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Original research article

Elevated plasma levels of cell-free mtDNA are associated with acute rejection following heart transplantation

Dana Dlouhá [1] *, Kristýna Janoušková [1], Šárka Chytilová [2], Jevgenija Vymětalová [3], Marianna Lukášová [3], Šárka Nováková [1], Eva Rohlová [4], Jaroslav A. Hubáček [1, 5]

[1] *Institute for Clinical and Experimental Medicine, Center of Experimental Medicine, Prague, Czech Republic.*
[2] *Institute for Clinical and Experimental Medicine, Department of Data Science, Prague, Czech Republic*
[3] *Institute for Clinical and Experimental Medicine, Cardio Center, Prague, Czech Republic*
[4] *Institute of Molecular Genetics of the Czech Academy of Sciences, Laboratory of Genomics and Bioinformatics, Prague, Czech Republic*
[5] *Charles University, First Faculty of Medicine, Third Department of Internal Medicine, Prague, Czech Republic*

Abstract
Acute rejection (AR) following heart transplantation (HTx) is a common complication, especially in the early post-HTx period. Mitochondrial DNA (mtDNA), released into circulation from stressed mitochondria, mimics ongoing immune activation and facilitates the release of pro-inflammatory substances. Our study aimed to assess cell-free mtDNA levels to identify early indicators of acute rejection progression.

The absolute concentration of cf-mtDNA (cp/μl) was measured in 77 adult patients using quantitative polymerase chain reaction. Blood samples (n = 300) were collected before their corresponding biopsy according to the timeline within the first year post-HTx. The median cf-mtDNA levels in samples with confirmed AR (n = 57) was higher compared to samples without diagnosed rejection (n = 210; Padj < 0.01). When acute cellular (ACR; n = 39) and antibody-mediated rejection (AMR; n = 18) were analyzed separately, only AMR demonstrated higher levels compared to samples without diagnosed rejection (Padj = 0.02). The highest cf-mtDNA levels were detected in samples collected during early post-HTx complications compared to samples without rejection and AR samples (for both Padj < 0.0001). Both ACR and AMR were observed throughout the one-year period, with the majority (3rd quartile) occurring during the first 200 days post-HTx. Post-HTx complications, such as graft dysfunction or acute kidney injury, were observed within the first 11 days, with the majority (71.4%) occurring within 5 days post-HTx. The presence of AR, and specifically AMR, is associated with elevated levels of cf-mtDNA. The increase in plasma cf-mtDNA levels strongly reflects the occurrence of early complications following HTx.

Keywords: Biomarker; cf-mtDNA; Rejection; Transplantation

Highlights:
- Allograft rejection still poses a risk for the survival of post-transplant patients.
- Mitochondrial DNA (mtDNA) is released into circulation from stressed mitochondria.
- Plasma cell-free mtDNA quantity may serve as an indicator of the graft's condition.

Abbreviations:
ACR, acute cellular rejection; AKI, acute kidney injury; AMR, antibody-mediated rejection; AR, acute rejection; BMI, body mass index; BNP, natriuretic peptide; cf-mtDNA, cell-free mtDNA; cf-nDNA, cell-free nuclear DNA; CRP, C-reactive protein; CVA, cerebrovascular accident; DCM, dilated cardiomyopathy; ddcfDNA, donor-derived cell-free DNA; ECMO, extracorporeal membrane oxygenation; EDTA, ethylenediaminetetraacetic acid; eGFR, estimated glomerular filtration rate; EMB; endomyocardial biopsy; GD, graft dysfunction; HTx, heart transplantation; ISHLT, International Society for Heart and Lung Transplantation; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; mtDNA, mitochondrial deoxyribonucleic acid; non-R, non-rejection; NYHA, New York Heart Association; PGD, primary graft dysfunction; RI, renal insufficiency; RVEF, right ventricular ejection fraction; SGD, secondary graft dysfunction; TIA, transient ischemic attack; Tx, transplantation.

* **Corresponding author:** Dana Dlouhá, Institute for Clinical and Experimental Medicine, Center of Experimental Medicine, Vídeňská 1958/9, 140 21 Prague 4, Czech Republic; e-mail: dadl@ikem.cz
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Introduction

Acute rejection (AR) is a significant medical phenomenon that occurs when the recipient's immune system identifies the transplanted graft as a foreign object and mounts an immune response against it. This reaction can lead to graft dysfunction (GD) or even complete failure of the transplanted organ if not appropriately managed. Over the years, the incidence of acute cellular rejection (ACR) has significantly declined, largely due to advancements in immunosuppressive therapies implemented in transplant (Tx) programs (Hertz et al., 2002). However, antibody-mediated rejection (AMR) has emerged as a critical concern, posing a substantial risk for all-cause mortality (Clerkin et al., 2016a; Coutance et al., 2015; Vaughn et al., 2018) and cardiovascular-related deaths (Kfoury et al., 2009). Additionally, AMR is associated with a range of serious allograft injuries that can severely impact heart function. These include conditions such as systolic dysfunction (Clerkin et al., 2016b; Coutance et al., 2018), restrictive physiology (Clemmensen et al., 2017), and cardiac allograft vasculopathy (Loupy et al., 2016; Wu et al., 2009). Endomyocardial biopsy (EMB) is considered the gold standard for diagnosing and monitoring heart transplant (HTx) rejection status (Shah et al., 2015). However, since EMB is an invasive procedure that carries rare but potentially serious complications, researchers have been seeking more accurate and less invasive methods for monitoring heart transplant rejection (Agbor-Enoh et al., 2021; De Vlaminck et al., 2014; Khush, 2021; Khush et al., 2019; Richmond et al., 2020).

Cell-free DNA, including cell-free nuclear DNA (cf-nDNA) and mitochondrial DNA (cf-mtDNA), is typically present in low concentrations in human blood, but during inflammation or organ damage, its levels can significantly increase (Lo et al., 2021). Cell-free DNA can serve as an important biomarker for monitoring patient health following organ Tx, providing insights into recovery and potential complications (De Vlaminck et al., 2014; Schütz et al., 2017). In recent years, donor-derived cell-free DNA (ddcfDNA) was described as a promising biomarker that could improve AR monitoring in heart transplant recipients (Agbor-Enoh et al., 2021; Khush et al., 2019; Richmond et al., 2020).

During HTx, the graft faces hypoxia and ischemia during preservation, leading to metabolic disruption and a heightened stress response (Li and Lan, 2023). Stressed mitochondria release fragments of mtDNA into the bloodstream, which mimics immune activation and triggers the release of pro-inflammatory substances (Newman and Shadel., 2023). This response can significantly impact the success of the graft and patient recovery. Recently, it was reported that higher levels of cf-mtDNA were associated with elevated inflammatory cytokines, indicating early GD in liver Tx (Pollara et al., 2018), and delayed graft function in kidney Tx (Jansen et al., 2020; Kim et al., 2019). Unfortunately, there is a lack of studies in HTx focused on cf-mtDNA detection. Bliksøen et al. (2012) demonstrated that focal myocardial necrosis from a myocardial infarction leads to the release of mtDNA fragments into the bloodstream, with plasma levels correlating to the extent of myocardial damage. The release of cfDNA including cf-mtDNA especially occurs at times of allograft injury, including AR. Cf-mtDNA quantity is abundant compared to cf-nDNA in plasma. A single cell contains hundreds to thousands of copies of mtDNA opposed to two copies of nuclear DNA (Chiu et al., 2023). We hypothesized that examining cf-mtDNA could offer a higher level of sensitivity than analyzing cf-nDNA. Our study aimed to assess cf-mtDNA levels as potential early indicators of AR progression.

