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# Absolute or Relative Quantification of Donor-derived Cell-free DNA in Kidney Transplant Recipients: Case Series

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**Background.** Donor-derived cell-free DNA (dd-cfDNA) is increasingly recognized as a valuable biomarker for acute transplant injury, with possible indications in the detection of cellular or humoral rejection and the guidance of immunosuppressive therapy. There is an ongoing debate on whether relative or absolute quantification of dd-cfDNA is more reliable for the detection of acute transplant injury. **Methods.** We retrospectively reviewed all 22 kidney transplant recipients who underwent dd-cfDNA measurements (percentage and absolute) between April 2020 and April 2021 at our institution. Of these, 9 (41%) showed discrepancies between absolute (cutoff: 50 copies/mL) and relative (cutoff: 0.5%) quantification in at least 1 dd-cfDNA measurement. **Results.** We report on 9 of 22 cases with discrepancies in relative and absolute quantification of dd-cfDNA, which were predominantly late posttransplant patients. We found bacterial and viral infections, as well as low leukocyte count from chronic myeloid leukaemia treatment, to be reasons for variability in total cell-free DNA (cfDNA), leading to inter- and intraindividual variability in relative dd-cfDNA quantification. When correlating dd-cfDNA quantification and biopsy results, as well as clinical course, our data indicate that relying solely on relative dd-cfDNA can lead to false-negative and false-positive results. **Conclusions.** In summary, these cases argue that absolute quantification of dd-cfDNA is better suited in patients with underlying conditions affecting total cfDNA levels and suggest using both absolute and relative dd-cfDNA together for higher reliability and interindividual comparability in the clinical setting. Especially for patients with chronic active antibody-mediated rejection, further studies on the use of dd-cfDNA are desirable.

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## INTRODUCTION

There is a need to improve personalized immunosuppression in solid organ transplantation to reduce premature graft loss.<sup>1,2</sup> In addition to monitoring immunosuppressive drug concentrations, biomarkers providing clinically actionable information are needed to improve the detection of rejection, asymptomatic graft injury including subclinical rejection, and underimmunosuppression. Assessment of minimal necessary drug exposure to guide tapering and prevent alloimmune activation is also important. The rationale

for using donor-derived cell-free DNA (dd-cfDNA) as a biomarker in transplantation is based on the fact that organ transplants are also genome transplants. This opens up the possibility to monitor graft health.<sup>3</sup>

So far, mainly relative dd-cfDNA quantification has been used,<sup>4</sup> which has the disadvantage of being affected by changes in recipient cell-free DNA (cfDNA). The total cfDNA (mainly stemming from the recipient) can be increased because of infections or autoimmune disorders or after exercise or psychological stress and can be decreased in leukopenia, among many other conditions.<sup>5-9</sup> More recently, methods for absolute

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quantification have been developed, which are not affected by recipient cfDNA variability.<sup>10,11</sup> In particular, most clinical studies have been done in patient cohorts early after transplantation,<sup>2</sup> but it has been shown recently that the total cfDNA drops long-term, which may be due to pharmacological influences of calcineurin inhibitors (CNI).<sup>12</sup> CNI seem to have a negative effect on cell stability.<sup>13</sup> Consequently, relative dd-cfDNA values are higher in patients in the late posttransplant period, presumably because of a decrease in apoptosis rate for white blood cells as immunosuppressant drug doses are tapered off.

Absolute dd-cfDNA measurements can be performed either by direct quantification of the dd-cfDNA<sup>11</sup> or by multiplying a primary relative quantification with the total cfDNA that is quantified in a separate (digital)-PCR, as used in this article.<sup>10</sup> More recently, a primary relative sequencing-based assay has been extended to identify atypical recipient cfDNA background levels.<sup>12</sup>

In this study, we present a case series of kidney transplant recipients (KTR), for whom we compared clinical events with results from absolute and relative dd-cfDNA quantifications.

## MATERIALS AND METHODS

We performed a retrospective review of all KTR who underwent dd-cfDNA testing (Chronix Biomedical, Göttingen, Germany) for clinical care between April 2020 and April 2021 at our institution. Indications for dd-cfDNA testing were previously diagnosed or suspected rejection, otherwise worsening kidney function, or a change of immunosuppressive regimen, among others. For patients with worsening kidney function or suspected rejection, measurements were performed at least once, and follow-up measurements were performed as indicated by the treating clinician. For patients who underwent a change of immunosuppressive regimen, measurements were performed 4 times (baseline and after 1, 3, and 6 mo). An abnormal dd-cfDNA result was defined as a value of  $>0.5\%$  or  $>50$  copies/mL, respectively.<sup>10</sup> All patients with at least 1 test showing a discrepancy between the relative and absolute quantifications of dd-cfDNA were included in the case series.

Donor characteristics evaluated included age, sex, and living versus deceased donation. Recipient characteristics evaluated included age, sex, cause of chronic kidney failure, type of dialysis, duration of dialysis, induction immunosuppressive regimen, early graft function, and time since transplantation. Delayed graft function was defined as the need for dialysis within 7 d after transplantation. Recipient serum creatinine, microalbuminuria, kidney biopsy results, dd-cfDNA levels, presence of donor-specific anti-HLA antibodies (DSA), and major clinical events after transplantation were examined and included in a comprehensive graphical case description for each patient. For illustration, red-shaded areas representing absolute dd-cfDNA and red lines representing relative dd-cfDNA were included in the graphs. Absolute and relative dd-cfDNA and total cfDNA, as well as the corresponding reference ranges, are provided in Table S1 (SDC, <http://links.lww.com/TXD/A375>).

The measurement of dd-cfDNA was performed as described previously.<sup>10,14</sup> In brief, for each patient, 4 informative single nucleotide polymorphisms (SNPs), defined as an SNP for which the recipient has a homozygous allelic state and the graft carries at least 1 heterozygous allele, were selected from a predefined set of 40 SNPs. These 4 SNPs were used to quantify the dd-cfDNA (%) concentration, defined

as donor alleles/(donor alleles + recipient alleles). Results for SNPs with heterozygous graft genotypes were corrected by a factor 2. Total cfDNA was extracted from up to 8 mL of plasma collected in certified blood collection tubes (Streck Corp, Omaha, Nebraska). The concentration was determined using droplet-digital PCR and was corrected for extraction loss and cfDNA fragmentation, as described previously.<sup>10</sup> The absolute concentration of dd-cfDNA per mL of plasma was calculated by multiplying total cfDNA (copies/mL) and dd-cfDNA (%). Reference ranges for total cfDNA in the post-transplant course were assessed in a cohort of 300 KTR, as described previously.<sup>13</sup>

The institutional review board of the ethics committee of Charité-Universitätsmedizin Berlin, Germany, approved the study (approval number EA2/144/20), and all procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients.

