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Master Biology of Disease

Exploring the use of cell-free nucleic acids as biomarkers for *ex vivo* heart function

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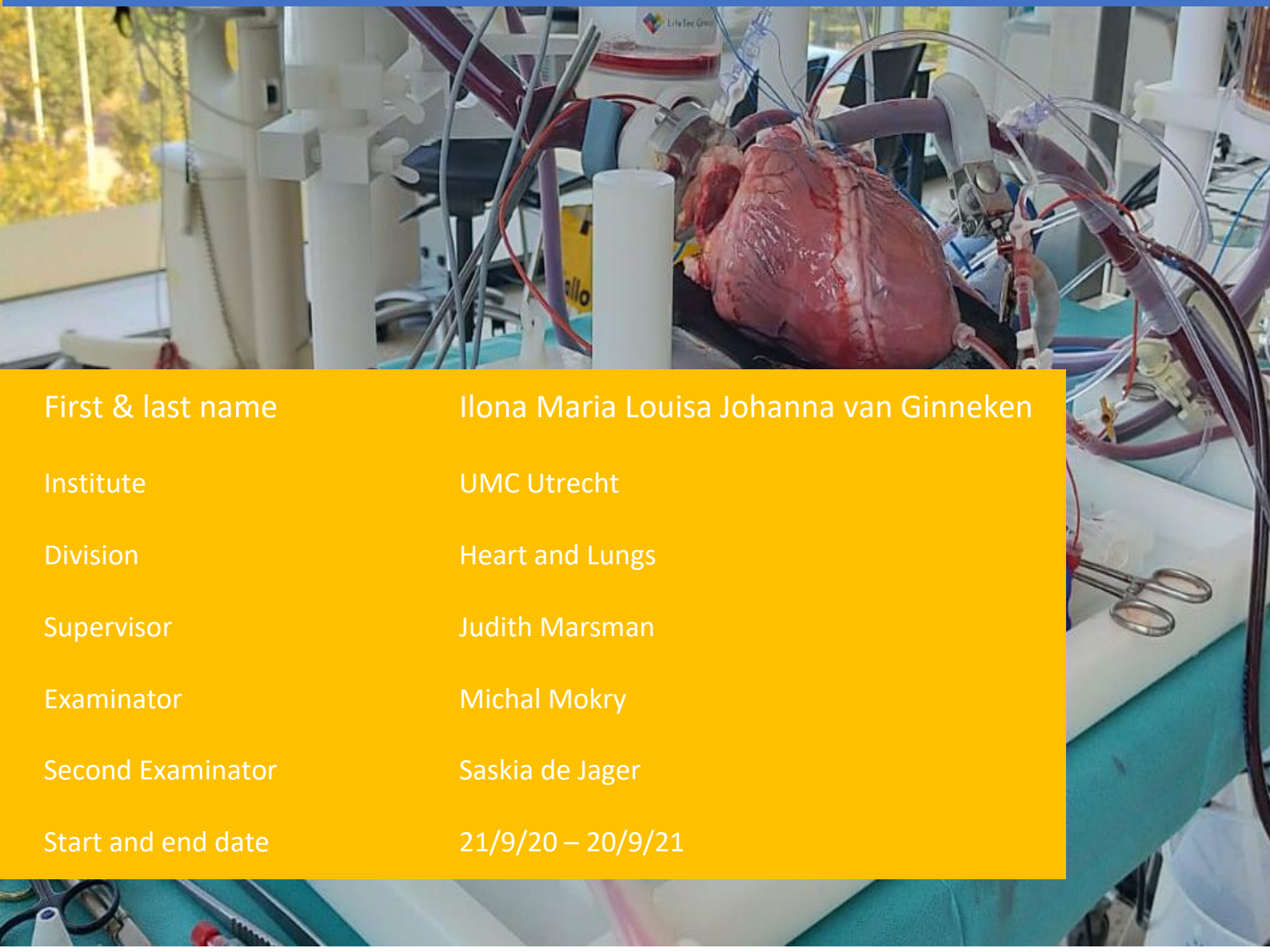

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Lekensamenvatting

Hart- en vaatziekten zijn wereldwijd doodsoorzaak nummer één en resulteren vaak in hartfalen, waarbij verminderde toevoer van zuurstof naar organen optreedt. Voor patiënten met eindstadium hartfalen blijft harttransplantatie de beste behandeling voor lange-termijn overleving met goede levenskwaliteit. Helaas zijn er meer patiënten die een harttransplantatie nodig hebben, dan beschikbare donorharten. Momenteel worden donorharten op ijs bewaard tot de transplantatie. Deze donorharten moeten binnen vier uur na overlijden getransplanteerd worden, omdat het complicatierisico daarna groot is door ondervonden schade. Om de bewaartijd van donorharten met behoud van hartfunctie te verlengen worden nieuwe bewaringstechnieken ontwikkeld.

In dit onderzoek werden varkensharten vier uur bewaard op ijs of in een machine met een daarvoor ontwikkelde vloeistof die door het hart werd gepompt. Koude doorstroming van het hart zorgt, net zoals bewaring op ijs, voor verlaagde energiebehoeften van het hart, maar vermindert ook ophoping van schadelijke stoffen. Deze bewaarmethode staat het toe om hartfunctie indirect te beoordelen via stoffen vrijgekomen in de doorstroomvloeistof. Lactaat is de belangrijkste bepalende factor tijdens machine perfusie voor het kiezen van te gebruiken harten voor transplantatie, omdat dit in eerder onderzoek voorspellend is gebleken voor harttransplantatie-uitkomsten. De betrouwbaarheid van lactaat als indicator voor harttransplantatie-uitkomsten wordt nog in twijfel getrokken, dus het is belangrijk dat nieuwe indicatoren worden onderzocht. In dit onderzoek hebben we bestudeerd of cel-vrij DNA en RNA (cf-DNA en cf-RNA), welke voorkomen in de doorstroomvloeistof, gebruikt kunnen worden voor het voorspellen van hartfunctie. Na koude bewaring werden de harten vier uur lang gekoppeld aan een warme perfusiemachine voor hart reperfusie. Hiermee werd de situatie na transplantatie nagebootst en was functionele beoordeling mogelijk. Tijdens koude en warme perfusie werden niveaus van zowel cf-DNA en cf-RNA als andere markers voor hartschade en ontsteking gemeten.

Cf-DNA is hoogstwaarschijnlijk afkomstig van stervende cellen. We hebben gekeken cf-DNA-niveaus afkomstig uit celkernen of mitochondriën. Dit zijn respectievelijk markers voor algemene cellulaire en mitochondriële schade. Cf-RNA, daarentegen, wordt voornamelijk door levende cellen vrijgelaten. Hierdoor wordt gedacht dat cf-RNA-niveaus de genexpressieprofielen van de cellen die het uitscheiden weerspiegelen. Hiermee kunnen we voorspellen welke cellen het RNA uitscheiden en of die cellen beschadigd zijn. Het onderzoek toonde aan dat totale en mitochondriële cf-DNA niveaus afnamen tijdens koude perfusie en toenamen tijdens warme perfusie, terwijl de nucleaire niveaus redelijk gelijk bleven. We zagen positieve correlatietrends van mitochondriële cf-DNA levels tijdens koude perfusie met lactaat levels tijdens warme perfusie. Ook zagen we negatieve correlatietrends tussen warme perfusie cf-DNA levels en linker atrium druk, wat een maat is voor de voorbelasting van het hart. Het gebrek aan sterke correlaties is verklaarbaar door het minimale verschil van functionele waardes tussen harten van verschillende kwaliteit. We zagen dat hartspiercellen de voornaamste bron waren van cf-RNA en dat biologische processen betrokken in energieproductie en metabolisme verslechterden tijdens zowel koude als warme perfusie. Genregulatie processen en immuunreacties werden opgewerkt tijdens koude en warme perfusie, respectievelijk.

Ons onderzoek indiceert dat cf-DNA- en/of cf-RNA levels mogelijk voorspellend zijn voor hartfunctie. Wellicht kunnen therapieën die mitochondriële schade beperken, het energiemetabolisme behouden, en/of een immuunreactie voorkomen, een mogelijke uitkomst bieden om donorharten langer te kunnen bewaren met hart machine perfusie.

Abstract

Due to the shortage of donor hearts, improvement of cardiac graft preservation remains important. Hypothermic machine perfusion (HMP) might increase preservation times compared to standard static cold storage (SCS) by reducing ischaemia-reperfusion injury and enabling biochemical assessment. Nuclear and mitochondrial cell-free DNA (cf-DNA) are markers for general cellular damage/death and mitochondrial damage, respectively. Therefore, cf-DNA in perfusate was expected to potentially reflect heart damage and thus serve as a biomarker for post-transplantation cardiac function. Cf-RNAs could reflect the gene expression profiles of the cells that released them. Therefore, cf-RNAs could potentially be used as biomarkers for cardiac function whilst also providing information about the role of specific cell types or processes throughout machine perfusion.

Porcine slaughterhouse hearts were subjected to 4-hour SCS or HMP, followed by 4-hour reperfusion on the Physioheart™ platform, a normothermic machine perfusion (NMP) set-up that enabled functional assessment. Total cf-DNA levels in perfusate samples from different time points during HMP and NMP were quantified using a Qubit fluorescence assay, whereas qPCR and ddPCR revealed their cf-nDNA and cf-mtDNA levels. Correlations between cf-DNA levels and functional parameters or other metabolic, damage, and inflammation markers were studied. Cf-mRNA sequencing data was used for cell type deconvolution, differential gene expression analysis, and gene set enrichment pathway analysis.

Total and mitochondrial cf-DNA levels decreased and increased during HMP and NMP, respectively, whereas nuclear cf-DNA levels remained unchanged. There were no significant differences between storage groups. Correlation data showed positive correlation trends for HMP cf-mtDNA levels with lactate levels during NMP and negative correlation trends of NMP cf-DNA levels with left atrial pressure. Cf-RNA data analysis revealed cardiomyocytes as its main source and showed affected biological processes involved in energy production and metabolism during HMP and NMP. Results showed that gene regulation processes and immune responses were elicited during HMP and NMP, respectively.

Together, these results indicate that cf-DNA and -RNA levels may predict cardiac function. Moreover, targeting mitochondrial and metabolic dysfunction or preventing immune responses during machine perfusion could facilitate donor heart preservation.

Introduction

Background heart transplantations

Cardiovascular diseases (CVDs) are the number one cause of death worldwide[1,2]. Of all CVD patients, roughly 65 million suffer from heart failure and this number is expected to rise even more in the upcoming years[3,4]. Heart failure is a complicated syndrome in which aberrant ventricular filling or blood ejection prevents the heart from supplying the body with an adequate blood flow [5]. To date, heart transplantation remains the most effective treatment for patients with end-stage heart failure who do not benefit from other treatments, as it facilitates long-term survival with a good quality-of-life[6]. However, the shortage of donor hearts remains a limiting factor for heart transplantation. This is because the number of patients on the waiting list continues to increase, whilst the number of performed heart transplants stays relatively unchanged[6–8]. Occasionally, a donor heart might arrive too late for the patient, as around thirty percent of the patients still die or become ineligible for heart transplantation within the first three years after entering the waiting list. This happens even when patients receive inotropic therapies and/or ventricular assist devices whilst being waitlisted[9–12]. There is room for improvement in reducing the donor shortage, as only one third of the available donor hearts is used for heart transplantation. This is due to the strict selection criteria regarding the donor's age, co-morbidities, and ischemic time[13–15].

Grounds for using *ex vivo* heart machine perfusion instead of static cold storage

The current standard preservation method for donor hearts is static cold storage (SCS), in which the coronary vessels are first flushed with a cold cardioplegic solution. Subsequently, hearts are stored on ice (4°C) in a bag containing preservation solution[16]. This preservation method elevates the risk of primary graft failure (PGF) and cardiac death due to ischemic damage. Ischemic damage is caused by the occurrence of anaerobic metabolism, which leads to increased acidosis and depleted ATP stores [17]. This contributes to reduced cellular function and causes cell death[18].

A total ischemic time that exceeds 4 hours is associated with an increased risk of PGF. Moreover, hearts that are preserved under ischemic conditions for longer than 6 hours are considered unsafe for use[8]. The available time between harvesting of the heart and transplantation is therefore restricted. Prolongation of the heart graft preservation time without the loss of cardiac function would be a compelling method for enlarging the donor pool. In addition, easing donor selection criteria could further help increase the donor pool. To realize these solutions, developments in the *ex vivo* coronary machine perfusion field have gained profound interest.

Ex vivo heart machine perfusion has several advantages over SCS, including a decreased total ischemic time, which allows for an increased total preservation time[19–22]. For example, human case reports of successfully transplanted hearts after total preservation times of more than 10 and 16 hours emerged with the use of *ex vivo* heart perfusion systems[23,24]. Since the optimal perfusion conditions are not established yet, optimisation of the perfusion conditions could further prolong the preservation time without loss of cardiac function[25–30]. Another benefit of *ex vivo* perfusion is that it allows for aerobic metabolism by continuously supplying the heart with oxygenated perfusate, thus possibly leading to maintenance of cellular ATP levels, membrane conditions, and better graft function[31–33]. Furthermore, continuous perfusion leads to the removal of toxic metabolites that are produced during ischemic conditions. It is for these reasons that machine perfusion has a protective effect against ischaemia reperfusion injury (IRI), leading to reduced cell death. In addition this perfusion leads to the mitochondria experiencing less damage during reperfusion due to reduced oxidative stress[18]. Machine perfusion can also provide clinicians with additional heart evaluation methods, as SCS does not allow for metabolic and functional assessment of the heart between harvesting and transplantation[34]. Furthermore, machine perfusion enables the administration of drugs to the heart to prevent further

myocardial damage and to restore cardiac function[35]. In addition, machine perfusion may stimulate the recovery of ischemic damage [31,32].

For SCS, only donation after brain death (DBD) hearts are considered safe to use. However, suitable brain death hearts have been vastly outnumbered by the recipients on transplant lists[36]. The benefits of machine perfusion may allow for the ease of donor selection criteria and may even enable the use of donation after circulatory death (DCD) hearts, which are considered to be high-risk, due to warm ischemic injury[37]. This is because machine perfusion protects these high-risk DCD grafts against the damaging effects of SCS and IRI. The study of Messer *et al.* showed promising results regarding an increase of nearly fifty percent in the number of heart transplantations[38].

Rationale behind utilization of *ex vivo* hypothermic heart machine perfusion

Hypothermic (4°C to 10°C) and normothermic machine perfusion (35°C to 37°C) are the two major developed *ex vivo* heart perfusion techniques. Up until now, no studies have been performed that directly compare HMP and NMP. Despite the advantages of NMP regarding prevention of low temperature-induced cardiomyocyte injury, improved evaluation of heart viability, and enhanced opportunity for drug delivery, NMP is considered to be expensive and complicated. This is due to the complex machines that are needed for normothermic blood perfusion to supply sufficient oxygen to the beating heart for metabolic processes [37,39–41]. Hypothermic machine perfusion is based on an (oxygenated) crystalloid solution that is delivered to the coronary arteries of the arrested heart[39]. Hypothermic conditions reduce the metabolic requirements of the heart in comparison to normothermic conditions[37]. This enables the heart to maintain more sufficient ATP levels, thereby slowing down the process of cell death[18]. Due to the reduced metabolic demands, a less complicated, cheaper machine can be used for HMP than for NMP, which comes with a lower risk of device malfunction. In addition, if the device malfunctions, the organ is stored in a cold, static manner, which further reduces the risk of heart damage in comparison to normothermic perfusion[42]. In preclinical studies that compared HMP to SCS, several preferable characteristics regarding ATP preservation, cell structure preservation, low lactate levels, reduced endothelial dysfunction, and preserved myocardial function were observed in the HMP group[32,33,43–47]. Moreover, the ongoing clinical studies show promising results for the HMP group regarding event-free survival and cardiac-related adverse effects when compared to the SCS group[48].

Assessment criteria for heart turn-down on machine perfusion

Currently, the Organ Care System (OCS) is the only perfusion machine apparatus that is used clinically for heart transplantation[38,40,49]. The OCS apparatus relies on NMP and keeps the heart in a beating, near-physologcial, but unloaded state. Therefore, functional assessment is not enabled. The donor hearts must thus be examined via visual inspection by the transplant team and via assessment of perfusate lactate levels, aortic pressure, and coronary flow[50]. The lactate levels are used as the main biomarker to determine whether a heart is still declined after OCS preservation, as it was a more specific and sensitive indicator of PGD compared to aortic pressure and coronary flow[51]. Lactate is the final product of anaerobic glycolysis and is therefore a measure of hypoxic metabolism[52]. However, the utility of lactate as main argument for determining transplantability after perfusion is questioned. In addition, there is no comparable lactate data available for static cold storage. Therefore, new reliable biomarkers that can predict cardiac function and viability on an *ex vivo* heart perfusion system are required.

Cell-free Nucleic Acids as biomarkers for *ex vivo* cardiac function

A variety of biomarkers in *ex vivo* heart perfusion for predicting cardiac viability have been suggested previously, including lactate dehydrogenase, ammonia, and troponin-I[53]. The lactate dehydrogenase (LD) enzyme converts pyruvate into lactate during anaerobic glycolysis and acts as a non-specific cellular injury marker[54]. However, due to the *ex vivo* isolated heart system, LD levels might indicate general heart injury. Ammonia is another metabolic marker, which could serve as a marker for

myocardial injury in the isolated heart system[55]. Moreover, myocardial injury is also reflected by the sensitive and specific damage marker Troponin I[56]. However, it should be noted that the findings for these markers with regards to cardiac viability are inconsistent.

Fast molecular diagnosis is an important aspect to ensure that the biomarkers have clinical potential for predicting cardiac viability during *ex vivo* cardiac perfusion. This creates the opportunity to explore the use of cell-free DNA and RNA in predicting heart function in an isolated heart system for the first time. Cell-free nucleic acids (cfNAs), such as nuclear DNA, mitochondrial DNA, messenger RNA, and non-coding RNAs, have increasingly gained attention for use in clinical applications including screening, diagnosis, prognosis, follow-up and treatment of pathological conditions, because they are available in non-invasive liquid biopsies, which includes perfusate[57]. The release mechanisms and potential applications during *ex vivo* heart machine perfusion of cf-DNA and -RNA are depicted in **Figure 1**. The main source of cf-DNA are apoptotic and necrotic cells, but the cf-DNA could also be derived by active release via secretion[58–60]. Other evidence suggests that cf-RNA is primarily derived from living cells[61,62].

Currently, cell-free DNA has already proven its diagnostic value in the field of prenatal testing for trisomies, tumor detection, and allograft rejection[63–70]. However, in these cases the diagnostic value of cf-DNA is dependent on differences in DNA sequences from different origins. This principle cannot be used to assess the donor heart damage during ex vivo heart machine perfusion, because this damage is not of genetic origin. Nevertheless, previous research showed that the cf-DNA concentration is also often elevated in patients with various pathological conditions related to tissue damage, including trauma, sepsis, immunological conditions, acute myocardial infarction, and acute coronary syndrome[71–80]. For example, the study of Antonatos, *et al.* showed that the cf-DNA levels were increased in acute myocardial infarction patients and positively correlated with the biomarkers that are currently used for assessing heart injury (e.g. troponin I), thereby indicating that plasma cf-DNA levels reflect the degree of myocardial damage[78]. Whether cf-DNA levels in perfusate can be used as damage and function marker for allografts before transplantation was investigated recently during *ex vivo* liver and lung perfusion[81–83]. The fact that these studies indicated that cf-DNA may be predictive for the functionality of several donor organs emphasizes the possibility that it might also be a promising marker for donor heart function.

The cf-DNA content is composed of DNA molecules of different sizes and forms. Cf-DNA can be found in extracellular membrane vesicles, can be bound to nucleosomes, lipids or lipoproteins, or can be available as free fragments[57,84–87]. The fragment sizes of cf-DNA might indicate the mechanism by which it was released. Apoptotic cell death results in release of shorter cf-DNA fragments (~80-200 bp) with a peak length of around 167 bp, because these DNA fragments are wrapped around nucleosomes, and lengths that are multiples of the nucleosomal unit [59,88,89]. The longer fragments (~10 kb) are derived from necrosis and are longer due to incomplete degradation [59,88,89]. Cf-DNA can be derived from both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA)[90]. Because cf-mtDNA is not protected by histones, the size of mtDNA fragments differs, often ranging from 20 to 100 bp[57]. Damaged cells and mitochondria contribute to the release of damage-associated molecular patterns (DAMPs), including cf-nDNA and cf-mtDNA [76,77,91–93]. The levels of cf-nDNA could be used as marker for general cellular damage and cell dealth. For that reason, it is considered to be an indicator of general DAMP release [94,95]. However, the effect of cf-nDNA on the immune response is still unknown. Increased levels of cf-mtDNA could indicate cell damage and mitochondrial damage[87] . Due to its bacterial ancestry, cf-mtDNA has gained attention as a DAMP. Cf-mtDNA might provoke an immune response that could lead to organ damage, thereby affecting primary graft outcome during heart transplantation[76,77,96]. Previous research already indicated that cf-mtDNA is a potential biomarker for several cardiovascular diseases that have underlying mitochondrial dysfunction, such as cardiac arrest, acute myocardial infarction, and atrial fibrillation[97–100]. Cf-DNA levels might be a good indicator of cardiac viability during *ex vivo* heart machine perfusion, since cf-DNA is derived from damaged and/or dying cells and injured mitochondria.

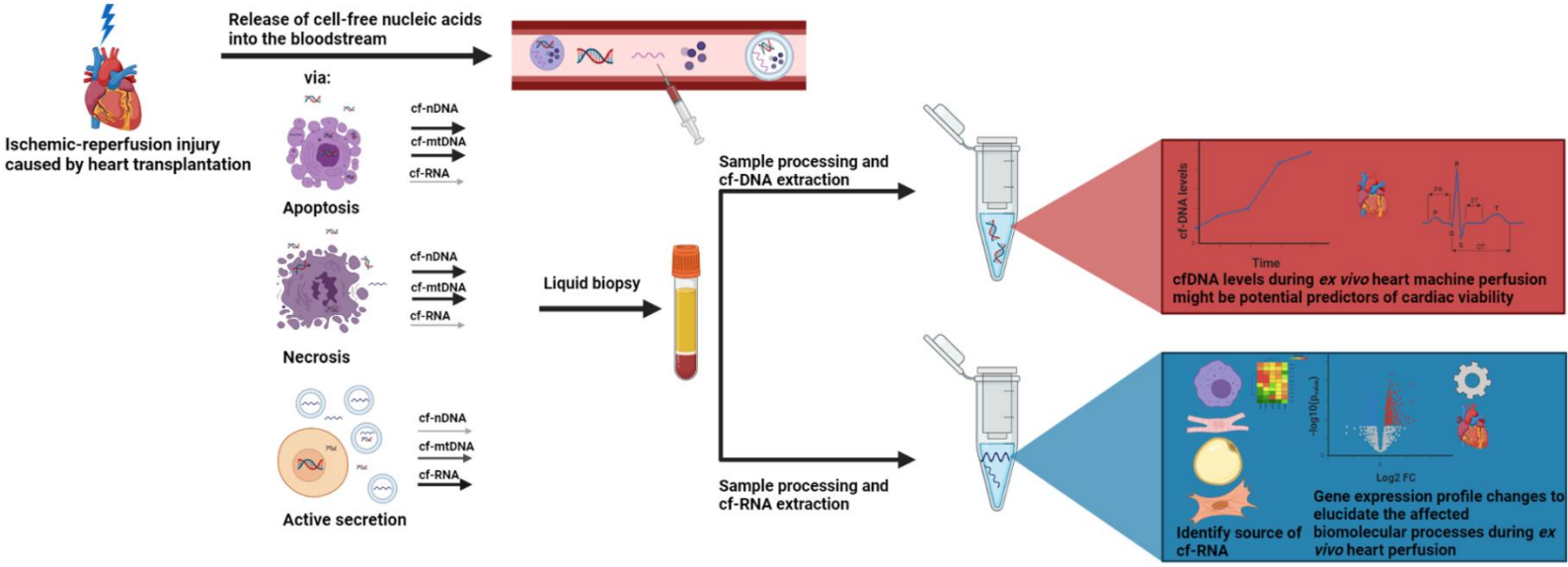
Unlike cf-DNA, cf-RNA is primarily derived from living cells and it is believed that cf-RNA reflects the gene expression patterns of the cells that release them[61,62]. Cf-RNA thus has the potential to give insights about the pathological condition of these cells. Cf-RNAs are protected from ribonuclease-mediated degradation due to their encapsulation within extracellular membrane vesicles (e.g. apoptotic bodies, exosomes, and microvesicles), or their association with proteins to form ribonucleoprotein complexes[71,101]. Up until now, most of the research towards cf-RNA in the cardiovascular damage biomarker discovery field regarding cf-RNA focussed on non-coding RNAs, especially miRNAs[102–105]. For example, Matton, *et al*. demonstrated the predictive value of specific miRNAs for liver injury and function in *ex vivo* liver perfusion[106]. Nevertheless, miRNAs comprise only a small portion of the transcriptome and only a small fraction of these miRNAs appears to be specific for a particular tissue or disease making it difficult to understand their function. On the other hand, long non-coding RNAs and messenger RNAs have well-established tissue-specific and/or disease-related gene expression patterns[107–109]. At present, no miRNA biomarkers are clinically available due to their low specificity. Moreover, insufficient reproducibility of existing studies into miRNA biomarkers, caused by unreliable miRNA levels that are affected by sample- and experiment-related components, impedes their clinical use[110].

Due to the fact that cf-mRNA has low abundance, their quantification is considered to be relatively complex[57]. However, a few recent studies performed cf-mRNA sequencing that enabled cf-mRNA profiling to gain a better understanding of the dynamic transcriptomal changes in pathological conditions including Alzheimer's disease, cancer, bone marrow transplantation, and nonalcoholic fatty liver disease[61,111–114]. For example, the clinical potential of cf-mRNA was reflected in a study of Yan, *et al*. where they identified a new biomarker for predicting Alzheimer's disease before its onset by examining the cf-RNA expression profiles over time[114]. In addition, Rotich, *et al.* performed gene-expression profiling on heart tissue biopsies obtained from porcine hearts mounted to an *ex vivo* heart perfusion system[115]. This study indicated that throughout the machine perfusion a molecular cardiac tissue injury response was provoked that correlated with cardiac function, caused by underlying gene expression phenotypes of inflammation, apoptosis and necrosis[115]. This indicates that cf-mRNAs might be promising potential biomarkers for predicting *ex vivo* heart function.

To be able to elicit its essential pump function, the heart consists of multiple cell types including cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells, pericytes, adipocytes, neuronal cells, and some immune cells[116]. Cardiomyocytes are the most prevalent cell type making up for most of the heart mass (around 70-80%)[117]. However, the heart has also high abundance of fibroblasts, pericytes and smooth muscle cells, and endothelial cells[116,118]. In addition, the adipose tissue contributes to about 20% of the heart mass[119]. By applying cell atlases that contain expression profiles of common cell types, cf-mRNA sequencing also enables the elucidation of the cell types that are

affected during *ex vivo* heart perfusion and how these proportions of cell type contribution change over time[120].

Figure 1: Schematic representation of the release mechanisms of cf-DNA and -RNA and their potential applications in *ex vivo* heart perfusion. Ischemic reperfusion injury caused by heart transplantation leads to the release of cf-DNA and -RNA. Apoptotic and necrotic cell death mechanisms are the major source of cf-nDNA and cf-mtDNA. Active secretion is primarily responsible for the release of cf-RNA. Moreover, active secretion is more likely to secrete cf-mtDNA than cf-nDNA, because cf-mtDNA can act as a damage signal. Cf-DNA and RNA can be assessed through a non-invasive liquid biopsy. After processing and extracting the cf-DNA, its levels can be quantified and used for prediction of *ex vivo* heart function and cardiac viability. After cf-RNA processing and extraction, gene expression profiles can be used to elucidate which cell types contribute to the release of the cf-RNA and which biomolecular processes are influenced during *ex vivo* cardiac perfusion.



Study design, hypotheses and aims

In this study, the aim is to explore the use of cf-DNA and -RNA in predicting heart function in an isolated heart perfusion system for the first time. The hearts were preserved for 4 hours using SCS or HMP. This was followed by 4 hours of NMP on the Physioheart™ platform,, which enables functional assessment and thus mimicksthe post-transplantation state. The effects of HMP on cf-DNA and -RNA levels were examined. Furthermore, the effect of 4 hours of HMP was compared to 4 hours of SCS in a porcine *ex vivo* heart preservation model in terms of cf-DNA and -RNA levels. In particular, the release of total, mitochondrial, and nuclear cf-DNA during HMP and NMP were examined. The total cf-DNA levels were quantified using the Qubit fluorescence assays, whereas the cf-nDNA and cf-mtDNA levels were quantified using qPCR and ddPCR. Because cf-nDNA and cf-mtDNA are markers of damaged and/or dying cells as well as injured mitochondria, it was hypothesized that cf-DNA levels increase over time during HMP, but also following reperfusion on an NMP platform. Furthermore, it was expected that HMP leads to decreased occurrence of IRI compared to SCS, resulting in higher cf-DNA levels in the SCS group than in the HMP group after heart reperfusion.

In addition, this study aimed to assess how the cf-DNA levels correlated with functional cardiac parameters (cardiac output, coronary flow, left atrial pressure, mean arterial pressure, cardiac index, and coronary flow index), measured after 4 hours of NMP. It was furthermore assessed whether the cf-DNA levels at different time points during HMP and NMP correlated with other known metabolic (lactate, lactate dehydrogenase, ammonia), damage (troponin-I), and inflammatory markers (TNF-α, and IL-6). This was all done to assess whether cf-DNA levels could predict cardiac function. In this study, the transcriptome profiles of the cf-mRNA during HMP and NMP were also determined using mRNA sequencing. The obtained unbiased gene expression profiles were compared to the expression profiles common cell types in the body, and to a specific heart cell dataset. This was assessed to elucidate which cells contributed to the release of the RNA and how these contributions changed throughout HMP and NMP. Moreover, the changes in gene expression profiles throughout HMP and

NMP were analysed to investigate which biomolecular processes were ongoing during *ex vivo* heart perfusion. It was expected to find changes in the gene expression of genes involved in energy metabolism, apoptosis, necrosis, and inflammation throughout machine perfusion, as this was also the case in the tissue expression profiles mentioned above.

Materials and Methods

Animals

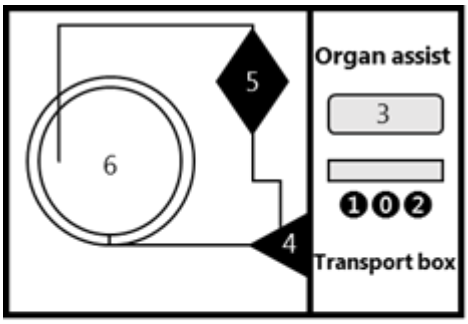
Eighteen hearts were harvested from slaughterhouse pigs sacrificed for human consumption (Dutch Landrace pigs), weighing about 110 kg. The procedures executed by the laboratory and slaughterhouse were in agreement with EC regulations 1069/2009 on the utilization of slaughterhouse animal materials for diagnostic and research purposes, supervised by the Dutch Government (Dutch Ministry of Agriculture, Nature and Food Quality). Moreover, these procedures were approved by the associated legal authorities of animal welfare (Food and Consumer Product Safety Authority).

Heart harvesting

The harvesting protocol for the hearts was identical in all slaughterhouse pigs, as was described previously[121,122]. Prior to the harvesting of the hearts, the pigs were stunned electrically to reach unconsciousness. Subsequently, the pigs were hung up by their hindlimbs for immediate exsanguination. During this procedure, no heparin was administered to the pigs. Subsequently, a minimum of 3 L blood from the same pig was obtained and heparinized with 5000 IU/L for usage during NMP. This was followed by creating an parasternal incision to open the thorax, allowing en-bloc removal of the heart and lungs. Immediate topical cooling was performed by putting them in cold saline (4°C). Next, the pericardium was opened and the pulmonary artery and aorta were cut downstream of the pulmonary artery bifurcation and first supra-aortic vessel, respectively. The aorta was cannulated for delivery of 1.8 L of heparinized (5000 IU/L) modified St. Thomas cardioplegic solution No. 2 at 4°C and a pressure of 80–100 mmHg. To guarantee the warm ischemic time threshold of 5 minutes was not passed, the harvesting time was measured. In addition, heart weight was measured. The hearts were randomly distributed to one of the two storage groups (SCS or HMP).

Hypothermic storage of the hearts

The hearts were stored on ice (n=7; static cold storage (SCS)) or placed on a hypothermic machine perfusion (HMP) system (n=11) for 3.5 hours. Hearts in the HMP group were placed onto a modified Kidney Assist-transport perfusion system (Organ Assist, Groningen, Groningen, The Netherlands). This system contains a reservoir, a rotary pump for the generation of a pressure-controlled pulsatile flow of 60 beats per minute and a maximum pressure of 35 mmHg, a control unit, and an oxygenator without active oxygenation (**Figure 2**). For HMP, 1.5 L of a hyperoncotic cardioplegic nutrition Steen Solution™ (LifeTec, Eindhoven, North Brabant, The Netherlands) was used to perfuse the hearts via the coronary arteries at a starting perfusion pressure of 25 mmHg and a temperature of 8°C[123]. This perfusion solution contained several hormonones, including insulin, T3/T4, cortisol, noradrenalin, and adrenalin, but lacked erythrocytes. Throughout HMP, the perfusion pressure was adapted to the lowest perfusion pressure within 20-25 mmHg that still maintained a coronary flow exceeding 100 mL/min. The temperature of the organ and the reservoir were cooled to around 8°C with ice. The SCS hearts were preserved in a bag with modified St. Thomas cardioplegic solution No. 2 at 4°C by storing them on ice. HMP was stopped at 3.5 hours to prepare the hearts under cold and cardioplegic conditions for resuscitation of the heart on the PhysioHeart™ platform (LifeTec) as previously described[121]. SCS hearts were prepared in the same manner as HMP hearts after 3.5 hours of storage.



0: OK; 1: increase pressure button;
2: decrease pressure button;
3: display; 4: pumphead;
5: oxygenator without oxygen flow;
6: reservoir.

