ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE CARDIO NEFRO TORACICHE

Ciclo 37

Settore Concorsuale: 06/A1 - GENETICA MEDICA

Settore Scientifico Disciplinare: MED/03 - GENETICA MEDICA

THE ASSOCIATION BETWEEN DONOR-DERIVED CELL-FREE DNA AND ACUTE REJECTION IN HEART TRANSPLANT PATIENTS

Presentata da: Bianca De Nicolo

Coordinatore Dottorato

Niccolò Daddi

Supervisore

Elena Bonora

Esame finale anno 2025

Borsa di dottorato del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005), risorse FSE REACT-EU, Azione IV.4 "Dottorati e contratti di ricerca su tematiche dell'innovazione" e Azione IV.5 "Dottorati su tematiche Green."

CUP: J35F21003340006

Abstract

Cardiac transplantation is currently the only effective therapy for patients with end-stage heart failure, a condition where the heart ability to pump blood is severely compromised, and no other treatment options remain. Despite its life-saving potential, heart transplantation is associated with several significant challenges, the most prominent of which are post-transplant complications. Among these, the risk of infections is heightened due to the immunosuppressive medications required to prevent organ rejection, while the possibility of graft rejection remains one of the primary concerns. Rejection occurs when the recipient's immune system recognizes the transplanted heart as foreign and mounts an immune response to destroy it.

The gold standard for diagnosing transplant rejection remains endomyocardial biopsy (EMB), where a small tissue sample is taken from the transplanted heart to examine the presence of rejection. However, this invasive procedure has several drawbacks, including patient discomfort, the need for multiple biopsies, and risks related to the procedure itself, such as bleeding and infection. Additionally, the process is costly due to the need for specialized equipment and histopathological analysis of the samples, making it a less-than-ideal solution for long-term monitoring of heart transplant recipients.

In light of these limitations, the study presented in this thesis explores the potential of a non-invasive approach for detecting transplant rejection: liquid biopsy. This technique involves analyzing blood samples to detect and quantify specific biomarkers of rejection, particularly donor-derived cell-free DNA (dd-cfDNA). dd-cfDNA is DNA that is released into the bloodstream when cells die, and its presence can indicate immune-mediated damage to the transplanted heart. Since dd-cfDNA is of donor origin, its levels in the recipient’s blood can be a direct reflection of transplant rejection, providing a more accessible and less invasive diagnostic method.

The methodology employed in this study uses NGS to analyze single nucleotide polymorphisms (SNPs), which are genetic variations that can distinguish between the donor's and the recipient's DNA. By sequencing these SNPs, the study can quantify the fraction of dd-cfDNA present in the patient’s bloodstream. Specialized software is then used to calculate the precise amount of dd-cfDNA, which serves as an indicator of ongoing rejection.

The study aims to establish a reliable and early biomarker for transplant rejection, which could potentially replace or complement the traditional biopsy method, offering a less invasive, more cost-effective alternative for monitoring transplant recipients over time.

Summary

1. Introduction

Cardiac Pathologies leading to End-Stage Heart Failure

End-stage heart failure (HF) can arise from a range of pathological conditions that progressively impair heart ability to pump blood effectively (*Sapna et al. 2023*). Among these, the most common are briefly mentioned in the following paragraphs.

1. Dilated Cardiomyopathy

Dilated cardiomyopathy is one of the leading causes of heart failure and the most frequent indications for heart transplantation. It is characterized by the dilation and weakening of the left ventricle *(Figure 1)*, resulting in reduced contractility *(Schultheiss et al. 2019)*. The clinical presentation of DCM is usually unspecific and /or unrelated to the underlying aetiology, ranging from dyspnea, swollen legs, ankles and stomach, fatigue and chest pain caused by reduced oxygen reaching the heart, to arrhythmia, acute decompensation or cardiogenic shock *(Schultheiss et al. 2019)*. Diverse aetiologies for DCM have been observed over decades of research that include genetic mutations, infections, inflammation, autoimmune diseases, exposure to toxins and endocrine or neuromuscular causes *(Schultheiss et al. 2019, Richardson et al. 1996)*. The most commonly reported causes of DCM are idiopathic and familial diseases.

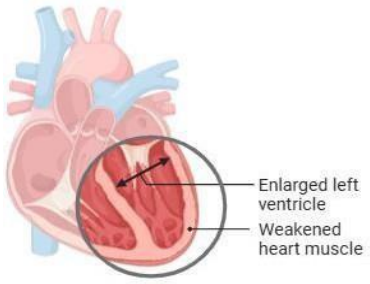


Figure 1: Dilated cardiomyopathy in human heart (From *Biorender.com*)

2. Ischemic Cardiomyopathy

Ischemic cardiomyopathy represents a systolic left ventricular dysfunction in the setting of obstructive coronary artery disease, and it is considered the most common cause of heart failure worldwide *(Del Buono et al. 2022, Felker et al. 2002)*. In CAD patients, left ventricular dysfunction is the result of irreversible loss of viable mass consequent to an acute myocardial infarction (AMI) *(Figure 2)*, occasionally combined with loss of contractility in ischemic, yet still viable, myocardium, better defined as hibernating myocardium *(Felker et al. 2002, Almeida et al. 2021).* This phenomenon carries a significant therapeutic and prognostic relevance, since loss of contractility in hibernating myocardium is potentially reversible after restoration of ischemia *(Page et al. 2015)*. Whatever the exact mechanism, patients with ischemic cardiomyopathy may show some symptoms of HF.

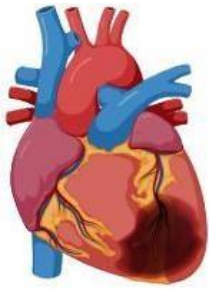


Figure 2: Myocardial infarction (From *Biorender.com*)

3. Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a genetic disorder characterized by cardiac hypertrophy, unexplained by the loading conditions, a non-dilated left ventricle and a normal or increased ejection fraction *(Marian et al. 2017)*. Cardiac hypertrophy is usually asymmetric *(Figure 3)*, most commonly involving the basal interventricular septum near the aortic valve *(Marian et al. 2017)*. In most patients, HCM has a relatively benign course *(Eriksson et al. 2002, Maron et al. 2015, Maron et al. 2014)*. However, HCM is also an important cause of sudden cardiac death, especially in adolescents and young adults *(Maron et al. 2009, Maron et al. 1996, Bagnall et al. 2016, Christiaans et al. 2010, O'Mahony et al. 2014)*. Unsupported ventricular tachycardia, syncope, a family history of sudden cardiac death, and severe cardiac hypertrophy are the main risk factors for sudden cardiac death. This complication can usually be avoided by implanting a cardioverter-defibrillator in appropriate high-risk patients *(Elliott et al. 2000, Spirito et al. 2014, Maron et al. 2016)*.

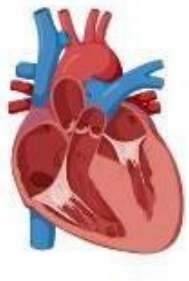


Figure 3: Hypertrophic heart (asymmetrical septal hypertrophy) *(*From *Biorender.com)*

4. Valvular Heart Disease

Valvular heart disease (VHD) is a commonly encountered abnormality in primary care *(Kisling et al. 2023)*. There are many causes of valvular heart disease: congenital, degenerative, infectious, traumatic, and many others. There is a wide variety of valvular heart diseases, and each valve has the ability to develop both regurgitation *(Figure 4)* and stenosis *(Figure 5)* through multiple mechanisms. Stenosis is the stiffening and narrowing of the valves, which reduces blood flow, while valve insufficiency, or regurgitation, occurs when the valves do not close properly and blood flows abnormally back into the heart chambers. In both cases, the heart has to work harder to maintain adequate circulation, eventually leading to enlarged and weakened ventricles *(Kisling et al. 2023)*. Unless corrected by valve repair or replacement, these pathologies are progressive to end-stage heart failure.

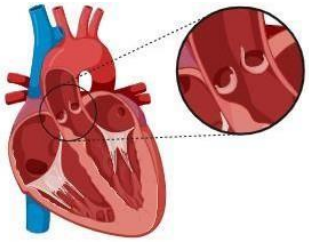


Figure 4: Aortic valve regurgitation
(From *Biorender.com)*

Figure 5: Aortic valve stenosis
(From *Biorender.com)*

5. Congenital Heart Disease

Congenital heart diseases (CHD) are the most common congenital malformations *(Bouma et al. 2017, van der Bom et al. 2011)*. CHD includes abnormalities in the structure of the heart present at birth. These defects occur in the fetus while it is developing in utero during pregnancy *(Sun et al. 2015)*. 1 in 100 children have heart defects due to genetic or chromosomal abnormalities, such as Down syndrome. These can include atrial or ventricular septal defects, valvular abnormalities, stenosis, abnormalities of the heart muscle, and a hole within the wall of the heart that causes a defect in blood flow, heart failure, and eventually death. Some of these malformations can be surgically corrected during childhood, whereas others lead to long-term complications such as heart failure *(Bouma et al. 2017, Zomer et al. 2011).* In many cases, heart transplantation is the only option to ensure a good quality of life and long-term survival *(Sun et al. 2015).*

6. Myocarditis

Myocarditis is an inflammatory process *(Figure 6)* of the heart muscle (myocardium), often caused by viral infections, such as Coxsackie B virus, or, less commonly, bacterial, fungal, or parasitic infections *(Kang et al. 2024, Bejiqi et al. 2019)*. The immune response to infection can damage cardiac tissue, impairing its contractile function and, in severe cases, leading to acute or chronic heart failure *(Bejiqi et al. 2019)*. While many cases of myocarditis resolve spontaneously or with medical therapy, severe forms can progress to irreversible cardiac damage, necessitating heart transplantation *(Kang et al. 2024)*.

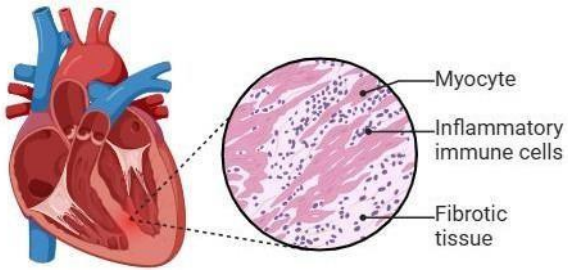


Figure 6: Myocardial inflammation (From *Biorender.com)*

7. Hypertensive Heart Disease

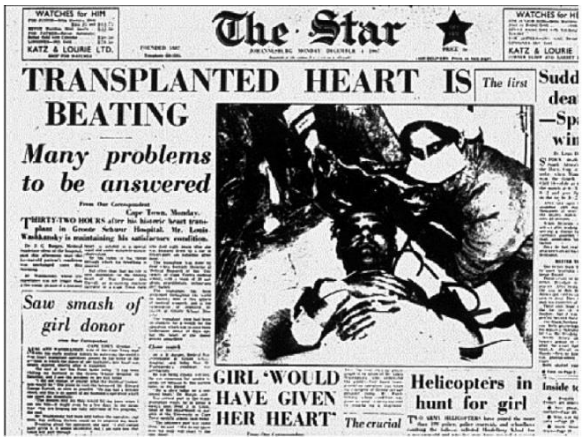
Hypertensive heart disease is the term applied to abnormalities of the heart-involving the structure and function of the left ventricle, left atrium, and intramural coronary arteries due to sustained elevated blood pressure *(Masenga et al. 2023, Drazner et al. 2011).* Left ventricular hypertrophy (LVH) is one of the earliest manifestations of hypertensive heart disease and is believed to be a compensatory mechanism to minimize increased ventricular wall stress and an intermediate pathological alteration in the progression of hypertensive heart disease *(Masenga et al. 2023, Dumitrescu et al. 2021).* However, LVH can progress to complications that include heart failure, arrhythmias, sudden cardiac arrest, ischemic stroke, end-stage renal disease (ESRD), and death *(Boner et al. 2005, Liao et al. 1995, Kim et al. 2018).*

Heart Transplantation

Regardless of the underlying cardiac pathology, the evolution toward end-stage heart failure involves symptoms such as dyspnea, extreme fatigue, peripheral edema, and reduced ability to perform daily physical activities *(Yancy et al. 2013, Metra et al. 1995, Braunwald et al. 2012).*

For patients in whom pharmacological treatments and mechanical support devices, including implantable defibrillators or ventricular assist devices, are ineffective and can no longer support adequate cardiac function, heart transplantation is a true lifeline and remains the only definitive treatment option, allowing restoration of an acceptable quality of life and significantly improved long-term survival rates *(Yancy et al. 2013, Lakhani et al. 2024, Vieira et al. 2020).*

The first human-to-human heart transplant was performed in 1967 by South African surgeon Dr. Christiaan Barnard *(Cooper et al. 2018)*. This pioneering procedure, which was performed on a patient who had terminal heart failure, was widely publicized *(Figure 7)* and inaugurated a new era in transplant medicine *(Cooper et al. 2018)*. The surgical endeavor was indeed successful, but the biggest medical challenge emerged in terms of immune rejection following transplantation. The patient died just 18 days later owing to complications from immunosuppression. In the following decades, the development of more effective immunosuppressive drugs, such as cyclosporine introduced in the 1980s, radically changed the field, with a significant increase in survival rates *(Novitzky et al. 1984, van Veldhuisen et al. 1984).* Nowadays, heart transplantation is an established procedure for treating end-stage heart failure *(Bounader et al. 2024)*.


The Star

TRANSPLANTED HEART IS BEATING

The first

Many problems to be answered

Saw smash of girl donor

GIRL 'WOULD HAVE GIVEN HER HEART'

The crucial

Helicopters in hunt for girl

Figure 7: The front page of a South African newspaper on the day following the first heart transplant (From *Cooper et al. 2018)*

Thousands of patients around the world undergo heart transplants every year, and their post-operative survival rates have been significantly improved compared to those in the early experimental years *(Nesseler et al. 2023)*. Current data suggest that more than 82% of patients survive the first-year post-transplant, while about 69% achieve five-year survival *(McCartney et al. 2017)*. A number of transplant recipients with a donated heart may live on for decades and return to near-normal quality of life.

However, despite continuous improvement in surgical techniques and drug treatments, transplant rejection is still a major complication *(Yamani et al. 2000)*. Rejection can occur in an acute form, within the first few months after transplantation, or in a chronic form, which can lead to irreversible organ dysfunction.

Lifelong noninvasive rejection monitoring in HT patients is a critical clinical need still poorly met in adults and even more for children and infants. Organ-transplant patients require life-long immunosuppression that must be controlled carefully to balance risk of allograft rejection and loss with immunosuppression-induced risks of infection, cancer, and other diseases *(Agbor-Enoh et al. 2021)*. In HT patients, this balance has been monitored through diagnostic modalities such as assessments of clinical symptomatology, viral loads and other microbiological indicators, immunosuppressive drug and procalcitonin blood levels,

echocardiography, cardiac magnetic resonance imaging, noninvasive measurements of levels of circulating donor-specific antibodies and cardiac derived proteins such as troponin and B-type natriuretic peptide hormone (NT-proBNP). Currently, monitoring of rejection relies mainly on surveillance or symptom-prompted application of endomyocardial biopsy (EMB) with or without concurrent coronary angiography, an invasive procedure that involves extracting small tissue samples from the transplanted heart for histological examination *(Holzhauser et al. 2023)*. This procedure is considered the "gold standard" for assessing cardiac allograft acute cellular rejection (ACR) and antibody-mediated rejection (AMR) due to its direct histological visualization of myocardial and/or intravascular inflammatory infiltration and cellular injury *(Agbor-Enoh et al. 2018, Agboe-Enoh et al. 2017, Agbor-Enoh et al. 2021)*, but it is associated with several limitations, including the risk of complications such as perforation, arrhythmias, and infection *(Farcas et al. 2024)*. In addition, biopsy provides only "retrospective" information; in other words, rejection is diagnosed when it is already in an active phase, thus limiting the possibility of preventing irreversible damage *(Iyer et al. 2011)*.

Classification of HT Rejection types

Over the past 50 years since the introduction of cardiac transplantation as a treatment, numerous systems have been used to classify the degree of rejection phenomena observed in recipients. It was only in 1990 that the ISHLT (*International Society for Heart and Lung Transplantation*) proposed a standardized classification for organ rejection, which remained in use for 15 years. In 2004, this classification was replaced by a revised and updated version, also developed by the ISHLT *(Stewart et al. 2005).*

A schematic comparison between these two classifications is provided in *Table 1* below.

1990 ISHLT Classification		**2004 ISHLT Classification**	
Grade 0	No ACR	**Grade 0**	No ACR
Grade 1A	Focal/ mild ACR Focal perivascular and/or interstitial infiltrate without myocyte damage	**Grade 1R**	Mild/low-grade ACR Interstitial and/or perivascular infiltrate with up to one focus of myocyte damage
Grade 1B	Diffuse, mild ACR Diffuse infiltrate without myocyte damage		
Grade 2	Focal, moderate ACR A focus of infiltrate with associated myocyte damage		
Grade 3A	Multifocal, moderate ACR A focus of infiltrate with associated myocyte damage	**Grade 2R**	Moderate/intermediate ACR Two or more foci of infiltrate with associated myocyte damage

Grade 3B	Diffuse, moderate ACR Diffuse infiltrate with myocyte damage	**Grade 3R**	Severe/high-grade ACR Diffuse infiltrate with multifocal myocyte damage, ± oedema, ± haemorrhage, ± vasculitis
Grade 4	Severe ACR Diffuse and polymorphous infiltrate with extensive myocyte damage, ± oedema, ± haemorrhage, ± vasculitis		

Table 1: Comparison between the ISHLT classifications of 1990 and 2004 *(Farcas et al. 2024)*. The ISHLT 2004 classification for ACR (acute cell-mediated rejection) is simplified compared to the previous one. Specifically, the number of rejection grades has been reduced to make it easier for pathologists to assign them to samples, following a progression from mild to severe rejection.

To increase the accuracy of rejection diagnosis, each endomyocardial biopsy requires a systematic evaluation of the endocardium and myocardium, as well as the interstitial tissue and intramural vessels *(Farcas et al. 2024).*

In the myocardium, cardiomyocytes are typically elongated, branched cells with a central nucleus, often containing external lipofuscin. The cardiomyocytes are connected to one another by junctions known as intercalated discs. Pathological changes, observed with hematoxylin-eosin staining, include hypertrophy with nuclear polyploidy, often seen in hypertrophic myocytes, atrophy, cytoplasmic vacuolization, myocytolysis, coagulative necrosis, and disorganization of cellular architecture *(Farcas et al. 2024)*.

The interstitial tissue provides information about the cellular components (fibroblasts, histiocytes, smooth muscle cells, myofibroblasts, mast cells, and adipocytes) and inflammatory infiltrates (lymphocytes, eosinophils, and neutrophils). Immunophenotyping can be performed if it is necessary to characterize the lymphocyte population. Suspicion of a post-transplant lymphoproliferative disorder or lymphoma should arise in the presence of evident morphological variation *(Farcas et al. 2024).*

Intramural vessels undergo changes during episodes of acute cell-mediated rejection and antibody-mediated rejection, as well as during chronic rejection in the form of vasculopathy. In antibody-mediated rejection, histopathological changes appear in the capillary bed, including endothelial growth and denudation, the presence of macrophages or neutrophils in the capillaries, interstitial edema, congestion, and hemorrhage. Fibrin is often found in the capillary bed as well. Immunopathological examination includes deposits of IgG, IgM, or IgA and positive staining for complement cascade components. Specific staining methods, such as immunohistochemical staining for CD31, CD34, CD45, CD68, and C4d, are among the most useful tools for diagnosing rejection *(Berry et al. 2013).*

Two essential components of the immune response are involved in rejection: the cellular component, which includes the action of lymphocytes, macrophages, mast cells, antigen-presenting cells (APCs), eosinophils, basophils, and neutrophils; and the molecular component, which includes antigens, the major histocompatibility complex (MHC), cytokines, adhesion molecules, receptors, enzymes, immunoglobulins, and complement with all its components *(Farcas et al. 2024).*

The major histocompatibility complex is encoded by more than 200 genes located on the short arm of chromosome 6, characterized by known polymorphisms and high allelic variability. This leads to highly divergent responses in the context of rejection phenomena. MHC antigens are divided into different classes: Class I, which includes the HLA-A, HLA-B, and HLA-C subgroups; Class II, which includes the DR, DQ, and DP subgroups, usually found in APCs and endothelial cells; and Class III, which includes molecules involved in humoral immunity. In heart transplantation and, more generally, in organ transplantation, MHC Class I and Class II antigens play a crucial role *(Söderlund et al. 2015, Ludhwani et al. 2024)*. Essentially, rejection occurs due to an incompatibility between the donor's and the recipient's MHC amino acids, so even the variability of a single amino acid can trigger a rejection response *(Farcas et al. 2024).*

The immune response is initiated when even a small percentage of T lymphocytes exhibit alloreactivity, meaning activation, proliferation, and differentiation in response to interaction

with cells from another individual. This reactivity is triggered by the recognition of a polymorphism different from the MHC molecules of the individual *(Janeway et al. 2001)*.

Based on the expected timing of onset, rejection can be classified into three types: hyperacute, which occurs within the first hours after transplantation, leading to graft dysfunction and most often to graft loss within 24 hours; acute, which is further divided into acute cell- mediated rejection (ACR) and antibody-mediated rejection (AMR); and chronic, also known as cardiac allograft vasculopathy (CAV) *(Farcas et al. 2024)*.

Hyperacute rejection is characterized by vascular congestion, thrombosis, and hyaline microthrombi, vasculitis with fibrinoid necrosis of small and medium vessels, edema, and interstitial inflammatory infiltrates of granulocytes. This condition occurs when preformed antibodies (PRA) against the donor's antigens are present, and it more frequently affects patients who have received multiple blood transfusions and women with multiple pregnancies *(Kobashigawa et al. 2009)*.

Acute-cellular rejection (ACR), or T-cell mediated rejection, is the primary target of maintenance immunosuppressive therapy. It presents as lymphocytic infiltration in the perivascular and interstitial compartments of the myocardium, leading to myocyte damage, necrosis, altered myocardial architecture, and graft dysfunction, causing 9% of deaths between the first and third year post-transplantation *(Labarrere et al. 2012)*. The highest frequency of ACR occurs within the first 3-6 months after transplantation. There are two distinct pathways for the recognition of alloantigens, or antigens foreign to the recipient:

1. **Direct pathway**, where T cells "directly" recognize intact non-self MHC molecules present on the donor's cell surfaces.
2. **Indirect pathway**, which involves the ability of T lymphocytes to recognize MHC molecules from the donor that are processed by antigen-presenting cells (APCs). The MHC molecules located on the APC surface present peptides in their grooves, which interact with the T-cell receptor (TCR). CD8+ T lymphocytes are responsible for recognizing class I peptide/MHC complexes, while CD4+ lymphocytes are involved in recognizing class II peptide/MHC complexes.

In addition to MHC molecules, there are also minor histocompatibility antigens that can trigger a CD4 or CD8-mediated lymphocytic response *(Ingulli 2010).* In this way, T lymphocytes activate an inflammatory response against the graft, leading to myocyte necrosis and graft failure *(Tan et al. 2007).*

Histopathological analysis of ACR rejection typically shows an inflammatory infiltrate *(Figure 8)*, predominantly consisting of lymphocytes, macrophages, and, in some cases, eosinophils. The presence of neutrophils suggests a different process, such as an infection, healing from ischemic injury, or antibody-mediated rejection (AMR) *(Farcas et al. 2024)*.

