Nephrol Dial Transplant, 2023, **0**, 1–4
https://doi.org/10.1093/ndt/gfad159
Advance access publication date: 17 July 2023

Advanced flow cytometric detection of endothelial cell chimerism in kidney transplants

Daphne M. Hullegie-Peelen, Martin J. Hoogduijn, Marjolein Dieterich, Dennis A. Hesselink and Carla C. Baan

Department of Internal Medicine, Nephrology and Transplantation, Erasmus MC Transplant Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands
Correspondence to: Daphne M. Hullegie-Peelen; E-mail: d.peelen@erasmusmc.nl

The prevalence and clinical implications of endothelial cell (EC) chimerism in solid organ transplants, including kidneys, is an area of ongoing debate [1]. It is hypothesized that EC chimerism may affect allograft rejection, immunological tolerance and long-term graft survival, but the extent of EC chimerism and the factors contributing to it in kidney transplants have not been fully established [2–6]. Efforts to investigate EC chimerism have been hampered by the lack of reliable and quantitative detection methods [1]. Here we present a technically improved method by using a flow cytometry-based approach. Previous studies have employed semi-quantitative approaches to assess EC chimerism, including immunohistochemistry (IHC), fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR). These methods have primarily focused on detecting recipient/donor discrepancies based on human leucocyte antigens (HLAs), blood group ABO antigens and sex [2–8]. However, IHC and FISH are limited by their low sensitivity, as illustrated by a Y-chromosome detection yield of only 40% in positive control samples [8, 9]. Also, interpretation of IHC and FISH can be subjective and the lack of standardized protocols has made it difficult to compare results between studies. While PCR offers enhanced sensitivity and quantification compared with IHC and FISH, its specificity for the target cell type is a limiting factor when working with tissue samples that consist of multiple cell types. For instance, when PCR is preceded by microdissection to isolate the target cell type from the tissue, there is a significant risk of including adjacent cell types, leading to potential contamination [7, 8]. In light of these limitations, there is a need for an improved method that can accurately detect and quantify EC chimerism in kidney transplants while also allowing for functional and phenotypic characterization of the EC population. This would provide essential information on the occurrence, extent and features of EC chimerism after kidney transplantation and contribute to the evaluation of its impact on graft outcomes.

Here, a flow cytometry-based approach was used to determine and quantify EC chimerism in kidney transplant nephrectomy specimens (Fig. 1A). Kidney transplant nephrectomies were collected in accordance with previously reported protocols at the Erasmus MC, University Medical Center, Rotterdam, The Netherlands, between December 2016 and August 2022 (Table 1, Fig. 1A) [10]. In brief, halved kidneys were obtained and dissected into small pieces, followed by enzymatic digestion with collagenase IV (1.1 mg/ml; Serva, Heidelberg, Germany), a series of filtration steps and Ficoll density gradient centrifugation [10]. Isolated cells were cryopreserved in liquid nitrogen (−195°C) until further use. On the day of the flow cytometry experiments, the cryopreserved cells were thawed and stained with fluorescently labelled antibodies against CD45 (haematopoietic cells), CD31 (endothelial cells) and CD144 (endothelial cells) (Fig. 1B). For the discrimination between donor and recipient, the HLA mismatches were identified for each sample. Subsequently, fluorescent antibodies against donor and/or recipient HLA class I were either commercially purchased or developed at the Leiden University Medical Center (Leiden, The Netherlands) and kindly provided by Dr S. Heidt [11]. Additionally, a live/dead marker was included. Detailed information for all antibodies used is provided in Supplementary Table 1. Following staining, the cells were measured on a FACSymphony A3 Cell Analyzer (BD Biosciences, Franklin Lakes, NJ, USA) and analysed with Kaluza Analysis 2.1 software (Beckman Coulter, Brea, CA, USA). The Kolmogorov–Smirnov test was used to examine the distribution of the data. An unpaired two-tailed *t*-test was used for comparison of two groups in normally distributed data and the Mann–Whitney U test was used for data that was not normally distributed. *P*-values $<.05$ were considered to be statistically significant.

