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META-ANALYSIS

Donor-derived cell-free DNA as a biomarker for rejection after kidney transplantation: a systematic review and meta-analysis

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SUMMARY

A systematic review and meta-analysis were performed to investigate the value of donor-derived cell-free DNA (dd-cfDNA) as a noninvasive biomarker in diagnosing kidney allograft rejection. We searched PubMed, Web of Science and the Cochrane Library for original research papers published between January 1994 and May 2020 on dd-cfDNA fractions in blood of kidney allograft recipients. A single-group meta-analysis was performed by computing pooled estimates for dd-cfDNA fractions using the weighted median of medians or quantile estimation (QE) approach. Weighted median differences in medians (WMDMs) and median differences based on the QE method were used for pairwise comparisons. Despite heterogeneity among the selected studies, the meta-analysis revealed significantly higher median dd-cfDNA fractions in patients with antibody-mediated rejection (ABMR) than patients without rejection or patients with stable graft function. When comparing patients with T cell-mediated rejection (TCMR) and patients with ABMR, our two statistical approaches revealed conflicting results. Patients with TCMR did not have different median dd-cfDNA fractions than patients without rejection or patients with stable graft function. dd-cfDNA may be a useful marker for ABMR, but probably not for TCMR.

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Key words
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Introduction

Kidney transplantation is the treatment of choice for patients with end-stage kidney disease. However, the allo-immune response induced by transplantation remains a major obstacle to graft success [1]. Fifteen per cent of transplant recipients develop acute kidney allograft rejection, which includes T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR) and mixed ABMR/TCMR [2]. TCMR has a relatively good prognosis if treated appropriately [3,4]. In contrast, ABMR has emerged as the major cause of late kidney allograft loss and as a challenging target to reduce transplant failure [3,5].

Early detection and accurate diagnosis of kidney allograft rejection, with an appropriate therapeutic strategy, is important for long-term survival of kidney transplants [6,7]. The current gold standard for diagnosing allograft rejection is a kidney allograft biopsy. However, kidney biopsies are expensive and invasive, limiting their repeatability and their interpretation is observer-dependent and sometimes difficult [6–10]. In addition, significant graft damage can already be present at the time of biopsy [7,11]. Serum creatinine is commonly used as a surrogate marker, but it is not specific and significant graft damage has often already occurred by the time an increase in serum creatinine is detected [6–9]. As such, there is an urgent need for novel biomarkers that outperform serum creatinine.

Many studies have been performed over the last few years to improve early, minimally invasive diagnosis of rejection in order to allow early therapeutic intervention [11]. One of the suggested biomarkers in these studies is donor-derived cell-free DNA (dd-cfDNA), which is expressed as a fraction of the total cell-free DNA [6,7,10,12–14]. Cell-free DNA consists of fragmented, degraded DNA that circulates in body fluids, such as plasma and urine [10,12,13]. The dd-cfDNA is released into the recipient's circulation as a result of allograft damage, probably via graft-cell apoptosis and necrosis [10,12]. The value of plasma dd-cfDNA as a noninvasive biomarker for kidney allograft rejection has been investigated in several studies focusing on ABMR, TCMR or both. As the results of these studies are conflicting, we conducted a systematic review and meta-analysis to further define the value of dd-cfDNA as a diagnostic biomarker for different types of kidney allograft rejection and more specifically ABMR.

Materials and methods

The systematic review and meta-analysis were reported according to the 'Meta-analysis Of Observational Studies in Epidemiology' (MOOSE) guidelines [15]. The clinical question was established according to the PECO (Population, Exposure, Comparator, Outcome) process: plasma dd-cfDNA (O) as a diagnostic marker for ABMR (E) versus TCMR, stable kidney function, or no rejection (C) in kidney transplant patients (P) [16]. This resulted in the following clinical question: What is the value of dd-cfDNA as a diagnostic marker for different types of kidney allograft rejection and more specifically ABMR?

Eligibility criteria

Studies were included if they described the level of cell-free DNA in the blood of kidney transplant patients, if the studies were written in English and published between 1 January 1994 and 18 May 2020. Studies focusing on urinary cell-free DNA were excluded. Cohort studies, case–control studies and cross-sectional studies were considered eligible. Meta-analyses, systematic reviews, case reports, editorials, brief reports, communications, conference/meeting abstracts and animal research studies were excluded. There was no restriction on the age of the participants in the included studies, nor on the number of kidney (re-)transplantations of the participants. However, studies including multi-organ recipients (e.g. kidney–pancreas recipients) were excluded. These criteria were predefined.

Search strategy

A systematic literature search in PubMed, the Web of Science and the Cochrane library was conducted with the following Mesh terms: 'cell free DNA AND kidney

transplantation' and 'cell free DNA AND kidney allograft'. This search was performed separately by two authors (KL/VW). The last search date was 18 May 2020.

Study selection

Studies were screened independently by two authors (KL/VW) in a two-step procedure. First, duplicates were removed and the studies screened by title and abstract. Subsequently, the full text of the remaining studies was reviewed.

Risk of bias in individual studies

The Methodological Index for Non-Randomized Studies (MINORS) was used to assess the methodological quality of the included studies [17]. Two authors (KL/VW) independently assessed the potential risk of bias of the selected studies based on this score. Consensus was reached by the two reviewers when they did not share the same opinion.

Data extraction

For every study that met the inclusion criteria, the following data were extracted: study design, sample characteristics (i.e. number of samples, type of blood collection tube, time between blood collection and centrifugation, dd-cfDNA quantification technique) and dd-cfDNA fractions expressed as median and interquartile range (IQR), whenever directly available, in various settings. If the latter summary statistics were unavailable, these measures were derived based on available information. Data were extracted by one author (VW) and verified by a second (KL).

Definitions of study groups

Single- and two-sample aggregate data meta-analyses were performed among the following patient groups: ABMR (patients with pure ABMR and/or mixed ABMR/TCMR), TCMR (patients with pure TCMR), stable patients (patients with stable graft function) and 'no rejection' (patients undergoing a kidney biopsy for clinical indications without histological signs of rejection). The stable patient group was defined slightly differently in the five studies: stable serum creatinine + no active rejection on protocol biopsy + no dd-cfDNA fluctuations [12]; samples collected during at least three consecutive visits, at which the patient had none of the described exclusion criteria (e.g. clinical suspicion of rejection, BKV infection, other active infection, unstable kidney function) [9]; no proteinuria within the preceding year or from discharge from the hospital + eGFR >40 ml/min/1.73 m^2 fluctuating within ±20% of the mean eGFR within the preceding year or from discharge from the hospital + HLA antibody negative or DSA positive but with normal histology on kidney biopsy [18]; well-functioning allografts and no clinical suspicion of rejection spanning ≥3 serial visits, defined by stable and acceptable serum creatinine values, no significant proteinuria and clinical stability [19]; or complete absence of injury on protocol biopsy [7].