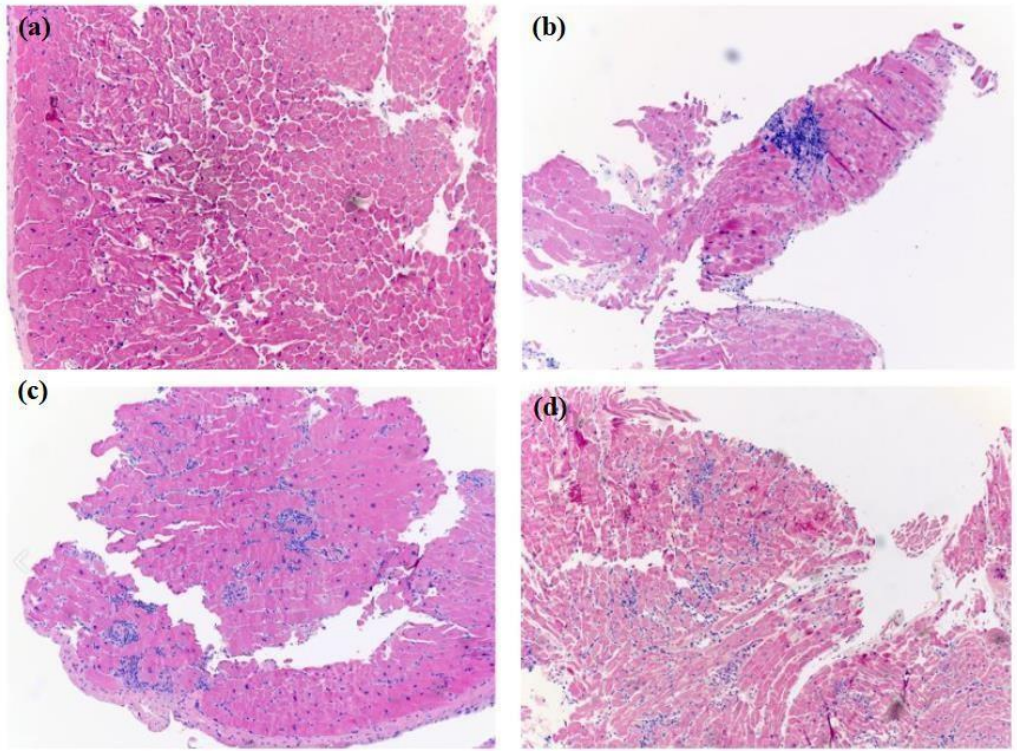


Figure 8: **Microscopic (histologic) images of ACR (a)** Grade 0R: normal myocardium and unremarkable endocardium; note the absence of perivascular or interstitial cellular infiltration and minimal myocyte damage. **(b)** Grade 1R: lymphocytic infiltrate is present within both the perivascular and interstitial compartments. Although the normal myocardial architecture has been disrupted, there is minimal myocyte damage; some myocyte splitting and branching can be observed in the central portion of the slide. **(c)** Grade 2R: in addition to a mild, more diffuse interstitial inflammatory infiltrate, there is a large, dense focus of inflammatory cells in the upper portion of the slide associated with multiple foci of myocyte damage; surrounding myocytes are hypereosinophilic and some have scalloped borders or pyknotic nuclei. **(d)** Grade 3R: diffuse inflammatory infiltrate is present throughout the sample associated with confluent multifocal myocyte injury and contraction band necrosis; myocardial edema (myocyte separation), interstitial hemorrhage (erythrocyte extravasation) and occasional neutrophils are present, particularly in the upper and central portions of the slide (Modified from *Musick et al. 2023).*

Antibody-mediated rejection (AMR), also known as humoral rejection or B-cell mediated rejection, was first described for heart transplantation in the late 1980s, and soon thereafter, pathological evidence supported the notion of a distinct rejection process, independent from the cellular mechanisms described for ACR *(Hammond et al. 1989)*. Active B lymphocytes present in the plasma produce antibodies specific to various antigens, such as proteins expressed on the surface of a transplanted heart. This immune response, driven by antibodies, damages the transplanted organ through complement cascade activation, compromising the organ and acting as a biochemical "amplifier" by interacting with other components of both the innate and adaptive immune responses, such as neutrophils, pro-inflammatory molecules, and cytokines *(Farcas et al. 2024).*

There are several risk factors for the development of AMR: blood transfusions or administration of blood products, pregnancies, repeated transplants, and, specifically for heart transplantation, the use of extracorporeal and intracorporeal devices for mechanical circulatory support *(Reed et al. 2006)*. Other potential risk factors for AMR development include female sex, high preformed antibodies (elevated cPRA values), CMV (cytomegalovirus) seropositivity, and a positive crossmatch between the donor and recipient *(Farcas et al. 2024).*

Clinical signs and symptoms that suggest acute rejection include dyspnea, edema, fatigue, nausea, fever, and arrhythmias *(Farcas et al. 2024)*. Typical histopathological changes *(Figure 9*) in AMR primarily affect the capillary bed, such as endothelial swelling and denudation, the presence of macrophages or neutrophils in the capillaries, interstitial edema, congestion, and, in severe cases, hemorrhage; fibrin may also be detected in the vessel bed in some cases. Immunopathological findings include deposits of IgG, IgM, or IgA and positive staining for complement cascade components *(Farcas et al. 2024).* The standardized nomenclature for the diagnosis of AMR according to the ISHLT is provided in *Table 2*.

Grade	Description	Histopathological results
pAMR0	Absence of pathological AMR	Negative histological and immunopathological studies
pAMR1 (H+)	Histological AMR only	Histological findings present and negative immunopathological studies
pAMR1 (I+)	Immunopathological AMR only	Negative histological results and positive immunopathological studies
pAMR2	Pathological AMR	Present both histological and immunopathological findings
pAMR3	Severe pathological AMR	Histological findings of interstitial haemorrhage, capillary fragmentation, mixed inflammatory infiltrates, pycnosis of endothelial cells and/or caryopses and marked oedema

Table 2: The 2013 ISHLT Classification for the pathological diagnosis of AMR *(Stewart et al. 2005)* identifies five grades of AMR (antibody-mediated rejection) based on histological and immunopathological analysis of endomyocardial biopsy samples. Stage pAMR0 corresponds to the absence of all the rejection signs mentioned above, while stage pAMR3, the highest grade, indicates the presence of all pathological signs of damage caused by a humoral immune response.

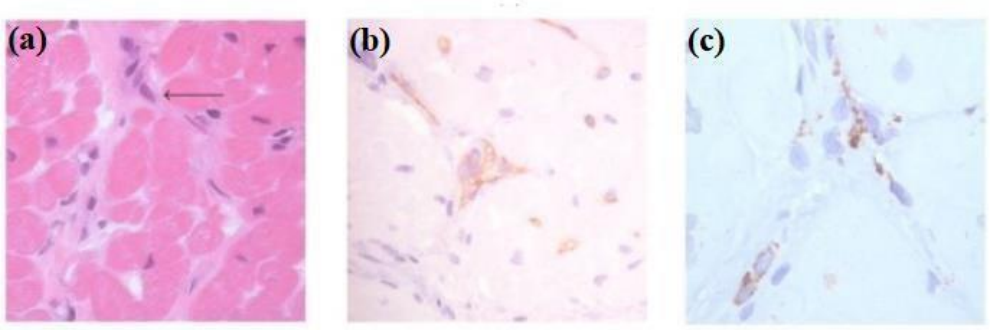


Figure 9: Examples of **histopathologic and immunopathologic biopsy findings of AMR** in heart transplantation. **(a)** A biopsy sample stained with hematoxylin and eosin shows evidence of endothelial cell swelling (arrow). **(b)** An immunoperoxidase stain shows diffuse C4d deposition in capillaries. **(c)** An immunoperoxidase stain confirms the presence of intravascular macrophages with positive staining for CD68 *(*Modified from *Chih et al. 2012).*

Significant rejection has been defined as **ACR grade ≥2R** and **AMR grade ≥pAMR1**, according to the International Society for Heart and Lung Transplantation classification.

Table 3 reports the principal differences between ACR and AMR.

Feature	ACR	AMR
Mechanism	T-cell-mediated	Antibody-mediated
Histological Features	Lymphocytic infiltrates	Microvascular injury, capillary endothelial swelling
Diagnostic Tools	EMB, histology	EMB, immunohistochemistry, DSA testing
Frequency	More common in the first year	Can occur at any time, often later post-transplant
Treatment	Intensified immunosuppression (corticosteroids, T-cell inhibitors)	Plasmapheresis, IVIG, rituximab, or complement inhibitors

Table 3: Key differences between ACR and AMR.

Both ACR and AMR require timely and appropriate management to preserve graft function and improve patient outcomes. The identification of non-invasive biomarkers is a promising avenue for enhancing the monitoring and classification of these rejection forms.

Limitations of Endomyocardial Biopsy: is its end near?

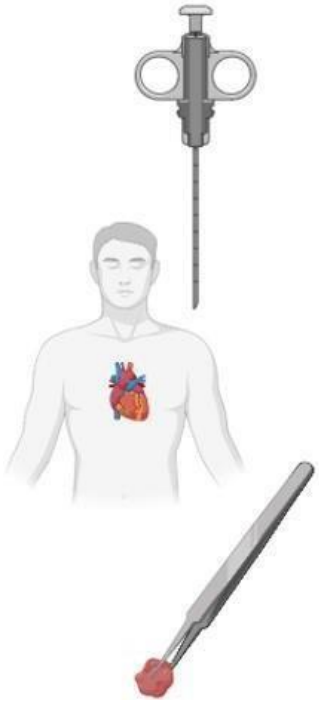


Figure 10: Endomyocardial biopsy (From *Biorender.com*)

EMB is the current gold standard for diagnosing rejection in heart transplant recipients, but it has significant limitations that raise the need for alternative monitoring strategies *(Holzhauser et al. 2023)*. First and foremost, EMB is an invasive procedure that involves the insertion of a catheter through a vein, typically the jugular, femoral or brachial veins, to extract small samples of myocardial tissue *(Figure 10)* from the transplanted heart *(Porcari et al. 2023, Schulz et al. 2015, Scaufele et al. 2015)*. This carries inherent risks, including vascular injury, arrhythmias, bleeding, infections, and - rarely - cardiac perforation *(Porcari et al. 2023, Yilmaz et al. 2020, Chimenti et al. 2013)*. Additionally, the procedure can cause discomfort and anxiety to patients, especially in pediatric recipients of EMB, for whom sedation or anesthesia may be necessary. Another major limitation of EMB is its spatial sampling bias *(Baandrup et al. 1982)*.

Small samples may not be representative of the whole myocardium, thus missing foci of localized or patchy rejection *(Baandrup et al. 1982)*. This is especially limited in AMR, which very often presents diffuse microvascular injury that may not be evident from biopsy. Moreover, the diagnostic value of EMB is highly dependent on the expertise of the pathologist interpreting the histological findings, further contributing to variability in interpretation *(Karameh et al. 2024, Sinagra et al. 2021).*

Logistically, EMB represents a resource-intensive procedure requiring specialized equipment, trained personnel, and often hospitalization, being less accessible in resource-constrained settings. The rather high frequency of biopsies, especially in the first-year post-transplant when the risk for rejection is highest, represents an additional significant burden that health care systems and the patients themselves have to bear. Furthermore, EMB reflects graft health retrospectively; thus, it cannot identify graft rejection before the development of histological changes. Rather, it is performed well after an active graft injury has been initiated *(Strecker et al. 2013)*. All the above considerations call for an increasing demand for a non-invasive, reliable, sensitive alternative to EMB in order to provide an intensive and dynamic monitoring of graft health.

Emerging non-invasive diagnostic techniques may challenge EMB gold standard monopoly on rejection monitoring. Of these, dd-cfDNA analysis has emerged as a highly promising modality *(Holzhauser et al. 2023)*. High levels of dd-cfDNA have shown to be associated with both ACR and AMR, offering a less invasive and possibly more sensitive method of diagnosing graft rejection *(Holzhauser et al. 2023)*.

Gene expression profiling tools, such as AlloMap, which monitors immune activation through gene expression patterns, also exist, although their sensitivity for AMR is still poor *(Agbor-Enoh et al. 2021, Deng et al. 2006, Pham et al. 2020, Kobashigawa et al. 2015, Crespo-Leiro et al. 2016).*

Advanced imaging modalities, such as cardiac magnetic resonance, provide supplementary detection of edema and fibrosis related to rejection for further reduction of EMB. The

combination of different techonologies may increase sensitivity and specificity, allowing comprehensive and less invasive surveillance of rejection.

Therefore, while non-invasive diagnostic tests remain far from fully replacing endomyocardial biopsy at this time, they surely represent a major step towards improving patient safety, comfort, and outcomes. All three aforementioned areas will be refined, normalized, and integrated into clinical service in future research. Consequently, the "end of endomyocardial biopsy," with all its foreseen promises for a new era in personalization and minimal invasiveness in post-transplantation care, may well prove to be within reach because of these progresses from endomyocardial biopsy alone, in heart transplant recipients *(Holzhauser et al. 2023)*.

Innovation of liquid biopsy and its potential application in post-transplant management

Despite advances in surgical techniques and immunosuppressive therapies, transplant recipients remain at risk of transplant rejection, a major cause of morbidity and mortality in these patients.

Although modern immunosuppressive regimens have reduced the frequency and severity of rejection episodes, monitoring remains a cornerstone of transplant care to detect rejection early and guide therapeutic adjustments *(Pilch et al. 2021)*.

Several studies have demonstrated the potential of dd-cfDNA analysis to detect rejection earlier than traditional methods, such as EMB or echocardiographic findings *(Keller et al. 2021)*. The ability to quantify dd-cfDNA provides clinicians with a dynamic, **real-time measurement of graft health**, enabling earlier therapeutic interventions to prevent irreversible graft damage *(Grskovic et al. 2016)*. Importantly, cfDNA analysis is non-invasive, requiring only a blood sample, which improves patient compliance and allows for more frequent monitoring compared to EMB *(Stewart et al. 2018)*.

The advent of **next-generation sequencing (NGS)** has revolutionized the field of molecular diagnostics by providing a highly sensitive and accurate platform for analyzing cfDNA *(Satam et al. 2023*). Using NGS, cfDNA can be differentiated into donor and recipient fractions through the analysis of **single nucleotide polymorphisms (SNPs)**, genetic markers that are unique to each individual *(Jackson et al. 2021, Snyder et al. 2011)*. The **CareDx AlloSeq cfDNA kit**, employed in this study, utilizes 202 SNPs to accurately quantify dd-cfDNA, offering a robust tool for monitoring graft health. This technology has the potential to not only identify rejection episodes but also to shed light on the molecular mechanisms of graft injury and recovery.

The new frontiers are addressed to evaluate the clinical utility of cfDNA analysis using NGS technology for monitoring rejection in heart transplant recipients, both adult and pediatric. Specifically, the focus is on correlating dd-cfDNA levels with episodes of ACR and AMR, thereby validating its potential as a **non-invasive alternative** to EMB. By analyzing the sensitivity, specificity, and predictive value of cfDNA for detecting rejection, this research seeks to establish its role as a reliable biomarker for routine post-transplant monitoring.

Beyond its diagnostic potential, cfDNA analysis offers the possibility of **individualized patient care** *(Sorbini et al. 2024)*. Unlike EMB, which provides a static histological assessment, cfDNA levels can be monitored longitudinally, providing insights into the progression or resolution of graft injury over time *(Sorbini et al. 2024, Gristina et al. 2022)*. This personalized approach aligns with the broader shift in medicine toward **precision healthcare**, where treatments and monitoring strategies are tailored to the unique needs of each patient.

The findings of this study are expected to have significant clinical implications. By reducing the reliance on EMB, cfDNA analysis could improve patient safety, enhance monitoring frequency, and optimize resource utilization in transplant care. Additionally, the early detection of rejection enabled by cfDNA might lead to **timelier therapeutic interventions**, improving both graft survival and patient outcomes *(Huang et al. 2019)*. However, the transition from experimental to routine clinical use requires further standardization of cfDNA testing protocols and thresholds, as well as validation across diverse patient populations.

Ultimately, this research seeks to advance the field of heart transplantation by providing a foundation for integrating **cfDNA-based liquid biopsies** into standard post-transplant care. By combining cutting-edge NGS technology with biomarker-driven monitoring, the goal is to pave the way for a less invasive, more effective approach to managing heart transplant recipients *(Biénkowski et al. 2020)*.

Cell-Free DNA (cfDNA): Definition and Applications

cfDNA generally refers to a DNA fragment with a double helix in the blood circulation *(Hu et al. 2021, Rolfo et al 2020)*, whose length is between 160− 180bp, and mainly around 167bp *(Hu et al. 2021, Jiang et al. 2015)*. This indicated that cfDNA is likely to be associated with nucleosomes, because the length of DNA wrapped around nucleosome (147bp) plus the length of linker DNA (20-50bp) nearly corresponded with cfDNA length *(Hu et al. 2021)*.

The release of cfDNA into blood flow circulation occurs through two main pathways:

1. **Apoptosis and Necrosis:** During cell death, nuclear DNA is cleaved into fragments and released into the bloodstream. Apoptotic cells produce smaller, regularly sized fragments, while necrotic cells release more variable and larger fragments
2. **Active Secretion:** Several cells, including tumor cells and cells under stress, actively release DNA into extracellular spaces.

Experiments have shown that the half-life of cfDNA in the circulation is between 4 and 30 minutes *(Hu et al. 2021, Bryzgunova et al. 2006)*, which means cfDNA analysis can be used to monitor disease in real time.

In healthy individuals, the concentration of cfDNA has been estimated at approximately 1− 10 ng/ml plasma *(Hu et al. 2021, Yao et al. 2016).* This concentration could be higher in patients with cancers *(Zill et al. 2018)* or other pathological conditions, such as inflammation *(Swarup et al. 2007)*, and autoimmunity *(Timmermans et al. 2016)*. In addition, age, sex, ethnicity and physiological parameters might also influence cfDNA concentration *(Cohen et al. 2018, Hummel et al. 2018).*

While the origins of cfDNA in mammals can derive from different and complex biological mechanisms, the detection of donor-derived cfDNA (dd-cfDNA) fraction is a sophisticated, non-invasive, and reliable temporal indicator of ongoing selective injury to the donor organ due to its short plasma half-life and the preponderance of donor-specific versus recipient-specific cfDNA generated from cellular apoptosis in transplant patients. In the context of transplantation, donor-derived cell-free DNA (dd-cfDNA) has gained significant attention as a potential biomarker for organ rejection.

The quantification and analysis of dd-cfDNA provide insights into the degree of cellular injury and turnover within the allograft, reflecting the dynamic interplay between the immune system and the transplanted organ.

Studies have demonstrated a correlation between elevated dd-cfDNA levels and the presence of acute cellular rejection, antibody-mediated rejection, and cardiac allograft vasculopathy *(Holzhauser et al. 2021)*. Key factors influencing dd-cfDNA levels include allograft rejection, ischemia-reperfusion injury, and other pathological processes affecting the transplanted heart. Mehlman et al. *(Mehlman et al. 2023)*, used a molecular microscopy approach in heart transplant management, underscoring the potential of dd-cfDNA as a noninvasive monitoring tool. This approach is based on molecular analysis to identify early changes in the heart tissue, facilitating timely diagnosis and more personalized treatment. It uses techniques such as genetic and proteomic profiling to detect cellular and molecular changes in the transplanted heart.

Similarly, Holzhauser et al. *(Holzhauser et al. 2023)*, provided practical guidance for noninvasive rejection surveillance, highlighting the role of dd-cfDNA in enhancing diagnostic accuracy and patient care *(Bohmer et al. 2023, Deshpande et al. 2022).*

The clinical applications of dd-cfDNA extend beyond rejection diagnosis to encompass prognostic, therapeutic monitoring, and risk stratification *(Henricksen et al. 2023, Kewcharoen et al. 2022*).

Moreover, advanced molecular diagnostic tools such as the Molecular Microscope Diagnostic System, have integrated dd-cfDNA analysis with other molecular markers to

provide a comprehensive view of graft health, demonstrating the real-world application of cfDNA in clinical settings, show casing its feasibility and effectiveness in routine transplant care *(Alam et al. 2022).*

dd-cfDNA can be particularly useful in detecting AMR, a form of rejection where antibody-mediated mechanisms target the graft vasculature. AMR is a significant cause of graft loss post-heart transplantation. It occurs when the recipient's immune system generates antibodies against the donor heart, leading to vascular injury and impaired graft function. Detecting AMR early is crucial as it can be asymptomatic and may not show immediate clinical signs *(Keller et al. 2021).*

Traditional biopsy methods can miss AMR due to sampling errors or the patchy nature of the lesion, whereas dd-cfDNA provides a more comprehensive assessment by reflecting the overall state of the graft.

Several studies investigated the role of circulating biomarkers, as microRNAs, alongside dd-cfDNA, to differentiate between cellular and antibody-mediated rejection, supporting the combined use of these biomarkers for more precise and accurate rejection diagnosis and detection of late manifestations of alloantibody-associated injury, underscoring its role in ongoing surveillance to prevent chronic rejection *(Agbor-Enoh et al. 2017, Shah et al. 2022).*

dd-cfDNA testing still faces challenges and limitations *(Knuttgen et al. 2022)*. Factors such as transplant-related variables, comorbidities, and concomitant infections can influence dd-cfDNA levels, leading to potential false-positive or false-negative results *(Verhoeven et al. 2022).*

Furthermore, the cost-effectiveness of dd-cfDNA testing remains a topic of debate *(Holzhauser et al. 2021)*. While initial studies suggest potential cost savings due to reduced EMB utilization and early rejection detection, comprehensive economic evaluations are needed to justify widespread adoption. Factors such as assay cost, reimbursement policies, and long-term outcomes must be considered in assessing the value of dd-cfDNA testing *(Alam et al. 2024, Feingold et al. 2023, Khush et al. 2019).*

Another area of interest is the impact of demographic factors on dd-cfDNA dynamics *(Shah et al. 2024).* Racial disparities in dd-cfDNA levels have been observed, with implications for transplant outcomes and personalized surveillance strategies, understanding the underlying mechanisms driving these differences is crucial for equitable post-transplant care *(DeFilippis et al. 2024).*

Moreover, the influence of viral infections, such as cytomegalovirus (CMV), on dd-cfDNA testing warrants investigation: active CMV infection has been shown to affect dd-cfDNA levels, potentially confounding rejection surveillance. Strategies to mitigate this interference, such as adjusting cfDNA thresholds or incorporating additional biomarkers, need to be explored *(Alam et al. 2024).*

dd-cfDNA testing represents a promising approach to non-invasive surveillance for heart transplant rejection; its ability to provide real-time information on allograft health, coupled with its minimally invasive nature, positions it as a valuable tool in the post-transplant management. Despite its promise, several challenges must be addressed before dd-cfDNA can be integrated into routine clinical practice. These include standardization of sample collection and processing protocols, validation of analytical platforms, and establishment of clinically relevant thresholds for rejection detection. Moreover, the influence of confounding factors such as comorbidities, medications, and surgical interventions on dd-cfDNA levels necessitates further investigation.

Nowadays, data regarding the clinical application of this method in a real-life setting are lacking. It was discovered that heart transplant patients have more dd-cfDNA than controls, albeit in variable percentages. For this reason, our study evaluated the presence of dd-cfDNA as an index of rejection by massively sequencing specific genomic polymorphisms. Moreover, we wanted to evaluate the correlation among cardiac hemodynamic parameters, ACR, AMR, and dd-cfDNA levels in a real-life European cohort of recipients of heart transplantation.

Pediatric Heart Transplant

Pediatric heart transplantation has additional and unique aspects from standard pediatric heart surgery and adult heart transplantation. The first pediatric cardiac transplant was performed on a newborn on December 6th, 1967, by Dr. Adrian Kantrowitz, three days after the first heart transplant on an adult *(Barnes et al. 2021, Bailey et al. 2011, Kantrowitz et al. 1968).* Unfortunately, the child died 612 hours post operatively. It was recognized that to have long term success, there was a need for effective immunosuppression. With the emergence of Cyclosporin in the 1970s, a new era began. Pediatric heart transplantation is a specialized patient population that has unique preoperative, intraoperative and postoperative aspects that continue to evolve over time. With constantly evolving testing, support options and medications, the future holds many promising options for this fragile patient group *(Barnes et al. 2021).*

Heart transplantation in children is a critical intervention for those suffering from severe heart conditions that cannot be managed through conventional medical or surgical treatments. Children may require a heart transplant for various reasons, including:

- Congenital heart disease: complex structural heart defects present at birth that cannot be corrected through surgery.
- Cardiomyopathy: diseases of the heart muscle that lead to decreased cardiac function.
- Life-threatening arrhythmias: persistent abnormal heart rhythms that pose significant health risks.

The decision to proceed with transplantation is based on the severity of the child's condition and their overall prognosis without the transplant. Pediatric heart transplantation accounts for approximately 14% of all heart transplants performed globally, reflecting its importance in this patient population *(Schweiger et al. 2015).*

Before being placed on the transplant list, children undergo extensive evaluations, including blood tests and imaging studies, to assess their suitability for transplantation. Factors such as age, weight, blood type, and overall health are considered *(Dipchand et al. 2018).*

Once listed, children may wait anytime from weeks to months for a suitable donor heart. The waiting time can vary significantly based on several factors, including the urgency of the case and donor availability. The mortality rate on the waiting list for pediatric patients is close to 25%, but thanks to the increasing use of ventricular assist devices, this has been reduced to 17% *(Power et al. 2024).*

The surgery typically lasts between four to six hours and involves several key steps. After surgery, patients are closely monitored in an intensive care unit (ICU) for any complications, including rejection of the new heart. Children will require lifelong follow-up care and immunosuppressive medications to prevent rejection *(Dipchand et al. 2018)*.

The prognosis for pediatric heart transplant recipients has improved significantly over the years. Current data indicate that more than 85% of children survive at least five years post-transplant. Infants tend to have the best long-term survival rates, with median survival times exceeding 20 years in some cases *(Dipchand et al. 2020)*.

2. Aim of the study

The primary objective of this study is to explore the effectiveness of dd-cfDNA as a non-invasive biomarker for monitoring acute cellular rejection (ACR) and antibody-mediated rejection (AMR) in heart transplant recipients. This research encompasses both adult and pediatric populations, aiming to provide a comprehensive evaluation of dd-cfDNA's utility across diverse age groups.

Additionally, the research aims to identify specific threshold values for donor-derived cfDNA (dd-cfDNA) that are indicative of ACR and AMR. Establishing these thresholds could lead to standardized protocols that enhance patient management and improve overall outcomes for heart transplant recipients.

