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Title:

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Date:

2019-04-01

Citation:

Whitlam, J. B., Ling, L., Skene, A., Kanellis, J., Ierino, F. L., Slater, H. R., Bruno, D. L. & Power, D. A. (2019). Diagnostic application of kidney allograft-derived absolute cell-free DNA levels during transplant dysfunction. American Journal of Transplantation, 19 (4), pp.1037-1049. <https://doi.org/10.1111/ajt.15142>.

Persistent Link:

<https://hdl.handle.net/11343/284664>

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Article type : O - Original Article

Diagnostic application of kidney allograft-derived absolute cell-free DNA levels during transplant dysfunction

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ajt.15142](https://doi.org/10.1111/ajt.15142)

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Abbreviations

ABMR = composite diagnosis of acute antibody mediated rejection and chronic active antibody mediated rejection

AMR = antibody mediated rejection

aAMR = acute antibody mediated rejection

AUC = area under the curve

aCMR = acute cellular mediated rejection

bCMR = borderline cellular mediated rejection

caAMR = chronic active antibody mediated rejection

cfDNA = cell-free DNA

CI = confidence interval

CMR = cellular mediated rejection

CNI = calcineurin inhibitor

CV = coefficient of variation

DNA = deoxyribonucleic acid

gd-cfDNA = graft-derived cell-free DNA

IQR = inter-quartile range

NOS = not otherwise specified

PCR = polymerase chain reaction

ROC = receiver operator characteristic

ABSTRACT

Graft-derived cell-free DNA (donor-derived cell-free DNA) is an emerging marker of kidney allograft injury. Studies examining the clinical validity of this biomarker have previously used the graft fraction, or proportion of total cell-free DNA that is graft-derived. The present study evaluated the diagnostic validity of absolute measurements of graft-derived cell-free DNA, as well as calculated graft fraction, for the diagnosis of graft dysfunction. Plasma graft-derived cell-free DNA, total cell-free DNA and graft fraction were correlated with biopsy diagnosis as well as individual Banff scores. 61 samples were included in the analysis. For the diagnosis of antibody mediated rejection, the receiver-operator characteristic area under the curves of graft-derived cell-free DNA and graft fraction were 0.91 (95% CI 0.82-0.98) and 0.89 (95% CI 0.79-0.98), respectively. Both measures did not diagnose borderline or type 1A cellular mediated rejection. Graft fraction was associated with a

broader range of Banff lesions, including lesions associated with cellular mediated rejection, while graft-derived cell-free DNA appeared more specific for antibody mediated rejection. Limitations of this study include a small sample size and lack of a validation cohort. The capacity for absolute quantification, and lower barriers to implementation of this methodology recommend it for further study.

1 Introduction

An unmet need in kidney transplantation is the ability to identify rejection non-invasively, so permitting frequent testing, facilitating early diagnosis and treatment, as well as reducing the requirement for unnecessary graft biopsy. To address this problem, a wide variety of putative biomarkers are under investigation, many of which employ multi-analyte technologies within the fields of genomics, transcriptomics, proteomics and metabolomics.

Within genomics, the detailed description of common genetic variation between individuals, combined with technologies that permit measurement of low concentrations of target DNA sequence, has permitted distinction and quantification of DNA sequences unique to the allograft within the plasma of the recipient. This has been termed donor-derived cell-free DNA (dd-cfDNA), but it is perhaps more accurately called graft-derived cell-free DNA (gd-cfDNA).

Cell-free DNA (cfDNA) is fragmented, degraded DNA that is detectable in body fluids, including plasma and urine. The majority of detectable cfDNA in plasma is thought to arise from cell-turnover within the haematopoietic system.¹ Total cfDNA levels may rise in a variety of physiologic and pathologic states. These include exercise, malignancy, sepsis, myocardial infarction, stroke and critical illness.^{2,3} Higher levels of total cfDNA have been associated with increased severity or poorer prognosis in some of these diseases.^{2,3}

In transplantation, it has been proposed that injury to the allograft results in increased release of gd-cfDNA into recipient plasma via graft-cell apoptosis and necrosis. Whilst the clinical validity of gd-cfDNA within kidney transplantation has been explored with variable success for some time, it is only recently that larger scale prospective clinical studies have been undertaken to support diagnostic validity for this biomarker and recommend translation into clinical practice.⁴⁻⁶

To date, there has been one large published prospective clinical study evaluating the diagnostic validity of plasma gd-cfDNA for allograft dysfunction in kidney transplantation. Bloom et al. used single nucleotide polymorphisms (SNPs) and sequencing of cfDNA to calculate the graft fraction

(proportion of total cfDNA that is graft-derived) in 102 adult kidney transplant recipients with graft dysfunction, and observed higher levels in AMR and more severe cellular mediated (CMR) rejection.⁶ A possible limitation of the method raised by this group is the potential for confounding of graft fraction by concurrent elevations in total cfDNA, an issue also observed in non-invasive prenatal testing.⁷

The present study employed droplet digital PCR and highly heterozygous copy number variation (CNV) DNA sequences to generate a negative background against which absolute quantification of gd-cfDNA could be performed. The objective was to prospectively evaluate the performance of gd-cfDNA for diagnosis of AMR in an adult kidney transplant cohort with allograft dysfunction who were undergoing graft biopsy. To permit comparison with other approaches, graft fraction was also calculated and evaluated alongside gd-cfDNA, and the performance of both methods described.

2 Materials and Methods

2.1 Study Design

A non-experimental prospective cross-sectional study design was used. Study sample size was calculated using a method described by Buderer et al. with a maximum clinically-acceptable two-tailed 95% confidence interval width of 0.1, estimated disease prevalence of 0.3, expected sensitivity of 0.9, and expected specificity of 0.8.⁸ Recruitment occurred at two transplanting centres in Melbourne, Australia. Institutional ethics approval was obtained at both sites and all participants provided written informed consent. Adult kidney transplant recipients undergoing standard-of-care allograft biopsy for investigation of graft dysfunction were included in the study. Exclusion criteria were multi-organ transplant, pregnancy, biopsy within 3 weeks of transplant, and critical illness at the time of biopsy. A separate group of samples were collected concurrently for assay development and validation.

2.2 Histopathologic Diagnosis

Allograft biopsy was performed according to local practice using ultrasound-guided needle biopsy. Histopathologic examination of biopsy samples was undertaken by institutional transplant pathologists that were blinded to study participation. Allograft pathology was classified and reported according to the Banff schema.⁹ Mixed rejection and 'suspicious for AMR' diagnoses were classified under the corresponding AMR diagnosis. In addition to Banff diagnoses, individual Banff scores were collected.

2.3 Plasma cfDNA Extraction

Blood was collected in two 10ml Streck Cell-Free DNA blood tubes (Streck, Omaha, NE, USA) by peripheral venesection within a week of renal biopsy. Samples were stored at room temperature. Plasma separation was performed within 7 days of sample collection by centrifugation for 10 minutes at 1,600 x g, following which the supernatant was centrifuged for 10 minutes at 16,000 x g. Plasma was combined and stored at -80 °C until cfDNA extraction. cfDNA extraction was performed using the QIAamp® Circulating Nucleic Acid Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions, and an elution volume of 50 µL distilled water per column. A maximum of 5 mL plasma was used per cfDNA extraction column, after which the eluted cfDNA was combined (a single cfDNA pool per participant-biopsy) prior to storage at -80 °C.