Figure 2: ***Schematic representation of the hypothermic machine perfusion circuit (unpublished Figure from Selma Kaffka genaamd Dengler).***

Normothermic machine perfusion for reperfusion and functional assessment of the hearts

After 4 hours of hypothermic storage, the hearts were mounted onto the PhysioHeart™ platform (LifeTec) for reperfusion and functional assessment of the hearts, as was described previously[121,122]. The aorta and left atrium were linked to PhysioHeart platform, which facilitated coronary perfusion in both the non-working Langendorff mode and in working mode. In addition, this enabled the heart to fill with perfusate in working mode. The venous blood was returned to the reservoir via the cannulated pulmonary artery. Furthermore, the cannulation of this artery allowed for coronary flow measurements. The NMP system was primed with perfusate consisting of modified Krebs solution (1.5 L) and normothermic heperanized blood (5000 IU/L, 3L) from the same pig. To manage physiological levels of blood, glucose levels, pH and calcium levels, glucose, insulin, sodium bicarbonate and calcium chloride were manually administered.

During the first hour of NMP, the hearts were perfused in Langendorff mode through the aorta, maintaining a coronary perfusion pressure of 80 mmHg. During Langendorff mode, the aortic valves were closed, which allowed for perfusion of the coronary arteries. The hearts were first perfused in Langendorff mode to recover contractile myocardial activity. When the hearts demonstrated abnormal contractions, lidocaine was administered intracoronary and the hearts were defibrillated with 30 J. During Langerdorff mode, the coronary flow was measured and the electrical activity of the hearts was tracked by connecting the right ventricle to temporary pacing leads (Medtronic Inc., Minneapolis, Minnesota, USA). These leads were also used to pace the heart at 90-110 beats per minute (BPM), which is the regular heart rhythm of pigs, when necessary. The 500 mL of the perfusate that ran through the circuit once, named the reperfusate, was removed from the circulation. The remaining 4 L of perfusate was recirculated during the rest of the perfusion.

After one hour in Langendorff mode, the hearts were transitioned into working mode by opening up the preload and afterload of the circuit. During working mode, the perfusate from the preload flows into the heart via the left atrium and the left ventricle pumps the perfusate into the afterload. The preload (atrial pressure) and afterload (aortic pressure) were regulated to generate mean loads of 10-20 mmHg and 100-110 mmHg, respectively. The associated pressures and flows were monitored. When the cardiac output (CO) dropped below 3.5 L/min or when the left atrial pressure (LAP) rose above 15 mmHg, dobutamine was infused in ascending dosages with steps of 1.2 mL/h, beginning at 2.4 mL/h and ending at a maximum of 6 mL/h when needed. If dobutamine administration did not help in maintaining the CO and LAP, the associated pressures and flows were maintained at physiological values and manually adjusted to adhere to the optimal clinical pump function of the heart, as was previously described by Schampeart *et al*.[124] Initially, hearts were removed from the platform if the CO still dropped below 3.0 L/min and the LAP remained above 20 mmHg. Later, additional hearts were preserved using SCS and these hearts where preserved for the full 4 hours of NMP, even if they did not meet these criteria.

Assessment of the mean CO and coronary flow (CF) was done by placing two ultrasound flow probes (SonoTT™ Clamp-On Transducer, em-tec GmbH, Finning, Germany) after the afterload and the pulmonary artery. Mean arterial pressure (MAP) and left atrial pressure (LAP) were measured using pressure sensors (P10EZ-1™; Becton Dickinson Medical, Franklin Lakes, USA). The MAP was calculated by doubling the diastolic aortic pressure together with the systolic aortic pressure divided by 3. The change of left ventricular pressure over time (dP/dT) was calculated to evaluate the cardiac function in means of cardiac contractility (dP/dTmax; maximum dP/dT) and diastolic relaxation (dP/dTmin; minimum dP/dT). The left ventricular pressure was assessed using a pressure wire (PressureWire4, Radi Medical Systems, Uppsala, Sweden) that was transapically inserted into the ventricle with a needle. Furthermore, the total cardiac output was determined by calculating the sum of the aortic and coronary flow. The cardiac index (CI) and coronary flow index (CFI) were determined by correcting CO and CF for harvesting weight. These functional assessments took place at predetermined time points (90, 120, 180, and 240 minutes) throughout NMP. After 4 hours of HMP, the hearts were halted by adding 10 mL of potassium chloride solution (1 mmol/mL) to the perfusate.

Collection and processing of perfusate samples

Perfusate samples from 18 *ex vivo* porcine hearts (7 HMP, 11 SCS; different NP numbers) were collected at 5 minutes (T0.05), 90 minutes (T1.5) and 4 hours (T4) for hypothermic perfusion and at baseline (T02), after the perfusion fluid has run through once (T02R; reperfusate), 1 hour (T60), 2 hours (T120), and 4 hours (T240) for normothermic perfusion (**Figure 3**). For HMP samples, 10 mL consisting of 5 mL perfusate plus 5.5 mL PBS was collected, and for NMP samples 10 mL of perfusate was collected into Cell-Free DNA BCT® CE and Cell-Free RNA BCT® CE (STRECK) tubes. To get rid of cells and cell debris, the samples were centrifuged at 300 *g* for 20 minutes and 1800 *g* for 15 minutes at room temperature for the cf-DNA and cf-RNA samples, respectively. The supernatant was collected into 15-mL Falcon tubes. This supernatant was centrifuged for 5000 *g* for 10 minutes and 2800 *g* for 15 minutes at room temperature for the DNA and RNA samples, respectively. The upper plasma layer was aliquoted into cryo vials and Eppendorf tubes and stored at -80°C preceding extraction.

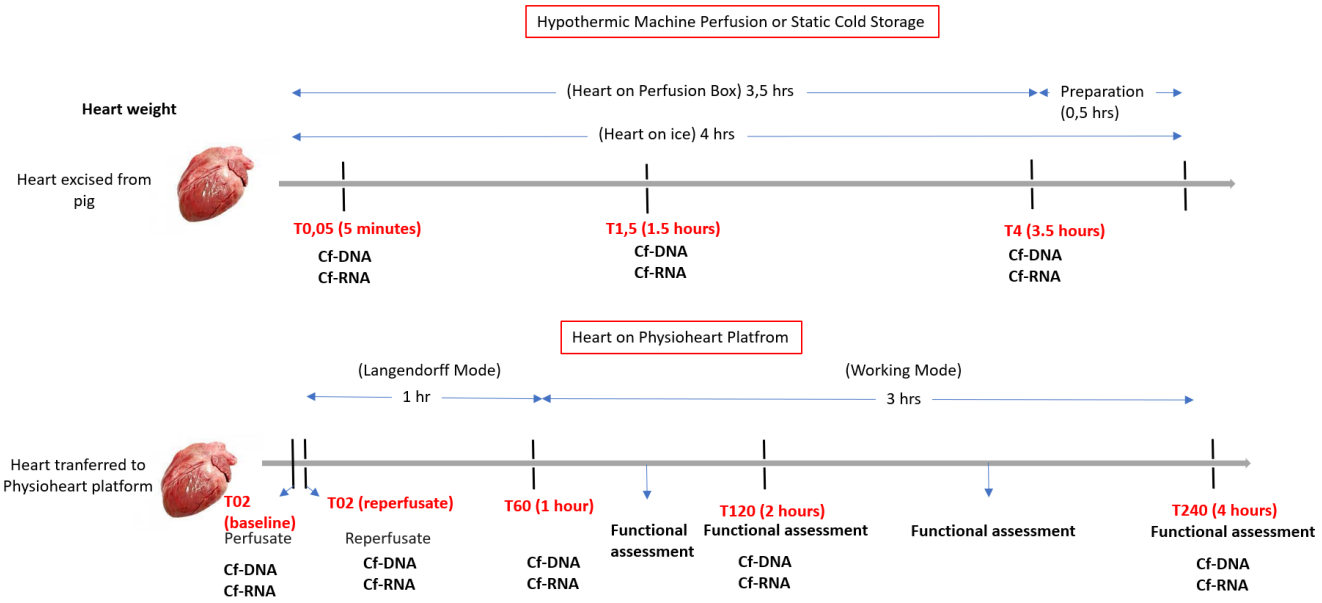


***Figure 2:* Schematic representation of the timeline of the experimental set-up and the perfusate collection sampling (adapted from an unpublished figure of Mudit Mishra).**

For protocol optimization and validation, the effect of centrifugation forces on cf-DNA fragment size was examined by comparing the abovementioned two-step centrifugation to a three-step centrifugation. The third centrifugation step was at 15,000 *g* for 5 minutes at room temperature. For this, the T0.05, and T1.5 NP03, and T02Rb, T60 and T120 NP17 samples were used. The fragment size of these samples was determined using the 2100 Agilent Bioanalyzer and the Agilent High Sensitivity DNA chip, according

to the manufacturer's instructions. The electropherograms of the DNA patterns were obtained using the Agilent 2100 Expert software.

The cold perfusate samples for the other metabolic and damage markers (ammonia, lactate , lactate dehydrogenase, and troponin I) were taken and analysed at the same time points as cf-DNA, but lacked the T60 time point. For the inflammatory marker TNF-α, samples were collected and analysed at identical time points as the cf-DNA samples, whereas for IL-6 only perfusate samples were analysed during all NMP corresponding time points, except for T60.

DNA extraction

For protocol optimization and validation, the effect of proteinase K (ProtK) incubation on cf-dna yield was examined by comparing the standard QIAGEN QIAamp MinElute ccfDNA kit (Qiagen, Hilden, Germany) protocol with 10 minute ProtK incubation to 60 minute protK incubation at 60°C. For this, the T02 and T02Ra NP07, T240c NP16, and T02, T120, and T240 HFP04 were used. The HFP samples were samples from the hemofiltration pilot studies, but these were not used for further analysis in this report. In addition, the fragment size of the T02 and T02Ra NP07, and T240c NP16 were determined using the 2100 Agilent Bioanalyzer to investigate the effect of ProtK incubation time.

The cell-free DNA was extracted from the plasma perfusate samples using the QIAGEN QIAamp MinElute ccfDNA kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. However, one modification was made, namely an increase in ProtK incubation time from 10 minutes at room temperature to 60 minutes at 60°C. Briefly, 4 mL and 2 mL plasma perfusate per sample were incubated with the appropriate volume of ProtK and bead binding buffer for 1 hour at 60°C for hypothermic and normothermic perfusion samples, respectively. After the ProtK incubation, the samples were cooled down on ice for 5 minutes. Subsequently, the magnetic bead suspension was added in the recommended ratio, followed by mixing on a roller mixer for 10 minutes at room temperature. The tubes containing the magnetic beads with bound cf-dna were placed onto a magnet rack for their collection and the supernatant was discarded. The pellet was dissolved in 200 µL bead elution buffer to elute the cf-dna from the beads. The pre-eluate, mixed with 300 µL of buffer ACB, was transferred onto a QIAamp UCP MinElute column for optimal adsorption of the cf-dna onto the column membrane. To remove any residual contaminants, the columns were washed with 500 µL ACW2. Subsequently, the membrane was dried at 56°C for 3 minutes to prevent ethanol carry-over. Finally, the cell-free DNA was eluted in 20 µL ultra-clean water and a re-elution was performed. The extracted DNA samples were stored at -20°C until further use.

The cf-dna concentration was quantified before and after cf-dna extraction by using the Qubit Fluorometer 3.0 (Invitrogen, Carlsbad, CA, USA), and Qubit dsDNA HS (Molecular Probes, Eugene, OR, USA) and Qubit dsDNA BR (Molecular Probes, Eugene, OR, USA) assay kits according to the manufacturer's instructions. The cf-dna concentrations were obtained from a calibration curve generated with the Qubit standards.

mtDNA and nDNA quantification by qPCR

cfnDNA and cfmtDNA were quantified by quantitative polymerase chain reaction (qPCR) using the BioRad CFX96 Real-time system (Bio-Rad Laboratories, Hercules, CA, USA). For this, specific primers to amplify the nuclear GAPDH gene and unique mitochondrial sequences were designed using Primer3 and BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the NCBI database (NC_010447.5 chr5:64129678-64135194 and NC_000845.1). BLAST was used to investigate which parts of the mitochondrial chromosome do not overlap with other pig chromosomes. Nuclear DNA primers were designed to sequences of the *GAPDH* gene at chromosome 5, as this region is one of the most conserved ones. Mitochondrial DNA primers were designed to location mt-chr:5000-5500. The primers were purchased at Integrated DNA technologies (Coraville, Iowa, USA) and their sequences are displayed in **Table 1.**

Table 1: ***Primer sets used for the amplification of cell-free nuclear and mitochondrial DNA with the qPCR assay***

Name	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon length
N1	GTAAAGTCGCGAGTAGCCGA	GTGCACATTGGCAGAACCAG	75 bp
N2	CACCAAGCTCACCTGACGAT	CCATGTTTGTGATGGGCGTG	70 bp
MT3	TATCGGGCCCATACCCCG	ATGAGTAGTCAGTGTGAGCTGATT	134 bp
MT4	TCATAACAGTAATGTCCGGAACCA	TCAGATGAGTAGTCAGTGTGAGC	62 bp

For their use in qPCR reactions, the cf-dna eluates were diluted 6- and 100-fold with Tris-HCl (5 mM, pH 8.5) for the cold and warm perfusion samples, respectively. The composition of the 10 µL standard reaction volume used in the qPCR assay is shown in **Table 2.**

Table 2: ***The composition of the 10 µL standard reaction volumed used in the qPCR assay***

Reagent	Volume per reaction	Final concentration
2X PerfeCTa SYBR Green Super Mix Mix (Quantabio, Gaithersburg, MD, USA)	5 µL	1X
3 µM of forward primer	1 µL	300 nM
3 µM of reverse primer	1 µL	300 nM
RNase/DNAse free water	2 µL	
Cf-DNA sample	1 µL	

The cycling conditions used in the qPCR assays are outlined in **Table 3.** The primer specificity was confirmed with a melt-curve analysis. In addition, the efficiency of the assays was determined by generating 5-point standard curves, based on a 1:8 dilution series of a perfusate sample ranging from 5 to 1.2 x 10^{-3} ng/µL (including 5, 6.25 x 10^{-1}, 7.8 x 10^{-2}, 9,8 x 10^{-3}, and 1.2 x 10^{-3} ng).

Table 3: ***The PCR thermal cycler profile used in qPCR assays for the quantification of cell-free mitochondrial and nuclear DNA in the perfusate samples.***

Stage	Temperature	Time	Cycles
DNA polymerase activation	95 °C	3 minutes	1 cycle
Denaturation **Annealing** **Extension**	95 °C 60 °C 72 °C	10 seconds 10 seconds 30 seconds	40 cycles
Melt curve	95 °C 65 – 95 °C with 0.5 °C increments	10 seconds 5 seconds per temperature	1 cycle

Moreover, calibration curves for all primer sets were generated using a 7-point 2-fold dilution series from approximately 1 x 10^5 to 1 x 10^2 copies/µL (including 1* 10^5 - 5* 10^4 - 1* 10^4 - 5* 10^3 - 1* 10^3 - 5* 10^2 - 1* 10^2 copies/µL) of each PCR amplicon product. The samples were analysed in duplicate and the mean of this was used in subsequent analyses.

mtDNA and nDNA quantification using ddPCR

The cf-mtDNA and cf-nDNA levels were also quantified using ddPCR. The primers and probes used during ddPCR were designed in the same way and around the same chromosomal locations as for qPCR. They were obtained from Integrated DNA technologies with FAM or HEX probes **(Table 4).**

Table 4: ***Primer sets used for the amplification of cell-free nuclear and mitochondrial DNA with the ddPCR assay***

Name	Forward primer 5'-3'	Reverse primer 5'-3'	Probe 5'-3'	Amplicon length
N1	GAGCTTGACG AAGTGGTCGT	CCAGGTTGTGTCCTG TGACT	[FAM]-TGAGGGCAATGCCAG CCCCAGCATCAA -[IABkFQ]	94 bp
N2	ATTTCCTCCTC CTCGCACAA	GCAGGATGGGAGCTT TTCAC	[FAM]-AGCCTGGCTTCCCAG CACAGCCACAAA -[IABkFQ]	79 bp
MT1	AATGCCTGCCC AGTGACA	GGAGAACAAGTGATT ATGCTACC	[HEX]-ACGGCCGCGGTATTCT GACCGTGCAAA -[IABkFQ]	76 bp
MT2	AAACCCCGCCT GTTTACCAA	TTGCACGGTCAGAAT ACCGC	[HEX]-AGAGGCAATGCCTGC CCAGTGACACCAGT -[IABkFQ]	106 bp

For the ddPCR assays, standard reaction volumes of 22 µL were made (**Table 5**) and applied in droplet generation using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad Laboratories). For the use in ddPCR reactions, the cf-DNA eluates were diluted 12-fold with RNAse/DNAse free water. In the no template controls (NTCs), the cf-DNA samples were replaced by RNAse/DNAse free water.

Table 5: ***The composition of the 22 µL standard reaction volumed used in the ddPCR assay***

Reagent	Volume per reaction	Final concentration
2X ddPCR Supermix for Probes (No dUTP)	11 µL	1X
20 µM of each forward primer	1 µL * 2 = 2 µL	±910 nM
20 µM of each reverse primer	1 µL * 2 = 2 µL	±910 nM
5 µM of each probe	1 µL * 2 = 2 µL	±227 nM
RNase/DNAse free water	1 µL	
Cf-DNA sample	4 µL	

Prior to PCR, the plates were heat-sealed with foil using the PX1 PCR Plate Sealer (Bio-Rad laboratories). Temperature gradient assays with perfusate cf-DNA samples were performed to find the optimal PCR thermal cycler profile for the final ddPCR assays that were performed on a T100™ Touch Thermal Cycler (Bio-Rad Laboratories) (**Table 6**).

Table 6: ***The PCR thermal cycler profile used in ddPCR assays for the absolute quantification of cell-free mitochondrial and nuclear DNA in the perfusate samples***

Stage	Temperature	Time	Cycles
DNA polymerase activation	95 °C	10 minutes	1 cycle
Denaturation **Annealing**	95 °C 64.5 °C	30 seconds 1 minute	40 cycles
Enzyme deactivation	98 °C	10 minutes	1 cycle
Infinite hold	12 °C	∞	N/A
Ramp rate was set to 2.5 °C/sec			

Subsequently, the fluorescence of each droplet was determined using the QX200 Droplet Reader (Bio-rad laboratories). The samples were analysed in duplicate and the mean of the positive droplets was

utilized to estimate the absolute cf-nDNA and cf-mtDNA levels. Data quality was guaranteed by removing wells in which less than 10,000 droplets were generated from further analysis. The data was analysed with QuantaSoft v1.7.4.0917 software (Bio-Rad Laboratories). A fluorescence threshold of 1500 for both channels, which was determined based on the negative droplet clusters in the NTC samples, was used to calculate the positive droplet concentrations in all perfusate samples. The results were expressed as copies per mL of perfusate sample.

Statistics

Both the standard protocol and the longer protK incubation protocol were performed on 6 individual samples, each of which were taken at different time points or involved different experimental set-ups. Therefore, the cf-DNA yield of the standard protocol was considered to be 100% and the yield of the longer ProtK incubation protocol was expressed as a percentage of the yield of the standard protocol. To investigate whether longer ProtK incubation resulted in a significantly different cf-DNA yield, a paired t-test was performed. To compare the difference of the cf-DNA levels between the SCS and HMP groups at each time point, unpaired t-tests were performed. In addition, it was examined whether the different cf-DNA levels significantly changed over time using repeated measures ANOVA (no missing values) or mixed effects model (missing values) analyses. Because the reperfusate samples are separate measurements, they were excluded from the over time analyses. For all these analyses, a p-value below 0.05 was considered to be significant.

The data for correlation analysis was normalised by subtracting the T02 (baseline) measurement values from all the other time point measurements during NMP for the cf-DNA and other metabolic and damage biomarker levels (ammonia, lactate, lactate dehydrogenase, and troponin-I). Subsequently, these laboratory measurement values were corrected by dividing them by the individual heart weight multiplied by the average heart weight of all hearts. Spearman's rank correlation coefficients for the correlations between cf-DNA levels and functional data were calculated using the GGally package in Rstudio (**Supplementary Code 1**). In addition, the Spearman's rank correlation coefficients were also calculated to assess the association between the cf-DNA levels and other laboratory markers levels (ammonia, lactate, lactate dehydrogenase, troponin-I, TNF-α, and IL-6) (**Supplementary Code 1**). Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.

RNA extraction

The cell-free RNA was extracted from 2.5 and 1.5 mL plasma perfusate samples and whole blood samples, respectively, using the Plasma/Serum cfc-RNA Advanced Purification Kit (Norgen Biotek, Thorold, Canada) following the manufacturer's protocol. Briefly, 2.5 and 1.5 mL perfusate were first incubated for 5 minutes with Binding Buffer A. After centrifugation, the supernatant was discarded and the pellet was resuspended in Elution Solution C. Subsequently, ProtK was added and the samples were incubated for 30 minutes at room temperature. Until this step in the protocol, all reagents were added to the perfusate in the recommended ratio. After incubation, 200 µL Slurry E and 1.5 mL Lysis Buffer A were added to the samples and incubated for 2 minutes at room temperature to perform sample lysis. Subsequently, to precipitate the nucleic acids, 2 mL of 96-100 percent ethanol was added to the samples and incubated for 2 minutes at room temperature, after which the samples were transferred to the columns. Subsequently, the columns were washed twice with 600 µL Wash Solution A. The cell-free RNA was then eluted in 250 µL Elution Solution A. To lyse the eluted RNA samples, 500 µL Lysis Buffer A was added. Nucleic acids were precipitated by adding 750 µL of 96-100 percent ethanol and samples were applied to the microcolumns. To obtain maximal removal of leftover DNA, the optional On-Column DNA Removal Protocol was performed by applying 50 µL RNase-free DNase I solution to the column, after which the flowthrough was reapplied. Subsequently, the column was incubated at 30°C for 15 minutes. Next, the second wash-step was performed by adding 600 µL Wash Solution A. Cell-free RNA was eluted in 25 µL of Elution Solution A and stored at -20°C until further use.

RNA sequencing

RNA sequencing was performed on 29 samples of 4 different hearts (2x HMP (WB, T0.05, T1.5, T4, T02, T02R, T60, T120, and T240 from NP01 and NP06) and 2x SCS (WB (only from NP24), T02, T02R, T60, T120, and T240 from NP21 and NP24). 3' mRNA sequencing libraries were prepared by

the Epigenomics facility at the UMC Utrecht by Noortje van den Dungen. 50 ng or the maximal volume was used as input for library preparation. Bulk cfRNA sequencing libraries were prepared according to the CEL-seq2 protocol[125]. This method enables direct counting of unique mRNA molecules in the perfusate samples by capturing the 3'-end of polyadenylated RNA and by utilizing unique molecular identifiers (UMIs) barcodes. The resulting cDNA libraries were sequenced on the Illumina NextSeq2000 platform (Utrecht Sequencing Facility, Utrecht, The Netherlands) to obtain paired-end reads with read lengths of 25 and 75 bp.

The sequencing read mapping and quality filtering was performed by Michal Mokry (https://github.com/mmokry). Briefly, the obtained reads were demultiplexed and mapped against the pig reference genome (ENSEMBL: SScrofa11.1; https://www.ncbi.nlm.nih.gov/genome/?term=pig) using the Burrows-Wheeler Aligner (0.7.13)[126]. When multiple reads aligned to the same genes and had an identical unique molecular identifier (UMI, 6 bp long), they were considered a single read.

Quality control RNA sequencing data

To filter out the samples that had low total raw counts and represented background noise, the column sum for each sample was calculated in Rstudio (**Supplementary Code 2 and 3**). In addition, for each sample the gene coverage was examined by calculating the amount of genes that had at least 5 reads. RNA sequencing samples were excluded from further data analysis when their total raw read counts were below the threshold of 25,000 and/or when their gene coverage was lower than 1500 genes.

Furthermore, a principal component analysis (PCA) based on the 500 most variable genes of the remaining RNA sequencing data was performed to examine whether time, heart number (NPnr), and/or storage conditions were responsible for the variation in the transcriptome. RNA sequencing data was regularized log transformed before it was used for PCA using the DESeq2 package in Rstudio.

Differential gene expression analysis

Differential gene expression analysis was performed using the DEseq2 package (**Supplementary code 4**). For this, the read count data was used and genes that had 10 or less reads across all samples were removed from further downstream DEG analysis. To analyse which genes demonstrated a consistent change in expression over the time-course of *ex vivo* machine perfusion, the DESeq2 Wald test analysis was performed with time as a continuous variable in the design formula. The HMP and NMP samples were analysed separately in this DEG analysis. In addition, it was examined whether there were genes that had a condition-specific effect over time by performing a likelihood ratio test (LRT) using a full model that included the condition (SCS or HMP), time and the interaction between them. The reduced model used in this test consisted of condition and time as predictive variables. The Benjamini-Hochberg multiple-testing correction method was executed in all DEG analyses to derive the adjusted p-values and an adjusted p-value of 0.1 was used as significance cut-off. The DEG analyses were performed on time-normalised data, meaning that each time point were divided by the mean of all time points and the Log2 fold changes were per unit.

Pathway analysis

Gene Ontology (GO) gene set enrichment and pathway analysis were performed using the clusterProfiler package in Rstudio (**Supplementary Code 4**). For these analyses, the genes that had a total count below 10 across all samples were also included. For these gene set enrichment and pathway analyses, the genes were ranked based on dividing the -log10(p-value) by the direction (positive or negative) of the Log2 fold change. Adjusted P-values were acquired by applying the Benjamini-Hochberg multiple-testing correction method.

Cf-RNA deconvolution and cell type annotation

SingleR was used to deconvolute the cell types of origin of cf-RNA. Because there are no available pig reference sets for cell type annotation, the pig gene names were converted to human gene names by using the biomaRt package in Rstudio. The counts of genes that had an identical human ensembl gene code were added up. The same accounts for genes that had a similar human gene name. In

addition, counts per million (CPM) normalization was applied to the count data before proceeding with cell type annotation (**Supplementary Code 5**).

Subsequently, the sources of the cf-RNA were estimated by correlating the gene expression of 43 human pure stroma and immune cell types from the merged Blueprint/ENCODE built-in reference to the gene expression profiles of the perfusate samples using SingleR (**Supplementary Code 5**) [127–129]. The Blueprint/ENCODE built-in reference, which is based on the normalized expression values of 259 bulk RNA-seq samples, was accessed via the celldex package. In addition, data was obtained from https://singlecell.broadinstitute.org/single_cell/study/SCP498/transcriptional-and-cellular-diversity-of-the-human-heart#study-download to create a custom reference set that included 17 observed heart cell types (**Supplementary Code 6**)[116]. The Normalized Sparse Expression Matrix data was read into R and the data was converted into a Seurat Object. Before the cf-RNA sequencing data was used in SingleR to annotate cell types, the reference data was transitioned into a SingleCellExperiment using the Seurat package. Subsequently, the Blueprint/ENCODE built-in reference and custom heart reference were used together for cell type annotation by comparing the scores across the references (**Supplementary Code 6)**. Here, the annotated cell type was based on the label with the highest score across both references. For this, the scores across the identified marker subset were recalculated to establish comparable scores across both references originating from an identical gene set.

Results

Optimisation of cf-dna recovery from perfusate samples

In initial explorative studies (unpublished data by Judith Marsman), it was observed that the perfusate samples contained a relatively high contribution of high molecular weight fragment sizes compared to previously reported plasma size distribution profiles. Large fragment sizes are regarded as an indication of contamination by intracellular genomic DNA. Therefore, the effect of centrifugation forces on the cf-DNA fragment size profiles was determined by comparing a two-step to a three-step centrifugation protocol.

As the HMP samples resulted in unusable cf-DNA fragmentation patterns due to their low initial sample concentration, they are not shown. **Figures 4A, 4B, and 4C** illustrate that the three-step centrifugation protocol resulted in a highly similar size distribution profile as the two-step centrifugation protocol across different normothermic cf-DNA perfusate samples. Therefore, the two-step centrifugation protocol was used for the processing of perfusate samples in further experiments.

In general, the bioanalyzer fragment size profiles of all normothermic perfusate samples showed the largest peak between 150 and 200 base pairs, which demonstrates the presence of mono-nucleosomal fragments. In addition, smaller peaks were observed at a size of 300 to 350 base pairs and 475 to 525 base pairs. This inidates the presence of di-, and tri-nucleosomal subunits. These peaks indicate the presence of cf-DNA derived from apoptotic cell death. In addition, different amounts of large cf-DNA fragments with lengths of 1-10 kb were observed. These fragment sizes might indicate cf-DNA obtained from active secretion. Necrotic cell death is likely to be responsible for the peak observed at around 10 kb. The electropherograms of this one replicate showed that over the time course of NMP, the relative amount of multi-nucleosomal and larger fragments increases.

In addition, it was examined whether 1 hour ProtK incubation at 60°C of the perfusate samples resulted in higher cf-dna yield than the standard 10 minutes ProtK incubation at room temperature that is described in the QIAamp MinElute ccfDNA protocol. There was intervened in this step, because denaturation and digestion of the proteins present in the perfusate is an essential step for efficient cf-dna extraction. The obtained cf-DNA concentrations significantly increased by 38% after ProtK incubation at 60°C for 1 hour compared to the standard protocol (**Supplementary Figure 1**). The effect of longer ProtK incubation on cf-DNA fragment size was also examined and the electropherograms showed no clear difference in the fragment size profiles (**Supplementary Figure 2**). Together, these results led to the use of 1 hour ProtK incubation at 60°C for cf-DNA extraction in further experiments.

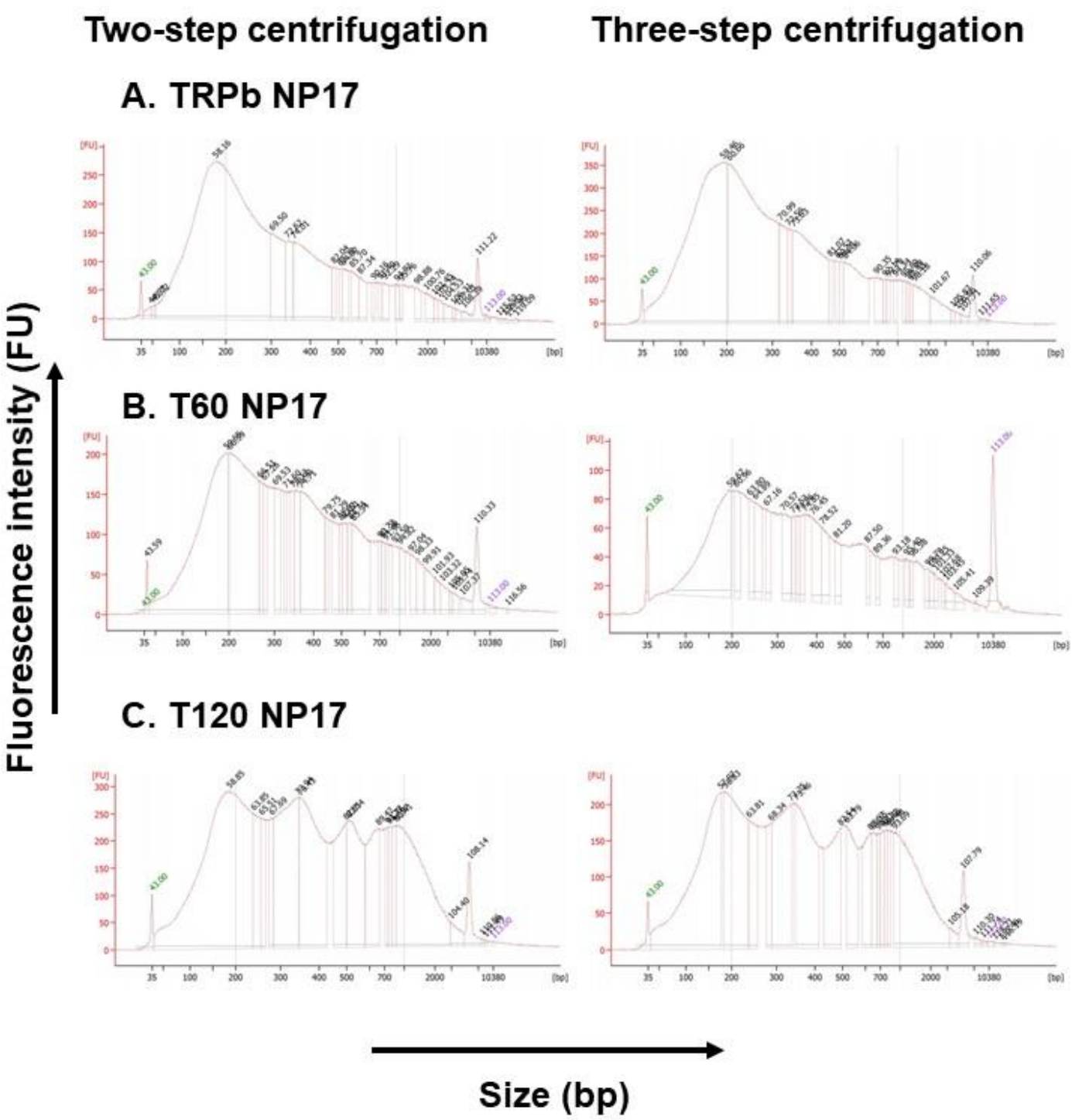


Figure 4: ***Electropherogram fragment size distribution profiles of perfusate cf-dna after two-step or three-step centrifugation protocols*** *The cf-DNA fragment distribution profiles of A) T02Rb NP17, B) T60 NP17, and C) T120 NP17 with a two- and three-step centrifugation protocol were investigated using the Agilent BioAnalyzer 2100. The different centrifugation protocols resulted in comparable fragment size distribution profiles. The X-axis depicts the fragment size (bp), whereas the Y-axis displays the fluorescence intensity (FU). The peaks at 35 (green) and 10380 (purple) base pairs are derived from the two internal High Sensitivity DNA markers. The non-marker peaks reflect mono-, di-, or tri-nucleosomal fragment sizes, as well as longer fragments (1-10 kb).*

Total cf-DNA levels decrease over time during HMP, corresponding to a decrease in cf-mtDNA levels, but not cf-nDNA levels

The total, mitochondrial, and nuclear cf-DNA levels were quantified to explore their predictive value for cardiac function during and following HMP. During HMP, the total cf-DNA levels were measured by the Qubit fluorescence method immediately after the hearts were mounted onto the machine, and after 1.5 and 3.5 hours of HMP. The total cf-DNA concentration in the perfusate samples showed a significant decrease over time ($p = 0.0239$) during HMP (**Figure 5A**). Moreover, the cf-nDNA and cf-mtDNA levels were examined by qPCR using two specific amplicons for both different locations of the nuclear GAPDH gene (N1 and N2) and unique locations of the porcine mitochondrial DNA (MT3 and MT4). Double amplicons were used for cf-nDNA and cf-mtDNA to account for sequence inequalities of the individual slaughterhouse pigs. As shown in **Figure 5B**, qPCR demonstrated that the initial peak of cf-mtDNA levels was followed by a significant drop in these cf-mtDNA levels (MT3 qPCR $p = 0.0137$, and MT4 qPCR $p = 0.0083$) throughout HMP. The cf-nDNA levels, however, did not significantly change over time (N1 qPCR $p = 0.2652$ and N2 qPCR) during HMP (**Figure 5B**). ‘

Furthermore, the cf-nDNA and cf-mtDNA levels were quantified using ddPCR, which is a more absolute quantification method compared to qPCR. Here, there was also made use of the two amplicons specific for the nuclear GAPDH gene (N1 and N2) and the porcine mitochondrial chromosome (MT1 and MT2). As depicted in **Figure 5C**, the ddPCR also showed a significant decrease of cf-mtDNA levels (MT1 ddPCR $p = 0.0355$ and MT2 ddPCR $p = 0.0280$) during HMP. The cf-nDNA levels also did not significantly change over time (N1 ddPCR $p = 0.6792$ and N2 ddPCR $p = 0.9891$) during HMP according to the ddPCR results (**Figure 5C**). In general, it was observed that cf-mtDNA levels were higher than the cf-nDNA levels in the perfusate samples. This difference was more pronounced in the ddPCR data, as the mitochondrial and nuclear amplicons showed more overlap in ddPCR than in qPCR analysis. Together, these results showed that total cf-DNA levels decreased during HMP due to a selective decrease in cf-mtDNA levels, but not cf-nDNA levels.