To achieve these goals, the study will differentiate between donor-derived cfDNA and recipient-derived cfDNA. The analysis will focus on 202 single nucleotide polymorphisms (SNPs) selected using the AlloSeq cfDNA kit from CareDx, specifically designed for transplant rejection monitoring. These SNPs will serve as biomarkers to distinguish the donor's cfDNA from that of the recipient, offering a clear measure of immune activity and rejection status.

3. Materials and Methods

Ethics Statement

This study was evaluated by the Central Emilia Wide Area Ethical Committee of the Emilia-Romagna Region (CE-AVEC) who was assigned the following number: **79/2014/U/Sper**. The CE-AVEC approved the consent for data processing and all other patient information.

To participate in the study, each patient provided written informed consent and consent for the processing of their personal data. All documents and data collected were handled in accordance with current regulations and Good Clinical Practice (GCP) guidelines. Data collected in the Clinical Research Form (CRF) were processed in a pseudonymized manner, with subjects identified by a unique number/code.

Adult heart transplant patients were recruited from the Heart Failure and Transplant Unit at IRCCS Azienda Ospedaliero-Universitaria di Bologna (IRCCS AOUBO) in Italy, as part of the CLIN-HEART project. This prospective study was evaluated by the Central Emilia Wide Area Ethical Committee of the Emilia-Romagna Region (CE-AVEC). Heart transplant patients enrolled in CLIN-HEART project were classified into one of the following categories: A) Patients undergoing follow-up biopsies according to the protocol used at our center (monitoring group); B) Patients exhibiting clinical signs or symptoms of graft dysfunction (positive control group); C) Patients who have been immunologically stable for over 5 years, with normal graft function, no coronary artery disease, and on low doses of anti-rejection therapy (negative control group).

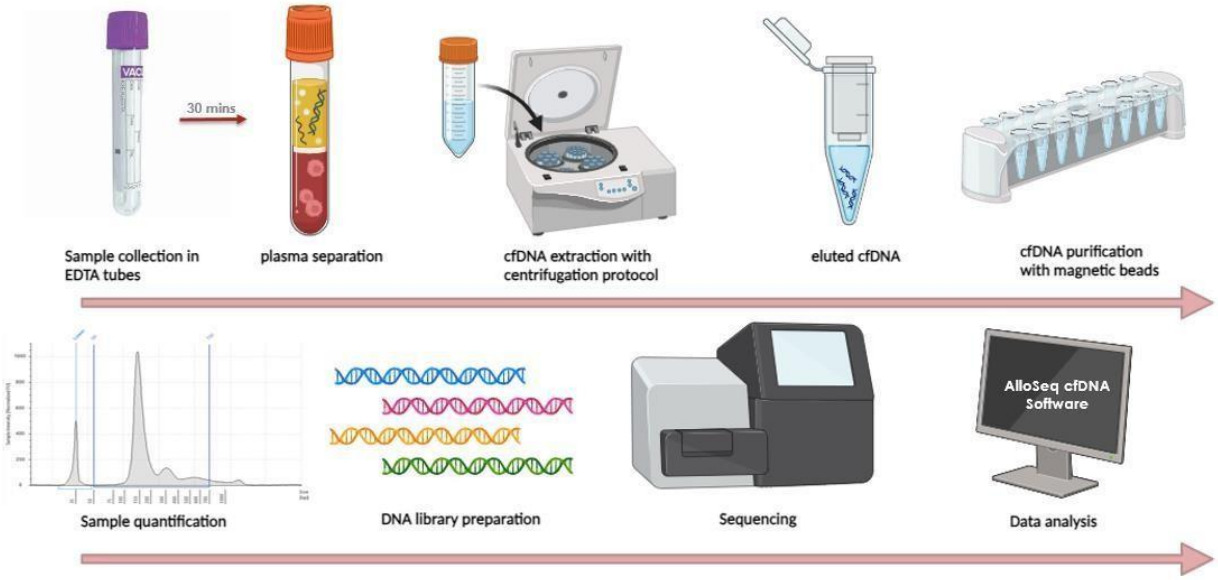


Figure 11: Experimental workflow (From *Biorender.com*)

Blood Draw Processing

Figure 11 shows the experimental workflow, which we examine in more detail below. Blood draw is conducted alongside myocardial biopsies, following a scheduled anti-rejection protocol or in response to patient-reported symptoms indicative of potential rejection. This dual approach ensures that cfDNA levels are monitored both as a preventive measure and as a response to clinical symptoms, increasing the chances of early detection of graft rejection.

To prevent blood coagulation, samples are collected in **EDTA tubes**, which help maintain the integrity of cfDNA. Once collected, the sample is transferred to a 15 ml Falcon tube and centrifuged at 5000 xg for 10 minutes at 4 °C. This step is essential for isolating cellular components into a pellet at the tube bottom, leaving a layer of plasma on top, which may appear yellow-orange due to slight hemolysis. This color can indicate early blood cell breakdown, which, if extensive, might interfere with cfDNA analysis. Careful handling and processing are required to avoid additional hemolysis that could compromise cfDNA quality and potentially introduce cellular DNA contaminants into the plasma. The plasma layer is then carefully separated and stored in 2 ml Nunc Cryovial tubes (ThermoScientific, Waltham), which are suitable for freezing at -80 °C. Processing the sample within 30 minutes

of collection is essential to prevent cfDNA degradation, as it has a half-life of approximately 30 minutes. This rapid processing helps maintain cfDNA quality for accurate downstream analysis, reflecting early biological changes associated with tissue damage or rejection.

An alternative to EDTA tubes is the use of specialized cfDNA-stabilizing tubes, such as Streck Cell-Free DNA BCT® (Streck, La Vista, NE) and PAXgene Blood ccfDNA Tubes (Qiagen, Hilden). These stabilizing tubes contain preservative agents that inhibit enzymatic degradation of cfDNA, significantly prolonging sample integrity at room temperature and allowing for extended handling and transport times without the need for immediate centrifugation or freezing. Streck Tubes, in particular, can stabilize cfDNA for up to seven days at room temperature, providing a practical solution in clinical and research settings where immediate sample processing is challenging. Additionally, the Streck Tubes' formulation minimizes cellular lysis, reducing the risk of cellular DNA contamination in the plasma and thereby improving the specificity of cfDNA measurements. Using these stabilizing tubes not only facilitates flexible clinical workflows but also ensures that high-quality cfDNA can be consistently obtained, even when samples are collected in decentralized locations or need to be transported to central laboratories for analysis. This increased flexibility and reliability can improve the applicability of cfDNA as a biomarker in real-world settings, helping to make cfDNA analysis a feasible alternative to invasive biopsy methods for transplant rejection monitoring.

cfDNA Extraction

The extraction of cfDNA from plasma is performed using the *Quick-cfDNA Serum & Plasma Kit* (Zymo Research, Orange). This kit provides all necessary reagents and columns for the protocol, ensuring a streamlined and reproducible process.

The following steps illustrate the protocol:

1. **Plasma Aliquoting**: The plasma is transferred from the Cryovial tube into 1.5 ml Eppendorf tubes, aliquoting approximately 1 ml per tube to ensure manageable sample volumes.

2. **Digestion Buffer Addition**: S&P 5X Digestion Buffer is added according to the proportions listed in *Table 4*, ensuring an addition of 50 µl for every 200 µl of sample.

Sample Volume	S&P 5X Digestion Buffer
200 µl	50 µl
1 ml	250 µl
3 ml	750 µl
5 ml	1.25 ml

Table 4: addition of Digestion Buffer

3. **Proteinase K Addition**: Proteinase K is added according to the specified ratio of 20 µl per 200 µl of plasma, and mix thoroughly, as indicated in *Table 5*.

Sample Volume	Proteinase K
200 µl	20 µl
1 ml	100 µl
3 ml	300 µl
5 ml	500 µl

Table 5: addition of Proteinase K

4. **Incubation**: Samples are incubated at 55 °C for 30 minutes to facilitate enzymatic digestion of proteins and other cellular materials, freeing the cfDNA from protein complexes.
5. **Binding Buffer Addition**: The S&P DNA Binding Buffer is added at the ratio indicated in *Table 6*:

Sample Volume	S&P DNA Binding Buffer
200 µl	540 µl
1 ml	2.7 ml
3 ml	8.1 ml
5 ml	13.5 ml

Table 6: addition of DNA Binding Buffer

6. **Column Preparation**: The Reservoir is attached to the Zymo-Spin III-S Column and placed in a 50 ml Falcon tube. 10 ml of the sample are transfered to the column and are centrifuge at 1000 xg for 10 minutes. The flow-through is discarded and the centrifugation is repeated, if necessary, until the entire sample has passed through the column.
7. **Washing Steps**:
    a. The reservoir is removed and the column is transferred to a clean Collection Tube.
    b. 400 µl of S&P DNA Prep Buffer are added to the column and centrifuge at ≥ 10,000 xg for 30 seconds, then, the flow-through is discarded.
    c. 700 µl of S&P DNA Wash Buffer are added and centrifuge at ≥ 10,000 xg for 30 seconds, then, the flow-through is discarded.
    d. 400 µl of S&P DNA Wash Buffer are added and centrifuge at maximum speed for 1 minute.
    e. A final dry spin is performed at maximum speed for 1 minute to remove any residual wash buffer.
8. **Elution**: The column is transferred to a new 1.5 ml microcentrifuge tube. 50 µl of Elution Buffer are carefully added directly onto the column matrix without puncturing it. After incubation of the column at room temperature for 3 minutes, the column is centrifuged at maximum speed for 30 seconds to elute the cfDNA.

    At the end of the extraction process, a final volume of 50 µl of eluted cfDNA is obtained, containing the total cfDNA extracted from plasma. This sample can be stored at -80 °C for long-term storage or can be used directly for the next analytical steps.

cfDNA Purification

The extracted cfDNA sample requires further purification. Although the initial kit provides a relatively high extraction quality, residual genomic DNA may still be present, which could interfere with sequencing results. This step is essential for purifying the cfDNA from genomic DNA, which typically consists of fragments ranging from **150 to 170 base pairs** in length.

The purification process uses magnetic beads that selectively bind to DNA. These beads are stored at +4 °C and should be balanced at room temperature for about 30 minutes prior to use. Proper resuspension of the beads is also essential for efficient binding. The purification protocol is as follows:

1. **Addition of AMPure XP Beads**: 30 µl of AMPure XP Beads (Beckman Coulter, Brea) are added to the purified cfDNA sample (80 µl). The sample is vortexed and incubated for 10 minutes at room temperature to allow cfDNA binding to the beads.
2. **Initial Magnetic Separation**: The microtubes are placed on a magnetic rack and incubated for an additional 5 minutes. During this time, the magnetic beads will migrate to one side of the tube, attracted by the magnet. The supernatant is carefully transferred to a new microtube, leaving behind the beads, which contain potential contaminant genomic DNA.
3. **Second Beads Addition**: 160 µl of AMPure XP Beads are added to the supernatant, vortexed to mix, and incubated again for 5 minutes at room temperature.
4. **Secondary Magnetic Separation**: The microtubes are placed back on the magnetic rack for 3 minutes. The beads will once again attach to the side of the tube, allowing to discard the supernatant carefully.
5. **Ethanol Washes**:
    a. 500 µl of freshly prepared 70% ethanol are added to the microtube to wash the beads. The microbead is rotated 180 degrees on the magnetic rack, then returned to its original position once the microbeads have moved to the opposite side.
    b. When the beads realign on the tube wall, the supernatant is carefully discarded.
    c. The ethanol wash is repeated with another 500 µl of 70% ethanol.
    d. Residual ethanol is allowed to air dry for a few minutes to ensure that no ethanol residue remains that could interfere with downstream processes.
6. **Elution of Purified cfDNA**:
    a. 30 µl of Resuspension Buffer (Illumina, San Diego), which is compatible with NGS sequencing, are added to the dried beads, vortexed and incubated for 2 minutes at room temperature.

b. The microtubes are placed back on the magnetic rack and waited for the microspheres to separate from the supernatant.
c. The supernatant, which now contains the purified cfDNA, is carefully transferred into new microtubes for storage at -80 °C until sequencing.

This purification step minimizes the presence of genomic DNA and other contaminants, improving the quality of the cfDNA sample for subsequent sequencing analysis.

Quantification of cfDNA using Qubit HS dsDNA and Agilent Bioanalyzer 2100

After the cfDNA has been purified, it is essential to quantify and assess the quality of the extracted sample before proceeding with sequencing. Accurate quantification ensures that the cfDNA concentration is suitable for downstream applications, while the quality check helps to confirm the presence of cfDNA-specific fragment sizes and the absence of genomic DNA contamination.

- **Quantification with Qubit High Sensitivity (HS) dsDNA Assay**:

The Qubit 4 Fluorometer (Thermo Fisher Scientific) with the Qubit High Sensitivity dsDNA Assay Kit is used for accurate quantification of low concentration cfDNA. This assay is highly sensitive and specifically designed for double-stranded DNA, making it ideal for cfDNA quantification.

To perform the assay, 2 µl of the cfDNA sample is mixed with the Qubit working solution, which contains fluorescent dyes that bind selectively to double-stranded DNA. After a short incubation period (approximately 2 minutes), the fluorescence intensity is measured using the Qubit Fluorometer, and the instrument calculates the DNA concentration in ng/µl. This result allows for precise control over the amount of cfDNA used in sequencing or other applications.

The kit, which must be stored at **+4 °C**, is balanced to room temperature for about **30 minutes** before use. The kit includes:

- **dsDNA HS Reagent**, containing a fluorescent dye that binds to double-stranded DNA (dsDNA).
- **dsDNA HS Buffer**, used to prepare the working solution.
- **dsDNA HS Standards #1 and #2**, for calibration.

1. **Preparation of the Working Solution**:
    a. **199 µl of Buffer** are combined with **1 µl of Reagent** per sample.
    b. The mix is prepared for the number of samples plus one (to account for standards and excess).
2. **Preparation of Standards and Samples**:
    a. For the standards: **190 µl of the working solution** are mixed with **10 µl of Standards #1 and #2**.
    b. For the samples: **198 µl of the working solution** are mixed with **2 µl of each sample**.
3. **Measurement**:
    a. The solutions are vortexed and incubated in the dark for **2 minutes**.
    b. The standards are measured first to generate a calibration curve, followed by the unknown samples. The concentration is displayed in **ng/µl**.

- **Quality Analysis with Agilent Bioanalyzer 2100**:

The Agilent Bioanalyzer 2100, using the High Sensitivity DNA Kit, provides a detailed assessment of cfDNA fragment sizes and overall quality.

In this assay, 1 µl of the cfDNA sample is loaded into the Bioanalyzer, which applies electrophoretic separation to visualize the DNA fragment distribution.

The Bioanalyzer generates an electropherogram, displaying cfDNA fragment peaks within the expected range of 150-170 bp. This range is characteristic of cfDNA and reflects its origin

from apoptotic cells. The presence of a sharp peak at this size indicates a high-quality cfDNA sample with minimal contamination from larger, genomic DNA fragments.

If additional peaks are present, especially at larger fragment sizes (e.g., >500 bp), this may indicate genomic DNA contamination, which could interfere with accurate sequencing results. In such cases, an additional purification step may be necessary to remove these contaminants.

Using both Qubit and Bioanalyzer allows for a dual approach to quality control: Qubit quantifies total cfDNA concentration, while the Bioanalyzer provides visual confirmation of fragment size distribution. This combined method ensures that only high-quality, correctly sized cfDNA is used for subsequent analysis, increasing the reliability and sensitivity of cfDNA-based diagnostics.

Amplification of SNPs via multiplex PCR for barcoding using the AlloSeq cfDNA Kit

The **AlloSeq cfDNA kit** (CareDx) is a cutting-edge solution for non-invasive monitoring of allograft rejection in transplant patients. It leverages the analysis of donor-derived cell-free DNA (dd-cfDNA) through a highly multiplexed PCR approach targeting **202 specific SNPs**. These SNPs were carefully selected based on their elevated minor allele frequency (MAF) in many populations and minimal interference from other genomic regions, making them ideal markers for differentiating between donor and recipient cfDNA.

This process combines amplification, barcoding, and library preparation.

Input cfDNA

The workflow requires high-quality **purified cfDNA** for a total of **10 ng** of cfDNA, for optimal results. We onbserved that samples with a quantity lower than 10 ng were not sequenced adequately and thus were excluded from the subsequent analysis. The concentration and quality of the input DNA are verified beforehand using Qubit and the

Agilent Bioanalyzer 2100 to ensure suitability for amplification. This step is critical because the success of multiplex PCR depends on the quality and integrity of the cfDNA. Samples with insufficient cfDNA or those contaminated with genomic DNA may result in amplification failure or low-quality libraries.

Multiplex PCR Amplification

The central feature of the AlloSeq cfDNA workflow is its **multiplex PCR**, which amplifies **202 SNPs** simultaneously in a single reaction.

To prepare the libraries for sequencing, **24 samples** are loaded onto a 96-well PCR plate. Among these 24 wells, 1-2 wells are usually reserved for control samples of known dd-cfDNA fraction. These controls consist of previously sequenced samples and are used to verify the success of the sequencing run. Each sample must be uniquely labeled with **two indexes** (a combination of indexes from two distinct sets), to enable sample-specific identification during sequencing data analysis. These indexes, provided in the kit, are strategically paired to ensure no overlap among samples.

Indexing Setup

The indices used are from two sets:

- **A500 series**: A501, A504, A505, A508
- **A700 series**: A701, A702, A703, A704, A706, A710

For each well, **4 µl of two different indices** are added:

- One index from the A500 series
- One index from the A700 series

This dual-index system ensures that each sample on the plate is tagged with a **unique combination** for downstream bioinformatics processing.

Preparation of the PCR Reaction

The PCR reaction is prepared in each well using the following components, provided in the AlloSeq cfDNA kit:

PCR Master Mix Composition (per sample)

13 µl of AlloSeq cfDNA PCR Mix

0.8 µl of AlloSeq cfDNA PCR Enzyme

2.2 µl of AlloSeq cfDNA SNP Primer Pool

A Master Mix is prepared for all samples to ensure consistency and minimize pipetting errors. For each well:

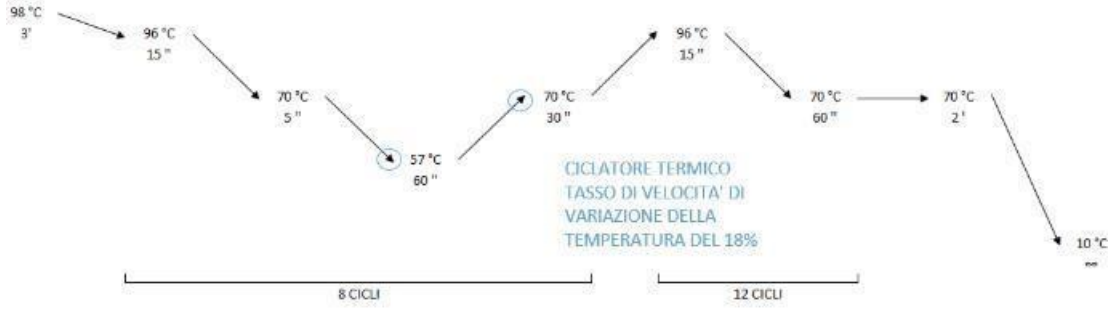
- **16 µl of Master Mix** is aliquoted.
- **16 µl of previously diluted cfDNA sample** is added.

Plate Setup

- The PCR plate is sealed with a **microseal** (Eppendorf, Hamburg).
- The plate is vortexed for 5 seconds to ensure proper mixing of reagents.
- A brief centrifugation is performed for 30 seconds at 1000 xg to remove bubbles and ensure all reagents are at the bottom of the wells.

Thermocycling Parameters

The PCR plate is then placed in a thermal cycler, and the following program is used:



Upon completion of the PCR run, the amplified products are ready for further purification and quality control steps.

Library preparation and clean up

Following the SNP amplification through multiplex PCR, the next step involves pooling the amplified samples for sequencing. To create the pool, **5 µl of each amplified sample from the 24 wells** are combined, resulting in a final volume of **120 µl**. This pooled volume contains all indexed libraries, ensuring the inclusion of every sample for sequencing.

Purification of the Pooled Libraries

To remove excess reagents from the previous PCR reaction, the pooled libraries undergo a further purification step using magnetic beads, as previously described. This step is crucial to eliminate PCR contaminants.

1. **Addition of Beads**:
    a. 100 µl of Purification Beads are added to the pooled sample.
2. **Incubation**:
    a. The pooled sample is vortexed for 5 seconds and incubated at room temperature for 5 minutes.
3. **Magnetic Separation**:
    a. The microtube is placed on the magnetic rack and incubated for 5 minutes.
    b. The supernatant is carefully removed, avoiding disturbance of the beads.
4. **Ethanol Wash**:
    a. 1 mL of 80% ethanol is added and then removed after 30 seconds.
    b. The ethanol wash step is repeated twice.
5. **Drying**:
    a. The residual ethanol is allowed to evaporate completely at room temperature.
6. **Resuspension**:
    a. 125 µl of AlloSeq Resuspension Buffer are added to the beads, vortexed for 5 seconds and incubated at room temperature for 5 minutes.
7. **Magnetic Separation**:
    a. The tube is placed back on the magnetic rack and incubated for 5 minutes.

b. 120 µl of the supernatant are transferred into a new microtube.

8. **Repeat Purification**:
    a. A second round of purification is performed using the same protocol.
    b. During this second purification, the beads are resuspended in 35 µl of AlloSeq Resuspension Buffer.
    c. At the end, 32 µl of the final supernatant are transferred into a new microtube, ensuring no beads are carried over.

These purification steps ensure that the pooled libraries are free from residual reagents and contaminants, providing clean DNA suitable for high-quality sequencing.

Pool quantification, denaturation and dilution

Once the pooled library has been purified, it must be prepared for loading onto the sequencing platform. This involves **quantification**, **dilution**, and **denaturation** of the library to ensure that the DNA concentration and structure are optimal for sequencing.

Prior to dilution and denaturation, the concentration of the pooled library is measured using the **Qubit dsDNA High Sensitivity Assay Kit**. This kit is the same kit described above.

- **4 µl of the pooled library** are used for duplicate measurements to ensure accuracy.
- The average of the two readings is calculated to determine the final concentration.
- The concentration in ng/µl is converted to **nM** using the following formula:

$$\text{concentration in nM} = \frac{\text{average Qubit concentrations in } \frac{ng}{\mu L}}{660\frac{g}{mol} * \text{average library size in bp}} \cdot 1000$$

660 g/mol *181,917 bp/ 1000 = 120,065

660 is a factor derived from the relationship between the molecular weight in g/mol and the conversion between weight and concentration units (ng and nM).

To prepare the DNA for sequencing, the double-stranded DNA (dsDNA) is denatured into single strands using freshly prepared **0.2 N NaOH**.

1. **Preparation of 0.2 N NaOH**:
    a. 4 µl of 2 N NaOH stock solution (stored frozen) is diluted with 36 µl of nuclease-free water.
2. **Denaturation Protocol**:
    a. Equal volumes of the diluted library (e.g., 10 µl of 4 nM library) and 0.2 N NaOH are combined.
    b. The mixture is incubated at room temperature for 5 minutes to denature the DNA.

Illumina sequencing requires the final pool concentration to be **20 pM**. The final preparation includes:

- A volume of the denatured pool ensuring a 20 pM concentration in a total volume of 1 ml.
- 5 µl of 0.2 N NaOH to stabilize the denatured DNA.
- HT1 Reagent (Hybridization Buffer) to dilute the library to the final volume of 1 ml.

This final preparation ensures the library is at the optimal concentration for sequencing, producing the right number of clusters for high-quality data and adequate SNP coverage.

Loading the Cartridge for Sequencing by Synthesis (SBS)

The final step in library preparation involves loading the library onto the **MiSeq Illumina NGS sequencer at high coverage (>1000X)** *(Figure 12)*. This process relies on Sequencing by Synthesis (SBS), a method where fluorescently labeled nucleotides are incorporated and detected in real time. The sequencing occurs on a solid substrate (flow cell Kit V3 150 Cycles PE), where clusters of identical DNA molecules are generated by bridge-PCR prior to the sequencing itself.

Monitoring and Output

- During sequencing, data on base calling, cluster density, and overall run performance are displayed in real-time.
- The run typically takes 24–48 hours depending on the platform and read length.
- Upon completion, the raw sequencing data (.bcl files) are processed into FASTQ format for downstream analysis.

All experiments reported here were performed on a MiSeq machine.

Data Output through the AlloSeq cfDNA Software

At the end of sequencing, raw data (fastq files) are demultiplexed using the corresponding indices and are used for subsequent analysis using the AlloSeq software, which estimated the percentage of dd-cfDNA in each sample. A template with the id code of the sample, corresponding index sequences and sequencing machine used is provided by the user alongside the fastq files.

Results are exported in **Excel format** after the analysis. Specifically, the data obtained with this analysis re the following:

- **dd-cfDNA quantification (%),** calculated as the ratio between donor cfDNA and recipient cfDNA using the 202 SNPs.
- Parameters such as **coverage** and **uniformity** are indicated, to ensure diagnostic accuracy, enabling reliable differentiation between donor and recipient SNPs.

