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PRECISION MEDICINE IN SOLID ORGAN TRANSPLANTATION: DONOR-DERIVED CELL-FREE DNA AS A VALUABLE NON-INVASIVE MARKER OF GRAFT INJURY AND REJECTION

Supervisors

Professor Antonio Amoroso

Professor Silvia Deaglio

Candidate

Monica Sorbini

Coordinator: Professor Giuseppe Matullo

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Scientific-Disciplinary Sector: MED/03

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Abstract

Circulating cell-free DNA (cfDNA) refers to small fragments of DNA molecules released after programmed cell death and necrosis in several body fluids such as blood, saliva, urine, and cerebrospinal fluid. The discovery of cfDNA has revolutionized the field of non-invasive diagnostics in the oncologic field, in prenatal testing, and in organ transplantation. Despite the potential of cfDNA and the solid results published in recent literature, cfDNA analysis is still considered a research marker to be further validated, and very few centers are implementing its analysis in the real-life assistance.

The aim of this thesis was to develop an easy and feasible method to quantify cfDNA released from the graft after solid organ transplantation, based on digital droplet PCR (ddPCR) and exploiting the genetic polymorphism in the *HLA-DRB1* gene.

Four different cohorts of patients receiving heart, lung or liver transplantation were recruited and evaluated for cfDNA presence. Results were published in esteemed journals in the transplant field and confirmed the potential of cfDNA as a biomarker of graft damage and rejection. Moreover, our method performed similarly compared to Next-generation sequencing approaches but resulted more feasible and cost-effective.

To improve the performance of the test and increase the number of patients to whom the test can be offered, we designed and optimize a more complete version of the assay, including all *HLA-DRB1* alleles and 4 probes for *HLA-DQB1* gene, that can cover potentially the totality of HLA-mismatched transplants.

Introduction

Circulating cell-free DNA

Cell-free DNA (cfDNA) is represented by double-stranded extracellular DNA fragments released into the bloodstream after apoptosis and necrosis processes in physiological and pathological situations. It was first described in 1948[1] when Mandel and Matais detected the presence of DNA in plasma samples from healthy and affected individuals. Later, its presence was detected in patients affected by Systemic Lupus Erythematosus[2], cancer patients[3], transplant recipients[4], and pregnant women[5]. CfDNA originates from many sources within the body and can be isolated from various body effluents such as blood, urine, effusions, and cerebrospinal fluid[6]. In healthy conditions, it derives mainly from blood cells[7,8], but it can arise from inflammatory cells, tumor cells, fetal cells crossing the placenta during pregnancy, or can be released from graft cells after solid organ transplantation[9]. Human plasma DNA consists of a mixture of DNA fragments of different sizes, mostly ranging between 100 and 200 base pairs[10,11], with a peak at 166 bases; this peculiar length was related to the nucleosomal structure[12,13], as during cell death process, proteins associated with DNA seem to protect short fragments from degradation. However, smaller (<100 bases) or larger fragments of several kilobases have also been reported[14–16] and associated respectively to mitochondrial and necrotic origin[13,17]. CfDNA concentration in blood widely ranges between undetectable to high concentration (up to 100 ng/mL) in healthy subjects[13,17], but it is known that its levels can be affected by many individual conditions, as age, BMI, circadian rhythm[17], exercise[8], inflammation[9], infections[18–20], and pharmacologic treatment[9], that tend to increase cfDNA presence.

Since the discovery of cfDNA, its potential applications in various fields have been continuously explored. The application of cfDNA analysis, which is defined “liquid biopsy”, consents to monitor pathological conditions in oncologic, prenatal and transplantation fields in a non-invasive and revolutionary method[13].

Technical issues for high-quality cfDNA analysis

The relevance of correct sampling

Performing a liquid biopsy means in the practice the retrieving of cfDNA from a body fluid, mostly peripheral blood. However, the rapid turnover and short half-life of cfDNA[13,17,21,22] require proper sampling, considering the relative low concentration of this marker. Most studies were performed using EDTA BD vacutainer[23–27], which does not preserve blood cells from apoptosis and release of genomic DNA, affecting the quantity and quality of cfDNA itself[20,28,29] if plasma is not rapidly separated from the corpuscular part[17]. To prevent cfDNA degradation and its dilution into genomic DNA, *ad hoc* collection tubes are available from different companies (Qiagen, Germany, Roche, Switzerland, and Streck, USA), which were successfully used in some studies[30–33]. Their main advantage is that tubes keep cfDNA stable and free from genomic contamination for up to 14 days, improving the performance of the following research studies, drug discovery, and assay development.

Technical comparison of cfDNA analysis methods

Advancements in technology, particularly the advent of quantitative PCR (qPCR) and next-generation sequencing (NGS), significantly enhanced the detection sensitivity and precision of cfDNA analysis. Methods for cfDNA analysis are generally divided in NGS and non-NGS approaches (Figure 1).

NGS-based methods

NGS-based approaches have the potential to simultaneously sequence thousands of targets. Considering the Illumina technology, its high accuracy and flexibility made it the most spread platform for cfDNA analysis compared to competitors, such as Ion Torrent, Oxford Nanopore, and Pacific Biosciences, which are still limited by their technical features that do not apply properly with short cfDNA fragments[34–36].

In NGS workflow, DNA samples are amplified targeting hundreds or thousands of single nucleotide polymorphisms (SNPs)[37–41] selected depending on the application field, then DNA fragments are tagged by adaptors and indexed before being sequenced with an elevated depth that consents sensitive results after bioinformatics analyses. Assay types can vary according to the aim of the analysis, moving from tagged-amplicon deep sequencing (TAm-Seq) if the target sequence has been previously characterized[42,43], to cancer personalized profiling by deep sequencing (CAPP-Seq)[39,42], to whole genome bisulfite sequencing (WGBS-Seq) for DNA methylation analysis[44,45], and to whole exome (WES) or genome sequencing (WGS), that provide a comprehensive evaluation of tumor mutations, identifying potential oncogenes and tumor suppressor genes, deleterious alterations and variants of unknown significance[42,46,47]. However, WES and WGS are limited by low sensitivity, excessive time and cost, and difficulties in the interpretation of results[6].

For accurate detection of low-abundance targets, as in the case of liquid biopsy in which the fraction of target DNA within a cfDNA sample is potentially poorly represented, deep sequencing is necessary to provide the required sensitivity[48]. Recent improvements in sequencing instrumentation offer options with extremely high coverage depth for large portions of the entire genome in a single sample[34]. Although the cost of performing NGS has decreased considerably[49], this method can have a relatively consistent cost with a long turnaround time (often at least 3 days) and with variable sensitivity. Indeed, when assays are designed to cover several genetic targets, the comprehensive nature of NGS can provide value in efficiency and cost reduction, while NGS is more expensive and time-consuming when analyzing a small number of variants or samples[50]. Moreover, NGS does not provide an absolute quantification of cfDNA meant as the total number of DNA copies[24,51–64].

Non-NGS methods

Real-time or qPCR, microarrays and digital PCR (dPCR) are included in non-NGS methods and offer faster and less expensive detection option compared to NGS. These methods are generally used to detect and quantify the presence of known specific mutation or polymorphisms in cfDNA samples[18,24,26,65–68]. However, to enhance assay sensitivity, improved PCR approaches were developed. To identify single base changes or short deletion, amplification-refractory mutation system (ARMS-PCR) exploits sequence-specific PCR primers that allow amplification of DNA only when the target is contained within the sample, thus lowing the limit of detection in comparison with conventional PCR[67,69]. The same results can be obtained by peptide nucleic acid (PNA) clamp PCR, which prevent nucleic acid amplification of wild-type DNA, increasing the amplification of the mutant DNA[70,71]. Another alternative is the co-amplification at lower denaturation temperature-based PCR (COLD-PCR), which results in the enhancement of both known and unknown minority alleles during PCR, irrespective of mutation type and position. This method is based on exploitation of the critical temperature at which mutation-containing DNA is preferentially melted over wild type[67].

To increase the number of targets that can be examined simultaneously, PCR can be coupled with mass spectrometry. After amplification, PCR products are analyzed with mass spectrometry, searching for dozens of target mutations in a single reaction with great sensitivity[72–75].

Besides encouraging results, qPCR efficiency may be affected by variations in amplification. Furthermore, qPCR measures the fluorescence accumulation of the amplified product and requires normalization to a standard curve or to a reference, resulting in a relative quantification. The main difference between qPCR and dPCR is that, unlike conventional amplification, the reaction in dPCR is partitioned into thousands of sub-reactions, allowing absolute quantitation and high sensitivity. DPCR was first described in 1992 by Sykes et al., who changed standard amplification with the

integration of limiting dilution, end-point PCR, and Poisson statistics[76]. While partitioning the samples in thousands of independent amplification reactions, dPCR reach higher accuracy and an absolute quantification of the target, which is determined by Poisson statistics. The evolution of Sykes method was achieved by Vogelstein and Kinzler who added the detection of the target through fluorescent probes to the partitioning of the sample[77]. Current dPCR technology uses reagents and workflows similar to those used for most standard TaqMan probe-based assays with a smaller sample requirement, reducing cost and preserving precious samples. The methods described by Sykes, Vogelstein and Kinzler have been improved and are commercially available as different platform. dPCR amplification can be performed on a microfluidic chip[78–80], microarrays[81] or spinning microfluidic discs[82], or can be based on oil-water emulsions[83–86]. Moreover, dPCR technology enables high-throughput analysis with reduced cost compared with other methods while maintaining great sensitivity and accuracy. Moreover, because cfDNA is poorly concentrated in plasma, repeated testing on different sample aliquots may not be possible. DPCR can overcome this limit, since it consents accurate detection and quantitation without separate calibration reactions[83], resulting in a reagent and sample saving. Compared with commercial qPCR assays[57], dPCR assays achieve a better limit of detection as well as a more accurate result.

However, dPCR shows practical drawbacks. The number of targets that can be detected is significantly lower compared to NGS-based methods due to the possibility to multiplex from 2 to a maximum of 6 fluorophores using the most innovative instruments. Moreover, limitations in droplet-to-droplet volume uniformity can influence quantification accuracy and reproducibility, but fluidics-based dPCR may offer an opportunity to overcome this limitation[87,88]. Then, PCR efficiency can vary due to different amplicon lengths[89], as longer amplicons are amplified less efficiently, which might result in underestimation of the true cfDNA value[90]. Similarly, Dauber et al. demonstrated that cfDNA concentration was 5 times higher when using smaller amplicons compared with larger

amplicons[66]. Therefore, the use of short amplicon is recommended for the accurate quantification of cfDNA to avoid underestimation of the target.

NGS and dPCR techniques demonstrated to consents similar results in different application fields. The comparison on kidney transplant recipient samples highlighted no significant differences in the detection of cfDNA, with a significant association between the measurements obtained with both methods[91]. Moreover, lower limits of quantification were similar and in line with what is already reported in literature[92], even though NGS method resulted more sensitive in the lower range than the dPCR method[91]. The quantification of mixed chimerism after hematopoietic stem cell transplantation appeared to be feasible with both methodologies conserving high performances in terms of sensitivity, reproducibility, and linearity[93]. Conversely, dPCR better performed in the detection of *KRAS* mutation in the oncologic field, with high sensitivity and specificity[94], and a limit of quantification 10-fold lower compared to NGS[95].

A great advantage of dPCR is the possibility to obtain the absolute concentration of the target, expressed as copies/µL or copies/mL, which is not influenced by fluctuations in the background cfDNA, derived from the patient. Indeed, NGS results can be expressed only in a cfDNA percentage that can be biased and underestimated as a consequence of physiological or pathological conditions of the subject (e.g., concomitant infections, BMI, exercise, etc.)[8,9,17–20]. The use of cfDNA as a concentration has also been shown to be superior to the ratio as a biomarker for allograft rejection[96].

In contrast with amplification-based method, an imaging single DNA molecules method for high precision cfDNA detection was developed. In VANADIS assay (PerkinElmer, Waltham, MA), DNA fragments are labeled with fluorescent oligonucleotides specific for precise genetic targets, then circularized and copied multiple times before being placed on a 96-well nanofilter microplate and analyzed by imaging[97]. This assay is now applied to prenatal screening with high accuracy[98,99]. Since

this method does not require DNA amplification and sequencing, is easily implemented in any laboratory, scalable and fully automated.

The use of cfDNA in the oncological, prenatal, and transplantation field

Tumor-derived cfDNA

In oncology, cfDNA analysis offers a non-invasive method for cancer detection, monitoring of the treatment response, and detecting minimal residual disease by analysing the genetic alterations present in circulating tumor DNA (ctDNA), allowing personalized treatment strategies[100,101]. Currently, the most common use of ctDNA analysis is in therapy selection and patient stratification based on the likelihood of response to targeted therapies[102–107] through the search of specific mutation markers for resistance or sensitivity such as tyrosine kinase inhibitors, inhibitors of programmed death-1[108], programmed death ligand-1[109] and cytotoxic T-lymphocyte-associated protein 4[110]. Through ctDNA analysis is, therefore, possible to differentiate and predict immune checkpoint blockade response patterns[111–114], characterize the tumor heterogeneity[115], and early detect resistance for targeted therapy and chemotherapy[40,116–122]. Le et al. reported that the success of the immunotherapy is associated to a genome-wide microsatellite instability (MSI), as cancer cells deficient of the mismatch repair system are more sensitive to treatment[123,124]. Of consequence, MSI represents another promising approach to monitor tumor progression, leading to a more personalized medicine through ctDNA analysis, in addition to the detection of mutations associated with recurrence or *de novo* drug resistance[125–127].

Another important and recent use of ctDNA is the approximation of tumor burden[40,128] enabled as the ctDNA quantity is directly associated with the number of tumor cells present in the body. Dawson et al. reported that ctDNA showed superior sensitivity with changes in tumor burden compared to other circulating biomarkers[40].

CtDNA screening can be used in combination with physical exams, chest X-ray, and computed tomography scan to monitor healthy individual or the at-risk population. However, the possibility of early cancer screening via ctDNA diagnostics[129–131] is delayed by a notable presence of cancer-associated mutations in healthy population[132] that make challenging to develop diagnostic tests with high sensitivity and specificity in asymptomatic subjects, with the exception for well-known strongly causative mutations[6] in *TP53*, *KRAS*, *NRAS*, and *BRAF* genes. Methylation markers have also been proposed for the detection of early cancer, with the advantage of discriminating the tissue of origin of the cfDNA based on the tissue-specific methylation pattern[133–136]. Despite promising results from clinical trials as STRIVE (NCT03085888), PATHFINDER (NCT04241796) and SUMMIT (NCT03934866) studies[137], and from the application of several panels of methylation markers based on ctDNA detection (e.g. PanSeer Assay[138], CancerSEEK[139], and cfMeDIP[140]), which proposed to develop and validate non-invasive test for early diagnosis of many different cancer types, none of them has already been translated into clinical practice and still need further validation.

Fetal cell-free DNA

In prenatal testing, the analysis of fetal cfDNA in maternal blood has revolutionized the field, allowing for non-invasive prenatal screening for chromosomal abnormalities and fetal aneuploidies in alternative to more invasive methods as karyotyping and FISH on fetal blood, chorionic villus sampling or amniocentesis[141–143]. In 1997, Lo et al. were the first to find fetal cfDNA in maternal plasma and serum[5]. They demonstrated that its concentration in maternal blood increases with gestational age and it is suitable for pregnancy tests due to the fast clearance after the end of the pregnancy[144]. Fetal fraction is estimated as the proportion of placental cfDNA copies in the maternal cfDNA, and is known to vary by maternal BMI, gestational age, and other biological factors[145,146]. CfDNA can be analyzed with a simple blood sampling from the pregnant woman[141], thus called non-

invasive prenatal test (NIPT). Fetal cfDNA can be detected in maternal plasma as early as 5–7 weeks[147] and then increases during gestation[148].

During non-invasive prenatal screening, maternal and fetal cfDNA are discriminated by genetic polymorphisms. CfDNA analysis can be performed either by NGS[149–152], dPCR[90,153] and microarrays[154,155], searching for pathological variations with a targeted or a genome-wide approach. As targeted method[149], we refer to the search for trisomies, microdeletion and genetic alteration in specific genomic loci, as chromosomes 9, 13, 16, 18, 21, 22, X, and Y, which are mostly involved in aneuploidy and chromosome alteration leading to potential genetic disorders[150–152,156,157]. In contrast, genome wide approaches include the analysis of genetic sequences distributed across the entire fetal genome[149]. Moreover, it is possible to non-invasively determine fetal sex, genotype the fetal rhesus D blood group antigen (RHD), and detecting variants involved in paternally or *de novo* inherited disorders[153].

Twin pregnancies represented a limitation to this method that was overcome recently. In dizygotic twin pregnancies, SNPs provide specific genetic information from each fetus. The fetal percentage of cfDNA in maternal plasma for women with twin pregnancies is higher than in singleton pregnancies[158,159]. Furthermore, in monozygotic pregnancies, NIPT findings for genetic conditions will be similar to that of singletons as both fetuses nearly always have the same genotype[160]. In contrast, in dizygotic twins usually only one fetus will be affected with an aneuploidy; cfDNA corresponding to each twin can be identified by SNPs profile and analyzed separately[161].

To date, a low fetal fraction is the most important cause of no-call results in cfDNA screening and is reported to cause test failure in up to 6.1% of tests performed[146,162,163]. A poor concentration of fetal cfDNA can result from an increase of the maternal fraction, due to physiological conditions of the mother[8,17].

Donor-derived cell-free DNA

In the late 1990s, innovative studies began investigating the presence of donor-derived circulating free DNA (dd-cfDNA) in transplant recipients[164]. Researchers hypothesized that detecting and quantifying this DNA fraction could serve as a non-invasive method to monitor graft status and detect rejection allowing for timely intervention and improved patient outcomes. Clinical studies highlighted the potential of dd-cfDNA as a biomarker for detecting rejection episodes earlier and with a greater sensitivity than traditional methods, sparking considerable interest among transplant clinicians and stimulating further research. This advancement laid the foundation for personalized medicine approaches in transplantation, with tailored treatment strategies and less invasive monitoring methods. Besides fragment length analysis[26,165,166] and total cfDNA quantification[18,26], transplantation outcome and graft status are usually monitored by quantifying the donor DNA contribution. Dd-cfDNA is discriminated from the cfDNA of the recipient by exploiting genetic polymorphisms spread across the genome, which consent to the differentiation between the donor and the recipient DNA. Indeed, allelic variations in polymorphic regions are identified as specific for the donor subject since they are not shared with the recipient patient. In the past, the first approach to analyze cfDNA was the detection of the Y chromosome in the plasma of female patients that received an organ from a male donor obtained by Lo and colleagues in 1998 by detecting the *SRY* gene[164]. A similar technique was employed later by Lui and Macher[167,168] in heart, liver and kidney recipients. However, the detection of the Y chromosome can be informative only in sex-mismatched donor-recipient pairs. Since the limit at the application of this method, more widely applicable approaches were developed. Most of them are based on a panel of short-tandem repeats (STRs), variable-number tandem repeats (VNTRs), SNPs, or insertion-deletion polymorphisms (INDELs) chosen as polymorphic enough to distinguish all the possible donor and recipient pairs[23,25,27,32,33,53–56,58–66,96,169–172]. One possible method to inform whether cfDNA is from the donor or the recipient is

to identify SNPs that are homozygous in both the donor and recipient but different between the two[171]. This characteristic leads to the necessity of genotype the genomic DNA of the subjects by qPCR prior to performing dd-cfDNA quantification[23,27], in order to identify informative loci. Polymorphisms are carefully selected with a high probability of being non-identical between two individuals regardless of their ancestral origin, considering minor allele frequency (MAF), polymerase error rate, heterozygosity, linkage, and fixation index, using data derived from large population genetics studies[37]. However, this procedure requires DNA from the donor, which may not be available, as in the case of transplants performed years earlier. Two ways were proposed to overcome this genotyping step. The first one is a NGS-based method defined "random approach" (in contrast to the "targeted approaches" described above that uses preselected SNPs[23,37,58,173]) that makes use of adapter ligation followed by sequencing[52,174] and does not require genotyping. Indeed, informative polymorphisms are identified after the library sequencing associating by default the minor allele to the donor. The second method is based on HLA typing. All transplant centers perform HLA locus analysis of donor and recipient to identify the best match for the transplant. Therefore, HLA typing is always available and can be exploited to discriminate donor cfDNA from that of the recipient. To date, four papers have been published applying this method to different organ transplants, with valuable results[30,31,175,176]. The limit of this method is the impossibility to detect dd-cfDNA in cases donor and recipient are HLA matched, but this limitation could be mitigated by detecting other informative targets[17]. Moreover, the frequency of acute rejection, graft dysfunction and loss is lower in HLA-matched transplants compared to unmatched transplants[177,178].

Dd-cfDNA has been tested in many different transplantation types, mainly represented by kidney[179–182] and heart transplant[30,53,183–185] followed by lung[31,54,165], liver[23,25,172,186,187] and, rarely, pancreas[188–190]. Differences in dd-cfDNA percentage were observed among graft types, reflecting the actual size and cell turnover specific for each organs[191], similar to what is reported for the ctDNA variations

associated to the tumor burden. Nevertheless, it revealed as potential biomarker not only of acute rejection, as it correlated nicely with biopsy-proven rejection, but more generally with graft damage, post-transplant complications and infections. Therefore, dd-cfDNA consensus is increasing and its analysis may be progressively integrated in the routine post-transplant management of patients, thanks to its non-invasive characteristics and powerful performances, with the final goal of avoiding unnecessary biopsy when there is no suspicion of rejection. Indeed, this enhancement will preserve graft tissue from the invasive biopsy injury, improving graft function and transplant survival.

Kidney transplant

Over the past few years, research has focused heavily on the kidney, as it is a highly transplanted organ affected by several complications in the post-operative phase, including surgical events, infections and rejection[192]. The gold standard method to detect kidney allograft rejection is histology obtained via needle biopsy, which is limited by cost, potential complications, and patient discomfort[193]. For these main reasons, dd-cfDNA analysis has been explored as a non-invasive alternative.

Results from the evaluation of dd-cfDNA percentages during the first week after transplant revealed a high donor DNA presence after the surgery followed by a rapid decrease to low levels at post operative day 7 (POD7)[194,195]. After this stabilization, dd-cfDNA is reported to significantly increase in association to rejection events; moreover, cfDNA outperformed antibody-mediated rejection (ABMR) detection compared to T cell-mediated rejection (TCMR)[196]. This could be explained by the fact that cell lysis caused by the antibody-mediated complement activation causes a more massive release of intracellular debris and DNA compared to TCMR, resulting in less detectable cfDNA quantity[52]. The majority of studies performed on kidney recipients set the positive threshold for rejection at 1% of dd-cfDNA, with a wide range of values between 0.43% and 2-3%, depending on the selected technique of analysis and the examined cohort[191,197]. Moreover, Cedars-Sinai Medical

Center researchers are currently studying the use of dd-cfDNA (NCT03859388) in assessing the treatment response for chronic ABMR, which is diagnosed considering the presence of donor-specific antibodies (DSA), evaluating the serum creatinine values and performing renal biopsies[198]. However, DSA modulation is not considered a specific marker of treatment response, since only a fraction of ABMR patients will experience a reduction in DSA amount, or can present with high DSA titer despite no evidence of reduced renal function or biopsy-proven rejection[199].

Finally, undetectable or very low dd-cfDNA levels have a strong negative predictive value for rejection, confirmed by several groups[198,199]. As a result, the primary clinical utility of dd-cfDNA introduction in the clinic will be ruling out rejection suspicion and confidently avoiding unnecessary biopsy[191].

Heart and lung transplant

Heart and lung transplantation are complex surgical procedures that offer a lifeline to individuals suffering from end-stage heart or lung diseases. While these procedures can significantly improve a patient's quality of life and increase their chances of survival, they are limited by important post-transplant complications. The first one is represented by acute rejection and infections, experienced between 10% and 30% of patients within the first year after transplant in the last decade[200,201]. As heart and lung tissue biopsy is an extremely invasive procedure with a high risk of possible complications[202,203], dd-cfDNA analysis represented a valid alternative to diagnose acute rejection. Dd-cfDNA trend is similar to that presented by the kidney; the cut-off percentages are around 1% in the bilateral lung transplant[204] but much lower in the heart transplant (usually between 0.1% and 0.3%), reflecting the relative small mass and reduced cell turnover that characterize this organ[37,53]. Feingold et al. reported the successful application of the dd-cfDNA quantification as an alternative procedure to monitor acute rejection in a cohort of pediatric heart transplant recipients[60]. They offered this test to patients considered not at risk of rejection, and observed that the 81% of

surveillance biopsies could be postponed or not performed when dd-cfDNA was below the threshold, without any adverse outcome for the children.

