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Research article

Mitochondrial DNA levels in perfusate and bile during *ex vivo* normothermic machine correspond with donor liver quality

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ABSTRACT

Ex vivo normothermic machine perfusion (NMP) preserves donor organs and permits real-time assessment of allograft health, but the most effective indicators of graft viability are uncertain. Mitochondrial DNA (mtDNA), released consequent to traumatic cell injury and death, including the ischemia-reperfusion injury inherent in transplantation, may meet the need for a biomarker in this context. We describe a real time PCR-based approach to assess cell-free mtDNA during NMP as a universal biomarker of allograft quality. Measured in the perfusate fluid of 29 livers, the quantity of mtDNA correlated with metrics of donor liver health including International Normalized Ratio (INR), lactate, and warm ischemia time, and inversely correlated with inferior vena cava (IVC) flow during perfusion. Our findings endorse mtDNA as a simple and rapidly measured feature that can inform donor liver health, opening the possibility to better assess livers acquired from extended criteria donors to improve organ supply.

1. Introduction

To meet the expanding disparity between donor supply and recipient demand, liver grafts from extended criteria donors (ECD) are increasingly transplanted [1,2]. ECD organs are derived from higher-risk donors and include those subjected to prolonged periods of hypo-oxygenation, including those originating from donation after circulatory death (DCD) [3]. ECD organs are more susceptible to ischemia reperfusion injury (IRI) during transplant [2], and their recipients are more likely to experience early allograft dysfunction (EAD), a major contributor to morbidity and mortality after liver transplantation [4]. The use of livers acquired from ECD is clearly

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Table 1
Mitochondrial gene target primers and corresponding primer-specific standard curve amplicon sequence.

Mitochondrial gene target	Primer sequence	Oligonucleotide sequence for standard curve (5'to 3'sense strand*, **)
CytB [27]	F: ATGACCCCAATACGCA AAAT R: CGAAGTTTCATCATGCGGAG	AAGAACACCA**ATGACCCCAATACGCAAAAT**TAACCCCCTAATAAA ATTAATTAACCGCTCATTCATCGACCTCCCCACCCCATCCA ACAT**CTCCGCATGATGAAACTTC**GGCTCA CTCCT
COXI [38]	F: TCATCTGTAGGCTCATTC R: GCGATCCATATAGT CACT	AAACATCCTA**TCATCTGTAGGCTCATTC**ATTTCTCTAACAGC AGTAATATTAATAATTTTCATGATTTGAGAAGCCTTCGCTTCGAAGCGA AAAGTCCTAATAGTAGAAGAACCCTCC ATAAACCTGG**AGTGACTATATGGATGCC**CCCCACCCTA
ND1 [38]	F: GCTACGACCAACTCATAC R: GAATGCTGGAGATT GTAATG	CCCCGATTCC**GCTACGACCAAC** **TCATAC**ACCTCCTATGAAAAAACTTCCTACCACTCACCCTAGCATTACT TATATGATATGTCTCCATACC**CATTACAAT** **CTCCAGCATTC**CCCCCTCAAAC
ND6 [38]	F: CCATCGCTGTAGTATATCCAA R: TCGGGTGTGTTATTA TTCTGA	TCCTCAATAG**CCATCGCTGTAGTATA** **TCCAA**AGACAACCATCATTCCCCCTAAATAAATTAAAAAAACTATTAAACCCATATAA CCTCCCCCAAAAT**TCAGAATAATAACACACCCGA**CCACACCGCT

CytB, cytochrome B; Cytochrome oxidase I, COX I; ND1, NADH-ubiquinone oxidoreductase chain 1; ND6, NADH-ubiquinone oxidoreductase chain 6; F, forward; R, reverse.
*Primer binding sites within oligonucleotide sequences are shown in bold.
**The given oligonucleotide amplicon sequence for each primer pair corresponds to the 5'to 3'sense strand i.e., the information needed to order this product from a commercial supplier. The oligonucleotide used for generation of the standard curve for qPCR assays consists of a double-stranded (sense and anti-sense strands) DNA product.

warranted, and timely measurements of liver health before and during liver transplantation (LT) might help to inform effective post-transplant management.

The traditional method of organ preservation via static cold storage (SCS) has not been changed to accommodate ECD grafts, which are more vulnerable to damage during SCS; this leads to the discard of potentially transplantable organs [5,6]. Normothermic machine perfusion (NMP) recapitulates physiologic circulation, temperature, and the metabolic environment, and consequently reduces hepatic injury in ECD grafts, compared with SCS [7–10]. Metabolites, oxygen, and circulation are supplemented using a machine that continuously circulates a perfusate fluid through the organ [3,11,12]. NMP permits real-time measurements of organ viability, function, and tissue damage that inform post-surgical risk and prognosis, that may permit prophylactic interventions to maintain allograft health post-transplant [3,11,12]. The ability to predict the extent of tissue injury and graft function during NMP could inform post-transplant management, but there is not yet a universally accepted, reliable approach to assess liver vitality during NMP. Traditional metrics like transaminase concentration, bile production, and lactate clearance may not be as sensitive in the NMP setting: the system is a closed circuit, not all organs produce bile during perfusion, and lactate can be concurrently produced and degraded to varying extents [10,13–18]. Whether a graft is ultimately transplanted or discarded is dependent upon donor risk factors and to a degree, real-time subjective assessment by clinicians [19,20].