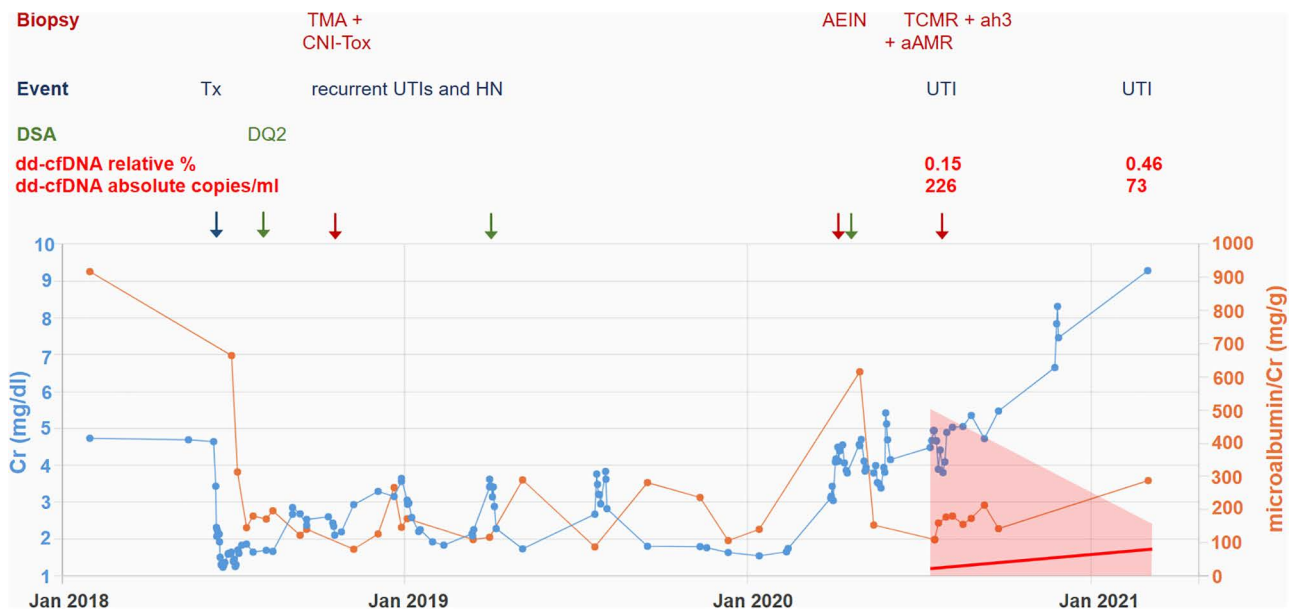
## RESULTS

During the study period, 22 patients underwent clinically indicated dd-cfDNA measurements, of which 9 showed at least 1 test with a discrepancy between the absolute and relative quantifications of dd-cfDNA. Figures 1 through 9 depict the clinical course, Table 1 shows donor and recipient characteristics for each of those patients, and Table S2 (SDC, <http://links.lww.com/TXD/A375>) contains demographic data of the total cohort.

For 5 of 9 patients, relative quantification showed normal values, whereas absolute dd-cfDNA levels were increased. In 4 of these 5 cases, biopsy or clinical course suggested ongoing transplant injury, indicating false-negative results by relative quantification alone. Relative quantification can be false-negative when total cfDNA is increased. In our case series, we found total cfDNA to be increased because of bacterial urinary tract infection (2 of 5 patients), viral infection (2 of 5 patients; cytomegalovirus and COVID-19), and suspected thrombotic microangiopathy (TMA) (1 of 5 patients).

For the remaining 4 of 9 patients, absolute quantification was borderline or normal, whereas relative dd-cfDNA was increased. All patients had either active (aAMR) or chronic active antibody-mediated rejection (caAMR) in previous biopsies. With no follow-up biopsy, it was hard to decide whether transplant injury was still present. Clinical course showed stable serum creatinine in 3 of 4 patients, no or decreasing proteinuria in 2 of 4 patients, and increasing proteinuria in the remaining 2 cases.

One patient exhibited very low total cfDNA below the fifth percentile of the reference population, leading to absolute quantification of 22 copies/mL, which is below the cutoff, whereas relative quantification was 1.42% and above the cutoff.<sup>13</sup> For this patient, a sound explanation for low total cfDNA was possible, being low leukocyte count due to chronic myeloid leukaemia (CML) and nilotinib treatment. Despite the fact that no repeated biopsy was performed, the clinical course suggested no ongoing activity of antibody-mediated rejection (AMR) in this patient at the time of dd-cfDNA measurement. Although creatinine rose to 1.5 mg/dL at the time of biopsy, it stabilized at 1.3 mg/dL after increasing immunosuppressive medication. Therefore, we concluded that this patient has a false-positive result from relative quantification and



**FIGURE 1.** Case 1: false-negative relative quantification due to high total cfDNA from urinary tract infection. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. aAMR, active antibody-mediated rejection; AEIN, acute eosinophilic interstitial nephritis; ah3, arteriolar hyalinosis grade 3 according to Banff 2018 classification; CNI-Tox, calcineurin inhibitor toxicity; Cr, creatinine; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific anti-HLA antibodies; HN, hydronephrosis; Jan, January; TCMR, acute T cell-mediated rejection; TMA, thrombotic microangiopathy; Tx, transplantation; UTI, urinary tract infection.

**TABLE 1.**

**Donor and recipient characteristics**

Case	Donor age (years/sex)	Living vs deceased donor	Recipient age (years/sex)	Cause of kidney failure	Dialysis type	Years on dialysis	Induction therapy	DGF (yes/no)	Time after KTx (mo)
1	52/male	Living, ABO incompatible	30/female	SLE	HD	1	Rituximab, basiliximab	no	26–32
2	33/female	Living, ABO incompatible	42/female	HTN	HD	1	Rituximab, basiliximab	no	45
3	43/female	Living, ABO compatible	32/female	HUS	n/a	n/a	Basiliximab	no	130–137
4	33/female	Living, ABO compatible	34/male	IgAN	PD	2	Basiliximab	no	63–70
5	64/male	Deceased	57/male	DM	HD	8	Basiliximab	yes	4–10
6	42/male	Living, ABO compatible	27/male	Reflux	HD	<1	Basiliximab	no	99
7	47/male	Living, ABO compatible	32/male	IgAN	HD	1	Basiliximab	no	94–101
8	56/female	Living, ABO compatible	53/male	unknown	PD	1	Basiliximab	no	191–200
9	51/female	Deceased	70/male	HTN	HD	7	Basiliximab	no	157–161

DGF, delayed graft function; DM, diabetic nephropathy; HD, hemodialysis; HTN, hypertensive nephropathy; HUS, hemolytic uremic syndrome; IgAN, IgA nephropathy; KTx, Kidney Transplantation; n/a, not applicable (preemptive); PD, peritoneal dialysis; SLE, systemic lupus erythematosus.

that AMR was not active anymore. This is supported by the fact that creatinine did not fall below 1.3 mg/dL over a period of 6 mo. This suggests that the episode of AMR left chronic damage, as baseline creatinine level in this patient rose from 1.0 mg/dL to 1.3 mg/dL after the rejection episode, but no acute injury was present at the time of dd-cfDNA measurement (cf. Case 6).

