Cell-free DNA : an upcoming biomarker in transplantation

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Running title:

Cell-free DNA as a transplantation biomarker

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Abbreviations:

µg	microgram
AR	acute rejection
AST	aspartate transaminase
ATN	acute tubular necrosis
AUC	area under the curve
BKVN	BK viral nephropathy
bp	base pairs
CAI	chronic allograft injury
cfDNA	cell-free DNA
ChrY	chromosome Y
CMV	cytomegalovirus
CND	copy number deletion
ddcfDNA	donor-derived cell-free DNA
ddPCR	digital droplet PCR
EDTA	ethylenediaminetetraacetic acid
GE	genome equivalents
H	heart
HTP	heart transplant patient
K	kidney
kb	kilobase
KPTP	kidney pancreas transplant patient
kSORT	kidney solid organ response test
KTP	kidney transplant patient

L	liver
LTP	liver transplant patient
MOF	multiple organ failure
MPSS	massive parallel shotgun sequencing
n	number in group
ng	nanogram
N.M.	not mentioned
NTX	drug-induced nephrotoxicity
P	pancreas
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
ROC	receiver operating characteristic
SNP	single nucleotide polymorphism
SRY	sex-determining region of chromosome Y
TMA	thrombotic microangiopathy
Tr-DNA	transrenal DNA
Tx	transplantation
UTI	urinary tract infection

ABSTRACT

After organ transplantation, donor-derived cell-free DNA (ddcfDNA) can be detected in the recipient's blood and urine. Different ddcfDNA quantification techniques have been investigated but a major breakthrough was made with the introduction of digital droplet PCR and massive parallel sequencing creating the opportunity to increase the understanding of ddcfDNA kinetics after transplantation. The observations of increased levels of ddcfDNA during acute rejection and even weeks to months before histologic features of graft rejection point to a possible role of ddcfDNA as an early, non-invasive rejection marker. In this review we summarize published research on ddcfDNA in the transplantation field thereby elaborating on its clinical utility.

Major references that will be used:

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MAIN BODY TEXT

Introduction

Current monitoring of the graft

After transplantation of any organ type, the graft is susceptible to different pathologies including (opportunistic) infection, disease recurrence and acute rejection. In addition, drug-induced toxicity is an important cause of graft dysfunction in kidney and liver transplantation (1, 2). The incidence of acute rejection varies between graft types, with the highest incidence of acute rejection after intestinal, heart, and lung transplantation (approximately 55%, 30-45%, and 35-40%, respectively) (3-5). After liver, simultaneous kidney-pancreas, and kidney transplantation, acute rejection is less frequent with incidences of 13-30%, 20%, and 12-14%, respectively within 2 years after transplantation (6-8). In the case of pancreas, kidney and liver transplantation, the measurement of blood markers (lipase/amylase, serum creatinine and liver enzymes, respectively) is recommended as routine post-transplantation monitoring (1, 9, 10). However, in the field of kidney transplantation, deterioration of graft function cannot be detected until significant graft damage has occurred (11) and after cardiac transplantation, the monitoring of cardiac enzymes is not recommended because of the poor sensitivity of these markers in the diagnosis of acute rejection (12), pointing to an urgent need of alternative biomarkers for monitoring graft status.

The importance of non-invasive graft monitoring is further emphasized as subclinical rejection may occur: these cases can exclusively be detected with (invasive) surveillance or protocol biopsies (13-17). Currently, to our knowledge, AlloMap is the only commercially available non-invasive organ transplant monitoring test (18). This gene expression profiling assay has been investigated and validated in cardiac transplant recipients and has been included in the current heart transplantation guidelines to rule out the presence of a moderate to severe acute rejection in low-risk patients between 6 months and 5 years after transplantation (12). In the field of kidney transplantation, the

use of the kSORT (kidney solid organ response test) - a peripheral blood gene set assay – has been investigated to identify patients at high risk of acute rejection (19). However, as discussed recently (20), more studies are needed to validate its predictive ability.

Interest in cell-free DNA

Since the discovery of donor-derived cell-free DNA (ddcfDNA) in the recipient's blood and urine, clinical interest in genomic cell-free DNA (cfDNA) within the transplantation field is growing (21, 22). The release of ddcfDNA in the recipient's blood and urine secondary to cell damage in the graft makes these molecules potential biomarkers of graft health. In contrast with aforementioned organ-specific molecular rejection prediction assays, ddcfDNA may be used as a universal marker that is applicable in the monitoring of the health of any solid organ transplant.