Tissue damage, including liver IRI [21], results in uncontrolled release of damage-associated molecular patterns (DAMPs) such as high-mobility group box-1 (HMGB1) [22], S100 family proteins [23], and adenosine triphosphate [24,25]. When released to the extracellular space, DAMPs can enter the circulation and signal broadly for inflammation [1,26]. In the systemic circulation or local tissue microenvironment, DAMPs derived from mitochondria (mitoDAMPs), such as formyl peptides and unmethylated mitochondrial DNA (mtDNA), can generate an inflammatory response [21,27–29], while arginine depletion by arginase can interfere with normal lymphocyte function [30].

Because mitochondria contain a separate genome, mtDNA can be measured in patient biofluids to estimate mitoDAMP burden [27]. MtDNA is emerging as a biomarker in patient biofluids including plasma [31], urine [32], and *ex vivo* lung perfusate [33], and its quantity is associated with severity of ischemic stroke [31], organ damage [32], primary graft dysfunction [33], sepsis [27], traumatic injury [34], and intensive care unit mortality [35]. Unfortunately, implementation of mtDNA quantification for widespread clinical use is hindered by the lack of universal and accessible methods for its rapid quantification.

We developed a quantitative real-time PCR (qPCR) assay that as a cost-effective and transferrable method for the absolute quantification of mtDNA. This assay uses mtDNA-specific primers alongside standard curves created with commercially available synthetic DNA to enable quantification of mtDNA copy number. Using our method, we quantified mtDNA in the perfusate fluid and bile of donor livers undergoing NMP. We demonstrate that the mtDNA concentration is significantly correlated with primary warm ischemia time (WIT), donor lactate, donor international normalized ratio (INR), and inferior vena cava (IVC) flow during perfusion, supporting the conclusion that mtDNA measurement post-procurement and during NMP can inform allograft health and transplant risk.

2. Results

2.1. Absolute quantification of extracellular mtDNA by qPCR

There remains an unmet need for a suitable quantitative metric of graft damage during NMP [13–15] and for standardized mtDNA