Figura 12: MiSeq Illumina sequencer (From *Biorender.com)*

Statistical analysis

Non-parametric Mann-Whitney tests were performed as reported in the corresponding *"Results"* sections and figure legends, using GraphPad Prism software version 8.0.2 (GraphPad). P-values < 0.05 were considered to indicate a statistically significant difference between two groups.

4. Results

During the period from 2015 to 2024 at the Heart Failure and Transplantation Unit of IRCCS Azienda Ospedaliero-Universitaria di Bologna (IRCCS AOUBO), 142 patients, as reported in *Tables 7, 8* and *9* and in *Table 13* and *14* in *"Appendix"*, received an endomyocardial biopsy (EMB) by protocol or by cause. Simultaneously with the EMB, these patients had blood drawn for dd-cfDNA analysis. The protocol followed was as described in *"Materials and Methods"*.

After cfDNA extraction, all the samples obtained were organized in subsequent sequencing run. The majority of sequencing runs were successful, but some samples failed, mainly because of the low DNA input used (in *Appendix*).

Quantification of extracted cfDNA with Qubit HS dsDNA

The following data were entered in *Tables 7, 8* and *9*:

- ID (**Alloseq** and **HT** refer to adult patients, whereas **CardioPed** refers to pediatric ones)
- Date of birth (when known)
- Date of transplant (when known)
- Date of biopsy
- Qubit HS ds DNA (ng/µl)
- Quantity: 10 ng in 16 µl
- The amount of H_20 to be added to reach the volume (16 µl)

It is important to note that this study only includes retrospective patients and samples from the first 4 weeks post-HT were not included because it is known that in the first month post-

transplant, cfDNA levels are significantly higher due to inflammation caused from the transplant itself.

First sequencing run

ID	Date of birth	Date of transplant	Date of biopsy	Concentration Qubit HS ds DNA (ng/μl)	10 ng in 16 μl	H_20
Alloseq1	20/8/1954	4/9/2012	20/2/2017	0.893	11.20	4.80
Alloseq2	2/8/1970	23/10/2012	16/3/2017	0.708	14.12	1.88
Alloseq3	5/7/1957	28/2/2012	24/1/2022	0.666	15.02	0.98
Alloseq4	18/6/1967	7/2/2014	27/8/2015	0.545	18.34	dry
Alloseq5	5/12/1958	27/5/1998	16/4/2018	0.53	18.86	dry
Alloseq6	12/12/1947	14/4/2013	15/3/2018	0.356	28.08	X
Alloseq7	26/10/1955	2/9/2016	15/2/2017	1.66	6.02	9.98
Alloseq8	31/7/1957	24/11/2016	2/3/2017	2.13	4.69	11.31
Alloseq9	28/05/1955	13/2/2016	10/11/2016	0.313	31.94	X
Alloseq10	11/01/1964	16/3/2016	1/12/2016	0.859	11.64	4.36
Alloseq11	9/11/1967	7/12/2016	23/1/2017	0.259	38.61	X
Alloseq12	13/6/1957	4/4/2016	20/10/2016	1.51	6.62	9.38
Alloseq13	1/1/1955	18/6/2015	21/11/2016	0.653	15.31	0.69
Alloseq14	11/9/1976	5/11/2016	10/8/2017	0.66	15.15	0.85
Alloseq15	7/7/1981	16/2/2017	7/8/2017	0.755	13.25	2.75
Alloseq16	9/11/1967	7/12/2016	1/6/2017	3.36	2.98	13.02
Alloseq17	1/9/1951	17/7/2016	11/5/2017	0.983	10.17	5.83
Alloseq18	1/1/1955	18/6/2015	20/7/2017	1.31	7.63	8.37
Alloseq19	9/11/1960	18/5/2017	17/8/2017	4.74	2.12	13.88
Alloseq20	18/10/1960	17/5/2016	8/5/2017	0.634	15.77	0.23
Alloseq21	11/9/1976	5/11/2016	4/5/2017	2.94	3.40	12.60
Alloseq22	27/8/1961	12/12/2016	4/9/2017	2.22	4.50	11.50
Alloseq23	27/10/1955	2/9/2016	31/8/2017	5.47	1.83	14.17
Alloseq24	27/6/1962	2/7/2017	28/12/2017	2.46	4.07	11.93
Alloseq25	19/4/1976	26/9/2017	18/12/2017	3.34	2.99	13.01
Alloseq26	28/5/1951	22/5/2015	7/12/2017	0.849	11.78	4.22
Alloseq27	14/09/1961	16/9/2017	12/3/2018	2.28	4.39	11.61

Alloseq28	27/6/1962	2/7/2017	5/4/2018	1.59	6.29	9.71
Alloseq29	9/6/1975	15/11/2016	21/5/2018	0.556	17.98	dry
Alloseq30	9/11/1960	18/5/2017	17/5/2018	0.99	10.10	5.90
Alloseq31	26/8/1975	13/1/2018	16/4/2018	1.64	6.10	9.90
Alloseq32	22/10/1971	12/2/2017	5/2/2018	0.516	19.37	dry
Alloseq33	5/04/1977	30/3/2016	22/3/2018	1.66	6.02	9.98
Alloseq34	2/12/1954	21/11/2017	24/5/2018	3.44	2.91	13.09
Alloseq35	24/3/1956	6/2/2018	2/5/2018	4.16	2.40	13.60
Alloseq36	10/5/1959	24/5/2017	7/6/2018	0.459	21.78	dry
Alloseq37	13/6/1957	4/4/2016	8/10/2018	0.661	15.13	0.87
Alloseq38	20/3/1958	2/8/2018	12/9/2018	4.09	2.44	13.56
Alloseq39	31/7/1957	24/11/2016	13/9/2018	0.747	13.39	2.61
Alloseq40	18/5/1961	3/3/2015	29/10/2018	0.364	27.47	dry
Alloseq41	17/9/1970	3/10/2016	29/10/2018	1.45	6.90	9.10
Alloseq42	29/3/1984	4/4/2018	22/10/2018	0.793	12.61	3.39
Alloseq43	29/3/1984	4/4/2018	10/1/2019	too low	/	/
Alloseq44	4/8/1956	20/5/2014	19/11/2018	0.501	19.96	dry
Alloseq45	9/11/1967	7/12/2016	14/6/2018	0.378	26.45	dry
Alloseq46	11/9/1976	5/11/2016	25/11/2021	2.68	3.73	12.27

Table 7: Data from 46 HT adult patients. Four samples are highlighted in red because they did not reach a sufficient cfDNA quantity after extraction. From our experience, samples below 10 ng are not included in the sequencing run because they are not properly sequenced. The remaining 42 samples had sufficient quantities of circulating DNA. Subsequently, they were run on the *Agilent 2100 Bioanalyzer* to evaluate their quality, and then, 24 samples (23 samples and 1 control sample) were selected for the first sequencing run.

Second sequencing run

ID	Date of birth	Date of transplant	Date of biopsy	Concentration Qubit HS ds DNA (ng/µl)	10 ng in 16 µl	H_20
Alloseq53	8/12/1955	17/10/2014	5/12/2016	0.853	11.72	4.28
Alloseq54	24/10/1967	28/5/2015	8/7/2015	2.86	3.49	12.51
Alloseq55	21/6/1957	13/12/2016	16/1/2017	2.61	3.83	12.17
Alloseq56	1/9/1951	17/7/2016	12/1/2017	0.527	18.97	dry
Alloseq57	16/4/1982	23/2/2017	22/5/2017	1.86	5.37	10.63
Alloseq58	31/1/1958	9/7/2017	16/8/2017	1.35	7.4	8.6
Alloseq59	9/6/1975	15/11/2016	18/5/2017	0.536	18.65	dry
Alloseq60	22/10/1971	12/2/2017	8/5/2017	0.604	16.55	dry
Alloseq61	22/10/1971	12/2/2017	22/5/2017	0.673	14.85	1.15
Alloseq62	27/8/1961	12/12/2016	15/6/2017	2.15	4.65	11.35
Alloseq64	8/1/1975	18/11/2017	21/12/2017	0.967	10.34	5.66
Alloseq65	16/4/1982	23/2/2017	24/8/2017	0.3	16	/
Alloseq67	2/12/1954	21/11/2017	1/3/2018	0.902	11.08	4.92
Alloseq68	11/1/1964	16/3/2016	22/3/2018	0.456	21.92	dry
Alloseq73	14/7/1972	12/9/2017	18/10/2018	0.838	11.93	4.07
Alloseq74	31/1/1958	9/7/2017	25/6/2018	1.08	9.25	6.75
Alloseq75	5/2/1985	04/3/2018	30/8/2018	1.55	6.45	9.55
Alloseq76	10/5/1959	24/5/2017	22/6/2017	2.28	4.38	11.62
Alloseq77	26/8/1975	13/1/2018	12/2/2018	1.32	7.57	8.43
Alloseq78	4/12/1972	22/2/2018	22/3/2018	0.622	16.07	dry
Alloseq79	5/2/1985	4/3/2018	28/4/2018	3.33	3	13
CardioPed3	/	/	30/3/2022	0.39	25.64	dry
CardioPed4	/	/	12/4/2022	0.143	16	/
CardioPed5	/	/	17/5/2022	0.347	28.82	dry

Table 8: Data from 24 patients. Of these 24 patients, 21 are adult patients (Alloseq53-Alloseq79), while 3 are pediatric ones (CardioPed3-CardioPed5). All samples are found to have sufficient quantifications.

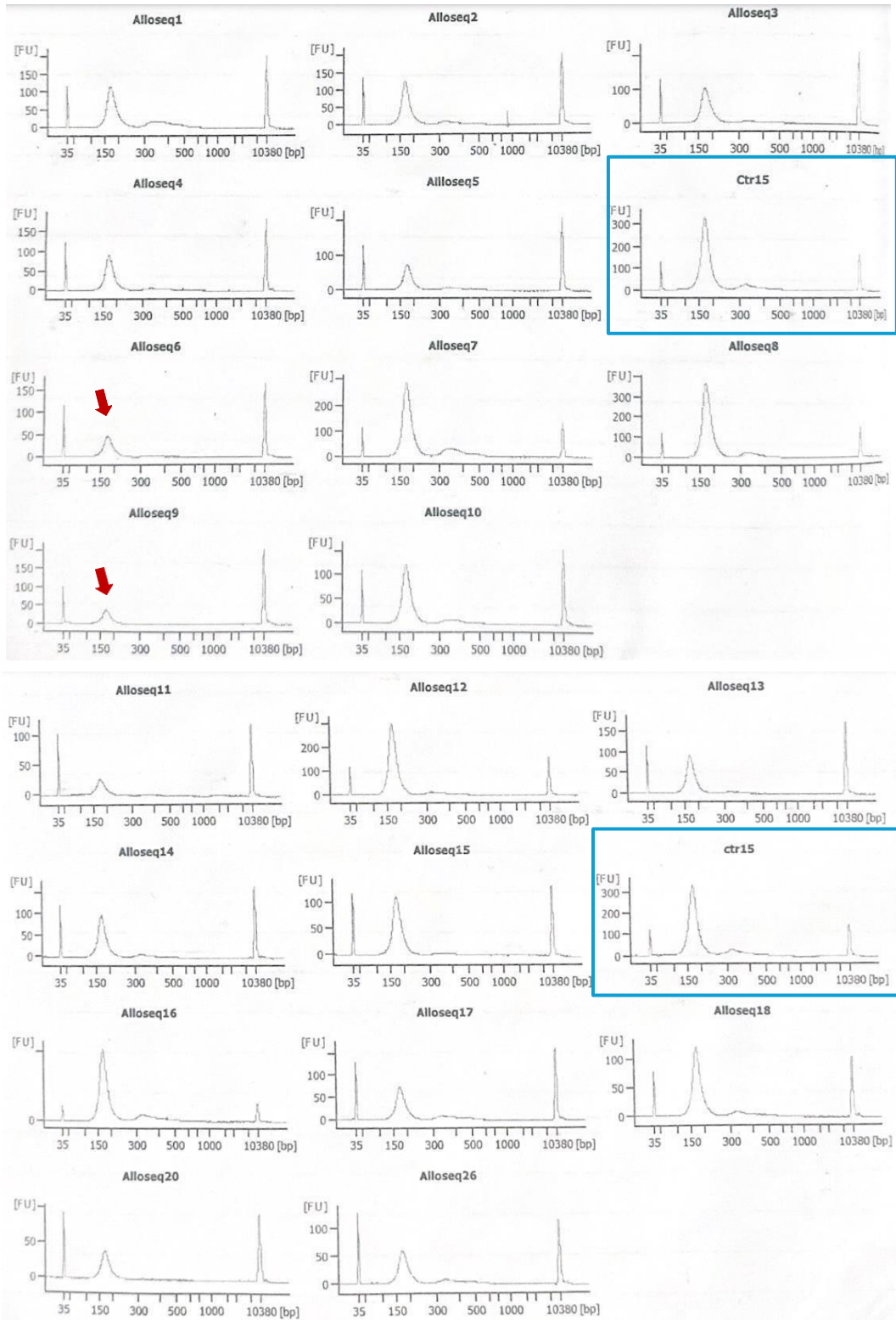
Third sequencing run

ID	Date of birth	Date of biopsy	Concentration Qubit HS ds DNA (ng/µl)	10 ng in 16 µl	H_20
HT1	/	15/4/2024	3.17	3.15	12.85
HT2	/	15/4/2024	1.14	8.77	7.23
HT4	/	17/4/2024	1.06	9.43	6.57
HT6	/	22/4/2024	2.66	3.75	12.25
HT9	/	22/4/2024	0.359	27.8	dry
HT10	/	22/4/2024	1.86	5.37	10.63
HT12	/	24/4/2024	4.50	2.22	13.78
HT16	/	2/5/2024	0.811	21.59	dry
HT17	/	2/5/2024	0.463	25.64	dry
CardioPed2	/	8/3/2022	4.6	2.18	13.82
CardioPed6	31/5/2005	25/5/2022	1.67	5.99	10.01
CardioPed9	14/9/1995	10/11/2022	11.3	0.89	15.11
CardioPed10	15/9/2009	14/11/2022	3.18	3.15	12.85
CardioPed11	3/2/1987	22/11/2022	1.73	5.79	10.21
CardioPed12	27/9/1998	14/11/2022	3.67	2.73	13.27
CardioPed15	20/5/2016	9/1/2023	2.03	4.93	11.07
CardioPed16	23/3/2003	10/2/2023	2.16	4.63	11.37
CardioPed17	19/4/2005	14/2/2023	1.45	6.90	9.10
CardioPed19	2/11/2001	21/3/2023	1.74	5.75	10.25
CardioPed20	15/9/2009	3/5/2023	2.28	4.39	11.61
CardioPed27	2/2/2002	30/1/2024	0.356	28.08	dry
CardioPed29	/	9/4/2024	0.383	26.1	dry
CardioPed30	/	23/4/2024	0.589	16.97	/
Alloseq55	21/6/1957	16/1/2017	3.83	2.61	13.39

Table 9: Data from 24 patients. Of these 24 patients, 9 are adult patients (HT1-HT17), while 14 are pediatric ones (CardioPed2-CardioPed30). Alloseq 55 was used as a positive control sample. All samples are found to have sufficient quantifications.

Qualitative assessment of cfDNA samples with Agilent 2100 Bioanalyzer

As described in *Materials and Methods*, the qualitative assessment of cfDNA samples is performed using *the Agilent 2100 Bioanalyzer*. The primary peak of interest appears around 150 bp, corresponding to the average length of cfDNA fragments. An additional critical feature is the absence of significant contamination by genomic DNA, which is confirmed by the lack of peaks at lengths greater than 150 bp. This method also provides quantitative insights: the area under each peak is directly proportional to the fluorescence emitted by the DNA, making it possible to corroborate the quantitative data obtained with *Qubit*. *Figure 13* shows the electropherograms of the 42 samples included in *Table 7*.


Alloseq1
Alloseq2
Alloseq3
Alloseq4
Alloseq5
Ctr15
Alloseq6
Alloseq7
Alloseq8
Alloseq9
Alloseq10
Alloseq11
Alloseq12
Alloseq13
Alloseq14
Alloseq15
ctr15
Alloseq16
Alloseq17
Alloseq18
Alloseq20
Alloseq26
[FU]
35 150 300 500 1000 10380 [bp]

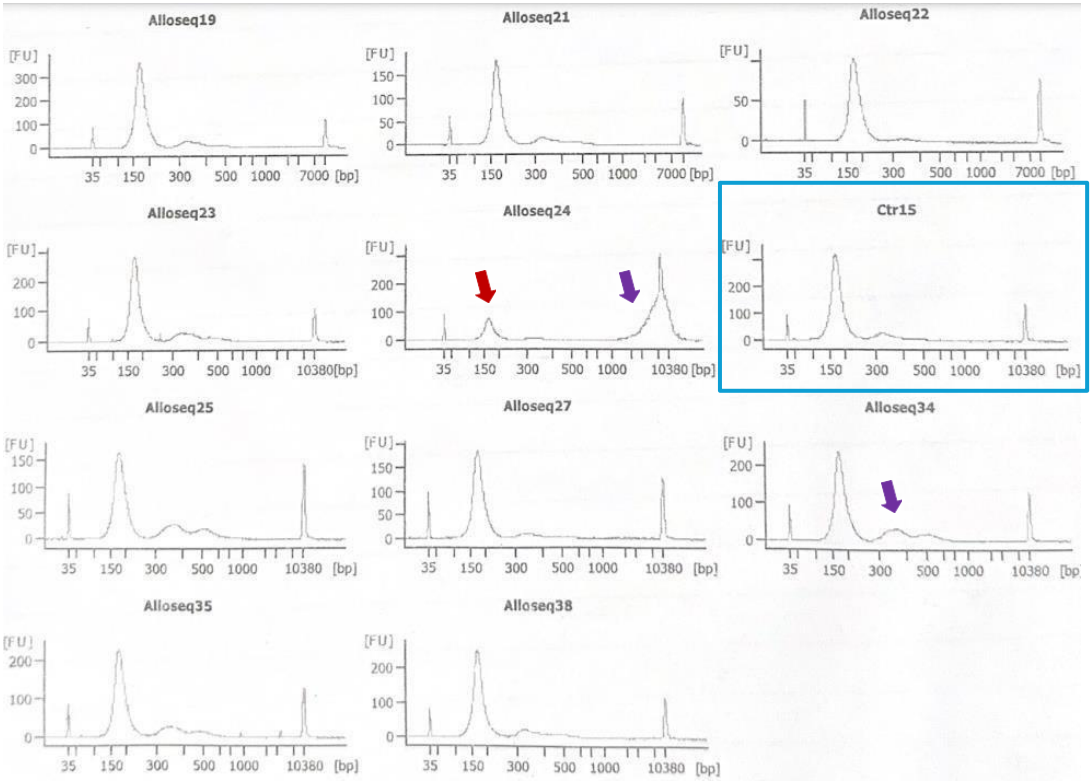


Figure 13: Electropherograms of the extracted and quantified samples for the first sequencing run. As seen in this figure, the electropherograms in blue boxes indicate sample "Ctr15," which represents our positive control. In samples AlloSeq6-9-24, the red arrow indicates a peak with low fluorescence (< of 50), suggesting a low amount of cfDNA. Also, in samples AlloSeq 24 and 34, the purple arrow indicates genomic DNA contamination. This contamination is evident by the presence of peaks at lengths other than 150 bp. For the above reasons, samples 6-9-24 did not pass "quality control" and thus were excluded from the sequencing run.

Plate Layout

As described in *"Materials and Methods"*, each sample must be uniquely labeled with two indices which contain the specific Illumina regions complementary to the sequencing primers (i5 and i7). A combination of indices from two separate sets: A500 series and A700 series also enables sample-specific identification during sequencing data analysis. These indices are strategically paired to ensure that there is no overlap between samples. In *Figure 14*, an example of how the 24 samples included in the first sequencing run were uniquely indexed is shown.

PLATE LAYOUT		01	02	03	04	05	06
		A701	A702	A703	A704	A706	A710
A	A501	Alloseq1	Alloseq2	Alloseq3	Alloseq4	Alloseq5	Alloseq7
B	A504	Alloseq8	Alloseq10	Alloseq12	Alloseq13	Alloseq14	Alloseq15
C	A505	Alloseq16	Alloseq17	Alloseq18	Alloseq19	Alloseq20	Alloseq21
D	A508	Alloseq22	Alloseq23	Alloseq25	Alloseq26	Alloseq19bis	CTR15

Figure 14: Plate layout with the 24 indexed samples that were selected for the first sequencing run.

AlloSeq cfDNA Assay

The AlloSeq cfDNA kit and the AlloSeq cfDNA Software (collectively referred to as AlloSeq cfDNA Assay) is a next generation sequencing (NGS) based assay that measures single-nucleotide polymorphisms (SNPs) to accurately quantify dd-cfDNA (%) in the plasma of solid organ transplant recipients, in this case, cardiac transplant patients.

Contraindications for use are as follows:

- Recipients of a transplant from a monozygotic (identical) twin
- Recipients of multiple transplanted organs from the same donor
- When more than two genomes are present in the recipient plasma (more than recipient + donor), contribution of cfDNA from each genome is not differentiated by the test unless genomic DNA samples from n-1 genomes are provided as reference samples. This includes:
  - Recipients of multiple transplanted organs from different donors
  - Recipients who are pregnant
  - Recipients of a bone marrow / hematopoietic transplant
- The AlloSeq cfDNA assay has been validated to the required measuring intervals of 0 to less than 50% (without recipient or donor genotype) when the minor contributor can be attributed to the donor fraction, and of 0 to 100% (with recipient or donor genotype, in order to successfully distinguish between donor and recipient cell free DNA within a given sample) when the minor contributor cannot be attributed to either fraction. Therefore, testing of a genomic DNA sample (either from donor or recipient, n-1) needs to be included into the AlloSeq Software analysis for accurate quantification of dd-cfDNA values in transplant recipients.
- The AlloSeq cfDNA assay results from recipients with malignancy should be interpreted cautiously, notably for those affecting blood cell counts or blood composition.

AlloSeq cfDNA Assay should NOT:

- Be used within 30 days following blood transfusion that contains white blood cells (washed or leukocyte-depeleted RBCs are acceptable).
- Be performed on patients within 24-48h following a biopsy.

AlloSeq cfDNA Assay results are provided by **AlloSeq cfDNA Software**.

Figure 15, which is divided into 6 sections, shows the workflow of the AlloSeq cfDNA Software:

- **Recipient information** *(Section 1)*

Recipient information includes: date of birth, demographic data, date of blood draw, and date of potential contraindications/ limitations (i.e. pregnancy, transfusion). Transplant relationship must be assigned to each sample before analysis. The accuracy of this information should be verified when interpreting the AlloSeq cfDNA Assay results.

- **Longitudinal graph with AlloSeq Assay results** *(Section 2)*

Serial AlloSeq cfDNA results are plotted on the longitudinal graph to assist with evaluating patient results over time. Summary graph together with other laboratory test results is possible (i.e. DSA MFI value, serum creatinine levels, etc.).

- **Track recipient timeline including treatment events** *(Section 3)*

The specimen collection date for the current result (day, month, and year), are shown together with other relevant clinical events.

- **Current AlloSeq cfDNA Assay result** *(Section 4)*

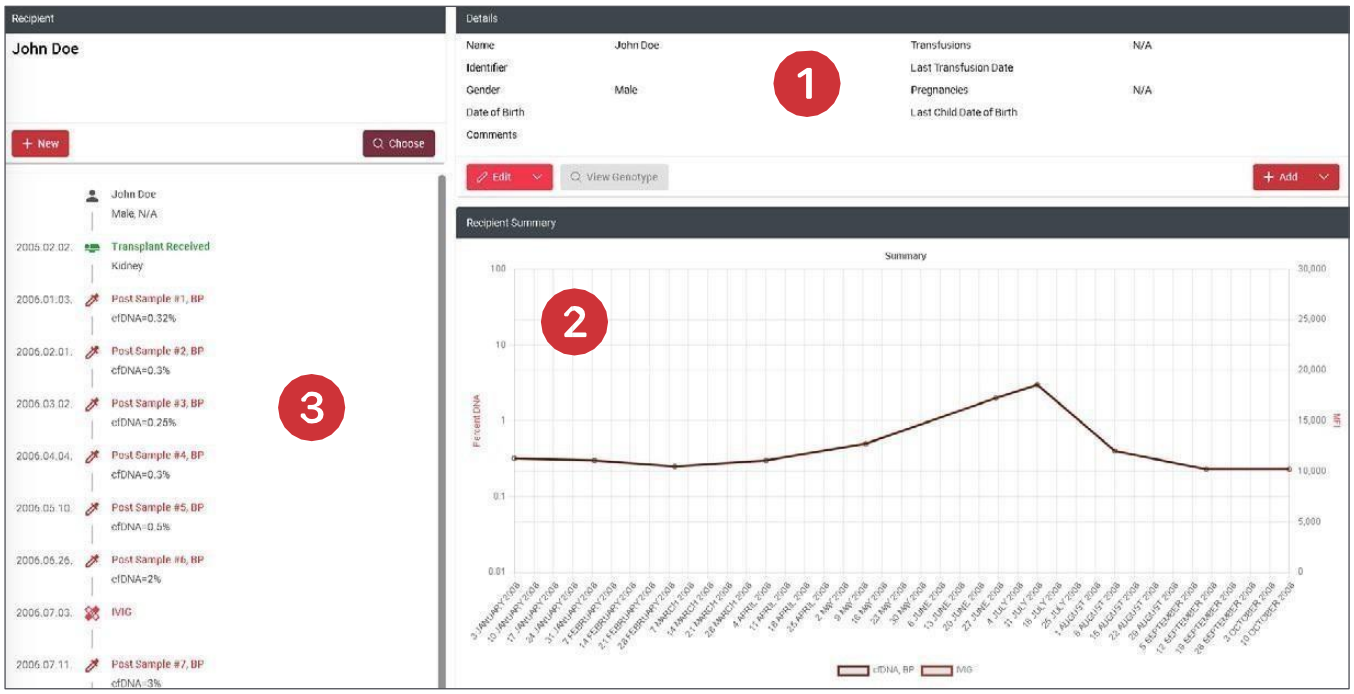
The current AlloSeq cfDNA Assay result is shown as the percent of donor cfDNA and recipient cfDNA of the total cell free DNA (cfDNA).