Materials and methods

Patient characteristics

Patients who underwent HTx from June 2015 to May 2022 at the Cardio Center of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic, were included in the study. A cohort of 77 patients (women 32.5%; age = 51.2 ± 11.6 years) was analyzed. The basic characteristics of the patients are shown in Table 1.

Table 1. Demographics of the patients

Characteristics	Graft recipients
n (females %)	25 (32.5%)
Age, years	51.2 ± 11.6
BMI (kg/m^2)	26.0 ± 4.1
Ever smokers (%)	49 (63.6%)
Diabetes mellitus (%)	18 (23.4%)
Hypertension (%)	40 (51.9%)
Hyperlipidemia (%)	34 (44.2%)
RI (%)	21 (27.3%)
CVA/ TIA (%)	16 (20.8%)
NYHA classification IV (%)	31 (40.3%)
LVEF <30% (%)	67 (87%)
RVEF <30% (%)	18 (23.4%)
Etiology of non-ischemic DCM (*n*)	43 (55.8%)
Etiology of ischemic DCM (*n*)	22 (28.6%)
Congenital heart defects	5 (6.5%)
Others	7 (9.1%)
LVAD implant before Tx, %	21 (27.3%)
Days of LVAD support (days)	532 (415.5)
Rejection occurrence	
ACR Grade 0	13 (16.9%)
ACR Grade 1	41(53.2%)
ACR Grade 2	6 (7.8%)
ACR Grade 3	1 (1.3%)
pAMR1	12 (15.6%)
pAMR2	4 (5.2%)
Other post-HTx complications	25 (32.5%)
AKI	22 (88%)
Combined AKI + GD	10 (40%)
PGD	8 (32%)
SGD	5 (20%)
Exitus within the 1st year post-HTx	3 (3.9%)

Note: ACR, acute cellular rejection; AKI, acute kidney injury; AMR, antibody-mediated rejection; BMI, body mass index; CVA, cerebrovascular accident; DCM, dilated cardiomyopathy; GD, graft dysfunction; NYHA, New York Heart Association; PGD, primary graft dysfunction; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; RI, renal insufficiency; RVEF, right ventricular ejection fraction; SGD, secondary graft dysfunction; TIA, transient ischemic attack. Categorical data are presented as the number (%), and continuous data are presented as the means ± SDs.

Patients were subjected to standard clinical practice. Histology, immunohistochemistry, and EMB were performed according to standard protocols and guidelines (Berry et al., 2013; Cooper et al., 2007; Stewart et al., 2005). Rejection grading was performed according to International Society for Heart and Lung Transplantation (ISHLT) consensus statement (Berry et al., 2013; Stewart et al., 2005). ACR grade 0 occurred in 13 patients; ACR grade 1R in 41 patients; ACR grade 2R in 6 patients; and ACR grade 3R in 1 patient. pAMR 1 (H+) in 12 patients; pAMR 2 in 4 patients. Early GD was defined according to the ISHLT consensus definition (Kobashigawa et al., 2014).

Corticosteroids and tacrolimus were administered as initial immunosuppressive therapy in all subjects. Biochemical parameters were analyzed through standard routine procedures in the central biochemical laboratory of the institute. Biochemical markers of heart and renal function or graft damage, such as troponin, brain-type natriuretic peptide (BNP), C-reactive protein (CRP), estimated glomerular filtration rate (eGFR), and creatinine was collected for each patient and compared with other analyzed markers.

Criteria for exclusion from the study

Exclusion criteria for the study included: i) repeated HTx (N = 2), ii) pregnancy (N = 0), iii) cancer (N = 0), and iv) insufficient blood sampling during planned endomyocardial biopsy (N = 5).

Grouping of samples before the analysis

According to the clinicians' recommendations, the plasma samples were categorized into the following groups: non-R (including ACR and AMR grade 0 and ACR grade 1A/1B, n = 210), ACR (including samples with diagnosed ACR grade ≥2; n = 39), and AMR (including samples with diagnosed pAMR grade 1 (H+) and pAMR grade 2; n = 18). Samples with other post-HTx complications (n = 25) were samples drawn during the extracorporeal membrane oxygenation (ECMO, n = 4) support and/or continuous renal replacement therapy (CRRT; n = 24). Patients with ECMO were diagnosed with PGD (n = 2) or SGD (n = 2). Three of them required both CRRT and ECMO support. Each sample was categorized based on the most prominent feature observed, meaning that in cases where rejection and post-HTx complications were present, the sample was classified as post-HTx complication (6 cases). Similarly, when both ACR and AMR were detected, the sample was classified as AMR (1 case). Due to the small sample sizes, samples of patients with dual chamber pacemaker implantation (n = 1); or diagnosed with post-drug pancytopenia (incl. thrombocytopenia; n = 3); sepsis and cholecystitis (n = 1), chronic leukocytosis (n = 1), 3rd degree atrioventricular block (n = 1), and sample drawn immediately after EMB (n = 1) were finally excluded from the statistical analysis.

Blood sample collection

Blood samples (10 ml) were collected in EDTA-containing tubes according to the timeline for performing EMBs (before their corresponding biopsy) (Suppl. Table S1). Blood samples were centrifuged at 1500 × g for 15 min at room temperature in the first step. In the second step, plasma was centrifuged at 16 000 × g for 10 min at 5 °C. Samples were processed within 30 min of blood collection, aliquoted into RNase-free tubes, and stored at –80 °C before cfDNA extraction.

Cf-mtDNA analysis

CfDNA including cf-mtDNA, was extracted from 400 µl of plasma using a Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit slurry (cat. no. 50600; Norgen Biotek). CfDNA was eluted in a final volume of 100 µl. Absolute quantification of the MT-ND5 and MT-CO3 human mitochondrial genes was conducted using a qPCR instrument (Bio-Rad; CFX384). In total, 300 plasma samples were measured.

Cf-mtDNA measurement

PCR reaction mix in a volume of 10 µl containing 5 µl of TATAA SYBR GrandMaster Mix (2×) (TATAA Biocenter AB), 2 µl of eluted cfDNA, 0.4 µl of primer mixture (10 µM; Generi Biotech; for primer sequences, see Suppl. Table S2), and 2.6 µl of nuclease-free water. The samples were analyzed in two replicates. The thermocycling conditions were 95 °C for 30 s and 35 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 s. Melting curve analysis was used to evaluate the specificity of the assay. A dilution series from a known concentration of DNA template was prepared and analyzed together with the samples. A series of seven 10-fold dilution points in four replicates was used to create a standard curve for calculating the concentration of cf-mtDNA in the patient samples. The qPCR concentration results were converted to a plasma-equivalent concentration (cp/µl) using the following equation:

$$C_{cf-mtDNA} = \frac{Q \cdot V_{elution}}{V_{cfDNA}} \cdot \frac{1}{V_{plasma}}$$

where Ccf-mtDNA is the concentration of the target within the plasma in cp/µl; Q is the quantity of cf-mtDNA measured by qPCR in cp/rxn; Velution is the volume of cfDNA solution obtained from extraction (100 µl); VcfDNA is the volume of cfDNA used in the qPCR (2 µl); and Vplasma is the volume of plasma used for cfDNA extraction (400 µl).