2.4 Assay Design

A total of 38 CNV loci were identified using a population genomics approach to maximise informativity, as described previously.^{10,11} ddPCR primers and Zen double-quenched probes (Integrated DNA Technologies, Baulkham Hills, Australia) were designed within each of the CNV loci in the panel. All targets were within agenic or intronic regions. A non-polymorphic control assay was also developed within the angiotensin I converting enzyme (*ACE*) gene (Online Mendelian Inheritance of Man no. 106180). Analytical validation (manuscript in preparation) limit of blank 0 copies/mL, limit of detection ~6 copies/mL, limit of quantification (inter- and intra-assay coefficient of variation < 50%) ~8-10 copies/mL, and linear performance demonstrated from 8 to 1280 copies/mL.

2.5 Droplet Digital PCR

Extracted cfDNA was run against the CNV assays using the Bio-Rad QX200 Droplet Digital System (Bio-Rad Laboratories, Gladesville, Australia). 31 of the 38 CNV assays developed as well as the *ACE* non-polymorphic control were run in a duplex arrangement using paired 6-carboxyfluorescein and hexachloro-fluorescein labelled hydrolysis probes. The PCR reaction consisted of 12.5 µL of Bio-Rad ddPCR Supermix for Probes (no deoxyuridine triphosphate), 2.25 µL of 10 µmol/L forward and reverse primers for both duplexed assays, 0.65 µL each of the 10 µmol/L duplexed probes, 2.5-5 µL of extracted cfDNA and distilled water to make the reaction volume 25 µL. PCR-cycling conditions were 95 °C for 10 minutes, 44 cycles of 94 °C for 30 seconds, 60 °C for 1 minute, 98 °C for 10 minutes, then an indefinite hold at 4 °C. ddPCR data were processed using the Bio-Rad QuantaSoft software (version 1.6). The method for assigning ddPCR thresholds for droplet positivity, as well as representative ddPCR 1D and 2D plots, are presented in the Supplementary Material and Figures S1-S2.

2.6 Calculation of Plasma Concentration

CNV assay concentrations within the PCR reaction volume measured by ddPCR were converted to the equivalent concentration in copies/mL of plasma that underwent cfDNA extraction (Supplementary Material).

2.7 Genotyping & cfDNA Measurement

Genotyping and cfDNA measurement was performed blinded to biopsy diagnosis. Recipient positive markers (CNV loci that are present within the recipient genome) were genotyped based upon their concentration relative to the *ACE* non-polymorphic marker concentration, and other genotypes (Figure S3). The median recipient one-copy marker concentration was calculated as the total cfDNA concentration. Detectable markers at concentrations less than 10% of total cfDNA concentration were genotyped as graft informative markers (Figure S3). No donor genomic DNA or non-chimeric recipient genomic DNA was therefore required for genotyping. The median graft informative assay concentration was calculated as the gd-cfDNA concentration. Graft fraction was calculated by dividing the gd-cfDNA concentration by total cfDNA concentration. Inter-assay coefficient of variation (CV) was calculated by dividing the standard deviation by the mean.

2.8 Diagnostic Performance Assessment

Receiver operator characteristic (ROC) area under the curves (AUC) were calculated for gd-cfDNA and graft fraction for the diagnosis of acute AMR (aAMR), and a composite diagnosis of aAMR and chronic active AMR (caAMR). ROC curves were compared between gd-cfDNA and graft fraction using the DeLong method and a one sided significance test. In comparisons with serum creatinine ROC curves, a bootstrap test was employed to compare against curves with an AUC < 0.5. Diagnostic performance parameters were calculated using diagnostic thresholds that were calculated to maximise sensitivity and specificity.¹²

2.9 Statistical Methods

Non-parametric distributions were compared using the Wilcoxon rank sum test (two groups), and the Kruskal-Wallis rank sum test (multiple groups). Post-hoc analysis for the latter was performed using Dunn's test with Holm-Šidák adjustment. Spearman's rank correlation was used to assess relationships between ordinal and continuous variables. Pearson's product-moment correlation was used to measure relationships between continuous variables. A *p* value < 0.05 was considered significant. All statistical analyses were performed using R.¹³

3 Results

3.1 Study Cohort

Seventy (20mL) blood samples meeting study criteria were collected. Two samples were duplicated by rescheduled biopsies, two samples were from non-diagnostic biopsies, four samples failed cfDNA extraction, and one sample was damaged in transport. Sixty-one sample results, collected from fifty-five patients were, therefore, used for the study (Figure 1).

The participant (Table 1, Table S1) and sample (Table 2, Table S2) characteristics are presented. The cohort was predominantly Caucasian, deceased donor recipients, and recipients of first grafts. The graft dysfunction indication for biopsy was creatinine in 92%, proteinuria in 3% and both in 2%. The median creatinine at sample collection was 165 (IQR 120-184) $\mu\text{mol/L}$, and median eGFR was 40 (IQR 29-54) ml/min . Duration since transplant was less than 6 months for 23% of samples, 6-12 months for 20%, 1-5 years for 20%, 5-10 years for 15% and more than 10 years in 23%. At the time of sample collection, the predominant immunosuppression regimen was prednisolone (92%), mycophenolate (82%) and tacrolimus (79%).

ABMR was more likely in participants recruited from the one recruitment centre ($p = 0.02$), recipients of third grafts ($p = 0.03$) and individuals biopsied for creatinine (rather than proteinuria, $p = 0.02$). There were no cohort differences between the aAMR group and no aAMR groups.

3.2 Assay Genotyping

Assay genotyping yield and informative marker frequencies, including the effect of donor-recipient relationship and gd-cfDNA concentration, are presented in the Supplementary Material. Median inter-assay CV for graft informative, recipient 1-copy and recipient 2-copy assays was 47% (IQR 36-63%), 14% (IQR 11-19%) and 10% (IQR 7-13%) respectively. The inter-assay CV inversely correlated with the logarithm of the concentration ($r = -0.70$, $N = 159$, $p < 0.0001$) (Figure S8).

3.3 Cell-Free DNA and Biopsy Diagnosis

There were 22 biopsies with Banff rejection diagnoses, including 6 borderline CMR (bCMR), 3 acute CMR (aCMR), 3 caAMR and 10 aAMR. Two suspicious for AMR diagnoses were included under the corresponding AMR diagnosis. Non-rejection diagnoses included 1 BK nephropathy, 2 transplant glomerulopathy, 1 non-specific mild inflammation, 5 calcineurin inhibitor (CNI) toxicity, 2 recurrent primary disease, and 28 negative biopsies.