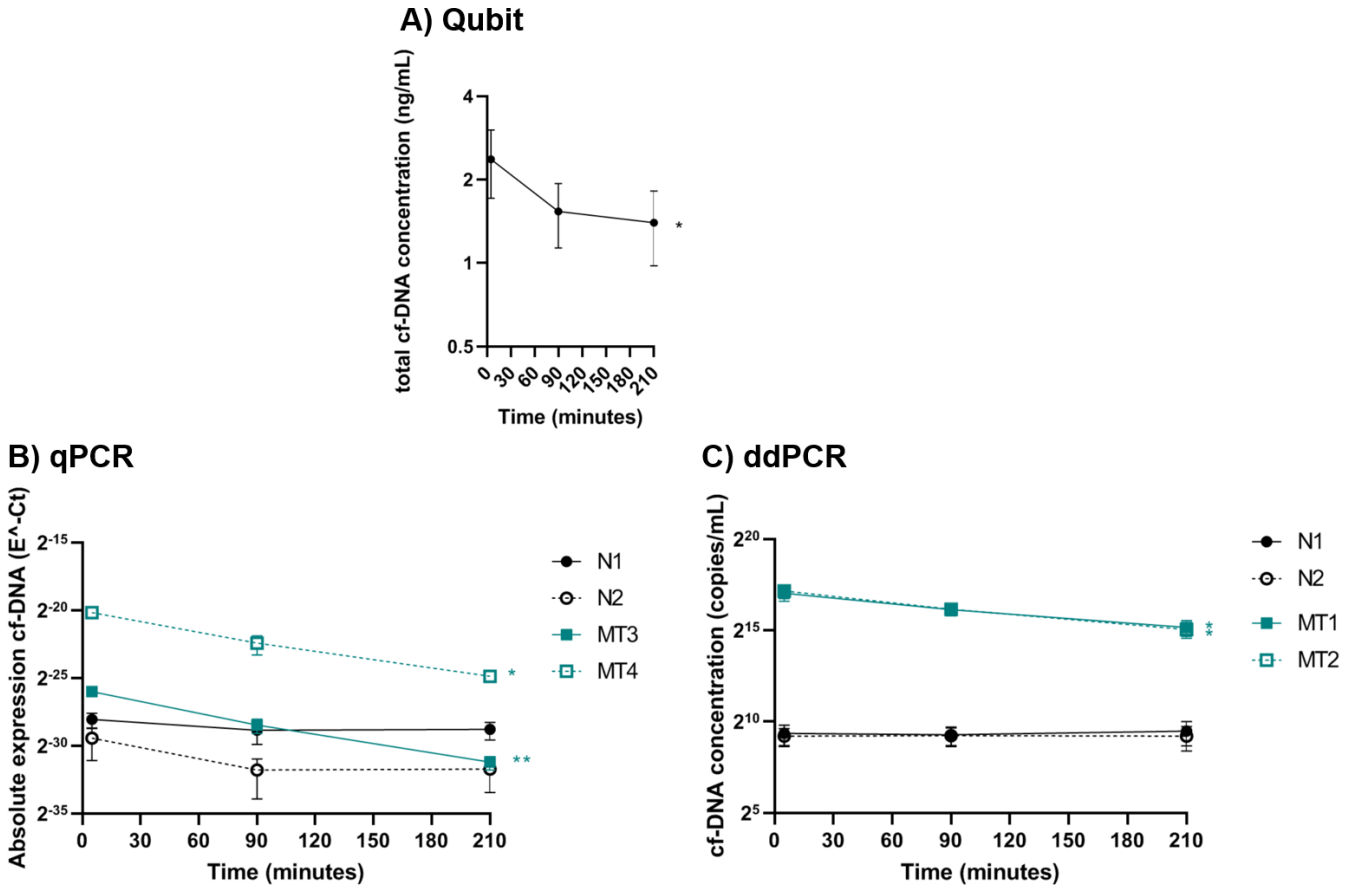


Figure 5: ***Total, mitochondrial and nuclear cf-DNA level changes during ex vivo hypothermic porcine heart machine perfusion.*** *The total cf-DNA levels were quantified using A) Qubit, whereas the mitochondrial and nuclear cf-DNA levels were quantified using B) qPCR , and C) ddPCR. For the quantification of cf-nDNA and cf-mtDNA, two amplicons on different locations of the nuclear GAPDH gene (N1 qPCR, N2 qPCR, N1 ddPCR, and N2 ddPCR) and two amplicons on unique locations of the mitochondrial pig chromosome (MT3 qPCR, MT4, qPCR, MT1 ddPCR, and MT2 ddPCR) were used in both qPCR and ddPCR . The perfusate cf-DNA levels were determined after the heart was mounted to the machine (5 minutes), and at 1.5 and 3.5 hours of hypothermic machine perfusion. A log2 scale was used on the y-axis. The cf-DNA levels are depicted as the actual mean and standard error of the mean (SEM). Repeated measures one-way ANOVA or mixed effects model analyses were performed to assess the change of cf-DNA levels over time and are marked with * $p < 0.05$ and ** $p < 0.01$.*

Total, mitochondrial and possibly nuclear cf-DNA levels increase over time throughout NMP, but do not differ between SCS and HMP hearts

To examine the potential of total perfusate cf-DNA levels during NMP as predictor for *ex vivo* cardiac function, the total cf-DNA perfusate concentrations of the HMP hearts were first compared to those of the SCS hearts (**Figure 6A**). During NMP, the total cell-free DNA levels for both the SCS and HMP hearts increased over time. However, this increase was only significant for the SCS hearts ($p = 0.0054$) and not HMP hearts ($p = 0.1129$). In addition, there were no significant differences in total cf-DNA levels between the SCS and HMP hearts at any of the time points according to the Qubit fluorescence assay.

Next, it was investigated whether this increase in total cf-DNA levels was caused by the release of mitochondrial or nuclear DNA. The qPCR results showed that there was an increase in cf-mtDNA levels of both the MT3 and MT4 amplicon for SCS hearts as well as HMP hearts, as depicted in **Figure 6B.** However, this increase was only significant for the SCS group (MT3 qPCR $p = 0.0052$ and MT4 qPCR $p = 0.0193$) and not for the HMP group (MT3 qPCR $p = 0.1957$ and MT4 qPCR $p = 0.1700$). As illustrated in **Figure 6B**, the cf-nDNA levels did not significantly elevate over time for both storage conditions according to qPCR. Comparable to the total cf-DNA levels, both cf-mtDNA and cf-nDNA levels did not significantly differ at any time point between the SCS and HMP groups for any of the qPCR amplicons (**Figure 6**).

Subsequently, the cf-mtDNA and cf-nDNA levels were absolutely quantified using ddPCR. In the ddPCR results, a trend of higher cf-mtDNA concentrations compared to cf-nDNA levels in the perfusate samples was observed (**Figure 6C**). During NMP, the cf-mtDNA levels were significantly increased in a time-dependent manner in the SCS group (MT1 ddPCR $p = 0.0044$ and MT2 ddPCR $p = 0.0023$), but there was no significant increase over time observed in the HMP hearts (MT1 ddPCR $p = 0.1699$ and MT2 ddPCR $p = 0.1632$) as shown in **Figure 6C**. Moreover, the cf-nDNA N2 amplicon levels were significantly elevated in the SCS group (N1 ddPCR $p = 0.1095$ and N2 ddPCR $p = 0.0151$). The cf-nDNA levels in the HMP preservation group did not significantly increase over time (N1 ddPCR $p= 0.2828$ and N2 ddPCR $p = 0.2141$). Similar to the qPCR results, no significant differences were observed between the SCS and HMP groups at any of the sampling time points.

Taken together, these results demonstrated that total cf-DNA levels increased over time during NMP. This was caused by a selective increase in cf-mtDNA levels and less by an increase in cf-DNA levels. In most cases, the increase in cf-nDNA levels was not significant. Moreover, there were no significant differences between the two preservation methods at any of the time points during reperfusion of the heart using a NMP set-up.

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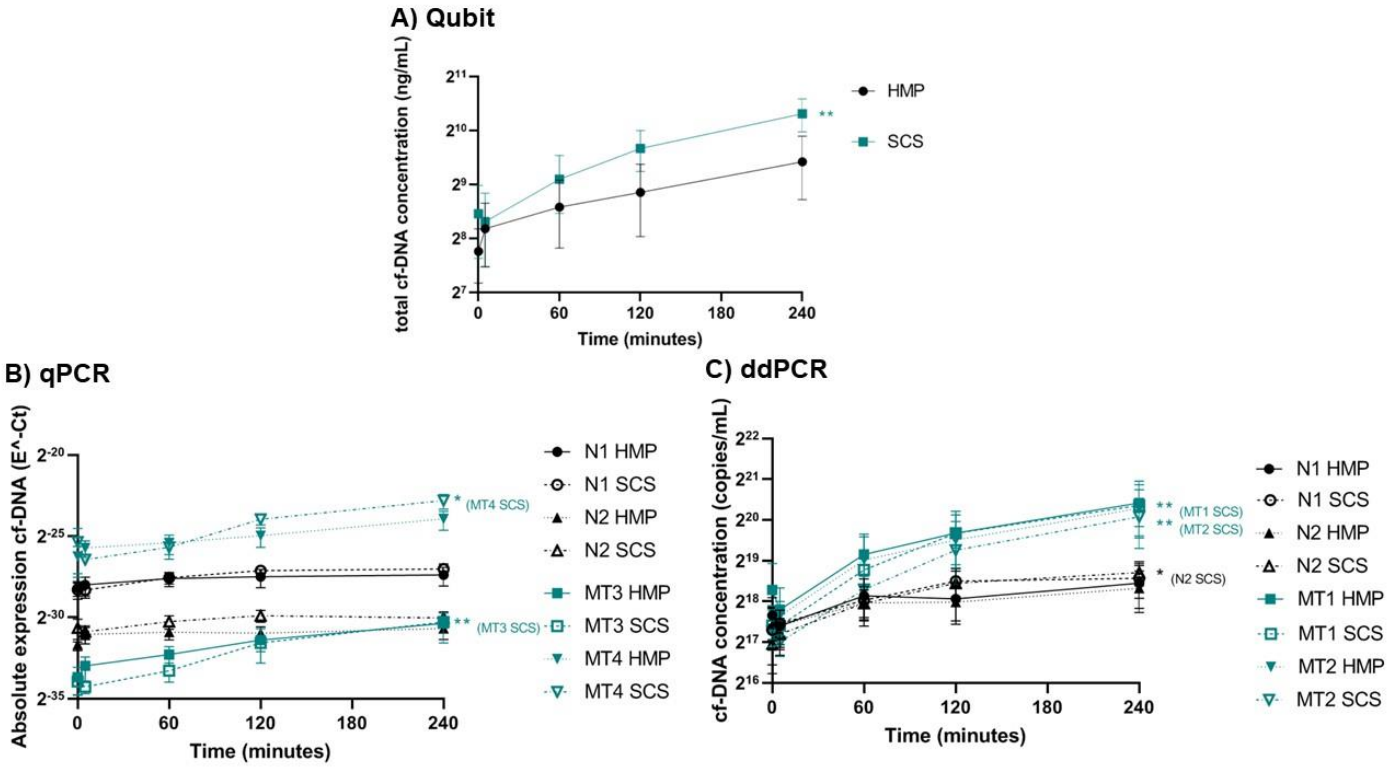


***Figure 6*: Total, mitochondrial and nuclear cf-DNA level changes during ex vivo normothermic porcine heart machine perfusion for SCS and HMP preserved hearts.** *The total cf-DNA levels were quantified using A) Qubit, whereas the mitochondrial and nuclear cf-DNA levels were quantified using B) qPCR , and C) ddPCR. For the quantification of cf-nDNA and cf-mtDNA, two amplicons on different locations of the nuclear GAPDH gene (N1 qPCR, N2 qPCR, N1 ddPCR, and N2 ddPCR) and two amplicons on unique locations of the mitochondrial pig chromosome (MT3 qPCR, MT4, qPCR, MT1 ddPCR, and MT2 ddPCR) were used in both qPCR and ddPCR . The perfusate cf-DNA levels were determined in unused perfusate (T=0), reperfusate (T=5), and at 60, 120 and 240 minutes of normothermic machine perfusion. A log2 scale was used on the y-axis. The cf-DNA levels are depicted as the actual mean and standard error of the mean (SEM). Repeated measures one-way ANOVA or mixed effects model analysis were performed to assess change of cf-DNA levels over time and are marked with * $p < 0.05$ and ** $p < 0.01$.*

Cf-DNA levels during HMP did not correlate with cardiac functional parameters

There is a lack of reliable biomarkers that can predict post-transplantation cardiac viability on a machine perfusion system that keeps the heart in a unloaded state prior to transplantation. Therefore, the potential use of cf-DNA levels as biomarkers for *ex vivo* heart function during HMP was explored. This was done by correlating weight-normalized the HMP cf-DNA levels to the functional cardiac parameters (**Figure 7**) using Spearman's rank correlation tests. For the functional cardiac parameters, cardiac output (CO), coronary flow (CF), left atrial pressure (LAP), mean arterial pressure (MAP), maximum and minimum rate of left ventricular pressure change (dP/dTmax and dP/dTmin), cardiac index (CI) and coronary flow index (CFI), and the measurement values at T240 (NMP) were used. LAP, MAP, dP/dTmax, and dP/dTmin are measures of preload, afterload, diastolic function, and myocardial contractility, respectively. The cf-DNA levels were normalized for heart weight, because larger hearts are likely contain more cells that can undergo damage and were thus expected to release more cf-DNA. Regarding all the cardiac functional parameters, no significant correlations between total, mitochondrial, and nuclear cf-DNA levels and CO, CF, LAP, MAP, dP/dTmax, dP/dTmin, CI, and CFI were observed throughout the HMP period according to the Spearman's rank correlations (**Figure 7**). These results demonstrated that cf-DNA levels during HMP were not able to predict cardiac function following reperfusion.

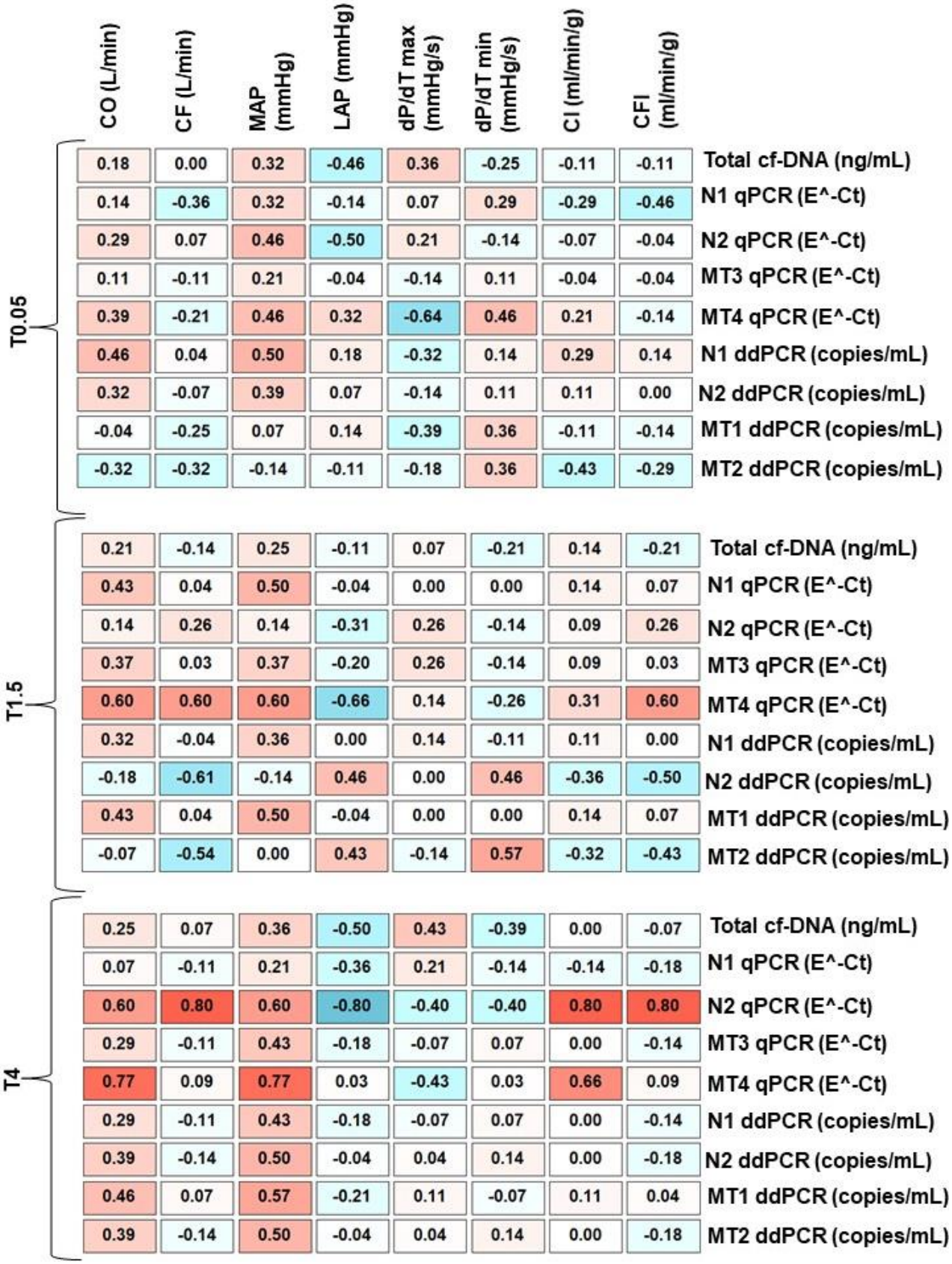


Figure 7: ***Spearman correlation matrix of cf-DNA levels during HMP with functional cardiac parameters.*** *The matrix depicts the Spearman's correlation coefficient of weight-normalized HMP total, nuclear and mitochondrial cf-DNA levels at 5 minutes (T0.05), 1,5 (T1.5), and 3.5 (T4) hours with the cardiac functional parameters measured at T240 (NMP). The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.*

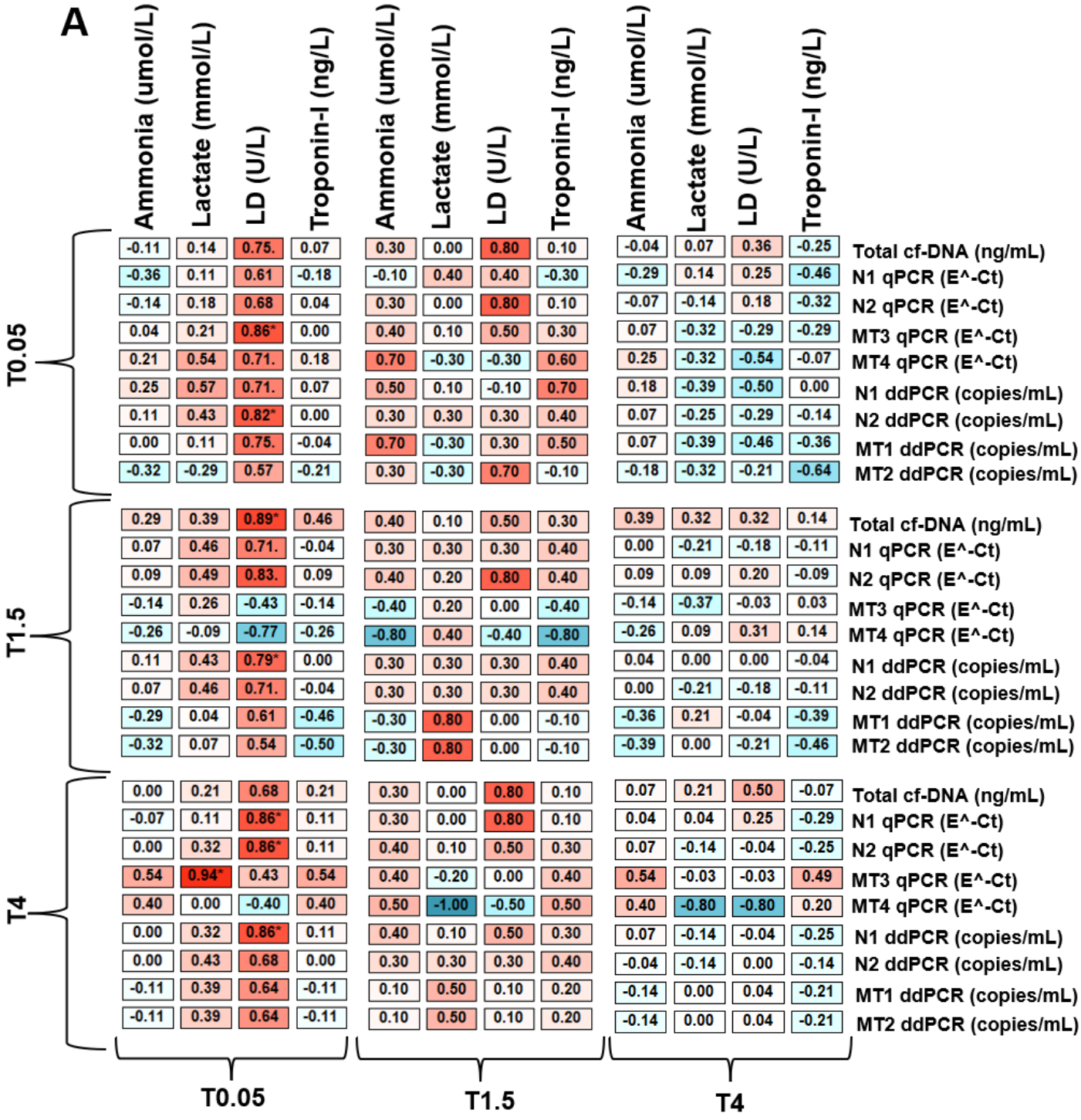
Cf-DNA levels during HMP positively correlated with HMP lactate dehydrogenase levels, and NMP ammonia and lactate levels

The predictive capacity of HMP cf-DNA levels for cardiac injury and heart function was also investigated by assessing their correlations with the levels of regularly used cardiac metabolic and damage markers (lactate, lactate dehydrogenase (LD), ammonia, troponin-I). Perfusate lacate is currently the best indicator of post-transplantation cardiac viability during machine perfusion preservation. Moreover, persistent high lactate serum levels post-transplantation are also an indicator of mortality after heart transplantation. LD in the perfusate indicates general heart injury and troponin-I reflects myocardial injury. Perfusate ammonia might indicate metabolic dysfunction in the heart due to the inactivity of the heart, and reflect myocardial damage. Ammonia is not very well investigated in heart transplantation, but is known to be produced by hearts that experience stress, injury, and/or cell death.

Spearman rank correlation tests were performed between the cf-DNA levels measured at different time points during HMP and the other metabolic and damage markers measured at the same time points (**Figure 8A**) and at different time points during NMP (**Figure 8B**). In **Figure 8A**, it was illustrated that several of the HMP total cf-DNA, cf-nDNA and cf-mtDNA levels at the different time points were positively correlated with lactate dehydrogenase measured at T0.05. More specifically, total cf-DNA levels at T0.05 and T1.5, N1 and N2 qPCR amplicon levels at T1.5 and T4, MT3 and MT4 qPCR amplicon levels at T0.05, N1 and N2 qPCR amplicon levels at T0.05, T1.5 and T4 (only N1), and MT1 ddPCR amplicon levels at T0.05 showed significant positive correlations with T0.05 LD levels. No significant correlations were found for cf-DNA levels with any of the metabolic or damage markers measured at T1.5 and T4.

The HMP mt-DNA levels measured at T0.05 showed significant positive correlations with lactate levels after 2 hours of NMP (ΔT120), except for the MT4 qPCR amplicon levels (**Figure 8B**). In addition, the ddPCR cf-mtDNA levels at T0.05 and T1.5 were found to have a significant positive correlation with lactate levels after 4 hours of NMP (ΔT240). As illustrated in **Figure 8B**, the HMP cf-mtDNA levels at T0.05, except for the MT1 ddPCR amplicon, showed significant positive correlations with reperfusate ammonia levels. The ddPCR cf-nDNA levels were also found to have a significant positive correlation with these reperfusate ammonia levels. Significant positive correlations between ddPCR cf-mtDNA levels measured after 1.5 (T1.5) and 3.5 (T4) hours of HMP and reperfusate ammonia were also observed. Aside from the N2 qPCR amplicon, the T1.5 cf-nDNA levels showed a significant positive correlation with reperfusate ammonia levels. Moreover, the N2 qPCR amplicon and N1 ddPCR amplicon levels were found to have a positively correlate with ammonia in the reperfusate. The HMP N1 qPCR amplicon levels at T0.05 showed significant positive correlations with ammonia levels measured at 2 and 4 hours of NMP. According to ddPCR, there were also significant positive correlations between T1.5 and T4 HMP cf-mtDNA levels and ammonia measured after 4 hours of NMP. The N2 qPCR and N1 ddPCR amplicon levels measured at T1.5 and N2 ddPCR amplicon levels measured at T4 revealed positive significant correlations with these ΔT240 ammonia levels. T1.5 and T4 ddPCR cf-mtDNA levels also correlated with the ΔT240 ammonia levels. A significant negative correlation of the cf-mtDNA ddPCR amplicon levels with LD and troponin-I levels was measured at T0.05. Moreover, MT4 qPCR amplicon levels showed a negative correlation with LD measured after 2 and 4 hours of NMP.

Taken together, these results indicated that HMP cf-mtDNA levels might predict lactate levels during reperfusion of the heart using NMP. Therefore, cf-mtDNA levels might aid in assessing post-transplantation cardiac viability during machine perfusion.


A
Ammonia (umol/L)
Lactate (mmol/L)
LD (U/L)
Troponin-I (ng/L)
T0.05
T1.5
T4
Total cf-DNA (ng/mL)
N1 qPCR (E^-Ct)
N2 qPCR (E^-Ct)
MT3 qPCR (E^-Ct)
MT4 qPCR (E^-Ct)
N1 ddPCR (copies/mL)
N2 ddPCR (copies/mL)
MT1 ddPCR (copies/mL)
MT2 ddPCR (copies/mL)

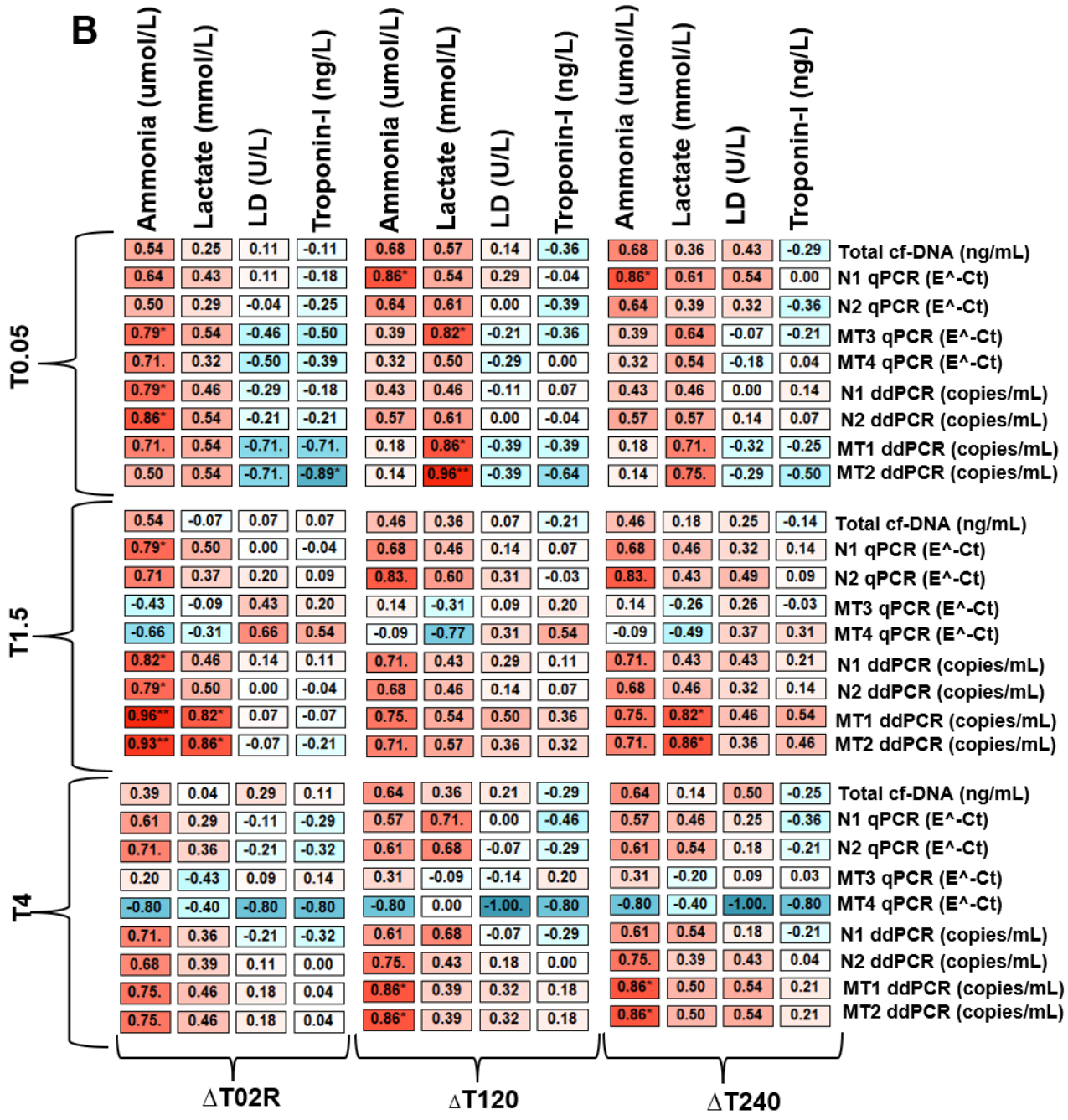


Figure 8: ***Spearman correlation matrix of cf-DNA levels during HMP with other cardiac metabolic and damage markers.*** *The matrix depicts the Spearman's correlation coefficients of weight-normalized HMP total, nuclear and mitochondrial cf-DNA levels at 5 minutes (T0.05), 1,5 (T1.5), and 3.5 (T4) hours with the weight-normalized levels of other metabolic and damage markers, ammonia, lactate, lactate dehydrogenase (LD), and troponin-I, measured during A) HMP measured at T0.05, T1.5, and T4 and B) NMP measured in the reperfusate (ΔT02R) and at 2 (ΔT120) and 4 hours (ΔT240). The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.*

No clear correlation trends between HMP cf-DNA levels and inflammatory markers were observed

Since it is believed that cf-dna plays an important role in regulating the immune response, Spearman's correlations between the cf-dna levels during HMP and levels of inflammatory markers (TNF-α and IL-6) during NMP were also examined. For this, TNF-α levels were normalised for the T02 baseline. It should be noted that IL-6 levels were not normalised for baseline levels, because T02 IL-6 values were below the detection threshold of the assay. In addition, weight-normalisation was performed for all measurements. As shown in **Figure 9**, there was a significant positive correlation between the MT4

qPCR amplicon levels at T0.05 and TNF-α levels in the reperfusate. Furthermore, a significant positive relationship between cf-nDNA levels of the N1 ddPCR amplicon and TNF-α levels measured at 4 hours of NMP was observed. The ddPCR cf-mtDNA levels at T1.5 showed positive correlations with ΔT240 TNF-α levels. From the HMP cf-DNA levels at T4, only the MT3 qPCR amplicon cf-mtDNA levels showed a negative correlation with IL6 levels at T240(**Figure 9**). These results demonstrated that there were no clear correlation trends that were persistent over time between HMP cf-DNA levels and inflammatory marker levels following reperfusion.