Data analysis and statistical methods

For each study, information was extracted regarding dd-cfDNA fractions (median and IQR) for the aforementioned patient groups. More specifically, five out of nine studies had the median and IQR for all groups readily available in the manuscript [9,10,18–20]. For two studies, additional median and IQR values were extracted based on available boxplots using WEBPLOTDIGITIZER v4.2 [6,7]. For the two remaining studies published by our own group, raw data were used to calculate medians and IQRs [8,12].

Since the papers by Bromberg *et al.* and Bloom *et al.* [6,19] were substudies from one large cohort study, both papers were considered as one study. Bromberg *et al.* selected the stable kidney transplant recipients, whereas Bloom *et al.* described the patients with abnormalities at indication biopsy (e.g. types of rejection or injury other than rejection like pyelonephritis) [6,19]. The same counts for both papers by Gielis *et al.*: in the paper of 2018, stable kidney transplant recipients were reported and in the paper of 2019 patient groups with different types of rejection and patients with other injury than rejection (e.g. pyelonephritis and BK viral infection) were described [8,12].

Subsequently, a median-based approach [WMDM] and a quantile estimation [QE] method were used to perform a meta-analysis. All statistical analyses were performed using the statistical software R version 3.6.0 [21].

Results

Study selection

The database search yielded a total of 412 hits in Web of Science (n = 220), Cochrane Library (n = 24) and

PubMed ($n = 168$). After removing duplicates, 249 records remained. After screening the title and abstract for the exclusion criteria, 43 studies were eligible for full-text review. The full-text studies were then screened, excluding 29 additional studies of which 17 were reviews without original data (Fig. 1).

The 14 remaining studies were included in this systematic review, nine of which were included in the meta-analysis. The full study selection process is shown in Fig. 1.

Study characteristics

The characteristics of the studies included in this systematic review are provided in Table 1. All studies were observational studies published during the last decade, including eight prospective cohort studies [6,8,9,12,19,22–24], one retrospective cross-sectional study [7], four prospective cross-sectional studies [10,13,18,25] and one study in which the data collection method was not described because of privacy or ethical restrictions, resulting in an unknown study design [20]. Nine studies used Cell-Free DNA Blood Collection Tubes (BCT®) [6,8,10,12,13,19,22–24], two studies used EDTA tubes [9,25], and the three remaining studies did not specify the method of blood collection [7,18,20]. Single nucleotide polymorphism (SNP) based quantification techniques were used in most studies, either sequencing technology [6–8,12,13,18–20,22–24] or digital droplet PCR [9,10]. One study applied the quantitative real-time PCR targeting insertion/deletion polymorphism (INDEL) technique [25].

In six studies, the fraction of dd-cfDNA was estimated separately in patient groups with ABMR together with mixed ABMR/TCMR or only mixed ABMR/TCMR, which were combined into a single 'component ABMR' group for further meta-analysis [6–8,10,18,20]. Four of these six studies also estimated dd-cfDNA levels in patients with isolated TCMR and in patients without rejection [6–8,20]. The latter four studies contained 58 samples from patients with a component of ABMR, 35 samples from patients with isolated TCMR and 225 samples from patients without kidney allograft rejection [6–8,20].

Risk of bias within studies

The MINORS score revealed a quality score of 86% (12 out of 14), which is 'high' or 'moderate', as shown in Table 2 and Table S1.

Synthesis of results

An overview of the full data extraction for the meta-analysis is given in Table 3.

One-sample meta-analysis

For each of the previously defined groups, (i) stable patients; (ii) patients without rejection at indication biopsy; (iii) patients with pure TCMR; (iv) patients with a component ABMR), pooled median estimates were calculated according to the WMM and QE approaches (Fig. 2). Five studies determined the fraction of dd-cfDNA in stable patients ($n = 1149$ samples), resulting in median [95% CI] fractions of 0.29% [0.21, 0.45] for the WMM method and 0.36% [0.22, 0.51] for the QE method [7,9,12,18,19]. In the ABMR group ($n = 89$ samples), a nearly 10-fold higher median value (WMM: 2.50% [1.40, 2.90]; QE: 2.15% [1.48, 2.82]) was found when combining the results from six separate studies [6–8,10,18,20]. Four of these six studies also described dd-cfDNA fractions in TCMR patients ($n = 35$ samples) and patients without rejection ($n = 225$ samples), resulting in a WMM of 0.27% [0.26, 2.69] in TCMR patients and 0.57% [0.30, 0.67] in patients without rejection [6–8,20]. Alternatively, the QE method resulted in median estimates of 0.38% [0.06, 0.70] in TCMR patients and 0.46% [0.29, 0.62] in patients without rejection.

Two-sample meta-analysis

Subsequently, the difference in medians was considered an effect measure in this meta-analysis (Fig. 3). Median dd-cfDNA fractions were significantly higher in patients with ABMR than patients without rejection [$n = 283$ samples; WMDM: 1.89% (1.02, 2.60); QE: 1.64% (0.50, 2.77); Fig. 3a]. Compared with stable patients, patients with ABMR showed also significantly higher median dd-cfDNA fractions [$n = 793$ samples; WMDM: 2.30% (1.80, 2.69); QE: 2.06% (1.32, 2.80); Fig. 3b]. When comparing patients with TCMR and patients with ABMR, the QE method showed higher median dd-cfDNA fractions in patients with ABMR [$n = 93$ samples; QE: 1.49% (0.36, 2.63)], though no significant difference was found between these groups based on the WMDM approach [$n = 93$ samples; WMDM: 1.13% (−0.13, 2.64); Fig. 3c]. Patients with TCMR did not have different median dd-cfDNA fractions compared to patients without rejection [$n = 260$ samples; WMDM: −0.04% (−0.15, 2.02); QE: −0.03% (−0.37, 0.31);