**Case Descriptions**

**Case 1**

Case 1 includes a 30-y-old female patient with a complex posttransplant course; multiple urinary tract infections, ureteral calcification, and recurring hydronephrosis occurred (Figure 1). As early as 1 mo after transplantation, the patient developed DSA. The first posttransplant biopsy showed TMA and CNI toxicity, leading to a switch to belatacept. Additionally, because of her pregnancy wish, a switch from mycophenolate to azathioprine was performed. In the following kidney biopsy, acute

eosinophilic interstitial nephritis was found, probably caused by azathioprine. Because no pregnancy had occurred in the meantime, the patient was treated with a steroid pulse, and mycophenolate was reinitiated. Kidney function further deteriorated, and a repeated biopsy showed acute T cell-mediated rejection Banff IA (i3, t3, v0) as well as aAMR (g0, ptc2, C4d2, cg0). There was a switch from belatacept to tacrolimus; steroid pulse therapy for the cellular rejection component, plasma exchange, rituximab, and intravenous immunoglobulins for the humoral component were administered. Because kidney function did not improve despite rejection treatment and severe arteriolar hyalinosis (ah3) was present as well in the latest biopsy, a rescue switch to, once again, belatacept with low-dose tacrolimus was performed. Because of worsening kidney function, dd-cfDNA measurements were performed.

One and 7 mo after the last biopsy and rejection therapy, we still found elevated absolute dd-cfDNA (226 copies/mL and 73 copies/mL) despite normal relative dd-cfDNA (0.15% and

0.46%, respectively). Despite deteriorating kidney function, the patient refused a further follow-up biopsy as well as changes in immunosuppressive medication. Ongoing transplant injury was supported by previous biopsy-proven acute cellular rejection Banff IB, aAMR, and progressive deterioration of kidney function. In the first sample, a total cfDNA concentration of 150 667 copies/mL was measured and was highly elevated above the 95th percentile of the time-posttransplant-matched reference group (27 089 copies/mL). This increase in total cfDNA was attributed to recurrent urinary tract infections.

## Case 2

Case 2 includes a 42-y-old female patient with COVID-19 after kidney transplantation (Figure 2). A kidney transplant biopsy performed because of worsening kidney function showed infection-associated interstitial nephritis, which was suspected to be associated with COVID-19. We performed dd-cfDNA measurements 2 wk after kidney biopsy. Absolute quantification indicated acute transplant injury (214 copies/mL), whereas relative quantification was normal (0.38%). The total cfDNA concentration was 56 316 copies/mL, with the 95th percentile of the time-posttransplant matched reference group being 15 113 copies/mL. The suspected cause for increased total cfDNA was concomitant COVID-19 disease with increased immune activation and leukocyte turnover.

## Case 3

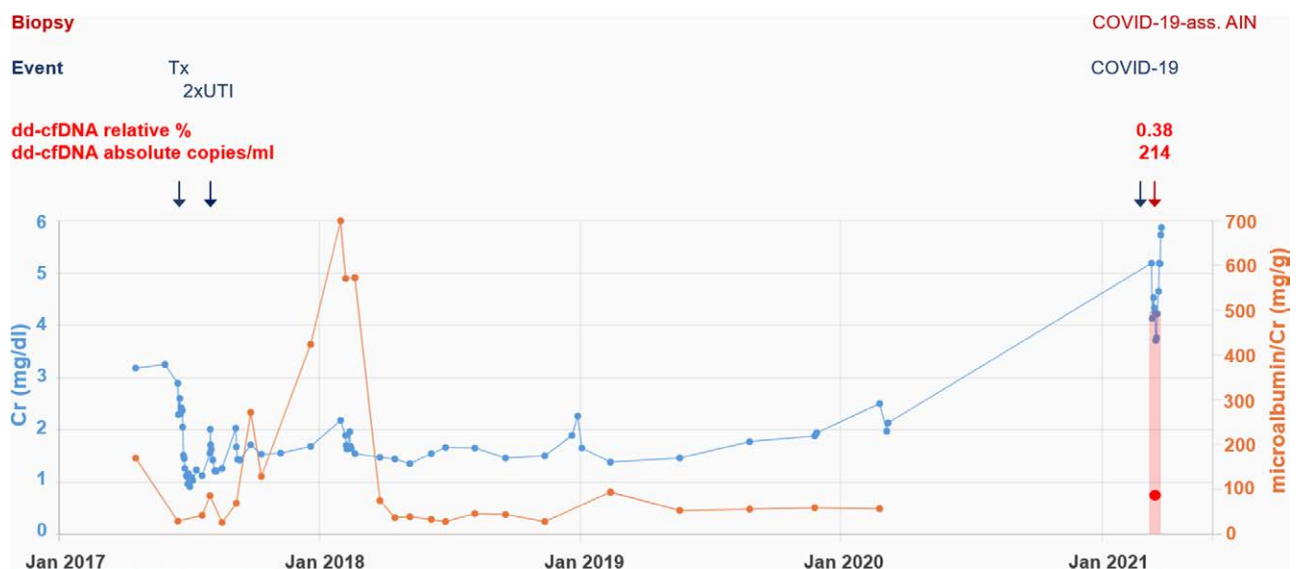
A 32-y-old female patient received a repeated diagnosis of caAMR, and the patient received treatment with plasma exchange and intravenous immunoglobulins in October 2019 (Figure 3). A follow-up biopsy in April 2020 showed ongoing caAMR without peritubular capillaritis (ptc0, C4d0) but with glomerulitis (g2) and advanced chronic changes (cg3). Because of severe arteriolar hyalinosis (ah3), a rescue switch to a CNI-free regimen was performed. Dd-cfDNA was measured repeatedly afterward starting 2 wk after the last biopsy.

Absolute quantification showed persisting transplant injury, which was supported by the creatinine course and latest biopsy. Relative quantification was below the cutoff (0.5%) for the initial 3 measurements. This was attributed to an increased amount of total cfDNA due to recurrent urinary tract infections. The total cfDNA ranged from 20 541 to 30 000 copies/mL for the first 3 measurements, whereas the 95th percentile was 16 234 copies/mL for the time-posttransplant-matched reference group. The last measurement showed increased dd-cfDNA for both relative and absolute quantification because total cfDNA normalized to 4056 copies/mL. Because the patient had already received therapy for both caAMR and severe arteriolar hyalinosis, no follow-up biopsy was performed because of the lack of therapeutic options. Because kidney function further deteriorated (creatinine rising from 3.8 mg/dL to 5.9 mg/dL, albumin-creatinine ratio gradually decreasing from 5.0 to 2.6 g/g), ongoing activity of caAMR was suspected from the clinical course.