Different mechanisms accounting for DNA release in the circulation have been postulated, including apoptosis (23-26), necrosis (26), and active secretion (27-29). Observations in gender-mismatched bone marrow and solid organ transplantation models point to hematopoietic cells as primary source of cfDNA in the plasma, with non-hematopoietic cells accounting for a minority of the free circulating DNA (24, 30, 31). After crossing the kidney barrier by glomerular filtration, low molecular weight DNA fragments can appear in the urine, called 'transrenal DNA' (Tr-DNA), whereas higher molecular weight DNA is derived from cells shed from the urinary tract or other urinary cells (32, 33) (Figure 1 and Table 1). The possibility to isolate cfDNA from serum, plasma, and urine for ddcfDNA quantification may give rise to the development of novel non-invasive tests for the diagnosis of graft damage linked to acute rejection. In this review we summarize published research on ddcfDNA in the transplantation field thereby elaborating on its clinical utility.

Cell-free DNA isolation and quantification: technical aspects

After transplantation, low concentrations of cfDNA can be detected in the recipient's plasma, serum and urine (Figure 1). As only a small fraction of this total cfDNA is graft-derived, quantification of ddcfDNA is challenging. Different pre-analytic and methodological factors must be taken into account to minimize cell degradation and subsequent release of background recipient DNA (Table 1). In the past 20 years, ddcfDNA quantification techniques have been investigated which differ in terms of applicability, sensitivity, turnaround times and instrumentation costs (Table 2). While amplification of Y-chromosome genes in the recipient's plasma and urine has been widely used in gender-mismatched transplantations (21, 22, 30, 31, 34-39), this strategy is limited to about 25% of the transplant population. A more universal approach is the use of informative genetic polymorphisms whereby a particular allele differs or is absent in the donor genome compared to the recipient genome; in addition to the quantification of donor-specific HLA DNA (40) and copy number deletion polymorphisms (41), single nucleotide polymorphisms (SNPs) can be targeted to determine the percentage of ddcfDNA by counting donor and recipient's bases at informative SNP sites with digital droplet polymerase chain reaction (ddPCR) (42-44) or massive parallel shotgun sequencing (MPSS) (34, 45).

Kinetics of ddcfDNA levels in recipients with stable grafts

The ddcfDNA kinetics seem to follow an L-shaped curve with high percentages in the immediate post-engraftment phase followed by a swift decrease to a stable baseline level. The high ddcfDNA levels immediately after transplantation may be caused by ischemia-reperfusion injury (40, 42, 43). If this is the case, higher levels of ddcfDNA are expected in recipients with grafts of deceased donors. However, no studies have been published comparing ddcfDNA kinetics in recipients transplanted with a graft of a living donor versus a deceased donor. Residual blood cells or cells loosely associated with the graft might be an alternative source of ddcfDNA.

In liver transplant recipients, plasma ddcfDNA fractions reaching 90% of total cfDNA were observed immediately after transplantation, and ddcfDNA levels decreased rapidly thereafter with a half-life of 24-48 hours, to < 15% at day 10 (42). High serum ddcfDNA levels (2000-72 000 ng/ml) were found in liver transplant recipients a few minutes after liver reperfusion, which dropped to low baseline levels within a few days when there were no complications (Table 3) (39).

Similar ddcfDNA kinetic curves have been observed in plasma (37) and urine (35, 37, 59) from kidney transplant recipients and in serum from recipients of a simultaneous kidney-pancreas transplant (40). One study reported a high inter-patient variability of urinary ddcfDNA levels and showed no correlation of urinary ddcfDNA levels with donor age, duration of cold ischemia or donor source (38). Another study reported a median urinary ddcfDNA fractional concentration of 8.7% in kidney recipients with stable graft function (Table 3) (22).

In heart transplant recipients, De Vlaminck and colleagues reported a mean plasma ddcfDNA fraction of 3.8% on the first day post-transplant, which declined within one week to a baseline level of 0.06% for the following year during stable graft conditions (45). Other studies reported plasma ddcfDNA fractions of approximately 0.9% in cardiac recipients with stable graft function (34, 42) (Table 3).

The amount of ddcfDNA release in the blood seems to be organ-specific, as Beck and colleagues reported mean plasma ddcfDNA fractions in heart, kidney, and liver transplant recipients with stable graft function of 0.9%, 1.2%, and 3.5%, respectively (42) (Table 3), thereby confirming previous observations in gender-mismatched transplantation models (31). As previously proposed (42), these differences might be related to organ size and cell regeneration rate.

