The use of plasma donor-derived cell-free DNA to monitor acute rejection after kidney transplantation

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Abstract

Background

After transplantation, cell-free DNA derived from the donor organ (ddcfDNA) can be detected in the recipient's circulation. We aimed to investigate the role of plasma ddcfDNA as biomarker for acute kidney rejection.

Methods

From 107 kidney transplant recipients, plasma samples were collected longitudinally after transplantation (day 1 – 3 months) within a multicenter set-up. Cell-free DNA from the donor was quantified in plasma as a fraction of the total cell-free DNA by next generation sequencing using a targeted, multiplex polymerase chain reaction (PCR)-based method for the analysis of single nucleotide polymorphisms.

Results

Increases of the ddcfDNA% above a threshold value of 0.88% were significantly associated with the occurrence of episodes of acute rejection ($p = 0.017$), acute tubular necrosis ($p = 0.011$) and acute pyelonephritis ($p = 0.032$). ROC analysis revealed an equal AUC of the ddcfDNA% and serum creatinine of 0.64 for the diagnosis of acute rejection.

Conclusion

Although increases in plasma ddcfDNA% are associated with graft injury, plasma ddcfDNA does not outperform the diagnostic capacity of the serum creatinine in the diagnosis of acute rejection.

Keywords: donor-derived cell-free DNA, biomarker, acute kidney rejection, kidney transplantation

1. Introduction

After renal transplantation, graft injury can be related to several conditions including ischemia/reperfusion, infection or acute rejection[1]. Monitoring of the graft by measurement of serum creatinine lacks specificity as increases in the serum creatinine early after transplantation might be caused by intrinsic renal processes but also by transient conditions such as volume depletion or acute calcineurin inhibitor toxicity[2]. Histologic evaluation of a biopsy remains the gold standard method to diagnose acute rejection, although there are limitations of this procedure including its invasiveness, cost and inter-observer variability[3,4]. Furthermore, often, significant graft damage is already present at the time of biopsy[5]. Therefore, there is an unmet clinical need of sensitive, non-invasive markers that allow for the detection of acute rejection in an early stage.

Donor-derived cell-free DNA (ddcfDNA) has been proposed as a candidate biomarker for graft injury after solid organ transplantation as its release might be associated with graft cell damage[6]. In heart and liver transplant recipients with an acute rejection, increased plasma fractions of ddcfDNA (proportion of circulating cell-free DNA that is donor-derived) were observed compared to recipients with stable graft function thereby suggesting a role for ddcfDNA as biomarker of rejection[7,8]. Currently, little is known about the role of ddcfDNA as monitoring tool for *kidney* allograft rejection.

The main aim of this study was to investigate if ddcfDNA levels in kidney transplant recipients could serve as a possible biomarker of renal allograft rejection. Therefore, we designed a prospective, observational multicenter study in which ddcfDNA was quantified in serial plasma samples of renal transplant recipients.

2. Materials and Methods

2.1 Study design

The study was approved by the Ethics committees of the Antwerp University Hospital (file number 14/30/308) and the Ghent University Hospital (file number 2014-1200). The study was in accordance with the Helsinki Declaration (2013). Adult patients who received a kidney transplantation were enrolled in a multicenter, prospective, observational clinical study at the Antwerp University Hospital and the Ghent University Hospital between October 2014 and March 2017 after providing a written informed consent. The Antwerp University Hospital included all consecutive patients who received a kidney transplantation, except for patients with a history of non-kidney transplantation, multi-organ transplant recipients and patients who preferred not to participate in the study. In addition to these exclusion criteria, patients who were referred from general hospitals were not included at the University Hospital of Ghent to ensure a complete follow-up of three months.

2.2 Sample collection

After renal transplantation, blood samples were collected in Cell-Free DNA BCT® collecting tubes (Streck, Nebraska, U.S.) at 10 time points: day 1, day 3, week 1, week 2, week 3, week 4, week 6, week 8, week 10 and month 3. Additional blood samples were collected simultaneously with a protocol biopsy procedure and during hospital admission for a rise in serum creatinine and/or the performance of an indication biopsy. From each recipient, a whole blood EDTA sample was collected before transplantation to isolate genomic DNA (gDNA). From each deceased donor, genomic DNA was provided by the HILA (Histocompatibility and Immunogenetic Laboratory, Belgian Red Cross Flanders, Mechelen, Belgium) and from each living donor, a whole blood EDTA sample or buccal swab (Isohelix, Kent, U.K) was collected after a written informed consent to isolate gDNA.