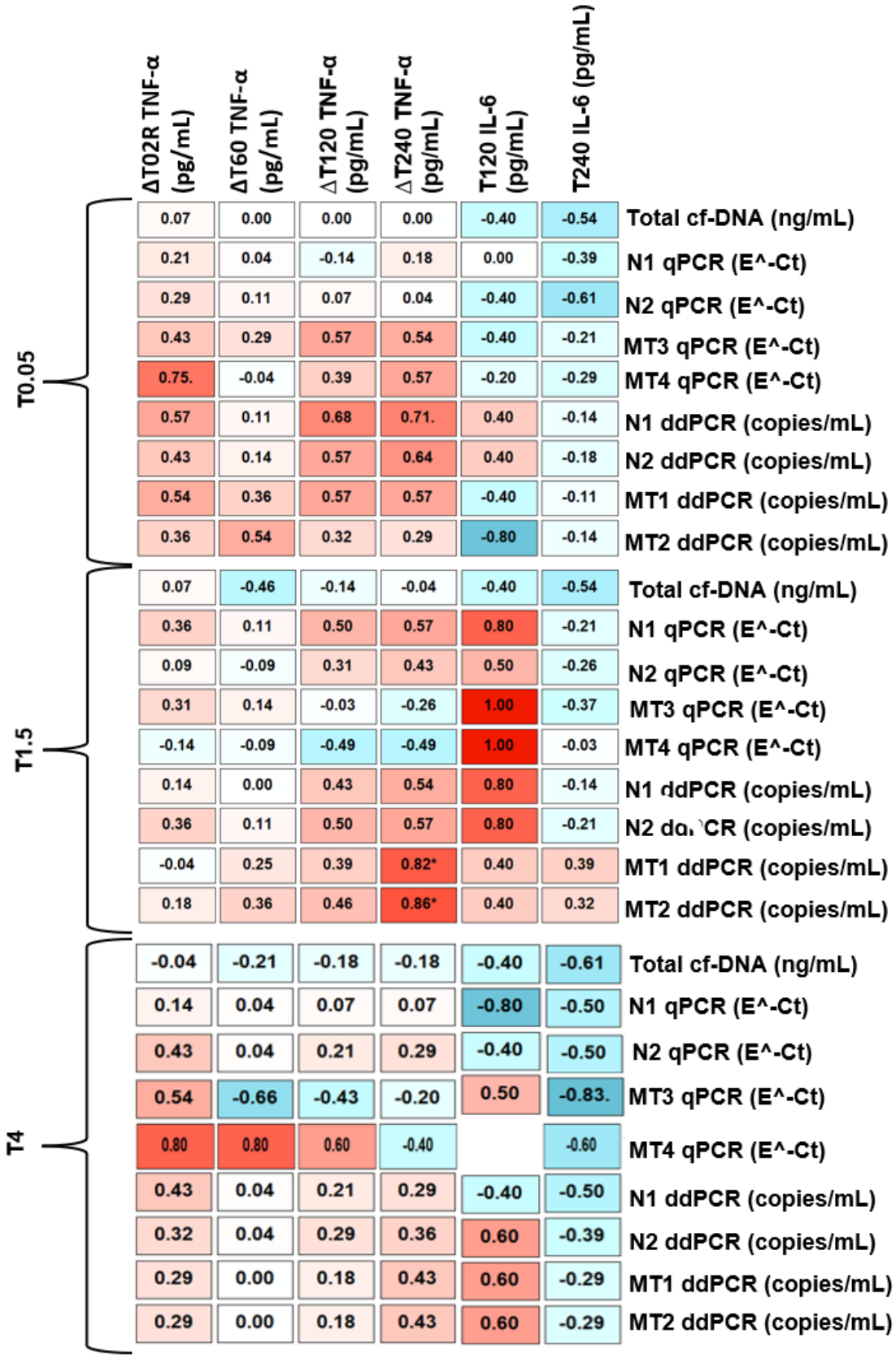


Figure 9: ***Spearman correlation matrix of cf-DNA levels during HMP with inflammatory marker levels during NMP.*** *The Spearman correlation matrix* depicts the Spearman's correlation coefficients between weight-normalised cf-DNA levels at *5 minutes (T0.05), 1,5 (T1.5), and 3.5 (T4) hours with the weight-normalised levels of the inflammatory markers, TNF-α and IL-6, across NMP in the reperfusate, and after 1 (ΔT60), 2 (ΔT120) and 4 (ΔT240) hours. The TNF-α were normalised for the baseline values measured at T02. The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.*

Cf-DNA levels during reperfusion of the heart were found to negatively correlate with functional cardiac parameters, especially after 2 hours of NMP

It was also tested whether cf-DNA levels could serve as an indicator of *ex vivo* heart function during NMP by perfoming Spearman's rank correlation tests (**Figure 10**). The data was normalised by weight and the T02 baseline values. As depicted in **Figure 10**, the perfusate (ΔT02R) MT1 ddPCR amplicon levels showed a positive correlation with LAP and dP/dTmin. In addition, cf-nDNA and cf-mtDNA reperfusate qPCR amplicon levels showed significant negative correlations with dP/dTmax, and positive correlations with dP/dTmin. As illustrated in **Figure 10**, these correlations seem to be stronger for cf-mtDNA levels. After 1 hour of NMP (ΔT60), negative correlations between cf-nDNA N1 qPCR and N1 ddPCR amplicon levels and LAP were observed (**Figure 10**).

After 2 hours of normothermic machine perfusion (ΔT120), total cf-DNA levels negatively correlated with CF with CF, MAP, LAP, CI, and CFI (**Figure 10**). All the T120 cf-nDNA levels showed negative correlations with MAP. Moreover, the cf-nDNA and the cf-mtDNA levels of the qPCR amplicons were negatively correlated with LAP. As is illustrated in **Figure 10**, other negative correlations included the N1 ddPCR amplicon levels with CO, cf-nDNA qPCR levels with CF, and N1, N2 and MT2 ddPCR amplicon levels with CI. The total cf-DNA levels and cf-nDNA levels at 4 hours of NMP (ΔT240) showed significant negative correlations with LAP, as depicted in **Figure 10**.

The Spearman correlations of NMP cf-DNA levels demonstrated that there were mostly negative correlations with cardiac functional parameters. In particular, negative correlation trends of total cf-DNA and cf-nDNA with LAP, a measure of preload, were observed. Moreover, the cf-DNA levels at 2 hours of NMP seem to be the best predictors of cardiac function post-transplantation.

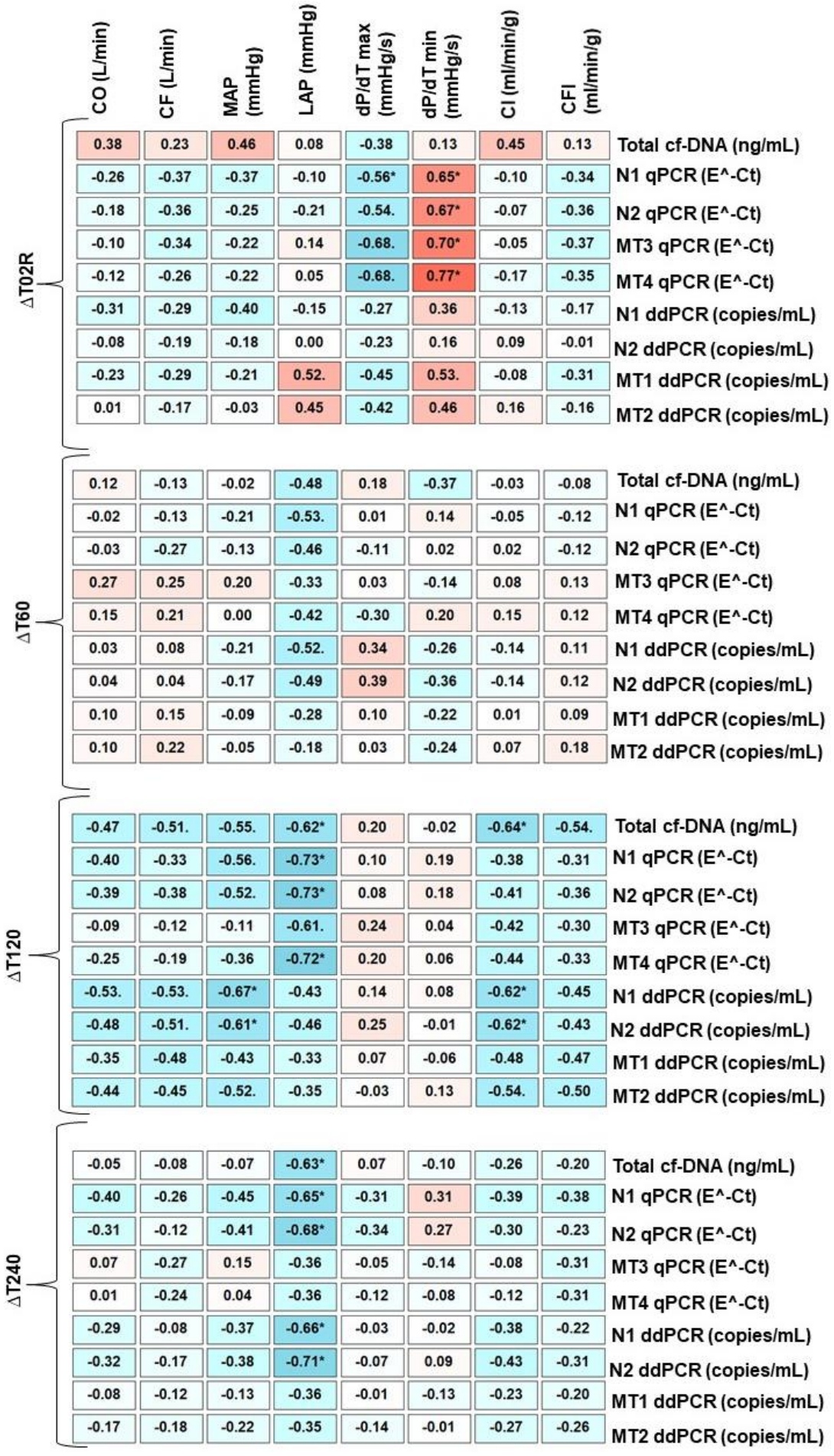

CO (L/min)
CF (L/min)
MAP (mmHg)
LAP (mmHg)
dP/dT max (mmHg/s)
dP/dT min (mmHg/s)
CI (ml/min/g)
CFI (ml/min/g)
ΔT02R
0.38 0.23 0.46 0.08 -0.38 0.13 0.45 0.13 Total cf-DNA (ng/mL)
-0.26 -0.37 -0.37 -0.10 -0.56* 0.65* -0.10 -0.34 N1 qPCR (E^-Ct)
-0.18 -0.36 -0.25 -0.21 -0.54. 0.67* -0.07 -0.36 N2 qPCR (E^-Ct)
-0.10 -0.34 -0.22 0.14 -0.68. 0.70* -0.05 -0.37 MT3 qPCR (E^-Ct)
-0.12 -0.26 -0.22 0.05 -0.68. 0.77* -0.17 -0.35 MT4 qPCR (E^-Ct)
-0.31 -0.29 -0.40 -0.15 -0.27 0.36 -0.13 -0.17 N1 ddPCR (copies/mL)
-0.08 -0.19 -0.18 0.00 -0.23 0.16 0.09 -0.01 N2 ddPCR (copies/mL)
-0.23 -0.29 -0.21 0.52. -0.45 0.53. -0.08 -0.31 MT1 ddPCR (copies/mL)
0.01 -0.17 -0.03 0.45 -0.42 0.46 0.16 -0.16 MT2 ddPCR (copies/mL)
ΔT60
0.12 -0.13 -0.02 -0.48 0.18 -0.37 -0.03 -0.08 Total cf-DNA (ng/mL)
-0.02 -0.13 -0.21 -0.53. 0.01 0.14 -0.05 -0.12 N1 qPCR (E^-Ct)
-0.03 -0.27 -0.13 -0.46 -0.11 0.02 0.02 -0.12 N2 qPCR (E^-Ct)
0.27 0.25 0.20 -0.33 0.03 -0.14 0.08 0.13 MT3 qPCR (E^-Ct)
0.15 0.21 0.00 -0.42 -0.30 0.20 0.15 0.12 MT4 qPCR (E^-Ct)
0.03 0.08 -0.21 -0.52. 0.34 -0.26 -0.14 0.11 N1 ddPCR (copies/mL)
0.04 0.04 -0.17 -0.49 0.39 -0.36 -0.14 0.12 N2 ddPCR (copies/mL)
0.10 0.15 -0.09 -0.28 0.10 -0.22 0.01 0.09 MT1 ddPCR (copies/mL)
0.10 0.22 -0.05 -0.18 0.03 -0.24 0.07 0.18 MT2 ddPCR (copies/mL)
ΔT120
-0.47 -0.51. -0.55. -0.62* 0.20 -0.02 -0.64* -0.54. Total cf-DNA (ng/mL)
-0.40 -0.33 -0.56. -0.73* 0.10 0.19 -0.38 -0.31 N1 qPCR (E^-Ct)
-0.39 -0.38 -0.52. -0.73* 0.08 0.18 -0.41 -0.36 N2 qPCR (E^-Ct)
-0.09 -0.12 -0.11 -0.61. 0.24 0.04 -0.42 -0.30 MT3 qPCR (E^-Ct)
-0.25 -0.19 -0.36 -0.72* 0.20 0.06 -0.44 -0.33 MT4 qPCR (E^-Ct)
-0.53. -0.53. -0.67* -0.43 0.14 0.08 -0.62* -0.45 N1 ddPCR (copies/mL)
-0.48 -0.51. -0.61* -0.46 0.25 -0.01 -0.62* -0.43 N2 ddPCR (copies/mL)
-0.35 -0.48 -0.43 -0.33 0.07 -0.06 -0.48 -0.47 MT1 ddPCR (copies/mL)
-0.44 -0.45 -0.52. -0.35 -0.03 0.13 -0.54. -0.50 MT2 ddPCR (copies/mL)
ΔT240
-0.05 -0.08 -0.07 -0.63* 0.07 -0.10 -0.26 -0.20 Total cf-DNA (ng/mL)
-0.40 -0.26 -0.45 -0.65* -0.31 0.31 -0.39 -0.38 N1 qPCR (E^-Ct)
-0.31 -0.12 -0.41 -0.68* -0.34 0.27 -0.30 -0.23 N2 qPCR (E^-Ct)
0.07 -0.27 0.15 -0.36 -0.05 -0.14 -0.08 -0.31 MT3 qPCR (E^-Ct)
0.01 -0.24 0.04 -0.36 -0.12 -0.08 -0.12 -0.31 MT4 qPCR (E^-Ct)
-0.29 -0.08 -0.37 -0.66* -0.03 -0.02 -0.38 -0.22 N1 ddPCR (copies/mL)
-0.32 -0.17 -0.38 -0.71* -0.07 0.09 -0.43 -0.31 N2 ddPCR (copies/mL)
-0.08 -0.12 -0.13 -0.36 -0.01 -0.13 -0.23 -0.20 MT1 ddPCR (copies/mL)
-0.17 -0.18 -0.22 -0.35 -0.14 -0.01 -0.27 -0.26 MT2 ddPCR (copies/mL)

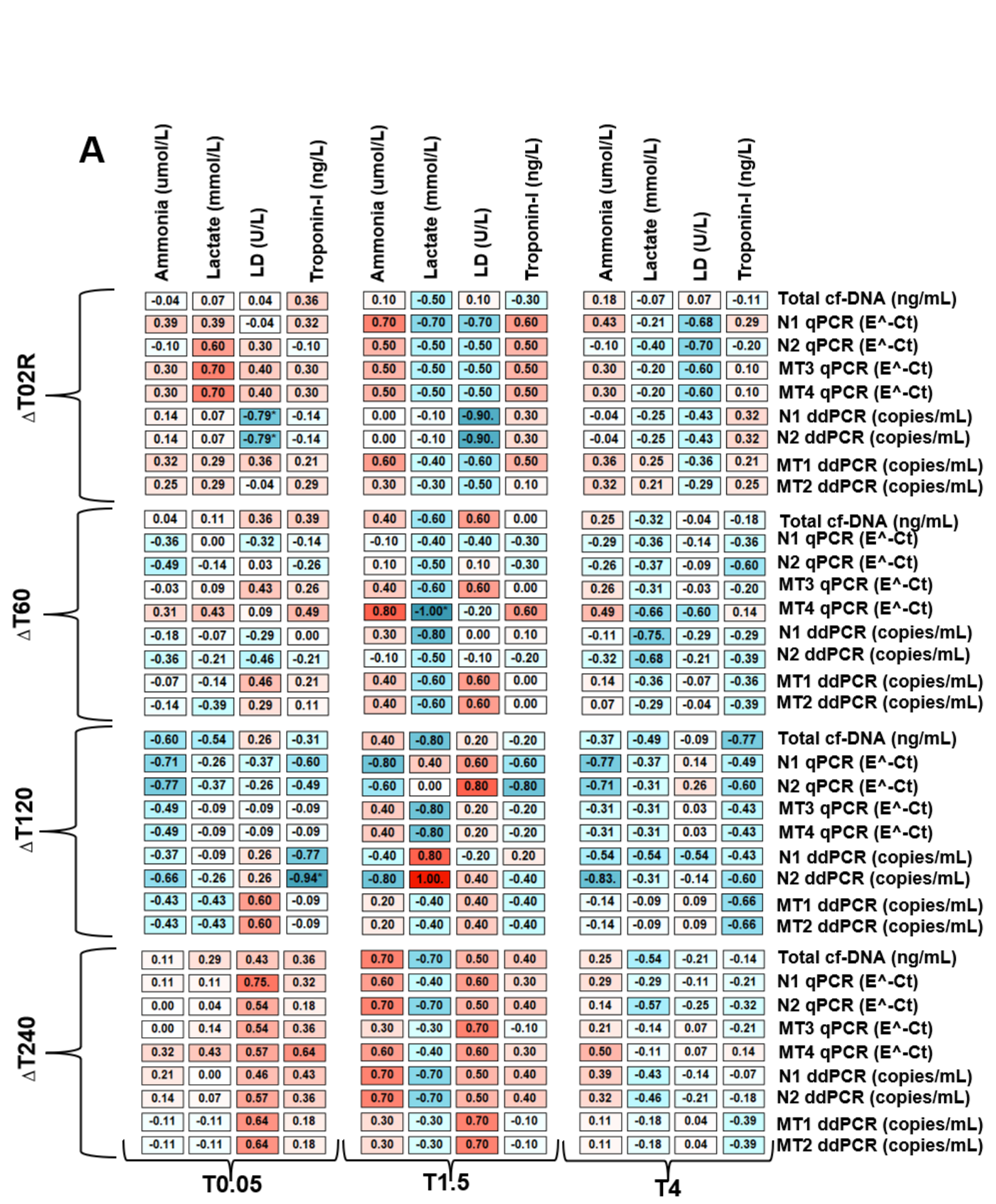
Figure 10: ***Spearman correlation matrix of cf-DNA levels during heart reperfusion with functional cardiac parameters.*** *The matrix depicts the Spearman's correlation coefficient of baseline(T02)- and weight-normalized total, nuclear and mitochondrial cf-DNA levels after reperfusion (∆T02R) and at 1 (∆T60) , 2 (∆T120) and 4 (∆T240) hours of NMP with the cardiac functional parameters measured at T240 (NMP). The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.*

Several significant negative and positive correlations were found between the NMP cf-DNA levels and other metabolic and damage markers measured across HMP and NMP

Spearman's correlation tests were also performed to assess whether there were correlations between the cf-DNA levels during NMP and the other metabolic and damage markers (ammonia, lactate, LD, and troponin-I) measured during HMP and NMP (**Figures 11A and 11B**). The ddPCR cf-nDNA reperfusate levels showed significant negative correlations with LD measured at T0.05 and T1.5 (**Figure 11A**). Moreover, the ∆T60 MT4 qPCR and N1 ddPCR were found to negatively correlate with lactate levels at T1.5 and T4, respectively. The ∆T120 N2 ddPCR amplicon levels showed negative correlations with T0.05 troponin-I and T4 ammonia levels, as well as a positive correlation with T1.5 lactate levels. Furthermore, a negative correlation was found between the ∆T240 N1 qPCR amplicon levels and T0.05 LD levels.

As illustrated in **Figure 11B**, total cf-DNA reperfusate levels showed a significant negative correlation with ammonia at ∆T120 and lactate in the reperfusate and at ∆T120. Moreover, significant negative correlations were observed between the cf-mtDNA levels at 1 hour of NMP and ammonia, LD, and troponin-I at 4 hours of NMP, except for the MT3 qPCR amplicons levels with ammonia and troponin-I. ∆T120 cf-mtDNA levels were found to have a significant positive correlation with lactate at that same time point and had a significant negative correlation with troponin-I at ∆T240. ∆T120 N1 qPCR and MT4 qPCR amplicon levels also showed positive correlations with ammonia levels at the same time point. Cf-nDNA after 4 hours of NMP showed significant negative correlations with reperfusate LD and troponin-I. Furthermore, the total cf-DNA and ddPCR cf-mtDNA levels also were significantly negatively correlated with reperfusate troponin-I. The N1 and MT4 qPCR amplicon levels were found to have a significant negative correlation with reperfusate lactate levels.

Together, the results demonstrated that there were several positive and negative correlations of the cf-DNA levels during heart reperfusion (NMP) and levels of other metabolic and damage markers during both HMP and NMP. However, the correlation trends seemed to be inconsistent over time, except for the negative correlations of cf-mtDNA levels and ∆T240 troponin-I levels.


A
Ammonia (umol/L)
Lactate (mmol/L)
LD (U/L)
Troponin-I (ng/L)
ΔT02R
ΔT60
ΔT120
ΔT240
Total cf-DNA (ng/mL)
N1 qPCR (E^-Ct)
N2 qPCR (E^-Ct)
MT3 qPCR (E^-Ct)
MT4 qPCR (E^-Ct)
N1 ddPCR (copies/mL)
N2 ddPCR (copies/mL)
MT1 ddPCR (copies/mL)
MT2 ddPCR (copies/mL)
T0.05
T1.5
T4

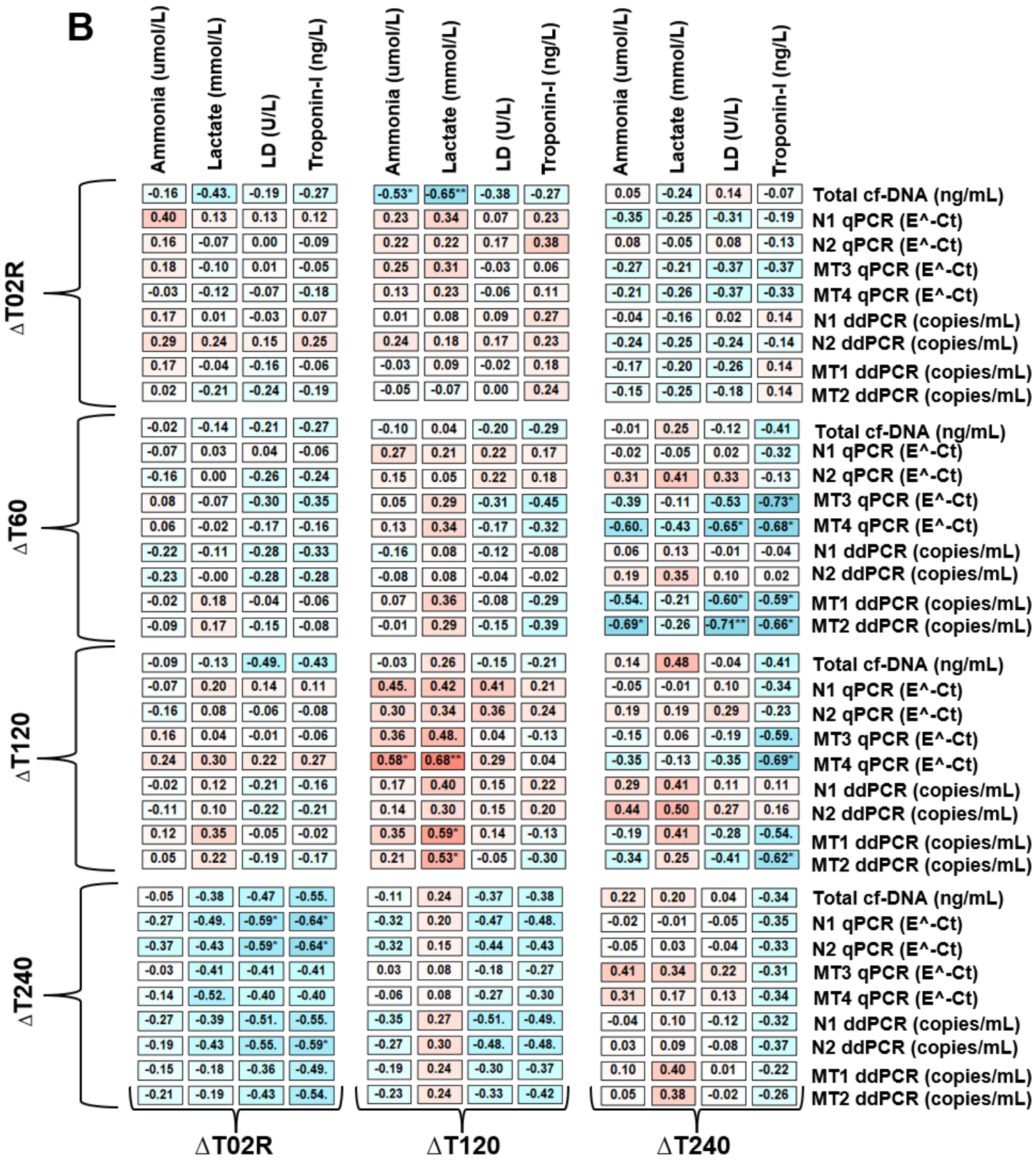


Figure 11: ***Spearman correlation matrix of cf-DNA levels during heart reperfusion with other cardiac metabolic and damage markers.*** *The matrix depicts the Spearman's correlation coefficient of baseline(T02)- weight-normalized NMP total, nuclear and mitochondrial cf-DNA levels in the reperfusate (ΔT02R) , and after 1 (ΔT60), 2 (ΔT120) and 4 (ΔT240) hours with the*

weight-normalized levels of other metabolic and damage markers, ammonia, lactate, lactate dehydrogenase, and troponin-I, measured during A) HMP measured at T0.05, T1.5, and T4 and B) NMP measured at ∆T02R, ∆T120, ∆T240. The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1, p < 0.05, ** p <0.01, and *** p < 0.001.*

No clear correlation trends between NMP cf-DNA levels and inflammatory markers were observed

The reperfusate cf-nDNA of the N1 qPCR and N2 ddPCR amplicons and cf-mtDNA levels of the MT3 qPCR amplicon showed significant positive correlations with the TNF-α levels at the same time point (**Figure 12**). Total cf-DNA reperfusate levels were found to have a significant negative correlation with NMP TNF-α levels at ∆T60, ∆T120, and ∆T240. According to the ddPCR data, there was a significant positive correlation of cf-nDNA reperfusate levels with IL-6 levels at T240 and a significant negative correlation of the cf-mtDNA reperfusate levels and ∆T120. The Spearman's correlation results showed that the qPCR ∆T60 cf-mtDNA levels were negatively associated with T240 IL-6 levels (**Figure 12**). Only the MT3 qPCR amplicon levels also showed a significant negative correlation with IL-6 levels at T120. In addition, a significant positive correlation between MT4 amplicon levels and reperfusate TNF-α levels was found. ∆T120 cf-nDNA levels were found to have significant positive correlations with ∆T240 TNF-α and for the N2 amplicon also with T120 IL-6 levels, according to the ddPCR data.The ddPCR MT2 amplicon levels also showed a positive correlation with T120 IL-6 levels. In addition, the MT4 qPCR amplicon levels showed a positive correlation with TNF-α levels in the reperfusate and after 1 hour of NMP. Cf-mtDNA levels of the MT1 ddPCR amplicon also showed a positive correlation with ∆T60 TNF-α levels.At 4 hours of NMP, the total cf-DNA levels and cf-mtDNA qPCR amplicon levels were significantly negatively correlated with IL-6 levels at T240.

Altogether, these correlations demonstrated that cf-DNA following reperfusion using NMP showed several significant positive and negative correlations with inflammatory markers. These correlations, however, did not have trends that persisted over time.

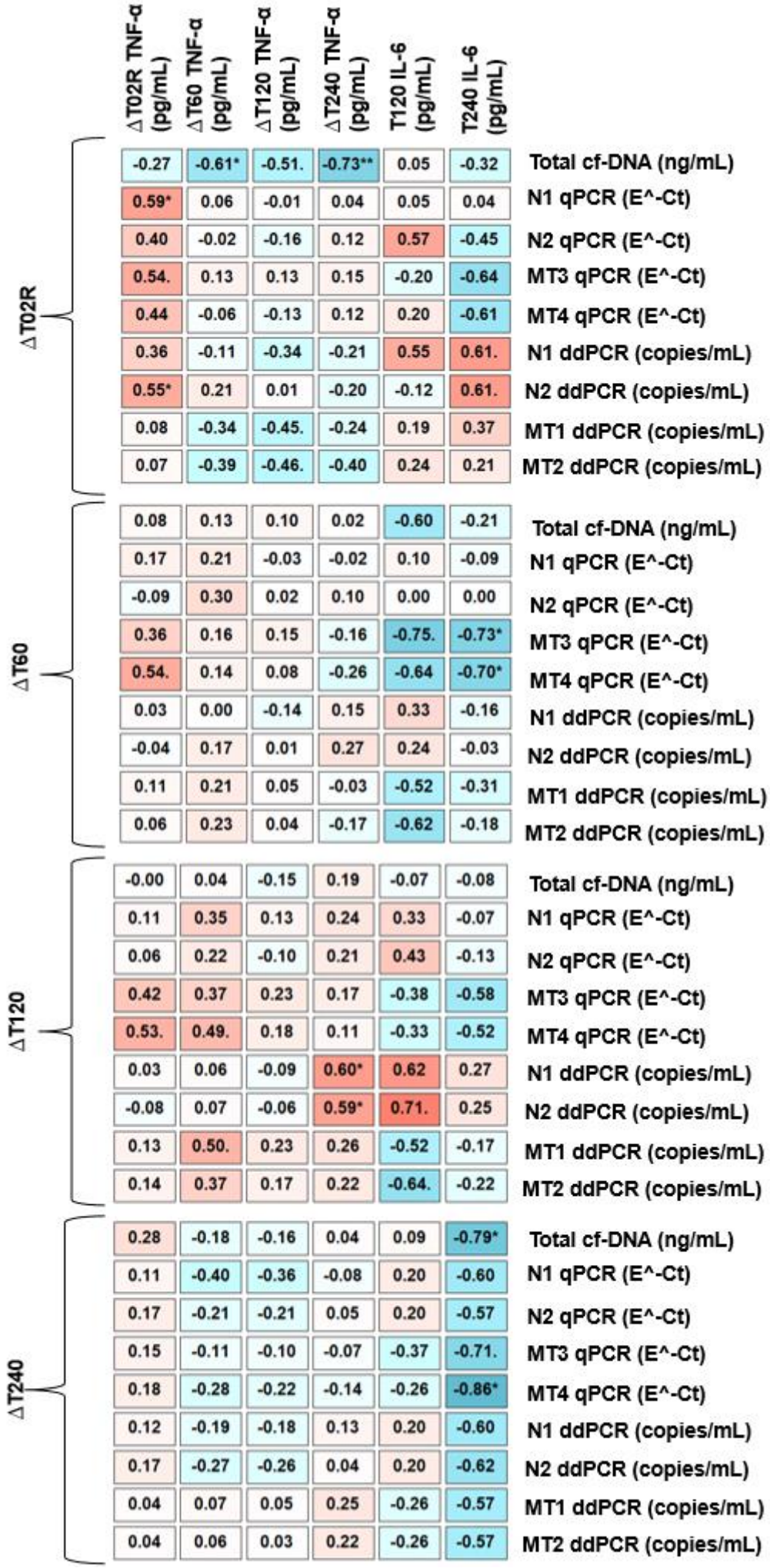

ΔT02R TNF-α (pg/mL)
ΔT60 TNF-α (pg/mL)
ΔT120 TNF-α (pg/mL)
ΔT240 TNF-α (pg/mL)
T120 IL-6 (pg/mL)
T240 IL-6 (pg/mL)
ΔT02R
-0.27 -0.61* -0.51. -0.73** 0.05 -0.32 Total cf-DNA (ng/mL)
0.59* 0.06 -0.01 0.04 0.05 0.04 N1 qPCR (E^-Ct)
0.40 -0.02 -0.16 0.12 0.57 -0.45 N2 qPCR (E^-Ct)
0.54. 0.13 0.13 0.15 -0.20 -0.64 MT3 qPCR (E^-Ct)
0.44 -0.06 -0.13 0.12 0.20 -0.61 MT4 qPCR (E^-Ct)
0.36 -0.11 -0.34 -0.21 0.55 0.61. N1 ddPCR (copies/mL)
0.55* 0.21 0.01 -0.20 -0.12 0.61. N2 ddPCR (copies/mL)
0.08 -0.34 -0.45. -0.24 0.19 0.37 MT1 ddPCR (copies/mL)
0.07 -0.39 -0.46. -0.40 0.24 0.21 MT2 ddPCR (copies/mL)
ΔT60
0.08 0.13 0.10 0.02 -0.60 -0.21 Total cf-DNA (ng/mL)
0.17 0.21 -0.03 -0.02 0.10 -0.09 N1 qPCR (E^-Ct)
-0.09 0.30 0.02 0.10 0.00 0.00 N2 qPCR (E^-Ct)
0.36 0.16 0.15 -0.16 -0.75. -0.73* MT3 qPCR (E^-Ct)
0.54. 0.14 0.08 -0.26 -0.64 -0.70* MT4 qPCR (E^-Ct)
0.03 0.00 -0.14 0.15 0.33 -0.16 N1 ddPCR (copies/mL)
-0.04 0.17 0.01 0.27 0.24 -0.03 N2 ddPCR (copies/mL)
0.11 0.21 0.05 -0.03 -0.52 -0.31 MT1 ddPCR (copies/mL)
0.06 0.23 0.04 -0.17 -0.62 -0.18 MT2 ddPCR (copies/mL)
ΔT120
-0.00 0.04 -0.15 0.19 -0.07 -0.08 Total cf-DNA (ng/mL)
0.11 0.35 0.13 0.24 0.33 -0.07 N1 qPCR (E^-Ct)
0.06 0.22 -0.10 0.21 0.43 -0.13 N2 qPCR (E^-Ct)
0.42 0.37 0.23 0.17 -0.38 -0.58 MT3 qPCR (E^-Ct)
0.53. 0.49. 0.18 0.11 -0.33 -0.52 MT4 qPCR (E^-Ct)
0.03 0.06 -0.09 0.60* 0.62 0.27 N1 ddPCR (copies/mL)
-0.08 0.07 -0.06 0.59* 0.71. 0.25 N2 ddPCR (copies/mL)
0.13 0.50. 0.23 0.26 -0.52 -0.17 MT1 ddPCR (copies/mL)
0.14 0.37 0.17 0.22 -0.64. -0.22 MT2 ddPCR (copies/mL)
ΔT240
0.28 -0.18 -0.16 0.04 0.09 -0.79* Total cf-DNA (ng/mL)
0.11 -0.40 -0.36 -0.08 0.20 -0.60 N1 qPCR (E^-Ct)
0.17 -0.21 -0.21 0.05 0.20 -0.57 N2 qPCR (E^-Ct)
0.15 -0.11 -0.10 -0.07 -0.37 -0.71. MT3 qPCR (E^-Ct)
0.18 -0.28 -0.22 -0.14 -0.26 -0.86* MT4 qPCR (E^-Ct)
0.12 -0.19 -0.18 0.13 0.20 -0.60 N1 ddPCR (copies/mL)
0.17 -0.27 -0.26 0.04 0.20 -0.62 N2 ddPCR (copies/mL)
0.04 0.07 0.05 0.25 -0.26 -0.57 MT1 ddPCR (copies/mL)
0.04 0.06 0.03 0.22 -0.26 -0.57 MT2 ddPCR (copies/mL)

Figure 12: ***Spearman correlation matrix of cf-DNA levels during NMP with inflammatory marker levels during NMP.*** *The Spearman correlation matrix* depicts the Spearman's correlation coefficients between baseline (T02) – and weight-normalised cf-DNA levels in the reperfusate (ΔT02R) and at 1 (ΔT60), 2 (ΔT120) and 4 (ΔT240) *hours with the weight-normalised levels of the inflammatory markers, TNF-α and IL-6, across NMP in the reperfusate, and after 1 (only for TNF-α), 2 and 4 hours. The TNF-α were normalised for the baseline values measured at T02. The colors (blue, white, and red) indicate the size and direction of the Spearman's correlation values ranging from -1 to 1, with red and blue indicating the strongest positive and negative correlation values, respectively. Significant correlations are marked with .p < 0.1,* p < 0.05, ** p <0.01, and *** p < 0.001.*

Quality control of cf-RNA seq data

All collected time samples from 2 SCS and 2 HMP replicates were subjected to CEL-seq2 after RNA extraction to examine whether this mRNA-sequencing method enables usable cf-mRNA transcriptome profiles. Sample-level quality control on the count-data is a crucial part in RNA sequencing data analysis to guarantee that only the useful samples are used in downstream analysis. cf-RNA-seq data may contain more variability than tissue biopsy RNA-seq data, because cf-RNA processing and extraction protocols are not optimized and standardized yet. In addition, variability in cf-mRNA seq data can be explained by the fact that the extracellular space is abundant in nucleases. Quality control was performed by assessing the total raw counts and gene coverage per sample (**Supplementary Figure 3**). **Supplementary Figure 3** depicts that the samples had a lot of variability in the total raw counts and gene coverage. The whole blood and T02 NP21 samples were excluded from further analysis, because they did not reach the thresholds of 25,000 total raw read counts and 1500 covered genes with more than 5 reads. Furthermore, principal component analysis (PCA) was performed on the samples to examine their similarity and to investigate to what extend time, storage conditions, or heart replicate (NP number) contributed to the variation. PCA of the 500 most variable genes of the residual samples showed that about 80% and 3% of the variability in the RNA sequencing data was explained by the first and second principal components, respectively (**Supplementary Figure 4**). NP number (**Supplementary Figure 4A**) and storage condition (**Supplementary Figure 4B**). did not result in separation of the samples, indicating that these factors did not explain the variability of the first and second principal component. The results indicate that the variation in the first principal component was explained by time, because separation between HMP and NMP samples between earlier and later time points during NMP occurred (**Supplementary Figure 4C**). Therefore, time was treated as a continuous variable in further analysis.