Cell-free DNA and graft injury

Various studies have been published on plasma, serum, and urinary levels of total and donor-derived cfDNA after kidney, kidney-pancreas, liver, and heart transplantation during different graft-associated complications (Table 3). Several research groups focused on the value of cell-free DNA as a rejection biomarker (22, 34, 37, 45), while others aimed to evaluate the role of cell-free DNA as a more general biomarker of organ integrity (38-40, 42, 43).

Kidney and kidney-pancreas allografts

After kidney transplantation, Garcia Moreira and coworkers showed early increases of total cfDNA levels during acute rejection and systemic infection and to a lesser extent during local infections, acute tubular necrosis and drug-induced nephrotoxicity, compared to patients with stable graft function (37) (Table 3). In addition, ddcfDNA levels were increased during acute rejection and graft infection episodes (37).

To investigate the role of urinary ddcfDNA as a surrogate marker of kidney transplant injury, Sigdel and coworkers compared urinary ddcfDNA levels between kidney recipients with BK viral nephropathy (BKVN), chronic allograft injury, and acute rejection (38). Polyomavirus nephropathy remains a common complication after kidney transplantation with a prevalence of approximately 10% in kidney transplant recipients. The differentiation from acute rejection is challenging both on clinical and pathological grounds (60). Because urinary ddcfDNA levels during BKVN did not differ

significantly from those in patients with acute rejection in the study of Sigdel and coworkers, the authors concluded that urinary ddcfDNA can be used as a sensitive biomarker of acute allograft injury (38). With a cut-off of 3 or more urinary chromosome-Y copies/µg creatinine, a sensitivity of 81% and specificity of 75% was reached in the diagnosis of allograft injury.

To the best of our knowledge, no studies in the field of kidney transplantation have investigated the role of a urinary tract infection (UTI) – the most common complication after renal transplantation - on urinary ddcfDNA levels. In non-transplanted patients, a significantly higher urinary total cfDNA level was found in patients suffering from UTI compared to healthy controls (61), but no differentiation between pyelonephritis and lower UTI was made. High urinary total cfDNA levels were also found in renal transplant patients with urinary sepsis (37). Based on the current data (32, 33, 58), we would expect increased levels of small Tr-ddcfDNA fragments in case of pyelonephritis, and larger donor-derived urinary tract cfDNA during a lower UTI.

In simultaneous kidney-pancreas transplantation, increased levels of serum ddcfDNA were found in patients suffering from acute rejection and a modest increase in a patient with thrombotic microangiopathy, which points to a possible role of ddcfDNA as a non-invasive biomarker of pancreas-kidney allograft injury (40).

Liver allografts

Beck and colleagues showed that the plasma ddcfDNA fraction was between 5 to 10% at day 10 post-transplantation in stable liver transplant recipients whereas in case of rejection it remained approximately 20% and gradually increased to 55-60% (42) (Table 3). Conventional biomarkers including aspartate transaminase (AST) levels were more variable during acute rejection compared to the ddcfDNA fraction. The occurrence of a liver hematoma, CMV infection, and acute rejection led to increased ddcfDNA fractions up to 90% compared to 10% during stable graft function (43). Macher and coworkers showed higher total cfDNA and ddcfDNA serum levels in patients with

damage to the liver transplant (acute rejection, hepatic arterial and venous thrombosis, and profound cholestasis ending in multiple organ failure), as compared to recipients with stable graft function. In contrast, increases in total cfDNA levels but not ddcfDNA were observed in patients with complications that did not compromise the donated organ (biliary peritonitis and surgical wound infection) (39).

Subtherapeutic tacrolimus trough levels resulted in increased ddcfDNA fractions up to 66% (43), possibly caused by immunological graft damage. The role of ddcfDNA in the establishment of minimal effective tacrolimus trough concentrations has been proposed by Oellerich and coworkers thereby creating the possibility of a personalized immunosuppressive treatment (44).

Could cfDNA serve as a biomarker of acute rejection?

The use of total cfDNA as a rejection biomarker has been evaluated by Garcia Moreira and colleagues (37). As both the occurrence of acute rejection and systemic infection led to marked increases in total cfDNA levels (55 303 and 40 400 GE/ml, respectively), total cfDNA values were combined with a biochemical parameter of systemic infection (procalcitonin) thereby increasing the diagnostic specificity for acute rejection from 85% to 98%.