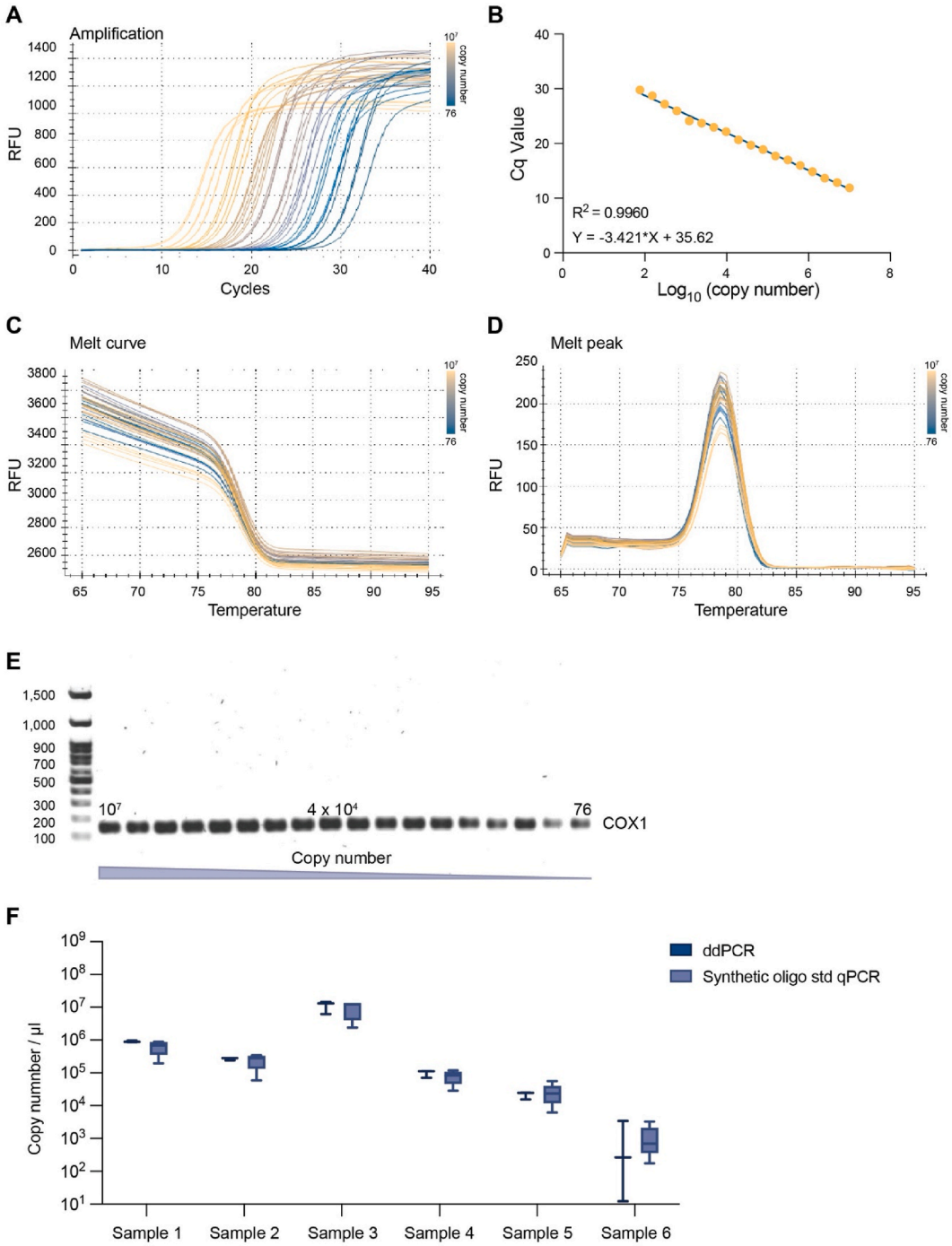

Fig. 1. Mitochondrial DNA is quantitated using gene target-specific synthetic oligonucleotide standard curves. SYBR green-based qPCR was performed with standard curves composed of serially diluted synthetic oligonucleotides corresponding to each primer-specific amplicon. (A) Representative qPCR amplification curves are shown for a two-fold serial dilution of oligonucleotides corresponding to the COX I-amplicon, from 10^7 to 76 copy numbers. (B) Standard curve formed by linear regression of C_q values plotted against $\log_{10}$ (copy number) of serially diluted COX I amplicon oligonucleotides (C) Representative qPCR melt curves and (D) melt peaks corresponding to the COX I amplification curves shown in A. (E) Gel electrophoresis of qPCR products corresponding to amplification curves for the COX I standard curve shown in A-D. (F) Comparison of mtDNA quantification from five healthy donor plasma samples using ddPCR, qPCR with a CytB-specific synthetic oligonucleotide standard curve (Synthetic oligo std qPCR). Each method of quantitation was performed over three independent qPCR experiments, each with a minimum of two technical replicates per sample. Bars/lines represent means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

quantification. We developed a qPCR-based protocol that amplifies mtDNA using targeted primers alongside serially diluted synthetic oligonucleotides for absolute quantification of extracellular mtDNA by qPCR (Table 1). This synthetic oligonucleotide standard curve establishes an internal technical control, and we find consistent quantitation values obtained over multiple experiments and different qPCR instruments (data not shown). Fig. 1A and B shows a typical standard curve composed of serially diluted synthetic oligonucleotides of the COX I amplicon **(Table 1)**, corresponding melt curve analysis (Fig. 1C and D), and gel electrophoresis (Fig. 1E).

Droplet digital PCR (ddPCR) allows quantitation of DNA via the fractionation of a single PCR reaction mixture into thousands of emulsified droplets and represents the "gold standard" for quantification of nucleic acid [36,37]. We used ddPCR-based quantification to validate our qPCR-based technique. We compared quantification of CytB copy number in healthy donor plasma samples by ddPCR, qPCR with an amplicon-specific oligonucleotide standard curve for CytB (Fig. 1F). The synthetic oligonucleotide standard curve accurately reproduced concentration values measured by ddPCR with no significant differences noted, endorsing our qPCR-based quantitation technique as a cost-effective and accurate method for mtDNA quantitation.

2.2. Study cohort

We collected clinical information and samples from 29 donor livers (8 donation after circulatory death [DCD], 21 neurological determination of death [NDD]). Each were preserved on NMP and subsequently transplanted, from 2015 to 2018 during a clinical trial of *ex vivo* NMP (Table 2). Data pertaining to donor liver ID numbers 1 to 10, which were collected December–February 2015, has been published previously [11]. Information pertaining to NMP parameters and perfusate biochemistry is available in Table 3.

2.3. Quantitation of mtDNA in serial NMP perfusate samples and bile

Perfusate samples (n = 29 donor livers, multiple time points per liver for a total of n = 84 samples) were quantitated via qPCR for each of the mtDNA genes studied: *CytB, COXI, ND1* and *ND6*. Each of the four genes amplified similarly, confirming the internal validity of our test. We observed strong positive correlations between all individual mtDNA targets, with the greatest concordance between ND1 and COX I ($R^2 = 0.93$, $p < 0.0001$), ND6 and COX I ($R^2 = 0.92$, $p < 0.0001$), and ND6 and ND1 ($R^2 = 0.98$, $p < 0.0001$) (Fig. 2). Quantitation values for all serially collected NMP perfusate samples for each donor liver are shown in Fig. 3A–D. Corresponding qPCR products showed target-specific amplification when assessed by agarose gel electrophoresis **(Supplementary Figs. 1A–D)**. For nearly all donors, mtDNA concentrations remained stable throughout all timepoints measured, and no significant

Table 2
Donor and recipient demographics and characteristics.

