Circulating donor-specific cytotoxic T lymphocytes with high avidity for donor human leukocyte antigens in pediatric and adult cardiac allograft valved conduit recipients

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Abstract

Objective: Specific immunological responses may be involved in the process of cryopreserved allograft valved conduit (AVC) degeneration, which is more frequently seen in young recipients. Rejection of heart and corneal allografts is preceded by an increase in the fraction of cytotoxic T lymphocytes (CTL) with high avidity for donor human leukocyte antigens (HLA) circulating in both peripheral blood and the affected graft. These donor-specific high-avidity CTLs are regarded as the destructive cells capable of causing graft damage. To monitor the precursors of these cells (CTLp) in young and adult AVC recipients, in vitro quantitative tests were performed on sequentially taken blood samples to quantify CTLp frequencies and their avidity for donor antigens.

Method: Six children and nine adults who received a cryopreserved AVC in the period between 1994 and 1997 were included in the study. From these patients, two to six blood samples were obtained up to 3 years after valve implantation. The number of circulating CTLp present within the peripheral blood mononuclear cell (PBMC) population was determined by limiting dilution analysis (LDA). The fraction of CTLp with high avidity for donor HLA class I was determined by addition of CD8 monoclonal antibodies (mAb) during the cytotoxic phase of the assay. Third-party stimulator cells were used to verify the donor-specificity of the response.

Results: The number of donor-specific CTLp increased significantly in the period 6–12 months after AVC implantation, while third-party-specific CTLp frequencies were not affected. Additionally, we found a significant increase of the high-avidity fraction of CTLp directed against donor antigens as early as during the first 6 months after AVC implantation. The fraction of high-avidity CTLp remained significantly higher post- compared with pre-implantation, even after 12 months. We observed no significant difference in the kinetics of CTLp frequencies between pediatric and adult AVC recipients. Conclusion: Implantation of cryopreserved human AVC induces an increase in the total number of circulating CTLp directed against donor HLA class I in both adults and children. The shift towards more destructive high-avidity CTLp in the peripheral blood indicates their potential damaging effect towards the heart valve allograft.

Keywords: Immune response; Cytotoxic T lymphocyte; Cardiac allograft valved conduit; Child; Adult

1. Introduction

Allograft valved conduits (AVC) or heart valve allografts have been used in the last three decades as biological valve prosthesis in the surgical treatment of acquired or congenital heart valve diseases. Several follow-up studies of AVC recipients have revealed good clinical results in the adult population, although in the long run the majority of the grafts showed primary tissue failure [1]. In the pediatric population however, the observed early onset of graft failure resulting in allograft replacement is a serious problem [2]. A specific immunological response of the recipient against the AVC has been suggested as one of the main causes for allograft degeneration, because AVC implantation is performed without HLA or blood group matching and in the absence of immunosuppressive therapy. Morphological studies of early and late AVC explants have demonstrated the involvement of immune competent cells like T and B lymphocytes as well as macrophages in the valve leaflets and arterial wall of AVC [3,4]. Induction of donor-specific antibodies against HLA class I and II antigens after AVC implantation also confirms activation of cellular and
humoral immune responses [5]. A causal relationship between an active immune response of the recipient and rejection of the allograft is difficult to prove, since histological confirmation of rejections are not possible.

In heart transplantation, the fraction of precursor cytotoxic T lymphocytes (CTLp) with high avidity for donor antigens within the total CTLp population, was found to be increased in peripheral blood of the recipients and in myocardial tissue during rejection [6]. A similar increase in the fraction of donor-specific high-avidity CTL was found in patients rejecting their corneal grafts [7]. Therefore, these CTLp with high avidity for donor antigens are regarded as the major effector cells capable of causing graft damage. Since there is a difference in clinical outcome between pediatric and adult AVC recipients it has been suggested that children are able to mount a more vigorous immune response [2,3].

In an attempt to explain the possible differences in immune response between children and adults, which could lead to destruction of a human AVC, we studied the kinetics of the frequency of CTLp and their avidity in peripheral blood of pediatric and adult AVC recipients.

