J Cell Immunol. 2026;8(1):1–10.

Journal of Cellular Immunology Review Article

Spectrum of Non-Invasive Biomarkers for Acute Cellular Rejection Surveillance in Heart Transplantation

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Received date: January 11, 2026, **Accepted date:** February 03, 2026

Citation: Talapaneni S, Hu R, Burke V, DeMarais N, Korutla L, Vallabhajosyula P. Spectrum of Non-Invasive Biomarkers for Acute Cellular Rejection Surveillance in Heart Transplantation. J Cell Immunol. 2026;8(1):1–10.

Abstract

Objective: Heart transplantation remains the definitive treatment for patients with advanced heart failure, but post-transplant complications such as acute cellular rejection (ACR) continue to limit long-term outcomes. The current standard for ACR surveillance is endomyocardial biopsy, which is invasive and associated with procedural risks, variability in interpretation, high healthcare utilization costs, and poor patient tolerability. For these reasons, there is increasing interest in identifying non-invasive biomarkers that can reliably detect ACR. This review details the range of non-invasive methods for detecting ACR and to provide practical guidance for clinicians considering a transition to less invasive monitoring protocols following heart transplantation.

Methods: We conducted a narrative review of published literature focusing on non-invasive biomarkers for ACR in heart transplantation. We specifically focused on evaluating donor-derived cell-free DNA, gene expression profiling, plasma microRNA, metabolomics, cytokines and chemokines, and extracellular vesicle approaches, with emphasis on clinical applicability, diagnostic performance, and limitations.

Results: Our review identified several promising non-invasive biomarkers for ACR detection, with donor-derived cell-free DNA and gene expression profiling showing the most advanced clinical integration. Emerging approaches such as microRNA profiling, metabolomics, cytokine or chemokine panels, and extracellular vesicle analysis demonstrate potential in early-phase studies but require further validation. Most biomarkers exhibited high negative predictive value, though sensitivity and positive predictive values remained variable across platforms along with their applications in the early perioperative and late follow-up periods. None of the biomarker platforms distinguish ACR from antibody mediated rejection. Practical challenges including cost, assay standardization, and limited availability continue to impact widespread adoption.

Conclusion: Non-invasive blood-based biomarkers show promise for improving acute cellular rejection surveillance in heart transplant recipients. While markers such as dd-cfDNA, extracellular vesicles, and microRNAs have demonstrated encouraging early results, their clinical use remains limited by variable inadequate sensitivity, low positive predictive value, inability to distinguish ACR from AMR, and lack of standardization. Ongoing investigations are expected to refine their diagnostic performance and guide their integration into routine clinical care.

Keywords: Acute cellular rejection, Non-invasive biomarkers, Transplantation immunology

Introduction

Heart transplantation remains a life-saving therapy for patients with end-stage heart failure. However, despite many advancements in care, acute rejection (AR) and immunosuppression related complications remain the primary drivers of morbidity and mortality in heart transplant patients [1,2]. The current gold standard for monitoring AR and guiding immunosuppression treatment strategies is endomyocardial biopsy (EMB) of the cardiac allograft [3]. While effective, this invasive procedure has several limitations, including procedural risks, patient discomfort, sampling variability,

variability in interpretation, high resource utilization, and that it is not amenable to easy, repeated serial testing [4,5]. Thus, there is a critical need for development of non-invasive biomarkers for AR surveillance in heart transplantation

Acute cellular rejection (ACR) is mediated by recipient alloreactive T-cells and occurs more frequently than antibody mediated rejection (AMR). Highest rates of ACR occur during the first six months after heart transplantation, which is associated with increased risk of development of cardiac allograft vasculopathy [3,4]. Biomarker development for noninvasive surveillance of ACR has remained a very active area of research over the past sixty years. More recently, advances in molecular profiling, proteomics, transcriptomics, and other high-throughput technologies have yielded some promising candidates for clinical use [7]. Non-invasive methods such as donor-derived cell-free DNA (dd-cfDNA), immune cell phenotyping, and metabolomic signatures are gaining traction as potential biomarkers of ACR. However, these new developments also have limitations, including suboptimal sensitivity, specificity and low positive predictive value [8].