2.3 Cell-free DNA (cfDNA) and genomic DNA extraction

Within 2 days after collection, blood samples were centrifuged following a 2-step centrifugation protocol (1600g for 10 min and 3200g for 20 min at room temperature) to remove the cells. Within 6 months of storage at -80°C, plasma samples were thawed at room temperature and cfDNA was extracted as previously described[9]. Genomic DNA was extracted from EDTA blood samples or buccal swabs by a standard salting-out procedure[10] or using a Buccalyse DNA extraction kit (Isohelix), respectively.

2.4 Quantification of donor-derived cell-free DNA

Fractions of ddcfDNA in plasma samples were quantified as a proportion of total circulating cell-free DNA using the Sequido assay (Multiplicom, Agilent Technologies). This assay enables a targeted amplification of 1027 single nucleotide polymorphisms (SNPs) containing amplicons for subsequent next generation sequencing (NGS). For specifications of the assay workflow, we refer to Gielis *et al*.[9].

2.5 Histologic evaluation of renal biopsies

All protocol and indication biopsies were evaluated centrally and blinded from the results of ddcfDNA% measurements by a single experienced nephropathologist (A. Dendooven, M.D., PhD) at the Antwerp University Hospital according to the Banff 2013 classification of allograft rejection[11,12].

2.6 Clinical data collection and definitions

2.6.1 Histological diagnoses

Acute rejection episodes were diagnosed based on the clinicopathological diagnosis of an indication or protocol biopsy, thereby including borderline treated rejection episodes, episodes of T cell-mediated (TCMR) (Banff I, II, III), antibody-mediated rejection (ABMR) or combined TCMR and ABMR rejection episodes. The diagnosis of acute tubular necrosis was made based on histology, and defined in the absence of acute rejection.

2.6.2 Clinical diagnoses

Delayed graft function was defined as a need of dialysis within the first week after transplantation[13]. Treated urinary tract infections were registered, including cases of symptomatic lower urinary tract infections and pyelonephritis (chills, fever and positive urinary culture with/without positive blood cultures). A BK viral (BKV) infection was defined as a progressive increase in viral DNA load (diagnosed with PCR) requiring an adjustment of the maintenance immunosuppressive treatment. A CMV infection was defined as a progressive increase in viral DNA load (PCR) requiring anti-viral treatment in a therapeutic dose. Episodes of edema or fluid retention were registered when intravenous diuretic treatment was required. Pre-renal acute kidney injury was defined as an increase in serum creatinine with a decrease in serum creatinine following fluid treatment. Calcineurin inhibitor (CNI)-induced serum creatinine increases were diagnosed when they resulted in lowering of the CNI dose.

2.7 Statistical analysis

Sample size calculation: According to the actual incidences, 15% of the kidney transplant recipients develop a graft rejection. Based on a 0.9% to 2.8% increase in donor-derived cell-free DNA in the rejection group compared to the stable group with a standard deviation of 1.2% in the stable group and 1.8% in the rejection group[14,15] and a power of 80%, at least 54 patients needed to be included in the stable group and at least 10 patients needed to be included in the rejection group using the t-statistic test for comparing the mean of a continuous measurement in two samples (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). We additionally included 40% more patients to compensate for drop-outs.

Plasma ddcfDNA% increases: Increased plasma ddcfDNA% measurements were defined as any ddcfDNA% value exceeding the 0.88% threshold that was previously established in stable kidney transplant patients[9], while values equal or below were considered as stable ddcfDNA% measurements. As ddcfDNA% decreases exponentially after transplantation to reach a stable ddcfDNA threshold value on average 10 days after transplantation[9], we only analyzed ddcfDNA increases from day 10 onwards.

We investigated the association of ddcfDNA increases with adverse events that occurred three days before to seven days after the plasma ddcfDNA% measurement with a Fisher's exact test with an adjustment of the p-value according to a Bonferroni correction for multiple testing.

A Spearman correlation analysis or ANOVA was performed to determine the correlation between clinical parameters and the ddcfDNA%, depending on the type of parameter.

ddcfDNA% as a biomarker of rejection: Plasma samples that were collected three days before to three days after a renal biopsy procedure and before any anti-rejection treatment were included (further called 'paired biopsies'). The association of the individual Banff lesions with ddcfDNA% was established with a Mann-Whitney U test. A receiver operating characteristic (ROC) analysis was performed and the associated area under the curve (AUC) values were calculated using the ROCR package with either the ddcfDNA% or serum creatinine as a predictive value. Confidence intervals were calculated using a bootstrapping algorithm (pROC library, 100 iterations and 90% CI). A DeLong test was performed to analyze differences between AUC values.