Nineteen kidney transplant nephrectomies were included in this study. Kidney transplants were mainly explanted due to untreatable acute or chronic rejection (58%) (Table 1). All kidneys had ceased functioning at the time of explantation. Recipients were either on dialysis prior to nephrectomy or initiated dialysis shortly thereafter. On average, 9627 living kidney–derived endothelial cells (range 366–35 219 cells) were acquired per sample for flow cytometric analysis. In 11 of 19 (58%) kidney transplants, EC of both donor and recipient origin were detected (Fig. 1B and C). The proportion of recipient-derived cells within the EC population varied considerably, with a median proportion of 1.5% (range 0.2–69.3%; Fig. 1C). To confirm that the HLA antibodies are able to discriminate between donor and recipient origin, the origin of $CD45^+$ lymphocytes was investigated. This analysis showed that $CD45^+$ lymphocytes were predominantly derived from the recipient, as expected (Fig. 1B). We then investigated whether clinical characteristics could explain the variation in the degree of EC chimerism. A significant association was observed between the time elapsed since transplantation and the presence of EC chimerism. Specifically, EC chimerism was only detected in kidneys that were explanted >100 days after transplantation (Fig. 1D). Furthermore, EC chimerism was negatively associated with recipient age, with

Received: June 1, 2023; Editorial decision: July 8, 2023

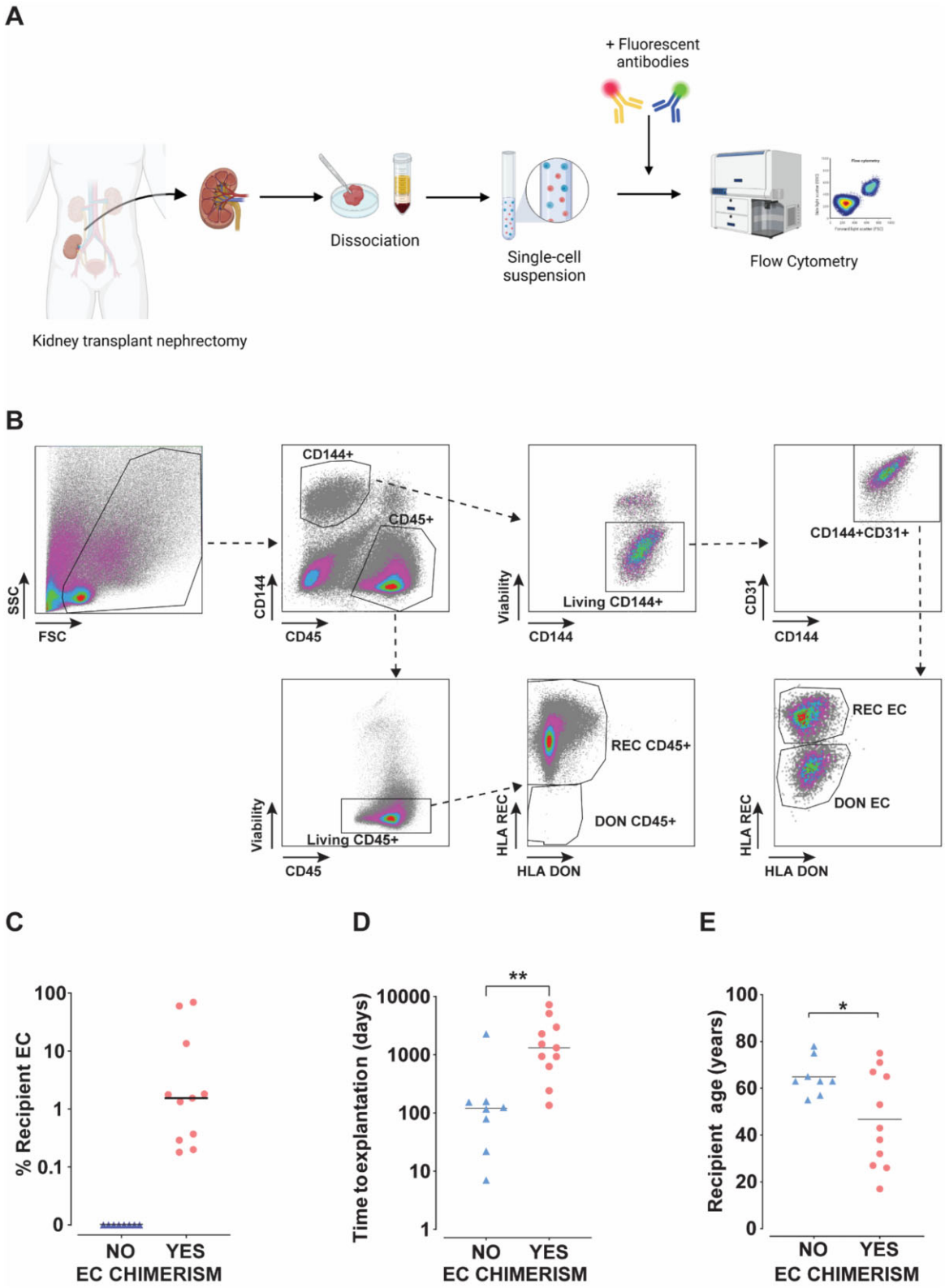

Figure 1: EC chimerism in kidney transplants assessed with a flow cytometry–based approach. **(A)** Overview of the experimental procedure. Created with BioRender.com. **(B)** Gating strategy for assessing the origin of EC and CD45$^+$ lymphocytes in kidney transplant nephrectomy specimens. **(C)** Prevalence of EC chimerism in the 19 included samples. Among the samples with EC chimerism, the median proportion of recipient-derived cells was 1.54%. **(D)** Time between transplantation and explantation in non-chimeric versus chimeric samples (median 121 and 1313 days, respectively; Mann Whitney U test $P = .003$). **(E)** Recipient age in non-chimeric versus chimeric samples (65 and 47 years, respectively; unpaired t-test $P = .03$).

Table 1: Baseline characteristics of kidney transplant nephrectomies ($N = 19$)

Variables	Values
Recipient characteristics	
Recipient age at nephrectomy (years), median (IQR)	63 (41–66)
Female, *n* (%)	8 (42)
First transplant, *n* (%)	15 (79)
Primary kidney disease, n (%)	
Hypertensive nephropathy	3 (16)
Diabetic nephropathy	5 (26)
Glomerulonephritis	2 (11)
Polycystic kidney disease	2 (11
Reflux nephropathy	1 (5)
Other	5 (26)
Unknown	1 (5)
Transplant characteristics	
Donor age (years), median (IQR)	63 (54–71)
Donor type, *n* (%)	
Living	5 (26)
Donation after brain death	6 (32)
Donation after circulatory death	8 (42)
HLA mismatches broad (HLA-A, -B and -DR), median *n* (IQR)	4 (3–5)
Time to explantation (days), median (IQR)	629 (130–1891)