In a recent study published by Pedini and colleagues, an easy algorithm based on dd-cfDNA was proposed to manage acute rejection and infections in lung transplant recipients[165]. By applying a positive threshold of 1.72%, the test showed a negative predictive value of 91% and a positive predictive value of 72% in distinguishing lung injury from a stable condition, and by integrating the length of the fragments, the positive predictive value raised to 100%.

Liver and pancreas transplant

Recent studies followed patients after liver transplantation measuring the percentage of dd-cfDNA, which assessed around 5 to 10% in stable conditions[171,172]. As the liver represent the 2% of the body weight, it releases a stronger cfDNA amount compared to the examples reported above. Indeed, during the first week after transplant, dd-cfDNA levels are high, reaching more than the 50% of total cfDNA amount[172]. As for the other graft types, biopsy-proven rejection causes a significant increase in dd-cfDNA compared to patients without signs of hepatic damage[23]. Correlation with markers of hepatic function was also considered, and dd-cfDNA resulted significantly associated with transaminase levels, bilirubin and hepatic enzymes as gamma-glutamyltransferase (GGT)[24], underlying its potential as an additional specific marker of the liver function.

Pancreas transplantation has been less investigated compared to other organs, but in the last years it gained visibility and interest in the field since graft biopsies are challenging and associated with morbidity and loss[205]. Median dd-cfDNA in stable patients assessed at 0.28%, and positive threshold set around 1%, similarly to the kidney[188,189]. This biomarker outperformed other markers of pancreas damage as lipase and amilase that are considered poor sensitive[190] in the post-transplant monitoring of pancreas injury.

Aim of the study

Given the potential applications of cfDNA, this biomarker is increasing the general agreement among clinicians in oncology, prenatal and transplantation fields. Despite the encouraging results, however, the cfDNA analysis is not a reality as it is exploited in a relatively small number of centers, and it is still considered a research marker to be further validated.

The main aim of this study was to develop an easy and feasible method to quantify cfDNA released from the graft after solid organ transplantation, based on ddPCR and exploiting the genetic polymorphism in the *HLA-DRB1* gene. Since HLA typing is part of the routine pre-transplant management of donor and recipient, we were able to distinguish the cfDNA from the two individuals without an additional genotyping step. Thanks to these features, the turnaround time of analysis was 1 day, and the cost/sample was maintained lower compared to other approaches based on NGS and thus affordable from the Public Health System.

The second aim was to evaluate the cfDNA presence in plasma samples from different cohorts of patients receiving heart, lung or liver transplantation, and characterize the circulating DNA kinetics in these recipients. Plasma samples were collected at specific time points during the routine post-transplant management of recipients, when the graft status was assessed for rejection and failure by needle biopsy and clinical examinations. Results were obtained by applying our ddPCR-based method, and the diagnostic power of cfDNA was evaluated by associating dd-cfDNA quantifications to biopsy investigations and clinical examinations collected contextually with liquid biopsies.

Materials and methods

Patients' recruitment and Ethics Committee approval

The prospective studies presented in this thesis were approved by the Ethics Committee of the Città della Salute e della Scienza University Hospital of Turin. Transplant recipients which fulfilled inclusion criteria were enrolled in the studies after the sign of an informed consent. Exclusion criteria were refusal or inability to provide informed consent, any form of substance abuse, psychiatric disorders or conditions that may complicate communication between investigator and patient. Four different cohorts were selected and recruited: a first pilot cohort of adult heart transplant recipients (n=19), a second cohort of adult lung recipients (n=30), a pediatric cohort of heart recipients (n=29) and a cohort of adult liver transplant recipients (n=51). Patient data were pseudonymized using an alphanumeric ID and all sensitive information was conserved on the RedCap online platform (https://www.medcap.unito.it/redcap/index.php) and used for analysis.

CfDNA collection and purification

CfDNA was obtained from plasma samples collected anytime patients underwent graft tissue biopsy and clinical examination during routine post-transplant management of acute rejection and graft failure. Samples were collected at specific time points defined based on the routine management of patients, described below in the histological section of Materials and Methods. Peripheral blood samples were collected using PAXgene Blood ccfDNATubes (#768165, Qiagen, Hilden, Germany) and plasma was separated through centrifugation at 2000xg for 15 minutes. Then, plasma aliquots were stored in the TESEO Biobank of the Department of Medical Sciences of the University of Turin until further processed. CfDNA extraction was performed starting from 1ml of plasma using the QIAmp MinElute ccfDNA Mini Kit (#55204, Qiagen, Hilden, Germany) following manifacturer's instruction and eluted in 30ul of Ultraclean water. Total cfDNA quantification was obtained using the Qubit dsDNA HS Assay Kit (Q32854, Invitrogen, Waltham, MA) on a Qubit Fluorometer. Quality assessment

was obtained using the High Sensitivity DNA kit on a 2100 Bioanalyzer instrument (Cat. N. 5067-4626, Agilent Technologies, Santa Clara, CA).

DdPCR assay

Dd-cfDNA quantification was obtained using the HLA Expert Design Assay (Bio-Rad Laboratories, Inc. Hercules, California, USA) as previously described[175]. Briefly, up to 2ng/20µl of cfDNA was pre-amplified to enrich samples and then 2µl of the pre-amplified cfDNA were analyzed by ddPCR targeting the genetic polymorphisms present between donor and patient at *HLA-DRB1* gene. Donor and recipient HLA typing was performed at Immunogenetics and Transplant Biology Service, Città della Salute e della Scienza, Torino, Italy, as part of the routine management before transplantation without the need to perform any additional genotyping before dd-cfDNA quantification. If donor and recipient pairs resulted HLA-matched, they were excluded from analysis. Fluorescence for each probe was measured by the QX100 or QX200 Droplet Reader and results were analyzed using Quantasoft Version 1.7.4.0917 software or QX Manager 1.2 Standard Edition (Bio-Rad Laboratories, Inc, Hercules, California, USA). The positive threshold was established based on the background of no template control (NTC) samples, whereas cfDNA or genomic DNA from HLA-typed donors, loaded at known concentrations, was used as a positive control. All measurements were done in triplicate. Donor cfDNA contribution was expressed as a percentage of the total cfDNA, and results were correlated to clinical information.

Histological and immunohistochemical analysis

Endomyocardial biopsies (EMBs)

Surveillance EMBs were performed with a different schedule in adult and children recipients. Adult patients were biopsied once per week within the first month after transplant, then every two weeks between month 2 and 3, once per month between month 4 and 6, and then once every 2 months up to 1-year follow-up (Figure 3). In contrast, pediatric heart recipients were biopsied within 1-3

months post-transplantation, then once in the first year and later only one EMB were collected per year, unless a high suspicion of rejection was present. EMBs were evaluated by a single Pathologist and graded according to the revised International Society for Heart and Lung Transplantation (ISHLT) biopsy grading system[206] for acute cellular rejection (ACR) and to the ISHLT working formulation for antibody-mediated rejection (AMR)[207]. Immunohistochemical testing for C4d was performed on every biopsy specimen (CD68 was tested only in case of AMR suspicion).

Transbronchial biopsies (TBBs)

Surveillance lung allograft bronchoscopy and TBBs were performed at 4, 8, 12- and 18-months post-transplant. In addition, bronchoscopy and TBB were performed whenever there was clinical suspicion of rejection or pulmonary infection. The Working Formulation of the ISHLT criteria[208] was applied by experienced transplant pathologists to diagnose and grade all graft TBBs. In particular, the diagnosis of rejection is based on the presence of perivascular and interstitial inflammatory cell infiltrates. Subendothelial infiltration/endotheliitis was also considered relevant for the final diagnosis of acute rejection. Based on the histological extent of injury and inflammation, rejection was graded as absent (grade A0), minimal (grade A1), mild (grade A2), moderate (grade A3), or severe (grade A4). Grade A2 is generally considered a threshold for therapeutic intervention. Morphological (e.g., neutrophilic margination, neutrophilic capillaritis, and acute lung injury with or without hyaline membrane deposits) and immunohistochemical (i.e., C4d deposition in interstitial alveolar capillaries) features of AMR were assessed and graded according to ISHLT and Banff recommendation[209–211].

Hepatic biopsies

Liver core-biopsy samples were formalin fixed, quickly processed through microwave hybrid processing technology, paraffin embedded, and 3 µm-thick serial slices were cut. Nine sections at different depth levels were stained with hematoxylin and eosin, 3 with Sirius-red stain to highlight

collagen fibers, 3 with Periodic acid–Schiff–diastase and 3 with Perls stain for iron deposits assessment. Other special stains and immunohistochemical reactions were performed according to diagnostic needs. In liver biopsy collected at Post_TX (1–2 hours after liver graft reperfusion into the recipient), the ischemia-reperfusion damage and steatosis were evaluated and overall graded as absent/mild/moderate/severe[212,213]. In subsequent follow-up biopsies, portal inflammation, biliary duct injury and endothelitis were scored and combined in the rejection activity index (RAI)[214]; acute rejection diagnosis was formulated and graded according to the Banff 2016 classification. Fibrosis was evaluated and staged using the Ishak scoring system[215] and/or liver allograft fibrosis semiquantitative scoring system (LAFSc)[216]. All the other possible pathological findings (e.g. cholestasis, cholangitis) were reported.

DSA evaluation

Serum samples collected during the routine post-transplant management were assessed for *de novo* DSA by Luminex method with commercially available SAB kits (LS1A04 and LS2A01 assays, One Lambda, West Hills, CA). The results were expressed as mean fluorescence intensity (MFI, cut-off positive value >1,000). All pre-transplant crossmatches were performed by flow-cytometry (FACSLyric, BD Biosciences) on T and B lymphocytes using historical and current sera. Donor lymphocytes were isolated from peripheral blood.

Statistical analysis

Continuous variables are reported as median and interquartile range or mean ± standard deviation (SD) as appropriate, whereas categorical variables are indicated as number and percentage. Groups were compared using the Kolmogorov-Smirnov nonparametric test, Mann-Whitney test or unpaired t-test based on the gaussian distribution of samples. The correlation between two continuous variables was analyzed using the nonparametric Spearman test or Pearson r test where appropriate. Receiver operating characteristic (ROC) curves were calculated using the Wilson-Braun method. P-

values lower than of 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism version 8.0.2.

Results

"HLA-DRB1 mismatch-based identification of donor-derived cell free DNA (dd-cfDNA) as a marker of rejection in heart transplant recipients: A single-institution pilot study"

Sorbini et al., Journal of Heart and Lung Transplantation, 2021 doi: 10.1016/j.healun.2021.05.001

Optimization of a ddPCR method to quantify dd-cfDNA based on *HLA-DRB1* gene

The first step of the project was to set up the ddPCR assay to quantify dd-cfDNA from plasma samples exploiting the *HLA-DRB1* polymorphisms between donor and recipient. The list of *HLA-DRB1* probes used for this work is shown in Table 1. Starting from the ddPCR protocol reported by Zou et al.[175] and the HLA Expert Design assays datasheet from Bio-Rad Laboratories, Inc. we identified 55°C as the most suitable temperature for discriminating positive droplets from the negative background with an optimal logarithmic dilution curve (Figure 2). These conditions were then adopted for all subsequent assays. Probe specificity was confirmed by testing each control DNA against the full probe set and confirming a positive signal only with the probe corresponding to its *HLA-DRB1* allele. This version of the assay was defined FAM singleplex method, as it was applied to detect only one target allele per well. The dd-cfDNA contribution was then obtained by calculating the ratio between the mean donor cfDNA copies and the total of cfDNA copies, and expressed as a percentage.

Workflow of analysis

After patients recruitment, plasma samples were been collected before surgery and considered as reference (negative) pre-transplant sample. After transplantation, blood samples were collected at day 7 and then every time the patient underwent EMB, unless noted otherwise (Figure 3). CfDNA quantity and quality were determined prior to ddPCR to exclude degraded samples. Mean cfDNA concentration was 1.6 ng/ml from 1 ml of plasma, and average DNA fragment length ranged

between 150 and 200 bp. Points of analysis in which the patient was not experiencing complications and had a negative EMB (0R score, 60 samples) are referred to as “stable condition” and were used for comparison with other time points. A total of 232 cfDNA samples were evaluated using the ddPCR assay (mean of 12 samples/patient). At least 2 ng of cfDNA were pre-amplified to increase the total number of molecules tested and then amplified by ddPCR. Probes were selected based on HLA typing performed as part of the routine pre-transplant management, and donor- and recipient-specific reactions were carried out in different wells of the same plate by applying the FAM singleplex method described above. Probe fluorescence was normalized based on negative template controls (NTC) and healthy donor sample signals. Dd-cfDNA was quantified as the ratio between mean donor copies and mean total copies, that is, the sum of recipient and donor copies, and expressed in percentage as fraction abundance.

Correlation between dd-cfDNA fraction abundance, ischemia-reperfusion injury and infections

Between July 1st, 2019 and June 30th, 2020, 24 patients underwent a heart-only transplant at our Institution. Nineteen of them were enrolled in this study and their demographic and clinical features are reported in Tables 2 and 3. Out of the 5 patients that could not be included in the analysis, 2 had mismatched alleles that were not covered by our probe panel (*HLA-DRB1*14* and *HLA-DRB1*10*), while the other 3 donor-recipient pairs were fully matched for *HLA-DRB1*, making it impossible to distinguish the donor from the recipient. All the included patients were adults (mean age 50.9±14.8 years) and predominantly males (n = 15, 78.9%). Idiopathic dilated cardiomyopathy (DCM) was the most recurrent diagnosis in the cohort (n = 9, 47.4%), followed by ischemic DCM (n = 5, 26.3%). Six patients (31.6%) were former smokers. Other frequent comorbidities were dyslipidemia (n = 5, 26.3%), metabolic diseases (n = 4, 21.1%), arterial hypertension (n = 4, 21.1%) and the previous implantation of a left ventricular assist device (LVAD, n = 4, 21.1%). Mean donor age was 38.4±11.5 years, and all donors were beating heart cadavers. One week after surgery all patients showed a

significant rise in dd-cfDNA fraction abundance (mean dd-cfDNA was 2.08±2.09%, p<0.0001) compared to stable conditions, which is in line with the known ischemia-reperfusion injury (Figure 4A). However, duration of ischemia (mean of 174.3±44.77 minutes) and post-operative support (intra-aortic balloon pump was required in R#1, R#6, R#14 and R#19, whereas R#14 and R#17 needed temporary extracorporeal membrane oxygenation) were not directly related to the fraction abundance (Figure 5A). Immunosuppressive therapy in the induction phase included thymoglobulin and steroids. Immunosuppression maintenance was based on calcineurin inhibitors (mainly cyclosporine) plus antimetabolite agents (mycophenolate) and corticosteroids. During post-transplant hospitalization (mean time of 32.2 days, with a minimum of 15 and a maximum of 65), 5 episodes of infection occurred: Pseudomonas aeruginosa (R#2 and R#19), Klebsiella pneumonia (R#8 and R#14) and Serratia marcescens(R#4). These episodes were not related to meaningful modifications in dd-cfDNA fraction abundance compared to stable conditions (Figure 4B). During the follow-up period, cytomegalovirus (CMV) replication was detected in 7 patients without clinical signs of infection. In addition, 5 patients tested positive for SARS-CoV-2 but none of them showed severe complications despite immunosuppressive treatment, and all of them recovered. Dd-cfDNA levels did not show significant modifications during viral infections (0.66±1.07%; Figure 4B) compared to stable conditions. Three patients died during follow-up. Causes of death were intracerebral bleeding in post-operative day (POD) 51 (R#2), sepsis in POD 156 (R#11), cardiac arrest in infectious pneumonia in POD 162 (R#9). R#2 never experienced significant rejection episodes and cerebrovascular events were related to severe infection. Donor DNA was stable at all follow-up time points as well as at the time of death (Figure 5A). R#9 had persistent SARS-CoV-2 positive nasopharyngeal swabs. He suffered from mild respiratory insufficiency requiring oxygen therapy. Laboratory findings showed persistent leukopenia and thrombocytopenia. Post-mortem investigation of heart specimens revealed features of 1R ACR together with endothelialitis and

capillary thrombosis, which were related to a significant increase in dd-cfDNA (Figure 5A). R#11 tested positive for SARS-CoV-2 5 weeks after transplantation without symptoms and his nasopharyngeal swab resulted negative 6 weeks later. During follow-up, EMB showed persistence of significant ACR that was treated with intravenous steroids and thymoglobulin. Repeated rejection events were related to spikes in dd-cfDNA.

Correlation between dd-cfDNA fraction abundance and rejection markers

We then correlated dd-cfDNA fraction abundance to the histological evaluation performed on EMBs collected at the same time as blood samples. Regarding ACR, EMBs were classified as 0R if negative, as 1R in case of mild rejection and as 2-3R in case of moderate/severe events (Figure 5B). A total of 130 EMB samples were collected (mean of 6.8 EMBs/patients), 69 of them (54.3%) were classified as 0R and 61 as 1R (46.9%). No routine biopsies were classified as >1R and none of them showed signs of AMR. Autopsy investigation on R#11 identified a 2R grade rejection. EMBs collected from patients who showed a linear profile with undetectable or a very low amount of dd-cfDNA during follow up were generally classified as negative; patients who showed 1R events had a dd-cfDNA profile characterized by repeated peaks consistent with rejection episodes (Figure 5A). While the majority of 1R events were not treated since they did not affect heart function, in 2 cases (R#3 and R#5) the immunosuppressive regimen was modified, leading to a rapid decline in dd-cfDNA fraction abundance to baseline levels. Overall, the mean dd-cfDNA percentage at rejection was significantly higher than that of 0R time points (0.43±1.04% vs 1.71±3.10%, $p<0.0001$, Figure 4C). DSAs were tested in the serum of 15 patients (78.9%), with one patient (R#7) testing positive for anti-HLA-B*51 antibodies (mean fluorescence intensity = 1998). This patient did not show any signs of rejection at EMB and no significant modification of dd-cfDNA fraction abundance when DSAs were first observed. Notably, the two patients who died with features of ACR were DSA-negative.

Calculation of a cut-off value

Lastly, we tried to estimate a clinically relevant cut-off value to identify samples that could potentially raise a warning signal for the functional status of the transplanted heart. In order to get a clearer correlation between ddPCR assay results and biopsy grade, and to reduce the confounding factor due to ischemia-reperfusion damage, only dd-cfDNA quantifications starting from 6 weeks post-transplantation were considered. To assess the performance of the tests, a ROC curve was calculated (Figure 6). The obtained Area Under the Curve (AUC) was 0.72 (95%CI, 0.6089-0.7929). With a cut-off of 0.11%, dd-cfDNA showed 70.8% specificity (95% CI, 58.17%-81.40%) and 64.2% sensitivity (95% CI, 49.80%-76.86%) in distinguishing acute rejection from no rejection. Specifically, out of the 130 dd-cfDNA quantifications performed, 56 (43%) were above the 0.11% cut-off. Of them, 42 (75%) were paired to an EMB score of 1R, while the remaining 14 were paired to an EMB score of 0R.

"Validation of a Simple, Rapid, and Cost-Effective Method for Acute Rejection Monitoring in Lung Transplant Recipients"

Sorbini et al., Transplant International, 2022 doi: 10.3389/ti.2022.10546

Optimization of a multiplex ddPCR method

As a further step in the optimization of the ddPCR protocol, we considered to perform dd-cfDNA analysis by multiplexing donor- and recipient-specific probes in the same well. Combinations of cfDNAs (Figure 7) or genomic DNAs (data not shown) carrying different *HLA-DRB1* alleles were loaded at known concentrations (1%, 5%, 10%, and 50%) and quantified using the FAM singleplex method described above, or by combining FAM and HEX probes targeting the two alleles in the same reaction. Since the results were consistent between both methods, we concluded that dd-cfDNA quantification of both the donor and recipient in the same well was feasible, with a reduction in time and costs of analyses while maintaining comparable accuracy. Therefore, the multiplexed protocol was applied in the following dd-cfDNA analysis.

Post-transplant clinical management of lung recipients

All patients were admitted to a dedicated intensive care unit (ICU), allowing controlled ventilator weaning. Primary graft dysfunction (PGD), defined according to the International Society of Heart and Lung Transplantation (ISHLT) guidelines[217], was evaluated at the time of admission to the ICU and after 24 and 72 h. Immunosuppressive therapy during the induction phase included thymoglobulin (1 mg/kg/day for five days) and steroids. Immunosuppression maintenance was based on calcineurin inhibitors (mainly cyclosporine), antimetabolites (mycophenolate), and corticosteroids. After discharge, patients were followed up in our lung transplant day hospital using spirometry, blood gas analysis, and medical and radiologic examinations to assess lung function. Rejection events determined by histological and clinical examination were mainly treated with pulse

dose glucocorticoids (methylprednisolone 15/mg/kg/day for 3 days). Chronic lung allograft dysfunction (CLAD) was defined as a substantial and persistent decline (≥20%) in the forced expiratory volume in 1 s (FEV1) when compared with the post-transplant baseline[218], and based on its duration classified as possible (<3 weeks), probable (≤3 months) and definite (>3 months). Biochemical and microbiological evaluations on blood and bronchoalveolar lavage samples were performed routinely and in case of suspicion of bacterial and viral infections.

Characteristics of the recruited cohort

Thirty consecutive patients who underwent primary lung transplantation at our institution between July 1st, 2019 and March 31th, 2021 were recruited for this study (Table 4). In 28 out of 30 cases (93.3%), organs were recovered from heart-beating donors (13 males, 43.3%) with a mean age at death of 42.5±16.1. In the two remaining cases, donations occurred after circulatory death in patients aged 58 and 69 years, respectively. The mean waiting list time was 306.0 (range:3-1607) days, with a median of 226.5 days. The mean age at transplantation was 47.0±15.5. Twenty-one patients (70.0%) received a double lung transplant, four (13.3%) received a single lung transplant, and five (16.7%) received a bilateral lung transplant associated with another solid organ transplantation (one lung-heart, one lung-kidney, and three lung-liver-pancreas). Idiopathic pulmonary fibrosis (11 cases, 36.7%) was the most common disease, followed by cystic fibrosis (7 patients, 23.3%), and chronic obstructive pulmonary disease (6 cases, 20.0%). Nine patients (30.0%) received a transplant on an urgent basis, four (13.3%) received mechanical ventilation, and 6 (20.0%) received ExtraCorporeal Membrane Oxygenation (ECMO) before transplantation. 22 subjects (73.3%) presented with clinical signs of PGD of any grade within the first 72 hours after transplantation. Three patients (10.0%) experienced grade 3 PGD 72 hours after transplant. Lastly, four recipients (13.3%) received organs that underwent Ex-vivo Lung Perfusion (EVLP) before

transplantation. The mean total organ ischemia was 332.9±159.4 minutes. The median total hospital stay was 47.5 days, and none of the patients died before discharge.

Dd-cfDNA release is influenced by ischemia-reperfusion injury

In total, 372 plasma samples were obtained from 30 patients (mean 12,4 samples/patient). Samples were collected contextually with TBBs, spirometry test or hematological evaluation for infections. The mean dd-cfDNA percentages obtained at all times differed significantly between monopulmonary, bipulmonary, and combined transplant recipients, reflecting the number of donor cells present in the recipient (Figure 8A). In fact, mean donor DNA levels were lower in single-lung recipients (2.8±3.2%) than in double-lung (6.2% ±10.9%, p=0.02) or combined transplant recipients (13.3% ±16.2%, p<0.0001). During the first 2 weeks after transplantation, dd-cfDNA peaked (mean value 6.36±5.36%), in line with our previous results on heart recipients, demonstrating organ damage due to ischemia-reperfusion (Figure 8B). In patients without complications, the mean donor dd-cfDNA quantification slowly stabilized at two weeks after transplantation. To determine the baseline value to be used for comparisons, we selected 18 samples from 10 patients (1 monopulmonary, 1 combined, and 8 bipulmonary recipients) at a time when no sign of rejection, infection, or worsening of their clinical condition could be observed. The mean dd-cfDNA calculated from these samples (2.18%±3.26%) was considered as the baseline.