Differential gene expression and pathway analyses revealed downregulation of energy metabolism pathways throughout HMP and NMP, and upregulation of gene regulation (HMP) and inflammatory pathways (NMP)

To examine which genes were differentially expressed during HMP, DEG analysis was performed with time as a continuous variable. This was performed on data from perfusate samples of two HMP replicates per time point obtained at 5 minutes, 1.5 hours and 3.5 hours of HMP. 79 genes were significantly upregulated and 34 genes significantly downregulated (adjusted p-value < 0.1) during HMP (**Figure 13A**).

For the differential gene expression throughout NMP, 2 replicates from both the SCS and the HMP group were included in the analysis. To identify if there were any differences between SCS and HMP samples, differential gene expression analysis was performed between the two storage groups and this showed that there were no significant condition-specific differences over time (**Supplementary Figure 5**). Therefore, the samples of both storage conditions were combined. In this way, differential gene expression analysis with time as a continuous variable showed that 54 genes were significantly upregulated, whereas 30 were significantly downregulated (adjusted p-value < 0.1) during NMP, as depicted in **Figure 13B**.

Together, these results indicated that these differentially expressed genes might be predicters of cardiac function after transplantation.

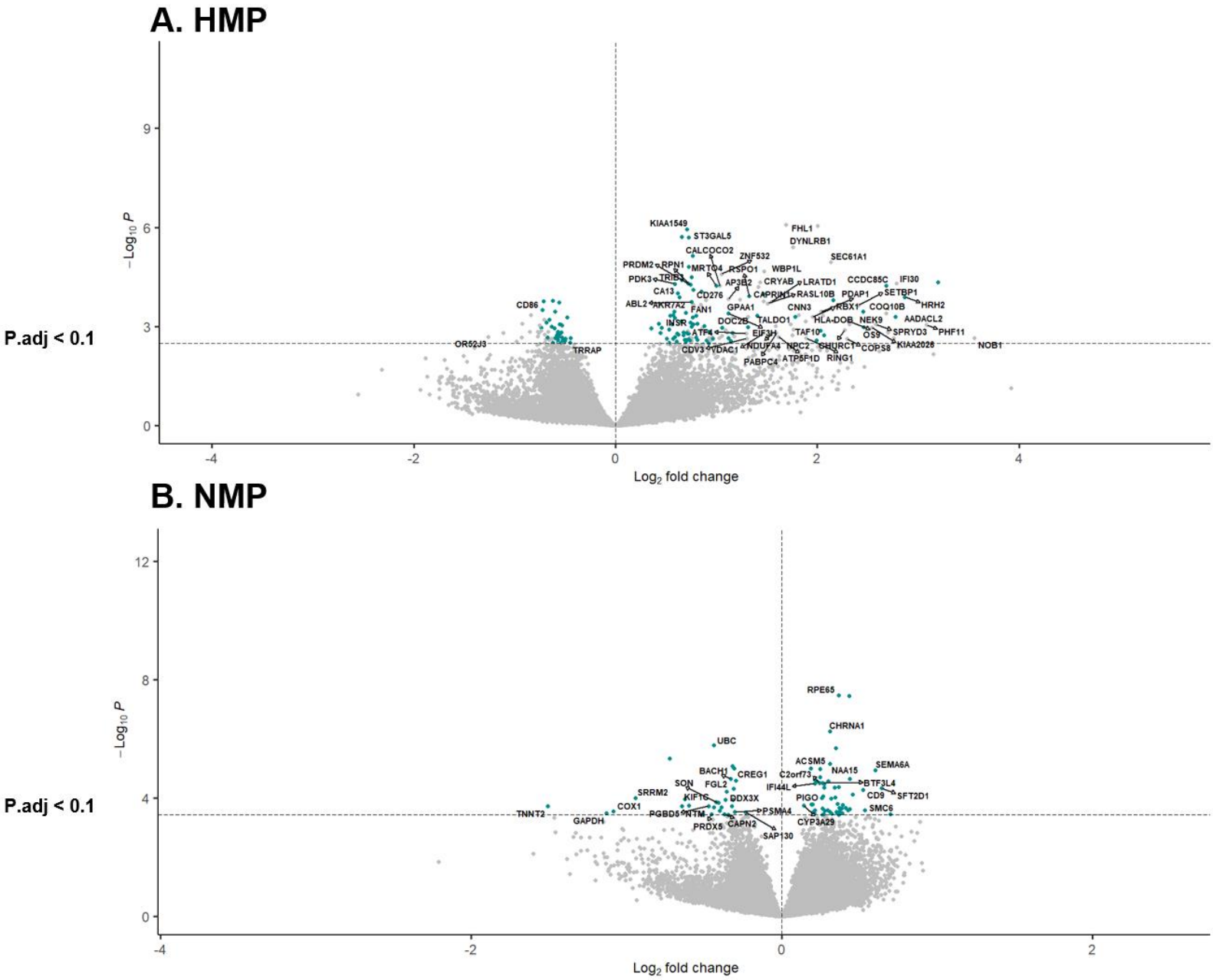


***Figure 13*: Volcano plots of identified differentially expressed genes of cf-mRNA in the perfusate over time during both hypothermic and normothermic ex vivo heart machine perfusion.** *Volcano plots illustrate the differentially expressed genes of cf-mRNA in perfusate samples calculated with the DESeq2 algorithm A) throughout hypothermic machine perfusion, and B) normothermic machine perfusion. Significant differentially expressed genes are depicted in blue-green and were considered significant for adjusted p-values lower than 0.1. The grey dots that were present in significant area of adjusted p-values lower than the 0.1 cut-off, indicated NA adjusted p-values. The x-axis depicts the log2 fold change, and the y-axis the -log10(p-value).*

Next, a GO gene set enrichment and pathway analysis was conducted to examine the biological role of these genes. The GO pathway analysis revealed that energy metabolism pathways, including oxidative phosphorylation and the electron transport chain, were suppressed during HMP, whereas GO terms involved in transcription, such as regulatory region nucleic acid binding, were activated (**Figure 14A**).

GO gene set enrichment and pathway analysis showed that pathways related to energy metabolism were also downregulated throughout NMP (**Figure 14B**). For example, the GO pathway analysis identified terms such as ‘ATP metabolic processes’ ,‘generation of precursor metabolites and energy’, and ‘cellular respiration’ as suppressed over the time-course of heart reperfusion using NMP. The enrichment of GO terms including ‘response to external biotic stimulus’, ‘immune response’, and

‘immune system process’ indicated that the heart responded to stimuli during NMP, and that an immune response was provoked.

Together, our cf-mRNA data indicates that the energy metabolism was disturbed during both HMP and NMP. During HMP, the heart might attempt to minimize ischemia-reperfusion injury by activating gene regulation processes, while throughout NMP an immune response was generated.

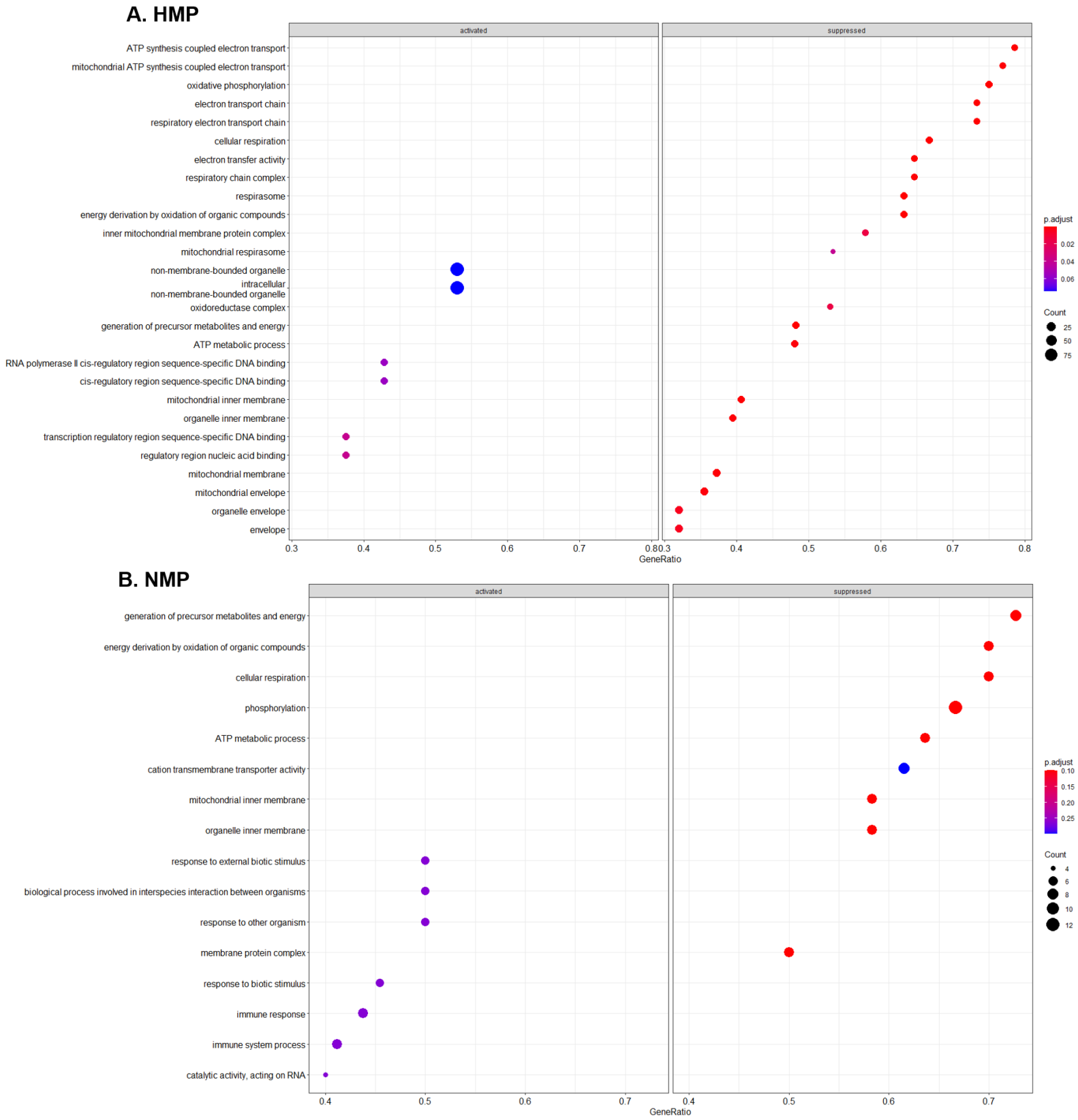


Figure 14: ***Gene ontology gene enrichment and pathway analysis throughout A) hypothermic and B) normothermic machine perfusion.*** *The dot plots illustrate the activated and/or suppressed pathways throughout HMP (A) and NMP (B). The y-axis shows the enriched GO terms. The x-axis depicts the GeneRatio, which is the number of genes affected from a certain gene set (count), divided by the total number of genes included in this gene set (setSize). The dot size indicates the gene count, whereas the dot color displays the Benjamini-Hochberg adjusted p-value of < 0.1 and < 0.3 for HMP and NMP, respectively.*

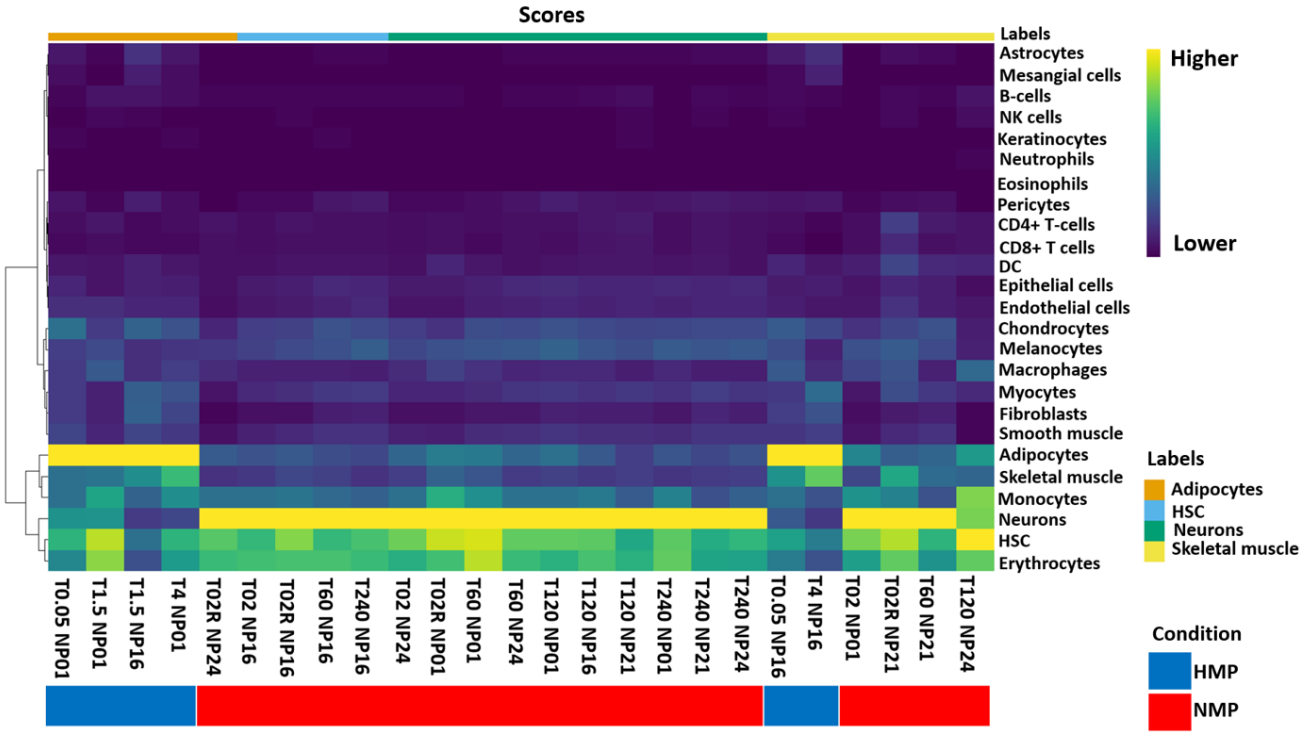
Ventricular cardiomyocytes and adipocytes are most likely major sources of cf-NA

Because RNA is very unstable, it is thought that the main source of cf-RNA that we can extract is derived from living cells, therefore it is believed that cf-RNA reflects the gene expression patterns of the cells that release them. To get an insight about which cells release the cf-RNA, cell type deconvolution using SingleR was performed using prevalent cell types of the body as a reference dataset. The origin of the cf-RNA is deciphered as SingleR compares the transcriptomic input data to transcriptomic reference data sets of pure cell types.

SingleR predicted that most of the cf-RNA was derived from adipocytes during HMP (**Figure 15A**). Skeletal muscle cells were also predicted to be major contributors to the cf-RNA content in the HMP samples. Moreover, the skeletal muscle cells also seem to release cf-RNA at the beginning of NMP (**Figure 15A**). In addition, neurons were identified as the main origin of the cf-RNA when the heart was in working mode during normothermic machine perfusion. Another potential source of cf-RNA during NMP were the hematopoietic stem cells (HSC). However, these cell types are not the most abundant cell types in the heart. Importantly, the deconvolution reference set did not contain cardiomyocytes. Therefore, it may not be an accurate reflection of which cell types release the cf-RNA. However, from these results it could be concluded that the main source of cf-RNA are not blood and/or immune cells.

Because the standard reference set for SingleR did not contain cardiomyocytes and other abundant cardiac cell types, deconvolution was subsequently performed with a reference set containing cell types found in the heart. Classification of these heart cell types was determined by single nuclear RNA-seq on human hearts[1]. Among the cardiac cell types, the ventricular cardiomyocytes I and cytoplasmic cardiomyocytes II were estimated to be the predominant sources of the cf-RNA (**Figure 15B**). In this case, cytoplasmic cardiomyocytes indicate cardiomyocytes that have a higher than normal number of reads in the exonic regions. Cytoplasmic cardiomyocytes II were predicted to be the main source of cf-RNA during HMP and earlier time points of NMP, whereas ventricular cardiomyocytes were most likely the main contributors at later time points of NMP. Furthermore, it was predicted that adipocytes also release cf-RNA throughout NMP. The results demonstrated that the most abundant cardiac cell type and the protective layer of the heart, the cardiomyocytes and adipose tissues, contributed the most to cf-RNA release.

A. Built-in Blueprint/ENCODE reference



B. Heart reference obtained from Tucker, *et al.*

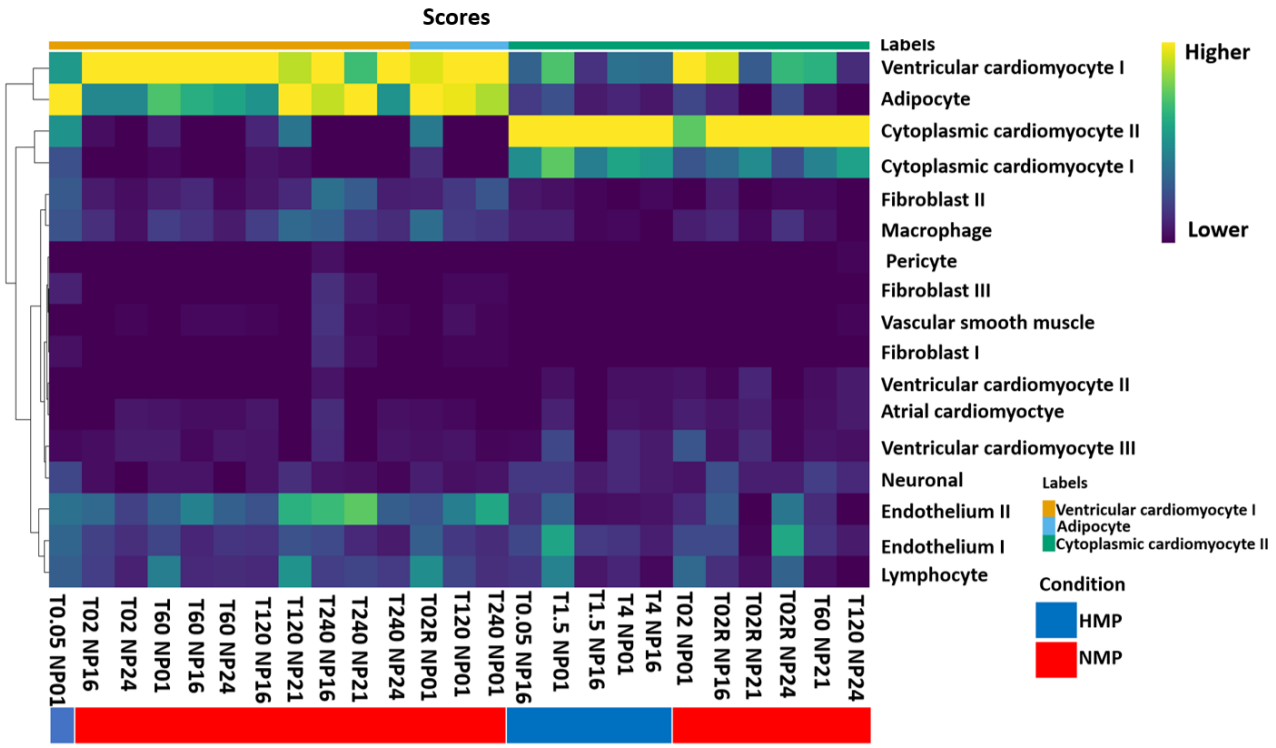


Figure 15: Deciphering the cell-type of origin of cf-RNA using SingleR. SingleR heatmap annotation scores obtained using the A) Blueprint/ENCODE built-in reference and B) cardiac cell type reference obtained from Tucker, et al[1]. The samples were depicted on the y-axis, whereas the x-axis represents the reference cell types. The cell type annotation scores were normalised and therefore the lowest score is 0 (blue) and highest score is 1 (yellow). Below the heatmap, the HMP and NMP perfusate samples were marked with blue and red labels, respectively. The annotated labels are depicted on top of the heatmap using different colours, indicating the adipocytes (orange), hematopoeietic stem cells (blue), neurons (green), and skeletal muscle cells (yellow) in A and ventricular cardiomyocytes I (orange), adipocytes (blue), and cytoplasmic cardiomyocytes II (green) in B.

To examine whether the gene expression profiles overlap more with cardiac cell types, both reference sets were combined. In this manner, the label with the highest score across both references is annotated. For this, the scores across the identified marker subset were recalculated to establish comparable scores across both references originating from an identical gene set.

When the reference sets were combined, ventricular cardiomyocytes were considered to be the main cell type of origin of both HMP and NMP perfusate cf-RNA (**Figure 16**). In addition, cf-RNA extracted from HMP samples was predicted to be derived from adipocytes, endothelial cells, and skeletal muscle cells (**Figure 16**). Almost all NMP samples had a gene expression profile that resembled the ventricular cardiomyocyte expression profile most. Only a few samples across NMP had higher label scores for adipocytes or cytoplasmic cardiomyocytes.

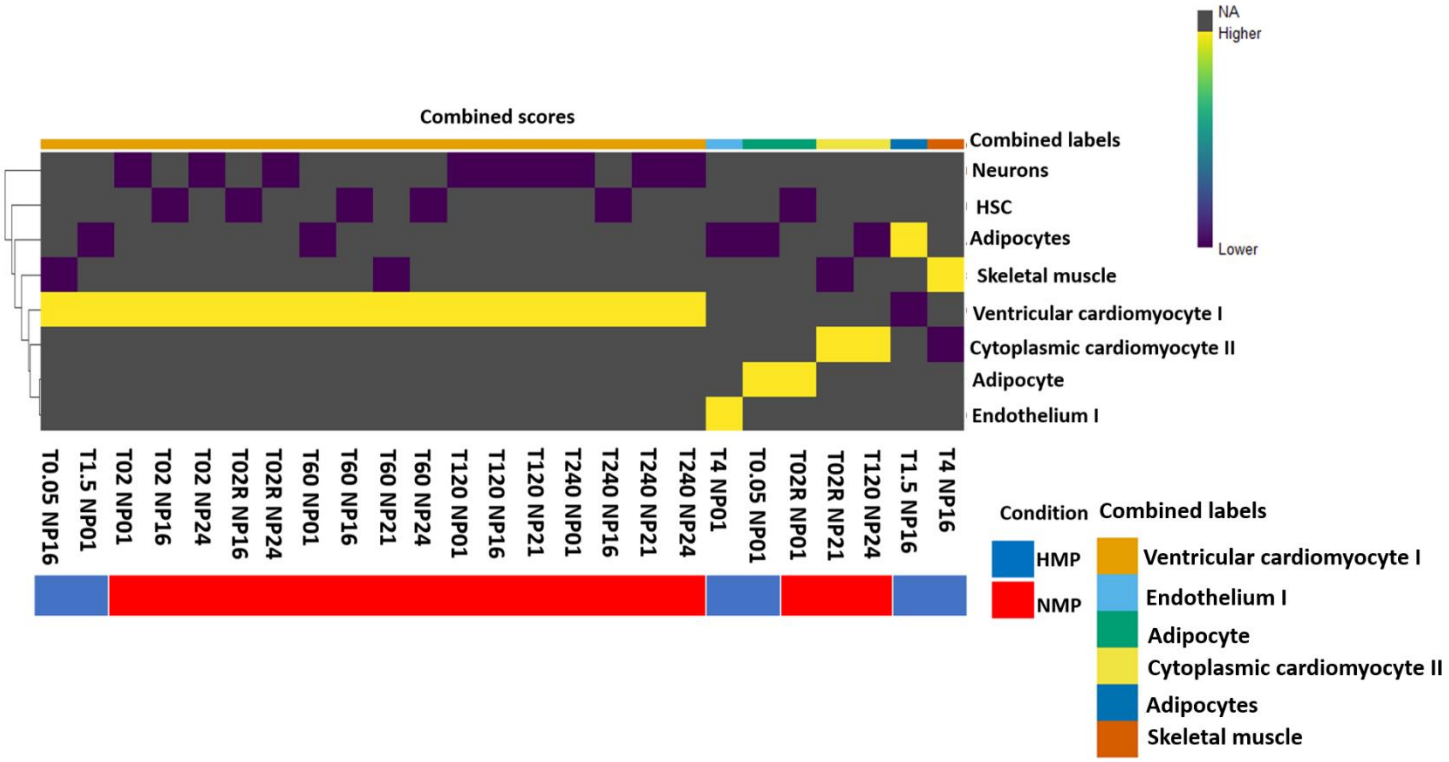


*Figure 16: **Deciphering the cell-type of origin of the cf-RNA using SingleR by combining the Blueprint/Encode and heart cell reference from Tucker, et al[1].** The combined cell type annotation scores were normalised and therefore the lowest score is 0 (blue) and highest score is 1 (yellow). Here, the combined cell type annotation scores were recalculated for only the labels that were predicted in the separate reference sets, and the other labels got NA assigned (grey boxes). The samples were depicted on the y-axis, whereas the x-axis represents the reference cell types. The cell type annotation scores were normalised and therefore the lowest score is 0 (blue) and highest score is 1 (yellow). Below the heatmap the HMP and NMP perfusate samples were marked with blue and red labels, respectively. The annotated labels based on the scores are depicted on top of the heatmap using different colours, indicating the ventricular cardiomyocytes (orange), endothelium I (blue), adipocytes (green), cytoplasmic cardiomyocytes II (yellow), adipocytes (dark blue), and skeletal muscle cells (red).*

Taken together, the data demonstrated that the predominant sources of cf-RNA during HMP were cardiomyocytes and adipocytes, whereas for NMP the predominant sources were the cardiomyocytes. This indicates that the cells that take up a large part of the heart mass or cover a large part of the heart were most likely to release their RNA into the perfusate.

Discussion

Heart transplantation remains the standard treatment for patients with end-stage heart failure. However, due to the lack of available donor hearts, *ex vivo* heart machine perfusion gained profound interest to replace SCS, as this method can prolong preservation times and increase the donor pool. Currently, the assessment of donor hearts during machine perfusion mainly relies on lactate levels, which display the best predictive power for heart graft dysfunction. Nevertheless, the use of lactate as a biomarker for this purpose remains debatable. Therefore, cf-DNA and -RNA were assessed to investigate if they could predict heart function and to gain a better mechanistical understanding of the biological processes that are affected during machine perfusion. In this study, cf-DNA and -RNA levels were examined during HMP, followed by experiments that compared the HMP and SCS groups in which hearts were mounted to an NMP system that mimics the post-transplantation state and that enabled functional assessment.

In the present study, the trends of total, mitochondrial, and nuclear cf-DNA levels in perfusate of *ex vivo* perfused slaughterhouse porcine hearts were investigated. Surprisingly, a decreasing trend in total and mitochondrial cf-DNA levels was observed during HMP. The high initial concentrations may be due to myocardial ischemic injury. The downward trend of total and mitochondrial DNA during *ex vivo* machine perfusion might indicate that hypothermic machine perfusion does not cause any additional damage to the hearts. However, the downward trend might also be a result of a significant amount of the DNA sticking to the tubing set of the perfusion system at low concentrations. In addition, the short cf-DNA fragments might become denatured when they are adsorbed to the tube walls[130]. Therefore, future studies should also investigate the effect of the tubing material on cf-DNA adsorption and denaturation.

In addition, it was examined whether SCS and HMP resulted in different cf-DNA levels and whether these levels could aid in examining cardiac damage and post-transplantation cardiac function. To this end, the cf-DNA levels and trends were assessed when the hearts were mounted for 4 hours onto the normothermic PhysioHeart™ perfusion platform. This platform is a working porcine heart model that simulates the human physiological state post-transplantation, following 4 hours of HMP or SCS. It was observed that the total and mitochondrial cell-free DNA levels increased over time, but this increase was only significant in the SCS hearts. There were no significant differences between those two groups at any time point during NMP. This indicates that a comparable amount of damage arises in hearts of different treatment groups, which was also reflected in the trends of other damage and metabolic markers that did not show any significant differences between the two storage groups (unpublished data from Selma Kaffka genaamd Dengler). This also adds up with the observations that except for survival based on CO, HMP did not result in beneficial functional outcomes in dP/dTmax, dP/dTmin, LAP, MAP, compared to SCS (unpublished data Selma Kaffka genaamd Dengler). Especially in ddPCR, it was observed that cf-mtDNA levels were higher than cf-nDNA levels. Besides the different types of damage that cf-nDNA (general cellular damage and cell death marker) and cf-mtDNA (mitochondrial damage marker) reflect, the differences between cf-nDNA and cf-mtDNA levels might be due to the fact that most cells contain a similar amount of genomic material, whereas the amount of mitochondria is variable between different cell types and even the same cell type[131,132]. It should, however, be noted that cardiomyocytes are often multinucleated or polyploid[133].

Furthermore, it was assessed whether cf-DNA levels correlated with functional cardiac parameter values measured after 4 hours on the PhysioHeart™ platform to examine whether these levels predicted cardiac function. The results demonstrate that across HMP, none of the weight-normalised cf-DNA levels showed a reliable predictive capacity for *ex vivo* cardiac function. During NMP, however, a negative correlation between the total and nuclear cf-DNA levels and LAP, a measure of preload, was found in the working mode. This is in agreement with the observation that LAP was found to be lower in the SCS compared to the HMP group, although this was not statistically significant (unpublished data Selma Kaffka genaamd Dengler). This might indicate that cf-DNA levels during heart reperfusion using NMP might be predictors of heart function, and therefore targeting cf-DNA prior to transplantation might have beneficial effects on preserving cardiac function.

Moreover, it was assessed whether cf-DNA levels correlated with commonly used damage and metabolic markers to examine their potential for predicting cardiac viability. Here, positive correlations

of HMP cf-DNA levels with LD at the beginning of HMP were observed, which can be explained by both markers serving as general heart damage markers in this isolated heart set-up[134]. Early HMP cf-DNA levels, especially the cf-mtDNA levels, were positively correlated with lactate levels when the heart was in working mode during NMP. Elevated levels of lactate and cf-mtDNA both indicate mitochondrial injury and disturbed metabolism. These findings are in agreement with the findings of Wang *et al*. and suggest that cf-mtDNA could be predictive of lactate levels[135]. Because post-transplantation lactate levels also are predictors of cardiac graft outcome, our results demonstrate that cf-mtDNA levels may predict post-transplantation cardiac function[136]. The apparent later onset of lactate release compared to cf-mtDNA release may be due to the accumulation of lactate in the tissue before its release into perfusate[43]. Nevertheless, this requires further study. The positive correlations found between HMP cf-DNA levels and ammonia during HMP seemed to precede the correlations of cf-DNA with lactate. This can be explained by the fact that ammonia contributes to energy metabolism dysfunction and therefore leads to elevated lactate levels[55,137,138].