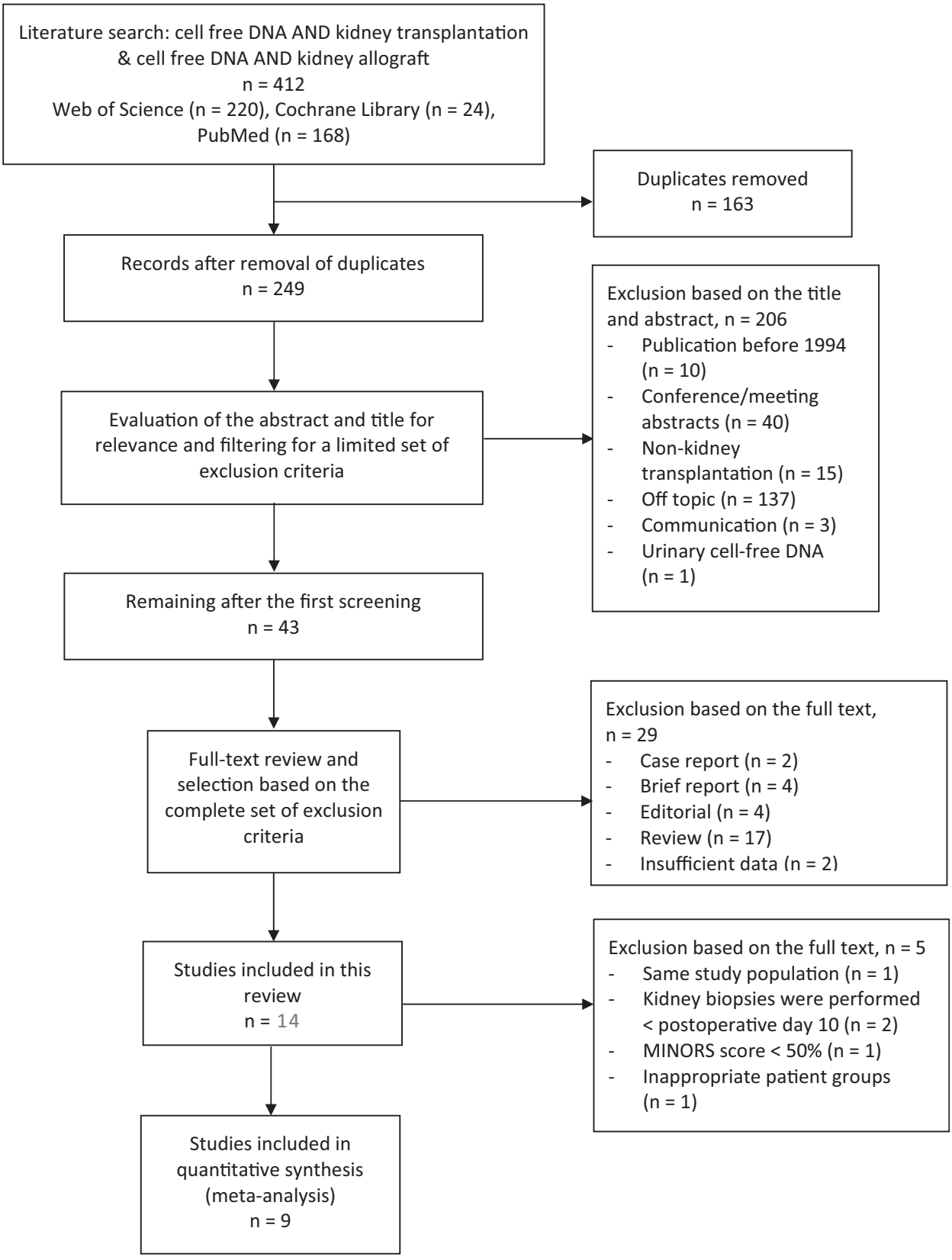

Figure 1 Flow diagram of study selection.

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Table 1. Methodological characteristics.

Author (year)	Study design	Study period	Scheduled post-transplant blood collection time points	Additional blood collection time points	Total samples	Samples used to analyse rejection vs. no rejection	Patients included for analysis of rejection vs. no rejection	Tube	Time until centrifugation after collection	dd-cfDNA quantification technique	dd-cfDNA Cut-off	Diagnosis of rejection
Bloom (2017) [6]	Prospective cohort study	Enrolment: April 2015–May 2016	Scheduled surveillance visits at months 1, 2, 3, 4, 6, 9, and 12 after transplantation	At time of indication biopsy	Not mentioned	107 plasma samples	102 KTR	Cell-free DNA BCT®	Within 7 days	Targeted amplification and sequencing of SNPs (Allosure)	1.0% Based on graphical image of sensitivity (%) and specificity (%) for dd-cfDNA to discriminate active ABMR vs. no active ABMR	TCMR: Banff 2007 criteria ABMR: Banff 2013 criteria
Bromberg (2017) [19]	Prospective cohort study Cf. Bloom [6]	Enrolment: April 2015–May 2016	Scheduled surveillance visits at months 1, 2, 3, 4, 6, 9, and 12 after transplantation	n.a.	380 plasma samples	n.a.	n.a.	Cell-free DNA BCT®	Within 7 days	Targeted amplification and sequencing of SNPs (Allosure)	1.0% (96th percentile) and 1.2% (97.5th percentile) (cut-off for the reference population with stable allograft function)	n.a.
Dauber (2019) [25]	Prospective cross-sectional study	n.a.	Postoperative day 6–39 (median 8)	At time of indication biopsy	29 plasma samples	29 plasma samples	29 KTR	Vacuette EDTA separator tubes	Within 150 min	Quantitative real-time PCR targeting (INDEL)	2.7% simultaneous maximization of sensitivity and specificity	Banff version not specified. Reference to the Banff 2015 kidney meeting report [36]
Gielis (2018) [12]	Prospective cohort study	October 2014–March 2017	Day 1–3 months after transplantation	n.a.	1036 plasma samples	n.a.	n.a.	Cell-free DNA BCT®	Within 2 days	Targeted multiplex PCR and sequencing of SNPs	0.88% Mean + 2SD	n.a.
Gielis (2019) [8]	Prospective cohort study	October 2014–March 2017	Day 1–3 months after transplantation	At time of protocol biopsy or indication biopsy	1036 plasma samples	101 plasma samples	107 KTR	Cell-free DNA BCT®	Within 2 days	Targeted multiplex PCR and sequencing of SNPs	0.88% Stable mean + 2SD	Banff 2013 criteria
Huang (2019) [20]	Observational Data are not publicly available because of privacy or ethical restrictions	August 4, 2017–September 14, 2018	n.a.	Within 30 days of an allograft biopsy	485 blood samples	n.a.	63 KTR	n.a.	n.a.	Allosure	0.74% and 1.0% Optimal cut-off ≥0.74%; 1.0% cf. Bloom [6]	Banff 2013 criteria
Jordan (2018) [22]	Prospective cohort study	April 2015–May 2016	Scheduled surveillance		1272 plasma samples	90 plasma samples	87 KTR	Cell-free DNA BCT®	Within 7 days	Targeted amplification	1.0% Cf. Bloom [6]	TCMR: Banff 2007 criteria

Table 1. Continued.