In addition to their limitations in diagnostic accuracy, current non-invasive biomarkers are not yet reliable for monitoring treatment response in patients with ACR [9]. While they may suggest the presence of rejection, they cannot consistently determine whether therapeutic interventions, such as intensification of immunosuppression, have successfully resolved the rejection episode. As a result, repeat EMB remains necessary to confirm histologic resolution of ACR, further underscoring the urgent need for biomarkers that not only detect rejection but also guide and monitor the efficacy of treatment in real time [9]. In this review, we summarize the current state of research in non-invasive biomarker development for detecting ACR in heart transplantation.

Donor-derived Cell-free DNA

Cellular death leads to release of DNA into peripheral blood. As ACR causes targeted cell death of allograft cardiomyocytes, some of its cellular DNA is released into circulation. Therefore, donor derived cell free DNA (dd-cfDNA) quantification may serve as a biomarker of ACR [10]. Although the DNA released into peripheral circulation from cellular death by apoptosis is far less than by cellular necrosis, there is strong evidence demonstrating the biomarker potential of this platform for ACR diagnosis [10]. Over the past twenty years, as dd-cfDNA has gained traction in transplant monitoring, many large cohort studies have been performed to evaluate dd-cfDNA's ability to diagnose ACR in heart transplant patients [10–18].

In a study by Deshpande *et al*., dd-cfDNA demonstrated strong concordance with clinical rejection and showed particular utility in predicting significant clinical outcomes after treatment for rejection [11]. More recently, Sorbini *et al*. demonstrated that dd-cfDNA levels were significantly higher in patients with ACR compared to controls without rejection, highlighting its potential as a non-invasive diagnostic tool [12]. These findings underscore the value of dd-cfDNA as a biomarker for monitoring rejection. Currently, there are two ways to detect dd-cfDNA: the first leverages genetic differences between donor and recipient, such as human leukocyte antigen (HLA) mismatches or single nucleotide polymorphisms (SNPs).The second on the other hand, quantifies dd-cfDNA levels using advanced methods like quantitative polymerase chain reaction (qPCR), digital droplet PCR, or whole genome sequencing [12–14].

The clinical potential of dd-cfDNA as an ACR biomarker has been investigated through multicenter studies, such as the Genomic Research Alliance for Transplantation (GRAfT). This prospective cohort study identified a 0.3% dd-cfDNA threshold with a negative predictive value (NPV) of 81% for detecting ACR or antibody mediated rejection (AMR) [15]. Additionally, Agbor-Enoh *et al*. observed that dd-cfDNA levels declined to a stable baseline of 0.13% by day 28 post-transplant but rose significantly during episodes of acute rejection, reaching a median of 0.38% compared to 0.03% in non-rejection states ($P<0.001$) [10]. Notably, elevated dd-cfDNA was detectable up to 5 months prior to biopsy-proven ACR and up to 3.2 months before AMR, highlighting its potential for early detection [10]. Although given the PPV of <20% in this study, it is difficult to ascertain the importance of a dd-cfDNA test to enable earlier detection. Using a dd-cfDNA threshold of 0.25%, the assay achieved a negative predictive value (NPV) of 99% for acute rejection, suggesting that this threshold could safely eliminate approximately 81% of routine biopsies [10]. Furthermore, dd-cfDNA levels were found to peak immediately after transplantation due to ischemia-reperfusion injury and then decline to a baseline (median ~0.13%) by day 28 post-transplant [10]. Increases in dd-cfDNA precede histologic diagnosis of ACR by approximately 0.5 months, and AMR by up to 3.2 months [10]. The study also found that dd-cfDNA levels were significantly higher in AMR compared to ACR, and that distinct fragmentomic signatures, such as increased guanosine-cytosine content and shorter DNA fragment sizes, could help differentiate between rejection subtypes [10].

Despite these advantages, dd-cfDNA is not without limitations. One key drawback is its low PPV and inability to distinguish ACR from AMR. Elevated dd-cfDNA levels can also be seen in other forms of graft injury, such as AMR or infection, potentially leading to false positives [10]. The sensitivity for ACR is only 70–75% [10]. As a result, while dd-cfDNA is a valuable screening tool, it cannot reliably distinguish between different causes of graft injury and should not be used as a standalone diagnostic test to rule in ACR. Furthermore, specificity for dd-cfDNA is around 80–85% [10]. Another limitation of dd-cfDNA is its relatively low positive predictive value (PPV),