Zhang and colleagues provided the first evidence of increased urinary total cfDNA concentrations during acute kidney rejection (Table 3). Different mechanisms accounting for increased total cfDNA levels were discussed including cellular destruction secondary to the rejection process, the release of DNA from the recipient's immune effector cells, and an alteration in glomerular permeability (22). Similar rises in urinary total cfDNA levels during acute kidney allograft rejection were reported in other studies (35, 37). Although Garcia Moreira and colleagues concluded that the measurement of ddcfDNA showed no better efficiency for the diagnosis of acute rejection compared to total cfDNA (37), this approach is less specific for graft injury as total cfDNA may rise during any process of increased cell-turnover in the recipient.

Studies investigating the role of ddcfDNA as a biomarker of acute rejection have been mainly performed within the cardiac transplantation field, thereby evaluating to which extent the measurement of ddcfDNA is discriminative of rejection as confirmed in the transplant biopsy. In the retrospective study by Snyder and colleagues (34), significantly higher ddcfDNA fractions (2.75 ± 1.81 %) were observed in patients with a biopsy-proven cardiac rejection (the true positive group) compared to other samples (0.92 ± 1.16%). ROC (receiver operating characteristic) analyses showed that at a threshold of 1.70% of ddcfDNA fraction a sensitivity of 83% and specificity of 84% were reached for the diagnosis of cardiac rejection (34). In a prospective study, De Vlaminck and colleagues (45) found significantly higher ddcfDNA fractions in heart transplant patients with T-cell- and antibody- mediated cardiac rejection compared to stable transplant recipients. Furthermore, ddcfDNA levels correlated with rejection severity. At a ddcfDNA fraction of 0.25%, a sensitivity and specificity of 58% and 93%, respectively, were reached for the diagnosis of an acute rejection with an area under the curve (AUC) of 0.83. In addition, ddcfDNA levels showed the best performance in distinguishing severe rejection events from no rejection (AUC = 0.95), and the lowest performance in distinguishing mild rejection from the absence of rejection (AUC = 0.60). The test performance of the ddcfDNA assay relative to the endomyocardial biopsy improved with time after transplantation, showing the best results beyond 4 months post-transplantation. De Vlaminck and coworkers showed significantly elevated ddcfDNA fractions in samples collected up to 5 months before the histological diagnosis of acute cardiac rejection, pointing to a potential role of ddcfDNA in the early diagnosis of transplant rejection (45).

How to implement ddcfDNA in clinical practice?

Although ddcfDNA is an interesting and promising marker of solid transplant organ health, much work still needs to be done before clinical implementation.
The studies performed to date mainly focused on detection and applicability of cfDNA as a biomarker. A few studies focused on quantification methods, searching for fast, easy, and affordable methods that are widely applicable in a transplantation setting (22, 34, 37-42, 45). Although much progress has been made in the ddcfDNA quantification techniques, several limitations impede the clinical implementation of the biomarker until now. Some of the techniques used to detect ddcfDNA (22, 37-39) are only suitable in female patients transplanted with a male donor organ. Other techniques are time consuming or less affordable (34, 42, 45), which makes them less attractive for routine monitoring of ddcfDNA. Other limitations of the currently published studies are the inclusion of relatively small study populations (22, 34, 35, 37-42) and the analysis of different targets, including total cfDNA (37, 39), ddcfDNA (34, 37, 39, 40, 42, 45) and Tr-DNA (22, 37, 38), making it difficult to propose recommendations for clinical practice. There is a need for large prospective studies in different types of solid organ transplantation along the line of the study by De Vlaminck and coworkers (45) to investigate the role of the three targets in the monitoring of transplant health. It would be interesting to investigate the role of donor-derived Tr-DNA, since urine is even more accessible than blood and larger sample volumes can be obtained. Since Tr-DNA consists of shorter DNA molecules, methodologic considerations need to be made to isolate these small urinary fragments in non-kidney transplant recipients (Figure 1 and Table 1). In contrast, after renal transplantation, urinary ddcfDNA can originate from the bloodstream and is filtered through the glomerular barrier, or it can be derived from dying cells shed from the donor urinary tractus (Figure 1).

As mentioned earlier, different technical measurements need to be taken into account for the isolation and quantification of ddcfDNA and Tr-DNA. In a few retrospective studies, plasma and serum samples from a repository have been used (34, 40), but these studies lack important pre-

analytical information related to sample collection, time between collection and processing, and storage time.