Author (year)	Study design	Study period	Scheduled post-transplant blood collection time points	Additional blood collection time points	Total samples	Samples used to analyse rejection vs. no rejection	Patients included for analysis of rejection vs. no rejection	Tube	Time until centrifugation after collection	dd-cfDNA quantification technique	dd-cfDNA Cut-off	Diagnosis of rejection
	Cf. Bloom [6]		visits at months 1, 2, 3, 4, 6, 9, and 12 after transplantation	At time of indication biopsy						and sequencing of SNPs (Allosure)		ABMR: Banff 2013 criteria
Oellerich (2019) [9]	Prospective cohort study	September 2013–October 2017	Day 1 until 1 year after kidney transplantation	≤6 days before time of biopsy	1349 plasma samples	430 plasma samples	189 KTR	EDTA tubes	Within 2 h of collection	Digital droplet PCR based on predefined SNPs	0.43% Identified as providing optimal discrimination between results obtained from stable phase and biopsy proven rejection patient groups by simultaneous optimization of sensitivity and specificity	Banff version not specified
Shen (2018) [23]	Prospective cohort study	January 3, 2018–February 15, 2018	3 h–14 days after kidney transplantation	n.a.	n.a.	n.a.	n.a.	Cell-free DNA BCT®	n.a.	Targeted SNP capture sequencing	n.a.	Banff 2015 classification of allograft rejection
Sigdel (2018) [7]	Retrospective cross-sectional study	n.a.	n.a.	At the time of an allograft biopsy or at various prespecified time intervals based on laboratory protocols	300 plasma samples	217 plasma samples	178 KTR	n.a.	n.a.	SNP-based massively multiplexed PCR (mmPCR)	1.0% Cf. Bloom [6]	Banff 2017 classification of allograft rejection with C4d stains for acute humoral rejection
Stites (2020) [24]	Prospective cohort study	June 2017–May 2019	n.a.	Prior to kidney biopsy and at the time of kidney biopsy	n.a. (79 patients)	n.a.	n.a.; only patients with TCMR 1A or borderline rejection were included	Cell-free DNA BCT®	n.a.	Targeted amplification and sequencing of SNPs (Allosure)	n.a.	TCMR: Banff 2017 criteria
Whitlam (2018) [10]	Prospective cross-sectional study	n.a.	n.a.	6 days before biopsy until 1 day after biopsy	70 plasma samples	61 plasma samples	55 KTR	Cell-free DNA BCT®	Within 7 days	Droplet digital PCR based on CNV assays	0.75% for the diagnosis ABMR	Banff version not specified reference to the revised (2013) Banff classification [37]

Table 1. Continued.

Author (year)	Study design	Study period	Scheduled post-transplant blood collection time points	Additional blood collection time points	Total samples	Samples used to analyse rejection vs. no rejection	Patients included for analysis of rejection vs. no rejection	Tube	Time until centrifugation after collection	dd-cfDNA quantification technique	dd-cfDNA Cut-off	Diagnosis of rejection
Zhang (2020) [18]	Prospective cross-sectional study	November 2016–September 2017	At admission-24 months post kidney allograft surgery or at the time of a kidney allograft biopsy	At the time of kidney allograft biopsy	37 plasma samples	37 plasma samples	37 KTR	n.a.	Within 4 h	Targeted sequencing of SNPs	1.0% for the diagnosis ABMR	Banff 2015 kidney meeting report [36]
Zhou (2019) [13]	Prospective cross-sectional study	June 2017–July 2018	n.a.	The blood sample was drawn on the same day as the kidney biopsy	32 plasma samples	32 plasma samples	30 KTR	Cell-free DNA BCT®	n.a.	Liquid hybridization technology of 6200 selected human SNP loci followed by next generation sequencing	n.a.	Not mentioned

ABMR, antibody-mediated rejection; ATN, acute tubular necrosis; BCT, blood collection tubes; CNV, copy number variation; dd-cfDNA, donor-derived cell-free DNA; EDTA, ethylenediamine tetraacetic acid; INDEL, insertion/deletion polymorphisms; KTR, kidney transplant recipients; n.a., not assessed; PCR, polymerase chain reaction; SD, standard deviation; SNP, single nucleotide polymorphism; TCMR, T cell-mediated rejection.

Table 2. Individual MINORS score: risk of bias for nonrandomized studies.

	Bloom 2017 [6]	Dauber 2019 [25]	Gielis 2018 [12]	Gielis 2019 [8]	Huang 2019 [20]	Jordan 2018 [22]	Oellerich 2019 [9]	Shen 2018 [23]	Sigdel 2018 [7]	Whitlam 2018 [10]	Zhou 2019 [13]	Bromberg 2017 [19]	Zhang 2020 [18]	Stites 2020 [24]
Clearly stated aim	2	2	2	2	2	2	2	1	2	2	2	2	2	2
Inclusion of consecutive patients	1	0	2	2	1	1	2	2	1	1	0	1	1	1
Prospective data collection	2	2	2	2	0	2	2	2	1	2	2	2	2	2
Endpoints appropriate to study aim	2	2	2	2	2	2	2	2	2	2	2	2	2	1
Unbiased assessment of study endpoint	2	0	2	2	0	2	2	0	2	2	0	2	2	1
Follow-up period appropriate to study aim	2	1	1	1	0	2	2	2	0	1	0	2	1	0
<5% loss to follow-up	1	0	2	2	1	1	2	2	1	1	0	1	2	0
Prospective calculation of study size	0	0	0	2	0	0	0	0	0	1	0	0	0	0
Adequate control group	2	2	n.a.	2	2	2	2	n.a.	2	2	2	n.a.	1	2
Contemporary groups	2	2	n.a.	2	2	2	2	n.a.	2	2	2	n.a.	2	2
Baseline equivalence of groups	1	1	n.a.	0	0	2	1	n.a.	1	2	0	n.a.	2	1
Adequate statistical analyses	2	1	n.a.	2	2	2	2	n.a.	2	2	0	n.a.	2	2
Total	19/24 79%	13/24 54%	13/16 81%	21/24 88%	12/24 50%	20/24 83%	21/24 88%	11/16 69%	16/24 67%	20/24 83%	10/24 42%	12/16 75%	19/24 79%	14/24 58%

n.a., not applicable.

*The items are scored 0 (not reported), 1 (reported but inadequate), or 2 (reported and adequate). The global ideal score is 16 for non-comparative studies and 24 for comparative studies. For noncomparative studies, the following score ranges are applied: 0–4, very low quality; 5–8, low quality; 9–12, moderate quality; and 13–16, high quality. For comparative studies, the corresponding scores are 0–6, very low quality; 7–12, low quality; 13–18, moderate quality; and 19–24, high quality.

Table 3. Full data extraction for meta-analysis.