which hovers around 8–20%, underscoring the importance of interpreting elevated values within the broader clinical context [4,10,19]. Additionally, dd-cfDNA detection relies on advanced molecular techniques, such as next-generation sequencing or digital droplet PCR, which may not be widely available across all transplant centers, potentially limiting access to this technology [12]. Timing also poses a challenge, dd-cfDNA levels are typically elevated in the immediate post-transplant period due to surgical and ischemic injury, making it unreliable for detecting rejection within the first month [10]. Furthermore, technical issues related to sample handling can affect test accuracy. For example, delayed processing of blood samples may result in contamination with recipient nuclear DNA due to cell lysis, even when using specialized blood collection tubes, compromising the integrity of the results [10]. In addition to these pre-analytical limitations, performance data show that both standard and expanded SNP dd-cfDNA tests have low sensitivity (39%, p = 1.0) but high specificity (82% and 84%, p = 1.0) for detecting ACR, while gene expression profiling (GEP) did not improve sensitivity and demonstrated even worse specificity compared to standard dd-cfDNA (p<.001) [16]. Moreover, Kim *et al.* found that dd-cfDNA levels rise above cutoff for ACR detection after 24 months following transplant which makes using dd-cfDNA less reliable for long term management [17].

Although extensive research has been performed with dd-cfDNA platforms, it has not replaced EMB as the gold standard. Overall, its high NPV enables ruling out ACR, but due to its low PPV and inability to distinguish ACR versus AMR, a positive test mandated by EMB is required for ACR diagnosis (**Table 1**). dd-cfDNA also has not been extensively studied to determine if it can enable noninvasive monitoring of treatment efficacy for ACR. Combining dd-cfDNA with other biomarkers, such as gene expression profiling or immune cell phenotyping, may enhance diagnostic accuracy [18].

Table 1. Summary of cfDNA studies.

Study	Sensitivity	Specificity	PPV	NPV	Conclusions
Agbor-Enoh *et al.*, Circulation 2021 [10]	89% (ACR), 100% (AMR)	78%	~20%	99.2% at 0.25% threshold	Unreliable in first month post-transplant due to elevated baseline; dd-cfDNA detectable up to 3.2 months before rejection. Concludes dd-cfDNA can reduce biopsy burden and differentiate ACR vs AMR using fragmentomics.
Deshpande *et al.*, Pediatr Transplant 2022 [11]	Not specified	Not specified	Not specified	High; dd-cfDNA correlated with clinical rejection	Used in pediatric patients; dd-cfDNA tracked resolution of rejection after treatment. Supports clinical concordance and post-rejection monitoring utility.
Sorbini *et al.*, JHLT 2021 [12]	84%	73%	Not specified	93%	dd-cfDNA higher in rejection; limited by requirement for HLA-DRB1 mismatch. Suggests potential for broader application with further validation.
Lo *et al.*, Lancet 1998 [14]	Proof-of-concept study	Not applicable	Not applicable	Not applicable	Early proof-of-concept showing donor DNA in plasma post-transplant. Laid groundwork for future cfDNA research.
Knüttgen *et al.*, Transplantation 2022 [13]	83%	78%	Not specified	High	Validated cfDNA as a clinical tool; noted influence of confounding factors (e.g., infection). Concludes cfDNA is useful but context-dependent.
Agbor-Enoh *et al.*, JHLT 2016 (GRAfT) [15]	Not specified	Not specified	Not specified	81% at 0.3% threshold	Early multicenter study establishing dd-cfDNA thresholds (0.3%) with moderate NPV. Suggested potential to reduce unnecessary biopsies.
North *et al.*, PLoS One 2020 [71]	High (assay validation)	High (assay validation)	Not specified	Not specified	Sample degradation due to delayed processing and nuclear DNA contamination noted. Emphasized importance of timely handling and proper storage.
Qian *et al.*, Curr Opin Organ Transplant 2022 [18]	Review summary of multiple studies	Review summary of multiple studies	~20%	Up to 99% in well-designed assays	Summarized field advances; emphasized dd-cfDNA's high NPV and limited PPV. Stressed integration of cfDNA with other biomarkers for rejection monitoring.