Storage time of plasma as a preanalytical factor

Because the patient selection for the study was retrospective and based on EMB results, there are differences in the time from plasma collection to the isolation of cf-mtDNA [plasma storage time (ST)]. The median ST duration was 1,187 (IQR = 736–1,795) days. Therefore, we investigated the ST as a preanalytical factor that could influence cf-mtDNA quantity in plasma. No clear linear relationship between ST and cf-mtDNA levels was observed; instead, a slight non-linear decreasing trend was detected (Fig. 1). While there was no significant effect of ST on the prediction of either ACR or AMR in the multivariate analysis, including ST as a non-linear covariate significantly improved the model's predictive performance for ACR.

Statistical analyses

Statistical analyses were performed utilizing R software (version 4.4.1). As both markers exhibited similar changes in cf-mtDNA concentration at post-HTx time points, with an overall Spearman correlation coefficient of 0.92 (Fig. 2A), the average cf-mtDNA quantity of both markers was used in subsequent analyses (referred to as "mean mtDNA"). To examine the impact of cf-mtDNA on ACR and AMR, we performed univariate analysis using the Kruskal–Wallis test with Dunn's *post hoc* test, as well as multivariate analysis through logistic regression. For logistic regression, ACR, AMR, and post-HTx complications were modeled separately with two approaches: i) using all post-HTx time points with patient-specific ran-

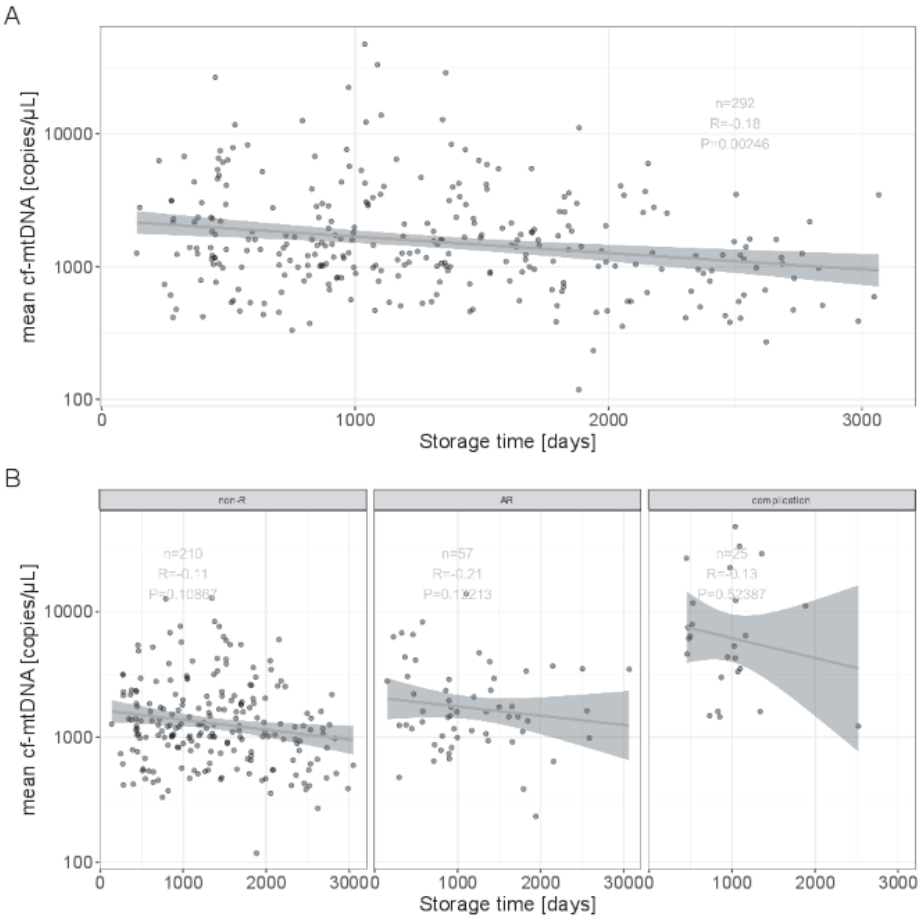


Fig. 1. Correlation between cf-mtDNA concentration and storage time of plasma. (**A**) all samples analyzed together (**B**) samples grouped to non-R, AR, and post-HTx.

dom effects, adjusting for storage time, results of previous follow-up (rejection/complication), and complications; and ii) restricting the analysis to the initial 0–9 days to predict future rejection events. Regression analyses were conducted using the following packages: rms (version 6.8-2), lme4 (version 1.1-35.5), and stats (version 4.4.1). A p value < 0.05 was considered statistically significant.

Results

Cf-mtDNA quantity and rejection (univariate analysis)

Both ACR and AMR were observed throughout the one-year period, with the majority (3rd quartile) occurring during the first 200 days post-HTx.

We measured two different markers (MT-ND5 and MT-CO3) to eliminate bias in the determination of cf-mtDNA concentration. We observed a similar trend in the amount of circulating cf-mtDNA for both markers during post-HTx days. As MT-ND5 and MT-CO3 were in strong correlation (Padj < 0.001; r = 0.92, Fig. 2A), for further analyses we used the means of the measured cf-mtDNA quantity. We observed a slight non-linear decreasing trend in cf-mtDNA concentration within the 1st year following HTx (see Fig. 2B).

The results of univariate analysis, utilizing all time points post-HTx, showed higher cf-mtDNA levels in AR samples with the median 1,586 (IQR = 1,022–2,938 cp/µl) compared to non-R samples with the median 1,209 (IQR = 778–2,026 cp/µl), (Padj < 0.01; Fig. 3A and 3B). When ACR and AMR were analyzed separately, only AMR with the median 1,913 (IQR = 1,064–4,109 cp/µl) demonstrated higher levels compared to non-R samples (Padj = 0.02). No significant difference or trend was observed when comparing ACR and non-R samples (Fig. 3C and 3D).

Occurrence of other post-HTx complications

Post-HTx complications, such as primary graft dysfunction (PGD) or acute kidney injury (AKI), were observed within the first 11 days, with the majority (71.4%) occurring within 5 days pos-HTx (see Suppl. Fig. S1). PGD manifested as severe ventricular dysfunction was diagnosed in eight patients during surgery. Secondary graft dysfunction manifested as pulmonary hypertension (N = 1), bleeding (N = 2), and by combined pulmonary hypertension and bleeding (N = 2). AKI was diagnosed in 22 patients.