Reason for removal, *n* (%)	
Ongoing rejection	11 (58)
To create space for a new kidney transplant	3 (16)
Other	5 (26)

*IQR = interquartile range.

EC chimerism consistently observed in recipients <50 years of age (Fig. 1E). Recipients' sex, a history of delayed graft function (DGF), a history of acute rejection, the length of cold ischaemia time during the transplantation procedure, donor age and donor type (living donation versus donation after brain death versus donation after circulatory death) were not associated with the presence or the degree of EC chimerism.

In this study we assessed an improved method for the detection of EC chimerism in kidney transplants that is both sensitive, specific and quantitative. The flow cytometry–based approach reported here overcomes the limitations of previously used methods, such as IHC, XY chromosome FISH and PCR. Additionally, flow cytometry allows for in-depth characterization of EC, such as measuring progenitor markers, activation markers or cytokines, which may contribute to unravelling the process of EC chimerism. Here we observed a prevalence of EC chimerism of 58% in kidney transplant nephrectomies. Consistent with earlier observations, a significant association existed between the time elapsed since transplantation and the presence of EC chimerism, supporting the notion that the development of EC chimerism requires a certain duration of time [6]. This finding is in line with the observation that 'early' factors such as the length of cold ischaemia and the occurrence of DGF were not associated with the development of EC chimerism. Furthermore, we observed an evident association between recipient age and EC chimerism, with a higher prevalence in younger recipients. It is plausible that the younger recipient population exhibits enhanced regenerative capacity [12]. Alternatively, age-related differences in immunological responses may contribute to the observed association [13].

The current study is limited by the exclusive inclusion of end-stage kidney transplants. Further investigation should include longitudinal biopsy samples, including early time points, to further explore the dynamics of EC chimerism and its impact on clinical events related to graft outcomes. However, whether sufficient cell numbers can be obtained from smaller tissue samples remains to be determined. Additionally, the potential presence of non-donor cells in the donor kidney prior to transplantation, due to previous pregnancies or blood transfusions, cannot be ruled out [14]. In conclusion, our flow cytometry-based method allows for quantification of EC chimerism in kidney transplants. It is believed that EC chimerism reduces the risk of rejection [1]. As a result, recipients displaying high levels of EC chimerism may be eligible for a reduction in their immunosuppressive medication.

SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

ACKNOWLEDGEMENTS

We would like to thank Dr S. Heidt for kindly providing the HLA antibodies used in this study.