Surprisingly, some negative correlations of NMP cf-DNA levels with for example troponin-I and LD, which are considered cardiac damage markers, were observed, especially during NMP. This correlation in the opposite direction than expected might also be explained that the difference in these levels between hearts with different qualities was rather small due to the fact that the functional difference was also small (unpublished data Selma Kaffka genaamd Dengler) .

Inflammation is a commonly occuring process during organ perfusion. It is also known that inflammation causes cell damage and cell death and vice versa[139–141]. Therefore, it was expected to find a two-way relationship between cf-DNA and the inflammatory markers. In the present study, the correlation between cf-DNA levels and inflammation was assessed. Several positive and negative correlations were found between cf-DNA levels and inflammatory markers, but there were no clear trends that persisted over time. Here, the release kinetics of cf-DNA and the inflammatory markers may also play a role. Future research should aim to get a deeper understanding of the mechanisms underlying the interplay of cf-DNA and the immune system, as this might offer therapeutic treatment options[139–141]. Treatment options that target DNA receptors that are involved in provoking an immune response could have a dual beneficial effect by reducing both inflammation and cf-DNA release and thus hold a promising treatment option for the future.

Cf-DNA has the potential to be used as a therapeutic target in the *ex vivo* heart perfusion system. Previous research showed that inhibiting cf-mtDNA by administration of DNAse I resulted in a reduced infarction size in an ischemia-reperfusion rat model[142]. Therefore, future research should also focus on applying therapeutics, such as DNAse I, during *ex vivo* heart perfusion to examine what their effects on cardiac function. This may provide opportunities to minimize the donor heart damage and to make more hearts eligible for transplantation.

The conducted study had several limitations, including a small sample size. The small sample size limited the statistical power of the study. Another limitation of the study was the use of a non-oxygenated hypothermic machine perfusion set-up. Previous research had already shown that oxygenation during HMP resulted in improved cardiac function and reduced oedema formation[143]. Although the unoxygenated HMP resulted in improved cardiac function in the HMP hearts, the switch to an oxygenated HMP system might provide bigger differences in cf-DNA levels and the other metabolic markers between the two different storage methods. It should be mentioned that the other biomarkers also did not show clear correlation trends with the functional parameters (unpublished data Selma Kaffka genaamd Dengler). Thus all hearts in this study accumulated damage over time, independent of the preservation method. Because the functional differences between the hearts was not large enough, the discovery of potential biomarkers in the present study was impeded.

Furthermore, the hearts that had a low cardiac output (≤ 3.0 L/min) at the beginning of the study were removed from the NMP platform, and were thus excluded before completion of the 4-hour NMP. This might have facilitated a misrepresentation of the measurements at later time-points, thereby benefitting the SCS results. Another limiting factor in the study design was the use of slaughterhouse pigs. Despite the advantages that slaughterhouse pig hearts offer concerning ethics, availability, costs, and patient population variability resemblance, the health status of slaughterhouse pig hearts is often negatively affected by uncontrolled breeding and nurturing circumstances[121,144]. In addition, the cell-

free nucleic acid levels, as well as the levels of the other damage and metabolic markers, might not only be affected by ischemic-reperfusion injury, but also by the uncontrolled harvesting procedure-related damage[122]. Moreover, these levels may be affected by reduced heart capacity, which is caused by the stress that slaughterhouse pigs experience during transport and slaughtering processes[122,145]. Therefore, it would be of profound interest to assess biomarker levels and their variability in experiments involving experimental pig hearts. Moreover, comparing such biomarker levels between experimental and slaughterhouse pig hearts aids the elucidation of the effects of the storage methods on these levels.

The hearts were reperfused using a NMP system (Langendorff first, followed by working mode) instead of an *in vivo* heart transplantation. In this manner, the perfusion solution is reused and therefore not cleared from several products (including inflammatory markers and cf-DNA) that could affect the affect heart function. The blood that was used to perfuse the hearts during NMP was thus suboptimal[43]. One of the possible solutions to create better circumstances is to add a leukocyte filter into the set-up, as these are also used in clinical settings [21,146]. In addition, hemofiltration might have beneficial effects on the NMP set-up. Hemofiltration gets rid of toxic metabolic products, as well as damage and inflammatory markers[43,147]. Moreover, the elimination of the excess of water might also make the reperfusion system more resemblant of the physiological state.

Another limitation of this study is that the hearts were only preserved for 4 hours with HMP or SCS. However, this is also the time that hearts are considered safe when preserved using static cold storage. As the long-term goal of machine perfusion is the prolongation of heart preservation times for heart transplantation, future research should aim to perform 24-hour experiments with a HMP set-up and compare these to 4 and 24 hours of SCS. This will give a better understanding of the marker levels when the functional differences between the groups are bigger. Nevertheless, to preserve the hearts during such a prolonged period of time, the HMP set-up requires improvements, such as the incorporation of continuous oxygenation into the system.

In addition to the cf-DNA levels, the corresponding fragment sizes should also be studied. The difference in fragment size between static cold storage and hypothermic perfused hearts was not explored in this study. However, previous research showed that cf-DNA size might give an indication about health status[148,149]. Its use in distinguishing the good from the bad functioning hearts and predicting cardiac viability might also have potential and thus should be explored. Other exploration options in the future include the assessment of epigenetic features of the cf-DNA (e.g. DNA methylation or cell-free histones and its modifications) as these might be more specific in distinguishing hearts viable enough for transplantation from those that are not[150]. This might be due to the fact that epigenetic features might give more insight in the pathological state of the cells.

Besides the use of cf-DNA as predictors for cardiac function during machine perfusion, there was also looked at the potential use of cf-RNA levels. This was done to investigate whether cf-RNA was a predictor of cardiac function and to see which cell types and biological processes are affected during machine perfusion. To the best of this author's knowledge, this was the first study that examined transcriptomic cf-mRNA profiling in an organ machine perfusion system. First, this study showed that CEL-seq2, a single-cell sequencing method, was able to identify and to quantify genes in the perfusate with a mean coverage of roughly 10,000 (>5 reads per gene)[125]. Based on the gene expression profiles, it was examined which cell types contributed most to the release of cf-RNA.

First, the cell type deconvolution was performed using a built-in reference and this showed that the major sources of cf-DNA were adipocytes (HMP + NMP), hematopoietic stem cells (NMP), neurons (NMP), and skeletal muscle cells (HMP + NMP). The enrichment of adipocytes during HMP and at the beginning of HMP might be explained by the fact that around 80 percent of the heart surface is surrounded with adipose tissue and that it has several roles concerning metabolism, thermoregulation, mechanical protection, and immunity[151]. It might especially be due to the protective effects on the heart that adipocytes elicit in response to hypothermia. Skeletal muscle enrichment could be caused by the fact that these cells have similarities with cardiomyocytes, which are not included in the reference set[152]. In addition, some of the NMP samples were enriched in hematopoietic stem cells, which can be explained, as the hearts were perfused with diluted blood perfusion fluid. The neuronal

enrichment may be caused by the disturbance of the intrinsic cardiac autonomic network, which plays a role in regulating cardiac function, predominantly by the harvesting procedure[153]. Technical issues of the analysis could also have resulted in wrong annotations, as the reference set for example does not contain all the important cell types present in the heart. In addition, some gene signatures can be assigned to different cell types, which causes enrichment of multiple cell types that are not necessarily all present. For this reason, the cell type deconvolution was also performed with a heart cell type reference set. Here, enrichment of cardiomyocytes and adipocytes was observed. In addition, an enrichment of endothelium, one of most prevalent cell types in the heart, was observed. However, this signal was seemingly overshadowed by the signals related to cardiomyocytes, which are even more abundant in the heart. When the references are combined by recomputing the highest score based on the labels that were enriched in the individual data sets and based on an identical gene sets. Therefore, become some genes are removed from analysis, labels might have switched when the references were combined. Altogether, the main finding was that main source of cf-RNA appeared to be cardiac cell types, and not immune and blood cells.

A disadvantage of cell type deconvolution using SingleR was that there were no built-in pig reference sets. Therefore, the cf-RNA seq data results had to be converted to the human ortholog genes. This probably resulted in loss of data, because not all the human orthologs are known.

Our study demonstrated that dozens of genes were differentially expressed during HMP, but also during the reperfusion of the hearts using NMP. Moreover, our data indicated that the affected genes during HMP were involved in biological processes and pathways that relate to functional recovery after heart transplantation [154]. More specifically, the results showed that pathways involved in maintaining the mitochondrial structure and energy metabolism were downregulated, whereas processes that regulate gene transcription were activated. During reperfusion of the heart with an NMP set-up, suppression of pathways related to energy metabolism was also observed and immune response associated pathways were activated. Despite the major negative impact that metabolic and mitochondrial dysfunction have on heart transplantation, the focus of previous studies did not lie on metabolic and mitochondrial condition of the donor hearts. Nevertheless, the administration of therapies that target this metabolic and mitochondrial dysfunction during machine perfusion and/or post-transplantation, in combination with an improved, oxygenated, HMP set-up, might provide beneficial effects on ameliorating cardiac function and viability. Our research emphasized the potential use of non-invasive perfusate cf-mRNA profiling for identifying markers that enable assessment cardiac function and identifying new potential therapeutic targets. Based on our research, future research should thus focus on applying these potential therapies during machine perfusion to assess how it affects cardiac function and viability.

Even though *ex vivo* heart machine perfusion might prolong the preservation times of donor hearts, the transplantation process remains time-restricted. Therefore, biomarkers for *ex vivo* heart function in the perfusate rely on rapid detection. Because RNA-seq is a time-consuming approach, it is only useful to detect candidate biomarkers. it is paramount to move the identified markers that are able to predict *ex vivo* cardiac function into the clinic, where they could help the transplant teams to decide whether the heart is transplantable or not. To this end, the RNA levels could be quantified by developing standardized RT-qPCR- or antibody-based detection methods. For example, ultra-rapid RT-qPCR that generates results within 15-30 minutes already exists and may be harnessed for this purpose[155,156].

As this was started as an explorative study to check whether cf-mRNA sequencing in the RNA would work, the sample size was a limiting factor of the study. Because only 2 replicates of both HMP and SCS were included in the cf-RNA seq study, no differentially expressed genes could be identified between the two different preservation methods over time. Therefore, the RNA-seq data set should be extended with the rest of the available samples from this experiment.

In this study, a CEL-seq2 protocol was performed, which is an mRNA-only sequencing method[125]. However, miRNAs might also be important players in the ischemic heart and could possibly even serve as a therapeutic target. For example, Ren *et al.* identified miR-320 as a key player in the development of cardiac ischemia-reperfusion injury[157]. In addition, the value of exploring the expression profile of miRNAs in a machine perfusion system was already proven by a study involving

an *ex vivo* lung perfusion system[158]. Therefore, it might be useful to perform Phospho-RNA-seq, PALM-seq (PolyAdenylation Ligation Mediated-Seq), or cf-RNA-seq in the future to cover a wider range of RNAs[159,160].

Future research should include the comparison between gene expression profiles of the cf-RNA and those of heart tissue biopsies. It would be highly advantageous if cf-RNA could replace the biopsy, because these biopsies often cause myocardial injury or are obtained from unrepresentative sites of the heart, such as the left atrial appendage[161]. Moreover, correlations between the cf-RNA transcriptome profiles and functional cardiac parameters should be assessed to get a better indication of cf-RNA profiles could predict cardiac function during machine perfusion.

cf-RNA is believed to be predominantly derived from living cells and therefore is thought to reflect the gene expression profile of the cells that secrete them. However, it should be noted that in our set-up, some of the cf-RNA might also as be released as cargo into vesicles for signalling purposes[162]. In this case, the cf-RNA does not reflect the gene expression of the cells that release them.

Besides the potential of targeting affected pathways during machine perfusion, cf-RNA, in general, might be used as a therapeutic target. Previous research namely indicated that RNAse I administration resulted in a reduced infarct size, a better cardiac function, and overall reduced cell death in an ischemia-reperfusion rat model by targeting the crosslink between cf-RNA and TNF-α[163].

Moreover, the important question remains whether cf-DNA would make a good biomarker. For one, cell damage and death, both marked by cf-DNA release, may not be the main problem in functional decline of the heart during transplantation. Moreover, cell damage and death are not considered to be accurate predictors of post-transplantation cardiac viability[53]. The levels of damage markers, such as cf-DNA, are subject to variability due to their potential release upon extended cold storage and during the harvesting procedure and perfusion set-up. Keeping this in mind, cardiac edema, for example, might have more potential as a measurement, as this was also found to be associated with PGD[23]. Moreover, the use of imaging techniques during HMP should be considered as predictors for post-transplantation cardiac viability, especially because HMP would enable imaging of the heart after its exposure to potential harmful events during storage[53]. It is expected that assembling a panel of several biomarkers would allow for the most accurate examination of hearts on machine perfusion prior to transplantation

In conclusion, this study demonstrated that HMP perfusate cf-mtDNA levels positively correlated with lactate levels during reperfusion of the heart using NMP. This indicates that cf-mtDNA levels in the perfusate might be able to predict post-transplantation cardiac function. In addition, the cf-DNA levels during NMP showed negative correlation trends with cardiac function. This illustrated that cf-DNA during reperfusion could predict cardiac function, but also suggests that it might be a potential therapeutic target that could be used to preserve improved cardiac function post-transplantation. Cf-RNA levels showed that biological processes involved in energy production and metabolism were affected during HMP and NMP. Furthermore, gene regulation processes and immune responses were activated during HMP and NMP, respectively. Altogether, the data demonstrated that targeting mitochondrial and metabolic dysfunction or preventing immune responses during machine perfusion may potentially facilitate better donor heart preservation in the future. Further studies are required for the validation of cf-DNA and cf-RNA as biomarkers for predicting post-transplantation cardiac function during machine perfusion. Moreover, combining cf-DNA and cf-RNA with other biomarkers may prove to be pivotal in obtaining the optimal donor heart assessment.

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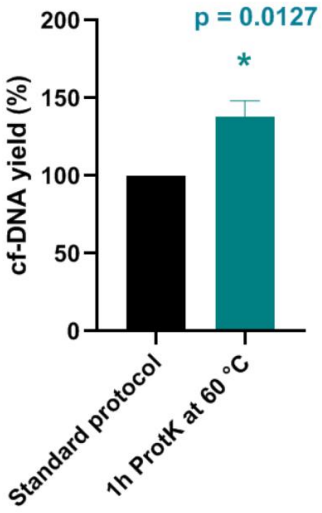
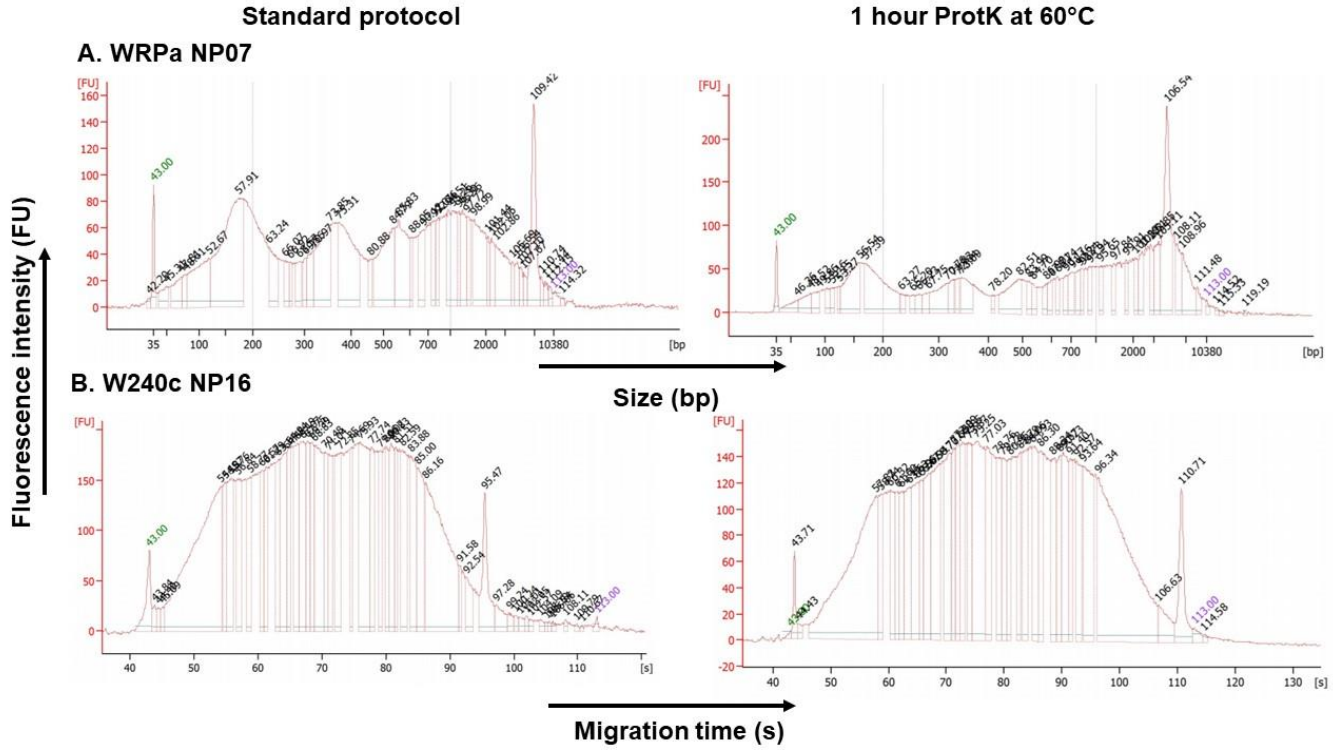
Supplementary materials

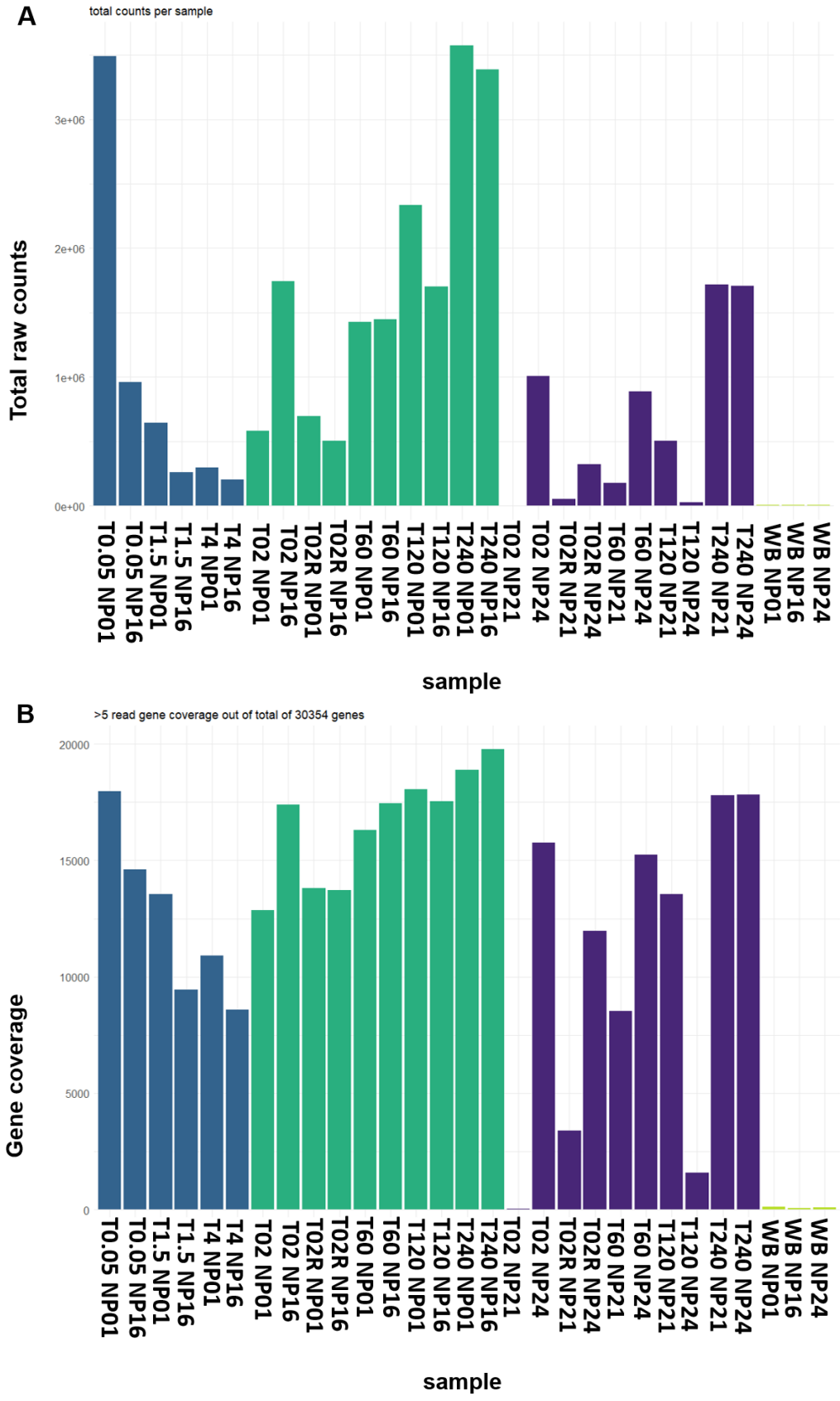


Supplementary Figure 1: ***The effect of different ProtK incubation protocols on cf-DNA yield.*** *The cf-DNA yield of the 1-hour ProtK incubation at 60°C protocol was expressed as a percentage of the standard protocol. The standard protocol refers to the QIAamp MinElute ccfDNA protocol in which the samples are incubated with ProtK for 10 minutes at room temperature. The results are illustrated as bars that represent the mean of cf-DNA yield from 6 replicate extractions using the standard or 1-hour ProtK protocol with error bars that indicate the standard error of the mean (SEM). The asterisk (*) represents a significant difference (paired t-test p-value <0.05) between the standard protocol and the 1-hour ProtK incubation.*

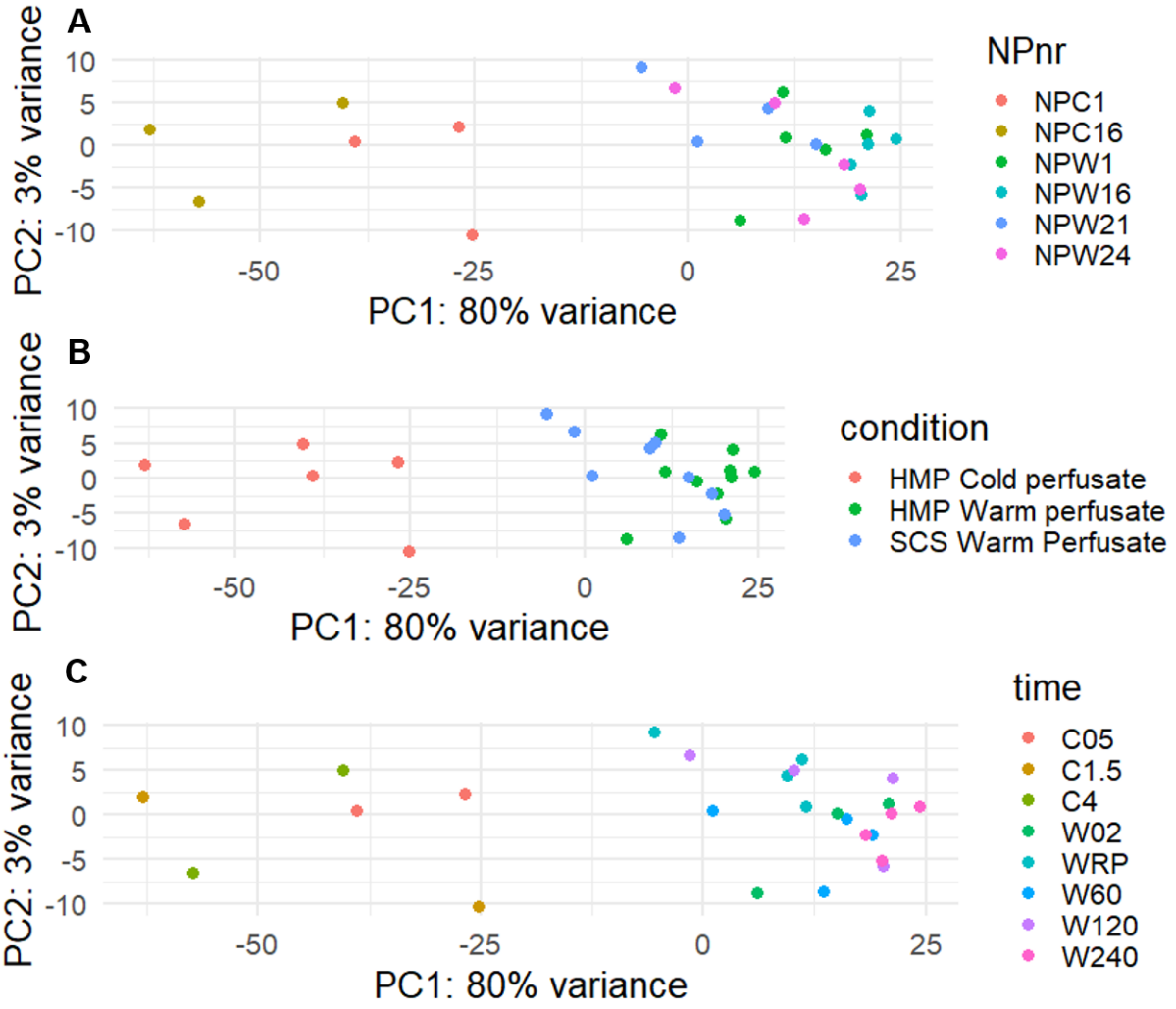


Supplementary Figure 2: ***Electropherogram fragment size distribution profiles of perfusate cfDNA after standard protocol ProtK or 1-hour ProtK incubation at 60 °C .*** *The cf-DNA fragment distribution profiles of A) T02Rb NP07, and B) T240 NP16 incubated with Proteinase K for 10 minutes at room temperature (standard protocol) or 1 hour at 60 °C, were examined using the Agilent BioAnalyzer 2100. The standard protocol was the QIAamp MinElute ccfDNA protocol. The different ProtK incubation protocols did not show clear differences in fragment size profiles. The X-axis depicts fragment size (bp) or the migration time (s) of the DNA, whereas the Y-axis displays the fluorescence intensity (FU). The peaks at 35 and 10380 base pairs are derived from*

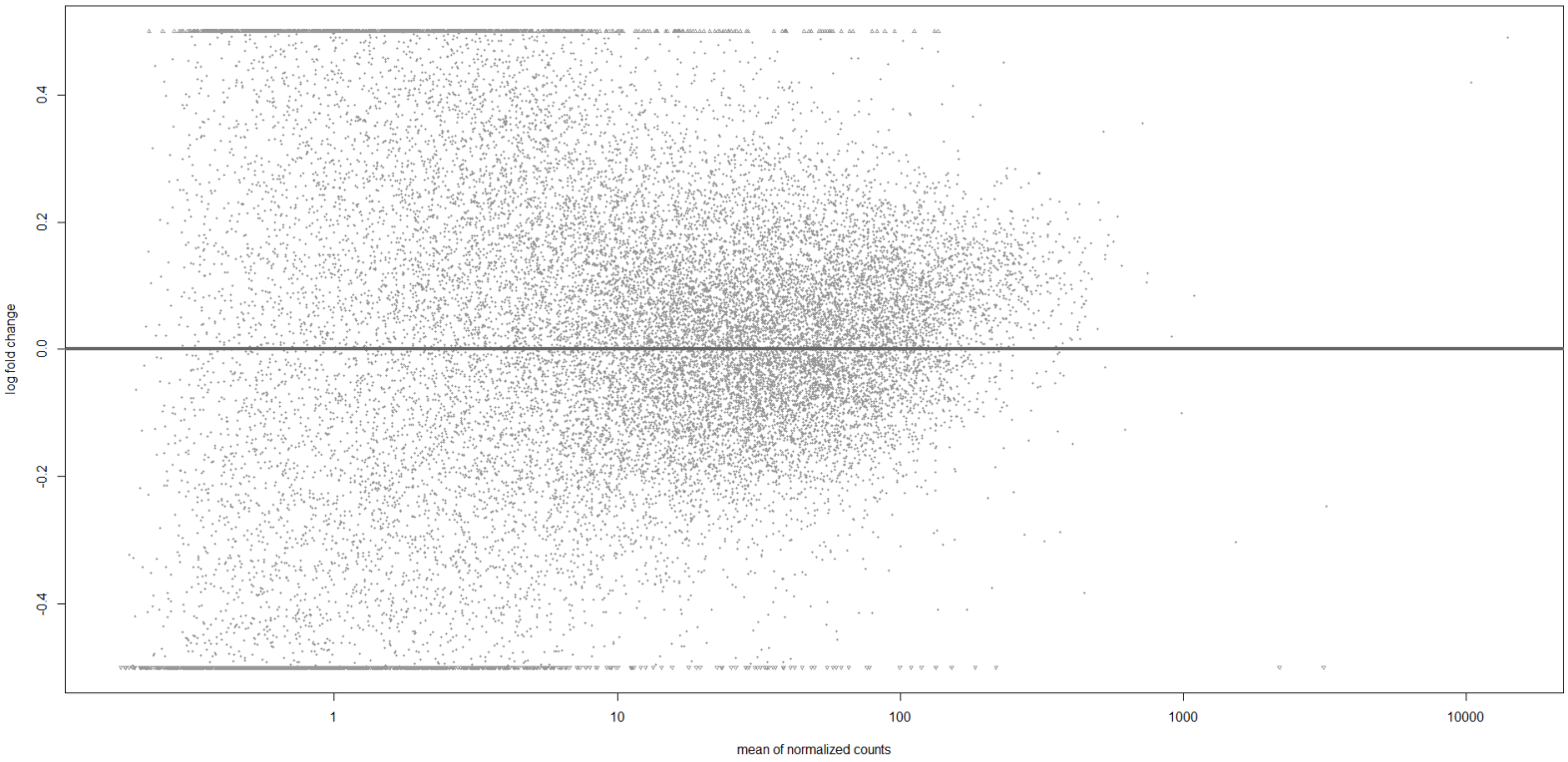
the two internal High Sensitivity DNA markers. The non-marker peaks reflect mono-, di-, or tri-nucleosomal fragment sizes, and also longer fragments (1-10 kb).