Study	Sensitivity	Specificity	PPV	NPV	Conclusions
Kim *et al.*, Am J Transplant 2025 [72]	86.5%	83.6%	33.58%	98.5%	Prospective study (n=187) introducing two-threshold algorithm combining dd-cfDNA fraction (0.26%) and donor-quantity score (18 copies/mL). Exceeding either threshold indicates positive result. Improved performance over single threshold approach with excellent NPV for ruling out rejection.
Richmond *et al.*, JHLT 2020 [36]	78.5%	76.9%	25.1%	97.3%	Multicenter prospective study (n=223 patients, 811 samples) including pediatric and adult recipients. Optimal threshold of 0.15% dd-cfDNA with AUC 0.86. Novel SNP-based test demonstrates good accuracy for non-invasive rejection surveillance. Cell lysis can affect results.

Gene Expression Profiling

By analyzing the transcriptional activity of immune-related genes in peripheral blood mononuclear cells, gene expression profiling (GEP) provides a potential window into the underlying immune activation associated with ACR. GEP is one of the most extensively studied surveillance strategies for ACR and is among the few with a clinically validated and FDA-approved test [20–22]. It is primarily employed to detect low to moderate grades of ACR in stable patients beyond 55 days post-transplant [4]. For example, the AlloMap test, which is FDA approved, utilizes a panel of 11 genes, including those involved in T-cell activation and inflammation, to stratify patients by rejection risk [23].

Recently, Pham *et al*. conducted a multicenter randomized clinical trial demonstrating that GEP of peripheral blood samples offers a viable alternative to routine EMB for monitoring rejection in cardiac transplant recipients [21]. Their findings indicated that GEP may be effective as a surveillance tool for patients at low risk for ACR after six months following transplant [21]. The trial revealed that compared to routine EMBs alone, treatment plans including GEP monitoring did not increase risk of adverse outcomes and significantly reduced the number of biopsies necessary for effective treatment [21]. However, the study has several limitations. First, the study population consisted primarily of stable, low-risk patients, which limits the generalizability of the findings to higher-risk or early post-transplant cohorts [24]. Additionally, only about 20% of eligible patients were enrolled in the study, raising the possibility of selection bias [24]. Furthermore, the study's noninferiority design allowed for a relatively wide margin, and the 95% confidence interval did not exclude the possibility of a 68% increase in primary events in the GEP group [21,24]. This statistical uncertainty limits the conclusiveness of the findings. Also, out of 34 rejection episodes in the GEP group, only six were detected solely by the gene-expression profiling test [21]. The majority were identified through clinical assessment or other means, suggesting that GEP may not be sufficient as a standalone monitoring tool. Lastly, the study had limited power to definitively conclude that GEP could replace routine biopsies, and the median follow-up period was 19 months [21].

In further support of GEP as a valid method for low grade ACR detection, Leiro *et al*. demonstrated that GEP could reliably identify the absence of moderate to severe rejection, with a high NPV of 98.1% to 100%, enabling the avoidance of biopsies in specific clinical contexts [25]. The CARGO II study reinforced prior findings by showing that GEP has a consistently high NPV across diverse patient populations, supporting its utility as a reliable, non-invasive tool for ruling out clinically significant rejection in stable heart transplant recipients [25]. These studies highlight the potential utility of GEP in risk stratification and ACR monitoring, particularly in low-risk patient populations.

Despite its strengths, the test demonstrated a low positive predictive value (2.0% to 4.7%) despite a high negative predictive value, meaning that positive results did not reliably indicate true rejection and often required confirmatory biopsies [25]. This may subject patients who do not have ACR to the procedural risks associated with EMB based on false positive GEP testing. Additionally, GEP is limited to the detection of acute cellular rejection and does not assess for antibody-mediated rejection (AMR), potentially missing other clinically important forms of allograft injury [26,27]. Finally, the test's utility is constrained by its dependence on biopsy for confirmation in cases of positive or ambiguous results, limiting its role as a standalone diagnostic tool [27]. Given some of these limitations associated with GEP, researchers are investigating the diagnostic utility of combining GEP with other platforms such as dd-cfDNA to improve accuracy.

MicroRNAs

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA molecules that regulate gene expression by inhibiting mRNA translation or promoting mRNA degradation [28]. By acting within complex miRNA-mRNA networks, miRNAs are able to modulate multiple genes simultaneously, as well as control biological pathways and cellular processes [29]. Their detectability in biofluids makes miRNAs highly attractive for clinical diagnostic applications [29]. Specifically, their inclusion in exosomes and microvesicles, binding to lipoproteins, and interactions with Argonaute 2 protein complex make them less susceptible to degradation by ribonucleases in blood [30,31]. Furthermore, their specificity for distinct biological processes means expression patterns of miRNAs may reflect molecular mechanisms associated with ACR, positioning them as potential biomarkers for transplant monitoring [32].