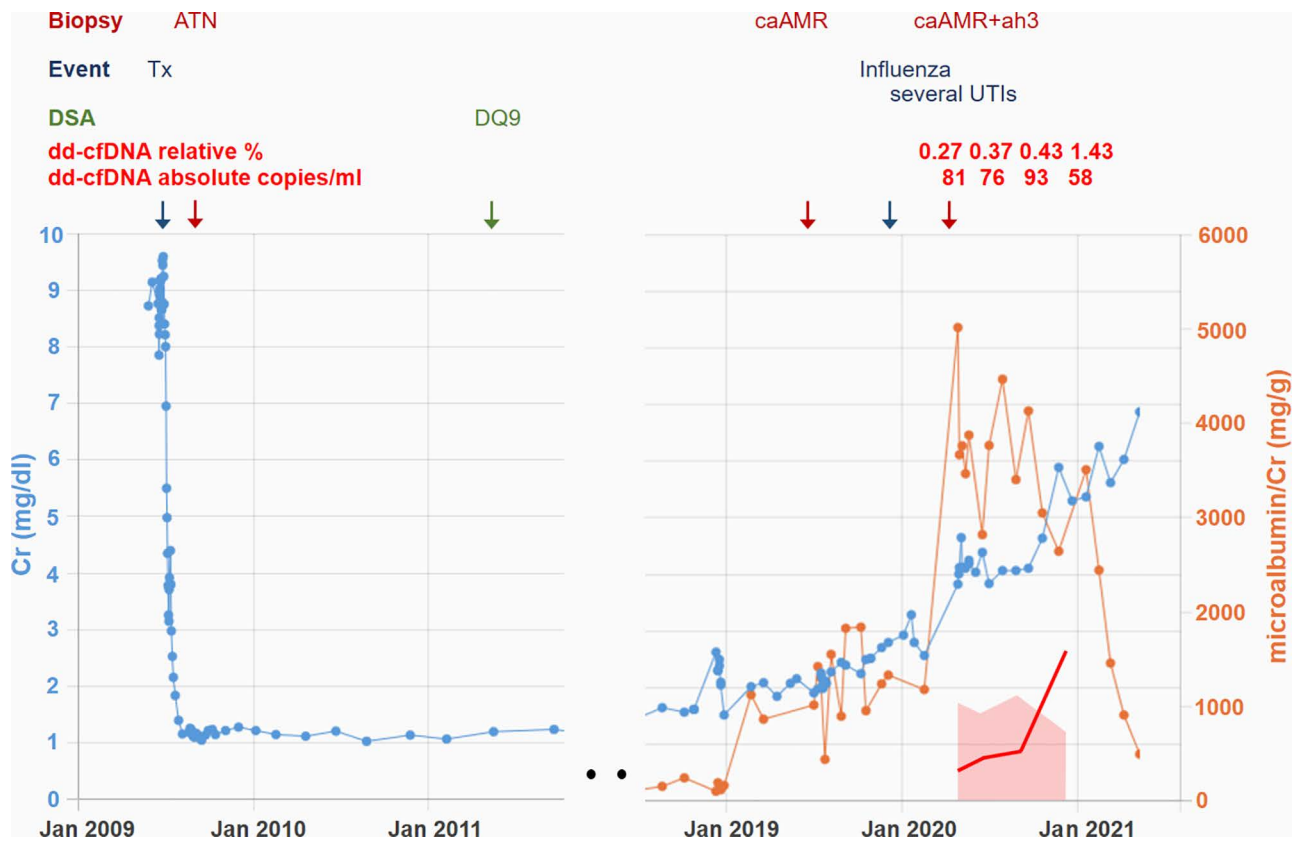
## Case 4

A 34-y-old male developed DSA 1.5 y after transplantation, and caAMR was diagnosed 3.5 y after transplantation, for which he received plasma exchange and rituximab (Figure 4). Five years after transplantation, the patient developed Coombs-negative hemolytic anemia and acute transplant failure, and clinical and biopsy-proven TMA was diagnosed. Because the patient was severely anemic after an unsuccessful treatment with eculizumab, a switch to belatacept was performed despite the fact that caAMR (ptc0, g2, cg3; other findings: v0, mm3, ah3) was still present in the latest kidney transplant biopsy. TMA resolved; the patient is in excellent condition, and kidney function stabilized (creatinine from maximum 3.8 mg/dL to 2.3 mg/dL, albumin-creatinine-ratio from maximum 1.8 to 0.38 g/g). Repeated measurements of dd-cfDNA were performed after the switch to belatacept, starting 1 mo after the last biopsy.

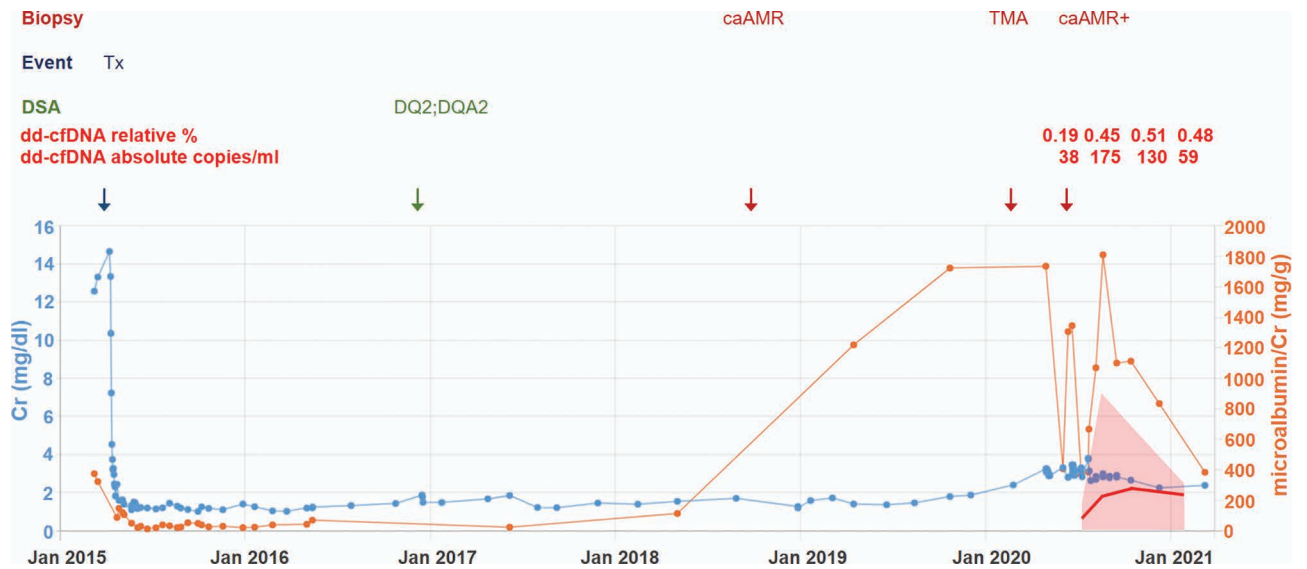
Absolute quantification showed resolving transplant injury after the switch to belatacept, which was explained by



**FIGURE 2.** Case 2: false-negative relative quantification in a patient with COVID-19 and biopsy-proven acute interstitial nephritis due to high total cfDNA from COVID-19. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment. COVID-19-ass. AIN, COVID-19-associated acute interstitial nephritis; Cr, creatinine; dd-cfDNA, donor-derived cell-free DNA; Jan, January; Tx, transplantation; UTI, urinary tract infection.