Gd-cfDNA concentration ranged from <6-72 copies/mL and varied between biopsy diagnoses ($\chi^2(5) = 22$, $p = 0.0006$) (Figure 2, left panel). Gd-cfDNA was significantly elevated in aAMR compared to (i) negative biopsy (30 (IQR 23-48) vs 7 (IQR 5-11) copies/mL, $p = 0.0004$) and (ii) bCMR (30 (IQR 23-48)

vs 4 (IQR 4-10) copies/mL, $p = 0.01$). There was also a trend toward elevation in aAMR compared to other group diagnoses (30 (IQR 23-48) vs 8 (IQR 6-17) copies/mL, $p = 0.051$). Combining aAMR and caAMR into one entity (ABMR), the distribution of gd-cfDNA concentrations was significantly higher than all other diagnoses combined (25 (IQR 22-35) vs 7 (IQR 5-12), $W = 59$, $p < 0.0001$) (Figure 3, left panel).

Total cfDNA concentration ranged from 177-20542 copies/mL and was not significantly different between biopsy diagnoses ($\chi^2(5) = 105$, $p = 0.07$) (Figure 2, middle panel), or for ABMR diagnosis (1266 (IQR 1073-2038) vs 1671 (IQR 968-4038), $W = 356$, $p = 0.45$) (Figure 3, middle panel). Extreme total cfDNA levels coincided with inflammatory illness, including *E. coli* bacteraemia (20542 copies/mL), haemoptysis (19651 copies/mL), unexplained fevers (17341 copies/mL), and foot inflammation (17341 copies/mL).

Graft fraction ranged from <0.04-7.6% and varied between biopsy rejection diagnoses ($\chi^2(5) = 28$, $p < 0.0001$) (Figure 2, right panel). Graft fraction was elevated in aAMR compared to (i) negative biopsy (2.4% (IQR 0.9-3.0%) vs 0.3% (IQR 0.1-0.6%), $p = 0.0002$), and (ii) bCMR (2.4% (IQR 0.9-3.0%) vs 0.37% (IQR 0.1-0.5%), $p = 0.046$). Graft fraction was also increased in caAMR compared to negative biopsy (2.5% (IQR 1.7-3.2%) vs 0.3% (IQR 0.1-0.6%), $p = 0.043$), and other compared to negative biopsy (1.0% (IQR 0.4-1.5%) vs 0.3% (IQR 0.1-0.6%), $p = 0.048$). For the combined diagnosis of ABMR, the distribution of graft fraction results was higher than all other diagnoses combined (2.5% (IQR 0.8-3.0%) vs 0.4% (IQR 0.1-0.8%), $W = 70$, $p < 0.0001$) (Figure 3, right panel).

There was moderate-strong correlation between gd-cfDNA levels and graft fraction ($r = 0.71$, $p < 0.0001$) (Figure S9). Half of the variance in graft fraction was not explained by gd-cfDNA levels ($r^2 = 0.50$).

The distribution of gd-cfDNA and graft fraction results for all samples (Figure S10), and negative biopsy samples alone (Figure S11), were not statistically significantly different if the sample was collected after biopsy compared to before biopsy (Supplementary Material).

With increasing time following transplantation, total cfDNA levels tended to be lower ($r = -0.32$, $p = 0.01$), but not gd-cfDNA ($r = 0.02$, $p = 0.85$) or graft fraction ($r = 0.17$, $p = 0.18$) (Figure S12). For negative biopsy samples alone, stable gd-cfDNA concentrations ($r = -0.12$, $p = 0.54$) and a decline in total cfDNA concentrations ($r = -0.37$, $p = 0.05$) resulted in a rise in graft fraction with increasing time following transplantation ($r = 0.43$, $p = 0.02$) (Figure S13).

3.4 Diagnostic Performance

ROC AUC of gd-cfDNA and graft fraction for diagnosis of aAMR were 0.92 (95% CI 0.84-1.00) and 0.85 (95% CI 0.73-0.97), respectively (Figure 4, left panel). For the composite diagnosis of ABMR, ROC AUC for gd-cfDNA and graft fraction were 0.91 (95% CI 0.82-0.98) and 0.89 (95% CI 0.79-0.98), respectively (Figure 4, right panel). Compared with graft fraction, there was a non-significant trend towards better performance of gd-cfDNA for diagnosis of aAMR (AUC 0.92 vs 0.85, $Z = 1.2$, $p = 0.11$) but not the composite diagnosis of ABMR (AUC 0.91 vs 0.89, $Z = 0.4$, $p = 0.36$).

Using a diagnostic threshold of 21 copies/mL for gd-cfDNA and 0.75% for graft fraction, the diagnostic odds ratio for aAMR was 69 (95% CI 7-630) and 10 (95% CI 2-50), respectively. Diagnostic odds ratios for ABMR were 21 (95% CI 4-109) and 17 (95% CI 3-85) using thresholds of 13 copies/mL and 0.75%, respectively. Sensitivity, specificity, predictive values and likelihood ratios for both measures in diagnosis of aAMR and ABMR are presented in Table 3.

Other group diagnoses that met both gd-cfDNA and graft fraction diagnostic cutoffs for ABMR were one case each of diabetic glomerulopathy (21 copies/mL, 2.4%) and mild non-specific chronic inflammation (47 copies/mL, 2.4%), and two cases of transplant glomerulopathy (14 copies/mL, 1.4%; 21 copies/mL, 1.4%). Additional diagnoses meeting graft fraction diagnostic cutoffs but not gd-cfDNA cutoffs for ABMR were two cases of chronic CNI toxicity (0.9%; 0.9%), and one case of BK nephropathy (1.5%).

3.5 Banff Scores

There were positive monotonic relationships between gd-cfDNA and Banff scores for interstitial inflammation ($r = 0.29$, $p = 0.03$), glomerulitis ($r = 0.48$, $p < 0.001$), peritubular capillaritis ($r = 0.45$, $p < 0.001$), c4d staining ($r = 0.28$, $p = 0.04$) and transplant glomerulopathy ($r = 0.27$, $p = 0.03$) (Figure 5).

Graft fraction was correlated with more Banff lesions, specifically interstitial inflammation ($r = 0.45$, $p < 0.001$), total inflammation ($r = 0.39$, $p = 0.004$), tubulitis ($r = 0.3$, $p = 0.02$), glomerulitis ($r = 0.38$, $p = 0.002$), peritubular capillaritis ($r = 0.39$, $p = 0.002$), c4d staining ($r = 0.39$, $p = 0.004$), transplant glomerulopathy ($r = 0.33$, $p = 0.009$), and interstitial fibrosis ($r = 0.29$, $p = 0.03$) (Figure 6).

3.6 Creatinine

There was no significant correlation between serum creatinine and gd-cfDNA ($r = -0.05$, $p = 0.72$), total cfDNA ($r = -0.07$, $p = 0.60$) and graft fraction ($r = 0.15$, $p = 0.24$) (Figure 7). The ROC AUC of serum creatinine for diagnosis of aAMR was 0.47 (95% CI 0.29-0.66), and for diagnosis of ABMR it was 0.59 (95% CI 0.42-0.75) (Figure 4). Compared to serum creatinine, gd-cfDNA and graft fraction performed better for diagnosis of aAMR (ROC AUC 0.92 vs 0.47, $D = 5.4$, $p < 0.001$; ROC AUC 0.85 vs

0.47, $D = 3.5$, $p < 0.001$) and ABMR (ROC AUC 0.91 vs 0.59, $Z = 3.0$, $p = 0.001$; ROC AUC 0.89 vs 0.59, $Z = 3.3$, $p < 0.001$).