2. Materials and methods

2.1. Cardiac allograft valved conduit

Aortic and pulmonary allograft valved conduits (AVC) consisting of the complete arterial root including semilunar valves, arterial wall and a minor muscular rim were obtained from multi-organ donors and non-heart beating donors within 24 h after circulatory arrest and from so called domino hearts from heart transplant recipients. Sterile preparation of the AVC was in conformity with the standard operating procedures of the Heart Valve Bank Rotterdam, The Netherlands. After dissection, the AVC were sterilized for 6 h at 37°C in Medium 199 (Bio-Whittaker, Verviers, Belgium) containing: 12 μg/ml vancomycin (Eli Lilly), 30 μg/ml flucytosin (Hoechst Pharma), 12 μg/ml amikacin (Bristol-Myers-Squibb), 12 μg/ml metronidazol (Rhone-Poulenc-Rorer) and 3 μg/ml ciprofloxacin (Bayer). Subsequently, the allografts were cryopreserved according to the standard cryopreservation protocol of the Heart Valve Bank Rotterdam (−1°C/min) in Medium 199 with 10% dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) and stored in the vapor phase of liquid nitrogen (−150 to −180°C). Before implantation, the allografts are rapidly thawed in a 37°C Medium 199 bath followed by stepwise dilution of the DMSO from the tissue in cold Medium 199.

2.2. Patients

Six children (aged under 16 years) and nine adults (older than 16 years), who received a cryopreserved cardiac AVC between June 1994 and September 1997, were included in the study after informed consent. The average age of the patients in the adult group was 40.2 years (range 25–57), and 9.3 years (range 3–16) in the children group. In the adult group, four patients received a cryopreserved aortic AVC and five patients an allograft of pulmonary origin. In all pediatric recipients, cryopreserved pulmonary AVC were used for reconstruction of the right ventricular outflow tract. Tables 1 and 2 summarize the characteristics of the patients and the indication for AVC implantation. During this study none of the patients were treated with long-term immunosuppressive therapy. The Medical Ethical Committee of the University Hospital Rotterdam and the Medical Faculty of the Erasmus University Rotterdam approved this study.

2.3. Peripheral blood mononuclear cells (PBMC) and spleen cell sampling

From each patient, two to six heparin-treated blood samples were obtained at different time points up to 3 years after AVC implantation. The first blood sample was

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Valve origin</th>
<th>Prim./sec. allograft</th>
<th>Blood transfusion</th>
<th>Indication</th>
<th>Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.E. M</td>
<td>52</td>
<td>Aorta</td>
<td>Primary</td>
<td>10 Ec200 Tc b</td>
<td></td>
<td>Bicuspid aortic valve insuff.</td>
<td>Root replacement</td>
</tr>
<tr>
<td>B.Y. M</td>
<td>57</td>
<td>Aorta</td>
<td>Primary</td>
<td>6 Ec1 Tc</td>
<td></td>
<td>Calcified tricuspid aortic valve insuff.</td>
<td>Root replacement</td>
</tr>
<tr>
<td>W.Y. F</td>
<td>41</td>
<td>Pulm.</td>
<td>Primary</td>
<td>3 Ec1 Tc</td>
<td></td>
<td>Fallot</td>
<td>Valve conduit reconstr. RVOT</td>
</tr>
<tr>
<td>R.Y. M</td>
<td>50</td>
<td>Aorta</td>
<td>Primary</td>
<td>5 Ec1 Tc</td>
<td></td>
<td>Bicuspid aortic valve insuff.</td>
<td>Root replacement</td>
</tr>
<tr>
<td>S.T. M</td>
<td>24</td>
<td>Pulm.</td>
<td>Primary</td>
<td>14 Ec3 Tc</td>
<td></td>
<td>Pulm. atresia and VSD</td>
<td>Valve conduit reconstr. RVOT</td>
</tr>
<tr>
<td>J.G. M</td>
<td>41</td>
<td>Pulm.</td>
<td>Primary</td>
<td>6 Ec1 Tc</td>
<td></td>
<td>Fallot</td>
<td>Valve conduit reconstr. RVOT</td>
</tr>
<tr>
<td>H.R. M</td>
<td>42</td>
<td>Aorta</td>
<td>Primary</td>
<td>5 Ec1 Tc</td>
<td></td>
<td>Bicuspid aortic valve insuff.</td>
<td>Root replacement</td>
</tr>
<tr>
<td>V.R. F</td>
<td>30</td>
<td>Pulm.</td>
<td>Primary</td>
<td>2 Ec1 Tc</td>
<td></td>
<td>Fallot</td>
<td>Valve conduit reconstr. RVOT</td>
</tr>
<tr>
<td>V.E. M</td>
<td>25</td>
<td>Pulm.</td>
<td>Secondary</td>
<td>10 Ec3 Tc</td>
<td></td>
<td>Aortic allograft stenosis and insuff.</td>
<td>Ross procedure</td>
</tr>
</tbody>
</table>