Supplementary Figure 3: ***Quality control of mRNA sequencing data by assessing total raw counts and gene coverage across all samples.*** *Bar plot visualisation of the total raw counts (A) and gene coverage (B) per sample. The gene coverage included genes that had at least 5 reads.*



Supplementary Figure 4: ***Principal component analysis (PCA) on regularized log-transformed (rlog) read counts from the 500 most variable genes for each sample.*** *The PCA plot was based on mRNA-sequencing data obtained using CEL-seq2. The PCA analysis was performed using the DESeq2 package in Rstudio. The first and second principle components explain 80 and 3% of the variance in the transcriptome data. The samples were separated by NP number (A), condition (B), and time (C).*



Supplementary Figure 5: ***MA plot generated using DESeq2 for the exploration of condition-specific changes over time during normothermic machine perfusion.*** *In the MA plot, the mean of normalised counts (x-axis) was plotted against the log2 fold changes (y-axis). An adjusted p-value cutoff of 0.1 was used for the detection of differentially expressed genes.*

Supplementary Code

Supplementary Code 1

```{r}
#read in the data of the cf-DNA levels, other metabolic markers, and functional cardiac parameters - not normalized - normalized by T02 - normalized by weight
setwd("~/MRP/RNA seq")
library(readxl)
correlationdata = read_excel('~/MRP/AllData_Correlation.xlsx', sheet = 1, col_names = TRUE)
correlationdataWN = read_excel('~/MRP/AllData_Correlation.xlsx', sheet = 2, col_names = TRUE)
library(tidyverse)
correlationdata <-  correlationdata %>% remove_rownames %>% column_to_rownames(var="Heart number")
correlationdataWN <- correlationdataWN[-c(19:23), ]
correlationdataWN <-  correlationdataWN %>% remove_rownames %>% column_to_rownames(var="Heart number")

#correlation of HMP cf-DNA levels with functional cardiac parameters -  weight normalised
correlationdataWNC0.05 <- correlationdataWN[-c(1:11),-c(1:6, 16:106, 111, 114, 117:175)]
correlationdataWNC0.05 <- correlationdataWNC0.05[c(1, 2, 4, 5, 3 , 6, 8, 7, 9, 10:17)]
correlationdataWNC1.5 <- correlationdataWN[-c(1:11),-c(1:19, 29:106, 111, 114, 117:175)]
correlationdataWNC1.5 <- correlationdataWNC1.5[c(1, 2, 4, 5, 3 , 6, 8, 7, 9, 10:17)]
correlationdataWNC4 <- correlationdataWN[-c(1:11),-c(1:32, 42:106, 111, 114, 117:175)]
correlationdataWNC4 <- correlationdataWNC4[c(1, 2, 4, 5, 3 , 6, 8, 7, 9, 10:17)]
# correlation of NMP cf-DNA levels with functional cardiac parameters normalised by T02, and weight normalised


correlationdataWNTRPT02N <- correlationdataWN[,-c(1:106, 111, 114, 117:123, 133:175)]
correlationdataWNTRPT02N <- correlationdataWNTRPT02N[c(9, 10, 12, 13, 11, 14, 16, 15, 17, 1:8)]
correlationdataWNT60T02N  <- correlationdataWN[,-c(1:106, 111, 114, 117:136, 146:175)]
correlationdataWNT60T02N <- correlationdataWNT60T02N[c(9, 10, 12, 13, 11, 14, 16, 15, 17, 1:8)]
correlationdataWNT120T02N  <- correlationdataWN[,-c(1:106, 111, 114, 117:145, 155:175)]
correlationdataWNT120T02N <- correlationdataWNT120T02N[c(9, 10, 12, 13, 11, 14, 16, 15, 17, 1:8)]
correlationdataWNT240T02N  <- correlationdataWN[,-c(1:106, 111, 114, 117:158, 168:175)]
correlationdataWNT240T02N <- correlationdataWNT240T02N[c(9, 10, 12, 13, 11, 14, 16, 15, 17, 1:8)]

#correlation of cf-DNA levels with other metabolic and damage markers - weight normalised and for NMP levels also T02 normalised
CorrelationwithMMWNC0.05 <- correlationdataWN[-c(1:11),-c(1:6,  20:28, 33:41, 46:132, 137:154, 159:167, 172:175)]
CorrelationwithMMWNC0.05 <- CorrelationwithMMWNC0.05[c(1, 2, 4, 5, 3 , 6, 8, 7, 9, 10:33)]
CorrelationwithMMWNC1.5 <- correlationdataWN[-c(1:11),-c(1:15, 33:41,  46:132, 137:154, 159:167,172:175)]
CorrelationwithMMWNC1.5 <- CorrelationwithMMWNC1.5[ c(5,6, 8, 9, 7, 10, 12, 11, 13, 1:4, 14:33)]
CorrelationwithMMWNC4<- correlationdataWN[-c(1:11),-c(1:15, 20:28, 46:132, 137:154, 159:167, 172:175)]
CorrelationwithMMWNC4<- CorrelationwithMMWNC4[ c(9,10, 12, 13, 11, 14, 16, 15, 17, 1:8, 18:33)]
CorrelationwithMMW02C <- correlationdataWN[,-c(1:15, 20:28, 33:41, 46:123, 137:154, 159:167, 172:175)]
CorrelationwithMMW02C <- CorrelationwithMMW02C[c(13, 14, 16, 17, 15, 18, 20, 19, 21, 1:12, 22:33)]
CorrelationwithMMW60C <- correlationdataWN[,-c(1:15, 20:28, 33:41, 46:132, 146:154, 159:167, 172:175)]
CorrelationwithMMW60C <- CorrelationwithMMW60C[c(17, 18, 20, 21, 19, 22, 24, 23, 25, 1:16, 26:33)]
CorrelationwithMMW120C <- correlationdataWN[,-c(1:15, 20:28, 33:41, 46:132, 137:145, 159:167, 172:175)]
CorrelationwithMMW120C <- CorrelationwithMMW120C[c(17, 18, 20, 21, 19, 22, 24, 23, 25, 1:16, 26:33)]
CorrelationwithMMW240C <- correlationdataWN[,-c(1:15, 20:28, 33:41, 46:132, 137:154, 172:175)]
```

```
CorrelationwithMMW240C  <- CorrelationwithMMW240C[c(21, 22, 24, 25, 23, 26, 28, 27, 29, 1:20, 30:33)]

#correlation of cf-DNA levels with inflammatory markers - weight normalised and for NMP levels also T02 normalised
CorrelationwithWNC0.05I <- correlationdataWN[-c(1:11),-c(1:6, 16:121, 124: 171)]
CorrelationwithWNC0.05I  <- CorrelationwithWNC0.05I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]
CorrelationwithWNC1.5I <- correlationdataWN[-c(1:11),-c(1:19, 29:121, 124:171)]
CorrelationwithWNC1.5I <- CorrelationwithWNC1.5I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]


ggpairs(CorrelationwithWNC4I   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold", vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

CorrelationwithWNC4I<- correlationdataWN[-c(1:11),-c(1:32, 42:121, 124:171)]
CorrelationwithWNC4I <- CorrelationwithWNC4I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]
CorrelationwithWNC4I  <- CorrelationwithWNC4I [, -c(5,14)]
CorrelationwithWNC4I<- correlationdataWN[-c(1:11),-c(1:32, 42:121, 124:171)]
CorrelationwithWNC4I <- CorrelationwithWNC4I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]
CorrelationwithWNC4I  <- CorrelationwithWNC4I [ -c(1:4,6:9, 14)]
CorrelationwithWNC4I<- correlationdataWN[-c(1:11),-c(1:32, 42:121, 124:171)]
CorrelationwithWNC4I <- CorrelationwithWNC4I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]
CorrelationwithWNC4I <- CorrelationwithWNC4I [-c(2:4), -c(5)]
CorrelationwithWNC4I<- correlationdataWN[-c(1:11),-c(1:32, 42:121, 124:171)]
CorrelationwithWNC4I <- CorrelationwithWNC4I[c(1, 2, 4, 5, 3, 6, 8, 7, 9, 12:15, 10, 11)]
CorrelationwithWNC4I <- CorrelationwithWNC4I [, -c(1:4, 6:13, 15)]

CorrelationwithW02CI <- correlationdataWN[,-c(1:121, 133:171)]
CorrelationwithW02CI <- CorrelationwithW02CI [c(3, 4, 6, 7, 5, 8, 10, 9, 11, 12:15, 1,2  )]

CorrelationwithW60CI <- correlationdataWN[,-c(1:121, 124:136 , 146:171)]
CorrelationwithW60CI <- CorrelationwithW60CI[c(3, 4, 6, 7, 5, 8, 10, 9, 11, 12:15, 1,2  )]
CorrelationwithW120CI <- correlationdataWN[,-c(1:121, 124:145, 155:171)]
CorrelationwithW120CI <- CorrelationwithW120CI[c(3, 4, 6, 7, 5, 8, 10, 9, 11, 12:15, 1,2  )]
CorrelationwithW240CI <- correlationdataWN[,-c(1:121, 124:158, 168:171)]
CorrelationwithW240CI <- CorrelationwithW240CI[c(3, 4, 6, 7, 5, 8, 10, 9, 11, 12:15, 1,2  )]
```

```{r}
# Made a function to perform Spearman correlations
my_fn <- function(data, mapping, use="pairwise", ...){

        # grab data
        x <- eval_data_col(data, mapping$x)
        y <- eval_data_col(data, mapping$y)

        # calculate correlation
        corr <- cor(x, y, method="spearman", use=use)

        # calculate colour based on correlation value
        # Here I have set a correlation of minus one to blue,
        # zero to white, and one to red
        # Change this to suit: possibly extend to add as an argument of `my_fn`
        colFn <- colorRampPalette(c("#3B9AB2", "white", "#F21A00"), interpolate ='spline')
```

```
            fill <- colFn(100)[findInterval(corr, seq(-1, 1, length=100))]

            ggally_cor(data = data, mapping = mapping, ...) +
              theme_void() +
              theme(panel.background = element_rect(fill=fill))
}
```


```{r}
library(ggplot2)
library(GGally)
ggpairs(correlationdataWNC0.05 , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNC1.5 , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNC4  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNTRPT02N  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNT60T02N , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNT120T02N  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(correlationdataWNT240T02N  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
```

```
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")
```

```{r}
ggpairs(CorrelationwithMMWNC0.05  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMWNC1.5  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMWNC4  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMW02C  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMW60C  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMW120C   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithMMW240C   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=4,color="black",fontface="bold",
vjust=0.1,digits=2 )
    )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")
```

```{r}
ggpairs(CorrelationwithWNC0.05I   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithWNC1.5I   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")


ggpairs(CorrelationwithWNC4I   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithW02CI  , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
    #this does spearman https://github.com/ggobi/ggally/issues/60 #,fontface="bold"
  )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithW60CI   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithW120CI   , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")

ggpairs(CorrelationwithW240CI    , labeller = label_wrap_gen(0, multi_line = TRUE),
    upper = list(
    continuous = wrap(my_fn, method = "spearman",size=6,color="black",fontface="bold",
vjust=0.1,digits=2 )
     )) +  theme(axis.text.x = element_text(angle = 90, hjust = 1))  +
  theme(strip.background =element_blank(), strip.placement = "outside")
```

Supplementary Code 2

Total number of read counts per sample and gene coverage

First the raw count data txt files were imported. I checked the # of total raw counts per sample and if each sample has an approx. equal # of covered reads. In most RNA-seq data sets at least 12-14k genes are covered. If a sample has less than this, it might be better to remove it from the analysis. I continued the analysis with the samples that had a total raw read count above 25,000 and gene coverage of 1500 genes that had at least 5 reads.

```{r setup DESeq2 data set, include=F}
Genetics = read.delim('~/MRP/RNA seq/cfRNA_combined_raw_counts.txt', row.names = 1)
colnames(Genetics) <- sub(".sam.counts", "", colnames(Genetics)) # remove .sam.counts from column names
colnames(Genetics) <- sub("__", "_", colnames(Genetics)) # remove other stuff
colnames(Genetics) <- sub("_$", "", colnames(Genetics)) # remove last _
colnames(Genetics) <- sub("NP15T4_warm", "NP15_T4_warm", colnames(Genetics)) # adjust
colnames(Genetics) <- sub("NP16T4_warm", "NP16_T4_warm", colnames(Genetics)) # adjust
colnames(Genetics) <- sub("T01", "T0", colnames(Genetics)) # adjust
GeneticsT = Genetics[,c(1:101)] # tissue
GeneticsCf = Genetics[,c(102:130)] # cfRNA
GeneticsTs =
Genetics[,c(c(1,3,4,5,2,6,8,9,7,15,17,16,31,33,34,35,32,46,48,49,50,47,56,58,59,60,57,61,63,64,65,62),c(10,12,13,14,11,18,20,21,19,22,24,25,23,26,28,29,30,27,36,38,39,40,37,41,43,44,45,42,51,53,54,55,52,66,67,68,69,70,71,72,73,74,77,76,78,75,79,82,81,83,80,84,87,86,88,85,89,92,91,93,90,94,97,96,98,95,99,101,100))] # sorted to get 7x HMP first, then 14x SCS
GeneticscfRNA = GeneticsCf[,c(24:29,1,2,20,21,13,14,5,6,9,10,3, 4, 22,23,15,16,7,8,11,12, 17:19)]
GeneticscfRNAfilter = GeneticsCf[,c(24:29,1,2,20,21,13,14,5,6,9,10,4,22,23,15,16,7,8,11,12)] # sorted to get 2x cold perfusion, then 2x HMP, then 2x SCS
# 3 is the W02 sample that is deleted and 17:19 are the whole blood samples that are deleted
for (i in c(1:25)){
  print(length(which(GeneticscfRNA[,i]==0)))
} # this outputs the # of genes that have 0 reads for each sample
nrow(GeneticscfRNA)
```

```{r QC plots}
# number of total counts per sample
sum <- data.frame(sample = colnames(GeneticscfRNA),sum = colSums(GeneticscfRNA))
sum$sample <- factor(sum$sample, levels = row.names(sum))
ggplot(sum, aes(x=sample,y=sum)) +
  geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 10), rep('#B8DE29FF',3))) +
  theme_minimal() + ggtitle("total counts per sample") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size = 12))

# quality check to see if each sample has an equal coverage of reads
cov <- c(1:29)
for (i in c(1:29)){
  cov[i] <- length(which(GeneticscfRNA[,i]>4))
  }
covData = data.frame(sample = colnames(GeneticscfRNA),cov)
covData$sample <- factor(covData$sample, levels = row.names(sum))
ggplot(covData, aes(x=sample,y=cov)) +
  geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 10), rep('#B8DE29FF',3))) +
  theme_minimal() + ggtitle(">5 read gene coverage out of total of 30354 genes") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size = 12))
```

```

```{r QC plots}
# number of total counts per sample
sum <- data.frame(sample = colnames(GeneticscfRNAfilter),sum = colSums(GeneticscfRNAfilter))
sum$sample <- factor(sum$sample, levels = row.names(sum))

ggplot(sum, aes(x=sample,y=sum)) +
   geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 9),
rep('#B8DE29FF',0))) +
 theme_minimal() + ggtitle("total counts per sample") +
 theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12))

# quality check to see if each sample has an equal coverage of reads
cov <- c(1:25)
for (i in c(1:25)){
 cov[i] <- length(which(GeneticscfRNAfilter[,i]>4))
 }
covData = data.frame(sample = colnames(GeneticscfRNAfilter),cov)
covData$sample <- factor(covData$sample, levels = row.names(sum))
ggplot(covData, aes(x=sample,y=cov)) +
 geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 9),
rep('#B8DE29FF',0))) +
 theme_minimal() + ggtitle("Gene coverage out of total of 30354 genes") +
 theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12))
```

PCA plots

I used rlog transformation as it inherently accounts for differences in sequencing depth, which is the case for these samples. Variance stabilising transformation is not good to use when there is a lot of variation in samples due to the experimental design. I could try vst if the samples with low sequencing depth are excluded.

I also removed genes that had less than a sum of 10 counts.

DESeq2 plots the PC1 and PC2 based on the top 500 most variable genes. I used the PCAtools package to get the other PCAs, which utilizes all genes.

```{r, include=F}
T = colnames(GeneticscfRNA)
T = sub("_*[^_]*$","",T)
T = gsub("a", "", T)
ColData = data.frame(row.names=colnames(GeneticscfRNA), condition = c(rep('HMP Cold
perfusate',6),rep('HMP Warm perfusate',10), rep('SCS Warm Perfusate', 10), rep('Whole Blood', 3)),
NPnr =
c(rep('NPC1',3),rep('NPC16',3),rep('NPW1',5),rep('NPW16',5),rep('NPW21',5),rep('NPW24',5),rep('WB'
,3)),time=T)

ColData
dds <- DESeqDataSetFromMatrix(countData = GeneticscfRNA,
                              colData = ColData,
                              design = ~ condition)
dds
dds$NPnr <- factor(dds$NPnr, levels = c("NPC1", "NPC16", "NPW1", "NPW16", "NPW21", "NPW24",
"WB")) # to order the levels, otherwise it will be on alphabetical order
```
```

```
dds$time <- factor(dds$time, levels = c("C05","C1.5","C4","W02","WRP",  "W60", "W120", "W240", "WB"))

dds <- dds[ rowSums(counts(dds)) > 10, ] # removed genes with a sum of 0-10 counts
#vsd <- vst(dds) # not good to use
rld <- rlog(dds) #rlog transformation inherently accounts for differences in sequencing depth
save.image("QC.RData")
```

```{r, include=F}
T1 = colnames(GeneticscfRNAfilter)
T1 = sub("_*[^_]*$","",T1)
T1 = gsub("a", "", T1)
ColData1 = data.frame(row.names=colnames(GeneticscfRNAfilter), condition = c(rep('HMP Cold perfusate',6),rep('HMP Warm perfusate',10), rep('SCS Warm Perfusate', 9), rep('Whole Blood', 0)), NPnr =
c(rep('NPC1',3),rep('NPC16',3),rep('NPW1',5),rep('NPW16',5),rep('NPW21',4),rep('NPW24',5),rep('WB',0)),time=T1)

ColData1
dds1 <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter,
                               colData = ColData1,
                               design = ~ condition)
dds1
dds1$NPnr <- factor(dds1$NPnr, levels = c("NPC1", "NPC16", "NPW1", "NPW16", "NPW21", "NPW24", "WB")) # to order the levels, otherwise it will be on alphabetical order
dds1$time <- factor(dds1$time, levels = c("C05","C1.5","C4","W02", "WRP", "W60", "W120",  "W240", "WB"))

dds1 <- dds1[ rowSums(counts(dds1)) > 10, ] # removed genes with a sum of 0-10 counts
#vsd <- vst(dds) # not good to use
rld1 <- rlog(dds1) #rlog transformation inherently accounts for differences in sequencing depth
save.image("QC.RData1")
```

Supplementary Code 3

Total number of read counts per sample and gene coverage

First the raw count data txt files were imported. I checked the # of total raw counts per sample and if each sample has an approx. equal # of covered reads. In most RNA-seq data sets at least 10.000 genes are covered. If a sample has less than this, it might be better to remove it from the analysis. I performed the quality control / PCA analyses for all samples, and also for the dataset with the excluded samples (thus only samples that had a total raw read count above 25,000 and gene coverage of 1500 genes that had at least 5 reads).

```{r QC plots}
# number of total counts per sample
sum <- data.frame(sample = colnames(GeneticscfRNA),sum = colSums(GeneticscfRNA))
sum$sample <- factor(sum$sample, levels = row.names(sum))
#br = c(brewer.pal(7,"Blues"),brewer.pal(7,"Reds"))
ggplot(sum, aes(x=sample,y=sum)) +
  geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 10),
rep('#B8DE29FF',3))) +
  theme_minimal() + ggtitle("total counts per sample") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12)) + labs(x = "sample", y = "total raw counts")

# quality check to see if each sample has an equal coverage of reads
cov <- c(1:29)
for (i in c(1:29)){
  cov[i] <- length(which(GeneticscfRNA[,i]>4))
  }
covData = data.frame(sample = colnames(GeneticscfRNA),cov)
covData$sample <- factor(covData$sample, levels = row.names(sum))
ggplot(covData, aes(x=sample,y=cov)) +
  geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 10),
rep('#B8DE29FF',3))) +
  theme_minimal() + ggtitle("Gene coverage out of total of 30354 genes") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12)) + labs(x = "sample", y = "gene coverage")
```
```{r}
# number of total counts per sample
sum <- data.frame(sample = colnames(GeneticscfRNAfilter),sum = colSums(GeneticscfRNAfilter))
sum$sample <- factor(sum$sample, levels = row.names(sum))
#br = c(brewer.pal(7,"Blues"),brewer.pal(7,"Reds"))
ggplot(sum, aes(x=sample,y=sum)) +
    geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 9),
rep('#B8DE29FF',0))) +
  theme_minimal() + ggtitle("total counts per sample") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12))

# quality check to see if each sample has an equal coverage of reads
cov <- c(1:25)
for (i in c(1:25)){
  cov[i] <- length(which(GeneticscfRNAfilter[,i]>4))
  }
covData = data.frame(sample = colnames(GeneticscfRNAfilter),cov)
covData$sample <- factor(covData$sample, levels = row.names(sum))
ggplot(covData, aes(x=sample,y=cov)) +
  geom_bar(stat='identity', fill=c(rep('#33638DFF',6),rep('#29AF7FFF',10), rep('#482677FF', 9),
rep('#B8DE29FF',0))) +
```

```
  theme_minimal() + ggtitle("Gene coverage out of total of 30354 genes") +
  theme(axis.text.x = element_text(angle = 60, hjust = 1, size = 10), axis.text.y = element_text(size =
12))
```

PCA plots

I used rlog transformation as it inherently accounts for differences in sequencing depth, which is the case for these samples. Variance stabilizing transformation is not good to use when there is a lot of variation in samples due to the experimental design. I could try vst if the samples with low sequencing depth are excluded.

I also removed genes that had less than a sum of 10 counts.

DESeq2 plots the PC1 and PC2 based on the top 500 most variable genes. I used the PCAtools package to get the other PCAs, which utilizes all genes.

```{r PCA function from DESeq2 adjustment for other PCAs, include = F}
DESeq2:::plotPCA.DESeqTransform
plotPCA1vs3 <- function (object, intgroup = "condition", ntop = 500, returnData = FALSE)
{
    rv <- rowVars(assay(object))
    select <- order(rv, decreasing = TRUE)[seq_len(min(ntop,
        length(rv)))]
    pca <- prcomp(t(assay(object)[select, ]))
    percentVar <- pca$sdev^2/sum(pca$sdev^2)
    if (!all(intgroup %in% names(colData(object)))) {
        stop("the argument 'intgroup' should specify columns of colData(dds)")
    }
    intgroup.df <- as.data.frame(colData(object)[, intgroup,
        drop = FALSE])
    group <- if (length(intgroup) > 1) {
        factor(apply(intgroup.df, 1, paste, collapse = ":"))
    }
    else {
        colData(object)[[intgroup]]
    }
    d <- data.frame(PC1 = pca$x[, 1], PC3 = pca$x[, 3], group = group,
        intgroup.df, name = colnames(object))
    if (returnData) {
        attr(d, "percentVar") <- percentVar[c(1,3)]
        return(d)
    }
    ggplot(data = d, aes_string(x = "PC1", y = "PC3", color = "group")) +
        geom_point(size = 3) + xlab(paste0("PC1: ", round(percentVar[1] *
        100), "% variance")) + ylab(paste0("PC3: ", round(percentVar[2] *
        100), "% variance")) + coord_fixed()
}

plotPCA2vs3 <- function (object, intgroup = "condition", ntop = 500, returnData = FALSE)
{
    rv <- rowVars(assay(object))
    select <- order(rv, decreasing = TRUE)[seq_len(min(ntop,
        length(rv)))]
    pca <- prcomp(t(assay(object)[select, ]))
    percentVar <- pca$sdev^2/sum(pca$sdev^2)
    if (!all(intgroup %in% names(colData(object)))) {
        stop("the argument 'intgroup' should specify columns of colData(dds)")
    }
```

```
    intgroup.df <- as.data.frame(colData(object)[, intgroup,
        drop = FALSE])
    group <- if (length(intgroup) > 1) {
        factor(apply(intgroup.df, 1, paste, collapse = ":"))
    }
    else {
        colData(object)[[intgroup]]
    }
    d <- data.frame(PC2 = pca$x[, 2], PC3 = pca$x[, 3], group = group,
        intgroup.df, name = colnames(object))
    if (returnData) {
        attr(d, "percentVar") <- percentVar[c(2,3)]
        return(d)
    }
    ggplot(data = d, aes_string(x = "PC2", y = "PC3", color = "group")) +
        geom_point(size = 3) + xlab(paste0("PC2: ", round(percentVar[1] *
        100), "% variance")) + ylab(paste0("PC3: ", round(percentVar[2] *
        100), "% variance")) + coord_fixed()
}

```

PCAs with the top 500 of most variable genes
time
```{r PCA time, message=FALSE}
# this only does PCA 1 and 2: plotPCA(vsd, intgroup=c("condition", "type"))
# intgroup is set at the 500 genes with the highest variance
pcaData <- plotPCA(rld, intgroup="time", returnData=TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
PCA1vs2 <- ggplot(pcaData, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData1vs3 <- plotPCA1vs3(rld, intgroup="time", returnData=TRUE)
percentVar1vs3 <- round(100 * attr(pcaData1vs3, "percentVar"))
PCA1vs3 <- ggplot(pcaData1vs3, aes(PC1, PC3, color=time)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3[2],"% variance")) +
  coord_fixed()  +
  theme_set(theme_minimal(base_size = 20))
pcaData2vs3 <- plotPCA2vs3(rld, intgroup="time", returnData=TRUE)
percentVar2vs3 <- round(100 * attr(pcaData2vs3, "percentVar"))
PCA2vs3 <- ggplot(pcaData2vs3, aes(PC2, PC3, color=time)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
PCA1vs2
PCA1vs3
PCA2vs3
ggarrange(PCA1vs3,PCA2vs3, legend = F, align = "h")
```
```{r}
# this only does PCA 1 and 2: plotPCA(vsd, intgroup=c("condition", "type"))
# intgroup is set at the 500 genes with the highest variance
pcaData1 <- plotPCA(rld1, intgroup="time", returnData=TRUE)
```

```
percentVar.1 <- round(100 * attr(pcaData1, "percentVar"))
PCA1vs2.1 <- ggplot(pcaData1, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar.1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar.1[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData1vs3.1 <- plotPCA1vs3(rld1, intgroup="time", returnData=TRUE)
percentVar1vs3.1 <- round(100 * attr(pcaData1vs3.1, "percentVar"))
PCA1vs3.1 <- ggplot(pcaData1vs3.1, aes(PC1, PC3, color=time)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3.1[2],"% variance")) +
  coord_fixed()  +
  theme_set(theme_minimal(base_size = 20))
pcaData2vs3.1 <- plotPCA2vs3(rld1, intgroup="time", returnData=TRUE)
percentVar2vs3.1 <- round(100 * attr(pcaData2vs3.1, "percentVar"))
PCA2vs3.1 <- ggplot(pcaData2vs3.1, aes(PC2, PC3, color=time)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3.1[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
PCA1vs2.1
PCA1vs3.1
PCA2vs3.1
ggarrange(PCA1vs3.1,PCA2vs3.1, legend = F, align = "h")
```

NPnr
```{r PCA NPnr, message=FALSE}
pcaData <- plotPCA(rld, intgroup="NPnr", returnData=TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
PCA1vs2 <- ggplot(pcaData, aes(PC1, PC2, color=NPnr)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData1vs3 <- plotPCA1vs3(rld, intgroup="NPnr", returnData=TRUE)
percentVar1vs3 <- round(100 * attr(pcaData1vs3, "percentVar"))
PCA1vs3 <- ggplot(pcaData1vs3, aes(PC1, PC3, color=NPnr)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData2vs3 <- plotPCA2vs3(rld, intgroup="NPnr", returnData=TRUE)
percentVar2vs3 <- round(100 * attr(pcaData2vs3, "percentVar"))
PCA2vs3 <- ggplot(pcaData2vs3, aes(PC2, PC3, color=NPnr)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
PCA1vs2
ggarrange(PCA1vs3,PCA2vs3, legend = F, align = "h")
```

```{r PCA NPnr, message=FALSE}
```

```
pcaData1 <- plotPCA(rld1, intgroup="NPnr", returnData=TRUE)
percentVar1 <- round(100 * attr(pcaData1, "percentVar"))
PCA1vs2.1 <- ggplot(pcaData1, aes(PC1, PC2, color=NPnr)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar1[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData1vs3.1 <- plotPCA1vs3(rld1, intgroup="NPnr", returnData=TRUE)
percentVar1vs3.1 <- round(100 * attr(pcaData1vs3.1, "percentVar"))
PCA1vs3.1 <- ggplot(pcaData1vs3.1, aes(PC1, PC3, color=NPnr)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3.1[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
pcaData2vs3.1 <- plotPCA2vs3(rld1, intgroup="NPnr", returnData=TRUE)
percentVar2vs3.1 <- round(100 * attr(pcaData2vs3.1, "percentVar"))
PCA2vs3.1 <- ggplot(pcaData2vs3.1, aes(PC2, PC3, color=NPnr)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3.1[2],"% variance")) +
  coord_fixed() +
  theme_set(theme_minimal(base_size = 20))
PCA1vs2.1
ggarrange(PCA1vs3.1,PCA2vs3.1, legend = F, align = "h")
```

HMP vs SCS

```{r PCA condition, message=FALSE}
br = c('mediumspringgreen','darkorchid1', 'deepskyblue', 'hotpink')
pcaData <- plotPCA(rld, intgroup="condition", returnData=TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
PCA1vs2 <- ggplot(pcaData, aes(PC1, PC2, color=condition)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar[2],"% variance")) +
  coord_fixed() +

  theme_set(theme_minimal(base_size = 20))
pcaData1vs3 <- plotPCA1vs3(rld, intgroup="condition", returnData=TRUE)
percentVar1vs3 <- round(100 * attr(pcaData1vs3, "percentVar"))
PCA1vs3 <- ggplot(pcaData1vs3, aes(PC1, PC3, color=condition)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3[2],"% variance")) +
  coord_fixed() +

  theme_set(theme_minimal(base_size = 20))
pcaData2vs3 <- plotPCA2vs3(rld, intgroup="condition", returnData=TRUE)
percentVar2vs3 <- round(100 * attr(pcaData2vs3, "percentVar"))
PCA2vs3 <- ggplot(pcaData2vs3, aes(PC2, PC3, color=condition)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3[2],"% variance")) +
  coord_fixed()  +
  theme_set(theme_minimal(base_size = 20))
```

```
PCA1vs2
ggarrange(PCA1vs3,PCA2vs3,legend = F, align = "h")
```

```{r}

pcaData1 <- plotPCA(rld1, intgroup="condition", returnData=TRUE)
percentVar1 <- round(100 * attr(pcaData1, "percentVar"))
PCA1vs2.1 <- ggplot(pcaData1, aes(PC1, PC2, color=condition)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar1[2],"% variance")) +
  coord_fixed() +

  theme_set(theme_minimal(base_size = 20))
pcaData1vs3.1 <- plotPCA1vs3(rld1, intgroup="condition", returnData=TRUE)
percentVar1vs3.1 <- round(100 * attr(pcaData1vs3.1, "percentVar"))
PCA1vs3.1 <- ggplot(pcaData1vs3.1, aes(PC1, PC3, color=condition)) +
  geom_point(size=2) +
  xlab(paste0("PC1: ",percentVar1vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar1vs3.1[2],"% variance")) +
  coord_fixed() +

  theme_set(theme_minimal(base_size = 20))
pcaData2vs3.1 <- plotPCA2vs3(rld1, intgroup="condition", returnData=TRUE)
percentVar2vs3.1 <- round(100 * attr(pcaData2vs3.1, "percentVar"))
PCA2vs3.1 <- ggplot(pcaData2vs3.1, aes(PC2, PC3, color=condition)) +
  geom_point(size=2) +
  xlab(paste0("PC2: ",percentVar2vs3.1[1],"% variance")) +
  ylab(paste0("PC3: ",percentVar2vs3.1[2],"% variance")) +
  coord_fixed() +

  theme_set(theme_minimal(base_size = 20))
PCA1vs2.1
ggarrange(PCA1vs3.1,PCA2vs3.1,legend = F, align = "h")
```

PCA1 and 2 with different numbers of most variable genes
```{r PCA different number of genes included}
# 1,000 genes with the highest variance
pcaData1k <- plotPCA(rld, intgroup="time", returnData=TRUE, ntop = 1000)
percentVar1k <- round(100 * attr(pcaData1k, "percentVar"))
K1 <- ggplot(pcaData1k, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar1k[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar1k[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 1,000") +
  theme_set(theme_minimal(base_size = 20))

# 2,000 genes with the highest variance
pcaData2k <- plotPCA(rld, intgroup="time", returnData=TRUE, ntop = 2000)
percentVar2k <- round(100 * attr(pcaData2k, "percentVar"))
K2 <- ggplot(pcaData2k, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar2k[1],"% variance")) +
```

```
  ylab(paste0("PC2: ",percentVar2k[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 2,000") +
  theme_set(theme_minimal(base_size = 20))

# 5,000 genes with the highest variance
pcaData5k <- plotPCA(rld, intgroup="time", returnData=TRUE, ntop = 5000)
percentVar5k <- round(100 * attr(pcaData5k, "percentVar"))
K5 <- ggplot(pcaData5k, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar5k[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar5k[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 5,000") +
  theme_set(theme_minimal(base_size = 20))

# All genes
pcaDataAll <- plotPCA(rld, intgroup="time", returnData=TRUE, ntop = nrow(rld))
percentVarAll <- round(100 * attr(pcaDataAll, "percentVar"))
Kall <- ggplot(pcaDataAll, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVarAll[1],"% variance")) +
  ylab(paste0("PC2: ",percentVarAll[2],"% variance")) +
  coord_fixed() + ggtitle(label = "All genes") +
  theme_set(theme_minimal(base_size = 20))
K1
K2
K5
Kall

ggarrange(K1,K2,K5,Kall, as_ggplot(get_legend(Kall, position = "right")), legend = F, align = "h")
```


```{r PCA different number of genes included}
# 1,000 genes with the highest variance


pcaData1k.1 <- plotPCA(rld1, intgroup="time", returnData=TRUE, ntop = 1000)
percentVar1k.1 <- round(100 * attr(pcaData1k.1, "percentVar"))
K1.1 <- ggplot(pcaData1k.1, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar1k.1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar1k.1[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 1,000") +
  theme_set(theme_minimal(base_size = 20))

# 2,000 genes with the highest variance
pcaData2k.1 <- plotPCA(rld1, intgroup="time", returnData=TRUE, ntop = 2000)
percentVar2k.1 <- round(100 * attr(pcaData2k.1, "percentVar"))
K2.1 <- ggplot(pcaData2k.1, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar2k.1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar2k.1[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 2,000") +
  theme_set(theme_minimal(base_size = 20))


# 5,000 genes with the highest variance
pcaData5k.1 <- plotPCA(rld1, intgroup="time", returnData=TRUE, ntop = 5000)
percentVar5k.1 <- round(100 * attr(pcaData5k.1, "percentVar"))
K5.1 <- ggplot(pcaData5k.1, aes(PC1, PC2, color=time)) +
```

```
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar5k.1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar5k.1[2],"% variance")) +
  coord_fixed() + ggtitle(label = "Top 5,000") +
  theme_set(theme_minimal(base_size = 20))

# All genes
pcaDataAll.1 <- plotPCA(rld1, intgroup="time", returnData=TRUE, ntop = nrow(rld1))
percentVarAll.1 <- round(100 * attr(pcaDataAll.1, "percentVar"))
Kall.1 <- ggplot(pcaDataAll.1, aes(PC1, PC2, color=time)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVarAll.1[1],"% variance")) +
  ylab(paste0("PC2: ",percentVarAll.1[2],"% variance")) +
  coord_fixed() + ggtitle(label = "All genes") +
  theme_set(theme_minimal(base_size = 20))

K1.1
K2.1
K5.1
Kall.1

ggarrange(K1.1,K2.1,K5.1,Kall.1, as_ggplot(get_legend(Kall.1, position = "right")), legend = F, align =
"h")
```

Supplementary Code 4

#load pig genes

```{r}

fix_genes <- . %>% # this doesn't work
  tbl_df %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         entrez=entrezgene,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

# update of fix_genes based on error messages
fix_genes <- . %>%
  tibble::as_tibble() %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

myattributes <- c("ensembl_gene_id", # removed entrezgenes, as it's not present in attributes)
                  "external_gene_name",
                  "chromosome_name",
                  "start_position",
                  "end_position",
                  "strand",
                  "gene_biotype",
                  "description")


# Pig
sscrofa <- useMart("ensembl") %>%
  useDataset(mart=., dataset="sscrofa_gene_ensembl") %>%
  getBM(mart=., attributes=myattributes) %>%
  fix_genes

rm(fix_genes, myattributes)


```

Analysis with time normalised - cold samples
Same analysis with centering and scaling time - this does not give any DESeq2 message. Time was divided by the mean of all time points (which is ~100). With this method the p-values are approximately the same (the lower digits are different) as without scaling time. The log2-fold changes are different, as it's reported per unit.