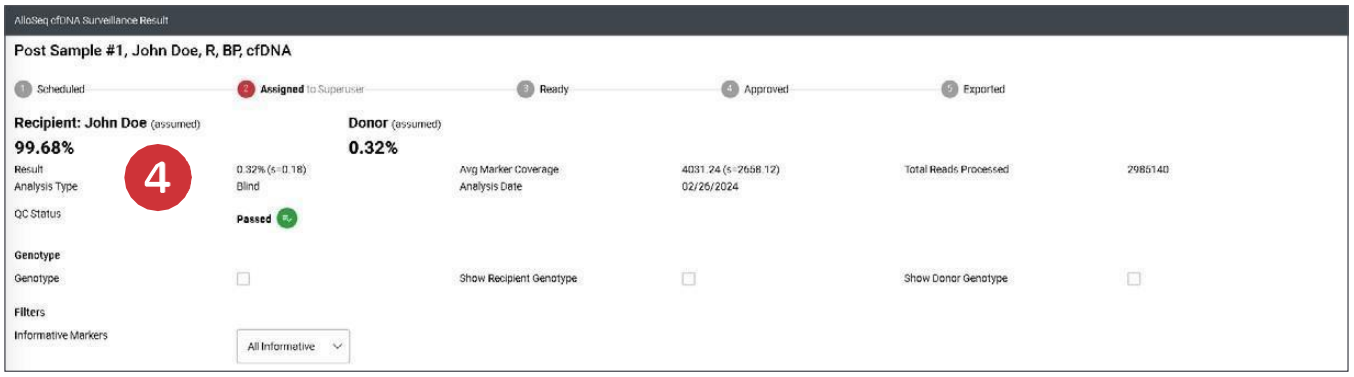
- **Quality metrics** *(Section 5)*

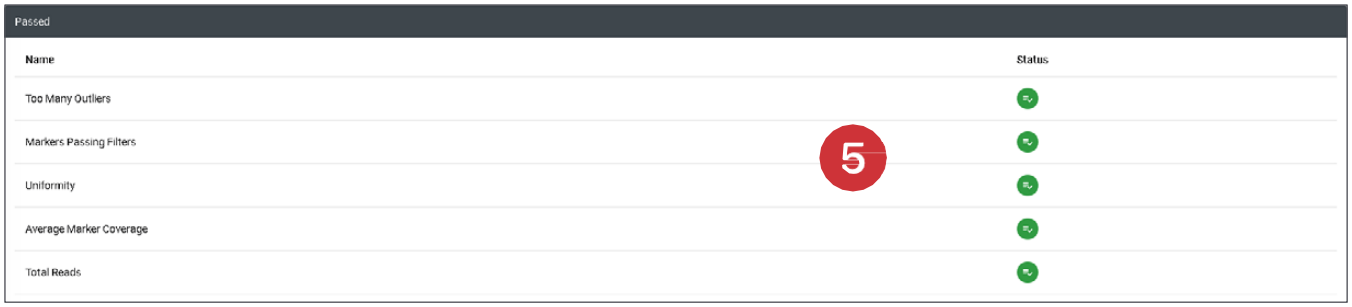
Quality metrics are embedded into the workflow. Detection of contamination or sample mix-up flag are included.

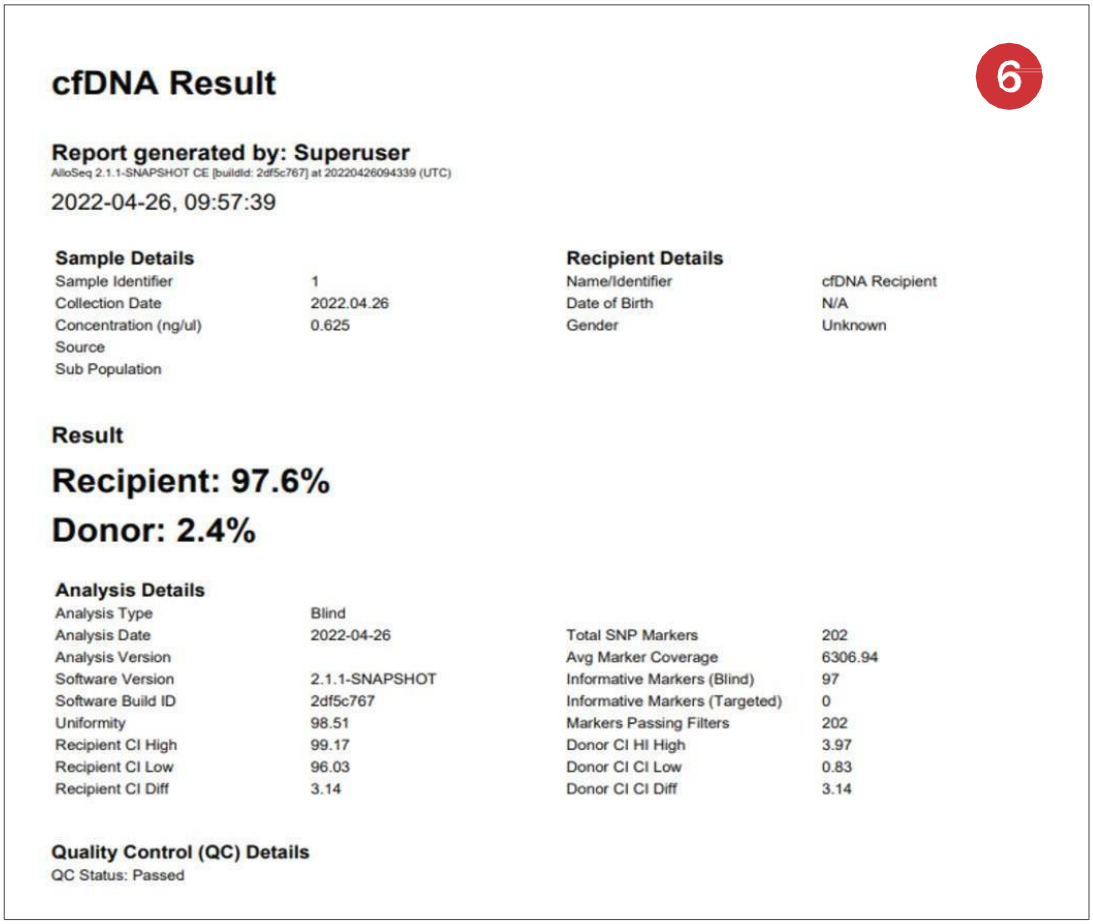
- **Result export** *(Section 6)*

The current AlloSeq cfDNA Assay result can be exported as a separate PDF file. Important information to assist with interpretation of the AlloSeq cfDNA Assay result is provided in the report. The **AlloSeq cfDNA result** is the **percent of dd-cfDNA of the total cfDNA** present in organ transplant recipients.


Recipient
John Doe
+ New
Choose
John Doe
Male, N/A
2005.02.02. Transplant Received
Kidney
2006.01.03. Post Sample #1, BP
cfDNA=0.32%
2006.02.01. Post Sample #2, BP
cfDNA=0.3%
2006.03.02. Post Sample #3, BP
cfDNA=0.26%
2006.04.04. Post Sample #4, BP
cfDNA=0.3%
2006.05.10. Post Sample #5, BP
cfDNA=0.5%
2006.06.26. Post Sample #6, BP
cfDNA=2%
2006.07.03. IVIG
2006.07.11. Post Sample #7, BP
cfDNA=3%
3
Details
Name John Doe
Identifier
Gender Male
Date of Birth
Comments
Transfusions N/A
Last Transfusion Date
Pregnancies N/A
Last Child Date of Birth
1
Edit
View Genotype
+ Add
Recipient Summary
Summary
2
Percent DNA
cfDNA, BP
IVIG


AlloSeq cfDNA Surveillance Result
Post Sample #1, John Doe, R, BP, cfDNA
1 Scheduled
2 Assigned to Superuser
3 Ready
4 Approved
5 Exported
Recipient: John Doe (assumed)
99.68%
Donor (assumed)
0.32%
4
Result 0.32% (s=0.18)
Analysis Type Blind
QC Status Passed
Avg Marker Coverage 4031.24 (s=2668.12)
Analysis Date 02/26/2024
Total Reads Processed 2986140
Genotype
Genotype
Show Recipient Genotype
Show Donor Genotype
Filters
Informative Markers
All Informative


Passed
Name
Status
Too Many Outliers
Markers Passing Filters
Uniformity
Average Marker Coverage
Total Reads
5



cfDNA Result

6

Report generated by: Superuser

AlloSeq 2.1.1-SNAPSHOT CE [buildId: 2df5c767] at 20220426094339 (UTC)

2022-04-26, 09:57:39

Sample Details

Sample Identifier	1
Collection Date	2022.04.26
Concentration (ng/ul)	0.625
Source	
Sub Population	

Recipient Details

Name/Identifier	cfDNA Recipient
Date of Birth	N/A
Gender	Unknown

Result

Recipient: 97.6%

Donor: 2.4%

Analysis Details

Analysis Type	Blind		
Analysis Date	2022-04-26	Total SNP Markers	202
Analysis Version		Avg Marker Coverage	6306.94
Software Version	2.1.1-SNAPSHOT	Informative Markers (Blind)	97
Software Build ID	2df5c767	Informative Markers (Targeted)	0
Uniformity	98.51	Markers Passing Filters	202
Recipient CI High	99.17	Donor CI HI High	3.97
Recipient CI Low	96.03	Donor CI CI Low	0.83
Recipient CI Diff	3.14	Donor CI CI Diff	3.14

Quality Control (QC) Details

QC Status: Passed

Figure 15: AlloSeq cfDNA Software workflow.

Data Output

After the sequencing run and the export of raw fastq data, they were used by the AlloSeq program to determine the percentage of dd-cfDNA. The data obtained with this analysis are reported in *Tables 10, 11* and *12:*

- dd-cfDNA (%)
- MeanCoverageAll Loci
- Uniformity (%)
- ACR
- AMR

dd-cfDNA (%),MeanCoverageAllLoci and Uniformity (%) are data derived from analysis with the *AlloSeq cfDNA Software*, whereas the data on the presence/absence of ACR and/or AMR are the results of the analysis of endomyocardial biopsies.

According to CareDx (AlloSeq cfDNA Assay), leading manufacturer of the kit and software used in this study, the applied **threshold for cardiac transplant rejection positivity is 0.25%**. This threshold is different for each transplanted organ.

Data Output of the first sequencing run

ID	dd-cfDNA (%)	MeanCoverageAllLoci	Uniformity (%)	ACR	AMR
Alloseq1	0.15%	2976	94	3A	/
Alloseq2	0.07%	2817	97	3A	pAMR0
Alloseq3	1.4%	3621	97	0	pAMR1
Alloseq4	0.46%	2418	96	1B	pAMR0
Alloseq5	0.23%	2812	97	0	/
Alloseq7	0.23%	4355	99	3A	pAMR1
Alloseq8	0.25%	3991	98	1A	/
Alloseq10	0.07%	3166	98	1A	/
Alloseq12	0.16%	4504	98	1B	/
Alloseq13	0.21%	3222	98	3A	pAMR0
Alloseq14	0.23%	3672	99	3A	pAMR1
Alloseq15	0.08%	3519	98	1A	/
Alloseq16	0.12%	3708	97	1A	/
Alloseq17	0.17%	1756	98	1B	/
Alloseq18	0.18%	1992	98	2	/
Alloseq19	0.16%	2042	98	3A	/
Alloseq19bis	0.12%	2146	98	3A	/
Alloseq20	0.28%	1407	96	1A	/
Alloseq21	0.55%	1939	98	1A	pAMR1
Alloseq22	0.07%	1411	97	0	/
Alloseq23	0.15%	920	92	1A	pAMR1
Alloseq25	0.15%	2165	98	1A	/
Alloseq26	0.21%	1509	98	0	/
CTR15	0.76%	764	96	0	/

Table 10: This table shows that all 24 samples passed quality control, none are found to have failed after AlloSeq cfDNA software analysis. Mean coverage and Uniformity are good in all samples. Regarding the percentages of dd-cfDNA, we can see that these are all below 1%, except for the Alloseq3, which has 1.4%. The last two columns of the table show the presence/absence of ACR and/or AMR with their respective grades. 7 patients show a significant degree of ACR (3A), 5, on the other hand, show a significant degree of AMR (pAMR1). Of these, 2 patients simultaneously show ACR and AMR of significant degree (Alloseq7 and Alloseq14).

Data Output of the second sequencing run

ID	dd-cfDNA (%)	MeanCoverageAllLoci	Uniformity (%)	ACR	AMR
Alloseq53	0.89%	1267	89	1A	pAMR1
Alloseq54	0.25%	1697	99	3A-3B	/
Alloseq55	0.98%	1907	100	1B	/
Alloseq56	1.5%	707	95	0	/
Alloseq57	0.96%	629	99	3A-3B	pAMR0
Alloseq58	0.23%	1821	99	3A-3B	pAMR0
Alloseq59	0.36%	2030	99	3A-3B	/
Alloseq60	0.91%	1740	99	3A-3B	/
Alloseq61	0.66%	1392	99	3A-3B	pAMR0
Alloseq62	0.20%	2178	99	2	/
Alloseq64	0.41%	2181	100	2	/
Alloseq65	1.2%	1804	99	1A	pAMR1
Alloseq67	0.63%	956	99	3A-3B	/
Alloseq68	0.78%	2042	98	3A-3B	/
Alloseq73	0.63%	1689	98	3A-3B	/
Alloseq74	0.27%	2358	100	2	/
Alloseq75	0.34%	1980	100	1B	pAMR0
Alloseq76	0.30%	2157	100	1B	/
Alloseq77	0.49%	1585	97	1B	/
Alloseq78	0.97%	1630	99	1B	/
Alloseq79	0.43%	2417	99	3A-3B	pAMR2
CardioPed3	3.6%	1157	97	0	pAMR0
CardioPed4	5.6%	1669	90	3B	pAMR0
CardioPed5	3.3%	652	99	0	pAMR2

Table 11: Results for all 24 samples, both adult and pediatric ones, passed quality control, none failed after AlloSeq cfDNA software analysis. Mean coverage and Uniformity are good in all samples. Regarding the percentages of dd-cfDNA, all samples are below 1%, except for the Alloseq56, which has 1.5% and Alloseq65 with 1.2%. In comparison, all three pediatric samples show a significantly higher percentage of dd-cfDNA than adult patients, which would indicate a greater release of circulating cfDNA and consequently a greater likelihood of rejection. Regarding the last two columns of the table, 10 adult patients show a significant degree of ACR (3A-3B), 3, instead, show a significant degree of AMR (pAMR1-2). Only one patient simultaneously

shows ACR and AMR of significant degree (Alloseq79). Important to note that Alloseq56, with a high percentage of dd-cfDNA (1.5%), which would indicate a high likelihood of rejection, has no rejection (neither ACR nor AMR). With regard to pediatric samples, CardioPed3, despite its high percentage of dd-cfDNA, does not show any rejection. On the other hand, CardioPed4 shows significant ACR (3B) and CardioPed5 shows significant AMR (pAMR2).

Data Output of the third sequencing run

ID	dd-cfDNA (%)	MeanCoverageAllLoci	Uniformity (%)	ACR	AMR
HT1	0.14%	3392.43	98	2	pAMR0
HT2	0%	0	NaN	3A	pAMR2
HT4	0.07%	2430.25	96	1A	/
HT6	0.22%	3630.41	97	1A	pAMR0
HT9	5.48%	94.5	100	1A	/
HT10	0.11%	4301.63	97	/	/
HT12	0.6%	5729.82	97	1A	pAMR1
HT16	3.38%	1983.68	78	1B	pAMR0
HT17	0.25%	3288.91	97	2	/
CardioPed2	0.14%	1548.84	97	0	pAMR0
CardioPed6	0.07%	2977.88	91	1A	pAMR0
CardioPed9	0.06%	4248.26	96	3A	pAMR0
CardioPed10	0.08%	5491.61	97	3A	pAMR1
CardioPed11	0.08%	5885.96	96	0	pAMR0
CardioPed12	0.73%	1218.44	78	0	pAMR0
CardioPed15	0.05%	7062.64	97	3A	pAMR1
CardioPed16	0.06%	7145.06	97	0	pAMR0
CardioPed17	0.1%	6353.44	99	1A	pAMR0
CardioPed19	2.3%	962.55	77	3A	pAMR0
CardioPed20	0.04%	5814.24	100	2A	pAMR0
CardioPed27	failed	0	NaN	1A	pAMR0
CardioPed29	2.46%	1515.03	77	/	/
CardioPed30	failed	0	NaN	1A	/
Alloseq55	1.18%	5603.16	100	1B	/

Table 12: Results for the 24 samples passed the quality control. 4 samples are found to have failed after AlloSeq cfDNA software analysis. In fact, no dd-cfDNA %, Mean coverage and Uniformity data are reported for 3 of the failed samples (HT2, CardioPed27 and CardioPed30). In the case of HT9, the software returns us a very high dd-cfDNA value (5.48%), and a very low coverage value (94.5), with an indication of failure at the end. The 4 samples in question were excluded from the final statistical analyses. For the remaining 20 samples, Mean coverage and Uniformity are good in all samples. Regarding the percentages of dd-cfDNA, all are below 1%, except for HT16, which has 3.38%, CardioPed19 with 2.3%, CardioPed29 with 2.46% and Alloseq55

(positive control) with 1.18%. HT16, despite this significantly above-average dd-cfDNA value, has no reported significant grade rejection. Regarding the last two columns of the table, none of the adult patients shows a significant degree of ACR, and only one adult patient shows a significant degree of AMR (pAMR1). For what concern pediatric patients, 4 samples show a significant degree of ACR. Of these 4 samples, only one shows a very high percentage of dd-cfDNA (CardioPed19 2.3%), while the other 3 have a particularly low dd-cfDNA %. CardioPed9 and CardioPed15, at the same time as the ACR, also show a significant degree of AMR.

ACR and AMR vs no rejection in adult HT patients

A statistical analysis was conducted to eliminate outliers, that is, all those values in a set of observations that are outliers and aberrant, clearly distant from other available observations. The samples eliminated as a result of this analysis are 5 and are: CTR15 0.76%, Alloseq55 0.98%, Alloseq56 1.50%, Alloseq78 0.97% and HT16 3.38%.

Thus, the data below were calculated from a total of 48 adult heart transplant patients.

Significant rejection was defined as **ACR grade ≥ 2R** and **AMR grade ≥ pAMR1**, according to the International Society for Heart and Lung Transplantation classification.

EMB results were as follows: 24 patients had no reported significant acute rejection (neither ACR nor AMR). In these patients, median dd-cfDNA levels were **0.20 %** [IQR 0.13 to 0.275%]. Significant ACR (grade ≥2R) was detected in 17 patients (41%, compared to 24 patients without rejection), in whom median dd-cfDNA levels were **0.25%** [IQR 0.185 to 0.645%] *(Figure 16)*. Significant AMR (grade ≥1) was detected in 9 patients (27%, compared to 24 patients without rejection, and 19 %, compared to No AMR patients), in whom median dd-cfDNA levels were **0.55%** [IQR 0.23 to 1.045%] *(Figure 17* and *18)*. Three samples simultaneously present ACR ≥2R and pAMR ≥1.

Starting from 30 days after HT, dd-cfDNA levels were higher in recipients with ACR (**p=0.0384**), as compared to those without *(Figure 19)*. The same is observed in patients with AMR, who have higher dd-cfDNA levels (**p=0.0031**), as compared to those without rejection *(Figure 20)*. AMR patients have higher dd-cfDNA levels (**p=0.0279**), as compared to No AMR recipients *(Figure 21).*

ACR vs no rejection

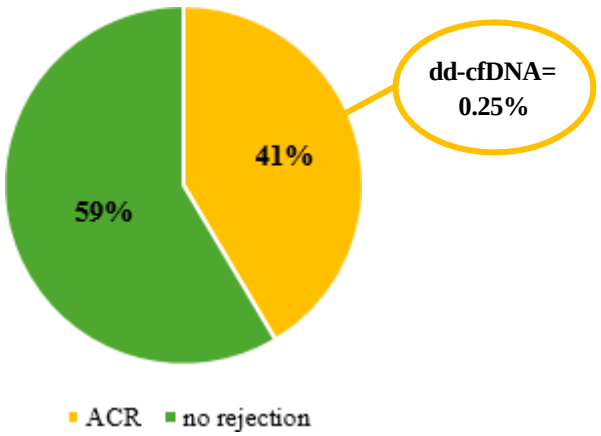


Figure 16: Significant ACR was detected in 17 patients (41%, compared to 24 patients without rejection), in whom median dd-cfDNA levels were **0.25%**.

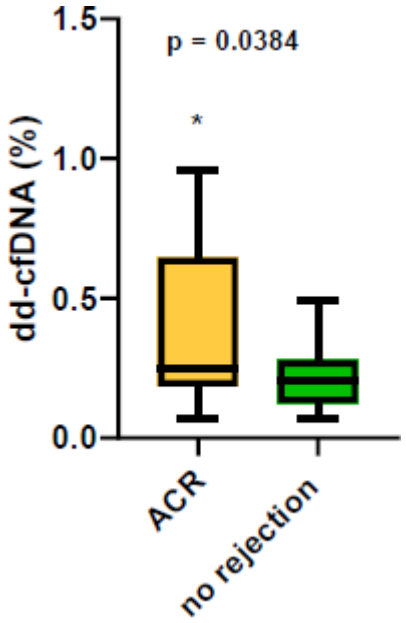


Figure 19: Analysis performed with the nonparametric Mann-Whitney test shows that dd-cfDNA values are significantly higher in ACR cases **(p=0.0384)**, compared with patients without rejection.

AMR vs no rejection

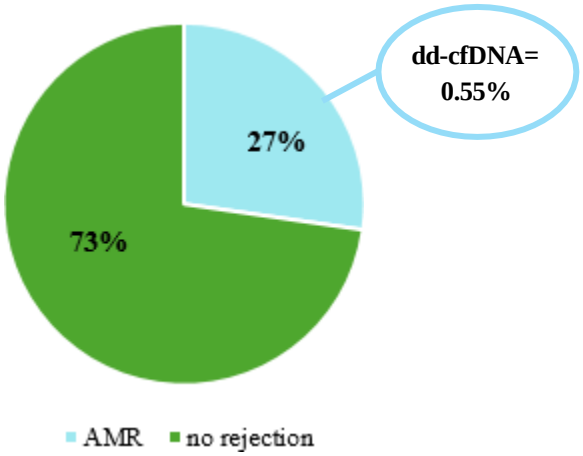


Figure 17: Significant AMR was detected in 9 patients (27%, compared to 24 patients without rejection), in whom median dd-cfDNA levels were **0.55%**.

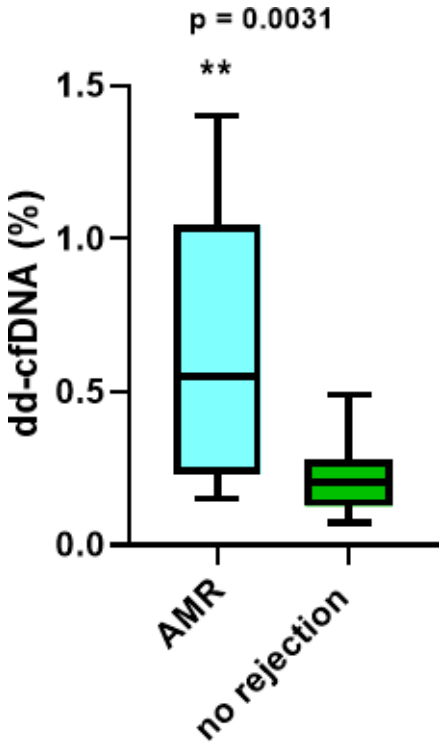


Figure 20: Analysis performed with a non-parametric Mann-Whitney test shows that dd-cfDNA values are significantly higher in AMR cases (**p=0.0031**), compared with no rejection patients.