Average Male: 7; 40.2; female: 2; 5; 8; aortic: 4; sec: 1

a Ec, erythrocyte concentration: 300 ml per unit.
b Tc, platelet concentration: 5 donors per unit.
obtained immediately before surgery. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and slowly frozen in 10% DMSO (Sigma) and 10% pooled human serum containing RPMI 1640 medium (Bio-Whittaker) up to $-80^\circ$C and transferred to $-140^\circ$C storage, awaiting functional in vitro tests.

Spleen cells were obtained by mechanical dissociation of small pieces of spleen from the AVC donor through a sieve in RPMI 1640 medium (Bio-Whittaker) containing 10 μg/ml DNase (Boehringer Mannheim, Mannheim, Germany) to prevent aggregation of cells. Subsequently, the cell suspension was filtrated through a 40-μm cell strainer (Falcon, Franklin Lakes, NJ) and washed. Thereafter, the spleen cells were centrifuged over a Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient, collected, washed and stored at $-80^\circ$C.

### 2.4. Limiting dilution analysis

Limiting dilution cultures were set up in 96-well U-bottom culture plates (Nunclon, Roskilde, Denmark) [8]. Twenty-four replicates of graded number of responder cells (recipient PBMC) were titrated in 7 steps of 1:1 dilutions starting from $8 \times 10^4$ to $625 \text{ PBMC per well}$. To study the specificity of the cytotoxic response two sets of dilution series were performed. In one series $8 \times 10^4$ irradiated (45 Gy) donor or donor HLA-matched (donor-like) spleen cells were added as stimulator cells. In the other series $8 \times 10^4$ irradiated (45 Gy) donor HLA-mismatched (third-party) spleen cells per well were used as stimulator cells. All wells contained a total volume of 200 μl culture medium, consisting of RPMI 1640 Dutch Modification (Bio-Whittaker), supplemented with 2 mM l-glutamine (Gibco BRL, Scotland, UK), 100 IU/ml penicillin (Bio-Whittaker), 100 μg/ml streptomycin (Bio-Whittaker) and 10% pooled human serum. After 3 days of culturing, 100 μl of the culture medium was refreshed with culture medium supplemented with 40 units/ml recombinant human interleukin-2 (Proleukin; Chiron BV, Amsterdam, The Netherlands).

### 2.5. Cell-mediated lymholyis

After 10 days, the microcultures were split in two equal parts (80 μl). One half of the split wells was tested for cytotoxicity in the absence of CD8 monoclonal antibodies (mAb) and the other half was tested in the presence of CD8 mAb. Each well was individually tested for cytolytic activity against 5 × $10^5$ Europium (Eu-DTPA; Fluka, Buchs, Switzerland and Sigma, St. Louis, MO) labeled target cells [9]. After donor stimulation, T-cell blasts of donor origin were used as targets, whereas third-party stimulated responder PBMC received T-cell blasts of third-party origin. After 4 h of incubation at $37^\circ$C in a humidified atmosphere with 5% CO₂, 20 μl of supernatant was harvested and transferred into 96-well flat-bottom microtiter plates with low background fluorescence (Fluoromunoplate, Nunclon). Subsequently, 100 μl Enhancement solution (Wallac, Turku, Finland) was added to each well and the fluorescence of the released Europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, Wallac, Finland). Fluorescence was expressed in counts/s. As a control for each target cell, spontaneous lysis (target cells + culture medium) and maximum lysis (target cells + 1% Triton X-100) was determined. The fraction of CTLp with high avidity for donor or third-party HLA class I antigens was calculated using the formula:

$$% \text{CTLp with high avidity} = 100% \times \frac{\text{CTLp frequency with CD8}}{\text{CTLp frequency without CD8}}$$