In samples drawn during early post-HTx complications, we observed elevated levels of cf-mtDNA with the median 6,105 (IQR = 3,326–11,749 cp/µl) compared to non-R samples (Padj < 0.0001; Fig. 3A and 3B). In some cases, AR was diagnosed alongside post-HTx complications (n = 6). These samples showed a slight increase of cf-mtDNA levels. However, the variance was large and no performed analysis confirmed

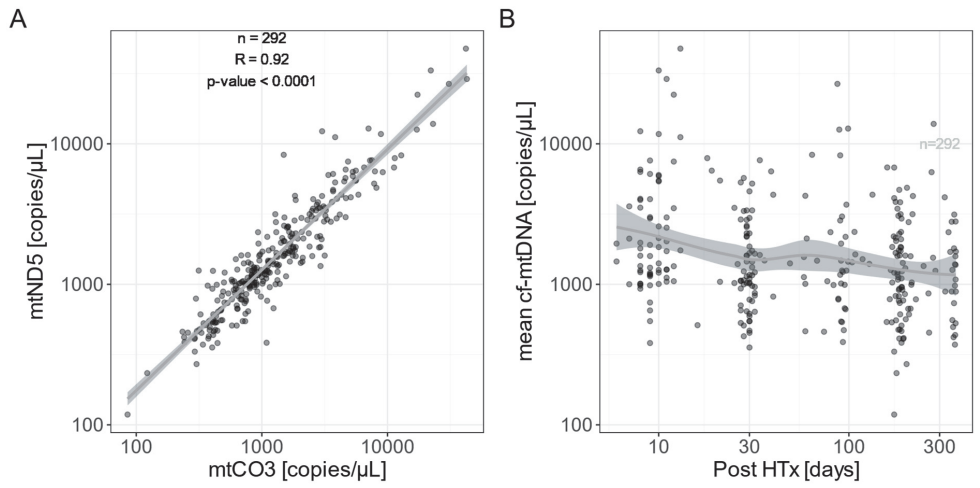


Fig. 2. Cf-mtDNA concentration during the post-HTx time (days). (**A**) Correlation between measured cf-mtDNA markers; (**B**) The decline of mean mtDNA levels within post-HTx days.

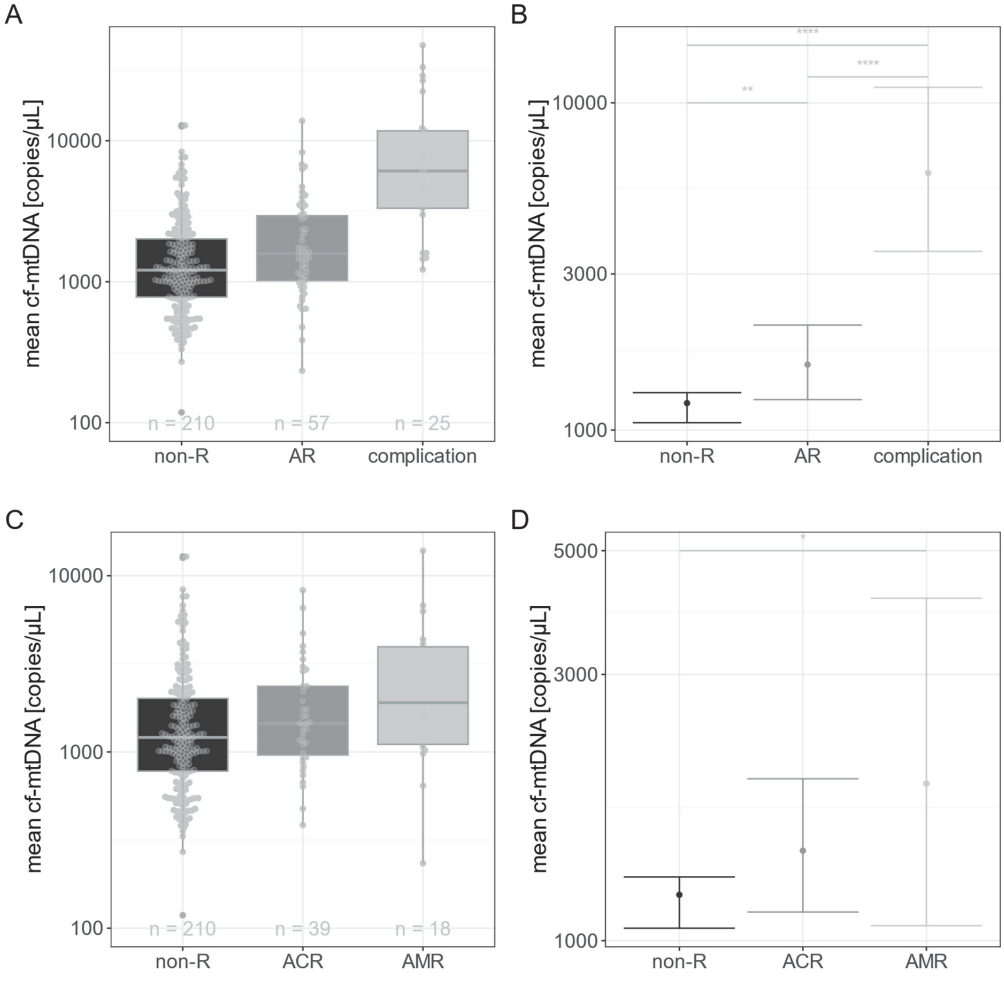


Fig. 3. Cf-mtDNA quantity. (**A**) and (**B**) in samples without rejection (non-R), with AR and with post-HTx complications; (**C**) and (**D**) in samples without rejection (non-R), with ACR and AMR. The results are given on the log10 scale. (**A**) and (**C**) boxes represent interquartile ranges, horizontal lines within boxes show medians, whiskers represent minimum and maximum as defined in methods, and dots are outliers. (**B**) and (**D**) point estimates are medians, and confidence intervals are calculated for the median values. The statistical significance of the results was noted as * for $P < 0.05$, ** for $P < 0.01$, and **** for $P < 0.0001$.

significant difference between rejection levels or significant effect of mean mtDNA on prediction of rejection. Post-HTx complications, with the cf-mtDNA median 5,315 (IQR = 2,993–11,749 cp/µl), exhibited a slight but statistically insignificant upward trend in cf-mtDNA levels when they co-occurred with AR, with the cf-mtDNA median 9,533 (IQR = 4,342–12,296 cp/µl), (Padj = 0.06; Fig. 4).

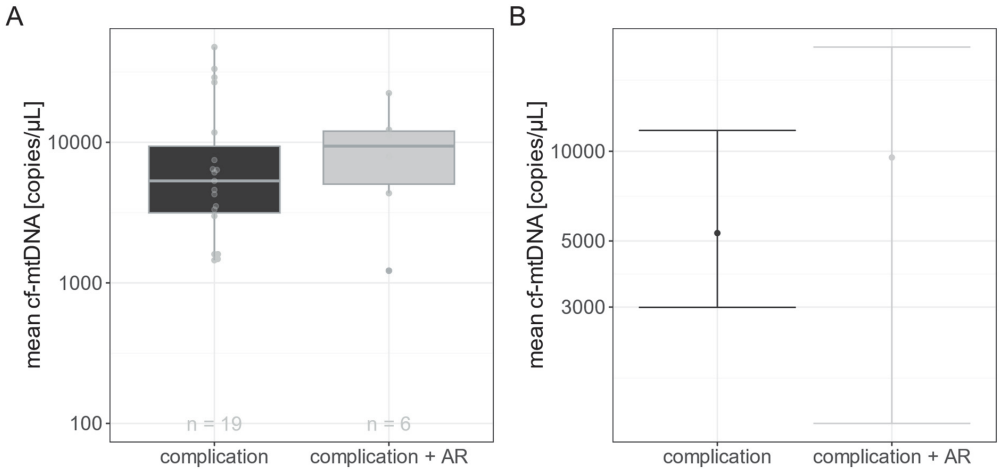


Fig. 4. Cf-mtDNA concentrations in subjects with post-HTx complication and post-HTx complication + AR. (**A**) boxes represent interquartile ranges, horizontal lines within boxes show medians, whiskers represent minimum and maximum as defined in methods and dots are outliers. (**B**) point estimates are medians and confidence intervals are calculated for the median values.