Acute rejection is followed by a significant increase of dd-cfDNA

A total of 20 out of 115 transbronchial biopsies (17.4%) scored positive for cellular rejection. Nine biopsies were classified as minimal grade (indicated as A1) and 11 as mild grade (indicated as A2, Figure 9A). No grade ≥A3 biopsies were observed during the follow-up period. Donor DNA levels were more elevated during AR events than under stable conditions (7.81±12.7%, p<0.0001, Figure 9B). In addition, levels varied significantly according to the severity of rejection; A1 events were related to a modest increase in donor DNA amount (mean value 5.74±10.0%, p=0.03), whereas A2

rejection caused a stronger increase (9.48±19.60%, p=0.008, Figure 9C). No biopsy showed morphological or immunohistochemical features of AMR, even though five patients (16.7%) developed DSA after transplantation and two of them had anti-HLA-DQ antibodies, which are generally associated with AMR. Of the 60 DSA tests performed, 38 (63.3%) were negative in accordance with negative biopsies, 2 (3.3%) were positive and associated with biopsy-proven A2 rejection, and 15 (25.0%) did not agree with the histochemical evaluation. The remaining five (8.4%) samples were not temporally related to graft tissue collection. Donor DNA percentages obtained from seven samples temporally close to DSA-positive sera were higher than those obtained from DSA-negative samples (nine samples). To avoid confounding factors that could affect the analysis, this statistical evaluation was performed considering only serum samples collected in the absence of documented infections and other evidence of graft damage not due to rejection (16 samples). Even if the number of samples included in the statistical analysis was limited, results reached significance when comparing stable conditions vs DSA-positive (p=0.01, Figure 9D). On the contrary, we could not observe significant differences between DSA-positive and DSA-negative samples. Finally, 3 patients (10.0%) experienced possible CLAD, 2 of whom showed clinical signs of bronchiolitis obliterans (BOS) and then recovered (Figure 9A), whereas the remaining patient developed a mixed form of BOS and restrictive allograft syndrome and died from severe pulmonary insufficiency caused by chronic rejection and pneumonia. All samples collected during these episodes showed elevated levels of dd-cfDNA (8.26±4.41%, p<0.0001, Figure 9C).

Respiratory tract infections were related to significant changes in dd-cfDNA levels

During follow-up, every patient experienced respiratory tract infections: bronchoalveolar lavage contained bacteria in 24 (80.0%), viruses in 23 (76.7%), and fungi in 11 (36.7%) cases, with specimens from 8 patients (26.7%) showing mixed contamination (Table 4). Among bacteria, the most frequent pathogens were *Pseudomonas aeruginosa* (12 specimens, 40.0%) and *Klebsiella pneumoniae* (5

specimens, 16.7%), while C*ytomegalovirus* (20 specimens, 66.7%) was the most common virus. Dd-cfDNA levels significantly increased during infectious episodes compared to stable conditions, with a slightly stronger increase observed during viral (7.70±14.20%, p=0.004) and mixed infections (13.7±23.5%, p=0.0007, Figure 9B and Figure 10A). Consistently, dd-cfDNA levels showed a positive association (r=0.37, p=0.0005) with C-reactive protein (CRP) blood levels, as determined by studying dd-cfDNA levels in 104 samples from 28 patients and collected close to CRP measurements during infection episodes (Figure 10B). One time point was excluded from the analysis because its CRP value was > 300 mg/L, representing a potential bias in the statistical analysis. Considering 5 mg/L as a clinical cut-off value, the same samples were divided into low and high CRP groups. With this classification, samples collected from patients with CRP levels ≥ 5 mg/L showed significantly higher mean dd-cfDNA percentages (9.91±16.4%) than low CRP samples (4.44±7.13%, p=0.004, Figure 10C).

Dd-cfDNA percentages correlate with respiratory function

Lung transplant function was assessed using spirometry. FEV1 was quantified using recipient characteristics for the normative equation and considered a respiratory function measure. A total of 114 liquid biopsies were collected close to the spirometry tests, and dd-cfDNA quantification was correlated with relative FEV1 percentages. As shown in Figure 11, there was a statistically significant inverse relationship between the two variables (r=-0.26, p=0.0054).

Accuracy of the test

ROC analysis was performed to assess the performance of this method. The AUC was 0.87 (95% confidence interval: 0.75-0.98, p<0.0001, Figure 12). With a cut-off value of 1.25%, dd-cfDNA had 80.7% sensitivity and 73.3% specificity for distinguishing AR from non-rejection. In particular, the test correctly identified 25 of the 31 biopsies classified as positive for rejection, and by excluding

samples in which rejection occurred together with infection, dd-cfDNA quantification was above the cut-off value in 14 of 16 (87.5%) biopsies.

"Routine monitoring of donor-derived cell-free DNA to avoid surveillance endomyocardial biopsies in pediatric heart transplant recipients"

Sorbini et al., Heliyon, under review

Post-transplant clinical management in children heart cohort

All patients included in the study were transplanted and followed-up in our Institution. During each follow-up visit, patients underwent clinical evaluation, electrocardiogram (ECG), echocardiogram and blood testing. Blood tests included dosage of N-terminal pro B-type natriuretic peptide (NT-proBNP) and high sensitive Troponin I (hs-Troponin I) levels: abnormal value for NT-proBNP was designated as ≥1000 ng/L and for hs-Troponin I as ≥3 ng/L based on previous studies[219–222] .

At clinical examination, signs and symptoms of heart failure were evaluated, including tachycardia, tachypnea, poor weight gain, decreased oral intake, dyspnea at rest or with exercise, fatigue, hepatomegaly, abdominal pain, nausea and vomiting, peripheral edema, jugular venous distention, poor perfusion.

Echocardiograms were interpreted by one of the Pediatric Cardiologists, measuring left ventricle (LV) dimensions, valves function and flow velocities, systolic and diastolic parameters, including ejection fraction (EF), shortening fraction, mitral annulus velocity by tissue Doppler imaging (TDI), isovolumetric relaxation time (IVRT) and right ventricle (RV) dimension and function, including tricuspid annular plane systolic excursion (TAPSE). Systolic dysfunction (EF reported as <50%), significantly increased mitral or tricuspid valve regurgitation, increase of TDI data, individual patient reduction in IVRT (>20%)[223,224] and/or new pericardial effusions were considered abnormal and consistent with a suspicion of rejection. ECG was evaluated by Pediatric Cardiologists, including sum of ECG total voltage (defined as sum of I, II, III, V1 and V6 voltages); ECG abnormalities, new

arrhythmias and individual patient ECG total voltage reduction (>20%)[225] were considered abnormal and consistent with a suspicion of rejection.

Description of the recruited cohort

Twenty-nine pediatric heart transplant recipients, 15 (55%) of whom females, were recruited at our Institution starting from February 2022 for the following 12 months (Tables 5 and 6). Mean age at recruitment was 11.0±4.8 years, and the most represented conditions leading to heart failure were DCM (n=15) and congenital heart disorders (CHD, n=12). During the first post-transplant year, patients were checked monthly, while in later years follow-ups decreased progressively up to one every four months. No death and no re-transplantation occurred during the study period; two patients were followed for cardiac allograft vasculopathy (CAV).

Correlation between dd-cfDNA values and clinical parameters

One hundred and fifty-eight blood samples were collected during outpatient visits, with an average of 5.4 samples/patient. We obtained an optimal DNA yield from all samples (mean cfDNA concentration 0.24 ng/µl, Table 6), despite the reduced volume of blood that was drawn from younger patients. Dd-cfDNA was monitored by ddPCR capturing polymorphisms in the *HLA-DRB1* locus, as described above.

No differences in dd-cfDNA percentages were observed in relation to patient or donor weight (p=0.54 and 0.39) and time after transplantation (p=0.34), indicating that these variables do not impact dd-cfDNA release (Figure 13A-C).

During the study time, a total of 28 EMBs were performed from 24 patients. 11 biopsies scored positive for rejection (39.3%), 9 of which were classified as ACR (all graded as 1R) and 2 as AMR (both pAMR2 score). Concomitant dd-cfDNA analysis were significantly different in samples showing no evidence of rejection (n=17, median 0%) compared to those presenting signs of rejection, globally considered (n=11, median 6.27%, p=0.0002, Figure 14A). Among the negative group, 4

samples from 3 patients resulted higher compared to the median value: the sample with the highest dd-cfDNA levels was an outlier related to a patient (ID 6) showing elevated values in 4 different samples, without any other clinical or laboratory parameter pointing to heart damage. The plasma showing dd-cfDNA levels of 8.37% contains DSAs and has elevated NT-proBNP and hs-Troponin I levels. The EMB performed at that time was negative for rejection, opening the question of whether the increase in dd-cfDNA was related to non-rejection caused cardiac injury, bad sampling or to the low sensitivity that affects EMB. From this same patient (ID 9), two more EMBs were collected; the first scored positive for AMR (pAMR2), then the patient was treated and the following biopsy resulted negative for rejection, with a decreasing dd-cfDNA value (1.73%). The last dd-cfDNA measure was related to a third patient (ID 5) dosed only once and in that occasion all parameters were negative. He entered the study in October 2022 and there was no further follow-up until the end of study period.

De-novo DSA analysis was performed in 154 samples, from 29 different patients. Results demonstrated the presence of DSA in 26 sera (16.9%), belonging to 6 different patients (Table 7). DSA were specific for HLA class II in 4 patients, for HLA class I in 1 patient, and 1 patient developed DSA specific for both HLA Class I and HLA Class II. Interestingly, only one patient developed signs of AMR in two different EMBs that both scored pAMR2. In the other 5 patients, no signs of AMR were found, but 3 presented signs of ACR at biopsy. No EMBs scored positive for complement deposition. The median value of dd-cfDNA was significantly increased in the DSA positive group compared to the DSA negative (0.06% vs 1.58%, p=0.0010, Figure 14B).

As further marker of heart damage, we measured NT-proBNP plasma levels in 155 samples from 29 patients, setting a threshold for graft injury at 1000 ng/L[219,220]. We found higher levels of dd-cfDNA in samples with ≥1000 ng/L NT-proBNP (median levels: 1.59%), compared to the <1000 ng/L group (median levels: 0.10%, p=0.02, Figure 14C). Hs-Troponin I values were further considered an indirect

marker of graft status, with a cut-off set at 3 ng/L[221,222,226,227]. The difference between median dd-cfDNA levels in low and high hs-Troponin I groups was highly significant (median dd-cfDNA: 0.04% vs 2.35%, respectively; p<0.0001, Figure 14D).

We then correlated dd-cfDNA levels to ECG and echocardiograms parameters that are considered associated with heart failure. Dd-cfDNA levels increased significantly in relation to reduced IVRT (p=0.0031, Figure 14E), which is generally associated to episodes of acute rejection[223,224], while no differences were observed according to the ECG voltage (p=0.48, Figure 14F).

Lastly, 9 samples were collected from 4 patients presenting clinical signs and symptoms of heart failure, mainly poor weight gain, decreased oral intake, dyspnea at rest or with exercise, fatigue, hepatomegaly, abdominal pain, nausea, vomiting and peripheral edema. In these samples, median dd-cfDNA was 5.24%, significantly higher compared to samples without any sign of failure (median dd-cfDNA: 0.14%, p=0.0018, Figure 14G). We also considered 11 sera from 4 patients, who received steroid treatment for ongoing rejection. Median dd-cfDNA% was 1.73% compared to 0.11% in the untreated group (147 samples, p=0.0017, Figure 14H). Collectively, these results show that dd-cfDNA is highly associated to every currently used marker of rejection, independent of the time elapsed since transplantation.

Determination of a rejection score based on dd-cfDNA

Next, we sought to calculate the performances of dd-cfDNA to distinguish between rejection and no-rejection, considering each laboratory and clinical marker and performing ROC analyses. ROC curve of dd-cfDNA related to EMB grade resulted in an AUC of 0.89 (95% CI 0.77-1.0; p=0.0005, Figure 15), similar to previously reported data on adult and pediatric patients[191]. With a cut-off of 0.55%, dd-cfDNA had 100% sensitivity and 76.5% specificity in distinguishing rejection from no rejection, with a Negative Predictive Value (NPV) of 100% and a Positive Predictive Value (PPV) of 73%. We then performed the same analysis considering other laboratory and clinical parameters:

DSA measurement resulted in an AUC of 0.69 (95% CI 0.58-0.80; p=0.0019), NT-proBNP resulted in an AUC of 0.64 (95% CI 0.51-0.77; p=0.03), hs-Troponin I resulted in an AUC of 0.73 (95% CI 0.63-0.83; p<0.0001), IVRT resulted in an AUC of 0.76 (95% CI 0.61-0.91; p=0.0060) and clinically defined heart failure resulted in an AUC of 0.78 (95% CI 0.63-0.94; p=0.0045, Figure 15).

We then applied 0.55% as a positive threshold for rejection to estimate the power of the test on samples from our recruited cohort. A total of 88 samples resulted under the positive threshold (median dd-cfDNA 0%, range 0-0.54). Among them, 13 were collected before a biopsy was performed, and none scored positive for rejection; DSA were present in 7 samples, NT-proBNP was above the threshold in 8 samples, hs-Troponin I was elevated in 4 samples, 2 samples were related to a reduced IVRT and only one sample was collected from a patient that was still under treatment for a past event of rejection. Globally, no samples were associated to signs of rejection and no modification in the immunosuppressive regimen was made.

In contrast, samples with dd-cfDNA% >0.55% were 70 (median dd-cfDNA 2.35%, range 0.59-20). Of them, 15 samples were collected before biopsies, and 11 resulted positive for rejection; DSA were present in 19 samples, NT-proBNP was elevated in 14 samples, hs-Troponin I was above the threshold in 22 cases, and 8 presented a reduced IVRT. Moreover, 10 samples from 4 patients were collected when additional steroid treatment for ongoing rejection was administered.

We then checked dd-cfDNA values according to the number of parameters positive for rejection (EMB, DSA, NT-proBNP, hs-Troponin I and heart failure). Of the 158 samples, 92 measurements did not present any marker of rejection (median dd-cfDNA 0.04%), 39 had one parameter indicating rejection (median dd-cfDNA 0.51%), 14 presented 2 parameters (median dd-cfDNA 1.24%), 11 samples presented 3 parameters (median dd-cfDNA 5.74%) and 2 samples presented 4 markers out 5 indicating rejection (median dd-cfDNA 7.36%), reflecting a step-wise increment of dd-cfDNA

related to the number of rejection and injury markers and, therefore, the severity of the allograft damage.

Lastly, we tried to integrate all clinical and hematological parameters to score the risk of rejection. We re-assessed the power of our test by applying 0.55% as a positive threshold for rejection. Considering all non-invasive blood parameters of heart injury to distinguish between healthy allograft and rejection, the NPV resulted in 100% and the PPV in 60%.

Relevant clinical cases of rejection

We selected 2 patients who developed CAV and were followed during the 12 months of the project; their dd-cfDNA values were dosed multiple times. The first patient is a 17 years-old girl with complex CHD, transplanted in 2006 (ID 13, Figure 16A).

After developing chronic allograft rejection in 2021 with heart failure, high NT-proBNP levels and discrete coronary stenosis (classified as CAV2 from ISHLT)[228], she was treated with angioplasty and switched to everolimus. In 2022, due to therapy non-compliance, she experienced chest pain and heart failure, requiring new angioplasty. She was enrolled in March, 2022 and DSA measurements were always negative during the entire follow-up. Dd-cfDNA% was evaluated monthly, and resulted above the threshold for rejection until patient condition stabilized after treatment in July 2022.

The second patient is a 5 years-old boy, transplanted in 2018 for DCM (ID9, Figure 16B). DSA were present since 2020 and were specific for HLA class II DQ antigens; one of them showed C1q binding capacity (DQ6, MFI=9223). In 2020-2021 he was treated with pulse steroids, i.v. Rituximab, intravenous immunoglobulin (IVIG) and Thymoglobulin. In the study period, he experienced two rejection events in September 2022 and January 2023, respectively. The first one scored for mixed ACR/AMR rejection (biopsy grade 1R(1A), pAMR2), and the second event scored for a pAMR2 grade rejection, with NT-proBNP and hs-Troponin I levels above the reference values. Cardiac catheterization revealed high filling pressure at September 2022 and January 2023; diffuse coronary

lesions and heart failure (ISHLT CAV3)[228] were present in January 2023. Biopsy-proven rejections were treated with Rituximab, Plasmapheresis, IVIG and Thymoglobuline. Dd-cfDNA values were over 0.55% in association to rejection events, and partially decreased after the two additional steroid treatment administered between July and October 2022 and between January and February 2023. In contrast, Figure 16C shows dd-cfDNA monitoring in 3 patients (ID 1, 14, 24) with no clinical signs of rejection and heart injury: their dd-cfDNA values were below the 0.55% cut-off in all measurements collected during follow-up time, reflecting the stable condition of the graft.

"Dd-cfDNA reflects ischemia-reperfusion injury and post-transplant complications in liver transplant recipients"

Sorbini et al., manuscript in preparation

Ischemia-reperfusion damage monitoring in liver transplant recipients

Fifty-one sequential liver transplant recipients were included in the study. Patients were transplanted at Città della Salute e della Scienza, Turin, Italy, and were enrolled after a signed informed consent. Patients were mainly males (n=42, 82.4%), with an average age at transplant of 58.0±8.8 years. In only one case a combined liver-kidney transplant was performed. Recipients and their donors' characteristics are listed in Table 8 and Table 9.

Clinical management

Pre-transplant liver perfusion was performed as follows. The dual-hypothermic oxygenated machine perfusion (D-HOPE) protocol was applied as described elsewhere[229,230]. Briefly, livers underwent an initial period of static cold storage using Celsior solution (IGL, Lissieux, France). The use of D-HOPE was systematic in grafts from DCD donors and was considered on a case-by-case basis in grafts from extended criteria DBD donors, considering donor and recipient characteristics, expected preservation time, and donor-recipient matching. Livers in the end-ischemic D-HOPE group were prepared on the backtable upon arrival at our transplant center and underwent a minimum of 90 min D-HOPE during recipient hepatectomy. D-HOPE was performed using the LiverAssist device (XVIVO, Goteborg, Sweden) primed with 3 L of Belzer MP solution (BridgeToLife, Northbrook, IL, USA) setting the pressure at 3–5 mmHg in the portal vein and at 25 mmHg in the hepatic artery. D-HOPE was not used for evaluation purposes, and all accepted grafts were eventually transplanted. Livers were flushed with chilled 5% albumin before implantation into the recipient.

The indication for normothermic machine perfusion (NMP) was shared between the procuring and transplanting surgeons and was based on donor characteristics, the macroscopic aspect of the liver, and histological findings. Only grafts with histologically proven macrovesicular steatosis ≥ 30% were included in this series. Graft histology was determined on liver biopsies obtained at procurement or before NMP, which were centrally reviewed by a single pathologist to homogenize MaS assessment. Perfusion was performed using the OrganOx Metra (OrganOx, Oxford, UK) device. Reconstruction of aberrant hepatic arteries, if any, was performed during backtable preparation, before connecting the liver to the NMP device. A total of 20–30 mEq of sodium bicarbonate was added to the perfusate to equilibrate pH during the priming phase. Perfusate composition and machine perfusion protocols for each device have been described elsewhere[231,232].

Post-transplant management was similar in both groups. Initial immunosuppression schedule included basiliximab as an induction, tacrolimus, steroids, and mycophenolate mofetil. Steroids were tapered and discontinued 3 months after the transplant. The introduction of everolimus was considered at 1-month follow-up in all patients without significant proteinuria or dyslipidemia. Transaminases, bilirubin and platelets levels were checked routinely according to the defined schedule to evaluate the outcomes of the transplant. Further imaging exams were obtained if clinically indicated. Post-reperfusion syndrome (PRS) was defined according to Aggarwal et al.[233] and Hilmi et al.[234]. PRS was defined as severe when associated with severe hemodynamic instability, persistent hypotension (more than 30% of the anhepatic level), asystole, or hemodynamically significant arrhythmias. Early allograft dysfunction (EAD) was defined according to the original publication[235].

A total of 522 blood samples (10.2 samples/patient) were collected at specific time points to evaluate the transplantation outcome both in the short and long term. For this purpose, samples were collected before and after organ reperfusion during surgery, daily between post-operative day

(POD) 1 and POD7, then at POD14, POD21, POD28, POD90, and POD180. Total cfDNA distribution shows a sharp peak corresponding to the post-surgery samples, followed by stabilization to baseline levels starting from POD4 (Figure 17A). On the contrary, dd-cfDNA percentages reached almost the totality of cfDNA in the immediate post-transplant time (mean POST_TX 86.72%) and slowly decreased during the first seven days without going lower than 21.35%, indicating an improvement of the surgical damage but still highlighting a situation of strong cellular stress (Figure 17B). Later, the percentage decreased to very low levels, setting around 6% at POD90 and POD180. Similarly, the dd-cfDNA copies/µl resulted elevated after the surgery to POD4 with great variability, then stabilized (Figure 17C).

Demographical distribution of dd-cfDNA

We evaluated the impact of demographical features on the dd-cfDNA release. First, no differences were observed between male and female recipients, even though we should consider the small number of women present in our cohort (Figure 18A). Moreover, we noticed that the mean ratio between the weight of the graft and that of the recipient was 2.0±0.4, reflecting an optimal allocation of the organs based on the recipient physical characteristics (Table 8). Of consequence, there were no differences in the dd-cfDNA release based on this ratio (Figure 18B). The same result was obtained considering the age of the donor; 60 yo was set as a threshold and there were no differences between the mean values of the two groups (Figure 18C). Lastly, we considered if the pre-transplant serum cross-matches of donor and recipient could influence the biomarker (Figure 18D). Again, no significant results were obtained, maybe due to the reduced number of positive cross-matches occurred.

Association with indicators of hepatocytolysis

Hepatocytolysis markers were evaluated daily in the first week after transplant, weekly up to POD28 and then at POD90 and POD180. Aspartate aminotranferase (AST) and alanine aminotransferase

(ALT) were strongly associated with the dd-cfDNA release between POD1 to POD7 ($p<0.0001$), while bilirubin and platelets were not found significantly correlated to the dd-cfDNA (Figure 19). On the other hand, considering hepatic markers from POD14 to POD180, we obtained a strong significant association between dd-cfDNA values and AST ($p=0.0021$), ALT ($p<0.0001$), gamma-glutamyltransferase (GGT, $p<0.0001$), alkaline phosphatase (ALP, $p=0.0043$) and bilirubin ($p=0.0174$), while platelets still did not reach a significant correlation ($p=0.82$, Figure 20).

Role of machine perfusion

Half of our cohort received a perfused graft based on the organ conditions. Machine perfusion, particularly hypothermic machine perfusion, has been found to improve liver transplant outcomes compared to conventional static cold storage, with reduced early allograft dysfunction, ischaemic cholangiopathy, non-anastomotic strictures, and graft loss, as well as shorter hospital stays and potential cost savings[236,237]. Our data were divided into perfused (n=26) and not-perfused organs (n=25) since only 3 livers were perfused by NMP, while the majority of grafts underwent D-HOPE (n=22). As expected, the dd-cfDNA percentages were the same in the two groups, suggesting the actual improvement in the hepatic function achieved through machine perfusion (Figure 21A), even though we do not have information about dd-cfDNA levels released from sub-optimal grafts that did not received perfusion, due to ethical reasons. In addition, we did not observe a significant difference in hepatocytolysis markers between these two groups (AST POD1_10: $p=0.60$; ALT POD1_10: $p=0.76$), enforcing our hypothesis (Figure 21B).

Dd-cfDNA recapitulated ischemia-reperfusion damage and early complications

Ischemia-reperfusion damage was evaluated by histopathological investigation performed during the surgery on the basis of steatosis and necrosis percentages. Patients were classified as mild (n=32), moderate (n=13), or severe injury (n=4), but for statistical reasons, we combined the latter two categories into a moderate/severe group. As we see from Figure 22A, the higher injury grade

caused a stronger dd-cfDNA release compared to the mild damage, resulting in a significant difference from POD1 to POD5, except from POD3 (p=0.07). We also obtained a significant correlation between the dd-cfDNA in the first 5 days after transplant and the percentage of necrosis (p=0.035, Figure 22B).

Patients were divided into EAD (n=16) and non-EAD (n=35) groups based on their hepatic function. We observed a slightly significant increase of dd-cfDNA at POD1 and POD2 in the EAD group, which then comply with the non-EAD group, in line with the clinical outcome of these patients (Figure 22C).

We also considered if severe PRS, which was present in 4 patients, can impact the dd-cfDNA. As expected, PRS patients had more increased dd-cfDNA% in the first days up to POD4, even though only POD2 reached weak significance (p=0.049)(Figure 22D).

We then divided patients into cases (n=24) or controls (n=27). Controls were defined as patients free from EAD and PRS with a mild ischemia injury at post-reperfusion biopsy. Consistently with our results, patients considered as cases showed dd-cfDNA levels significantly higher from POD1 to POD5 compared to controls, while from POD6 the two groups overlapped (Figure 22E).