Donor characteristics n = 29	Median [min-max; IQR] and/or n (%)
Donor risk index	1.5 [1.0–2.7; 0.7]
Donor type	8.0 (27.6)
DCD	21.0 (72.4)
NDD	
Donor age, years	38.0 [14.0–71.0; 31.5]
Donor sex	20.0 (72.4)
Male	8.0 (27.6)
Female	
Donor BMI, kg/m^2	25.8 [21.0–37.0; 4.7]
WIT, min	21.5 [14.0–52.0; 8.8]
CIT, min	325.0 [50.0–510.0; 176.3]
Pre-procurement donor liver function	
AST, U/L	33.0 [9.0–1434.0; 62.5]
ALT, U/L	39.5 [10.0–3558.0; 92.1]
ALP, U/L	59.0 [28.0–322; 30.0]
T Bili μmol/L	5.5 [0.2–28.0; 7.0]
Lactate, nmol/L	1.8 [0.9–5.1; 2.8]
INR	1.2 [0.9–1.8; 0.25]
Recipient Characteristics	
Recipient age, years	59.0 [34.0–74.0; 13.0]
Recipient sex	24.0 (85.7)
Male	4.0 (14.3)
Female	
MELD score	19.0 [7.0–43.0; 15.5]
EAD	5.0 (17.9)
Hospital stay, days	22.5 [8.0–121.0; 31.3]

Data are presented as median [minimum – maximum; interquartile range] and n (%) unless otherwise indicated.
DCD, donation after circulatory death; NDD, neurological determination of death; BMI, body mass index; WIT, primary warm ischemia time; CIT, cold ischemia time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T Bili, total bilirubin; INR, international normalized ratio; MELD, model for end-stage liver disease.

associations were observed between the change over time in mitoDAMP concentrations and clinical variables.

Bile samples (n = 16) were collected at the end of *ex vivo* NMP; not every graft produced bile while connected to the perfusion apparatus. MtDNA was quantitated in n = 10 bile samples from the cohort in which we measured perfusate mtDNA levels. The quantification of mtDNA in bile was performed for an additional group of six donor livers (ID numbers 30 to 35) for which perfusate samples were unavailable (Supplementary Table 1). Using the same qPCR approach, we quantitated mtDNA in bile and similarly found no significant differences between quantitation values for each mitochondrial gene (*CytB, COXI, ND1, ND6*), but note that this small cohort was underpowered to detect differences (Fig. 3E). As with perfusate samples, qPCR products showed target-specific amplification when assessed by agarose gel electrophoresis **(Supplementary Figs. 2A–D).**

2.4. Correlation of donor parameters with levels of mtDNA in bile and perfusate

To determine whether mtDNA levels in perfusate or bile correlated with donor liver health or clinical outcomes, regressions were performed between mtDNA levels and donor, NMP, or recipient parameters, where matched clinical data was available. Samples collected from the perfusate of each donor graft during *ex vivo* NMP varied in number and time of collection. To best approximate the health of the livers at the time of transplantation, we measured associations between clinical parameters and the final timepoint collected during *ex vivo* NMP of the donor liver. Values for linear regressions exploring potential correlations between NMP parameters, outcome variables, and average mtDNA copy number (both significant and non-significant) are available in Supplementary Table 3 and Supplementary Fig. 4.

Primary WIT is sustained only by DCD donor livers and begins when the organ becomes under-perfused prior to cessation of the heartbeat. This period is typically measured from when systolic blood pressure drops below 50 mmHg, until collection of the donor organ. We observed a positive association with COX I and CytB with increasing WIT (both $p = 0.04$; Fig. 4A). CIT (the time spent in static cold storage) exhibited a similar correlation between time and mtDNA concentrations, but this metric did not achieve statistical significance (COXI: $p = 0.05$ and CytB: $p = 0.13$; Fig. 4B). Donor lactate was positively associated with mtDNA levels in perfusate (CytB, $p = 0.012$; ND1, $p = 0.040$; Fig. 4C). Donor INR exhibited a significant positive association with mtDNA levels in both perfusate (CytB, $p = 0.003$; ND1, $p = 0.048$; Fig. 4D) and bile (CytB, $p = 0.048$; ND1, $p = 0.049$; ND6, $p = 0.032$; Fig. 4E). There were no significant associations between mtDNA copy number in perfusate or bile with circulating ALT, AST, or ALP in the donor.

We next investigated whether parameters monitored during NMP associate with mtDNA levels in perfusate and bile. IVC flow, for which lower values indicate less flexibility and higher vasculature resistance in donor livers, was associated with higher mtDNA levels in perfusate (CytB, $p = 0.005$; ND1, $p = 0.013$; ND6, $p = 0.036$; Fig. 4E). Neither portal or arterial flow, nor arterial or IVC pressure showed significant associations with mtDNA concentration. There was no significant association between donor type (DCD vs NDD), and concentration of mtDNA in perfusate or bile samples (Supplementary Fig. 3 A and B). Similarly, significant clinical associations (i.e. with MELD, hospital stay and EAD) were not identified in this cohort.

Taken together, our findings reveal a significant correlation between mtDNA released to perfusate and bile and clinical parameters of liver health. This supports further development of mtDNA quantification during NMP to predict outcomes for allograft recipients.

3. Discussion

Tissue damage leads to the release of mtDNA; extracellular mtDNA concentration correlates with outcomes in a broad range of pathologies [38]. MtDNA has been proposed as a biomarker to predict clinical outcomes in a variety of disease processes, but has not yet been translated for clinical use, possibly owing to a lack of universal approaches to quantify mtDNA. Here, we describe a novel approach to quantifying mtDNA using standard qPCR protocols and equipment, mtDNA-specific primers and standard curves created

Table 3
Normothermic machine perfusion parameters and perfusate biochemistry.