FUNDING

None declared.

AUTHORS' CONTRIBUTIONS

D.M.H.P., M.J.H., D.A.H. and C.C.B. participated in the design of the experiments and reviewed and edited the manuscript. D.M.H.P. and M.D. performed the experiments. D.M.H.P. analysed the data and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no competing interests to report regarding the present study. Outside of the submitted work, D.A.H. has received lecture fees and consulting fees from Astellas Pharma, AstraZeneca, Chiesi Pharma, Medincell, Novartis Pharma, Sangamo Therapeutics and Vifor Pharma; has received grant support from Astellas Pharma, Bristol-Myers Squibb and Chiesi Pharma (paid to his institution); and does not have employment or stock ownership at any of these companies, and neither does he have patents or patent applications.

REFERENCES

1. Koopmans M, Hovinga ICLK, Baelde HJ *et al.* Endothelial chimerism in transplantation: looking for needles in a haystack. *Transplantation* 2006;**82**:S25–9. https://doi.org/10.1097/01.tp.0000231446.41051.98
2. Kapessidou Y, Habran C, Buonocore S *et al.* The replacement of graft endothelium by recipient-type cells conditions allograft rejection mediated by indirect pathway CD4$^+$ T cells. *Transplantation* 2006;**81**:726–35. https://doi.org/10.1097/01.tp.0000184444.93108.d1
3. Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ *et al.* Endothelial cell chimerism after renal transplantation and vascular rejection. *Lancet* 2001;**357**:33–7. https://doi.org/10.1016/S0140-6736(00)03569-8

4. Ferlicot S, Vernochet A, Romana S *et al.* Microchimerism in renal allografts: clinicopathological associations according to the type of chimeric cells. *Histopathology* 2010;**56**:188–97. https://doi.org/10.1111/j.1365-2559.2009.03466.x
5. Murata H, Ratajczak P, Meignin V *et al.* Endothelial cell chimerism associated with graft rejection after Human lung transplantation. *Transplantation* 2008;**85**:150–4. https://doi.org/10.1097/01.tp.0000297246.63022.71
6. Tanaka Y, Haga H, Egawa H *et al.* Intragraft expression of recipient-type ABO blood group antigens: long-term follow-up and histological features after liver transplantation. *Liver Transpl* 2005;**11**:547–54. https://doi.org/10.1002/lt.20415
7. Kleeberger W, Versmold A, Rothämel T *et al.* Increased chimerism of bronchial and alveolar epithelium in Human lung allografts undergoing chronic injury. *Am J Pathol* 2003;**162**:1487–94. https://doi.org/10.1016/S0002-9440(10)64281-2
8. Mengel M, Jonigk D, Marwedel M *et al.* Tubular chimerism occurs regularly in renal allografts and is not correlated to outcome. *J Am Soc Nephrol* 2004;**15**:978–86. https://doi.org/10.1097/01.ASN.0000120369.92378.54
9. Grimm PC, Nickerson P, Jeffery J *et al.* Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. *N Engl J Med* 2001;**345**:93–7. https://doi.org/10.1056/NEJM200107123450203
10. de Leur K, Dieterich M, Hesselink DA *et al.* Characterization of donor and recipient CD8+ tissue-resident memory T cells in transplant nephrectomies. *Sci Rep* 2019;**9**:5984.
11. Mulder A, Kardol M, Regan J *et al.* Reactivity of twenty-two cytotoxic human monoclonal HLA antibodies towards soluble HLA class I in an enzyme-linked immunosorbent assay (PRA-STAT). *Hum Immunol* 1997;**56**:106–13. https://doi.org/10.1016/S0198-8859(97)00146-8
12. Heiss C, Keymel S, Niesler U *et al.* Impaired progenitor cell activity in age-related endothelial dysfunction. *J Am Coll Cardiol* 2005;**45**:1441–8. https://doi.org/10.1016/j.jacc.2004.12.074
13. Weyand CM, Goronzy JJ. Aging of the immune system. Mechanisms and therapeutic targets. *Ann Am Thorac Soc* 2016;**13(Suppl 5)**:S422–8. https://doi.org/10.1513/AnnalsATS.201602-095AW
14. Koopmans M, Hovinga ICLK, Baelde HJ *et al.* Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. *Am J Transplant* 2005;**5**:1495–502. https://doi.org/10.1111/j.1600-6143.2005.00858.x

Received: June 1, 2023; Editorial decision: July 8, 2023