Association of dd-cfDNA with the short and long-term outcome

Samples collected between POD14 and POD28 (n=55) were considered informative of the transplant short-term outcome. We observed that during this period, patients who experienced post-transplant complications related to the graft had higher dd-cfDNA percentages compared to patients that were considered stables (p<0.0001)(Figure 23A). The same result was obtained in the long-term period between POD90 and POD180 (n=52), with a stronger dd-cfDNA increment related to cases compared to controls (p=0.0002)(Figure 23B). We calculated ROC curves specific for short and long-term outcome, which resulted respectively AUC=0.91 (p<0.0001) and AUC=0.93

(p=0.0018). Keeping in mind the idea of maximizing the performance of our test, we selected 6.35% and 8.28% as positive dd-cfDNA thresholds for liver damage, respectively.

For-cause biopsies

Thirteen hepatic biopsies were performed on 12 patients for suspicion of acute rejection (n=7), cholangitis (n=5), and cholestasis (n=1). All of them resulted positive for hepatic damage. The dd-cfDNA corresponding to these events was analyzed and showed high levels compared to the median value of the cohort in the short and long-term outcome (4.67%) since negative biopsies were not available (Figure 24). Despite the reduced number of samples, this result confirms the role of dd-cfDNA as a marker of rejection and acute damage.

Developing and optimization of an improved and "home-made" version of ddPCR kit

During the analysis of our cohort of solid organ transplant recipients, we realized that there was a limited but relevant number of patients for whom dd-cfDNA levels could not be assessed since their *HLA-DRB1* alleles were not included in the commercial probe panel from Bio-Rad Laboratories, Inc. we used to perform analysis. In our experience, we can not offer our test to the 6.63% of patients for this reason. Indeed, this probe panel was designed to cover approximately 90% of the Caucasian population, as it does not include rarer but relevant alleles such as *HLA-DRB1*09, HLA-DRB1*10, HLA-DRB1*12,* and *HLA-DRB1*14*. To expand the coverage of the population and the potential of this ddPCR test, we designed a new complete panel of probes including all *HLA-DRB1* and *HLA-DQB1* alleles, together with the tech support of the Integrated DNA Technologies (IDT). The panel includes 16 probes, 12 for *HLA-DRB1* and 4 for *HLA-DQB1* gene (Table 10), labeled with FAM or HEX fluorophore, and a list of primers for the amplification of the region of interest. Primers and probes have been designed to target only a specific SNP, identified by the alignment of all genomic sequences. A patent registered on April 2022 (P022840IT-01 April 2022) protects oligonucleotide sequences and their application. The technical development of the panel was initially funded by the POC Instrument grant from Fondazione Links, LIFTT, and Compagnia di San Paolo.

Protocol optimization

Primers and probes were validated using genomic and circulating cfDNA samples, testing different concentrations and melting temperatures to obtain the best performances. We started by the selection of the best-performing annealing temperature by performing gradients from 65° to 57°C, that consented to obtain the most clear signal at 58°C. Moreover, we added an additional annealing step at 67°C to let probes interact specifically with their DNA target before reducing the temperature at 58°C allowing primers annealing. This step resulted necessary to improve ddPCR

yield. Primers and probe concentration was then adjusted from the recommended 900nM/250nM ratio to a final 500nM/250nM to enhance the positive signal (Figure 25).

Specificity and sensitivity assessment

To assess the specificity of our assay, all probes were tested on DNA samples with known DRB1 and DQB1 typing, and our results show that each probe only recognizes the template containing its specific sequence and not any other template (Figure 26). As a caveat, HLA-DQB1*06 is not included in the actual panel of probes because all the designs that we tested failed correct amplification and specificity tests.

Sensitivity was assessed by performing a two-fold serial dilution of gDNA samples, starting from 1 ng, and quantifying the number of DNA molecules by ddPCR. We were able to quantify the absolute copy number of the target even at very low concentrations (Figure 27).

Future perspectives

The further step for assay optimization is the multiplexing reaction performed on cfDNA samples stored in the TESEO Biobank of the Department of Medical Sciences of the University of Turin. This phase is still on going and aims to improve the performances of each assay by enhancing the positive signal and cleaning the background noise. Reaction conditions and oligonucleotide sequencing will be implemented considering the possible interaction between all the available 2-probe combinations within the multiplexed amplification reaction. Moreover, from April 2023 our license was shared with GenDX (Utrecht, The Netherlands), that is a company which develops and markets innovative diagnostic tests, software, services and educational products in close collaboration with business and academic partners. Part of the optimization and developmental process will be performed in collaboration with the company team, with the final aim to realize and distribute a validated kit for dd-cfDNA quantification.

Discussion

Solid organ transplantation is a life-saving medical procedure for patients with severe chronic diseases. Despite significant advancements in transplant efficacy, substantial challenges associated with post-transplant management remain, such as monitoring organ function and rejection. In this context, circulating cfDNA has emerged as a promising biomarker for assessing the status of transplanted organs. The present research aimed to comprehensively explore the role of cfDNA in the context of solid organ transplantation, focusing on the identification and quantification of dd-cfDNA and evaluating its clinical relevance in different types of organ transplant.

The first aim of the study was to develop an easy and feasible method to quantify dd-cfDNA based on ddPCR and exploiting the genetic polymorphism in the *HLA-DRB1* gene. Our decision to focus on ddPCR instead of NGS, which is the most validate approach available with a deep sensitivity, relied on the great feasibility of this PCR-based technique compared to sequencing. Indeed, the contained costs, high scalability (from a minimum of 1 sample to entire 96-well plates), easy implementation and rapid protocol together with great sensitivity and accuracy result in an efficient method affordable by the Public Health System. Moreover, by selecting the polymorphisms of the *DRB1* gene within the HLA locus, which is always typed before transplantation, we avoided the genotyping step required by other PCR-based methodologies, thus saving time and costs. Conversely, the decision to base our analysis on this genetic target forced us to exclude *HLA-DRB1*-matched transplants, which represent a small fraction of the total number of transplant recipients, at least considering heart, lung and liver transplantation. However, the frequency of rejection events in this group of patients is reported to be minimal[177,178], as the higher immunological compatibility plays a key role in preserving the graft tissue. Moreover, this limitation could be overcome by searching for other informative targets.

We first recruited a pilot cohort of adult heart recipients, as more than 10% of them suffers from acute rejection within the first year after transplant[200]. Since the gold standard method to diagnose acute rejection in the heart is EMB, a poor sensitive technique with a high risk of complications[202,203], a non-invasive alternative is strongly required to improve the quality of life of these patients, particularly in the case of children. For these reasons, we decided to optimize a ddPCR-based molecular method to easily diagnose acute rejection in heart recipients by quantifying dd-cfDNA obtained from liquid biopsies collected concomitantly to EMBs.

Results from the analysis of this pilot cohort indicate that dd-cfDNA levels are influenced by ischemia-reperfusion damage occurring in the first few days after transplantation, and by acute rejection, as detected by EMB. Our findings are in line with results previously obtained in two larger cohorts of heart transplant recipients[33,183], reporting significantly higher dd-cfDNA percentages in patients with biopsy-proven rejection compared to patients in stable conditions. Importantly, as in our cohort, mild infections or inflammation did not interfere with dd-cfDNA plasma levels, arguing in favor of assay specificity. Overall, despite the promising results of dd-cfDNA analysis, its efficiency should be improved to achieve a more robust clinical test. Increasing the number of samples analyzed and implementing the assay characteristics can help overcome the relatively high rate of false negative results in this analysis.

Similar results were obtained by analyzing dd-cfDNA in lung recipients. The mean donor DNA percentages showed a clear correlation with the amount of donor tissue transplanted; bilateral transplant samples showed values approximately double those of single-lung transplants. Moreover, samples from patients who received more than one organ presented a significantly higher amount of dd-cfDNA, reflecting the higher number of donor cells inside the recipient. All samples collected in the first 2 weeks after transplantation demonstrated high levels of dd-cfDNA, consistent with ischemia-reperfusion injury and in line with previous data reported in the

literature[54,238,239]. Donor DNA kinetics exhibited low percentages in samples collected from patients in stable conditions, whereas values increased significantly in relation to ACR episodes, with moderate A2 rejection associated with a stronger release than A1 rejection. In addition, samples from patients with clinical signs of possible CLAD showed the highest dd-cfDNA levels. In particular, one of the CLAD patients developed early A2 rejection after 1-month post-transplant, and then suffered from relapsing pneumonia and chronic rejection treated with immunosuppressive boluses and photopheresis. Finally, the patient developed severe pulmonary insufficiency as a consequence of graft failure and died on post-transplant day 343. His dd-cfDNA levels increased early and did not decrease even after immunosuppressive treatment, consistent with severe rejection. Regarding the other two cases of CLAD, no specimens were collected after the additional immunosuppressive treatment; therefore no information could be obtained about their dd-cfDNA variations. Morphological and immunohistochemical evaluations did not report evidence of AMR in any TBB collected during the follow-up period, even though seven recipients developed DSA. Although we observed a significant difference between DSA positive samples and stable conditions, the reduced number of samples do not allow any speculation about the value of dd-cfDNA as a biomarker of AMR. Remarkably, dd-cfDNA also increased in the presence of infection, in keeping with the notion that it is a marker of graft damage, independent of the cause[240]. Therefore, for optimal clinical use, dd-cfDNA quantification should be performed together with a set of biomarkers of infection and radiological examination of the lung. The finding of increased dd-cfDNA in the absence of any sign of infection should prompt biopsy evaluation of the transplanted lung. Thus, dd-cfDNA could reduce the number of biopsies in a population of patients with a high suspicion of rejection.

Then, we enrolled a pediatric cohort of heart transplant recipients with the main aim to understand if the introduction of dd-cfDNA dosage in the routine post-transplant management of these patients could improve their quality of life and preserve graft tissue by avoiding surveillance EMBs in the

absence of signs of rejection. We monitored dd-cfDNA using our test in pediatric patients undergoing follow-up at our Institution, for 12 months. Patients were included in the study regardless of the time of transplantation, enabling us to investigate dd-cfDNA levels also in the chronic setting. Results were then correlated to all other clinical and instrumental parameters usually considered in the follow-up.

The first result is that dd-cfDNA can be measured in every patient, including infants. The second result is that dd-cfDNA levels are strongly correlated to every pathological, clinical or laboratory parameter of rejection and heart damage, confirming its potential as a biomarker in this patient population. Based on these findings, we determined a threshold with a strong negative predictive value for rejection. We based our analysis on the correlation with EMB grade and our test best performed when the cut-off was set at 0.55% of dd-cfDNA, with a 100% sensitivity and 76.5% specificity. None of the 11 EMBs positive for rejection showed dd-cfDNA levels below the 0.55% cut-off, while in the EMB negative counterpart only 4/18 samples were above the cut-off. If we had applied this threshold to our patient population over the study period, 7 patients had dd-cfDNA levels consistently below the cut-off value. Among them, 5 underwent surveillance EMB as part of their routine follow-up, and all were negative for rejection. Based on our data, these 5 patients could have postponed their EMB. However, further studies are needed to understand if in the future dd-cfDNA quantification can help clinicians in their decision to perform or post-pone EMBs in stable patients. Moreover, as stated for adult heart recipients, the performance of the test should be improved by additional investigation to avoid false results related to bad sampling or to the low sensitivity that affects EMB.

Beside encouraging results, our study shows several limitations. First, the reduced number of patients and samples considered in the analysis, reflecting the experience of 12-month rejection monitoring program at our Institution. Secondly, the limited number of EMBs collected and

considered for positive cut-off determination, which is directly related to the small size of the recruited cohort and also to the fact that the time from transplant was >1 year, when acute rejection is reported to be less frequent[241]. Similar to this, the third limitation is the number of ACR and AMR events that occurred in our cohort, preventing the possibility to deeper investigate the dd-cfDNA differences between the two types of rejection.

The fourth and last examined cohort was enrolled with the aims to evaluate the dd-cfDNA trend after ischemia-reperfusion damage in liver transplantation and to investigate if this biomarker could be informative of the early hepatic injury and dysfunction. In contrast to heart and lung transplant, clinicians from Liver Transplant Unit were mostly concerned about EAD and short-term outcome rather than acute rejection, which indeed was present in a small fraction of patients.

The first result was the dd-cfDNA trend during the first 7 days after transplant. Similar to the other examined graft types, the biomarker was increased remarkably as a consequence of post-transplant ischemia-reperfusion damage, and significantly associated to the grade of injury. In addition, a relevant observation was the percentage of cfDNA released from the liver, which was considerably much higher compared to heart and lung, reflecting the actual size and cell turnover of this organ, in line with expectations. Moreover, the absence of differences due to demographical influence claims for the universal application of dd-cfDNA quantification.

The second result is the significant association between dd-cfDNA levels and signs of hepatic dysfunction and damage. Indeed, it correctly reflected the graft injury as a result of EAD, PRS, rejection and other clinical conditions that can affect liver recipient during post-transplant time, also confirmed by the correlation with other hematological markers of hepatic damage represented by transaminases and bilirubin. The lack of a significant association with platelets should be deeper investigated, as it could be caused by imbalance and blood transfusions following surgery and post-transplant complications.

A third important observation is the overlapping dd-cfDNA profile in perfused and non-perfused organs. Since machine perfusion was introduces to improve the hepatic function of extended criteria donors (ECD)[242], our findings is confirming the effectiveness of this technique, in line with publications reporting increased performances of perfused ECD organs compared to non-perfused[236].

Regarding the potential meaning of dd-cfDNA measurement in both short and long-term outcome of the transplant, our data are strongly limited by the small number of events occurred during the follow-up period in our cohort. The determination of a cut-off value able to distinguish stable patients from patients affected by graft-related complications, despite the significant statistics we obtained, should be validated with a larger sample size in order to define a robust and reliable threshold.

The challenging decision to design a home-made assay for dd-cfDNA quantification was driven by the need to expand the population of patients to whom offer this non-invasive test for rejection. Indeed, we observed that a little but relevant percentage of recipients was excluded from analysis because of the lack of a specific probe for their HLA typing. Moreover, since it would not be optimal to simply add the missing assays to an already validated commercial kit, we preferred to re-design the entire panel of probes starting from the alignment of the genomic sequences of *HLA-DRB1* and *HLA-DQB1* genes. We decided to focus on these two targets in line with the previous results obtained using the commercial kit from Bio-Rad Laboratories, Inc., but we added *DQB1* alleles since this locus is polymorphic enough to consent to cover the totality of the population with just few probes. Although we are aware of the linkage disequilibrium between DRB1 and DQB1 loci, we believe that the possibility of a wider set of available assays will consent to potentially cover the entire transplanted population.

After patent application, we tried to further develop our assay with supporting grants and collaborations with companies involved in the transplantation field. We licensed GenDX (Utrecht, The Netherlands), which is part of the Eurobio Scientific Group and is a leader in the field of high-resolution HLA typing and related molecular diagnostic testing. Working together, we believe that we will be able to develop an advanced version of our molecular tests to be distributed as an IVD product to clinicians within the transplantation centers to finally improve the quality of life of recipient patients.

Overall, our data demonstrated the feasibility of dd-cfDNA determination with an easy and cheap molecular method based on ddPCR that consented to obtain solid results from different graft types, correctly identifying tissue damage, acute rejection and graft dysfunction. The improvement that will be obtained from the collaboration with a molecular biology company should enforce the performances of our technical approach, helping us to achieve stronger and more precise results for a better post-transplant management to be offered to solid organ recipients.

In conclusion, cfDNA has emerged as a promising tool not only in the field of transplantation but also in oncology and prenatal testing. Its potential to provide valuable information regarding tumor burden and potential therapeutic targets, inherited disease, graft function, rejection, and other complications is revolutionizing the way we monitor and manage the patients. By analyzing specific genetic markers present in the circulating DNA, clinicians can obtain real-time insights into the status of the affected individual, allowing for timely intervention and improved patient outcomes. Furthermore, the non-invasive nature of cfDNA testing reduces the need for invasive investigation, minimizing patient discomfort and risk. With further research and technological advancements, cfDNA analysis will become an indispensable tool in advanced personalized medicine.

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Tables

Table 1. HLA Expert Design panel.

Probe ID	Target allele	Amplicon length
dHsaEXD29156242	*HLA-DRB1*01*	66
dHsaEXD93426015	*HLA-DRB1*03*	70
dHsaEXD67695788	*HLA-DRB1*04*	61
dHsaEXD41965561	*HLA-DRB1*07*	66
dHsaEXD16235334	*HLA-DRB1*08*	70
dHsaEXD80505107	*HLA-DRB1*11*	98
dHsaEXD54774880	*HLA-DRB1*13*	61
dHsaEXD29044653	*HLA-DRB1*15/16*	67

List of the *HLA-DRB1* probes used in droplet digital PCR assays. Target allele and amplicon length are indicated for each probe.

Table 2. Adult heart transplant cohort.

ID	Age	Sex	*HLA DRB1*	Disease	Comorbidities	LVAD	Ischemic time (minutes)	ECMO	Total hospital stay (days)	Immunosuppressive therapy	Post transplant infections	Cause of death	Transplant survival (days)	DSA
R #1	70	M	*HLA-DRB1*07*	valvular CMP	hypogonadism hyperthyroidism	no	174	no	30	cyclosporine, everolimus, corticosteroids	*CMV*	/	425	NA
D #1	47	F	*HLA-DRB1*04; HLA-DRB1*07*											
R #2	38	M	*HLA-DRB1*04; HLA-DRB1*12*	idiopathic DCM	ex-smoker	no	250	no	63	NA	*P. aeruginosa, MRSA*	intracerebral bleeding	51	NA
D #2	56	F	*HLA-DRB1*11; HLA-DRB1*14*											
R #3	66	M	*HLA-DRB1*07; HLA-DRB1*08*	ischemic DCM	dyslipidemia, ex-smoker, COPD, hyperthyroidism, steroid diabetes	yes	275	no	18	cyclosporine, mycophenolate, corticosteroids	/	/	371	not present
D #3	48	M	*HLA-DRB1*03; HLA-DRB1*15*											
R #4	63	M	*HLA-DRB1*03; HLA-DRB1*04*	idiopathic DCM	hypertension, CRD, ex-smoker	no	128	no	23	cyclosporine, mycophenolate, corticosteroids	*K. Oxytoca, S.marcescens*	/	356	not present
D #4	41	M	*HLA-DRB1*03; HLA-DRB1*13*											
R #5	54	M	*HLA-DRB1*07; HLA-DRB1*14*	idiopathic DCM	/	no	205	no	18	cyclosporine, mycophenolate, corticosteroids	*CMV*	/	342	not present
D #5	36	M	*HLA-DRB1*11; HLA-DRB1*12*											
R #6	44	M	*HLA-DRB1*01; HLA-DRB1*15*	idiopathic DCM	ex-smoker	no	220	no	38	tacrolimus, mycophenolate, corticosteroids	/	/	341	not present
D #6	26	M	*HLA-DRB1*04; HLA-DRB1*12*											
R #7	56	F	*HLA-DRB1*03; HLA-DRB1*07*	ischemic DCM	dyslipidemia	yes	209	no	21	cyclosporine, everolimus, corticosteroids	*CMV*	/	335	*HLA B51*
D #7	49	F	*HLA-DRB1*03; HLA-DRB1*11*											
R #8	51	M	*HLA-DRB1*03; HLA-DRB1*11*	ischemic DCM	dyslipidemia, hypertension, overweight	yes	216	no	33	cyclosporine, mycophenolate, corticosteroids	*K. Pneumoniae, CMV*	/	298	NA

D #8	29	M	*HLA-DRB1*01*										
R #9	66	M	*HLA-DRB1*03; HLA-DRB1*15*	idiopathic DCM	iatrogenic thyrotoxicosis	no	206	no	45	mycophenolate, corticosteroids	*CMV; SARS-CoV-2*	Cardiac arrest in infectious pneumonia	156	not present
D #9	49	F	*HLA-DRB1*11; HLA-DRB1*13*											
R #10	62	M	*HLA-DRB1*03; HLA-DRB1*16*	ischemic DCM	ex-smoker, dyslipidemia, hypertension, diabetes mellitus, OSAS, DVT	no	169	no	26	cyclosporine, mycophenolate, corticosteroids	*SARS-CoV-2, CMV*	/	224	NA
D #10	46	M	*HLA-DRB1*07; HLA-DRB1*11*											
R #11	62	M	*HLA-DRB1*08; HLA-DRB1*16*	cardiac amyloidosis	/	no	132	no	15	cyclosporine, mycophenolate, corticosteroids	*SARS-CoV-2, CMV*	Sepsis	162	not present
D #11	56	M	*HLA-DRB1*11*											
R #12	36	M	*HLA-DRB1*03*	idiopathic DCM	/	no	205	no	20	cyclosporine, mycophenolate, corticosteroids	/	/	77	not present
D #12	20	M	*HLA-DRB1*03; HLA-DRB1*07*											
R #13	18	M	*HLA-DRB1*04; HLA-DRB1*16*	idiopathic DCM	/	no	202	no	16	cyclosporine, mycophenolate, corticosteroids	*S.epidermidis; SARS-CoV-2*	/	196	not present
D #13	18	M	*HLA-DRB1*07; HLA-DRB1*11*											
R #14	57	M	*HLA-DRB1*03; HLA-DRB1*11*	ischemic DCM	ex-smoker, dyslipidemia, hyperthension, DVT	yes	678	yes	42	cyclosporine, mycophenolate, corticosteroids	*Clostridium difficile; S.haemoliticus; K.Pneumoniae; C.albicans*	/	178	not present
D #14	30	M	*HLA-DRB1*11; HLA-DRB1*15*											
R #15	53	M	*HLA-DRB1*11*	hypertrophic CMP	cerebral ischemia	no	204	no	17	cyclosporine, mycophenolate, corticosteroids	/	/	77	not present
D #15	35	M	*HLA-DRB1*04; HLA-DRB1*08*											
R #16	53	M	*HLA-DRB1*04; HLA-DRB1*11*	idiopathic DCM	ex-smoker	no	104	no	30	cyclosporine, mycophenolate/mycophenolic acid, corticosteroids	respiratory tract infections	/	188	not present
D #16	29	M	*HLA-DRB1*11; HLA-DRB1*15*											

R #17	18	F	*HLA-DRB1*11*	congenital CMP	/	no	NA	yes	NA	NA	*Sars-CoV-2*	/	146	not present
D #17	30	F	*HLA-DRB1*13; HLA-DRB1*14*											
R #18	56	F	*HLA-DRB1*01; HLA-DRB1*07*	idiopathic DCM	ex-smoker	no	166	no	18	cyclosporine, mycophenolate, corticosteroids	*VZV*	/	173	not present
D #18	38	F	*HLA-DRB1*11; HLA-DRB1*15*											
R #19	45	F	*HLA-DRB1*11*	Re-transplant	CRD, CAD, hyperthyroidism atrial tachycardia atrial fibrillation	no	199	no	65	cyclosporine, mycophenolate, corticosteroids	*S.haemolyticus, P.aeruginosa*	/	112	not present
D #19	46	F	*HLA-DRB1*04*											

List of all heart transplant donor-recipient pairs analyzed in this study. ID, age, sex, HLA-DRB1 typing, disease, comorbidities, hospitalization information and post-transplant features are listed. CMP: cardiomyopathy; DCM: dilated cardiomyopathy; COPD: chronic obstructive pulmonary disease; CRD: chronic renal disease; OSAS: obstructive sleep apnea syndrome; DVT: deep vein thrombosis; CAD: coronary artery disease; LVAD: left ventricular assist device; NA: not available; ECMO: extracorporeal membrane oxygenation; CMV: cytomegalovirus; MRSA: Methicillin-resistant Staphylococcus Aureus; VZV: Varicella-Zoster virus; DSA: donor-specific HLA antibodies.

Table 3. Adult heart cohort characteristics.

Variable	N
Analyzed patients	19
N. of samples	232
Samples per patient (mean)	12
Age at transplant	50.9 ± 14.8
Variable	**N (%)**
Sex	
Male	15 (78.9)
Female	4 (21.1)
Pretransplant diagnosis	
Idiopathic DCM	9 (47.4)
Ischemic DCM	5 (26.3)
Hypertrophic CMP	1 (5.3)
Congenital CMP	1 (5.3)
Valvular CMP	1 (5.3)
Cardiac amyloidosis	1 (5.3)
Re-transplant	1 (5.3)
Comorbidities	
Ex-smokers	6 (31.6)
LVAD	4 (21.1)
Dyslipidemia	5 (26.3)
Arterial hypertension	4 (21.1)
Metabolic diseases	4 (21.1)
Other	9 (47.4)
Post-transplant infections	
CMV	7 (36.8)
SARS-CoV-2	5 (26.3)
Other	6 (31.6)
None	4 (21.1)

Abbreviations: DCM, dilated cardiomyopathy; CMP, cardiomyopathy; LVAD, left-ventricular assist device; CMV, cytomegalovirus.

Table 4. Donors and recipients' characteristics of lung cohort.