4 Discussion

This study is the first to examine the assay performance of absolute quantification of gd-cfDNA for the diagnosis of Banff-classified kidney allograft rejection, and compare it with that of graft fraction. This was achieved using highly heterozygous CNV DNA sequences to facilitate mismatches in which the graft DNA contained 1- or 2-copies of the target sequence and the recipient had no copies for that respective sequence. This enabled gd-cfDNA to be quantified without interference from recipient DNA sequences. Compared with methods quantifying the relative proportion of one SNP allelotype against another, the present approach has theoretical advantages with respect to analytical sensitivity and specificity for absolute quantification. Additionally, unlike next generation sequencing-based platforms, there is no pre-amplification of cfDNA, with its potential to introduce bias.

The present study assayed gd-cfDNA and total cfDNA, and calculated from these the graft fraction. It demonstrated that both graft-specific measurements were higher in ABMR compared to non-ABMR and negative biopsies. There was no association between total cfDNA and these diagnoses. There was a non-significant trend towards better performance of gd-cfDNA for diagnosis of aAMR, and an association between gd-cfDNA and the Banff lesions associated with ABMR. The performance of gd-cfDNA and graft fraction for composite diagnosis of ABMR was comparable.

Graft fraction was also associated with Banff lesions associated with ABMR, but unlike gd-cfDNA, demonstrated association with some CMR lesions such as total inflammation, tubulitis and arterial inflammation, as well interstitial fibrosis. This suggests that the relative suppression of total cfDNA in these scenarios, whilst not helpful in isolation, adds additional diagnostic information to the graft fraction. Supporting this, only half of the variance in graft fraction in this study was accounted for by the gd-cfDNA concentration, implying total cfDNA concentration was responsible for the remainder. Thus, whilst gd-cfDNA may be a more specific marker for aAMR, graft fraction could provide more diagnostic value in caAMR and potentially more severe forms of CMR, although confounding of graft fraction by other pathologies (BK nephropathy, CNI toxicity) was observed. Considering both gd-cfDNA and graft fraction together may also provide additional diagnostic information and this requires further evaluation, in particular because study of the effect of CMR, BK nephropathy and CNI toxicity on both measures was limited by small numbers in this study.

The results of the present study support the findings of Bloom et al. in their examination of graft fraction for diagnosis of rejection in a similar cohort using a sequencing-based method and SNP

polymorphisms. In their study, diagnostic performance for a composite outcome of type $\geq$ IB CMR and ABMR was used (“active rejection”). The current study cohort included only two biopsies with type $\geq$ 1B CMR. One biopsy had type IB CMR and concurrent acute AMR with high gd-cfDNA (23 copies/mL) and graft fraction (1.1%); the other had type IIB CMR alone and no elevation in gd-cfDNA (7 copies/mL) but a high graft fraction (1.1%). Similar to the study of Bloom et al., no significant rise in graft fraction (or gd-cfDNA) was observed in type IA CMR and borderline CMR. This may reflect the limited sensitivity of these approaches for milder forms of inflammation, and/or the localisation of the cellular injury further away from the vascular space as compared to AMR. Excluding CMR, both methods appear to offer similar performance with respect to diagnosis of ABMR. The positive predictive value of both analytes for ABMR was relatively low but the negative predictive value was very high, suggesting that these tests perform best as “rule out” tests.

Notably, this study did not observe confounding of the diagnostic performance of graft fraction by concurrent elevation in total cfDNA levels arising from non-alloimmune processes, although the study was not powered to examine this.

The droplet digital PCR method employed for this work can be performed rapidly, with a turnaround time of less than 24 hours. This platform also permits a lower per sample cost and avoids access barriers compared with sequencing-based approaches. These characteristics favour application to serial monitoring in clinical practice as a marker of predominantly antibody-mediated allograft injury. Unlike other transcriptomic or proteomic biomarkers that measure alloimmune activity, the current understanding of cfDNA physiology proposes that gd-cfDNA release reflects graft injury itself (i.e. the end-organ effects of the alloimmune response). The high negative predictive value $\geq$ 95% could provide utility in monitoring response to treatment after a diagnosis of AMR, where serial biopsy is often employed. The 90% sensitivity of gd-cfDNA for aAMR could provide evidence of relapse in high pre-test probability circumstances. A negative gd-cfDNA test could also modify the risk:benefit of performing surveillance biopsy, particularly in those with a high biopsy risk.

The current study found moderate variation between informative markers at low gd-cfDNA concentrations within the clinically significant range. This finding supports the use of multiple informative markers, which independently measure gd-cfDNA and can be used to improve reliability of the result. Additionally, the method employed in this study assayed all markers from a panel of strategically informative markers across all samples. To maximise efficiency, reduce cost, and improve sensitivity, once informative markers have been identified from the first sample, future implementations could assay only selected informative markers for donor and recipient. This would permit use of higher DNA concentrations per assay reaction, improving sensitivity and precision at

the low gd-cfDNA concentrations observed in this study. Such an approach would also reduce the sample collection requirement to 9 ml blood for serial samples.

Key limitations of the present study arise from the small sample size and breadth of pathologies assessed. In particular, the number of cases of CMR, caAMR and BK nephropathy was small. Additionally, the study only assessed transplant recipients undergoing biopsy for cause. This was necessary to maximise the prevalence of rejection on the study cohort. The effect of DSA on the diagnostic performance of cfDNA was not included in the study design, and analysis was not performed due to incomplete DSA data at the time of biopsy. Further validation of the diagnostic thresholds and study of the performance of this biomarker in surveillance, and in serial monitoring during/after AMR treatment is warranted, since results of this work suggest utility in these scenarios.

ACKNOWLEDGMENTS

The authors thank Tom Harrington, Ranier Arolanda and Marina Katerelos for their assistance with sample processing and handling. The authors also thank Marieke Veenendaal for assistance with recruitment and sample collection.

The project was supported by a Roche Organ Transplant Research Foundation grant (529172731), and by the Victorian Government's Operational Infrastructure Support Program. Author J.B.W. received stipend support from Kidney Health Australia (SCH16 141516) and the Australian Government National Health and Medical Research Council (GNT1114931). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

J.B.W. and J.K. were responsible for recruitment and sample collection. J.B.W. and L.L. performed the laboratory work. J.B.W. performed the data analysis and wrote the manuscript. D.L.B. and H.S. provided scientific advice/oversight and edited the paper. Authors D.A.P., J.K., F.L.I. and A.S. provided clinical advice/oversight and edited the paper.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. D.L.B. and H.R.S. are co-inventors on a patent (WO2013/049892) covering the analytical method used in this study. The other authors have no conflicts of interest to disclose.

