH Lörrach, Germany). ΔΨm stability was assessed before and after uncoupling. Fluorescence-activated cell sorting (FACS) was performed according to the Lecoeur et al. [7]. The ratio of J-aggregate and JC1+ mitochondria determined ΔΨm.

Quantification of subcellular fractional cytochrome C (Cyt C)

The supernatant of disrupted tissue was collected. Total protein content was determined. Cytochrome C (Cyt C) content was analysed using the R&D systems rat/mouse Cyt C immunoassay (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Values were expressed as ng Cyt C/µg of protein.

Mitochondrial membrane integrity analysis

Citrate synthase (CS) activity was determined in mitochondria according to the Chemnitius et al. [8]. The ratio of latent and free CS activity determined the CS ratio (CSR). CS activity was measured at 405 nm. CStotal was determined after pre-incubation with 2.5% Triton X-100 and CSfree after pre-incubation with H2O.

Tissue MMP-9 activity

Lungs were homogenized and the supernatant’s protein content was determined after centrifugation. Forty micrograms of protein in SDS buffer was loaded on gels. After electrophoresis, washing and re-constitution, gels were developed and stained. Analysis was performed digitally. Values are expressed as densitometry units.

Assessment of neutrophil granulocyte sequestration by tissue myeloperoxidase activity (MPO) assay

Lungs were homogenized and centrifuged. The pellet was washed, centrifuged and incubated at 60°C and disrupted by repeated freeze-thaw cycles in hexaoleytrimethyl ammonium-bromide. After centrifugation, the supernatant’s protein content was assessed. Aliquots were added to the TMB substrate system (Sigma-Aldrich GmbH, Munich, Germany). The change in absorption at 655 nm was recorded. Results are expressed as means in mU MPO/µg protein.

Tissue water content (TWC) quantification

Weighing before and after drying overnight at 60°C quantified TWC from lungs. Values are expressed as percentage.

Differential determination of tissue glutathione (GSH, GSSG)

GSH and GSSG from tissue were quantified by the Arbor Assays DetectX® Glutathione Colorimetric Detection Kit (http://www.arborassays.com). Data are expressed as the GSH/GSSG ratio.

Statistical analysis

Results are expressed as means ± standard deviation (SD) or 95% confidence intervals (95% CI). A one-way analysis of variances (ANOVA) combined with the Tukey’s multiple comparison test (TMCT) or the Kruskal-Wallis (KW) test combined with Dunn’s multiple comparisons test (DMCT) was used to test for inter-group differences. Analysis of continuous data was performed using a repeated measure ANOVA combined with the Bonferroni’s multiple comparison test. A P-value < 0.05 was considered significant.

RESULTS

Gly preconditioning ameliorated IR-induced RCC II dysfunction

IR impairs the RCCs (Table 1). Compared with IR30/60, Gly-IR30/60 demonstrated reduced complex dysfunction at II–V (II–V
State3, respectively, 95% CI: 1.7–2.5 vs. 0.7–2.4, \( P > 0.05 \) and II–IV (II–IV ratio, 95% CI: 0.9–2.1 vs. 2.1–3.0, \( P < 0.05 \)).

**Gly preconditioning protected mitochondrial viability**

Controls demonstrated high mitochondrial viability, expressed by declines in light absorption (Fig. 1). Compared with sham, IR30/60 revealed significantly impaired viability \( (P < 0.001, \text{TMCT}) \) and in Gly-IR30/60 viability after IR was significantly preserved \( (P < 0.001, \text{TMCT}) \).