Study	Year	Patients, *n*	Samples, *n*	ABMR, *n*			ABMR and mixed ABMR, *n*	Mixed TCMR/ ABMR, *n*	Component ABMR, *n*	dd-cfDNA (%)* component ABMR, median [IQR]	TCMR total, *n*	dd-cfDNA (%)* TCMR total, median [IQR]
				Total	Aamr	caAMR						
Bloom *et al*. [6]‡	2017	102	107		6	10	16		16	2.90 [1.08–4.35]	11	0.26 [0.19–1.62]
Bromberg *et al*. [19]‡	2017	93	380									
Gielis *et al*. [8]†	2019	107	101	2			6	4	6	2.54 [0.49–15.82]	4	0.42 [0.32–1.01]
Gielis *et al*. [12]†	2018	42	294									
Huang *et al*. [20]	2019	63	?	22			24	2	24	1.40 [1.15–2.60]	10	0.27 [0.19–1.30]
Oellerich *et al*. [9]	2019	189	1359									
Sigdel *et al*. [7]	2018	178	217	16				12	12	2.56 [1.56–7.40]	10	2.69 [1.68–5.06]
Whitlam *et al*. [10]	2018	55	61				13		13	2.50 [0.80–3.00]		
Zhang *et al*. [18]	2020	37	37	18	11	7			18	2.40 [1.52–3.70]		

Study	Year	Patients, *n*	Samples, *n*	Stable, *n*	dd-cfDNA (%)* stable, median [IQR]	No rejection, *n*	dd-cfDNA (%)* no rejection, median [IQR]
Bloom *et al*. [6]‡	2017	102	107			80	0.30 [0.16–0.76]
Bromberg *et al*. [19]‡	2017	93	380	380	0.21 [0.12–0.39]		
Gielis *et al*. [8]†	2019	107	101			91	0.57 [0.36–0.91]
Gielis *et al*. [12]†	2018	42	294	294	0.45 [0.34–0.6]		
Huang *et al*. [20]	2019	63	?			29	0.38 [0.26–1.10]
Oellerich *et al*. [9]	2019	189	1359	408	0.29 [0.17–0.56]		
Sigdel *et al*. [7]	2018	178	217	55	0.26 [0.15–0.92]	25	0.67 [0.21–1.30]
Whitlam *et al*. [10]	2018	55	61				
Zhang *et al*. [18]	2020	37	37	12	0.60 [0.53–0.66]		

AAMR, active antibody-mediated rejection; ABMR, antibody-mediated rejection; caAMR, chronic active antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; IQR, interquartile range; TCMR, T cell-mediated rejection.

*In all the studies included in this meta-analysis, dd-cfDNA was measured as a fraction (%) of the total circulating cell-free DNA.

†The two papers of Gielis *et al*. [8,12] originated from the same time period and the same transplant centre. The data extracted from both papers were never used in the same analysis and patients were therefore not included twice. The 2018 paper only included stable patients [12], while from the 2019 paper, component ABMR, total TCMR and no rejection subgroups [8] were extracted from the total cohort.

‡Since the papers by Bromberg *et al*. and Bloom *et al*. [6,19] were substudies from one large cohort study, both papers were considered as one study. Bromberg *et al*. selected the stable kidney transplant recipients, whereas Bloom *et al*. described the patients with abnormalities at indication biopsy (e.g. types of rejection or injury other than rejection like pyelonephritis).

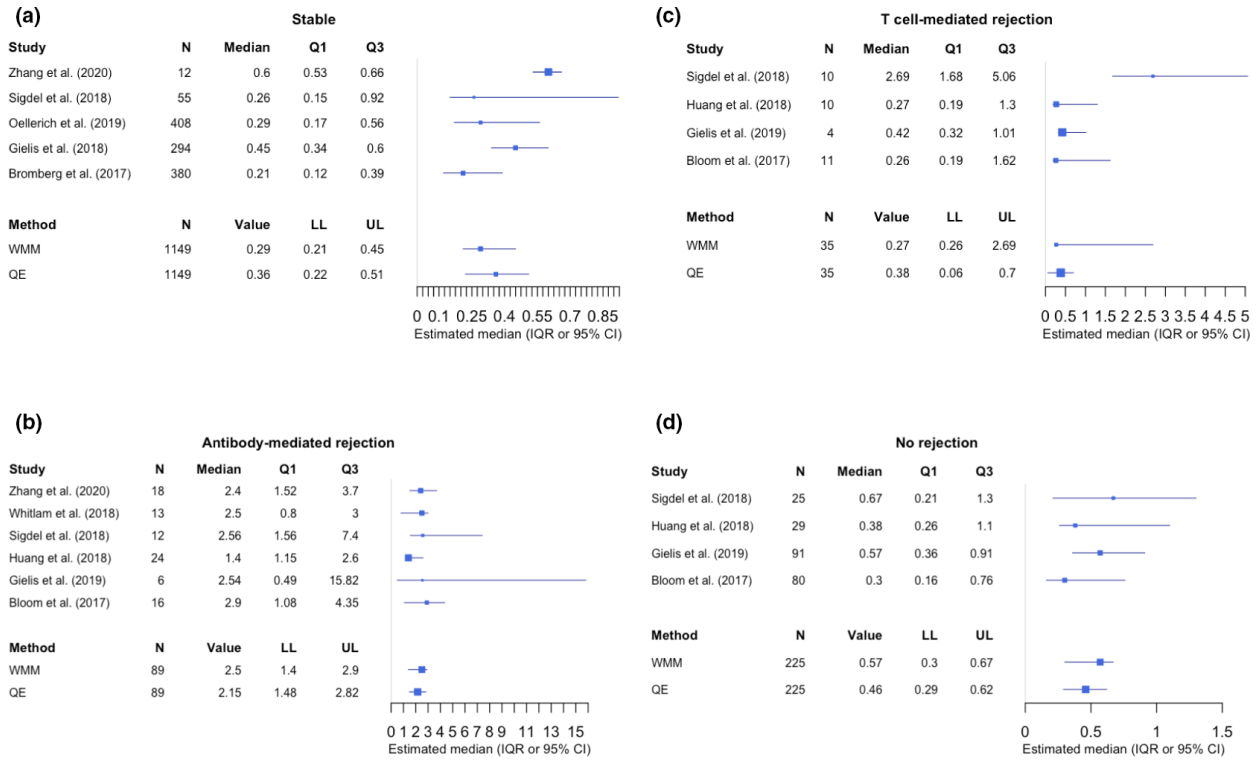

Figure 2 Pooled median estimates according to the weighted median of medians (WMM) and quantile estimation (QE) approach. Box sizes in the forest plots are proportional to the interquartile range (IQR) as a measure of study-specific variability in dd-cfDNA levels. Limits of the displayed intervals are defined as quartiles for the individual studies and as 95% confidence intervals (CIs) for the pooled results. Five studies calculated the fraction of dd-cfDNA in stable patients (n = 1149 samples), resulting in a median [95% CI] of 0.29% [0.21, 0.45] for the WMM method and 0.36% [0.22, 0.51] for the QE method (a). In the antibody-mediated rejection (ABMR) group (n = 89 samples), a remarkably higher median (WMM: 2.50% [1.40, 2.90]; QE: 2.15% [1.48, 2.82]) was found when combining the results from six separate studies (b). The dd-cfDNA fractions in T cell-mediated rejection (TCMR) patients (n = 35 samples) and patients without rejection (n = 225 samples) had a WMM of 0.27% [0.26, 2.69] and 0.57% [0.30, 0.67], respectively. Alternatively, the QE method resulted in an estimated median of 0.38% [0.06, 0.70] in TCMR patients and 0.46% [0.29, 0.62] in patients without rejection (c,d). LL, lower limit; *N*, number; Q1, first quartile; Q3, third quartile; UL, upper limit.