FIGURE LEGEND

Figure 1

Study sample flow diagram

Study inclusion and exclusion criteria were met for 70 samples, 9 of which were not included in the clinical study due to duplication and technical issues. The final study cohort included 61 samples from 55 participants. Of these, 13 samples were included in the ABMR cohort and, within this cohort, 9 samples were included in the aAMR cohort. The no aAMR cohort was therefore comprised of 48 no ABMR and 3 caAMR samples. The no ABMR cohort comprised 48 samples, 28 of which were negative biopsies with no other pathology evident.

Figure 2

Sample measurements of gd-cfDNA (left panel), total cfDNA (middle panel) and calculated graft fraction (right panel) grouped by biopsy diagnosis

gd-cfDNA levels were significantly elevated in aAMR compared to negative biopsy, bCMR and other diagnoses. In contrast, graft fraction levels in caAMR, aAMR and other diagnoses were significantly elevated compared to negative biopsies. Hollow circles represent individual results.

Figure 3

Sample measurements of gd-cfDNA (left panel), total cfDNA (middle panel) and calculated graft fraction (right panel) grouped by the presence (positive) or absence (negative) of ABMR

gd-cfDNA and graft fraction were significantly elevated in ABMR. Points are individual results and point shapes represent type of AMR. Suspicious for ABMR diagnoses had some of the lowest levels with the ABMR group (black points).

Figure 4

Receiver-operator characteristic curves for gd-cfDNA (black line), graft fraction (light gray line), and creatinine (dark gray dashed line) in the diagnosis of aAMR (left panel), and composite aAMR and caAMR (ABMR, right panel)

ROC AUC point estimates are presented. Compared with graft fraction, there was a non-significant trend towards better performance of gd-cfDNA for diagnosis of aAMR ($p = 0.11$) but not the composite diagnosis of ABMR ($p = 0.36$). Both measures performed better than serum creatinine for both diagnoses ($p \leq 0.001$).

Figure 5

The distribution of gd-cfDNA measurements for each Banff lesion and score

Hollow circles are individual results. The number of results included in the analysis (N), Spearman's rho (r), and the significance of the correlation (p) are presented for each Banff lesion. gd-cfDNA positively correlated with interstitial inflammation (i), glomerulitis (g), peritubular capillaritis (ptc), C4d staining (c4d), and transplant glomerulopathy (cg) lesions.

(Below figure)

Banff score lesions: i, interstitial inflammation; ti, total inflammation; t, tubulitis; v, arterial inflammation; g, glomerulitis; ptc, peritubular capillaritis; c4d, c4d deposition; ci, interstitial fibrosis; ct, tubular atrophy; cv, arterial intimal thickening; cg, transplant glomerulopathy; ah, arteriolar hyalinosis; mm, mesangial matrix increase.

Figure 6

The distribution of calculated graft fraction results for each Banff lesion and score

Hollow circles are individual results. The number of results included in the analysis (N), Spearman's rho (r), and the significance of the correlation (p) are presented for each Banff lesion. Graft fraction positively correlated with interstitial inflammation (i), total inflammation (ti), tubulitis (t), glomerulitis (g), peritubular capillaritis (ptc), C4d staining (c4d), transplant glomerulopathy (cg), and interstitial fibrosis (ci) lesions.

(Below figure)

Banff score lesions: i, interstitial inflammation; ti, total inflammation; t, tubulitis; v, arterial inflammation; g, glomerulitis; ptc, peritubular capillaritis; c4d, c4d deposition; ci, interstitial fibrosis; ct, tubular atrophy; cv, arterial intimal thickening; cg, transplant glomerulopathy; ah, arteriolar hyalinosis; mm, mesangial matrix increase.

Figure 7

Correlation of serum creatinine concentration with gd-cfDNA, total cfDNA and graft fraction

There was no correlation between serum creatinine concentration and cfDNA levels.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab of this article.

TABLES

Table 1

Participant characteristics.

	Participant Cohort (N=55)	No ABMR (N=42)	ABMR (N=13)	p-value ¹
Demographics				
Age at first sample (years)	53 (39-61)	55 (39-61)	49 (40-55)	0.49
Male sex	30 (55%)	23 (55%)	7 (54%)	>0.99
Race				0.32
Caucasian	44 (80%)	34 (81%)	10 (77%)	
Asian	7 (13%)	6 (14%)	1 (8%)	
Polynesian	2 (4%)	1 (2%)	1 (8%)	
Arabic	1 (2%)	0 (0%)	1 (8%)	
African	1 (2%)	1 (2%)	0 (0%)	
Primary disease				0.63
IgA nephropathy	14 (25%)	11 (26%)	3 (23%)	
Reflux nephropathy	8 (15%)	4 (10%)	4 (31%)	
Primary FSGS	6 (11%)	5 (12%)	1 (8%)	
Polycystic kidney disease	6 (11%)	4 (10%)	2 (15%)	
Secondary FSGS	4 (7%)	3 (7%)	1 (8%)	
Diabetes	3 (5%)	3 (7%)	0 (0%)	
Unknown	2 (4%)	2 (5%)	0 (0%)	
ANCA vasculitis	2 (4%)	1 (2%)	1 (8%)	
Hypertension	2 (4%)	2 (5%)	0 (0%)	
Other	8 (15%)	7 (17%)	1 (8%)	
Recruiting hospital				0.02
Hospital 1	49 (89%)	40 (95%)	9 (69%)	
Hospital 2	6 (11%)	2 (5%)	4 (31%)	
Transplant				
Graft number				0.03
First	51 (93%)	40 (95%)	11 (85%)	
Second	2 (4%)	2 (5%)	0 (0%)	
Third	2 (4%)	0 (0%)	2 (15%)	
Donor Type				>0.99

Deceased	40 (73%)	30 (71%)	10 (77%)	
Brain death	19 (35%)	14 (33%)	5 (38%)	
Circulatory death	13 (24%)	11 (31%)	2 (15%)	
Unknown	8 (15%)	5 (12%)	3 (23%)	
Living	15 (27%)	12 (29%)	3 (23%)	
Parent	7 (13%)	6 (14%)	1 (8%)	
Sibling	2 (4%)	2 (5%)	0 (0%)	
Related NOS	1 (2%)	0 (0%)	1 (8%)	
Not related	5 (9%)	4 (10%)	1 (8%)	
Matched donor-recipient sex	25/51 (49%)	17/39 (43%)	8/12 (66%)	0.20
ABO compatible	52 (95%)	40 (95%)	12 (92%)	0.56
DSA at transplant	10/47 (21%)	6/34 (18%)	4/13 (31%)	0.43
HLA mismatch				0.53
5-6	12 (22%)	8 (19%)	4 (31%)	
3-4	25 (45%)	21 (50%)	4 (31%)	
1-2	16 (29%)	12 (29%)	4 (31%)	
0	2 (4%)	1 (2%)	1 (8%)	
Induction agent				0.49
Basiliximab	42 (76%)	32 (76%)	10 (77%)	
None	4 (7%)	4 (10%)	0 (0%)	
OKT3	1 (2%)	1 (2%)	0 (0%)	
Unknown	8 (15%)	5 (12%)	3 (23%)	

Data ranges are presented as median (inter-quartile range).