All analyses were performed in R (v3.2.4). Boxplots were generated using IBM SPSS Statistics (v.24).

3. Results

3.1 Patients and samples

In total, 107 patients were enrolled into this study. The pre-transplantation, donor and transplantation characteristics of these recipients are shown in table 1. Of these recipients, 1036 plasma samples were collected at ten (84 recipients), nine (16 recipients) and eight (5 recipients) follow-up visits. In two recipients, plasma sampling was ended after six visits because of a transplant nephrectomy. Protocol biopsies were performed as planned in 57 recipients between week 10 and 3 months. A blood sample concomitant with the protocol biopsy was available for 43 patients. Supplementary blood samples

(total number of 46) were drawn from 28 transplant recipients hospitalized for acute graft dysfunction and/or indication biopsy.

3.2 Increases in plasma ddcfDNA%

A total number of 792 plasma samples were collected from day 10 onwards after transplantation. Increased ddcfDNA fractions were measured in 103 (13%) plasma samples. Samples from one recipient were excluded from the analysis as this subject never reached the 0.88% threshold value and remained at a higher ddcfDNA% throughout the entire study follow-up with median plasma ddcfDNA% of 1.76% (1.07% - 2.42%).

As shown in table 2, increases of the plasma ddcfDNA% were significantly associated with the occurrence of acute rejection ($p = 0.017$), pyelonephritis ($p = 0.032$) and episodes of acute tubular necrosis (not related to acute rejection; $p = 0.011$). In contrast, episodes of BKV or CMV infection, symptomatic lower urinary tract infection, fluid retention, pre-renal acute kidney injury or CNI-induced serum creatinine increases were not significantly associated with increases in the ddcfDNA fraction (table 2).

Importantly, of the 103 ddcfDNA increases that were measured from day 10 after transplantation onwards, 84 (82%) increases could not be explained by the occurrence of acute rejection, pyelonephritis, or acute tubular necrosis (table 2). Plasma ddcfDNA% did not correlate with kidney function (serum creatinine, $r = -0.04$, $p = 1.00$; eGFR, $r = 0.09$, $p = 0.16$) nor with markers of inflammation (C-reactive protein (CRP), $r = 0.04$, $p = 1.00$; neutrophils, $r = 0.06$, $p = 1.00$) during the post-transplantation from day 10 onwards.

3.3 ddcfDNA as biomarker for acute rejection

ddcfDNA% and histological diagnosis

In total, 81 plasma samples were analyzed to investigate if the ddcfDNA% might be useful to diagnose acute rejection after renal transplantation. Paired biopsies were scored as normal (respectively 41

(95.3%) and 24 (63.2%) protocol and indication biopsies), acute rejection (respectively 2 (4.7%) and 11 (28.9%) protocol and indication biopsies) or acute tubular necrosis (not in the context of acute rejection, including 3 (7.9%) indication biopsies). In figure 1, log transformed plasma ddcfDNA fractions are shown for different clinicopathological diagnoses. A median ddcfDNA% of 0.42% (0.09% - 2.05%) was measured in plasma samples collected paired with indication or protocol biopsies showing no rejection neither acute tubular necrosis lesions (n = 56), while the median plasma ddcfDNA% of the untreated borderline rejection episodes (n = 9) was 0.55% (0.27% - 1.54%). In recipients with a treated borderline rejection episode (n = 3), plasma ddcfDNA% ranged from 0.36 to 0.56% with a median ddcfDNA% of 0.48%. During 4 Banff IIA TCMR rejection episodes (3 recipients), a median ddcfDNA% of 0.42% (0.29% - 1.19%) was measured. The plasma fractions of the ddcfDNA exceeded the threshold value of 0.88% in all episodes of combined TCMR and ABMR (n = 4) with median ddcfDNA% of 6.97% (range 1.43% to 32.44%). In one recipient with two episodes of ABMR, no increased plasma ddcfDNA were measured, with median ddcfDNA% of 0.48% (range 0.37% to 0.53%) throughout the post-transplantation period from day 10 onwards. In this patient, however, dialysis was restarted at the first day after transplantation and a diagnosis of primary non-function led to a transplant nephrectomy 68 days after transplantation.

ddcfDNA% and individual Banff lesions

The presence of peritubular capillaritis was significantly associated with increased ddcfDNA% ($p = 0.02$) while histological signs of interstitial inflammation ($p = 0.13$), tubulitis ($p = 0.80$), glomerulitis ($p = 1.00$), or vasculitis ($p = 0.96$) were not significantly correlated. Furthermore, no significant association was observed with C4d positivity (defined as focal or diffuse C4d positivity on immunohistochemistry or diffuse positivity on immunofluorescence). No significant correlations were found between the ddcfDNA% and chronic Banff lesions (ci, ct, cv) or mesangial matrix (mm) increase nor with the presence of arteriolar hyalinosis (ah).