### 2.6. Target cells

T-cell blasts were obtained by culturing donor or third-party spleen cells for 7 days in culture medium supplemented with 1% phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI) and 10% v/v lymphocult-T (Biotest AG, Dreieich, Germany). Then, the T-cell blasts were labeled with Europium as reported previously by Bouma et al [9] These T-cell blasts can be used to determine donor HLA class I (but not class II) directed cytotoxicity [6].
2.7. CD8 inhibition

FK18 (gift of Dr. F. Koning, Department of Immunohematology and Blood Bank, University Hospital Leiden, The Netherlands) is a mouse anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule [10]. In this study, a concentration of approximately 1 μg IgG/well of FK18 mAb obtained from culture supernatant of the Tecnomouse system (Tecnomara-Integra Bioscience, Wallisellen, Switzerland) was used. This concentration inhibited the cytotoxic capacity of CD8-dependent CTLp clones but had no effect on cell lysis by CD8-independent CTLp clones [6].

2.8. Statistical analysis

Experimental wells were scored positive, if the counts of the cell well exceeded the mean counts/s + 3 × SD of the 24 wells in which only stimulator cells were present. For each responder cell concentration the number of negative wells were determined and used to calculate the frequency with a statistical program designed by Strijbosch et al. [11]. The CTLp frequency (expressed as the number of CTLp per 10⁶ PBMC) and the 95% confidence interval (CI) were calculated by the Jackknife procedure for maximal likelihood. The post-operative follow up is divided into three periods: 1–6 months, 6–12 months and > 12 months. When a time period included multiple blood samples of one patient the average of the analyzed frequencies was calculated. The significance of differences in CTLp frequencies or the percentage of high-avidity CTLp against donor or third-party antigens at different periods after AVC implantation was analyzed by the Mann–Whitney U-test. The paired Wilcoxon signed rank test was used to evaluate the frequency kinetics in both children and adult group. Two-sided P-values of 0.05 or less were considered significant.

3. Results

3.1. Follow-up of valve allograft recipients

From one adult (V.N.) and one pediatric (V.M.) AVC recipient the follow-up period was less than 6 months, because they stopped visiting the outpatient department at 3.5 months (Table 3). The mean follow-up period of the adult patients was 19.8 months (range 3.5–36.5 months) and 19.0 months (range 4.7–35.5 months) for the pediatric group. From two pediatric patients (H.Z., B.X.) we did not receive blood samples in the period 1–6 months and 6–12 months, respectively. From one adult patient (H.R.) the 1–6 months blood sample was not available. At the time of completion of this study all patients are still alive.

3.2. CTLp frequency in peripheral blood

The number of donor-specific CTLp per million PBMC, the CTLp frequency, of all 15 patients increased in time compared with the pre-operative value. This was not related to the amount of blood transfusions each patient received (Table 1). In the period 6–12 months after AVC implantation the CTLp frequencies were maximal and significantly higher than before implantation (P = 0.01; Table 3). In 12 patients the pre-implantation CTLp frequencies were below 50, and for three AVC recipients (H.R., S.T. and V.M.), they exceeded 100 CTLp per million PBMC. No significant increase of CTLp frequencies against third-party antigens was observed at 1–6 (P = 0.82), 6–12 (P = 0.41) and > 12 (P = 0.89) months after implantation (Fig. 1b), indicating the donor specificity of the CTLp response.