Table 1: Polarographic determination of respiratory complex activities

<table>
<thead>
<tr>
<th>Complex</th>
<th>Control</th>
<th>Sham 30/60</th>
<th>IR30/60</th>
<th>GLY-IR30/60</th>
<th>KW test</th>
</tr>
</thead>
<tbody>
<tr>
<td>I–V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State2 respiration</td>
<td>2.6 ± 0.5</td>
<td>2.6 ± 0.8</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.6</td>
<td>( P = 0.007 )</td>
</tr>
<tr>
<td>State3 respiration</td>
<td>3.1 ± 0.9</td>
<td>2.7 ± 0.9</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.7</td>
<td>( P = 0.008 )</td>
</tr>
<tr>
<td>II–V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State2 respiration</td>
<td>3.2 ± 0.4</td>
<td>3.3 ± 0.7</td>
<td>1.4 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>( P = 0.0005 )</td>
</tr>
<tr>
<td>State3 respiration</td>
<td>4.2 ± 1.2</td>
<td>4.2 ± 1.3</td>
<td>1.5 ± 0.7</td>
<td>2.1 ± 0.4</td>
<td>( P = 0.0007 )</td>
</tr>
<tr>
<td>III–V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State2 respiration</td>
<td>6.5 ± 1.5</td>
<td>6.2 ± 1.3</td>
<td>3.9 ± 0.9</td>
<td>4.6 ± 1.1</td>
<td>( P = 0.006 )</td>
</tr>
<tr>
<td>State3 respiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II–IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope I</td>
<td>3.6 ± 0.8</td>
<td>3.0 ± 1.1</td>
<td>1.1 ± 0.3</td>
<td>2.8 ± 0.8</td>
<td>( P = 0.007 )</td>
</tr>
<tr>
<td>Slope II</td>
<td>5.8 ± 1.0</td>
<td>5.8 ± 1.3</td>
<td>3.2 ± 0.9</td>
<td>3.7 ± 0.4</td>
<td>( P = 0.002 )</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.7 ± 0.5</td>
<td>1.9 ± 0.2</td>
<td>2.5 ± 0.6</td>
<td>1.5 ± 0.6</td>
<td>( P = 0.006 )</td>
</tr>
<tr>
<td>III–IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope I</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.6</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.6</td>
<td>( P = 0.31 )</td>
</tr>
<tr>
<td>Slope II</td>
<td>2.3 ± 0.7</td>
<td>2.6 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>2.4 ± 0.7</td>
<td>( P = 0.03 )</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.7</td>
<td>1.7 ± 0.3</td>
<td>2.0 ± 0.6</td>
<td>( P = 0.96 )</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>37.4 ± 6.5</td>
<td>33.1 ± 6.0</td>
<td>29.2 ± 4.4</td>
<td>21.9 ± 4.9</td>
<td>( P = 0.003 )</td>
</tr>
</tbody>
</table>

**Gly preconditoning limited IR-induced \( \Delta \Psi_m \) alterations**

Compared with control, IR30/60 demonstrated mitochondrial hyper-polarization (Fig. 2). In Gly-IR30/60, \( \Delta \Psi_m \) of energized mitochondria remained normal. \( \Delta \Psi_m \) was jeopardized by addition of CCCP. The decay of \( \Delta \Psi_m \) against time \((0 \text{ to } 3 \text{ min after CCCP})\) differed significantly between groups \( (P = 0.017, \text{ANOV A}) \). Decline in \( \Delta \Psi_m \) in IR30/60 was pronounced, when compared with control \( (P < 0.05, \text{TMCT}) \). In Gly-IR30/60, \( \Delta \Psi_m \) decay was insignificantly inhibited \( (P > 0.05, \text{TMCT}) \).

**Figure 1:** Mitochondrial viability by Ca\(^{2+}\)-induced mitochondrial swelling: presented data represent declines in light absorption after Ca\(^{2+}\)-induced mitochondrial swelling. Differences between study groups were highly significant \( (P < 0.0001) \). Compared with controls, mitochondrial viability was significantly impaired in IR30/60 \( (P < 0.001) \). Compared with IR30/60, GSH preconditioning (Gly-IR30/60) significantly protected mitochondrial viability during IR \( (P < 0.001, \text{TMCT}) \). Data are expressed as means ± SEM.

**Figure 2:** FACS analysis of \( \Delta \Psi_m \) in energized mitochondria and after uncoupling with CCCP: values reflect the ratio of J-aggregate\(^+\) mitochondria and JC1\(^+\) mitochondria. Compared with controls, membrane potential \( (\Delta \Psi_m) \) of energized mitochondria demonstrated hyper-polarization during IR (IR30/60). After uncoupling with CCCP, \( \Delta \Psi_m \). \( \Delta \Psi_m \) declined faster in IR30/60 mitochondria when compared with controls. Gly preconditioning (Gly-IR30/60) prevented hyper-polarization and accelerated decline after CCCP compared with IR30/60. Differences between groups reached statistical significance at 3 min after CCCP addition \( (P < 0.01) \). Data are expressed as means ± SEM.
Gly preconditioning did not ameliorate IR-related loss of Cyt C

Compared with control, Cyt C content was significantly reduced in IR30/60 (95% CI: 6.22 ± 7.90 vs. 2.50 ± 4.96, P < 0.05, TMCT). The Cyt C loss remained evident in Gly-IR30/60 when compared with IR30/60 (95% CI: 3.28–4.13 vs. 2.00–4.96, P > 0.05, TMCT).