Determination of the performance of ddcfDNA as an organ health marker or as a rejection biomarker relies on the histological evaluation of a biopsy specimen, currently the 'golden standard' for the diagnosis of transplant pathology. The imperfection of this golden standard (interobserver variability, sampling error) needs to be taken into account.
Since ddcfDNA at present seems to be a general marker of allograft pathology, its measurement in the recipient´s plasma or urine after transplantation should be implemented in a clinical algorithm for the early and non-invasive detection of acute allograft rejection and the exclusion of other graft-associated pathologies after transplantation. The diagnostic efficiency of ddcfDNA can be improved when combining its measurement with other parameters including immunosuppressive drug monitoring, immunological monitoring (measurement of donor-specific antibodies), and microbial screening (BKV infection). Based on the current data, plasma and urine ddcfDNA levels show marked increases both during acute rejection and graft infection (37, 38), pointing to the necessity of a combined viral monitoring strategy. Furthermore, a diagnostic threshold of ddcfDNA levels according to the type of transplanted organ should be considered, since baseline levels in recipients with stable graft function are organ specific (31, 42). As indicated by Gadi and coworkers, ddcfDNA offers potential as a marker for decision making: either to biopsy or to treat when biopsy is not possible (40). As discussed before (34), a combination of measuring ddcfDNA fractions in the blood with other non-invasive monitoring strategies like AlloMap after cardiac transplantation may be beneficial, since the former test reflects graft injury while the latter monitors host immunologic responses during acute rejection.

Obviously, before the implementation in clinical practice, larger clinical studies of a ddcfDNA based algorithm are needed alongside other molecular diagnostic methods of graft rejection (18, 19). In conclusion, whereas observational studies published so far point to ddcfDNA as a promising

marker in the transplantation field, randomized control studies are mandatory comparing a ddcfDNA-based monitoring strategy with the standard post-transplantation monitoring in terms of graft and patient survival.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

FIGURE LEGENDS

Figure 1

Title:

Origin of blood and urinary (donor-derived) cfDNA after solid organ transplantation

Legend:

After solid organ transplantation, donor-derived cell-free DNA circulates in the recipient's blood, and accounts for only a small fraction of total cfDNA (recipient plus donor-derived). Small cell-free DNA fragments from the blood circulation can be filtered through the glomerular barrier (A) and appear in the urine, also called 'transrenal DNA'. After kidney transplantation, high molecular weight donor-derived cell-free DNA derived from cells shed from the donor-derived urinary tract also appears in

the urine (B). After liver, pancreas or heart transplantation, only transrenal donor-derived cfDNA can be detected in the recipient's urine. cfDNA: cell-free DNA; L: liver; K: kidney; P: pancreas, H: heart.

TABLE LEGENDS

Table 1

Title:

Overview of different cell-free DNA biomarkers

Legend:

cfDNA: cell-free DNA; Tr-DNA: transrenal DNA; bp: base pairs; kb: kilobase; qPCR: quantitative polymerase chain reaction; ddPCR: digital droplet PCR; SNPs: single nucleotide polymorphisms; MPSS: massive parallel shotgun sequencing; EDTA: ethylenediaminetetraacetic acid.

Table 2

Title:

Overview of different ddcfDNA quantification techniques

Legend:

ddcfDNA: donor-derived cell-free DNA; qPCR: quantitative polymerase chain reaction; SRY: sex-determining region, Y-linked; CND: copy number deletion; ddPCR: digital droplet polymerase chain reaction; SNPs: single nucleotide polymorphisms; MPSS: massive parallel shotgun sequencing; GE: genome-equivalents; Sensitivity: the more '+' symbols, the more sensitive the assay; Cost instrumentation: the more '$' symbols, the more expensive the instrumentation.

Table 3

Title:

Overview of studies published on quantification of plasma, serum or urinary cfDNA levels or fractions in patients suffering from different post-transplant complications compared to a stable graft function

Legend:

ddcfDNA: donor-derived cell-free DNA; cfDNA: cell-free DNA; ref.: reference; Tx: transplantation; KTP: kidney transplant patients; HTP: heart transplant patients; LTP: liver transplant patients; KPTP: kidney-pancreas transplant patients; n: number in group; GE: genome equivalents; ChrY: chromosome Y; AR: acute rejection; ATN: acute tubular necrosis; NTX: drug-induced nephrotoxicity; CAI: chronic allograft injury; BKVN: BK viral nephropathy; TMA: thrombotic microangiopathy; MOF: multiple organ failure; N.M.: not mentioned. * Results are shown as median (p25, p75), ** Results are shown as median (min, max), *** Results are shown as mean (± standard deviation), **** Results are shown as mean (min-max).

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