Fig. 3d], nor compared with stable patients [n = 754 samples; WMDM: 0.05% (−0.03, 2.43); QE: 0.52% (−0.68, 1.72); Fig. 3e). When comparing patients without rejection with stable patients, patients without rejection showed significantly higher dd-cfDNA fractions [n = 925 samples; WMDM: 0.12% (0.09, 0.41); QE: 0.12% (0.04, 0.19); Fig. 3f]. A schematic overview of these results is depicted in Table 4.

The QE method enabled us to study between-study variation in differences in medians. The I^2 statistic was 40.1% (ABMR versus no rejection), 31.5% (ABMR versus TCMR) and 0% (TCMR versus no rejection; ABMR versus stable; No rejection versus stable), indicating moderate to low variation in the study results. When comparing the median dd-cfDNA fractions in patients with TCMR versus stable patients, the I^2 statistic was 84.2%, indicating a considerable heterogeneity. However, the chi-squared test for heterogeneity was borderline significant (P = 0.0492). A funnel plot was not constructed, since less than 11 studies were available for meta-analysis.

Sensitivity analysis

An analysis was performed with regard to the inclusion of results obtained by Gielis *et al.* [8], who found the largest variation in dd-cfDNA fractions in ABMR patients. In general, the impact of their study on the results of the meta-analysis appeared to be small, with comparable WMDM and pooled difference in medians based on the QE method in all comparative analyses (ABMR versus no rejection, ABMR versus stable, ABMR versus TCMR, TCMR versus no rejection; Appendix S1a–d).

The impact of including Sigdel *et al.*'s [7] results was also investigated, as it is the only retrospective study in

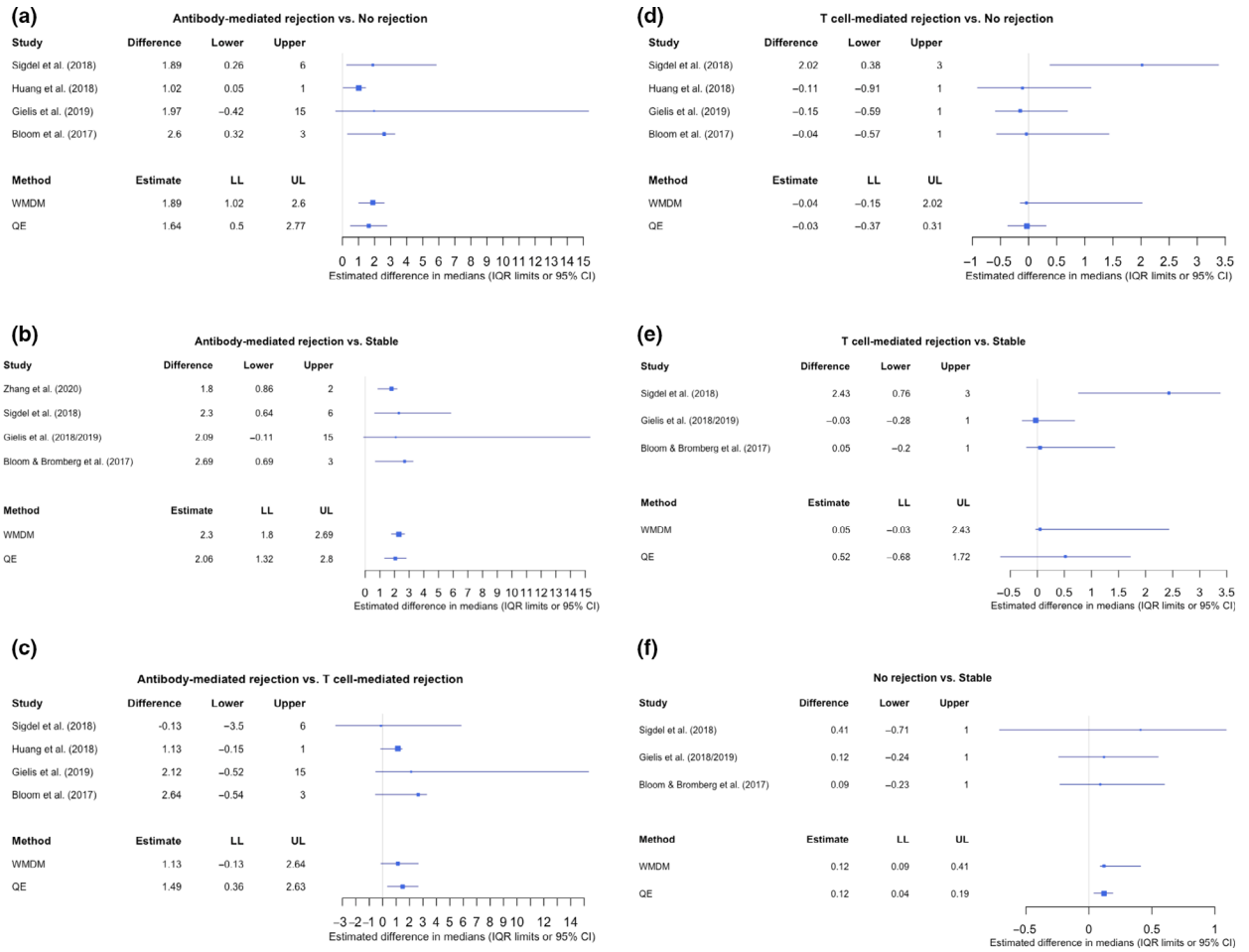

Figure 3 Results of the weighted median difference in medians (WMDM) and the quantile estimation (QE) method. Lower and upper values for the individual studies were calculated from IQR values obtained from group-specific values. Q3 in the second group was subtracted from Q1 in the first group to obtain a lower limit and Q1 in the second group from Q3 in the first group to obtain an upper limit. Median dd-cfDNA fractions appeared to be significantly higher in patients with antibody-mediated rejection (ABMR) compared to median fractions in patients without rejection (a) or stable patients (b). When comparing patients with T cell-mediated rejection (TCMR) and patients with ABMR, our two statistical approaches revealed conflicting results (c). Patients with TCMR did not have different median dd-cfDNA fractions than patients without rejection (d) or stable patients (e). Patients without rejection showed significantly higher dd-cfDNA fractions than stable patients (f). CI, confidence interval; IQR, interquartile range; LL, lower limit; *N*, number; Q1, first quartile; Q3, third quartile; UL, upper limit.