Receiver operating curve analysis

A ROC analysis was performed to investigate the test performance of the plasma ddcfDNA% to distinguish an acute rejection episode from a normal indication/protocol biopsy or biopsy with borderline untreated rejection episode or acute tubular necrosis (figure 2). An AUC of 0.64 (90% CI: 0.501 – 0.779) was observed, thereby showing a sensitivity and specificity of 38% and 85% respectively at a ddcfDNA% of 0.88%. As shown in figure 2, the measurement of the ddcfDNA% did not outperform the serum creatinine with an AUC of 0.64 (90% CI: 0.499-0.781, p = 1.00). Combining the two biomarkers might slightly improve the diagnostic capacity (AUC of 0.70, 90% CI: 0.552-0.842) for the diagnosis of acute rejection.

Longitudinal analysis

In all subjects with a combined TCMR and ABMR (n = 3), early increases in the ddcfDNA% were observed before the clinical-histopathological diagnosis of acute rejection. In these recipients, increases above the threshold value of 0.88% were measured 1 to 9 days before histological diagnosis. Rises in the serum creatinine were also present in this period, except for one case in which increases in ddcfDNA% were observed while serum creatinine remained stable. The courses of the ddcfDNA% together with serum creatinine of the three patients with a combined TCMR and ABMR are shown in figure 3.

4. Discussion

This study was designed to investigate the usefulness of plasma ddcfDNA for the detection of acute rejection after kidney transplantation. We found that increases in ddcfDNA% above a threshold value of 0.88% were associated with acute rejection, and also with acute pyelonephritis and acute tubular necrosis in the period from 10 days to 3 months after transplantation. However, only 18% of the increases in ddcfDNA% could be explained by the occurrence of these adverse events. Our data further

demonstrate that plasma ddcfDNA% does not outperform the serum creatinine for the diagnosis of acute rejection.

Increases in total cfDNA concentrations have been previously reported in renal transplant recipients suffering from local and systemic infection episodes and episodes of acute tubular necrosis compared to recipients with stable graft function[16], indicating that cell-free DNA can also be released by the recipient cells during these complications. The observation of an increase in the ddcfDNA fraction during acute pyelonephritis and acute tubular necrosis indicate that the release of cell-free DNA from the graft exceeds the release of cell-free DNA from recipient's cells during these events.

In the present study, increases in plasma ddcfDNA% in patients with a combined ABMR-TCMR were prominently present in our study, with median fractions of 6.97%. In these patients, increases above the threshold value of 0.88% were measured 1 to 9 days before histological diagnosis. In a recently published cross-sectional study[17], significantly higher ddcfDNA% were measured in plasma of recipients showing histological signs of acute or chronic active rejection compared to recipients without signs of active rejection in the renal biopsy. The presence of an ABMR resulted in higher plasma ddcfDNA% (median 2.9%) compared to TCMR (Banff ≥ IB) (median 1.2%). Plasma ddcfDNA% of patients with a TCMR type IA did not significantly differ from the control group of biopsies without evidence of active rejection (median 0.2% versus 0.3%). Using the same assay, Huang *et. al* found significantly increased ddcfDNA% in plasma samples from patients with ABMR and mixed ABMR compared to samples from patients without rejection (median 1.4% versus 0.38%) or isolated TCMR (0.27%)[18]. These phenotype-dependent increases in the ddcfDNA% are in line with our findings, where marked increases were observed during a combined rejection phenotype.

In the present study, we implemented the genomic donor and recipient DNA in the assay, so the precise ddcfDNA% could be measured. In our study, ddcfDNA fractions of 32% were measured in plasma samples from a patient with a mixed TCMR and ABMR and of 54% in a recipient with acute tubular necrosis. In contrast, in the study of Bloom *et al*., the genomic DNA of the donor was not implemented in the assay used to quantify donor fractions. Consequently, the upper limit of detection

of the assay was 25%[17,19]. From our results, it became clear that the plasma ddcfDNA% might increase to fractions higher than 25% in kidney transplant recipients.