ⁱ The *p*-value presented is the significance of differences between the ABMR and no ABMR groups. The Wilcoxon rank sum test, Fisher's exact test for count data, and the equality of proportions test were used for comparisons as indicated.

FSGS, focal segmental glomerulosclerosis; ANCA, anti-neutrophil cytoplasmic antibody; NOS, not otherwise specified; DSA, donor specific antibody; HLA, human leukocyte antigen; OKT3, muromonab-CD3

Table 2

Sample characteristics.

	Sample Cohort (N=61)	No ABMR (N=48)	ABMR (N=13)	p-value ⁱ
Sample Collection				
Time post-transplant (years)	2.6 (0.6-8.2)	2.3 (0.6-8.3)	4.0 (0.6-8.2)	0.91
Time post-transplant cohorts (years)				0.89
< 0.5	14 (23%)	11 (23%)	3 (23%)	
0.5-1	12 (20%)	10 (21%)	2 (15%)	
1-5	12 (20%)	10 (21%)	2 (15%)	
5-10	9 (15%)	6 (13%)	3 (23%)	
>10	14 (23%)	11 (23%)	3 (23%)	
Collection time from biopsy (days)				0.76
-6 (before biopsy)	1 (2%)	1 (2%)	0 (0%)	
-1	1 (2%)	1 (2%)	0 (0%)	

0 (day of biopsy)	57 (93%)	44 (92%)	13 (100%)	
+1 (after biopsy)	2 (3%)	2 (4%)	0 (0%)	
Clinical Characteristics				
Biopsy indication				0.02
Creatinine	56 (92%)	45 (93%)	11 (85%)	
Proteinuria	3 (3%)	3 (6%)	0 (0%)	
Both	2 (5%)	0 (0%)	2 (15%)	
Baseline creatinine (μmol/L) (N=60) ⁱⁱ	125 (102-156)	121 (102-157)	140 (121-156)	0.39
Baseline eGFR (ml/min) (N=60) ⁱⁱ	52 (42-65)	52 (43-65)	51 (33-65)	0.72
Creatinine (μmol/L)	165 (120-184)	159 (114-185)	168 (147-182)	0.34
eGFR (ml/min)	40 (29-54)	40 (29-54)	36 (29-43)	0.41
Indication urine protein:creatinine (g/mmol) (N=5)	0.21 (0.20-0.35)	0.21 (0.17-0.28)	0.48 (0.34-0.62)	0.80
Urine protein:creatinine (g/mmol) (N=40)	0.03 (0.01-0.09)	0.03 (0.01-0.07) (N=32)	0.09 (0.02-0.19) (N=8)	0.29
Any history of donor specific antibody	18/35 (51%)	11/24 (46%)	7/11 (64%)	0.47
Maintenance immunosuppression				
Prednisolone	56 (92%)	44 (92%)	12 (92%)	>0.99
Mycophenolate	50 (82%)	38 (79%)	12 (92%)	0.43
Azathioprine	6 (10%)	5 (10%)	1 (8%)	>0.99
Tacrolimus	48 (79%)	37 (77%)	11 (85%)	0.72
Ciclosporin	10 (16%)	9 (19%)	1 (8%)	0.67
mTOR	4 (7%)	4 (8%)	0 (0%)	0.57
Drug levels				
Tacrolimus level (μg/L) (N=48)	5.3 (4.2-6.3)	5.2 (4.2-6.0) (N=37)	5.7 (4.1-7.9) (N=11)	0.49
Ciclosporin level (μg/L) (N=10)	318 (207-490)	388 (200-521) (N=9)	227 (N=1)	0.8
Weight (kg) (N=56)	78 (69-85)	78 (68-85) (N=46)	80 (75-85) (N=10)	0.45

Data ranges are presented as median (inter-quartile range).

ⁱ The *p*-value presented is the significance of differences between the ABMR and no ABMR groups. The Wilcoxon rank sum test, Fisher's exact test for count data, and the equality of proportions test were used for comparisons as indicated.

ⁱⁱ One participant had not achieved a baseline creatinine by the time of sample collection.

eGFR, estimated glomerular filtration rate (CKD-EPI); mTOR, mammalian target of rapamycin.

Table 3

Diagnostic performance characteristics of gd-cfDNA and graft fraction for the diagnosis of aAMR and ABMR.

	Diagnosis of aAMR		Diagnosis of ABMR	
	gd-cfDNA > 21 copies/mL	Graft Fraction > 0.75%	gd-cfDNA > 13 copies/mL	Graft Fraction > 0.75%
Sensitivity	0.90 (0.55-1.00)	0.80 (0.44-0.97)	0.85 (0.55-0.98)	0.85 (0.55-0.98)
Specificity	0.88 (0.76-0.96)	0.71 (0.56-0.83)	0.79 (0.65-0.90)	0.75 (0.60-0.86)
PPV	0.60 (0.32-0.84)	0.35 (0.16-0.57)	0.52 (0.30-0.74)	0.48 (0.27-0.69)
NPV	0.98 (0.88-1.00)	0.95 (0.82-0.99)	0.95 (0.83-0.99)	0.95 (0.82-0.99)
PLR	7.65 (3.51-16.68)	2.72 (1.61-4.60)	4.06 (2.23-7.39)	3.38 (1.97-5.82)
NLR	0.11 (0.02-0.73)	0.28 (0.08-0.99)	0.19 (0.05-0.70)	0.21 (0.06-0.74)
DOR	68.5 (7.22-630)	9.60 (1.82-50.6)	20.9 (3.97-109)	16.5 (3.19-85.2)

Performance measures presented as value (95% confidence interval).

PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio;

DOR, diagnostic odds ratio.