AMR vs No AMR

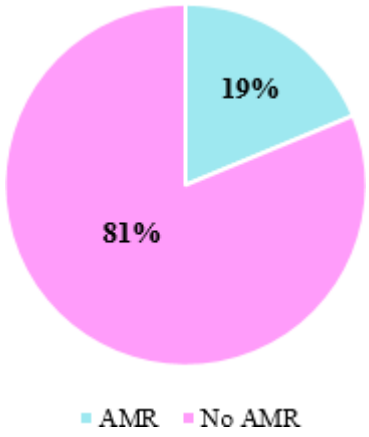


Figure 18: Significant AMR was detected in 9 patients (19%, compared to No AMR patients), in whom median dd-cfDNA levels were 0.55%.

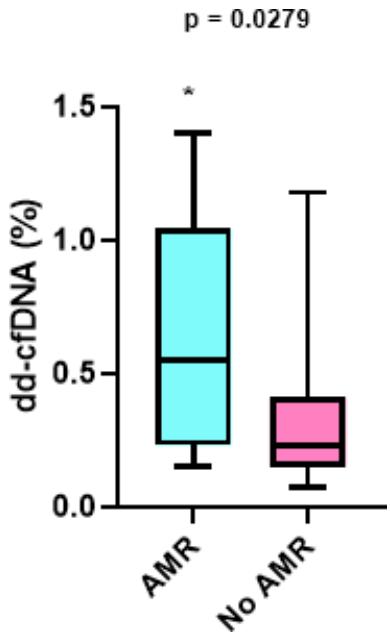


Figura 21: Analysis performed with a non-parametric Mann-Whitney test shows that dd-cfDNA values are significantly higher in AMR cases (**p=0.0279**), compared with No AMR patients.

ACR and AMR vs no rejection in pediatric HT patients

A statistical analysis was conducted to eliminate outliers. The samples eliminated as a result of this analysis are 2 and are: CardioPed5 3.30% and CardioPed29 2.46%.

Out of a total of 15 samples, 2 are found to have failed after analysis with the software, so data analysis is conducted on 13 samples.

Exactly as we have seen for adult patients, in pediatric HT patients, significant rejection was defined as **ACR grade ≥ 2R** and **AMR grade ≥ pAMR1**, according to the International Society for Heart and Lung Transplantation classification.

EMB results were as follows: 7 patients had no reported significant acute rejection (neither ACR nor AMR). In these patients, median dd-cfDNA levels were **0.08 %** [IQR 0.06 to 0.12%]. Significant ACR (grade ≥2R) was detected in 5 patients (42%, compared to 7 patients without rejection), in whom median dd-cfDNA levels were **0.08%** [IQR 0.06 to 2.95%] *(Figure 22)*. Significant AMR (grade ≥1) was detected in 3 patients (30%, compared to 7 patients without rejection), in whom median dd-cfDNA levels were **0.08%** [IQR 0.05 to 2.84%] *(Figure 23)*. Two samples simultaneously present ACR ≥2R and pAMR ≥1.

Starting from 30 days after HT, median dd-cfDNA levels were the same in recipients with ACR (**non-significant p-value**), as compared to those without rejection *(Figure 24)*. Although the data are not statistically significant, probably because of the small number of samples, it can be seen from *Figure 24* that dd-cfDNA values are much higher in patients presenting ACR, compared with cases without rejection, confirming the correlation between increased dd-cfDNA levels and rejection.

The patients presenting with significant grade AMR are only 3. This number appears to be too low to do a statistical analysis of correlation between dd-cfDNA levels and rejection.

ACR vs no rejection

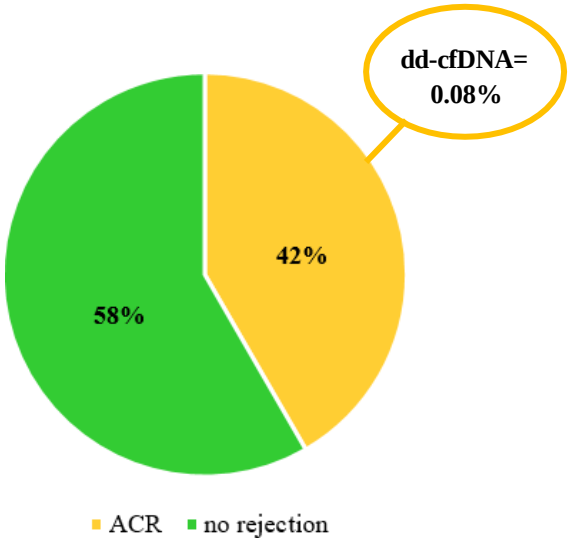


Figura 22: Significant ACR was detected in 5 patients (42%, compared to 7 patients without rejection), in whom median dd-cfDNA levels were **0.08%**.

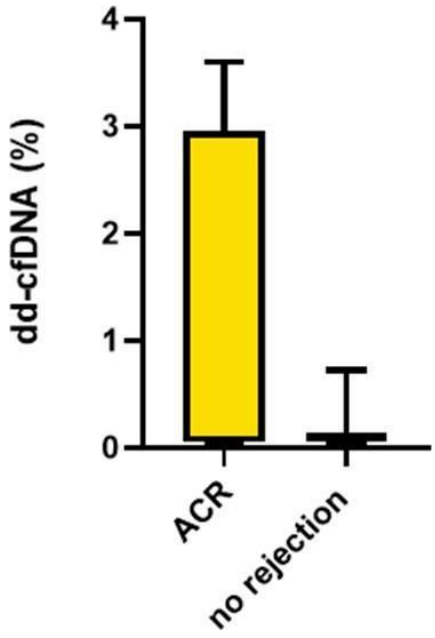


Figura 24: Analysis performed with the nonparametric Mann-Whitney test shows a non-significant difference in dd-cfDNA values between ACR cases and patients without rejection.

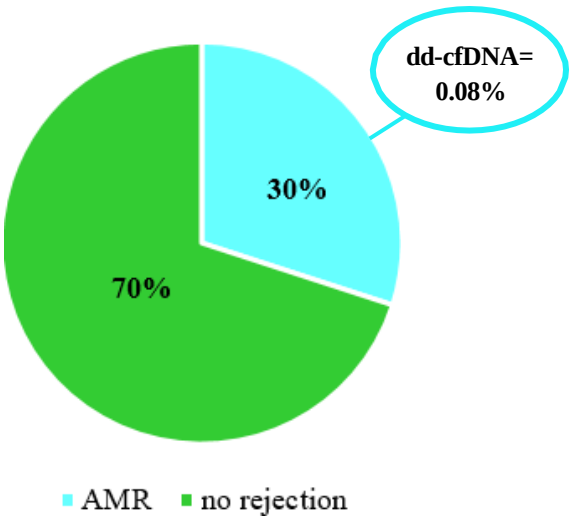


Figura 23: Significant AMR was detected in 3 patients (30%, compared to 7 patients without rejection), in whom median dd-cfDNA levels were **0.08%**.

5. Discussion

The present study reports the performance of a novel dd-cfDNA test (AlloSure Assay) in a cohort of HT patients.

Our results show a significant difference in the biomarker levels between adult HT patients with and without ACR and AMR, and a positive predictive value for a dd-cfDNA threshold of 0.25% in ACR (p=0.0384) and of 0.55% in AMR (p=0.0031). These findings align with those of previous studies using various dd-cfDNA tests. This supports the biomarker's role as a noninvasive tool for rejection surveillance in HT recipients, potentially reducing the number of EMB procedures currently performed.

Our study only included retrospective patients, all of whom underwent surveillance and for-cause EMB and blood sampling on the same day. We did not include any samples from the first 4 weeks post-HT, because it is well-known that in the first month post-transplant, dd-cfDNA levels are significantly higher due to inflammation caused from the transplant itself.

The test revealed excellent discrimination between no rejection and acute rejection, especially in the case of AMR, where the median dd-cfDNA value was 0.55%, thus well above the 0.20% corresponding to the median dd-cfDNA value of patients without rejection.

As also reported in the literature, the correlation between above-threshold dd-cfDNA levels and the diagnosis of ACR, is less clear-cut, perhaps also related to the high variability among pathologists in establishing this diagnosis, in fact, although significant, the difference between median dd-cfDNA levels of no rejection (0.20%) and median dd-cfDNA levels of ACR (0.25%) is lower than for patients presenting AMR.

It should be borne in mind that the assessment of dd-cfDNA levels is a method of early diagnosis, so that negative endomyocardial biopsy results, but associated with borderline or above-threshold dd-cfDNA levels, could fall into the category of those patients who do not

yet have visible rejection tissue damage, but whose dd-cfDNA values are already rising; to verify this would require targeted follow-up of a number of patients over a fairly long period of time.

Our study combined adult and pediatric patients. Unfortunately, in pediatric patients the data are not statistically significant. It is important to underline that the main difference we find between adult and pediatric HT samples relates to the volume of plasma that can be extracted: when it comes to pediatric patients, the greatest difficulty lies in the collection, which does not always allow for blood quantities of more than 5-6 ml, and this affects all downstream steps, starting with cfDNA extraction. It is also important to emphasise that the group of patients analysed is particularly limited, only 13 patients, compared to the adult HT group. All these reasons could be the cause of non-significant results. Nevertheless, we could still see how, in ACR cases, increased levels of dd-cfDNA were correlated with episodes of rejection and low levels of dd-cfDNA, were correlated with absence of rejection. In contrast, for patients with AMR, the number of cases was too low to perform the analysis. Given the small number of cases, new patients will be needed to validate this test on HT pediatric patients as well.

The relatively small sample size, particularly in pediatric patients, is a significant limitation to the statistical power and generalizability of the results obtained in the study. This problem is common in clinical trials involving specific and rare populations, such as pediatric cardiac transplant recipients, where patient availability is naturally limited. Addressing sample size limitation is critical to consolidating evidence on the use of dd-cfDNA in monitoring rejection, especially in pediatric populations. Promoting multicenter collaborations, leveraging meta-analyses and dedicated registries, and adopting innovative statistical methodologies represent concrete strategies to increase the statistical power and clinical relevance of future studies.

In the field of pediatric transplantation, collaboration between national and international centers is essential to overcome limitations related to small sample sizes and to improve the quality and standardization of care. A concrete example is the participation of the pediatric

hepatology and gastroenterology facility of ASST Papa Giovanni XXIII in Bergamo in the European network for pediatric transplantation, ERN TransplantChild, which promotes shared clinical studies and data exchange between European and non-European centers.

In Italy, the National Transplant Network coordinated by the National Transplant Center (NTC) runs specific programs for pediatric patients, such as the National Pediatric Program (PNP), which ensures priority in pediatric organ allocation and has contributed to a significant increase in the number of transplants in this age group. These national registries collect clinical, immunologic, and outcome data, creating a solid basis for observational studies and validation of biomarkers such as dd-cfDNA. Membership in such registries also facilitates participation in multicenter studies and enables monitoring of the effectiveness of large-scale therapeutic strategies.

Some HT samples were not valid due to several reasons (too low concentration, not enough quantity after purification, genomic DNA contaminations), and could not be used for analysis. Given that these events happened randomly, we believe they did not introduce any bias in our results.

This study assessed the accuracy, sensitivity, and specificity of dd-cfDNA to establish a robust correlation with histologically confirmed rejection episodes. By doing so, it aims to present dd-cfDNA as a viable alternative to the current standard of care, which predominantly relies on invasive endomyocardial biopsies (EMBs). A high negative predictive value associated with dd-cfDNA levels could empower clinicians to confidently rule out rejection without the need for invasive procedures.

Furthermore, the effectiveness of next-generation sequencing (NGS) as a high-throughput method for detecting and quantifying dd-cfDNA levels was evaluated.

In summary, this study aspires to validate dd-cfDNA as a pivotal biomarker in the surveillance of heart transplant rejection. By potentially replacing or reducing the frequency of invasive biopsies, dd-cfDNA analysis may significantly enhance patient comfort and safety while ensuring timely interventions, ultimately contributing to improved graft survival rates and quality of life for transplant recipients.

However, further studies are still needed to standardize protocols and precisely define the sensitivity and specificity levels of dd-cfDNA analysis in order to make it a routine clinical test. The combination of liquid biopsy and advanced DNA sequencing technologies represents a very promising area of research that could significantly improve the management of heart transplant recipients. Prospective randomized clinical trials are needed to determine if a dd- cfDNA surveillance strategy, alone or in combination with other biomarkers, is noninferior to EMB for acute rejection monitoring in HT patients.

A comprehensive understanding of how dd-cfDNA thresholds can be effectively standardized is crucial to ensure their reliable application in clinical practice. Standardization of dd-cfDNA thresholds requires a multi-pronged approach: robust meta-analytical evidence, harmonized measurement methods, dual-threshold algorithms, condition-specific cutoffs, standardized laboratory protocols, and broad clinical validation. This process will enable accurate, reproducible, and clinically actionable use of dd-cfDNA in transplant rejection monitoring.

A first approach consists of using meta-analysis and evidence-based cutoff selection. A recent meta-analysis of 13 studies (over 12,000 samples) identified significant variability in dd-cfDNA thresholds for detecting acute cardiac transplant rejection, with cutoffs ranging from 0.1% to 0.35%. By pooling sensitivity and specificity data and maximizing the Youden index, the analysis determined an optimal threshold of 0.218 (rounded to 0.22%) for clinical screening *(Shah et al. 2024)*. This approach uses statistical modeling to balance sensitivity and specificity, providing a data-driven basis for standardization.

Standardization requires consistent use of validated quantification techniques. dd-cfDNA can be measured as a fraction (%) or as absolute quantity (copies/ml), with each method having

strengths and limitations. Absolute quantification is less affected by fluctuations in recipient cfDNA (e.g., due to infection or inflammation), while fractional measurement is widely adopted but more susceptible to confounders. Clinical protocols should specify the preferred method and ensure laboratories use harmonized assays with defined performance characteristics.

Recent studies suggest combining both dd-cfDNA fraction and absolute quantity thresholds can improve diagnostic accuracy. For example, one approach *(Khush et al. 2024)* considers a sample at high risk for rejection if either the percentage or the absolute quantity exceeds a validated cutoff, thus minimizing false negatives in patients with high background cfDNA. This dual-threshold strategy can be incorporated into clinical guidelines to enhance reliability.

The optimal threshold may differ between types of rejection - ACR and AMR - as these conditions can produce different dd-cfDNA profiles. However, most current studies aggregate both types. Further research should stratify results by rejection subtype and validate distinct cutoffs for ACR and AMR, improving diagnostic precision.

Also very important is the standardization of pre-analytical and analytical protocols. Sample collection, processing, and storage should be standardized to reduce variability. Laboratories should participate in external quality assessment programs and use reference materials to calibrate assays.

Proposed thresholds must be validated in large, diverse, and prospective cohorts across multiple centers to ensure generalizability and reproducibility. This process should also account for population differences, comorbidities, and technical variables.

In discussing the role of dd-cfDNA for transplant rejection monitoring, it is valuable to highlight significant experiences from other solid organ transplants, such as kidney and lung transplants, where dd-cfDNA has also been extensively studied and applied.

- **Kidney Transplantation**

dd-cfDNA has emerged as a promising noninvasive biomarker for detecting kidney

transplant rejection. A systematic review and meta-analysis *(Xing et al. 2024)* evaluating its diagnostic accuracy included nine studies assessing ACR and twelve studies focusing AMR. For ACR, the pooled sensitivity was 59%, specificity 83%, and area under the receiver operating characteristic curve (AUROC) 0.80, while for AMR, sensitivity increased to 81%, specificity was 80%, and AUROC reached 0.87. The heterogeneity observed among studies was influenced by factors such as study design, patient age groups, and sample size. Despite some limitations in diagnosing ACR due to variability, dd-cfDNA demonstrated high diagnostic value for AMR. These findings are corroborated by a large multicenter observational study *(Aubert et al. 2024)*, involving 2,882 kidney transplant recipients across 14 centers in Europe and the United States. This study confirmed strong correlations between elevated dd-cfDNA levels and both ACR and AMR, with the addition of dd-cfDNA significantly improving diagnostic discrimination compared to standard clinical parameters (AUC increased from 0.777 to 0.821). Furthermore, dd-cfDNA proved useful in detecting subclinical rejection and monitoring response to anti-rejection therapy. Collectively, these data support dd-cfDNA as a sensitive and dynamic biomarker for kidney transplant surveillance, particularly effective in identifying AMR.

- **Lung Transplantation**

Although less extensively documented than kidney transplantation, dd-cfDNA is emerging as a promising biomarker in lung transplant recipients as well. A comprehensive meta-analysis *(Li et al. 2023)* was conducted by systematically reviewing studies from multiple databases, evaluating circulating dd-cfDNA levels in patients experiencing graft rejection, ACR and AMR compared to those without rejection. The analysis demonstrated that dd-cfDNA levels were significantly elevated in all rejection types, with standardized mean differences (SMD) of 1.78 for graft rejection overall, 1.03 for ACR, and 1.78 for AMR, indicating a robust association between increased dd-cfDNA and rejection episodes. Furthermore, dd-cfDNA showed strong diagnostic performance, distinguishing rejection from non-rejection with a pooled sensitivity of 87% and specificity of 82%, and an area under the receiver operating characteristic curve (AUROC) of 0.90. These findings support the clinical utility of circulating dd-cfDNA as a sensitive and non-invasive biomarker for early

detection and monitoring of graft rejection after lung transplantation, potentially enabling timely therapeutic interventions and improved graft outcomes.

The experiences in kidney and lung transplantation reinforce the potential of dd-cfDNA as a universal biomarker for solid organ transplant rejection. These fields provide valuable insights into assay standardization, threshold optimization, and integration with clinical parameters that can inform and accelerate the clinical adoption of dd-cfDNA monitoring in heart transplant recipients. Moreover, the demonstrated ability of dd-cfDNA to detect subclinical rejection and to monitor treatment response in other organs highlights its promise for improving post-transplant care and outcomes in cardiac transplantation.

6. Conclusions

The study discussed in this thesis follows in the footsteps of other similar studies conducted worldwide with the aim of better characterising the potential of dd-cfDNA as a biomarker and implementing it in clinical practice not only to effectively diagnose organ rejection, in this case cardiac rejection, at an early stage, but also to modulate immunosuppressive therapy in patients who do not present clinical symptoms of rejection and to seek a strategy to distinguish between the two types of rejection, ACR and AMR.

As a non-invasive, quantitative marker of allograft injury, dd-cfDNA provides promise as a safe, accurate, and feasible method of acute rejection monitoring in heart transplant recipients. While further studies are required to validate specific threshold values for routine clinical use, dd-cfDNA currently demonstrates the greatest potential as a surveillance monitoring tool, screening patients who would most benefit from preceding to biopsy. The further ongoing investigation will determine its role in the diagnosis of other forms of allograft injury, its potential to serve as a treatment target following episodes of acute rejection of infection, and the ability to serve as a prognostic marker for adverse long term outcomes. Advances in the use of dd-cfDNA rejection monitoring further realizes our quest for the development of precision medicine techniques in heart transplant recipients.

7. Appendix

Table 13 and *14* show the following data:

- ID (**Alloseq** refers to adult patients, whereas **CardioPed** refers to pediatric ones)
- Date of birth (when known)
- Date of transplant (when known)
- Date of biopsy
- Qubit HS ds DNA (ng/µl)
- Quantity: 10 ng in 16 µl
- The amount of H_20 to be added to reach the volume (16 µl)

The samples in *Tables 13* and *14* were sequenced, but both sequencing runs **failed**, resulting in empty fastq files.

ID	Date of birth	Date of transplant	Date of biopsy	Qubit HS ds DNA (ng/µl)	10 ng in 16 µl	H_20
Alloseq6	12/12/1947	14/4/2013	15/3/2018	0.356	28.08	X
Alloseq9	28/5/1955	13/2/2016	10/11/2016	0.313	31.94	X
Alloseq11	5/4/1977	30/3/2016	23/1/2017	0.259	38.61	X
Alloseq24	27/6/1962	2/7/2017	28/12/2017	0.168	59.52	X
Alloseq27	14/9/1961	16/9/2017	12/3/2018	2.28	4.39	11.61
Alloseq28	27/6/1962	2/7/2017	5/4/2018	1.59	6.29	9.71
Alloseq29	9/6/1975	15/11/2016	21/5/2018	0.556	17.98	dry
Alloseq30	9/11/1960	18/5/2017	17/5/2018	0.99	10.10	5.90
Alloseq31	26/8/1975	13/1/2018	16/4/2018	1.64	6.10	9.90
Alloseq32	22/10/1971	12/2/2017	5/2/2018	0.516	19.37	dry
Alloseq33	5/4/1977	30/3/2016	22/3/2018	1.66	6.02	9.98
Alloseq34	2/12/1954	21/11/2017	24/5/2018	3.44	2.91	13.09
Alloseq35	24/3/1956	6/2/2018	2/5/2018	4.16	2.40	13.60
Alloseq36	10/5/1959	24/5/2017	7/6/2018	0.459	21.78	dry
Alloseq37	13/6/1957	4/4/2016	8/10/2018	0.661	13.13	0.87
Alloseq38	20/3/1958	2/8/2018	12/9/2018	4.09	2.44	13.56

Alloseq39	31/7/1957	24/11/2016	13/9/2018	0.747	13.39	2.61
Alloseq40	18/5/1961	3/3/2015	29/10/2018	0.364	27.47	dry
Alloseq41	17/9/1970	3/10/2016	29/10/2018	1.45	6.90	9.10
Alloseq42	19/4/1976	26/9/2017	22/10/2018	0.793	12.61	3.39
Alloseq43	29/3/1984	4/4/2018	10/1/2019	0.387	25.83	X
Alloseq44	4/8/1956	20/5/2014	19/11/2018	0.501	19.96	dry
Alloseq45	9/11/1967	7/12/2016	14/6/2018	0.378	26.45	dry
Alloseq46	11/9/1976	5/11/2016	25/11/2021	0.187	53.47	X
Alloseq47	5/7/1957	28/2/2012	24/1/2022	0.935	10.70	5.30
Alloseq48	11/9/1976	5/11/2016	27/1/2022	1.56	6.42	9.58
Alloseq49	7/8/1969	16/3/2019	27/1/2022	11.3	0.89	15.11
Alloseq50	5/3/1968	13/8/2020	7/2/2022	4.35	2.30	13.70
Alloseq51	5/7/1957	28/2/2012	24/2/2022	2.89	3.47	12.53
CardioPed1	/	/	27/5/2023	0.548	18.24	dry
CardioPed2	22/4/1998	/	27/5/2023	4.6	2.18	13.82

Table 13: Data from 31 HT adult and pediatric patients. Six samples are highlighted in red because they did not reach a sufficient cfDNA quantity after extraction. Two other samples, **Alloseq50** and **CardioPed2**, underlined in green, were not included in the sequencing run because, after quantification by *Agilent 2100 Bioanalyzer*, they were repurified, due to the presence of a lot of genomic DNA *(Figure 25)*, and after repurification, the quantification at *Qubit HS dsDNA* was too low. The remaining 23 samples had sufficient quantities of cfDNA and, were selected for sequencing. Unfortunately, the sequencing run failed, with empty fastq files.

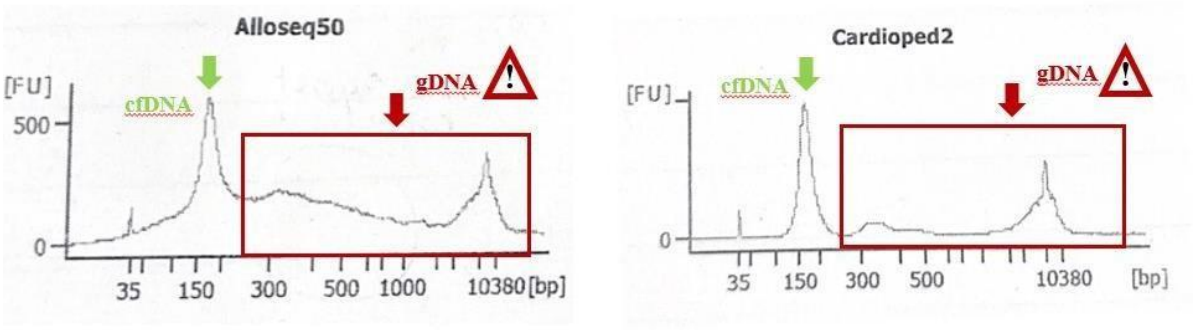


Figure 25: Typical profile of cfDNA with genomic DNA contaminations. gDNA contamination always leads to underestimated dd-cfDNA values.