Variable		Monopulmonary LTx N=4	Bipulmonary LTx N=21	Combined LTx N=5	Total N=30
Donor	**Age (y), mean±SD**	41.1±17.6	42.1±16.2	41.8±16.8	42.5±16.1
	Male sex, n (%)	3 (75.0)	7 (33.3)	3 (60.0)	13 (43.3)
	Cardiac death, n (%)	0 (0)	2 (9.52)	0 (0)	2 (6.70)
	Brain death, n (%)	4 (100)	19 (90.5)	5 (100)	28 (93.3)
	Ischemic time (minutes), mean±SD	352.0±181.1	331.0±157.5	342.4±170.8	332.9±159.4
Recipient	**Age (y), mean±SD**	48.2±17.2	47.0±15.7	46.7±17.1	47.0±15.5
	Male sex, n (%)	4 (100)	7 (33.3)	4 (80.0)	15 (50.0)
	Disease, n (%)				
	IPF	3 (75.0)	8 (38.1)	0 (0)	11 (36.7)
	CF	0 (0)	4 (19.0)	3 (60.0)	7 (23.3)
	COPD	0 (0)	5 (23.8)	1 (20.0)	6 (20.0)
	BOS	1 (25.0)	1 (4.8)	0 (0)	2 (6.7)
	Ciliary dyskinesia	0 (0)	2 (9.5)	0 (0)	2 (6.7)
	PH	0 (0)	1 (4.8)	1 (20.0)	2 (6.7)
	Total hospital stay (d), mean±SD	67.8±59.0	67.8±54.8	68.3±57.8	66.7±54.2
	CEC, n (%)	0 (0)	9 (42.9)	2 (40.0)	11 (36.7)
	ECMO, n (%)	0 (0)	10 (47.6)	2 (40.0)	12 (40.0)
	EVLP, n (%)	0 (0)	4 (19.0)	0 (0)	4 (13.3)
	Hemodynamic support, n (%)	3 (75.0)	19 (90.5)	5 (100)	27 (90.0)
	Dobutamine	0 (0)	11 (52.4)	5 (100)	13 (43.3)
	Noradrenaline	3 (75.0)	15 (71.4)	1 (20.0)	19 (63.3)
	iNO	1 (25.0)	5 (23.8)	2 (40.0)	8 (26.7)
	Pulmonary infections, n (%)	4 (100)	21 (100)	5 (100)	30 (100)
	Bacteria	1 (25.0)	18 (85.7)	5 (100)	24 (80.0)
	Virus	3 (75.0)	18 (85.7)	2 (40.0)	23 (76.7)
	Fungi	1 (25.0)	8 (38.1)	2 (40.0)	11 (36.7)

List of main features of donors and recipients included in the study. The number and percentage of subjects in each group are shown. LTx, lung transplant; SD, standard deviation; IPF, idiopathic pulmonary fibrosis; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; BOS, bronchiolitis obliterans; PH, pulmonary hypertension; CEC, extracorporeal circulation; ECMO, extracorporeal membrane oxygenation; EVLP, ex vivo lung perfusion; iNO, inhaled nitric oxide.

Table 5. Characteristics of the pediatric heart cohort.

ID	Gender	Age (y)	Diagnosis	Transplant date	Enrollment date	Time since transplant (y)	N of EMB	dd-cfDNA determinations
1	F	18.6	CHD	01/10/06	02/22/22	16.1	1	4
2	F	15.3	DCM	07/03/08	04/26/22	13.8	0	6
3	F	17.9	DCM	02/09/10	03/08/22	12.1	1	4
4	F	15.1	CHD	08/27/10	03/29/22	11.6	1	1
5	M	14.1	DCM	12/31/10	10/04/22	11.8	1	1
6	M	16.6	CHD	04/25/11	04/08/22	11.0	1	8
7	F	12.6	HCM	07/02/11	03/21/22	10.7	1	4
8	F	13.9	DCM	03/07/13	04/05/22	9.1	1	6
9	M	11.4	DCM	04/17/13	02/24/22	8.9	1	9
10	F	10.3	DCM	01/09/14	03/01/22	8.1	1	6
11	M	10.2	CHD	09/18/14	03/14/22	7.5	0	3
12	F	8.4	DCM	06/20/15	02/24/22	6.7	1	5
13	F	18.5	CHD	01/08/16	03/09/22	6.2	1	8
14	F	12.7	CHD	07/16/16	05/09/22	5.8	1	5
15	F	7.0	DCM	10/21/16	04/19/22	5.5	1	5
16	F	8.3	DCM	04/26/17	05/23/22	5.1	1	4
17	F	7.2	DCM	08/01/17	03/03/22	4.6	1	3
18	F	12.0	DCM	12/05/17	02/28/22	4.2	1	5
19	M	5.6	DCM	11/04/18	02/23/22	3.3	4	11
20	M	7.0	CHD	06/16/19	05/02/22	2.9	1	5
21	F	3.5	DCM	11/12/20	04/11/22	1.4	1	4
22	F	9.9	CHD	12/22/20	03/15/22	1.2	1	7
23	M	14.6	RCM	07/01/21	02/25/22	0.7	2	13
24	M	16.5	CHD	07/17/21	03/10/22	0.6	1	5
25	F	2.4	DCM	08/25/21	03/14/22	0.6	1	7
26	M	14.7	CHD	03/17/22	03/24/22	0.0	1	11
27	M	0.8	CHD	07/30/22	10/20/22	0.2	0	2
28	F	7.1	DCM	01/08/23	01/20/23	0.0	0	3
29	M	11.3	CHD	01/11/23	02/13/23	0.1	0	3

For each patient, gender, age, diagnosis, date of transplantand enrollment, and number of EMBs and dd-cfDNA samples are listed. Abbreviation: EMB: endomyocardialbiopsy, dd-cfDNA: donor-derived cell-free DNA, DCM: dilated cardiomyopathy, RCM: restrictivecardiomyopathy, HCM: hypertrophic cardiomyopathy, CHD, congenital heart defects.

Table 6. Demographical and clinical features of children recipients.

Patient		**29**
Male gender		**13 (44.8%)**
Age at transplant (mean±SD)		**4.2±4.4**
Age at recruitment (mean±SD)		**11.0±4.8**
Transplant survival (mean±SD)		**6.8±5.0**
Weight (mean±SD)		**33.6±16.2**
Diagnosis	DCM	**15 (51.7%)**
	RCM	**1 (3.4%)**
	HCM	**1 (3.4%)**
	CHD	**12 (41.4%)**
Positive cross-match		**5 (17.2%)**
Immunosuppression	Tacrolimus	**26 (89.7%)**
	Ciclosporin	**3 (10.3%)**
	MMF	**23 (79.3%)**
	Mycophenolic acid	**2 (6.9%)**
	Everolimus	**3 (10.3%)**
Samples		**158**
Mean sample/patient		**5.4**
Mean cfDNA ng/ul		**0.24**

Continuous variables are reported as mean ± standard deviation (SD), and categorical variables are indicated as number and percentage (%).

Abbreviation: DCM: dilated cardiomyopathy, RCM: restrictive cardiomyopathy, HCM: hypertrophic cardiomyopathy, CHD, congenital heart defects, MMF, mycophenolate mofetil.

Table 7. *De novo* donor-specific antibodies (DSA) in 26 sera from 6 pediatric patients.

ID patient	#Serum	Class	Antigen	MFI	AMR
4	**1**	**I**	A3	1811	no
29	**2**	**II**	DQ5	1521	no
		II	DQ8	2537	
		II	DR53	1759	
16	**3**	**II**	DR52	1322	no
9	**4**	**II**	DQ6	4326	no
		II	DQ7	5212	
	5	**II**	DQ6	3250	no
		II	DQ7	5186	
	6	**II**	DQ6	2840	no
		II	DQ7	4463	
	7	**II**	DQ6	2899	no
		II	DQ7	4673	
	8	**II**	DQ6	2458	yes
		II	DQ7	3175	
	9	**II**	DQ6	1792	no
		II	DQ7	1414	
	10	**II**	DQ6	2202	no
		II	DQ7	2612	
	11	**II**	DQ6	2279	no
		II	DQ7	3505	
	12	**II**	DQ6	2076	no
		II	DQ7	3296	
	13	**II**	DQ6	2596	yes
		II	DQ7	2917	
	14	**II**	DQ6	1139	no
23	**15**	**I**	A1	1325	no
		II	DQ8	2650	
	16	**II**	DQ8	3012	no
	17	**II**	DQ8	2988	no
	18	**II**	DQ8	2883	no
12	**19**	**II**	DR4	1660	no
	20	**II**	DR4	1682	no
	21	**II**	DR4	1745	no
	22	**II**	DR4	1363	no
		II	DR53	1316	
	23	**II**	DR4	2672	no
		II	DR53	1332	
	24	**II**	DR4	1971	no
	25	**II**	DR4	1565	no
	26	**II**	DR53	1803	no

Specificity for class I or II and relative mean fluorescent intensity (MFI) is reported for each serum resulted containing DSA. Biopsy-proven antibody-mediated rejection (AMR) and acute cellular rejection (ACR) are indicated.

Table 8. Liver recipients' characteristics.

RECIPIENTS							
		n	%			n	%
Patients		51		**CMV**		42	82.4
Gender	M	42	82.4	**Creatinine (mg/dl) (mean±SD)**	1±0.4		
	F	9	17.6	**Bilirubin (mg/dl) (mean±SD)**	5.3±10.3		
Blood group	A	25	49.0	**INR (mean±SD)**	1.6±0.5		
	B	5	9.8	**MELD (mean±SD)**	15.1±7.2		
	AB	3	5.9	**Pre-tx status**	Home	35	68.6
	0	18	35.3		Hospital	16	31.4
Age (mean±SD)	58±8.8			**Ischemia-reperfusion injury**	Mild	30	58.8
Combined Tx		1	2.0		Moderate	14	27.5
Disease prevalence	HCC	22	43.1		Severe	4	7.8
	HCV	15	29.4		NA	3	5.9
	HBV	6	11.8	**Ast peak (IU/L)(mean±SD)**	1381.1±1174.9		
	DELTA	2	3.9	**Alt peak (IU/L)(mean±SD)**	717.8±448.3		
	ALCI	28	54.9	**High bilirubin**		8	15.7
	NASH	13	25.5	**High INR**		5	9.8
	AUCI	4	7.8	**Creatinine_T2 (mg/dl) (mean±SD)**	1.5±0.7		
	METABOLIC	7	13.7	**Creatinine_T7 (mg/dl) (mean±SD)**	1.7±0.8		
	CHOLESTATIC	2	3.9	**EAD**	Yes	16	31.4
	OTHERS	3	5.9		No	35	68.6
Weight (kg) (mean±SD)	75.9±15			**AKI stage**	0	10	19.6
BMI (mean±SD)	25.7±4.3				1	25	49.0
BSA (m^2) (mean±SD)	1.9±0.2				2	13	25.5
Albumin (g/dl) (mean±SD)	3.3±0.7				3	3	5.9
				Induction therapy		37	72.5
Natriuremia (mmol/L) (mean±SD)	135±10			**Itu (days)**	6.4±11.6		
				Hospitalization (days)	19.9±27.2		

Categorical variables are indicated as numbers and percentages, whereas continuous variables are reported as mean and standard deviation (SD). Abbreviation: Tx, Transplant; HCC, Hepatocellular Carcinoma; HCV, Hepatitis C Virus; HBV, Hepatitis B Virus; DELTA, Hepatitis D Virus; ALCI, Alcoholic Cirrhosis; NASH, Non-Alcoholic Steatohepatitis; AUCI, Autoimmune Cirrhosis; BMI, Body Mass Index; BSA, Body Surface Area; CMV, Cytomegalovirus; INR, International Normalized Ratio; MELD, Mayo End Stage Liver Disease; EAD, Early Allograft Dysfunction; AKI, Acute Kidney Injury; Itu, Intensive Therapy Unit.

Table 9. Liver donors' characteristics.

DONORS							
		n	%			n	%
Patients		51		**BSA (m^2) (mean±SD)**	1.8±0.2		
Gender	M	29	56.9	**Natriuremia (mmol/L) (mean±SD)**	149.4±9.3		
	F	22	43.1	**Creatinine (mg/dl) (mean±SD)**	1.4±1.2		
Blood group	A	26	51	**AST (IU/L)(mean±SD)**	86.7±118.0		
	B	5	9.8	**ALT (IU/L)(mean±SD)**	62.7±84.9		
	AB	1	1.96	**GGT (IU/L)(mean±SD)**	65.5±83.8		
	0	19	37.3	**HBV**		6	11.8
Age (mean±SD)	62.7±13.4			**HCV**		6	11.8
Donor type	DBD	47	92.2	**CMV**		35	68.6
	DCD	4	7.84	**Ex-situ perfusion type**	No machine	25	49.0
Weight (mean±SD)	75.1±15.5				D-HOPE	22	43.1
BMI (mean±SD)	26.3±5.3				NMP_Organox	3	5.9
Graft weight (kg)(mean±SD)	1517.9±326.2			**Perfusion time**	98.5±161.6		
GRBRW (mean±SD)	2.0±0.4			**Graft type**	whole	51	100.0
Itu (days) (mean±SD)	5.3±6.2			**End lactate (mmol/L)**	2.8±3.0		
Diabetes		6	11.8	**Severe post-reperfusion syndrome**		4	7.8
Arrest		11	21.6				

Categorical variables are indicated as numbers and percentages, whereas continuous variables are reported as mean and standard deviation (SD). Abbreviation: BMI, Body Mass Index; GRBRW, Graft Versus Recipient Body Weight Ratio; Itu, Intensive Therapy Unit; BSA Body Surface Area; AST, Aspartase Aminotransferase; ALT, Alanine Aminotransferase; GGT, Gamma-Glutamyl Transferase; HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; CMV, Cytomegalovirus.

Table 10. List of patented assays for ddPCR analysis of dd-cfDNA samples.

	#	allele
HLA-DRB1 gene	1	01
	2	03
	3	04
	4	07
	5	08
	6	09
	7	10
	8	11
	9	12
	10	13
	11	14
	12	15/16
HLA-DQB1 gene	13	02
	14	03
	15	04
	16	05

List of ddPCR assays available and patented on April 2022 (P022840IT-01). Target alleles for *HLA-DRB1* and *HLA-DQB1* genes are indicated as the first field of HLA typing.

Figures

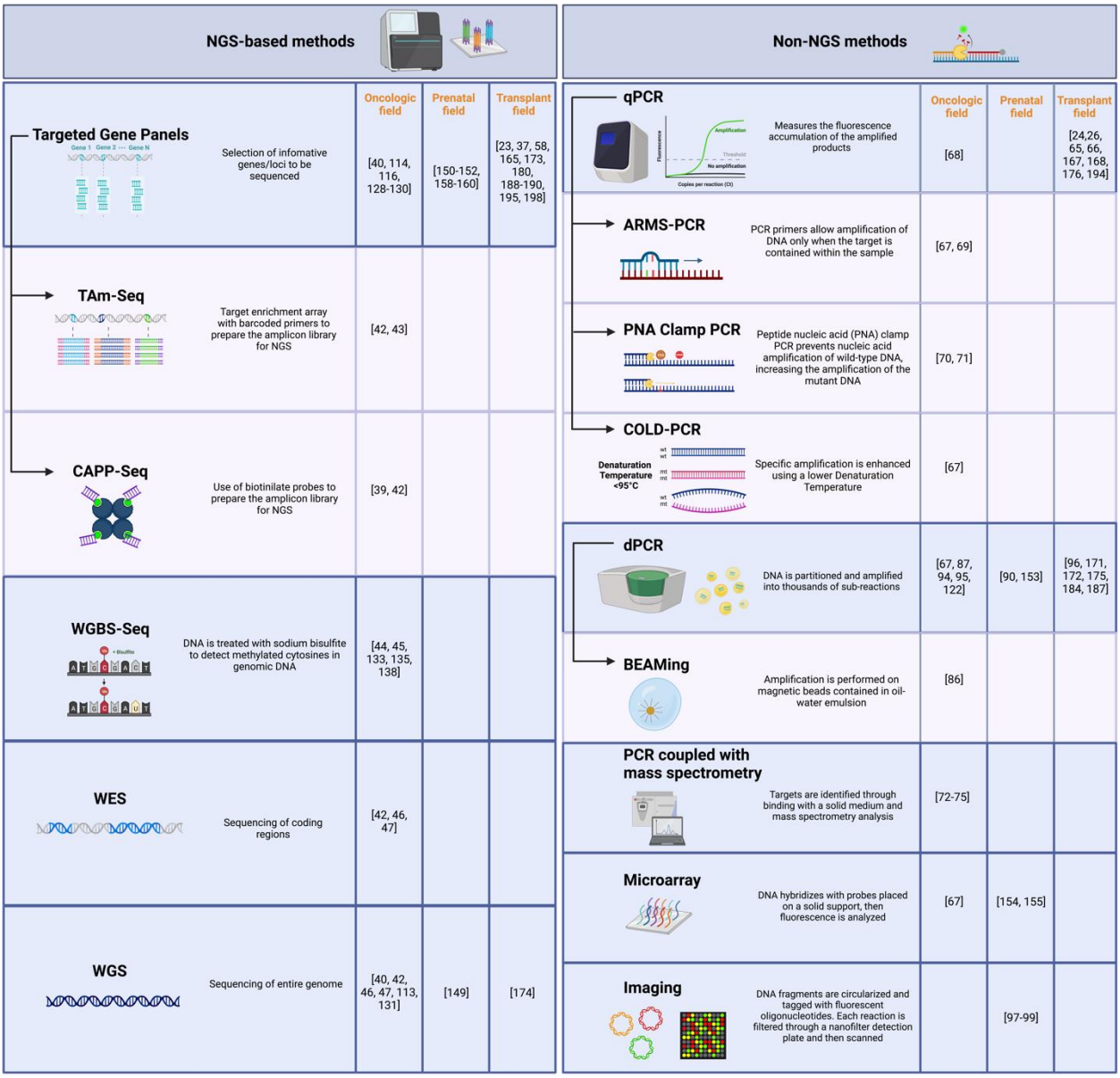

Figure 1. List of the NGS-based and non-NGS methods for cfDNA analysis. The different methodologies are divided according to their technological approaches. The main methods are highlighted in blue, while derived methods are indicated by arrows. References are listed by application field. NGS: Next-generation Sequencing; TAm-Seq: Tagged-amplicon Deep Sequencing; CAPP-Seq: Cancer Personalized Profiling by Deep Sequencing; WGBS-Seq: Whole Genome Bisulfite Sequencing; WES: Whole Exome Sequencing; WGS: Whole Genome Sequencing; qPCR: quantitative PCR; ARMS-PCR: Amplification Refractory Mutation System PCR; PNA Clamp PCR: Peptide Nucleic Acid Clamp PCR; COLD-PCR: Co-amplification at Lower Denaturation Temperature-based PCR; dPCR: digital PCR; BEAMing: Beads, Emulsion, Amplification, Magnetics PCR.

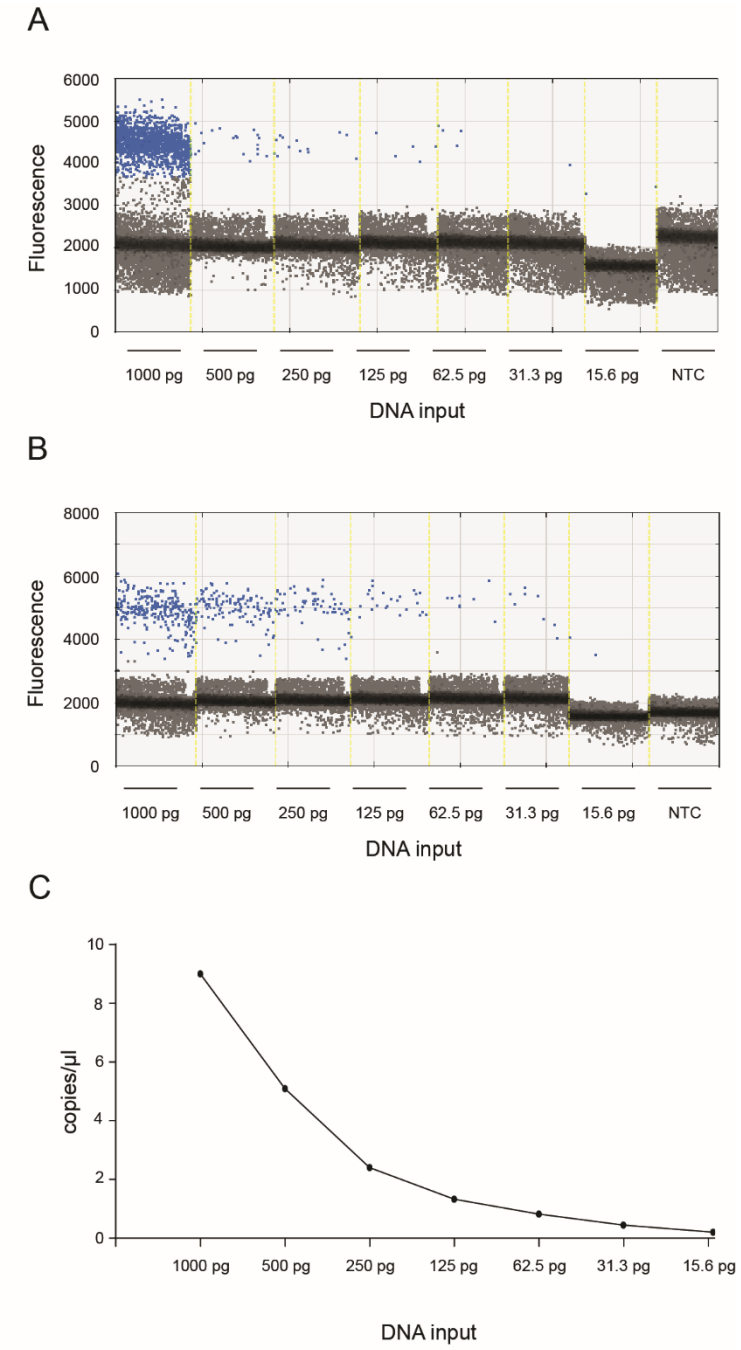

Figure 2. Set up of PCR amplification conditions. Genomic DNA was loaded from a starting concentration of 1000 pg and then was serially diluted to a final concentration of 15.6 pg. Probe fluorescence using the annealing temperature of 57 °C (A) and 55 °C (B) is compared. Blue dots indicate positive droplets and gray dots indicate the negative background. Target copies/ul at 55 °C are reported for each dilution point (C).

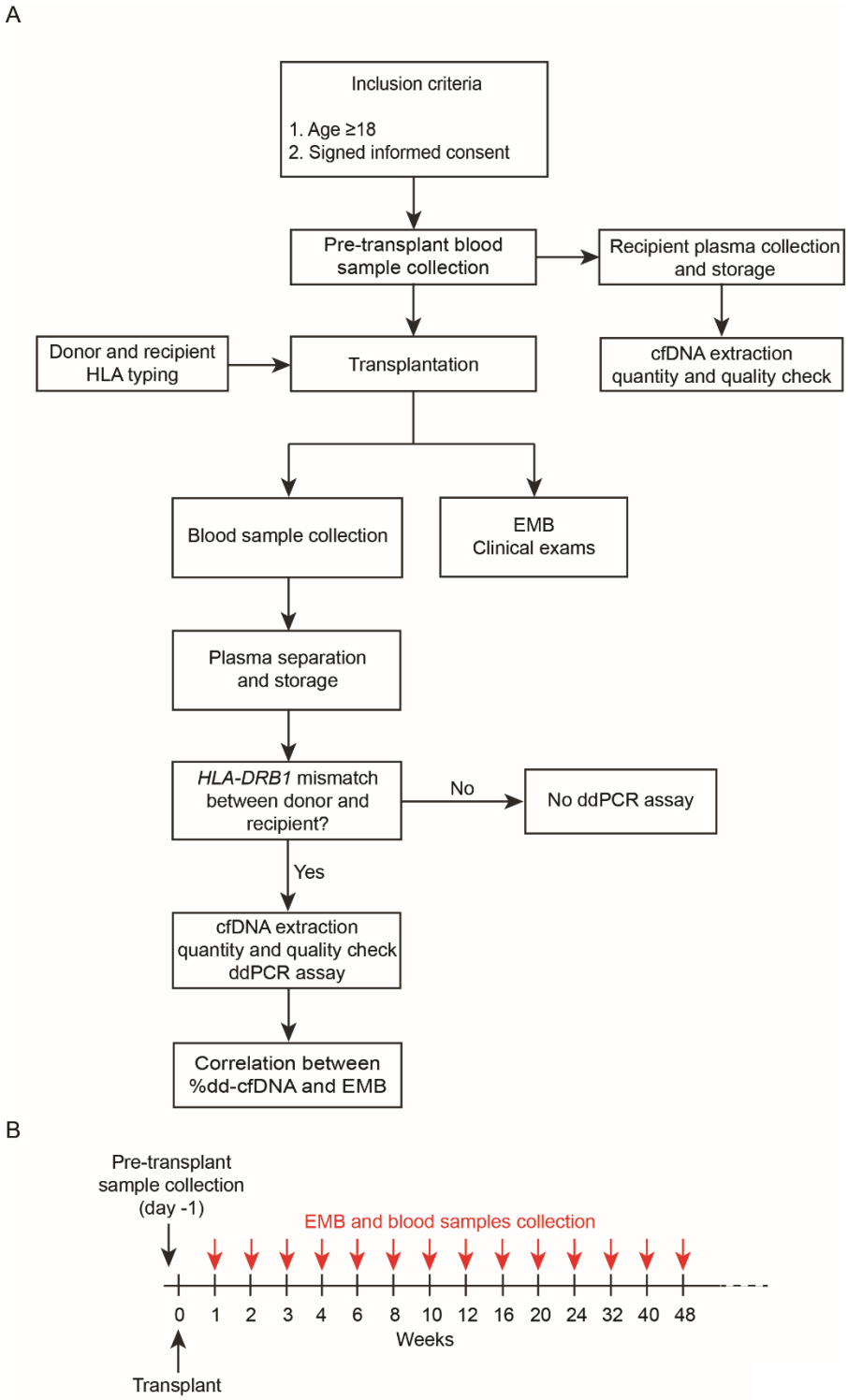

Figure 3. Flowchart of patient recruitment, sample collection and analysis. Patients were recruited based on specific inclusion criteria, and then a pre-transplant blood sample was collected and processed. After transplantation cfDNA was extracted and droplet digital PCR assay was performed. The last step was the correlation between fraction abundance and EMB (A). Timeline of sample collection (B).