Donor livers perfused by NMP	Median [min-max; IQR] or n (%)
NMP time (min)	520.5 [223.0–960.0; 321.2]
Average value during NMP	
IVC flow, L/minute	1.6 [1.0–2.0; 0.4]
Arterial flow, L/minute	0.6 [0.4–0.9; 0.1]
Portal flow, L/min	1.0 [0.4–1.2; 0.2]
IVC pressure mmHg	0.7 [0.0–6.3; 0.8]
Arterial pressure mmHg	66.0 [62.0–70.0; 2.8]
Bile production, mL/hour	14.5 [1.8–53; 12.9]
Peak value during NMP	
AST, U/L	920.0 [350.0–2600.0; 1992.0]
ALT, U/L	571.0 [205.0–13462.0; 1795.5]
T Bili μmol/L	18.0 [8.0–100.0; 12.0]
Lactate, nmol/L	5.6 [0.8–12.8; 5.4]

Data are presented as median [minimum – maximum; interquartile range] and n (%) unless otherwise indicated.
NMP, normothermic machine perfusion; IVC, inferior vena cava; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T Bili, total bilirubin.

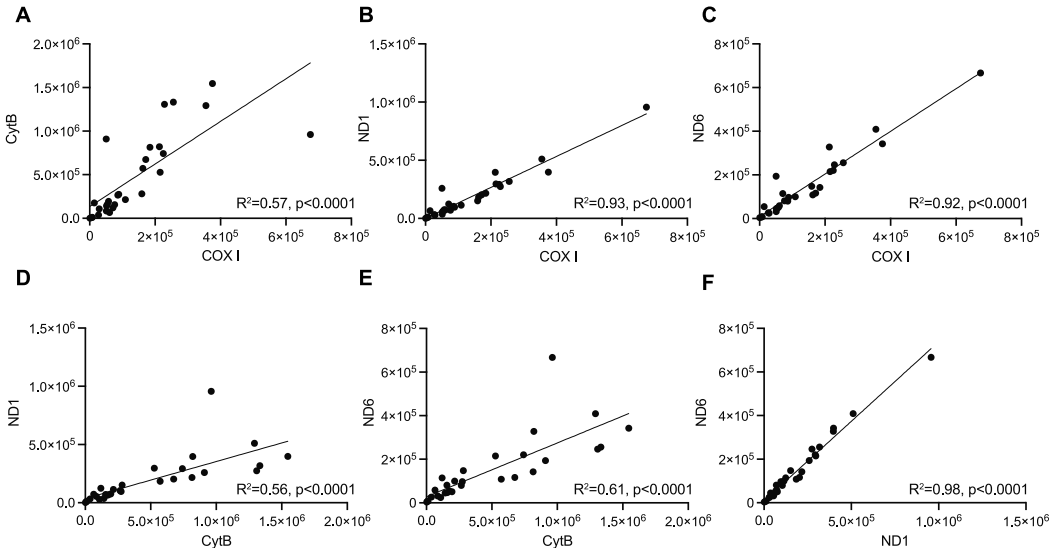

Fig. 2. MtDNA copy number in perfusate collected during *ex vivo* NMP of donor livers is consistent across gene targets. mtDNA copy number in perfusate from each donor liver was quantitated via qPCR with amplicon-specific standard curves for COX I, CytB, ND1, and ND6 at the final timepoint before transplantation. (A–F) Simple linear regressions were performed between pairs of mitochondrial gene targets at the last sequential NMP time point; correlations are reported as coefficient of determination (R^2). Quantitation was performed over three independent qPCR experiments, each with a minimum of two technical replicates per sample.

using commercially available synthetic DNA. We expect that this will enable quantification of mtDNA in the context of NMP to measure liver health with metrics that can be standardized across different centers.

Our method yields quantitative results comparable to ddPCR, is considerably less expensive, can produce results rapidly, and uses approaches and equipment available in most clinical laboratories. We demonstrate that this approach works equally well in samples of perfusate and bile from livers undergoing NMP, and can also be applied to human blood plasma. We find a positive association between mtDNA copy number in perfusate with donor and transplant features, including primary WIT, CIT, donor lactate, and NMP IVC flow. Similarly, donor INR was significantly correlated with mtDNA concentrations in both perfusate and bile. Altogether, this highlights the potential use of mtDNA measurements before and during NMP to inform post-transplant prognosis and care.

NMP is an attractive alternative to traditional static cold storage for preserving the vitality of organs destined for transplantation. NMP can mitigate IRI-induced liver damage sustained during SCS, a major advantage for marginal grafts sourced from extended-criteria donors because they are more susceptible to injury during LT and are being transplanted with increasing frequency to limit waitlist deaths [35]. With NMP, and unlike static cold storage, the metabolic activity of the donor graft is recapitulated *ex vivo*, which allows an extended window of preservation and an opportunity for therapeutic intervention and repair prior to transplantation into the recipient [11,12,39,40]. These interventions, both during and after transplantation, require agile assessments of graft viability and functionality; existing metrics have not yet been fully optimized, especially for ECD organs, which vary in quality and post-transplant performance [12].