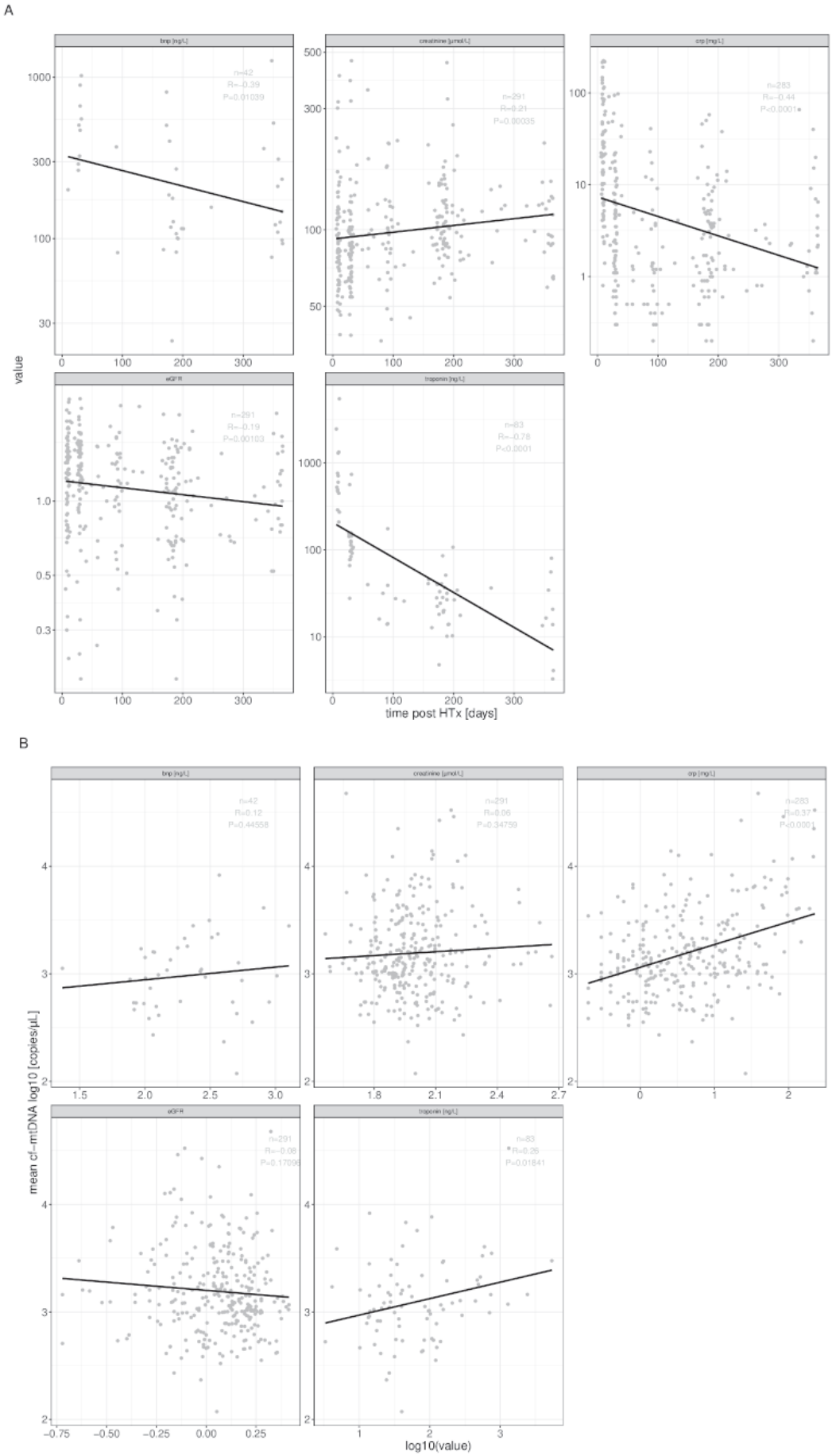
The potential of mean cf-mtDNA as a predictor of rejection (multivariate analysis)

Logistic regression models did not show a significant effect of mean cf-mtDNA levels on the prediction of ACR or AMR in either of the used modeling approaches. Cf-mtDNA was identified as a significant predictor for post-HTx complications when random effect for patients were included together with information about rejection of previous follow-up and storage time as covariates [odds ratio 8.85 (CI 8.8, 8.90), Padj < 0.001; see Suppl. Multivariate analysis.pdf].

Cf-mtDNA and biochemical parameters

Further investigations focused on the relationships between cf-mtDNA levels and biochemical markers of heart and renal function or damage. Post-HTx levels of troponin (R = –0.78), BNP (R = –0.39), CRP (R = –0.44), and eGFR (R = –0.19) showed a non-linear decreasing, and creatinine (R = 0.21) increasing trend (all parameters Padj < 0.05 – spearman correlation, see Fig. 5). Cf-mtDNA quantity correlated with CRP (Padj < 0.001; R = 0.37) and troponin levels (Padj = 0.02; R = 0.26), Fig. 5. Despite significant P-values, the R-values indicate a weak correlation, and the graph's distribution suggests either a non-linear relationship or no relationship at all.

In samples with post-HTx complications, creatinine levels were higher and eGFR lower compared to non-R (for both Padj < 0.001; Fig. 5). CRP levels were elevated in samples with post-HTx complications compared to non-R, as well as compared to AR samples (for both Padj < 0.0001). Additionally, troponin levels were higher in the group with early post-HTx complications compared to non-R and AR (for both Padj < 0.001). BNP levels in AR did not differ significantly from non-R (Padj = 0.08), likely due to the absence of serial BNP measurements.