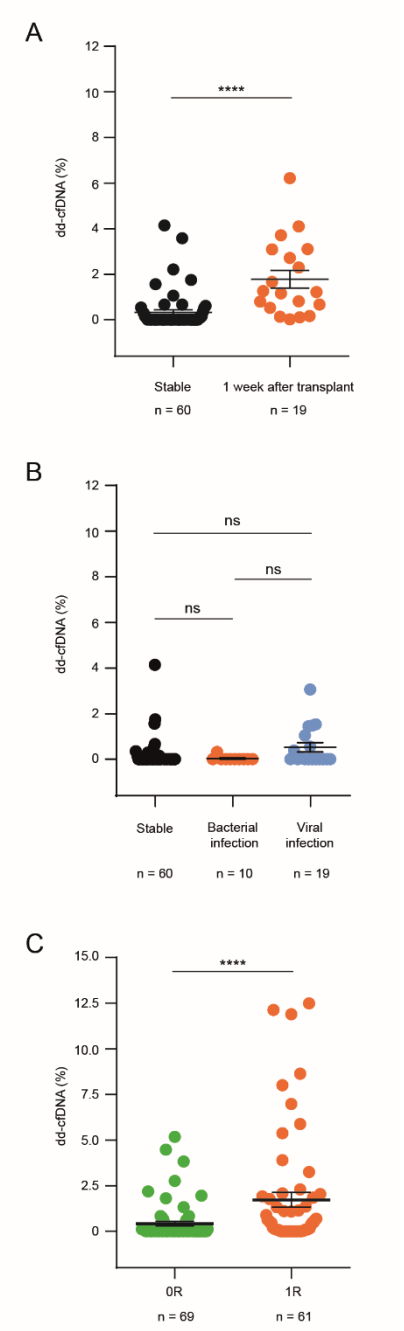

Figure 4. dd-cfDNA levels according to ischemia-reperfusion, infections, and acute rejection in adult heart recipients. (A) Dot plot showing dd-cfDNA percentage in stable conditions vs 7 days after transplantation. (B) Comparison between dd-cfDNA variations in stable conditions vs bacterial and viral infections. (C) Comparison between dd-cfDNA percentage and EMB scored as 0R and 1R. Data are presented as dot plots. The number of samples in each group is reported. Error bars represent SEM. P values were determined by Kolmogorov-Smirnov test (ns, not significant; ****, P<0.0001).

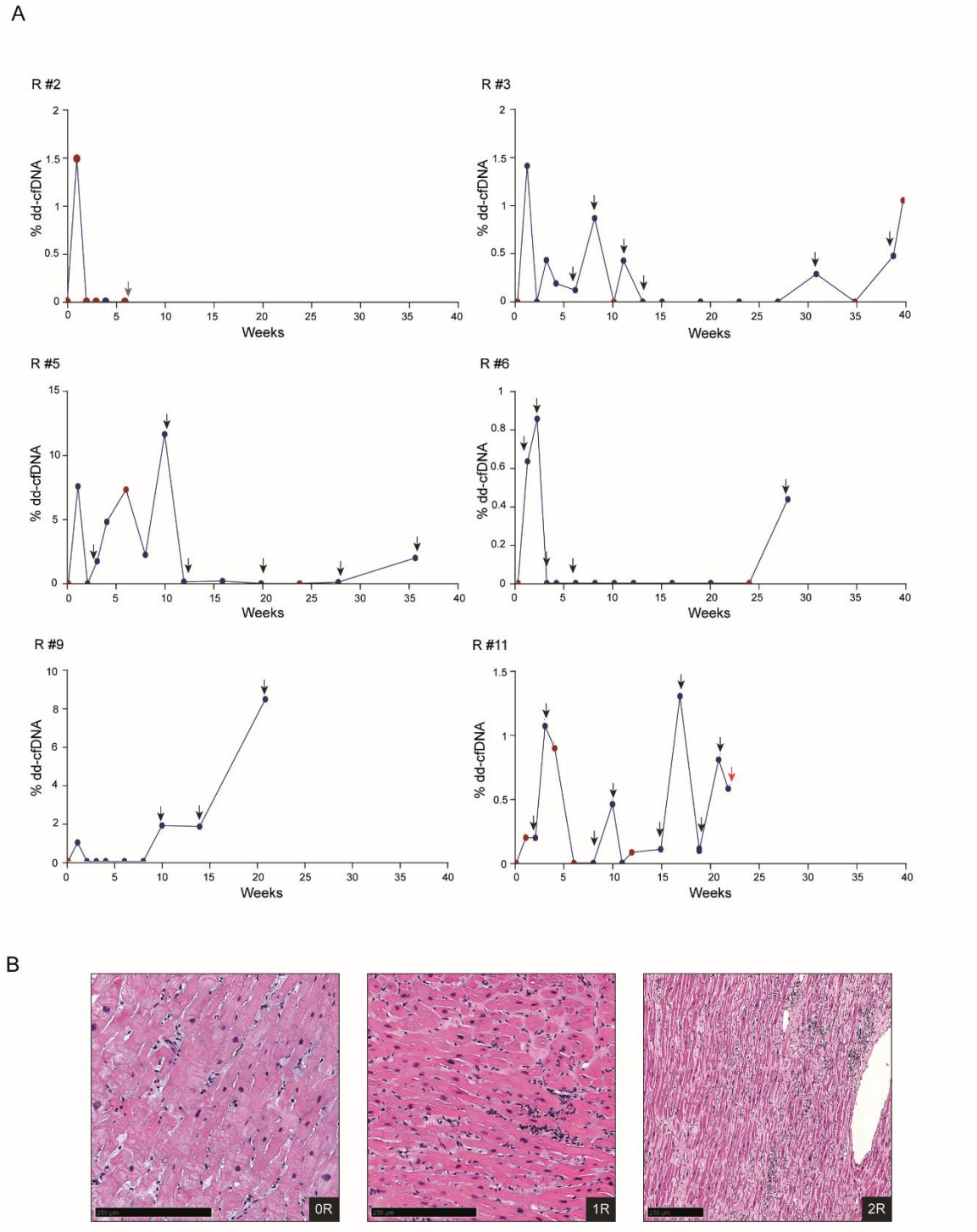

Figure 5. dd-cfDNA profile in representative adult heart recipients. dd-cfDNA levels during follow-up are shown for the indicated patients. After the initial peak due to ischemia-reperfusion, donor DNA levels decreased, only to increase again during 1R (black arrows) or 2R (red arrow) events. In R#2 the occurrence of intracerebral hemorrhage is shown as a gray arrow. Red dots indicate liquid biopsies collected without performing EMBs. (A). Hematoxylin and eosin staining corresponding to 0R, 1R and 2R according to the 2005 ISHLT guidelines. Scale bars 250µm (B).

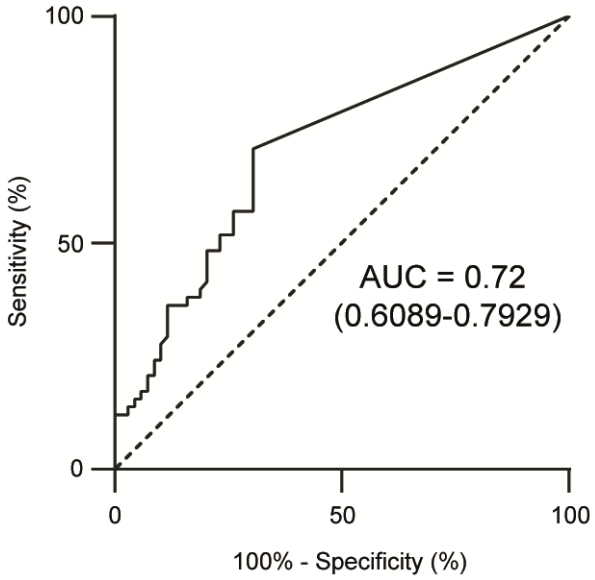

Figure 6. ROC curve for dd-cfDNA to identify rejection. AUC=0.72 (95% CI, 0.6089-0.7929).

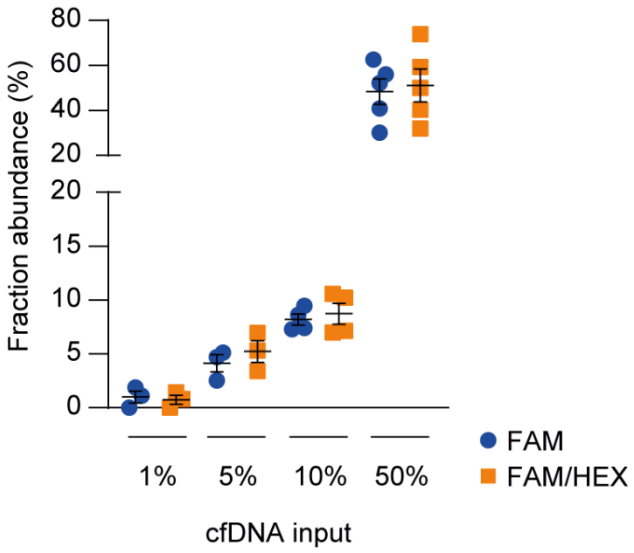

Figure 7. Technical comparison between HLA-DRB1 FAM and FAM/HEX probe panels. Serially diluted cfDNAs were spiked into a constant level of background cfDNA and quantified through droplet digital PCR assay using both the FAM-only and FAM/HEX methods. The total DNA concentration was 10 ng and the percentage of spiked DNA is shown in the graph. The results were reported as the mean fraction abundance. Error bars represent SEM. p-values were obtained using the Mann-Whitney nonparametric test.

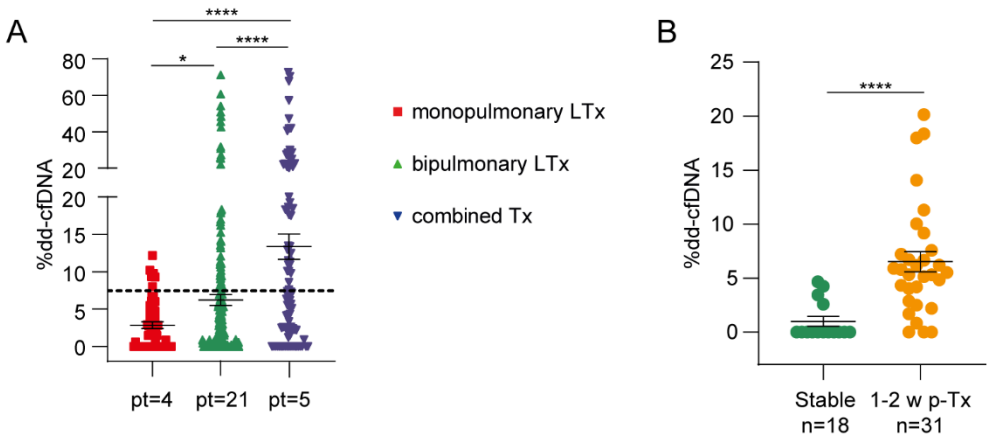

Figure 8. Dd-cfDNA release is influenced by the type of lung transplant and ischemia-reperfusion injury. (A) Dd-cfDNA quantification in monopulmonary, bipulmonary, and combined lung transplants (LTx). The number of patients (pt) is reported for each group. The dotted line represents the total average percentage of dd-cfDNA in all time measurements. (B) dd-cfDNA levels during the first 2 weeks after transplantation (31 measurements) were compared to stable condition samples (18 measurements from 10 patients). The number of samples (n) in each group is shown below. The results are reported as percentages and shown as dot plots. Error bars represent SEM. p-values were obtained using the Mann-Whitney nonparametric test.

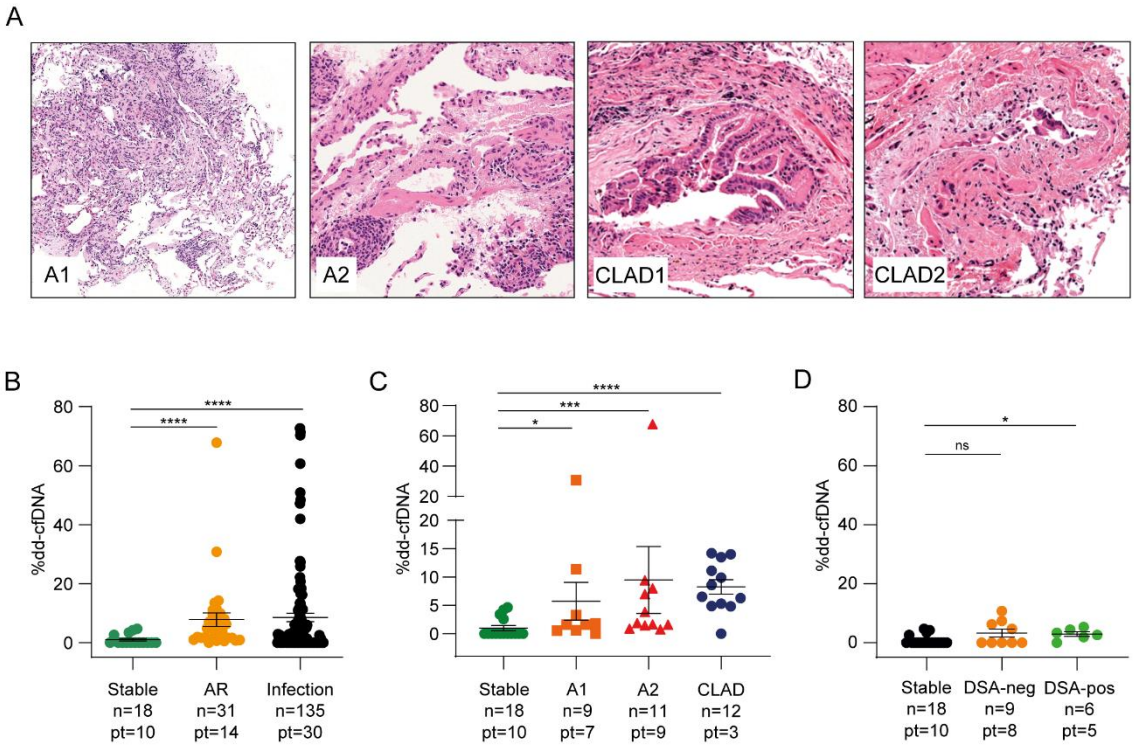

Figure 9. Acute rejection is followed by a significant increase of dd-cfDNA in lung recipients. (A) Histopathological features of acute rejection grades A1 and A2 and evidence of bronchiolar wall fibrosis with lumen narrowing (CLAD1) and epithelial damage (CLAD2) in patients with obliterative bronchiolitis syndrome (BOS-CLAD). Hematoxylin and eosin staining, A1 ×100 original magnification, A2 and CLAD ×200 original magnification. (B) dd-cfDNA values during acute rejection (AR) and infectious events
compared to stable conditions. (C) donor DNA levels in minimal (A1) and mild (A2) rejection and in chronic lung allograft dysfunction (CLAD) episodes. (D) dd-cfDNA percentages in DSA-negative and DSA-positive samples compared to those under stable conditions. The numbers of samples (n) and patients (pt) in each group are indicated. The results are reported as percentages and shown as dot plots. Error bars represent SEM. p-values were calculated using the Mann-Whitney nonparametric
test.

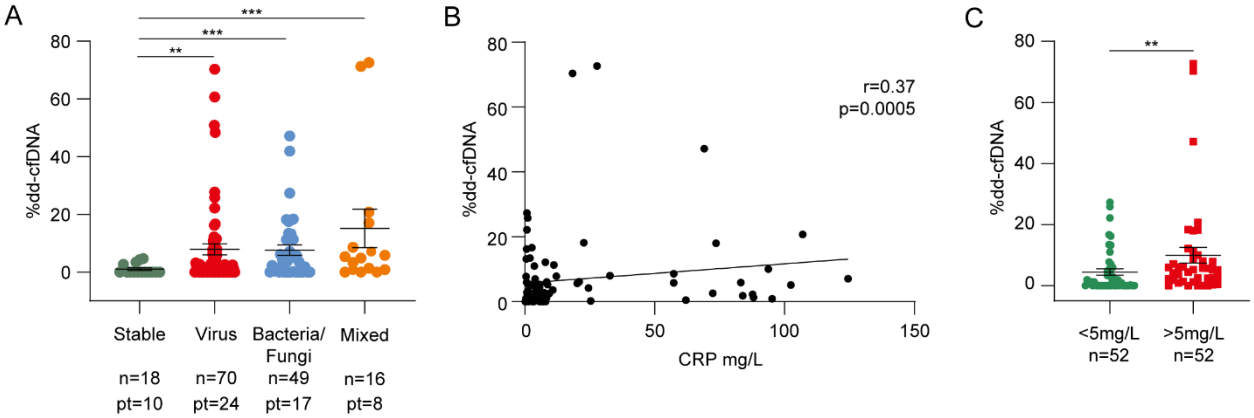

Figure 10. Respiratory tract infections cause dd-cfDNA release in the recipient bloodstream. (A) dd-cfDNA quantification related to infections divided into virus, bacteria/fungi, and mixed groups. The number of samples and patients (pt) from whom the samples were collected are shown for each category. (B) Linear regression between dd-cfDNA percentage and relative C-reactive protein (CRP) level (n = 104). Correlations were calculated using the nonparametric Spearman's test. (C) Differences between %dd-cfDNA in the low (<5 mg/L) and high (>5 mg/L) CRP samples. The results in panels (A) and (C) are reported as percentages and shown as dot plots. Error bars represent SEM. p-values were obtained using the Mann-Whitney nonparametric test.

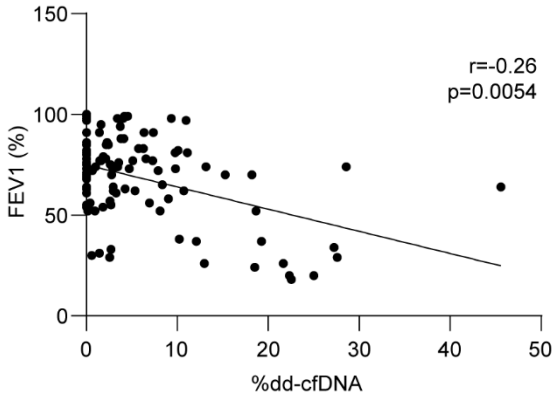

Figure 11. Dd-cfDNA is inversely related to respiratory function. Linear regression between forced expiratory volume in 1 s (FEV1) and related ddcfDNA levels (n = 114). FEV1 was calculated considering recipient characteristics for normative equations. Correlations were obtained using the nonparametric Spearman test.

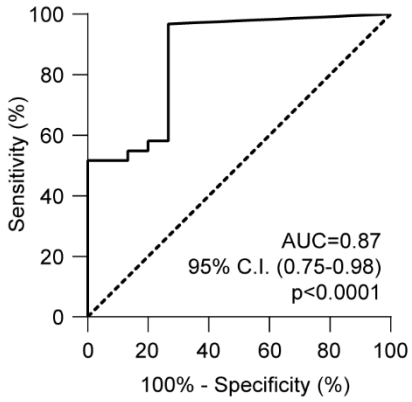

Figure 12. ROC analysis of *HLA-DRB1* droplet digital PCR assay. The ROC curve was obtained considering the dd-cfDNA values associated with rejection and no rejection. The curve was calculated using the Wilson-Braun method. Area under the curve (AUC) = 0.87 (95% C.I., 0.75–0.98).

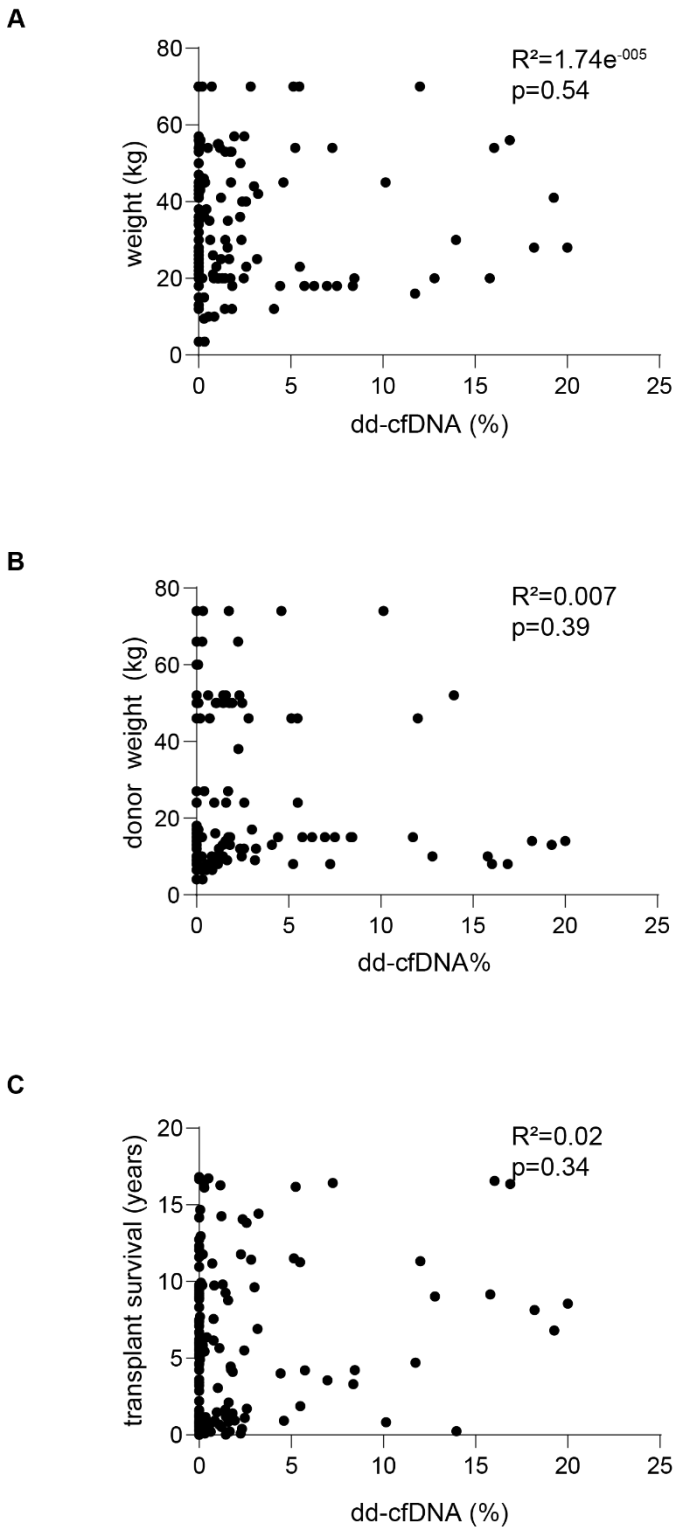

Figure 13. Dd-cfDNA level distribution based on patient (A) and donor weight (B) and transplant survival (C) in children. The correlation was performed using the nonparametric Spearman test. P-values are 0.54, 0.39 and 0.34, respectively.