3.3. Fraction of CD8-independent (high-avidity) CTLp

Similar to the total frequency of donor-specific CTLp, the fraction of CTLp with high avidity for donor antigens varied between the 15 patients (Fig. 2). In 13 out of 15 AVC recipients (86%) the percentage of donor-specific high-avidity CTLp post-operatively was higher compared with pre-implantation values. It should be noted that from the two patients (V.N., V.M.) in whom the fraction high-avidity CTLp remained unchanged, the follow-up period was incomplete (Table 3). The highest levels were observed at 6–12 months (P < 0.0001, Table 3). After the first 12 months the fraction high-avidity CTLp declined in five patients (Table 3). Nevertheless, the median value remained significantly higher compared with the pre-operative fraction (P = 0.01; Table 3).

Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre: ab</th>
<th>1–6 months (%)</th>
<th>6–12 months (%)</th>
<th>&gt; 12 months (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.E.</td>
<td>31/0</td>
<td>29/83</td>
<td>73/80</td>
<td>66/92</td>
</tr>
<tr>
<td>B.Y.</td>
<td>5/–</td>
<td>26/85</td>
<td>43/95</td>
<td>33/36</td>
</tr>
<tr>
<td>WY</td>
<td>35/26</td>
<td>50/56</td>
<td>83/78</td>
<td>40/35</td>
</tr>
<tr>
<td>R.Y.</td>
<td>41/46</td>
<td>67/70</td>
<td>79/95</td>
<td>55/49</td>
</tr>
<tr>
<td>S.T.</td>
<td>11/9</td>
<td>70/86</td>
<td>111/56</td>
<td>155/59</td>
</tr>
<tr>
<td>J.G.</td>
<td>18/33</td>
<td>73/33</td>
<td>51/100</td>
<td>107/88</td>
</tr>
<tr>
<td>V.R.</td>
<td>37/38</td>
<td>65/29</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>V.E.</td>
<td>9/–</td>
<td>17/65</td>
<td>98/70</td>
<td>123/88</td>
</tr>
<tr>
<td>B.U.</td>
<td>44/14</td>
<td>59/41</td>
<td>n.d.</td>
<td>41/44</td>
</tr>
<tr>
<td>H.A.</td>
<td>30/50</td>
<td>n.d.</td>
<td>46/91</td>
<td>n.d.</td>
</tr>
<tr>
<td>M.L.</td>
<td>5/–</td>
<td>12/42</td>
<td>30/33</td>
<td>15/20</td>
</tr>
<tr>
<td>K.H.</td>
<td>47/26</td>
<td>77/91</td>
<td>77/81</td>
<td>59/83</td>
</tr>
<tr>
<td>S.T.</td>
<td>126/74</td>
<td>220/61</td>
<td>596/82</td>
<td>n.d.</td>
</tr>
<tr>
<td>V.M.</td>
<td>236/32</td>
<td>419/31</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Median</td>
<td>30/5.32</td>
<td>51/62.5</td>
<td>75/80.5</td>
<td>66/59</td>
</tr>
</tbody>
</table>

* a, CTLp frequency, i.e. number of donor-specific CTLp per million PBMC. b, fraction of high-avidity CTLp expressed as percentage of the total CTLp frequency. n.d., not determined; −, not calculated because the CTLp frequency (a) was below 10 CTLp per million PBMC.

b Patients with 3 months follow-up.
3.4. Comparison of the frequency kinetics between pediatric and adult patients

The paired Wilcoxon signed rank test was used to determine the frequency kinetics of each patient group. In both pediatric and adult AVC recipients the CTLp frequencies increased in time (Fig. 1a). Statistical analysis of the pre- and highest post-implantation values revealed P-values of 0.03 and 0.004 for children and adults, respectively. The kinetics in pediatric AVC recipients was comparable to that in adults, because the absolute increase of the CTLp frequency after AVC implantation (i.e. ΔCTLp frequency) in children and adults was not significantly different during the three time periods, i.e. first and second 6 months and after the first year (Mann–Whitney U-test; \( P = 0.44, P = 0.21, P = 0.07 \), respectively).

In the adult group the median fraction donor-specific high-avidity CTLp increased significantly \(( P = 0.008 )\), whereas in the children group the increase was almost significant \(( P = 0.06 )\) due to the small numbers of patients.