As presented in table 2, only a minority (18%) of the ddcfDNA increases could be explained by the occurrence of either acute rejection, acute pyelonephritis or acute tubular necrosis in our cohort. Of course, these unexplained ddcfDNA increases compromise the role of ddcfDNA as an adequate rejection monitoring tool.

Based on our ROC analysis (including samples collected paired with a renal biopsy), the diagnostic capacity of the ddcfDNA to diagnose rejection was rather poor with a sensitivity and specificity of 38% and 85% respectively at a threshold of 0.88% ddcfDNA%. Furthermore, based on this sensitivity and specificity, the positive predictive value of the assay is expected to be poor and ranging from 22% to 39% according to an acute rejection risk between 10% and 20% respectively.

In kidney transplantation, using a threshold of 1% ddcfDNA, Bloom *et al*. likewise reported a rather poor sensitivity but acceptable specificity of 59% and 85% respectively for the discrimination of active rejection from no rejection (AUC 0.74)[17]. From these data, the authors concluded that a plasma ddcfDNA% below 1% reflects the absence of an active rejection and a ddcfDNA% exceeding 1% indicates a probability of an active rejection. Using the same ddcfDNA threshold, Huang *et. al* reported a sensitivity of 67.6% and specificity of 74.2% for the diagnosis of acute rejection[18]. It remains, however, remarkable that in both studies in approximately 25% of the samples without active rejection, the ddcfDNA% was higher than 1%.

With an AUC of 0.64, ddcfDNA% measurement does not outperform the diagnostic capacity of the serum creatinine for the distinction between rejection and no rejection in our study. Bloom *et al.* reported an AUC of only 0.54 for the serum creatinine, which was weaker compared to the ddcfDNA test performance[17]. However, the design of these two studies was different. While only samples paired with indication biopsy procedures were included in the Bloom study, samples paired with protocol biopsies were also included in our analysis.

Of course, future research is needed to investigate the value of ddcfDNA as an add-on to other minimal invasive biomarkers including DSAs[20], chemokines, and microRNAs[21].

We did not find an association between increased plasma ddcfDNA and the occurrence of BKV infection. In urine samples of renal transplant recipients, it was shown that increased amounts of ddcfDNA are present in renal transplant recipients with a BKV nephropathy compared to patients with stable graft function[14]. In our study, plasma ddcfDNA% remained below the threshold value in three cases of BKV nephropathy (positive PCR accompanied with viral cytopathogenic changes on biopsy). It might thus be possible that only urinary, not plasma ddcfDNA% reflects BKV infection.

In kidney transplant recipients, it has to be questioned whether increased levels of ddcfDNA are related to impaired kidney function rather than reflecting actual graft damage. In analogy with our previous findings in the early post-transplantation period[9], we did not observe a correlation between ddcfDNA% and kidney function (serum creatinine or eGFR) between 10 days and 3 months after transplantation. It has been shown that renal excretion is only a minor route of cell-free DNA clearance[22], and total cell-free DNA levels do not differ between patients with chronic kidney disease (CKD) and healthy controls, nor between patients with different stages of CKD[23-25]. Furthermore, in the present study, ddcfDNA was measured as a fraction of the total circulating cell-free DNA, not by quantification of absolute ddcfDNA levels. We may thus conclude that the the ddcfDNA% is not related to the renal function. It has to be considered that stable ddcfDNA values might differ between kidney transplant recipients thereby reflecting graft quality or recipient or transplantation characteristics. However, in a recent publication of our group, we did not find any significant correlation between recipient, donor transplantation characteristics and individual baseline ddcfDNA values in the same cohort of patients[9].

An important strength of this study is the longitudinal set-up and the evaluation of the impact of different graft-associated complications on the circulating ddcfDNA fraction. However, due to the limited numbers of rejection subtypes, we cannot make firm conclusions about the differences in plasma ddcfDNA fractions between rejection phenotypes.

In summary, although increases in plasma ddcfDNA% are associated with graft injury, plasma ddcfDNA does not outperform the diagnostic capacity of the serum creatinine in the diagnosis of acute rejection Our data suggest that the use of this ddcfDNA is limited by many unexplained increases that occur in renal transplant recipients during longitudinal follow-up. At this stage, the value of ddcfDNA as rejection biomarker therefore seems rather limited.

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Disclosures

Jurgen Del Favero and Joachim De Schrijver are affiliated to Multiplicom N.V.