Gly preconditioning did not protect from IR-induced mitochondrial destabilization

Compared with sham, IR30/60 demonstrated impaired mitochondrial stability, expressed by CSR (2.97 ± 5.64 vs. 0.55 ± 2.85, P < 0.01, TMCT). In Gly-IR30/60, CSR did not improve, when compared with IR30/60 (0.90 ± 1.76 vs. 0.55 ± 2.85, P > 0.05, TMCT).

Gly preconditioning limited IR-induced MMP-9 activation

In control, MMP-9 activity was low, with significant MMP-9 activation in IR30/60 (P < 0.001, TMCT; Fig. 3 left column). In Gly-IR30/60, MMP-9 activation was significantly ameliorated (P < 0.001, TMCT) after IR. Differences between Gly-IR30/60 and Sham remained insignificant (P > 0.05, TMCT).

Gly preconditioning did not inhibit neutrophil granulocytes sequestration

Between control and sham, differences of MPO activity remained insignificant (95% CI: 0.002–0.006 vs. 0.000–0.006, P > 0.05, TMCT). When compared with sham, MPO was substantially elevated in IR30/60 (95% CI: 0.011–0.031, P < 0.05, TMCT) and Gly-IR30/60 (95% CI: 0.008–0.035, P < 0.05, TMCT). Between Gly-IR30/60 and IR30/60, significant differences remained undetectable (P > 0.05, TMCT).

Gly preconditioning ameliorated pulmonary oedema formation

Regarding control, TWC consisted of 81.0% (95% CI: 78.9–83.1%) and of 80.6% (95% CI: 78.5–82.5%) in sham, respectively (Fig. 3, right column). In IR30/60, TWC accumulated to 86.9% (95% CI: 85.9–87.8%). Gly preconditioning limited TWC to 85.2% (95% CI: 84.5–85.8%). When compared with sham, TWC differed significantly in IR30/60 (P < 0.001, TMCT) and Gly-IR30/60 (P < 0.001, TMCT). Differences between IR30/60 and Gly-IR30/60 trended towards significance (95% CI: 84.5–85.8 vs. 85.9–87.8%).

Gly preconditioning did not inhibit pulmonary glutathione oxidation

GSH/GSSG in control (95% CI: 4.3–20.1) equalled ratios of sham (95% CI: 3.4–21.1). IR30/60 revealed a trend towards GSH/GSSG ratio decay (95% CI: 7.3–11.3). In Gly-IR30/60, IR-induced GSH oxidation was not ameliorated when compared with IR30/60 (95% CI: 5.0–13.6 vs. 7.3–11.3).

DISCUSSION

The previously described cyto-protective effect of Gly during pulmonary IR is poorly understood. This study demonstrates Gly-dependent amelioration of IR-induced mitochondrial injury and tissue decay. During IR, Gly preconditioning protected mitochondrial survival, ΔΨm and complex II-function and inhibited MMP-9 activation. However, it did not stabilize mitochondrial integrity or prevent from oxidation of GSH into GSSG. It also did not inhibit neutrophil sequestration after IR.

In rabbits, Sheth et al. [9] demonstrated that Gly-dependent improvement of the Cyt C oxidase activity, combined with an ameliorated cellular damage, improved micro-circulation and lowered cytokine liberation after liver IR. He concluded that Cyt C oxidase activity preservation resulted from a Gly-protected microcirculation, stabilizing electron transport to O2. In our previous study, we also have detected a Gly-dependent protection of pulmonary perfusion, supporting the findings of Sheth [1]. Ruiz-Meana et al. [10] demonstrated Gly-related protection from re-oxygenation-induced cell death in cardiomyocytes. During ischemia, cellular acidosis develops. With the initiation of reperfusion, a rapid washout of cellular acidic valences occurs, resulting in opening of the membrane transition pore (mMTP), followed by subsequent caspase activation and initiation of apoptosis [11]. It has been demonstrated that either an acidotic intra-cellular pH or an increased pyruvate level inhibit mMTP activation. Regarding Gly, our data of the mitochondrial swelling assay indicated an improved mitochondrial survival. Our results completely match those of Ruiz-Meana et al. [10]. Interestingly, they demonstrated a pH-independent pathway of Gly-mediated mMTP inhibition, since restoration of the acidic intra-cellular pH remained unaffected. In contrast, Kim et al. [12] demonstrated in isolated hepatocytes that induction of mitochondrial-dependent apoptosis remained unchanged by Gly, but necrotic cell death was substantially suppressed. We also observed improved mitochondrial survival after Gly-preconditioning but not a reduced Cyt C liberation. Hence, our data support the