FIGURES

Figure 1

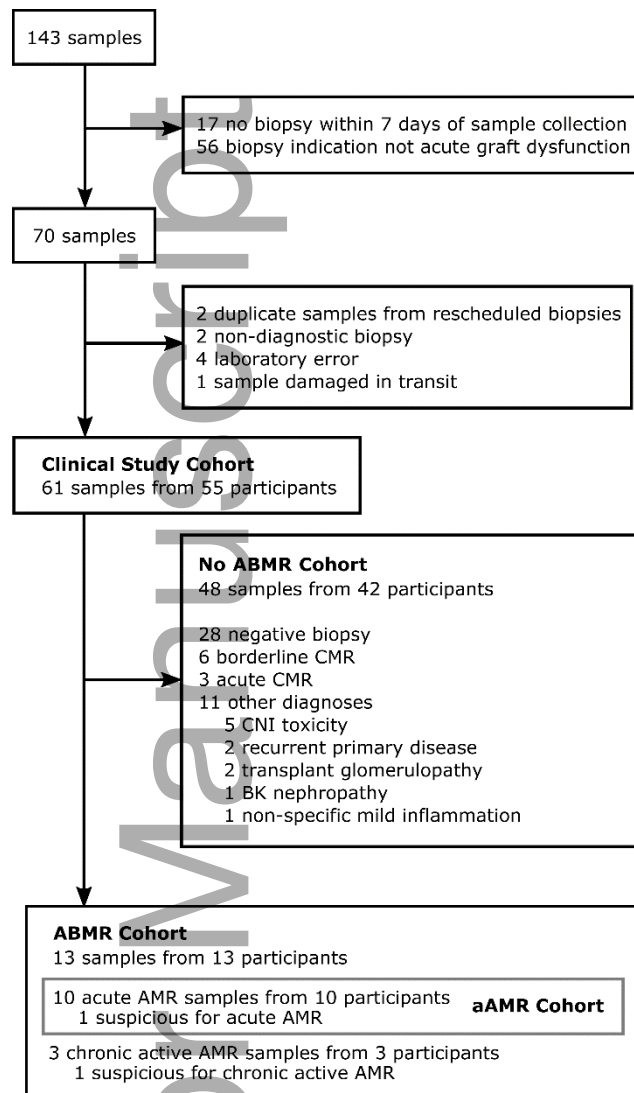


Figure 2

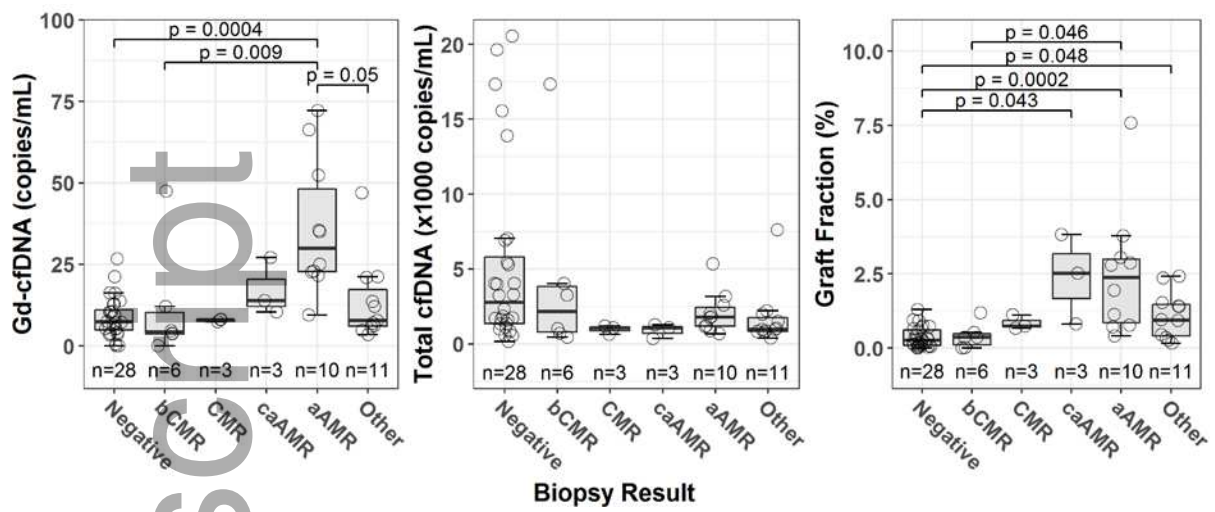


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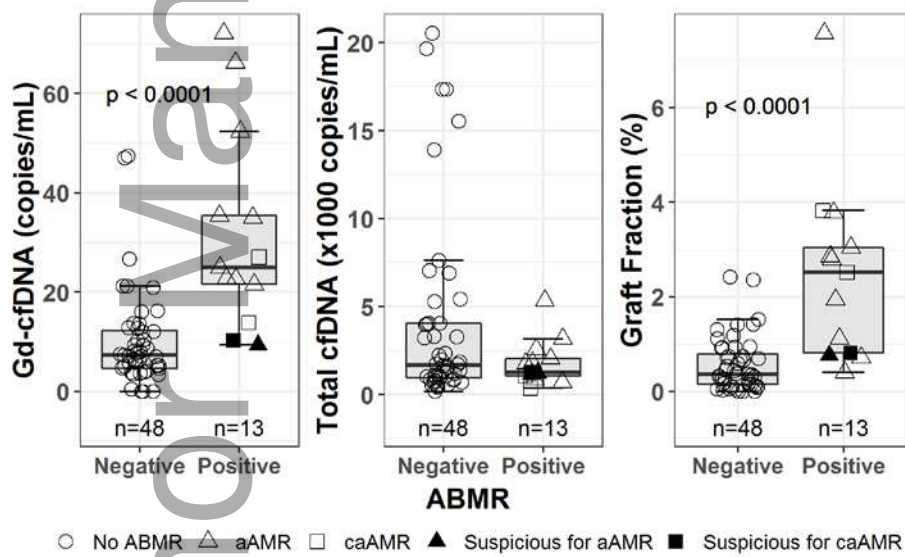


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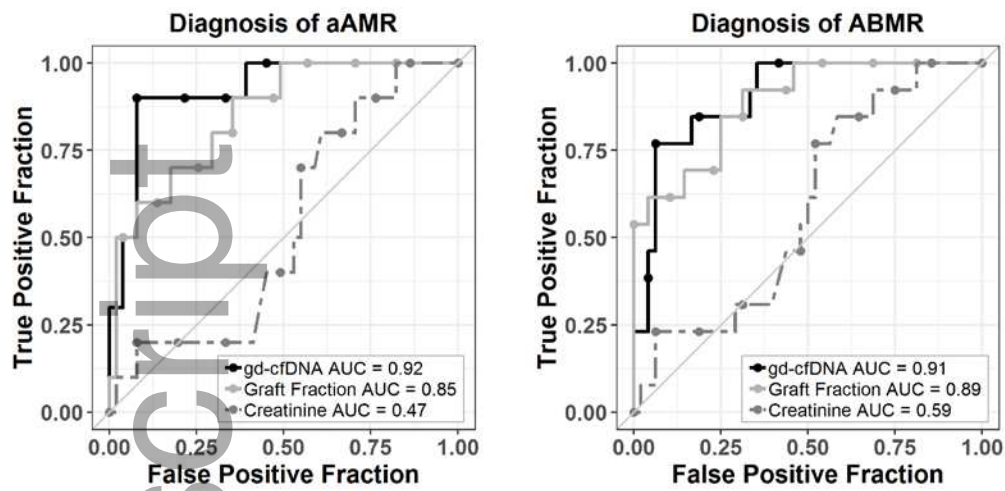