Authors' Contributions

Conceptualization (KJL, BYDW, JLB, JDF, DA), Funding acquisition (KJL, BYDW, JDF, DA), Data curation (EG), Investigation (EG, KJL, JDS, JDF), Methodology (EG, KJL, JDS, BYDW, JLB, JDF, DA), Project administration (EG, KJL), Supervision (KJL, BYDW, JLB, DA), Visualization (EG, KJL, PM, CB, KL), Writing original draft preparation (EG), Writing-review-editing (KJL, AD, PM, CB, KL, JDS, SVL, MPE, BYDW, JLB, JDF, DA), Resources (AD, SVL, WVB, MPE), Formal analysis (PM, CB, KL), Software (PM, CB, KL, JDS, JDF), Validation (JDS, JDF).

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References

1. Cooper JE, Wiseman AC. Acute kidney injury in kidney transplantation. *Curr Opin Nephrol Hypertens.* 2013;22(6):698-703.
2. Josephson MA. Monitoring and managing graft health in the kidney transplant recipient. *Clin J Am Soc Nephrol.* 2011;6(7):1774-1780.
3. Furness PN, Taub N, Convergence of European Renal Transplant Pathology Assessment Procedures P. International variation in the interpretation of renal transplant biopsies: report of the CERTPAP Project. *Kidney Int.* 2001;60(5):1998-2012.
4. Nankivell BJ, Chapman JR. The significance of subclinical rejection and the value of protocol biopsies. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2006;6(9):2006-2012.
5. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *Journal of the American Society of Nephrology : JASN.* 2007;18(4):1046-1056.
6. Gielis EM, Ledeganck KJ, De Winter BY, et al. Cell-Free DNA: An Upcoming Biomarker in Transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2015;15(10):2541-2551.
7. De Vlaminck I, Valantine HA, Snyder TM, et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Science translational medicine.* 2014;6(241):241ra277.
8. Beck J, Bierau S, Balzer S, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. *Clinical chemistry.* 2013;59(12):1732-1741.
9. Gielis EM, Beirnaert C, Dendooven A, et al. Plasma donor-derived cell-free DNA kinetics after kidney transplantation using a single tube multiplex PCR assay. *PloS one.* 2018;13(12):e0208207.
10. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic acids research.* 1988;16(3):1215.
11. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2014;14(2):272-283.
12. Sis B, Mengel M, Haas M, et al. Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2010;10(3):464-471.
13. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2009;9 (Suppl 3):S1-S157.
14. Sigdel TK, Vitalone MJ, Tran TQ, et al. A rapid noninvasive assay for the detection of renal transplant injury. *Transplantation.* 2013;96(1):97-101.
15. Snyder TM, Khush KK, Valantine HA, Quake SR. Universal noninvasive detection of solid organ transplant rejection. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(15):6229-6234.
16. Garcia Moreira V, Prieto Garcia B, Baltar Martin JM, Ortega Suarez F, Alvarez FV. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. *Clinical chemistry.* 2009;55(11):1958-1966.
17. Bloom RD, Bromberg JS, Poggio ED, et al. Cell-Free DNA and Active Rejection in Kidney Allografts. *Journal of the American Society of Nephrology : JASN.* 2017;28(7):2221-2232.

18. Huang E, Sethi S, Peng A, et al. Early clinical experience using donor-derived cell-free DNA to detect rejection in kidney transplant recipients. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2019.
19. Grskovic M, Hiller DJ, Eubank LA, et al. Validation of a Clinical-Grade Assay to Measure Donor-Derived Cell-Free DNA in Solid Organ Transplant Recipients. *The Journal of molecular diagnostics : JMD.* 2016;18(6):890-902.
20. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2012;12(5):1157-1167.
21. Eikmans M, Gielis EM, Ledeganck KJ, Yang J, Abramowicz D, Claas FFJ. Non-invasive Biomarkers of Acute Rejection in Kidney Transplantation: Novel Targets and Strategies. *Front Med (Lausanne).* 2018;5:358.
22. Yu SC, Lee SW, Jiang P, et al. High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. *Clinical chemistry.* 2013;59(8):1228-1237.
23. Garcia Moreira V, de la Cera Martinez T, Gago Gonzalez E, Prieto Garcia B, Alvarez Menendez FV. Increase in and clearance of cell-free plasma DNA in hemodialysis quantified by real-time PCR. *Clinical chemistry and laboratory medicine : CCLM / FESCC.* 2006;44(12):1410-1415.
24. Korabecna M, Opatrna S, Wirth J, et al. Cell-free plasma DNA during peritoneal dialysis and hemodialysis and in patients with chronic kidney disease. *Annals of the New York Academy of Sciences.* 2008;1137:296-301.
25. McGuire AL, Urosevic N, Chan DT, Dogra G, Inglis TJ, Chakera A. The impact of chronic kidney disease and short-term treatment with rosiglitazone on plasma cell-free DNA levels. *PPAR Res.* 2014;2014:643189.