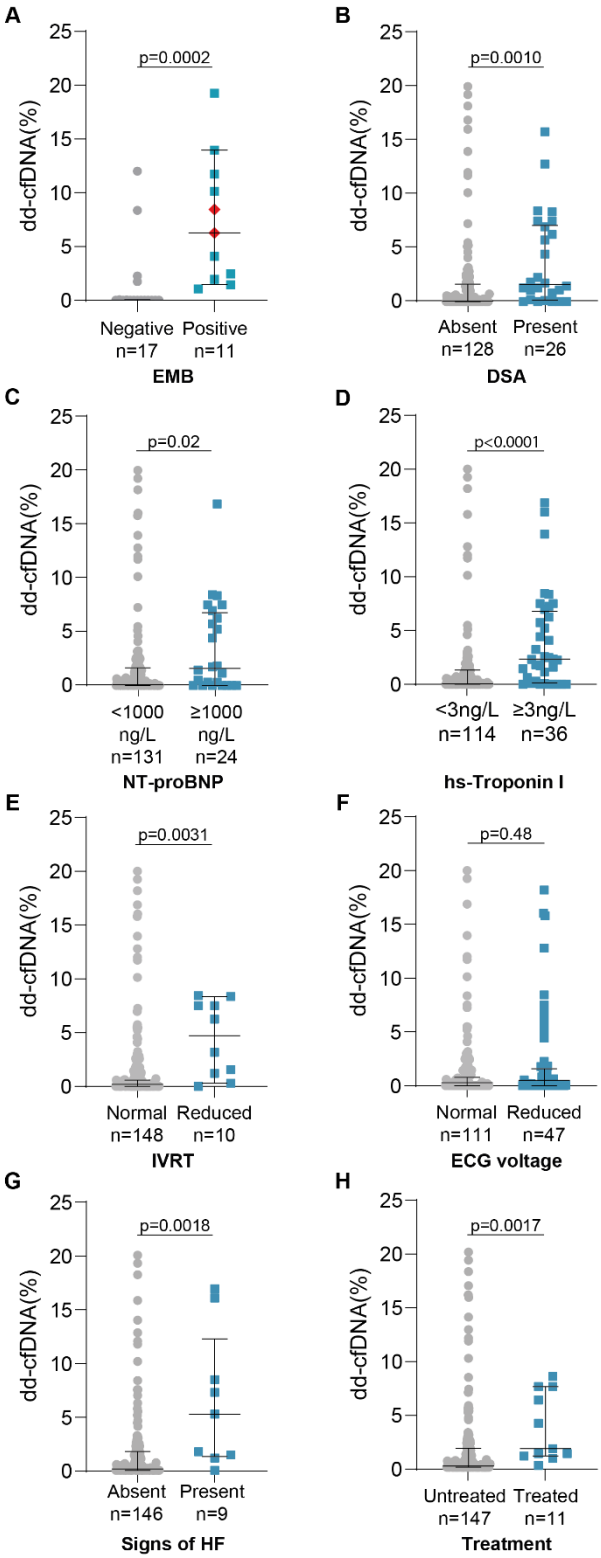

Figure 14. Correlation between dd-cfDNA values and clinical parameters of graft status in pediatric heart recipients. Dd-cfDNA percentages are plotted in each graph based on (A) EMB rejection grade, (B) serum DSA presence, (C) NT-proBNP levels, (D) hs-Troponin I blood levels, (E) IVRT, (F) ECG voltage, (G) clinical signs of heart failure, (H) for-cause treatment. The number of samples in each category is indicated below the graphs. EMBs that scored positive for AMR are highlighted in red in graph A. EMB: endomyocardial biopsy, DSA: donor-specific antibodies, NT-proBNP: N-terminal pro-Brain Natriuretic Peptide, hs-Troponin I: high sensitive Troponin I, IVRT: Isovolumic Relaxation Time, ECG: electrocardiogram, HF: heart failure.

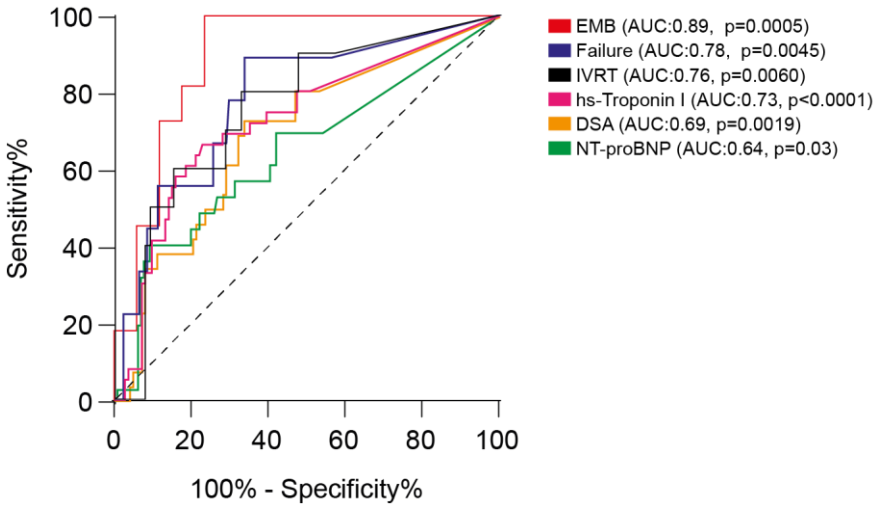

Figure 15. Dd-cfDNA Receiver Operator Characteristic (ROC) curves related to clinical and blood parameters of allograft injury. ROC analysis of rejection markers and clinical parameters of cardiac injury is calculated and compared. Area under the curve (AUC) and p-values are reported in the legend in brackets. EMB: endomyocardial biopsy, IVRT: Isovolumic Relaxation Time, hs-Troponin I: high sensitive Troponin I, DSA: donor-specific antibodies, NT-proBNP: N-terminal pro-Brain Natriuretic Peptide.

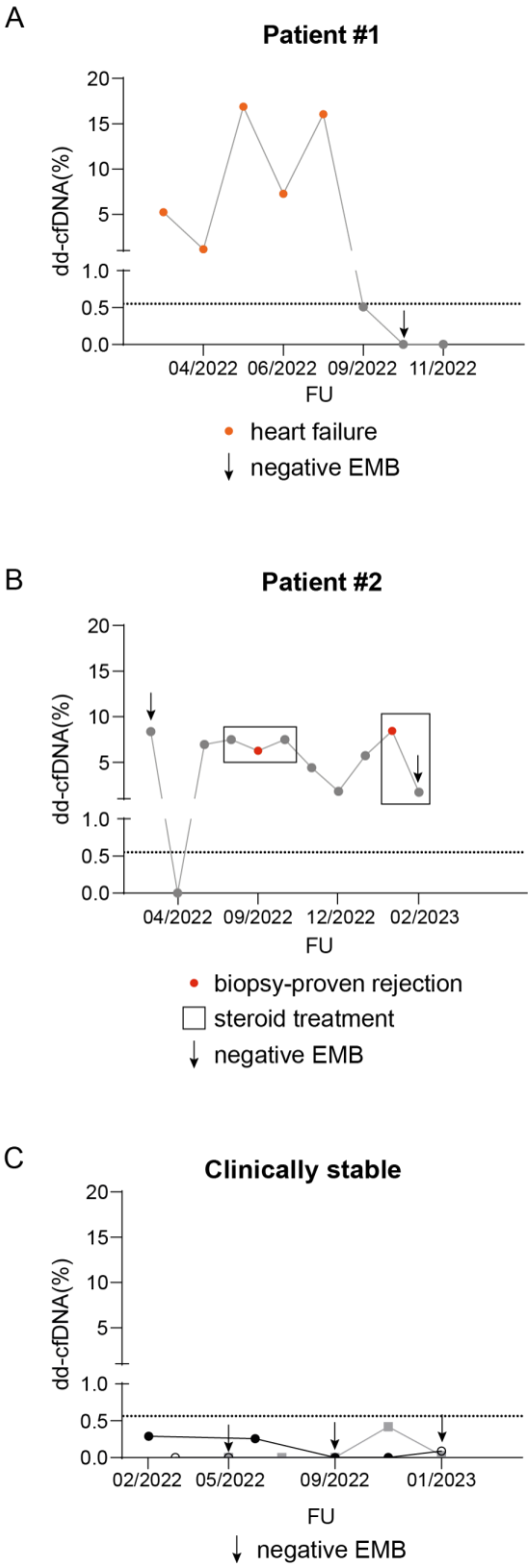

Figure 16. Dd-cfDNA trend in children with clinical signs of rejection or with healthy allograft. The percentage of dd-cfDNA is reported for each measurement performed. Samples were collected monthly during routine post-transplant follow-up. Clinically and biopsy-proven heart failure and rejection are highlighted in orange and red, respectively. EMBs that scored negative are indicated by a black arrow. Patient #1 (ID 13, panel A) experienced heart failure with high NT-proBNP levels and coronary lesions as a consequence of chronic allograft rejection, then stabilized after treatment with a decrease in dd-cfDNA%. Patient #2 (ID 9, panel B) had a mixed ACR/AMR rejection in September 2022 and an AMR rejection in January 2023, heavily treated. DSA were present during all follow-up time. NT-proBNP and hs-Troponin I levels were

above the reference values. Cardiac catheterization revealed high filling pressure and, at last follow-up, coronary allograft rejection and heart failure. In contrast, dd-cfDNA values of patients (N=3, ID 1, 14, 24, panel C) with no signs of rejection were all below the 0.55% cut-off. Dd-cfDNA%: donor-derived cell-free DNA percentage, FU: follow-up, EMB: endomyocardial biopsy, DSA: donor-specific antibodies, NT-proBNP: N-terminal pro-Brain Natriuretic Peptide, hs-Troponin I: high-sensitive-Troponin I, ACR: acute cellular rejection, AMR: antibody-mediated rejection.

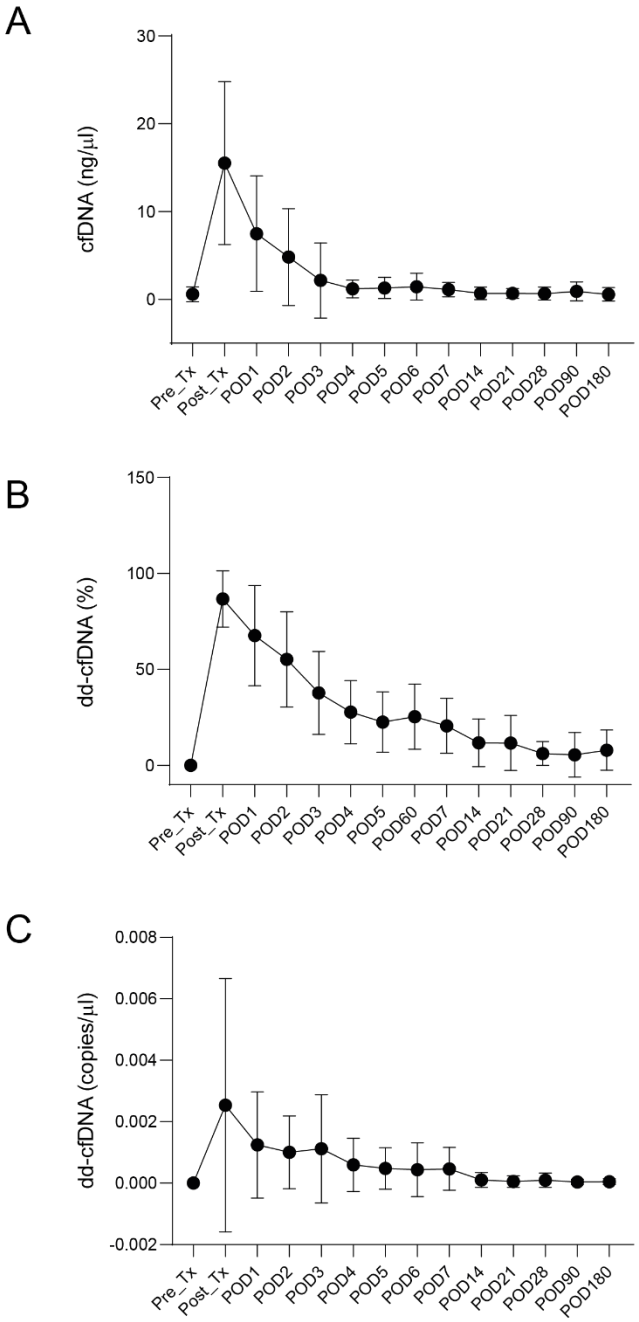

Figure 17. Total and donor-derived cell-free DNA trend in liver cohort. Mean and standard deviation of (A) total cfDNA, (B) dd-cfDNA percentage and (C) dd-cfDNA copies/µl are reported for the entire cohort (n=51). cfDNA: cell-free DNA; dd-cfDNA: donor-derived cell-free DNA; Tx: transplant; POD: post operative day.

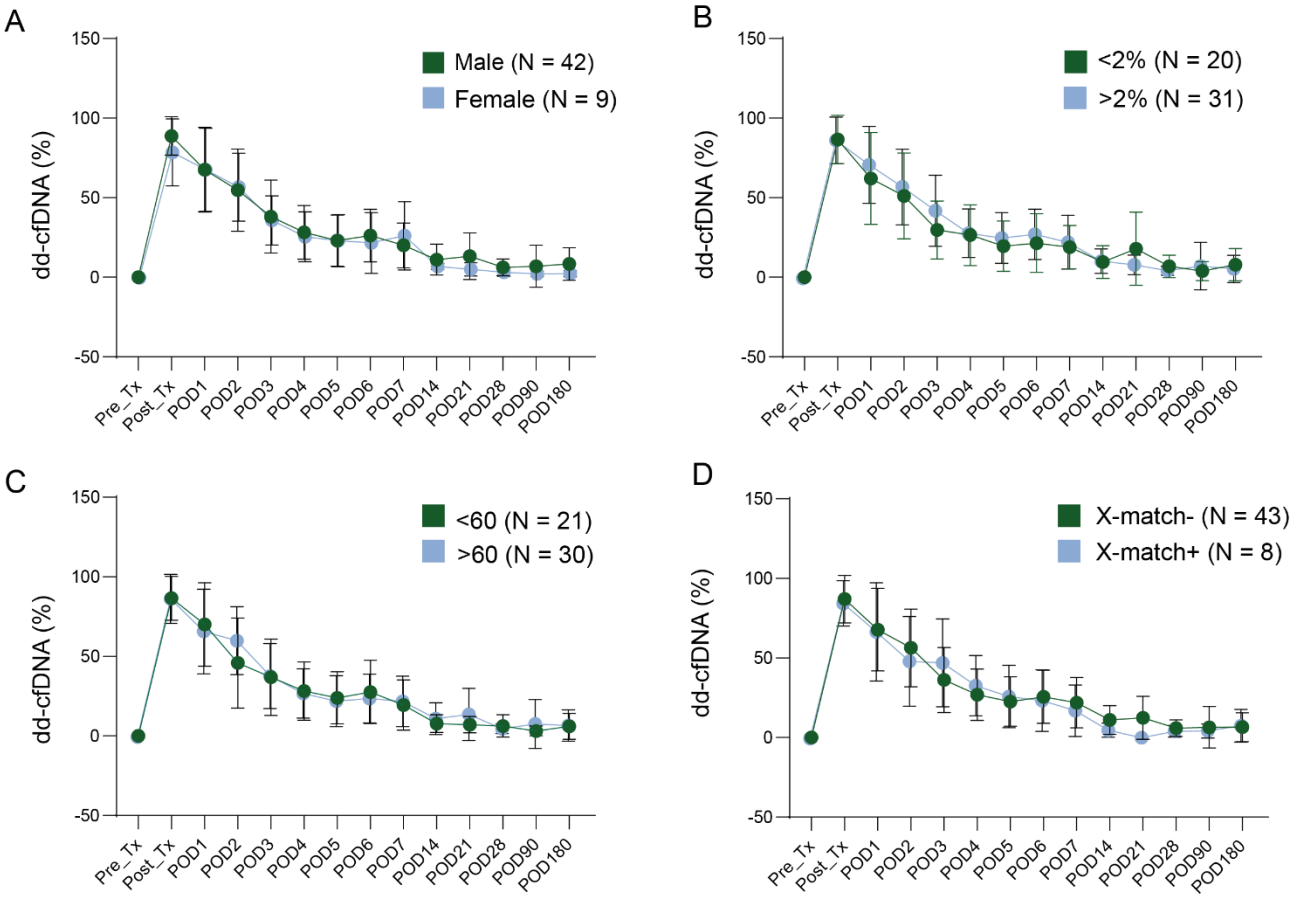

Figure 18. Demographical stratification of dd-cfDNA values in liver cohort. Patients were divided based on their gender (A), GRWR (B), donor age (C) and pre-transplant cross-match (D)**.** Each graph represent the mean and standard deviation of samples. dd-cfDNA: donor-derived cell-free DNA; Tx: transplant; POD: post operative day.

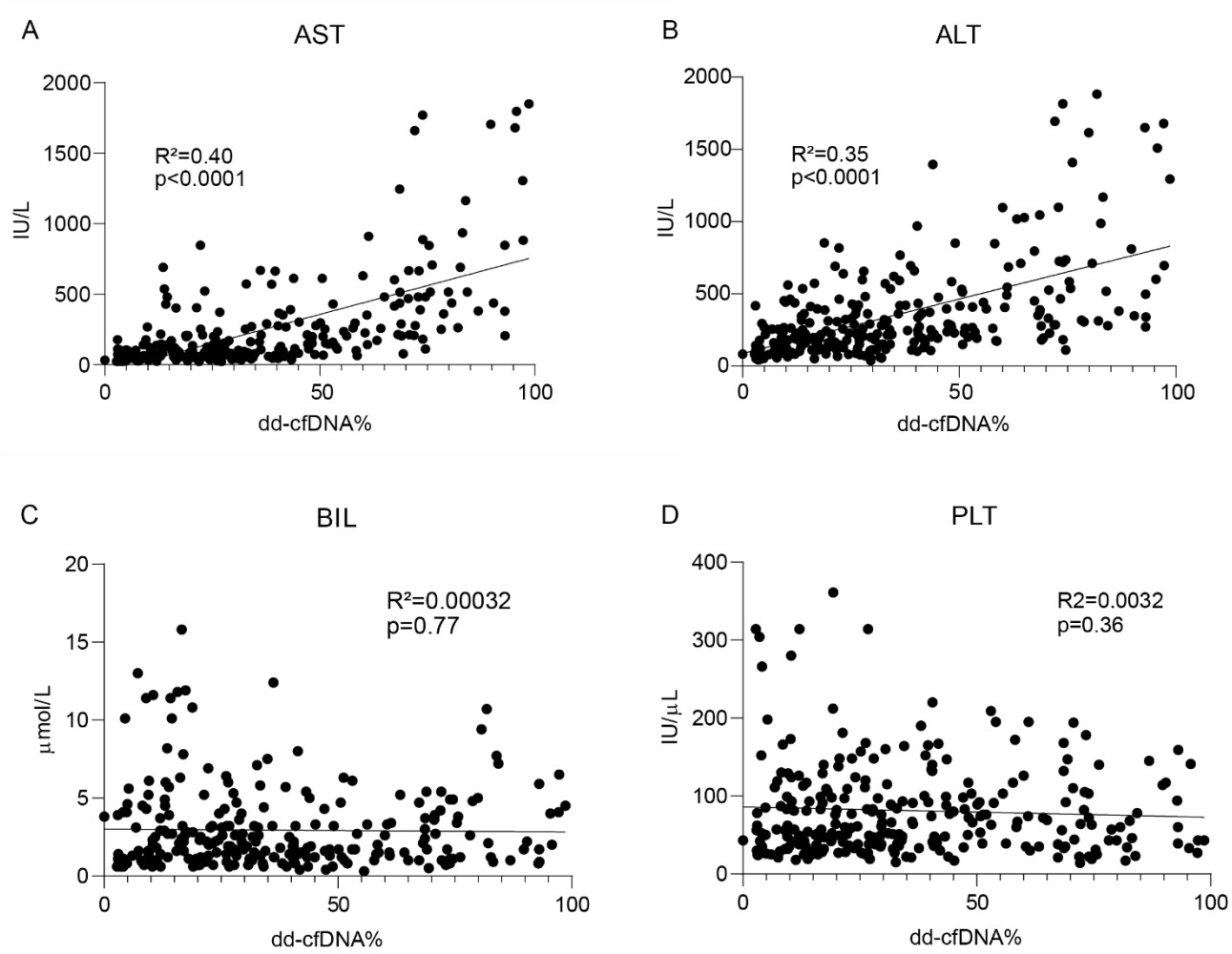

Figure 19. Hepatocytolysis markers and donor-derived cell-free DNA (dd-cfDNA) during the first week after transplant. Dd-cfDNA percentages were correlated with AST (A), ALT (B), BIL (C), and platelets (D). Correlation was performed with Pearson r test. R^2 and p-values are indicated in each graph. AST: aspartate aminotransferase; ALT: alanine aminotransferase; BIL: bilirubin; PLT: platelets.

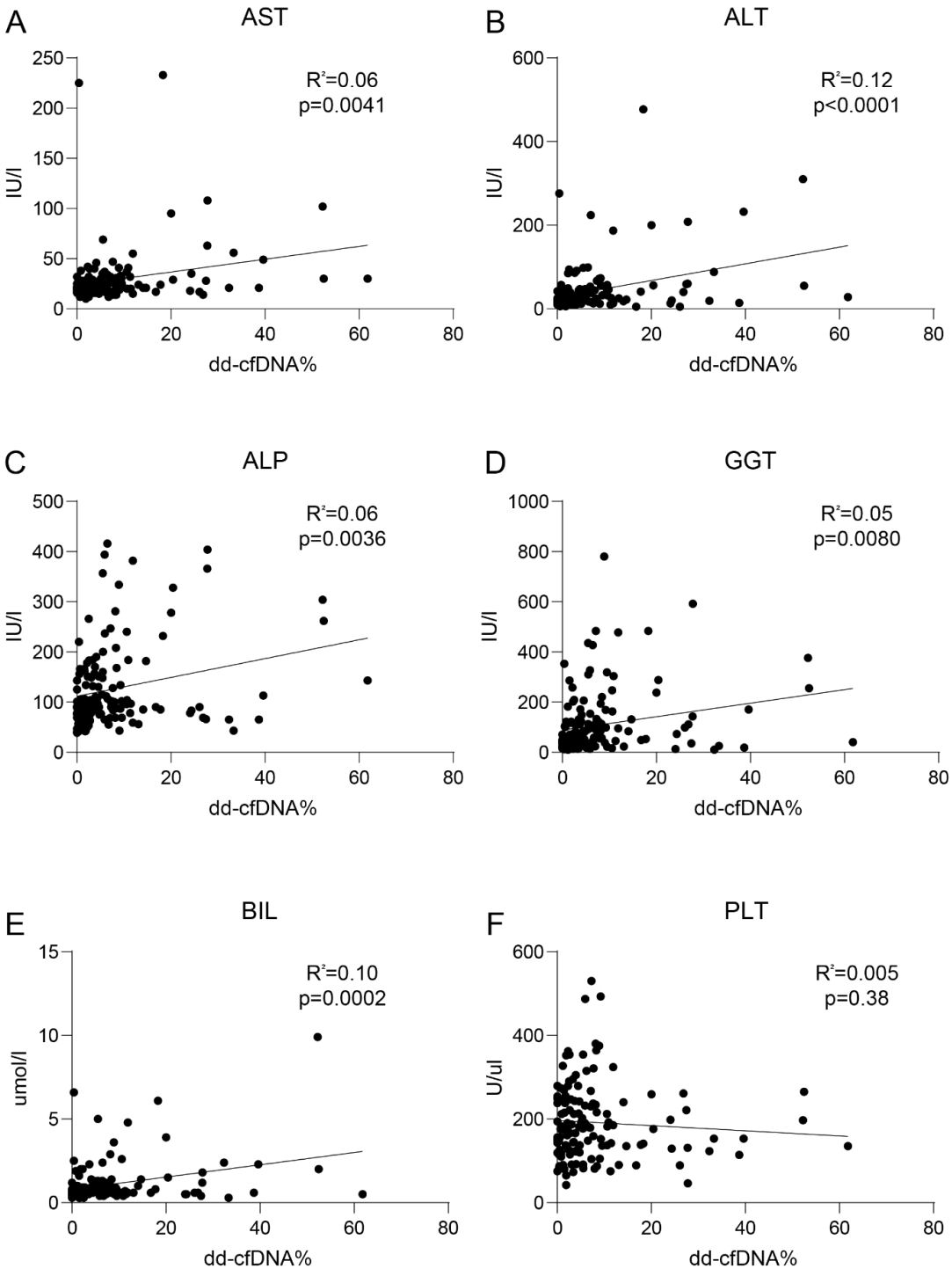

Figure 20. Hepatocytolysis markers are associated with donor-derived cell-free DNA (dd-cfDNA). Dd-cfDNA percentages were correlated with AST (A), ALT (B), ALP (C), GGT (D), BIL (E) and plateles (F). Correlation was performed with Pearson r test. R^2 and p-values are indicated in each graph. AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutaryl transferase; BIL: bilirubin; PLT: platelets.