Several studies have highlighted the diagnostic potential of miRNAs in ACR and AMR. Wei *et al*. identified that miR-182 is significantly elevated in peripheral blood mononuclear cells and plasma during graft rejection, suggesting its utility as a biomarker for graft status [33]. Constanso-Conde *et al*. validated miR-185a-5p as a marker for ACR, achieving an area under the curve (AUC) of 0.80 and a NPV of 98% in a cohort of 121 patients [34]. More recently, Kennel *et al*. identified elevated miR-139-5p, miR-151a-5p, and miR-186-5p levels as a marker of rejection, and miR-29c-3p as predictive of subsequent ACR development [35]. Collectively, these studies demonstrate that miRNA analysis holds promise as a candidate biomarker platform for ACR surveillance in heart transplantation.

However, despite growing evidence supporting the utility of microRNAs (miRNAs) as non-invasive biomarkers for detecting ACR and AMR, several limitations constrain their clinical adoption. A major challenge lies in the variability of identified miRNA signatures across studies, reflecting differences in patient populations, transplant types, immunosuppressive regimens, and assay methodologies. While some studies report high NPVs, such as 98% for miR-181a-5p, the PPVs are often unreported or modest, limiting their confirmatory power [34]. Moreover, the lack of standardized protocols for miRNA quantification and normalization introduces technical variability that hampers reproducibility [32]. Sample sizes are frequently small, and validation in large, multicenter cohorts remains scarce. Finally, the temporal relationship between miRNA expression and rejection episodes is incompletely understood, raising questions about their role as early predictive markers versus concurrent indicators of graft injury [32,33,36]. To address these limitations, ongoing research is exploring the integration of miRNA profiling with complementary biomarkers such as donor-derived cell-free DNA (dd-cfDNA) and proteomic signatures to enhance diagnostic precision and clinical applicability.

Proteomics and Metabolomics

Proteomics and metabolomics may serve as powerful tools for identification of unique protein and metabolite signatures associated with ACR versus allograft quiescence [37]. These signatures might enable biomarker discovery that may not be detectable through traditional diagnostic approaches. For instance, proteomics can detect alterations in extracellular matrix proteins and complement pathways, while metabolomics reveals disruptions in metabolic pathways like energy metabolism and oxidative stress during rejection episodes.

Cardiac troponins (cTn) T and I are proteins exclusively expressed in cardiomyocytes and are well-established biomarkers for myocardial injury due to their high sensitivity and specificity [38]. Advances in high-sensitivity immunoassays (hs-cTn) have enabled the detection of extremely low troponin concentrations, prompting investigation into their utility for ACR monitoring in heart transplant patients. Fitzsimons *et al*. evaluated the performance of cTn assays in detecting ACR and reported a sensitivity range of 8% to 100% and a specificity range of 13% to 88%. Notably, the positive predictive value (PPV) was generally low, often below 50%, indicating limited utility in confirming rejection when troponin levels were elevated. In contrast, the negative predictive value (NPV) was consistently higher, ranging from 79% to 100%, suggesting that normal cTn levels could be somewhat reassuring in excluding significant rejection [39,40].

However, significant heterogeneity in assay types, rejection grading criteria, sampling intervals, and cut-off thresholds undermined the reliability of cTn as a diagnostic tool. Furthermore, their 2022 study specifically assessed hs-cTnI in a cohort of heart transplant recipients and similarly concluded that hs-cTnI was not associated with biopsy-proven ACR (≥2R) [39]. The receiver operating characteristic (ROC) analysis yielded an area under the curve (AUC) of only 0.509, indicating no discriminatory capacity [39]. The authors suggested that elevated troponin levels in transplant recipients likely reflect non-rejection-related myocardial injury, such as ischemia-reperfusion injury, infections, or allograft vasculopathy, rather than immunologically mediated rejection [39]. Together, these studies underscore the lack of diagnostic specificity of cTn assays for ACR and support the conclusion that troponin, despite its sensitivity to myocardial injury, is not a reliable standalone biomarker for rejection surveillance in heart transplant patients.