Tables

Table 1

Title: Recipient, donor and transplantation characteristics of the entire study cohort

Number of recipients	**107**
Center 1 (n (%))	90 (84.1)
Center 2 (n (%))	17 (15.9)
Recipient characteristics (at transplantation)	
Male gender (n, (%))	79 (73.8)
Age (years)	51 (18-71)
Diabetes Mellitus type II (n, (%))	16 (15.0)
Obesity (BMI ≥ 30 kg/m²) (n, (%))	26 (24.3)
Current smoking (n, (%))	5 (4.7)
Chronic inflammatory disease/auto-immune disease	37 (34.6)
Primary renal disease (n (%))	
Glomerular disease	31 (29.0)
Chronic interstitial nephritis	12 (11.2)
Cystic disease	14 (13.1)
Renal vascular disease	15 (14.0)
Diabetes	4 (3.7)
Others	14 (13.1)
Not known	17 (15.9)
Renal replacement therapy before transplantation (n, (%))	101 (94.4)
Previous renal transplantation(s)	12 (11.2)
One (n, (%))	6 (5.6)
Two (n, (%))	6 (5.6)
PRA before transplantation (n, (%))	
0-4%	88 (82.2)
5-80%	12 (11.2)
>80%	7 (6.5)
Height (cm)	171.9 ± 9.1
Weight (kg)	79.5 ± 15.9
BMI (kg/m²)	26.8 ± 4.5
Donor characteristics	**n = 107**
Donor Type (n, (%))	
Living donor	13 (12.1)
Deceased donor	94 (87.9)
DBD	78 (72.9)
DCD	16 (15.0)
Donor age (years)	48 (16-72)
Height (cm)	173.3 ± 8.9
Weight (kg)	78.4 ± 15.5
BMI (kg/m²)	25.5 (17.7 - 46.9)
Serum creatinine (mg/dl)	0.7 (0.2 - 1.5)

Donor-recipient compatibility characteristics	
Number of HLA mismatches in HLA A and B (n, (%))	
0	8 (7.5)
1	24 (22.4)
2	50 (46.7)
3	23 (21.5)
4	2 (1.9)
Number of HLA mismatches in HLA DR (n, (%))	
0	28 (26.2)
1	73 (68.2)
2	6 (5.6)
Transplantation characteristics	
Ischemia times	
Cold ischemia time (hours)	11.9 (6.1)
2nd warm ischemia time (minutes)	30 (10 - 185)
Induction therapy (n, (%))	
IL2-RA	62 (57.9)
ATG	45 (42.1)
Maintenance immunosuppressive treatment	
Prednisolone (n, (%))	107 (100.0)
MMF/ Azathioprin/ Everolimus (n, (%))	101 (94.4) / 2 (1.9) / 4 (3.7)
Tacrolimus/ Cyclosporin (n, (%))	86 (80.4) / 21 (19.6)

Legend: n: number; BMI: Body mass index; PRA: panel reactive antibodies; DBD: donation after brain death; DCD: donation after circulatory death; HLA: Human leukocyte antigen; IL2-RA: Interleukin-2 receptor antagonist; ATG: Anti-thymocyte globulin; MMF: mycophenolate mofetil. Normally distributed continuous data are presented as a mean (± standard deviation, SD), not-normally distributed data are presented as median (min-max).