Figure 3: Left column: activity of MMP-9 determined by gelatin in vitro zymography. Control and sham did not differ significantly (P > 0.05). Differences between control or sham and IR30/60 reached a statistically significant level (P < 0.01; P < 0.01). Gly pre-conditioning effectively reduced MMP-9 activity during IR when compared with IR30/60 (P < 0.001). Values of IR30/60 equalled values of sham (P > 0.05). Data are expressed as means ± SEM. Right column: tissue water content determined from wet to dry weight ratio: tissue water content in either control or sham group remained low and was substantially elevated during IR (P < 0.001 both). Gly pre-conditioning led to an insignificantly reduced tissue water accumulation during IR (P > 0.05).
conclusion of Kim Gly protecting from necrosis but not from mitochondrial-dependent induction of apoptosis. 

ΔΨm alterations are the initial step of mitochondria-induced apoptosis [13]. We demonstrated ΔΨm alteration during IR, ameliorated by Gly. Data reflecting on the direct influence of Gly on ΔΨm remain limited. Pal et al. [14] demonstrated a mercury (Hg)-induced ΔΨm decay, successfully ameliorated by Gly, in a dose-dependent fashion. Gly also reduced lipid peroxidation and protein carbonylation and limited GSH oxidation, an effect we did not observe. Although Hg-induced GSH oxidation differs from IR-induced GSH oxidation, results of our experiment to some extent con

From IR-induced GSH oxidation, results of our experiment to some extent conflict with those of Pal.

Petrat et al. [15] demonstrated Gly-dependent amelioration of intestinal IR-induced MPO activity. We achieved similar results in pulmonary tissue after GSH preconditioning, as described earlier [4]. However, in our current experiment, Gly failed to inhibit neutrophil sequestration. Petrat et al. [15] described a dose-dependency, hence a dose modification of administered Gly might ameliorate MPO activity in our experiment.

CONCLUSION

Gly preconditioning is a promising strategy to pulmonary IR injury. Gly preconditioning protects (i) complex II activity, (ii) protects mitochondrial viability, and (iii) ameliorates early steps of mitochondria-induced apoptosis during pulmonary IR injury. Gly protects from collagen IV decay and limits oedema formation. A dose modification might also result in protection from neutrophil sequestration. Following studies will detect for apoptosis and necrosis during pulmonary IR and the influence of Gly on the cell-death pattern.

ACKNOWLEDGEMENTS

The authors thank Renate Wahn, Daniel Schäfer, Marcus Lesitner, Bernhard Gohrbandt and Heidi Linß. This article contains work of the medical thesis of Vendela Grußman.

Conflict of interest: none declared.

Funding

This work was funded by grants of the Interdisciplinary Center for Clinical Research of the University Hospital Würzburg (IZKF-project A-132N, 2010; http://www.izkf.uni-wuerzburg.de/).

REFERENCES


APPENDIX. CONFERENCE DISCUSSION

Dr F. Melfi (Pisa, Italy): Your previous paper demonstrated that the use of glycine, either in the flush preservation solution or as a pre-treatment in a pig single-lung transplantation model, decreases the severity of ischemia/reperfusion injury after 24 hours of cold ischaemic preservation and after 7 reperfusion hours. This was primarily represented by improved oxygenation. In the present study you focused on the effect of glycine preconditioning on mitochondrial integrity with tissue degradation, oedema, and neutrophil sequestration during pulmonary ischemia/reperfusion. The glycine preconditioning appears to be a promising strategy to ameliorate pulmonary tissue and mitochondrial damage.

My first question is, do you consider these results suitable in a clinical setting in the very near future? Secondly, how do you consider the use of gene therapy as a possible future strategy in the transplantation setting?

Dr Sommer: First of all, you can use it for clinical observations, but you have to obtain fresh tissue and you have to analyse this immediately. This is a major limitation. Second, gene therapy increasing, for instance, antioxidants in the cell might be a beneficial strategy for sure, protecting, for instance, complex II responsible for ROS generation in this setting.

Dr Melfi: In the setting of lung transplantation.

Dr Sommer: Yes.