**FIGURE 3.** Case 3: false-negative relative quantification due to high total cfDNA from urinary tract infection. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. ah3, arteriolar hyalinosis grade 3; ATN, acute tubular necrosis; caAMR, chronic active antibody-mediated rejection; Cr, Creatinine; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific anti-HLA antibodies; Jan, January; Tx, Transplantation; UTI, urinary tract infection.



**FIGURE 4.** Case 4: suspected false-negative relative quantification due to high total cfDNA from thrombotic microangiopathy. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. caAMR, chronic active antibody-mediated rejection; Cr, creatinine; DSA, donor-specific anti-HLA antibodies; dd-cfDNA, donor-derived cell-free DNA; Jan, January; RTX, rituximab treatment; TMA, Thrombotic microangiopathy; Tx, Transplantation.

resolving TMA. Relative quantification showed borderline results over time with respect to the cutoff of 0.5%. This was due to increased total cfDNA, which was ranging from 12292

to 38889 copies/mL, whereas the 95th percentile was 16234 copies/mL for the time-posttransplantation-matched reference group. Because of clinical improvement and the stabilization

of kidney function, no repeated biopsy was performed after the switch of immunosuppressive regimen, so there is no direct evidence of ongoing transplant injury at the time of dd-cfDNA measurement. The clinical course, previous biopsy, and total cfDNA levels support the interpretation that absolute quantification was a more accurate indication of graft injury in this case.

### Case 5

In a 57-y-old patient with type 1 diabetes mellitus, delayed graft function occurred after transplantation (Figure 5). Because of dual antiplatelet therapy for a recent myocardial infarction, there was no kidney transplant biopsy, but an empiric steroid pulse and rescue switch to a CNI-free regimen were performed. Dd-cfDNA levels were quantified repeatedly afterward to monitor for ongoing graft injury. Absolute and relative quantifications of dd-cfDNA aligned well until the last measurement. For this measurement, absolute quantification still suggested ongoing transplant injury, whereas relative quantification was normal. This was explained by changes in total cfDNA levels, which were ranging from 9274 to 11481 copies/mL for the first 3 measurements but increased to 31 200 copies/mL for the last measurement, whereas the median of the time-posttransplant–matched reference population was 6369 copies/mL. This increase in total cfDNA was attributed to cytomegalovirus infection and mild leukocytosis of  $10.4 \times 10^9/L$ .

### Case 6

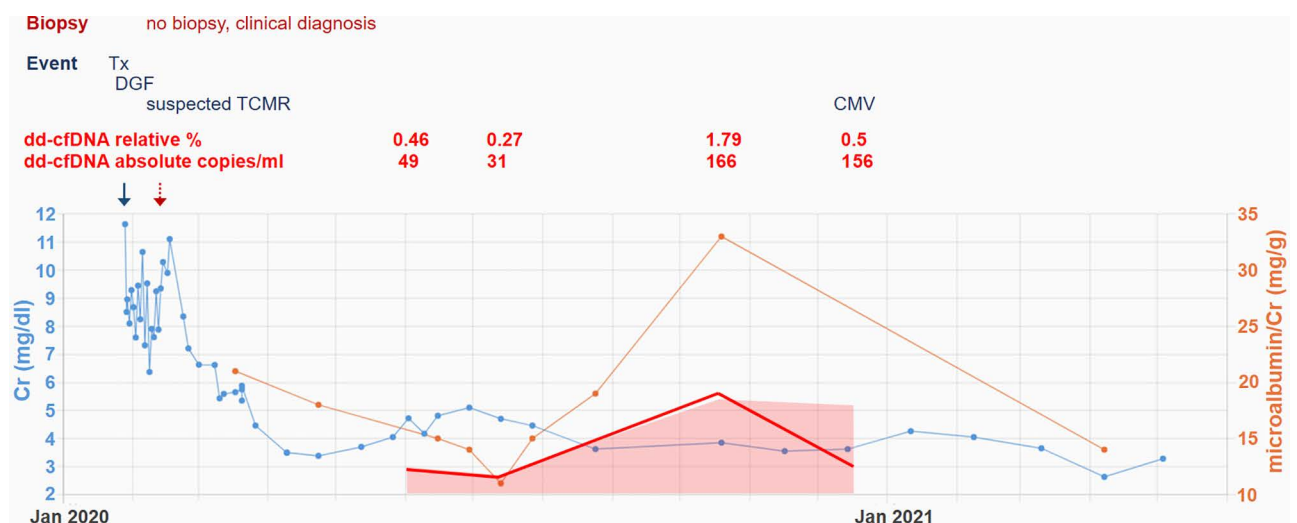
A 27-y-old male patient had an unremarkable post-transplant course until leukocytosis of up to  $50/nL$  developed 7 y after transplantation (Figure 6). A diagnosis of BCR-ABL positive CML was made. Azathioprine was discontinued, and therapy with nilotinib was initiated, leading to remission of CML after 9 mo. Concomitantly, creatinine rose from 1.0 to 1.5 mg/dL, and strong DSA were detected. A kidney biopsy showed aAMR with peritubular capillaritis and C4d positivity (g0, ptc2, C4d2, ci1, ct1, cv1, cg0, mm0, ah2, ti2), and azathioprine was reinitiated. This led to stabilization of kidney

function at a level of 1.3 mg/dL (no significant albuminuria present), suggesting some degree of irreversible damage. Dd-cfDNA was quantified to assess the ongoing transplant injury 3 mo after diagnosis of aAMR.