Table 2

Title: Overview of the number of samples with ddcfDNA% increases and their association with adverse events

Increased ddcfDNA	**Adverse event**		**p-value**
	Yes	No	
	Acute rejection		
Yes	9	94	**p = 0.017**
No	15	667	
	Acute pyelonephritis		
Yes	6	97	**p = 0.032**
No	7	675	
	Acute tubular necrosis		
Yes	4	99	**p = 0.011**
No	1	681	
	BKV infection		
Yes	1	102	p = 1.00
No	11	671	
	CMV infection		
Yes	9	94	p = 0.29
No	25	657	
	Symptomatic low urinary tract infection		
Yes	2	101	p = 1.00
No	15	667	
	Fluid retention		
Yes	4	99	p = 0.54
No	8	674	
	Pre-renal acute kidney injury		
Yes	3	100	p = 1.00
No	16	666	
	CNI-induced serum creatinine increases		
Yes	41	62	p = 1.00
No	224	458	

Legend: Increased ddcfDNA% were measured in 103 samples of a total amount of 785 samples collected from 106 recipients from day 10 onward transplantation. Samples from one recipient were excluded from the analysis as this subject never reached the 0.88% threshold value and remained at a higher ddcfDNA% throughout the entire study follow-up. Different crosstables are shown for each reported adverse event. Significant p-values ($p < 0.05$ with Fisher's exact test) are marked in bold.

Legends to figures

Figure 1

Title: Plasma donor-derived cell-free DNA fractions and clinicopathological diagnosis.

Legend: Boxplots of plasma ddcfDNA%. The horizontal dashed line represents the ddcfDNA threshold value 0.88% determined in stable transplant recipients[9]. In one recipient with two episodes of ABMR, no increased plasma ddcfDNA% were measured. In this patient, however, dialysis was restarted at the first day after transplantation and a diagnosis of primary non function led to a transplant nephrectomy 68 days after transplantation. The plasma ddcfDNA fraction was increased during three episodes of acute tubular necrosis (ATN). In one recipient with a ddcfDNA% increase to 53.64%, signs of acute calcineurin inhibitor toxicity were also present in the renal biopsy. In a second patient, ATN was diagnosed on an indication biopsy because of sudden anuria 12 days after transplantation. After an initial decrease in the ddcfDNA fraction to 0.72% on day 7, the ddcfDNA% increased to 3.00% during the episode of ATN. An indication biopsy was performed 10 days after transplantation in a third recipient because of a persistently poor kidney function. In this patient, also diagnosed with delayed graft function, a ddcfDNA% of 34.27% was measured on the first day after transplantation. The ddcfDNA% failed to decrease below threshold value within 10 days after transplantation, as the ddcfDNA% still amounted 3.42% at that moment.

ddcfDNA: donor-derived cell-free DNA; NC: no changes on indication/protocol biopsy; Bord-UT: Borderline untreated rejection episode; Bord-T: Borderline treated rejection episode; TCMR: T cell-mediated rejection; ABMR: antibody-mediated rejection episode; TCMR-ABMR: combined T cell-mediated and antibody-mediated rejection episode; ATN: acute tubular necrosis; n = number of episodes.

Figure 2

Title: Receiver operating curves

Legend: Receiver operating characteristic (ROC) curves of the plasma ddcfDNA%, serum creatinine and combined measurement of both biomarkers for the distinction of an acute rejection episode from a normal indication/protocol biopsy or biopsy with borderline untreated rejection episode or acute tubular necrosis (not in the context of acute rejection). The measurement of the ddcfDNA% did not outperform the serum creatinine (both AUC 0.64; p = 1.00). Combining both biomarkers results in a slightly improved diagnostic capacity (AUC 0.70) compared to the measurement of each biomarker alone.

Figure 3

Title: Courses of ddcfDNA% and serum creatinine of the three patients with a combined TCMR and ABMR

Legend: Courses of the ddcfDNA% (orange dots) and serum creatinine (blue dots) in three patients with a combined TCMR and ABMR. The vertical dashed line represents the time of histologic diagnosis. The asterisk on the X-axis represent the day of the first significant ddcfDNA% increase before the histologic diagnosis of acute rejection. In the patient presented in figure A, a rejection was diagnosed at day 21. Increases in the ddcfDNA% were measured already 9 days before acute rejection (day 12; ddcfDNA% of 2.41%). In the patient presented in figure B, two rejection episodes were diagnosed. A first rejection episode occurred at day 13 after transplantation and increased ddcfDNA% were measured 1 day before this episode (day 12; ddcfDNA% of 1.42%). A second episode occurred at day 23, with increased ddcfDNA% already 4 days before this episode (day 19; ddcfDNA% of 1.74%). In the patient presented in figure C, an acute rejection was diagnosed at day 24 after transplantation, with increased ddcfDNA% measured in a sample collected 3 days before this episode (day 21; ddcfDNA% of 10.28%). Furthermore, in this patient, ATN and calcineurin inhibitor toxicity was diagnosed on day 13 after transplantation. Increased ddcfDNA% (53.64% and 43.73%) were also measured during this event.