A
bnp [ng/L]
n=42
R=−0.39
P=0.01039
creatinine [µmol/L]
n=291
R=0.21
P=0.00035
crp [mg/L]
n=283
R=−0.44
P<0.0001
eGFR
n=291
R=−0.19
P=0.00103
troponin [ng/L]
n=83
R=0.78
P<0.0001
value
time post HTx [days]
B
bnp [ng/L]
n=42
R=0.12
P=0.44558
creatinine [µmol/L]
n=291
R=0.06
P=0.34759
crp [mg/L]
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eGFR
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R=−0.08
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P=0.01841
mean cf-mtDNA log10 [copies/µL]
log10(value)

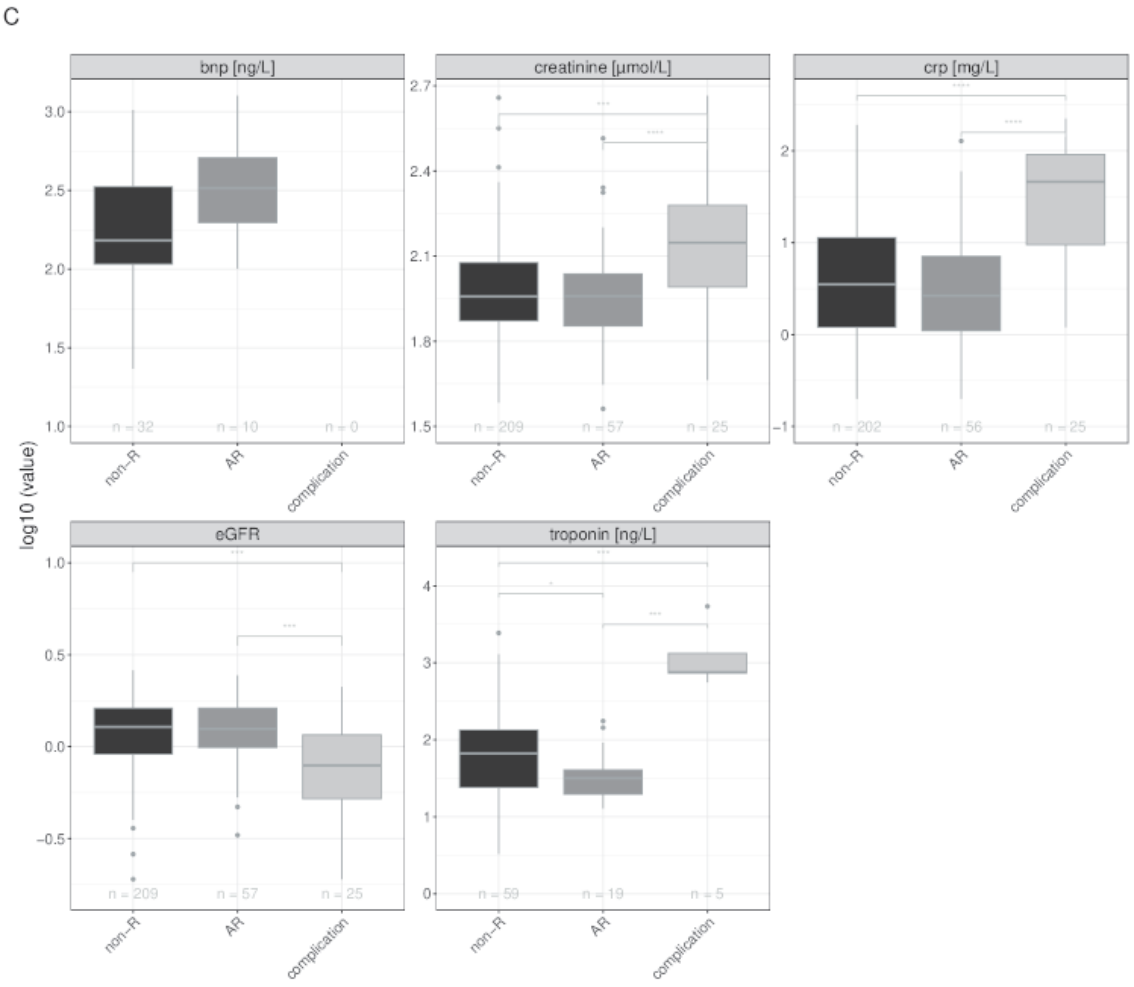

Fig. 5. Biochemical parameters. (**A**) during post-HTx days; (**B**) correlation with cf-mtDNA, and (**C**) according to group of samples when rejection/complications occurred. Boxes represent interquartile ranges, horizontal lines within boxes show medians, whiskers represent minimum and maximum as defined in methods, and dots are outliers. The statistical significance of the results was noted as * for $P < 0.05$, *** for $P < 0.001$, and **** for $P < 0.0001$.

Discussion

In our single-center study, we evaluated plasma cf-mtDNA concentrations as a non-invasive biomarker for AR detection in HTx patients. We found elevated cf-mtDNA quantity during diagnosed AR. When ACR and AMR were analyzed separately, higher levels of cf-mtDNA remained only during AMR. Recent studies indicate a significant correlation between elevated donor cf-mtDNA levels and complications like delayed graft function and AMR in kidney Tx (Han et al., 2019, 2021). Ibrahim et al. (2013) have also shown that increased cf-mtDNA is linked to PGD in lung transplants, highlighting the relevance of mtDNA in assessing transplant viability. Research specifically investigating changes in circulating mtDNA post-HTx is limited. In our recent study (Dlouha et al., 2025), we assessed ddcfDNA concentrations (cp/ml) as a biomarker for AR following HTx. When comparing the complementarity of cf-mtDNA and ddcfDNA, we observed a similar trend in the average amounts of both markers (see Suppl. Fig. S2). As we know from previous studies (Agbor-Enoh et al., 2021; De Vlaminck et al., 2014) and from our measurement of ddcfDNA (Dlouha et al., 2025), higher levels of ddcfDNA were found during AMR compared to ACR diagnosis. Our primary results indicate that using cf-mtDNA can potentially aid in AR detection. However, further analysis incorporating donor-specific cf-mtDNA may enhance measurement sensitivity and specificity.

Further, we found the highest levels of cf-mtDNA during the occurrence of early post-HTx complications, such as AKI or GD requiring extracorporeal membrane oxygenation (ECMO) and/or continuous renal replacement therapy or dialysis. As the majority of patients with post-HTx were affected by AKI, the increase of cf-mtDNA was probably the result of the patient's overall poor condition. This fact is supported by the finding of the highest levels of creatinine, troponin, and CRP, and the lowest value of eGFR during post-HTx complications compared to non-R or AR samples. The amount of cf-mtDNA could also be increased due to mechanic damage to the cells. Previous studies have indicated that increased concentrations of cf-mtDNA are associated with turbulent blood flow through ECMO (Bynum et al., 2017). Turbulent flow may activate clot-

ting cascades or lead to cell lysis (Thurairajah et al., 2018), but the exact mechanism behind mtDNA release in this context has not yet been investigated. Furthermore, exposure of blood to the artificial circuit during ECMO can provoke an inflammatory response (Doo et al., 2022). All focused biochemical parameters showed improvement one year after HTx, reflecting a better condition of the patients.

In our study, the length of plasma storage between the first and last samples varied by seven years (Fig. 1). Randeu et al. (2022) investigated several preanalytical variables, including sample storage time, and reported significantly higher levels of cf-mtDNA in more recently collected samples. While considerable research has been conducted on the preanalytical standardization of cfDNA measurements (El Messaoudi et al., 2013; Greytak et al., 2020; Meddeb et al., 2019; Ungerer et al., 2020), there is still a lack of knowledge regarding many of the preanalytical variables that affect cf-mtDNA measurements. Compared to nuclear DNA, cf-mtDNA molecules are not protected by histone proteins, making them more susceptible to degradation. Additionally, cf-mtDNA exhibits distinct dynamics in the extracellular environment (Randeu et al., 2022).