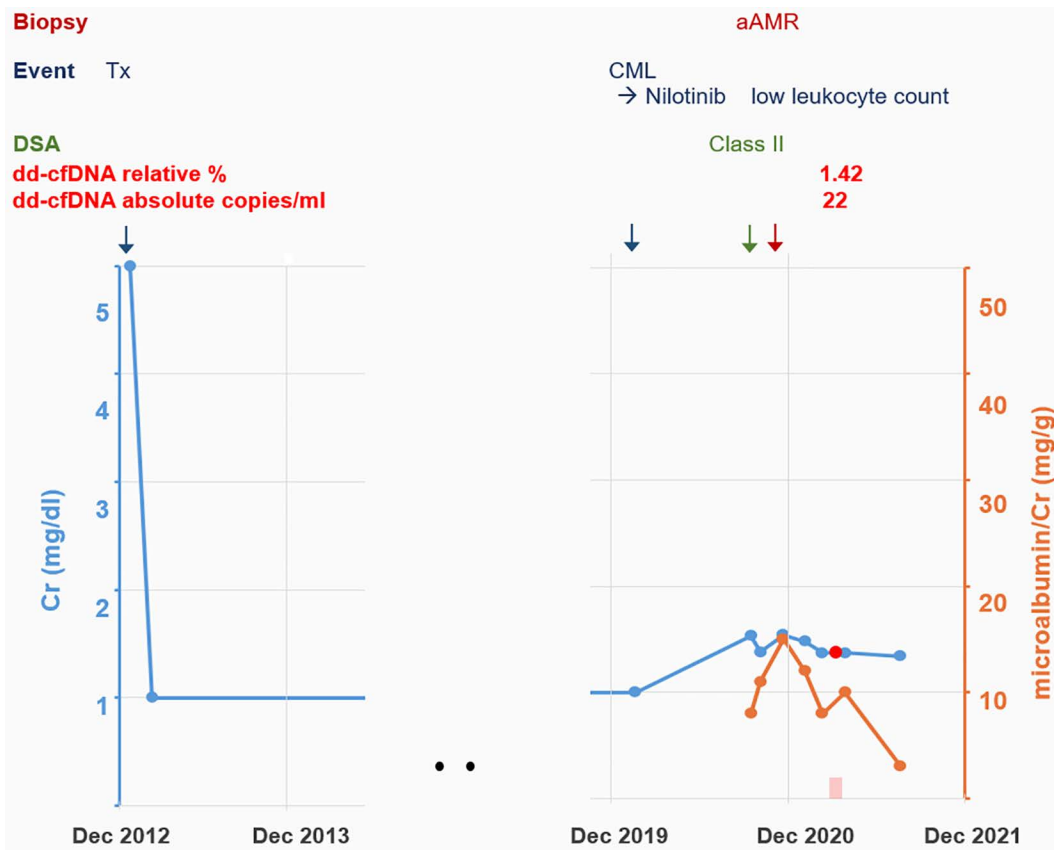
Although absolute quantification showed normal levels of dd-cfDNA (22 copies/mL), relative quantification indicated ongoing transplant injury (1.42%). In this patient, low total cfDNA levels of 1549 copies/mL were found, with the fifth percentile of the time-posttransplant–matched reference population being 2010 copies/mL. This was attributed to low leukocyte counts of  $4.6 \times 10^9/L$  due to CML and nilotinib treatment. Since no repeated biopsy was performed in this patient because of clinical stabilization, ongoing rejection cannot be ruled out. In our view, the clinical course suggests irreversible damage but not ongoing injury, which would align well with the absolute quantification.

### Case 7

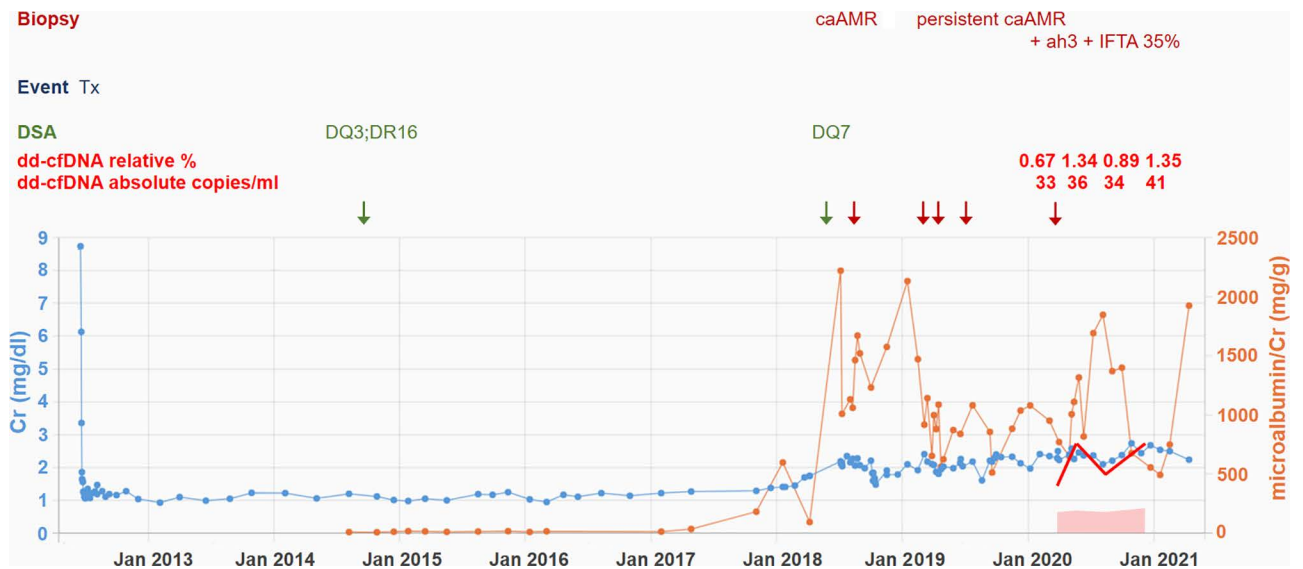
In a 32-y-old male patient, caAMR was found in repeated biopsies, the latest showing persisting signs of activity (g2, but ptc0), chronicity (cg3), as well as other findings (i2, t0, ci2, ct2, ah3) (Figure 7). Because kidney function stabilized and the latest biopsy showed severe arteriolar hyalinosis, a rescue switch to a CNI-free regimen was performed. Dd-cfDNA was measured repeatedly thereafter starting 1 mo after the last biopsy. Absolute quantification with borderline results suggested no significant transplant injury over time, whereas relative quantification indicated persisting injury. Since no follow-up biopsy was performed because of stable kidney function with respect to creatinine and proteinuria (ACR was stable at 0.7 g/g but showed a temporary rise to maximum 1.9, which was attributed to high blood pressure), we can neither confirm nor exclude ongoing transplant injury. Nevertheless, because absolute quantification was borderline and other authors already suggested that relative quantification could be of higher sensitivity in patients with caAMR,<sup>11</sup> this could denote a case where absolute quantification is less sensitive than relative quantification in detecting ongoing transplant injury. An additional reason for decreased



**FIGURE 5.** Case 5: false-negative relative quantification due to high total cfDNA from cytomegalovirus infection. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment. CMV, cytomegalovirus infection; Cr, creatinine; dd-cfDNA, donor-derived cell-free DNA; DGF, delayed graft function; Jan, January; TCMR, acute T cell-mediated rejection; Tx, transplantation.



**FIGURE 6.** Case 6: false-positive relative quantification due to very low total cfDNA from low leukocyte count from nilotinib treatment for chronic myeloid leukaemia. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. aAMR, active antibody-mediated rejection; CML, chronic myeloid leukaemia; Cr, creatinine; DSA, donor-specific anti-HLA antibodies; dd-cfDNA, donor-derived cell-free DNA; Tx, transplantation; Dec, December.



**FIGURE 7.** Case 7: Absolute quantification suggests no ongoing transplant injury, whereas relative quantification is above the cutoff. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. ah3, arteriolar hyalinosis grade 3; caAMR, chronic active antibody-mediated rejection; Cr, creatinine; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific anti-HLA antibodies; IFTA, interstitial fibrosis tubular atrophy; Jan, January; Tx, Transplantation.

dd-cfDNA release could be a high grade of interstitial fibrosis (ci2), which was described to be 35% in the last biopsy.

### Case 8

In the repeated biopsies of a 53-y-old male patient with caAMR, the latest biopsy chronic AMR (cAMR) with little activity (g1, ptc0) and advanced chronic changes (cg3), with other findings being (i0, t0, v0, ci1, ct1, ah3) (Figure 8). Due to severe arteriolar hyalinosis, a switch to CNI-free regimen was performed. Repeated measurements of dd-cfDNA were performed starting 1 mo after the last biopsy. Note that for this patient, 2 baseline measurements were performed.