Continuous circulation of perfusate in a static volume ensures that mtDNA, once released, remains within the system. The inherent stability and resistance to degradation of mtDNA make it an ideal and reliable indicator of perturbations that result in increasing concentrations, i.e., following tissue injury. The potential of mtDNA as an indicator for tissue damage can be effectively demonstrated in the example of WIT: even though our study only assessed a small sample of DCD donors ($n = 7$) and involved brief ischemic durations (<26 min), we observed that higher mtDNA levels were associated with extended periods of WIT. With NMP, we observed a considerable reduction in mtDNA levels as IVC flow increased. This observation implies that higher mtDNA levels correlate with the degree of vascular resistance during NMP, which is an indicator of graft injury. Notably, our study only included livers deemed healthy enough for transplantation; expanding this to include ECD and livers of marginal quality might further highlight the utility of mtDNA concentrations as a metric of organ vitality.

Common metrics of liver function during NMP include bile production and lactate levels, but these may be insufficient to predict liver health [14–18]. Non-functioning grafts may produce bile during NMP, and conversely, grafts that do not produce bile may still function following transplant. Similarly, livers that demonstrate lactate clearance during NMP may still exhibit primary non-function [16]. Lactate clearance occurs at the oxygen-rich periphery of the hepatic lobule (zone 1), which is less susceptible to ischemic insult than the rest of the organ. Lactate clearance can therefore be unaffected despite injury to more hypoxia-sensitive tissue, located furthest from oxygenated blood supply [41]. Altered clearance of liver enzymes further negates their use as a quantitative measure of tissue health, because clearance rates are confounded with production rates and inconsistent between grafts. We did not observe a correlation between levels of transaminases in perfusate and bile and mtDNA. This lack of correlation may arise from the distinct sources of these factors; transaminases are exclusively produced by hepatocytes, while mtDNA can be released by any cell containing