this meta-analysis. Blood samples were analysed retrospectively after an undefined time period between sampling and analysis, which may compromise the validity of the study findings. Overall, exclusion of Sigdel *et al.* led to higher WMDMs. The difference between ABMR and TCMR became significant after excluding this study, with a WMDM of 2.12% [1.13, 2.64] and a pooled difference in medians of 1.71% [0.37, 3.06] using the QE method, compared with a WMDM of 1.13% [−0.13, 2.64] and pooled estimate of 1.49% [0.36, 2.63] when including the study (Appendix S2 and Fig. 3). Concerning ABMR versus no rejection, comparable results were obtained after excluding the aforementioned paper, with a WMDM of 1.97% [1.02, 2.60] and a pooled difference in medians of 1.65% [0.25, 3.06], compared with 1.89% [1.02, 2.60] and 1.64% [0.50, 2.77], respectively, if the paper was included (Appendix S2 and Fig. 3). When comparing ABMR and stable kidney function, exclusion of Sigdel *et al.* also led to higher WMDMs, but identical pooled differences in medians [WMDM of 2.69% (1.80, 2.69) vs. 2.30% (1.80, 2.69); QE of 2.04% (1.27, 2.81) vs. 2.06% (1.32, 2.80); Appendix S2 and Fig. 3]. A sensitivity analysis of TCMR or no rejection versus stable kidney transplant patients was not possible, as only three studies (Sigdel *et al.* included) reported dd-cfDNA fractions in both stable kidney transplant patients and TCMR patients or patients without rejection [6–8,12,19]. Interestingly, the

Table 4. Overview of pairwise comparisons of median dd-cfDNA fractions (%) among different patient groups.

	TCMR	No rejection	Stable
ABMR	*n* = 93 samples WMDM 1.13% [−0.13 to 2.64] QE 1.49% [0.36–2.63]	*n* = 283 samples WMDM 1.89% [1.02–2.60] QE 1.64% [0.50–2.77]	*n* = 793 samples WMDM 2.30% [1.80–2.69] QE 2.06% [1.32–2.80]
TCMR		*n* = 260 samples WMDM − 0.04% [−0.15 to 2.02] QE − 0.03% [−0.37 to 0.31]	*n* = 754 samples WMDM: 0.05% [−0.03 to 2.43] QE: 0.52% [−0.68 to 1.72]
No rejection			*n* = 925 samples WMDM: 0.12% [0.09–0.41] QE: 0.12% [0.04–0.19]

Weighted median differences in medians (WMDMs) were calculated for pairwise comparisons of the median donor-derived cell-free DNA (dd-cfDNA) fractions among different patient groups. Results from the two statistical approaches [median-based approach (WMDM) and the quantile estimation (QE) method; % in favour of the first column] are reported with 95% confidence intervals in squared brackets. Significantly higher dd-cfDNA fractions were found in kidney transplant patients with antibody-mediated rejection (ABMR) compared to patients without rejection or stable patients, but results were inconclusive when comparing ABMR with T cell-mediated rejection (TCMR). Patients with TCMR did not have different dd-cfDNA fractions than stable patients or those without rejection. When comparing patients without rejection with stable patients, patients without rejection showed significantly higher dd-cfDNA fractions.

results of Sigdel *et al.* were different from the two other studies when comparing TCMR and stable kidney transplant patients: Sigdel *et al.* reported higher dd-cfDNA fractions in TCMR patients, whereas no significant difference was reported in the other two studies [6–8,12,19].

Discussion

In this systematic review and meta-analysis, the potential of dd-cfDNA as a noninvasive biomarker to distinguish between different types of kidney allograft rejection was explored. Higher dd-cfDNA fractions were found in kidney transplant patients with ABMR than patients without rejection or stable patients, but results were inconclusive when comparing ABMR and TCMR. Patients with TCMR did not have different dd-cfDNA fractions than patients without rejection or stable patients. To the best of our knowledge, this is the first systematic review and meta-analysis on dd-cfDNA in kidney allograft recipients.

Fourteen studies met the inclusion criteria, nine of which were included in the meta-analysis. All included studies were published over the last 3 years, underlining the emerging global interest in dd-cfDNA as a tool to monitor kidney allograft integrity. These studies reported conflicting results because of heterogeneity in study design, inclusion criteria and outcome measures. However, a meta-analysis using two different statistical approaches revealed significantly higher fractions of dd-cfDNA in patients with ABMR than patients without histological rejection or stable patients, underlining the potential of dd-cfDNA as a specific marker for ABMR.

These results are in line with previous studies. As described by Knight *et al.* [26], dd-cfDNA has the greatest discriminatory power in solid organ transplants with high grades of TCMR or ABMR. In cardiac transplant patients, higher median dd-cfDNA fractions were present during ABMR (5.8%) versus TCMR (0.39%) [27]. Similar findings were reported in lung transplant patients, as dd-cfDNA fractions were 5-times higher during ABMR than TCMR (5.4% vs. 1.1%), and even 20 times higher during ABMR than nonrejection time points [28,29]. To date, the underlying mechanisms explaining the difference in dd-cfDNA fractions between ABMR and TCMR have not been clarified. However, one may speculate that this difference could be because of differences in pathogenesis, with ABMR involving an interaction between the antibody and the allograft vascular endothelium, and TCMR having less vascular involvement. This may lead to a greater release in dd-cfDNA from damaged cells/tissues into the circulation when the lesions involve the kidney graft vasculature rather than the tubuli. Another reason for the difference may be a longer timeframe between the onset of damage and diagnosis in the case of ABMR [29].

Accurate biomarkers for the early detection of kidney allograft injury have not yet entered the clinical arena. Three of the nine studies included in this meta-analysis performed a ROC analysis to compare dd-cfDNA fractions and serum creatinine in the diagnosis of kidney allograft rejection [6,8,10]. Gielis *et al.* [8] evaluated the

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performance of plasma dd-cfDNA fractions compared with serum creatinine in distinguishing acute rejection from a combined group of patients with normal indication/protocol biopsy, borderline untreated rejection and acute tubular necrosis. They found equal diagnostic performance with similar areas under the curve (AUCs) of ~0.64. Bloom *et al.* [6] reported an AUC of 0.54 for serum creatinine and 0.74 for dd-cfDNA fractions, suggesting better discrimination of active rejection by measuring dd-cfDNA fractions. Notably, the study design differed between these two studies; Gielis *et al.* [6,8] included plasma samples paired with both indication and protocol biopsy, whereas Bloom *et al.* included only plasma samples paired with indication biopsies. Bloom *et al.* [6] also investigated the diagnostic performance of dd-cfDNA fractions and serum creatinine in discriminating ABMR from the absence of active ABMR. This resulted in an AUC of 0.87 for dd-cfDNA fractions versus 0.57 for serum creatinine. These results are in accordance with those of a third study [10] in which AUCs of 0.89 and 0.59 were reported for dd-cfDNA and serum creatinine, respectively. Huang *et al.* and Zhang *et al.* [18,20] also assessed the diagnostic accuracy of dd-cfDNA fractions for ABMR with similar results (AUC 0.82 and 0.90, respectively) to the three previous studies, but no comparison was made with serum creatinine.