ID	Date of birth	Date of biopsy	Qubit HS ds DNA (ng/µl)	10 ng in 16 µl	H_20
CardioPed6	31/5/2005	25/5/2022	1.67	5.99	10.01
CardioPed9	14/9/1995	10/11/2022	11.3	0.88	15.12
CardioPed10	15/9/2009	14/11/2022	3.18	3.14	12.86
CardioPed11	3/2/1987	22/11/2022	1.73	5.78	10.22
CardioPed12	27/9/1998	14/11/2022	3.67	2.72	13.28
CardioPed13	19/11/2004	12/12/2022	0.498	20.08	dry
CardioPed14	19/11/2004	12/12/2022	0.364	27.47	dry
CardioPed15	20/5/2016	9/1/2023	2.03	4.92	11.08
CardioPed16	23/3/2003	10/2/2023	2.16	4.62	11.38
CardioPed17	19/4/2005	14/2/2023	1.45	6.89	9.11
CardioPed18	25/12/2014	6/3/2023	0.382	26.17	dry
CardioPed19	2/11/2001	21/3/2023	1.74	5.74	10.26
CardioPed20	15/9/2009	3/5/2023	2.28	4.38	11.62
CardioPed23	12/8/2004	31/5/2023	0.632	15.82	0.18
CardioPed24	21/5/2011	2/8/2023	0.419	23.86	dry
CardioPed26	12/7/2000	20/12/2023	0.342	29.23	dry
Alloseq55	21/6/1957	16/1/2017	2.61	3.83	12.17
Alloseq62	27/8/1961	15/6/2017	2.15	4.65	11.35
Alloseq76	10/5/1959	22/6/2017	2.28	4.38	11.62
Alloseq80	/	5/12/2003	1.31	7.63	8.37

Table 14: Data from 20 HT adult and pediatric patients. All the samples had sufficient quantities of cfDNA and were therefore selected for sequencing. Unfortunately, the sequencing run failed, with empty fastq files (~1kb). Probably something went wrong during PCR or purification with the magnetic beads, or during indexing phase. Samples used as controls (Alloseq55-62-76), with a higher amount of cfDNA, are also empty.

8. Bibliography

- Agbor-Enoh S, Jackson AM, Tunc I, Berry GJ, Cochrane A, Grimm D, Davis A, Shah P, Brown AW, Wang Y, Timofte I, Shah P, Gorham S, Wylie J, Goodwin N, Jang MK, Marishta A, Bhatti K, Fideli U, Yang Y, Luikart H, Cao Z, Pirooznia M, Zhu J, Marboe C, Iacono A, Nathan SD, Orens J, Valantine HA, Khush K. Late manifestation of alloantibody-associated injury and clinical pulmonary antibody-mediated rejection: Evidence from cell-free DNA analysis. J Heart Lung Transplant. 2018 Jul;37(7):925-932. doi: 10.1016/j.healun.2018.01.1305. Epub 2018 Jan 31. PMID: 29500138.
- Agbor-Enoh S, Shah P, Tunc I, Hsu S, Russell S, Feller E, Shah K, Rodrigo ME, Najjar SS, Kong H, Pirooznia M, Fideli U, Bikineyeva A, Marishta A, Bhatti K, Yang Y, Mutebi C, Yu K, Kyoo Jang M, Marboe C, Berry GJ, Valantine HA; GRAfT Investigators. Cell-Free DNA to Detect Heart Allograft Acute Rejection. Circulation. 2021 Mar 23;143(12):1184-1197. doi: 10.1161/CIRCULATIONAHA.120.049098. Epub 2021 Jan 13. PMID: 33435695; PMCID: PMC8221834.
- Agbor-Enoh S, Tunc I, De Vlaminck I, Fideli U, Davis A, Cuttin K, Bhatti K, Marishta A, Solomon MA, Jackson A, Graninger G, Harper B, Luikart H, Wylie J, Wang X, Berry G, Marboe C, Khush K, Zhu J, Valantine H. Applying rigor and reproducibility standards to assay donor-derived cell-free DNA as a non-invasive method for detection of acute rejection and graft injury after heart transplantation. J Heart Lung Transplant. 2017 Sep;36(9):1004-1012. doi: 10.1016/j.healun.2017.05.026. Epub 2017 May 20. PMID: 28624139; PMCID: PMC7988434.
- Alam A, Van Zyl J, Paul Milligan G, Michelle McKean S, Patel R, Anne Hall S. Evolving the surveillance and workup of heart transplant rejection: A real-world analysis of the Molecular Microscope Diagnostic System. Am J Transplant. 2022 Oct;22(10):2443-2450. doi: 10.1111/ajt.17087. Epub 2022 May 24. PMID: 35514138.
- Alam AH, Van Zyl J, Shakoor HI, Farsakh D, Abdelrehim AB, Maliakkal N, Jamil AK, Patel R, Felius J, McKean S, Hall SA. The impact of active cytomegalovirus infection on donor-derived cell-free DNA testing in heart transplant recipients. Clin Transplant. 2024 Mar;38(3):e15287. doi: 10.1111/ctr.15287. PMID: 38477177.
- Almeida AG, Carpenter JP, Cameli M, Donal E, Dweck MR, Flachskampf FA, et al. Multimodality imaging of myocardial viability: an expert consensus document from the European Association of Cardiovascular Imaging (EACVI). Eur Heart J

Cardiovasc Imaging. 2021;22:e97–125.

- Aubert O, Ursule-Dufait C, Brousse R, Gueguen J, Racapé M, Raynaud M, Van Loon E, Pagliazzi A, Huang E, Jordan SC, Chavin KD, Gupta G, Kumar D, Alhamad T, Anand S, Sanchez-Garcia J, Abdalla BA, Hogan J, Garro R, Dadhania DM, Jain P, Mandelbrot DA, Naesens M, Dandamudi R, Dharnidharka VR, Anglicheau D, Lefaucheur C, Loupy A. Cell-free DNA for the detection of kidney allograft rejection. Nat Med. 2024 Aug;30(8):2320-2327. doi: 10.1038/s41591-024-03087-3. Epub 2024 Jun 2. PMID: 38824959; PMCID: PMC11333280.
- Baandrup U, Florio RA, Olsen EG. Do endomyocardial biopsies represent the morphology of the rest of the myocardium? A quantitative light microscopic study of single v. multiple biopsies with the King's bioptome. Eur Heart J. 1982 Apr;3(2):171-8. doi: 10.1093/oxfordjournals.eurheartj.a061281. PMID: 7044784.
- Bagnall RD, Weintraub RG, Ingles J, Duflou J, Yeates L, Lam L, Davis AM, Thompson T, Connell V, Wallace J, Naylor C, Crawford J, Love DR, Hallam L, White J, Lawrence C, Lynch M, Morgan N, James P, du Sart D, Puranik R, Langlois N, Vohra J, Winship I, Atherton J, McGaughran J, Skinner JR, Semsarian C. A prospective study of sudden cardiac death among children and young adults. The New England journal of medicine. 2016; 374:2441–2452. [PubMed: 27332903]
- Bailey LL. Origins of neonatal heart transplantation: an historical perspective. Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu. 2011;14(1):98-100. doi: 10.1053/j.pcsu.2011.01.004. PMID: 21444055.
- Barnes A, Gibson W. Pediatric heart transplant. Semin Pediatr Surg. 2021 Apr;30(2):151039. doi: 10.1016/j.sempedsurg.2021.151039. Epub 2021 Mar 21. PMID: 33992306.
- Bejiqi R, Retkoceri R, Maloku A, Mustafa A, Bejiqi H, Bejiqi R. The Diagnostic and Clinical Approach to Pediatric Myocarditis: A Review of the Current Literature. Open Access Maced J Med Sci. 2019 Jan 4;7(1):162-173. doi: 10.3889/oamjms.2019.010. PMID: 30740183; PMCID: PMC6352488.
- Berry GJ, Burke MM, Andersen C, Bruneval P, Fedrigo M, Fishbein MC, Goddard M, Hammond EH, Leone O, Marboe C, Miller D, Neil D, Rassl D, Revelo MP, Rice A, Rene Rodriguez E, Stewart S, Tan CD, Winters GL, West L, Mehra MR, Angelini A. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J Heart Lung Transplant. 2013 Dec;32(12):1147-62. doi: 10.1016/j.healun.2013.08.011. PMID: 24263017.
- Bieńkowski M, Pęksa R, Popęda M, Kołaczkowska M, Frankiewicz A, Żaczek AJ, Gruchała M, Biernat W, Siondalski P. Liquid biopsy for minimally invasive heart

transplant monitoring: a pilot study. J Clin Pathol. 2020 Aug;73(8):507-510. doi: 10.1136/jclinpath-2019-205926. Epub 2019 Dec 5. PMID: 31806731.

- Böhmer J, Wasslavik C, Andersson D, Ståhlberg A, Jonsson M, Wåhlander H, Karason K, Sunnegårdh J, Nilsson S, Asp J, Dellgren G, Ricksten A. Absolute Quantification of Donor-Derived Cell-Free DNA in Pediatric and Adult Patients After Heart Transplantation: A Prospective Study. Transpl Int. 2023 Oct 30;36:11260. doi: 10.3389/ti.2023.11260. PMID: 37965628; PMCID: PMC10641041.
- Boner G, Cooper ME, McCarroll K, Brenner BM, de Zeeuw D, Kowey PR, Shahinfar S, Dickson T, Crow RS, Parving HH; RENAAL Investigators. Adverse effects of left ventricular hypertrophy in the reduction of endpoints in NIDDM with the angiotensin II antagonist losartan (RENAAL) study. Diabetologia. 2005 Oct;48(10):1980-7. doi: 10.1007/s00125-005-1893-1. Epub 2005 Aug 5. PMID: 16082528.
- Bouma BJ, Mulder BJ. Changing Landscape of Congenital Heart Disease. Circ Res. 2017 Mar 17;120(6):908-922. doi: 10.1161/CIRCRESAHA.116.309302. PMID: 28302739.
- Bounader K, Flécher E. End-stage heart failure: The future of heart transplant and artificial heart. Presse Med. 2024 Mar;53(1):104191. doi: 10.1016/j.lpm.2023.104191. Epub 2023 Oct 26. PMID: 37898310.
- Braunwald E. Heart failure. JACC Heart Fail. 2013 Feb;1(1):1-20. doi: 10.1016/j.jchf.2012.10.002. Epub 2013 Feb 4. PMID: 24621794.
- Bryzgunova OE, Skvortsova TE, Kolesnikova EV, Starikov AV, Rykova EY, Vlassov VV, Laktionov PP. Isolation and comparative study of cell-free nucleic acids from human urine. Ann N Y Acad Sci. 2006 Sep;1075:334-40. doi: 10.1196/annals.1368.045. PMID: 17108229.
- Chih S, Chruscinski A, Ross HJ, Tinckam K, Butany J, Rao V. Antibody-mediated rejection: an evolving entity in heart transplantation. J Transplant. 2012;2012:210210. doi: 10.1155/2012/210210. Epub 2012 Mar 26. PMID: 22545200; PMCID: PMC3321610.
- Chimenti C, Frustaci A. Contribution and risks of left ventricular endomyocardial biopsy in patients with cardiomyopathies: a retrospective study over a 28-year period. Circulation. 2013 Oct 1;128(14):1531-41. doi: 10.1161/CIRCULATIONAHA.13.001414. Epub 2013 Sep 4. PMID: 24004501.
- Christiaans I, van Engelen K, van Langen IM, Birnie E, Bonsel GJ, Elliott PM, Wilde AA. Risk stratification for sudden cardiac death in hypertrophic cardiomyopathy: Systematic review of clinical risk markers. Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of

Cardiology. 2010; 12:313–321.

- Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, Douville C, Javed AA, Wong F, Mattox A, Hruban RH, Wolfgang CL, Goggins MG, Dal Molin M, Wang TL, Roden R, Klein AP, Ptak J, Dobbyn L, Schaefer J, Silliman N, Popoli M, Vogelstein JT, Browne JD, Schoen RE, Brand RE, Tie J, Gibbs P, Wong HL, Mansfield AS, Jen J, Hanash SM, Falconi M, Allen PJ, Zhou S, Bettegowda C, Diaz LA Jr, Tomasetti C, Kinzler KW, Vogelstein B, Lennon AM, Papadopoulos N. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018 Feb 23;359(6378):926-930. doi: 10.1126/science.aar3247. Epub 2018 Jan 18. PMID: 29348365; PMCID: PMC6080308.
- Cooper DKC. Christiaan Barnard-The surgeon who dared: The story of the first human-to-human heart transplant. Glob Cardiol Sci Pract. 2018 Jun 30;2018(2):11. doi: 10.21542/gcsp.2018.11. PMID: 30083541; PMCID: PMC6062759.
- Crespo-Leiro MG, Stypmann J, Schulz U, Zuckermann A, Mohacsi P, Bara C, Ross H, Parameshwar J, Zakliczyński M, Fiocchi R, Hoefer D, Colvin M, Deng MC, Leprince P, Elashoff B, Yee JP, Vanhaecke J. Clinical usefulness of gene-expression profile to rule out acute rejection after heart transplantation: CARGO II. Eur Heart J. 2016 Sep 1;37(33):2591-601. doi: 10.1093/eurheartj/ehv682. Epub 2016 Jan 7. PMID: 26746629; PMCID: PMC5015661.
- DeFilippis EM, Sweigart B, Khush KK, Shah P, Agbor-Enoh S, Valantine HA, Vest AR. Sex-specific patterns of donor-derived cell-free DNA in heart transplant rejection: An analysis from the Genomic Research Alliance for Transplantation (GRAfT). J Heart Lung Transplant. 2024 Jul;43(7):1135-1141. doi: 10.1016/j.healun.2024.03.001. Epub 2024 Mar 7. PMID: 38460620; PMCID: PMC11144097.
- Del Buono MG, Moroni F, Montone RA, Azzalini L, Sanna T, Abbate A. Ischemic Cardiomyopathy and Heart Failure After Acute Myocardial Infarction. Curr Cardiol Rep. 2022 Oct;24(10):1505-1515. doi: 10.1007/s11886-022-01766-6. Epub 2022 Aug 16. PMID: 35972638; PMCID: PMC9556362.
- Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, Kobashigawa J, Johnson FL, Starling RC, Murali S, Pauly DF, Baron H, Wohlgemuth JG, Woodward RN, Klingler TM, Walther D, Lal PG, Rosenberg S, Hunt S; CARGO Investigators. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. Am J Transplant. 2006 Jan;6(1):150-60. doi: 10.1111/j.1600-6143.2005.01175.x. PMID: 16433769.
- Deshpande SR, Zangwill SD, Kindel SJ, Schroder JN, Bichell DP, Wigger MA, Richmond ME, Knecht KR, Pahl E, Gaglianello NA, Mahle WT, Stamm KD,

Simpson PM, Dasgupta M, Zhang L, North PE, Tomita-Mitchell A, Mitchell ME. Relationship between donor fraction cell-free DNA and clinical rejection in heart transplantation. Pediatr Transplant. 2022 Jun;26(4):e14264. doi: 10.1111/petr.14264. Epub 2022 Mar 8. PMID: 35258162.

- Dipchand AI, Laks JA. Pediatric heart transplantation: long-term outcomes. Indian J Thorac Cardiovasc Surg. 2020 Aug;36(Suppl 2):175-189. doi: 10.1007/s12055-019-00820-3. Epub 2019 May 29. PMID: 33061202; PMCID: PMC7538525.
- Dipchand AI. Current state of pediatric cardiac transplantation. Ann Cardiothorac Surg. 2018 Jan;7(1):31-55. doi: 10.21037/acs.2018.01.07. PMID: 29492382; PMCID: PMC5827130.
- Drazner MH. The progression of hypertensive heart disease. Circulation. 2011 Jan 25;123(3):327-34. doi: 10.1161/CIRCULATIONAHA.108.845792. PMID: 21263005.
- Dumitrescu M, Constantin A, Nemecz AM, Drăgan E, Popov LD, Tanko G. Hypertension induces compensatory left ventricular hypertrophy by a mechanism involving gap junction lateralization and overexpression of CD36, PKC and MMP-2. Rom J Morphol Embryol. 2021 Jul-Sep;62(3):713-721. doi: 10.47162/RJME.62.3.08. PMID: 35263399; PMCID: PMC9019672.
- Elliott PM, Poloniecki J, Dickie S, Sharma S, Monserrat L, Varnava A, Mahon NG, McKenna WJ. Sudden death in hypertrophic cardiomyopathy: Identification of high-risk patients. Journal of the American College of Cardiology. 2000; 36:2212–2218. [PubMed: 11127463]
- Eriksson MJ, Sonnenberg B, Woo A, Rakowski P, Parker TG, Wigle ED, Rakowski H. Long-term outcome in patients with apical hypertrophic cardiomyopathy. Journal of the American College of Cardiology. 2002; 39:638–645. [PubMed: 11849863]
- Farcas AO, Stoica MC, Maier IM, Maier AC, Sin AI. Heart Transplant Rejection: From the Endomyocardial Biopsy to Gene Expression Profiling. Biomedicines. 2024 Aug 22;12(8):1926. doi: 10.3390/biomedicines12081926. PMID: 39200392; PMCID: PMC11351478.
- Feingold B, Rose-Felker K, West SC, Miller SA, Zinn MD. Short-term clinical outcomes and predicted cost savings of dd-cfDNA-led surveillance after pediatric heart transplantation. Clin Transplant. 2023 May;37(5):e14933. doi: 10.1111/ctr.14933. Epub 2023 Feb 23. PMID: 36779524.
- Felker GM, Shaw LK, O'Connor CM. A standardized defnition of ischemic cardiomyopathy for use in clinical research. J Am Coll Cardiol. 2002;39:210–8.
- Gristina V, Barraco N, La Mantia M, Castellana L, Insalaco L, Bono M, Perez A, Sardo D, Inguglia S, Iacono F, Cutaia S, Bazan Russo TD, Francini E, Incorvaia L,

Badalamenti G, Russo A, Galvano A, Bazan V. Clinical Potential of Circulating Cell-Free DNA (cfDNA) for Longitudinally Monitoring Clinical Outcomes in the First-Line Setting of Non-Small-Cell Lung Cancer (NSCLC): A Real-World Prospective Study. Cancers (Basel). 2022 Dec 6;14(23):6013. doi: 10.3390/cancers14236013. PMID: 36497493; PMCID: PMC9735435.

- Grskovic M, Hiller DJ, Eubank LA, Sninsky JJ, Christopherson C, Collins JP, Thompson K, Song M, Wang YS, Ross D, Nelles MJ, Yee JP, Wilber JC, Crespo-Leiro MG, Scott SL, Woodward RN. Validation of a Clinical-Grade Assay to Measure Donor-Derived Cell-Free DNA in Solid Organ Transplant Recipients. J Mol Diagn. 2016 Nov;18(6):890-902. doi: 10.1016/j.jmoldx.2016.07.003. Epub 2016 Oct 7. PMID: 27727019.
- Hammond EH, Yowell RL, Nunoda S, Menlove RL, Renlund DG, Bristow MR, Gay WA Jr, Jones KW, O'Connell JB. Vascular (humoral) rejection in heart transplantation: pathologic observations and clinical implications. J Heart Transplant. 1989 Nov-Dec;8(6):430-43. PMID: 2693662.
- Henricksen EJ, Moayedi Y, Purewal S, Twiggs JV, Waddell K, Luikart H, Han J, Feng K, Wayda B, Lee R, Shudo Y, Jimenez S, Khush KK, Teuteberg JJ. Combining donor derived cell free DNA and gene expression profiling for non-invasive surveillance after heart transplantation. Clin Transplant. 2023 Mar;37(3):e14699. doi: 10.1111/ctr.14699. Epub 2022 May 23. PMID: 35559582.
- Holzhauser L, Clerkin KJ, Fujino T, Alenghat FJ, Raikhelkar J, Kim G, Sayer G, Uriel N. Donor-derived cell-free DNA is associated with cardiac allograft vasculopathy. Clin Transplant. 2021 Mar;35(3):e14206. doi: 10.1111/ctr.14206. Epub 2021 Feb 6. PMID: 33368611; PMCID: PMC10040222.
- Holzhauser L, DeFilippis EM, Nikolova A, Byku M, Contreras JP, De Marco T, Hall S, Khush KK, Vest AR. The End of Endomyocardial Biopsy?: A Practical Guide for Noninvasive Heart Transplant Rejection Surveillance. JACC Heart Fail. 2023 Mar;11(3):263-276. doi: 10.1016/j.jchf.2022.11.002. Epub 2023 Jan 11. PMID: 36682960.
- Hu Z, Chen H, Long Y, Li P, Gu Y. The main sources of circulating cell-free DNA: Apoptosis, necrosis and active secretion. Crit Rev Oncol Hematol. 2021 Jan;157:103166. doi: 10.1016/j.critrevonc.2020.103166. Epub 2020 Nov 18. PMID: 33254039.
- Huang E, Sethi S, Peng A, Najjar R, Mirocha J, Haas M, Vo A, Jordan SC. Early clinical experience using donor-derived cell-free DNA to detect rejection in kidney transplant recipients. Am J Transplant. 2019 Jun;19(6):1663-1670. doi: 10.1111/ajt.15289. Epub 2019 Mar 29. PMID: 30725531.

- Hummel EM, Hessas E, Müller S, Beiter T, Fisch M, Eibl A, Wolf OT, Giebel B, Platen P, Kumsta R, Moser DA. Cell-free DNA release under psychosocial and physical stress conditions. Transl Psychiatry. 2018 Oct 29;8(1):236. doi: 10.1038/s41398-018-0264-x. PMID: 30374018; PMCID: PMC6206142.
- Ingulli E. Mechanism of cellular rejection in transplantation. Pediatr Nephrol. 2010 Jan;25(1):61-74. doi: 10.1007/s00467-008-1020-x. PMID: 21476231; PMCID: PMC2778785.
- Iyer A, Kumarasinghe G, Hicks M, Watson A, Gao L, Doyle A, Keogh A, Kotlyar E, Hayward C, Dhital K, Granger E, Jansz P, Pye R, Spratt P, Macdonald PS. Primary graft failure after heart transplantation. J Transplant. 2011;2011:175768. doi: 10.1155/2011/175768. Epub 2011 Aug 1. PMID: 21837269; PMCID: PMC3151502.
- Jackson AM, Amato-Menker C, Bettinotti M. Cell-free DNA diagnostics in transplantation utilizing next generation sequencing. Hum Immunol. 2021 Nov;82(11):850-858. doi: 10.1016/j.humimm.2021.07.006. Epub 2021 Sep 30. PMID: 34600770.
- Janeway, C.A., Jr.; Travers, P.; Walport, M.; Shlomchik, M.J. Immunobiology: The Immune System in Health and Disease, 5th ed.; The Major Histocompatibility Complex and Its Functions; Garland Science: New York, NY, USA, 2001. Available online: https://www.ncbi.nlm.nih.gov/books/NBK27156/
- Jiang P, Chan CW, Chan KC, Cheng SH, Wong J, Wong VW, Wong GL, Chan SL, Mok TS, Chan HL, Lai PB, Chiu RW, Lo YM. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci U S A. 2015 Mar 17;112(11):E1317-25. doi: 10.1073/pnas.1500076112. Epub 2015 Feb 2. PMID: 25646427; PMCID: PMC4372002.
- Kang M, Chippa V, An J. Viral Myocarditis. 2023 Nov 20. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 29083732.
- Kantrowitz A, Haller JD, Joos H, Cerruti MM, Carstensen HE. Transplantation of the heart in an infant and an adult. Am J Cardiol. 1968 Dec;22(6):782-90. PMID: 4880223.
- Karameh M, Meir K, Qadan A, Pappo O, Cohen D, Durst R, Amir O, Asleh R. Endomyocardial biopsy in clinical practice: the diagnostic yield and insights from a 5-year single-center experience. Hellenic J Cardiol. 2024 Mar 11:S1109-9666(24)00059-9. doi: 10.1016/j.hjc.2024.03.004. Epub ahead of print. PMID: 38479703.
- Keller M, Agbor-Enoh S. Donor-Derived Cell-Free DNA for Acute Rejection Monitoring in Heart and Lung Transplantation. Curr Transplant Rep. 2021;8(4):351-358. doi: 10.1007/s40472-021-00349-8. Epub 2021 Nov 5. PMID: 34754720;

PMCID: PMC8570240.