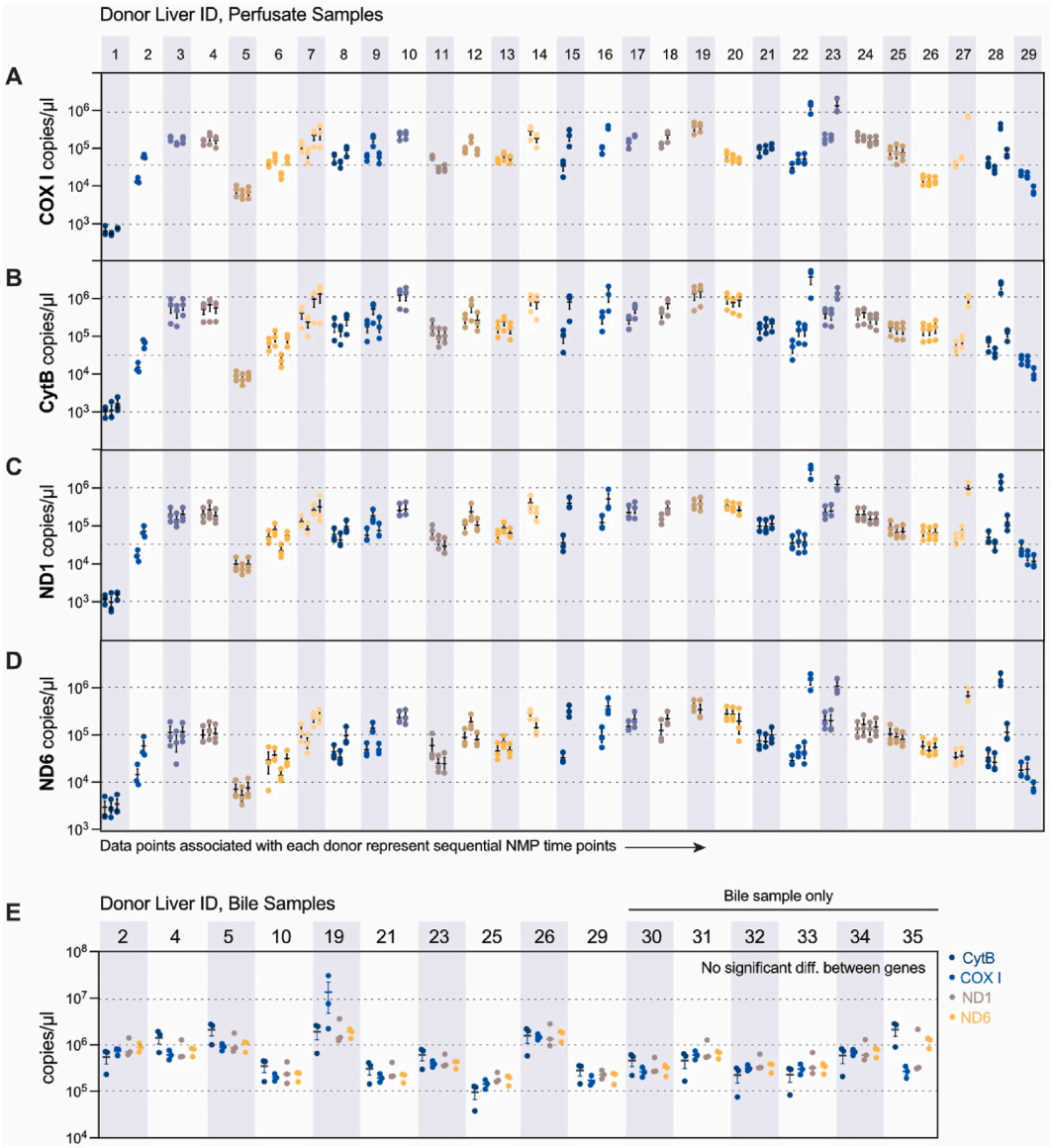

Fig. 3. Quantitation of mtDNA in perfusate and bile samples collected from donor livers during *ex vivo* NMP prior to transplant. MtDNA quantitation for perfusate samples collected at sequential NMP time points is shown for each donor liver and mtDNA gene target (A) COX I, (B) CytB, (C) ND1, or (D) ND6. Sequential samples for each patient (unique ID) are shown in the same vertical band; each data point from left to right represents earlier to later collection time points. qPCR products were subsequently assessed by agarose gel electrophoresis (Supplementary Fig. 1). (E) mtDNA levels for COX I, CytB, ND1, and ND6 in bile produced by a subset of donor livers during NMP. Bile samples were collected at the conclusion of NMP. Donor liver ID numbers correspond with ID numbers in A-D; bile production during NMP was not observed in every donor liver. Corresponding perfusate samples were unavailable for bile samples from donor liver ID numbers 30 to 35. qPCR products were subsequently assessed by agarose gel electrophoresis (Supplementary Fig. 2). Mean ± SEM is shown for each mtDNA gene target. Data represents three independent qPCR experiments, each with a minimum of two technical replicates per sample.

mitochondria - and the total copy number of mtDNA can differ between cells. Even though both are indicators of cellular damage, their ratios may differ since the numbers of their cell origins are not correlated.

Of particular importance for LT, the liver has a high mitochondrial content to support its metabolic activity [42]. Tissue damage and necrotic cell death compromise cellular integrity and lead to secretion of intracellular contents, including mitoDAMPs, and mtDNA, which is highly stable [27]. The degree of mitochondrial injury and metabolic performance, including mitochondrial respiration, during *ex vivo* NMP can serve as a predictor of liver function and viability prior to transplant. However, measurement of bioenergetic properties requires specialized equipment, such as fluorescence spectroscopy, high-resolution respirometry, or ion-pair chromatography [20,43–45]. Similarly, damage to the biliary tree assessed by measurement of pH, bicarbonate, and glucose levels in bile, may serve as predictors of post-transplant complications such as cholangiopathy and biliary structures. However, these

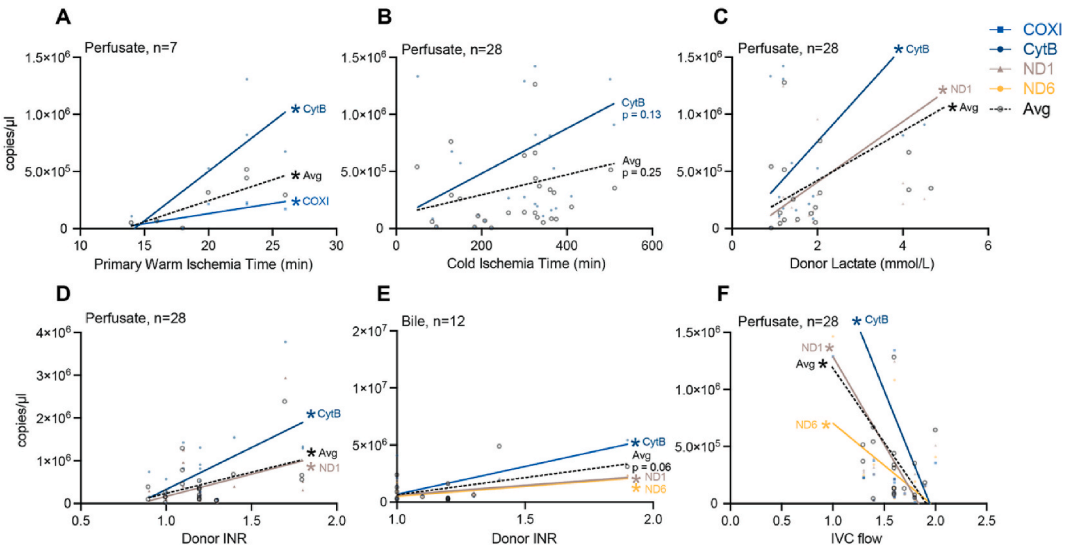

Fig. 4. MtDNA levels in perfusate and bile collected during *ex vivo* NMP are significantly associated with donor and perfusion parameters. Simple linear regressions were performed for continuous variables and mtDNA copy number collected at the final time point during *ex-vivo* NMP. Those with statistically significant regressions, or near statistical significance, and the average of the four mtDNA copy number values are plotted (dotted line). For samples where matched clinical data was available (as indicated), regressions were performed between mtDNA level in perfusate and (A) primary warm ischemia time (WIT) incurred by donor livers prior to *ex vivo* NMP, (B) cold ischemia time (CIT) incurred by donor livers prior to *ex vivo* NMP, and (C) donor lactate measured prior to *ex vivo* NMP. (D, E) association between mtDNA levels in perfusate (D) and bile (E) with donor INR measured prior to *ex vivo* NMP. Donor INR data was not available for every donor liver that produced bile. (F) association between mtDNA levels in perfusate and IVC flow during NMP. Each data point represents the average of three independent qPCR experiments, each with a minimum of two technical replicates per sample.