Thus far, all published studies have focused on a predefined cut-off for dd-cfDNA fraction to discriminate acute rejection. It would be interesting to investigate whether a relative increase in dd-cfDNA% points to acute rejection. A prospective study design with frequent sampling would be necessary to define a relevant increase in dd-cfDNA%. In addition, the absolute quantification of dd-cfDNA (copies/ml) could be an alternative method for detecting kidney allograft rejection. Two studies have investigated the use of absolute dd-cfDNA values, with conflicting results [9,10]. Oellerich *et al.* found the absolute quantification of dd-cfDNA to be superior to dd-cfDNA fraction in discriminating kidney allograft rejection. They argued that absolute dd-cfDNA values are not affected by changes in circulating recipient DNA and, therefore, are more reliable [9]. These conclusions were not confirmed by Whitlam *et al.* [10]. Further research is needed to confirm the value of absolute dd-cfDNA levels.

It is questionable whether a single biomarker would detect kidney allograft rejection with an excellent positive and negative predictive value. Therefore, several studies have focused on the development of a panel of biomarkers to improve their diagnostic accuracy. Roedder *et al.* [30] developed a 17-gene set (kSORT assay) to detect patients at high risk of acute rejection, and Van Loon *et al.* [31] described an 8-gene assay for minimally invasive diagnosis of ABMR. Suthanthiran *et al.* [32] reported a three-gene signature in urine cell pellets for diagnosing TCMR. These gene sets show promising potential. In addition, other biomarkers, such as urinary CXCL9 and CXCL10, donor-specific antibodies and eventually microRNAs, may be considered [11,22,33]. It may be of interest to investigate whether combining dd-cfDNA with other biomarkers improves its diagnostic capacity.

Limitations of the meta-analysis

The possibility of performing a classical meta-analysis was limited by several issues detected in the nine included papers [6–10,12,18–20]. Meta-analytic approaches for pooling (the differences in) means are well established in the literature, but studies reporting medians are often discarded or transformation-based methods are applied. In the latter, mean and standard deviation are derived from both the median and a measure of spread (e.g. IQR). Recently, McGrath *et al.* [34,35] proposed one- and two-sample aggregate data meta-analysis tools for pooling median estimates that outperform transformation-based approaches. These median-based methods are preferred, especially in the case of skewed outcome distributions, as with the dd-cfDNA fractions in the included studies [34,35]. Thus, medians and differences in medians were considered as effect measures in this meta-analysis, as sample medians provide a more robust measure of the central tendency of the underlying distribution. More specifically, the weighted medians of (study-specific) medians (WMMs) were computed as pooled estimates for the median dd-cfDNA fractions in the previously defined patient groups [35]. In a two-sample context, the weighted median differences in medians (WMDMs) were calculated for pairwise comparisons of the median dd-cfDNA fractions among the aforementioned groups. Weights were chosen proportional to the group-specific sample sizes (one-sample) or total sample sizes in both groups (two-sample), and approximate 95% confidence intervals (CIs) were constructed based on inverting the sign test while relying on the asymptotic normality of the test statistic and using the respective quantiles of the weighted study medians as lower and upper limits [34,35]. Notably, the WMM and WMDM methods implicitly assume fixed effects meta-analysis models, assuming that the (differences in) medians are coming

from a single underlying distribution, potentially underestimating the variability. In addition, in some meta-analyses, (weighted) median estimates are calculated based on effect measures obtained from a small number of studies. Therefore, one should be cautious when interpreting the pooled estimates. In addition to the median-based methods, a (parametric) quantile estimation (QE) method was used in which one relies on asymptotic normality of the distribution of the median or difference in medians and parametrically estimates the large sample variance in the median or difference in medians estimator. This offers the advantage of describing between-study variability in relation to within-study variation (expressed in terms of the I^2 statistic) over the above-mentioned median-based methods, but at the cost of specifying parametric distributions for the effect measure of interest. In general, however, the results based on the nonparametric median-based approaches and the QE method were similar, despite the limited number of studies. Nevertheless, the small number of studies, together with their limited number of samples and observational study designs, created a certain risk of bias. Only six out of nine studies (67%) included for meta-analysis revealed a high quality score using the MINORS.

Furthermore, heterogeneity was observed in study design (e.g. retrospective vs. prospective study designs, differences in blood collection method, dd-cfDNA quantification technique, Banff criteria to diagnose kidney allograft rejection) and patient inclusion (consecutive transplanted patients versus patients with an acute kidney event). In addition, different styles of statistical reporting were noted (mean ± SD or median with IQR or min-max). Moreover, in several studies, identically named pathology study groups contained a different composition of patients. For example, study groups termed ABMR could include pure ABMR and mixed ABMR/TCMR, or only mixed ABMR/TCMR. These groups were combined into one 'component ABMR' group in the meta-analysis, as our main purpose was to investigate whether the presence of ABMR characteristics is related to increases in dd-cfDNA. Besides, included studies reported no dd-cfDNA fractions for different grades of TCMR, making a distinction between dd-cfDNA fractions in low vs. high grades of TCMR impossible [6–8,20].

Despite this heterogeneity across the different studies, the QE method appropriately encompassed both within- and between-study variation when pooling the study-specific estimates using inverse variance weighting. Therefore, we conclude that our pooled estimates adequately reflect variation across and within the included studies.

Future perspectives

Although this meta-analysis showed higher dd-cfDNA fractions in patients with ABMR, several steps are necessary before considering the routine use of dd-cfDNA to monitor kidney allograft rejection. All currently published studies (Table 1) are observational cohort studies with limited sample sizes. Large multicentre studies are urgently needed. Furthermore, biomarker research for kidney transplant rejection needs to be performed in a more standardized manner (e.g. identical study design and statistical reporting), with comparisons of all relevant study groups [i.e. patients with stable graft function, patients with acute ABMR (ABMR only or mixed ABMR/TCMR), patients with acute TCMR, patients with nonrejection kidney injury]. In addition, several methods of using dd-cfDNA as a diagnostic tool for kidney allograft rejection should be taken into account, such as relative increase in dd-cfDNA%, a dd-cfDNA% threshold, absolute quantification of dd-cfDNA levels and the combination of dd-cfDNA with other biomarkers.

Conclusion

Even though the study populations are rather small and the studies are limited in number, we were able to clearly demonstrate that dd-cfDNA increases in case of ABMR but not in case of TCMR when compared to patients without rejection or stable patients. Further studies using a standardized approach in a multicentre setting are required to confirm these findings.

Authorship

KL and VW: performed the literature search independently, assessed the methodological quality and subsequently extracted data from the studies that met the inclusion criteria. PP, SA and NH: performed the meta-analysis. VW, KL and DA: drafted the manuscript. VW, PP, SA, NH, EG, RH, AM, DH, BDW, DA and KL: reviewed and approved the final version of the manuscript.

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Conflict of interest

Dennis Hesselink reported grants and personal fees from Astellas Pharma and Chiesi Pharma, outside the submitted work. The other authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Individual MINORS score: rationale for risk of bias judgements.

Appendix S1. Sensitivity analyses (excluding Gielis *et al.*).

Appendix S2. Sensitivity analyses (excluding Sigdel *et al.*).

Appendix S3. MOOSE checklist.

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