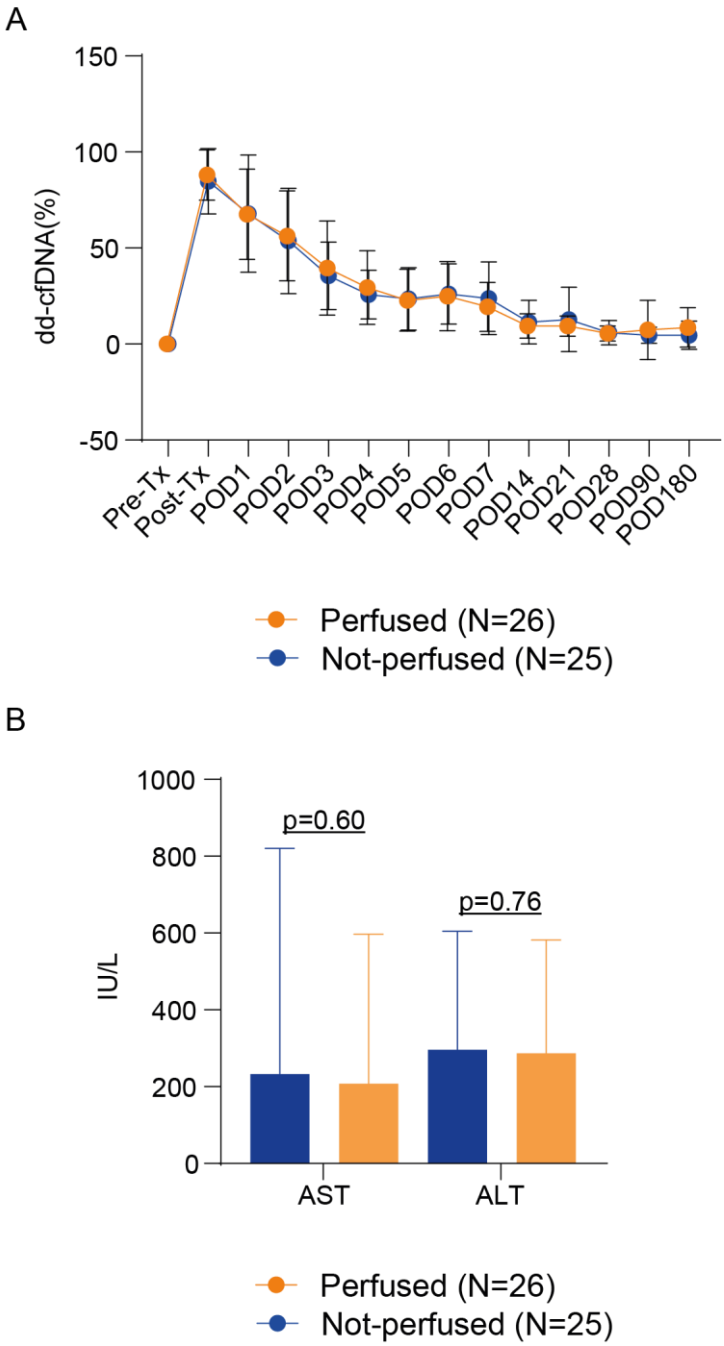

Figure 21. Machine perfusion effect on donor-derived cell-free DNA (dd-cfDNA) and transaminase release. No differences can be observed in dd-cfDNA percentages between perfused (n=26) and not-perfused (n=25) groups (A). The same results are obtained by comparing aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values from the same patients (B). Mean values and standard deviation are reported in both graph. Tx: transplantation; POD: post-operative day.

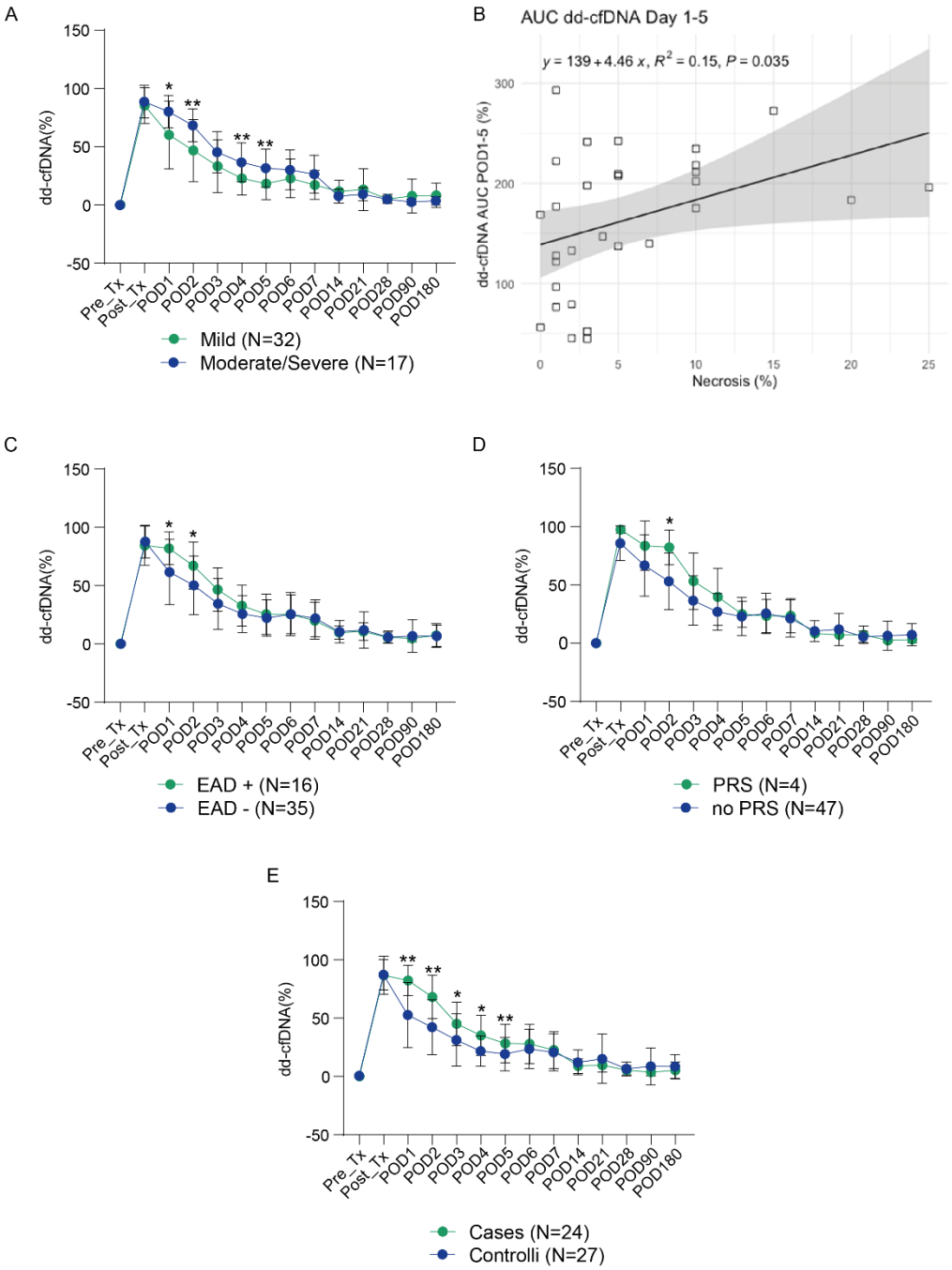

Figure 22. Donor-derived cell-free DNA (dd-cfDNA) recapitulates post-transplant ischemia injury, graft necrosis, early allograft dysfunction (EAD), and post-reperfusion syndrome (PRS) in liver recipients. Dd-cfDNA is augmented in association with ischemia-reperfusion damage (A) and necrosis (B) evaluated by biopsy. EAD (C) and PRS (D) patients show higher dd-cfDNA percentages in POD1 and POD2 compared to stable patients. Overall, patients presenting these 4 signs of early liver damage have significantly increased dd-cfDNA levels up to POD5 (E). Graphs show mean and standard deviation values. P-values are indicated as *: p<0.01, **:p<0.001. Tx: transplantation; POD: post-operative day.

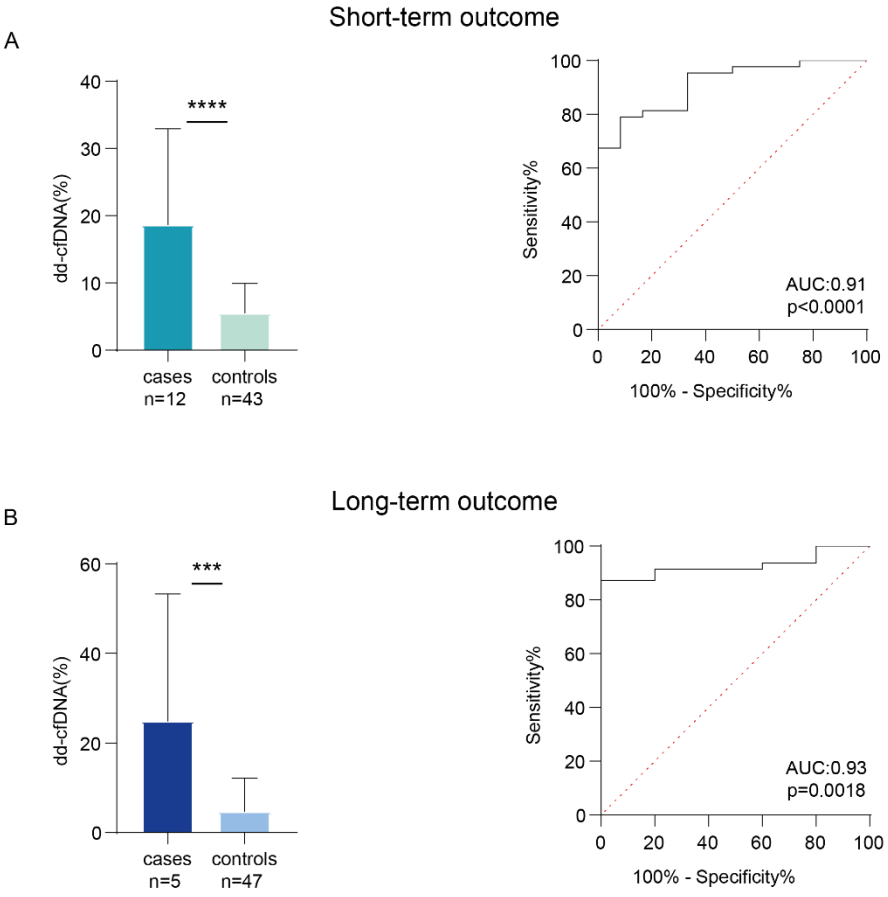

Figure 23. Short and long-term outcome of liver transplant cohort. Cases are represented by samples collected concomitantly to signs of hepatic distress and failure. Short-term period refers to events that happened between POD14 and POD28, while long-term refers to events that occurred between POD90 and POD180. The number of samples in each group is reported below graphs. P-value is expressed as ***:p<0.001, ****:p<0.0001. dd-cfDNA: donor-derived cell-free DNA; AUC: area under the curve.

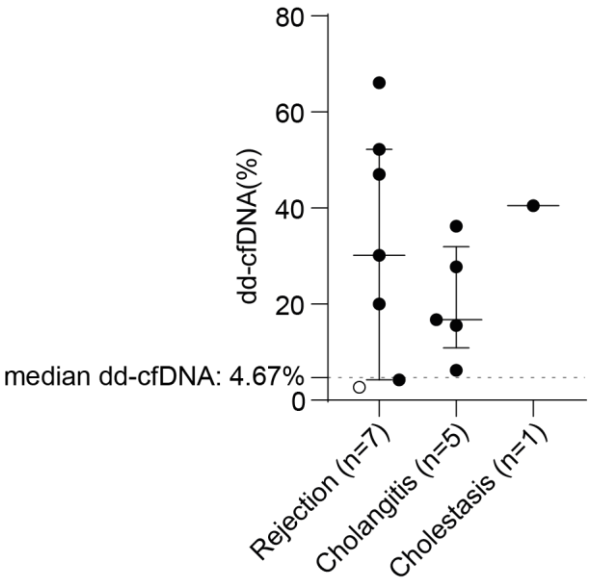

Figure 24. Donor-derived cell-free DNA (dd-cfDNA) in for-cause liver biopsies (n=13). Dd-cfDNA quantifications related to for-cause biopsies are higher compared to the median dd-cfDNA levels in the short and long-term outcome of the cohort.

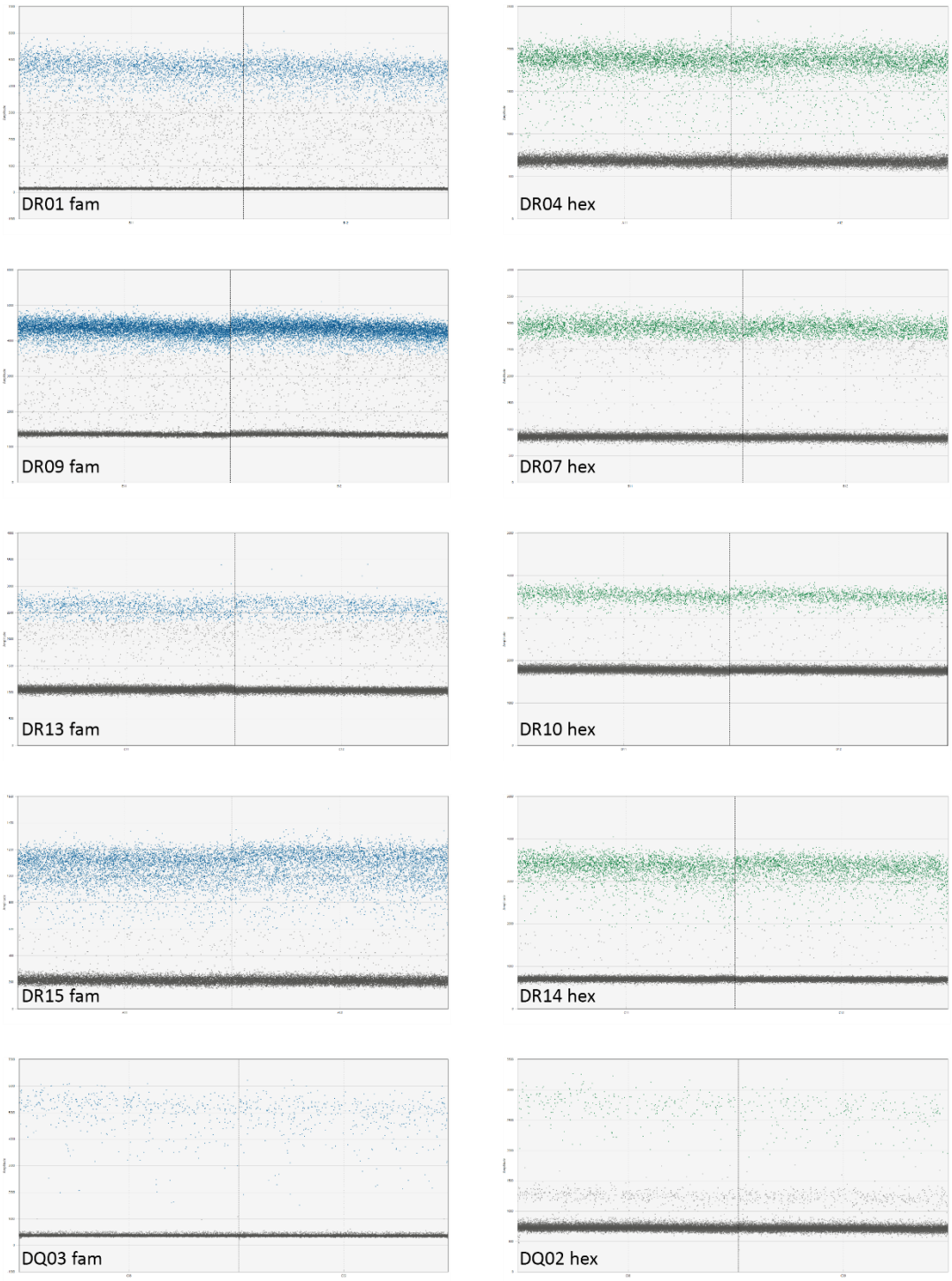

Figure 25. Validation of *HLA-DRB1* and *HLA-DQB1* probes. Experiments were performed using genomic DNA samples specific for each probe. The positive droplets are represented as colored dots (blue: FAM; green: HEX) and the negative background is shown as grey dots.

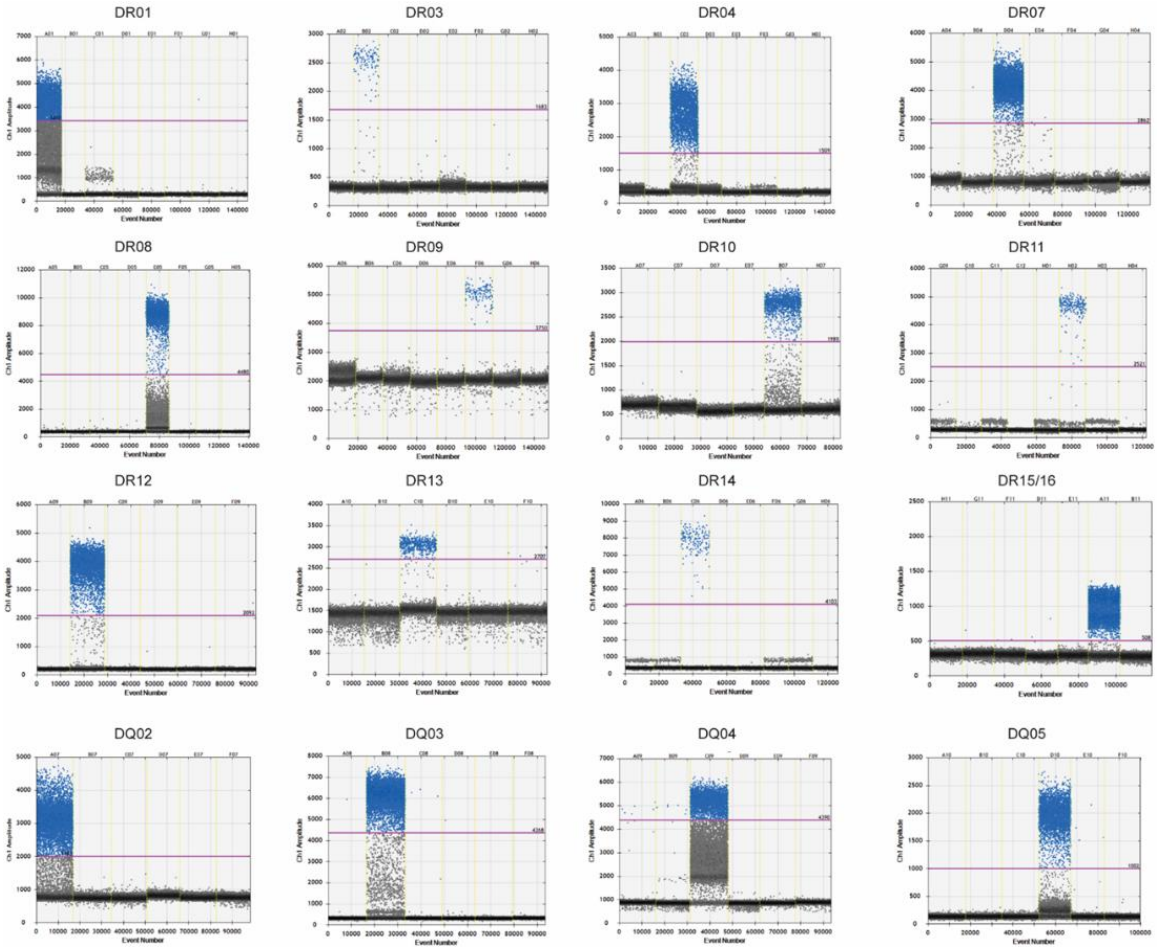

Figure 26. Specificity assay. Probes labelled with FAM fluorophore were tested against samples different *HLA-DRB1* and *HLA-DQB1* alleles, but amplification was obtained only in presence of the target allele.

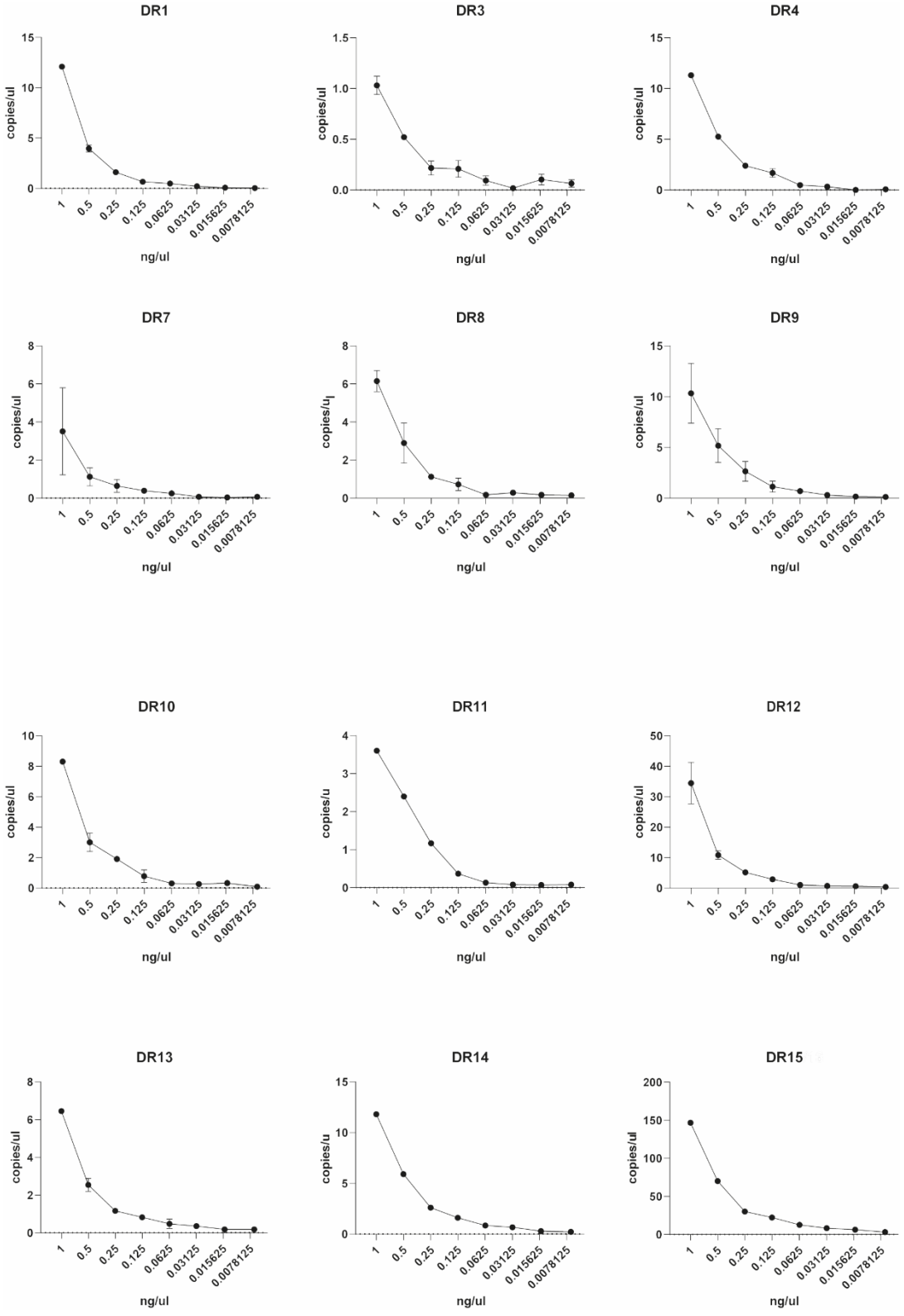

Figure 27. Sensitivity assay. Two-fold serial dilution of DNAs were quantified using *HLA-DRB1* set of probes. DNA starting concentration was 1 ng.