- Kewcharoen J, Kim J, Cummings MB, Leitner KB, Suzuki EMB, Banerjee D, Lum CJ. Initiation of noninvasive surveillance for allograft rejection in heart transplant patients > 1 year after transplant. Clin Transplant. 2022 Mar;36(3):e14548. doi: 10.1111/ctr.14548. Epub 2021 Dec 13. PMID: 34843112.
- Khush K, Hall S, Kao A, Raval N, Dhingra R, Shah P, Bellumkonda L, Ravichandran A, Van Bakel A, Uriel N, Patel S, Pinney S, DePasquale E, Baran DA, Pinney K, Oreschak K, Kobulnik J, Shen L, Teuteberg J. Surveillance with dual noninvasive testing for acute cellular rejection after heart transplantation: Outcomes from the Surveillance HeartCare Outcomes Registry. J Heart Lung Transplant. 2024 Sep;43(9):1409-1421. doi: 10.1016/j.healun.2024.05.003. Epub 2024 May 15. PMID: 38759766.
- Khush KK, Patel J, Pinney S, Kao A, Alharethi R, DePasquale E, Ewald G, Berman P, Kanwar M, Hiller D, Yee JP, Woodward RN, Hall S, Kobashigawa J. Noninvasive detection of graft injury after heart transplant using donor-derived cell-free DNA: A prospective multicenter study. Am J Transplant. 2019 Oct;19(10):2889-2899. doi: 10.1111/ajt.15339. Epub 2019 Apr 8. PMID: 30835940; PMCID: PMC6790566.
- Kim YH, Her AY, Choi BG, Choi SY, Byun JK, Baek MJ, Ryu YG, Park Y, Mashaly A, Jang WY, Kim W, Choi JY, Park EJ, Na JO, Choi CU, Lim HE, Kim EJ, Park CG, Seo HS, Rha SW. Impact of left ventricular hypertrophy on long-term clinical outcomes in hypertensive patients who underwent successful percutaneous coronary intervention with drug-eluting stents. Medicine (Baltimore). 2018 Aug;97(35):e12067. doi: 10.1097/MD.0000000000012067. PMID: 30170421; PMCID: PMC6392834.
- Kisling A, Gallagher R. Valvular Heart Disease. Prim Care. 2024 Mar;51(1):95-109. doi: 10.1016/j.pop.2023.08.003. Epub 2023 Sep 7. PMID: 38278576.
- Klarich KW, Attenhofer Jost CH, Binder J, Connolly HM, Scott CG, Freeman WK, Ackerman MJ, Nishimura RA, Tajik AJ, Ommen SR. Risk of death in long-term follow-up of patients with apical hypertrophic card.
- Knüttgen F, Beck J, Dittrich M, Oellerich M, Zittermann A, Schulz U, Fuchs U, Knabbe C, Schütz E, Gummert J, Birschmann I. Graft-derived Cell-free DNA as a Noninvasive Biomarker of Cardiac Allograft Rejection: A Cohort Study on Clinical Validity and Confounding Factors. Transplantation. 2022 Mar 1;106(3):615-622. doi: 10.1097/TP.0000000000003725. PMID: 33653997.
- Kobashigawa J, Mehra M, West L, Kerman R, George J, Rose M, Zeevi A, Reinsmoen N, Patel J, Reed EF; Consensus Conference Participants. Report from a consensus conference on the sensitized patient awaiting heart transplantation. J Heart Lung Transplant. 2009 Mar;28(3):213-25. doi: 10.1016/j.healun.2008.12.017. PMID:

19285611; PMCID: PMC3878294.

- Kobashigawa J, Patel J, Azarbal B, Kittleson M, Chang D, Czer L, Daun T, Luu M, Trento A, Cheng R, Esmailian F. Randomized pilot trial of gene expression profiling versus heart biopsy in the first year after heart transplant: early invasive monitoring attenuation through gene expression trial. Circ Heart Fail. 2015 May;8(3):557-64. doi: 10.1161/CIRCHEARTFAILURE.114.001658. Epub 2015 Feb 19. PMID: 25697852.
- Labarrere CA, Jaeger BR. Biomarkers of heart transplant rejection: the good, the bad, and the ugly! Transl Res. 2012 Apr;159(4):238-51. doi: 10.1016/j.trsl.2012.01.018. Epub 2012 Feb 14. PMID: 22424428.
- Lakhani HA, Lizarzaburo Penafiel LS, Fakhoury M, Seide M, Duran S PX, See JW, Dhillon DK, Shah S, Khan AM, Nimmagadda M, Susmitha T, Rai M. Heart Transplantation and Left Ventricular Assist Devices: Long-Term Prognosis and Effects on Mental Health. Cureus. 2024 Sep 5;16(9):e68691. doi: 10.7759/cureus.68691. PMID: 39371854; PMCID: PMC11452842.
- Li Y, Liang B. Circulating donor-derived cell-free DNA as a marker for rejection after lung transplantation. Front Immunol. 2023 Oct 11;14:1263389. doi: 10.3389/fimmu.2023.1263389. PMID: 37885888; PMCID: PMC10598712.
- Liao Y, Cooper RS, Mensah GA, McGee DL. Left ventricular hypertrophy has a greater impact on survival in women than in men. Circulation. 1995 Aug 15;92(4):805-10. doi: 10.1161/01.cir.92.4.805. PMID: 7641360.
- Ludhwani D, Abraham J, Sharma S, Kanmanthareddy A. Heart Transplantation Rejection. 2024 Aug 11. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 30725742.
- Marian AJ, Braunwald E. Hypertrophic Cardiomyopathy: Genetics, Pathogenesis, Clinical Manifestations, Diagnosis, and Therapy. Circ Res. 2017 Sep 15;121(7):749-770. doi: 10.1161/CIRCRESAHA.117.311059. PMID: 28912181; PMCID: PMC5654557.
- Markus H, Contente-Cuomo T, Farooq M, Liang WS, Borad MJ, Sivakumar S, Gollins S, Tran NL, Dhruv HD, Berens ME, Bryce A, Sekulic A, Ribas A, Trent JM, LoRusso PM, Murtaza M. Evaluation of pre-analytical factors affecting plasma DNA analysis. Sci Rep. 2018 May 9;8(1):7375. doi: 10.1038/s41598-018-25810-0. PMID: 29743667; PMCID: PMC5943304.
- Maron BJ, Doerer JJ, Haas TS, Tierney DM, Mueller FO. Sudden deaths in young competitive athletes: Analysis of 1866 deaths in the united states, 1980–2006. Circulation. 2009; 119:1085– 1092. [PubMed: 19221222]
- Maron BJ, Ommen SR, Semsarian C, Spirito P, Olivotto I, Maron MS. Hypertrophic

cardiomyopathy: Present and future, with translation into contemporary cardiovascular medicine. Journal of the American College of Cardiology. 2014; 64:83–99. [PubMed: 24998133]

- Maron BJ, Rowin EJ, Casey SA, Lesser JR, Garberich RF, McGriff DM, Maron MS. Hypertrophic cardiomyopathy in children, adolescents, and young adults associated with low cardiovascular mortality with contemporary management strategies. Circulation. 2016; 133:62– 73. [PubMed: 26518766]
- Maron BJ, Rowin EJ, Casey SA, Link MS, Lesser JR, Chan RH, Garberich RF, Udelson JE, Maron MS. Hypertrophic cardiomyopathy in adulthood associated with low cardiovascular mortality with contemporary management strategies. Journal of the American College of Cardiology. 2015; 65:1915–1928. [PubMed: 25953744]
- Maron BJ, Shirani J, Poliac LC, Mathenge R, Roberts WC, Mueller FO. Sudden death in young competitive athletes. Clinical, demographic, and pathological profiles. Jama. 1996; 276:199–204. [PubMed: 8667563]
- Masenga SK, Kirabo A. Hypertensive heart disease: risk factors, complications and mechanisms. Front Cardiovasc Med. 2023 Jun 5;10:1205475. doi: 10.3389/fcvm.2023.1205475. PMID: 37342440; PMCID: PMC10277698.
- McCartney SL, Patel C, Del Rio JM. Long-term outcomes and management of the heart transplant recipient. Best Pract Res Clin Anaesthesiol. 2017 Jun;31(2):237-248. doi: 10.1016/j.bpa.2017.06.003. Epub 2017 Jun 23. PMID: 29110796.
- Mehlman Y, Valledor AF, Moeller C, Rubinstein G, Lotan D, Rahman S, Oh KT, Bae D, DeFilippis EM, Lin EF, Lee SH, Raikhelkar JK, Fried J, Theodoropoulos K, Colombo PC, Yuzefpolskaya M, Latif F, Clerkin KJ, Sayer GT, Uriel N. The utilization of molecular microscope in management of heart transplant recipients in the era of noninvasive monitoring. Clin Transplant. 2023 Dec;37(12):e15131. doi: 10.1111/ctr.15131. Epub 2023 Oct 28. PMID: 37897211.
- Metra M, Teerlink JR. Heart failure. Lancet. 2017 Oct 28;390(10106):1981-1995. doi: 10.1016/S0140-6736(17)31071-1. Epub 2017 Apr 28. PMID: 28460827.
- Nesseler N, Mansour A, Cholley B, Coutance G, Bouglé A. Perioperative Management of Heart Transplantation: A Clinical Review. Anesthesiology. 2023 Oct 1;139(4):493-510. doi: 10.1097/ALN.0000000000004627. PMID: 37458995.
- Novitzky D, Cooper DKC, Barnard CN. Reversal of acute rejection by cyclosporine in a heterotopic heart transplant. J Heart Transplant. 1984;3:117120.
- O'Mahony C, Jichi F, Pavlou M, Monserrat L, Anastasakis A, Rapezzi C, Biagini E, Gimeno JR, Limongelli G, McKenna WJ, Omar RZ, Elliott PM. Hypertrophic Cardiomyopathy Outcomes I. A novel clinical risk prediction model for sudden cardiac death in hypertrophic cardiomyopathy (hcm risk-scd). European heart journal. 2014; 35:2010–2020. [PubMed: 24126876]

- Page BJ, Banas MD, Suzuki G, Weil BR, Young RF, Fallavollita JA, et al. Revascularization of chronic hibernating myocardium stimulates myocyte proliferation and partially reverses chronic adaptations to ischemia. J Am Coll Cardiol. 2015;65:684–97.
- Pham MX, Teuteberg JJ, Kfoury AG, Starling RC, Deng MC, Cappola TP, Kao A, Anderson AS, Cotts WG, Ewald GA, Baran DA, Bogaev RC, Elashoff B, Baron H, Yee J, Valantine HA; IMAGE Study Group. Gene-expression profiling for rejection surveillance after cardiac transplantation. N Engl J Med. 2010 May 20;362(20):1890-900. doi: 10.1056/NEJMoa0912965. Epub 2010 Apr 22. PMID: 20413602.
- Piening BD, Dowdell AK, Zhang M, Loza BL, Walls D, Gao H, Mohebnasab M, Li YR, Elftmann E, Wei E, Gandla D, Lad H, Chaib H, Sweitzer NK, Deng M, Pereira AC, Cadeiras M, Shaked A, Snyder MP, Keating BJ. Whole transcriptome profiling of prospective endomyocardial biopsies reveals prognostic and diagnostic signatures of cardiac allograft rejection. J Heart Lung Transplant. 2022 Jun;41(6):840-848. doi: 10.1016/j.healun.2022.01.1377. Epub 2022 Feb 3. PMID: 35317953; PMCID: PMC9133065.
- Pilch NA, Bowman LJ, Taber DJ. Immunosuppression trends in solid organ transplantation: The future of individualization, monitoring, and management. Pharmacotherapy. 2021 Jan;41(1):119-131. doi: 10.1002/phar.2481. Epub 2020 Dec 30. PMID: 33131123; PMCID: PMC8778961.
- Porcari A, Baggio C, Fabris E, Merlo M, Bussani R, Perkan A, Sinagra G. Endomyocardial biopsy in the clinical context: current indications and challenging scenarios. Heart Fail Rev. 2023 Jan;28(1):123-135. doi: 10.1007/s10741-022-10247-5. Epub 2022 May 14. PMID: 35567705; PMCID: PMC9107004.
- Power A, Sweat KR, Roth A, Dykes JC, Kaufman B, Ma M, Chen S, Hollander SA, Profita E, Rosenthal DN, Barkoff L, Chen CY, Davies RR, Almond CS. Contemporary Pediatric Heart Transplant Waitlist Mortality. J Am Coll Cardiol. 2024 Aug 13;84(7):620-632. doi: 10.1016/j.jacc.2024.05.049. PMID: 39111968.
- Reed EF, Demetris AJ, Hammond E, Itescu S, Kobashigawa JA, Reinsmoen NL, Rodriguez ER, Rose M, Stewart S, Suciu-Foca N, Zeevi A, Fishbein MC; International Society for Heart and Lung Transplantation. Acute antibody-mediated rejection of cardiac transplants. J Heart Lung Transplant. 2006 Feb;25(2):153-9. doi: 10.1016/j.healun.2005.09.003. PMID: 16446213.
- Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, Olsen E, Thiene G, Goodwin J, Gyarfas I, Martin I, Nordet P. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. Circulation. 1996 Mar

1;93(5):841-2. doi: 10.1161/01.cir.93.5.841. PMID: 8598070.

- Rolfo C, Cardona AF, Cristofanilli M, Paz-Ares L, Diaz Mochon JJ, Duran I, Raez LE, Russo A, Lorente JA, Malapelle U, Gil-Bazo I, Jantus-Lewintre E, Pauwels P, Mok T, Serrano MJ; ISLB. Challenges and opportunities of cfDNA analysis implementation in clinical practice: Perspective of the International Society of Liquid Biopsy (ISLB). Crit Rev Oncol Hematol. 2020 Jul;151:102978. doi: 10.1016/j.critrevonc.2020.102978. Epub 2020 May 5. Erratum in: Crit Rev Oncol Hematol. 2020 Oct;154:103058. doi: 10.1016/j.critrevonc.2020.103058. PMID: 32428812.
- Sapna F, Raveena F, Chandio M, Bai K, Sayyar M, Varrassi G, Khatri M, Kumar S, Mohamad T. Advancements in Heart Failure Management: A Comprehensive Narrative Review of Emerging Therapies. Cureus. 2023 Oct 4;15(10):e46486. doi: 10.7759/cureus.46486. PMID: 37927716; PMCID: PMC10623504.
- Satam H, Joshi K, Mangrolia U, Waghoo S, Zaidi G, Rawool S, Thakare RP, Banday S, Mishra AK, Das G, Malonia SK. Next-Generation Sequencing Technology: Current Trends and Advancements. Biology (Basel). 2023 Jul 13;12(7):997. doi: 10.3390/biology12070997. Erratum in: Biology (Basel). 2024 Apr 24;13(5):286. doi: 10.3390/biology13050286. PMID: 37508427; PMCID: PMC10376292.

scenarios. Heart Fail Rev. 2023 Jan;28(1):123-135. doi: 10.1007/s10741-022-10247-

- Schäufele TG, Spittler R, Karagianni A, Ong P, Klingel K, Kandolf R, Mahrholdt H, Sechtem U. Transradial left ventricular endomyocardial biopsy: assessment of safety and efficacy. Clin Res Cardiol. 2015 Sep;104(9):773-81. doi: 10.1007/s00392-015-0844-1. Epub 2015 Apr 2. PMID: 25832352.
- Schultheiss HP, Fairweather D, Caforio ALP, Escher F, Hershberger RE, Lipshultz SE, Liu PP, Matsumori A, Mazzanti A, McMurray J, Priori SG. Dilated cardiomyopathy. Nat Rev Dis Primers. 2019 May 9;5(1):32. doi: 10.1038/s41572-019-0084-1. PMID: 31073128; PMCID: PMC7096917.
- Schulz E, Jabs A, Gori T, Hink U, Sotiriou E, Tschöpe C, Schultheiss HP, Münzel T, Wenzel P. Feasibility and safety of left ventricular endomyocardial biopsy via transradial access: Technique and initial experience. Catheter Cardiovasc Interv. 2015 Oct;86(4):761-5. doi: 10.1002/ccd.25834. Epub 2015 Feb 3. PMID: 25586731.
- Schweiger M, Stiasny B, Dave H, Cavigelli-Brunner A, Balmer C, Kretschmar O, Bürki C, Klauwer D, Hübler M. Pediatric heart transplantation. J Thorac Dis. 2015 Mar;7(3):552-9. doi: 10.3978/j.issn.2072-1439.2015.01.38. PMID: 25922739; PMCID: PMC4387410.
- Shah k, Han D, Kim IC, Stern L, Kransdorf E, Chang D, Hamilton M, Nikolova A, Kittleson M, Patel J, Berman D, Kobashigawa J. Optimal threshold of donor-derived

cell-free DNA for detection of cardiac transplant rejection: a meta-analysis. Circulation. 2024 Nov 11. doi: 10.1161/circ.150.suppl_1.4145247.

- Shah P, Agbor-Enoh S, Bagchi P, deFilippi CR, Mercado A, Diao G, Morales DJ, Shah KB, Najjar SS, Feller E, Hsu S, Rodrigo ME, Lewsey SC, Jang MK, Marboe C, Berry GJ, Khush KK, Valantine HA; GRAfT Investigators. Circulating microRNAs in cellular and antibody-mediated heart transplant rejection. J Heart Lung Transplant. 2022 Oct;41(10):1401-1413. doi: 10.1016/j.healun.2022.06.019. Epub 2022 Jun 28. PMID: 35872109; PMCID: PMC9529890.
- Shah P, Agbor-Enoh S, Lee S, Andargie TE, Sinha SS, Kong H, Henry L, Park W, McNair E, Tchoukina I, Shah KB, Najjar SS, Hsu S, Rodrigo ME, Jang MK, Marboe C, Berry GJ, Valantine HA; GRAfT Investigators. Racial Differences in Donor-Derived Cell-Free DNA and Mitochondrial DNA After Heart Transplantation, on Behalf of the GRAfT Investigators. Circ Heart Fail. 2024 Apr;17(4):e011160. doi: 10.1161/CIRCHEARTFAILURE.123.011160. Epub 2024 Feb 20. PMID: 38375637; PMCID: PMC11021168.
- Sinagra G, Porcari A, Fabris E, Merlo M. Standardizing the role of endomyocardial biopsy in current clinical practice worldwide. Eur J Heart Fail. 2021 Dec;23(12):1995-1998. doi: 10.1002/ejhf.2380. Epub 2021 Nov 21. PMID: 34755442.
- Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol. 2017 Sep;14(9):531-548. doi: 10.1038/nrclinonc.2017.14. Epub 2017 Mar 2. PMID: 28252003.
- Snyder TM, Khush KK, Valantine HA, Quake SR. Universal noninvasive detection of solid organ transplant rejection. Proc Natl Acad Sci U S A. 2011 Apr 12;108(15):6229-34. doi: 10.1073/pnas.1013924108. Epub 2011 Mar 28. PMID: 21444804; PMCID: PMC3076856.
- Söderlund C, Rådegran G. Immunosuppressive therapies after heart transplantation--The balance between under- and over-immunosuppression. Transplant Rev (Orlando). 2015 Jul;29(3):181-9. doi: 10.1016/j.trre.2015.02.005. Epub 2015 Feb 28. PMID:25812489.
- Sorbini M, Carradori T, Togliatto GM, Vaisitti T, Deaglio S. Technical Advances in Circulating Cell-Free DNA Detection and Analysis for Personalized Medicine in Patients' Care. Biomolecules. 2024 Apr 19;14(4):498. doi: 10.3390/biom14040498. PMID: 38672514; PMCID: PMC11048502.
- Spirito P, Autore C, Formisano F, Assenza GE, Biagini E, Haas TS, Bongioanni S, Semsarian C, Devoto E, Musumeci B, Lai F, Yeates L, Conte MR, Rapezzi C, Boni L, Maron BJ. Risk of sudden death and outcome in patients with hypertrophic

cardiomyopathy with benign presentation and without risk factors. The American journal of cardiology. 2014; 113:1550–1555. [PubMed: 24630786]

- Stewart CM, Tsui DWY. Circulating cell-free DNA for non-invasive cancer management. Cancer Genet. 2018 Dec;228-229:169-179. doi: 10.1016/j.cancergen.2018.02.005. Epub 2018 Mar 11. PMID: 29625863; PMCID: PMC6598437.
- Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, Andersen CB, Angelini A, Berry GJ, Burke MM, Demetris AJ, Hammond E, Itescu S, Marboe CC, McManus B, Reed EF, Reinsmoen NL, Rodriguez ER, Rose AG, Rose M, Suciu-Focia N, Zeevi A, Billingham ME. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant. 2005 Nov;24(11):1710-20. doi: 10.1016/j.healun.2005.03.019. Epub 2005 Jun 20. PMID: 16297770.
- Strecker T, Rösch J, Weyand M, Agaimy A. Endomyocardial biopsy for monitoring heart transplant patients: 11-years-experience at a german heart center. Int J Clin Exp Pathol. 2013;6(1):55-65. Epub 2012 Nov 20. PMID: 23236543; PMCID: PMC3515982.
- Sun R, Liu M, Lu L, Zheng Y, Zhang P. Congenital Heart Disease: Causes, Diagnosis, Symptoms, and Treatments. Cell Biochem Biophys. 2015 Jul;72(3):857-60. doi: 10.1007/s12013-015-0551-6. PMID: 25638345.
- Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids--a promising, non-invasive tool for early detection of several human diseases. FEBS Lett. 2007 Mar 6;581(5):795-9. doi: 10.1016/j.febslet.2007.01.051. Epub 2007 Feb 2. PMID: 17289032.
- Tan CD, Baldwin WM 3rd, Rodriguez ER. Update on cardiac transplantation pathology. Arch Pathol Lab Med. 2007 Aug;131(8):1169-91. doi: 10.5858/2007-131-1169-UOCTP. PMID: 17683180.
- Timmermans K, Kox M, Scheffer GJ, Pickkers P. Plasma Nuclear and Mitochondrial DNA Levels, and Markers of Inflammation, Shock, and Organ Damage in Patients with Septic Shock. Shock. 2016 Jun;45(6):607-12. doi: 10.1097/SHK.0000000000000549. PMID: 26717107.
- van der Bom T, Zomer AC, Zwinderman AH, Meijboom FJ, Bouma BJ, Mulder BJ. The changing epidemiology of congenital heart disease. Nat Rev Cardiol. 2011 Jan;8(1):50-60. doi: 10.1038/nrcardio.2010.166. Epub 2010 Nov 2. PMID: 21045784.
- van Veldhuisen DG, van den Berg MP, Novitzky D, Cooper DKC. Cyclosporin A in the management of patients with heart transplants. Initial experience at Groote Schuur Hospital. S Afr Med J. 1984;66:557562.

- Verhoeven JGHP, Hesselink DA, Peeters AMA, de Jonge E, von der Thüsen JH, van Schaik RHN, Matic M, Baan CC, Manintveld OC, Boer K. Donor-Derived Cell-Free DNA for the Detection of Heart Allograft Injury: The Impact of the Timing of the Liquid Biopsy. Transpl Int. 2022 Mar 21;35:10122. doi: 10.3389/ti.2022.10122. PMID: 35387397; PMCID: PMC8977404.
- Vieira JL, Ventura HO, Mehra MR. Mechanical circulatory support devices in advanced heart failure: 2020 and beyond. Prog Cardiovasc Dis. 2020 Sep-Oct;63(5):630-639. doi: 10.1016/j.pcad.2020.09.003. Epub 2020 Sep 21. PMID: 32971112.
- Wang, Y.C.; Herskowitz, A.; Gu, L.B.; Kanter, K.; Lattouf, O.; Sell, K.W.; Ahmed-Ansari, A. Influence of cytokines and immunosuppressive drugs on major histocompatibility complex class I/II expression by human cardiac myocytes in vitro. Hum. Immunol. 1991, 31, 123–133.
- Xing Y, Guo Q, Wang C, Shi H, Zheng J, Jia Y, Li C, Hao C. Donor-derived cell-free DNA as a diagnostic marker for kidney-allograft rejection: A systematic review and meta-analysis. Biomol Biomed. 2024 Feb 20;24(4):731-740. doi: 10.17305/bb.2024.10049. PMID: 38386614; PMCID: PMC11293223.
- Yamani MH, Starling RC. Long-term medical complications of heart transplantation: information for the primary care physician. Cleve Clin J Med. 2000 Sep;67(9):673-80. doi: 10.3949/ccjm.67.9.673. PMID: 10992625.
- Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL; American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. Circulation. 2013 Oct 15;128(16):e240-327. doi: 10.1161/CIR.0b013e31829e8776. Epub 2013 Jun 5. PMID: 23741058.
- Yao W, Mei C, Nan X, Hui L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. Gene. 2016 Sep 15;590(1):142-8. doi: 10.1016/j.gene.2016.06.033. Epub 2016 Jun 16. PMID: 27317895.
- Yilmaz A, Kindermann I, Kindermann M, Mahfoud F, Ukena C, Athanasiadis A, Hill S, Mahrholdt H, Voehringer M, Schieber M, Klingel K, Kandolf R, Böhm M, Sechtem U. Comparative evaluation of left and right ventricular endomyocardial biopsy: differences in complication rate and diagnostic performance. Circulation.

2010 Aug 31;122(9):900-9. doi: 10.1161/CIRCULATIONAHA.109.924167. Epub 2010 Aug 16. PMID: 20713901.

- Zill OA, Banks KC, Fairclough SR, Mortimer SA, Vowles JV, Mokhtari R, Gandara DR, Mack PC, Odegaard JI, Nagy RJ, Baca AM, Eltoukhy H, Chudova DI, Lanman RB, Talasaz A. The Landscape of Actionable Genomic Alterations in Cell-Free Circulating Tumor DNA from 21,807 Advanced Cancer Patients. Clin Cancer Res. 2018 Aug 1;24(15):3528-3538. doi: 10.1158/1078-0432.CCR-17-3837. Epub 2018 May 18. PMID: 29776953.
- Zomer AC, Verheugt CL, Vaartjes I, Uiterwaal CS, Langemeijer MM, Koolbergen DR, Hazekamp MG, van Melle JP, Konings TC, Bellersen L, Grobbee DE, Mulder BJ. Surgery in adults with congenital heart disease. Circulation. 2011 Nov 15;124(20):2195-201. doi: 10.1161/CIRCULATIONAHA.111.027763. Epub 2011 Oct 10. PMID: 21986279.