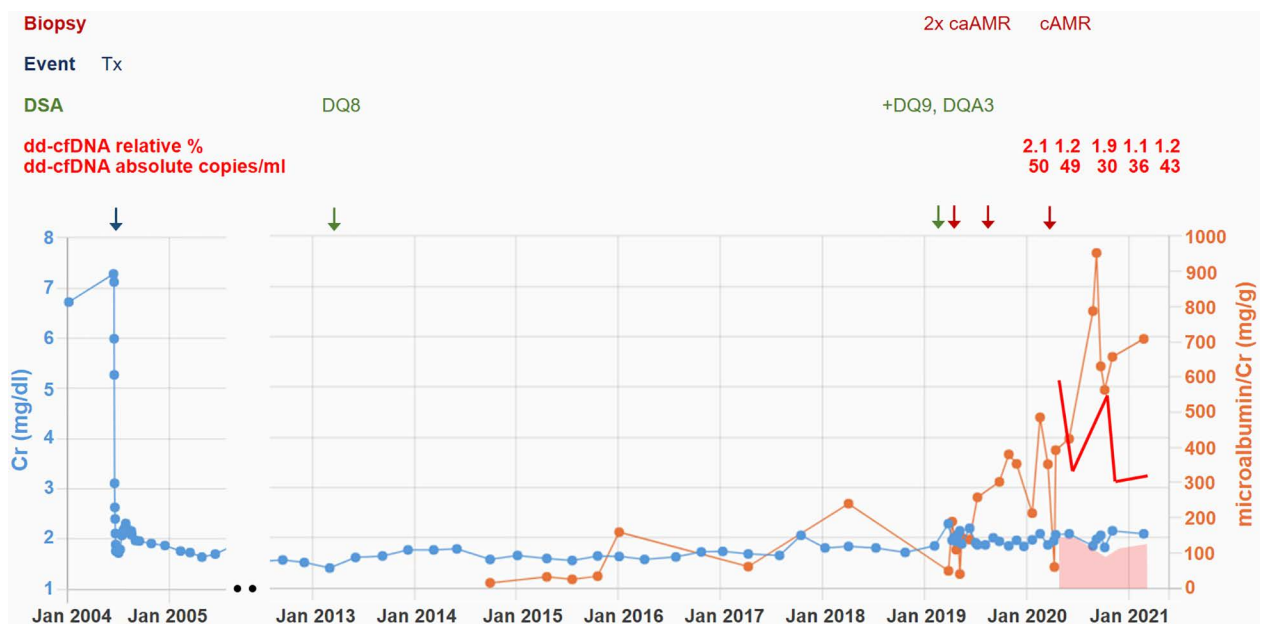
Absolute quantification showed borderline results below the cutoff of 50 copies/mL, whereas relative quantification indicated ongoing transplant injury. In the third measurement, when absolute quantification showed 30 copies/mL, but relative quantification showed 1.9%, the total cfDNA of 1579 copies/mL was found with the fifth percentile of the time-posttransplant-matched reference population being 2010 copies/mL. The low total cfDNA may have contributed to the increase of relative dd-cfDNA. Again, as in case 7, because of the stable creatinine course and only slightly increasing proteinuria (ACR 0.4 to 0.7g/g during the observation period), we did not perform a follow-up biopsy in this patient. Therefore, ongoing activity of AMR can neither be confirmed nor excluded. Although stable creatinine course and Banff grades g1 and ptc0 in the latest biopsy argue in favor of little activity of caAMR, rising albuminuria may point toward ongoing transplant injury. On the other hand, albuminuria may be increased because of the cessation of CNI and may not be related to possible caAMR activity at all. This case demonstrates a limitation of dd-cfDNA in such complex patients, as even with comprehensive data available, it is hard to decide whether absolute or relative quantification is more reliable without performing kidney biopsy.

### Case 9

In a 70-y-old male patient with caAMR (v0, g1, ptc0, cg2, mm1, ah2) and progressive deterioration of kidney function, medication nonadherence was suspected (Figure 9). Because of the rise in albuminuria and creatinine, dd-cfDNA was assessed. Absolute and relative quantification indicated ongoing transplant injury for the first 2 measurements, which was supported by the clinical course (creatinine rising from 2.9 mg/dL to 4.2 mg/dL, ACR falling from 3.0 to 0.3 g/g). For the last measurement, although absolute quantification was borderline (43 copies/mL), relative quantification (0.78%) was above the cutoff. Nevertheless, both absolute and relative levels showed a decline in the third measurement. We attributed this decline to advanced fibrosis (estimated glomerular filtration rate <15 mL/min/1.73m<sup>2</sup>). Again, because no follow-up biopsy was performed due to the lack of therapeutic options in this patient with advanced caAMR, there is no “gold standard” to compare dd-cfDNA measurement against. In fact, relative quantification may be of a higher sensitivity with respect to the cutoffs used in this analysis. More importantly, this case points toward a limitation of dd-cfDNA measurement in patients with advanced fibrosis. In such cases, dd-cfDNA in general is less elevated because less vital tissue is present that is capable of releasing cfDNA.