Figure 5

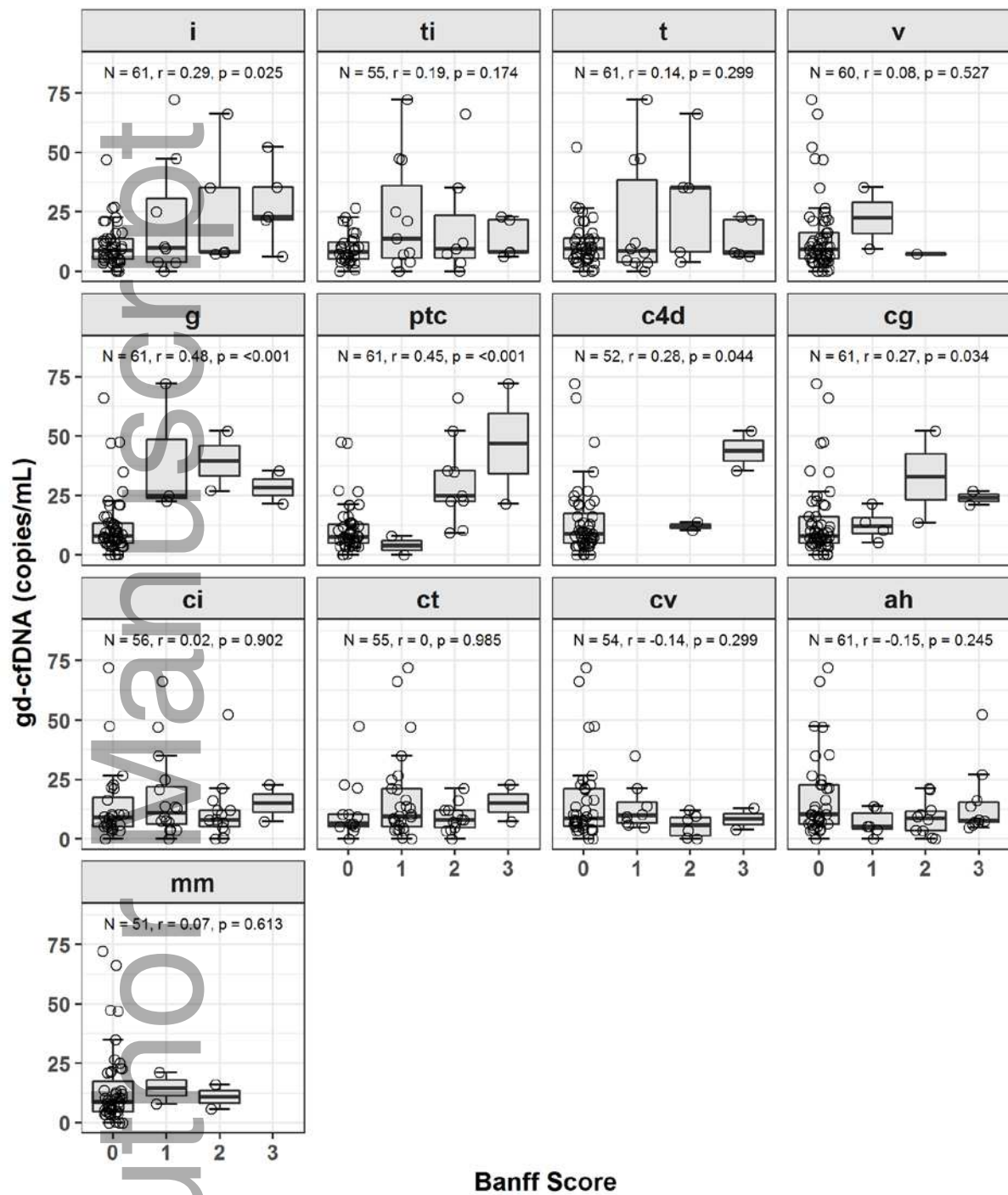


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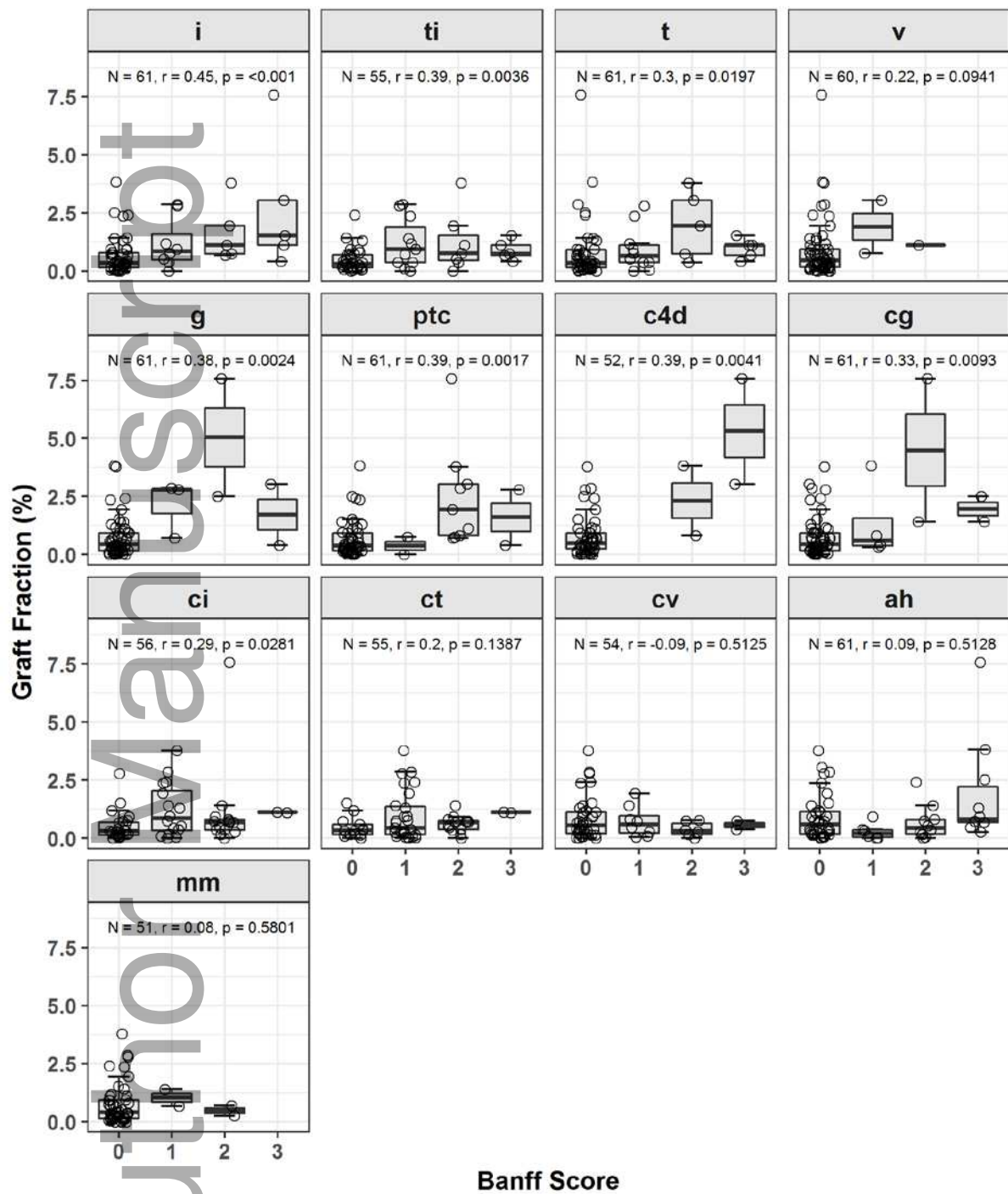


Figure 7