Note that I use 210 minutes for T4, because HMP is stopped at 3.5 hours to prepare the heart for NMP.

```{r time scaled, include=T}
t <- c(0,90,210)
tn <- t/mean(t)
ColDataContN = data.frame(row.names=colnames(GeneticscfRNAfilter[, 1:6]), time = rep(tn,each=2), condition = c(rep('HMP Cold perfusate',6)), NPnr = c(rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1)))
ddsColdN <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter[,1:6],
                              colData = ColDataContN[1:6,],
                              design = ~ time)
ddsColdN <- ddsColdN[ rowSums(counts(ddsColdN)) > 10, ] # removed genes with a sum of 0-10 counts
ddsTimeColdN = DESeq(ddsColdN)
resTimeColdN <- results(ddsTimeColdN)
resTimeColdN
resTimeColdNOrdered <- resTimeColdN[order(resTimeColdN$pvalue),]
summary(resTimeColdN)
sum(resTimeColdN$padj < 0.1, na.rm=TRUE)
plotMA(resTimeColdN, ylim=c(-2,3), main="Differentially expressed genes") # standard

```

```{r}
ensembl1 <- useEnsembl(biomart = "ensembl",
                       dataset = "sscrofa_gene_ensembl")

genesCold <- rownames(resTimeColdN)
G_listCold <- getBM(filters= "ensembl_gene_id", attributes= c("ensembl_gene_id", "external_gene_name",
"description", "entrezgene_id"),values=genesCold,mart= ensembl1)

dim(G_listCold)
length(unique(G_listCold$ensembl_gene_id))

G_listCold %>%
   add_count(ensembl_gene_id) %>%
   dplyr::filter(n>1)

G_listCold <- G_listCold %>% distinct(ensembl_gene_id, .keep_all = TRUE)

library(tidyverse)
G_listCold <- G_listCold %>%
    remove_rownames() %>%
    column_to_rownames(var = 'ensembl_gene_id')


 deCold <- merge(as.data.frame(resTimeColdN), G_listCold, by=0, all=TRUE)  # merge by row names (by=0 or by="row.names")
 #de[is.na(de)] <- 0                # replace NA values
 deCold
```

```{r}
  #BiocManager::install('EnhancedVolcano')
```

```
library(EnhancedVolcano)
EnhancedVolcano(deCold,
    lab = deCold$external_gene_name,
    x = 'log2FoldChange',
    y = 'pvalue',
    pCutoff = 0.1,
    pCutoffCol = 'padj',
    labSize = 4.0,
    drawConnectors = TRUE,
    #maxoverlapsConnectors = Inf,
    widthConnectors = 0.75,
    labCol = 'black',
    labFace = 'bold',

    FCcutoff = 0.0,
     col=c('gray', 'gray', 'gray', 'darkcyan'),
    colAlpha = 1,
    gridlines.major = FALSE,
    gridlines.minor = FALSE)
```
The grey dots in the significant field indicate NA padjust values.

Analysis with time normalised - warm samples

```{r}


t1 <- c(0,5,60, 120, 240)
tn1 <- t1/mean(t1)
ColDataContN1 = data.frame(row.names=colnames(GeneticscfRNAfilter[, 7:25]), time =
c(rep(tn1,each=2), rep(tn1[1],1 ), rep(tn1[2:5], each=2)), condition = c(rep('HMP Warm perfusate',10),
rep('SCS Warm perfusate', 9)), NPnr =
c(rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP1
6',1),rep('NP1',1),rep('NP16',1), rep('NP24',1), rep('NP21', 1), rep('NP24', 1), rep('NP21', 1),
rep('NP24', 1),rep('NP21', 1), rep('NP24', 1),rep('NP21', 1), rep('NP24', 1)))
ddsWarmN <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter[,7:25],
                              colData = ColDataContN1[1:19,],
                              design = ~ time)
ddsWarmN <- ddsWarmN[ rowSums(counts(ddsWarmN)) > 10, ] # removed genes with a sum of 0-10
counts
ddsTimeWarmN = DESeq(ddsWarmN)
resTimeWarmN <- results(ddsTimeWarmN)
summary(resTimeWarmN)
plotMA(resTimeWarmN, ylim=c(-2,2), main="Differentially expressed genes") # standard
resTime.tidyN <- cbind(tidy.DESeqResults(resTimeWarmN),counts(ddsTimeWarmN))
resTime.tidyN[,3] <- round(resTime.tidyN[,3],digits = 3)
resTime.tidyN[,7] <- signif(resTime.tidyN[,7],digits = 3)
resTime.tidy.namesN <- resTime.tidyN %>%
  arrange(p.adjusted) %>%
  dplyr::filter(p.adjusted<0.1) %>%
  inner_join(sscrofa, by=c("gene"="ensgene")) %>%

dplyr::select(names(resTime.tidyN[1]),names(sscrofa[c(2,7)]),names(resTime.tidyN[2:7]),names(resTi
me.tidyN[8:26]))
resTime.tidy.namesN[,c(2:5,8:9)] %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")
```

```{r}
genesWarm <- rownames(resTimeWarmN)
G_listWarm <- getBM(filters= "ensembl_gene_id", attributes= c("ensembl_gene_id",
"external_gene_name",
"description", "entrezgene_id"),values=genesWarm,mart= ensembl1)

dim(G_listWarm)
length(unique(G_listWarm$ensembl_gene_id))

G_listWarm %>%
    add_count(ensembl_gene_id) %>%
    dplyr::filter(n>1)

G_listWarm <- G_listWarm %>% distinct(ensembl_gene_id, .keep_all = TRUE)

library(tidyverse)
G_listWarm <- G_listWarm %>%
     remove_rownames() %>%
     column_to_rownames(var = 'ensembl_gene_id')


 deWarm <- merge(as.data.frame(resTimeWarmN), G_listWarm, by=0, all=TRUE)  # merge by row
names (by=0 or by="row.names")
 #de[is.na(de)] <- 0                # replace NA values
 deWarm
```

```{r}
EnhancedVolcano(deWarm,
    lab = deWarm$external_gene_name,
    x = 'log2FoldChange',
    y = 'pvalue',
    pCutoff = 0.1,
    pCutoffCol = 'padj',
    labSize = 4.0,
    drawConnectors = TRUE,
    widthConnectors = 0.75,
    labCol = 'black',
    labFace = 'bold',

    FCcutoff = 0,
      col=c('gray', 'gray', 'gray', 'darkcyan'),
    colAlpha = 1,
    gridlines.major = FALSE,
    gridlines.minor = FALSE)
```

```{r}
t <- c(0,90,210)
tn <- t/mean(t)
ColDataContN = data.frame(row.names=colnames(GeneticscfRNAfilter[, 1:6]), time = rep(tn,each=2),
condition = c(rep('HMP Cold perfusate',6)), NPnr =
c(rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1)))
```

```
ddsColdN <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter[,1:6],
                              colData = ColDataContN[1:6,],
                              design = ~ time)
#ddsColdN <- ddsColdN[ rowSums(counts(ddsColdN)) > 10, ] # removed genes with a sum of 0-10
counts
ddsTimeColdN = DESeq(ddsColdN)
resTimeColdN <- results(ddsTimeColdN)
resTimeColdN
resTimeColdNOrdered <- resTimeColdN[order(resTimeColdN$pvalue),]
summary(resTimeColdN)
sum(resTimeColdN$padj < 0.1, na.rm=TRUE)
plotMA(resTimeColdN, ylim=c(-2,3), main="Differentially expressed genes") # standard
# export for GSEA. These were done based on all genes, so genes with <10 counts per row were not
removed.
# Two methods: apeglm schrunken log2 FCs and a combi of FC and p-value. Regarding the latter:
people either multiply the sign of the Log2FC and -log10(p-value), or divide it (doesn't matter which
way).See https://www.biostars.org/p/279097/
res_apeglmCold <- lfcShrink(ddsTimeColdN, coef = "time", type="apeglm")
plotMA(res_apeglmCold, ylim=c(-0.5,2), main="Differentially expressed genes")
head(res_apeglmCold)
resTimeColdN$lfcShrink <- res_apeglmCold$log2FoldChange
resTimeColdN$fcsign <- sign(resTimeColdN$log2FoldChange)
resTimeColdN$logP <- -log10(resTimeColdN$pvalue)
resTimeColdN$metric <- resTimeColdN$logP/resTimeColdN$fcsign
res_OrderedCold <- resTimeColdN[order(resTimeColdN$pvalue),]
head(res_OrderedCold,10)
tail(res_OrderedCold,10)
nrow(res_OrderedCold)
final <- na.omit(resTimeColdN)
nrow(final)
finalOrdered <- final[order(final$metric),]
plot(finalOrdered$metric)
length(which(finalOrdered$metric<0))
length(which(finalOrdered$metric>0))
head(final[order(final$pvalue),])
tail(final[order(final$pvalue),])
metricCold <- data.frame(gene=row.names(final), final$metric)
lfcShrinkCold <- data.frame(gene=row.names(final), final$lfcShrink)
options(scipen = 999) # switch off printing e numbers
write.table(metricCold,file="resTimeContinuousWaldCold_metric.rnk",sep = "\t",row.names = F,
col.names = F,quote = F)
write.table(lfcShrinkCold,file="resTimeContinuousWaldCold_lfcShrink.rnk",sep = "\t",row.names = F,
col.names = F,quote = F)
options(scipen = 0)
```

```{r}
ensembl1 <- useEnsembl(biomart = "ensembl",
                       dataset = "sscrofa_gene_ensembl")
metricCold2 <- metricCold %>% remove_rownames %>% column_to_rownames(var="gene")

genesColdmetric <- rownames(metricCold2)
G_listColdmetric <- getBM(filters= "ensembl_gene_id", attributes= c( "ensembl_gene_id",
"external_gene_name", "entrezgene_id"),values=genesColdmetric,mart= ensembl1)


G_listColdmetric %>%
   add_count(ensembl_gene_id) %>%
   dplyr::filter(n>1)
```

```
G_listColdmetric<- G_listColdmetric %>% distinct(ensembl_gene_id, .keep_all = TRUE)

library(tidyverse)
G_listColdmetric <- G_listColdmetric %>%
    remove_rownames() %>%
    column_to_rownames(var = 'ensembl_gene_id')


finalmetricCold <- merge(as.data.frame(metricCold2), G_listColdmetric, by=0, all=TRUE)  # merge by
row names (by=0 or by="row.names")
 #de[is.na(de)] <- 0               # replace NA values
 finalmetricCold
```

```{r}

library(clusterProfiler)
## Remove any NA values
deCold_entrezmetric <- subset(finalmetricCold , is.na(entrezgene_id) == FALSE)


## Remove any Entrez duplicates
deCold_entrezmetric<- deCold_entrezmetric[which(duplicated(deCold_entrezmetric$entrezgene_id)
== F), ]
geneCold_matrixmetric <- deCold_entrezmetric$final.metric
names(geneCold_matrixmetric) <- deCold_entrezmetric$entrezgene_id



geneCold_matrixmetric = sort(geneCold_matrixmetric, decreasing = TRUE)

head(geneCold_matrixmetric)

```
GO gene enrichment and pathway analysis
```{r}
gseColdmetric <- gseGO(geneList = geneCold_matrixmetric,
            ont = "ALL",
            pvalueCutoff = 0.1,
            pAdjustMethod = "BH",
            OrgDb = org.Ss.eg.db
            )
require(DOSE)
dotplot(gseColdmetric, showCategory =25, split = ".sign") + facet_grid(.~.sign)


```



```{r export for GSEA II, include= F}
t1 <- c(0,5,60, 120, 240)
tn1 <- t1/mean(t1)
ColDataContN1 = data.frame(row.names=colnames(GeneticscfRNAfilter[, 7:25]), time =
c(rep(tn1,each=2), rep(tn1[1],1 ), rep(tn1[2:5], each=2)), condition = c(rep('HMP Warm perfusate',10),
rep('SCS Warm perfusate', 9)), NPnr =
c(rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP16',1),rep('NP1',1),rep('NP1
```

```
6',1),rep('NP1',1),rep('NP16',1), rep('NP24',1), rep('NP21', 1), rep('NP24', 1), rep('NP21', 1),
rep('NP24', 1),rep('NP21', 1), rep('NP24', 1),rep('NP21', 1), rep('NP24', 1)))
ddsWarmN <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter[,7:25],
                           colData = ColDataContN1[1:19,],
                           design = ~ time)
#ddsWarmN <- ddsWarmN[ rowSums(counts(ddsWarmN)) > 10, ] # removed genes with a sum of 0-
10 counts
ddsTimeWarmN = DESeq(ddsWarmN)
resTimeWarmN <- results(ddsTimeWarmN)
summary(resTimeWarmN)
plotMA(resTimeWarmN, ylim=c(-2,2), main="Differentially expressed genes") # standard
# export for GSEA. These were done based on all genes, so genes with <10 counts per row were not
removed.
res_apeglmW <- lfcShrink(ddsTimeWarmN, coef = "time", type="apeglm")
plotMA(res_apeglmW, ylim=c(-0.7,0.7), main="Differentially expressed genes")
head(res_apeglmW)
resTimeWarmN$lfcShrink <- res_apeglmW$log2FoldChange
resTimeWarmN$fcsign <- sign(resTimeWarmN$log2FoldChange)
resTimeWarmN$logP <- -log10(resTimeWarmN$pvalue)
resTimeWarmN$metric <- resTimeWarmN$logP/resTimeWarmN$fcsign
res_OrderedW <- resTimeWarmN[order(resTimeWarmN$pvalue),]
head(res_OrderedW,10)
tail(res_OrderedW,10)
nrow(res_OrderedW)
finalW <- na.omit(resTimeWarmN)
nrow(finalW)
finalOrderedW <- final[order(finalW$metric),]
plot(finalOrderedW$metric)
length(which(finalOrderedW$metric<0)) #339
length(which(finalOrderedW$metric>0)) #1176

head(finalW[order(finalW$pvalue),])
tail(finalW[order(finalW$pvalue),])
metricW <- data.frame(gene=row.names(finalW), finalW$metric)
lfcShrinkW <- data.frame(gene=row.names(finalW), finalW$lfcShrink)
options(scipen = 999)
write.table(metricW ,file="resTimeContinuousWaldWarm_metric.rnk",sep = "\t",row.names = F,
col.names = F,quote = F)
write.table(lfcShrinkW,file="resTimeContinuousWaldWarm_lfcShrink.rnk",sep = "\t",row.names = F,
col.names = F,quote = F)
options(scipen = 0)
```

```{r}
ensembl1 <- useEnsembl(biomart = "ensembl",
                       dataset = "sscrofa_gene_ensembl")
metricW2 <- metricW %>% remove_rownames %>% column_to_rownames(var="gene")

genesW <- rownames(metricW2)
G_listW <- getBM(filters= "ensembl_gene_id", attributes= c(
"ensembl_gene_id","external_gene_name", "entrezgene_id"),values=genesW,mart= ensembl1)


G_listW %>%
   add_count(ensembl_gene_id) %>%
   dplyr::filter(n>1)

G_listW<- G_listW %>% distinct(ensembl_gene_id, .keep_all = TRUE)
```

```
library(tidyverse)
G_listW <- G_listW %>%
    remove_rownames() %>%
    column_to_rownames(var = 'ensembl_gene_id')


finalmetricW <- merge(as.data.frame(metricW2), G_listW, by=0, all=TRUE)  # merge by row names
(by=0 or by="row.names")
 #de[is.na(de)] <- 0               # replace NA values
 finalmetricW
```

```{r}

library(clusterProfiler)
## Remove any NA values
deW_entrez <- subset(finalmetricW , is.na(entrezgene_id) == FALSE)


## Remove any Entrez duplicates
deW_entrez<- deW_entrez[which(duplicated(deW_entrez$entrezgene_id) == F), ]
geneW_matrix <- deW_entrez$finalW.metric
names(geneW_matrix) <- deW_entrez$entrezgene_id



geneW_matrix = sort(geneW_matrix, decreasing = TRUE)

head(geneW_matrix)

```



```{r}
gseW <- gseGO(geneList = geneW_matrix,
             ont = "ALL",
             pvalueCutoff = 0.3,
             pAdjustMethod = "BH",
             OrgDb = org.Ss.eg.db
             )
require(DOSE)
dotplot(gseW, showCategory = 25, split = ".sign") + facet_grid(.~.sign)


```


Analysis using the LRT test

Temperature-specific differences over time
Full model: temperature + time + temperature:time. Reduced model: temperature + time. This will give the genes that have a temperature-specific effect over time, either by going in different direction or by having a different log2-FC over time. The log2-FC output is the difference between the log2-FC over time in cold and warm samples. Note therefore that this will not give small p values to genes that moved up or down over time in the same way in both temperatures.
See
https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#interactions
for example explanations.

```{r LRT temperature-specific}
ddsLRTwarm <- DESeqDataSetFromMatrix(countData = GeneticscfRNAfilter[,7:25],
                              colData = ColDataContN1[1:19,],
                              design = ~ condition + time + condition:time)
ddsLRTwarm <- ddsLRTwarm[ rowSums(counts(ddsLRTwarm)) > 10, ] # removed genes with a sum of 0-10 counts
ddsLRTwarm = DESeq(ddsLRTwarm, test = "LRT", reduced = ~ condition + time)
resLRTwarm <- results(ddsLRTwarm)
resLRTwarm
summary(resLRTwarm)
plotMA(resLRTwarm, ylim=c(-0.5, 0.5))


res.tidyN <- cbind(tidy.DESeqResults(resLRTwarm),counts(ddsLRTwarm))
res.tidyN[,3] <- round(res.tidyN[,3],digits = 3)
res.tidyN[,7] <- signif(res.tidyN[,7],digits = 3)
res.tidy.namesN <- res.tidyN %>%
  arrange(p.adjusted) %>%
  dplyr::filter(p.adjusted<0.1) %>%
  inner_join(sscrofa, by=c("gene"="ensgene")) %>%

dplyr::select(names(res.tidyN[1]),names(sscrofa[c(2,7)]),names(res.tidyN[2:7]),names(res.tidyN[8:26]))
res.tidy.namesN[,c(2:5,8:9)] %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")
#write.csv(res.tidy.namesN, file = 'resTemp-specific_LRT_Padjusted_U0.9.csv')
```

Supplementary Code 5

First the raw count data txt files were imported. Michal created these; first the barcodes and UMIs were sorted, then he mapped the reads to the transcriptome of S. Scrofa using bwa. I checked the # of total raw counts per sample and the read coverage across genes (**Supplementary Code 2**). I continued the analysis with the samples that had a total raw read count above 25,000 and gene coverage of 1500 genes that had at least 5 reads.

The # of genes in the count file: 30354 (at least 1 read in any of the 29 samples).
Total # of genes in pig: 31908.
Total # of genes in human: 67128

``` {r add pig genes, include=F}
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
BiocManager::install("biomaRt")
library(biomaRt)

fix_genes <- . %>% # this doesn't work
  tbl_df %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         entrez=entrezgene,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

# update of fix_genes based on error messages
fix_genes <- . %>%
  tibble::as_tibble() %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

myattributes <- c("ensembl_gene_id", # removed entrezgenes, as it's not present in attributes)
                  "external_gene_name",
                  "chromosome_name",
                  "start_position",
                  "end_position",
                  "strand",
                  "gene_biotype",
                  "description")


# Pig
sscrofa <- useMart("ensembl") %>%
  useDataset(mart=., dataset="sscrofa_gene_ensembl") %>%
  getBM(mart=., attributes=myattributes) %>%
  fix_genes

rm(fix_genes, myattributes)
```

```
fix_genes <- . %>% # this doesn't work
  tbl_df %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         entrez=entrezgene,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

# update of fix_genes based on error messages
fix_genes <- . %>%
  tibble::as_tibble() %>%
  distinct %>%
  rename(ensgene=ensembl_gene_id,
         symbol=external_gene_name,
         chr=chromosome_name,
         start=start_position,
         end=end_position,
         biotype=gene_biotype)

myattributes <- c("ensembl_gene_id", # removed entrezgenes, as it's not present in attributes)
                  "external_gene_name",
                  "chromosome_name",
                  "start_position",
                  "end_position",
                  "strand",
                  "gene_biotype",
                  "description")

# human
hsapiens <- useMart("ensembl") %>%
  useDataset(mart=., dataset="hsapiens_gene_ensembl") %>%
  getBM(mart=., attributes=myattributes) %>%
  fix_genes

rm(fix_genes, myattributes)

```


Top 20 expressed genes per sample
Ordered on counts from high-low for the cf-RNA samples.


```{r}
GeneticscfRNA2 = data.frame(gene=row.names(GeneticscfRNAfilter),GeneticscfRNAfilter[,1:25])
colnames(GeneticscfRNA2)
colnames(sscrofa)
GeneticscfRNA.tidy.names <- GeneticscfRNA2 %>%
  inner_join(sscrofa, by=c("gene"="ensgene")) %>%
  dplyr::select(gene, symbol, C05_NP01, C05_NP16, C1.5_NP01, C1.5_NP16, C4_NP01, C4_NP16,
W02_NP01, W02_NP16, W02_NP24, WRPa_NP01, WRPa_NP16, WRP_NP21, WRP_NP24,
W60_NP01, W60_NP16, W60_NP21, W60_NP24, W120_NP01, W120_NP16, W120_NP21,
W120_NP24, W240a_NP01, W240a_NP16, W240_NP21, W240_NP24,biotype, description)
```

```{r top expressed genes per sample}
GeneticscfRNA.tidy.names %>%
  arrange(desc(C05_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(C05_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(C1.5_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(C1.5_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(C4_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")

GeneticscfRNA.tidy.names %>%
  arrange(desc(C4_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")



GeneticscfRNA.tidy.names %>%
  arrange(desc(W02_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W02_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")



GeneticscfRNA.tidy.names %>%
  arrange(desc(W02_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(WRPa_NP01)) %>%
  head(20) %>%
```

```
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(WRPa_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(WRP_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(WRP_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W60_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W60_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W60_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W60_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W120_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W120_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W120_NP21)) %>%
  head(20) %>%
```

```
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W120_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W240a_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W240a_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W240_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


GeneticscfRNA.tidy.names %>%
  arrange(desc(W240_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


```


Top expressed heart-specific genes
I downloaded a list from the human protein atlas with genes that are enriched in heart. Settings: cell_type_category_rna:Cardiomyocytes;Cell type enriched,Group enriched,Cell type enhanced AND sort_by:tissue specific score.

665 out of 880 heart-specific genes are found in our pig gene count file.

```
``` {r heart specific genes,include = F}
HeartGenes = read.delim('proteinatlas_6aacaf9d.tsv', row.names = 1)
colnames(HeartGenes[,1:10])
head(HeartGenes[1:50])
HeartGenes = data.frame(HeartGene = row.names(HeartGenes),HeartGenes)
HeartGenesS <- GeneticscfRNA.tidy.names  %>%
  inner_join(HeartGenes, by=c("symbol"="HeartGene")) %>%
  dplyr::select(gene, symbol,C05_NP01, C05_NP16, C1.5_NP01, C1.5_NP16, C4_NP01, C4_NP16, W02_NP01, W02_NP16,  W02_NP24, WRPa_NP01, WRPa_NP16, WRP_NP21, WRP_NP24, W60_NP01, W60_NP16, W60_NP21, W60_NP24, W120_NP01, W120_NP16, W120_NP21, W120_NP24, W240a_NP01, W240a_NP16, W240_NP21, W240_NP24,  RNA.tissue.specificity.score, Gene.description) #RNA.tissue.specific.NX, RNA.single.cell.type.specific.NX, RNA.blood.cell.specificity
nrow(HeartGenesS) #665
nrow(HeartGenes) #880
```
```

top 20 heart-specific genes based on tissue specificity score
```` {r list top expressed heart genes}
# top 20 heart-specific genes based on tissue specificity score
HeartGenesS %>%
  arrange(desc(RNA.tissue.specificity.score)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")
```
```` {r top expressed heart genes per sample}

HeartGenesS %>%
  arrange(desc(C05_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(C05_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(C1.5_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(C1.5_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(C4_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")

HeartGenesS %>%
  arrange(desc(C4_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")



HeartGenesS %>%
  arrange(desc(W02_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W02_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")



HeartGenesS %>%
````

```
  arrange(desc(W02_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(WRPa_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(WRPa_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(WRP_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(WRP_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W60_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W60_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W60_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W60_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W120_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
```

```
  arrange(desc(W120_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W120_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W120_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W240a_NP01)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W240a_NP16)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W240_NP21)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")


HeartGenesS %>%
  arrange(desc(W240_NP24)) %>%
  head(20) %>%
  pander::pandoc.table(split.table=100, style="rmarkdown")
```

```
``` {r write tables, include = F}
HeartGenesS2 <- Genetics.tidy.names  %>%
  inner_join(HeartGenes, by=c("symbol"="HeartGene")) %>%
  dplyr::select(gene, symbol, C05_NP01, C05_NP16, C1.5_NP01, C1.5_NP16, C4_NP01, C4_NP16,
WB_NP01, WB_NP16, WB_NP24, W02_NP01, W02_NP16, W02_NP21, W02_NP24, WRPa_NP01,
WRPa_NP16, WRP_NP21, WRP_NP24, W60_NP01, W60_NP16, W60_NP21, W60_NP24,
W120_NP01, W120_NP16, W120_NP21, W120_NP24, W240a_NP01, W240a_NP16, W240_NP21,
W240_NP24, RNA.tissue.specificity.score, Gene.description, RNA.tissue.specific.NX,
RNA.single.cell.type.specific.NX, RNA.blood.cell.specificity)
write.table(HeartGenesS2,file="HeartGenes.txt",sep = "\t",row.names = F, col.names = T,quote = F)

```
```

convert pig genes to human genes

```{r}
ensembl1 <- useEnsembl(biomart = "ensembl",
                       dataset = "sscrofa_gene_ensembl")
```

```
humancfRNA <- getBM( mart = ensembl1,
      filters = "ensembl_gene_id",
      values = GeneticscfRNA.tidy.names$gene,
      attributes = c("ensembl_gene_id", "external_gene_name", "hsapiens_homolog_ensembl_gene",
"hsapiens_homolog_associated_gene_name")
      )
```

```{r}
library(dplyr)
finalcfRNA <- humancfRNA %>%
  inner_join(GeneticscfRNA.tidy.names, by=c("ensembl_gene_id" = "gene"))
```

```{r}
BiocManager::install("EDASeq")
library("EDASeq")
```

Total count normalization

I calculated the total count per sample using colSums

```{r}
colSums(finalcfRNA[,c(6:30)])
```

I summed up the counts for genes that have the similar human ensembl gene code.

```{r}
sumfinalcountstrycfRNA <-
finalcfRNA %>% group_by(hsapiens_homolog_ensembl_gene,
hsapiens_homolog_associated_gene_name) %>%
  summarize_at(.vars = vars(C05_NP01, C05_NP16, C1.5_NP01, C1.5_NP16, C4_NP01, C4_NP16,
W02_NP01, W02_NP16, W02_NP24, WRPa_NP01, WRPa_NP16, WRP_NP21, WRP_NP24,
W60_NP01, W60_NP16, W60_NP21, W60_NP24, W120_NP01, W120_NP16, W120_NP21,
W120_NP24, W240a_NP01, W240a_NP16, W240_NP21, W240_NP24),
              .funs = c(sum = "sum"))
```

I removed the row that does not have a homolog human gene.

```{r}
cfRNAwithhomolog <- sumfinalcountstrycfRNA[-c(1),]
colSums(cfRNAwithhomolog[,c(3:27)])
```

I summed up the counts for genes that have the similar human gene name

```{r}
sumfinalcountcfRNA <- cfRNAwithhomolog %>%
group_by(hsapiens_homolog_associated_gene_name) %>%
```

```
  summarize_at(.vars = vars(C05_NP01_sum, C05_NP16_sum, C1.5_NP01_sum, C1.5_NP16_sum,
C4_NP01_sum, C4_NP16_sum,  W02_NP01_sum, W02_NP16_sum,  W02_NP24_sum,
WRPa_NP01_sum, WRPa_NP16_sum, WRP_NP21_sum, WRP_NP24_sum, W60_NP01_sum,
W60_NP16_sum, W60_NP21_sum, W60_NP24_sum, W120_NP01_sum, W120_NP16_sum,
W120_NP21_sum, W120_NP24_sum, W240a_NP01_sum, W240a_NP16_sum, W240_NP21_sum,
W240_NP24_sum),
              .funs = c(sum = "sum"))
```

I removed the row that does not have a homolog human gene.

```{r}
cfRNAwithhumangenename <- sumfinalcountcfRNA[-c(1),]
colSums(cfRNAwithhumangenename[,c(2:26)])
```

I calculated the total count per sample using colSums

```{r}
cfRNAtotalcountpersample <- colSums(cfRNAwithhumangenename[ , c(2:26)])
```

total counts per sample per million

```{r}

totalcountspersamplepermillioncfRNA <- cfRNAtotalcountpersample / 1e6
```

I divided the gene counts / total count per sample per million

```{r}
cfRNAsumfinalcount<- cfRNAwithhumangenename  %>% mutate (C05_NP01 =
C05_NP01_sum_sum / totalcountspersamplepermillioncfRNA[1], C05_NP16 = C05_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[2], C1.5_NP01 = C1.5_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[3], C1.5_NP16 = C1.5_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[4], C4_NP01 = C4_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[5], C4_NP16 = C4_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[6],  W02_NP01 = W02_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[7], W02_NP16 = W02_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[8], W02_NP24 = W02_NP24_sum_sum /
totalcountspersamplepermillioncfRNA [9], WRPa_NP01 = WRPa_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[10], WRPa_NP16 = WRPa_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[11], WRP_NP21 = WRP_NP21_sum_sum /
totalcountspersamplepermillioncfRNA[12], WRP_NP24 = WRP_NP24_sum_sum /
totalcountspersamplepermillioncfRNA[13], W60_NP01 = W60_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[14], W60_NP16 = W60_NP16_sum_sum /
totalcountspersamplepermillioncfRNA[15], W60_NP21 = W60_NP21_sum_sum /
totalcountspersamplepermillioncfRNA[16], W60_NP24 = W60_NP24_sum_sum /
totalcountspersamplepermillioncfRNA[17], W120_NP01 = W120_NP01_sum_sum /
totalcountspersamplepermillioncfRNA[18], W120_NP16 =
W120_NP16_sum_sum/totalcountspersamplepermillioncfRNA[19], W120_NP21 =
```

```
W120_NP21_sum_sum / totalcountspersamplepermillioncfRNA[20], W120_NP24 =
W120_NP24_sum_sum/totalcountspersamplepermillioncfRNA[21], W240a_NP01 =
W240a_NP01_sum_sum / totalcountspersamplepermillioncfRNA[22], W240a_NP16 =
W240a_NP16_sum_sum / totalcountspersamplepermillioncfRNA[23], W240_NP21 =
W240_NP21_sum_sum / totalcountspersamplepermillioncfRNA[24], W240_NP24 =
W240_NP24_sum_sum / totalcountspersamplepermillioncfRNA[25]

                                        )
```

```{r}
CPMcfRNA <- dplyr::select(cfRNAsumfinalcount, hsapiens_homolog_associated_gene_name,
C05_NP01, C05_NP16, C1.5_NP01, C1.5_NP16, C4_NP01, C4_NP16,W02_NP01, W02_NP16,
W02_NP24, WRPa_NP01, WRPa_NP16, WRP_NP21, WRP_NP24, W60_NP01, W60_NP16,
W60_NP21, W60_NP24, W120_NP01, W120_NP16, W120_NP21, W120_NP24, W240a_NP01,
W240a_NP16, W240_NP21, W240_NP24)
```

```{r}
install.packages("writexl")
library("writexl")
```

```{r}
write_xlsx(CPMcfRNA,"~/MRP/CPMcfRNA.xlsx" )
```

SingleR
```{r}
BiocManager::install("SingleR")
library(SingleR)
BiocManager::install("celldex")
library(celldex)
BiocManager::install("scRNAseq")
library(scRNAseq)

BiocManager::install("scater")
library(scater)
```

```{r}
hpca.se <- HumanPrimaryCellAtlasData()
hpca.se
reference <- BlueprintEncodeData()
reference
```

Converting our data to a SingleCellExperiment
```{r}

dfCPMcfRNA <- as.data.frame(CPMcfRNA)
```

```
CPMcfRNA2 <- dfCPMcfRNA[-1]
row.names(CPMcfRNA2)<- dfCPMcfRNA$hsapiens_homolog_associated_gene_name




CPMcountscfRNA <- SingleCellExperiment(assays = list(counts = CPMcfRNA2))
CPMcountscfRNA


```

```{r}


 pred.cfRNAseq <- SingleR( test = CPMcountscfRNA, ref = hpca.se,assay.type.test = "counts", labels = hpca.se$label.main)
pred.cfRNAseq

table(pred.cfRNAseq$labels)

 pred1.cfRNAseq <- SingleR( test = CPMcountscfRNA, ref = reference,assay.type.test = "counts", labels = reference$label.main)
pred1.cfRNAseq

table(pred.cfRNAseq$labels)
table(pred1.cfRNAseq$labels)

```

```{r}
library(scRNAseq)
BiocManager::install("scuttle")
library(scuttle)

plotScoreHeatmap(pred.cfRNAseq)
plotScoreHeatmap(pred1.cfRNAseq, show_colnames = TRUE)

```

Supplementary Code 6

As the standard reference set for SingleR did not contain cardiomyocytes and other cardiac cell types, deconvolution was next performed with cell types found in the heart 'Transcriptional and Cellular Diversity of the Human Heart'.

```{r}
HeartTranscriptome <- ReadMtx( mtx = "~/MRP/RNA seq/gene_sorted-matrix/gene_sorted-matrix.mtx", features = "~/MRP/RNA seq/gene_sorted-matrix/genes_v2.tsv", cells = "~/MRP/RNA seq/gene_sorted-matrix/barcodes.tsv" )
SeuratHeartTranscriptome <- CreateSeuratObject(counts = HeartTranscriptome)
 metadataHeartTranscriptome <- read.delim("~/MRP/RNA seq/gene_sorted-matrix/meta.data.v3.txt")
 metadataHeartTranscriptome <- metadataHeartTranscriptome[-1,]


 #trying to couple the metadata to the Seuratobject

rownames(metadataHeartTranscriptome) <- metadataHeartTranscriptome[,1] #Assigning row names from 1st column
metadataHeartTranscriptome[,1] <- NULL #Removing the first column
metadataHeartTranscriptome
HeartTranscriptomeSeurat <- AddMetaData(SeuratHeartTranscriptome, metadataHeartTranscriptome)
head(HeartTranscriptomeSeurat@meta.data)
```

```{r}
resultsHeart <- SingleR(test = CPMcountscfRNA, ref = as.SingleCellExperiment(HeartTranscriptomeSeurat), assay.type.test = "counts", labels = HeartTranscriptomeSeurat$Cluster)

resultsHeart
```

```{r}
library(scRNAseq)
library(scuttle)
plotScoreHeatmap(resultsHeart, show_colnames = TRUE)
```

Then, I combined the two reference sets and this technique involves performing classification separately within each reference, and then collating the results to choose the label with the highest score across references.

```{r}
resultsHeartCombined <- SingleR(test = CPMcountscfRNA, ref = list(as.SingleCellExperiment(HeartTranscriptomeSeurat), reference), assay.type.test = "counts", labels = list(HeartTranscriptomeSeurat$Cluster, reference$label.main))

resultsHeartCombined
```

```{r}
plotScoreHeatmap(resultsHeartCombined, show_colnames = TRUE)

```

```{r}
table(resultsHeartCombined$labels)
table(resultsHeartCombined$reference)
```

```
```