measurements require specific diagnostic instruments [46]. Our protocol for mtDNA quantitation's rapid and straightforward nature is worth highlighting, as it offers both sampling convenience and user friendliness. Since it measures features of genomic DNA, there is no need for a reverse transcription step, allowing for immediate analysis using qPCR. Results are available in a few hours, and the necessary equipment and expertise is readily available in most clinical laboratories. Future iterations could expedite the process further, especially where more specialized instrumentation is available. For example, using optics-based molecular testing, such as loop-mediated isothermal amplification, would enable mtDNA measurement in the operating room, or even integration into NMP apparatuses and return results within minutes [47].

MtDNA concentration may inform expected post-surgical sequelae and illuminate opportunities for precision medicine in patients undergoing liver transplantation. While further validation will be required to incorporate mtDNA metrics in clinical scenarios, we demonstrate its feasibility and pose considerations for future opportunities to integrate these measures as post-surgical decision making aids. Our intention is to demonstrate this technique, however, in its current state, the measurement of mitochondrial DNA (mtDNA) levels does not offer immediate feedback for real-time clinical decision-making. Nearly all biomarkers in pre-clinical research require time, but as they are adopted for clinical use, technologies advance to minimize this period.

Our novel and accurate approach to mtDNA concentration likely also has implications beyond NMP and LT, where tissue damage is a prominent feature. Quantifying mtDNA is a promising avenue for assessing organ health with diverse applications across clinical scenarios. In preventive medicine, the measurement of mtDNA holds potential for enhancing cancer screening efforts, particularly for tumor subtypes that manifest without overt signs or symptoms. This is especially pertinent in cases where traditional screening methods are limited, such as ovarian or pancreatic cancers. Beyond preventative medicine and screening, mtDNA assessment may have implications for monitoring treatment efficacy in various medical conditions. In autoimmune disorders, cancer treatment, neurodegenerative diseases, metabolic disorders, and instances of drug toxicities, tracking changes in mtDNA levels could provide insights into cellular stress and response to therapeutic interventions. This multifaceted application underscores the potential of mtDNA quantification as a versatile tool in the realm of personalized medicine, offering a nuanced understanding of organ health across a spectrum of clinical contexts.

4. Limitations of the study

In this exploratory study, we demonstrate the feasibility of an accurate and scalable test for mtDNA measurement in all fluids generated during *ex vivo* NMP and provide proof-of-principle for its utility in the clinical setting of LT with NMP. We acknowledge that this work has inherent limitations, but hope that description of our method and cohort will enable further research at other sites, and for other indications. The serial perfusate and bile samples quantified in this study resulted from a preliminary single-center clinical trial of *ex vivo* liver perfusion prior to transplantation, and therefore, our work was performed with a relatively small sample size. To

extrapolate and translate our findings, follow-up studies with larger, more diverse, cohorts are warranted. Secondly, serial perfusate samples were collected at different timepoints between donor livers [11]; for most donors, we found that mtDNA concentrations were stable throughout the transfusion period, and by limiting our analysis to the final mtDNA sample taken, we expect to have ascertained the health of the liver as it was transplanted. Nevertheless, expansion of this cohort might be expected to include more instances where mtDNA concentrations increase over time, and it will be interesting to explore whether these interval changes reflect liver health and stress. Only a single timepoint was available for bile samples, and not every donor liver produced bile during NMP, limiting sample size. Therefore, this warrants a larger validation cohort to reach stronger conclusions and support clinical utility.

Of note, all donor livers included in this cohort were successfully transplanted and deemed to be of sufficient quality for transplantation; marginal grafts, including those deemed unsuitable for transplant, may have yielded higher mtDNA levels and therefore revealed stronger correlations with clinical outcomes. Indeed, future validation studies should prioritize inclusion of high risk and discarded organs to increase the scope and translatability of mtDNA quantification. We predict that data from additional donor livers, collected at standardized time intervals, will reveal further correlations with mtDNA copy number and would strengthen our preliminary findings. To fully assess the clinical predictive capabilities of this mtDNA quantitation protocol, a larger multi-center cohort, adequately powered to address outcome variables, is required.

CRediT authorship contribution statement

Lauren P. Westhaver: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sarah Nersesian:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Riley J. Arseneau:** Validation, Writing – original draft, Writing – review & editing. **Joshua Hefler:** Data curation, Investigation, Validation, Writing – review & editing. **Breanna KV Hargreaves:** Conceptualization, Writing – original draft, Formal analysis, Validation. **Alexander Edgar:** Validation. **Yara Azizieh:** Formal analysis, Writing – original draft. **Nerea Cuesta-Gomez:** Writing – original draft. **Dayne L. Izquierdo:** Data curation. **AM James Shapiro:** Conceptualization, Data curation, Resources, Writing – review & editing, Supervision. **Boris L. Gala-Lopez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Jeanette E. Boudreau:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27122.

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