Additionally, Linda *et al*. assessed biomarkers such as NT-proBNP, troponin T, and C-reactive protein and found that their levels prior to acute rejection did not reliably predict ACR occurrence [41]. Taken together, these studies underscore the current limitations of troponin-based assays in diagnosing

ACR at this juncture. However, future analyses and integration of proteomic and metabolomics data with other platforms may enhance diagnostic accuracy and reliability.

Cytokine and Chemokine Profiling

Cytokine and chemokine profiling may provide a valuable approach for detecting ACR in heart transplant recipients by reflecting the immune response associated with ACR. These soluble mediators, such as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and chemokines like CXCL9 and CXCL10, play central roles in T-cell activation, recruitment, and inflammatory signaling [42]. Specifically, the odds of identifying ACR on endomyocardial biopsy increased significantly with rising concentrations of the inflammatory markers CXCL10 and CD5 [43]. A two-fold increase in CXCL10 levels was associated with more than double the odds of detecting ACR (odds ratio [OR] = 2.2; 95% confidence interval [CI], 1.3–3.6), while a similar increase in CD5 levels was linked to a nearly five-fold elevation in the odds of ACR (OR = 4.7; 95% CI, 1.7–12.9) [43]. These findings suggest that both CXCL10 and CD5 may serve as valuable non-invasive biomarkers for the detection and monitoring of acute cellular rejection, potentially aiding in the early identification of allograft inflammation and guiding timely clinical interventions.

Furthermore, Whang *et al*. found that cytokines and chemokines are differentially expressed in patients with ACR when compared to control [44]. Kong *et al*. found that CXCL10+Gbp2+ subcluster of cardiac fibroblasts (CFBs) were enriched within grafts at the later phase of acute rejection after heart transplantation [45]. These CFBs exhibit strong interferon responsiveness and high expression of chemokines, indicating their role in immune cell recruitment and activation. This highlights the potential of targeting CXCR3 for therapeutic intervention in transplant rejection. Additionally, the expression of the chemokine receptor CXCR3 and its ligand IP-10 has been strongly associated with episodes of acute rejection. Specifically, the presence of $CXCR3^+$ T cells and elevated levels of IP-10 within endomyocardial biopsy specimens has shown a significant correlation with histopathological evidence of acute rejection [46]. The odds of identifying acute rejection were markedly increased in the presence of $CXCR3^+$ T cells (odds ratio [OR] = 11.73) and IP-10 (OR = 4.05), indicating a strong association between these immune markers and rejection pathology [46]. These associations were highly significant ($p<0.001$), underscoring their potential utility as biomarkers for detection and risk stratification in heart transplant recipients [46]. However, it is unclear whether the changes noted in the allograft biopsy translate to significant changes in the blood.

Despite the promising associations of cytokines and chemokines such as CXCL10 and CD5 with ACR, several limitations hinder their routine clinical application. One major concern is specificity, these biomarkers can be elevated in various inflammatory conditions beyond ACR, potentially leading to false-positive results [43,47]. Additionally, their expression exhibits temporal variability, with levels fluctuating over time and not always aligning with histopathological findings, making the timing of sample collection critically important [45]. Moreover, the immune response in ACR is inherently complex, involving a broad network of cells and signaling pathways, and measuring a limited subset of cytokines or chemokines may fail to capture the full immunological landscape [45,48]. Technical challenges further complicate cytokine use, as accurate quantification requires specialized laboratory techniques and consistent sample handling, both of which can introduce variability [49]. Finally, the effects of immunosuppressive therapy, commonly administered in transplant patients, can alter biomarker profiles, making it difficult to distinguish true rejection from drug-induced changes [50]. Therefore, while cytokine and chemokine profiling holds promise for detecting ACR, these limitations highlight the need for further validation and standardization before it can be widely adopted in clinical practice.

Extracellular Vesicles

Extracellular vesicles (EVs), including exosomes, are being extensively investigated as promising biomarkers for several disease states, especially in cancer and neurodegenerative disorders [51–55]. Their ability to carry bioactive molecules reflective of the conditional states of their cells of origin, thus offering a rich, noninvasive source of molecular information makes them ideal candidates for non-invasive diagnostic and prognostic tools [37]. Recent studies have demonstrated their clinical potential across a wide range of conditions, including cardiovascular disease, central nervous system disorders, and regenerative medicine applications, highlighting their versatility as both biomarkers and therapeutic agents [51–55].