Our study has several limitations. We quantified total cf-mtDNA, which does not specifically distinguish donor-derived cf-mtDNA. Various pathological conditions in donors and recipients can lead to increased cf-mtDNA levels after HTx. The small sample size hindered our ability to evaluate key graft outcomes like graft failure and acute cellular rejection. Unfortunately, there was a lack of serial biochemical measurements for some parameters, such as BNP or troponin. A weak correlation between some biochemical parameters and cf-mtDNA could be impacted by this factor.

Conclusion

In conclusion, the presence of AR, specifically AMR, is associated with elevated levels of cf-mtDNA. The increase in plasma circulating cf-mtDNA levels strongly reflects the incidence of early post-HTx complications.

Authors' contributions

The original draft was written by *DD* and *JAH*. *JV* and *DD* designed and directed the study. *JV* and *ML* summarized the clinical characteristics, contributed to the interpretation of the results, and edited the article. *ER* and *KJ* designed and performed the experiments, analyzed the data, and wrote the article. *DD* and *ER* observed and verified all the raw data. *SN* coordinated and created the clinical databases. *SC* performed the statistical analysis. *JAH* performed the data review. All authors read and approved the final version of the manuscript.

Data availability

The datasets generated and analyzed during the current study are not publicly available for privacy reasons but are available from the corresponding author upon reasonable request. Raw qPCR data are available in the Mendeley Data repository, https://data.mendeley.com/datasets/k34r564y3j/1

Ethics approval

The study was approved on June 15, 2015 (approval no. G-15-06-15) by the Ethics Committee of the Institute of Clinical and Experimental Medicine and Thomayer Hospital with Multicenter Competence in Prague, Czech Republic. The study was performed according to the Declaration of Helsinki (2000) of the World Medical Association. All subjects signed informed consent forms to participate in the study.

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Conflict of interest

The authors have no conflict of interest to declare.

References

Agbor-Enoh S, Shah P, Tunc I, Hsu S, Russell S, Feller E, et al. (2021). Cell-Free DNA to Detect Heart Allograft Acute Rejection. Circulation 143(12): 1184–1197. DOI: 10.1161/CIRCULATIONAHA.120.049098.

Berry GJ, Burke MM, Andersen C, Bruneval P, Fedrigo M, Fishbein MC, et al. (2013). The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J Heart Lung Transplant 32(12): 1147–1162. DOI: 10.1016/j.healun.2013.08.011.

Bliksøen M, Mariero LH, Ohm IK, Haugen F, Yndestad A, Solheim S, et al. (2012). Increased circulating mitochondrial DNA after myocardial infarction. Int J Cardiol 158(1): 132–134. DOI: 10.1016/j.ijcard.2012.04.047.

Bynum J, Meyer A, Darlington DN, McIntosh C, Peltier G, Taylor AS, et al. (2017). Increased mitochondrial DNA in an ECMO model is associated with loss of platelet function. Blood 130(Suppl. 1): 1126. DOI: 10.1182/blood.V130.Suppl_1.1126.1126.

Chiu RW, Chan LY, Lam NY, Tsui NB, Ng EK, Rainer TH, Lo YM (2023). Quantitative analysis of circulating mitochondrial DNA in plasma. Clin Chem 49(5): 719–726. DOI: 10.1373/49.5.719.

Clemmensen TS, Koefoed-Nielsen P, Jensen LA, Poulsen SH, Holm NR, Løgstrup BB, et al. (2017). Donor-Specific Antibodies Are Associated with Micro- and Macrovascular Coronary Disease, Restrictive Myocardial Damage, and Poor Outcome in Heart-Transplanted Patients. Clin Transplant 31(9). DOI: 10.1111/ctr.13033.

Clerkin KJ, Farr MA, Restaino SW, Zorn E, Latif F, Vasilescu ER, et al. (2016a). Donor-Specific Anti-HLA Antibodies with Antibody-Mediated Rejection and Long-Term Outcomes Following Heart Transplantation. J Heart Lung Transplant 36(5): 540–545. DOI: 10.1016/j.healun.2016.10.016.

Clerkin KJ, Restaino SW, Zorn E, Vasilescu ER, Marboe CC, Mancini DM (2016b). The Effect of Timing and Graft Dysfunction on Survival and Cardiac Allograft Vasculopathy in Antibody-Mediated Rejection. J Heart Lung Transplant 35(9): 1059–1066. DOI: 10.1016/j.healun.2016.04.007.

Cooper LT, Baughman KL, Feldman AM, Frustaci A, Jessup M, Kuhl U, et al. (2007). American Heart Association; American College of Cardiology; European Society of Cardiology. The role of endomyocardial biopsy in the management of cardiovascular disease: a scientific statement from the American Heart Association, the American College of Cardiology, and the European Society of Cardiology. Circulation 116(19): 2216–2233. DOI: 10.1161/CIRCULATIONAHA.107.186093.

Coutance G, Ouldamar S, Rouvier P, Saheb S, Suberbielle C, Bréchot N, et al. (2015). Late Antibody-Mediated Rejection after Heart Transplantation: Mortality, Graft Function, and Fulminant Cardiac Allograft Vasculopathy. J Heart Lung Transplant 34(8): 1050–1057. DOI: 10.1016/j.healun.2015.03.002.

Coutance G, Van Aelst L, Hékimian G, Vidal C, Rouvier P, Saheb S, et al. (2018). Antibody-Mediated Rejection Induced Cardiogenic

Shock: Too Late for Conventional Therapy. Clin Transplant 32(5): e13253. DOI: 10.1111/ctr.13253.

De Vlaminck I, Valantine HA, Snyder TM, Strehl C, Cohen G, Luikart H, et al. (2014). Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. Sci Transl Med 6(241): 241ra77. DOI: 10.1126/scitranslmed.3007803.

Dlouha D, Chytilova S, Vymetalova J, Rohlova E, Lukasova M, Novakova S, Hubacek JA (2025). Increased Donor-Derived Cell-Free DNA as a Predictor for the Early Detection of Antibody-Mediated Rejection Following Heart Transplantation. Clin Transplant 39(6): e70209. DOI: 10.1111/ctr.70209.

Doo I, Staub LP, Mattke A, Haisz E, Seidler AL, Alphonso N, Schlapbach LJ (2022). Diagnostic Accuracy of Infection Markers to Diagnose Infections in Neonates and Children Receiving Extracorporeal Membrane Oxygenation. Front Pediatr 9: 824552. DOI: 10.3389/fped.2021.824552.

El Messaoudi S, Rolet F, Mouliere F, Thierry AR (2013). Circulating cell free DNA: Preanalytical considerations. Clin Chim Acta 424: 222–230. DOI: 10.1016/j.cca.2013.05.022.

Greytak SR, Engel KB, Parpart-Li S, Murtaza M, Bronkhorst AJ, Pertile MD, Moore HM (2020). Harmonizing Cell-Free DNA Collection and Processing Practices through Evidence-Based Guidance. Clin Cancer Res 26(13): 3104–3109. DOI: 10.1158/1078-0432.CCR-19-3015.