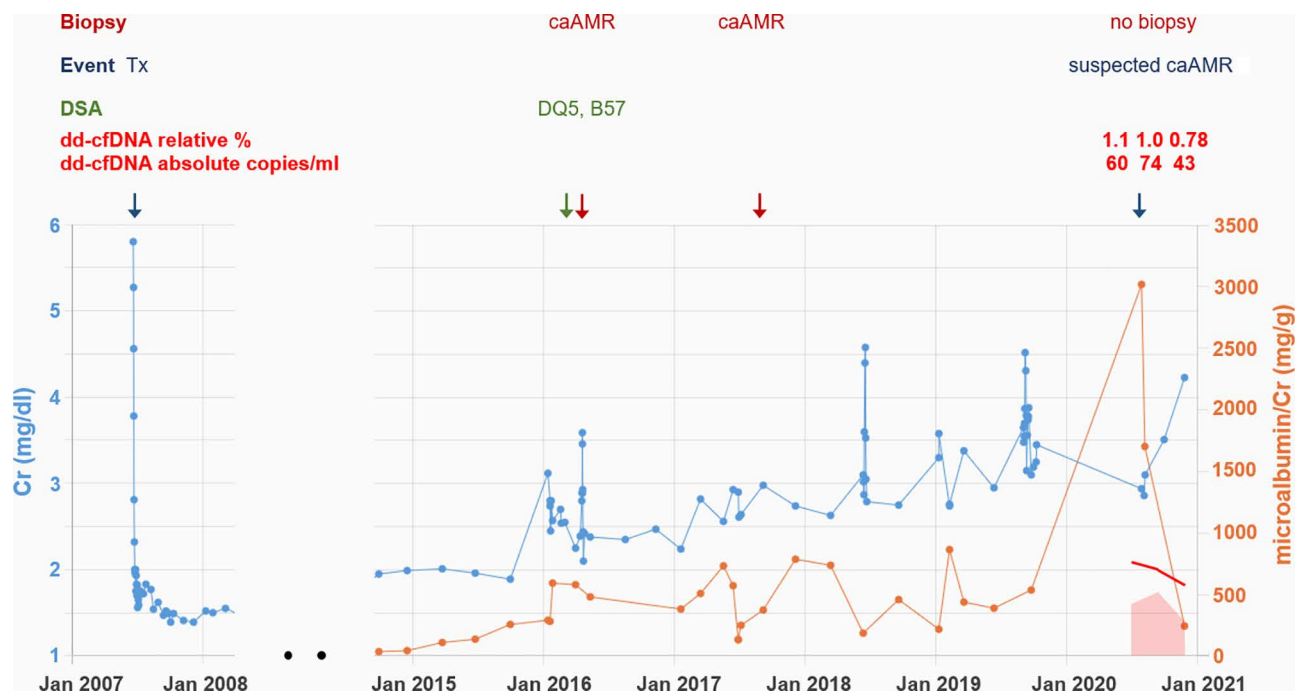
## DISCUSSION

This case series demonstrates several important limitations of relative, absolute, and dd-cfDNA quantification in general. First, we show that discrepancies between absolute and relative quantifications of dd-cfDNA occur frequently (9 out of 22 patients analyzed) in a real-life kidney transplant cohort mainly consisting of long-term transplanted patients. Second, such discrepancies are in large part due to graft unrelated increases in total cfDNA, which can lead to false-negative



**FIGURE 8.** Case 8: Absolute quantification shows borderline results, whereas relative quantification is above the cutoff. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. caAMR, chronic active antibody-mediated rejection; cAMR, chronic antibody mediated rejection; Cr, creatinine; DSA, donor-specific anti-HLA antibodies; dd-cfDNA, donor-derived cell-free DNA; Jan, January; Tx, transplantation.





**FIGURE 9.** Case 9: In the last measurement, absolute quantification is already below the cutoff, whereas relative quantification is still above. Clinical course suggests progressive fibrosis and deterioration of kidney function. Arrows indicate the time when certain clinical events occurred: brown, kidney transplant biopsies; blue, clinical events or treatment; green, donor-specific HLA antibody occurrence. caAMR, chronic active antibody-mediated rejection; Cr, Creatinine; DSA, donor-specific anti-HLA antibodies; dd-cfDNA, donor-derived cell-free DNA; Jan, January; Tx, Transplantation.

results of relative dd-cfDNA measurements, but can also occur because of a decrease in total cfDNA, which, in opposition, can lead to false-positive results of relative dd-cfDNA measurements. On the other hand, in some patients with caAMR, relative quantification may be of a higher sensitivity when compared with absolute quantification, as suggested by other authors as well.<sup>11</sup> The pathophysiology of caAMR is not well defined, and performance of dd-cfDNA for this diagnosis is an area for further research.

Total cfDNA is increased under several circumstances—bacterial or viral infection, leukocytosis, or an increased amount of activated leukocytes, as well as tissue damage due to hematological or oncological diseases, among others. Because bacterial and viral infections occur frequently in KTR, there are strong intra- and interindividual variations of total cfDNA levels. Recently, Bunnapradist et al described serial dd-cfDNA measurements in a KTR suffering from COVID-19, showing excessively increased levels of total cfDNA and corresponding low relative dd-cfDNA.<sup>12</sup> We can support and further expand this finding by presenting a case of a KTR with COVID-19 and biopsy-proven acute interstitial nephritis, in whom absolute levels of dd-cfDNA indicated transplant injury while relative dd-cfDNA was below the threshold of 0.5%.

On the other hand, total cfDNA levels below the normal range will lead to false-positive results of relative dd-cfDNA quantification. We suspect this in 1 case, where CML and nilotinib treatment led to a low leukocyte count of  $4.6 \times 10^9/L$ , resulting in total cfDNA levels below the fifth percentile of the reference population.<sup>13</sup>

Still, relative quantification of dd-cfDNA occasionally indicated transplant injury while absolute levels were normal. As stated earlier, this can be due to a higher sensitivity of relative quantification in a patient population with caAMR. Other

potential benefits of relative quantification are its insensitivity to preanalytical variables (eg, DNA extraction efficiency) and its insensitivity to changes in the rate of cfDNA degradation in blood circulation. On the other hand, there is evidence that a decrease of total cfDNA over time results in an apparent increase of relative dd-cfDNA in stable KTR, an effect static thresholds cannot account for. This was shown in a reference population of over 300 patients, where median total cfDNA levels decreased from 6369 copies/mL at 12 mo to 5256 copies/mL at 24 mo, which went down to 4419 copies/mL at 60 mo after transplantation. This was paralleled by a decrease of 95th percentile of total cfDNA from 35 628 copies/mL at 12 mo to 16 234 copies/mL at 60 mo. These changes in total cfDNA can result in an apparent increase in relative dd-cfDNA over time.<sup>13</sup>

Applying those reference ranges, we have noticed for the 9 patients forming our case group that 39% (11 of 28) of total cfDNA values were outside of the expected range.<sup>13</sup> Total cfDNA outside the reference range could be a warning sign that relative quantification could be erroneous, and absolute quantification should be used instead.

Our study has a number of limitations. (i) We present a case series from a retrospective review of a subgroup from all KTR who received dd-cfDNA measurements at our institution. Additionally, the total cohort is rather small (consisting of 22 patients). (ii) Our cohort mainly consists of late post-transplant patients, most of which had acute events prompting further diagnostics. This is in contrast to most studies performed so far that studied patients in the early posttransplant period with graft dysfunction, where the proportion of stable patients is lower than in the general transplant population. This could overestimate the effects of states affecting total cfDNA. On the other hand, with growing availability of dd-cfDNA testing, it is important to detect and understand

possible confounders to the test. We therefore deem it necessary to further analyze how dd-cfDNA performs in such complex and multimorbid transplant patients. (iii) Because only indication biopsies were performed in our cohort, there was no consistent “gold-standard” of biopsy to compare against at the time of dd-cfDNA measurement for all patients. Instead, we used clinical and laboratory parameters as a surrogate to judge whether ongoing rejection was present or not, especially in patients with underlying AMR. Although this is a major limitation of our study, it does not limit our main conclusion, which is that in some cases, changes of total cfDNA affect the interpretation of relative quantification of dd-cfDNA. (iv) The time between the latest biopsy and first dd-cfDNA measurement varied from 2 wk to 3 y, which complicates the interpretation of dd-cfDNA together with biopsy results.

Despite those shortcomings, this case series provides anecdotal evidence that relative quantification of dd-cfDNA alone can be misleading in certain cases. Hence, we suggest including all 3 parameters (relative dd-cfDNA, total cfDNA, and absolute dd-cfDNA) in a comprehensive analysis as suggested by other authors as well.<sup>11</sup> That way, misinterpretation of relative dd-cfDNA can be prevented, and altered levels of total cfDNA will improve additional understanding of the patients' pathophysiological changes.

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