The diagnostic potential of EVs for ACR surveillance in heart transplant patients has been investigated by several groups. Castellani *et al.* investigated the utility of EVs as a link between allograft phenotypes, biochemical indices, and histological parameters for the detection of ACR. Their findings revealed a significant increase in circulating EV concentration and a decrease in their diameter in patients experiencing ACR and AMR [56]. Specific EV surface markers, such as CD3, CD2, ROR1, SSEA-4, HLA-I, and CD41b, were identified as discriminants between controls and ACR patients, while markers like HLA-II, CD326, CD19, CD25, CD20, ROR1, SSEA-4, HLA-I, and CD41b differentiated controls from AMR patients [56]. A diagnostic model built upon differential EV-marker expression achieved an accuracy of 86.5% in an independent validation cohort [56]. Additionally, the Mean fluorescence intensity (MFI) for EV-

carried HLA-I, CD2, and SSEA-4 displayed a sensitivity of 100% in the diagnosis of ACR, with specificities ranging between 63.6% and 87.9% [56].

Kennel *et al*. explored the proteomic profiles of serum-derived exosomes during rejection episodes, identifying 15 differentially expressed proteins associated with the ACR process [57]. These proteins included several with known immunologic relevance, suggesting they may reflect ongoing immune activation during rejection. Principal component analysis demonstrated distinct clustering between rejection and non-rejection samples, underscoring the discriminatory potential of exosomal protein signatures [57]. Together, these studies highlight the potential promise of EV-based diagnostics for ACR surveillance in heart transplantation, offering a non-invasive alternative to endomyocardial biopsy. Notably, eight of the differentially expressed proteins were found to play direct roles in immune and inflammatory pathways, supporting their biological plausibility as biomarkers [57]. Together, these studies highlight the potential promise of EV-based diagnostics for ACR surveillance in heart transplantation.

Our group has been investigating the diagnostic potential of EVs in ACR for the past decade [49,58–68]. Our work has focused on studying the quantitative cargo profiles of enriched sub-populations of EVs as biomarkers of ACR and AMR [49,66]. In the animal model, we demonstrated that these subpopulations of EVs are specifically altered during ACR [60,61]. Extrapolating these results to the clinical setting, our group investigated cell specific EVs in heart transplant patients, which demonstrated ACR detection with high diagnostic accuracy [69]. Accordingly, we reported that a composite panel of small EV cargoes for donor heart small EVs and T-cell small EVs demonstrated a sensitivity and specificity >95% for ACR detection [66,69]. Therefore, future studies may enable the clinical translation potential of cell specific small EV platforms.

A key strength of EVs lies in their stability in biological fluids [49,66]. This stability coupled with their ability to preserve their cargo under various conditions, makes EVs suitable for non-invasive, real-time monitoring [70]. Additionally, EVs provide a snapshot of immune cell dynamics and allograft status, enabling the identification of rejection-specific molecular signatures [70]. However, one of the challenges in implementing EVs as a diagnostic tool is the need for standardization of EV isolation and characterization methods, which can vary widely and impact the reproducibility of findings. Moreover, the small size and heterogeneity of EV populations can complicate their analysis, and the specificity of EV-derived markers for ACR compared to other forms of graft dysfunction is still under investigation. Additionally, successful translation into clinical practice will require further validation in large, multicenter studies, along with the development of standardized, high-throughput platforms for EV analysis. However, EV-based diagnostics hold promise as novel biomarkers for ACR surveillance in heart transplantation.

Conclusion

Emerging evidence suggests that non-invasive, blood-based biomarker platforms hold significant promise for monitoring cardiac allograft health. Potential biomarkers such as dd-cfDNA, EVs, and miRNAs have shown encouraging results and warrant further investigation. Ongoing large-scale studies aim to refine the diagnostic performance of these biomarkers and establish standardized protocols for their clinical application. Prospective multicenter clinical trials are also needed to evaluate their utility as rule-out tests for rejection and as tools for guiding immunosuppressive therapy.

The lack of standardized methodologies and protocols has hindered the widespread adoption of biomarkers in post-transplant care. Comparatively low PPV and sensitivity have precluded the widespread use of these noninvasive platforms, as EMB remains the gold standard. While individual biomarkers show promise, future research needs to explore the synergistic potential of combining these biomarkers to enhance diagnostic accuracy.

Funding

None.

Conflict of Interest

PV is consultant for Terumo Aortic, Edwards Lifesciences.

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