Han F, Sun Q, Huang Z, Li H, Ma M, Liao T, et al. (2021). Donor plasma mitochondrial DNA is associated with antibody-mediated rejection in renal allograft recipients. Aging (Albany NY) 13(6): 8440–8453. DOI: 10.18632/aging.202654.

Han F, Wan S, Sun Q, Chen N, Li H, Zheng L, et al. (2019). Donor Plasma Mitochondrial DNA Is Correlated with Posttransplant Renal Allograft Function. Transplant 103(11): 2347–2358. DOI: 10.1097/TP.0000000000002598.

Hertz MI, Taylor DO, Trulock EP, Boucek MM, Mohacsi PJ, Edwards LB, Keck BM (2002). The registry of the international society for heart and lung transplantation: nineteenth official report-2002. J Heart Lung Transplant 21(9): 950–970. DOI: 10.1016/s1053-2498(02)00498-9.

Ibrahim M, Strah H, Huang H, Krupnick A, Kreisel D, Hachem R, et al. (2013). Circulating Mitochondrial DNA Is Elevated in Patients with Immediate Primary Graft Dysfunction. J Heart Lung Transplant 32(4): S33. DOI: 10.1016/j.healun.2013.01.885.

Jansen MPB, Pulskens WPC, Uil M, Claessen N, Nieuwenhuizen G, Standaar D, et al. (2020). Urinary mitochondrial DNA associates with delayed graft function following renal transplantation. Nephrol Dial Transplant 35(8): 1320–1327. DOI: 10.1093/ndt/gfy372.

Kfoury AG, Hammond ME, Snow GL, Drakos SG, Stehlik J, Fisher PW, et al. (2009). Cardiovascular Mortality among Heart Transplant Recipients with Asymptomatic Antibody-Mediated or Stable Mixed Cellular and Antibody-Mediated Rejection. J Heart Lung Transplant 28(8): 781–784. DOI: 10.1016/j.healun.2009.04.035.

Khush KK (2021). Clinical utility of donor-derived cell-free DNA testing in cardiac transplantation. J Heart Lung Transplant 40(6): 397–404. DOI: 10.1016/j.healun.2021.01.1564.

Khush KK, Patel J, Pinney S, Kao A, Alharethi R, DePasquale E, et al. (2019). Noninvasive detection of graft injury after heart transplant using donor-derived cell-free DNA: A prospective multicenter study. Am J Transplant 19(10): 2889–2899. DOI: 10.1111/ajt.15339.

Kim K, Moon H, Lee YH, Seo JW, Kim YG, Moon JY, et al. (2019). Clinical relevance of cell-free mitochondrial DNA during the early postoperative period in kidney transplant recipients. Sci Rep 9(1): 18607. DOI: 10.1038/s41598-019-54694-x.

Kobashigawa J, Zuckermann A, Macdonald P, Leprince P, Esmailian F, Luu M, et al. (2014). Consensus Conference participants. Report from a consensus conference on primary graft dysfunction after cardiac transplantation. J Heart Lung Transplant 33(4): 327–340. DOI: 10.1016/j.healun.2014.02.027.

Li Q, Lan P (2023). Activation of immune signals during organ transplantation. Signal Transduct Target Ther 8(1): 110. DOI: 10.1038/s41392-023-01377-9.

Lo YMD, Han DSC, Jiang P, Chiu RWK (2021). Epigenetics, fragmentomics, and topology of cell-free DNA in liquid biopsies. Science 372(6538): eaaw3616. DOI: 10.1126/science.aaw3616.

Loupy A, Toquet C, Rouvier P, Beuscart T, Bories MC, Varnous S, et al. (2016). Late Failing Heart Allografts: Pathology of Cardiac Allograft Vasculopathy and Association With Antibody-Mediated Rejection. Am J Transplant 16(1): 111–120. DOI: 10.1111/ajt.13529.

Meddeb R, Pisareva E, Thierry AR (2019). Guidelines for the Preanalytical Conditions for Analyzing Circulating Cell-Free DNA. Clin Chem 65(5): 623–633. DOI: 10.1373/clinchem.2018.298323.

Newman LE, Shadel GS (2023). Mitochondrial DNA Release in Innate Immune Signaling. Annu Rev Biochem 92: 299–332. DOI: 10.1146/annurev-biochem-032620-104401.

Pollara J, Edwards RW, Lin L, Bendersky VA, Brennan TV (2018). Circulating mitochondria in deceased organ donors are associated with immune activation and early allograft dysfunction. JCI Insight 3(15): e121622. DOI: 10.1172/jci.insight.121622.

Randeu H, Bronkhorst AJ, Mayer Z, Oberhofer A, Polatoglou E, Heinemann V, et al. (2022). Preanalytical Variables in the Analysis of Mitochondrial DNA in Whole Blood and Plasma from Pancreatic Cancer Patients. Diagnostics (Basel) 12(8): 1905. DOI: 10.3390/diagnostics12081905.

Richmond ME, Zangwill SD, Kindel SJ, Deshpande SR, Schroder JN, Bichell DP, et al. (2020). Donor fraction cell-free DNA and rejection in adult and pediatric heart transplantation. J Heart Lung Transplant 39(5): 454–463. DOI: 10.1016/j.healun.2019.11.015.

Schütz E, Fischer A, Beck J, Harden M, Koch M, Wuensch T, et al. (2017). Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: A prospective, observational, multicenter cohort study. PLoS Med 14(4): e1002286. DOI: 10.1371/journal.pmed.1002286.

Shah KB, Flattery MP, Smallfield MC, Merinar G, Tang DG, Sheldon EH, et al. (2015). Surveillance Endomyocardial Biopsy in the Modern Era Produces Low Diagnostic Yield for Cardiac Allograft Rejection. Transplantation 99(8): e75–e80. DOI: 10.1097/TP.0000000000000615.

Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, et al. (2005). Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant 24(11): 1710–1720. DOI: 10.1016/j.healun.2005.03.019.

Thurairajah K, Briggs GD, Balogh ZJ (2018). The source of cell-free mitochondrial DNA in trauma and potential therapeutic strategies. Eur J Trauma Emerg Surg 44(3): 325–334. DOI: 10.1007/s00068-018-0954-3.

Ungerer V, Bronkhorst AJ, Holdenrieder S (2020). Preanalytical variables that affect the outcome of cell-free DNA measurements. Crit Rev Clin Lab Sci 57(7): 484–507. DOI: 10.1080/10408363.2020.1750558.

Vaughn GR, Jorgensen NW, Law YM, Albers EL, Hong BJ, Friedland-Little JM, Kemna MS (2018). Outcome of Antibody-Mediated Rejection Compared to Acute Cellular Rejection after Pediatric Heart Transplantation. Pediatr Transplant 22(1). DOI: 10.1111/petr.13092.

Wu GW, Kobashigawa JA, Fishbein MC, Patel JK, Kittleson MM, Reed EF, et al. (2009). Asymptomatic Antibody-Mediated Rejection after Heart Transplantation Predicts Poor Outcomes. J Heart Lung Transplant 28(5): 417–422. DOI: 10.1016/j.healun.2009.01.015.