Comparison of the delta (Δ) increases of the fraction high-avidity CTLp in children versus adults, using the Mann–Whitney U-test, showed no significant differences in the increase during the three observation periods \(( P = 0.13, P = 0.08, P = 0.80 )\).

4. Discussion

Since the introduction of cardiac AVCs as biological substitutes in valve replacement surgery, the risk of tissue degeneration due to immunological reactions has been a matter of debate. Viability preservation of AVCs has been suggested to improve durability [12], while others promote preservation of the non-vital collagenous network as an important criteria for prolonged allograft durability [13]. Cryopreservation represents a cell- and tissue-protective preservation method, which preserves the cellular viability as well as the stromal structure. The question regarding the loss of the endothelium expressing HLA class I and II molecules remains unclear, since conflicting results have been
Finally, Hoekstra et al. demonstrated donor-specific aortic valve explants were free from any cellular component preserved pulmonary valve allograft explants, while the within less than 8 months after implantation [4]. Others valve allografts explanted due to structural failure eration [13]. On the other hand, Rajani and colleagues endothelial or connective tissue cells. They suggested that valve allografts, which all appeared non-viable without morphological studies of clinical valve allograft explants. [5]. However, contradictory reports have clonotypic for donor HLA class I antigens. The preferential promote an increase in the number of circulating CTLp and its high-avidity fraction within pediatric and adult patients. The small number of pediatric patients and the lack of patients, who reached defined end of analysis (i.e. re-operation or death due to severe valve degeneration) are both definite limitations of this single-center study. Apart from expanding the study population, further studies concerning the clinical consequences of such immunological activity have to be performed using relevant clinical parameters for structural valve failure [24]. In conclusion, cryopreserved cardiac AVCs are able to promote an increase in the number of circulating CTLp specific for donor HLA class I antigens. The preferential expansion of the high-avidity fraction of CTLp that we have found, may reflect an ongoing process of cell-mediated destruction of the valve allograft tissue in situ, which ultimately may lead to or contribute to primary structural failure. We observed no differences in the kinetics of CTLp frequency between pediatric and adult recipients. 

Acknowledgements

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References


Appendix A. Conference discussion

**Mr J. Pepper (London, UK):** You have shown an increase in donor-specific cytotoxic cells, and then you say that they are responsible for damage to the homograft. Allografts tend to deteriorate in the manner of calcification, but have you actually demonstrated a link between these cytotoxic cells and cell damage? It is still a hypothesis and you have not really demonstrated histological damage directly due to these cytotoxic T cells, have you?

**Dr Oei:** No, we did not. Our next step of the study is to correlate the data found in the present study with the clinical parameters that may resemble rejection of valve allografts. But we make this statement based on an indirect correlation. What we have seen in heart transplantation and corneal transplantation is that during a histological proven rejection episode, these high-avidity cytotoxic T lymphocytes are increased in the peripheral blood, and based on what we have seen here, we suppose that these destructive cells are attacking the allograft.

**Dr C. Yankah (Berlin, Germany):** Congratulations for this interesting study and presentation. I would like to know whether you could detect anti-endothelial antibodies and whether your homografts actually had viable endothelial cells, and whether you could also explain how long these cytotoxic T lymphocytes remain in the patients and whether you found in your explants, anti-endothelial antibodies at your immunohistological studies?

**Dr Oei:** Our rate of explants is at this moment very low, so we are at this moment still collecting material to investigate. So I hope in the future to come back with an answer on this question.

**Dr M. Antunes (Coimbra, Portugal):** From the clinical point of view, this means viability of the cells, I suppose.

**Dr Oei:** Yes, there must be viable cells present in the valve allografts that are expressing at least HLA class I antigens for inducing such a cellular immune response.

**Dr Antunes:** Would it be different if you had non-viable homografts such as those preserved at 4°C, for example?

**Dr Oei:** At our valve bank in Rotterdam we have a cryopreservation technique which is standardized, which includes 6 h incubation with a cocktail of antibiotics at 36°C, and it seems that this cryopreservation method is leaving the allograft viable.

**Dr Antunes:** Should we start immunosuppressing our patients?

**Dr Oei:** I think it is a little bit too early to do that.